

## Detection of Diverse and High Molecular Weight Nesprin-1 and Nesprin-2 Isoforms Using Western Blotting

James Carthew and Iakowos Karakesisoglou

### Abstract

Heavily utilized in cell and molecular biology, western blotting is considered a crucial technique for the detection and quantification of proteins within complex mixtures. In particular, the detection of members of the nesprin (nuclear envelope spectrin repeat protein) family has proven difficult to analyze due to their substantial isoform diversity, molecular weight variation, and the sheer size of both nesprin-1 and nesprin-2 giant protein variants (>800 kDa). Nesprin isoforms contain distinct domain signatures, perform differential cytoskeletal associations, occupy different subcellular compartments, and vary in their tissue expression profiles. This structural and functional variance highlights the need to distinguish between the full range of proteins within the nesprin protein family, allowing for greater understanding of their specific roles in cell biology and disease. Herein, we describe a western blotting protocol modified for the detection of low to high molecular weight (50–1000 kDa) nesprin proteins.

**Key words** LINC complex, Nesprins, Nuclear envelope, Polyvinylidene difluoride, SDS-PAGE, SYNE, Western blotting

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### 1 Introduction

The LINC (Linker of Nucleoskeleton and Cytoskeleton) complex is a structural bridge spanning the entire nuclear envelope of eukaryotic cells, serving as a functional connection between the nuclear interior and the cytoskeleton [1, 2]. At its core are KASH- and SUN-domain protein associations. Proteins that are members of the vertebrate KASH-domain family are known as nesprins (nuclear envelope spectrin repeat proteins). Nesprins are encoded by the *SYNE* genes and display a plethora of isoforms, which are structurally and functionally diverse. These isoforms are generated via alternative initiation, termination, and splicing. As a consequence, nesprins vary drastically in their modular organization, including in their spectrin repeat copy number, ABD (actin-binding domain), PBD (plectin-binding domain), and KASH (Klarsicht, ANC-1, and *SYNE* homology) domain compositions [3].

The ability to detect and distinguish between this wide range of isoforms is crucial to the advancement of nesprin research. With key roles in directed cell migration, nuclear structure, ciliogenesis, DNA damage repair, and cellular signalling pathways, nesprins exhibit a high degree of functional diversity [3]. Not surprisingly, nesprin knockout mice exhibit severe neuromuscular and cardiac disease pathologies [3–5], high-frequency hearing loss [6], and memory defects [7]. This phenotypic diversity suggests that advanced analytical methods are required to comprehensively investigate the roles of distinct nesprin isoforms and paralogues within the cell.

Western blot analysis relies on the separation of proteins by molecular weight via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [8–11] and subsequent protein transfer to a membrane. Existing western blot protocols accurately detect proteins between 10 and 250 kDa; however, proteins with broader molecular weight ranges, such as nesprins (between 20 and 1000 kDa), require additional adaptations. These include the use of polyacrylamide gels fortified with agarose [12] and gradient gels ranging from 3 to 15 % acrylamide [13–15], in order to aid in their acquisition. Protein transfer can be achieved via a variety of techniques including diffusion [9], vacuum-assisted solvent flow [16], and electrophoretic transfer [11]. For the transfer of larger molecular weight proteins (>250 kDa), adapted electrophoretic transfer methods have been developed, one of which will be described within this protocol. Using the method described herein, it has been possible to accurately resolve nesprin proteins ranging from 20 to 1000 kDa. This was achieved through the adaptation of a gradient polyacrylamide gel system, which allows for direct comparisons to be made between nesprin isoform variants.

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## 2 Materials

All solutions described should be prepared using purified deionized water and analytical grade reagents unless otherwise stated. Prepare and store all reagents at room temperature (unless indicated otherwise). When disposing of waste materials and solutions, follow your respective guidelines and COSHH (control of substances hazardous to health) forms as to their safe disposal.

### 2.1 Cell/Tissue Lysis

1. Sterile filtered, cell culture grade PBS.
2. Protein lysis buffer (RIPA): 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % SDS (sodium dodecyl sulphate), 1 % Nonidet P-40, 0.5 % sodium-deoxycholate, and protease inhibitors (*see Note 1*).

3. Protease inhibitor cocktail (PIC, 100×): Once made into solution, aliquots should be stored at  $-20^{\circ}\text{C}$ .
4. Cell scrapers.
5. 1 mL syringes.
6. 24G  $\times$   $\frac{3}{4}$  in. disposable hypodermic needles.
7. Laemmli sample buffer (5×): 0.25 M Tris-HCl, pH 6.8, 20 % glycerol, 4 % SDS, 1.43 M  $\beta$ -mercaptoethanol and 0.2 % bromophenol blue (*see Note 2*).

## 2.2 SDS-PAGE

1. Gel Caster: (Hoefer Inc.) Dual gel caster for mini vertical units.
2. Gel electrophoresis running chamber: (Hoefer Inc.) SE260 Mighty Small II. Deluxe Mini Vertical Electrophoresis Unit with 1.5 mm thick spacers (*see Note 3*).
3. 10.6 cm  $\times$  10.1 cm glass plate: (Web Scientific).
4. Aluminium back plate: (Web Scientific).
5. 25 mL Gradient mixer: (VWR).
6. Peristaltic pump.
7. 3 % Polyacrylamide resolving gel stock: For 100 mL, combine 25 mL of 1.5 M Tris-HCl, pH 8.8 with 10 mL [30 %] acrylamide (*see Note 4*). Add a further 1 mL of [10 %] SDS and top up with 64 mL  $\text{H}_2\text{O}$ . Stock solution should be stored at  $4^{\circ}\text{C}$  for maximum 1 month.
8. 15 % Polyacrylamide resolving gel stock: For 100 mL, combine 25 mL of 1.5 M Tris-HCl, pH 8.8 with 50 mL [30 %] acrylamide (*see Note 4*). Add a further 1 mL [10 %] SDS and top up with 24 mL  $\text{H}_2\text{O}$ . Store the stock solution at  $4^{\circ}\text{C}$  for 1 month.
9. 4 % Polyacrylamide stacking gel stock: For 100 mL, combine 20 mL of 0.5 M Tris-HCl, pH 6.8 with 13.3 mL [30 %] acrylamide (*see Note 4*). Add a further 1 mL [10 %] SDS and top up with 65.6 mL  $\text{H}_2\text{O}$ . Store the stock solution at  $4^{\circ}\text{C}$  for 1 month.
10. SDS gel running buffer (10×): 0.25 M Tris-HCl, pH 8.3, 2 M glycine, 1 % SDS.
11. Ammonium persulfate (APS): 10 % solution in  $\text{H}_2\text{O}$ . Leave one aliquot for use and store the rest at  $-20^{\circ}\text{C}$ .
12. *N,N,N',N'*-tetramethylethylenediamine (TEMED). Store at  $4^{\circ}\text{C}$ .

## 2.3 Immunoblotting

1. Nesprin-2 affinity-purified C-terminal antibodies (pAbK1; [17, 18]).
2. Anti-Rabbit POD (Horseradish Peroxidase) secondary antibodies.

3. Prestained protein ladder.
4. Western blot transfer buffer: 25 mM Tris-Base, 190 mM glycine, 0.1 % SDS, 10 % ethanol.
5. Western blot transfer tank: e.g. Electroblothing unit BTV100 (VWR).
6. Whatman paper.
7. Polyvinylidene difluoride (PVDF) membrane: Immobilon-P 0.45  $\mu\text{m}$  pore size (*see Note 5*).
8. Wet transfer compression cassette (VWR).
9. Fiber pads (VWR).
10. Blocking solution: 5 % skim milk powder in PBS.
11. Tween-20 washing buffers: Two buffers are utilized containing 0.1 and 0.3 % Tween-20 in PBS.
12. Enhanced chemiluminescence (ECL) detection solution: For 20 mL, add 17.7 mL H<sub>2</sub>O followed by 2 mL of 1 M Tris-HCl, pH 8.5. Add a further 200  $\mu\text{L}$  of 250 mM luminol (3-Aminophthalhydrazide) and 89  $\mu\text{L}$  of 90 mM *p*-Coumaric acid. Just before usage, add 6.1  $\mu\text{L}$  of 30 % H<sub>2</sub>O<sub>2</sub>. Luminol and *p*-Coumaric acid stock solutions are prepared in dimethyl sulfoxide (DMSO), stored at ambient temperature and protected from light.
13. High performance chemiluminescence films.

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### 3 Methods

All methods described below should be undertaken at room temperature unless otherwise specified.

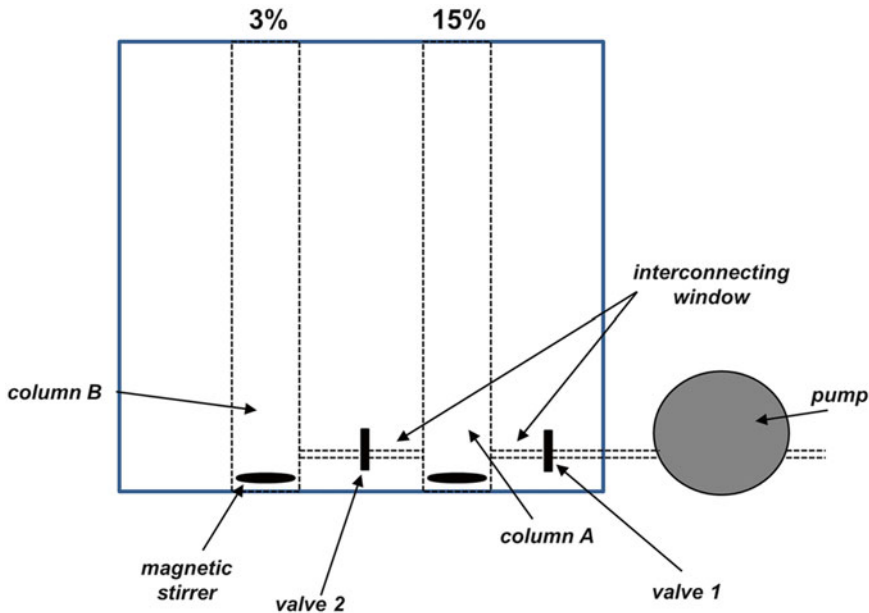
#### 3.1 *Sample Preparation: From Cell Culture to Cell Lysates*

1. For a T-75 culture flask at 70 % cell confluency: prepare 500  $\mu\text{L}$  ice-cold RIPA buffer, combine with 5  $\mu\text{L}$  of 100 $\times$  concentrated PIC (protease inhibitor cocktail), and then leave on ice in an Eppendorf tube.
2. Take the T-75 flask from the cell incubator, aspirate the media, and wash the cells three times for 1 min each with ice-cold cell culture grade PBS.
3. Perform all subsequent processes on ice to prevent autolysis.
4. Remove the PBS and add the ice-cold RIPA buffer containing PIC. Evenly distribute the solution across the surface while using a cell scraper to dislodge cells (*see Note 6*).
5. Dislodge all cells from the flask surface and transfer the lysate into a pre-cooled Eppendorf tube and leave on ice for 15 min.

6. Perform a mechanical breakdown of the lysate during the 15 min incubation on ice using a sterile 1 mL syringe fitted with a 24G  $\times$   $\frac{3}{4}$  in. disposable hypodermic needle. Syringe sample 15 times to ensure lysate homogenization, efficient protein extraction, and genomic DNA shearing (*see Note 7*).
7. Centrifuge the resulting lysate at 4 °C for 15 min at 12,000  $\times g$  to pellet the remaining cell debris.
8. Carefully remove the supernatant and place into a new Eppendorf tube (*see Note 8*).
9. Add 120  $\mu$ L of 5 $\times$  concentrated Laemmli sample buffer to the extracted supernatant and then incubate at 99 °C for 4 min (*see Note 9*).
10. After incubation, store samples at -20 °C (*see Note 10*).
11. Equal loading of cell lysates can be obtained by protein quantitation assays (e.g. BCA, Lowry) before Laemmli sample buffer addition, or by our preferred method of assessing Coomassie blue staining after SDS-PAGE (*see Note 11*).

**3.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

1. Assemble the gel casting equipment using the 10.6 cm  $\times$  10.1 cm glass and aluminium back plates and the spacers. Add water to ensure that no leakage occurs across the bottom of the caster (*see Note 12*).
2. For large molecular weight proteins such as nesprins, utilize a gradient gel system (Fig. 1), to produce 3–15 % acrylamide gradient gels. Alternatively, commercially available precast gradient gels can be used.
3. Mix 5.5 mL of 15 % polyacrylamide stock with 18.3  $\mu$ L of 10 % APS and 9.7  $\mu$ L TEMED in a 50 mL conical flask, and insert solution into column A. Once the solution is inserted into column A, mix 5.5 mL of 3 % polyacrylamide stock with 18.3  $\mu$ L 10 % APS and 9.7  $\mu$ L TEMED in a 50 mL conical flask, then load the solution into column B (*see Note 13*). Elevate the gradient maker and then activate the magnetic stirrers and the peristaltic pump (50 rpm). Open valve 1 first, followed by valve 2. As the 3 % acrylamide is pulled from column B to column A, the 15 and 3 % acrylamide solutions mix, forming a gradual decrease in % acrylamide from the base of the gel to the top (*see Note 14*). Ensure enough space is allowed for the gel comb.
4. Gently pour 2 mL isobutanol over the gel to ensure a level gel surface is formed (*see Note 15*).
5. Pour off the isobutanol once the resolving gel is set (ca. 30 min). In a separate conical tube, combine 3 mL of 4 % polyacrylamide stock with 30  $\mu$ L 10 % APS and 16  $\mu$ L TEMED to



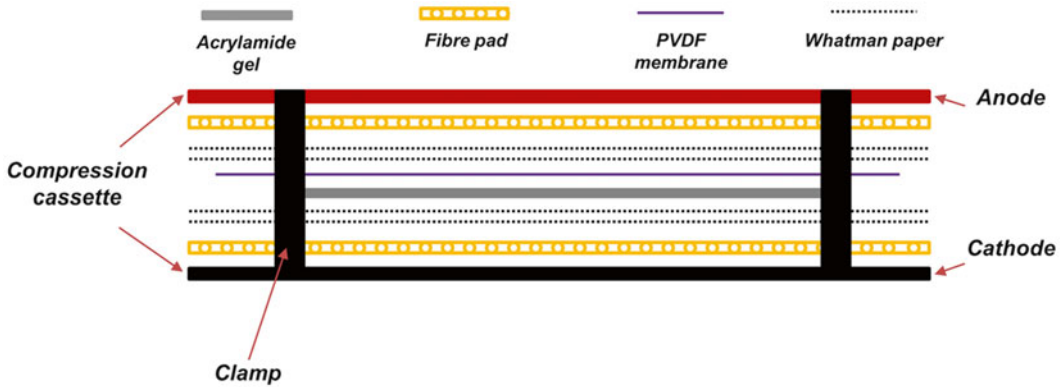
**Fig. 1** Schematic depicting the self-casting gradient mixer system. Both the 15 and 3% acrylamide solutions mix within column A as the pump draws liquid to the gel caster. This produces an acrylamide concentration gradient of 15% at the base of the gel, which decreases to 3% at the top

prepare the stacking gel solution. Pour the stacking gel solution onto the resolving gel and add the comb (*see Note 16*).

6. Thaw the sample aliquots produced in Subheading 3.1 and load equal amounts of protein alongside a standard protein ladder.
7. After loading, electrophorese the samples at a constant voltage of 80–100 V for 20 min. Increase the voltage to 120 V and electrophorese the samples until the dye front has reached the base of the gel (*see Note 17*).
8. Immediately following electrophoresis, carefully separate the glass plates using a spatula or similar tool, leaving one side of the gel still attached to one glass plate. Transfer the gel to a separate container and submerge in transfer buffer for 1 min (*see Note 18*).
9. Cut 4 sections of Whatman paper and 1 section of PVDF membrane to the same size as the gel. Before transfer, activate the PVDF membrane by immersing for 15 s in methanol, followed by a 2 min wash in distilled H<sub>2</sub>O and a final 5 min wash in transfer buffer.

### 3.3 Tank (Wet) Western Blot Electro-Transfer

1. Following protein separation via SDS-PAGE, wet electrotransfer is conducted. An example of the final transfer set up using a compression cassette and fiber pads is shown in Fig. 2.



**Fig. 2** Schematic indicating the Whatman paper, acrylamide gel, and PVDF membrane “sandwich” assembly in the compression cassette. The assembly order is as follows; (1) 1 fiber pad, (2) 2 pieces of transfer buffer-soaked Whatman paper, (3) acrylamide gel, (4) PVDF membrane, (5) 2 pieces of transfer buffer-soaked Whatman paper, (6) 1 fiber pad

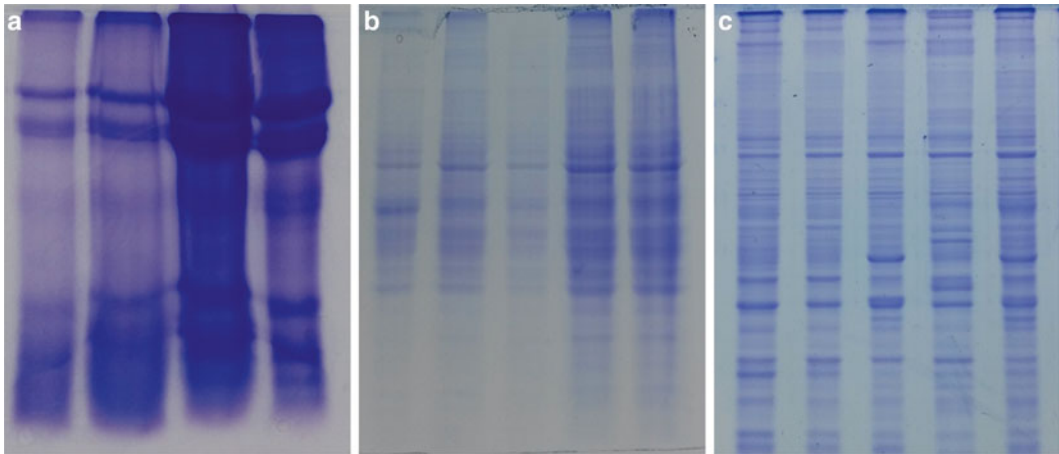
2. Place 1 fiber pad followed by 2 Whatman paper layers, soaked with western blot transfer buffer, in the compression cassette. Then gently place the acrylamide gel across the surface of the Whatman paper (*see Note 18*).
3. Carefully place the PVDF membrane across the surface of the gel (*see Note 19*).
4. Add a further 2 Whatman paper layers soaked in transfer buffer, and the final fiber pad (*see Note 20*).
5. Place the compression cassette into an electrophoretic transfer tank filled with 2 L western blot transfer buffer.
6. For lower molecular weight proteins (<100 kDa), transfer can be conducted for 2 h at a constant voltage of 50 V. For large molecular weight proteins (>250 kDa), transfer is conducted at a constant voltage of 10 V for a minimum of 18 h, followed by a further 2 h at a constant voltage of 45 V (*see Note 21*).

### 3.4 Membrane Blocking and Immuno-Blotting

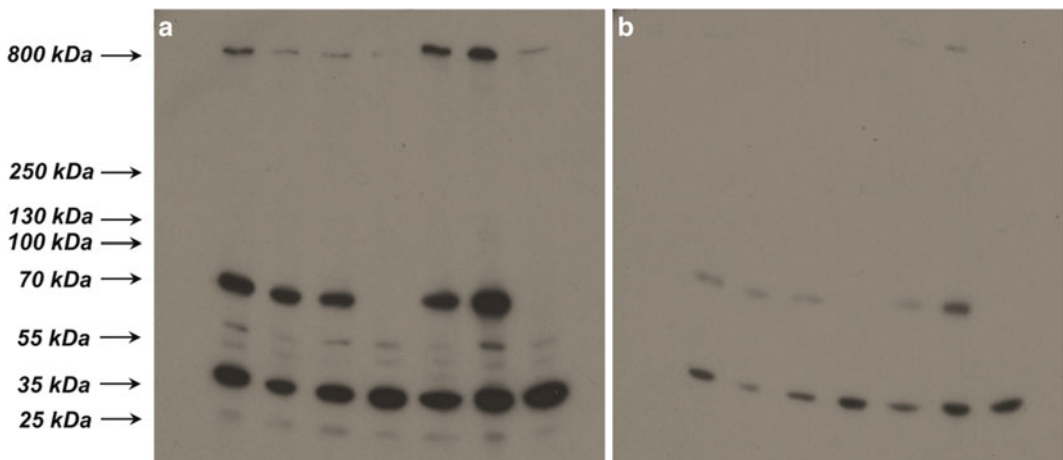
1. Following transfer, disassemble the compression cassette and label the prestained molecular weight standards and gel positioning on the PVDF membrane (*see Note 22*).
2. Wash the PVDF membrane in methanol for 10 s, then sandwich in Whatman paper and leave to dry for 15 min (*see Note 23*).
3. Incubate the membrane in blocking solution for 30 min at ambient temperature or overnight at 4 °C to reduce unspecific antibody binding to the membrane.
4. Rinse the membrane two times for 15 s each time in PBS + 0.1 % Tween-20, followed by a single 5 min wash in PBS + 0.1 % Tween-20.

5. Add primary antibody, diluted in blocking solution, to the membrane. For lower molecular weight proteins of <120 kDa, antibodies are incubated for 1 h at room temperature. However, for larger proteins of >120 kDa, overnight primary antibody incubation at 4 °C using a rocking shaker is required.
6. Wash the membrane three times with PBS + 0.1 % Tween-20, 10 min each time.
7. Dilute the secondary antibody in blocking buffer and incubate for 1 h at room temperature using a rocking shaker.
8. Wash the membrane initially three times with PBS + 0.3 % Tween-20, for 5 min each time.
9. Wash an additional three times using PBS + 0.1 % Tween-20, for 5 min each time (*see Note 24*).
10. Remove the washing solution from the membrane and carefully drain excess liquid with a paper towel (*see Note 25*).
11. Place the freshly made ECL detection solution onto the membrane for 1 min. Remove the excess solution and leave a thin film of ECL solution lining the surface of the membrane.
12. Wrap the membrane carefully within Saran wrap, place it into a standard film cassette, and overlay blot with a photographic film (*see Note 26*). Films should be developed at a range of exposures to ensure adequate documentation.
13. Label the molecular weights of the reference standard on the film.
14. To ensure equal loading has been achieved, standard western blots against GAPDH,  $\beta$ -actin, or  $\beta$ -tubulin are initially performed. At least 2–3 loading control proteins should be assessed as proteome variability between cell lines, and varying cell culture conditions can lead to differential expression between these standards.
15. Protein smearing is an issue which occurs during western blotting and often results from incomplete DNA shearing and lysate homogenization during Subheading 3.1, step 6 in this protocol. Figure 3 displays representative images of variable protein smearing states in which panel (a) displays extreme smearing and protein overloading, while panel (b) displays reduced smearing and unequal total protein loading. The protein smearing effects seen in Fig. 3b can be removed through re-syringing the sample and adjusting the total protein content (Fig. 3c).
16. See pan-nesprin-2 western blot examples in Fig. 4. Panel (a) displays the simultaneous detection of both giant (~800 kDa) and low molecular weight (~25 kDa) nesprin-2 isoforms. The same gel developed at a lower exposure time can be seen in panel (b), highlighting how lower nesprin isoforms vary in their expression relative to nesprin-2 giant.





**Fig. 3** Coomassie blue-stained SDS-PAGE gels, displaying varying protein lysate qualities and quantities. Panel (a) displays protein overloading and lysates containing cell debris. Panel (b) displays less drastic smearing, recoverable through re-syringing and centrifugation. Panel (c) displays optimal experimental conditions



**Fig. 4** Nesprin 2 western blot analysis of various cell lines using polyacrylamide gradient gels, displaying both 2 min (a) and 30 s (b) exposure times. The western blot was executed utilising the pAbK1 anti-nesprin-2 affinity-purified C-terminal polyclonal antibody [17, 18]. Nesprin isoforms between 25 and 800 kDa are attained, displaying dissimilar expression patterns. Our detection method therefore provides increased accuracy when assessing isoform variability across broad molecular weight ranges. The lanes represent samples from the following cell lines: HeLa (cervical cancer), SW620 and SW480 (colon adenocarcinoma), MDA-MB-231 and MCF7 (breast cancer epithelia), HaCaT (immortalized keratinocytes), and COS7 (African green monkey fibroblast-like kidney cells)

## 4 Notes

1. Once prepared, the RIPA (RadioImmuno-Precipitation Assay) buffer must be protected from light. Leave one 10 mL aliquot on ice for immediate use, and store remaining aliquots at  $-20^{\circ}\text{C}$ .

2. One aliquot should remain at ambient temperature for immediate use and remaining aliquots should be stored at  $-20\text{ }^{\circ}\text{C}$ . SDS precipitates at  $4\text{ }^{\circ}\text{C}$ , so Laemmli buffer must always be warmed prior use.
3. 1.5 mm spacers are preferred over thinner spacers for the detection of large molecular weight proteins.
4. Acrylamide solution used contains 30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide stock (37.5:1).
5. For the specific detection of nesprin proteins, PVDF membranes are preferred over nitrocellulose due to their increased protein-binding affinity and re-probing potential.
6. Care should be taken not to remove the cell culture flask from the ice while scraping cells from the surface. Ensure all cells are removed from the surface of the cell culture flask as if too few cells are dislodged, the overall protein concentration may be too low for successful detection. Roughly 2 min of thorough scraping is enough to achieve complete removal of cells. As a rough estimate, cell debris should be seen within the lysis buffer when tilted at  $45^{\circ}$ .
7. Draw the lysis solution into the syringe before the needle tip is attached. Further care should be taken during the first few syringing-steps; the high viscosity of the protein solution can result in high pressure, causing the hypodermic needle to fall off the syringe, resulting in sample loss.
8. Care should be taken not to disturb the pellet. If a distinct pellet is not seen, re-centrifuge the sample to ensure complete separation.
9. Seal the Eppendorf tubes securely with a locking clip during boiling to prevent sample loss.
10. Continual freeze/thaw cycles will compromise lysate quality; ensure enough sample aliquots are produced at this stage in order to avoid this.
11. Coomassie blue staining provides an early indication of total protein quantity and lysate quality, as cellular debris, unsheared genomic DNA, and pellet fragments can be detected as smears. If smears do appear, samples can be re-syringed and/or sonicated prior centrifugation at  $12,000\times g$  for 15 min.
12. Spacers, glass, and aluminium back plates should be thoroughly cleaned with ethanol and dried before use. Residual dust and dirt can cause gels to stick to the plates, making it difficult to remove the gels for transferring. Use flawless plates and ensure proper alignment.
13. When mixing APS and TEMED with the acrylamide stocks, ensure the solution is mixed thoroughly so that a uniformed gel is produced. If bubbles appear, gently tap the solution to remove them.

14. Stirrer and peristaltic pump speeds should be kept to a minimum. Increasing both speeds can cause the two acrylamide solutions to mix too quickly, compromising the quality of the gradient gel.
15. Ensure the isobutanol is evenly distributed across the surface of the gel.
16. Mix the solution quickly as the mixture sets rapidly. As the comb is inserted, take care that no bubbles form across the bottom of the wells as this can result in unequal electrophoretic migration of the proteins within the resolving gel.
17. The lower voltage used initially helps to ensure all proteins within the sample collect together at the bottom of the wells, allowing proper protein separation to occur in the resolving gel.
18. Care must be taken not to distort or break the acrylamide gel.
19. It is best to correctly position the PVDF membrane onto the gel on the first attempt.
20. It is important that no bubbles form between the gel and PVDF membrane, as this will impair protein transfer. To avoid this, use a 10 mL pipette to gently roll over the Whatman paper surface.
21. The tank can overheat during overnight transfers, resulting in distorted gel morphology, and hence, an abnormal western blot pattern (*see* Fig. 4, nesprin-2 low molecular weight isoform “smiling” effects). To counteract this, the blotting procedure should be performed in a cold room at 4 °C.
22. Noting ladder marker positioning allows accurate molecular weights to be assigned to each western blot signal after the procedure is completed.
23. At this stage, the membrane can be left for several days within the Whatman paper at 4 °C without further processing.
24. If the western blot analysis yields high background, increase the washing times and the PBS + 0.3 % Tween-20 washing steps.
25. It is important to ensure that the membrane is not completely dried at this stage. Only the excess washing solution should be removed from the membrane.
26. The Saran wrap helps ensure that the membrane doesn't dry out during the final western blot stages, as some proteins will require film exposures of up to 30–45 min.

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