



## STEM CELL LABORATORY (STCL)



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Hematopoietic Progenitor Cell Assay (HPCA) - Fresh and Thawed Clinical Products

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## STCL-PROC-022

### HEMATOPOIETIC PROGENITOR CELL ASSAY (HPCA) – FRESH AND THAWED CLINICAL PRODUCTS

#### 1 PURPOSE

- 1.1 Hematopoietic progenitor cell assays are used to quantitate the frequency of granulocyte, macrophage, erythrocyte, megakaryocyte, T-lymphocyte, B-lymphocyte, or blast cell precursors that are present in cells obtained from fresh, bone marrow, peripheral blood, leukapheresis, or other unusual sources of hematopoietic precursors.

#### 2 INTRODUCTION

- 2.1 Progenitor cell assays allow analysis of hematopoiesis at an earlier level than that of the standard morphologic and histochemical techniques. In this assay, a single cell suspension of fresh mononuclear cells is plated in duplicate in a semisolid medium enriched with hematopoietic growth factors, cytokines and other supplements that support cell proliferation and differentiation. After a 14-16 day incubation period under specific conditions, colonies arising from progenitor cells are identified and enumerated microscopically.

#### 3 SCOPE AND RESPONSIBILITIES

- 3.1 The Medical Technologist performing the assay is responsible for following the SOP as written. The Medical Directors and Laboratory Manager are responsible for ensuring that the requirements of this procedure are successfully met.
- 3.1.1 Specimen requirements
- 3.1.1.1 A sterile single suspension of mononuclear cells from a fresh BM, PB, or PSCs, is delivered to the HPCA area in the STCL. The specimen must be accompanied by a STCL Clinical HPCA Worksheet, a manual differential worksheet (if applicable), and extra ISBT 128 barcode labels.

#### 4 DEFINITIONS AND ACRONYMS

4.1	STCL	Stem Cell laboratory
4.2	HPCA	Human Progenitor Cell Assay
4.3	IMDM	Iscoe's Modified Dulbecco's Medium with 2% FBS
4.4	CFU-GM	Colony Forming Unit granulocyte/macrophage
4.5	CFU-GEMM	Colony Forming Units granulocyte-erythrocyte-macrophage and megakaryocyte
4.6	CFU-BFUE	Burst-forming unit erythroid
4.7	PSC	Peripheral Stem Cell

4.8	BM	Bone Marrow
4.9	BM-OR	Bone Marrow-Operating Room
4.10	BM-POST CS	Bone Marrow- Post process in CS-3000
4.11	PB	Peripheral Blood
4.12	BSC	Biological Safety Cabinet
4.13	TNCC	Total nucleated cell count

## 5 MATERIALS

5.1	Reagents:	Manufacturer / Catalog #
5.1.1	MethoCult 4434 Medium	Stem Cell Technologies/ Cat# 4434
5.1.2	IMDM with 2% FBS	Stem Cell Technologies/ Cat# 7700
5.1.3	Sterile water 1L	Sigma/ Cat# W3500-1L
5.2	Supplies:	
5.2.1	3 ml sterile syringes with luer lock tip	BD Biosciences/ Cat# 309657
5.2.2	Blunt-end needle, 16G	Stem Cell Technologies/ Cat# 28110
5.2.3	24-well Costar cell culture plates	Corning Inc./ Cat# 3524
5.2.4	Specimen ID barcodes	Computype/ Cat# 1018184
5.2.5	Permanent marker	Sharpie/ Cat# 30001
5.2.6	Sterile 12 x 75 polystyrene tubes	Port City Diagnostics/ Cat# T2063STR
5.2.7	Sterile 15 ml conical tubes	Port City Diagnostics/ Cat# U1100SRGFT
5.2.8	Sterile 200µl pipette tips	Port City Diagnostics/ Cat# 7509-96RS
5.2.9	Sterile serological pipettes	Port City Diagnostics/ 1ml Cat# SER-0010-S01 2ml Cat#SER-0020-S01 5ml Cat#SER-0050-S01
5.2.10	2 hole filters	Fisher Scientific 10ml Cat# SER-0100-S01
5.2.11	Plastic cytofunnel	Shandon/ Cat# 59910021
5.2.12	Cytoclip- slide clip	Shandon/ Cat# 59910052M
5.2.13	Frosted microscope slide	Cardinal Health/ Cat# M6146



## 6 EQUIPMENT

6.1	Barcode scanner	Barcode scanner
6.2	Inverted Microscope	Olympus IMT-2
6.3	Thermo Scientific CO2 Incubators	HERAcell 150 SN# 225658 & SN# 225659
6.4	Vortex Mixer	Isotemp Plus SN# Z01J464990ZJ VWR/ Mini Vortexer MV1
6.5	DIFFCOUNT electronic cell counter	Modulus Data Systems/ SN# 319806
6.6	Stamping Clock	Lathem 1000E/ SN# 1E014032
6.7	3 channel traceable timer	Fisher Scientific/ SN# 111878606 / SN# 111878753
6.8	Class II Biological Safety Cabinet	NUAIRE 425-600/ SN# 123044050508
6.9	Micropipettes	Baker SG400/ SN# SL29877V Rainin/ 20µl : SN# M11689G 20µl: SN# I0985053K 200µl: SN# I0984537K 200µl: SN# M10263E 1000µl: SN# R73215A 1000µl: SN#H0962198K
6.10	Shandon Cytospin 2	Shandon SN# MA103807N
6.11	Olympus microscope BH-2	Olympus SN# H3260700135

## 7 SAFETY

- 7.1 All procedures for cell processing and set-up of cell culture assays should be performed using sterile technique under a biohazard safety cabinet certified for Level II handling of biological materials, and universal handling precautions. When handling a biological hazardous substance, appropriate personal protective equipment (PPE), must be worn as the primary barrier of protection. PPE may include, but is not limited to face protection, lab coats and gloves. Appropriate PPE should be donned before handling potentially hazardous biological materials and removed immediately and replaced if gross contamination of the equipment occurs.

## 8 PROCEDURE

- 8.1 In the typical duplicate assay, 100 µl of cells added to the syringe containing 500 µl of MethoCult to yield a final cell density of  $1 \times 10^4$  cells/well with approximately 250µl/well (~100 µl of total volume dead space is assumed to be lost in syringe). For optimal time management, at the beginning of your shift, the HPCA technologist may prepare the 1.0 ml IMDM tubes, 0.5 ml MethoCult syringes and culture plates ahead of time and store for later use. (Refer to section

8.13, Notes on preparation of MethoCult and IMDM media, for detailed instructions).

- 8.2 A single cell suspension of mononuclear cells from fresh BM, PB or PSC will be counted on the hematology analyzer (XS-1000i), and the total nucleated cell count will be recorded on the Clinical Sample Form by the processing technician. The specimen will be delivered to the HPCA area along with the Clinical Sample Form, extra barcode labels for that specimen and a Manual Differential Form, when applicable
- 8.3 The STCL Clinical HPCA Worksheet will be time stamped by the processing technician at the time the sample is delivered to the work area. The form will include Patient's name, Duke history number, product type, barcode ID, and time and date of collection. The donor's name and/or history # should also be provided if the cells are from an allogeneic product.
- 8.4 Remove reagent supplies from the refrigerator at this time 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 min).

- 8.5 Specimen ID is verified and sample is logged in the designated notebook in the HPCA area as follows:

<b>BARCODE LABEL</b>	Doe, John	DU0000	PSC #1 @ 1200
	(patient's name)	(Duke History #)	(specimen type/ log-in time)

- 8.6 If a manual differential is requested, prepare a cytoprep slide at this time to add specimen at the time of plating. **(Refer to Procedural Notes 8.12 for detailed instructions).**
- 8.7 The specimen and worksheet are delivered to the plating area.
- 8.8 If more than one specimen is being processed at one time, a rack must be assigned to each specimen with a maximum of 3 specimens under the hood at one time.
- 8.9 The HPCA technologist will calculate the amount needed for plating fresh samples at a concentration of  $1 \times 10^4$  cells/well (or  $2 \times 10^5$ /ml) or for thawed samples plating at a concentration of  $2 \times 10^4$  cells/well (or  $4 \times 10^5$ /ml) in duplicate. Record the information on the STCL Clinical HPCA Worksheet along with his/her initials.
- 8.10 Calculations will vary depending on the total nucleated cell count. For example, if a sample has a cell count of **200.0 x 10<sup>6</sup>** or lower calculate as follows:



Example:  $TNCC = 189.6 \times 10^6$

$$\frac{2 \text{ (number of wells)}}{1896.0 \text{ (cell count} \times 10^5)} \times 1000 = 1.1 \text{ } \mu\text{l of sample required for plating}$$

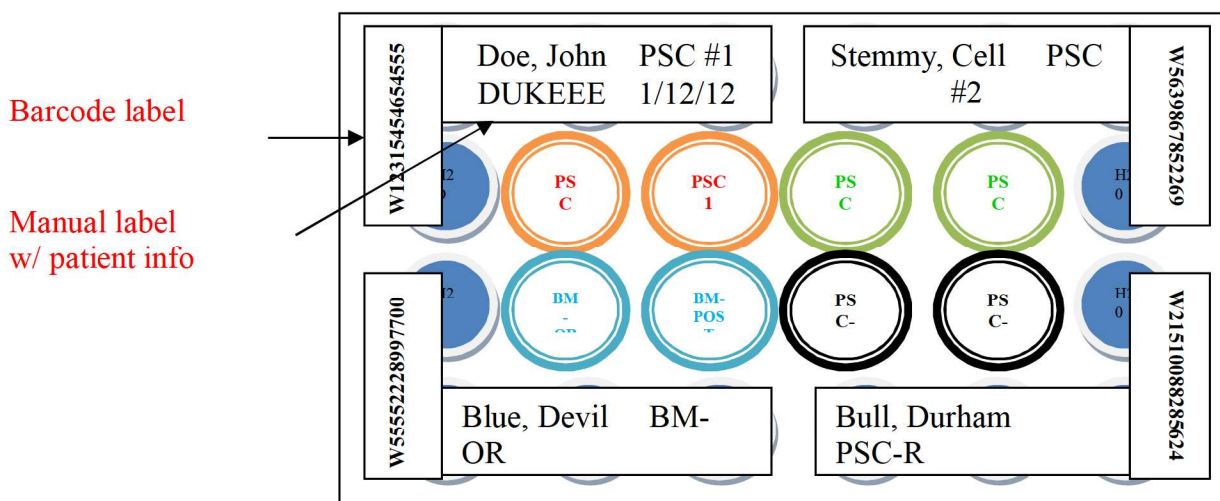
(round to 1 decimal place)

**\* If the cell count is  $> 200.0 \times 10^6$  skip to section 8.11 of this procedure (Serial Dilution).**

- 8.10.1 Place a unique barcode ID label on the 1.0 ml IMDM tube, the culture plate and on the sample rack. In addition, label tube with patient's last name and specimen type. If a plate is not ready for use, add ~ 1ml of sterile water to each of the outer wells of the plate to allow humidity to be evenly distributed on the plate to keep the cell mixture from drying. Plates may be prepared ahead of time, label with preparation date and initials. Store plate at room temperature under BSC until ready for use.
- 8.10.2 Vortex specimen tube to mix cells at high speed (~3000 rpms) for 1-5 seconds.
- 8.10.3 Using aseptic technique, pipette the calculated amount of cells into the 1.0 ml IMDM tube; rinse the tip 3-5 times and vortex to mix for 1-3 seconds at high speed.  
  
**NOTE:** If the total volume of sample to be plated is  $\geq 50$  uls, remove that same calculated volume from the 1 ml IMDM tube and discard to maintain a cell-to-diluent ratio of approximately 1 ml. This practice will also minimize over dilution of the sample.
- 8.10.4 Remove the needle from the 0.5 methocult syringe and transfer 100 $\mu$ l of the cell mixture (cells + 1.0 ml IMDM), into the syringe. In a single motion, pull down on the syringe plunger with your left hand as you dispense the 100 $\mu$ l of the cell mixture into the top of the syringe using a 200 $\mu$ l micropipette on your right hand.
- 8.10.5 Mix cell suspension within the syringe with an up/down movement of the plunger 5-10 times. Be careful not to expel any sample while mixing.
- 8.10.6 Replace blunt needle on the syringe and allow it to stand for at least 5 minutes to allow the bubbles to rise to the top. Set a timer for 5 minutes to alert you when the sample is ready for plating. Avoid letting the mixture to sit for longer than 15 minutes.
- 8.10.7 At this time, you may begin preparing the next specimen for plating. Refer to steps **(8.10.4 – 8.10.6)**.
- 8.10.8 At the end of the 5 minutes, remove the blunt needle from the syringe and lift off the lid of the culture plate. Plate in duplicate by dispensing ~250 $\mu$ l into each of the designated wells one drop a time per well, until the entire cell mixture is gone.
- 8.10.9 On a 24-well Costar culture plate identify the plate's section selected for plating in duplicate with the patient's name, Duke history #, specimen type, date and unique barcode ID.

**NOTE:** Each culture plate can accommodate up to four clinical samples. Since multiple samples may be plated in adjacent wells on the same plate, extreme care must be taken to label each set of patient samples thoroughly.

## 24 well cell culture plate containing 4 clinical samples plated in duplicate.



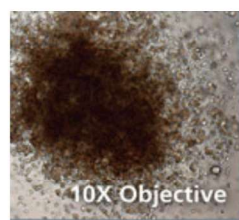
- 8.10.10 Replace the lid on the plate. Swirl plate gently to evenly cover the bottom of each well and remove any visible bubbles using aseptic technique.
- 8.10.11 If the sample also needs flow cytometry testing, deliver the sample to the flow area at this time along with extra barcode labels.
- 8.10.12 If a cytoprep was performed, deliver slide holder to the Shandon Cytospin and stain slide on the Wescor hematology stainer.
- 8.10.13 Place the culture plate in a humidified 37°C incubator in 5% CO<sub>2</sub> for 14-16 days.
- 8.10.14 At the end of the incubation period, remove the plates to be scored from the incubator and recover the worksheet from the designated binder.
- 8.10.15 Confirm sample identity by comparing barcodes on the plate and of the form, as well as patient's name and history number.
- 8.10.16 On the STCL Clinical HPCA Worksheet, record the counts for each colony type in each of the wells. Also record the date the plate was read and the scoring technologist's name.
  - 8.10.16.1 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:

### 8.10.16.1.1 BFU-E ( erythroid)

- Bright red or brown

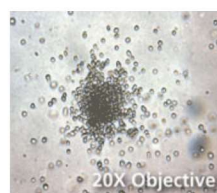


- 200 cells/burst
- A multi centric burst in counted as a single entity
- Cells in each portion of a burst are tightly packed
- Colonies can appear as one compact cluster or with multiple clusters
- Cells from different individual centers of a burst that are closest to the center of the mass of the whole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



#### 8.10.16.1.2 CFU-GM (granulocyte and macrophage)

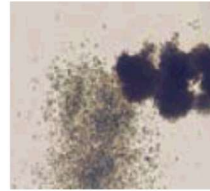
- Colorless, sometimes granular or “glossy”
- Uniform in size and usually tightly packed
- Individual cells can be distinguished, particularly at the edge of the colony
- 40 cells/ colony
- Macrophage colonies are larger and more spread out than granulocyte colonies
- Cells are typically spread out but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



#### 8.10.16.1.3 CFU-GEMM (granulocyte, erythroid, macrophage and megakaryocyte)



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



\*For assistance in recognition for various colony types, refer to the Atlas of Hematopoietic Colonies from Cord Blood and the Atlas for Hematopoietic colonies in Health and Disease located in the HPC area.

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

- 8.10.17 Place the completed STCL Clinical HPCA Worksheet in the Lab Manager's mailbox fso results can be entered in the Lab Information System (Cerner).

## 8.11 SERIAL DILUTION

- 8.11.1 If the cell count is > **200.0 x10<sup>6</sup>** then a serial dilution must be performed to avoid overgrowth of cell cultures. In order to perform a serial dilution, prepare two (2) tubes of 1.0 ml Of IMDM in each. (For detailed instructions see the notes on preparation on IMDM and MethoCult media).
- 8.11.2 Label each tube with the unique barcode label and patient's last name and specimen type.
- 8.11.3 Calculate dilution as follows:

Example: TNCN= 360.0 x 10<sup>6</sup>

$$\frac{1}{36.0 \text{ (cell count x } 10^7)} \times 1000 = 27.8 \text{ } \mu\text{l of sample (round to 1 decimal place) required for serial dilution}$$

- 8.11.4 Add the calculated amount of cells x 10<sup>7</sup>, to the first tube containing 1.0 ml of IMDM.
- 8.11.5 Vortex the sample at high speed (~ 3000 rpm), for 1-5 seconds. Transfer 20  $\mu$ l of the cell suspension from this tube (IMDM/cell mixture) into the second 1 ml IMDM tube. Vortex the sample for 1-3 seconds at high speed. Proceed to step **8.10.4** in the procedure using the tube containing (1ml IMDM + 20 $\mu$ l of cell mixture). Write on sample sheet as follows:

Cell concentration plated:  $1 \times 10^7 = 27.8 \text{ } \mu\text{l} / 2 \times 10^5 = 20 \mu\text{l}$ .

## 8.12 Preparation of Cytopreps

8.12.1 Dilute cells to  $2 \times 10^5$ / ml

8.12.2 Prepare cytospin assembly:

8.12.2.1 Label frosted end of slide with patient's name, history number, sample type, date, and attach a unique barcode label to the slide.

<b>Doe, John</b> DUK0000 PSC #1 1/12/12	W23164598889000	
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8.12.2.2 In the silver cytoclip, place the slide with label facing up.

8.12.2.3 Over the slide, place a 2 hole filter card

8.12.2.4 Over the filter card, place a plastic sample chamber funnel and ensure the slide and filter lay flat against the silver assembly.

8.12.2.5 Lock the silver assembly by snapping the stainless steel spring into place. The spring action keeps the components in place so the whole sample chamber assembly can be handled as a unit.

8.12.2.6 Pipet 200 uls of diluted cells into the sample chamber funnel; lay silver assembly on its side to prevent the fluid from flowing back onto the filter until it is ready for centrifugation.

8.12.2.7 The slide assembly is not sterile so do not allow the pipette tip to touch while delivering the specimen in order to maintain aseptic technique.

8.12.2.8 Deliver the slide assembly to the Cytospin centrifuge area and place the slide inside the sample carousel. Make sure the carousel is balanced and replace the carousel cover. Lock the cover and place carousel inside the centrifuge. Close centrifuge lid.

8.12.2.9 Start the centrifuge cycle by pressing START. The centrifuge has been pre-programmed to spin for 6 minutes at 600 rpm.

8.12.2.10 At the end of the spin cycle, the audible alarm will signal the operator the completion of the spin cycle. Open the lid of the cytospin by pressing the button on the top left.

8.12.2.11 Remove the slide assembly. Unlock the springs of the silver holder. Place the plastic sample chamber in the "dirty" bin,



discard the 2 hole filter and allow slide to air dry for at least 5 minutes before staining.

8.12.2.12 Stain slide in the Wescor stainer, place a coverslip over the stained area and perform a manual differential.

8.12.2.13 Record results on the Manual Differential form for clinical specimens and place in the Lab Manager's mailbox so the results can be entered into the EMMES computer system.

### 8.13 Notes on Preparation of the MethoCult and IMDM Media

8.13.1 This procedure is intended for the thawing of a complete 100 ml MethoCult or IMDM media bottle.

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

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

8.13.3 **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.

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

8.13.5 Aliquot complete media into 15cc conical tubes and make sure the tubes are capped tightly to maintain sterility.

8.13.6 Label each tube with the name of media, aliquot date, expiration date (from the time of thaw), and tech initials.

8.13.7 Media is stable until the expiry date on the manufacturer's label when stored at -20°C or for one month at 2-8°C.

8.13.8 You may choose to prepare MethoCult syringes and IMDM tubes for daily use from a 15cc conical tube and allow them to sit in the refrigerator until ready for use.

8.13.9 Preparation of **MethoCult** syringes for fresh plating fresh clinical samples:

8.13.10 Remove a 12ml MethoCult aliquot tube from the refrigerator and shake well. Let tube sit for 5 min to allow bubbles to rise to the top.

8.13.11 Using aseptic technique, attach a blunt-end needle to a 3 ml sterile syringe. To remove the air from the syringe, place the needle below the surface of the MethoCult medium and draw up approximately 0.5 ml, gently depress the plunger and expel the medium completely.

8.13.12 Draw up the desired amount for plating, **0.5 ml**, until no air space is visible. Recap syringe and store at 2-8°C for later use. Label rack with preparation date, expiration date and tech initials.

8.13.13 Preparation of **IMDM** tubes for plating fresh clinical samples:



- 8.13.13.1 Remove a 12ml IMDM aliquot tube from the refrigerator.
- 8.13.13.2 Using a sterile 1ml or 2ml volumetric pipette, prepare 12 x 75 sterile tubes by adding **1.0 ml** of IMDM to each tube.
- 8.13.13.3 Label each tube with the amount of IMDM dispensed and store at 2-8°C until ready for use. Label rack with preparation date, expiration date and tech initials.

## 9 RELATED DOCUMENTS/FORMS

- 9.1 STCL-PROC-022 FRM 1 STCL Clinical HPCA Worksheet
- 9.2 STCL-PROC-022 FRM2 Media Lot-to-Lot QA Release Log
- 9.3 STCL-PROC-022 FRM3 Reagent Verification Log
- 9.4 STCL-PROC-022 FRM4 HPCA Quarterly Competency Assessment
- 9.5 STCL-PROC-022 FRM5 HPCA Repeat Analysis
- 9.6 STCL-PROC-022 FRM6 HPCA Specimen Logsheet
- 9.7 STCL-PROC-022 FRM7 HPCA Worksheet for NMDP Biobank Samples
- 9.8 STCL-PROC-022 JA1 Tech Manual HPCA – Methocult
- 9.9 STCL-PROC-022 JA2 Separation of Cells Using a Ficol-Gradient
- 9.10 STCL-PROC-022 JA3 Thawing of Frozen Products for HPCA Testing
- 9.11 STCL-FORM-064 Manual Differential Worksheet – Clinical Products

## 10 REFERENCES

- 10.1 Kurtzberg J, Denning SM, Nycum L, Singer KH, Haynes BF. Immature human thymocytes can be driven to differentiate into non lymphoid lineages by cytokines from thymic epithelial cells. Proc Natl Acad Sci USA 86: 7575-7579, 1989.
- 10.2 American Association of Blood Banks. Standards for Hematopoietic Progenitor Cell and Cellular Product. Current edition.
- 10.3 Foundation for the Accreditation of Hematopoietic Cell Therapy (FACT) and Netcord. International Standards for Cord Blood Collection, Processing, Testing, Banking, Selection and Release Current edition.
- 10.4 MethoCult Technical Manual. Stem Cell Technologies, OCT 2009.
- 10.5 Catherine Nissen-Druey, Basel Andre Tichelli, Basel Sandrine Meyer-Monard, Atlas of Hematopoietic Colonies in Health and Disease. Stem Cell Technologies Inc., 2005.

**11 REVISION HISTORY**

<b>Revision No.</b>	<b>Author</b>	<b>Description of Change(s)</b>
08	B. Waters-Pick	<ul style="list-style-type: none"><li>• Added documents and names to Section 9 Related Documents/Forms associated with this parent document</li><li>• Added form # of manual differential form to Section 9</li><li>• Created NEW document <i>STCL-PROC-022 (FRM7) HPCA Worksheet for NMDP Biobank Samples</i> as a result of a recent CCBB Program NMDP Audit recommendation</li></ul>

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All dates and times are in Eastern Time.

**STCL-PROC-022 Hematopoietic Progenitor Cell Assay (HPCA) - Fresh and Thawed Clinical Products****Author**

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Name/Signature	Title	Date	Meaning/Reason
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**Medical Director**

Name/Signature	Title	Date	Meaning/Reason
Joanne Kurtzberg (KURTZ001)		21 Mar 2023, 05:38:16 PM	Approved

**Quality**

Name/Signature	Title	Date	Meaning/Reason
Isabel Storch De Gracia (IMS19)		22 Mar 2023, 04:07:23 PM	Approved

**Document Release**

Name/Signature	Title	Date	Meaning/Reason
Sandra Mulligan (MULLI026)		23 Mar 2023, 10:25:09 PM	Approved