ELSEVIER

Quality control of antibodies for assay development

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Antibodies are used as powerful tools in basic research, for example, in biomarker identification, and in various forms for diagnostics, for example, identification of allergies or autoimmune diseases. Due to their robustness and ease of handling, immunoassays are favourite methods for investigation of various biological or medical questions. Nevertheless in many cases, additional analyses such as mass spectrometry are used to validate or confirm the results of immunoassays. To minimize the workload and to increase confidence in immunoassays, there are urgent needs for antibodies which are both highly specific and well validated. Unfortunately many commercially available antibodies are neither well characterized nor fully tested for cross-reactivities. Adequate quality control and validation of an antibody is time-consuming and can be frustrating. Such validation needs to be performed for every assay/application. However, where an antibody validation is successful, a highly specific and stable reagent will be on hand. This article describes the validation processes of antibodies, including some often neglected factors, as well as unspecific binding to other sample compounds in a multiparameter diagnostic assay. The validation consists of different immunological methods, with important assay controls, and is performed in relation to the development of a diagnostic test.

Introduction

Since the invention of *in vitro* production of monoclonal antibodies by Köhler and Milstein in 1975 [1], they have become favoured tools in basic research as well as in diagnostics. Their target specific binding and their robustness and stability according to the ambient temperature enable easy and rapid detection of a variety of different analytes. But besides the well-known pregnancy or allergy tests, there are only a few immunoassays which are routinely used and most deliver only qualitative statements. When looking more closely into the topic, many users report unspecific binding of the antibodies [2,3]. Another issue found in publications is poor reproducibility [3,4]. These characteristics make an extensive quality control and validation procedure for every specific application necessary. Unfortunately little information can be found in the literature or is provided by suppliers of commercial antibodies [2,3]. Nevertheless within recent years, efforts have been made to improve the knowledge base of antibodies. Initiatives such as Biocompare (http://www.biocompare.com), Antibodypedia (http://www.antibodypedia.com) or Antibody Resource (http://www.antibodyresource.com) and academic projects such as the Human Proteome Atlas (http://www.proteinatlas.org) offer information concerning antibody type, reactivity and applications. The data provided could be a good starting point to avoid unnecessary and cost-intensive experiments. We believe that deliberate antibody validation and quality control is essential and a prerequisite to develop a reliable immunoassay with the potential to be deployed in diagnostics.

To emphasize the importance of antibody quality control, we have chosen the detection of drug abuse as an example. The drugs under investigation were amphetamine (PubChem CID 5826),

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methamphetamine (PubChem CID 10836), MDMA (3,4-methylenedioxy-methamphetamine, PubChem CID 1615), THC (tetrahydrocannabinol, PubChem CID 16078), PCP (phencyclidine, PubChem CID 6468), methadone (PubChem CID 4095), morphine (PubChem CID 5288826), cocaine (PubChem CID 446220) and benzoylecgonine (PubChem CID 448223). This scenario is medically relevant and represents a difficult case of antibody validation due to the small size and structural similarity of the different analyte molecules. Starting with a set of nine different drugs to be implemented in one assay, the corresponding antibodies were validated by ELISA (enzyme-linked immunosorbent assay), Western blot analysis and on protein microarrays. A first screening of the antibodies was performed by direct ELISA and Western blot and later compared to findings on protein microarrays. We considered an antibody as validated if the following conditions were met: Specific binding to the target and no detectable cross-reactivity to chemically or structurally related molecules.

- No unspecific binding to assay compounds, for example, biological medium or blocking solution.
- Stable antigen–antibody reaction, as shown by technical and biological replicates with CVs $< 20\%$ (GTFCh – Society of Toxicological and Forensic Chemistry, ICH – International Conference on Harmonisation).

Here we outline the significance of antibody quality control and highlight some issues which are often overlooked, but which may influence the performance of an assay.

Material and methods

Reagents and equipment

For immobilization, BSA–drug conjugates from Fitzgerald Industries International USA (Amphetamine–BSA 80-IA22; Methamphetamine–BSA 80-IM59; MDMA–BSA 80-1044; THC–BSA 80-IT63; PCP–BSA 80-IP10; Methadone–BSA 80-IM55, Morphine–BSA 80-IM50; Cocaine–BSA 80-1034; Benzoylecgonine–BSA 80-IB31) were used. On the basis of the ratio of BSA to drug molecules provided by the manufacturer, the relative drug concentration of the conjugates was calculated. In the competitive assays, pure drugs dissolved in methanol (LGC Standards, UK) were used. As sample medium, undiluted human serum (UTAK, USA) was employed. The drug specific antibodies were purchased from Acris Antibodies, Germany (anti-amphetamine antibody AM31389PU-N; anti-THC antibody BM2701), Fitzgerald Industries International, USA (anti-cocaine antibody 10-1030) and Abcam, UK (anti-PCP antibody ab20457; anti-morphine antibody ab23357) and chosen such that all were detected with the same fluorescent labeled secondary anti-mouse antibody (anti-mouse IgG Alexa Fluor 555, Invitrogen, USA; A-21422). For dilutions and washing steps, PBS-T (phosphate buffered saline with 0.05% Tween 20) and a carbonate buffer (28.6 mM Na_2CO_3, 72.13 mM $NaHCO_3$, pH 9.56) were used. Unless noted otherwise BSA–drug conjugates and antibodies were used from the same batch to avoid variations.

For ELISA experiments, black 96-well plates (Nunc, Fisher Scientific, USA) were used. Western blotting was performed on nitrocellulose membranes (GE-Healthcare, UK) and for protein microarrays epoxy slides were produced in-house. The microarrays were produced by a non-contact spotting system with a piezo-nozzle (M2-Automation, Germany). The nozzle diameter was 100 μm and parameters of voltage, frequency, pulse duration and pressure were adjusted, such that a 120 pL droplet was formed. In total, 10 droplets of each sample were applied to the surface. As read-out systems for fluorescence detection, a Typhoon-Scanner (GE-Healthcare, UK; Western blot), microplate reader (BMG Labtech, Germany) and Axon Scanner (Molecular Devices, USA) were used.

Immunoassays

For Western blot analysis, 12%-SDS-PAGE was performed. The BSA–drug conjugates were diluted in PBS-T and 300 ng of each was applied to the gel. Following electrophoresis the samples were transferred to a nitrocellulose membrane using a semi-dry protocol. The transfer was controlled by a reversible Ponceau-S (AppliChem, Germany) staining. Blotting and blocking were performed at room temperature (RT) and the antibody incubation at 4°C. During each incubation step the membrane was gently shaken. Unless stated otherwise, antibodies were diluted 1:1000 in 1%-milk powder. After each incubation step, the membrane was washed briefly 3× with PBS-T. After a blocking step with 10%-milk powder (BioRad, USA) for one hour, incubation with a primary antibody was performed for 90 min. Finally, the membrane was incubated for 60 min with the fluorescent labeled secondary antibody and scanned.

For ELISAs and protein microarrays the BSA–drugs conjugates were diluted in carbonate buffer. In the case of ELISAs the MTP was incubated for 2 h at RT. Blocking was performed for 60 min with 2% BSA or undiluted serum. If not otherwise specified the antibodies were applied in a 1:1000 dilution in PBS-T or undiluted serum.

Protein microarrays were vacuum sealed and stored overnight at 4 °C after spotting. For the incubation steps, a Whatman slide holder (GE Healthcare, UK) with 16 incubation chambers was used. After immobilization, free binding sites were blocked for 60 min with either a 2% BSA solution, undiluted blank serum or an ethanolamine solution (Sigma, USA). Unless noted otherwise, the antibodies were diluted 1:100 in PBS-T or undiluted serum.

For ELISAs and protein microarrays primary antibodies were incubated for 90 min and the secondary antibody was applied for 60 min before reading. For both methods 3 replicates of each sample were analyzed, after each step, 3 short washes with 1xPBS-T were performed and all steps were done at RT on a shaker.

Data analysis

The mean (or median) of the replicates of each sample was calculated. The buffer was set as background signal and subtracted from the sample signals. For ELISAs the mean of the signals was used and for microarray analysis the median was calculated of the spots [5]. The signal intensities of the included negative controls BSA and blank serum should be at a similar level to the buffer if no cross-reactivity occurred. This means no unspecific binding to assay compounds, for example, sample medium, drugs or buffer was detectable. For analysis of the microarray data, GenePix 7 software (Molecular Devices, USA) was used.

Results

In the following section, effects which occurred during antibody validation are described. The validation process consisted of

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