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Dot-blotting: A quick method for expression analysis of recombinant proteins

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Abstract

Expressing recombinant proteins in heterologous host cells is a prerequisite for purification and other downstream processes. Cell cultures require a protein expression test to optimize incubation time, temperature, and additives (like chemical inducers) to identify the best growth conditions with maximum recombinant protein yield. However, running SDS-PAGE followed by western blotting is cumbersome, and results are not quick. Here, I describe a simple protocol to quickly check the presence of recombinant protein in cell cultures using a dot-blot experiment. The cells can be rapidly lysed and directly spotted on the nitrocellulose membrane. Then, the membrane is incubated with an HRP conjugated antibody raised against the affinity tag present on the recombinant protein to confirm the protein expression by chemiluminescence. It takes less than an hour to get results. This method rapidly investigates recombinant protein expression in different cell lines and tests other variables.

BASIC PROTOCOL 1: Protein expression analysis for eukaryotic systems

BASIC PROTOCOL 2: Protein expression analysis for bacterial systems

Keywords

recombinant protein; protein expression; purification; dot-blot; nitrocellulose; western blotting

Introduction

Ever since recombinant DNA technology has revolutionized biology as we know it, the expression of recombinant proteins is one of the first analyses required before any other downstream experiments(Papaneophytou & Kontopidis, 2014; Rosano & Ceccarelli, 2014). Unfortunately, the 'one size fits all' philosophy does not work for recombinant proteins. Each gene requires optimization to identify ideal conditions for optimal protein production(Packiam, Ramanan, Ooi, Krishnaswamy, & Tey, 2020). Recombinant proteins expressed in eukaryotic or prokaryotic heterologous host cells require several optimization processes before it can be successfully produced. Crucial factors like expression host type (eukaryote or prokaryote), strain type, vector design (promoters and enhancers), and growth

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conditions (temperature, pH, media) can significantly influence protein expression(Francis & Page, 2010; Gutierrez-Gonzalez et al., 2019).

The bacterial expression system is the most commonly used system for recombinant protein production(Tripathi & Shrivastava, 2019). The bacterial strains are relatively easy to handle and grow. Genetic manipulations are also easy and less time-consuming in bacterial cells. In a single experiment, a lot of bacterial strains and growth conditions can be tested to identify ideal conditions for recombinant protein expression. However, trying a lot of growth conditions for protein expression is a bottleneck. Samples separated by electrophoresis are blotted onto a membrane before being tested for the presence of recombinant proteins. This is a lengthy but unavoidable step. Moreover, only a few conditions can be tested in one experiment.

Eukaryotic protein expression poses another set of challenges. It takes more time and effort to grow and maintain eukaryotic cells(Almo & Love, 2014). In addition, the recombinant proteins accumulate in low abundance often, which makes the detection process more tedious. Dot-blotting offers a quick and easy solution to several of the time and effort-consuming steps in detecting recombinant protein expression in a high-throughput fashion(Qi et al., 2018; Rupprecht et al., 2010; Stott, 1989). The technique is like western blotting without the separation of samples by electrophoresis(Ortega Ibarra, Cifuentes-Castro, Medina-Ceja, & Morales-Villagran, 2021). The samples can be spotted directly onto a membrane in an array format. Several experimental conditions can be tested at once to identify the recombinant protein expression using immunodetection methods like chemiluminescence, fluorescence, or colorimetry(Goldman, Harper, & Speicher, 2016; Ortega Ibarra et al., 2021). Following are two basic protocols for a dot-blot experiment for eukaryotic and prokaryotic recombinant protein expression systems.

BASIC PROTOCOL 1: Protein expression analysis for eukaryotic systems

Insect and mammalian cell culture systems are the two most used eukaryotic recombinant protein expression systems(He, Wang, & Yan, 2014). These cells serve as heterologous hosts to produce recombinant proteins that require eukaryotic protein folding mechanisms(Ailor & Betenbaugh, 1999). Insect cells, *Spodoptera frugiperda* (sf9), and *Tricoplusia ni* (Tni) provide robust protein expression with least technical challenges(Mishra, 2020). They are relatively easy to grow and are infected by engineered baculoviruses containing the gene of interest. Apart from insect cells, HEK293 human cells are also a good host of recombinant protein production.

Here, I show the detection of recombinant protein expression using the dot-blot method while comparing it with a traditional method comprising Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by western blot analysis. Insect cells (sf9) are used as an example to demonstrate this protocol for eukaryotic recombinant protein expression systems.

Materials:

Cell Culture

Sf9 cells, Thermo Fisher Scientific, cat. no: 12659017

Sf900 III SFM insect cell culture media, Thermo Fisher Scientific, cat. no: 12658019

Grace's Insect Medium, Unsupplemented, Thermo Fisher Scientific, cat.no: 11595030

FuGENE® HD Transfection Reagent, Promega, cat.no. E2311

Gibco™ Antibiotic-Antimycotic, cat. no:15240112

DH10Bac™ Gibco, cat. no: 10361012

PureYield™ Plasmid Midiprep System, Promega, cat. no: A2492

Kanamycin, Sigma-Aldrich, cat. no: K0254–20ML

Isopropyl β-D-1-thiogalactopyranoside (IPTG), GoldBio, cat. no: I2481

Bluo-Gal, substrate for β-galactosidase, Thermo Fisher Scientific, cat. no: 15519028

Gentamycin sulphate, Millipore Sigma, cat. no: G1397

Tertacyline, Millipore Sigma, cat. no: 58346

Luria Bertani (LB) broth, Gibco™ , Thermo Fisher Scientific, cat. no: J75854.A1

LB Agar, Powder, Thermo Fisher Scientific, cat.no. 22700025

I-PER™ Insect Cell Protein Extraction Reagent, Thermo Fisher Scientific, cat. no: 89802

pFastBac His6 MBP N10 TEV LIC cloning vector (4C) was a gift from Scott Gradia (Addgene plasmid # 30116)

SDS-PAGE

(All materials are purchased from Thermo Fisher Scientific)

NuPAGE™ 12%, Bis-Tris 1.0 mm, Mini Protein Gels, cat.no. NP0341BOX

Novex™ Tris-Glycine SDS sample buffer (2X), cat. no: LC2676

Novex™ Tris-Glycine SDS running buffer (10X), cat. no: 28362

Dot-blot and western blot materials

(All materials are purchased from Thermo Fisher Scientific)

Nitrocellulose membrane, cat. no: 88024

1X Phosphate-Buffered Saline, 0.1% v/v Tween(R) 20 detergent (PBST), cat. no: 28352

Non-fat dried milk, cat. no: LP0033B

Tris-glycine transfer buffer, cat. no: LC3675

Pierce™ ECL Western Blotting Substrate, cat.no: 32106

6X-His Tag Monoclonal Antibody, HRP conjugate, cat. no: PA1-983B-HRP

Recombinant baculovirus generation—Following are the steps to generate recombinant baculovirus harboring the His6-Maltose Binding Protein (MBP) gene. For more information, please refer to Bac-to-bac Baculovirus expression system, Thermo Fisher Scientific, cat.no.10359016.

1. Transform DH10Bac™ competent cell with pFastBac-His6-MBP plasmid as per Bac-to-Bac® insect cell meditated protein expression protocol.
2. Perform blue/white colony selection on IPTG (40 μg/ml) and Bluo-gal (100 μg/ml) containing LB Agar plates in presence of Kanamycin (50 μg/ml), Tetracycline (10 μg/ml), and Gentamycin (7 μg/ml) antibiotics. Blue and white colonies were observed after 48 hours post transformation upon incubation of the LB-agar plates at 37 °C.
3. Re-streak positive white colonies on a fresh LB agar plate to avoid false positives.
4. Culture a positive white colony overnight in 100ml Luria Bertani (LB) media at 37 °C.
5. Purify recombinant bacmid with PureYield™ Plasmid Midiprep System. 105.7 ng/μl recombinant bacmid DNA yielded from this process.
6. Prepare glycerol stocks of the DH10Bac™ cell containing the recombinant bacmid and store at −80°C. For more details for glycerol stock preparation please refer to this protocol, https://www.addgene.org/protocols/create-glycerol-stock/.
7. Seed 8×10^5 sf9 cells with >95% viability onto 6 well tissue culture plate at the time of transfection. Keep the cells in hood with air flow for the entire transfection process.
8. Replace the cell culture media with Grace's Insect Medium, Un-supplemented.
9. Mix 1 μg of the purified His6-MBP bacmid DNA with FuGENE® HD Transfection Reagent in 1:3, DNA(μg): transfection reagent(μl) ratio. Use (if necessary) pFastBac™-Gus expressing β-glucuronidase (Thermo Fisher Scientific) or pFastBac™HT-CAT expressing chloramphenicol acetyltransferase (Thermo Fisher Scientific) as positive expression control for transfection.
10. Incubate the mixture for 30 minutes at room temperature.
11. Add the mixture in dropwise fashion to the Sf9 cells.
12. Incubate the cells at 27 °C with gentle rocking for 4 hours.
13. Replace the media with new Sf900 III SFM insect cell culture media with 1x Antibiotic-Antimycotic.
14. Incubate the cells at 27 °C with gentle rocking for 72 hours.

15. Post 72 hours incubation observe cells under the microscope for viral infection. Phenotypically, there is a significant 20–50% increase in the diameter of the virus infected cells. Increase in the size of nuclei, almost filling the cell. Virus infected cells have arrested growth and division. In addition, budding is more prominent in significant population of lysed cells.

16. Separate the medium containing the recombinant virus from the sf9 cells by centrifugation at 300 × g for 3 minutes. This first generation of virus was named as P0.

17. Infect 2×10^6 sf9 cells/ml with 100 µl of P0 virus for 72 hours in a 10ml suspension culture at 25 °C in a 50 ml conical flask. This virus generation (P1) was used for dot-blot experiment.

Detection of recombinant protein expression by dot-blotting—Following are the steps. Figure1A is a diagrammatical representation of the dot-blotting process.

18. Infect 10 ml culture of 2×10^6 /ml sf9 cell with 100 µl of P1 virus and incubate at 27 °C for 72 hours.

19. Collect the virus infected cells by centrifugation of 500 µl of cell culture at 300 × g for 3 minutes at 4°C.

20. Suspend the cells in 50 µl of I-PER™ lysis buffer for 2 minutes.

21. Clarify the lysate by centrifugation at 16000 × g for 5 minutes.

22. Prepare the nitrocellulose membrane by marking a 1 cm^2 grid with a pencil. Wet the membrane in water and transfer buffer, respectively.

23. Optional step: Quantify total protein using standard Bradford or BCA protein quantification assay.

24. Apply 2 µl of the clarified lysate (approximately 20µg) directly to the nitrocellulose membrane in the center of each grid. Place the membrane on a blotting paper and allow the samples to soak. Dry the membrane for 30 seconds.

25. Block the membrane with 5% w/v non-fat milk dissolved in 1X PBST for 10 minutes.

26. Incubate the membrane with HRP conjugated 6X-His tag monoclonal antibody in 1:5000 ratio, dissolved in 10ml of 1X PBST, for 30 minutes.

27. Wash the membrane with 1X PBST, two times for 5 minutes.

28. Incubate the membrane with ECL reagent for 2 minutes and observe/record the chemiluminescence with the BioRad ChemiDoc imaging system.

Western blotting control experiment—Perform a western blot control along with the dot-blot to compare the results using the following steps:

SDS-PAGE

29. Add 1X sample loading buffer to 50 µl of clarified lysate.
30. Boil the samples for 2 minutes at 95 °C.
31. Load 10µl samples onto a 12% polyacrylamide denaturing gel. Run the gel at 100V constant for 90 minutes at room temperature.

Western blot

32. Remove the gel from the cassette after electrophoresis. Keep the gel hydrated with transfer buffer.
33. Place the nitrocellulose membrane on top of the gel. Assemble a sandwich between a set of presoaked (in transfer buffer) filter papers followed by two sets of presoaked blotting pads.
34. Place this sandwich in the XCell II™ blotting module (Thermo Fisher Scientific).
35. Fill the inner and outer chambers of the blot module with pre-chilled (4 °C) transfer buffer.
36. Perform transfer with 25V constant for 90 minutes in an ice bath.
37. Remove the nitrocellulose membrane upon completion of the transfer and incubate it with blocking buffer containing 5% w/v non-fat milk dissolved in 1X PBST for 1 hour.
38. Incubated the membrane with HRP conjugated 6X-His tag monoclonal antibody in 1:5000 ratio, dissolved in 10ml of 1X PBST for 1 hour.
39. Wash the membrane with 1X PBST, three times for 5 minutes.
40. Use BioRad ChemiDoc imaging system to image/record chemiluminescence upon incubation with ECL reagent for 2 minutes.

Quick sample preparation protocol for dot-blotting—Some shortcuts can be implemented to reduce the sample processing times for dot-blotting experiments.

Approach 1: Lyse the sf9 cells with I-PER™ and directly apply a couple of microliters of lysate onto the nitrocellulose membrane (Figure 2A and B). Blot signal obtained was similar the clarified lysate application.

Approach 2: Seed approximately 10,000 cells (2–3 µl) directly after centrifugation and suspension in 1xPBS (Phosphate-buffered Saline, Thermo Fisher Scientific, cat.no. J60801.K2) onto the nitrocellulose membrane. Lyse the cells on the membrane by soaking the membrane with I-PER™ for a couple of minutes. Place a filter paper under the membrane to ensure the soaking of excess liquids. Process the sample for immunodetection as per the above-described protocol (Figure 2C and D). Dot-blot signal obtained from this method has high background or could be faint, but this is the quickest dot-blotting approach.

BASIC PROTOCOL 2: Protein expression analysis for bacterial systems

The bacterial protein expression system is relatively straight forward as compared to eukaryotic systems. A plethora of bacterial host strains are available at our disposal for testing optimal expression of the recombinant proteins. Additionally, by tweaking different physical parameters including, media, temperature, inducer (IPTG, arabinose) concentration and other additives like glycerol we can easily optimize the expression of the recombinant proteins

I use *E coli* BL21 based expression system for the following dot-blot protocol which can used for any bacterial cell/strain based recombinant protein expression system.

Materials

Cell Culture

E coli BL21 competent cells, S.O.C media, New England Biolabs, cat no: C2527H

Luria Bertani (LB) broth, Gibco™ , Thermo Fisher Scientific, cat. no: J75854.A1

LB Agar, Powder, Thermo Fisher Scientific, cat.no. 22700025

Kanamycin, Sigma-Aldrich, cat. no: K0254–20ML

Isopropyl β-D-1-thiogalactopyranoside (IPTG), GoldBio, cat. no: I2481

pET His6 MBP TEV LIC cloning vector (1M) was a gift from Scott Gradia (Addgene plasmid # 29656)

B-PER™ Bacterial Protein Extraction Reagent, Thermo Fisher Scientific, cat no. 78243

The SDS-PAGE, dot-blotting, and western blotting materials are same as described in the basic protocol 1.

Transformation protocol—Use following steps to transform the *E coli* BL21cells with the pET His6 MBP plasmid.

1. Incubate 10 ng of the plasmid with *E coli* BL21chemically competent cells for 15 minutes on ice bath.
2. Heat shock the cells at 42 °C for 30 seconds and immediately place it on the ice bath for 1 minute.
3. Add 250 µl of room temperature S.O.C media (Gibco) to the competent cells.
4. Incubate the cells at 37 °C for 45 minutes with vigorous shaking.
5. Plate 50 µl of cultured cells onto a LB agar 100mm plate containing 50 µg/ml kanamycin and incubate overnight for colony formation at 37 °C.
6. After overnight incubation and bacterial colony formation store the plate at 4°C (approximately 6 hours before bacterial culture). Keep plate at 4°C for prolonged period (up to 2 weeks) for storage purposes.

Cell Culture

7. Pick a single bacterial colony from the transformation plate to inoculate 5ml of LB media containing 50 μg/ml kanamycin.
8. Culture the cells overnight in a shaker incubator at 37 °C.
9. Use 100 μl of the overnight grown primary culture to inoculate a 50ml LB media with 50 μg/ml kanamycin at 37 °C. Monitor the cell growth by optical density (OD) at 600nm using a spectrophotometer.
10. At OD of 0.6 at 600nm (generally obtained within 3–4 hours of cell culture) remove cells from the incubator and bring down the temperature of the culture to 30 °C.
11. Induce the culture with 1mM of IPTG. Set aside an un-induced sample for experimental control.
12. Incubate the culture overnight in a shaking incubator at 30 °C for recombinant protein expression.

Dot-blotting experiment

13. Spin down the bacterial cultures by centrifugation at 2000 × g at 4°C.
14. Suspend the cells in 500 μl of B-PER™ buffer for 5 minutes.
15. Clarify the lysate by centrifugation at 16000 × g for 5 minutes.

All additional steps of the dot-blotting protocol (step 1–11), SDS-PAGE (1–3), and western blotting (step 1–9) are same as described in basic protocol 1.

Quick sample preparation for dot-blotting from bacterial cell culture: Some shortcuts can be implemented to reduce the sample processing times for dot-blotting experiments.

Approach 1: Apply the lysate directly onto the nitrocellulose membrane without centrifugation (Figure 3C).

Approach 2: Lyse the cell on the membrane using B-PER™ reagent to subsequently perform dot-blot (Figure 3D)

Reagents and solutions

IPTG stock solution: Dissolve 238mg on IPTG powder in 1ml of water to make 1M stock solution. Adjust for volume and store at −20 °C.

Tetracycline stock solution: Make is 10mg/ml stock solution in water, store at −20 °C.

LB broth: Add 25g of LB powder to 1 liter of water. Autoclave the solution at 121°C for 20 minutes to sterilize. Store at room temperature.

Blocking buffer: Add 5%w/v of non-fat milk into 1xPBST solution. Always make this buffer fresh.

Commentary

Background Information:

Dot-blotting is a versatile assay to detect nucleic acids and proteins. It has been extensively used for screening monoclonal antibodies, protein quantification, nucleic acid detection and protein-protein interactions(Brown, 2001; Mishra et al., 2021; Morcol & Subramanian, 1999; Ortega Ibarra et al., 2021; Ren et al., 2018; Rupprecht et al., 2010). The method essentially entails deposition of test samples directly onto a membrane(Stott, 1989). Nitrocellulose is the most used membrane material for dot-blots, other options include polyvinylidene difluoride (PVDF) and nylon membranes(Ortega Ibarra et al., 2021). The samples are subsequently processed to develop a signal that can be recorded to perform qualitative or quantitative analysis. DNA and RNA are shown to be quantified using dot-blotting(Brown, 2001). Proteins are also quantified using several approaches like antibody-based detection or dye-based identification/quantification. Imido black, Ponceau S, Coomassie blue, and MemCode are some of the commonly used dyes for dot-blot assays(Helbing, Bohm, Oraha, Stabenow, & Cui, 2022; Morcol & Subramanian, 1999).

In this study I describe a basic dot-blot protocol that can be used to analyze recombinant protein expression in both eukaryotic and prokaryotic heterologous hosts. Recombinant protein expression is one of the foremost prerequisites for most protein-based biochemical or structural analyses. Large quantities of recombinant proteins can be generated using standard commercially available bacterial and eukaryotic expression systems with affinity tags that can be used for downstream purification and biochemical analysis. Protein expression is empirically fine-tuned by tweaking a set of physical (temperature, pH, MOI) and/or chemical (media and additives) parameters. A lot of analysis is involved in these experiments. Dot-blotting provides a quick and easy qualitative test for protein expression. The preferred method for recombinant protein detection for this protocol is using specific antibodies against the affinity tags that are incorporated at the N-or C- terminus of the proteins. 6x His-tag is the most common affinity tag that can be used for dot-blotting. FLAG, HA, STREP, V5, and HALO are some other affinity tags that can be successfully used for dot-blotting. FLAG and HA especially provide high specificity for recombinant proteins that are expressed in low abundance. Previously purified or commercially available affinity tagged proteins can serve as positive control for these assays and can also be used for empirical optimization of the antibody. I have described the detection of recombinant proteins in these protocols using Horse Radish Peroxidase conjugated antibodies, however, Alkaline phosphatase conjugated antibodies can also be used for chemiluminescence based detection.

Advantages of dot-blotting: Following are some of the critical advantages of a dot-blotting test for recombinant protein expression.

1. It is a quick ON or OFF test and unlike the traditional SDS-PAGE, western blot analysis it is less time consuming.
2. It can be used to test many experimental conditions in high throughput.
3. A minimal amount (2 μl) of sample is required for analysis.

4. No special considerations for any post-translational modifications or protein associated co-factors is required. Thes modifications can be address by a secondary analysis using traditional SDS-PAGE and western blotting.
5. It can help save time that can be used for further analysis of the narrowed-down test conditions using the traditional western blotting.
6. It can be used to test protein-protein interactions by blotting for other partner proteins of a complex after affinity purification or in cell lysate.
7. It can serve as a rapid test before a large-scale purification to ensure the cell pellet expresses the desired recombinant protein.
8. Secretory recombinant proteins can also be analyzed by dot-blotting. In this case the media may/may not be concentrated depending upon protein abundance to be used in a same dot-blot protocol as described above to achieve desirable results.
9. Membrane proteins can also be analyzed using the same dot-blot protocol.
10. The method is relatively straightforward and requires minimal optimization.

Limitations of dot-blotting: Following are some of the significant drawbacks of this method.

1. The dot blot cannot determine the molecular weight of the recombinant protein as the samples are not resolved. It can only show the protein's presence in the cell lysate. However, sometimes it is essential to know whether the protein expressed is of the right size (molecular weight). To test that an SDS-PAGE analysis is required.
2. Antibody cross-reactivity can also be a problem. If an antibody with a high background is used for the dot blot analysis, it would be hard to differentiate the genuine protein expression from an artifact. So, it is essential to use a highly specific antibody for a dot-blots to work reliably.
3. Quantitative analysis for recombinant protein expression can a be challenging.
4. The technique is also incapable of determining denatured, partially expressed, and degraded proteins.

Dot-blotting provides a relatively straightforward approach for finding the optimal growth condition for recombinant protein expression. Its advantages weigh more than the shortcoming of the technique. This methodology can be easily applied without any technical expertise and sophisticated equipment. Moreover, a simple dot-blot experiment can be performed using the common reagents for SDS-PAGE and western blotting. Its modular nature can be convenient for testing a lot of bacterial strains for hard-to-express proteins in an array format. Analysis of common bottlenecks like recombinant protein solubility observed in the bacterial protein expression systems can also be analyzed by dot-blotting. For the eukaryotic expression systems, different host cells, incubation times, inoculum quantity (virus or DNA) can be tested easily by dot-blotting. When coupled with western

blotting, it can be a powerful tool to pinpoint the optimal growth conditions for recombinant protein expression.

Critical Parameters:

Basic Protocol 1: Following are the factors that influence the basic protocol 1 and require special attention to achieve robust results.

1. Blue/white bacterial colony screening is a critical step for identification of positive recombinant DNA containing bacmids. Always use freshly made LB agar plates with freshly made recommended additives for optimal results.
2. The insect cells should be >90% viable for a transfection experiment. Use a hemocytometer or an automated cell counter to count viable cells.
3. Use a fresh stock of lipid-based transfection reagent (FuGENE-HD).
4. Several ratios (1:3, 1:4, 1:5) of bacmid DNA (μg): FuGENE-HD (μl) reagent can be used to achieve optimal transfection results.
5. Phenotypically, changes in the virus infected cells should be carefully monitored. In some instances incubation of an additional 24 hours can lead to optimal protein expression.
6. Samples should always be kept at 4 °C after cell lysis.
7. Protease inhibitors can be used if significant recombinant protein degradation is observed.
8. Blotting paper should be placed under the nitrocellulose membrane to absorb excess liquids and to prevent diffusion of the material on the membrane.
9. Antibody concentration should be empirically determined.

Basic Protocol 2: Following are the factors that influence the basic protocol 2 and require special attention to achieve robust results.

1. Only use fresh buffers made with ultra-pure quality MilliQ water (resistivity 18.2 MΩ.cm at 25 °C).
2. Glassware and plastics should be thoroughly washed with deionized water before use.
3. Fingers should not touch the dot-blot nitrocellulose membranes at any time. Clean forceps should be used for maneuvering membranes.
4. Special precautions should be taken to keep the membranes wet at all times.
5. Antibody concentrations should be empirically adjusted. Always use an antibody with high specificity that will result in low backgrounds. To attain stringency, washing steps can also be changed (increased or decreased).

Troubleshooting:

Basic Protocol 1: Some of the common problems, their possible causes and solution with the basic protocol 1 are discussed in Table 1.

Basic Protocol 2: Table 2 shows some common problematic causes and their potential solutions for basic protocol 2.

Understanding Results : Figure 1A shows the flow scheme for the dot-blotting experiment. The dot-blot analysis clearly shows a positive expression of the recombinant protein in the virus infected sf9 cells. Figure1B shows the sf9 cell uninfected by virus does not express the recombinant His6-MBP protein. The traditional western blotting experiments produce comparable results to the dot-blotting, Figure1C. However, the dot-blotting results can be observed within an hour, including sample processing time. A comparable signal was observed by dot-blotting the lysate without clarification (Figure 2B). However, the signal observed was very faint when the insect cells were directly applied on the membrane (Figure 2D). These results suggest that lysis can influence the results. Cell debris present on the membrane can cause noisy chemiluminescence. Ideally a hard spin (16000 × g) for 5 minutes after lysis will clarify the lysate from cell membrane debris and nuclei acids to give optimal dot-blotting results.

The results from the dot-blot experiment (Figure 3 A and B) clearly show the expression of the recombinant protein in IPTG induced *E coli* BL21 cell. The western blot control experiment validates the presence of this recombinant protein. Direct application of cells on membrane result in noisy signal due presence of cell debris and nucleic acids on the membrane surface (Figure 3 A, C, and D). It is advisable to clarify lysate for optimal results.

Application of dot-blot analysis in eukaryotic cell cultures:

1. Same protocol can be used to test recombinant protein expression in HEK293T cells (data not shown). Commercially available protein extraction buffers like M-PER™ for mammalian cells (cat no. 78501) and Y-PER™ for yeast cells (cat.no.78991) (Thermo Fisher Scientific) can also be used to quickly release the proteins from cells for dot-blotting.
2. To test the Multiplicity of Infection (MOI) for optimal protein expression. MOI is the ratio of the virus to the host cell. It significantly affects protein expression. Dot-blot can be used to test different MOIs to calculate the optimal quantity of viral stock for infection.
3. To optimize cell culture incubation time post viral infection. Recombinant protein accumulation times in eukaryotic cells vary from 24 to 96 hours post viral infection. Dot-blotting provides an excellent tool for quick assessment of time-driven expression analysis.
4. To test secretory proteins. Dot-blotting the media can be a quick test for successful recombinant protein secretion.

Application of the dot-blot analysis in bacterial systems:

1. To detect recombinant protein expression in different bacterial stains for choosing an ideal strain for soluble protein expression.
2. To quickly test different temperature and inducer (IPTG) concentrations for optimal protein production.
3. To quickly test the solubility of the recombinant proteins.

Time Consideration:

1. Sf9 cell culture take 72 hours post virus infection for optimal recombinant protein expression.
2. The dot-blot protocol will take <1 hour for results, starting from cell harvesting to blot imaging as compared to the traditional SDS-PAGE western blotting experiments that will require a minimum of 4–6 hours.
3. Bacterial cell culture will require generally an overnight incubation after induction. This process can be completed within 3 hours in some cases.
4. 1 hour is sufficient for obtaining a dot-blot signal.

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Data Availability Statement:

Data is available upon request from the author.

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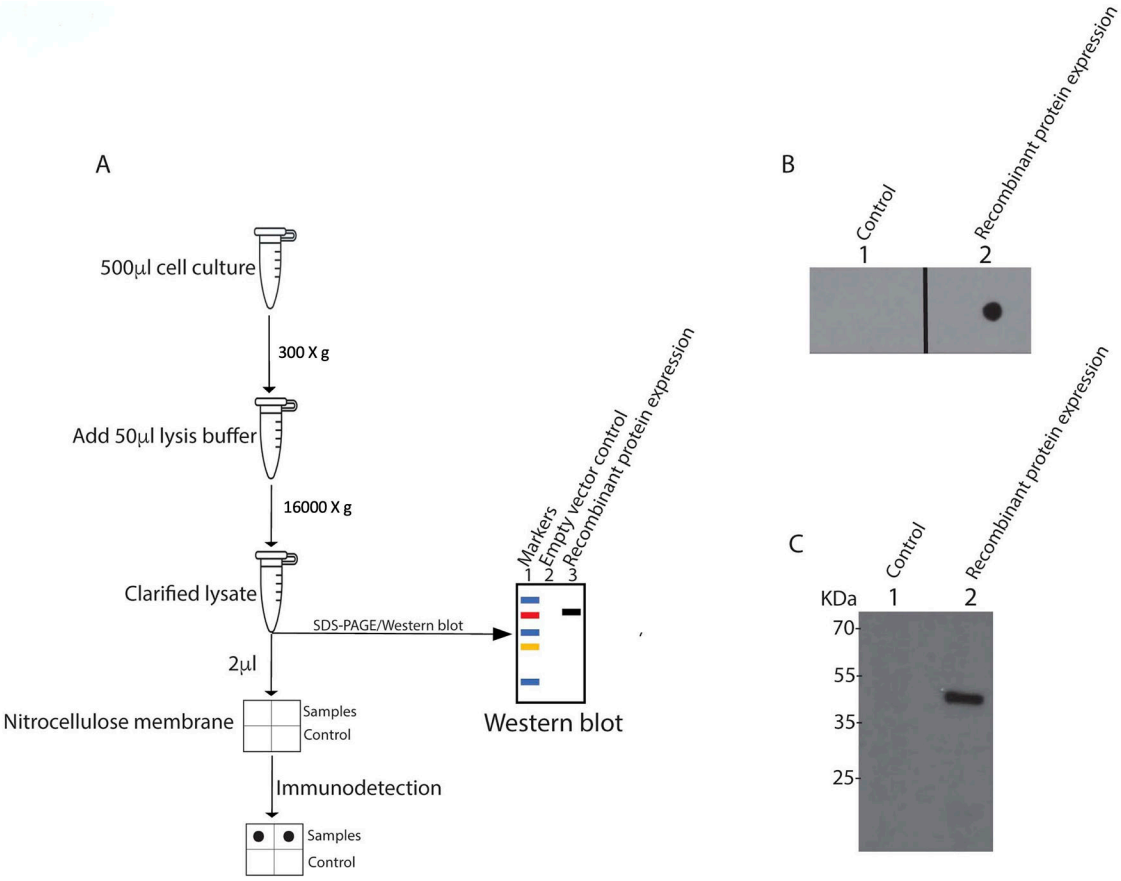

Figure1:

Steps for dot-blotting: (A) A flowchart showing the steps of sample preparation for dot-blotting. (B) Immunodetection results of the recombinant protein expression in a dot-blot experiment. Lane 1: Uninfected sf9 cell clarified lysate (obtained after centrifugation) control, and Lane 2: Insect cell clarified lysate infected with recombinant baculovirus. (C) Western blot experiment for recombinant protein expression. Lane 1: Uninfected insect cell lysate, control, and Lane 2: Insect cell lysate infected with recombinant baculovirus.

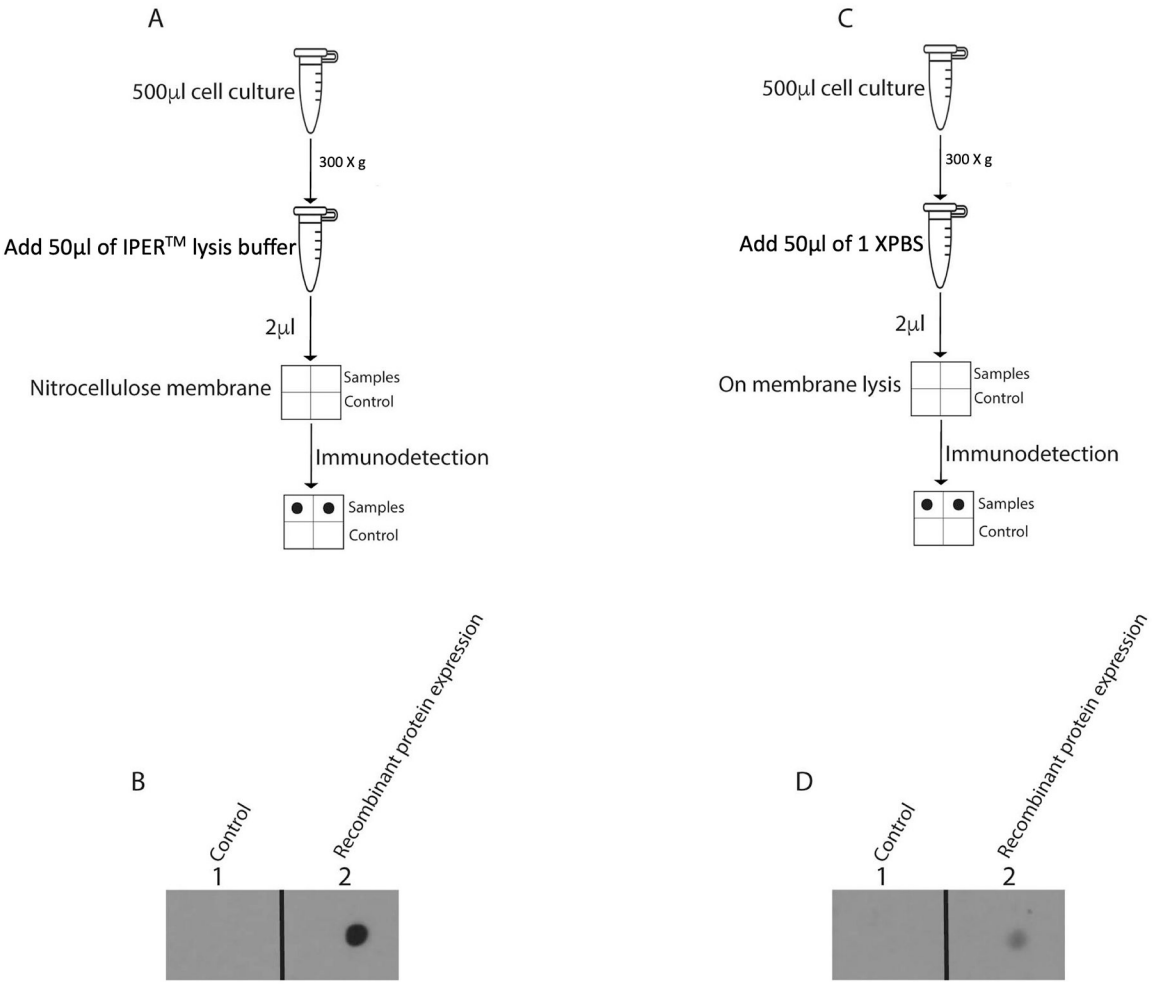

Figure2:

Quick sample preparation method for dot-blotting. (A) A flowchart showing the steps for quick sample preparation without lysate clarification step. (B) Immunodetection results for recombinant protein expression by whole cell lysate blotting. Lane 1: Uninfected sf9 cell lysate, control, and Lane 2: Insect cell lysate infected with recombinant baculovirus. (C) A flowchart showing the steps for quick sample preparation by direct cell dot-blotting. (D) Results for recombinant protein expression by direct cell dot-blotting. Lane 1: Uninfected insect cells, control, and Lane 2: Insect cells infected with recombinant baculovirus.

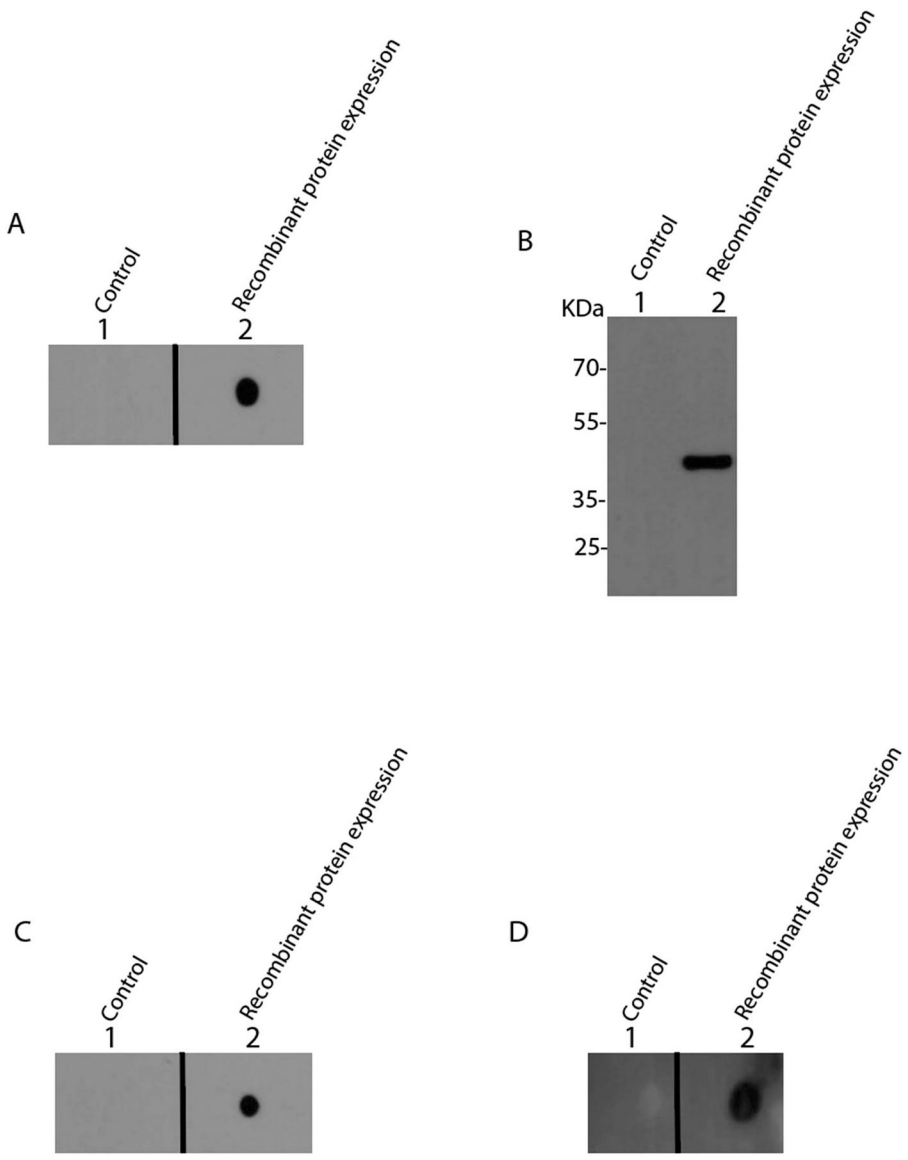

Figure3:

Dot-blotting results from bacteria: (A) Immunodetection results of the recombinant protein expression in a dot-blot experiment. Lane 1: Uninduced *E coli* BL21 clarified lysate, control, and Lane 2: IPTG induced *E coli* BL21 clarified lysate expressing HisX6-MBP protein. (B)Western blot experiment for recombinant protein expression. Lane 1: Uninduced clarified lysate, control, and Lane 2: induced clarified lysate. (C) Immunodetection results for recombinant protein expression by whole cell lysate blotting. Lanes 1 and 2: Uninduced and induced cell lysate, respectively. (D) Results for recombinant protein expression by direct cell dot-blotting. Lanes 1 and 2: Uninduced and induced bacterial cells, respectively.

Table 1:

Troubleshooting Guide for Basic Protocol 1

Problem	Possible Cause	Solution
No dot-blot signal	**1** No/low abundant recombinant protein expression **2** Bad antibody **3** Protein/affinity tag degradation **4** Bad ECL reagent	**1** Check protein expression/abundance/ affinity tag degradation by traditional western blotting, SDS-PAGE-Coomassie or silver-nitrate staining. **2** Change the antibody stock. Use an Alkaline Phosphatase conjugated antibody. **3** Change the antibody ratio to 1:2500 or 1:1000. **4** Change the ECL reagent. Use more sensitive ECL-prime reagent.
Poor/ diffused signal	**1** Bad antibody/ratio **2** Diffused sample **3** Bad reagents and buffers	**1** Change the antibody stock, change antibody concentration and incubation times. **2** Use filter paper to soak access liquid when spotting the membrane. **3** Make new buffers and reagent. Use fresh milk stock for membrane blocking.
Noisy signal/ high background	**1** Insufficient blocking **2** Diffused sample **3** Presence of contaminants, nucleic acids, antibody-cross-reacting proteins, and cell debris/insufficient lysis **4** High amount of antibody used	**1** Use fresh milk stock and increase blocking incubation times. **2** Use filter paper under the membrane while spotting sample. **3** Clarify the lysate, pipette carefully to avoid cell debris, centrifuge twice, filter the lysate. Check by traditional western blotting for antibody cross-reactivity with non-specific proteins. Lower the antibody concentration.

Table 2:

Troubleshooting Guide for Basic Protocol 2

Problem	Possible cause	Solution
No dot-blot signal	**1** Consider all causes discussed in table 1. **2** No induction of protein **3** Protein not expressed in BL21 cell	**1** Consider all solutions mentioned in table 1. **2** Check IPTG concentration and use fresh stock. Check OD of cell culture before inducing. **3** Empirically optimize induction temperatures. **4** Try other *E coli* expression host strains.
Control also shows dot-blot signal	**1** Leaky protein expression **2** Non-specific antibody interactions **3** In-sufficient washing **4** Use of an affinity tag that is endogenously expressed	**1** Optimize induction parameters. Use a different expression host strain. **2** Use a different antibody, empirically determine antibody concentration. **3** Increase PBST wash steps. Use 0.2% v/v Tween-20 in PBST. **4** Endogenously expressed affinity tag like Maltose Binding Protein (MBP) are not ideal for dot-blotting. Carefully choose affinity tags while designing constructs
Weak dot-blot signal	Consider all points mentioned in table 1	Consider all solutions discussed in table 1.
High background	Consider all points mentioned in table 1	Consider all solutions discussed in table 1.