Collecting Blood and Isolating PBMCs for iPS Cell Reprogramming

Introduction: Blood Collection and Infectious Disease Testing

All persons collecting and handling any blood samples should be appropriately trained regarding (A) the safe handling and administration of blood and blood components, (B) bloodborne pathogen safety and exposure controls (tasks and procedures to minimize human exposure to such pathogens), and (C) any and all other requirements under all applicable laws and regulations and guidance as to best safety practices.

Note: *For clarification on this protocol, email support@cellulardynamics.com and request the MyCell® Products Webinar: Getting Started.*

A trained phlebotomist must collect blood samples using Vacutainer tubes included in the MyCell Blood Donor Collection Kit.

All blood samples must undergo infectious disease testing (IDT):

- Ship fresh blood within 24 hours of collection to a certified testing laboratory.

Certifications Accepted for IDT	Location
CLIA	United States
ISO15189	International Countries
DAP	British Colombia, Canada
IQMH	Ontario, Canada
CCKL	Netherlands

Note: *Check with the certified testing laboratory to determine proper storage, handling, and timeframes*.

- The blood must be determined free of HBV, HCV, and HIV.

IDT Name	Relative Current Procedural Terminology (CPT) Code
Hepatitis B Surface Antigen	87340
Hepatitis C Antibody	86803
HIV Antigen and Antibody	87389

- IDT results must be received by Cellular Dynamics International (CDI) before shipment of samples to CDI.
- In the case of a positive IDT result, do not ship the donor's samples to CDI.

Required Equipment and Consumables

The following equipment and consumables are required for collecting blood and isolating peripheral blood mononuclear cells (PBMCs).

Item	Vendor	Catalog Number
Equipment		
Biological Safety Cabinet with UV Lamp	Multiple Vendors	
Hemocytometer or Automated Cell Counter[1]	Multiple Vendors	
Liquid Nitrogen Storage Unit	Multiple Vendors	
Mechanical Freezer Capable of Maintaining -80°C	Multiple Vendors	
Mechanical Refrigerator Capable of Maintaining 4°C	Multiple Vendors	
Mr. Frosty Freezing Container	Nalgene	5100-0001
Pipettors	Multiple Vendors	
Tabletop Centrifuge with Proper Adaptors for Vacutainer Tubes and Capable of Maintaining 4°C[2]	Multiple Vendors	
Consumables		
MyCell Blood Donor Collection Kit[3,4,5]	Cellular Dynamics International (CDI)	
15 ml Conical Centrifuge Tubes	Multiple Vendors	
50 ml Conical Centrifuge Tubes	Multiple Vendors	
Cryogenic Tubes	Nalgene	5000-1020
CryoStor CS10 Freeze Medium	BioLife Solutions	210102
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Invitrogen	14190
Ethanol, 70%	Multiple Vendors	
Isopropyl Alcohol, 100%	Multiple Vendors	
Serological Pipettes	Multiple Vendors	
Trypan Blue	Life Technologies	15250

1 CDI recommends using a Cellometer Auto T4 Cell Counter (Nexcelom) or Countess Automated Cell Counter (Life Technologies).

2 CDI recommends using a USA E8 Fixed Speed Centrifuge (LW Scientific, Cat. No. E8C-U8AF-1503) or an equivalent centrifuge.

3 Do not discard any materials included in the kit as they may be used for sample identification and shipment.

4 Store the gel packs at 4°C until ready to use. Do not freeze the gel packs.

5 Store the Vacutainer tubes at room temperature and use before the expiration date printed on the tube. The kit includes Vacutainer CPT (BD Biosciences, Cat. No. 362761), SST (BD Biosciences, Cat. No. 367977), and Lithium Heparin (BD Biosciences, Cat. No. 367884) tubes.

Workflow

Blood samples are collected in Vacutainer tubes: 1 Lithium Heparin tube for IDT and DNA extraction, 1 SST tube for IDT, and 4 CPT tubes for reprogramming. Plasma is removed from the 1 Lithium Heparin tube and sent for IDT, and the remaining contents in the tube are snap-frozen and sent to CDI for DNA extraction. The 1 SST tube is sent for IDT. The blood in the 4 CPT tubes are processed for PBMC isolation, and the frozen PBMCs are sent to CDI for reprogramming.

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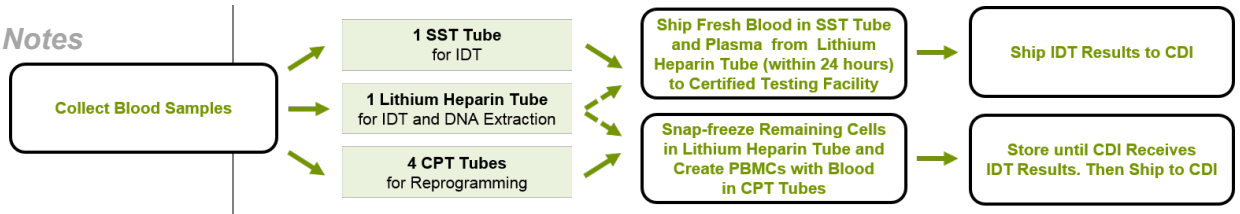

Methods

Collecting Blood Samples

The MyCell Blood Donor Collection Kit includes the Vacutainer Lithium Heparin, SST, and CPT tubes for blood samples. The kit also includes the MyCell Donor Sample and MyCell Incoming PBMC labels with CDI-assigned ID and project numbers for sample identification.

The phlebotomist should collect 1 Lithium Heparin tube (4 ml/tube), 1 SST tube (4 ml/tube), and 4 CPT tubes (8 ml/tube) from each donor.

Note: *Store the Vacutainer tubes at room temperature until ready to perform blood collection. Use before the expiration date printed on the tube.*

1. Label each Vacutainer tube with a CDI-provided MyCell Donor Sample label (Figure 1).

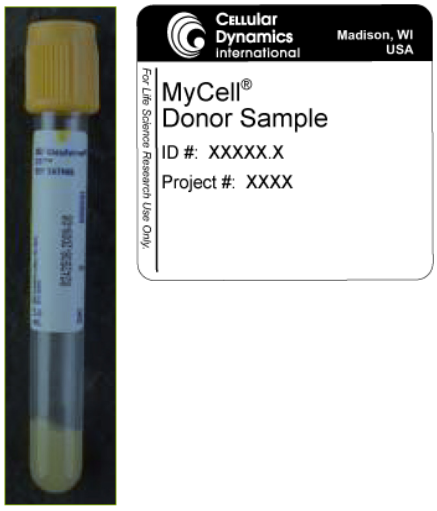

Figure 1: Labeling the Vacutainer Tubes
Apply a CDI-provided MyCell Donor Sample label to each tube to ensure proper identification.

2. Collect the blood samples by venipuncture using standard blood drawing procedures. Immediately invert the Lithium Heparin tubes 8 - 10 times to ensure complete mixing. Invert the SST and CPT tubes 5 times.
3. Place the Lithium Heparin tube on ice while the CPT and SST tubes are incubating at room temperature. Incubate the CPT and SST tubes at room temperature for 30 - 120 minutes.

   Note: *See Appendix A for recommendations on properly handling and processing SST tubes.*

4. Centrifuge the Lithium Heparin, SST, and CPT tubes at room temperature using the speed and duration, as specified below, for the centrifuge's rotor type:

   Note: *See the manufacturer's instructions for adaptors needed to centrifuge Vacutainer tubes.*

   Note: *Ensure the rotor is correctly balanced to achieve proper blood separation.*

Vacutainer Tube	Speed (x g)	Duration (min)	
		Swinging Bucket Rotor	Fixed Angle Rotor
CPT Tube	1500 - 1800	20	10
SST Tube	1100 - 1300	10	15
Lithium Heparin Tube	1100 - 1300	10	15

5. Inspect the tubes to ensure that proper blood separation was achieved (Figure 2).

   Note: *If the blood did not separate into phases, ensure the rotor is balanced and centrifuge the tubes once more; however, be aware there will be a reduction in the number of PBMCs isolated. If the blood still does not separate, collect a fresh sample from the same donor.*

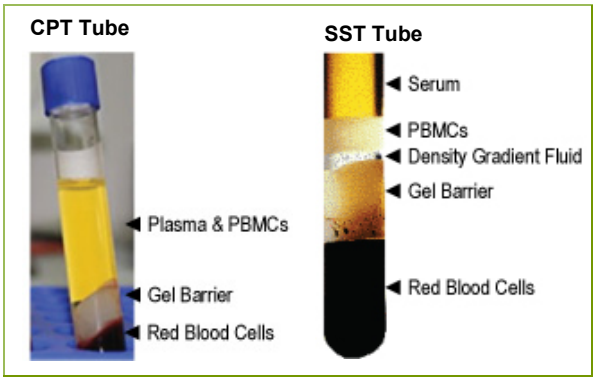

Figure 2: Vacutainer CPT and SST Tubes after Blood Centrifugation
Blood in the tubes separates into phases upon centrifugation.

6. Maintain the tubes on ice until ready to perform PBMC isolation.

Isolating and Freezing PBMCs

PBMC isolation and freezing are performed on the blood samples collected in the CPT tubes.

Perform the following procedure in a biological safety cabinet using sterile technique, making sure to maintain all reagents on ice.

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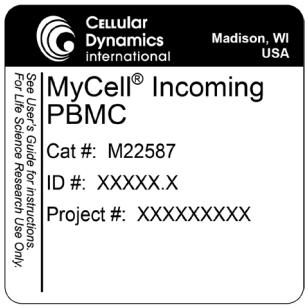
7. Label 3 cryogenic tubes per donor with a CDI-provided MyCell Incoming PBMC label.



8. Equilibrate the tabletop centrifuge at 4°C.
9. Equilibrate one 50 ml conical centrifuge tube and 3 cryogenic tubes per donor, the CryoStor CS10 freeze medium, and D-PBS at 4°C.

Maintain materials and reagents on ice throughout the following isolation steps.

10. Spray the CPT tubes with 70% ethanol, carefully wipe, and maintain on ice in a biological safety cabinet to minimize contamination.
11. Spray the 50 ml conical centrifuge tube with 70% ethanol, carefully wipe, and maintain on ice in the biological safety cabinet.

Use sterile technique throughout the following isolation steps.

12. Carefully remove the cap from the CPT tube and gently pour the plasma and PBMCs above the gel barrier (Figure 2) into an ice-cold 50 ml conical centrifuge tube. Maintain on ice.
13. Repeat step 6 for the remaining 3 CPT tubes to pool the plasma and PBMCs from the 4 CPT tubes per donor into the same 50 ml conical centrifuge tube (~30 ml volume/donor).

Note: *From the same donor, 4 CPT tubes will be processed into a single 50 ml conical centrifuge tube.*

14. Using a serological pipette, rinse each CPT tube with 2 ml of ice-cold D-PBS by gently dispensing the D-PBS on the tube walls.

Dispensing the D-PBS too forcefully can dislodge pieces of the gel barrier.

15. Transfer the 2 ml of D-PBS rinse and any residual cells from the 4 CPT tubes to the 50 ml conical centrifuge tube containing plasma and PBMCs.

Note: *Avoid transferring large pieces of the gel barrier and any residual red blood cells that did not separate to the 50 ml conical centrifuge tube containing the plasma and PBMCs.*

16. Add ice-cold D-PBS to the 50 ml conical centrifuge tube containing plasma and PBMCs to achieve a final volume of 50 ml/tube.

17. Remove an aliquot to count the fraction of viable PBMCs using an automated cell counter.

    Note: *See Appendix B for settings for the CDI-recommended Cellometer Auto T4 Cell Counter or Countess Automated Cell Counter. If using another cell counter, determine the appropriate settings for PBMC counting before blood collection.*

    Note: *See Appendix C for instructions on manually counting cells using a hemocytometer.*

    Note: *The expected number of PBMCs from 4 CPT tubes is between 4 x 10^7 and 2 x 10^8. If the number of PBMCs is too low, email mycell@cellulardynamics.com for assistance or see the appropriate MyCell contract to determine if alternate cell numbers are acceptable due to the donor's history.*

18. Transfer the 50 ml conical centrifuge tube from the biological safety cabinet to the tabletop centrifuge, maintaining the tube on ice.

19. Centrifuge the cells at 600 x g at 4°C for 10 minutes.

    Perform the following procedure in a biological safety cabinet using sterile technique.

20. Add 100% isopropyl alcohol to the fill line on the Mr. Frosty freezing container according to the manufacturer's instructions.

21. Ensure each cryogenic tube is labeled with a CDI-provided MyCell Incoming PBMC label.

22. Aspirate the supernatant, being careful not to disturb the cell pellet. Resuspend the cell pellet in 3.2 ml of ice-cold CryoStor CS10 freeze medium. Gently mix.

23. Incubate the cell suspension on ice for 20 minutes.

24. Dispense the cell suspension equally among the 3 ice-cold cryogenic tubes per donor (~1 ml/tube).

    Note: *The minimum number of PBMCs per tube is 10 x 10^6. If ≥10 x 10^6 but ≤20 x 10^6 PBMCs are obtained, split the suspension equally between 2 cryogenic tubes. More than 1 tube of PBMCs is preferred whenever possible.*

25. Transfer the cryogenic tubes to the Mr. Frosty freezing container filled with isopropyl alcohol and store the container at -80°C.

    Note: *PBMCs can be stored in the Mr. Frosty freezing container filled with isopropyl alcohol at -80°C for up to 7 days before shipping. If storing for a longer period of time, transfer the cryogenic tubes to a liquid nitrogen storage unit.*

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Aliquoting Plasma and Snap-freezing Cell Pellet from the Lithium Heparin Tube

1. Label a 15 ml conical centrifuge tube with a CDI-provided MyCell Donor Sample label.
2. Transfer the plasma layer from the Lithium Heparin tube to the 15 ml conical centrifuge tube. Maintain this tube on ice until ready for shipment to the certified testing laboratory.

   Note: *The laboratory will use this plasma for reflex IDT if necessary.*
3. Snap-freeze the remaining cell pellet in the Lithium Heparin tube. Store the tube at -80°C until ready for shipment to CDI.

Do not discard this cell pellet. You will ship it along with the PBMCs to CDI.

Sample Shipment

Shipping Samples for IDT and Submitting IDT Results to CDI

IDT results must be received by CDI before the shipment of the frozen PBMCs and cell pellet to CDI.

1. Check the submission requirements of the certified testing laboratory that will perform the IDT.
2. Ship both the SST tube and the 15 ml conical centrifuge tube containing the plasma on ice within 24 hours of blood collection.
3. When you receive the IDT results from the certified testing laboratory, redact the donor's personal information and add the corresponding CDI-assigned ID number from the MyCell Sample Donor label to the document.
4. Insert the results into and then seal the 9 x 12 inch envelope.
5. Ship the envelope by FedEx **for overnight delivery** to:

   Cellular Dynamics International, Inc.
   c/o MyCell Project Management
   525 Science Drive
   Madison, WI 53711 USA
6. Email the FedEx tracking number to logistics@cellulardynamics.com and mycell@cellulardynamics.com to enable CDI to track the shipment.

Shipping Samples for DNA Extraction and Reprogramming

Ship the cryogenic tubes containing PBMCs and the Lithium Heparin tube containing the cell pellet on dry ice or in a liquid nitrogen container by FedEx or World Courier **for overnight delivery**. Please ensure arrival at CDI on or before the Thursday of the same week.

To ship on dry ice:

1. Maintain the cryogenic tubes containing PBMCs and the Lithium Heparin tube containing the cell pellet at -80°C until ready to ship.
2. Place the insulated shipper in an outer shipping box.
3. Add dry ice to the bottom of the insulated shipper.
4. Place the cryogenic tubes in the small vial carton. Wrap the Lithium Heparin tube in bubble wrap.
5. Place the carton containing the cryogenic tubes and the wrapped Lithium Heparin tube in the sealable plastic bag.
6. Place the sealable plastic bag in the center of the insulated shipper on top of the dry ice. Surround the sealable plastic bag with enough dry ice to fill the insulated shipper. Ensure the sealable plastic bag remains in the center of the insulated shipper when adding the dry ice.
7. Seal the insulated shipper's lid.
8. Ship by FedEx or World Courier **for overnight delivery** to:

   Cellular Dynamics International, Inc.
   c/o MyCell Project Management
   525 Science Drive
   Madison, WI 53711 USA
9. Email the FedEx or World Courier tracking number to logistics@cellulardynamics.com and mycell@cellulardynamics.com. This notification to enables CDI to track and prepare for delivery of the shipment.

To ship in a liquid nitrogen container:

Note: *See the manufacturer's instructions as necessary.*

1. Maintain the cryogenic tubes containing PBMCs and the Lithium Heparin tube containing the cell pellet at -80°C until ready to ship.
2. Charge the liquid nitrogen container.
3. Place the tubes in the liquid nitrogen container.
4. Ship by FedEx or World Courier **for overnight delivery** to:

   Cellular Dynamics International, Inc.
   c/o MyCell Project Management
   525 Science Drive
   Madison, WI 53711 USA
5. Email the FedEx or World Courier tracking number to logistics@cellulardynamics.com and mycell@cellulardynamics.com. This notification to enables CDI to track and prepare for delivery of the shipment.

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Appendices

Appendix A. Proper Handling of Vacutainer SST Tubes

See the next page for the November 2005 issue of Tech Talk (courtesy of Becton, Dickinson and Company), which details how to handle and process Vacutainer SST tubes.

Tech Talk

Volume 4, No. 2 November 2005

Author: Lena Arzoumanian

BD Global Technical Services receives many questions about BD products. To address these questions, we have developed a periodic news bulletin called "Tech Talk."

Q. What is the importance of properly processing a BD Vacutainer® SST™ Tube?

A. The preparation of blood samples is a critical first step in the testing process. By understanding the components of the product and adhering to the following processing instructions, the facility may dramatically *IMPROVE SPECIMEN INTEGRITY*, resulting in a *QUALITY SPECIMEN*, a *QUALITY RESULT*, and ultimately assisting the doctor in providing *QUALITY TREATMENT* to patients.

Components of the BD Vacutainer® SST™ Tube

TUBE TYPES
Plus Plastic – Polyethylene Terephthalate (PET); Glass – Soda Lime Glass

CLOSURE TYPES
BD Hemogard™ Closure; Conventional Stopper

ADDITIVES
Clot Activator – Micronized Silica Particles; Barrier – Polymer Gel; Silicone Coating

In general, glass is a natural clotting agent. The blood will clot due to contact activation causing the initiation of the clotting mechanism.

Plastic tubes require the clot activator, which helps accelerate the blood clotting mechanism.

Silicone and clot activator are applied to the interior surface of the tube.

- A silicone coating on the walls of most serum tubes reduces the adherence of red cells to the tube wall.[1,2]
- The clot activator helps accelerate the blood clotting mechanism.[1]
- The density of the polymer gel causes it to move upward during centrifugation to the serum-clot interface, where it forms a barrier separating serum from the clot.[1]

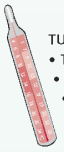
The Importance of Proper Handling and Processing of the BD Vacutainer® SST™ Tube

PRECENTRIFUGATION

FILL tubes to the stated draw volume to ensure the proper blood-to-additive ratio.

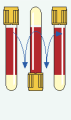
Allow the tube to fill until the vacuum is exhausted and blood flow ceases.[3]

Most BD SST™ tubes have a 12-month shelf life.



TUBE STORAGE

- Tubes should be stored at 4-25°C (39-77°F).
- Tubes should not be used beyond the designated expiration date.
- The expiration date is assigned to ensure adequate vacuum, barrier, or additive performance.



MIX the tube by 5 complete inversions.

- Mixing is critical to achieving appropriate clotting times and clot formation.
- Mixing facilitates dispersion of the silica into the blood, assisting the clotting process.
- Inadequate mixing may result in incomplete clotting.

30 minutes

CLOT for 30 minutes in a vertical position in a tube rack. Observe a dense clot.

- The recommended 30-minute minimum clotting time for the BD SST™ tube is based upon an intact clotting process.
- Insufficient clotting (short clotting time) can result in the formation of fibrin. This fibrin formation may interfere with barrier formation.
- Samples from certain populations of patients with impaired coagulation may require longer than 30 minutes to clot in a BD SST™ tube:
  - Blood from patients on anticoagulant therapy (e.g., Coumadin®) may require longer clotting time.
  - Blood from patients on high doses of heparin may not clot at all.
  - Certain diseases may require longer blood clotting times (e.g., liver disease).
  - Multiple Myeloma—the isolated myeloma globulin inhibits all three stages of fibrin formation: the proteolytic action of thrombin on fibrinogen, the aggregation of fibrin monomers, and the stabilization of fibrin by cross-linkages in the gamma and alpha chains.[4]

It is recommended that serum be physically separated from contact with cells as soon as possible with a **maximum time limit of 2 hours from the time of collection**, unless conclusive evidence indicates that longer contact times do not contribute to error of the results.[1,8]

CENTRIFUGATION

The gel exhibits thixotropic properties (such that it is semi-solid under static conditions and becomes less viscous when a force is applied), enabling it to flow during centrifugation. Separator gels are designed with a specific density that falls between those of the serum and cells, thus determining the location of the interface.[5]

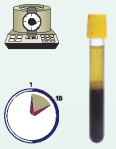
Complete and adequate barrier formation is time, temperature and *g*-force dependent.

Uniformity of the barrier is time dependent.

- An incomplete barrier could result from shortened centrifugation times.
- For a horizontal (swing-bucket) centrifuge, the recommended spin time is 10 minutes.
- For a fixed-angle centrifuge, the recommended spin time is 15 minutes.

A minimum *g*-force is required to get the gel moving, thus the recommendation is 1000 *g*.[1,5]

- During the centrifugation process, centrifugal forces are applied to the gel in the tube. At comparable *g*-force settings, the horizontal centrifuge is more efficient at gel barrier formation than a fixed angle centrifuge, due to a higher axial force setting on the gel.



10 or 15 minutes

- The quality of the barrier formed from fixed-angle centrifugation also depends upon the angle of the centrifuge head. Barriers formed in fixed-angle centrifuges contain a bias angle relative to the angle of the head. These barriers are typically thinner than horizontal barriers, because the gel must cover a greater cross-sectional area in the tube.

The flow properties of the barrier material are temperature dependent.[1]

- Gel flow may be impeded if chilled before or during centrifugation. To optimize flow and prevent heating during centrifugation, set refrigerated centrifuges to 25°C (77°F).[1]

Centrifugation recommendations:

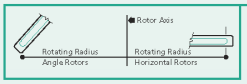
Product	RCF (*g* force)	Time (min) Swing-Bucket	Time (min) Fixed-Angle Bucket
BD SST™ Glass Tube	1000-1300	10	15
BD SST™ Plus - 13 mm	1100-1300	10	15
BD SST™ Plus - 16 mm	1000-1300	10	15
BD SST™ Transport Tubes	1100-1300	15	15

Conversion of RCF to RPM (radius in inches)

Radius (Inches)	Speed–RPM Min.	Speed–RPM Max.	Radius (Inches)	Speed–RPM Min.	Speed–RPM Max.
3	3500	4000	7	2300	2600
4	3000	3500	8	2100	2400
5	2700	3100	9	2000	2300
6	2500	2800	10	1900	2200

Conversion of RCF to RPM (radius in centimeters)

Radius (cm)	Speed–RPM Min.	Speed–RPM Max.	Radius (cm)	Speed–RPM Min.	Speed–RPM Max.
8	3300	4000	16	2300	2600
10	3000	3400	18	2200	2500
12	2700	3100	20	2100	2400
14	2500	2900	25	1900	2100



Radius is the rotational radius of the centrifuge as measured from the center of the rotor head to the outside bottom of the rotor bucket (when the rotor bucket is held at 180 degrees).[1,8]

POSTCENTRIFUGATION

The specimen in the original tube should be centrifuged one time. Tubes should not be recentrifuged once the barrier is formed. A potential for inaccurate test results is possible.

Analytes from cellular leakage/exchange, accentuated by clot retraction, will then be centrifuged into the serum being used for testing. If recentrifugation is required for improved serum quality, then aspirate serum into a properly labeled clean test tube.

References:

1. BD Vacutainer® Evacuated Blood Collection System product insert available from www.bd.com/vacutainer/productinserts
2. Bush V, Skobe C, Dubrowny N, Cohen R, Pando S, Mohammad F. Assessment of Cellular Contamination of Serum for Routine Chemistry Analytes [abstract]. Clin Chem 1997
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4. Soria J, Soria C, Samama M, Fine JM, Bousser J. Analysis of a fibrin formation abnormality in a case of multiple myeloma. *Scand J Haematol.* 1975; 15(3): 207-218
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7. Hira K, Ohtani Y, Rahman M, Noguchi Y, Shimbo T, Fukui T. Pseudohypokalaemia caused by recentrifugation of blood samples after storage in gel separator tubes. *Ann Clin Biochem.* 2001 July; 38 (Pt 4): 386-390
8. CLSI, Procedures for the handling and processing of blood specimens; approved guideline. 3rd ed. H18-A3, Vol. 24 No. 38

Please call BD Global Technical Services for clinical support material.

BD Global Technical Services: 1.800.631.0174 **BD Customer Service:** 1.888.237.2762

BD Diagnostics
Preanalytical Systems
1 Becton Drive
Franklin Lakes, NJ 07417
www.bd.com/vacutainer

Notes

Appendix B. Settings for CDI-recommended Automated Cell Counters

- Cellometer Auto T4 Cell Counter
  - *Cell type program*: PBMC_Human_Fresh
  - *Parameters*: 4 - 22 µm diameter; small cell decluster
- Countess Automated Cell Counter
  - *Sensitivity*: 5
  - *Minimum Size*: 5 µm
  - *Maximize Size*: 20 µm
  - *Circularity*: 80

Appendix C. Manual Cell Count Using a Hemocytometer

The following equipment and consumables are required for manually counting PBMCs using a hemocytometer.

Item	Vendor	Catalog Number
Equipment		
Micropipettors	Multiple Vendors	
Microscope	Multiple Vendors	
Consumables		
Acetic Acid	Multiple Vendors	
Bleach	Multiple Vendors	
Microcentrifuge Tubes	Multiple Vendors	
Sterile Micropipettor Tips	Multiple Vendors	

Preparing the Cell Dilution in Trypan Blue

Perform the following procedure in a biological safety cabinet using sterile technique.

1. Transfer 20 µl of cell suspension from the 50 ml conical centrifuge tube to a microcentrifuge tube using a sterile micropipettor fitted with a sterile tip.
2. Add the appropriate volume of 0.4% trypan blue to the microcentrifuge tube to achieve a desired dilution. Examples of dilution ratios are as follows:
   - 1:5 (20 µl of cell suspension + 80 µl of trypan blue)
   - 1:2 (20 µl of cell suspension + 20 µl of trypan blue)
3. Gently mix the tube.

Loading the Cell Dilution into a Hemocytometer

1. Clean the hemocytometer and a cover glass with 70% ethanol using lens paper. Allow the hemocytometer to air-dry.

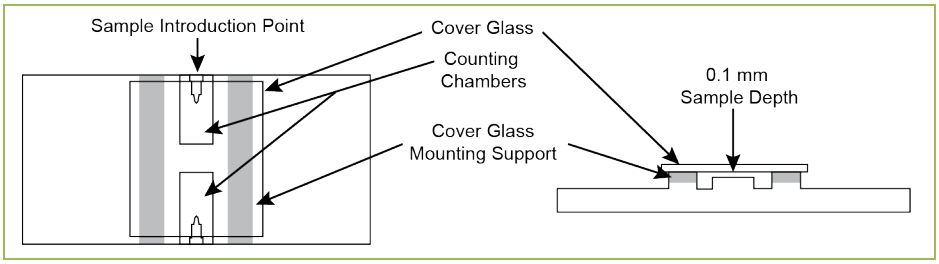

Figure 4: Standard Hemocytometer
A hemocytometer consists of two counting chambers. A cover glass mounting support on either side of the counting chambers ensures proper positioning of the cover glass to create a 0.1 mm sample depth. The cell dilution is loaded through the corresponding sample introduction point.

2. Incubate the cell dilution for at least 1 minute (but no more than 15 minutes) to allow for complete staining of the non-viable cells.

   Note: *Do not count cells incubated for longer than 15 minutes as non-specific staining of viable cells may have occurred. If cells are not counted within 15 minutes, prepare a fresh cell dilution before continuing.*

3. Place a cover glass on the cover glass mounting supports (Figure 4).
4. Swirl or tap the tube to thoroughly mix the cell dilution before plating. A well-mixed sample is critical for accurate counting.
5. Place a 10 µl drop of the cell dilution at the edge of the sample introduction point using a micropipettor. The cell dilution flows into the counting chamber by capillary action until it is completely filled. Do not overfill the counting chamber.

   Note: *Do not count if the counting chamber is overfilled or if the cover glass moved after plating. Clear and clean the counting chamber and cover glass before reloading the sample.*

6. Wait 30 seconds to allow all cells to settle in the same plane before counting.

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Counting PBMCs

1. Place the hemocytometer on the microscope stage. Focus on the cells and counting grid using a low power lens.

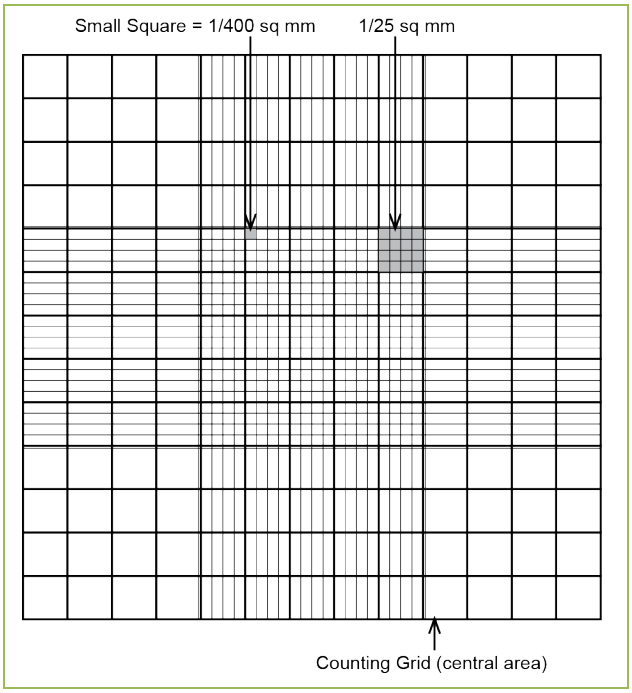

Figure 5: Counting Grid
Each counting chamber on a hemocytometer contains a counting grid consisting of 4 large corner squares of 1 mm^2 that are further divided into 16 smaller squares.

2. Scan the counting chamber, noting cell distribution, unusual or excessive cellular debris, or high numbers of red blood cells. If the red blood cell contamination is high enough to interfere with counting, prepare a new cell dilution.

   Note: *If red blood cell contamination is present, use a 1:1 mixture of trypan blue and 2% acetic acid to dilute the PBMC suspension. Acetic acid will lyse the red blood cells*.

3. Count the cells in the 4 large corner squares (Figure 5) using a high power lens. Count the viable and non-viable cells separately, noting the following:

   - Viable cells appear as bright, unstained spheres surrounded by a halo. Non-viable cells will be stained blue.
   - Red blood cells appear more spherical and slightly smaller than PBMCs. Small irregular-shaped particles are platelets and debris. Do not count red blood cells or platelets.
   - Cells can lie on the left, right, top, or bottom borders of the large corner squares. To prevent over-counting cells, count only cells that lie on either the left or right border (but not both) and cells that lie on either the top or bottom border (but not both). Count the same two borders in each large corner square.

Calculating the Cell Density and Viability

Use the cell counts from the hemocytometer to calculate the cell density and viability in the PBMC suspension.

1. Determine the dilution factor (DF):

$$DF = \frac{Volume\ of\ PBMC + Volume\ of\ Trypan\ Blue}{Volume\ of\ PBMC}$$

2. Determine the cell density:

$$Cell\ Density\ \left(\frac{cells}{ml}\right) = \frac{Total\ Cells\ Counted}{Number\ of\ Large\ Squares\ Counted}\ x\ DF\ x\ 10^4$$

3. Determine the cell viability:

$$Viability\ (\%) = \frac{Viable\ Cell\ Count}{Viable\ Cell\ Count + Non\text{-}viable\ Cell\ Count}\ x\ 100$$

Cleaning the Hemocytometer

1. Soak the hemocytometer and cover glass in 10% bleach for 30 minutes.
2. Rinse the hemocytometer and cover glass with tap water followed by 70% ethanol.
3. Allow the hemocytometer to air dry. Lens paper may be used to wipe the surface of the counting chamber and cover glass.

   Note: *Carefully handle the hemocytometer and cover glass to prevent scratches. Scratches may visually interfere with counting and compromise the accuracy of the cell count.*

4. Store the hemocytometer and cover glass in a covered container.

Notes

Revision History
Version 1.0: December 2016
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