Best Practices for PBMC Isolation from a Leukapheresis Product

Refer to *PBMC Isolation From Leukapheresis SOP

2018 ACTG Annual Network Meeting

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IQA Immunology Quality Assessment Program An NIH, NIAID, DAIDS Program

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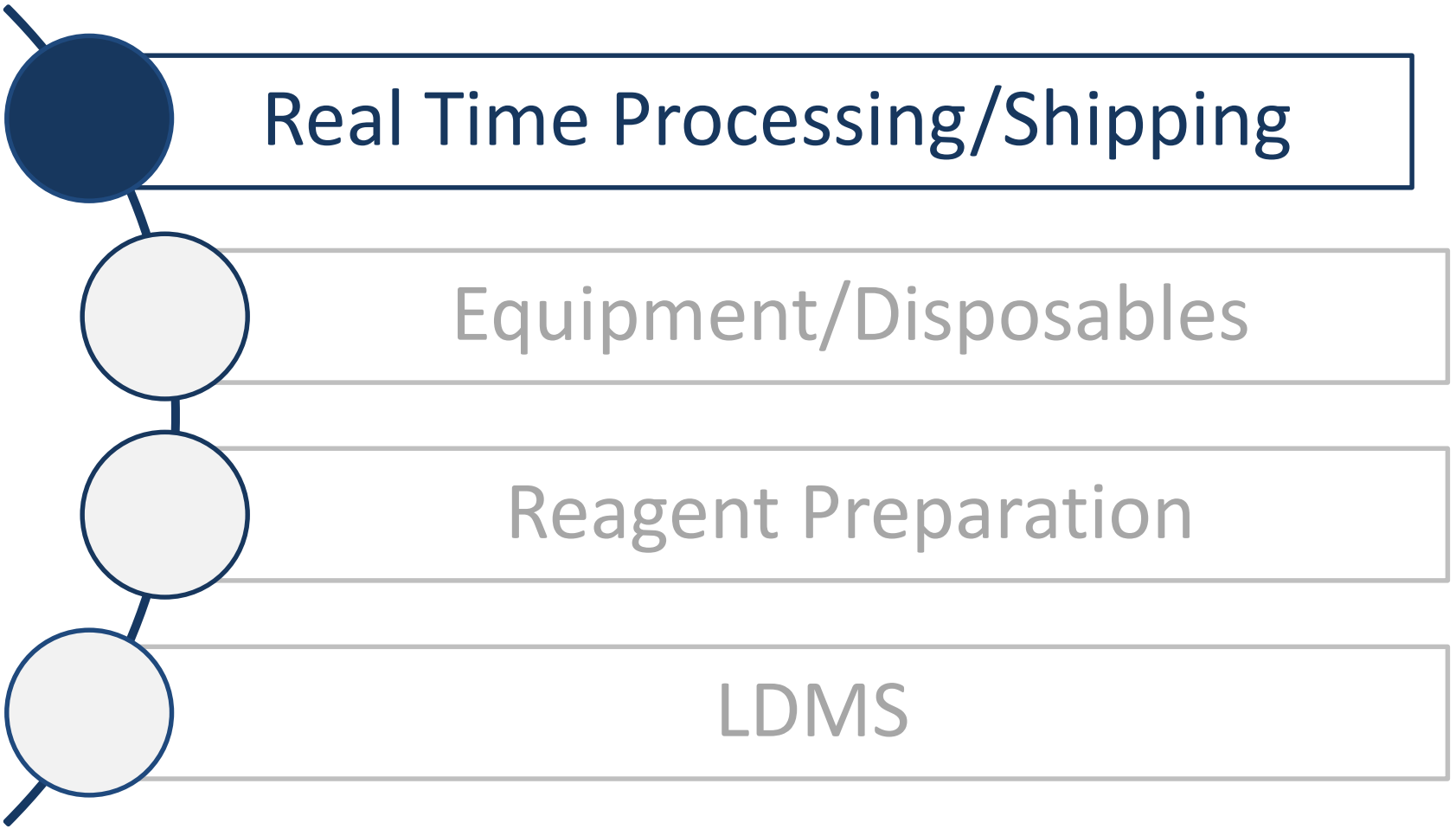
*https://www.hanc.info/SearchCenter/Pages/results.aspx?k=pbmc&cs=This%20Site&u=https%3A%2F%2Fwww.hanc.info

Leukapheresis Preparation



Real Time Processing of the Leukopak

Refer to Section 9_PBMC Isolation From Leukapheresis

- Transport the leukopak to the processing laboratory under ambient conditions (15 to 30°C).
- Use an insulated container to avoid temperature extremes, include absorbent material, and comply with local specimen transport requirements.
- Leukopak specimens should be processed by the laboratory processing unit as soon as possible upon receipt.

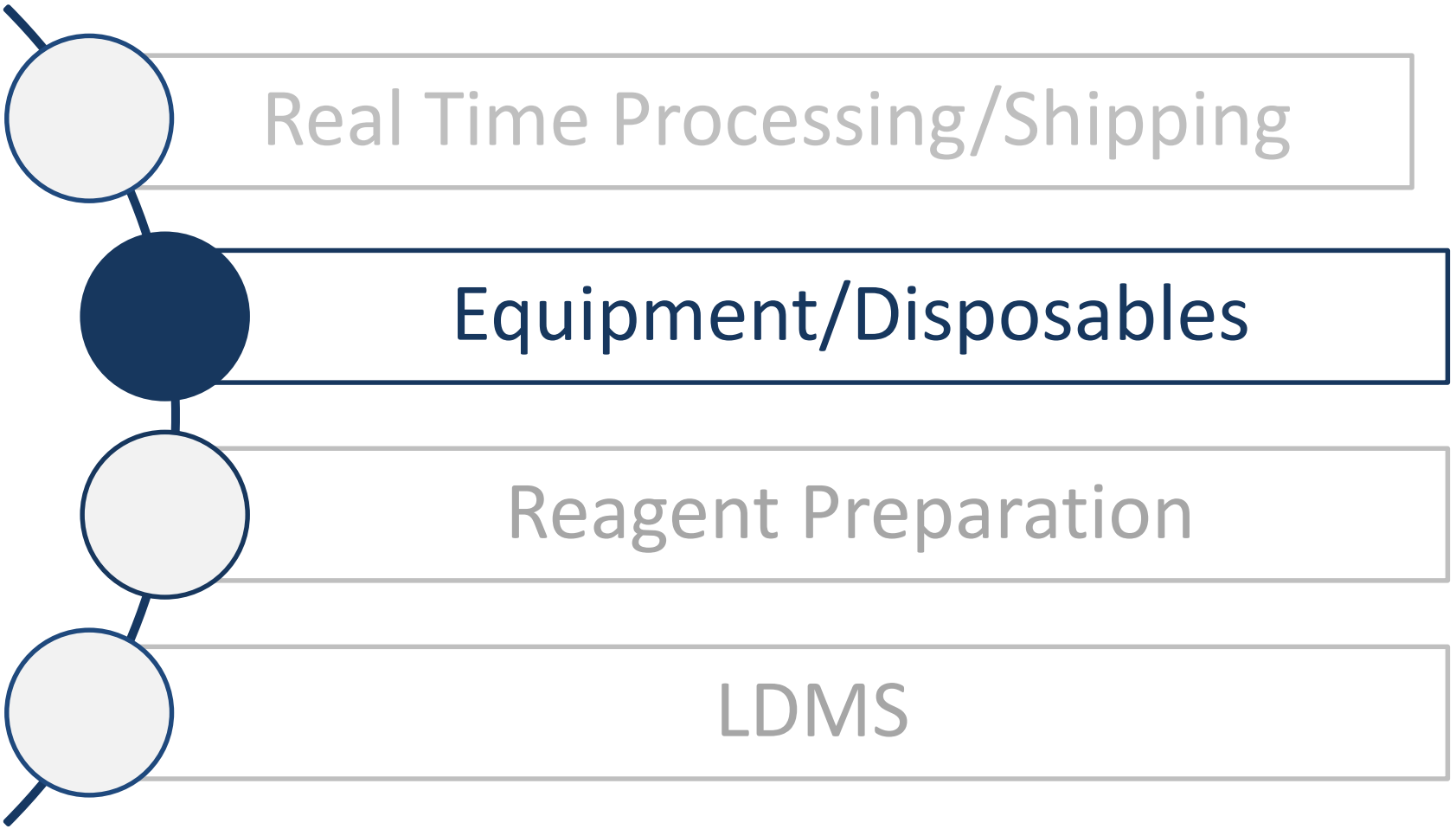
Shipping of the Leukopak

Refer to Section 9 and 10_PBMC Isolation From Leukapheresis

- Leukopaks being sent to a processing laboratory via overnight shipping must be packaged properly to help maintain constant temperature conditions during transit.
- If shipping (follow the instructions provided in the protocol support documents, LPC and/or MOP as the required conditions will vary with the analyte).
- The use of a temperature monitor is recommended to record shipping conditions.
- Package the leukopak material using *ACTG/IMPAACT guidelines for shipping a package under packing instructions 650 for Biological Substances, Category B (UN3373).
- Track the shipment to ensure proper delivery of the specimen.

*https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx

Leukapheresis Preparation



Equipment Preparation & Processing

Refer to Section 11_PBMC Isolation From Leukapheresis

Before receiving a leukapheresis product, check that all equipment listed in Section 11 of the *PBMC Isolation from a Leukapheresis SOP* are present and functional.

-Class II biosafety cabinet (BSC)

-Centrifuge, low-speed (capable of 300 to 1000 x g), with swinging bucket rotor and sufficient capacity for twenty 50mL conical centrifuge tubes

-Micropipettes, range 20, 200, 1000mL

-Pipet-Aid (cordless preferred)

--80°C freezer (-65 to -95°C); for short-term PBMC storage

-2 to 8°C refrigerator or Ice Bath for CPS

Cryopreservation Equipment

Refer to Section 11.4_PBMC Isolation From Leukapheresis

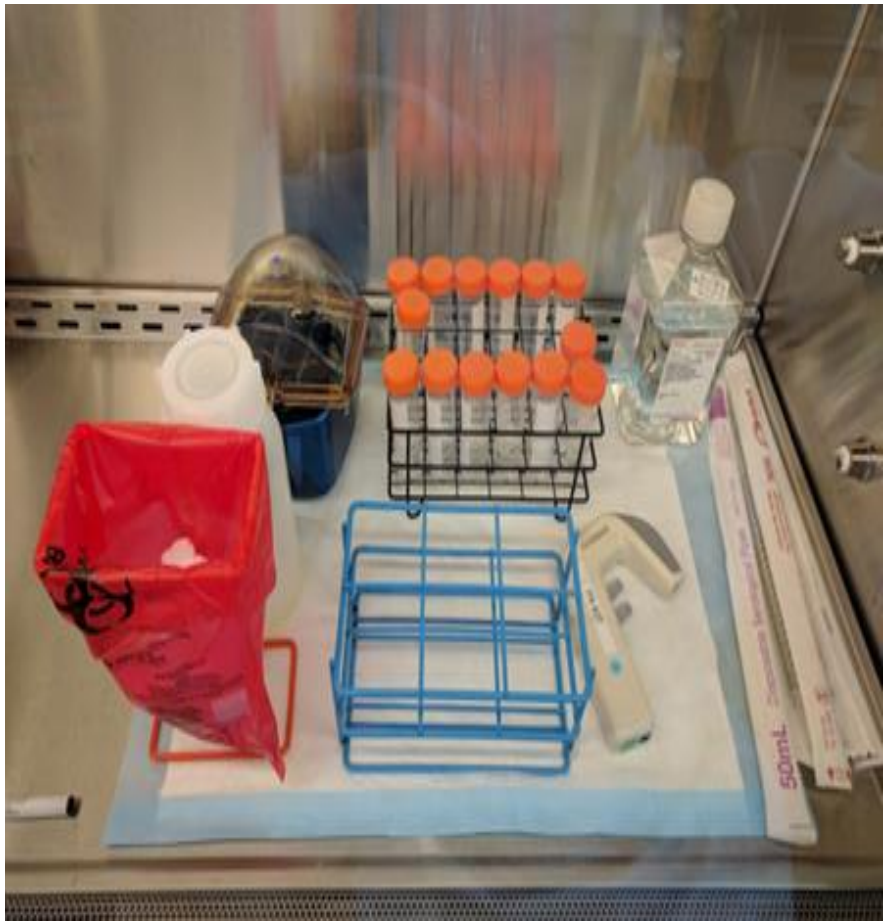
- In a typical 1.5-3 hour leukapheresis procedure, 3-10 x10^9 PBMCs can be isolated.

- Ensure sufficient capacity is available in approved cryopreservation vessels and freezer to accommodate the large number of aliquots that will be generated.

If the laboratory uses Mr. Frosty units, verify the isopropanol status. An example log can be found in the appendices of the Cross-Network PBMC Processing SOP at https://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx.

Disposables

Refer to Section 12_PBMC Isolation From Leukapheresis



Before receiving a leukapheresis product, properly prepare all required disposables listed in Section 12 of the *PBMC Isolation from a Leukapheresis SOP*.

-(20) 50mL conical tubes labeled with PTID and adding 15mL of Density gradient Media (DGM) in each tube

-(20) 50mL conical tubes labeled with PTID for washes

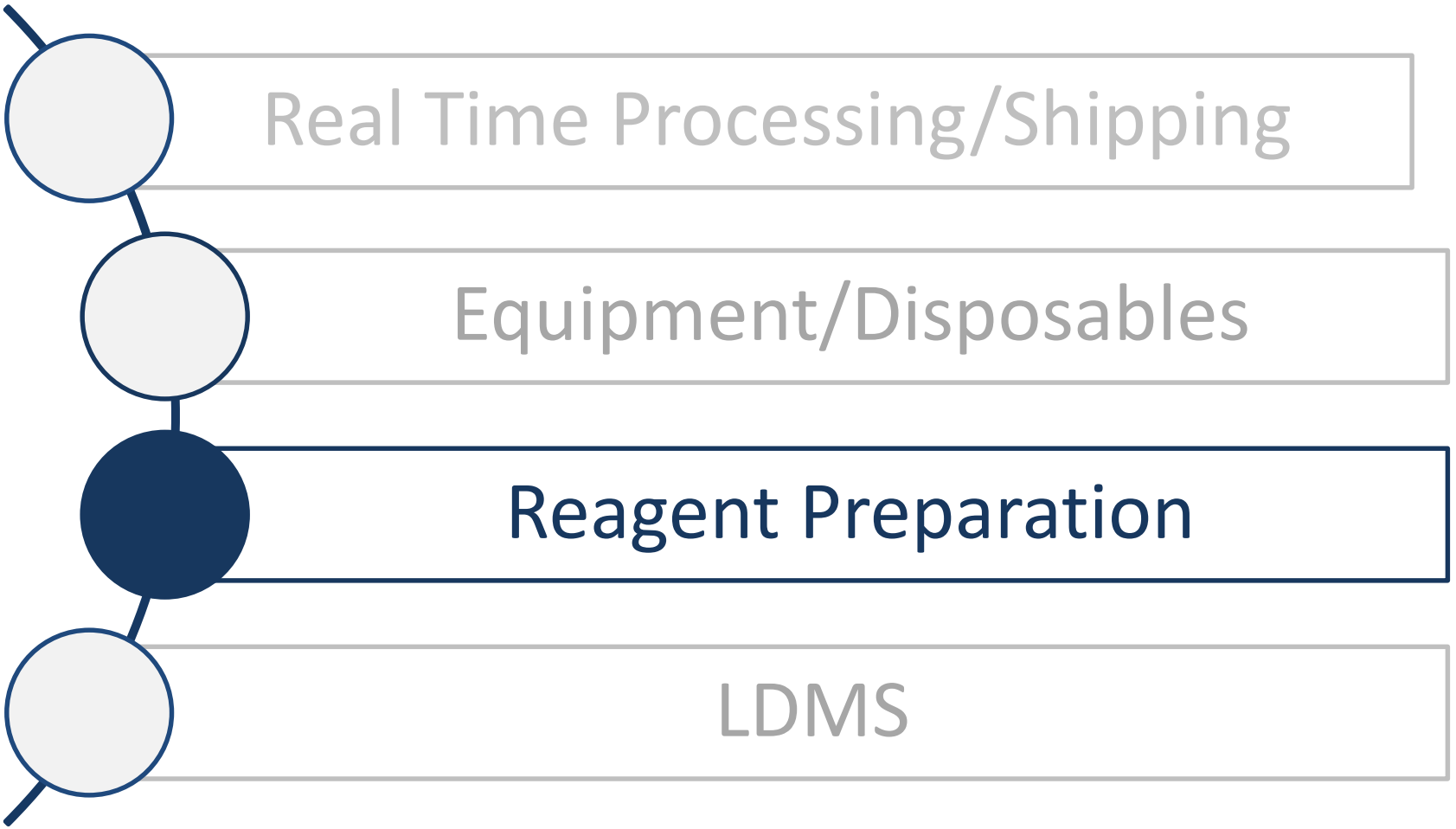
-Serological pipets, disposable, 1, 5, 10, 25, 50mL, sterile

-Pipet tips, 20, 100, 200, 1000 mL, sterile

-Cryogenic vials (cryovials), 1.8 to 2mL

-Sterile disposable bottles (250mL and 500mL) or flasks, 45mm neck

Leukapheresis Preparation



Reagents Required at **15-30°C**

Refer to Section 14_PBMC Isolation From Leukapheresis

Wash Diluent Reagent (WDR)

1.077g/ml Density Gradient Media (DGM)

Dimethyl sulfoxide (DMSO)

Trypan Blue (If performing a manual count)

Fetal Bovine Serum (FBS) Preparation

-Heat-Inactivated FBS (HI-FBS)

Refer to Section 15_PBMC Isolation From Leukapheresis

- Thaw in the refrigerator (2 to 8°C) overnight, or for several hours at room temperature.
- Do not allow HI-FBS to sit at room temperature any longer than necessary to complete the thawing process.
- Once thawed HI-FBS must be stored at 4°C until Cryopreservation Solution (CPS) production.
- The volume of FBS that will be needed will depend on the number of cryopreserved PBMCs and the final cell concentration required by the protocol.

Cryopreservation Solution (CPS)

Refer to Section 15_PBMC Isolation From Leukapheresis

- Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill (2-8°C) the CPS.
- Determine the volume of FBS (VFBS) needed using the expected number of PBMC for cryopreservation (Nc) and the final cell concentration (Cc) plus approximately 10%.

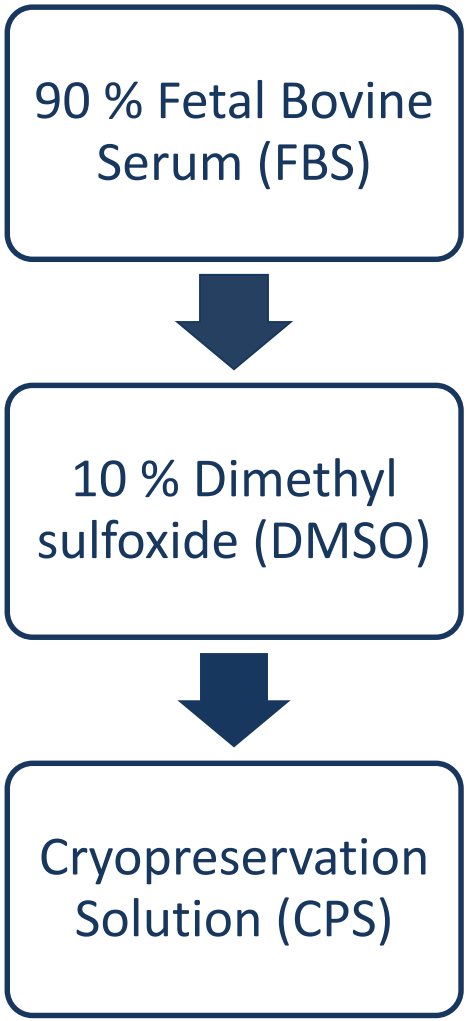
VFBS = Nc/Cc

*Example: If the protocol expects 1 x 10^9 cryopreserved PBMC at 10 x 10^6 cells/mL, VFBS = (1.1 x 10^9 cells)/(10 x 10^6 cells/mL) = 110mL.

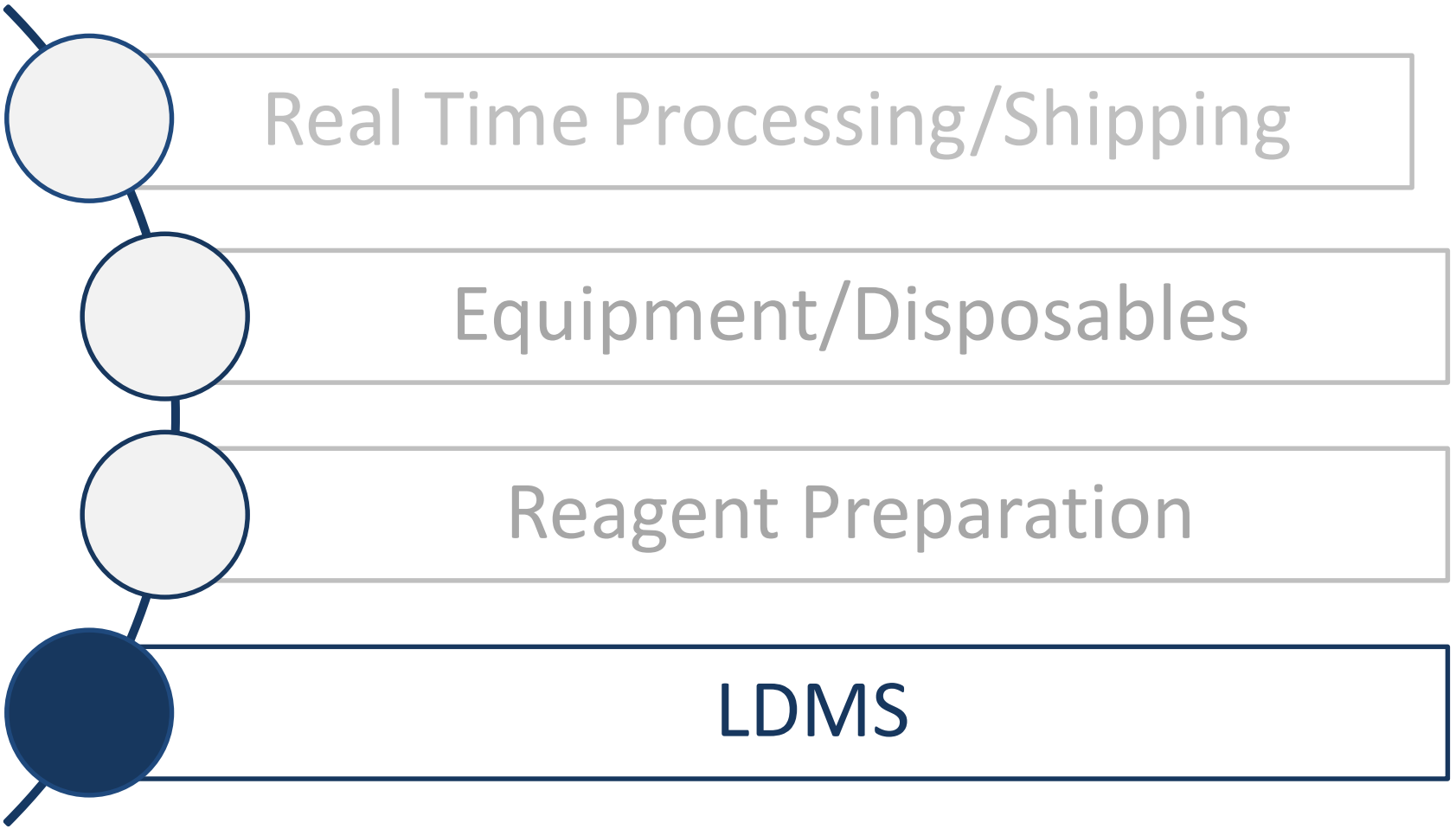
- Determine the volume of DMSO (VDMSO) that will be needed.

VDMSO = VFBS/9, rounded to the nearest 0.1 mL

*Example: If VFBS = 110 mL, VDMSO = 110/9 = 12.2 mL.



Leukapheresis Preparation



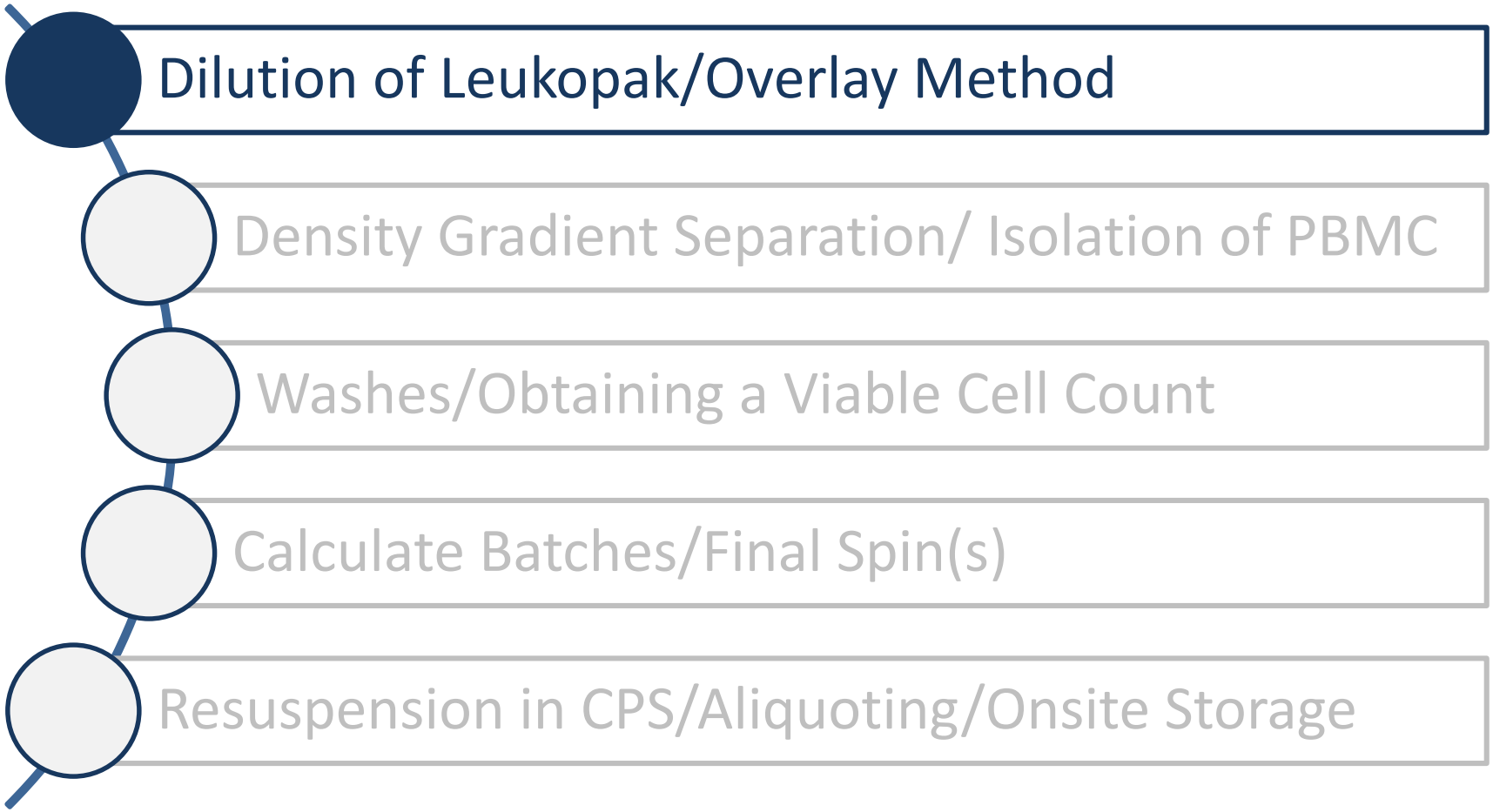
LDMS Entry

Refer to Section 18_PBMC Isolation From Leukapheresis

- Once the leukapheresis procedure is started, the clinic should FAX a copy of the CRF providing the PID, protocol, date and start time of the collection.
- Based on the CRF information, the processing laboratory can log the Leukopak into the LDMS, generate the expected number of aliquot labels, affix the labels to the cryovials and QC the label process.
- Estimate the number of labels and cryovials that will be needed using the volume of Cryopreservation Solution (CPS).
- If a LDMS field does not accommodate the number obtained, enter the information into the comments field and contact LDMS user support, ldmshelp@fstrf.org.

Leukopak Processing

Refer to* PBMC Isolation from Leukapheresis SOP



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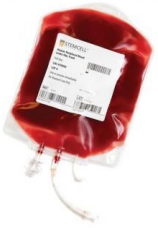
*https://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx

Dilution of Leukopak

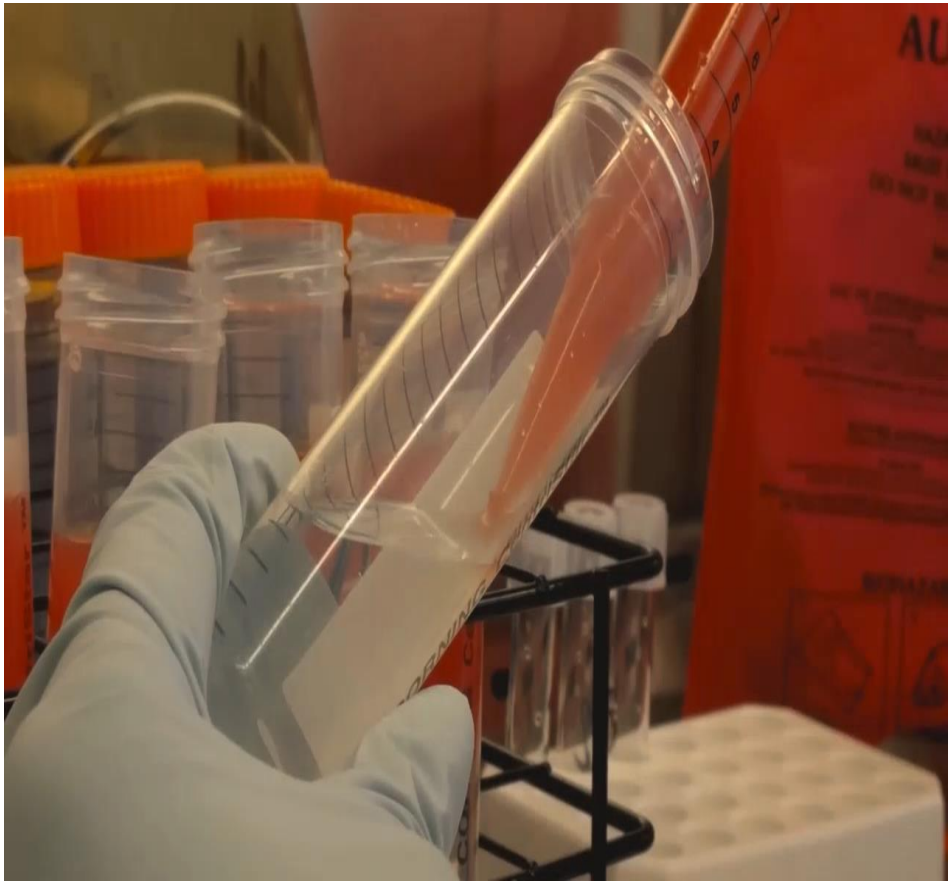


Refer to Section 19.5_PBMC Isolation From Leukapheresis

- Cut one of the tubing port with disinfected scissors and pour through the tubing into a sterile flask or bottle.
- Determine and record the approximate blood volume (typically 150 – 200mL).
- Add sufficient Wash Diluent Reagent (WDR) to dilute the leukopak to 600mL total.
- Mix the blood well with a serologic pipet 3-4 times; avoid creating bubbles.

Overlay of Leukopak

Refer to Section 19.5_PBMC Isolation From Leukapheresis

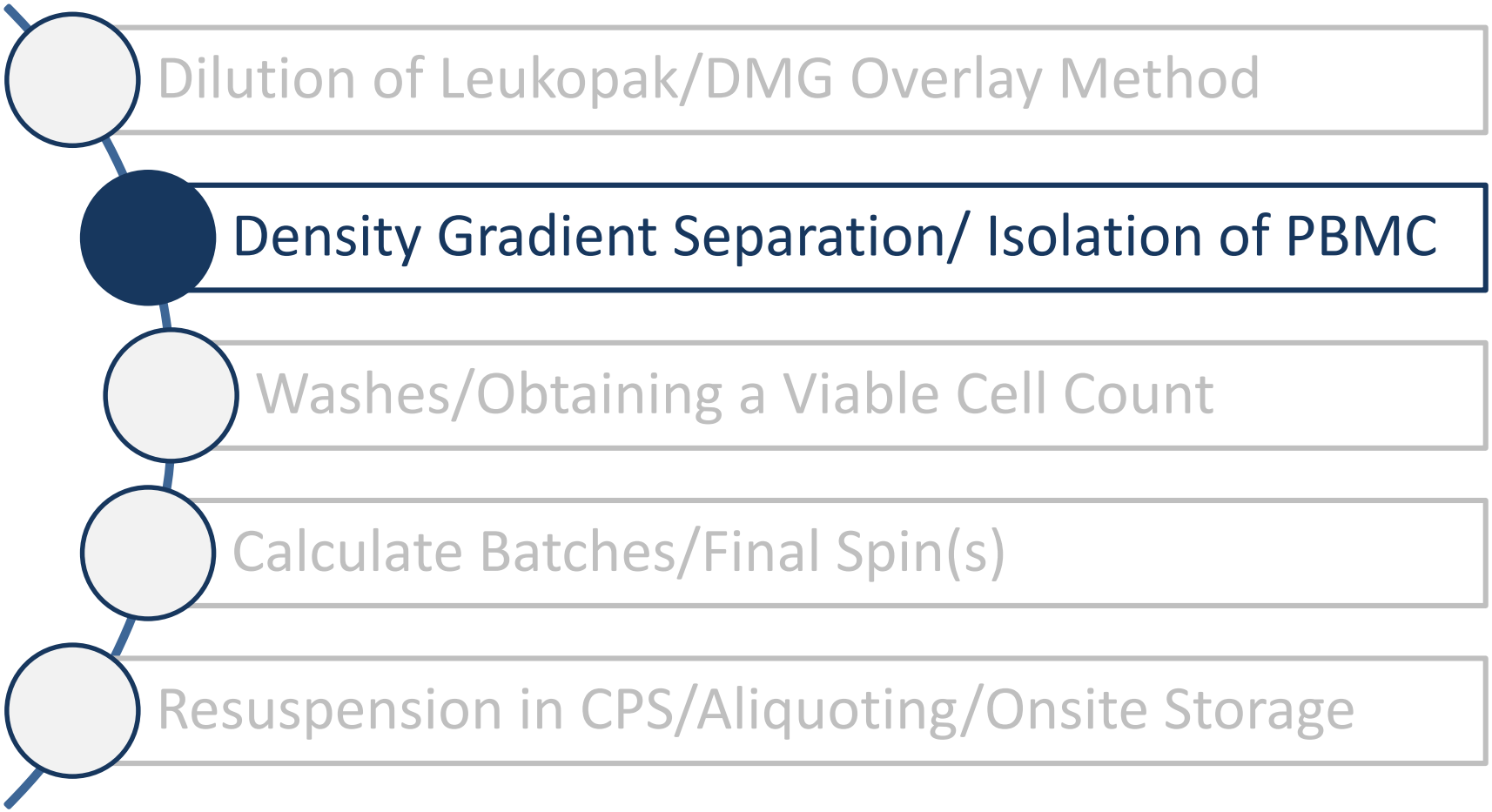


- Carefully and slowly overlay 30mL of the diluted leukopak on top of each of the (20) 50mL conicals containing 15mL *DGM
  - -The Diluted Leukopak is going to be less viscous than whole blood, turn pipette aid on low
  - - Mix diluted leukopak in between the overlay process
- Gently allow the diluted leukopak to flow down the side of the tube and pool on top of the *DGM surface without breaking the surface plane. Tilting the conical tube to approximately 45° angle, often helps this process.
- Centrifuge at 400 x g for 30 min at 15 to 30°C with the Brake OFF

*DMG-Density Gradient Media

Leukopak Processing

Refer to* PBMC Isolation from Leukapheresis SOP



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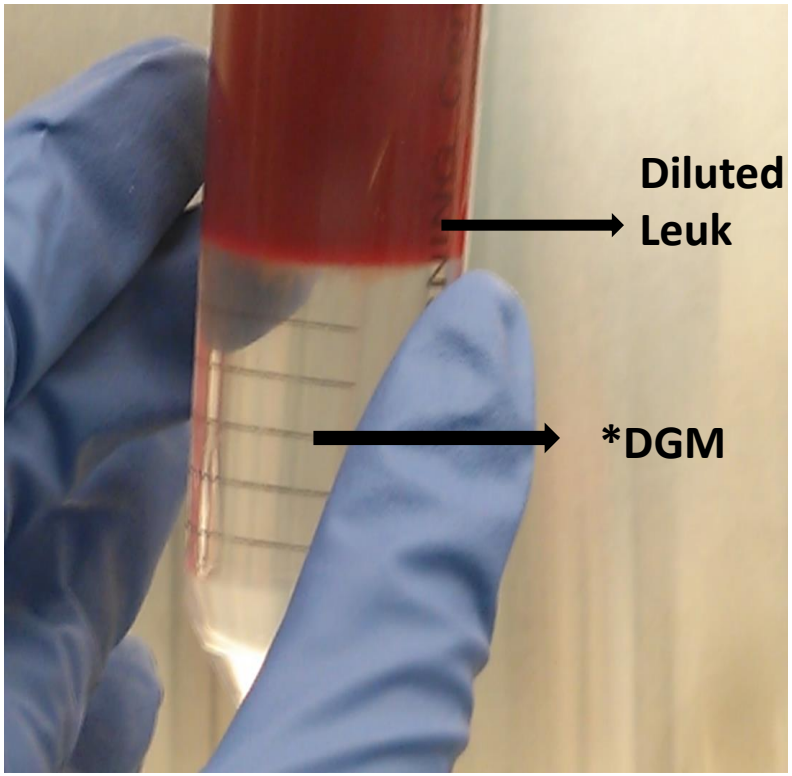
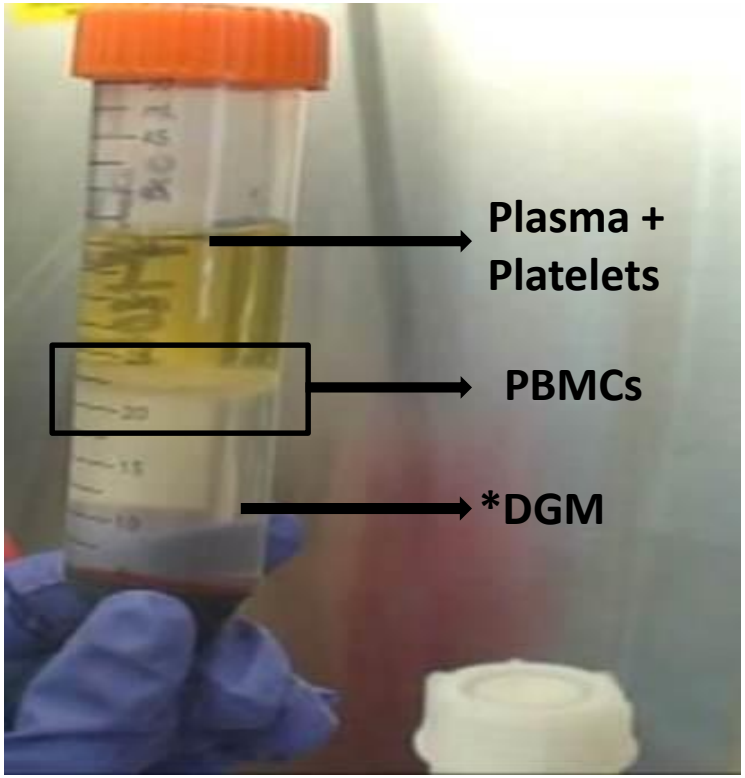
Density Gradient Separation

Refer to Section 19.6_PBMC Isolation From Leukapheresis

Layers *Before* Density Gradient Separation Spin



Layers *After* Density Gradient Separation Spin

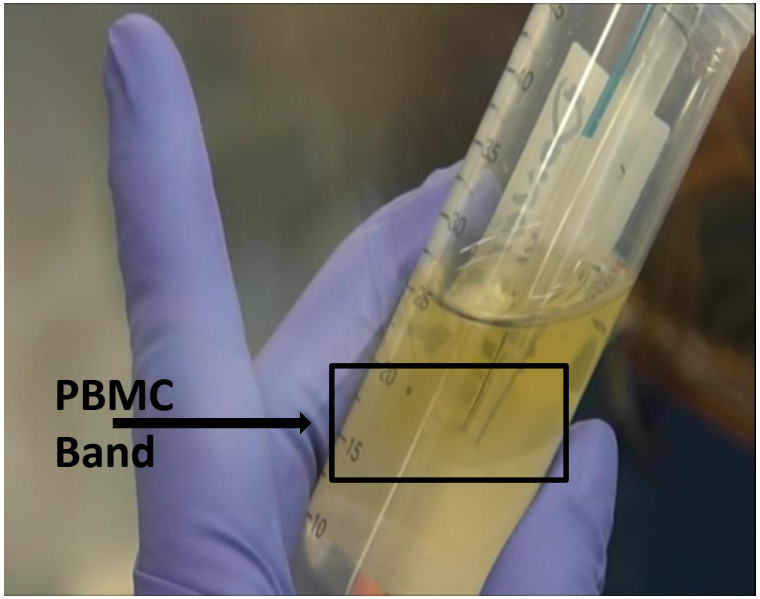


*DMG-Density Gradient Media

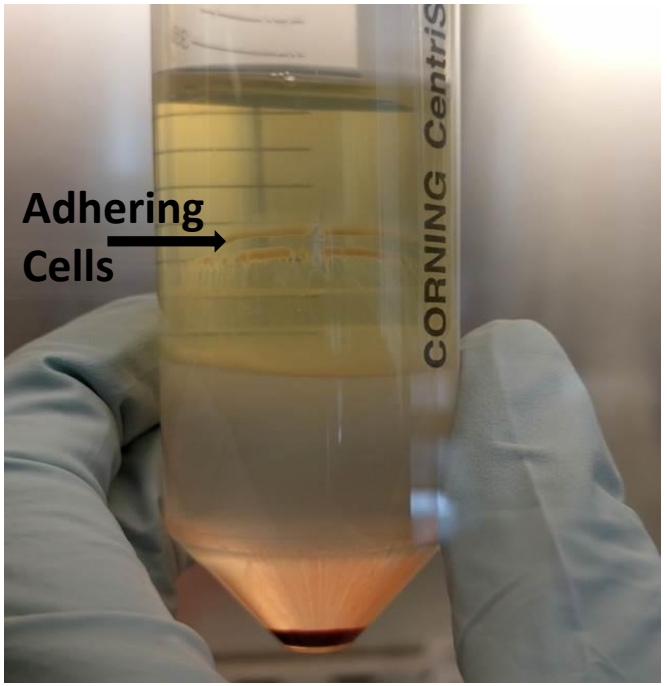
PBMC Isolation

Refer to Section 19.6_PBMC Isolation From Leukapheresis

Collect the PBMC band without removing excess amounts of Plasma and/ or *DGM, limiting cell contamination, with a sterile pipet.



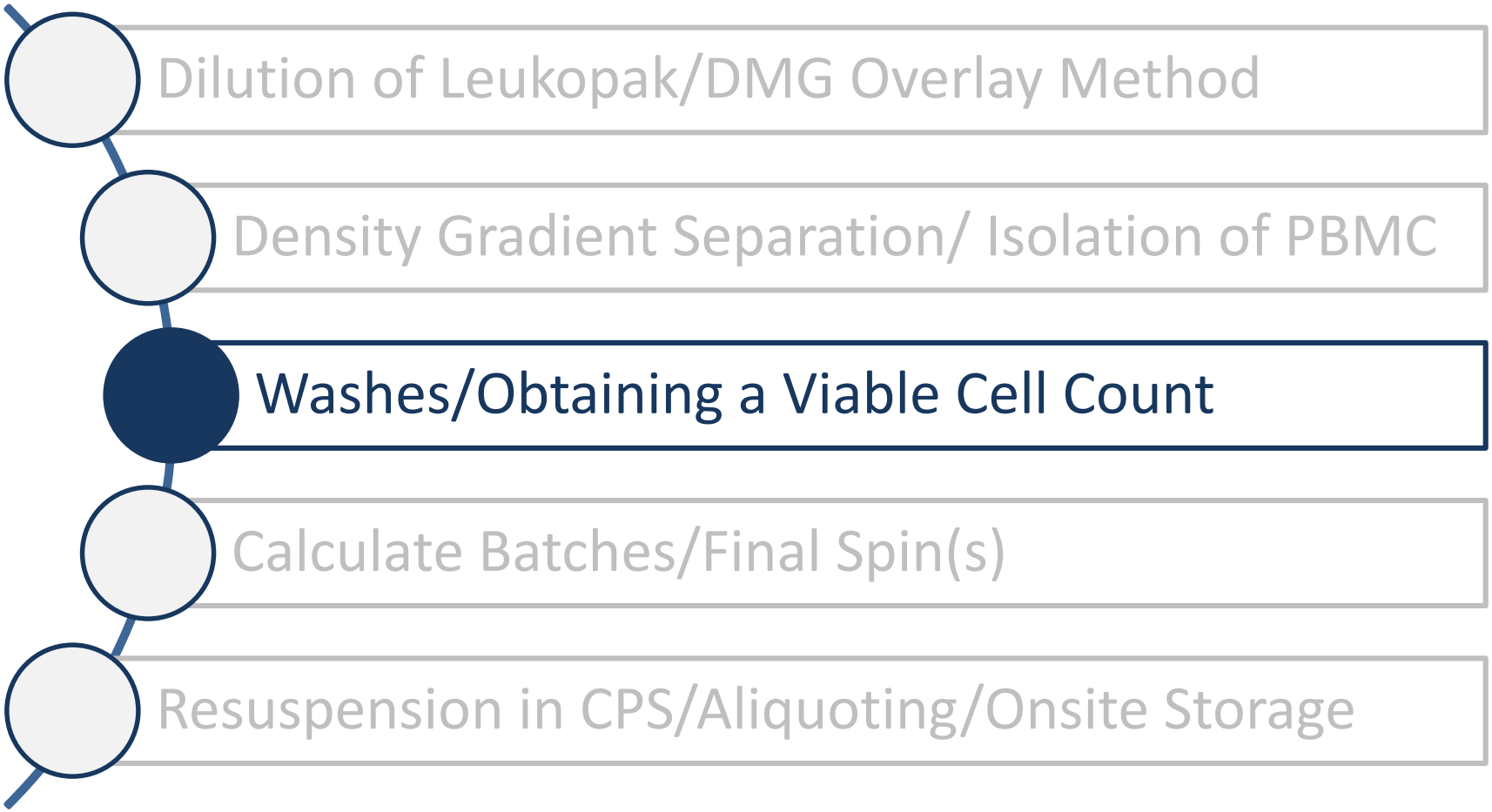
Do NOT scrape cells that are adhering to the plastic tube; these adherent cells are generally not PBMCs



*DMG-Density Gradient Media

Leukopak Processing

Refer to* PBMC Isolation from Leukapheresis SOP



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PBMC Washes

Refer to Section 20_PBMC Isolation From Leukapheresis

PBMC Wash #1

- Transfer the collected cells from one 50 mL conical gradient tube into a new 50 mL conical wash tube.
- Q.S. each (20) wash tubes to approximately 45mL by adding *WDR.
- Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
- Quickly Decant without disturbing the cell pellets. The pellets will be quite large and relatively “loose” compared to routine PBMC pellets from whole blood.

PBMC Wash #2

- Re-suspend each pellet in 5mL of *WDR.
- Combine the pellet suspensions from four (4) 50mL conical tubes into one tube.
- This step condenses the cells from 20 tubes to five (5) tubes and Q.S. each wash tube to approximately 45mL by adding *WDR.
- Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).

*WDR- Wash Diluent Reagent

Obtain the Viable Cell Count

Refer to Section 20.3_PBMC Isolation From Leukapheresis

- Re-suspend each of the 5 cell pellets in 10mL of *WDR and combine into one sterile container.
- Q.S. to 200mL with *WDR and mix gently. It is important to be as accurate as possible because the cell count will be based on a resuspension volume of 200mL.
- Mix cells gently, but thoroughly, before sampling for the cell count.
- Transfer a small volume (<100 µL) of the resuspended cells to a small tube for counting.

*WDR- Wash Diluent Reagent

Viable Cell Counting

Refer to Section 20_PBMC Isolation From Leukapheresis

The Quality Expectation of freshly isolated PBMC viability should be > 95%

If an automated cell counter is used that is not capable of distinguishing viable cells, **viability** must be determined with a manual cell counting chamber.

Automated counts may be run once, though duplicate counts are preferred.

Be aware of the automated cell counters counting range.

Viable Cell Count Dilution

Refer to Section 20.3._PBMC Isolation From Leukapheresis

Manual Counting Method

- It is likely this aliquot will require an additional 1:100 dilution if performing manual cell counts.
- Manual counts using a hemacytometer should count the four large corner squares (1mm2).
- Document any additional dilution volumes used for counting the cells.
- Determine the total number of cells and the percent viability.

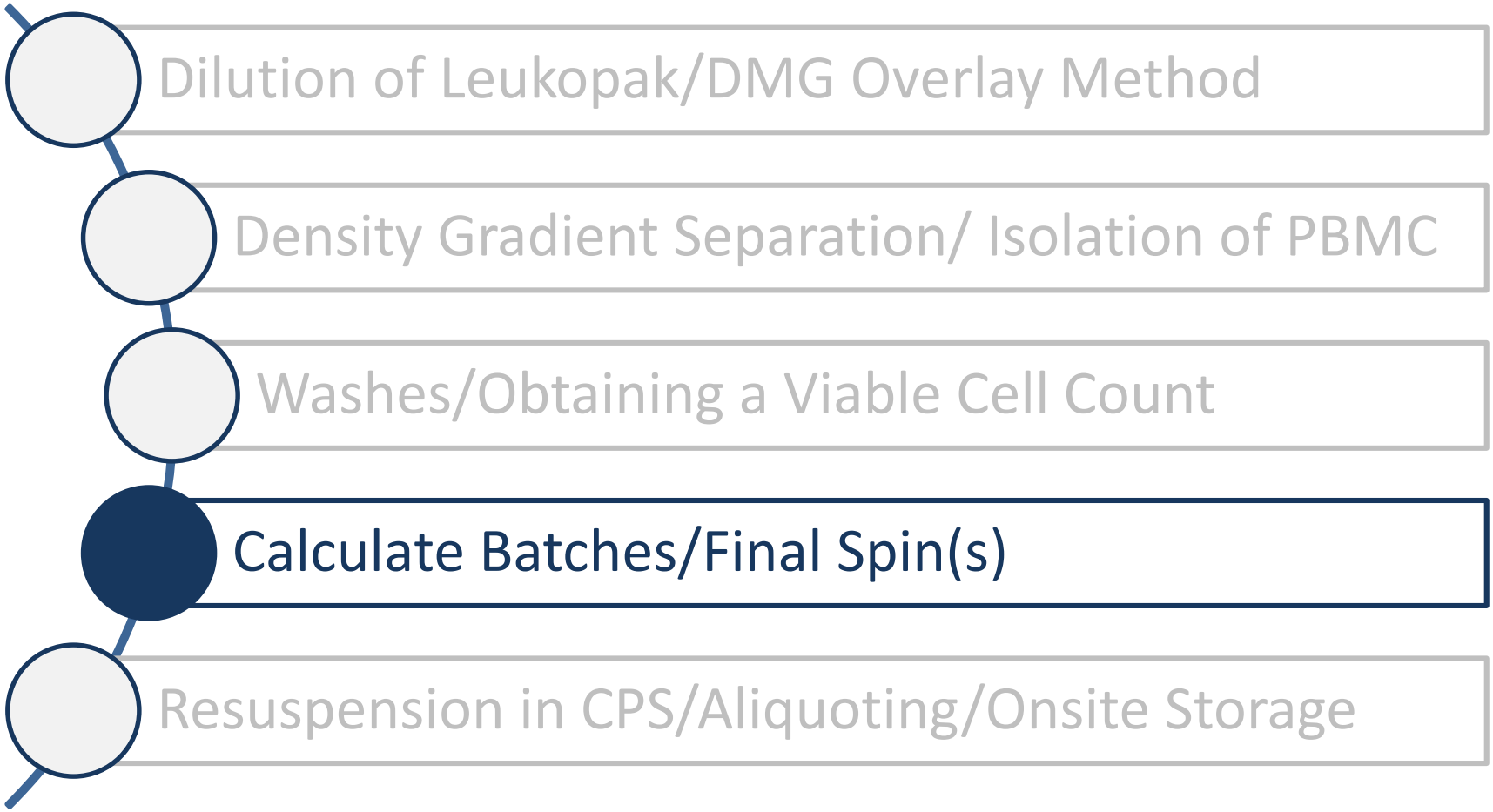
Example of Serial Dilution

- Remove 100 µL of cell suspension
- Add 160µL of *WDR each to 2 tubes/wells and 50µL of Trypan blue in a third tube/well.
- Transfer 40 µL of the cell suspension to the first tube/well with 160µL of *WDR. Mix well.
- Transfer 40 µL of this cell suspension to the second tube/well with 160 µL of *WDR. Mix well.
- Transfer 50 µL of this diluted suspension to the tube/well with 50µL of 0.4% Trypan blue. Mix well.

*WDR-Wash Diluent Reagent

Leukopak Processing

Refer to* PBMC Isolation from Leukapheresis SOP



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Calculating Batch Size

Refer to Section 20.6.2_PBMC Isolation From Leukapheresis

- Before the final spin the appropriate batch size (B) for centrifuging and aliquoting the cells will need to be determined.
- Determine the number of whole batches (NBw) and partial batches (NBp) that will be needed.

$NB = Nc/(Cc \times Va \times B)$

Nc = Expected number of PBMC for cryopreservation (cells)

Cc= Final Cell Concentration per protocol (cell/mL)

Va= Volume of each aliquot (mL)

B= Batch, size of the cryopreservation vessel

Example:

$Nc = 1.1 \times 10^9$ cells

$Cc = 10 \times 10^6$ cells/mL

Va = 1.5 mL

B = 24

Example Continued:

$NB = 1.1x\ 10^9/(10 \times 10^6 \times 1.5 \times 24) = 3.06$

NBw= 3 Whole Batches

NBp= 0.06 Partial Batch

Note: If the whole batches will yield the number of cells required by the protocol plus an additional 5%, there is no need to process the partial batch.

In this example, it would be unnecessary to process the partial batch.

Resulting in,

NBw =24 x3=70 aliquots at $15x10^6$ cell/aliquot

Distribution of Cells for Final Centrifugation

Refer to Section 20.6.3_PBMC Isolation From Leukapheresis

- For each whole batch (Vw) and each partial batch (Vp), calculate the volume of harvested cells that will be distributed to each conical centrifuge tube for final centrifugation.

To calculate a Whole Batch (Vw):

Vw = Va x Cc x B/Ch,

**rounded to the nearest 0.1 mL*

Va= Volume of each aliquot (mL)

Cc= Cell Concentration per protocol (cell/mL)

B= Batch, size of the cryopreservation vessel

Ch=Concentration of cells harvested (cell/mL)

Example:

Va = 1.5 mL

Cc = 10 x 10^6 cells/mL

B = 24

Ch= 44.5x10^6 cell/mL

Vw = (1.5 x 10 x 10^6 x 24)/(44.5 x 10^6) = 8.08 mL, rounded to 8.1mL for each whole batch

To calculate a partial batch (Vp):

Vp = NBp x Vw

**rounded to the nearest 0.1 mL*

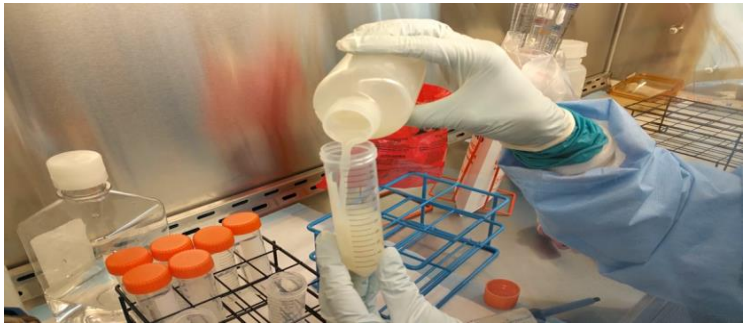
Example:

Vw = 8.1mL

NBp = .73

Vp =.73 x 8.1 =5.91 mL, rounded to 5.9 mL

- 1 conical centrifuge tube for each whole batch and each partial batch.
- Batches are to be processed one at a time.



Final Centrifugation

Refer to Section 20.6.3 _PBMC Isolation From Leukapheresis

• Before the final spin,

Determine the final resuspension volume of *CPS for each whole batch tube (Vfw) and each partial batch tube (Vfp).

To Calculate the volume of CPS For *Vfw* :

Vfw = Va x B

**rounded to the nearest 0.1 mL*

Example:

Va = 1.5 mL

B = 24

Vfw = 1.5 x 24 = 36 mL of CPS for each whole batch

To Calculate the volume of CPS For *Vpw :*

Vpw = NBp x Vfw

**rounded to the nearest 0.1 mL*

Bp = .73

Vfw = 36mL

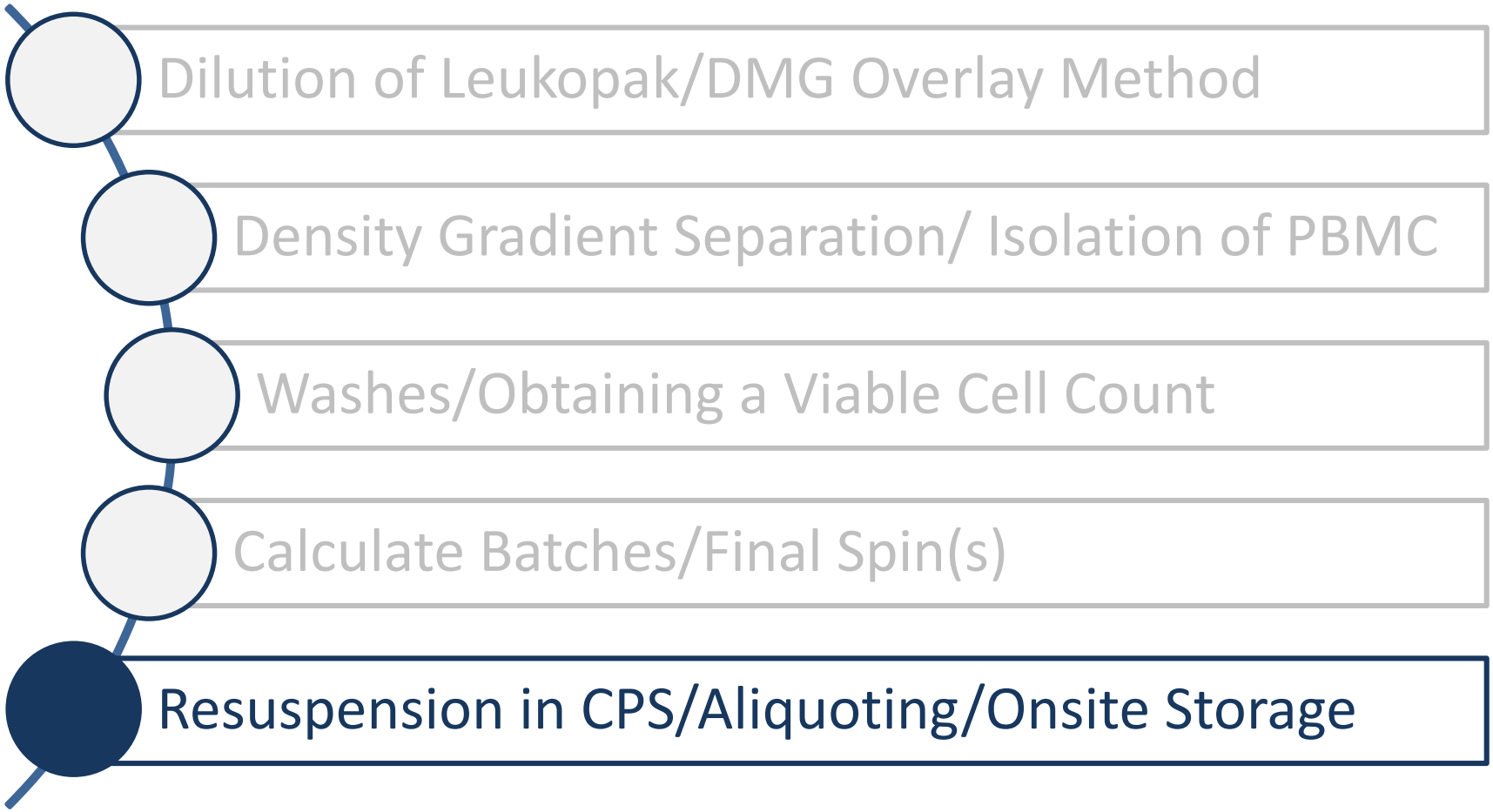
Vfp = .73 x 36 = 26.28mL, or 26.3mL.

- Label each conical centrifuge tube with the PTID, the volume of harvested cells that will be distributed to the tube (Vw/Vp), and the final resuspension volume of *CPS (Vfw/Vfp).
- Gently mix the flask of harvested cells prior to each distribution.
- Distribute the harvested cells among the prepared conical centrifuge tubes as was determined for batch size.
- Place the conical centrifuge tube(s) containing one batch of cells in the centrifuge and spin at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
- Do not allow cells to sit in the centrifuge once spinning has stopped.

*CPS- Cryopreservation Solution

Leukopak Processing

Refer to* PBMC Isolation from Leukapheresis SOP



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Resuspension in CPS/Aliquoting

Refer to Section 20.7._PBMC Isolation From Leukapheresis

- Remove and discard the *WDR supernatant. Gently re-suspend the cell pellet(s) by flicking or pipetting.

- Gently re-suspend each pellet(s) in the volume of prepared *CPS (Vfw/Vfp),add the *CPS to the resuspended cells with continuous swirling.

- Work *quickly* once the *CPS has been added.

- Mix the cells well before refilling the repeater pipettor or in between every 10 aliquots.

- While one batch of cells is being resuspended in *CPS, aliquoted, and transferred to the controlled-rate freezing vessel, the next batch of cells can be in the centrifuge.

- Do not allow the cells to be in contact with DMSO for longer than 10 minutes before placing in the freezer.
- The use of Wet ice is allowed.
- Immediately transfer all cryovials to the controlled-rate freezing vessel.

*WDR-Wash Diluent Reagent
*CPS- Cryopreservation Solution

Onsite Temporary Storage in a -70/-80°C Freezer

Refer to Section 21.0_PBMC Isolation From Leukapheresis

After Cryopreservation

- Transfer the cryovials from the controlled-rate cooling system to the designated storage location in a -70/-80°C freezer the cryovials after a minimum of 4 hours for NALGENE® Mr. Frosty and biocision® CoolCell or overnight for StrataCooler ® Cryo.
- The cold-chain must be maintained during all transfer steps to avoid damage to the cells.
- Use a dry ice transfer pan. Make sure the cryovial freezer box is deeply covered on all sides with dry ice. Work rapidly and efficiently to minimize cryovial exposure to ambient temperature.
- Ship on dry ice within 4 weeks of cryopreservation. Make sure the dry ice shipper is completely full of dry ice.

Challenges Observed by the IQA

Out of Range Viable Recovery	Cellular Contamination
	Counting/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

*CPS- Cryopreservation Solution

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- Chelsea Johnson