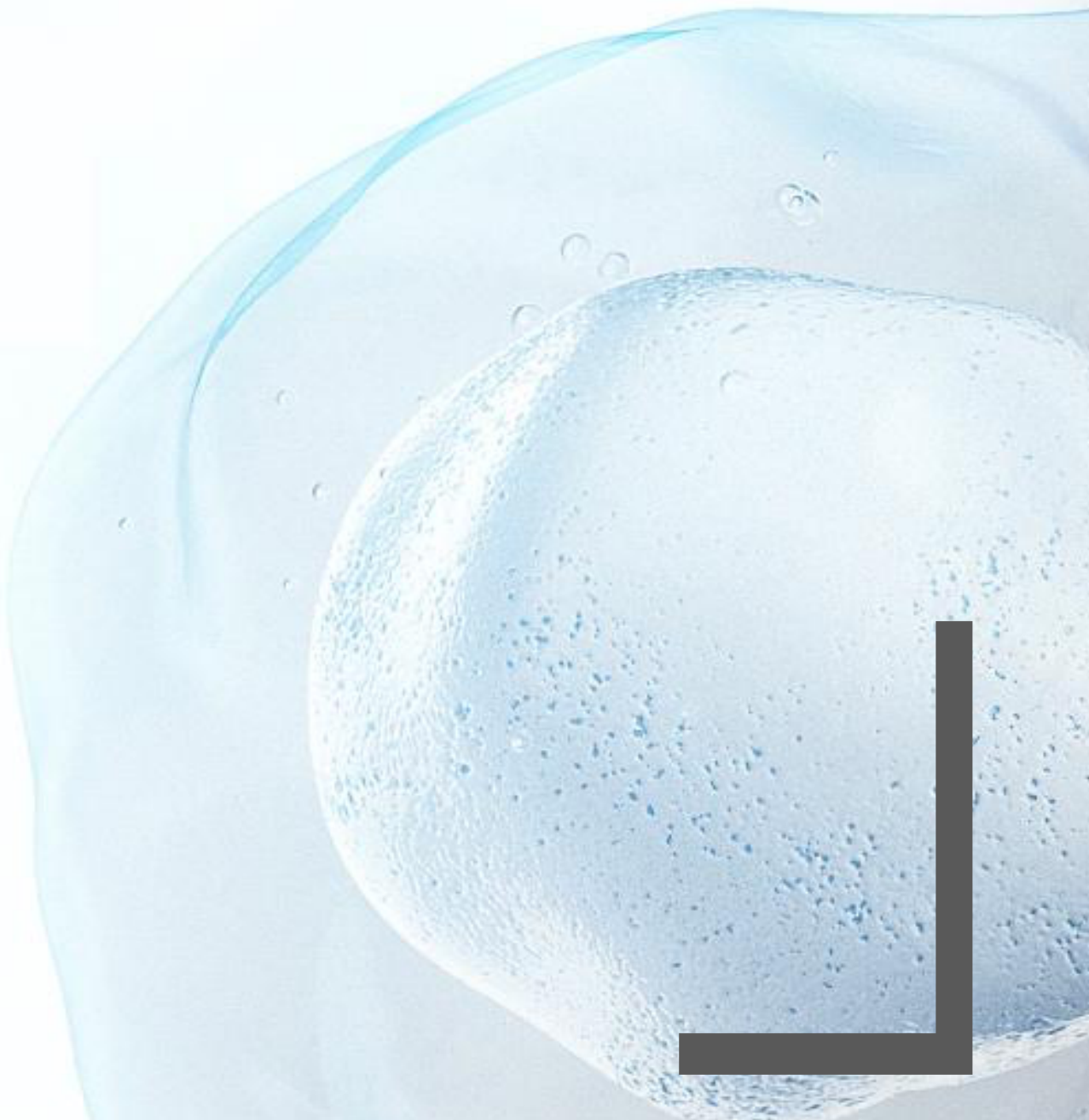
USER MANUAL

PIXELGEN PROXIOME KIT v2

IMMUNO 155 v2

Proximity Network Assay for Cell Surface Analysis

PROXIMM0028, PROXIMM00216, PROXIMM00232



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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
RCA	Rolling Circle Amplification
RCP	Rolling Circle Product
RT	Room Temperature
SPRI	Solid Phase Reversible Immobilization
UMI	Unique Molecular Identifier

About this User Manual

This User Manual describes the experimental procedure using the Pixelgen Proxiome Kit v2, Immuno 155 v2 (PROXIMM0028, PROXIMM00216, PROXIMM00232) in detail.

Technical Support

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

1. Product Description

The intended use of the Pixelgen Proxiome Kit v2, Immuno 155 v2, 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 neighboring 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 Pixelgen Proxiome Kit v2 is available in three configurations, sized by the number of reactions (samples) that can be processed:

- 8 reactions, product number PROXIMM0028
- 16 reactions, product number PROXIMM00216
- 32 reactions, product number PROXIMM00232

Following PFA fixation, samples are hashtag-stained in groups of 8 and combined into a pool. Consequently, the 8-reaction configuration yields 1 pool, while 16- or 32- reaction workflows yield 2 and 4 pools, respectively.

The core steps of the Proximity Network Assay by the Proxiome Kit v2 are illustrated below.

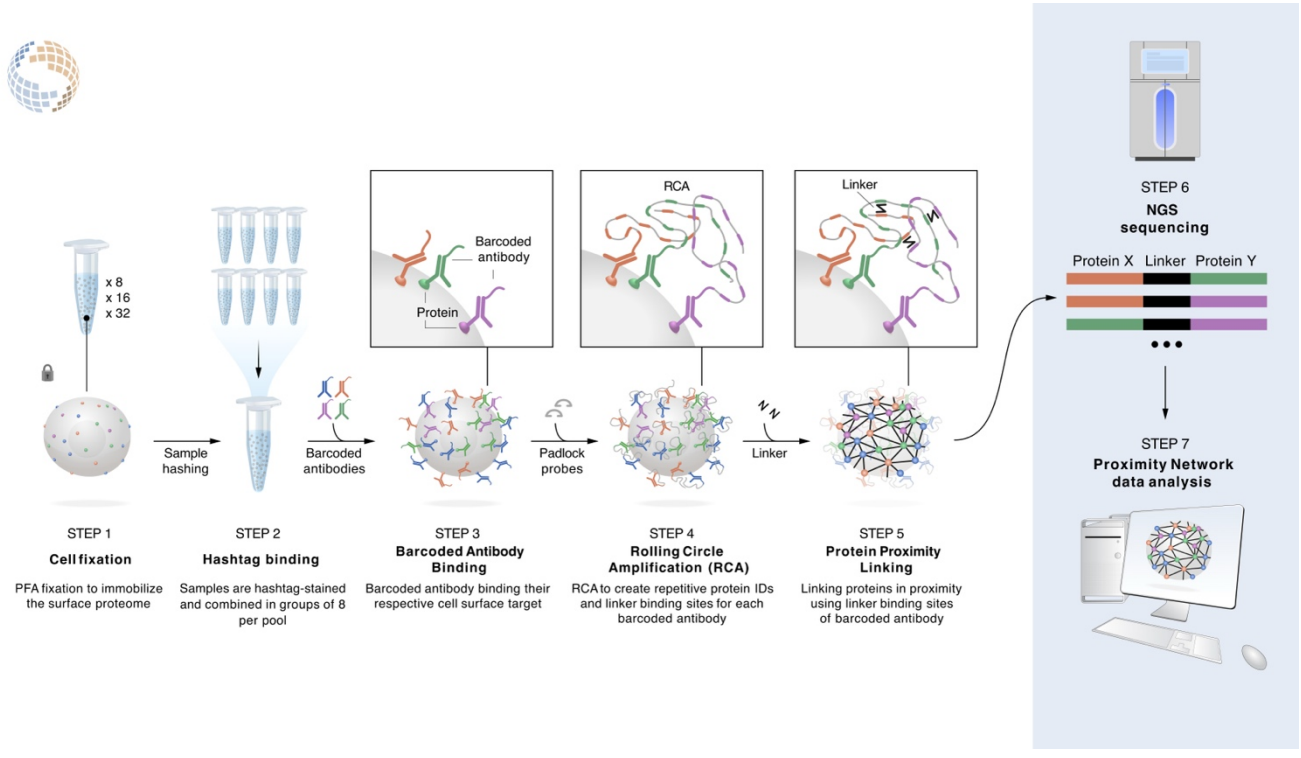


Figure 1. Overview of the Proximity Network Assay workflow and core steps.

Reagents supplied with the Pixelgen Proxiome Kit v2

Reagents included in the kit are listed below in Table 1. Each kit contains sufficient reagents for processing the designated number of reactions depending on the selected configuration. The reagents are supplied in four individual boxes, with storage temperature and expiration date stated on the label of each box. The kit includes one Akura™ Spheroid Microplate (two plates in the 32-reaction kit configuration) specifically designed to minimize pellet interaction and reduce the required cell input. The additional plate in the 32-reaction kit configuration provides flexibility for customers processing and fixing samples over two days. This plate is used in the first part of the protocol as described below.

> **NOTE:** All reagents for the Pixelgen Proxiome Kit v2, Immuno 155 v2, are lot specific and reagents from different kit lots should not be combined.

Product number: PROXIMM0028, PROXIMM00216, PROXIMM00232

Table 1. Reagents supplied in the individual kit boxes and their storage temperatures. Bullet point colors correspond to the reagent lid color. The chosen configuration will be specified on each reagent included in the kit.

Box 1 store at 4°C	Box 2 store at 4°C	Box 3 store at -20°C	Box 4 store at -20°C
● Hashtag Set 1 – PP099_1	● Antibody Panel – PP101	● Padlock Oligos – BD085	● Blocking Solution (1 tube) – BD093 *
● Hashtag Set 2 – PP099_2	● 2nd Antibody – PP100	● Gap-fill Solution – BD091	● Blocking Solution (2 tubes) – BD093 **
● Hashtag Set 3 – PP099_3	● Wash Buffer – BD084	● Gap-fill Ligase – EE088	● Blocking Solution (4 tubes) – BD093 ***
● Hashtag Set 4 – PP099_4	● Quenching Solution – BD094	● Gap-fill Polymerase – EE089	● Pre-amp Primers – IP086
● Hashtag Set 5 – PP099_5		● RCA Solution – BD095	● PCR Master Mix – MM092
● Hashtag Set 6 – PP099_6		● RCA Polymerase – EE090	● Primers 1 – IP087_1 *
● Hashtag Set 7 – PP099_7		● Proximity Oligos – BD096	● Primers 2 and 3 – IP087_2 and IP087_3 **
● Hashtag Set 8 – PP099_8		● Digestion Solution – BD097	● Primers 4-5-6 and 7 – IP087_4, IP087_5, IP087_6 and IP087_7 ***
		● Digestion Enzymes – EE098	

* Reagents included in the 8-reaction kit configuration, which yields 1 pool.
** Reagents included in the 16-reaction kit configuration, which yields 2 pools.
*** Reagents included in the 32-reaction kit configuration, which yields 4 pools.

The Akura™ Spheroid Microplate is a registered trademark owned by InSphero AG.

Additional requirements

A list of equipment, reagents and consumables required to perform the Proximity Network Assay can be found below. The suggested suppliers and part numbers noted are equivalent to equipment used during optimization and validation of the assay or those verified through proven field performance.

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 rcf)	Centrifuge 5418 R - microcentrifuge	Eppendorf	5418 R
Centrifuge PCR tubes (1000 rcf)	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
Single pipettes: ● 0.5 - 2.5 µl ● 2 - 20 µl ● 20 - 200 µl ● 100 - 1000 µl	Research® plus	Eppendorf (Avantor)	613-5893 613-5928
Multichannel pipettes: ● 0.5 - 10 µl ● 10 - 100 µl ● 30 - 300 µl		Eppendorf	3125000010 3125000036 3125000060
Reagent reservoirs	Corning® Costar reagent reservoirs	Sigma-Aldrich®	CLS4870-200EA
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

Automated cell counter or	Countess 3 FL automated cell counter or	Invitrogen	AMQAF2000
or	LUNA-BX7 automated cell counter	Logos Biosystems	LOG-L90001
Hemocytometer or	Counting Chambers Bürker-Türk	Karl Hecht Assistent®	40445
Single-use hemocytometer	Millicell® Disposable Hemocytometer	Sigma-Aldrich®	MDH-2N1-50PK

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

Consumables			
Description	Product name	Suggested Supplier	Part 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
1.5 ml low adhesion tube	Eppendorf Tubes® low adhesion	BIOplastics™	B74030
Pipette tips: 10 µl 20 µl 200 µl 1000 µl	OMNITIP™ Sterile, filter tips	ULPlast Sp.z.o.o.	 83240 86240 81240 85240
Parafilm	Parafilm® M	VWR	291-0057

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

Reagents			
Description	Product name	Suggested Supplier	Part 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
Magnetic beads	SPRIselect, 60 ml or AMPure XP beads	Beckman-Coulter Life science	B23318 A63880
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)	Thermo Fisher	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.

** Required for the OPTIONAL: Freezing and thawing of fixed cells, *STEP 1.5 - Freezing of PFA-fixed cells*.

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 resting- and stimulated- PBMCs, as well as leukemia and lymphoma cell lines.

Workflow overview and steps

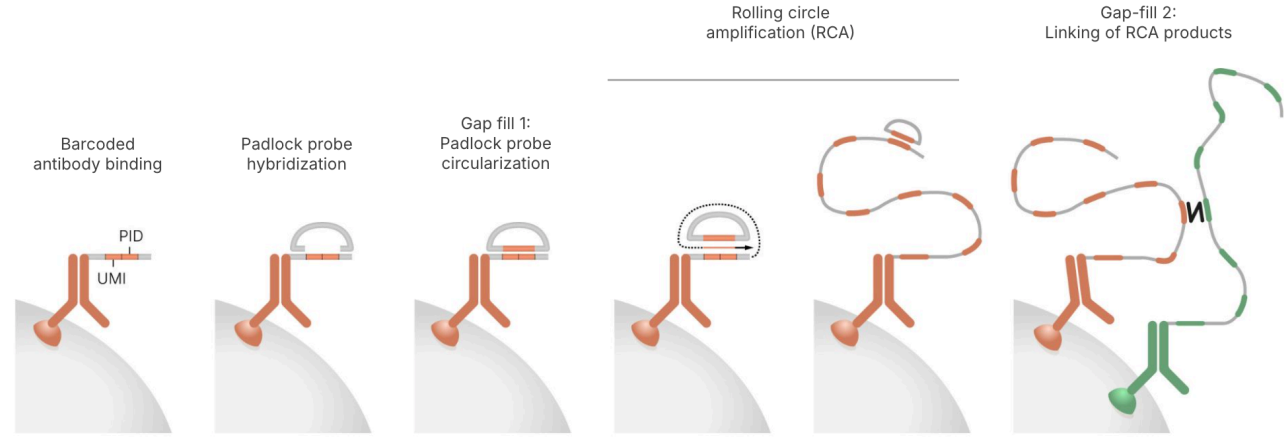


Figure 2a. Molecular description of core steps in the Proximity Network Assay.

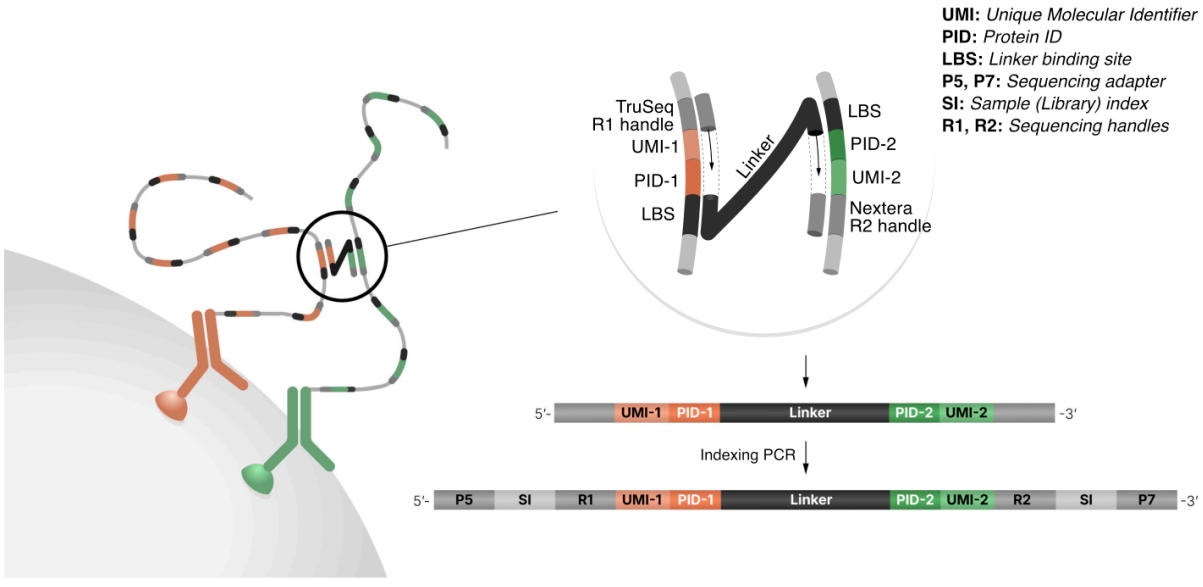


Figure 2b. Zoom-in on Gap-fill 2 (Linking of RCPs) and PCR steps in the Proximity Network Assay.

Table 3. Workflow steps and time needed.

Step	Description	Duration (16-reaction kit configuration)
1	**Cell Fixation:** **1.1** Preparation of the Akura™ Spheroid Microplate (practice) **1.2** Blocking wells of the Akura™ Spheroid Microplate **1.3** Cell preparation (in Akura™ Spheroid Microplate) **1.4** PFA fixation (in Akura™ Spheroid Microplate)	4-6 h (Duration might vary depending on the number of processed reactions)
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	**Hashtag Binding:** **2.1** Cell blocking (in Akura™ Spheroid Microplate) **2.2** Hashtag binding (in Akura™ Spheroid Microplate) **2.3** Hashtag-stained sample pooling (in PCR tubes)	3 h
3	**Barcoded Antibody Binding:** **3.1** Barcoded antibody binding **3.2** 2nd antibody binding	ON + 1 h
STOP	– Alt. stopping point: Up to 2 days at 4°C	
4	**Protein Proximity Network Generation:** **4.1** Padlock probes hybridization **4.2** Gap-fill 1 (padlock probe circularization)	4 h
STOP	– Alt. stopping point: Up to 3 days at 4°C	
	4.3 Rolling circle amplification (RCA) **4.4** Gap-fill 2 (linking of RCA products)	
STOP	– Alt. stopping point: Up to 3 days at 4°C	
5	**PCR Amplification and Indexing:** **5.1** Manual cell counting **5.2** Enzymatic digestion	4 h
STOP	– Alt. stopping point: Up to 3 days at 4°C	
	5.3 Pre-amplification PCR **5.4** Pre-amplification PCR clean-up	
STOP	– Alt. stopping point: -20°C for long periods of time	
	5.5 Indexing PCR **5.6** Indexing PCR clean-up	
STOP	– Alt. stopping point: -20°C for long periods of time	
6	**NGS Preparation:** **6.1** Quality control and quantification **6.2** Library pooling **6.3** Sequencing using Illumina® platforms	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-blocked wells of the Akura™ Spheroid Microplate and PCR tubes during fixation, hashtag binding and barcoded antibody binding to retain enough cells. The cell input range to *STEP 5 - PCR Amplification and Indexing* needs to be a maximum of 8 000 cells to ensure software compatibility. An input of 8 000 cells per pool is recommended to ensure high statistical power during data analysis by providing at least 1 000 cells of each of the 8 hashed samples within the pool. Automated cell counters are suitable for all counting steps, however, manual counting is recommended for STEP 5 to increase accuracy.

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

NOTE: The *STEP 2.2 - Hashtag Binding* supports up to 1 000 000 cells per sample. If you do not plan to freeze any remaining cells, you can proceed directly through STEP 2.2 without any intermediate cell counting step and count directly at *STEP 2.3 Hashtag-stained sample pooling*.

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	50 000 - 1 000 000 cells	50 000 - 1 000 000 cells	Automated cell counter OK
2.2 Hashtag Binding	30 000 - 1 000 000 cells	30 000 - 1 000 000 cells	Not required if not freezing remaining cells
2.3 Hashtag-stained sample pooling	80 000 - 250 000 cells	250 000 cells*,**	Automated cell counter OK
4. Protein Proximity Network Generation	40 000 - 100 000 cells	50 000 cells*	Automated cell counter OK
5. PCR Amplification and Indexing	1 000 - 8 000 cells*	8 000 cells	Recommended manual cell counting***

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

** High precision is required during this counting step as inaccuracies will directly impact the final sample proportion representation

*** Automated cell counter is also supported in this step

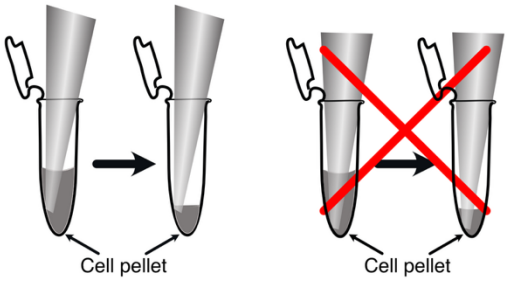
Indexing

During the Indexing PCR step (STEP 5.5), each pool is assigned with unique index sequences, converting it into a sequencing-ready NGS library. Each NGS library can then be combined with other NGS libraries for multiplexed sequencing on the same Illumina flow cell (*STEP 6.2 - Library pooling*). It is critical that each NGS library within the same sequencing run is assigned with unique index to ensure correct demultiplexing.

See Appendix 3 for a complete list of index sequences. When combining Proxiome NGS libraries with externally generated libraries, verify that all indices are unique across the entire sequencing run to prevent index collisions.

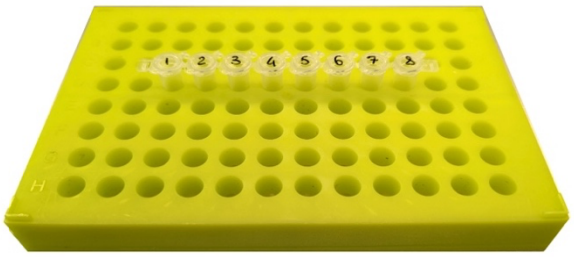
Important instructions

- 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].
- The Akura™ Spheroid Microplate design minimizes the interaction with the cell pellet, allowing for efficient use of **multichannel pipettes**. When discarding liquid, use an oblique (tilted) angle with tips against the inner ledge to avoid pellet disturbance. When pipetting reagents and washing buffers, hold the pipette vertically to ensure complete cell resuspension.
- Never aspirate close to the bottom of the PCR tubes during liquid removal in wash steps - the **pellet will not be visible** when working with low cell numbers.

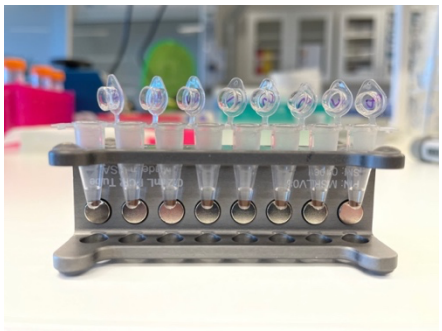


- Centrifugation should be performed using a **swinging bucket rotor**, as usage of fixed-angle rotors increases cell loss.
- When working with PCR tubes, be careful and **keep the tubes vertical** after centrifugation to not disturb the cell pellet, as it could otherwise lead to increased cell loss.
- Ensure all samples and pools have **equal volumes**, as repeated washing steps in the protocol can cause discrepancies. We recommend preparing one PCR test tube with 25 µl of 1x PBS and inspect tube levels visually. If necessary, adjust with the appropriate buffer for that step without disturbing the cell pellet.

- **Centrifugation of PCR tubes/strip** can be performed either using adapters for PCR tubes, or by putting the PCR tubes/strip in a PCR tube rack and centrifuging with a rotor for microplates.



- Do not vortex samples.
- During the magnetic beads cleanup using SPRI beads or AMPure XP beads, visually confirm the clearing of the solution before proceeding to the next step.



- To ensure proper mixing, aspirate at least **50%** of the total reagent volume when pipetting up and down.
- Keep all reagents **on ice** once thawed, unless otherwise stated.
- Return enzymes, antibodies and buffers to their corresponding **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 fixed using paraformaldehyde (PFA) and quenched with **Quenching Solution**. Before beginning with the protocol, it is important to get familiar with the Akura™ Spheroid Microplate which will be used during the first steps. The wells of the plate will be blocked with BSA which helps reducing cell loss in STEP 1 and 2. Akura™ Spheroid Microplates are used to minimize cell pellet disturbance enabling the efficient use of well-calibrated multichannel pipettes during washing steps. Additionally, the flat-bottom design of the plates facilitates the visualization of cells under the microscope allowing for cell verification at any stage of the protocol.

1.1 Preparation of the Akura™ Spheroid Microplate (practice)

PREPARATION: Verify the plate orientation before starting; clearly mark the A1 well corner to facilitate orientation and avoid plate rotation errors.

NOTE: 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 reaction mixes or washing buffers hold the pipette vertically to ensure cells are fully resuspended. For more detailed instructions, please watch our video tutorial [2].

NOTE: If excessive bubbles form during resuspension or pipetting, spin the plate briefly (400 rcf before PFA cell fixation, 700 rcf after PFA cell fixation).

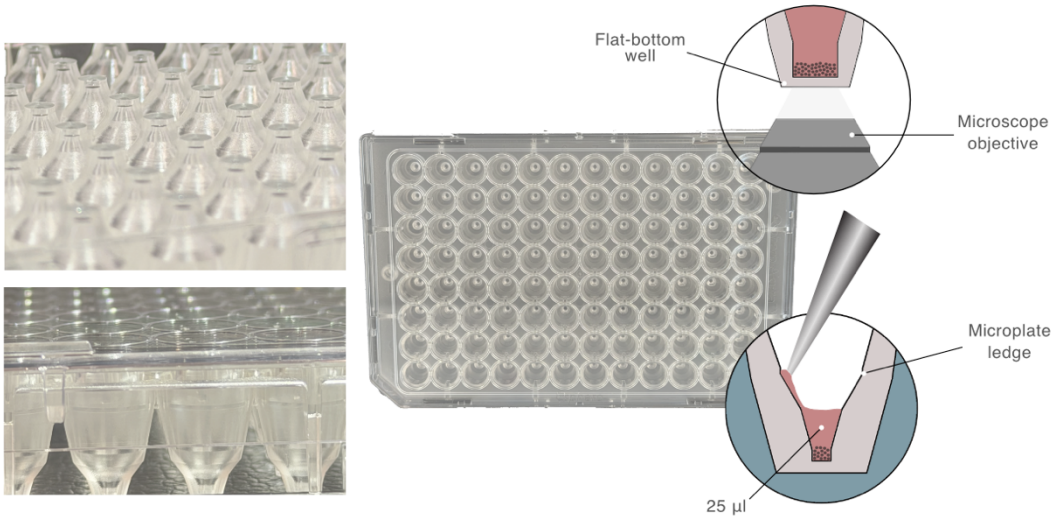


Figure 3. Overview of the Akura™ Spheroid Microplate.

A. ☐ Choose the last row on the plate (i.e., H) and practice using the multichannel pipette in this setup. Attach 8 tips to the pipette and get a feel for the ledge in each well.

B. ☐ Dispense 150 µl of **1x PBS** into the wells then aspirate 125 µl, leaving 25 µl behind.

C. ☐ Take a moment to visually confirm what the remaining volume looks like.

D. ☐ If necessary, repeat C, until feeling comfortable with the technique and ready to proceed with the actual protocol.

1.2 Blocking wells of the Akura™ Spheroid Microplate

PREPARATION: Prepare a fresh solution of 0.5% BSA in 1x PBS. The volume depends on the number of samples processed. Store the solution at 4°C and use it within 1 week. The BSA should be protease-free and of ≥98% purity.

NOTE: Each sample will require preparation of one BSA-blocked well.

A. ☐ Add 180 µl of the **1x PBS + 0.5% BSA** solution to each empty well (1 sample/well).

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

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

NOTE: If not used immediately, store the blocked plate at 4°C up to 48h.

1.3 Cell preparation (in Akura™ Spheroid Microplate)

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 any residual serum-supplemented solution, then resuspend in 1x PBS.

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 count in *Appendix 1*. Please refer to the "Best Practices for Cell Preparation" document if viability is lower than 70% [1].

B. ☐ For each sample, transfer 50 000 - 1 000 000 cells to a BSA-blocked well of the plate, and add **1x PBS** for a total volume of 150 µl per sample. **IMPORTANT!** Arrange samples in rows of 8. This grouping is required and will facilitate the subsequent addition of the 8-**Hashtag Set** and the group-based pooling.

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

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

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

1.4 PFA fixation (in Akura™ Spheroid Microplate)

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.

F. ☐ 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.

G. ☐ Incubate for 15 min at RT.

H. ☐ 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.

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

J. ☐ Carefully discard 160 µl of supernatant from each of the 185 µl quenched sample without disturbing the cell pellet, leaving behind 25 µl of supernatant.

K. ☐ 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

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

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

N. ☐ 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 - Hashtag Binding*. If planning to store cells at 4°C or frozen at -80°C, continue with step U.

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

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

Q. ☐ 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 1x PBS for up to 5 days or frozen at -80°C (see optional below) until proceeding with STEP 2. We recommend sealing the Akura™ Spheroid Microplate with parafilm when storing it at 4°C to reduce the evaporation of the liquid.

OPTIONAL: Freezing and thawing of PFA-fixed cells

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

1.5 Freezing of PFA-fixed cells

PREPARATION: Prepare a fresh freezing solution of 5% DMSO and 95% FBS. The volume depends on the number of samples processed.

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

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

C. ☐ Add 125 µl **freezing solution** and pipette up and down 10 times.

D. ☐ Transfer the 150 µl cell solution to a cryotube.

E. ☐ Add 150 µl **freezing solution** to the empty well and pipette up and down 10 times to wash any remaining cells.

F. ☐ Transfer the 150 µl wash to the cryotube. Total of 300 µl in each cryotube.

G. ☐ Add 200 µl **freezing solution** to the 300 µl cell solution. Total of 500 µl in each tube.

H. ☐ Place the cryotubes in a cryogenic box and transfer to -80°C until further use.

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

1.6 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. The volume depends on the number of samples processed.

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 well of the plate per sample by following the steps in *1.2 - Blocking wells in the Akura™ Spheroid Microplate.*

I. ☐ Put the frozen cryotubes in a 37°C bath for up to 5 min (confirm that the cell solution has thawed).

J. ☐ Transfer the thawed 500 µl cell solution to a 1.5 ml Eppendorf tube.

K. ☐ 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.

Wash 1

L. ☐ Transfer the 500 µl solution to the Eppendorf tube. Total of 1 000 µl in each tube.

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

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

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

Wash 2

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

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

R. ☐ Pipette up and down 10 times and transfer the 50 µl cell suspension to a BSA-blocked well in the Akura™ Spheroid Microplate, per sample. **IMPORTANT!** Arrange samples in rows of 8. This grouping is required and will facilitate the subsequent addition of the 8-**Hashtag Set** and the group-based pooling.

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

T. ☐ Transfer the 100 µl solution to the blocked well in the plate. Total of 150 µl in each well.

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

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

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

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

Up to 24 hours at 4°C

NOTE: At this step, cells can be kept at 4°C storage for 24 hours. We recommend sealing the Akura™ Spheroid Microplate with parafilm when storing it at 4°C to reduce the evaporation of the liquid.

STEP 2 - Hashtag Binding

STEP 2 consists of blocking cells, binding of the Hashtag Sets followed by pooling of the samples and moving to BSA-blocked PCR tubes for *STEP 3 - Barcoded Antibody Binding*.

IMPORTANT! From this step forward, ensure that cells are fully resuspended during mixing by pipetting vigorously (10 times in ~4 seconds). Gentle pipetting mixing may result in cell aggregate formation.

2.1 Cell blocking (in Akura™ Spheroid Microplate)

A. ☐ Thaw the **Blocking Solution**, vortex to mix, then quick spin.

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

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

D. ☐ Add 125 µl **Blocking Solution** and pipette up and down 10 times.

E. ☐ Incubate for 15 min at 4°C.

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

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

H. ☐ 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

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 125 µl of **1x PBS + 0.5% BSA** on top of the 25 µl sample. Proceed with *STEP 2.2 - Hashtag binding* (in Akura™ Spheroid Microplate) if not freezing remaining cells.

OPTIONAL: Freezing remaining cells not needed for the following steps

NOTE: Since the minimum cell input to the next step is 30 000 cells, remaining cells can be frozen using the optional protocol at STEP 1.5 and kept at -80°C storage for up to 3 months.

☐ To determine the remaining cell number, 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 3 months at -80°C

2.2 Hashtag binding (in Akura™ Spheroid Microplate)

NOTE: The supported cell input range is 30 000 – 1 000 000 cells.

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

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

C. ☐ Centrifuge each **Hashtag Set** vial at 17 000 rcf for 10 min at RT or 4°C, to pull down any aggregates to the bottom of the vial.

D. ☐ Prepare a PCR strip and transfer the following volumes of each **Hashtag Set** into each tube. **IMPORTANT!** Do not aspirate near the bottom of the centrifuged Hashtag Set vials as it contains aggregates.

Kit configuration	Volume to transfer in a PCR strip
8 reactions	30 µl
16 reactions	60 µl
32 reactions	110 µl

E. ☐ Using a multichannel pipette, transfer 25 µl of each **Hashtag Set** into each 25 µl sample in the plate (final volume of 50 µl), and pipette up and down 10 times. When working with the 16- or 32-reaction kit configurations, add the 8 **Hashtag Sets** to the first row of 8 samples and then repeat for the other rows/groups. **IMPORTANT!** Add each set at the same time as variations in timing might impact the final results.

F. ☐ Incubate 10 min at 4°C.

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

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 of supernatant.

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

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

N. ☐ 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. **IMPORTANT!** High precision is required during this counting step as inaccuracies will directly impact the final sample proportion representation.

2.3 Hashtag-stained sample pooling (in PCR tubes)

PREPARATION: Block one PCR tube for every pool of 8 samples by following the steps in *1.2 - Blocking wells in the Akura™ Spheroid Microplate.*

PREPARATION: Prepare one PCR test tube with 25 µl of 1x PBS and use it as volume reference when ensuring all pools have equal volumes.

NOTE: The supported cell input range is 80 000 – 250 000 cells. We recommend loading 250 000 cells per pool in 1x PBS + 0.5% BSA to a total volume of 200 µl (as described below).

IMPORTANT! Combine up to 8 samples into a single BSA-blocked PCR tube; these will be processed as a single pool in all subsequent steps. If processing 16 or 32 samples, combine them into 2 or 4 pools, respectively. Each tube represents one pool for all the downstream steps. It is critical to combine each sample into the final pool by equal cell number to ensure equal representation between all the samples.

O. ☐ Combine individually hashtag-stained samples by **equal cell number** into the appropriate tube as follows (see Figure 4):

- If the pooled volume is ≤ 200 µl, combine samples directly into a BSA-blocked PCR tube and proceed to step 2.3 - P.
- If the pooled volume is > 200 µl, combine samples into a BSA-blocked 1.5 ml Eppendorf® tube prepared following the steps in 1.2 - *Blocking wells in the Akura™ Spheroid Microplate*, by filling it with **1x PBS + 0.5% BSA** at least equal to the required pooled volume. Centrifuge at 700 rcf for 4 min at RT. Remove supernatant leaving 50 µl, resuspend cells and transfer to a BSA-blocked PCR tube. Rinse the 1.5 ml Eppendorf® tube with 50 µl **1x PBS + 0.5% BSA** to collect remaining cells and transfer to the BSA-blocked PCR tube for a final volume of 100 µl. Proceed to STEP 2.3 - P.

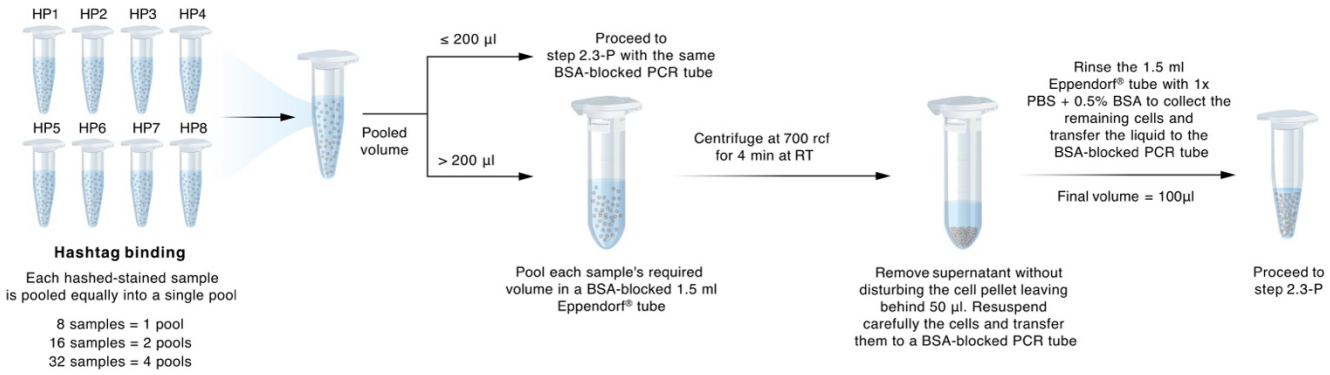


Figure 4. Hashtag-stained sample pooling scheme.

P. ☐ Bring up the volume to 200 µl with **1x PBS + 0.5% BSA.**

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

R. ☐ Carefully discard 175 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.

IMPORTANT REMINDER! Use the 25 µl test tube to visually inspect the volume of each pool. Adjust if necessary but do not disturb the pellet. Equal volumes are important for the upcoming steps.

STEP 3 - Barcoded Antibody Binding

STEP 3 consists of binding 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.

NOTE: If using spike-ins, add them to the Antibody Panel before starting this step.

3.1 Barcoded antibody binding

A. ☐ Centrifuge the **Antibody Panel** vial at 17 000 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 ready to proceed with the next step.

B. ☐ Add 25 µl of the centrifuged **Antibody Panel** solution to each 25 µl pool (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

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

3.2 2nd antibody binding

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

Wash 1

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 of supernatant.

G. ☐☐☐ Add 125 µl **1x PBS + 0.5% BSA** on top of each 25 µl pool and pipette up and down 10 times.

Wash 2, 3, 4

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 of supernatant.

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

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

L. ☐ Add 25 µl of **2nd Antibody** to each 25 µl pool and pipette up and down 10 times.

M. ☐ Incubate each pool for 10 min at 4°C.

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

Wash 5

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

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

Q. ☐ Add 125 µl **Wash Buffer** on top of each 25 µl pool and pipette up and down 10 times.

Wash 6

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

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

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

U. ☐ 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 1x PBS for up to 2 days before proceeding with the next steps of the protocol.

STEP 4 - Protein Proximity Network Generation

In this step of the protocol, RCPs are created from the barcoded antibodies bound to each cell, followed by linking together neighboring RCPs in spatial proximity using a gap-fill ligation reaction.

Reminder: vortex to mix, then quick spin all reagents when indicated. Pipette up and down 5 times or vortex to mix after preparing the Master mix.

4.1 Padlock probes hybridization

NOTE: The supported cell input range is 40 000 - 100 000 cells. We recommend loading 50 000 cells per pool in 1x PBS to a total volume of 100 µl (as described below).

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

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

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

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

E. ☐ Dispense 25 µl **Padlock Oligos** to each 25 µl pool and pipette up and down 10 times. Total of 50 µl in each tube.

F. ☐ Incubate each pool in a thermal cycler for 30 min at 45°C.

G. ☐ Remove from the thermal cycler, add 100 µl **Wash Buffer** to each pool and pipette up and down 10 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 of supernatant.

J. ☐ Add 125 µl of **Wash Buffer** and pipette up and down 10 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 of supernatant.

4.2 Gap-fill 1 (padlock probe circularization)

A. ☐ Thaw the **Gap-fill Solution**, vortex to mix, then quick spin**.** 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 performed 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 Gap-fill Ligase and Gap-fill 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 #	8-reaction kit configuration	16-reaction kit configuration	32-reaction kit configuration
Box3 -20°C	Gap-fill Solution	BD091	25.3 µl	50.6 µl	101.2 µl
Box3 -20°C	Gap-fill Ligase	EE088	1.1 µl	2.2 µl	4.4 µl
Box3 -20°C	Gap-fill Polymerase	EE089	1.1 µl	2.2 µl	4.4 µl
	Total		27.5 µl	55 µl	110 µl

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

C. ☐ Place each pool on ice and dispense 25 µl **Gap-fill** master mix to each 25 µl pool and pipette up and down 10 times. Total of 50 µl in each tube.

D. ☐ Incubate each pool in a thermal cycler for 30 min at 25°C.

E. ☐ Add 100 µl of **Wash Buffer** to each pool at RT and pipette up and down 10 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 of supernatant.

H. ☐ Add 125 µl of **Wash Buffer** to each pool at RT and pipette up and down 10 times. 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 of 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 each pool tubes to ensure they have equal volume. Adjust if necessary but do not disturb the pellet. Equal volumes are important for the upcoming step.

4.3 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**, vortex to mix, then quick spin. 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 #	8-reaction kit configuration	16-reaction kit configuration	32-reaction kit configuration
Box3 -20°C	RCA Solution	BD095	24.75 µl	49.5 µl	99 µl
Box3 -20°C	RCA Polymerase	EE090	2.75 µl	5.5 µl	11 µl
	Total		27.5 µl	55 µl	110 µl

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

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

E. ☐ Incubate each pool in a thermal cycler for 15 min at 30°C.

F. ☐ Put each pool on ice directly after the incubation.

G. ☐ Vortex the **Proximity Oligos** to mix, then quick spin.

H. ☐ Directly add 100 µl **Proximity Oligos** to each pool on ice and pipette up and down 10 times. Total of 150 µl in each tube.

I. ☐ Incubate each pool 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 10 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 10 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.

4.4 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 #	8-reaction kit configuration	16-reaction kit configuration	32-reaction kit configuration
Box3 -20°C	Gap-fill Solution	BD091	25.3 µl	50.6 µl	101.2 µl
Box3 -20°C	Gap-fill Ligase	EE088	1.1 µl	2.2 µl	4.4 µl
Box3 -20°C	Gap-fill Polymerase	EE089	1.1 µl	2.2 µl	4.4 µl
	Total		27.5 µl	55 µl	110 µl

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

C. ☐ Put each pool on ice and dispense 25 µl **Gap-fill** master mix to each 25 µl pool and pipette up and down 10 times. Total of 50 µl in each tube.

D. ☐ Incubate each pool in a thermal cycler for 30 min at 25°C.

E. ☐ Add 100 µl of **Wash Buffer** to each pool at RT and pipette up and down 10 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 of supernatant.

H. ☐ Add 125 µl **Wash Buffer** and pipette up and down 10 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 pool and pipette up and down 10 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 5 - PCR Amplification and Indexing

In this step of the protocol, the amplicons generated from each pool are enzymatically digested, then amplified by PCR and indexed to produce individual NGS libraries carrying a unique barcode. NGS libraries are subsequently quality controlled, quantified and combined by library pooling for sequencing.

5.1 Manual cell counting

GUIDANCE: See *Appendix 2* for more detailed information regarding cell counting. We recommend setting up the PCR with 8 000 cells which ensures each individual sample in the pool is represented by at least 1 000 cells. Note that the number of cells loaded into the PCR dictates the required number of PCR cycles and will directly impact the total read requirement and the sample representation.

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

A. ☐ We recommend counting cells using a hemocytometer or a single-use counting chamber. Mix each pool by pipetting up and down 10 times before taking an aliquot for counting. To ensure accuracy, count cells using two separate aliquots to achieve duplicate counts. We recommend staining cells with Trypan Blue or Erythrosin B to improve the accuracy of the counting. If the cell concentration is too high to count accurately, dilute each pool in **Wash Buffer** ensuring that a minimum of 2 µl is pipetted when transferring cells to a new PCR tube as described in the following step.

B. ☐ For each pool, transfer 8 000 cells into a new PCR tube and add **Wash Buffer** for a final volume of 15.5 µl. See *Appendix 2* for an aliquot example table. **NOTE:** If the volume exceeds 15.5 µl, centrifuge at 700 rcf for 4 min at RT and then remove the excess supernatant to a final volume 15.5 µl.

5.2 Enzymatic digestion

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

B. ☐ Prepare **Digestion** master mix as indicated in Table 8, directly before use by combining the reagents in the order listed below, maintain on ice or +4°C.

Table 8. **Digestion** master mix preparation.

Lid color, box #, box storage	Component	Article #	8-reaction kit configuration	16-reaction kit configuration	32-reaction kit configuration
Box3 -20°C	Digestion Solution	BD097	2.2 µl	4.4 µl	8.8 µl
Box3 -20°C	Digestion Enzymes	EE098	2.75 µl	5.5 µl	11 µl
	Total		4.95 µl	9.9 µl	19.8 µl

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

D. ☐ Put each pool on ice and dispense 4.5 µl **Digestion** master mix to each 15.5 µl pool and pipette up and down 10 times. Total of 20 µl in each tube.

E. ☐ Perform digestion using the following program in a thermal cycler (Table 9).

Table 9. Digestion program.

Lid temperature	Reaction Volume	Run Time
105°C	20 µl	~ 35 min
Step	**Temperature**	**Time**
1	37°C	00:30:00
2	85°C	00:05:00
3	4°C	Hold

Up to 3 days at 4°C

NOTE: The enzymatic digestion product can be stored at 4°C for up to 3 days before proceeding with the next steps of the protocol.

5.3 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 11.

B. ☐ Thaw **Pre-amp Primers**, vortex to mix, then quick spin. 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 10, directly before use by adding the reagents in the order listed below, and maintain at RT until use.

Table 10. **Pre-amplification PCR** master mix preparation.

Lid color, box #, box storage	Component	Article #	8-reaction kit configuration	16-reaction kit configuration	32-reaction kit configuration
Box4 -20°C	Pre-amp Primers	IP086	5.5 µl	11 µl	22 µl
Box4 -20°C	PCR Master Mix	MM092	27.5 µl	55 µl	110 µl
	Total		33 µl	66 µl	132 µl

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

D. ☐ Dispense 30 µl **Pre-amplification PCR** master mix to each 20 µl pool after digestion and pipette up and down 10 times or vortex to mix. Total of 50 µl in each tube.

E. ☐ Perform pre-amplification PCR using the following PCR program (Table 11). If SPRI/AMPure XP beads are stored in the fridge, place them at RT 30 min before performing step 5.4.

Table 11. Pre-amplification PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	50 µl	~ 18 min	
Step	**Temperature**	**Time**	**Number of cycles**
1	98°C	00:10:00	1
2	98°C	00:00:15	4 cycles
3	72°C	00:00:30	
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.

5.4 Pre-amplification PCR clean-up

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

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

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

C. ☐ Add 25 µl **10 mM Tris pH 8** to each pool. Total of 50 µl in each tube.

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

E. ☐ Incubate for 5 min at RT.

F. ☐ Place each pool on a PCR tube magnet and incubate for 2 min or until the beads have settled against the tube wall and the solution clears.

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

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

I. ☐☐ While on the magnet, carefully remove the ethanol without disturbing beads.

J. ☐ Repeat steps H-I for a total of two washes.

K. ☐ Quick-spin each pool 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.

L. ☐ Remove each pool from the magnet.

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

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

O. ☐ Place each pool on the magnet and wait until the beads have settled against the tube wall.

P. ☐ 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.

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

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.

5.5 Indexing PCR

In this step, unique index sequences are introduced into each pool during PCR, generating an NGS library from each pool. This step is critical for correct demultiplexing during data analysis. The resulting indexed NGS libraries are ready for quality control, quantification and library pooling prior to sequencing. If combining Proxiome NGS libraries with externally generated libraries, ensure that all index combinations are unique across the entire sequencing run to prevent index collisions.

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

A. ☐ Pre-program a PCR system with the PCR program denoted in Table 12.

B. ☐ Thaw the PCR Index **Primers** needed, vortex to mix, then quick spin. Thaw the **PCR Master Mix** vial, quick spin and pipette up and down 5 times to mix.

C. ☐ For each pool, transfer **10 µl** of pre-amplified, purified product to fresh PCR tubes. The remaining volume can be stored at -20°C as backup.

D. ☐ Add 15 µl of one unique PCR Index **Primers** to each 10 µl pool. Make a note of the index primer used for each pool.

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

F. ☐ Perform PCR using the following PCR program (Table 12).

Table 12. PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	50 µl	~ 10 min	
Step	**Temperature**	**Time**	**Number of cycles**
1	98°C	00:01:00	1
2	98°C	00:00:15	1 000 cells: 8 cycles 4 000 cells: 6 cycles 8 000 cells: 5 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.

5.6 Indexing PCR clean-up

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

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

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

B. ☐ Add 50 µl of resuspended SPRI/AMPure XP bead mixture to each indexed pool (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 indexed pool 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 remove the ethanol, without disturbing beads.

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

I. ☐ Quick-spin the indexed pool 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 indexed pool from the magnet.

K. ☐ Resuspend beads by adding 31 µl of **10 mM 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 indexed pool on the magnet and wait until the beads have settled against the tube wall.

N. ☐ The supernatant now contains the eluted NGS library. 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 6 - NGS Preparation

6.1 Quality control and quantification

Before proceeding to sequencing, each library needs to be:

- Quality controlled (to confirm the correct size of approximately 271 bp)
- Quantified, diluted and pooled

Table 13 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 13. List of instruments which 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 (Thermo Fisher)	❌	✅
Gel Electrophoresis with TBE gel, 200V for 25 min	✅	❌

The size of the generated PCR product is 271 bp. To convert a concentration reading from ng/µl to nM, a conversion factor of 5.59 can be used:

Library concentration [ng/µl] x 5.59 = X nM

6.2 Library pooling

Equimolar pooling of NGS libraries is recommended in most cases. This can for example be achieved by diluting each library to the same concentration, followed by combining equal volumes of each normalized library (see *Appendix 3* for details and an equimolar pooling calculation example). For further details, refer to the Illumina guidelines for library concentration normalization [3] or use the **PNA library pooling table** [4] available on our website.

6.3 Sequencing using Illumina® platforms

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 using the following loading concentrations:

Table 14. Recommended loading concentrations.

NextSeq 1000/2000 XLEAP/SBS chemistry	P1/P2: 650 pM P3/P4: 488 pM
NovaSeq X, NovaSeq X Plus	140 pM

NOTE: These loading recommendations are for first-time users and can later be titrated to your specific use case.

When sequencing libraries on other Illumina platforms (e.g., NovaSeq 6000) we recommend following Illumina's guidelines for the loading concentration of "100% PhiX libraries". If sequencing on other platforms like AVITI (Element Biosciences), Ultima Genomics or MGI please contact us at support@pixelgen.com.

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:

Table 15. Recommended sequencing depths.

Cell line	Read depth required
PBMCs & stimulated PBMCs	300 000 reads / cell
Cell lines	500 000 reads / cell

Table 16 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 **pooling indexing sequences.**

Table 16. Sequencing parameters.

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%

NOTE: When combining libraries generated with the Pixelgen Proxiome Kit v2 with other library types, the PhiX concentration may be reduced, as the overall run complexity is increased.

When processing data using Pixelator, users must explicitly choose a panel version. Please see our software webpage [5] for additional description on data processing using Pixelator and nf-core/pixelator [6].

See the table below for which panel version to use:

Table 17. Data processing panel version.

Product version	Panel version	Panel name
Proxiome v2	Immuno 155-v2.0	proxiome-v2-immuno-155-v2.0

Table 17 lists the standard panel file version to use with the Pixelator software for data processing. If you have spiked additional barcoded antibodies into the standard Antibody Panel, a different panel file is required. Please check the Pixelator software webpage for detailed explanation. For any questions, please contact us at support@pixelgen.com.

4. References

1. https://www.pixelgen.com/wp-content/uploads/2026/06/best-practices-for-cell-preparation-v1.02.pdf
2. Tutorial video: Handling of Akura™ Spheroid Microplate
3. https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001252
4. PNA library pooling table
5. https://software.pixelgen.com/
6. https://software.pixelgen.com/nf-core-pixelator/introduction/

Appendix

Appendix 1

Cell counting

Table A1. Cell counts and viability per reaction for STEP 1 and STEP 2.3. If using the 32-reaction kit configuration please use the continuation of the Table A1 on the next page to record all data.

		STEP 1 Before fixation				STEP 2.3 Before hashtag-stained sample pooling		
	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
8-reaction kit	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
16-reaction kit	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							

32-reaction kit

32-reaction kit

	STEP 1 Before fixation				STEP 2.3 Before hashtag-stained sample pooling		
Sample	Cell conc.	Volume	Cell count	Viability	Cell conc.	Volume	Cell count
Example	$6*10^6$ cells/ml	150 µl	$6*10^6*10^{-3}*150$ = 900 000 cells	95%	$4.5*10^6$ cells/ml	150 µl	$4.5*10^6*10^{-3}*150$ = 675 000 cells
17							
18							
19							
20							
21							
22							
23							
24							
25							
26							
27							
28							
29							
30							
31							
32							

Table A2. Cell counts per pool for STEP 4 and STEP 5. The 8-reaction kit configuration yields a single pool, while the 16- and 32- reaction kit configurations yield 2 and 4 pools, respectively.

	STEP 4 Before Protein Proximity Network Generation			STEP 5 PCR Amplification (manual cell counting)		
Pool	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	$4.5*10^5$ cells/ml	100 µl	$4.5*10^5*10^{-3}*100$ = 45 000 cells
1						
2						
3						
4						

Appendix 2

Cell counting considerations

Counting of cells before fixation (STEP 1), the hashtag-stained sample pooling (STEP 2.3) and Protein Proximity Network Generation (STEP 4), 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 5 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.

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. We recommend staining cells with Trypan Blue or Erythrosin B to improve the accuracy of the counting. In our experience, not all single-use counting chambers work accurately and we strongly recommend using the suggested product in Table 2a if using a single-use hemocytometer, to ensure accurate counting, duplicate counts for each pool are recommended. If the cell concentration is too high to count accurately, dilute each pool in **Wash Buffer** ensuring that a minimum of 2 µl is pipetted when transferring cells to the new PCR tube.

Aliquoting before pre-amplification PCR: example

Table A3, displays an example of two pools with concentrations of 300 and 600 cells/µl and how to dilute them in **Wash buffer** to achieve 8 000 cells per pool in 15.5 µl total.

Table A3. Example table of how to dilute cells in STEP 5, to achieve 8 000 cells per pool for sequencing.

Pool	Cell concentration	Sample volume needed	Wash buffer needed	Total
Pool 1	300 cells/µl	8 000/300 = 26.6 µl	N/A*	26.6 µl*
Pool 2	600 cells/µl	8 000/600 = 13.3 µl	15.5-13.3 = 2.2 µl	13.3+2.2 = 15.5 µl

*If the volume needed exceeds 15.5 µl, centrifuge at 700 rcf for 4 min at RT and then remove the excess of supernatant to a final volume 15.5 µl.

Appendix 3

QC of NGS library

The expected size of the PCR product is 271 bp. The presence of PCR products of the intended size can be confirmed using either gel electrophoresis or fragment analyzer assays.

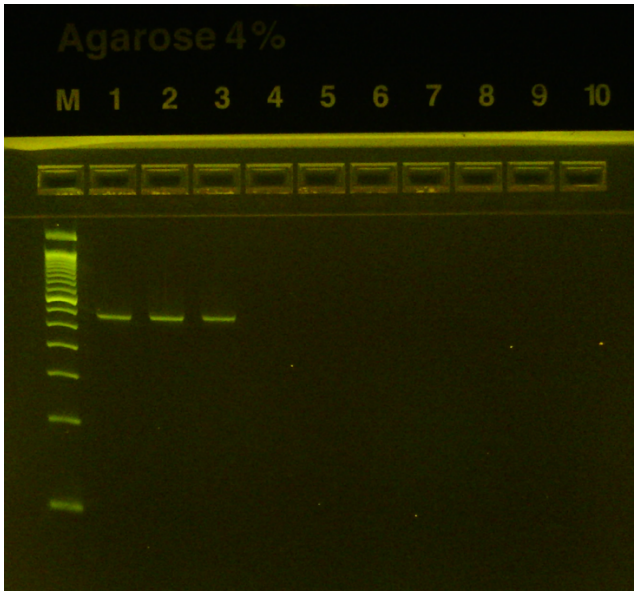


Figure A1. Quality control example of a successfully amplified PCR product. E-Gel Ex 4% Agarose Gel. Lane 1: unstimulated PBMCs, Lane 2: PHA stimulated PBMCs, Lane 3: PMA stimulated PBMCs. Lane M: Ladder, 20 µl of 50 bp DNA ladder (Thermo Fisher, 10416014), diluted 1:50.

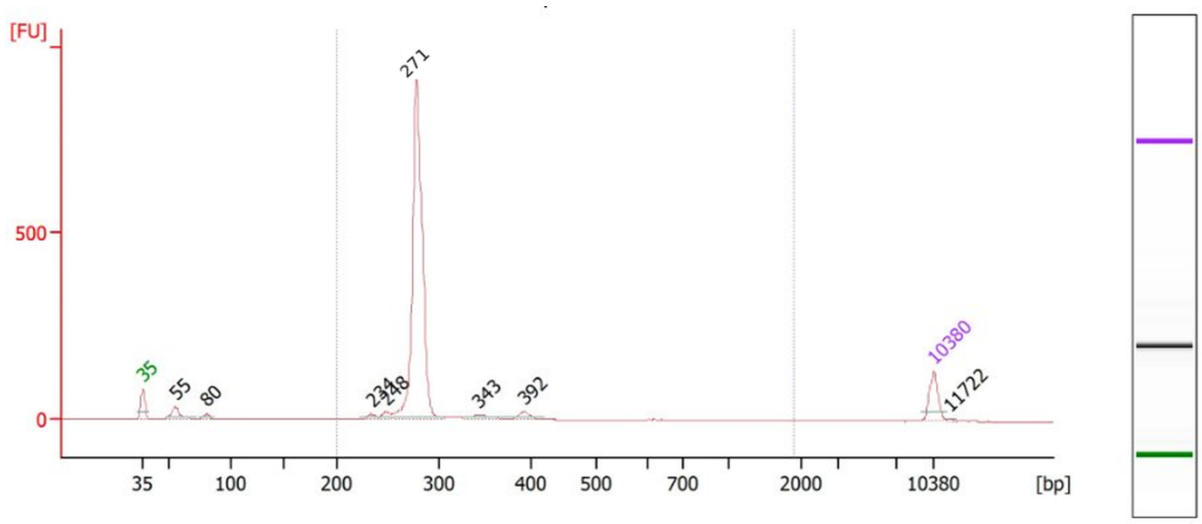


Figure A2. Quality control example using **BioAnalyzer™, High Sensitivity DNA kit (Agilent)**, showing the expected size of the final PCR product. The size measured by the instrument might vary by ±10% from the expected 271 bp.

Sequencing read calculation: example

Table A4, describes how to calculate the total number of reads necessary for your experiment, using an example of 1 (8 reaction) or 4 (32 reactions) pools with 8 000 cells per pool. 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 15 refer 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 4 pools with 8 000 cells per pool.

Nr. of pools	Reads/cell	Cells/pool	Total nr of cells	Total nr of reads needed
1 (8 reaction)	300 000	8 000	1x8 000 = 8 000	8 000x300 000 = 2.4B
4 (32 reactions)	300 000	8 000	4x8 000 = 32 000	32 000x300 000 = 9.6B

Pooling strategies for NGS

There are two strategies for multiplexing libraries that can affect how the sequencing reads are allocated between each pool:

1. **Equimolar pooling (recommended):** An equal number of moles are multiplexed together for each pool in a library, resulting in an equal number of sequenced reads for each pool, regardless of the number of unique molecules present in each pool.

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

Equivolume pooling strategy (2) can result in insufficient read depth for some pools if there is a high variability in the number of unique molecules between the pools. It is therefore recommended to perform equimolar pooling (1) which ensures equal sequencing read distribution per pool. To help you with the pooling please use the **PNA library pooling table** [4] available on our website.

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

Pool	Conc. ng/ul	Conc. nM	Pool needed for 5nM dilution	Tris buffer needed	Volume from each pool to combine
Pool 1	3 ng/ul	3*5.59=16.77 nM	5*20/16.77=5.96ul	20-5.96=14.04ul	10ul
Pool 2	4 ng/ul	4*5.59=22.36 nM	5*20/22.36=4.47ul	20-4.47=15.53ul	10ul
…					

Indexing sequences

Table A6. List of indexing sequences supplied with the reagent kit.

Index name	i7 index	i5 index* (forward)	i5 index* (reverse complement)
Primer 1	TTCTCCAA	TTGTGGAA	TTCCACAA
Primer 2	CACCTACT	GGAGGTGT	ACACCTCC
Primer 3	ACAACCAC	AATTCGTG	CACGAATT
Primer 4	GAGAACCT	GGTTGCTC	GAGCAACC
Primer 5	ACTTACCG	TAGGATGA	TCATCCTA
Primer 6	CTAGCAGA	TAGACACG	CGTGTCTA
Primer 7	AGCCTGTC	ACACTGAT	ATCAGTGT

* 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/SHBasicalARE/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).