Protocol for totalseqB hashing labelling for cells

TotalSeq antibodies should be titrated to determine the optimal labelling concentration prior to use. To do so, see protocol at the end.

10XG protocol CG000426 (section 2.1) now allows to perform labelling in 96-well plates.

Hashing can be performed only with very high-quality samples. If nuclei/cells break after the hashing, the antibodies will be released in the resuspension buffer, contaminating the droplets of other intact cells/nuclei and compromising the results of the full experiment.

For **nuclei**, consult the specific protocol, which is in a separate document.

For **fragile cells**

- In case of fragile cells perform only 3 washes instead of 4.
- Recommended antibody incubation time is 10 min.

Preparation

1. Prepare necessary amount of **PBS, 10% FBS**. Keep it **on ice**. **Filter FBS before use**.
2. Prepare 50 ul of each **antibody mix** in **PBS, 10% FBS**, on **ice** and then store at 4°.
3. Use the **amount of antibody** based on previous titration. Perform an intermediary dilution of the antibody in PBS, 10% FBS if the volume to be added is lower than 0.5 ul (e.g. for adding 0.125ug of a 0.5ug/ul antibody stock, first do a 2x dilution of the stock - 0.5ul stock antibody + 0.5ul Wash and resuspension buffer - and use 0.5ul of that dilution.
4. Centrifuge antibody before adding to cells (see protocol below).

Labelling

5. Transfer cells (**0.5x10^6 cells** - 0.2-2 x 10^6 cells according to 10XG) to a new 5-ml tube
6. Add chilled PBS + 10% FBS for a total 1 ml volume.
7. Centrifuge cells at 4°C.
    a. *Optimization of centrifugation speed/time may be needed based on cell type. Larger or fragile cell types may require slower centrifugation speeds.*
    b. *Normally centrifugation is 150-400 rcf, 5-10 min.*
    c. *Use of swinging-bucket rotor is strongly recommended for higher cell recovery.*
8. Remove the supernatant without disturbing the pellet.
9. Resuspend cell pellet in 50 µl chilled PBS + 10% FBS.
10. Blocking
    a. Human cells -> add 5 µl Human TruStain FcX (total volume = 50 µL). Gently pipette mix and incubate for 10 min at 4°C.
    b. Mouse cells -> add 0.5 µL of TruStain FcX PLUS (anti-mouse CD16/32) blocking reagent (total volume = 50 µL). Gently pipette mix and incubate for 10 min at 4°C.
11. During blocking, centrifuge the TotalSeq antibodies at **14,000 g** at **4°C** for **10 minutes** and carefully pipette out the required antibody, avoiding the bottom of the tube. **This is critical to ensure the removal of protein aggregates**.
12. Add the TotalSeq antibody to the 50 µL blocked cell suspension. Total volume: 100 µl.
13. Gently pipette mix 10x (pipette set to 90 µl).

14. Incubate at 4°C for the amount of time tested during the titration (normally 10 or 30 min).
    a. *If using FACS antibodies, incubate without light exposure.*
    b. *Biolegend advises 30 min incubation. If successfully tested during titration, use shorter incubation time (e.g. 10min), especially for fragile cells.*
    c. *Recommended incubation temperature for most sample types is 4°C. However, incubation temperature is sample type dependent and should be chosen accordingly.*

Cell washing

Proceed to cell washing:

1. **Wash 1**: add 3.5 mL of chilled PBS, 10% FBS
2. Gently pipette mix.
3. Centrifuge at **4°C** for 5 minutes at 150-300 rcf depending on your sample type.
4. Thoroughly decant the wash buffer
5. **Wash 2**: resuspend the pellet in 3.5 mL of chilled PBS, 10% FBS.
6. **Transfer** to a new 5-ml tube (**important step to get rid of unbound antibody sticking to the sides of the original tube** - this transfer step reduces the unbound antibody in the cell suspension).
7. Centrifuge at **4°C** for 5 minutes at 150-300 rcf depending on your sample type.
8. Thoroughly decant the wash buffer.
9. **Wash 3:** resuspend the pellet in 3.5 mL of chilled PBS, 10% FBS.
10. Gently pipette mix.
11. Centrifuge at **4°C** for 5 minutes at 150-300 rcf depending on your sample type.
12. Thoroughly decant the wash buffer.
13. **Wash 4** (skip in case of **fragile cells**)**:** resuspend the pellet in 3.5 mL of chilled PBS, 10% FBS.
14. Gently pipette mix.
15. Centrifuge at **4°C** for 5 minutes at 150-300 rcf depending on your sample type.

Final resuspension

15. After the final wash, remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.
16. Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled PBS, 10% FBS to obtain the concentration required depending on the targeted cells.
17. Gently mix the cells by pipetting.
18. Slowly filter cells through 40 µm Flowmi Cell Strainer into a low protein binding microcentrifuge tube.

    Note: 40 µm Flowmi Cell Strainer may be too small for some sample types, use 70um strainer in that case.
19. Count cells and assess cell viability.
20. Dilute cells with chilled PBS, 10% FBS as necessary for appropriate input into the 10x Chromium chip.
21. Proceed immediately to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology.

Protocol for totalseq-fluorescent titration prior hashing labelling

Antibodies preparation

Note: Check concentration of Fluorescent control antibodies, because they may be at different concentrations than hashing antibodies.

Note: For mouse and human the hashing antibodies are a mix of 2 clones, so we need to add a mixture of both clones (note: mixing 0.2ug/ul + 0.2ug/ul still makes a 0.2ug/ul mix). For nuclear pore proteins antibodies, there is only 1 clone.

Note: Prepare also the same mix for the **isotypes controls** (but only plan for the highest amount, not for all dilutions).

1. Decide amount of antibody you want to test: the recommended amount of hashing antibody is 0.1-0.25 µg per sample.
2. While the official protocol suggests to incubate cells with antibodies for 30 minutes, during titration we suggest to try also shorter incubation times (e.g. 10 minutes). If they work, it is better to use shorter times, to preserve cell integrity.
3. Prepare 50 ul of each antibody mix on ice and then store at 4° protected from light:
    a. for adding more than 0.25ug to the final labelling use undiluted fluorescent antibody.
    b. For adding less antibodies, first do a dilution of the fluorescent stock accordingly in PBS 10%FBS. E.g. for adding 0.125ug, first do a 2x dilution of the stock: 0.5ul stock antibody + 0.5ul PBS+10%BSA.
4. Keep the antibodies in the dark

Once the antibodies are ready, follow the protocol for hashing and, after filtering the cells with a flowmi 40um (or larger if 40um is not suitable), go to FACS.

Notes

Protocol based on TotalSeq-B or -C with 10x Feature Barcoding Technology protocol from Biolegend and 10XG protocol (CG000149, Rev D), articles (https://kb.10xgenomics.com/hc/en-us/articles/8992191577741-Can-I-perform-Cell-Hashing-in-the-5-workflow and https://kb.10xgenomics.com/hc/en-us/articles/360041942012-How-can-I-optimize-my-TotalSeq-antibody-labeling-protocol)

Version log

- v1.0-v1.1 (01.09.2024-12.12.2024): initial version & many small changes
- v1.2 (20.12.2024): small change
- v1.3 (22.01.2025): use 0.2U/ul RNase inhibitor for washes/resuspension of nuclei. Changed the final conc. of cells/nuclei needed
- v1.4 (27.02.2025): fewer washes, 10 min incubation and extra care for nuclei. Smaller washes volume. Added reference to paper for nuclei hashing in note
- v1.5 (14.03.2025): for nuclei, added to follow the specific protocol. Removed mention to nuclei in this protocol. Added mention to fragile cells
- v1.6 (24.06.2025): preparation part made clearer. CG000426 for labelling in 96-well plates.