USER MANUAL

PIXELGEN SINGLE CELL SPATIAL PROTEOMICS KIT

IMMUNOLOGY PANEL 2, Human **v2**

PXGIMM002

PIXELGEN
TECHNOLOGIES

UM00002 MPX v2 User Manual version 1.01

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List of Abbreviations

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

About this User Manual

This User Manual describes the experimental procedure using the Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel 2, Human v2 (#PXGIMM002) in detail.

Technical Support

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

Version Update (v1.01)

Changes made in UM00002 MPX v2 User Manual (v1.01):

- Typo corrected in *STEP 2 - Antibody-Oligo Conjugate (AOC) binding*, step N.

1. Product Description

The Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel 2, Human v2 reagent kit can be used to study surface protein expression and spatial arrangement on paraformaldehyde fixed human immune cells in suspension.

The technology used in this kit is called Molecular Pixelation (MPX). The core steps of the technology are illustrated below.

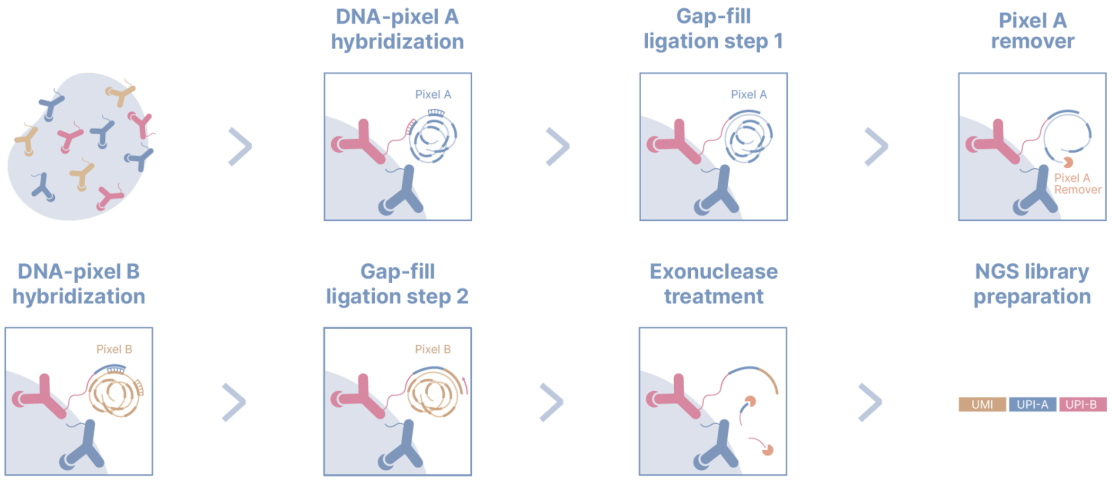


Figure 1. Overview of Molecular pixelation workflow and core steps.

Reagents supplied with the Molecular Pixelation 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. Storage temperature and expiration date for components are stated on the label of each box.

> Note All reagents for the Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel 2, Human v2 are lot specific and reagents from different kit lots should not be combined.

Product number: PXGIMM002

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

Box 1 PXGIMM002PP store at 4°C	Box 2 PXGIMM002E store at -20°C	Box 3 PXGIMM002IP store at -20°C
• AOC Panel - PP043 • 2nd antibody - PP028 • Wash Buffer - PP016	• Blocking buffer - PP009 • Pixel A - PP015A • Pixel A Diluent - BD019A • Pixel B - PP015B • Pixel B Diluent - BD019B • Gap-fill Buffer - BD018 • Gap-fill Ligase - EE020 • Gap-fill Polymerase - EE021 • Pixel A Remover - EE022 • Exonuclease - EE023	• PCR Master Mix - MM025 • Primer 1 - IP024_1 • Primer 2 - IP024_2 • Primer 3 - IP024_3 • Primer 4 - IP024_4 • Primer 5 - IP024_5 • Primer 6 - IP024_6 • Primer 7 - IP024_7 • Primer 8 - IP024_8

Additional Requirements

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

Third party Instrumentation needed

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
Thermocycler/ PCR system	ProFlex™ 3x 32-well PCR System	ThermoFisher®	4484073
Pipettes: • 0.5 - 2.5 µl • 2 - 20 µl • 20 - 200 µl • 100 - 1000 µl	Research® plus	Eppendorf®	 J70399L O89082L N23237L N44241L
Hemocytometer or	Counting Chambers Bürker-Türk	Karl Hecht Assistent®	40445
Single-use hemocytometer	Millicell® Disposable Hemocytometer	Sigma-Aldrich®	MDH-2N1-50PK
Magnet for PCR tubes	DynaMag™-96 Side Magnet	ThermoFisher®	12331D
Light microscope	Microscope trinocular inverted	Sagitta®	63335
PCR product size and quantification instrument	BioAnalyzer™ with a 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	ThermoFisher®	10010-023
Paraformaldehyde, methanol-free*	Paraformaldehyde 16% Aqueous Sol.	Electron Microscopy Sciences	15710
AMPure beads	AMPure XP reagent, 60mL	Beckman-Coulter® Life science	A63881
TE buffer	Tris-EDTA buffer solution, BioUltra, for molecular biology, pH 8.0	Sigma-Aldrich®	93283

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

2. General guidelines & information

Cell input requirements

The protocol consists of several steps where a certain range of cell input is required (Table 3.). Due to cell losses during fixation and overall protocol washing steps, it is important to stay within the range to ensure that enough cells remain.

Validation of cell inputs to fixation step has been confirmed for up to 1 million cells per reaction, AOC binding step has been validated for cell inputs up to 500 000 cells and the Pixelation step has been validated for up to 100 000 cells per reaction. The cell input range to PCR needs to be within 300 to 1000 cells to ensure software compatibility.

500 000 - 1 000 000 cells are typically needed as input to fixation due to substantial cell loss during the fixation process. However, a demonstrated protocol is available for working with low cell numbers, down to 50 000 cells as input to fixation. Please contact support for more information. Note that the specified cell numbers for fixation below are the total number of cells (live + dead cells). After fixation, it is normal that most cells will appear dead.

Table 3. Cell inputs and counting requirements for different steps of the protocol.

Step	Cell input range per reaction	Recommended cell input per reaction	Counting procedure	Counting, accuracy
Fixation	500 000 - 1 000 000 cells*	500 000 - 1 000 000 cells	Automated cell counter OK	Required, estimation
AOC binding	50 000 - 500 000 cells	200 000 cells	Automated cell counter OK	Optional, estimation
Pixelation	10 000 - 100 000 cells	20 000 cells	Automated cell counter OK	Required, precise
Exonuclease and PCR	300 - 1 000 cells, dependent on experimental set-up**	1 000 cells	Manual	Required, precise

*A demonstrated protocol is available for working with low cell numbers, down to 50 000 cells as input to fixation. Please contact support for more information.

** The number of cells input to PCR will directly impact the read requirements.

Cell counting considerations

Counting of cells after fixation and AOC binding, prior to Pixelation can be performed using either a hemocytometer or using automated cell counters as the cell numbers are higher during this stage of the workflow.

However, it is important to accurately count and dilute cells prior to Exonuclease & PCR step, 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 at this stage, we have found automatic cell counters such as the Countess Automated cell counter (ThermoFisher) to **not** be reliable and could require a significant portion of the reaction to be used for counting.

We therefore highly recommend using manual counting with a hemocytometer or single-use counting chamber when counting cells prior to the Exonuclease step of the Pixelation 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. We therefore 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.

Sample indexing during PCR

The reagent kit is supplied with 8 PCR primer sets, each primer set containing a unique sample index barcode combination, allowing for pooling of samples for sequencing on the same Illumina flow cell (Table 4). It is critical that each individual sample sequenced together in the same run has a unique sample index combination.

Table 4. List of sample indexes supplied with the reagent kit.

index name	i7 index	i5 index* (forward)	i5 index** (reverse complement)
i1	CAATGTGG	CCAACACT	AGTGTTGG
i2	AGTTGGCT	CAGTCACA	TGTGACTG
i3	AACGTGAT	TAGTCTCG	CGAGACTA
i4	AACCGAGA	AACGCACA	TGTGCGTT
i5	AGAGTCAA	CAGGTAAG	CTTACCTG
i6	CACTTCGA	ACCATAGG	CCTATGGT
i7	TTTGGGTG	ACACCTCA	TGAGGTGT
i8	ATGTGAAG	CAACACAG	CTGTGTTG

* Forward i5 index should be used for NextSeq® 1000, 2000 (using **v2** Sample Sheet), NovaSeq® 6000 (**v1.0** reagent kits), HiSeq® 2000, 2500, MiSeq®, MiniSeq® (rapid reagents kits)

** Reverse complement i5 index should be used for NovaSeq® 6000 (**v1.5** reagent kits), NextSeq® 1000, 2000 (using **v1** Sample Sheet), NextSeq® 500, 550, HiSeq® 3000, 4000, X, MiniSeq® (standard reagents), iSeq® 100

If pooling samples prepared using this protocol together with other types of samples that were prepared using some other library preparation workflow, please ensure that sample indexes are **unique** amongst all samples aimed to be sequenced together to avoid sample index collisions.

Quantification and pooling of NGS library

Since each sample has been barcoded with a unique sample index combination during the PCR step, they can be pooled and sequenced together on the same Illumina flow cell. There are some considerations for how to pool each sample that will affect how the sequencing read amounts are distributed between each sample.

Each sample contains a number of unique molecules generated throughout the workflow (originating from the AOCs bound to each cell), and amplified during PCR. Depending on the nature of the sample (cell type, degree of stimulation / activation etc), the number of unique molecules can vary between samples.

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.

3. Workflow overview

The protocol described, shows how to perform the full workflow from live cells in suspension to a sequencing-ready NGS library. This protocol has been demonstrated and validated using peripheral blood mononuclear cells (PBMC), Raji and Jurkat cell lines.

Workflow illustration

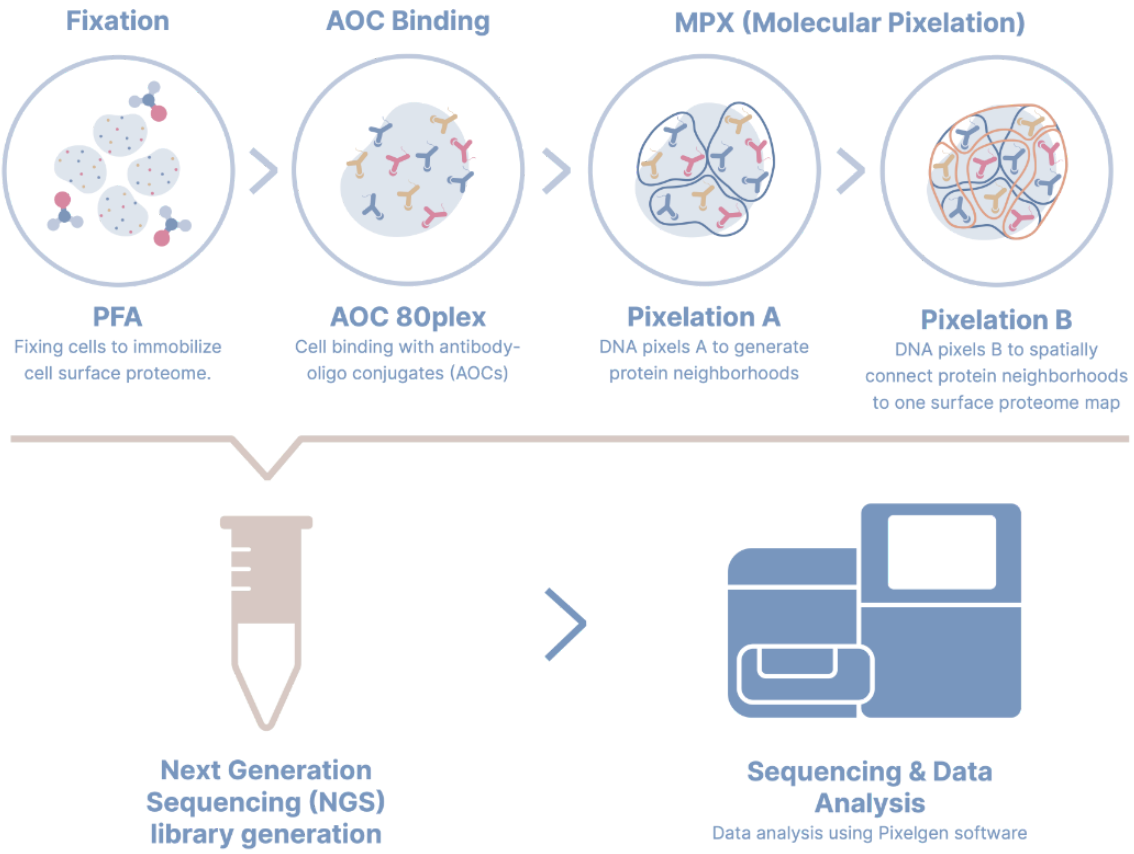


Figure 2. Overview of Molecular pixelation workflow.

There are 4 main steps in the protocol, starting from live cells in suspension to sequence-ready NGS library. Table 5. describes the workflow and outlines each step and the approximate time needed.

Table 5. Workflow steps and time needed.

Step	Description	Duration
1	**Cell preparation:** • Cell fixation using PFA • Cell blocking and PFA quenching using Blocking Buffer 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	1-2 h
2	**Antibody-oligo conjugate (AOC) binding:** • Binding of Antibody-oligo conjugates (AOCs) • Stabilization of bound AOCs using 2nd antibody • Cell counting and dilution STOP – Alt. stopping point: Up to 2 days at 4°C	3 h
3.1	**Pixelation (Pixelation A and B):** • Hybridization of pixel set A • Gap-fill ligation A • Enzymatic removal of pixel set A – Alt. stopping point: Up to 1 h at 4°C • Hybridization of pixel set B • Gap-fill ligation step B STOP – Alt. stopping point: Up to 16 hours at 4°C	4-5 h
3.2	**Pixelation (Exonuclease and PCR):** • Cell counting using hemocytometer and dilution • Exonuclease degradation of incomplete amplicons • PCR amplification and sample indexing STOP – Alt. stopping point: -20°C for long periods of time	3-4 h
4	**NGS preparation:** • PCR product cleanup using AMPure beads • Quantification and QC of NGS library	1-2 h

4. Tips & Best Practices

General good lab practice

- Label tubes before starting protocol.
- Ensure centrifuge is balanced prior to loading samples.
- Use calibrated pipettes.
- It is recommended to process a maximum of 8 reactions in parallel.
- All incubations higher or equal to 37°C should have a heated lid temperature of 105°C.
- Aspirate at least 50% of the total reagent volume when pipette mixing to ensure proper mixing.
- A multichannel pipette can be used for wash buffer addition prior to centrifugation during wash steps. If doing so, ensure equal volumes are dispensed to each tube. To remove the liquid after centrifugation during wash steps, a single pipette needs to be used to not disturb the cell pellet.
- Make sure to never aspirate close to the bottom of the tube during liquid removal in wash steps - pellet will not be visible when working with low cell numbers.

General reagent handling

- 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.
- Do not vortex samples or reagents.
- 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.
- 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.
- Use the necessary precautions when handling paraformaldehyde (PFA) as it is a CMR substance (Carcinogenic, Mutagenic, or toxic to Reproduction).

5. Protocol

STEP 1 - Cell preparation

During Step 1 of the workflow, live cell suspensions are fixated using paraformaldehyde (PFA) and blocked using the Blocking solution.

Cell preparation

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 automated cell counter (e.g., Countess II Automated Cell Counter), hemocytometer or other cell counting device, aiming at >90% viability.
- [ ] B. For each sample, transfer 500 000 - 1 million cells in separate PCR tubes, and add 1x PBS for a total volume of 150 µl per sample.
- [ ] C. Centrifuge at 400 rcf for 4 min at room temperature (RT).
- [ ] D. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] E. Add 125 µl of 1x PBS on top of the 25 µl cell suspension, 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 to preserve the pellet.
- [ ] H. Add 65 µl of 1x PBS on top of the 25 µl cell suspension, gently pipette up and down 10 times or until the cells are uniformly suspended and proceed to Cell fixation and blocking.

Cell fixation and blocking

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

Note Prepare a fresh solution of 2% v/v PFA solution (methanol free) in 1xPBS. A volume of 100 µl is needed per sample (90µl + extra). Use the solution within 2 hours, and store in dark until use.

- [ ] A. Add 90 µl of the **2% PFA solution** to each sample and pipette up and down 10 times.
- [ ] B. Incubate for 15 min at RT.
- [ ] C. Centrifuge at 700 rcf for 4 min at RT.
- [ ] D. From each 180 µl fixation sample, carefully discard 155 µl supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant.
- [ ] E. Invert the **Wash Buffer** tube 10 times to mix. Add 125 µl of **Wash Buffer** on top of the 25 µl cell suspension and pipette up and down 10 times.
- [ ] F. Centrifuge at 700 rcf for 4 min at RT.
- [ ] G. Carefully discard 135 µl supernatant without disturbing the cell pellet, leaving behind 15 µl of supernatant.
- [ ] H. Quick spin the **Blocking Buffer** and pipette it up and down 5 times to mix.
- [ ] I. Add 165 µl **Blocking Buffer** and pipette up and down 10 times.
- [ ] J. Incubate for 15 min at 4°C.
- [ ] K. Centrifuge at 700 rcf for 4 min at RT.
- [ ] L. Carefully discard 155 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] M. Add 125 µl of **Wash Buffer** on top of the 25 µl cell suspension and pipette up and down 10 times.
- [ ] N. Centrifuge at 700 rcf for 4 min at RT.
- [ ] O. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.

- [ ] P. Add 125 µl of 1x PBS on top of the 25 µl cell suspension.
- [ ] Q. Optional: Determine the cell concentration after PFA fixation, using either hemocytometer or other cell counting device (e.g. Countess II Automated Cell Counter).

POSSIBLE
Up to 5 days at 4°C or up to 3 months at -80°C if performing freezing protocol

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 AOC binding.

OPTIONAL: Freezing and thawing of fixed cells

This is an optional step, if planning to do AOC binding within 5 days, please proceed to *STEP 2 - Antibody-Oligo Conjugate (AOC) binding*.

The fixed and blocked cells can be frozen in -80°C for longer storage, up to 3 months. This part of the protocol describes the freezing and thawing procedure.

Freezing of PFA-fixed cells

Note Prepare a fresh freezing solution of 5% DMSO and 95% FBS. A volume of 600 µl per sample is needed (500 µl + extra).

- [ ] A. Centrifuge at 700 rcf for 4 min at RT and remove 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl.
- [ ] B. Add 125 µl freezing solution and pipette up and down 10 times.
- [ ] C. Transfer the 150 µl cell solution to a cryotube.
- [ ] D. Add 350 µl freezing solution to the 150 µl cell solution.
- [ ] E. 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.

Thawing of PFA-fixed, frozen cells

- [ ] F. Put the cryotubes in a 37°C bath for up to 5 minutes (confirm that the cell solution has thawed).
- [ ] G. Transfer the thawed 500 µl cell solution to a 1.5 ml Eppendorf tube.
- [ ] H. Add 500 µl **Wash buffer** to the empty cryotube and pipette up and down 10 times to wash any remaining cells.
- [ ] I. Transfer the 500 µl wash to the Eppendorf tube. Total of 1000 µl in each tube.
- [ ] J. Centrifuge at 700 rcf for 4 min at RT.
- [ ] K. Remove 950 µl supernatant without disturbing the cell pellet, leaving behind 50 µl.
- [ ] L. Add 950 µl **Wash buffer** and pipette up and down 10 times.
- [ ] M. Centrifuge at 700 rcf for 4 min at RT.
- [ ] N. Remove 950 µl supernatant without disturbing the cell pellet, leaving behind 50 µl.
- [ ] O. Add 100 µl 1x PBS to resuspend the pellet.
- [ ] P. Optional: Determine the cell concentration after thawing, using either hemocytometer or other cell counting device (e.g. Countess II Automated Cell Counter).

POSSIBLE
Up to 24 hours
at 4°C

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

STEP 2 - Antibody-Oligo Conjugate (AOC) binding

Step 2 contains binding of Antibody-oligo conjugates (AOCs) to 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 Pixelation steps.

> Note From this step and forward, make sure cells become resuspended during pipette mixing steps by mixing vigorously. Too gentle pipette-mixing may result in cell aggregate formation.

AOC binding

> Note The recommended starting point is to load 200 000 cells per sample in 1xPBS to a total volume of 150 µl (as described below).

- [ ] A. Centrifuge the vial containing **AOC panel** solution at 17000 rcf for 10 min at RT or 4°C, to pull down any aggregates to the bottom of the **AOC panel** vial.
- [ ] B. Meanwhile, mix each sample containing cells by pipetting up and down 10 times.
- [ ] C. Transfer 200 000 fixed cells to new PCR tubes and add 1x PBS for a total volume of 150 µl per sample.
- [ ] D. Centrifuge at 700 rcf for 4 min at RT.
- [ ] E. Carefully discard 125 µl sample supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] F. Add 25 µl of the centrifuged **AOC panel** solution to each 25 µl sample, to a final volume of 50 µl, and pipette up and down 10 times. Make sure to avoid aspirating near the bottom of the centrifuged **AOC panel** vial.
- [ ] G. Incubate for 45 min at 4°C.
- [ ] H. Add 100 µl **Wash Buffer** on top of the 50 µl cell suspension and pipette up and down 10 times.
- [ ] I. Centrifuge at 700 rcf for 4 min at RT.
- [ ] J. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.

- [ ] K. Add 125 µl **Wash Buffer** on top of the 25 µl cell suspension and pipette up and down 10 times.
- [ ] 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 supernatant to preserve the pellet.
- [ ] N. Repeat steps K-M 2 more times, for a total of 4 washes (including H-J). It is important to perform in total 4 washes to completely remove any remaining unbound AOCs present in the solution.
- [ ] O. Quick spin the **2nd Antibody** and pipette it up and down 5 times to mix.
- [ ] P. Add 25 µl of **2nd Antibody** to the 25 µl cell pellet and pipette up and down 10 times.
- [ ] Q. Incubate for 30 min at 37°C.
- [ ] R. Add 100 µl **Wash Buffer** on top of the 50 µl cell suspension and pipette up and down 10 times.
- [ ] S. Centrifuge at 700 rcf for 4 min at RT.
- [ ] T. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] U. Add 125 µl **Wash Buffer** on top of the 25 µl cell suspension and pipette up and down 10 times.
- [ ] V. Centrifuge at 700 rcf for 4 min at RT.
- [ ] W. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] X. Resuspend the cell pellet in 75 µl of 1xPBS and pipette up and down 10 times. Total of 100 µl in each tube.
- [ ] Y. Determine the cell concentration for each sample using either hemocytometer or other cell counting device (e.g Countess II Automated Cell Counter). Mix by pipetting up and down 10 times before taking an aliquot for counting.

POSSIBLE
Up to 2 days at 4°C

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 Pixelation (20 000 cells).

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 3.1 - Pixelation (Pixelation A and B)

In this step of the protocol, a series of DNA hybridization and enzymatic treatments are performed to incorporate DNA-pixel barcodes onto AOC oligos bound to the cells, forming the amplicons that can be amplified by PCR and finally sequenced.
Reminder: quick spin all reagents and pipette each reagent tube up and down 5 times to mix before preparing the Master mix.

DNA-Pixel A hybridization

Note The recommended starting point is to load 20 000 cells per sample in 1xPBS to a total volume of 25 µl (as described below).

Note The protocol can be paused for up to 1h between the steps Pixel A remover and DNA-Pixel B hybridization.

- [ ] A. Dilute cells in fresh PCR tubes to 800 cells/µl in 1x PBS for a total of 25 µl (20 000 cells in total per sample).

Note If the cell concentration is lower than 800 cells/µl, centrifuge sample at 700 rcf for 4 min at RT and remove e.g. 50% of the volume to get 2x original concentration.

- [ ] B. Thaw the **Pixel A Diluent** and **Pixel A**, and prepare **Pixel A** hybridization master mix as indicated in Table 6. directly before use by combining the reagents in the order listed below, and maintain at RT.

Table 6. **Pixel A** hybridization 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	Pixel A Diluent	BD019A	16.5 µl	66 µl	132 µl
Box2 -20°C	Pixel A	PP015A	11 µl	44 µl	88 µl
	Total		27.5 µl	110 µl	220 µl

- [ ] C. Quick spin the **Pixel A** hybridization master mix and pipette up and down 5 times to mix.

- [ ] D. Dispense 25 µl **Pixel A** hybridization master mix to each 25 µl sample and pipette up and down 10 times. Total of 50 µl in each tube.
- [ ] E. Incubate the samples in a thermal cycler for 15 min at 55°C.
- [ ] F. Remove the samples from the thermal cycler, add 100 µl **Wash Buffer** to each sample and pipette up and down 10 times. Total of 150 µl in each tube.
- [ ] G. Centrifuge at 700 rcf for 4 min at RT.
- [ ] H. Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- [ ] I. To each sample, add 125 µl of **Wash Buffer** and pipette up and down 10 times.
- [ ] 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 supernatant to preserve the pellet.

Gap-fill ligation step 1

- [ ] A. Thaw the **Gap-fill Buffer** and prepare **Gap-fill** master mix as indicated in Table 7. directly before use by combining the reagents in the order listed below and maintain at RT. Keep the **Gap-fill Buffer** on ice or at 4°C, until used at the second Gap-fill ligation step 2 of the protocol (put the enzymes back at -20°C until second Gap-fill ligation step).

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 Buffer	BD018	25.3 µl	101.2 µl	202.4 µl
Box2 -20°C	Gap-fill Ligase	EE020	1.1 µl	4.4 µl	8.8 µl
Box2 -20°C	Gap-fill Polymerase	EE021	1.1 µl	4.4 µl	8.8 µl
	Total		27.5 µl	110 µl	220 µl

- [ ] B. Quick spin the **Gap-fill** hybridization master mix and pipette up and down 5 times to mix.
- [ ] C. Dispense 25 µl **Gap-fill** hybridization master mix to each 25 µl sample and pipette up and down 10 times. Total of 50 µl in each tube.
- [ ] D. Incubate the samples in a thermal cycler for 20 min at 37°C.
- [ ] E. Add 100 µl of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 µl in each tube.
- [ ] 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 to preserve the pellet.

Pixel A remover

- [ ] A. Prepare **Pixel A Remover** master mix according to Table 8. directly before use by adding the reagents in the order listed below and maintain at RT.

Table 8. **Pixel A Remover** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box1 +4°C	Wash Buffer	PP016	26.4 µl	105.6 µl	211.2 µl
Box2 -20°C	Pixel A Remover (enzyme)	EE022	1.1 µl	4.4 µl	8.8 µl
	Total		27.5 µl	110 µl	220 µl

- [ ] B. Quick spin the **Pixel A Remover** master mix and pipette up and down 5 times to mix.
- [ ] C. Dispense 25 µl **Pixel A Remover** master mix to each 25 µl sample and pipette up and down 10 times. Total of 50 µl in each tube.
- [ ] D. Incubate the samples in a thermal cycler for 15 min at 37°C.
- [ ] E. Add 100 µl of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 µl in each tube.
- [ ] 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 to preserve the pellet.

Note If pausing for up to 1 h at this step, resuspend the pellet by pipetting up and down 10 times before putting the samples at 4°C.

DNA-pixel B hybridization

- [ ] A. Thaw the **Pixel B Diluent** and **Pixel B**, and prepare **Pixel B** hybridization master mix as indicated in Table 9. directly before use by adding the reagents in the order listed below and maintain at RT.

Table 9. **Pixel B** hybridization 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	Pixel B Diluent	BD019B	16.5 µl	66 µl	132 µl
Box2 -20°C	Pixel B	PP015B	11 µl	44 µl	88 µl
	Total		27.5 µl	110 µl	220 µl

- [ ] B. Quick spin the **Pixel B** hybridization master mix and pipette up and down 5 times to mix.
- [ ] C. Dispense 25 µl **Pixel B** hybridization master mix to each 25 µl sample and pipette up and down 10 times. Total of 50 µl in each tube.
- [ ] D. Incubate the samples in a thermal cycler for 15 min at 55°C.
- [ ] E. Add 100 µl **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 µl in each tube.
- [ ] 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 to preserve the pellet.
- [ ] H. To each sample, add 125 µl **Wash Buffer** and pipette up and down 10 times.

- [ ] 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 to preserve the pellet.

Gap-fill ligation step 2

- [ ] A. Prepare **Gap-fill** master mix as indicated in Table 10. directly before use by adding the reagents in the order listed below and maintain at RT. Return remainders of the Gap-fill Buffer and enzymes to -20°C storage.

Table 10. **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 Buffer	BB018	25.3 µl	101.2 µl	202.4 µl
Box2 -20°C	Gap-fill Ligase	EE020	1.1 µl	4.4 µl	8.8 µl
Box2 -20°C	Gap-fill Polymerase	EE021	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. Dispense 25 µl **Gap-fill** master mix to each 25 µl sample and pipette up and down 10 times. Total of 50 µl in each tube.
- [ ] D. Incubate the samples in a thermal cycler for 20 min at 37°C.
- [ ] E. Add 100 µl of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 µl in each tube.
- [ ] 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 to preserve the pellet.
- [ ] H. To each sample, add 125 µl of **Wash Buffer** and pipette up and down 10 times.

- [ ] 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 to preserve the pellet.
- [ ] K. Add 50 µl **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 75 µl in each tube.

POSSIBLE
Up to 16 hours
at 4°C

Note At this step, cells can be stored at 4°C for up to 16 hours before proceeding with the next steps of the protocol.

STEP 3.2 - Pixelation (Exonuclease & PCR)

Exonuclease treatment

- [ ] A. Count cells using a hemocytometer or single-use counting chamber. To ensure accuracy, count cells in replicates (2 reproducible counts). See *2. General Guidelines and Information: cell counting considerations* for more detailed information regarding cell counting.

- [ ] B. For each sample, transfer 300 to 1000 cells (depending on experimental set-up) in **Wash Buffer** to a final volume of 7 µl, in new PCR tubes. See *Appendix 1* for an example table.

Note If the cell concentration is lower than 143 cells/µl, centrifuge sample at 700 rcf for 4 min at RT and remove e.g. 50% of the volume to get 2x original concentration.

- [ ] C. Prepare **Exonuclease** master mix as indicated in Table 11. directly before use by adding the reagents in the order listed below, pipette up and down 10 times and maintain at RT until use.

Table 11. **Exonuclease** 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 Buffer	BB018	7.7 µl	30.8 µl	61.6 µl
Box2 -20°C	Exonuclease	EE023	1.1 µl	4.4 µl	8.8 µl
	Total		8.8 µl	35.2 µl	70.4 µl

- [ ] D. Dispense 8 µl **Exonuclease** master mix to each 7 µl cell sample containing cells and pipette up and down 5 times to mix, total of 15 µl in each tube.

- [ ] E. Incubate in a thermal cycler at 37°C for 30 min, followed by 75°C for 10 min (Table 12).

Table 12. Incubation protocol for **Exonuclease** treatment.

Lid temperature	Reaction Volume	Run Time
105°C	15 µl	40 min
Step	**Temperature**	**Duration**
1	37°C	00:30:00
2	75°C	00:10:00
3	4°C	Hold

Sample index PCR

Sample indexes are introduced during PCR, allowing for pooling and sequencing multiple reactions in the same sequencing run. The reagent kit is supplied with 8 different sample indexes. Please see Paragraph 4 “General Guidelines & Information'' in this document, for more detailed description and considerations related to sample indexing.

> Note 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 13.
- [ ] B. Thaw the **PCR Master Mix** vial (box 3) completely prior to use. Quick spin the **PCR Master Mix** and pipette up and down 5 times to mix.
- [ ] C. Add 5 µl of one unique **PCR index primer** (box 3) to each 15 µl exonuclease treated sample. Make a note of the primer index used for each sample.
- [ ] D. Add 20 µl of **PCR Master Mix** and pipette up and down 10 times to mix. Total of 40 µl per sample.
- [ ] E. Perform PCR using the following PCR program (Table 13.).

Table 13. PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	40 µl	~ 42 min	
Step	**Temperature**	**Time**	**Number of cycles***
1	98°C	00:01:00	1
2	98°C	00:00:15	1000 cells: 13 cycles 500 cells: 14 cycles 300 cells: 15 cycles
3	60°C	00:00:30	
4	72°C	00:00:40	
5	72°C	00:05:00	1
6	4°C	Hold	

* Adjust number of PCR cycles with number of cells input to PCR reaction.

POSSIBLE
Up to 24 hours at 4°C or long term storage at -20°C

Note The PCR products can be stored at 4°C for up to 24 hours or at -20°C for long term storage.

Note **Quality control** (QC) can be performed at this step, using an aliquot of the PCR product for gel electrophoresis. QC can also be performed after the AMPure beads clean-up, using instruments such as BioAnalyzer™, Tapestation™ or Fragment Analyzer™. See section *STEP 4 - Quantification and quality control of purified PCR products* for more information.

STEP 4 - NGS preparation

AMPure XP beads clean-up

The PCR products are purified using Ampure XP SPRI beads prior to NGS sequencing to remove primers, salts, dNTPs etc. Two consecutive rounds of AMPure cleanup is performed to ensure all primers have been removed, as any remaining primers will negatively affect sequencing results.

Third party Consumables and hardware required:

- AMPure XP (Beckman Coulter, cat. no. A63880)
- Invitrogen™ DynaMag™-96 Side Magnet (ThermoFisher, cat. no. 12331D)
- TE Buffer (10 mM Tris-Acetate pH 8.0, 1 mM EDTA) for DNA elution
- Freshly prepared 70% ethanol

Note Place Agencourt AMPure XP beads at RT for 30 min before use, as recommended by Beckman Coulter.

Note Always use freshly prepared 70% ethanol, as recommended by Beckman Coulter. Prepare 1 ml (720 µl + extra) 70% ethanol per sample.

Note It is important to perform two rounds of cleanup for complete removal of PCR primers.

- [ ] A. Vortex the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.
- [ ] B. For each sample, transfer 30 µl of PCR product to fresh PCR tubes. The remaining volume can be stored at -20°C as backup.
- [ ] C. Add 45 µl (1.5x bead:sample ratio) of resuspended AMPure bead mixture to each sample. Pipette mix 10 times to form a homogenous mixture.
- [ ] D. Incubate for 5 min at RT.

- [ ] E. Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.
- [ ] F. Carefully discard the supernatant, without disturbing beads.
- [ ] G. Add 180 µl of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- [ ] H. Carefully remove the supernatant, without disturbing beads.
- [ ] I. Add 180 µl of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- [ ] J. Carefully discard the supernatant, without disturbing beads.
- [ ] K. Completely remove the residual ethanol and air-dry beads with lid open until any remaining ethanol has evaporated.

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 the PCR tubes from the magnet.
- [ ] M. Resuspend dried beads in 30 µl of TE Buffer and incubate for 2 min at room temperature to elute the PCR product from the beads.
- [ ] N. Place the PCR tube on the magnet and incubate for 2 min, until the beads have settled against the tube wall.
- [ ] O. Carefully transfer the 30 µl supernatant to new PCR tubes, without disturbing beads. Do not discard the supernatant in this step as it contains eluted product.
- [ ] P. Initiate cleanup round 2 by vortexing the Agencourt AMPure XP bottle and adding 45 µl (1.5x bead:sample ratio) of resuspended AMPure bead mixture to each 30 µl elution. Pipette mix 10 times to form a homogenous mixture.
- [ ] Q. Incubate for 5 min at RT.
- [ ] R. Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.
- [ ] S. Carefully discard the supernatant, without disturbing beads.
- [ ] T. Add 180 µl of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- [ ] U. Carefully discard the supernatant, without disturbing beads.

- [ ] V. Add 180 µl of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- [ ] W. Carefully discard the supernatant, without disturbing beads.
- [ ] X. Completely remove the residual ethanol and air-dry beads with lid open until any remaining ethanol has evaporated.

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.

- [ ] Y. Remove the PCR tubes from the magnet.
- [ ] Z. Resuspend dried beads in **20 µl** of TE Buffer and incubate for 2 min at room temperature to elute the PCR product from the beads.
- [ ] AA. Place the PCR tube on the magnet and incubate for 2 min, until the beads have settled against the tube wall.
- [ ] BB. Carefully transfer the 20 µl supernatant to new PCR tubes, without disturbing beads. Do not discard the supernatant in this step as it contains the purified PCR product.

The purified PCR products can either be prepared for sequencing directly, or stored at -20°C until use.

Quantification and quality control of purified PCR products

Before proceeding to NGS sequencing, samples need to be:

- Quality controlled (to confirm the correct size of approximately 268 bp)
- Quantified, diluted and pooled

Table 14. 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 14. 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 (ThermoFischer)	❌	✅
Gel Electrophoresis with TBE gel, 200V for 25 min	✅	❌

The size of the generated PCR product is approximately 268 bp. To convert a concentration reading from ng/µl to nM, a conversion factor of 5.65 can be used:

Library concentration [ng/µl] x 5.65 = X nM

After QC and quantification, samples should be diluted and pooled. One method for performing **equimolar pooling** is to normalize each sample to the same concentration, followed by pooling by equal volumes of each normalized sample. The required concentration of the final pool for Illumina sequencing depends on the Illumina platform used. Creating a 2 nM pool will be sufficient for most Illumina platforms. For additional information, see Illumina guidelines for normalizing library concentrations [1].

The generated pool can be quantified again to ensure it is at the intended concentration.

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 recommended loading concentration for "PhiX libraries", as suggested by Illumina.

NGS sequencing using 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 to increase the diversity and base quality of low diversity regions. Please consult Illumina documentation for PhiX spiking.

Insufficient amount of sequencing reads per reaction will result in poor data quality.

Each reaction, corresponding to 300-1000 cells, should be given at least **120 000 reads/cell** to ensure good performance and to take into account cell counting variability.

Sequencing on NextSeq® 1000/2000, NovaSeq® 6000 and NovaSeq® X Plus has been validated.

Table 15. 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 but will result in trimming of these additional bases during downstream Pixelator data processing.

Table 15. Sequencing parameters.

Sequencing depth	> 120 000 read pairs per 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*

* Assuming sample indexes provided in reagent kit are used

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

6. References

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

Appendix 1

Dilution before Exonuclease treatment: example

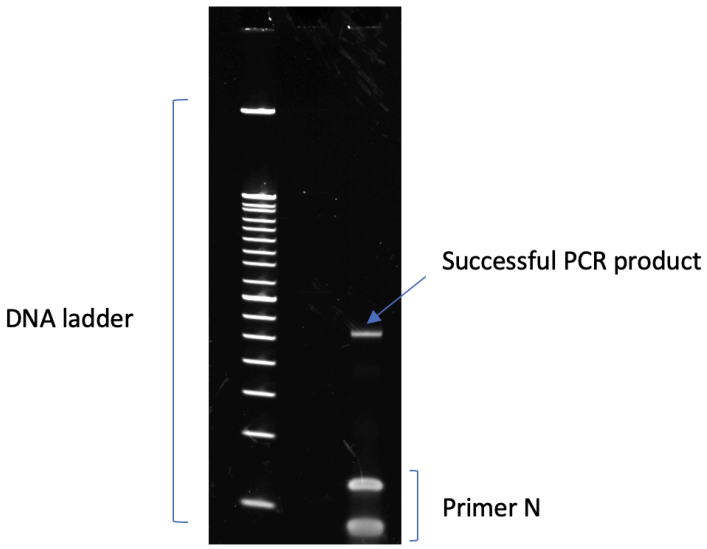
Appendix Table 1. displays an example of two samples with concentrations of 160 and 200 cells/µl and how to dilute them in Wash buffer to achieve 1000 cells per reaction in 7 µl total.

Appendix Table 1. Example table of how to dilute cells before Exonuclease treatment, to achieve 1000 cells per reaction for sequencing.

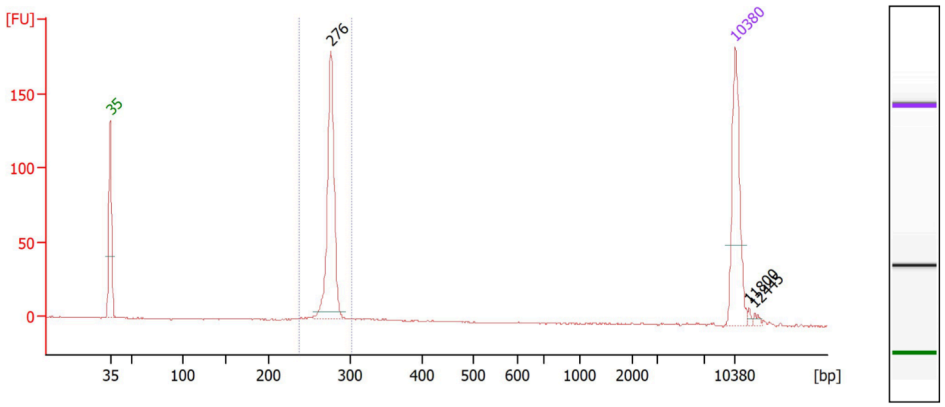
Sample	Cell concentration	Sample volume needed	Wash buffer needed	Total
Sample 1	160 cells/µl	1000/160 = 6.25 µl	7-6.25 = 0.75 µl	6.25+0.75 = 7 µl
Sample 2	200 cells/µl	1000/200 = 5 µl	7-5 = 2 µl	5+2 = 7 µl

QC of NGS library size

The expected size of the PCR product is 268 bp. The presence of PCR products of the intended size can be confirmed using either gel electrophoresis or fragment analyzing assays.



Appendix Figure 1. An example of a successfully amplified PCR product prior to AMPure cleanup using TBE gel electrophoresis. To the left - 50 bp DNA ladder, to the right - 268 bp PCR product.



Appendix Figure 2. An example of a successfully amplified PCR product after AMPure cleanup using BioAnalyzer. The peak of the product is expected at around 268 bp.