Slightly altering your immunoblotting protocol could resolve issues of non-specific background ‘noise’.

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Standard protocols for resolving proteins by SDS-PAGE electrophoresis followed by Western blotting are pretty straightforward. In general, after running an SDS-PAGE gel and transferring separated proteins to a solid membrane, the membrane is incubated with a primary antibody that binds specifically to a particular protein. After a few short washes, a Secondary antibody is added which is both specific to your Primary and conjugated to a Horse Radish Peroxidase (HRP) tag. By tossing in a peracid (which catalyzes a reaction with the HRP), light is generated that can be detected on photographic film. The stronger the signal, the more protein.

All of this is meant to be pretty specific, and it can be - but not all the time.

Recently, I have been performing immunoprecipitations using various antibodies. The principal behind immunoprecipitation is to coat beads with an antibody, then incubate the coated beads with your protein. Once a bead-antibody-protein complex has formed, washing will isolate only the proteins that have bound to the antibody. Boiling and reducing this complex will break apart this interaction leaving you with your separated protein of interest along with your (now reduced) antibody light (25kDa) and heavy chains (50kDa).

However the procedure is not always this simple or specific. Often, there are other proteins which are isolated using this procedure which include: a) proteins that bind to the beads themselves, b) proteins that bind to the protein of interest, and c) proteins that are present in the antibody stock. Now a) and b) can be enough to give ‘unexpected’ protein bands on a Western blot but c) can be particularly troubling as it depends on how diligent a company is when they purify their antibody - something which can vary from company to company. Of course controls can be performed to look at a), b), and c), but sometimes blots can give a whole spectrum of proteins that are often difficult to explain. Sadly, very often these ‘unexpected’ protein bands are shrugged off as ‘non-specific binding’ and disregarded entirely - especially if they are far away from the size of the protein of interest. However, what do you do when it is difficult to distinguish, from all of this background ‘noise’, the protein that your antibody has pulled out?

Recently I have encountered this dilemma using some non-commercial antibodies which I’ve found to have high levels of these ‘extra’ proteins. Since these proteins were very close to the size of my protein, I needed to differentiate between my primary ‘specific’ binding and any secondary ‘non-specific’ background noise. Put simply,
before I got excited if I saw a band, I wanted to know if it was my protein or not.

To determine this, I have slightly altered my Western blotting protocol so that, after blocking my membrane, I incubate it in my secondary first (Fig. 1A) and then expose the blot to film. Surprisingly, the secondary alone picked up a number of large proteins that were shipped along with the antibody. These proteins, determined by protein sequencing, turned out to be various macroglobulins and complement factors that also seem to bind with high affinity to the beads used for the immunoprecipitations (Fig 1B). By next adding primary and then secondary antibodies, the specific or ‘primary-dependent’ binding of the primary could be differentiated from this secondary-only binding (Fig. 1C)².

Since I now have a deep seeded mistrust of the purity of antibodies and I fear getting excited about a band that could be an irrelevant protein, I have adopted this additional step every time I perform an immunoprecipitation or Western blot. While adding only an hour or so to the standard protocol, this slight alteration can help a great deal in determining the specificity of your primary antibody and perhaps increase your confidence that the band you are seeing is in fact the protein you are looking for – and not just non-specific binding of your secondary.

**Figure 1.** Immunoblotting with secondary first (before primary) can help identify some issues of non-specific secondary ‘noise’. Revised protocol for using secondary first (A). Exposure of blot after incubation with secondary only (B) yields bands ranging from 160kDa to 100kDa (later determined by sequencing to be macroglobulins). The same blot after incubation with primary, then the secondary (c) yields identification of the protein of interest – distinguishable from proteins bound non-specifically by secondary antibody.
1. Generally, conditioned media from cells generated to over-express an antibody are run through a bead column – similar to an immunoprecipitation – and then the bound antibody is eluted off the column. However, how a company generates this antibody can vary (ie, using insect or mammalian cells) as well as the methods used to purify the antibody. This can make a big difference in what additional proteins may be present in the antibody as it is shipped.

2. These experiments in Figure 1 were performed with antibodies, not commercially available, that were generated by 4 different companies. All were raised to detect Receptor X and selected for in vivo use because they inhibit the binding of Receptor X’s ligand. Their use in vivo has been to test whether inhibiting Receptor X can inhibit tumor growth. In general, these antibodies are not different from those commercially available and used in Westerns – yet it is possible that these antibodies may not be as pure as those you buy commercially.

A stem cell (SC) is a cell from an embryo or adult that can self-renew and differentiate into specialized cells such as neurons, skin or muscle. Embryonic stem (ES) cells are considered to be “blank slates” as they typically exhibit little or no differentiation, and are considered to have more therapeutic potential than adult SCs. Adult SCs are found in the bone marrow, gut, and blood, whereas ES cells are obtained from the inner cell mass during early embryogenesis, resulting in the destruction of the embryo. Due to the elementary properties of SCs, research in this field shows tremendous promise for regenerating or replacing failed tissues and organs. Moreover, a recently published scientific article by Hubner and colleagues (2003) demonstrated that mouse ES cells could form oocytes in culture, expanding their potential utility from regenerative to reproductive medicine. Assuming that this research is translatable to humans, having the ability to produce artificial, viable oocytes in culture from human ES cells would prevent the exploitation of women for egg donations, expand research on causes of infertility and improve assisted reproductive technologies.

The approach taken by Hubner et al. (2003) involved inserting a DNA construct carrying a germ cell-specific green fluorescent protein (GFP) into cultured mouse ES cells. GFP was under the control of a modified Oct4 regulatory sequence deleted in conserved enhancer elements that restrict expression to germ cells. Artificially derived oocytes were characterized by the formation of follicular structures and an examination of oocyte-specific gene expression (zona pellucida (ZP) 1-3) revealed similar expression patterns between naturally and artificially derived oocytes.

Although this study validated that the SCs created cells resembled oocytes, it did not demonstrate that the eggs were functional and capable of being fertilized and developing into embryos suitable for implantation. Interestingly, the authors found that only two of the three oocyte-specific ZP genes in cultured oocytes were expressed properly, which may explain why artificially derived oocytes have fragile zonae. Although the popular press has implied that similar studies can be done with human ES cells, such experiments have yet to be reported.

The ability of SCs to differentiate into tissue or gametes has the potential to impact both reproductive and regenerative medicine. Assuming that human ES cells can form oocytes in culture, the Hubner et al. (2003) study provides a model for studying egg development without using donated oocytes from women. In this sense, an unlimited source of raw material for reproductive research will be available. An unlimited source of fresh oocytes may help infertile women who have problems producing oocytes or whose oocytes are incapable of being fertilized. Using artificially derived oocytes may also obviate the need for donated eggs and the concerns that women be exploited for egg donations. Furthermore, the option of using artificially derived oocytes may be a safer alternative to other currently available options. For example, couples undergoing in vitro fertilization (IVF) require women to stimulate egg release from the ovaries using super-ovulation drugs, which have been reported to cause