HTAPP_Dissociation of human neuroblastoma tumors to a single-cell suspension for single-cell RNA-seq using Papain

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ABSTRACT

This protocol was used for the dissociation of fresh pediatric neuroblastoma samples from patient tumors or orthotopic patient derived xenografts. Using this method, we were able to prepare highly viable (>90%) single-cell suspensions compatible with droplet-based single-cell RNA-Seq technology (Slyper et al).

For the Human Tumor Atlas Pilot Project (HTAPP), this protocol has been successfully applied to neuroblastoma resections collected from abdominal and paraspinal sites.

GUIDELINES

Store papain kit at 4° C.
MATERIALS

- PDS Kit, Papain Vial
  Worthington Biochemical Corporation
  Catalog #LK003178

- Falcon™ 15mL Conical Centrifuge
  Tubes Fisher Scientific Catalog #14-959-53A

- Aspen Surgical™ Bard-Parker™ Protected
  Disposable Scalpel No. 10 Fisher
  Scientific Catalog #02-688-78

- A1000 Pipettor MIDSCI Catalog #A-1000

- Pipet-aid pipette controller
  Drummond Scientific Catalog #4-000-110

- Falcon 50mL Conical Centrifuge Tubes
  Fisher Scientific Catalog #14-959-49A

- AvantGuard 1250 microliter filter
tips MIDSCI Catalog #AV1250-H

- Falcon 5mL sterile serological pipets
  Fisher Scientific Catalog #13-675-22

- Falcon 10mL sterile serological pipets
  Fisher Scientific Catalog #13-675-20

- Sterile 40 micron nylon strainer
  Fisher Scientific Catalog #08-771-1

- Trypan blue 0.4% solution
  Fisher Scientific Catalog #15-250-061

- 1.5 mL microcentrifuge tubes
  Fisher Scientific Catalog #05-408-129

- Thermo Scientific Sorvall Legend XTR
  Centrifuge Fisher Scientific Catalog #75-217-420

- Bright-Line Hemacytometer
  Fisher Scientific Catalog #02-671-5

- A100 pipetter Contributed by
  users Catalog #A-100

- AvantGuard 100 microliter filter
tips MIDSCI Catalog #AV1000

- Falcon 60 mm tissue-culture treated dishes
  Fisher Scientific Catalog #08-772B
SAFETY WARNINGS

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

BEFORE START INSTRUCTIONS

- Warm water bath to 37° C.
- Allow EBSS (vial #1), albumin-ovomucoid inhibitor mixture (vial #4), papain (vial #2) and DNase I (vial #3) to warm to room temperature for 15 min.
- If you are starting with a new kit, add 32 ml of Earle’s balanced salt solution (EBSS; PDS kit vial #1) to albumin-ovomucoid inhibitor (vial #4).

Reagent Preparation

1. Prepare reagents (1 set for each sample):

1.1 Add 5 mL of Earle's Balanced Salt Solution (vial #1) to papain (vial #2). Allow papain to dissolve for 15 min at 37° C.

1.2 Add 500 µL of Earle's Balanced Salt Solution (vial #1) to DNase I (vial #3). Mix using micropipette.

1.3 Add 250 µL from DNase I solution (vial #3) to papain solution (vial #2).

Tissue dissociation

2. Obtain tumor tissue. Use 100-500 mg of tissue per papain vial. Use a disposable scalpel to mince the sample into small pieces (approximately 1 mm fragments).
Room temperature Process tissue quickly to avoid viability loss (mincing should take less than 5 min)

**Note:** We typically receive tissue on ice from the repository. Tissue is processed immediately upon receipt. Once dissociation has started, we do not recommend putting tissue back onto ice (temperature fluctuations lead to tissue degradation).

**Note:** We typically mince tissue on a 60 mm sterile tissue-culture treated plate.

3 Move minced tissue into a sterile 15 mL Falcon conical tube, and transfer papain-DNase mixture from vial #2 to the conical tube. Incubate conical tube in water bath at 37 °C for 15 min.

4 Triturate tissue by pipetting up and down 10-15 times using a 10 mL sterile serologic pipette.

5 Continue dissociation by incubating tube in the water bath for another 15 min.

6 Repeat trituration by pipetting up and down 10-15 times using a 10 mL sterile serologic pipette.

**Note:** The cell suspension should look cloudy with chunks of debris.

7 Filter cell suspension through a 40 µm cell strainer into a 50 mL Falcon conical tube.

**Note:** Debris will be captured on the cell strainer, while the filtrate should appear cloudy.

8 Rinse filter with 5 mL Earle's Balanced Salt Solution (vial #1).

9 Centrifuge cell suspension at 500 g for 5 min at room temperature.
During centrifugation, prepare the two 15 mL Falcon conical tubes containing:

### 10.1 Resuspension buffer:
- 2.7 mL Earle’s Balanced Salt Solution (vial #1)
- 300 µL Albumin-ovomucoid inhibitor solution (vial #4)
- 150 µL DNase I solution (vial #3)
- Room temperature

### 10.2 Density gradient:
- 5 mL Albumin-ovomucoid inhibitor solution (vial #4)
- Room temperature

After centrifugation (step #9), remove supernatant carefully without disturbing the pellet. *Tip: We typically use suction to aspirate the first 9 mL of supernatant, followed by a micropipette to remove the last 0.5-1 mL of supernatant (to avoid suctioning the pellet). If the pellet is very small or difficult to see, we recommend transferring the top 9.5 mL of supernatant to a tube labeled ‘supernatant 1’ as a backup.*

Re-suspend pellet with 3 mL resuspension buffer (from step #10.1), and gently mix using a 5 mL serologic pipette.

Carefully layer resuspended cells over density gradient (from step #10.2).

Centrifuge at 100 g for 6 min at room temperature.
15 Carefully remove supernatant without disturbing the pellet.  
*Tip: We typically use suction to aspirate the first 7 mL of supernatant, followed by a micropipette to remove the last 0.5-1 mL of supernatant (to avoid suctioning the pellet). If the pellet is very small or difficult to see, we recommend transferring the top 7.5 mL of supernatant to a tube labeled 'supernatant 2' as a backup.*

16 Re-suspend pellet in 10 mL Earle’s Balanced Salt Solution and filter through a 40 µm cell strainer into a sterile 50 mL Falcon conical tube.

17 Mix 10 µL of single-cell suspension with 10 µL of Trypan blue solution, and load 10 µL onto hemacytometer.

18 Count and report the number of viable single cells.  
*Tip: We recommend proceeding only with samples that have >80% viability. Low viability samples will have higher cell clumping, and ambient RNA will complicate downstream single-cell RNA-sequencing analysis.*

19 If necessary, dilute cells prior to loading onto 10x Genomics 3’ single cell RNA-sequencing workflow.  
*Tip: We aim to load 10,000 cells per channel. We recommend trying to dilute cells to a concentration 1000 cells/µL.*

20 Keep diluted cell suspension on ice, and proceed immediately to 10x Genomics single-cell RNA-sequencing workflow.