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Semiquantitative Western Blot (quantification of SMN protein levels)

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1. OBJECTIVE

Semiquantitative western blot is a method to quantify expression level of specific proteins. After protein solutions are extracted either from cell cultures or from diverse tissues, the protein concentration can be easily determined; equal amounts of proteins are loaded onto a SDS-PAGE. SDS-PAGE allows separation of proteins according to their molecular weight. Transfer of proteins (blotting) to a nitrocellulose membrane (or PVDF) is necessary for subsequent antibody detection of the proteins of interest. Membranes are then incubated with a specific antibody recognizing the target protein, and subsequently with a secondary antibody, which is HRP-conjugated (or labeled with a fluorescent conjugate, e.g. for LiCor system). Incubation with a chemiluminescent substrate permits the detection of the amount of protein (level of luminescence or fluorescence) either by chemiluminescence films or direct detection through optical documentation systems. The quantification of the band intensity can be used to determine specific protein levels in the tested cells or tissues. Detection of a second "housekeeping" protein is necessary to control for variability in protein loading between samples.

2. SCOPE AND APPLICABILITY

Semiquantitative western blot is a method widely used to quantify the expression level of specific proteins. Here, we describe the method to quantify SMN protein levels in cells or tissues. This method is very robust and highly reproducible.

3. CAUTIONS

The most critical step during protein extraction process is to keep samples on ice to avoid protein degradation. Thus, the use of protease-inhibitors is strongly recommended. (Recommendation: dissolve 1 tablet Complete Mini (*Roche, #11836153001*) in 7ml RIPA buffer). It is important to note that the use of protease inhibitors interferes with the Bradford protein quantification assay; consequently, we recommend using the *BCA Protein Assay Kit* (*Pierce* Cat. #23225) following the manufacturer's protocol.

When developing chemiluminescence films, it is absolutely necessary that the protein bands are not saturated to guarantee an exact quantification of the protein levels. Therefore, it might be useful to determine the optimal exposure time. Moreover, to compare different protein samples, quantification of β-actin within the respective samples should be used to normalize inter-sample loading variability.

4. MATERIALS

4.1 Chemicals:

α-SMN, monoclonal IgG1, (MANSMA7, clone 1B12), *Developmental Studies Hybridoma Bank University of Iowa,* Ref.: Exp. Cell Res. 256, 365-374.

α-β-actin, monoclonal IgG2a, Cat.# A 5316 *Sigma*

Horseradish peroxidase conjugated goat anti-mouse IgG, # 115-035-000, *Dianova*

SuperSignal West Pico Chemiluminiscent Substrate Kit*, Pierce*

Precision Plus Protein All Blue Standards, # 161-0373, *Bio-Rad*

Milk powder (Instant Nonfat Dry Milk), *supermarket*

Alternative: Nonfat dried milk powder, # A0830,0500, *AppliChem*

Sodiumdodecylsulfate (SDS)*, AppliChem*

DOC (Deoxycholic acid), # D2510, *Sigma*

IGEPAL, # I3021, *Sigma*

Ponceau S, # P3504, *Sigma*

Acrylamide: Acrylamide/Bisacrylamide 29:1 (30%), *Biorad*

Ammoniumpersulfate, *AppliChem*

TEMED: N,N,N',N' – Tetramethyl-Ethylendiamine, *AppliChem*

Tris: Tris-(hydroxymethyl)-Aminomethane, *Roth*

Tris-Base: Tris-(hydroxymethyl)-Aminomethane, *Sigma*

Tween 20: Polysorbat 20, *Caelo*

β-Mercaptoethanol, *AppliChem*

Glycine, *AppliChem*

Glycerol, *AppliChem*

Bromophenol-Blue, *AppliChem*

Methanol, *AppliChem*

Acetic Acid glacial, *AppliChem*

Hyperfilm ECL (GE healthcare) or equivalent

Bovine Serum Albumin (powder or solution), *Sigma*

4.2 Solutions:

Ammonium Persulfate (APS) solution (10%):	for 10 ml:
APS	1.0 g
deionized H_2O	to a final volume of 10 ml
	store in 1ml aliquots at -20°C
Blocking solution (6%):	for 100 ml:
Nonfat dry milk	6 g
TBS Tween buffer	to a final volume of 100 ml
	Prepare fresh every time
Bradford solution:	for 1l:
Coomassie Brilliant Blue G250	100 mg
H_3PO_4 (85%)	100 ml
Ethanol (95%)	50 ml
deionized H_2O	to a final volume of 1 l
	store at 4°C
Electrophoresis buffer (10x)	for 1l
Tris (Base)	30.29 g
Glycine	144.13 g
SDS	10 g
deionized H_2O	to final volume of 1 l
Laemmli buffer for SDS-PAGE (2x):	for 100 ml:
Tris-Base	0.757 g
Glycerol	20 ml
Bromphenol Blue	10 mg
SDS	6 g
deionized H_2O	to a final volume of 90 ml

Prior to use, add 10 ml of β-Mercaptoethanol .

(Laemmli buffer with β-Mercaptoethanol must be stored at 4°C for no longer than 2 weeks)

Ponceau solution:	for 100 ml:
0.5% Ponceau S	0.5 g
1% Acetic acid glacial	1 ml
deionized H_2O	to a final volume of 100 ml
RIPA buffer:	for 50 ml:
150 mM NaCl	1.5 ml 5 M NaCl
1% IGEPAL	5 ml 10% IGEPAL
0.5% DOC (Deoxycholic acid)	2.5 ml 10% DOC
0.1% SDS (Sodium Dodecyl Sulfate)	0.5 ml 10% SDS
50 mM Tris (pH 8.6)	2.5 ml 1 M Tris (pH 8.6)
deionized H_2O	to a final volume of 50 ml
TBS Tween buffer:	for 5 l:
20 mM Tris	12.1 g Tris
137 mM NaCl	40.0 g NaCl
0.5% Tween 20	25 ml Tween 20
deionized H_2O	to a final volume of 5 l
	adjust to pH 7.56
Transfer buffer:	for 5 l:
Tris-Base	12.1 g
Glycine	56.3 g
Methanol	1000 ml
deionized H_2O	to a final volume of 5 l
Separation gel for SDS-PAGE 12%:	for 1 gel:
deionized H_2O	1.7 ml
acrylamide-bisacrylamide mix (29:1, 30%)	2.0 ml
Tris (1.5M, pH 8.8)	1.3 ml
SDS (10%)	0.05 ml
APS (10%)	0.05 ml

TEMED	0.002 ml
Stacking gel for SDS-PAGE:	for 1 gel:
deionized H_2O	1.4 ml
acrylamide-bisacrylamide mix (29:1, 30%)	0.33 ml
Tris (1 M, pH 6.8)	0.25 ml
SDS (10%)	0.02 ml
APS (10%)	0.02 ml
TEMED	0.002 ml

4.3 Equipment:

Imaging systems:

Chemidoc XRS, *Biorad*

Gel Doc 2000, *Biorad*

Electrophoresis system:

Mini-Protean 3 Cell, *Biorad*

Western Blot transfer chamber:

Mini Trans-Blot Cell, *Biorad*

Autoradiography cassette:

Developer Cassette 18x24cm, *Siemens*

Developer machine:

CURIX 60, *Agfa*

Power supply:

PowerPac 1000, *Biorad*

5. METHODS

Extraction of proteins from mouse organs

Humanely sacrifice the laboratory mouse - either by decapitation (young mice) or by gasification (CO_2 inhalation) (adult mice), or drug overdose (depends upon the end users' approved animal protocol). Open the body and quickly remove the organs of interest. Snap freeze the freshly dissected organs in liquid nitrogen.

To extract proteins from mouse organs, transfer about 300 mg of the respective organ into 150 µl of RIPA buffer (Sigma) (may be supplemented with protease inhibitors) and homogenize thoroughly using a T-10 Basic Ultra Turrax Homogenizer (IKA). Perform all steps on ice to avoid enzymatic degradation of proteins. To remove cellular debris and contaminating DNA, transfer the homogeneous lysate into a micro-centrifuge and spin at 12,000 rpm at 4°C for 20 min. After centrifugation, transfer the protein containing supernatant into a new Eppendorf tube. Use the protein lysates either directly for experiments or store at -80°C. Aliquots of protein extracts would be recommended to reduce freeze/thaws and protein degradation.

Extraction of proteins from cell cultures

To harvest protein from cell lines, remove culture medium and wash cells twice in 1 x PBS buffer (without Ca^{2+}, Mg^{2+}). To lyse cells, add 50 µl RIPA buffer directly onto a 10 cm petri dish (or culture flask). To complete the lysis, keep dishes on ice for 20 min (it is recommended to rock the plate from time to time to ensure that no area of the plate becomes dry). To remove cellular debris and contaminating DNA, transfer the homogeneous cell lysate into a 1.5 ml eppendorf tube and place into a micro-centrifuge and spin at 12,000 rpm at 4°C for 20 min. After centrifugation, transfer the protein containing supernatant into a new eppendorf tube. Use the protein lysates either directly for experiments or store at -80°C.

Protein concentration determined with the BCA Protein Assay kit

To quantify proteins, the BCA protein assay kit is recommended as protease inhibitors interfere with accurate quantification by the Bradford technique (Bradford 1976). Follow the manufacturer's specifications.

Discontinuous denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins according to their molecular weight, it is necessary to denature proteins with an anionic detergent SDS (sodium dodecylsulfate). Since SDS adds a negative charge to denatured proteins, the protein's own charge is masked so that proteins can be separated according to their molecular weight (Laemmli 1970).

First, prepare and pour the 12% polyacrylamide separating gel solution between two glass plates (thickness: 0.5 mm). Add 70% ethanol on top of the polyacrylamide solution until the gel is polymerized.

Second, remove the ethanol and pour the 5% polyacrylamide stacking solution on top of the separating gel. Because the stacking gel contains less polyacrylamide than the separating gel, the pore size is larger. Moreover, the stacking gel is prepared with a Tris-buffer of lower pH. Taken together, the stacking gel allows proteins to stack into a single sharp band shortly after penetrating into the gel. Proteins are then resolved in the separating gel which possesses tighter pores. As soon as the electrical current is applied across the gel, negatively charged proteins begin to migrate. Lower molecular weight proteins will more easily pass through the pores in the gel, while larger ones will have more difficulty and migrate more slowly. The use of a system with discontinuous buffers and gels with different pore sizes enhances the sharpness of the protein bands.

Third, supplement protein samples containing 7.5 μg of protein with Laemmli buffer and boil at 95°C for 5 min to ensure that proteins are denatured. Finally, load each sample into an individual lane of the polyacrylamide gene. In a separate lane, load marker proteins of known molecular weight (Precision Plus Protein All Blue Standards, Biorad) to estimate the molecular weight of the proteins of interest to be detected in the protein samples under investigation. Gels are run at 50-120 V in an electrophoresis chamber using 1 x electrophoresis buffer; run until the blue marker (Laemmli buffer) reaches the bottom of the gel.

Transfer of proteins to nitrocellulose membrane by Western blotting

To make proteins available for antibody detection, proteins must be transferred from the acrylamide gel onto a nitrocellulose (or PVDF) membrane [Protran BA 83 Cellulosenitrat (E), Whatman]. First, equilibrate the membrane and soak two fiber pads and two Whatman filter papers in pre-cooled (4°C) transfer buffer. Transfer the polyacrylamide gel onto a soaked Whatman filter paper, gently place the nitrocelluose (or PVDF) membrane on top of the gel making sure to remove all air bubbles. Cover with the second Whatman filter paper and place the sandwich between the pre-soaked fiber pads. The gel sandwich is put into a cassette which is then introduced into the Mini Trans-Blot Cell (Biorad) apparatus. Transfer proteins at a current of 30 V overnight at 4°C. Negatively charged proteins move and adhere onto the membrane while maintaining the size specific organization they had within the gel. Consequently, proteins are now exposed on a solid thin surface easily accessible for chemiluminescence detection.

Ponceau staining of proteins on nitrocellulose membranes

To confirm uniform transfer of proteins, stain the nitrocellulose membrane in a Ponceau solution for 30 s. The Ponceau dye stains positively charged amino acid side chains red. A homogeneous ladder of protein bands and the absence of air bubbles are indicative of

a good transfer. Wash the membrane in TBS-Tween buffer several times to remove Ponceau dye before proceeding to immunostaining.

Immunostaining of membranes with antibodies and detection of signals with chemiluminescent reagents

After transfer, total proteins are bound to the nitrocellulose membrane. To avoid background signals due to nonspecific interactions of the respective antibodies with the nitrocellulose membrane, block nonspecific binding sites with a 6% blocking solution containing non-fat dry milk in TBS-Tween buffer for 3 h at 4°C. This step is followed by incubating the membrane with the primary antibody against the target protein of interest. For the SMN monoclonal antibody, dilute the primary antibody in 5 ml of pre-cooled 1-2% non-fat dry milk in TBS-Tween for a final concentration of 1:1,000 and incubate the nitrocellulose membrane in the antibody solution in a 50 ml reaction tube overnight on a roller shaker at 4°C (you may also use flat plastic boxes and a platform shaker for incubation steps). On the next day, add the β-actin monoclonal antibody. For this dilute the primary β-actin antibody in 5 ml of pre-used (above mentioned) SMN antibody solution to a final concentration of 1:10,000 and incubate for 1h on a roller shaker at 4°C. To remove unbound primary antibody, wash the membrane 5 times for 5 min each in TBS-Tween. The secondary antibody, which is linked to the horseradish peroxidase reporter enzyme and is able to bind to the primary antibody, is incubated together with the membrane as described for the primary antibody. Again, wash the membrane 5 times for 5 min each in TBS-Tween. Detection of proteins bound by the antibody of interest is accomplished by chemiluminescence. For this purpose, membranes are incubated with 8 ml SuperSignal® West Pico Chemiluminescent Substrate (Pierce) for 5 min. This substrate will emit light when exposed to the horseradish peroxidase bound to the secondary antibody. Emitted light is then detected by chemiluminescence films (Hyperfilm ECL, Amersham). The image should be analyzed by densitometry using the Multi-Analyst Version 1.1 software, supplied by BioRad to evaluate the optical density and relative amount of the protein of interest.

Dilution and incubation time of antibodies:

Anti-β-Actin, monoclonal mouse (Sigma, Cat.# A 5316) 1:10,000 for 1 h

Anti-SMN, monoclonal mouse, (DSHB, MANSMA7, clone 1B12), 1:1,000 overnight

5.1 Quick Protocol Overview (in order):

Pouring the gels:

- prepare a 12% SDS-polyacrylamide gel solution:

	1 gel (5ml)
1. H_2O	1.7 ml
2. 30% Acrylamide (29:1)	2 ml
3. 1.5 M Tris pH 8.8	1.3 ml
4. 10% SDS	50 µl
5. 10% APS	50 µl
6. TEMED	2 µl

- mix all ingredients and add TEMED last!
- immediately pour the gel between clean glass plates mounted in an electrophoresis chamber
- cover the polyacrylamide solution with 70% EtOH to create a straight flat gel surface
- wait until gel polymerizes (use the excess polyacrylamide solution as a "polymerization control")
- when gel is polymerized decant the EtOH and dry the plates
- prepare the stacking gel as follows (add TEMED last!):

	1 gel (2ml)
1. H_2O	1.4 ml
2. 30% Acrylamide (29:1)	330 µl
3. 1 M Tris pH 6.8	250 µl
4. 10% SDS	20 µl
5. 10% APS	20 µl
6. TEMED	2 µl

- after pouring the stacking gel, immediately insert the appropriate gel comb into the stacking gel solution, making sure not to introduce air bubbles
- wait until gel polymerizes (use the excess solution as "polymerization control")
- carefully remove the comb

- place the gel plate into the electrophoresis chamber and add electrophoresis buffer (1x)

Preparing the protein samples:

- add 5 µl Laemmli (2x) to 7.5 µg protein
- denature protein lysates 5 min at 95°C
- cool protein lysates in a cold ice water bath
- spin down (to remove condensation)

Loading protein samples onto gel:

- load each sample into a single slot
- avoid using the outermost lanes (protein band might run slanted)
- load 7.5 µl All-Blue Protein-Standard in a separate lane for MW determination

Running the gel:

- start at 50V
- when the blue running front has entered separation gel, adjust current to 80V
- after 45 minutes, may increase current to 100-120V
- when the bromophenol blue dye front has run out of the gel, stop the current and set up the Western blotting

Blotting:

- cut membrane and filter paper to adequate size (gel size)
- equilibrate membrane, filter paper and pads in transfer buffer at room temperature
- carefully remove gel from the glass plate
- take care of the right orientation; make sure to remove air bubbles between gel and membrane!
- setup blotting chamber (add optional cool pack and magnetic stir bar)
- fill blotting chamber with transfer buffer
- place blotting chamber on magnetic stirrer in cold room (4°C)
- run blot at 30V overnight in cold room

Antibody staining:

- disassemble blotting chamber
- wash membrane in TBS-Tween

- perform Ponceau-staining
- wash in TBS-Tween until Ponceau staining completely disappears
- block in 6% Blocking solution (1.5g milk powder in 25 ml TBS-Tween per blot)
- block for 3-4 hours or overnight at 4°C (shaking)
- remove blocking solution
- Incubate membrane with 5ml 1-2% milk powder-TBS-Tween and SMN antibody (conc. 1:1,000) in 50ml-tube
- incubate overnight on a roller shaker at 4°C
- Add β-actin antibody (conc. 1:10,000) to the pre-used SMN-antibody solution in 50ml-tube
- incubate 1 hour on a roller shaker at 4°C
- wash membrane 5 times for 5 min each in TBS-Tween (shaking at room temperature)
- incubate blot in 50ml-tube with 5ml 1-2% milk powder-TBS-Tween and 1:10,000 antibody (HorseRaddish-Peroxidase conjugated α-mouse) for detection of primary mouse antibody
- incubate 1-2 hours on a roller shaker at 4°C
- wash membrane 5 times for 5 min in TBS-Tween (shaking at room temperature)
- prepare chemiluminescence solution as recommended by manufacturer (mix 4ml solution A + 4ml of solution B)
- incubate membrane with chemiluminescence solution (8ml) for 5 min
- expose to chemiluminescence film in an film cassette
- develop film in developer machine

Quantification

- Scan adjusted (not saturated!) bands using ChemiDoc (or equivalent) scanner
- Quantify the densitrometric intensity of each band
- Use Excel or similar software to determine ratio of SMN intensity: β-actin intensity of each lane

6. EVALUATION AND INTERPRETATION OF RESULTS

Data evaluation can be performed by Excel or similar software to determine the ratio between SMN intensity and β-actin intensity in each lane. Each experiment should be performed at least three times (at least 3 cell culture dishes or three animals). If three independent values for each treatment (concentration or time point) are available, significance can be determined by using the Student´s t-test. If $p<0.05$, the difference of the respective values are interpreted as statistically significant.

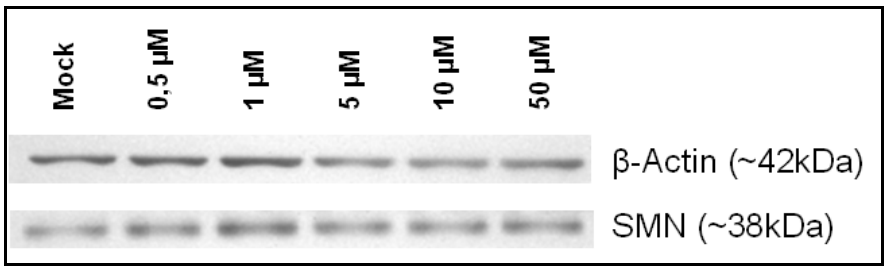

Fig. 1: Example Western Blot. Proteins were prepared from a SMA-patient fibroblast cell line. Cells were treated with no (mock) or increasing concentrations of HDACi ST17 (from 0.5 to 50 µM) for 64 h. The nitrocellulose-membrane was incubated with α-β-Actin and α-SMN antibodies as described above. Running-sizes of the respective proteins are given.

7. REFERENCES

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8. APPENDIX

Abbreviations:

APS	ammonium persulfate
ECL	Electrochemiluminescence
h	hour
HRP	Horseradish peroxidase
l	liter
M	molar
m	milli
min	minutes
ml	milliliter
mM	millimolar
nm	nanometer
p	probability (statistical significance)
PAGE	polyacrylamide gel electrophoresis
pH	power of hydrogen
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
µ	micro
µl	microliter
µM	micromolar
µm	micrometer