

Immunology Quality Assessment Cryopreservation Proficiency Testing Program

Best Practices for PBMC Processing

2015 ACTG Network Annual Meeting

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IQA Cryopreservation PT Program

- The IQA Cryopreservation PT Program measures viability and viable recovery of the processed PBMC samples at participating DAIDS-supported laboratories on a quarterly basis to ensure sample integrity
- The optimization of PBMC processing is an absolute necessity to ensure continued success in the development of vaccines and treatments designed to elicit cellular immunity
- Utilizing the best practices for handling, processing, and cryopreserving PBMCs results in a pure population with minimal cellular contamination



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Be Prepared



Equipment

Reagents

Cryopreservation Solution (CPS)



Assemble all processing supplies in the BSC
prior to processing using aseptic technique

Reagents

Density Gradient Media (DGM)

Wash Diluent Reagent (WDR)

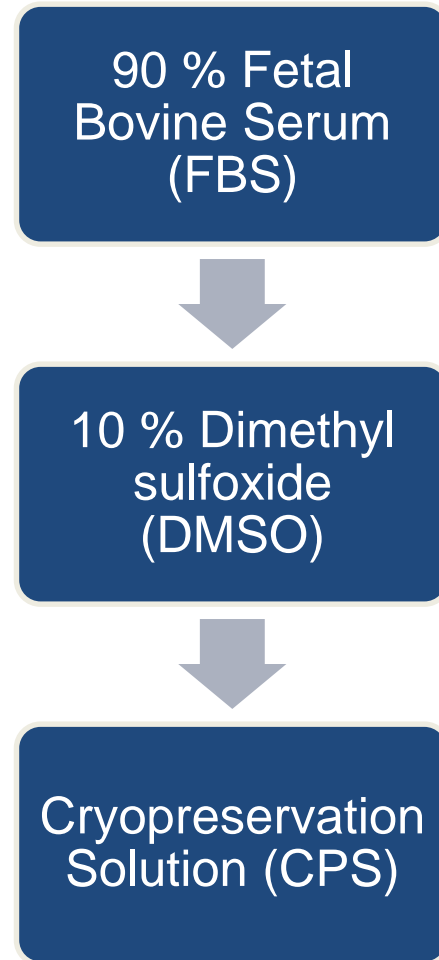
Fetal Bovine Serum (FBS)

Dimethyl sulfoxide (DMSO)

0.4% Trypan Blue Solution

Cryopreservation Solution (CPS)

- CPS must be prepared in advance and chilled in the refrigerator (2°C to 8°C) for at least 30 minutes or in an ice bath for at least 15 minutes prior to use
- CPS can be stored at 2°C to 8°C for <18 hours, label accordingly



PBMC Processing



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PBMC Processing Overview

Dilute/Overlay and PBMC Isolation



Wash PBMCs and Obtain the Viable Cell Count and Viability

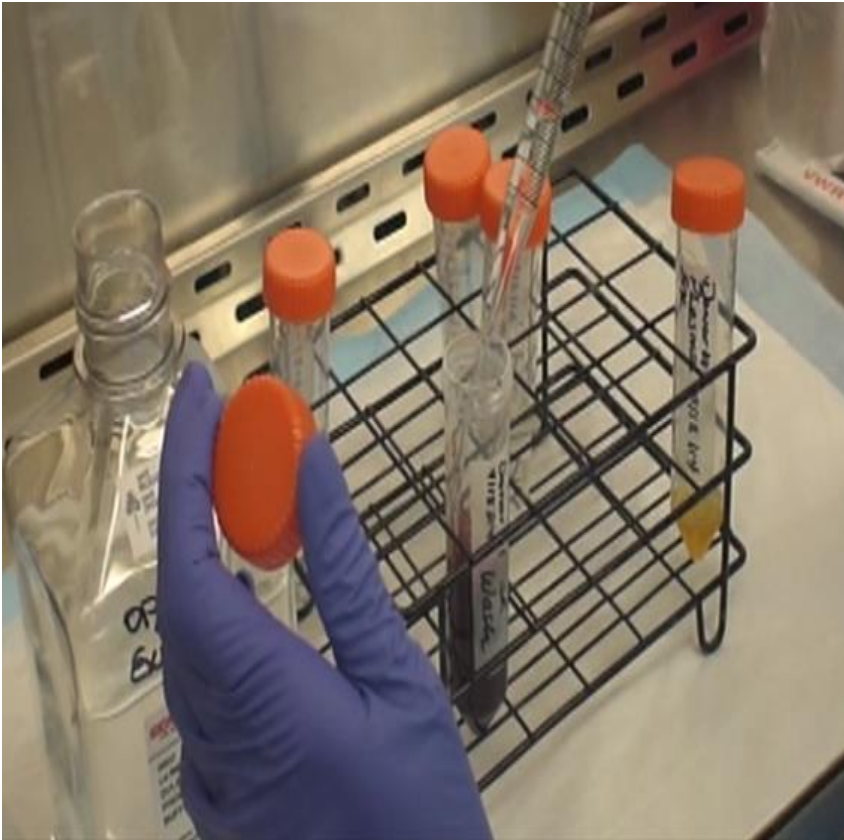


Suspend PBMCs in Accurate Volume of Cryopreservation Solution (CPS)



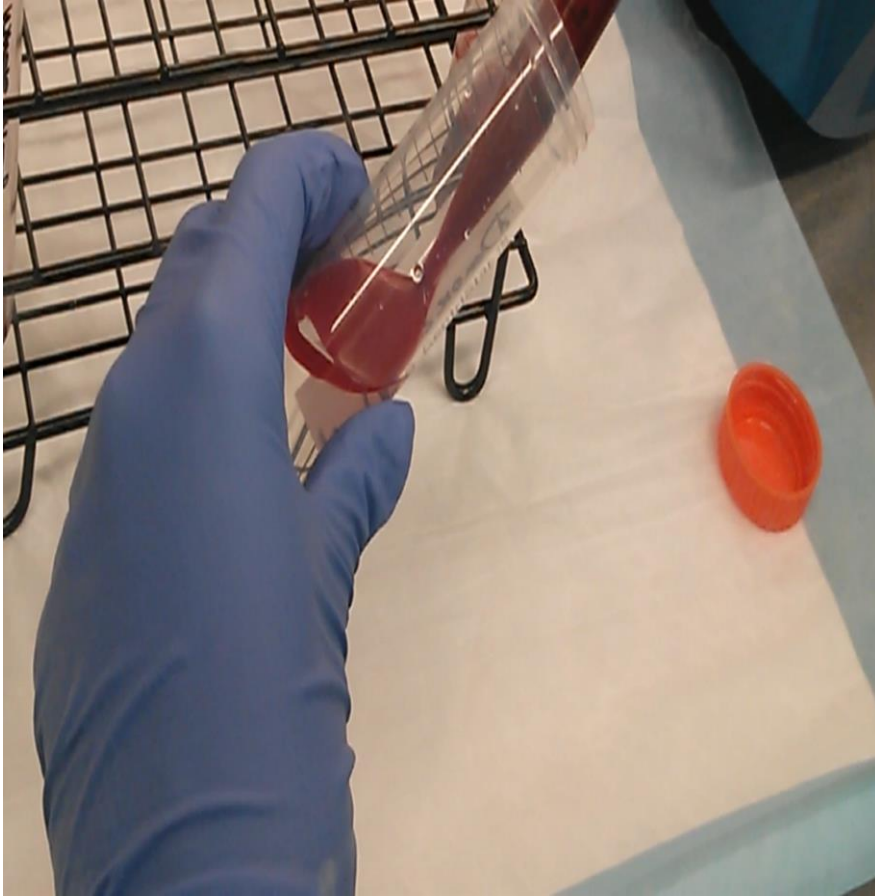
Cryopreservation and On Site Storage

Dilution

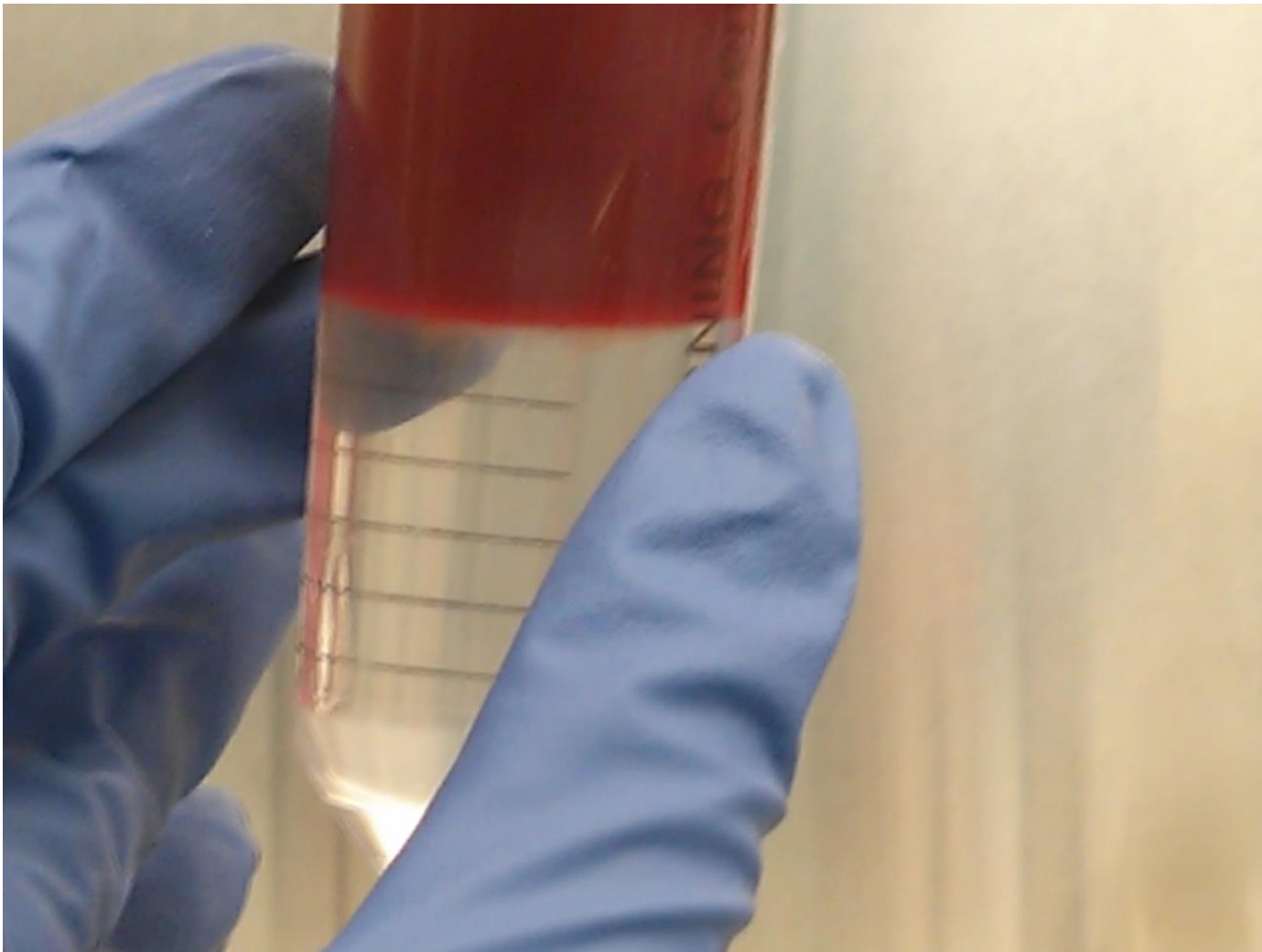


- The blood to WDR dilution allows for the cells to be dispersed in solution, suitable for the density gradient
- The volume of DGM and WDR will depend on the ratio of DGM to diluted blood recommended by the manufacturer

The Overlay Process



- Allow the tip of the serological pipette to touch the interface or place slightly above the DGM then gently start overlaying the diluted blood product as slow as possible
- Do not disrupt the layer



Hold the tubes in an upright position and gently transfer to the centrifuge as not to disrupt the layers.

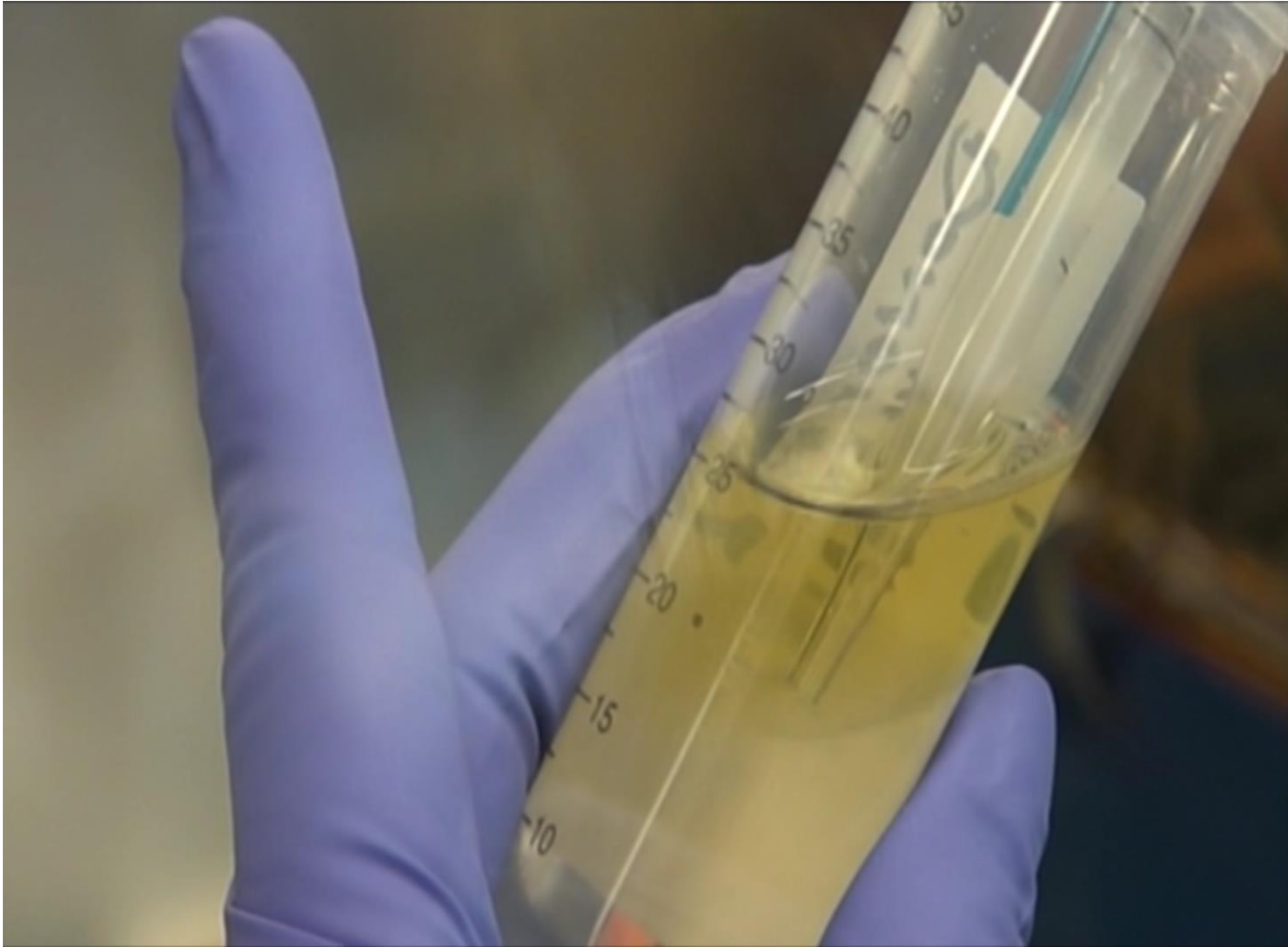
PBMC Density Centrifugation

- The centrifuge brake must be turned OFF and use swinging buckets for the separation to be clean and to maximize retrieval of the PBMCs, if the brake is on it will disrupt the layers.
- Centrifuge at 400 x g for 30 minutes at 15°C to 30°C with the Brake OFF, or as outlined in the package insert that accompanies the DGM.



Once the centrifuge has completely stopped carefully remove the layered tubes from the centrifuge as not to disrupt the layers and place in the BSC.

Isolation of Buffy Coat



Buffy Coat Isolation

Avoid harvesting the platelet aggregates that form on the tube wall adjacent to the PBMC layer

Avoid removing excess amounts of supernatant and/ or separation media with the PBMC band to limit contamination from plasma proteins and granulocytes

If the cell layer is not visible, confirm that the centrifuge is operating properly it may be necessary to re-centrifuge tube

Document any problems and/or actions taken according to network and laboratory requirements

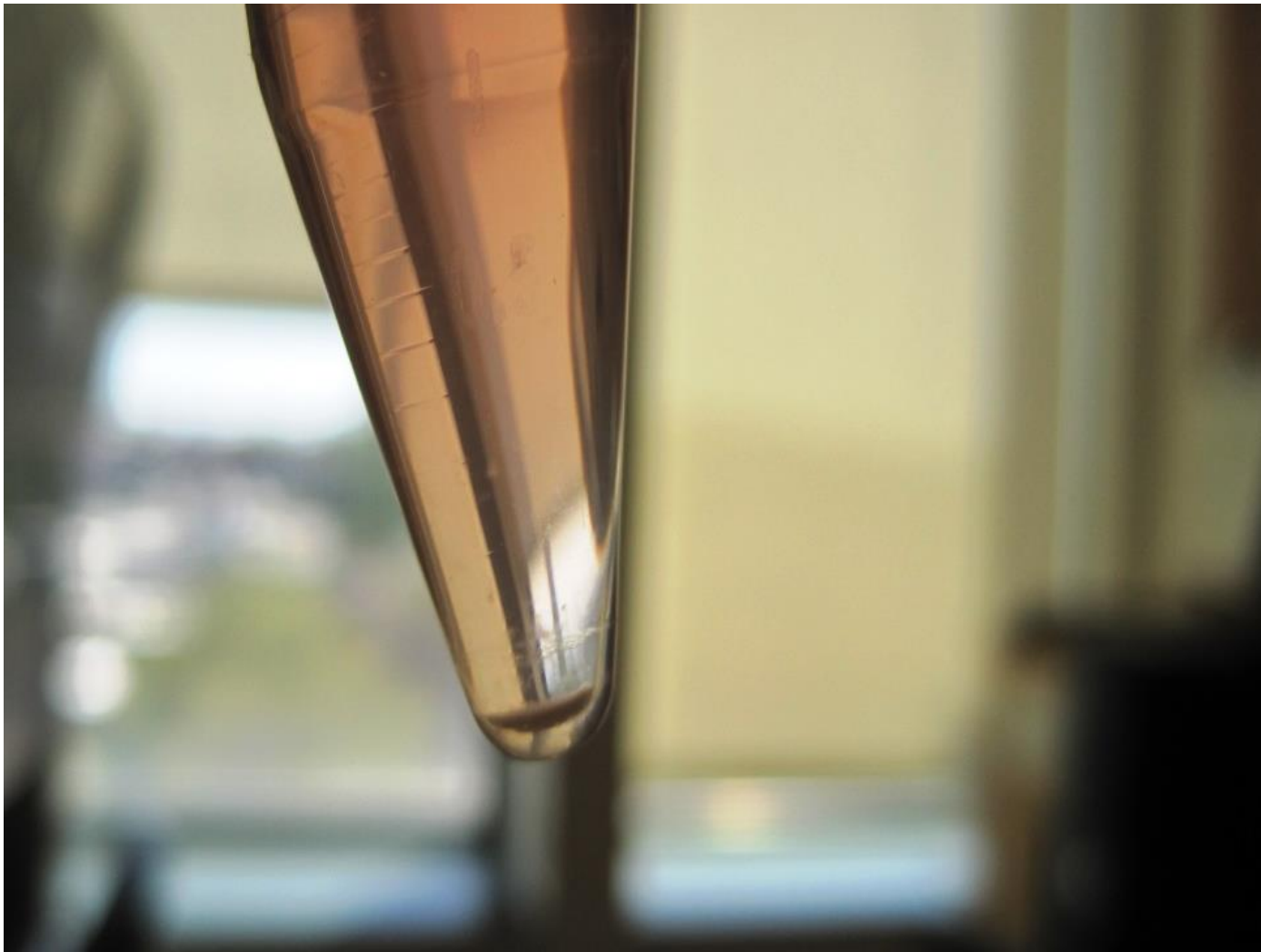
Wash PBMCs



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Use the number of washes according to the study protocol, fully re-suspend the PBMC pellet during each wash.

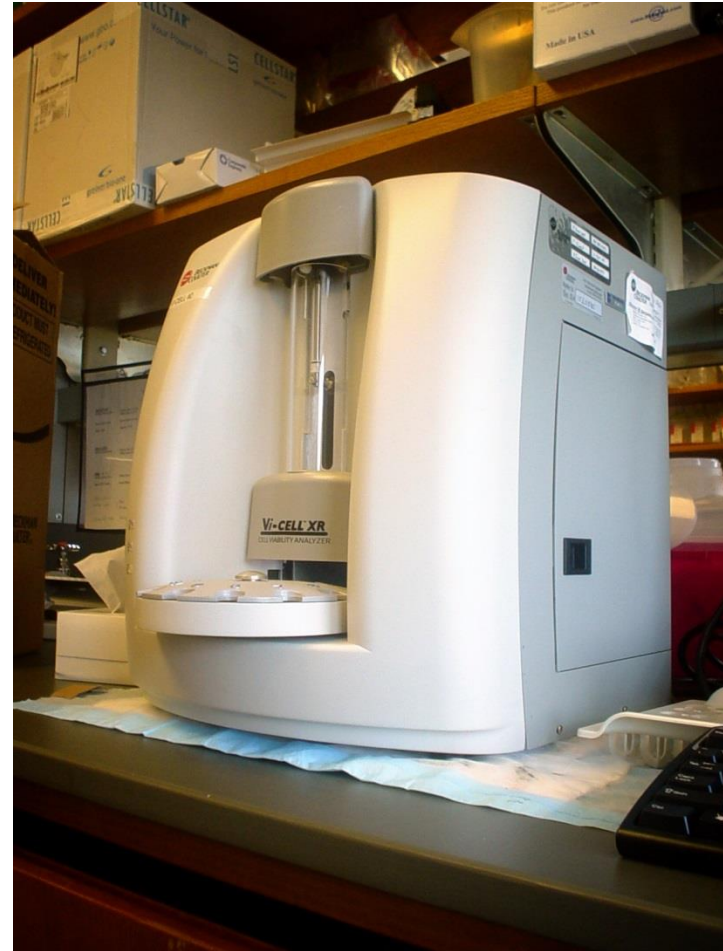


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Viable Cell Counts



Manual Counting Sample Quality Control

The cell counts between the four quadrants should agree within $\pm 15\%$

Allow the cell suspension to sit in the Trypan Blue for at least one minute before plating to allow for complete staining of the non-viable cells

Allow the sample to settle for 30 seconds until the cells are all in the same plane when viewed through the microscope

Do not count cells left standing in Trypan Blue for longer than 15 minutes; after 15 minutes nonspecific staining of viable cells may occur

Manual Counting Sample Quality Control

Significant calculation errors can occur if the cell concentration is too low or too high

Cells should be evenly distributed
Overlapping or clumping cells indicates the need to re-dilute and recount

This ensures the even distribution of cells throughout the hemacytometer and an accurate count of the total viable cells

Cryopreservation of PBMCs

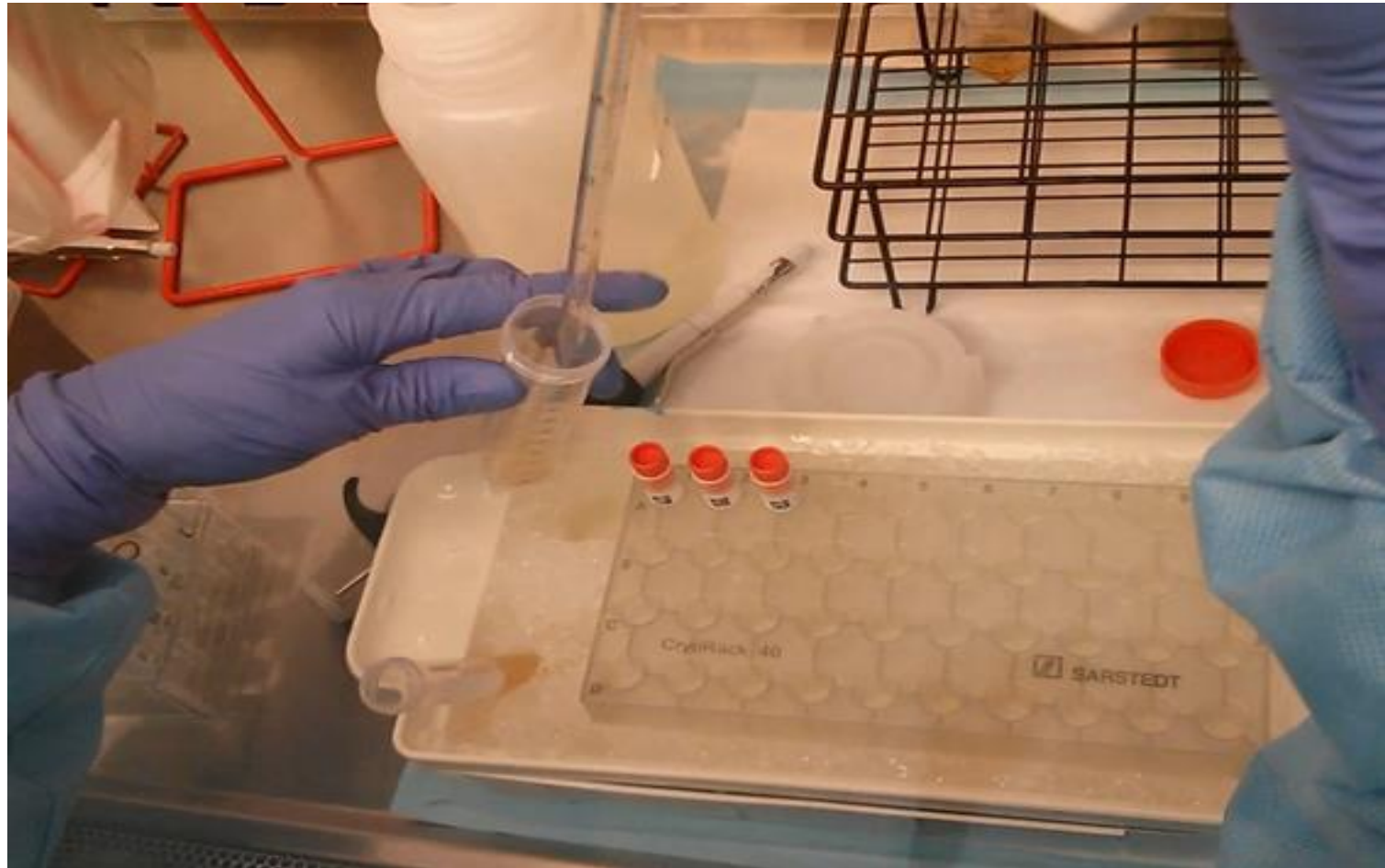


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Gently add the accurate volume of CPS to the re-suspended cells with continuous swirling.

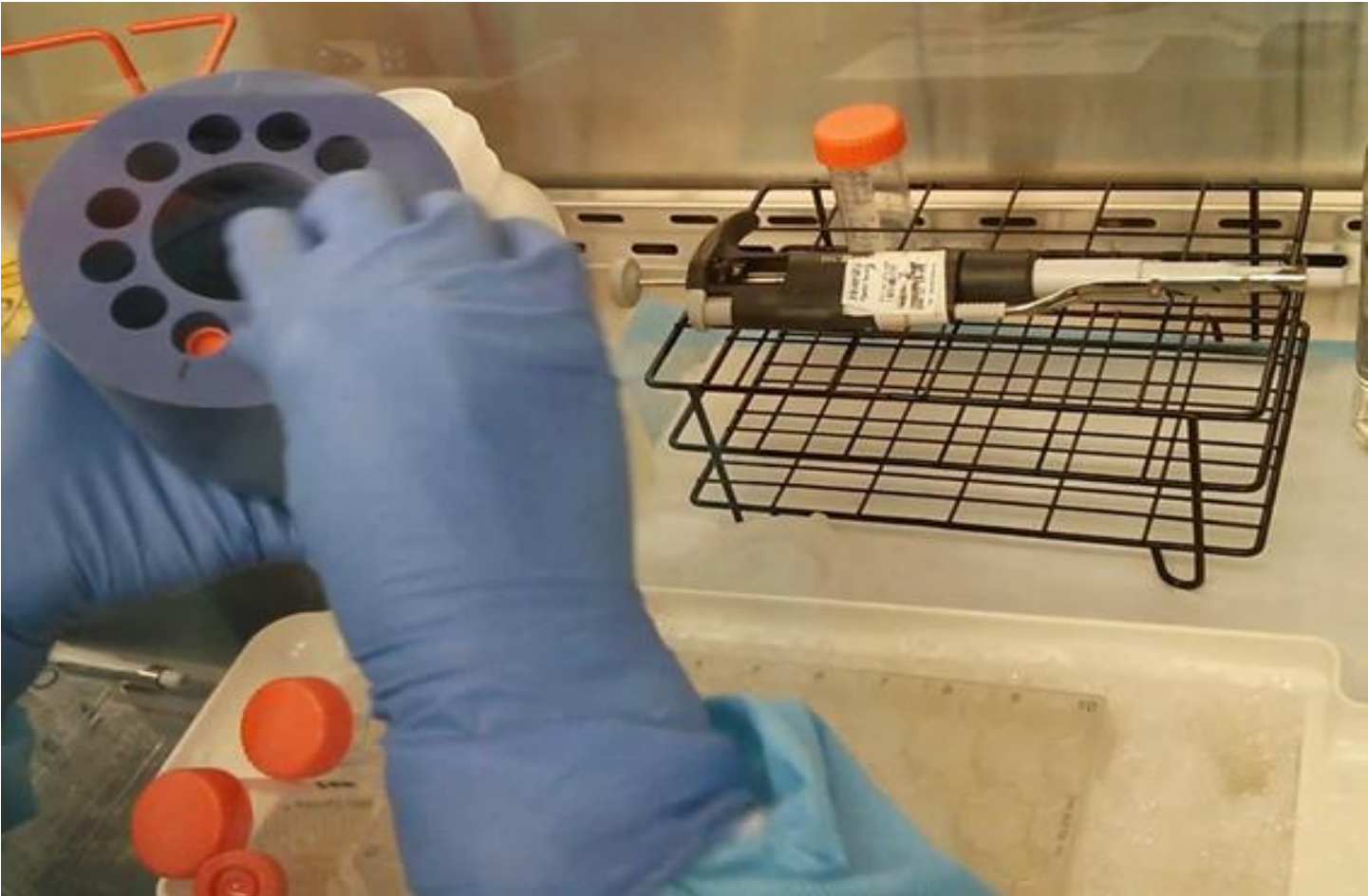


Addition of CPS

Check all calculations/dilutions

Keep the PBMCs cryovials on an ice bath during the addition of CPS and during aliquoting process

Mix CPS + PBMC cell suspension gently and thoroughly during the aliquoting process



After the addition of CPS, the PBMCs should be frozen immediately.

On Site Storage

After the PBMCs are aliquoted the final cryopreservation step is to freeze gradually at a rate of approximately 1°C per minute. Rate controlled freezing is important so that rapid intracellular freezing and excess extracellular ice formation is avoided.

Freezing Containers



Rate-
controlled
freezer



NALGENE
® Mr.
Frosty



Strata Cooler
®



Biocision®
CoolCell

On Site Storage

Take care to ensure that the cold chain temperature is consistently maintained during the long/short term transfer process

Keep freezing container in a secure location within the $-70^{\circ}\text{C}/-80^{\circ}\text{C}$ freezer, away from door/possible temperature fluctuation

Follow all laboratory and network guidelines and document any out of control incidences

Challenges and Recommendations



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Challenges Observed by the IQA

Out of Range Viable Recovery

- Cellular Contamination
- Calculation errors
- Dilution errors
- Poor mixing / aliquoting

Out of Range Viability

- CPS made incorrectly
- Processing time
- Use of Expired Reagents
- Lack of Cold Chain Method

Good PBMC Processing Recommendations

Prior to PBMC processing:

- Confirm the freezer and centrifuge is in proper working condition and up-to-date PM has been performed
- Clean and set up BSC for the processing procedure
- Check all reagent expiration dates; do not use expired reagents
- Confirm that DGM is at room temperature and protected from light
- Prepare the estimated volume of CPS in advance

During PBMC processing:

- Follow the Cross-Network PBMC Processing SOP; document deviations
- Process PBMCs in a timely manner and work quickly, but not hastily
- Use accurate and precise pipetting techniques
- Handle sample gently, but make sure to mix well and re-suspend your pellet thoroughly
- Maintain the cold chain temperature

Acknowledgments



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Questions?

Thank You!



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