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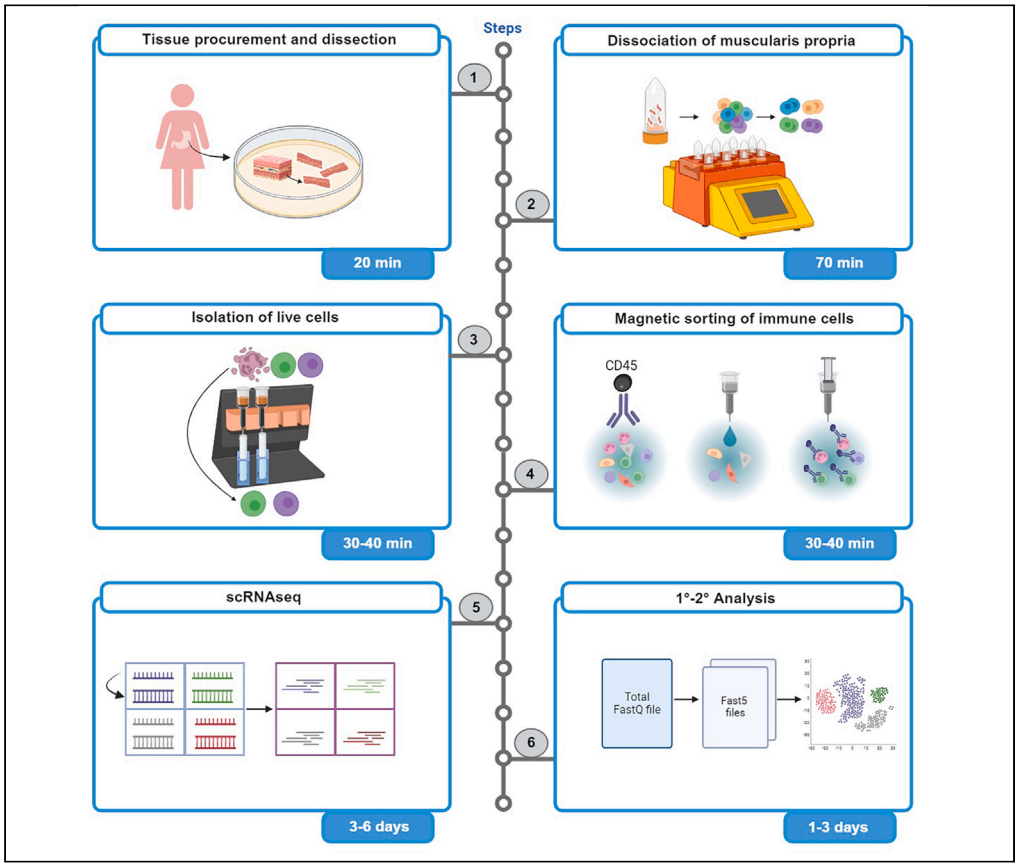
Protocol for isolating immune cells from human gastric muscularis propria for single-cell analysis



Understanding the diversity of gastrointestinal (GI) immune cells, especially in the muscularis propria, is crucial for understanding their role in the maintenance of enteric neurons and smooth muscle and their contribution to GI motility. Here, we present a detailed protocol for isolating single immune cells from the human gastric muscularis propria. We describe steps for tissue preservation, dissection, and dissociation of the muscularis propria. We then detail procedures for magnetic sorting of $CD45^+$ cells and single-cell RNA sequencing (scRNA-seq) analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for preserving and separating human gastric muscularis propria

Standardized protocol to dissociate human gastric body muscle tissue

Guide to isolating single $CD45^+$ cells by magnetic sorting for scRNA-seq analysis

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Protocol for isolating immune cells from human gastric muscularis propria for single-cell analysis

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SUMMARY

Understanding the diversity of gastrointestinal (GI) immune cells, especially in the muscularis propria, is crucial for understanding their role in the maintenance of enteric neurons and smooth muscle and their contribution to GI motility. Here, we present a detailed protocol for isolating single immune cells from the human gastric muscularis propria. We describe steps for tissue preservation, dissection, and dissociation of the muscularis propria. We then detail procedures for magnetic sorting of CD45$^+$ cells and single-cell RNA sequencing (scRNA-seq) analysis. For complete details on the use and execution of this protocol, please refer to Chikkamenahalli et al.[1]

BEFORE YOU BEGIN

Understanding the diversity of immune cells in the muscularis propria of the GI tract is crucial for understanding their role in regulating gut motility through interactions with multiple cell types including Interstitial Cells of Cajal, neurons, smooth muscle cells, and glial cells.[2–6] Therefore, this protocol was developed to determine the immune cell repertoire within the gastric muscularis propria. Our goal in developing this protocol was to investigate the heterogeneity and changes of macrophages proportions and related signalling pathways in the context of idiopathetic gastroparesis.[1] For this purpose, we used gastric muscularis propria from patients with idiopathic gastroparesis and control tissue obtained from surgical waste of patients undergoing bariatric surgery. In this protocol, we identified the optimal tissue storage buffer and time to maximize cell yield and quality. We also optimized the gentleMACS dissociation protocol to efficiently extract single-cell suspensions from the gastric muscularis propria. We isolated the live immune cells (CD45$^+$ cells) by column-based microbeads isolation kits. Finally, the required cells were used for library preparation and subsequently for single cells RNA Sequencing analysis (Figure 1).

Institutional permissions

All participants provided informed consent for the procurement and use of gastric tissue. The study received approval from the Mayo Clinic IRB (07–003371). Twenty Caucasian females, with an average age of 46 $\pm$ 15 SD controls and 49 $\pm$ 15 SD idiopathic gastroparesis patients, participated in this study.

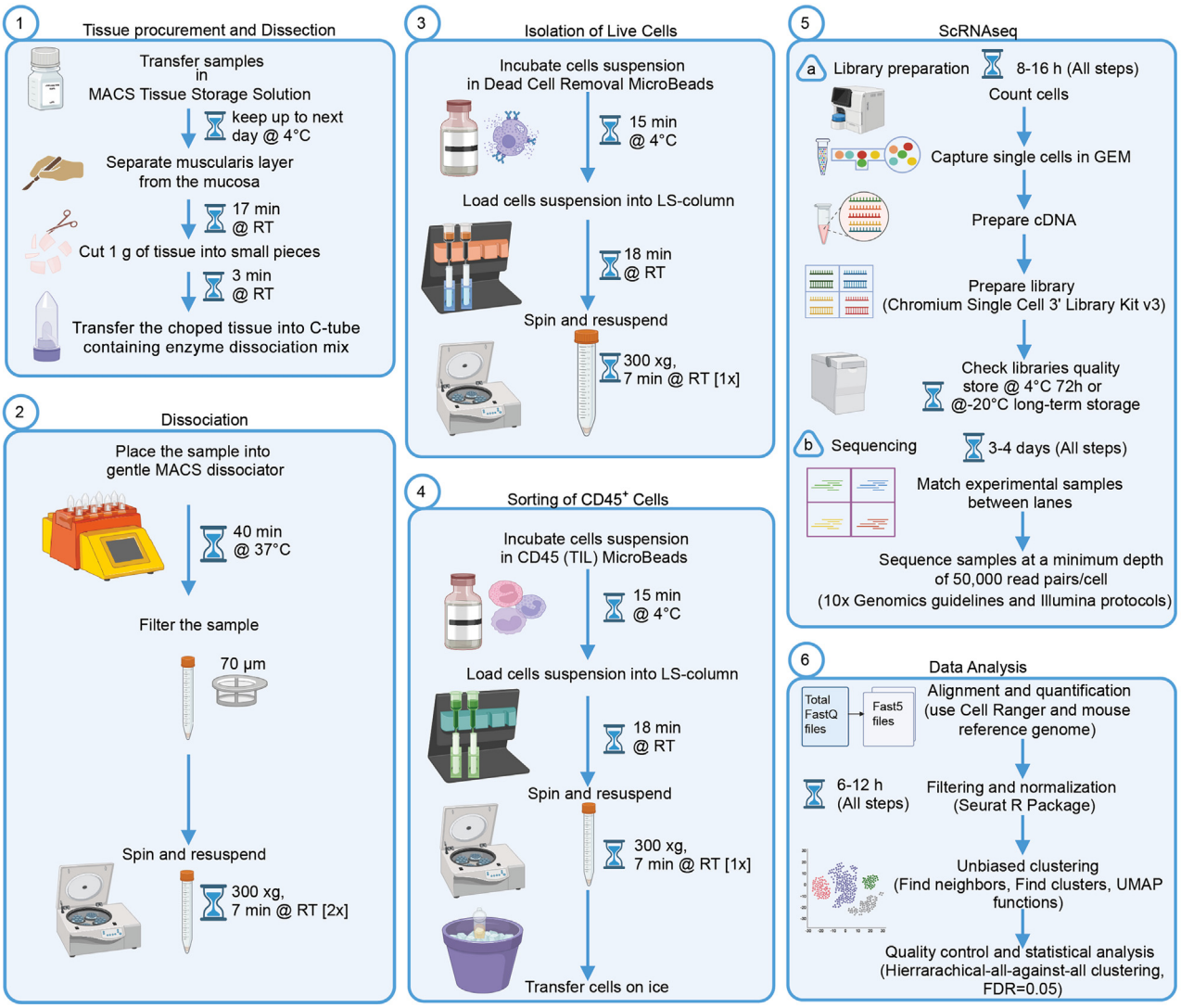

Figure 1. Illustration of protocol overview
GEM, gel bead-in-emulsion.

Prepare the media and Sylgard dishes

Timing: 30 min (for step 1)

Timing: 10 min (for step 2)

Timing: 5 min (for step 3)

Timing: 1 h (for step 4)

1. Prepare the individual enzyme stocks.

   Note: Enzyme stock may be prepared the day before tissue processing.

2. Prepare the enzyme dissociation mix.

Note: The enzyme dissociation mix should be prepared on the same day of experiment.

3. Prepare a wash buffer containing phosphate-buffered saline, bovine serum albumin and EDTA (PEB buffer).

This section outlines the procedure for preparing the dissection plate.

4. Prepare the Sylgard dishes as follows:

Note: Prepare the dishes 3–7 days prior to the use. One container of Sylgard should make about 24 dishes of 100 mm × 20 mm and six of 150 mm × 25 mm dish.

a. Pour small container of Curing agent into base and mix well.
b. Transfer the Sylgard into a 500 mL beaker and then pour it into the petri dishes.
c. Place petri dishes in desiccator and apply vacuum for a few hours or overnight, to remove air bubbles.

Transfer samples

5. Collect and store the tissue in MACS Tissue Storage Solution (Figure 1.1). Troubleshooting 2 and 3.

Pause point: Tissue can be stored for up to 24 h post collection at 4°C. It is ideal to process as soon as possible within that timeframe.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human CD45 (TIL) MicroBeads (Dilute 1 part microbeads with 9 parts cell suspension in PEB buffer)	Miltenyi Biotec	130-118-780
Biological samples		
Gastric muscularis biopsies	Human	N/A
Chemicals, peptides, and recombinant proteins		
MACS tissue storage solution	Miltenyi Biotec	130-100-008
AutoMACS rinsing solution	Miltenyi Biotec	130-091-222
MACS BSA stock solution	Miltenyi Biotec	130-091-376
Multi Tissue Dissociation kit 1	Miltenyi Biotec	130-110-201
LS + positive selection column, 25 pc	Miltenyi Biotec	130-042-401
Dead Cell Removal Kit (dilute 1 part microbeads with 0.666667 part cell suspension in PEB buffer)	Miltenyi Biotec	130-090-101
gentleMACS C tubes	Miltenyi Biotec	130-093-237
Pre-separation filters, 30 µm, 50 pc	Miltenyi Biotec	130-041-407
MACS SmartStrainer; 70 µm	Miltenyi Biotec	130-098-462
Sylgard 184 Silicone Elastomer Kit Clear 0.5 kg (1.1LB) kit	Krayden	DC4019862
1x PBS, pH 7.4	Corning	21-040-cv
BSA	Sigma-Aldrich	A7906-100G
EDTA	Sigma-Aldrich	EDS-100G
Qiagen Buffer EB (elution buffer)	QIAGEN	19086
Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1, 16 rxns	10× Genomics	1000121
Chromium Next GEM Chip G Single Cell Kit, 48 rxns	10× Genomics	1000120

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Single Index Kit T Set A, 96 rxns	10× Genomics	1000213
Qubit high sensitivity dsDNA quantification assays	Thermo Fisher Scientific	Q32854
Kapa DNA quantification reagents	Kapa Biosystems	KK4824
Illumina cBot and HiSeq 3000/4000 PE Cluster Kit	Illumina	PE-410-1001
HiSeq 3000/4000 sequencing kit	Illumina	FC-410-1002
Experimental models: Organisms/strains		
Species	Human	N/A
Sex	Females	N/A
Deposited data		
Gene expression	GEO	GEO: GSE252126
Other		
gentleMACS Octo Dissociator with heaters	Miltenyi Biotec	130-096-427
OctoMACS Separator	Miltenyi Biotec	130-042-109
MACS MultiStand	Miltenyi Biotec	130-042-303
Vi-Cell XR cell viability analyzer	Beckman Coulter	N/A
Agilent Bioanalyzer high sensitivity DNA chips	Agilent	N/A

MATERIALS AND EQUIPMENT

Enzyme stock preparation		
Reagent	Size	Amount
Enzyme D	80–160 mg	3.0 mL Serum-free DMEM
Enzyme R	20–35 mg	2.7 mL Serum-free DMEM
Enzyme A	5–15 mg	1.0 mL Buffer A
Total	N/A	N/A

Note: For Enzyme D, close vial, and invert periodically for at least 5 min. Before pipetting Enzyme R, invert the tube carefully to prevent precipitation. For Enzyme A, reconstitute only by inversion or flicking the tube. Store lyophilized enzymes and buffer A at 4°C. The composition of these enzymes D, R, A, and buffer A is not described by Miltenyi. Can store all re-constituted enzymes aliquots at −20°C for no more than 6 months.

Alternatives: Use serum free-RPMI 1640 as an alternative to DMEM medium.

Enzyme dissociation mix preparation		
Reagent	Final concentration	Amount
Enzyme D	1:101.6	25.0 µL
Enzyme R	1:203.3	12.5 µL
Enzyme A	1:811.7	3.13 µL
Serum-free DMEM medium	N/A	2,500 µL
Total	N/A	2,540.63 µL

Note: Thaw the enzymes D, R, and A at 22°C. Add above specified volumes to gentleMACS C Tubes.

PEB buffer preparation		
Reagent	Final concentration	Amount
MACS BSA Stock Solution [PBS+10% BSA]	0.5% BSA	75 mL
AutoMACS Rinsing Solution [PBS+ 2 mM EDTA, pH 7.2]	N/A	1,450 mL
Total	N/A	1,525 mL

Note: Store at 2°C–8°C for up to expiry date of MACS BSA Stock Solution.

Human stomach muscularis propria dissociation program on gentleMACS dissociators	
Program	
Steps	Condition
1	Temp ON
2	Spin 200 rpm, 10″
3	Spin −200 rpm, 2″
4	Spin 200 rpm, 2″
5	Spin −200 rpm, 2″
6	Spin 200 rpm, 10″
7	Spin −200 rpm, 2″
8	Spin 200 rpm, 8″
9	Spin −20 rpm, 40′0″
10	Spin 200 rpm, 10″
11	Spin −200 rpm, 2″
12	Spin 200 rpm, 2″
13	Spin −200 rpm, 2″
14	Spin 200 rpm, 10″
15	Spin −200 rpm, 2″
16	Spin 200 rpm, 8″
17	Temp OFF
18	END

(″) and (′) represent seconds and minutes, respectively.

Note: This program was customized specifically to dissociate human gastric muscularis propria tissue. You may adjust the spin speed and the timing for each step based on the tissue type. Thinner tissues may require lower spin speed and shorter duration. The strength of enzyme may also determine the required overall dissociation time. Troubleshooting 1, 2, and 3.

Dyna-Bead clean-up						
Components	Volume 1x (μL)	2.2x	3.3x	4.4x	6.6x	8.8x
Cleanup Buffer	182	400.4	600.6	801	1,201.2	1,602
Dynabeads MyOne SILANE	8	17.6	26.4	35	52.8	70
Reducing Agent B	5	11	16.5	22	33	44
Nuclease free Water	5	11	16.5	22	33	44
Total	200	440	660	880	1,320	1,760

Elution solution 1		
Components	Volume 1x (μL)	10x
Buffer EB	98	980
10% Tween 20	1	10
Reducing Agent B	1	10
Total	100	1,000

cDNA amplification mix						
Components	Volume 1x (μL)	2.2x	3.3x	4.4x	6.6x	8.8x
Amp Mix	50	110	165	220	330	440
cDNA Primers	15	33	49.5	66	99	132
Total	65	143	214.5	286	429	572

cDNA amplification protocol and recommended cycle range		
Lid temperature	Reaction volume	Run time
105°C	100 μL	~30–45 min
Step	Temperature	Time
1	98°C	3 min
2	98°C	15 s
3	63°C	20 s
4	72°C	1 min
5	Go to step 2; see table below for total number of cycles	
6	72°C	1 min
7	4°C	hold

Note: This protocol is recommended by the manufacturer.

Recommended starting point for cycle number optimization based on targeted cell number	
Targeted cell recovery	Total cycles
<500	13
500–6,000	12
>6,000	11

Note: This table is based on recommendations by the manufacturer.

Note: The number of cDNA cycles should be decreased when sampling a high number of cells.

Library PCR reagent volumes						
Components	Volume 1x (μL)	2.2x	3.3x	4.4x	6.6x	8.8x
Amplification Mix	50	110	165	220	330	440
SI Primer	10	22	33	44	66	88
Total	60	132	198	264	396	528

Library PCR cycling protocol		
Lid temperature	Reaction volume	Run time
105°C	100 μL	~25–40 min
Step	Temperature	Time
1	98°C	45 s
2	98°C	20 s
3	54°C	30 s
4	72°C	20 s
5	Go to step 2; see table below for total number of cycles	
6	72°C	1 min
7	4°C	Hold

Note: This protocol is recommended by the manufacturer.

Recommended cycle numbers based on cDNA input	
cDNA input	Total cycles
0.25–25 ng	14–16
25–150 ng	12–14
150–500 ng	10–12
500–1,000 ng	8–10

(*Continued on next page*)

Continued	
cDNA input	Total cycles
1,000–1,500 ng	6–8
>1,500 ng	5

Note: This table is based on recommendations by the manufacturer.

STEP-BY-STEP METHOD DETAILS

Dissection of full-thickness gastric tissue to obtain muscularis propria

Timing: 20 min

This section outlines the steps necessary to isolate the muscularis propria (Figures 1.1 and 2A).

1. Use forceps and scalpel to cut mucosa off and obtain the muscularis propria. See Methods video S1. Troubleshooting 2.
   a. Position the tissue with the muscularis propria facing up, secure it in a petri dish filled with Sylgard.

   Note: Sometimes it is easier to flip the mucosa layer upwards, depending on whichever orientation allows for easier separation of muscularis propria from mucosa layer.

   Note: The muscularis propria may contract and curl the tissue; this can be alleviated by stretching out and pinning the edges of the mucosa.

   b. Carefully cut out the muscularis propria.

   Note: Perform this step while keeping the tissue in the tissue storage solution.

   c. Weigh the muscularis propria tissue and divide it into pieces of 1 g each. Troubleshooting 2.
   d. Cut the 1 g tissue into 3–4 mm small pieces before continuing with the procedure (Figure 2B).

   Note: Avoid cutting the tissue pieces too small as that may make it difficult to dissociate. Very small tissue pieces may not come in close contact with the paddles in the gentleMACS C Tube that is necessary for efficient dissociation.

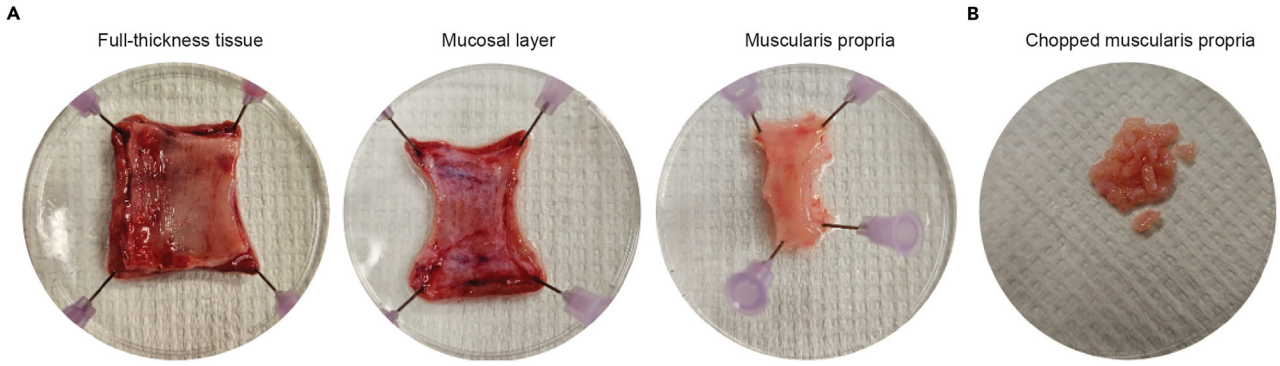

Figure 2. Isolation of gastric muscularis propria
(A) The photos display full thickness and separated muscularis propria and mucosa layer.
(B) Photo shows 1 g of tissue chopped into 3–4 mm pieces for dissociation.

2. Transfer the tissue pieces into each gentleMACS C Tube and ensure the lid is securely closed.
3. Each gentleMACS C Tube should contain no more than 1 g of tissue.

Tissue dissociation

Timing: 70 min

This section delineates the steps for dissociating single cells from the gastric muscularis propria (Figure 1.2).

4. Securely attach the gentleMACS C Tubes to the gentleMACS Dissociators (Figure 3A).
5. Start dissociation program on the gentleMACS dissociators for up to 40 min. Troubleshooting 1.

Note: Ensure all tissue pieces are immersed in the medium at the bottom of the tube (Figure 3B).

6. Remove the tubes from the dissociator. Troubleshooting 1. Then, proceed with the following steps while processing samples at 22°C.

Note: Prior to proceeding, check carefully if the tissue was well dissociated i.e., complete tissue homogenization with absence of large tissue pieces. However, you may still observe the presence of large connective tissue (Figure 3C).

a. Place a SmartStrainer (70 μm), on top of a 15 mL conical tube.
b. Pre-wet the strainer with 1 mL of DMEM medium.
c. Transfer the dissociated tissue into the strainer.

Note: If filter clogs, use serological pipette to gently stir and aid passage through the filter.

d. In the gentleMACS C Tube, add 10 mL of DMEM medium, close the lid, mix gently, and pour it into the strainer.
e. Centrifuge the cells at 300 × *g* for 7 min at 4°C.
f. Remove the supernatant and resuspend the cells in 10 mL of PEB buffer.
g. Centrifuge again at 300 × *g* for 7 min at 22°C.

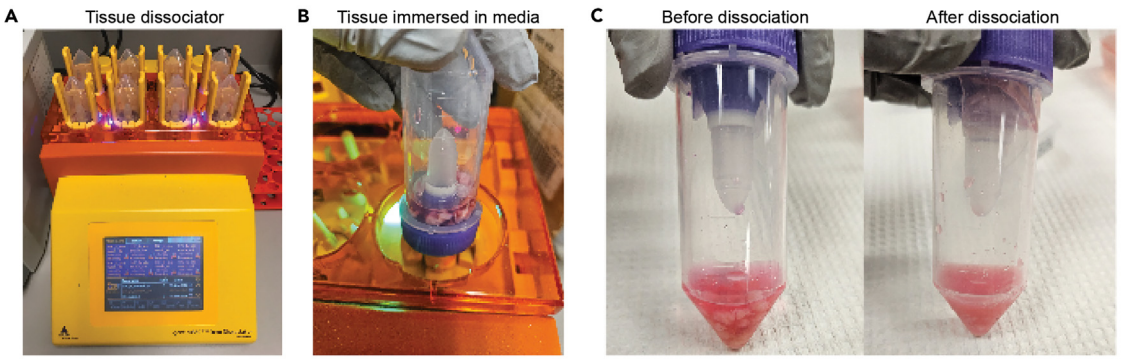

Figure 3. Dissociation of gastric muscularis propria into single cells
(A) Photo depicts gentleMACS Tissue Dissociator setup.
(B) Photo shows that every tissue piece needs to be covered with dissociation media and positioned at the paddles.
(C) The photos of gentleMACS C Tubes containing tissue sample before and after dissociation.

Dead cell removal

Timing: 30–40 min

This section describes the steps for isolating live cells from the overall cell suspension (Figure 1.3).

7. Discard the supernatant and resuspend the cells in 100 μL of PEB buffer.
8. Add 150 μL of Dead Cell Removal Microbeads and incubate the sample for 15 min at refrigerator 4°C.
9. During incubation, prepare the set up for next step as follows:
   a. Prepare 0.97x Binding Buffer by diluting 750 μL of 20x Binding Buffer Stock solution in 14.75 mL of double distilled water.
   b. Place a LS column in the OctoMACS Separator.
   c. Place a 15 mL conical tube below the column and position a yellow pre-separation filter on top of the LS column.
   d. Pre-wet the filter and LS column by adding 1 mL of 0.97x Binding Buffer and allow the volume to pass through the filter.
10. After incubating the cells with the Dead Cell Removal Microbeads.
    a. Directly add the cell suspension into 2 mL of 0.97x Binding Buffer.
    b. Load it into the filter.
    c. Allow complete passage through the LS column.
11. Rinse the LS column three times with 3 mL of 0.97x Binding Buffer each time.
12. Remove the conical tube and centrifuge the cells at 300 × *g* for 7 min at 22°C.
13. Remove the supernatant and resuspend the cells in 90 μL of PEB buffer.

Magnetic labelling and isolation of $CD45^+$ cells

Timing: 30–40 min

This section outlines the procedure for achieving high-purity isolation of immune cells using CD45 microbeads (Figure 1.4).

14. Add 10 μL of CD45 (TIL) MicroBeads into the 90 mL of cell suspension, thoroughly mix, and incubate in the refrigerator (4°C) for 15 min.
15. During incubation, prepare the set up for next step as follows:
    a. Place a LS column in the OctoMACS Separator.
    b. Place a 15 mL conical tube below the column and position a yellow pre-separation filter on top of the LS column.
    c. Pre-wet the filter and LS column by adding 1 mL of 1x Binding Buffer.
16. After incubating cells with CD45 (TIL) MicroBeads.
    a. Directly add the cell suspension with 2 mL of 1x Binding Buffer.
    b. Load it into the filter.
    c. Allow complete passage through the LS column.
17. Rinse the LS column three times with 3 mL of 1x Binding Buffer each time.
18. Remove the LS column along with the filter from the OctoMACS Separator and place it on a new 15 mL conical tube.
19. Add 5 mL of PEB buffer into the filter and then remove the filter.
20. Place the plunger atop the LS column and gently press to elute the $CD45^+$ cells.
21. Add another 5 mL of PEB buffer to the 15 mL conical tube and centrifuge the cells at 300 × *g* for 7 min at 22°C.
22. Remove the 10 mL of supernatant and resuspend the cells in 500 μL of PBS containing 2 mM EDTA and 0.04% BSA.

Library preparation

Timing: 8–12 h (for steps 23–48)

Timing: 3–4 days (for steps 49 and 50)

This section provides the steps for preparing gastric muscularis single immune cells for library preparation (Figure 1.5A).

23. Count the number of isolated $CD45^+$ cells and determine their viability using Vi-Cell XR Cell Viability Analyzer. Troubleshooting 7.

Alternatives: Cell counting on a hemocytometer and light microscope.

24. Thaw the Gel Beads.

Note: Equilibrate to 25°C.

25. Prepare the cDNA master mix using Chromium Single Cell 3′ v3 library kit as per manufacturer's instructions.
26. Mix a proper volume of live cells according to concentration with the cDNA master mix based on the desired number of cells to be captured.
27. Add the cell suspension and master mix, thawed Gel Beads, and partitioning oil to a Chromium Single Cell B chip.
28. Load the filled chip into the Chromium Controller.

Note: process each sample and capture the individual cells within the sample into uniquely labeled Gel Beads-In-Emulsion (GEMs).

29. Collect the GEMs from the chip and transport them to the bench to undergo reverse transcription (Figure 4A). Troubleshooting 4.
30. Gather Recovery Agent from Chip B kit at 25°C.
    a. Thaw frozen reagents from the Chromium Single Cell 3' GEM Kit v3 (module 1; 10x Genomics).
    b. Equilibrate most reagents to 25°C.
    c. Keep Amp mix on ice.
31. Prepare Dyna-bead mix, Elution Solution 1, and cDNA Amplification master mix for the Post GEM–RT Cleanup & cDNA amplification steps.
    a. Complete the GEM dissolution and clean up, followed by 12 cycles of cDNA amplification.

Note: Cycle numbers vary based on the cell number targeted. Refer to manufacturer's instructions for more detail.

    b. After cDNA amplification, continue into the cDNA clean-up step or store.

Pause point: Safe Stopping Point – Store at 4°C for up to 72 h; Freeze at −20°C for up to a week.

32. Complete a clean-up step using SPRIselect beads.
    a. Measure all cleaned cDNA pools and proceed with a portion of the pooled cDNA for library construction or store. Troubleshooting 5.

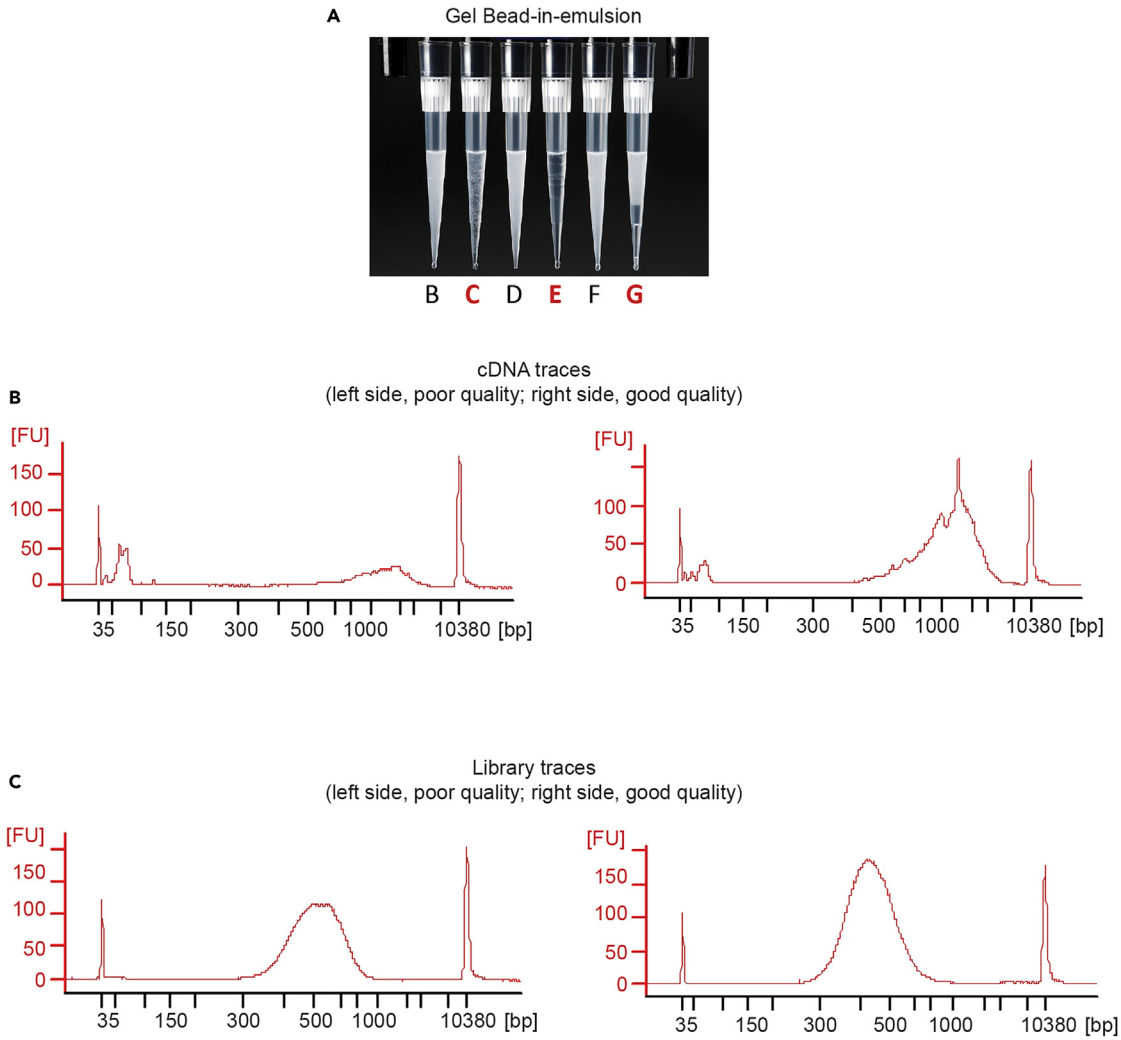

Figure 4. Potential results of single cell library preparation

(A) Photo displays various examples emulsions quality. Proper emulsion examples: pipette tips B, D and F. Partially emulsified GEMs: pipette tip C. Failed emulsion: pipette tip E (contains no emulsion). Partial emulsion: pipette tip G (contains a half-volume, indicating that there was a clog on the chip). This emulsion may work to create usable cDNA, but the cDNA yield will potentially be much lower.

(B) Agilent traces of cDNAs. (Left) Example of a poor-quality cDNA (flat peak indicates low cDNA yield). (Right) Example of a good quality cDNA (sharp peak represent high cDNA yield). Normal range for cDNA fragment size is 400–9,000 bases.

(C) Agilent traces of libraries. (Left) Example of a poor-quality library. (Right) Example of a good quality library. FU, fluorescence units.

Pause point: Safe Stopping Point – Store at 4°C for up to 72 h; Freeze at −20°C for up to a week.

Note: The resulting cDNA contains a pool of uniquely barcoded molecules.

33. Thaw frozen reagents from the Chromium Single Cell 3′ Library Kit v3 (module 2; 10x Genomics).

Note: Equilibrate most reagents to 25°C.

Note: Keep Fragmentation enzyme, DNA ligase and Amp mix on ice.

34. Prepare the Fragmentation, End Repair and A-tailing master mixes using Chromium Single Cell 3′ v3 library kit as per manufacturer's instructions.

Note: Keep all samples and master mixes on ice.

35. Add to a new strip tube 15 μL Fragmentation master mix, 10 μL of sample, and 25 μL of Buffer EB. Complete the Fragmentation step.
36. Clean samples and complete a double-sided size selection using SPRIselect beads.
    a. Add 60 μL of beads to reaction volume.
    b. Incubate at 25°C for 5 min, place on magnet and retain the supernatant.
37. Add 10 μL beads to supernatant, incubate at 25°C for 5 min and place on magnet.
38. Remove supernatant and wash two times with 80% ethanol.
    a. Use Buffer EB (elution buffer) to elute sample. Refer to manufacturer's instructions for more detail.
    b. Continue into the Adaptor Ligation step.
39. Prepare the Adaptor Ligation master mix using Chromium Single Cell 3′ v3 library kit (10x Genomics) per manufacturer's instructions.
40. Add 50 μL Adaptor Ligation master mix to each sample.
    a. Complete the Adaptor Ligation step and clean the samples using SPRIselect beads.
    b. Continue into the Sample Index PCR step.
41. Thaw and centrifuge the Single Index plate (plate T set A).

Note: Equilibrate to 25°C.

Note: The Single Index plate contains standard Illumina sequencing primers and unique i7 indices. Each well represents a unique index. Designate 1 unique well for each sample preparation. Record the well ID.

Note: Our libraries were prepared using the single index plates. But currently, dual indices are recommended as it offers more flexibility in sharing a flow cell with other library types.

42. Prepare the Sample Index PCR master mix.
43. Add 60 μL PCR master mix to each sample.
44. Add 10 μL of a unique index well to the designated sample. Pipette mix 5 times.

Note: PCR cycle numbers are dependent on the quantity of cDNA used to begin the library preparation, so refer to the cDNA measurements to determine the starting input.

45. Complete the Sample Index PCR step for all samples.
46. Continue into the PCR clean-up step or store.

Note: Individual samples may need different cycle numbers and so will need to be separated from each other. Label sample tubes and record cycle numbers carefully.

Pause point: Safe Stopping Point – Store at 4°C for up to 72 h; Freeze at −20°C for up to a week.

47. Complete a double-sided size selection clean-up step using SPRIselect beads. Refer to manufacturer's instructions for more detail.
48. Measure the concentration of cDNA libraries using Qubit High Sensitivity assays, Agilent Bioanalyzer High Sensitivity chips, and Kapa DNA Quantification reagents. Refer to Figure 4C and to troubleshooting 5.

Note: Safe Stopping Point – Store at 4°C for up to 72 h; Store at −20°C for long-term storage.

Pause point: Store library samples at −20°C for up to 6 months.

This section provides general guidance on how to perform single cell RNA sequencing (Figure 1B).

Note: The sequencing protocol may be changed depending on resources availability, cell type (s) of interest and the sample type.

Note: Our Libraries were sequenced at 60,000 fragment reads per cell. The flow cells were sequenced as 100 × 2 paired end reads on an Illumina HiSeq 4000. However, HiSeqs are no longer supported by Illumina.

49. Ensure sequencing quality by matching distribution of experimental groups between lanes to prevent batch effects.
50. Sequence the libraries at a minimum of 50,000 read pairs per cell in accordance with 10x Genomics' sequencing requirements.

Note: It is recommended to use the NovaSeq 6000 or NovaSeq X for sequencing, as their higher outputs enable the interrogation of larger cell numbers.

Note: Sequencing should be performed according to manufacturer's standard protocol for the specific instrument and flow cell type.

Note: Sequencing instruments and software versions will vary depending on the institution.

Data analysis

Timing: 6–12 h

This section outlines the protocol for initial data processing and quality control (Figure 1.6).

51. First perform the alignment feature and quantification as follows using the latest Cell Ranger Suite from 10× Genomics.
    a. Run Cell Ranger mkfastq function of Cell Ranger to demultiplex the raw base call files of the Illumina BCL output folder into unaligned fastq sequencing files.
    b. Run Cell Ranger count function of Cell Ranger using the reference gene expression data from 10× Genomics for the human hg38. Troubleshooting 6.

```
1. $cellranger count --id=$id --sample=$s --transcriptome=$ref --include-introns=true --fastqs=
$fq --jobmode=slurm --maxjobs=24 --mempercore=16000

2. Reference transcriptome file downloaded from 10xGenomics (e.g. refdata-gex-GRCh38-2020-A)
```

Note: Inputs are the species-specific reference database, sample name, directory containing fastq files, output directory name, and include introns flag set to true.

Note: You can find more details regarding the Cell Ranger data generated by this protocol in Chikkamenahalli et al.[1]

Note: The following steps utilize the R package Seurat normalize and cluster the scRNA-seq data. An example analysis is provided on the Seurat GitHub page.

c. Import data: load the filtered feature matrices into the Seurat R package for filtering and normalization.

```
1. dataMat <- Read10X(data.dir=$output_dir)
```

d. Clean data: Subset the data to cells with reads mapped to greater than or equal to 200 genes, and less than 40% of reads mapping to mitochondrial genes.

```
1. dataMat <- CreateSeuratObject(counts=dataMat, project=$id, min.cells=3, min.features=200)

2. dataMat[["percent.mt"]] <- PercentageFeatureSet(object = dataMat, pattern = "^MT-|^mt-")

3. dataMat <- subset(dataMat, subset= nFeature_RNA > 200 & nFeature_RNA < 300000 & percent.mt < 40)
```

e. Transform data: log Normalize the counts using the NormalizeData function.

```
1. dataMat <- SCTransform(dataMat, variable.features.n = 3000,vars.to.regress = 'percent.mt', verbose = FALSE,return.only.var.genes = FALSE)

2. dataMat <- NormalizeData(dataMat, verbose = FALSE)
```

f. Filter data: Subset the genes using the FindVariableFeatures function.

Note: This step requires at least 3 cells to have expression of a gene.

```
1. dataMat <- FindVariableFeatures(dataMat, selection.method = "vst", nfeatures = 2000)
```

g. Scale data: Scale the gene expression counts using the ScaleData function.

```
1. dataMat <- ScaleData(dataMat, features=all.genes)
```

h. Cluster and visualize data: perform unbiased clustering using the FindNeighbors, FindClusters, and RunUMAP functions.

```
1. dataMat <- FindNeighbors(dataMat, reduction = "pca", dims = 1:30)

2. dataMat <- FindClusters(dataMat, resolution = resolution)

3. dataMat <- RunTSNE(dataMat, reduction = "pca", dims = 1:30)

4. dataMat <- RunUMAP(dataMat, reduction = "pca", dims = 1:30)
```

i. Export data: convert the final normalized read counts to logTP10K + 1 expression values.

```
1. write.table(log(as.matrix(dataMat@assays$SCT@counts)+1), paste0($output_dir,'/normcount.txt'), row.names = T, col.names = T, quote=F, sep='\t')
```

52. Next, proceed with quality control.
    a. Compare the quality metrics output from the Cell Ranger suite to known batches and technical groups, to identify sources of non-biological variation.
    b. Create two matrices.
        i. In the first matrix, arrange the samples as the columns and all Cell Ranger quality metrics, both categorical and quantitative, as the rows.
        ii. In the second matrix, organize the samples as the columns and batch/technical group designations as the rows (e.g., processing batch or frozen/fresh collection).

c. Perform Hierarchical-all-against-all (HAllA) clustering of the two matrixes to identify statistically significant relationships between the two-matrices using an FDR of 0.05 as the threshold for significance.

Note: Batches or categories with statistically significant correlations to quality metrics may need batch correction prior to further analysis.

EXPECTED OUTCOMES

Our protocol should reliably produce cell yields ranging from 85,000 to 2,000,000 cells per sample with viability range between 70% and 100% $CD45^+$ cells. In our study, the twenty-three samples ranged from 0.4 to 6.0 g/sample. Based on the published output of a NovaSeq 6000 S4 lane and our recommendation of 50k read pairs/cell, 40,000 to 50,000 cells per lane can be sequenced. For a NovaSeq X 10B lane with the same read depth, up to 25,000 cells per lane can be sequenced. Thus, the yield of cells from our protocol is sufficient for subsequent scRNA-seq analysis to identify immune cell types and their transcriptomic profiles.

LIMITATIONS

Whereas our protocol proposes average of reliable cell number and viability, the range of cell number and viability is dependent on tissue quantity and quality. Although our procedures were fine-tuned for isolating immune cells from stomach muscularis propria, they were not used for the isolation of other cell types, such as neurons, smooth muscles, and interstitial cells of Cajal. Additionally, this protocol was not optimized for using tissue from other anatomical regions of the alimentary canal. Given that the thickness of the different gut regions varies as does the amount of connective tissue, optimization of the dissociation protocol will be needed for other parts of the gut. This protocol was used to capture 2000–3000 $CD45^+$ cells per sample. The number of captured cells and reads depth may vary depending on the research goal. For example, investigating rare population require high cell number, while studying subtle changes between experimental conditions require high reads depth.[7]

TROUBLESHOOTING

Problem 1

Gastric muscularis propria is not fully dissociated (See step 5).

Potential solution

- Increase dissociation timing on the gentleMACS Dissociators. You may run the sample for an additional 5–10 min. However, overly long dissociation can decrease cellular viability (See step 5 and Human stomach muscularis propria dissociation program on gentleMACS Dissociators).

Problem 2

Low cell yields.

This may result from several reasons, including insufficient dissociation indicated by the presence of fully undissociated tissue pieces, low tissue weight, and the use of the wrong enzymes or enzyme degradation. Conversely, harsh dissociation may lead to low cell yield by compromising the cells viability.[8] Moreover, low tissue quality, delayed tissue preservation after biopsy, long dissection duration and considerable delay before tissue processing may be considered as contributing factors as they may affect the cells viability and hence results into low cell yields (See step 5 and step 1).

Potential solution

- Increase tissue weight up to 1 g.
- See potential solutions for improving cells viability and tissue quality in solutions related to problem 3.

Problem 3

Low cell viability.

This problem may arise from poor tissue handling or storage before experiments (See step 5) or harsh dissociation or due to cellular damaged caused by high enzymes concentrations.[9] (See step 2).

Potential solution

- We evaluated two different tissue storage buffers including F12 medium and MACS Tissue Storage Solution. The MACS Tissue Storage Solution was better, as it improved the cells viability.
- You may reduce the concentration of working enzyme solution or the incubation time.[8] We did assess different strengths of the enzymes cocktail and dissociation time, and cells viability was improved by reducing the both enzymes strength and incubation time.
- Maintain the proposed dissection time in this protocol (See step 1).

Problem 4

Poor emulsification (Figure 4A) (See step 29).

If there is a clog on the chip, there may be no emulsification product recovered or there may be a failed emulsification product recovered. A poor or failed emulsion will not yield good cDNA or a proper library downstream. A partial emulsion may yield good cDNA, but the quantity will be low.

Potential solution

- High viability samples with no debris typically do not clog the chips. Samples with debris can cause clogs. Only load high viability samples onto the chip.
- Be sure that there is no floating debris from reagents or consumables. Shards of plastic or clumps of flocky reagents will not be counted during cell QC, but they may be big enough (or inflexible enough) to clog the microchannels in the chip.
- Perform careful pipetting during the chip loading step.
- Keep the chip holder containing the chip on a flat lab bench surface. Do not keep a bench pad or bench paper beneath the chip holder. It could cause a static charge that could affect sample flow in the chip.
- Be sure to fill all wells in correct order with correct reagents, per manufacturer's instructions.
- Do not jostle chip or chip holder once chip is filled. Carefully place chip into controller to begin partitioning step.

Problem 5

- Low cDNA yield (See step 32). This problem may result if not enough cells were targeted for partitioning/capture (Figure 4B). Also, if there was a poor emulsion, there may not have been enough cells partitioned/captured, and so there may be a poor cDNA yield.
- Poor library (See step 48). A poor library may be created because of an incorrect amount of input cDNA, or an input cDNA of poor quality. If there was very little cDNA input, the subsequent library may not measure well enough to sequence (Figure 4C). If there is very little cDNA input, there may be over-cycling at the index PCR stage. The library QC may look acceptable, but the library may not sequence well (over-amplification; too many repeated sequences; not a true representation of original sample). Last, if there were an improper cDNA measurement, an incorrect amount of cDNA could have been used to create the library.

Potential solution

- If the reason for low cDNA yield is a poor emulsion, the experiment may need to be repeated. Occasionally, a poor emulsion will yield enough cDNA, but many times, it will not.

- During library preparation, the number of PCR cycles may need to be raised.
- If there are lower numbers of cells targeted, or potentially lower numbers of cells partitioned/captured, the number of PCR cycles during cDNA amplification can be increased.
- Some cell types will inherently contain lower levels of RNA, so they will yield lower levels of cDNA. It may be necessary to increase the number of PCR cycles by 1 or 2 cycles to obtain enough cDNA to create a proper library.
- Remeasure the original cDNA and repeat the library preparation steps.

Problem 6

Poor mapping and alignment to genome (See step 51 and Figure 4).

Lower than expected mapping percentage can be due to using the wrong reference genome for alignment.

Potential solution

- Double checking the reference genome against the source of the samples may identify a discrepancy.
- Rerun Cell Ranger using the correct genome.

Problem 7

High duplication percentage in samples (See step 23).

The duplication percentage of reads in a sample is >80%. Duplication is usually the result of low molecular diversity of the starting library input.

Potential solution

- Regenerate libraries from a fresh RNA extraction

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Madhusudan Grover (Grover.Madhusudan@mayo.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Shefaa AlAsfoor (Alasfoor.Shefaa@mayo.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The gene expression data generated during this study are available at Gene Expression Omnibus, GEO: GSE252126.

This study does not report original code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103258.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.A., C.E.B., E.J., V.A.S., F.R., G.C., L.C.H., J.A.P., K.J.C., J.S.L., S.J.M., S.D., G.F., and M.G.; investigation, S.A.; writing – original draft, S.A., E.J., C.E.B., V.A.S., and F.R.; writing – review and editing, S.A., C.E.B., E.J., V.A.S., F.R., G.C., L.C.H., J.A.P., K.J.C., J.S.L., S.J.M., S.D., G.F., and M.G.; visualization, S.A., V.A.S., F.R., and E.J.; supervision, M.G.

DECLARATION OF INTERESTS

M.G. has received investigator-initiated grant funding from Takeda Pharmaceuticals and clinical trial funding from Alexza and Donga Pharmaceuticals. He serves as a consultant for Alfasigma, Ardelyx, and Evoke Pharmaceuticals.

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