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🌐 Cell lysis plus depletion single-nucleus RNA-seq (CL-D snRNAseq)

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ojasvi chaudhary¹, Mia Steinberg¹, Grant Duclos¹, Peter Gathungu¹, Manisha Rao¹, Rogelio Aguilar¹, Varsha Shankarappa¹, Chris Rands¹, Xi Chen¹, Rebecca Halpin¹, Elizabeth Galery¹, Joseph Boland¹, Maurizio Scaltriti¹, Brian Dougherty¹, Asaf Rotem¹

¹Astrazeneca



ojasvi chaudhary

Astrazeneca

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We use this protocol and it's working

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Abstract

PAXgene Blood RNA Tubes (Qiagen) are commonly used for the direct collection of whole blood from donors. These tubes contain a proprietary solution that stabilizes RNA in blood cells, allowing for long-term storage at -80°C . For routine preparation of frozen PAXgene blood specimens for single-nucleus RNA sequencing (snRNA-seq), it has been demonstrated that nuclei must be isolated and RNA from these nuclei must be profiled using a specific, reproducible protocol. Here, we present a protocol focused on the isolation of nuclei from frozen PAXgene blood specimens, followed by library preparation using the 10x Genomics Single Cell Gene Expression Solution. Additionally, we describe the use of CRISPR-based digestion to remove cDNA generated from globin gene RNA, optimizing the quality of sequencing data.

Guidelines

ENVIRONMENT

This protocol must be utilized in a BSL-2 laboratory facility.

Materials

 CG000315_ChromiumNextGEMSin... 4.3MB

EQUIPMENT AND MATERIALS for nuclei isolation

A	B	C
Reagents / Consumables / Equipment	Details	Supplier / Cat. No.
UltraPure Distilled Water	Reagent for making cell lysis buffer	Invitrogen/ThermoFisher / Cat. No. 10977015
Sodium chloride solution 5M	Reagent for making cell lysis buffer	Sigma / Cat. No. 59222C
Trizma hydrochloride solution	Reagent for making cell lysis buffer	Sigma / Cat. No. T2194
Magnesium chloride solution 1M	Reagent for making cell lysis buffer	Sigma / Cat. No. M1028
10% Tween 20	Reagent for making cell lysis and wash buffer	Bio-Rad / Cat. No. 1662404
RNase Inhibitor, Murine	Reagent for making cell lysis and wash buffers	New England BioLabs / Cat. No. M0314L
1X Dulbecco's Phosphate Buffered Saline (DPBS), no calcium, no magnesium (sterile filtered, suitable for cell culture)	Reagent for making wash buffer	Gibco/ThermoFisher / Cat. No. 14190144
10% MACS BSA Stock Solution	Reagent for making wash buffer	Miltenyi Biotec / Cat. No. 130-091-376
Digitonin		
(Heat to 65°C and make multiple aliquots of 20 ul. Store for long term at 4°C. Each aliquot can be used multiple times)	For lysis buffer	Thermofisher / Cat. No. BN2006
Nonidet P-40		
(or use IGEPRAL CA-630 as substitute. Freshly prepare 10% solution)	For lysis buffer	Sigma / Cat. No. 74385
Sigma / Cat. No. i8896		
Dithiothreitol (DTT)	For lysis and wash buffer	Sigma / Cat. No. 646563
100µm Cell Strainer	For debris removal	Corning / Cat. No. 431752

A	B	C
40µm Cell Strainer	For debris removal	Corning / Cat. No. 431750
Cellometer Auto 2000 Cell Viability Counter	For measuring cell concentration and viability	Nexcelom Bioscience

Table continued -

A	B	C
Reagents / Consumables / Equipment	Details	Supplier / Cat. No.
<ul style="list-style-type: none"> Pipette controller Sterile serological pipettes (5 ml, 10 ml & 50 ml, polystyrene) Single channel pipette (P20, P200, P1000) Filtered sterile pipette tips (P20, P200, P1000) 	For handling liquids	General
Centrifuge Requirements: <ul style="list-style-type: none"> Capable of temperature control at 4°C. Capable of centrifugation at 3000 RCF. Equipped with swinging bucket rotor. Centrifuge carriers and inserts should be of the size specific to the tubes used.	To pellet nuclei	General
Centrifuge Requirements: <ul style="list-style-type: none"> Capable of temperature control at 4°C. Capable of centrifugation at 3000 RCF. Equipped with swinging bucket rotor. Centrifuge carriers and inserts should be of the size specific to the tubes used.	To pellet nuclei	General
Conical tubes (50ml)	Liquid collection & centrifugation	General
<ul style="list-style-type: none"> Kimwipes (small, 8.4 × 4.4 in) 	To cover sterile tweezers	Kimberly-Clark / Cat. No. 34120

Table 1. Lysis Buffer Preparation (all volumes in ml)

A	B	C	D
Final Volume:	1ml	2ml (4 reactions)	4ml
H2O	0.835	1.67	3.34

	A	B	C	D
	5M NaCl	0.002	0.004	0.008
	1M Tris-HCl pH7.4	0.010	0.020	0.040
	1M MgCl ₂	0.003	0.006	0.012
	10% Tween-20	0.010	0.020	0.040
	Freshly prepared 10% NP40 or Substitute solution	0.010	0.020	0.040
	Digitonin	0.002	0.004	0.008
	10% MACS BSA Stock Solution	0.100	0.200	0.400
	DTT	0.001	0.002	0.004
	---	---	---	---
	+RNase Inhibitor	0.025	0.050	0.100

Table 2. Lysis Wash Buffer Preparation (all volumes in ml)

	A	B	C	D	E	F
	Final Volume:	4ml	8ml	12ml	16ml (4 reactions)	20ml
	H ₂ O	3.4	6.8	10.2	13.6	17
	5M NaCl	0.008	0.016	0.024	0.032	0.040
	1M Tris-HCl pH7.4	0.040	0.080	0.120	0.160	0.200
	1M MgCl ₂	0.012	0.024	0.036	0.048	0.060
	10% Tween	0.040	0.080	0.120	0.160	0.200
	DTT	0.004	0.008	0.012	0.016	0.020
	10% MACS BSA Stock Solution	0.400	0.800	1.2	1.6	2
	---	---	---	---	---	---
	+RNase Inhibitor	0.100	0.200	0.300	0.400	0.500

Table 3. General Wash Buffer (PBS with 0.04% BSA) Preparation (all volumes in ml)

	A	B	C	D	E	F	G	H
Final Volume:	1 ml	25 ml	50 ml	75 ml	100 ml	150 ml (4 reactions)	200 ml	
PBS	0.996	24.9	49.8	74.7	99.6	149.4	199.2	
10% BSA (Miltenyi)	0.004	0.1	0.2	0.3	0.4	0.6	0.8	
---	---	---	---	---	---	---	---	---
+RNase Inhibitor	0.001	0.025	0.05	0.075	0.1	0.15	0.2	

Table 4. H2O with RNase Inhibitor (all volumes in ml)

	A	B	C	D	E	F	G	H
Final Volume:	1 ml	25 ml (4 reactions)	50 ml	75 ml	100 ml	150 ml	200 ml	
H2O	0.999	24.975	49.95	74.925	99.9	149.85	199.8	
---	---	---	---	---	---	---	---	---
+RNase Inhibitor	0.001	0.025	0.05	0.075	0.1	0.15	0.2	

Additional materials for Nucleus Concentration Measurement:

- AO/PI Cellometer ViaStain AOPI Staining Solution
- Cellometer PD100 Counting Chambers

EQUIPMENT AND MATERIALS for globin RNA depletion

A	B	C
Reagents/consumable /Equipment	Details	Supplier/Cat. No.
CRISPRclean RNA depletion kit	For CRISPR digestion of cDNA	Jumpcode Genomics, KIT1024
Library construction kit	For Library preparation	10x Genomics, 1000190 or 1000196
0.2 ml PCR 8-tube strips (TempAssure)	For reactions and clean-ups	USA scientific, 1402-4700
Vortex	For mixing	General
Microcentrifuge	For spinning down	General

	A	B	C
	PCR Thermal Cycler	To carry out reactions	Bio-Rad C1000, 1851148, or equivalent thermal cycler
	cDNA-amplified product	Input material	Supplied from workflow
	SPRIselect	For cleaning up DNA fragments	Beckman coulter, B23318
	Ethanol	To wash SPRI beads	Sigma, E7023-500ML
	10x Magnetic Separator	For SPRI clean-up	10x Genomics, 230003
	Buffer EB	For SPRI product elution	Qiagen, 19086
	UltraPure Distilled Water	Reagent for making 80% ethanol	Invitrogen/ThermoFisher / Cat. No. 10977015

Table 5. SPRIselect Preparation (all volumes in ml)

	A	B	C	D	E	F
		2 Samples	4 Sample	8 Samples	16 Samples	24 Samples
	SPRIselect	0.250	0.500	1	2	3

Table 6. Ribonucleoprotein (RNP) Complex Preparation (all volumes in µl)

	A	B	C	D	E
	Reagent	1 reaction	2 reactions	4 reactions	8 reactions
	10x Cas9 Buffer	1.1 µl	2.2 µl	4.4 µl	8.8 µl
	RNase Inhibitor	1.1 µl	2.2 µl	4.4 µl	8.8 µl
	Guide RNA	1.76 µl	3.52 µl	7.04 µl	14.08 µl
	Cas9	0.99 µl	1.98 µl	3.96 µl	7.92 µl
	Nuclease-Free Water	4.07 µl	8.14 µl	16.28 µl	32.56 µl
	Total Volume	9.02 µl	18.04 µl	36.08 µl	72.16 µl

Table 7: Sample Index PCR Cycle Number Guidelines

	A	B
	Input cDNA in nanograms (ng)	Index PCR cycles
	200 ng or greater	11
	20ng to 200 ng	12
	Less than 20 ng	14

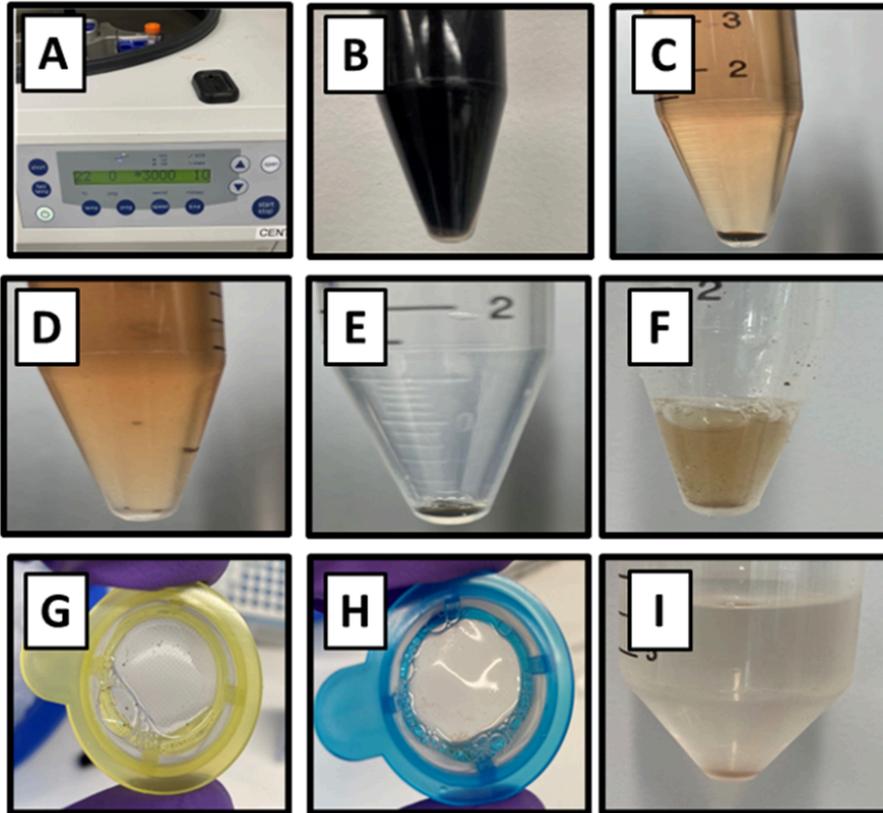
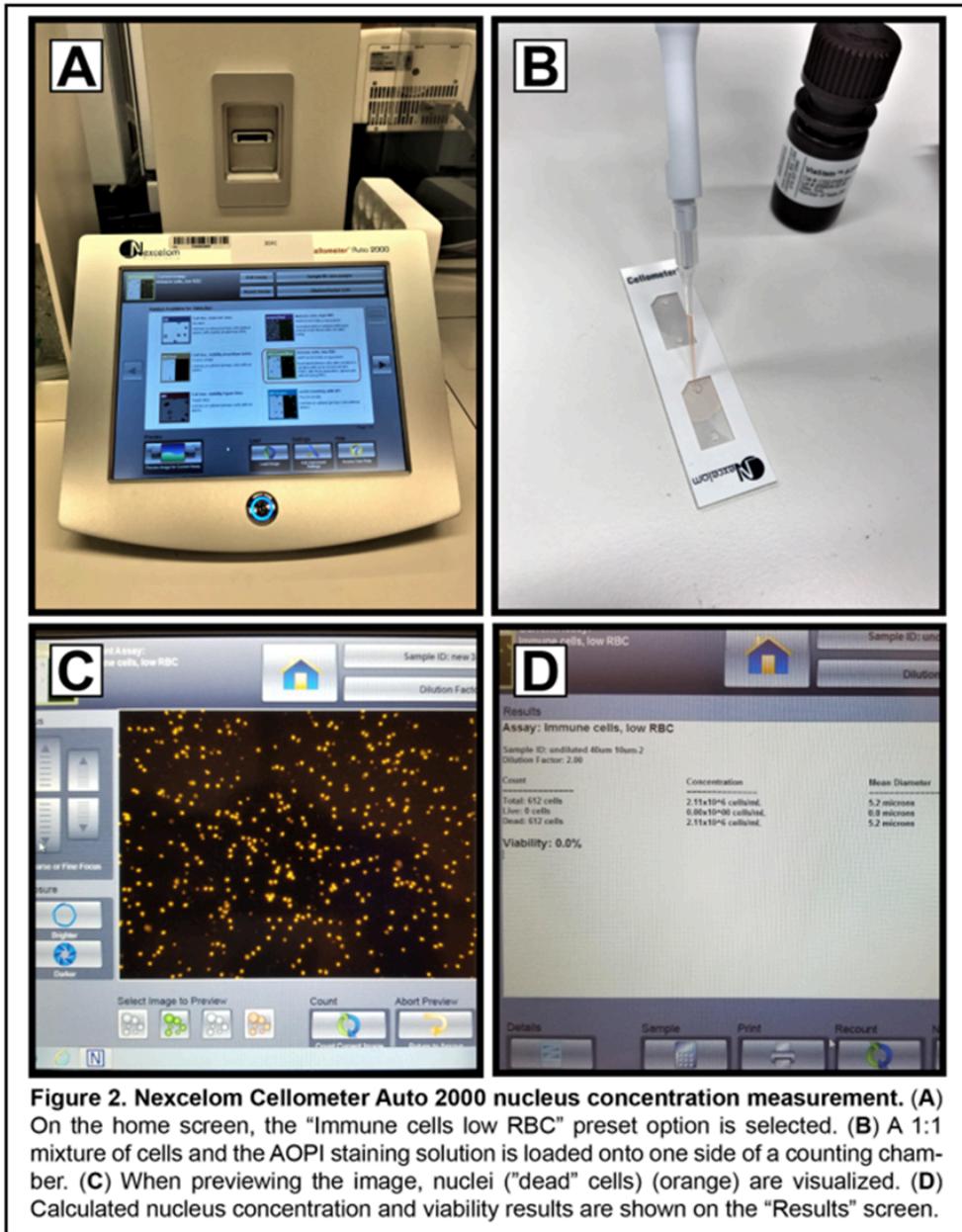


Figure 1: Nuclei preparation from PAXgene RNA blood. (A) Centrifuge is set at 3000 RCF for 10 minutes. **(B)** Notice Gray pellet of PAXgene blood after centrifugation. **(C)** After adding H₂O with RNase Inhibitor to pellet and **(D)** Resuspension. **(E)** Removal of H₂O with RNase Inhibitor **(F)** Shows resuspension of pellet in lysis buffer. **(G)** Picture of debris on 100 μ m filter **(H)** Debris on 40 μ m filter **(I)** Pellet during washes after filtration.



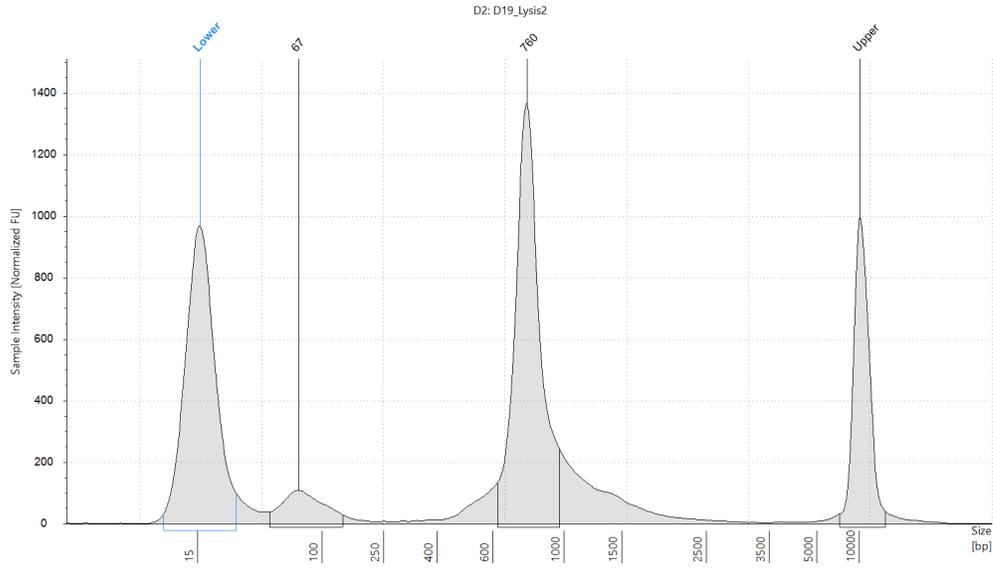
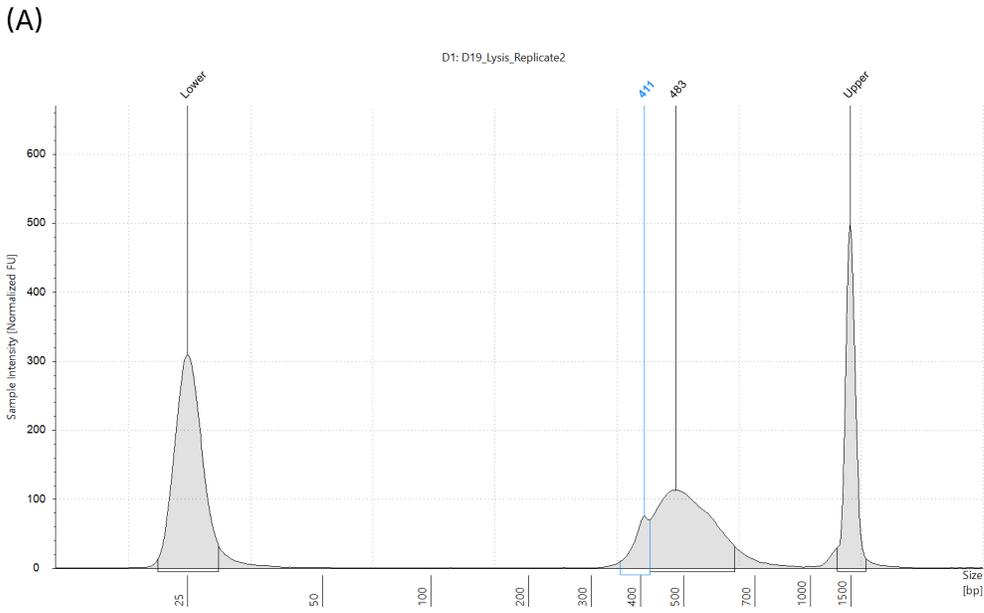


Figure 3: cDNA amplified product from PAXgene blood nuclei. Characteristic sharp peak at ~750 base pairs (bp).



(B)

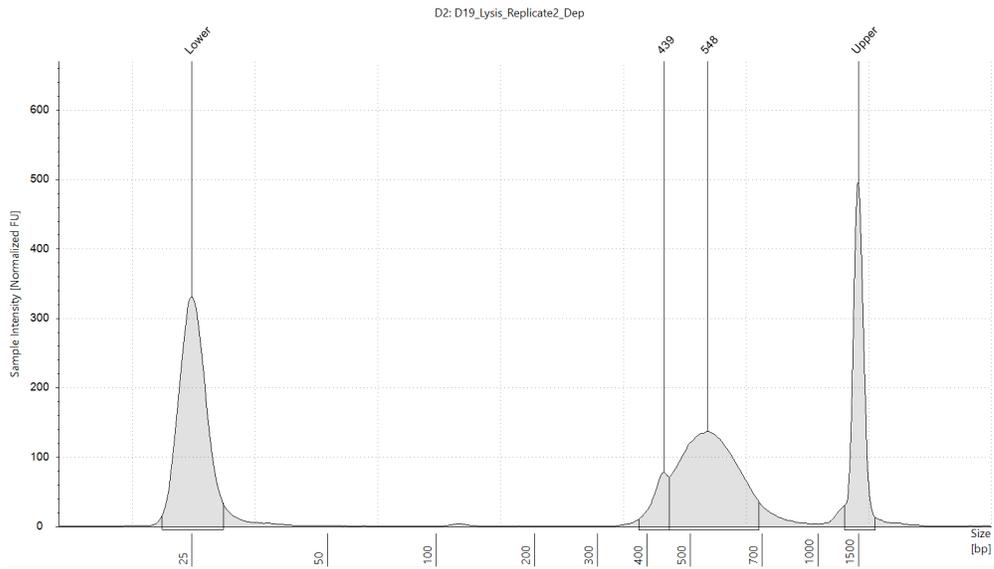


Figure 4: Final library from PAXgene blood single nucleus sequencing before (A) and after (B) depletion of globin genes. The depleted libraries have larger average size.

Troubleshooting

PROCESS FLOW

- 1 (I) Frozen PAXgene Blood Nucleus Isolation (II) 10X Genomics GEX GEM Generation and Barcoding (III) 10X Genomics GEX cDNA Amplification (IV) 10X Genomics GEX Library Construction Part A (V) Globin RNA Depletion (VI) 10X Genomics GEX Library Construction Part B

(I) FROZEN PAXgene blood nucleus isolation ADVANCED PREPARATION INSTRUCTIONS

18h

- 2 One day prior to protocol initiation, transfer frozen specimen(s) (stored in -80°C freezer) to 4°C .
 - 2.1 Incubate at 4°C overnight (at least 16 hours) to thaw. 16h 
 - 2.2 After overnight 4°C incubation, transfer specimen(s) to room temperature (RT).
 - 2.3 Incubate at RT for 2 hours. 2h 
 - 2.4 **Note:** It is recommended that an individual user does NOT exceed simultaneous processing of 4 specimens. Delays in processing due to excessive sample numbers may irrevocably reduce sample quality. If an experiment requires greater than 4 specimens, multiple rounds of sample preparation are recommended.

(I) FROZEN PAXgene blood nucleus isolation PREPARATION INSTRUCTIONS

1h

- 3 Ensure that the temperature of the swinging-bucket centrifuge is RT. 
- 4 Prepare Lysis Buffer for cell lysis on ice (4°C) (at least 500 μl per sample). See Table 1 (Materials) to guide Lysis Buffer preparation (range of final volumes). Immediately prior to use, add 25 μl RNase inhibitor per 1 ml Lysis Buffer (1U of RNase inhibitor per 1 μl Lysis buffer) and mix well by pipetting 10 times.  
- 5 Prepare Lysis Wash Buffer on ice (4°C) (at least 4 ml per sample). See Table 2 (Materials) to guide Lysis Wash Buffer preparation (range of final volumes).  



Immediately prior to use, add 25 ul RNase inhibitor per 1 ml Wash Buffer (1U of RNase inhibitor per 1ul Wash buffer) and mix well by pipetting 10 times.

- 6 Prepare General Wash Buffer (PBS with 0.04% BSA) on ice (4°C).
See Table 3 (Materials) to guide General Wash Buffer preparation (range of final volumes).
Immediately prior to use, add 1ul RNase inhibitor per 1 ml General Wash Buffer (40U of RNase inhibitor per 1 ml Wash Buffer) and mix well by pipetting 10 times.



- 7 Prepare H2O with RNase Inhibitor on ice (4°C).
See Table 4 (Materials) to guide H2O with RNase inhibitor preparation (range of final volumes).
Immediately prior to use, add 1ul RNase inhibitor per 1 ml H2O (40U of RNase inhibitor per 1 ml Wash Buffer) and mix well by pipetting 10 times.



(I) Frozen PAXgene Blood Nucleus Isolation - Preparation for Nucleus Extraction (refer to figure 1)

40m

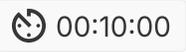
- 8 Invert thawed PAXgene blood specimen 10 times.
- 9 Using a 10ml serological pipette, pipette the full sample volume 10 times to mix.
- 10 Transfer 4ml thawed PAXgene blood to a new 15ml conical tube.
- 11 Centrifuge the 15ml tube containing the 4ml sample volume at for at RT.
- 12 Using a 5ml serological pipette, gently remove the supernatant and transfer to an empty tube (in case of accidental pellet aspiration) leaving a volume of 0.5ml.
- 13 ♦ **Note:** A large dark red pellet should be clearly visible post-centrifugation.
- 14 Using a 5ml serological pipette, resuspend pellet in 4ml H2O with RNase inhibitor and pipette 10 times to mix.
- 15 ♦ **Note:** If the pellet does not fully dissolve during resuspension, proceed to Step 7.



10m

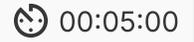




- 16 Centrifuge the 15ml tube containing the 4ml sample volume at  3000 x g, 25°C for  00:10:00 . 10m
- 17 Using a 5ml serological pipette, gently remove the supernatant and transfer to an empty tube (in case of accidental pellet aspiration) (if not possible to completely remove the supernatant without disturbing the pellet, leave a volume of 0.1ml).
- 18 ♦ **Note:** Only perform Step 8 one sample at a time and proceed **immediately** to “Nucleus Extraction” – do not allow the pellet to dry out after supernatant removal.

(I) Frozen PAXgene Blood Nucleus Isolation - Nucleus Extraction (refer to figure 1)

10m

- 19 Using a wide-bore P1000 tip, resuspend the pellet in 350ul Lysis Buffer and pipette the full sample volume 10 times to mix.
♦ **Note:** Mix the sample well and ensure that the pellet is fully dissolved.
- 20 Incubate on ice ( 4 °C) for  00:05:00 . 5m
- 21 Immediately after incubation, using a 5ml serological pipette, add 3.5ml Lysis Wash Buffer (to stop the lysis) and pipette the full sample volume 10 times to mix. 
- 22 ♦ **Note:** If the pellet does not fully dissolve during resuspension, please proceed to “III. Nucleus Purification” regardless.

Frozen PAXgene Blood Nucleus Isolation - Nucleus Purification (refer to figure 1)

1h

- 23 All steps related to Nucleus Purification starting steps 19 must be performed on ice.
- 24 Place a 100um Falcon Cell Strainer on a new 50ml conical tube.
- 25 Using a wide-bore P1000 tip, wet the surface of the 100um Falcon Cell Strainer with 1ml General Wash Buffer (cover the entire surface of the strainer).

- 26 Using a 5ml serological pipette, pipette the full sample volume (~4ml) through the 100um Falcon Cell Strainer.
 - ◆ **Note:** To minimize sample loss, firmly press the 5ml serological pipette against the cell strainer when ejecting the volume.
- 27 Using a wide-bore P1000 tip, wash the 15ml tube (that previously contained the suspended sample) with 1ml General Wash Buffer, then pipette the volume through the 100um Falcon Cell Strainer. Repeat 2 more times with General Wash Buffer (3 washes in total – the resulting volume in the 50ml conical tube is ~8ml).
- 28 Place a 40um Falcon Cell Strainer on a new 50ml conical tube.
- 29 Using a wide-bore P1000 tip, wet the surface of the 40um Falcon Cell Strainer with 1ml General Wash Buffer (cover the entire surface of the strainer).
- 30 Using a 10ml serological pipette, pipette the ~8ml sample volume (previously filtered through the 100um strainer) through the 40um Falcon Cell Strainer.
- 31 Using a wide-bore P1000 tip, wash the 50ml conical tube (that previously contained the 100um strainer flow-through) with 1ml General Wash Buffer, then pipette the volume through the 40um Falcon Cell Strainer. Repeat 2 more times with General Wash Buffer (3 washes in total – the resulting volume in the 50ml conical tube is ~12ml).
- 32 Centrifuge the 50ml tube containing the ~12ml sample volume at 500 x g, 4°C for 00:05:00 .
- 33 Using a 10ml serological pipette gently remove the supernatant and transfer to an empty tube (in case of accidental pellet aspiration) leaving a volume of 1ml.
- 34 ◆ **Note:** Pellet may NOT be visible and due to instability, use caution when removing supernatant (i.e., extremely slow aspiration is required).
- 35 Add 10ml General Wash Buffer to the sample pellet and resuspend by pipetting 5 times.
- 36 Centrifuge the 50ml tube containing the 10ml sample volume at 500 x g, 4°C for 00:05:00 .
- 37 Using a 10ml serological pipette gently remove the supernatant and transfer to an empty tube (in case of accidental pellet aspiration) leaving a volume of 0.5ml. Completely



5m





remove supernatant if pellet is visible.

38 ♦ **Note:** Pellet may NOT be visible and due to instability, use caution when removing supernatant (i.e., extremely slow aspiration is required).

39 Resuspend the pellet in the residual 0.5ml volume.

(I) Frozen PAXgene Blood Nucleus Isolation - Nucleus Concentration Measurement (refer to figure 2)

20m

40 • **Technical Note:**

- Cell concentration must be between 1×10^5 and 1×10^7 for accurate measurement by the Cellometer Auto 2000.
- High sample-to-sample variability in nucleus yield is expected.
- Using the following workflow, all isolated nuclei should be detectable as “Dead” cells (orange fluorescence) with very few detectable “Live” cells (green fluorescence).

41 Collect the following materials before proceeding:

- AO/PI Cellometer ViaStain AOPI Staining Solution
- Cellometer PD100 Counting Chambers

42 On the Cellometer Auto 2000, select the “Immune cells, low RBC” pre-set option.

43 For each aliquot, use a wide-bore P1000 pipette tip to thoroughly mix nuclei 10 times immediately prior to use.

44 Pipette 20ul nuclei into a new 1.5ml Eppendorf tube.

45 Pipette 20ul AOPI solution into the same 1.5ml Eppendorf tube that contains 20ul nuclei.

46 Using a pipette set to 20ul, mix the cells & AOPI 5 times, then immediately load 20ul of that mixture into one side of a counting chamber.

47 Insert the loaded side of the counting chamber into the “sample slot” of the Cellometer Auto 2000.

48 Select “Preview Image for Current Assay” at the bottom-left of the screen.



- 49 Upon previewing the image, the “bright-field” will be visualized by default.
- 50 Adjust the focus of the image using the “Course Focus” or “Fine Focus” options on the left side of the Preview screen.
- 51 Along the bottom of the preview screen, select the option depicting orange cells to visualize nuclei (confirm that the image is in focus – adjust if needed).
- 52 Along the bottom of the preview screen, select the option depicting green cells to visualize live cells (it is expected that very few live cells will be detectable).
- 53 Select “Count” / “Count Current Image” to calculate cell concentration and viability.
- 54 Following the calculation, the “Results” screen will be depicted.
Record total cell concentration and viability as follows:
 - Under “Concentration”, to the right of “Total” (under “Count”): record this value (total cell concentration) (“# x 10⁴ cells/mL”)
 - To the right of “Viability”: record this value (percentage of live cells) (“#%”)All cells should be dead stained in PI. The viability should be 0% or very close to 0%.

(II-VI) Introduction

- 55 When generating single-nucleus (sn) RNA-Seq data from frozen PAXgene blood specimens, ambient RNA derived from red blood cells (RBCs) has been detected in white blood cell nuclei. Much of this contamination can be attributed to a small number of RBC-specific globin genes: HBB, HBA1, HBA2, and HBD. Importantly, it has been shown that a CRISPR-based approach can modify the amplified complementary DNA (cDNA) used to generate sequencing libraries compatible with both the 10x Genomics workflow and the Illumina sequencing platform. For routine preparation of snRNA-Seq data from frozen PAXgene blood specimens, a CRISPR-based strategy can be used to deplete RBC-derived globin RNA from amplified cDNA following a specific, reproducible protocol. This protocol is integrated directly within the standard 10x Genomics gene expression workflow (following step 3.4 in protocol CG000315) and focuses on selective CRISPR digestion of cDNA generated from RNA specific to HBB, HBA1, HBA2, and HBD, followed by SPRI clean-up.

(II-VI) ADVANCED PREPARATION INSTRUCTIONS

20m



- 56 Jumpcode Genomics KIT1024 includes two boxes.Box 1 (store at -20°C) contains: 10x Cas9 Buffer (REA1040), Cas9 (REA1039), RNase Inhibitor (REA1041), Nuclease-Free Water (REA1042). Box 2 (store at -80°C) contains: Guide RNA for Globin (REA1053)
- 57 After the initial thaw of the Guide RNA for Globin (REA1053), prepare aliquots of 10 µl (≤5 reactions) and store at -80°C.
Note: When subsequently using guide RNA aliquots, DO NOT exceed 3 freeze-thaw cycles.
- 58 Set up the following thermal cycler program for "CRISPR Digestion":
a. 37°C for 60 minutes (with lid temperature set to 37°C)
b. 4°C infinite hold
- 59 Prepare SPRIselect aliquots according to "Table 5. SPRIselect Preparation".

(II-VI) PREPARATION INSTRUCTIONS

30m

- 60 **Note:** Prior to preparing aliquots, fully resuspend SPRIselect stock by vortexing and ensure that the solution is a consistent brown colour.
Note: Pay close attention to the elution volumes throughout this protocol (volumes are lower than those specified in the standardized 10X Genomics workflow).
- 61 Freshly prepare 80% ethanol using UltraPure distilled water for each experimental day.
- 62 Prior to initiation of CRISR Digestion, thaw Nuclease-Free Water at room temperature for 10 minutes.
- 63 Prior to initiation of CRISR Digestion, transfer the following reagents to thaw on ice for 10 minutes:
- Guide RNA for Globin aliquot
 - 10x Cas9 Buffer
 - Cas9
 - RNase Inhibitor
- Note:** This protocol has been validated on Chromium Next GEM Single Cell 3' Kit v3.1 (10x Genomics, user manual CG000315 Rev E). This protocol is likely applicable other 10x Genomics gene expression products, including Chromium Next GEM Single Cell 3' HT Kit v3.1 (10x Genomics, user manual CG000416 Rev D), Chromium Next GEM Single cell 5' Kit v2 (10x Genomics, user manual CG000331), Chromium Next GEM Single Cell 5' HT Kit

v2 (10x Genomics, user manual CG000423 Rev C) and Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (CG000338 Rev F), but it has not been validated.

(II-VI) PROCESS INSTRUCTION

1h 44m

- 64 **Note:** Pay close attention to the elution volumes throughout this protocol (volumes are lower than those specified in the standardized 10X Genomics workflow).
- 65 Perform "Post Ligation Cleanup" according to 10X Genomics user manual CG000315 Rev E (Step 3.4), but elute post-ligation product in 16 ml Buffer EB and transfer 15 ml eluted product to a new 0.2 ml tube strip. 
- Note:** The elution volume will be 16 μ l instead of 30.5 μ l (as written in 10X Genomics' user manual. Attached in materials). Revised Step 3.4 (j-m) in brief:
- j. Add 16 μ l Buffer EB to resuspend the SPRI beads with bound post-ligation product.
 - k. Incubate for 2 minutes.
 - l. Place on the 10x Magnetic Separator (in low position) (incubate 2 minutes)
 - m. Transfer 15 μ l eluted product to new 0.2 ml tube strip.
- 66 Prepare CRISPR digestion reaction, referred to by the manufacturer as "Ribonucleoprotein (RNP) complex" in an RNase-free tube according to "Table 6: RNP Complex Preparation".
- Note:** After thawing reagents on ice, DO NOT vortex - finger-tap tubes to mix and spin down.
- 67 Gently pipette mix the RNP master mix. Centrifuge briefly to collect the contents on the bottom of the tube.
- 68 Incubate at room temperature for  00:10:00 . 10m
- 69 Add 1.5 μ l 10x Cas9 Buffer to 15 μ l of post-ligation product (in 0.2 ml PCR tube strip). And add 8.2 μ l RNP to bring total volume to 24.7 μ l.
- 70 Using a P20 pipette, pipette mix 10 times and centrifuge for 2 seconds.
- 71 Transfer sample to thermocycler and start program "CRISPR Digestion".  01:00:00 1h
- 72 Immediately after 60 minutes are over, transfer reaction tube to ice for 2  00:02:00 minutes. 2m



- 73 Briefly centrifuge (2-5 seconds) the CRISPR Digestion reaction tube
- 74 Add 25.3 μ l Nuclease-Free Water to the CRISPR Digestion reaction, then pipette mix 10 times.
- 75 Thoroughly resuspend SPRI beads by vortexing. Add 30 μ l of resuspended beads (0.6X) to digestion mix. Pipette mix 10 times and incubate for  00:10:00 at room temperature. After 5 minutes of incubation (midway), pipette mix 10 times. 10m
- 76 Place the tube strip on 10x Magnetic Separator at high position and allow solution to clear ( 00:03:00). Set the pipette to 80 μ l and slowly remove the supernatant. **Note:** DO NOT disturb the beads – only supernatant should be removed. 3m
- 77 Add 200 μ l of 80% ethanol to the beads while tube is on the 10x Magnetic Separator and incubate for 30 seconds.
- 78 Set the pipette to 200 μ l, then remove and discard ethanol. Repeat step 5 for a total of 2 washes. **Note:** DO NOT disturb the beads - only ethanol should be removed.
- 79 Briefly centrifuge the tube strip (1-2 seconds), then place the tube strip on the 10x Magnetic Separator at low position. Using P20 pipette, completely remove all the ethanol. **Note:** DO NOT disturb the beads - only ethanol should be removed. Leave the cap open after ethanol has been removed.
- 80 Air dry for  00:05:00 . 5m
- 81 Remove the tube strip from the 10x Magnetic Separator and add 31 μ l of Buffer EB to the beads.
- 82 Fully resuspend the beads in Buffer EB and incubate for  00:10:00 at room temperature. After 5 minutes of incubation (midway), pipette mix 10 times. 10m
- 83 Place the tube strip on the 10x Magnetic Separator at high position and incubate for  00:02:00 (allow solution to clear). Transfer 30 μ l of the supernatant to a new 0.2 ml 2m



tube strip - this product will be used for "Sample Index PCR" according to 10X Genomics user manual CG000315 Rev E (Step 3.5).

- 84 Perform "Sample Index PCR" according to 10X Genomics user manual CG000315 Rev E (Step 3.5). See guidelines for index cycles in "Table 7: Sample Index PCR Cycle Number Guidelines".

Note: For optimal results stay within 20 ng to 200 ng input and 12 index PCR cycles.

- 85 Perform "Post Sample Index PCR Double Sided Size Selection" according to 10X Genomics user manual CG000315 Rev E (Step 3.6), but elute final library in 21 ml Buffer EB and transfer 20 ml eluted product to a new 0.2ml tube strip.

Note: The elution volume will be 21 μ l instead of 35.5 μ l (as written in 10X Genomics' user manual). Revised Step 3.6 (m-p) in brief:

m. Add 21 μ l Buffer EB to resuspend the SPRI beads with bound PCR product.

n. Incubate for  00:02:00

o. Place on the 10x Magnetic Separator (in low position) (incubate for  00:02:00)

p. Transfer 20 μ l eluted product to new 0.2 ml tube strip.

- 86 Figure 3 shows cDNA amplified product. And Figure 4 shows final library trace on tapestation.

2m

Protocol references

<https://www.biorxiv.org/content/10.1101/2025.03.06.641241v1.article-metrics>