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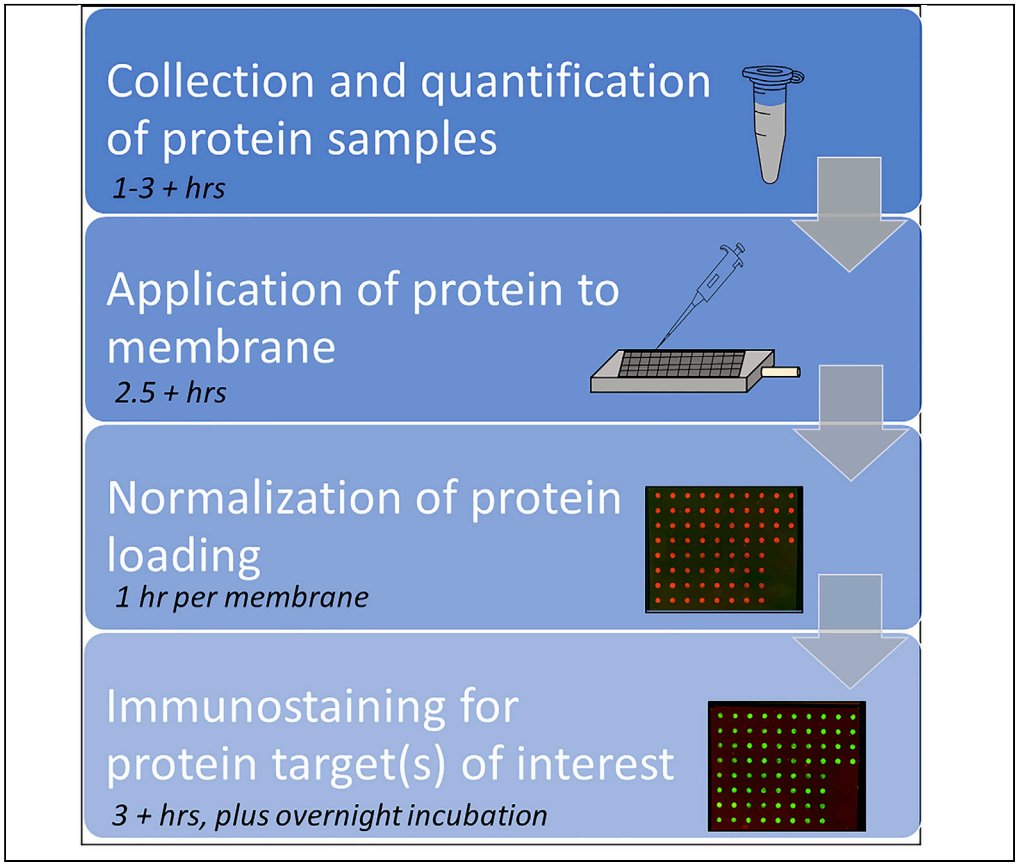
Protocol for High-Throughput Screening of Neural Cell or Brain Tissue Protein Using a Dot-Blot Technique with Near-Infrared Imaging



This protocol describes the preparation of protein fractions from brain regions or neural cell cultures for the high-throughput quantification of target proteins using a dot-blot technique with near-infrared detection. This method also allows for the simultaneous detection of two different protein targets of interest, in addition to their normalization with total protein.

Anna C. Chlebowski, Glen E. Kisby

achlebowski@westernu.edu (A.C.C.)
gkisby@westernu.edu (G.E.K.)

HIGHLIGHTS

Efficient preparation of protein samples from brain regions or neural cell cultures

Normalization of the same membrane as antibody detection for increased accuracy

Simultaneous detection of two protein targets and their normalization

Detection of near-infrared wavelength antibodies for increased sensitivity

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Protocol for High-Throughput Screening of Neural Cell or Brain Tissue Protein Using a Dot-Blot Technique with Near-Infrared Imaging

Anna C. Chlebowski[1,2,*] and Glen E. Kisby[1,3,*]

[1]Western University of Health Sciences College of Osteopathic Medicine of the Pacific- Northwest, Lebanon, OR 97355, USA

[2]Technical Contact

[3]Lead Contact

*Correspondence: achlebowski@westernu.edu (A.C.C.), gkisby@westernu.edu (G.E.K.)
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SUMMARY

Dot blotting allows for the rapid screening of a larger number of samples and/or targets than more traditional methods, such as a western blot or in-tissue-based methods. We have developed a dot-blot assay specifically for use with a LiCor Odyssey CLx imager, which allows for sensitive detection of proteins in the infrared range. Here, we provide a detailed protocol for the preparation of brain tissue and neural cell culture lysates for analysis of protein targets by dot blotting.

BEFORE YOU BEGIN

Option 1: Cell Collection and Preparation

Timing: 1+ h, depending on number of samples

1. Cultivate cell type as appropriate. Further details on basic cell culture should be sought elsewhere.
2. Once cell cultures are ready for collection (80%–90% confluency), media over the cells is aspirated, accutase (STEMCELL™ Technologies or Sigma) is then added and the cells treated for 5 min in a CO_2 incubator at 37°C.
   a. Check for detachment by light microscopy after 5 min and incubate for an additional 5 min for adequate detachment (*if necessary*).
   b. 1 mL of accutase is sufficient for one well of a 6-well plate or 500 μL/well of a 12-well plate.
   c. Other dissociation methods can be used as appropriate for cell type and preferred culture protocols.
3. Using a pipette tip, transfer the detached cells into a microcentrifuge tube (1.7 mL or larger, depending on size of well plate).
4. Using a fresh pipette tip, rinse each well with the same volume of cell culture media or DMEM (500 μL/12-well plate, 1 mL/6-well plate) and then add it to the tube containing the detached cells. Repeat for all sample wells.
5. Centrifuge the tubes at 750 × *g* for 5 min at 20°C–22°C (room temperature).
6. Carefully aspirate all media over the cell pellet and then transfer tubes to an ice bucket.
   a. Cell pellets can be washed with PBS prior to snap-freezing. Care should be taken to not agitate or aspirate the cell pellet. This step is routinely omitted when the cell pellet is small, to minimize loss of material.

Pause Point: At this point, cell pellets can be snap-frozen in liquid nitrogen and stored at −80°C.

7. Resuspend cell pellet in 25 μL of cOmplete™ Mini EDTA-Free Protease Inhibitor cocktail (Roche).
   a. To make the protease inhibitor cocktail, dissolve 1 cOmplete™ Mini EDTA-free tablet in 10 mL PBS (pH 7.4) and mix well.
8. Homogenize the samples on ice by sonication (we use a Qsonica, Newton, CT), to obtain a homogeneous solution.
   a. Program used: Pulse 1 s on/5 s off at 20% amp.
   b. Typically 2–4 pulses is adequate to achieve homogenization. Monitor for a uniformly turbid solution and no visible cell clumps.
   c. Place the microcentrifuge tube in a 50 mL conical tube containing ice to prevent overheating of samples during sonication.
   d. Wash sonicator tip with ddH_2O before starting, between samples, and after sonication.
   e. Store cell homogenates on ice.

Note: Wear hearing protection as appropriate for the sonication instrument used.

9. Centrifuge cell homogenates for 1 min at 750 × *g*.
10. Transfer the supernatant to a clean microcentrifuge tube and store the remaining insoluble material at −80°C.
11. Bring final volume of the supernatant to 150 μL with PBS.

Note: Final volume of 150 μL was appropriate for the amount of protein collected from 200,000 or more neural stem cells (NPCs). Smaller numbers of cells may warrant a smaller final volume. Likewise, a larger volume or additional dilutions may be required for samples with a higher cell count and/or protein yield.

12. Diluted cell supernatants are stored at −80°C until protein quantification.

Pause Point: Samples can be stored at −80°C before and/or after protein quantification. However, we recommend analyzing supernatants after centrifugation to minimize freeze-thaw effects on samples.

Option 2: Brain Tissue Preparation

Timing: 3+ h, depending on number of samples

13. Freshly frozen brain tissue is placed in a microcentrifuge tube (previously weighed) and then immediately placed on dry ice. The tube containing the brain tissue is weighed and the wet weight determined. All tubes are kept on ice during this procedure.

Note: Murine hemi-brains were dissected on ice into cortex, hippocampus, and cerebellum and each region placed in separate tubes before processing the tissue samples.

14. Add extraction buffer to each sample tube containing tissue (see recipe for extraction buffer in Table 1).

Note: The amount of extraction buffer depends on the brain region: 500 μL for cortex, 200 μL for hippocampus, and 50 μL for cerebellum from the hemi-brain of an adult mouse. Volumes may need to be adjusted for other regions.

Table 1. Extraction Buffer Recipe for the Extraction of Proteins from Brain Tissue

Reagent	Final Concentration	Amount
Tris HCl (100 mM) pH 7.8	10 mM	1.0 mL
Dithiothreitol (DTT) (50 mM)	0.5 mM	0.1 mL
$MgCl_2$ (50 mM)	5.0 mM	1.0 mL
Adenosine triphosphate (ATP)	3.08 mg/mL	30.8 mg
cOmplete™ Mini Protease inhibitor, EDTA-free	1×	1 tablet
ddH_2O	n/a	7.8
Total	**n/a**	**10.0**

15. Homogenize the tissue on ice by sonication (Qsonica, Newton, CT), to obtain a uniform turbid solution.
    a. Program used: Pulse 1 s on/5 s off at 30% amp.
    b. 2–10 pulses with the sonicator may be required, depending on the amount and type of tissue used. Monitor for a uniformly turbid solution and no visible tissue chunks.
    c. Place the microcentrifuge tube in a 50 mL conical tube containing ice to prevent overheating of samples during sonication.
    d. Wash sonicator tip with ddH_2O before starting, between samples, and after sonication.
    e. Store cell homogenates on ice.
16. After sonication, place the tissue homogenates on ice for 30 min to lyse the tissue.
17. Transfer tissue lysates to a new 1.7 mL microcentrifuge tube (previously weighed). Weigh tube to determine the wet brain tissue weight.
18. Centrifuge the lysates at 15,000 × *g* for 90 min at 4°C.
19. Transfer the supernatant into a new tube (soluble proteins). The pellet (insoluble fraction) is stored at −80°C until use.
20. Supernatants are stored at −80°C until protein quantification.
    a. Use excess extraction buffer as diluent for protein quantification technique of choice.

Pause Point: Supernatants can be stored at −80°C before and/or after protein quantification. However, we recommend analyzing supernatants after centrifugation to minimize freeze-thaw effects on samples.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
IRDye® 700 and 800 Secondary antibodies	LiCor	Species specific, see https://www.licor.com/bio/reagents/irdye-secondary-antibodies for options
Chemicals, Peptides, and Recombinant Proteins		
Intercept® (TBS) Blocking Buffer	LiCor	Cat# 927-60001
Tris-buffered saline (TBS)	ThermoFisher	Cat# 28358
Tween®-20	Sigma Aldrich	Cat# P9416
Tris HCl (100 mM) pH 7.8	MP Biomedicals	Cat# 816124
Dithiothreitol (DTT) (50 mM)	Sigma Aldrich	Cat# D-0632
$MgCl_2$ (50 mM)	Sigma Aldrich	Cat# M8266
Adenosine triphosphate (ATP)	Sigma Aldrich	Cat# A6419
cOmplete™ Mini Protease inhibitor, EDTA-free®	Roche/Sigma Aldrich	Cat# 11836170001

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Revert® 700 Total Protein Stain Kit	LiCor	Cat# 926-11010
Software and Algorithms		
Image Studio™ software	LiCor	n/a
Other		
0.45 μM nitrocellulose membrane	Bio-Rad	Cat# 1620115
Bio-Dot™ Microfiltration System	Bio-Rad	Cat# 1703938
LiCor Odyssey CLx imager	LiCor	Model 9140
Centrifuge	n/a	n/a

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr Glen Kisby (gkisby@westernu.edu).

Materials Availability

This study did not generate any unique materials or reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

MATERIALS AND EQUIPMENT

Primary antibodies as appropriate for sample/targets of interest should be selected, and the appropriate IRDye® Secondary antibodies (LiCor) selected. IR-wavelength secondary antibodies are also available from other vendors and could potentially be utilized as well.

For generation of neural stem cells and neurons, iPSCs (cell line ND50031 from RUCDR Infinite Biologics) were differentiated using protocols commercially available from STEMCELL™ Technologies. Similar cells can be purchased commercially or derived through other differentiation processes.

Tris-buffered saline (TBS) and TBS + 0.1% Tween-20 (TBST) are required. These can be made in-house, or purchased commercially (as listed in the Key Resources Table). This buffer system is for the extraction of soluble native proteins from cells or tissues. If analysis of other protein fractions is desired, alternate buffer formulations can be used as appropriate. 1x TBS should contain 25 mM Tris and 0.15 mM NaCl, buffered to pH 7.6.

The Bradford Assay (Bio-Rad, Cat# 5000201) can be used for protein quantification, but any other similar method could also be used.

The Bio-Dot® Microfiltration Apparatus (Bio-Rad) is made of polysulfone with a silicone gasket, designed for the application of samples to a membrane using vacuum. Adapters are available for the application of either 96 "dots" in a 12x8 array, or as rectangular "slots" in a 6x8 array. The current protocol is designed for use with the 96 "dot" array, but could be modified for the slot array (Bronowicka-Szydełko et al., 2020). Details on setup and cleaning of the apparatus can be found in the product manual (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1706545.pdf). Similar vacuum apparatuses are available through other manufacturers (Guillemin et al., 2009), the specific user manuals for these should be referenced for any necessary modifications.

Alternatively, dots can be applied freehand, as has been published by other laboratories (Au - Chunhui et al., 2018, Tizon et al., 2010). In this case, the user should take care to keep the membrane as clean as possible (the infrared detection system is more sensitive to background contamination than other methods such as chemiluminescence (Schutz-Geschwender et al., 2004)). A template, such as a pipette tip rack, would be helpful for aligning the dots on the membrane in a uniform manner, as analysis in the ImageStudio™ software can be done using a 96-well template rather than individual selection of each dot (Wehr and Levine, 2012).

Note: PVDF membranes are not recommended for use with the Bio-Dot® Microfiltration Apparatus, as per the product manual. Specific inquiries regarding membrane types other than nitrocellulose should be investigated with the manufacturer.

⚠ CRITICAL: Methanol (CAS# 67-56-1) is required for the Revert™ 700 Total Protein Stain Kit. Avoid direct skin contact and inhalation of vapors of methanol and Revert™ Kit reagents. We recommend keeping the kit reagents contained in a chemical safety hood or a closed container. Use appropriate personal protective equipment (gloves, eye protection) when handling these reagents.

⚠ CRITICAL: The Revert™ 700 Total Protein Stain Kit components contain acetic acid (CAS# 64-19-7) and sodium hydroxide (CAS# 1310-73-2). Avoid contact with skin, eyes, and inhalation of vapors. We recommend keeping the kit reagents in a chemical safety hood or a closed container. Review the SDS document for these reagents before use. Use appropriate personal protective equipment (gloves, eye protection) when handling of these reagents.

STEP-BY-STEP METHOD DETAILS

Sample Loading onto Membranes

Timing: 2.5+ h/membrane, plus thawing samples

Following protein determination, samples are loaded on a nitrocellulose membrane by manual pipetting and allowed to dry thoroughly prior to staining. Careful dilution of samples and application to the membrane is important for uniform results. The use of a 96-well dot array allows for high-throughput determination of a larger number of samples (96 individual samples, or 48 samples analyzed in duplicate).

1. Thaw protein samples on ice. Quantify using a Bradford Assay or similar technique for total protein quantification.
2. Dilute the protein sample before applying to the membrane.
   a. Each sample must be diluted to a final concentration of 1 μg of protein in a total volume of 10 μL (see Troubleshooting section for method optimization and selection of protein loading amount).
   b. Store the diluted samples on ice before loading on the membrane.

Note: We recommend replicates of each sample be applied to the membrane for greater accuracy. We also suggest making 5–10 μL extra of each sample solution before application to ensure an adequate volume for all dots.

3. Using scissors cleaned with alcohol, cut a nitrocellulose membrane (0.45 μm) to size for the required number of samples to be dotted.
4. Place the cut membrane(s) in a container with a lid (e.g., clean pipette tip box) and cover them with TBS.

5. Allow the membrane(s) to rehydrate for a minimum of 10 min before applying samples.

Note: Multiple membranes can be placed in the same box for rehydration with TBS.

6. Place a pre-wetted membrane in the 96-well Bio-Dot® Microfiltration Apparatus (Bio-Rad) according to the manufacturer's instructions. If less than 96 samples are applied to a membrane, cover any un-used wells with tape and seal well.
7. Connect the vacuum line and leave it open to the atmosphere during sample application.

Note: When setting up the Bio-Dot® Apparatus, the membrane should be marked to keep track of the orientation of samples. A notch or small hole(s) made with a clean tweezer along the edge of the membrane is ideal.

8. Add 100 μL TBS to each well of the membrane in the Bio-Dot® apparatus.
9. Turn on the vacuum, and with a gloved finger, gently block the vacuum line (open to the atmosphere) to slowly increase the vacuum on the Bio-Dot® apparatus. Make sure that the TBS is vacuumed thoroughly before applying samples. Detailed information for the operation of the vacuum valve of the apparatus can be found in the product manual.
10. Add 10 μL of sample to each well of the membrane. Take care not to puncture the membrane during application with a pipette tip. Replace the pipette tip between each sample.
11. After all samples are loaded on the membrane, cover the Bio-Dot® apparatus to prevent dust or dirt contamination (foil or a pipette lid box works well). Allow the apparatus to sit for a minimum of 1 h without vacuum to allow protein samples to bind.
12. After 1 h, turn on the vacuum and using a gloved finger to block the vacuum line (open to the atmosphere) to gently pull any remaining sample through the membrane.
13. Use a pair of clean tweezers to remove the membrane from the apparatus. Place the clean membrane on an elevated surface to dry for a minimum of 1 h.

Note: We suggest placing the membrane on the hinge of a clean pipette tip box (with lid) to allow optimal air flow and then the membrane placed in a laminar flow hood to prevent contamination from dust or dirt.

14. After the membrane is dried, it can be stained with Revert® 700 Total Protein Stain or it can be stored for up to one week at 4°C.

Pause Point: For long term storage, dotted membranes can be stored at 4°C for up to one week prior to staining and immunoprobing.

Revert® 700 Total Protein Stain

Timing: 1 h per membrane

The Revert® 700 Total Protein Stain (LiCor) allows for the normalization of the protein dotted on each well of the membrane. The final antibody signal is normalized against the Revert® signal to allow for more accurate quantification of total amount of applied protein and allows for the simultaneous detection of two protein targets. Re-imaging of the membrane after removing the Revert® stain accounts for residual background, especially when using two antibodies for a sample, or for detection of low-abundance proteins.

Important: Once the Revert® staining of the membrane is started, you must continue through each of the steps to completion (i.e., incubation with antibodies and imaging). Repeated drying of the membrane or storage in buffer for an extended period of time is not recommended.

15. Place each membrane in a clean, small container with a lid to allow the membrane/solution to gently move on a rocker. Large containers will require excess reagents (e.g., Revert®, antibodies).

Note: For large membranes (e.g., >48 dots), a clean pipette tip box with lid works well. Small membranes (<16 dots) can be stained or immunoprobed in sterile petri dishes (>35 mm). Boxes designed for incubating western blots are suitable for medium-size membranes (16–48 dots). Volumes need to be adjusted for the type of container (e.g., a pipette tip box or container of similar size) used for the membrane.

Note: LiCor does not recommend placing multiple membranes in a container for staining with Revert® or antibodies.

16. Add methanol to Revert® kit reagents as described by the manufacturer. Appropriate volumes to add are indicated for each reagent.

Note: Revert® 700 Total Protein Stain kit reagents need to be kept in a chemical safety hood when open and the waste disposed to minimize exposure to fumes.

17. Rinse membrane briefly with 15 mL ultrapure water before staining.
18. Add 5–10 mL (enough volume to just cover the membrane) Revert® 700 Total Protein Stain to the membrane and incubate for 5 min with gentle rocking. Keep container lid closed or if possible, place the rocker in a chemical safety hood to reduce exposure to fumes. Blue dots should become visible for protein samples that were applied to the membrane.

Note: Lack of visible blue dots could indicate issues with protein application to the membrane.

19. Decant the Revert® stain into a waste container.
20. Add 10–15 mL of Revert® Wash solution to the membrane and incubate for 30–60 s with gentle shaking/agitation.
21. Decant the wash solution into a waste container and wash the membrane for 30–60 s in 15 mL ultrapure water. Decant wash solution into a waste container.
22. Immediately after staining, the membrane(s) are imaged on a LiCor Odyssey® CLx near IR imager. Recommended scan settings are: medium scan quality, 84 μm resolution, and a 0.0 mm offset.
    a. For a faster run time, a resolution of 169 μm can be used instead of 84 μm. We prefer the higher resolution and slightly longer run time for better dot visualization.
    b. Use a silicone mat and roller (LiCor) to remove any bubbles or liquid on the membrane.
    c. Intensities and contrast values for both channels can be optimized for each image/experiment without impacting quantification.
    d. See Troubleshooting section for more information on problems with the Revert® stain.

Note: Clean imaging surface with alcohol prior to and after imaging the membrane.

23. After imaging the stained membrane, add 5–10 mL of the Revert® 700 Destain solution and incubate for 5–7 min at 20°C–22°C with gentle rocking. The visible blue dots from the Revert® stain should rapidly diminish and disappear during destaining.
24. Decant the Revert® Destain solution and briefly rinse the membrane with 15 mL ultrapure water.
25. Re-image the destained membrane using the same imaging parameters (see step 22).
26. After imaging the membrane, immediately block the membrane and incubate with primary antibody(s).

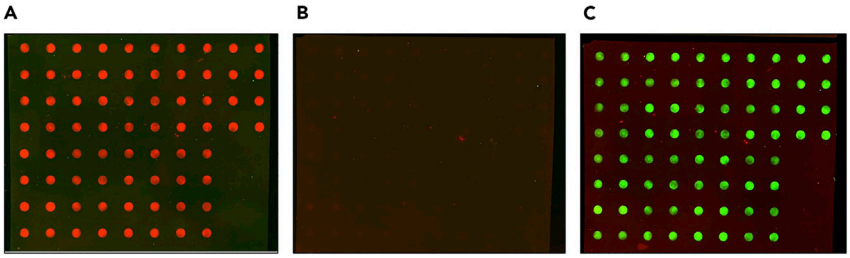

Figure 1. Representative Images at Each Critical Step of the Dot Blot Assay

A membrane was dotted with neuronal cell lysates (1 μg protein/dot) and imaged following staining with Revert® 700 Total protein stain (shown red) (A), after removal of the Revert® stain (B), and after incubation with primary and secondary antibodies (shown green) (C). Note the intensity of the dots following staining with Revert® are relatively uniform indicating that samples were accurately applied to the membrane. Primary antibody used was mouse-anti Map2 (Invitrogen, MA5-12826) at 1:1,000 dilution. Secondary antibody was goat-anti-mouse IRDye® 800 at a 1:10,000 dilution (LiCor). The quantification of these membranes and an example workflow is demonstrated in Table 2.

Incubation with Antibodies

Timing: 1 h, ~16–24 h overnight incubation, 2+ h next day (+30 min/membrane)

Following incubation with blocking buffer, the membrane(s) is incubated sequentially with a primary antibody and then an IRDye® conjugated secondary antibody for detection at 800 nm. If two targets are desired, the secondary antibody to the more-abundant protein is detected at 700 nm to minimize interference from residual Revert® stain (see Troubleshooting section).

27. Incubate membrane(s) with 10 mL Intercept® (TBS) Blocking Buffer (LiCOr) for 45–60 min at 20°C–22°C with gentle rocking.
28. Decant the blocking buffer and add the primary antibody solution to the membrane.
29. Incubate the membrane 16–24 h at 4°C with gentle rocking.
    a. Primary antibodies should be made in Intercept® blocking buffer:TBS with 0.01% Tween-20 (50:50).
    b. If detection of two protein targets is desired, both primary antibodies can be applied and incubated simultaneously.

Note: Dilutions of each antibody should be determined by user. Suggested dilutions for western blotting are often appropriate, but the user should consult the data sheets for each antibody.

Optional: Primary and secondary antibodies can also be diluted in 100% Intercept® (TBS) Blocking Buffer, but we recommend diluting the Intercept® buffer to minimize reagent use.

30. Decant the primary antibody solution and wash the membrane successively 3 × 10 min with 15 mL of TBS +0.1% Tween-20 (TBST) at 20°C–22°C with gentle rocking.

Note: Primary antibody can be saved and re-used depending on dilution concentration and protein abundance, ideally within a few days to minimize antibody degradation or bacterial contamination of the buffer.

31. Add the secondary antibody to the immunoprobed membrane and incubate for 1 h at 20°C–22°C.
    a. Dilute the IRDye® secondary antibody(s) in Intercept blocking buffer: TBS with 0.01% Tween-20 (50:50) to a final concentration of 1:10,000.

Table 2. Example of Data Quantification and Analysis

700 nm Revert® signal									
39325.63	47854.84	43274.98	43533.29	41409.51	38262.79	39320.73	40580.71	48671.24	48028.9
40101.15	40803.05	43633.96	46954.63	41187.27	39185.38	41820.01	44945.08	51517.1	49714.33
39130.48	39431.51	41083.66	41974.86	36102.52	35537.79	41391.7	40769.94	44075.72	45503.02
42817.7	36250.27	36829.37	38899.21	33379.94	34572.8	44005.65	40887.78	44576.13	46629.25
46787.11	40068.7	32025.12	32182.57	32564.8	35213.75	34041.37	35230.14		
43513.12	38902.95	34201.8	33869.79	38262.18	35114.08	35140.95	35699.27		
79585.73	67318.86	36241.09	37316.02	45459.24	41305.18	33846.3	33753.52		
45646.42	43645.91	41595.6	43134.48	43042.87	41227.75	40312.23	39034.98		
800 nm Revert® signal after destain									
542.3281	546.135	541.2319	537.6279	534.8091	533.4812	528.686	530.0818	530.0691	530.1543
537.7957	537.7644	536.2605	534.9106	532.8384	530.3384	527.7983	528.7622	530.957	527.2891
539.6426	540.4126	537.0103	534.4797	534.1409	533.1497	527.7388	527.1843	525.5657	524.0273
536.2266	530.8677	530.1199	528.6519	525.6045	525.4082	526.0254	526.3159	525.0593	525.4895
538.4771	534.8564	534.2458	530.8733	530.2805	528.0095	524.8201	537.4849		
540.7908	540.9287	538.4631	534.0232	531.7529	529.0176	528.0061	526.1147		
549.5657	550.1379	537.6619	538.8743	535.8604	532.7209	531.6387	533.2056		
540.8818	541.7168	542.0713	539.4387	533.8689	532.4343	531.7932	530.7268		
800 nm antibody signal									
1974.495	2255.098	2605.119	2595.216	2381.56	2364.609	2441.422	2555.386	2762.412	2753.495
1860.999	1983.541	1901.76	1943.954	2120.027	2118.661	2808.831	2844.216	2647.968	2515.487
1698.695	1923.18	2825.785	2832.818	2055.783	2037.267	2011.492	2147.915	2644.119	2452.068
1832.993	1878.122	2455.607	2695.125	1832.613	1865.734	2672.099	2579.503	3100.659	3056.216
1939.647	2147.758	1865.78	1928.732	2486.749	2602.941	1776.811	1739.599		
2315.652	2437.313	2128.267	2018.61	2123.796	2142.003	2748.91	2618.123		
3267.77	2896.804	1858.057	1969.232	2846.689	2698.491	2026.776	1921.47		
2065.46	1988.486	2292.146	2388.771	2870.855	2895.53	2051.392	2357.373		
(800 nm antibody signal) minus (700 nm Revert®destain signal)									
1432.167	1708.963	2063.887	2057.588	1846.75	1831.128	1912.736	2025.304	2232.343	2223.34
1323.203	1445.776	1365.5	1409.043	1587.188	1588.322	2281.033	2315.454	2117.011	1988.198
1159.053	1382.768	2288.774	2298.338	1521.642	1504.117	1483.753	1620.731	2118.553	1928.041
1296.767	1347.254	1925.487	2166.473	1307.009	1340.326	2146.074	2053.187	2575.6	2530.726
1401.17	1612.901	1331.534	1397.859	1956.468	2074.931	1251.99	1202.114		
1774.862	1896.384	1589.804	1484.587	1592.043	1612.986	2220.904	2092.008		
2718.204	2346.666	1320.395	1430.358	2310.829	2165.77	1495.138	1388.265		
1524.578	1446.77	1750.075	1849.332	2336.987	2363.095	1519.598	1826.646		
[(800 nm antibody signal) minus (700 nm Revert®destain signal)] / (Revert® 700 nm signal)									
0.036418	0.035711	0.047692	0.047265	0.044597	0.047857	0.048644	0.049908	0.045866	0.046292
0.032997	0.035433	0.031294	0.030009	0.038536	0.040534	0.054544	0.051517	0.041093	0.039992
0.02962	0.035068	0.05571	0.054755	0.042148	0.042324	0.035847	0.039753	0.048066	0.042372
0.030286	0.037165	0.052281	0.055695	0.039156	0.038768	0.048768	0.050215	0.05778	0.054273
0.029948	0.040253	0.041578	0.043435	0.060079	0.058924	0.036779	0.034122		

0.040789	0.048747	0.046483	0.043832	0.041609	0.045936	0.0632	0.058601		
0.034154	0.034859	0.036434	0.038331	0.050833	0.052433	0.044174	0.041129		
0.0334	0.033148	0.042074	0.042874	0.054294	0.057318	0.037696	0.046795		

Quantification of the membranes shown in Figure 2, with the suggested flow for analysis. Final consolidation of replicates and statistical analysis will depend on the particular experimental design. Values in the table are oriented the same as the images of the membranes. Quantification of the images is copied from the exported Excel files following image acquisition, and consolidated to a single spreadsheet for analysis.

b. If doing two protein targets, incubation with both secondary antibodies can be done simultaneously.

Note: Membranes must be kept in the dark during steps 31 through the imaging step. We recommend covering the container with foil if it is not opaque.

Note: For the detection of one protein, we recommend using the 800 nm channel for best sensitivity. For the detection of two proteins, we recommend using the more-abundant protein for the 700 nm channel. Additional support can be found for different applications can be found on the LiCor website.

32. Decant the secondary antibody solution and wash the membrane 2 × 10 min each with 15 mL TBST, and 1 × 15 min with 15 mL TBS.
33. Re-image membrane using the same imaging parameters (see step 22).

EXPECTED OUTCOMES

Using either protein extraction method should yield solutions with quantifiable amounts of protein. The user may need to adjust the volumes if substantially larger or smaller amounts of cells/tissues need to be used. Examples of protein amounts from cell cultures are 50–100 μg from 2 × 10^5 hNPCs, 150–300 μg from 2 × 10^5 hNPCs differentiated into neurons for 28 days, and 15–25 μg protein from

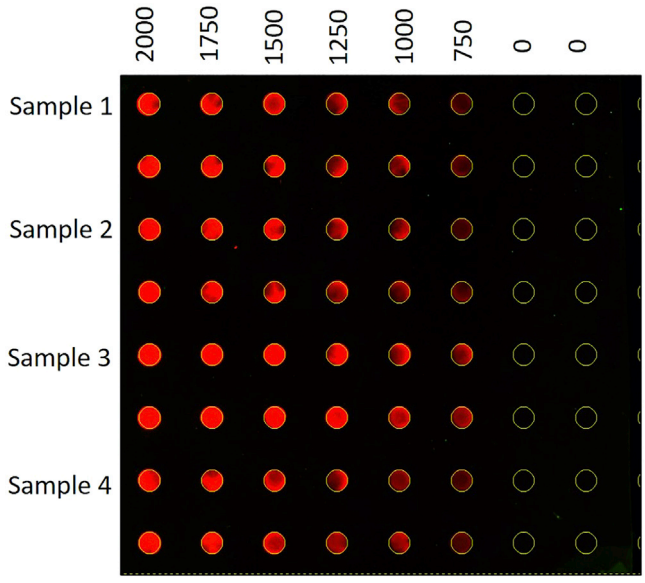

Figure 2. Optimal Application of Samples for Accurate Quantification

Following incubation with the Revert® 700 Total Protein Stain (red) and quantification using Image Studio™, dots are selected for quantification (yellow circles) to designate protein signal from background. The membrane was dotted with a dilution series of murine cerebellar extracts (note that protein concentrations for sample 3 are slightly higher). Note the uniform intensities of dots in each column (same amount of protein/dot).

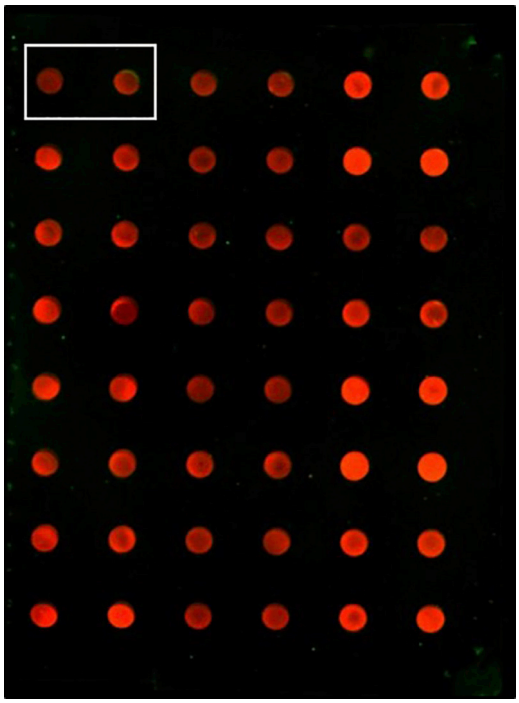

Figure 3. Poor Sample Loading Leads to Inconsistent Revert® Staining

Each dot applied to the membrane was thought to contain 1 μg protein, based on previous quantification of murine brain region tissue extracts. Imaging of the Revert® stained membrane revealed variable intensities among samples. Note that adjacent technical replicates (white box) of the samples was relatively uniform indicating that the samples were incorrectly quantified. Re-quantification of the samples is required prior to their application to a new membrane.

1×10^5 mature astrocytes. From dissected hemi-brains of adult mice, protein amounts averaged 2–3 mg from the cortex, 400–600 μg from the hippocampus, and 1–2 mg from the cerebellum. Optimization of tissue/cell preparation protocols may be necessary for other cell/tissue types and should be optimized by the user.

Following completion of this protocol for determination of proteins, the membrane will have been quantified three times: first with Revert® Total Protein Stain for normalization, second to account for residual Revert® background, and third for quantification of protein target(s). Representative images of these three critical steps are shown in Figure 1. Analysis of these three images is described in the Quantification and Statistical Analysis section, and is shown in Table 2.

Expected signal intensities following Revert® staining are in the 40–100K range, for 1 μg protein dotted. Following Revert® destain, values should drop to below 5,000. Signals following antibody staining will be dependent on the individual target of interest, but signals in the 1,000–6,000 range were common.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of the scanned images was done using Image Studio™ software from LiCor. Each sample dot is encircled (this can be done using a multiwell plate template provided in the ImageStudio™ software) to designate sample area from background (Figure 2), and the data exported as an Excel spreadsheet. The LiCor website (https://www.licor.com/bio/support/) provides tutorials on the use of this software for image analysis and quantification of stained or immunoprobed membranes.

Data is presented as (antibody signal - residual Revert® signal)/(Revert® signal) for each dot. An example of the workflow for analysis is shown in Table 2. Values from analysis of membranes will be assay-dependent, examples of expected quantification values are given in the Expected Outcomes section. While the analysis can be completed without the additional imaging step following Revert® destain, this additional quantification allows for more accurate background subtraction, especially if quantification of a protein target in the 700 nm channel is desired.

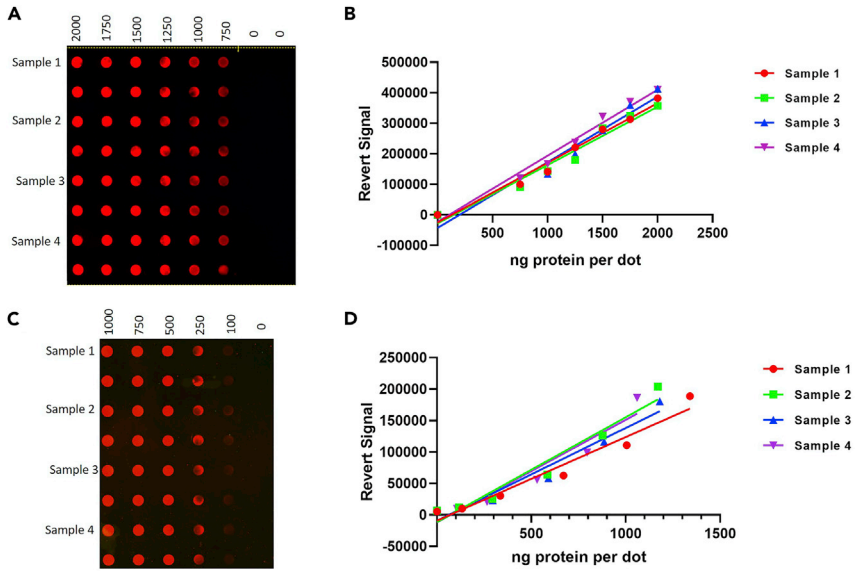

Figure 4. Determination of Revert® 700 Total Protein Stain Signal Linearity

Protein homogenates from the cerebral cortex of adult mice were diluted and then applied (in duplicate) to a membrane before analysis using the Revert® Total Protein Stain at a high (A and B) or a low dilution range (C and D) (Note: concentrations in C are approximate whereas concentrations in (D) are exact). Linear fit of the Revert® quantification of the membrane from both dilution ranges are shown (B, D). Linear fits for the higher dilution range were R^2=0.96 and higher, whereas R^2 values for the lower dilution range were between 0.91 and 0.95. Images (A) and (C) were captured separately so visual comparison should be avoided.

Multiple technical replicates (as reasonably possible) should be used, based on the total number of samples to be analyzed and the volume of each sample available. If the protein dilutions are made and applied to the membrane properly, variability between technical replicates should be low. If high variability is observed, refer to the Troubleshooting section. Variability between technical replicates for properly applied samples should be within + 5% of the average value. Between separately prepared and diluted protein samples applied to different membranes, variability should be below 15%.

LIMITATIONS

Quantification of low abundant proteins will be challenging using this method, because the cell or tissue lysate is not separated prior to staining/immunoprobing and the mixture of proteins in each dot is complex. Optimization of the amount of protein applied to the membrane may increase sensitivity. Given the rapidity of this assay and the ability to quickly troubleshoot issues, we suggest trying a dilution series with higher protein loading per dot to optimize the experimental conditions for each protein target.

TROUBLESHOOTING

Problem

Inconsistent staining with the Revert® Total Protein Stain.

Potential Solution

If there is inconsistency between technical replicates, this suggests that the problem is with either the dilution of the samples or the pipetting of the samples on the membrane. To minimize inconsistency among technical replicates, ensure samples are well-mixed prior to application to the membrane, and use proper pipetting technique to apply samples to the membrane. Due to the nature of manual pipetting, small variations between technical replicates are expected, and will be

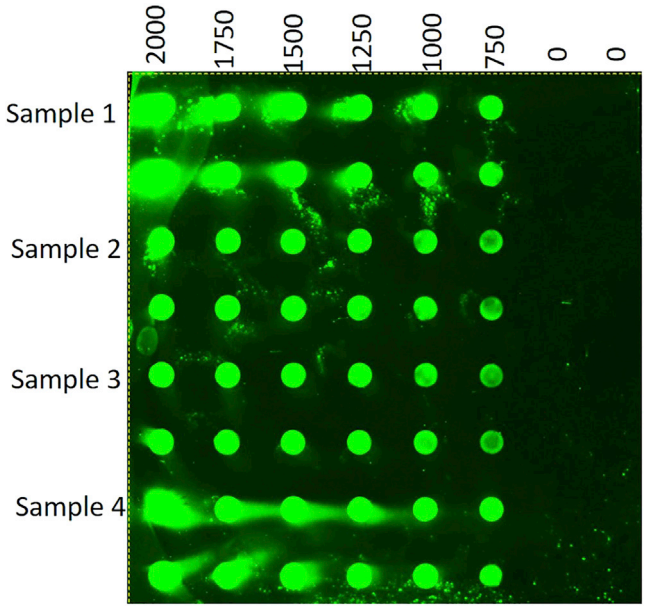

Figure 5. Representative Image of a Protein Overloaded Membrane

Protein homogenates from the cerebral cortex of adult mice were diluted, dotted on a membrane and imaged following detection of tau5 (Invitrogen, MA5-12808) at a 1:1,000 dilution (imaging of the Revert stained membrane in Figure 4). Secondary antibody was goat-anti-mouse IRDye® 800 at a 1:10,000 dilution (LiCor). Note the smearing of the dots in samples 1 and 4 (mice that over-expresses human tau, htau) vs. samples 2 and 3 (wild type mice) indicate a high level of tau protein and thus, quantification will be less accurate. To avoid smearing of dots for samples with high-abundance proteins, we recommend applying 1 μg protein/dot.

accounted for during the Revert® normalization analysis. Deviation between technical replicates should be below 5%. If the technical replicates are consistent, but there is variation among the different samples, the protein quantification or dilution of the samples was done incorrectly. For an example of an incorrectly quantified and loaded membrane, see Figure 3. Consequently, the samples should be re-quantified and applied to another membrane.

Problem

Quantification of the antibody is low or too high.

Potential Solution

Verify that the primary antibody has been previously used for blotting-applications with the reported concentration of the diluted antibody. Dilution of the protein sample is also an alternative approach. Quantification of the Revert® signal is robust and linear at 500 ng per dot and above (Figure 4). We suggest as a starting amount that 1 μg protein be loaded per dot because higher amounts caused the protein samples to smear between samples for highly-abundant targets (Figure 5). For detection of less-abundant targets, application of higher amounts (> 1 μg) may be required to get good signal and can be tried as needed. The binding capacity of a nitrocellulose membrane is 80–100 $\mu g/cm^2$. The protein loaded per dot should not exceed this amount (~10 μg/dot).

Problem

Residual signal from the Revert® stain was detected after destaining the membrane.

Potential Solution

We consistently saw 95%–97% removal of the Revert® stain when following the protocol as described (Figure 1). Quantification of the Revert® stain in the 700 nm channel should yield values

in the 40–100 K range. Following destain, values in the 700 nm channel should be below 5,000. If the Revert® stain is not efficiently removed (step 23), the incubation time of the reversal solution should be increased to 10 min. More residual signal is routinely detected in the 700 nm than the 800 nm channel, although a small amount will be detected in the 800 nm channel (quantification in the 800 nm channel following destain should be <600). For this reason, antibody detection should be done in the 800 nm channel, as well as the extra imaging step following Revert® destain, so that residual Revert® stain can be accurately determined. Accurate levels of the residual Revert® increases the accuracy of the signal intensities from the primary antibodies in the 700 nm or 800 nm channels.

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AUTHOR CONTRIBUTIONS

A.C.C. was responsible for conducting experiments and manuscript preparation. G.E.K. provided oversight and insight during experiments and manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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