STEM CELL LABORATORY (STCL)

DOCUMENT NUMBER: STCL-SOP-059

DOCUMENT TITLE:
STEMvision" Automated Colony Counting and Enumeration of Hematopoietic Progenitor Cells in Thawed Umbilical Cord Blood

DOCUMENT NOTES:
Document required for the BLA.

Document Information

Revision: 01	**Vault:** STCL-Processing-rel
Status: Release	**Document Type:** STCL

Date Information

Creation Date: 27 Apr 2018	**Release Date:** 09 Apr 2021
Effective Date: 09 Apr 2021	**Expiration Date:**

Control Information

Author: KS137	**Owner:** WATER002
Previous Number: None	**Change Number:** STCL-CCR-417

STCL-SOP-059
STEMVISION™ AUTOMATED COLONY COUNTING AND ENUMERATION OF HEMATOPOIETIC PROGENITOR CELLS IN THAWED UMBILICAL CORD BLOOD

1 PURPOSE

1.1 The purpose of this procedure is to outline the steps associated with thawing DMSO nunc vials in preparation for performing the colony-forming unit assay and subsequent colony counting using the STEMVision™ automated instrument.

2 INTRODUCTION

2.1 STEMVision™ is an automated instrument and computer system designed specifically for imaging, classifying and counting hematopoietic colonies produced by human progenitor cells in the colony-forming unit (CFU) assay. It separately counts colonies derived from erythroid burst-forming units (BFU-E), myeloid progenitor cells (colony-forming units granulocyte, macrophage (CFU-GM)), or multipotent progenitor cells (colony-forming units granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM), that develop in conventional 14 day CFU assays of umbilical cord blood (UCB) cells.

2.2 In order for the STEMVision™ software to accurately count and identify colonies, red blood cell (RBC) background in the sample must be minimized. A hematocrit of less than 1% in the dishes to be counted is recommended. For this assay, RBCs will be removed using ErythroClear™. A single cell suspension is then plated in triplicate at a cell density, 5.0 x 10^4 cells per well, in an enriched, semi-solid media containing methylcellulose using a six well SmartDish™. The plate is then incubated in a 37°C with 5% CO2 incubator for 14-16 days. The semi-solid media minimizes the movement of cells in the culture, ensuring that the daughter cells derived from a single progenitor stay in close proximity to each other. At the end of the incubation period, the plate will be acquired and analyzed using the STEMVision™ automated colony counter.

3 SCOPE AND RESPONSIBILITIES

3.1 The designated, trained laboratory staff are responsible for ensuring that the requirements of this procedure are successfully met.

4 DEFINITIONS/ACRONYMS

4.1	CFU	Colony Forming Unit
4.2	HPCA	Hematopoietic Progenitor Cell Assay
4.3	BSC	Biosafety Cabinet
4.4	UCB	umbilical cord blood
4.5	CBU	cord blood unit

4.6	CCBB	Carolinas Cord Blood Bank
4.7	ISBT	International Society of Blood Transfusion
4.8	RBC	Red Blood Cell
4.9	WBC	White Blood Cell
4.10	BFU-E	Burst forming unit erythroid- primitive erythroid progenitors that give rise to large multi-clustered colonies due to their proliferative capacity
4.11	CFU-GM	Colony-forming unit-granulocyte, macrophage
4.12	CFU-GEMM	Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte
4.13	IMDM	Iscove's Modified Dulbecco's Medium with 2% FBS

5 MATERIALS

5.1 Umbilical cord blood vials and their associated ISBT128 barcode ID labels.

5.2 ALDEFLUOR® assay buffer (*StemCell Technologies #01702*) *or equivalent*

5.3 ErythroClear Red Blood Cell Depletion Reagent Kit (*StemCell Technologies #01738*) *or equivalent*

5.4 Alcohol prep pad, 1" X 1", sterile

5.5 Serological pipettes 2 ml, 5 ml, 10 ml (*and 2 ml aspirating pipettes*), sterile

5.6 Gauze squares 4" x 4", sterile

5.7 Methocult (*Stem Cell Technologies, #H4434*) or equivalent

5.8 Antibiotic/Antimycotic reagent (*e.g. Invitrogen #15240-096*)

5.9 35 mm SmartDish™ *or equivalent*

5.10 5 ml round bottom tubes *or equivalent*

5.11 9 X 9 Tissue Culture Plate *or equivalent*

5.12 35 mm Tissue Culture Dish *or equivalent*

5.13 3 ml Sterile Syringe *or equivalent*

5.14 16 Gauge Blunt End Needle *or equivalent*

5.15 5 ml Sterile Polystyrene Round Bottom Tubes *or equivalent*

5.16 15 ml Conical Tubes *or equivalent*

6 EQUIPMENT

6.1 Biosafety Cabinet, Class II (BSC)

6.2 Various size single channel pipettes to deliver 1-1000 microliter (□l) volumes, appropriate sterile tips for each size pipette (e.g. Rainin)

6.3 37°C water bath

6.4 37°C, humidified, 5% CO2 Incubator

6.5 Vacuum pump source with aspiration waste bottle and tubing

6.6 Vortex

6.7 Ice bucket

6.8 4°C Refrigerator (2-8°C)

6.9 -20°C Freezer (-10 to -30°C)

6.10 Inverted Phase Contrast microscope

6.11 Tally counter (*for counting colonies*)

6.12 Sysmex hematology analyzer (cell counter) or equivalent (for WBC count)

6.13 STEMVision™

7 SAFETY

7.1 The cells used in this method are from humans, therefore all specimens should be handled as though they are potentially infectious (i.e. “universal precautions" should be taken). The minimum personal protective equipment required is gloves and lab coat.

8 PROCEDURE

8.1 Paperwork Preparation

8.1.1 Print 4 copies of ISBT128 barcode ID labels per CBU by scanning the barcodes using an on-demand printer.

8.1.2 Place the appropriate barcode sticker for each cord blood segment to be tested on 6-Well SmartDish HPCA Worksheet FRM1.

8.1.3 Record all lot numbers and expiration dates of reagents in use for the day on 6-well SmartDish HPCA Worksheet FRM1. This should be done as reagents are used.

8.2 Reagent Preparation

8.2.1 Prior to the day of the assay, one bottle of Methocult media should be removed from freezer and thawed in the refrigerator overnight. The next day, 1 ml of antibiotics should be added to the bottle. The bottle should be shaken and stored in the refrigerator. The media is ready for use once the bubbles have floated to the top.

8.2.2 Nunc vials should be picked up from the designated location. Nunc vials should be stored on dry ice until use.

8.2.3 Remove ALDEFLUOR buffer and media from the refrigerator to allow them to come to room temperature.

NOTE: All media should be allowed to come to room temperature before use but should not sit for more than 1 hour.

8.2.4 Remove a tube of ErythroClear from the refrigerator and place on ice.

8.2.5 Inside the BSC, prepare a 15 ml tube for each segment to be tested and label them with an ISBT128 number. Label two microfuge tubes for each segment with an ISBT128 number. Label one 5 ml tube for each segment.

8.2.6 Add 100 µl of ALDEFLUOR buffer to each 15 ml tube.

8.2.7 Label another 5 ml tube with daily test number. This tube will be used for the 1:10 dilution of the blood in Cellpack solution for counting on the Sysmex.

8.3 Removal of Blood from Nuncs Vials

Reminders:

- Nunc vials should stay on dry ice until immediately ready for thawing.
- Handle only one nunc vial at a time in the BSC.
- One nunc vial should be removed from dry ice, thawed, cleaned, and have the blood removed and placed in Dextran/Albumin before beginning to thaw the next vial.
- Transport segments on dry ice in transport box to laboratory where assay is to be performed
- Each nunc vial should be handled separately.
- Once the blood from one nunc vial has reached the step of the ten minute incubation, the next nunc vial can be started.

8.3.1 Remove a nunc vial from the dry ice. Verify match of the ID on the vial with the ISBT128 labels on the paperwork and 15 ml tube.

8.3.2 Thaw the nunc vial in a 37◦C water bath for 1 minute.

8.3.3 Remove from water bath.

8.3.4 Wipe the vial off with alcohol prep pad.

8.3.5 Open the vial in the BSC.

8.3.6 Verify with a pipet that the sample is slushy. If it is, proceed to next step. If the vial is not thawed, close the vial and return to water bath for 30 seconds. Continue with previous steps until the tube is thawed.

8.3.7 Remove 100 µl of blood with a pipette.

8.3.8 Add to corresponding 15 ml tube and mix be gently shaking.

8.3.9 Incubate the tube for 10 minutes at room temperature.

8.3.10 Add additional 200 µl of buffer to the 15 ml conical tube and shake the tube gently side-to-side at the bottom to mix the contents.

8.3.11 Incubate at room temperature for 3 minutes.

8.3.12 Add additional 400 µl of buffer to the 15 ml conical tube and shake the tube gently side-to-side at the bottom to mix the contents.

8.3.13 Incubate at room temperature for 3 minutes.

8.3.14 Add additional 200 µl of buffer to the 15 ml conical tube and shake the tube gently side-to-side at the bottom to mix the contents.

8.3.15 Add 350 µl of diluted blood to the corresponding microfuge tube.

8.3.16 Add 175 µl of ErythroClear reagent to the microfuge tube.

8.3.17 Mix with the pipet.

8.3.18 Let the microfuge tube sit for one minute at room temperature.

8.3.19 Place the microfuge tube in the ErythroClear magnet.

8.3.20 Keep the tube in the magnetic field for one minute.

8.3.21 With a 1 mL pipette, carefully pipet off 500 µl of cell suspension. Avoid disturbing the RBC pellet on the side of the tube. Pipette the sample into the second microfuge tube.

8.4 Sysmex enumeration of WBCs

8.4.1 For each required HPCA, pipette 20 µl blood to 180 µl Cellpack in labeled tubes.

8.4.2 Perform Sysmex cell counts. Print out paper copy of count results for each segment and attach copies to paperwork.

8.5 STEMvision™ plating.

8.5.1 Methocult media has been formulated to allow the addition of cells to methocult at a 1:10 ratio.

8.5.2 To determine the new volume of diluted cells required to plate at a density of 5.0×10^4 per well, perform the following calculations

8.5.2.1 50,000 cells per well

8.5.2.2 1.1 ml of media per well

8.5.2.3 4.4 ml of media total

8.5.2.4 Therefore 250.000 total WBC cells will be needed. This amount must be in 500 µl of a 10X solution.

8.5.2.5 Calculation based off Sysmex count: 250000 divided by Sysmex count of WBC concentration (10^3/µl) divided by 10000.

Example: Sysmex count is WBC 0.59 [10^3/µl].

250,000/0.59/10,000-=42.37 µl.

8.5.2.6 Transfer the calculated amount of RBC depleted sample into a sterile round bottom tube.

8.5.2.7 Add IMDM to make the volume 500 µl.

Example: 42.37 µl of sample plus 457.63 µl of IMDM.

8.5.2.8 Mix 10X cell suspension by vortexing at high speed (~3000 rpms) for 1-3 seconds.

8.5.3 Set-up CFU Assay

8.5.3.1 Transfer 400 µl of 10X cell suspension into 4.0 ml aliquot of methocult media.

8.5.3.2 Vortex vigorously at high speed (~3000 rpm) for 10 seconds.

8.5.3.3 Allow bubbles to rise to the top by letting the sample sit for 5 minutes.

8.5.3.4 Attach an ISBT barcode label to the side of the SmartDish plate to correspond to the row into which the sample is being plated.

8.5.3.5 Use a sterile syringe attached to a 16-Guage blunt- end needle to dispense the Methocult mixture into the culture dish. Expel air from the syringe by drawing 1 ml of mixture into the syringe and fully expelling it.

8.5.3.6 Draw the Methocult mixture back up into the syringe and dispense a volume of 1.1 ml into each of the 3 wells of a 35 mm SmartDish as follows

8.5.3.6.1 Hold the syringe in one hand and remove the lid with the other hand. Position the syringe over the center of each well without touching the dish. Dispense 1.1 ml into each well and replace lid.

8.5.3.7 Distribute the media evenly across the surface of each well by gently tilting the dish to allow the media to attach to the wall of the wells on all sides.

8.5.3.8 Add 3.0 ml of sterile water to the center of the 6-well SmartDish in between the wells.

8.5.3.9 Place the SmartDish inside a larger 9 X 9 plate and add a 25 mm tissue culture dish filled with water. The SmartDishes should remain covered and the additional dish should be uncovered. Two SmartDishes will fit in the 9 X 9 plate.

8.5.3.10 Incubate at 37◦C in 5% CO2 for 14 days. Score using the STEMVision™ automated instrument and computer system.

8.6 Colony Scoring

8.6.1 If scoring using the STEMvision™ analyzer, please refer to the STEMvision™ Technical Manual for detailed set-up, acquisition and analysis instructions.

8.6.2 On day 14 after plating cells (following steps 8.5.7 or 8.6.3.10) count colonies as described in section 8.1.22 in STCL-SOP-052 Hematopoietic Progenitor Cell Assay (HPCA) Cord Blood Bank Products, and record the numbers of each colony type for each well on the 6-well SmartDishes in STCL-SOP-059 FRM1, 6-Well SmartDish HPCA Worksheet FRM1.

8.6.3 Count each well on inverted microscope.

8.6.4 For accuracy, compare barcodes from plate and worksheets.

8.6.5 Calculations

8.6.5.1 Calculate the average number of each colony type (BFU-E, CFU-GM and CFU-GEMM) in all four wells by calculating the average and record it on worksheet using the formula below, i.e. # BFU-E in well 1 + # BFU-E in well 2 + # BFU-E in well 3 ÷ 3 =AVERAGE BFU-E per well.

8.6.5.2 Calculate the total CFU by using the average number of all three colony types in all three wells by using formula below and record on worksheet. Average BFU-E + Average CFU-GM + Average CFU-GEMM = Total All CFU per well.

8.6.5.3 Calculate the average number of each colony type per 10^5 cells seeded using the average for all three wells for each colony type multiplying that number by 2 and recording the results on the worksheet. This calculation assumes the standard number of 5 x 10^4 cells/well was plated.

8.6.5.4 Average # BFU-E (per 2 x10^4 cells) x 2 = Average # BFU-E per 10^5 cells plate.

Based on: 1x10^5 ÷ 5 x 10^4 = 0

8.6.5.5 To calculate per 10^5 Total CFU sum per 10^5 for all three colony types using formula below.

Per 10^5 BFU-E + per 10^5 CFU-GM + per 10^5 CFU-GEMM = per 10^5 Total CFU

9 RELATED DOCUMENTS/FORMS

9.1 Hematopoietic Progenitor Cell Assay (HPCA) Cord Blood Bank Products: STCL-SOP-052.

9.2 6-Well SmartDish HPCA Worksheet: STCL-SOP-059 FRM1

10 REFERENCES

10.1 Instrument manuals and SOP for use of Sysmex Hematology Analyzer.

10.2 STEMVision™ Technical Manual

10.3 Methocult® Technical Manual

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
01	K. Shoulars	New Document.

Signature Manifest

Document Number: STCL-SOP-059 **Revision:** 01

Title: STEMvision™ Automated Colony Counting and Enumeration of Hematopoietic Progenitor Cells in Thawed Umbilical Cord Blood

Effective Date: 09 Apr 2021

All dates and times are in Eastern Time.

STCL-SOP-059 STEMvision™ Automated Colony Counting, Enumeration of HP Cells in Thawed Umbilical CB

Author

Name/Signature	Title	Date	Meaning/Reason
Kevin Shoulars (KS137)		29 Jan 2019, 10:43:06 AM	Approved

Manager

Name/Signature	Title	Date	Meaning/Reason
Barbara Waters-Pick (WATE02)		01 Feb 2019, 09:36:34 AM	Approved

Medical Director

Name/Signature	Title	Date	Meaning/Reason
Joanne Kurtzberg (KURTZ001)		01 Feb 2019, 10:40:13 AM	Approved

Quality

Name/Signature	Title	Date	Meaning/Reason
Richard Bryant (RB232)		01 Feb 2019, 11:50:54 AM	Approved

Document Release

Name/Signature	Title	Date	Meaning/Reason
Sandra Mulligan (MULLI026)		26 Mar 2021, 08:44:10 PM	Approved