Version 2

Aug 28, 2020

# HTAPP_Dissociation of human ovarian cancer resection 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.bhbhj2j6

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ABSTRACT

This protocol is used for the dissociation of human ovarian cancer specimens resected from the ovary and omentum to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology. It is based on the Miltenyi Human Tumor Dissociation Kit, which was previously reported to successfully dissociate ovarian tumors for multiparametric mass cytometry (CyTOF) (Gonzalez et al, 2018).

For the Human Tumor Atlas Pilot Project (HTAPP) it has been successfully applied to ovarian cancer resections from the ovary and omentum and captured a diversity of cells including malignant and non-malignant 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.

DOI

dx.doi.org/10.17504/protocols.io.bhbhj2j6

PROTOCOL CITATION

Isaac Wakiro, Sara Napolitano, Jingyi Wu, Sébastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev 2020. HTAPP_Dissociation of human ovarian cancer resection to a single-cell suspension for single-cell RNA-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bhbhj2j6



CREATED

Jun 09, 2020

LAST MODIFIED

Aug 28, 2020

PROTOCOL INTEGER ID

37961

GUIDELINES

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

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| ACK Lysing Buffer | A1049201 | Thermo Fisher Scientific |
| Trypan Blue solution 0.4% | T8154-20ML | Sigma Aldrich |
| RPMI 1640 Medium | 11875101 | Life Technologies |
| TrypLE Express Enzyme (1X) | 12604013 | Life Technologies |
| PBS 1X PH 7.4 | 10010049 | Life Technologies |
| 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 |
| MACS SmartStrainers (70 µm) | 130-098-462 | Miltenyi Biotec |
| MACS SmartStrainers (100 µm) | 130-098-463 | Miltenyi Biotec |
| Eppendorf Tubes™ 5.0 mL | 14-282-305 | Fisher Scientific |
| MACS SmartStrainers (30 µm) | 130-098-458 | Miltenyi Biotec |
| UltraPure™ BSA (50 mg/mL) | AM2616 | Ambion |
| Tumor Dissociation Kit human | 130-095-929 | Miltenyi Biotec |
| NanoEnTek Inc. Disposable Hemocytometer | C-CHIP | Westnet |

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 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 0.5X TrypLE by diluting 1X TrypLE with calcium and magnesium free PBS. This solution can be prepared in advance and stored at 4˚C for several weeks.
- 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.
- Resuspend enzymes H, R, and A following instructions from Miltenyi Human Tumor Dissociation Kit data sheet.
- Prepare dissociation mix immediately before use as described in the table below and keep on ice. The



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suggested amount is 2.36 mL per 50-200 $mm^3$ resection, but this may be adjusted depending on the size of the resection. Record in the table the volumes used.

| Reagent | Volume for 50-200 mm3 resection (µL) | Volume prepared (µL) |
|---|---|---|
| **RPMI 1640** | 2,200 | |
| **Enzyme H** | 100 | |
| **Enzyme R** | 50 | |
| **Enzyme A** | 12.5 | |

## Sample Description and Allocation

1 Report sample processing information.

**4 °C Wet Ice**

Sample ID:

Anatomical Site of Resection (e.g., Ovary, Omentum):

Date:

Media Used for Transportation:

Person Processing:

2 Transfer sample to a Petri dish with cold PBS (or RPMI 1640 without phenol red) kept on ice in order 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.

**4 °C Wet Ice**

Describe Sample Appearance:

Insert Annotated Picture(s) of Sample:

3 Transfer the resection to a Petri dish with cold RPMI 1640 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**

Describe Tumor Dissection:

Dimensions of Dissected Tumor (mm):

Insert Annotated Picture(s) Documenting Tumor Dissection:



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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 3x3x3 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**

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 3x3x3 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 2.36 mL cold dissociation mix. Report time at which dissociation starts.

*Tip: The suggested amount of 2.36 mL works well on 50-200 $mm^3$ resections, but may be reduced or increased for smaller or larger resections, respectively.*

**4 °C Wet Ice**

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 1.36 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. If spring scissors are not available, scalpels can be used instead.*

**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*



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*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 RPMI 1640.
*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.*

**4 °C Wet Ice**

13 Centrifuge at 450 g for 7 minutes in 4˚C pre-cooled centrifuge. This long spin helps to get rid of fat more efficiently than subsequent short spins, which is particularly important for tissue resected from the omentum.

**00:07:00 Centrifugation**

**4 °C**

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 of ACK Lysing Buffer. Proceed quickly to the next step.



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*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 #15 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 Cell Clump Dissociation (Step 25) | Quality Control after Optional Debris Removal (Step 26) |
|---|---|---|---|
| **Number of Viable Single Cells Counted** | | | |



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| | | | |
|---|---|---|---|
| **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)** | | | |

## **[Optional]** Cell Clump Dissociation

25 If the proportion of cell clumps is greater than 5%, dissociate them as described below. Otherwise, go to Step 26.
*Tip: Avoid exceeding two rounds of cell clump dissociation using TrypLE, as this may result in important loss of cell viability. If large cell clumps persist after the first round of dissociation, use 1X instead of 0.5X TrypLE for the second round.*

25.1 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**

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

25.3 Resuspend pellet in 200 µL of 0.5X TrypLE.
**4 °C Wet Ice**

25.4 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**

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

25.6 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**



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

**4 °C Wet Ice**

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

**4 °C Wet Ice**

25.9 Repeat steps 23-24.

**Quality Control**

**[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: 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.

**4 °C Wet Ice**

26.2 Filter through 35 µm FACS tube cell strainer.
*Tip: Alternately, 30 µm MACS SmartStrainer can be used to minimize cell loss.*

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

**4 °C Wet Ice**



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

**4 °C Wet Ice**

26.8 Repeat steps 23-24.

**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:

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:



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