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HPAP Processing Protocol V.2

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Michael Betts[1], Gregory Golden[1]

[1]University of Pennsylvania

Michael Betts: betts@mail.med.upenn.edu
Gregory Golden: Gregory.Golden1@Pennmedicine.upenn.edu

Human Islet Research Ne…

Sandy Beshir
City of Hope

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

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Abstract

Early in life, a combination of environmental insults and genetic pre-disposition results in an autoimmune reaction to pancreatic b-cells in the Islets of Langerhans, leading to b-cell destruction and a lack of insulin production. Over time, near-complete loss of insulin production leads to Type I Diabetes (T1D), an often-deadly disease that requires lifetime treatment. Many studies have focused on immune system components that drive T1D autoimmunity, particularly the immune cells that infiltrate the islets of Langerhans. However, acute insulitis is extremely difficult to detect, as T1D pancreas samples are rare and individual islet destruction is thought to happen rapidly and sporadically. Therefore, studies have focused on autoimmune imprints within memory T and B cells that may develop after insulitis. Memory T and B cells that target b-cells would likely reside in lymph nodes that drain the pancreas and are potentially detectable in circulation or in the spleen. The Human Pancreas Analysis Program has organized a protocol to isolate single immune cells from the blood, spleen, and peri-pancreatic lymph nodes (PLNs) from healthy, pre-diabetic, and T1D patients. Herein, we describe the standardized protocol of single cell isolation from blood, spleen, PLNs, mesenteric LNs, and superior mesenteric LNs.

Materials

- ACK Lysing Buffer 100mL **Quality Biological Catalog #**118-156-721
- Collagenase-D, 10mg/mL
- Dimethyl sulfoxide 100mL **Sigma Aldrich Catalog #**D2650-100ML
- DNase I Recombinant, RNAse free (10000U/mL) **Sigma Aldrich Catalog #**4716728001
- Fetal Bovine Serum 500mL **Gemini Bioproducts Catalog #**100-500
- Ficoll Paque Plus 500mL **Ge Healthcare Catalog #**17-1440-03
- L-glutamine 200mM 100mL **Corning Catalog #**25-005-CI
- Penicillin/streptomycin 10000 U each 100mL **Lonza Catalog #**DE17-602E
- PFA: 4% in PBS
- RPMI 1640,1x with L-glutamine,1L **Corning Catalog #**10-040-CM
- Conical, 50mL
- Cryovials 2mL **Corning Catalog #**430488
- Freezing container, Corning CoolCell
- Sterile Forceps
- gentleMACS C Tubes **Miltenyi Biotec Catalog #**130-093-237
- gentleMACS Dissociator **Miltenyi Biotec Catalog #**130-093-235
- MACS SmartStrainers 70µm **Miltenyi Biotec Catalog #**130-098-462
- MACS SmartStrainers 100µm **Miltenyi Biotec Catalog #**130-098-463
- SepMate-50 **Stemcell Technologies Catalog #**85450
- Sterile Scalpel
- Sterile Syringe, 5mL

1 **BLOOD**

1. Spin down vials at 2000rpm for 15min at room temperature

2. Aliquot plasma into cryovials and freeze and store at -80 °C ; toss remaining plasma

3. Measure volume of spun down blood and transfer to a new conical tube

4. Using a 1:1 ratio of spun blood to R10 media, wash vials and transfer to the conical with the spun down blood
a. R10 media = RPMI + 10% fetal bovine serum + 2mM L-Glutamine + 100 U/mL Penicillin + 100 µg/mL Streptomycin

5. In a SepMate™-50 conical, add 15 mL of ficoll below the physical barrier

6. Slowly add the blood to the top of the ficoll, taking care not to mix the two

7. Spin down at 1200 g for 10min at room temperature

8. Once separated, pour the supernatant with the PBMCs into a fresh conical

9. Spin down at 500 g for 7min

10. Pour off supernatant and resuspend the pellet in ~2mL ACK Lysis buffer

11. Incubate at room temperature for 5min and then quench with 18 mL R10 media

12. Spin down at 500 g for 7min

13. Resuspend pellet in 10 mL of media

14. Count cells

15. Spin down at 500 g for 7min

16. Pour off supernatant and resuspend the pellet in FBS + 10% DMSO at 5-10 million cells per mL

17. Freeze in 1 mL aliquots at -80 °C in a Mr. Frosty freezing container for 24-48hrs

18. For long term storage, store in liquid nitrogen

2 LYMPH NODE PROCESSING

1. Prepare media by adding 50 µL DNase + 50 mL R10 media into one conical for each tissue type

2. Weigh tissue in a culture dish and record mass

3. Add 10 mL of prepared media to the culture dish

4. Place tissue in dish and gently cut away the fat

5. Rinse with 10 mL of prepared media

6. For histology:

a. Cut off a small piece of tissue (~1-2cm) and place in 50 mL freshly prepared PBS + 4% PFA solution

b. Write date and time on the tube and store for ~24hrs at RT

c. Transfer to 50 mL conical with 80% EtOH and store at 4 °C

7. Place tissue pieces in a 70µm cell strainer and grind the tissue with the top end of a 5 mL syringe

8. Strain media in plate through the cell strainer into a fresh 50 mL conical

9. Rinse plate several times with remaining media and strain through the cell strainer into the conical

10. Spin down at 500 g for 7min

11. Resuspend pellet in 10 mL of R10 media

12. Count cells

13. Spin down at 500 g for 7min

14. Pour off supernatant and resuspend the pellet in FBS + 10% DMSO at 5-10 million cells per mL

15. Freeze in 1 mL aliquots at -80 °C in a Mr. Frosty freezing container for 24-48hrs

16. For long term storage, store in liquid nitrogen

3 SPLEEN PROCESSING

1. Prepare media by adding 50 µL DNase + 50 µL Collagenase + 50 mL R10 media

2. Weigh tissue in a culture dish and record mass

3. Cut tissue into ~2cm pieces

4. Dissociate using a gentleMACS with the tissue suspended in prepared media

5. Incubate for 15min at 37 °C on a rotator

6. Repeat gentleMACS dissociation

7. Strain suspension through a 100µM filter

8. Spin down at 500 g for 10min

9. Pour off supernatant and mix in ~10mL ACK Lysis buffer

10. Let sit for 5min and then quench with 40 mL of R10 media

11. Strain suspension through a 70µM filter

12. Spin down vials at 500 g for 10min at room temperature

13. Add 10 mL ficoll to a clean 50 mL conical

14. Resuspend cells and very carefully add to the top of the ficoll

15. Spin down at 2200rpm for 20min with the brake off

16. Resuspend cells with 10 mL of R10 media

17. Dilute 10 µL of the suspension in 990 µL of PBS

18. Count cells and multiply by 1000 to obtain the final cell count

19. Spin down at 500 g for 10min

20. Pour off supernatant and resuspend the pellet in 20-30mL FBS + 10% DMSO

21. Freeze in 20-30 aliquots at -80 °C in a Mr. Frosty freezing container for 24-48hrs

22. For long term storage, store in liquid nitrogen