FIXATION AND FREEZING PROTOCOL WITHOUT KIT ACCESS

Fixation and freezing protocol without access to the Pixelgen Proxiome Kit v2



PIXELGEN TECHNOLOGIES

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This protocol describes how to prepare, fix and freeze the cells for the Proximity Network Assay, without access to the Pixelgen Proxiome Kit v2. The fixation can be performed either in PCR tubes or in the Akura™ Spheroid Microplate, which is available separately from Pixelgen Technologies. BSA, glycine, methanol-free paraformaldehyde and 1x PBS are required to perform these steps. DMSO and FBS are also required if freezing the fixed cells.

EQUIPMENT, REAGENTS AND CONSUMABLES NEEDED

Equipment			
Description	Product name	Suggested Supplier	Catalog number
Centrifuge	Mega Star 4.0R	VWR®	521-2664
Single pipettes: • 0.5 - 2.5 µl • 2 - 20 µl • 20 - 200 µl • 100 - 1000 µl Multichannel pipettes: • 0.5 - 10 µl • 10 - 100 µl • 30 - 300 µl	Research® plus	Eppendorf (Avantor) / Eppendorf	613-5893, 613-5928, 3125000010, 3125000036, 3125000060
Automated cell counter or Hemocytometer or Single-use hemocytometer	Countess 3 FL automated cell counter or LUNA-BX7 automated cell counter or Counting Chambers Bürker-Türk or Millicell® Disposable Hemocytometer	Invitrogen Logos Biosystems Karl Hecht Assistent® Sigma-Aldrich®	AMQAF2000 LOG-L90001 40445 MDH-2N1-50PK

Reagents			
Description	Product name	Suggested Supplier	Catalog number
1x PBS	PBS, pH 7.4	Gibco™	10010-023
Paraformaldehyde, methanol-free*	Paraformaldehyde 16% Aqueous Sol.	Electron Microscopy Sciences or Thermo Fisher	15710 28906/28908
1M Glycine solution	1M Glycine solution	Sigma-Aldrich®	67419-1ML-F
Bovine Serum Albumin, protease-free, ≥98% purity	Bovine Serum Albumin	Sigma-Aldrich®	A3294
Fetal bovine serum	FBS, heat-inactivated	Sigma-Aldrich®	F9665
Dimethyl sulfoxide	DMSO	Sigma-Aldrich®	D2438

* It is important to use methanol-free paraformaldehyde as methanol can permeabilize the cell membrane and promote protein denaturation.

Consumables			
Description	Product name	Suggested Supplier	Catalog number
0.2 ml PCR tubes or	FastGene® PCR Tubes 0.2 ml	Nippon Genetics Europe	FG-021
0.2 ml PCR tube strips or	8-well PCR tube strips 0.2 ml with cap strips	Nippon Genetics Europe	FG-088WF
0.2 ml PCR tube strips	PCR strip tubes, Axygen®	Corning	PCR-0208-AF-C
Akura microplate*	Akura™ Spheroid Microplate	Pixelgen Technologies	PROX_plate
Pipette tips: 10 µl 20 µl 200 µl 1000 µl	OMNITIP™ Sterile, filter tips	ULPlast Sp.z.o.o.	 83240 86240 81240 85240
Cryotubes	Cryotubes	Sarstedt	72.380.992
Parafilm	Parafilm® M	VWR	291-0057

* The Akura™ Spheroid Microplate is a registered trademark owned by InSphero AG.
NOTE: Store the plate at room temperature upon arrival, as indicated on the package.

CHOICE OF FIXATION VESSEL:

- Cells can be fixed either in PCR tubes or in the Akura™ Spheroid Microplate (available from Pixelgen Technologies, catalog number PROX_plate). Choose the vessel based on the number of cells available per sample:
    - **PCR tubes:** 500 000 - 1 000 000 cells per sample.
    - **Akura™ Spheroid Microplate:** 50 000 - 1 000 000 cells per sample.
- All fixation volumes, centrifugation speeds and incubation times are identical in both vessels. Where a step refers to a PCR tube, use one plate well per sample when working with the Akura™ Spheroid Microplate.
- The fixation vessel determines the freezing route: cells fixed in PCR tubes are frozen in cryotubes (*section 4A - Freezing in cryotubes*); cells fixed in the plate are frozen in the plate (*section 4B - Freezing in the Akura™ Spheroid Microplate*).

PREPARATIONS:

- Prepare a fresh solution of 0.5% BSA in 1x PBS, per sample. A volume of 550 µl per sample is needed (430 µl + extra). The BSA should be protease-free and of ≥98% purity. The volume depends on the number of samples processed.

- Prepare a fresh solution of 2% v/v PFA solution (methanol free) in 1x PBS. A volume of 100 µl is needed per sample (80 µl + extra). Use the solution within 2 hours, and store in dark until use. The total volume depends on the number of samples processed.

- Prepare a fresh freezing solution of 5% DMSO and 95% FBS. A volume of 600 µl per sample is needed for *section 4A - Freezing in cryotubes* (475 µl + extra), or 150 µl per well (125 µl + extra) for *section 4B - Freezing in the Akura™ Spheroid Microplate*. The total volume depends on the number of samples processed.

Important instructions:

- **Cell handling** can directly impact phenotype, making it critical to minimize cellular stress throughout the cell preparation workflow. Please refer to the "Best Practices for Cell Preparation" document for guidelines on optimized cell preparation and thawing of live cells prior to running your reactions [1].
- We recommend **randomizing the order of your samples** from each experimental condition to minimize potential batch effects.
- **Never aspirate close to the bottom** of the PCR tube during liquid removal in wash steps - follow the liquid surface upon aspiration, as the pellet may not be clearly visible.
- Centrifugation should be performed using a **swinging bucket rotor**, as usage of fixed-angle rotors increases cell loss.
- Be careful and **keep the PCR tubes vertical** after centrifugation to not disturb the cell pellet, as it could otherwise lead to increased cell loss.
- Ensure all samples have **equal volume**, as repeated washing steps in the protocol can cause discrepancies. Inspect tube or well levels visually, and if necessary, adjust with appropriate buffer for that step without disturbing the cell pellet.
- **Centrifugation of PCR tubes** can be performed either using adapters for PCR tubes, or by putting the PCR tubes in a PCR tube rack and centrifuging with a rotor for microplates. The Akura™ Spheroid Microplate is centrifuged directly using a rotor for microplates.

When performing the fixation in the Akura™ Spheroid Microplate:

- The Akura™ Spheroid Microplate design minimizes interaction with the cell pellet, allowing efficient use of multichannel pipettes.
- When **discarding liquid**, position the pipette tips on the inner ledge of the wells and hold the pipette at an oblique (tilted) angle to avoid disturbing the cell pellet.
- When pipetting reagents and washing buffers, hold the pipette **vertically** to ensure cells are fully resuspended. For more detailed instructions, please watch our video tutorial [2].
- If **excessive bubbles** form during resuspension or pipetting, spin the plate briefly (400 rcf before PFA cell fixation, 700 rcf after PFA cell fixation).

1. Blocking PCR tubes or plate wells with BSA

NOTE: Each sample will need preparation of one BSA-blocked PCR tube or plate well.

A. ☐ Add 180 µl of the **1x PBS + 0.5% BSA** solution to each empty PCR tube or plate well.

B. ☐ Incubate for a minimum of 15 min at 4°C.

C. ☐ Remove the liquid completely and air-dry the tubes with lid open, or the plate wells, for 3-5 min at room temperature (RT).

NOTE: If not used immediately, store the coated PCR tubes or plates at 4°C for up to 48 h.

2. Cell preparation

PREPARATION: Prepare one PCR test tube (or one plate well, if using the Akura™ Spheroid Microplate) with 25 µl 1x PBS. Keep aside and use as volume reference when ensuring all actual samples have equal volumes.

NOTE: Visually inspect the cell suspension for cell aggregates or debris as these can contribute to inaccurate cell counting. If needed, filter the cell suspension using a cell strainer to remove large aggregates. Please refer to the *"Best Practices for Cell Preparation"* document available on our website for detailed instructions and guidelines on cell preparation and thawing of live cells [1].

NOTE: It is important to pipette the cell suspension gently throughout this part of the protocol.

NOTE: When processing multiple batches of cells, keep suspensions (e.g., after thawing) in a serum-supplemented solution (e.g., 1x PBS + 0.5% BSA) at 4°C to ensure stability before proceeding with cell preparation. Minimize storage time at 4°C as it can negatively impact results.

PREPARATION: When ready, wash the cells with 1x PBS prior to counting to remove residual of the serum-supplemented solution, then resuspend in 1x PBS.

A. ☐ Count the cells using either automated cell counter (e.g., Countess 3 FL automated cell counter), hemocytometer or other cell counting device.

B. ☐ For each sample, transfer 500 000 - 1 000 000 cells to the BSA-blocked PCR tubes, or 50 000 - 1 000 000 cells to the BSA-blocked plate wells, and add **1x PBS** for a total volume of 150 µl per sample. **IMPORTANT!** When using the Akura™ Spheroid Microplate, arrange samples in rows or columns of 8, as the cells remain in the same wells through fixation, freezing and thawing, and the position of each sample determines both the Hashtag Set it receives and the pool it is combined into in the UM002: User Manual Proxiome Kit v2 Cell Surface.

C. ☐ Centrifuge at 400 rcf for 4 min at RT.

D. ☐ Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl to preserve the cell pellet.

E. ☐ Add 55 µl **1x PBS** on top of the 25 µl cell suspension and gently pipette up and down 10 times.

3. Cell fixation with paraformaldehyde (PFA)

PREPARATION: Prepare a fresh solution of 2% v/v PFA solution (methanol-free) in 1x PBS. Use the solution within 2 hours, and store in dark until use. The volume depends on the number of samples processed.
NOTE: Use the necessary precautions when handling PFA solution since it is a CMR substance (Carcinogenic, Mutagenic, or toxic to Reproduction).

NOTE: Following PFA fixation, cells may still appear as 'live' by automated counters. This occurs because our mild fixation protocol preserves membrane integrity, limiting viability dyes from penetrating the cell. Consequently, always use the 'Total Count' setting to accurately quantify the fixed cells in your reactions.

- **A.** ☐ Add 80 µl of **2% PFA/1x PBS** (final concentration of 1%) to each 80 µl sample and pipette up and down 10 times.
- **B.** ☐ Incubate for 15 min at room temperature (RT).
- **C.** ☐ Quench the PFA by adding 25 µl of **1M Glycine solution** on top of the 160 µl fixation solution and pipette up and down 10 times.
- **D.** ☐ Centrifuge at 700 rcf for 4 min at RT.
- **E.** ☐ Carefully discard 160 µl supernatant from each of the 185 µl quenched sample without disturbing the cell pellet, leaving behind 25 µl of supernatant.
- **F.** ☐ Add 125 µl **1x PBS + 0.5% BSA** on top of the 25 µl cell suspension and pipette up and down 10 times.
- **G.** ☐ Centrifuge at 700 rcf for 4 min at RT.
- **H.** ☐ Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl to preserve the pellet.
- **I.** ☐ Add 125 µl **1x PBS + 0.5% BSA** on top of the 25 µl cell suspension and pipette up and down 10 times.
- **J.** ☐ Centrifuge at 700 rcf for 4 min at RT.
- **K.** ☐ Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl to preserve the pellet.
- **L.** ☐ Resuspend the pellet by adding 125 µl 1x PBS. Total of 150 µl in each tube or well.



Up to 5 days at 4°C
Up to 3 months at -80°C

NOTE: At this step, cells can be stored at 4°C in 1x PBS for up to 5 days or frozen at -80°C. If not freezing the cells, continue with *STEP 2 - Hashtag Binding* in the UM002: User Manual Proxiome Kit v2 Cell Surface. Cells fixed in PCR tubes must first be transferred to a BSA-blocked well of the Akura™ Spheroid Microplate, prepared as described in section 1. Cell counting is not required at this step, as the cells are counted at *STEP 2.3 - Hashtag-stained sample pooling*. When storing the Akura™ Spheroid Microplate at 4°C, we recommend sealing it with parafilm to reduce the evaporation of the liquid. If freezing the cells, continue with *section 4 - Freezing of PFA-fixed cells* below.

4. Freezing of PFA-fixed cells

NOTE: Both freezing routes use the **freezing solution** of 5% DMSO and 95% FBS. The total volume depends on the number of samples processed. Follow *section 4A - Freezing in cryotubes* if the cells were fixed in PCR tubes, or *section 4B - Freezing in the Akura™ Spheroid Microplate* if the cells were fixed in the plate.

4A - Freezing in cryotubes

- **A.** ☐ Centrifuge the samples with fixed cells at 700 rcf for 4 min at RT and remove 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl.
- **B.** ☐ Add 125 µl **freezing solution** and pipette up and down 10 times.
- **C.** ☐ Transfer the 150 µl cell solution to a cryotube.
- **D.** ☐ Add 150 µl **freezing solution** to the empty PCR tube and pipette up and down 10 times to collect any remaining cells.
- **E.** ☐ Transfer the collected 150 µl to the cryotube. Total of 300 µl in each cryotube.
- **F.** ☐ Add 200 µl **freezing solution** to the 300 µl cell solution. Total of 500 µl in each tube.
- **G.** ☐ Place the cryotubes in a cryogenic box and transfer to -80°C until further use.

NOTE: At this step, cells can be kept in -80°C storage for up to 3 months.

4B - Freezing in the Akura™ Spheroid Microplate

- **A.** ☐ Centrifuge the plate with fixed cells at 700 rcf for 4 min at RT.
- **B.** ☐ Remove 125 µl supernatant from each well without disturbing the cell pellet, leaving behind 25 µl.
- **C.** ☐ Add 125 µl **freezing solution** to each well.
- **D.** ☐ Pipette up and down 10 times.
- **E.** ☐ Seal the plate with parafilm to reduce the evaporation of the liquid.
- **F.** ☐ Transfer the plate to -80°C until further use.

NOTE: At this step, the plate can be kept in -80°C storage for up to 3 months.

HOW TO PROCEED

Once ready to run the Proximity Network Assay using the Pixelgen Proxiome Kit v2, continue in the UM002: User Manual Proxiome Kit v2 Cell Surface. Cells frozen in cryotubes (section 4A) are thawed following *STEP 1.6A - Thawing from cryotubes*. Cells frozen in the plate (section 4B) are thawed following *STEP 1.6B - Thawing in the Akura™ Spheroid Microplate*.

REFERENCES

[1] Best Practices for Cell Preparation

[2] Tutorial video: Handling of Akura™ Spheroid Microplate

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United States Patent Number 12,123,050, Patent Application Serial Numbers (US20230027467A1, WO2024069322A1, 63/414,883, 63/439,839).

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