iGEM2014 – Microbiology – BMB – SDU	
Title: Western Blotting **SOP number:** SOP0011 **Version number:** 1	**Date issued:** 2013.09.25 **Review date:** **Written by:** Victoria Mikkelsen

1. Purpose

To analyze expression of proteins/ show the presence of a protein

2. Area of application

E.coli strain

3. Apparatus and equipment

Apparatus/equipment	Location (Room number)	Check points	Criteria for approval/rejection

4. Materials and reagents – their shelf life and risk labelling

Name	Components	Supplier / Cat. #	Room (hallway storage)	Safety considerations

5. QC – Quality Control

6. List of other SOPs relevant to this SOP

iGEM2014_SOP0002_v01_MM_E.coli_growth_culture_exp
iGEM2014_SOP0001_v01_MM_ON_culture_of_E.coli

7. Environmental conditions required

8. Procedure

Preparation of samples:

1. Grow cells in liquid media and extract cells corresponding to one mL at OD600=0.2. (i.e. if the OD600 is different from 0.2, divide 0.2 by the measured OD600).
2. Spin cells down 2 minutes at 14,000 rpm.
3. Resuspend the pellet in 100 µL Sample Buffer
4. If the samples are used immediately after this, they set them in the heating block at 80° C for 10 minutes. Otherwise store in the freezer and heat before use.

Semi-dry blotting of proteins and Western blot

Use gloves.

5. Run an SDS-PAGE, use a prestained protein marker. Before stopping the gel, prepare 6 pieces of Whatman 3MM paper and 1 piece of membrane (**Immobilon P**) corresponding to the size of the gel to be blotted. Wash the blotting apparatus in dH_2O and wipe off with paper towels.
6. Stop the gel, separate glass plates and cut away stacking gel. Remove all small gel pieces; if necessary flush the gel with dH_2O.
7. Equilibrate the separation gel for 5 min in transfer buffer (discard the buffer). Soak the membrane in 100 % methanol 1-2 min, transfer to transfer buffer and equilibrate for 5 min (reuse methanol).
8. Assembly of transfer sandwich:
   - Move 3 pieces of Whatman paper held together through transfer buffer and place on the anode. With a glass pipette, roll from the middle and to the edges to remove air bobbles.
   - Place the equilibrated membrane on the sandwich. Remove air bobbles.
   - Place the equilibrated protein gel on the sandwich. Moisten with fresh transfer buffer. Remove air bobbles. Remove any gel part that is outside the sandwich. Again remove air bobbles.
   - Move 3 pieces of Whatman paper held together through transfer buffer and place on top of the sandwich. Moisten with transfer buffer and remove air bobbles.
   - Place cathode on top of the sandwich and connect the blotting apparatus to itself.
9. Blot at 0.8 mA/cm^2 for 1 hour. Note the voltage, it should increase during blotting.

10. Disconnect the apparatus and carefully remove the cathode without disturbing the sandwich. Check if the protein marker is visible on the membrane. If not; moisten the sandwich with transfer buffer and continue blotting for 30-60 minutes.
11. Disassembly of the sandwich:
    - Mark the protein marker with a pen and cut one corner of the membrane to mark the protein side and orientation. Discard paper and protein gel.
    - It might be a good idea to stain the membrane with Ponceau S. Otherwise transfer the membrane directly to blocking solution.
12. Ponceau S staining (reversible staining of proteins):
    - Make a 0.1 % Ponceau S solution in 1 % acetic acid.
    - Stain for a few min, remove staining solution (can be reused) and wash the membrane with dH_2O, keep an eye on protein bands on the membrane. Remove staining by washing several times in dH_2O. Residual staining should not interfere with western blotting.

Western blotting (classic method)

All steps demands shaking and is performed at RT. All solutions are discarded. Use a volume of solution so that the membrane is covered and can move freely.

1. Block the membrane in TTBS + 5 % nonfat dry milk for 2-4 h at RT or O.N. at 4 °C.
2. Wash the membrane 2x5 min in TTBS.
3. Dilute primary antibody in TTBS + 2 % dry milk. Incubate 2 hours. This solution can be reused for 1-2 weeks, store at 4 °C.
4. Wash the membrane 2x5 min in TTBS and then 15 min in TTBS
5. Dilute HRP-conjugated secondary antibody in TTBS + 2 % dry milk. Incubate for 60 min.
6. Wash the membrane 15 min in TTBS and then 4x5 min in TTBS.
7. Detection with chemiluminescence:
    - Mix according to manufacturer just before use. Pour over membrane (protein side up) for 1 min, remove the membrane and wrap in Vita Wrap. Avoid air bobbles and excess reagent. Store chemiluminescence reagent at 4 °C
    - Expose X-ray film to the membrane on time. Develop and adjust exposure time. Use same developing time. Chemiluminescence lasts for 15-30 min.
8. Save membrane. Before reusing, soak the membrane in 100 % methanol, strip for primary and secondary antibody.

Western blotting (Snap-ID)

Make sure the “membrane” in the cassette for the Snap-ID is intact. Fill up water and ice for the motor. Use tweezers to move the membrane.

1. Prepare appr. 50 mL TTBS + 0.3 % dry milk for each membrane. Dilute the primary and the secondary antibody in 3 mL TTBS + 0.3 % dry milk.
2. Place membrane in Snap-ID cassette, protein side up. Block the membrane in 30 mL TTBS + 0.3 % dry milk (pour 30 mL into cassette, turn on suction and wait until the membrane looks “dry”).

3. Incubate with primary antibody for 10 min. Distribute with pipette so that the membrane is covered. Turn on suction.
4. Wash 3 times in 30 mL TTBS (as blocking).
5. Incubate with secondary antibody for 10 min. Turn on suction.
6. Wash 3 times in 30 mL TTBS.
7. Place membrane in large petridish – protein side up – and pour chemiluminescence (Biorad detection system, mix prior to use) over the membrane. It has to be covered completely. Incubate 1 min. Drag the membrane on the edge of the petridish to get rid of the chemiluminescence reagent (collect and reuse for 1-2 weeks, store at 4 °C). Wrap the membrane in Vita Wrap and place in cassette. Bring to developing room and expose the X-ray film for 1 min. Develop and adjust exposing time if needed. Soak the film in dH_2O, then dry for ½ hour at 37 °C.
8. Stripping of the membrane: Boil dH_2O. Put membrane in container, pour with boiling water and continue boiling in microwave oven for 2 min.
9. The membrane can be stored at 4 °C.

9. Waste handling

Chemical name	Concentration	Type of waste (C, Z…)	Remarks

10. Time consumption

- 4 hours

11. Scheme of development

Date / Initials	Version No.	Description of changes

12. Appendices

Transfer buffer

1 x	39 mM glycin	10 x	29.3 g glycin
	48 mM TrisBase		58.1 g Trisbase

0.0375 % SDS	3.75 g SDS
20 % methanol	dH_2O until 1000 mL

Dilution of 10 x stock
100 mL 10 x stock
200 mL methanol
700 mL dH_2O

TBS

1 x	20 mM TrisHCL	10 x	24.2 g TrisBase
	137 mM NaCl		80 g NaCl
	pH 7.6		Add 500 mL dH_2O and adjust pH to 7.6.
			Add dH_2O until 1000 mL

TTBS (0.05 % tween 20 in 1 x TBS)
Dilute 100 mL 10 x TBS in 900 mL dH_2O and add 0.5 mL 100 % Tween 20. Prepare fresh.

TTBS + dry milk
Weigh desired amount of dry milk and dissolve by stirring. Prepare fresh.

Sample Buffer
0.5 M Tris/HCl pH 6.8, 10% SDS, 150 mM DTT, 14% glycerol, 0.025% bromophenolblue.