Best Practices for Cell Preparation

Cell handling can directly impact phenotype, making it critical to minimize cellular stress throughout the cell preparation workflow. This document contains tips and guidelines for optimized cell preparation and thawing of live cells prior to running your samples with the Pixelgen Proxiome Kit v2. It serves as an extension of the UM002: User Manual Proxiome Kit v2 Cell Surface (v1.00).

Sample randomization

When processing samples we recommend randomizing the order of samples from each of your experimental conditions to minimize any potential batch effects. This includes randomizing samples both for the *PFA fixation* step and the *Hashtag-stained sample pooling* during the Pixelgen Proxiome Kit v2.

Platelet removal

To ensure high purity for samples isolated from blood, effective removal of platelet contamination is a critical step before fixation, as significant contamination can interfere with downstream results.

We recommend preventing this by performing multiple rounds of gentle washing to separate larger cells from smaller, lighter platelets. Centrifuge your samples at 120 x g for 10 minutes with the centrifuge brake turned off. This gentle force pellets your target cells while leaving the majority of platelets in the liquid supernatant. Repeat this slow centrifugation wash 2-3 times for optimal purity.

Red blood cell (RBC) removal

If the samples have significant RBC contamination, we recommend removing them before proceeding with the fixation step as they can interfere with the final results. Efficient RBC lysis can be achieved using e.g. the RBC lysis buffer 10x (eBiosciences, Cat. no. 00-4300-54).

Cell clumping

For the most accurate cell counts, it is important to remove any visible cell aggregates or debris before fixation. We recommend visually inspecting your sample and, if clumps are present, filtering the cell suspension through a cell strainer (e.g., 40-70 µm) to remove them.

Cell dissociation

Some *in vitro* cultured immune cells may adhere to plastic and might require dissociation before fixation. We have successfully dissociated activated T-cells and breast cancer tumor-spheroids using Accutase for 15 min at 37°C with pipetting every 5 min. While some epitopes might be lost, we found Accutase to be gentler than trypsin.

Dead cell removal kit

If during cell preparation viability is lower than 70% we recommend using a dead cell removal kit. High amounts of dead cells in the samples can impact the downstream results. We recommend using: EasySep Dead Cell Removal (Annexin V) kit from Stem Cell Technologies (Cat. no. 17899) or Dead Cell Removal Kit from Miltenyi Biotec (Cat. no. 130-090-101).

Rare cell types

The maximum number of cells/sample to input to the sequencing step with the Proximity Network Assay (PNA) is 1 000 cells. If your cells of interest are rare (<5%), an enrichment step before fixation may be required. This can be achieved using positive/negative selection with magnetic beads or FACS sorting. When choosing the strategy, please be aware that positive selection might use an antibody that can block the target epitope needed for detection with the Pixelgen Proxiome Kit v2.

Recommended protocol for thawing of live cells

The Pixelgen Proxiome Kit v2 supports up to 64 samples per run. Follow the guidelines below to maintain cell quality across all samples.

Note:
Live human samples might contain infectious agents. Treat samples with care, avoid spills and splashes, and protect yourself from droplets by working in a hood until cells are fixed. All supernatant from live cells should be disposed of in a closed container and preferably decontaminated with bleach. If a spill occurs,

wipe the liquid with a dry paper towel, followed by water, bleach, and finally 70% ethanol. Sterilize the hood and all objects in the hood carefully after use with 70% ethanol.

IMPORTANT POINTS:

- During cell preparation, we recommend thawing cells in batches of a maximum of 8 cryovials at a time to ensure consistent handling and minimize variability between samples.
- When using the 4x8 configuration (32 reactions), we recommend processing samples over two days (16 reactions per day), making use of one of the two Akura™ Spheroid Microplates included with this configuration each day.
- Keep cells at +4°C in 1x PBS + 0.5% BSA while thawing all the samples. We have tested the storage of cells at +4°C for up to 6 h.
- Keep the cells on ice during the counting step to maintain viability.
- Remember to randomize the order of the samples during the processing.

RECOMMENDED PROTOCOL:

Pre-warm at 37°C **1x PBS + 0.5% BSA** before starting with the following recommended protocol.

1. Minimize exposure of cryovials to room temperature when removing from storage.
2. Thaw live cell cryovials at 37°C (approximately 1-2 minutes). Avoid having cryovials completely thawed. Some ice should still be visible.
3. Wipe the outside of cryovial with 70% ethanol.
4. Transfer the content of the **cryovial** to a 15 ml Falcon tube.
5. Rinse the **cryovial** with 1 ml **1x PBS + 0.5% BSA** to recover residual cells and carefully add it **dropwise** onto the cells in the 15 ml Falcon tube.
6. Add 8 ml of **1x PBS + 0.5% BSA** to the 15 ml Falcon tube to wash out DMSO. We recommend adding the first 1-2 ml **dropwise** and the rest at a slightly faster pace. Pipette up and down.
7. Spin down at **300 x g** for **5 min**.
8. Carefully remove supernatant without disturbing the cell pellet.
9. Add 10 ml of **1x PBS + 0.5% BSA** and pipette up and down.
10. Spin down at **300 x g** for **5 min**.
11. Carefully remove supernatant without disturbing the cell pellet.
12. Resuspend cells in 1 ml of **1x PBS + 0.5% BSA** and keep at +4°C while thawing other **cryovials**.
13. Once all **cryovials** have been thawed, perform an additional wash and resuspend cells in 1 ml of **1x PBS** to remove carrier protein before fixation. Proceed with STEP 1 - 1.3 of the **UM002: User Manual Proxiome Kit v2 Cell Surface (v1.00)**.