

**GENERAL LABORATORY METHODS**

**Method Selection**

The inclusion of a method in this edition of the *Technical Manual* is a subjective decision of the authors and editors. Exclusion from the current edition does not necessarily indicate that a method’s use is prohibited. However, some procedures were removed because the chemicals used in the procedures could present a safety risk or the methods are no longer needed or applicable. Readers are cautioned when referring to procedures in previous editions because they have not been reviewed for content and safety since publication in these editions.

Methods given here are reliable, straightforward, and representative of current practice. Personnel should always consult the instructions from the manufacturer of a specific reagent and follow the directions provided.

Although the investigation of unusual problems often requires flexibility in thought and methodology, the adoption of uniform methods for routine procedures in the laboratory is imperative. In order for laboratory personnel to have reproducible and comparable results in a test procedure, it is essential that everyone in the laboratory perform the same procedure in the same manner.

**General Notes**

The methods outlined in the following sections are *examples* of acceptable procedures. Other acceptable procedures may be used by facilities if desired. To the greatest extent possible, the written procedures should conform to the *Laboratory Documents: Development and Control; Approved Guideline* developed by the Clinical and Laboratory Standards Institute. As indicated in Title 21 of the *Code of Federal Regulations* (CFR), Part 606.65(e), the manufacturer’s instructions (eg, product insert) for reagents and supplies licensed by the Food and Drug Administration (FDA) must be followed. Alternative procedures and exceptions can require FDA approval as described in 21 CFR 640.120. Alternative procedures must be validated using appropriate controls and incorporated into a standard operating procedure before approval by the medical director. It is important to use Standard Precautions when appropriate.

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| Reagent Preparation | Many procedures include formulas for reagent preparation. Labels for reagents prepared in-house must contain the following:   * Name of solution. * Date of preparation. * Expiration date (if known). * Storage temperature and/or conditions. * Mechanism to identify the person who prepared the solution. * Universal hazardous substance label. |

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| Temperatures | Whenever specific incubation or storage temperatures are given, the following ranges are considered satisfactory:   |  |  | | --- | --- | | **Stated Temperature** | **Acceptable Range** | | 4 C | 2-8 C | | Room temperature | 20-24 C | | 37 C | 36-38 C | | 56 C | 54-58 C | |

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| Centrifugation Variables | Centrifugation speeds (relative centrifugal force) and times should be standardized for each piece of equipment. (See the quality control methods.) |

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| Reference | Quality management system: Development and management of laboratory documents. (CLSI Document QMS02-A6, 6th edition.) Wayne, PA: Clinical and Laboratory Standards Institute, 2013. |

**Use of These Methods**

These methods are provided in a convenient electronic format so that blood banks, transfusion services, and cellular therapy laboratories may adopt them with or without customization. Such facilities may do so without prior permission from the copyright holder, AABB. However, other publishers, content aggregators, course pack developers, and website administrators must obtain permission in advance for use of the material.

**METHOD 1-1. SHIPPING HAZARDOUS MATERIALS**

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| Principle | It is the responsibility of the shipper of biologic or infectious material to properly classify, package, label, and document the substance being shipped.  For transport by mail of infectious materials, clinical specimens, or biologic products, the United States Postal Service (USPS) Hazardous, Restricted, and Perishable Mail Regulations must be followed.1 For interstate transport of infectious materials by ground or air, the United States Department of Transportation (DOT) regulations apply.2 Most air carriers apply the International Air Transport Association (IATA)3 regulations and the technical instructions of the International Civil Aviation Organization (ICAO).4 These agencies adopt the recommendations of the United Nations (UN) Committee of Experts on the Transport of Dangerous Goods for the international transport of infectious substances and clinical specimens.  The Centers for Disease Control and Prevention (CDC)5 and the IATA6 provide packing and labeling requirements for shipments of infectious materials. Facilities should also consult their local carriers for additional requirements. |

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| Procedure | **Step** | **Action** |
| 1 | *Classify the type of hazard:* If inadvertent exposure to human specimens occurs during shipment, the level of risk can be categorized.   * Category A substances contain infectious agents in a form that can cause permanent disability or life-threatening or fatal disease when otherwise healthy humans or animals are exposed to the substance. Examples include Ebola virus in any form, hepatitis B virus (HBV) in culture, and human immunodeficiency virus (HIV) in culture. The proper shipping name and UN numbers are as follows:   **Infectious Substance, affecting humans**, UN 2814;  or **Infectious Substance, affecting animals** *only*, UN 2900.   * Category B substances contain or are suspected to contain infectious agents but do not meet the criteria for Category A. Examples include routine blood specimens from patients or donors who are known to be infected with HIV or HBV. The proper shipping name and UN number are as follows:   **Biological Substance, Category B**, UN 3373.   * Exempt substances are those that do not contain infectious substances or are unlikely to cause disease in humans or animals. Patient samples that contain a Category B infectious substance and are transported for the purpose of research, diagnosis, investigational activities, or disease treatment or prevention are also considered exempt if they are transported by a private or contract carrier in a motor vehicle used exclusively to transport such materials. Examples of exempt substances include specimens from patients who are not suspected of having an infectious disease, blood components for transfusion, cellular therapy products for infusion, and reagents that have undergone pathogen inactivation. The shipping container is labeled as follows:   **Exempt human specimen**, or Exempt animal specimen. |
| 2 | *Package and label according to the hazard category*: Both the International Air Transport Association (IATA) and the US Department of Transportation (DOT) provide detailed instruction on the amount of material that may be contained in one package, the types of packaging materials that may be used, and proper methods of packing for each hazard category.   * It is the shipper’s responsibility to ensure that materials are packaged properly. The carrier will inspect the package for proper markings and labels, but they generally will not open the box to verify packaging. * Packaging requirements apply to both air and land transportation. * Category A and B packages must bear the proper shipping name and UN label on the box. * There are additional requirements for shipping specimens in dry ice or liquid nitrogen. If specimens are shipped in dry ice, the outer package must allow the release of carbon dioxide gas. Advance arrangements with the carrier are required when more than 2.3 kg of dry ice is contained in one package. The outer container must be marked with “Carbon dioxide, solid” or “Dry ice.” |
| 3 | *Examples:* Selected examples of specimen classification, applicable packaging, instructions, and labels are given in the table below. |
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| **References** | 1. Publication 52: Hazardous, restricted, and perishable mail. Section 3 - Hazardous materials, mailability by hazard class, Section 346. Washington, DC: US Postal Service, 2020 (revised annually). [Available at <https://pe.usps.com/text/pub52/pub52c3_024.htm> (accessed February 13, 2020).] 2. Code of federal regulations. Title 49, CFR Part 171-180, and Title 42, CFR Part 73. Washington, DC: US Government Publishing Office, 2019 (revised annually). [Available at [www.ecfr.gov](http://www.ecfr.gov).] 3. IATA. Dangerous goods regulations. 61st ed. Montreal, Canada: International Air Transport Association, 2020 (revised annually). [Available at <https://www.iata.org/en/publications/dgr/> (accessed February 13, 2020).] 4. Technical instructions for the safe transport of dangerous goods by air. Documents 9284-AN/905. 2019-2020 ed. Montreal, Canada: International Civil Aviation Organization, 2018. [Available at [www.icao.int/](http://www.icao.int/) (see catalog of publications; Document 9284, ISBN 978-92-9249-075-1)]. 5. Centers for Disease Control and Prevention shipping instructions for specimens collected from people who may have been exposed to chemical agents. Atlanta, GA: CDC, 2018 (updated regularly). [Available at <https://emergency.cdc.gov/labissues/specimens_shipping_instructions.asp>.] 6. Dangerous Goods Panel, International Civil Aviation Organization. Guidance document: Infectious substances. Montreal, Canada: International Air Transport Association, 2020. [Information available at [www.iata.org](http://www.iata.org)/ and [www.icao.int/](http://www.icao.int/) (accessed February 25, 2020).] |

**METHOD 1-2. MONITORING TEMPERATURE DURING SHIPMENT OF BLOOD**

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| **Principle** | Some form of temperature indication or monitoring is desirable when shipping blood. The temperature of the contents of a shipping container used for whole blood or liquid-stored red cell components can be ascertained when the shipment is received, as follows: |

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| Procedure | **Step** | **Action** |
| 1 | Open the shipping container and promptly place the sensing end of a calibrated liquid-in-glass or electronic thermometer between two bags of blood or components (labels facing out) and secure the “sandwich” with two rubber bands. |
| 2 | Close the shipping container. |
| 3 | After approximately 3 to 5 minutes, read the temperature. |
| 4 | If the temperature has exceeded the acceptable range, quarantine the units until their appropriate disposition can be determined. |

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| **Notes** | Other suitable methods for monitoring shipments are as follows:   1. Use time/temperature indicators, one such indicator per shipping carton. These indicators will change color or show another visible indication if the temperature has exceeded the acceptable range. 2. Place a “high-low” thermometer in the shipping container. This simple, reusable thermometer measures and records the highest and lowest temperatures during any period. |

**METHOD 1-3. TREATING INCOMPLETELY CLOTTED SPECIMENS**

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| **Principle** | Fibrin generation may continue in serum separated from incompletely clotted blood, especially during incubation at 37 C. The fibrin generation produces strands of protein that entrap red cells and that make it difficult to evaluate agglutination. Blood from patients who have recently received heparin may not clot at all, and blood from patients with excessive fibrinolytic activity may reliquefy or may contain protein fragments that interfere with examination for agglutination. |

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| **Materials** | 1. Thrombin: dry human/bovine thrombin or thrombin solution (50 units/mL in saline). 2. Glass beads. 3. Protamine sulfate: 10 mg/mL in saline. 4. Epsilon aminocaproic acid (EACA): 0.25 g/mL in saline. |

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| Procedure | **Step** | **Action** |
| 1 | *To accelerate clotting*: Either of the following techniques may be used:   1. Add to the specimen the amount of dry thrombin that adheres to the tip of an applicator stick or 1 drop of thrombin solution per mL of whole blood or serum. Allow 10 to 15 minutes for the clot to form. Use standard centrifugation to separate the clot and serum. 2. Gently agitate the separated serum with small glass beads, at 37 C, for several minutes. Then, use low speed centrifugation to pellet the glass beads. Transfer the serum to another tube. |
| 2 | *To neutralize heparin:* Protamine sulfate can be added to the specimen to neutralize heparin; however, excess protamine promotes rouleaux formation and, in great excess, will inhibit clotting.   1. Add 1 drop of protamine sulfate solution to 4 mL of whole blood, and wait 30 minutes to evaluate the effect on clotting. If clotting does not occur, add additional protamine sparingly. 2. Note: protamine sulfate may work more rapidly when briefly incubated (5-10 minutes) at 37 C. |
| 3 | *To inhibit fibrinolytic activity:* Add 0.1 mL of EACA to 4 mL of whole blood. |

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| **Notes** | 1. The use of anticoagulated (eg, acid-citrate-dextrose or EDTA) collection tubes may help to avoid the problem of incompletely clotted specimens. The use of anticoagulated specimens must be validated in accordance with each standard operating procedure. 2. Because preparations of human thrombin may contain red cell antibodies, test results must be carefully observed for false-positive reactions. Quality control should be performed on thrombin reagents before, or concurrent with, their use to identify those with contaminating antibodies. 3. Each laboratory should validate the performance of treated samples to determine that the samples behave as expected in the test system used after treatment. |

**METHOD 1-4. SOLUTION PREPARATION PROCEDURE**

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| **Principle** | The basic definitions, calculations, and instructions given below serve as a review of simple principles necessary for solution preparation.   1. Mole, gram-molecular weight: Weight, expressed in grams equal to the atomic or molecular weight of the substance. |
|  | 1. Molar solution: A one-molar (1 M) solution contains one mole of solute in a liter of solvent. The solvent is assumed to be distilled or deionized water unless otherwise indicated. |
|  | 1. Gram-equivalent weight: Weight, in grams, of a substance that will produce or react with 1 mole of hydrogen ion. |
|  | 1. Normal solution: A one-normal (1 N) solution contains one gram-equivalent weight of solute in a liter of solution. |
|  | 1. Percentage solutions: The percentage designation of a solution gives the weight or volume of solute present in 100 units of total solution. Percentage can be expressed as follows: 2. Weight/weight (w/w), indicating grams of solute in 100 g of solution. 3. Volume/volume (v/v), indicating milliliters of solute present in 100 mL of solution. 4. Weight/volume (w/v), indicating grams of solute in 100 mL of solution. Unless otherwise specified, a solution expressed in percentage can be assumed to be w/v. |
|  | 1. Water of crystallization, water of hydration: Molecules of water that form an integral part of the crystalline structure of a substance. A given substance may have several crystalline forms, with different numbers of water molecules intrinsic to the entire molecule. The weight of this water must be included in calculating molecular weight of the hydrated substance. |
|  | 1. Anhydrous: The salt form of a substance with no water of crystallization. 2. Atomic weights (rounded to whole num­bers): H, 1; O, 16; Na, 23; P, 31; S, 32; Cl, 35; K, 39. 3. Molecular weights:   HCl: 1 + 35 = 36; NaCl: 23 + 35 = 58  KCl: 39 + 35 = 74  H2O: (2 × 1) + 16 = 18  NaH2PO4: 23 + (2 × 1) + 31 + (4 × 16) = 120  NaH2PO4 • H2O: 23 + (2 × 1) + 31 + (4 × 16) + (2 × 1) + 16 = 138  KH2PO4: 39 + (2 × 1) + 31 + (4 × 16) = 136  H2SO4: (2 × 1) + 32 + (4 × 16) = 98 |
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| **Examples** | 1. Molar solution:   1 M KH2PO4 = 136 g of solute made up to 1 L.  0.15 M KH2PO4 = (136 × 0.15) = 20.4 g of solute made up to 1 L.  0.5 M NaH2PO4 = (120 × 0.5) = 60 g of solute made up to 1 L. |
|  | 1. Molar solution with hydrated salt:   0.5 M NaH2PO4 • H2O = (138 × 0.5) = 69 g of the monohydrate crystals made up to 1 L. |
|  | 1. Normal solutions:   1 N HCl = 36 g of solute made up to 1 L. One mole HCl dissociates into one mole H+, so gram-equivalent weight and gram-molecular weight are the same.  12 N HCl = (36 × 12) = 432 g of solute made up to 1 L.  1 N H2SO4 = (98 ÷ 2) = 49 g of solute made up to 1 L. One mole H2SO4 dissociates to give two moles of H+, so the gram-equivalent weight is half the gram-molecular weight. |
|  | 1. Percentage solution:   0.9% NaCl (w/v) = 0.9 g of solute made up to 100 mL of solution. |

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| **Notes** | 1. Accurate results require accurate preparation of reagents. It is important to carefully read and follow all instructions and labels. 2. Weigh only quantities appropriate for the accuracy of the equipment. The operator’s manual should give these specifications. |
|  | 1. Prepare the largest volume that is practical. There is greater accuracy in measuring larger volumes than smaller volumes. If a reagent balance is accurate to ± 0.01 g, the potential error in weighing 0.05 g (50 mg) will be 20%, whereas the potential error in weighing 0.25 g (250 mg) will be only 4%. If the solution retains its activity when stored appropriately, it is usually preferable to prepare a large volume. If the solution deteriorates rapidly, smaller volumes may be preferred to reduce waste. |
|  | 1. Note whether a substance is in the hydrated or anhydrous form. If the instructions give solute weight for one form, and the available reagent is in another form, be sure to adjust the measurements appropriately. For example, if instructions for 0.5 M NaH2PO4 call for 60 g, and the reagent is NaH2PO4 • H2O, find the ratio between the weights of the two forms. The molecular weight of NaH2PO4 • H2O is 138, and the molecular weight of NaH2PO4 is 120. Therefore, the ratio is 138 ÷ 120 = 1.15. Multiply the designated weight by the ratio (60 g × 1.15 = 69 g) to obtain the final weight needed. |
|  | 1. Dissolve the solute completely before making the solution to the final volume. This is especially important for substances, such as phosphates, that dissolve slowly. For example, to make 500 mL of 0.15 M KH2PO4: 2. Weigh 10.2 g of solute in a weighing boat or glass [(0.15 × 136) ÷ 2] because only 500 mL will be made. 3. Place 350 mL of water in a 500-mL volumetric flask on a magnetic stirrer. Add the stirring bar and adjust it to a slow, steady stirring speed. 4. Add 10.2 g of salt, then rinse the boat with several aliquots of water until no salt remains. Numerous small-volume rinses remove adherent material more effectively than a few larger volumes. Add the rinse water to the material in the flask and stir until the salt has completely dissolved. 5. If pH measurement is unnecessary, add water to the 500-mL mark, adjusting the volume for the stirring bar, and mix thoroughly. For solutions needing pH adjustment, see the next step. |
|  | 1. Adjust the pH of the solution before bringing it to its final volume so that the addition of water (or other solvent) does not markedly change the molarity. For example, to bring 500 mL of 0.1 M glycine to a pH of 3: 2. Add 3.75 g of glycine (H2NCH2COOH: molecular weight, 75) to 400-475 mL of water in a beaker. Dissolve completely, using a magnetic stirrer. 3. Add a few drops of concentrated (12 N) HCl and measure pH after acid is thoroughly mixed. Continue adding HCl until pH is 3.0. 4. Transfer the solution to a 500-mL volumetric flask. Rinse beaker and stirring bar with aliquots of water, adding the rinse water to the flask. Use the rinses to contribute to the total 500-mL volume. 5. Measure the pH of the solution at final volume. |

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| **References** | 1. Remson ST, Ackerman PG. Calculations for the medical laboratory. Boston, MA: Little, Brown & Co., 1977. 2. McPherson RA, Pincus MR, eds. Henry’s clinical diagnosis and management by laboratory methods. 23rd ed. Philadelphia: Elsevier, 2016. |

**METHOD 1-5. SERUM DILUTION PROCEDURE**

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| **Principle** | Serum is sometimes diluted in saline or other diluents to determine its relative antibody concentration. It is customary to express the dilution as 1 part of serum *contained* in the total number of parts of the dilution. For example, to test the serum at one-tenth its original concentration, a dilution of 1 part in 10 may be made by mixing 1 mL of serum with 9 mL of saline. The *final volume is 10*, and the dilution is expressed as a 1-in-10 dilution. The diluted material contains one-tenth (1/10 or 0.1) of the unmodified serum. It is often customary to report the titer of an antibody as the reciprocal of the highest dilution that retains a 1+ agglutination. Therefore, serum that reacts at a dilution of 1/32 is considered to have a titer of 32. |

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| Procedure | **Step** | **Action** |
| 1 | To dilute an existing dilution:   1. A new higher dilution can be prepared from diluted material by adding more diluent. The formula for calculating either the new higher final dilution or the amount of diluent to add to obtain a higher final dilution is as follows: 2. *Example:* Serum dilution is 1 in 2, and volume of serum dilution is 1.0 mL. If 4.0 mL of saline is added, the new final dilution will be   X = 10, or a 1-in-10 dilution |
|  | 2 | To dilute a dilution to a specified volume:   1. The formula for calculating the volume of diluent to add to a dilution to achieve a certain quantity of a new higher final dilution is as follows: 2. *Example:* Present serum dilution is 1 in 2, total final volume is 100 mL, and new final serum dilution is 1 in 10. To make up a final volume of 100 mL of a 1-in-10 dilution, 20 mL of serum (diluted 1 in 2) will have to be added:   X = 20, or 20 mL of serum (dilution of 1 in 2) must be added to 80 mL of diluent to obtain 100 mL of a 1-in-10 dilution. |

**METHOD 1-6. DILUTION OF PERCENTAGE SOLUTIONS PROCEDURE**

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| **Principle** | Serologic tests may require the use of solutions at concentrations that are different from the concentration offered by the manufacturer. Precise calculations are needed to dilute the original volume and concentration to the volume and concentration desired. |

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| Procedure | **Step** | **Action** |
| 1 | Dilutions can be prepared from more concentrated solutions by use of the following formula:  (Volume1 × Concentration1) =  (Volume2 × Concentration2) V1 × C1 = V2 × C2  where V1 and C1 represent original volume and concentration, and V2 and C2 represent final desired volume and concentration. |
| 2 | *Example:* 30% albumin is available, but 2 mL of 6% albumin is needed. The proper dilution can be calculated as follows:  V1 × 30 = 2 × 6  30V1 = 12  V1 = 12 ÷ 30 = 0.4  Therefore, mix 0.4 mL of 30% albumin with 1.6 mL saline to obtain 2.0 mL of 6% albumin, or for small-volume use, mix 4 drops 30% albumin with 16 drops saline to obtain 20 drops of 6% albumin. |

**METHOD 1-7. PREPARING A 3% RED CELL SUSPENSION**

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| **Principle** | A 3% red cell suspension is a common reagent in many serologic procedures. The suspension need not be exactly 3%; an approximation achieves the appropriate serum-to-cell ratio for most test procedures and for an adequate number of red cells so one can read and grade the reactions. The following steps are intended to help an individual gain confidence in approximating a 3% red cell suspension visually, both as a suspension of cells and in the appropriate size of the cell pellet achieved after centrifugation. |
| **Materials** | 1. Whole blood sample. 2. Test tubes. 3. Disposable pipettes (1-mL and 10-mL serologic). 4. Saline. 5. Centrifuge (3000 rpm or equivalent). 6. Commercially prepared 3% reagent red cell suspension. |

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| Procedure | **Step** | **Action** |
|  | *To prepare 10 mL of a 3% red cell suspension:* |
| 1 | Transfer at least 1 mL of whole blood to a 10-mL tube. |
| 2 | Wash the red cells in saline or phosphate-buffered saline (PBS), centrifuging for 5 minutes to pellet the cells. Repeat two or three times. The final supernate should be clear and should be completely removed by aspiration. |
| 3 | Transfer 0.3 mL of the washed red cells to a tube with 9.7 mL of saline, PBS, or Alsever’s solution. |
| 4 | Cap or cover the tube with parafilm. Thoroughly mix the red cells and saline by gently inverting the tube several times. |
| 5 | To compare the color and density of the suspension by eye, transfer a volume of the prepared suspension to a 10 × 75 mm tube. Also transfer a similar volume of a known 3% red cell suspension (eg, commercial reagent red cell suspension) to another 10 × 75 mm tube. Hold the two tubes in front of a light source to compare them. |
| 6 | To compare the size of the cell pellet expected from a 3% red cell suspension, transfer 1 drop of the prepared suspension to a 10 × 75 mm tube. Similarly, transfer 1 drop of a known 3% commercial reagent red cell suspension to another 10 × 75 mm tube. Centrifuge the tubes in a serologic centrifuge, using the spin time designated for “saline.” The size of the two cell pellets should be similar. |

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| Note | For best results use red cell suspensions only on the day of preparation unless stability for a longer time has been validated. |

**METHOD 1-8. PREPARING AND USING PHOSPHATE BUFFER**

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| Principle | Mixtures of acids and bases can be prepared at specific pH values and used to buffer (render) other solutions to that pH. The following procedure includes a method for preparing phosphate-buffered saline (PBS), which can be used as a diluent in serologic tests. |

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| Reagents | 1. Prepare acidic stock solution (solution A) by dissolving 22.16 g of NaH2PO4 • H2O in 1 L of distilled water. This 0.16 M solution of the monobasic phosphate salt (monohydrate) has a pH of 5.0. 2. Prepare alkaline stock solution (solution B) by dissolving 22.7 g of Na2HPO4 in 1 L of distilled water. This 0.16 M solution of the dibasic phosphate salt (anhydrous) has a pH of 9.0. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare working buffer solutions of the desired pH by mixing appropriate volumes of the two solutions. A few examples are as follows:   |  |  |  | | --- | --- | --- | | **pH** | **Solution A** | **Solution B** | | 5.5 | 94 mL | 6 mL | | 7.3 | 16 mL | 84 mL | | 7.7 | 7 mL | 93 mL | |
| 2 | Check the pH of the working solution before using it. If necessary, add small volumes of acid solution A or alkaline solution B to achieve the desired pH. |
| 3 | To prepare PBS of a desired pH, add one volume of phosphate buffer at that pH to nine volumes of normal saline. |

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| References | 1. Hendry EB. Osmolarity of human serum and of chemical solutions of biologic importance. Clin Chem 1961;7:156-64. 2. Bain B, Bates I, Laffan M, Lewis S. Dacie and Lewis practical haematology. 11th ed. London, England: Churchill Livingston, 2012. |

**METHOD 1-9. READING AND GRADING TUBE AGGLUTINATION**

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| Principle | The purpose of grading reactions is to allow comparison of reaction strengths. This purpose is beneficial in detecting multiple antibody specificities or antibodies exhibiting dosage. |

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| **Materials** | 1. Centrifuged serologic tests for agglutination. 2. Agglutination viewer. |

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| Procedure | **Step** | **Action** |
| 1 | Gently shake or tilt the tube to resuspend the red cell button in the tube. The tilt technique uses the solution meniscus to gently dislodge the red cell button from the wall of the tube. |
| 2 | Observe the way that cells are dispersed from the red cell button. |
| 3 | Record reactivity by comparing the agglutinates to the descriptions in the table below. The reactivity should be assessed when the red cells have been completely resuspended from the button. |

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| **Interpretation** | **Interpretation of Agglutination Reactions**   |  |  |  | | --- | --- | --- | | Macroscopically Observed Findings | Designation | Score | | One solid agglutinate | 4+ | 12 | | Several large agglutinates | 3+ | 10 | | Medium-size agglutinates, clear background | 2+ | 8 | | Small agglutinates, turbid background | 1+ | 5 | | Very small agglutinates, turbid background | 1+w | 4 | | Barely visible agglutination, turbid background | w+ or +/− | 2 | | No agglutination | 0 | 0 | | Mixtures of agglutinated and unagglutinated red cells (mixed field) | mf |  | | Complete hemolysis | H |  | | Partial hemolysis, some red cells remain | PH |  | |
| **Notes** | 1. For uniformity and reproducibility, the grading of agglutination reactions should be standardized among all members of the laboratory staff. 2. The grading system should be described in a written procedure available to all staff. 3. Some systems use assigned numeric values (scores) for the observed reactions. 4. The grading system above is not necessarily applicable to column agglutination and solid-phase technologies. For appropriate grading of reactions in newer technologies, the package insert should be consulted. |

**RED CELL TYPING METHODS**

If the antigens on a donor’s red cells are not an identical match to those of the recipient, transfused blood is capable of inducing an antibody response in the recipient. Thus, it is important to identify the antigenic substances on both the donor’s red cells and those of the intended recipient.

The most immunogenic and clinically important antibodies are those directed against antigens of the ABO and Rh blood groups. Donor blood samples are routinely typed for ABO and Rh at the time of donation. The ABO group is confirmed when the Red Blood Cell unit is received in the hospital’s transfusion service, and the Rh type is confirmed on units labeled Rh negative. Recipient samples are typed before transfusion.

**METHOD 2-1. DETERMINING ABO GROUP OF RED CELLS—SLIDE TEST**

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| Principle | Because of the dire clinical consequences associated with ABO incompatibilities, ABO typing and ABO compatibility testing remain the foundation of pretransfusion testing and an important component of typing before transplantation. Detailed discussions are found in Chapters 10 and 17 of the AABB *Technical Manual*, 20th edition. |

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| Specimen | The reagent manufacturer’s instructions must be consulted before slide tests are performed; some manufacturers recommend performing slide tests with whole blood, whereas others specify the use of red cell suspensions of lighter concentrations prepared in saline, serum, or plasma. |

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| Reagents | 1. Anti-A. 2. Anti-B. 3. Anti-A,B (optional). |

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| Procedure | **Step** | **Action** |
| 1 | Place 1 drop of anti-A on a clean, labeled glass slide. |
| 2 | Place 1 drop of anti-B on a separate clean, labeled glass slide. |
| 3 | Place 1 drop of anti-A,B on a third slide—if parallel tests are to be performed with this reagent—or on a single, clean, labeled slide if this test is the only one performed. |
| 4 | Add to each drop of reagent on the slides 1 drop of well-mixed suspension (in saline, serum, or plasma) of the red cells to be tested. (Consult the reagent manufacturer’s instructions to determine the correct cell concentration to be used.) |
| 5 | Mix the reagents and red cells thoroughly, using a clean applicator stick for each reagent. Spread the mixture over an area approximately 20 mm × 40 mm. |
| 6 | Gently tilt the slide from side to side continuously for up to 2 minutes. Do not place the slide over a heated surface, such as an Rh viewbox, during this period. |
| 7 | Read, interpret, and record the results of the reactions on all slides. |

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| --- | --- |
| Interpretation | 1. Strong agglutination of red cells in the presence of any ABO typing reagent constitutes a positive result. 2. A smooth suspension of red cells at the end of 2 minutes is a negative result. 3. Samples that give weak or doubtful reactions should be retested using a tube test, rather than a slide test. |

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| Notes | 1. All reagents must be used in accordance with the manufacturer’s instructions. 2. Slide testing imposes a greater risk of exposure to infectious samples. Personnel should follow safety measures detailed in the facility’s procedures manual. 3. Slide testing is not suitable for detection of ABO antibodies in serum or plasma. |

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| Reference | 1. Olsson ML, Westman JS. ABO and other carbohydrate blood group systems. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:297-327. |

**METHOD 2-2. DETERMINING ABO GROUP OF RED CELLS AND SERUM—TUBE TEST**

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| Principle | Because of the dire clinical consequences associated with ABO incompatibilities, ABO typing and ABO compatibility testing remain the foundation of pretransfusion testing and an important component of typing before transplantation. Detailed discussions are found in Chapters 10 and 17 of the AABB *Technical Manual*, 20th edition. |

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| Specimen | The reagent manufacturer’s package insert must be consulted to determine specific specimen requirements. Generally, clotted or anticoagulated blood samples may be used for ABO testing. The red cells may be suspended in autologous serum, plasma, or saline, or they may be washed and resuspended in saline. |

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| Reagents | 1. Anti-A. 2. Anti-B. 3. Anti-A,B (optional). 4. 2% to 5% suspension of A1, A2, and B red cells. The red cells can be obtained commercially or prepared daily by the testing laboratory. Note: The use of A2 cells is optional. |

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| Procedure | **Step** | **Action** |
|  | *Testing Red Cells* |
| 1 | Place 1 drop of anti-A in a clean, labeled test tube. |
| 2 | Place 1 drop of anti-B in a separate, clean, labeled tube. |
| 3 | Place 1 drop of anti-A,B in a third clean, labeled tube, if tests are to be performed with this reagent. |
| 4 | To each tube, add 1 drop of a 2% to 5% suspension (in saline, serum, or plasma) of the red cells to be tested. Alternatively, the equivalent amount of red cells can be transferred to each tube with clean applicator sticks. |
| 5 | Gently mix the contents of the tubes; then centrifuge for the calibrated spin time. |
| 6 | Gently resuspend the cell buttons, and examine them for agglutination. |
| 7 | Read, interpret, and record the test results. Compare the red cell test results with those obtained in the serum or plasma tests (see below). |
|  | *Testing Serum or Plasma* |
| 1 | Add 2 or 3 drops each of serum or plasma to two clean, labeled test tubes. |
| 2 | Add 1 drop of A1 reagent red cells to the tube labeled A1. |
| 3 | Add 1 drop of B reagent red cells to the tube labeled B. |
| 4 | Add A2 red cells to a third appropriately labeled tube with 2 or 3 drops serum or plasma, if this optional test is being performed. |
| 5 | Gently mix the contents of the tubes; then centrifuge for the calibrated spin time. |
| 6 | Examine the serum overlying the red cell buttons for evidence of hemolysis. Gently resuspend the cell buttons, and examine them for agglutination. |
| 7 | Read, interpret, and record test results. Compare serum test results with those obtained in testing red cells (see above). |

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| Interpretation | 1. Agglutination of tested red cells and either hemolysis or agglutination in tests with serum or plasma constitute positive test results. 2. A smooth cell suspension after resuspension of the cell button is a negative test result. 3. Interpretation of serum or plasma and red cell tests for ABO is given in the table below:      1. Any discrepancy between the results of the tests with serum or plasma and red cells should be resolved before an interpretation is recorded for the patient’s or donor’s ABO group. 2. Mixed-field agglutination should be investigated for possible cause. |

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| Notes | 1. All reagents must be used in accordance with the manufacturer’s instructions. 2. Positive reactions characteristically show 3+ to 4+ agglutination by reagent ABO antibodies; reactions between test serum and reagent red cells are often weaker. The serum tests may be incubated at room temperature for 5 to 15 minutes to enhance weak reactions. A discussion of weakly reactive samples is found in Chapter 10 of the AABB *Technical Manual*, 20th edition. |
|  |  |
| Reference | 1. Olsson ML, Westman JS. ABO and other carbohydrate blood group systems. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:297-327. |

**METHOD 2-3. DETERMINING ABO GROUP OF RED CELLS AND SERUM—MICROPLATE TEST**

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| --- | --- |
| Principle | Because of the dire clinical consequences associated with ABO incompatibilities, ABO typing and ABO compatibility testing remain the foundation of pretransfusion testing and an important component of typing before transplantation. Detailed discussions are found in Chapter 10 and 17 of the AABB *Technical Manual*, 20th edition.  Microplate techniques can be used to test for antigens on red cells and for antibodies in serum. A microplate can be considered as a matrix of 96 “short” test tubes; the principles that apply to hemagglutination in tube tests also apply to tests in microplates. |

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| Specimen | The reagent manufacturer’s package insert must be consulted before testing is performed to determine specific specimen requirements. Generally, clotted or anticoagulated blood samples may be used for ABO testing. Some manufacturers recommend performing tests with whole blood; others recommend that red cells be suspended in autologous serum, plasma, or saline, or washed and resuspended in saline. |

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| Equipment | 1. Dispensers (optional): Semiautomated devices are available for dispensing equal volumes to a row of wells. |
|  | 1. Microplate readers (optional): Automated photometric devices are available that read microplate results by the light absorbance in U-shaped bottom wells to differentiate between positive and negative test results. The microprocessor component of the reader interprets the reactions and prints the blood testing results. The manufacturer’s instructions for the collection and preparation of serum or plasma and cell specimens must be followed. |
|  | 1. Centrifuges: Special plate carriers can be purchased to fit common table-top centrifuges. Appropriate conditions must be established for each centrifuge. The following times and relative centrifugal forces, expressed as *g*, are suggested. Consult the manufacturer’s directions for specific information. 2. *For a flexible U-shaped-bottom microplate:* 700 × *g* for 5 seconds for red cell testing and serum or plasma testing. 3. *For a rigid U-shaped-bottom microplate:* 400 × *g* for 30 seconds for red cell testing and serum or plasma testing. |

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| Reagents | 1. Anti-A. 2. Anti-B. 3. Anti-A,B. Note: Use of this reagent is optional. 4. 2% to 5% suspension of group A1, A2, and B reagent red cells. The red cells can be obtained commercially or prepared daily by the testing laboratory (see Method 1-7). Note: The use of A2 red cells is optional. |

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| Procedure | **Step** | **Action** |
|  | *Testing Red Cells* |
| 1 | Place 1 drop of anti-A and 1 drop of anti-B in separate clean wells of a U-bottom microplate. If tests with anti-A,B are to be performed, add this reagent to a third clean well. |
| 2 | Add 1 drop of a 2% to 5% saline suspen­sion of red cells to each well containing blood typing reagent. |
| 3 | Mix the contents of the wells by gently tapping the sides of the plate. |
| 4 | Centrifuge the plate at the appropriate conditions established for the centrifuge. |
| 5 | Resuspend the cell buttons by manually tapping the plate or with the aid of a mechanical shaker, or place the plate at an angle for the tilt-and-stream method. |
| 6 | Read, interpret, and record results. Compare red cell test results with those obtained in testing serum or plasma. |
|  | *Testing Serum or Plasma* |
| 1 | Add 1 drop of serum or plasma under test to each well. |
| 2 | Add 1 drop of a 2% to 5% suspension of A1 and B reagent red cells to separate clean wells of a U-bottom microplate. If an optional test on A2 red cells will be performed, add A2 red cells to a third well. |
| 3 | Mix the contents of the wells by gently tapping the sides of the plate. |
| 4 | Centrifuge the plate at the appropriate conditions established for the centrifuge. |
| 5 | Resuspend the red cell buttons by manually tapping the plate or using a mechanical shaker, or place the plate at an angle for the tilt-and-stream method. |
| 6 | Read, interpret, and record results. Compare test results on serum or plasma with those obtained in testing red cells. |

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| Interpretation | 1. Agglutination in any well of red cell tests or hemolysis or agglutination in any well of a serum test will constitute positive results. 2. A smooth suspension of red cells after resuspension of the cell button is a negative test result. 3. The interpretation of ABO tests is given in the table below:   **Routine ABO Grouping**   |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **Reaction of Red Cells**  **with Antisera**  **(Red Cell Grouping)** | | | **Reaction of Serum with Reagent Red Cells (Serum Grouping)** | | | | | | **Interpreta-tion** | | | | **Prevalence (%) in US Population** | | | | **Anti-A** | | **Anti-B** | | **A1 Cells** | | **B Cells** | | **O Cells** | | **ABO Group** | **European**  **Ethnicity** | | | **African Ethnicity** | | | | 0 | 0 | | + | | + | | 0 | | 0 | | | 45 | | | 49 | | | + | 0 | | 0 | | + | | 0 | | A | | | 40 | | | 27 | | | 0 | + | | + | | 0 | | 0 | | B | | | 11 | | | 20 | | | + | + | | 0 | | 0 | | 0 | | AB | | | 4 | | | 4 | | | 0 | 0 | | + | | + | | + | | Bombay\* | | | Rare | | | Rare | |   + = agglutination; 0 = no agglutination.  \*H null phenotype (see section on H antigen).   1. Any discrepancy between results on cell and serum or plasma tests should be resolved before an interpretation is recorded for the patient’s or donor’s ABO group. |

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| Notes | 1. Many manufacturers supply ABO or Rh typing reagents that are licensed by the Food and Drug Administration for use as undiluted reagents in microplate tests. 2. Microplates may be rigid or flexible, with either U-shaped or V-shaped bottoms. U-shaped-bottom plates are more widely used because results can be read either after centrifuging the plate and observing the characteristics of resuspended red cells or by observing the streaming pattern of the red cells when the plate is placed at an angle. Either reading technique permits estimation of the strength of agglutination. 3. To enhance weak serum or plasma reactions, the plates may be incubated at room temperature for 5 to 10 minutes; then the centrifugation, reading, and recording steps may be repeated. |

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| Reference | 1. Olsson ML, Westman JS. ABO and other carbohydrate blood group systems. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:297-327. |

**METHOD 2-4. INITIAL INVESTIGATION OF ABO GROUPING DISCREPANCIES PROCEDURE**

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| Principle | To be considered valid, the results of red cell grouping and serum grouping should agree. This method describes a general approach to the initial investigation of an ABO grouping discrepancy caused by either missing reactions or unexpected positive reactions. Detailed discussions on ABO grouping are found in Chapters 10 and 14 of the AABB *Technical Manual*, 20th edition. |

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| Procedure | **Step** | **Action** |
| 1 | Repeat ABO typing on the same sample. If initial tests were performed on red cells resuspended in serum or plasma, testing should be repeated after washing red cells several times with saline. This retest will eliminate many of the problems associated with plasma proteins or autoantibody. |
| 2 | Test a new sample. A new sample for testing should be requested when the ABO discrepancy reflects a disagreement between the current test results and a previous test result on record or when specimen contamination is suspected. |
| 3 | Review the patient’s medical history for medical conditions that could alter or interfere with ABO typing. This review can include the following:   1. Medical diagnosis. 2. Historical blood group. 3. Transfusion history. 4. Transplantation history. 5. Current medications. |
| 4 | Review the results of plasma testing against autologous red cells and group O red cells in the antibody screen to evaluate potential interference by autoantibodies or alloantibodies. A direct antiglobulin test (Method 3-14) may be helpful. |

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| Reference | 1. Olsson ML, Westman JS. ABO and other carbohydrate blood group systems. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:297-327. 2. Borge PD Jr, Mansfield PM. The positive direct antiglobulin test and immune-mediated hemolysis. In: Cohn C, Delaney M, Johnson S, Katz L,eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:429-55. |

**METHOD 2-5. DETECTING WEAK A AND B ANTIGENS AND ANTIBODIES BY COLD TEMPERATURE ENHANCEMENT**

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| Principle | Prolonged incubation at low temperatures can enhance antibody binding and detection of weak ABO antigens and antibodies. Because it is often unclear whether an ABO discrepancy is the consequence of weak antigens or of antibodies, testing both red cells and serum in parallel is recommended. |

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| Specimen | 1. Washed red cells to investigate missing red cell antigens. 2. Serum or plasma to investigate missing isoagglutinins. |

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| Reagents | 1. Monoclonal or polyclonal anti-A, anti-B, and anti-A,B. 2. A1, A2, B, and O reagent red cells (serum investigations). 3. 6% albumin. |

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| Procedure | **Step** | **Action** |
|  | 1 | Place 1 drop of anti-A in a clean, labeled test tube. |
|  | 2 | Place 1 drop of anti-B in a separate, clean, labeled test tube. |
|  | 3 | Place 1 drop of anti-A,B in a third clean, labeled test tube. |
|  | 4 | To each tube, add 1 drop of a 2% to 5% suspension (in saline, serum, or plasma) of the red cells to be tested. Alternatively, the equivalent amount of red cells can be transferred to each tube with clean applicator sticks. |
| 5 | Incubate all tubes for 30 minutes at room temperature. |
| 6 | Centrifuge tubes according to reagent manufacturer’s directions. |
| 7 | Gently resuspend cell buttons and examine for agglutination. |
| 8 | If no agglutination is observed, incubate tubes for 15 to 30 minutes at 4 C. |
| 9 | Centrifuge and examine for agglutination. |

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| Interpretation | 1. No interpretation can be made if the 6% albumin control for spontaneous agglutination is positive or if cold autoantibody or alloantibody is detected. 2. Additional information on resolving ABO discrepancies is found in Chapter 10 of the AABB *Technical Manual*, 20th edition. |

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| Notes | 1. The manufacturer’s directions for any reagent should be consulted regarding limitations of assay conditions for testing. 2. When testing a patient’s red cells, it is recommended to also incubate cells with 6% albumin as a control to detect spontaneous or autoagglutination. During testing of a patient’s plasma, group O reagent red cells should be included to detect cold autoantibody or alloantibody. |
| Reference | 1. Olsson ML, Westman JS. ABO and other carbohydrate blood group systems. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:397-327. |

**METHOD 2-6. CONFIRMING WEAK A AND B ANTIGENS USING ENZYME-TREATED RED CELLS**

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| Principle | Enzyme treatment of red cells can enhance antigen-antibody reactions to ABO and other carbohydrate antigens. |

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| Specimens | 1. Washed, untreated autologous red cells. 2. Washed, enzyme-treated (ficin, papain, or bromelin) autologous red cells. |

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| Reagents | 1. Monoclonal or polyclonal anti-A, anti-B, and anti-A,B. 2. Reagents for enzyme treatment of red cells (see methods for preparing ficin and papain enzyme stocks). 3. Group O, enzyme-treated red cell control. |

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| Procedure | **Step** | **Action** |
| 1 | Place 1 drop of anti-A in a clean, labeled test tube. |
|  | 2 | Place 1 drop of anti-B in a separate, clean, labeled test tube. |
|  | 3 | Place 1 drop of anti-A,B in a third clean, labeled test tube. |
|  | 4 | To each tube, add 1 drop of a 2% to 5% suspension (in saline, serum, or plasma) of the red cells to be tested. Alternatively, the equivalent amount of red cells can be transferred to each tube with clean applicator sticks. |
| 5 | Set up ABO tube test for enzyme-treated group O red cells as a control. |
| 6 | Incubate for 30 minutes at room temperature. |
| 7 | Centrifuge tubes according to reagent manufacturer’s directions. |
| 8 | Gently resuspend red cell buttons and examine for agglutination. |

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| Interpretation | Test results can be considered valid *only* if no reactivity is observed with the group O, enzyme-treated red cell control. Reactivity by anti-A, -B, or -A,B reagents with the enzyme­treated group O red cell control indicates excess enzyme treatment. No ABO interpretation can be made if the group O control is positive. |

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| Notes | 1. Red cell samples to be tested include untreated and enzyme-treated red cells. 2. When no agglutination is observed after 30 minutes at room temperature, a modified procedure using enzyme-treated red cells and 15 to 30 minutes of incubation at 4 C may be used to further enhance detection of weak A and B expression. |

**METHOD 2-7. CONFIRMING WEAK A OR B SUBGROUP BY ADSORPTION AND ELUTION**

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| Principle | Some weak ABO subgroups are too weak to be detected by direct agglutination, even after cold temperature and antibody enhancement. The presence of A antigens, B antigens, or both requires adsorbing anti-A or anti-B to red cells, followed by elution of bound antibody. The eluate is then evaluated for the presence of anti-A and anti-B by testing against A1 or B reagent red cells. |

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| Specimen | Red cells to be tested. |

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| Reagents | 1. Human anti-A and/or anti-B. Because some monoclonal ABO typing reagents are sensitive to changes in pH and osmolarity, the reagents may not be suitable for use in adsorption or elution tests. 2. Eluting agent: See heat elution and Lui freeze-thaw elution (Methods 4-3 and 4-4). 3. Group O red cell samples (three examples). 4. Group A1 or B red cell samples as appropriate (three examples). |

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| Procedure | **Step** | **Action** |
| 1 | Wash 1 mL of red cells to be tested at least three times with saline. Remove and discard supernatant saline after the last wash. |
| 2 | Add 1 mL of reagent anti-A (if weak A variant suspected) or 1 mL reagent anti-B (if weak B variant suspected) to washed red cells. |
| 3 | Mix red cells with antibody, and incubate at 4 C for 1 hour, mixing occasionally. |
| 4 | Centrifuge mixture to pack the red cells. Remove all supernatant reagent. |
| 5 | Transfer red cells to a clean test tube. |
| 6 | Wash red cells at least eight times with large volumes (10 mL or more) of cold (4 C) saline. Save an aliquot of the final wash supernatant fluid, and test it in parallel with the eluate. |
| 7 | Use an elution method suitable for recovery of ABO antibodies [eg, heat (Method 4-3) or Lui freeze-thaw elution methods (Method 4-4)]. |
| 8 | Test the eluate and the final wash solution (from Step 6), in parallel, against three examples of group O red cells and three examples of red cells of the appropriate ABO type (A1 or B cells). Add 2 drops of eluate or wash to 1 drop of red cells, and examine for agglutination after immediate centrifugation. |
| 9 | If no agglutination is observed after centrifugation, incubate for 15 to 30 minutes at room temperature and centrifuge. |
| 10 | If no agglutination is observed after room-temperature incubation, incubate at 37 C (15 minutes) and perform an indirect antiglobulin test. |

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| Interpretation | 1. The presence of anti-A or anti-B in the eluate indicates the presence of A or B antigen on test red cells. The results of the eluate are valid only if the following occur: 2. The eluate is reactive with all three antigen-positive cells, at any phase. 3. The eluate is nonreactive with all three group O red cells. 4. The final wash solution is nonreactive with all six cells tested. 5. Nonreaction of the eluate with antigen-positive red cells may indicate that the test red cells did not express A or B antigen. Alternatively, the lack of reaction could reflect failure to perform the eluate correctly. 6. Reactivity in the eluate with some or all of both antigen-positive and group O red cells indicates recovery of some other or additional antibody in the process. 7. If the wash solution is reactive with antigen-positive red cells, the eluate results are invalid. The reaction can occur if unbound reagent antibody was not adequately removed by washing before the elution step or by dissociation of bound antibody during the wash process. 8. Adsorption and elution of A1, B, or O red cells, or all three, can be performed and tested in parallel as positive and negative controls. |

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| Reference | 1. Beattie KM. Identifying the cause of weak or “missing” antigens in ABO grouping tests. In: The investigation of typing and compatibility problems caused by red blood cells. Washington, DC: AABB, 1975:15-37. |

**METHOD 2-8. TESTING SALIVA FOR A, B, H, Lea, AND Leb ANTIGENS**

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| Principle | Approximately 78% of all individuals possess the Se gene that governs the secretion of water-soluble ABH antigens into all body fluids except cerebrospinal fluid. Such secreted antigens can be demonstrated in saliva by inhibition tests with ABH and Lewis antisera. The importance of testing for ABO, H, and Lewis antigens is described in Chapter 10 of the AABB *Technical Manual*, 20th edition. |

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| Specimen | 1. Collect 5 to 10 mL of saliva in a small beaker or wide-mouth test tube. Most individuals can accumulate this amount in several minutes. To encourage salivation, the person may chew wax, paraffin, or a clean rubber band, but not gum or anything else that contains sugar or protein. 2. Centrifuge saliva at 900 to 1000 × *g* for 8 to 10 minutes. 3. Transfer the supernate to a clean test tube, and place it in a boiling waterbath for 8 to 10 minutes to inactivate salivary enzymes. 4. Recentrifuge at 900 to 1000 × *g* for 8 to 10 minutes, remove clear or slightly opalescent supernatant fluid, and discard the opaque or semisolid material. Dilute the supernatant fluid with an equal volume of saline. 5. Refrigerate, if testing is to be done within several hours. If testing will not be done on the day of collection, freeze the sample and store it at –20 C. Frozen samples retain activity for several years. |

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| Reagents | 1. Human (polyclonal) anti-A and anti-B. 2. Anti-H lectin from *Ulex europaeus* obtained commercially or prepared by saline extraction of *U. europaeus* seeds. 3. Polyclonal (rabbit, goat, or human) anti-Lea. There are no published data on the suitability of monoclonal Lewis antibodies. 4. A1 and B red cells, as used in Method 2-2. 5. Group O, Le(a+b–) red cells. 6. Frozen or fresh saliva from persons known to be secretors or nonsecretors, to use as positive and negative controls. (See Notes.) |

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| Procedure | **Step** | **Action** |
|  | *Selection of Blood Grouping Reagent Dilution* |
| 1 | Prepare doubling dilutions of the appropriate blood typing reagent: anti-A, anti-B, anti-H to determine ABH secretor status, or anti-Lea to determine secretor status (see Method 3-15). |
| 2 | To 1 drop of each reagent dilution, add 1 drop of 2% to 5% saline suspension of red cells [A, B, O, or Le(a+) as appropriate]. |
| 3 | Centrifuge each tube and examine macroscopically for agglutination. |
| 4 | Select the highest reagent dilution that gives 2+ agglutination. |
|  | *Inhibition Test for Secretor Status* |
| 1 | Add 1 drop of appropriately diluted blood grouping reagent to each of four tubes. For ABH studies, the tubes should be labeled “Secretor,” “Nonsecretor,” “Saline,” and “Unknown.” For Lewis studies, the labels will be “Lewis positive,” “Lewis negative,” “Saline,” and “Unknown.” |
| 2 | Add 1 drop of the appropriate saliva to each of the “Secretor,” “Nonsecretor,” and “Unknown” tubes, and 1 drop of saline to the tube marked “Saline.” |
| 3 | Mix the contents of the tubes. Incubate the tubes for 8 to 10 minutes at room temperature. |
| 4 | Add 1 drop of 2% to 5% saline suspension of washed indicator red cells to each tube [A, B, O, or Le(a+), as appropriate]. |
| 5 | Mix the contents of the tubes. Incubate the tubes for 30 to 60 minutes at room temperature. |
| 6 | Centrifuge each tube and inspect each red cell button macroscopically for agglutination. |

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| Interpretation | 1. Agglutination of indicator red cells by antibody in tubes containing saliva indicates that the saliva does not contain the corresponding antigen. 2. The failure of known antibody to agglutinate indicator red cells after incubation with saliva indicates that the saliva contains the corresponding antigen. 3. The failure of antibody in the saline control tube to agglutinate indicator red cells invalidates the results of saliva tests; the failure usually reflects use of reagents that are too dilute. Redetermine the appropriate reagent dilution, as described earlier, and repeat the testing. 4. For further interpretation, see table below:  |  |  |  |  |  | | --- | --- | --- | --- | --- | | Interpretation of Saliva Testing | | | | | | Testing with Anti-H | | | | | | Unknown Saliva | *Se* Saliva (H Substance Present) | Non-*Se* Saliva (H Substance  Not Present) | Saline (Dilution  Control) | Interpretation | | 2+ | 0 | 2+ | 2+ | Nonsecretor of H | | 0 | 0 | 2+ | 2+ | Secretor of H | | Testing with Anti-Lea | | | | | | Unknown Saliva | Le-Positive  Saliva | Le-Negative  Saliva | Saline (Dilution  Control) | Interpretation | | 2+ | 0 | 2+ | 2+ | Lewis-negative | | 0 | 0 | 2+ | 2+ | Lewis-positive\* | | \*A Lewis-positive person shown to be a secretor of ABH can be assumed to have Leb as well as Lea in saliva. Le(a+) persons who are *sese* and do not secrete ABH substance will have only Lea in saliva. | | | | | |

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| Notes | 1. For ABH status, use saliva from previously tested *Se* and *sese* persons. For Lewis testing, use saliva from a person whose red cells are Le(a+b–) or Le(a–b+) as the positive control; use saliva from a Le(a–b–) person as the negative control. Aliquots of saliva from persons of known secretor status may be frozen for later use. 2. This screening procedure can be adapted for the semiquantitation of blood group activity by testing serial saline dilutions of saliva. The higher the dilution needed to remove inhibitory activity, the more blood group substance is present in the saliva. Saliva should be diluted before it is incubated with antibody. To detect or to measure salivary A or B substance in addition to H substance, the same procedure can be used with diluted anti-A and anti-B reagents. The appropriate dilution of anti-A or anti-B is obtained by titrating the reagent against A1 or B red cells, respectively. 3. A Lewis-positive person shown to be a secretor of A, B, and H can be assumed to have Leb as well as Lea in his or her saliva. A Le(a+) person who does not secrete A, B, or H substances lacks the Se gene and will have only Lea in his or her saliva. |

**METHOD 2-9. CONFIRMING ANTI-A1 IN AN A2 OR WEAK A SUBGROUP**

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| Principle | A2 and weak A subgroups can possess an anti-A1 in serum or plasma, which will react with A1 reagent cells during reverse or serum grouping. Anti-A1 is a common cause of ABO discrepancies in A2 and weak A subgroups. |

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| Specimen | Red cells and serum or plasma to be evaluated. |

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| Reagents | 1. *Dolichos biflorus* lectin (anti-A1). 2. Group A1, A2, and O control red cells. |

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| Procedure | **Step** | **Action** |
|  | *Testing Red Cells* |
| 1 | Add 1 drop of A1 lectin to each test and control tube. |
| 2 | Add 1 drop of 2% to 5% saline suspension of red cells (Method 1-7) to the appropriate tubes. |
| 3 | Centrifuge 15 seconds. |
| 4 | Examine and record agglutination. |
|  | *Testing Serum/Plasma (see Methods 2-2 and 2-3)* |
| 1 | Serum should be tested against several examples (eg, two each) of A1, A2, and O red cells using established serum grouping methods. |

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| Interpretation | 1. The lectin should strongly agglutinate A1 red cells (3+ to 4+) but should not agglutinate A2 or O red cells. Group A red cells that fail to agglutinate with the lectin can be considered A2 or another weak A subgroup. 2. Anti-A1 in the patient’s serum will agglutinate all A1 samples. Anti-A1 will not agglutinate autologous, A2, or group O red cells. If the patient’s serum agglutinates group A2 or group O red cells, another cause for unexpected reactivity should be investigated. 3. If commercial lectin preparations are used, the manufacturer’s directions should be followed for appropriate testing method and controls. |

**METHOD 2-10. RESOLVING ABO DISCREPANCIES CAUSED BY UNEXPECTED ALLOANTIBODIES**

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| Principle | Some alloantibodies (eg, anti-P1 and anti-M) are reactive at room temperature. Unexpected positive reactions resulting in an ABO discrepancy can occur if A1 or B reagent red cells, or both, used for serum grouping are positive for the antigen. |

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| Procedure | **Step** | **Action** |
| 1 | Test the patient’s serum or plasma with antibody detection red cells at room temperature. If a cold-reactive alloantibody is identified, phenotype reagent A1 and B red cells for the presence of the antigen if the information is not available from the manufacturer. |
| 2 | Test serum or plasma against A1 and B red cells lacking the specific antigen of interest. |
| 3 | If the antibody detection test result is negative at room temperature, the patient may possess an alloantibody to a low-prevalence antigen present on A1 or B reagent red cells. Retest the serum or plasma with other randomly selected A1 and B red cell samples. |

**METHOD 2-11. DETERMINING SERUM GROUP WITHOUT CENTRIFUGATION**

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| Principle | Strongly reactive cold autoantibodies, such as anti-I and anti-IH, can agglutinate adult red cells, including reagent red cells, at room temperature. With few exceptions, agglutination by these cold agglutinins is weaker than that caused by anti-A and anti-B. One method to identify anti-A and anti-B in the presence of cold antibodies is by using a “settled reading.” |

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| Specimen | Serum or plasma to be evaluated. |

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| Reagents | A1, B, and O reagent red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Warm serum and reagent red cells to 37 C. |
| 2 | Add 2 to 3 drops serum to prelabeled (A1, B, O) clean test tubes. |
| 3 | Add 1 drop of the appropriate reagent red cells to each of the labeled tubes. |
| 4 | Mix contents and incubate at 37 C for 1 hour. |
| 5 | Remove and examine for agglutination. Do not centrifuge samples (settled reading). |

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| Notes | 1. Weak examples of anti-A and anti-B may not be detected by this method. 2. If the group O red cell control shows agglutination, no valid conclusion can be made regarding ABO type. |

**METHOD 2-12. DETERMINING Rh(D) TYPE—SLIDE TEST**

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| Principle | The Rh system is highly immunogenic and complex with numerous polymorphisms and clinically significant alleles. Because of the dire clinical consequences, pretransfusion Rh testing remains second only to ABO testing in importance. |

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| Specimen | Red cells from clotted or anticoagulated blood samples may be used. The red cells may be suspended in autologous serum, plasma, or saline or may be washed and resuspended in saline. Slide tests produce optimal results with a higher concentration of red cells than required for tube tests. |

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| Reagents | The reagent anti-D must specifically indicate that it is suitable for slide tests. The manufacturer’s instructions will indicate the type of reagent control to use. |

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| Cautions | Slide testing imposes a much greater risk of biohazard exposure. Personnel should follow safety measures detailed in the facility’s procedures manual. Evaporation of the reaction mixture can cause the red cells to aggregate and be misinterpreted as agglutination. Tests for weak D expression cannot be performed by slide testing. |

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| Procedure | **Step** | **Action** |
| 1 | Prewarm the glass slides on an Rh viewbox to 40 to 50 C before testing. |
| 2 | Place 1 drop of anti-D onto a clean, labeled slide. |
| 3 | Place 1 drop of the appropriate control reagent, if needed, onto a second labeled slide. Follow the manufacturer’s instructions for the reagent control. For tests using a low-protein anti-D, a negative result on slide testing with anti-A or anti-B serves as the control reaction. |
| 4 | To each slide, add 2 drops of a well-mixed 40% to 50% suspension of red cells in serum or plasma. |
| 5 | Use a clean applicator stick to thoroughly mix the cell suspension and reagent over an area approximately 20 mm × 40 mm. |
| 6 | Place the slide(s) on the viewbox and tilt it gently while continuously observing for agglutination. Inspect for macroscopic agglutination and read within 2 minutes. Do not mistake drying of the reaction mixture or rouleaux for agglutination. |
| 7 | Interpret and record the results. |

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| Interpretation | 1. Agglutination with anti-D and no agglutination on the control slide constitute a positive test result and indicate that the red cells are D positive. 2. No agglutination with either anti-D or the control suggests the red cells are D negative. Testing by an indirect antiglobulin procedure (IAT; see Method 2-15) will detect weak expression of D not detected on slide testing. 3. If agglutination is observed on the control slide, results of anti-D testing must not be interpreted as positive without further testing. 4. Drying around the edges of the mixture must not be confused with agglutination. |
| Reference | 1. Peyrard T, Wagner FF. The Rh system. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:329-54. |

**METHOD 2-13. DETERMINING Rh(D) TYPE—TUBE TEST**

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| Principle | The Rh system is highly immunogenic and complex with numerous polymorphisms and clinically significant alleles. Because of the dire clinical consequences, pretransfusion Rh testing remains second only to ABO testing in importance. |

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| Specimen | Red cells from clotted or anticoagulated blood samples may be used. The red cells may be suspended in autologous serum, plasma, or saline or may be washed and resuspended in saline. |

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| Reagents | Suitable reagents include low-protein monoclonal reagents and high-protein polyclonal reagents. The manufacturer’s instructions will indicate the type of reagent control to use. |

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| Procedure | **Step** | **Action** |
| 1 | Place 1 drop of anti-D into a clean, labeled test tube. Note: The addition of the reagent to the tube *before* the addition of the red cell suspension acts as a visual check for the presence of the anti-D to eliminate false-negative reactions caused by failure to add the reagent. |
| 2 | Place 1 drop of the appropriate control reagent to a second labeled tube. |
| 3 | Add 1 drop of a 2% to 5% suspension of the red cells in saline, serum, or plasma. Alternatively, an equivalent amount of red cells to be tested can be transferred to each tube with a clean applicator stick. |
| 4 | Mix gently and centrifuge for the time, and at the speed, specified by the manufacturer. |
| 5 | Gently resuspend the red cell button and examine for agglutination. If red cells were added by transfer with an applicator stick, addition of 1 drop of saline will aid resuspension of the cell button. |
| 6 | Grade reactions and record the test and control results. |

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| Interpretation | 1. Agglutination in the anti-D tube, combined with a smooth suspension in the control tube, indicates that the red cells are D positive. 2. No agglutination of the red cells in both the anti-D and the control tubes is a negative test result. A sample from a patient may be designated as D negative at this point. AABB *Standards for Blood Banks and Transfusion Services* requires donor blood and infants of mothers being evaluated for Rh Immune Globulin to be tested further for the presence of weak D antigen. 3. A negative tube test result with anti-A and/or anti-B is a valid negative control if a low-protein anti-D reagent is used for testing. 4. Agglutination in the control tube invalidates the test. Methods for removal of IgM or IgG antibody from the red cells may be required. See Methods 2-17 through 2-21. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:33,51. 2. Peyrard T, Wagner FF. The Rh system. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:329-54. |

**METHOD 2-14. DETERMINING Rh(D) TYPE—MICROPLATE TEST**

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| Principle | The Rh system is highly immunogenic and complex with numerous polymorphisms and clinically significant alleles. Because of the dire clinical consequences, pretransfusion Rh testing remains second only to ABO testing in importance. |

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| Specimen | Follow the manufacturer’s instructions. Automated methods may require the use of samples drawn into a specific anticoagulant. |

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| Reagents | Use only anti-D reagents approved for use in microplate tests. Consult the manufacturer’s instructions for specific reagents, equipment, and proper controls. |

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| Procedure | **Step** | **Action** |
| 1 | Place 1 drop of anti-D reagent into a clean well of the microplate. If the reagent requires use of an Rh control, add 1 drop of the control to a second well. |
| 2 | Add 1 drop of a 2% to 5% saline suspension of red cells to each well. |
| 3 | Mix the contents by gently tapping the sides of the plate. |
| 4 | Centrifuge the plate at the appropriate conditions according to the manufac­turer’s instructions. |
| 5 | Resuspend the cell buttons by manually tapping the plate or with the aid of a microplate shaker, or place the plate at an angle for the tilt-and-stream method. |
| 6 | Examine for agglutination, read, interpret, and record the results. |
| 7 | To enhance weak reactions, incubate tests with negative results at 37 C for 15 to 30 minutes and repeat Steps 4 to 6. |

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| Interpretation | 1. Agglutination in the anti-D well, combined with a smooth suspension in the control well, indicates that the red cells are D positive. 2. No agglutination of the red cells in both the anti-D and the control wells is a negative test result. A sample from a patient may be designated as D negative at this point. AABB *Standards for Blood Banks and Transfusion Services* requires donor blood and infants of mothers being evaluated for Rh Immune Globulin to be tested further for the presence of weak D antigen. 3. A negative test result with anti-A and/or anti-B is a valid negative control if a low-protein anti-D reagent is used for testing. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:33,51. 2. Peyrard T, Wagner FF. The Rh system. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:329-54. |  |

**METHOD 2-15. TESTING FOR WEAK D**

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| Principle | Some weak D antigens are recognized only by an indirect antiglobulin test (IAT) procedure. AABB *Standards for Blood Banks and Transfusion Services* requires detection of weak D when typing donor units, but this is not required for pretransfusion testing of patients’ samples. |

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| Specimen | Follow the manufacturer’s instructions. |

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| Reagents | 1. Antihuman globulin reagent, either polyspecific or anti-IgG. 2. IgG-coated control cells. |

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| Procedure | **Step** | **Action** |
| 1 | If a tube test was performed for the direct anti-D test, the same tube may be used for the weak D test if an appropriate reagent was used. Go to step 5. |
| 2 | Place 1 drop of anti-D in a clean, labeled test tube. |
| 3 | Place 1 drop of the appropriate control reagent in a second, labeled test tube. |
| 4 | To each tube, add 1 drop of 2% to 5% saline-suspended red cells. |
| 5 | Mix and incubate the test and control tubes according to the reagent manufacturer’s directions. This is typically 15 to 30 minutes at 37 C. |
| 6 | If desired, centrifuge and read after incubation by gently resuspending the red cell button, and examine for agglutination. |
| 7 | Wash the red cells at least three times with saline. |
| 8 | Add antiglobulin reagent according to the manufacturer’s directions. |
| 9 | Mix gently and centrifuge according to the calibrated spin time for the centrifuge. |
| 10 | Gently resuspend and examine for agglutination, grade, and record. |
| 11 | Add IgG-coated control cells to confirm the validity of negative antiglobulin test results. |

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| Interpretation | 1. Agglutination in the anti-D tube, combined with a smooth suspension in the control tube, indicates that the red cells are D positive. It is incorrect to report the results as “weak D positive” or “D negative, weak Dpositive.” 2. No agglutination of the red cells in both the anti-D and the control tubes is a negative test result. 3. It is permissible to use a direct antiglobulin test on the test cells as a control, but an IAT procedure with an Rh or albumin control reagent is preferable because this ensures that all reagent components that might cause a false-positive result are represented. 4. Agglutination at any phase in the control tube invalidates the test, and no interpretation can be made. IgG removal from the red cells may be helpful (see Methods 2-20 and 2-21). |

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| Note | Not every anti-D reagent is suitable for the weak D test. Consult the manufacturer’s package insert for test procedures and appropriate controls. |

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| Reference | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:33,51. |

**METHOD 2-16. PREPARING AND USING LECTINS**

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| Principle | Saline extracts of seeds react with specific carbohydrates on red cell membranes and make useful typing reagents that are highly specific at appropriate dilutions. |
| Reagents | Seeds may be obtained from health-food stores, pharmacies, or commercial seed companies. The seeds should be raw. |

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| Procedure | **Step** | **Action** |
| 1 | Grind the seeds in a food processor or blender until the particles look like coarse sand. A mortar and pestle may be used, or seeds can be used whole. |
| 2 | In a large test tube or small beaker, place ground seeds and three to four times their volume of saline. (Seeds vary in the quan­tity of saline they absorb.) |
| 3 | Incubate at room temperature for 4 to 12 hours, stirring or inverting occasionally. |
| 4 | Transfer supernatant fluid to a centrifuge tube, and centrifuge for 5 minutes to obtain a clear supernatant. Collect and filter the supernatant fluid, and discard the seed residue. |
| 5 | Test dilutions of the extract to find the dilution for the desired activity. Deter­mine the activity of the extract with the appropriate red cells, as below. |
|  | *For Dolichos biflorus:*   1. Add 1 drop of 2% to 5% saline suspension of known A1, A2, A1B, A2B, B, and O red cells to appropriately labeled tubes. 2. Add 1 drop of the extract to each tube. 3. Centrifuge for calibrated time. 4. Inspect for agglutination and record results. 5. The lectin should agglutinate A1 and A1B red cells but not A2, A2B, B, or O red cells. The native extract often agglutinates all the red cells tested. To make the product useful for reagent purposes, add enough saline to the extract so that there is 3+ or 4+ agglutination of A1 and A1B red cells, but not of A2, A2B, B, or O red cells. |
|  | *For Ulex europaeus:*   1. Add 1 drop of 2% to 5% saline suspension of known A1, A2, A1B, B, and O red cells to appropriately labeled tubes. 2. Add 1 drop of extract to each tube. 3. Centrifuge for the calibrated time. 4. Inspect for agglutination and record results. 5. The strength of the agglutination should be in the order of O>A2>B>A1>A1B. 6. Dilute extract with saline, if necessary, to a point that O red cells show 3+ or 4+ agglutination, A2 and B red cells show 1+ to 2+ agglutination, and A1 or A1B red cells are not agglutinated. |

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| Interpretation | 1. If commercially made lectins are used, follow the manufacturer’s instructions. 2. The anticipated reactions with various types of polyagglutinable red cells are shown in the table below:  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | | Reactions between Lectins and Polyagglutinable Red Cells | | | | | | |  | T | Th | Tk | Tn | Cad | | *Arachis hypogaea\** | + | + | + | 0 | 0 | | *Dolichos biflorus*† | 0 | 0 | 0 | + | + | | *Glycine max* (soja) | + | 0 | 0 | + | + | | *Salvia sclarea* | 0 | 0 | 0 | + | 0 | | *Salvia horminum* | 0 | 0 | 0 | + | + | | \*T and Th cells give weaker reactions with *Arachis* after protease treatment; Tk reactivity is enhanced after protease treatment.  †A and AB cells may be reactive because of anti-A reactivity of *Dolichos* lectin. | | | | | | |

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| Notes | 1. Diluted extract of *Dolichos biflorus* agglutinates A1 red cells but not A2. *Ulex europaeus* extract reacts with the H determinant; it agglutinates in a manner proportional to the amount of H present (O>A2>B>A1>A1B red cells). 2. Other lectins useful for special purposes include *Arachis hypogaea* (anti-T), *Glycine max* (anti-T, -Tn), *Vicia graminea* (anti-N), and the *Salvia* lectins (*S. horminum*, anti-Tn/Cad; *S. sclarea*, anti-Tn). 3. To investigate red cell polyagglutination, prepare and test the red cells with *Arachis*, *Glycine, Salvia*, and *Dolichos* lectins. 4. To facilitate grinding hard seeds, one should cover the seeds with saline and soak them for several hours before grinding. The container used for soaking should not be tightly closed because some beans release gas during the soaking process, which could cause the container to explode. 5. The saline extracts may be stored in the refrigerator for several days; they may be stored indefinitely if frozen. 6. Tests should include a positive and negative control. |

**METHOD 2-17. REMOVING AUTOANTIBODY BY WARM SALINE WASHES**

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| Principle | Red cells heavily coated with autoantibodies can spontaneously agglutinate or autoagglutinate and lead to false-positive reactions with anti-A, -B, and -D. Washing red cells with warm saline will often remove sufficient autoantibody to allow determination of ABO and Rh type. |

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| Specimen | Red cells with spontaneous agglutination or autoagglutination interfering with red cell antigen typing. |

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| Reagents | 1. Warm isotonic saline. 2. Monoclonal or polyclonal anti-A and anti-B. 3. Control reagent such as 6% albumin. |

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| Procedure | **Step** | **Action** |
| 1 | Warm red cell suspension to 37 C for 15 minutes to 1 hour. |
| 2 | Wash cells with warm (37 C) saline several times to remove autoantibody. |
| 3 | Type washed red cells with anti-A, anti-B, and anti-D and 6% albumin as described in methods for determining ABO group (Methods 2-1 to 2-3). If the control is still positive, see methods for dissociating antibody molecules from the red cells (Methods 2-18 through 2-21). |

**METHOD 2-18. USING SULFHYDRYL REAGENTS TO DISPERSE AUTOAGGLUTINATION**

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| Principle | Red cells heavily coated with IgM autoantibodies can spontaneously agglutinate during centrifugation, leading to false-positive reactions in red cell typing and direct antiglobulin (DAT) tests. Dithiothreitol (DTT) or 2-mercaptoethanol (2-ME) can reduce the disulfide bonds of IgM molecules, decreasing their polyvalency and ability to directly agglutinate red cells. |

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| Specimen | Red cells with IgM autoagglutination interfering with red cell antigen typing. |

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| Reagents | 1. 0.01 M DTT: 0.154 g of DTT dissolved in 100 mL of phosphate-buffered saline (PBS) at pH 7.3; store at 4 C. 2. 0.1 M stock 2-ME: 0.7 mL of a 14 M stock solution of 2-ME diluted in 100 mL of PBS at pH 7.3; 2-ME should be stored in a dark glass container at 4 C. 3. PBS at pH 7.3. 4. Antigen-positive control red cells for antigen typing to be treated in parallel. 5. Red cell typing antisera. 6. 6% albumin (control reagent). |

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| Procedure | **Step** | **Action** |
| 1 | Wash red cells three times with saline, and dilute to a 50% concentration in PBS. |
| 2 | Add an equal volume of 0.01 M DTT in PBS or 0.1 M 2-ME in PBS to the red cell suspension. |
| 3 | Incubate at 37 C for 15 minutes (DTT) or 10 minutes (2-ME). |
| 4 | Wash red cells three times in saline, and dilute to 2% to 5% suspension in saline. |
| 5 | Test the treated cells with 6% albumin (immediate-spin test) to make sure the cells do not spontaneously agglutinate. If the test result is negative, the red cells are now ready for use in red cell typing tests. |

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| Notes | 1. Treated red cells should not agglutinate in 6% albumin. 2. Antigen-positive control red cells should be strongly and equally reactive with typing reagent before and after treatment. 3. This procedure is normally used only for ABO forward typing, Rh determination, and the DAT. 4. KEL system antigens may be weakened or destroyed by DTT and 2-ME treatment. Jsa and Jsb may be more sensitive than other KEL antigens to this concentration of DTT. |

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| Reference | 1. Judd WJ, Johnson S, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 2-19. USING GENTLE HEAT ELUTION TO TEST RED CELLS WITH A POSITIVE DAT RESULT**

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| Principle | Red cells that are heavily coated with IgG may spontaneously agglutinate in high-protein reagents and will cause false-positive antihuman globulin (AHG) test results. For red cell antigen typing, it may be necessary to dissociate antibody from the cells by elution without damaging membrane integrity or altering antigen expression. The gentle heat elution procedure used to prepare immunoglobulin-free red cells differs from procedures that are intended to recover active antibody. |

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| Specimen | Test red cells with a positive direct antiglobulin test (DAT) result. |

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| Reagents | AHG. |

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| Procedure | **Step** | **Action** |
| 1 | Place one volume of washed antibody-coated red cells and three volumes of normal saline in a test tube of appropriate size. In another tube, place the same volumes of saline and washed red cells positive for the antigen under test. This step will provide a check that the elution technique does not destroy the antigen reactivity. |
| 2 | Incubate the contents of both tubes at approximately 45 C for 10 to 15 minutes. The tubes should be agitated frequently. The time of incubation should be roughly proportional to the degree of antibody coating, as indicated by the strength of antiglobulin reactivity. |
| 3 | Centrifuge the tubes and discard the supernatant saline. |
| 4 | Test the red cells for the degree of antibody removal by comparing results of a DAT on the treated red cells with the DAT on untreated red cells. If the antibody coating is reduced but still present, Steps 1 through 3 can be repeated; the control red cells should be subjected to a similar second treatment. |
| 5 | Test the treated red cells for the desired antigen. |

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| Notes | 1. This procedure may be unnecessary if IgM monoclonal reagents are available; such reagents cause direct agglutination and are not usually affected by bound immunoglobulin. 2. As with untreated patients’ red cells, results of antigen testing in recently transfused patients should be interpreted with caution because of the potential presence of donor red cells. |

**METHOD 2-20. DISSOCIATING IgG BY CHLOROQUINE FOR ANTIGEN TESTING OF RED CELLS WITH A POSITIVE DAT RESULT**

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| Principle | Red cells giving a positive direct antiglobulin test (DAT) result cannot be tested accurately with blood typing reagents that require an indirect antiglobulin technique. Under controlled conditions, chloroquine diphosphate dissociates IgG from the red cell membrane with little or no damage to its integrity. Use of this procedure permits complete phenotyping of red cells coated with warm-reactive autoantibody, including tests with reagents solely reactive by indirect antiglobulin techniques. |

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| Specimen | Red cells with a positive DAT resulting from IgG coating. |

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| Reagents | 1. Chloroquine diphosphate solution prepared by dissolving 20 g of chloroquine diphosphate in 100 mL of saline. Adjust to pH 5.1 with 1 N NaOH, and store at 2 to 8 C. 2. Control red cells carrying a single-dose expression of antigens for which the test samples are to be phenotyped. 3. Anti-IgG antiglobulin reagent. |

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| Procedure | **Step** | **Action** |
| 1 | To 0.2 mL of washed IgG-coated cells, add 0.8 mL of chloroquine diphosphate solution. Similarly treat the control sample. |
| 2 | Mix and incubate at room temperature for 30 minutes. |
| 3 | Remove a small aliquot (eg, 1 drop) of the treated test cells and wash them four times with saline. |
| 4 | Test the washed cells with anti-IgG. |
| 5 | If this treatment has rendered the cells nonreactive with anti-IgG, wash the total volume of treated test cells and control cells three times in saline and make a 2% to 5% suspension in saline to use in subsequent blood typing tests. |
| 6 | If the treated red cells are reactive with anti-IgG after 30 minutes of incubation with chloroquine diphosphate, Steps 3 and 4 should be repeated at 30-minute intervals (for a maximum incubation period of 2 hours), until the sample tested is nonreactive with anti-IgG. Then proceed as described in Step 5. |

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| Notes | 1. Chloroquine diphosphate does not dissociate complement proteins from the cell membrane. If red cells are coated with both IgG and C3, only anti-IgG should be used in tests performed after chloroquine treatment. |
|  | 1. Incubation with chloroquine diphosphate should not extend beyond 2 hours. Prolonged incubation at room temperature or incubation at 37 C may cause hemolysis and loss of red cell antigens. |
|  | 1. Some denaturation of Rh antigens may occur. |
|  | 1. Many serologists test chloroquine-treated control cells for each antigen of interest. Select control cells that are positive for the antigen corresponding to the antisera that will be used to type the patient’s cells. |
|  | 1. Chloroquine diphosphate may not completely remove antibody from sensitized red cells. DAT results on red cells from some persons, particularly those with a strongly positive initial test result, may only be diminished in strength. |
|  | 1. In addition to its use for removal of autoantibodies, this method can be used for removal of Bg (HLA)-related antigens from red cells. Appropriate Bg controls should be used. |
|  | 1. If a commercial kit is used, manufacturer’s instructions should be followed for testing and controls. |

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| References | 1. Edwards JM, Moulds JJ, Judd WJ. Chloroquine dissociation of antigen-antibody complexes: A new technique for phenotyping red blood cells with a positive direct antiglobulin test. Transfusion 1982;22:59-61. 2. Swanson JL, Sastamoinen R. Chloroquine stripping of HLA-A,B antigens from red cells (letter). Transfusion 1985;25:439-40. |

**METHOD 2-21. USING ACID GLYCINE/EDTA TO REMOVE ANTIBODIES FROM RED CELLS**

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| Principle | Acid glycine/EDTA can be used to dissociate antibody molecules from red cell membranes. The procedure is routinely used for blood typing tests or adsorption procedures. All common red cell antigens can be detected after treatment with acid glycine/EDTA except antigens of the KEL system, Bg antigens, and Er antigens. Thus, red cells treated in this manner cannot be used to determine these phenotypes. |

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| Specimen | Red cells giving a positive direct antiglobulin test (DAT) result. |

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| Reagents | 1. 10% EDTA prepared by dissolving 2 g of disodium ethylenediamine tetraacetic acid (Na2EDTA) in 20 mL of distilled or deionized water. 2. 0.1 M glycine-HCl buffer (pH 1.5) prepared by diluting 0.75 g of glycine to 100 mL with isotonic (unbuffered) saline. Adjust the pH to 1.5 using concentrated HCl. 3. 1.0 M TRIS-NaCl prepared by dissolving 12.1 g of Tris(hydroxymethyl)aminomethane (TRIS) and 5.25 g of sodium chloride (NaCl) to 100 mL with distilled or deionized water. |

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| Procedure | **Step** | **Action** |
| 1 | Wash the red cells to be treated six times with isotonic saline. |
| 2 | In a test tube, mix together 20 volumes of 0.1 M acid glycine-HCl (pH 1.5) with five volumes of 10% EDTA. This mixture is the acid glycine/EDTA reagent. |
| 3 | Place 10 volumes of washed red cells in a clean tube. |
| 4 | Add 20 volumes of acid glycine/EDTA. |
| 5 | Mix the contents of the tube thoroughly. |
| 6 | Incubate the mixture at room temperature for no more than 2 to 3 minutes. |
| 7 | Add one volume of 1.0 M TRIS-NaCl, and mix the contents of the tube. |
| 8 | Centrifuge at 900 to 1000 × *g* for 1 to 2 minutes; then aspirate and discard the supernatant fluid. |
| 9 | Wash the red cells four times with saline. |
| 10 | Test the washed red cells with anti-IgG. If nonreactive with anti-IgG, the cells are ready for use in blood typing or adsorption procedures. If the DAT is still positive, one additional treatment can be performed. |

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| Notes | 1. Overincubation of red cells with acid glycine/EDTA causes irreversible damage to red cell membranes. |
|  | 1. Include a parallel control reagent, such as 6% bovine albumin or inert plasma, when typing treated red cells. |
|  | 1. Use anti-IgG, not a polyspecific antiglobulin reagent, in Step 10. |
|  | 1. Many serologists test acid glycine/EDTA-treated control red cells for each antigen of interest. Select control red cells that are positive for the antigen corresponding to the antisera that will be used to type the patient’s cells. |
|  | 1. If a commercial kit is used, manufacturer’s instructions should be followed for testing and controls. |

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| References | 1. Louie JE, Jiang AF, Zaroulis CG. Preparation of intact antibody-free red cells in autoimmune hemolytic anemia (abstract). Transfusion 1986;26:550. 2. Champagne K, Spruell P, Chen J, et al. EDTA/ glycine-acid vs. chloroquine diphosphate treatment for stripping Bg antigens from red blood cells (abstract). Transfusion 1996;36 (Suppl):21S. 3. Reid ME, Lomas-Francis C, Olsson M. The blood group antigen factsbook. 3rd ed. London, UK: Elsevier Academic Press, 2012. |

**METHOD 2-22. SEPARATING TRANSFUSED FROM AUTOLOGOUS RED CELLS BY SIMPLE CENTRIFUGATION**

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| Principle | Newly formed autologous red cells generally have a lower specific gravity than transfused red cells and will therefore concentrate at the top of the column of red cells when blood is centrifuged in a microhematocrit tube. This provides a simple method for recovering autologous red cells in a blood sample from recently transfused patients. |

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| Specimen | Red cells from whole blood collected into EDTA. |

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| Materials | 1. Microhematocrit centrifuge. 2. Plain (not heparinized) glass or plastic hematocrit tubes. 3. Sealant. |

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| Procedure | **Step** | **Action** |
| 1 | Wash the red cells three times in saline. For the last wash, centrifuge them at 900 to 1000 × *g* for 5 to 15 minutes. Remove as much of the supernatant fluid as possible without disturbing the buffy coat. Mix thoroughly. |
| 2 | Fill 10 microhematocrit tubes to the 60-mm mark with well-mixed washed red cells. |
| 3 | Seal the ends of the tubes by heat or with sealant. |
| 4 | Centrifuge all tubes in a microhematocrit centrifuge for 15 minutes. |
| 5 | Cut the microhematocrit tubes 5 mm below the top of the column of red cells. This 5-mm segment contains the least dense, hence youngest, circulating red cells. |
| 6 | Place the cut microhematocrit tubes into larger test tubes (10 or 12 × 75 mm), add saline, and mix well to flush the red cells from the microhematocrit tubes. Then, either 1) centrifuge them at 1000 × *g* for 1 minute and remove the empty hematocrit tubes or 2) transfer the saline-suspended red cells to a clean test tube. |
| 7 | Wash the separated red cells three times in saline before resuspending them to 2% to 5% in saline for testing. |

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| Notes | 1. Separation is better if the sample is obtained 3 or more days after transfusion rather than shortly after transfusion. 2. The red cells should be mixed continuously while the microhematocrit tubes are being filled. 3. Separation techniques are effective only if the patient is producing normal or above­normal numbers of reticulocytes. This method will be ineffective in patients with inadequate reticulocyte production. 4. Some red cell antigens may not be as strongly expressed on reticulocytes as on older cells. Particular attention should be given to determinations of the E, e, c, Fya, Jka, and Ge antigens. 5. Red cells from patients with hemoglobin S or spherocytic disorders are not effectively separated by this method (see Method 2-23 for an alternative procedure). |

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| References | 1. Reid ME, Toy P. Simplified method for recovery of autologous red blood cells from transfused patients. Am J Clin Pathol 1983;79:364-6. 2. Vengelen-Tyler V, Gonzales B. Reticulocyte rich RBCs will give weak reactions with many blood typing antisera (abstract). Transfusion 1985;25:476. |

**METHOD 2-23. SEPARATING TRANSFUSED FROM AUTOLOGOUS RED CELLS IN PATIENTS WITH HEMOGLOBIN S DISEASE**

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| Principle | Red cells from patients with sickle cell disease, either hemoglobin SS or SC, are resistant to lysis by hypotonic saline, in contrast to red cells from normal persons and those with hemoglobin S trait. This procedure permits isolation of autologous red cells from patients with hemoglobin SS or SC disease who have recently been transfused. |

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| Specimen | Red cells to be evaluated. |

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| Reagents | 1. Hypotonic saline (0.3% w/v NaCl): NaCl, 3 g; distilled water to 1 L. 2. Normal saline (0.9% w/v NaCl): NaCl, 9 g; distilled water to 1 L. |

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| Procedure | **Step** | **Action** |
| 1 | Place 4 or 5 drops of red cells into a 10 or 12 × 75-mm test tube. |
| 2 | Wash the cells six times with 0.3% NaCl, or until the supernatant fluid no longer contains grossly visible hemoglobin. For each wash, centrifuge at 1000 × *g* for 1 minute. |
| 3 | Wash the cells twice with 0.9% NaCl to restore tonicity. For each wash, centrifuge at 200 × *g* for 2 minutes to facilitate removal of residual stroma. |
| 4 | Resuspend the remaining intact red cells to a 2% to 5% concentration for phenotyping. |

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| Notes | 1. Larger volumes, for use in adsorption studies, can be processed in a 16 × 100-mm test tube. 2. When using the hypotonic saline technique, one must be careful to remove the stroma from the lysed cells because stroma can adsorb typing serum and produce false-negative typing results. |

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| Reference | 1. Brown D. A rapid method for harvesting autologous red cells from patients with hemoglobin S disease. Transfusion 1988;28:21-3. |

**ANTIBODY DETECTION, IDENTIFICATION, AND COMPATIBILITY TESTING METHODS**

Pretransfusion compatibility testing begins with the type and screen procedure. The recipient’s ABO group and Rh type are determined first; then a screening procedure is used to detect any unexpected non-ABO blood group antibodies that may be present.

If the screening test reveals the presence of an antibody, the specificity of that antibody is determined by an antibody identification panel. Once the specificity of the antibody has been identified, donor units of the appropriate ABO group and Rh type are screened for the corresponding antigen. Units that are negative for that antigen are crossmatched with the recipient to ensure compatibility.

**METHOD 3-1. USING IMMEDIATE-SPIN COMPATIBILITY TESTING TO DEMONSTRATE ABO INCOMPATIBILITY**

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| Principle | Compatibility testing is performed in order to prevent transfusion of incompatible donor red cells that might result in an immune-mediated hemolytic transfusion reaction. Details on the general principles of compatibility testing are found in Chapter 17 of the AABB *Technical Manual.* |

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| Specimen | Patient serum or plasma may be used. The age of the specimen must comply with the pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services.*1 |

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| Reagents | 1. Normal saline. 2. Donor red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare a 2% to 5% suspension of donor red cells in normal saline or EDTA saline.  Some serologists using serum for testing prefer to suspend the donor red cells in EDTA saline because high-titer anti-A or anti-B can initiate complement coating, which can cause steric hindrance of agglutination.2 The use of a patient’s sample collected in EDTA is an alternative approach to prevent this phenomenon. |
|  | 2 | Label a tube for each donor red cell suspension being tested with the patient’s serum. |
| 3 | Add 2 drops of the patient’s serum or plasma to each tube. |
| 4 | Add 1 drop of the suspension of donor red cells to the appropriate test tube. |
| 5 | Mix the contents of the tube(s) and centrifuge according to the calibration of the centrifuge. |
| 6 | Examine the tube(s) for hemolysis, gently resuspend the red cell button(s), and examine for agglutination. |
| 7 | Read, interpret, and record test results. |

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| Interpretation | 1. Agglutination or hemolysis constitutes a positive (incompatible) test result. 2. A smooth suspension of red cells after resuspension of the red cell button constitutes a negative result and indicates a compatible immediate-spin crossmatch. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Judd WJ, Steiner EA, O’Donnell DB, Oberman HA. Discrepancies in ABO typing due to prozone: How safe is the immediate-spin cross-match? Transfusion 1988;28:334-8. 3. Alquist CR, Harm SK. Transfusion-service-related activities: Pretransfusion testing and storage, monitoring, processing, distribution, and inventory management of blood components. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:503-35. |

**METHOD 3-2. SALINE INDIRECT ANTIGLOBULIN TEST PROCEDURE**

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| Principle | An indirect antiglobulin test (IAT) demonstrates in-vitro reactions between red cells and antibodies, and is used in antibody detection, antibody identification, crossmatching, and blood group phenotyping. |

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| Specimen | Serum or plasma may be used. The age of the specimen must comply with pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services*. |

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| Reagents | 1. Normal saline. 2. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 3. Group O antibody detection cells. Pooled group O antibody-detection cells may be used only for donor testing. Testing of patient samples must be performed with unpooled cells. 4. A 2% to 5% suspension of donor red cells in saline. 5. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Add 2 drops of serum or plasma to properly labeled tubes. |
| 2 | Add 1 drop of 2% to 5% saline-suspended reagent group O red cells or donor red cells to each tube and mix. |
| 3 | Centrifuge and observe for hemolysis and agglutination. Grade and record the results. |
| 4 | Incubate at 37 C for 30 to 60 minutes. |
| 5 | Centrifuge and observe for hemolysis and agglutination. Grade and record the results. |
| 6 | Wash the red cells three or four times with saline, and completely decant the final wash. |
| 7 | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. |
| 8 | Centrifuge and observe for agglutination. Grade and record the results. |
| 9 | Confirm the validity of negative results by adding IgG-coated red cells. |

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| Interpretation | 1. The presence of agglutination/hemolysis after incubation at 37 C constitutes a positive test result. 2. The presence of agglutination after addition of AHG constitutes a positive test result. 3. Antiglobulin test results are negative when no agglutination is observed after initial centrifugation followed by agglutination with the addition of IgG-coated red cells and centrifugation. If the IgG-coated red cells are not agglutinated, the negative result is invalid and the test must be repeated. |

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| Controls | 1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak examples of antibody. 2. Control sera can be prepared from reagent grade typing sera diluted with 6% bovine albumin to give 2+ reactions by an IAT. Human sources of IgG antibodies are also acceptable. |

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| Notes | 1. The incubation times and the volume and concentration of red cells indicated are those given in the literature. Individual laboratories may choose to standardize techniques with somewhat different values. Other limitations when modifying procedures are mentioned in Chapter 17 of the AABB *Technical Manual*. In all cases, the manufacturer’s package insert should be consulted before modifying a procedure. |
|  | 1. Step 3 may be omitted to avoid the detection of antibodies reactive at room temperature. |
|  | 1. Steps 6 through 9 should be performed without interruption. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Alquist CR, Harm SK. Transfusion-service-related activities: Pretransfusion testing and storage, monitoring, processing, distribution, and inventory management of blood components. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:503-35. |

**METHOD 3-3. ALBUMIN OR LISS-ADDITIVE INDIRECT ANTIGLOBULIN TEST PROCEDURE**

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| Principle | An indirect antiglobulion test (IAT) demonstrates in-vitro reactions between red cells and antibodies, and is used in antibody detection, antibody identification, crossmatching, and blood group phenotyping. The albumin method may reduce repulsive forces between cells and thus promote agglutination. Use of a LISS additive accelerates antibody binding to red cells. |

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| Specimen | Serum or plasma may be used. The age of the specimen must comply with pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services*. |

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| Reagents | 1. Bovine albumin (22%). 2. LISS, available commercially for this use. 3. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 4. Group O antibody detection cells. Pooled group O antibody-detection cells may be used only for donor testing. Testing of patient samples must be performed with unpooled cells. 5. A 2% to 5% suspension of donor red cells in saline. 6. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Add 2 drops of serum or plasma to properly labeled tubes. |
| 2 | Add an equivalent volume of 22% bovine albumin or LISS additive (unless the manufacturer’s directions state otherwise). |
| 3 | Add 1 drop of a 2% to 5% saline-suspended reagent or donor red cells to each tube and mix. |
| 4 | For albumin, incubate at 37 C for 30 to 60 minutes. For LISS, incubate for 10 to 15 minutes per the manufacturer’s directions. |
| 5 | Centrifuge and observe for hemolysis and agglutination. Grade and record the results. |
| 6 | Wash the red cells three or four times with saline, and completely decant the final wash. |
| 7 | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. |
| 8 | Centrifuge and observe for agglutination. Grade and record the results. |
| 9 | Confirm the validity of negative results by adding IgG-coated red cells. |

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| Interpretation | 1. The presence of agglutination/hemolysis after incubation at 37 C constitutes a positive test result. 2. The presence of agglutination after addition of AHG constitutes a positive test result. 3. Antiglobulin test results are negative when no agglutination is observed after initial centrifugation followed by agglutination with the addition of IgG-coated red cells and centrifugation. If the IgG-coated red cells are not agglutinated, the negative result is invalid and the test must be repeated. |

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| Controls | 1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak examples of antibody. 2. Control sera can be prepared from reagent-grade typing sera diluted with 6% bovine albumin to give 2+ reactions by an IAT. Human sources of IgG antibodies are also acceptable. |

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| Notes | 1. The incubation times and the volume and concentration of red cells indicated are those given in the literature. Individual laboratories may choose to standardize techniques with somewhat different values. In all cases, the manufacturer’s package insert should be consulted before modifying a procedure. 2. Steps 6 through 9 should be performed without interruption. |
| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Alquist CR, Harm SK. Transfusion-service-related activities: Pretransfusion testing and storage, monitoring, processing, distribution, and inventory management of blood components. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:305-35. |

**METHOD 3-4. LISS-SUSPENSION INDIRECT ANTIGLOBULIN TEST PROCEDURE**

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| Principle | An indirect antiglobulion test (IAT) demonstrates in-vitro reactions between red cells and antibodies, and is used in antibody detection, antibody identification, crossmatching, and blood group phenotyping. Low-ionic-strength saline (LISS), which has reduced ionic strength compared to that of normal saline, accelerates antibody binding to red cells. |

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| Specimen | Serum or plasma may be used. The age of the specimen must comply with pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services*. |

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| Reagents | 1. LISS, available commercially for this use. 2. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 3. Group O antibody detection cells. Pooled group O antibody-detection cells may be used only for donor testing. Testing of patient samples must be performed with unpooled cells. 4. A 2% to 5% suspension of donor red cells in saline. 5. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Wash reagent or donor red cells three times in normal saline, and completely decant the saline. |
| 2 | Resuspend the cells to a 2% to 3% suspension in LISS. |
| 3 | Add 2 drops of serum to a properly labeled tube. |
| 4 | Add 2 drops of LISS-suspended red cells, mix, and incubate at 37 C for 10 to 15 minutes per the manufacturer’s directions. |
| 5 | Centrifuge and observe for hemolysis and agglutination by gently resuspending the red cell button. Grade and record results. |
| 6 | Wash the red cells three or four times with saline, and completely decant the final wash. |
| 7 | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. |
| 8 | Centrifuge and observe for agglutination. Grade and record the results. |
| 9 | Confirm the validity of negative results by adding IgG-coated red cells. |

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| Interpretation | 1. The presence of agglutination/hemolysis after incubation at 37 C constitutes a positive test result. 2. The presence of agglutination after addition of AHG constitutes a positive test result. 3. Antiglobulin test results are negative when no agglutination is observed after initial centrifugation followed by agglutination with the addition of IgG-coated red cells and centrifugation. If the IgG-coated red cells are not agglutinated, the negative result is invalid and the test must be repeated. |

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| Controls | 1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak examples of antibody. 2. Control sera can be prepared from reagent-grade typing sera diluted with 6% bovine albumin to give 2+ reactions by an IAT. Human sources of IgG antibodies are also acceptable. |

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| Notes | 1. The incubation times and the volume and concentration of red cells indicated are those given in the literature. Individual laboratories may choose to standardize techniques with somewhat different values. In all cases, the manufacturer’s package insert should be consulted before modifying a procedure. |
|  | 1. Steps 6 through 9 should be performed without interruption. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Alquist CR, Harm SK. Transfusion-service-related activities: Pretransfusion testing and storage, monitoring, processing, distribution, and inventory management of blood components. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:305-35. |

**METHOD 3-5. PEG INDIRECT ANTIGLOBULIN TEST PROCEDURE**

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| Principle | An indirect antiglobulion test (IAT) demonstrates in-vitro reactions between red cells and antibodies, and is used in antibody detection, antibody identification, crossmatching, and blood group phenotyping. The polyethylene glycol (PEG) method accelerates the binding of antibody to red cells by steric exclusion of water molecules in the diluent. |

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| Specimen | Serum or plasma may be used. The age of the specimen must comply with pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services*. |

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| Reagents | 1. PEG, available commercially or made as follows: to 20 g of 3350 MW PEG, for 20% w/v, add phosphate-buffered saline (PBS) pH 7.3, resulting in volume of 100 mL. 2. Antihuman globulin (AHG) reagent. Anti-IgG, rather than polyspecific AHG, must be used. 3. Group O antibody detection cells. Pooled group O antibody-detection cells may be used only for donor testing. Testing of patient samples must be performed with unpooled cells. 4. A 2% to 5% suspension of donor red cells in saline. 5. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | For each red cell sample to be tested, mix 2 drops of test serum, 4 drops of 20% PEG in PBS, and 1 drop of a 2% to 5% suspension of red cells or follow the manufacturer’s instructions if using commercially prepared PEG. |
| 2 | Incubate at 37 C for 15 minutes. |
| 3 | DO NOT CENTRIFUGE. |
| 4 | Wash the red cells four times with saline, and completely decant the final wash. |
| 5 | Add anti-IgG to the dry red cell button according to the manufacturer’s directions. Mix well. |
| 6 | Centrifuge and observe for agglutination. Grade and record the results. |
| 7 | Confirm the validity of negative results by adding IgG-coated red cells. |

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| Interpretation | 1. The presence of agglutination/hemolysis after incubation at 37 C constitutes a positive test result. 2. The presence of agglutination after addition of anti-IgG constitutes a positive test result. 3. Antiglobulin test results are negative when no agglutination is observed after initial centrifugation followed by agglutination with the addition of IgG-coated red cells and centrifugation. If the IgG-coated red cells are not agglutinated, the negative result is invalid and the test must be repeated. |
| Controls | 1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak examples of antibody. 2. Control sera can be prepared from reagent-grade typing sera diluted with 6% bovine albumin to give 2+ reactions by an IAT. Human sources of IgG antibodies are also acceptable. |
| Notes | 1. The incubation times and the volume and concentration of red cells indicated are those given in the literature. Individual laboratories may choose to standardize techniques with somewhat different values. In all cases, the manufacturer’s package insert should be consulted before modifying a procedure. |
|  | 1. In this procedure, centrifugation after 37 C incubation is omitted because red cells will not resuspend readily. 2. Use anti-IgG rather than polyspecific AHG to avoid unwanted positive reactions caused by C3-binding autoantibodies. Precipitation of serum proteins when PEG is added appears to be related to elevated serum globulin levels. The problem becomes apparent when the IgG-coated red cells are nonreactive or unexplained weak reactions are detected.2 At least four washes of the red cells at AHG phase, with agitation, will fully resuspend the red cells and usually prevent the problem from occurring. Alternatively, the test may have to be repeated using a method that does not use PEG. 3. The manufacturer’s instructions should be followed for the proper use of commercial PEG solutions. 4. Steps 4 through 7 of the IAT should be performed without interruption. |
|  |  |
| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Hoffer J, Koslosky WP, Gloster ES, et al. Precipitation of serum proteins by polyethylene glycol (PEG) in pretransfusion testing. Immunohematology 1999;15:105-7. 3. Alquist CR, Harm SK. Transfusion-service-related activities: Pretransfusion testing and storage, monitoring, processing, distribution, and inventory management of blood components. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:305-35. |

**METHOD 3-6. PREWARMING PROCEDURE**

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| --- | --- |
| Principle | Prewarming may be useful in the detection and identification of red cell antibodies that bind to antigen only at 37 C. |

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| Specimen | Serum or plasma may be used. The age of the specimen must comply with pretransfusion specimen requirements in AABB *Standards for Blood Banks and Transfusion Services*.1 |

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| Reagents | 1. Normal saline. 2. Anti-IgG. 3. Commercially available group O antibody detection red cells. Pooled group O antibody detection red cells may be used only for donor testing. Testing of patient samples must be performed with unpooled red cell samples. 4. IgG-coated red cells. |

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| --- | --- |
| Cautions | The use of the prewarming technique for testing sera of patients with cold-reactive autoantibody activity that may mask the presence of clinically significant antibodies has become controversial.2,3 It has been shown to result in decreased reactivity of some potentially significant antibodies, and weak antibodies can be missed.4 The technique should be used with caution and not used to eliminate unidentified reactivity. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Prewarm a container of saline to 37 C. |
| 2 | Label one tube for each reagent or donor sample to be tested. |
| 3 | Add 1 drop of 2% to 5% saline-suspended red cells to each tube. |
| 4 | Place the tubes containing red cells and a tube containing a small volume of the patient’s serum and a pipette at 37 C; incubate for 5 to 10 minutes. |
| 5 | Using the prewarmed pipette, transfer 2 drops of prewarmed serum to each tube containing prewarmed red cells. Mix without removing tubes from the incubator. |
| 6 | Incubate at 37 C for 30 to 60 minutes. |
| 7 | Without removing the tubes from the incubator, fill each tube with prewarmed (37 C) saline. Centrifuge and wash three or four times with 37 C saline. |
| 8 | Add anti-IgG according to the manufacturer’s directions. |
| 9 | Centrifuge and observe for reaction. Grade and record the results. |
| 10 | Confirm the validity of negative results by adding IgG-coated red cells. |

|  |  |
| --- | --- |
| Notes | 1. The prewarming procedure will not detect alloantibodies that agglutinate at 37 C or lower and are not reactive in the antiglobulin phase. If detection of these antibodies is desired, testing and centrifugation of a duplicate tube at 37 C is required. If time permits, a tube containing a prewarmed mixture of serum and cells can be incubated at 37 C for 60 to 120 minutes, and the settled red cells can be examined for agglutination by resuspending the button without centrifugation. 2. Cold-reactive antibodies may not be detectable when room temperature saline instead of 37 C saline is used in the wash step.3 The use of room temperature saline may avoid the elution of clinically significant antibody(ies) from reagent red cells that can occur with the use of 37 C saline. Some strong cold-reactive autoantibodies, however, may still react and therefore require the use of 37 C saline to avoid their detection. 3. Strong cold-reactive autoantibodies may be reactive in prewarmed tests; other techniques such as cold allo- or autoadsorption or dithiothreitol treatment of plasma may be required to detect underlying clinically significant antibodies. |
| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Judd WJ. Controversies in transfusion medicine. Prewarmed tests: Con. Transfusion 1995;35:271-5. 3. Mallory D. Controversies in transfusion medicine. Prewarmed tests: Pro—why, when, and how—not if. Transfusion 1995;35:268-70. 4. Leger RM, Garratty G. Weakening or loss of antibody reactivity after prewarm technique. Transfusion 2003;43:1611-14. |

**METHOD 3-7. DETECTING ANTIBODIES IN THE PRESENCE OF ROULEAUX—SALINE REPLACEMENT**

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| --- | --- |
| Principle | Patient samples with abnormal concentrations of serum proteins, altered serum-protein ratios, or high-molecular-weight volume expanders can aggregate reagent red cells and can mimic agglutination. Rouleaux are red cell aggregates that adhere along their flat surfaces, giving a “stacked coin” appearance microscopically. |

|  |  |
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| Specimen | Serum or plasma to be evaluated. |

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| Reagents | 1. Saline. 2. A1, B, and O reagent red cells. |

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| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | After routine incubation and resuspension, proceed with the following steps if the appearance of the resuspended red cells suggests rouleaux formation. The saline replacement technique is best performed by the test tube method. |
| 2 | Recentrifuge the serum (or plasma) or cell mixture. |
| 3 | Remove the serum, leaving the red cell button. |
| 4 | Replace the serum with an equal volume of saline (2 drops). |
| 5 | Resuspend the red cell button gently, and observe for agglutination. Rouleaux will disperse when suspended in saline. True agglutination is stable in the presence of saline. |

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| Notes | 1. In some instances, simple dilution of serum 1:3 with saline is sufficient to prevent rouleaux and to detect ABO isoagglutinins. 2. Review of the patient’s recent medical history and other laboratory results may be helpful (eg, history of multiple myeloma). |

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| --- | --- |
| Reference | 1. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998:1135. |

**METHOD 3-8. PREPARING FICIN ENZYME STOCK, 1% W/V**

|  |  |
| --- | --- |
| Principle | Ficin can destroy or weaken some red cell antigens, yet ficin-treated red cells may show enhanced reactivity with antibodies to other antigens. Ficin is used in pretransfusion testing when a pattern of weak reactions fails to indicate specificity, or when the presence of an antibody is suspected but cannot be confirmed. |

|  |  |
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| Reagents | 1. Dry ficin powder, 1 g. 2. Phosphate-buffered saline (PBS), pH 7.3. 3. Phosphate buffer, pH 5.4. |

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| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Place 1 g of powdered ficin in a 100-mL volumetric flask. Handle the ficin carefully; it is harmful if it gets in the eyes or is inhaled. It is desirable to wear gloves, mask, and apron, or to work under a hood. |
| 2 | Add PBS, pH 7.3, to 100 mL to dissolve the ficin. Agitate vigorously by inversion, rotate for 15 minutes, or mix with a magnetic stirrer until mostly dissolved. The powder will not dissolve completely. |
| 3 | Collect clear fluid, either by filtration or centrifugation, and prepare small aliquots. Store the aliquots at –20 C or colder. Do not refreeze a thawed solution. |

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| Note | The ficin preparations used in blood banking differ from lot to lot. Each time a stock enzyme solution is prepared, its reactivity should be tested, and incubation periods should be standardized for optimal effectiveness. |

|  |  |
| --- | --- |
| Reference | 1. Er LS, Bailey DJ. Identification of antibodies to red cell antigens. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:389-428. |

**METHOD 3-9. PREPARING PAPAIN ENZYME STOCK, 1% W/V**

|  |  |
| --- | --- |
| Principle | Papain can destroy or weaken some red cell antigens, yet papain-treated red cells may show enhanced reactivity with antibodies to other antigens. Papain is used in pretransfusion testing when a pattern of weak reactions fails to indicate specificity, or when the presence of an antibody is suspected but cannot be confirmed. |

|  |  |
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| Reagents | 1. L-cysteine hydrochloride (0.5 M), 0.88 g in 10 mL distilled water. 2. Dry papain powder, 2 g. 3. Phosphate buffer (0.067 M at pH 5.4), prepared by combining 3.5 mL of Na2HPO4 and 96.5 mL of KH2PO4. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Add 2 g of powdered papain to 100 mL of phosphate buffer (pH 5.4). Handle papain carefully; it is harmful to mucous membranes. Use appropriate protective equipment. |
| 2 | Agitate enzyme solution for 15 minutes at room temperature. |
| 3 | Collect clear fluid by filtration or centrifugation. |
| 4 | Add L-cysteine hydrochloride, and incubate solution at 37 C for 1 hour. |
| 5 | Add phosphate buffer (pH 5.4) to final volume of 200 mL. Store aliquots at −20 C or colder. Do not refreeze aliquots. |

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| --- | --- |
| Note | The papain preparations used in blood banking differ from lot to lot. Each time a stock enzyme solution is prepared, its reactivity should be tested, and incubation periods should be standardized for optimal effectiveness. |

|  |  |
| --- | --- |
| Reference | 1. Er LS, Bailey DJ. Identification of antibodies to red cell antigens. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:389-428. |

**METHOD 3-10. STANDARDIZING ENZYME PROCEDURES**

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| Principle | For a two-stage enzyme procedure, the optimal treatment time must be determined for each new lot of stock solution. The following technique for ficin can be modified for use with other enzymes. |

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| Reagents | 1. 1% stock solution of ficin in PBS, pH 7.3. 2. Several sera known to lack unexpected antibodies. 3. Anti-D that agglutinates only enzyme-treated D+ red cells and does not agglutinate untreated D+ red cells. 4. Anti-Fya of moderate or strong reactivity. 5. D+ and Fy(a+b–) red cell samples. 6. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 7. IgG-coated red cells. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Prepare 0.1% ficin by diluting one volume of stock ficin solution with nine volumes of PBS, pH 7.3. |
| 2 | Label three tubes: 5 minutes, 10 minutes, and 15 minutes. |
| 3 | Add equal volumes of washed red cells and 0.1% ficin to each tube. |
| 4 | Mix and incubate at 37 C for the time designated. Incubation times are easily controlled if the 15-minute tube is prepared first, followed by the 10- and 5-minute tubes at 5-minute intervals. Incubation will be complete for all three tubes at the same time. |
| 5 | Immediately wash the red cells three times with large volumes of saline. |
| 6 | Resuspend treated red cells to 2% to 5% in saline. |
| 7 | Label four tubes for each serum to be tested: untreated, 5 minutes, 10 minutes, and 15 minutes. |
| 8 | Add 2 drops of the appropriate serum to each of the four tubes. |
| 9 | Add 1 drop of the appropriate red cell suspension to each of the labeled tubes. |
| 10 | Mix and incubate at 37 C for 15 minutes. |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 11 | Perform the IAT:   |  |  | | --- | --- | | a. | Wash the red cells three or four times with saline, and completely decant the final wash. | | b. | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. | | c. | Centrifuge and observe for agglutination. Grade and record the results. | | d. | Confirm the validity of negative results by adding IgG-coated red cells. | |  |  | |

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| --- | --- |
| Interpretation | 1. The table below shows possible results with D+, Fy(a+b–) cells and the sera indicated. In this case, the optimal incubation time would be 10 minutes. Incubation for only 5 minutes does not completely abolish Fya activity or maximally enhance anti-D reactivity. Incubation for 15 minutes causes false-positive AHG reactivity with inert serum. 2. If incubation for 5 minutes overtreats the red cells, it is preferable to use a more dilute working solution of enzyme than to reduce incubation time, because it is difficult to accurately monitor very short incubation times. Additional tests can evaluate a single dilution at different incubation times, or a single incubation time can be used for different enzyme dilutions. |

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| --- | --- | --- | --- | --- |
| Hypothetical Results with D+, Fy(a+b–) Red Cells | | | | |
| **Cells and**  **Enzyme** |  | Inert  Serum | Anti-D | Anti-Fya |
| Untreated | 37 C incubation | 0 | 0 | 0 |
|  | Antihuman globulin (AHG) test | 0 | 1+ | 3+ |
| 5 minutes | 37 C incubation | 0 | 1+ | 0 |
|  | AHG test | 0 | 2+ | 1+ |
| 10 minutes | 37 C incubation | 0 | 2+ | 0 |
|  | AHG test | 0 | 2+ | 0 |
| 15 minutes | 37 C incubation | 0 | 2+ | 0 |
|  | AHG test | W+ | 2+ | W+ |

**METHOD 3-11. EVALUATING ENZYME-TREATED RED CELLS**

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| --- | --- |
| Principle | After optimal incubation conditions have been determined for a lot of enzyme solution, treated red cells should be evaluated before use to demonstrate that they are adequately, but not excessively, modified. Satisfactory enzyme treatment should produce red cells that are directly agglutinated by an antibody that reacts only by IAT with untreated cells; however, enzyme-treated red cells should not be agglutinated or aggregated by inert serum. |

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| --- | --- |
| Specimen | Enzyme-treated red cells. |

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| --- | --- |
| Reagents | 1. Sera known to contain antibody that will agglutinate enzyme-treated red cells. 2. Sera free of any unexpected antibodies. 3. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 4. IgG-coated red cells. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Select an antibody that agglutinates enzyme-treated red cells positive for the antigen but gives only AHG reactions with unmodified red cells. Many examples of human-source anti-D behave in this way. |
| 2 | Add 2 drops of the selected antibody-containing serum to a tube labeled “positive.” |
| 3 | Add 2 drops of a serum free of unexpected antibodies to a tube labeled “negative.” |
| 4 | Add 1 drop of 2% to 5% suspension of enzyme-treated red cells to each tube. |
| 5 | Mix and incubate 15 minutes at 37 C. |
| 6 | Centrifuge and resuspend the red cells by gentle shaking. |
| 7 | Examine macroscopically for the presence of agglutination. |
| 8 | Perform the IAT on the tube labeled “negative.”   |  |  | | --- | --- | | a. | Wash the red cells three or four times with saline, and completely decant the final wash. | | b. | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. | | c. | Centrifuge and observe for agglutination. Grade and record the results. | | d. | Confirm the validity of negative results by adding IgG-coated red cells. | |  |  | |
| Interpretation | There should be agglutination in the “positive” tube and no agglutination in the “negative” tube. If agglutination occurs in the “negative” tube, the red cells have been overtreated; if agglutination does not occur in the “positive” tube, treatment has been inadequate. | |

**METHOD 3-12. ONE-STAGE ENZYME PROCEDURE**

|  |  |
| --- | --- |
| Principle | Enzymatic digestion of red cell antigens selectively removes certain antigens, while preserving or enhancing the reactivity of others. |

|  |  |
| --- | --- |
| Specimen | Serum or plasma to be tested. |

|  |  |
| --- | --- |
| Reagents | 1. Reagent red cells. 2. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 3. IgG-coated red cells. |

|  |  |  |
| --- | --- | --- |
| Procedure | Step | Action |
| 1 | Add 2 drops of serum to an appropriately labeled tube. |
| 2 | Add 2 drops of a 2% to 5% saline suspension of reagent red cells. |
| 3 | Add 2 drops of 0.1% papain solution and mix well. |
| 4 | Incubate at 37 C for 15 minutes. |
| 5 | Centrifuge; gently resuspend the red cells and observe them for agglutination. Grade and record the results. |
| 6 | Perform the IAT.   |  |  | | --- | --- | | a. | Wash the red cells three or four times with saline, and completely decant the final wash. | | b. | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. | | c. | Centrifuge and observe for agglutination. Grade and record the results. | | d. | Confirm the validity of negative results by adding IgG-coated red cells. | |  |  | |
| 7 | To ensure that the enzyme is performing properly, a control should be included every time enzyme testing is performed. A control procedure is given in Method 3-11. |

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| Notes | 1. An alternative method for Steps 4 and 5 is to incubate the serum and enzyme-treated cells at 37 C for 60 minutes and to examine the settled cells for agglutination without centrifugation. This examination can be useful for serum with strong cold-reactive agglutinins and can sometimes prevent false-positive results. 2. Microscopic examination is not recommended for routine use and is particularly inappropriate with enzyme-enhanced tests; false-positive reactions will often be detected. 3. Enzyme preparations are available commercially. The manufacturer’s directions should be followed for appropriate use and quality control. |

|  |  |
| --- | --- |
| References | 1. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998. 2. Judd WJ, Johnson S, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 3-13. TWO-STAGE ENZYME PROCEDURE**

|  |  |
| --- | --- |
| Principle | Enzymatic digestion of red cell antigens selectively removes certain antigens, while preserving or enhancing the reactivity of others. |

|  |  |
| --- | --- |
| Specimen | Serum or plasma to be tested. |

|  |  |
| --- | --- |
| Reagents | 1. Reagent red cells. 2. Antihuman globulin (AHG) reagent. Polyspecific or anti-IgG may be used unless otherwise indicated. 3. IgG-coated red cells. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Prepare a diluted enzyme solution (papain or ficin) by adding 9 mL of PBS, pH 7.3, to 1 mL of stock enzyme. |
| 2 | Add one volume of diluted enzyme to one volume of packed, washed reagent red cells. See Method 3-11. |
| 3 | Incubate at 37 C for the time determined to be optimal for that enzyme solution. |
| 4 | Wash treated red cells at least three times with large volumes of saline, and resuspend the red cells to a 2% to 5% concentration in saline. |
| 5 | Add 2 drops of serum or plasma to be tested to an appropriately labeled tube. |
| 6 | Add 1 drop of 2% to 5% suspension of enzyme-treated red cells. |
| 7 | Mix and incubate for 15 minutes at 37 C. |
| 8 | Centrifuge; gently resuspend the red cells and observe for agglutination. Grade and record the results. |
| 9 | Perform the IAT.   |  |  | | --- | --- | | a. | Wash the red cells three or four times with saline, and completely decant the final wash. | | b. | Add AHG to the dry red cell button according to the manufacturer’s directions. Mix well. | | c. | Centrifuge and observe for agglutination. Grade and record the results. | | d. | Confirm the validity of negative results by adding IgG-coated red cells. | |  |  | |
| 10 | To ensure that the enzyme is performing properly, a control should be included every time enzyme testing is performed (see Method 3-11). |

|  |  |
| --- | --- |
| Notes | 1. An alternative method for Steps 7 and 8 is to incubate the serum and enzyme-treated cells at 37 C for 60 minutes and to examine the settled cells for agglutination without centrifugation. This examination can be useful for serum with strong cold-reactive agglutinins and can sometimes prevent false-positive results. 2. Microscopic examination is not recommended for routine use and is particularly inappropriate with enzyme-enhanced tests; false-positive reactions will often be detected. 3. Either papain or ficin may be used in a two-stage procedure. 4. Enzyme preparations are available commercially. The manufacturer’s directions should be followed for appropriate use and quality control. |

|  |  |
| --- | --- |
| References | 1. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998. 2. Judd WJ, Johnson S, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 3-14. PERFORMING A DIRECT ANTIGLOBULIN TEST**

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| Principle | The direct antiglobulin test (DAT) can determine if red cells have been coated in vivo with immunoglobulin, complement, or both. It is used primarily for the investigation of hemolytic transfusion reactions, hemolytic disease of the fetus and newborn, autoimmune hemolytic anemia, and drug-induced immune hemolysis. Further details on the principles of the DAT are found in Chapter 14 of the AABB *Technical Manual*. |

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| Specimen | Red cells from an EDTA-anticoagulated blood sample. |

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| Reagents | 1. Antihuman globulin (AHG) reagent: polyspecific antiglobulin reagent, anti-IgG, anti- complement antisera. 2. A control reagent (eg, saline or 6% albumin) is required when all antisera tested give a positive result. 3. IgG-coated red cells. 4. Complement-coated red cells, if instructed by the manufacturer. |

|  |  |  |
| --- | --- | --- |
| Procedure | **Step** | **Action** |
| 1 | Dispense 1 drop of a 2% to 5% suspension of red cells into a tube for each antiglobulin reagent or control to be tested. |
| 2 | Wash each tube three or four times with saline. Completely decant the final wash. |
| 3 | Immediately add antisera and mix. For the amount of antisera required, refer to the manufacturer’s directions. |
| 4 | Centrifuge according to the manufacturer’s directions. For anticomplement, the manufacturer may indicate a delay before centrifugation. |
| 5 | Examine the cells for agglutination. Grade and record the reaction. |
| 6 | If using polyspecific AHG or anticomplement, incubate nonreactive tests at room temperature if indicated by the manufacturer; then centrifuge and read again. |
| 7 | Confirm the validity of negative results as indicated by the manufacturer (eg, add IgG-coated red cells to tests containing anti-IgG). |
| 8 | Centrifuge according to the manufacturer’s directions. |
| 9 | Examine the cells for agglutination and record the reaction. |

|  |  |
| --- | --- |
| Interpretation | 1. The DAT result is positive when agglutination is observed either after immediate centrifugation or after the centrifugation that followed room-temperature incubation. IgG-coated red cells usually give immediate reactions, whereas complement coating may be more easily demonstrable after incubation.1,2 Monospecific AHG reagents are needed to confirm which globulins are present. |
|  | 1. The DAT result is negative when no agglutination is observed at either test phase and the IgG-coated cells added in Step 7 are agglutinated. If the IgG-coated cells are not agglutinated, the negative DAT result is considered invalid, and the test must be repeated. A negative DAT result does not necessarily mean that the red cells have no attached globulin molecules. Polyspecific and anti-IgG reagents detect 150 to 500 molecules of IgG per cell, but patients may still experience autoimmune hemolytic anemia when IgG coating is below this level.2 |
|  | 1. No interpretation can be made if the control reagent is reactive. This may indicate the presence of a strong cold autoagglutinin or spontaneous agglutination due to warm-reactive IgM or IgG antibodies. Warming the red cells to 37 C and/or washing with warm (37 C) saline should resolve reactivity due to cold autoagglutinins. Spontaneous agglutination requires treatment of the red cells with dithiothreitol or 2-aminoethylisothiouronium bromide (see Method 3-18). |

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| Notes | 1. Steps 2 through 5 should be performed without interruption. 2. Initial testing may be performed with polyspecific reagent only. If the DAT result is negative with polyspecific reagent, no further testing is necessary. If the DAT result is positive with polyspecific reagent, perform the DAT with monospecific reagents, anti-IgG, and anticomplement, to determine which globulins are present. 3. Additional washes may be needed when testing cord blood samples contaminated with Wharton’s jelly. |

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| --- | --- |
| References | 1. Klein HG, Anstee DJ. Mollison’s blood transfusion in clinical medicine. 12th ed. Oxford: Wiley-Blackwell, 2014. 2. Petz LD, Garratty G. Immune hemolytic anemia. Philadelphia: Churchill-Livingstone, 2004. 3. Borge PD Jr, Mansfield PM. The positive direct antiglobulin test and immune-mediated hemolysis. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:429-55. |

**METHOD 3-15. ANTIBODY TITRATION PROCEDURE**

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| Principle | Titration is a semiquantitative method used to determine the concentration of antibody in a serum sample or to compare the strength of antigen expression on different red cell samples. The usual applications of titration studies are as follows: 1) estimating antibody activity in alloimmunized pregnant women to determine whether and when to perform more complex invasive investigation of the fetal condition; 2) elucidating autoantibody specificity; 3) characterizing antibodies as having high titer and low avidity, traits common in antibodies to antigens of the KN and CH/RG systems, Csa, and JMH; and 4) observing the effect of sulfhydryl reagents on antibody behavior, to determine immunoglobulin class (IgG or IgM). |

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| --- | --- |
| Specimen | Serum or plasma antibody to be titrated. |

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| Reagents | 1. Red cells that express the antigen(s) corresponding to the antibody specificity(ies), in a 2% to 5% saline suspension. Uniformity of red cell suspensions is very important to ensure comparability of results. 2. Saline. (Note: Dilutions may be made with albumin if desired.) |

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| --- | --- | --- |
| Procedure | **Step** | **Action** |
|  | The master dilution technique for titration studies is as follows: |
| 1 | Label 10 test tubes according to the serum dilution (eg, 1:1, 1:2, etc). A 1:1 dilution means one volume of serum undiluted; a 1:2 dilution means one volume of serum in a final volume of two, or a 50% solution of serum in the diluent. |
| 2 | Deliver one volume of saline to all test tubes except the first (undiluted, 1:1) tube. |
| 3 | Add an equal volume of serum to each of the first two tubes (undiluted and 1:2). |
| 4 | Using a clean pipette, mix the contents of the 1:2 dilution several times, and transfer one volume into the next tube (the 1:4 dilution). |
| 5 | Continue the same process for all dilutions, using a clean pipette to mix and transfer each dilution. Remove one volume of diluted serum from the final tube, and save it for use if further dilutions are required. |
| 6 | Label 10 tubes for the appropriate dilutions. |
| 7 | Using separate pipettes for each dilution, transfer 2 drops of each diluted serum into the appropriately labeled tubes, and add 2 drops of a 2% red cell suspension. Alternatively, for convenience, add 1 drop of a 3% to 4% suspension of red cells as supplied by the reagent manufacturer, although this method is less precise. |
| 8 | Mix well and test by a serologic technique appropriate to the antibody (see Chapter 13). |
| 9 | Examine test results macroscopically; grade and record the reactions. The prozone phenomenon may cause reactions to be weaker in the more concentrated serum preparations than in higher dilutions. If one is to avoid misinterpretation of results, it may be preferable to examine first the tube containing the most dilute serum and then to proceed through the more concentrated samples to the undiluted specimen. |

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| Interpretation | 1. Observe the highest dilution that produces 1+ macroscopic agglutination. The titer is reported as the reciprocal of the dilution level (eg, 32—*not* 1 in 32 or 1:32). (See table below.) If there is agglutination in the tube containing the most dilute serum, the endpoint has not been reached, and additional dilutions should be prepared and tested. |
|  | 1. In comparative studies, a significant difference in titer is three or more dilutions. Variations in technique and inherent biologic variability can cause duplicate tests to give results that differ by one dilution in either direction. Serum containing antibody at a true titer of 32 may show, on replicate tests, the endpoint in the 1:32 tube, the 1:64 tube, or the 1:16 tube. |
|  | 1. Titer values alone can be misleading if the strength of agglutination is not also evaluated. The observed strength of agglutination can be assigned a number, and the sum of these numbers for all tubes in a titration study represents the score, another semiquantitative measurement of antibody reactivity. The arbitrarily assigned threshold for significance in comparing scores is a difference of 10 or more between different test samples (see table below). |
|  | 1. Antibodies with high-titer and low-avidity characteristics generally have a titer greater than 64, with most tubes showing consistently weak reactivity. |
|  | 1. The table below shows the results obtained with three sera, each of which shows no more agglutination after 1:256 dilution. The differences in score, however, indicate considerable variation in strength of reactivity. |

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| Examples of Antibody Titers, Endpoints, and Scores | | | | | | | | | | | | | |
|  | Reciprocal of Serum Dilution | | | | | | | | | | |  |  |
|  |  | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | Titer\* | Score |
| Sample #1 | Strength | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ | ± | ± | 0 | 64(256) |  |
| Score | 10 | 10 | 10 | 8 | 8 | 8 | 5 | 3 | 2 | 0 |  | 64 |
| Sample #2 | Strength | 4+ | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 1+ | ± | 0 | 128(256) |  |
| Score | 12 | 12 | 12 | 10 | 10 | 8 | 8 | 5 | 3 | 0 |  | 80 |
| Sample #3 | Strength | 1+ | 1+ | 1+ | 1+ | ± | ± | ± | ± | ± | 0 | 8(256) |  |
| Score | 5 | 5 | 5 | 5 | 3 | 3 | 3 | 2 | 2 | 0 |  | 33 |
| \*The titer is often determined from the highest dilution of serum that gives a reaction 1+ (score 5). This reaction may differ significantly  from the titration endpoint (shown in parentheses), as with the reactions of an antibody with high-titer, low-avidity characteristics,  manifested by Sample #3. | | | | | | | | | | | | | |

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| Notes | 1. Investigation of the fetal condition is discussed in Chapter 23 of the AABB *Technical Manual*, 20th edition. Elucidating autoantibody specificity is discussed in Chapter 14. 2. Titration is a semiquantitative technique. Technical variables greatly affect the results, and care should be taken to achieve the most uniform practices possible. 3. Measurements are more accurate with large volumes than with small volumes; a master dilution technique (see earlier) gives more reliable results than individual dilutions for a single set of tests. The volume needed for all planned tests should be calculated, and an adequate quantity of each dilution should be prepared. |
|  | 1. Careful pipetting is essential. Pipettes with disposable tips that can be changed after each dilution are recommended. |
|  | 1. The age, phenotype, and concentration of the test red cells will influence the results. |
|  | 1. The optimal time and temperature of incubation, and the time and force of centrifugation, should be used consistently. |
|  | 1. When the titers of several antibody-containing sera are to be compared, all of them should be tested against red cells (preferably freshly collected) from the same donor. If this is not possible, the tests should use a pool of reagent red cells from donors of the same phenotype. Comparisons are valid only when specimens are tested concurrently. |
|  | 1. When a single serum is to be tested against different red cell samples, all red cell samples should be collected and preserved in the same manner and diluted to the same concentration before use. Material from the master dilution should be used for all the tests. Comparisons are valid only when specimens are tested concurrently. |
|  | 1. When performing a titration for anti-D for hemolytic disease of the fetus and newborn, see Method 5-3. |
|  | 1. Other titration methods have been described that may show less variation.1 |

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| Reference | 1. AuBuchon JP, de Wildt-Eggen J, Dumont LJ, et al. Reducing the variation in performance of antibody titrations. Arch Pathol Lab Med 2008;132:1194-201. |

**METHOD 3-16. USING SULFHYDRYL REAGENTS TO DISTINGUISH IgM FROM IgG ANTIBODIES**

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| Principle | Treating IgM antibodies with sulfhydryl reagents abolishes both agglutinating and complement-binding activities. Observations of antibody activity before and after sulfhydryl treatment are useful in determining immunoglobulin class. Sulfhydryl treatment can also be used to abolish IgM antibody activity to permit detection of coexisting IgG antibodies. A discussion of this principle is found in Chapter 13 of the AABB *Technical Manual*. |

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| Specimen | 2 mL of serum or plasma to be treated. |

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| Reagents | 1. Phosphate-buffered saline (PBS) at pH 7.3. 2. 0.01 M dithiothreitol (DTT) prepared by dissolving 0.154 g of DTT in 100 mL of pH 7.3 PBS. Store at –18 C or lower. |

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| Procedure | **Step** | **Action** |
| 1 | Dispense 1 mL of serum or plasma into each of two test tubes. |
| 2 | To one tube (labeled dilution control), add 1 mL of pH 7.3 PBS. |
| 3 | To the other tube (labeled test), add 1 mL of 0.01 M DTT. |
| 4 | Mix and incubate at 37 C for 30 to 60 minutes. |
| 5 | Test the DTT-treated and dilution control samples in standard procedures. |

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| Interpretation | 1. Reactivity in the dilution control serum and no reactivity in the DTT-treated serum indicates an IgM antibody. 2. Reactivity in the dilution control serum and in the DTT-treated serum indicates an IgG antibody or an IgG and IgM mixture. Titration studies may be necessary to distinguish between them (see table below). 3. No reactivity in the dilution control serum indicates dilution of weak antibody reactivity and an invalid test. |

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| Effect of Dithiothreitol on Blood Group Antibodies | | | | | | |
|  | Reciprocal of Serum Dilution | | | | |  |
| **Test Sample** | 2 | 4 | 8 | 16 | 32 | Interpretation |
| Serum + DTT | 3+ | 2+ | 2+ | 1+ | 0 | IgG |
| Serum + PBS | 3+ | 2+ | 2+ | 1+ | 0 |
| Serum + DTT | 0+ | 0+ | 0+ | 0+ | 0 | IgM |
| Serum + PBS | 3+ | 2+ | 2+ | 1+ | 0 |
| Serum + DTT | 2+ | 1+ | 0+ | 0+ | 0 | IgG + IgM\* |
| Serum + PBS | 3+ | 2+ | 2+ | 1+ | 0 |
| \*May also indicate only partial inactivation of IgM. Note: DTT = dithiothreitol; IgG = immunoglobulin G; IgM = immunoglobulin M; PBS = phosphate-buffered saline. | | | | | | |

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| Control | A serum or plasma sample known to contain an IgM antibody should be treated and tested in parallel. |

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| Notes | 1. 2-mercaptoethanol can also be used for this purpose. 2. Sulfhydryl reagents used at low concentration may weaken antigens of the Kell system. For investigation of antibodies in the Kell system, it may be necessary to use other methods. 3. Gelling of a serum or plasma sample may be observed during treatment with DTT. This gelling can occur if the DTT has been prepared incorrectly and has a concentration above 0.01 M. Gelling may also occur if serum and DTT are incubated too long. An aliquot of the sample undergoing treatment can be tested after 30 minutes of incubation; if the activity thought to be caused by IgM has disappeared, there is no need to incubate further. Gelled samples cannot be tested for antibody activity because overtreatment with DTT causes the denaturation of all serum proteins. |

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| Reference | 1. Klein HG, Anstee DJ. Mollison’s blood transfusion in clinical medicine. 12th ed. Oxford: Wiley-Blackwell, 2014. |

**METHOD 3-17. USING PLASMA INHIBITION TO DISTINGUISH ANTI-CH AND -RG FROM OTHER ANTIBODIES WITH SIMILAR CHARACTERISTICS**

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| Principle | Binding of CH/RG antibodies to red cells is readily inhibited by plasma from CH/RG+ individuals. This is useful as an aid to identification of these antibodies. |

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| Specimen | Serum or plasma to be tested. |

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| Reagents | 1. Reactive red cell samples. 2. A pool of six or more normal plasma samples. 3. 6% bovine albumin. 4. Anti-IgG. 5. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare serial twofold dilutions of test serum in saline. The dilution range should be from 1:2 to 1:512, or to one tube beyond the known titer. The volume prepared should be not less than 0.3 mL for each red cell sample to be tested. |
| 2 | For each red cell sample to be tested, place 2 drops of each serum dilution into each of two sets of appropriately labeled 10 or 12 × 75-mm test tubes. |
| 3 | To one set, add 2 drops of pooled plasma to each tube. |
| 4 | To the other set, add 2 drops of 6% albumin to each tube. |
| 5 | Gently agitate the contents of each tube and incubate the tubes at room temperature for at least 30 minutes. |
| 6 | Add 1 drop of a 2% to 5% suspension of red cells to each tube. |
| 7 | Gently agitate the contents of each tube and incubate the tubes at 37 C for 1 hour. |
| 8 | Wash the cells four times in saline, add anti-IgG, and centrifuge according to the manufacturer’s directions. |
| 9 | Resuspend the cell buttons and examine for agglutination; confirm all nonreactive tests microscopically. Grade and record the results. |
| 10 | Confirm the validity of negative results by adding IgG-coated red cells. |

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| Interpretation | 1. Inhibition of antibody activity in the tubes to which plasma has been added suggests anti-Ch or anti-Rg specificity; this inhibition is often complete. 2. The presence of partial inhibition suggests the possibility of additional alloantibodies. This can be tested by preparing a large volume of inhibited serum and testing it against a reagent red cell panel to see if the nonneutralizable activity displays antibody specificity. 3. Lack of reactivity in the control (6% albumin) indicates dilution of weakly reactive antibody and an invalid test. |

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| Notes | 1. Antibodies to other plasma antigens may also be partially inhibited by plasma.1 2. Adsorption with C4-coated red cells is an alternative procedure that may be used for identifying anti-Ch or anti-Rg and for detecting underlying alloantibodies.2 |

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| References | 1. Reid ME, Lomas-Francis C, Olsson M. The blood group antigen factsbook. 3rd ed. San Diego: Elsevier Academic Press, 2012. 2. Ellisor SS, Shoemaker MM, Reid ME. Adsorption of anti-Chido from serum using autologous red blood cells coated with homologous C4. Transfusion 1982;22:243-5. |

**METHOD 3-18. TREATING RED CELLS USING DTT OR AET**

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| Principle | Dithiothreitol (DTT) and 2-aminoethylisothiouronium bromide (AET) are efficient reducing agents that can disrupt the tertiary structure of proteins by irreversibly reducing disulfide bonds to free sulfhydryl groups. Without tertiary structure, protein-containing antigens can no longer bind antibodies that are specific for them. Red cells treated with DTT or AET are not reactive with antibodies in the KEL blood group system, most antibodies in the KN system, or most examples of anti-LWa, -Yta, -Ytb, -Doa, -Dob, -Gya, -Hy, and -Joa. These inhibition techniques may be helpful in identifying some of these antibodies or in determining if a serum contains additional underlying alloantibodies. |
| Specimen | Red cells to be tested. |

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| Reagents | 1. Prepare 0.2 M DTT by dissolving 1 g of DTT powder in 32 mL of phosphate-buffered saline (PBS), pH 8.0. Divide it into 1-mL volumes, and freeze aliquots at –18 C or colder. 2. PBS at pH 7.3. 3. Prepare 6% AET by dissolving 0.6 g of AET in 10 mL distilled water, and bring the pH to 8 by slowly adding 5 N NaOH. 4. Red cells known to be positive for the antigen in question and, as a control, red cells known to be positive for K, which is consistently disrupted by DTT or AET. 5. Anti-K, either in reagent form or strongly reactive in a serum specimen. |

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| Procedure—DTT | **Step** | **Action** |
| 1 | Combine four volumes of the prepared DTT solution (0.2 M DTT, pH 8.0) with one volume of PBS-washed, packed red cells to be treated. |
| 2 | Incubate at 37 C for 30 to 45 minutes. |
| 3 | Wash four times with PBS. Slight hemolysis may occur; if hemolysis is excessive, repeat the procedure using fresh red cells and a smaller volume of DTT (eg, two or three volumes). |
| 4 | Resuspend the cells to a 2% to 5% suspension in PBS. |
| 5 | Test DTT-treated cells with serum containing the antibody in question. Test K+ red cells with anti-K. |

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| Procedure—AET | **Step** | **Action** |
| 1 | Combine four volumes of the 6% AET solution with one volume of washed, packed red cells to be treated. |
| 2 | Incubate for 20 minutes at 37 C. |
| 3 | Wash treated cells with PBS five to seven times or until the supernatant is clear. |
| 4 | Resuspend the cells to a 2% to 5% suspension in PBS. |
| 5 | Test AET-treated cells with serum containing the antibody in question. Test K+ red cells with anti-K. |

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| Interpretation | 1. The control K+ red cells should give negative reactions when tested with anti-K; if not, the DTT or AET treatment has been inadequate. Other antigens in the KEL system can also serve as the control. 2. If reactivity of the test serum is eliminated, the suspected antibody specificity may be confirmed. Enough red cell samples should be tested to exclude most other clinically significant alloantibodies. |

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| Note | Treatment of red cells with 0.2 M DTT or 6% AET will denature or weaken all antigens of the KEL, YT, LW, DO, and KN systems. Lower concentrations of DTT may selectively denature particular blood group antigens (ie, 0.002 M DTT will denature only Jsa and Jsb antigens, while other KEL antigens will not be affected). This property may aid in certain antibody investigations. |

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| References | 1. Advani H, Zamor J, Judd WJ, et al. Inactivation of Kell blood group antigens by 2-aminoethyl­isothiouronium bromide. Br J Haematol 1982;51:107-15. 2. Branch DR, Muensch HA, Sy Siok Hian S, Petz LD. Disulfide bonds are a requirement for Kell and Cartwright (Yta) blood group antigen integrity. Br J Haematol 1983;54:573-8. |

**METHOD 3-19. NEUTRALIZING ANTI-Sda WITH URINE**

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| Principle | To confirm anti-Sda in a serum sample, urine from a known Sd(a+) individual (or a pool of urine specimens) can be used to inhibit the antibody reactivity. |

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| Specimen | Serum or plasma suspected of containing anti-Sda. |

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| Reagents | 1. Urine from a known Sd(a+) individual, or from a pool of at least six individuals of unknown Sda type, and prepared as follows: Collect urine and immediately boil it for 10 minutes. Cool. Using 10-mm-interior-diameter cellulose membrane tubing (12,400 MW cut off), dialyze it against phosphate-buffered saline (PBS), pH 7.3, at 4 C for 48 hours. Change PBS several times. Centrifuge. Dispense supernatant into aliquots, which can be stored at –20 C until thawed for use. 2. PBS, pH 7.3. |

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| Procedure | **Step** | **Action** |
| 1 | Mix equal volumes of thawed urine and test serum. |
| 2 | Prepare a dilution control tube containing equal volumes of serum and PBS. |
| 3 | Prepare a urine control tube by mixing equal volumes of thawed urine and PBS. |
| 4 | Incubate all tubes at room temperature for 30 minutes. |
| 5 | Mix 1 drop of each test red cell sample with 4 drops from each of the tubes: neutralized serum, serum with PBS, and urine with PBS. Test each one using standard procedures. |

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| Interpretation | 1. Persistent agglutination in the serum sample incubated with urine means either that partial or no neutralization was achieved or that underlying antibodies are present. Microscopic examination may be helpful; agglutination caused by anti-Sda has a refractile, mixed-field appearance on microscopic examination. 2. No agglutination in the neutralized tube with persistent agglutination in the dilution control tube and absence of hemolysis and agglutination in the urine control tube indicate that the antibody has been neutralized and is quite probably anti-Sda. 3. The absence of agglutination in the dilution control tube means that the dilution in the neutralization step was too great for the antibody present, and the results of the test are invalid. 4. The urine control tube provides assurance that no substances in the urine are agglutinating or damaging the red cells. |

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| Notes | 1. Urine may also contain ABO and LE blood group substances, depending upon the ABO, LE, and secretor status of the donor. 2. Urine known to lack Sda substance, or saline, should be used a dilution control. |

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| Reference | 1. Judd WJ, Johnson S, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 3-20. ADSORPTION PROCEDURE**

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| Principle | Antibodies can be removed from a serum sample by adsorption. It may be possible to harvest bound antibody by elution or examination of the absorbed serum for antibody(ies) remaining after the adsorption process. |

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| Specimen | Serum or plasma containing antibody to be adsorbed. |

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| Reagents | Red cells (eg, autologous or allogeneic) that express the antigen corresponding to the antibody specificity to be adsorbed. |

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| Procedure | **Step** | **Action** |
| 1 | Wash the selected red cells at least three times with saline. |
| 2 | After the last wash, centrifuge the red cells at 800 to 1000 × *g* for at least 5 minutes, and remove as much of the supernatant saline as possible. Additional saline may be removed by touching the red cell mass with a narrow piece of filter paper. |
| 3 | Combine appropriate volumes of the packed red cells and serum, and incubate at the desired temperature for 30 to 60 minutes. |
| 4 | Mix the serum or cell mixture periodically throughout the incubation phase. |
| 5 | Centrifuge the red cells at 800 to 1000 × *g* for 5 minutes to pack cells tightly. Centrifuge at the incubation temperature, if possible, to avoid dissociation of antibody from the red cell membranes. |
| 6 | Transfer the supernatant fluid, which is the adsorbed serum, to a clean test tube. If an eluate is to be prepared, save the red cells. |
| 7 | Test an aliquot of the adsorbed serum, preferably against a reserved unused aliquot of the red cells used for adsorption, to see if all antibody has been removed. |

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| Interpretation | If reactivity remains, the antibody has not been completely removed. No reactivity signifies that antibody has been completely adsorbed. |
| Notes | 1. Adsorption is more effective if the area of contact between the red cells and serum is large. Use of a large-bore test tube (13 mm or larger) is recommended. 2. Multiple adsorptions may be necessary to remove an antibody completely; however, each successive adsorption increases the likelihood that the serum will be diluted and unadsorbed antibodies weakened. 3. Repeat adsorptions should use a fresh aliquot of red cells and not the red cells from the earlier adsorption. 4. Enzyme pretreatment of adsorbing red cells can be performed to increase antibody uptake for enzyme-resistant antigens. |
| Reference | 1. Judd WJ, Johnson S, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 3-21. USING THE AMERICAN RARE DONOR PROGRAM**

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| Principle | The American Rare Donor Program (ARDP) helps to locate blood products for patients requiring rare or unusual blood. The ARDP maintains a database of rare donors submitted by AABB-accredited immunohematology reference laboratories (IRLs) or American Red Cross IRLs. Donors are considered rare because of the absence of a high-prevalence antigen, the absence of multiple common antigens, or IgA deficiency. |

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| Procedure | **Step** | **Action** |
| 1 | A hospital blood bank, transfusion service, or blood center identifies a patient who needs rare blood. |
| 2 | The institution contacts the nearest AABB-accredited or Red Cross IRL to supply the needed blood. |
| 3 | If the IRL cannot supply the blood, it contacts the ARDP. *All* requests to the ARDP *must* come from an AABB-accredited or Red Cross IRL (or another rare donor program). Requests received directly from a nonaccredited facility will be referred to the nearest accredited institution. |
| 4 | The institution contacting the ARDP (requesting institution) *must* confirm the identity of the antibody(ies) by serologic investigation or by examining the serologic work performed by another institution. |
| 5 | ARDP staff search their database for centers that have identified donors with the needed phenotype and contact the centers for availability of units. The ARDP staff give the name(s) of the shipping center(s) to the requesting institution. |
| 6 | The requesting and shipping institutions should discuss and agree on charges and testing requirements before units are shipped. |
| 7 | If an initial search does not result in a sufficient number of units, the following mechanisms can be used by the ARDP staff to obtain needed units: 1) communicating with all ARDP participating centers, alerting them to search their inventories and/or recruit donors matching the needed phenotype, or 2) contacting other rare donor files such as those administered by the World Health Organization, Japanese Red Cross, or similar organizations. |

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| Notes | 1. All requests to the ARDP must originate from an AABB-accredited or Red Cross IRL to ensure that the patient in question has been accurately evaluated and reported. 2. All shipping and rare-unit fees are established by the shipping institution. |

**METHODS FOR INVESTIGATING A POSITIVE DAT RESULT**

**Eluates**

After an eluate is prepared, it should be tested by an appropriate technique for the type of antibody being investigated. Eluates prepared for the detection of IgG antibodies should be incubated at 37 C and converted to the antiglobulin test. Heat eluates prepared for detection of IgM antibodies may first be incubated at room temperature for 15 to 30 minutes and, if nonreactive, then incubated at 37 C, centrifuged, read for agglutination, and subsequently converted to the antiglobulin phase. IgM agglutinins may not be detectable at the antiglobulin phase.

Supernatant fluid from the final wash of the red cells to be eluted should be tested in parallel with the eluate and found to be nonreactive to ensure that antibody detected in the eluate is only red-cell-bound antibody and not free antibody from the plasma. Also, transferring the red cells to a clean tube before the elution step eliminates the possibility of dissociating any plasma antibody that may have nonspecifically bound to the test tube during preparation.

**Immune Hemolytic Anemia Serum/Plasma Methods**

Included in this section are methods used to remove warm or cold autoantibody reactivity (eg, adsorptions) so that alloantibody detection tests and diagnostic tests for differentiating the immune hemolytic anemias can be performed. A discussion of the immune hemolytic anemias is found in Chapter 14 of the AABB *Technical Manual*.

**METHOD 4-1. COLD-ACID ELUTION PROCEDURE**

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| Principle | Elution of antibodies at low pH is probably the result of disruption of the electrostatic bonds in proteins and changes to the tertiary structure. This method is suitable for recovery of warm-reactive auto- and alloantibodies. |

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| Specimens | 1. Red cells washed four to six times with large volumes of saline. 2. Supernatant saline from the final wash of the red cells to be eluted. |

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| Reagents | 1. Glycine-HCl (0.1 M, pH 3.0), prepared by dissolving 3.75 g of glycine and 2.922 g of sodium chloride in 500 mL of deionized or distilled water. Adjust the pH to 3.0 with 12 N HCl. Store at 4 C. Use chilled. 2. Phosphate buffer (0.8 M, pH 8.2), prepared by dissolving 109.6 g of Na2HPO4 and 3.8 g of KH2PO4 in approximately 600 mL of deionized or distilled water and adjusting the final volume to 1 L. Adjust the pH, if necessary, with either 1 N NaOH or 1 N HCl. Store at 4 C (see Note 1). 3. NaCl, 0.9%, at 4 C. Use chilled. |

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| Procedure | **Step** | **Action** |
| 1 | Place the glycine-HCl and the saline in an ice waterbath. |
|  | 2 | Place 1 mL of red cells in a 13 × 100-mm test tube and chill in an ice waterbath for 5 minutes before adding the glycine-HCl. |
|  | 3 | Add 1 mL of chilled saline and 2 mL of chilled glycine-HCl to the red cells. |
|  | 4 | Mix and incubate the tube in an ice waterbath (0 C) for 1 minute. |
|  | 5 | Quickly centrifuge the tube at 900 to 1000 × *g* for 2 to 3 minutes. |
|  | 6 | Transfer the supernatant eluate into a clean test tube, and add 0.1 mL of pH 8.2 phosphate buffer for each 1 mL of eluate. |
|  | 7 | Mix and centrifuge at 900 to 1000 × *g* for 2 to 3 minutes. |
|  | 8 | Transfer the supernatant eluate into a clean test tube, and test it in parallel with the supernatant saline from the final wash. |

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| Notes | 1. Phosphate buffer will crystallize during storage at 4 C. Redissolve it at 37 C before use. 2. Acidity may cause hemolysis of the reagent red cells used in testing the eluate. Addition of 22% bovine albumin (one part to four parts of eluate) may reduce such hemolysis. |

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| References | 1. Judd WJ, Johnson ST, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. 2. Rekvig OP, Hannestad K. Acid elution of blood group antibodies from intact erythrocytes. Vox Sang 1977;33:280-5. |

**METHOD 4-2. GLYCINE-HCl/EDTA ELUTION PROCEDURE**

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| Principle | Dissociation of antibodies from red cells enables the identification of auto- or alloantibodies. Elution methods used in conjunction with adsorption techniques are also useful in detecting weak antigen expression on the adsorbing red cells, as well as in separating mixtures of antibodies against red cell antigens. |

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| Specimen | Red cells positive by the direct antiglobulin test (DAT) washed six times with large volumes of saline (saving the last wash). |

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| Reagents | 1. Disodium EDTA (10% w/v): Na2EDTA 10 g; add distilled water to 100 mL. 2. Glycine-HCl (0.1 M at pH 1.5): 0.75-g glycine diluted to 100 mL with 0.9% NaCl; adjust to pH 1.5 with 12 N HCl. 3. TRIS-NaCl (1 M): Tris(hydroxymethyl)aminomethane (TRIS) or TRIZMA BASE, 12.1 g; NaCl, 5.25 g; distilled water to 100 mL. 4. Supernatant saline from the final wash of the red cells to be tested. |

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| Procedure | **Step** | **Action** |
| 1 | In a test tube, mix together 20 volumes (eg, drops) of 0.1 M glycine-HCl buffer and five volumes of 10% EDTA. This mixture is the eluting solution. |
|  | 2 | In a 12 × 75-mm tube, place 10 volumes of red cells. |
|  | 3 | Add 20 volumes of the eluting solution to the red cells, mix well, and incubate at room temperature for 2 minutes. Do not overincubate. |
|  | 4 | Add 1 volume of TRIS-NaCl, mix, and immediately centrifuge the tube at 900 to 1000 × *g* for 60 seconds. |
|  | 5 | Transfer the supernatant eluate into a clean test tube, and carefully adjust it dropwise to pH 7.0 to 7.4 with 1 M TRIS-NaCl. The pH can be checked with pH paper. |
|  | 6 | Centrifuge at 900 to 1000 × *g* for 2 to 3 minutes to remove the precipitate. |
|  | 7 | Transfer the supernatant eluate into a clean test tube and test it in parallel with the supernatant saline from the final wash. |

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| Notes | 1. Once the red cells have been rendered DAT negative, they may be tested for the presence of blood group antigens, except those in the KEL system and Era, as those antigens are denatured by glycine-HCl/EDTA. Wash the red cells at least three times in saline before use. 2. Red cells modified with glycine-HCl/EDTA may be treated with a protease and used in autologous adsorption studies. 3. Overincubation with the eluting solution (Step 3) will irreversibly damage the red cells. 4. TRIS-NaCl is very alkaline, and only a few drops should be required to attain the desired pH (Step 5). 5. Aliquots of the reagents can be stored frozen, and one tube of each can be thawed just before use. The 10% EDTA may precipitate when stored at 2 to 8 C. 6. Stored eluate (4 C or frozen) may be more stable if albumin is added (3 volumes of 22% bovine albumin for every 10 volumes of eluate). If albumin is added to the eluate, the albumin also should be added to the last wash. |

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| Reference | 1. Byrne PC. Use of a modified acid/EDTA elution technique. Immunohematology 1991;7:46-7. |

**METHOD 4-3. HEAT ELUTION PROCEDURE**

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| Principle | Heat elution uses an increase in temperature to dissociate antibodies from red cells. This method is best suited for the investigation of ABO hemolytic disease of the fetus and new­born and for the elution of IgM antibodies from red cells. It should not be used routinely for the investigation of IgG auto- or alloantibodies. |

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| Specimens | 1. Red cells positive by the direct antiglobulin test (DAT), washed four to six times with large volumes of saline (see Note). 2. Supernatant saline from the final wash of the red cells to be eluted. |

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| Reagent | 6% bovine albumin. |

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| Procedure | **Step** | **Action** |
| 1 | Mix equal volumes of washed packed cells and 6% bovine albumin in a 13 × 100-mm test tube. |
|  | 2 | Place the tube at 56 C for 10 minutes. Agitate the tube periodically during this time. |
|  | 3 | Centrifuge the tube at 900 to 1000 × *g* for 2 to 3 minutes. |
|  | 4 | Immediately transfer the supernatant eluate into a clean test tube, and test in parallel with the supernatant saline from the final wash. |

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| Note | For optimal recovery of cold-reactive antibodies, the red cells should be washed in ice-cold saline to prevent dissociation of bound antibody before elution. |

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| References | 1. Judd WJ, Johnson ST, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. 2. Landsteiner K, Miller CP Jr. Serological studies on the blood of primates. II. The blood groups in anthropoid apes. J Exp Med 1925;42:853-62. |

**METHOD 4-4. LUI FREEZE-THAW ELUTION PROCEDURE**

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| Principle | As red cells freeze, extracellular ice crystals form that attract water from their surroundings. This increases the osmolarity of the remaining extracellular fluid, which then extracts water from the red cells. The red cells shrink, resulting in lysis. As the membranes are disrupted, antibody is dissociated. This method is used primarily for the investigation of ABO hemolytic disease of the fetus and newborn. |

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| Specimens | 1. Red cells washed four to six times with large volumes of saline. 2. Supernatant saline from the final wash of the red cells to be eluted. |

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| Procedure | **Step** | **Action** |
| 1 | Mix 0.5 mL of the red cells to be tested with 3 drops of saline in a test tube. |
|  | 2 | Cap the tube, then rotate the tube to coat the tube wall with cells. |
|  | 3 | Place the tube in a horizontal position in a freezer at –6 C to –70 C for 10 minutes. |
|  | 4 | Remove the tube from the freezer and thaw it quickly with warm, running tap water. |
|  | 5 | Centrifuge for 2 minutes at 900 to 1000 × *g*. |
|  | 6 | Transfer the supernatant eluate to a clean test tube, and test it in parallel with the supernatant saline from the final wash. |

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| References | 1. Judd WJ, Johnson ST, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. 2. Feng CS, Kirkley KC, Eicher CA, et al. The Lui elution technique: A simple and efficient method for eluting ABO antibodies. Transfusion 1985;25:433-4. |

**METHOD 4-5. COLD AUTOADSORPTION PROCEDURE**

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| Principle | Although most cold autoantibodies do not cause a problem in serologic tests, some potent cold-reactive autoantibodies may mask the concomitant presence of clinically significant alloantibodies. In these cases, adsorbing the serum in the cold with autologous red cells can remove the autoantibody, permitting detection of underlying alloantibodies. In the case of most nonpathologic cold autoantibodies, a simple quick adsorption of the patient’s serum with enzyme-treated autologous red cells will remove most cold antibody. |
| Specimens | 1. 1 mL of serum or plasma to be adsorbed. 2. One or more 1-mL aliquots of autologous red cells. See Notes to determine number of aliquots. |

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| Reagents | 1. 1% cysteine-activated papain or 1% ficin. 2. Phosphate-buffered saline (PBS), pH 7.3. 3. 0.2 M DTT prepared by dissolving 1 g of DTT in 32.4 mL of pH 7.3 PBS. Dispense into 3-mL aliquots and store at –18 C or colder. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare ZZAP reagent2 by mixing 0.5 mL of 1% cysteine-activated papain with 2.5 mL of 0.2 M DTT and 2 mL of pH 7.3 PBS. Alternatively, use 1 mL of 1% ficin, 2.5 mL of 0.2 M DTT, and 1.5 mL of pH 7.3 PBS. |
|  | 2 | Add 2 mL of ZZAP reagent to 1 mL of autologous red cells. There is no need to wash the red cells before treatment. Mix and incubate at 37 C for 30 minutes. |
|  | 3 | Wash the cells three times in saline. Centrifuge the last wash for at least 5 minutes at 900 to 1000 × *g* and remove as much of the supernatant saline as possible. |
|  | 4 | To the tube of ZZAP-treated red cells, add 1 mL of the autologous serum. Mix and incubate at 4 C for 30 minutes. |
|  | 5 | Centrifuge at 900 to 1000 × *g* for 4 to 5 minutes and transfer the serum into a clean tube. |
|  | 6 | Steps 2 through 5 may be repeated if the first autoadsorption does not satisfactorily remove the autoantibody activity. See Note 2. |
|  | 7 | After the final adsorption, test the serum with reagent red cells for alloantibody activity. |

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| Notes | 1. Washing the red cells with warm (37 C) saline before treatment will help to dissociate cold autoantibody from the red cells. Performing autoadsorption with red cells treated with ZZAP, a combination of proteolytic enzyme and dithiothreitol (DTT), is more efficient. Complement is removed in addition to IgM and IgG,1 and the red cells are simultaneously enzyme-treated, thereby enhancing the uptake of free autoantibody from the serum. 2. Sufficient cold autoantibody can usually be removed in one or two adsorptions. 3. If the reactivity of the autoantibody is not diminished, the target autoantigen may have been destroyed by either the enzyme or the DTT. The adsorption should be repeated against untreated autologous red cells washed several times in warm saline. |

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| References | 1. Branch DR. Blood transfusion in autoimmune hemolytic anemias. Lab Med 1984; 15:402-8. 2. Branch DR, Petz LD. A new reagent (ZZAP) having multiple applications in immunohematology. Am J Clin Pathol 1982;78:161-7. 3. Judd WJ, Johnson ST, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 4-6. DETERMINING THE SPECIFICITY OF COLD-REACTIVE AUTOAGGLUTININS**

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| Principle | Cold-reactive autoagglutinins are usually IgM, which binds to red cells in the lower temperature of the peripheral circulation and causes complement components to attach to the red cells. As the red cells circulate to warmer areas, the IgM dissociates but the complement remains. A discussion of the specificity of cold-reacting autoantibodies is found in Chapter 14 of the AABB *Technical Manual*. |

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| Specimens | 1. Serum or plasma, separated at 37 C from a blood sample maintained and/or allowed to clot at 37 C, or plasma, separated from an anticoagulated sample after periodic inversion at 37 C for approximately 15 minutes. 2. Autologous red cells. |

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| Reagents | Test red cells of the following phenotypes:   1. A pool of two or more examples of adult group O I adult red cells; these can be the reagent cells routinely used for alloantibody detection. 2. Group O i cord red cells. 3. The patient’s own (autologous) red cells, washed at least three times with 37 C saline. 4. Red cells of the same ABO group as the patient, if the patient is not group O. If the patient is group A or AB, use both A1 and A2 cells. 5. Saline or phosphate-buffered saline (PBS), pH 7.3. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare serial twofold dilutions of the serum or plasma in saline or PBS. The dilution range should be from 1 in 2 to 1 in 4096 (12 tubes), and the volumes prepared should be more than the total volume needed to test all of the desired red cells. For example, diluting 0.4 mL of serum with 0.4 mL of saline would be sufficient to test three red cell examples. See Notes 1 and 2. |
|  | 2 | Label a set of 12 tubes with the dilution (eg, 2, 4, 8, etc) for each kind of red cells to be tested (eg, adult, cord, autologous). |
|  | 3 | Dispense 2 drops of each dilution into the appropriate tubes. |
|  | 4 | Add 1 drop of a 3% to 5% saline suspension of each red cell sample to the appropriate set of tubes. |
|  | 5 | Mix and incubate at room temperature for 30 to 60 minutes. |
|  | 6 | Centrifuge for 15 to 20 seconds at 900 to 1000 × *g*. Examine the tubes one by one macroscopically for agglutination, starting with the set of tubes at the highest dilution for each cell tested (ie, read all the tubes for each dilution as a set). Grade and record the results. |
|  | 7 | Incubate the tubes at 4 C for 1 to 2 hours. |
|  | 8 | Centrifuge for 15 to 20 seconds at 900 to 1000 × *g*. Immediately place the tubes in a rack in an ice waterbath. Examine the tubes as in Step 6. Grade and record the results. |

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| Interpretation | 1. The table below summarizes the reactions of the commonly encountered cold-reactive autoantibodies.  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | | Typical Relative Reactivity Patterns of Cold Autoantibodies | | | | | | |  | Antibody Specificity | | | | | | **Red Cells** | Anti-I | Anti-i | Anti-IT | Anti-IH | Anti-Pr | | O I adult | + | 0/↓ | 0/↓ | + | + | | O i cord | 0/↓ | + | + | ↓ | + | | O i adult | 0/↓ | + | 0/↓ | ↓ | + | | A1 I adult | + | 0/↓ | 0/↓ | ↓ | + | | Autologous | + | 0/↓ | 0/↓ | ↓ | + | | O I enzyme-treated | ↑ | ↑ | ↑ | ↑ | 0 | | + = reactive; 0 = nonreactive; ↓ = weaker reaction; ↑ = stronger reaction. | | | | | |  1. In cold agglutinin syndrome, anti-I is seen most frequently, but anti-i may also be encountered. When cord cells react stronger than adult cells, the specificity may be anti-i, but adult i red cells need to be tested to confirm that these reactions are due to anti-i and not anti-IT. Some examples of anti-I are more strongly reactive with red cells that have a strong expression of H antigen (eg, O and A2 cells); such antibodies are called anti-IH. 2. Rarely, the specificity may be anti-Pr, which should be suspected if all the cells tested are equally reactive. Anti-Pr can be confirmed by testing enzyme-treated cells; anti-Pr is not reactive with enzyme-treated cells, whereas anti-I and anti-i react better with enzyme-treated cells. Anti-Pr is equally reactive with untreated red cells of I or i phenotypes. |

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| Notes | 1. It is important to use separate pipettes or pipette tips for each tube when preparing serum dilutions because the serum carried from one tube to the next when a single pipette is used throughout may cause falsely high titration endpoints. The difference can convert a true titer of 4000 to an apparent titer of 100,000 when the use of separate pipettes is compared with the use of a single pipette. |
|  | 1. Serum dilutions can be prepared more accurately with large volumes (eg, 0.5 mL) than with small volumes. |
|  | 1. Potent examples of cold-reactive autoantibodies generally do not show apparent specificity until titration studies are performed; this specificity may not even be apparent with dilutions at room temperature or 4 C. In such circumstances, tests can be incubated at 30 to 37 C. Differential reactivity may be more apparent if incubation times are prolonged and agglutination is evaluated after settling, without centrifugation. Settled readings are more accurate after a 2-hour incubation. |
|  | 1. This procedure can be used to determine both the titer and the specificity. If incubations are started at 37 C (set up prewarmed, ie, all reactants at 37 C before combining) and readings are taken sequentially after incubation at each temperature (eg, 37 C, 30 C, room temperature, 4 C), the specificity, titer, and thermal amplitude of the autoantibody can be determined with a single set of serum dilutions. |
|  | 1. If testing will also be performed at 30 C and 37 C, include in parallel a test of the neat (undiluted) serum. |

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| Reference | 1. Petz LD, Garratty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004. 2. Borge PD Jr, Mansfield PM. The positive direct antiglobulin test and immune-mediated hemolysis. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:429-55. |

**METHOD 4-7. COLD AGGLUTININ TITER PROCEDURE**

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| Principle | Cold-reactive autoantibodies, if present at very high titers, may suggest a pathologic cold agglutinin disease. This may result in overt hemolysis and systemic symptoms and may indicate underlying B-cell hematologic neoplasia. |

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| Specimen | Serum or plasma, separated at 37 C from a sample maintained and/or allowed to clot at 37 C, or plasma, separated from an anticoagulated sample after periodic inversion at 37 C for approximately 15 minutes. |

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| Reagents | 1. A pool of two or more examples of washed group O I adult red cells (eg, antibody detection cells). 2. Phosphate-buffered saline (PBS), pH 7.3. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare serial twofold dilutions of the patient’s serum or plasma in PBS. The dilution range should be from 1 in 2 to 1 in 4096 (12 tubes). See Notes 1 and 2. |
|  | 2 | Mix 2 drops of each dilution with 1 drop of a 3% to 5% suspension of red cells. |
|  | 3 | Mix and incubate at 4 C for 1 to 2 hours. |
|  | 4 | Centrifuge the tubes for 15 to 20 seconds at 900 to 1000 × *g*, then place the tubes in a rack in an ice waterbath. Examine the tubes one by one macroscopically for agglutination, starting with the tube at the highest dilution. Grade and record the results. |

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| Interpretation | 1. The titer is the reciprocal of the highest serum dilution at which macroscopic agglutination is observed. Titers above 64 are considered elevated, but hemolytic anemia resulting from cold-reactive autoagglutinins rarely occurs unless the titer is ≥1000. Titers below 1000 may be obtained when the autoantibody has a different specificity (eg, anti-i) or if the cold agglutinin is of the less-common low-titer, high-thermal-amplitude type. 2. If the patient has a positive direct antiglobulin test (DAT) result because of complement only and has clinical signs of hemolytic anemia, specificity and thermal amplitude studies should be performed. |

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| Notes | 1. It is important to use separate pipettes for each tube when preparing serum dilutions because the serum carried from one tube to the next when a single pipette is used throughout may cause falsely high titration endpoints. 2. Serum dilutions can be prepared more accurately with large volumes (eg, 0.5 mL) than with small volumes. |

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| Reference | 1. Petz LD, Garratty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004. |

**METHOD 4-8. ADSORBING WARM-REACTIVE AUTOANTIBODIES USING AUTOLOGOUS RED CELLS**

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| Principle | Warm-reactive autoantibodies in serum may mask the concomitant presence of clinically significant alloantibodies. Adsorption of the serum with autologous red cells can remove autoantibody from the serum, permitting detection of underlying alloantibodies. However, autologous red cells in the circulation are coated with autoantibody. Autologous adsorption of warm-reactive autoantibodies can be facilitated by dissociating autoantibody from the red cell membrane, thereby uncovering antigen sites that can bind free autoantibody to remove it from the serum. |

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| Specimens | 1. 1 mL of serum or plasma (or eluate) to be adsorbed. 2. One or more 1-mL aliquots of autologous red cells. See Note 3. |

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| Reagents | 1. 1% cysteine-activated papain or 1% ficin. 2. Phosphate-buffered saline (PBS), pH 7.3. 3. 0.2 M DTT prepared by dissolving 1 g of DTT in 32.4 mL of pH 7.3 PBS. Dispense into 3-mL aliquots and store at –18 C or colder. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare ZZAP reagent by mixing 0.5 mL of 1% cysteine-activated papain with 2.5 mL of 0.2 M DTT and 2 mL of pH 7.3 PBS. Alternatively, use 1 mL of 1% ficin, 2.5 mL of 0.2 M DTT, and 1.5 mL of pH 7.3 PBS. |
|  | 2 | Add 2 mL of ZZAP reagent to each of two tubes containing 1 mL of packed red cells. There is no need to wash the red cells before treatment. Mix and incubate at 37 C for 30 minutes with periodic mixing. |
|  | 3 | Wash the red cells three times in saline. Centrifuge the last wash for at least 5 minutes at 900 to 1000 × *g* and remove as much supernatant saline as possible. |
|  | 4 | Add serum to an equal volume of ZZAP-treated red cells, mix, and incubate at 37 C for approximately 30 to 45 minutes. |
|  | 5 | Centrifuge and carefully remove serum. |
|  | 6 | If the original serum reactivity was only 1+, proceed to step 7; otherwise, repeat Steps 4 and 5 once more using the once ­adsorbed patient’s serum and a second aliquot of ZZAP-treated cells. See Note 3. |
|  | 7 | Test the adsorbed serum against group O reagent cells. If reactivity persists, repeat Steps 4 and 5. |

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| Interpretation | One or two adsorptions ordinarily remove sufficient autoantibody so that alloantibody reactivity, if present, is readily apparent. If the twice-autoadsorbed serum reacts with defined specificity, as shown by testing against a small antibody identification panel, then the defined specificity of the antibody is probably an alloantibody. If the serum reacts with all cells on the panel, 1) additional autoadsorptions are necessary, 2) the serum contains antibody to a high-prevalence antigen, or 3) the serum contains an autoantibody (eg, anti-Kpb) that does not react with ZZAP-treated cells and thus will not be adsorbed by this procedure. To check this latter possibility, test the reactive autoadsorbed serum against reagent cells that have been pretreated with the ZZAP reagent. |

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| Notes | 1. ZZAP treatment destroys all KEL system antigens and all other antigens that are destroyed by proteases (eg, M, N, Fya, and Fyb), as well as the antigens of the LW, YT, DO, and KN systems. If the autoantibody is suspected to have specificity to a high-prevalence antigen in any of these blood groups, an alternative procedure is to perform autoadsorption with untreated autologous cells or autologous cells treated only with 1% ficin or 1% cysteine-activated papain. |
|  | 1. Cold autoantibodies reactive at room temperature can also be present in the serum of about 35% of patients with warm-reactive autoantibodies. Removal of these cold antibodies can be facilitated by placing the serum and cell mixture at 4 C for about 15 minutes after incubation at 37 C. |
|  | 1. As a guide, when the original serum reactivity is 1+ in the low-ionic-strength saline indirect antiglobulin test (LISS-IAT), usually only one adsorption is required. Antibodies with 2+ to 3+ reactivity will generally be removed in two to three adsorptions. Performing greater than four adsorptions increases the risk of diluting alloantibody reactivity. |
|  | 1. Some autoantibody can be dissociated by a gentle heat elution for 3 to 5 minutes at 56 C. Subsequent treatment of the cells with enzymes enhances the adsorption process by removing membrane structures that otherwise hinder the association between antigen and antibody. The most effective procedure involves the use of ZZAP reagent,1 a mixture of a proteolytic enzyme and the sulfhydryl reagent dithiothreitol (DTT). ZZAP removes immunoglobulins and complement from the red cells and enhances the adsorption process. |
|  | 1. Red cells from patients transfused within the last 3 months should not be used for autoadsorption because transfused red cells present in the circulation are likely to adsorb the alloantibodies that are being sought. |

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| Reference | 1. Branch DR, Petz LD. A new reagent (ZZAP) having multiple applications in immunohematology. Am J Clin Pathol 1982;78:161-7. |

**METHOD 4-9. ADSORBING WARM-REACTIVE AUTOANTIBODIES USING ALLOGENEIC RED CELLS**

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| Principle | Adsorption of serum with selected red cells of known phenotypes will remove autoantibody and leave alloantibodies to most common blood group antigens. The specificity of the antibodies that remain after adsorption can be confirmed by testing against a panel of reagent red cells. This procedure can be used to detect underlying alloantibodies if the patient has been recently transfused, or if insufficient autologous red cells are available. |

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| Specimen | Serum/plasma containing warm-reactive autoantibodies or eluate from direct antiglobulin test (DAT)-positive cells. |

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| Reagents | 1. 1% cysteine-activated papain or 1% ficin. 2. ZZAP reagent (papain or ficin plus 0.2 M DTT). 3. Phosphate-buffered saline (PBS), pH 7.3. 4. Adsorbing red cells *when the patient’s phenotype is unknown:* Group O red cells of the phenotypes R1R1, R2R2, and rr; one of these cells should be Jk(a–), and one should be Jk(b–). Additionally, if the red cells are to be enzyme-treated only, at least one of the samples should also be K–; the cells can be treated with enzyme or ZZAP to denature other antigens (see table below and Note 1).     Adsorbing red cells *when the patient’s phenotype is known:* The red cells can be selected to match the patient’s phenotype, or at least they should have the same RH and JK phenotypes if the cells can be treated with enzyme or ZZAP to denature other antigens.  The red cells can be reagent cells or from any blood specimen that will yield a sufficient volume of red cells. See Note 2. Reserve a sample of these red cells to test for completeness of adsorption (Step 7). |

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| Procedure | **Step** | **Action** |
| 1 | Wash 1 mL of each red cell specimen once in a large volume of saline, centrifuge to pack the cells, and remove the supernatant saline. There is no need to wash packed red cells before treatment with ZZAP. |
|  | 2 | To each volume of washed packed cells, add one volume of 1% enzyme solution or two volumes of working ZZAP reagent. Mix the cells by inverting several times. |
|  | 3 | Incubate at 37 C: 15 minutes for enzyme or 30 minutes for ZZAP. Mix periodically throughout incubation. |
|  | 4 | Wash the red cells three times with large volumes of saline. Centrifuge at 900 to 1000 × *g* for at least 5 minutes and remove the last wash as completely as possible to prevent dilution of the serum. |
|  | 5 | For each of the red cell specimens, mix one volume of treated cells with an equal volume of the patient’s serum and incubate at 37 C for 30 minutes, mixing occasionally. |
|  | 6 | Centrifuge at 900 to 1000 × *g* for approximately 5 minutes and harvest the supernatant serum. |
|  | 7 | Test the sample(s) of adsorbed serum against the cells (untreated) used for adsorption, respectively, for completeness of adsorption. If reactivity is present, repeat Steps 5 through 7 until no reactivity remains. Consider the phenotype of the adsorbing red cells when evaluating completeness of adsorption; reactivity may persist if it is directed to an antigen destroyed by enzyme or DTT treatment; for example, anti-Fya in the adsorbed serum will be reactive with untreated Fy(a+) adsorbing red cells if they were treated for adsorption. The three samples of adsorbed serum can then be tested against antibody detection/panel cells and the results compared for demonstration of persisting and removed antibody activity. |

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| Notes | 1. The s antigen may not be denatured by a particular enzyme or ZZAP solution. The s antigen status of the adsorbing red cells may need to be considered. 2. If the autoantibody is very strong, three or more aliquots of adsorbing cells should be prepared. As a guide, when the original serum reactivity is 1+ in the low-ionic-strength saline indirect antiglobulin test (LISS-IAT), usually only one adsorption is required. Antibodies with 2+ to 3+ reactivity will generally be removed in two to three adsorptions. Performing greater than four adsorptions increases the risk of diluting alloantibody reactivity. The use of a higher proportion of cells to serum/eluate may enhance adsorption effectiveness. 3. A visible clue to the effectiveness of adsorption is clumping of the enzyme- or ZZAP-treated cells when they are mixed with the serum, especially when strong antibodies are present. 4. Because the treated red cells will lack the antigens destroyed by DTT and/or enzymes, adsorption with untreated red cells may be tried if the autoantibody is not removed with treated red cells. 5. Treating the adsorbing cells with enzyme or ZZAP typically enhances the adsorption process. In addition, the treated red cells will lack the antigens destroyed by dithiothreitol (DTT) and/or enzymes. |

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| References | 1. Branch DR, Petz LD. A new reagent (ZZAP) having multiple applications in immunohematology. Am J Clin Pathol 1982;78:161-7. 2. Judd WJ, Johnson ST, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. |

**METHOD 4-10. POLYETHYLENE GLYCOL ADSORPTION PROCEDURE**

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| Principle | Polyethylene glycol (PEG) enhances the adsorption of antibody by untreated red cells. Testing the adsorbed aliquot against a panel of red cells can identify the specificity of antibodies that remain after adsorption. This method can be used for both autologous and allogeneic adsorption. |

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| Specimen | Serum or plasma to be tested. |

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| Reagents | 1. PEG, 20% (20 g PEG, 3350 MW, in 100 mL of PBS, pH 7.3) or commercial PEG enhancement reagent. 2. Autologous red cells or ABO-compatible allogeneic red cells of known phenotype (see table below). Reserve a sample of these red cells to test for completeness of adsorption (Step 5). | |
| Procedure | **Step** | **Action** |
| 1 | Wash aliquots of red cells in large volumes of saline three times and centrifuge for 5 to 10 minutes at 1000 × *g*. Remove all residual saline. |
|  | 2 | To 1 volume (eg, 1 mL) of red cells, add 1 volume of serum and 1 volume of PEG. Mix well and incubate at 37 C for 15 minutes. |
|  | 3 | Centrifuge the serum/PEG/cell mixture for 5 minutes and harvest the adsorbed serum/PEG mixture. |
|  | 4 | To test the adsorbed serum, add 4 drops of the serum/PEG mixture to 1 drop of test red cells, incubate for 15 minutes at 37 C, and proceed to the antiglobulin test with anti-IgG. The larger volume of serum tested (4 drops) is required to account for the dilution of the serum by the PEG. See Notes 3 and 4. |
|  | 5 | To check for completeness of adsorption, test the adsorbed serum against the red cells used for the adsorption. If positive, repeat the adsorption by adding the adsorbed serum to a fresh aliquot of red cells but do not add additional PEG. If the test result was negative, test the adsorbed serum with a panel of cells. |

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| Notes | 1. Red cells for adsorption may be chemically modified (eg, with enzymes or ZZAP) before adsorption if denaturation of antigens is desired. |
|  | 1. Although many laboratories successfully use the PEG adsorption method, some serologists have reported a weakening or loss of antibody reactivity in some samples when compared with results obtained using a different technique. To accommodate this potential weakening of antibody reactivity, some serologists test 6 drops of the PEG-adsorbed serum. Testing with double-dose antigens will also increase sensitivity. |
|  | 1. Test the adsorbed serum on the day it was adsorbed. Weak antibody reactivity may be lost upon storage of PEG-adsorbed sera, possibly as a result of precipitation of the protein noticeable after 4 C storage. |
|  | 1. Agglutination of the adsorbing red cells does not occur when PEG is used; therefore, there is no visible clue to the efficiency of the adsorption process. As a guide, when the original serum reactivity is 1+ in the low-ionic-strength saline indirect antiglobulin test (LISS-IAT), usually only one adsorption is required. Antibodies with 2+ to 3+ reactivity will generally be removed in two adsorptions. |

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| References | 1. Leger RM, Garratty G. Evaluation of methods for detecting alloantibodies underlying warm autoantibodies. Transfusion 1999;39:11-16. 2. Leger RM, Ciesielski D, Garratty G. Effect of storage on antibody reactivity after adsorption in the presence of polyethylene glycol. Transfusion 1999;39:1272-3. |

**METHOD 4-11. PERFORMING THE DONATH-LANDSTEINER TEST**

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| Principle | IgG autoantibodies that cause paroxysmal cold hemoglobinuria (PCH) act as biphasic hemolysins in vitro. The IgG autoantibodies bind to the red cells at cold temperatures, and as the test is warmed to 37 C, complement is activated and lysis of the red cells occurs. The patient for whom this procedure should be considered is one with a positive direct antiglobulin test (DAT) result due to C3; demonstrable hemoglobinemia, hemoglobinuria, or both; and no evidence of autoantibody activity in the serum or the eluate made from the DAT-positive cells. A discussion of PCH is found in Chapter 14 of the AABB *Technical Manual.* |

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| Specimen | Serum separated from a freshly collected blood sample maintained at 37 C. See Note 1. |

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| Reagents | 1. Freshly collected pooled normal sera known to lack unexpected antibodies, to use as a source of complement. 2. 50% suspension of washed group O red cells that express the P antigen (eg, antibody detection cells). |

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| Procedure | **Step** | **Action** |
| 1 | Label three sets of three 10 × 75-mm test tubes as follows: A1-A2-A3; B1-B2-B3; C1-C2-C3. |
|  | 2 | To tubes 1 and 2 of each set, add 10 volumes (eg, drops) of the patient’s serum. |
|  | 3 | To tubes 2 and 3 of each set, add 10 volumes of fresh normal serum. |
|  | 4 | To all tubes, add one volume of the 50% suspension of washed P-positive red cells and mix well. |
|  | 5 | Place the three “A” tubes in a bath of melting ice for 30 minutes and then at 37 C for 1 hour. |
|  | 6 | Place the three “B” tubes in a bath of melting ice and keep them in melting ice for 90 minutes. |
|  | 7 | Place the three “C” tubes at 37 C and keep them at 37 C for 90 minutes. |
|  | 8 | Gently mix and centrifuge all tubes. Examine the supernatant fluid for hemolysis. |

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| Interpretation | The Donath-Landsteiner test result is considered positive when the patient’s serum, with or without added complement, causes hemolysis in the tubes that were incubated first in melting ice and then at 37 C (ie, tubes A1 and A2) and there is no hemolysis in any of the tubes maintained throughout at 37 C (ie, tubes C1, C2) or in melting ice (ie, tubes B1, B2). The A3, B3, and C3 tubes serve as controls of the normal sera complement source and should not manifest hemolysis. |

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| Notes | 1. To avoid loss of antibody by autoadsorption before testing, the patient’s blood should be allowed to clot at 37 C, and the serum separated from the clot at this temperature. 2. Active complement is essential for demonstration of the antibody. Because patients with PCH may have low levels of serum complement, fresh normal serum should be included in the reaction medium as a source of complement. 3. If a limited amount of blood is available (eg, from young children), set up tubes A­1, A2, A3, C1, and C2; if there is only enough serum for two tests (ie, 20 drops), set up tubes A2, A3, and C2. 4. To demonstrate the P specificity of the Donath-Landsteiner antibody, ABO-compatible p red cells should be tested in a second set of tubes A-1, A-2, and A-3. No lysis should develop in these tubes, confirming the P specificity of the antibody. |

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| References | 1. Judd WJ, Johnson ST, Storry JR. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. 2. Bain B, Bates I, Laffan M, Lewis S. Dacie and Lewis practical haematology. 11th ed. London, England: Churchill Livingston, 2012. 3. Borge PD Jr, Mansfield PM. The positive direct antiglobulin test and immune-mediated hemolysis. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:429-55. |

**METHOD 4-12. DETECTING DRUG ANTIBODIES BY TESTING DRUG-TREATED RED CELLS**

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| Principle | Some drugs, principally penicillin and many cephalosporins, can induce an IgG immune response that can be detected by testing red cells treated with the drug. The preparation of the drug used should, to the extent possible, be the same as what was given to the patient. Antibodies to either penicillin or cephalothin may be cross-reactive with cells treated with the other drug (ie, penicillin antibodies may attach to cephalothin-treated cells and vice versa). Antibodies to other cephalosporins may be reactive with cephalothin-treated cells. |

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| Specimen | Serum or plasma *and* eluate (and last wash) to be studied. |

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| Reagents | 1. 0.1 M sodium barbital buffer (BB) at pH 9.6 to 9.8, prepared by dissolving 2.06 g of sodium barbital in 80 mL of distilled or deionized water. Adjust the pH to between 9.6 and 9.8 with 0.1 N HCl. Bring total volume to 100 mL. Store at 2 to 8 C. 2. Phosphate-buffered saline (PBS), pH 7.3. 3. Drug (eg, penicillin, cephalosporin). 4. Washed, packed, group O red cells. 5. Normal sera/plasma (antibody free) as a negative control. 6. Positive control serum/plasma, if available. 7. Antihuman globulin or anti-IgG. 8. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare drug solutions just before use. See Notes 1 and 2.   1. For penicillin-treated cells, dissolve 600 mg of penicillin in 15 mL of BB. This high pH is optimal, but if the buffer is unavailable, PBS, pH 7.3, can be used. Add 1 mL of red cells. In a separate tube, prepare control cells by adding 1 mL of untreated red cells (without the drug) to 15 mL of the same buffer. Incubate both tubes for 1 hour at room temperature with occasional mixing. Wash three times and prepare a 5% suspension in PBS. 2. For cephalosporin-treated cells, dissolve 400 mg of the drug in 10 mL of PBS, pH 7.3. Add 1 mL of red cells. In a separate tube, prepare control cells by adding 1 mL of untreated red cells (without the drug) to 10 mL of PBS. Incubate both tubes for 1 hour at 37 C with occasional mixing. Wash three times and prepare a 5% suspension in PBS. |
|  | 2 | Label two sets of tubes (drug-treated and untreated) for each sample to be tested: serum, eluate, last wash, PBS, negative control sera/plasma, and positive control. If the drug is known to cause nonimmunologic protein adsorption, also test a 1-in-20 dilution of the patient’s serum and the controls (negative and positive). |
|  | 3 | Add 2 or 3 drops of each sample to the appropriate tube. |
|  | 4 | Add 1 drop of a 5% saline suspension of drug-treated red cells to one set of tubes. Add 1 drop of a 5% saline suspension of untreated red cells to the second set of tubes. |
|  | 5 | Incubate the tubes at 37 C for 60 minutes. Centrifuge and examine for hemolysis and agglutination. Record the results. |
|  | 6 | Wash the cells four times in saline, and test by an indirect antiglobulin technique using polyspecific antihuman globulin or anti-IgG reagent. Centrifuge and examine for agglutination. Record the results. |
|  | 7 | Confirm the validity of negative test results by adding IgG-coated red cells. |

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| Interpretation | Reactivity (hemolysis, agglutination, and/or positive indirect antiglobulin test result) with drug-treated cells, but not with untreated cells, indicates that drug antibodies are present (see Note 4). No hemolysis will be seen in tests with plasma or the eluate.  Negative results without a positive control can be interpreted to mean only that drug antibodies were not detected. The drug may or may not be bound to the test red cells. |

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| Notes | 1. The volume of drug-treated red cells can be scaled down as long as the ratio of the 40 mg/mL drug solution to red cells is constant (eg, 120 mg penicillin in 3 mL BB plus 0.2 mL red cells, or 100 mg cephalosporin in 2.5 mL PBS plus 0.25 mL red cells). 2. Drug-treated red cells may be kept in PBS at 4 C for up to 1 week; however, there may be some weakening of drug coating upon storage. Drug-treated and untreated red cells may also be stored frozen. 3. Cephalosporins do not require a high pH for optimal coating of red cells. In fact, a lower pH (ie, pH 6 to 7) decreases nonspe­cific protein adsorption seen when a high pH buffer is used. The least amount of nonspecific protein adsorption by drug-treated red cells will occur if a pH 6.0 buffer is used, but this leads to a slight decrease in coating by the drug. 4. To control for nonspecific protein adsorption of normal sera observed with some cephalosporins (eg, cephalothin), test the control sera and the test serum at a 1-in-20 dilution in PBS. Normal sera diluted 1 in 20 generally do not react nonspecifically. Thus, reactivity of the diluted serum with the drug-treated cells but not with the untreated cells indicates that drug antibody is present. 5. When antibodies are not detected with drug-treated red cells, test for drug antibodies in the presence of the drug. Antibodies to some third-generation cephalosporins (eg, ceftriaxone) do not react with drug-treated red cells. 6. For drugs other than penicillin and the cephalosporins, refer to published reports for the method used to treat the red cells. Normal sera may contain weak antibodies to penicillin or cephalosporins, presumably as a result of environmental exposure. 7. Reactivity of the eluate against the penicillin­-treated red cells and no reactivity of the eluate against the uncoated red cells are definitive of a penicillin-induced positive DAT result. |

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| Reference | 1. Petz LD, Garratty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004. 2. Leger RM, Arndt PA, Garratty G. How we investigate drug-induced immune hemolytic anemia. Immunohematology 2014;30:85-94. |

**METHOD 4-13. DETECTING DRUG ANTIBODIES BY TESTING IN THE PRESENCE OF DRUG**

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| Principle | Some drug antibodies are demonstrable when the patient's serum is tested in the presence of soluble drug (or metabolite) against untreated or enzyme-treated red cells. In the past, this has been referred to as the “immune complex” method, although the actual mechanism has not been proven.1 |

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| Specimen | The patient’s serum. |

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| Reagents | 1. The drug under investigation, in the same form (powder, tablet, capsules) that the patient is receiving. 2. Phosphate-buffered saline (PBS) at pH 7.0 to 7.4. 3. Fresh, normal serum known to lack unexpected antibodies, as a source of complement. 4. Pooled group O reagent red cells, 5% sus­pension: one aliquot treated with a proteolytic enzyme, and one untreated. 5. Polyspecific antihuman globulin reagent. 6. IgG-coated red cells. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare a 1-mg/mL solution of the drug in PBS. Centrifuge to remove any particulate matter, and adjust the pH of the supernatant fluid to approximately 7 with either 1 N NaOH or 1 N HCl, as required, if the pH is below 5 or above 8. |
|  | 2 | Label two sets of three tubes (untreated and enzyme-treated) for the following test mixtures:   1. Patient’s serum + drug. 2. Patient’s serum + PBS. 3. Patient’s serum + complement (normal serum) + drug. 4. Patient’s serum + complement (normal serum) + PBS. 5. Normal serum + drug. 6. Normal serum + PBS. |
|  | 3 | Add two volumes (eg, 2 drops) of each component to the appropriate tubes (eg, 2 drops of serum + 2 drops of drug). |
|  | 4 | Add 1 drop of a 5% saline suspension of untreated group O reagent red cells to one set of tubes. Add 1 drop of a 5% saline suspension of enzyme-treated group O reagent red cells to the second set of tubes. |
|  | 5 | Mix and incubate at 37 C for 1 to 2 hours, with periodic gentle mixing. |
|  | 6 | Centrifuge, examine for hemolysis and agglutination, and record the results. |
|  | 7 | Wash the cells four times in saline, and test with a polyspecific antiglobulin reagent. |
|  | 8 | Centrifuge, examine for agglutination, and record the results. |
|  | 9 | Confirm the validity of negative test results by adding IgG-coated red cells. |

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| Interpretation | Hemolysis, direct agglutination, or positive indirect antiglobulin tests can occur together or separately. Reactivity in any of the tests containing the patient’s serum to which the drug was added, and absence of reactivity in the corresponding control tests containing PBS instead of the drug, indicate that antibody to the drug is present. See Note 4. |

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| Notes | 1. The drug may be more easily dissolved by incubation at 37 C and vigorous shaking of the solution. If the drug is in tablet form, crush it with a mortar and pestle and remove any visible outer tablet coating material before adding PBS. |
|  | 1. Not all drugs will dissolve completely in PBS. Consult the manufacturer or a reference such as the Merck Index for the solubility of the drug in question. A previous report of drug-induced immune hemolytic anemia resulting from the drug in question may provide information on the drug solution preparation. |
|  | 1. When available, a serum or plasma known to contain antibody with the drug specificity being evaluated should be included as a positive control. |
|  | 1. Tests without the drug added may be positive if autoantibodies or circulating drug­antibody immune complexes are present in the patient’s sample. Autoantibody reactivity would be persistent over time, whereas circulating immune complexes are transient. |
|  | 1. Testing with enzyme-treated red cells and the addition of fresh normal serum as a source of complement may increase the sensitivity of the test. |
|  | 1. If testing in the presence of the drug and tests with drug-treated red cells are noninformative, consider testing with a metabolite of the drug.2 |

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| References | 1. Petz LD, Garratty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004. 2. Johnson ST, Fueger JT, Gottschall JL. One center’s experience: The serology and drugs associated with drug-induced immune hemolytic anemia—a new paradigm. Transfusion 2007;47:697-702. 3. Leger RM, Arndt PA, Garratty G. How we investigate drug-induced immune hemolytic anemia. Immunohematology 2014;30:85-94. |

**METHODS FOR HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN**

Sensitizing exposures to fetal red cell antigens can occur during pregnancy through bleeding from the fetal to maternal circulation. Such sensitizing events may occur at the time of delivery, miscarriage, abortion, or invasive procedures such as amniocentesis.

The fetus does not have a competent immune system and therefore cannot make red cell alloantibodies. However, the mother who is sensitized can make antibodies against the fetal antigens. Any maternally produced IgG may cross the placenta and hemolyze the fetal red cells. Testing for hemolysis is necessary to determine the correct treatment (eg, Rh Immune Globulin, exchange transfusion).

**METHOD 5-1. TESTING FOR FETOMATERNAL HEMORRHAGE—THE ROSETTE TEST**

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| Principle | This test detects D+ red cells in the blood of a D– woman whose fetus or recently delivered infant is D+. When reagent anti-D is added to maternal blood containing D+ fetal cells, fetal red cells become coated with anti-D. When D+ reagent cells are subsequently added, easily visible rosettes are formed, with several red cells clustered around each antibody-coated D+ red cell. |

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| Specimen | A 2% to 5% saline suspension of washed red cells from a maternal blood sample. |

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| Reagents | Prepared reagents are commercially available. The steps below can be used for in-house preparation.   1. Negative control: a 2% to 5% saline suspension of washed red cells known to be D–. 2. Positive control: a 2% to 5% saline suspension of a mixture containing approximately 0.6% D+ red cells and 99.4% D– red cells. The positive control can be prepared by adding 1 drop of a 2% to 5% suspension of D+ control cells to 15 drops of a 2% to 5% suspension of washed D– control cells. Mix well, then add 1 drop of this cell suspension to 9 drops of the 2% to 5% suspension of D– red cells. Mix well. 3. Indicator red cells: a 2% to 5% saline suspension of group O, R2R2 red cells. Either enzyme-treated or untreated cells in an enhancing medium can be used. 4. High-protein reagent anti-D serum. Some monoclonal/polyclonal blended reagents are unsuitable for use in this method. The antisera selected for use should be evaluated for suitability before incorporation into the test procedure. |

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| Procedure | **Step** | **Action** |
| 1 | To each of three test tubes, add 1 drop (or the volume specified in the manufacturer’s instructions) of reagent anti-D. |
|  | 2 | Add 1 drop of maternal cells, negative control cells, and positive control cells to the appropriately labeled tubes. |
|  | 3 | Incubate at 37 C for 15 to 30 minutes, or as specified by the manufacturer’s instructions. |
|  | 4 | Wash cell suspensions at least four times with large volumes of saline, to remove all unbound reagent anti-D. Decant saline completely after last wash. |
|  | 5 | To the dry cell button, add 1 drop of indicator cells and mix thoroughly to resuspend them. |
|  | 6 | Centrifuge the tubes for 15 seconds at 900 to 1000 × *g*. |
|  | 7 | Resuspend the cell button and examine the red cell suspension microscopically at 100× to 150× magnification. |
|  | 8 | Examine at least 10 fields and count the number of red cell rosettes in each field. |

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| Interpretation | The absence of rosettes is a negative result. With enzyme-treated indicator cells, up to one rosette per three fields may occur in a negative specimen. With untreated indicator cells and an enhancing medium, there may be up to six rosettes per five fields in a negative test. The presence of more rosettes than these allowable maxima constitutes a positive result, and the specimen should be examined using a test that quantifies the amount of fetal blood present.  The presence of rosettes or agglutination in the negative control tube indicates inadequate washing after incubation, allowing residual anti-D to agglutinate the D+ indicator cells. A strongly positive result is seen with red cells from a woman whose Rh phenotype is weak D rather than D–; massive fetomaternal hemorrhage may produce an appearance difficult to distinguish from that caused by a weak D phenotype, and a quantitative test for fetal cells should be performed. If the infant’s cells are shown to be weak D, a negative result on the mother’s specimen should be interpreted with caution. In this situation, a quantitative test that does not rely on D antigen expression should be performed. |

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| Notes | 1. Although the number of rosettes is roughly proportional to the number of D+ red cells present in the original mixture, this test provides only qualitative information about fetal-maternal admixture. Specimens giving a positive result should be subjected to further testing to quantify the number of fetal cells. 2. The acid-elution procedure and flow cytometry are acceptable choices. If a commercial test is available, the directions in the package insert should be followed. |

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| Reference | 1. Sebring ES, Polesky HF. Detection of fetal maternal hemorrhage in Rh immune globulin candidates. Transfusion 1982;22:468-71. |

**METHOD 5-2. TESTING FOR FETOMATERNAL HEMORRHAGE—MODIFIED KLEIHAUER-BETKE TEST**

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| Principle | Fetal hemoglobin resists elution from red cells under acid conditions, whereas adult hemoglobin is eluted. When a thin blood smear is exposed to an acid buffer, hemoglobin from adult red cells is leached into the buffer so that only the stroma remains; fetal cells retain their hemoglobin and can be identified by a positive staining pattern. The approximate volume of fetomaternal hemorrhage can be calculated from the percentage of fetal red cells in the maternal blood film. |

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| Specimen | Maternal anticoagulated whole blood sample. |

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| Reagents | Prepared reagents are commercially available in kits. The directions below can be used for in-house preparations.   1. Stock solution A (0.1 M of citric acid): C6H8O7•H2O, 21.0 g, diluted to 1 liter with distilled water. Keep refrigerated. 2. Stock solution B (0.2 M of sodium phosphate): Na2HPO4•7H2O, 53.6 g, diluted to 1 liter with distilled water. Keep refrigerated. 3. McIlvaine’s buffer, pH 3.2: Add 75 mL of stock solution A to 21 mL of stock solution B. Prepare fresh mixture for each test. This buffer mixture should be brought to room temperature or used at 37 C. 4. Erythrosin B, 0.5% in water. 5. Harris hematoxylin (filtered). 6. 80% ethyl alcohol. 7. Positive control specimen: Ten parts of anticoagulated adult blood, mixed with one part of anticoagulated ABO-compatible cord blood. 8. Negative control specimen: Anticoagulated adult blood. |

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| Procedure | **Step** | **Action** |
| 1 | Prepare very thin blood smears, diluting blood with an equal volume of saline. Air dry. |
|  | 2 | Fix the smears in 80% ethyl alcohol for 5 minutes. |
|  | 3 | Wash the smears with distilled water. |
|  | 4 | Immerse the smears in McIlvaine’s buffer, pH 3.2, for 11 minutes at room temperature or 5 minutes at 37 C. This reaction is temperature sensitive. |
|  | 5 | Wash the smears in distilled water. |
|  | 6 | Immerse the smears in erythrosin B for 5 minutes. |
|  | 7 | Wash the smears completely in distilled water. |
|  | 8 | Immerse the smears in Harris hematoxylin for 5 minutes. |
|  | 9 | Wash the smears in running tap water for 1 minute. |
|  | 10 | Examine the smears dry using 40× magnification, count a total of 2000 red cells, and record the number of fetal cells observed. |
|  | 11 | Calculate the percentage of fetal red cells in the total counted. |

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| Interpretation | 1. Fetal cells are bright pink and refractile; normal adult red cells appear as very pale “ghosts.” 2. The conversion factor used to indicate the volume (as mL of whole blood) of fetomaternal hemorrhage is the percentage of fetal red cells observed times 50. |

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| Note | The accuracy and precision of this procedure are poor, and decisions regarding Rh Immune Globulin (RhIG) dosage in massive fetomaternal hemorrhage should be made with this understanding. If there is a question regarding the need for additional RhIG, it is preferable to administer another dose to prevent the risks of undertreatment. (See table below for dosage.)  A screenshot of a cell phone  Description automatically generated  Notes:  1. Based on a maternal blood volume of 5000 mL.  2. 1 vial of 300 µɡ (1500 IU) is needed for each 15 mL of fetal red cells or 30 mL of fetal whole blood. |

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| Reference | 1. Sebring ES. Fetomaternal hemorrhage—incidence and methods of detection and quantitation. In: Garratty G, ed. Hemolytic disease of the newborn. Arlington, VA: AABB, 1984:87­118. |

**METHOD 5-3. USING ANTIBODY TITRATION STUDIES TO ASSIST IN EARLY DETECTION OF HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN**

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| Principle | In pregnancy, antibody titration is performed to identify women with significant levels of antibodies that may lead to hemolytic disease of the fetus and newborn (HDFN) and, for low-titer antibodies, to establish a baseline for comparison with titers found later in pregnancy. Titration of non-Rh antibodies should be undertaken only after discussion with the obstetrician about how the data will be used in the clinical management of the pregnancy. The significance of titers has been sufficiently established only for anti-D (using a saline technique). |

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| Specimen | Serum for titration (containing potentially significant unexpected antibodies to red cell anti­gens), 1 mL. If possible, test the current sample in parallel with the most recent previously submitted (preceding) sample from the current pregnancy. |

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| Materials | 1. Antihuman IgG: need not be heavy-chain specific. 2. Isotonic saline. 3. Volumetric pipettes, or equivalent: 0.1- to 0.5-mL delivery, with disposable tips. 4. Red cells: group O reagent red cells, 2% suspension. (See Note 2 regarding the selection of red cells for testing.) Avoid using Bg+ red cells because they may result in falsely high values, especially with sera from multiparous women. 5. IgG-coated red cells. |

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| Quality Control | 1. Test the preceding sample in parallel with the most recent sample. 2. Prepare the dilutions using a separate pipette for each tube. Failure to do so will result in falsely high titers because of carryover. 3. Confirm all negative reactions with IgG-coated red cells (see Step 9 below). |

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| Procedure | **Step** | **Action** |
| 1 | Using 0.5-mL volumes, prepare serial twofold dilutions of serum in saline. The initial tube should contain undiluted serum and the doubling dilution range should be from 1 in 2 to 1 in 2048 (total of 12 tubes). |
|  | 2 | Place 0.1 mL of each dilution into appropriately labeled test tubes. |
|  | 3 | Add 0.1 mL of the 2% suspension of red cells to each dilution. Alternatively, for convenience, add 1 drop of a solution of a 3% to 4% suspension of red cells as supplied by the reagent manufacturer, although this method is less precise. |
|  | 4 | Gently agitate the contents of each tube; incubate at 37 C for 1 hour. |
|  | 5 | Wash the red cells four times with saline; completely decant the final wash supernatant. |
|  | 6 | To the dry red cell buttons thus obtained, add anti-IgG according to the manufacturer’s directions. |
|  | 7 | Centrifuge as for hemagglutination tests. |
|  | 8 | Examine the red cells macroscopically; grade and record the reactions. |
|  | 9 | Add IgG-coated red cells to all negative tests; recentrifuge and examine the tests for macroscopic agglutination; repeat the testing if the tests with IgG-coated red cells are nonreactive. |

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| Interpretation | The titer is reported as the reciprocal of the highest dilution of serum at which 1+ agglutination is observed. Depending on the antibody specificity, a titer of ≥16 (this value may vary according to the laboratory) is considered significant and may warrant further monitoring for HDFN. For instance, some laboratories consider any amount of anti-K antibody clinically significant. |

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| Notes | 1. Antibody titration is a semi-quantitative method of determining antibody concentration. Serial twofold dilutions of serum are prepared and tested for antibody activity. The reciprocal of the highest dilution of plasma or serum that gives a 1+ reaction is referred to as the titer (ie, 1 in 128 dilution; titer = 128). 2. The selection of the most suitable phenotype of red cells to use when performing titration studies for HDFN is controversial. Some workers select red cells that have the strongest expression of antigen, such as R2R2 for anti-D. Others select red cells with the phenotype that would be expected in fetal circulation—ie, red cells that express a single dose of the antigen, such as R1r for testing for anti-D. Whichever viewpoint is followed, it is important that the laboratory be consistent and use red cells of the same phenotype for future titrations to test the same patient’s serum. 3. Some titration methods have been proposed that may show less variation, although no one method has been shown to be superior.6,7 4. Titration studies should be performed upon initial detection of the antibody. Save an appropriately labeled aliquot of the serum (frozen at –20 C or colder) for comparative studies with the next submitted sample for each sample received. 5. When the titer (eg, ≥16) and the antibody specificity have been associated with HDFN, it is recommended that repeat titration studies be performed every 2 to 4 weeks, beginning at 18 weeks’ gestation. 6. Each institution should develop a policy to ensure a degree of uniformity in reporting and interpreting antibody titers. 7. For antibodies to low-prevalence antigens, consider using putative paternal red cells, having established that they express the antigen in question. 8. Do not use enhancement techniques [albumin, polyethylene glycol, low-ionic-strength saline (LISS)] or enzyme-treated red cells because falsely elevated titers may be obtained. Gel testing is not recommended. 9. LISS should not be used as a diluent in titration studies; nonspecific uptake of globulins may occur in serum-LISS dilutions. 10. Failure to obtain the correct results may be caused by 1) incorrect technique, notably failure to use separate pipette tips for each dilution, or 2) failure to adequately mix thawed frozen serum. |

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| References | 1. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998:1067-9. 2. Judd WJ, Luban NLC, Ness PM, et al. Prenatal and perinatal immunohematology: Recommendations for serologic management of the fetus, newborn infant, and obstetric patient. Transfusion 1990;30:175-83. 3. Judd WJ, Johnson ST, Storry J. Judd’s methods in immunohematology. 3rd ed. Bethesda, MD: AABB Press, 2008. 4. Judd WJ. Practice guidelines for prenatal and perinatal immunhematology, revisited. Transfusion 2001;41:1445-52. 5. Judd WJ for the Scientific Section Coordinating Committee. Guidelines for prenatal and perinatal immunohematology. Bethesda, MD: AABB, 2005. 6. AuBuchon JP, de Wildt-Eggen J, Dumont LJ, et al. Reducing the variation in performance of antibody titrations. Arch Pathol Lab Med 2008;132:1194-201. 7. Bachegowda LS, Cheng YH, Long T, Shaz BH. Impact of uniform methods on interlaboratory antibody titration variability: Antibody titration and uniform methods. Arch Pathol Lab Med 2017;141:131-8. |

**BLOOD COLLECTION, COMPONENT PREPARATION, AND STORAGE METHODS**

Whole blood collection from the donor and subsequent processing of the donation into separate components are critical steps in the transfusion chain of events. Careful attention to proper technique is a key factor in ensuring optimal care of the donor with minimized risk and optimal benefit to the recipient.

Plasma proteins and cellular components require different storage conditions to preserve function and viability. Appropriate storage temperatures must be maintained, monitored, and recorded.

**METHOD 6-1. SCREENING FEMALE DONORS FOR ACCEPTABLE HEMOGLOBIN LEVEL—COPPER SULFATE METHOD**

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| Principle | This method estimates the hemoglobin content of blood from its specific gravity. A drop of blood in contact with copper sulfate solution of specific gravity 1.053 becomes encased in a sac of copper proteinate, which prevents dispersion of the fluid or any change in specific gravity for about 15 seconds. If the specific gravity of the blood is higher than that of the solution, the drop will sink within 15 seconds; if not, the drop will hesitate and remain suspended or rise to the top of the solution. A specific gravity of 1.053 corresponds to a hemoglobin concentration of 12.5 g/dL. Note that this concentration is only appropriate for testing females by FDA guidelines. A different solution would need to be used for males. |

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| Reagents and Materials | 1. Copper sulfate solution at specific gravity 1.053, available commercially. Store in tightly capped containers to prevent evaporation. Keep solution at room temperature or bring to room temperature before use. 2. Sterile gauze, antiseptic wipes, and sterile lancets. 3. Containers for the disposal of sharps and other biohazardous materials. 4. Capillary tubes and dropper bulbs or a device to collect capillary blood without contact. |

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| Procedure | **Step** | **Action** |
| 1 | Into a labeled, clean, dry tube or bottle, dispense a sufficient amount (at least 30 mL) of copper sulfate solution to allow the drop to fall approximately 3 inches. Change the solution daily or after 25 tests. Be sure that the solution is adequately mixed before beginning each day’s determinations. |
|  | 2 | Clean the site of the skin puncture thoroughly with antiseptic solution and wipe it dry with sterile gauze. |
|  | 3 | Puncture the finger firmly, near the end but slightly to the side, with a sterile, disposable lancet or spring-loaded, disposable needle system. A good free flow of blood is important. Do not squeeze the puncture site repeatedly because this may dilute the drop of blood with tissue fluid and lower the specific gravity. |
|  | 4 | Collect the blood in a capillary tube without allowing air to enter the tube. |
|  | 5 | Let one drop of blood fall gently from the tube at a height about 1 cm above the surface of the copper sulfate solution. |
|  | 6 | Observe for 15 seconds. |
|  | 7 | Dispose of lancets and capillary tubes in appropriate biohazard containers. Dispose of gauze appropriately; gauze contaminated with droplets of blood that subsequently dry such that the item is stained but not soaked or caked may be considered nonhazardous. |

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| Interpretation | 1. If the drop of blood sinks, the female donor’s hemoglobin is at an acceptable level for blood donation. 2. If the drop of blood does not sink, the donor’s hemoglobin may not be at an acceptable level for blood donation. If time and equipment permit, it is desirable to perform a quantitative measurement of hemoglobin or hematocrit. |

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| Notes | 1. This is not a quantitative test; it shows only whether the prospective donor’s hemoglobin is below or above the acceptable level of 12.5 g/dL. This hemoglobin level is acceptable for a female donor only; acceptable level for a male is 13.0 g/dL.1 2. False-positive reactions are rare; donors whose drop of blood sinks nearly always have an acceptable hemoglobin level. False-negative reactions occur fairly commonly and can cause inappropriate deferral.2,3 Measuring hemoglobin by another method or determining hematocrit sometimes reveals that the prospective donor is acceptable. 3. A certificate of analysis from the manufacturer should be obtained with each new lot of copper sulfate solution. 4. Used solution should be disposed of as biohazardous or chemical material because of the blood in the container. Refer to local and state laws regarding disposal. 5. Use care to prevent blood from contaminating work surfaces, the donor’s clothing, or other persons or equipment. 6. Cover the container between uses to prevent evaporation. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Lloyd H, Collins A, Walker W, et al. Volunteer blood donors who fail the copper sulfate screening test: What does failure mean, and what should be done? Transfusion 1988;28: 467-9. 3. Morris MW, Davey FR. Basic examination of blood. In: Henry JB, ed. Clinical diagnosis and management by laboratory methods. 20th ed. Philadelphia: WB Saunders, 2001:479-519. |

**METHOD 6-2. PREPARING THE DONOR’S ARM FOR BLOOD COLLECTION**

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| Principle | Iodophor compounds, or other antiseptic compounds, are used to clean the venipuncture site before blood collection. |

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| Materials | 1. Scrub solution: Disposable povidone-iodine scrub 0.75% or disposable povidone-iodine swab stick 10%; available in prepackaged single-use form. 2. Preparation solution: 10% povidone-iodine; available in prepackaged single-use form. 3. Sterile gauze. |

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| Procedure | **Step** | **Action** |
| 1 | Apply tourniquet or blood pressure cuff; identify venipuncture site; then release tourniquet or cuff. |
|  | 2 | Scrub area at least 4 cm (1.5 inches) in all directions from the intended site of venipuncture (ie, 8 cm or 3 inches in diameter) for a minimum of 30 seconds with 0.7% aqueous solution of iodophor compound. Excess foam may be removed, but the arm need not be dry before the next step. |
|  | 3 | Starting at the intended site of venipuncture and moving outward in a concentric spiral, apply “prep” solution; let stand for 30 seconds or as indicated by manufacturer. |
|  | 4 | Cover the area with dry, sterile gauze until the time of venipuncture. After the skin has been prepared, it must not be touched again. Do not repalpate the vein at the intended venipuncture site. |

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| Notes | 1. Detailed instructions are specific to each manufacturer and should be followed as indicated. The procedure above is written in general terms as an example. 2. For donors sensitive to iodine (tincture or povidone preparations), another method (eg, ChloraPrep 2% chlorhexidine and 70% isopropyl alcohol) should be designated by the blood bank physician. Green soap should not be used. 3. For donors sensitive to both iodine and chlorhexidine, a method using only isopropyl alcohol could be considered. The preferred procedure is the use of a 30-second up-and-down scrub, followed by enough time for the skin to dry. A second scrub is then applied. This method may require a variance from the US Food and Drug Administration or other applicable regulatory agency. |
| Reference | 1. Goldman M, Roy G, Fréchette N, et al. Evaluation of donor skin disinfection methods. Transfusion 1997;37:309-12. |

**METHOD 6-3. COLLECTING BLOOD AND SAMPLES FOR PROCESSING AND TESTING**

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| Principle | Blood for transfusion and accompanying samples are obtained from prominent veins on the donor’s arm, usually in the area of the antecubital fossa. |

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| Materials | 1. Sterile collection bag containing anticoagulant, with integrally attached tubing and needle. 2. Metal clips and hand sealer or dielectric sealer. 3. Balance or automated system to monitor volume of blood drawn. 4. Sterile gauze, arm scrub, and instruments (scissors, hemostats, forceps). 5. Test tubes for sample collection. 6. Device for stripping tubing. 7. Dielectric sealer (optional). | |
| Procedure | **Step** | **Action** |
| 1 | Inspect the collection bag for any defects or discoloration. Inspect the anticoagulant for particulates. |
|  | 2 | Prepare the collection bag and test tubes with the donor identification number. |
|  | 3 | Confirm the donor’s identity against the donor record, collection bag, and test tubes. |
|  | 4 | Position the collection bag below the donor’s arm.   1. If a balance system is used, be sure the counterbalance is level and adjusted for the amount of blood to be drawn. Hang the bag, and route the tubing through the pinch clamp. 2. If a balance system is not used, be sure to monitor the volume of blood drawn. 3. Unless metal clips and a hand sealer are used, make a very loose overhand knot in the tubing. |
|  | 5 | Apply a hemostat to the tubing near the needle. Do this before uncapping the needle to prevent air from entering the line. |
|  | 6 | Prepare the donor’s arm for blood collection (see method for donor arm preparation). |
|  | 7 | Apply tourniquet, or inflate blood pressure cuff. Ask the donor to open and close his or her hand until the previously selected vein is again prominent. |
|  | 8 | Uncap the sterile needle, and perform the venipuncture immediately.   1. Once the bevel has penetrated the skin, palpation of the skin above the needle stem may be performed with a gloved finger, provided the needle is not touched. 2. When the needle position is acceptable, tape the tubing to the donor’s arm to hold the needle in place, and cover the site with sterile gauze. 3. A clean, skillful venipuncture is essential for collection of a full, clot-free unit. |
|  | 9 | Release the hemostat for blood flow. Open the temporary closure between the interior of the bag and the tubing. |
|  | 10 | Ask the donor to open and close his or her hand slowly every 10 to 12 seconds during collection. |
|  | 11 | Fill the test tubes for donor testing by a method that prevents contamination of the contents of the bag.  This process can be done in several ways.   1. If the blood collection bag contains an in-line needle, make an additional seal with a hemostat, metal clip, hand sealer, or tight knot made from a previously prepared loose knot just distal to the in-line needle. Open the connector by separating the needles. Insert the proximal needle into a processing test tube, remove the hemostat, allow the tube to fill, and clamp the tubing. The donor needle is now ready for removal. 2. If the blood collection bag contains an in-line processing tube, be certain that the processing tube or pouch is full when the collection is complete and that the original clamp is placed near the donor needle. The entire assembly may now be removed from the donor. 3. If a straight-tubing assembly set is used, the following procedure should be followed. Place a hemostat on the tubing, allowing about four segments between the hemostat and the needle. Pull tight the loose overhand knot made in Step 4c. Release the hemostat, and strip a segment of the tubing free of blood between the knot and the needle (about 1 inch in length). Reapply the hemostat, and cut the tubing in the stripped area between the knot and the hemostat. Fill the required tube(s) by releasing the hemostat, and then reclamp the tubing with the hemostat. Because this system is open, Biosafety Level 2 precautions should be followed. 4. If a blood collection set that includes a sample diversion pouch is used, the following procedure should be followed. A hemostat is applied above the Y-junction before phlebotomy. Immediately after the phlebotomy, the hemostat is released to allow the blood to flow to the pouch. The sample pouch should be positioned below the donor’s arm. Ensure that blood is not flowing into the tubing leading to the collection bag. Fill the pouch with 30 to 35 mL of blood or as recommended by the manufacturer. Once the pouch is filled, apply a hemostat or Robert’s clamp to the tubing between the donor needle and the pouch. Break the in-line cannula in the tubing that leads from the donor needle to the collection bag to allow the blood to flow into the collection bag. 5. Sample tubes are collected from the pouch as follows. Sample tubes must be collected within 4 minutes or as recommended by the manufacturer from the time the hemostat is applied to stop the flow into the pouch. The manufacturer-supplied access device is inserted into the sampling site of the pouch by clockwise rotation. A sample tube is allowed to slide into the access device with a straight motion, and the tube is allowed to fill until the blood flow spontaneously stops. These steps are repeated for all sample tubes. |
|  | 12 | Check the labels on the sample tubes against the collection container after filling. |
|  | 13 | Monitor the blood being collected for mixing, volume, and timing.   1. Mixing may be done by hand (approximately every 45 seconds) or by continuous mechanical mixing. 2. If a balance is used, the device will interrupt blood flow after the proper amount has been collected. Generally, devices that are designed to mix the blood during collection also permit interruption of the flow when the preset amount is collected. One mL of blood weighs at least 1.053 g, indicated by the minimum allowable specific gravity for donors. The volumes and weights for 450-mL and 500-mL collection bags are given in the table below. |
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|  | 1. The time required for collection can be monitored by indicating the time of phlebotomy or the maximal allowable time (start time plus 15 minutes) on the donor’s record. Units requiring more than 15 minutes to draw may not be suitable for preparation of platelets, Fresh Frozen Plasma (FFP), or Cryoprecipitated AHF. |
|  | 14 | Monitor the donor throughout the donation process.   1. Blood flow. Be sure blood flow remains fairly brisk so that coagulation activity is not triggered. If there is continuous, adequate blood flow and constant agitation, rigid time limits are not necessary. 2. Adverse events. The donor should never be left unattended during or immediately after the donation. |
|  | 15 | Stop the blood flow when the appropriate volume has been collected.   1. Deflate the cuff or remove the tourniquet. 2. Clamp the tubing. 3. Remove the needle from the donor’s arm. 4. Apply pressure over the gauze, and ask the donor to raise his or her arm (elbow straight) and to hold the gauze firmly over the phlebotomy site with the other hand. |
|  | 16 | Discard the needle assembly into a biohazard container designed to prevent the accidental injury to, and contamination of, personnel. |
|  | 17 | Tend the donor’s phlebotomy site and move the donor to the canteen area. |
|  | 18 | Recheck numbers on the container, processing tubes, donation record, and retention segment. |
|  | 19 | Strip donor tubing as completely as possible into the bag, starting at the seal.   1. To prevent the blood from clotting in the tubing, work quickly. 2. Invert the bag several times to mix the contents thoroughly; then allow the tubing to refill with anticoagulated blood from the bag. 3. Repeat this procedure. |
|  | 20 | Seal the tubing attached to the collection bag into segments.   1. Leave the segment number clearly and completely readable. 2. Attach a unit identification number to at least one segment to be stored as a retention segment. 3. Knots, metal clips, or a dielectric sealer may be used to make segments suitable for compatibility testing. 4. It must be possible to separate segments from the unit without breaking sterility of the bag. 5. If a dielectric sealer is used, the knot or clip should be removed from the distal end of the tubing after creating a hermetic seal. |
|  | 21 | Reinspect the container for defects. |
|  | 22 | Place blood at appropriate temperature based on the components to be made from the unit. |

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| Notes | 1. Detailed instructions are specific to each manufacturer and should be followed as indicated. The procedure above is written in general terms as an example. 2. If the needle is withdrawn and venipuncture is attempted again, preparation of the site must be repeated as in the method for arm preparation for blood collection, and blood is drawn into a new collection set. 3. In addition to routine blood donor phlebotomy, this procedure may be adapted for use in therapeutic phlebotomy. 4. AABB *Standards for Blood Banks and Transfusion Services* requires that a diversion pouch be used for any blood collection from which platelets will be prepared. 5. Whole blood from which platelet concentrate will not be prepared should be placed at 1 to 6 C after collection unless it must be transported from the collection site to the processing laboratory. In that case, the blood must be placed in a temporary storage area having sufficient coolant capacity to cool the blood continuously toward a range between 1 and 10 C until the blood arrives at the processing laboratory. If platelets are to be prepared, blood should not be chilled but should be stored in a manner intended to reach a temperature of 20 to 24 C until platelets are separated. Platelets must be separated within 8 hours after collection of the unit of Whole Blood or within the time frame specified in the directions for use of the blood collecting, processing, and storage system. |
| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:24. 2. Smith LG. Blood collection. In: Green TS, Steckler D, eds. Donor room policies and procedures. Arlington, VA: AABB, 1985:25-45. 3. Huh YO, Lightiger B, Giacco GG, et al. Effect of donation time on platelet concentrates and fresh frozen plasma. Vox Sang 1989;56:21-4. 4. Sataro P. Blood collection. In: Kasprisin CA, Laird-Fryer B, eds. Blood donor collection practices. Bethesda, MD: AABB, 1993:89-103. |

**METHOD 6-4. PREPARING RED BLOOD CELLS FROM WHOLE BLOOD**

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| Principle | Red Blood Cells (RBCs) are obtained by removal of supernatant plasma from centrifuged whole blood. The volume of plasma removed determines the hematocrit of the component. |

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| Materials | 1. Freshly collected whole blood, obtained by phlebotomy. Collect blood in a collection unit with integrally attached transfer container(s). 2. Plasma extractor. 3. Metal clips and hand sealer. 4. Instruments (scissors, hemostats). 5. Dielectric sealer (optional). 6. Refrigerated centrifuge. 7. Scale. |

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| Procedure | **Step** | **Action** |
| 1 | If platelet-rich plasma will not be prepared, centrifuge whole blood using a “heavy” spin, with a temperature setting of 4 C. Heavy spin using 5000 × *g* for 5 minutes or 5000 × *g* for 7 minutes (plus deceleration time) should generally be sufficient. Each individual laboratory must establish its own parameters. If one is to calculate relative centrifugal force (RCF) in *g*, the formula given below should be used.    Centrifuge whole blood using a “light” spin if platelet-rich plasma is to be harvested. Light spin consisting of 2000 × *g* for 3 minutes (plus deceleration time) should generally be sufficient. |
|  | 2 | Place the primary bag containing centrifuged blood on a plasma expressor, and release the spring, allowing the plate of the expressor to contact the bag. |
|  | 3 | Temporarily clamp the tubing between the primary and satellite bags with a hemostat; if a mechanical sealer will not be used, make a loose overhand knot in the tubing. |
|  | 4 | If two or more satellite bags are attached, apply the hemostat to allow plasma to flow into only one of the satellite bags. Penetrate the closure of the primary bag. A scale, such as a dietary scale, may be used to measure the expressed plasma. Remove the appropriate amount of plasma to obtain the desired hematocrit. An automated expressor may also be used for this purpose. |
|  | 5 | Reapply the hemostat when the desired amount of supernatant plasma has entered the satellite bag. Seal the tubing between the primary bag and the satellite bag in two places. |
|  | 6 | Check that the satellite bag has the same donor number as that on the primary bag, and cut the tubing between the two seals. |

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| Notes | 1. When RBCs are preserved in citrate-phosphate-dextrose-adenine-1 (CPDA-1), maximal viability during storage requires an appropriate ratio of cells to preservative. A hematocrit of 80% or lower in CPDA-1 RBC units ensures the presence of adequate glucose for red cell metabolism for up to 35 days of storage. 2. If blood was collected in a single bag, modify the above directions as follows: Before centrifugation, attach a transfer bag to the Whole Blood unit using a sterile connection device. Alternatively, afterplacing the bag on the expressor, apply a hemostat to the tubing of a sterile transfer bag, aseptically insert the cannula of the transfer bag into the outlet port of the bag of blood, release the hemostat, and continue as outlined above. However, the expiration date will change as a result of the open system. 3. For 450 mL of whole blood collection, the removal of 230 to 256 g (225 to 250 mL) of plasma and preservation of the red cells in the anticoagulant-preservative solution will generally result in a red cell component with a hematocrit between 70% and 80%. Correspondingly, for 500 mL of whole blood collection, the removal of 256 to 281 g (250 to 275 mL) of plasma and the preservation of the red cells in the anticoagulant-preservative solution will generally result in a red cell component with a hematocrit between 70% and 80%. 4. The use of an additive solution allows removal of a greater volume of plasma in Step 4. After the plasma has been removed, the additive solution is allowed to flow from the attached satellite bag into the red cells. This process will result in a hematocrit of 55% to 65%. Be sure that an appropriate label and dating period are used. Follow the manufacturer’s instructions. |

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| Reference | 1. Formula for calculating relative centrifugal force. Boston, MA: Naval Blood Research Laboratory. |

**METHOD 6-5. PREPARING PRESTORAGE RED BLOOD CELLS LEUKOCYTES REDUCED FROM WHOLE BLOOD**

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| Principle | Red Blood Cells (RBCs) are obtained by removal of supernatant plasma from centrifuged whole blood. The volume of plasma removed determines the hematocrit of the component. The red cells are filtered using a special leukocyte reduction filter. |

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| Materials | 1. Freshly collected whole blood, obtained by phlebotomy. Collect blood in a collection unit with integrally attached transfer container(s). 2. Plasma extractor. 3. Metal clips and hand sealer. 4. Instruments (scissors, hemostats). 5. Dielectric sealer (optional). 6. Refrigerated centrifuge. 7. Scale. 8. In-line leukocyte reduction filter (if collection system does not include one). | |
| Procedure | **Step** | **Action** |
| 1 | Before centrifugation, the anticoagulated whole blood may be filtered by hanging the container upside down and allowing the blood to flow through an in-line filter by gravity into a secondary container. The steps in a method for the preparation of RBCs are then followed. |
|  | 2 | The anticoagulated whole blood may be centrifuged with the in-line filter attached. After centrifugation, the plasma is expressed. The additive solution (AS) is added, and the red cells in the AS are filtered by gravity, as in Step 1 above. |
|  | 3 | A red cell component prepared using a red cell preparation method either in residual anticoagulated plasma or in additive solution (AS-1, AS-3, AS-5) may have a secondary container with an in-line filter attached using a sterile connection device. Filtration can proceed according to the manufacturer’s directions using gravity, as in Step 1. The timing of this filtration is often within 24 hours of collection but can be up to 5 days or as directed by the manufacturer of the filter. |
|  | 4 | Red cells that are leukocyte reduced are labeled “Red Blood Cells Leukocytes Reduced.” There is no specific label for prestorage leukocyte reduction. |
| Notes | 1. In most cases, red cell leukocyte reduction filters licensed in the United States remove platelets to some degree. Anticoagulated whole blood may be filtered, from which only platelet-poor plasma (leukocyte reduced) and red cells may be made. However, the Food and Drug Administration (FDA) has approved a platelet-sparing, whole blood leukocyte reduction filter. 2. Alternatively, the red cells may be filtered in additive solution, potentially allowing the preparation of platelets, plasma, and red cells. Non-leukocyte-reduced red cells may also undergo leukocyte reduction after preparation by attaching a leukocyte reduction filter connected to a storage container using a sterile connection device. 3. If the collection system does not include an in-line filter, a sterile connection device can be used to attach a leukocyte reduction filter to the collection system. The filter should be used according to the manufacturer’s directions. 4. Generally, whole-blood-derived platelets can be manufactured only before leukocyte reduction. However, FDA-approved, platelet-sparing, whole blood leukocyte reduction filters are available. | |

**METHOD 6-6. USING HIGH-CONCENTRATION GLYCEROL TO CRYOPRESERVE RED CELLS—MERYMAN METHOD1**

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| Principle | Cryoprotective agents make possible the long-term (10 or more years) preservation of red cells in the frozen state. High-concentration glycerol is particularly suitable for this purpose. This method addresses Red Blood Cells (RBCs) collected in a 450-mL bag. |

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| Materials | 1. Donor blood, collected into citrate-phosphate-dextrose (CPD), citrate-phosphate-dextrose-dextrose (CP2D), citrate-phosphate-dextrose-adenine-1 (CPDA-1), or an additive solution (AS). 2. Complete all blood processing on units intended for freezing. 3. RBCs preserved in CPD, CP2D, or CPDA-1 may be stored at 1 to 6 C for up to 6 days before freezing. 4. RBCs preserved in AS-1 and AS-3 may be stored at 1 to 6 C for up to 42 days before freezing. 5. For RBCs that have undergone rejuvenation see manufacturer’s package insert. 6. RBCs that are in any preservative solution and that have been entered for processing must be frozen within 24 hours of puncturing the seal. 7. Storage containers, either polyvinyl chloride (PVC) or polyolefin bags. 8. 6.2 M of glycerol lactate solution (400 mL). 9. Cardboard or metal canisters for freezing. 10. Hypertonic (12%) NaCl solution. 11. 1.6% NaCl, 1 liter for batch wash. 12. Isotonic (0.9%) NaCl with 0.2% dextrose solution. 13. 37 C waterbath or 37 C dry warmer. 14. Equipment for batch or continuous-flow washing, to deglycerolize cells frozen in high-concentration glycerol. 15. Freezer tape. 16. Freezer (–65 C or colder). |

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| Procedure | **Step** | **Action** |
|  |  | *Preparing RBCs for Glycerolization* |
|  | 1 | Prepare RBCs from Whole Blood units by removal of supernatant anticoagulant-preservative or additive solution. Weigh the RBC unit to be frozen, and obtain the net weight of the red cells. The combined weight of the cells and the collection bag should be between 260 g and 400 g. |
|  | 2 | Underweight units can be adjusted to approximately 300 g either by the addition of 0.9% NaCl or by the removal of less plasma than usual. Record the weight; if applicable, document the amount of NaCl added. |
|  | 3 | Record the Whole Blood number, the ABO group and Rh type, the anticoagulant, the date of collection, the date frozen, the expiration time, and the identity of the person performing the procedure. If applicable, document the lot number of the transfer bag. |
|  | 4 | Warm the red cells and the glycerol to at least 25 C by placing them in a dry warming chamber for 10 to 15 minutes or by allowing them to remain at room temperature for 1 to 2 hours. The temperature must not exceed 42 C. |
|  | 5 | Apply a “Frozen Red Blood Cells” label to the freezing bag in which the unit will be frozen. The label must include the name of the facility freezing the unit, Whole Blood number, ABO group and Rh type, and the expiration date. The label must also include tracking for the date collected, the date frozen, and the cryoprotective agent used. |
|  |  | *Glycerolization* |
|  | 1 | Document the lot numbers of the glycerol; the freezing bags; and, if used, the 0.9% NaCl. |
|  | 2 | Place the container of red cells on a shaker, and add approximately 100 mL of glycerol as the red cells are gently agitated. |
|  | 3 | Turn off the shaker and allow the cells to equilibrate, without agitation, for 5 to 30 minutes. |
|  | 4 | Allow the partially glycerolized cells to flow by gravity into the freezing bag. |
|  | 5 | Add the remaining 300 mL of glycerol slowly in a stepwise fashion, with gentle mixing. Add smaller volumes of glycerol for smaller volumes of red cells. The final glycerol concentration is 40% w/v. Remove any air from the bag. |
|  | 6 | Allow some glycerolized cells to flow back into the tubing so that segments can be prepared. Preferably, two segments should be prepared so that the unit may be crossmatched and/or phenotyped before a decision to thaw. |
|  | 7 | Maintain the glycerolized cells at temperatures between 25 and 32 C until freezing. The recommended interval between removing the RBC unit from refrigeration and placing the glycerolized cells in the freezer should not exceed *4 hours.* |
|  |  | *Freezing and Storage* |
|  | 1 | Place the glycerolized unit in a cardboard or metal canister, and place it flat in a freezer at –65 C or colder. |
|  | 2 | Label the top edge of the canister with freezer tape marked with the Whole Blood number, the ABO group and Rh type, and the expiration date. |
|  | 3 | Do not bump or handle the frozen cells roughly. |
|  | 4 | The freezing rate should be less than 10 C/minute. |
|  | 5 | Store the frozen RBCs at –65 C or colder for up to 10 years. For blood of rare phenotypes, a facility’s medical director may wish to extend the storage period. The unusual nature of such units and the reason for retaining them past the routine 10-year storage period must be documented. |
|  |  | *Thawing and Deglycerolizing* |
|  | 1 | Put an overwrap on the protective canister containing the frozen cells, and place it in either a 37 C waterbath or 37 C dry warmer. |
|  | 2 | Agitate it gently to speed thawing. The thawing process takes at least 10 minutes. The temperature of the thawed cells should be 37 C. |
|  | 3 | After the cells have thawed, use a commercial instrument for batch or continuous-flow washing to deglycerolize cells. Follow the manufacturer’s instructions. |
|  | 4 | Record the lot numbers and manufacturer of all the solutions and software used. Apply a “Deglycerolized Red Blood Cells” label to the transfer pack; be sure that the label includes identification of the collecting facility, the facility preparing the deglycerolized cells, the ABO group and Rh type of the cells, the Whole Blood number, and the expiration date and time. |
|  | 5 | Dilute the unit with a quantity of hypertonic (12%) NaCl solution appropriate for the size of the unit. Allow it to equilibrate for approximately 5 minutes. |
|  | 6 | Wash the cells with 1.6% NaCl until deglycerolization is complete. Approxi­mately 2 liters of wash solution are required. See method on checking for residual glycerol. |
|  | 7 | Suspend the deglycerolized cells in isotonic (0.9%) NaCl with 0.2% dextrose. |
|  | 8 | Fill the integrally attached tubing with an aliquot of cells sealed in such a manner that it will be available for subsequent compatibility testing. |
|  | 9 | Deglycerolized RBCs must be stored at 1 to 6 C for no longer than 24 hours. (A closed system has been licensed that allows storage of deglycerolized RBCs at 1 to 6 C for 2 weeks. The closed system deglycerolization requires that the glycerolization step also be performed by a closed system in accordance with the manufacturer’s instructions.) |

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| Notes | 1. An aliquot of the donor’s serum or plasma should be frozen and stored at –65 C or colder for possible future use if new donor screening tests are implemented. |
|  | 1. When new donor screening tests have been implemented and stored units do not have aliquots available for testing, the units may have to be issued with a label stating that the test has not been performed. The reason for distributing an untested component should be documented. If a specimen from the donor is obtained and tested after the unit was stored, the date of testing should be noted on the unit when it is issued. |
|  | 1. Glycerolization and deglycerolization of leukocyte-reduced RBCs obtained from 500-mL Whole Blood units and stored in AS-1 and AS-3 have been successful.2 In-vivo recoveries were greater than 80%, and chromium-51 tagged t1/2 value was greater than 40 days for the two additive solutions. The amount of glycerol added to red cells may be adjusted to achieve 40% w/v concentration. For this calculation, the assumption is made that each 100 mL of glycerol contains 57 g of glycerol. |

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| References | 1. Meryman HT, Hornblower M. A method for freezing and washing RBCs using a high glycerol concentration. Transfusion 1972;12:145-56. 2. Bandarenko N, Hay SN, Holmberg J, et al. Extended storage of AS-1 and AS-3 leukoreduced red blood cells for 15 days after deglycerolization and resuspension in AS-3 using an automated closed system. Transfusion 2004;44:1656-62. |

**METHOD 6-7. USING HIGH-CONCENTRATION GLYCEROL TO CRYOPRESERVE RED CELLS—VALERI METHOD1**

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| Principle | Red Blood Cells (RBCs) collected in an 800-mL primary collection bag in citrate-phosphate­dextrose-adenine (CPDA-1) and stored at 1 to 6 C for 3 to 38 days can be biochemically rejuvenated and frozen with 40% w/v glycerol in the 800-mL primary container. See the manufacturer’s instructions for a rejuvenation method. |

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| Materials | 1. Quadruple plastic bag collection system with 800-mL primary bag. 2. Hand sealer clips. 3. Empty, 600-mL polyethylene cryogenic vials [eg, Corning 25702 (Corning Incorporated Life Sciences) or Fisher 033746 (Thermo Fisher Scientific)]. 4. Sterile connection device with wafers. 5. Freezer tape. 6. 600-mL transfer bag. 7. 50 mL of Red Blood Cell Processing Solution [Rejuvesol (Citra Labs)]. 8. Heat-sealable 8" × 12" plastic bags. 9. Y-type rejuvenation set. 10. 500 mL of glycerolyte 57 solution (Fenwal 4A7833) or 500 mL of 6.2 M glycerolization solution (Cytosol PN5500). 11. Labels—Red Blood Cells Frozen Rejuvenated. 12. Corrugated cardboard storage box (7" × 5.5" × 2" outside dimensions). 13. Heat sealing device. 14. Plastic bag for overwrapping. |

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| Procedure | **Step** | **Action** |
|  |  | *Preparation of RBCs for Glycerolization* |
|  | 1 | Collect 450 mL of whole blood in the primary bag. Invert the bag, fold it about 2 inches from the base, secure the fold with tape, and place the bag upright in a centrifuge. Centrifuge and remove all visible supernatant plasma. The hematocrit of the RBC unit must be 75% ± 5%. |
|  | 2 | Store RBCs at 1 to 6 C in the 800-mL primary bag, along with the adapter port on the tubing that connects the primary bag and transfer pack. |
|  | 3 | Centrifuge the stored cells to remove all visible plasma before undertaking rejuvenation. The gross weight and net weight of the red cells should not exceed 352 g and 280 g, respectively. |
|  | 4 | Transfer the plasma to the integrally connected transfer pack, fold the integral tubing, and replace the hand sealer clip (not crimped). |
|  | 5 | Using a sterile connection device, attach an empty 600-mL transfer pack to the integral tubing of the primary collection bag. |
|  | 6 | If desired, transfer 1 mL of plasma to each of three cryogenic vials to be used for future testing. |
|  |  | *Biochemical Modification of the Cells*2 |
|  | 1 | Aseptically insert the vented spike of the Y-type rejuvenation set into the rubber stopper of a 50-mL Red Blood Cell Processing Solution bottle. Join the tubing of the Y-type rejuvenation set with integral tubing of the primary collection bag using a sterile docking device. Alternatively, aseptically insert the coupler of the set into one of the administration ports of the primary collection bag. |
|  | 2 | Elevate the Red Blood Cell Processing Solution approximately 28 inches above the primary collection bag |
|  | 3 | Squeeze the drip chamber to prime the system and open the slide clamp on the Y-type rejuvenation set. Allow the entire contents to flow into the primary bag while gently agitating the bag. |
|  | 4 | After all of the solution has been transferred, close the slide clamp and heat-seal the tubing between the solution and the primary bag three times. The second tubing of the Y-set is used to add glycerol (see below). |
|  | 5 | Completely overwrap the 800-mL primary bag, the integrally connected empty transfer pack, and the coupler of the Y-type harness; incubate them in a 37 C waterbath for 1 hour. |
|  |  | *Glycerolization* |
|  | 1 | Remove the numbered crossmatch segments, leaving the initial segment and number attached to the collection bag. Weigh the unit. |
|  | 2 | Using the values shown in table below, determine the amount of glycerol to be added, on the basis of the gross or net weight of the unit. |
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|  | 3 | Aseptically insert the coupler of the rejuvenation harness into the outlet port of the rubber stopper on the glycerol solution bottle. For the Fenwal harness only, insert a filtered airway needle into the vent portion of the glycerol bottle stop­per. |
|  | 4 | Place the bag on a shaker. Add the amount of glycerol shown in the table above for the first volume while the bag is shaking at low speed (180 oscillations/ minute). |
|  | 5 | Equilibrate the mixture for 5 minutes without shaking, and add the second volume. Equilibrate it for 2 minutes. Add the third volume of glycerol, using vigorous manual shaking. |
|  | 6 | Heat-seal the tubing between the empty bottle of glycerol and the tubing proximal to the adapter port. Ensure that the transfer pack remains integrally attached to the primary collection bag. |
|  | 7 | Centrifuge the mixture of red cells and glycerol, and transfer all visible supernatant glycerol to the transfer pack, resuspend, and mix. Because the supernatant glycerol is removed before freezing, only two salt solutions (the hypertonic 12% saline and the 0.9% saline-0.2% dextrose solution) will be used in the deglycerolization process. Note that this step differs from the one in the Meryman method. |
|  | 8 | Seal the tubing 4 inches from the primary collection bag, detach the transfer pack containing the supernatant fluid, and discard it. |
|  | 9 | Affix an overlay blood component label, the facility label, and an ABO/Rh label. Record the expiration date on the label. |
|  | 10 | Weigh the unit just before freezing, and record the weight. |
|  | 11 | Fold over the top portion of the primary bag (approximately 2 inches). Place the primary bag into a plastic bag overwrap, and heat-seal the outer bag across the top so that there is as little air as possible between the bags. |
|  | 12 | Place one vial of plasma and the plastic bag containing the glycerolized red cells in the cardboard box. Store the other two vials, suitably identified, at –65 C or colder for future testing, if needed. |
|  | 13 | Affix a “Frozen Rejuvenated Red Blood Cells” label, an ABO/Rh label, a facility label, and the original unit number on the outside of the box. Record separately or affix on the cardboard box the collection, freezing, and expiration dates. |
|  | 14 | Freeze the unit in a –80 C freezer. No more than 4 hours should be allowed to elapse between the time the unit was removed from the 4 C refrigerator and the time the cells are placed in the –80 C freezer. |
|  |  | *Thawing and Deglycerolization* |
|  | 1 | Put an overwrap on the protective canister containing the frozen cells, and place it in either a 37 C waterbath or 37 C dry warmer. |
|  | 2 | Agitate it gently to speed thawing. The thawing process takes at least 10 minutes. The temperature of the thawed cells should be 37 C. |
|  | 3 | After the cells have thawed, use a commercial instrument for batch or continuous-flow washing to deglycerolize cells. Follow the manufacturer’s instructions. |
|  | 4 | Record the lot numbers and manufacturer of all the solutions and software used. Apply a “Deglycerolized Red Blood Cells” label to the transfer pack; be sure that the label includes identification of the collecting facility, the facility preparing the deglycerolized cells, the ABO group and Rh type of the cells, the Whole Blood number, and the expiration date and time. |
|  | 5 | Dilute the unit with a quantity of hypertonic (12%) NaCl solution appropriate for the size of the unit. Allow it to equilibrate for approximately 5 minutes. |
|  | 6 | Wash the cells with 1.6% NaCl until deglycerolization is complete. Approxi­mately 2 liters of wash solution are required. To check for residual glycerol, see Method 6-8. |
|  | 7 | Suspend the deglycerolized cells in isotonic (0.9%) NaCl with 0.2% dextrose. |
|  | 8 | Fill the integrally attached tubing with an aliquot of cells sealed in such a manner that it will be available for subsequent compatibility testing. |
|  | 9 | Deglycerolized RBCs must be stored at 1 to 6 C for no longer than 24 hours. (A closed system has been licensed that allows storage of deglycerolized RBCs at 1 to 6 C for 2 weeks. The closed-system deglycerolization requires that the glycerolization step also be performed by a closed system in accordance with the manufacturer’s instructions.) |

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| Note | Glycerolization and deglycerolization of leukocyte-reduced RBCs obtained from 500-mL Whole Blood units and stored in AS-1 and AS-3 have been successful.2 In-vivo recoveries were greater than 80%, and chromium-51 tagged t1/2 value was greater than 40 days for the two additive solutions.3 The amount of glycerol added to red cells may be adjusted to achieve 40% w/v concentration. For this calculation, the assumption is made that each 100 mL of glycerol contains 57 g of glycerol. |

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| References | 1. Valeri CR, Ragno G, Pivacek LE, et al. A multi-center study of in vitro and in vivo values in human RBCs frozen with 40% (wt/vol) glycerol and stored after deglycerolization for 15 days at 4 C in AS-3: Assessment of RBC processing in the ACP 215. Transfusion 2001;41:933-9. 2. Rejuvesol package insert. Braintree, MA: Citra Labs, 2013. [Available at <http://www.citra-labs.com/fileLibrary/FL7000-rejuvesol.pdf> (accessed March 6, 2020).] 3. Bandarenko N, Hay SN, Holmberg J, et al. Extended storage of AS-1 and AS-3 leukoreduced red blood cells for 15 days after deglycerolization and resuspension in AS-3 using an automated closed system. Transfusion 2004;44:1656-62. |

**METHOD 6-8. CHECKING THE ADEQUACY OF DEGLYCEROLIZATION OF RED BLOOD CELLS**

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| Principle | Glycerolization of red cells for frozen storage creates a hyperosmolar intracellular fluid, which must be restored to physiologically compatible levels before the cells are transfused. Inadequately deglycerolized red cells will be hemolyzed by contact with normal saline, or with serum or plasma if subjected to crossmatching. |

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| Materials and Equipment | 1. Semiautomated instrument for deglycerolizing cryopreserved Red Blood Cells (RBCs). 2. Transparent tubing, as part of disposable material used to deglycerolize individual unit. 3. Color comparator, available commercially. |

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| Procedure | **Step** | **Action** |
|  |  | *Final Wash Method* |
|  | 1 | Interrupt the last wash cycle at a point when wash fluid is visible in the tubing leading to the disposal bag. |
|  | 2 | Hold the comparator block next to an accessible segment of tubing and against a well-lighted white background. |
|  | 3 | Note coloration of the wash fluid, which should be no stronger than the block, indicating 3% hemolysis (ie, 3% of the red cells are hemolyzed). |
|  | 4 | If the level of hemolysis is excessive, continue the wash process until the color is within acceptable limits. |
|  | 5 | Record observation for the individual unit and for the quality assurance program. |
|  | 6 | If unacceptable hemolysis occurs repeatedly, document the corrective action. |
|  |  | *Other Methods for Quality Control of Deglycerolized RBCs* |
|  | 1 | Hand-held refractometer: A hand-held refractometer may be used according to the manufacturer’s instructions. A small amount of supernatant is transferred to the measuring prism, and the instrument is held against a light source. The refraction value should be less than 30 to ensure that the glycerol level is less than 1 g%. |
|  | 2 | Osmolality: Osmometer can be used to measure osmolality according to the manufacturer’s instructions. A small amount of supernatant is transferred to an osmometer cuvette, and osmolality of the sample is measured. The value should not exceed 400 mOsm/kg H2O to ensure that the residual glycerol concentration is less than 1 g%. |

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| Notes | 1. During deglycerolization, the last solution in contact with the cells is normal saline that contains low concentration of dextrose. The easiest way to determine adequacy of glycerol removal is to determine the level of free hemoglobin (mg/dL) in the final wash. 2. An adequate estimate of hemolysis can be achieved by comparing the color of the final wash fluid with the blocks in a commercially available color comparator. Alternatively, normal saline can be added to an aliquot of deglycerolized cells, and the color of the supernatant fluid can be evaluated against the color comparator. |

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| References | 1. Quality control of deglycerolized red blood cells. Boston, MA: Naval Blood Research Laboratories, 2007. 2. Umlas J, O’Neill TP. Use of refractive index to measure the adequacy of glycerol removal from previously frozen erythrocytes. Transfusion 1980;20:720-4. |

**METHOD 6-9. PREPARING FRESH FROZEN PLASMA FROM WHOLE BLOOD**

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| Principle | Plasma is separated from cellular blood elements and is frozen to preserve the activity of labile coagulation factors. Plasma must be placed in the freezer within 8 hours or the time frame specified in the directions for use of the blood collecting, processing, and storage system (see Note). |

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| Materials | 1. Freshly collected whole blood obtained by phlebotomy, in a collection unit with integrally attached transfer container(s). 2. Metal clips and hand sealer. 3. Instruments (scissors, hemostats). 4. Dielectric sealer (optional). 5. Plasma extractor. 6. Freezing apparatus. 7. Refrigerated centrifuge. 8. Scale. |

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| Procedure | **Step** | **Action** |
|  | 1 | Centrifuge blood soon after collection, using a “heavy” spin (see method for preparation of RBCs). Use a refrigerated centrifuge at 1 to 6 C unless also preparing platelets (see method for preparation of platelets from whole blood). |
|  | 2 | Place the primary bag containing centrifuged blood on a plasma extractor, and place the attached satellite bag on a scale adjusted to zero. Express the plasma into the satellite bag, and weigh the plasma. |
|  | 3 | Seal the transfer tubing with a dielectric sealer or metal clips but do not obliterate the segment numbers of the tubing. Place another seal nearer the transfer bag. |
|  | 4 | Label the transfer bag with the unit number before it is separated from the original container. Affix a Fresh Frozen Plasma (FFP) component label and record the volume of plasma on the label. See Note. |
|  | 5 | Cut the tubing between the two seals. The tubing may be coiled and taped against the plasma container, leaving the segments available for any testing desired. |
|  | 6 | Store the plasma at –18 C or colder within 8 hours of phlebotomy; within 6 hours of phlebotomy if anticoagulated with ACD; or as specified by the applicable regulatory authority. |

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| Note | If plasma is frozen within 24 hours but after more than 8 hours, the plasma must be labeled appropriately as Plasma Frozen Within 24 Hours After Phlebotomy (PF24). |

**METHOD 6-10. PREPARING CRYOPRECIPITATED AHF FROM WHOLE BLOOD**

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| Principle | Coagulation factors (fibrinogen, Factor VIII, Factor XIII, vWF, and fibronectin) can be concentrated from freshly collected plasma by cryoprecipitation. Cryoprecipitation is accomplished by slow thawing of Fresh Frozen Plasma (FFP) at 1 to 6 C. Provided that refreezing can occur within 1 hour, multiple units of cryoprecipitate may be combined using a closed system into a pooled product. |
| Materials | 1. FFP (>200 mL) with at least one integrally attached transfer container. 2. Metal clips and hand sealer OR dielectric sealer. 3. Clean instruments (scissors, hemostats). 4. Plasma extractor. 5. Refrigerated centrifuge. 6. Freezing apparatus: Suitable freezing devices include: 1) blast freezers or mechanical freezers capable of maintaining temperatures of –18 C or colder, 2) dry ice, or 3) an ethanol dry ice bath. In a bath of 95% ethanol and chipped dry ice, freezing will be complete in about 15 minutes. 7. 1 to 6 C circulating waterbath or refrigerator. 8. Scale. 9. Multi-lead pooling harness (optional). 10. Sterile connection device. |

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| Procedure | **Step** | **Action** |
|  |  | *Preparing Cryoprecipitate* |
|  | 1 | Allow FFP to thaw at 1 to 6 C by placing the bag in a 1 to 6 C circulating waterbath or in a refrigerator. If thawed in a waterbath, use a plastic overwrap (or other means) to keep container ports dry. |
|  | 2 | When the plasma has thawed to the desired consistency, separate liquid plasma from the cryoprecipitate by one of the procedures below:   1. Centrifuge the plasma at 1 to 6 C using a “heavy” spin. (See method for preparation of Red Blood Cells.) Remove the supernatant plasma:    1. Hang the bag in an inverted or elevated position and allow the separated plasma to flow rapidly into the transfer bag, leaving the cryoprecipitate adhering to the sides of the primary bag. To prevent the cryoprecipitate from dissolving and flowing out of the bag, separate the cryoprecipitate from the plasma promptly. Ten to 15 mL of supernatant plasma may be left in the bag for resuspension of the cryoprecipitate after thawing. Cryoprecipitate may be refrozen immediately or pooled.    2. Place the thawed plasma in a plasma extractor with the bag in an upright position. Allow the plasma to be expressed from the cryoprecipitate until approximately 10-15 mL of plasma remains in the original container. Cryoprecipitate may be refrozen immediately or pooled. 2. Place the thawing plasma in a plasma extractor when approximately one-tenth of the contents are still frozen. With the bag in an upright position, allow the supernatant plasma to flow slowly into the transfer bag, using the ice crystals at the top as a filter. The cryoprecipitate paste will adhere to the sides of the bag or to the ice. Seal the bag when ~90% of supernatant plasma has been removed. Refreeze the cryoprecipitate immediately (pooling not recommended). |
|  |  | *Pooling Cryoprecipitate* |
|  | 1 | Select the desired number of products to pool. All products should be the same ABO (Rh matching is not necessary). |
|  | 2 | Massage and resuspend the cryoprecipitate in the supernatant plasma. |
|  | 3 | Prepare the pool:   1. If using a multi-lead pooling harness, follow the manufacturer’s directions for preparing the pooled product. 2. In the absence of a pooling harness:    1. Use a sterile connection device to attach two of the cryoprecipitate products together.    2. Drain the cryoprecipitate into one of the containers.    3. Attach a third cryoprecipitate to the product bag containing the pool.    4. Drain the third cryoprecipitate into the product bag containing the pool.    5. Repeat iii. and iv. until all of the desired components are drained into one bag.    6. Use the last empty bag to burp any excess air from the pooled product.    7. Detach the empty bag from the pool. |
|  |  | *Freezing and Storing Cryoprecipitate* |
|  | 1 | The cryoprecipitate should be refrozen within 1 hour of being removed from the refrigerated centrifuge or waterbath. |
|  | 2 | Store at −18 C or colder, preferably −30 C or colder, for up to 12 months after collection. |

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| Note | Cryoprecipitated AHF may be prepared from FFP at any time within 12 months of collection. Cryoprecipitated AHF expires 12 months from the date of phlebotomy, not from the date it was prepared. Pooled cryoprecipitate expires 12 months from the earliest phlebotomy date. Method supplied courtesy of Indiana Blood Center. |

**METHOD 6-11. THAWING AND POOLING CRYOPRECIPITATED AHF**

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| Principle | Cryoprecipitated AHF should be rapidly thawed at 30 to 37 C but should not remain at this temperature once thawing is complete. The following method permits rapid thawing and pooling of this component. |

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| Materials | 1. Circulating waterbath at 37 C (waterbaths designed for thawing plasma are available commercially, as are specially designed dry heat devices). 2. Medication injection ports. 3. Sterile 0.9% NaCl for injection. 4. Syringes and needles. |

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| Procedure | **Step** | **Action** |
|  | 1 | Cover the container with a plastic overwrap to prevent contamination of the ports with unsterile water, or use a device to keep the containers upright with the ports above water. Place container in 37 C waterbath. |
|  | 2 | Resuspend the thawed precipitate carefully and completely, either by kneading it into the residual 10 to 15 mL of plasma or by adding approximately 10 mL of 0.9% NaCl and gently resuspending. |
|  | 3 | Pool by inserting a medication injection site into a port of each bag. Aspirate contents of one bag into a syringe, and then inject into the next bag. Use the ever-increasing volume to flush each subsequent bag of as much dissolved cryoprecipitate as possible until all contents are in the final bag. |

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| Notes | 1. Thawed Cryoprecipitated AHF prepared for transfusion must be stored at room temperature. If pooled for immediate transfusion, it must be administered within 4 hours. Thawed single units, if not entered, must be administered within 6 hours of thawing if intended for replacement of Factor VIII. Pools of thawed individual units may not be refrozen. 2. Prepooled Cryoprecipitated AHF may be prepared by pooling 4 to 10 units at the time of initial preparation and will have an expiration date of 1 year (see method for preparation of cryoprecipitate). No diluent can be added to the product before freezing. Pooling is generally performed with the use of a sterile connection device but may use an “open” system. Prestorage pooled Cryoprecipitated AHF has an expiration time of 4 hours when an “open” system is used, or 6 hours once thawed if prepared using a sterile connection device. Pooled Cryoprecipitated AHF should be frozen within 1 hour of preparation and may be stored for 1 year from the date of collection. In accordance with AABB *Standards for Blood Banks and Transfusion Services*, the pool must contain a minimum of 150 mg of fibrinogen and 80 IU of coagulation Factor VIII times the number of components in the pool. The pool must be labeled with ABO/Rh. If 1 unit in a pool is Rh positive, then the pool must be labeled Rh positive. Thawed pools may not be refrozen. |

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| References | 1. Joint UKBTS/NIBSC Professional Advisory Committee. Cryoprecipitate pooled, leucocyte depleted. In: Guidelines for the blood transfusion service in the United Kingdom. 7th ed. Sheffield, UK: National Blood Service, 2005. [Available at <http://www.transfusionguidelines.org.uk/index.asp?Publication=RB&Section=25&pageid=969> (accessed April 5, 2020).] 2. Smith KJ, Hodges PA. Preparation of pooled cryoprecipitate for treatment of hemophilia A in a home care program. Transfusion 1984;24:520-3. 3. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:31. 4. Code of federal regulations. Title 21, CFR Part 640.54. Washington, DC: US Government Publishing Office, 2019 (revised annually). |

**METHOD 6-12. PREPARING PLATELETS FROM WHOLE BLOOD**

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| Principle | Platelets can be prepared by the platelet-rich plasma (PRP) method or by the buffy-coat method. In the PRP method, PRP is separated from whole blood by “light-spin” centrifugation, the platelets are concentrated by “heavy-spin” centrifugation, and the supernatant plasma is subsequently removed. In the buffy-coat method, whole blood is centrifuged at “high speed” with subsequent collection of the buffy coat. The buffy coat is then centrifuged at “low speed” to concentrate platelets and to remove red cells and white cells. Both methods are described below. |

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| Materials | 1. Freshly collected whole blood obtained by phlebotomy, in a collection unit with two integrally attached transfer containers. The final container must be made of a plastic approved for platelet storage. Keep blood at room temperature (20 to 24 C) before separating PRP from the red cells. This separation must take place within 8 hours of phlebotomy or within the time frame specified in the directions for use of the blood collecting, processing, and storage system. 2. In-line filter (if preparing prestorage leukocyte-reduced components). 3. Metal clips and hand sealer. 4. Instruments (scissors, hemostats). 5. Plasma extractor. 6. Dielectric sealer (optional). 7. Centrifuge, calibrated. 8. Scale. 9. Rotator. |

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| Procedure | **Step** | **Action** |
|  |  | *For Preparation of PRP Platelets* |
|  | 1 | Do not chill the blood at any time before or during platelet separation. If the temperature of the centrifuge is 1 to 6 C, set the temperature control of the refrigerated centrifuge at 20 C, and allow the temperature to rise to approximately 20 C. Centrifuge the blood using a “light” spin (see method for preparation of Red Blood Cells). |
|  | 2 | Express the PRP into the transfer bag intended for platelet storage. Seal the tubing twice between the primary bag and Y connector of the two satellite bags and cut between the two seals. Place the red cells at 1 to 6 C. |
|  | 3 | Centrifuge the PRP at 20 C using a “heavy” spin (see method for preparation of Red Blood Cells). |
|  | 4 | Express the platelet-poor plasma into the second transfer bag, and seal the tubing. Some plasma should remain on the platelet button for storage, but no exact volume can be designated. AABB *Standards for Blood Banks and Transfusion Services*1 requires that sufficient plasma remain with the platelet concentrate to maintain the pH at 6.2 or higher for the entire storage period. This pH level usually requires a minimum of 35 mL of plasma when storage is at 20 to 24 C, but 50 to 70 mL is preferable. |
|  | 5 | The platelet concentrate container should be left stationary, with the label side down, at room temperature for approximately 1 hour. |
|  | 6 | Resuspend the platelets in either of the following ways:   1. Manipulate the platelet container gently by hand to achieve uniform resuspension. 2. Place the container on a rotator at room temperature. The slow, gentle agitation should achieve uniform resuspension within 2 hours. |
|  | 7 | Maintain the platelet suspension at 20 to 24 C with continuous gentle agitation. |
|  | 8 | Platelets should be inspected before issue to ensure that no platelet aggregates are visible. |
|  |  | *For Preparation of Buffy-Coat Platelets*2 |
|  | 1 | Whole blood should be stored at 20 to 24 C before centrifugation. |
|  | 2 | Centrifuge whole blood at “high” speed [eg, 2800 × *g* for 11.5 minutes using Beckman J6ME centrifuge (Tritech, Inc).] |
|  | 3 | Remove supernatant plasma from the top of the container, and red cells from the bottom of the container of a specially designed collection set manually or by using an automated instrument. Approximately 50 mL of buffy coat remains in the bag. |
|  | 4 | Pool buffy coats from 4 to 6 units and centrifuge at “low” speed (eg, 700 × *g* for 5 minutes using Beckman J6ME centrifuge). Transfer supernatant PRP manually or by an automated instrument into platelet storage bag. Filter platelets to remove leukocytes during the transfer. |
|  |  | *For Preparation of Prestorage Leukocyte-Reduced Platelets* |
|  |  | Prestorage leukocyte-reduced (LR) platelets may be prepared from whole blood using in-line filtration of the PRP. The resulting intermediate product is a filtered PRP, from which LR platelet concentrate and LR plasma may be manufactured. |

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| Note | The supernatant plasma may be frozen promptly and stored as Fresh Frozen Plasma (FFP) if the separation and freezing are completed within the time frame specified in the directions for use of the blood collecting, processing, and storage system. The volume of FFP prepared after platelet preparation will be substantially less than that prepared directly from whole blood. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020:31-32. 2. Turner CP, Sutherland J, Wadhwa M, et al. In vitro function of platelet concentrates prepared after filtration of whole blood or buffy coat pools. Vox Sang 2005;88:164-71. 3. Sweeney JD, Holme S, Heaton WAL, Nelson E. Leukodepleted platelet concentrates prepared by in-line filtration of platelet rich plasma. Transfusion 1995:35:131-6. 4. Sweeney JD, Kouttab N, Penn LC, et al. A comparison of prestorage leukoreduced whole blood derived platelets with bedside filtered whole blood derived platelets in autologous stem cell transplant. Transfusion 2000;40:794-800. |

**METHOD 6-13. REMOVING PLASMA FROM PLATELETS (VOLUME REDUCTION)**

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| Principle | Although optimal storage of platelets requires an adequate volume of plasma, a few patients may not tolerate large-volume infusion. Stored platelets may be centrifuged and much of the plasma removed shortly before transfusion, but appropriate resuspension is essential. The platelets must remain at room temperature, without agitation, for 20 to 60 minutes before resuspension into the remaining plasma. Transfusion must take place within 4 hours of the time the platelet bag was entered. Volume reduction can be performed on individual or pooled units. |

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| Materials | 1. Apheresis Platelets or Platelets prepared from whole blood. 2. Metal clips and hand sealer. 3. Scissors, hemostats. 4. Dielectric sealer (optional). 5. Centrifuge, calibrated. 6. Plasma extractor. |

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| Procedure | **Step** | **Action** |
|  | 1 | Pool platelets, if desired, into a transfer pack, using standard technique. Single platelet concentrates may need volume reduction for pediatric recipients. Apheresis components may be processed directly. |
|  | 2 | Centrifuge at 20 to 24 C, using one of the following protocols:   1. 580 × *g* for 20 minutes 2. 2000 × *g* for 10 minutes. 3. 5000 × *g* for 6 minutes. |
|  | 3 | Without disturbing the contents, transfer the bag to a plasma extractor. Remove all but 10 to 15 mL plasma from single units, or somewhat more volume, proportionately, from a pool or from a component prepared by apheresis. |
|  | 4 | Mark expiration time on bag as 4 hours after the time the unit was entered or units were pooled. |
|  | 5 | Leave bag at 20 to 24 C without agitation for 20 minutes if centrifuged at 580 × *g*, or for 1 hour if centrifuged at 2000 or 5000 × *g*. |
|  | 6 | Resuspend platelets. |

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| Notes | 1. No consensus exists regarding the optimal centrifugation condition. One study1 found 35% to 55% platelet loss in several units centrifuged at 500 × *g* for 6 minutes, compared with 5% to 20% loss in units centrifuged at 5000 × *g* for 6 minutes or 2000 × *g* for 10 minutes. Centrifugation at 2000 × *g* for 10 minutes is recommended, to avoid any risk that a higher centrifugal force might inflict on the plastic container. A study by Moroff et al2 found mean platelet loss to be less than 15% in 42 units centrifuged at 580 × *g* for 20 minutes. High *g* forces are of theoretical concern because they may damage the platelets when they are forced against the wall of the container and also increase the possibility of container breakage. 2. If a sterile connection device is used for removing plasma from a hemapheresis component or individual platelet concentrate, the unit is considered functionally closed and it is not necessary to impose the 4-hour expiration interval required for entered platelets. However, no data exist to support storage of reduced-volume platelet concentrates; therefore, it is preferable to transfuse them as soon as possible. 3. Reduced-volume platelet concentrates may not be distributed as a licensed product. 4. In the United States, platelets that have been pooled must be used within 4 hours of entering the units, whether or not they have been volume reduced, unless pooled as part of an FDA-cleared closed system. In such a case, expiration is 5 days following collection of the oldest unit of the pool. Pooled platelets may not be distributed as a licensed product. |

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| References | 1. Simon TL, Sierra ER. Concentration of platelet units into small volumes. Transfusion 1984;24:173-5. 2. Moroff G, Friedman A, Robkin-Kline L, et al. Reduction of the volume of stored platelet concentrates for use in neonatal patients. Transfusion 1984;24:144-6. |

**TRANSPLANTATION OF CELLS AND TISSUE METHODS**

As with the other sections of methods, these cellular therapy methods are representative of the many specific techniques currently used in facilities worldwide.

**METHOD 7-1. INFUSING CRYOPRESERVED HEMATOPOIETIC CELLS**

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| Principle | The purpose of this procedure is to outline the steps to be performed before the infusion of cryopreserved hematopoietic progenitor cells (HPCs) can be started, as well as the steps necessary to infuse the cells. The method presented below should be considered a representative example of methods in current use. |

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| Materials and Equipment | 1. Standard IV setup. 2. Bags of NaHCO3 and KCl combined at a 50:20 ratio mEq/L. 3. Urine output toilet insert. 4. pH sticks. 5. 650 mg acetaminophen. 6. 1 mg/kg diphenhydramine—limit = 50 mg. 7. Antiemetics. 8. Thawed HPCs (see laboratory procedure for thawing details). 9. Standard infusion set (blood administration set may be used). 10. Patient infusion record. 11. *Circular of Information for the Use of Cellular Therapy Products.* |

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| Procedure | **Step** | **Action** |
| 1 | If the product volume is greater than 300 mL, hydration should be initiated with 0.25% normal saline and NaHCO3 at least 3 hours before infusion. The NaHCO3 is added to ensure alkaline urine. The goal is to achieve a urine output of 2 to 3 mL/kg/ hour and a urine pH of 7.0. |
|  | 2 | At 15 to 30 minutes before HPC infusion, medicate with the following:   1. Diphenhydramine, 1 mg/kg (maximum = 50 mg). 2. Antiemetics, as necessary. |
|  | 3 | Repeat medications if additional cells are to be infused after a break of more than 4 hours. |
|  | 4 | Infuse thawed HPC aliquots rapidly (50 mL over 5-10 minutes) to minimize clumping. |
|  | 5 | In double CB transplants, thaw and infuse units independently. Do not begin to thaw the second unit before infusion of the first unit has been completed and any infusion reactions have resolved. |
|  | 6 | Maintain an infusion record with vital signs and adverse events, as appropriate. |

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| Notes | 1. Infusion protocols should be defined in the appropriate (nursing and/or laboratory) procedures manual. Medical staff administering the product need to be familiar with adverse signs of blood transfusion: fever, chills, dyspnea, bronchospasm, hypotension, cyanosis, rash or hives, chest or back pain, and any other change in condition. Dimethylsulfoxide (DMSO) toxicity is the most common complication of cryopreserved HPC product administration. Symptoms result from histamine release and include flushing, rash, chest tightness, nausea and vomiting, and cardiovascular instability. The *Circular of Information for the Use of Cellular Therapy Products* contains detailed descriptions of the side effects and hazards related to the infusion of HPC products.1 HPCs should not be infused through leukocyte reduction filters. However, depending on the protocol, cells may be filtered through a standard blood administration set—for example, a 170-micron filter. |
|  | 1. Adverse reactions to the infusion of HPC products are documented on a “Report of Adverse Reaction Form.” A copy should be kept in the laboratory. These forms are completed by nursing and laboratory staff. Certain adverse reactions related to HPC administration are required to be reported to the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA). The definitions of reportable adverse reactions and the requirements for reporting them are specified in the *Code of Federal Regulations* (21 CFR 1271.350).2 Updated information about CBER reporting requirements may be found on the FDA website.3 |
|  | 1. If the total infusion volume contains greater than 1 g/kg of DMSO, it is recommended that the product be infused over 2 days. For reference, 100 mL of cryopreserved cells in a 10%-DMSO-based cryoprotectant contains 10 g of DMSO. Consult the cellular therapy laboratory medical director if the product exceeds this limit. |

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| References | 1. AABB, America’s Blood Centers, American Red Cross, American Society for Apheresis, American Society for Blood and Marrow Transplantation, College of American Pathologists, Cord Blood Association, Foundation for the Accreditation of Cellular Therapy, ICCBBA, International Society for Cellular Therapy, Joint Accreditation Committee of ISCT and EBMT, National Marrow Donor Program, World Marrow Donor Association. Circular of information for the use of cellular therapy products. Bethesda, MD: AABB, October 2018. 2. Code of federal regulations. Title 21, CFR Part 1271.350. Washington, DC: US Government Publishing Office, 2019 (revised annually). 3. Food and Drug Administration. Biological product deviations: Includes human tissue and cellular and tissue-based product (HCT/P) deviation reporting. Silver Spring, MD: CBER Office of Communication, Outreach, and Development, 2020. [Available at https://www.fda.gov/vaccines-blood-biologics/report-problem-center-biologics-evaluation-research/biological-product-deviations (accessed April 6, 2020).] |

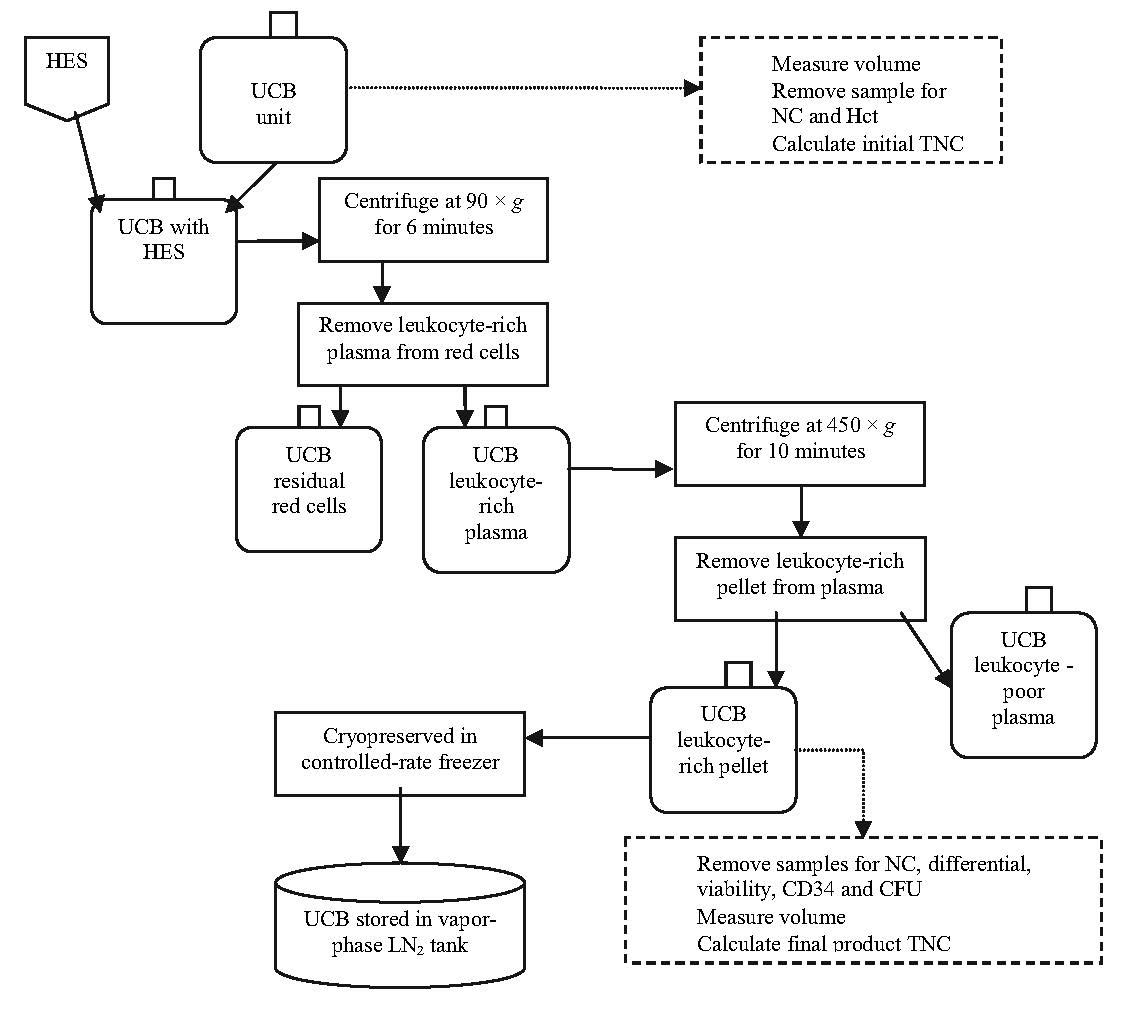
**METHOD 7-2. PROCESSING UMBILICAL CORD BLOOD**

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| Principle | The method below, representative of several methods in current use, reduces red cell content and concentrates leukocyte content in cord blood units. It minimizes the final product volume (optimizing storage space) and cryoprotectant volumes (decreasing potential infusion-related complications). |

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| Materials and Equipment | 1. Collected umbilical cord blood. 2. Hydroxyethyl starch (HES; 6% w/v) or an alternative red cell sedimenting agent. 3. Blood component centrifuge. 4. Plasma expressor. 5. Transfer bags. 6. Sterile dimethylsulfoxide (DMSO). 7. Controlled-rate freezer. 8. Liquid-nitrogen freezer. 9. Automated hematology analyzer. 10. Flow cytometry facilities. |

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| Procedure | **Step** | **Action** |
| 1 | Combine the cord blood product (5 parts) in a bag with HES (1 part). |
|  | 2 | Allow red cells to sediment for 30 minutes or centrifuge the mixture at 90 × *g* for 6 minutes with no brake. |
|  | 3 | Express the leukocyte-rich plasma into a secondary bag. |
|  | 4 | Centrifuge the leukocyte-rich plasma at 450 × *g* for 10 minutes. |
|  | 5 | Express the leukocyte-reduced plasma into a third bag. |
|  | 6 | Cryopreserve the remaining leukocyte pellet (approximate volume: 20-23 mL) using a controlled-rate freezer (final product concentration: 10% DMSO and 1% dextran 40). |
|  | 7 | Store the product in a liquid-nitrogen storage container in either liquid or vapor phase (<–150 C). |
|  | 8 | Quality control parameters that should be measured include nucleated cell count and hematocrit of the starting umbilical cord unit, and nucleated cell count, leukocyte differential, viability, CD34 count, and CFU on an aliquot from the final product before addition of cryoprotectant. |

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| Note | Summary diagram of umbilical cord blood processing procedure. |



CFU = colony-forming unit; Hct = hematocrit; HES = hydroxyethyl starch; LN2 = liquid nitrogen; NC = nucleated cells; TNC = total nucleated cells.

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| References | 1. McCullough J, McKenna D. Management of umbilical cord blood at the transplant center. In: Broxmeyer HE, ed. Cord blood: Biology, transplantation, banking, and regulation. Bethesda, MD: AABB Press, 2011:585-94. 2. Rubenstein P, Dobrilla L, Rosenfield RE, et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. Proc Natl Acad Sci U S A 1995;92:10119-22. |

**METHOD 7-3. INVESTIGATING ADVERSE EVENTS AND INFECTIONS FOLLOWING TISSUE ALLOGRAFT USE**

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| Principle | Transplanting surgeons are responsible for reporting to the hospital tissue service any recipient infection where there is a likelihood or suspicion that the infection originated from a contaminated tissue allograft.  Early reporting allows actions to take place to determine the source of contamination, to develop corrective actions, and to limit further spread of infection. Early reporting also facilitates the identification and evaluation of other patients who may have received contaminated tissue from the same donor and who may have experienced a similar infection.  Hospital tissue services are responsible for investigating adverse events and infections and for reporting those occurrences to the tissue processor and supplier. |

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| Materials | Adverse event file. |

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| Procedure | **Step** | **Action** |
|  | *Quarantine, Record-Keeping, and Notification* |
|  | 1 | Immediately sequester, quarantine, and cease further distribution of any existing, unused tissue allografts from the same donor, if any are known to be in the hospital tissue service’s inventory. Tissue allografts from the same donor or processing lot can be identified by a donor-specific numerical identifier that can be obtained from the tissue supplier. Unused tissue from the same donor may be needed to test for contamination and should be quarantined but not destroyed until all investigations are complete. |
|  | 2 | If quarantined tissue allografts from the same donor are later cleared of possible contamination, there should be a documented quality assurance review and approval by the tissue service’s medical director before they are removed from quarantine and placed in releasable inventory. |
|  | 3 | Initiate an investigation and open an adverse event file. The adverse event file should contain a record of all investigative actions and the final report after it has been reviewed and approved by the hospital tissue service’s medical director. |
|  | 4 | Notify the hospital tissue service’s medical director as soon as possible. Immediate actions by the medical director should include a review of the patient’s illness and a discussion with the transplant surgeon to confirm the type of infection and possible contributing factors and to confirm that the allograft is suspected to be involved with causing the infection. |
|  | 5 | Notify the tissue processor (name is in package insert) and tissue supplier (if not the same) promptly so appropriate actions, such as quarantining tissues from the same donor, can take place and the tissue supplier (tissue bank, tissue processor, tissue distribution intermediaries) can conduct its own investigation as to the possible origin of the contaminant from an infected donor or from acquisition during tissue recovery, tissue processing, or storage. |
|  | 6 | Notify hospital risk management, especially if the infection is likely to be from the allograft. |
|  | 7 | If a serious infection is determined to have been caused by the allograft, notify The Joint Commission. |
|  | 8 | If the recipient is seriously or fatally ill from an allograft, the hospital tissue service should consider voluntary notification of the Food and Drug Administration (FDA) through MedWatch (notification is mandatory for tissue processors and suppliers). |
|  |  | *Hospital Investigation* |
|  | 1 | Cooperate with the ongoing investigation by the tissue processor. |
|  | 2 | With the aid of the hospital tissue service’s medical director and hospital infection control, investigate whether patient or hospital factors could have contributed to the infection. |
|  | 3 | If the infection is viral (eg, human immunodeficiency virus, hepatitis C virus, hepatitis B virus), consider the presence of behavioral risk factors in the tissue allograft recipient (eg, hemophiliac receiving coagulation factors, injecting drug user, prostitute, male who had sex with another male, transfusion recipient, etc). |
|  | 4 | Investigate whether the patient’s infection could have been hospital-acquired. This is particularly important when the infection is bacterial or fungal. In collaboration with the hospital’s infection control or epidemiology service, the hospital tissue service needs to determine whether the infection could have been acquired from the hospital environment, supplies, equipment, or staff. Determine whether there have been recent outbreaks of infections by the same organism. An investigation and assessment by the hospital infection control service, a clinical infectious disease specialist, or both may be needed. The state department of health can also be involved if needed. |
|  | 5 | Request a report of the tissue supplier’s investigation. If the tissue supplier discovers contamination of the tissue and its source, the investigation in the hospital can be brief. |
|  | 6 | If the infection may be allograft associated, conduct a sentinel event investigation. If the source of the infection is uncertain or is determined to be hospital acquired rather than allograft acquired, the hospital should conduct a root cause analysis, look for contributing factors, and implement corrective and preventive actions, as needed. |
|  |  | *Confidentiality* |
|  | 1 | During the investigation of an adverse event, pertinent confidential patient medical information may be shared with the investigative teams of the hospital, the public health department, and the tissue supplier (in accordance with the Health Insurance Portability and Accountability Act). |
|  | 2 | The tissue supplier is required to investigate adverse events reported to the supplier and report those activities to the FDA. |
|  |  | *Review of Supplier Certification* |
|  | 1 | If the tissue allograft is determined to be the probable or confirmed cause of the recipient infection, review the qualifications of the tissue supplier. Obtain and review information to determine whether tissues from that supplier are less safe or less effective than others. Information can be obtained by consulting the FDA (eg, MedWatch reports, FDA inspection findings), by interviewing the tissue supplier quality assurance officer or medical director, and by determining whether there have been infections in other patients who received tissues from the same tissue bank. |
|  | 2 | If the tissue supplier or tissue processor refuses to provide needed information, quarantine all other tissue allografts in the hospital inventory previously obtained from the supplier. Suspend that supplier’s certification and approvals and obtain tissue from other suppliers. |
|  |  | *Final Assessment* |
|  | 1 | After the investigation is complete, prepare a written final report identifying the cause of the patient’s infection and determining whether the tissue allograft was unlikely, likely, probably, or confirmed to be the cause of the infection. The investigation documents, conclusion, final report, and any corrective actions should be reviewed and approved in writing by the hospital tissue service’s medical director. Place the final report in the adverse event file. |
|  | 2 | If corrective actions were taken, an evaluation should be made at a later date to document that actions are in place and are effective. |
|  |  | *Final Reports to Others* |
|  | 1 | A report of the investigation written by, or reviewed and approved by, the tissue service’s medical director should be provided to the following entities:   1. The transplant surgeon who originally reported the infection. 2. The tissue processor and supplier. 3. The hospital tissue services committee. |
|  | 2 | If the hospital tissue service determines that either 1) the recipient infection was caused by the allograft or 2) the organism was hospital acquired, and that 3) the infection is fatal, life-threatening, or has resulted in permanent impairment of a body function or permanent damage to a body structure, then notify The Joint Commission and conduct a sentinel event investigation. |
|  | 3 | If a transmitted contagious infection is diagnosed in the recipient and is report­able according to state law, notify the state department of health in writing. |
|  | 4 | It is not mandatory for the hospital to report adverse events to the FDA. How­ever, if the hospital tissue service determines that the adverse event caused by the tissue 1) involves a communicable disease; 2) is fatal, life-threatening, or results in permanent impairment of a body function or permanent damage to a body structure; or 3) necessitates medical or surgical intervention, then the hospital can voluntarily notify the FDA by Med-Watch (21 CFR 1271.350). MedWatch reports are accepted online (see <http://www.fda.gov/Safety/MedWatch>); by telephone at 1-888-463-6332; by FAX at 1-800-FDA-0178; or by mail to MedWatch, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD 20993. |

**QUALITY CONTROL METHODS**

Quality control (QC) testing of blood components and the equipment used in their preparation is an important part of process control. The failure to meet QC criteria can serve as an indicator of unexpected suboptimal reagents or material. The timely detection of QC failures enables early identification and resolution of a manufacturing problem.

QC testing is necessary to comply with regulatory requirements and voluntary standards. Those requirements and standards are considered to be minimum criteria and any facility may establish more stringent requirements.

**Thermometers**

Thermometers used during laboratory testing and in the collection (donor eligibility), processing, and storage of blood components and reagents should be calibrated and standardized to ensure accurate indication of temperatures. Calibration should be performed at temperatures close to the temperature at which the thermometers will be used. Each thermometer should be calibrated before initial use and periodically thereafter, as well as any time there is reason to suspect change or damage. Calibration must be verified for all thermometers, including those described as “self-calibrating.”

**Blood Storage Equipment Alarms**

Blood storage refrigerators and freezers must be equipped with a system for continuous temperature monitoring and an audible alarm. If a storage unit goes into alarm, it is essential that personnel know the appropriate actions to take. Standard operating procedures (SOPs) for such events must be available and personnel must be trained to initiate these actions if the temperature cannot be corrected rapidly. The alarm on each storage unit must be checked periodically for proper functioning. Periodic checks are performed consistent with the manufactuerer’s instructions to ensure the equipment is in good operating condition. Because alarms may be disconnected or silenced during repairs, it is also prudent to verify alarm functioning after repairs. Personnel competence with these actions must be maintained.

The high and low temperatures of alarm activation must be checked and the results recorded. AABB *Standards for Blood Banks and Transfusion Services* requires that the alarm be set to activate at a temperature that will allow appropriate intervention before blood or components reach unacceptable conditions. Because of the diversity of equipment available, it is not possible to give specific instructions for all applicable alarm systems. If the equipment user’s manual does not provide suitable directions for testing the alarm, consult the manufacturer or other equipment storage expert. The facility SOPs must include a detailed description of the method(s) in local use. (Quality control testing intervals are listed in Chapter 1 of the AABB *Technical Manual*.)

**METHOD 8-1. VALIDATING COPPER SULFATE SOLUTION**

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| Principle | Copper sulfate solution can be validated for use in screening female donors by observing the behavior (sinking or floating) of drops of blood of known hemoglobin concentration. |

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| Materials | 1. Copper sulfate—specific gravity 1.053. 2. Capillary tubes. 3. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Obtain several (three to six, if possible) blood samples with known hemoglobin levels. Samples should include hemoglobin levels slightly above and below 12.5 g/ dL. |
|  | 2 | Gently place a drop of each blood sample into a vial of copper sulfate solution with a stated specific gravity of 1.053. |
|  | 3 | Record the date of testing; the manufacturer, lot number, and expiration date of the copper sulfate; sample identity; the test results; the interpretation of pass or fail; and the identity of the person performing the test. |
|  | 4 | Document the corrective action taken if the results are outside acceptable limits. |

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| Interpretation | The copper sulfate is validated for use if the drops of all blood samples with hemoglobin at or above 12.5 g/dL sink and those with hemoglobin levels below 12.5 g/dL float. |

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| Reference | 1. Philips RA, Van Slyke DD, Hamilton PB, et al. Measurement of specific gravities of whole blood and plasma by standard copper sulfate solutions. J Biol Chem 1950;183:305-30. |

**METHOD 8-2. CALIBRATING LIQUID-IN-GLASS LABORATORY THERMOMETERS**

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| Principle | Thermometers used during laboratory testing and in the collection (donor eligibility), processing, and storage of blood components and reagents must be calibrated and standardized to ensure accurate indication of temperatures. |

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| Materials | 1. National Institute of Standards and Technology (NIST)-certified thermometer or thermometer with NIST-traceable calibration certificate 2. Thermometer to be calibrated. 3. Suitable container (eg, 250-500 mL beaker). 4. Water. 5. Crushed ice. 6. 37 C waterbath. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Before choosing a thermometer for a particular application, consider all the governing factors; be sure that the thermometer will be used at its proper immersion; and follow the manufacturer’s instructions for its proper use. When using a certified thermometer, read and follow the applicable notes. Be sure to include any correction factors noted on the certificate for the NIST-traceable thermometer and apply them in calculations. |
|  | 2 | Categorize the thermometers by key factors, such as immersion, increments, and temperature of intended use. Test them in groups, comparing similar thermometers. Do not attempt to compare dissimilar thermometers in a single procedure. |
|  | 3 | Number each thermometer being tested to identify each thermometer (eg, place a numbered piece of tape around the top of each thermometer or use the manufacturer’s serial number). |
|  | 4 | Perform calibration with water at a temperature close to that which the thermometer will monitor. |
|  | 5 | To calibrate at 37 C, place the thermometers to be tested and the NIST thermometer at a uniform depth in a standard 37 C waterbath, making sure that the tips of all devices are at the same level in the liquid.1 |
|  | 6 | To calibrate at 1 to 6 C, fill a suitable container with water. Add crushed ice until the approximate desired temperature is reached. Place the thermometers to be tested and the NIST thermometer at a uniform depth in the water/ice mixture, making sure that the tips of all devices are at the same level and are in the liquid, not the upper ice. |
|  | 7 | Stir constantly in a random motion until the temperature equilibrates, approximately 3 to 5 minutes. |
|  | 8 | Observe temperatures. Record each thermometer’s identification and results. |
|  | 9 | Complete the calibration record with the date of testing and identity of the person who performed the test. |
| Notes | 1. Acceptance criteria depend on the level of precision required, but for most blood banking applications, agreement within 1 C between the two thermometers may be considered acceptable. If the reading varies by more than one degree from the standard, the thermometer may be returned to the distributor (if newly purchased), labeled with the correction factor (degrees different from the NIST thermometer) that must be applied to each reading, or discarded. 2. If a thermometer is to be used for temperatures over a range greater than a few degrees (eg, 10 degrees), a three-point calibration should be performed. Use water of appropriate temperature. Test at temperatures just below, just above, and at the midway point of intended use. 3. Over time, liquid-in-glass thermometers may give a different reading at a given temperature because of permanent changes in the volume of the bulb related to relaxation of the glass.1. 4. Thermometers should be observed routinely for any split in the column because this will cause inaccurate readings. The methods for reuniting the separation can be found in CLSI Standard I2-A2.2 When this occurs, document corrective action and recalibrate the thermometer. 5. Each thermometer should be calibrated before initial use and periodically thereafter, as well as any time there is reason to suspect change or damage. | |

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| References | 1. Wise JA. A procedure for the effective recalibration of liquid-in-glass thermometers. NIST special publication 819. Gaithersburg, MD: National Institute of Standards and Technology, 1991. 2. Temperature calibration of water baths, instruments, and temperature sensors. 2nd ed; approved standard I2-A2 Vol. 10 No. 3. Wayne, PA: CLSI, 1990. |

**METHOD 8-3. CALIBRATING ELECTRONIC ORAL THERMOMETERS**

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| Principle | Thermometers used during laboratory testing and in the collection (donor eligibility), processing, and storage of blood components and reagents should be calibrated and standardized to ensure accurate indication of temperatures. |

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| Materials | 1. National Institute of Standards and Technology (NIST)-certified thermometer or thermometer with NIST-traceable calibration certificate. 2. Thermometer to be calibrated. 3. Suitable container (eg, 250-500 mL beaker). 4. Water. 5. Crushed ice. 6. 37 C waterbath. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Use any of the following methods to verify calibration:   1. Follow manufacturer’s instructions for verifying calibration. 2. Use a commercially available calibration device by following the instructions provided by the device’s manufacturer. 3. Calibrate the thermometer by inserting the probe in a waterbath that has a temperature in the range to be tested using a NIST-certified thermometer. |
|  | 2 | A result is acceptable if the readings agree within acceptable tolerances. If not, document and remove from use. |
|  | 3 | Record the date of testing, thermometer identification numbers, temperature readings, and the identity of the person performing the test. |

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| Notes | 1. Calibration should be performed at temperatures close to the temperature at which the thermometers will be used. 2. Each thermometer must be calibrated before initial use and periodically thereafter, as well as any time there is reason to suspect change or damage. 3. Calibration must be verified for all electronic thermometers, including those described as “self-calibrating.” |

**METHOD 8-4. TESTING REFRIGERATOR ALARMS**

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| Principle | Blood storage refrigerators must be equipped with a system for continuous temperature monitoring and an audible alarm. The alarm must be set to activate at a temperature that will allow appropriate intervention before blood or components reach unacceptable conditions. |

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| Materials | 1. Calibrated thermometer. 2. Pan large enough to hold the thermocouple container. 3. Water. 4. Crushed ice. 5. Table salt. 6. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Verify that the alarm circuits are operating, the alarm is switched on, and the starting temperature is 1 to 6 C. Immerse an easy-to-read calibrated thermometer in the container with the alarm thermocouple. |
|  |  | *For Low Activation* |
|  | 2 | Place the container with the thermocouple and thermometer in a pan containing an ice and water slush at a temperature of –4 C or colder. To achieve this temperature, add several spoonfuls of table salt to the slush. |
|  | 3 | Close the refrigerator door to avoid changing the temperature of the storage compartment. Keep the container in the pan of cold slush, and gently agitate it periodically until the alarm sounds. |
|  | 4 | Record this temperature as the low-activation temperature. |
|  |  | *For High Activation* |
|  | 5 | Place the container with thermocouple and thermometer in a pan containing cool water (eg, 12 to 15 C). |
|  | 6 | Close the refrigerator door. Allow the fluid in the container to warm slowly, with occasional agitation. |
|  | 7 | Record the temperature at which the alarm sounds as the high-activation temperature. |
|  | 8 | Record the date of testing, the refrigerator identification, the thermometer identification, and the identity of the person performing the test. |
|  | 9 | If temperatures of activation are too low or too high, take appropriate corrective actions such as those suggested by the manufacturer, record the nature of the corrections, and repeat the alarm check to document that the corrections were effective. |

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| Notes | 1. Refrigerator temperatures may increase above acceptable limits for several reasons, including the following: improperly closed door, insufficient refrigerant, compressor failure, dirty or blocked heat exchanger, or loss of electrical power. 2. The thermocouple for the alarm should be easily accessible and equipped with acord long enough so that it can be manipulated easily. 3. The thermocouple for the continuous temperature monitor need not be in the same container as that of the alarm. If it is in the same container, a notation should be made in the records that explains any out-of-range temperature registered as a result of the alarm check. 4. When the temperatures of alarm activation are checked, the temperature change should occur slowly enough so that the measurements and recording are accurate. Too rapid a change in temperature may give the false impression that the alarm does not sound until an inappropriate temperature is registered. 5. The low temperature of activation should be greater than 1 C (eg, 1.5 C); the high temperature of activation should be less than 6 C (eg, 5.5 C). 6. AABB *Standards for Blood Banks and Transfusion Services*1 requires that the alarm be set to activate at a temperature that will allow appropriate intervention before blood or components reach unacceptable conditions. 7. Alarms should sound simultaneously at the site of the refrigerator and at the location of remote alarms, when employed. If remote alarms are used, the alarm check should include a verification that the alarm sounded at the remote location. 8. Directions for such events should be available in a conspicuous location, and personnel should be trained to initiate these actions if the temperature cannot be corrected rapidly. 9. The amount of fluid in which the thermocouple is immersed must be no larger than the volume of the smallest component stored in that refrigerator. The thermocouple may be immersed in a smaller volume, but this means that the alarm will go off with smaller temperature changes than those registered in a larger volume of fluid. Excessive sensitivity may create a nuisance. 10. With the one-time assistance of a qualified electrician, the required refrigerator alarm checks of units with virtually inaccessible temperature probes can be performed with an electrical modification cited by Wenz and Owens.2 11. The alarm on each storage unit must be checked periodically for proper functioning. Periodic checks are performed with the manufacturer’s instructions to ensure the equipment is in good operating condition. Because alarms may be disconnected or silenced during repairs, it is also prudent to verify alarm functioning after repairs. 12. If the equipment user’s manual does not provide suitable directions for testing the alarm, consult the manufacturer or other equipment storage expert. The facility standard operating procedures must include a detailed description of the method(s) in local use. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Wenz B, Owens RT. A simplified method for monitoring and calibrating refrigerator alarm systems. Transfusion 1980;20:75-8. 3. Quinley ED, Grace PC. Quality management systems: Principles and practice. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:29-31. |

**METHOD 8-5. TESTING FREEZER ALARMS**

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| Principle | Blood freezers must be equipped with a system for continuous temperature monitoring and an audible alarm. The alarm must be set to activate at a temperature that will allow appropriate intervention before blood or components reach unacceptable conditions. |

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| Materials | 1. Protection for the freezer contents (eg, a blanket). 2. Calibrated thermometer or thermocouple independent from that built into the system. 3. Warm water or an oven mitt. 4. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Protect frozen components from exposure to elevated temperatures during the test. |
|  | 2 | Use a thermometer or thermocouple, independent from that built into the system that will accurately indicate the temperature of alarm activation. Compare these readings with the temperatures registered on the recorder. |
|  | 3 | Warm the alarm probe and thermometer slowly (eg, in warm water, by an ovenmitt-covered hand, exposure to air). The specific temperature of activation cannot be determined accurately during rapid warming, and the apparent temperature of activation will be too high. |
|  | 4 | Record the temperature at which the alarm sounds, the date of testing, the identity of the person performing the test, the identity of the freezer and calibrating instrument, and any observations that might suggest impaired activity. |
|  | 5 | Return the freezer and the alarm system to their normal conditions. |
|  | 6 | If the alarm sounds at too high a temperature, take appropriate corrective actions such as those suggested by the manufacturer, record the nature of the correction, and repeat the alarm check to document that the corrections were effective. |

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| Notes | 1. Freezer temperatures may rise to unacceptable levels for a variety of reasons. Common causes of rising temperatures include the following: improperly closed freezer door or lid, low level of refrigerant, compressor failure, dirty or blocked heat exchanger, or loss of electrical power. 2. Alarms should sound simultaneously at the site of the freezer and at the location of the remote alarms, when employed. If remote alarms are used, the alarm check should include a verification that the alarm sounded at the remote location. 3. AABB *Standards for Blood Banks and Transfusion Services* requires that the alarm be set to activate at a temperature that will allow appropriate intervention before blood or components reach unacceptable conditions. 4. Directions for such events should be available in a conspicuous location, and personnel should be trained to initiate these actions if the temperature cannot be corrected rapidly. |
|  | 1. Test battery function, electrical circuits, and power-off alarms more frequently than the activation temperature. Record function, freezer identification, date, and identity of person performing the testing. |
|  | 1. For units with the sensor installed in the wall or in air, apply local warmth to the site or allow the temperature of the entire compartment to rise to the point at which the alarm sounds. Remove the frozen contents or protect them with insulation while the temperature rises. |
|  | 1. For units with the thermocouple located in antifreeze solution, pull the container and the cables outside the freezer chest for testing, leaving the door shut and the contents protected. |
|  | 1. For units with a tracking alarm that sounds whenever the temperature reaches a constant interval above the setting on the temperature controller, set the controller to a warmer setting and note the temperature interval at which the alarm sounds. |
|  | 1. Liquid-nitrogen freezers must have alarm systems that activate at an unsafe level of contained liquid nitrogen. |

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| References | 1. Gammon R, ed. Standards for blood banks and transfusion services. 32nd ed. Bethesda, MD: AABB, 2020. 2. Quinley ED, Grace PC. Quality management systems: Principles and practice. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:29-31. |

**METHOD 8-6. CALIBRATING CENTRIFUGES FOR PLATELET SEPARATION**

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| Principle | Successful preparation of platelet concentrates requires adequate but not excessive centrifugation; the equipment used must perform in a consistent and dependable manner. |

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| Materials | 1. Freshly collected whole blood, obtained by phlebotomy into a bag with two integrally attached transfer containers. 2. A specimen of blood from the donor, anticoagulated with EDTA and collected in addition to the specimens drawn for routine processing. 3. Metal clips and hand sealer or dielectric sealer. 4. Clean instruments (scissors, hemostats, tubing stripper). 5. Plasma extractor. 6. Centrifuge suitable for preparation of platelet concentrates. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
|  | *For Preparation of PRP* |
|  | 1 | Perform a platelet count on the anticoagulated specimen. |
|  | 2 | Calculate and record the number of platelets in the Whole Blood unit: platelets/μL × 1000 × mL of whole blood = number of platelets in whole blood. |
|  | 3 | Prepare PRP at a selected speed and time. (See method for preparation of Red Blood Cells (RBCs) or see guidance provided by the centrifuge manufacturer.) |
|  | 4 | Place a temporary clamp on the tubing so that one satellite bag is closed off. Express the PRP into the other satellite bag. Seal the tubing close to the primary bag, leaving a long section of tubing, or the “tail.” Disconnect the two satellite bags from the primary bag. Do not remove the temporary clamp between the satellite bags until the platelets are prepared (see next section). |
|  | 5 | Strip the tubing and “tail” several times so that they contain a representative sample of PRP. |
|  | 6 | Seal off a segment of the “tail” and disconnect it so that the bag of PRP remains sterile. |
|  | 7 | Perform a platelet count on the sample of PRP in the sealed segment. Calculate and record the number of platelets in the bag of PRP: platelets/μL × 1000 × mL of PRP = number of platelets in PRP. |
|  | 8 | Calculate and record the percentage of yield: (number of platelets in PRP × 100) divided by (number of platelets in whole blood) = % yield. |
|  | 9 | Repeat the above process three or four times with different donors, using different speeds and times of centrifugation; compare the yields achieved under each set of test conditions. |
|  | 10 | Select the shortest time and lowest speed combination that results in the highest percentage of platelet yield without unacceptable levels of red cell content in the PRP. |
|  | 11 | Record the centrifuge identification, the calibration settings selected, the date, and the identity of the person performing the calibration. |
|  |  | *For Preparation of Platelets* |
|  | 1 | Centrifuge the PRP (as prepared above) at a selected time and speed to prepare platelets. (See method for preparation of RBCs, or see the guidance provided by the centrifuge manufacturer.) |
|  | 2 | Remove the temporary clamp between the two satellite bags, and express the supernatant plasma into the second attached satellite bag, leaving approximately a 55- to 60-mL volume in the platelet bag. Seal the tubing, leaving a long section of tubing attached to the platelet bag. |
|  | 3 | Allow the platelets to rest for approximately 1 hour. |
|  | 4 | Place the platelets on an agitator for at least 1 hour to ensure that they are evenly resuspended. Platelet counts performed immediately after centrifugation will not be accurate. |
|  | 5 | Strip the tubing several times, mixing tubing contents well with the contents of the platelet bag. Seal off a segment of the tubing and disconnect it, so that the platelet bag remains sterile. |
|  | 6 | Perform a platelet count on the contents of the segment. |
|  | 7 | Calculate and record the number of platelets in the concentrate: platelets/μL × 1000 × mL of platelets = number of platelets in platelet concentrate. |
|  | 8 | Calculate and record the percentage of yield. |
|  | 9 | Repeat the above process with PRP from different donors, using different speeds and times of centrifugation; compare the yields achieved under each set of test conditions. |
|  | 10 | Select the shortest time and lowest speed combination that results in the highest percentage of platelet yield in the platelet concentrate. |
|  | 11 | Record the centrifuge identification, the calibration settings selected, the date performed, and the identity of the person performing the calibration. |

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| Notes | 1. It is not necessary to perform functional recalibration of a centrifuge unless the instrument has undergone adjustments or repairs, or unless component quality control indicates that platelet countshave fallen below acceptable levels. However, timer, speed, and temperature calibrations of the centrifuge should occur on a regularly scheduled basis. Suggested quality control performance intervals are found in Chapter 1 of the AABB *Technical Manual*. 2. Each centrifuge used for preparing platelets must be calibrated individually. Use the conditions determined to be optimal for each instrument. 3. Each centrifuge used to prepare platelets should be calibrated upon receipt and after adjustment or repair. Functional calibration of the centrifuge for both the preparation of platelet-rich plasma (PRP) from whole blood and the subsequent preparation of platelet concentrates from PRP can be performed during the same procedure. |
|  | 1. When counting platelet samples on an instrument intended for whole blood, it may be necessary to use a correction factor to obtain accurate results. 2. When determining the appropriate time and speed of centrifugation, one should consider other products that will be prepared from the whole blood. Final size and hematocrit of red cell and plasma volume made available for further processing are important factors to consider. 3. In one study,1 the average number of platelets in a typical Whole Blood unit was 1.14 × 1011. This figure was based on an average 238,000/μL platelet count of blood donors and an average 478 mL of whole blood collected per unit. PRP from such units contained 8.3 × 1010 platelets, and the platelet concentrates contained 9 × 1010 platelets.1 Average yield in a platelet concentrate was 69%.1 4. In one study,2 platelet concentrate samples collected in dry K2EDTA (1.5 mg/mL) and then counted in a flow cell cytometer gave higher platelet counts than the samples collected without the EDTA—probably the result of microaggregates in samples without EDTA. |

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| References | 1. Kahn R, Cossette I, Friedman L. Optimum centrifugation conditions for the preparation of platelet and plasma products. Transfusion 1976;16:162-5. 2. McShine R, Das P, Smit Sibinga C, Brozovic B. Effect of EDTA on platelet parameters in blood and blood components collected with CPDA­1. Vox Sang 1991;61:84-9. 3. Quinley ED, Grace PC. Quality management systems: Principles and practice. In: Cohn C, Delaney M, Johnson S, Katz L, eds. Technical manual. 20th ed. Bethesda, MD: AABB, 2020:29-31. |

**METHOD 8-7. CALIBRATING A SEROLOGIC CENTRIFUGE FOR IMMEDIATE AGGLUTINATION**

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| Principle | Each centrifuge should be calibrated upon receipt, after adjustments or repairs, and periodically. Calibration evaluates the behavior of red cells in solutions of different viscosities, not the reactivity of different antibodies. |

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| Materials | 1. Test tubes, 10 × 75 mm or 12 × 75 mm (whichever size is routinely used in the laboratory). 2. Worksheet for recording results. 3. For saline-active antibodies:  * Serum from a group A person (anti-B) diluted with 6% albumin to give 1+ macroscopic agglutination (3 mL of 22% bovine albumin + 8 mL of nor­mal saline = 6% bovine albumin). See method for dilution of percentage solutions. * *Positive control:* Group B red cells in a 2% to 5% saline suspension. * *Negative control:* Group A red cells in a 2% to 5% saline suspension.  1. For high-protein antibodies:  * Anti-D diluted with 22% albumin to give 1+ macroscopic agglutination. * *Positive control:* D+ red cells in a 2% to 5% saline suspension. * *Negative control:* D– red cells in a 2% to 5% saline suspension. |

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| Procedure | **Step** | **Action** |
| 1 | For each set of tests (saline and high-protein antibodies), label five test tubes for positive reactions and five for negative reactions. |
|  | 2 | In quantities that correspond to routine use, add diluted anti-B to each of 10 tubes for the saline test and add diluted anti-D to each of 10 tubes for the high-protein test. Add serum and reagents in quantities that correspond to routine use. |
|  | 3 | Add the appropriate control cell suspension to one set of tubes (one positive and one negative tube for the saline test, and one positive and one negative tube for the high-protein antibody test). Centrifuge immediately for the desired time interval (eg, 10 seconds). |
|  | 4 | Observe each tube for agglutination and record observations. (See example in table below.) |
|  | 5 | Repeat Steps 2 and 3 for each time interval (eg, 15, 20, 30, and 45 seconds). Do not allow cells and sera to incubate before centrifugation. |
|  | 6 | Select the optimal time of centrifugation, which is the shortest time required to fulfill the following criteria:   1. The supernatant fluid is clear. 2. The cell button is clearly delineated and the periphery is sharply defined, not fuzzy. 3. The cell button is easily resuspended. 4. Agglutination in the positive tubes is as strong as determined in preparing reagents. 5. There is no agglutination or ambiguity in the negative tubes. |
|  | 7 | Record centrifuge identification, the times selected, the date, and the identity of the person performing the calibration. |

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| Interpretation | An example of readings from Step 4 and Step 6 follows:  Example of Serologic Centrifuge Test Results\*   |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | |  | Time in Seconds | | | | | | **Criteria** | 10 | 15 | 20 | 30 | 45 | | Supernatant fluid is clear | No | No | Yes | Yes | Yes | | Cell button is clearly delineated | No | No | No | Yes | Yes | | Cells are easily resuspended | Yes | Yes | Yes | Yes | Yes | | Agglutination is observed | ± | ± | 1+ | 1+ | 1+ | | Negative tube is negative | Yes | Yes | Yes | Yes | Resuspends roughly | | \*The optimal time for centrifugation in this example is 30 seconds. | | | | | | |

**METHOD 8-8. CALIBRATING A SEROLOGIC CENTRIFUGE FOR WASHING AND ANTIGLOBULIN TESTING**

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| Principle | Tests in which antihuman globulin (AHG) serum is added to red cells may require centrifugation conditions different from those for immediate agglutination. Centrifugation conditions appropriate for both washing and AHG reactions can be determined in one procedure. |

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| Materials | 1. AHG reagent, unmodified. 2. Saline, large volumes. 3. Test tubes, 10 × 75 mm or 12 × 75 mm (whichever size is routinely used in the laboratory). 4. Worksheet for recording results. 5. *Positive control:* a 2% to 5% saline suspension of D+ red cells incubated for 15 minutes at 37 C with anti-D diluted to give 1+ macroscopic agglutination after addition of AHG. 6. *Negative control:* a 2% to 5% suspension of D+ red cells incubated for 15 minutes at 37 C with 6% albumin. (Note: D– red cells incubated with diluted anti-D may also be used as a negative control.) |

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| Procedure | **Step** | **Action** |
| 1 | Prepare five test tubes containing 1 drop of positive cells and five tubes containing 1 drop of negative control cells. |
|  | 2 | Fill tubes with saline and centrifuge them in pairs, one positive and one negative, for different times (eg, 30, 45, 60, 90, and 120 seconds). The red cells should form a clearly delineated button, with minimal cells trailing up the side of the tube. After the saline has been decanted, the cell button should be easily resuspended in the residual fluid. The optimal time for washing is the shortest time that accomplishes these goals. |
|  | 3 | Repeat washing process on all pairs three more times, using time determined to be optimal. |
|  | 4 | Decant supernatant saline thoroughly. |
|  | 5 | Add AHG to one positive control test tube and one negative control test tube. Centrifuge immediately for the desired interval (eg, 10 seconds). |
|  | 6 | Observe each tube for agglutination and record observations. |
|  | 7 | Repeat Steps 5 and 6 for each interval (eg, 15, 20, 30, and 45 seconds). Do not allow cells and AHG to incubate before centrifugation. |
|  | 8 | Select optimal time as in immediate agglutination procedure. |
|  | 9 | Record centrifuge identification, the times selected, the date, and the identity of the person performing the calibration. |

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| Interpretation | An example of readings from Step 6 and Step 8 follows:  Example of Serologic Centrifuge Test Results\*   |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | |  | Time in Seconds | | | | | | **Criteria** | 10 | 15 | 20 | 30 | 45 | | Supernatant fluid is clear | No | No | Yes | Yes | Yes | | Cell button is clearly delineated | No | No | No | Yes | Yes | | Cells are easily resuspended | Yes | Yes | Yes | Yes | Yes | | Agglutination is observed | ± | ± | 1+ | 1+ | 1+ | | Negative tube is negative | Yes | Yes | Yes | Yes | Resuspends roughly | | \*The optimal time for centrifugation in this example is 30 seconds. | | | | | | |

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| Notes | 1. This procedure does not monitor the completeness of washing; use of IgG-coated cells to control negative AHG reactions provides this check. This procedure addresses only the mechanics of centrifugation. 2. Periodic recalibration is performed to verify that the timing in use continues to be the optimal timing. This may be accomplished by using a shortened version of the procedures outlined above. For example, use the current timing for a particular centrifuge and each medium and those times just above and just below the current timing. |

**METHOD 8-9. TESTING AUTOMATIC CELL WASHERS**

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| Principle | Antihuman globulin (AHG) is inactivated readily by unbound immunoglobulin. The red cells to which AHG will be added must be washed free of all proteins and suspended in a protein-free medium. A properly functioning cell washer must add large volumes of saline to each tube, resuspend the cells, centrifuge them adequately to avoid excessive red cell loss, and decant the saline to leave a dry cell button. |

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| Materials | 1. Test tubes routinely used in the laboratory, 10 × 75 mm or 12 × 75 mm. 2. Additive routinely used to potentiate antigen-antibody reactions. 3. Human serum, from patient or donor. 4. IgG-coated red cells known to give a 1 to 2+ reaction in antiglobulin testing. 5. Normal saline. 6. AHG reagent, anti-IgG or polyspecific. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | To each of 12 tubes, add potentiator and human serum in quantities that correspond to routine use, and 1 drop of IgG-coated red cells. |
|  | 2 | Place the tubes in a centrifuge carrier, seat the carrier in the cell washer, and start the wash cycle. |
|  | 3 | After addition of saline in the second cycle, stop the cell washer. Inspect the contents of all tubes. There should be an approximately equal volume of saline in all tubes; some variation is acceptable. Tubes should be approximately 80% full, to avoid splashing and cross-contamination. (Refer to manufacturer’s instructions for specific requirements.) Record observations. |
|  | 4 | Observe all tubes to see that the red cells have been completely resuspended. Record observations. |
|  | 5 | Continue the washing cycle. |
|  | 6 | After addition of saline in the third cycle, stop the cell washer and inspect tubes as above. Record observations. |
|  | 7 | Complete the wash cycle. |
|  | 8 | At the end of the wash cycle, inspect all tubes to see that saline has been completely decanted and that each tube contains a dry cell button. Record observations. |
|  | 9 | Add AHG according to the manufacturer’s directions, centrifuge, and examine all tubes for agglutination. If the cell washer is functioning properly, the size of the cell button should be the same in all tubes. All tubes should show the same degree of agglutination. Record observations. |
|  | 10 | Record identity of centrifuge, the date of testing, and the identity of the person performing the testing. |

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| Notes | 1. Further investigation is needed if: 2. The amount of saline varies significantly from tube to tube or cycle to cycle. 3. The cell button is not resuspended completely after being filled with saline. 4. Any tube has weak or absent agglutination in the antiglobulin phase. 5. Any tube has a significant decrease in the size of the cell button. 6. Cell washers that automatically add AHG should also be checked for uniform addition of AHG. In Step 9 above, AHG would be added automatically, and failure of addition would be apparent by absence of agglutination. The volume of AHG should be inspected and found to be equal in all tubes. The volume of AHG delivered automatically by cell washers should be checked monthly to ensure that it is as specified in the manufacturer’s directions and that delivery is uniform in all tubes. 7. Some manufacturers market AHG colored with green dye for use in automated cell washers so that it will be immediately obvious if no reagent has been added. |

**METHOD 8-10. MONITORING CELL COUNTS OF APHERESIS COMPONENTS**

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| Principle | When cellular components are prepared by apheresis, it is essential to determine cell yields without compromising the sterility of the component. |

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| Materials | 1. Component collected by apheresis. 2. Metal clips and hand sealer or dielectric sealer. 3. Tubing stripper. 4. Clean instruments (scissors, hemostats). 5. Test tubes. 6. Cell-counting equipment. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Ensure that the contents of the apheresis component bag are well mixed. |
|  | 2 | Strip the attached tubing at least four times, mixing the contents of the tubing with the contents of the bag, to ensure that the contents of the tubing accurately represent the entire contents of the bag. |
|  | 3 | Seal a 5- to 8-cm (2- to 3-inch) segment distal to the collection bag. There should be approximately 2 mL of fluid in the segment. Double-seal the end of the tubing next to the component bag and detach the segment. |
|  | 4 | Empty the contents of the segment into a suitably labeled tube. |
|  | 5 | Determine and record cell counts in cells/mL.   1. For results reported as cells/μL, change values to cells/mL by multiplying by 1000 (or 103). 2. For results reported as cells/L, change values to cells/mL by dividing by 1000 (or 103). |
|  | 6 | Multiply cells/mL by the volume of the component, in mL, to obtain total cell count in the component. |
|  | 7 | Record component’s identity, the date, and the identity of the person performing the testing. |

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| Note | Refer to the manufacturer’s directions for any additional requirements. |

**METHOD 8-11. COUNTING RESIDUAL WHITE CELLS IN LEUKOCYTE-REDUCED BLOOD AND COMPONENTS—MANUAL METHOD**

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| Principle | The residual white cell content of leukocyte-reduced (LR) whole blood and components can be determined using a large-volume hemocytometer. |

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| Materials | 1. Hemocytometer chamber with 50-μL counting volume [eg, Nageotte Brite Line Chamber (Biotrans GmbH)]. 2. 0.01% Turk’s solution. 3. Red cell lysing agent (eg, Zapoglobin, Coulter Electronics, Brea, CA), for red-cell-containing components only. 4. Pipettor (40 μL and 100 μL) with disposable pipette tips. 5. Talc-free gloves, clean plastic test tubes, plastic petri dish, and filter paper. 6. Light microscope with 10× ocular lens and 20× objective. 7. Worksheet for recording results. |

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| Procedure | **Step** | **Action** |
| 1 | Dilute and stain LR blood and component samples as follows:   1. For red-cell-containing components: 2. Pipette 40 μL of lysing agent into a clean test tube. 3. Place a representative sample of the component to be tested in a clean test tube. The hematocrit of the sample to be tested should not exceed 60%. 4. Pipette 100 μL of the sample into the tube containing 40 μL of lysing agent. Rinse the pipette several times to mix the two fluids, until the pipette tip is no longer coated with intact red cells. 5. Pipette 360 μL of 0.01% Turk’s solution into the mixture, and mix fluids by pipetting up and down several times. The final volume is now 500 μL. 6. For platelets: 7. Place a representative sample of the platelet in a clean test tube. 8. Pipette 100 μL of the platelet sample into a clean test tube. 9. Pipette 400 μL of 0.01% of Turk’s solution into the 100 μL of platelets, and mix fluids by pipetting up and down several times. The final volume is now 500 μL. |
|  | 2 | Fit the hemocytometer with a cover slip; using a pipette, load the mixture until the counting area is completely covered but not overflowing. |
|  | 3 | Cover the hemocytometer with a moist lid to prevent evaporation (a plastic petri dish into which a piece of damp filter paper has been placed works well), and let it rest undisturbed for 10 to 15 minutes to allow the white cells to settle in the counting area of the chamber. |
|  | 4 | Remove the moist lid, place the hemocytometer on the microscope and—using a 20× objective—count the white cells present in the entire 50-μL volume of the counting chamber. White cells appear as intact cells that are refractile with a blue gray color. |
|  | 5 | Calculate and record results.   1. White cell concentration:   leukocytes/μL = (cells counted/50 μL) × 5 where 50 μL is the volume counted and 5 is the dilution factor resulting from the addition of lysing agent and Turk’s solution.   1. Total white cell content of the LR component:   leukocytes/component = leukocytes/μL × 1000 μL/mL × volume in mL of the component. |
|  | 6 | Record the component’s identity, the date obtained, and the identity of the person performing the testing. |

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| Notes | 1. For red-cell-containing components, the red cells in the aliquot to be counted are first lysed; 0.01% Turk’s solution is used to stain the leukocyte nuclei. 2. The Nageotte counting chamber has a volume 56 times that of the standard hemocytometer. Accuracy of counting is improved by examining a larger volume of minimally diluted specimen, compared with standard counting techniques. 3. White cells deteriorate during refrigerated storage; counts on stored blood or red cell components may give inaccurate results. 4. Use of talc-free gloves is recommended because talc particles that contaminate the counting chamber can be misread as white cells. 5. Filtration of the Turk’s solution (0.22 micron) is recommended if the counting chamber demonstrates a large amount of particulates. 6. The accuracy of the counting method can be validated from a reference sample with a high white cell content that has been quantified by another means. This reference sample can be used for serial dilutions in blood or a component that has been rendered extremely leukocyte reduced by two passages through a leukocyte-reduction filter. Counts obtained on the serially diluted samples can be compared with the expected concentration derived by calculation. 7. This counting technique is not known to be accurate at concentrations lower than 1 white cell/μL. |

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| References | 1. Lutz P, Dzik WH. Large-volume hemocytometer chamber for accurate counting of white cells (WBCs) in WBC-reduced platelets; vali­dation and application for quality control of WBC-reduced platelets prepared by apheresis and filtration. Transfusion 1993;33:409-12. 2. Dzik WH, Szuflad P. Method for counting white cells in white cell-reduced red cell concentrates (letter). Transfusion 1993;33:272. |

Appendices

|  |  |  |
| --- | --- | --- |
| Appendix 1. Normal Values in Adults\*† | | |
| **Determination** | **SI Units** | **Conventional Units** |
| Alanine aminotransferase |  |  |
| Males | 21-72 U/L at 37 C | 21-72 U/L at 37 C |
| Females | 9-52 U/L at 37 C | 9-52 U/L at 37 C |
| Bilirubin, total | 0-22 µmol/L | 0.2-1.3 mg/dL |
| Haptoglobin | 0.3-2.0 g/L | 30-200 mg/dL |
| Hematocrit |  |  |
| Males | 0.44-0.53 | 44.2-53% |
| Females | 0.36-0.49 | 36-49% |
| Hemoglobin (Hb) |  |  |
| Males | 148-178 g/L | 14.8-17.8 g/dL |
| Females | 126-159 g/L | 12.6-15.9 g/dL |
| Hemoglobin A2 | 0.020-0.035 total Hb | 2.0-3.5% total Hb |
| Hemoglobin F | 0-0.021 total Hb | 0-2.1% total Hb |
| Hemoglobin (plasma) | 0-97 mg/L | 0-9.7 mg/dL |
| Immunoglobulins |  |  |
| IgG | 7.68.0-16.32 g/L | 768-1632 mg/dL |
| IgA | 0.6-4.0 g/L | 68-408 mg/dL |
| IgM | 0.3-2.6 g/L | 35-263 mg/dL |
| IgD | ≤15 mg/L | ≤15.3 mg/dL |
| IgE | ≤214 IU/L | ≤214 kU/L |
| Methemoglobin | 0-1.9 total Hb | 0-1.9% total Hb |
| Platelet count | 150-450 × 109/L | 150,000-450,000/µL |
| Red cells |  |  |
| Males | 4.17-6.14 × 1012/L | 4.17-6.14 × 106/µL |
| Females | 4.08-5.47 × 1012/L | 4.08-5.47 × 106/µL |
| Reticulocyte count |  |  |
| Males | 47-152 × 109/L | 47-152 × 103/µL |
| Females | 47-127 × 109/L | 47-127 × 103/µL |
| Viscosity, relative | 1.10-1.80 × water | 1.10-1.80 cP × water |
| White cells | 4.3-11.3 × 109/L | 4.3-11.3 × 103/µL |

\*The normal ranges can vary from one laboratory to another, and depend on the local population, equipment,

test method, and conditions of testing.

**†**Data compiled from ARUP Laboratories, Salt Lake City, Utah.

|  |  |  |  |
| --- | --- | --- | --- |
| Appendix 2. Selected Normal Values in Children\* | | | |
|  |  | Bilirubin (total) | |
| **Age** |  | **SI Units** | **Conventional Units** |
| Cord | Preterm | <30 mmol/L | <1.8 mg/dL |
|  | Term | <30 mmol/L | <1.8 mg/dL |
| 0-1 day | Preterm | <137 mmol/L | <8 mg/dL |
|  | Term | <103 mmol/L | <6 mg/dL |
| 1-2 days | Preterm | <205 mmol/L | <12 mg/dL |
|  | Term | <137 mmol/L | <8 mg/dL |
| 3-7 days | Preterm | <274 mmol/L | <16 mg/dL |
|  | Term | <205 mmol/L | <12 mg/dL |
| 7-30 days | Preterm | <205 mmol/L | <12 mg/dL |
|  | Term | <120 mmol/L | <7 mg/dL |
| Thereafter | Preterm |  |  |
|  | 0 days-20 years | 10-24 mmol/L | 0.6-1.4 mg/dL |
|  | After 20 years | 3-22 mmol/L | 0.2-1.3 mg/dL |
|  | Term | <17 mmol/L | <1 mg/dL |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Age | Hemoglobin (g/dL) | | White Blood Cells (× 109/L) | |
|  | Male | Female | Male | Female |
| ≤3 days | 13.5-22.5 | | 8.04-15.4 | 8.16-14.56 |
| 4-7 days | 13.5-19.5 | | 8.04-15.4 | 8.16-14.56 |
| 8-14 days | 12.5-20.5 | | 8.04-15.4 | 8.16-14.56 |
| 15-30 days | 10.0-18.0 | | 7.8-15.91 | 8.36-14.42 |
| 31-60 days | 9.0-13.5 | | 8.14-14.99 | 7.05-14.68 |
| 61-180 days | 9.5-13.5 | | 6.51-13.32 | 6.0-13.25 |
| 6 months to <2 years | 10.5-13.5 | | 5.98-13.51 | 6.48-13.02 |
| 2 to <6 years | 11.5-13.5 | | 5.14-13.38 | 4.86-13.18 |
| 6 to <12 years | 11.5-15.5 | | 4.31-11.0 | 4.27-11.4 |
| 12 to <18 years | 13.0-16.0 | 12.0-16.0 | 3.84-9.84 | 4.19-9.43 |
| ≥18 years | 13.5-17.5 | 12.0-16.0 | 3.91-8.77 | 4.37-9.68 |
|  | Platelet count: 150,000-400,000 μL | | | |

*(Continued)*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Age** | **IgG** | | **IgM** | | **IgA** | |
| Newborn | 831-1231 mg/dL | | 6-16 mg/dL | | <3 mg/dL | |
| 1-3 months | 312-549 mg/dL | | 19-41 mg/dL | | 8-34 mg/dL | |
| 4-6 months | 241-613 mg/dL | | 26-60 mg/dL | | 10-46 mg/dL | |
| 7-12 months | 442-880 mg/dL | | 31-77 mg/dL | | 19-55 mg/dL | |
| 13-24 months | 553-971 mg/dL | | 35-81 mg/dL | | 26-74 mg/dL | |
| 25-36 months | 709-1075 mg/dL | | 42-80 mg/dL | | 34-108 mg/dL | |
| 3-5 years | 701-1157 mg/dL | | 38-74 mg/dL | | 66-120 mg/dL | |
| 6-8 years | 667-1179 mg/dL | | 40-80 mg/dL | | 79-169 mg/dL | |
| 9-11 years | 889-1359 mg/dL | | 46-112 mg/dL | | 71-191 mg/dL | |
| 12-16 years | 822-1070 mg/dL | | 39-79 mg/dL | | 85-211 mg/dL | |
|  | | **Activated Partial Thromboplastin Time** | |  | | **Prothrombin Time** | |
| Preterm | | 70 seconds | |  | | 12-21 seconds | |
| Term | | 45-65 seconds | |  | | 13-20 seconds | |
| \*Data compiled from:  The Harriet Lane Handbook. 15th ed. St. Louis, MO: Mosby, 2000.  The Children’s Hospital of Philadelphia. Hematology normal values. Book 21-21.010P. Core laboratory procedure manual. | | | | | | | |

|  |  |
| --- | --- |
| Appendix 3. Typical Normal Values in Tests of Hemostasis and Coagulation (Adults) | |
| **Test** | **Normal Value** |
| Activated partial thromboplastin time | 24-35 seconds |
| Bleeding time | N/A |
| Coagulation factors | N/A |
| Fibrin degradation products | <5 mg/L |
| Fibrinogen | 150-430 mg/dL |
| Plasma D-dimers | 0.0-0.4 µg/mL |
| Protein C | 83-168% |
| Protein S (total) | 57-131% |
| Prothrombin time | 11.6-13.8 seconds |
| Thrombin time | 14.7-19.5 seconds |

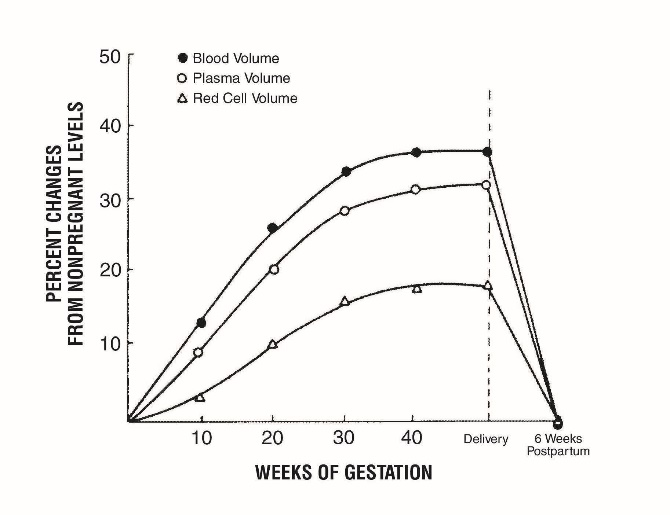
Reprinted with permission from Henry JB. Clinical diagnosis and management by laboratory methods. 23rd ed. Philadelphia: Elsevier, 2017.

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| --- | --- | --- | --- | --- | --- | --- | --- |
| Appendix 4. Coagulation Factor Values in Platelet Concentrates | | | | | | | |
| **Factor/Protein** | Normal Range | **Day 0** | **Day 1** | **Day 2** | **Day 3** | **Day 4** | **Day 5** |
| II % | 78-122 | 104 | 91-96 | 96 | 85-94 | 90 | 90 |
| V % | 47-153 | 78-98 | 69-78 | 50 | 36-47 | 28 | 24-35 |
| VII % | 51-168 | 108 | 93-117 | 88 | 80-103 | 75 | 72 |
| VIII % | 48-152 | 68-126 | 85-99 | 76 | 68-76 | 75 | 39-70 |
| IX % | 62-138 | 72-105 | 100-106 | 95 | 91-98 | 93 | 63-97 |
| X % | 58-142 | 66-101 | 93-94 | 92 | 85-88 | 84 | 60-83 |
| XI % | 52-148 | 91-111 | 106-108 | 103 | 96-98 | 101 | 86-110 |
| XII % | 46-126 | 117 | 107-112 | 116 | 106-123 | 123 | 131 |
| C % | 57-128 | 106 | 102 | 101 | 98 | 99 | 100 |
| S % | 83-167 | 95 | 75 | 61 | 40 | 32 | 31 |
| Antithrombin % | 88-126 | 103 | 99 | 101 | 102 | 103 | 97 |
| Plasminogen % | 60-140 | 140 | 133 | 126 | 122 | 124 | 117 |
| Fibrinogen mg/dL | 198-434 | 217-308 | 278-313 | 310 | 265-323 | 302 | 221-299 |
| Ristocetin -cofactor % | 50-150 | 106 | 124 | 125 | 133 | 116 | 127 |
| Note: Coagulation factor % = 100 × coagulation factor units/mL. Reproduced with permission from Brecher ME, ed. Collected questions and answers. 6th ed. Bethesda, MD: AABB, 2000. | | | | | | | |

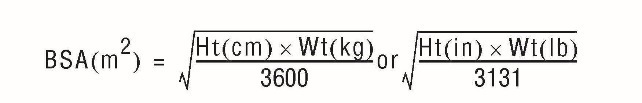
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Appendix 5. Approximate Normal Values for Red Cell, Plasma, and Blood Volumes | | | | |
|  | Infant1 | | Adult2 | |
|  | Premature | Term Birth at 72 hours | Male | Female |
| Red Cell Volume mL/kg | 50 | 40 | 26 | 24 |
| Plasma Volume mL/kg | 58 | 47 | 40 | 36 |
| Blood Volume mL/kg | 108 | 87 | 66 | 60 |

The adult values should be modified to correct for:

1. Below age 18: increase values by 10%.
2. Weight loss:
   1. Marked loss within 6 months—calculations made at original weight.
   2. Gradual loss over a longer time—calculations made at present weight and raised 10% to 15%.
3. Obese and short: values are reduced by 10%.
4. Elderly: values are reduced by 10%.
5. Pregnancy3:



Estimation of Body Surface Area4:



Blood Volume (BV)5:

BV = 2740 mL/m2—males

BV = 2370 mL/m2—females

Hematocrit6:

Venous hematocrit = HV (blood obtained by vein or finger puncture)

Whole-body hematocrit = HB

HB = (Hv) × (0.91)

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Appendix 6. Blood Group Antigens Assigned to Systems

In 1980, the International Society of Blood Transfusion (ISBT) formed a Working Party on Terminology for Red Cell Surface Antigens. The task of this group was to devise a uniform nomenclature that would be both eye and machine readable. The numeric system proposed by this group was not intended to replace traditional terminology but, instead, to enable communication using computer systems where numbers are necessary. It also provides a genetical classification for blood groups. ISBT terminology uses uppercase letters and Arabic numbers for system and antigen codes. Each system, collection, or series of antigens is given a number (eg, ABO system = 001), and each antigen within the system is given a number (eg, A = 001, B = 002). Sinistral zeros may be omitted. Thus, in ISBT terminology, the A antigen would be written using computer code as 001001, or using the system symbol as ABO1.

The Working Party meets biennially in conjunction with the ISBT International Scientific Congress to update assignment of antigens to systems, collections, and series. The table below lists the blood group systems and the antigens assigned to those systems. Other red cell antigens are assigned to collections and to series of high- and low-prevalence antigens. Although all terms in the table are acceptable, the *Technical Manual* and *TRANSFUSION* choose to use traditional terminology for blood group antigens in most cases. Further information on blood group terminology, which antigens are assigned to the collections, and the series of high- and low-prevalence antigens can be found in the references.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **System (Symbol/Number)** | **Antigen**  **(ISBT Number)** |  |  |  |
| ABO (ABO/001) | A (ABO1) |  |  |  |
|  | B (ABO2) |  |  |  |
|  | A,B (ABO3) |  |  |  |
|  | A1(ABO4) |  |  |  |
| MNS (MNS/002) | M (MNS1) | Me (MNS13) | Dantu (MNS25) | ERIK (MNS37) |
|  | N (MNS2) | Mta (MNS14) | Hop (MNS26) | Osa (MNS38) |
|  | S (MNS3) | Sta (MNS15) | Nob (MNS27) | ENEP (MNS39) |
|  | s (MNS4) | Ria (MNS16) | Ena (MNS28) | ENEH (MNS40) |
|  | U (MNS5) | Cla (MNS17) | EnaKT (MNS29) | HAG (MNS41) |
|  | He (MNS6) | Nya (MNS18) | ‘N’ (MNS30) | ENAV (MNS42) |
|  | Mia (MNS7) | Hut (MNS19) | Or (MNS31) | MARS (MNS43) |
|  | Mc (MNS8) | Hil (MNS20) | DANE (MNS32) | ENDA (MNS44) |
|  | Vw (MNS9) | Mv (MNS21) | TSEN (MNS33) | ENEV (MNS45) |
|  | Mur (MNS10) | Far (MNS22) | MINY (MNS34) | MNTD (MNS46) |
|  | Mg (MNS11) | sD (MNS23) | MUT (MNS35) | SARA (MNS47) |
|  | VR (MNS12 | MIT (MNS24) | SAT (MNS36) | KIPP (MNS48) |
|  |  |  |  | JENU (MNS49) |
| P1PK (P1PK/003) | P1 (P1PK1) | Pk (P1PK3) | NOR (P1PK4) |  |
| Rh (RH/004) | D (RH1) | hrS (RH19) | Rh35 (RH35) | FPTT (RH50) |
|  | C (RH2) | VS (RH20) | Bea (RH36) | MAR (RH51) |
|  | E (RH3) | CG (RH21) | Evans (RH37) | BARC (RH52) |
|  | c (RH4) | CE (RH22) | Rh39 (RH39) | JAHK (RH53) |
|  | e (RH5) | DW (RH23) | Tar (RH40) | DAK (RH54) |
|  | f (RH6) | c-like (RH26) | Rh41 (RH41) | LOCR (RH55) |
|  | Ce (RH7) | cE (RH27) | Rh42 (RH42) | CENR (RH56) |
|  | CW (RH8) | hrH (RH28) | Crawford (RH43) | CEST (RH57) |
|  | CX (RH9) | Rh29 (RH29) | Nou (RH44) | CELO (RH58) |
|  | V (RH10) | Goa (RH30) | Riv (RH45) | CEAG (RH59) |
|  | EW (RH11) | hrB (RH31) | Sec (RH46) | PARG (RH60) |
|  | G (RH12) | Rh32 (RH32) | Dav (RH47) | CEVF (RH61) |
|  | Hro (RH17) | Rh33 (RH33) | JAL (RH48) | CEWA (RH62) |
|  | Hr (RH18) | HrB (RH34) | STEM (RH49) |  |
| Lutheran (LU/005) | Lua (LU1) | Lu8 (LU8) | Lu17 (LU17) | LUGA (LU24) |
|  | Lub (LU2) | Lu9 (LU9) | Aua (LU18) | LUAC (LU25) |
|  | Lu3 (LU3) | Lu11 (LU11) | Aub (LU19) | LUBI (LU26)  LUYA (LU27)  LUNU (LU28)  LURA (LU29) |
|  | Lu4 (LU4) | Lu12 (LU12) | Lu20 (LU20) |  |
|  | Lu5 (LU5) | Lu13 (LU13) | Lu21 (LU21) |  |
|  | Lu6 (LU6) | Lu14 (LU14) | LURC (LU22) |  |
|  | Lu7 (LU7) | Lu16 (LU16) | LUIT (LU23) |  |
| Kell (KEL/006) | K (KEL1) | K13 (KEL13) | K24 (KEL24) | KASH (KEL34) |
|  | k (KEL2) | K14 (KEL14) | VLAN (KEL25) | KELP (KEL35) |
|  | Kpa (KEL3) | K16 (KEL16) | TOU (KEL26) | KETI (KEL36) |
|  | Kpb (KEL4) | K17 (KEL17) | RAZ (KEL27) | KHUL (KEL37) |
|  | Ku (KEL5) | K18 (KEL18) | VONG (KEL28) | KYOR (KEL38) |
|  | Jsa (KEL6) | K19 (KEL19) | KALT (KEL29) | KEAL (KEL39) |
|  | Jsb (KEL7) | Km (KEL20) | KTIM (KEL30) |  |
|  | Ula (KEL10) | Kpc (KEL21) | KYO (KEL31) |  |
|  | K11 (KEL11) | K22 (KEL22) | KUCI (KEL32) |  |
|  | K12 (KEL12) | K23 (KEL23) | KANT (KEL33) |  |
| Lewis (LE/007) | Lea (LE1) | Leab (LE3) | ALeb (LE5) |  |
|  | Leb (LE2) | LebH (LE4) | BLeb (LE6) |  |
| Duffy (FY/008) | Fya (FY1) | Fy3 (FY3) | Fy6 (FY6) |  |
|  | Fyb (FY2) | Fy5 (FY5) |  |  |
| Kidd (JK/009) | Jka (JK1) | Jkb (JK2) | Jk3 (JK3) |  |
| Diego (DI/010) | Dia (DI1) | WARR (DI7) | Vga (DI13) | Tra (DI19)\* |
|  | Dib (DI2) | ELO (DI8) | Swa (DI14) | Fra (DI20) |
|  | Wra (DI3) | Wu (DI9) | BOW (DI15) | SW1 (DI21) |
|  | Wrb (DI4) | Bpa (DI10) | NFLD (DI16) | DISK (DI22) |
|  | Wda (DI5) | Moa (DI11) | Jna (DI17) |  |
|  | Rba (DI6) | Hga (DI12) | KREP (DI18) |  |
| Yt (YT/011) | Yta (YT1)  Ytb (YT2) | YTEG (YT3)  YTLI (YT4) | YTOT (YT5) |  |
| Xg (XG/012) | Xga (XG1) | CD99 (XG2) |  |  |
| Scianna (SC/013) | Sc1 (SC1) | Sc3 (SC3) | STAR (SC5) | SCAN (SC7) |
|  | Sc2 (SC2) | Rd (SC4) | SCER (SC6) |  |
| Dombrock (DO/014) | Doa (DO1) | Hy (DO4) | DOMR (DO7) | DODE (DO10) |
|  | Dob (DO2) | Joa (DO5) | DOLG (DO8) |  |
|  | Gya (DO3) | DOYA (DO6) | DOLC (DO9) |  |
| Colton (CO/015) | Coa (CO1) | Cob (CO2) | Co3 (CO3) | Co4 (CO4) |
| Landsteiner- Wiener  (LW/016) | LWa (LW5) | LWab (LW6) | LWb (LW7) |  |
| Chido/Rodgers (CH/RG/017) | Ch1 (CH/RG1) | Ch4 (CH/RG4) | WH (CH/RG7) |  |
|  | Ch2 (CH/RG2) | Ch5 (CH/RG5) | Rg1 (CH/RG11) |  |
|  | Ch3 (CH/RG3) | Ch6 (CH/RG6) | Rg2 (CH/RG12) |  |
| H (H/018) | H (H1) |  |  |  |
| Kx (XK/019) | Kx (XK1) |  |  |  |
| Gerbich (GE/020) | Ge2 (GE2) | Wb (GE5) | Dha (GE8) | GEAT (GE11) |
|  | Ge3 (GE3) | Lsa (GE6) | GEIS (GE9) | GETI (GE12) |
|  | Ge4 (GE4) | Ana (GE7) | GEPL (GE10) |  |
| Cromer (CROM/021) | Cra (CROM1) | Esa (CROM6) | GUTI (CROM11) | CROZ (CROM16) |
|  | Tca (CROM2) | IFC (CROM7) | SERF (CROM12) | CRUE (CROM17) |
|  | Tcb (CROM3) | WESa (CROM8) | ZENA (CROM13) | CRAG (CROM18) |
|  | Tcc (CROM4) | WESb (CROM9) | CROV (CROM14) | CROK (CROM19) |
|  | Dra (CROM5) | UMC (CROM10) | CRAM (CROM15) | CORS (CROM20) |
| Knops (KN/022) | Kna (KN1) | Sl1(KN4) | Sl2 (KN7) |  |
|  | Knb (KN2) | Yka (KN5) | Sl3 (KN8)\* |  |
|  | McCa (KN3) | McCb (KN6) | KCAM (KN9)  KDAS (KN10) |  |
| Indian (IN/023) | Ina (IN1) | INFI (IN3) | INRA (IN5) |  |
|  | Inb (IN2) | INJA (IN4) | INSL (IN6) |  |
| Ok (OK/024) | Oka (OK1) | OKGV (OK2) | OKVM (OK3) |  |
| Raph (RAPH/025) | MER2 (RAPH1) |  |  |  |
| John Milton Hagen (JMH/026) | JMH (JMH1) | JMHL (JMH3) | JMHM (JMH5) | JMHN (JMH7) |
|  | JMHK (JMH2) | JMHG (JMH4) | JMHQ (JMH6) |  |
| I (I/027) | I (I1) |  |  |  |
| Globoside (GLOB/028) | P (GLOB1) |  |  | PX2 (GLOB4) |
| Gill (GIL/029) | GIL (GIL1) |  |  |  |
| Rh-Associated Glycoprotein (RHAG/030) | Duclos (RHAG1) | Ola (RHAG2) | DSLK\* (RHAG3) |  |
| FORS (FORS/031) | FORS1 |  |  |  |
| JR (JR/032) | Jra (JR1) |  |  |  |
| Lan (LAN/033) | Lan (LAN1) |  |  |  |
| VEL (VEL/034) | Vel (VEL1) |  |  |  |
| CD59 (CD59/035) | CD59.1 |  |  |  |
| Augustine (AUG/036) | AUG1 | Ata (AUG2) | ATML (AUG3) | ATAM (AUG4) |
| KANNO (KANNO/037) | KANNO1 |  |  |  |
| Sid (SID/038) | Sda (SID1) |  |  |  |
| CTL2 (CTL2/039)\* | CTL2.1\* | Rif \* |  |  |

\*Provisional; as the *Technical Manual* went to press, the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology meeting to approve CTL as blood group 039 was delayed. See ISBT website for updates.

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**APPENDIX 7.** Examples of Blood Group System Gene, Antigen, and Phenotype Symbols in Conventional and International Society of Blood Transfusion Terminology

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **System** | **Genes** | **Antigens** | **Phenotypes** | |
| ABO | *ABO* | A A1 B | A A1 A2 B A1B | |
| Rh | *RHD*  *RHCE* | D C E c e  RH1 RH2 RH3  RH4 RH5 | D+ C+ E–c+e+  RH:1,2,-3,4,5 | |
| MNS | *GYPA*  *GYPB* | M N S s  MNS1 MNS2  MNS3 MNS4 | M+ N+ S– s+  MNS:1,2,–3,4 | |
| Lewis | *FUT3* | Lea Leb  LE1 LE2 | Le(a+) Le(a–b+)  LE:–1,2 | |
| Kell | *KEL* | K k Kpa Kpb Jsa Jsb  KEL1 KEL2 KEL3  KEL4 KEL5 KEL6 | K– k+ Kp(a+b+) Js(a–b+)  KEL:–1,2,3,4,-5,6 | |
| Kidd | *JK* | Jka Jkb Jk3  JK1 JK2 JK3 | Jk(a+b–) Jk:3  JK:1,–2,3 | |
| Daniels GL, Fletcher A, Garratty G, et al. Blood group terminology 2004. From the ISBT Committee on Terminology for Red Cell Surface Antigens. Vox Sang 2004;87:304-16. | | | |

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| Appendix 8. Examples of Correct and Incorrect Terminology\* | | |
| **Term Description** | **Correct Terminology** | **Incorrect Terminology** |
| Phenotype | Fy(a+) | Fya+, Fy(a+), Fya(+), Fya(+), Duffya+, Fy(a)  Duffya -positive |
| Phenotype | Fy(a+b–) | Fya+b–, Fy(a+b–), Fya(+)b(–), Fya(+)b(–) |
| Antibody | Anti-Fya | Anti Fya, Anti-Duffy |
| Antigen | K | Kell (name of system), K1 |
| Antibody | Anti-k | Anti-Cellano, anti-K2 |
| Phenotype | KEL:1, KEL:–2 | KEL1+, K1+, KEL(1), K(1), KEL1–, KEL1-negative, K1-negative |
| Phenotype | A RhD+, B RhD–  A RhD-positive,  A RhD-negative | A+ (means positive for A antigen)  B– (means negative for B antigen) |
| Phenotype | M+ N– | M(+), MM (implies unproven genotype) |
| Phenotype | RH:–1,–2,–3,4,5 | RH:–1,–2,–3,+4,+5  RH:–1,–2,–3, 4+,5+ |
| Antigen Lea Lewis a  Leb Lewis b  Antibody Anti-Lea Anti-Lewis a, anti-Lewis  \*Modified from Issitt L. Blood group nomenclature. In: Blood groups: Refresher and updates (syllabus). Bethesda, MD: AABB, 1995.  Note: The examples shown may not represent the only correct terminologies. In the Rh system, for example, use of CDE terminology is also acceptable and is more commonly used. | | |
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| Appendix 9. Distribution of ABO/Rh Phenotypes by Race or Ethnicity | | | | | | | | | |
|  |  | Phenotype Distribution (%)† | | | | | | | |
| **Race or  Ethnicity** | **Number** | **O Rh+** | **O Rh–** | **A Rh+** | **A Rh–** | **B Rh+** | **B Rh–** | **AB Rh+** | **AB Rh–** |
| White non-Hispanic | 2,215,623 | 37.2 | 8.0 | 33.0 | 6.8 | 9.1 | 1.8 | 3.4 | 0.7 |
| Hispanic‡ | 259,233 | 52.6 | 3.9 | 28.7 | 2.4 | 9.2 | 0.7 | 2.3 | 0.2 |
| Black non-Hispanic | 236,050 | 46.6 | 3.6 | 24.0 | 1.9 | 18.4 | 1.3 | 4.0 | 0.3 |
| Asian§ | 126,780 | 39.0 | 0.7 | 27.3 | 0.5 | 25.0 | 0.4 | 7.0 | 0.1 |
| North American Indian | 19,664 | 50.0 | 4.7 | 31.3 | 3.8 | 7.0 | 0.9 | 2.2 | 0.3 |
| All donors | 3,086,215 | 39.8 | 6.9 | 31.5 | 5.6 | 10.6 | 1.6 | 3.5 | 0.6 |
| \*Used with permission from Garratty G, Glynn SA, McEntire R, et al for the Retrovirus Epidemiology Donor Study. ABO and Rh(D) phenotype frequencies of different racial/ethnic groups in the United States. Transfusion 2004;44:703-6.  †Percentages may not add up to 100.0% because of rounding. ‡Hispanic includes Mexican (68.8%), Puerto Rican (5.0%), Cuban (1.6%), and other Hispanic donors (24.6%). §Asian includes Chinese (29.8%), Filipino (24.1%), Indian (13.8%), Japanese (12.7%), Korean (12.5%), and Vietnamese (7.1%) donors. | | | | | | | | | |

**Abbreviations**

AATB American Association of Tissue Banks

ACD acid-citrate-dextrose

ACE angiotensin-converting enzyme

ADP adenosine diphosphate

AET 2-aminoethylisothiouronium

AHF antihemophilic factor

AHG antihuman globulin

AHTR acute hemolytic transfusion reaction

AIDS acquired immune deficiency syndrome

AIHA autoimmune hemolytic anemia

ALDH aldehyde dehydrogenase

ALT alanine aminotransferase

AML acute myelogenous leukemia

AMR antibody-mediated rejection

ANH acute normovolemic hemodilution

AORN Association of periOperative Registered

Nurses

APC antigen-presenting cell

aPTT activated partial thromboplastin time ARDP American Rare Donor Program

AS additive solution

ASFA American Society for Apheresis

ASHI American Society for Histocompatibility and Immunogenetics

ATP adenosine triphosphate

BCR B-cell receptor

BLA biologics license application

BPD biological product deviation

BSA bovine serum albumin or body surface area

BSC biological safety cabinet

BSL-1 Biosafety Level 1

CAP College of American Pathologists

CAS cold agglutinin syndrome

CBER Center for Biologics Evaluation and Research

CCI corrected count increment

CD clusters of differentiation

CDC Centers for Disease Control and Prevention

cDNA complementary deoxyribonucleic acid

CDRH Center for Devices and Radiological Health

CFR Code of Federal Regulations

CFU colony-forming unit

CGD chronic granulomatous disease

cGMP current good manufacturing practice cGTP current good tissue practice

cGy centiGray

CHIKV chikungunya virus

CI confidence interval

CIDP chronic inflammatory demyelinating polyneuropathy

CJD Creutzfeldt-Jakob disease

CLIA Clinical Laboratory Improvement Amendments

CLSI Clinical and Laboratory Standards Institute

CML chronic myelogenous leukemia

CMS Centers for Medicare and Medicaid

Services

CMV cytomegalovirus

CNS central nervous system

CP2D citrate-phosphate-dextrose-dextrose

CPD citrate-phosphate-dextrose

CPDA-1 citrate-phosphate-dextrose-adenine-1 CR complement receptor

CREG cross-reactive group

C/T crossmatch/transfusion

CV coefficient of variation

DAF decay-accelerating factor

DAT direct antiglobulin test

DDAVP deamino-D-arginine vasopressin

DHQ donor history questionnaire

DHTR delayed hemolytic transfusion reaction

DIC disseminated intravascular coagulation DMSO dimethylsulfoxide

DNA deoxyribonucleic acid

DOT (US) Department of Transportation 2,3-DPG 2,3-diphosphoglycerate

DRG diagnosis-related group

DSTR delayed serologic transfusion reaction

DTT dithiothreitol

EACA epsilon aminocaproic acid

EBAA Eye Bank Association of America

ECMO extracorporeal membrane oxygenation

ECV extracorporeal volume

EDTA ethylenediaminetetraacetic acid

EIA enzyme immunoassay

ELBW extremely low birthweight

ELISA enzyme-linked immunosorbent assay

EPO erythropoietin

FACT Foundation for the Accreditation of Cellular Therapy

FcR Fc gamma receptor

FDA Food and Drug Administration

FFP Fresh Frozen Plasma

FMH fetomaternal hemorrhage

FNAIT fetal/neonatal alloimmune

thrombocytopenia

FNHTR febrile nonhemolytic transfusion reaction

FTA-ABS fluorescent treponemal antibody absorption test

G-CSF granulocyte colony-stimulating factor GalNAc N-acetylgalactosamine

GM-CSF granulocyte-macrophage colony- stimulating factor

GMP good manufacturing practice

GPIa glycoprotein Ia

GPA glycophorin A

GPB glycophorin B

GPC glycophorin C

GPD glycophorin D

GTP good tissue practice

GVHD graft-vs-host disease

Gy Gray

HAV hepatitis A virus

HAZMAT hazardous material

Hb hemoglobin

HBc hepatitis B core antigen

HBsAg hepatitis B surface antigen

HBV hepatitis B virus

Hct hematocrit

HCT/Ps human cells, tissues, and cellular and tissue-based products

HCV hepatitis C virus

HDFN hemolytic disease of the fetus and newborn

HES hydroxyethyl starch

HHS (US) Department of Health and Human Services

HIT heparin-induced thrombocytopenia

HIV human immunodeficiency virus

HNA human neutrophil antigen

HPA human platelet antigen

HPC hematopoietic progenitor cell

HPC(A) HPCs from apheresis (HPC, Apheresis) HPC(C) HPCs from cord blood (HPC, Cord

Blood)

HPC(M) HPCs from marrow (HPC, Marrow)

HSC hematopoietic stem cell

HSCT hematopoietic stem cell transplantation HTLV-I human T-cell lymphotropic virus, type I

HTR hemolytic transfusion reaction

HUS hemolytic uremic syndrome

IAT indirect antiglobulin test

IATA International Air Transport Association ICAM-1 intercellular adhesion molecule-1

ID identification or individual donation

Ig immunoglobulin

IL-1 interleukin-1 alpha

IL-1 interleukin-1 beta

IL-2 interleukin-2

IM intramuscular

IND investigational new drug

INR international normalized ratio iPSCs induced pluripotent stem cells

IRL immunohematology reference

laboratory

IS immediate spin

ISBT International Society of Blood Transfusion

ISO International Organization for Standardization

ITP immune thrombocytopenia

IU international unit

IV intravenous

IVIG intravenous immune globulin

LDH lactate dehydrogenase

LDL low-density lipoprotein

LISS low-ionic-strength saline

LN2 liquid nitrogen

LR leukocyte-reduced

MAC membrane attack complex

2-ME 2-mercaptoethanol

MF mixed field

MHC major histocompatibility complex

MNC mononuclear cell

MoAb monoclonal antibody

MPHA mixed passive hemagglutination assay mRNA messenger ribonucleic acid

MSC mesenchymal stem cell

MSDS material safety data sheet

MSM male who has sex with another male

NAIT neonatal alloimmune thrombocytopenia

NAN neonatal alloimmune neutropenia

NAT nucleic acid testing

NHLBI National Heart, Lung, and Blood Institute

NIH National Institutes of Health

NIPA nonimmunologic protein adsorption

NK natural killer

NMDP National Marrow Donor Program

NRC Nuclear Regulatory Commission

NRF National Response Framework

OSHA Occupational Safety and Health

Administration

p probability

PAD preoperative autologous (blood) donation

PBM patient blood management

PBS phosphate-buffered saline

PCH paroxysmal cold hemoglobinuria

PCR polymerase chain reaction

PEG polyethylene glycol

PF24 Plasma Frozen Within 24 Hours After Phlebotomy

PF24RT24 Plasma Frozen Within 24 Hours After Phlebotomy Held at Room Temperature Up to 24 Hours After Phlebotomy

PPE personal protective equipment

PRA panel-reactive antibody

PRCA pure red cell aplasia

PRP platelet-rich plasma

PRT pathogen reduction technology

PT prothrombin time or proficiency testing

PTP posttransfusion purpura

PTT partial thromboplastin time

PVC polyvinyl chloride

QA quality assessment or quality assurance

QC quality control

QSE Quality System Essential

RBCs Red Blood Cells (blood donor unit)

RFLP restriction fragment length polymorphism

rFVIIa recombinant Factor VIIa

Rh Rhesus factor

RHAG Rh-associated glycoprotein RhIG Rh Immune Globulin

RIBA recombinant immunoblot assay

RIPA radioimmunoprecipitation assay

RNA ribonucleic acid

RPR rapid plasma reagin (serologic test for syphilis)

RT room temperature or reverse transcriptase

SCF stem cell factor

SD standard deviation or solvent/detergent

SNP single nucleotide polymorphism

SOP standard operating procedure SPRCA solid-phase red cell adherence

TA transfusion-associated

TACO transfusion-associated circulatory overload

TCR T-cell receptor

TMA transcription-mediated amplification TNCs total nucleated cells

TNF- tumor necrosis factor alpha

TPE therapeutic plasma exchange

TPO thrombopoietin

TRALI transfusion-related acute lung injury

TSE transmissible spongiform

encephalopathy

TTP thrombotic thrombocytopenic purpura

UCB umbilical cord blood

UDP uridine diphosphate

UNOS United Network for Organ Sharing

USC United States Code

vCJD variant Creutzfeldt-Jakob disease VLBW very low birthweight

vWD von Willebrand disease

vWF von Willebrand factor

WAIHA warm autoimmune hemolytic anemia

WB whole blood or Western blot

WBC white blood cell

WHO World Health Organization

WNV West Nile virus

ZIKV Zika virus