OPINION ARTICLE

REVISED Quality of histone modification antibodies undermines chromatin biology research [version 2; referees: 3 approved]

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v2 **First published:** 28 Oct 2015, **4**:1160 (doi: 10.12688/f1000research.7265.1)
Latest published: 24 Nov 2015, **4**:1160 (doi: 10.12688/f1000research.7265.2)

Abstract

Histone post-translational modification (PTM) antibodies are essential research reagents in chromatin biology. However, they suffer from variable properties and insufficient documentation of quality. Antibody manufacturers and vendors should provide detailed lot-specific documentation of quality, rendering further quality checks by end-customers unnecessary. A shift from polyclonal antibodies towards sustainable reagents like monoclonal or recombinant antibodies or histone binding domains would help to improve the reproducibility of experimental work in this field.

This article is included in the Antibody validations channel.

Open Peer Review

Referee Status: ✓ ✓ ✓

	Invited Referees 1	2	3
REVISED version 2 published 24 Nov 2015	✓ report		✓ report
version 1 published 28 Oct 2015	? report	✓ report	? report

1 **Andrew Bradbury**, Los Alamos National Laboratory USA

2 **Scott B. Rothbart**, Van Andel Institute USA

3 **Shohei Koide**, University of Chicago USA

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How to cite this article: Kungulovski G and Jeltsch A. **Quality of histone modification antibodies undermines chromatin biology research [version 2; referees: 3 approved]** *F1000Research* 2015, **4**:1160 (doi: 10.12688/f1000research.7265.2)

Grant information: Work in the authors' lab has been supported by the DFG JE 252/26-1.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors declared no competing interests.

First published: 28 Oct 2015, **4**:1160 (doi: 10.12688/f1000research.7265.1)

REVISED **Amendments from Version 1**

We like to thank all reviewers for their insightful comments. The reviewers' comments and suggestions were incorporated into the revised manuscript. In brief, we have added a request that new polyclonal antibodies should get new catalogue numbers and old specificity data from one batch must not be transferred to the next. In this respect we added a reference to Voskuil (2014). We included a hint that the validation method should reflect the intended application of antibodies. We discuss the costs associated with improved quality control and now also refer to Bradbury & Plückthun (2015a and 2015b) when proposing a move away from polyclonal antibodies. Finally, we discuss the option to publish the sequences of recombinant affinity reagents to ensure long term reproducibility.

See referee reports

The lack of reproducibility is widely recognized as a serious issue in contemporary research (see (Buck, 2015; Freedman & Inglese, 2014; Freedman *et al.*, 2015; McNutt, 2014a; McNutt, 2014b) and the Nature special "Challenges in irreproducible research" April 2, 2013). In molecular biology, the quality of antibodies has been identified and highlighted as one of the most recurring stumbling blocks that undermine the quality and validity of experimental results (Baker, 2015; Bordeaux *et al.*, 2010; Bradbury & Plückthun, 2015a; Bradbury & Plückthun, 2015b). This issue is even more pervasive in the field of molecular epigenetics and chromatin biology, where antibodies for various types of histone post translational modifications (PTMs) have been single-handedly used to translate the language of histone modifications into experimentally observable properties. Because of this, most of what we know about the distribution, role and function of histone modifications so far has been passed through an antibody as essential mediator.

Raising a specific histone modification antibody is not a trivial task; this is mostly due to the hypermodified state of the histone tail, coupled with the minute size and the chemical relatedness of many histone modifications and similarities in the amino acid sequence of the modified residues. The antibody has to be able to discriminate between the unmodified and the modified state of the targeted amino acid residue, as well as between different forms of modifications (e.g. acetylations of different lysine residues, mono-, di- and trimethylation of lysine residues, or symmetric and asymmetric methylation of arginine residues). Moreover, the presence of an adjacent modification might prevent binding of an antibody to the target modification, causing false negative results. In addition, the antibody should bind the modified amino acid residue only at defined modification sites on the target protein, which implies that not only the modification but also the amino acid sequence must be recognized. This is particularly difficult for some histone modifications such as methylation or acetylation of H3K9 and H3K27 which occur within an identical amino acid context (ARKS motif) and make the readout of the target peptide sequence outside of this central motif vital as well.

In spite of the intricate task of producing histone modification antibodies and their crucial role in chromatin biology, surprisingly, they remain insufficiently characterized. In line with this, numerous scientific groups have alarmingly raised concerns about the promiscuous behavior of some histone modification antibodies and undocumented effects of secondary modifications (Bock *et al.*, 2011; Egelhofer *et al.*, 2011; Hattori *et al.*, 2013; Kungulovski *et al.*, 2014; Nishikori *et al.*, 2012; Rothbart *et al.*, 2015). As mentioned above, the situation in chromatin biology is exceptional, because of the role of histone PTM antibodies as the sole research tool in this field. As a consequence, elaborate quality control criteria for histone PTM antibodies were put forward to ensure the integrity of research (Egelhofer *et al.*, 2011; Kungulovski *et al.*, 2015; Landt *et al.*, 2012). To increase transparency, at least two databases for deposition of antibody quality data from researchers were put in place (http://compbio.med.harvard.edu/antibodies/; http://www.histoneantibodies.com/) (Egelhofer *et al.*, 2011; Rothbart *et al.*, 2015). However, in spite of being heroic attempts, these and similar databases have only a limited value, because most of the antibodies used in chromatin biology are polyclonal, and lab experience over the last years has demonstrated that the specificity data obtained for one batch of antibody do not necessarily reflect the properties of another one (Kungulovski *et al.*, 2014), a caveat which is still often ignored by naïve end-users. Related to this the practice of some antibody manufactures of retaining catalog numbers for new polyclonal antibody lots is unacceptable and misleading as well as the practice to use historical data sheets for antibodies to which they do not apply (Voskuil, 2014).

The necessary quality control steps for histone modification antibodies (Egelhofer *et al.*, 2011; Kungulovski *et al.*, 2015; Landt *et al.*, 2012) currently burden the individual antibody user with high costs and workload. Given that antibodies are expensive reagents, which are of no use without appropriate quality documentation, these efforts must be redirected from the end-customer to the manufacturers of antibodies. Herein, we urgently ask the vendors and manufacturers of antibodies to provide the necessary product sheets for all types of antibodies on a regular basis, including quality control documentation for each batch of polyclonal and each catalog number of recombinant or monoclonal antibodies. Results of the following validation tests must be provided to enable the end-user finding the information, which is particularly relevant for the intended application of an antibody:

1. Combinatorial profiling of specificity with peptide arrays or similar high-throughput methods. If possible, profiling of specificity with recombinant and semisynthetic nucleosomes harboring different modifications.

2. Western blot results with native (as positive control) and recombinant histones (as negative control).

3. Western blot results with native histones or nuclear extracts from cells where the responsible histone modifying enzyme has been deleted or depleted (mammalian cells) or mutant histones (yeast).

4. Reproducibility of ChIP-seq data and high correlation with similar validated ChIP-seq datasets.

As proposed by others (Bradbury & Plückthun, 2015a; Bradbury & Plückthun, 2015b) end-users should consider boycotting companies not complying with this demand, or at least stay away from products lacking a proper lot-specific documentation. While one may expect

that better quality control will increase the prices of commercial antibodies, end-customers will not be forced to conduct their own quality control and they will not waste money for non-functional antibodies, so that the overall final costs may not be much higher. Moreover, the value of the obtained data will increase massively with better antibody validation.

The batch-to-batch variability of critical properties like cross reactivity or inhibition by secondary marks makes the application of polyclonal antibodies intrinsically unsustainable, because experiments cannot be reproduced after the corresponding batch of an antibody is sold out. As a consequence of this, rigorously speaking, large data sets in chromatin biology exist in a "grey" area outside of natural science, since it is impossible to repeat the underlying experiments. In a long-term perspective, a shift away from polyclonal antibodies towards alternative reagents, which can be produced at constant quality, would help to reduce the necessary financial and workload efforts associated with quality control of polyclonal antibodies and ensure sustainability (Bradbury & Plückthun, 2015a; Bradbury & Plückthun, 2015b). This applies to high quality monoclonal antibodies, recombinant antibodies (Hattori *et al.*, 2013) or analogous recombinant reading domains (Kungulovski *et al.*, 2014). This will not only help to reduce costs in chromatin research in the long run (once obtained, the documentation will be valid for all lots) but also help to standardize the affinity reagents used and ease the lab-to-lab comparison of data. The recombinant reagents are particularly promising, because their sequences can be published, which ensures full transparency and reproducibility. Of note, in chromatin biology native reading domains designed by nature to specifically recognize relevant histone PTM marks are available as an alternative to antibodies (Kungulovski *et al.*, 2014), which is an advantage over other fields, where recombinant production of antibodies is the only technical solution to the issue of reproducible performance and long term availability of these essential research reagents.

Author contributions

GK and AJ wrote the paper. All authors have seen and agreed to the final content of the manuscript.

Competing interests

The authors declared no competing interests.

Grant information

Work in the authors' lab has been supported by the DFG JE 252/26-1.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Open Peer Review

Current Referee Status: ✓ ✓ ✓

Version 2

Referee Report 18 January 2016

doi:10.5256/f1000research.7991.r11772

Shohei Koide
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The authors concentrate on their core message for better characterization and documentation and for shifting toward renewable reagents. It is important to note that more characterization would be better but demanding the inclusion of assays of limited predictive power would increase the cost of antibodies without substantially benefiting the end users.

"Moreover, we are not convinced that the development of special conditions by each lab is an advisable development, because in many cases these may not be fully documented which - again - would undermine reproducibility. While it is self-evident that the researchers are ultimately responsible for the validity of all their results, lab internal quality certifications in our view are not the best way to proceed in an ever growing experimental field."

This is not what meant in my review. Instead, I mean that what an end user studies would always represents novel conditions (different cells, different culture conditions, different number of cells, etc.). So, strictly speaking, there is no guarantee that even a highly validated antibody functions in the actual experiment. The field would benefit greatly by establishing better internal controls that are included in each experiment, particularly for ChIP.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 04 January 2016

doi:10.5256/f1000research.7991.r11774

Andrew Bradbury
Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA

The authors have addressed my concerns

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 19 November 2015

doi:10.5256/f1000research.7827.r10962

Shohei Koide
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This paper succinctly reviews antibody-related problems that have been widely recognized in the biological and biomedical community, in a specific context of anti-histone posttranslational modification (PTM) antibodies and their uses in epigenetic research. The authors correctly emphasize the large negative impact of the batch-to-batch variation of anti-histone PTM antibodies and its consequence ('large data sets in chromatin biology exist in a “grey” area outside of natural science, since it is impossible to repeat the underlying experiments.'). I generally agree with the current challenges described in this paper, but it could be improved by addressing the following points.

An important omission in the paper is the limited recognition by users of potentially large effects of the mismatch between validation methods and real applications. We can perhaps agree that antibodies should ideally be validated in a manner that closely mimics how it is used in the actual application. Validation methods can be divided into two general types based on their formats: one in which antibodies are immobilized and captured antigens are detected ("immunoprecipation (IP) type") and the other in which antigens are immobilized and captured antibodies are detected ("blotting type"). Peptide arrays and Western blotting fall into the latter blotting-type validation methods, in which peptides or denatured proteins are localized, often at high density, on a solid support and binding of dilute antibody samples is detected. To state the obvious, IP-type methods are suited for validating antibodies for IP-type applications (IP, ChIP), whereas blotting-type methods are suited for validating antibodies for blotting-type applications (Western, immunostaining).

Potential problems arise when the validation format is different from the application format. It is not easy to predict whether antibodies validated using blotting-type methods perform well in IP-type applications such as ChIP, and vise versa. Egelhofer *et al*. (2011) reported that more than 20% of antibodies that have been validated to be specific in peptide blots still fail in ChIP experiments. In typical IP applications where an antibody is immobilized on a solid support, antibody affinity is a critical parameter. In contrast, affinity is not critical in blotting type applications, because the bivalent format of the conventional antibody (i.e. two antigen-binding sites per molecule) helps boost binding (the so-called avidity effect). Indeed, it has been found that an antibody that looked good on peptide arrays performed poorly in IP and conversely another antibody that did not look good on peptide arrays performed exceedingly well in IP (Nishikori *et al.,* 2012). Similarly, an antibody (Active Motif 39156) performed well in IP validation using semi-synthetic nucleosomes ("IceChIP"), although it appeared cross-reactive in peptide-array validation and in Western blotting (Rothbart *et al.*, 2015). Further complications arise from the fact that spot intensities in array-type experiments are not quantitatively correlated with the strengths of the measured interactions (see, for

examples, Stiffler *et al*., 2006; Hause *et al.* 2012).

Another omission is that validation and actual results depend on experimental conditions and accordingly an antibody validated under one set of conditions may not perform as well under a different set of conditions. Practitioners of immunoblotting are all familiar with the need for "optimizing" conditions for their own experiments. Similar optimizations are needed for other types of applications for which desired outcomes are less obvious. Furthermore, the abundance of the antigen of interest relative to off targets influences the outcome. Even a highly selective and potent antibody may not sufficiently enrich extremely rare antigens. Accordingly, for IP-type applications, mass spectroscopy-based validation using IP with input materials similar to those used in actual experiments (Peach *et al*., 2012; Hattori *et al.,* 2013; Marcon *et al.* 2015) and IP of semi-synthetic nucleosomes (Grzybowski *et al.,* 2015) are better suited as validation methods.

This paper should emphasize more that the end user must critically evaluate limitations of validation methods caused by format mismatches and/or variations in experimental conditions. I do not agree with this paper's recommendations that a single set of information be provided with any antibody regardless of its intended use.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 24 Nov 2015

Albert Jeltsch, Uni Stuttgart, Germany

We agree with the general conclusions of this reviewer that validation methods and protocols need to be adjusted to the planned applications. However, it should be noticed that the main aim of this short opinion paper was to further raise concerns and contribute to a move towards better documentation and sustainable reagents in chromatin research. The current state is that antibody documentation is widely insufficient and the reagents are often not sustainable. We propose to improve this situation step by step. It would be an important advance for the field to have a panel of standardized quality documentation data available for each antibody lot. This includes data in different formats, which will allow users to select the most relevant information for their experiments. To take up the point of this reviewer, we included a hint that validation methods must reflect the intended application of antibodies.
Defining the best format of quality checks will be an ongoing challenge for the entire field. We already expressed a preferences for the application of recombinant modified nucleosomes as validation method in chromatin biology in the original version of the manuscript. Another very important improvement for the field would be to have more recombinant affinity reagents, which are fully sustainable.

However, we like to mention that we are not fully convinced by the statement that the differences in technical formats (blotting vs. pull-down) is the only or main reason of differences in antibody performance between certain assays. One additional, very critical, difference is that short peptides are used in many validation assays (for practical reasons) but real applications deal with the pull-down of full histone tails. Moreover, we are not convinced that the development of special conditions by each lab is an advisable development, because in many cases these may not be fully documented which - again - would undermine reproducibility. While it is self-evident that the researchers are ultimately responsible for the validity of all their results, lab internal quality certifications in our view are not the best way to proceed in an ever growing experimental field.

Competing Interests: No competing interests were disclosed.

Referee Report 03 November 2015

doi:10.5256/f1000research.7827.r10993

Scott B. Rothbart
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Antibody reliability in biomedical research is of utmost importance. The quality of these reagents in chromatin biology applications is of particular concern given their position as essential tools for most techniques characterizing the cellular abundance and genomic distribution of histone post-translational modifications (PTMs).

I agree with the Kungulovski and Jeltsch that increased accountability needs to be demanded from companies who sell histone PTM antibodies, and their four recommended quality control measures are reasonable expectations. In addition, it should also be noted that the practice of retaining catalog numbers for new polyclonal antibody lots is unacceptable and misleading.

Moreover, and particularly in light of the increased awareness of antibody concerns in the field, experimentalists and epigenome consortium leaders (e.g., ENCODE, BLUEPRINT) should be more rigorous in their own evaluation of histone PTM antibodies when choosing a reagent for their study. Antibody specificity data and lot numbers used should also be standard requests from journal editors and reviewers.

Accountability clearly needs to come from all parties if we are to continue benefiting from the use of these affinity tools in chromatin research.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 12 Nov 2015

Albert Jeltsch, Uni Stuttgart, Germany

- *"I agree with the Kungulovski and Jeltsch that increased accountability needs to be demanded from companies who sell histone PTM antibodies, and their four recommended quality control measures are reasonable expectations. In addition, it should also be noted that the practice of retaining catalog numbers for new polyclonal antibody lots is unacceptable and misleading."*

  Reply: We have added the sentence to the paper "that the practice of retaining catalog numbers for new polyclonal antibody lots is unacceptable and misleading". Thanks a lot for this helpful suggestion.

- *"Moreover, and particularly in light of the increased awareness of antibody concerns in the field, experimentalists and epigenome consortium leaders (e.g., ENCODE, BLUEPRINT) should be more rigorous in their own evaluation of histone PTM antibodies when choosing a reagent for their study. Antibody specificity data and lot numbers used should also be standard requests from journal editors and reviewers. Accountability clearly needs to come from all parties if we are to continue benefiting from the use of these affinity tools in chromatin research."*

  Reply: We like to mention, that in our view more responsibility in structured product

documentation lies at the side of the supplier. Quality checks done by end-customers are an emergency action, but they will not solve the problem of long-term and lab-to-lab reproducibility. Also they put all financial pressure on the end customer or a product, which is not common practice in other parts of the economy.

Competing Interests: No competing interests were disclosed.

Referee Report 02 November 2015

doi:10.5256/f1000research.7827.r10961

Andrew Bradbury
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Paragraph 2 should also indicate that antibodies purportedly recognizing PTMs at specific sites, need to have their recognition specificity also tested against the same PTM at different sites, in the same protein, or others, and with the same core sequence or others. Many so-called specific phosphotyrosine antibodies actually recognize the phosphotyrosine modification independently of its sequence context.

"In spite of the intricate task of producing histone modification antibodies and their crucial role in chromatin biology, surprisingly, they remain to be insufficiently characterized" should be changed to "In spite of the intricate task of producing histone modification antibodies and their crucial role in chromatin biology, surprisingly, they remain insufficiently characterized"

"the specificity data obtained for one batch of antibody do not necessarily reflect the properties of another one" Voskuil (Commercial **antibodies** and their validation. Version 2. F1000Res. 2014 Oct 2 [revised 2014 Oct 15];3:232. doi: 10.12688/f1000research.4966.2. eCollection 2014) describes the relatively unknown practice of some antibody manufacturers to use historical data sheets for antibodies to which they do not apply. This should also be mentioned and cited.

The recommendations in paragraph 4 are commendable. However, if manufacturers are expected to carry this out on every lot they sell, the author must acknowledge that the cost of antibodies will have to increase.

"As proposed by others (Bradbury & Pluckthun, 2015) end-users should consider boycotting companies not complying with this demand, or at least stay away from products lacking a proper lot-specific documentation." Actually, this was not our main proposal. Our main point (amplified in Getting to reproducible antibodies: the rationale for sequenced recombinant characterized reagents. **Bradbury** AR, Plückthun A. Protein Eng Des Sel. 2015 Oct;28(10):303-5. doi: 10.1093/protein/gzv051) was that we should move away from the use of polyclonal antibodies altogether and use only well characterized sequenced recombinant antibodies. Only in this way can we ensure antibody reproducibility.

In the last paragraph, the author indicates that recombinant antibodies (or other proteins) may solve this problem. However, this will only be the case if such binders can be unequivocally identified, which will only occur if sequences can be referred to unambiguously. Otherwise, as antibody companies are bought and sold, catalog numbers will change and it may become difficult to reproduce experiments, because it will not be clear which original antibody was used.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 12 Nov 2015

Albert Jeltsch, Uni Stuttgart, Germany

- *“Paragraph 2 should also indicate that antibodies purportedly recognizing PTMs at specific sites, need to have their recognition specificity also tested against the same PTM at different sites, in the same protein, or others, and with the same core sequence or others. Many so-called specific phosphotyrosine antibodies actually recognize the phosphotyrosine modification independently of its sequence context.”*

  Reply: We agree. This point was mentioned in paragraph 2 on p. 2 using methylation at H3K9 and H3K27 as an example. “Another difficulty is that some histone modifications such as methylation or acetylation of H3K9 and H3K27 lie within an identical amino acid context (ARKS motif), which makes the readout of the target peptide sequence outside of this central motif very important as well.” We have modified this paragraph to make the point clearer and better reflect what the reviewer was asking for: “Moreover, the antibody should bind the modified amino acid residue only at defined modification sites on the target protein, which implies that not only the modification but also the amino acid sequence must be recognized. This is particularly difficult for some histone modifications such as methylation or acetylation of H3K9 and H3K27 which occur within an identical amino acid context (ARKS motif) and make the readout of the target peptide sequence outside of this central motif vital as well.”

- *"In spite of the intricate task of producing histone modification antibodies and their crucial role in chromatin biology, surprisingly, they remain to be insufficiently characterized" should be changed to "In spite of the intricate task of producing histone modification antibodies and their crucial role in chromatin biology, surprisingly, they remain insufficiently characterized"*

  Reply: This has been changed as proposed.

- *"the specificity data obtained for one batch of antibody do not necessarily reflect the properties of another one" Voskuil (Commercial antibodies and their validation. Version 2. F1000Res. 2014 Oct 2 [revised 2014 Oct 15];3:232. doi: 10.12688/f1000research.4966.2. eCollection 2014) describes the relatively unknown practice of some antibody manufacturers to use historical data sheets for antibodies to which they do not apply. This should also be mentioned and cited.*

  Reply: Thank you. This point and reference has been added.

- *“The recommendations in paragraph 4 are commendable. However, if manufacturers are expected to carry this out on every lot they sell, the author must acknowledge that the cost of antibodies will have to increase.”*

Reply: Please note in the original manuscript on p. 2 we stated that a shift to sustainable reagents (i.e. away from polyclonal antibodies) “would help to reduce the necessary financial and workload efforts associated with quality control of polyclonal antibodies and ensure sustainability”, which partially addressed this point. We now added an additional sentence stating “While one may expect that better quality control will increase the prices of commercial antibodies, end-customers will not be forced to conduct their own quality control and they will not waste money for non-functional antibodies, so that the overall final costs may not be much higher.” to incorporate this request more explicitly.

- "As proposed by others (Bradbury & Pluckthun, 2015) end-users should consider boycotting companies not complying with this demand, or at least stay away from products lacking a proper lot-specific documentation." Actually, this was not our main proposal. Our main point (amplified in Getting to reproducible antibodies: the rationale for sequenced recombinant characterized reagents. Bradbury AR, Plückthun A. Protein Eng Des Sel. 2015 Oct;28(10):303-5. doi: 10.1093/protein/gzv051) was that we should move away from the use of polyclonal antibodies altogether and use only well characterized sequenced recombinant antibodies. Only in this way can we ensure antibody reproducibility.”

  Reply: We have added the citation to this very insightful paper now also at the corresponding place in our manuscript and also added the Prot. Eng. Des. Sel. reference. “As already proposed, in a long-term perspective, a shift away from polyclonal antibodies towards alternative reagents, which can be produced at constant quality, would help to reduce the necessary financial and workload efforts associated with quality control of polyclonal antibodies and ensure sustainability (Bradbury & Plückthun, 2015, Nature 518, 27-29; Bradbury & Plückthun, 2015, Prot. Eng. Des. Sel. 28, 303-305).”

- *“In the last paragraph, the author indicates that recombinant antibodies (or other proteins) may solve this problem. However, this will only be the case if such binders can be unequivocally identified, which will only occur if sequences can be referred to unambiguously. Otherwise, as antibody companies are bought and sold, catalog numbers will change and it may become difficult to reproduce experiments, because it will not be clear which original antibody was used.”*

  Reply: This is a valid point. We have added on sentence to stress this: “The recombinant reagents would be particularly promising, because their sequences can be published, which ensures full transparency and reproducibility.”

Competing Interests: No competing interests were disclosed.