Cite seq and hashing preparation protocol

This protocol is a close adaptation from the original cite seq authors and can be found at:

<https://citeseq.files.wordpress.com/2019/02/cite-seq_and_hashing_protocol_190213.pdf>

The cite where the link is provided can be found here:

<https://cite-seq.com/protocols/>

Another source of information that has been used is the Biolegend protocol for the CiteSeq C T/B/NK antibody kit:

https://www.biolegend.com/en-us/global-elements/pdf-popup/totalseq-c-human-tbnk-cocktail-19094?filename=TotalSeq-C Human TBNK Cocktail.pdf&pdfgen=true

**Major errors** in reagents: the TotalSeq C T/B/NK kit had been frozen when it should be stored at 4-8°C.

Minor changes:

* the hashing solution is provided in 10 uL PBS per donor and is thus used as a part of the base for resuspension of the cite seq antibody mix.
* No tween was included. In the original protocol, there is a 0.01% tween addition to the staining buffer. This was removed, as it is not deemed necessary by the original authors, and as it due to its high viscosity and toxicity could bring in unwanted variability into the experiment.
* FCS was used instead of BSA in the staining buffer, as the latter could not be obtained in a sterile format in the laboratory.

*Material:*

Frozen cells from 8 myasthenia patients

RPMI+8% FCS (thawing medium)

PBS+2%FCS (staining buffer)

Sterile U-bottom 96-well polystyrene plate

15 ml falcon tubes

50 ml falcon tubes

40 uM strainers

Sterile capped 4 ml polypropylene BD Falcon FACS tubes

1.5 ml autoclaved Eppendorf tubes

Cell strainer, 40 uM.

Biolegend Truestain FcX solution

*Procedure:*

1. Pre-warm thawing medium to 37°C.
2. Thaw all eight samples in waterbath.
3. Carefully add 1 ml to each tube drop by drop while sample is being shaken by a XXX (cannot find the word!) that has been placed in the hood.
4. When all four has gotten 1 ml, repeat, until each tube contains 5 ml of fluid.
5. Add 9 ml thawing buffer.
6. Spin samples at 350 G for 8 minutes at a temperature falling from 22 to 4°C during the spin.
7. Decant supernatant.
8. Add 1 ml staining buffer and resuspend cells.
9. Add 13 staining buffer
10. Spin samples at 350 G for 8 minutes
11. While spinning, count the cells.
12. Remove the supernatant and adjust the concentration with staining buffer to reach 25 000 000 cells/ml.
13. Move 40 uL to a well in the U-bottom plate. NB! Make sure that the wells that are used are well separated. In this case, the cells were distributed so that they were not closer than 3 wells to any other cell.
14. Add 10 uL Fc block from BIoLegend.
15. Incubate for 10 minutes at 4°C
16. While incubating, remove cite-seq antibodies from fridge, and spin the T/B/NK cocktail in its lyophilized format for 30 seconds at 10000
17. Add 40 uL of staining buffer to each of the lyophilized antibody mixes.
18. Add one hashing antibody solution to each lyophilized vial, making sure to keep track of the label.
19. Add the hashing/totalseq mixture to the cells, the right one to each donor.
20. Incubate for 30 minutes at 4°C.
21. Spin for 3 minutes at 350 G.
22. Gently remove supernatant by tilting the plate and aspiring the fluid with a 200 uL pipette.
23. Resuspend in 200 uL staining buffer.
24. Spin for 3 minutes at 350 G.
25. Gently remove supernatant by tilting the plate and aspiring the fluid with a 200 uL pipette.
26. Resuspend in 200 uL staining buffer.
27. Spin for 3 minutes at 350 G.
28. Gently remove supernatant by tilting the plate and aspiring the fluid with a 200 uL pipette.
29. Resuspend in 200 uL staining buffer.
30. Put the solution through a 40 uM cell strainer set over a 50 ml Falcon tube.
31. Rinse the cell strainer with 2x200 uL staining buffer containing DAPI diluted 1:1000.
32. Move the solution from the 50 ml Falcon tube to a 4 ml polypropylene FACS tube.
33. Proceed to sorter.
34. Post sorting, spin the Eppendorf and remove the supernatant to a volume that corresponds to 2 million cells/ml, believing that the concentration on the sorter is correct.

**Discussion**

There was a small delay due to a sorter booking mistake, but after this was solved, and the whole experiment instead sorted on the BD Influx instead of the Sony SH800, it all looked well. The cells were in better shape than expected: even in the case of the worst donor from 2013, it was possible to sort 57% of all events, then excluding both debris and dead cells. The major issue was however the freezing of the TotalSeq antibodies. It is however likely that a large fraction of them should survive, and if so, and we can still use the CD4 and CD8 stainings, then life is good.