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# HTAPP\_Dissociation of human primary lung cancer resection to a single-cell suspension for single-cell RNA-seq V.2

In 1 collection

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Michal Slyper<sup>1,2</sup>, Avinash Waghray<sup>3</sup>, Julia Waldman<sup>1,2</sup>, Isaac Wakiro<sup>4</sup>, Mei-Ju Su<sup>4,2</sup>, Sébastien Vigneau<sup>4</sup>, Asaf Rotem<sup>4,2</sup>, Bruce Johnson<sup>4,2</sup>, Orit Rozenblatt-Rosen<sup>1,2</sup>, Aviv Regev<sup>1,5,6,7</sup>

<sup>1</sup>Broad Institute; <sup>2</sup>Human Tumor Atlas Pilot Project; <sup>3</sup>Massachusetts General Hospital;

<sup>4</sup>Dana-Farber Cancer Institute; <sup>5</sup>Human Tumor Atlas Project; <sup>6</sup>Massachusetts Institute of Technology;

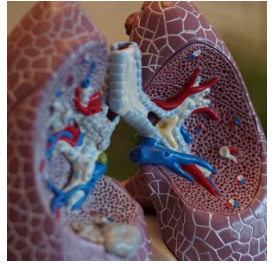
<sup>7</sup>Howard Hughes Medical Institute

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Judit Jane-Valbuena

The Broad Institute



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**Protocol status:** Working

We use this protocol and it's working

**Created:** June 08, 2020

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Protocol Integer ID: 37960

## Abstract

This protocol is used for the dissociation of human primary lung cancer resections to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology.

For the Human Tumor Atlas Pilot Project (HTAPP) it has been successfully applied to non-small cell adenocarcinoma from which it was able to capture a diversity of cell types, including malignant (epithelial) and non malignant cells such as mesenchymal, endothelial, myeloid, and lymphoid cells. In some instances, this protocol can be combined with a **protocol for CD45-cell depletion**, which is used for fast depletion of immune cells for enrichment of malignant and stromal cells.

Description of this protocol and guidance for testing and selecting methods for processing other tumor and sample types can be found in **Slyper et al.**

## Guidelines

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



## Materials

### MATERIALS

- ✕ ACK Lysing Buffer **Thermo Fisher Scientific Catalog #A1049201**
- ✕ PBS, pH 7.4 **Thermo Fisher Scientific Catalog #10010049**
- ✕ Pronase from *Streptomyces griseus* **Sigma Aldrich Catalog #10165921001**
- ✕ Dispase II (neutral protease grade II) **Sigma Aldrich Catalog #4942078001**
- ✕ Collagenase A from *Clostridium histolyticum* **Sigma Aldrich Catalog #10103578001**
- ✕ Collagenase Type 4 100MG **Thermo Fisher Scientific Catalog #NC9836075**
- ✕ Elastase from Porcine Pancreas **Thermo Fisher Scientific Catalog #NC9301601**
- ✕ DNase I from bovine pancreas **Sigma Aldrich Catalog #11284932001**
- ✕ Trypan Blue solution 0.4% **Sigma Aldrich Catalog #T8154-20ML**
- ✕ 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**
- ✕ Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap 25/Pack 500/Case **Corning Catalog #352235**
- ✕ Pipet-Lite LTS Pipette L-1000XLS **Rainin Catalog #17014382**
- ✕ Pipet-Lite LTS Pipette L-200XLS **Rainin Catalog #17014391**
- ✕ Pipet-Lite LTS Pipette L-20XLS **Rainin Catalog #17014392**
- ✕ 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**
- ✕ Aspen Surgical™ Bard-Parker™ Protected Disposable Scalpel **Fisher Scientific Catalog #02-688-78**
- ✕ HBSS no calcium no magnesium **Thermo Fisher Scientific Catalog #14170112**
- ✕ MACS SmartStrainers (70 µm) **Miltenyi Biotec Catalog #130-098-462**
- ✕ MACS SmartStrainers (100 µm) **Miltenyi Biotec Catalog #130-098-463**
- ✕ Eppendorf Tubes™ 5.0 mL **Fisher Scientific Catalog #14-282-305**

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

✕ UltraPure™ BSA (50 mg/mL) **Ambion Catalog #AM2616**

✕ NanoEnTek Disposable Hemocytometer **Westnet Catalog #C-CHIP**

## Safety warnings

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

## Before start

- Set centrifuge to 4°C.
- Set hybridization oven with rotator to 37°C.
- Label one 50 mL conical tubes as “Supernatant 1” and one 15 mL conical tube as “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, keep on ice. This solution can be prepared in advance and stored at 4°C for several weeks.
- Prepare dissociation mix immediately before use as described in the table below and keep on ice. The suggested amount is 3 mL per 25-200 mm<sup>3</sup> resection, and may be adjusted depending on the size of the resection. Record in the table the volumes used.

Reagent	Stock concentration (mg/mL)	Final concentration (µg/mL)	Volume for 3 mL Mix (µL)	Volume Prepared (µL)
HBSS	-	-	2692	
Collagenase A	150	1500	30	
Collagenase IV	100	100	3	
DNase I	10	100	30	
Dispase II	10	100	30	
Elastase	1	9.2	27.6	
Pronase	20	1250	187.5	

## Sample Description and Allocation

- 1 Report sample processing information.

 4 °C Wet Ice

### Note

Sample ID:

Date:

Media Used for Transportation:

Person Processing:

- 2 Transfer sample to a Petri dish with cold PBS (or HBSS without phenol red) 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, often with dark spots in lung cancer. Necrotic regions tend to be soft and crumbly.

 4 °C Wet Ice

### Note

Describe Sample Appearance:

Insert Annotated Picture(s) of Sample:

- 3 Transfer the resection to a Petri dish with cold HBSS 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.

 4 °C Wet Ice

### Note

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 10x Genomics Single-Cell RNA-seq system are 3×3×3 mm or larger. Describe the decisions taken to allocate the sample, including the dimensions of each piece, and document the allocation process with an annotated picture.

 4 °C Wet Ice

#### Note

Describe Sample Allocation:

Dimensions of Tissue Piece Allocated (mm):

Insert Annotated Picture(s) Documenting Sample Allocation:

## Tissue Dissociation

- 5 If the dimensions of the tumor piece are greater than 3×3×3 mm, cut into smaller fragments using safety scalpels.

 4 °C Wet Ice

- 6 Transfer the tumor piece or fragments to a 5 mL Eppendorf tube containing 3 mL cold dissociation mix. Report time at which dissociation starts.

*Tip: The suggested amount of 3 mL works well on 25-200 mm<sup>3</sup> resections, but may be reduced or increased for smaller or larger resections, respectively.*

 4 °C Wet Ice

#### Note

Volume of Dissociation Mix Used (mL):

Dissociation Start Time:


- 7 Mince the tissue 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 sample should be minced in a Petri dish within 1 mL dissociation mix before transferring the resulting tissue fragment suspension to a 5 mL Eppendorf tube containing an additional 2 mL of dissociation mix.*

 20 °C Proceed quickly at room temperature

- 8 Incubate for 10 minutes at 37°C, with rotation at approximately 14 rpm.


*Tip: Wrap the lid of the Eppendorf tube with Parafilm to prevent any leakage, and use lab tape to secure tubes on the rotator.*

 00:10:00 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 1 mL tip.


*Tip: Do not put the tube on ice at this step as repeated, drastic changes in temperature may be detrimental to cell viability. Pipette down pushing against the wall of the tube for optimal dissociation. If fragments are too large for pipetting, continue mincing with spring scissors before pipetting.*

 20 °C Proceed quickly at room temperature

- 10 Incubate for another 10 minutes at 37°C, with rotation at approximately 14 rpm.

*Tip: Wrap the lid of the Eppendorf tube with Parafilm to prevent any leakage, and use lab tape to secure tubes on the rotator.*

 00:10:00 Enzymatic Dissociation, Part 2

 37 °C Hybridization Oven 14 rpm

- 11 Continue dissociation by pipetting up and down 20–30 times using a pipette with 1 mL tip. No or only very small fragments should be visible by eye and the solution should appear cloudy.

*Tip: Pipette down pushing against the wall of the tube for optimal dissociation. If fragments are too large for pipetting, continue mincing with spring scissors before pipetting.*


 20 °C Proceed quickly at 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 10 mL cold HBSS.

*Tip: If the mixture appears to have a lot of undigested tissue pieces or mucus, which may clog the 70 µm strainer, use a 100 µm strainer instead.*

 4 °C Wet Ice

- 13 Centrifuge at 400 g for 5 minutes in 4°C pre-cooled centrifuge. This long spin helps to get rid of fat more efficiently than subsequent short spins.

 00:05:00 Centrifugation

 4 °C Centrifuge

## Red Blood Cell Removal

- 14 Report pellet color (e.g., red, pink, white) in the first row of the table at Step 20.

 4 °C Wet Ice



- 15 Carefully transfer supernatant to the 50 mL "Supernatant 1" tube kept on ice, making sure to remove any fat and to not disturb the pellet.  
*Tip: If any fat is visible, aspirate and discard it before transferring the remaining of the supernatant using a different pipette tip.*
- 4 °C Wet Ice
- 16 Resuspend pellet in 300-500 µL cold ACK Lysing Buffer to lyse red blood cells. If cells are in a 15 mL tube, transfer them to a 1.7 mL Eppendorf tube kept on ice.  
*Tip: The volume of ACK should be adjusted to the size and color of the pellet and may be increased up to 1 mL if the pellet is large or extremely bloody.*
- 4 °C Wet Ice
- 17 Incubate for 1 minute on ice.
- 00:01:00 ACK Red Blood Cell Lysis
- 4 °C Wet Ice
- 18 Mix in a volume of PBS equal to twice the volume ACK Lysing Buffer. 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 1.7 mL Eppendorf tube (500 µL - 1 mL) and proceed quickly to the next step.*
- 4 °C Wet Ice
- 19 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.*
- 00:00:08 Centrifugation
- 4 °C Centrifuge
- 20 If the pellet is pink or red, revealing a significant portion of red blood cells, repeat steps 15-19. For each round of ACK Lysing Buffer treatment, report the ACK Lysing Buffer volume, treatment duration, and pellet color in the table below.  
*Tip: Avoid exceeding three rounds of 1 min ACK Lysing Buffer treatment, as this may result in important loss of cell viability.*
- [go to step #14](#) if pink or red pellet

Repeat	ACK Lysing Buffer Volume (µL)	ACK Lysing Buffer Treatment Duration (min)	Pellet Color
0	NA	NA	




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

🧊 4 °C Wet Ice

- 22 Resuspend in 50 µL cold PBS with 0.4% BSA.

🧊 4 °C Wet Ice

## Quality Control

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

- 24 Count and report the number of viable single cells, dead single cells, cell doublets or clumps, and whether debris are present, then calculate additional quality control metrics below. Take picture if possible.

	Initial Quality Control	Quality Control after Optional Debris and Cell Clumps Removal (Step 25)
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)		

#### Note

Insert Picture for Initial Quality Control:

Insert Picture for Final Quality Control (if additional cleanup was performed as described in Step 25):

## [Optional] Debris and Cell Clumps Removal

- 25 If the quantity of debris or cell clumps is too high to load on 10x Genomics Single-Cell RNA-seq system and the number of cells is at least double from 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.*

- 25.1 Resuspend in 500  $\mu$ L cold PBS with 0.4% BSA.

 4 °C Wet Ice

- 25.2 Filter through 35  $\mu$ m FACS tube cell strainer.

*Tip: Alternately, 30  $\mu$ m MACS SmartStrainer can be used to minimize cell loss.*

 4 °C Wet Ice

- 25.3 Wash filter with an additional 500  $\mu$ L of cold PBS with 0.4% BSA to recover as many cells as possible.

 4 °C Wet Ice

- 25.4 Transfer to Eppendorf tube.

 4 °C Wet Ice

- 25.5 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.*

 00:00:08 Centrifugation

 4 °C Centrifuge



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

🧊 4 °C Wet Ice

- 25.7 Resuspend in 50 µL cold PBS with 0.4% BSA.

🧊 4 °C Wet Ice

- 25.8 Repeat steps 23-24.

➡ go to step #23 Quality Control

## Loading on 10x Genomics Single-Cell RNA-seq system

- 26 If necessary, adjust the concentration before proceeding to loading on 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%.*

🧊 4 °C Wet Ice

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

### Note

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: