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A Simplified Technique for Western-Blot Analysis of Whole-Cell or -Tissue Protein Extracts

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Abstract

Western-blot analysis is a very powerful and widely used technic for protein analysis and quantification. However, most of the sample preparation procedures involved a centrifugation step after lysis, in order to remove insoluble materials. These pellets are insoluble DNA/chromatin complexes and proteins, like integral membrane ones, or tightly chromatin-bound transcription and replication factors. Therefore, discarded as pellets, these proteins are rarely considered and analyzed, generating indeed several artefacts.

We present here a simple technic to extract and analyze total proteins by western-blot, from cell sample or tissues. This technic simply involves the lysis of the sample in SDS 1%/NaCl 0.5M/Tris 10 mM pH 7.5, at room temperature with protease inhibitors, and the direct protein dosage and western-blot analysis. As an example, we analyzed the quality of the technic by detecting ATAD3, a mitochondrial integral membrane protein which is mainly insoluble in 1% triton/0.5M NaCl.

This technic avoids therefore all the possibilities to lose part or all of the target protein because of the high level of lysis/solubilization and the suppression of clarifying centrifugation step. Also, limitations of the procedure are discussed.

Introduction

The Western-blot technic was invented in 1979 by both George Stark's group at Stanford University, as Jaime Renart et al. [1] of Harry Towbin's et al. [2] group in Basel, Switzerland, including also Towbin et al. [2] (at Friedrich Miescher Institute) and Theophil Staehelin et al. [2] (at Hoffman-La Roche) and as well as by W. Neal Burnette et al. [3], working in Robert Nowinski's lab at the Fred Hutchinson Cancer Research Center in Seattle, giving it the name of the technique. From the start of its use, the success was so big that today Western-blot is the most spread and informative technic in the field of biology research, even such as microscopy.

The Western-blot technic offers the way to detect and quantify unique protein forms with a high level of sensitivity. This depends of course of the preponderance of the target protein in the cell-tissue extract, as of the affinity of the antibody used. Therefore, considering only one protein, the limit of detection can be as small as 0.1 ng of protein.

Whatever, and as ever, like for all analytical techniques, numerous artefacts can exist. Of course, the quality of the antibody is essential. Its specificity (the unicity of the epitope) and its preservation are both important. Also, other questions are sources of artefacts. A typical example is the overloading of the target protein which can be a substantial problem and which is not known enough. If the amount of protein sample is too big, the quantity of the target protein can be over the one of the antibody (Figure 1). Then, the antibody will fix but not being in excess it may fix randomly and not precisely regarding the quantity of target on each lanes. It is therefore important to refer to a titration curve and never work at the plateau. To avoid this problem easily, it is very simple to load and analyze different amounts of extracts and check if the signal appears proportional. Another important point to be considered is how the protein of interest is spread on the gel/membrane, in order to avoid having too compact bands where the antibody will not diffuse easily in the middle of which, reducing the signal significantly. Then the target protein band should be spread enough, not to be too thin.

Of course, this rule is exactly the same concerning the secondary, enzyme-linked antibodies. For both, the range of detection could be finally of a range of two logs (100 times of sensitivity).

Among the few other sources of artefact, a second example, even rarest to encounter, is due to

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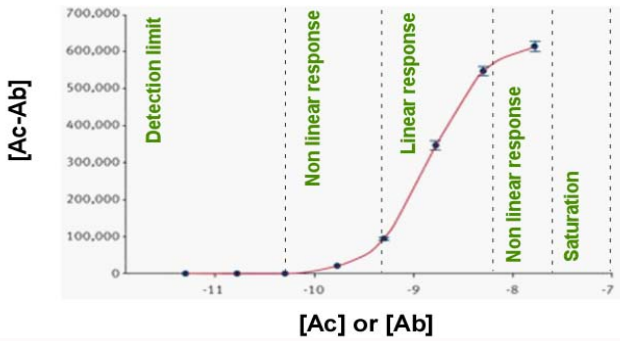

Figure 1: Ab/Ag titration curve. Theoretical Western-blot dose-response signal is presented. [Ac] or [Ab] are respectively the concentrations of antibody or of antigen used, [Ac-Ab] is the concentration of immune complex, corresponding to the Western-blot signal.

the use of anti-peptide antibodies, commonly used today. If raised against peptide, un-modified peptides containing a phosphorylation site, it is very probable that the antibody would not recognize the same epitope when phosphorylated, showing by the way how much anti-peptide antibodies can be specific [4].

A last and main example of artefacts, among the others, is the extraction/solubilization efficiency, the purpose of the technic presented here.

In most protein extraction protocol, all indeed, we perform a "cleaning" step, a clearing centrifugation just after the lysis. Either with RIPA buffer, or with all other lysis buffers, the pellet which is obtained here is of quite important size. This represents chromatin, with tightly bound-insoluble materials like could be transcription/replication factors, but also dense and membrane associated structures like could be insoluble supra-complex [5].

As a proof, a case of study on which I worked on for long time is the mitochondrial protein ATAD3 [6]. Probably involved in contact sites between endoplasmic reticulum and mitochondria, as well as in matrix systems, ATAD3 has been shown to be essential for mitochondrial biogenesis [7]. As it has been observed [8], ATAD3 is only half soluble in lysis buffer containing 0.5M NaCl and 1% of mainly used detergents, like Triton X-100. Also, as even a more well-known protein, some forms of p21-waf1 are also pelleted in these clearing procedures.

Of course, approaches like immuno-precipitations clearly required a clarified extract, not to spin down insoluble complexes while performing precipitation. Also, the affinity column procedure solves this problem properly.

Knowing this, it appears that for a quantitative measurement of a target protein a total protein extract is definitively required.

Using ATAD3 as an example of highly insoluble protein, we propose here a method for protein extraction without cleaning/clearing step, allowing a direct Western-blot analysis of whole total protein extracts, from biopsies and cultured cells.

Materials and Methods

Cells

The human U373 cell line used in this study were purchased from American Type Culture Collection (ATCC, USA). U373 cells were cultured in a Dulbecco's Modified Eagle's Medium (DMEM, Gibco

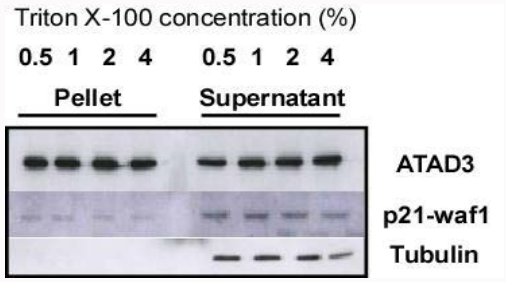

Figure 2: Solubilization as says for ATAD3 and p21-waf were performed with different concentrations of Triton X-100.

TM, USA) containing 10% FBS (Biowest, EU) at 37°C and 5% CO_2. Mouse tissues were taken from C57Bl6 male mice according to ethic rules.

Lysis buffer, whole cell lysis extract

Approximately 30 mg of biopsies (muscle, adipose tissue, liver, brain and heart), or 3.106 cultured cells (treated directly on dishes) are mixed with 10 volumes (W/V) of lysis buffer (SDS 1%, NaCl 150 mM, Tris 10 mM pH 8, protease inhibitor cocktail (Sigma™), bromo-phenol blue 1%), at room temperature (to avoid SDS precipitation). For cultured cells, the lysate is recovered from culture dishes with a rubber-policeman, transfer to Eppendorf™ tubes and proceed directly for the sonication step. For tissues biopsies, the lysis is proceeding using a dounce-homogeneizer until to see no tissue debris, then the sample is subject to sonication. Sonication is performed on ice at a power of 100 watts, for 3 times 15 seconds.

Spectrophotometric measurement

For protein concentration dosage, we proceeded as with normal samples (Biorad assay). We used BSA to obtain standard titration range assay, by diluting BSA in the lysis buffer. Lysis buffer is therefore use as the blank and protein concentration of samples can be extrapolated from BSA etalon curve.

Western-blot assays

Similar protein amounts, similar cells numbers or identical µg of biopsies can be load on the wells, depending of the normalization used. If disulfide bonds need to be reduced, β Mercapto-ethanol or DTT can be added to the sample before heating 5 min at 98°C and proceed to the standard Western-blot procedure.

Results

Analysis of the efficiency of the extraction technic

In order to show the efficiency and the necessity of the method in some cases, we studied the detection of ATAD3, because this protein is a typical integral protein case study, highly resistant to extraction/solubilization [8], and p21-waf1, a replication regulator that can associate tightly to chromatin complexes. To show that ATAD3 and p21-waf1 are currently partially resistant to extraction, we used U373 cells to compare the solubilization levels of these proteins after lysis and centrifugation in extraction buffer (NaCl 0.5M, Tris pH 8 10 mM and protease inhibitors) containing different concentration of Triton X-100. As we can see in Figure 2, ATAD3 is approximately 70% resistant to solubilization with 0.5% Triton. Also, 40% of ATAD3 resist to solubilization with 4% Triton. P21-waf1 was observed more soluble, but as it can be seen, approximately 20% remain in the insoluble material. As a control, Tubulin is shown completely soluble in these buffers.

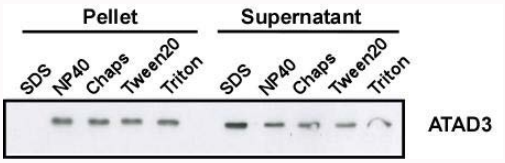

Figure 3: Solubilization as says for ATAD3 were performed with different detergents used at a concentration of 1%.

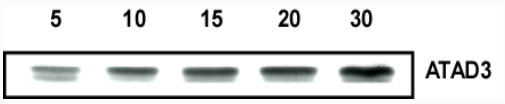

Figure 4: Quantitative analysis of ATAD3 immuno-detection using different amount of mouse brain biopsies (from 5 to 30 mg).

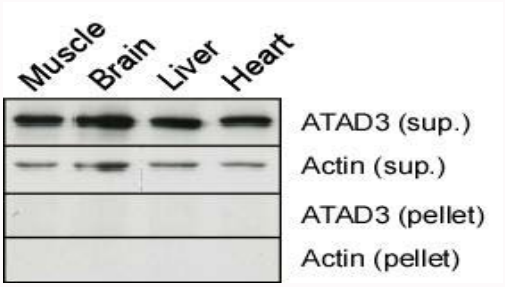

Figure 5: Qualitative analysis of ATAD3 immuno-detection extracted from different tissues. Supernatant and 10000g pellet of the Laemli lysis buffer were analyzed.

To explore deeper the question, we tested a set of commonly used detergents (SDS, NP40, Chaps and Tween 20) with the same lysis buffer (NaCl 0.5M, Tris 10 mM pH 7.5). As can be seen in Figure 3, ATAD3 resists to extraction by strong detergents, like Triton X100 and Chaps. Also, as can be expected, ATAD3 is soluble in 1% SDS.

Of course, the obligation to use high level of detergent (1%) and specially SDS or Chaps, is a problem because at 4°C they precipitate, avoiding thus reliable protein dosages. Then, the only inconvenient of the method is the need to work at room temperature. However, using protease inhibitors in the lysis buffer we never observed protein degradation following this process. Therefore, SDS is, as expected, a powerful detergent to extract ATAD3 from cell samples.

Concentration range

In order to show the reliability of the method, we extracted proteins from 5 mice brain samples of different weights (exactly approximately 5, 10, 15, 20, 30 mg/300 µl of 1% SDS-lysis buffer) and analyzed ATAD3 level of same volume (20 µl) of the 5 total extracts (loadings are therefore of 333, 666, 999, 1332 and 1998 µg each). As can been seen on Figure 4, the signals obtained appear proportional over the range of analysis, then reliable for the different amount of biopsies used here.

Quality of extraction in different tissues, cells

To compare the efficiency of the lysis in different tissues, we used muscles, liver, heart and brain from C57B6j mouse. To verify the efficiency of the extraction, a centrifugation was performed after lysis and both pellets and supernatants were analyzed by Western-blot. The result is presented Figure 5. As it can be seen with all organs, the extraction protocol allows the full recovery of ATAD3 proteins.

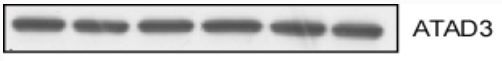

Figure 6: Qualitative analysis of ATAD3 immuno-detection extracted from different pieces of the same biopsies prepared independently.

Efficient dosage of proteins/tissue weight and efficient detection of different proteins

To better appreciate the reliability of the method, different extracts of the same biopsy (mouse muscle) were prepared independently and detected by Western-blot (Figure 6). As it can be observed, the Western-blot signal appears clearly the same for the different extractions, and the procedure appears therefore highly reproductive.

Discussion

Using this new technic for Western-blot analysis, by producing a whole cells/tissue lysate in SDS 1%, NaCl 150 mM and Tris 10 mM pH 8, we were able to prove that the extraction procedure is complete, even for highly insoluble proteins like ATAD3 or p21-waf1 proteins. This technic is efficient with all the organs tested (brain, muscle, liver and heart) and is highly reliable in term of repeatability. This method is quick and allows the measurement of proteins concentration even if the Bromophenol blue stain is used at start in the lysis buffer. No degradation was never observed, even working at room temperature, by the help of high detergent level as of protease inhibitors cocktail.

Since the whole/total extracts contain DNA, the sonication step could be necessary if performing a highly concentrated lysate or optional for low concentrated lysates. However, this step is very rapid. As shown, the ratio “biopsy mass”/“lysis buffer volume” is fine for a range of5 to 30 mg/300 µl of lysis buffer, but this can be up to 100 mg/300 µl for a low represented protein target. Finally, reducing agent can be used or not during the extraction procedure.

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