Western Blotting of Proteins

This is a description of Western Blotting using the Bio-Rad Mini Trans-Blot apparatus and chromogenic or chemiluminescence detection of protein-antibody complexes.

1. Transfer of proteins

- Remove gel from SDS-PAGE apparatus, remove stacking gel, and soak separation gel for 15min in Blotting Buffer.
- Prewet Nitrocellulose transfer membrane in H$_2$O. Prewet PVDF membrane 15s in methanol, then 2min in H$_2$O. Then soak membrane in Blotting Buffer for 10min.
- Set up transfer assembly in a shallow box filled with Blotting Buffer (kept at 4°C in advance) keeping everything wet during assembly and avoiding to trap air bubbles between each layer:

  | Clear Panel | Fiber Pad |
  | Thick Filter Paper | Gel |
  | Transfer Membrane | Thick Filter Paper |
  | Fiber Pad | Gray Panel |

- Close Gel Holder Cassette and place in Transfer Unit filled with Blotting Buffer in the direction: (+) Anode - Gray Panel - Transfer Membrane - Gel - Clear Panel - Cathode (-) (We are using the opposite orientation of clear and gray than described in the Bio-Rad manual because assembly is easier this way. Therefore place the Gel Holder Cassette in the Buffer Tank so that the clear panel of the holder is facing the red cathode electrode panel!)
- When the Gel Holder Cassettes and the Bio-Ice cooling unit (kept at -20°C in advance) are in place fill the gel box with Blotting Buffer to completely cover the cassettes. Insert a stir bar, place unit on stirrer, and start transfer.
- Transfer 60min at 100V constant voltage corresponding to 200-250 mA. (Different conditions might be needed for specific applications or when using different transfer buffers).
- Disassemble the transfer assembly, label Transfer Membrane with a pencil (stain gel after transfer to check efficiency).

2. Immunodetection of proteins using alkaline phosphatase (AP) conjugates

- Place Transfer Membrane in a box just slightly larger than the membrane with the side next to the gel in the transfer assembly facing upwards. Place box on a shaker (100rpm for incubations, 125rpm for washing steps).
- Wash membrane 10min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) to block non-specific binding.
- Incubate membrane ≥ 60min at RT (or o/n at 4°C) in 10ml TBSTM (TBSTB) containing Primary Antibody.
- Wash membrane 4 x 5min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) containing Alkaline Phosphatase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
- Wash membrane 3 x 5min with 20ml TBST, 1 x 5min with 20ml TBS, 1 x 5min with 20ml AP Buffer.
- Add 10ml AP Substrate and incubate until desired result is obtained (usually 10-60min).
- Wash membrane 10min with H$_2$O to stop the reaction and dry membrane on the air.

3. Immunodetection of proteins using horseradish peroxidase (HRP) conjugates

- Place Transfer Membrane in a box just slightly larger than the membrane with the side next to the gel in the transfer assembly facing upwards. Place box on a shaker (100rpm for incubations, 125rpm for washing steps).
- Wash membrane 10min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) to block non-specific binding.
- Incubate membrane ≥ 60min at RT (or o/n at 4°C) in 10ml TBSTM (TBSTB) containing Primary Antibody.
- Wash membrane 4 x 5min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) containing Horseradish peroxidase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
- Wash membrane 3 x 5min with 20ml TBST and 2 x 5min with 20ml TBS.
- Add 10ml HRP Substrate and incubate until desired result is obtained (usually 10-60min).
- Wash membrane 10min with H$_2$O to stop the reaction and dry membrane on the air.
4. Immunodetection of proteins using chemiluminescence reagents

- Place Transfer Membrane in a box just slightly larger than the membrane with the side next to the gel in the transfer assembly facing upwards. Place box on a shaker (100rpm for incubations, 125rpm for washing steps).
- Wash membrane 10min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) to block non-specific binding.
- Incubate membrane ≥ 60min at RT (or o/n at 4°C) in 10ml TBSTM (TBSTB) containing Primary Antibody.
- Wash membrane 4 x 5min with 20ml TBST.
- Incubate membrane 60min at RT in 10ml TBSTM (TBSTB) containing Horseradish Peroxidase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
- Wash membrane 4 x 5min with 20ml TBST.
- Detection: - For each 65x90mm membrane prepare 2ml of detection reagent by mixing 1ml of Detection Reagents 1 and 2 (ECL) or 2ml Solution A and 50µl Solution B (ECL Plus).
- Drain excess buffer from the membrane by placing it on a sheet of filter paper.
- Place the membrane on a sheet of foil and add the detection reagent. Incubate for 1min (ECL reagent) or for 5min (ECL Plus).
- Drain off excess detection reagent by placing the membrane briefly on a sheet of filter paper. Wrap the membrane in Saran wrap.
- Immediately expose the membrane to autoradiography film for 1min. Develop the film and expose the membrane again for a time that gives a satisfactory signal based on the result of the first exposure.
- Store the wrapped membrane at 4°C for reprobing.

- Stripping and reprobing:
  - Incubate the membrane for 30min at 50°C in Stripping Buffer with occasional agitation.
  - Wash membrane 3 x 5min with 20ml TBST.
  - Incubate membrane 60min at RT in 10ml TBSTM.
  - Add the Primary Antibody and continue with the detection protocol outlined above.

5. Testing of antibody dilutions with membrane strips

- Run a SDS-PAG with a preparative comb loading 200µg (200µl) of a protein mixture (or less if pure proteins are used) per gel. Transfer proteins to Transfer Membrane as described above.
- Place Transfer Membrane in a box just slightly larger than the membrane with the side next to the gel in the transfer assembly facing upwards.
- Wash membrane 10min with 20ml TBST.
- Incubate membrane 60min at RT in 20ml TBSTM (TBSTB).
- Wash membrane 2 x 10min with 20ml TBST.
- Dry membrane on the air, label so that upper side and top can be identified.
- Cut strips of ca. 5mm from the membrane, store unused strips at 4°C, stain one strip with PageBlue for reference.
- For incubations place strips in Mini-Incubation Trays (Bio-Rad) with the protein side facing upwards. During incubations and washings the strips must float freely in the channels. This is best achieved by placing the trays on a rotary shaker. Solutions are added with a Multipette (Eppendorf) and removed by vacuum-aspiration.
- Rewet dried strips in 2ml TBST.
- Incubate strips ≥ 60min at RT (or o/n at 4°C) in 1ml TBSTM (TBSTB) containing Primary Antibody.
- Wash strips 4 x 5min with 2ml TBST.

5a. Alkaline phosphatase detection:

- Incubate strips 60min at RT in 1ml TBSTM (TBSTB) containing Alkaline Phosphatase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
- Wash strips 3 x 5min with 2ml TBST, 1 x 5min with 2ml TBS, 1 x 5min with 2ml AP Buffer
- Add 1ml AP Substrate and incubate until desired result is obtained (usually 10-60min).
- Wash strips 10min with H₂O to stop the reaction and dry strips on the air.

5b. Horseradish peroxidase detection:

- Incubate strips 60min at RT in 1ml TBSTM (TBSTB) containing Horseradish Peroxidase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
- Wash strips 3 x 5min with 2ml TBST and 2 x 5min with 2ml TBS.
- Add 1ml HRP Substrate and incubate until desired result is obtained (usually 10-60min).
- Wash strips 10min with H₂O to stop the reaction and dry strips on the air.

5c. Chemiluminescence detection:

- Incubate strips 60min at RT in 1ml TBSTM (TBSTB) containing Horseradish Peroxidase conjugated Secondary Antibody (eg. goat-anti-rabbit Ab) (dilution as specified by the supplier).
• Wash strips 5 x 5 min with 2ml TBST.
• Incubate strips for precisely 1 min in 1 ml ECL detection reagent (mix 500µl Reagents 1 and 2) or 5 min in 1 ml ECL Plus reagent (mix 1 ml Solution A and 25µl Solution B).
• Drain excess reagent by placing the strips briefly on a sheet of filter paper and wrap the strips in Saran wrap.
• Immediately expose the strips to autoradiography film. Develop the film and expose the strips again for a time that gives a satisfactory signal based on the result of the first exposure.
• Store the wrapped filter strips at 4°C for reprobing.

6. Reagents

• Transfer Membranes: (size 65x90mm) Hybond-C extra (Amersham): supported nitrocellulose membrane, pore size 0.45µm, binding capacity 80-100µg/cm², wet in H₂O
Hybond-P (Amersham): polyvinylidene difluoride membrane, pore size 0.45µm, binding capacity 170-200 µg/cm², wet in methanol
Immobilon-P (Millipore): polyvinylidene difluoride membrane, pore size 0.45µm, binding capacity 170-200µg/cm², wet in methanol

• 10 x Blotting Buffer: 250mM Tris 30.3g
1920mM Glycine 144g
0.1% SDS 1g
H₂O to 1000ml
pH=8.3 (unadjusted!)

• Blotting Buffer: 25mM Tris 100ml 10 x Blotting Buffer
192mM Glycine 10 ml
0.01% SDS 200ml
H₂O to 1000ml
pH=8.3 (unadjusted!) (store at 4°C)

• 10 x TBS: 500mM Tris 60.55g
1500mM NaCl 87.66g
H₂O to 1000ml
pH=7.5 (HCl)

• TBS: 50mM Tris 100ml 10 x TBS
150mM NaCl to 1000ml with H₂O
pH=7.5

• TBST: TBS pH=7.5 100ml 10 x TBS
0.1 % Tween 20 1ml Tween 20
H₂O to 1000ml

• TBSTM: TBST 50ml
2% Non-Fat Dry Milk (Bio-Rad or Maresi) 1g
dissolve well, store at 4°C (not longer than one week)

• TBSTB: TBST 50ml
1% Bovine Serum Albumin (BSA; Roche) 1g
dissolve well, store at 4°C (not longer than one week)

• AP Buffer: 100mM Tris 12.11g
100mM NaCl 5.84g
5mM MgCl₂ 6 H₂O 1.02g
H₂O to 1000ml
pH=9.5 (HCl)

• NBT: 100mg Nitro Blue Tetrazolium (Sigma N6876) in 2ml 70% DMF (store at 4°C)

• BCIP: 100mg 5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine salt (Sigma B8503) in 2ml 100% DMF (store at 4°C)

• AP Substrate: AP Buffer 10ml
NBT 5µl
BCIP 15µl

• HRP Substrate: TBS 9ml
0.5% 4-Chloro-1-naphthol in Methanol 1ml
30% H₂O₂ 10µl
• ECL detection reagents (Amersham):
  Mix equal volumes of ECL detection reagents 1 and 2
  Reagent 1 = 0.005% H_2O_2 [1.67µl 30%H_2O_2/10ml H_2O]
  (the mixed reagents can be reused for several days when stored at 4°C)

• ECL Plus detection reagents (Amersham):
  Mix 1000 µl Reagent A and 25 µl Reagent B
  (use immediately, do not store)

• Stripping Buffer:
  62.5mM Tris.Cl pH=6.8
  2% SDS
  100mM 2-Mercaptoethanol
  H_2O
  1.25ml 1M Tris.Cl pH=6.8
  2ml 20% SDS
  140µl 2-Mercaptoethanol
  to 20ml
  (prepare fresh before use)

• Anti-Mouse IgG - Alkaline Phosphatase Conjugate (Sigma A-3562) (store at 4°C)
• Anti-Rabbit IgG - Alkaline Phosphatase Conjugate (Sigma A-3687) (store at 4°C)
• Anti-Chicken IgG - Peroxidase conjugated (Sigma A-9046) (store at -20°C, once thawed at 4°C)
• Anti-Goat IgG - Peroxidase Conjugate (Sigma A-5420) (store at -20°C, once thawed at 4°C)
• Anti-Mouse IgG - Peroxidase Conjugate (Dako P-0447) (store at 4°C)
• Anti-Rabbit IgG - Peroxidase Conjugate (Sigma A-0545) (store at -20°C, once thawed at 4°C)
• Anti-Rat IgG - Peroxidase Conjugate (Sigma A-5795) (store at -20°C, once thawed at 4°C)
• Primary Antibody:
  Polyclonal (serum, affinity purified) or Monoclonal (culture supernatant, purified)
  Test suitable dilution, Store at 4°C or -20°C (depends on individual stability)

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