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Perspective: The Antibody Validation Webinar Series from The Antibody Society

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Abstract

In the wake of the reproducibility crisis and the discussions on how commercially available antibodies as research tool contribute to it, the Antibody Society developed a series of 10 webinars to address the issues involved. The webinars were delivered by the speakers who are from both academic and commercial backgrounds. This report highlights the

problems, and offers solutions to help the scientific community appropriately identify the right antibodies and to validate them for their research and development projects.
Despite the various solutions proposed here, there is no silver bullet. Each antibody must be verified based on the content of the product sheet, and subsequently through experimentation to confirm integrity, specificity and selectivity. Verification needs to focus on the precise application and tissue/cell type for which the antibody will be used, and all verification data must be reported openly. The discussed approaches all have caveats, so a combination of solutions must be considered.

Introduction

Extensive discussions and publications about the reproducibility crisis[1] and the confusion and complexities associated with the global market for commercial research tool antibodies[2,3], generated calls for robust strategies on antibody validation[4,5,6]. This resulted in several scientific publications[7,8,9] and international meetings of stakeholders[10,11,12]. Some significant issues emerged and were adequately addressed, but the dissemination to, and especially implementation by, the broader scientific community has been a challenge.
So, during an international meeting in 2018[12], we decided to highlight specific concerns and ideas for practical improvements in a series of online seminars (webinars). From November 2019 to February 2020, fifteen of us convened to create a ten-part series of webinars that was supported and broadcast by The Antibody Society. The webinars, freely accessible on the Antibody Society Internet page (https://www.antibodysociety.org/learningcenter/), highlight many of the problems and suggest possible solutions to improve reproducibility in research involving antibodies to detect proteins, although no single solution (silver bullet) was identified.
Manufacturers, vendors and scientists all share the responsibility to ensure the antibodies are fit for purpose. In this perspective, we give an overview of the problems identified, possible solutions, and future developments that were highlighted in the webinars. With this contribution, we hope to eliminate research tool antibodies as a cause of irreproducible research.

Reproducibility crisis

The well-known publication of the Amgen study in 2012[1], demonstrated that 47 research claims out of 53 from top tier publications were not reproducible. This study, and others at the same time, has prompted many discussions, publications and meetings to address the underlying mechanisms. The Amgen study identified the following six principal factors (‘Begley’s six criteria’):
1 Studies must be blinded (they hardly ever are)
2 All results must be shown (commonly, inconvenient data are omitted)
3 Repeated experiments (hardly ever reported)

4 Inclusion of positive and negative controls (hardly ever reported)
5 Validation of reagents (if done at all, usually omitted)
6 Robust analysis of the data (robustness rarely addressed)

The validation of reagents has received much attention since this study, with many discussions and publications (including these webinars) focused on research tool antibodies. The most frequent mistake made with antibodies being used as research reagents is that their specificity is not experimentally verified before use. Especially when antibodies are purchased from a large vendor, users assume that the vendor has verified the performance of the reagent sold, and that their reputation is a sufficient assurance. This lack of vigilance has resulted in the widespread use of cross-reactive antibodies, inaccurate data sets, a catastrophic waste of funds and time, and significantly slowed progress in medical science. Worse still is the "opportunity cost" associated with well-meaning investigators following up spurious research findings. The damage incurred by use of improperly validated antibodies becomes worse when such research reagents find their way into the clinic as established tools for biomarker detection, thus damaging and invalidating costly clinical trials[13,14]. Global spending on protein-binding reagents (primarily antibodies) was estimated at $1.6 billion in 2015, and if up to 50% of commercial antibodies were improperly validated or inactive before use[13], $800 million per annum would potentially have been wasted. By 2019 the global market size had risen to an estimated $ 3.4 billion[15], with a proportionate increase in the estimate of research waste due to poorly validated antibodies. However, this is probably a substantial underestimate of the real cost of poorly validated research antibodies, given that the ubiquitous use of antibodies for many/most research procedures and the cost of irreproducible research in 2015 was estimated to be $28 billion per annum in the USA alone[16].
Examples of cross-reactive antibodies erroneously used in clinical settings were mentioned in the webinars and are summarised in Table1.

Target	**Antibody IDs**	**Biomarker**	**Cross-reactions**
EpoR (EPOR)	M20 and C20	tumour cells	HSP70[17]
ER-β (ESR2)	12 out of 13	breast cancer	WDCP, POU2F1, multiple[18]
HER2 (ERBB2)	2 out of 3	breast cancer	HER4[19]
ERCC1	8F1	Prognostic	CCT-alpha[20]
CDK1	A17	Cancer	Cep152[21]

Table 1. Some cross-reactive antibodies erroneously used to identify therapeutically relevant clinical biomarkers caused devastating personal and financial damage to science and medical progress.

The anti-Erythropoietin receptor (EpoR/EPOR) antibodies M20 and C20 (no longer on the market) were rabbit anti-peptide polyclonal antibodies, promoted as useful for the detection of EpoR in tumor sections by immunohistochemistry (IHC), and reported in several publications. However, rigorous validation demonstrated that they could not detect EpoR in IHC at all, thus implying cross-reaction with other irrelevant

proteins. In fact, the antibodies showed signals in IHC on sections of EpoR$^{-/-}$ knock out mouse embryos[17]. Rather than EpoR, the dominant protein the antibody C20 detected was actually HSP70 in western blotting (WB)[17]. HSP70 and EpoR share the amino acid sequence that was used as immunizing peptide to raise the polyclonal antibody.

Estrogen receptor beta (ER-β/ESR2) was deemed a biomarker for breast cancer based on strong signals given by several antibodies, including the often-used monoclonal antibodies PPG5/10 and 14C8. However, neither normal breast tissue, nor any stage of breast cancer, exhibit any ER-β mRNA expression, while ER-α (ESR1) is expressed[18]. Only one rarely used antibody out of the 13 previously validated antibodies tested was specific for the ER-β protein. The others, including the widely used antibodies PPG5/10 and 14C8, were variously cross-reactive and gave signals in overtly ER-β negative cell lines. In 2017, eight breast cancer clinical trials were based on ER-β as key biomarker, all using antibodies that have since been shown not to be specific for ER-β! The question is whether the results of such trials can be considered valid, since they employed such cross-reactive antibodies.

HER2-type breast cancer is diagnosed based on the elevated expression of human epidermal growth factor receptor 2 (HER2/ERBB2). Diagnostics are first performed by IHC of biopsy material using a HER2-specific antibody. When results are ambiguous, gene expression is tested by *in situ* hybridisation. However, in 2011, when three available pharmaco-diagnostic antibodies were evaluated by testing for cross-reactivity to the other HER proteins, only one antibody was selective, while the other two cross-reacted with HER4 (ERBB4)[19].

The levels of ERCC1 (GeneID 2067) are used as a prognostic biomarker in chemotherapy of non-small-cell lung cancer (NSCLC), using IHC with the monoclonal antibody 8F1. However, problems with 8F1 emerged when it was demonstrated to cross-react with CCT-α (GeneID 5130, PCYT1A)[20]. While 8F1 is no longer used, the original observations are now explained by its reactivity with CCT-α/PCYT1A, which appears to be a useful marker for selecting the treatments of NSCLC and of head and neck squamous cell carcinoma (HNSCC)[20].

Mouse monoclonal antibody A17 for Cyclin-dependent kinase 1 (CDK1, CDC2) has been cited in hundreds of papers as a cancer biomarker. However, A17 cross-reacts with another nuclear protein, CEP152, both in WB and in immunocytochemistry (ICC)[21], leaving the question of whether CDK1, CEP152 or either, are indeed valid biomarkers.

These few examples beg the question of whether the papers published, using these cross-reactive antibodies, need revision or retraction. They also emphasize another major issue: the literature offers no alert processes or repercussions for un-retracted publications, nor does it allow tagging of the use of invalidated antibodies or flagging for irreproducible results.

Antibody problems dissected

The above examples represent only a small sample from the literature and demonstrate how using antibodies as tools for biomarker detection can potentially fail when their fundamental characteristics are not fully appreciated. We have here examined the complexities of antibodies and highlight the different aspects that need rigorous scrutiny.

Specificity:

An antibody is a protein of the immunoglobulin (Ig) family. Most commercial antibodies used for research and for the detection of clinical biomarkers are of the isotype IgG. Proteins, including IgG, have a natural affinity for binding to other proteins, so they adhere to each other randomly and fleetingly at low affinity, while there may be a more stable and functional interaction with much higher affinity. Such defined interaction may be highly specific, as antibodies have so evolved to act; the greater the difference in affinity between the low and the high affinity interactions, the greater the antibody specificity. Antibodies specifically bind to a particular site on a protein, referred to as the epitope. Therefore, antibody specificity is defined by its affinity to the epitope on its designated target protein compared to its affinity to other epitopes. However, a proper dilution of the antibody is required to preclude a highly specific antibody from binding to unrelated proteins at lower affinity. When an antibody binds to other unrelated proteins (that contain similar epitopes) at equal or similar affinity, the specificity of the antibody is low. When it binds to only one defined epitope, but that epitope is shared with several proteins, the antibody is still specific to that epitope but not to one particular protein (as illustrated in the case of EpoR and HSP70 mentioned above).

The specificity of an antibody is determined by the molecular characteristics of the Ig (as discussed below), and by those of the antigen, including the epitope’s degree of folding/unfolding. The ability of the antibody to recognise the target protein is lost when the epitope is either masked or destroyed due to post-translational modifications or due to changes introduced during a particular sample preparation. Therefore, antibody-antigen interactions depend strictly on experimental context. In addition, the integrity and specificity of the antibody can be negatively affected when its production and purification processes are not carefully controlled. Commercially available research-grade antibodies would fail rigorous validation if they showed lack of specificity (failing to recognise the intended target protein).

Selectivity:

An antibody is selective when, at optimal dilution / concentration and under specified experimental conditions, it binds exclusively to its target protein in a complex mixture of proteins. Under these conditions, such an antibody either does not bind to similar proteins or to proteins with similar epitopes present, or is prevented from doing so by their absence. The antibody binds to a unique epitope (i.e. is mono-specific), when it is not accessible on any other protein under the prevailing experimental conditions. Selectivity does not only depend on the concentration or dilution of the antibody, but also on the relative levels of the target

protein and the levels of similar proteins with which the antibody may cross-react. Thus, selectivity is determined by the experimental conditions of the assay.

Antibodies targeting native folded proteins may well show different selectivity when used for detection of unfolded, or partially unfolded proteins. Unfolding may unmask the epitope in another protein, or hide it in the designated target. Similarly, antibodies against denatured proteins may show different selectivity when used for detection of folded proteins. The level of unfolding of proteins differs in WB, IHC, ICC and in antigen-coated microwells for enzyme-linked immunosorbent assay (ELISA). The level of natively folded protein may differ in samples used in immunoprecipitation (IP), in capture ELISA, and in multiplex systems. In microwells for ELISA, the level of correct folding can depend on the size and chemical characteristics of the protein, the plastic surface of the well, and the pH and ionic conditions at which adsorption and assay are performed. It is worth noting that a higher level of selectivity can be enforced when antibodies are used in a dual-recognition combination, as in sandwich assays (two antibodies per protein), which can enhance the reliable detection of a target antigen. In such cases it may be acceptable to use a less specific (polyclonal) antibody i.e. to capture, combined with a highly specific (monoclonal) antibody i.e. to detection.

From this it follows that:

1. Demonstrating the selectivity of an antibody is an essential aspect of validation.
2. Validation needs to be performed in each application where an antibody is used.
3. Validation needs to be performed in samples containing varying, experimentally relevant concentrations and ratios of intended target and non-intended off-target proteins.

Chemical fixation and subsequent antigen retrieval as in IHC can affect selectivity, depending on the epitope to be detected. Hence, the antibody performance depends on the quality of sample preparation. Like specificity (ability to correctly detect the target epitope), selectivity (ability to differentiate from similar epitopes) can also depend on the method of IgG purification (see ***Manufacturing specifics*** below), on the choice of antigen used to generate or screen for the antibody, and on the degree of denaturation of the target protein in the assay being used. Multiplex bead-based antibody arrays[22,23] under both native (ELISA-like) and denatured (WB-like) conditions reveal that most tested commercial antibodies are neither selective (i.e. they cross-react with off-target proteins containing the target epitope), nor specific (i.e. they cross-react with off-target proteins not containing the target epitope). The designated target protein is usually present in the top five proteins detected, but is seldom the protein most strongly bound. This observation was made even from the proteins derived from a single molecular weight (webcast #8a, slide 21).

In summary, an antibody can be specific for an epitope, but still lacks selectivity when it cross-reacts to other proteins with identical or similar epitopes depending on the type of sample preparation.

Clonality:

Antisera contain a polyclonal mixture of antibodies of different specificities and affinities. An ever-changing proportion of non-specific antibodies from animal to animal causes inevitable inconsistency between the sera. The *in vitro* diagnostics industry mitigates this problem by immunizing in parallel a large number of animals with the same antigen to obtain a 'gold standard' antiserum pool. Affinity purification of polyclonal antibodies reduces, but does not eliminate, inconsistency from batch to batch. And because polyclonal antibodies detect a multiplicity of epitopes, a more defined antigen will lead to improved consistency between batches. Polyclonal antibodies raised against peptides and subsequently peptide affinity-purified, will theoretically have the level of specificity and consistency approaching those of monoclonal antibodies. However, the immunizing peptides have to meet size criteria (too small a peptide loses uniqueness of sequence; a length over 10-15 amino acids creates too many epitopes) and the amino acid sequences need to be unique to avoid sharing epitopes with other proteins. A lack of antigen size restrictions will result in cross-reactivity, like the anti-EpoR antibodies cross-reacting with HSP70 shown in Table 1.

On the other hand, many researchers consider a monoclonal antibody to be homogenous by virtue of its production by a monoclonal hybridoma, and assume it to be a single antibody with specificity to one epitope. However, one study showed that about 30% of the hybridomas tested express additional light chains, often derived from the cell-fusion partner, thus rendering the expressed antibodies non-specific[24]. Monoclonal antibodies can also be derived from a hybridoma expressing more than one heavy chain allele, or from a hybridoma arising from a fusion to two different B cells, thus producing a mixture of specificities. In either case mono-specificity is lost. But it must be emphasized that the observed cross-reactivity of many monoclonal antibodies is an *intrinsic* molecular property of the homogeneous IgG molecule, which cannot be purified away. In other words, even a "strictly" monoclonal antibody can still be non-specific.

Both a polyclonal and a monoclonal antibody may react with off-target proteins, and on occasions even more strongly than with the intended target protein (non-selective reactivity). Hence, both polyclonal and monoclonal antibodies require stringent validation before use.

Mortality:

Polyclonal antibodies can only be maintained by the continuous bleeding and eventually sacrifice of many animals. Hybridoma cell lines, by contrast, are long-lived. However, they can become genetically unstable over time, and may be inadvertently lost during re-cloning. Therefore, antibodies produced by hybridomas may change or become unavailable. A polyclonal antibody can be reproduced in a relatively consistent way, provided each batch is antigen affinity purified and the Quality Control (QC) criteria are easy to test. Such consistency mostly requires a well-defined immunogen, but each polyclonal batch will be more or less different and some will fail in QC.

Applicability:

The choice of antibody needs to be made strictly in the context of the type of experiments it is required for. Antibodies against low abundance proteins are often less versatile (i.e. fit for few assay-types and sometimes not useful in any, possibly because the target protein is unstable), and antibodies specific for post-transcriptional modifications (such as phosphorylation) are rarely selective for the intended protein of interest. Low-affinity antibodies will not work well as capture antibodies. The required selectivity of the antibody is not only determined by the chosen antigen and the dilution/concentration of the antibody, but also by the intended application. Therefore, it is the intended user-application that determines the optimal type of antibody to use, provided that there is a choice, and it should guide the design of the antigen used by the manufacturer to generate it.

Availability:

Antibodies from original manufacturers often get rebranded and may appear in several catalogues[2]. By adding data from the vendor's lab or from the vendor's customers, or by altering the name, the same antibody may appear in multiple identities in different catalogues. Because such duplicate products are hard to recognise, scientists run the risk of buying identical antibodies from different catalogues as comparators. Also, historical data may be retained on the product sheet, while they no longer relate to the current batch sold by the rebranding vendor.

It is often the case that well-validated antibodies (especially polyclonal antibodies which are generated in finite amounts) are sold out and become no longer available, or may be replaced without this fact being clearly communicated. Finally, there is the risk of not finding the best antibody when the many choices from the larger catalogues overwhelm the few, possibly much better, antibodies from smaller catalogues.

Confusion:

All these issues mean that scientists have a hard time finding the optimal commercial antibodies for their specific projects. Once they try one or a few unsuccessfully, they may give up, while the best ones remain hidden in the masses of available catalogue items. This may lead to projects being aborted for no good reason. Unlike other types of reagents, most antibodies are not molecularly fully defined (unless they are recombinant with disclosed variable region sequences: see below), and they are sold on the basis of claimed performance rather than physical identity. As discussed, polyclonal antibodies are molecularly undefined, but even hybridoma-derived monoclonal antibodies may have un-predictabilities as discussed above. In addition, there are many instances where valuable clones from academia become the victims of institutional brain-death and are summarily discarded following a scientist's retirement.

Here is a list of other confounding factors:

- Manufacturers package labelling inconsistent with content.
- Sale of undefined antibody quantity, or sale by volume (antiserum, hybridoma-conditioned media) rather than by functional antibody concentration
- Concealed manufacturer (via Original Equipment Manufacturer or OEM agreement)

- Renaming of clones
- Batch-to-batch inconsistencies
- Product sheets with historical data, not reflecting the latest batch

Communication:
If a disappointed user doesn’t tell the providers and the community that an antibody is "bad", these products will contaminate the market. It should be part of the scientist’s duties to verify and report the validity of the reagents used. There is still little incentive within the system for scientists to perform high-quality reproducible science. If the scientific community and its reward systems were more focused on quality, then the sales of poor-quality antibodies might be suppressed.

Solutions

Now that we have dissected the problems with research antibodies and we have seen the consequences when antibodies are not properly validated, we suggest a set of possible solutions that we have discussed to mitigate these problems.

Rigorous reporting:
When a scientist identifies problems with an antibody reagent, the provider should be informed immediately. All providers with a good reputation will try to resolve issues when their product is non-performing. There is usually a money-back guarantee, and in many cases a replacement sample solves the problem. However, on publication, authors routinely continue to not provide sufficient information to clearly identify the reagents, and especially the antibody reagents, they are using. While this has been improving[25], most journals still permit publications lacking even that minimal information (i.e. without: catalogue number; lot/batch number[26]) that would allow easy and unambiguous identification of the reagent described. The omissions of sufficient reagent details and validation data to support its use in the assay, potentially prevent others from reproducing the published research data. Thus, any intent an author might have to warn a provider or to allow the community to reproduce their experiments often lacks the information necessary for that warning to be effective. This is especially critical for research tool antibodies, because, as noted, they are currently not identified by their primary protein sequence.
To help alleviate the problem, publishers have been urged to respond[27] and several now demand a separate section for reagent specifics and in some cases also their validation. The need for an authentication of key biological/chemical resources has prompted the creation of the **R**esearch **R**esource **ID**entifiers (RRID)[28]. Publishers increasingly insist on the use of this unique identification system, and since 2014 authors have added 240,000 RRIDs to their papers, mainly consisting of antibody RRIDs[25]. The RRID system, to be accessed through SciCrunch or directly through the Antibody Registry, brings together identical antibodies with different brands under one RRID. However, OEM rebranding is not always visible

due to confidentiality agreements, which potentially leads to different RRIDs for the same antibody. Furthermore, RRID does not discriminate between batches/lots to reveal different characteristics between them, as is often seen with polyclonal antibodies. Despite these shortcomings, the RRID system is an important first step towards reproducibility and enables the building of a notification system that will more easily alert both companies and other scientists of potential antibody problems. It is clearly a positive development that antibody vendors are increasingly using RRIDs on their product pages.

Literature citations:
In addition to the information provided by the antibody catalogues, there is a wealth of information on how research tool antibodies have been used in the scientific literature. Resources such as CiteAb ranks antibodies based on the number of citations[29]. CiteAb allows users to filter for host species, reactive species, application, clonality, and conjugation form. Antibodies with the most citations are by inference the most successful, as they have generated the most published data. However, such inference comes with several caveats: Antibodies with the highest number of citations tend be the oldest in the market, although much younger antibodies of much higher quality might be available with fewer or no citations. As we saw in Table 1, top-cited antibodies do not necessarily show specificity or selectivity when properly validated. This historical lack of attention led us to webinar examples like the top-cited BACE1 monoclonal antibody failing in a knock-out WB (webcast #7b, slide 12), and a CDK2 monoclonal antibody with 690 citations showing 10 times weaker signals than another antibody with only a single citation (webcast #9, slides 22/23). In most publications up until the very recent past, cited commercial antibodies have not been properly identified, lacking even catalogue numbers, let alone batch and aliquot coding[26]. Such ambiguity causes confusion about the identity of the antibody described.
CiteAb mitigate such caveats by providing validation data when available. This allows researchers to select both the most cited antibodies and well-validated antibodies. Researchers must then still validate them side by side in their own laboratory for their own specific experiments.

How to find the right antibody in this large and complex market?
As a starting point, there is a range of online search tools available to generate a short list of candidate commercial antibodies for the scientist to choose from. In addition to the discussed SciCrunch and CiteAb, we mention Antibodypedia, Antibodyresource, Benschsci, Biocompare, Labome, and Linscott's Directory as resources for consultation. Once the shortlist has been created, the following three criteria need to be noted:
1 The product data sheet must convince the scientist that the supplied antibody will meet the expectations.
2 The antibody performance must be consistent from purchase to purchase
3 The antibody must be available for the foreseeable future

The product sheet should provide direct data for the first, and initial pointers (see below) for the second and third criteria. All the relevant details on the product sheet are either manufacturing specifics or performance specifics[25] and are summarised in Table 2.

Manufacturing specifics	**Performance specifics**
Catalogue number and batch/clone number	Application claims
Names and symbols of target protein	Data confirming successful use in applications
GeneID and/or SwissProt accession	Titre in ELISA
Host species and isotype	Successful usage claims in literature
Antigen and epitope	Positive controls (tissues; cell types; cell lines)
Purification method	Negative controls
Formulation (buffer components)	Data confirming selectivity
Quantity, known (mg) or unknown (ml)	Data confirming molecular integrity

Table 2. Data to be expected on the antibody product sheets separated by manufacturing and performance specifics.

There are precious few vendors/manufacturers who provide product sheets showing all of the items in Table 2. Because of this lack of clarity, it is the scientist's responsibility to be vigilant and to avoid purchasing antibodies with critical omissions in the product sheet. Both categories come with pitfalls that the scientist must be aware of:

Manufacturing specifics: It is essential to not pick an antibody based on the name of its target, but only based on the target GeneID or SwissProt accession. This is because some proteins share identification symbols. For example: OCT2 is shared between Gene ID 5452 (POU2F2) and 6582 (SLC22A2) and CCT-α is shared between GeneID 5130 (PCYT1A) and 6950 (TCP1).

When a monoclonal antibody is sought, the clone identifier should prevail over the catalogue number or product name so as to avoid buying a re-cloned product with characteristics different from the original. However, the typical two-letter two-digit identifier (usually reflecting the position of the identified clone in the ELISA plate) may fail to represent a unique clone (e.g. clone P1F6 is used for antibodies against ITGB5 and BCL6).

Batch coding is critical, especially for polyclonal antibodies, because of intrinsic batch-to-batch variations. A batch coding might be applied to a purified polyclonal antibody, but the amount of IgG may not reflect the titre of specific antibody in such a product, as this may change from batch to batch. Batch variations also exist among monoclonal antibodies, especially when they are offered un-purified as conditioned culture media or crude ascites.

The purification grade is another factor to consider. Some antibodies are affinity-purified using Protein A/G (which captures all IgG), or using the antigen (which captures only target-specific antibodies). Many products are offered as an IgG-enriched fraction (via ammonium sulphate precipitation). Such differences in purification grades may have profound consequences on the performance of the reagents. One must also be aware how the antibody was raised: against the entire protein, against a specific domain or a specific subdomain, or against a peptide of the protein of interest. This affects selectivity and specificity in unpredictable ways dependent on experimental context. Importantly, this will affect whether an unfolded form or the folded form of the protein will be recognized. And finally, there is the formulation in which the product is delivered. Products containing carrier protein such as bovine serum albumin, or anti-microbials like azide, may interfere with coupling of the antibody to a stationary phase or to a reporting enzyme or fluorophore. Also, sufficient added glycerol allows storage at -20˚C without rendering the antibody frozen solid. Repeated freeze-thaw cycles can denature and inactivate antibodies and should be avoided (e.g. by storage at 4˚C or freezing down small aliquots). These considerations may all influence consistency between purchases (criterion 2 above).

Until recently, a project involving antibodies started with the choice between a hybridoma-derived monoclonal antibody and a polyclonal antibody. As discussed, for long-term consistency (criterion 3 above), monoclonal antibodies are preferable, though, as noted in the above section, even monoclonal antibodies are not always immortal for guaranteed future supply. Recent technologies for the production of recombinant antibodies have finally allowed such a guarantee (see **Recombinant Antibodies** below).

Performance specifics: There are some critical performance questions the scientist needs an answer to before deciding to make a purchase. A product sheet may claim that the antibody is fit for a certain application, but do the data (if any are present at all) support that claim? Are such data on the product sheet or in the literature, and do they make scientific sense? Showing unvalidated staining of a cancer section does not demonstrate fit-for-purpose, nor is there any proof of specificity when entire cells light up either in fluorescence microscopy, or in flow cytometry on a single cell line. Being fit for an application may be supported when a cell membrane protein is visibly stained at the cell membrane or a nuclear protein is visibly stained in the nucleus. The precise experimental protocols under which the presented data were obtained must be disclosed. If not, how can a scientist reproduce the data to verify a product’s integrity? The product sheet may show comparisons of the antibody with a ‘gold standard’ antibody, with expressing and non-expressing cells or tissues, or with other members of the same protein family to demonstrate selectivity. All such comparisons need to be done in parallel and at the same optimal antibody dilution (to satisfy criterion 1).

Antibody validation:

As proposed by Uhlén et al[9], antibody validation may be approached by the so-called five pillars principle:

1 Orthogonal study (compare mRNA with protein expression)
2 Independent antibody control (using a ‘gold standard’ antibody)
3 Recombinant expression (great for selectivity at elevated levels, not so great for specificity at native levels)
4 Immuno-mass spectrometry (great for specificity, not so great for selectivity)
5 Genetic strategies (knock-down and knock-out studies)

Based on this, one ideal validation would combine pillars 3 and 4, as they complement each other for specificity and selectivity assessments. However, this approach only works when both pillars are readily available. If not, one might have to compare with a ‘gold standard’ antibody, if one exists, or pursue the orthogonal approach– bearing in mind that mRNA expression levels correlate poorly with protein expression for certain proteins (see *Immunochemistry* below). Using the recombinant expression pillar on its own does not suffice: Comparing reactivity to several overexpressed members of the same protein family adds value to addressing selectivity, but this does not replace the assessment of selectivity in cells with much lower native target protein levels. In such experiments, unspecific staining may render the same antibody useless. When an antibody is required for studying cell lines, or cultured cells, a potent tool for antibody validation is the use of the genetic strategy. Small interfering RNA (siRNA) introduced via transfection can reduce (knockdown) the mRNA expression levels from the gene of interest. Lowered mRNA expression levels can be compared with the protein levels detected by the antibody. Reduction of both mRNA expression and protein levels may help validate the antibody specificity (confirming it recognized the protein of interest in that specific cell line). However, since multiple mRNAs can be affected, this technology has the potential to confuse the results. Furthermore, it does not rule out that another related protein may be detected in a different cell line. So, selectivity is not assessed in this approach. Also, proteins with long half-lives can remain at high levels despite their mRNA being silenced by siRNA. In such case this strategy is less useful. In principle, the advantages of this approach appears to be multifold: It is fast (72-96h turnaround time), robust (compare multiple cellular backgrounds in parallel), highly sensitive (detection at low expression levels), and accurate (but with use of proper controls: No Template Control [NTC]; multiple siRNAs; mock transfection; treated cell, etc). However, since siRNA has off-target effects there is still the possibility of non-specific silencing of unintended proteins. Such a risk is mitigated by comparing several target sequences, as well as having proper controls.

An alternative to siRNA knockdown is the knockout (KO) approach. The CRISPR-Cas9 system (a prokaryotic defence mechanism to remove phage DNA from its genome), can be used to delete an epitope or to introduce a frameshift mutation into the genome. This leads to non-functional protein expression (when both copies of the gene in a diploid cell line are

successfully targeted). This enables screens for selective monoclonal antibodies by testing multiple specific clones for off-target binding. This approach is most effective when the KO is introduced into a cell line known to express other members of the same protein family. Antibodies validated in a KO cell line still need validation in the tissue or cell type needed in each new project. KO cell lines are now being increasingly used by antibody producers for primary validation. A selection of commercial KO cell lines is currently available with the matching parental cell line as a control, and with a lead time of fewer than 7 days. A custom-made KO cell line (when biologically feasible) has a lead time of approximately 12 weeks. When available, KO mice allow for comparisons in different tissue types. However, many genes do not allow the generation of systemic KO mice, and antibodies not cross-reactive in mouse cannot be validated this way. While CRISPR-Cas9 technology has greatly facilitated the generation of KO mice, it may still take a year or more to generate a colony of KO mice suitable for experiments.

In general, for antibody validation the following recommendations are made:

- Always include relevant positive and negative controls for validation of each batch
- Always first repeat the results of the product datasheet to make sure the antibody has not lost its integrity.
- Validate in the application in which the antibody is used
- Validate in the tissue type or cell type in which the antibody is used
- Use the validated antibodies at their optimal dilutions/concentrations
- Compare results obtained with different antibodies from different sources

It is important to note that even so-called standard applications used for antibody validation can present unexpected problems, for example, the frequently used WB and IHC:
For Western blot (WB): When quantitative analysis of band intensities is required, gel loading, band detection and intensity normalization are all non-trivial. Between X-ray film, colorimetric, chemiluminescence, and CCD camera for detection, the signal generation, linearity and saturation levels are different[30], thus affecting outcomes and experimental interpretations. When identical blots are labelled with the same antibody at the same dilution, differences are also observed between incubations in phosphate-buffered saline and Tris-buffered saline[30]. The detection of so-called house-keeping proteins (HKP) as loading controls has limited value as their expression levels vary between different tissues. Quantifying the protein loading using a total protein stain as a reference is preferable to internal HKP. Purified proteins as standards need to be loaded in much lower quantities than lysates to stay under saturating signal levels. It is important to use both technical replicates (e.g. same lysate used multiple times), and biological replicates (different lysates of same cell type) and to be beware that observed molecular weight (MW) may not correspond to

calculated MW due to post-translational modification (PTM), cleavage, etc. It is not hard to find antibodies performing anomalously in WB. An investigation in 2015 showed that five commercially available antibodies against interferon-stimulated gene 15 (ISG15) each gave different results with respect to the semi-quantification of ISGylated (ISG15 labelled) proteins in young and old rat hearts[31]

For immunochemistry (IHC/ICC): Under semi-native or native conditions (frozen sections, or unfixed cells), the antibodies must bind the target protein in its near-native conformation. In IHC however, the protein may need to be detected after fixation, embedding, and with post-translational modification, cleavage, or interacting proteins each specific to that cell / tissue type. During sample preparation, epitopes may have to be retrieved: fixation derivatizes certain amino acids, thus potentially destroying epitopes, while cross-linking reduces antibody access into tissue. Orthogonal studies do not suffice, when proteins are spatially separated from their mRNA. For example, when the protein is secreted, or when in neuronal cells the mRNA is in the cell body but the protein is trafficked through the cell's processes. As such, no matter what validation approach is used (genetic, orthogonal, etc.) it is crucial to perform the validation on the type of tissue and under the sample preparation conditions that will be used in the experiments.

When studying surface proteins on cells that express Ig receptors, epitope-independent signals arising from these Ig receptors can be reduced by the use of $F(ab')_2$ antibody fragments, or of negative isotype control in combination with Ig from a different species as a blocking agent.

For protein arrays: Protein arrays can be excellent for addressing the specificity and selectivity of antibodies, depending on the composition of the array. The greater the number of arrayed proteins, the higher the degree of selectivity analysis that can be achieved; arrays with over 20,000 full length human proteins are now available, covering much of the human proteome[32]. Shared epitopes can be identified through cross-reactivity to other members of the same protein family. For each antibody, the optimal dilution can be identified by the maximal specific signal with a minimal signal of cross-reactivity. The practical implications of cross-reactivity revealed by largescale screening on protein arrays will depend on the particular application. In principle, the arrays can be used for detection of reactivity against proteins in either native or denatured states. However, the extent of native conformation in each individual protein across the entire array, and the way the proteins were manufactured (usually by recombinant expression in *Escherichia coli*, or in yeast) need to be considered.

Recombinant antibodies:

Recombinant antibodies are monoclonal antibodies cloned and artificially expressed in a cell line. The recombinant antibody is immortal by virtue of its defined DNA sequence. This unique identifier, at least in principle, fundamentally distinguishes recombinant antibodies from other commercial antibodies.

Antibodies bind the epitope on the target protein via their antigen binding site, also called the paratope. This is built from parts of the variable (V) regions of the light chain (VL) and the heavy chain (VH) at the tips of each arm of the Ig molecule. The smallest antibody element with a complete antigen binding site is the Fv fragment, comprising only the VL and VH regions. To stabilize Fv fragments, either the V regions are connected to a soluble and flexible peptide (creating an scFv - single chain fragment variable), or the constant (C) domains are added to the Fv to create a Fab fragment[33].

Recombinant antibodies may be derived from a hybridoma cell line by cloning the V regions from the cell line into an IgG expression vector. Alternatively, specific V regions (scFvs or Fabs), selected by virtue of their binding activities from highly diversified phage display libraries, are subsequently re-cloned into IgG expression vectors. Recombinant antibodies with known V-region DNA sequences are immortal because these sequences can be re-synthesised to recreate the antibodies using the expression vector[33]. Depending on the frequency of usage and the quantities needed, recombinant antibodies can be expressed either in transiently (e.g. in HEK293 cells) or continuously (e.g. in CHO) producing cell lines.

Recombinant antibodies solve many of the problems of hybridoma-derived monoclonal antibodies or polyclonal antibodies described above, and in addition, they can even give stronger signals than their parental hybridoma-derived version[24]. Despite the dramatic benefits of recombinant antibodies, a few realities should be noted: costs are high and lead times long to clone V regions for a new recombinant antibody from a hybridoma, as does the need to test each VH / VL combination to find the optimal candidate. However, many companies now clone from hybridomas at reasonable costs. Generating recombinant antibodies from *in vitro* display libraries (phage or yeast) requires access to a very high-quality library and to high-quality antigen for display-selection, both of which are critical for success. Poor quality libraries or targets lead to poor quality antibodies, and if high quality libraries and targets are available, the lead time to generate a recombinant antibody is faster than approaches based on immunization.

It is unfortunate that in general, V region sequences are currently not disclosed (to avoid copycat synthesis and pirating of non-patented sequences) for commercially available recombinant antibodies. While patenting the sequence would protect the organization that has invested in quality control, the profit margins in the reagent market are apparently too small to justify this.

It must be emphasised that, just as for all other antibodies, recombinant antibodies still need to be fully validated for the specific experiments in which they are required in a project. While recombinant antibodies provide solutions both to the identifiability problem and to the risk of losing a validated hybridoma antibody, validation and QC is of course required for each production batch.

Alternative affinity-reagents:

Several affinity binders based on scaffold proteins have been designed and used as alternatives to antibodies[34], including Adnectins, Affibodies, Affimers, Anticalins, Bicyclic peptides, DARPins, Fynomers, Kunitz domains, Monobodies, etc. The general concept for each is similar: a stable scaffold protein is used to display diversified amino acid sequences at exposed surface sites, and the affinity binders are selected using an appropriate display platform (phage, yeast or ribosome display). Because of the small size and their low-cost production, such alternatives to antibodies are being used in clinical trials[34], and will likely soon enter the commercial tool affinity binder market. As with antibodies, validation for specific purposes and batch quality control remain necessary.
DARPins may be especially interesting due to their high stability and high production levels when expressed in bacteria, their potential multivalence, possibility of site-specific conjugations, and their potential picomolar affinities[35]. They are a cost-effective solution for large-scale production (the costs of generating a new DARPin are similar to making a custom monoclonal antibody), for detection or interference inside targeted cells, and for multiplex applications. They have been successfully used for CAR-T cells, for viral manipulations and for cytoplasmic markers. Like for many scaffolds, currently the research market is not yet served commercially, but only through academic collaborations. Because of the attractive profit margins, commercial use has been focused on therapeutics.
In addition to protein-based binders, chemically stabilised oligonucleotides, known as aptamers, have also been successfully used. Large aptamer libraries allow screening for specificity and selectivity reminiscent of recombinant antibody fragment libraries. However, they are (still) too costly for fundamental research applications and to date they are mainly applied in drug and biomarker discovery[36].

Conclusions

The webinars highlighted several methods to identify and to validate optimal antibodies from the complex commercial market for integrity, specificity and selectivity with respect to the required specific applications. The choice of the optimal antibody depends on the experimental conditions it is to be used for (and the antigen that was used to generate and select it). For example, an antibody excellent for WB may be ineffective in other applications such as IHC, ICC or ELISA and *vice versa*. All verification procedures have caveats and therefore a combination of the suggested methods should be applied for each experimental context. It is also important for researchers to select antibodies that are expected to be available in the long-term, and to fully described what these are, and how they have been validated for their experiments, so that these can always be repeated by other researchers.

Discussed subjects	**Webcasts**
Cross-reactive antibodies with clinical implications	#2a, #2b, #3a, #9
Specificity	#1, #2b, #3a, #4b, #5, #6a, #6b,

	#7a, #7b, #8a, #8b, #10
Selectivity	#1, #3a, #4b, #5, #6a, #7b, #8a, #8b, #9
Clonality	#1, #3a, #4b, #9
Availability	#3a, #4b
Rigorous reporting	#2a, #4a, #6a
Literature citations	#3b, #4a, #9
Finding the right antibody	#3a, #3b, #4a, #4b, #8a, #9
Antibody validation	#1, #2a, #2b, #5, #6a, #6b, #7a, #7b, #8a, #9
KO / KD of gene expression	#5, #7a
Assays for native vs denatured proteins	#1, #3a, #5, #6a, #6b, #8b
Protein arrays	#8a, #8b
Recombinant antibodies	#1, #7b, #9
Alternative affinity-binders	#10

Table 3. Overview of the several aspects highlighted in the webinars (a and b refer to the first and second talks in a webinar).

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Disclosure statement
JLAV, and AS are founder and employees resp. of companies selling antibodies
AB and ADC are founders of online antibody search tools
ARMB is CSO of a company selling antibody libraries and selection services
TH is an employee of a company selling knockout cell lines and knockdown reagents
MJT is the founder of a company providing protein array sales and services
AP is founder of companies developing antibody and affinity-binder libraries.

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