CATEGORY:	Laboratory Work Instructions	**SOP Number**: Lymph Node_Rutgers	Version 1.0
TITLE:	**Separation and Freezing Technique for Lymph Node Mononuclear Cells from Donor Lymph Nodes at ITN Central Cell Processing Core Facility**		

1.0 Title

Separation and Freezing Technique for Lymph Node Mononuclear Cells from Donor Lymph Nodes at ITN Central Cell Processing Core Facility

2.0 Purpose

The purpose of this SOP is to describe the steps involved in the isolation and cryopreservation of lymph node mononuclear cells from a donor lymph node using the Ficoll method.

3.0 Definitions and Abbreviations

LMNC	Lymph node Mononuclear Cells
FCS	Fetal Calf Serum
PBS	Phosphate Buffer Saline
DMSO	Dimethyl Sulfoxide

4.0 Equipment / Supplies and Reagents

4.1 Equipment / Supplies

	Name	Vendor	Catalog Number
4.1.1	Sieve base	PGC Scientific	76-0161-00
4.1.2	Sieve # 80 (8” Tall)	PGC Scientific	75-9002-39
4.1.3	60 ml Syringe	No specific vendor required	
4.1.4	Tweezers	No specific vendor required	
4.1.5	Scalpel	No specific vendor required	

		4.1.6	- 80 Freezer	No specific vendor required	
		4.1.7	Conical Tube, 50 ml, Sterile, Falcon	Falcon 352074	Fisher # 14-432-22
		4.1.8	Centrifuge: Beckman Coulter Allegra 6R - Rotor Gh 3.8A or comparable	No specific vendor required	
		4.1.9	ViCell XR	Beckman Coulter	
		4.1.10	Liquid nitrogen Tank	Thermo Forma	8030
		4.1.11	Rate Controlled Cell Freezer	In place	
		4.1.13	Pipette Aid	Drummond 4-000-100	Fisher # 13-681-19
		4.1.14	Vials, Cryogenic, Sterile, 1.8 ml	NUNC	345418
		4.1.15	Class II Biological Safety Cabinet	Forma Scientific or equivalent	Model 1286
		4.1.16	250 ml Container (receiver for a cell culture filter)	No specific vendor required	
		4.1.17	Hematology Mixer Model M26125	Barnstead/Thermoline	Fisher # 12-814-2
		4.1.18	Set of Pipettes	No specific vendor required	
		4.1.19	Cell Strainer, 40 µm	BD Falcon	352340
	4.2	Reagents			
			Name	**Vendor**	**Catalog Number**
		4.2.1	RPMI Human Media 1640 (1X), liquide, with Glutamax-I and HEPES buffer	Invitrogen	72400-120
		4.2.3	Dulbecco's PBS sterile, Ca++, Mg++ Free	Invitrogen	14190-250
		4.2.4	Fetal Calf Serum (FCS)	No specific vendor required	
		4.2.5	Ficoll	GE Healthcare	17-1440-03

		4.2.6	5,000 units Penicillin/5,000 µg Streptomycin, 100 ml	Invitrogen	15070-063
		4.2.7	VersaLyse, 100 tests	Beckman Coulter	IM3648
		4.2.8	7X® Cleaning Solution 1 Gal. (M)	MP Biomedicals	097667093
		4.2.9	ViaCheck Concentration Controls (1E6, 4E6, & 8E6)	Polysciences, Inc.	24627, 24628, & 24629
		4.2.10	ViaCheck Viability Controls (0%, 50%, & 100%)	Polysciences, Inc.	24622, 24623, & 24626
		4.2.11	ViCell™ Reagent Quad Pack	Beckman	383721
		4.2.12	Freezing Medium B, 20% DMSO		Provided by RUCDR
		4.2.13	HuAB serum • Tissue culture tested • Heat inactivated • Filtered	Provided by ITN	
		4.2.14	Trypan Blue	Fisher Biotech	BP1302-10
		4.2.15	Bleach	Clorox	

IMPORTANT:
Keep all reagents at recommended storage conditions. Allow reagents to equilibrate to room temperature before use.
Do not work with chilled reagents so as to maintain the membrane lipid fluidity of the cells.

5.0	**Procedures**	
	IMPORTANT: **All work needs to be performed under a biological safety cabinet observing biosafety regulations using sterile technique whenever working with open containers.**	
	Note: Lymph node samples will be shipped to the Central Cell Processing Core from the transplant trial sites in the even that spleen specimens are not available. Process all lymph nodes together according to the steps below.	
	5.1	Preparation of the lymph node sample

		5.1.1	Wet the sieve with 20 ml of RPMI with 10% FCS and 1% antibiotics. Place the sieve on top of the receiving container and then place the lymph node(s) sample(s) into the sieve.
		5.1.2	While the lymph node(s) are in the sieve, slice the lymph node(s) into thin (~1/8 in) slices with the scalpel. Clean and discard any fatty tissue from the lymph node slices.
		5.1.3	Gradually press the lymph node slices through the sieve with the back of the 60 ml syringe so that it all goes easily through sieve. At the same time steadily pour small incremental quantities of RPMI with 10% FCS and 1% antibiotics using a total of 100 ml to make sure cells are washed through. You will see that some parts of the lymph node will stay on the sieve after this step is done.
		5.1.4	Add another 25 ml of RPMI with 10% FCS and 1% antibiotics to the sieve and press the tissue through one more time.
		5.1.5	Put drained matter into a sterile 250 ml container/receiver. Let it sit for 5 minutes.
		5.1.6	Replace the receiving container under the sieve and pour all of the contents of the 250 ml container back into the sieve and gradually press the unsuspended white matter through the screen.
		5.1.7	Add 60 ml RPMI with 10% FCS and 1% antibiotics to the 250 ml container in order to rinse the remaining matter from it. Then pour this suspension over the remaining white matter in the sieve.
		5.1.8	Gently press the white matter through the sieve.
		5.1.9	Aliquot the strained material from the receiving container into 50 ml tubes at a volume of 10 ml each.
	5.2	LMNC Isolation using Ficoll	
		5.2.1	Underlay 10 ml Ficoll into each tube. Since 10 ml was aliquoted into each tube in Step 5.1.9 you will now have a 1:1 proportion of reagent volume to the sample volume
		5.2.2	Centrifuge at RT for 30 minutes at 2000 rpm with the **brake off.**
		5.2.3	For each prep, using a sterile serological pipette aspirate the interphase layer of cells along with the upper layer of serum and place them into a fresh 50 ml Falcon Tube. It is possible to combine 2-3 50 ml tubes together at this step.
		5.2.4	For each prep, add PBS up to 45 ml mark on the tube.
		5.2.5	Centrifuge this mixture for 7 minutes at 1500 rpm with the **brake on - low.**

		5.2.6	Discard the supernatant and re-suspend all of the cell pellets in 10 ml of 10% FCS prepared in RPMI (see Appendix A).
		5.2.7	Centrifuge cells for 5 minutes at 1500 rpm with the **brake on- -low**.
	5.3	When the centrifuge stops, take the tubes out and pour off the liquid. Re-suspend the pellet by tapping the tube.	
	5.4	Add 6 ml of Human AB serum to the tube.	
	5.5	Filter cell suspension through the 40uM BD Cell strainer.	
	5.6	Counting Cells	
		5.6.1	**Perform cell counts according to the steps in Section 6.0 of this SOP. Make sure to perform a red blood cell lysis prior to performing the cell count.**
	5.7	Centrifuge cells for 5 minutes at 1500 rpm with the **brake on - low**.	
	5.8	While cells are in the centrifuge, label the appropriate number of 1.8 ml cryotubes per patient based upon the cell count. Each cryotube will contain approximately 10 x 10^6 LMNCs.	
	5.9	Preparation for the Cryopreservation of Cells	
		5.9.1	When the centrifuge stops, take the tubes out adjust volume of the remaining solution to a cell concentration of 20x10^6 cells/ml of Human AB serum.
		5.9.2	Mix the LMNCs in the tube by gently tapping the side of the tube. **Do not use a pipette.**
		5.9.3	Add a volume of Freezing Medium B that is equal to that of the Human AB serum from step 5.9.1 to the tube containing the LMNCs. This will bring the cell concentration to 10x10^6 cells/ml.
		5.9.4	Once mixing is complete, quickly aliquot LMNC suspension into the pre-labeled 1.8 ml cryovials. Aliquot only 1.0 ml of cells suspension per cryovial. **DO NOT** freeze more than 10 x 10^6 cells per vial. **NOTE: Pipette gently to minimize sheer forces!**
	5.10	Freeze the cryovials using the appropriate Rutgers Cell and DNA Repository protocol for effective cryopreservation. The protocol used is dependent upon the freezer used.	
		5.10.1	The *ThermoForma* freezer requires the use of Rutgers Cell and DNA Repository protocol "Loading and Starting ThermoForma Control Rate Freezer, Version T.F.2.1"
		5.10.2	The *Planer Kryo 10 Series III* freezer requires the use of one of two Rutgers Cell and DNA Repository protocols depending on which equipment will be used to store the ITN cells: "Loading and Starting

			Planer Kryo 10 Series III Control Rate Freezer (MR3 Computer), Version MR3.1.2" or "Loading and Starting Planer Kryo 10 Series III Control Rate Freezer (MRV Computer), Version M.R.V. 1.2"
NOTE ON CELL YEILDS: Cells recovered will depend on the number and size of the lymph nodes you receive. There are other variables will affect the recovery, including the amount of time between procurement and processing and the subject's health and age.			
6.0	ViCell™ XR Viability Analyzer Operation and Analysis of LMNCs		
	SAFETY CAUTION **If injuries occur due to chemical exposure or a reagent spill occurs, IMMEDIATELY notify a supervisor and REHS at 732-445-2550. If accident occurs after hours, call Rutgers Police at 6-911. Give them the following information:** *Nature of accident *Your name and location (building and room numbers) *Your telephone number **The ViCell™ should be left on at all times, unless it is being serviced by a Beckman Coulter service technician.** **Do not place fingers near the carousel while the machine is operating to avoid injury. If a tube becomes jammed in the carousel remove it with forceps only and never by hand.**		
	6.1	Wear all appropriate personal protective equipment.	
	6.2	ViCell™ XR setup	
		6.2.1	Ensure that the computer attached to the ViCell™ XR is turned on.
		6.2.2	Double click on the "ViCell XR" icon to activate the software.
		6.2.3	Listen for the pump to turn on. The system will then go to idle mode as indicated on the Instrument Status and Control window.
		6.3.4	Observe the "Runs Left" indicator: If the number of runs left on the machine is less than the number of samples-plus-three, the reagent quad pack must be changed prior to use and the liquid waste container along with the used-cup bin must be emptied (see Appendix C for this procedure.)
		6.3.5	Ensure that a control has been run within the last 24 hours by checking the ViCell™ XR Usage Log. If a control has been performed proceed to section 6.4. If it is necessary to run a control, proceed to section 6.3.
	6.3	*Running Control*	
		6.3.1	Aliquot 0.5-1.0 ml of each concentration and viability controls into a sample cup and insert into the next available carousel position.

.		6.3.2	Double click on the “Control” icon on the left of the screen to display the login screen.	
		6.3.3	Ensure that the data in the “Current Lot” display corresponds with the control lot being used. If it does match, proceed to section 6.4. If it does not match, perform the following steps:	
			6.3.3.1	Single click on the “New Lot” button.
			6.3.3.2	Enter the following information as below.

Field Name	Data to Enter
Name	Concentration Control
Cell Type	Control
Assay Parameter	Total cell /ml
Acceptance Limits	1E6, 4E6, or 8E6
Comments	New Lot
Current Lot Fields	Information Obtained From Bottle

Minimum Diameter (µm)	2
Maximum Diameter (µm)	50
Minimum Circularity	0.9
Number of Images	50
Aspirate Cycles	2
Trypan Blue Mixing Cycles	3
Cell Brightness (%)	70
Cell Sharpness	75
Viable Cell Spot Brightness (%)	55
Viable Cell Spot Area (%)	1.0
De-cluster Degree	Low

6.3.3.3 Settings for Viability Control

Field Name	Data to Enter
Name	Viability Control
Cell Type	Control
Assay Parameter	Viability
Acceptance Limits	0%, 50%, or 100%
Comments	New Lot
Current Lot Fields	Information Obtained From Bottle

Minimum Diameter (µm)	5
Maximum Diameter (µm)	50
Minimum Circularity	0.9
Number of Images	50
Aspirate Cycles	2
Trypan Blue Mixing Cycles	3
Cell Brightness (%)	85
Cell Sharpness (%)	100
Viable Cell Spot Brightness (%)	60
Viable Cell Spot Area (%)	3.0
De-cluster Degree	Low

		6.3.4	Click “OK.”
		6.3.5	Click “Start Query.”
		6.3.6	When the query is complete, observe the graph that is displayed on the computer screen and insure that the data for the current date is within acceptable parameters. If the data is acceptable proceed with section. If it is not within acceptable parameters and troubleshooting methods recommended in the operator manual does not help, contact a Beckman Coulter service technician for service.
		6.3.7	Record the control run in the ViCell™ XR Usage Log.
	6.4	*Loading and Running Samples*	

<table>
<tr><td></td><td></td><td colspan="3">NOTE: Steps 6.4.1, 6.4.2, and 6.4.3 are performed aseptically under a class II biological safety cabinet.</td></tr>
<tr><td></td><td></td><td>6.4.1</td><td colspan="2">Add 0.450 ml of VersaLyse to a sample cup.</td></tr>
<tr><td></td><td></td><td>6.4.2</td><td colspan="2">Remove 50 µl of LMNC suspension from the flask and add it to the sample cup.</td></tr>
<tr><td></td><td></td><td>6.4.3</td><td colspan="2">Incubate for 10 minutes at RT and use Vi-Cell cell counter according to manufacturer’s instructions.</td></tr>
<tr><td></td><td></td><td>6.4.4</td><td colspan="2">Place the sample cup(s) into an empty carousel position and record the cell line number(s) in a notebook.</td></tr>
<tr><td></td><td></td><td>6.4.5</td><td colspan="2">Click the “Cell Type” icon in the tool bar window located on the left of the screen.</td></tr>
<tr><td></td><td></td><td>6.4.6</td><td colspan="2">In the tool bar to the right, select “Lymphoblastoid.”</td></tr>
<tr><td></td><td></td><td></td><td>6.4.6.1</td><td>Ensure the following parameters are met in the Cell Type window.
<table>
<tr><td>Minimum Diameter (microns)</td><td>7</td></tr>
<tr><td>Maximum Diameter (microns)</td><td>20</td></tr>
<tr><td>Number of Images</td><td>50</td></tr>
<tr><td>Aspirate Cycles</td><td>2</td></tr>
<tr><td>Trypan Blue Mixing Cycles</td><td>3</td></tr>
<tr><td>Cell Brightness (%)</td><td>85</td></tr>
<tr><td>Cell Sharpness</td><td>100</td></tr>
<tr><td>Viable Cell Spot Brightness (%)</td><td>65</td></tr>
<tr><td>Viable Cell Spot Area (%)</td><td>3.5</td></tr>
<tr><td>Minimum Circularity</td><td>0.1</td></tr>
<tr><td>De-cluster Degree</td><td>Low</td></tr>
</table>
IMPORTANT: If the parameters are not acceptable, contact a supervisor before continuing.</td></tr>
<tr><td></td><td></td><td>6.4.7</td><td colspan="2">Click the “Log In Sample” button to open the log in window.</td></tr>
</table>

			6.4.7.1	On the screen, select the carousel position for the sample cup and enter the respective cell line number under "Sample ID." (This step and steps 6.4.7.2, 6.4.7.3, and 6.4.7.4 must be repeated for each sample cup being run.) **Note:** The following characters may not be entered in the sample ID field: [ ] = : /
			6.4.7.2	Set dilution factor to 10.0.
			6.4.7.3	Enter any appropriate comments into the comment field.
			6.4.7.4	If the results are to be printed or saved to a Microsoft Excel file, select the appropriate box and/or file.
			6.4.7.5	Once all positions are recorded click "Start Queue." **IMPORTANT: Do not leave the ViCell™ XR while it is running. Sample cups can become jammed in the carousel and cause damage to the instrument. Use only forceps to remove jammed cups.**
		6.4.8	Once queue has been processed, the results can be recorded from the Previous Run Results window or accessed by opening "file," then opening "run," and opening the desired run.	
		6.4.9	After all samples have been analyzed, select "file" and "exit" to terminate the ViCell™ XR program.	

APPENDIX A

1X Dulbecco's PBS (DPBS)

HuAB serum: Human AB serum (GeminiBiotech), tested for assay compatibility, heat inactivated and filtered. It can be thawed and refrozen up to 3 times or can be thawed and kept at 4°C for up to one month.

Freezing Medium B: 20% DMSO (Sigma D2650) in heat inactivated HuAB serum, filtered. It can only be thawed one time and must be used the same day.

DMSO Hybri-Max tm Sigma D2650, graded for sensitive cells.
It is not primarily the toxicity of DMSO itself, but the osmotic shock caused by DMSO rapidly that harms the cells most. Also, the viability and functionality of the cells is much better if DMSO is added at room temperature.

200 ml of RPMI with 10% FCS

20 ml FCS

180 mL RPMI Medium 1640

Preparation of the 1% vol/vol antibiotic solution in RPMI1640

Mix 1 ml aliquot of Penicillin / Streptomycin solution (purchased from Gibco/Invitrogen)

With 99 ml of RPMI 1640. This will give you final 1 % volume to volume of antibiotic solution in RPMI 1640.

For future use: Prepare 5 ml aliquots of the antibiotics solution to be used for each 500 ml RPMI bottle. Aliquots of the antibiotics should be stored frozen at -80ºC until use

Appendix B

To calculate the correct centrifuge speed for a given RCF, use the following formula:

$$\text{RPM Speed Setting} = \sqrt{((RCF) \times (100{,}000) / (1.12) \times (r))}$$

Appendix C

Sieve and sieve base cleaning procedure

- Put sieve and sieve base in the sink and spread with 10% bleach and leave it for 5 min
- Rinse with tap water
- Clean with detergent **(7X® CLEANING SOLUTION)** as follow:
- Prepare a 1% solution in tap water and gentle scrubbing with a soft brush.
- Drain the used solution and rinse several times with tap water (warm is better).
- Leave them submerged into a recipient with tap water for at least 1 h
- Final-rinse using distilled water.
- Place cleaned items on clean bench-paper and allow to air dry.
- Send for sterilization.

Appendix D

Installing New Quad Pack

1. Open the reagent compartment door on the right side of the ViCell™ XR and remove the color coded caps from the empty reagent quad pack.
2. Install the color coded caps onto the new quad pack, ensuring that the color coding matches correctly.
3. Place new quad pack into the ViCell™ XR.
4. Remove the liquid waste bottle from the ViCell™ XR.
5. Install an empty 1 liter bottle onto the waste tube cap in the ViCell™ XR. Save the cap from the bottle.
6. Open the old quad pack and dispose remaining reagents into the liquid waste bottle that was just removed from the machine. Recap the full liquid waste bottle with the cap from the new empty bottle and dispose of the bottle as per the *Rutgers University Chemical Hygiene Guide*.
7. Remove and the used cup receptacle from the ViCell™ XR and empty it into the bio-hazardous waste container. Return the cup receptacle to the ViCell™ XR once empty.
8. Record quad pack change and/or prime into the ViCell™ XR Usage Log.

Appendix E

Deviations that must be recorded:

dev_code	dev_description
CCP-11	Lost all pellet while aspirating supernatant (CPT and Ficoll).
CCP-12	Layers did not separate properly during centrifugation (CPT and Ficoll).
CCP-13	ITN SOP violation noted.
KIT-1	Expired tube used
KIT-2	Incorrect kit used, or supplies taken from another kit.
KIT-3	Incorrect or non ITN tube used.
LBL-1	Specimen unidentifiable due to no label on specimen and no requisition form or shipping log
LBL-2	Specimen appears mislabeled, contents don't match specimen type
LBL-3	Tube label does not match requisition form
LBL-4	Tube label not on specimen, but found in shipping box
LBL-5	Illegible handwriting on tube label
LBL-6	Missing information on tube label
LBL-7	ITN label not used, but specimen identifiable
LBL-8	Incorrect label orientation
LBL-9	Patient identifying information on tube label.
NON-1	No deviation assigned due to no deviation noted
OTH-1	Other not specified
PRO-12	Primary tube or aliquot not completely filled
PRO-14	Received tube was not spun before shipping to the Core (CPT only)
PRO-3	Specimen hemolyzed, not separated, or clotted
PRO-4	Specimen not processed in adequate time per SOP
REQ-3	Requisition form not included with specimen
REQ-4	Incomplete / incorrect information on requisition form
REQ-5	Requisition form difficult to read
REQ-6	Specimen not found on requisition form
REQ-7	Incorrect specimen identifying information assigned on the requisition form
REQ-8	Specimen on requisition form not included in shipment
TEM-3	Specimen arrives colder than expected
TEM-6	Specimen not properly stored at specified temperature and conditions
TRA-1	Specimen arrives at laboratory from site > 36 hours from collection
TRA-2	Specimen sent to the wrong location
TRA-4	Specimen leaked or broken
TRA-8	Shipment lost in transit
TRA-9	ITN shipping procedures not followed
TRA-10	No Shipment notification.