

# Chapter 2

## Antibodies That Work Again and Again and Again

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### Abstract

In the past few years significant concern has been raised about the quality and reproducibility of antibodies used in numerous scientific publications. In this chapter we discuss some of the biggest contributing factors to the “antibody problem” from both the commercial production side, as well as the end-users side. Specifically we argue that Western blot data should be used to provide a reliable initial indication of antibody quality, as well as a guide to distinguish between multiple offerings for antibodies to the same target. Secondly, we describe a set of best practices for antibody manufacturers to employ that will eliminate most of the variability in polyclonal antibodies. Taken together these proposals provide a way to significantly improve both the quality and the reproducibility of commercial antibodies.

**Key words** Antibody validation, Antibody specificity, Antibody variability, Western blot, Recombinant proteins, Cell lysate, Phospho-specific antibodies, Lot-to-lot variations

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### 1 Introduction

In the past few years there have been a number of articles in several major journals that discuss what has become known as the “antibody problem” [1, 2]. In addition there have been numerous other articles that identify specific antibodies which do not exhibit specificity for their expected target [3–6]. These articles focus heavily on the significant reproducibility and quality issues experienced with commercial antibodies and the lack of validation done to ensure consistency when working with commercial antibodies. These reports typically argue that insufficient antibody validation was performed in the original publications and only later when proper validation methods were employed did it become apparent that the antibodies used in the original publications lacked specificity.

An additional factor contributing to the “antibody problem” is that even when an antibody is found to be specific, the variability of different lots, or batches, of that antibody makes it virtually impossible to reproduce previous work with the antibody [2, 7, 8]. These considerable problems with lot-to-lot variability and antibody quality have been implicated as playing an important role in the

reproducibility crisis identified by Begley and Ellis [9]. Therefore, both antibody validation and antibody variability must be dealt with in order to resolve the irreproducibility issue in studies using antibodies.

Some have argued that the problems with antibodies are so severe that an entirely new approach to antibody production, namely the use of recombinant antibodies, should be employed. In our opinion, such an untested approach that will cost billions of dollars is not warranted. Recombinant antibody technique has been in use for more than two decades and very few antibodies that have been generated with this technique perform better than or even as well as antibodies that are prepared as traditional polyclonal or monoclonals. In a project cited in a recent article in *Nature* [1], reagents used in 10,000 biomedical papers published since 2006 were examined. In these studies polyclonal antibodies were used in 1293 studies, monoclonals were used in 755 papers and there was only one paper in the 10,000 that used a recombinant antibody!

We argue that there are straightforward changes that can be made in classical antibody production and characterization that will go a very long way towards ameliorating many of the antibody problems. In this chapter we focus on the type of changes we envisage to address the “antibody problem”. We concentrate in this review on measures that should be undertaken to improve the quality and the reproducibility of commercial antibodies. Thus, we propose a set of best practices that antibody manufacturers should follow to help to address the “antibody problem.” We also provide a set of guidelines that should enable antibody users to identify high quality antibodies that will provide consistent and reproducible results for end-users. In so doing we do not attempt to provide a comprehensive review of complete validation protocols. Such protocols are clearly application specific. Moreover, resolving the many controversies concerning what constitutes antibody validation in techniques such as immunohistochemistry (IHC) and immunoprecipitation (IP) is simply beyond our scope. Rather, we are proposing not perfect solutions but rather a very good set of straightforward solutions to the “antibody problem” that are readily accessible to virtually all antibody users and manufacturers.

As discussed above, there are two main issues contributing to the “antibody problem”: antibody validation, and antibody variability. In the next two sections to follow we first discuss antibody validation and then turn to antibody variability in Subheading 3.

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## 2 Antibody Validation

We have been working with antibodies for quite some time and have had many opportunities to observe antibodies that do and do not work, or antibodies that react specifically with the target of interest and those that do not. When antibodies work, the outcome often

leads to the development of important new insight into a specific protein's role in normal or disease function. In contrast, months of frustration and false leads are usually all that results when using antibodies that do not work. What is desperately needed is a good way for antibody users to identify antibodies that work. In the pages to follow we try to provide a very useful way to address this need.

## **2.1 How to Determine if an Antibody Works**

There are four basic procedures that have typically been used to validate the specificity of the antibody. These are Western blot (WB), knockout, double antibody, and IP coupled with MS. Of these, the WB is by far the most widely used measure of antibody specificity [10–15]. Fortunately almost any lab is able to do a WB of the cell or tissue lysate of interest or is able to get help from a colleague to perform this validation test. Thus, WB provides an absolutely critical form of antibody validation; as it is the only one of the four possible validation techniques that is available to almost all antibody users.

The primary value of the WB is that one is able to detect not only antibody binding, but also the  $M_r$  of the protein that is being bound. Many other methods for labeling tissue fail to provide an independent confirmation that the antibody signal being generated is due to reaction with the protein of interest. For imaging techniques such as IHC, immunofluorescence (IF), immunocytochemistry (ICC) etc. the immunolabeling signal may reflect binding to the protein of interest or to another protein that is distinct from the protein of interest. These methods have no internal control to validate the specificity of the binding in the image. Similarly ELISAs, flow cytometry, and immunoprecipitation (IP) lack internal controls that allow for confirmation that the signal exhibited in the assays reflects the presence of the protein of interest, rather than an interaction with some other nonspecific protein.

An additional critical function of WBs is to provide information about whether the antibody also binds isoforms or nonspecific proteins in addition to the protein of interest. This information provides useful insight into the antibody's suitability in other applications. While not a perfect measure, WB results have widely been shown to predict the success of an antibody in other applications [10, 12, 16–20]. In addition, weak labeling present in WB typically is correlated with weak staining in IHC [10]. Lastly, WBs can be performed in almost any laboratory and users can expect that every antibody manufacturer can provide WB data for every antibody they sell. Consequently one should question the specificity of any antibody product that does not show WB data in its product data sheet.

## **2.2 What Does It Mean When an Antibody “Does Not Work” in Western Blots?**

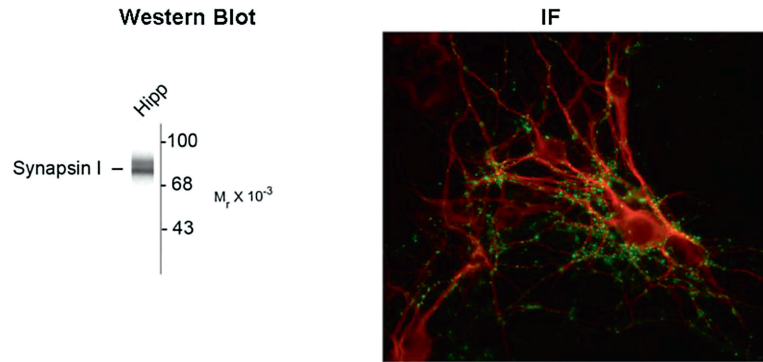
We started wondering about this question after a colleague told us about a company that “validates” all of their antibodies by immunostaining. We examined the company's website and in some cases WBs showing the labeling of a single band were also presented. But, for the vast majority of products on the site, only the

apparently innocuous phrase “does not work in WB” was seen. What does this mean? It is our opinion that for many antibody users (especially people who do not do WB and use various immunostaining protocols like IF or IHC) this failure to “work” in WB is often interpreted as something akin to “don’t worry about the WB data, see if the antibody ‘works’ in immunostaining.” However, any end-user of an antibody that “does not work in WB” should be very worried indeed. This is because in the overwhelming majority of cases “does not work in WB” means the antibody labels many different proteins in a WB. It is extremely rare to get an antibody that labels nothing in a WB. So remember when you read the words “does not work in WB” it should be translated as “this antibody probably cross reacts with a number of different proteins.”

WBs are by no means a perfect validation tool. In some cases, antibodies may work in WB and not in staining or the converse may be true [3, 4, 6]. Moreover, a WB may show labeling of multiple proteins; while under different imaging conditions an antibody may label only the protein of interest (as confirmed for example by imaging material from knockout animals). These caveats do not however diminish the strength of the foregoing arguments. In a single experiment, a WB can provide critical information regarding an antibody’s specific reactivity with the protein of interest. As such, it provides convincing initial data about an antibody’s specificity or lack thereof towards its protein of interest.

For the reasons cited above, we argue that WB is the best method to use as a first step in validating a new antibody. Moreover, virtually every antibody supplier can perform WBs on their antibodies. WB data can provide a reliable indication of the antibody quality, as well as a guide to distinguish between multiple offerings for antibodies to the same target. A smart antibody user will always check to see WB data before buying an antibody. We recommend purchasing only those antibodies that show specificity with WB data. Moreover, if an antibody supplier indicates that the antibody does not work in WB but offers only an IHC image as validation, **BUYER BEWARE!**

Given the aforementioned, it is remarkable that so many immunostaining papers are published with no antibody validation at all, not even a simple WB. The failure to use WBs as a validation tool for immunostaining most likely results from a widespread misunderstanding in the research community about the relevance of WB data to immunostaining. Some have suggested that the detection environment in WB, with the denaturing effects of SDS, is so different from that in immunostaining as to make WB data irrelevant to immunostaining. However, as emphasized by Forsström et al. [21] it is important to understand that both WB and IHC use denaturing conditions. While exceptions do exist [3, 4, 6], a large body of evidence points to a high correlation between positive WB data for an antibody and good data for the same antibody in IHC,

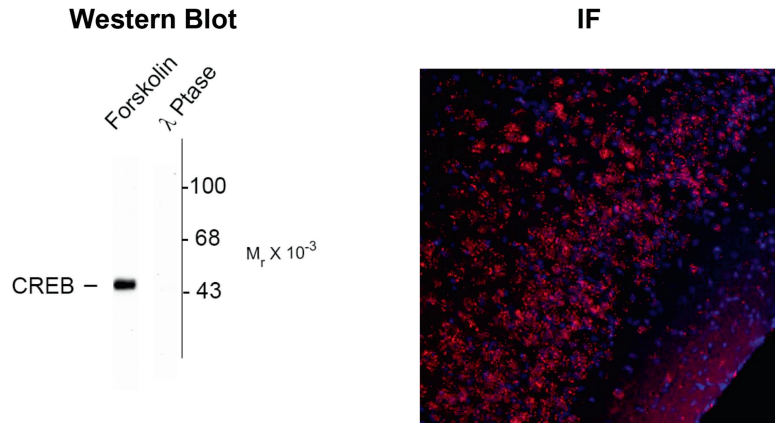


**Fig. 1** Labeling of the same synapsin I polyclonal antibody in Western blot and immunofluorescence (IF). At the left a Western blot of 10  $\mu$ g of a hippocampal lysate (Hipp) showing specific immunolabeling of the ~78 kDa synapsin I doublet (Synapsin Ia and Ib). The image at the right shows immunofluorescent labeling of cultured caudate neurons showing punctuate synaptic labeling of synapsin I (*green*) and diffuse cellular labeling of microtubule associated protein (*red*). Cells and photo courtesy of QBM Cell Science

ICC and IF [12, 14, 16, 17]. As argued by Kurien et al. [14] “immunoblotting is a must to determine specificity of antibodies used for Immunohistochemistry (IHC).” The Journal of Endocrinology and the Journal of Histochemistry and Cytochemistry both editorialize in favor of using WB as an initial antibody screen [22, 23]. Both argue that any antibody that yields multiple bands in WBs raises a critical red flag and that the antibody should not be used in IHC unless some other test can be used to validate the antibody.

After making over 500 antibodies over the past few decades, we have found that more than 90% of the antibodies that gave a single band signal in WB also gave a good signal immunostaining. See for example Fig. 1 where WB and IHC staining of an antibody to synapsin I, a neuron specific synaptic vesicle associated protein, is shown. As shown clearly in the figure, the synapsin antibody specifically labels only the synapsin I doublet in the WB. Similarly the IF image shows the same synapsin antibody exhibiting specific punctuate labeling characteristic of the localization of the synaptic vesicle associated protein. Similarly Fig. 2 shows WB and IHC staining of a phosphospecific antibody for ser133 of CREB, a cellular transcription factor. As with the synapsin antibody the phospho-CREB antibody exhibits specific labeling in both the WB and in IF in the rat pyriform cortex.

So in summary, antibody validation by WB is certainly not perfect. However, it is important to realize that WBs provide very important validation tool particularly given the fact that no other validation method is available for most targets. The ideal antibody validation tool is of course the use of knock out animals in the



**Fig. 2** Labeling of the same P-CREB Ser133 polyclonal antibody in Western blot and Immunofluorescence (IF). At the left a Western blot of 15  $\mu$ g of rat hippocampal slice lysate showing phospho-specific immunolabeling of the ~45 kDa CREB. The lysate from slices incubated in forskolin is shown in the left lane. The lysate incubated in  $\lambda$  phosphatase is shown in the right lane of the Western blot. The figure at the right shows immunofluorescent staining of rat pyriform cortex showing extensive labeling of P-CREB Ser133 in *red*. Nuclear staining of DAPI is shown in *blue*

immunological methods of interest (however *see* [24, 25] for some limitations on the use of knockouts in antibody validation). Thus when knockouts are available they should almost always be used in preference over WB. Unfortunately, knockouts are available for only a very small percentage of the protein targets of interest. Consequently, it seems illogical to let the fact that WB validation is not a perfect validation tool to limit its use as a very good antibody validation tool. This is particularly true at a time when the results of many antibody studies being published use antibodies with little or no validation, leading to data that is flawed and cannot be reproduced. Having said that, if WBs are going to be used as a validation tool it is essential that best practices be utilized in the WB assay. In the section below we discuss these best practices and how to avoid pitfalls in using WB for antibody validation.

### 2.3 Why Recombinant Proteins Make Poor Antibody Validation Tools

Antibody validation is a topic that has garnered a great deal of attention lately in discussions of the problem with the lack of reproducibility in science. One type of WB antibody validation that should be avoided uses purified recombinant proteins. In this type of study a purified recombinant protein is run on a WB and then the labeling of the new antibody is examined. Given that only a single protein is run on the blot, this “validation” study offers very little information about the specificity or sensitivity of the antibody. Additionally, the blot obviously provides no information



about whether the antibody recognizes other nonspecific proteins since no other protein is present in the blot. Moreover, absent any information about the relevance of the amount of recombinant protein used compared to the amount of endogenous protein in situ, the experiment does not even validate the ability of the antibody to bind to the protein of interest in a tissue of interest. Consequently, this WB method should not be used to demonstrate antibody quality.

A similar issue occurs in experiments in which a target protein is overexpressed in cells that do not normally contain the protein [26]. In such experiments the lysate containing the overexpressed protein is examined via WB along with the control non-expressing cells. This protocol has the advantage of a negative control in the non-expressing cells and this is certainly a plus. However, within overexpressing cells protein expression is invariably present at levels far beyond that seen endogenously. Accordingly, little information can be gained about the ability of the antibody to specifically label the protein of interest in situ.

Thus WBs with a recombinant protein or an overexpressed protein do little to answer the two key questions about antibody quality: (1) Does the antibody possess the sensitivity to recognize the antigen endogenously in a tissue of interest? and (2) Is the antibody binding specific for the antigen of interest in situ? Nevertheless it is not unusual to see such data used to validate an antibody in product data sheets or even in refereed publications. **So it is important to look carefully at any antibody validation blots to be sure that a cell lysate and not simply a purified or an overexpressed protein is being analyzed.**

In the example above we described a situation in which a recombinant protein based assay was used to give a false positive validation. It is also not uncommon to see a recombinant protein based assay provide a **false negative** result, i.e., to falsely invalidate an antibody. Such experiments are most commonly seen with phospho-specific antibodies. Such antibodies can be extremely valuable tools as they permit one to evaluate the phosphorylation state of a single phosphorylation site on a specific protein. A critical question in the validation of such antibodies is whether they are indeed phospho-specific. A recombinant protein with the phosphorylation site of interest mutated to a non-phosphorylatable amino acid is run alongside the recombinant protein in a WB. The antibody of interest is then tested for binding in this assay. The binding of an antibody to the non-phosphorylatable mutant in this type of an assay has been used as evidence to invalidate the antibody's phospho-specificity. However, this type of data does not actually substantiate these claims. A phospho-specific antibody will always have at least a finite affinity for the non phospho-site.

Thus when protein levels are saturated with tens of micrograms of the mutant protein, some binding is highly likely. In order to use such an assay for validation it is necessary to do a very detailed dose response with multiple concentrations of both the phosphorylated and the non-phosphorylated mutant protein. Meticulous attention must also be paid to the endogenous concentration of the protein of interest in situ and also its level of phosphorylation at the site of interest in the target tissue. Determining these values is always quite problematic. Accordingly, the use of such a validation technique is not recommended particularly when other, much more relevant, validation assays are available.

The most common assay to test for phosphospecificity is a WB performed with control and phosphatase treated endogenous tissue lysates. In this assay the antibody is validated if it labels a single band at the correct  $M_r$  in the control lysate and if the labeling is absent in the phosphatase treated lysate. This very straightforward form of validation can also provide detailed quantitative information about total and phosphorylated protein levels when whole tissue sample are used.

One issue raised in a preceding paragraph is the fact that a finite affinity of a phospho-specific antibody for the non-phosphoprotein will always exist. Such binding of the phospho-antibody to the non-phosphorylated protein can be quite problematic during the production of a phospho-specific antibody. When purifying a phospho-specific antibody, occasionally a very good phospho-specific antibody may fail to flow through a non-phosphorylated peptide column due to its nonspecific binding to the column. To avoid failures in this kind of negative selection experiment, it is essential to optimize the antibody to peptide ratio on the non-phosphorylated affinity column.

**Summary** In summary we argue that significant progress in antibody validation could be achieved by some rather straightforward actions by both antibody manufacturers and antibody users. We urge antibody manufacturers to always provide WB data on every antibody. A WB of a tissue or cell lysate is the most widely used test of antibody specificity [10–15]. Additionally, WBs can readily be performed by every antibody manufacturer. Antibody users should follow the advice of numerous authors including editorials in the *Journals of Histochemistry and Cytochemistry*, as well as *Endocrinology*, and preferentially select antibodies that give a single band at the appropriate  $M_r$  in a WB of a cell or tissue lysate. However, even if one can find a good antibody, a critical issue with antibody variability remains. The next section attempts to address that issue.



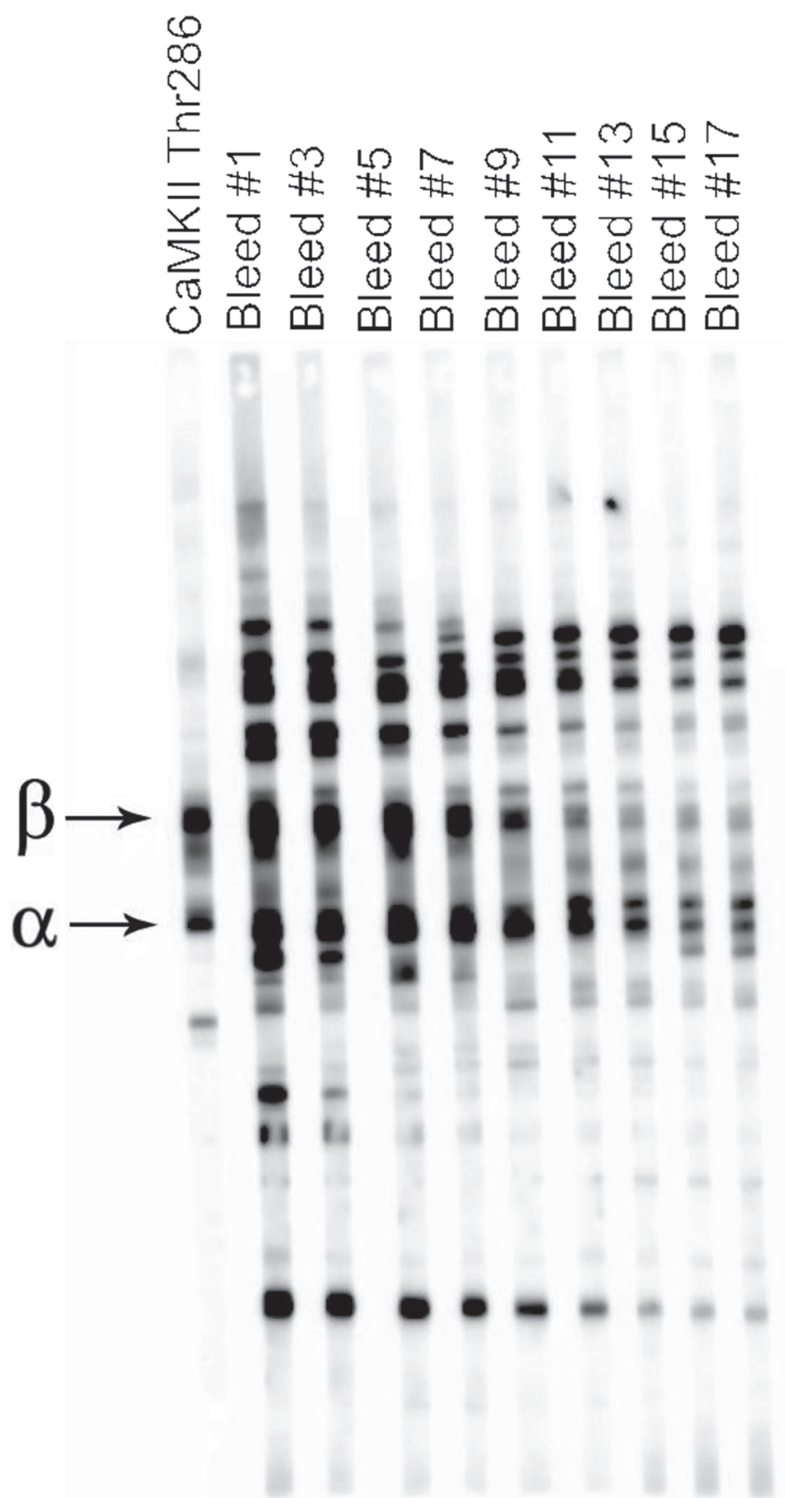
### 3 Antibody Variability

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In the preceding section we focused on antibody quality and described how WBs provide a critical initial assessment of an antibody's specificity for its target protein. In the current section we discuss how to address the second critical issue with antibodies, namely antibody variability. Antibody variability has been repeatedly cited as a critical issue plaguing reproducibility in research over the past several years [1, 2].

A typical issue with antibody variability occurs when a new good antibody is created and a strong demand develops for the antibody. Subsequently numerous antibody manufacturers take notice and create a competing antibody. This in turn creates an oversaturated market of monoclonal and polyclonal antibodies directed towards the same target. These antibodies, however, are inherently different from the original because they do not come from the same animal as the original antibody; therefore validation performed on the original antibody does not pertain to these new antibodies. One way to deal with this problem was recently suggested by Andrew Chalmers and his colleagues [7]. They argue that all publications using commercial antibodies should report the name of the supplier and the catalog number of the antibody used. That way even if a supplier sells many varieties of the antibody a researcher will be able to order the same antibody that was used in the publication. Subsequently Bandrowski et al. [8] proposed an even more detailed and efficient antibody identification protocol with their Research Resource Identifiers (RRIDs), which are based on accession numbers assigned by an authoritative database. These suggestions are being incorporated into the instructions to authors in more and more journals to help others to replicate and expand on results.

Even though these actions would enable researchers to more readily identify an antibody used in a particular publication, significant sources of variability still exist with antibodies that have the same catalog number. The first such source of antibody variability is lot-to-lot or batch variability. This type of antibody variability occurs when one buys the same antibody with the same catalog number from the same manufacturer, yet still encounters large variability in different lots of the antibody. Lot-to-lot variations in polyclonal antibodies are due primarily to the fact that different lots of an antibody are often obtained from different bleeds of the same animal. The titer and specificity of antibody present in polyclonal serum varies significantly from one bleed to another. The typical extent of bleed-to-bleed variation can be seen in Fig. 3, where several bleeds from one rabbit were compared to one another. The intensity of immunolabeling of the alpha (~50 kDa) and beta (~60 kDa) subunits of Calcium Calmodulin Kinase II



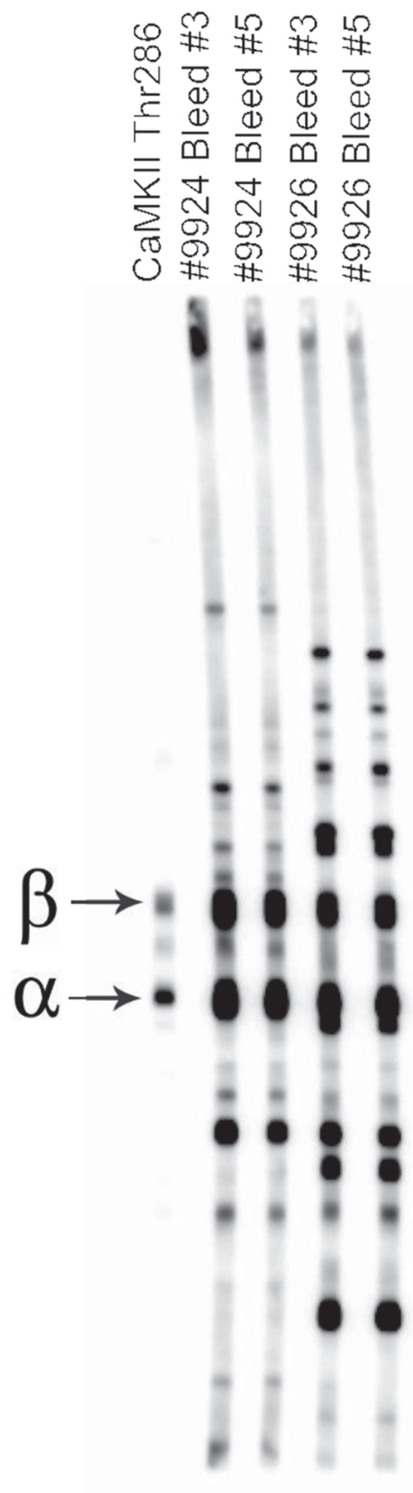
**Fig. 3** Differences in immune response demonstrated through various bleeds of the same animal. Western blot showing differences in serum collected at varying times of one rabbit's lifespan. Marked differences in specific labeling of the  $\alpha$  (~50 kDa) and  $\beta$  (~60 kDa) subunits of Calcium Calmodulin Kinase II (CamKII) phosphorylated at thr306 can be seen across the several bleeds. Additionally, significant variances in cross-reactivity with other nonspecific proteins can also be seen in the different bleeds

(CamKII) phosphorylated at thr306 varies dramatically across the several bleeds, as does cross-reactivity with other nonspecific proteins present in the serum. Even if affinity purification of the serum is performed, the marked differences in starting material often result in large differences in the concentrations of both specific and nonspecific antibodies in the affinity purified material. Even more dramatic variability in the same catalog number can occur when a manufacturer substitutes antibodies from a different animal entirely. As shown in Fig. 4, two rabbits immunized with the same immunogen directed at CamKII phosphorylated at Thr306 produce very different immune responses. Animal #9924 serum has far less nonspecific banding present than animal #9926. In order to limit confusion and reproducibility concerns, if new animals are being used to recreate an antibody, a new catalog number and new RRID should be issued for the product as it cannot be identical to the original antibody.

### **3.1 Straightforward Solution**

Although some have argued that this key issue of lot-to-lot variation experienced with polyclonal antibodies cannot be solved and therefore researchers must buy up as much of a good working antibody as they can get their hands on [2, 22], there is actually a very straightforward solution to eliminating polyclonal antibody variability. By screening each bleed from immunized animals and pooling the serum that shows good labeling for the desired target, a large homogeneous stock of serum can be created for each product. Instead of purifying from one bleed to the next and not knowing what the yield and quality of antibody will be, purifying from a homogeneous pool of serum would ensure that the quality and yield of antibody was virtually invariant. Thus basically all lot-to-lot variation can be eliminated by purifying each lot of a particular antibody from its homogeneous pool of serum. It is important to note that for most antibodies a single rabbit can produce a stable 10–15 year supply of antibody. However, in the rare case when the pool of antibody does become exhausted, it should go without saying that any new antibody generated for the target should be assigned a new catalog number and new RRID should be issued for the new product.

There are some who argue that the only way to truly solve the reproducibility crisis is for researchers to discontinue the use of polyclonal antibodies entirely and instead only use monoclonal or recombinant antibodies because of a flawed idea that monoclonal and recombinant antibodies possess an innate homogeneity and specificity that can easily be controlled [22, 28, 29]. Variability with monoclonal antibodies happens more frequently than was first assumed and it seems unclear whether the antibodies are in fact exactly the same from year to year [2]. Though it is often assumed monoclonal antibodies are more specific than a polyclonals [29, 30] because monoclonals are derived from a single B



**Fig. 4** Differences in immune response demonstrated through various bleeds of two different animals. Western blot showing differences in serum collected on two separate dates from two rabbits (#9924 and #9926) immunized with the same antigen corresponding to amino acid residues surrounding the phosphor-Thr<sup>306</sup> found in rat CaM Kinase II. Significant difference in specific labeling of the  $\alpha$  (~50 kDa) and  $\beta$  (~60 kDa) subunits of Calcium Calmodulin Kinase II (CaMKII) phosphorylated at thr306 can be seen between the two animals. Additionally, varying patterns of nonspecific banding can be seen between the two rabbits. Rabbit #9926 has a substantial amount of nonspecific banding that is not present in the bleeds from rabbit #9924

lymphocyte clone, numerous studies have shown that polyclonal antibodies often exhibit superior specificity [29, 31, 32]. This has sometimes been attributed to polyclonal antibodies' heterogeneous nature and their ability to recognize a variety of epitopes, making them less susceptible to slight changes on a single or a small number of epitopes [29].

An additional argument against abandoning polyclonal antibodies and switching to solely monoclonal and recombinant antibodies is the cost and time involved in producing these types of antibodies. Custom monoclonal or recombinant antibody production is most often far more expensive when compared to the cost of creating custom polyclonal antibodies. Furthermore, production time of monoclonal antibodies is typically more than twice as long as that for polyclonal antibodies. It is hard to justify the significant increase of cost and production time of monoclonal and recombinant antibodies when the specificity and versatility of them is often equitable and in many cases has even been shown to be inferior to that of polyclonal antibodies [29, 32]. And finally, more users clearly prefer polyclonal antibodies as shown in the study cited in *Nature* [1] where over 2000 published papers using antibodies were examined and of these 65% used polyclonal antibodies, 35% used monoclonal antibodies, and only .05% used a recombinant antibody.

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## 4 Best Practices for Antibody Use

Even when an antibody has been validated extensively, major problems in studies using such an antibody may occur if experimenters fail to use best practices. In particular there are numerous procedures involved in preparing the samples for antibody analysis that can have dramatic effects on the expression or localization of the protein target to be analyzed with antibodies. If these procedures vary within an experiment so too may the antibody signal obtained. In this final section we review some of the best practices and also key sample preparation procedures that should be utilized. It cannot be emphasized enough how important it is to keep these various conditions constant within experiments. Moreover, it is essential for reproducibility that all of these procedures (many of which are thought to be routine and relatively unimportant) be completely described so others can follow the procedures correctly. It is not enough to simply have an antibody that works, one must also be able to replicate the procedures used with the antibody in question.

### 4.1 *Animal Sacrifice*

The method in which an animal is euthanized or a cell line is harvested can greatly and differentially affect expression of individual proteins within the sample [33]. The differing effects of sacrifice on a protein's expression are of even greater concern when testing

for post-translation modifications such as phosphorylation. A number of studies have shown that the method of sacrifice (e.g., CO<sub>2</sub> narcosis or cervical dislocation), the absence or presence of anesthesia and the time elapsed after sacrifice may increase or decrease protein expression levels and/or levels of protein phosphorylation [34–40]. In order to limit these factors it is very important to precisely control the method of sacrifice and the time elapsed from sacrifice to tissue solubilization. It is also critical to use a lysis buffer that effectively eliminates alterations in protein expression and phosphorylation. We recommend the following lysis buffer for solubilization of all samples to be subsequently analyzed in WB.

1 % SDS.

10 mM Tris-HCl pH 8.0.

1 mM EDTA.

Buffers with less SDS such as RIPA (0.1 % SDS) and others with only non-ionic detergents should be scrupulously avoided as they fail to terminate protein degradation and dephosphorylation and also fail to completely solubilize some proteins, especially proteins in the synaptic junctions in brain [41].

## **4.2 Tissue Harvesting**

When working with cell lines, the technique used to harvest cells is also a potential source for significant variation in data. When cells are harvested enzymatically by trypsinization or mechanically by scraping, it takes a number of minutes to completely detach the cells from the dish. During this time membrane permeability, protein phosphorylation, and expression and metabolic activity all can undergo modifications [42–44]. In order to block any changes in protein expression or phosphorylation while harvesting cells, it is recommended that the following protocol be used.

1. Aspirate off cell media and discard.
2. Wash cells with PBS, aspirate and discard. Add appropriate amount of heated (90 °C) buffer to completely cover plate surface.

Lysis buffer:

1 % SDS.

10 mM Tris-HCl pH 8.0.

1 mM EDTA.

3. Aspirate lysed cells into small tube.
4. Rinse plate with small amount of lysis buffer to remove any remaining adherent cells.
5. Sonicate cells for 5 s.
6. Heat sample at ~95 °C for 10 min.



### **4.3 Subcellular Fractionation**

Proteins in their native states are embedded in their natural environments where they are associated with other proteins, biological macromolecules and other matrix materials [45]. Subcellular fractionation of cellular material is an extremely useful process for exploring these protein associations. When performing such fractionation it is obviously essential to avoid any treatments that will alter protein association such as SDS or other solubilizing agents. However, this also makes it possible for changes in protein expression, phosphorylation, and/or association to occur during the fractionation. Consequently it is critical to keep fractionation time constant for all comparisons and to attempt to minimize any alterations during the fractionation by using low temperature, protease and phosphatase inhibitors whenever possible. Most importantly it is essential to solubilize the resultant fractions in the SDS lysis buffer (1 % SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) as soon as possible at the end of the fractionation. When testing with purified fractions it is essential that absolute yield and/or amount of protein lost are taken into consideration when trying to determine quantitative information, otherwise any data gather can only be considered qualitative [13].

### **4.4 Protein Solubilization**

Proteins are generally insoluble and must be denatured in order to bring them into solution [45]. Solubilization buffers typically contain a number of additives (chaotropes, detergents, reducing agents, buffers, salts, and ampholytes) and previous research has shown that the composition and gross physicochemical properties of the lysis buffer can significantly alter the solubility of proteins [45]. Additionally, the physicochemical properties of proteins such as average charge and molecular weight have been shown to greatly affect a protein's solubility [45, 46]. However, when planning to use antibodies in WB the key issue is efficient solubilization of the cellular proteins and rapid termination of any protein degradation and/ or dephosphorylation. Thus, it is very important to avoid lysis buffers that contain only non-ionic detergents, as such detergents fails to terminate proteolysis and dephosphorylation and they also fail to solubilize many proteins, especially proteins in the synapse [41]. Even RIPA buffer, which does contain 0.1 %, fails to completely solubilize synaptic proteins. Therefore, we recommend the above cited lysis buffer (1 % SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) for solubilizing all samples to be tested in WB.

### **4.5 Western Blots**

As discussed in Subheading 1 above, WBs are an extremely valuable tool for antibody validation. However, they can also be used to compare various experimental samples in absolute quantitative and semiquantitative terms. In order for both validation and quantification to be obtained in WB, it is essential to both follow best practices and to also adequately describe such procedures in the Methods. This will also benefit the reproducibility of such research. Below we discuss some of these best practices.

#### 4.5.1 *Transfer Efficiency*

A frequently overlooked variable in WB experimental design is the variation that may occur in protein transfer efficiency. Differences in transfer efficiency have been shown to result in a two- to fourfold increase or decrease in signal between gel lanes [47]. If excessive protein is loaded on a gel, it begins to layer on top of the protein already bound to the surface of the transfer membrane. Since primary antibodies will bind primarily to the surface layer of the transferred protein, this further hinders the ability to quantify the total amount of target protein present in a sample [48]. It is important to take these factors concerning transfer efficiency into account in order to decrease variability and increase reproducibility of data collected.

#### 4.5.2 *Blocking Buffer*

Another potential source of variability when using antibodies in Western blotting originates from the type of blocking buffer used. Before any of the transferred proteins can be probed with antibodies for detection, the membrane they were transferred onto must be incubated in blocking buffer to saturate any free binding sites to prevent noise created from nonspecific binding of the primary antibody [30]. The most common blocking buffers consist of either 3–5 % BSA or 5 % nonfat dried milk (NFDM) diluted in TBST. While NFDM is often preferred, milk contains casein, which has been shown to interfere with some results [30]. Due to this, BSA is often considered a preferred blocking buffer when working with phosphoproteins [30]. However, in some cases blocking with BSA can lead to detection of additional banding that is not present when same sample were blocked with NFDM [15]. When working with a new antibody it is recommended that both BSA and NFDM be tested to determine which is buffer gives the best signal strength and quality.

The use of housekeeping proteins, such as GAPDH, beta actin and tubulin, as loading controls is another factor that may lead to variability within Western blotting results [15, 48]. Due to their relatively high abundance, housekeeping proteins have a limited dynamic range and are not linear at high protein concentrations [15, 48, 49]. Additionally, housekeeping proteins can be variably expressed between the experimental conditions, thus their usefulness for the normalization of Western blots has been brought into question [48]. In order to avoid these issues, it has been proposed that the total lane density of transferred protein on the membrane be used for normalization purposes [48, 50, 51].

#### 4.5.3 *Quantitative Western Blots*

Obtaining true quantitative analysis of a target protein requires careful consideration of the total amount of protein loaded. A common mistake made in WB is loading too much protein, often times in an attempt to successfully detect lowly expressed proteins [13, 15, 48]. Though this rationale is understandable, a number of studies have shown that quantitative analysis of poorly expressed

proteins can often be obtained when smaller amounts of protein are loaded [13, 15]. Additional research has demonstrated that above certain loading levels the band intensities observed actually underestimate the amount of protein loaded [13]. Loading excess protein also increases the chance of nonspecific binding of antibodies [15]. Calibration curves are extremely useful in determining the appropriate amount of protein to load in order for genuine qualitative analysis to occur [13].

Determining the upper and lower limits of detection for each experiment is a very important step in quantifying data. *Absolute quantification* of a target protein present in a given sample can be obtained by using a calibration curve consisting of known amounts of the purified protein from the same host species (e.g., recombinant or purified from tissue). When using this method it is also necessary to validate the WB system in order to make sure that the protein in both its purified form and that present within the sample are detected with similar efficacy. Once validated, the system can be used to determine the absolute amount of the given protein present in a sample by comparing it to the standard curve generated using known amounts of the protein of interest [13].

Two approaches can be used in order to determine *relative quantification*. First, a range of known standards (e.g., different masses of a particular tissue homogenate) can be run on the same gel as the samples of interest, and the band densities of samples A and B then compared to those on the calibration curve constructed from the range of standards. Alternatively, a 3–5 point calibration curve can be created using the samples themselves and the slopes of linear regressions for the two samples compared [13].

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## 5 Summary

In the past few years significant concern has been raised about the quality and reproducibility of antibodies used in numerous scientific publications. In this chapter we discuss some of the biggest contributing factors to the “antibody problem” from both the commercial production side as well as the end-users side. Specifically we argue that Western blot data should be used to provide a reliable initial indication of antibody quality, as well as a guide to distinguish between multiple offerings for antibodies to the same target. Secondly, we describe a set of best practices for antibody manufacturers to employ that will eliminate most of the variability in polyclonal antibodies. Taken together these proposals provide a way to significantly improve both the quality and the reproducibility of commercial antibodies. This is admittedly not a perfect solution to the antibody, but we believe it is a very good start.

## References

1. Baker M (2015) Antibody Anarchy: A Call to Order *Nature (Lond)* 527:545–551
2. Marx V (2013) Finding the right antibody for the job. *Nat Methods* 10:703–707
3. Yu W, Hill WG (2013) Lack of specificity shown by P2Y6 receptor antibodies. *Naunyn Schmiedebergs Arch Pharmacol* 386:885–981
4. Herrera M, Sparks MA, Alfonso-Pecchio AR et al (2013) Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor protein. *Hypertension* 61:253–258
5. Milner R, Wombwell H, Eckersley S et al (2013) Validation of the BRCA1 antibody MS110 and the utility of BRCA1 as a patient selection biomarker in immunohistochemical analysis of breast and ovarian tumors. *Virchows Arch* 462:269–279
6. Back JH, Darlington CL, Smith PF et al (2013) Antibody testing for brain immunohistochemistry: brain immunolabeling for the cannabinoid CB(2) receptor. *J Neurosci Methods* 216:87–95
7. Helsby MA, Fenn JR, Chalmers AD (2013) Reporting research antibody use: how to increase experimental reproducibility. *F1000Res* 2:153
8. Bandrowski A, Brush M, Grethe JS et al (2015) The resource identification initiative: a cultural shift in publishing. *F1000Res* 4:134
9. Begley CG, Ellis LM (2012) Drug development: raise standards for preclinical cancer research. *Nature (Lond)* 483:531–533
10. Algenas C, Agaton C, Fagerberg L et al (2014) Antibody performance in western blot applications is context-dependent. *Biotechnol J* 9:435–445
11. Burry RW (2011) Controls for immunocytochemistry: an update. *J Histochem Cytochem* 59:6–12
12. Egelhofer TA, Minoda A, Klugman S et al (2011) An assessment of histone-modification antibody quality. *Nat Struct Mol Biol* 18:91–93
13. Murphy RM, Lamb GD (2013) Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *J Physiol* 591: 5823–5831
14. Kurien BT, Dorri Y, Dillon S, Dsouza A et al (2011) An overview of Western blotting for determining antibody specificities for immunohistochemistry. *Methods Mol Biol* 717:55–67
15. Gilda JE, Ghosh R, Cheah JX et al (2015) Western blotting inaccuracies with unverified antibodies: need for a Western Blotting Minimal Reporting Standard (WBMRS). *PLoS One* 10:e0135392
16. Schuster C, Malinowsky K, Liebmann S et al (2012) Antibody validation by combining immunohistochemistry and protein extraction from formalin-fixed paraffin-embedded tissues. *Histopathology* 60:E37–E50
17. Sawicka M, Pawlikowski J, Wilson S et al (2013) The specificity and patterns of staining in human cells and tissues of p16INK4a antibodies demonstrate variant antigen binding. *PLoS One* 8:e53313
18. Ishikawa K, Miyamoto M, Yoshioka T et al (2013) Method for the validation of immunohistochemical staining using SCID mouse xenografts: expression of CD40 and CD154 in human non-small cell lung cancer. *Oncol Rep* 29:1315–1321
19. Kunzel F, Peschke R, Tichy A et al (2014) Comparison of an indirect fluorescent antibody test with Western blot for the detection of serum antibodies against *Encephalitozoon cuniculi* in cats. *Parasitol Res* 113:4457–4462
20. Holmseth S, Zhou Y, Follin-Arbelet VV et al (2012) Specificity controls for immunocytochemistry: the antigen preadsorption test can lead to inaccurate assessment of antibody specificity. *J Histochem Cytochem* 60:174–187
21. Forsstrom B, Axnas BB, Rockberg J et al (2015) Dissecting antibodies with regards to linear and conformational epitopes. *PLoS One* 10:e0121673
22. Saper CB (2009) A guide to the perplexed on the specificity of antibodies. *J Histochem Cytochem* 57:1–5
23. Gore AC (2013) Editorial: antibody validation requirements for articles published in endocrinology. *Endocrinology* 154:579–580
24. Lorincz A, Nusser Z (2008) Specificity of immunoreactions: the importance of testing specificity in each method. *J Neurosci* 28: 9083–9086
25. Watanabe M, Fukaya M, Sakimura K, Manabe T, Mishina M, Inoue Y et al (1998) Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield. *Eur J Neurosci* 10:478–487
26. Marchalant Y, Brownjohn PW, Bonnet A et al (2014) Validating antibodies to the cannabinoid CB2 receptor: antibody sensitivity is not evidence of antibody specificity. *J Histochem Cytochem* 62:395–404
27. Jensen BC, Swigart PM, Simpson PC (2009) Ten commercial antibodies for alpha-1-

- adrenergic receptor subtypes are nonspecific. *Naunyn Schmiedebergs Arch Pharmacol* 379: 409–412
28. Bradbury A, Pluckthun A (2015) Reproducibility: standardize antibodies used in research. *Nature (Lond)* 518:27–29
29. Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F (2005) Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J* 46:258–268
30. He J (2013) Theory and applications of ligand binding, ELISA and related techniques In: “The immunoassay handbook, 4th edn.” Wild, D(ed). Elsevier Science, UK
31. Smedley RC, Patterson JS, Miller R et al (2007) Sensitivity and specificity of monoclonal and polyclonal immunohistochemical staining for West Nile virus in various organs from American crows (*Corvus brachyrhynchos*). *BMC Infect Dis* 7:1–9
32. Rhodes KJ, Trimmer JS (2006) Antibodies as valuable neuroscience research tools versus reagents of mass distraction. *J Neurosci* 26: 8017–8020
33. Hwang CK, Iuvone PM (2013) Technical brief: a comparison of two methods of euthanasia on retinal dopamine levels. *Mol Vis* 19: 1122–1124
34. Lin L-H, Chen LL, Zirrollo JA et al (1992) General anesthetics potentiate gamma-aminobutyric acid actions on gamma-aminobutyric acid<sub>A</sub> receptors expressed by *Xenopus* oocytes: lack of involvement of intracellular calcium. *J Pharmacol Exp Ther* 263:569–578
35. Bickler PE, Buck LT, Hansen BM (1994) Effects of isoflurane and hypothermia on glutamate receptor-mediated calcium influx in brain slices. *Anesthesiology* 81:1461–1469
36. Martin DC, Plagenhoef M, Abraham J et al (1995) Volatile anesthetics and glutamate activation of *N*-methyl-D-aspartate receptors. *Biochem Pharmacol* 49:809–817
37. Goebel SM, Alvestad RM, Coultrap SJ et al (2005) Tyrosine phosphorylation of the *N*-methyl-D-aspartate receptor is enhanced in synaptic membrane fractions of the adult rat hippocampus. *Mol Brain Res* 142:65–79
38. Wang Y, Zhang Y, Hu W et al (2015) Rapid alteration of protein phosphorylation during postmortem: implication in the study of protein phosphorylation. *Sci Rep* 5:15709
39. Gu J, Hacker GW (2012) Modern methods in analytical morphology. Springer Science & Business Media, New York
40. Grant SG, Husi H (2001) Proteomics of multiprotein complexes: answering fundamental questions in neuroscience. *Trends Biotechnol* 19:S49–S54
41. Husi H, Grant SG (2001) Isolation of 2000-kDa complexes of *N*-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *J Neurochem* 77:281–291
42. Batista U, Garvas M, Nemec M et al (2010) Effects of different detachment procedures on viability, nitroxide reduction kinetics and plasma membrane heterogeneity of V-79 cells. *Cell Biol Int* 34:663–668
43. Bjorge JD, Jakymiw A, Fujita DJ (2000) Selected glimpses into the activation and function of Src kinase. *Oncogene* 19:5620–5635
44. Bi H, Krausz KW, Manna SK et al (2013) Optimization of harvesting, extraction, and analytical protocols for UPLC-ESI-MS-based metabolomic analysis of adherent mammalian cancer cells. *Anal Bioanal Chem* 405:5279–5289
45. Ngoka LC (2008) Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. *Proteome Sci* 6:30
46. Davis GD, Elisee C, Newham DM et al (1999) New fusion protein systems designed to give soluble expression in *Escherichia coli*. *Biotechnol Bioeng* 65:382–388
47. Gomes AV, Liu N (2013) Making Western blot quantification more reliable. *Genetic Engineering and Biotechnology News*
48. Taylor SC, Berkelman T, Yadav G et al (2013) A defined methodology for reliable quantification of Western blot data. *Mol Biotechnol* 55:217–226
49. Dittmer A, Dittmer J (2006) Beta-actin is not a reliable loading control in Western blot analysis. *Electrophoresis* 27:2844–2845
50. Colella AD, Chegenii N, Tea MN et al (2012) Comparison of Stain-Free gels with traditional immunoblot loading control methodology. *Anal Biochem* 430:108–110
51. Romero-Calvo I, Ocon B, Martinez-Moya P et al (2010) Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 401:318–320





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