Version 2

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HTAPP_Processing human ovarian cancer ascites 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.bhbkj2kw

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ABSTRACT

This protocol is used to process human ovarian cancer ascites to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology.

A majority of ascites samples have a high proportion (>90%) of CD45-expressing (CD45+) cells, thus greatly reducing the ability to capture non-immune and malignant cells. To address this issue, we have included a CD45+ cell depletion step to reduce the number of CD45+ cells. In such samples, where the proportion of immune cells is very high, a large proportion of both CD45+ and CD45- cells can be recovered post-depletion, allowing simultaneous assessment of the immune and non-immune microenvironment. In cases with a lower initial proportion of CD45+ cells, however, non-immune cells tend to be dominant following CD45+ cell depletion.

Of note, many ascites samples contain multicellular aggregates, or spheroids, which pose challenges during sample processing and dissociation. Larger aggregates are removed through an initial 70 µm straining (Step 14) whereas smaller residual aggregates, when present, can be removed through a final 30 µm straining (optional Step 20). Yet, we have encountered instances where small aggregates cannot be efficiently removed through straining. This protocol is not optimized for those cases. In addition, we would like to note that ascites can be quite variable and further optimization of this protocol may be needed to handle cases with atypical characteristics.

For the Human Tumor Atlas Pilot Project (HTAPP), this protocol was successfully applied to 4 ovarian cancer ascites samples.

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.bhbkj2kw

PROTOCOL CITATION

Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, Sébastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev 2020. HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bhbkj2kw

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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
ACK Lysing Buffer	A1049201	Thermo Fisher Scientific
PBS, pH 7.4	10010049	Thermo Fisher Scientific
UltraPure™ BSA (50 mg/mL)	AM2616	Thermo Fisher Scientific
Flex-Tube® 1.5 mL PCR clean colorless	022364120	Eppendorf
Falcon® 15 mL High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Rack 500/Case	352097	Corning
MACS SmartStrainers (70 µm)	130-098-462	Miltenyi Biotec
MACS SmartStrainers (30 µm)	130-098-458	Miltenyi Biotec
Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap	352235	Corning

SAFETY WARNINGS

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

BEFORE STARTING

- Set centrifuge to 4˚C.
- 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.
- Follow "Before Start" instructions for the CD45+ cell depletion sub-protocol (Step 15).

Sample Description and Allocation

1 Report sample processing information.

4 °C Wet Ice

Sample ID:

Date:

Person Processing:

2 Report the volume and appearance of the sample, including a picture.

4 °C Wet Ice

Sample Volume (mL):

Citation: Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, SÃÂ©bastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev (08/28/2020). HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbkj2kw

Describe Sample Appearance:

Insert Picture(s) of Sample:

3 If required, aliquot some of the ascites for different assays and document the allocation process. For single-cell RNA-seq, we recommend starting with at least 200 mL of ascites since a large number of cells will be lost during CD45+ cell depletion. This protocol assumes that ascites have been collected in four 50 mL conical tubes.

4 °C Wet Ice

Describe Sample Allocation:

4 Spin down the tubes at 580 g for 5 min in 4°C pre-cooled centrifuge.

00:05:00 Centrifugation

4 °C Centrifuge

Red Blood Cell Removal

5 Report the cell pellet color (e.g., red, pink, white) in the first row of the table at Step 11.

4 °C Wet Ice

6 Aspirate off and discard supernatant.

4 °C Wet Ice

7 Resuspend each cell pellet in 1-5 mL cold ACK Lysing Buffer. If cell suspensions are in different tubes, combine them into a smaller number of 50 mL or 15 mL tubes without exceeding one third of the volume of the tubes. For instance, four 5 mL cell suspensions (20 mL in total) can be combined in two 50 mL tubes, and four 1 mL cell suspensions (4 mL in total) can be combined in one 15 mL tube.
Tip: The volume of ACK should be adjusted to the size and color of the cell pellets. Combining cell pellets helps minimizing cell loss while also reducing processing time.

4 °C Wet Ice

8 Incubate on ice for 3 minutes.

00:03:00 ACK Red Blood Cell Lysis

4 °C Wet Ice

9 Mix in a volume of PBS equal to twice the volume of ACK Lysing Buffer and proceed quickly to the next step.

4 °C Wet Ice

10 Centrifuge at 580 g for 5 minutes in 4˚C pre-cooled centrifuge.

00:05:00 Centrifugation

4 °C Wet Ice

11 If the pellet is pink or red, revealing a significant portion of red blood cells, repeat steps 6-10. For each round of ACK

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Lysing Buffer treatment, report the ACK Lysing Buffer volume, treatment duration, and pellet color in the table below.
Tip: Avoid exceeding three rounds of 3 min ACK Lysing Buffer treatment, as this may result in important loss of cell viability.

go to step #6 if pink or red pellet

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

12 Aspirate off and discard supernatant.

4 °C Wet Ice

13 Resuspend the cell pellet in 20 mL cold PBS.

4 °C Wet Ice

14 Filter the cell suspension through a 70 µm cell strainer into a 50 mL conical tube. Wash strainer with 20 mL cold PBS to recover as many cells as possible.

4 °C Wet Ice

CD45+ Cells Depletion

15 Deplete CD45+ cells using the protocol below, with the aim to balance the proportion of immune and non-immune cells. This step may be skipped for samples known to already contain a large enough proportion of non-immune cells.

HTAPP_Depletion of CD45+ cells from ovarian cancer ascites single cell suspensions for single-cell RNA-Seq
by Judit Jane-Valbuena,
The Broad Institute
PREVIEW RUN

15.1 Mix 5 µL of red blood cell-free ascites single-cell suspension with 5 µL Trypan blue and load on hemocytometer. The ascites cell suspension can be obtained following the "HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq" protocol.

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

	Quality Control
Number of Viable Single Cells Counted	
Number of Dead Single Cells Counted	
Number of Cell Aggregates or Doublets Counted	
Concentration of Viable Single Cells (cells/µL)	
Concentration of Dead Single Cells (cells/µL)	
Concentration of Cell Aggregates or Doublets (doublets/µL)	

Citation: Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, SÃÂ©bastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev (08/28/2020). HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbkj2kw

Volume of Single Cell Suspension (µL)	
Total Number of Viable Single Cells	
Proportion of Single Cells that are Viable (%)	
Proportion of Cell Aggregegages or Doublets (%)	
Description of debris (if any)	

Insert Picture:

15.3 Centrifuge at 580 g for 5 minutes in 4˚C pre-cooled centrifuge.

00:05:00 Centrifugation

4 °C Wet Ice

15.4 Remove supernatant as completely as possible without disrupting the cell pellet.

4 °C Wet Ice

15.5 If cell number is lower than 10 million, resuspend the cell pellet in 80 µL MACS buffer. If cell number is higher than 10 million cells, resuspend the cell pellet in 80 µL MACS buffer for every 10 million cells.

4 °C Wet Ice

15.6 Add 20 µL CD45 microbeads per 80 µL MACS buffer.

4 °C Wet Ice

15.7 Incubate on ice or in 4°C refrigerator for 15 minutes.

00:15:00 Labeling

4 °C Wet Ice

15.8 During the incubation, prime an LS column: insert the column into a MidiMACS separator and let 3 mL of MACS buffer flow through the column, collecting it in the 15 mL "Wash" tube. Once the column is primed, discard the 15 mL "Wash" tube and position the 15 mL "CD45 negative fraction" tube under the column.

15.9 Following incubation (Step 7), resuspend the labeled cell suspension with 900 µL cold MACS buffer per 100 µL of suspension.

4 °C Wet Ice

15.10 Spin the suspension at 500 g for 4 minutes in 4˚C pre-cooled centrifuge.

00:04:00 Centrifugation

4 °C Centrifuge

15.11 Remove supernatant as completely as possible without disrupting the labeled cell pellet.

4 °C Wet Ice

Citation: Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, SÃÂ©bastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev (08/28/2020). HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbkj2kw

15.12 Resuspend the pellet (up to 100 million cells) in 500 µL cold MACS buffer.

4 °C Wet Ice

15.13 Transfer the labeled cell suspension to the primed LS column on the MidiMACS separator and collect flow-through in the 15 mL "CD45 negative fraction" tube. This CD45 depleted cell suspension can be used in the "HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq" protocol.
Tip: Cell recovery may be increased by washing the column up to three times with 3 mL of MACS buffer.

Room temperature

Quality Control

16 Centrifuge at 580 g for 5 minutes in 4˚C pre-cooled centrifuge.

00:05:00 Centrifugation

4 °C Centrifuge

17 Discard supernatant and resuspend cell pellet in 50 µL PBS with 0.4% BSA.

4 °C Wet Ice

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

19 Count and report the number of viable single cells, dead single cells, cell doublets or aggregates, 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 Aggregate Removal (Step 20)
Number of Viable Single Cells Counted		
Number of Dead Single Cells Counted		
Number of Cell Aggregates or Doublets Counted		
Concentration of Viable Single Cells (cells/µL)		
Concentration of Dead Single Cells (cells/µL)		
Concentration of Cell Aggregates 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 Aggregates 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 20):

[Optional] Debris and Cell Aggregate Removal

Citation: Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, SÃÂ©bastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev (08/28/2020). HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbkj2kw

20 If the quantity of debris or cell aggregates is too high to load on the 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 aggregates. 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.
4 °C Wet Ice

20.1 Resuspend in 500 µL cold PBS with 0.4% BSA.
4 °C Wet Ice

20.2 Filter through 35 µm FACS tube cell strainer.
Tip: Alternately, a 30 µm MACS SmartStrainer can be used to minimize cell loss.
4 °C Wet Ice

20.3 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

20.4 Transfer to Eppendorf tube.
4 °C Wet Ice

20.5 Repeat Steps 16-19.
Quality Control

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

21 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 aggregates lower than 5%.

22 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: Benjamin Izar, Parin Shah, Mei-Ju Su, Isaac Wakiro, Sara Napolitano, Jingyi Wu, SÃÂ©bastien Vigneau, Asaf Rotem, Orit Rozenblatt-Rosen, Bruce Johnson, Aviv Regev (08/28/2020). HTAPP_Processing human ovarian cancer ascites to a single-cell suspension for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.bhbkj2kw