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Research paper

Evaluation of methods to reduce background using the Python-based ELISA_QC program

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ABSTRACT

Almost all immunological approaches [immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), Western blot], that are used to quantitate specific proteins have had to address high backgrounds due to non-specific reactivity. We report here for the first time a quantitative comparison of methods for reduction of the background of commercial biotinylated antibodies using the Python-based ELISA_QC program. This is demonstrated using a recombinant humanized anti-cocaine monoclonal antibody. Several approaches, such as adjustment of the incubation time and the concentration of blocking agent, as well as the dilution of secondary antibodies, have been explored to address this issue. In this report, systematic comparisons of two different methods, contrasted with other more traditional methods to address this problem are provided. Addition of heparin (HP) at 1 μg/ml to the wash buffer prior to addition of the secondary biotinylated antibody reduced the elevated background absorbance values (from a mean of 0.313 ± 0.015 to 0.137 ± 0.002). A novel immunodepletion (ID) method also reduced the background (from a mean of 0.331 ± 0.010 to 0.146 ± 0.013). Overall, the ID method generated more similar results at each concentration of the ELISA standard curve to that using the standard lot 1 than the HP method, as analyzed by the Python-based ELISA_QC program. We conclude that the ID method, while more laborious, provides the best solution to resolve the high background seen with specific lots of biotinylated secondary antibody.

1. Introduction

Since the development of the ELISA in the early 1970s by Eva Engvall and Peter Perlmann, this technique has been widely used to quantitate drugs, antibodies and disease markers in medical and pharmaceutical studies (Engvall and Perlmann, 1971). Our laboratory has routinely used this assay to quantitate our humanized anti-cocaine antibody in pharmacokinetic studies (Paula et al., 2004; Norman et al., 2007; Norman et al., 2014; Wetzel et al., 2017a, 2017b). We use an indirect ELISA method, where the hapten conjugate is first adsorbed onto a 96-well plate and the anti-cocaine antibody is detected using a secondary antibody conjugated to biotin in a biotin-streptavidin reaction. The endpoint is measured by the alkaline phosphatase (linked to streptavidin) hydrolysis of the substrate para-nitrophenyl phosphate (pNPP). The importance of a reliable secondary antibody cannot be over-emphasized for any immunological method. If a well-characterized and historically reliable secondary antibody suddenly begins to give extremely high backgrounds, considerable time and resources can be depleted before the problem is diagnosed and resolved. In addition, if unnoticed for long it could also create erroneous and irreproducible data leading to incorrect conclusions.

Problems with antibodies have been extensively reviewed (Saper, 2005; Couchman, 2009; Voskuil, 2014). Difficulties caused by lot-to-lot variations in commercial antibodies from the same manufacturer are also well-documented (Kim et al., 2012, Voskuil, 2017). Issues with commercial antibodies are so widespread that there is an antibody portal that can be used to evaluate antibodies (Björling and Uhlén, 2008). Thus, problems with antibodies are a common occurrence and it is crucial to run appropriate controls and to diligently scrutinize experimental results obtained. Historically, in most research laboratories, the utility of a specific standard curve is only for the day of the assay. Only when there is a dramatic change in either the assay development time being too rapid or too slow do the standard curves get seriously scrutinized. This is why we recently developed and published an easy-to-use Python-based ELISA_QC program to rapidly and quantitatively check the quality and reproducibility of the data obtained (Wetzel et al., 2017a, 2017b). In a clinical translational laboratory that is performing preclinical studies, these measures are critical to success.

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Some common causes of high background in an ELISA are unclean containers, incorrect or inadequate blocking conditions and reagents as well as the inappropriate working concentrations of specific antibodies (Buchwalow et al., 2011; Voskuil, 2014). When an issue persists after the most common causes are carefully eliminated, there is the need to re-evaluate strategies to address and resolve the problem. It was this predicament that led us to develop different strategies to resolve the issue. Our laboratory has used an affinity purified goat antihuman IgG-gamma chain specific biotinylated antibody in an ELISA for over ten years, which indicates the quality and reproducibility of this commercial antibody over a long time. However, toward the end of 2015, the aliquots of this lot that were in use were exhausted and problems were noted when using the new lots of the antibody from the same commercial source. Negative controls using the hapten conjugate and the biotinylated secondary antibody in the absence of the primary antibody clearly showed at least a two-fold increase in optical density (OD) values from those previously measured. Testing multiple lots showed differences in the intensity of the backgrounds, with all lots resulting in OD values two-fold or greater than previous levels. Lot-to-lot variations in commercial antibodies are common problems as documented by numerous literature reports described above. Therefore, it is critical to continuously devise, adapt, and document methods to resolve such issues for the benefit of the scientific community. In this report, we compare two different approaches to resolve this problem, namely the heparin wash (HP) and the immunodepletion (ID) methods.

2. Materials and methods

2.1. Materials

Recombinant humanized anti-cocaine monoclonal antibody, h2E2 (will be referred to as primary antibody from now on) was manufactured by Catalent PharmaSolutions (Madison, WI). Goat anti-human IgG gamma chain specific antibody, biotinylated (GAH, catalog # B1140), streptavidin conjugated to alkaline phosphatase (strep-AP) and para-nitrophenyl phosphate (pNPP) were purchased from Sigma (catalog # N3254, St. Louis, MO). GAH lot numbers used were lot 1 (O85H8920), lot 2 (SLBJ2092V), lot 3 (O48K6141) and lot 4 (SLBG6977V). Benzoylecgonine 1,4-butanediamine-BSA (BE-BSA) was synthesized in-house (Paula et al., 2004). Polyvinyl 96-well ELISA plates were purchased from Thermo Fisher Scientific. Standard curves were created by the SoftMax Pro software using a SpectraMax M3 Multimode Microplate reader. Heparin sodium injection, USP (NDC # 25021-400-30, Lot # WH 442N) was purchased from Sagent Pharmaceuticals (Schaumburg, IL).

2.2. Methods

2.2.1. Basic ELISA

ELISA was carried out according to a previously described method (Paula et al., 2004). The primary antibody was quantified by binding it to the BE-BSA conjugate (2 μg/ml) on a 96-well ELISA plate. The BE-BSA conjugate diluted in 1 mM Tris-EGTA, pH 7.4 was adsorbed onto the plates, at room temperature for 1 h, which were washed × twice and then blocked using BSA-Tris buffer (0.5% BSA in 10 mM Tris, 140 mM NaCl, and 0.02% NaN_3, pH 7.2) for 15 min. The primary antibody bound to BE-BSA was detected by incubation with the secondary GAH. The colorimetric signal due to biotin-bound streptavidin-AP conjugate hydrolysis of pNPP was recorded as optical density (OD) at 405 nm. All the steps, except the incubation with substrate, were carried out at room temperature for 1 h. After the incubation with primary antibody, all subsequent washes were done with BSA-PBS buffer (0.5% BSA, 10 mM sodium phosphate, 145 mM NaCl, 1.5 mM $MgCl_2$, 0.05% Triton X-100, and 0.02% NaN_3, pH 7.2). All standard curves of different h2E2 antibody concentrations (0 to 0.4 μg/ml) were assayed in triplicate. Negative controls without primary antibody and other reagent controls such as the BSA wash buffer and 1 mM Tris-EGTA were included on every plate. OD values obtained without the primary antibody were subtracted from all the readings obtained and will be referred to as the negative control.

2.2.2. Biotinylated anti-human IgG gamma chain specific antibody (GAH) lots, dilutions & blocking conditions

Different lots of commercial GAH were evaluated at the same 1:300 dilution to select the lot with the lowest background. Different concentrations of BSA ranging from 0.5% to 4% were evaluated for blocking. Three different blocking times 15, 30 and 60 min were also tested. GAH was tested at dilutions of 1:300, 1:500, 1:1000 and 1:2000. All experiments were performed at least twice in triplicate.

2.2.3. Immunodepletion (ID) method

After adsorbing BE-BSA (2.5 μg/ml) onto a plate for 1 h at room temperature, 100 μl of the undiluted commercial GAH was applied to the antigen-adsorbed wells for 30 min while shaking at room temperature. GAH was then collected and reapplied to a fresh antigen-adsorbed well, and this process was repeated a total of six times. Subsequently, the undiluted GAH depleted in this way was diluted 300-fold and subjected to another six rounds of exposure to an antigen-adsorbed well. GAH prepared this way was designated ID-GAH and was then used in the basic ELISA protocol in place of the untreated GAH normally used for the protocol. The number of rounds of immuno-depletion was determined by testing to see if background was reduced to standard levels attained with lot 1 GAH after every three rounds.

2.2.4. Heparin wash (HP) method

In this method, the only change was the use of a heparin (1 μg/ml in BSA-Tris buffer) wash for 10 mins for the third wash after the incubation with primary antibody and before application of GAH. This was adapted from Pesce et al. (1986). The concentration of heparin was chosen after screening a few different concentrations (data not shown).

2.2.5. Statistical analyses

Experiments with different GAH lots (2–4) and dilutions were performed twice. Data shown for the negative controls for lot 1, ID and HP are from 24 separate experiments. Negative control means for lots 2, 3 and 4 are from two experiments. Data from negative control values in lots 1 to 4, ID and HP methods were not normally distributed according to the Kolmogorov Smirnov test. Therefore, they were compared using a Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's analysis if $p < 0.05$. A two-way ANOVA followed by Tukey's analysis was used to compare the two different conditions (concentration and blocking time) tested for BSA. For each method, a total of 20 experiments were analyzed by the Python-based ELISA_QC program that we published earlier (Wetzel et al., 2017a, 2017b) and is posted on Github (https://github.com/hanna133/ELISA_QC).

3. Results

3.1. Reduction of negative control OD

3.1.1. Biotinylated anti-human IgG gamma chain specific antibody (GAH) lot comparison

Different lots of commercial GAH were tested to evaluate resultant ELISA background levels. Data shown in Fig. 1 is obtained from the mean of the OD values obtained in negative control of four different lots. A comparison of the mean OD values obtained for the negative controls from the original lot 1 with 3 different subsequently obtained lots is shown in Fig. 1, panel A. No significant differences were seen amongst lots 2, 3 and 4, but lot 1 was significantly different from the three other lots (Fig. 1A) based on Kruskal-Wallis one-way analysis of variance on ranks.