USER MANUAL

PIXELGEN PROXIOME KIT

IMMUNO 155

Proximity Network Assay for Cell Surface Analysis

PROXIMM001

PIXELGEN
TECHNOLOGIES

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List of Abbreviations

- **BSA** Bovine Serum Albumin
- **CMR** Substance Carcinogenic, Mutagenic, or toxic to Reproduction
- **DMSO** Dimethyl Sulfoxide
- **FBS** Fetal Bovine Serum
- **NGS** Next Generation Sequencing
- **PBS** Phosphate-Buffered Saline
- **PBMC** Peripheral Blood Mononuclear Cell
- **PCR** Polymerase Chain Reaction
- **PFA** Paraformaldehyde
- **RT** Room Temperature
- **SPRI** Solid Phase Reversible Immobilization

About this User Manual

This User Manual describes the experimental procedure using the Pixelgen Proxiome kit, Immuno 155 (PROXIMM001) in detail.

Technical Support

For technical support, please contact Pixelgen Technologies at support@pixelgen.com

1. Product Description

The intended use of the Pixelgen Proxiome kit, Immuno 155, is for spatial profiling of immune cell surface proteins at nanoscale resolution. This is achieved by forming an interconnected spatial network between barcoded antibodies bound to each cell. A Rolling Circle Amplification product (RCP) is generated from each antibody, which enables hybridization of linker oligos that connect neighbouring RCPs on each cell. Each RCP contains multiple copies of a unique molecular identifier (UMI). Sequences from two UMIs are incorporated into a hybridized linker oligo via a gap-fill ligation reaction, followed by sequencing of the generated molecules. From the set of sequenced molecules (each representing a link between two neighboring proteins) a Proximity Network is generated from each single cell. This interconnected spatial network cell is analyzed by spatial statistics to infer the nanoscale organization of each assayed protein.

The core steps of the Proximity Network Assay by the Proxiome Kit are illustrated below.

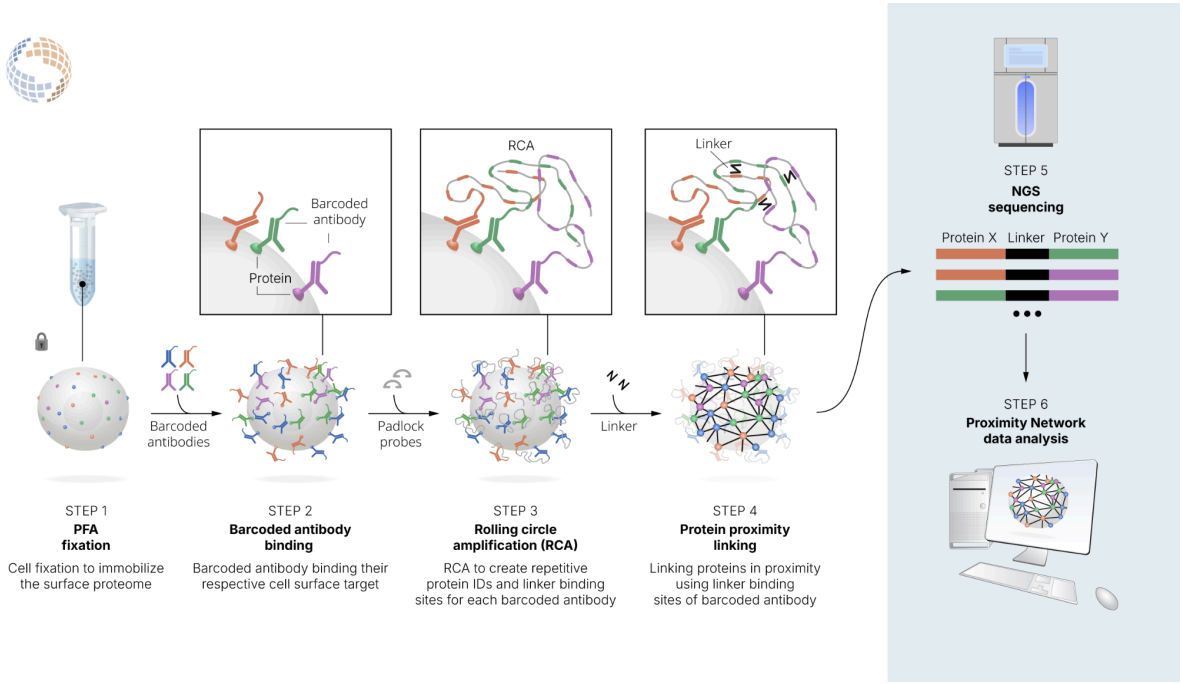


Figure 1. Overview of the Proximity Network Assay workflow and core steps.

Reagents supplied with the Pixelgen Proxiome kit

Reagents included in the kit are listed below in Table 1. Each kit contains reagents sufficient for processing of 8 reactions. The reagents are supplied in three individual boxes, with storage temperature and expiration date stated on the label of each box.

> **NOTE:** All reagents for the Pixelgen Proxiome Kit, Immuno 155, are lot specific and reagents from different kit lots should not be combined.

Product number: PROXIMM001

Table 1. Reagents supplied in the individual kit boxes and their storage temperatures. Bullet point colors correspond to the reagent lid color.

Box 1 PROXIMM001_box1 store at 4°C	Box 2 PROXIMM001_box2 store at -20°C	Box 3 PROXIMM001_box3 store at -20°C
Antibody Panel - PP056	Blocking Solution - BD059	Primers 1 - IP050_1 *
2nd Antibody - PP028	Padlock Oligos - BD045	Primers 2 - IP050_2 *
Wash Buffer - BD044	Gap-fill Solution - BD057	Primers 3 - IP050_3 *
Quenching Solution - BD066	Gap-fill Ligase - EE052	Primers 4 - IP050_4 *
	Gap-Fill Polymerase - EE053	Primers 5 - IP050_5 *
	RCA Solution - BD046	Primers 6 - IP050_6 *
	RCA Polymerase - EE054	Primers 7 - IP050_7 *
	Proximity Oligos - BD047	Primers 8 - IP050_8 *
	PCR Master Mix - MM058	
	Pre-amp Primers - IP049	

* Primers for sample index PCR come in 3 different sets. **Set 1** contains unique Primers 1 - 8, **set 2** contains unique Primers 9 - 16 and **set 3** contains unique Primers 17 - 24. This is to easily enable sequencing of up to 24 Proximity Network Assay samples in the same flowcell. See *Appendix 3* for primer index sequences.

Additional Requirements

Below is a list of equipment, reagents and consumables required to perform the Proximity Network assay. The suggested suppliers and part numbers noted are equivalent to equipment used during optimization and validation of the assay.

Table 2a. List of suggested third party equipment needed to perform the analysis workflow.

Equipment			
Description	Product name	Suggested Supplier	Part number
Centrifuge with rotor for 2 mL micro tubes (17 000 x *g*)	Centrifuge 5418 R - microcentrifuge	Eppendorf	5418 R
Centrifuge PCR tubes (1000 x *g*)	Mega Star 4.0R	VWR®	521-2664
Mini centrifuge	Mini Centrifuge	Nippon Genetics Europe	NG002B
Thermocycler/ PCR system	ProFlex™ 3x 32-well PCR System	Applied Biosystems™	4484073
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 Hemocytometer or Single-use hemocytometer	Countess 3 FL automated cell counter Counting Chambers Bürker-Türk Millicell® Disposable Hemocytometer	Invitrogen Karl Hecht Assistent® Sigma-Aldrich®	AMQAF2000 40445 MDH-2N1-50PK
Magnet for PCR tubes	0.2 mL PCR 8 Strip Magnetic Separator 5 µL ~ 0.2 mL Volume	Permagen®	MSRLV08
Light microscope	Microscope trinocular inverted	Sagitta	63335

PCR product size and quantification instrument	BioAnalyzer™ with High Sensitivity DNA kit	Agilent	G2939A

Table 2b. List of suggested third party consumables needed to perform the workflow.

Consumables			
Description	Product name	Suggested Supplier	Part number
0.2mL PCR tubes	FastGene® PCR Tubes 0.2mL	Nippon Genetics Europe	FG-021
1.5mL low adhesion tube	Eppendorf Tubes® low adhesion	BIOplastics™	B74030
Pipette tips: 10 µl 200 µl 1000 µl	OMNITIP™ Sterile, filter tips	ULPlast Sp.z.o.o.	 83240 81240 85240

Table 2c. List of suggested third party reagents needed to perform the workflow.

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	15710
SPRI beads	SPRIselect, 60 mL	Beckman-Coulter Life science	B23318
Tris buffer	Tris (1 M), pH 8.0, RNase-free	Invitrogen™	AM9855G
Absolute ethanol	Ethanol absolute, 1 L	VWR™	20821.310
Bovine Serum Albumin, ≥98% purity	Bovine Serum Albumin	Sigma-Aldrich®	A3294
Nuclease Free Water	Nuclease-Free Water (not DEPC-Treated)	ThermoFisher	AM9932

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

2. Workflow and Guidelines

The protocol outlines the complete assay workflow, from live cells in suspension to a sequencing-ready NGS library. It has been demonstrated and validated using PBMCs as well as leukemia and lymphoma cell lines.

Workflow Overview and Steps

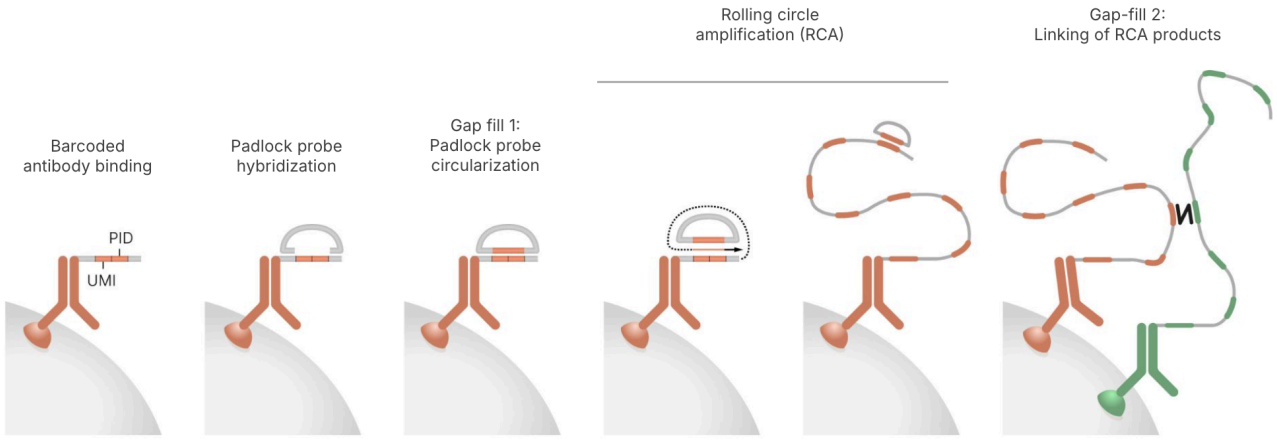


Figure 2a. Molecular description of core steps in the Proximity Network assay.

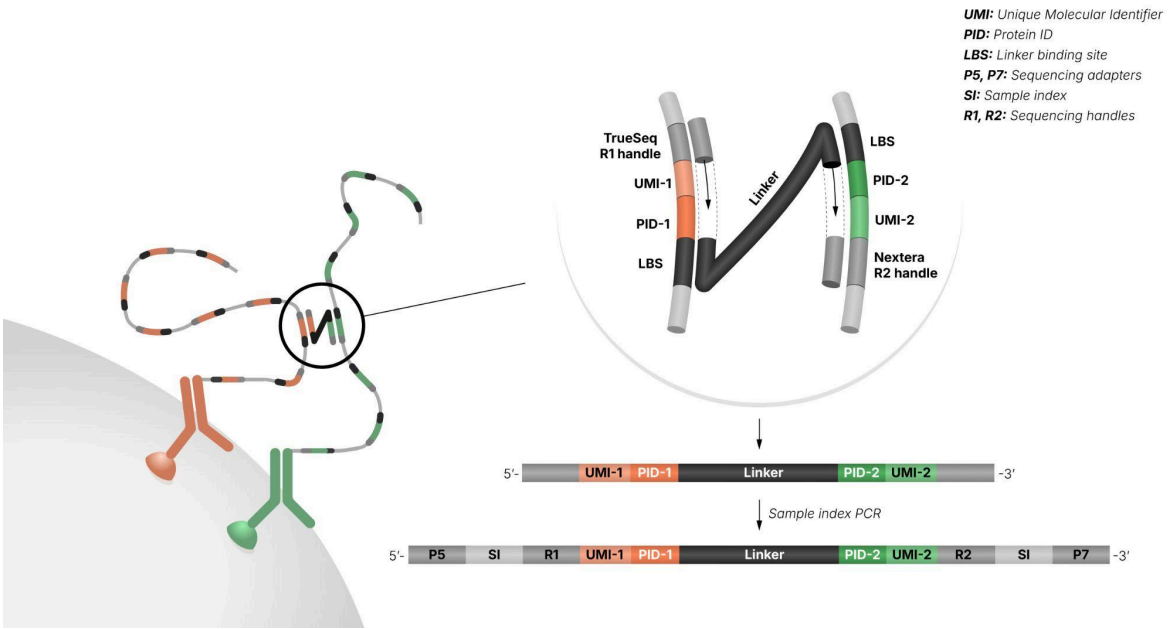


Figure 2b. Zoom-in on Gap-fill 2 (Linking of RCA products) and PCR steps in the Proximity Network assay.

There are **5 main steps** in the protocol, starting from live cells in suspension to sequence-ready NGS library. Table 3. describes these main steps of the workflow and outlines the approximate time needed.

Table 3. Workflow steps and time needed.

Step	Description	Duration
1	**Cell Fixation:** **1.1** Blocking tubes with BSA **1.2** Cell preparation **1.3** PFA fixation	2 h
STOP	– Alt. stopping point: Up to 5 days at 4°C or up to 3 months at -80°C if performing the optional freezing protocol	
2	**Barcoded Antibody Binding:** **2.1** Cell blocking **2.2** Antibody binding **2.3** 2nd antibody binding	2 h + ON + 2.5 h
STOP	– Alt. stopping point: Up to 2 days at 4°C	
3	**Protein Proximity Network Generation:** **3.1** Padlock probes hybridization **3.2** Gap-fill 1 (padlock probe circularization)	5 h
STOP	– Alt. stopping point: Up to 3 days at 4°C	
	3.3 Rolling circle amplification (RCA) **3.4** Gap-fill 2 (linking of RCA products)	
STOP	– Alt. stopping point: Up to 3 days at 4°C	
4	**PCR Amplification and Sample Indexing:** **4.1** Manual cell counting **4.2** Pre-amplification PCR **4.3** Pre-amplification PCR clean-up	5 h
STOP	– Alt. stopping point: -20°C for long periods of time	
	4.4 Sample index PCR **4.5** Sample index PCR clean-up	
STOP	– Alt. stopping point: -20°C for long periods of time	
5	**NGS Preparation:** **5.1** Quality control and quantification **5.2** Pooling **5.3** NGS sequencing using the Illumina® platform	2 h

Cell input requirements

The protocol consists of several steps where a certain range of cell input is required (Table 4.). Due to **cell losses** during fixation and washing steps, it is important to stay within the validated range and use BSA blocking during fixation and antibody binding to retain enough cells. The cell input range to *STEP 4 - PCR Amplification and Sample Indexing* needs to be within 200 to 1000 cells to ensure software compatibility. 1000 cells are recommended, to increase statistical significance during data analysis. Automated cell counters can be used for all counting steps except STEP 4 where manual counting is required, due to low cell concentrations not generating accurate counts.

Tables in *Appendix 1* can be used for counting and viability documentation.

Table 4. Cell inputs and counting requirements for different steps of the protocol.

STEP	Supported cell input range per reaction	Recommended cell input per reaction	Counting procedure
1. Cell Fixation	500 000 - 1 000 000 cells	500 000 - 1 000 000 cells	Automated cell counter OK
2. Barcoded Antibody Binding	50 000 - 250 000 cells	250 000 cells**	Automated cell counter OK
3. Protein Proximity Network Generation	20 000 - 100 000 cells	50 000 cells**	Automated cell counter OK
4. PCR Amplification and Sample Indexing	200 - 1 000 cells*	1 000 cells	Manual cell counting

* It is possible to proceed to PCR Amplification and Sample Indexing with 200 - 1000 cells if less than 1000 cells remain. Please note that the number of cells input to PCR will require different PCR cycles and will directly impact the total read requirement.

** Use the same cell input across all the samples to minimize data output variation between samples.

Sample indexing

Each primer contains a unique sample index barcode combination, allowing for pooling of samples for sequencing on the same Illumina flow cell. It is critical that each individual sample sequenced together in the same run has a unique sample index. See *Appendix 3* for a complete list of the sample index sequences.

If planning to pool the samples together with other types of samples that were prepared using other library preparation workflows, please ensure that sample indices are **unique** amongst all samples aimed to be sequenced together, to avoid sample index collisions.

Important instructions

- Never aspirate close to the bottom of the tube during liquid removal in wash steps - the **pellet will not be visible** when working with low cell numbers.
- 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.
- It is recommended to process a maximum of **8 reactions in parallel**.
- Do not vortex samples.
- A **multichannel pipette** can be used to add wash buffer before centrifugation during wash steps; ensure equal volumes are dispensed. To remove liquid after centrifugation, use a single pipette to avoid disturbing the cell pellet.
- To ensure proper mixing, aspirate at least **50%** of the total reagent volume when pipetting up and down.
- Pulse-spin all reagent tubes before opening to pull down any liquid that may be present under the lid. Thoroughly **mix reagents before use** by pipetting up and down 5 times.
- Keep all reagents **on ice** once thawed, unless otherwise stated.
- Return enzymes, antibodies and buffers to their **storage** directly after use to minimize time exposed to elevated temperatures.
- All incubations ≥ 25°C should have a heated **lid temperature of 105°C.**
- **Label** both the side and the top of the PCR tubes throughout the protocol. Heat on the lid during incubations may smudge or remove the top label.

3. Protocol

STEP 1 - Cell Fixation

During *STEP 1* of the workflow, live cell suspensions are fixated using paraformaldehyde (PFA) and quenched with **Quenching Solution**. As an initial step, PCR tubes are blocked with BSA, which helps to reduce cell loss in *STEP 1* and *STEP 2*.

1.1 Blocking Tubes with BSA

PREPARATION: Prepare a fresh 30 ml solution of 0.5% BSA in 1x PBS. Store the solution at 4°C and use within 1 week. The BSA should be of ≥98% purity.

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 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).

1.2 Cell Preparation

PREPARATION: Prepare one PCR test tube with 15 µL 1x PBS and 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: Check visually 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.

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

A. ☐ Count the cells using either an automated cell counter (e.g., Countess II Automated Cell Counter) or hemocytometer. Fill in the viability and cell counts in *Appendix 1*.

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

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 supernatant.

E. ☐ Add 125 µl of **1x PBS** on top of the 25 µl sample, gently pipette up and down 10 times.

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 supernatant.

H. ☐ Add 55 µl of **1x PBS** on top of the 25 µl sample, gently pipette up and down 10 times.

1.3 PFA fixation

PREPARATION: Prepare a fresh 1 ml solution of 2% v/v PFA solution (methanol free) in 1x PBS. Use the solution within 2 hours, and store in dark until use.

NOTE: Use the necessary precautions when handling PFA solution since it is a CMR substance (Carcinogenic, Mutagenic, or toxic to Reproduction).

A. ☐ Add 80 µl of the **2% PFA solution** (final PFA concentration of 1%) to each 80 µl sample and pipette up and down 10 times.

B. ☐ Incubate for 15 min at RT.

C. ☐ Quench the PFA by adding 25 µl of **Quenching 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. ☐ From each 185 µl quenched sample, carefully discard 160 µl supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

F. ☐ Add 125 µl of **1x PBS + 0.5% BSA** on top of the 25 µl sample and pipette up and down 10 times.

Wash 1

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.

I. ☐ Add 125 µl **1x PBS + 0.5% BSA** on top of the 25 µl cell suspension and pipette up and down 10 times.

NOTE: At this step, if planning to continue directly with the protocol, proceed to STEP 2 - Barcoded Antibody Binding. If planning to store cells at 4°C or frozen at -80°C, continue with step J.

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.

L. ☐ Resuspend the pellet by adding 125 µl **1x PBS.** Total of 150 µl in each tube.

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 1xPBS for up to 5 days or frozen at -80°C (see optional below) until proceeding with STEP 2.

OPTIONAL: Freezing and thawing of fixed cells

This is an optional step for storage up to 3 months at -80°C. If planning to perform Antibody Binding within 5 days, please proceed to *STEP 2 - Barcoded Antibody Binding*.

1.4 Freezing of PFA-fixed cells

PREPARATION: Prepare a fresh 5 ml freezing solution of 5% DMSO and 95% FBS.

- **A.** ☐ Count the cells using either an automated cell counter or hemocytometer. Mix by pipetting up and down 10 times before taking an aliquot for counting.
- **B.** ☐ Centrifuge the samples at 700 rcf for 4 min at RT.
- **C.** ☐ Carefully discard 115 µl supernatant (if 10 µl was used for counting) without disturbing the cell pellet, leaving behind 25 µl supernatant.
- **D.** ☐ Add 125 µl **freezing solution** and pipette up and down 10 times.
- **E.** ☐ Transfer the 150 µl cell solution to a cryotube.
- **F.** ☐ Add 150 µl **freezing solution** to the empty PCR tube and pipette up and down 10 times to wash any remaining cells.
- **G.** ☐ Transfer the 150 µl wash to the cryotube. Total of 300 µl in each cryotube.
- **H.** ☐ Add 200 µl **freezing solution** to the 300 µl cell solution. Total of 500 µl in each tube
- **I.** ☐ 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 3 months.

1.5 Thawing of PFA-fixed, frozen cells

PREPARATION: Prepare a fresh 50 ml solution of 0.5% BSA in 1x PBS. Store the solution at 4°C and use within 1 week. The BSA should be of ≥98% purity.

PREPARATION: Prepare one 1.5 ml Eppendorf test tube with 50 µL 1x PBS. Keep aside and use as volume reference when ensuring all actual samples have equal volumes.

PREPARATION: Block one PCR tube per sample by following the steps in *1.1 Blocking tubes with BSA*.

- **J.** ☐ Put the frozen cryotubes in a 37°C bath for up to 5 min (confirm that the cell solution has thawed).
- **K.** ☐ Transfer the thawed 500 µl cell solution to a 1.5 ml Eppendorf tube.
- **L.** ☐ Add 500 µl **1x PBS + 0.5% BSA** solution to the empty cryotube and pipette up and down 10 times to wash any remaining cells.
- **M.** ☐ Transfer the 500 µl solution to the Eppendorf tube. Total of 1000 µl in each tube.

N. ☐ Centrifuge at 700 rcf for 4 min at RT.

Wash 1

O. ☐ Remove supernatant without disturbing the cell pellet (approximately 950 µl), leaving behind 50 µl.

P. ☐ Add 950 µl **1x PBS + 0.5% BSA** solution and pipette up and down 10 times.

Wash 2

Q. ☐ Centrifuge at 700 rcf for 4 min at RT.

R. ☐ Remove 950 µl supernatant without disturbing the cell pellet, leaving behind 50 µl.

S. ☐ Pipette up and down 10 times and transfer the 50 µl cell suspension to a newly BSA blocked PCR tube, per sample.

T. ☐ Add 100 µl **1x PBS + 0.5% BSA** solution to the empty Eppendorf tube and pipette up and down 10 times.

U. ☐ Transfer the 100 µl solution to the PCR tube. Total of 150 µl in each tube.

NOTE: At this step, if planning to continue directly with the protocol, proceed with STEP 2 - Barcoded Antibody Binding. If planning to store cells at 4°C, continue with step V.

V. ☐ Centrifuge at 700 rcf for 4 min at RT.

W. ☐ Remove 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl.

X. ☐ Add 125 µl **1x PBS** and pipette up and down 10 times. Total of 150 µl in each tube

Up to 24 hours at 4°C

NOTE: At this step, cells can be kept at +4°C storage for 24 hours.

STEP 2 - Barcoded Antibody Binding

Step 2 consists of blocking cells, binding of Barcoded Antibodies to respective protein targets, and their stabilization on cells using a secondary antibody. Cells are counted and diluted at the end of this part of the workflow prior to starting the Protein Proximity Network Generation.

IMPORTANT! From this step forward, make sure cells become resuspended during mixing steps by mixing vigorously (10 times in ~3 seconds). Too gentle pipette-mixing may result in cell aggregate formation.

2.1 Cell Blocking

A. ☐ Centrifuge the samples at 700 rcf for 4 min at RT.

B. ☐ Carefully discard 135 µl supernatant without disturbing the cell pellet, leaving behind 15 µl of supernatant. **IMPORTANT:** Be careful not to aspirate too much volume and use the 15 µl test tube to visually inspect the sample tubes to ensure they have equal volume. Adjust if necessary but do not disturb the pellet.

C. ☐ Thaw the **Blocking solution**, quick spin and vortex to mix.

D. ☐ Add 165 µl **Blocking Solution** and pipette up and down 10 times.

E. ☐ Incubate for 15 min at 4°C.

F. ☐ During the incubation, centrifuge the **Antibody Panel** vial at 17000 rcf for 10 min at RT or 4°C, to pull down any aggregates to the bottom of the vial. After centrifugation place the vial at 4°C until STEP 2.2

G. ☐ Centrifuge the blocked cells at 700 rcf for 4 min at RT.

H. ☐ Carefully discard 155 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

I. ☐ Add 125 µl of **1x PBS + 0.5% BSA** on top of the 25 µl sample and pipette up and down 10 times.

Wash 1

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 supernatant.

L. ☐ Add 125 µl of **1x PBS + 0.5% BSA** on top of the 25 µl sample.

M. ☐ Count the cells using either an automated cell counter or hemocytometer. Mix by pipetting up and down 10 times before taking an aliquot for counting.

2.2 Antibody binding

PREPARATION: Block one PCR tube per sample by following the steps in *1.1 Blocking tubes with BSA*.

NOTE: The supported cell input range is 50 000 - 250 000 cells. We recommend loading 250 000 cells per sample in 1xPBS + 0.5% BSA to a total volume of 150 µl (as described below).

A. ☐ For each sample, mix by pipetting up and down 10 times, and transfer 250 000 cells to the newly blocked PCR tube. Add **1x PBS + 0.5% BSA** for a total volume of 150 µl per sample. **IMPORTANT!** Use the same cell input across all the samples to minimize data output variation between samples.

B. ☐ Centrifuge at 700 rcf for 4 min at RT.

C. ☐ Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant. **IMPORTANT REMINDER!** Use the 25 µl test tube to visually inspect the sample tubes to ensure they have equal volume. Adjust if necessary but do not disturb the pellet. Equal volumes are important for the upcoming step.

D. ☐ Add 25 µl of the centrifuged **Antibody Panel** solution to each 25 µl sample (final volume of 50 µl), and pipette up and down 10 times. **IMPORTANT!** Do not aspirate near the bottom of the centrifuged Antibody Panel vial as it contains aggregates.

ON INCUBATION

E. ☐ Incubate overnight (16h-20h) at 4°C.

2.3 2nd antibody binding

A. ☐ Add 100 µl **1x PBS + 0.5% BSA solution** on top of each 50 µl overnight incubated sample and pipette up and down 10 times.

Wash 1

B. ☐ Centrifuge at 700 rcf for 4 min at RT.

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

D. ☐☐☐ Add 125 µl **1x PBS + 0.5% BSA solution** on top of the 25 µl sample and pipette up and down 10 times.

Wash 2, 3, 4

E. ☐☐☐ Centrifuge at 700 rcf for 4 min at RT.

F. ☐☐☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

G. ☐ Repeat steps D-F 2 more times, for a total of 4 washes (including A-C). It is important to perform 4 washes to fully remove any unbound antibodies present in the solution.

H. ☐ Quick spin the **2nd Antibody** and pipette it up and down 5 times to mix.

I. ☐ Add 25 µl of **2nd Antibody** to the 25 µl sample and pipette up and down 10 times.

J. ☐ Incubate for 30 min at 37°C.

Wash 5

K. ☐ Invert the **Wash Buffer** tube 5 times to mix. Add 100 µl **Wash Buffer** on top of the 50 µl cell suspension and pipette up and down 10 times. The wash buffer can be kept at RT throughout the entire protocol.

L. ☐ Centrifuge at 700 rcf for 4 min at RT.

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

Wash 6

N. ☐ Add 125 µl **Wash Buffer** on top of the 25 µl sample and pipette up and down 10 times.

O. ☐ Centrifuge at 700 rcf for 4 min at RT.

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

Q. ☐ Resuspend the cell pellet in 75 µl of **1x PBS** and pipette up and down 10 times. Total of 100 µl in each tube.

NOTE: Make sure to not use too big of a portion of the sample for counting, as that may result in a deficit of cell input to *STEP 3*.

R. ☐ Count the cells using either an automated cell counter or hemocytometer. Mix by pipetting up and down 10 times before taking an aliquot for counting.

Up to 2 days at 4°C

NOTE: At this step, cells can be stored at 4°C in 1xPBS for up to 2 days before proceeding with the next steps of the protocol.

STEP 3 - Protein Proximity Network Generation

In this step of the protocol, RCA products are created from the barcoded antibodies bound to each cell, followed by linking together neighbouring RCA products in spatial proximity using a gap-fill ligation reaction.
Reminder: quick spin all reagents and vortex when indicated. Pipette up and down 5 times to mix after preparing the Master mix.

3.1 Padlock Probes Hybridization

NOTE: The supported cell input range is 20 000 - 100 000 cells. We recommend loading 50 000 cells per sample in 1xPBS to a total volume of 100 µl (as described below).

A. ☐ For each sample, mix by pipetting up and down 10 times, and transfer 50 000 cells per sample to new PCR tubes. Add **1x PBS** for a total volume of 100 µl per sample. **MPORTANT!** Use the same cell input across all the samples to minimize data output variation between samples.

B. ☐ Centrifuge at 700 rcf for 4 min at RT.

C. ☐ Carefully discard 75 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

D. ☐ Thaw the **Padlock Oligos**, quick spin and vortex to mix.

E. ☐ Dispense 25 µl **Padlock Oligos** to each 25 µl sample and pipette up and down 5-6 times. Total of 50 µl in each tube.

F. ☐ Incubate the samples in a thermal cycler for 30 min at 45°C.

G. ☐ Remove the samples from the thermal cycler, add 100 µl **Wash Buffer** to each sample and pipette up and down 5-6 times. Total of 150 µl in each tube.

Wash 1

H. ☐ Centrifuge at 700 rcf for 4 min at RT.

I. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

J. ☐ Add 125 µl of **Wash Buffer** and pipette up and down 5-6 times.

Wash 2

K. ☐ Centrifuge at 700 rcf for 4 min at RT.

L. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

3.2 Gap-fill 1 (Padlock Probe Circularization)

A. ☐ Thaw the **Gap-fill Solution**, quick spin and vortex to mix**.** Prepare **Gap-fill** master mix as indicated in Table 5. directly before use by combining the reagents in the order listed below, maintain on ice or +4°C. If the following steps of the protocol are run on the same day, keep the **Gap-fill Solution** tube on ice or at 4°C, until used at the Gap-fill 2 step of the protocol (put the ligase and polymerase back at -20°C until the second Gap-fill step).

Table 5. **Gap-fill** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box2 -20°C	Gap-fill Solution	BD057	25.3 µl	101.2 µl	202.4 µl
Box2 -20°C	Gap-fill Ligase	EE052	1.1 µl	4.4 µl	8.8 µl
Box2 -20°C	Gap-fill Polymerase	EE053	1.1 µl	4.4 µl	8.8 µl
	Total		27.5 µl	110 µl	220 µl

B. ☐ Quick spin the **Gap-fill** master mix and pipette up and down 5 times to mix.

C. ☐ Put the samples on ice and dispense 25 µl **Gap-fill** master mix to each 25 µl sample and pipette up and down 5-6 times. Total of 50 µl in each tube.

D. ☐ Incubate the samples in a thermal cycler for 30 min at 25°C.

E. ☐ Add 100 µl of **Wash Buffer** to each sample at RT and pipette up and down 5-6 times. Total of 150 µl in each tube.

Wash 1

F. ☐ Centrifuge at 700 rcf for 4 min at RT.

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

H. ☐ Add 125 µl of **Wash Buffer** to each sample at RT and pipette up and down 5-6times. Total of 150 µl in each tube.

Wash 2

I. ☐ Centrifuge at 700 rcf for 4 min at RT.

J. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant.

Up to 3 days at 4°C

NOTE: At this step, cells can be stored at 4°C for up to 3 days before proceeding with the next steps of the protocol. If stopping here, remember to put the Gap-fill Solution back at -20°C.

IMPORTANT REMINDER! Visually inspect the sample tubes to ensure they have equal volume. Adjust if necessary but do not disturb the pellet. Equal volumes are important for the upcoming step.

3.3 Rolling Circle Amplification (RCA)

A. ☐ Thaw the **Proximity Oligos** and maintain at RT. This reagent will be needed during steps G-H.

B. ☐ Thaw the **RCA solution**, quick spin and vortex to mix. Prepare **RCA** master mix as indicated in Table 6. directly before use by combining the reagents in the order listed below, maintain on ice or +4°C.

Table 6. **RCA** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box2 -20°C	RCA Solution	BD046	24.75 µl	99 µl	198 µl
Box2 -20°C	RCA Polymerase	EE054	2.75 µl	11 µl	22 µl
	Total		27.5 µl	110 µl	220 µl

C. ☐ Quick spin the **RCA** master mix and pipette up and down 5 times to mix.

D. ☐ Put the samples on ice and dispense 25 µl **RCA** master mix to each 25 µl sample and pipette up and down 5-6 times. Total of 50 µl in each tube.

E. ☐ Incubate the samples in a thermal cycler for 15 min at 30°C.

F. ☐ Put the samples on ice directly after the incubation.

G. ☐ Quick spin the **Proximity Oligos** and vortex to mix.

H. ☐ Directly add 100 µl **Proximity Oligos** to each sample on ice and pipette up and down 5-6 times. Total of 150 µl in each tube.

I. ☐ Incubate the samples in a thermal cycler for 30 min at 45°C.

J. ☐ Centrifuge at 700 rcf for 4 min at RT.

K. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

L. ☐ Add 125 µl **Wash Buffer** and pipette up and down 5-6 times.

Wash 1

M. ☐ Centrifuge at 700 rcf for 4 min at RT.

N. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

O. ☐ Add 125 µl **Wash Buffer** and pipette up and down 5-6 times.

Wash 2

P. ☐ Centrifuge at 700 rcf for 4 min at RT.

Q. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

3.4 Gap-fill 2 (Linking of RCA Products)

A. ☐ Prepare **Gap-fill** master mix as indicated in Table 7. directly before use by combining the reagents in the order listed below, maintain on ice or +4°C.

Table 7. **Gap-fill** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box2 -20°C	Gap-fill Solution	BD057	25.3 µl	101.2 µl	202.4 µl
Box2 -20°C	Gap-fill Ligase	EE052	1.1 µl	4.4 µl	8.8 µl
Box2 -20°C	Gap-fill Polymerase	EE053	1.1 µl	4.4 µl	8.8 µl
	Total		27.5 µl	110 µl	220 µl

B. ☐ Quick spin the **Gap-fill** master mix and pipette up and down 5 times to mix.

C. ☐ Put the samples on ice and dispense 25 µl **Gap-fill** master mix to each 25 µl sample and pipette up and down 5-6 times. Total of 50 µl in each tube.

D. ☐ Incubate the samples in a thermal cycler for 30 min at 25°C.

E. ☐ Add 100 µl of **Wash Buffer** to each sample at RT and pipette up and down 5-6 times. Total of 150 µl in each tube.

Wash 1

F. ☐ Centrifuge at 700 rcf for 4 min at RT.

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

H. ☐ Add 125 µl **Wash Buffer** and pipette up and down 5-6 times.

Wash 2

I. ☐ Centrifuge at 700 rcf for 4 min at RT.

J. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

K. ☐ Add 75 µl of **Wash Buffer** to each sample and pipette up and down 5-6 times. Total of 100 µl in each tube.

Up to 3 days at 4°C

NOTE: At this step, cells can be stored at 4°C for up to 3 days before proceeding with the next steps of the protocol.

STEP 4 - PCR Amplification and Sample Indexing

In this step of the protocol, the generated amplicons are amplified by PCR and sample indexed in preparation for NGS sequencing.

4.1 Manual Cell Counting

GUIDANCE: See *Appendix 2* for more detailed information regarding cell counting. It is possible to proceed to PCR Amplification and Sample Indexing with 200 - 1000 cells if less than 1000 cells remain. Please note that the number of cells input to PCR will require different PCR cycles and will directly impact the total read requirement.

PREPARATION: Prepare 3 ml 10mM Tris pH 8 in Nuclease Free Water.

A. ☐ Count cells using a hemocytometer or a single-use counting chamber. Mix each sample by pipetting up and down 10 times before taking an aliquot for counting. To ensure accuracy, count cells using 2 separate aliquots to achieve duplicate counts.

B. ☐ For each sample, transfer 1000 cells in a new PCR tube and add **Wash Buffer** for a final volume of 20 µl. See *Appendix 2* for an aliquot example table. **NOTE:** When working with cell lines, PCR can be set up with 500 cells to decrease the total amount of reads required for sequencing. See step 5.3 *NGS Sequencing using the Illumina® platform*.

4.2 Pre-amplification PCR

NOTE: The PCR Master Mix may contain precipitates. Once thawed, pipette the PCR Master Mix up and down 10 times and avoid aspirating any small leftover precipitate when preparing the mix.

A. ☐ Pre-program a PCR system with the PCR program denoted in Table 9.

B. ☐ Thaw **Pre-amp Primers**, quick spin and vortex to mix. Thaw the **PCR Master Mix** vial, quick spin and pipette up and down 5 times to mix. Prepare **Pre-amplification PCR** master mix as indicated in Table 8. directly before use by adding the reagents in the order listed below, and maintain at RT until use.

Table 8. **Pre-amplification PCR** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box2 -20°C	Pre-amp Primers	IP049	5.5 µl	22 µl	44 µl
Box2 -20°C	PCR Master Mix	MM058	27.5 µl	110 µl	220 µl
	Total		33 µl	132 µl	264 µl

C. ☐ Quick spin the **Pre-amplification** master mix and pipette up and down 5 times to mix.

D. ☐ Dispense 30 µl **Pre-amplification PCR** master mix to each 20 µl sample containing cells and pipette up and down 5 times to mix, total of 50 µl in each tube.

E. ☐ Perform pre-amplification PCR using the following PCR program (Table 9.). If SPRI beads are stored in the fridge and not in RT: place the SPRI beads at RT 30 min before performing step 4.3.

Table 9. Pre-amplification PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	50 µl	~ 23 min	
Step	**Temperature**	**Time**	**Number of cycles**
1	98°C	00:10:00	1
2	98°C	00:00:15	7 cycles
3	67°C	00:00:20	
4	72°C	00:00:30	
5	72°C	00:01:00	1
6	4°C	Hold	

Up to 72 hours at +4°C
Long term storage at -20°C

NOTE: The pre-amplified PCR products can be stored at 4°C for up to 72 hours or at -20°C for long term storage.

4.3 Pre-amplification PCR clean-up

PREPARATION: Prepare 5 ml of freshly made 80% ethanol in Nuclease Free Water.

A. ☐ Vortex the SPRI beads bottle to resuspend magnetic particles that may have settled.

B. ☐ For each sample, transfer 25 µl of PCR product to fresh PCR tubes. The remaining volume can be stored at -20°C as backup.

C. ☐ Add 75 µl **10mM Tris pH 8** to each sample. Total of 100 µl in each tube.

D. ☐ Add 60 µl of resuspended SPRI bead mixture to each sample. Pipette up and down 10 times to mix or until the mixture becomes homogenous.

E. ☐ Incubate for 5 min at RT.

F. ☐ Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall. Do not discard the supernatant.

G. ☐ While on magnet, carefully **transfer** the supernatant to a new PCR tube, without disturbing the beads. Discard the empty tubes containing beads.

H. ☐ Vortex the SPRI beads bottle to resuspend magnetic particles that may have settled and add 40 µl of resuspended SPRI bead mixture to each sample. Pipette up and down 10 times to mix or until the mixture becomes homogenous.

I. ☐ Incubate for 5 min at RT.

J. ☐ Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.

K. ☐ While on magnet, carefully **discard** the supernatant, without disturbing beads.

L. ☐☐ While on the magnet, add 180 µl of 80% ethanol to the bead pellet and incubate for 30 sec.

M. ☐☐ Carefully discard the supernatant, without disturbing beads.

N. ☐ Repeat steps L-M for a total of two washes.

O. ☐ Quick-spin the samples briefly. Place the tubes on the magnet and remove any remaining ethanol with a P10 pipette.

NOTE: Avoid over-drying the beads as this could negatively affect DNA recovery. The dried patch of beads will show some cracks if over-dried.

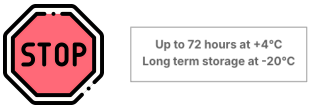
P. ☐ Remove the PCR tubes from the magnet.

Q. ☐ Resuspend beads by adding 31 µl of **10mM Tris pH 8** and pipette up and down 10 times to mix or until the mixture becomes homogenous.

R. ☐ Incubate for 1 min at RT to elute the PCR product from the beads.

S. ☐ Place the PCR tube on the magnet and wait until the beads have settled against the tube wall.

T. ☐ The supernatant now contains the eluted, pre-amplified product. While on magnet, carefully **transfer** the 30 µl supernatant to new PCR tubes, without disturbing beads. Discard the empty tubes with beads.



NOTE: The clean pre-amplified PCR products can be stored at 4°C for up to 72 hours or at -20°C for long term storage.

4.4 Sample Index PCR

Sample indices are introduced during PCR, allowing for pooling and sequencing multiple reactions in the same sequencing run. Please see section 2. Workflow and Guidelines: *Sample Indexing* for a more detailed description and considerations related to sample indexing.

IMPORTANT! Make sure to use different index primers for each sample and note which sample index was used for each sample.

A. ☐ Pre-program a PCR system with the PCR program denoted in Table 10.

B. ☐ Thaw the **PCR index primers** needed (box 3), quick spin and vortex to mix. Thaw the **PCR Master Mix** vial, quick spin and pipette up and down 5 times to mix.

C. ☐ For each sample, transfer **5 µl** of pre-amplified, purified sample to fresh PCR tubes. The remaining volume can be stored at -20°C as backup.

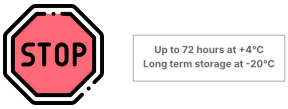
D. ☐ Add 20 µl of one unique **PCR index primer** (box 3) to each 5 µl sample. Make a note of the primer index used for each sample.

E. ☐ Add 25 µl of **PCR Master Mix** and pipette up and down 10 times to mix. Total of 50 µl per sample.

F. ☐ Perform PCR using the following PCR program (Table 10.).

Table 10. PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	50 µl	~ 15 min	
Step	**Temperature**	**Time**	**Number of cycles**
1	98°C	00:01:00	1
2	98°C	00:00:15	1000 cells: 7 cycles 500 cells: 8 cycles 200 cells: 9 cycles
3	63°C	00:00:20	
4	72°C	00:00:30	
5	72°C	00:01:00	1
6	4°C	Hold	



NOTE: The PCR products can be stored at 4°C for up to 72 hours or at -20°C for long term storage.

4.5 Sample Index PCR clean-up

The PCR products are purified using SPRI beads prior to NGS sequencing to remove primers, salts, dNTPs etc.

PREPARATION: Prepare 5 ml of freshly made 80% ethanol in Nuclease Free Water.

A. ☐ Vortex the SPRI beads bottle to resuspend magnetic particles that may have settled.

B. ☐ Add 50 µl of resuspended SPRI bead mixture to each sample (total volume of 100 µl). Pipette up and down 10 times to mix or until the mixture becomes homogenous.

C. ☐ Incubate for 5 min at RT.

D. ☐ Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.

E. ☐ While on magnet, carefully **discard the supernatant**, without disturbing the beads.

F. ☐☐ Add 180 µl of 80% ethanol to the bead pellet while on the magnet and incubate for 30 sec.

G. ☐☐ While on magnet, carefully discard the supernatant, without disturbing beads.

H. ☐ Repeat steps F-G for a total of two washes.

I. ☐ Quick-spin the samples briefly. Place the tubes on the magnet and remove any remaining ethanol with a P10 pipette.

NOTE: Avoid over-drying the beads as this could negatively affect DNA recovery. The dried patch of beads will show some cracks if over-dried.

J. ☐ Remove the PCR tubes from the magnet.

K. ☐ Resuspend beads by adding 31 µl of **10mM Tris pH 8** and pipette up and down 10 times to mix or until the mixture becomes homogenous.

L. ☐ Incubate for 1 min at RT to elute the PCR product from the beads.

M. ☐ Place the PCR tube on the magnet and wait until the beads have settled against the tube wall.

N. ☐ The supernatant now contains the eluted, final product. While on magnet, carefully **transfer** the 30 µl supernatant to new PCR tubes, without disturbing beads. Discard the empty tubes with beads.

NOTE: The clean PCR products can be stored at 4°C for up to 72 hours or at -20°C for long term storage.

STEP 5 - NGS Preparation

5.1 Quality Control and Quantification

Before proceeding to NGS sequencing, samples need to be:

- ☐ Quality controlled (to confirm the correct size of approximately 277 bp)
- ☐ Quantified, diluted and pooled

Table 11. below describes what kits and instruments that can be used for this purpose. It is important to do both QC and quantification, i.e. if an instrument is used that only fulfills one of the purposes, another instrument needs to be included to perform the other.

Table 11. List of instruments that can be used for product quality control and/or quantification.

Instrument	Quality control	Quantification
BioAnalyzer™ with a High Sensitivity DNA kit (Agilent)	✅	❌
Fragment Analyzer™	✅	❌
TapeStation™	✅	❌
Qubit™ 4 Fluorometer with the Qubit™ dsDNA High Sensitivity (HS) Assay Kit (ThermoFisher)	❌	✅
Gel Electrophoresis with TBE gel, 200V for 25 min	✅	❌

The size of the generated PCR product is approximately 277 bp. To convert a concentration reading from ng/µl to nM, a conversion factor of 5.45 can be used:

Library concentration [ng/µl] x 5.45 = X nM

5.2 Pooling

Equimolar pooling of indexed samples is recommended in most cases. This can e.g. be achieved by diluting each sample to the same concentration, followed by pooling equal volumes of each normalized sample (see *Appendix 3* for details and an equimolar pooling calculation example). Creating a 5 nM pool is typically sufficient for most Illumina platforms. For additional information, see Illumina guidelines for normalizing library concentrations [1].

5.3 NGS Sequencing using the Illumina® platform

The sequenced amplicons contain regions of low diversity sequences, which can cause a quality drop on Illumina sequencing systems. Therefore, each sequenced library should be spiked with **15% PhiX**. Please consult Illumina documentation for PhiX spiking.

The **loading concentration** for Illumina sequencing depends on the sequencing platform used. Illumina offers recommendations for loading concentrations for different library types.

- We recommend following Illumina's guidelines for the loading concentration of "PhiX libraries".

Sequencing requirements are dependent on cell type. Cells with high surface protein expression will have higher sequencing requirements to ensure well connected cell networks and overall data quality. We recommend sequencing:

- PBMC samples at **250 000 reads/cell**, excluding PhiX (250M total reads per 1000 cells)
- Cell line samples at **500 000 reads/cell**, excluding PhiX (500M total reads per 1000 cells or 250M total reads per 500 cells).

Sequencing on NextSeq® 1000/2000 has been validated. **NOTE:** this read recommendation is for first time users and can later be titrated to your specific use case.

Table 12. denotes the recommended sequencing parameters. 44 cycles for read1 and 78 cycles for read2 are minimum values. Additional cycles may be used for read1 and read2 and will result in trimming of the additional bases during downstream Pixelator data processing. **An example** on how to calculate the total number of reads needed for an experiment can be found in *Appendix 3*, together with **sample index sequences.**

Table 12. Sequencing parameters.

Sequencing depth	PBMCs: 250 000 reads/cell Cell lines: 500 000 reads/cell
Sequencing type	Paired-end
Paired-end sequencing read configuration (minimum values)	Read1: 44 cycles Read2: 78 cycles i7 index: 8 cycles i5 index: 8 cycles
PhiX	15%

Please see Pixelator tutorials [2] for description on data processing.

4. References

1. https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001252

2. https://software.pixelgen.com

Appendix

Appendix 1

Counting Tables

Table A1. Cell counts and viability per sample.

	STEP 1 Before fixation				STEP 2 Before Antibody Binding		
Sample	Cell conc.	Volume	Cell count	Viability	Cell conc.	Volume	Cell count
Example	$7*10^6$ cells/mL	150 µL	$7*10^6*10^{-3}*150$ = 1 050 000 cells	95%	$5*10^6$ cells/mL	150 µL	$5*10^6*10^{-3}*150$ = 750 000 cells
1							
2							
3							
4							
5							
6							
7							
8							
...							

Table A2. Cell counts per sample.

	End of STEP 2 Antibody Binding			End of STEP 3 Protein Proximity Network Creation		
Sample	Cell conc.	Volume	Cell count	Cell conc.	Volume	Cell count
Example	$1*10^6$ cells/mL	100 µL	$1*10^6*10^{-3}*100$ = 100 000 cells	$3*10^5$ cells/mL	100 µL	$3*10^5*10^{-3}*100$ = 30 000 cells
1						
2						
3						
4						
5						
6						
7						
8						
...						

Appendix 2

Cell counting considerations

Counting of cells before fixation and after antibody binding, can be performed using either a hemocytometer or an **automated cell counter** as the cell numbers are higher during these stages of the workflow.

However, it is important to accurately count and aliquot cells in STEP 4 prior to pre-amplification, as these products will be taken forward to sequencing and inaccurate cell numbers may result in poor data quality due to insufficient sequencing read amounts. Due to the low cell numbers and concentration at this stage, we have found several automatic cell counters such as the Countess Automated cell counter (ThermoFisher) to **not** be reliable.

We therefore highly recommend using **manual counting** with a hemocytometer or single-use counting chamber when counting cells prior to the pre-amplification PCR step of the workflow. Follow counting guidelines associated with the hemocytometer or single-use counting chamber type used. In our experience, not all single-use counting chambers work accurately and we strongly recommend to use the suggested product in Table 2a. if using a single-use hemocytometer. To ensure accurate counting, duplicate counts for each reaction is recommended.

Aliquoting before pre-amplification PCR: example

Table A3. displays an example of two samples with concentrations of 160 and 200 cells/µl and how to dilute them in Wash buffer to achieve 1000 cells per reaction in 20 µl total.

Table A3. Example table of how to dilute cells in STEP 4, to achieve 1000 cells per reaction for sequencing.

Sample	Cell concentration	Sample volume needed	Wash buffer needed	Total
Sample 1	160 cells/µl	1000/160 = 6.25 µl	20-6.25 = 13.75 µl	6.25+13.75 = 20 µl
Sample 2	200 cells/µl	1000/200 = 5 µl	20-5 = 15 µl	5+15 = 20 µl

Appendix 3

QC of NGS library size

The expected size of the PCR product is 277 bp. The presence of PCR products of the intended size can be confirmed using either gel electrophoresis or fragment analyzing assays.

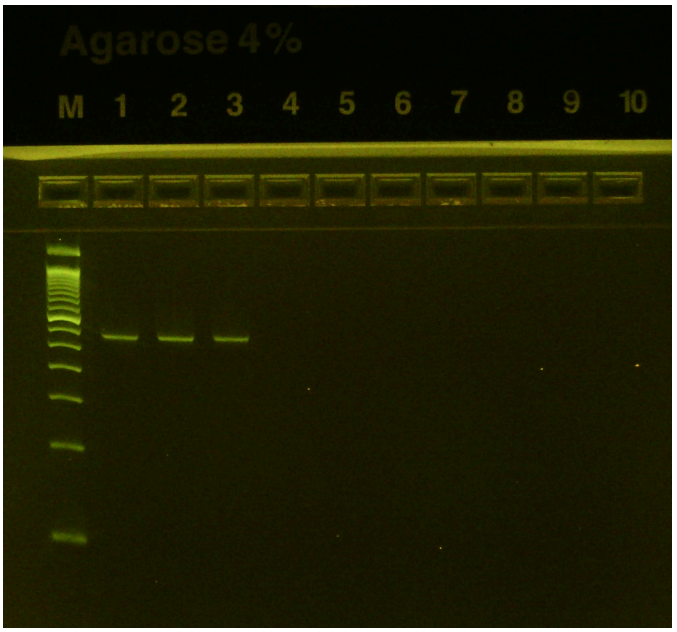


Figure A1. Quality control example of a successfully amplified PCR product. E-Gel Ex 4% Agarose Gel. Lane 1: unstimulated PBMCs, Lane 2: PHA stimulated PBMCs, Lane 3: PMA stimulated PBMCs. Lane M: Ladder, 20ul of 50bp DNA ladder (Thermo Fisher, 10416014), diluted 1:50.

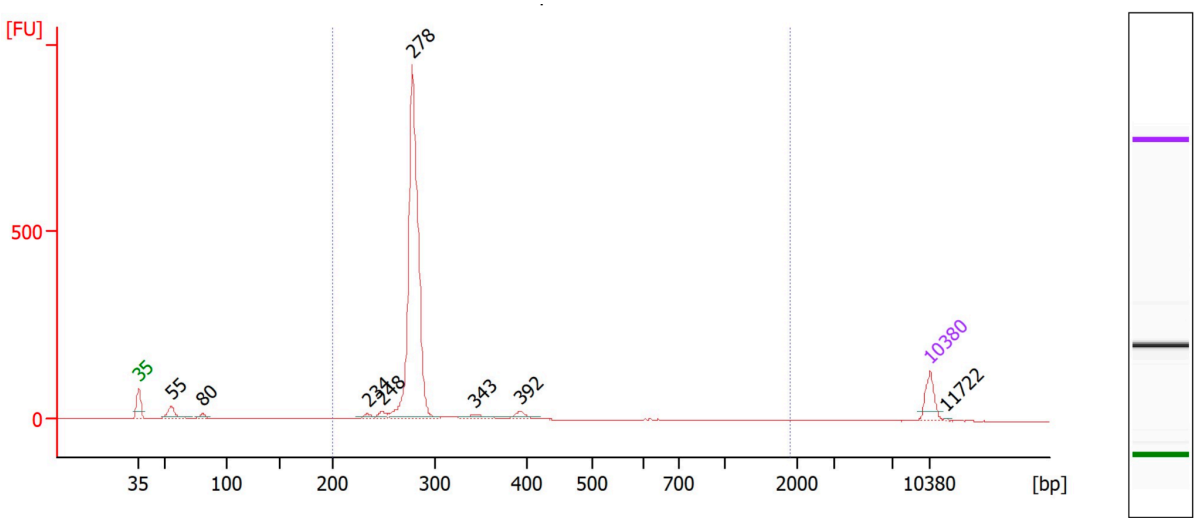


Figure A2. Quality control example using **BioAnalyzer™, High Sensitivity DNA kit (Agilent)**, showing approximately the expected size of the final PCR product.

Sequencing read calculation: example

Table A4. describes how to calculate the total number of reads necessary for your experiment, using an example of 1 or 8 reactions with 1 000 cells per reaction. Make sure to select an Illumina sequencing reagent kit that provides enough reads to satisfy reads/cell requirements for the total number of cells sequenced. Illumina flowcell output is sometimes specified by both their single-end read and paired-end read output. One single end read = 1 cluster = 1 read pair = 2 paired end reads. The read/cell requirements in Table 1 refers to the single-end read output. Contact support@pixelgen.com if you have any questions.

Table A4. Sequencing calculation example for an experiment of 1 or 8 reactions with 1 000 cells per reaction.

Nr of reactions	Reads/cell	Cells/reaction	Total nr of cells	Total nr of reads needed
1	250 000	1000	1×1 000 = 1 000	1 000×250 000 = 250M
8	250 000	1000	8×1 000 = 8 000	8 000×250 000 = 2B

Pooling strategies for NGS library

There are two strategies for pooling samples that can affect how the sequencing reads are allocated between each sample:

1. **Equimolar pooling (recommended):** An equal number of moles are pooled together for each sample, resulting in an equal number of sequenced reads for each sample, regardless of the number of unique molecules present in each sample. Each sample is quantified and diluted to the same concentration separately, before pooled together.

2. **Equivolume pooling:** An equal volume of purified PCR product is pooled together for each sample. The pool is then quantified and diluted for sequencing. This strategy will result in an equal number of reads per unique molecule, regardless of the sample, which means that the number of sequencing reads per sample will be proportional to the number of unique molecules.

Equivolume pooling strategy (2) can result in insufficient read depth for some samples if there is a high variability in the number of unique molecules between the samples. It is therefore recommended to perform equimolar pooling (1) which ensures equal sequencing read distribution per sample.

Table A5. Dilution example for equimolar pooling, first creating 5nM sample dilutions of 20uL, followed by creating a 5nM pool by combining 10uL of each 5nM sample dilution (in this example, a final pool volume of 40uL).

Sample	Conc. ng/uL	Conc. nM	Sample needed for 5nM dilution	Tris buffer needed	Volume from each sample to pool
Sample 1	3 ng/uL	3*5.45=16.35 nM	5*20/16.35=6.1uL	20-6.1=13.9uL	10uL
Sample 2	4 ng/uL	4*5.45=21.8 nM	5*20/21.8=4.6uL	20-4.6=15.4uL	10uL
Sample 3	5 ng/uL	5*5.45=27.25 nM	5*20/27.25=3.7uL	20-3.7=16.3uL	10uL
Sample 4	3 ng/uL	3*5.45=16.35 nM	5*20/16.35=6.1uL	20-6.1=13.9uL	10uL
...					

Sample index sequences

Table A6. List of sample indexes supplied with the reagent kit.

index name	i7 index	i5 index* (forward)	i5 index* (reverse complement)
Primer 1	TGGTTGTT	AACTGTAT	ATACAGTT
Primer 2	TGTACCTT	CAGGCATT	AATGCCTG
Primer 3	TCTGCTGT	AAGGCGAT	ATCGCCTT
Primer 4	TTGGAGGT	GCGTCGAA	TTCGACGC
Primer 5	TCGAGCGT	GAACGACA	TGTCGTTC
Primer 6	TGATACGT	GGCAAGCA	TGCTTGCC
Primer 7	TGCATAGT	GTAACCGA	TCGGTTAC
Primer 8	TGCGATCT	GCTATGGA	TCCATAGC
Primer 9	TTCCTGCT	GACACTTA	TAAGTGTC
Primer 10	TACAGGAT	GGTTGGAC	GTCCAACC
Primer 11	TGTGGTTG	TCAGATTC	GAATCTGA
Primer 12	TTCCATTG	TATGCCAG	CTGGCATA
Primer 13	TAACGCTG	TGGCTCAG	CTGAGCCA
Primer 14	TTGGTATG	TCATTGAG	CTCAATGA
Primer 15	TGAACTGG	TGTATGCG	CGCATACA
Primer 16	TACTTCGG	TCCAGTCG	CGACTGGA
Primer 17	TCCAGTCG	TACTTCGG	CCGAAGTA
Primer 18	TGTATGCG	TGAACTGG	CCAGTTCA
Primer 19	TCATTGAG	TTGGTATG	CATACCAA
Primer 20	TGGCTCAG	TAACGCTG	CAGCGTTA
Primer 21	TATGCCAG	TTCCATTG	CAATGGAA
Primer 22	TCAGATTC	TGTGGTTG	CAACCACA
Primer 23	GGTTGGAC	TACAGGAT	ATCCTGTA
Primer 24	GACACTTA	TTCCTGCT	AGCAGGAA

* The forward orientation of i5 indices should be used in most cases, unless manually creating a sample sheet to be analyzed with bcl2fastq. For guidance on which orientation to use, we refer to the Illumina resource:

https://support-docs.illumina.com/SHARE/AdapterSequences/Content/SHARE/AdapterSeq/Overview.htm

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