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Detection of connexins in liver cells using sodiumdodecylsulfate polyacrylamide gel electrophoresis and immunoblot analysis

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Summary

Since connexin expression is partly regulated at the protein level, immunoblot analysis represents a frequently addressed technique in the connexin research field. The present chapter describes the set-up of an immunoblot procedure, including protein extraction and quantification from biological samples, gel electrophoresis, protein transfer and immunoblotting, which is optimized for analysis of connexins in liver tissue. In essence, proteins are separated on a polyacrylamide gel using sodiumdodecylsulfate followed by transfer of proteins on a nitrocellulose membrane. The latter allows specific detection of connexins with antibodies combined with revelation through enhanced chemiluminescence.

Keywords

connexins; protein extraction; sodiumdodecylsulfate polyacrylamide gel electrophoresis; immunoblot analysis; antibody; enhanced chemiluminescence

1 Introduction

Renart, Reiser and Stark were the firsts to describe a rapid and sensitive method for the separation and detection of proteins using diffusion over polyacrylamide gels and antibodies (1). Later on, Towbin reported transfer of proteins in an electrical field (2). This approach was optimized by Brunette, who introduced the designation western blot, playing on the Southern blot and northern blot methods used to detect DNA and RNA, respectively (3). Throughout the years, the immunoblot technique underwent significant advances with respect to the sensitivity, robustness and flexibility (4). At present, the western blot technique is widely used because of its specific advantages in comparison with other protein detection methods, such as mass spectrometry and enzyme-linked immunosorbent assay (ELISA). Overall, the immunoblot procedure has a great specificity and is cheap in comparison with mass spectrometry. Moreover, western blotting can detect proteins in small amounts of sample. On the other hand, semi-quantitative densitometric analysis is typically

used to quantify protein expression on a blot, being less accurate than the ELISA detection method (5). Connexin (Cx) half-life is very short (*i.e.* 1.5 to 5 h) compared to other transmembrane proteins (6–10). Connexin mRNA translation can be modified through microRNAs or RNA-binding proteins and may be altered through trafficking and degradation mechanisms (11, 12).

As such, 6 connexin proteins gather to form a hemichannel in a single cell and 2 hemichannels of adjacent cells can connect to establish gap junctions (13). The latter are important communication channels between neighbouring cells, providing the passage of a number of molecules, such as cyclic adenosine monophosphate, adenosine triphosphate, inositol triphosphate, and ions, all which play important roles in liver homeostasis (14–16). The liver was the first organ in which connexins have been identified and hence several research efforts over the last decades have been focused on the analysis of these particular gap junction proteins (17–20). In this regard, detection of connexin proteins using immunoblot analysis is a valuable method in liver research.

In this chapter, an immunoblot procedure optimized for analysis of hepatic connexins, specifically Cx26, Cx32 and Cx43, is outlined. The procedure basically consists of 2 steps, namely sodiumdodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Adding SDS, a strong anionic detergent, to protein extracts will result in an equal distribution in charge *per* unit mass. When applying voltage to the polyacrylamide gel, proteins will migrate at different speed and will be separated according to their molecular weight (21). Subsequently, separated proteins can be transferred onto a membrane, typically made of nitrocellulose, polyvinylidene difluoride or polyvinylpyrrolidone, upon establishment of an electrical field (22). In order to reduce nonspecific binding of antibodies on the membrane in the next step, blots are incubated in a solution containing bovine serum albumin (BSA), nonfat dry milk or casein (23). Visualisation of the protein can be achieved by a number of methods, of which enhanced chemiluminescence (ECL) is the most popular one. ECL is a very sensitive method and can be used for relative quantification of protein abundance on the membrane by detecting horseradish peroxidase from antibodies. The reaction product produces luminescence (Fig. 1), which can be captured on photographic film, and this is correlated with the amount of target protein on the membrane (24).

2 Materials

All solutions should be prepared with ultrapure water (*i.e.* purified and deionized water with a constant sensitivity of 18 M Ω at 25°C).

2.1 Protein extraction

1. Ethylenediaminetetraacetic acid (EDTA) solution: 0.5 M EDTA in water. Adjust to pH 8.0 (*see* Note 1). This solution can be stored for 6 months at 4°C.
2. Lysisbuffer (*see* Note 2): *Prior* to use, add 100 μ L EDTA solution and 100 μ L 100x protease and phosphatase inhibitor cocktail to 9.8 mL radio-immunoprecipitation assay buffer.

3. Centrifuge suitable for cooling at 4°C.
4. Sonicator (*i.e.* for cell culture samples) or mixer (*i.e.* for liver tissue samples).
5. Rotator.

2.2 Protein quantification assay (see Note 3)

1. Bicinchoninic acid (BCA) working reagent: *Prior* to use, mix BCA reagent A with BCA reagent B in a ratio 50:1. The solution turns blue. BCA working reagent can be stored for several days in a closed container at room temperature.
2. BSA standards (Table 1). Keep on ice.
3. Flat-bottom 96-well plate.
4. Multiplate reader.
5. Incubator.

2.3 Preparation of gels (Table 2)

1. Tris buffer 1: 1.5 M Tris in water. Adjust to pH 8.8. This solution can be stored for 6 months at 4°C.
2. Tris buffer 2: 0.5 M Tris in water. Adjust to pH 6.8. This solution can be stored for 6 months at 4°C.
3. Polyacrylamide solution: 30% acrylamide/bisacrylamide 37.5:1 solution (*see* Note 4).
4. SDS solution: 10% SDS in water. This solution can be stored for 6 months at room temperature.
5. Ammoniumpersulfate (APS) solution: 10% APS in water. The solution is prepared *ex tempore* and should be kept on ice.
6. Tetramethylethylenediamine (TEMED).
7. Isobutanol solution: 1:3 isobutanol/water solution. Shake and let the solution rest after preparation for at least 30 min.
8. Glass plates.
9. Combs.
10. Filterpaper.

2.4 SDS-PAGE

1. Bromophenol blue solution: 0.5% bromophenol blue in water. This solution can be stored for 6 months at 4°C.
2. Sample buffer: Mix 2.5 mL Tris buffer 2, 2 mL SDS solution, 2 mL glycerol, 200 µL bromophenol blue solution and 2.35 mL water. This

solution can be stored at -20°C for 2 weeks. 1/10 β -mercaptoethanol is added *prior* to use (*see* Note 5).

3. Running buffer: 24.9 mM Tris, 192 mM glycine, 3.5 mM SDS in water. About 1 L *per* 2 gels is needed. Prepare *ex tempore*.
4. Protein marker (*see* Note 6).
5. Electrophoresis tank.
6. Power supply source.

2.5 Protein transfer

1. Transferbuffer: 24.9 mM Tris, 192 mM glycerol, 20% methanol in water. Prepare *ex tempore*.
2. Nitrocellulose membrane (*see* Note 7).
3. Ponceau S solution: 0.03 M Ponceau S, 1.84 M trichloroacetic acid and 1.18 M 5-sulfosalicylic acid dihydrate in water. This solution can be re-used when stored for 6 months at room temperature.
4. Electrophoresis tank.
5. Power supply source.

2.6 Immunoblotting and protein detection

1. 10x Tween-supplemented Tris-buffered phosphate-buffered saline (TBST): 0.20 M Tris, 1.36 M NaCl, 9 mM Tween 20 in water. Adjust to pH 7.6. This solution can be stored for 6 months at 4°C.
2. 1x TBST: Dilute 10x TBST 10 times in water. Prepare *ex tempore*.
3. Blocking buffer: 5% milk powder in 1x TBST (*see* Note 8). Prepare *ex tempore*.
4. Appropriate primary and secondary antibody: Polyclonal anti-Cx26 antibody produced in rabbit (Thermo Scientific, USA), polyclonal anti-Cx32 primary antibody produced in rabbit (Sigma, USA), polyclonal anti-Cx43 primary antibody produced in rabbit (Sigma, USA). Polyclonal goat anti-rabbit immunoglobulins/horseradish peroxidase secondary antibody (Dako, Denmark) (*see* Note 9).
5. Pierce™ ECL western blotting substrate (Thermo Scientific, USA): Mix 2 mL peroxide solution with 2 mL luminol enhancer solution *ex tempore*.
6. Fixation solution.
7. Development solution.
8. Photographic films.

2.7 Stripping of the immunoblot

Restore™ western blot (Thermo Scientific, USA).

3 Methods

3.1 Protein extraction

1. Add 10 µL of lysisbuffer *per* mg liver tissue or 100 µL *per* cell culture dish with a diameter of 10 cm (*see* Note 10).
2. For cultured cells, sonicate 30 s with 50% pulse while keeping the cells on ice (*see* Note 11). For liver tissue samples, homogenize using a mixer and keep on ice.
3. Shake for 15 min on a rotator at 4°C (*see* Note 12).
4. Centrifuge at 14000*xg* for 15 min at 4°C.
5. Transfer the supernatant to a new tube and store at -80°C.

3.2 Protein quantification assay

1. All samples, standards and blanks should be measured in triplicate and should be kept on ice.
2. Prepare the BSA standards (Table 1) and the BCA working reagent.
3. Pipet 10 µL of each properly diluted sample (*see* Note 13) and BSA standards in the respecting wells of a flat-bottom 96-well plate. Add 200 µL of the BCA working reagent to each well. Use 210 µL BCA working reagent as a blank. Mix the plate thoroughly on a plate shaker for 30 s.
4. Protect the plate from light and incubate at 37°C for 30 min.
5. Cool the plate to room temperature and measure the absorbance at 562 nm on the multiplate reader.
6. Subtract the average absorbance measurement of the blank standard from the measurements of all other standards and samples. Prepare a standard curve by plotting the average absorbance measurement for each BSA standard as a function of concentration expressed in µg/mL. Use the standard curve to determine the protein concentration of each sample by interpolation.

3.3 Preparation of gels (*see* Note 14)

1. Prepare the gel solution (*see* Note 15) in a conical flask (Table 2).
2. Pour the separation gel solution between the glass plates and add a thin layer of isobutanol solution. The polymerisation process starts instantly. Let rest for 30 min at room temperature.
3. Prepare the stacking gel solution (Table 2).

4. Remove the isobutanol solution using a filterpaper, wash 3 times with water and pour the stacking gel solution on top of the separation gel. Correctly place the combs into the gel and let rest for at least 30 min at room temperature.
5. Remove the combs and rinse the wells 3 times with water.

3.4 SDS-PAGE

1. Thaw the protein samples on ice before starting.
2. Calculate the volume needed to load 50 µg protein from each sample (*see* Note 16) on the gel based on the respective protein concentration. Dilute until 10 µL with lysisbuffer and add 10 µL sample buffer. All samples should be kept on ice during preparation.
3. Install the gel cassette into the electrophoresis tank (*see* Note 17) and add running buffer between the gels and in the tank (*see* Note 18). Spin the samples shortly before loading on the gel and shortly mix protein ladder solution. Load 5 µL of the protein ladder (*see* Note 6) in the first well and load samples (*i.e.* 20 µL) in the other wells. Empty wells are loaded with a mixture of 10 µL lysisbuffer and 10 µL sample buffer (*see* Note 19).
4. Close the electrophoresis tank, connect to the voltage source and start separation by applying a constant voltage of 150V. Stop when the bromophenol blue line reaches the end of the gel (*see* Note 20).

3.5 Protein transfer (*see* Note 21)

1. Cut a nitrocellulose membrane fit to the size of the gel and let soak in cold transferbuffer together with the blottingpaper and sponges.
2. Take the cassette out of the tank, open and remove the stacking gel using a spatula (*see* Note 22). Put the separation gel in the transfer recipient with the nitrocellulose membrane underneath. Add blottingpaper on the membrane and on the gel. Keep in mind that the gel is the top layer while the membrane forms the bottom layer. Remove all air bubbles by rolling over using a roller. Add a sponge to each side and put in a cassette. Add transferbuffer to the tank and apply a constant voltage of 150V for 1 h (*see* Note 23).
3. Remove the membrane from the cassette and incubate with Ponceau S solution for 3 min while shaking. Ponceau S will bind to all proteins and allows assessing the efficacy of the transfer and checking equal loading (*see* Note 24).
4. Rinse the membrane 3 times with water and shake with TBST until all Ponceau S is removed from the membrane.

3.6 Immunoblotting and protein detection

1. Block the nitrocellulose membrane for 1 h in blocking buffer at room temperature on a shaker. Make sure that the blotting side is faced up.
2. Incubate with the primary antibody (*see Note 25*) diluted in blocking buffer overnight at 4°C on a shaker (*see Note 26*).
3. Wash the membrane 3 times 10 min with TBST on a shaker.
4. Incubate with the secondary antibody solution for 1 h at room temperature (*see Note 27*).
5. Wash 3 times 10 min with TBST on a shaker (*see Note 28*).
6. Prepare the Pierce™ ECL western blotting substrate solution and incubate with the membrane for 1 min at room temperature.
7. Place the photographic film on the membrane and close the cassette for an appropriate period of time (*see Note 29*).
8. Remove the film and place for 1 min in fixation solution, rinse shortly with water and expose for 1 min to development solution. Rinse shortly with water and let dry (*see Note 30*).

3.7 Stripping of the immunoblot (*see Note 31*)

1. If necessary, rehydrate the membrane by immersing in TBST.
2. Incubate the membrane in Restore™ western blot stripping buffer for 5 to 15 min at room temperature.
3. Wash 3 times 5 min with TBST on a shaker.
4. Check the efficiency of the stripping process by re-incubation of the membrane with the previously used secondary antibody, followed by washing with TBST and developing of the immunoblot.
5. If no signal is observed, proceed with re-incubation of the membrane with the antibody of interest.

3.8 Processing of results

1. If stripping of the membrane is necessary (*see Note 31*), redevelop the membrane as described in 3.6 with appropriate primary and secondary antibody of a suitable housekeeping protein (*see Note 32*).
2. Analyze and quantify the protein signals on the membrane using Quantity One 1D Analysis Software (Bio-Rad, USA) by semi-quantitative densitometry for both the target proteins and housekeeping proteins (*see Note 33*).
3. Correct the target protein measurements to the housekeeping protein results in each lane of the membrane (*see Note 33*).

4. Statistically process the results using an appropriate test (*see* Note 34).

4 Notes

1. pH adjustment of a solution is commonly performed by using HCl. A concentrated HCl solution (*i.e.* 37%) can be used for this purpose, yet it can be useful to use a less concentrated HCl solution (*i.e.* 5%) to avoid a sudden drop in pH.
2. The selection of an appropriate lysis buffer is of utmost importance. It should be optimally designed to efficiently extract the protein(s) of interest, whilst leaving their antigenic sites intact. Particularly the use of a suitable detergent for protein extraction and solubilisation should be taken into account as well as addition of a proper set of protease and phosphatase inhibitors (25). Some antibodies will only recognize a protein in its native non-denatured form and will not detect a protein that has been extracted with a denaturing detergent, such as SDS, deoxycholate, Triton X-100 and NP-40. In general, the following range of variables should be optimized: salt concentration 0 to 1 M, non-ionic detergent 0.1 to 2%, ionic detergents 0.01 to 0.5%, divalent cation concentration 0 to 10 mM, EDTA concentration 0 to 5 mM and pH 6 to 9 (26).
3. A diverse array of protein quantification assays is currently available. It is of major importance to select the correct assay based on the type of sample and concentration range of protein in the sample, since each test has its specific limitations (27, 28). For cultured hepatocytes and freshly isolated liver tissue, it is recommended to use the BCA assay, as it provides a good combination of specificity and ease of use. The BCA assay is a spectrophotometric assay based on the alkaline reduction of cupric ions to cuprous ions mediated by proteins. This is followed by chelation and colour development by the BCA reagent. BSA is used as a standard because of its great working range and stability (29).

Immunoglobulin G could also be used as a standard against which the concentration of protein in samples is normalized (30).
4. Use gloves and be careful when handling acrylamide, as it is a neurotoxic and carcinogenic agent (31–33).
5. Add β -mercaptoethanol to the sample buffer in a well-ventilated area, as it is considered toxic and can cause irritation to the nasal cavity, respiratory tract and skin.
6. A molecular weight marker should be loaded onto the gel along with the analyzed samples in order to enable the determination of the protein size and to monitor the progress of the electrophoretic run. Precision Plus Protein™ Kaleidoscope™ Prestained Standards (Bio-Rad, USA) is a suitable protein marker for the detection of connexin proteins, since it allows monitoring of electrophoretic separation, molecular weight seizing

and blot transfer efficacy on SDS-PAGE gels by fluorescence. The choice of an appropriate molecular weight marker depends on the protein detection method and molecular weight range of the connexin of interest. Bio-Rad (USA) provides a series of protein markers, such as prestained protein ladders for visual assessment during electrophoresis and transfer, unstained protein ladders for coomassie blue and zinc staining, and western blotting standards for fluorescent visualization and colorimetric or chemiluminescent immunodetection on western blots.

7. There are 3 types of blotting membranes available, namely nitrocellulose, which is the most popular one, polyvinylidene fluoride, which requires pretreatment in methanol for 1 to 2 min, and polyvinylpyrrolidone. Nitrocellulose membranes are typically used for low molecular weight protein detection, while polyvinylpyrrolidone is more suitable for northern blotting. All types of membranes can be stripped and reprobed.
8. When preparing blocking buffer, make sure that all milk powder is dissolved by stirring the solution for about 20 min. This solution can be used up to 5 days when stored at 4°C. It is recommended to mix again before use.
9. The decision regarding whether to use polyclonal or monoclonal antibodies depends on a number of factors. Polyclonal antibodies can be generated more rapidly and at less expense, and are more stable over a broad pH and salt concentration range. In contrast, monoclonal antibodies are homogenetic, which can be useful for detection of changes in phosphorylation status. However, small changes in the structure of the epitope can affect the effectiveness of monoclonal antibodies (34).
10. For measurement of connexins *via* immunoblotting, it is strongly recommended to use 10 cm diameter cell culture dishes with hepatocytes plated at a density of 0.57×10^5 cells/cm² (*i.e.* a total of 4.4×10^6 hepatocytes) in order to obtain sufficient amounts of total cellular protein. 70 to 100 µl of lysis buffer is recommended for a total amount of 4.4×10^6 hepatocytes. It is advised to start with a lower volume of the lysis buffer and to observe the colour of cell homogenate upon resuspending the cell pellet. Extremes, such as transparency or brownish colour of the cell lysate, indicating too high or too low dilution respectively, should be avoided. For liver tissue, 10 µL lysisbuffer per mg should be sufficient.
11. Keeping the cells on ice will prevent protein degradation by heat produced from sonication.
12. Shaking on a rotator is not strictly necessary, but it increases the yield of the protein extraction.
13. Dilution of protein extracts for quantification with the BCA assay needs to be optimized. When adding 10 µL lysisbuffer per mg tissue or when using 100 µL lysisbuffer for a total amount of 4.4×10^6 hepatocytes cultured in

10 cm diameter cell culture dishes, a 1/20 or 1/40 dilution is usually sufficient.

14. Appropriate results have been obtained with PROTEAN® TGX Stain-Free™ Precast gels (Bio-Rad, USA) for the detection of connexin proteins. These gels are based on a novel modification of the Laemmli system (*i.e.* Tris, glycerol and SDS), which increases gel matrix stability, allows the use of Tris-glycerol-SDS running buffer and offers rapid UV detection of connexin proteins using a stain-free imaging system, such as ChemiDoc™ MP. Moreover, an optimal resolution of connexins is obtained in the 10 to 100 kDa molecular weight range. The Laemmli system operates at pH 9.5, which can cause deamination and alkylation of proteins. Therefore, the Bis-Tris gel system can be a suitable alternative, as this system has a lower pH of 7.0. The Bis-Tris system ensures increased protein stability, decreased protein modifications and a faster running time (35).
15. Depending on the connexin of interest, a different polyacrylamide percentage is considered during gel preparation, in particular 10% gel for Cx43, 12% gel for Cx32 and Cx26 (Table 2). It is recommended to add TEMED and APS solution as final ingredients, as they initiate gel formation instantly.
16. The total amount of protein loaded onto the gel must be optimized. It is dictated by cellular abundance of the protein of interest as well as reactivity of the primary antibody used. Overall, the scarcer the protein and/or the lower affinity of the antisera, the higher the amount of total cellular proteins that should be loaded in order to avoid false negatives. It is recommended to start with 50 µg total cellular proteins per lane and to include a positive (*e.g.* transfected cell line) and a negative (*e.g.* tissue from connexin-deficient animals) control. Exceeding this amount should be avoided, as this will result in gel overloading and creation of protein 'smudges' instead of bands.
17. When using PROTEAN® TGX Stain-Free™ Precast Gels (Bio-Rad, USA), gently remove the combs and the green tape from the gel cassette before use.
18. Make sure that the running buffer between the cassettes and in the electrophoresis tank do not make contact with each other, as this may interfere with the voltage potential.
19. It can be useful to add a negative control between the weight marker and the first sample, as there might be some interference. It is critical to fill all the wells of the gel. If not, this could lead to so-called smiling of the pattern.
20. The running time should be optimized and will depend on the molecular weight of the connexin under investigation. The goal is to spread as much

as possible the molecular weight area where the protein of interest is located.

21. The traditional wet blotting method requires water, which helps to prevent the generation of oxygen from the copper anode resulting in a more consistent protein transfer without causing band distortion. The alternative semi-dry blotting uses high-field strength and currents to reduce the transfer time of proteins from gels onto membranes within 7 min (28).
22. When using PROTEAN® TGX Stain-Free™ Precast Gels (Bio-Rad, USA), carefully open the cassette using a spatula and check the loading of the gel by UV detection with ChemiDoc™ MP using the stain-free activation. The latter allows total protein staining which can serve as superior alternative for loading control with housekeeping proteins (36).
23. Transfer of connexin proteins can be efficiently performed by using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, USA), which allows rapid and efficient transfer, and the Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Pack (Bio-Rad, USA), which contains an appropriate buffer, membrane and filterpaper. High molecular weight proteins (*i.e.* 200 to 250 kDa) can sometimes remain on the gel because of the rapid transfer. This usually poses no problem for the detection of connexin proteins, as their molecular weight ranges from 26 to 60 kDa. Transfer time of connexins depends on the molecular weight of the protein to be analyzed. As a rule of thumb, the higher the molecular weight, the higher the voltage and the longer the running time. It is also critical to reduce the production of heat during immunoblotting, as this may harm the integrity of the proteins. This can be done by adding a cooling element.
24. Not only transfer efficacy can be tested with Ponceau S. Indeed, it also has shown to be a good loading control even superior to housekeeping proteins (36).
25. A plethora of companies have primary connexin antibodies available, including Sigma, Abcam, Thermo Scientific, Santa Cruz, EMD Millipore, Genetex, ProteinTech, Alomone, Biocompare, Life Technologies and Merck Millipore.
26. Concentrations of both primary and secondary antibody solutions should be optimized for each connexin of interest. It is recommended to test different concentrations of the primary antibody and keep the concentration of the secondary antibody low to avoid non-specific binding. Other possibilities to be included during optimization are incubation for 1 h at 37°C or 2 h at room temperature.
27. Most commonly used are secondary antibodies linked to horseradish peroxidase, an enzyme which catalyzes the oxidation of luminol, producing light (37). However, other detection methods are also available,

such as the use of a fluorochrome or a colorimetric substrate (38). Detection *via* fluorescence is performed if high sensitivity is required, typically when there are 2 different targets on 1 blot. Dyes like fluorescein and rhodamine are often used, but have numerous limitations, including relatively low fluorescence intensity and tendency to photobleach. Colorimetry is simple and the most cost-effective method of detection. Commonly used substrates include 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt and nitro-blue tetrazolium chloride (39).

28. Inefficient washing was shown to be the primary reason for high background in western blots. Lowering the non-ionic detergent (*i.e.* Tween 20) concentration in antisera dilution buffer (*i.e.* 0.02 to 0.005%) and rising the concentration of the detergent in the wash buffer (*i.e.* 0.5%) could improve the results (40).
29. The exposure time of the membrane to the film must be experimentally optimized. It is important that several exposures are produced to ensure that the response is within a linear range of the photographic film. One should aim at obtaining the sharpest bands possible that still have a greyish appearance. This is one of the advantages of the ChemiDoc™ MP (Bio-Rad, USA), which allows detection of overexposed bands.
30. Cx43 is the only connexin protein for which posttranslational phosphorylation can be detected in western blotting using anti-Cx43 primary antibody produced in rabbit (Sigma, USA) (Fig. 2). Additionally, some connexins undergo partial proteolysis during protein extraction, which may lead to detection at other molecular weight values than suggested by the designation. The best example is Cx32, for which cDNA sequencing predicts a molecular weight of 32 kDa, but that is usually found at 27 kDa in immunoblot analysis (Fig. 2).
31. Stripping of the membrane should be avoided as it results in gradual loss of blotted proteins from the membrane. It is therefore advised to directly proceed with re-incubation of the membrane, unless the protein of interest and the loading control protein have approximately the same molecular weight or when species-based interference is expected between antisera used for the detection of the target protein and the loading control. When using the stain-free loading control, stripping of the membrane is not necessary since no housekeeping genes are used.
32. It is advisable to proceed with re-incubation of the membrane with an antibody directed against a typical housekeeping protein, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -actin or α -tubulin (Table 3), which serves as a loading control. Housekeeping proteins should show constant protein expression patterns between different tissue types. It is required to check if all lanes in the gel were evenly loaded with sample, especially when a comparison must be made between the expression levels of a protein in different samples. Detection

of housekeeping proteins is also useful to check for equal transfer from the gel to the membrane across the whole gel. The loading control bands can be used to quantify the protein amounts in each lane by means of densitometry.

33. From a statistical point of view, it is strongly recommended to include at least 3 biological and 3 technical repeats. With exposures captured on photographic film, one can utilize densitometric methods to measure relative quantities of a target protein as well as loading control protein and subsequently compare to a control (*e.g.* specific time point or untreated sample). Bio-Rad Quantity One and Image Lab™ Software (Bio-Rad, USA) are available for image analysis of bands on photographic film and PROTEAN® TGX Stain-Free™ Precast Gels (Bio-Rad, USA) respectively.
34. Results can be processed and evaluated by 1-way analysis of variance followed by *post hoc* Bonferroni tests for testing of 3 or more groups. When comparing only 2 groups, a t-test is sufficient.

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Figure 1.

Figure 2.

Table 1

BSA standard	Water (μL)	Stock (μL)	Final concentration (μg/mL)
A	0	300 from stock	2000
B	125	375 from stock	1500
C	325	325 from stock	1000
D	175	175 from B	750
E	325	325 from C	500
F	325	325 from E	250
G	325	325 from F	125
H	400	100 from G	25

Table 2

	Separation gel (7.5%)	Separation gel (10%)	Separation gel (12%)	Separation gel (15 %)	Stacking gel (4%)
Water (mL)	4.85	4.00	3.35	2.35	3.05
1.5 M Tris buffer (mL)	2.50	2.50	2.50	2.50	-
0.5 M Tris buffer (mL)	-	-	-	-	1.25
Polyacrylamide solution (mL)	2.50	3.33	4.00	5.00	0.65
SDS solution (mL)	0.10	0.10	0.10	0.10	0.050
APS solution (mL)	0.050	0.050	0.050	0.050	0.025
TEMED (mL)	0.015	0.015	0.015	0.015	0.015

Table 3

Loading control	Sample type	Molecular weight (kDa)	Remarks
Beta actin	Whole cell / cytoplasmic	43	Not suitable for skeletal muscle samples. Changes in cell-growth conditions and interactions with extracellular matrix components may alter actin protein synthesis (41)
GAPDH		37	Some physiological factors, such as hypoxia and diabetes, increase GAPDH expression in certain cell types (42, 43)
Tubulin		55	Tubulin expression may vary according to resistance to antimicrobial and antimitotic drugs (44, 45)
VDAC1/Porin	Mitochondrial	31	
COXIV		16	
Lamin B1	Nuclear	66	Not suitable for samples devoid of nuclear envelope
TBP		38	Not suitable for samples devoid of DNA