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Human Thymus single cell dissociation protocol - Teichmann Lab

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Teichmann lab

Nadav Yayon

This protocol comes as is with the purpose of knowledge sharing and open science, however there is no guarantee this protocol will work although we strongly believe it will.

This Protocol is intended for human Thymus single cell dissociation.
It includes tissue preservation and handling, Enzymatic dissociation, FACS an MACS enrichments.
Developed In the Teichmann lab at the Sanger institute, Wellcome Gemone Campus, UK

VK, CS and JP contributed equally

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human, FACS, MACS, dissociation, Liberase, single cell, thymic

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Note: Please follow safety and MSDS guidelines for all included reagents

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Tick List Before Starting

1. RPMI
2. PBS
3. EDTA
4. DMSO
5. FBS
6. RNAlater
7. cryovials
8. petri dishes
9. scalpels and handles
10. Sharp forceps
11. Cell strainer (70um)
12. RBC lysis buffer, eBioscience™ (00-4333-57);

Enzymes

1. Liberase TH stock [M]**2.5 mg/mL** – Roche, 05401135001
2. Trypsin – EDTA (0.25%) – Gibco, 25200056
3. DNaseI (1000X) – Roche, 4716728001
4. Collagenase type IV (stock 50mg/ml current stock might be different) – Gibco, 17104019

Other components

1. Cell counting: C-Chips and Trypan Blue
2. 5'v2 10x GEM Kit (+Beads)
3. Sony Sorting Chip - (130 µm nozzle size)

For Magnetic cell sorting:

1. MidiMACS Separator (Miltenyi Biotec)
2. LS columns MACS, Miltenyi Biotec https://www.miltenyibiotec.com/GB-en/products/macs-cell- separation/columns/ls-columns.html# 130-042-401 * 25 columns (130-042-401)
3. CD45 MicroBeads, human (130-045-801) 2mL total (10 mkl per 10^7 cells)
4. CD326 (EpCAM) MicroBeads, human (130-061-101)

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Materials and reagents

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Same Day Buffers - Make fresh

5 **DM1** - Digestion media I:

a) **9.35 ml** RPMI
b) **640 ul** type IV collagenase stock (stock 50mg/ml from freezer -20°C, final conc. 3.2mg/ml)
c) **10 ul** DNase I stock aliquot (DNase I aliquot from -20°C, use at 1:1000 dilution)
d) Prewarm in water bath

DM2 - Digestion media II:

a) **9.79 ml** RPMI
b) **200 ul** Liberase TH (2.5mg/ml stock, 50X, final 50ug/ml)
c) **10 ul** DNase I stock aliquot
d) Prewarm in water bath

Stop solution: 2% FBS in RPMI (**40 ml** RPMI + **800 ul** FBS), Keep on ice

FACS buffer:

a) **49.55 ml** PBS

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b) **250 ul** 100% FBS
c) **200 ul** 0.5M EDTA (final 0.5% FBS + 2mM EDTA)
d) Keep on ice

Working Protocol

6 When sample arrives measure weight and size, and take note of time of arrival, patient number and storage type.

Histology (Optional):
Divide sample 2 ways (½ for histology – ½ for dissociation)
- 1/3 for snap freezing
- 1/3 for OCT mounting
- 1/3 for FFPE

Tissue dissociation:
Divide sample 4 ways:
- 1/4
  -- for 10% DMSO 90% FBS freezing (small chunks of 3-4mm)
  -- Keep small part for RNAlater – place 1 ml of RNAlater in cryovial, add sample, top up with more RNAlater, store in - 80°C freezer
- 1/4 for collagenase type IV digestion (**DM1**)
- 2/4 for liberase TH digestion (**DM2**)

7 1. Prepare hood (wipe with 70% EtOH); bring sterile petri dishes and scalpels and digestion solution

2. Place tissue onto petri dish (in the hood), remove any attached fat. If fat tissue is not clear proceed with whole sample.

3. Mince the tissue and put it into 2 x 50ml falcon tube filled with DM1or DM2 (depending on size of tissue (2-3
cm2)– add appropriate volume of digestion media i.e. if big add 40ml)
a. Do not use pipet tips as this can be sticky
b. Use scalpel to carry minced tissue
c. Wash scalpel with digestion solution to put all remaining tissue into the falcon tube

4. Incubate at 37°Con the rotator (put on middle speed, check the tissue every 10 minutes). Don't go more than 1
hour. If liberase doesn't look digested then top up with double the concentration of liberase & dnaseI.

5. Filter through strainer (leave the undigested tissue to settle to bottom) and add 10ml of **stop solution**. Leave aside
on ice and label either "with DM1" or "with DM2". Cells digested with DM1 will be banked (in 90% FBS/10% DMSO)
AFTER SORTING. Cells digested with DM2 will be FACS sorted for EpCAM+ (i.e. thymic

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epithelial cells)

6. Combine the undigested tissue from DM1 and DM2 and add Trypsin – EDTA (0.25%) (pre-warm ~15mL Trypsin at
37°C) for further digestion (limit to 15min) – add DNAseI at 1000x concentration.

7. Incubate at 37°C on the rotator (put on middle speed, check the tissue every 10 minutes).

8. While trypsin digestion is occurring, spin down the 50ml tubes from digestion I and digestion II in 500g at 4°C for
5min to pellet the cells.

9. Leave 20mL RBC lysis buffer at room temp.

10. If the cells appear visibly red, resuspend cell pellet in 10ml 1x RBC lysis buffer. Incubate for 3-5min at RT. Then
top up with PBS to 40ml. Strain through strainer. Centrifuge 500g for 5min.

11. Resuspend the cells in 5ml of stop solution. Count cells. Cells digested with trypsin will be FACS sorted for
CD45- (i.e. stromal cells)

12. Once trypsin digestion is done, spin down the 50ml tube in 500g at 4C for 5min to pellet the cells.

13. If the cells appear visibly red, resuspend cell pellet in 5ml 1x RBC lysis buffer. Incubate for 3-5min at RT. Then top
up with PBS to 40ml. Strain through strainer. Centrifuge 500g for 5min.

14. Resuspend the cells in 5ml of stop solution. Count cells.

8 MACS enrichment

In the next steps prepare 3 vials and profile them on 5' 10X V(D)J:
a.Total cell fraction;
b.CD45 depleted cell fraction;
c.EPCAM enriched cell fraction.
Note: Use LS (enrichment) columns for both CD45 depletion and EPCAM enrichment.

Magnetic sorting (CD 45+ depletion and EPCAM+ enrichment)

15. Prepare solutions & equipment:
    a. MACS Buffer: PBS + 0.5% BSA + 2mM EDTA: keep cold and degase
    b. LS MACS columns and separators
    c. CD45 MicroBeads, human (130-045-801) 2mL total (10 mkl per 10^7 cells)
    d. CD326 (EpCAM) MicroBeads, human (130-061-101)

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*Use cells up to 10^7 (*for higher volumes, scale up) with 20 ul of MicroBeads (magnetic labelling);
** Use cells up to 10^8 (*for higher volumes, scale up) with 500 ul of buffer (separation);

16. Determine cell numbers which would be used for the total fraction, sorting of EPCAM+ & CD45- cells, separate it in three vials. Following on that, centrifuge cells that will be used for magnetic sorting at 300*g for 10min -> resuspend cell pellet in 80ul of buffer per 10^7 cells;

17. Add 20ul of CD45/ EPCAM MicroBeads per each 10^7 cells -> Mix well and incubate for 15 mins (CD45) and 30 min (EPCAM) at 4'C. (in the refrigerator)

18. Wash cells by adding 1-2 ml buffer per 10^7 cells and centrifuge at 300g for 10min.

19. Discard supernatant.

20. Resuspend up to 10^8 cells in 500ul buffer (*for higher volumes, scale up).

Enrichment and depletion with LS columns

21. Prepare 2*LS Columns (CD45 – and EPCAM +) by rinsing with 3mL of degassed buffer. Discard effluent and change collection tube (use LS column immediately).
22. Apply CD45 and EPCAM stained cell suspensions onto the appropriate columns:
!(CD45- fraction) Collect cells that pass through (unlabeled cell fraction)

23. Wash columns by adding 3 ml of buffer 3 times (CD45) or 4 times (EPCAM), waiting for column reservoir to be empty. **!(CD45- fraction)** Collect washing buffer.

24. Remove column from the separator and place it on collection tube, add 5ml of MACS buffer onto the column, immediately flush out fraction with magnetically labelled cells:
!(EPCAM+ fraction)
25. Spin down the cells, resuspend in small volume and count for 10X loading.

9 FACS staining and sorting

Spin down cells from digestion II and trypsin, and resuspend in FACS buffer (5M in 50ul). For EpCAM+ need 50M (so 500uL). For CD45- take all cells from trypsin digest.

15. FACS staining (in 1.5ml Ependorfs) Vortex bottle of Ultra Comp Beads beforehand and invert up and down a few

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times.

16. Add FACS buffer and cells.

17. Add 5ul TruStain. Mix and incubate at 4 degrees for 10min. (prepare tubes with beads)

18. Add mix of antibodies (2ul each) and incubate further for 30min.

Dilute DAPI stock solution 1:100 i.e. take 1ul of stock solution and add 99ul water to make final concentration
0.14mM. Then add 2ul of diluted DAPI per 100ul of sample. Incubate for 15min.

19. Add 1ml FACS buffer, centrifuge 300g for 3min. Then resuspend cells in 1ml FACS buffer.

20. Bring 15ml tubes with 1ml 2% FBS in PBS buffer for collection.

	FACS buffer	CD45 BV785	EPCAM PE	Cells	TruStain	DAPI
Digestion II + all stain	39ul	2ul	2ul	50ul cells	5ul TruStain	2ul 1:100 DAPI
Digestion trypsin + all stain	39ul	2ul	2ul	50ul cells	5ul TruStain	2ul 1:100 DAPI
Beads + CD45	100ul FACS buffer	1ul		1 drop beads		
Beads + EPCAM	100ul FACS buffer		1ul	1 drop beads		
Cells + DAPI	50 ul			50ul cells		2ul 1:100 DAPI
Unstained cells	50 ul			50ul cells		

21. FACS Sorting: 130 Nozzle size
a.Trypsin digested cells - sort for DAPI-EpCAM+ cells + DAPI-EpCAM-CD45-
b.Using DM2 cells-sort for DAPI-EpCAM+ cells + DAPI-EpCAM-CD45-

We want at least 40,000 EpCAM+ cells and 40,000 CD45- cells

10x loading

22. Spin down sample (loose half of cells) optimal concentration 1,000 cells/ul. If less than 40ul then 'resuspend' in
40ul (use 6ul for counting). Transfer as many cells as possible for loading if table has blank cell for target cell
recovery.

For unsorted i.e. total -> liberase TH (because we only took 50 million)

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Aim for: 2 total, 2 CD45-, 1-2 EpCAM+

Appendix

10 Reconstitution for liberase TH

Reconstitute the lyophilized enzyme with injection-quality sterile water or tissue-dissociation buffer. Do not add serum or other components such as albumin or protease inhibitors, to the dissociation buffer. Enzyme stability is reduced at higher concentrations and warmer temperatures (4 °C). Avoid both the above conditions.

Reconstitute the entire vial. Do not weigh individual aliquots of the lyophilizate. The introduction of moisture into the vial results in a decline in enzymatic activity.

Place vial on ice to rehydrate the lyophilized enzyme.
Gently agitate the vial at 2 to 8 °C until enzyme is completely dissolved (max. 30 min).

2 ml (1 vial with 5 mg–10 mg pack size), 10 ml (1 vial with 50 mg–100 mg pack size)

Weight	Stock concentration	Vol. water	Aliquots
5mg	2.5mg/ml	2ml	5 x 400ul

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