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Preparation of Single Cell Suspensions of the Intra-Epithelial Layer and Lamina Propria from Human Intestinal Tissue

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ABSTRACT

This protocol describes a method for the isolation of the immune cells, structural and epithelial cells, and progenitors from the epithelial layer and the lamina propria of human gut sections of about one gram of 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. This protocol can be used for any section of the intestinal tract from duodenum to distal colon.

ATTACHMENTS

dzhibk587.pdf

MATERIALS

Materials:

- Fisherbrand™ Sterile Syringes for Single Use **Fisher Scientific Catalog #14955459**
- Benzonase nuclease **Sigma Aldrich Catalog #E1014-5KU**
- Dulbeccos phosphate-buffered saline (DPBS) **Gibco - Thermo Fischer Catalog #14190144**

Protocol status: Working
We use this protocol and it's working

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Penicillin-Streptomycin-Glutamine (100X) **Thermo Fisher Catalog #10378016**

Thermo Scientific™ Nunc™ 50mL Conical Sterile Polypropylene Centrifuge Tubes **Fisher Scientific Catalog #12-565-271**

Thermo Scientific™ 250mL Conical Centrifuge Tube **Fisher Scientific Catalog #12566441**

DTT (Dithiothreitol) **Cell Signaling Technology Catalog #7016**

Gibco™ IMDM (Iscoves Modified Dulbeccos Medium) **Fisher Scientific Catalog #12-440-053**

Gibco™ Fetal Bovine Serum qualified Australia **Fisher Scientific Catalog #10-099-141**

UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**

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

Ficoll-Paque™ PLUS Media **Fisher Scientific Catalog #45-001-749**

Collagenase D **Sigma Aldrich Catalog #11088882001**

Mr. Frosty™ Freezing Container **Fisher Scientific Catalog #5100-0001**

DNASE 1 100MG **Fisher Scientific Catalog #NC9709009**

CryoStor CS10 100ML **Fisher Scientific Catalog #NC9930384**

Corning™ Externally Threaded Cryogenic Vials **Fisher Scientific Catalog #09-761-71**

5mL Falcon™ Round-Bottom Polypropylene Test Tubes **Fisher Scientific Catalog #14-959-11A**

Solution 13 AO – DAPI **Chemometec Catalog #910-3013**

NC-Slide A8™ box with 25 Slides **Chemometec Catalog #942-0003**

Equipment:

- Multi-Axle-Rotating Mixer
- Centrifuge
- Cell Counter - NC-3000
- Surgical scissors
- Scale

Preparing Mediums and Buffers

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

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

Table 1.

2 Create the following DPBS-FBS Solution in a bottle of DPBS by using the table below:

	A	B	C	D
	Component	Volume (mL)	Starting Conc.	Final Conc.
	DPBS	500	-	-
	FBS	25	100%	5%

Table 2.

3 Create the following IMDM-FBS-PSQ-EDTA-DTT Media in a 500mL bottle of IMDM by using the table below:

	A	B	C	D
	Component	Volume (mL)	Starting Conc.	Final Conc.
	IMDM	500	-	-
	FBS	50	100%	10%
	Penicillin-Streptomycin-Glutamine	5	100X	100X
	EDTA	10	0.5M	10mM
	DTT	1	1M	2mM

Table 2.

4 Create the following **DPBS-FBS-EDTA Solution** in a bottle of DPBS by using the table below:

	A	B	C	D
	Component	Volume (mL)	Starting Conc.	Final Conc.
	DPBS	500	-	-
	FBS	25	100%	5%
	EDTA	1	0.5M	10mM

Table 2.

Tissue Preparation

5 Use a surgical scissors to remove about 7 cm - 8 cm of intestinal tissue section from the mysentary. Remove any remaining mysentary from the intestinal tissue.

6 Gently massage the chyme or fecal matter out of the tissue over a bucket.

7 Cut open the tissue on a tray containing cold DPBS-FBS Solution and add the tissue to a 250 mL conical with 100 mL of cold DPBS-FBS Solution and using a forceps gently agitate the tissue to remove yellow/brown chyme, fecal matter and/or mucus.

8 Discard the DPBS-FBS solution into a bucket, replace with 100 mL of cold DPBS-FBS Solution and continue to wash the tissue until the DPBS-FBS Solution is no longer brown – when it has been successfully cleaned the DPBS-FBS Solution it should appear cloudy yellow/white. Depending upon how clean the tissue is this may take numerous washes (anywhere between 3-10, perhaps more).

Tissue Dissociation – Epithelial Stripping (IE Fraction)

1h

9 Add 1±0.2 grams of the cleaned intestinal tissue to a 50 mL centrifuge tube and record the weight below:
Total weight. _____g.

Note

NOTE: Going beyond the 1±0.2 grams of tissue without concomitantly increasing the number of tubes reduces the efficacy of the eventual enzymatic digest and lowers yields.

10 Add 20 mL of Room temperature IMDM-FBS-PSQ-EDTA-DTT Media to the tissue-containing 50 mL tube. Incubate on a shaker for 00:30:00 at 37 °C. 30m

11 Filter the cell suspension through a [M] 100 micromolar (µM) filter into a 50 mL conical, rinse the tissue and the filter with 20 mL of DPBS-FBS Solution. Set the cell suspension aside at 4 °C. Place the remaining tissue into a back into its original 50 mL conical.

12 Re-add 20 mL IMDM-FBS-PSQ-EDTA-DTT Media to the tissue and incubate on a shaker for 00:30:00 at 37 °C. 30m

13 Filter the cell suspension through a [M] 100 micromolar (µM) filter into a 50 mL conical, rinse the tissue and the filter with 20 mL of DPBS-FBS Solution. Set the cell suspension aside at 4 °C. Place the remaining tissue into a back into its original 50 mL conical.

Tissue Dissociation – Lamina Propria Digestion (LP Fraction) 32m

14 Add 5 mL of Room temperature IMDM (NO ADDITIVES! Just the base media formulation) to the tube and use a scissors to chop the tissue into a fine “mash”.

15 Add 40 mL of Room temperature IMDM (NO ADDITIVES) and spike in 0.400 mL of Collagenase D, and 0.400 mL of DNAse to the tube to begin the enzymatic digestion. Place on a shaker for 00:30:00 at 37 °C. 30m

16 After digestion, add 0.500 mL of EDTA [M] 0.5 Molarity (M) pH 8.0 to the digested cell suspensions and incubate for 00:02:00 at 20 °C. 2m

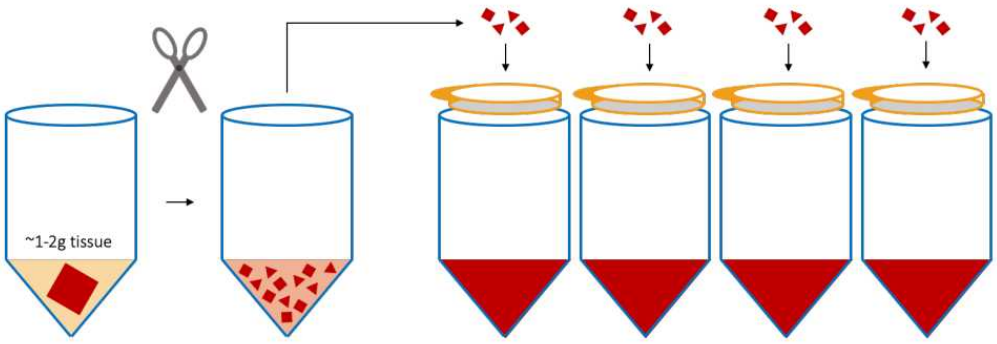

Figure 1. Steps 4.4.4 through 4.4.5.

17 Distribute and filter the mash of tissue over [M] 100 micromolar (µM) cell strainers above 50 mL tubes (about 4 filters/gram of tissue).

Note

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.

18 Apply pressure with the black rubber bottom or the plastic end of a 10 mL 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. When finished, combine the tubes of cell suspension and proceed to the next section.

Ficoll-Paque 1h 20m

19 Centrifuge the cell suspensions (EL and LP fractions) for 00:10:00 at 400 x g at 20 °C 10m

20 Remove the EL and LP supernatants and combine the cell pellets down to a single 50 mL, 1 g /tube, keep the fractions distinct, add 10 mL with Room temperature **IMDM (NO ADDITIVES)**.

21 Add 10 µL of benzonase/1 gram of tissue to the EL and LP fractions and incubate at 37 °C 30m 00:30:00.

22 Add 15 mL of IMDM (NO ADDITIVES) to the cell suspension, spike in 0.250 mL of EDTA [M] 0.5 Molarity (M) pH 8.0 to all tubes.

23 Filter both the EL and LP cell suspensions through a [M] 100 micromolar (µM) cell strainer.

24 Layer 25 mL of cell suspension (both IE and LP fractions) on top of 15 mL of Ficoll-Paque Media PLUS.

25 Spin for 00:20:00, 1200 x g at 20 °C with 4 acceleration and 0 brake, evenly distribute 20m tubes across the entire rotor to prevent wobbling (use all four buckets if possible as opposed to just two).

26 For both fractions, remove the mononuclear cell layer with a transfer pipet and transfer to a separate 10m 50 mL tubes. Add cold DPBS-FBS-EDTA Solution to a final volume of 50 mL and centrifuge the cell suspensions for 00:10:00 at 400 x g, 4 °C.

27 Remove the supernatant and re-suspend the cell pellet in 50 mL cold DPBS-FBS-EDTA Solution 10m centrifuge the cell suspension for 00:10:00 at 120 x g, 4 °C.

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

Cell Count

29 **IE Fraction** - 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
$cell\ number\ (cells/mL) * viability(\%) * final\ volume\ (mL) = total\ viable\ cells$
Total Viable Cells: _____

30 **LP Fraction** - 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
$cell\ number\ (cells/mL) * viability(\%) * final\ volume(mL) = total\ viable\ cells$
Total Viable Cells: _____

Freeze-down

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

32 Aliquot cells for analysis or experimentation, and then freeze down cells in up to 5 x 10^6 aliquots using Cryostor CS10 Medium, a Mr. Frosty, and a -80 °C freezer (1 mL - 1.5 mL aliquots, round down to the nearest 5 million cells and discard/freeze/use any left over cells). Record the number of vials frozen: _____.