Antibody Conjugation Kit for PhenoCode™ Signature

CATALOG # PCSP0200

SUPPLIED MATERIALS

The Antibody Conjugation Kit for PhenoCode™ Signature is designed to enable the conjugation of an antibody of interest to an Akoya barcode for compatibility with PhenoCode Signature assays. This kit contains sufficient reagents to perform up to 2 conjugation reactions. Each conjugation reaction requires 50 µg of purified, Bovine Serum Albumin (BSA)-free antibody and yields approximately 100 µL of conjugated antibody. Barcoded antibody can be used with PhenoCode Signature panels to stain human formalin-fixed paraffin-embedded (FFPE) tissue on the Leica™ BOND RX™ autostainer. Two conjugations yield an adequate amount of antibody to stain a total of 20 slides (2 batches of 10 slides). It is recommended to use conjugated antibodies at the vendor-recommended concentration and titrate when needed. For antibodies that do not have a vendor-recommended concentration, start at a 1:100 dilution and titrate if needed. Conjugation should be performed at least 2 days prior to use in the PhenoCode Signature assay to avoid high levels of nuclear background staining. Gel electrophoresis may be performed to determine the success of conjugation. Materials needed to perform gel electrophoresis are not included in this kit. See the Verification of Conjugated Antibody section for details.

Note: This kit is not needed for the use of PhenoCode Signature antibodies sold by Akoya Biosciences since they are already barcoded.

Component #	Description	Units	Storage Notes	Shipment & Storage Temps
200032	Reduction Solution 1	1	Single-use reagent, discard after opening	-20°C
200028	Reduction Solution 2	1	N/A	4°C
200033	Filter Blocking Solution	1	N/A	4°C
200029	Conjugation Solution	1	N/A	4°C
240103	Barcode BX078	2	Single-use reagent, discard after opening	-20°C
200031	Antibody Storage Solution	1	N/A	4°C
200030	Purification Solution	1	N/A	4°C
PCSD078	PhenoCode Signature Detector HX078	1	N/A	-20°C

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MATERIALS NOT PROVIDED

Required for 20 slides:

Type	Item Description & Minimum Volume	Recommended Vendor, Catalog #
Antibody	BSA-free purified antibody 50 µg per conjugation reaction, 100 µg total	Customer Choice
Consumables	Amicon Ultra-0.5 Centrifugal Filter Unit, 50KDa	Millipore Sigma, UFC505096
	Water - General Molecular Biology Grade, Nuclease Free, 1 mL	GROWCELLS, NUPW-1000
	Gibco™ 1X PBS, pH 7.4, 1 mL	Thermo Fisher Scientific, 10010-023
	Protein LoBind® PCR tubes, 1.5 mL	Eppendorf, 0030 108 442
	Bucket of ice for antibodies	N/A
Equipment	Ultracentrifuge for 1.5 mL tubes	N/A
	NanoDrop™ spectrophotometer	Thermo Fisher Scientific
	Vortex (optional)	N/A

Note: If performing verification using gel electrophoresis, see Page 6 for additional materials required.

EXPERIMENT OVERVIEW

Antibody is added to the top of a 50 kDa molecular weight cut-off (MWCO) filter unit. Centrifugation results in the accumulation of concentrated antibody solution in the filter and flow-through in the bottom of the tube. Flow-through solution should be discarded after each step as instructed. A 50 kDa MWCO filter must be used since other filters will likely result in poor purification, poor conjugation, and/or loss of barcoded antibody.

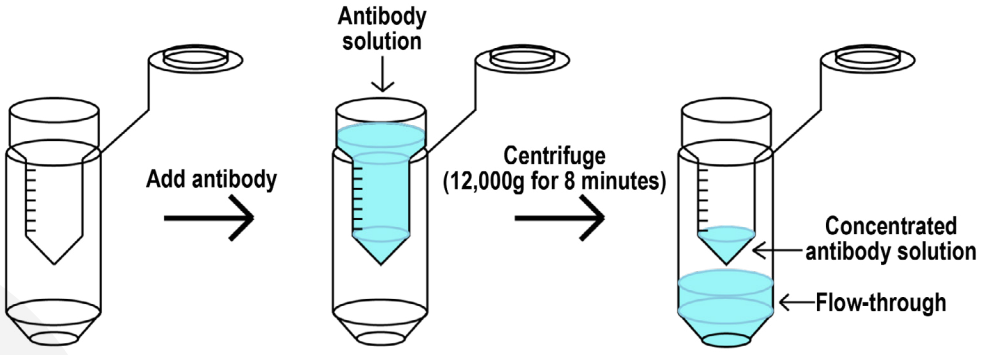

FIGURE 1. Experiment overview using 50 kDa MWCO filter unit.

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ANTIBODY SELECTION & PURIFICATION

Identify & Test Unconjugated Antibody

It is critical to identify the best-suited antibody clone and verify positive staining by using the unconjugated, purified antibody on the tissue of interest. To determine antibody specificity, perform chromogenic 3, 3'-diaminobenzidine (DAB) staining. It is recommended to use positive and negative controls when performing DAB staining if possible. Use antibodies with a stock concentration of 0.45 mg/mL or higher.

Purification

Purchase purified antibodies suspended in PBS or similar buffer that is free of carrier proteins and other preservatives. If the antibody of interest is not commercially available in a purified form, a purification step must be performed before conjugation. Carriers such as BSA, gelatin, and glycerol must be removed prior to conjugation. The presence of sodium azide does not interfere with conjugation and does not need to be removed if present.

ANTIBODY CONJUGATION

The success of custom conjugation is highly dependent on the ratio of antibody to barcode. Often, labeled antibody concentrations are provided in a range of values or the concentration is unknown.

1. Measure and calculate the concentration of the selected purified antibody using a NanoDrop spectrophotometer if the concentration is unavailable.
2. Prepare a solution containing 50 µg of unconjugated antibody with a total volume of at least 100 µL. If the stock antibody concentration is greater than 0.5 mg/mL, add 1X PBS to reach a final volume of 100 µL. No additional PBS is needed if the stock antibody concentration is 0.5 mg/mL or less.
3. Retrieve the following reagents from 4°C storage and bring to room temperature (RT):
   - Reduction Solution 2
   - Filter Blocking Solution
4. Label a 50 kDa MWCO filter unit for each antibody.
5. Add 500 µL of Filter Blocking Solution to the top of each filter and centrifuge the filter units at 12,000 g for 2 minutes at RT. See Figure 1.
6. Discard the flow-through in the tube and use a pipette to remove all the liquid on top of the filter.
7. Add the unconjugated antibody solution prepared in Step 2 to the filter.
8. Centrifuge the filter units at 12,000 g for 8 minutes at RT. Discard the flow-through.
9. Retrieve Reduction Solution 1 from -20°C storage and bring to RT.
10. Prepare the Antibody Reduction Master Mix according to the number of antibody conjugation reactions planned using Table 1 below.

TABLE 1. Required Volume of Antibody Reduction Master Mix

Reagent	Volume per Number of Conjugation Reactions	
	1 Reaction	2 Reactions
Reduction Solution 1 (µL)	6.6	13.2
Reduction Solution 2 (µL)	275	550
Antibody Reduction Master Mix Total Volume (µL)	281.6	563.2

11. Add 260 µL of Antibody Reduction Master Mix to the top of each filter. Close the lid and vortex the filter unit for 2-3 seconds.
12. Incubate at RT for exactly 30 minutes.

Note: Exceeding 30 minutes will result in irreparable damage to antibodies.

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13. After the 30-minute incubation, retrieve the following reagents from storage:
    - Barcode BX078 (retrieve from -20°C storage)
    - Conjugation Solution (retrieve from 4°C storage)
14. Centrifuge the filter unit at 12,000 g for 8 minutes at RT. Discard the flow-through.
15. Add 450 µL of Conjugation Solution to the top of each filter and centrifuge the filter unit at 12,000 g for 8 minutes at RT. Discard the flow-through.
16. Resuspend each BX078 barcode in 10 µL of nuclease-free water (NF H_2O).
17. Add 210 µL of Conjugation Solution to each suspended BX078 barcode. Gently pipette to mix.
18. Add the BX078 barcode solution to the top of each filter. Close the lid and vortex the filter unit for 3 seconds.
19. Incubate for 2 hours at RT.
20. After the 2-hour incubation, retrieve the following reagents from 4°C storage:
    - Antibody Storage Solution
    - Purification Solution

Note: Keep the Purification Solution cold while handling.

21. Set aside a 7 µL aliquot of conjugated antibody solution from each filter; this will be used later to run a verification gel. See the Verification of Conjugated Antibody section for details.
22. Centrifuge conjugated antibody in the filter units at 12,000 g for 8 minutes at 4°C. Discard the flow-through.
23. Add 450 µL of Purification Solution to the top of each filter.
24. Centrifuge the filter units at 12,000 g for 8 minutes at 4°C. Discard the flow-through.
25. Repeat Steps 23-24 an additional 2 more times for a total of 3 purifications.

Note: Some antibodies may require additional centrifugation (2 more minutes each time). Check the volume level in the filter until the residual volume is around 20-30 µL. The top of the filter contains the conjugated antibody solution.

26. Add 100 µL of Antibody Storage Solution to each filter containing the conjugated antibody.
27. Label a new tube for each antibody; these are the antibody collection tubes.
28. Remove the filter from the filtrate collection tube and place the labeled empty tube upside-down on top of the filter.
29. Invert the filter units to collect the conjugated antibody.
30. Centrifuge the filter units at 3,000 g for 2 minutes at 4°C. The final volume in the tube should be approximately 120-130 µL.
31. Transfer the conjugated antibody solution to a sterile, low protein binding screw-top tube for storage.

Note: Conjugated antibody can be stored at 4°C for up to 1 year. Following the conjugation procedure, do not use the conjugated antibodies for tissue staining within 48 hours. High levels of background nuclear staining may occur if used within this timeframe.

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VERIFICATION OF CONJUGATED ANTIBODY

Additional Equipment & Reagents

TABLE 2. Materials Required for Verifying Conjugation with Gel Electrophoresis

Type	Item	Recommended Vendor	Catalog #
Reagents & Protein Gel	NuPAGE™ LDS Sample Buffer (4X)	Thermo Fisher Scientific	NP0008
	NuPAGE Sample Reducing Agent (10X)	Thermo Fisher Scientific	NP0009
	NuPAGE 4-12% Bis-Tris Protein Gels	Thermo Fisher Scientific	NP0321BOX
	Novex™ Sharp Pre-Stained Protein Standard – 3.5-260 kDa	Thermo Fisher Scientific	LC5800
	NuPAGE MOPS SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0001
	Novex SimplyBlue™ SafeStain	Thermo Fisher Scientific	LC6065
	ddH_2O	N/A	N/A
	Water - General Molecular Biology Grade, Nuclease Free, 1 mL	GROWCELLS	NUPW-1000
Instrumentation	XCell SureLock™ Mini-Cell Electrophoresis System	Thermo Fisher Scientific	EI0001
	Mini Dry Bath	N/A	N/A
	Shaker	N/A	N/A
	Microwave	N/A	N/A

Sample Preparation & Gel Procedures

1. Prepare samples per Table 3 below.

TABLE 3. Reagent Preparation for Gel Electrophoresis

Reagent	Conjugated Antibody Sample Volume	Unconjugated Antibody Sample Volume
Antibody	Conjugated Antibody: 7 µL	Unconjugated Antibody: 2 µL* (1 mg/mL) or 2 µg
NF H_2O	6 µL	11 µL*
NuPAGE LDS Sample Buffer	5 µL	5 µL
NuPAGE Sample Reducing Agent	2 µL	2 µL
Total Volume	20 µL	20 µL

*Depends on the antibody concentration.

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2. Denature samples at 95°C in a dry bath for 5 minutes.
3. Prepare 1X SDS running buffer by adding 25 mL of NuPAGE MOPS SDS Running Buffer (20X) to 475 mL of ddH_2O.
4. Prepare NuPAGE 4-12% Bis-Tris Protein Gel according to the manufacturer's package instructions.
5. Fill gel tank with the 1X SDS running buffer according to the manufacturer's guidance.
6. Load 5 µL of a pre-stained protein standard (3.5-260 kDa) to the first well in the gel to determine molecular weight.
7. Load 15-20 µL of each unconjugated and conjugated sample to subsequent wells in the gel.
8. Run the gel at 200 V for 45 minutes until completion.
9. Remove the gel from the plastic cassette and place in a container filled with ddH_2O.
10. Rinse the gel 3 times with ddH_2O.
11. Gently transfer the gel into a microwavable container filled with ddH_2O.

Note: Ensure the gel is covered with ddH_2O.

12. Microwave the gel until the first boiling bubbles form (approximately 50-60 seconds); drain.
13. Stain the gel with Novex SimplyBlue SafeStain (2-3 presses to dispense enough to cover the gel).
14. Microwave the gel again until the first boiling bubbles form (approximately 50-60 seconds).
15. Place the gel on a shaker for 10 minutes.
16. Drain Novex SimplyBlue SafeStain buffer into a biohazard waste container.
17. Rinse gel with ddH_2O and microwave until the first boiling bubbles form (approximately 50-60 seconds).
18. Cool the gel down until it stops steaming (approximately 5 minutes).
19. Repeat Steps 17-18 an additional 2 more times for a total of 3 washes.
20. Place the gel on a white plastic sheet to capture the image. The protein bands should be visible in the gel. See the example in Figure 2.

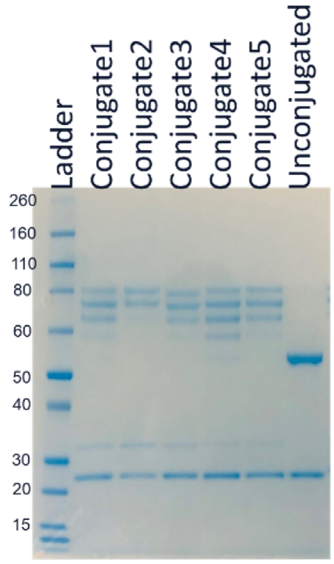

FIGURE 2. The gel demonstrates successful barcode conjugations. From left to right: the protein standard (Ladder); 1-5 barcoded, conjugated antibodies (Conjugates 1-5); and the unconjugated antibody (Unconjugated) in the far-right lane.