FIXATION AND FREEZING PROTOCOL WITHOUT KIT ACCESS

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

PIXELGEN TECHNOLOGIES

Fixation and freezing without kit access

This protocol describes how to prepare, fix and freeze the cells for the Proximity Network Assay, without access to the Pixelgen Proxiome Kit v2. 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	Part number
Centrifuge PCR tubes (1000 rcf)	Mega Star 4.0R	VWR®	521-2664
Pipettes: • 0.5 - 2.5 µl • 2 - 20 µl • 20 - 200 µl • 100 - 1000 µl	Research® plus	Eppendorf	J70399L O89082L N23237L N44241L
Automated cell counter or	Countess 3 FL automated cell counter or	Invitrogen	AMQAF2000
	LUNA-BX7 automated cell counter	Logos Biosystems	LOG-L90001
or Hemocytometer or	Counting Chambers Bürker-Türk	Karl Hecht Assistent®	40445
Single-use hemocytometer	Millicell® Disposable Hemocytometer	Sigma-Aldrich®	MDH-2N1-50PK

Reagents			
Description	Product name	Suggested Supplier	Part number
1xPBS	PBS, pH 7.4	Gibco™	10010-023
Paraformaldehyde, methanol-free*	Paraformaldehyde 16% Aqueous Sol.	Electron Microscopy Sciences or ThermoFisher	15710 28906/28908
Glycine 1M solution	Glycine 1M solution	Sigma-Aldrich®	67419-1ML-F
Bovine Serum Albumin, ≥98% purity	Bovine Serum Albumin	Sigma-Aldrich®	A3294
Nuclease Free Water	Nuclease-Free Water (not DEPC-Treated)	ThermoFisher	AM9932
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	Part number
0.2mL PCR tubes or	FastGene® PCR Tubes 0.2mL	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
Pipette tips: 10 µl 20 µl 200 µl 1000 µl	OMNITIP™ Sterile, filter tips	ULPlast Sp.z.o.o.	 83240 86240 81240 85240

PREPARATIONS:

- Prepare a fresh solution of 0.5% BSA in 1x PBS, per sample. A volume of 1.5 ml per sample is needed (825 µl + extra). The BSA should be 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 1xPBS. A volume of 100 µl is needed per sample (90µl + 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 (500 µl + extra). The total volume depends on the number of samples processed.

Important instructions:

- **Never aspirate close to the bottom** of the 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 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 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.

1. Blocking tubes with BSA

NOTE: Each sample will need preparation of one BSA blocked PCR tube.

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

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

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

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

2. Cell preparation

PREPARATION: Prepare one PCR test tube 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.

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.

D. ☐ Count the cells using either automated cell counter (e.g., Countess II Automated Cell Counter), hemocytometer or other cell counting device.

E. ☐ For each sample, transfer 500 000 - 1 000 000 cells to the BSA-blocked PCR tubes, and add **1x PBS** for a total volume of 150 µl per sample.

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

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

H. ☐ 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 **1 M 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**.

M. ☐ Determine the cell concentration for each sample using either a hemocytometer or other cell counting devices (e.g. Countess II Automated Cell Counter). Mix by pipetting up and down 10 times before taking an aliquot for counting.

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 (v1.00). If freezing the cells, continue with *STEP 4 - Freezing of PFA-fixed cells* below.

4. Freezing of PFA-fixed cells

NOTE: The total volume of the freezing solution depends on the number of samples processed.

- **A.** ☐ Centrifuge the samples with fixed cells at 700 rcf for 4 min at RT and remove supernatant (125 µl - volume used for counting) 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 wash any remaining cells.
- **E.** ☐ Transfer the 150 µl wash 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℃ until further use.

NOTE: At this step, cells can be kept in -80℃ storage for 3 months.

HOW TO PROCEED

Once ready to run the Proximity Network Assay using the Pixelgen Proxiome Kit v2, please continue with **STEP 1.6 Thawing of PFA-fixed, frozen cells** of the UM002: User Manual Proxiome Kit v2 Cell Surface (v1.00).

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