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HTAPP_Dissociation of human metastatic breast cancer core needle biopsy to a single-cell suspension for single-cell RNA-seq V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bhbjj2kn

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ABSTRACT

This protocol is used for the dissociation of human metastatic breast cancer core needle biopsies to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology (*Slyper et al*).

For the Human Tumor Atlas Pilot Project (HTAPP) it has been successfully applied to biopsies collected from breast, liver and lymph node.

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*.

DOI

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PROTOCOL CITATION

Michal Slyper, Julia Waldman, Jingyi Wu, Abhay Kanodia, Sébastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev 2020. HTAPP_Dissociation of human metastatic breast cancer core needle biopsy to a single-cell suspension for single-cell RNA-seq. **protocols.io** dx.doi.org/10.17504/protocols.io.bhbjj2kn

KEYWORDS

single-cell RNA-seq, biopsy, dissociation, breast cancer

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GUIDELINES

- Keep sample on ice and use cold reagents unless noted otherwise.

- Report information as indicated in the protocol.

MATERIALS

NAME	CATALOG #	VENDOR
RPMI 1640 Medium	11875093	Thermo Fisher Scientific
TrypLE	12604013	Thermo Fisher Scientific
ACK Lysing Buffer	A1049201	Thermo Fisher Scientific
Liberase™ TM Research Grade	5401119001	Millipore Sigma
PBS, pH 7.4	10010049	Thermo Fisher Scientific
DNase I from bovine pancreas	11284932001	Millipore Sigma
Noyes Spring Scissors - Tungsten Carbide	15514-12	Fine Science Tools
Flex-Tube® 1.5 mL PCR clean colorless	022364120	Eppendorf
Tips RT-LTS-A-10µL-/F/L-960/10	30389226	Rainin
Tips RT-LTS-A-200µL-/F/L-960/10	30389240	Rainin
Tips RT-LTS-A-1000µL-/F-768/8	30389212	Rainin
Falcon® 100 mm TC-treated Cell Culture Dish 20/Pack 200/Case Sterile	353003	Corning
Falcon® 15 mL High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Rack 500/Case	352097	Corning
Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap 25/Pack 500/Case	352235	Corning
Pipet-Lite LTS Pipette L-1000XLS	17014382	Rainin
Pipet-Lite LTS Pipette L-200XLS	17014391	Rainin
Pipet-Lite LTS Pipette L-20XLS	17014392	Rainin
Centrifuge 5430 R refrigerated with Rotor FA-45-30-11 incl. rotor lid keypad 120 V/50 – 60 Hz (US)	022620601	Eppendorf
Shake n Stack™ Hybridization Ovens	6241	Thermo Fisher Scientific
Aspen Surgical™ Bard-Parker™ Protected Disposable Scalpel	02-688-78	Fisher Scientific
Eppendorf Tubes™ 5.0 mL	14-282-305	Fisher Scientific
Nunc CryoTube Vials	375418	Thermo Fisher Scientific
MACS SmartStrainers (30 µm)	130-098-458	Miltenyi Biotec
UltraPure™ BSA (50 mg/mL)	AM2616	Thermo Fisher Scientific
NanoEnTek Inc. Disposable Hemocytometer	C-CHIP	NanoEnTek Inc.

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 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 RPMI 1640 with 10% FBS and keep on ice. This solution can be prepared in advance and stored at 4˚C for several weeks.
- 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 dissociation mix immediately before use as described in the table below and keep on ice. The suggested amount is 1 mL per core for 18 gauge (~0.8 mm diameter), 10-20 mm long cores, and may be

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adjusted depending on the size and number of cores. Record in the table the volumes used.

Reagent	Stock concentration (mg/mL)	Final concentration (µg/mL)	Volume for 1 mL Mix (µL)	Volume Prepared (µL)
RPMI 1640	-	-	950	
DNAse I	10	100	10	
Liberase TM	2.5	100	40	

Sample Description

1 Report sample processing information.

4 °C Wet Ice

Sample ID:

Date:

Anatomical Site of the Biopsy:

Number of Biopsy Cores:

Core(s) Priority Number:

Media Used for Transportation:

Person Processing:

2 Transfer the sample to a Petri dish with cold PBS (or RPMI without phenol red) kept on ice in order to better visualize its composition. Take a picture of each core alongside a ruler, and describe its appearance (e.g., is it fragmented or necrotic?).

4 °C Wet Ice

Describe Sample Appearance:

Core(s) Dimensions (mm):

Insert Picture(s) of Sample:

Tissue Dissociation

3 Transfer the sample to a 2 mL screw cap tube (e.g., cryotube) or Eppendorf tube containing 1 mL cold dissociation mix 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 the volume of dissociation mix is larger than 1.5 mL, dissociation may be performed in a 5 mL (instead of 2 mL) Eppendorf tube.

4 °C Wet Ice

Volume of Dissociation Mix Used (mL):

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Dissociation Start Time:

4 Mince the sample with spring scissors to <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 within <1 mL dissociation mix before transferring the resulting tissue fragment suspension to a 2 mL screw cap vial.

20 °C Proceed quickly at room temperature

5 Incubate for 10 minutes at 37˚C, with rotation at approximately 14 rpm.
Tip: If using an Eppendorf tube, wrap the tube's lid with Parafilm to prevent any leakage. Use lab tape to secure tubes on the rotator.

00:10:00 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 1 mL tip.
Tip: Do not put the tube on ice 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

7 Incubate for another 10 minutes at 37˚C, with rotation at approximately 14 rpm.
Tip: If using an Eppendorf tube, wrap the tube's lid with Parafilm to prevent any leakage. Use lab tape to secure tubes on the rotator.

00:10:00 Enzymatic Dissociation, Part 2

37 °C Hybridization Oven, 14 rpm

8 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

9 Centrifuge at 300 g for 4 minutes in 4˚C pre-cooled centrifuge. This long spin helps to get rid of fat and debris more efficiently than subsequent short spins.

00:04:00 Centrifugation

4 °C Centrifuge

Red Blood Cell Removal

10 Report the cell pellet color (e.g., red, pink, white) in the first row of the table at Step 16.

4 °C Wet Ice

11 Carefully transfer the supernatant to the 15 mL “Supernatant 1” tube kept on ice, making sure to remove any fat and to not 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.

4 °C Wet Ice

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12 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.
4 °C Wet Ice

13 Incubate for 1 minute on ice.
00:01:00 ACK Red Blood Cell Lysis
4 °C Wet Ice

14 Mix in a volume of PBS equal to twice the volume of 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 1.7 mL Eppendorf tube (500 µL - 1 mL) and proceed quickly to the next step.
4 °C Wet Ice

15 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

16 If the cell pellet is pink or red, revealing a significant portion of red blood cells, repeat steps 11-15. 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 loss of cell viability.
if pink or red pellet

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

Cell Clump Dissociation

17 Carefully transfer the supernatant to the 15 mL “Supernatant 2” tube kept on ice without disturbing the cell pellet.
4 °C Wet Ice

18 Resuspend the cell pellet in 100 µL TrypLE per core.
4 °C Wet Ice

19 Hold tube in hand for 1 min while constantly pipetting up and down using a pipette with 200 µL tip.
Tip: Pipette down pushing against the wall or bottom of the tube for optimal dissociation.
00:01:00 TrypLE Treatment

20 Inactivate TrypLE by mixing with 200 µL of cold RPMI 1640 with 10% FBS per 100 µL TrypLE.
4 °C Wet Ice

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21 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

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

4 °C Wet Ice

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

4 °C Wet Ice

Quality Control

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

25 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 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):

[Optional] Debris and Cell Clumps Removal

26 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.

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26.1 Resuspend the cell pellet in 500 µL cold PBS with 0.4% BSA.

4 °C Wet 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 to the tube. Flow cytometry tubes with 35 µm cell strainer snap cap can also be used but typically result in lower cell recovery.

4 °C Wet Ice

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

4 °C Wet Ice

26.4 Transfer to Eppendorf tube.

4 °C Wet 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.

00:00:08 Centrifugation

4 °C Centrifuge

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

4 °C Centrifuge

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

4 °C Centrifuge

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 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

28 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:

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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: Michal Slyper, Julia Waldman, Jingyi Wu, Abhay Kanodia, SÃÂ©bastien Vigneau, Asaf Rotem, Bruce Johnson, Orit Rozenblatt-Rosen, Aviv Regev (08/02/2020). HTAPP_Dissociation of human metastatic breast cancer core needle biopsy to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbjj2kn