Detection of proteins of interest in cell lysates.

CMCI (Center for molecular and Cellular Intervention)
University Medical Center Utrecht

Written By

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<tbody>
<tr>
<td>Robert van der Burgh</td>
<td>PhD student</td>
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Conformation

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<tr>
<td>Prof.dr. A.B.J. Prakken</td>
<td>Co-Chair CMCI</td>
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Changes from last version

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Detection of proteins of interest in cell lysates.

1. **Subject**
This Standard Operation Procedure (SOP) describes a method to detect proteins of interest in cell lysates.

2. **Application**
Studying the expression of proteins or protein modifications like phosphorylation and ubiquitination

3. **Definitions and Abbreviations**
- TBS-T = Tris buffered saline with tween
- PBS = Phosphate buffered saline
- RT = room temperature
- AB = Antibody
- BSA = Bovine serum albumin
- w/v = weight/volume
- v/v = volume/volume
- PVDF = Polyvinylidene Fluoride
- SDS = Sodium dodecyl sulfate
- APS = Ammoniumpersulfate
- BME = β-mercaptoethanol
- Ab = Antibody
- TEMED = N,N,N,N'-tetramethylethylenediamine
- Tris = 2-Amino-2-(hydroxymethyl)-1,3-propanediol
- BPB = Bromophenolblue

4. **Principle**
When cells are subjected to any kind of change in its direct environment (activation, inhibitor treatment, proliferation, differentiation, etc) the protein expression profile in cells changes. With Western blotting it is possible to detect the protein of interest and compare its expression profile in different circumstances. Proteins are first separated on size with gel electrophoresis. In this step proteins are denatured by a strong detergent, usually SDS. The binding of SDS to the denatured protein ensures all proteins have the same mass to charge ratio. This allows them to be separated on mass by running through a polyacrylamide gel. After size separation the proteins are transferred to a protein binding membrane. This membrane is incubated with specific Abs against the target protein, followed by a secondary Ab that is labeled with a fluorochrome. The signal intensity of the Ab with the correct size confirms the presence and amount of the protein.
Detection of proteins of interest in cell lysates.

![Chemical structure of SDS](image)

5. **Safety precautions**

Treat every sample containing human material such as AB serum and the lymphocyte samples as infectious material. Wear disposable gloves.

- **β-Mercaptoethanol**
  - Toxic, dangerous to the environment
- **Acrylamide/bis solution 29:1**
  - Toxic
- **Ammoniumpersulfate**
  - Oxidizing, harmful
- **TEMED**
  - Corrosive, flammable
- **96% Ethanol**
  - Flammable
- **Acetic Acid**
  - Corrosive
- **Hydrochloric acid**
  - Corrosive
- **2-Propanol**
  - Flammable, harmful

6. **Reagents**

6.1 **Chemicals**

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6.2 Solutions

5 M NaCl
For 1L: 292.2 g NaCl, add 950 ml water, stir until NaCl is completely dissolved, add water to a total volume of 1L.

30% Tween (v/v)
For 500 ml: 166.67 ml Tween, 333.33 water ml. Stir with closed bottle, foams extensively.
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1.5 M Tris pH 8.8
For 1L: 181.71 g Tris, add 850 ml water, set pH to 8.8 with 37% hydrochloric acid, add water to a total volume of 1L.

1.0 M Tris pH 8.0
For 1L: 121.14 g Tris, add 850 ml water, set pH to 8.0 with 37% hydrochloric acid, add water to a total volume of 1L.

1.0 M Tris pH 6.8
For 200 ml: 24.23 g Tris, add 150 ml water, set pH to 6.8 with 37% hydrochloric acid, add water to a total volume of 200 ml.

Ponceau S staining solution
For 250 ml:
0.1% BPB (w/v)
For 10 ml: 0.01 g BPB, dissolve in 10 ml water.

10% APS (w/v)
For 3 ml: 0.3 g ammoniumpersulfate, add 3 ml water, store at 4°C for up to two months.

6.3 Buffers

Laemmli buffer (lysis): 2% SDS, 10% glycerol, 62.5 mM Tris pH 6.8
For 50 ml: 5 ml 20% SDS, 5.75 ml 87% Glycerol, 3.125 ml 1M Tris pH 6.8, add H₂O to ml.
After protein concentration determination, supplement with 5% BME and 0.002% BPB to get 1X sample buffer

Sample buffer: 2% SDS, 10% glycerol, 62.5 mM Tris pH 6.8, 5% BME, 0.05% BPB
For 10 ml 5X: 0.5 ml 20% SDS, 0.32 ml 1M Tris pH 6.8, 5.75 87% glycerol, 100 µl 0.1% BPB, 2.5 ml BME (fumehood!), add water to a total volume of 10 ml. Store in 0.5 ml aliquots at -20°C, can be frozen and thawed multiple times. When using the 5X sample buffer, work in fumehood.

Running buffer: 25 mM Tris pH 8.3, 192 mM glycline, 0.1% SDS
For 2L 10X: 60.75 g Tris, 288.27 g Glycine, 10 ml 20% SDS, add 1840 ml water, set pH to 8.3 with 37% hydrochloric acid, add water to a total volume of 2L.

Blotting buffer: 25 mM Tris pH 8.3, 192 mM glycline, 20% (v/v) Ethanol
For 2L 10X: 60.75 g Tris, 288.27 g Glycine, add 1850 ml water, set pH to 8.3 with 37% hydrochloric acid, add water to a total volume of 2L.
For 5L 1X: 500 ml 10X Blotting buffer, 1L 96% Ethanol, add water to a total volume of 5L.

Blocking buffer: 5% ELK or 5% BSA in TBS-T
For 5 ml: 0.25 g ELK/BSA in 5 ml TBS-T.

Antibody buffer: 0.5% ELK/BSA in TBS-T + Ab
For 5 ml: 1 ml 5% ELK/BSA, 4 ml TBS-T, add Ab at desired concentration.
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TBS-T: 150 mM NaCl, 10 mM Tris pH 8.0, 0.3% Tween
For 5 L: 150 ml 5 M NaCl, 50 ml 1 M Tris pH 8.0, 50 ml 30% Tween, add water to a total volume of 5L.

6.4 Antibodies

Primary Antibody: Protein/modification specific

Secondary Antibody:
Li-COR IRDye 680 Donkey anti-Rabbit IgG
Li-COR IRDye 800CW Donkey anti-Mouse IgG

7. Equipment and Accessories tools

7.1 Equipment
- Microcentrifuge (Eppendorf, Germany)
- Biorad mini trans-blot cell (Biorad)
- Biorad mini PROTEAN system (Biorad)
- Pipets 10-1000 µl (Gilson, The Hague, The Netherlands)
- Shaker at RT
- Roller bank at RT
- Roller bank at 4°C
- Odyssey Imaging system (Li-COR)
- Heat block at 95°C

7.2 Accessories
- 50 ml sterile polypropylene conical tubes (Falcon/ Becton Dickinson, Erembodegem, Belgium, 352070)
- Disposable gloves (Kimberly Clark, Zaventem, Belgium)
- Easyload 10 µl (741015), 200 µl (741035) and 1000 µl (741000) sterile pipettips (Greiner Bio-one, Germany)
- Aluminum foil
- Plastic trays ~ 12 x 8 cm

8. Samples

8.1 Sample Collection
Western blotting can be done on a large variety of cells. The minimum cell number per sample is highly dependent on the expression of the protein or the abundance of the modification. For proteins with high or moderate expression 250,000 cells is usually sufficient. For low expressed protein or modifications 750,000 cells is recommended.

8.2 Sample Processing
All handlings of the sample before cell lysis should be done in a biohazard safety
Detection of proteins of interest in cell lysates.

9. Procedure

1. Wash cells 2x with PBS to remove all serum from cells
2. Lyse cells by adding lysis buffer (~50 µl per 100,000 cells) and boil samples for 10 min at 95°C. Lysates can be stored for a long time at -20°C
3. Determine protein content in samples by either Lowry protein assay or BCA protein assay.
4. Prepare gel casting system by washing the glass plates with soap followed by 70% ethanol. Dry the plates with a soft tissue. Make sure there are no more stains on the glass as this will cause impurities in the gel
5. Place the glass plates in the gel casting holder, and add small amount of water between the plates to check for leaks. If there are no leaks remove the water with a strip of whatman filter paper
6. Prepare the mix for the running gel. Determine the desired gel percentage and add the reagents in a 50 ml tube. The amounts are listed on the paper near the RT-PCR cycler. All solutions can be found on the shelf above the "molecular corner", except for the acrylamide/bis mix and 10% APS which are in the door of middle refrigerator. Add the APS last because it starts the gel polymerization. The amount needed for a gel depends on the thickness (1.0 mm ~ 7ml, 1.5 ~11 ml). The gel system requires 2 gels to be run at once, so prepare two gels (second gel doesn’t have to be loaded with sample)
7. Cast the gel mix between the glass plates. Make sure there is enough room left at the top for the comb and stacking gel. Add 250 µl of isopropanol on top of the gel. This removes bubbles and creates a level top of the gel. Save the leftover of the gel mix as an indicator for when the gel is polymerized.
8. Leave the gel for ~20 min at RT
9. Check if the gel has polymerized and remove the isopropanol with a strip of whatman paper
10. Prepare the stacking gel mix (~4 ml for two gels), add it to the top of the running gel and place the comb between the plates
11. Leave the gel for ~15 min at RT
12. Put the gels in the PROTEAN gel system (combs directed inward), an add 1X running buffer to the inner compartment. Make sure there is no running buffer leaking from this compartment as it will disrupt the electric current
13. Dilute samples if necessary (~30-40 µg of protein/slot, equal amount of protein per slot) and add BME and BPB to the samples and load them on the gel. (Max 32 µl for 1.0 mm, max 65 µl for 1.5 mm, for a 10 slot gel). Reserve 1 slot for the protein ladder (middle freezer, third drawer). Load 2 ~ 3 µl of ladder
14. Run the gel at 100 V until all the samples have cleared the stacking gel, then increase the voltage to 150 V
15. Stop running the gel when the separation for the protein is sufficient. The blue line can be used as an indication; it runs at an approximate weight of 17 kD on a 12% gel. It runs on a lower weight with higher percentage gels. The marker is often also visible on the gel
16. Transfer the gel to the blotting system. Pre-wet the sponges and Whatman paper in Blot buffer and soak the PVDF in 96% alcohol for 2-3 minutes. Stack in the following order:
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i. White plastic (top, (+))
ii. Sponge
iii. Whatman paper (2x)
iv. PVDF membrane
v. Polyacrylamide gel
vi. Whatman paper (2x)
vii. Sponge
viii. Black plastic (bottom, (-))

NB: The PVDF membrane binds proteins. Always wear gloves when handling the membrane. Try to reduce bending and folding of the membrane as this will increase background signal.

17 Put the stacked gels in the blot system, add a block of ice and fill to the brim with blot buffer. Blot for 1.5 hour with a constant current of 300 mA.
18 Remove the membranes from the blotting system. (OPTIONAL) Put the membrane in a small tray and soak in ponceau S for a minute. Save the excess of ponceau S as it can be used multiple times. Wash the membrane several times with dH₂O. This step is a control to see if the blotting procedure was successful. Ponceau S binds reversibly to proteins; if the transfer was successful bands should be visible in all the lanes. Wash several times more with dH₂O to remove all ponceau S.
19 Block the membrane by soaking it for 30 min to 1 hour at RT in 5% ELK or BSA in TBS-T.
20 Wash membranes 3 times 10 minutes with TBS-T.
21 Incubate membrane with primary Ab in 0.5% ELK/BSA in TBS-T overnight at 4°C. Dilute antibody to manufacturer’s instructions. It is possible to detect two different proteins at the same time if the second primary Ab comes from a different host species than the first. If this is the case the other Ab can be added to this solution.
22 Wash membrane 3 times 10 min with TBS-T.
23 Incubate membrane with secondary Ab in 0.5% BSA in TBS-T for 1 hour at RT in the dark. Dilute the secondary Ab 1:20.000 (right refrigerator, top shelf on the door in silver bag. Red = α-rabbit, yellow = α-mouse). If a second Ab was used at step 21, both secondary Abs should be added. This solution can be used several times and should be stored in the dark at 4°C. Stored this way it can be kept for 2.5 weeks.
24 Wash membrane 4 times 10 minutes with TBS-T.
25 Wash membrane once 30 min with TBS-T.
26 Measure fluorescent intensity on the Odyssey.
27 The membrane can be stored indefinitely when dried and protected from light. If further steps are done with the membrane (e.g. stripping or additional probing) the membrane can be stored up to a week in PBS.

10. Processing of the Results

The images from the Odyssey contain the raw data. This data can be analyzed afterwards on the PCs in the Coffer lab. It is possible to quantify the intensity of the bands (with background correction) to compare protein levels. The images can also be enhanced visually (e.g. change contrast, brightness, color, etc.), this does not changes the raw data. The Odyssey can also scan at different resolutions to get resolution suitable for publications.
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11. **Documentation**

   Document experiment in laboratory notebook.

12. **Accuracies and Precision**

   As long as the signal is above the detection limit, it is possible to quantify the fluorescent intensity. Background should not go above 250 (green) or 500 (red). If background is higher, try longer and/or more wash steps. Or increase dilution of the primary and secondary Abs. keep in mind that intensity of bands can change with different Abs.

13. **Quality Control**

   A marker should be run on each gel as a size control. Also it is good practice to take along a positive and (if possible) a negative control.

14. **Literature**


*** END ***