USER MANUAL

PIXELGEN PROXIOME v2 CUSTOM CONJUGATION KIT

PROXCUST001, PROXCUST002, PROXCUST003



PIXELGEN
TECHNOLOGIES

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List of Abbreviations

BSA	Bovine Serum Albumin
CMR	Substance Carcinogenic, Mutagenic, or toxic to Reproduction
FBS	Fetal Bovine Serum
PBS	Phosphate-Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde
QC	Quality control
RT	Room Temperature

About this User Manual

This User Manual describes the experimental procedure using the Pixelgen Proxiome v2 Custom Conjugation Kit, (PROXCUST001, PROXCUST002 and PROXCUST003) in detail.

Technical Support

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

1. Product Description

The Pixelgen Proxiome Kit v2 offers a 155-plex panel for spatial profiling of immune cell surface proteins at nanoscale resolution. When customers need to include a custom target of interest not covered by the standard panel, they can conjugate their own antibody using this kit.

This protocol provides step-by-step instructions for customers to validate and conjugate custom antibodies. The protocol covers:

- Flow cytometry-based antibody validation (live and fixed cells)
- UV-mediated antibody–oligonucleotide conjugation
- Quality control (QC) of conjugates by SDS-PAGE

The final product will be ready-to-use conjugated antibodies that will be spike-in into the Antibody Panel of the Pixelgen Proxiome Kit v2 before proceeding with STEP 3 as described in the UM002: User Manual Proxiome Kit v2 Cell Surface.

This protocol is intended for researchers with standard cell culture and flow cytometry experience and supports conjugation of up to two antibodies.

NOTE: This protocol guides you through antibody validation by flow cytometry prior to conjugation (PART A), and then through the conjugation and QC steps (PART B). Validating your antibody before conjugation is strongly recommended to confirm target-specific staining with both live and fixed cells.

Each kit supports conjugation of up to two **monoclonal antibodies** of your choice, with one barcode assigned to each antibody; each barcode is available in two versions, A and B. Three kit versions are available:

- Version 1 is designed for conjugation of two mouse IgG1 antibodies, catalog number PROXCUST001.
- Version 2 is designed for conjugation of two antibodies with any host/subclass other than mouse IgG1, catalog number PROXCUST002.
- Version 3 is designed for conjugation of one mouse IgG1 antibody and one antibody with any other host/subclass, catalog number PROXCUST003.

Disclaimer: Although Version 1 is designed for antibodies with a mouse IgG1, some mouse IgG1 antibodies may show lower conjugation efficiency than expected. Conjugation performance can vary depending on the individual antibody clone, purity, and other antibody-specific properties. Customers may still choose to proceed with mouse IgG1 antibodies, but please note that performance efficiency cannot be guaranteed. Please check the "oYo-Link® Antibody Compatibility" table linked in *Section 5 - References*, to check your antibody(ies) compatibility with the conjugation method provided in this protocol [1].

IMPORTANT! Use only unconjugated monoclonal antibodies. Polyclonal antibodies are incompatible with the Pixelgen Proxiome Kit v2.

The core steps of the Pixelgen Proxiome v2 Custom Conjugation Kit are illustrated in Figure 1 below.

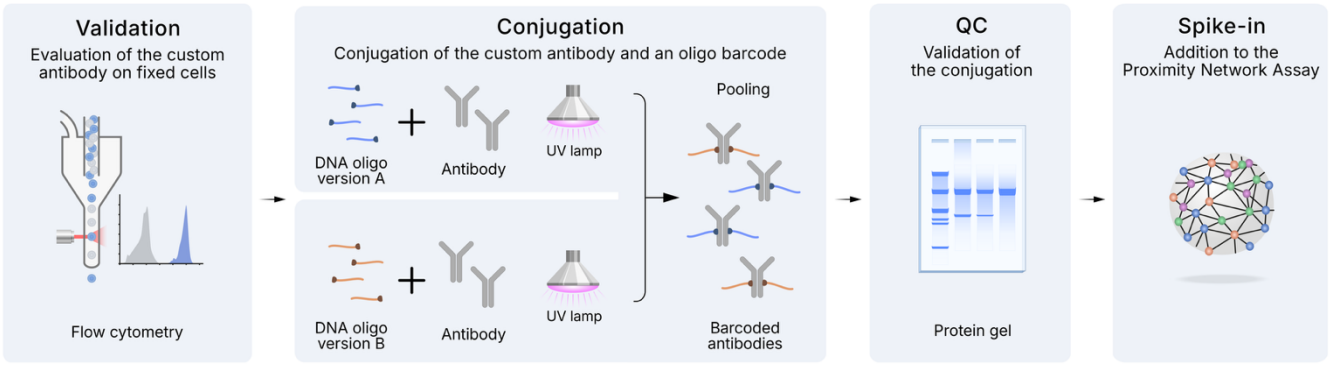


Figure 1. Overview of the Pixelgen Proxiome v2 Custom Conjugation Kit workflow and core steps.

Reagents supplied with the Pixelgen Proxiome v2 Custom Conjugation Kit

Each kit includes sufficient reagents to conjugate up to two monoclonal antibodies of your choice. The reagents are supplied in one box, identified by its catalog number, with the storage temperature and expiration date indicated on the label. Each box contains five tubes: two barcodes (BC), each provided in two versions (Oligo A and Oligo B), and one Storage Solution.

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

Catalog number:
PROXCUST001: Pixelgen Proxiome v2 Custom Conjugation Kit, 2 x mouse IgG1
PROXCUST002: Pixelgen Proxiome v2 Custom Conjugation Kit, 2 x other host/subclass
PROXCUST003: Pixelgen Proxiome v2 Custom Conjugation Kit, 1 x mouse IgG1 and 1 x other host/subclass

Table 1. Reagents supplied in the box and the storage temperatures. Bullet point colors correspond to the reagent lid color.

Box Content PROXCUST001	Box Content PROXCUST002	Box Content PROXCUST003
store at -20°C	store at -20°C	store at -20°C
Storage Solution BD062	Storage Solution BD062	Storage Solution BD062
oYo-Link® BC3 – Oligo A BO109_A	oYo-Link® BC1 – Oligo A BO107_A	oYo-Link® BC2 – Oligo A BO108_A
oYo-Link® BC3 – Oligo B BO109_B	oYo-Link® BC1 – Oligo B BO107_B	oYo-Link® BC2 – Oligo B BO108_B
oYo-Link® BC4 – Oligo A BO110_A	oYo-Link® BC2 – Oligo A BO108_A	oYo-Link® BC4 – Oligo A BO110_A
oYo-Link® BC4 – Oligo B BO110_B	oYo-Link® BC2– Oligo B BO108_B	oYo-Link® BC4 – Oligo B BO110_B

The oYo-Link® oligonucleotides are manufactured and supplied by AlphaThera, Inc.

Additional Requirements

A list of equipment, reagents and consumables required to perform the Pixelgen Proxiome v2 Custom Conjugation Kit can be found below. The suggested suppliers and catalog 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	Catalog number
Centrifuge	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
Long-wavelength UV light source (~365 nm) *	UV lamp LEDPX2 from AlphaThera (international compatibility)	AlphaThera	AT8001-D
Tank to run gels	XCell SureLock™ Mini-Cell	Thermo Fisher	EI0001
Power Unit	PowerEase Touch 120W Power supply	Thermo Fisher	PS0121
Gel Imager	GelDoc Go Gel Imaging System with White Sample Tray	Bio-Rad	12009077

* oYo-Link® products are compatible with a wide range of long-wavelength UV photo-crosslinking devices operating at 365 nm. Please refer to the “Compatible photocrosslinking devices” list available on the AlphaThera website for suitable alternatives [2].

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

Consumables			
Description	Product name	Suggested Supplier	Catalog number
0.2 ml PCR tubes or	FastGene® PCR Tubes 0.2 ml	Nippon Genetics Europe	FG-021
0.2 ml PCR tube strips or	8-well PCR tube strips 0.2 ml with cap strips	Nippon Genetics Europe	FG-088WF
0.2 ml PCR tube strips	PCR strip tubes, Axygen®	Corning	PCR-0208-AF-C
0.5 ml low adhesion tube	Protein LoBind® Tubes	Eppendorf	0030108094
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	Catalog number
1x PBS	PBS, pH 7.4	Gibco™	10010-023
Paraformaldehyde, methanol-free*	Paraformaldehyde 16% Aqueous Sol.	Electron Microscopy Sciences or Thermo Fisher	15710 28906/28908
Bovine Serum Albumin, protease-free, ≥98% purity	Bovine Serum Albumin	Sigma-Aldrich®	A3294
Fc blocking	Human TruStain FcX or Human Fc Block	Biolegend or BD Biosciences	422301 564220
Fetal Bovine Serum	FBS, heat-inactivated	Sigma-Aldrich®	F9665
1M Glycine solution	1M Glycine solution	Sigma-Aldrich®	67419-1ML-F
EDTA	EDTA (0.5 M), pH 8.0, RNase-free	Thermo Fisher	AM9261
2nd Antibody**	Anti-mouse PE	Thermo Fisher	P852
Protein Gel	NuPAGE 4-12% BisTris Mini Protein gel, 15-well	Thermo Fisher	NP0336BOX
Sample Buffer	NuPAGE™ LDS Sample Buffer (4X)	Thermo Fisher	NP0007
Running Buffer	NuPAGE™ MOPS SDS Running Buffer (20X)	Thermo Fisher	NP0001
Protein ladder for gel	Mark12™ Unstained Standard	Thermo Fisher	LC5677
Deionized Water	Water Deionized	Sigma-Aldrich®	38796-1L
Coomassie Blue	ReadyBlue Protein gel stain	Sigma-Aldrich®	RSB-1L

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

** If your primary antibody is a mouse isotype, we recommend using this secondary antibody for Flow cytometry validation.

2. Workflow and Guidelines

The protocol outlines the complete assay workflow, from flow cytometry validation of antibodies to ready to spike-in conjugated antibodies in the Antibody Panel in STEP 3 of the UM002: User Manual Proxiome Kit v2 Cell Surface.

Workflow Overview and Steps

The workflow is divided into PART A - Flow Cytometry Validation of Antibodies and PART B - Custom Antibody Conjugation. It has been demonstrated and validated using resting and stimulated PBMCs.

Table 3. Workflow steps and time needed for **PART A - Flow Cytometry Validation of Antibodies**.

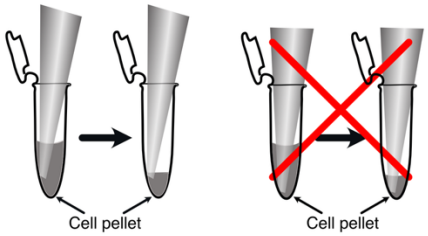
Step	Description	Duration
1	Cell Preparation: 1.1 Harvest, count and wash cells 1.2 PFA fixation and quenching 1.3 Cell blocking	1.5 h
2	Primary Antibody Staining: 2.1 Primary antibody(ies) binding on live and fixed cells	1.5 h
3	2nd Antibody Staining: 3.1 2nd antibody binding	1 h
4	OPTIONAL – Proximity Network Assay (PNA) simulation: 4.1 Incubate fixed, stained cells under PNA-like conditions	1 h
5	Evaluation of the results: 5.1 Evaluation of flow cytometry results	

Table 4. Workflow steps and time needed for **PART B – Custom Antibody Conjugation**.

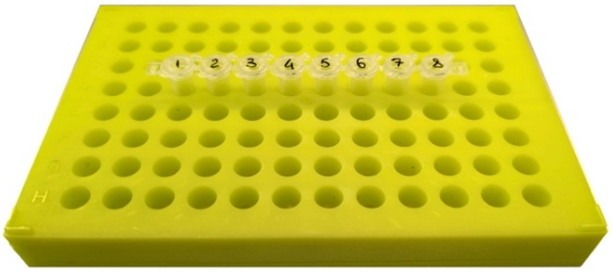
Step	Description	Duration
6	Antibody(ies) preparation and conjugation reaction: 6.1 Antibody(ies) conjugation	2.5 h
7	Quality control of conjugation efficiency 7.1 Gel electrophoresis 7.2 Gel staining 7.3 Evaluation of the results	2 h

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 for optimized cell preparation prior to running your reactions [3].
- 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.
- 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.
- 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 oligonucleotides, antibodies and buffers to their respective 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. PART A - Flow Cytometry Validation of Antibodies

STEP 1 - Cell Preparation

During STEP 1 of the workflow, cells are harvested, washed, and counted. Before beginning the protocol, it is important to block the PCR tubes with BSA, which helps reduce cell loss. Once ready, the cells are transferred to the BSA-blocked PCR tubes, fixed with PFA, quenched with glycine, and blocked with an Fc blocker. Suggested Fc blocking reagents are listed in Table 2c.

1.1 Harvest, count and wash cells

PREPARATION: Use 500 000 – 1 000 000 live cells per staining condition. Harvest cells under appropriate experimental conditions; handle gently to preserve viability and surface integrity. Count cells using an automated counter or hemocytometer before starting.

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 protease-free and of ≥98% purity.

Table 5 below presents a suggested experimental design with eight conditions for live and fixed cells, including the desired antibody(ies) of interest and appropriate controls such as isotype control, secondary antibody-only, and unstained samples.

Table 5. Suggested experimental design.

Sample	Condition	Antibody
1	Live	Target antibody
2	Live	Isotype control
3	Live	Secondary antibody only
4	Live	Unstained
5	PFA-Fixed	Target antibody
6	PFA-Fixed	Isotype control
7	PFA-Fixed	Secondary antibody only
8	PFA-Fixed	Unstained

PREPARATION: Start by blocking each PCR tube with **1x PBS + 0.5% BSA** which helps reducing cell loss.

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

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

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

NOTE: If not used immediately, store the coated BSA-blocked tubes at 4°C up to 48h.

D. ☐ When ready, transfer 500 000 – 1 000 000 cells into BSA-blocked PCR tubes. Add **1x PBS** to a total volume of 150 µl per sample.

E. ☐ Centrifuge at 400 rcf for 4 min at room temperature (RT).

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

G. ☐ Add 125 µl of **1x PBS** and pipette up and down 10 times.

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

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

NOTE: If you follow the experimental design suggested in Table 5: for live-cell Conditions 1-4, proceed directly to *STEP 1.3 - Cell blocking*; for PFA-fixed Conditions 5-8, first perform *STEP 1.2 - PFA fixation and quenching*.

IMPORTANT! Always compare fixed cells to live cells — fixation frequently alters epitope conformation and may affect antibody binding.

1.2 PFA fixation and quenching (PFA fixed conditions only)

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

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

A. ☐ Add 55 µl of **1x PBS** and pipette up and down 10 times gently (total volume 80 µl).

B. ☐ 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.

C. ☐ Incubate for 15 min at RT. Pipette gently up and down 10 times during incubation.

D. ☐ Quench the PFA by adding 25 µl of **1M Glycine solution on top of the 160 µl fixation solution** and pipette up and down 10 times.

E. ☐ Centrifuge at 700 rcf for 4 min.

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

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

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

I. ☐ Repeat steps G–H one additional time.

J. ☐ Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl of supernatant. Proceed to *STEP 1.3 - Cell Blocking*.

1.3 Cell blocking

NOTE: Block cells to reduce nonspecific antibody binding, particularly via Fc receptors.

PREPARATION: Prepare 50 ml of Staining Buffer (**2% FBS + 2 mM EDTA in 1x PBS**).

PREPARATION: Prepare Fc Blocking Reagent as suggested by the vendor in Staining Buffer.

A. ☐ Add 75 µl of **Fc Blocking Reagent** to each sample.

B. ☐ Incubate for 15 min at 4°C (follow the manufacturer's recommendation or your in-house validated conditions).

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

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

E. ☐ Add 75 µl of **Staining Buffer**. Cells are now in 100 µl.

F. ☐ 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. Confirm you have at least 250 000 cells per condition before proceeding.

G. ☐ Bring the final count to 250 000 in 25 µl of **Staining Buffer**. Proceed with *STEP 2 - Primary Antibody Staining*.

STEP 2 - Primary Antibody Staining

During STEP 2 of the workflow, the target antibody(ies) and the isotype control are first diluted to the working concentration and then added to both live and fixed cells. Cells are then washed to remove unbound antibodies and reduce background.

2.1 Primary antibody(ies) binding on live and fixed cells

NOTE: Always include the correct isotype control matching the species and subclass of your primary antibody (e.g., mIgG1, mIgG2a, or mIgG2b). Ensure all dilutions are made in Staining Buffer.

A. ☐ Dilute both the target antibody(ies) and the isotype control to 2 µg/ml in **Staining Buffer**.

B. ☐ Add 25 µl of primary antibody for a final concentration of 1 µg/ml.

C. ☐ Incubate at 4°C for 40 min. NOTE: If your primary antibody is already conjugated with a fluorophore, protect from light.

D. ☐ After primary antibody incubation, add 100 µl of **1x PBS + 0.5% BSA** to each sample and pipette up and down 10 times.

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 the supernatant.

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

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

I. ☐ Repeat steps F–H two more times (for a total of 4 washes).

J. ☐ After the final wash, carefully discard 125 µl supernatant, without disturbing the cell pellet, leaving behind 25 µl of the supernatant. Proceed to *STEP 3 - 2nd Antibody Binding*.

NOTE: If your primary antibody(ies) is/are already conjugated with a fluorophore, proceed directly to *OPTIONAL STEP 4 - Proximity Network Assay (PNA) simulation* or directly to *STEP 5 - Evaluation of the results*.

STEP 3 - 2nd Antibody Binding

During STEP 3 of the workflow, cells are stained with a conjugated secondary antibody for detection by flow cytometry. After incubation, cells are washed to remove unbound secondary antibody and resuspended in staining buffer prior to acquisition.

3.1 2nd antibody binding

NOTE: If your primary antibody is a mouse isotype, use the suggested anti-mouse PE 2nd Antibody listed in Table 2c, or an equivalent 2nd Antibody validated for flow cytometry. If using other fluorophores than PE, the flow cytometry results might be affected.

A. ☐ Prepare the 2nd Antibody (e.g., anti-mouse PE, Thermo Fisher Cat. No. P852) at a dilution of 1:250 in the **Staining Buffer**.

B. ☐ Add 25 µl of diluted 2nd Antibody to each sample.

C. ☐ Incubate at 4°C for 40 min, protected from light.

D. ☐ After 2nd antibody incubation, add 100 µl of **1x PBS + 0.5% BSA** to each sample and pipette up and down 10 times.

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 the supernatant.

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

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 the supernatant.

J. ☐ Add 125 µl of **Staining Buffer** and pipette up and down 10 times. Acquire samples on the flow cytometer.

NOTE: For PFA-fixed conditions only, you may reserve half of the cells after 2nd antibody staining for additional validation. In that case, proceed to *OPTIONAL STEP 4 - Proximity Network Assay (PNA) simulation*. If proceeding directly to *STEP 5 - Evaluation of the results*, make sure the cells are resuspended in a final volume of 150 µl.

OPTIONAL STEP 4 - Proximity Network Assay (PNA) simulation

During OPTIONAL STEP 4, fixed and stained cells are exposed to temperatures similar to those used in the Proximity Network Assay to assess whether antibody staining is maintained after heat exposure.

4.1 Incubate fixed, stained cells under PNA-like conditions

A. ☐ Transfer the reserved cells (75 µl) to a new PCR tube.

B. ☐ Add 75 µl of **1x PBS** and pipette up and down 10 times.

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

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

E. ☐ Add 125 µl of **1x PBS** and pipette up and down 10 times.

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 the supernatant.

H. ☐ Add 25 µl of **1x PBS** and pipette up and down 10 times.

I. ☐ Incubate the cells at 45°C for 30 min in a thermocycler.

J. ☐ After incubation, add 100 µl of **1x PBS** to each sample and pipette up and down 10 times.

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 the supernatant.

M. ☐ Add 125 µl of **1x PBS** and pipette up and down 10 times.

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

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

P. ☐ Add 125 µl of Staining Buffer. Acquire samples on the flow cytometer.

IMPORTANT! Compare the change in signal-to-noise ratio before and after heating at 45°C during *STEP 5 - Evaluation of the results*.

STEP 5 - Evaluation of the results

At this stage of the workflow, after acquiring cytometry data from both live and fixed cells, evaluate the results.

5.1 Evaluation of flow cytometry results

Your antibody is suitable for conjugation when the following criteria are met:

1) Clear separation of the stained target antibody(ies) signal from unstained and isotype control populations (see Figure 2 below).
2) An acceptable signal-to-noise ratio (S/N ratio) in both live and fixed conditions.
3) The antibody retains binding after fixation; however, some antibodies may lose part or all of their signal after fixation, as shown in Figure 3 and 4 below.
4) Minimal or no specific staining in the isotype control.
5) No significant non-specific signal in the secondary-only control.

IMPORTANT! If the antibody does not pass flow cytometry validation, do not proceed to conjugation. Contact us at support@pixelgen.com if you have any questions.

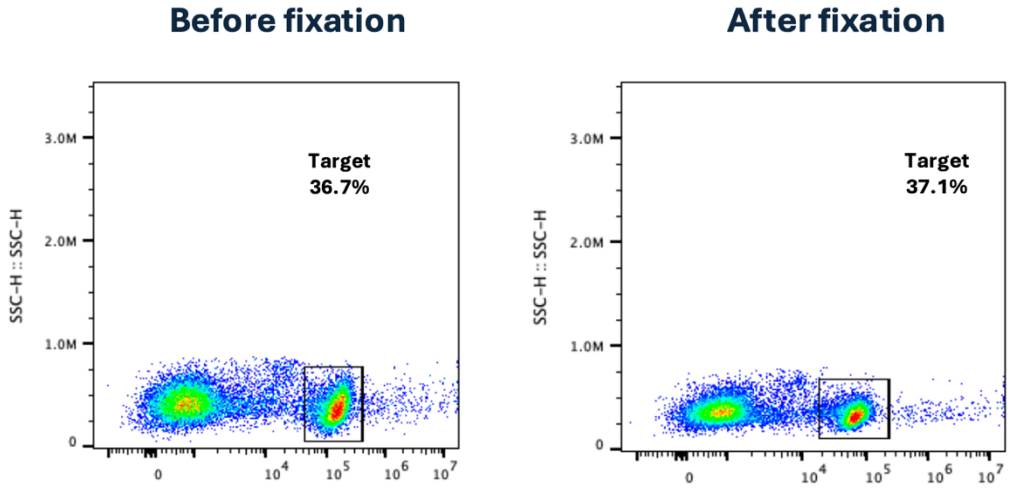


Figure 2. Representative example of a target exhibiting strong S/N ratio performance before and after fixation

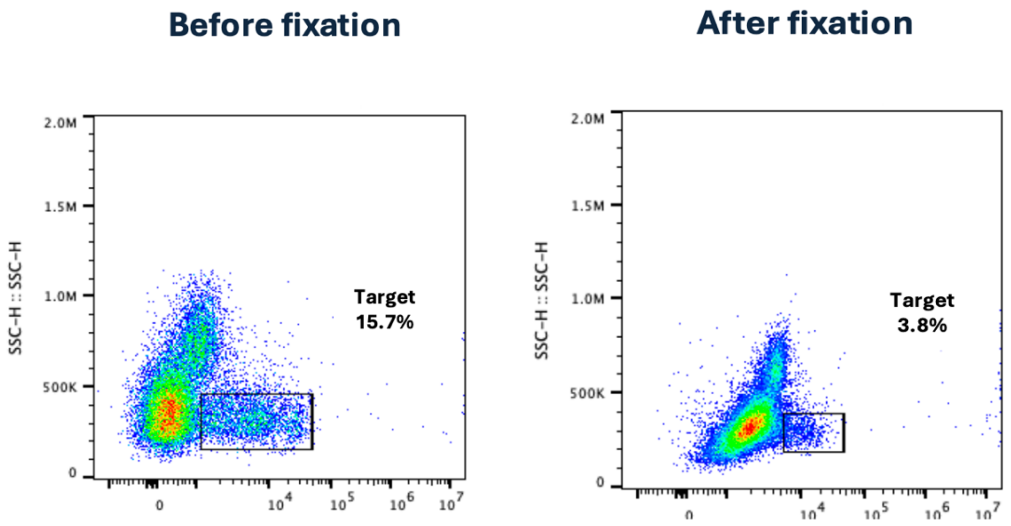


Figure 3. Representative example of a target exhibiting partial loss in signal following fixation

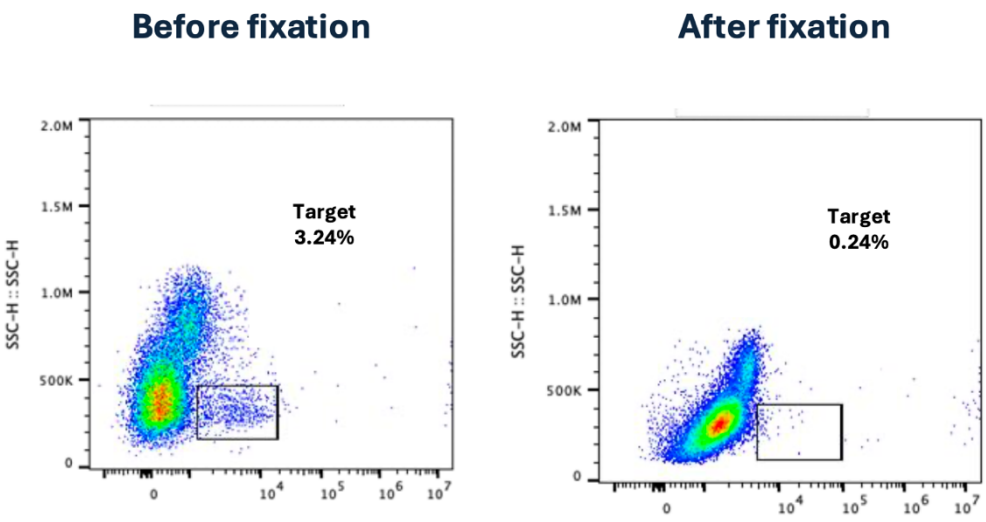


Figure 4. Representative example of a target exhibiting complete loss in signal following fixation

NOTE: Proceed to conjugation only after successful flow cytometry validation. Before proceeding to *STEP 6 – Antibody(ies) preparation*, ensure that antibody concentration is at least 500 µg/ml before starting.

4. PART B - Custom Antibody Conjugation

STEP 6 - Antibody(ies) preparation

During STEP 6, the validated antibody(ies) are first diluted to the working concentration in 1x PBS and then mixed with the conjugation oligonucleotides provided in the kit. The resulting mixture is subsequently exposed to UV light to initiate conjugation.

6.1 Antibody(ies) conjugation

NOTE: Proceed to conjugation only after successful flow cytometry validation. Ensure the antibody concentration is at least 500 µg/ml before starting.

NOTE: Use the necessary precautions when handling a UV light source.

A. ☐ Dilute the antibody(ies) to a final concentration of 500 µg/ml in **1x PBS** if necessary. If the antibody already has a concentration of 500 µg/ml, directly pipette the amount needed from the stock tube.

NOTE: If the antibody needs to be diluted, a minimum volume of 15 µl is needed to cover conjugation and QC of antibody conjugation.

B. ☐ Thaw the oYo-Link® barcodes from the kit. Vortex to mix and quick-spin the tubes.

C. ☐ Each antibody must be conjugated to one unique barcode. Each barcode has 2 versions: Oligo A and Oligo B. Your antibody must be conjugated with each oligo in a separate reaction/tube as follow. IMPORTANT! Label the tube on the cap only. Side labels will block the UV light source used in the conjugation reaction.

Box storage	Component	Volume of oligo to add	Volume of **antibody 1** to add
-20°C	oYo-Link® BCX- Oligo A	10 µl	5 µl
-20°C	oYo-Link® BCX - Oligo B	10 µl	5 µl

Box storage	Component	Volume of oligo to add	Volume of **antibody 2** to add
-20°C	oYo-Link® BCY - Oligo A	10 µl	5 µl
-20°C	oYo-Link® BCY - Oligo B	10 µl	5 µl

D. ☐ Seal each tube securely with parafilm.

E. ☐ Place tubes on their side on ice under a long-wavelength UV light source (~365 nm) as shown in Figure 5 below.

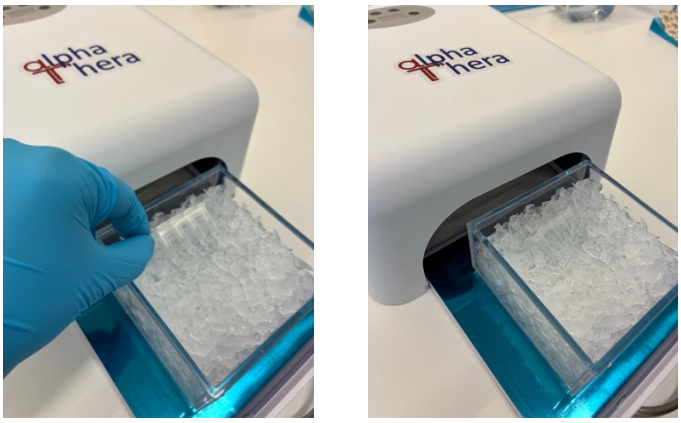


Figure 5. Positioning of the tubes under the UV light source during conjugation.

F. ☐ Incubate for 2 hours.

IMPORTANT! The ice will melt during the 2-hour UV incubation. Ensure all tubes are tightly capped and properly sealed with parafilm to prevent evaporation or contamination.

G. ☐ Remove the tubes from the box, dry them and quick-spin the tubes.

H. ☐ Store the Custom Barcoded Antibodies up to 3 months at 4°C. **Do not freeze**.

STEP 7 - Quality control of the conjugation efficiency

During STEP 7, SDS-PAGE gel is used to confirm successful conjugation of the antibody to the oYo-Link® barcodes. Successful conjugation is indicated by a band shift to a higher molecular weight compared with the unconjugated antibody control as shown in Figure 5 below. No reducing agents are used in this protocol.

7.1 Gel electrophoresis

A. ☐ Prepare **1x MOPS SDS Running Buffer**: mix 25 ml of NuPAGE™ MOPS SDS Running Buffer (20X) with 475 ml of deionized water.

B. ☐ Prepare the following mix for each unconjugated antibody (control) and conjugate (A or B):

Component	Volume to add
Sample (unconjugated antibody or conjugated antibody)	2 µl
NuPAGE™ LDS Sample Buffer (4X)	2.5 µl
1x PBS	5.5 µl
Total volume	**10 µl**

C. ☐ Pipette up and down 5 times to mix, then quick-spin each tube.

D. ☐ Incubate samples in a thermocycler at 70°C for 10 min.

E. ☐ Assemble the gel tank. Fill with sufficient **1x MOPS SDS Running Buffer** to cover the gel.

F. ☐ Load 7 µl of protein ladder into one well.

G. ☐ Load 7 µl of each prepared sample into separate wells.

H. ☐ Run at a constant 175 V for 40 minutes.

7.2 Gel staining

NOTE: Use the necessary precautions when handling Coomassie Blue staining solution.

I. ☐ When the run is over, carefully remove the gel from the cassette and transfer to a Coomassie Blue staining solution.

J. ☐ Ensure the gel is fully submerged. Stain for approximately 1 hour, protected from light, or until clear bands are visible.

K. ☐ Image the gel using a gel imaging system.

7.3 Evaluation of the results

Evaluate the gel as follows:

1) Successful conjugation: conjugate bands appear at higher molecular weight than the unconjugated antibody control, indicating oligo attachment. See Figure 6 below.
2) Failed/suboptimal conjugation: Conjugate bands appear at the same molecular weight as the control – Repeat conjugation. See Figure 7 below.
3) Band quality: bands should be sharp and well-resolved. Diffuse or multiple bands may indicate aggregation or incomplete reaction.

IMPORTANT! If the conjugation QC fails, do not use the conjugates in downstream experiments. Contact us at support@pixelgen.com if you have any questions and for troubleshooting assistance.

Once the antibodies have passed the conjugation QC, proceed to spike them in following the Barcoded Antibodies spike-in Pixelgen Proxiome Kit v2 protocol available on our website.

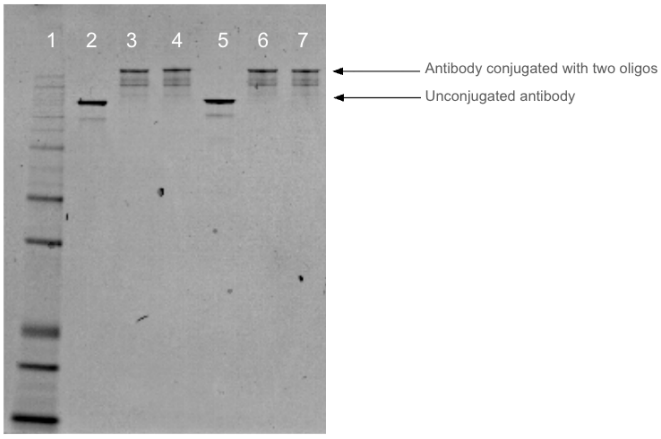


Figure 6. Representative example of a NuPAGE 4-12% BisTris Mini Protein gel, 15-well stained with Coomassie Blue showing a successful conjugation. **(1)** Protein ladder - Mark12™ Unstained Standard, **(2)** Unconjugated antibody 1, **(3) - (4)** Conjugated antibody 1 with Oligo A and Oligo B respectively, **(5)** Unconjugated antibody 2, **(6) - (7)** Conjugated antibody 2 with Oligo A and Oligo B respectively.

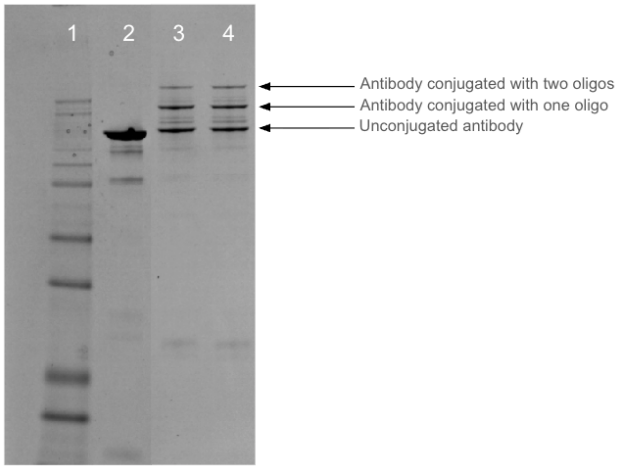


Figure 7. Representative example of a NuPAGE 4-12% BisTris Mini Protein gel, 15-well stained with Coomassie Blue showing a failed/suboptimal conjugation. **(1)** Protein ladder - Mark12™ Unstained Standard, **(2)** Unconjugated antibody 1, **(3) - (4)** Conjugated antibody 1 with Oligo A and Oligo B respectively.

5. References

1. Antibody and Buffer Compatibility
2. Compatible Photocrosslinking Devices
3. Best Practices for Cell Preparation

Appendix

Appendix 1 – Storage and Handling

- Validated unconjugated antibodies should be stored according to manufacturer recommendations (typically at 4°C or -20°C).
- Barcoded antibodies should be stored at 4°C and can be kept up to 3 months. **Do not freeze**.
- Barcodes (oligonucleotides) should be stored at -20°C. Avoid repeated freeze-thaw cycles.
- Always use fresh PFA solution during the fixation step. Discard unused PFA in accordance with your institution's chemical waste guidelines.

Appendix 2 – Troubleshooting Guide

Issue	Possible Cause	Recommended Action
High background in flow cytometry	Insufficient blocking or washing	Increase Fc block incubation time; perform additional wash cycles.
No signal from target antibody	Antibody not recognizing target; wrong cell type or condition	Verify antibody specificity; check cell viability; confirm target expression.
No band shift on SDS-PAGE after conjugation	UV conjugation failed; incorrect oligo or antibody concentration	Confirm antibody concentration is 500 µg/ml; check UV light source output; repeat conjugation.
Smearing or diffuse bands on gel	Sample degradation or aggregation	Check sample preparation conditions; ensure no reducing agents are present.

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

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