Preparation of Single Cell Suspension from Human Lymph Node Tissue

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1. Abstract

This protocol describes a method for the isolation of pan-lymphocytes, pan-myeloid cells, and progenitors from human lymph node tissue. By providing defined media formulations, volumes at each step, and a defined dilution factor for density centrifugation, it yields consistent single-cell suspensions across samples.

2. Materials

10mL Syringe (Fisher Scientific, Cat. No.: 14-955-459)

Dulbecco's Phosphate Buffered Saline (DPBS) (Fisher Scientific, Cat. No.: 14-190-144)

Penicillin-Streptomycin-Glutamine (100X) (Fisher Scientific, Cat. No.: 10-378-016)

50mL Centrifuge Tube (Fisher Scientific, Cat. No.: 12-565-271)

Iscove's Modified Dulbecco's Medium (IMDM) (Fisher Scientific, Cat. No.: 12-440-053)

Fetal Bovine Serum (FBS) (Fisher Scientific, Cat. No.: 10-099-14)

EDTA 0.5M pH 8.0 (Fisher Scientific, Cat. No.: 15-575-020)

100µM cell strainer (Fisher Scientific, Cat. No.: 50-146-1428)

Ficoll-Paque™ PLUS Media (Fisher Scientific, Cat. No.: 45-001-749)

Collagenase D (2.5g) (Sigma, Cat. No.: 11088882001)

DNase (100mg) (Fisher Scientific, Cat. No.: NC9709009)

Mr. Frosty (Fisher Scientific, Cat. No.: 51000001)

Cryostor CS10 (Fisher Scientific, Cat. No.: NC9930384)

Cryogenic Vials (Fisher Scientific, Cat. No.: 09-761-71)

5mL Falcon™ Round-Bottom Polypropylene Tubes (Fisher Scientific, Cat. No.: 14-959-11A)

Solution 13 AO/DAPI (Chemometec, Cat. No.: 910-3013)

NC-Slide A8 (Chemometec, Cat. No.: 942-0003)

Falcon™ Plastic Disposable Transfer Pipets (Fisher Scientific, Cat. No.: 13-680-50)

3. Equipment

Multi-Axle-Rotating Mixer/Shaker with Temperature Control

Centrifuge

Cell Counter - NC-3000

Surgical scissors

Scale

4. Protocol

4.1. Preparing Mediums and Buffers

4.1.1. Create the following **IMDM-FBS-PSQ Media** in a 500mL bottle of IMDM by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.**
IMDM	500		-
Penicillin-Streptomycin-Glutamine	5	100X	1X
FBS	50	100%	10%

Table 1.
**Final Concentration is approximate.*

4.1.2. Create the following **DPBS-FBS-EDTA Solution** in a bottle of DPBS without calcium and magnesium by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.**
DPBS	500	-	-
FBS	25	100%	5%
EDTA	1	0.5M	1mM

Table 2.
**Final Concentration is approximate.*

4.2. Tissue Dissociation

4.2.1. Clean lymph nodes of fat and connective tissue post dissection, record the site below.

_____

4.2.2. Add up to 2 ± 10% grams of cleaned lymph node tissue to per 50mL centrifuge tube – record the total weight below.

_____g

NOTE: Going beyond the 2 grams of tissue per tube reduces the efficacy of the enzymatic digest and lowers yields.

4.2.3. Add 5mL of room temperature IMDM **(NO ADDITIVES! Just the base media formulation)** to each tube and use a scissors to chop the tissue into a fine "mash".

4.2.4. Add 35mL of room temperature IMDM **(NO ADDITIVES! Just the base media formulation)** and spike in 0.400mL of Collagenase D, and 0.400mL of DNAse to the tube to begin the enzymatic digestion. Place on a shaker or rotator for 30 minutes at 37°C.

4.2.5. After digestion, add 0.500mL of EDTA 0.5M pH 8.0 to the digested cell suspensions and incubate for 2 minutes at 20°C.

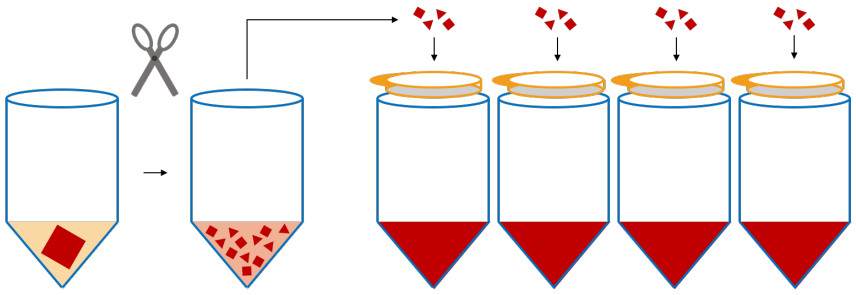

Figure 1. Steps 4.2.3 through 4.2.7.

4.2.6. Distribute and filter the mash of tissue over 100µM cell strainers above 50mL tubes (about 4 filters/2 grams of tissue).

NOTE: Cell yields and ease of pushing through the filter are increased by using multiple filters/gram of tissue, default to using more filters to decrease processing time, and increase yields.

4.2.7. Apply pressure with the black rubber bottom or the plastic end of a 10mL syringe plunger to any remaining, partially digested tissue on the cell strainers, and intermittently wash through with DPBS-FBS-EDTA Solution from a transfer pipet – the aim is to push and wash through the tissue until only light pink/white/grey connective tissue remains. When finished, combine the tubes of cell suspension and proceed to the next section.

4.3. Ficoll-Paque

4.3.1. Centrifuge the cell suspensions for 10 minutes at 400 × g at 20°C.

4.3.2. Remove the supernatants and combine the cell pellets down to a single 50mL tube, top to 50mL with room temperature IMDM-FBS-PSQ Media, spike in 0.500mL of EDTA 0.5M pH 8.0.

4.3.3. Filter the cell suspension through a 100µM cell strainer.

4.3.4. In two 50mL tubes, layer 25mL of cell suspension on top of 15mL of Ficoll-Paque Media PLUS.

4.3.5. Spin for 20 minutes, 1200 x g at 20°C with 4 acceleration and 0 brake, evenly distribute the tubes across the entire rotor to prevent wobbling (use all four buckets if possible as opposed to just two).

4.3.6. Remove the mononuclear cell layer from both tubes with a transfer pipet and combine in one 50mL tube. Add cold DPBS-FBS-EDTA Solution to a final volume of 50mL and centrifuge the cell suspension for 10 minutes at 400 × g, 4°C.

4.3.7. Remove the supernatant and re-suspend the cell pellet in 50mL cold DPBS-FBS-EDTA Solution and centrifuge the cell suspension for 10 minutes at 120 x g, 4°C.

4.3.8. Remove the supernatant and re-suspend the cell pellet in cold 10mL IMDM-FBS-PSQ Media.

4.4. Cell Count

4.4.1. Count cells, and viability by using the NC-3000 cell counter. Calculate total viable cells and record below:

cell number: _____cells/mL,_____% viable

final volume: _____mL

$$cell\ number\left(\frac{cells}{mL}\right) * viability(\%) * final\ volume(mL) = total\ viable\ cells$$

Total Viable Cells: _____

4.5. Analysis and Freeze-down

4.5.1. **(Optional QC)** Aliquot 2 x 10^6 cells to a 5mL Falcon tube and place on ice for subsequent flow cytometric analysis.

4.5.2. Aliquot cells for analysis or experimentation, and then freeze down cells in up to 3 x 10^7 aliquots using Cryostor CS10 Medium, a Mr. Frosty, and a -80°C freezer (1-1.5mL aliquots, round down to the nearest 20 million cells and discard/freeze/use any left over cells). Record the number of vials frozen: _____.