Indiana University Health	**Original Creation Date:** 02/11/2013	**Publication Date:** 03/06/2023
	Owner: Dave Schwering (Manager-Quality)	**Next Review:** 03/06/2025
	Category: Labs AHC	
	Education: Level 1	

Approval Signatures: Dave Schwering (Manager-Quality) (03/06/2023), Muhammad Idrees (Physician) (03/06/2023)

Procedure Flow Cytometric Acquisition and Analysis of CD34 and CD3 Using the BD FACSCalibur Flow Cytometer

I. PURPOSE/PRINCIPLE

To detail the procedure for the acquisition and analysis of CD34+ and CD3+ using the BD FACSCalibur Flow Cytometer.

II. SCOPE

This procedure encompasses all fresh and thawed products processed by the CTL including, but not limited to: HPC, Apheresis, HPC, Marrow, HPC, Cord Blood, MNC, Apheresis and peripheral blood. It further details the ordering and resulting of peripheral CD34+ in Cerner. All CTL Technologists will comply with this policy.

Any exceptions to this policy must be approved by the IU Health Cellular Therapy Laboratory Processing Facility Medical Director or Processing Facility Director.

III. EQUIPMENT/REAGENTS/SUPPLIES

Equipment:

BD FACSCalibur Flow Cytometer SN: E2910 Macintosh Mini OSX Yosemite v10.10.1

Cell Quest Pro software version 6.1 Printer: Lexmark CS510de

Reagents:

BD FACSFlow (Sheath Fluid)

BD Stem Cell Control Kit (High and Low Levels) CD Chex Plus Control

BD CD3 APC

BD Stem Cell Enumeration Kit containing the following reagents:

- BD Stem Cell Reagent (CD45 FITC/CD34PE)
- 7-AAD reagent
- 10X ammonium chloride lysing solution
- BD Trucount Tubes

Supplies:

Kimwipes

Repeater pipette and tips Single channel pipettes and tips Ice Water Bath

Vortex Timer

PBS pH 7.4

Sterile Water

50 mL conical tube

IV. SAFETY PRECAUTIONS

- Follow all Biohazard and chemical safety policies as defined in DPLM Safety Program.
- Always wear required PPE:
- Gloves and impermeable lab coats are required at all times when performing this procedure
- Eye/Face protection is required when handling open liquids.
- 7-AAD is a potential carcinogen. Avoid contact with skin and eyes.
- 7-AAD contains dimethyl sulfoxide. It is harmful if swallowed. Dimethyl sulfoxide freely penetrates the skin and may carry dissolved chemicals into the body. It is irritating to eyes, respiratory system and skin. Avoid contact with skin and eyes. Wear suitable PPE.

V. SPECIMEN REQUIREMENTS

- All specimens must be labeled for adequate identification:
  - Minimum: Patient name and MRN.
  - On Study: Study # and Subject #.
- The following are acceptable sample types but may not be all inclusive:
  - HPC, Apheresis; HPC, Cord Blood; HPC, Marrow; Peripheral Blood
  - MNC, Apheresis; NK, Apheresis Cells
- Peripheral blood specimens may be collected in the following anticoagulants:
  - K_2EDTA (BD Vacutainer® lavender top)
  - K_3EDTA (BD Vacutainer® lavender top)
  - ACD-A (BD Vacutainer® yellow top)
  - Sodium Heparin (BD Vacutainer® green top)
- Apheresis and bone marrow products may be collected in ACD-A, heparin, CPD or any combination of the three.
- Thawed samples must be stained within 2 hours of thaw.
  - Cryopreserved HPC, Apheresis or HPC, Cord Blood samples prepare according to SOP PM 012 Procedure: Thaw and Wash of Cellular Therapy Products .
  - Cryovials: prepare according to SOP RT 007 Procedure Colony Forming Unit Assay .
- Sample integrity evaluation:
  - All peripheral blood samples must be analyzed within 24 hours.
  - All product samples should be analyzed upon receipt.
    - In cases where an NMDP sample arrives late, the product may be stored over night at 2-6° C.
  - Samples will be stored for up to one week, at 2-6° C.
  - Hemolyzed samples are unacceptable.

        - Control material should be evaluated upon receipt for hemolysis.
        - Also, check for suboptimal shipping temperature and exposure to temperatures outside manufacturer's defined ranges.
            - For any unacceptable material, proceed per SOP QC 003 Supply Inventory .
    - Clotted samples are unacceptable.
- Notify CTL Medical Director if a sample is unacceptable.
    - Follow instructions given by CTL Medical Director.
    - Document sample disposition in the activity log.
    - Report to ordering physician and request new sample if applicable.
    - Complete a CTL Occurrence Report Form F-228b.
- Adding any portion of an aliquoted sample or product back to the original container is prohibited.

VI.CALIBRATION/VERIFICATION

Calibration and/or verification procedures are not performed by the user. The analyzer is verified to be correct during annual PM's performed by the manufacturer. Latron beads are used daily to ensure that the analyzer is functioning properly and that no shifting has occurred.

VII. QUALITY CONTROL

A. Control Assay Frequency:

i. BD Stem Cell Controls must be prepared to verify instrument setup and performance, and data analysis in the following situations:

1. Each time the cytometer is turned on for patient sample characterization.
2. After BD Biosciences field service engineer performs preventative or other maintenance or repair.

ii. Streck CD Chex Plus CD3 control must be prepared only when there is an allogeneic patient, or need for CD3 acquisition or analysis.

B. Control Storage and Handling:

i. Control material should be stored at 2-6° C upon arrival. Refer to SOP QC 016 Procedure FACSCalibur Calibration, QC and Maintenance .

ii. The control materials should be allowed to stand at room temperature, approximately 10-15 minutes, prior to use.

iii. Control material should be rolled back and forth in the hand and inverted several times to ensure a well mixed sample.

iv. BD Stem Cell Control:

1. Unopened vials are good until the expiration stated on the box.
2. Opened vials are good for 12 thermal cycles (uses).
3. Upon opening BD Stem Cell Control complete a BD Stem Cell Control label (Attachment 2) by completing lot #, expiration date, date opened and tech initials and place it on the box.
4. Each time the controls are removed from the refrigerator is considered one use.
    a. The box label will be marked to indicate each use.
5. BD Stem Cell Control label is located on the shared drive.

v. Streck CD Chex Plus Control:

1. Unopened vials are good until the expiration stated on the vial.
2. Opened vials are stabile for 30 days.

VIII. PROCEDURE

A. Preparing Lysing Solution:

i. Each day prepare enough 1X ammonium chloride lysing solution for use.

ii. To make, dilute 1 part of 10X ammonium chloride solution with 9 parts of sterile water in a labeled 50 mL conical tube (See Attachment 1 for label).

1. Store and use at room temperature.

2. Once prepared dilution is good for 24 hours.

B. Control and Patient panel set up (Attachment 3):

i. MAbs should NOT be pipetted ahead of usage.

ii. For each control and patient specimen, label a BD Trucount tube, for patients an interim label PST 008 *Product and Reagent Labeling* should be used.

1. It is good laboratory practice to color code interim labels if multiple samples are assayed simultaneously, to help distinguish each patient and help to prevent sample mix up.

2. Controls should be labeled with the level (if applicable) and/or type of control; ie CD 34 High, CD34 Low, or CD3.

iii. Before use, verify that the BD Trucount bead pellet is under the metal retainer at the bottom of the tube. If this is not the case, discard the BD Trucount tube and replace it with another. DO NOT transfer beads to another tube.

1. Use tubes within 1 hour after removal from the foil pouch.

2. Refer to step O for other limitations/precautions with the Trucount tubes.

iv. Further inspect the tubes for any cracks that may prevent tube pressurization and result in acquisition failure.

v. Label each tube with the MAb and/or stain to be added:

1. For Stem Cell controls label:

a. CD45 FITC / CD34 PE (or SCR)

2. For CD3 controls label:

a. SCR / CD3 APC

3. For Patient samples label:

a. SCR / 7-AAD / CD3 APC (if performing)

C. Staining Control and Patient Cells:

i. BD SCR Addition:

1. Pipette 20 µL of the BD SCR into the bottom of the tube.

a. Pipette just above the stainless steel retainer of the BD Trucount tube, do not touch the bead pellet.

b. NOTE: Always change tips between tubes. Discard tips in an appropriate biohazard container.

c. Avoid contamination of any MAb as the reagents are costly.

i. Any tips exposed to one MAb must not be placed into a bottle of another MAb.

ii. Viability Stain Addition:

1. Pipette 20 µL of 7-AAD reagent into patient ONLY tubes.

iii. CD3 Reagent Addition (if applicable):

1. Pipette 5 µL of CD3 APC into each tube.

iv. Cell Addition Control:

1. Pipette 100 µL of well mixed control material into each tube.
    a. Pipette the BD Stem Cell high and low controls into their respective tubes.
    b. Pipette Streck CD Chex Plus for CD3 control.

v. Cell Addition Patient Sample:
    1. A WBC count on all patient samples is required. If the WBC count is >40.0 E+03 cells/µL dilute
    2. the specimen with PBS.
        a. Note the dilution factor as this will be used later for calculations in the BD CellQuest Pro software.
    3. Pipette 100 µL of a well-mixed specimen (WBC count < 40.0E+03 cells/µL) onto the side of the tube, just above the retainer.
    4. NOTE: Avoid smearing the specimen down the side of the tube. IF any specimen remains on the side of the tubes, it will not be stained with the regent but can be resuspended by the lysing solution and therefore can affect results.
    5. Accurate pipetting is critical when using a BD Trucount tube.

vi. Cap each tube and vortex gently to mix.

vii. Incubate for 20 minutes in the dark at room temperature, inside a closed drawer is acceptable.

viii. Add 2 mL of room temperature 1X ammonium chloride lysing solution, prepared in Step A, to each tube to lyse red blood cells.

ix. Cap each tube and vortex gently to mix.

x. Incubate for 10 minutes in the dark at room temperature, inside a closed drawer is acceptable.

xi. Immediately, place tubes on wet ice in the dark until ready to acquire samples. Samples must be acquired within 1 hour of lysing.

D. Instrument Set Up:

i. Start the BD FACSCalibur instrument and then turn on the computer. The power button is on the right side of the back of the computer.

ii. Set up the cytometer using BD CaliBRITE beads and BD FACSComp software with 3-color or 4-color lyse/no-wash (LNW) settings. Verify that all parameters pass (Refer to SOP QC 016 Procedure FACSCalibur Calibration, QC and Maintenance for procedure).
    1. 3-color beads will be used when only CD45/CD34 is being performed.
    2. 4-color beads will be needed when CD3 APC is added to the tube for acquisition and analysis.

iii. Quit BD FACSComp software.

iv. Open the BD Stem Cell Kit Template
    1. Location: BD FacStation HD→CTL→CD34 Pt Acq and Analysis Tru Count

v. Select Acquire→Connect to Cytometer. ((Refer to Attachment 4 for Macintosh keyboard shortcuts.)

vi. Select Cytometer→Instrument Settings.
    1. Click Open in the Instrument Settings window.
    2. Select BD FacStation HD→BD Files→Instrument Settings Files→Calib File.LNW. Double Click.
    3. Click Set. Once the settings have been uploaded, select done.

vii. Under Cytometer select the following:
    1. Detector/Amps
    2. Threshold
    3. Compensation

4. Status

viii. Under Acquire select the following:

1. Counters

a. Expand to view the 3 sources and mode.

i. The first source should be set to reject debris.

ii. The second source should be set to collect viable.

E. Optimizing and Adjusting Compensation:

i. Refer to Attachment 5 for Gate definitions and Attachment 6 for Plot definitions (template).

ii. Place the prepared High control on the SIP and press RUN and HI on the cytometer.

iii. In the Acquisition Control window, select the Setup checkbox and then click Acquire to begin instrument optimization.

iv. Plot 1 is ungated and is used to include all $CD45^{dim}$ to $CD45^{bright}$ events and excludes debris, platelets, and unlysed RBCs (which all are $CD45^{-}$).

v. If needed adjust R1 leukocyte gate to extend above the top of Plot 1 to include high SSC events that are $CD45^{+}$. Adjust the left side of the gate to include all $CD45^{+}$ cells including dim $CD45^{+}$ events that are $CD34^{+}$. The right side can extend to the edge of the plot.

vi. In Plot 1, adjust polygon region R5 around the lymphocytes. Include only as many events as necessary to define the viable lymphocyte population displayed in Plot 6.

vii. In the Threshold window, change FL3 to FL1.

viii. Monitor Plot 1(FLA vs SSC) while adjusting the FL1 threshold as necessary, to exclude debris without excluding any CD45 cells. This value should be set around 300.

ix. Observe Plot 6 (FSC vs SSC) and if necessary, adjust the FSC gain in the Detectors/Amps window, so that the viable lymphocytes fall between channels 400 and 600.

1. R7 is an acquisition exclusion gate used to exclude debris. R7 should not exceed FSC channel 200 and SSC channel 200 and should not encroach the lymphocyte population, or cells in this region will be excluded from the data file.

x. In the Compensation window, note the initial value for FL3-%FL2. Increase this value by 4. For example, if the starting value is 12.0, increase the value to 16.0.

1. Failure to increase compensation can result in inaccurate data and an underestimation of viable CD 34+ cells.

xi. Observe the two 7-AAD vs SSC Plots 7, and 8. Plot 8 is ungated and Plot 7 is gated on the CD34 total gate. Both plots display R8. Because the control sample does not contain 7-AAD in the tube, all the CD34 cells fall in the negative gate (R8).

1. On the ungated Plot 8, adjust the R8 to include all the 7-AAD negative cells.

2. On the gated Plot 7, verify that the CD34 cells are all within R8. Adjust the FL3-%FL2 if any $CD34^{+}$ cells are outside the gate.

xii. Plot 2 displays viable CD45 cells (G1 or plot 1) and is used to identify CD34+ cells. Adjust R2 to include all $CD34^{+}$ events.

xiii. Plot 3 displays G2 (plot 2) cells and is used to further define the viable $CD34^{+}/CD45^{+}$ cell population.

1. Adjust R3 to include only those events that form a cluster with low to intermediate SSC and $CD45^{dim}$ expression.

2. Adjust R3 to exclude any lymphocytes or monocytes seen to the right of the stem cell cluster.

xiv. Plot 4 displays G3 (R3 and G2) cells and is used to identify viable stem cells ($CD34^{+}$).

1. Adjust R4 to include only those events that form a cluster with low to intermediates SSC and medium to high FSC.

2. This plot can be gated on R1 and R2 and R3 to display Total $CD34^{+}$ cells instead of viable $CD34^{+}$ cells.

xv. Plot 5 is ungated multicolor plot used to identify beads R6.

xvi. Plot 9 displays CD3 of viable CD45. Adjust R10 to encompass the entire CD3 population.

xvii. Plot 10 is used to see the flow of the cells and determine if there is something impeding the flow.

xviii. For additional gating strategy refer to the BD Stem Cell Enumeration Application Guide. (pg 22).

F. Acquisition and Storage Criteria For Control Samples:

i. Samples that complete the acquisition in less than 15 minutes must meet the following conditions to be reported:

1. At least 100 viable CD34 events
2. At least 1,000 beads
3. At least 75,000 viable CD45 events

ii. Browser Set Up:

1. Data Storage: Under Directory, select BD FacStation HD→CTL→ BD Stem Cell Control
   a. Select the current year and month
      i. Create new month/year as needed.
   b. Create a new folder and label with the current date. Select choose.
   c. Assign the following sample and patient information for consistency
      i. Control Sample
         1. Sample ID:
            a. Lot Number
            b. Date
         2. Patient ID:
            a. High Control, Low Control, CD Chex Plus CD3 Control, or High Control with 7-AAD.

iii. Verify the Trucount bead number in the template. Change as needed.

1. To change bead count number, activate the Trucount box on the template. (Click on box so the black squares appear in the corners of the box.)
2. From the tool bar click on Stats. From the drop down menu select edit expression. Enter the new bead count in the box as it appears on the Trucount tube package; make sure to use the bead count and not the lot number as they are close to each other on the packaging.
3. Click OK when completed.

iv. Verify the dilution factor. For control samples the dilution factor should always be 1.00.

G. Acquiring Controls:

i. Gently vortex the High BD Stem Cell control and place it on the SIP.

ii. Click Acquire.

1. For both the High and Low BD Stem Cell controls, acquire and save 75,000 viable $CD45^+$ events each.

iii. Add 20 µL 7-AAD to the high control, place in the dark for 10 minutes.

iv. Acquire the Low BD Stem Cell control.

v. Acquire the CD Chex Plus CD3 control (if needed). Acquire and save 20,000 viable CD45+ events.

vi. To test the performance of the 7-AAD reagent, use the High BD Stem Cell control stained with 7-AAD. Click Aquire, ensure your population of cells in Plot 8 is now outside R8 (or 7-AAD^+). Acquire approximately 10,000 total events, and click pause, then click save.

H. Analyzing Controls:

i. The BD Stem Cell Enumeration Kit is an acquisition and analysis template. Control samples can be analyzed immediately after acquiring before moving on to the next sample to acquire.

ii. Upload the data file into the template.

iii. CD34 Control Analysis:

1. For both the High and Low BD Stem Cell controls, move the right border of R8 against the lymphocytes, monocytes, and granulocytes.
2. Check each plot and verify that the gates enclose the appropriate populations.
3. Using the viable CD34 of viable CD45 result box, verify that results are within the established range for the process controls being used. Document the results electronically on BD Stem Cell Control Form F-154.
    a. All $CD34^+$ controls must be within the BD Stem Cell Control Kit assay values and expected ranges.
        i. One of these controls should be at (or near) clinical decision levels.
        ii. Document on F-055:
            1. Control lot number, expiration date, tech initials, and "Y" if QC passed.
4. For the BD Stem Cell high control stained with 7-AAD reagent, verify that most of the cells appear outside Region R8. If the population is outside R8, document "pass" on F-154.
    a. If the population is not outside R8, repeat steps G.iii and G.vi replacing high control with low control.
    b. If it still does not pass open a new bottle of 7-AAD and re-stain one level of control.
5. Prior to printing results, check to make sure:
    a. There is a minimum of 75,000 viable CD45 events.
    b. There is a minimum 100 viable CD34 events.
    c. The Trucount bead number is correct.
    d. The dilution factor is correct.
6. Print and save both levels of control and the high control stained with 7-AAD.
    a. A PDF file will be created for each final analyzed sample so that a permanent record is saved.
    b. Command P or File ->Print can be used.
    c. Do not hit the print button on this screen. On the bottom left there is a blue arrow, select open in PDF preview.
    d. A PDF will be opened. Go to File Save. Save under the appropriate folder (CTL Patient Reports or Control Reports) re-name the file as necessary (the default folder and file name will be wrong).
    e. After saving the PDF preview box will still be open. Go to File Print to print the PDF.
    f. This will make sure the printout and saved file match and have the same date and time stamp as required.
    g. Staple all printouts together, write "QC OK", date, and initial. Place the printouts in the QC folder.
7. If a control fails:
    a. Determine if instrument, sample or staining related.
        i. If necessary, stain another level of control.
    b. If problem is instrument related and cannot be resolved call BD Biosciences for further instructions.
    c. Document control failure on F-055.

d. IF the QC does not pass, do NOT proceed, both levels of QC must be in range to report out patient results.

l. CD3 Control Analysis:

i. Upload the file into the template.

ii. In plot 1, adjust the lymphocyte gate R5 around the lymphocyte population.

iii. Activate plot 1.

iv. Pull up the inspector (apple F).

v. Change gate from "no gate" to "viable lymphs = R5 and R8".

vi. In plot 9, adjust R10 around the CD3 population.

vii. Verify the results are within the Streck range.

viii. Document the results electronically on form F-098, and place into correct folder.

ix. If the control fails:

1. Rerun the sample again.

2. If problem is instrument related and cannot be resolved call BD Biosciences for further instructions.

3. QC must be in range to report patient results.

x. Acquisition and Storage Criteria For Patient Samples:

xi. Samples that complete the acquisition in less than 15 minutes must meet the following conditions to be reported:

1. At least 100 viable CD34 events. Note: Not required for peripheral CD3 analysis.

2. At least 75,000 viable CD45 events

3. At least 1,000 bead events.

xii. Browser Set Up:

1. Data Storage: Under Directory, select BD FacStation HD→CTL→CTL Patient (current quarter/year).

a. For new patients, select "New Folder", type in the patient name (Last, First), select create.

b. For each collection select "New Folder" and label with the collection's unique alpha-numeric identifier. (For peripheral blood samples without unique identifiers, use date of collection.) Select "Create" and "Choose".

i. If acquiring more than one sample on any collection (unique identifier) designate the folders accordingly (ex: W2276 16 005000 Post Filter, W2276 16 005000 Post Heta).

c. Assign the following sample and patient information for consistency.

i. Sample ID:

1. DIN

2. Date of collection

ii. Patient ID:

1. Patient name: Last, First

2. Product Type

xiii. Verify the Trucount bead number in the template. Change as needed.

1. See step F.iii.1-3, for changing bead number.

xiv. Enter the dilution Factor.

1. To enter the dilution factor activate the box. From the tool bar, click on Stats. From the drop down menu, select edit expression.

2. After entering the dilution factor used to set up the flow sample, click OK.

xv. Starting from the instrument settings used for the controls, place the prepared patient sample on the SIP and press RUN and HI on the cytometer.

xvi. In the Acquisition Control window, select the Setup checkbox and then click Acquire to begin instrument optimization.

xvii. Following Steps E.iii-xviii, optimize and adjust compensations for the patient sample.

1. Pay special attention to plot 6 as it may need adjusted when going from control samples to patient samples or between different types of patient samples (ex. Bone marrow, cord, peripheral blood).

xviii. While in setup, observe the CD34 population in Plot 2 and Plot 3.

1. If the population is large, set the Acquisition parameters to collect 75,000 viable CD45 events.
2. If the population is small, set the Acquisition parameters to collect 150 viable CD34 events.
3. CD3 is not a rare event. The appropriate number of CD3 events will be acquired if I.xviii.1-2 is met.

xix. Once completed, deselect the setup checkbox and begin acquisition.

J. Analyzing Patient Samples:

i. The BD Stem Cell Enumeration Kit is an acquisition and analysis template. Patient samples can be analyzed immediately after acquiring before moving on to the next sample to acquire.

ii. Upload the patient data file into the template.

iii. Check each plot and verify that the gates enclose the appropriate populations, adjust as needed.

1. Plot 1: R5 should be around the lymphocyte population.
2. Plot 2: R2 should be around the CD34 population.
3. Plot 3: R3 should be around the $CD45^{dim}$ population, (R1 can be adjusted to the left to reveal the entire population if needed.)
4. Plot 8: R8 move the right border of R8 against the lymphocytes, monocytes, and granulocytes.
5. Plot 9: R10 should be around the CD3 population, if the population trails upward, should only be around the majority of the population.

iv. Prior to printing results, check to make sure:

1. There is a minimum of 75,000 viable CD45 events.
2. There is a minimum 100 viable CD34 events.
3. The Trucount bead number is correct.
4. The dilution factor is correct.

v. See H.iii.6.a-g for printing instructions.

K. Calculating Product/Peripheral Blood Results:

i. Calculate the fresh product $CD34^+$ content:

1. Use the Viable CD34 of Viable CD45% box on the printout.
2. $$\frac{[\text{Product total WBC} \times (\text{viable CD34+ relative \%} \div 100)]}{\text{patient weight in kg}}$$
3. Recipient weight must be used in allogeneic product calculations.
   a. Example:

   Total WBC = 1878.98E+08

   Recipient Wt = 76.6 kg

   $CD34^+$% = 0.59

b. $\frac{1878.98E+08 \times 0.59}{100} = 1.11E+09$

c. $\frac{1.11E+09}{76.6} = 14.5E+06\ CD34^{+}/kg$

4. Record the viable CD34 results on an Estimated Viable Results label (Attachment 7) and place on the analysis.
    a. Complete the label by recording the recipient weight, Total WBC, Viable $CD34^{+}$ events, and calculate the $CD34^{+}/kg$.
5. Record the viable CD34 of viable CD45 % on the F-030a/b Interpretive Flow Cytometric Report.

ii. Calculate the fresh product CD3 content:

1. Use the % Gated CD3 (this is Viable CD3 of Viable CD45).
    a. $\frac{[\text{Product total WBC} \times (\text{viable CD3+ relative \%} \div 100)]}{\text{patient weight in kg}}$
    b. Recipient weight must be used in allogeneic product calculations.
        i. Example:
        ii. Total WBC = 1878.98 E+08
        iii. Recipient Wt = 76.6 kg
        iv. $CD3^{+}\%$ = 20.20
        v. $\frac{1878.98\ E+08 \times 20.20}{100} = 3.80\ E+10$
        vi. $\frac{3.80\ E+10}{76.6} = 5.0\ E+08\ CD3^{+}/kg$
2. Record the viable CD3 results on an estimated viable results label (Attachment 7) and place on the analysis.
    a. Complete the label by recording the recipient weight, Total WBC, Viable $CD3^{+}$ events, and calculate the $CD3^{+}/kg$.
3. Record the viable CD3 of viable CD45 % on the F-030a.

iii. Calculating previously frozen product $CD34^{+}$ and $CD3^{+}$ content:

1. Flow cytometry will not be performed on post thaw samples for HPC, Apheresis, MNC, Apheresis, or HPC, Marrow.
    a. Post thaw CD34 calculations will be based on the percentage of CD34 prior to freezing and the viable TNC after thaw via trypan blue according to SOP RT-003 Procedure Trypan Blue Cell Viability .
        i. Example:
        ii. Post Thaw Viable TNC = 1572.35E+08
        iii. Recipient Wt = 76.6 kg
        iv. $CD34^{+}\%$ = 0.59 prior to freezing
        v. $1572.35E+08 \times 0.59 = 9.28E+08$

100

vi. $\frac{9.28E+08}{76.6}$ = 12.1E+06 $CD34^+$/kg

2. Due to the processing of Cord Blood Units at other facilities prior to freezing, flow cytometry will be performed on HPC, Cord products.
    a. For the HPC, Cord, the **UNCORRECTED** Total WBC and post thaw CD34% will be used for the calculations as described in steps K.i-ii.
3. The calculations are the same as above for CD3 from thawed products.

iv. Peripheral Blood $CD34^+$ Calculation:

1. Use the Viable CD34 of Viable CD45%
    a. Calculate the number of $CD34^+$ events per µL:
        i. $\frac{\text{WBC E+03/ µL x viable } CD34^+ \text{ relative \%}}{100}$
        ii. Example:
        iii. WBC = 8.9 E+03 / µL
        iv. CD34+% = 0.06
        v. $\frac{8.9 \text{ E+03/ µL x } 0.06}{100}$ = 5 cells per µL

v. Peripheral Blood $CD3^+$ Calculation:

1. Use the Viable CD3 of Viable CD45%
2. Calculate the number of $CD3^+$ events per µL:
    a. $\frac{\text{WBC E+03/ µL x viable } CD3^+ \text{ relative \%}}{100}$
    b. Example:
    c. WBC = 8.9 E+03 / µL
    d. CD3+% = 12.07
    e. $\frac{8.9 \text{ E+03/ µL x } 12.07}{100}$= 1074 cells per µL

vi. Product Mid Sample $CD34^+$ Calculation:

1. Calculate the product mid sample $CD34^+$ content:
2. Use the product volume provided by Apheresis
    a. Divide by 1.027 to correct for specific gravity.
3. Use the Viable CD34 of Viable CD45% box on the printout.
4. $\frac{\text{[Product total WBC x (viable CD34+ relative \% ÷ 100)]}}{\text{patient weight in kg}}$
5. Recipient weight must be used in allogeneic product calculations.
    a. Example:

    Volume documented by Apheresis = 259 mL

    Corrected volume = 259 mL/1.027 = 252 mL

    WBC = 393.00e+06

Total WBC = 990.36E+08

Recipient Wt = 76.6 kg

$CD34^+$% = 0.59

b. $\frac{990.36E+08 \times 0.59}{100} = 5.84E+08$

c. $\frac{5.84E+08}{76.6} = 7.6E+06\ CD34^+/kg$

vii. Product Mid Sample $CD3^+$ Calculation:

1. Calculate the product mid sample $CD3^+$ content:
2. Use the product volume provided by Apheresis
   a. Divide by 1.027 to correct for specific gravity.
3. Use the % Gated CD3 (this is Viable CD3 of Viable CD45).
4. $\frac{\text{[Product total WBC x (viable CD3+ relative \% ÷ 100)]}}{\text{patient weight in kg}}$
5. Recipient weight must be used in allogeneic product calculations.
   a. Example:

   Volume documented by Apheresis = 259 mL

   Corrected volume = 259 mL/1.027 = 252 mL

   WBC = 393.00e+06

   Total WBC = 990.36E+08

   Recipient Wt = 76.6 kg

   $CD3^+$% = 14.52

   b. $\frac{990.36E+08 \times 14.52\%}{100} = 1.44E+10$

   c. If requested calculate the CD3/kg $\frac{1.44e+10}{76.6} = 1.9E+08\ CD3^+/kg$

viii. All results should be verified by a second technologist before reporting.

1. Second technologist needs to not only review the results of the calculations, but also the
   a. Trucount bead number and the dilution factor.
   b. They should further check that at least 100 viable CD34 events and at least 75,000 viable CD45 events were collected.
   c. In the event a second technologist is not available (ex: after hours incoming NMDP) the technologist responsible for the product will double check all calculations using the 2nd tech review label prior to reporting the estimated values to the Medical Director or on call physician.
      i. The results will be verified by a second technologist the first thing the next business day.

L. Computer Back-up

i. Back-up will be performed weekly at a minimum and documented on the F-055 FacsCalibur Log.

ii. Obtain the MacDisk 1 or MacDisk 2 from the QA department.

1. Disks will be held in a secure area not in the vicinity of the analyzer when not in use.
2. Disks are in a waterproof fireproof safe.
3. The disks are encrypted and password protected.

iii. Plug the disk into the Mac Mini

1. A pop-up will appear stating that a disk drive has been inserted that is not readable, press ignore.
    a. Do Not press initialize or the data on the disk could be erased.

iv. There is a very small bullseye at the top right of the screen by the wifi symbol.

1. This will open a program called M3 Bitlocker Loader
2. When you double click it will bring up an icon of the Mac diskinserted.
3. Click on the icon
    a. Enter password when prompted.
4. This will bring up a window of the MacDisk that is inserted.

v. Open another window by double clicking the BD FacsStation HD icon.

1. Go to the CTL folder
2. Highlight the folder called Control Reports
3. Click on the icon that looks like a gear at the top of the open window
4. Choose the option to Duplicate
5. A copy of the folder will be made
6. Drag the Control Reports Copy folder to the MacDisk window.

vi. Repeat steps L.v.1-6 for the CTL Patient Reports folder.

vii. Close all windows except the M3 Bitlocker Loader screen.

1. Click on the icon triangle with the line below it that is labeled as eject.
2. This allows the disk to be safely removed from the computer.

viii. Return the portable disk to the QA department.

M. Limitations

i. For accuracy in determining absolute counts, it is critical that pipetting is done with precision following the SOP.

ii. Using an incorrect bead count from the BD Trucount tubes pouch label will result in inaccurate absolute counts.

iii. Erroneous results will occur if the tubes are not placed immediately on wet ice in the dark until acquired or if samples are not run on a flow cytometer within 1 hour of lysing.

iv. Insufficient mixing of samples will result in inaccurate results.

v. Do not use the reagent if you observe any change in appearance. Precipitation or discoloration indicates instability or deterioration.

vi. The antibody reagent contains sodium azide as a preservative; however care should be taken to avoid microbial contamination, which can cause erroneous results.

vii. BD SCR, 7-AAD, and 10X ammonium chloride all must be stored at 2-6°C. Do NOT use past the expiration date shown on the label.

viii. BD Trucount tubes can be stored at room temperature.

1. Examine the desiccant each time you open the pouch.
    a. If the desiccant has turned from blue to lavender, discard the remaining tubes.
2. An unopened pouch is stable until the expiration date shown on the packaging.

3. Do NOT use tubes past the expiration date shown on the package.
4. Use tubes within 30 days after opening the pouch.

IX.REPORTING RESULTS

A. All results should be verified by a second technologist before reporting.
   i. Second technologist needs to not only review the results of the calculations, but also the
   ii. Trucount bead number and the dilution factor.
      1. They should further check that at least 100 viable CD34 events and at least 75,000 viable CD45 events were collected.
   iii. In the event a second technologist is not available (ex: after hours incoming NMDP) the technologist responsible for the product will double check all calculations using the 2nd tech review label prior to reporting the estimated values to the Medical Director or on call physician.
      1. The results will be verified by a second technologist the first thing the next business day.

B. Pre $CD34^+$ Cerner Order Entry & Resulting:
   i. Pre $CD34^+$ Order Entry:
      1. Log into Cerner.
      2. Click on Department Order Entry icon (earth with an arrow).
      3. In the "Medical Record Number" field, enter the MRN of the patient and press enter.
      4. Select the appropriate encounter for the patient.
      5. In the "Orderable" field, enter "Pre CD34". Select the "Pre $CD34^+$ Panel" by pressing enter.
      6. Enter the following for the order:
         a. Enter the ordering physician.
         b. Select the appropriate label printer.
         c. Select the "Specimen receive location" as "CT Lab Receiving".
         d. For "Order Date/Time", leave the current date & time (default).
         e. For "Requested Date/Time", leave the current date and change the time to at least one minute following the current time.
         f. For "Received Date/Time", leave the current date and change the time to at least one minute following the "Requested Date/Time".
         g. Submit the order.
         h. Note the accession number (for result entry).

C. Pre $CD34^+$ Result Entry and Verification:
   i. Log into Cerner.
   ii. Click on the Accession Result Entry icon (paper and pencil).
   iii. Enter the accession # (press enter for current Julian date to automatically be entered; then enter remaining 5 digits).
   iv. Click Retrieve Button.
   v. Enter the sample collection date.
      1. This is a free-text field. Use the following date format: MM/DD/YY.
   vi. Enter the Pre $CD34^+$ result (# cells per µL).
   vii. Click Perform.
   viii. A second technologist will verify the results are correct:
      1. Follow steps IX.C.i-iv.

a. Click Verify.

D. To View Results:

i. Log into Cerner.

ii. Click on the Order Result Viewer icon (person with a magnifying glass).

iii. Select the radio button for "Accession".

iv. Enter the accession # (press enter for current Julian date to be entered automatically; then enter remaining 5 digits) and click OK.

v. Double click on the order to view.

E. Pre $CD3^+$ Cerner Order Entry & Resulting:

i. Pre $CD3^+$ Order Entry:

1. Log into Cerner
2. Click on Department Order Entry icon (earth with an arrow).
3. In the "Medical Record Number" field, enter the MRN of the patient and press enter.
4. Select the appropriate encounter for the patient.
5. In the "Orderable" field, enter "Pre CD3". Select the "Pre $CD3^+$ Panel" by pressing enter.
6. Enter the following for the order:
    a. Enter the ordering physician.
    b. Select the appropriate label printer.
    c. Select the "Specimen receive location" as "CT Lab Receiving".
    d. For "Order Date/Time", leave the current date & time (default).
    e. For "Requested Date/Time", leave the current date and change the time to at one minute following the current time.
    f. For "Received Date/Time", leave the current date and change the time to at least one minute following the "Requested Date/Time".
    g. Submit the order.
    h. Note the accession number (for result entry).

F. Pre $CD3^+$ Result Entry and Verification:

i. Log into Cerner.

ii. Click on the Accession Result Entry icon (paper and pencil).

iii. Enter the accession # (press enter for current Julian date to automatically be entered; then enter remaining 5 digits).

iv. Click Retrieve Button.

v. Enter the sample collection date.

1. This is a free-text field. Use the following date format: MM/DD/YY.

vi. Enter the Pre $CD3^+$ result (# cells per µL).

vii. Click Perform.

viii. A second technologist will verify the results are correct:

ix. Follow steps O.2.a-d.

x. Click Verify.

G. Product Mid Sample CD34/CD3 result Entry and Verification:

i. Mid Product samples will be ordered by the Apheresis provider assigned to the donor.

ii. Log into Cerner.

iii. Click on the Accession Result Entry icon (paper and pencil).

iv. Enter the accession # (press enter for current Julian date to automatically be entered; then enter remaining 5 digits).

v. Click Retrieve Button.

vi. Double-Click on the Result field.

1. A text box will pop up.

a. Enter the following as applicable

a. DIN#

b. Date Collected (use MM/DD/YYYY format)

c. Time Collected

d. Product volume at time of sampling

e. Blood Volumes Processed (mLs)

f. Product WBC/mL

g. Mid Sample TNC

h. Mid Sample CD34+%

i. Mid Sample CD34+ Total

j. Mid Sample CD34+/kg

k. Mid Sample CD3+%

l. Mid Sample CD3+ Total

m. Mid Sample CD3+/kg

vii. Click Perform.

viii. A second technologist will verify the results are correct:

1. Follow steps IX.G.i-v.

a. Verify that all information is correct.

b. Click Verify.

H. To View Results:

i. Log into Cerner.

ii. Click on the Order Result Viewer icon (person with a magnifying glass).

iii. Select the radio button for "Accession".

iv. Enter the accession # (press enter for current Julian date to be entered automatically; then enter remaining 5 digits) and click OK.

v. Double click on the order to view.

I. To View Results:

i. Log into Cerner.

ii. Click on the Order Result Viewer icon (person with a magnifying glass).

iii. Select the radio button for "Accession".

iv. Enter the accession # (press enter for current Julian date to be entered automatically; then enter remaining 5 digits) and click OK.

v. Double click on the order to view.

X. ACTION TO BE TAKEN IF METHOD BECOMES INOPERABLE/

- Follow CTLQA 015 Procedure Equipment Management to initiate a service call.
- The BD FACSVia flow cytometer is used as a back-up analyzer for cellular therapy product analysis only.
  - Refer to SOP RT 014 Flow Cytometric Acquisition and Analysis Using the BD FACSVia .

XI. CLINICAL SIGNIFICANCE

Transplantation of hematopoietic progenitor cells is used increasingly in the treatment of blood disorders, malignancies, and genetic abnormalities. Progenitor cells are rare and are found primarily in the bone marrow with extremely low frequencies in peripheral blood, however with the arrival of mobilization regimens, peripheral blood has become the preferred source of stem cells. The CD34 antigen is present on immature hematopoietic precursor cells (progenitor cells). An accurate measure of the CD34 cell count is necessary for dose requirement protocols in stem cell transplantation. Flow cytometric applications for $CD34^+$ cell identification and enumeration provide a rapid, quantitative and reproducible method to evaluate the progenitor cell population.

The single tube assay is performed by staining the sample with the reagent in individual BD Trucount tubes for absolute counts. When a sample is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to the cell surface. Additionally, the lyophilized pellet in the BD Trucount tube dissolves, releasing a known number of fluorescent beads.

Non-viable cells stain positive with the nucleic acid dye 7-AAD due to their compromised cell membrane. Ammonium chloride is added to lyse erythrocytes before the sample is acquired on the FACSCalibur.

During analysis of the sample, the concentration of viable $CD34^+$ cells and viable $CD45^+$ cells, and the percentage of viable $CD34^+$ cells in the viable $CD45^+$ cell population, are calculated.

The CD45 antigen is present on all human leukocytes and is weakly expressed on hematopoietic progenitor cells. The CD34 antigen is present on immature hematopoietic precursor cells and all hematopoietic colony- forming cells in bone marrow and blood, including unipotent and pluripotent progenitor cells.

BD Trucount tubes are used with this procedure. By adding the reagent and the sample directly to the BD Trucount tube, and using a Lyse/No-Wash procedure, the absolute count of the cell population of interest can be directly determined.

The CD3 antigen is part of the T cell receptor complex on mature T-lymphocytes. During analysis of allogeneic samples the percentage of viable $CD3^+$ in the viable $CD45^+$ cell population is calculated.

For abbreviations and proper names of products refer to SOP PST 008 Procedure: Product and Reagent Labeling .

Refer to SOP QC 016 Procedure FACSCalibur Calibration, QC and Maintenance for control verification, reagent verification and maintenance of the FACSCalibur flow cytometer.

Class II or Class III monoclonal antibodies must be used for the detection of CD34. BD Stem Cell Enumeration kit used with this procedure has Class III antibodies.

XII. REFERENCES

AABB Technical Manual, current edition.

AABB Standards for Cellular Therapy Product Services, current edition. BD Biosciences Stem Cell Enumeration Application Guide

BD Biosciences package inserts:

BD Stem Cell Control

BD Stem Cell Enumeration Kit

CAP Flow Cytometry Standards, current edition.

FACT, Standards for Cellular Therapy Product Collection, Processing, and Administration, current edition.

XIII. ATTACHMENTS/ADDENDUMS

Attachment 1: 1X Ammonium Chloride Lysing Solution Preparation Label

Attachment 2: BD Stem Cell Control Label

Attachment 3: BD Stem Cell Enumeration Kit Panel

Attachment 4: Mac keyboard short cuts

Attachment 5: Gate Definitions

Attachment 6: Plot Definitions (Template) Attachment 7: Estimated Viable Results Label

F-030a Interpretive Flow Cytometric Report Product Analysis

F-030b Interpretive Flow Cytometric Report- Peripheral Blood CD34+

F-030c Interpretive Flow Cytometric Report- Peripheral Blood CD3+

F-055 FACSCalibur Log

F-098 Streck CD Chex Plus Flow Cytometry Control

F-154 BD Stem Cell Control Kit Data Sheet

F-228 CTL Occurrence Report

XIV. DEFINITIONS

7-AAD: 7-Amino-actinomycin D

AABB: Association for the Advancement of Blood & Biotherapies

ACD-A: Acid Citrate Dextrose solution A

APC: Allophycocyanin

BD: Becton Dickinson

CAP: College of American Pathologists

CD: Cluster of Differentiation

CD##: The fluorescent antibody coupled to the specified CD cell antigen

CTL: Cellular Therapy Laboratory

DIN: Donation Identification Number

DPLM: Department of Pathology and Laboratory Medicine

FACT: Foundation for the Accreditation of Cellular Therapy

FCS: Flow Cytometry Standard

FITC: Fluorescein isothiocyanate

FL 1, 2, 3, 4: Fluorescence parameters 1, 2, 3, 4

FSC: Forward Scatter

BD FacStation HD: Macintosh hard drive

HPC: Hematopoietic Progenitor Cells

ISHAGE: The International Society of Hematotherapy and Graft Engineering

IUHPL: Indiana University Health Pathology Lab

MAb: Monoclonal Antibody

MNC: Mononuclear Cells

NMDP: National Marrow Donor Program

OSX: Operating System X (Cell Quest Pro Software)

PBS: Phosphate Buffered Saline

PE:	Phycoerythrin
PPE:	Personal Protective Equipment
PMT:	Photo Multiplier Tube
QC:	Quality Control
RT:	Room Temperature
SCR:	Stem Cell Reagent
SIP:	Sample Injection Port
SOP:	Standard Operating Procedure
SSC:	Side Scatter

PROCEDURE #:

RT 012