Protein transfer from SDS-PAGE to nitrocellulose membrane using the Trans-Blot SD cell (Western).

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Summary: Try to detect whether his tagged protein is present in a protein gel by transferring the protein bands to a nitrocellulose membrane and probing by using anti-his antibody.

Materials/Reagents/Equipment	**Vendor/Recipe**
Reagents	
Mini Protean unit for running mini SDS/PAGE gel	Use 10% PAGE for 60 kDa protein.
Trans-Blot SD cell	BIO-RAD #170-3940
Towbin Transfer Buffer	25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3
Towbin Transfer Buffer/0/1% SDS if bands are not transferring to NC	25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, **0.1% SDS**
TTBS: Tris.Tween.Buffered Saline	20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 % Tween 20
Nitrocellulose membrane (n/c)	Amersham #RPN203D-350N, tall cabinet by desk.
Mini Blot size Blotting paper	BIO-RAD #170-3966: located at-350N, tall cabinet by desk.
Mouse anti-his antibody 1st Antibody	Freezer VI, 10 µl/eppendorf. Add to 30 ml of TTBS (1:3000). Can be reused 10 times, keep at 4C. $170 Amersham 27-4710-01
ECL Anti mouse IgG conjugated to horseradish peroxidase (sheep) 2nd Antibody	Refrigerator VI, door GE Healthcare (formerly Amersham) NXA931 $164
TMB stabilized substrate for HRP	Bench 350 N, shelf above the bench Promega W4121 $77
Prestained Protein Marker	NE Biolabs #P7708S $90

EH&S No special requirements.

1. Run SDS-PAGE with protein samples. Use Prestained dye molecular weight markers (6 ul for mini gel), Freezer VI. Boil protein standard for 5 min. It is a good idea to run the same samples that will be blotted in duplicate and the duplicate is stained with Coomassie blue (this will show you what the samples looked like).
2. Prepare transfer buffer and equilibrate gel in buffer for 20 min to remove SDS. To make 1L of Towbin Transfer Buffer (25mM Tris, 192mM glycine, 20% methanol, pH8.3) dissolve 3.03 g Tris and 14.4 g glycine in ddH2O, add 200 ml of methanol, adjust volume to 1 L with ddH2O. **Do not add acid or base to adjust pH. If bands do not transfer well, you can add 0.1% SDS to Towbin Transfer Buffer.**
3. Cut n/c membrane to gel size and wet in Towbin Transfer Buffer.

Transfer of protein bands to n/c membrane:

Remove safety cover from Trans-Blot SD cell and prepare gel sandwich as follows:
Bottom: anode metal plate: Place in this order:
Pre-wet filter paper in Towbin Transfer Buffer
Pre-wet n/c membrane
Equilibrated PAGE gel
Pre-wet filter paper in Towbin Transfer Buffer
Note: Roll out air bubbles between layers, using glass pipet, glass rod or 13 mm plastic tube used for reading Bradfords.

4. Secure top cathode plate and safety cover.
5. Run blot. Mini gel: 15 V for 30 min for entire mini gel or 12V for 20 min for half a mini gel. After transferring, stain the gel with Coomassie blue to make sure transfer has been successful.
6. Turn off the power supply and open the lid. With a pencil, gently mark the top of each well on the n/c membrane. Remove n/c membrane and place in a clean sandwich box. Rinse the unit with water, dry with paper towels, air dry, close.

Use a clean sandwich box for all of the following steps:

7. Place n/c membrane in TTBS + 3% dry milk (50 ml) for 1 hr, room temperature. Place on a gel shaker at room temperature. This blocks all areas of n/c membrane that do not have any protein bound to it.
8. Rinse membrane with TTBS (20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 % Tween 20).
9. Incubate membrane for 40 min with anti-his antibody (Amersham 27-4710-01) that has been diluted 1:3000 with TTBS. This antibody binds to his tagged proteins (10 ul of antibody: 30 ml of TTBS)
10. Rinse for 20 min in TTBS.
11. Incubate membrane with secondary antibody for 40 min: Anti mouse IgG-HRP from GE Healthcare (formerly Amersham) NXA931, diluted 1:1000 in TTBS (20 ul antibody:20 ml TTBS). This antibody will bind to the first antibody.
12. Rinse for 20 min in TTBS.
13. Developing: Use 1 -2 ml of Promega TMB stabilized substrate for HRP, W4121.

Use enough developer to just cover the top of the membrane. Wait until signal appears (~ 5 min).

17. Rinse membrane with water to stop the reaction, semi-dry, take picture in BioRad Chemidoc.
18. Sensitivity of this assay: 50-100 ng per band in our hands but in published reports it should be 10-20 ng.

Amount of time it takes to do a Western: 5 hrs

Amount of time it takes to do a Dot blot: ~3 hrs (Dot blot entails applying 1 ul of sample onto nitrocellulose paper and then following the rest of the procedure).

Chemidoc of n/c membrane:

1. While n/c is still wet, take to BioRad Chemidoc, Rm 308.
2. Store image on a ZIP.
3. Place n/c on white platform. Select EPI white. Trans UV for gel.
4. File: Quantity One: ChemiDoc XRS
5. Live Focus
6. Freeze
7. Auto expose

If you want to change exposure, push up and down arrows on manual exposure.

8. File: Export as a TIFF image
9. Export View excl overlay
10. Export
11. Save on ZIP

Towbin Transfer Buffer

25 mM Tris	3.03 g Tris
192 mM glycine	14.4 g glycine
20% methanol, pH 8.3	20 % methanol

Bring up to 1 L with water
DO NOT ADD acid or base to adjust pH

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Towbin Transfer Buffer/0.1 % SDS

25 mM Tris	3.03 g Tris
192 mM glycine	14.4 g glycine
20% methanol, pH 8.3	20 % methanol

1 g SDS
Bring up to 1 L with water
DO NOT ADD acid or base to adjust pH

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TTBS: Tris.Tween.Buffered Saline

20 mM Tris, pH 7.5	20 ml 1 M Tris
0.1 M NaCl	100 ml 1 M NaCl
0.1 % Tween 20	1 ml Tween 20

Up to 1 L with water, adjust pH

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