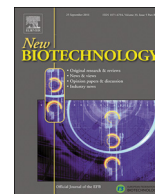




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Antibody validation: a view from the mountains

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ABSTRACT

Validation of antibodies and other protein binders is a subject of pressing concern for the research community and one which is uppermost in the minds of all who use antibodies as research and diagnostic reagents. Assessing an antibody's fitness for purpose includes accurate ascertainment of its target specificity and suitability for the envisaged task. Moreover, standardised procedures are essential to guarantee sample quality in testing procedures. The problem of defining precise standards for antibody validation has engendered much debate in recent publications and meetings, but gradually a consensus is emerging. At the 8th Alpbach Affinity Proteomics workshop (March 2017), a panel of leaders in the antibody field discussed suggestions which could bring this complex but essential issue a step nearer to a resolution. 'Alpbach recommendations' for best practice include tailoring binder validation processes according to the intended applications and promoting greater transparency in publications and in the information available from commercial antibody developers/providers. A single approach will not fit all applications and end users must ensure that the reported validation holds for their specific requirements, highlighting the need for adequate training in the fundamentals of antibody characterisation and validation across the user community.

Introduction

Ensuring correct antibody validation is a subject of primary concern for research and clinical users, commercial producers, journal publishers and database curators of antibodies and antibody-linked research alike. It was a key area of discussion at the 8th Alpbach workshop on Affinity Proteomics held in March 2017 (<https://affinityproteomicsalpbach.com/>), as reflected in the contributions in this Special Issue. The overarching objective of antibody-based research and diagnostics is to be able to select “the correct reagent for a particular target for a specific application” [1]. Much of the discussion in recent years has centred around the frequent reports that many research antibodies fail to live up to expectations, e.g. by not recognising the protein they are supposed to, by recognising a different protein instead of, or in addition to, the desired target, or functioning in some applications but not others [2]. The Alpbach meeting was reminded at the outset (Mathias Uhlén, Stockholm) that the Human Protein Atlas (www.proteinatlas.org) [3] has examined some 55,000 polyclonal antibodies

(pAbs) and 5000 monoclonals (mAbs) and found that, although many were validated in western blotting, only about 50% worked satisfactorily in the applications and sample preparation conditions used to generate the Atlas (immunohistochemistry and immunocytochemistry) [4]. Such frequent reports of failings have created a degree of uncertainty and confusion around antibody reagents as well as documented errors in published work, e.g. [5–9]. As a result, regardless of arena and application, a tremendous amount of time, effort and expense is still expended in antibody-based experiments that have the potential to yield erroneous (false positive/false negative) results, or alternatively in extensive measures aimed at ensuring that the properties of reagent antibodies from commercial or academic sources are indeed relevant and appropriate. Where such reagents can be thoroughly tested there is at least the hope that, for future users, careful documentation and transparent disclosure of the results will enhance the utility and reliability of research performed using them. All too often the onus for checking reagents does indeed fall on the users, especially when the required validations have not been performed

Abbreviations: mAb, monoclonal antibody; pAb, polyclonal antibody; WB, western blot; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; IHC, immunohistochemistry; ICC, immunocytochemistry; IP, immunoprecipitation; IWGAV, International Working Group on Antibody Validation; MS, mass spectrometry; MIAPAR, Minimal Information about a Protein Affinity Reagent; PTM, post-translational modification; RRID, Research Resource Identifier

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adequately or reported transparently by the developers/suppliers. This also applies for antibodies that have been extensively validated and transparently reported, but for which the intended use falls outside those for which validation has been performed and/or reported.

Recent publications and meetings in the USA [10] and UK [11] as well as Alpbach have highlighted some of the practical challenges and uncertainties surrounding the development of universal standards for antibody validation. Important aspects are how such standards may impact the use of antibodies and other protein binders by the research community on the one hand and on the business of antibody development and distribution on the other. Issues around antibodies in research can also be seen as part of a larger drive to enhance rigour, reliability and transparency of biomedical research in general [12–18]. The aim (applicable to validation) is to ensure research reproducibility, with transparency as the means to achieve it.

Following in this journal issue, authored by Alpbach participants, the Discussion article by Simon Goodman [19] relates his vexed personal experience of using commercial antibody reagents and the complexities at different levels surrounding their fitness for purpose. He argues for a number of remedies, including greater use of recombinant antibodies, more transparency on the part of (certain) producers and ultimately preparedness of individual users to carry out their own validations in their technique of choice. Ulf Landegren and colleagues [20] follow up with their thoughts on the importance of achieving detection specificity sufficient to measure very low concentrations of target proteins such as troponin and other leakage markers, and how this may be achieved by proper design of diagnostic assays. Considerations of specificity also permeate the other articles in this Special Issue.

Characterisation and validation of antibodies

Antibody characterisation and validation can be regarded as parallel requirements which go hand in hand in determining the properties governing the use of antibodies in different applications.

Characterisation encompasses the basic attributes which are the core information for any antibody, namely the nature of the antibody molecule and the specific preparation in which it is being supplied (serum, purified IgG, affinity-purified, etc.), knowledge of its binding specificity (identity of the target recognised at both the whole molecule and epitope levels), cross-reactivity (identity of non-target reactants and the extent of off-target binding), affinity binding constant (both equilibrium and kinetic parameters), the antibody sequence and ultimately its combining site structure when complexed with the target. Typical characterisation methods include enzyme-linked immunosorbent assays (ELISA, target reactivity), surface plasmon resonance (SPR, affinity determination), peptide arrays (epitope mapping), protein arrays (specificity screens), variable (V)-gene cloning (sequencing) and X-ray crystallography (native and complexed structure determinations).

Validation extends these properties to the criterion of ‘suitability for particular applications’, which in the case of antibodies are legion and include immunoprecipitation (IP), western blotting (WB), sandwich assays, immunohistochemistry (IHC), immunocytochemistry (ICC), flow cytometry, proximity ligation, intracellular and *in vivo* action, and many others [21]. On the face of it, the high failure rate among antibody reagents in these techniques may seem surprising: the major distinction between native, fixed and denatured protein structures is well known, requiring appropriate reagents for those categories, but within them a reliable outcome with a target-specific binder would, perhaps naïvely, be anticipated. If that were the case, only three types of test would be needed, IP, IHC/ICC and WB. However, applications are increasingly specialised so that other considerations (e.g. how exactly the sample is fixed or denatured, the composition and complexity of the sample, incubation conditions, etc.) can moderate antibody functionality [4,22–24]. Moreover, target proteins of the same primary sequence may exhibit subtle cell- or tissue-specific differences which

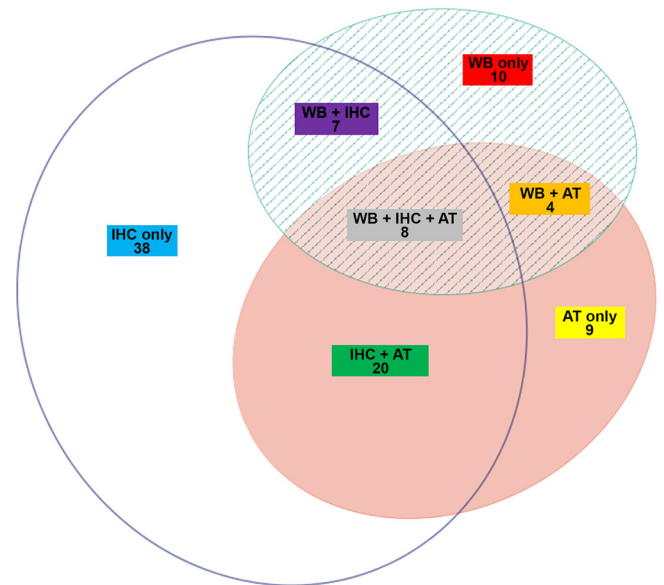


Fig. 1. Application-specific validation of target-specific mAbs. The Euler diagram depicts subsets of ELISA-positive mAbs that exhibit efficacy and specificity in applications on native brain tissue. WB: Western blot. IHC: conventional IHC on brain sections. AT: Immunofluorescence labelling of brain sections prepared for array tomography.

can alter their conformations and their epitopes, such as post-translational modifications (PTMs, e.g. glycosylation, phosphorylation), interacting proteins, etc. As antibodies are themselves proteins, their conformational diversity and PTMs can also impact their binding characteristics [25], whether produced recombinantly or from natural sources. Thus, specificity, the most familiar property of antibodies, and its thorough and exact delineation for every case, turns out to be a major part of the problem. Validation of specificity for the ‘real world’ applications in which it will be used, namely against the target expressed at endogenous levels in cells and tissues, is the ultimate consideration that, from a practical standpoint, exceeds simple characterisation performed against a single, or set of, purified or exogenously overexpressed targets.

We provide one example (Fig. 1) of a dataset showing application-specific differences in validation of a sizeable collection of target-specific monoclonal antibodies (mAbs). In this case, 96 mAbs were selected on the basis of overall immunoreactivity in ELISA, either against cells overexpressing the target protein or purified target protein. This entire set of ELISA-positive mAbs was then assayed for efficacy and specificity in distinct applications on native brain tissue samples, namely WB, IHC and specialised IHC in the form of plastic embedded sections prepared for array tomography [26]. These results underscore that distinct mAbs (or for that matter any other binder type) may be suitable for a particular assay, but unsuitable for another even highly related assay, and that validation needs to be performed for each intended purpose. They also speak to the need for transparent reporting of the exact nature of any prior antibody validation, enabling it to be thoughtfully evaluated in relation to the user’s needs.

These issues are not restricted to antibodies but are equally applicable to non-antibody protein binding molecules, such as DARPins, aptamers, monobodies, affimers and other molecular entities [27], all of which exert an effect through their ability to bind to a protein target. Moreover, they hold true as much for binders made through recombinant technologies as for the classical pAbs and mAbs. Purified pAbs often have excellent monospecificity, especially when affinity purified, and in the Human Protein Atlas validation results for a large

number are presented transparently. While, in principle, mAbs have the capacity to be more specific, they sometimes exhibit entirely unexpected strong cross-reactions, where an epitope other than that intended fortuitously interacts well with the combining site. Although this has the potential to be amplified in polyclonal preparations, in which the constituent antibodies could each exhibit distinct properties, cross-reactivity in a pAb may be diluted out in many cases by being a mixed population, while reactivity with the target is common to all its components. In some cases, mAb cross-reactivity has been analysed by X-ray crystallography; indeed, it is possible to select for useful bispecific interactions in the same combining site [28]. It is perhaps too early to understand fully the specificity characteristics of the non-antibody binders where a smaller range has been produced, but for which many of the same principles will likely hold.

The antibody validation problem

There is no one particular use of antibodies: they reach into every aspect of biological and biomedical research, and immunoassay methods of one form or another are commonplace in virtually every type of research project. Different techniques make their own distinct demands of antibody reagents, but with shared underlying principles, essentially of specificity, cross-reactivity, affinity, functionality and having the right tool for the right job, all viewed in the context of how the antibody will be used, including details of sample preparation. While there will be instances of anticipated cross-reactivity, such as between different isoforms of the same protein, each containing the epitope, there is also the unpredictable cross-reactivity to nonrelated proteins, as well as binding to other material in the sample that could associate with antibody molecules in a manner distinct from *bona fide* antibody-antigen interaction. An important parameter in all the methods is the condition of the antigen target, e.g. whether in a native, fixed or denatured state, with the devil being in the details (see also pre-analytical considerations below). Unfortunately, essential characterisation and validation information on the potential antibody reagent and the state of the target is frequently incomplete, may be commercially biased or even completely lacking (on grounds of commercial secrecy), including even the most fundamental detail of the immunogen employed to generate the antibody. Thus, the need for standards of antibody validation and agreed procedures for determining it are now well recognised. Moreover, validation information is not static, but needs to be confirmed or updated for every different form and batch of the product even when recombinant; hence the importance of recording the exact origin of the antibody, including batch identification, in publications and databases. Reproducibility of research with antibodies depends on their correct validation, but testing them is context dependent, which is the nub of the problem.

User surveys can be useful in trying to define the level of concern for this problem across the research community. In one case [29] a survey highlighted an association between willingness to carry out in-house validations and number of years of research experience of the respondents, drawing the conclusion that young researchers often either lacked awareness of its importance or saw validation as an obstacle rather than a necessity in their own experimental work. The conclusion was that a focus on education and training of junior researchers is needed to make them aware of the issue and ensure they are prepared to make the effort to satisfy themselves that the reagents they use perform as intended.

Sample quality standards in the pre-analytical phase

A particular instance of the need for quality control standards in respect of the sample rather than the antibody applies in the initial phase of the analytical process, including sample collection, handling, labelling and storage, especially in the clinical field where it is of utmost importance to ensure that correct and reproducible results with

antibody reagents are achieved. The pre-analytical phase is considered one of the most vulnerable parts of immunoassays in general, highlighting the need for harmonisation between different centres. If a sample is not handled correctly at the beginning of the analytical process, all subsequent antibody validation approaches will be compromised [30]. This was illustrated at the Alpbach workshop in connection with the reverse phase protein array (RPPA) technology (Karl-Friedrich Becker, Munich). RPPA may have an important clinical application for analysis of signalling networks and protein biomarkers in cancer tissues, but only if it can be guaranteed that the sample collection procedure follows identical standards within different institutions, since differences in handling will potentially cause unpredictable changes in protein and phosphoprotein profiles [31,32]. Similar considerations apply to the effect on plasma protein determinations of pre-analytical blood collection and handling procedures, with temperature and pre-centrifugation delay of the blood sample being critical factors [33]. Standardisation of pre-analytical procedures is also particularly relevant in personalised medicine applications, where fast and accurate results could make a significant difference in the treatment approach to be followed. The EU consortia SPIDIA and its follow-up SPIDIA4P have proposed a number of CEN (European Committee for Standardization) technical specifications and ISO (International Organization for Standardization) global standards that provide guidelines for standardisation in the pre-analytical phase. Several of these documents are already available, with more expected in the coming years [34].

A range of solutions: agreeing on validation guidelines

A number of initiatives have suggested broad guidelines for antibody validation as well as detailed context-dependent measures. Three active groups offering solutions which can be highlighted are The European Monoclonal Antibody Network (EuroMABNet) [35], the International Working Group on Antibody Validation (IWGAV) [1] and the Global Biological Standards Institute (GBSI) [36].

EuroMABNet (www.euromabnet.com), comprising a network of well experienced academic laboratories engaged in mAb production, provide in their ‘practical guide’ [35] a very useful introduction to manoeuvring through the antibody jungle, tackling the validation issue stepwise from the ground up. Their article offers much helpful advice on, among others, obtaining background information on immunogen selection and design, the choice of antibody types in relation to envisaged techniques, finding highly rated suppliers and features of different databases. The point that the onus for validation is on the user is made repeatedly and is coupled with a set of criteria and instructions for researchers to use in-house for each antibody, tailored to the identity of the antigen and the demands of the technique to be used. The importance of confirming reactivity with the endogenous antigen is emphasised, an aspect now increasingly being confirmed with gene edited cell lines or knockout animal models.

A major contribution to bringing validation methods fully up to date has been made by the IWGAV in defining five sets of criteria or “validation pillars”, making use of cutting edge developments in genomics and proteomics [1]. The approach is to seek correlations between the detection and abundance of antigen, as determined by the antibody in question, with (a) the effect of specific gene disruption or mRNA knockdown, (b) use of antibody-independent methods, (c) results obtained using independent antibodies against different target epitopes, (d) the expression pattern of tagged proteins and (e) target identification by immunocapture linked to mass spectrometry (MS). Any of these criteria, preferably in combination, could be applied as appropriate validations for particular technique applications. While each assessment method has its pros and cons, the aim is to allow for effective assessment of specificity, potential cross-reactivity and functionality *in the context of the desired samples and applications*, complementing the properties defined in primary characterisation. Acceptance of the pillar criteria could also make for greater uniformity in the important matter

of fully reporting the properties of antibody reagents in publications and databases, as discussed below.

A dedicated meeting, Antibody Validation: Standards, Policies, and Practices, was held in September 2016 at Asilomar, California, organised by GBSI in conjunction with The Antibody Society [36]. It focused less on the details of validation methods and more on developing standards to certify antibodies, with multiple panel discussions and small working groups on particular immunoassay techniques. The aspiration was that a clearer understanding of the landscape of antibody validation would form the foundation of proposals for concrete standards regarding what constitutes a properly validated (and then certified) antibody. The attendees included leading academic biomedical researchers, commercial producers, journal editors and others advocating training, and the organisers went to some lengths to encourage audience engagement. Breakout sessions with working groups focused on developing certification standards for specific applications, including WB, IHC, IP and sandwich assays, were followed by electronic polling sessions which sought to come to consensus on their recommendations. This resembled the survey approach (above), but on-the-spot consensus proved quite difficult to reach, with strong voices counter to almost any approach proposed. Consequently, it was deemed that further deliberations among working groups and selected panels would be needed. In the meantime, the outcome of the meeting has been presented in the form of ‘consensus principles’ and an extended report [36], largely based on the IWGAV methodology.

One problem encountered, which goes back to the sheer variety of antibody applications, is the diversity of different criteria among groups of stakeholders. As an example, in IHC the needs for those in the clinical pathology arena are very different from those engaged in basic research, such that antibodies certified for one purpose would likely fail in the other, due to dramatic differences in sample preparation in terms of fixation. Similar anomalies exist for other techniques.

A suggestion promoted at the Asilomar meeting was in favour of a ‘pick the winner’ strategy with a scoring system for each antibody measured against a predefined set of rules and ultimately with each antibody listed in catalogues or databases according to its score [36,37]. “Workshop attendees generally agreed that a system for scoring of antibodies for their overall quality and performance, and individual characteristics would be useful” [36]. This is a point of contrast with the IWGAV and EuroMAbNet approach of defining good quality antibodies through formal assessment criteria, with each antibody classified according to whether or not it fulfils the relevant criteria. The design of a universal scoring system raises questions of objectivity, scope, value and implementation, and the concept is likely to prove controversial, although even a compilation of the criteria used to validate individual antibodies would be valuable.

Minimal information initiative: MIAPAR

Reporting guidelines for unambiguously describing an antibody or other binder in its functional context, setting out the information required and a format for reporting, were provided a few years ago in the ‘minimal information’ style as MIAPAR or ‘Minimal Information about a Protein Affinity Reagent’ [38], developed within the EU ProteomeBinders project [39]. “The key principle in MIAPAR is that an affinity binder is designed/developed to recognise a target, and unambiguous description of the binder requires description of its design/development, production, and evaluation as a molecular tool.” [40]. In parallel, ProteomeBinders also proposed a Proteomics Standard Initiative for Protein Affinity Reagents (PSI-PAR) as a global community standard format for the representation and exchange of protein affinity reagent data [41]. The aim of MIAPAR was to permit the reliable reporting and identification of affinity reagents, their targets and applications; in short, a complete description of the antibody as a molecular reagent. Thus, a MIAPAR reporting document would include details of the antibody’s production, its characterisation features, the validations

performed describing its use in applications, and experimental references. The reported information is structured so as to allow for entry into databases and enable useful querying and automated data analysis and could be used in commercial catalogues or public databases. Authors submitting a paper including a new antibody or other affinity reagent would be requested to complete a MIAPAR form requiring the information, while subsequent publications could reference the MIAPAR description. Thus far, the MIAPAR format and recommendations, as well as similar attempts to establish a uniform convention for antibodies and other affinity proteomics reagents, have not been embraced by the community or publishers, and journals may be unwilling to impose a stringent requirement. Nevertheless, the MIAPAR criteria can provide a model and framework for eventual adoption by journals.

Databases

Antibody databases can make an important contribution to validation and provide users with comparisons and recommendations. This is particularly important where, as frequently arises, researchers can choose between a large number of alternative antibodies for the proteins they study and require, as much as possible, some objective means to select the best products for their particular use. Two which were highlighted at Alpbach were CiteAb (www.citeab.com) (Andrew Chalmers, Bath) and pAbmAbs (<http://pabmabs.com/wordpress>) (Simon Glerup, Aarhus). CiteAb [42] mines the scientific literature, “combined with human validation”, for publications citing the use of individual antibodies, to generate a comprehensive searchable database of literature citations, together with suppliers, blog and news. Identification of citations is made much easier if the product code is mentioned, as well as the company name, in the Materials section of a publication (see below); thus, a significant complicating factor is that the same antibody can be sold by different suppliers under different codes. Searching for an antibody in CiteAb leads to a guide to reported applications. Whereas CiteAb does not provide evaluations from individual users, in pAbmAbs antibodies are evaluated by feedback from users who submit short reports to the website; the antibodies are ranked according to a star rating system with links to the reviews. Such consumer reports are clearly interesting and often valuable, but there will be an element of taking feedback on trust, especially if the skills of the reporting researcher are not known and the precise conditions of each assay performed are not sufficiently detailed.

Alpbach panel discussion

The antibody validation debate was continued at the 8th Alpbach workshop with a panel comprising academic researchers, publishers and commercial providers.*(see footnote) The panel was also invited to speculate on the future of antibodies. The following summarises points made in the discussion.

Validation principles

- While producers have a responsibility to provide as much detail on the origin and evaluation of their products as possible, including what has and has not been tested, as well as on the precise nature of the product provided, ultimately the onus is on the user to ensure the required validation has been performed.
- The now widely held view was affirmed that antibody specificity is context-dependent and that antibodies can only be properly validated in the application techniques and under the conditions in which they will ultimately be used. Validation should use the most modern technology available and be described in transparent detail (Mathias Uhlén, Stockholm). It should be noted that the conditions of an application assay will affect the properties of both the target and the antibody.
- As a corollary, success or failure in the use of a given binder may

only hold for the application in which it was tested and with samples prepared under those specific conditions and may not translate to other applications or other sample preparation conditions.

- Merely showing that an antibody has low binding to an off-target protein may not be sufficient specificity validation without considering the relative concentrations of the proteins in the samples to be tested: a weak cross-reactivity may take on great significance if the cross-reactant is present in the sample at a much higher concentration than the true target. Hence results of tests for specificity of protein detection must take into account the vast concentration ranges of endogenous target molecules which exist in practice in biological samples. This challenge is prominent in the measurement of leakage protein markers that can signal the presence of damage to specific tissues (Ulf Landegren, Uppsala).
- There is now available a variety of affinity reagent types (pAbs, mAbs, recombinant, scaffolds, *etc.*) and in different forms (antiserum, affinity purified, *etc.*) which will work in different applications and have inherent differences in regard to reproducibility (*e.g.* the need to revalidate every batch of pAbs). Nevertheless, each class of binders has their own importance in affinity-based research. The panel endorsed the desirability of knowing the binder sequences for unambiguous identification and favoured a greater use of recombinants, which would mean in principle that sequence identity is readily available and is expected to lead to greater experimental reproducibility, by allowing researchers to ensure that they are using the same molecular entities. (Andrew Bradbury, Los Alamos; Andreas Plückthun, Zurich). However, commercial suppliers, although starting to embrace recombinants, are very reluctant to reveal their antibody sequences in order to protect their products.
- There would be advantages in a more quantitative proteomics approach to replace WB, with the data made publicly available in a format that could be subjected to meta-analysis, in keeping with the theme that reproducibility comes from transparency. (Fridtjof Lund-Johansen, Oslo)
- For use of IP-MS to define antibody specificity and cross-reactions, it would be beneficial to store data in a public database of antibodies used against various cell lines and other biological samples. The challenge would be to produce a suitable format for the complex raw data so as to be comprehensible by a wider audience (Susanne Gräslund, Stockholm).
- Given that researchers still buy many poor quality antibodies, the question is whether it is feasible to implement standardised testing of a very large number of reagents in a large number of companies, and whether this can be done at an acceptable cost. There was also scepticism over the possible grading of antibodies by a universal scoring system [36,37], which was regarded as potentially complicated and difficult to distil from large amounts of data.
- The fact that the same antibody can often be obtained from different suppliers, using different identification codes, confounds good faith efforts to replicate results with two independent reagents, as well as wasting valuable time and resources for researchers comparing what appear to be independent antibodies, for specificity and efficacy in their experimental system, but which in reality are the same antibody from different sources.

Publication of antibody-based results and requirements of authors

- Publishers are in a unique position to play a role in improving antibody validation by determining what authors will be required to include in the Methods sections of submitted publications. The main requirements are compliance with agreed guidelines, transparency in describing validation conditions and outcomes, and traceability, *i.e.* ability to identify reagents, including batches, and certified conditions of use. As a minimum reporting standard, which should be required by all journals, the vendor and catalogue number should always be reported for mAbs, together with a lot number for pAbs. It

would also be appropriate to introduce a section on validation of key reagents into the Methods and encourage inclusion of detailed protocols. However, it is difficult for journals to mandate validation information or for staff to check it.

- Beyond the straightforward statement of supplier, batch, *etc.*, the situation regarding publishing guidelines is complex. According to Natalie de Souza (Chief Editor, Nature Methods), while journals can support and promote reagent validation and sharing, it is questionable whether they can respond effectively to the challenge of changing author behaviour. Major journals have taken a strong lead in raising the problem and increasing awareness. They also have the leverage to insist on standards but, with so many disparate publications, enforcing such standards across the entire field will be difficult. Moreover, antibodies are not a clearly defined field in themselves, their use is diverse, and they have no single user community, presenting a challenge of implementation of uniform standards. There is also the matter of limited editorial resources, so that where journals do request specific information and references, the rules are not strictly enforced. Until validation guidelines are agreed within the community, mandating inclusion of specific validation steps is unlikely.
- The issue of limited editorial resources was also made by Janice Reichert (The Antibody Society, Editor in Chief of mAbs) who pointed out that, while mAbs (the journal) in common with other journals requires, as a minimum, basic vendor information including lot numbers, any more advanced publication standards introduced for antibody validation must also be practical. She suggested that author checklists could be introduced, which would first require agreement on establishing the criteria and secondly place extra work on editorial staff and reviewers in going over the checklist data. The introduction of checklists could also have an educational effect on authors in alerting them to the issues.
- There is also a burden on reviewers and, as cross-reactive antibodies may yield incorrect results, it is reasonable to ask what type of evidence they should request to confirm results and to what extent researchers can cite manufacturer testing. The question was posed by Fridtjof Lund-Johansen (Oslo), who also asked whether criteria need to be uniform for all results or just for those that are used to support the main conclusion. In reply, Natalie de Souza (Nature Methods) pointed out that editorial judgement will be needed, according to the importance of the antibody to the particular work. At one extreme, the antibody may be a new reagent and central to the conclusions of the study, in which case validation should be part of the paper. At the other extreme, the use of a particular antibody is peripheral to the main story, is of commercial origin and already well-documented in the literature, in which case simply reporting the details of the commercial product could be sufficient. It is also appropriate to ask reviewers with appropriate expertise to check the information on reagents, similar to the practice with statistics.
- According to Lynn Sherrer (Elsevier, publisher of New Biotechnology), although there are time constraints in the publishing process and enforcement will not be easy, if publishers adopt reasonable standards that are mandatory, with feedback from the stakeholders, authors will adapt to them. Notwithstanding the concern that some may decide not to publish in a journal that has stringent standards for open data and transparency, most would continue to try to publish in high-impact journals. It is also an onus on the research community to become engaged when their members do not provide full and appropriate information and to be proactive in using feedback mechanisms to report and question reproducibility.
- Another useful reference point for antibody citation discussed by Christine Ferguson (previously joint Chief Editor PLOS Biology) is the Research Resource Identifier or RRID [43,44], assigned *via* the Antibody Registry (<http://antibodyregistry.org/>). The aim of RRIDs is to enable unambiguous identification of the antibody to which it

refers; the RRID link is inserted into the paper and allows people to ask questions like “which other papers use this same antibody?” A significant advantage of such a central antibody registration system is that it reflects information about the product no matter what happens to the supplier name or if the product itself is discontinued; however it was pointed out that RRIDs currently do not include the lot number and that identical antibodies from different suppliers may be given different RRIDs. Altogether (as of March 2017) ~2000 papers have been published citing ~20,000 RRIDs, most of these publications containing references to antibodies as well as other research resources for which RRIDs have been generated (mouse strains, software, etc.). Currently PLOS requests but does not mandate RRIDs [45], but since uptake to date is low, the possibility of mandating is under consideration. Cell Press is another publisher advocating the inclusion of RRIDs in manuscripts (<https://www.cell.com/rrid>). However, it remains the case that acceptance of, and adherence to, any universal system of transparent antibody documentation and reporting has not yet been accomplished.

The future binder landscape

Recombinants or more of the same?

A major area of recent discussion has been the benefits, over standard pAb and mAb reagents, of using antibodies and other binders produced by recombinant technologies, or by conventional means and then converted into recombinant form [46,47]. This potentially disruptive technology is linked to the advantages in validation, reproducibility, replication of results and transparency that would follow from the use of sequenced recombinant reagents and could greatly expand the applications possible today. Andreas Plückthun (Zurich) advocated the use of recombinant binders with known sequences, citing several reasons, principally access by the user to the gene, which would enable different types of ‘next generation’ experiments to be pursued in antibody engineering, such as development of novel binder formats, new site-specific coupling and labelling techniques, as well as rapid adjustment of affinity and cross-reactivity, among others. Sequence knowledge will also lead to greater application of computational modelling and docking for elucidation of epitopes. Notwithstanding the reluctance of suppliers to provide antibody sequences, sequence determination from the protein itself by MS is becoming a practical and affordable methodology, with cost expected to reduce further with time. This would allow thereby reverse engineering of conventional mAbs into recombinant forms, and although unlikely to be adopted by individual scientists, it could form the basis for disruption in the research antibody supply market. New recombinants will be added to the market, including the gene made to order, with the option for the binder to be produced at relatively low cost by the user. On the other hand, the prospect of systematically converting conventional mAbs into recombinants is more distant, because of the resistance of commercial providers and the additional cost. Andrew Bradbury (Los Alamos) also pointed out that the output of hybridomas are often non-homogeneous due to additional immunoglobulin chains, mostly light chains, and that only about 65% of 187 hybridomas screened in a recent survey had a single V_H/V_L combination [48]; if antibodies are recombinant their inherent properties will remain constant. In time, using an unsequenced antibody would be viewed as unacceptable as using an unsequenced plasmid. Indeed several companies are moving to position themselves in the recombinant antibody arena. In the long run, recombinants may not be cheaper, will still require the same level of validation, and some customers will prefer the familiarity of pAbs and mAbs; nevertheless they will enable improved reproducibility and consistency and will extend the possibilities available to researchers.

Assays using more than one binder

It is often pointed out that pairwise binding of affinity reagents

greatly augments specificity over what can be achieved with single binders (Ulf Landegren, Uppsala). This goes back to the familiar sandwich assay which uses two binders against different epitopes for capture and detection; the same principle is employed in the *in situ* and solid phase proximity ligation assays invented by Landegren and colleagues [49] and can be expanded even further to three binders in a novel format [50,51]. Employing the principle that the performance of affinity reagents needs to be measured in the contexts in which they are intended to be used means that binders intended to be applied in pairs should be evaluated as such both in solution and *in situ*. Hence ensuring distinct epitope recognition (absence of inter-antibody competition) becomes critical.

Large scale protein readouts

While the cost of DNA analyses (e.g. single nucleotide polymorphism identification) has decreased by orders of magnitude, this trend is much less evident in protein determinations. A future scenario envisaged for antibody use is the possibility of analysing thousands of proteins in millions of samples of plasma and other biospecimens cheaply and accurately (Ulf Landegren, Uppsala). This will require new standards for several steps, including:

- pre-analytical collection, treatment and storage of samples (see above);
- development and selection of reagents (more rapid, more cheaply, new constructs);
- novel high throughput assay formats; and
- inexpensive readouts with absolute quantification.

Implementation of such large-scale protein analyses will have a significant impact on disease prevention, diagnostics, drug development and selection, and ‘wellness’ monitoring (see also [20] for further discussion of these issues).

Alpbach recommendations

While establishing a single set of universal standards or guidelines was not a goal of this workshop, a set of overall recommendations emerged from the panel discussion (see Box 1). They are not intended to be and assuredly will not be the final word on this subject and the debate over the best way to validate and report reagent antibodies will continue. In fact, future instalments of both the Bath Antibody Validation (<http://www.antibodyvalidation.co.uk/>) and Alpbach Affinity Proteomics (<https://affinityproteomicsalpbach.com/>) meetings will assuredly touch on many of these issues. The frequent and open discussion of these important topics will shape how biomedical researchers view and use antibodies and other affinity reagents in their own research, and the standards employed by developers, suppliers and other entities (reviewers, publishers, funders) that hold a stake in the pursuit of rigorous, reproducible and transparently reported research.

***Footnote:**

Panel participants were Andrew Bradbury (Los Alamos), Andreas Plückthun (Zurich), Mathias Uhlén (Stockholm), Fridtjof Lund-Johansen (Oslo), Susanne Gräslund (Stockholm), Karl-Friedrich Becker (Munich), Ulf Landegren (Uppsala), Alejandra Solache (Abcam), Natalie de Souza (Nature Methods), Janice Reichert (mAbs), Lynn Sherrer (Elsevier), Christine Ferguson (PLOS Biology), Andrew Chalmers (Bath) and Simon Glerup (Aarhus), with Jim Trimmer providing a final summation and Mike Taussig as moderator.

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Box 1

Alpbach antibody validation recommendations.

- 1 Validation is application and context specific. While antibodies in general are all-purpose reagents, any individual antibody must be validated as a fit-for-purpose reagent. The “5 pillars” of the IWGAV [1] provide an agreed-upon framework for validation testing.
- 2 Exhibiting efficacy and specificity in one application against samples prepared under one set of conditions does not necessarily hold across other applications, or even in the same application in which samples are prepared differently. Hence a single criterion of validation is unlikely in general to be indicative of fitness across all applications.
- 3 Test conditions affect the properties of both the antibody and the target. Pre-analytical sample quality control is essential, since correct sample handling is crucial for validation experiments. Variations in sample preparation conditions can significantly alter the structure of the target protein and the chemical properties of its amino acid side chains, which can profoundly affect antibody epitope recognition and binding. Where possible, equivalently prepared sample material should be used in both testing and analysis.
- 4 Validation against cells/tissues expressing endogenous targets in their native environment is crucial to interpretation of antibody-based results. Extensive validation of specificity and affinity against purified recombinant proteins, while informative, may hold little predictive value when the antibody is used in native tissue, due to numerous factors, including orders of magnitude differences in expression levels of on- versus off-targets, cell-specific PTMs and interacting proteins that can fundamentally impact antibody binding.
- 5 Evaluation of specificity and cross-reactivity must take into account the vast concentration ranges of proteins in biological samples, as off-targets with low apparent reactivity *in vitro* may nevertheless show significant binding at concentrations pertaining *in vivo*.
- 6 Transparent reporting by antibody developers of the details of antibody generation and validation is essential to guiding end users towards reagents relevant to their particular research needs. This includes information on: the immunogen; the specific applications evaluated; the sample nature and preparation conditions in those applications; the form of the antibody and concentrations tested; and positive and negative results. It is key to distinguishing end user problems arising from ‘off-label use’ versus failures in manufacturing/QC resulting in the antibody lot distributed being substantially different from that originally validated.
- 7 End users share the responsibility for transparent reporting in their published work, providing accurate and comprehensive details on how they used the antibody compared with its original validation, and any in-house validation performed, should their use differ substantially from previous validations. Transparency is essential for reproducibility of results.
- 8 Training researchers in the basics of antibodies, their use and validation, and its transparent reporting, is key to enhancing reliability and reproducibility of antibody-based research.

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Declaration of interests

Michael Taussig is the founder and CEO and Claudia Fonseca is COO at Cambridge Protein Arrays Ltd., a company which performs antibody specificity profiling.

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