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Multi tissue processing for single cell sequencing of human immune cells

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1

dx.doi.org/10.17504/protocols.io.bz4qp8vw

Cambridge University

dbr dbr

This protocol has been developed for the simultaneous processing of multiple human tissues to extract immune cells for single cell RNA sequencing using the 10X platform, and ideal for atlasing projects. Included in this protocol are the steps needed to go from tissue to loading the 10X Chromium for single cell RNA sequencing and includes the hashtag and CiteSeq labelling of cells as well as the details needed to stimulate cells with PMA+I.

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DOI

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protocol

The gut processing protocol has been taken from: James, K.R., Gomes, T., Elmentaite, R. et al. Distinct microbial and immune niches of the human colon. Nat Immunol 21, 343–353 (2020). https://doi.org/10.1038/s41590-020-0602- The skin processing protocol has been taken from: Human skin single cell dissociation on Protocols.io https://www.protocols.io/view/human-skin-single-cell-dissociation-ripd4dn

Human, tissue, single cell RNA sequencing, Atlas

protocol ,

Nov 16, 2021

Nov 23, 2021

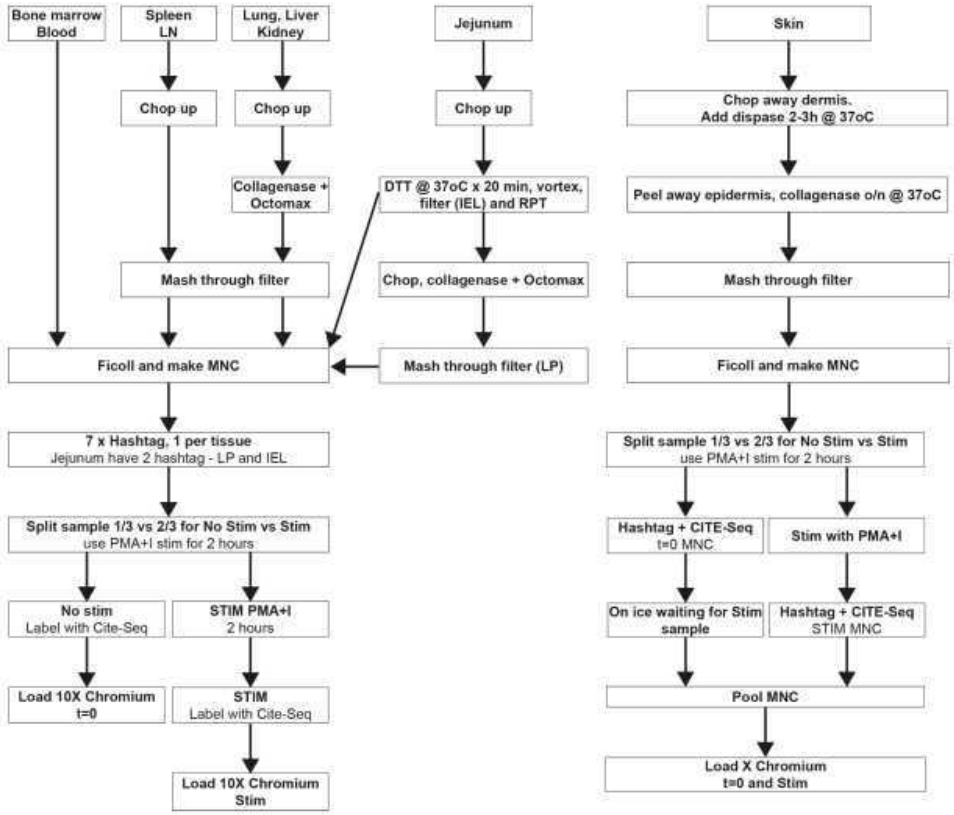
Nov 16, 2021 renuka.s

Nov 22, 2021 dbr

55152

This protocol has been optimised for extracting immune cells from small pieces of tissue (around 10g or less) and has divided tissues into five categories depending on how much mechanical or chemical digestion is needed to enable extraction of immune cells, as shown in the workflow diagram. Blood and bone marrow need no processing. Lymphoid tissues like spleen and lymph nodes require a gentle mashing to make a cell suspension. Non-lymphoid tissues like lung, liver and kidney require both a mechanical and collagenase digestion. The gut and skin require more specialised protocols and use published protocols from the James and Hanifa laboratories.
We include an activation step using PMA+I for 2 hours, however this stimulation condition will need optimising depending on the hypothesis being tested.

Workflow



Reagent

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

X-VIVOTM 15 Serum-free Hematopoietic Cell Medium **Lonza Catalog #BE02-060Q**

Fetal Bovine Serum **Merck Millipore Catalog #F7524**

Dulbecco's Phosphate Buffered Saline **Merck Millipore Catalog #D8537-500ML**

Gibco™ Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Scientific Catalog #15260037**

UltraPure 0.5M EDTA, pH 8.0 **Thermo Fisher Scientific Catalog #15575-038**

Roche DTT 14-Dithiothreitol **Sigma Aldrich Catalog #10197777001**

USB Dithiothreitol (DTT) 0.1M Solution **Thermo Fisher Scientific Catalog #707265ML**

Collagenase IV, Merck, C7926-100MG

Dispase® II protease **Sigma Aldrich Catalog #D4693-1G**

Benzonase® Nuclease Purity > 90% **Merck Millipore Catalog #70746-4**

Ficoll Paque Plus 500mL **Ge Healthcare Catalog #17-1440-03**

eBioscience™ Cell Stimulation Cocktail (500X) **Thermo Fisher Catalog #00-4970-93**

Hashtags, Biolegend, Total-C

TotalSeq™-C Human Universal Cocktail V1.0 **BioLegend Catalog #399905**

Solutions to make:

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

A	B	C	D
Solution	Base	Reagent 1	Reagent 2
X-vivo + 1% FBS	49.5 ml x-vivo	0.5 ml FBS	2.5 µl Benzonase
PBS + 0.04% BSA	500 ml PBS	2.66 ml	7.5% BSA
X-vivo +5 mM EDTA + 2 mM DTT + 1% FBS	48.5 ml x-vivo + 1% FBS	0.5 ml of 0.5M EDTA	1 ml of 100 mM DTT

Flowmi® Cell Strainers
Cell Strainers

Flowmi® **BAH136800040**

3ml Syringe, disposable, sterile,
3ml Syringe, disposable, sterile,

Terumo **GS574**

70 µm Cell Strainer
Cell Strainer

Falcon **352350**

White, Sterile, Individually Packaged

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gentleMACS™ C Tubes
C Tubes
gentleMACS™ 130-093-237

Equipment
In addition to the regular equipment found in a Containment level 2 laboratory you will need:

- Miltenyi Gentlemacs
- **37 °C** incubator
- 10X Chromium instrument

Citations
The gut processing protocol has been taken from:

James, K.R., Gomes, T., Elmentaite, R. et al. (2020). Distinct microbial and immune niches of the human colon. Nature Immunology. http://10.1038/s41590-020-0602

The skin processing protocol has been taken from:
Human skin single cell dissociation on Protocols.io https://www.protocols.io/view/human-skin-single-cell-dissociation-ripd4dn

Tissue to cell suspension - Bone Marrow and Blood

1 No processing, go straight to ficoll layering.

2 Place **On ice** until other tissues have caught up.

Tissue to cell suspension - Lymphoid Tissues (Spleen, Lymph node)

3 Mash the lymphoid tissue through a **70 µm** filter placed on top of a **50 mL** falcon, using the plunger from a **2 mL** syringe as a pestle.

4

Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

5 Depending on the size of the tissue, top up the filtered cell suspension to **30 mL** to **50 mL** with x-vivo + 1% FBS.

6 Place **On ice** until other tissues have caught up.

Tissue to cell suspension - Non-lymphoid tissue (Lung, Liver, Kidney) 32m 22s

7 We receive around **5 g** of tissue and the protocol will need to be scaled up if more tissue is being processed.

> Do not overload the Gentlemacs as this will reduce cell yield.

8 Chop up the tissue with scissors into **0.5 cm** pieces.

9 Do not overload the Gentlemacs C-tube, with no more than **2.5 g** of tissue.

10 

Transfer to Gentlemacs tube and add **2.5 mL** of collagenase and **2.5 mL** x-vivo.

11 Run the following programme that takes **00:32:00** . 32m

11.1 Loop .
- Loop .(1/3)
- Loop. (2/3)
- Loop. (3/3)

11.2  12s

Ramp **900 rpm** **00:00:12** .

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

11.3 1s

Spin **700 rpm, 00:00:01** .

11.4 8s

Ramp **1000 rpm, 00:00:08** .

11.5 1s

Spin **1500 rpm, 00:00:01** .

11.6 4s

Spin **1900 rpm, 00:00:04** .

11.7 1s

Spin **1500 rpm, 00:00:01** .

11.8 3s

Spin **1900 rpm, 00:00:03** .

11.9 Temperature on **37 °C** and loop.

- loop (1/2).
- loop (2/2).

11.10 15m

Spin **50 rpm, 00:15:00** .

11.11 20s

Spin **350 rpm, 00:00:20** .

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12 

Add **20 µL** of **0.5 millimolar (mM)** EDTA (**2 millimolar (mM)** final conc.) per **5 mL** of collagenase to neutralise and shake to mix.

13 Pour and scrape digested tissue into a **70 µm** cell strainer placed on top of a **50 mL** falcon.

14 Use the plunger of a **2 mL** syringe to mash tissue through the filter, like a pestle.

15 

Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.

16 Depending on the size of the tissue, top up the filtered cell suspension to **30 mL** to **50 mL** with x-vivo + 1% FBS.

17 Place **On ice** until other tissues have caught up.

Tissue to cell suspension - Jejunum 1h 32m

18 

Protocol adapted from from Kylie James “Distinct microbial and immune niches of the human colon”, Nature Immunology, 2020. We receive around 5g of tissue and the protocol will need to be scaled up if more tissue is being processed. Do not overload the digestion steps as this will reduce cell yield.

Wash jejunum with PBS + 0.04% BSA to remove any chime.

19 Chop up the jejunum with scissors into **0.5 cm** pieces.

 30m

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

20

Transfer to a **50 mL** falcon tube and add **10 mL** of x-vivo + **2 millimolar (mM)** DTT + **5 millimolar (mM)** EDTA + 1% FBS and put in the **37 °C** incubator for **00:20:00** and shake after **00:10:00** .

21

Put jejunum chemical digest through a **70 µm** filter on top of a **50 mL** falcon and rinse with **10 mL** of x-vivo + 1% FCS.

22

The wash through from the filter contains the IEL cells, keeping the falcon **On ice** .

23 Scrape tissue from the filter back into a **50 mL** falcon and repeat the digest with **10 mL** x-vivo + **2 millimolar (mM)** DTT + **5 millimolar (mM)** EDTA + 1% FBS and place back in the **37 °C** incubator for **00:20:00** , and shake after **00:10:00** .
30m

24

Put jejunum digest through a **70 µm** filter on top of the **50 mL** falcon containing the IEL cells and rinse with **10 mL** of x-vivo + 1% FCS. Keep the IEL cells **On ice** .

25 Scrape tissue from the filter into a Gentlemacs C tube and digest with **2.5 mL** of collagenase and **2.5 mL** of x-vivo and run the programme called 'Sarah' takes **00:32:00** , with various mixing speeds.
32m

26

Add **20 µL** of **0.5 millimolar (mM)** EDTA (**2 millimolar (mM)** final conc) per **5 mL** of collagenase to neutralise and shake to mix.

27 Pour and scrape digested tissue into a **70 µm** cell strainer placed on top of a **50 mL** falcon.

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28 Use the plunger of a **2 mL** syringe to mash tissue through the filter, like a pestle.

29 

Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue, cells that pass through the filter are **LP cells**.

30 Depending on the size of the tissue, top up the filtered cell suspension to **30 mL** to **50 mL** with x-vivo + 1% FBS.

31 Place **On ice** until other tissues have caught up.

Tissue to cell suspension - Skin 1h 32m

32 **Protocol from Haniffa Lab, Newcastle University https://www.protocols.io/view/human-skin-single-cell-dissociation-ripd4dn**

Depending on when tissues arrive, skin can either be set up the night before and then will be processed with all the other tissues. Or will have to be processed the next day.

- If processed the same day as other tissues then will be hashtagged with all other tissues.
- If processed the next day will have to follow the same procedure but the unstim will wait on ice until the stim catches up and can be loaded on 1 lane of 10x.
  - **5 µL** of CITE-Seq will need to be left from the processing of the other tissues.

Chop into ~ **0.5 cm2** sized pieces. Remove as much dermis from each as possible using a razor blade - be careful, extremely sharp. Discard the dermis layer.

33  5h

Incubate the retained skin in dispase for **02:00:00** to **03:00:00** at **37 °C** , to allow the epidermis to be stripped.

34 Separate the epidermis from the dermis using fine forceps. These can be kept separate or processed

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together.

35

Wash in PBS.

36 3h

Add collagenase at 3X the volume of the tissue and incubate at **37 °C** **Overnight** .

37

Add **20 µL** of **0.5 millimolar (mM)** EDTA (**2 millimolar (mM)** final conc) per **5 mL** of collagenase to neutralise and shake to mix.

38

Scrape the digested skin and media into a **70 µm** filter on top of a **50 mL** falcon.

39 Use the plunger from a **2 mL** syringe to mash the skin through the filter, like a pestle.

40

Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.

41 Depending on the size of the tissue, top up the filtered cell suspension to **30 mL** to **50 mL** with x-vivo + 1% FBS.

42 Place **On ice** until other tissues have caught up, or if processing the next day alone proceed with cell count and ficoll.

Cell suspension to MNC - Wash cell suspension 10m

43 10m

Once all the tissues have reached a cell suspension, spin at **600 x g, 00:10:00** .

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44 Pour off supernatant and resuspend in x-vivo + 1% FBS, the volume to resuspend depends what you are going to layer over ficoll.

45 There is no exact science to the layering but as a guide:

a. Spleen - **90 mL**

b. Lymph nodes - **7 mL**

c. Non-lymphoid tissue - up to **60 mL**

d. Skin - **7 mL**

Cell suspension to MNC - Ficoll 1h 5m

46

Number of ficoll tubes to be used depends on the size of the tissue and the cell pellet.

Bone marrow **10 mL** + **20 mL** x-vivo layer on **15 mL** ficoll per **50 mL** falcon.

47 Blood up to **15 mL** + **15 mL** x-vivo layer on **15 mL** ficoll per **50 mL** falcon.

48 Spleen **30 mL** cells suspension over **15 mL** ficoll per **50 mL** falcon x3.

49 Lymph nodes **7 mL** cells suspension over **8 mL** ficoll per **15 mL** falcon.

50 Non-lymphoid tissue depending on the size of the cell pellet up to **30 mL** cell suspension over **5 mL** ficoll x2.

51 Skin **7 mL** cells suspension over **8 mL** ficoll per **15 mL** falcon.

52 1h 5m

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Spin tubes at **400 x g, 00:25:00** with slow deceleration. Takes around **00:40:00** to run.

Cell suspension to MNC - CD66b and RBC depletion 16m 30s

53

1. It is good to remove granulocytes (expressing CD66b) and RBC from each sample where required as these cells do not provide useful single cell sequencing information.
2. Use Stem Cell CD66b positive selection kit (17882) to remove granulocytes from each sample and Stemcell RBC depletion reagent (18170).

In a **15 mL** falcon, add from **0.5 mL** to **3 mL** of sample (up to 5 million cells).

54 3m

Add **25 µL** of CD66b positive selection cocktail, mix and incubate for **00:03:00** at **Room temperature** .

55 30s

Vortex RapidSpheres for **00:00:30** .

56 3m

Add **25 µL** of RapidSpheres, mix and incubate for **00:03:00** at **Room temperature** .

57

Add **25 µL** of RBC depletion reagent per **1 mL** of sample and mix.

58 5m

Immediately place the samples on a magnet for **00:05:00** .

59

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Carefully pipette off the supernatant to a fresh tube and place **On ice** .

This contains the cells you want, DO NOT discard.

60 5m

Wash the beads with **5 mL** of PBS 1% FBS + **1 millimolar (mM)** EDTA and place back on the magnet for **00:05:00** .

61 Collect supernatant and add to the fresh tube in **step 60**.

62 Throw away the leftover tube with beads as this contains the granulocytes and RBC.

Cell suspension to MNC - Count Cells 16m 30s

63 Count cells from each tissue after ficoll (and CD66b / RBC depletion).

64

Make sure cells are well mixed and count with trypan blue. If count all 25 squares of the haemocytometer, then:
Cell count x dilution factor x volume x 10,000 = Total cell count.

Hashtag, CITE-Seq and stimulation - Hashtag 1h 5m

65 Take at least 500k MNC per tissue (use 750k to 1 million cells if available) into a **1.5 mL** lo-bind eppendorf.

66 5m

Spin cells at **600 x g, 00:05:00** , remove as much supernatant as possible and resuspend in **50 µL** PBS+0.04% BSA.

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67 Record which hashtag is used for which tissue.

68  10m

Add **5 µL** FC block and incubate at **4 °C** for **00:10:00** .

69  10m

Spin each hashtag at **14000 x g, 00:10:00** .

70 

Add **0.5 µL** of hashtag to each tube.

71  30m

Incubate at **4 °C** for **00:30:00** .

72 **Make up lyophilised CITE-Seq antibodies** (see - **section CITE-Seq section**)

73  5m

Top up to **500 µL** with PBS + 0.04% BSA, and spin at **600 x g, 00:05:00** , and remove supernatant.

74  5m

Wash cells with **500 µL** with PBS + 0.04% BSA, and spin at **600 x g, 00:05:00** , and remove supernatant.

75 

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Resuspend in **100 µL** of PBS + 0.04% BSA.

Hashtag, CITE-Seq and stimulation - Count cells 1h 5m

76 Count cells from each tissue after the Hashtag washes as there will be cell loss, and if a particular tissue has fewer cells than needed, then repeat the hashtag process with more cells.

77 Make sure cells are well mixed and count with trypan blue. If count all 25 squares of the haemocytometer, then:
Cell count x dilution factor x volume x 10,000 = Total cell count.

Hashtag, CITE-Seq and stimulation - Pool MNC from all tissues and split for Unstim and Stim (if required) 5m

78 Use the post hashtag cell counts to pool MNC from each tissue at equal cell number, based on what the lowest count is, into a **1.5 mL** lo-bind eppendorf.

79 Ideally you want 300k - 400k from each tissue. Record the total volume.

80 

Flick to mix the cells really well.

81 Remove ⅓ of the cell volume to a new **1.5 mL** tube and label as Unstim and top up to **500 µL** with PBS + 0.04% BSA. Spin at **600 x g, 00:05:00** and proceed to the **CITE-Seq section**. 5m

82 To the remaining ⅔ of pooled MNC, label the tube as Stim and top up to **1 mL** with x-vivo + 1% FCS and proceed to **MNC stimulation**.

Hashtag, CITE-Seq and stimulation - MNC Stimulation with PMA+I 3h 10m

83  2h

- We are using a PMA+I stimulation which we have optimised to assess early activation events,

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and depending on the hypothesis being tested may need to be a different stimulant and time point.

Get the MNC stim on as it takes **02:00:00** .

84 Pool culture in MNC in **1 mL** of x-vivo + 1% FBS for **02:00:00** at **37 °C** with **2 µL** of cell stim cocktail (PMA+I). Flick tube to mix cells every **00:30:00** to **00:40:00** . 3h 10m

85 Culture MNC at no more than 2 million cells per ml.

- Use more than one **1.5 mL** tube if needed.
- Cell stim cocktail (PMA+I, eBioscience) is 1:500 stock.

86  2h

Incubate for **02:00:00** , move to Cite-Seq of stimulated cells.

Hashtag, CITE-Seq and stimulation - CITE-Seq 3h 10m

87 

Make up lyophilized CITE-Seq antibodies - each vial is enough for 500k cells, but will use 1 vial for up to 2 million cells.

87.1 Spin lyophilised reagent at **10000 x g, 00:00:30** . 30s

87.2 

Add **27.5 µL** Cell staining buffer to the lyophilised CITE-Seq reagent and briefly vortex.

87.3  5m

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Incubate at **Room temperature** for **00:05:00** .

87.4 30s

Briefly vortex again, then spin at **10000 x g, 00:00:30** .

87.5 Transfer entire volume to a lo-bind PROTEIN tube.

87.6 10m

Spin at **14000 x g, 4°C, 00:10:00** .

87.7 Store in the fridge until ready to use.

88 5m

Spin the unstim pool MNC at **600 x g, 00:05:00** and remove supernatant.

89

Resuspend cells in **50 µL** of PBS + 0.04% BSA.

90

No need to add FC block, as already done at hashtag stage.

90.1 10m

If not hashtagged already, then add **5 µL** FC block for **00:10:00** at **4 °C** .

91 30m

Add **10 µL** of CITE-Seq 130Ab and incubate at **4 °C** for **00:30:00** .

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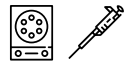
Keep the remaining CITE-Seq reagent for the stimulated sample and the skin if processed the next day.

92 30m

(Take 10x reagent out of the freezer to warm up to Room temperature , during CITE-Seq incubation. It takes 00:30:00 to warm up to Room temperature .)

93 5m



Top up to **500 µL** with PBS + 0.04% BSA, and spin at **600 x g, 00:05:00** , and remove supernatant.

94 5m

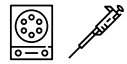
Wash cells with **500 µL** with PBS + 0.04% BSA, and spin at **600 x g, 00:05:00** , and remove supernatant.

95

Resuspend cells in **250 µL** PBS + 0.04% BSA and put through a flowmi filter. Rinse out **1.5 mL** tube with **250 µL** PBS + 0.04% BSA, and put this through the same Flowmi filter.

TIP - use a second **1 mL** pipette tip, so can keep Flowmi filter on original tip, remove from pipette and pipette second **250 µL** wash into the top of the tip with the filter. Reattach the pipette and wash through the filter.

96 5m



Spin at **600 x g, 00:05:00** , and remove supernatant.

97 Resuspend in **100 µL** of PBS + 0.04% BSA.

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Hashtag, CITE-Seq and stimulation - Count cells 3h 10m

98 Count the unstim pooedl MNC sample.

99 Make sure cells are well mixed and count with trypan blue.

100 **2 µL** of cells to **8 µL** of Trypan blue. If count all 25 squares of the haemocytometer, then: Cell count x 5 x 0.1 x 10,000 = Total cell count.

Hashtag, CITE-Seq and stimulation - Load unstim for 10x

101 Load cells at 1,000 cells per **1 µL** (Max 2,000 cells / µl).

102 Dilute the sample (if needed).

103 Load 15,000 cells per tissue, 30,000 cells per 10x GEM reaction.

> So for 6 tissues, it would be 90,000 cells over 3 10x GEMs.

Hashtag, CITE-Seq and stimulation - Cite-Seq of stimulated cells 3h 20m

104 2h 5m

After the **02:00:00** stimulation, spin the stim pool MNC at **600 x g, 00:05:00** and remove supernatant.

105

Resuspend cells in **15 µL** of PBS + 0.04% BSA.

> No need to add FC block as already done at hashtag stage.

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106 30m

Add **12.5 µL** of CITE-Seq 130Ab and incubate at **4 °C** for **00:30:00** .

107 30m

(**Take 10x reagent out of the freezer to warm up to Room temperature , during CITE-Seq incubation. It takes 00:30:00 to warm up to Room temperature** .)

108 5m

Top up to **500 µL** with PBS + 0.04% BSA, and spin at **600 x g, 00:05:00** , and remove supernatant.

109 5m

Wash cells with **500 µL** with PBS + 0.04% BSA, and spin at **600 rpm, 00:05:00** , and remove supernatant.

110

Resuspend cells in **250 µL** PBS + 0.04% BSA and put through a flowmi filter. Rinse out **1.5 mL** tube with **250 µL** PBS + 0.04% BSA, and put this through the same Flowmi filter.

TIP - use a second 1ml pipette tip, so you can keep Flowmi filter on the original tip, remove from pipette and pipette second **250 µL** wash into the top of the tip with the filter. Reattach the pipette and wash through the filter.

111 5m

Spin at **600 x g, 00:05:00** , and remove supernatant.

112

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Resuspend in **100 µL** of PBS + 0.04% BSA.

Hashtag, CITE-Seq and stimulation - Count cells

113 Count the Stim pooled MNC sample.

114 

Make sure cells are well mixed and count with trypan blue.

115 **2 µL** of cells to **8 µL** of Trypan blue. If count all 25 squares of the haemocytometer, then: Cell count x 5 x 0.1 x 10,000 = Total cell count.

Hashtag, CITE-Seq and stimulation - Load stim for 10x

116 Load cells at 1,000 cells per **1 µL** (Max 2,000 cells / µl).

117 Dilute the sample (if needed) in PBS + 0.04% BSA.

118 Load 15,000 cells per tissue, 30,000 cells per 10x GEM reaction.

> So for 6 tissues, it would be 90,000 cells over 3 10x GEMs.

Flow, Freezing and RNA from remaining cells - Remaining cells put in RLT 5m

119 When all the 10x GEMs have been processed and they look good, pellet any leftover pooled unstim or stim MNC at **600 rpm, 00:05:00** and take off supernatant. 5m

120 Flick to resuspend dry pellet and resuspend in **350 µL** of Qiagen RLT buffer.

121 

Quickly vortex and freeze at **-80 °C** until ready to extract the RNA.

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

Flow, Freezing and RNA from remaining cells - Flow cytometry

122 Run a flow panel to QC the sample and get proportions of the major cell types. Stain ~500k per tissue with the desired panel of antibodies.

This is an example flow cytometry panel, however may need to be adjusted depending on the flow cytometer available:
a. CD3 - Percp Cy5.5
b. CD19 - APC
c. CD56 - PE 10
d. CD4 - PE Cy7
e. CD14 - FITC
f. CD16 - BV421
g. CD8-APCCy7

123 Fix cells and store at **4 °C** until they can be analysed.

Flow, Freezing and RNA from remaining cells - Freeze down excess cells 35m

124  5m

Any cells that are not going for 10x or flow cytometry can be frozen down.

Spin cells at **600 x g, 00:05:00** , and remove as much supernatant as possible.

125 Flick to resuspend cell pellet.

126 

Add cell freezing media dropwise, until ~ 10 million cells per ml.

127 

Flick to mix, and transfer to labelled NUNC tubes.

Citation: Daniel Rainbow, Sarah Howlett, Lorna Jarvis, Joanne Jones Multi tissue processing for single cell sequencing of human immune cells https://dx.doi.org/10.17504/protocols.io.bz4qp8vw

128 30m

Put NUNC tubes in a Mr Frosty and store at **-80 °C** **Overnight** .

129 Next day, transfer to LN2 storage.

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