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Protein Extraction and western blotting

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Protein Extraction

1 Whole cell protein was extracted from cells by treating cell pellets with Radio Immunoprecipitation Assay buffer (RIPA buffer: 1M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 50mM pH 7.4 Tris in ddH_2O) was prepared according to the Cold Spring Harbor and supplemented with 1µl of Protease Inhibitor Cocktail from Roche per 100µl of RIPA buffer. RIPA buffer was added at a concentration of 500µl per ~2x10^6 cells.

2 Samples were gently agitated by shaking for 30 minutes at 4°C to allow for the breakdown of cells, then were centrifuged at max speed (>14,000 rcf) at 4°C for 20 minutes.

3 The supernatant containing the protein component was transferred to a fresh microcentrifuge tube to be loaded immediately into an SDS-PAGE gel or stored at -20°C.

SDS-PAGE gel

4 In preparation for running protein samples on the SDS-PAGE gel a 1.5mm glass spacer plate and cover plate were sandwiched together in a casting clamp and placed upon a gel casting stand (all from Bio Rad).

5 The volume within the plates was filled with water and allowed to sit for several minutes to ensure that there was no leakage. Once it was confirmed that there were no leaks the water was poured out.

6 10ml of 12% acrylamide lower running gel was prepared consisting of 4.2ml of 29:1 acrylamide/bis-acylamide (Bio Rad), 2.5ml of 4x lower gel buffer (1.5M Tris and 0.4%SDS in distilled water, pH of 8) and 3.3ml of distilled water. This was mixed thoroughly then 40µl of 10% ammonium persulfate (prepared freshly each time) as well as 10µl of TEMED from Fisher Scientific was added.

7 The lower running gel solution was immediately poured into the gel casting plates to within 1cm of the top. The final cm of the plates was filled with 100% isopropanol. The lower gel solidified for a minimum of 30 minutes.

8 3ml of upper gel mixture consisting of 0.75ml 29:1 acrylamide/bis-acylamide, 0.45ml of 4x upper gel buffer (0.5M Tris and 0.4%SDS in distilled water, pH of 6.8), 1.8ml of distilled water, 10µl of of 10% ammonium persulfate and 5µl of TEMED(Fisher Scientific) was prepared once the lower gel had solidified.

9 The isopropanol covering the lower gel was poured off completely, exposing the top of the lower gel. The upper gel mixture was poured on top, filling the remaining centimeter in the gel plates. A 1.5mm gel comb that accompanied the spacer plate was then inserted into the top of the plates. This upper gel mixture solidified over the course of 30 minutes.

10 The solid gel and its glass case were removed from the casting stand and clamp and were fitted into the electrode assembly (Bio Rad) with the short plates facing inwards; the comb was then removed. If only one gel was being run then a mock spacer plate and short plate combination was inserted on the other side of the electrode assembly with short plate on the inside. This created a watertight container that was fitted into the Bio Rad buffer tank and filled to overflowing with Running buffer.

11 Running buffer was produced 1 liter at a time by diluting 100ml of 10x Running buffer (0.25M Tris, 1.92M Glycine and 1% w/v SDS in distilled water) in 900ml distilled water. The tank itself was then filled with the remaining Running buffer.

12 Once the gel was prepared for electrophoresis, 15µl of each protein extract was mixed with 2X SDS gel-loading buffer (4% SDS, 0.2% bromophenol blue, 20% glycerol and 200mM dithiothreitol) according to the Cold Spring Harbor protocol and the resulting mixture was heated to 95°C for 2-3 minutes

13 The protein mixture was loaded into each well of the gel, and 5µl BenchMark Pre-stained Protein Ladder from Thermo Fisher was loaded into one of the wells abutting the samples.

14 The gel was run at 180 volts until the bromophenol blue dye in the various samples had nearly reached the bottom of the gel but had not run off. The bottom band of the Pre-stained Protein Ladder corresponded to ~10kDa and so it was ensured that there was always a significant gap between this band of the ladder and the bottom of the gel to ensure that the histones had not been run off the gel.

Western blotting

15 Transfer buffer was prepared by combining 100ml of 10x Transfer buffer (0.25M Tris and 1.9M glycine in distilled water and stored at 4°C), 200ml of methanol and 700ml of distilled water.

Citation: t.a.mcdougall (03/23/2021). Protein Extraction and western blotting. https://dx.doi.org/10.17504/protocols.io.bqubmwsn

16 The gel was removed from the tank, electrode assembly and spacer plates. The gel was immersed for 5 minutes in cold Transfer buffer and the transfer system was prepared.

17 The Bio Rad transfer holder cassette was arranged with the black frame (which would face the negative electrode) on one side and the clear frame (which would face the positive electrode) on the other. A foam pad from Bio Rad was placed on top of the black frame and upon that was placed a piece of the filter paper supplied in the Nitrocellulose/Filter Paper Sandwich from Thermo Fisher.

18 The now soaked gel was placed carefully upon that piece of filter paper so as to be completely supported by it and not touching any part of the sponge beneath.

19 Carefully and using metal tweezers, the 0.2µm nitrocellulose was first briefly soaked in Transfer buffer then placed on top of the gel. Pressure was applied with a smooth flat implement to remove any gaps that might exist between the nitrocellulose and gel and a small amount of transfer buffer was poured on top to ensure the nitrocellulose was completely soaked.

20 The other piece of filter paper was placed on top of the nitrocellulose to cover it completely. An additional piece of spongy foam was then placed on top of the filter paper. The clear frame was then folded over and fastened to the black frame and the cassette was fitted into the Bio Rad Core Assembly Module for protein transfer with the black of the cassette facing the black side of the module.

21 The Core Assembly Module was inserted into the Bio Rad protein transfer tank and a cold ice pack was placed in the transfer tank as well. The tank was then filled with Transfer buffer and was hooked up to a Bio Rad PowerPac HC power supply.

22 Protein transfer was carried out at 90 volts for one hour at 4°C with constant gentle agitation of the Transfer buffer using a magnetic stir bar to ensure dispersion of heat.

23 After the one hour the protein bound nitrocellulose membrane was transferred into a container filled with 40ml of blocking buffer consisting of Tris buffered saline (TBS) with 0.1% v/v Tween-20 and 3% Bovine Serum Albumin (BSA: 1.2 grams in 40ml TBS).

24 The membrane was gently rocked back and forth for one hour in the blocking buffer to prevent the non-specific absorption of antibodies into the nitrocellulose.

25 After blocking, the nitrocellulose was placed inside a 50ml falcon tube containing 20ml of primary antibody solution (3% w/v BSA, 0.1% v/v Tween-20 in TBS plus the relevant antibodies). The primary antibodies used in the cited study are shown here.

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A	B	C	D	E	F
Primary western blotting antibodies					
Target	**Company**	**Catalog #**	**Lot #**	**Host species**	**Dilution**
H3K27me3	Diagenode	C15410069	A1824D	rabbit	1:500
H2AK119ub	Sigma-Aldrich	05-678	2591849	mouse	1:2000
H4	Upstate	25296	07-108	rabbit	1:2000
β-actin	Invitrogen	MA5-15739	TC263471	mouse	1:5000

26 The membrane was incubated in this solution overnight at 4°C on a tube rotator to allow even coating of the entire surface of the membrane with antibodies.

27 The next day the membrane was retrieved from its falcon tube and was washed four times for 5 minutes each in room temperature TBST (0.1% v/v Tween-20 in TBS). During each wash the nitrocellulose membrane was placed on a rocker to help wash off any unbound proteins.

28 The nitrocellulose membrane was then placed in a container containing 20ml of secondary antibody solution (3% w/v BSA, 0.1% v/v Tween-20 in TBS plus the secondary antibody of interest. The membrane was incubated in the secondary antibody solution for one hour at room temperature on a gently rocking rocker.

A	B	C	D	E	F
Secondary fluorescent western blotting antibodies					
Target	Company	Catalog #	Lot #	Host species	Dilution
Rabbit antibodies	LI-COR	925-68071	B81113-01	goat	1:10,000
Mouse antibodies	LI-COR	925-32210	926-32201	goat	1:10,000

29 Following this secondary incubation, the membrane was washed twice with TBST then once in TBS for 5 minutes each at room temperature.

30 The protein and antibody bound membrane was then imaged at the relevant wavelengths using an LI-COR Odyssey machine from BioAgilytix and the software package, Image Studio.

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