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Chapter 6

Western Blotting Against Tagged Virulence Determinants to Study Bacterial Pathogenicity

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Abstract

Western blotting is a common approach to detect the presence of a target protein in biological samples or proteins mixture using specific antibodies. This method is also useful to study regulation of virulence determinants by analyzing changes in protein expression between different genetic backgrounds or under varying environmental conditions. To avoid the need to raise specific antibodies for each studied protein, commercial antibody against commonly used peptidic epitopes can be utilized if the right target tagged version is constructed. Here we describe a C-terminal fusion between a protein of interest and the two hemagglutinin A (2HA) tag. The tagged protein is cloned into a low-copy number vector and expressed under its native promoter in *Salmonella enterica*. Then, the expression of the tagged protein can be analyzed by Western blotting and commercially available anti-2HA antibodies.

Key words Western blotting, Gel electrophoresis, Protein tagging, Immunoblotting, Hemagglutinin, Antibodies

1 Introduction

Western blotting (also known as immunoblotting) is a well-established analytical technique used to detect a particular protein in a complex mixture or biological samples (cell lysate, cellular fractionation, conditional media, etc.) based on antigen–antibody specific recognition [1]. Western blotting involves a polyacrylamide gel electrophoresis (PAGE) and transfer of the separated proteins onto a special membrane [typically nitrocellulose or polyvinylidene difluoride (PVDF)] in a process called blotting. The membrane-transferred proteins are accessible to a primary antibody binding, followed by a secondary antibody conjugated with a catalytic enzyme (e.g., horseradish peroxidase) that is used in a detection reaction.

Nevertheless, since raising and using a specific primary antibody against individual proteins of interest is time-consuming and expensive, one may use commercially available antibodies against

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common peptide epitopes (tags) such as FLAG, glutathione-S-transferase (GST), histidine (His), or hemagglutinin A (HA). The tag of selection is fused to the protein of interest, usually at the C- or the N-terminus. This approach provides high specificity and versatility, but requires a prior step of cloning and expression of the tagged target in the appropriate bacterial host under its native promoter. The recombinant tagged version of the protein can be cloned into a low-copy number vector under its native promoter or replace the chromosomal locus of the target gene (*see* **Note 1**).

The commonly used HA tag (YPYDVPDYA) is derived from the binding domain of the *Influenza* hemagglutinin protein and contains a high composition of charged residues comprising a strong antibody recognition site. Here, we describe a protocol to construct a C-terminal fusion between a target protein of interest and a 2HA tag and determine the expression of this tagged protein in different backgrounds of *S. enterica*. To demonstrate this protocol, we show the construction of the *Salmonella* flagellin subunit FliC tagged with a 2HA epitope.

2 Materials

2.1 2HA-Tagged Protein Fusion

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Bacterial genomic DNA purification kit.
3. Plasmid mini extraction kit.
4. Hot start high fidelity DNA polymerase.
5. Oligonucleotide primers.
6. DNA Gel Extraction Kit.
7. Appropriate restriction enzymes.
8. Low-copy number cloning vector (*see* **Note 2**).
9. T4 DNA ligase.
10. Competent *Escherichia coli* DH5α cells.
11. Oligonucleotides for sequencing:

For pWSK29/pWSK129:

'M13/Puc forward' (5'-GTTTTCCCAGTCACGACGTTG-3')

'M13/Puc primer reverse' (5'-AGCGGATAACAATTTCACACAGGA-3')

For pACYC184:

'pACYC184 forward' (5'-CACCGGAAGGAGCTGACTG-3')

'pACYC184 reverse' (5'-GTAGCACCTGAAGTCAGCCC-3')

Table 1
Reagents required for casting the polyacrylamide separation gel

% gel	6%	8%	10%	12%	15%
Separation range (kDa)	60–200	40–100	20–70	20–60	10–40
Acrylamide–Bis (mL)	2.3	3.0	4.0	4.5	5.6
ddH_2O (mL)	8.7	7.9	6.9	6.3	5.2
TEMED (μL)	12	9	6	6	6

2.2 SDS-Polyacrylamide Gel

1. SDS-PAGE running buffer (For 1 L 10×): 10 g SDS, 30.3 g Tris, 144.1 g Glycine.
2. SDS-PAGE sample buffer (2×): 1.5 mL of 1 M Tris pH 6.8, 1.2 mL of 10% SDS, 6 mL of 50% glycerol, 1.5 mL of β-mercaptoethanol, 0.18 mg of bromophenol blue. Aliquot into 1 mL portions and store at −20 °C.
3. SDS-PAGE resolving gel (15 mL for 2 mini gels): 40% Acrylamide–Bis solution (37.5:1; according to Table 1), ddH_2O (according to Table 1), 3.8 mL of 1.5 M pH 8.8 Tris–HCl, 150 μL of 10% SDS, 150 μL of 10% ammonium persulfate (APS) and N, N, N′, N′-tetramethylethylenediamine (TEMED, according to Table 1 and *see* **Notes 3** and **4**).
4. 5% stacking gel (6 mL for 2 mini gels): 0.75 mL of 40% Acrylamide–Bis solution (37.5:1), 4.4 mL of ddH_2O, 0.75 mL of 1 M pH 6.8 Tris–HCl, 60 μL of 10% SDS, 60 μL of 10% APS, and 6 μL of TEMED (*see* **Notes 3** and **5**).

2.3 Immunobloting

1. Transfer buffer: 3.03 g of Tris, 14.4 g of glycine, 0.375 g of SDS. Dissolve all three reagents in 500 mL of dH_2O. Add 200 mL of methanol (*see* **Note 6**) and adjust to 1 L with dH_2O.
2. TBS (10×): dissolve 80 g NaCl, 2 g KCl, and 30 g Tris in 700 mL dH_2O. Adjust the pH to 7.4 with concentrated HCl and complete the volume to 1 L.
3. Washing buffer (TBST): 250 μL of Tween 20 in 1 L of 1× TBS.
4. Blocking buffer: 5% skim milk powder in TBST.
5. Detection reagent: ECL Western Blotting Detection Reagent.
6. Transparency film sheets.

2.4 Antibodies

1. Mouse monoclonal antibody [HA.C5] against HA tag. Add 20 μL of the antibody in 20 mL of blocking solution.
2. Mouse monoclonal [8E2/2] against Dnak. Add 3 μL of the antibody in 30 mL of blocking solution.
3. Goat polyclonal secondary antibody against mouse IgG- H&L (HRP). Add 3 μL of the antibody in 15 mL of blocking solution.

3 Methods

3.1 Construction of 2HA-Tagged Proteins Fusion

1. Isolate the relevant bacterial DNA, which will be used as the PCR template by a bacterial genomic DNA purification kit.
2. Isolate the cloning plasmid (e.g., pWSK29, pWSK129, or pACYC184) using a plasmid mini extraction kit.
3. Amplify the 2HA DNA sequence using a PCR and primers, which include appropriate restriction sites at the 5′ and 3′ ends of the sequence (*see* **Note 7**). Make sure to also introduce a stop codon (e.g., TAA) downstream to the 2HA tag if a C-terminus tag is constructed.
4. Clone the 2HA DNA sequence into the cloning vector of choice, using the flanked restriction sites.
5. Amplify by PCR the target gene of interest (without the stop codon) including the upstream regulatory region, using primers that include appropriate restriction sites. The restriction site at the 3′-end should be the same as the restriction site, which is located upstream from the cloned tag in a way that the cloning will result in an in-frame C-terminus tag fusion (*see* **Note 8**).
6. Separate the PCR products in an agarose gel and purify the PCR products using DNA gel extraction kit.
7. Digest the cloning plasmid (containing the 2HA tag) and the insert (target gene) with the same pair of restriction enzymes. Purify the digested DNA after the restriction reaction (*see* **Note 9**).
8. Ligate the insert into the cloning plasmid using T4 DNA ligase at 16 °C for overnight.
9. Transform the ligation product into competent *Escherichia coli* DH5α cells.
10. Isolate the recombinant clone after plating on selective LB agar plates.
11. Verify the clone by restriction analysis followed by sequencing to make sure that no mutation has been introduced during PCR amplification and that the tag is inserted in-frame.
12. Transform the recombinant construct into the appropriate *Salmonella* background.

3.2 Preparation of Protein Fractions

1. Grow *Salmonella* culture under the desired growth conditions under appropriate selection.
2. Measure the cultures OD_{600} and normalize all cultures to the same optical density (*see* **Note 10**).
3. Centrifuge the cultures at 11,000 × *g* for 2 min at room temperature in a microcentrifuge and remove the supernatant carefully using a pipette. Resuspend the pellets in 150–200 μL of 1× SDS-PAGE sample buffer. Boil the samples for 5 min and place on ice immediately.

3.3 Electrophoretic Wet Transfer and Blotting

1. Load the samples on the SDS-PAGE gel including molecular weight markers and run the gel at constant current of 30 mA in running buffer until the loading dye reaches the bottom of the gel.
2. Gently, separate the glass cast, take out the acrylamide gel and equilibrate in transfer buffer to remove any remnants of electrophoresis buffer salts.
3. Place the electrophoretic wet transfer tank onto a magnetic stir plate (*see* **Note 11**).
4. For each gel, cut one piece of polyvinylidene fluoride (PVDF) membrane and two pieces of Whatman filter paper to the dimensions of the gel (e.g., 7.5 × 8.5 cm for mini gels).
5. Place the PVDF membrane in methanol for 1 min, then soak the membrane for 2 min in ddH_2O and then place it in a transfer buffer.
6. Build the gel and membrane sandwich inside the gel holder cassette using two filter papers (7.5 × 8.5 cm) and two fiber pads (sponges) soaked with transfer buffer.
7. Place a wet fiber pad on top of the black side of the cassette immersed in transfer buffer.
8. Place the following components between the two wings of the gel holder from the anode to the cathode: fiber pad, Whatman filter paper, PVDF membrane, gel, filter paper, and fiber pad (Fig. 1). Use a clean glass test tubes to remove any air bubbles caught between the layers of the sandwich and to ensure proper contact between the gel and membrane by rolling it over the sandwich layers.
9. Close and lock the cassette, insert it into the transfer tank with the latch side up and with the black wing facing the black electrode plate (cathode).
10. Place the cassette inside the chamber and fill it with cold transfer buffer until the gels and membranes are submerged under transfer buffer (~1.5 L).
11. Put a stirrer magnet at the bottom of the chamber, and turn on the stirrer. Set the power to100 V and transfer the proteins for 1 h.
12. After the blotting is completed, disassemble the tank transfer system and gently open the gel and membrane sandwich. Rinse the membrane briefly in ddH_2O to ensure that no residual gel shards adhere to the membrane and incubate the membrane in fresh blocking buffer for 1 h with gentle shaking at room temperature.
13. Incubate the membrane with the primary antibody solution, e.g., anti-2HA tag or anti-DnaK as a loading control for 1 h at room temperature or for overnight at 4 °C.

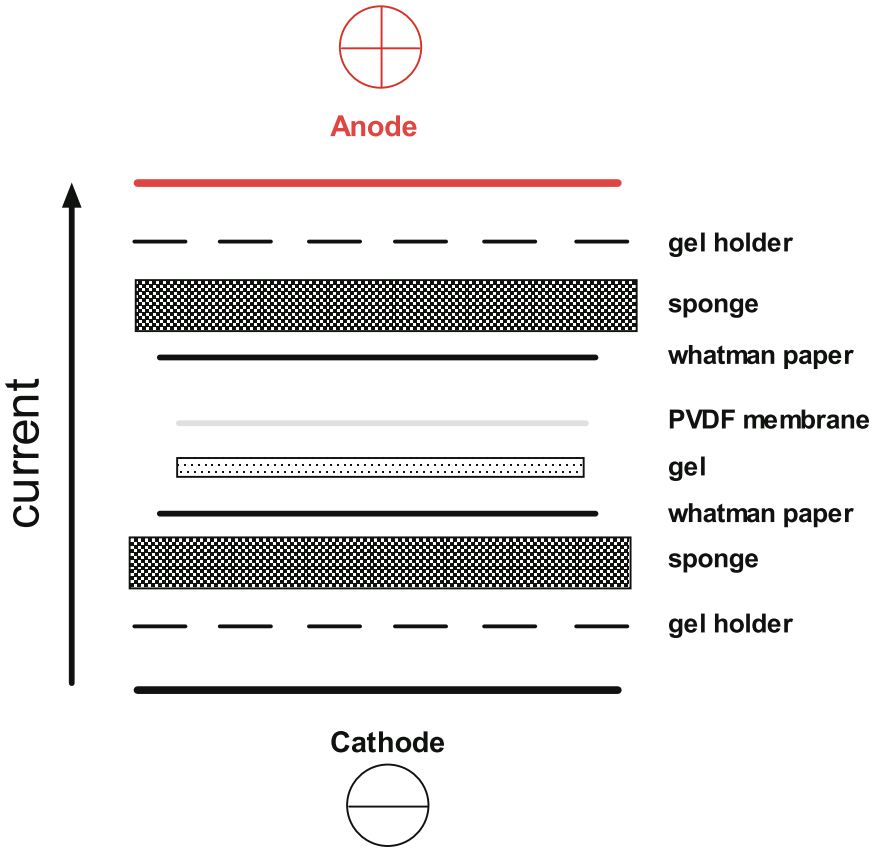

Fig. 1 The assembly of the transfer sandwich. A schematic representation of Western blotting "sandwich," used for proteins blotting from an acrylamide gel to the PVDF membrane during a wet transfer

14. Wash the membrane for 5 min with the washing buffer under gentle shaking and repeat this washing step four times.
15. Incubate the membrane with the secondary antibody, e.g., goat anti-mouse conjugated with peroxidase diluted in blocking buffer for 1 h at room temperature, with gentle shaking.
16. Wash the membrane with washing buffer four times (5 min each time).
17. Prepare 2 mL of the enhanced chemiluminescence (ECL) Western Blotting Substrate (used for peroxidase substrate, by mixing 1 mL of each reagent) in a tube. Place the membrane onto one sheet of transparency film (transferred proteins facing up) and pour the 2 mL of the ECL substrate on top of the membrane. Place immediately the second plastic film to cover, make sure that the ECL substrate evenly covers the entire membrane surface and incubate for 1 min at room temperature.
18. Place the plastic films wrapping the membrane on a paper towel and squeeze out excess of the ECL reagent using Kimwipes disposable wipers and place the membrane inside an X-ray cassette for the detection of the signals using X-ray film (Fig. 2).

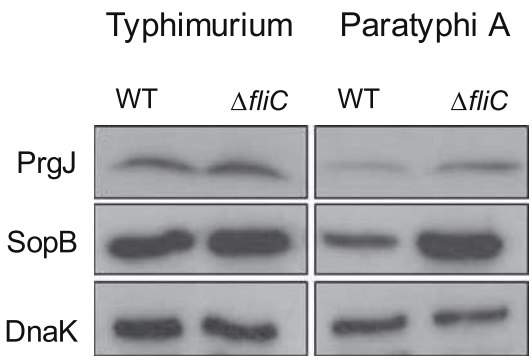

Fig. 2 The absence of flagellin induces SPI-1 gene expression in *S.* Paratyphi A. SDS-PAGE Western blot analysis of bacterial cell lysate from *S.* Typhimurium and *S.* Paratyphi A wild type (WT) and a *fliC* mutant (Δ*fliC*) expressing SopB-2HA or PrgJ-2HA (two SPI-1 effector proteins). Protein fractions were probed using anti-HA antibodies and anti-DnaK as a loading control. This analysis showed higher expression of SPI-1 effectors in the absence of *fliC* in *S.* Paratyphi A compared to *S.* Typhimurium. This figure is reproduced from Elhadad et al. [2] with permission from the publisher (ASM Journals).

4 Notes

1. For most purposes expressing the tagged protein under the native regulatory region from a low-copy number vector will provide reliable data. However, one can also use the λ-red recombinase method and replace the gene of interest in the chromosome with a tagged version as described in reference [3].
2. For *S. enterica* or *E. coli*, we normally use the pWSK29, pWSK129 [4], or pACYC184 [5] vectors.
3. When preparing the SDS-PAGE resolving and stacking gel add the APS and the TEMED last, because together they catalyze the polymerization of acrylamide and bisacrylamide. Mix well and quickly pipet the gel solution into the gap between the glass plates.
4. When pipetting the resolving gel, do not forget to allow space for the stacking gel (~0.5 cm lower from the end of the gel comb) and then add water onto the top of the gel until it overflows. This will create a smooth interface between the stacking and resolving layers and protect the gel from ambient oxygen, which inhibits acrylamide polymerization.
5. When the polymerization of the resolving gel is completed (about 30 min), pipet the stacking gel solution until it overflows and insert a gel comb immediately without trapping air bubbles under the teeth of the comb.
6. Use high-quality analytical grade methanol.

7. Add 4 nucleotides, (we usually add AAAA) upstream and downstream to the restriction sites in the forward and reverse primers, respectively. This allows optimal digest of the restriction sites in the obtained amplicon.
8. In certain cases the C-terminus of a protein may be important for its function and a C-terminal fusion cannot be constructed. In such cases an N-terminus tag can be considered. Under such circumstances, make sure the protein is not secreted and that the N-terminus is not cleaved by the Sec system.
9. It is recommended to purify the cut inserts by standard ethanol precipitation (we use 2.5 volume of ice-cold ethanol absolute; 0.1 volume of 3 M sodium acetate pH 5.2; and 5 μg of yeast tRNA). The digested cloning vector has to be separated in an agarose gel and purified by a gel extraction kit in order to exclude uncut molecules.
10. Normalization of total proteins amounts in each sample can also be determined using standard protein concentration assays (e.g., Bradford).
11. Electrophoretic wet transfer of the proteins from the gel to a polyvinylidene fluoride (PVDF) membrane should be done under cold temperature (standard cold room is not cold enough), if you do not use a cooling system for the tank transfer system, one may fill a large box (at least 30 × 30 cm) with crushed ice and put the transfer chamber inside.

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References

1. Kurien BT, Scofield RH (2015) Western blotting: an introduction. Methods Mol Biol 1312:17–30
2. Elhadad D, Desai P, Rahav G et al (2015) Flagellin is required for host cell invasion and normal *Salmonella* pathogenicity island 1 expression by *Salmonella enterica* serovar Paratyphi a. Infect Immun 83:3355–3368
3. Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L (2001) Epitope tagging of chromosomal genes in *Salmonella*. Proc Natl Acad Sci U S A 98:15264–15269
4. Wang RF, Kushner SR (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene 100:195–199
5. Rose RE (1988) The nucleotide sequence of pACYC184. Nucleic Acids Res 16:355–356