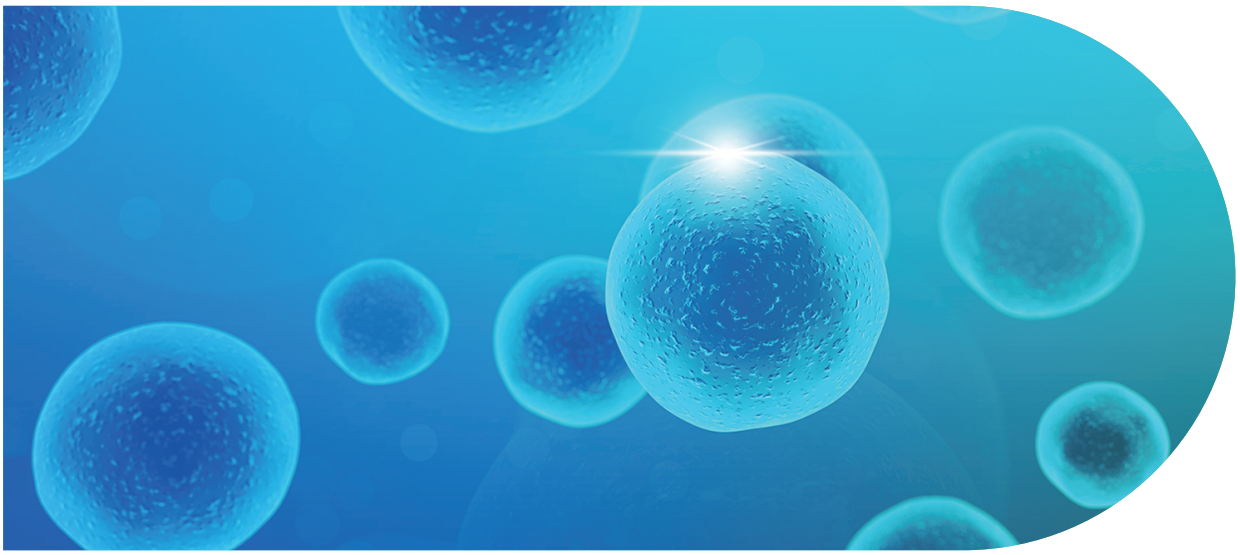
Novogene



Sample Preparation Demo Protocols for Cell Suspensions

Advancing Genomics, Improving Life

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1 Overview

Note: We are pleased to announce that we have created a brochure that provides useful information and tips on preparing cell suspensions. The brochure covers topics such as cell isolation, viability, and concentration.

The brochure is based on the protocols and guidelines from 10X Genomics and peer-reviewed reference. We have cited the relevant sources from 10X Genomics in the brochure, and we acknowledge their contributions to the advancement of scRNA-seq technology.

However, the brochure is not limited to 10X Genomics products or platforms. The brochure is compatible for any cell samples that can be used for scRNA-seq, regardless of the manufacturer or vendor. The brochure aims to help researchers and technicians who are interested in performing scRNA-seq experiments with any type of cell samples.

We hope that you will find the brochure helpful and informative. If you have any questions or feedback, please feel free to contact us at any time. Thank you for your attention and interest.

Solid tissues or large cell aggregates must be dissociated into separate single cells before proceeding with loading to the Chip for generating single cell GEMs; the dissociation could be performed by using mechanical or enzymatic dissociation, cell sorting, or other cell isolation techniques. Novogene is delighted to share some demo protocols collected from the 10x Genomics website and peer-reviewed papers to help you better comprehend the preparation of single cell suspension, especially for some typical species and their tissues.

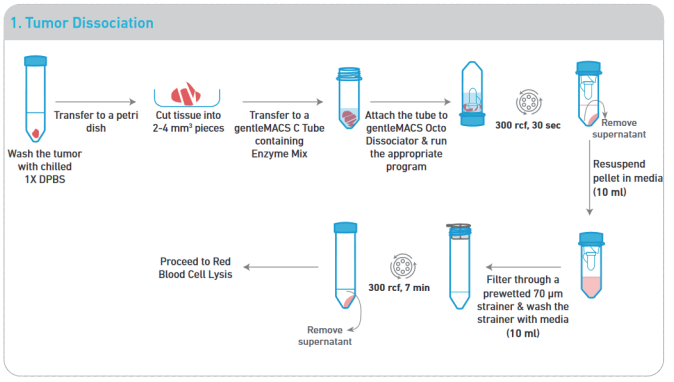
Notably, the dissociation effect should be largely affected by specie types, tissue types and tissue statues, so modifications to these demonstrated protocols may be necessary for different sample types (e.g. dissociation time, resuspension buffer, enzyme concentration, centrifugation speed and time).

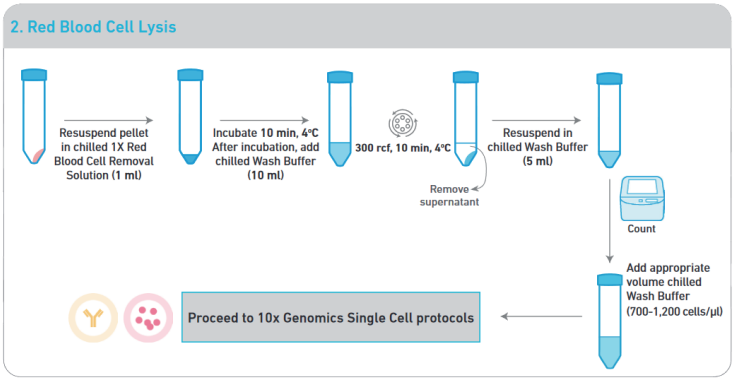
We recommend sending either fresh or frozen tissue cryopreserved, or fresh cell suspensions to Novogene for preparing single cells, as it is preferable to load the Chip immediately after obtaining the qualified single cells. To avoid the sample quality problem caused by improper preservation and shipping methods, Novogene specially offers Preservation and Shipping Instructions to convey your samples across the shipping waves. We strongly recommend that you check Page 19, Section 4 , before you collect and send samples to Novogene. The instruction is based on a summary of various samples under different conditions.

2 Demonstrated Protocols from 10x Genomics

2.1 Mouse Tumor Dissociation for Cell suspensions

Protocol Overview





2.1.1 Tumor Dissociation

a. Wash the tumor tissue in a 50 ml centrifuge tube by adding 10 ml chilled 1X DPBS.
b. Place in a petri dish and cut the tumor tissue into small pieces of 2~4 mm^3.
c. Prepare enzyme mix in a gentleMACS™ C Tube by adding 2.35 ml RPMI 1640 or DMEM, 100 µl Enzyme D, 50 µl Enzyme R, and 12.5 µl Enzyme A.
d. Transfer the tumor tissue pieces to the C Tube containing the enzyme mix.
e. Tightly close the C Tube and attach it upside down to a sleeve of a gentleMACS™ Octo Dissociator with Heaters.
f. Select the Dissociator program based on tumor tissue texture (soft, medium or hard tissue).
g. Run the program 37C_m_TDK_1 for melanoma and colon tumor (soft and medium tissue respectively). Run the program 37C_m_TDK_2 for breast tumor (hard tissue).
h. At the end of the run, detach the C Tube from the Dissociator.
i. Centrifuge at 300 RCF for 30 sec at room temperature.
j. Remove the supernatant without disturbing the cell pellet.
k. Add 10 ml RPMI 1640 or DMEM and gently pipette mix to resuspend the cell pellet.
l. Filter the cell suspension through a prewetted 70-µm MACS® SmartStrainer placed on a 50 ml centrifuge tube.
m. Wash the strainer with 10 ml RPMI 1640 or DMEM and collect the wash in the tube with the cell suspension.
n. Centrifuge the cell suspension at 300 RCF for 7 min at room temperature.
o. Remove supernatant without disturbing the cell pellet.
p. Proceed immediately to the next step 2.1.2 for Red Blood Cell Lysis.

2.1.2 Red Blood Cell Lysis

a. Add 1 ml chilled 1X Red Blood Cell Removal Solution to the cell pellet from step 3.1n and gently pipette mix to resuspend the cells.
b. Incubate for 10 min at 4°C
c. Add 10 ml chilled Wash Buffer.
d. Centrifuge at 4°C at 300 RCF for 10 min.
e. Remove supernatant without disturbing the cell pellet.
f. Add 5 ml chilled Wash Buffer and gently pipette mix to resuspend the cell pellet.
g. Determine the cell concentration using a Countess® II FL Automated Cell Counter or hemocytometer.
h. Add the appropriate volume of chilled Wash buffer to the cell suspension and gently pipette mix to achieve the target cell concentration of 700 to 1200 cells/µl ($7x10^5$ to $1.2x10^6$ cells/ml).
i. Proceed immediately with the 10x Genomics Single Cell protocol.

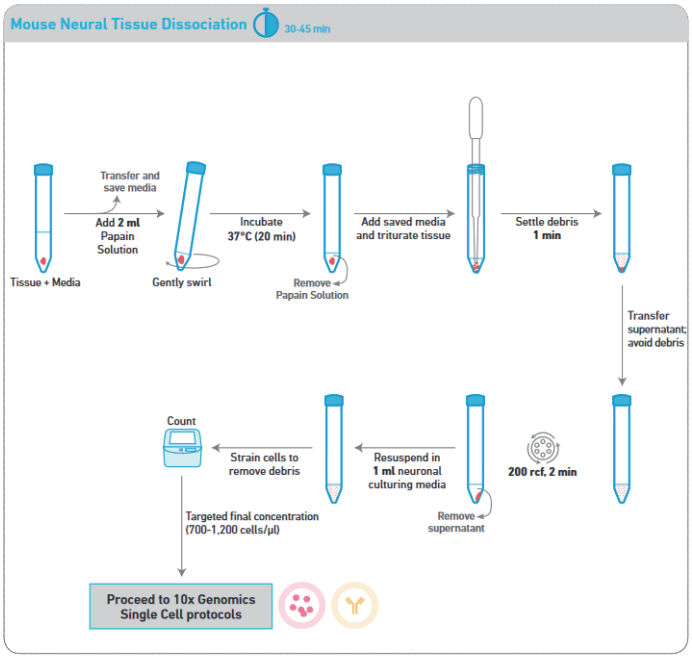
Reference

Technical Note – Tumor Dissociation for Single Cell RNA Sequencing. Document Number CG000147, 10x Genomics, (2022, December 7).

More information including Reagents and Consumables can be found by clicking here.

2.2 Mouse Embryonic Neural Tissue Dissociation for Cell suspensions

Protocol Overview



2.2.1 Tissue lysis and cleaning nucleus

a. Using a 1000 µl wide-bore pipette tip, gently transfer the tissue along with the Hibernate E®/B27®/GlutaMAX™ (HEB) medium to a new 15 ml conical tube and wait until the tissue is settled at the bottom of the tube.
b. Transfer the HEB medium from the tissue to a new 15 ml conical tube, leaving only enough medium to cover the tissue. Keep the HEB medium on ice for step d.
c. Add 2 ml chilled Lysis Buffer to the tissue and lyse the tissue on ice for 15 min. Gently swirl to mix, repeat 2 to 3 times during the incubation.
d. Add the HEB medium saved from step b back to the lysed tissue.
e. Aspirate the tissue and the HEB medium into a fire polished silanized Pasteur pipette and immediately dispense the contents back into the tube. Triturate with 5 to 7 passes of the tissue through the pipette.
f. Centrifuge the nuclei at 500 RCF for 5 min at 4°C.
g. Remove the supernatant without disrupting the nuclei pellet.
h. Using a regular-bore pipette tip, add 1 ml Nuclei Wash and Resuspension Buffer and gently pipette mix 8 to 10 times.
i. Use a 40 µm Flowmi™ Cell Strainer to remove cell debris and large clumps. Transfer to a 2 ml centrifuge tube.
j. Centrifuge the nuclei at 500 RCF for 5 min at 4°C.
k. Remove the supernatant without disrupting the nuclei pellet.
l. Using a regular-bore pipette tip, add 1 ml Nuclei Wash and Resuspension Buffer and gently pipette mix 8 to 10 times.
m. Repeat steps i to k.
n. Using a regular-bore pipette tip, add 180 µl Nuclei Wash and Resuspension Buffer and gently pipette mix 8 to 10 times.
o. Proceed directly to Myelin Removal.

2.2.2 Removal of Myelin

ⓐ Add 20 µl Myelin Removal Beads II to the resuspended nuclei from step 2.4n. Mix thoroughly with a wide-bore pipette tip. Do not vortex it.
ⓑ Incubate for 15 min at 4°C.
ⓒ Meanwhile, prepare an LS column with a 3 ml LS Column Calibration Buffer.
ⓓ After incubation is complete, dilute the nuclei suspension (containing Myelin Removal Beads II) with 5 ml Nuclei Wash and Resuspension Buffer (using a 10 ml serological pipette) and gently pipette mix 5 times.
ⓔ Centrifuge the nuclei at 500 RCF for 10 min at 4°C.
ⓕ Remove the supernatant without disrupting the nuclei pellet.
ⓖ Resuspend the pelleted nuclei in 1 ml Nuclei Wash and Resuspension Buffer.
ⓗ Apply the resuspended nuclei to the LS column.
ⓘ Wash the column twice with 1 ml Nuclei Wash and Resuspension Buffer.
ⓙ Collect the effluent in one 5 ml Eppendorf tube.
ⓚ Centrifuge the nuclei at 500 RCF for 5 min at 4°C.
ⓛ Remove the supernatant without disrupting the nuclei pellet.

ⓜ Using a regular-bore pipette tip, add 500 µl Nuclei Wash and Resuspension Buffer or an appropriate volume to the pelleted nuclei to achieve the target nuclei concentration of 1000 nuclei/µl (1 x 10^6 nuclei/ml). Gently pipette mix 8 to 10 times or until nuclei are completely suspended.
ⓝ Use a cell strainer to remove cell debris and large clumps. For low volume, a 40 µm Flowmi™ Tip Strainer is recommended to minimize loss of sample volume.
ⓞ Determine the nuclei concentration using a Countess® II FL Automated Cell Counter or hemocytometer.
ⓟ If the nuclei concentration is < 500 nuclei/µl (5 x 10^5 nuclei/ml), adjust the volume accordingly.
ⓠ Once the target nuclei concentration of 1000 nuclei/µl (1 x 10^6 nuclei/ml) is obtained, place the nuclei on ice.
ⓡ Proceed immediately with the 10x Genomics Single Cell Protocol and minimize the time between nuclei preparation and chip loading.

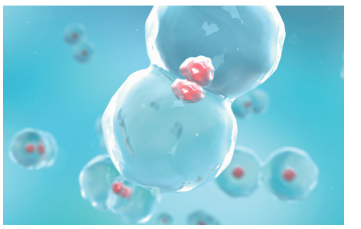
Reference

Technical Note – Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing, Document Number CG00055, 10x Genomics, (2022, July 13).

More information including Reagents and Consumables can be found by clicking here.

2.3 Isolation of Single Cell Suspensions from Cultured Cell Lines for Cell suspensions

2.3.1 Cell Harvesting – Suspension Cell Lines



a. Determine the cell concentration using a Countess® II Automated Cell Counter.
b. The target cell concentration is 3 x 10^5 – 1 x 10^6 cells/ml with >viability 90%. If the cell concentration is > 6 x 10^5 cells/ml, transfer 1.5 ml of the cell suspension to a 2 ml Eppendorf tube. If the cell concentration is < 6 x 10^5 cells/ml, transfer 1.5 ml to two 2 ml Eppendorf tubes for a total of 3 ml of cell suspension.

2.3.2 Cell Harvesting – Adherent Cell Lines

a. Using a 10 ml serological pipette, remove and discard the culture medium.
b. Using a 10 ml serological pipette, add 1.5 ml 0.25% Trypsin-EDTA solution to the flask and briefly rinse the cell layer to eliminate residual serum. Immediately discard the used trypsin solution.
c. Using a 10 ml serological pipette, add 4 to 5 ml 0.25% Trypsin-EDTA solution to cover the cell layer and incubate the flask at 37°C for 5 – 15 min until the cells detach from the flask surface.
d. Using a 10 ml serological pipette, add 10 ml culture medium to stop digestion.
e. Using a 10 ml serological pipette, gently and thoroughly mix the trypsin solution and the culture medium by gently pipette mixing 5 to 10 times to break up clumps of cells.
f. Transfer the cell suspension to a 50 ml conical tube.
g. Centrifuge cells at 250 RCF for 5 min.
h. Using a 10 ml serological pipette, discard supernatant without disrupting the cell pellet.
i. Using a 1000 µl regular-bore pipette tip, add 1 ml culture medium and resuspend the pelleted cells by gently pipette mixing 10 times or until the cell are completely resuspended.
j. Using a 10 ml serological pipette, add 2 – 5 ml culture medium (depending on the cell line yield) and gently pipette mix 5 times.
k. Place a 30 µm cell strainer (or a strainer with an appropriate pore size for the cell type) on top of a 50 ml conical tube and filter the cell suspension into the 50 ml tube.
l. Thoroughly mix the cells and determine the cell concentration using a Countess® II Automated Cell Counter.
m. If the cell concentration is > 6 x 10^5 cells/ml, transfer 1.5 ml of the cell suspension to a 2 ml Eppendorf tube. If the cell concentration is < 6 x 10^5 cells/ml, transfer 1.5 ml to two 2 ml Eppendorf tubes for a total of 3 ml of cell suspension.

2.3.3 Cell Washing

a. Centrifuge cells in a 2 ml Eppendorf tube at 150 RCF for 3 min.
b. Remove supernatant without disrupting the cell pellet.
c. Using a wide-bore pipette tip, add 1 ml 1X PBS with 0.04% BSA to each tube and gently pipette mix 5 times and invert tubes to resuspend the cell pellet. Pool the tubes if necessary.
d. Centrifuge cells at 150 RCF for 3 min.
e. Remove supernatant without disrupting the cell pellet.
f. Using a wide-bore pipette tip, add 1 ml 1X PBS with 0.04% BSA to each tube and gently pipette mix 5 times and invert tubes to resuspend the cell pellet.
g. Centrifuge cells at 150 RCF for 3 min.
h. Remove supernatant without disrupting the cell pellet.
i. Using a regular-bore pipette tip, add around 500 µl 1X PBS with 0.04% BSA or appropriate volume such that cell concentration is above 7 x 10^5 cells/ml. Gently pipette mix 10 to 15 times or until the cells are completely suspended.
j. Use a cell strainer to remove cell debris and large clumps. For low volume cell suspensions, a FlowmiTM Tip Strainer is recommended for minimal loss of sample volume.
k. Determine the cell concentration using a Countess® II Automated Cell Counter.
l. The target cell concentration is 7 x 10^5 cells/ml (700 cells/µl). If the cells are over concentrated, adjust the volume accordingly and re-count.
m. Once the target cell concentration is obtained, place the cells on ice.
n. Proceed with the 10x Genomics Single Cell Protocol.

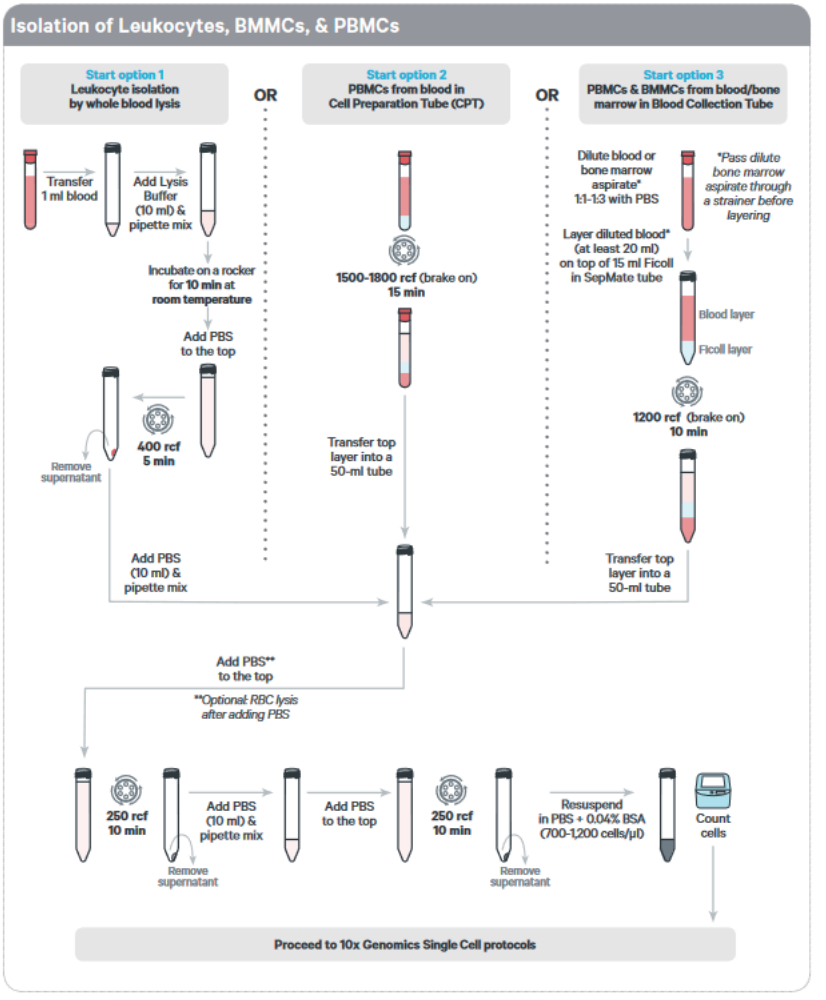
Reference

Technical Note – Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing, Document Number CG00054, 10x Genomics, (2022, December 7).

More information including Reagents and Consumables can be found by clicking here.

2.4 Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Cell suspensions

Protocol Overview



Start Option 1: Leukocyte isolation by whole blood lysis

- Transfer 1 ml whole blood into a 50 ml tube.
- Add 10 ml 1x RBC lysis buffer.
- Incubate on a rocker for 10 min at room temperature.
- Add PBS to the top of the tube.
- Centrifuge at 400 RCF for 5 min.
- Pour off the supernatant. Add 10 ml PBS. Gently pipette mix 10x to resuspend the pellet.
- Add PBS to the top of the 50 ml tube.
- Proceed directly to either optional step g for additional RBC lysis.

Start Option 2: PBMCs from blood collected in BD Vacutainer CPT

- Centrifuge at 1,500 --1,800 RCF (brake on) for 15 min at room temperature in a horizontal rotor (swing-out head). If transporting samples, centrifugation is recommended prior to transportation.
- Proceed directly to step f.

Start Option 3: PBMCs & BMMCs from blood/ bone marrow collected in BD Vacutainer Blood Collection Tube

- Add 15 ml Ficoll-Paque® PLUS (1.077 mg/ml) to a SepMate™ tube through the SepMate™ insert center hole without introducing bubbles.
- Dilute collected blood 1:1-1:3 with 1x PBS (e.g. 10 ml blood diluted with 10 to 30 ml PBS). The minimum recommended input volume for a 50 ml SepMate™ tube is 20 ml diluted blood. For bone marrow aspirate, pass the diluted aspirate through a 70 µm strainer to remove debris.
- Pipette diluted blood slowly down the side of the SepMate™ tube.
- Centrifuge at 1,200 RCF for 10 min (brake on). For samples collected > 24 hr before processing, centrifugation for 20 min is recommended.
- Pour top layer into a new 50 ml centrifuge tube in single smooth motion.
- Add PBS to the top of the 50 ml tube.
- OPTIONAL For RBC Lysis: Centrifuge at 400 RCF for 5 min. Pour off the supernatant and resuspend the cell pellet in 10 ml 1x RBC lysis buffer. Incubate on a rocker for 10 min at room temperature. Add PBS to the top of the 50-ml tube with the sample.
- Centrifuge at 250 RCF for 10 min at room temperature.
- Pour off the supernatant. Add 10 ml PBS. Gently pipette mix 10x to resuspend the pellet.
- Add PBS to the top of the 50 ml tube.
- Centrifuge at 250 RCF for 10 min at room temperature.
- Pour off the supernatant.
- Resuspend cell pellet in equal volume PBS / PBS+0.04% BSA to original whole blood volume. For example, if the input was 10 ml whole blood, resuspend in 10 ml PBS for ~$1x10^6$ cells/ ml.
- Determine cell concentration and viability using an Automated Cell Counter (Countess® II or Cellaca MX) or a hemocytometer. Cellaca MX with ViaStain™ AOPI Staining Solution should be used for this protocol.
- If needed, add an appropriate volume of PBS/ PBS+0.04% BSA to obtain a concentration of 700-1,200 cells/µl.
- Once the final cell concentration is achieved, maintain cells at room temperature. DO NOT place on ice as it may result in granulocyte lysis.
- Proceed immediately to the 10x Genomics Single Cell protocol.

Reference

Technical Note – Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing, Document Number CG000392, 10x Genomics, (2022, July 13).

More information including Reagents and Consumables can be found by clicking here.

3 Demonstrated Protocols from Published Literatures

3.1 Mouse Tissue

3.1.1 Mouse Tumor Mode: Melanoma, Breast Cancer, Lung Adenocarcinoma, Colon Cancer, Fibrosarcoma

Method: Mouse Tumor Dissociation Kit (Miltenyi)

a. Each mouse tumor was harvested when the tumor size reached 100 to 200 mm^3.
b. Each sample was minced and digested with reagents from Mouse Tumor Dissociation Kit (Miltenyi) according to the manufacturer's instructions.
c. Cells were resuspended at $2x10^5$ cells/mL in PBS-0.04% BSA. Each sample was processed individually and run in technical duplicates.
d. For each sample (except CT26 and MC-38) one replicate was enriched for CD45 positive cells.
e. Live CD45 positive cells were sorted with BD Aria after staining with FITC-CD45 (Biolegend) and 7-AAD.
f. Single cell suspensions of all samples were resuspended in PBS-0.04% BSA at $5x10^5$ cells/mL and barcoded with a 10x Chromium Controller (Kumar et al., 2018).

Reference

Kumar, M. P., Du, J., Lagoudas, G., Jiao, Y., Sawyer, A., Drummond, D. C., Lauffenburger, D. A., & Raue, A. (2018). Analysis of single-cell RNA-seq identifies cell-cell communication associated with tumor characteristics. *Cell reports,* 25(6), 1458–1468.

3.1.2 Mouse Colon

Method: Enzyme digestion

a. Colons were processed individually in parallel. Caecal pouches, mesenterium and fat were first detached and discarded.
b. The remaining colons were opened vertically and cut into 1 cm fragments.
c. These were incubated at 37°C in RPMI with 0.1% BSA and 5 mM EDTA with horizontal shaking for 40 minutes to detach epithelial crypts.

d The crypt containing supernatant was discarded.

e Fresh RPMI with 0.1% BSA and 5 mM EDTA was added, and a further 15 minutes of incubation at 37 °C with horizontal shaking was performed to further deplete the epithelium.

f The tissue fragments were then washed and incubated in RPMI with added FCS (10%), HEPES (15 mM) and Collagenase VIII (100 U/mL, Sigma Aldrich) for 60 minutes at 37 °C with horizontal shaking.

g The resulting supernatant was passed through a 70 µm strainer and single cells were pelleted by centrifugation at 500 g for 8 minutes.

h Percoll gradient centrifugation was performed to remove non-cellular debris. (Physiological 100% Percoll was made by combining 9 parts Percoll with 1-part 10X PBS).

i The cell pellets were resuspended in RPMI with 30% Percoll (GE Healthcare) and the resulting suspension layered over PBS with 70% Percoll in a 15 ml Falcon.

j Centrifugation at 900 g for 20 minutes (4 °C) was performed and the 30% / 70% interface layer was collected (Kinchen et al., 2018).

Reference

Kinchen, J., Chen, H. H., Parikh, K., Antanaviciute, A., Jagielowicz, M., Fawkner-Corbett, D., Ashley, N., Cubitt, L., Mellado-Gomez, E., & Attar, M. (2018). Structural remodeling of the human colonic mesenchyme in inflammatory bowel disease. *Cell, 175* (2), 372–386.

3.1.3 Mouse Brain

Method: Enzyme digestion

ⓐ Mice were deeply anaesthetized and perfused transcardially with 30 ml of ice-cold phosphate-buffered saline (PBS). For whole-brain isolation, mice were decapitated and brains were placed on ice-cold Roswell Park Memorial Institute (RPMI) 1640 medium(Gibco™).

ⓑ Brains were cut into small pieces followed by the addition of enzyme mix (30 U ml−1 DNAse I (Roche), 10 U ml−1 collagenase type I (Worthington) and 400 U ml−1 collagenase type IV (Worthington) diluted in 1× Hanks' buffered salt solution (Gibco™)).

ⓒ Following 20 min at 37 °C, tissue was crushed with a syringe plunger and homogenized via trituration using standard serological pipettes.

ⓓ The solution was filtered twice over a 100 μm nylon filter and centrifuged (Van Hove et al., 2019).

Reference

Van Hove, H., Martens, L., Scheyltjens, I., De Vlaminck, K., Pombo Antunes, A. R., De Prijck, S., Vandamme, N., De Schepper, S., Van Isterdael, G., & Scott, C. L. (2019). A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. *Nature neuroscience,* 22(6), 1021–1035.

3.1.4 Mouse Adipose

Method: Enzyme digestion

ⓐ Following tissue dissection, adipose tissues were washed with PBS, minced, and digested with type 2 collagenase in Hanks' balanced salt solution containing sodium bicarbonate, 10 mM HEPES and 0.5% fatty acid free bovine serum albumin for 30 minutes at 37 °C.

ⓑ Dissociated cells were filtered through a cell strainer, and washed with a PBS buffer containing 1 mM EDTA, 2.5 mM HEPES, and 10% heat-inactivated fetal bovine serum.

ⓒ Centrifuged at 500 x g for 10 minutes at 4 °C.

ⓓ Removing the supernatant, pellets containing the stromal vascular faction were incubated in red blood cell lysis buffer for 5 minutes at room temperature.

ⓔ Cellular debris was removed by gradient centrifugation in Iodixanol solution overlaid with FACS buffer.

ⓕ Cells recovered in the FACS buffer layer were pelleted, washed, and resuspended in PBS containing 5% FF-BSA (Burl et al., 2018).

Reference

Burl, R. B., Ramseyer, V. D., Rondini, E. A., Pique-Regi, R., Lee, Y.-H., & Granneman, J. G. (2018). Deconstructing adipogenesis induced by β3-adrenergic receptor activation with single-cell expression profiling. *Cell metabolism, 28* (2), 300–309.

3.1.5 Mouse Small Intestine Epithelium

Method: Enzyme digestion

a The small intestine of mice was isolated and rinsed in cold PBS.

b The tissue was opened longitudinally and sliced into small fragments roughly 2 mm in length. Epithelial cells from the follicle-associated epithelia were isolated by extracting small sections (0.2 – 0.5 cm) containing Peyer's patches from the small intestine of mice.

c The tissue was incubated in 20 mM EDTA-PBS on ice for 90 min, shaking every 30 min.

d The tissue was then shaken vigorously and the supernatant was collected as fraction 1 in a new conical tube. The tissue was incubated in fresh EDTA–PBS and a new fraction was collected every 30 min.

e Fractions were collected until the supernatant consisted almost entirely of crypts.

f The final fraction (enriched for crypts) was washed twice in PBS, centrifuged at 300g for 3 min, and dissociated with TrypLE express (Invitrogen) for 1 min at 37 °C.

g The single-cell suspension was then passed through a 40-μm filter and stained for FACS for scRNA-seq or used for organoid culture (Haber et al., 2017).

Reference

Haber, A. L., Biton, M., Rogel, N., Herbst, R. H., Shekhar, K., Smillie, C., Burgin, G., Delorey, T. M., Howitt, M. R., & Katz, Y. (2017). A single-cell survey of the small intestinal epithelium. *Nature, 551*(7680), 333–339.

3.1.6 Mouse Heart

Method: Manual operation

ⓐ Embryos were dissected in cold PBS (Life Technologies, CAT# 14190250), de-yolked and placed in PBS/1% FBS (ThermoFisher Scientific, CAT# 10439016) solution on ice until dissociation.

ⓑ Dissected cardiac tissue was incubated in 200 µl TrypLE™ (ThermoFisher Scientific, 12563029) for 5 minutes.

ⓒ Triturated with a 200 µl pipette tip, and incubated for 5 min.

ⓓ The TrypLE™ solution was quenched with 600 µl PBS with 1% FBS.

ⓔ Cells were filtered through a 70 µm cell strainer (BD Falcon, 08-771-2), centrifuged at 150g for 3 min.

ⓕ Resuspended in 35 µl PBS with 1% FBS (de Soysa et al., 2019).

Reference

de Soysa, T. Y., Ranade, S. S., Okawa, S., Ravichandran, S., Huang, Y., Salunga, H. T., Schricker, A., Del Sol, A., Gifford, C. A., & Srivastava, D. (2019). Single-cell analysis of cardiogenesis reveals basis for organ-level developmental defects. *Nature, 572* (7767), 120–124.

3.1.7 Mouse Cardiac Outflow Tract

Method: Enzyme digestion

ⓐ Washed the dissected tissue with DPBS.

ⓑ Digested them with Pierce™ Primary Cardiomyocyte Isolation Kit (Thermo Fisher #88281) according to the manufacturer's instructions.

ⓒ The obtained suspensions were filtered using a 40-um strainer to remove any cell debris or large clumps.

ⓓ Cell viability and concentration were measured by Countstar.

ⓔ Cells were finally diluted to 1X106 cells per milliliter by adding appropriate volumes of HBSS with 0.04% BSA (Liu et al., 2019).

Reference

Liu, X., Chen, W., Li, W., Li, Y., Priest, J. R., Zhou, B., Wang, J., & Zhou, Z. (2019). Single-cell RNA-seq of the developing cardiac outflow tract reveals convergent development of the vascular smooth muscle cells. *Cell Reports, 28*(5), 1346–1361.

3.1.8 Mouse Liver

Method: Enzyme digestion

ⓐ The liver was perfused in situ with calcium-free HBSS containing 0.2mg/mL EDTA, followed by sequential perfusion with 0.4mg/mL pronase (Sigma, P5147) and 0.2% collagenase type II (Worthington, LS004196).

ⓑ The liver was minced and further digested with HBSS containing 0.2% collagenase type II, 0.4 mg/mL pronase and 0.1mg/mL DNase I (Roche, R104159001) in 37°Cwater bath with shaking for 20 min.

ⓒ Digestion was terminated with DMEM containing 10% serum.

ⓓ The resulting liver cell suspension was centrifuged at 50 g for 3 min to remove hepatocytes and passed through 30 um nylon cell strainers.

ⓔ Treatment with 0.8% NH4Cl to lyse red blood cells.

ⓕ This nonparenchymal cell(NPC) suspension was centrifuged, resuspended in HBSS, and subjected to density gradient centrifugation using 20% OptiPrep™ (Axis Shield, 1114542) to remove dead cells.

ⓖ Cell viability was confirmed by trypan blue exclusion (Xiong et al., 2019).

Reference

Xiong, X., Kuang, H., Ansari, S., Liu, T., Gong, J., Wang, S., Zhao, X.-Y., Ji, Y., Li, C., & Guo, L. (2019). Landscape of intercellular crosstalk in healthy and NASH liver revealed by single-cell secretome gene analysis. *Molecular cell, 75* (3), 644–660.

3.2 Human Tissue

3.2.1 Human Urine

Method: Manual operation

ⓐ Midstream urine samples were collected from patients with LN before kidney biopsy. The total urine volume (15 to 90 ml) was split into 2 50-ml Falcon tubes.

ⓑ Urine cells were pelleted by centrifugation at 200g for 10 min, and then resuspended in 1 ml cold X-VIVO10 medium (Lonza BE04-743Q).

ⓒ Cells were transferred to a microcentrifuge tube, washed once in 1 ml X-VIVO10 medium and then resuspended in 0.5 ml cold CryoStor® CS10.

ⓓ Cells were transferred into a 1.8 ml cryovial, placed in a Mr. Frosty™ Freezing Container stored at −80 °C overnight and then transferred into liquid nitrogen.

ⓔ For downstream analyses, cryopreserved urine cells were rapidly thawed by vigorous shaking in a 37 °C water bath, transferred into warm RPMI/10% FBS, centrifuged at 300g for 10 min and resuspended in cold HBSS/1% BSA (Arazi et al., 2019).

Reference

Arazi, A., Rao, D. A., Berthier, C. C., Davidson, A., Liu, Y., Hoover, P. J., Chicoine, A., Eisenhaure, T. M., Jonsson, A. H., & Li, S. (2019). The immune cell landscape in kidneys of patients with lupus nephritis. *Nature immunology, 20* (7), 902–914.

3.2.2 Human Pleural Effusion

Method: Manual operation

a. Pleural effusion samples collection and experiment were carried out in accordance with guidelines and protocols that were approved by the Ethics and Scientific Committees of both hospitals.
b. 10 ml of PE was first filtered by a membrane with a pore size of around 100 mm, followed by centrifuging at 500 g for 5 min to separate cell pellets.
c. 1 ml of red blood cell lysing buffer (BD) was then added to lyse red blood cells for 5 min, followed by centrifuging at 500 g for 5 min.
d. The nucleated cell pellet was re-suspended and washed with HBSS.
e. After cell counting ~500,000 cells were obtained (Tang et al., 2017).

Reference

Tang, Y., Wang, Z., Li, Z., Kim, J., Deng, Y., Li, Y., Heath, J. R., Wei, W., Lu, S., & Shi, Q. (2017). High-throughput screening of rare metabolically active tumor cells in pleural effusion and peripheral blood of lung cancer patients. *Proceedings of the National Academy of Sciences, 114* (10), 2544–2549.

3.2.3 Human Ascites

Method: Density Gradient Centrifugation

a. Up to 1L of ascites was collected in sterile culture flasks.
b. After centrifuging ascites for 10 min at 2,000 rpm, the cell pellet was resuspended in an appropriate volume of ascites supernatant
c. Ascites Mononuclear Cells were separated by density gradient centrifugation using Lympholyte (Cedarlane) or Ficoll-Paque (Biochrom AG, Berlin, Germany).
d. Ascites Mononuclear Cells were washed twice in PBS, counted and used immediately (Lutz et al., 2019).

Reference

Lutz, P., Jeffery, H. C., Jones, N., Birtwistle, J., Kramer, B., Nattermann, J., Spengler, U., Strassburg, C. P., Adams, D. H., & Oo, Y. H. (2019). NK Cells in Ascites From Liver Disease Patients Display a Particular Phenotype and Take Part in Antibacterial Immune Response. *Frontiers in immunology, 10,* 1838.

3.2.4 Human Colorectal Tumor

Method: MACS Tumor Dissociation Kit

ⓐ Tumors and adjacent normal tissues were cut into approximately 1-mm3 pieces in the RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum (FBS; Sciencell)

ⓑ Enzymatically digested with MACS Tumor Dissociation Kit (Miltenyi Biotec) for 30 min on a rotor at 37 °C, according to the manufacturer's instruction.

ⓒ The dissociated cells were subsequently passed through a 40-μm cell-strainer (BD) and centrifuged at 400g for 10 min

ⓓ After the supernatant was removed, the pelleted cells were suspended in Red Blood Cell Lysis Buffer (Solarbio) and incubated on ice for 2 min to lyse red blood cells

ⓔ After washing twice with PBS (Invitrogen), the cell pellets were re-suspended in sorting buffer (PBS supplemented with 1% FBS) (Zhang et al., 2018).

Reference

Zhang, L., Yu, X., Zheng, L., Zhang, Y., Li, Y., Fang, Q., Gao, R., Kang, B., Zhang, Q., & Huang, J. Y. (2018). Lineage tracking reveals dynamic relationships of T cells in colorectal cancer. *Nature, 564* (7735), 268–272.

3.2.5 Human Brain

Method: Worthington Papain Dissociation System kit

ⓐ Individual brain organoids were dissociated into a single-cell suspension using the Worthington Papain Dissociation System kit (Worthington Biochemical). A detailed description of the dissociation protocol is available at Protocol Exchange33.

ⓑ Dissociated cells were resuspended in ice-cold PBS containing 0.04% BSA (Sigma) at a concentration of 1,000 cells/μl (Velasco et al., 2019).

Reference

Velasco, S., Kedaigle, A. J., Simmons, S. K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., & Regev, A. (2019). Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature, 570*(7762), 523–527.

3.2.6 Human Kidney

Method: Miltenyi Kit

a Kidney biopsies were taken by pathologists from normal and tumor regions. Where clinically permissible, separate cortical, medullary, pelvic, and ureteric biopsies were obtained. Or a biopsy from the interface of medulla and cortex was taken.

b Tissues were sliced into approximately 30mm3 pieces of tissue and digested for 30 min at 37°C with agitation in a digestion solution containing 25μg/ml Liberase™ TM(Roche) and 50μg/ml DNase (Sigma) in RPMI (Gibco™).

c Following incubation samples were transferred to a C tube (Miltenyi Biotec) and processed on a gentle MACS (Miltenyi Biotec) on programme spleen 4 and subsequently lung 2.

d The resulting suspension was passed through a 70μm cells strainer (Falcon), washed with PBS and live cells enriched using a Dead Cell Removal kit (Miltenyi Biotec) as per manufactuers' instructions.

e Enriched live cells were washed and counted using a hemocytometer with trypan blue. For fetal samples, whole kidneys were dissociated into single cell suspension following rapid 30 min collagenase treatment. Live, single cells were enriched for FACS-sorted DAPI- cell with further enrichment of immune cells by CD45 expression. Overall it took 5-6 hours from obtaining biopsies to generate single cell suspensions run on the Chromium 10X device (Young et al., 2018).

Reference

Young, M. D., Mitchell, T. J., Vieira Braga, F. A., Tran, M. G., Stewart, B. J., Ferdinand, J. R., Collord, G., Botting, R. A., Popescu, D.-M., & Loudon, K. W. (2018). Single-cell transcriptomes from human kidneys reveal the cellular identity of renal tumors. *science, 361*(6402), 594–599.

4 Novogene Preservation and Shipping Instruction

4.1 Human & Animal Tissue Samples

4.1.1 Sending in snap/fresh-frozen

Using freshly collected samples is preferred. Quickly cut tissues into smaller pieces (50~100 mg) on ice. Please do NOT rinse tissues prior to freezing. Freeze the tissue pieces by directly submerging them for ~1min in isopentane in a liquid nitrogen bath. THEN transfer the frozen tissue to pre-chilled RNase-free 1.5 mL/2.0 ml EP tubes or freezing tubes with a screw cap. Seal tubes with sealing film and store in liquid nitrogen or at -80°C immediately. To ensure RNA preservation, it is recommended to complete this step within 3 minutes upon tissue removal from a living body. Aliquot and store the sample in several tubes to avoid degradation due to repeated freezing and thawing. For long term storage (>2 days), it is strongly recommended to store tissue samples in liquid nitrogen to avoid degradation. If liquid nitrogen storage is not available, store sample at -80C or colder.

4.1.2 Sending in fresh

If sending fresh organ or tissue samples without snap-freezing, please use a commercial RNA protection reagent (we recommend MACS® Tissue Storage Solution) and follow the corresponding manufacturer's recommendations. Using freshly collected samples is preferred. It is recommended to rinse freshly extracted tissue sample from living animals immediately with precooled RNase-free water, removing blood, unwanted tissues (such as hair and connective tissue), and other contaminants. Ship with ice packs for fresh tissues.

4.1.3 Shipping Instructions

For tissues, aliquot in amounts of at least 50mg. Although only one aliquot is required, it is recommended to send a second aliquot as a backup.
Place tubes into a 50 ml conical tube. Print the shipping form and fill it out. Place form in a sealed plastic bag and include it in the shipment container.

- Ship the materials in a polystyrene box (with at least a 1" thick walls and minimal inside dimensions of 8" x 6" x4"). 5LB dry ice per day for frozen tissues. / Sufficient **ice packs for** fresh tissues stored in Tissue Storage Solution.
- Apply Dry Ice labels on outside box for frozen tissues.
- Send the package by next-day delivery service (FedEx Priority Overnight or UPS Next Day Air). Email *Technical Support* the tracking number and the shipment delivery date. We recommend packages shipped on Monday or Tuesday, avoiding shipments arriving on Saturdays, Sundays, or national holidays.

Note: Please inform ***Technical Support*** **one week ahead of the shipping**, which is required for scheduling an effective checking in for your samples.

4.2 Human & Animal Single Cell Samples

We can accept single cell suspensions with enough cells from cultured cell lines, cell sorting platforms like Fluorescence-activated Cell Sorting (FACS), dissociated tissues or other cell isolation methods. The samples need to be frozen properly prior to shipping with dry ice.

4.2.1 Freezing Cells (recommended method)

ⓐ Grow enough cells to seed/maintain a backup culture at your facility after removing 3 million viable cells for shipment. Flasks may be pooled if necessary/ Aspirate enough cell suspension (3 million cells) dissociated from animal tissues or sorted by cell sorting platforms.

ⓑ Calculate total number of cells; pipette first to gently break up cell aggregates.

ⓒ Centrifuge for 10 min at 120 × g (860 rpm in GH 3.8A rotor) at 4°C.

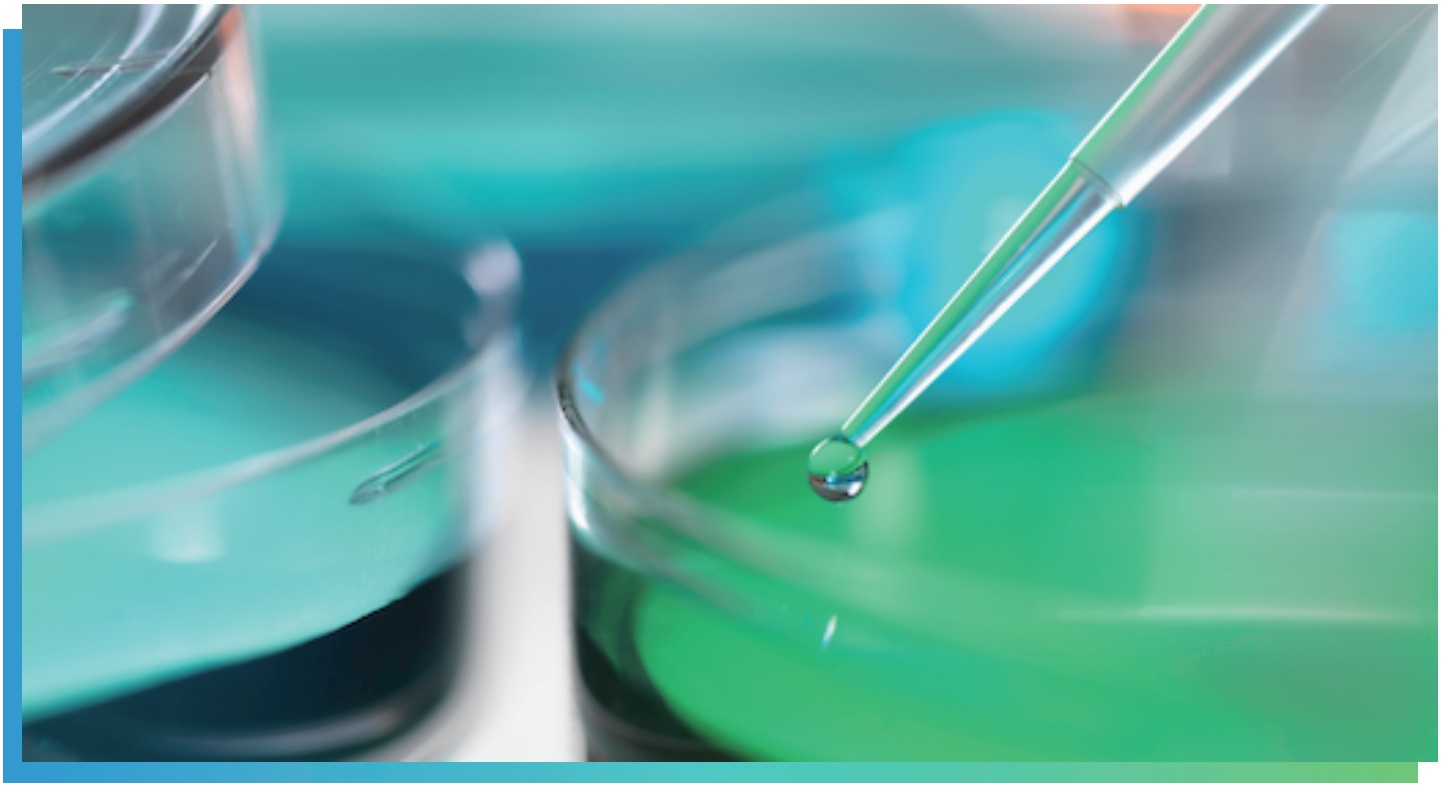
ⓓ Resuspend pellet in freezing media at 1.5x 106 cells/ml. Example Freezing media: RPMI 1640, 20% FBS, and 6% DMSO

ⓔ Make 1 ml aliquots in cryovials.

ⓕ Freeze overnight at -1°C/min in -80°C freezer using freezing chamber containing fresh isopropanol.

ⓖ Transfer cryovials to liquid nitrogen tank for storage.

Note: It is highly recommended to count cells before freezing, using a hemocytometer or other devices.



4.2.2 Shipping Instructions

- For frozen cells, aliquot in amounts of at least 1.5 million cells. Although only one aliquot is required, it is recommended to send a second aliquot as a backup.
- Place cryovials inside a 50 ml conical tube. Print the shipping form and fill it out. Place the form in a sealed plastic bag and include it in the shipment container.
- Ship the materials in a polystyrene box (with at least a 1" thick walls and minimal inside dimensions of 8" x 6" x4"), 5LB dry ice per day.
- Apply Dry Ice labels on the outside box.
- Send the package by next-day delivery service (FedEx Priority Overnight or UPS Next Day Air). Email Technical Support the tracking number and the shipment delivery date. We recommend packages shipped on Monday or Tuesday, avoiding shipments arriving on Saturdays, Sundays, or national holidays.

Note: Please inform ***Technical Support*** **one week ahead of the shipping**, which is required for scheduling an effective checking in for your samples.

Novogene Corporation Inc.

2921 Stockton Blvd. Suite 1810, Sacramento CA 95817

Follow us on LinkedIn

☎ 916-252-0068-383 inquiry_us@novogene.com www.novogene.com

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