Western blot

Dot blotting: the key to optimizing your western blot

Did you know that in some cases, using less antibody can result in more signal? Or, conversely, that using more antibody can result in less signal?

Western blots may need to be optimized if you are faced with one of these challenges (learn more at Western Blot Troubleshooting and Tips and Tricks):

- Weak or no signal
- High background
- Nonspecific or diffuse protein bands

The simplicity and efficiency of the dot blot procedure make it a valuable tool for optimizing antibody concentration.

A dot blot is a simplified version of a western blot that is performed to optimize antibody concentrations and help ensure accurate and reliable western blot results. By considering an antibody's specific activity and affinity, as well as the amount of antigen present, optimal antibody concentrations can be determined using this technique.

This antibody optimization method **helps reduce the time and complexity** of the experimental workflow that would otherwise be needed to assess antigen–antibody interactions for further western blot experiments. Additionally, a dot blot requires lower sample and antibody volumes compared to western blots, helping to make it more **cost-effective**, especially when working with limited or precious samples.

Figure 1 shows a schematic diagram of an indirect sandwich assay dot blot. First, a sample consisting of a protein extract, purified protein, or even a synthetic peptide is spotted directly onto a membrane in a dot or small circle format, as opposed to being first separated by gel electrophoresis, which is standard in western blotting. The protein-spotted membrane is then blocked to prevent nonspecific binding, incubated with primary and/or secondary antibodies, and followed with chemiluminescent or fluorescent protein detection.

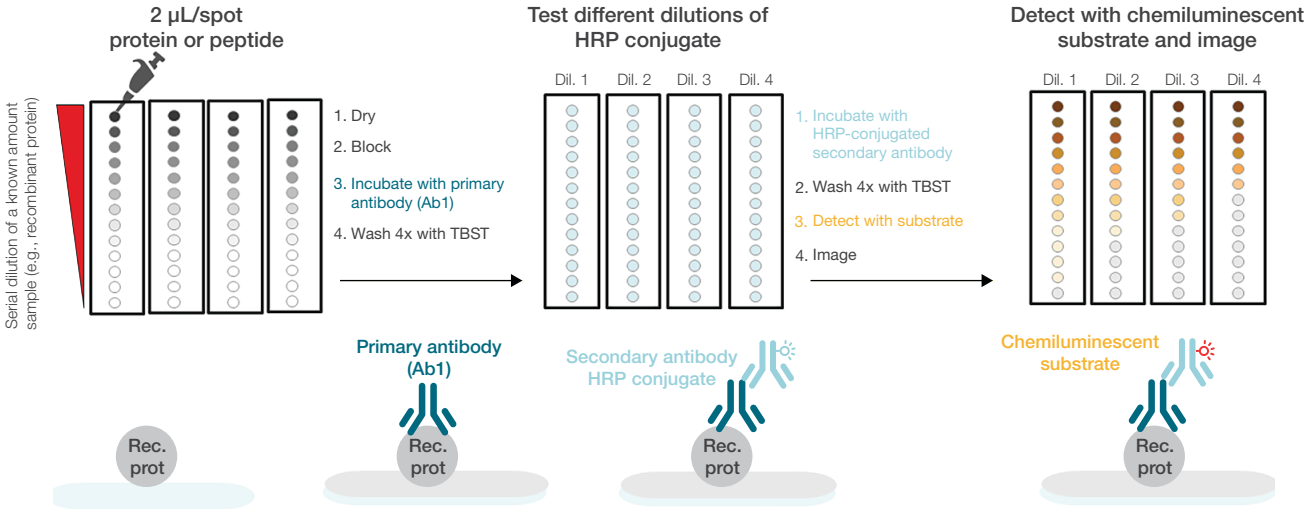

Figure 1. Schematic representation of the indirect sandwich assay using primary and secondary antibodies.

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The membranes are spotted with serial dilutions of a known amount of sample, such as a recombinant protein. After drying and blocking, they are incubated with a primary antibody. Following incubation, the membranes are washed four times with Thermo Scientific™ TBST (TBS buffer with Tween™ detergent) to remove any unbound primary antibody.

Next, different dilutions of the species-specific, HRP-conjugated secondary antibody are added. The secondary antibody binds to the primary antibody, forming a sandwich complex. The membranes are washed again four times with TBST to remove any unbound secondary antibody.

Finally, a chemiluminescent detection substrate is added to the membranes. The HRP enzyme conjugated to the antibody acts on the substrate, resulting in the emission of light. The membrane strips are imaged to visualize the presence and distribution of the target protein on the membrane.

Figure 2 shows a schematic diagram of a direct sandwich assay dot blot. First, the membranes are spotted with serial dilutions of a known amount of sample, such as rabbit IgG. The membranes are dried and blocked to prevent nonspecific binding. A single wash with TBST is performed to remove any residual debris or unbound sample.

Next, the membranes are incubated with different dilutions of the species-specific, HRP-conjugated secondary antibody. The secondary antibody directly recognizes and binds to the target protein or antibody on the membrane, forming a sandwich complex. Following incubation with the secondary antibody, the membranes are washed three times with TBST to remove any unbound secondary antibody.

Finally, a chemiluminescent detection substrate is added to the membranes. The HRP enzyme conjugated to the antibody acts on the substrate, resulting in the emission of light. The membrane strips are imaged to visualize the presence and distribution of the target protein or antibody on the membrane.

Materials

To perform either an indirect or direct sandwich assay dot blot, the following materials are needed:

- Nitrocellulose membrane
- Antigen-containing sample or primary antibody
- Primary and secondary antibodies
- Blocking buffer
- Wash buffer containing Tween-20 detergent (e.g., TBS/PBS with 0.05% Tween-20)
- Chemiluminescent substrate for horseradish peroxidase (HRP)-driven systems
- CCD camera or film to capture chemiluminescence or fluorescence signal

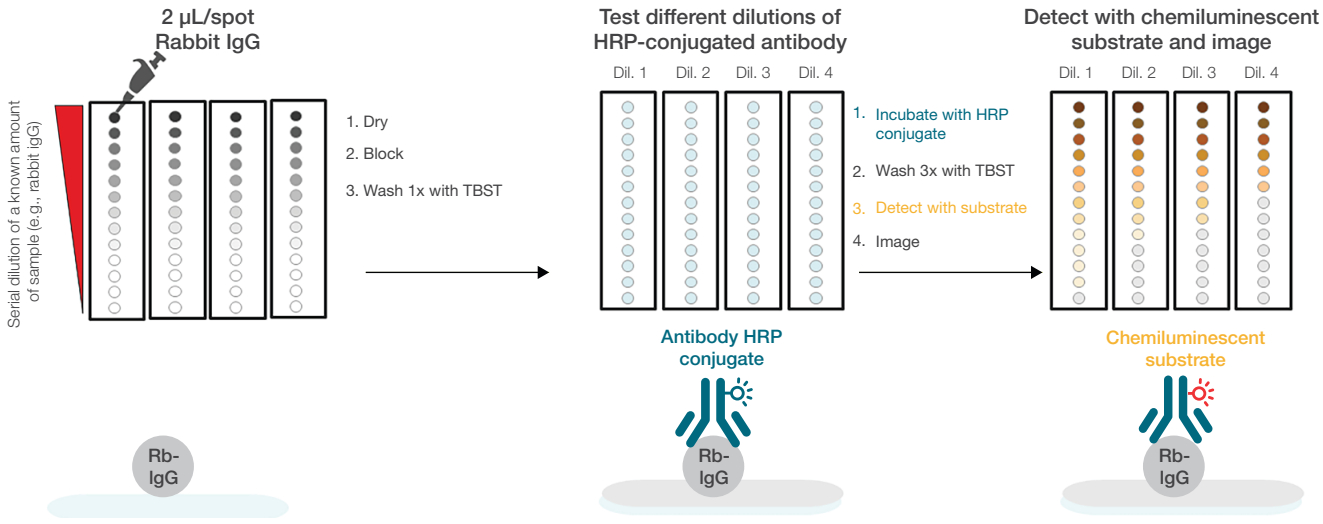

Figure 2. Schematic representation of the direct sandwich assay using secondary antibody.

Procedure

1. Prepare sample dilutions in either TBS or PBS buffer. In general, it is advisable to test a range of 1–50 µg of protein for cell lysate samples. However, for purified protein samples, a range of 5–500 ng is recommended. It is important to note that these ranges are provided as recommendations only, because the optimal signal for certain samples may fall outside these ranges. For HRP-driven detection, see Table 1.
2. Prepare separate nitrocellulose membrane strips for each primary/secondary antibody condition. (Nitrocellulose is recommended because it can be used dry when dotting samples.) Typically, one to three concentrations of the primary antibody, as well as one to three concentrations of the secondary antibody, should be tested. Membranes can be labeled with a pencil for easy membrane distinction. Test antibody concentrations within the recommended range provided by the supplier. For Invitrogen™ antibodies, this information can usually be found on the antibody product page and/or data sheet. In cases where the manufacturer does not supply a recommendation, test a range of dilutions from 1:100 to 1:1,000 (for an antibody stock of 1 mg/mL). Similarly, secondary antibodies should be tested within the recommended dilution range provided by the supplier. If the supplier does not provide a recommendation, test a range of dilutions from 1:1,000 to 1:10,000.
3. Dot sample dilutions onto dry nitrocellulose membrane strips on a flat surface. Dotting approximately 2 µL of each dilution will keep dot sizes as small as possible. Placing membranes on a template over a light box can assist in easier tracking of the dotted dilutions.
4. Allow the membranes to dry for 10–15 minutes or until no visible moisture is observed.
5. Block the spotted membranes in blocking buffer for 30 minutes at room temperature with gentle agitation. A blocking buffer selection guide can be found at **Blocking Buffers for Western Blot and ELISA**.
6. Prepare primary antibody dilutions in blocking buffer. Refer to Table 1 for suggested primary antibody dilutions.
7. Add primary antibody dilutions to the membrane strips, and incubate for 1 hour at room temperature with gentle agitation.
8. Wash the membrane strips for 5 minutes with a sufficient volume of wash buffer that easily flows over the membranes during agitation. Repeat this wash step three more times, for a total of four **washes**.
9. Prepare secondary antibody dilutions in wash buffer. Refer to Table 1 for suggested secondary antibody dilutions.
10. Add the secondary antibody dilutions to the membrane strips, and incubate for 30 minutes at room temperature with gentle agitation.
11. Wash the membrane strips as described in step 8. For HRP chemiluminescent detection, proceed to the following step. For fluorescent detection, skip to step 15.
12. Prepare the chemiluminescent substrate working solution (WS), ensuring a sufficient volume for complete coverage of the membrane strips to prevent their drying during incubation.
13. Incubate the membrane strips in the substrate WS with gentle agitation at room temperature for the time indicated in the instructions for the chemiluminescent substrate.
14. Remove the membrane strips from the substrate WS and place them in a plastic sheet protector.
15. Image the membranes on an imaging device or expose them to film.

Table 1. Chemiluminescent substrate selection guide.

	Thermo Scientific™ Pierce™ ECL Western Blotting Substrate	Thermo Scientific™ SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate	Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific™ SuperSignal™ West Atto Ultimate Sensitivity Substrate
Sensitivity	Low to mid picogram	Low picogram to high femtogram	Mid femtogram	Low to mid femtogram	Low femtogram to high attogram
Antibody dilution (calculated based on stock concentration of 1 mg/mL)	Primary: 1:100 to 1:5,000 (0.2–10 µg/mL)	Primary: 1:1,000 to 1:5,000 (0.2–1.0 µg/mL)	Primary: 1:1,000 to 1:25,000 (0.04–1.0 µg/mL)	Primary: 1:1,000 to 1:25,000 (0.04–1.0 µg/mL)	Primary: 1:1,000 to 1:5,000 (0.2–1.0 µg/mL)
	Secondary: 1:1,000 to 1:15,000 (0.07–1.0 µg/mL)	Secondary: 1:25,000 to 1:150,000 (0.007–0.04 µg/mL)	Secondary: 1:50,000 to 1:250,000 (0.004–0.02 µg/mL)	Secondary: 1:100,000 to 1:500,000 (0.002–0.01 µg/mL)	Secondary: 1:100,000 to 1:250,000 (0.004–0.01 µg/mL)
Select when:	Detecting high-abundance protein targets and sample is abundant	Performing routine, daily applications	Performing quantitative western blotting	Target is low-abundance with previous system optimization	Detecting extremely low-abundance targets or when sample and antibodies are limited

Enhance your western blotting experiments with the SuperSignal West Pico PLUS, Dura, Femto, and Atto chemiluminescent substrates, which offer exceptional sensitivity compared to traditional ECL substrates. The SuperSignal West substrates revolutionize protein detection by significantly reducing the amount of primary antibody required to enable optimal results. With enhanced sensitivity, you can achieve robust signals with lower primary antibody concentrations, which can result in substantial cost savings without compromising data quality.

Other resources:

Protein gel electrophoresis and western blotting education center

Blocking buffers for western blot and ELISA

Western blot antibody dilution calculator

Antibodies for western blotting

Western blot tool

Western blot protocols and recipes

Chemiluminescent substrate selection guide

Related Thermo Scientific™ products:

Nitrocellulose membranes

BupH™ Phosphate-Buffered Saline Packs

StartingBlock™ Blocking Buffer

Blocker™ Casein

Blocker™ BSA

Pierce™ Concentrated Buffer Stocks (10X and 20X)

SuperSignal™ West Pico PLUS Chemiluminescent Substrate

SuperSignal™ West Femto Maximum Sensitivity Substrate

SuperSignal™ West Dura Extended Duration Substrate

SuperSignal™ West Atto Ultimate Sensitivity Substrate

Primary antibodies for western blot

Secondary antibodies for western blot

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