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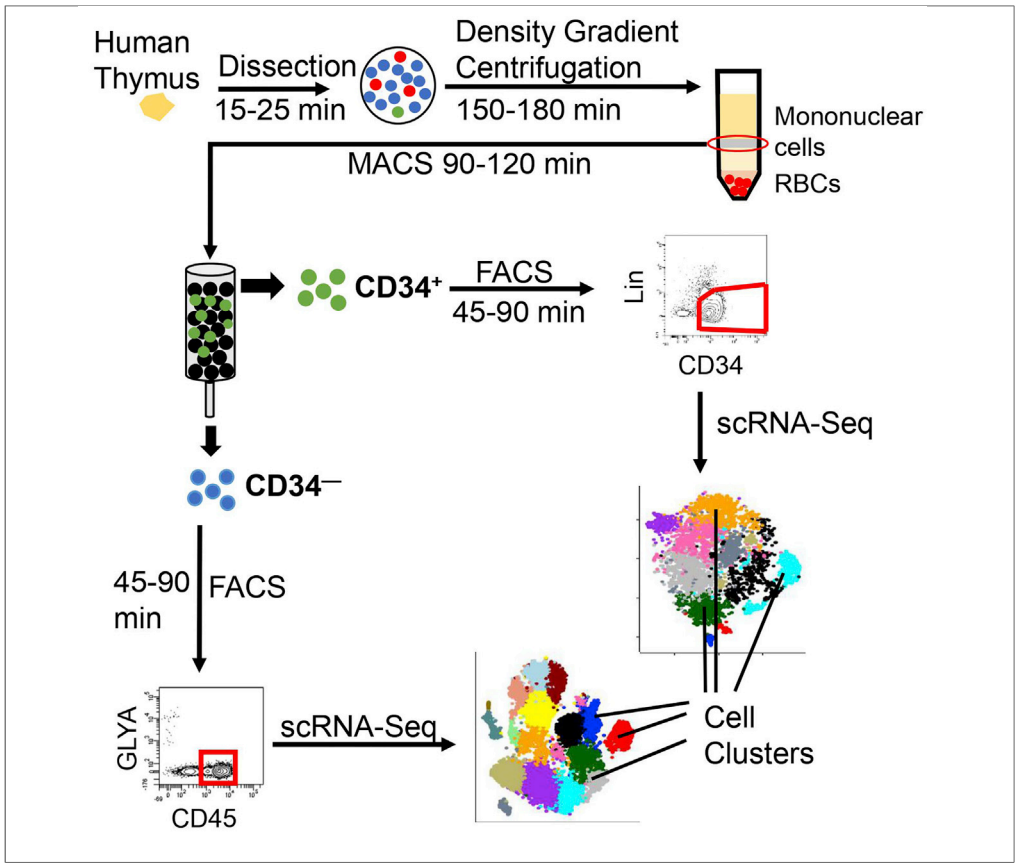
Processing Human Thymic Tissue for Single Cell RNA-Seq



Single cell RNA sequencing of human thymic cells is dependent on isolation of highly pure and viable cell populations. This protocol describes the isolation of $CD34^+$ progenitor and more differentiated $CD34^-$ fractions from post-natal thymic tissue to study thymopoiesis. $CD34^+$ cells represent <1% of thymic cells, so this protocol uses magnetic- followed by fluorescence-activated cell separation to isolate highly enriched $CD34^+$ cells.

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HIGHLIGHTS

Protocol for processing of human thymus for single cell RNA-seq

Thymus dissection and density gradient centrifugation isolate mononuclear cells

Magnetic-activated cell separation isolates $CD34^+$/$CD34^-$ cells

Fluorescence-activated cell separation isolates populations for RNA-seq

Le et al., STAR Protocols 1, 100090
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Processing Human Thymic Tissue for Single Cell RNA-Seq

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SUMMARY

Single cell RNA sequencing of human thymic cells is dependent on isolation of highly pure and viable cell populations. This protocol describes the isolation of $CD34^+$ progenitor and more differentiated $CD34^-$ fractions from post-natal thymic tissue to study thymopoiesis. $CD34^+$ cells represent <1% of thymic cells, so this protocol uses magnetic- followed by fluorescence-activated cell separation to isolate highly enriched $CD34^+$ cells.

For complete details on the use and execution of this protocol, please refer to Le et al. (2020).

BEFORE YOU BEGIN

Timing: 30–45 min

1. See Materials and Equipment for preparation of needed materials
2. Set centrifuge to 20°C–22°C (room temperature)
3. Adjust cell counter settings (see step 9 Note for additional information)

⚠ CRITICAL: Human thymus samples should be obtained using an Institutional Review Board (IRB) approved protocol. Human samples are considered to be potentially infectious and should be handled in Biosafety Level II cabinets using standard aseptic precautions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD4 FITC (clone: RPA-T4)	BD Biosciences	Cat#: 555346; RRID: AB_395751
CD8 PerCP (clone: SK1)	BD Biosciences	Cat#: 347314; RRID: AB_400280
CD3 APC (clone: UCHT1)	BD Biosciences	Cat#: 561810 RRID: AB_10893350

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD45 APC/Cy7 (clone: HI30)	Biolegend	Cat#: 304014 RRID: AB_314402
Glycophorin A PE (clone: 11E4B-7-6 (KC16))	Beckman Coulter	Cat#: IM2211 RRID: AB_131213
CD34 PerCP-Cy5.5 (clone: 8G12)	BD Biosciences	Cat#: 347203 RRID: AB_400266
CD4 APC (clone: RPA-T4)	BD Biosciences	Cat#: 555349 RRID: AB_398593
CD8 APC (clone: SK1)	Biolegend	Cat#: 344722 RRID: AB_2075388
CD235a APC (clone: GA-R2)	BD Biosciences	Cat#: 551336 RRID: AB_398499
CD7 FITC (clone 4H9)	BD Biosciences	Cat#: 347483 RRID: AB_400309
CD1a PE (clone: HI149)	BD Biosciences	Cat#: 555807 RRID: AB_396141
Biological Samples		
Human Thymus	Children's Hospital Los Angeles	N/A
Chemicals, Peptides, and Recombinant Proteins		
Ficoll Paque Plus	Millipore-Sigma	Cat#: GE17-1440-03
DAPI	Invitrogen	Cat#: D1306
Immune Globulin Intravenous (Human)	Carimune	Cat#: 44206-417-06
autoMACS Rinsing Solution	Miltenyi Biotec	Cat#: 130-091-222
MACS BSA Stock Solution (10%)	Miltenyi Biotec	Cat#: 130-091-376
Fetal Bovine Serum	Gemini Bio	Cat#: 100-106
Penicillin/Streptomycin	Gemini Bio	Cat#: 400-109
Acetic Acid	Fisher Healthcare	Cat#: A38-500
DAPI	Invitrogen	Cat#: D1306
Trypan Blue	Fisher Healthcare	Cat#: 15250-061
DMSO	Fisher Healthcare	Cat#: BP231-100
DPBS	Fisher Healthcare	Cat#: 14190-235
Critical Commercial Assays		
CD34 MicroBead Kit UltraPure, human	Miltenyi Biotec	Cat#: 130-100-453
Software and Algorithms		
BD FACSDiva Software	BD Biosciences	https://www.bdbiosciences.com/en-us
Other		
BD FACSAria Laser Setup: 4-laser (488/561/633/405 nm)	BD Biosciences	https://www.bdbiosciences.com/en-us
70 µm Cell Strainers	Fisher Healthcare	Cat#: 22363548
10 mL Syringe	Fisher Healthcare	Cat#: 14955-459
Scalpels	Fisher Healthcare	Cat#: 29552
LS Columns	Miltenyi Biotec	Cat#: 130-042-401
30 µm Pre-Separation Filters	Miltenyi Biotec	Cat#:130-041-407
Magnet Stand	Miltenyi Biotec	Cat#: 130-042-303
Magnet	Miltenyi Biotec	Cat#: 130-042-302

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
FACS Tubes (Filter Cap)	Fisher Healthcare	Cat#: 08-771-23
FACS Tubes	Fisher Healthcare	Cat#: 14-959-2A
BD FACSAria	BD Biosciences	N/A
Countess II FL Automated Cell Counter	Thermo Fisher Scientific	AMQAF1000

MATERIALS AND EQUIPMENT

Miltenyi Buffer	Amount
autoMACS Rinsing Solution (99.5%)	475 mL
MACS 10% BSA Stock Solution (0.5%)	25 mL
Total	**500 mL**

D10	Amount
DMEM (89%)	222.5 mL
Fetal Bovine Serum (10%)	25 mL
Penicillin/Streptomycin (1%)	2.5 mL
Total	**250 mL**

3% Acetic Acid (3% AA)	Amount
Distilled H_2O (97%)	9.7 mL
100% Acetic acid (3%)	0.3 mL
Total	**10 mL**

DPBS + 1% FBS	Amount
DPBS (99%)	495 mL
Fetal Bovine Serum (1%)	5 mL
Total	**500 mL**

Freezing Medium (if Cryopreserving Cells)	Amount
Fetal Bovine Serum (90%)	18 mL
DMSO (10%)	2 mL
Total	**20 mL**

Blocking Buffer (if Thawing Cells)	Amount
DPBS (95%)	9.5 mL
Immune Globulin Intravenous (Human) (5%)	0.5 mL
Total	**10 mL**

DAPI (5 mg/mL)	Amount
DAPI (lyophilized)	10 mg
Distilled H_2O	2 mL
Total	5 mL

Note: DAPI (5 mg/mL) should be further diluted to 50 μg/mL with additional distilled H_2O and stored in leak-proof, opaque tubes.

Note: Buffers need to be vacuum filtered through 0.22 μm filters to prevent contamination and degassed to prevent excess air bubbles (which could block columns during the magnetic separation step). We recommend using Miltenyi buffer and D10 that are no more than 2 weeks old to minimize the risk of contamination.

⚠ CRITICAL: Use calcium and magnesium free DPBS to prevent inhibition of enzymatic reactions during library preparation for sequencing

STEP-BY-STEP METHOD DETAILS

Thymus Dissection

Timing: 15–25 min

This section describes how to break down the thymus into the cells that will be used in the next sections. While we have successfully isolated $CD34^+$ cells from thymuses processed up to 24 h after surgical collection, for best results and when isolating $CD34^-$ cells we recommend beginning the process of cell isolation within 2 h of surgical collection. Following surgical collection, the thymus should be immediately placed in D10 in a sterile container, and the container should be kept on ice until processing is begun.

1. Decontaminate working spaces and equipment with UV light and 70% ethyl alcohol before beginning.
2. Put the thymus into a petri dish and add 10 mL DPBS to the petri dish. Place a cell strainer in the petri dish with the thymus (do not place the thymus in the strainer until it has been sliced).
3. Add 10 mL 20°C–22°C (room temperature) D10 to a 50 mL centrifuge tube and place a cell strainer on top of the tube.

⚠ CRITICAL: D10 will maintain cell viability during the dissection process. Keep the cells at 20°C–22°C (room temperature) when processing (this is important for the density gradient centrifugation step).

4. In the petri dish, hold the thymus with the tweezers and slice the thymus with the scalpel.

Note: Depending on the size of the thymus, not all of it needs to be used (to save time). A 1 $inch^3$ piece of thymus yields 1–5 billion cells based on how finely it is sliced. 1 billion thymus cells yields roughly 1 million $CD34^+$ cells.

5. Repeatedly cut the thymus until it is in 1 cm chunks and place the chunks into the cell strainer in the petri dish.
6. Hold the cell strainer and mash the chunks with the flat end of a 5 mL syringe piston (the piston should be removed from the syringe) to filter the cells into the petri dish and create a cell suspension in DPBS. The DPBS in the petri dish will turn cloudy as cells are released into it.

7. Pipette the cell suspension into the 50 mL centrifuge tube through the cell strainer to further remove clumps.

   Note: Clumps of thymus cells may clog the pipette tip; break or cut the tip of the pipette to increase the bore size of the pipette inlet, thereby preventing clogging.

8. Add additional DPBS to the petri dish and continue to use the syringe piston to mash chunks through the cell strainer until the mashing does not release more cells into the dish. Transfer the cell suspension to the 50 mL tube.

   Note: Highest cell numbers were achieved with very fine slicing, additional 20–30 mL DPBS, and 10–20 min of mashing.

Density Gradient Centrifugation

Timing: 150–180 min

Density gradient centrifugation is done to separate mononuclear cells from red blood cells (RBCs), neutrophils, and dead cells. Ficoll with a density 1.077 g/mL is used in this protocol.

Note: We have successfully performed bulk RNA-seq and differentiation studies of cells isolated from human thymi without using density gradient centrifugation (Casero et al., 2015, Ha et al., 2017).While we expect that density gradient centrifugation could be omitted if fluorescence-activated cell separation (FACS) is used to remove dead cells and RBCs prior to single cell RNA-seq, we have used density gradient centrifugation for isolation of thymic cells in all single cell RNA-seq experiments in order to minimize dead cells and RBCs. Directly proceed from step 9 to step 27 and use the cell count from step 9 for calculating buffer, blocking reagent, and microbead amounts in steps 29 and 30 if omitting density centrifugation.

⚠ CRITICAL: Ficoll should be at 20°C–22°C (room temperature) to maintain ideal density for separation of layers.

9. Count the cells using a method that excludes RBCs (pre-Ficoll cell count).

   Note: We use acetic acid to lyse RBCs in the aliquot of cells used for counting. We typically dilute a 10 μL aliquot of the cell suspension in 3% acetic acid (AA) (1:500–1,000) for counting on a hemocytometer. Other methods such as automated cell counter approaches that exclude RBCs can be used for cell counting. However, since thymus cells tend to be smaller than the default cell size settings on some automated cell counters, the cell size settings on automatic counters may need to be adjusted to accurately count thymus cells.

10. Calculate the number of cells needed for the experiment and dilute the sample to 20 million cells/mL with DPBS.

    Note: Using higher cell concentrations per tube may result in poor cell separation and recovery. Use a 2:1 volume ratio of diluted cells to Ficoll; we use 50 mL centrifuge tubes in this protocol (30 mL diluted cells and 15 mL Ficoll per tube).

11. Calculate the number of 50 mL centrifuge tubes needed and add 15 mL Ficoll to each tube.
12. Before adding cells to each prepared Ficoll tube, mix the cells by pipetting to ensure even concentration of cells.
13. Slowly layer 30 mL of the cells (600 million cells) onto the Ficoll in each tube; avoid mixing the cells with the Ficoll.

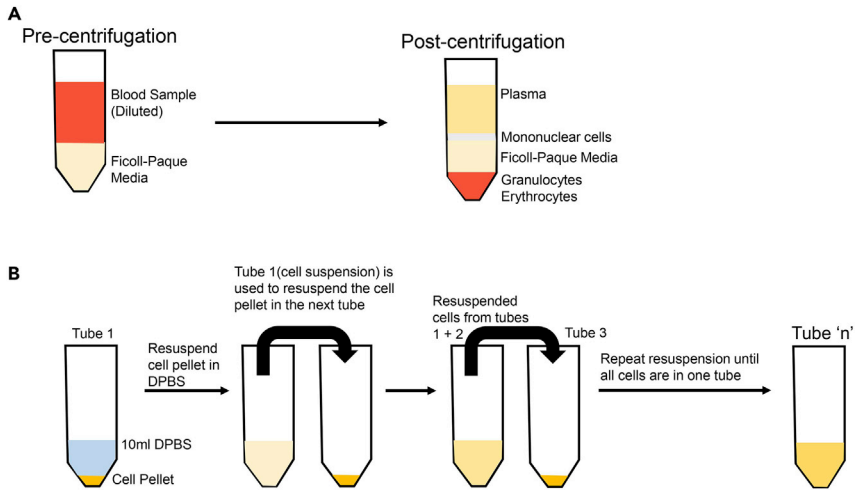

Figure 1. Density Gradient Centrifugation for Isolation of Human Thymic Mononuclear Cells

(A) Expected layers created by density gradient centrifugation (step 15).

(B) Pooling cell pellets from multiple tubes following density gradient centrifugation (step 24).

⚠ CRITICAL: Cell recovery will decrease if the Ficoll has been overly disturbed. When layering, pipette very slowly while directly touching the top of the Ficoll. As the cell layer rises, raise the pipette above the Ficoll and you may pipette more quickly.

14. Keep the Ficoll tubes with cells at 20°C–22°C (room temperature) while adding cells to additional Ficoll tubes.
15. Centrifuge all tubes at (400 × *g*, 30 min, 20°C–22°C (room temperature), No Brake (Decel=Off)).

⚠ CRITICAL: It is critical that the brake is off otherwise the buffy layer of mononuclear cells will be disrupted and recovery will decrease.

16. Once centrifugation is complete, set the temperature of the centrifuge to 4°C.
17. Aspirate and discard the upper layer containing plasma, leaving 10–15 mL of plasma above the mononuclear cell layer (See Figure 1A for details).

Note: While it is okay to have plasma with the cells, post-Ficoll cell recovery drastically decreases if too much Ficoll is collected, which is why it is important to leave some of the plasma layer in the tube while collecting the buffy layer.

18. Collect the buffy layer containing the mononuclear cells and transfer to a new 50 mL centrifuge tube.
19. Repeat the process with each additional tube, transferring each buffy layer of cells to a separate, new tube.

⚠ CRITICAL: The buffy layer from each tube needs to be transferred to a separate new tube to prevent the presence of an excessive amount of Ficoll during centrifugation; an excessive amount of Ficoll will prevent cells from pelleting.

20. Bring each tube up to 50 mL with DPBS and centrifuge (300 × *g*, 10 min, 4°C, Low Brake)

⚠ CRITICAL: From this point on, cells must be kept on ice or refrigerated and reagents that have been precooled on ice or at 4°C should be used to minimize cell death.

21. Transfer the supernatant to a new container. The supernatant from all the tubes can be combined into one container. Place the container on ice.

Note: If the cells have not formed a pellet (due to excess Ficoll), it is possible to recover them with an additional dilution with DPBS and centrifugation but viability and cell number will likely decrease.

22. Add 10 mL Miltenyi Buffer to one of the tubes (tube 1) and re-suspend the pellet
23. Transfer the 10 mL of re-suspended cells to the next tube (tube 2) and re-suspend the cell pellet in tube 2 in this 10 mL.
24. Continue the sequential transfer of the 10 mL of re-suspended cells to each of the remaining tubes to re-suspend all the cell pellets so that at the end of step 24 all of the cells are combined into a single tube in 10 mL of Miltenyi Buffer (See Figure 1B for details).
25. Add an additional 10 mL Miltenyi Buffer to the first tube and transfer similarly to steps 22–24 to recover any remaining cells (i.e., at the end of this step, all cells should be in one tube in 20 mL Miltenyi Buffer).
26. Count the recovered cells using a method that excludes RBCs (post-Ficoll cell count).

Note: Expected post-Ficoll cell count recovery is 30%–70% of the pre-Ficoll cell count. Minimization of processing times and Ficoll carry over with the buffy layer increases cell recovery. We count cells on a hemocytometer using 3% AA to lyse red cells (see note in step 9 for details and alternative counting methods). If the post-Ficoll count is lower than expected then the supernatant saved in step 21 could be centrifuged to attempt retrieval of cells that did not pellet in step 20 due to excessive Ficoll carry over.

27. Bring up the volume of cells to 50 mL with Miltenyi Buffer and centrifuge (300 × *g*, 10 min, 4°C, Low Brake).
28. Remove the supernatant and proceed with the column purification.

Isolation of $CD34^+$ and $CD34^-$ Cells via Magnetic-Activated Cell Separation (MACS)

Timing: 90–120 min

$CD34^+$ cells can be isolated from mononuclear cells with this process

⚠ CRITICAL: All cells and reagents should be kept on ice during this process to prevent cell death

29. Re-suspend the cells in 300 µL of Miltenyi Buffer for every 400 million cells.
30. Add 100 µL of FCR blocking reagent and 100 µL of Anti-CD34 (Anti-Hapten) Microbeads for every 400 million cells. Mix by gently tapping the tube.

Note: The manufacturer recommends using 300 µL of buffer, 100 µL of FCR blocking reagent, and 100 µL of microbeads per 100 million cells. However, we have found the lower ratios of reagent volumes (buffer, blocking reagent, microbeads) to cell number mentioned in steps 29 and 30 to be effective.

31. Incubate the cells in a refrigerator for 30 min.
32. Remove the cells from the refrigerator, bring up to 50 mL with Miltenyi Buffer, and centrifuge (300 × *g*, 10 min, 4°C, Low Brake).
33. While the cells are spinning, attach the magnet to the magnetic stand in the biosafety cabinet, place the LS column in the magnet holder, and the pre-separation filter in the LS column.
    a. Place a 50 mL tube under the column to collect $CD34^-$ cells. The $CD34^-$ cells will be in the flow through from the column.

b. Equilibrate the column by pipetting 3 mL Miltenyi Buffer into the pre-separation filter.

Note: Limit the number of cells per LS Column to two billion. Use multiple columns if necessary (e.g., use two columns for 4 billion cells).

34. Remove the supernatant and re-suspend cells in 5 mL Miltenyi Buffer.
35. Add the re-suspended cells to the pre-separation filter/column.
36. Rinse the centrifuge tube with 3 mL Miltenyi Buffer to recover remaining cells.
37. Wait for the first 5 mL of cells to completely pass through the column first, then add the next 3 mL of rinsed cells to the pre-separation filter/column.
38. After the rinsed cells have passed through, wash the pre-separation filter/column 6 times with 3 mL Miltenyi Buffer.

Note: This process will take approximately 45 min.

⚠ CRITICAL: When adding a new wash, wait until the previous wash has completely filtered through. It is important to do 5–6 washes for maximum purity. The rate at which the Miltenyi Buffer passes through the column decreases with each wash. During the final two washes, the buffer will drip through the column slowly (20 min per wash in some cases) - this is normal. If the Miltenyi Buffer takes more than 20 min during the 5th wash then skip the final wash to minimize the time the $CD34^+$ cells stay in the column. If the Miltenyi Buffer stops flowing through the column, it is possible to remove the excess buffer (that is not in the bead area of the column) and continue with step 39.

39. After the last wash is completed, remove the magnet, magnet stand, and pre-separation filter. Keep $CD34^-$ cells on ice. Perform a viable cell count for $CD34^-$ cells.
40. Place the magnetic column over a new 15 mL conical and add 5 mL Miltenyi Buffer into the column.
41. Insert the plunger into the column and push hard to extract the $CD34^+$ cells from the magnetic beads into the 15 mL conical tube.
42. Perform a viable cell count for $CD34^+$ cells.

Note: We use trypan blue and a hemocytometer for counting viable cells. The expected yield of $CD34^+$ cells is 0.2%–0.5% of the post-Ficoll cell count. An automated cell counter (e.g., ThermoFisher Countess II) can be used for this step.

43. Immediately proceed with FACS for isolation of highly enriched populations of viable $CD34^+$ or $CD34^-$ cells for single cell RNA-seq.

Pause Point: The $CD34^+$ cells can be cryopreserved and FACS sorting for further isolation of these cells for single cell RNA-seq can be performed at a later time. Post-thaw cell recovery will be approximately 30%–50% of the pre-freeze cell count. Cryopreserved and freshly prepared $CD34^+$ cells have yielded similar results in single cell RNA-seq studies done in our lab (Le et al., 2020). $CD34^-$ thymic cells predominantly consist of $CD4^+CD8^+$ (double positive, DP) cells. Less than 10% of DP cells in the thymus undergo positive selection and survive while the remaining DP cells undergo apoptosis (Klein et al., 2014). Due to this high physiological death rate among $CD34^-$ cells, we recommend using fresh $CD34^-$ cells rather than cryopreserved $CD34^-$ cells for FACS isolation of cells for single cell RNA-seq in order to maximize the viability of the input cells for single cell RNA-seq. See Optional Sections: Cryopreserving Isolated Cells and Thawing Cells for additional details.

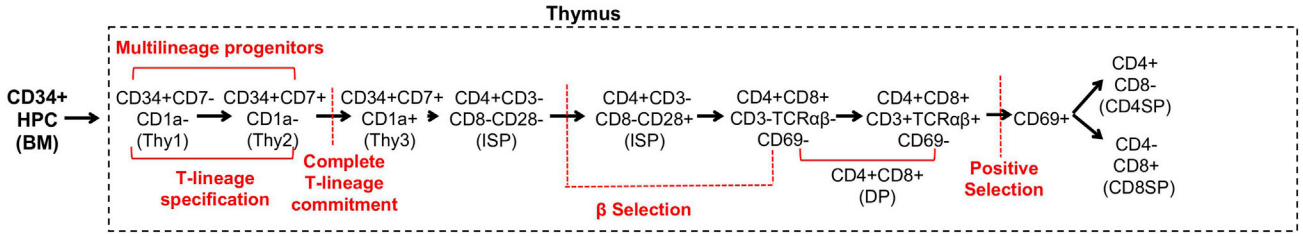

Figure 2. Schema for Human Thymopoiesis

HPC, hematopoietic progenitor cells; ISP, immature single positive cells; DP, double positive cells; SP, single positive cells.

FACS Sorting for Single Cell RNA-Seq

Timing: 45–90 min

This section is for sorting live ($DAPI^-$) $CD34^+$ progenitor ($CD34^+Lin^-$), $CD34^-$ leukocyte ($CD34^-CD45^+GlyA^-$), CD4 single positive ($CD3^+CD4^+CD8^-$), and CD8 single positive ($CD3^{+-}CD4^-CD8^+$) cells for single cell RNA-seq (intended input cell concentration of 400–1,500 cells/μL for library preparation). Lin: CD4, CD8, and Glycophorin A (Gly A) (GlyA for removing RBCs).

The hematopoietic progenitor cells that migrate from the bone marrow and initiate T-cell differentiation in the human thymus are characterized by expression of CD34. The initial stages of thymopoiesis are marked by two processes, the induction of expression of T-lineage genes (specification) and the loss of alternative (non-T) lineage potentials (commitment). The earliest thymic progenitors ($CD34^+CD7^-CD1a^-$, Thy1) possess myelo-erythroid and full lymphoid (B, T, and NK) potential (Hao et al., 2008). Successive stages of commitment are marked by the sequential upregulation of CD7 ($CD34^+CD7^+CD1a^-$, Thy2) and CD1a and a progressive loss of alternative lineage potentials, resulting in the generation of $CD34^+CD7^+CD1a^+$ (Thy3) cells, the earliest fully committed progenitors, which subsequently give rise to immature single positive (ISP, $CD3^-CD4^+CD8^-$) cells. ISP expressing a rearranged TCR (T-cell receptor) β chain proliferate via pre-TCR signaling and differentiate into double positive (DP, $CD4^+CD8^+$) cells (β-selection). Only the DP expressing a TCRαβ receptor reactive to a self-peptide/MHC (major histocompatibility antigen) complex survive (positive selection) and differentiate into mature naïve single positive $CD3^+$T cells ($CD4^+$ or $CD8^+$) (Figure 2). Cells with high TCR reactivity to self-peptides are eliminated via negative selection (Plum et al., 2008).

Note: All cells and reagents should be kept on ice during this section of the protocol to prevent cell death.

44. Separate 20 million $CD34^-$ cells, 20,000 $CD34^+$ cells (unstained control), and the remaining $CD34^+$ cells.
45. Centrifuge the $CD34^+$ and $CD34^-$ cell suspensions (300 × *g*, 4°C, 5 min).
46. While the cells spin, prepare the following antibody mixes:

Mix A (for up to 20 million cells, scale up accordingly):

Antibody	Volume
CD45 APC-Cy7	5 μL
Glycophorin A PE	10 μL
CD4 FITC	20 μL
CD8 PerCP	20 μL
CD3 APC	5 μL

Mix B (for up to 2.5 million cells, scale up accordingly):

Antibody	Volume
CD34 PerCP-Cy5.5	10 μL
CD4 APC	2.5 μL
CD8 APC	2.5 μL
CD7 FITC	10 μL
CD1a PE	10 μL
CD235a APC (Diluted)[a]	2.5 μL

[a]Dilute CD235a APC antibody 1:6 in distilled water before use

Note: Preparation of an antibody mix reduces the cell loss associated with pipetting multiple antibodies into a low volume of cells. It is always recommended to create a mix when using multiple antibodies.

47. Aspirate the supernatant to the following volumes:
    a. $CD34^-$: 40 μL buffer/20 million cells
    b. $CD34^+$: 20 μL buffer/2.5 million cells

Note: If using cryopreserved $CD34^+$ cells then the $CD34^+$ cells need to be blocked in 40 μL of blocking buffer for 5 min on ice prior to the addition of flow cytometry antibodies. Blocking reduces non-specific binding of antibodies to cells. The addition of FCR blocking reagent when processing cells for MACS (step 30) obviates the need for a separate blocking step prior to staining cells with flow cytometry antibodies if performing FACS using fresh cells directly after MACS.

48. Add the appropriate antibody mix to the cells and incubate for 15 min at 4°C (in the fridge, covered with foil).
    a. Antibody Mix A: add to $CD34^-$ cells
    b. Antibody Mix B: add to $CD34^+$ cells. CD7 and CD1a antibodies were included to enable additional enrichment for the earliest thymic progenitor (Thy 1: $CD34^+CD7^-CD1a^-$) cells
49. Add 3 mL PBS + 1% FBS to each tube and strain the cell suspension into a new Filter FACS tube.

Note: We recommend loosening the filter cap before adding the cell suspension in order to aid filtration. Be careful when loading the cell suspension to prevent overflow since the cell suspension does not usually filter immediately. If the cell suspension is stuck in the filter cap, lightly tap the bottom of the tube against the floor of the biosafety cabinet. Tap repeatedly until the cell suspension begins to filter before adding more cells.

50. Centrifuge (300 × *g*, 4°C, 5 min) and bring down volume of supernatant to achieve concentration of roughly 5 million - 10 million cells/mL.

Note: We use an aliquot of stained cells for area scaling and setting sort gates

51. To enable exclusion of dead cells, add 2.5 μL sterile DAPI a few minutes prior to starting FACS.

Note: The DAPI is only added just prior to FACS in order to prevent overstaining that can occur with prolonged exposure of cells to DAPI

52. Sort desired populations into 100% FBS in sterile, capped tubes and transfer onto ice.

Note: See Figures 3 and 4 for gating strategies.

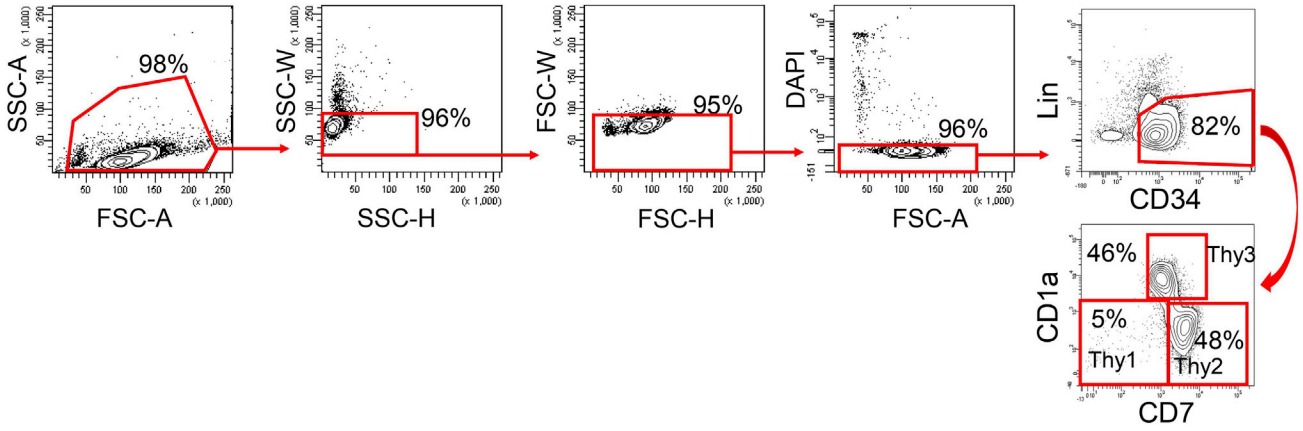

Figure 3. Gating Strategy for Sorting CD34$^+$Lin$^-$ and CD34$^+$CD7$^-$CD1a$^-$ Cells

Thy1, CD34$^+$CD7$^-$CD1a$^-$; Thy2, CD34$^+$CD7$^+$CD1a$^-$; Thy3, CD34$^+$CD7$^+$CD1a$^+$. Figure reprinted with permission from Le et al. (2020).

Note: A 70 μm nozzle is recommended to minimize sort time and dilution of the FBS in the collection tube from sheath fluid volume carry over, which can negatively affect cell viability. Cells should be run on the flow cytometer at a rate of 500–3,000 events/s during FACS.

⚠ CRITICAL: 100% FBS is used to maintain the viability of the sorted cells. Collection tubes should be inverted to coat the sides of the tube with FBS, free of air bubbles, and on ice prior to sorting. For best results, the preparation of cells for downstream droplet based single cell RNA-seq (10x or InDrop) should be completed within 30 min after FACS to prevent excess cell death. Cells and reagents should also be kept on ice to prevent cell death.

Note: Due to sheath volume carry over from the cytometer, up to 5 × 10^5 cells can be sorted into 300 μL of 100% FBS in a 1.5 mL Eppendorf tube. Higher numbers of cells should be sorted

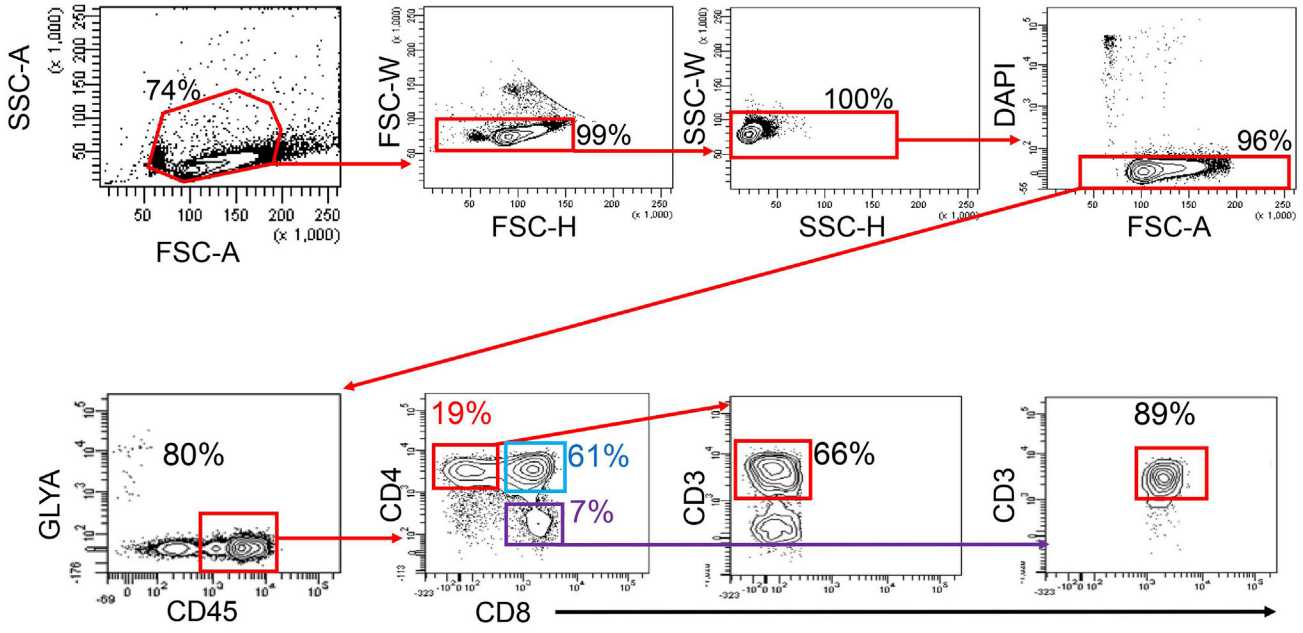

Figure 4. Gating Strategy for Sorting CD34$^-$CD45$^+$GlyA$^-$, CD3$^+$CD4$^+$CD8$^-$, and CD3$^+$CD4$^-$CD8$^+$ Cells

Figure reprinted with permission from Le et al. (2020).

into 1 mL 100% FBS in a 5 mL FACS tube. The maximum number of cells that can be sorted into a 1.5 mL Eppendorf tube will be lower if using a larger nozzle. After the sort, mix each collection tube by inversion to suspend the cells in FBS. We typically sort 400,000 $CD34^+Lin^-$ cells into a 1.5 mL Eppendorf tube and 1 million $CD34^-CD45^+GlyA^-$ cells into a FACS tube.

Note: Since the earliest thymic progenitor population ($CD34^+CD7^-CD1a^-$) represents less than 5% of $CD34^+$ cells, additional $CD34^+CD7^-CD1a^-$ cells can be sorted into the collection tube for $CD34^+Lin^-$ cells to ensure adequate representation of the earliest progenitors in the single cell RNA-seq data for $CD34^+$ cells. We typically sort an additional 10,000 $CD34^+CD7^-CD1a^-$ into the same collection tube into which 400,000 $CD34^+Lin^-$ cells have been previously sorted.

53. Transfer the sorted cells to a 15 mL conical tube. Rinse out any leftover cells in the original collection tube with additional Miltenyi Buffer and transfer to the 15 mL conical tube.
54. Bring the volume up to 15 mL with Miltenyi Buffer.
55. Centrifuge (300 × *g*, 5 min, 4°C, Standard Brake) and then aspirate the supernatant to leave behind a volume of ~40 μL.
56. Bring the volume up to 4 mL with D10.
57. Remove the filter cap from a Filter FACS Tube, hold it over a 15 mL conical tube, and filter the cell suspension into the 15 mL conical.

Note: The cell suspension needs to be concentrated for single cell RNA-seq and it is easier to aspirate from a 15 mL conical compared to a Filter FACS Tube. Trying to aspirate to volumes below 200 μL in a Filter FACS Tube may lead to loss of cells.

58. Centrifuge (300 × *g*, 5 min, 4°C, Standard Brake) and aspirate the supernatant down to a volume of 50–80 μL.

Note: Cell loss can be avoided by using a P200 to aspirate when the volume is below 500 μL in the conical

59. Re-suspend the cell pellet with a P20 or P200 micropipette to avoid cell loss.
60. Count cells using a cell counter and a hemocytometer and proceed with single cell RNA-seq library preparation.

⚠ CRITICAL: An accurate cell count is critical for successful library preparation with droplet based single cell RNA-seq protocols (e.g., 10x, InDrop). Refer to the protocol for the 10x or InDrop kit that you are using to determine the ideal input cell concentration needed for library preparation. We have used this protocol for isolation of $CD34^+$ and $CD34^-$ cells for the downstream generation of 10x (v2 and V3.2) and InDrop (v2 and v3) libraries.

Note: $CD34^-$ thymic cells tend to be small in size making default settings for cell detection on some electronic cell counters (e.g., ThermoFisher Countess II) inaccurate. Cell counter settings should be adjusted for $CD34^-$ and $CD34^+$ human thymic cells prior to use. A hemocytometer count done in parallel is recommended. Cell counts may vary by two fold between the hemocytometer and automated counts done on the same sample. Larger variations indicate improper settings on the electronic counter or counting errors when using the hemocytometer. Electronic cell counter results were used in this protocol for determining the input volume of the cell suspension for library preparation.

Note: Standard hemocytometers and the Countess II require 10 μL of sample to be loaded, so use a 1:1 ratio of cells to Trypan blue (5 μL cell sample + 5 μL Trypan blue). The optimal dilution

for other electronic cell counters may vary, so use the lowest amount of cell sample to meet the recommended dilution.

Cryopreserving Isolated Cells (Optional)

Timing: 10 min

If time is a limiting factor, it is possible to cryopreserve $CD34^+$ cells following MACS (Pause Point at step 43) and resume processing at a later time.

Note: To maximize cell viability, use FBS + 10% DMSO that was prepared no more than a week ago.

61. Centrifuge cells (300 × *g*, 5 min, 4°C, Standard Brake) and remove as much supernatant as possible without disturbing the cells.
62. Add FBS + 10% DMSO to cells to a volume of 1 mL per tube and re-suspend cells

Note: Post-thaw cell recovery is best when vials are frozen at 2–5 million cells per vial.

63. Aliquot cells into cryovials and into a freezing container before transferring to a −80°C freezer.

Note: DMSO is toxic to cells - cryovials should be transferred to a −80°C within 2 min of adding the FBS + 10% DMSO.

64. After 24–48 h, transfer the vials from the freezing container into a container in a liquid nitrogen freezer.

Thawing Cells (Optional)

Timing: 10 min

This section outlines how to thaw cryopreserved MACS enriched $CD34^+$ cells for further processing. If cryopreserved cells are used then proceed with the protocol starting from step 44.

65. Warm D10 to 20°C–22°C (room temperature) by leaving it in a low-light area or in a heated water bath for 5–10 min.

Note: To maximize cell viability, the D10 should be no more than 2 weeks old. Using 20°C–22°C (room temperature) D10 is important in order to prevent cell death due to sudden temperature changes.

66. Remove the cryovial containing 1 mL of frozen cells from the liquid nitrogen freezer and immediately place it into a 37°C water bath.
67. Thaw the cells by gently swirling the vial in the 37°C water bath until just a tiny sliver of ice remains in the vial (1–2 min).
68. Spray the outside of the vial with 70% ethanol and transfer the vial into the biosafety cabinet to prevent contamination.
69. Transfer the cells to a 15 mL conical.
70. Slowly add the 20°C–22°C (room temperature) D10 in dropwise fashion into the tube containing the thawed cells to bring the volume up to 15 mL. Shake tube while adding D10.

Table 1. Expected Outcomes

Outcome	Result
Post dissection cell recovery	A 1inch^3 piece of thymus yields 1–5 billion cells
Post-Ficoll cell recovery	30%–70% of the pre-Ficoll cell count
Post-column cell recovery of CD34$^+$ cells	0.2%–0.5% of the post-Ficoll cell count, >80% viability on the post-column hemocytometer count
Viability of CD34$^-$ cells on the post-column hemocytometer count	Viability on the post-column count is typically lower than that seen for CD34$^+$ cells and can be as low as 50%
Post-MACS purity of CD34$^+$ cells	50%–90% (seen by flow cytometry analysis of CD34$^+$ cells from step 41)
Post-FACS sort CD34$^+$ viability (Countess)	Viability >90%
Post-FACS sort CD34$^+$ viability (Hemocytometer)	Viability >90%
Post-FACS sort CD34$^-$ viability (Countess)	Viability >90%
Post-FACS sort CD34$^-$ viability (Hemocytometer)	Viability >90%

Frequency of Thy 1, 2, and 3 vary between 0.1%–5%, 20%–80%, and 10%–60%, respectively. Frequencies for these populations across 25 donors along with donor age and sex are listed in Table S1. Older children (>1 year of age) tend to show a higher frequency of Thy3 cells compared to infants (<1 year of age) (Figure 5).

Note: It is important to add the 20°C–22°C (room temperature) D10 slowly in order to prevent sudden changes in the temperature of the cells, which could lower viability. If thawing multiple vials, up to two vials of frozen cells can be pooled per 15 mL conical. Thawing more than two vials is not recommended due to the amount of DMSO present in freezing medium; if the DMSO is not properly diluted, cell viability will decrease.

71. Centrifuge cell samples at 300 × *g*, 5 min, 20°C–22°C (room temperature).
72. Aspirate as much of the supernatant as possible without disturbing the cell pellet to remove as much DMSO as possible.
73. Gently re-suspend the cells in appropriate media or buffer for downstream studies.
74. Cell recovery after thawing is 30%–50% of frozen cells, we recommend performing a cell count after resuspending cells in the appropriate buffer or medium.

EXPECTED OUTCOMES

See Table 1 and Table 2 for step-by-step expected outcomes. See Table S1 and Figure 5 for expected frequencies of Thy 1, 2, 3.

Table 2. Example Results

Outcome	Result
Pre-Ficoll cell count	9.7 billion
Post-Ficoll cell count	5.1 billion
Number of cells stained with CD34 microbead kit	5.1 billion
Post-column CD34$^+$ cell count	13 million CD34$^+$ cells
FACS	Sorted 400,000 CD34$^+$Lin$^-$ cells + 10,000 CD34$^+$CD7$^-$CD1a$^-$ cells into one collection tube; 1 million CD34$^-$CD45$^+$GlyA$^-$ cells into another collection tube
Post-FACS sort CD34$^+$ viability (Countess)	446 cells/μL, 95% viability
Post-FACS sort CD34$^+$ viability (Hemocytometer)	640 cells/μL, 90% viability
Post-FACS sort CD34$^-$ viability (Countess)	1,000 cells/μL, 94% viability
Post-FACS sort CD34$^-$ viability (Hemocytometer)	1,920 cells/μL, 90% viability

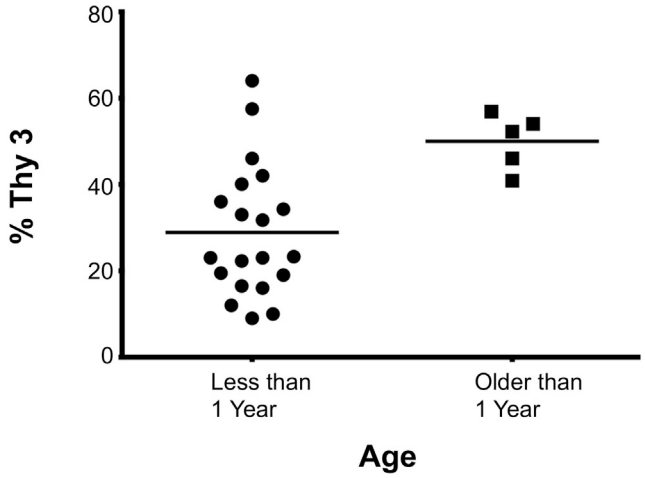

Figure 5. Frequency of Thy3 (CD34+CD7+CD1a+) Cells among CD34+Lin- Cells in Infants (<1 year) and Older Children (>1 year)

Lin, CD4, CD8, GlyA. Each data point represents a different donor. Horizontal line, mean.

LIMITATIONS

The protocol described here is expensive, labor intensive, and time consuming. Due to the rarity of $CD34^+$ cells in the human thymus, a large number of thymic cells need to be processed to obtain adequate numbers of $CD34^+$ cells for downstream studies and it is critical that the isolation procedures are done with utmost precision. This protocol has not been tested for thymuses collected post-mortem and there is no stopping step when isolating $CD34^-$ cells for single cell RNA-seq.

TROUBLESHOOTING

Problem 1

No distinct buffy layer after Ficolling.

Potential Solutions

(1) Ficoll not at 20°C–22°C (room temperature) (2) Ficoll layer broken when adding cell layer (3) Centrifuge brake engaged (4) Poor cell viability: this can be especially seen when the thymus is processed more than 48 h after surgical collection.

Problem 2

Lower than expected post-Ficoll cell recovery.

Potential Solutions

(1) Too many cells loaded per tube in step 13 (2) Buffy layer not properly aspirated in steps 17–19 (3) Buffy layers from multiple tubes directly combined into the same new tube in steps 18–19 resulting in excessive Ficoll carry over. (4) Excessive Ficoll carry over when extracting the buffy layer. The presence of excess Ficoll during subsequent centrifugation steps can prevent cells from pelleting. The supernatant saved in step 21 could be centrifuged to attempt retrieval of cells that did not pellet in step 20 due to the presence of excess Ficoll.

Problem 3

Lower than expected $CD34^+$ cell recovery/purity

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Potential Solutions

(1) Too many cells loaded into magnetic column in step 35 (see note in step 33) (2) Not enough washes performed in step 38 (3) Dead cells clogging the column- this can be seen when the thymus sample is processed more than 48 h after surgical collection or there is cell death during prior processing steps due to prolonged processing times or improper temperature conditions.

Problem 4

FACS shows poor viability of cells or low post-sort cell yields.

Potential Solutions

(1) Cell death due to temperature issues, old buffers, improper centrifugation/cytometer setup (2) Prolonged sort time or processing time for prior steps (3) Sorted cells were not immediately processed for downstream studies (4) Sides of the collection tube were not coated with FBS.

Problem 5

Low quality sequencing data.

Potential Solutions

(1) Incorrect cell numbers or cell concentration used during the input step of library preparation due to inaccurate cell counts (2) Low viability of cells.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Chintan Parekh (cparekh@chla.usc.edu).

Materials Availability

Deidentified thymuses from children undergoing cardiac surgery were collected through a Children's Hospital Los Angeles IRB approved protocol. This protocol does not generate unique reagents.

Data and Code Availability

Single cell RNA-seq data are available at the Gene Expression Omnibus (GEO) (accession number GSE139042, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139042).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100090.

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AUTHOR CONTRIBUTIONS

L.J., collection and assembly of data (performance of experiments), data analysis and interpretation, and manuscript writing; V.L.H., collection and assembly of data (performance of experiments) and data analysis and interpretation; A.L., FACS sorting, collection, and assembly of data (performance of experiments), and data analysis and interpretation; P.C., conception and design, collection and

assembly of data (performance of experiments), data analysis and interpretation, manuscript writing, and final approval of manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Casero, D., Sandoval, S., Seet, C.S., Scholes, J., Zhu, Y., Ha, V.L., Luong, A., Parekh, C., and Crooks, G.M. (2015). Long non-coding RNA profiling of human lymphoid progenitor cells reveals transcriptional divergence of B cell and T cell lineages. Nat. Immunol. *16*, 1282–1291.

Ha, V.L., Luong, A., Li, F., Casero, D., Malvar, J., Kim, Y.M., Bhatia, R., Crooks, G.M., and Parekh, C. (2017). The T-ALL related gene BCL11B regulates the initial stages of human T-cell differentiation. Leukemia *31*, 2503–2514.

Hao, Q.L., George, A.A., Zhu, J., Barsky, L., Zielinska, E., Wang, X., Price, M., Ge, S., and Crooks, G.M. (2008). Human intrathymic lineage commitment is marked by differential CD7 expression: identification of CD7- lympho-myeloid thymic progenitors. Blood *111*, 1318–1326.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol. *14*, 377–391.

Le, J., Park, J.E., Ha, V.L., Luong, A., Branciamore, S., Rodin, A.S., Gogoshin, G., Li, F., Loh, Y.E., Camacho, V., et al. (2020). Single-cell RNA-Seq mapping of human thymopoiesis reveals lineage specification trajectories and a commitment spectrum in T cell development. Immunity *52*, 1105–1118.e9.

Plum, J., De Smedt, M., Leclercq, G., Taghon, T., Kerre, T., and Vandekerckhove, B. (2008). Human intrathymic development: a selective approach. Semin. Immunopathol. *30*, 411–423.