STEM CELL LABORATORY (STCL)

DOCUMENT NUMBER: STCL-SOP-056

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

DOCUMENT NOTES:
Document required for the BLA.

Document Information

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STCL-SOP-056
STEMvision™ AUTOMATED COLONY COUNTING AND ENUMERATION OF HEMATOPOIETIC PROGENITOR CELLS IN FRESH UMBILICAL CORD BLOOD

1 PURPOSE

1.1 One surrogate method of the hematopoietic cell number, engraftment potential and overall survival after transplantation of a sample of cord blood is its content of hematopoietic colony forming cells. This assay is dependent on the ability of a single cell to divide and differentiate, forming clusters of cells (colonies), in a semi-solid media containing appropriate growth factors and formulated for the optimal growth and quantification of erythroid progenitors, granulocyte/macrophage progenitors, and multi-potential granulocyte, erythroid, macrophage and megakaryocyte progenitors.

2 INTRODUCTION

2.1 STEMvision™ is an automated instrument and computer system that is designed specifically for imaging, classifying (or "scoring") 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 dish 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 HetaSep™. A single cell suspension is then plated in triplicate at a low cell density, 1.25 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.

2.3 In the event that a fresh cord blood specimen shows contamination or no growth, refer to *STCL-SOP-059 STEMVision Automated Colony Counting and Enumeration of Hematopoietic Progenitor Cells in Thawed Umbilical Cord Blood* so repeat testing can be completed, if appropriate.

3 SCOPE AND RESPONSIBILITIES

The Medical Director, STCL Manager and designated 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	BFU-E	Burst Forming Unit Erythroid
4.3	CFU-GM	Colony Forming Unit granulocyte-macrophage
4.4	UCB	Umbilical Cord Blood
4.5	RBC	Red Blood Cell
4.6	˚C	Degrees Celsius
4.7	CO2	Carbon Dioxide
4.8	ISBT	International Society of Blood Transfusion
4.9	STCL	Stem Cell Laboratory
4.10	PBS	Phosphate Buffered Saline
4.11	IMDM	Iscove's Modified Dulbecco's Medium with 2% FBS
4.12	mL	milliliter
4.13	mm	millimeter
4.14	CCBB	Carolinas Cord Blood Bank
4.15	ID	Identification
4.16	HPCA	Hematopoietic Progenitor Cell Assay
4.17	µL	microliter
4.18	BSC	Biological Safety Cabinet
4.19	RPM	Revolutions per Minute
4.20	WBC	White Blood Count

5 MATERIALS

5.1 Reagents

- 5.1.1 Dulbecco's Phosphate Buffered Saline (PBS)
- 5.1.2 HetaSep™
- 5.1.3 Methocult®
- 5.1.4 Iscove's Modified Dulbecco's Medium with 2% FBS (IMDM)
- 5.1.5 Sterile Deionized Water

5.2 Supplies

- 5.2.1 15 mL and 50 mL Conical Tubes
- 5.2.2 Sterile Micro Centrifuge Tubes
- 5.2.3 Sterile Pipette Tips
- 5.2.4 Timer

5.2.5 Calculator

5.2.6 5 mL Sterile Polystyrene Round Bottom Tubes

5.2.7 14 mL Sterile Polystyrene Round Bottom Tubes

5.2.8 3 mL Sterile Syringe

5.2.9 16 Gauge Blunt End Needle

5.2.10 35mm SmartDish™

5.2.11 9 x 9 Tissue Culture Plate

5.2.12 35mm Tissue Culture Dish

5.2.13 Gloves

5.2.14 Permanent Marker

6 EQUIPMENT

6.1 2 - 8˚C Refrigerator

6.2 Biological Safety Cabinet

6.3 Pipettes

6.4 Vortex

6.5 37˚C CO2 Incubator

6.6 STEMvision™

7 SAFETY

7.1 Wear all appropriate personal protective equipment when handling any/all potentially hazardous blood and body fluids to include, but not limited to, lab coat, gloves, goggles, etc.

7.2 All procedures for cell processing and set-up of cell culture assays should be performed using sterile technique under a biological safety cabinet certified for Level II handling of biological materials

8 PROCEDURE

8.1 A sterile, fresh UCB, that has been red cell and volume reduced will be provided for testing; the sample will be accompanied by additional adhesive ISBT barcode labels, *FLOW-FORM-012 Graft Characterization*, *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*, and *FLOW-GEN-012 FRM5 Stem Cell Laboratory Flow Cytometry Worksheet*.

8.2 Specimen Delivery and Log-in

8.3 For optimal time management at the beginning of the shift, the technologist may prepare IMDM and MethoCult® tubes ahead of time for later use. (For detailed instructions, see section 8.7 Notes on Preparation and Storage of MethoCult® and IMDM Media).

8.3.1 Cord blood samples are delivered to the STCL by CCBB staff in a designated container accompanied by UCB documents *FLOW-FORM-012 Graft Characterization*, *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*, and *FLOW-GEN-012 FRM5 Stem Cell Laboratory Flow Cytometry Worksheet*. Extra ISBT barcode labels, slides to be stained and a manual differential worksheet *STCL-PROC-035 (FRM1) Manual Differential (Slide Method) – CBUs for CCBB Program*, if applicable, will also accompany the sample(s). CCBB staff will date and time stamp the top page of each UCB packet of documents in an effort to track the delivery time in the STCL. More than one cord blood sample may be delivered at one time.

8.3.2 Remove reagent supplies from the refrigerator to allow them to come to room temperature. Set a timer for 15 minutes to alert you when the reagents are ready for use.

NOTE: All media should be allowed to come to room temperature before use, at least 15 minutes and no longer than 1 hour.

8.3.3 The technologist will verify the sample ISBT label with extra ISBT barcodes, and pre-labeled UCB documents. For internal labeling purposes, a letter will be assigned to each cord blood sample beginning with the first letter of the alphabet. This letter will be transcribed onto all the extra ISBT barcode labels (including the one on the sample tube) and the documents provided for that sample. If more than 26 samples are received in one day, the alphabet designation resets using a double lettering system (ie. A, B, C…, AA, BB, etc). Record the study ID of the technologist responsible for plating the sample on form *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*.

8.3.4 Log sample(s) in the HPCA log including, unique ISBT bar code label, specimen type, time the sample was stamped and delivered by CCBB and time HPCA technologist logged the specimen in before plating.

8.3.5 Deliver sample(s), documents and ISBT labels to the plating area.

8.4 Working Sample Preparation-Isolation of RBCs by Sedimentation over HetaSep™

8.4.1 Label a 0.5 mL sterile micro centrifuge tube with UCB ISBT label.

8.4.2 Dispense 150 µL of PBS to the micro centrifuge tube.

8.4.3 Vortex fresh UCB sample at high speed for 3-5 seconds and add 50 µL of well mixed UCB sample to the 150 µL of PBS. Mix cell solution by pipetting up and down gently 5 times (avoid bubbles).

8.4.4 Add 40 µL of HetaSep™ and mix cell solution again by pipetting 5 times.

NOTE: Multiple samples may be processed at one time for increased workflow efficiency.

8.4.5 Seal the micro centrifuge tube by snapping the cap well.

8.4.6 Incubate at 37°C for 20 minutes. Following the incubation, handle the tubes carefully, so as not to disturb the layers.

NOTE: DO NOT allow more than 30 minutes to pass from the start of the incubation before proceeding to the next step. If more than 30 minutes has passed, mix the layers by pipetting and repeat the 20 minute incubation.

8.4.7 Transfer as much of the top layer as possible to a clean micro centrifuge tube without disturbing the red cell pellet. Once all the sample has been removed, gently mix the cell solution to obtain an even cell suspension. A volume of 180 µL or more should be recovered (depending on the hematocrit of the starting sample).

8.5 Calculate sample volume required for plating

8.5.1 It has been determined that a starting sample volume of 50 µL, results in a sample dilution factor of 0.208. This number must be taken into account when calculating the amount of sample to be used for plating.

8.5.2 MethoCult® media has been formulated to allow the addition of cells to MethoCul®t at a 1:10 ratio in order to maintain the viscosity of the medium. In addition, a volume of 1.1 mL of the cell suspension in MethoCult® should be dispensed into each well (x 3), of a 35mm SmartDish™ cell culture plate.

8.5.3 To determine the new volume of post processed sample required to plate a density of 1.25×10^4 cells per well in triplicate, perform the calculations as follows:

8.5.3.1 To prepare a 10X working cell suspension to be added to the MethoCult®:

$$\frac{\text{(total volume of 10X suspension) X (desired concentration of suspension)}}{\text{WBC X } 10^6}$$

Example: $\frac{(1.0\text{ mL}) \quad X \quad (250{,}000\text{ cells/mL})}{46.9 \times 10^6\text{ cells/mL}} = 5330.5\text{ cells/mL or } 5.3\text{ Ml}$

- Divide this volume by the 0.208 dilution factor constant of the Hetasep™ step

  µL / 0.208 = 25.5 µL of UCB required for testing

- Transfer calculated amount of Hetasep™ treated sample into a sterile round bottom tube containing IMDM.

  NOTE: The total volume of your 10X suspension must be 1.0 mL.

  Example: 25.5 µL of sample + 974.5 µL of IMDM

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

8.6 Set-up CFU Assay

8.6.1 Transfer 400µL of 10X cell suspension into 4.0 mL aliquot of MethoCult® media, (4 mL will provide enough total volume to plate 1.1 mL of cell suspension in triplicate).

8.6.2 Vortex vigorously at high speed (~ 3000 rpms) for 8-10 seconds.

8.6.3 Set a timer for 5 minutes to allow the mixture to sit undisturbed to allow bubbles to rise to the top. Avoid letting the cell mixture sit for longer than 15 minutes before plating.

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

NOTE: If the SmartDish™ is labeled with barcodes placed on the lid, the lid will have to be removed prior to imaging.

8.6.5 At this time, you may proceed with the preparation of the next sample if needed.

8.6.6 At the end of the 5 minutes, use a sterile syringe attached to a 16-guage blunt-end needle, to dispense the Methocult® mixture containing cells into the culture dish. To expel the air from the syringe, place the needle below the surface of the solution and draw up approximately 1 mL. Gently depress the plunger and expel medium completely.

8.6.7 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:

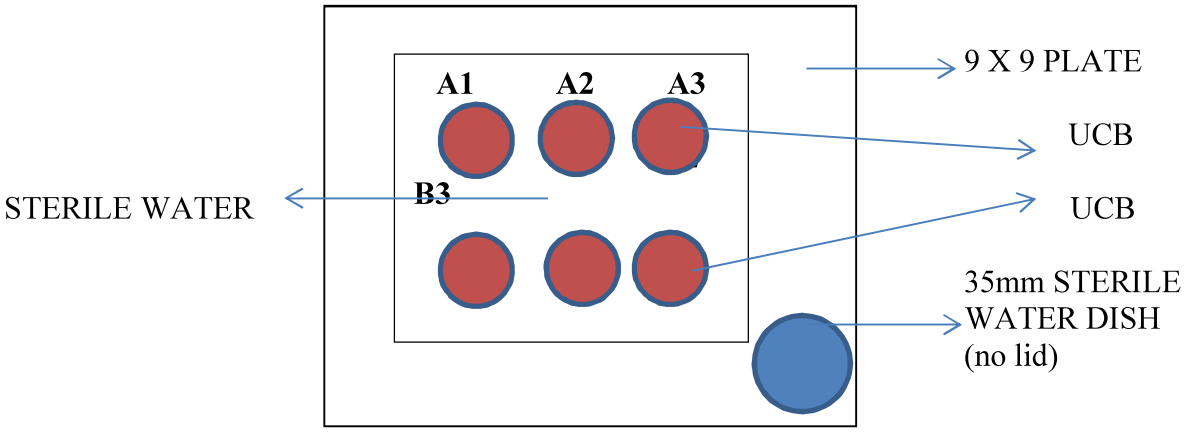
8.6.7.1 While holding the syringe containing the MethoCult®/cell mixture in one hand, remove the lid of a 35 mm dish with the opposite hand. Position the syringe over the center of the dish without touching the syringe to the dish. Dispense 1.1 mL and replace lid.

8.6.7.2 Repeat the dispensing procedure for the next 2 wells, if plating in triplicate.

8.6.8 Distribute the medium evenly across the surface of each well by gently tilting and rotating the dish to allow the medium to attach to the wall of the wells on all sides.

8.6.9 Add approximately 3.0 mL of sterile water to the 6 well SmartDish™ in between the wells to help maintain humidity during incubation. Place SmartDish™ inside a larger 9 x 9 plate and add an additional 35 mm water dish. (See diagram below). Cover plate and place in the incubator. More than one SmartDish™ may be placed in the 9 x 9 plate.

8.6.10 Incubate at 37°C, in 5% CO_2, for 14-16 days. Proceed to score manually or using the STEMvision™ automated instrument and computer system.



8.7 Scoring the CFU Assay

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

8.7.1 If scoring manually, remove the plates to be scored from the incubator and recover the UCB documents with *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*. Remove only the number of plates that can be scored within one hour. If unable to score a plate within the established 14-16 day range, please note on the comment section of *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*, the reason for reading outside this range (ie. holiday, vacation, etc.).

8.7.2 Confirm unit identity by comparing the ISBT barcode number on the plate to the ISBT barcode number on the form.

8.7.3 On *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*, record the number of cells plated per well (1.25×10^4), the date and the study ID of the technologist reading the plate.

8.7.4 Colony growth is enumerated using a high-quality inverted microscope with a blue filter to enhance the color of hemoglobinized erythroblasts. Use the following criteria when scoring a sample:

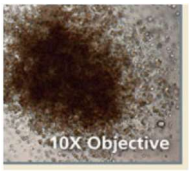
8.7.4.1 Place the plate on the inverted microscope stage and adjust the focus under low power (2X objective). Consider the overall colony appearance, distribution, background, plane of view and general morphology.

8.7.4.2 Count the colonies in each well and differentiate as follows:

8.7.4.2.1 BFU-E (erythroid)

- Bright red or brown
- > 200 cells in a single or multiple clusters
- A multi-centric burst is counted as a single entity

- Cells in each portion of a burst are tightly packed
- Colonies can appear as one compact cluster or with multiple clusters
- Cells form different individual centers of a burst that are closest to the center of the mass of the whole BFU-E tend to be in the same focal plane as those from adjoining centers.

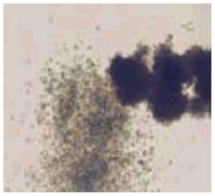


8.7.4.2.2 CFU-GM (granulocyte and macrophage)

- Colorless, sometimes granular or "glossy"
- Uniform in size and usually tightly packed
- Individual cells can be distinguished, particularly at the edge of the colony.
- At least 20 cells/colony
- Macrophage colonies are larger and more spread out than granulocyte colonies.
- Cells are typically spread out but may have macrophages clustered together in the center of the colony, making it appear dark.

8.7.4.2.3 CFU-GEMM (granulocyte, erythroid, macrophage and megakaryocyte) *

- Often large and have a larger capacity to proliferate (at least 500 cells)
- Erythroid cells tend to be in the center and surrounded by non-erythroid cells



***NOTE:** For assistance in recognition for various colony types, refer to the *Atlas of Hematopoietic Colonies from Cord Blood.*

NOTE: If the colony count is greater than 100 colonies per plate, score the plate as >100.

8.7.5 The technologist reading the plate records the counts for each colony type, their Study ID, and the date the plate was read on *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*. The total nucleated cell count and the average colony count (from each triplicate plating) will be used to calculate the total number of CFU-GM (x 10^5), CFU-GEMM (x 10^5) and BFU-E (x 10^5) colonies. Verify the calculations by recording the date and initials on the back of *STCL-SOP-052 (FRM1) Progenitor Cell Assay Form*.

8.7.6 The completed UCB documents (*FLOW-FORM-012 Graft Characterization, STCL-SOP-052 (FRM1) Progenitor Cell Assay Form, and FLOW-GEN-012 FRM5 Stem Cell Laboratory Flow Cytometry Worksheet*) will be delivered to designated STCL staff so results can be entered in the CCBB EMMES database.

8.8 Quality Control

8.8.1 Proficiency Testing

8.8.1.1 Fresh or frozen cord blood proficiency testing samples will be performed quarterly by the delegated technologists trained in plate enumeration and plating. These samples will be submitted to Stem Cell Technologies for review and grading. These blinded samples will be used to determine reproducibility between the technologists and all participating centers.

8.8.2 Quarterly Inter-Lab QA

8.8.2.1 Choose one cord blood sample from the daily workload and have all technologists trained in HPCA plating plate this sample according to *STCL-SOP-052 Hematopoietic Progenitor Cell Assay (HPCA) – Cord Blood Bank Products*. To minimize variables, all technologists must plate within a two-hour time and store in the same incubator.

8.8.2.2 At the end of the 14-16 day incubation period, plates will be read and scored. The same technologist will read all plates for standardization purposes.

8.8.2.3 Each technologist's plate readings should agree within 80% of the scoring technologists.

8.8.2.4 If the counts exceed a 20% difference, the scoring technologist and identified plating technologist will undergo a review of technique and a new sample will be repeated.

8.8.2.5 If there is more than one scoring technologist, each scoring technologist will read each other's plate in addition to their own to determine reproducibility between them. Overall morphology of the colonies must also be evaluated as

adequate or unacceptable. The total colony count must agree within 80% from each other. All four numbers will be used to establish an acceptable range of colony growth for the QA sample.

Example: **Sample X**

Scoring Tech 1 - plate1 Total colony count	Scoring Tech 2 - plate 2 Total colony count
Scoring Tech 1 = 80	Scoring Tech 1 = 89
Scoring Tech 2 = 85	Scoring Tech 2 = 88

Mean for sample X = 85.5 (86)

Range for sample X = 86 +/- 20% = 86 +/- 17 (69 – 103)

8.8.2.6 Summarize data in QA form *STCL-PROC-022 FRM 4 HPCA Quarterly Competency Assessment*. For a better visual interpretation, plot data in a graph and provide all documents to the lab manager for review.

8.9 Notes on Preparation and storage of MethoCult® and IMDM Media

8.9.1 Follow this procedure when thawing an entire bottle of 100 mL MethoCult® or IMDM media.

8.9.1.1 Thaw media bottle overnight under refrigeration (2 - 8°C) or at room temperature until thawed.

NOTE: Do NOT thaw medium at 37°C. The methylcellulose in frozen MethoCult® will not be homogeneous and small lumps may be present if the MethoCult® is thawed rapidly at 37°C.

8.9.1.2 Shake the MethoCult® bottle vigorously until the media becomes opaque with bubbles. Due to the viscosity of the MethoCult® media, it is necessary to shake the bottle vigorously before aliquoting.

8.9.1.3 Let the bottle stand for at least 5 minutes before aliquoting.

8.9.1.4 Thawed media is stable for one month when stored at 2-8°C.

8.9.2 Preparation of MethoCult® tubes for fresh UCB plating:

8.9.2.1 In the BSC, aliquot the entire bottle of MethoCult® media by adding 4 mL of media to 14 mL sterile Falcon round bottom tubes. Cap tubes securely to maintain sterility.

8.9.2.2 Label each tube with the name of the media, aliquot date, expiration date, and technologist initials. Store at 2-8°C. These tubes are ready for daily use.

8.9.2.3 Allow media to come to room temperature before plating, at least 15 minutes and no longer that 1 hour. Based on manufacturer's recommendations, the media should NOT remain at room temperature for a prolonged period of time.

8.9.3 Preparation of IMDM media for fresh UCB plating:

8.9.3.1 In the BSC, aliquot the entire bottle of IMDM media by adding 12 mL of media into 15 mL conical tubes. Cap tubes securely to maintain sterility.

8.9.3.2 Label each tube with the name of the media, aliquot date, expiration date, and technologist initials. Store at 2-8°C. These tubes are ready for daily use.

8.9.3.3 Allow media to come to room temperature before plating, at least 15 minutes and no longer that 1hour. Based on manufacturer's recommendations, the media should NOT remain at room temperature for a prolonged period of time.

9 RELATED DOCUMENTS/FORMS

9.1 FLOW-FORM-012 Graft Characterization

9.2 FLOW-GEN-012 FRM5 Stem Cell Laboratory Flow Cytometry Worksheet

9.3 STCL-PROC-022 FRM4 HPCA Quarterly Competency Assessment

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

9.5 STCL-SOP-052 FRM1 Progenitor Cell Assay Form

9.6 STCL-SOP-052 FRM2 Hematopoietic Progenitor Cell (HPCA) CCBB Samples Repeat Analysis

9.7 STCL-SOP-056 FRM OOS – Colony Forming Unit Assay

9.8 STCL-SOP-056 JA1 Operation of STEMvision™ Automated Colony-Forming Cell Assay Analyzer JA1

9.9 STCL-SOP-056 JA2 STEMvision™ Automated Colony-Forming Unit (CFU) Assay Reader Technical Manual JA2

9.10 STCL-SOP-056 JA3 Flow Diagram for Processing Fresh Cord Blood Samples Using STEMvision™ Analyzer

9.11 STCL-SOP-056 JA4 STEMvision Instrument Quick Guide JA4

9.12 STCL-SOP-059 STEMVision Automated Colony Counting and Enumeration of Hematopoietic Progenitor Cells in Thawed Umbilical Cord Blood

10 REFERENCES

10.1 STEMvision™ Technical Manual

10.2 MethoCult® Technical Manual

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)

02	B. Waters-Pick	• Added Section 2.3 to address repeat testing using thawed samples • Inserted Section 8.1 to address specimen provided • Review to include references to OOS of CFU assay and its html form. Added STCL-SOP-056 form and Job aids to section 9.

Signature Manifest

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All dates and times are in Eastern Time.

STCL-SOP-056 STEMvision Automated Colony Counting and Enumeration of HPC in Fresh UCB

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