Experimental Protocol for Validating PrecisionAb™ Antibodies Using the V3 Western Workflow™

Protocol

10049129

MATERIALS

- Protein samples and reagents
  - Endogenous lysates
  - 1 M Dithiothreitol (DTT)
- Electrophoresis gels, reagents, and equipment
  - 4–15% Criterion™ TGX Stain-Free™ Precast Gels, Bio-Rad cat. #567-8083
  - Criterion™ Cell, Bio-Rad cat. #165-6001
  - PowerPac™ Universal Power Supply, Bio-Rad cat. #164-5070
  - Precision Plus Protein™ All Blue Standards Value Pack, Bio-Rad cat. #161-0393
  - 1x Tris/glycine/SDS (TGS; running buffer), Bio-Rad cat. #161-0732
  - 2x Laemmli Sample Buffer, Bio-Rad cat. #161-0737
- Transfer membranes, reagents, and equipment
  - Trans-Blot® Turbo™ Transfer System, Bio-Rad cat. #170-4150
  - Trans-Blot Turbo Midi PVDF Transfer Pack, Bio-Rad cat. #170-4157
- Western blotting reagents and equipment
  - Primary antibodies (from the AbD Serotec® catalog)
  - Secondary antibodies (see antibody data sheet)
  - 10x Tris-buffered saline (TBS), Bio-Rad cat. #170-6435
  - 10% Tween 20, Bio-Rad cat. #161-0781
  - 1x TBS + 1% casein (blocking buffer), Bio-Rad cat. #161-0782
  - 1x TBS + 0.1% Tween 20 (TBST)
- Imaging reagents and equipment
  - ChemiDoc™ MP System, Bio-Rad cat. #170-8280
  - Clarity™ Western ECL Substrate, Bio-Rad cat. #170-5061
  - Image Lab™ Software, Bio-Rad cat. #170-9690

Antibodies are one of the most commonly used tools in life science research and are critical to obtaining a successful western blot. As there are no standardized guidelines or methods for determining the validity of antibodies, it is imperative to thoroughly evaluate them before the data they generate can be considered trustworthy. For an antibody to be considered validated and to be added to the PrecisionAb product line, it must be shown to be specific, sensitive, and reproducible. At Bio-Rad, we do this using our rapid and comprehensive V3 Western Workflow, which provides the highest level of quality control and ensures that our PrecisionAb Antibodies deliver industry-leading performance. This protocol describes our validation process and gives you, the user, an opportunity to verify our results yourself.

For guidance on how to adopt this protocol to your specific needs, please see the section titled “Protocol Optimization.”

Procedure

Gel setup and running

1. Prepare the validation lysates by adding 200 µl Laemmli Sample Buffer, 10 µl 1 M DTT, and 190 µl deionized water.
2. Boil validation lysates at 95°C for 5 min, and vortex to mix. Spin the samples briefly in a centrifuge to collect any condensed liquid.
3. Assemble the Criterion Cell according to its instruction manual and attach to the PowerPac Universal Power Supply.
4. Place the Criterion TGX Stain-Free Gel into the electrophoresis chamber.
5. Fill the chambers up to the fill line with running buffer and rinse the wells of the gel with the buffer.
6. Load the validation lysates (35 µg) and Precision Plus Protein All Blue Standards (5 µl) onto the gel.

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7 Run the gel for 18–20 min at 300 V. Check that the dye front has reached the bottom of the gel.

Note that the loading amounts and running times will vary based on gel size and well volume. For more information on available gel types, refer to **bio-rad.com/tgx**. To adapt the protocol for different gel types, please see the section titled "Protocol Optimization."

Stain-free imaging and transfer to blot membranes

1 After electrophoresis, remove the gel from the electrophoresis cell and rinse the cassette briefly with deionized water to remove SDS.

2 Carefully remove the gel from the plastic cassette.

3 Place the gel on the sample stage of the ChemiDoc MP Imager, and start the Image Lab Software.

4 Capture an image of the gel using the following steps:
– Select New Protocol → Single Channel…
– Click Select… and choose Protein Gels → Stain-Free Gels from the dropdown menu
– For Gel Activation, choose Gels Used in Blotting (1 min)
– For Gel Type, choose Bio-Rad Criterion Gel
– For Image Exposure, choose Intense Bands
– Select Position Gel and center the blot in the Live View window
– Select Run Protocol

5 After image is captured, save a copy to your desktop or USB key.

6 Open a Trans-Blot Turbo Midi PVDF Transfer Pack and place the pad with the membrane on the base of the transfer cassette.

7 Place the gel on top of the membrane, place the top pad on the gel, and gently roll out any air bubbles.

8 Place the lid on the cassette base and lock it.

9 Insert the cassette into the Trans-Blot Turbo instrument and select a transfer protocol based on the target MW:
– If target MW is ≤100 kD, select Protocol: List → Bio-Rad → (1 midi or 2 mini) → MIXED MW
– If target MW is >100 kD, select Protocol: List → Bio-Rad → (1 midi or 2 mini) → HIGH MW

10 After the transfer is complete, disassemble the blotting sandwich and place the blot in 15 ml blocking buffer + 0.1% Tween 20.

Note that the transfer times and settings will vary depending on the gel size used. Refer to the owner's manual for the correct settings.

Blocking and antibody incubations

1 Incubate the blot in blocking buffer + 0.1% Tween 20 at room temperature (RT) for 30 min with gentle agitation.

2 Dilute each appropriate primary antibody 1:1,000 in 15 ml of blocking buffer.

3 Incubate the blot in the primary antibody and blocking buffer solution overnight at 4°C with gentle agitation.

4 Rinse the blot with 20 ml TBST at RT for 5 min. Repeat for a total of five washes.

5 Dilute the appropriate secondary antibody (see Table 1 in the "Protocol Optimization" section) in 15 ml blocking buffer according to the recommendations on the antibody product page.

6 Incubate the blot in secondary antibody at RT for 1 hr with gentle agitation.

7 Rinse the blot with 20 ml TBST at RT for 5 min. Repeat for a total of five washes.

8 After the final wash step, keep the blot in TBST while preparing the chemiluminescent solution.

Chemiluminescent development

1 Mix the components of the Clarity Western ECL Substrate Kit in a 1:1 ratio. Prepare 0.1 ml of solution per cm^2 of membrane.

2 Place the membrane in the substrate solution and let the membrane develop for 5 min.

3 Place the membrane, protein side up, on the sample stage of the ChemiDoc MP Imager and start Image Lab Software.

4 Capture an image of the blot using the following steps:
– Select New Protocol → Single Channel…
– For Application, choose Chemi
– For Gel Type, choose Bio-Rad Criterion Gel
– For Image Exposure, choose Intense Bands
– Select Run Protocol

5 After image is captured, save a copy to your desktop or USB key.

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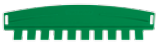
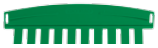
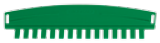
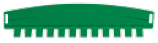
Protocol Optimization

For our validation experiments, we used larger midi formats. However, if you are using a mini gel, make the following adjustments to your protocol.

Gel setup and running

Due to the differences in well volume, you will need to load different amounts of validation lysate. Use the following tables to determine the correct volumes.

Mini-PROTEAN® TGX Stain-Free™ Precast Gels

Description	 10-Well 30 µl	 10-Well 50 µl	 15-Well 15 µl	 12-Well 20 µl	 8+1-Well 30 µl
Recommended load volume, µl	25	25	15	20	25
			Catalog #		
7.5%	456-8023	456-8024	456-8026	456-8025	456-8029
10%	456-8033	456-8034	456-8036	456-8035	456-8039
12%	456-8043	456-8044	456-8046	456-8045	456-8049
4–15%	456-8083	456-8084	456-8086	456-8085	456-8089
4–20%	456-8093	456-8094	456-8096	456-8095	456-8099
8–16%	456-8103	456-8104	456-8106	456-8105	456-8109
Any kD™	456-8123	456-8124	456-8126	456-8125	456-8129

Criterion TGX Stain-Free Precast Gels

Description	 12+2-Well 45 µl	 18-Well 30 µl	 26-Well 15 µl
Recommended load volume, µl	35	25	15
		Catalog #	
7.5%	567-8023	567-8024	567-8025
10%	567-8033	567-8034	567-8035
12%	567-8043	567-8044	567-8045
18%	567-8073	567-8074	567-8075
4–15%	567-8083	567-8084	567-8085
4–20%	567-8093	567-8094	567-8095
8–16%	567-8103	567-8104	567-8105
10–20%	567-8113	567-8114	567-8115
Any kD	567-8123	567-8124	567-8125

All formats are available as both ten packs (catalog numbers listed) and two packs. To order as a two pack, add an "S" to the end of the catalog number for the corresponding ten pack.

Blocking and antibody incubations

- Dilute the primary antibody 1:1,000 in 10 ml blocking buffer
- After overnight incubation at 4°C, rinse the blot in 15 ml TBST at RT for 5 min. Repeat for a total of five washes
- Dilute the secondary antibody in 10 ml blocking buffer using the recommended dilutions listed in Table 1
- After 1 hr incubation at RT, rinse the blot in 15 ml TBST for 5 min. Repeat for a total of five washes

Table 1. Recommended secondary antibody dilutions.

Primary Antibody Host	Secondary	AbD Serotec Catalog #	Recommended Dilution*
Mouse	Goat anti-mouse IgG:HRP	STAR207P	1:10,000
Rabbit	Goat anti-rabbit IgG:HRP	STAR208P	1:10,000
HuCAL®	Goat anti-human IgG F(ab')2:HRP	STAR126P	1:2,500
Rat	Goat anti-rat IgG:HRP	STAR72	1:10,000
Goat	Donkey anti-sheep/goat IgG:HRP	STAR88P	1:10,000
Sheep	Rabbit anti-sheep IgG:HRP	5184-2504	1:10,000

* See antibody product page at **abdserotec.com** for specific recommendations.

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