Apr 18, 2021

HTAPP_Test protocol_Dissociation of primary neuroblastoma resection to a single-cell suspension for single-cell RNA-seq (using papain and ACK)

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1 Works for me dx.doi.org/10.17504/protocols.io.brvqm65w

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ABSTRACT

This protocol was used as part of the Human Tumor Atlas Pilot Project's (HTAPP) efforts to establish a working protocol for the dissociation of human neuroblastoma resections to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology. It is a testing protocol and we do not recommend using it for the intended purpose. Instead, for processing human neuroblastoma tumors, we recommend using the papain-based protocol established by Patel et al.

DOI

dx.doi.org/10.17504/protocols.io.brvqm65w

PROTOCOL CITATION

Sara Napolitano, Jingyi Wu, Michal Slyper, Sébastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev 2021. HTAPP_Test protocol_Dissociation of primary neuroblastoma resection to a single-cell suspension for single-cell RNA-seq (using papain and ACK). **protocols.io** https://dx.doi.org/10.17504/protocols.io.brvqm65w

CREATED

Jan 27, 2021

LAST MODIFIED

Apr 18, 2021

PROTOCOL INTEGER ID

46736

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**

☒ 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**

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

SAFETY WARNINGS

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

BEFORE STARTING

Citation: Sara Napolitano, Jingyi Wu, Michal Slyper, SÃÂ©bastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev (04/18/2021). HTAPP_Test protocol_Dissociation of primary neuroblastoma resection to a single-cell suspension for single-cell RNA-seq (using papain and ACK). https://dx.doi.org/10.17504/protocols.io.brvqm65w

This protocol was part of HTAPP's optimization efforts and is not recommended for the dissociation of human neuroblastoma tissues. We recommend using the papain-based protocol established by Patel et al.

- 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 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:

Media Used for Transportation:

Person Processing:

Citation: Sara Napolitano, Jingyi Wu, Michal Slyper, SÃÂ©bastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev (04/18/2021). HTAPP_Test protocol_Dissociation of primary neuroblastoma resection to a single-cell suspension for single-cell RNA-seq (using papain and ACK). https://dx.doi.org/10.17504/protocols.io.brvqm65w

2 Transfer sample to a Petri dish with cold PBS kept on ice to better visualize its composition. Take a picture of the resection alongside a ruler and annotate its different regions. Tumors are typically stiff and light-colored. Necrotic regions tend to be soft and crumbly.

On ice

Describe Sample Appearance:

Insert Picture(s) of Sample:

3 Transfer the resection to a Petri dish with cold PBS kept on ice and dissect the tumor from non-tumor tissue using a scalpel. Describe the decisions taken to dissect the sample and document them with an annotated picture.

On ice

Describe Tumor Dissection:

Dimensions of Dissected Tumor (mm):

Insert Annotated Picture(s) Documenting Tumor Dissection:

4 If required, divide the tumor using a scalpel and allocate pieces to different assays following each assay's requirements. Recommended dimensions to obtain enough cells for the 10x Genomics Single-Cell RNA-seq system are 3x3x3 mm or larger. Describe the decisions taken to allocate the sample, including each piece's dimensions, and document the allocation process with an annotated picture.

On ice

Describe Sample Allocation:

Dimensions of Tissue Piece Allocated (mm):

Insert Annotated Picture(s) Documenting Sample Allocation:

Tissue Dissociation

5 If the tumor piece's dimensions are greater than 3x3x3 mm, cut into smaller fragments using safety scalpels.

On ice

6 Transfer the tumor piece or fragments to a 5 mL Eppendorf tube containing 3 mL cold Papain and DNAse solution. Report time at which dissociation starts.
Tip: The suggested amount of 3 mL works well on 25-200 mm3 resections but may be reduced or increased for smaller or larger resections, respectively.

On ice

Volume of Dissociation Mix Used (mL):

Dissociation Start Time:

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7 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 tumor piece should be minced in a Petri dish with 1-2 mL of the dissociation mix before transferring the resulting tissue fragment suspension to a tube with the remaining dissociation mix.

Room temperature **Proceed quickly**

8 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**

9 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**

10 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**

11 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

12 Filter through a 70 µm cell strainer set on a 15 mL conical tube kept on ice to get rid of fragments. Wash strainer with 5 mL cold PBS.
Tip: If the mixture appears to have a lot of undigested tissue pieces, which may clog the 70 µm strainer, use a 100 µm strainer instead.

On ice

13 Centrifuge at 300 g for 5 minutes at room temperature. This long spin helps to get rid of fat more efficiently than subsequent short spins.

300 x g, Room temperature , 00:05:00

Cleanup

14 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

15 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

Resuspend cell pellet in 3 mL of the DNase Albumin Ovomucoid Inhibitor Solution prepared in Step 14.

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16 **Room temperature**

17 Transfer to two 1.5 mL Eppendorf tubes and 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

18 Report the cell pellet color (e.g., red, pink, white) in the first row of the table at Step 24. 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 19). If the cell pellet is white, continue to Quality Control (Step 25).
On ice

[Optional] Red Blood Cell Removal with ACK Lysing Solution

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

20 Resuspend cells in 300-500 µL cold ACK Lysing Buffer to lyse red blood cells. If cells are in separate tubes, combine them in one single 1.5 mL Eppendorf tube.
Tip: The ACK volume *should be adjusted to the cell pellet's size and color and may be increased up to 1 mL if the pellet is large or extremely bloody.*
On ice

21 Incubate for 1 minute on ice.
00:01:00 ACK Red Blood Cell Lysis
On ice

22 Mix in a PBS volume 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

23 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

24 If the cell pellet is pink or red, revealing a significant portion of red blood cells, repeat Steps 19-23. 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 #19 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

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

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On ice

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

On ice

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

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

A	B	C
	Initial Quality Control	**Quality Control after Optional Debris and Cell Clumps Removal (Step 29)**
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 29):

[Optional] Debris and Cell Clumps Removal

29 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.

29.1 Resuspend in 500 µL cold PBS with 0.4% BSA.

On ice

29.2 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

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

On ice

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29.4 Transfer to Eppendorf tube.

On ice

29.5 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

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

On ice

29.7 Resuspend in 50 µL cold PBS with 0.4% BSA.

On ice

29.8 Repeat steps 27-28.

go to step #27 Quality Control

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

30 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

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

Time of Loading:

Person Loading:

Single-Cell RNA-seq Kit Used:

Concentration of Viable Cells Loaded (cells/µL):

Number of Cells Loaded per Channel:

Number of Channels Loaded:

Citation: Sara Napolitano, Jingyi Wu, Michal Slyper, SÃÂ©bastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev (04/18/2021). HTAPP_Test protocol_Dissociation of primary neuroblastoma resection to a single-cell suspension for single-cell RNA-seq (using papain and ACK). https://dx.doi.org/10.17504/protocols.io.brvqm65w