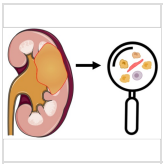

Jul 07, 2020

Human Kidney Tumor Dissociation for single-cell genomics

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2 Works for me dx.doi.org/10.17504/protocols.io.2n2gdge

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ABSTRACT

This protocol was developed to dissociate (clear cell) renal cell carcinoma tumors in order to study single cell genomics using the Chromium Single Cell Gene Expression technology (10X Genomics). Starting from fresh biological material, the protocol was optimized to ensure fast and reproducible processing, both of which are very important criteria in single-cell are analyzes. This protocol allowed us to obtain biologically relevant high-quality data. It can easily be adapted according to the following optional steps: i) multiple sampling in several macroscopic areas of the tumor; ii) depletion of CD45+ cell populations; iii) and, progressive freezing of the dissociated tumor cells for biobanking.

DOI

dx.doi.org/10.17504/protocols.io.2n2gdge

PROTOCOL CITATION

Evrard Bertrand, Saout Judikael, Lardenois Aurelie, Kammerer-Jacquet Solène-Florence, Rioux-Leclercq Nathalie, Chalmel Frederic 2020. Human Kidney Tumor Dissociation for single-cell genomics. **protocols.io** dx.doi.org/10.17504/protocols.io.2n2gdge

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

KEYWORDS

Clear cell renal cell carcinoma; single-cell genomics; tissue dissociation; cell viability

CREATED

May 07, 2019

LAST MODIFIED

Jul 07, 2020

PROTOCOL INTEGER ID

22970

MATERIALS

NAME	CATALOG #	VENDOR
C Tube	130-096-334	Miltenyi Biotec
gentleMACS Dissociator	130-093-235	Miltenyi Biotec
Tumor Dissociation Kit human	130-095-929	Miltenyi Biotec

Tumor Preparation

1

This step should be designed according to the biological question. As this protocol was developed to catch the tumor heterogeneity, each tumor was sampled in multiple areas.

Material

Material	Supplier Info
Petri Dish, 100 mm	Corning (430591)
Biopsy Punch 8mm	Dutscher (030740)
Ice	

2 Transport the fresh tumor resection on ice and collect evenly distributed punch biopsies in multiple macroscopic areas.

- Avoid all fibrous and necrotic areas. If these areas are of interest, please consider using Fluorescence Activated Cell Sorting (FACS) after dissociation to sort viable cells (not tested).

- Up to 9 biopsies per tumor were routinely collected (*in the case of radical nephrectomies*).

3 Place each biopsy onto 100mm Petri Dish, on ice

Depending on the amount of tissue, put aside parts of each biopsy for further complementary analyses.

- Number and identify each punch biopsy as well as its following match samples.
- Cut each biopsy in three parts (¼, ¼, ½) consisting of approximately ¼ for formaldehyde treatment and immunohistochemistry staining; ¼ for snap freezing, -80°C storage and further DNA/RNA extraction; and ½ for tissue dissociation of tumor punches.

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Tumor Dissociation

4

This core step consists of a combined mechanical and enzymatic disaggregation of the tumor tissue. It requires GentleMACS Dissociator devices.

Material

Material	Supplier Info
Tumor Dissociation Kit, Human	Miltenyi Biotec (130-095-929)
Gentle MACS Octo Dissociator	Miltenyi Biotec (130-096-427)
GentleMACS C tubes	Miltenyi Biotec (130-096-334)
MACS SmartStrainers 70 µm	Miltenyi Biotec (130-098-462)
RPMI 1640	Thermofisher (11835030)
Surgical Blade	Dutscher (132522)
Forceps	Dutscher (711200)
50ml Falcon Tubes / 15ml Falcon Tubes	Dutsher (352098 / 352097)
1.5 ml Eppendorf tube	Eppendorf (0030 108.035)
Trypan Blue	SIGMA Aldrich (T8154)
Malassez Chamber	Dutscher (140501)
Petri Dish, 100 mm	Corning (430591)

5 In the petri dish, pool the punch biopsies dedicated to further dissociation.

Do not exceed 1g of total tumor tissue.

6 Prepare Digest Mix ON ICE by adding into a gentleMACS C Tube 4.7ml RPMI 1640, 200µl Enzyme H, 100µl Enzyme R and 25µl Enzyme A.

7 In the petri dish, pour 500ml Digest Mix onto the punch biopsies.

8 Gently mince the punch biopsies into small pieces of about 2 mm^3 using a scalpel.

9 Using forceps, gently transfer tissue fragments into the gentle MACS C tube containing the Digest Mix (be careful not to crush the fragments). Add the remaining digest mix solution from the petri dish and tightly close C Tube.

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10 Run the gentle MACS Octo Dissociator Program “37_h_TDK_1”RUN **01:00:00**

In case you own the gentleMACS^{TM}Dissociator, please successively run programs h_tumor_01, h_tumuor_02 and h_tumor_03 with 2x 30 minutes of incubation at 37°C under continuous rotation between programs 01-02 and programs 02-03.

11 Humidify the MACS SmartStrainer 70 µm with 5ml RPMI 1640 above a 50ml Falcon Tube.

12 Apply cell suspension through the strainer.

13 Rinse the remaining cells in the MACS C tube and reapply it through the strainer.

14 Wash the strainer and complete the suspension to a final volume of 40ml RPMI 1640.

15 Centrifuge cell suspension **300 x g** **00:05:00**

Red Blood Cell (RBC) Lysis Protocol

16

In this step, red blood cells are lysed and washed away as they represent irrelevant cells for the study.

Material	Supplier Info
RBC Buffer (10x)	BioLegend (420301)
Desionized water	Invitrogen (10977035)
DPBS	Thermofisher (14190250)

17 Take advantage of the centrifugation duration to prepare 1X RBC Lysis Buffer on ice: dilute 1ml 10X RBC Lysis Buffer to 1X working concentration with 9ml deionized water.

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18 Discard the supernatant, gentlgy resuspend the cell pellet in 1ml RBC Lysis Buffer and quickly transfer it into a new 50ml Falcon.

19 Add the remaining RBC Lysis Buffer.

20 Incubate on Ice for 4 min with occasional shaking.

21 Stop the reaction by diluting the active RBC Lysis Buffer with 30ml 1X PBS.

22 Centrifuge cell suspension **300 x g** **05:00:00**

23 Discard the supernatant and resuspend the pellet in 1ml RPMI 1640

Optional:
- Take 20µl from cell suspension for counting and microscopic quality control of the dissociation using trypan blue in a Malassez Chamber.
- 0.3 million cells were used for Flow Cytometry Assays to measure viability with 7-amino-actinomycin (7AAD), as well as CD45 (immune) and CD34 (endothelial) positive cells..

Low Centrifugation

24

This step aims at clearing the suspension from debris and dead cells while preserving tumor cells. When working on cancerous tissues, we recommend not using Annexin V-based dead cell removal kits as they also remove altered but yet viable cells and presumed pre-apoptotic tumor cells that often represent relevant populations in the framework of single-cell studies. Successive low centrifugations were chosen instead as they allow to recover a higher proportion of tumor cells. This solution was also preferred over FACS sorting because of its significant time savings.

Material	Supplier Info
15ml Falcon Tubes	Dutsher (352097)
1.5 ml Eppendorf tube	Eppendorf (0030 108.035)
RPMI 1640	Thermofisher (11835030)

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25 Transfer the cells in a 15ml Falcon tube and complete the suspension to a final volume of 12ml RPMI 1640.

26 Centrifuge cell suspension at 800g **150 x g** **00:06:00**

27 Discard the supernatant and resuspend the pellet in 1 ml of RPMI 1640.

28 Transfer cells in a new 15ml Falcon Tube and repeat the low centrifugation step.

CD45 Positive Cells Depletion

29

- This step is optional and depends on the targeted cell population. It aims at enriching the cell suspension with tumor and stromal cells by depleting the population of leucocytes, mainly consisting of circulating lymphocytes, which may represent the vast majority of the cells after dissociation.
- The following volumes were adapted to a maximum population of 10^7 cells. In case you recover too much cells, please adapt the volumes according to the following ratios.

Material	Supplier Info
CD45(TIL) MicroBeads	Miltenyi Biotec (130-118-780)
EDTA 0.5 M	Sigma Aldrich (03690)
BSA	Sigma Aldrich (A7906)
DPBS	Thermofisher (14190250)
RPMI 1640	Thermofisher (11835030)
LS MACS Columns	Miltenyi Biotec (130-042-401)
MACS Separator	Miltenyi Biotec (130-090-976)

30 Prepare 10ml MACS Buffer solution containing PBS, 0.5% BSA and 2 mM EDTA.

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31 Resuspend cell pellet (up to 10^7 cells) in 80 µl of MACS Buffer, and mix well by gently pipetting 10 to 15 times.

32 Add 20 µl CD45 micro beads, mix and incubate in fridge **4 °C** **00:15:00**

33 Add 400 µl of MACS Buffer, mix by pipetting

34 Place LS column in QuadroMACS Magnetic Cell Separator and prepare it by rinsing with 3ml MACS Buffer (discard effluent).

35 Apply cell suspension onto the column and immediately collect flow-through containing unlabeled cells in 1.5ml Falcon tube.

36 Rinse the column twice with 1ml of MACS Buffer, collect and pool the effluent.

37 Centrifuge cell suspension **300 x g** **00:05:00**

38 Discard the supernatant and resuspend the pellet in 1.5ml tubes with 250µl RPMI 1640.

Optionnal:
- take 20µl from cell suspension for counting and microscopic quality control do the dissociation using trypan blue in a Malassez Chamber.
- 0.25 million cells were used for Flow Cytometry Assays to measure viability with 7-amino-actinomycin (7AAD), as well as CD45 (immune) and CD34 (endothelial) positive cells.

Expected Result

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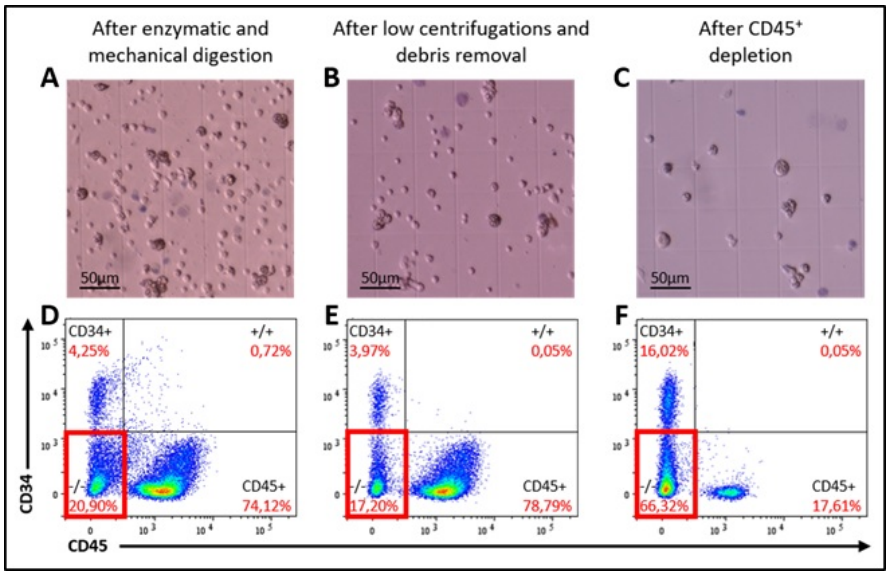
39



Cell dissociation control by Blue Trypan staining and flow cytometry. After GentleMacs dissociation (A, D), after Low Centrifugation (B, E) and after CD45+ depletion (C,F). CD34 expression reveals endothelial cells and CD45 expression reveals immune cells. Red squares indicate the proportions of CD45-/CD34- cells containing tumor cells and part of stromal cells.

Application

40 Single Cell experiment

If cell viability is higher than 70%, cells can then be processed for scRNA-seq on 10X genomics platform. Gently resuspend the cells in appropriate volume of PBS, 0.04% BSA and proceed to 10X protocol.

41 Freezing samples and biobanking

- This step aims at storing the tumor cells after dissociation in order to allow their delayed study. This can be useful for the study of asynchronous samples such as matched primary and metastatic tumors.
- We tested this approach and were able to obtain high quality and comparable data in single-cell RNA-sequencing of fresh versus frozen samples (data not shown).
- Transfer enough cells (0.1 to 1 Million) in 1ml freezing solution consisting of 700µl RPMI 1640, 200µl Fetal Bovine Serum and 100µl DMSO). Immediately place the cells at -20°C for one hour before gradually freezing and storing them at -80°C overnight (~12h). Then, transfer cells to liquid nitrogen for long-term storage.

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