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HTAPP_Dissociation of primary neuroblastoma core needle biopsy to a single-cell suspension for single-cell RNA-seq (using papain, density gradient, and optionally ACK)

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1 Works for me dx.doi.org/10.17504/protocols.io.bkgnktve

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ABSTRACT

This protocol was used for the dissociation of human neuroblastoma core needle biopsies to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology for the Human Tumor Atlas Pilot Project. For processing human neuroblastoma tumors, however, we now recommend using the papain-based protocol established by Patel et al.

DOI

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GUIDELINES

- Keep sample on ice and use cold reagents unless noted otherwise.
- Report information as indicated in the protocol.

MATERIALS TEXT

MATERIALS

☒ BSA nuclease-free 50mg ml **Ambion Catalog #AM2616**

☒ Gibco™ (Phosphate Buffered Saline) Solution, pH 7.4 (PBS) **Fisher Scientific Catalog # 10010-049**

☒ Trypan Blue Solution 0.4% Sterile-filtered **Sigma Aldrich Catalog #T8154**

☒ ACK Lysing Buffer **Thermo Fisher Scientific Catalog #A1049201**

☒ Noyes Spring Scissors - Tungsten Carbide **Fine Science Tools Catalog #15514-12**

☒ Flex-Tube® 1.5 mL PCR clean colorless **Eppendorf Catalog #022364120**

☒ Tips RT-LTS-A-10µL-/F/L- 960/10 **Rainin Catalog #30389226**

☒ Tips RT-LTS-A-200µL-/F/L- 960/10 **Rainin Catalog #30389240**

☒ Tips RT-LTS-A-1000µL-/F- 768/8 **Rainin Catalog #30389212**

☒ Falcon® 100 mm TC-treated Cell Culture Dish 20/Pack 200/Case Sterile **Corning Catalog #353003**

☒ Falcon® 15 mL High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Rack 500/Case **Corning Catalog #352097**

☒ NanoEnTek Inc. Disposable Hemocytometer **Westnet Catalog #C-CHIP**

☒ Centrifuge 5430 R refrigerated with Rotor FA-45-30-11 incl. rotor lid keypad 120 V/50 – 60 Hz (US) **Eppendorf Catalog #022620601**

☒ Shake n Stack™ Hybridization Ovens **Thermo Fisher Scientific Catalog #6241**

☒ MACS SmartStrainers (70 µm) **Miltenyi Biotec Catalog #130-098-462**

☒ MACS SmartStrainers (100 µm) **Miltenyi Biotec Catalog #130-098-463**

☒ Papain Dissociation System **Worthington Biochemical Corporation Catalog #LK003510**

☒ Nunc CryoTube Vials **Thermo Fisher Scientific Catalog #375418**

☒ MACS SmartStrainers (30 µm) **Miltenyi Biotec Catalog #130-098-458**

SAFETY WARNINGS

Follow general lab safety and institutional guidelines for working with human samples and sharps.

BEFORE STARTING

- Set centrifuge to 4˚C.
- Set hybridization oven with rotator to 37˚C.
- Label two 15 mL conical tubes as “Supernatant 1” and “Supernatant 2” and keep on ice. These tubes will be used to collect supernatant before and after red blood cell removal, respectively, and prevent accidental loss of

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cells. If needed, cells can be recovered from the supernatants by centrifugation using settings from the protocol.

- Store ACK Lysing Buffer at 4˚C or cool down on ice.
- Prepare PBS with 0.4% BSA and keep on ice. This solution can be prepared in advance and stored at 4˚C for several weeks.
- Prepare Worthington Papain Dissociation System according to manufacturer's instructions, summarized below:

A	B	C
Solution	**Preparation**	**Storage**
EBSS	If the EBSS solution (Vial 1) is alkaline (red or purple color), equilibrate with 95% O2:5% CO2 to reach physiological pH (orange color).	Store at 4˚C.
Albumin Ovomucoid Inhibitor Solution	Add 32 mL of EBSS (Vial 1) to the Albumin Ovomucoid Inhibitor vial (Vial 4). Allow the contents to dissolve for 10 minutes at room temperature before gently mixing. If the solution is alkaline (red or purple color), equilibrate with 95% O2:5% CO2 to reach physiological pH (orange color). Mix gently before using.	Store at 4˚C.
Papain Solution	Add 5 mL of EBSS (Vial 1) to a Papain vial (Vial 2). Allow the contents to dissolve at 37˚C for 10 minutes or until the solution is clear before gently mixing. If the solution is alkaline (red or purple color), equilibrate with 95% O2:5% CO2 to reach physiological pH (orange color).	Use promptly after reconstitution and do not store longer than the duration of the experiment. Several vials are provided in the kit.
DNase Solution	Add 500 µL of EBSS (Vial 1) to a DNase vial (Vial 3). Allow the contents to dissolve for 5 minutes at room temperature before gently mixing.	Use promptly after reconstitution and do not store longer than the duration of the experiment. Several vials are provided in the kit.
Papain and DNAse Solution	Add 250 µL of reconstituted DNAse Solution to the vial containing the reconstituted Papain Solution. Mix gently. The final concentration is approximately 20 units/mL Papain and 0.005% DNase.	Use promptly after preparation and do not store longer than the duration of the experiment. Several vials are provided in the kit.

Tissue Description

1 Report sample processing information.

Sample ID:

Date:

Time Received:

Anatomical Site of the Biopsy:

Number of Biopsy Cores:

Core(s) Priority Number:

Media Used for Transportation:

Person Processing:

2 Transfer sample to a Petri dish with cold PBS kept on ice to better visualize its composition. Take a picture of each biopsy alongside a ruler, and describe its appearance (e.g., is it fragmented or necrotic?).

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On ice

Describe Sample Appearance:

Core(s) Dimensions (mm):

Insert Picture(s) of Sample:

Tissue Dissociation

3 Transfer biopsy to a 2 mL screw cap vial containing 1 mL cold Papain and DNAse solution per core. Report time at which dissociation starts.
Tip: The suggested amount of 1 mL per core works well on 18 gauge (~0.8 mm diameter), 10-20 mm long cores, but may be reduced or increased for smaller or larger cores, respectively. If dissociation mix volume is larger than 1.5 mL, dissociation may be performed in a 5 mL Eppendorf tube.

On ice

Volume of Dissociation Mix Used (mL):

Dissociation Start Time:

4 Mince the biopsy with spring scissors into <0.5 mm fragments for approximately 3 min.
Tip: If spring scissors are not available, scalpels can be used instead. In this case, the biopsy should be minced in a Petri dish with <1 mL dissociation mix before transferring the resulting tissue fragment suspension to a 2 mL screw cap vial.

Room temperature **Proceed quickly**

5 Incubate for 12:30 minutes at 37˚C, with rotation at approximately 14 rpm.
Tip: If using an Eppendorf tube, wrap the lid with Parafilm to prevent any leakage. Use lab tape to secure tubes on the rotator.

00:12:30 **Enzymatic Dissociation, Part 1**

37 °C **Hybridization Oven, 14 rpm**

6 Continue dissociation by pipetting up and down 20-30 times using a pipette with a 1 mL tip.
Tip: Do not put the tube on ice as repeated drastic temperature changes may be detrimental to cell viability. If fragments are too large for pipetting, continue mincing with spring scissors or enlarge the 1 mL tip's opening by cutting its extremity with scissors or a scalpel.

Room temperature **Proceed quickly**

7 Incubate for another 12:30 minutes at 37˚C, with rotation at approximately 14 rpm.

00:12:30 **Enzymatic Dissociation, Part 2**

37 °C **Hybridization Oven, 14 rpm**

8 Continue dissociation by pipetting up and down until the tissue disaggregates, which may take up to 5 minutes. Start by using a pipette with a regular 1 mL tip, then continue using a 1 mL tip bent 2-3 mm from its extremity to increase tissue shearing. No or only very small fragments should be visible by eye, and the solution should appear cloudy.
Tip: Do not put the tube on ice as repeated drastic temperature changes may be detrimental to cell viability. If fragments are too large for pipetting, continue mincing with spring scissors or enlarge the 1 mL tip's opening by cutting its extremity with scissors or a scalpel. If the tissue does not disaggregate, additional enzymatic dissociation may be added by 5 minutes increments.

Room temperature

9 Transfer to a 1.5 mL Eppendorf tube and centrifuge at 300 g for 5 minutes at 4˚C. This long spin helps to get rid of fat more efficiently than subsequent short spins.

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300 x g, 4°C, 00:05:00

Cleanup with Discontinuous Density Gradient

10 During the centrifugation, prepare the DNase Albumin Ovomucoid Inhibitor Solution as follows:

2.7 mL EBSS (Vial 1)

300 µl Albumin Ovomucoid Inhibitor Solution (Vial 4)

150 µl DNase Solution (Vial 3)

Room temperature

11 Carefully transfer supernatant to the 15 mL "Supernatant 1" tube kept on ice, making sure to remove any fat that may be floating on top and not to disturb the cell pellet.
Tip: If any fat is visible, aspirate and discard it before transferring the remaining of the supernatant using a different pipette tip.

Room temperature

12 Resuspend cell pellet in 600 µL of the DNase Albumin Ovomucoid Inhibitor Solution prepared in Step 10.

Room temperature

13 Prepare discontinuous density gradient by adding 1 mL of Albumin Ovomucoid Inhibitor Solution (Vial 4) into a 1.5 mL Eppendorf tube and carefully layering the cell suspension on top.

Room temperature

14 Centrifuge at 70 g for 6 minutes at room temperature.

70 x g, Room temperature , 00:06:00

15 Report the cell pellet color (e.g., red, pink, white) in the first row of the table at Step 21. If the cell pellet is red or pink, indicating the presence of red blood cells, proceed to the optional red blood cell removal with ACK Lysing Solution (Step 16). If the cell pellet is white, continue to Quality Control (Step 22).

On ice

[Optional] Red Blood Cell Removal with ACK Lysing Solution

16 Carefully transfer supernatant to the 15 mL "Supernatant 1" tube kept on ice, making sure to not disturb the cell pellet.

On ice

17 Resuspend the cell pellet in 200-500 µL cold ACK Lysing Buffer to lyse red blood cells.
Tip: The volume of ACK should be adjusted to the size and color of the cell pellet and may be increased up to 1 mL if the cell pellet is large or extremely bloody.

On ice

18 Incubate for 1 minute on ice.

00:01:00 ACK Red Blood Cell Lysis

On ice

19 Mix in a volume of PBS equal to twice the volume ACK Lysing Buffer and proceed quickly to the next step.
Tip: If more than 500 µL ACK Lysing Buffer was used, mix in the largest volume of PBS that can safely fit in a 1.5 mL Eppendorf tube (500 µL - 1 mL) and proceed quickly to the next step.

On ice

20 Centrifuge for 8 sec at 4˚C using short spin setting, with centrifugal force ramping up to (but not exceeding) 11,000 g. Proceed quickly to the next step.
Tip: Do not spin for a longer duration or at a higher centrifugal force, as this would result in cell death.

11000 x g, 4°C, 00:00:08 , Short Spin Centrifugation

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21 If the cell pellet is pink or red, revealing a significant portion of red blood cells, repeat Steps 16-20. For each round of ACK Lysing Buffer treatment, report the ACK Lysing Buffer volume, treatment duration, and cell pellet color in the table below.
Tip: Avoid exceeding three rounds of 1 min ACK Lysing Buffer treatment, as this may result in an important loss of cell viability.

go to step #16 if pink or red cell pellet

A	B	C	D
Repeat	**ACK Lysing Buffer Volume (µL)**	**ACK Lysing Buffer Treatment Duration (min)**	**Cell Pellet Color**
0	NA	NA	

Quality Control

22 Carefully transfer supernatant to the 15 mL "Supernatant 2" tube kept on ice without disturbing the cell pellet.

On ice

23 Resuspend the cell pellet in 50 µL cold PBS with 0.4% BSA.

On ice

24 Mix 5 µL of single-cell suspension with 5 µL Trypan blue and load on a hemocytometer.

25 Count and report the number of viable single cells, dead single cells, cell doublets or clumps, and whether debris are present. Take a picture if possible.

A	B	C
	Initial Quality Control	**Quality Control after Optional Debris and Cell Clumps Removal (Step 26)**
Number of Viable Single Cells Counted		
Number of Dead Single Cells Counted		
Number of Cell Clumps or Doublets Counted		
Concentration of Viable Single Cells (cells/µL)		
Concentration of Dead Single Cells (cells/µL)		
Concentration of Cell Clumps or Doublets (doublets/µL)		
Volume of Single Cell Suspension (µL)		
Total Number of Viable Single Cells		
Proportion of Single Cells that are Viable (%)		
Proportion of Cell Clumps or Doublets (%)		
Description of debris (if any)		

Insert Picture for Initial Quality Control:

Insert Picture for Final Quality Control (if additional cleanup was performed as described in Step 26):

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[Optional] Debris and Cell Clumps Removal

26 If the quantity of debris or cell clumps is too high to load on the 10x Genomics Single-Cell RNA-seq system and the number of cells is at least double what is required to load, strain the sample as described below.
Tip: Samples should typically have less than 5% cell clumps. If enough cells (e.g., at least four times the number of cells to be loaded), strain only half of the sample and keep the other half as a backup.

26.1 Resuspend in 500 µL cold PBS with 0.4% BSA.
On ice

26.2 Filter through a 30 µm MACS SmartStrainer into a 15 mL tube.
Tip: To maximize cell recovery, pipette residual liquid from underneath the strainer and transfer it to the tube. Flow cytometry tubes with a 35 µm cell strainer snap cap can also be used but typically result in lower cell recovery.
On ice

26.3 Wash filter with an additional 500 µL of cold PBS with 0.4% BSA to recover as many cells as possible.
On ice

26.4 Transfer to Eppendorf tube.
On ice

26.5 Centrifuge for 8 sec at 4˚C using short spin setting, with centrifugal force ramping up to (but not exceeding) 11,000 g. Proceed quickly to the next step.
Tip: Do not spin for a longer duration or at a higher centrifugal force, as this would result in cell death.
11000 x g, 4°C, 00:00:08 , Short Spin Centrifugation

26.6 Carefully transfer supernatant to the 15 mL “Supernatant 2” tube kept on ice without disturbing the cell pellet.
On ice

26.7 Resuspend in 50 µL cold PBS with 0.4% BSA.
On ice

26.8 Repeat steps 24-25.
go to step #24 Quality Control

Loading on 10x Genomics Single-Cell RNA-seq System

27 If necessary, adjust the concentration before proceeding to load on the 10x Genomics Single-Cell RNA-seq system, following 10x Genomics recommendations.
Tip: 8,000-10,000 live cells are typically loaded per channel. Optimal cell recovery is achieved for concentrations between 800 and 1,200 cells/µL but deviations from that range are acceptable (see 10x Technical Note on this topic). Furthermore, it is recommended that viability be higher than 60% and the proportion of cell clumps lower than 5%.
On ice

28 Report the information listed below about loading on the 10x Genomics Single-Cell RNA-seq system, including the number and concentration of cells per channel.

Time of Loading:

Person Loading:

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Single-Cell RNA-seq Kit Used:

Concentration of Viable Cells Loaded (cells/µL):

Number of Cells Loaded per Channel:

Number of Channels Loaded:

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