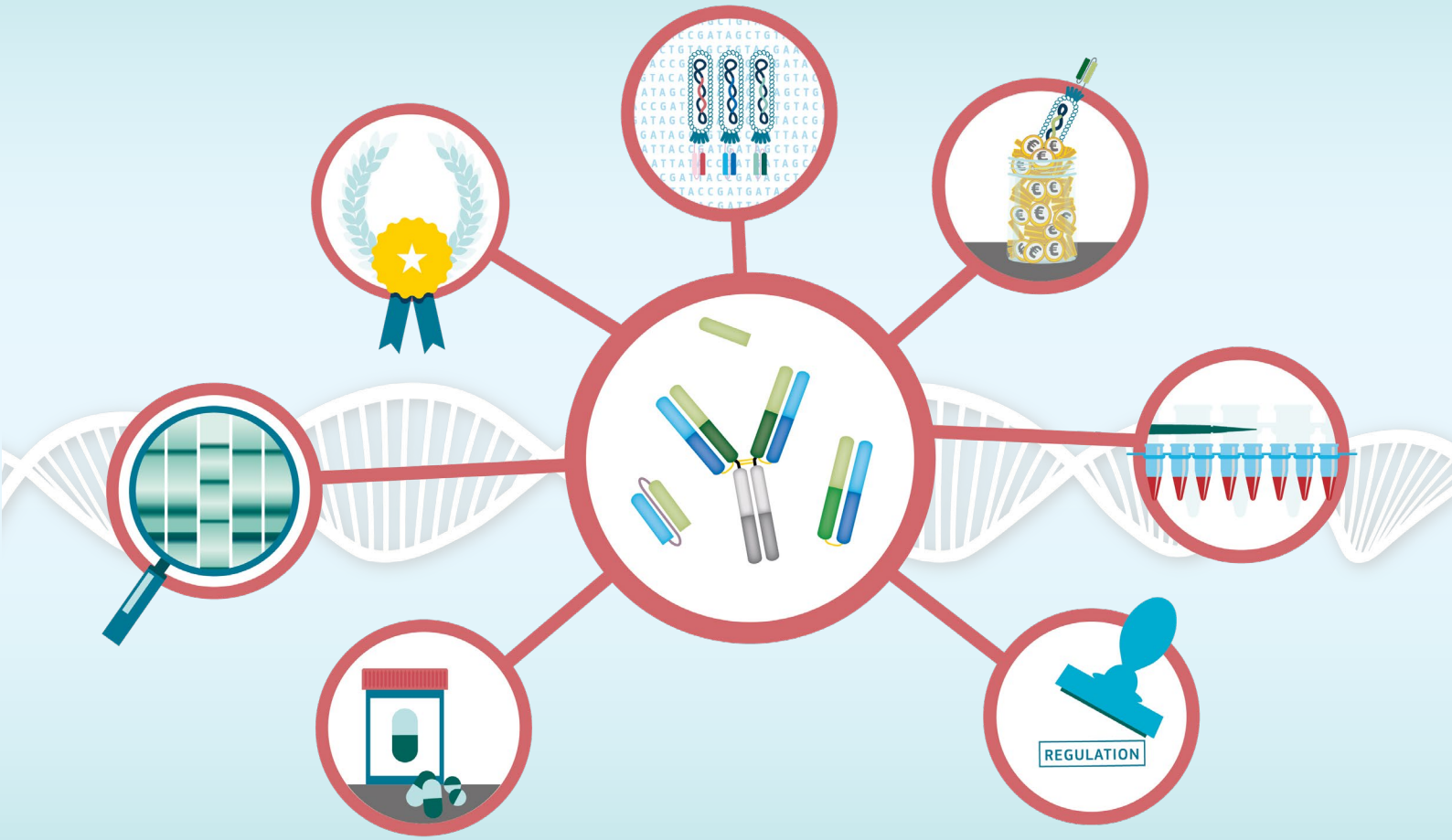
European Commission

EURL ECVAM Recommendation on Non-Animal-Derived Antibodies



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Joint Research Centre

EURL ECVAM Recommendations

The aim of a EURL ECVAM Recommendation is to provide the views of the *EU Reference Laboratory for alternatives to animal testing (EURL ECVAM)* on the scientific validity of alternative test methods, to advise on possible applications and implications, and to suggest follow-up activities to promote alternative methods and address knowledge gaps. During the development of its Recommendation, EURL ECVAM typically mandates the *EURL ECVAM Scientific Advisory Committee (ESAC)* to carry out an independent scientific peer review which is communicated as an ESAC Opinion and Working Group report. In addition, EURL ECVAM consults with other Commission services, EURL ECVAM's advisory body for *Preliminary Assessment of Regulatory Relevance (PARERE)*, the *EURL ECVAM Stakeholder Forum (ESTAF)* and with partner organisations of the *International Collaboration on Alternative Test Methods (ICATM)*.

Contact information

European Commission, Joint Research Centre (JRC), Chemical Safety and Alternative Methods Unit (F3)
Address: via E. Fermi 2749, I-21027 Ispra (VA), Italy
Email: *JRC-F3-ENQUIRIES@ec.europa.eu*

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JRC SCIENCE FOR POLICY REPORT

EURL ECVAM Recommendation on Non-Animal-Derived Antibodies

EURL ECVAM Recommendation
on Non-Animal-Derived Antibodies

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1. Introduction

Antibodies are binding molecules (affinity reagents) that have a high specificity for their unique target (antigen) and are crucial tools for research, diagnostics, therapeutics and regulatory procedures. Animals are still used for the generation of monoclonal and polyclonal antibodies as well as other types of affinity reagents despite the availability of technologies for their development and production that do not entail the use of animals. The EU Directive 2010/63/EU on the protection of animals used for scientific purposes does not allow the use of animal-based methods when other methods not entailing the use of animals exist, which would allow obtaining the results sought (Articles 4 "Principle of replacement, reduction and refinement" and 13 "Choice of method").

The EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) avails of the scientific opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) about specific issues related to alternative methods. In 1998, based on the available evidence, ESAC concluded that for all levels of monoclonal antibody production, scientifically acceptable *in vitro* methods (i.e. use of hybridomas) were practicably available and that these methods were either better than, or equal to, the *in vivo* (ascites) production method in terms of antibody quality. Thus, the ESAC stated in 1998 that *in vivo* production of monoclonal antibodies by the ascites method was no longer scientifically necessary, except in rare cases[1]. Nevertheless, the recent statistical information published by the European Commission on the use of animals for scientific purposes in the EU show an increase by 65% in the use of animals for monoclonal antibody production by the mouse ascites method between 2015 (27333 animals used) and 2017 (45024 animals used). It is unclear from the non-technical project summaries how the ascites method could be justified and as a consequence, why such projects could still be authorised in EU countries.

Although animal-use statistics do not indicate the total number of animals used for generation and production of antibodies beyond the ascites method, it is well known that animal immunisation is still widely used for the development of hybridomas for monoclonal antibody production and that the largest proportion of animals used is for producing polyclonal antibodies. It is estimated that close to 1 million animals are used per year for antibody development and production in the EU alone. Several factors contribute to the slow transition from animal-derived to non-animal-derived affinity reagents used by the scientific community. Commercial availability of non-animal-derived affinity reagents is limited since the majority of providers still generate antibodies by animal immunisation. Moreover, many misconceptions exist in the scientific community about the quality and validity of non-animal-derived affinity reagents; a problem that is compounded by a lack of education and training opportunities for users to gain a better understanding and appreciation of non-animal-derived affinity reagents and how they can ultimately benefit their work.

2. ESAC review and EURL ECVAM position

In March 2018, EURL ECVAM asked ESAC to review the scientific validity of non-animal-derived antibodies and non-antibody affinity reagents used for research and applications in regulatory and diagnostic fields. Based on its independent scientific peer review, the ESAC produced an Opinion on the on the "Scientific validity of replacements for animal-derived antibodies" (Annex 1), supported by an ESAC Working Group report (Annex 2).

In its review, the ESAC focused on non-animal-derived antibodies generated by phage-display, where a bacteriophage is genetically modified to display an antibody, since this is the most mature technology. As noted by the ESAC, the 2018 Nobel Prize in Chemistry was awarded "for the phage display of peptides and antibodies". The phage-display technology for antibody production involves the use of large collections of

1 *https://tsar.jrc.ec.europa.eu/test-method/tm1998-04*

recombinant forms of antibody analogues or of miniaturised antibodies that contain at least part of the antigen-binding site of an antibody such as single variable heavy (VH) domain, single-chain fragment variable (scFv) and fragment antigen binding (Fab). In this approach, large binder libraries are presented on the surface of a phage (i.e. phage display), which permits the selection of peptides or proteins with high affinity and specificity for virtually any target. Such recombinant antibodies produced by phage display are already widely used across all fields of antibody application.

The ESAC also briefly discussed new alternative binders that are being introduced in the market, such as aptamers, affimers, DARPINs. These alternative binders can be based on peptides, proteins, ribonucleic acids, or single-stranded DNA. Although these new molecules are already being applied in diagnostics, antibodies are still the molecules of choice for many applications. The ESAC recommended that a more detailed review of such alternative affinity reagents is conducted in the future once more data become available (see Annex 2 - ESAC Working Group report).

Based on the available scientific literature, application examples and the experts' own extensive experience, the ESAC concluded that non-animal-derived antibodies are mature reagents generated by a proven technology that are not only equivalent to animal-derived antibodies, but in many respects can offer significant scientific advantages and economic benefits (see Annex 1 - ESAC Opinion and Annex 2 – ESAC Working Group report). Non-animal-derived antibodies are well-defined and better reagents that will improve the reproducibility and relevance of scientific procedures and lead to more efficient and effective use of research funds. Animal-derived antibodies typically suffer from batch-to-batch variability and many show low specificity. Such issues can be easily addressed through the use of non-animal-derived antibodies obtained by phage-display technology. Equally, antibodies used for regulatory applications, e.g., reagents used in toxicity tests or for the quality control of biologicals, should be non-animal-derived. This will enhance reproducibility of results and the sustainable supply of reagents.

Animal-derived polyclonal antibodies make up a large proportion of animals used today for antibody production and, therefore, present serious ethical concerns. However, they can be produced using defined mixtures of sequence-defined recombinant antibodies developed from universal phage display libraries, thereby avoiding the use of animals. These so-called "multiclonal" antibodies[2] have been recently shown to exceed the performance of the monoclonal products[3]. These recent developments also show that non-animal-derived "multiclonal" antibodies with superior quality (e.g., lower unspecific reactions) and higher reproducibility over animal-derived polyclonal antibodies can and should be generated. It is therefore possible to combine the best features of monoclonal and polyclonal antibodies in a completely animal-free and defined product.

Even though the ESAC review did not cover the field of therapeutic applications, EURL ECVAM considers that non-animal-derived antibodies are also a suitable alternative in this field. In fact, monoclonal affinity reagents approved for therapeutic applications are nowadays exclusively recombinant, well characterised because of strict regulations, and stably produced in large amounts. However, while several of these affinity reagents are non-animal-derived, most of them are still generated by immunising animals without a clear need or justification.

Therefore, taking into consideration the ESAC Opinion on the scientific validity of replacements for animal-derived antibodies, EURL ECVAM recommends that animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications. The provisions of Directive 2010/63/EU should be respected and EU countries should no longer authorise the development and production of antibodies through animal immunisation, where robust, legitimate scientific justification is lacking.

2 *https://abcalis.com/multiclonals*
3 *https://www.abcalis.com/releases/ABCALIS_TechNote_Multiclonals.pdf*

"EURL ECVAM recommends that animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications. In the EU, the provisions of Directive 2010/63/EU should be respected and EU countries should no longer authorise the development and production of antibodies through animal immunisation, where robust, legitimate scientific justification is lacking."

3. Breaking down misconceptions about non-animal-derived affinity reagents

Availability, quality and relevance of non-animal-derived antibodies

- Non-animal-derived antibodies are available from catalogues. Companies and other organisations offer custom generation of non-animal-derived antibodies (see Section V in the Appendix of Annex 2 - ESAC Working Group report).
- There is a wealth of scientific literature describing various approaches for generating non-animal-derived antibodies, for example, building of universal recombinant antibody gene libraries, antigen selection and production (see Section 10 of Annex 2 - ESAC Working Group report).
- Non-animal-derived antibodies can be stably produced in unlimited amounts as the example of approved therapeutics shows.
- Non-animal-derived antibodies are equivalent to animal-derived antibodies for the vast majority of applications. There are no general or systematic disadvantages of non-animal-derived antibodies with respect to properties such as affinity, stability/shelf life, and specificity. On the contrary, they can offer significant scientific advantages (see "Scientific benefits" below).
- To date most of the recombinant antibody gene libraries have been built using human antibody sequences as a blueprint; however, there are no known limits concerning the choice of the species for building such libraries and there are some examples available.

Equipment, expertise, resources

- Only standard laboratory equipment and consumables are needed for generating non-animal-derived antibodies.
- Laboratories familiar with modern molecular, cell and microbiology techniques should be able to generate non-animal-derived antibodies once adequately trained.
- Development of a universal recombinant library requires a significant time investment, ranging from a few months to a couple of years, which is very much dependent on the approach used, level of expertise and quality control; however, once the library is developed, this supplies a diversity of antibody candidates equivalent to a lifetime supply of animals and removes any ongoing cost of animal care, where relevant.
- However, there is a significant benefit in time: selection of antibodies using a universal recombinant library can be performed in a few weeks, while the generation of animal-derived monoclonal antibodies needs several months.

Economic benefits

- Generation costs of non-animal-derived antibodies are comparable to the generation of monoclonal antibodies by immunisation.

- The financial impact of producing animal-derived polyclonal or monoclonal antibodies of questionable quality and using them in research and regulatory applications to obtain results that are potentially meaningless is high and far exceeds the costs of generating well-defined non-animal-derived affinity reagents.

- It is estimated that many hundreds of millions of euro are inadvertently spent annually by the biomedical research community on non-specific and badly defined animal-derived antibodies. Moreover, significant losses are also being incurred as a consequence through associated waste of time and resources and the follow-up of potentially misleading research results (see Section 6 of Annex 2 – ESAC Working Group report).

Scientific benefits

- Many of the animal-derived antibodies, in particular, polyclonal antibodies, have limitations with both specificity and reproducibility. The generation methods for animal-derived antibodies have not significantly improved in response to these known issues over the past 40 years, nor have they been rigorously quality-controlled in light of the above problems.

- In contrast, non-animal-derived monoclonal antibodies can be generated with superior properties and their properties can further be improved by *in vitro* evolution after initial generation.

- "Multiclonal" antibodies exceeding the performance of monoclonal products and with superior quality (e.g., lower unspecific reactions) over animal-derived polyclonal antibodies from catalogues can also be produced using defined mixtures of sequence-defined recombinant antibodies developed from universal phage display libraries[4,5].

- *In vitro* antibody selection against a target antigen can be tightly controlled to enrich clones with desired properties. For example, one can do the affinity selection with the exact biochemical conditions under which the antibody will be used, and therefore, select only antibodies that are functional at these conditions.

- The sequences of non-animal-derived antibodies are typically determined as part of their isolation process. This ensures unlimited supply with antibodies of the same quality and increases the reproducibility of scientific experiments.

- The genetic sequence of a non-animal-derived antibody can be modified to add a multitude of features including a variety of antibody formats and detection systems (e.g., fusion to tags, enzymes, etc.). The possibility of adding a detection system of choice, in particular, has the advantage of decreasing the need for labelled secondary antibodies. One can also envision the development of a non-animal-derived universal labelled secondary antibody.

- While most animal-derived antibodies are in the immunoglobulin G (IgG) format, recombinant technologies offer additional opportunities to generate a variety of formats, e.g., full-length IgG functionally indistinguishable from animal-derived IgGs in all typical applications, or antibody fragments.

- Non-animal-derived antibodies can be used in typical immune-analysis applications, for example, western blotting, immunohistochemistry, flow cytometry, immunosorbent assays, immunoprecipitation, and multiplexed planar or bead arrays.

4 *https://abcalis.com/multiclonals*
5 *https://www.abcalis.com/releases/ABCALIS_TechNote_Multiclonals.pdf*

4. How can the EU promote the generation and use of non-animal-derived antibodies?

Education and training

- Competency of all users and developers/producers of antibodies in non-animal technologies such as phage display could be increased/improved by offering training, e.g., webinars, e-learning, hands-on training courses. Such training should also be included in courses on laboratory animal science.

- The abundance of scientific literature describing approaches for the development, selection and production of non-animal-derived antibodies as well as the experiences of users, developers/producers should be leveraged.

- Education and training should extend beyond end-users, to include suppliers, funders, ethical review bodies, publishers, and other bodies.

- Education should also be used to convey the scientific and economic limitations of the animal-based methods of antibody production, challenging the widely held belief that animal models are the "gold standard".

Authorisation of projects in the light of 2010/63/EU

- Since phage display is a mature and proven non-animal technology for the development and production of reliable and relevant antibodies or affinity reagents, projects requesting authorisation for the use of animals for these purposes should systematically be challenged (in line with Articles 4 and 13 of Directive 2010/63/EU) and rejected by the authorising bodies where robust, legitimate scientific justification is lacking. In the light of the ESAC Opinion (Annex 1) and the ESAC Working Group report (Annex 2), no scientifically justified exceptions could be identified.

- Antibody production using the ascites method should no longer be acceptable under any circumstances.

Publicly and privately funded research projects in the EU (i.e., EU funding, national funding and private funding)

- In the interest of ethical standards and quality of science, any newly generated antibody (or more generally any binding reagent) should be detailed in a funding application, and, where possible, be non-animal-derived. Robust, legitimate scientific justification should be provided for the use of animals to generate and produce antibodies.

- Any binding reagent used or purchased for the project should ideally be non-animal-derived (i.e., not requiring animal immunisation) and this should also be specified in a funding application. Existing, sequenced and well-characterised hybridomas may continue to be used to produce recombinant monoclonal antibodies *in vitro* as animals are no longer involved.

- A transition towards the use of non-animal-derived antibodies in the scientific community will be promoted if funding opportunities increase with the use of non-animal-derived affinity reagents, creating incentive for the use of non-animal-derived products.

Provision of funding to fully characterise affinity reagents generated in EU (and US NIH)-funded programmes

- EU (and US NIH)-funded programmes have already generated thousands of affinity reagents without using animals, which should be disseminated to researchers (see Section III in the Appendix of Annex 2 – ESAC Working Group report). Further funding and efforts are needed to prioritise and fully characterise these reagents and make them available to scientists.

5. What should antibody manufacturers/suppliers do?

- Manufacturers/suppliers should replace the animal-derived antibodies available in their catalogues (especially those produced by the ascites method) by non-animal-derived affinity reagents and clearly describe their origin and development in the accompanying product literature. Existing, sequenced and well-characterised hybridomas may continue to be used to produce recombinant monoclonal antibodies *in vitro* as animals are no longer involved in the production of the antibodies.

- Manufacturers/suppliers should establish a rapid phasing-out timescale (with regularly reviewed targets and deadlines) and provide regular updates to the authorities (under Directive 2010/63/EU) until animals are no longer used for antibody generation and production.

- Manufacturers/suppliers should form partnerships, e.g., with other companies, academics, non-governmental organisations and/or government agencies, to increase the production and availability of non-animal-derived antibodies.

- All catalogue affinity reagents should be unambiguously labelled to show whether they are animal-derived or not.

6. What should end-users do?

- End-users should search for and specifically request well-defined, non-animal-derived affinity reagents from suppliers to address their research/regulatory questions and coordinate to develop a platform to assist in future searches. Higher demand will increase the production and eventually decrease the costs.

- Academic institutions should coordinate efforts to establish non-animal-derived universal recombinant libraries and development and production services to support research activities.

7. What should editors, reviewers and publishers do?

- Editors, reviewers and publishers of scientific publications should demand higher quality in antibody-based research, strive for increased scientific standards and adopt unified validation standards for affinity reagents used in their publications. Authors of scientific reports or articles should be requested to state the source of the affinity reagents used, whether they are animal-derived or non-animal-derived, and how their quality and relevance (affinity and specificity) were controlled/ensured. If animal-derived antibodies have been used, clear justification should be provided.

- Manuscripts with results obtained using poor quality and/or undefined antibodies should be systematically rejected.

- Applying stricter journal policies of challenging and eventually not accepting manuscripts with data generated with animal-derived antibodies where a non-animal alternative could have been used would further accelerate a transition towards the use of non-animal-derived antibodies by the scientific community. Editors and publishers should nevertheless adopt a reasonable timeframe for applying such stricter policies considering the widespread use of animal-derived antibodies in research and the fact that antibody storage 'shelf life' may range from several weeks to many years.

Annex 1

ESAC OPINION

ON THE SCIENTIFIC VALIDITY OF REPLACEMENTS FOR ANIMAL-DERIVED ANTIBODIES

EURL ECVAM Scientific Advisory Committee (ESAC)

Core Members

- Dr. Chantra ESKES (ESAC Chair)
- Prof. Paula M. ALVES
- Dr. Rebecca CLEWELL
- Prof. Emanuela CORSINI
- Prof. Ian COTGREAVE
- Prof. Annette KOPP-SCHNEIDER
- Dr. José Maria NAVAS ANTÓN
- Prof. Aldert PIERSMA
- Dr. Carl WESTMORELAND

Ad-hoc Members

- Prof. Carl BORREBAECK
- Dr. Andrew BRADBURY
- Prof. Stefan DÜBEL
- Dr. Alison GRAY
- Dr. Hans-Joachim KNAPPIK
- Prof. Andreas PLÜCKTHUN

The EURL ECVAM Scientific Advisory Committee (ESAC) was requested by EURL ECVAM to review the available proof of the scientific validity of antibodies and non-antibody affinity reagents, used in research, regulatory applications and diagnostics, generated using animal-free technologies. An ESAC working group (WG) was established for this purpose, which delivered an ESAC WG report (Annex 2).

At its 45th meeting, held on 3-5 June 2019 at EURL ECVAM, Ispra, Italy, the non-Commission members of ESAC unanimously endorsed the following opinion, which was based on the existing scientific literature, application examples and the experts' own extensive experience as detailed in the ESAC WG report (Annex 2).

1 Non-animal-derived antibodies are mature reagents generated by a proven technology.

Non-animal-derived antibodies, in particular those generated by display of combinatorial antibody gene libraries, have matured to a point where, for the vast majority of applications, namely all known applications where non-animal-derived antibodies have been developed, they are equivalent to animal-derived antibodies. There is a solid body of evidence demonstrating that there are no general or systematic disadvantages of non-animal-derived antibodies with respect to properties such as affinity, stability/shelf life, and specificity.

Non-animal-derived antibodies can be provided in all well-known molecular formats (e.g., Immunoglobulin G) and therefore with properties indistinguishable from animal-derived antibodies. Moreover, they can be produced in additional molecular formats that can offer significant advantages (see below).

Many scientists are unaware of the fact that non-animal-derived antibodies are already successfully used in approved therapeutics, diagnostic applications and available from catalogues as research reagents. Generation of non-animal-derived antibodies is also available as a commercial service. EU- and US NIH-funded programmes have already generated thousands of monoclonal affinity reagents without the use of animals.

2 Non-animal-derived antibodies offer significant additional scientific benefits.

Non-animal-derived antibodies are superior defined reagents because their polypeptide sequences are established as part of the generation process. This is not the case for animal-derived antibodies (including monoclonal antibodies), the sequences of which are unknown, unless specific efforts are made to determine them.

The knowledge of the sequence provides a unique identifier as well as an unlimited and sustainable supply, which will improve experimental reproducibility. For example, in contrast to a not uncommon loss of hybridoma clones that normally cannot be regenerated, an identical non-animal-derived antibody or scaffold can always be generated from its known sequence. Furthermore, it makes these reagents amenable to adaptation to end users' assays, for example by freely choosing the Fc-part to fit the individual user's detection system.

Methods such as phage display, for which the 2018 Nobel Prize in Chemistry was awarded, offer further opportunities, like the use of other binder scaffolds, and allow the guided selection of essential characteristics, including specificity, compatibility to certain assay conditions, cross-reactivities, stability or affinity, which cannot be accomplished by animal immunisation. In summary, biochemical properties can be improved by *in vitro* evolution.

3 Non-animal-derived antibodies should be promoted.

It is the experts' opinion that adoption of these technologies in research has been too slow because there are still only a few providers that offer such reagents for the research market; the majority of developers are focused on therapeutic applications. Although intellectual property rights have been an issue in the past, most key patents have now expired. Today, the upfront cost of generating non-animal-derived recombinant reagents is about the same as traditional monoclonal antibodies, but significantly more than traditional polyclonal antibodies. However, there are multiple issues associated with polyclonal antibodies, which have a direct negative impact on the cost of research. The financial impact of producing and using questionable-quality polyclonal or monoclonal antibodies in research and regulatory applications is high and far exceeds the costs of the reagents themselves, considering the costs of generating results that are potentially meaningless.

The experts identified a lack of awareness about the current status of non-animal-derived antibodies, resulting in significant scientific misconceptions regarding these reagents. Education on the properties, availability and specific additional advantages of non-animal-derived affinity reagents is needed. Training on the implementation of these technologies must also be improved.

4 CONCLUSION

The experts conclude on the scientific evidence that non-animal-derived antibodies are able to replace animal derived antibodies in the vast majority of applications. Moreover, well-characterised, recombinant affinity reagents will improve the reproducibility of science and positively impact society.

Annex 2

ESAC WORKING GROUP REPORT

ON THE SCIENTIFIC VALIDITY OF REPLACEMENTS FOR ANIMAL-DERIVED ANTIBODIES

EURL ECVAM Scientific Advisory Committee (ESAC)

ESAC Working Group

- Dr. Rebecca CLEWELL (WG Chair)
- Dr. Carl WESTMORELAND
- Prof. Carl BORREBAECK
- Dr. Andrew BRADBURY
- Prof. Stefan DÜBEL
- Dr. Alison GRAY
- Dr. Hans-Joachim KNAPPIK
- Prof. Andreas PLÜCKTHUN

Executive summary

Antibodies bind to and thereby identify specific targets of interest in very complex environments of other biomolecules. At present, almost all (polyclonal or monoclonal) antibodies are produced by immunising animals. They are used in immunoanalysis applications such as Western blotting, immunohistochemistry (IHC), immunocytochemistry (ICC), flow cytometry, immunosorbent assays (ELISA) and immunoprecipitation, and being relied upon by all market sectors such as in the development of therapeutics, in diagnostics, in research discovery and in regulatory applications.

Representing the largest fraction of commercial antibody reagents, polyclonal antibodies are characterised by multiple epitope recognition. They often have high overall antibody affinity against the target antigen, offer good sensitivity, are inexpensive and quick to produce. However, they suffer issues of batch-to-batch variation, low specificity and high detection background.

Monoclonal antibodies from hybridomas are in principle characterised by single epitope recognition and are thought to bind with high specificity to only one unique epitope, with reduced probability of cross-reactivity. Nonetheless, many monoclonal antibodies used in research were found to still cross-react with other molecules. Moreover, a recent study has shown that almost a third of randomly chosen hybridomas contained one or more additional productive heavy or light chains that interfered with specificity or sensitivity (Bradbury *et al.*, 2018). In addition, hybridoma clones can die off and be lost.

The resounding impact on research, of using non-specific antibody reagents, in terms of wasted cost, time and resources, or the repercussion on diagnosis and health management, is tremendous. Whilst the reagents used in most clinical trials and especially in medical procedures cleared by the US Food and Drug Administration or the European Medicines Agency are extremely reliable, such abnormalities go largely unnoticed in cases where antibodies are not validated to the same degree. At an annual worldwide spending of $1.6 billion on the antibody market, and with only 5-49% functionality being reported in monoclonal reagents alone, a staggering annual loss of at least $800 million to the biomedical research community is estimated, without counting the additional and unaccounted cost for waste in materials, time, money and follow-on research.

Recombinant antibodies can be animal-derived: generated by cloning the antibody-encoding DNA of hybridomas or by cloning of antibody gene repertoires from B-cells of immunised animals. Recombinant antibodies can also be non-animal-derived, i.e., obtained without using animals: from “universal” gene repertoires of non-immunised (naïve) donor B-cells, from completely synthetic universal antibody libraries, or from cloning the antibody DNA from human donor B-cells, e.g., from a patient after an infection.

Libraries obtained from an immunised animal are already enriched for antibodies that bind a specific antigen but require the time-consuming generation of a new library for each new antigen of interest. In contrast, universal libraries are independent of any living organism and can be used time and again to enrich specific antibodies.

Such non-animal-derived antibodies have been proven as suitable replacements for applications that currently employ animal-derived antibodies. It is worthy of note that the use of non-animal-derived antibodies in therapeutic applications is well established. Non-animal-derived antibodies are selected *in vitro* from large gene libraries predominantly by phage and yeast display, and less commonly by ribosome, bacterial or mammalian cell display. Such libraries are typically built using immunoglobulin domains as scaffolds, but there is an increasing list of publications where libraries are built on non-antibody scaffolds. This report focusses on non-animal-derived antibodies produced by phage display, since they are leading in respect of technological maturation and compatibility to the usual scientific applications, with large bodies of evidence supporting their use and fewest perceived hurdles to rapid implementation, but the field is rapidly developing and diversifying.

Non-animal-derived, naïve or synthetic, universal libraries can contain an enormous repertoire of structurally different candidate antibody genes (typically more than 10^{10} independent clones). This diversity is equal to or even larger than that of a naïve immune system prior to affinity maturation, and thus provides similar or even better opportunities to identify a specific binder against any target antigen. Non-animal-derived antibodies are routinely sequenced after isolation and therefore unambiguously identifiable, which allows a consistent, reproducible and sustainable supply. Furthermore, their sequence can be immortalised in a computer file. These factors offer the benefit of improved quality of scientific data over that of animal-derived antibodies. The *in vitro* antibody

selection against a target antigen can be tightly controlled to enrich clones with desired properties such as epitope specificity, stability, expression yield or affinity. The genetic sequence can be modified to add a multitude of features including a variety of antibody formats and detection systems. In addition, the availability of the sequence allows for applications such as functioning as intrabodies, expression on cells as chimeric antigen receptors and redirection of viruses to infect particular cell types. Non-animal-derived recombinant antibodies may also be used for multiple epitope recognition where animal-derived polyclonal antibodies are traditionally used.

Once a large non-animal-derived antibody library is established, it can replace a lifetime supply of immunised animals. Generation costs of antibodies from such a library are comparable to the generation of monoclonal antibodies by immunisation, but there is a significant benefit in time: while the generation of monoclonal antibodies requires several months, the selection of antibodies using a naïve recombinant library can be performed in a few weeks.

In the view of the expert panel, non-animal-derived antibodies are currently not used more frequently due to: a propensity toward existing methods; a lack of awareness of the current scientific status; perceived economic and contractual constraints; limited accessibility to entities producing such molecules; and significant scientific misconceptions.

In order to support higher scientific quality, meet ethical standards and ensure the limitations of animal-derived antibodies are overcome, it is recommended that the use of non-animal-derived antibodies is endorsed by government authorities, funding agencies and publishers. In conjunction with stringent validation and quality control, antibody reagents should be unambiguously identifiable by sequence. Furthermore, to ensure that scientific and ethical standards are met, the EU Governments could allocate subsidies to antibody producers or customers requiring custom antibody production, who satisfy eligibility criteria. Alternatively, or additionally, non-profit centres (akin to the academic animal facilities of today and the academic DNA synthesis and peptide synthesis facilities of the past) may provide such recombinant binder generation services to the community for a transition period, until the commercial sector takes over. To ensure researchers are incentivised, regulatory standards should also reflect the high quality scientific and ethical core values. If these commitments are made, scientists will be motivated to move away from animal-derived antibodies and be able to contribute to achieving the best possible scientific standards by making use of non-animal-derived, well characterised antibodies.

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1. Background

Antibodies are fascinating proteins characterised by their ability to identify and bind to specific targets of interest in very complex environments of other biomolecules (akin to finding the needle in the haystack). Therefore, they can be used to protect our health by detecting a vast range of molecules in health care management, biomedical research and development, consumer safety testing, product claims, cosmetics efficacy testing, or food and environmental contamination. Today, almost all antibodies in research are made by immunising animals. Antibodies can be isolated from most higher-order animals. For practical reasons, the majority of antibodies are made by immunising rabbits, drawing blood and collecting polyclonal antibodies from the serum. The other major portion is made by immunising mice, collecting their spleen cells and fusing them to cancer cells to create hybridomas secreting monoclonal antibodies (Figure 1). They are then grown in cell culture to collect the tissue culture supernatant or from the ascites fluid of the peritoneal cavity of further mice (in countries where this is still allowed). Antibody genes from immunised animals can be subsequently cloned to allow recombinant expression. For example, Abcam lists over 10,000 antibodies as recombinant (*https://www.abcam.com/primary-antibodies/recombinant-antibodies*), mostly rabbit monoclonals. However, such methods always rely on an immunised animal to start with.

For more than 20 years, methods have existed to isolate antibodies from non-animal-derived libraries of antibody genes by purely biotechnological methods, e.g., from universal phage display libraries (Figure 1). No animals are used for this method. The EURL ECVAM Scientific Advisory Committee (ESAC) was requested to review the available evidence for the scientific validity of replacements for animal-derived antibodies, used in research and diagnostics, generated using animal-free technologies. An ESAC working group was established comprising experts from academia and industry. Expertise within the panel covered antibody generation with animal and non-animal technologies, antibody engineering and antibody use in many immuno-analysis applications, and their use in different market sectors including diagnostics, therapeutics, research discovery and regulatory toxicity testing.

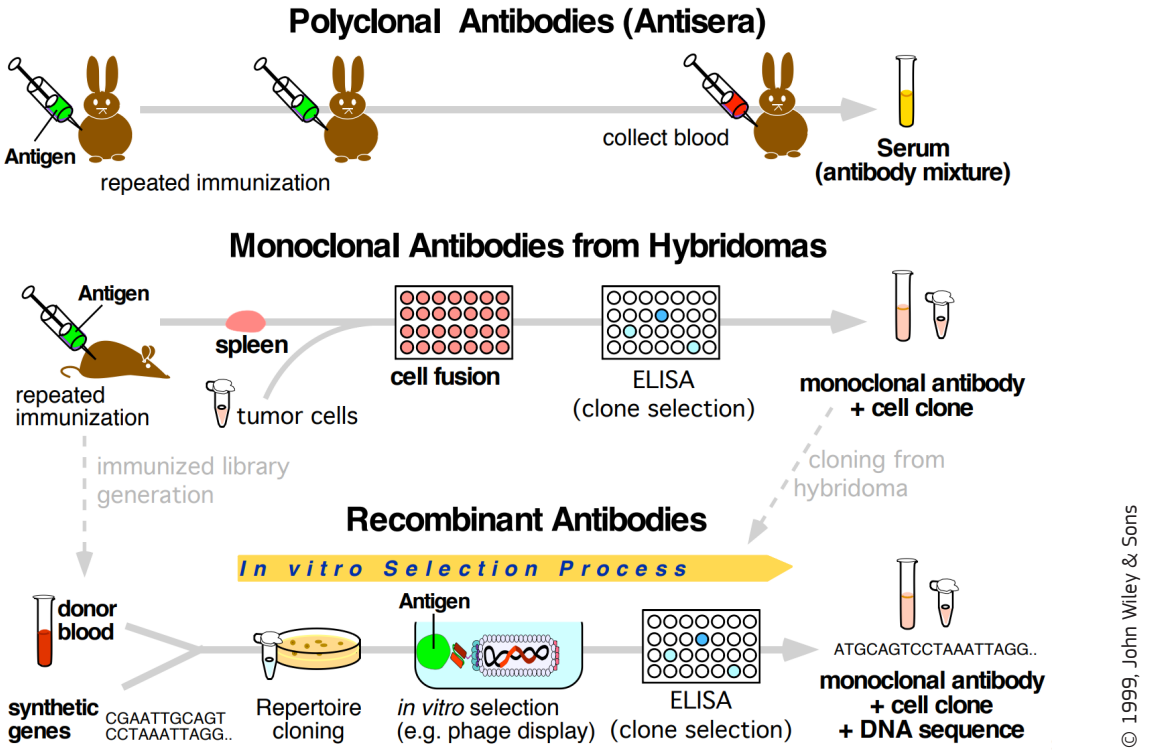

Figure 1: (Reprinted with permission from Breitling and Dübel, 1999, *Recombinant Antibodies*, © 1999, John Wiley & Sons) The three ways to generate polyclonal antisera, hybridoma-derived monoclonal antibodies and recombinant monoclonal antibodies. Recombinant antibodies are non-animal-derived, if no animals were immunised to obtain their encoding deoxyribonucleic acid (DNA). Recombinant antibodies can also be made from animal materials, by employing antibody gene repertoires from immunised animals for phage display or other *in vitro* selection methods, or by cloning of the antibody genes from hybridoma cells (dotted arrows).

2. Definitions

Terminology related to animal-derived and non-animal-derived methods, as they are used in the current report, are defined below.

Polyclonal antibodies ("polyclonals") are either sera or immunoglobulin (Ig)-fractions collected from the blood of immunised animals (Figure 1). They are a collection of different immunoglobulin molecules that include a fraction reacting against a specific antigen of interest. Polyclonal antibodies typically react with several different epitopes of the antigen.

Monoclonal antibodies ("monoclonals") are traditionally defined as being derived from the supernatant of hybridoma cells (see below). In theory, they contain a single antibody species from a single B-cell lineage but may be contaminated by additional antibody chains. An antibody sequence is typically not known but can be determined. Recombinant antibodies are also monoclonal, but in the scope of this report, the term "monoclonal" will be used to describe hybridoma-derived antibodies.

Hybridomas are artificial fusions between a myeloma cancer cell line and B-cells of an immunised animal producing one, or at most a few different antibodies. They are the production source of monoclonal antibodies.

Recombinant antibodies are produced by cells transfected with DNA encoding one antibody. Consequently, they are invariably sequence-defined and represent the best-defined type of affinity reagents with respect to specificity and unlimited availability, i.e., repeatability and reproducibility of experiments. Recombinant antibodies can be animal-derived: generated by cloning the antibody encoding deoxyribonucleic acid (DNA) of hybridomas; or by cloning of antibody gene repertoires from B-cells of immunised animals. Recombinant antibodies can also be non-animal-derived: from "universal" antibody gene libraries generated by cloning the entire antibody gene repertoires of non-immunised (naïve) human donor B-cells or from completely synthetic genes.

Animal-derived antibodies – Includes any antibodies isolated or produced using methods where animal immunisation is required. This includes monoclonals, polyclonals and recombinant methods relying on immunised animals as starting material.

Non-animal-derived antibodies – Includes antibodies that were derived from *in vitro* recombinant libraries without the use of animal immunisation at any phase of library generation or antibody production. Such antibodies might be identical in structure to full-length immunoglobulin G (IgG), or they may encompass parts of an antibody such as fragment antigen-binding (Fab), single-chain fragment variable (scFv), single-chain fragment variable dimerised by the fragment constant (Fc) domain (scFv-Fc), disulphide-bond stabilised fragment variable (dsFv), camelid-derived variable heavy (VH) chain fragment (VHH), variable new antigen receptor (vNAR), etc. (some examples are given in Figure 2).

Non-antibody affinity reagents – Non-antibody polypeptides or oligonucleotides that bind to specific targets with high affinity. These technologies include a large variety of scaffolds, including, for example, designed ankyrin repeat proteins (DARPins), affibodies, anticalins, armadillo repeat proteins, etc. They are invariably produced from large synthetic libraries and thus not derived from animals.

Recombinant libraries are large collections of animal-derived or non-animal-derived antibody genes, or non-antibody affinity reagents. In this report, they refer to those not originating from immunised animals. They are the typical starting point for selection of non-animal-derived antibodies or other affinity reagents. The antibody proteins are expressed from the library by using techniques such as phage, yeast or ribosome display. Libraries used in the generation of non-animal-derived antibodies from non-immunised sources are referred to as "synthetic", "universal" or "naïve" to distinguish them from technologies that require immunisation of animals.

Antibody fragments are derived from immunoglobulin molecules and contain at least part of the antigen-binding site of an antibody (Figure 2). Not all domains of a full-length antibody are necessary for antigen binding. The fragment variable (Fv) portion consisting of just the variable domains of heavy and light chain provide the entire antigen binding function, while the other domains provide effector function (e.g., binding to Fc receptors, antibody dependent cytotoxicity, opsonisation and complement determined cytotoxicity). There are also examples of antibody fragments consisting of only the single variable heavy (VH) domain that still show high specificity and sensitivity in antigen binding (Figure 2).

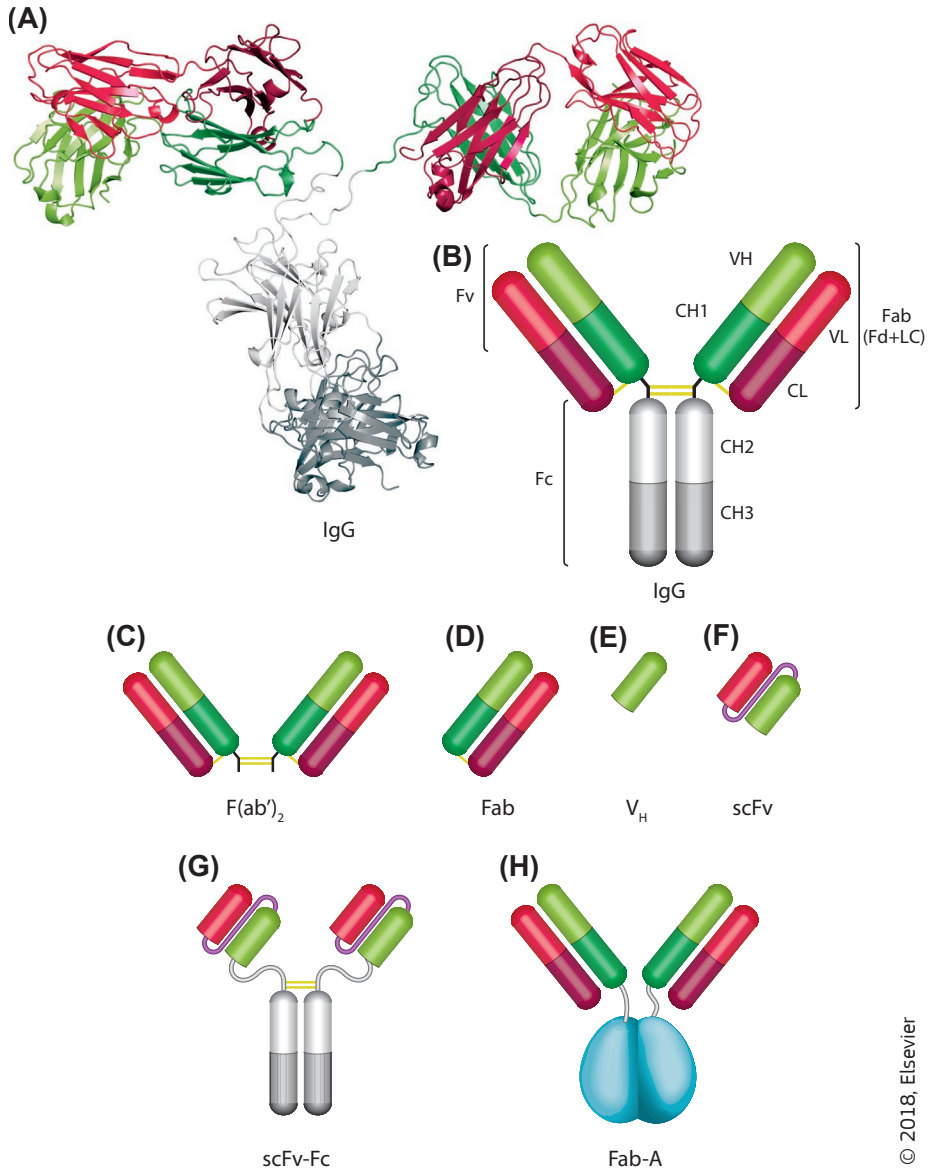

Figure 2: (Reprinted with permission from Hentrich *et al.*, 2018, Monoclonal antibody generation by phage display: *History, state-of-the-art and future.* In: Handbook of Immunoassay Technologies: Approaches, Performances and Applications, © 2018, Elsevier) Recombinant antibody formats. (A) Crystal structure of a full-length mammalian immunoglobulin (IgG) antibody. (B)-(H): Schematic representation of different antibody formats. Domains are coloured as in (A) (B) Full-length IgG antibody where V=variable, C=constant, H=heavy, L=light, Fab=fragment antigen binding. Disulphide bonds are dark yellow. (C) Bivalent F(ab')2 (D) Monovalent Fab. (E) Single-domain antibody such as VHH. (F) Single-chain fragment variable (scFv). (G) scFv-Fc fusions are scFvs dimerised by the Fc domain. (H) Fab-A is with the fusion of alkaline phosphatase, that can be directly detected using a colorimetric substrate and is an example of a wide variety of tags that can be directly fused to the antibody fragment, pre- or post-selection.

3. Scope

The ESAC working group was asked to provide a report on the scientific validity of the replacements for animal-derived antibodies used in current non-therapeutic applications. These replacements could include both non-animal-derived antibodies and non-antibody affinity reagents at various stages of development and acceptance. The ESAC working group focused on non-animal-derived antibodies, since they are leading in respect of technological maturation and compatibility with the usual scientific applications, with large bodies of evidence supporting their use and fewest perceived hurdles to rapid implementation. It was noted, however, that there would be value in summarising non-antibody affinity reagents as additional sources for replacement of animal-derived antibodies in this report and convening a separate working group to review them in greater depth. The use of non-animal-derived antibodies in therapeutic applications is well established.

4. Current status of animal-derived antibodies

Polyclonal antibodies often have high overall antibody affinity against the target antigen, offering good sensitivity for detection of proteins that are present in low quantities in a sample. Polyclonals are inexpensive and quick to produce. However, since a polyclonal antibody represents a collection of antibodies from different B-cells that recognise multiple epitopes on the same antigen with a mixture of other antibodies to unknown antigens, only 0.5–5% of the antibodies from the serum sample of an immunised animal bind to their intended target (Bradbury and Plückthun, 2015a). Consequently, high background is often observed. For example, this can be seen by the reactivity of the polyclonal serum used in a study by Russo *et al.* (2018) with the negative control. Immunopurification (chromatographic purification using the desired antigen as ligand) offers some benefit, if it is possible to immobilise the antigen in the correct steric orientation, i.e., when the binding site regions are not blocked by the chemical bonds introduced for immobilisation. Although unwanted polyclonal antibody specificity can be improved by such methods, the resulting antibody mixture still remains undefined in terms of epitope specificity, as the both composition of the mixture as well as the sequences of the individual antibodies are not known. Immunopurification is not usually done due to high cost and low yields.

More importantly, variability between different batches of polyclonals produced in different animals at different times presents a major obstacle to their reliability, such that even a particularly well-performing batch may be difficult to reproduce by a subsequent immunisation. This batch-to-batch variability has inevitable consequences on the reproducibility of experiments that rely on polyclonal antibodies for protein detection.

As mentioned, polyclonals have a high potential for cross-reactivity due to multiple epitope recognition. For example, a lack of target specificity was identified for 49 polyclonals against 19 different targets (subtypes of G-protein-coupled receptors) (Michel *et al.*, 2009). Although technically possible (Cheung *et al.*, 2012), there is no easy way to determine the sequences of the antibodies included in polyclonal sera.

Monoclonal antibodies (Köhler and Milstein, 1975), by contrast, are derived from a single antibody-producing B-cell and therefore are thought to bind with high specificity to only one unique epitope, with reduced probability of cross-reactivity. Provided that the original clone remains available, it is possible to reproduce the original antibody. It is also possible to produce large quantities of identical antibody and routinely achieve batch-to-batch homogeneity. Monoclonal antibodies are significantly more expensive to produce than polyclonals (about ten times), requiring more time to produce and develop the hybridoma. Although it is possible to sequence the antibody genes of a hybridoma cell line, there are still only few monoclonal antibodies, with known sequences available for research applications.

Researchers are typically unaware that quite a number of hybridoma-derived monoclonal antibodies are actually not monospecific. Nonetheless, the literature is littered with reports of non-specific monoclonal antibodies. There are two fundamental reasons for their lack of specificity (see below for details): the particular antibody may react with more than one target molecule; and the hybridoma cell may contain additional antibody chains (Bradbury *et al.*, 2018). By way of illustration, two widely used commercial mouse monoclonal antibodies against a master cell cycle regulator, cyclin-dependent kinase, were found to cross-react with a centrosomal protein due to partial overlapping amino acid sequence similarities between the epitope regions of the two antigens. This casts serious doubt over the findings of over 200 publications where this antibody has been used (Lukinavičius *et al.*, 2013). In a 2008 study, fewer than half of around 6,000 routinely used commercial antibodies (comprising both monoclonals and polyclonals) recognised only their specified targets (Berglund *et al.*, 2008). A 2014 study analysed 13,000 monoclonal and polyclonal antibodies in Western blotting and found that only 45% of the antibodies yielded supportive staining (Algenas *et al.*, 2014).

As mentioned, beside a potentially intrinsic lack of specificity, one other root cause is thought to be due to additional light chains from various origins, including the myeloma fusion partner, expression of more than one allele and *in vitro* evolution and fusion of more than one spleen B-cell to the partner cell line during hybridoma production. The presence of additional chains reduces antibody specificity and deteriorates the signal to noise ratio. In a study of 185 random hybridomas in a large multicentre study (seven labs from five countries), the lack of monospecificity was attributed to the genetic heterogeneity existing in a third (31.9%) of monoclonal antibodies (Bradbury *et al.*, 2018).

As a consequence of the procedure employed for hybridoma generation, monoclonal antibodies raised in animals are vulnerable to such abnormalities, if

not sequenced and produced recombinantly. The resounding impact of using non-specific antibody reagents in terms of wasted cost, time and resources, as well as the repercussion on diagnosis and health management, is immeasurable and the true picture remains largely unknown. Whilst the reagents used in most clinical trials and especially in medical procedures cleared by the US Food and Drug Administration or the European Medicines Agency are extremely reliable, such abnormalities go largely unnoticed in cases where antibodies are not validated to the same degree.

To reiterate, a true monoclonal antibody (devoid of additional chains from the hybridoma) can still be of low specificity. Therefore, it is essential to subject any antibody and non-antibody affinity reagent to stringent quality control and validation, as described elsewhere (Bradbury and Plückthun, 2015a-c). The demands for specificity testing for animal-derived and non-animal-derived antibodies and non-antibody affinity reagents are identical. The key difference is that recombinant products are molecularly defined and thus can be uniquely linked to the product properties.

5. The current status of non-animal-derived antibodies

5.1 Formats

Antibody libraries for *in vitro* antigen selections can be generated from the activated B-cells of immunised animals (Krebber *et al.*, 1997; Ridder *et al.*, 1995; Muyldermans, 2001), from non-activated (naïve) B-cells to create naïve antibody gene repertoires, or from carefully designed "synthetic" repertoires (Yan *et al.*, 2014; Knappik *et al.*, 2000; Hust and Dübel, 2004). Immunised antibody libraries are enriched for antibodies that bind a specific antigen but require that a new library be generated for each new antigen of interest. Such immune libraries have been constructed from virus-infected patients to isolate neutralising antibodies, as well as from cancer patients to isolate tumour specific antibodies (Geyer *et al.*, 2012). Also, the great majority of VHH domain antibodies are created from immunised camelids, with subsequent library construction and phage display (see below).

An increasing number of different non-animal-derived antibodies are in development, have been developed or are commercialised. They are generated independently of any living immune system and are available to replace animal immunisation techniques for a wide range of applications. Non-animal-derived antibodies are selected *in vitro* by phage, ribosome, yeast or cell display. Such libraries typically use the antibody scaffold, but there is an increasing list of publications where non-antibody scaffolds are used, generated either from nucleic acids or protein scaffolds into which diversified loops are inserted to form the antigen binding site (Skerra, 2007), or other diversification strategies have been used (see Section 8).

Non-immunised or synthetic "universal" libraries contain an enormous repertoire (typically more than 10^{10}) of different candidate antibody genes. This equates to structural diversity of antibodies that is equal to or even larger that of any naïve immune system prior to affinity maturation and thus provides similar or even better chances to identify a specific binder against any target antigen (Bradbury *et al.*, 2011). Once such a library is established, it can be used for decades and replaces a lifetime supply of immunised animals.

The recombinant antibodies isolated from the libraries can be converted into any antibody format, including IgG, the essential function being centred on the binding region in a supporting framework region. Consequently, various less-bulky antibody formats have been developed, compatible with bacterial expression systems (Figure 2). For example, the approved therapeutic antibody drug blinatumomab is solely composed of two scFv fragments fused by a linker peptide. The antibody drugs ranibizumab and certolizumab pegol are Fab fragments (see IMGT at *http://www.imgt.org/mAb-DB/query*; or DrugBank at *https://www.drugbank.ca/*).

A particular comment is needed for nanobodies (derived from camelid VHH domains), as the overwhelming majority is still obtained by immunising llamas or camels; yet only recently, synthetic libraries have been reported that appear to perform equivalently (Crepin *et al.*, 2017; Moutel *et al.*, 2016; Yan *et al.*, 2014) and can avoid the immunisation of animals. Many nanobodies have long protruding loops and may thus be suitable to access cavities in targets. In general, they are less aggregation prone than scFv fragments, particularly those of murine antibodies, making some fusion proteins easier to produce.

Synthetic "universal" libraries have been mostly built using human antibody sequences as blueprint, as the intention was mostly to develop human antibody therapeutics. There are some examples for libraries built on non-human antibody scaffolds (e.g., Sommavilla *et al.*, 2010; Moutel *et al.*, 2016; McMahon *et al.*, 2018) but there are no known limits concerning the choice of species for building such libraries.

5.2 Principles of Action

Antibodies can be selected by a variety of methods (Bradbury *et al.*, 2011). Here, we focus on phage display (Figure 3), the most widely adopted system, the development of which was honoured with the Nobel Prize in 2018.

The design of a non-animal-derived antibody universal phage display library and selection of binders from it adopts and adapts the same mechanistic principles of nature that have been relied upon to produce animal-derived antibodies, prior to and following immunisation, thereby mimicking and surpassing the whole animal immune response through the careful control of *in vitro* parameters (Fuchs *et al.*, 1992). These include: combinatorial diversification to create the hypervariable binding regions; selection of antibody-antigen binding partners; potential counterselection against antigens that should not be cross-reactive; and optional affinity maturation for

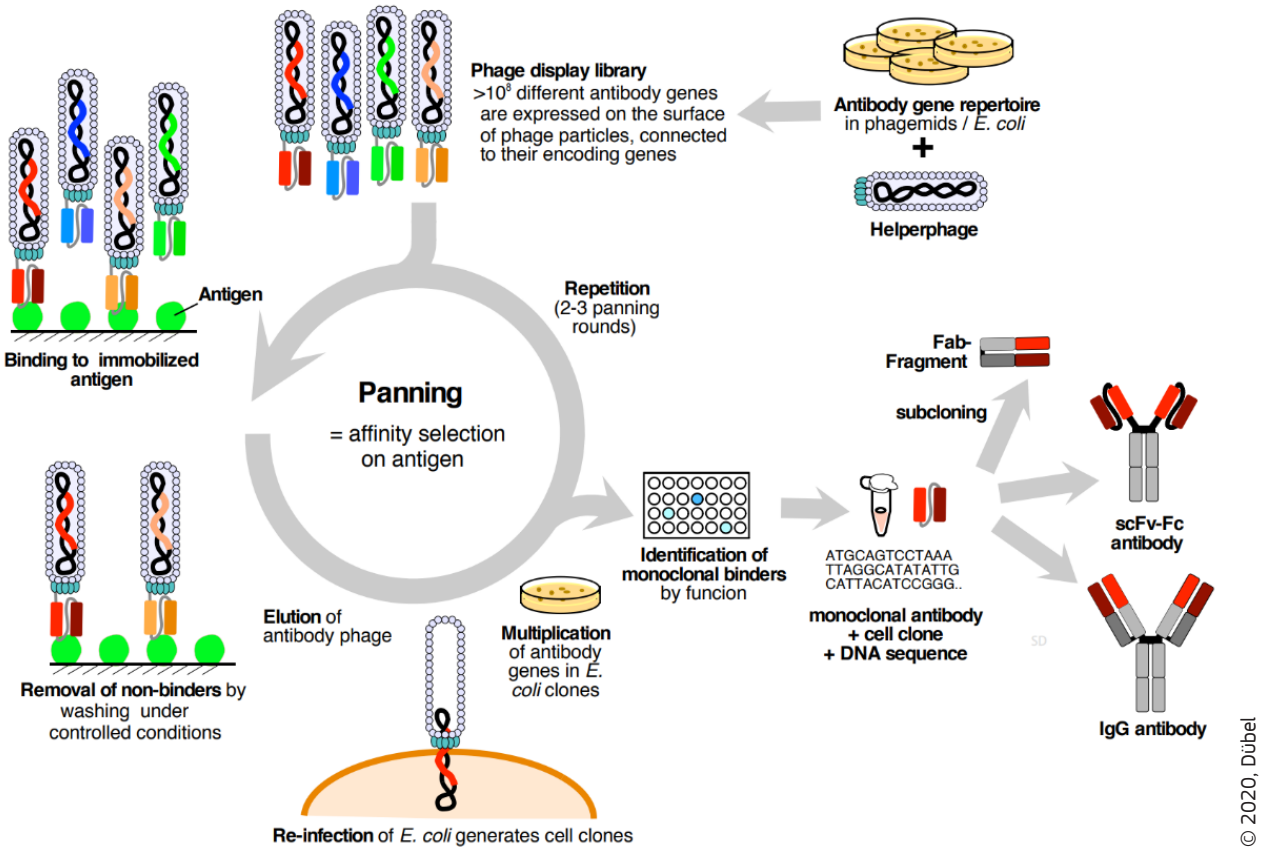

Figure 3: (© 2020, Dübel under CC BY 4.0) Antibody phage display.

improved sensitivity (Figure 3). In consequence, these mechanisms create at least the same repertoire, diversity, structure and function as is achieved by using immunised animals.

The principle of combinatorial diversification, otherwise known as recombination of variable (V), diversity (D) and joining (J) segments is relied upon by nature, pre-immunisation, to create a theoretical diversity of at least 10^{12} potential antibody gene sequences (Alberts *et al.*, 2002). The number of peripheral blood B-cells in a healthy adult human is in the order of 5×10^9 (Briney *et al.*, 2019). Using the same principle of combinatorial diversification for *in vitro* antibody library construction, genetic sequences of antibody binding regions are manually re-combined (synthetic) or amplified from B-cell isolated DNA derived from human blood (non-activated B-cells), using standard molecular, cell and micro-biology techniques. The genetic sequence of billions of different polymerase chain reaction (PCR) amplified antibody genes, with high diversity in the binding regions, are subsequently cloned into a phagemid vector (Breitling *et al.*, 1991) and fused to filamentous phage minor coat protein gene III, which ensures that antibodies will eventually be expressed on the surface of a phage particle, fused to a phage coat protein and thereby exposed for antigen recognition. Introduced into a laboratory strain of the *Escherichia (E.) coli* bacterial host, a universal antibody DNA library of the chosen format, e.g., scFv or Fab, is created, with diversity only limited by the transformation efficiency of *E. coli*. This has yielded libraries with over 10^{11} independent transformants (CAT2 library, Lloyd *et al.*, 2009; Ylanthia® library, Tiller *et al.*, 2013). Infecting the *E. coli* culture with helper phages leads to production of phages that carry the antibody on their surface and the corresponding antibody gene information in the packaged phagemid, thereby linking the antibody genotype with its phenotype. The phage particles can be harvested and used for the isolation of specific antibodies by phage display panning (Schirrmann *et al.*, 2011; Barbas *et al.*, 1991; Altshuler *et al.*, 2010).

Once a phage display library is constructed and the antibodies expressed as proteins, selecting the right antibody to bind to the antigen from the enormous repertoire of possible antibody binding sites might seem a daunting prospect, analogous to searching for the needle in the haystack. In fact, this selection process also resembles that which takes place in nature and the practice of isolating antibodies secreted by hybridomas. For animal-derived monoclonal

antibody production, a B-lymphocyte population, enriched by cells secreting antibodies to the target of interest (or antigen), is isolated from the spleen of immunised animals and fused with myeloma cells to produce an immortal hybridoma cell. Each cell will (in theory) only secrete identical antibodies specific for one epitope. Antibody candidates are selected by detection of antigen-bound secreted antibodies from each cell with the immobilised antigen, and positive clones are subsequently expanded in tissue culture or in the ascites fluid of the mammalian peritoneal cavity (Nelson *et al.*, 2000). In phage display, the vast collection of phages displaying the antibodies encoded in the library is brought in contact with the (immobilised) antigen of interest. The antibody-carrying phages that show affinity to the antigen are captured and the non-specific phages are washed off, a process referred to as "panning". Finally, the bound phages, containing the corresponding genetic information for the displayed antibody, are eluted and used to infect new *E. coli* cells. The infected *E. coli* culture is grown to produce new copies of the antibody-displaying phages that were eluted in the previous panning round. After typically 3 rounds of panning, the enrichment of phages displaying antibodies that bind to the target of interest is so strong that individual phage-infected *E. coli* cells can be identified and isolated. Positive cells are grown to express antibody fragments such as scFv or Fab (mono- or bivalent), or the antibody genes are re-formatted to, for instance, IgG or scFv-Fc and produced in mammalian cell culture.

The immune system has evolved cellular machinery for selecting those binding specificities that are needed to meet the challenge of an invading antigen, specifically amplifying those antibodies until they become prevalent in the total antibody population (Petrenko and Smith, 2000). Non-animal-derived antibodies have all the target recognition qualities of animal-derived antibodies. *In vitro* selection of the phage-displayed antibody against the desired antigen similarly interrogates the functionality, i.e., affinity and stability of potential binding partners, which can be selected for under increasingly stringent conditions such as competition with closely related antigens, pH or concentration. The specificity can then be tested and verified.

In nature, during the course of a host's immune response, the antibody affinity to the antigen continues to increase due to somatic hypermutation focusing on the hypervariable, antigen-binding region of the antibody genes, followed by competitive clonal selection. Similar mechanisms have been developed for affinity maturation of antibodies obtained from naïve or synthetic recombinant libraries. These include designed or PCR-based random mutagenesis, or variable chain shuffling to name a few. They generate a vast repertoire of antibody variants from the starting clone (or clones), which are then used as starting point for high-stringency selection procedures to enrich for high-affinity variants (Teng and Papavasiliou, 2007; Roskos *et al.*, 2007). Improvements in other antibody characteristics such as stability, pharmacokinetic parameters, effector parameters, tissue penetrating capacity of antibodies and their immunogenicity are possible (Altshuler *et al.*, 2010; Igawa *et al.*, 2011) by carefully adjusting the *in vitro* selection conditions (see Section 7). While affinity in immunised animals is thought to be limited to ~100 picomolar (pM) by the physiological mechanisms of B-cell activation, *in vitro* generated affinities in the low pM range can often be achieved (Batista and Neuberger, 1998; Zahnd *et al.*, 2004; Luginbühl *et al.*, 2006; Geyer *et al.*, 2012).

5.3 Antibodies used in typical laboratory immuno-analysis applications

The main immuno-analysis applications used today are Western blotting, immunohistochemistry (IHC), immunocytochemistry (ICC), flow cytometry, immunosorbent assays (ELISA) and immunoprecipitation (Uhlen *et al.*, 2016). Other areas, such as multiplexed planar or bead arrays are mostly based on the ELISA or fluorescence principle. Non-animal-derived antibodies have been generated and used in all those applications (Borrebaeck, 2017). In some publications, direct comparison of non-animal-derived antibodies with animal-derived antibodies in typical immuno-analysis applications, has been performed. In addition, affinity ligands obtained from scaffold libraries have found additional applications in affinity purification because of their inexpensive production, robustness and wide resistance to a range of pHs, all of which are desired properties for column regeneration and immobilisation processes (Yu *et al.*, 2017; Hansen *et al.*, 2017). Furthermore, smaller non-animal-derived antibodies such as nanobodies or affinity ligands obtained from scaffold libraries have been frequently generated for super-resolution microscopy (Pleiner *et al.*, 2015). For *in vivo* imaging applications, they have been found useful as the smaller size leads to high tumour uptake and shortens *in vivo* half-life due to fast renal clearance and therefore improves signal-to-background ratios (Helma *et al.*, 2015; Bedford *et al.*, 2017). The availability of sequences enables the site-specific conjugation of labels in these cases, which is another advantage (Jost and Plückthun, 2014). Some of the affinity ligands have progressed to late stage clinical trials for therapy (for clinical trials numbers, see

Section 8 below). They have also been used in various diagnostics applications, although their clinical use in routine applications still awaits validation. Some examples for use of non-animal-derived antibodies in the main research applications are given below.
For Western blotting, a direct comparison between conventionally produced and non-animal-derived antibodies for a set of proteomics targets had been performed by Ohara *et al.* (2006). The authors concluded that non-animal-derived antibodies are well suited for Western blot analysis and compare favourably with the polyclonal antibodies used in this study regarding sensitivity and specificity. Other laboratories have reported Western blotting results, using non-animal-derived antibodies or aptamers to be comparable or superior to blots probed with traditional antibodies (Cho *et al.*, 2011; Lazzarotto *et al.*, 1997; Martin *et al.*, 2013; Ramos *et al.*, 2007, 2010; Shin *et al.*, 2010; Webber *et al.*, 2014). As mentioned below (see Section 6.5), the traditional polyclonal secondary antibodies can be replaced as well (Pleiner *et al.*, 2018; also see *https://abcalis.com/multiclonals*).

Antibodies that work in IHC staining of formalin-fixed paraffin-embedded (FFPE) tissues are generally difficult to generate, as the cognate antigen undergoes massive modification during formalin-fixation. Using *in vitro* antibody selection processes, such as phage display, FFPE tissue can be used directly for selection of antibodies. There are several examples in the literature where phage display antibody selection has been successfully performed for generation of IHC-positive non-animal-derived antibodies (see Figure 8, see Section 7.3). For instance, a differential guided selection against mantle-cell lymphoma FFPE tissue sections with depletion on normal human tonsil tissue yielded IHC positive antibodies for vimentin (Jarutat *et al.*, 2007). Protocols for this method have been published (Haaf *et al.*, 2018). Similar methods have been applied to select non-animal-derived antibodies for staining of fresh frozen tissue sections. Using a peptide antigen comprising the epitope of the well-characterised Ki-67 antibody led to the generation of non-animal-derived antibodies that performed well in IHC on FFPE tissue sections, showing a Ki-67 equivalent staining pattern (Jarutat *et al.*, 2006). Comparison of non-animal-derived antibodies with polyclonal antibodies generated against the same antigens and testing in IHC has also been investigated (Ohara *et al.*, 2006). While the polyclonal antibodies mostly showed various extents of non-specific binding on the analysed tissues, the non-animal-derived antibodies could be used to specifically detect the proteins in brain and other tissues. Use of affinity ligands obtained from scaffold libraries have also been explored in IHC, exemplified by DARPins (Theurillat *et al.*, 2010; Boersma, 2018) or affimers (Bedford *et al.*, 2017). An anti-human epidermal growth factor receptor 2 (HER2) affibody is today commercially available for use in IHC (Bedford *et al.*, 2017).

Flow cytometry is another main immuno-analysis application that is highly dependent on antibodies. These reagents are typically directed against a cell surface marker and are labelled with different fluorescent dyes. Non-animal-derived antibody generation, using either purified antigen or whole cells in differential panning set-ups have been described. Antibodies for flow cytometry may bind to cellular Fc-receptors, thereby creating background staining. The recombinant nature of non-animal-derived antibodies can provide a solution by either producing the antibodies without the Fc part or by including mutations that suppress Fc-receptor binding (LALA, TM, or N297A mutations), which have been investigated on human antibody Fc regions but can also be transferred to other species (Hentrich *et al.*, 2018), demonstrating the versatility of non-animal-derived antibodies (Vincent and Zurini, 2012; Liu *et al.*, 2017; also see *https://absoluteantibody.com/antibody-resources/antibody-engineering/fc-engineering/*). Scaffold proteins such as DARPins can be efficiently expressed in bacteria directly as green fluorescent protein (GFP) fusions and thus used directly in flow cytometry, also in identifying cell-binding and overlapping epitopes (Andres *et al.*, 2019).

Antibodies are also commonly used for immunoprecipitation to capture proteins from their cellular environment to identify binding partners or post-translational modification. Non-animal-derived antibodies have been generated for use in immunoprecipitation experiments (Colwill *et al.*, 2011). The study concluded that non-animal-derived antibodies were able to capture proteins from cell lysates, similar as animal-derived antibodies. In another study, non-animal-derived antibodies were generated against peptides to three proteins, empirically determined as proteolytic fragments observed by mass spectrometry, to be used as capture reagents in multiple reaction monitoring (MRM) experiments (Whiteaker *et al.*, 2014). The resulting antibodies were tested in both Fab and IgG formats and compared to high-affinity monoclonal benchmark antibodies from mouse or rabbit generated against the same peptides by immunisation. The performance of the non-animal-derived antibodies in the immuno-MRM application was found to be excellent.

A special form of immunoprecipitation is the chromatin immunoprecipitation (ChIP) application, in which antibodies binding to posttranslational modification

of histones are used. Non-animal-derived antibodies have been generated against a trimethylated lysine residue of histone H3 (H3K9me3) by Hattori *et al.* (2013). The authors first tested eight commercial animal-derived antibodies against this target and found all but one to be of poor quality. The only one with good quality was a polyclonal antibody lot that was no longer available. Therefore, they selected and affinity-matured an antibody from a universal library, which showed excellent performance in ChIP. In addition, they created a negative control by mutating four key residues of this antibody (Hattori *et al.*, 2013). This antibody is commercially available (Diagenode, product C15500003).

Non-animal-derived antibodies have been generated for producing antibody arrays, which are used to measure and quantify multiple biomarkers in parallel. A microarray platform composed of 349 non-animal-derived antibodies formatted as single-chain variable fragments (scFvs) directed against 156 antigens has been published (Mellby *et al.*, 2018). It was successfully used to identify a biomarker signature associated with pancreatic stage I/II cancer, useful for early detection in cancer patients. For building high-density antibody arrays, non-purified probes are to be directly purified, oriented and coupled in a generic one-step procedure directly on the chip (Steinhauer *et al.*, 2006). Here again, the recombinant nature of non-animal-derived antibodies enables site-directed addition of affinity tags, such as the His- or Myc-tag for affinity capture of non-animal-derived antibodies to the chip surface (Steinhauer *et al.*, 2006). Other array formats such as multiplexed bead arrays typically use antibody sandwich pairs for analyte quantification. During both animal-derived and non-animal-derived antibody generation, the initial screening of candidate clones is most often done in ELISA format due to the high throughput that this assay allows. Sandwich pairs are then identified in a second screening step, whereby positive antibodies from the first screen are tested one by one for their ability to build sandwich pairs. *In vitro* selection methods, such as phage display, enable guided selection strategies with the aim of selecting a matching detection antibody to a complex of antigen bound to a capture antibody (Hentrich *et al.*, 2018).

5.4 *In vitro* diagnostics and other applications

Non-animal-derived antibodies are commonly used in commercial *in vitro* diagnostic assays, both as active components of the immunoassay or as controls and calibrators. Some of the distinct advantages that they can offer in this area are exemplified below.

As non-animal-derived antibodies are often derived from *human* antibody libraries, they can be used as alternative to human disease state sera, as the calibrator material. Sera can be replaced by recombinant phage display antibodies with the desired specificity and reformatted to the human isotype of choice (Hentrich *et al.*, 2018). A major challenge with serological testing is access to consistent and unlimited control reagents to provide assay quality control and facilitate data consolidation. Often clinical positive sera are difficult to source and use as routine, inter-laboratory reagents. It has been demonstrated that non-animal-derived antibodies are suitable control reagents for serological and autoimmune testing (Knappik *et al.*, 2009, Golden *et al.*, 2016).

The ability to perform *in vitro* selection on antigen complexes has been used to develop non-animal-derived antibodies that are difficult to obtain by animal immunisations. A highly specific antibody against the complex formed between an anti-morphine antibody and morphine was selected from a naïve scFv phage display library (Pulli *et al.*, 2005). The complex-specific antibody was subsequently used in a fluorescence resonance energy transfer (FRET) assay to detect morphine bound by the anti-morphine antibody, thereby avoiding the competitive ELISA format that is typically used in immunoassays for the detection of small compounds. Such an assay can avoid the problem of false positive results that can arise from the use of a competitive assay format. Another recent application of this selection strategy is the generation of highly specific non-animal-derived antibodies that bind to a complex of antibody drug bound to the drug target (Harth *et al.*, 2018). Such specificities are used in pharmacokinetic studies during development of antibody therapeutics, as they enable quantification of human antibody drugs in serum samples without using the complicated bridging assay format.

Other advantages of non-animal-derived antibodies are evident from the sections above: they allow consistent, reproducible and sustainable supply; assay conditions can be taken into account by adaptation of the *in vitro* selection process; and the selection of stable antibodies is possible. For instance, the prolonged incubation at 37°C or repeated cycles of treatment with highly chaotropic guanidine hydrochloride was used to select scFvs with high stability for the detection of C-reactive protein (CRP) incorporated into a lab-on-a-chip system as a point-of-care device to rapidly detect

the acute phase of bacterial-induced inflammation (Al-Halabi *et al.*, 2013). A stable recombinant scFv-Fc construct allowed the development of a sandwich-ELISA, integrated on a biosensor portable device, to monitor *Legionella* concentration in water for human use, as a rapid on-site alterative to the standard detection approach that relies on lengthy cultivation and analysis of bacterial cultures (Kuhn *et al.*, 2017). The possibility of affinity maturation for increased assay sensitivity and the realisation of large multiplexed assay concepts (Borrebaeck, 2017) is to mention but a few of the capabilities of non-animal-derived antibodies, since they are not dependent on the antibodies that an animal decides to generate.

5.5 Validation of large data sets exemplifying the performance of non-animal-derived antibodies in different applications and from various sources

Although there is no accepted standard for the provision of data to clarify the performance characteristics for an antibody-antigen interaction, attempts have been made to codify the minimum information required for antibody performance (Gloriam *et al.*, 2010; Uhlen *et al.*, 2016). Nonetheless, adoption of such proposals has been slow, and information on antibodies tends to be generated on an *ad hoc* basis. Data may therefore be supplied by various immuno-analysis techniques. Evidence of the antibody-antigen binding characteristics typically provided in commercial catalogues includes the visual identification of a pattern of localised tissue staining (IHC), or the identification of a target at the expected molecular weight under reducing conditions (Western blotting). These are not statistically quantifiable measurements. Alternatively, a modification in the expression of a molecule can be marked by the intensity of fluorescence or enzymatically induced colour change marker, conjugated as a tag to the antibody and compared to an off-target negative control (ELISA). Often, this approach is used as the primary screen for identifying positive clones in antibody development, after several rounds of panning using universal recombinant libraries or following animal immunisation and extraction of B-cells. The initial screen is a routine procedure to eliminate non- or low-specificity monoclonal binders and is performed for all monoclonal antibodies, regardless of their source (animal or non-animal-derived). Selection produces high numbers of clones of varied specificity and affinity. At this stage, duplicate sequences and clones that do not meet the set criteria can be eliminated.

It is important to understand that the performance of an antibody in one application may bear no relation to its performance in another. This is primarily due to the fact that some assays require an obligate denatured state (Western blots), others an obligate native state (Fluorescence-activated cell sorting; FACS), others are dependent on the imposed experimental conditions (IHC), yet others can be adapted (ELISA). In aggregate, this means that this can render data difficult to compare outside of a consistent quality control framework that would otherwise serve to facilitate comparisons of antibodies from different sources for use in different applications.

Several large-scale antibody generation studies have met this challenge by providing large data sets, using quantifiable methods of analyses such as the rate constant of association (k_{on}), rate constant of dissociation (k_{off}), or equilibrium dissociation constant (K_D) of a binding partnership as measure of affinity, e.g. determined by surface plasmon resonance (SPR) analysis. Affinity data may also more crudely be determined by ELISA determination of the antibody concentration at which binding is detected, or by the mean fluorescence intensity (MFI) following analysis of the flow cytometric localisation of binding to a single cell target. Some data sources may even identify the specific epitope employed (epitope mapping). Importantly, the performance of the antibody is not only defined by favourable affinity to its target, but also by recognition of the target modified during the specific application, and by the lack of significant interaction with other substances that may interfere in the relevant assay, all of which have to be tested independently.

Examples are cited throughout the report giving detailed analyses of non-animal-derived antibodies performing in all known immuno-analysis applications. Additionally, a selection of data has been extracted from published sources to demonstrate the feasibility of their generation in large numbers. Further, evidence of their development for clinical use is included. The data examples are derived from a range of different phage display library sources, from various countries and different antibody formats (see Appendix, Section IV).

6. Why are non-animal-derived antibodies not already being used more frequently?

Major barriers to the uptake of non-animal-derived antibodies exist that have impacted on the evolution of antibody development and account for the erroneous beliefs that have hindered their widespread use. These include: a propensity toward existing methods; a lack of awareness of the current status; perceived economic and contractual constraints; limited accessibility to resources; and significant scientific misconceptions.

6.1 Propensity for existing methods

Many researchers consider the problems that they experience with catalogue antibodies (see Section 4 and Goodman *et al.*, 2018) as unavoidable, i.e., a molecular feature of antibodies in general. This misconception arises from the day-to-day experiences of most researchers using catalogue antibodies and the widespread use of polyclonal antibodies that show the greatest specificity problems. Monoclonal antibodies can also suffer specificity issues (see Section 4) although they are generally better. The question arises as to why researchers continue to use antibodies that are so problematic. This was addressed by Bradbury *et al.* (2011) who concluded that researchers are generally unaware of recombinant ways to make antibodies, a problem reinforced by the lack of companies providing recombinant antibody selection services for the non-therapeutic market, and scientific misconceptions on the quality of antibodies generated by display methods which reflect early experiences in the field.

The generation methods for animal-derived antibodies have not significantly improved in response to these known issues over the past 40 years, nor have they been rigorously quality-controlled in light of the above problems. As a result, there is a perception that these problems are inherent to antibodies in general and that their shortcomings must be accepted. It would therefore stand to reason that a different method of making antibodies would not improve the outcome. However, the unique advantages of recombinant antibodies, such as being sequence defined, has been reported in high-profile papers so there is a solid body of data to amend this perception.

6.2 Lack of awareness

Recombinant antibodies can be produced by cloning the DNA of animal-derived hybridomas (Bradbury *et al.*, 2018), obtaining B-cell repertoires from immunised animals (Krebber *et al.*, 1997, Ridder *et al.*, 1995, Muyldermans, 2001), or creating completely animal-free synthetic (Knappik *et al.*, 2000; Tiller *et al.*, 2013; Säll *et al.*, 2016) or naïve human B-cell universal libraries (Hust and Dübel, 2014; Mondon *et al.*, 2008). During the early years (1990's), recombinant antibodies were typically cloned from hybridomas. Despite optimism in the scientific community, there were difficulties in producing some of the clones as scFv fragments (see Figure 2) in bacteria, using the newly developed systems of the day. Further, a fraction of these hybridoma-derived scFv fragments were unstable proteins - an inherent property of some of the mouse variable domains (Wörn and Plückthun, 2001). Thus, these scFv fragments were not user-friendly, i.e., they could not be used in typical applications without additional effort and know-how. Furthermore, users needed to be educated that detection had to be achieved by using an anti-tag antibody and not by an anti-Fc polyclonal antibody — since they lacked an Fc region. Even though the detection would ultimately be identical (typically, by a linked enzyme or fluorophore to the detection antibody), there was the perception that the polyclonal anti-Fc serum would result in higher sensitivity.

In addition, some companies sold premature products and commercial kits that did not work, which added to the negative image. Furthermore, early antibody libraries did not produce antibodies with affinities as high as those derived from immunisation and the problems with mouse scFv protein stability resulted in a perception bias against display methods of antibody generation that has persisted despite twenty-five years of development and maturity.

Today, recombinant antibodies can be generated with superior properties and their properties can further be improved by *in vitro* evolution after initial generation (see Section 9). Still confusing for many users, however, are the larger number of possible recombinant antibody formats. While most animal-derived antibodies are in the IgG format, recombinant technologies offer additional opportunities to generate a variety of formats, a few of which are depicted in Figure 2 (see Section 2). Often overlooked is the fact that recombinant antibodies can easily be made also as full-length IgG, giving rise to parameters that render them functionally indistinguishable from

animal-derived IgGs in all typical applications, with the added benefit of sequence identity (see Section 8) preserving their monospecificity and unlimited supply. In certain applications, the use of antibody fragments instead of full-length IgG is an advantage.

The IgG molecules used as approved therapeutics - the application with the highest quality standards - are stably produced in large amounts and are now exclusively recombinant. Some of the approved therapeutics have been made using animal-free methods, while about half have been derived from immunised mice and then "humanised". Several marketed therapeutic antibodies take advantage of smaller formats such as the Fab or the scFv format.

To address educational needs, an abundance of different approaches to non-animal-derived antibody generation and selection methods are described in the literature including reviews, methodologies and research, indicating that sufficient educational resources exist. A simple PubMed analysis (Figure 4) reveals that an abundance of publications exists, indicating that there is no shortage of learning opportunities. A wealth of literature describing the shortcomings of animal-derived antibody approaches also exists (for example, see Couchman 2009). However, the scientific community would benefit from a greater focus on training and education to improve competency. Not every laboratory is familiar with modern molecular cloning and selection technologies and only very few institutions have a service facility. In contrast, animal houses are easily accessible and the cell culture work for making hybridomas is more familiar to biology laboratories.

6.3 Perceived economic and contractual constraints

In the past, few companies provided the necessary resources to produce non-animal-derived antibodies for the non-therapeutic market. Although this was perceived as lack of utility, this was more related to the attraction of the more lucrative therapeutics market to this sophisticated technology and thus driven by investment opportunities. Consequently, non-animal-derived antibodies were simply not available, neither off-the shelf nor custom-made (with very few exceptions). In addition to the focus on the therapeutics market, IP restrictions prevented more widespread commercialisation of the technology. Highly publicised patent fights in the therapeutic/pharma world, regarding recombinant antibody generation platforms and the exorbitant price tags of company deals, gave the impression that the technology was challenging, exclusive and very expensive, creating economic and contractual constraints. However, all initial recombinant antibody generation patents have now expired in the EU, making the technology available to all.

Although initial naïve recombinant library development requires a significant time investment, once the library is developed, this supplies a diversity of antibody candidates equivalent to a lifetime supply of animals and abolishes the ongoing cost of animal care. Generation costs of monoclonal non-animal-derived antibodies are similar to, or even cheaper than, those of monoclonal animal-derived antibodies because they are faster and only require standard laboratory equipment and consumables. This clearly underlines that antibody selection from universal libraries can replace animal use in this competitive market. As demand increases, technologies are expected to improve and further price reductions are anticipated. Beside cost, there is a significant benefit in time. While the generation of monoclonal antibodies needs several months, the selection of antibodies using a naïve recombinant library can be performed in a few weeks.

Polyclonal antibodies not only represent the largest fraction of commercially available research antibodies, but also are typically chosen by budget-restrained researchers over monoclonal reagents because of their significantly lower generation cost (currently about one tenth of a hybridoma antibody). However, as mentioned above, polyclonals are often problematic in terms of specificity and reproducibility.

At an annual worldwide spend of $1.6 billion on the antibody market and with only 5-49% functionality being reported in monoclonal reagents alone, a staggering annual loss to the biomedical research community of up to $800 million for the purchase of non-functional antibodies alone is estimated. The use of non-specific and poorly defined antibodies elicits an additional and unaccounted for waste in materials, time, money and follow-on research (Bradbury and Plückthun, 2015a; 2015c).

6.4 Limited accessibility to resources

Due to a lack of awareness, contractual constraints and a subsequent lack of demand, as well as lower profit margins, few companies have been motivated to invest in or market recombinant antibodies and affinity reagents other than for the therapeutics market. Fortunately, that situation is changing and some companies are now generating non-animal-

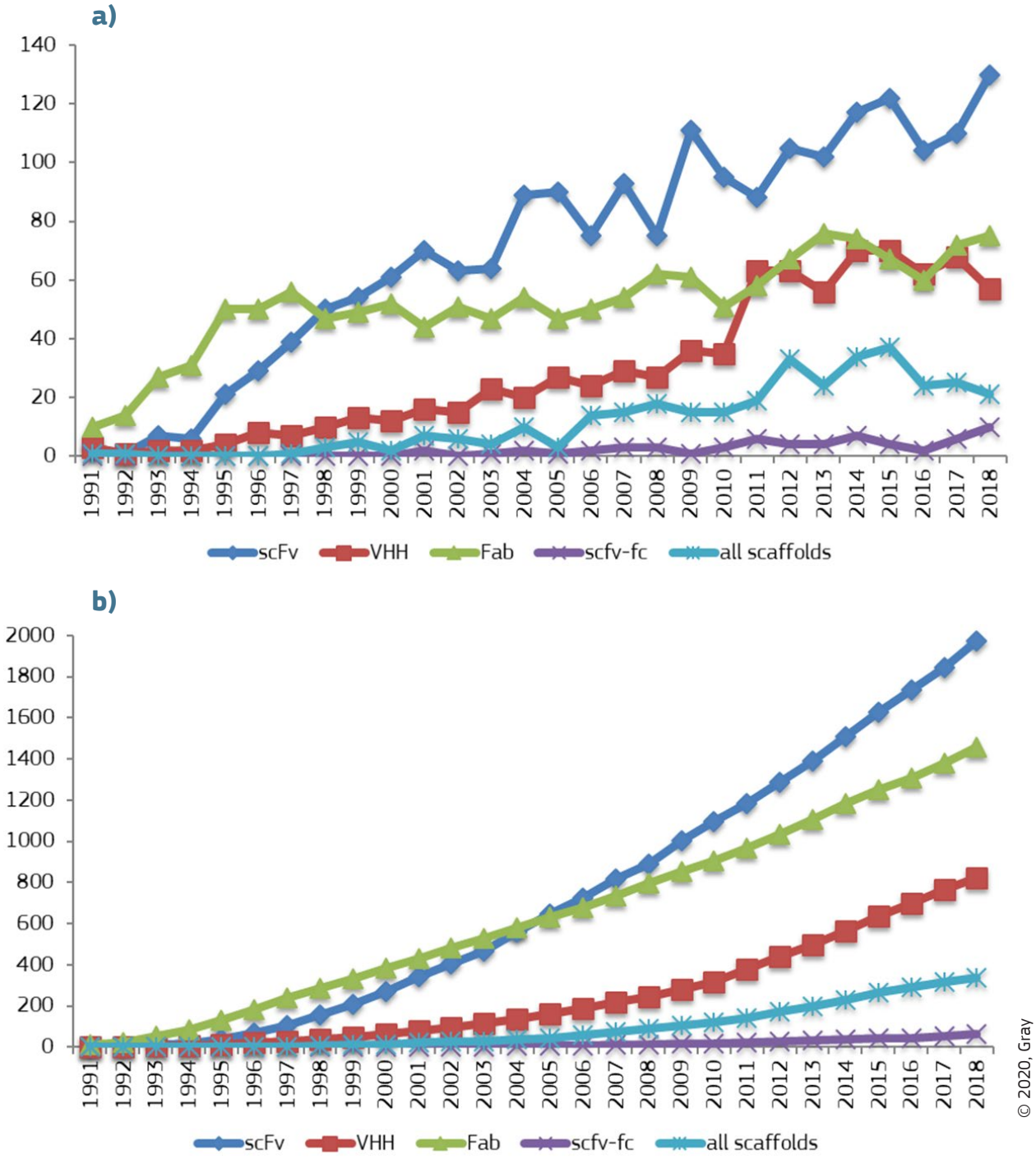

Figure 4: (© 2020, Gray under CC BY 4.0) Graphs showing **a)** the annual and **b)** the cumulative number of publications (1991 to present), for the recombinant antibody formats described in Figure 2, and scaffolds, using search terms: phage display OR ribosome display OR yeast display. Scaffolds included DARPin(s) or Designed Ankyrin Repeat Protein(s), monobody/ies, affilin(s), affitin(s), avimer(s), fynomer(s), FN3, cystine knot(s), abdurin or adhiron, affimer, adnectin, affibody, anticalin or lipocalin, bicyclic peptide or macrocyclic peptide or peptide macrocycle, or dipeptide.

derived research antibodies for customers (see Appendix, Section V). However, it is important to be aware that some companies may offer recombinant antibodies advertised as "animal free" that were actually originally derived from animal sources, i.e., derived from hybridomas or libraries obtained from immunised animals, e.g., *http://www.enzolifesciences.com/browse/products/by-product-type/antibodies/recombinant-antibodies/*.

Recombinant antibodies made entirely by non-animal methods are viable products in the research reagent market (see Appendix, Section IV and Section V). There are even examples of non-animal-derived research antibodies in catalogues that were made explicitly by phage display due to the inability to obtain antibodies with the required sophisticated functionality by animal immunisation, with some even sold as if they were derived by immunisation. Examples of such specificities include the anti-DJ-1 antibody AbD03055 that recognises the DJ-1 protein only when oxidised at cysteine residue 106, whereby the oxidative state at C106 determines the biological activities of DJ-1. The antibody has been selected from a recombinant library using a peptide comprising the oxidised cysteine 106 for selection and simultaneously blocking the library with a peptide of the same sequence but containing a reduced cysteine 106 (Ooe *et al.*, 2006,). Another example is the anti NS2B-NS3 antibody AbD05320 that inhibits the active site of the NS2B-NS3 proteinase from West Nile Virus. Here the recombinant library was blocked with a mutant NS2B-NS3 construct in which a histidine residue had been replaced by alanine within the NS2B-NS3 active site (H51A). This substitution mutation only altered the active site, keeping all other putative epitopes of the wild-type enzyme intact. Hence the mutant could be used to block members of the antibody library that bind to epitopes outside the active site (Shiryaev *et al.*, 2010). Another example is the anti-human SMAD3 antibody ABC0204 obtained by phage display panning using competition with the non-phosphorylated epitope to select antibodies that recognise a single epitope only if this is phosphorylated (Blokzijl *et al.*, 2016).

It is also largely unknown that thousands of non-animal-derived recombinant antibody reagents have successfully been made and used in academic consortia (see Appendix, Section III).

Additional research areas that could be enabled (see Section 7) by recombinant antibodies require that the user can obtain the DNA of the antibody from the company that generated the antibody. Such applications include fusion on the surface of cells, e.g., chimeric antigen receptors on T-cells (CAR-T cells), for redirecting viruses, or for studying cell biology with intrabodies, to name a few examples (see Section 7.5). However, even among the companies that generate antibodies by display methodologies, very few make the DNA available for free. As long as this is the case, non-profit organisations or consortia (see Section 9) still have an important role to play.

6.5 Scientific misconceptions

An important source of scientific view-points on the reliability of non-animal-derived antibodies are expressed in the publicly available Non-Technical Project Summaries under Article 43 of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes (*http://ec.europa.eu/environment/chemicals/lab_animals/nts_en.htm*), produced by each Member State. Opinions have also been collected through LinkedIn forums and personal discussions with users and producers in the scientific community. Misconceptions that non-animal-derived antibodies may not fulfil the requirement of certain demanding applications, in comparison with the natural immune response, reliability in terms of cross-reactivity, stability, affinity, avidity specificity and functionality, and multi-epitope recognition are discussed below and briefly addressed in Table 1. More detailed examples of successfully developed antibodies from non-animal sources are presented in Section 8 and in the Appendix.

Certain scientific limitations that are common to both animal-derived and non-animal-derived affinity reagents exist due to the complexity of the natural antibody-antigen binding relationship. These difficulties arise due to conformational challenges presented by the antigen or the availability of the epitope. For example, complex biological samples, e.g., viruses or whole cells, present a challenge because it may be difficult to expose or access the desired epitope. In applications where the target has undergone considerable modification, e.g., denaturation during Western blotting (see Section 5) or denaturation/chemical crosslinking during IHC, the epitope may no longer be displayed in its original form. Instead new epitopes become exposed. It should be noted that some degree of modification is likely in most applications. Therefore, different antibodies will be needed for different applications, independent of the method of generation.

Certain molecules, such as carbohydrates, have low immunogenicity due to the absence of the rich structural and chemical diversity typical of proteins and peptides. Animal-derived antibodies

Technical factor	Recombinant antibody expression from naïve phage display libraries	Monoclonal antibody production	Polyclonal antibody production
Reliance on animals	None. Not reliant on in vivo immune response. One off development of library, renewable resource, equivalent to lifetime supply of animals	Derived from animal immunization: new animals required for each new target.	Derived from animal immunization; new animals required for each new target, higher volumes, new batches.
Facilities required	Cell (mammalian and bacterial) culture facilities, standard equipment for molecular biology and protein analysis.	Animal housing facilities upkeep, immunization and animal care, cell (mammalian) culture facilities, standard equipment for protein analysis.	
Time required	6 months or less for one off development of library. Weeks for selection and clonal expansion of antibody candidates, from existing libraries.	6-8 months (protein antigen) or 12-15 months (smaller, less immunogenic molecules), for development of immune response, fusion and characterization.	2-4 months for development of immune response, harvest and purification.
Reliability of candidate selection	High. Acquisition of rare specificities to targets away from the influence of the natural immune response , e.g., against conserved epitopes, toxins, pathogens, non-immunogenics, closely related epitopes or cross reactivities.	Varied. Natural immunity may limit response to certain targets including toxins, pathogens, non-immunogenic molecules and closely related epitopes. May require conjugation to carrier protein and careful identification strategy. Antibodies vulnerable to negative selection.	
Antibody specificity	Excellent. Achieved by exposure of antibody candidates to multiple solution targets of interest for reduced cross reactivity or DNA directly and readily accessible for post selection affinity maturation based techniques	Varied. Dependent upon immunization response and efficiency of clonal selection procedure	Varied. Dependent upon immunization response
Affinity	nM-uM: optimised by design of biological milieu and can be improved to pico or femto molar level by affinity maturation based techniques	nM-uM: limited by immune response and affinity ceiling effect of the B-cell response.	Undefined: due to multiple epitope recognition and limited by immune response
Validation issues	Easy access to sequencing data for high level scientific validation. Immortal expression of antibody to target of interest achievable by expression in cell line. Resulting excellent lot-to-lot consistency means re-validation not required.	Sequencing data rarely acquired. Hybridoma produces indefinite supply of selected antibody but random mutation / genetic drift can occur. May exhibit poor binding characteristics or specificity	Non-specific binders and batch to batch variation (even in the same animal) meaning that they may not recognise the same target and each batch must be re-validated. May exhibit poor binding characteristics or specificity
Formats	ScFv or Fab fragment compatible with bacterial expression systems. Post selection engineering produces various formats including whole antibody monoclonal isotypes (IgG, IgA, IgE), scFv-Fc or polyclonal (by random mutation based principles), compatible with all standard laboratory applications	Various formats available reliant upon the output generated by the immune response and success of the purification process	No control over format
Quantity antigen required	1-100 µg (selection by ELISA)	1.5-2 mg protein (immunisation and selection by ELISA)	500 µg protein (immunisation and selection by ELISA)
Antibody generation pipeline	Amenable to automation and high throughput, multiple target selection and miniaturization	High throughput possible for selection of candidates, only after lengthy immunization process	Not amenable to high throughput
Therapeutic applications	100% humanization since derived from human lymphocytes or synthetic. Immunogenicity lower, making these more probable candidates for therapeutic applications	Up to 95% humanization possible. High immunogenicity against therapeutic candidates (HAMA) requiring resolution by humanization or deimmunization strategies.	Not applicable
Expertise	Requires knowledge of molecular biology techniques (PCR, cloning, vector design, sequencing, immuno-detection and competence in a range of specialist mammalian / bacterial cell culture techniques	Requires competence/ training in animal care and handling, extensive knowledge of immunization strategies, immuno-detection and competence in a range of specialist cell culture techniques	

Table 1: (Reprinted under CC BY-NC-ND 4.0 from Gray *et al.*, 2016, *Trends in Biotechnology*, 34, p. 965-966, © 2016, Gray, Sidhu, Chandrasekera, Hendriksen and Borrebaeck published by Elsevier) Technical Comparison of Phage Display and Animal-Derived Antibodies.

suffer additional restrictions because of the T-cell-independent antibody response to carbohydrates and failure to elicit memory responses. This can result in the production of low affinity and difficult to work with immunoglobulin M (IgM) antibodies to these molecules. (Heimburg-Molinaro and Rittenhouse-Olson, 2009). New technological developments to solve this problem are discussed in Section 1.

A commonly expressed viewpoint is that in order to exploit the full potential of the immune repertoire, a whole animal immune response to an antigen is required. There is a large body of data showing that this is not the case (see Section 7). The underlying mechanistic, functional, structural and diversity-generating similarities are discussed in Section 5 to clarify how the non-animal-derived antibody development strategy has adopted and adapted the essential principles of the naturally evolving immune system during the generation of antibody candidates against a specific target.

A challenging issue for animal-derived antibody development is the generation of antibodies to self-antigens or when there are high sequence homologies between the human and the respective animal-derived antigen. Similarly, a commonly expressed, but erroneous, viewpoint is that recombinant libraries cannot deliver antibodies against antigens from the same species. While it is difficult to raise mouse monoclonal antibodies to mouse antigens, a synthetic library is not influenced by the tolerance mechanisms that wipe out B-cells producing self-antibodies during the immune response. Consequently, many human antibodies against human antigens have been successfully made from such libraries, many for clinical use, and a number of these have already been approved for therapy. Further, European Union funded academic consortia have demonstrated the generation of hundreds of human antibodies to human proteins, e.g., Affinomics, see Appendix, Section III. The misconception that recombinant antibodies have lower affinities than animal-derived antibodies originated from the early years of recombinant antibody technology where most studies were executed with smaller libraries of lower functional diversity than routine today. There is clear evidence that antibody libraries with a larger number of functional antibody genes result in antibodies with higher intrinsic affinities (see Figure 3.5 in Hentrich *et al.*, 2018). Early libraries were not sufficiently large to contain antibodies with affinities like mouse monoclonal antibodies.

This misconception of lower affinity was also amplified by confounding the intrinsic affinity measured from bacterially derived monovalent antibody fragments (typically scFv or Fab) with the apparent affinity (avidity) measured from bivalent IgGs. Consequently, monovalent antibody fragments generated from naïve libraries, or cloned from hybridoma cell lines, failed to achieve the same functional affinities as the natural bivalent antibody when directly compared in typical binding assays such as ELISA, western blot or SPR, even though the underlying true affinities (of the monovalent arm) may have been identical. Today recombinant antibodies can easily be produced in the bivalent IgG format or in many other bi- and multivalent formats (Plückthun and Pack, 1997; Frenzel *et al.* 2017), which can be produced in a variety of hosts.

In vitro selection systems used with recombinant libraries also present unique opportunities to optimise the panning conditions or affinity-mature to resolve any unforeseen binding issues as well as improve them beyond the typical values expected of traditional IgGs. As a rule of thumb, with modern naïve libraries, after selection, non-animal-derived antibodies can be expected to achieve binding affinities at least similar to those commonly observed in rat and mouse monoclonal antibodies (low nanomolar [nM] range). Sub-nanomolar affinities for antibodies obtained directly from sufficiently large naïve libraries have been frequently reported (Table 3.1. in Hentrich *et al.*, 2018). After affinity maturation, non-animal-derived antibodies can achieve affinities similar or better than those sometimes observed for rabbit monoclonal antibodies (sub-nM to low pM range).

For certain applications, polyclonal antibodies derived from serum and capturing the whole animal immune response are preferred, such as when multiple epitope recognition is required. It is assumed that non-animal-derived approaches cannot meet this requirement. However, *in vitro* selection strategies routinely identify multiple antibody candidates against the antigen's various epitopes during the panning procedure. For example, Russo *et al.* (2018) isolated 11 highly specific scFv-human Fc anti-zebrafish cadherin antibodies binding to at least 5 different epitopes of recombinant zebrafish cadherin. Recombinant approaches allow the choice between the selection of individual clones with the most appropriate binding characteristics to create a monoclonal reagent against a specific target, or to focus on a multiple epitope approach for applications such as complex or highly mutagenic targets (Haurum, 2006; Alvarenga *et al.*, 2014). For applications where polyclonals have historically been relied upon, e.g., scorpion venom neutralisation, it should not be assumed that this approach is exclusive to the application, since it

is also possible to neutralise the closely related toxins by using a monoclonal approach (Riaño-Umbarila *et al.*, 2005). Polyclonals have been used historically for the generation of secondary reagents that recognise multiple binding sites within the Fc region of the primary antibody, for the purpose of fusion tag signal amplification. Recently it was demonstrated, however, that recombinant antibody fragments (VHH fragments in this case) made against all mouse IgG subclasses and rabbit IgG can outperform polyclonal secondary reagents (Pleiner *et al.*, 2018). To assist with the availability of one such VHH secondary reagent, the sequences and methods have been made accessible to the public and the expression plasmids are available on Addgene (Ewers, 2018). Those VHH reagents were developed by immunisations of llama and are therefore animal-derived. However, they prove that monoclonal secondary reagents with superior quality over polyclonals can be generated.

A non-animal derived alternative to polyclonal antibodies for signal detection and amplification are "multiclonal" antibodies recognising the IgG Fc region (*https://abcalis.com/multiclonals/*). Here, up to seventeen recombinant sequence defined monoclonal antibodies derived by phage display from universal libraries are combined to provide multiple epitope recognition, thus allowing to replace current animal derived secondary antibodies.

7. New opportunities made available using non-animal-derived antibodies

Since the natural immune response is mimicked and adapted during recombinant library selections, the previous limitations observed with animal-derived antibodies are avoided and replaced by a much more controlled generation of binding reagents.

7.1 Non-animal-derived antibodies are invariably sequence defined as part of their isolation

The sequences of non-animal-derived antibodies are typically determined as part of their isolation process, whereas this feature, i.e., known sequence, cannot be acquired for polyclonals, and only with significant additional effort for animal-originating hybridoma-derived monoclonal antibodies. Consequently, the sequences of antibodies contained in a polyclonal serum sample are never known and will change when preparing subsequent batches. The sequences of hybridoma-derived antibodies are rarely known.

Sequencing is an integral part of recombinant antibody generation during the initial identification of a clone. In consequence, once the sequence has been documented, the cell clone could be lost, but the corresponding antibody can be easily reconstituted by gene synthesis to obtain the identical specificity and binding profile. This factor allows unlimited reproducibility of scientific data, very much in contrast to classical polyclonal research data which can never been repeated exactly once the limited amount of animal blood serum is depleted. Monoclonal antibody producing hybridoma cell lines require (expensive) ultra-deep freezing for long-term storage and a large number of monoclonal antibodies described in the literature are thought to have been lost already, also limiting the reproducibility of the respective experiments (Bradbury and Plückthun, 2015a). The logistics of creating redundant storage of cell lines should be compared with the trivial logistics of redundantly storing a computer file of the sequence.

Since during *in vitro* antibody selection, the gene encoding the antibody is cloned at the same time as the antibody is selected, simple subcloning steps after *in vitro* selection permit the creation of constructs with added functionalities, as depicted in Figure 5.

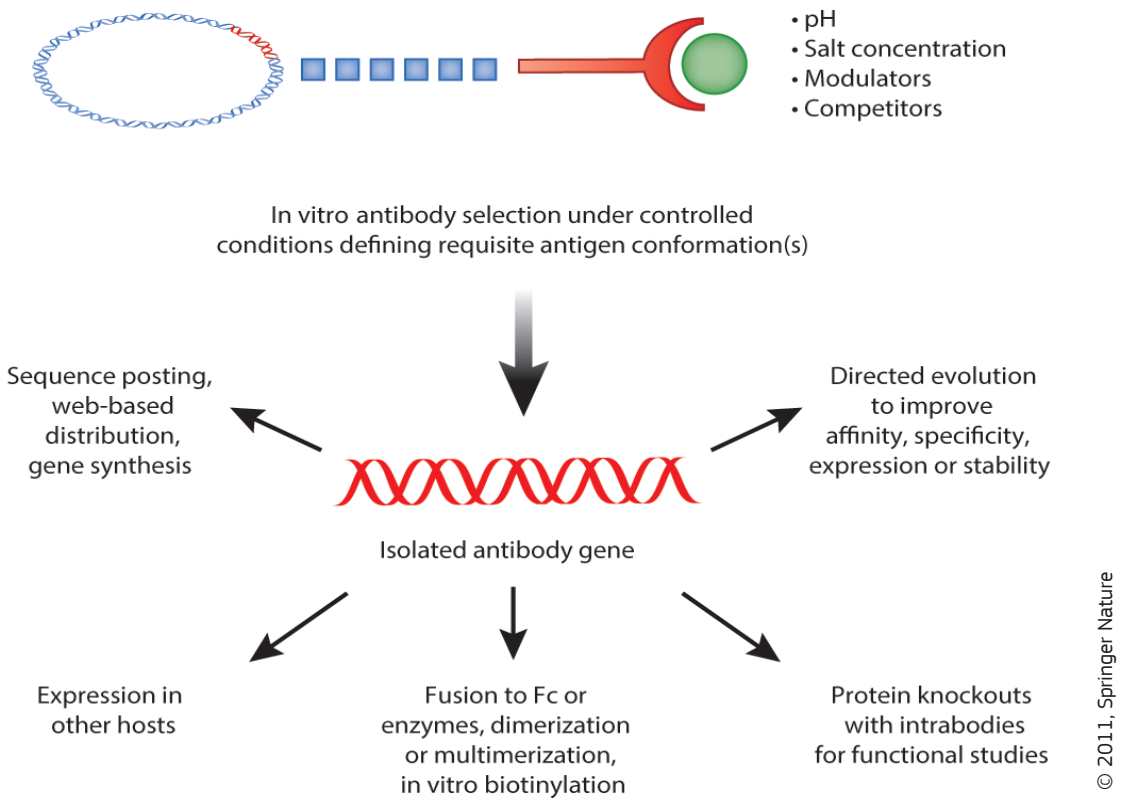

Figure 5: (Reprinted under CC BY-ND from Bradbury *et al.*, 2011, *Nature Biotechnology,* 29, p. 246 © 2011, Springer Nature) The unique capabilities of *in vitro* selection offer advantages over the immunisation of animals for antibody generation. The direct coupling of the antibody and its encoding gene is characteristic of all display methodologies, including phage, yeast and ribosome display.

7.2 Unique features of antibodies enabled by display technologies

By permitting control over selection and screening conditions, display technologies allow the generation of antibodies against defined antigen conformations or epitopes. For instance, the inclusion of competitors can direct selection toward specific targets. Antibody specificities can be broadened or narrowed by appropriate selection and screening (Figure 6).

7.3 More flexibility in the use of antibodies

In contrast to animal-derived antibodies, the researcher using recombinant antibodies is not limited by the choice of a particular detection system. All *in vitro* selection systems immediately provide the genes and corresponding sequences of antibodies selected against a particular target. This provides ready access to additional antibody formats by simple subcloning and expression. Functions adopted using this "gene-based" approach includes dimerisation, multimerisation and fusions to enzymes, tags or fluorescent proteins. Fusion to bacterial alkaline phosphatase is a particularly useful example of improved functionality (Weiss and Orfanoudakis, 1994). Antibody fragments can also be transformed into full-length antibodies, or scFv-Fc fusions, which are very similar in many aspects (Bradbury *et al.*, 2011). The genetic fusion of antibody genes to DNA encoding Fc parts from different species permits selection of the conventional secondary detection system in many immunoassays. For example, antibodies selected from the same universal human antibody phage display can carry either a mouse, rat, human, rabbit or other Fc part (Figure 7). Alternatively, or additionally, it can carry any variety of tags, including those for site-directed biotinylation. Recent developments in protein ligation technologies such as the Sortase system (Schmohl and Schwarzer, 2014) or the SpyTag system (Reddington and Howarth, 2015) circumvent the re-cloning step by enabling site-specific attachment of Fc parts or other moieties directly to the antibody fragment obtained from library selections.

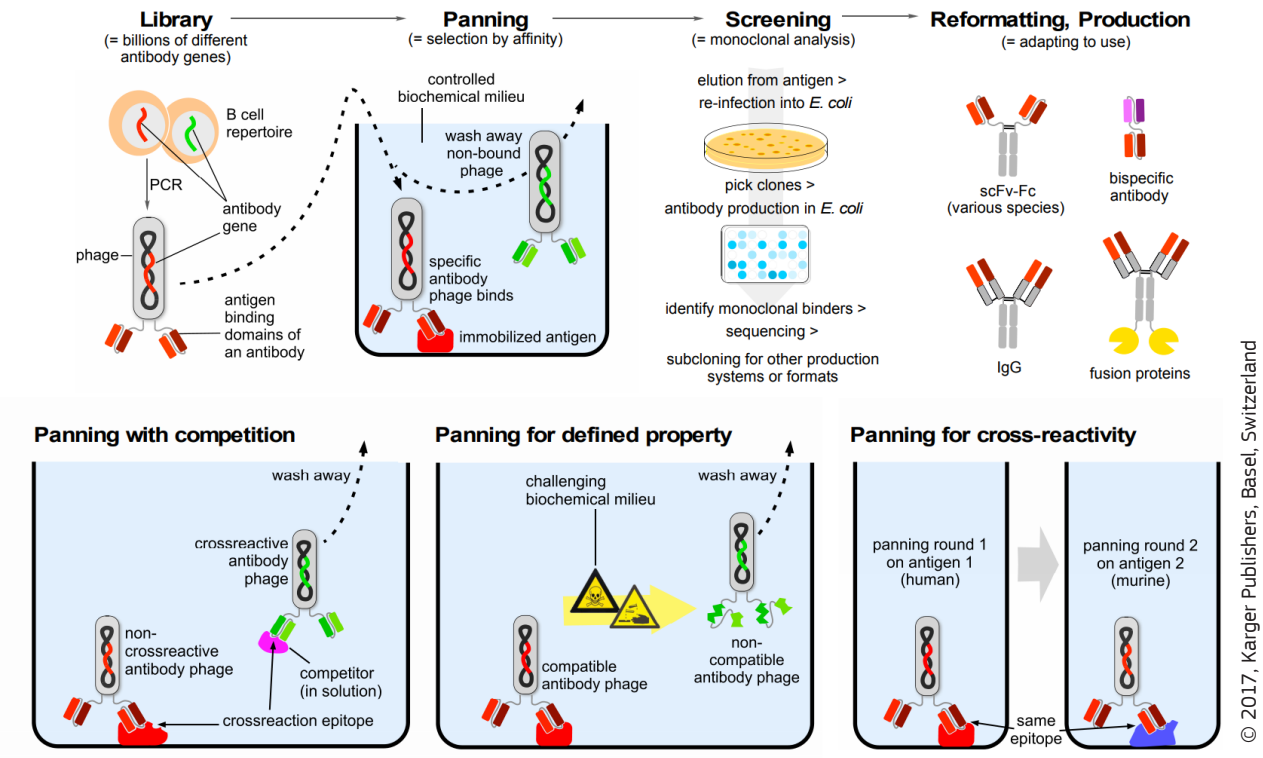

Figure 6: (Reprinted with permission from Frenzel *et al.*, 2017, *Tranfusion Medicine and Hemotherapy,* 44, p. 313, © 2017, Karger Publishers, Basel, Switzerland). Antibody phage display. In an antibody phage particle, the antibody gene and the function it encodes (antigen binding) are physically linked. This allows the affinity selection of monoclonal human antibodies in the test tube (a process named "panning"), e.g. panning in the presence of soluble competitor to deplete cross-reacting antibodies, panning under defined biochemical conditions selects only antibodies that are functional at these conditions, or sequential panning rounds on two different but homologous antigens allow to functionally select antibodies that bind to a structural feature common to the two proteins.

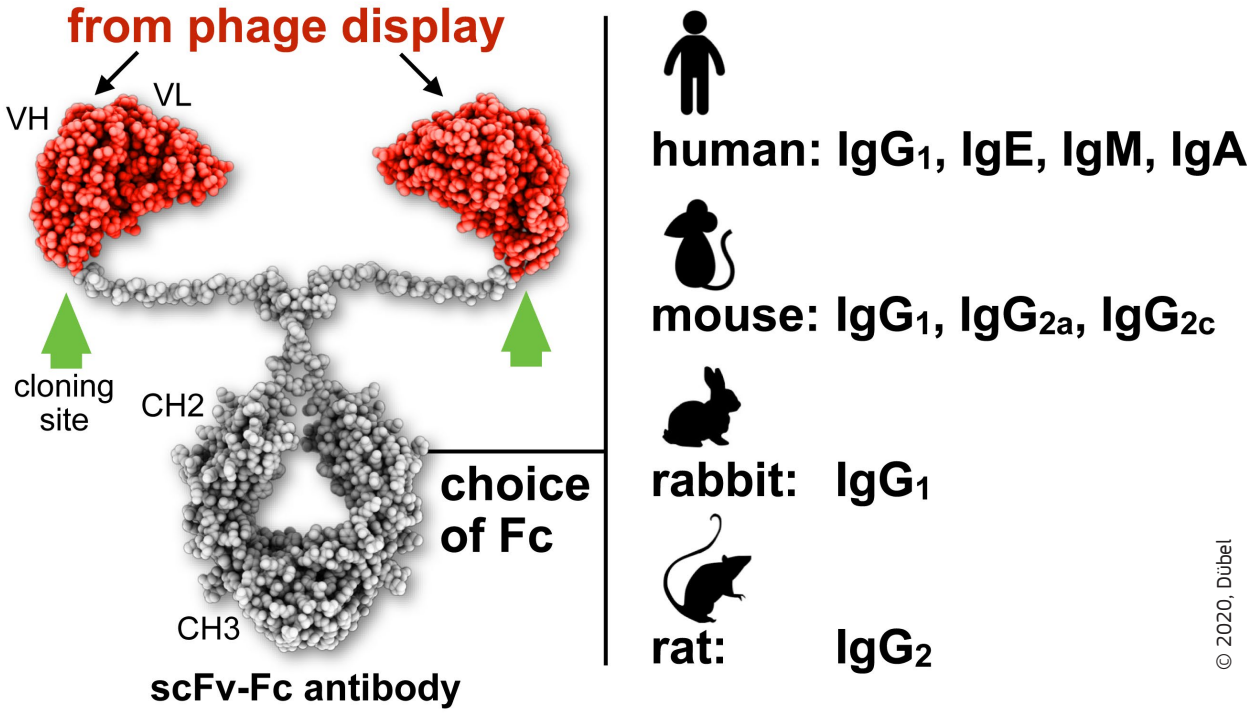

Figure 7: (© 2020, Dübel under CC BY 4.0) Animal-free generation of antibodies functioning as if generated from different animals: The scFv-Fc antibody format has been shown to be functionally equivalent to full IgG in a large number of applications, including the most widely used ones (immunoblot, immunohistochemistry/ immunofluorescence, FACS) (as shown by hundreds of examples within the EU funded Program Affinomics, see Appendix, Section III). It allows a simple combination of the antigen binding site (defining the antibody specificity) with different Fc parts for detection.

For example, this allows visualising three different biomolecules in a cell by using three different colours, which are recognised by three different antibodies carrying Fc parts from three different species (Figure 8). The possibility to engineer Fc regions to carry mutations that suppress Fc-receptor binding (LALA, TM, or N297A mutations) and therefore reduce background in flow cytometry experiments has been mentioned above (see Section 7.1). Respective antibody variants using the same recombinant binding site with different Fc parts for detection are already commercially available.

Even more colours could be added, e.g., by using biotinylation tags and streptavidin, plus other tags in addition, including direct GFP fusions. This cannot be achieved with typical state-of-the-art animal-derived antibodies.

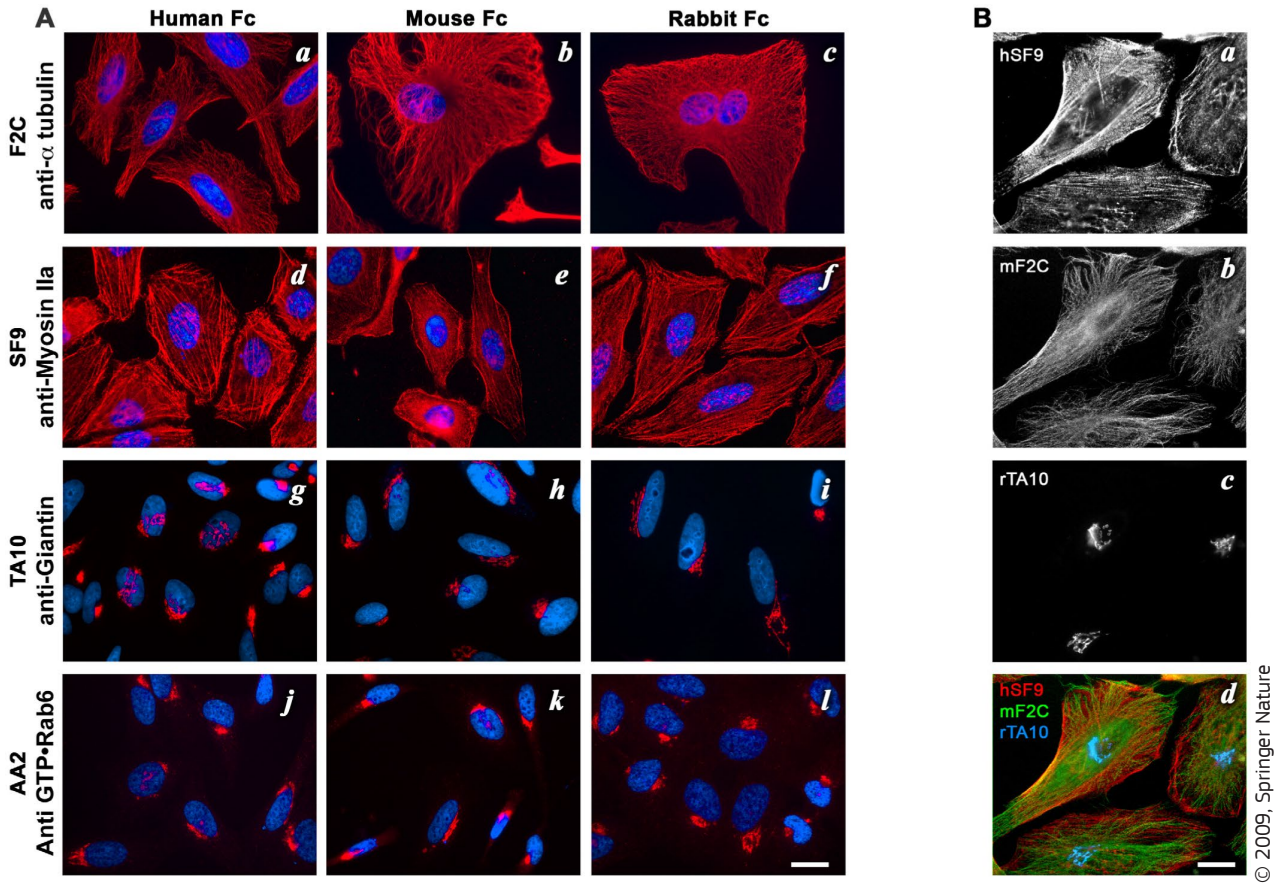

Figure 8: (Reprinted under CC By 2.0 from Moutel *et al.*, 2009, *BMC Biotechnology,* 9, p. 5, © 2009, Springer Nature) Immunofluorescence using multi-species variants of recombinant antibodies. A: The scFv-Fc F2C (a-c), SF9 (d-f), TA10 (g-i) and AA2 (j-l) in their human (a, d, g, j), mouse (b, e, h, k) or rabbit (c, f, i, l) versions can be used in immunofluorescence to detect their respective target proteins with similar efficiency (red). Bar = 20 µm. B: Using the multi-species approach, antibodies can be produced fused to any of the three IgG species. This allows a large diversity of multiplexing. In this example, myosin, tubulin and giantin were co-detected in HeLa cells using the human version of SF9 (a), the mouse version of F2C (b) and the rabbit version of TA10 (c) respectively. The three labellings are shown overlaid in (d). Bar = 20 µm.

7.4 Improving antibody affinity and specificity

After initial generation, non-animal-derived antibodies can be improved in many aspects by *in vitro* evolution of the encoding DNA. *"Libraries of mutagenized variants can be created and the same selection process repeated to yield variants with superior specificity and affinity. The improvement of antibody affinity to picomolar levels has become relatively routine, with one study describing an antibody that reached femtomolar affinity after in vitro maturation. These affinities are far higher than those of antibodies obtained by immunisation, which are usually limited to ~100 pM by the physiological mechanism of B-cell activation."* (from Bradbury *et al.*, 2011). Even parameters of the binding kinetics can be optimised by directed evolution and appropriate selection parameters to fit the experimenter's need, like the association and dissociation rates shown in the following example (Figure 9). The physical constraints and experimental set-ups needed are well understood (Zahnd *et al.*, 2010).

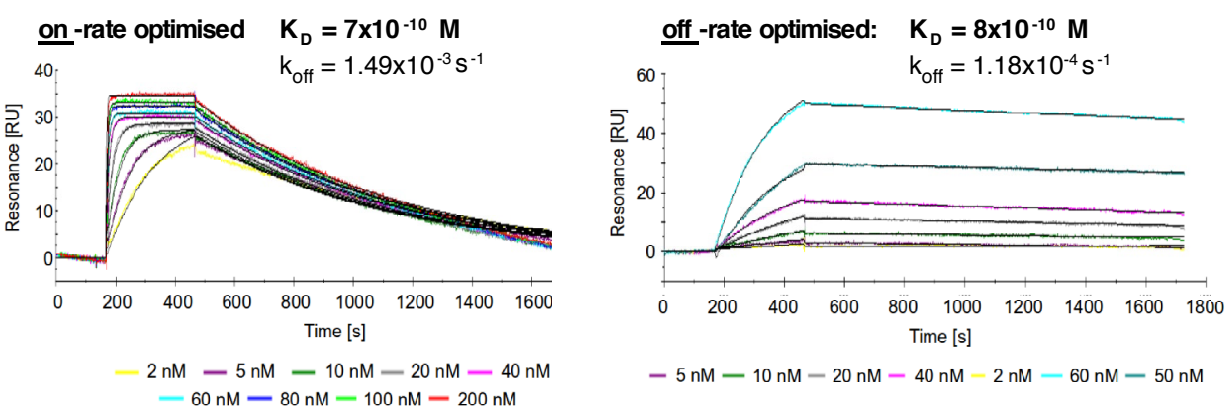

Figure 9: (Reprinted with permission from Frenzel *et al.*, 2017, *Tranfusion Medicine and Hemotherapy,* 44, p. 316, © 2017, Karger Publishers, Basel, Switzerland) An example for selective variation of association and dissociation rate constants observed upon light chain shuffling and selection using phage display with different selection parameters. The heavy chain of an antibody candidate was re-shuffled with the entire light chain repertoire of the naïve human library. After screening with different conditions, antibodies with different binding kinetics were obtained while maintaining the original high affinity, as determined by surface plasmon resonance.

7.5 Additional applications enabled by the recombinant nature of antibodies selected *in vitro*

Since the sequence is known and the DNA encoding the antibody is available from the start, the antibody fragments from animal-free generation methods can also be used right away in applications typically not possible for animal-derived antibodies, e.g., the use as intrabodies by expression in cells bearing the antigen, thus generating a protein knock down phenotype (for extensive review see Marschall *et al.*, 2015) the expression on cells, e.g., to create CAR-T cells (June *et al.*, 2018) or to redirect viruses to infect particular cell types (Buchholz *et al.*, 2015).

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8. Non-antibody affinity reagents or "scaffolds"

Ever since affinity reagents could be generated from a fully synthetic gene library, research had focused on substitution of the IgG protein framework by other protein scaffolds that could provide certain advantages in particular applications, whilst retaining the highly advantageous principles of diversity, selection and evolution that are characteristic of all affinity reagents (see Section 5). In other words, as an alternative to replacing animal-derived IgGs with recombinant IgGs (or Fab and scFv fragments), other protein scaffolds were designed to exploit the additional opportunities provided by protein engineering with greater ease. Thus, the main justification of using non-antibody scaffolds over recombinant antibodies is to extend the application range of binding reagents (for review, see Škrlec *et al.*, 2015), as well as exploiting their generally more robust nature, being advantageous for all applications. A particular advantage of several scaffolds is their highly efficient and inexpensive production in *E. coli*, providing cost-effective access to applications where larger quantities are needed, e.g., affinity purification of targets, or structural biology. Due to the extremely straightforward bacterial expression and purification, even inexperienced laboratories can express and purify these affinity proteins themselves, permitting the shipment of plasmids instead of proteins, or even only electronically transmit just the DNA sequence.

Several scaffolds are free of cysteines and fold well in the cytoplasm of cells, facilitating their application as intrabodies that work in the reducing environment of the cytoplasm. Domains devoid of beta-sheets are generally more robust against aggregation. Fusion proteins of such scaffolds with GFP, viral surface proteins or transmembrane anchors usually fold reproducibly well and are thus well expressed in any host. This is not always the case with beta-sandwich domains such as those from antibodies. For the same reason, robust scaffold proteins can also be homo- and hetero-dimerised and -oligomerised in many formats and geometries. These are well expressed in bacteria, where they generally fold well, while this is often only possible in eukaryotic hosts for antibody domains.

The best proof of appropriate specificity and affinity of such scaffold proteins is the fact that several of them have been used in drug discovery and are now in late-stage clinical trials (Simeon and Chen, 2018), which require specificity and good biophysical properties. The scaffolds that have reached clinical stage are also non-immunogenic. The flip side is that, at the time of writing, the companies developing these scaffolds do this for therapeutic applications only, entirely for economic considerations. However, the reagents are available to researchers through collaborations with several academic laboratories and facilities that have generated them for non-commercial purposes, some in high throughput (Appendix, Section V.b). Since numerous publications have greatly increased awareness and demand, it is only a matter of time until they will become commercially abundant.

In principle, every protein can be converted into a library of highly diverse binding proteins, but to achieve the desired properties for enabling the applications listed above, a particularly stable scaffold having a suitably large interaction surface and a well-conceived randomisation strategy needs to be used. A large number of non-antibody scaffolds have been explored (Binz *et al.*, 2005; Caravella and Lugovskoy, 2010; Gebauer and Skerra, 2009; Gilbreth and Koide, 2012; Simeon and Chen, 2018; Weidle *et al.*, 2013; Jost and Plückthun, 2014) and we direct the reader to the above excellent reviews summarising many of these aspects.

By way of example, Designed Ankyrin Repeat Proteins (DARPins) may serve to illustrate a mature technology (Plückthun, 2015). Different libraries have been made (see Binz *et al.*, 2004; Schilling *et al.*, 2014) and selections have been carried out with ribosome display (Dreier and Plückthun, 2012), phage display (Steiner *et al.*, 2008) and yeast display (Schütz *et al.*, 2016). Binders against several hundred targets have been created, largely within academic collaborations for research purposes using high-throughput ribosome display in selections with 92 targets in parallel. Several DARPins are in late-phase clinical trials (NCT03418532, NCT03136653, NCT02194426, NCT03084926, NCT03539549, NCT02462486, NCT02186119, NCT02462928, NCT02181517, NCT02181504) with constructs containing one through five different fused DARPins. Clinical applications of these DARPins was possible because of their lack of immunogenicity.

The published use of reagent DARPins have ranged from immunohistochemistry (Theurillat *et al.*, 2010), large scale affinity purification (Hansen *et al.*, 2017), SPR immobilisation (Hansen *et al.*, 2017), virus retargeting (Bender *et al.*, 2016; Friedel *et al.*, 2015; Friedrich *et al.*, 2013; Hanauer *et al.*, 2016; Hartmann *et al.*, 2018; Münch *et al.*, 2011; Münch *et al.*, 2015; Schmid *et al.*, 2018), intracellular sensors (Kummer *et al.*, 2013; Kummer *et al.*, 2012), crystallisation chaperones (Batyuk *et al.*, 2016; Wu *et al.*, 2017; Wu *et al.*, 2018), or research on cancer mechanisms

(Schwill *et al.*, 2019; Tamaskovic *et al.*, 2016; Tamaskovic *et al.*, 2012), to name a few examples.

Other scaffolds may have advantages for particular applications, e.g., anticalins (derived from lipocalins) have a rather deep pocket and may be advantageous in binding smaller biomolecules (Rothe and Skerra, 2018); while armadillo repeat proteins bind extended protein chains and may not only be useful for the detection of linear epitopes but also be developed into a modular sequence recognition technology (Hansen *et al.*, 2018; Hansen *et al.*, 2016; Reichen *et al.*, 2014) that would eventually avoid the selection against every individual target altogether, with great cost-saving potential.

Currently, one obstacle to a wider adoption of non-antibody scaffolds is the present lack of commercial availability, which is due to the companies' business models. This may change rapidly, as some of the key patents will expire soon, but today respective catalogue research reagents are still rare. Thus, most academic research has come from collaborations with the generators of the various scaffolds. However, the scaffolds certainly offer promising features which may boost their application to replace animal-derived antibodies in the future. While their generation and quality-control cost is very similar to non-animal-derived antibodies, the *subsequent protein* production, i.e., the expression and purification from an existing gene) could be significantly cheaper, for example when based on high-yielding bacterial expression. This can drive the price below that of monoclonals today, and with further technology development, as well as the prospect of a modular sequence recognition technology (Hansen *et al.*, 2018; Hansen *et al.*, 2016; Reichen *et al.*, 2014), perhaps even below those of polyclonals.

9. Future directions

The generation of non-animal-derived antibodies and other affinity ligands offers a superior scientific resource compared to animal-derived polyclonal and monoclonal antibodies. Their selection can be controlled *in vitro*, they can be generated against "self-" molecules and against toxins, their sequences are known, they can be engineered and their recombinant nature provides a consistent reproducible resource. Commercial producers, government authorities, funding agencies and academics, worldwide, have the opportunity to place far greater emphasis on antibody generation and validation by using non-animal-derived antibodies and scale down production of antibodies by methods that rely on animal immunisation. Here also lies the opportunity to improve awareness, cost, training and education, accessibility, which will automatically drive up demand for all non-animal-derived affinity reagents.

There is limited awareness of the fact that several thousands of animal-derived antibodies exist against the same popular targets. It has been calculated that there are 3483 protein targets with 100 or more antibodies and 42 proteins with over 1000 antibodies to each of the targets. The record is held by epidermal growth factor receptor (EGFR) with 8537 antibodies from 58 providers (*https://www.antibodypedia.com*). Duplication of many of the more popular targets reflects that the commercial market is driven to those targets of high demand — obviously a self-fulfilling prophecy, as researchers will work on targets with available reagents. A "reset" with new batches of recombinant reagents may have the potential of "evening out" the research on different and new therapeutic targets, provided appropriate subsidies are in place to reward such broader approaches. The relation between supply and demand is bidirectional.

Creating awareness and educating the typical user of research antibodies about the above misconceptions and current status is best achieved by two components: by making such non-animal-derived reagents conveniently available (see below); and by generating an increased number of successful case studies, ideally matched to convince today's patchwork of quite diverse research communities. Unfortunately, as of today, the reagents for this endeavour are simply not yet available off-the shelf. While they can all be custom-made, commercial efforts in generating such molecules have focused almost exclusively on therapeutic products and so the industrial capacity for producing research reagents is still low. Such custom-made non-animal-derived products will have to compete for some time with animal-derived catalog antibodies. Since the price cannot currently be undercut, the recombinant product must be positioned for its higher quality and greater opportunities (see Section 7 and Section 8).

There is also still ample potential to make recombinant research antibodies generated by phage display even cheaper, and probably even more so for other non-antibody scaffolds based on other selection technologies. One effective direction currently being followed is the creation of specialised binding domains, derived from low-affinity enzymes or binding domains, improved through directed evolution, to exploit already existing binding specificities, e.g., to detect ubiquitin-modified proteins (Tandem Ubiquitin Binding Entities (TUBEs), lifesensors.com), ADP-ribosylated proteins (Leutert *et al.*, 2018), or glycosylated proteins (Hu *et al.*, 2015). It is not necessary to have the same scaffold or the same universal library for all target molecules — for the end user, the assay will work identically.

The EU has already made some significant progress to support the development of recombinant affinity reagents: it has invested significant funding for several consortia which included tasks to parallelise and miniaturise *in vitro* antibody and binder generation. The goal to reach cost levels comparable to animal-derived monoclonal antibodies has already been achieved by these consortia (Appendix, Section III). Yet, with high probability of success, further large reductions could be achieved by further parallelisation and miniaturisation and by applying this technology to larger numbers so that a significantly lower cost of goods can be envisaged here.

The reason why such further improvements have not been pursued already by companies is a vicious circle: Profit margins and sales volumes per product in the research reagent markets are small and cannot fund substantial technological developments. In the development of therapeutics, the contribution of binder generation to the overall cost of clinically developing a therapeutic is almost a rounding error, such that there is no incentive for such technology development, either. As long as the misconceptions among potential customers exist that "phage display" antibodies are unreliable and expensive, such investments are still unattractive for companies, leading in turn to fewer examples of success stories. Indeed, some scientists are of the impression that recombinant antibodies must have bad properties, only based on the fact that there are so few in the catalogues.

Demand is also curbed by the limited availability: the current *commercial* cost of custom-made

antibody generation by recombinant methods is somewhat higher than the generation of traditional monoclonals, meaning that customers, guided by budget constraints, are choosing to accept a sub-standard research tool (see Section 6.3). As mentioned, commercial efforts in generating such molecules have gone almost exclusively into therapeutic products. Although costs will decrease as antibody generation by *in vitro* methods becomes more common-place, an impetus is needed during the transitional phase. To ensure scientific and ethical standards are met, the EU Governments could allocate subsidies to antibody producers or customers requiring custom antibody production, who satisfy eligibility criteria. Alternatively or additionally, non-profit centres (akin to the academic animal facilities of today and the academic DNA synthesis and peptide synthesis facilities of the past) may provide such recombinant binder generation services to the community for a transition period, until the commercial sector takes over. To ensure researchers are incentivised, regulatory standards should also reflect the high quality scientific and ethical core values.

Since there is a wide-spread acceptance of the limitations of antibodies, which are considered "intrinsic", fuelling the continued production of animal-derived antibodies and limiting the potential for non-animal-derived antibodies to gain a greater foothold in the market, the demand for a scientifically superior resource has to be created. This can only come from government authorities, funding agencies and publishers, supporting higher quality scientific, regulatory and ethical standards. For example, one proposal is that all newly generated binding reagents proposed in funding applications or described in published papers should be non-animal-derived, from naïve universal recombinant libraries or other non-animal-derived suitably engineered sources and defined at the sequence level. This would parallel the instilled standard that has existed for the past few decades, namely that gene sequences and coordinates for new protein structures be deposited and made publicly available. Making sequenced well-characterised reagents and ensuring all binding reagents in published papers are recombinant and defined at the sequence level, in conjunction with stringent quality control of the reagents, has previously been called for (Bradbury and Plückthun, 2015a) and is reiterated here.

Where the requirement for high scientific quality, reliability and consistency over long periods of time is of paramount importance, i.e., where it is anticipated that antibodies may be destined for use in diagnostics, batch testing of biologicals or otherwise regulated test methods, or as a tool for analysis, the use of animal-derived monoclonal or polyclonal antibodies can be avoided. In this case, the use of *in vitro* universal libraries enables sequencing data from candidate clones to be routinely collected and logged, avoiding that clones may succumb to any of the problems described in Section 4. Similarly, the results of biomedical research can influence the development of diagnostic tests and therapeutic development. Therefore, researchers carry an enormous responsibility to contribute to reproducible standards that follows the same requirements.

If these commitments are made, scientists will not want to use animal-derived antibodies and demand will follow. The absence of the adopted standards will lead to market disadvantages, which will encourage competitors beyond European legislative control to raise their standards.

10. References

Al-Halabi, L., Black, A., Michalzik, M., Fröde, D., Büttgenbach, S., Hust, M., Schirrmann, T. and Dübel, S. (2013). Recombinant antibody fragments allow repeated measurements of C-reactive protein with a quartz crystal microbalance immunosensor. *MAbs, 5*(1):140–149. doi:*10.4161/mabs.22374*

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, R. (2002). The Generation of Antibody Diversity. In: Molecular Biology of the Cell. 4th edition. Garland Science, New York. Available at: *https://www.ncbi.nlm.nih.gov/books/NBK26860*

Algenas, C., Agaton, C., Fagerberg, L., Asplund, A., Björling, L., Björling, E., Kampf, C., Lundberg, E., Nilsson, P., Persson, A., Wester, K., Pontén, F., Wernérus, H., Uhlén, M., Ottosson Takanen, J. and Hober, S. (2014). Antibody performance in western blot applications is context-dependent. *Biotechnology Journal, 9*(3):435-445. doi:*10.1002/biot.201300341*

Altshuler, E.P., Serebryanaya, D.V. and Katrukha, A.G. (2010). Generation of recombinant antibodies and means for increasing their affinity. *Biochemistry (Moscow),* 75(13):1584-1605. doi:*10.1134/S0006297910130067*

Alvarenga, L.M., Zahid, M., di Tommaso, A., Juste, M.O., Aubrey, N., Billiald, P. and Muzard, J. (2014). Engineering venom's toxin-neutralising antibody fragments and its therapeutic potential. *Toxins, 6*(8):2541-67. doi:*10.3390/toxins6082541*

Andres, F., Lamele, L., Meyer, T., Stüber, J.C., Kast, F., Gherardi, E., Niemann, H.H. and Plückthun, A. (2019). Inhibition of the MET kinase activity and cell growth in MET-addicted cancer cells by bi-paratopic linking. *Journal of Molecular Biology, 431*(10):2020-2039. doi:*10.1016/j.jmb.2019.03.024*

Barbas, C.F., Kang, A.S., Lerner, R.A. and Benkovict, S.J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: The gene III site. *Proceedings National Academy Science of the United States of America* 88(18):7978-7982. doi:*10.1073/pnas.88.18.7978*

Batista, F. and Neuberger, M. (1998). Affinity dependence of the B-cell response to antigen: a threshold, a ceiling and the importance of off-rate. *Immunity, 8*(6):751-759. doi:*10.1016/S1074-7613(00)80580-4*

Batyuk, A., Wu, Y., Honegger, A., Heberling, M.M. and Plückthun, A. (2016). DARPin-based crystallisation chaperones exploit molecular geometry as a screening dimension in protein crystallography. *Journal of Molecular Biology, 428*(8):1574-1588. doi:*10.1016/j.jmb.2016.03.002*

Bedford, R., Tiede, C., Hughes, R., Curd, A., McPherson, M.J., Peckham, M. and Tomlinson, D.C. (2017). Alternative reagents to antibodies in imaging applications. *Biophysical Reviews, 9*(4):299–308. doi:*10.1007/s12551-017-0 278-2*

Bender, R.R., Muth, A., Schneider, I.C., Friedel, T., Hartmann, J., Plückthun, A., Maisner, A. and Buchholz, C.J. (2016). Receptor-targeted Nipah virus glycoproteins improve cell-type selective gene delivery and reveal a preference for membrane-proximal cell attachment. *PLoS Pathogens, 12*(6):e1005641. doi:*10.1371/journal.ppat.1005641*

Berglund, L., Björling, E., Oksvold, P., Fagerberg, L., Asplund, A., Szigyarto, C.A., Persson, A., Ottosson, J., Wernérus, H., Nilsson, P., Lundberg, E., Sivertsson, A., Navani, S., Wester, K., Kampf, C., Hober, S., Pontén, F. and Uhlén, M. (2008). A genecentric Human Protein Atlas for expression profiles based on antibodies. *Molecular & Cellular Proteomics, 7*(10):2019-2027. doi: *10.1074/mcp.R800013-MCP200*

Binz, H.K., Amstutz, P., Kohl, A., Stumpp, M.T., Briand, C., Forrer, P., Grütter, M.G. and Plückthun, A. (2004). High-affinity binders selected from designed ankyrin repeat protein libraries. *Nature Biotechnology, 22*(5):575-582. doi:*10.1038/nbt962*

Binz, H.K., Amstutz, P. and Plückthun, A. (2005). Engineering novel binding proteins from nonimmunoglobulin domains. *Nature Biotechnology, 23*(10):1257-1268. doi:*10.1038/nbt1127*

Blokzijl, A., Zieba, A., Hust, M., Schirrmann, T., Helmsing, S., Grannas, K., Hertz, E., Moren, A., Chen, L., Soderberg, O., Moustakas, A., Dübel, S. and Landegren, U. (2016). Single chain antibodies as tools to study TGF-β regulated SMAD proteins in proximity ligation-based pharmacological screens. *Molecular & Cellular Proteomics, 15*(6):1848-1856. doi:*10.1074/mcp.M115.055756*

Boersma, Y.L. (2018). Advances in the application of designed ankyrin repeat proteins (DARPins) as research tools and protein therapeutics. In: Udit A. (Eds) Protein Scaffolds. Methods in Molecular Biology, vol 1798, pp. 307-327. Humana Press, New York, NY. doi:*10.1007/978-1-4939-7893-9_23*

Borrebaeck, C.A.K. (2017). Precision diagnostics: moving towards protein biomarker signatures of clinical utility in cancer. *Nature Reviews Cancer, 3*:199-204. doi:*10.1038/nrc.2016.153*

Bradbury, A.R.M., Sidhu, S., Dübel, S. and McCafferty, J. (2011). Beyond natural antibodies: the power of *in vitro* display technologies. *Nature Biotechnology, 29*(3):245-254. doi:*10.1038/nbt.1791*

Bradbury, A. and Plückthun, A. (2015a). Reproducibility: Standardize antibodies used in research. *Nature, 518*(7537):27-29. doi:*10.1038/518027a*.

Bradbury, A.R.M. and Plückthun, A. (2015b). Getting to reproducible antibodies: the rationale for sequenced recombinant characterized reagents. *Protein Engineering, Design and Selection, 28*(10):303–305. doi:*10.1093/protein/gzv051*

Bradbury, A.M. and Plückthun, A. (2015c). Antibodies: Validate recombinants once. *Nature, 520*(7547):295. doi:*10.1038/520295b*

Bradbury, A.R.M., Trinklein, N., Thie, H., Wilkinson, I., Tandon, A., Anderson, S., Bladen, C., Jones, B., Aldred, S., Bestagno, M., Burrone, O., Maynard, J., Ferrara, F., Trimmer, J., Görnemann, J., Glanville, J., Wolf, P., Frenzel, A., Wong, J., Koh, X., Eng, H., Lane, D., Lefranc, M., Clark, M. and Dübel, S. (2018). When monoclonal antibodies are not monospecific: Hybridomas frequently express additional functional variable regions. *MAbs, 10*(4):539-546. doi:*10.1080/19420862.2018.1445456*

Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I. and Little, M. (1991). A surface expression vector for antibody screening. *Gene, 104*(2):147-153. doi:*10.1016/0378-1119(91)90244-6*

Breitling, F. and Dübel, S. (1999). Recombinant Antibodies. 161 pages. John Wiley and Sons, Inc., New York N.Y.

Briney, B., Inderbitzin, A., Joyce, C. and Burton, D.R. (2019). Commonality despite exceptional diversity in the baseline human antibody repertoire. *Nature, 566*(7744):393-397. doi:*10.1038/s41586-019-0879-y*

Buchholz, C.J., Friedel, T. and Buning H. (2015). Surface-engineered viral vectors for selective and cell type-specific gene delivery. *Trends in Biotechnology, 33*(12):777-790. doi:*10.1016/j.tibtech.2015.09.008*

Caravella, J. and Lugovskoy, A. (2010). Design of next-generation protein therapeutics. *Current Opinion in Chemical Biology, 14*(4):520-528. doi:*10.1016/j.cbpa.2010.06.175*

Cheung, W.C., Beausoleil, S., Zhang, X., Sato, S., Schieferl, S., Wieler, J., Beaudet, J., Ramenani, R., Popova, L., Comb, M., Rush, J. and Polakiewicz, R. (2012). A proteomics approach for the identification and cloning of monoclonal antibodies from serum. *Nature Biotechnology, 30*(5):447-452. doi:*10.1038/nbt.2167*

Cho, S.J., Woo, H.M., Kim, K.S., Oh, J-W. and Jeong, Y.J. (2011). Novel system for detecting SARS coronavirus nucleocapsid protein using an ssDNA aptamer. *Journal of Bioscience and Bioengineering, 112*(6):535-540. doi:*10.1016/j.jbiosc.2011.08.014*

Colwill, K., Renewable Protein Binder Working Group and Gräslund, S. (2011). A roadmap to generate renewable protein binders to the human proteome. *Nature Methods, 8*(7):551-558. doi:*10.1038/nmeth.1607*

Couchman, J.R. (2009). Commercial antibodies. The good, bad and really ugly. *Journal Histochemistry and Cytochemistry, 57*(1):7-8. doi:*10.1369/jhc.2008.952820*

Crepin, R., Veggiani, G., Djender, S., Beugnet, A., Planeix, F., Pichon, C., Moutel, S., Amigorena, S., Perez, F., Ghinea, N. and de Marco, A. (2017). Whole-cell biopanning with a synthetic phage display library of nanobodies enabled the recovery of follicle-stimulating hormone receptor inhibitors. *Biochemical and Biophysical Research Communications, 493*(4):1567-1572. doi:*10.1016/j.bbrc.2017.10.036*

Dreier, B. and Plückthun, A. (2012). Rapid selection of high-affinity binders using ribosome display. In: Douthwaite J., Jackson R. (Eds) Ribosome Display and Related Technologies. Methods in Molecular Biology (Methods and Protocols), vol 805, pp 261-268. Springer, New York, NY. doi:*10.1007/978-1-61779-379-0_15*

European Union (2010). Directive 2010/63/EU of the European Parliament and of the Council (22 September 2010) on the protection of animals used for scientific purposes. Official Journal of the European Union L276:33-79.

Ewers, H. (2018). Open-source recombinant monoclonal secondary nanobodies. *Journal of Cell Biology, 217*(3):809–811. doi:*10.1083/jcb.201802025*

Frenzel, A., Kügler, J., Helmsing, S., Meier, D., Schirrmann, T., Hust, M. and Dübel, S. (2017). Designing human antibodies by phage display. *Tranfusion Medicine and Hemotherapy, 44*(5):312-318. doi:*10.1159/000479633*

Friedel, T., Hanisch, L.J., Muth, A., Honegger, A., Abken, H., Plückthun, A., Buchholz, C.J. and Schneider, I.C. (2015). Receptor-targeted lentiviral vectors are exceptionally sensitive toward the biophysical properties of the displayed single-chain Fv. *Protein Engineering, Design & Selection, 28*(4):93-106. doi:*10.1093/protein/gzv005*

Friedrich, K., Hanauer, J.R., Prüfer, S., Münch, R.C., Volker, I., Filippis, C., Jost, C., Hanschmann, K.M., Cattaneo, R., Peng, K.W., Plückthun, A., Buchholz, C., Cichutek, K. and Mühlebach, M. (2013). DARPin-targeting of measles virus: unique bispecificity, effective oncolysis and enhanced safety. *Molecular Therapy*, *21*(4):849-59. doi:*10.1038/mt.2013.16*

Fuchs, P., Dübel, S., Breitling, F., Braunagel, M., Klewinghaus, I. and Little, M. (1992). Recombinant human monoclonal antibodies: Basic principles of the immune system transferred to *E. coli*. *Cell Biophysics, 21*(1-3):81-92. doi:*10.1007/BF02789480*

Gebauer, M. and Skerra, A. (2009). Engineered protein scaffolds as next-generation antibody therapeutics. *Current Opinion in Chemical Biology, 13*(3):245-55. doi:*10.1016/j.cbpa.2009.04.627*

Geyer, C.R., McCafferty, J., Dübel, S., Bradbury, A. and Sidhu, S. (2012). Recombinant antibodies and *in vitro* selection technologies. In: Proetzel G., Ebersbach H. (Eds) Antibody Methods and Protocols. Methods in Molecular Biology (Methods and Protocols), vol 901, pp 11-32. Humana Press, Totowa, NJ. doi:*10.1007/978-1-61779-931-0_2*

Gilbreth, R.N. and Koide, S. (2012). Structural insights for engineering binding proteins based on non-antibody scaffolds. *Current Opinion in Structural Biology, 22*(4):413-20. doi:*10.1016/j.sbi.2012.06.001*

Gloriam, D.E., Orchard, S., Bertinetti, D., Björling, E., Bongcam-Rudloff, E., Borrebaeck, C.A., Bourbeillon, J., Bradbury, A.R., de Daruvar, A., Dübel, S., Frank, R., Gibson, T.J., Gold, L., Haslam, N., Herberg, F.W., Hiltke, T., Hoheisel, J.D., Kerrien, S., Koegl, M., Konthur, Z., Korn, B., Landegren, U., Montecchi-Palazzi, L., Palcy, S., Rodriguez, H., Schweinsberg, S., Sievert, V., Stoevesandt, O., Taussig, M.J., Ueffing, M., Uhlén, M., van der Maarel, S., Wingren, C., Woollard, P., Sherman, D.J. and Hermjakob, H. (2010). A community standard format for the representation of protein affinity reagents. *Molecular & Cellular Proteomics, 9*(1):1-10. doi:*10.1074/mcp.M900185-MCP200*

Golden, A., Stevens, E.J., Yokobe, L., Faulx, D., Kalnoky, M., Peck, R., Valdez, M., Steel, C., Karabou, P., Banla, M., Soboslay, P., Adade, K., Tekle, A., Cama, V., Fischer, P., Nutman, T., Unnasch, T., de los Santos, T. and Domingo, G. (2016). A recombinant positive control for serology diagnostic tests supporting elimination of *Onchocerca volvulus*. *PLoS Neglected Tropical Diseases, 10*(1): e0004292. doi:*10.1371/journal.pntd.0004292*

Goodman, S. (2018). The antibody horror show: an introductory guide for the perplexed. *New Biotechnology, 45*:9-13. doi:*10.1016/j.nbt.2018.01.006*

Gray, A., Sidhu, S., Chandrasekera, P., Hendriksen, C. and Borrebaeck, C. (2016). Animal-friendly affinity reagents: Replacing the needless in the haystack. *Trends in Biotechnology, 34*(12):960-969. doi:*10.1016/j.tibtech.2016.05.017*

Hanauer, J.R., Gottschlich, L., Riehl, D., Rusch, T., Koch, V., Friedrich, K., Hutzler, S., Prüfer, S., Friedel, T., Hanschmann, K.M., Münch, R., Jost, C., Plückthun, A., Cichutek, K., Buchholz, C. and Mühlebach, MD. (2016). Enhanced lysis by bispecific oncolytic measles viruses simultaneously using HER2/neu or EpCAM as target receptors. *Molecular Therapy Oncolytics, 3*:16003. doi:*10.1038/mto.2016.3*

Hansen, S., Tremmel, D., Madhurantakam, C., Reichen, C., Mittl, P.R. and Plückthun, A. (2016). Structure and energetic contributions of a designed modular peptide-binding protein with picomolar affinity. *Journal of the American Chemical Society, 138*(10):3526-3532. doi:*10.1021/jacs.6b00099*

Hansen, S., Stüber, J.C., Ernst, P., Koch, A., Bojar, D., Batyuk, A. and Plückthun, A. (2017). Design and applications of a clamp for Green Fluorescent Protein with picomolar affinity. *Scientific Reports, 7*(1):16292. doi:*10.1038/s41598-017-15711-z*

Hansen, S., Ernst, P., König, S.L.B., Reichen, C., Ewald, C., Nettels, D., Mittl, P.R.E., Schuler, B. and Plückthun, A. (2018). Curvature of designed armadillo repeat proteins allows modular peptide binding. *Journal of Structural Biology, 201*(2):108-117. doi:*10.1016/j.jsb.2017.08.009*

Harth, S., Ten Haaf, A., Loew, C., Frisch, C., Knappik, A. (2018). Generation by phage display and characterisation of drug-target complex-specific antibodies for pharmacokinetic analysis of biotherapeutics. *MAbs, 11*(1):178-190. doi:*10.1080/19420862.2018.1538723*

Hartmann, J., Münch, R.C., Freiling, R.T., Schneider, I.C., Dreier, B., Samukange, W., Koch, J., Seeger, M.A., Plückthun, A. and Buchholz, C.J. (2018). A library-based screening strategy for the identification of DARPins as ligands for receptor-targeted AAV and lentiviral vectors. *Molecular Therapy Methods & Clinical Development, 10*:128-143. doi:*10.1016/j.omtm.2018.07.001*

Hattori, T., Taft, J.M., Swist, K.M., Luo, H., Witt, H., Slattery, M., Koide, A., Ruthenburg, A.J., Krajewski, K., Strahl, B.D., White, K.P., Farnham, P.J., Zhao, Y. and Koide, S. (2013). Recombinant antibodies to histone post-translational modifications. *Nature Methods, 10*(10):992-995. doi:*10.1038/nmeth.2605*

Haurum, J. (2006). Recombinant polyclonal antibodies-the next generation of antibody therapeutics. *Drug Discovery Today, 11*(13-14):655-660. doi:*10.1016/j.drudis.2006.05.009*

Heimburg-Molinaro, J. and Rittenhouse-Olson, K. (2009). Development and characterisation of antibodies to carbohydrate antigens. In: Packer N.H., Karlsson N.G. (Eds) Glycomics. Methods in Molecular Biology (Methods and Protocols), vol 534, pp 341–357. Humana Press, Totowa, NJ. doi:*10.1007/978-1-59745-022-5_24*

Helma, J., Cardoso, M.C., Muyldermans, S. and Leonhardt, H. (2015). Nanobodies and recombinant binders in cell biology. *Journal of Cell Biology, 209*(5):633-644. doi:*10.1083/jcb.201409074*

Hentrich, C., Ylera, F., Frisch, C., Ten Haaf, A. and Knappik, A. (2018). Monoclonal antibody generation by phage display. History, state-of-the-art and future; pp 47-80. In: S. Vashist, T. Luong (Eds) Handbook of Immunoassay Technologies: Approaches, Performances and Applications. Academic Press. doi:*10.1016/B978-0-12-811762-0.00003-7*

Hu, D., Tateno, H. and Hirabayashi, J. (2015). Lectin engineering, a molecular evolutionary approach to expanding the lectin utilities. *Molecules, 20*(5):7637-7656. doi:*10.3390/molecules20057637*

Hust, M. and Dübel, S. (2004). Mating antibody phage display with proteomics. *Trends in Biotechnology, 22*(1):8-14. doi:*10.1016/j.tibtech.2003.10.011*

Igawa, T., Tsunoda, H., Kuramochi, T., Sampei, Z., Ishii, S. and Hattori, K. (2011) Engineering the variable region of therapeutic IgG antibodies. *MAbs, 3*(3):243-52. doi:*10.4161/mabs.3.3.15234*

Jarutat, T., Frisch, C., Nickels, C., Merz, H. and Knappik, A. (2006). Isolation and comparative characterisation of Ki-67 equivalent antibodies from the HuCAL phage display library. *Biological Chemistry, 387*(7):995-1003. doi:*10.1515/BC.2006.123*

Jarutat, T., Nickels, C., Frisch, C., Stellmacher, F., Hofig, K.P., Knappik, A. and Merz, H. (2007). Selection of vimentin-specific antibodies from the HuCAL phage display library by subtractive panning on formalin-fixed, paraffin-embedded tissue. *Biological Chemistry, 388*(6):651-658. doi:*10.1515/BC.2007.070*

Jost, C. and Plückthun, A. (2014). Engineered proteins with desired specificity. DARPins, other alternative scaffolds and bispecific IgGs. *Current Opinion in Structural Biology, 27*:102-112. doi:*10.1016/j.sbi.2014.05.011*

June, C.H., O'Connor, R.S., Kawalekar, O.U., Ghassemi, S. and Milone, M.C. (2018). CAR T cell immunotherapy for human cancer. *Science, 359*(6382):1361-1365. doi:*10.1126/science.aar6711*

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wölle, J., Plückthun, A. and Virnekäs, B. (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomised with trinucleotides. *Journal of Molecular Biology, 296*(1):57-86. doi:*10.1006/jmbi.1999.3444*

Knappik, A., Capuano, F., Frisch, C., Ylera, F. and Bonelli, F. (2009). Development of recombinant human IgA for anticardiolipin antibodies assay standardisation. *Annals of the New York Academy of Sciences, 1173*: 190–198. doi:*10.1111/j.1749-6632.2009.04749.x*

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*:495–497. doi:*10.1038/256495a0*

Krebber, A., Bornhauser. S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R. and Plückthun, A. (1997). Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *Journal of Immunological Methods, 201*(1):35-55. doi:*10.1016/s0022-1759(96)00208-6*

Kuhn, P., Thiem, S., Steinert, M., Purvis, D., Lugmayr, V., Treutlein, U., Plobner, L., Leiser, R.M., Hust, M. and Dübel, S. (2017). Human anti-lipopolysaccharid (LPS) antibodies against Legionella with high species specificity. *Human Antibodies, 26*(1):29-38. doi:*10.3233/HAB-170318*

Kummer, L., Parizek, P., Rube, P., Millgramm, B., Prinz, A., Mittl, P.R., Kaufholz, M., Zimmermann, B., Herberg, F.W. and Plückthun, A. (2012). Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries. *Proceedings of the National Academy of Sciences of the United States of America, 109*(34):E2248-57. doi:*10.1073/pnas.1205399109*

Kummer, L., Hsu, C.W., Dagliyan, O., Macnevin, C., Kaufholz, M., Zimmermann, B., Dokholyan, N.V., Hahn, K.M. and Plückthun, A. (2013). Knowledge-based design of a biosensor to quantify localised ERK activation in living cells. *Chemistry & Biology, 20*(6):847-856. doi:*10.1016/j.chembiol.2013.04.016*

Lazzarotto, G., Maine, P., Dal Monte, A., Ripalti. and Landini, M. (1997). A novel Western blot test containing both viral and recombinant proteins for anticytomegalovirus immunoglobulin M detection. *Journal of Clinical Microbiology, 35*:393-397.

Leutert, M., Menzel, S., Braren, R., Rissiek, B., Hopp, A.K., Nowak, K., Bisceglie, L., Gehrig, P., Li, H., Zolkiewska, A., Koch-Nolte, F. and Hottiger, M.O. (2018). Proteomic characterisation of the heart and skeletal muscle reveals widespread arginine ADP-ribosylation by the ARTC1 ectoenzyme. *Cell Reports, 24*(7):1916-1929. e5. doi:*10.1016/j.celrep.2018.07.048*

Liu, H., Saxena, A., Sidhu, S. and Wu, D. (2017). Fc engineering for developing therapeutic bispecific antibodies and novel scaffolds. *Frontiers in Immunology, 8*:38. doi:*10.3389/fimmu.2017.00038*

Lloyd, C., Lowe, D., Edwards, B., Welsh, F., Dilks, T., Hardman, C. and Vaughan, T. (2009). Modelling the human immune response: performance of a 1011 human antibody repertoire against a broad panel of therapeutically relevant antigens. *Protein Engineering, Design and Selection, 22*(3):159–168. doi:*10.1093/protein/gzn058*

Luginbühl, B., Kanyo, Z., Jones, R.M., Fletterick, R.J., Prusiner, S.B., Cohen, F.E., Williamson, R.A., Burton, D.R. and Plückthun, A. (2006). Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation. *Journal of Molecular Biology, 363*(1):75-97. doi:*10.1016/j.jmb.2006.07.027*

Lukinavičius, G., Lavogina, D., Gönczy, P. and Johnsson, K. (2013). Commercial Cdk1 antibodies recognise the centrosomal protein Cep152. *Biotechniques, 55*(3), 111-114. doi:*10.2144/000114074*

Marschall, A.L.J., Dübel, S., Böldicke, T. (2015) Specific knockdown of protein function by intrabodies. *MAbs, 7*(6):1010-1035. doi:*10.1080/19420862.2015.1076601*

Martin, M., García-Hernández, M., García-Recio, E., Gómez-Chacón, G., Sánchez-López, M. and González, V. (2013). DNA aptamers selectively target Leishmania infantum H2A protein. *PLoS One, 8*(10): e78886. doi:*10.1371/journal.pone.0078886*

McMahon, C., Baier, A.S., Pascolutti, R., Wegrecki, M., Zheng, S., Ong, J.X., Erlandson, S.C., Hilger, D., Rasmussen, S.G.F., Ring, A.M., Manglik, A. and Kruse, A.C. (2018). Yeast surface display platform for rapid discovery of conformationally selective nanobodies. *Nature Structural and Molecular Biology, 25*(3):289-296. doi:*10.1038/s41594-018-0028-6*

Mellby, L., Nyberg, A., Johansen, J., Wingren, C., Nordestgaard, B., Bojesen, S., Mitchell, B., Sheppard, B., Sears, R. and Borrebaeck, C. (2018). Serum biomarker signature-based liquid biopsy for diagnosis of early-stage pancreatic cancer. *Journal of Clinical Oncology, 36*(28):2887-2894. doi:*10.1200/JCO.2017.77.6658*

Michel, M.C., Wieland, T. and Tsujimoto, G. (2009). How reliable are G-protein-coupled receptor antibodies? *Naunyn-Schmiedeberg's Archives of Pharmacology, 379*(4):385-8. doi:*10.1007/s00210-009-0395-y*

Mondon, P., Dubreuil, O., Bouayadi, K. and Kharrat, H. (2008). Human antibody libraries: a race to engineer and explore a larger diversity. *Frontiers in Bioscience, 13*:1117-29. doi:*10.2741/2749*

Moutel, S., El Marjou, A., Vielemeyer, O., Nizak, C., Benaroch, P., Dübel, S. and Perez, F. (2009). A multi-fc species system for recombinant antibody production. *BioMedCentral Biotechnology, 9*:14. doi:*10.1186/1472-6750-9-14*

Moutel, S., Bery, N., Bernard, V., Keller, L., Lemesre, E., de Marco, A., Ligat, L., Rain, J.C., Favre, G., Olichon, A. and Perez, F. (2016). NaLi-H1: A universal synthetic library of humanised nanobodies providing highly functional antibodies and intrabodies. *eLife, 5*:e16228. doi:*10.7554/eLife.16228*

Münch, R.C., Mühlebach, M.D., Schaser, T., Kneissl, S., Jost, C., Plückthun, A., Cichutek, K. and Buchholz, C.J. (2011). DARPins: an efficient targeting domain for lentiviral vectors. *Molecular Therapy, 19*(4):686-693. doi:*10.1038/mt.2010.298*

Münch, R.C., Muth, A., Muik, A., Friedel, T., Schmatz, J., Dreier, B., Trkola, A., Plückthun, A., Buning, H. and Buchholz, C.J. (2015). Off-target-free gene delivery by affinity-purified receptor-targeted viral vectors. *Nature Communications, 6*:6246. doi:*10.1038/ncomms7246*

Muyldermans, S. (2001). Single domain camel antibodies: current status. *Reviews in Molecular Biotechnology, 74*(4):277-302. doi:*10.1016/s1389-0352(01)00021-6*

Nelson, P.N., Reynolds, G.M., Waldron, E.E., Ward, E., Giannopoulos, K. and Murray, P.G. (2000). Demystified... Monoclonal antibodies. *Molecular Pathology, 53*(3): 111–117. doi:*10.1136/mp.53.3.111*

Ohara, R., Knappik, A., Shimada, K., Frisch, C., Ylera, F. and Koga, H. (2006). Antibodies for proteomic research. Comparison of traditional immunisation with recombinant antibody technology. *Proteomics, 6*(9):2638-2646. doi:*10.1002/pmic.200500579*

Ooe, H., Iguchi-Ariga, S.M. and Ariga, H. (2006). Establishment of specific antibodies that recognise C106-oxidised DJ-1. *Neuroscience Letters, 404*(1-2):166-169. doi:*10.1016/j.neulet.2006.05.031*

Petrenko, V.A. and Smith, G.P. (2000). Phages from landscape libraries as substitute antibodies. *Protein Engineering, 13*(8):589–592. doi:*10.1093/protein/13.8.589*

Pleiner, T., Bates, M., Trakhanov, S., Lee, C.T., Schliep, J.E., Chug, H., Böhning, M., Stark, H., Urlaub, H. and Görlich, D. (2015). Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. *Elife, 4*, e11349. doi:*10.7554/eLife.11349*

Pleiner, T., Bates, M. and Görlich, D. (2018). A toolbox of anti-mouse and anti-rabbit IgG secondary nanobodies. *Journal of Cell Biology, 217*(3):1143-1154. doi:*10.1083/jcb.201709115*

Plückthun, A. and Pack, P. (1997). New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology, 3*(2):83-105. doi:*10.1016/s1380-2933(97)00067-5*

Plückthun, A. (2015). Designed Ankyrin Repeat Proteins (DARPins): Binding proteins for research, diagnostics and therapy. *Annual Review of Pharmacology and Toxicology, 55*:489-511. doi:*10.1146/annurev-pharmtox-010611-134654*

Pulli, T., Hoyhtya, M., Soderlund, H. and Takkinen, K. (2005). One-step homogeneous immunoassay for small analytes. *Analytical Chemistry, 77*(8):2637-2642. doi:*10.1021/ac048379l*

Ramos, E., Piñeiro, D., Soto, M., Abanades, D., Martín, M., Salinas, M. and González, V.M. (2007). A DNA aptamer population specifically detects Leishmania infantum H2A antigen. *Laboratory Investigation, 87*(5):409-416. doi:*10.1038/labinvest.3700535*

Ramos, E., Moreno, M., Martín, M., Soto, M. and Gonzalez, V. (2010). *In vitro* selection of *Leishmania infantum* H3-binding ssDNA aptamers. *Oligonucleotides* 20:207-213. doi:*10.1089/oli.2010.0240*.

Reddington, S.C., and Howarth, M. (2015). Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher. *Current Opinion in Chemical Biology, 29*:94-99. doi:*10.1016/j.cbpa.2015.10.002*

Reichen, C., Hansen, S. and Plückthun, A. (2014). Modular peptide binding: From a comparison of natural binders to designed armadillo repeat proteins. *Journal of Structural Biology, 185*(2):147-62. doi:*10.1016/j.jsb.2013.07.012*

Riaño-Umbarila, L., Juárez-González, V.R., Olamendi-Portugal, T., Ortíz-León, M., Possani, L.D. and Becerril, B. (2005). A strategy for the generation of specific human antibodies by directed evolution and phage display. An example of a single-chain antibody fragment that neutralises a major component of scorpion venom. *The FEBS Journal, 272*(10):2591-2601. doi:*10.1111/j.1742-4658.2005.04687.x*

Ridder, R., Schmitz, R., Legay, F. and Gram, H. (1995). Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast Pichia pastoris. *Biotechnology* (NY), *13*(3):255-260. doi:*10.1038/nbt0395-255*

Roskos, L., Klakamp, S., Liang, M., Arends, R. and Green, L. (2007). Molecular Engineering II: Antibody Affinity (Chapter 7, pp 145-168). In: Handbook of Therapeutic Antibodies. Dübel, S. (Ed). WILEY-VCH Verlag GmbH & Co. KGaA.

Rothe, C. and Skerra, A. (2018). Anticalin((R)) proteins as therapeutic agents in human diseases. *BioDrugs, 32*(3):233-243. doi: *10.1007/s40259-018-0278-1*

Russo, G., Theisen, U., Fahr, W., Helmsing, S., Hust, M., Köster, R. and Dübel, S. (2018). Sequence defined antibodies improve the detection of Cadherin 2 (N-Cadherin) during zebrafish development. *New Biotechnology, 45*:98-112. doi:*10.1016/j.nbt.2017.12.008*

Säll, A., Walle, M., Wingren, C., Müller, S., Nyman, T., Vala, A., Ohlin, M., Borrebaeck, C.A.K. and Persson, H. (2016). Generation and analyses of human synthetic antibody libraries and their application for protein microarrays. *Protein Engineering, Design and Selection, 29*(10): 427-437. doi: *10.1093/protein/gzw042*

Schilling, J., Schöppe, J. and Plückthun, A. (2014). From DARPins to LoopDARPins: novel LoopDARPin design allows the selection of low picomolar binders in a single round of ribosome display. *Journal of Molecular Biology ,426*(3):691-721. doi:*10.1016/j.jmb.2013.10.026*

Schirrmann, T., Meyer, T., Schütte, M., Frenzel, A. and Hust, M. (2011). Phage display for the generation of antibodies for proteome research, diagnostics and therapy. *Molecules, 16*(1):412-426. doi:*10.3390/molecules16010412*

Schmid, M., Ernst, P., Honegger, A., Suomalainen, M., Zimmermann, M., Braun, L., Stauffer, S., Thom, C., Dreier, B., Eibauer, M., Kipar, A., Vogel, V., Greber, U., Medalia, O. and Plückthun, A. (2018). Adenoviral vector with shield and adapter increases tumor specificity and escapes liver and immune control. *Nature communications, 9*:450. doi:*10.1038/s41467-017-02707-6*

Schmohl, L. and Schwarzer, D. (2014). Sortase-mediated ligations for the site-specific modification of proteins. *Current Opinion in Chemical Biology, 22*:122-8. doi:*10.1016/j.cbpa.2014.09.020*

Schütz, M., Batyuk, A., Klenk, C., Kummer, L., de Picciotto, S., Gulbakan, B., Wu, Y., Newby, G.A., Zosel, F., Schöppe, J., Sedlák, E., Mittl, P., Zenobi, R., Wittrup, K. and Plückthun, A. (2016). Generation of fluorogen-activating designed ankyrin repeat proteins (FADAs) as versatile sensor tools. *Journal of Molecular Biology, 428*(6):1272-1289. doi:*10.1016/j.jmb.2016.01.017*

Schwill, M., Tamaskovic, R., Gajadhar, A.S., Kast, F., White, F.M. and Plückthun, A. (2019). Systemic analysis of tyrosine kinase signaling reveals a common adaptive response program in a HER2-positive breast cancer. *Science signaling, 12*(565):eaau2875. doi:*10.1126/scisignal.aau2875*

Shin, S., Kim, I, Kang, W., Yang, J. and Hah, S. (2010). An alternative to Western blot analysis using RNA aptamer-functionalised quantum dots. *Bioorganic & Medicinal Chemistry Letters, 20*(11):3322-3325. doi:*10.1016/j.bmcl.2010.04.040*

Shiryaev, S.A., Radichev, I.A., Ratnikov, B.I., Aleshin, A.E., Gawlik, K., Stec, B., Frisch, C., Knappik, A. and Strongin, A.Y. (2010). Isolation and characterisation of selective and potent human Fab inhibitors directed to the active-site region of the two-component NS2B-NS3 proteinase of West Nile virus. *The Biochemical Journal, 427*(3):369-376. doi:*10.1042/BJ20100074*

Simeon, R. and Chen, Z. (2018). *In vitro*-engineered non-antibody protein therapeutics. *Protein & Cell, 9*:3-14. doi:*10.1007/s13238-017-0386-6*

Skerra, A. (2007). Alternative non-antibody scaffolds for molecular recognition. *Current Opinion in Biotechnology, 18*(4):295-304. doi:*10.1016/j.copbio.2007.04.010*

Škrlec, K., Štrukelj, B. and Berlec, A. (2015). Non-immunoglobulin scaffolds: a focus on their targets. *Trends in Biotechnology, 33*(7):408-18. doi:*10.1016/j.tibtech.2015.03.012*

Sommavilla, R., Lovato, V., Villa, A., Sgier, D. and Neri, D. (2010). Design and construction of a naïve mouse antibody phage display library. *Journal of Immunological Methods, 353*(1-2):31-43. doi:*10.1016/j.jim.2010.01.003*

Steiner, D., Forrer, P. and Plückthun, A. (2008). Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *Journal of Molecular Biology, 382*(5):1211-27. doi:*10.1016/j.jmb.2008.07.085*

Steinhauer, C., Wingren, C., Khan, F., He, M., Taussig, M.J. and Borrebaeck, C.A. (2006). Improved affinity coupling for antibody microarrays. Engineering of double-(His)6-tagged single framework recombinant antibody fragments. *Proteomics, 6*(15):4227–4234. doi:*10.1002/pmic.200600036*

Tamaskovic, R., Simon, M., Stefan, N., Schwill, M. and Plückthun, A. (2012). Designed ankyrin repeat proteins (DARPins) from research to therapy. *Methods in Enzymology, 503*:101-134. doi:*10.1016/B978-0-12-396962-0.00005-7*

Tamaskovic, R., Schwill, M., Nagy-Davidescu, G., Jost, C., Schaefer, D.C., Verdurmen, W.P., Schaefer, J.V., Honegger, A. and Plückthun, A. (2016). Intermolecular biparatopic trapping of ErbB2 prevents compensatory activation of PI3K/AKT via RAS-p110 crosstalk. *Nature Communications, 7*:11672. doi:*10.1038/ncomms11672*

Ten Haaf, A., Gattenlöhner, S. and Tur, M.K. (2018). Antibody selection on FFPE tissue slides. In: Hust M., Lim T. (Eds) Phage Display. Methods in Molecular Biology, vol 1701, pp 381-391. Humana Press, New York, NY. doi:*10.1007/978-1-4939-7447-4_21*

Teng, G. and Papavasiliou, F.N. (2007). Immunoglobulin somatic hypermutation. *Annual Review of Genetics, 41*:107-120. doi: *10.1146/annurev.genet.41.110306.130340*

Theurillat, J.P., Dreier, B., Nagy-Davidescu, G., Seifert, B., Behnke, S., Zurrer-Hardi, U., Ingold, F., Plückthun, A. and Moch, H. (2010). Designed ankyrin repeat proteins: a novel tool for testing epidermal growth factor receptor 2 expression in breast cancer. *Modern Pathology, 23*(9):1289-1297. doi:*10.1038/modpathol.2010.103*

Tiller, T., Schuster, I., Deppe, D., Siegers, K., Strohner, R., Herrmann, T., Berenguer, M., Poujol, D., Stehle, J., Stark, Y., Heßling, M., Daubert, D., Felderer, K., Kaden, S., Kölln, J., Enzelberger, M. and Urlinger, S. (2013). A fully synthetic human Fab antibody library based on fixed VH/VL framework pairings with favorable biophysical properties. *MAbs, 5*(3):445-70. doi:*10.4161/mabs.24218*

Uhlen, M., Bandrowski, A., Carr, S., Edwards, A., Ellenberg, J., Lundberg, E., Rimm, D., Rodriguez, H., Hiltke, T., Snyder, M. and Yamamoto, T. (2016). A proposal for validation of antibodies. *Nature Methods, 13*(10): 823–827. doi:*10.1038/nmeth.3995*

Vincent, K.J. and Zurini, M. (2012). Current strategies in antibody engineering: Fc engineering and pH-dependent antigen binding, bispecific antibodies and antibody drug conjugates. *Biotechnology Journal, 7*(12):1444-1450. doi:*10.1002/biot.201200250*

Webber, J., Stone, T., Katilius, E., Smith, B., Gordon, B., Mason, M., Tabi, Z., Brewis, I. and Clayton, A. (2014). Proteomics analysis of cancer exosomes using a novel modified aptamer-based array (SOMAscan™) platform. *Molecular & Cellular Proteomics, 13*(4):1050-64. doi:*10.1074/mcp.M113.032136*

Weidle, U.H., Auer, J., Brinkmann, U., Georges, G. and Tiefenthaler, G. (2013). The emerging role of new protein scaffold-based agents for treatment of cancer. *Cancer Genomics Proteomics, 10*(4):155-168

Weiss, E. and Orfanoudakis, G. (1994). Application of an alkaline phosphatase fusion protein system suitable for efficient screening and production of Fab-enzyme conjugates in *Escherichia coli*. *Journal of Biotechnology, 33*(1):43-53. doi:*10.1016/0168-1656(94)90097-3*

Whiteaker, J.R., Zhao, L., Frisch, C., Ylera, F., Harth, S., Knappik, A. and Paulovich, A.G. (2014). High-affinity recombinant antibody fragments (Fabs) can be applied in peptide enrichment immuno-MRM assays. *Journal of Proteome Research, 13*(4):2187-2196. doi:*10.1021/pr4009404*

Wörn, A. and Plückthun, A. (2001). Stability engineering of antibody single-chain Fv fragments. *Journal of Molecular Biology, 305*(5):989-1010. doi:*10.1006/jmbi.2000.4265*

Wu, Y., Batyuk, A., Honegger, A., Brandl, F., Mittl, P.R.E. and Plückthun, A. (2017). Rigidly connected multispecific artificial binders with adjustable geometries. *Scientific Reports, 7*(1):11217. doi:*10.1038/s41598-017-11472-x*

Wu, Y., Honegger, A., Batyuk, A., Mittl, P.R.E. and Plückthun, A. (2018). Structural basis for the selective inhibition of c-Jun N-terminal kinase 1 determined by rigid DARPin-DARPin fusions. *Journal of Molecular Biology, 430*(14):2128-2138. doi:*10.1016/j.jmb.2017.10.032*

Yan, J., Li, G., Hu, Y., Ou, W. and Wan, Y. (2014). Construction of a synthetic phage-displayed Nanobody library with CDR3 regions randomised by trinucleotide cassettes for diagnostic applications. *Journal of Translational Medicine, 12*:343. doi:*10.1186/s12967-014-0343-6*

Yu, X., Yang, Y.P., Dikici, E., Deo, S.K. and Daunert, S. (2017). Beyond antibodies as binding partners. The role of antibody mimetics in bioanalysis. *Annual Review of Analytical Chemistry, 10*(1):293–320. doi:*10.1146/annurev-anchem-061516-045205*

Zahnd, C., Sarkar, C.A. and Plückthun, A. (2010). Computational analysis of off-rate selection experiments to optimise affinity maturation by directed evolution. *Protein Engineering, Design and Selection, 23*(4):175-184. doi:.*10.1093/protein/gzp087*

11. List of Abbreviations

CAR-T cells	Chimeric antigen receptor T cells
ChIP	Chromatin immunoprecipitation
DARPins	Designed Ankyrin Repeat Proteins
DNA	Deoxyribonucleic acid
dsFv	Disulfide-bond stabilised Fv
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESAC	EURL ECVAM Scientific Advisory Committee
EU	European Union
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
Fab	Fragment antigen-binding
Fab-A	Fab fused with alkaline phosphatase
FACS	Fluorescence-activated cell sorting
Fc	Fragment constant
FFPE tissue	Formalin-fixed paraffin-embedded tissue
FRET	Fluorescence resonance energy transfer
Fv	Fragment variable
GFP	Green fluorescent protein
HER2	Human epidermal growth factor receptor 2
Ig	Immunoglobulin
IgG	Immunoglobulin G
IHC	Immunohistochemistry
MFI	Mean fluorescence intensity
MRM	Multiple reaction monitoring
nM	Nanomolar
PCR	Polymerase chain reaction
pM	Picomolar
scFv	Single-chain fragment variable
scFv-Fc	Single-chain fragment variable dimerised by the fragment constant (Fc) domain
SPR	Surface plasmon resonance
VH	Variable heavy
VHH	Single variable heavy domain (heavy chain only antibody from camelids)
vNAR	Variable new antigen receptor

12. Appendix

Case studies

I Non-animal-derived antibodies in clinical use

The following link summarises phage display-derived antibodies that are approved for therapy or in clinical development. The vast majority being non-animal-derived (synthetic, semi synthetic or non-immunised/naïve): *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5058633/table/t0001/?report=objectonly*. A selection of these antibodies is described in more detail in the corresponding review (Frenzel *et al.*, 2016), demonstrating different aspects of the phage display technology and its development over the last 25 years. For further information, also see *https://www.antibodysociety.org/* and Nelson *et al.*, 2010.

II Use of antibodies in regulatory procedures: The human Cell Line Activation Test (h-CLAT; OECD TG442E)

Skin sensitisation is the regulatory endpoint aiming at the identification of chemicals able to elicit an allergic response in susceptible individuals. Following repeated exposure to a sensitising agent, the adverse health effect of allergic contact dermatitis (ACD) may be provoked. Thus, the development of ACD is characterised by two distinct phases: the induction of specialised immunological memory following the initial exposure to an allergen, called sensitisation, and elicitation of the clinical allergic response following subsequent exposure to the allergen. Regulatory adopted animal-based tests, which are part of Council Regulation No 440/2008 (EU, 2008), include: Local lymph node assay, LLNA (OECD TG 429; OECD, 2010a), and its non-radioactive modifications, LLNA-DA (OECD TG 442A; OECD, 2010b) and LLNA-BrdU Elisa (OECD TG 442B; OECD, 2018a); Guinea pig maximisation test, GPMT, by Magnusson and Kligman (OECD TG 406; OECD, 1992); and Buehler occluded patch test in the guinea pig (OECD TG 406; OECD, 1992).

The human Cell Line Activation Test (h-CLAT) is cited as an alternative to animal-based test methods for the measure of skin sensitisation. The h-CLAT measures the expression of the CD86 and CD54 membrane markers in THP-1 cells, a human monocytic leukaemia cell line used as a surrogate model for dendritic cells. The expression levels of the markers are measured by flow cytometry following 24 hours of exposure to eight serial concentrations of test chemical selected on the basis of a pre-determined CV75, i.e. the concentration of test chemical that allows 75% of cell survival). The test method is designed to discriminate between sensitising and non-sensitising chemicals whereby chemicals are classified as sensitisers if the relative fluorescence intensity (RFI) of either CD86 and/or CD54 exceeds a defined threshold, i.e. RFI CD86≥150 and RFI CD54≥200, compared to the vehicle control wells at any tested concentration, in at least two out of three independent measurements. The h-CLAT is expected to be used in conjunction with other methods to be able to replace the current animal tests. Such complimentary information may come from other testing methods that address other key events involved in skin sensitisation. Examples of such alternative methods might include the Direct Peptide Reactivity Assay (DPRA-OECD TG 442C; OECD, 2015) and/or the Keratinosens assay (OECD TG 442D; OECD, 2018b).

However, whilst classed as an animal replacement method, the h-CLAT has been developed using several animal components including foetal calf serum and animal-derived antibodies. XCellR8 is a UK-based, GLP-accredited laboratory, specialising in animal-free safety and efficacy tests for the cosmetics, personal care and chemical industries. In response to direct requests from companies in the cosmetics and personal care sectors, XCellR8 adapted the h-CLAT method by replacing the animal components 10% FBS/FCS and the mouse anti human CD54 and CD86 monoclonal antibodies with 10% Human Serum and custom nonanimal-derived anti-CD54 and anti-CD86 antibodies produced by Bio-Rad using the HuCAL® technology (Human Combinatorial Antibody Library). To assess the performance of the HuCAL antibodies, quality control ELISA and dissociation rate assessment (k_{off} ranking) were used as selection criteria.

XCellR8 sought to obtain regulatory acceptance of the adapted method by submitting a proposal to the OECD Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) detailing the inclusion of an adapted h-CLAT method into OECD TG 442E (OECD, 2018c) which would allow global use of a novel animal-free integrated approach to testing and assessment for skin sensitisation. The project proposal was accepted by the WNT at its annual meeting in April 2018.

XCellR8 demonstrated the satisfactory performance of the adapted h-CLAT method in passing the acceptance criteria as specified in OECD TG 442E (OECD, 2018c), where: 9/10 CV75 values were within range; 10/10 substances were correctly classified as sensitisers or non-sensitisers, and 8/10 substances met the EC150 and EC200 ranges specified and now awaits a decision regarding the regulatory acceptance of the adapted h-CLAT test method.

XCellR8s OECD proposal aims to replace animal products with human reagents to achieve xeno-free culture conditions and to provide the cosmetics industry, including its supply chain, with truly animal-product-free versions of the *in vitro* test methods for skin sensitisation, while maximising the scientific relevance of the methods for human safety. Further to this line of reasoning, is the significance of replacing the animal-derived antibodies with higher scientific quality, sequence defined antibodies, as described throughout this report, to make available a more scientifically reliable regulatory test method and instil a standard, which should be adopted in the future development of validated alternative test methods. Although in this case, the test method is subject to proficiency testing, the specificity of the original antibodies is undetermined and the sequence of the antibodies is undefined. If the hybridoma is lost in the future, the test method will be compromised. Whilst the adapted h-CLAT test method serves as a good example of the replacement of animal-derived antibodies by phage display antibodies in a regulatory test method, it is hoped that the extensive assessment, review and acceptance process can be avoided in the future by adding the incorporation non-animal-derived antibody as a pre-requisite in future test method developments.

III European Union (EU) / US National Institute of Health (NIH) projects demonstrating the feasibility of generating large numbers of antibodies

A major objective of the post-genome era is to detect, quantify and characterise all relevant human proteins in tissues and fluids in health and disease. Substantial funding has been committed to pan-European and US based initiatives to generate sets of well characterised and harmonised renewable binding reagents (including non-animal-derived antibodies and scaffolds) for the human proteome. Renewable binding reagents in this context refer to reagents that are recombinantly produced from a known sequence. The majority of these clones are not yet used today due to a lack of follow up studies for individual characterisation.

III.a The Renewable Protein Binder Working Group

A forerunner of AFFINOMICS, this proof of concept exercise, part-organised by The Structural Genomics consortium, *https://www.thesgc.org/*, was set up to raise binders in a coordinated fashion against 20 individual Src Homology 2 (SH2) domains. Eleven international collaborating groups showed that it was possible to move quickly to a productive outcome. Over 500 renewable binders of different types were made (165 monoclonal antibodies, 340 unique recombinant single-chain fragments variable (scFV), which were confirmed to work in ELISA, Western blot, immunohistochemistry (IHC) and on microarrays of folded SH2 domains or protein fragments (PrESTs). The binders were produced in an impressively short space of time (3 months), 25% had better than 10 nM affinities and some could discriminate very closely related targets. This project was important in demonstrating that a binder-raising project, although on a relatively small scale in regard to target number, could be carried out efficiently (Colwill *et al.*, 2011; Mersmann *et al.*, 2010).

III.b Proteome binders - A European Infrastructure of Ligand Binding Molecules Against the Human Proteome (1 March 2006 - 31 May 2010): *https://cordis.europa.eu/project/rcn/78471/factsheet/en*

Co-oordinated by Babraham Bioscience Technologies, this project integrated existing infrastructures, reviewed technologies and high throughput production methods, standardised binder-based tools and applications, assembled bioinformatics and established a database schema for a central binders repository. The project comprised the planning phase for the coordinated systematic development, production, resource management and quality control of a European resource infrastructure of characterised and standardised ligand-binding reagents under the 7th Framework Programme of the European Commission. These included antibodies (animal-derived and non-animal-derived antibodies), novel protein scaffolds and nucleic acid aptamers, directed against the entire human proteome (Taussig *et al.*, 2007).

III.c Affinity Proteome - Advanced affinity tools and technologies for high throughput studies of the human proteome (1 March 2009 - 29 February 2012): *https://cordis.europa.eu/project/rcn/90292/reporting/en*

Following on from the planning phase, this project focused on production of recombinant binders (antibody fragments, Designed Ankyrin Repeat Proteins [DARPins], aptamers) and advanced detection methodologies (microarrays, proximity ligation, intrabodies) for analysis of proteins in two critical signal transduction pathways, namely the mitogen-activated protein (MAP) kinase and transforming growth factor beta (TGF-beta) pathways. Specifically, the objectives were to define and produce targets, by recombinant expression in bacteria or peptide synthesis. Then, to select the binders and establish methods for their characterisation and adaptation to application systems. Since the pathways investigated are frequently disturbed in major diseases, the availability of the binders generated in the project intended to be of benefit in medical research and development of new diagnostic assays. The project addressed issues of throughput, sensitivity, cost, validation and quality control, to develop technical solutions to a level where they could be exploited as commercial products. Some of the non-animal-derived antibodies made in this consortium are commercially available from Abcalis (*https://abcalis.com/*), with plans to add more in the future.

III.d Affinomics - Protein Binders for Characterisation of Human Proteome Function: Generation, Validation, Application (1 April 2010 - 31 March 2015): *https://cordis.europa.eu/project/rcn/94419/reporting/en*

Affinomics initiated the generation of a proteome-wide binder collection and to this end the consortium integrates the expertise and technologies available in 19 leading European centres and one small and medium-sized enterprise (SME) in order to create an efficient pipeline for target and binder production validation and quality control. The pipeline included traditional polyclonal and monoclonal antibodies, but was strongly geared toward phage display and ribosome display for recombinant binder formats to increase throughput and reduce cost. The biomedical focus was on the characterisation of 5 classes of proteins involved in signal transduction pathways in normal and cancer cells and consisted of 1429 target proteins, 2900 antigens and 5595 binders to kinases, phosphatases, SH2 domains, proteins mutated in cancer and plasma biomarkers. Some of the non-animal-derived antibodies made in this consortium are commercially available from Abcalis (*https://abcalis.com/*), with plans to add more in the future.

III.e The US NIH Common Fund Protein Capture Reagents programme RM-10-017 / RFA RM-10: *https://commonfund.nih.gov/proteincapture*

The goal of the US Common Fund's Protein Capture Reagents program is to develop a community resource of renewable, high-quality protein capture reagents, with a focus on the creation of transcription factor reagents and testing next generation capture technologies. The ultimate aim is to understand the critical role the multitude of cellular proteins play in development, health and disease. These resources will support a wide-range of research and clinical applications by enabling the isolation and tracking of proteins of interest. Its database at *https://proteincapture.org* lists 350 entries for highly validated recombinant antibodies. These are produced by the Recombinant Antibody Network (RAN), which is a consortium of three expert centres at the University of Chicago, University of California San Francisco (UCSF) and the University of Toronto (*http://recombinant-antibodies.org*). A further 1172 animal-derived monoclonal antibodies are contributed by The Johns Hopkins University (JHU) and CDI Laboratories, Inc. (CDI). Therefore, the focus on recombinant antibody production is relatively small (Blackshaw *et al.*, 2016).

III.f The Antibody Factory (2004-2006)

Based at The Technical University of Braunschweig, Germany, this Federal Ministry of Education and Research (BMBF) funded pilot initiative produced and commercialised user-friendly, cost-efficient "design antibodies" using phage display from non-animal-derived sources to produce 461 recombinant monoclonals (scFv) from naïve libraries to 204 antigens. The final report is available in German: *https://edocs.tib.eu/files/e01fb08/585656304l.pdf*.

III.g ATLAS of protein expression group at the Sanger Institute (2002-2007)

Funded by the Wellcome Trust, this group constructed a high quality phage display library containing over 10^{10} human antibodies and used it to generate, select, screen and sequence over 38,000 recombinant antibodies to 292 antigens, yielding over 7,200 unique clones. As many as 4,400 antibodies were characterised by specificity testing and detailed sequence analysis and the data/clones were made available online and to buy from Geneservice in Cambridge, UK, now Source Bioscience (Schofield *et al.*, 2007). Full details of the ATLAS scFV Library, (Source Species: mouse or human; vector: pSANG14-3F; host Species: *Escherichia coli*; Host Strain: TG1) including a database listing 2400 clones from 135 unique targets, can be found at *https://www.sourcebioscience.com/products/life-sciences-research/clones/artificial-antibody-libraries/atlas-scfv-library/*.

Project	Lab	Binder format	no. binders	Targets
Affinomics	Braunschweig	scFv-Fc	898	191
Affinomics	Lund	scFv	1068	206
Affinomics	Zürich	DARPin	185	185
Affinity Proteome	Braunschweig	scFv	122	40
Affinity Proteome	Zürich	DARPin	8	8
Sanger	Sanger	scFv	7236	292
Antibody Factory	Braunschweig	scFv	461	204
SGC-Pilot	SH2	scFv/Fab	340	20
TOTAL			10318	1146

Table I: Summary of various international consortia supporting large scale production and characterisation of non-animal-derived antibodies; scFv = Single-chain fragment variable; scFv-Fc = scFv dimerised by the fragment constant (Fc) domain; DARPin = designed ankyrin repeat proteins; Fab = fragment-antigen binding.

IV Data supporting Section 5.5 of the main text: Validation of large data sets exemplifying the performance of non-animal-derived antibodies in different applications and from various sources

IV.a Example 1: From Collwill *et al.,* (2011) and Mersmann *et al.,* (2010)

The following data were generated as part of an unfunded pilot study organised by the Structural Genomics Consortium (SGC). In the pilot study, 20 SH2 domain proteins were selected as antigens due to their broad interest to the scientific community. They are also a challenging test set, owing to their high degree of sequence and structural similarities. Antigens were distributed to researchers in five laboratories for antibody generation. One group generated monoclonal antibodies by using hybridoma technology. The other groups generated recombinant fragment antigen-binding (Fab) or scFv reagents using phage display. Antibody candidates were analysed by enzyme-linked immunosorbent assay (ELISA) to determine the selectivity of binding for the cognate SH2 domain over other SH2 domains and scored as positive if the signal for their cognate targets was ten times above the off-target background. A subset of the ELISA data is shown in the following figure (Figure I).

Figure I: (Reprinted with permission from Mersmann *et al.*, 2010, *New Biotechnology* 27, p. 122, © 2009, Elsevier B.V.) ELISA data from 12 SH2 antigens by one of the groups generating scFv antibodies (gene libraries HAL4/7), shown in graphical form: Single clone antigen ELISA data of the first screening campaign on microtiter plates. 92–96 antibody clones were randomly picked (per antigen). Bars showing the binding to the panning antigen are in green, to negative control antigen (hen egg lysozyme) in brown.

Antibodies that passed primary validation were further analysed by surface plasmon resonance to determine on rate (k_{on} or k_a), off rate (k_{off} or k_d) and dissociation constant (K_D) as shown in the following table (Table II). As a measure of their specificity to their antigen at its specified molecular weight, a subset of the data from analysis by immunoblot is also shown. The data is derived from three phage display libraries: a synthetic Fab library (Sidhu and Koide laboratories, Toronto, Canada), a human naïve scFv library (McCafferty laboratory, Cambridge, UK) wand another human naïve scFv library (Dübel laboratory, Braunschweig, Germany), both derived from human donors.

Target[a]	Binder name[b]	Alternate name	Affinity maturation	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	Experimental validation
ABL1	ABL1_JM_scFv_1	JM_043_1H10	Naive	4.84×10^4	3.63×10^{-4}	7.51×10^{-9}	
	ABL1_JM_scFv_2	JM_043_1G05	Naive	2.15×10^4	1.48×10^{-3}	6.86×10^{-8}	
ABL2	ABL2_JM_scFv_1	JM_061_2E04	Naive	2.10×10^4	5.44×10^{-5}	2.59×10^{-9}	
BCAR3	BCAR3_SS_Fab_9	BCAR3-9	Matured	3.31×10^4	1.96×10^{-3}	5.94×10^{-8}	
BTK	BTK_JM_scFv_1	JM_060_2D01	Naive	7.99×10^5	1.65×10^{-3}	2.07×10^{-9}	
CRK	CRK_JM_scFv_1	JM_070_1E02	Matured	1.78×10^4	4.65×10^{-4}	2.61×10^{-8}	
	CRK_JM_scFv_2	JM_070_1H05	Matured	1.32×10^4	7.12×10^{-4}	5.41×10^{-8}	
	CRK_JM_scFv_3	JM_070_1E08	Matured	2.15×10^4	5.20×10^{-4}	2.42×10^{-8}	
	CRK_SD_scFv_1	dm140-K-H2	Naive	9.11×10^4	3.00×10^{-3}	3.30×10^{-8}	Failed ectopic protein IP
	CRK_SD_scFv_2	dm140-K-H10	Naive	6.72×10^5	3.00×10^{-2}	4.84×10^{-8}	Ectopic protein IP only
	CRK_SS_Fab_9	CRK-9	Matured	4.21×10^4	1.04×10^{-4}	2.47×10^{-9}	Endogenous protein IP
	CRK_SS_Fab_10	CRK-10	Matured	7.13×10^3	4.78×10^{-4}	6.71×10^{-8}	
	CRK_SS_Fab_11	CRK-11	Matured	2.80×10^4	2.59×10^{-4}	9.25×10^{-9}	Failed ectopic protein IP
FYN	FYN_SS_Fab_2	FYN-2	Naive	4.50×10^5	9.13×10^{-4}	2.03×10^{-9}	
GRAP2	GRAP2_SK_Fab_1		Naive	1.36×10^5	2.28×10^{-3}	1.68×10^{-8}	
	GRAP2_SK_Fab_2		Naive	8.28×10^4	6.06×10^{-3}	7.32×10^{-8}	
	GRAP2_SK_Fab_3		Naive	9.31×10^4	1.07×10^{-3}	1.15×10^{-8}	
GRB2	GRB2_AS_mAB_3	GRB2_11H3	Naive	6.72×10^5	3.30×10^{-4}	4.92×10^{-10}	Failed ectopic protein IP
	GRB2_JM_scFv_1	JM_047_2A08	Naive	1.21×10^4	2.68×10^{-3}	2.22×10^{-7}	
	GRB2_JM_scFv_2	JM_047_2A02	Naive	1.67×10^4	3.86×10^{-3}	2.30×10^{-7}	
	GRB2_JM_scFv_3	JM_047_2A10	Naive	9.72×10^4	7.91×10^{-4}	8.13×10^{-9}	Failed ectopic protein IP
	GRB2_JM_scFv_4	JM_047_2B02	Naive	9.78×10^3	2.27×10^{-3}	2.32×10^{-7}	
	GRB2_SK_Fab_1		Naive	1.17×10^5	3.00×10^{-2}	2.96×10^{-7}	
	GRB2_SK_Fab_2		Naive	2.42×10^5	1.45×10^{-3}	5.99×10^{-9}	Ectopic protein IP only
	GRB2_SK_Fab_3		Naive	1.88×10^5	2.14×10^{-3}	1.14×10^{-8}	Endogenous protein IP
	GRB2_SS_Fab_2	GRB2-2	Naive	5.65×10^4	4.08×10^{-3}	7.22×10^{-8}	
	GRB2_SS_Fab_4	GRB2-4	Matured	7.14×10^4	2.25×10^{-3}	3.16×10^{-8}	
	GRB2_SS_Fab_5	GRB2-5	Matured	9.84×10^4	2.78×10^{-3}	2.83×10^{-8}	
	GRB2_SS_Fab_6	GRB2-6	Matured	1.96×10^5	8.43×10^{-3}	4.29×10^{-8}	
LCK	LCK_SS_Fab_6	LCK-6	Matured	1.16×10^4	1.82×10^{-3}	1.57×10^{-7}	
LYN	LYN_JM_scFv_1	JM_065_1E12	Matured	2.53×10^5	2.27×10^{-3}	8.98×10^{-9}	Failed ectopic protein IP
	LYN_JM_scFv_2	JM_065_1E07	Matured	2.62×10^5	2.66×10^{-3}	1.01×10^{-8}	Failed ectopic protein IP
	LYN_JM_scFv_3	JM_065_1E03	Matured	3.12×10^5	2.19×10^{-3}	7.02×10^{-9}	Failed ectopic protein IP
	LYN_SD_scFv_1	dm130-F-A9	Naive	9.30×10^4	5.72×10^{-4}	6.15×10^{-9}	Ectopic protein western
	LYN_SS_Fab_1	AP03-43j	Naive	1.91×10^5	1.46×10^{-3}	7.61×10^{-9}	
	LYN_SS_Fab_2	LYN-4	Naive	2.54×10^5	2.27×10^{-3}	8.95×10^{-9}	Ectopic protein IP and western
	LYN_SS_Fab_3	LYN-7	Matured	1.23×10^5	3.96×10^{-3}	3.22×10^{-8}	
NCK1	NCK1_JM_scFv_1	JM_067_1E03	Matured	3.52×10^5	8.97×10^{-3}	2.55×10^{-8}	Failed endogenous protein IP
	NCK1_JM_scFv_2	JM_075_1B09	Matured	1.74×10^5	3.00×10^{-2}	1.47×10^{-7}	Failed endogenous protein IP
	NCK1_JM_scFv_3	JM_067_1F09	Matured	1.25×10^5	4.04×10^{-3}	3.22×10^{-8}	Failed endogenous protein IP
PIK3R1 C	PIK3R1C_SS_Fab_2	PIK3R1C-2	Matured	9.76×10^4	4.46×10^{-3}	4.56×10^{-8}	
	PIK3R1C_SS_Fab_3	PIK3R1C-3	Matured	2.91×10^4	6.84×10^{-3}	2.35×10^{-7}	
PLCG1 C	PLCG1C_SS_Fab_4	PLCG1C-4	Matured	9.22×10^4	2.82×10^{-3}	3.06×10^{-8}	
	PLCG1C_SS_Fab_5	PLCG1C-5	Matured	5.71×10^4	2.38×10^{-3}	4.17×10^{-8}	
	PLCG1C_SS_Fab_6	PLCG1C-6	Matured	2.14×10^5	1.90×10^{-3}	8.84×10^{-9}	
PTPN11 C	PTPN11C_JM_scFv_1	JM_069_1C02	Matured	8.83×10^5	9.14×10^{-3}	1.03×10^{-8}	
	PTPN11C_JM_scFv_2	JM_069_1A11	Matured	1.46×10^5	1.21×10^{-3}	8.33×10^{-9}	
	PTPN11C_SS_Fab_5	PTPN11C-5	Naive	1.80×10^4	8.91×10^{-3}	4.96×10^{-7}	
RASA1 C	RASA1C_JM_scFv_1	JM_056_2A03	Naive	4.06×10^5	2.23×10^{-3}	5.48×10^{-9}	
	RASA1C_JM_scFv_2	JM_056_2A10	Naive	3.70×10^4	4.20×10^{-3}	1.14×10^{-7}	
	RASA1C_SD_scFv_1	dm124-Q-H1	Naive	1.60×10^6	4.56×10^{-4}	2.85×10^{-10}	Failed endogenous protein IP
	RASA1C_SD_scFv_2	dm124-Q-H8	Naive	5.08×10^4	0.13	2.61×10^{-6}	
SH2D1A	SH2D1A_JM_scFv_1	JM_051_2B04	Naive	2.72×10^5	3.20×10^{-3}	1.18×10^{-8}	
	SH2D1A_JM_scFv_2	JM_051_2B09	Naive	6.21×10^3	4.40×10^{-3}	7.08×10^{-7}	
	SH2D1A_SS_Fab_4	SH2D1A-4	Naive	2.79×10^5	4.00×10^{-2}	1.56×10^{-7}	
	SH2D1A_SS_Fab_9	SH2D1A-9	Matured	2.58×10^5	2.00×10^{-2}	8.48×10^{-8}	
SHC1	SHC1_AS_mAb_1	SHC1_9E11	Naive	2.32×10^5	1.74×10^{-4}	7.49×10^{-10}	Endogenous protein IP
	SHC1_AS_mAb_2	SHC1_2H6	Naive	4.16×10^5	1.35×10^{-3}	3.25×10^{-9}	Endogenous protein IP
	SHC1_JM_scFv_1	JM_072_1A10	Matured	1.21×10^5	5.82×10^{-4}	4.80×10^{-9}	Endogenous Protein IP
	SHC1_JM_scFv_2	JM_072_1A01	Matured	3.53×10^4	3.04×10^{-3}	8.61×10^{-8}	
	SHC1_JM_scFv_3	JM_072_1C04	Matured	2.86×10^4	9.28×10^{-4}	3.24×10^{-8}	
	SHC1_SD_scFv_2	dm122-G-B6	Naive	7.49×10^4	3.82×10^{-4}	5.10×10^{-9}	Failed endogenous protein IP
	SHC1_SD_scFv_3	dm122-G-B9	Naive	9.60×10^4	1.79×10^{-3}	1.86×10^{-8}	
	SHC1_SD_scFv_4	dm122-G-C9	Naive	9.64×10^4	4.39×10^{-4}	4.56×10^{-9}	Failed endogenous protein IP
SYK	SYK_JM_scFv_1	JM_054_2A04	Naive	4.86×10^3	3.00×10^{-2}	5.58×10^{-6}	
VAV1	VAV1_JM_scFv_1	JM_066_1E03	Matured	6.59×10^4	1.59×10^{-3}	2.42×10^{-8}	
	VAV1_JM_scFv_2	JM_066_1E09	Matured	8.03×10^4	1.76×10^{-3}	2.19×10^{-8}	
	VAV1_JM_scFv_3	JM_066_1H05	Matured	5.39×10^4	2.26×10^{-3}	4.20×10^{-8}	
ZAP70	ZAP70_SS_Fab_1	AP03-17t	Naive	1.48×10^4	2.33×10^{-3}	1.58×10^{-7}	
	ZAP70_SS_Fab_2	AP03-17ab	Matured	4.29×10^4	1.90×10^{-3}	4.42×10^{-8}	
	ZAP70_SS_Fab_7	AP03-17cd	Matured	6.27×10^4	2.79×10^{-3}	4.44×10^{-8}	
	ZAP70_SS_Fab_8	AP03-17ef	Matured	1.00×10^4	2.72×10^{-3}	2.70×10^{-7}	

k_a, on rate; k_d, off rate; and IP, immunoprecipitation.
[a]For a summary of tested clones, see Supplementary Table 1 . [b]Standardized binder name: SH2 domain target_principal investigator initials_Binder type_number. Sequences in FASTA format are available in Supplementary Figure 4 .

Table II: (Reprinted with permission from Collwill *et al.*, 2011, *Nature Methods*, 8, p. 553, © 2011, Springer Nature) Surface plasmon resonance analysis and biological validation of 73 non-animal-derived antibodies.

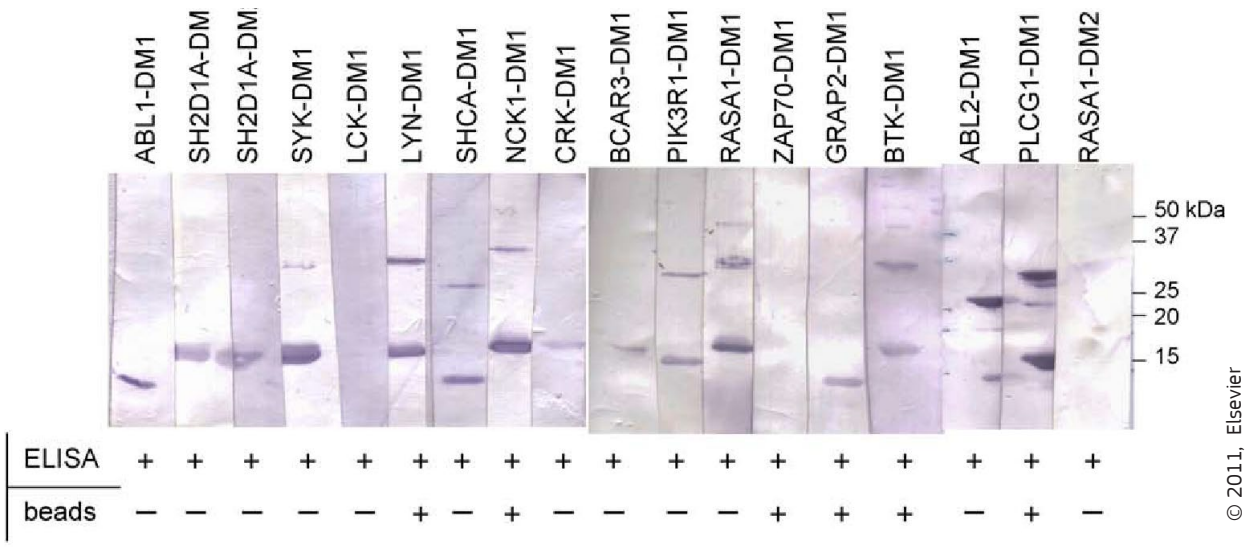

Figure II: (Reprinted with permission from Mersmann *et al.*, 2010, *New Biotechnology*, 27, p. 125, © 2011, Elsevier) Immunoblot data from SH2 antigens by one of the groups generating scfv antibodies where 1 µg of each SH2 domain was run on a 15% SDS-PAGE gel. ScFv fragments were used to stain immunoblots of their corresponding antigens. Detection: anti-myc-tag-antibody followed by an anti-mouse AP conjugate. Staining with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP).

IV.b Example 2: Data sets of commercially available non-animal-derived antibodies from Abcalis, Adipogen and Bio-Rad (other sources are available):

Abcalis: *https://abcalis.com/*. This data set lists (currently) 16 non-animal-derived antibodies (scFv-Fc-antibodies generated after selecting scFv from universal naïve gene libraries HAL4/7/8 and HAL9/10 derived from human B cells) that have been identified as positive by primary ELISA screen. In each case, the original citation is given where the antibody performance is demonstrated by a range of immuno-analysis techniques, often including tissue stainings, fluorescence-activated cell sorting (FACS) or even detailed functional assays, like antigen neutralisation. Most have been mapped to bind a specific epitope sequence and the binding affinity (K_D) of some is given.

Adipogen: *https://adipogen.com/pub/media/wysiwyg/Catalogs/PDFs/INSIGHTS_Recombinant_Antibodies_2019.pdf*. This data set lists 20 different antibody clones (RecMAbs™) produced without the use of animals, developed by antibody phage display technology using two different human naïve scFv antibody gene libraries obtained through collaboration with two research groups from different countries. The selected monoclonal scFv, identified as positive by primary ELISA screen, were cloned into an appropriate vector containing a Fc portion of interest and then produced in mammalian cells to generate an immunoglobulin G (IgG) like scFv-Fc fusion protein. *https://adipogen.com/antibodies.html?apg_product_type=118&p=4*.

Bio-Rad: *https://www.bio-rad-antibodies.com/primary-antibodies-monoclonal-polyclonal.html#isotype=HuCAL%20Fab%20bivalent|HuCAL%20Fab%20monovalent*. This data set uses the Human Combinatorial Antibody Library (HuCAL®) which is a fully human, synthetic monoclonal antibody library in Fab format. Selected non-animal-derived antibodies were validated by a variety of end points including ELISA, immunoblot, immunoprecipitation, flow cytometry and IHC. Data for the immuno- analysis techniques employed and their corresponding references, where possible, are given for 158 different HuCAL antibody clones. Those converted to IgG format are not included in this link.

IV.c Example 3: From Frenzel *et al.* (2016)

To illustrate the suitability of non-animal-derived antibodies for clinical use, the table in the link below (open access source) lists 60 antibodies from naïve, synthetic or semi synthetic scFv or Fab libraries from various

sources. These antibodies are evaluated in clinical studies or already approved for therapy by the European Medicines Agency or the United States Food and Drug Agency.
https://doi.org/10.1080/19420862.2016.1212149 or
https://www.tandfonline.com/doi/figure/10.1080/19420862.2016.1212149?scroll=top&needAccess=true.

IV.d Example 4: From Huang *et al.* (2015)

To test a high-throughput synthetic antibody generation system, human Src Homology 3 (SH3) protein domains were selected as antigens against which antibodies were selected using a synthetic Fab-phage library (Library F, University Toronto, Canada) with a highly diverse (3×10^{10} unique members) repertoire Positive Fab clones were identified as having a cognate antigen ELISA signal at least 10-fold greater than the signal against three negative controls. Since some clones selected in the primary screen could have the same sequence, clones were DNA sequenced which revealed 216 unique Fab-phage clones, each of which bound specifically to one of 58 SH3 protein domains. The selected Fab antibody ID, its unique peptide sequence and its association to the corresponding antigen is shown in the following table (Table III). As a measure of affinity, k_{on}, k_{off} and K_D data is given for 6 of the antibodies.

SH3 domain	Fab ID	k_{on} ($\times 10^4$ $M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$ s^{-1})	K_d (nM)	CDRL3 107	108	109	110	111	112	113	114	115	116	CDRH1 30	35	36	37	38	39	CDRH2 55	56	57	58	59	62	63	64	65	66	CDRH3 107	108	109	110	111	111	111	111	112	112	112	112	112	113	112	112	112	112	113	114	115
	F001				H	Y	S	-	-	V	H	P	I		I	Y	Y	S	Y	I	S	I	Y	S	Y	Y	S	Y	T	Y	S	P	S	Y	S	W	G	P	Y	-	Y	G	G	P	S	G	F	A	M
	F002				F	W	-	-	-	-	G	P	I		I	S	Y	S	S	I	S	I	S	S	Y	S	G	S	T	Y	Y	P	Y	Y	G	-	-	-	-	-	-	-	-	-	G	Y	A	L
	F003	8.99	3.62	40.3	F	Y	-	-	-	-	S	P	I		F	S	S	S	S	I	S	I	S	P	Y	S	G	S	T	Y	Y	F	Y	Y	H	-	-	-	-	-	-	-	-	V	V	G	L
RASA1-1/1	F003.1	22.2	0.014	0.07	F	Y	-	-	-	-	S	P	I		I	S	S	S	S	M	S	I	S	P	Y	S	G	S	T	Y	Y	F	Y	Y	H	-	-	-	-	-	-	-	-	V	V	G	L
	F003.2	47.3	0.012	0.03	F	Y	-	-	-	-	S	P	I		I	S	S	S	S	I	S	I	S	P	Y	S	G	S	T	Y	Y	F	Y	Y	H	-	-	-	-	-	-	-	-	V	V	G	L
	F004				S	G	Y	-	-	-	S	P	I		I	S	Y	S	S	M	S	I	S	S	Y	S	G	S	T	Y	Y	S	Y	Y	H	-	-	-	-	-	-	-	-	Y	A	G	A	M
	F005				W	F	A	-	-	-	S	P	I		I	Y	Y	S	S	I	S	I	S	S	Y	S	G	Y	T	Y	Y	S	Y	Y	G	-	-	-	-	-	-	-	-	H	Y	G	A	L
	F006				F	W	-	-	-	-	G	P	I		I	S	Y	S	S	I	S	I	S	S	Y	S	G	S	T	Y	Y	P	Y	Y	G	-	-	-	-	-	-	-	-	-	G	Y	A	L
	F007				G	A	A	Y	-	G	S	L	I		L	Y	Y	Y	S	M	S	I	S	S	Y	S	S	Y	T	S	Y	S	A	A	W	S	-	-	-	-	-	-	G	P	Y	S	G	M
BAIAP2L2-1/1	F008				S	W	-	-	-	-	F	P	I		I	S	Y	S	S	M	S	I	Y	P	S	Y	G	Y	T	Y	A	Y	S	-	-	-	-	-	-	-	-	-	-	-	-	A	F
	F009				S	V	S	-	-	W	V	P	F		I	S	S	Y	Y	I	S	I	Y	S	Y	Y	G	Y	T	S	G	W	V	-	-	-	-	-	-	-	-	-	-	-	A	G	M
	F010				F	S	W	-	-	-	G	L	I		L	Y	S	S	S	M	S	I	Y	P	S	Y	G	Y	T	S	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	L
	F011				S	F	-	-	-	-	W	P	I		I	Y	Y	Y	S	M	S	I	S	S	Y	Y	S	S	T	Y	A	A	A	A	G	W	S	-	-	-	-	-	P	A	G	Y	S	G	L
	F012				S	H	S	-	-	A	S	L	F		I	S	Y	Y	S	M	S	I	Y	S	S	Y	G	Y	T	Y	G	P	G	P	A	Y	-	-	-	-	-	-	-	-	-	Y	F	A	M
	F013				S	W	G	-	-	-	G	L	I		L	S	Y	S	Y	M	S	I	Y	S	S	Y	S	Y	T	Y	A	P	G	P	Y	-	-	-	-	-	-	-	-	-	Y	H	G	L
	F014				S	P	Y	-	-	-	V	P	F		I	Y	Y	Y	S	I	S	I	Y	S	Y	Y	G	Y	T	Y	S	Y	W	Y	-	-	-	-	-	-	-	-	-	G	Y	G	M
BAIAP2-1/1	F015				S	S	Y	-	-	-	S	L	I		I	S	S	Y	S	I	Y	I	S	P	Y	S	G	Y	T	Y	G	S	F	F	F	A	-	-	-	-	-	V	Y	G	G	G	L
	F016				P	S	Y	-	-	F	G	L	I		L	S	S	Y	S	M	Y	I	S	P	S	S	G	Y	T	S	T	V	R	G	S	K	K	P	-	-	-	Y	F	S	G	W	A	M
	F017				G	V	Y	-	-	-	G	L	F		I	Y	Y	S	S	M	Y	I	S	P	Y	Y	S	Y	T	S	H	P	S	Y	-	-	-	-	-	-	-	-	-	Y	W	G	L
	F018				S	Y	S	V	W	Y	Y	P	F		L	Y	Y	Y	Y	I	S	I	Y	P	Y	S	S	Y	T	Y	V	P	G	Y	G	Y	-	-	-	-	-	-	F	S	G	M
	F019				W	V	G	-	-	-	P	L	I		I	Y	Y	S	S	M	S	I	Y	P	Y	Y	S	Y	T	Y	Y	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	L
	F020				G	V	Y	-	-	-	G	L	F		I	Y	Y	S	S	M	Y	I	S	P	Y	Y	S	Y	T	S	H	P	S	Y	-	-	-	-	-	-	-	-	-	Y	W	G	L
	F021				V	S	S	Y	-	F	W	L	I		I	S	S	Y	S	M	S	I	S	S	S	Y	G	Y	T	Y	V	S	P	S	S	-	-	-	-	-	-	-	-	-	V	W	G	M
	F022				G	A	S	P	F	G	H	L	F		L	S	S	S	Y	M	S	I	Y	P	Y	Y	S	Y	T	Y	S	S	S	-	-	-	-	-	-	-	-	-	-	-	G	G	M
	F023				G	W	-	-	-	-	F	L	I		L	S	S	S	Y	M	S	I	Y	P	Y	Y	S	Y	T	Y	S	S	S	-	-	-	-	-	-	-	-	-	-	-	G	G	M
BTK-1/1	F024				Y	V	H	Y	F	A	G	L	I		L	Y	S	S	Y	M	S	I	S	S	S	Y	G	S	T	Y	S	W	A	-	-	-	-	-	-	-	-	-	-	-	Y	G	L
	F025				V	S	S	Y	-	Y	W	L	F		F	S	S	S	S	I	S	I	S	S	S	Y	G	Y	T	Y	T	V	R	G	S	K	K	P	-	-	-	Y	F	S	G	W	A	M
	F026				V	Y	S	Y	-	S	W	L	F		F	S	S	S	S	I	S	I	S	S	S	Y	G	Y	T	Y	T	V	R	G	S	K	K	P	-	-	-	Y	F	S	G	W	A	M
	F027				W	S	-	-	-	-	G	L	I		L	Y	S	Y	S	I	S	I	Y	P	Y	Y	G	Y	T	Y	S	G	G	S	S	H	Y	-	-	-	-	-	P	S	Y	S	A	M
	F028				W	P	G	-	-	Y	G	L	I		F	S	S	S	S	I	S	I	Y	P	Y	Y	G	Y	T	Y	A	Y	S	F	-	-	-	-	-	-	-	-	-	V	G	L
TEC-1/1	F029				W	Y	H	S	G	F	Y	P	I		I	Y	S	S	S	M	S	I	S	P	Y	S	S	Y	T	Y	R	W	A	W	-	-	-	-	-	-	-	-	-	H	A	G	L
	F030				G	Y	F	-	-	-	G	L	I		L	Y	Y	Y	S	M	Y	I	Y	S	Y	Y	G	Y	T	Y	S	A	W	P	S	H	P	S	H	W	G	F	S	P	S	A	W	A	M
	F031				S	S	Y	-	-	-	S	L	I		F	S	S	S	S	I	S	I	Y	P	Y	Y	G	S	T	S	Y	S	Y	H	W	P	W	G	G	-	Y	H	Y	V	G	Y	G	A	M
SORBS1-1/3	F032				G	S	W	-	-	-	W	P	I		L	Y	Y	Y	Y	I	S	I	Y	S	S	Y	G	Y	T	Y	G	V	W	G	Y	G	A	G	S	-	-	H	S	S	Y	Y	S	A	I
	F033				S	F	Y	P	S	W	G	L	F		I	S	Y	Y	S	M	S	I	S	S	S	S	Y	T	S	S	V	Y	G	Y	S	S	G	Y	W	-	Y	Y	A	A	H	W	Y	A	M
	F034				S	S	W	Y	G	P	S	L	I		L	Y	Y	Y	S	M	S	I	Y	P	S	S	S	Y	T	Y	Y	A	H	S	Y	-	-	-	-	-	-	-	-	-	Y	Y	A	G	F
	F035				G	S	W	-	-	G	H	L	I		I	S	Y	Y	Y	M	S	I	S	S	S	Y	G	Y	T	Y	W	Y	Y	W	-	-	-	-	-	-	-	-	-	-	S	G	L
	F036				S	Y	-	-	-	-	Y	P	I		L	Y	Y	Y	S	M	S	I	Y	P	Y	Y	G	Y	T	Y	Y	G	V	-	-	-	-	-	-	-	-	-	-	-	-	S	G	L
	F037				S	Y	S	-	-	-	P	P	F		I	Y	Y	S	S	I	S	I	Y	S	Y	S	G	Y	T	Y	G	Y	G	-	-	-	-	-	-	-	-	-	-	-	-	G	G	F
	F038				S	Y	-	-	-	-	Y	L	F		I	S	S	S	S	M	S	I	Y	P	Y	Y	G	Y	T	Y	A	Y	S	W	-	-	-	-	-	-	-	-	-	-	S	Y	A	L
SORBS2-1/3	F039				Y	Y	Y	Y	-	H	Y	L	I		I	S	S	S	S	M	S	I	Y	P	Y	Y	G	Y	T	Y	S	Y	V	S	-	-	-	-	-	-	-	-	-	-	A	A	L
	F040				S	A	Y	G	-	F	S	P	I		L	S	Y	Y	Y	M	S	I	S	S	S	Y	G	Y	T	Y	Y	G	Y	Y	-	-	-	-	-	-	-	-	-	-	G	G	L
	F041				G	W	-	-	-	-	Y	L	F		L	S	S	Y	S	M	S	I	Y	P	Y	Y	S	Y	T	S	A	S	Y	Y	G	-	-	-	-	-	-	-	-	-	S	Y	A	F
	F042				Y	Y	Y	Y	-	H	Y	L	I		I	S	S	S	S	M	S	I	Y	P	Y	Y	G	Y	T	Y	S	Y	V	S	-	-	-	-	-	-	-	-	-	-	A	A	L
	F043				F	P	Y	-	-	Y	Y	P	I		I	S	S	S	S	I	S	I	Y	P	Y	S	G	S	T	Y	Y	Y	A	G	Y	Y	S	-	-	-	-	-	Y	G	Y	P	Y	A	L
	F044				S	S	Y	-	-	-	S	L	I		F	S	S	S	S	I	Y	I	Y	P	Y	S	G	Y	T	Y	S	V	A	Y	Y	-	-	-	-	-	-	-	-	G	W	W	A	F
	F045				S	A	F	-	-	-	F	P	F		L	S	Y	Y	Y	M	S	I	S	S	S	Y	S	Y	T	S	G	Y	Y	S	W	G	A	H	-	-	-	F	S	H	H	V	W	A	M
GRAP2-	F046	32.5	0.664	2.04	Y	S	A	-	-	H	S	L	I		L	Y	Y	Y	S	I	S	I	S	S	S	Y	G	Y	T	Y	V	G	Y	-	-	-	-	-	-	-	-	-	-	A	M
CRK-1/2	F047	7.27	0.907	12.5	Y	G	G	H	Y	H	Y	P	I		L	Y	S	Y	S	M	S	I	Y	S	Y	S	S	Y	T	Y	A	S	Y	W	A	S	S	A	P	P	S	Y	S	Y	S	Y	W	G	M
	F048	22.4	4.84	21.6	S	H	F	-	-	G	G	L	F		L	Y	Y	S	Y	M	S	I	S	S	S	Y	G	Y	T	Y	P	S	H	W	S	-	-	-	-	-	-	-	-	A	W	G	I
	F049				S	H	F	-	-	G	G	L	F		I	S	Y	S	S	I	S	I	Y	P	Y	S	G	Y	T	Y	W	P	G	Y	H	Y	-	-	-	-	-	-	S	S	A	Y	G	L
DNMBP-4/6	F050				P	A	S	Y	Y	W	S	P	F		I	Y	Y	S	S	M	S	I	Y	P	Y	S	G	Y	T	S	H	Y	-	-	-	-	-	-	-	-	-	-	-	-	G	A	M
	F051				V	Y	S	-	-	-	G	L	I		I	S	S	Y	S	M	Y	I	Y	S	Y	S	G	Y	T	S	S	Y	G	P	Y	G	S	-	-	-	-	-	G	G	Y	W	Y	G	L
	F052				S	Y	Y	-	-	Y	H	L	I		L	Y	S	Y	Y	M	S	I	Y	P	Y	Y	G	Y	T	Y	Y	P	Y	A	A	W	-	-	-	-	-	-	P	A	Y	G	F
	F053				W	G	Y	S	V	Y	S	P	I		I	Y	Y	S	S	M	S	I	Y	S	Y	Y	G	Y	T	S	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	M
	F054				W	G	S	-	-	G	F	P	I		I	Y	S	Y	S	M	S	I	Y	S	Y	Y	S	S	T	Y	G	S	W	S	Y	Y	S	S	S	S	H	H	F	G	A	H	P	G	L
	F055				V	W	-	-	-	-	Y	P	I		L	Y	S	Y	S	M	S	I	S	S	Y	S	G	Y	T	S	Y	G	V	G	Y	P	Y	-	-	-	-	-	F	Y	Y	Y	F	G	L
	F056	34.1	6.68	19.6	V	A	P	W	S	Y	Y	P	F		L	Y	S	S	Y	M	S	I	S	S	S	Y	G	Y	T	Y	W	V	Y	Y	Y	Y	S	G	-	-	-	-	-	Y	Y	F	-	G	L
	F057				F	W	G	-	-	-	G	S	L	F		I	S	S	S	Y	I	Y	I	S	S	Y	S	G	Y	T	Y	A	G	W	Y	Y	Y	G	Y	-	-	-	-	G	G	W	S	G	I
ITSN2-2/5	F058				S	S	Y	-	-	-	S	L	I		F	S	S	S	S	I	Y	I	Y	S	Y	Y	G	Y	T	S	S	G	G	W	Y	-	-	-	-	-	-	-	-	W	Y	A	I
	F059				Y	S	S	Y	-	Y	A	L	I		L	S	Y	Y	Y	I	S	I	Y	P	Y	S	S	G	S	T	Y	A	Y	V	-	-	-	-	-	-	-	-	-	-	-	Y	G	L
	F060				S	S	Y	-	-	-	S	L	I		I	S	Y	S	S	I	S	I	Y	P	S	Y	G	S	T	Y	V	S	W	H	-	-	-	-	-	-	-	-	-	-	S	G	L
	F061				S	S	Y	-	-	-	S	L	I		F	S	S	S	S	I	S	I	Y	P	Y	Y	G	S	T	Y	G	V	A	P	S	P	Y	-	-	-	-	S	V	S	F	S	G	L
	F062				P	W	-	-	-	-	S	P	I		I	S	Y	Y	S	I	S	I	S	P	S	S	G	Y	T	S	Y	G	H	S	H	G	G	S	Y	-	W	G	G	G	Y	Y	Y	A	L
ITSN1-3.5	F063	43.1	1.83	4.26	Y	G	V	-	-	F	H	P	I		L	Y	S	S	Y	M	S	I	Y	P	Y	Y	S	Y	T	S	S	W	Y	-	-	-	-	-	-	-	-	-	-	A	F
	F064				Y	G	V	-	-	F	H	P	I		I	S	S	S	Y	I	Y	I	Y	P	S	Y	G	Y	T	S	S	W	Y	-	-	-	-	-	-	-	-	-	-	A	I

Table III: (Reprinted with permission from John Wiley and Sons: Huang *et al.*, 2015, *Protein Science* 24, p. 1892, © 2015, The Protein Society) Complementarity-determining regions (CDR) sequences and binding kinetics of Fabs. Positions randomised within each of the CDRs are shown at the top of each column numbered according to ImMunoGeneTics (IMGT) standards. For each SH3 domain antigen, abbreviated protein codes corresponding to the Uniprot recommended gene codes are provided to unambiguously identify the parent protein, and its position relative to the total number of SH3 domains in the parent protein is denoted numerically (e.g., ITSN2-2/5 represents the second of five SH3 domains in the protein ITSN2). The binding parameters k_{on} (on rate), k_{off} (off rate) and K_D (affinity constant) were determined from kinetic analysis of Fabs binding to immobilised antigens measured by surface plasmon resonance. Sequences are shown for 65 Fabs raised against 12 antigens.

IV.e Example 5: From Hornsby *et al.* (2015)

This multicentre, NIH funded project (U54 HG006436) was launched by Rutgers University, the Structural Genomics Consortium (SCG) and the Recombinant Antibody Network (RAN) to rapidly develop and implement a high-throughput pipeline designed to generate high quality renewable recombinant antibodies. It describes an industrialised platform to generate antigens and validated recombinant antibodies focusing on proteins involved in chromatin biology including 346 transcription factors (representing >18 protein domain folds) and 211 epigenetic antigens. The study utilised a highly stable and diverse synthetic Fab scaffold developed at University Toronto, Canada (libraries E and F). These cloned antibodies are available to the academic community for research purposes through the recombinant-antibodies.org (RAN) to allow a more system-wide analysis of transcription factors and chromatin biology. A summary table (Table IV) for the 670 Fabs that passed primary and secondary validation criteria is shown below. For a more detailed account, go to *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4597156/*, supplementary materials, table S1 4.

Antibodies passed primary validation if they were able to bind their cognate immobilised antigen in the presence of 20nM of the same competing soluble antigen (competitive ELISA) and achieve a ratio (OD650/min of competitive binding divided by the OD650/min of direct binding) of < 0.5. Antibodies passed secondary validation if they were able to bind to their parent antigen in a cellular lysate (spiked IP) since lysate may contribute to high-level non-specific binding and reduce specificity. Non-specific binding was estimated by the reduction in the direct binding (ELISA EC50) of antibody to antigen in the presence of non-cognate protein from baculovirus extract.

Domain type	Primary validation			Secondary validation		
	Total # of antigens	Passing antigens	Domain success (%)	Total # of antigens	Passing antigens	Domain success (%)
BRD	37	32	86	2	1	50
BTB	10	9	90	7	6	86
bZip	11	11	100	4	0	0
Cupin	3	3	100	NT	NT	
DUF	2	2	100	NT	NT	
Fork Head	5	4	80	3	3	100
HDAC	3	2	67	NT	NT	
HMG Box	10	8	80	5	2	40
Homeobox	6	5	83	15	5	33
Hormone Recep.	6	5	83	5	3	60
JMJ	12	11	92	3	3	100
Misc	141	122	87	50	34	68
PHD	15	12	80	3	0	0
PWWP	7	7	100	2	1	50
SCAN	46	46	100	40	35	88
SET	31	21	68	5	2	40
SIR2	4	4	100	3	1	33
ZNF	148	105	71	46	12	26
Sum	537	435	81	193	108	56

Table IV: (Reprinted under CC BY 4.0 from Hornsby *et al.*, 2015, *Molecular & Cellular Proteomics*, 14, p. 2841, © 2015, American Society for Biochemistry and Molecular Biology) Ability of antigens to produce primary and secondary validated Fabs as a function of antigen domain. Of the 537 TF antigens, representing at least 18 discrete domain types, 435 (81%) successfully generated sequence unique Fabs that passed competition ELISA. For 193 antigens with Fabs entering secondary validation, 108 (56%) passed the Spiked-IP validation test. The best performing domains are the Forkhead, SCAN, JMJ, and BTB domains with success rates of 100%, 88%, 100%, and 86% respectively.

IV.f Example 6: From Schofield *et al.* (2007)

This study describes the generation of non-animal-derived antibodies on a genome-wide scale in order to gain information about protein interaction, expression, modification and sites of action by using renewable, defined and homogenous reagents. As phage display technology can be adapted to a 96-well format, hundreds of antigens can be handled in parallel and the entire selection process can take as little as two weeks. This approach demonstrated that generating synthetic antibodies on a proteome-wide scale is an achievable goal.

The scFv library was generated using B-lymphocytes from 42 human peripheral blood donations and 1 tonsil and cloned into PSANG4 vector. Antibodies were selected to 404 antigen targets (representing 280 genes), the majority (214/280) being cell surface receptors. Large-scale generation of 4,400 antibodies were characterised to 292 antigens by ELISA. Detection sensitivity was measured for 100 antibodies to 10 antigens using a bead based flow cytometry assay. Representative histograms for antigen Jagged-1 (a ligand for Notch receptors expressed on embryonic stem cells) are given below to show that performance in the bead assay correlates with ability to detect endogenous levels of antigen (Figure III). This shows that signal intensity varies with different antibodies and this, in turn, corresponds to the performance ranking found in the bead based assay.

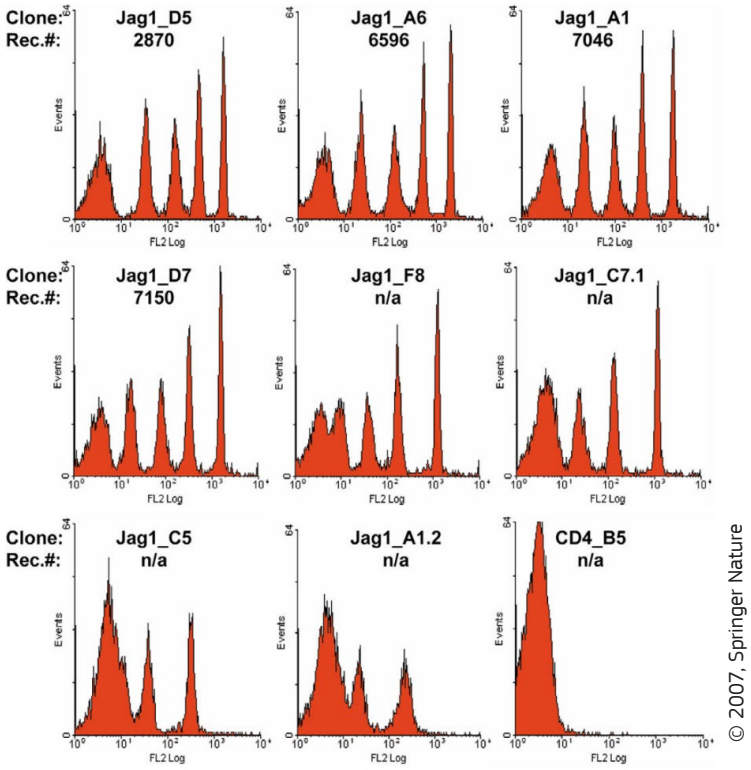

Figure III: (Reprinted under CC BY 4.0 from Schofield *et al.*, 2007, *Genome Biology*, 8, p. R254.8, © 2007, Springer Nature) Flow cytometry calibration beads with varying number of anti-human Fc antibodies were coated with Jagged-1-Fc fusion to yield antigen display levels of 29,000, 83,000, 204,000 and 619,000 Jagged-1 molecules/bead. These were labelled with a panel of different recombinant antibodies raised against Jagged-1 and binding was detected with labelled anti-FLAG antibodies. The resulting histograms are shown, giving different levels of sensitivity. In Jag1_D5 for example, five peaks are visible corresponding to uncoated beads and each of the four antigen coated beads. In the case of Jag1_C5, where there is lower sensitivity, only the two beads with highest density are resolved while the others merge with that of the uncoated bead. Where all four beads are clearly resolved, the theoretical limit of detection of receptors per bead (Rec. #) is calculated. The primary peak and CD4_B5 respectively represent fluorescence in the absence of antibody and non-specific antibody binding negative controls.

The bead assay was applied to 90 other antibodies representing nine different target genes. In this experiment beads were coated with antigen at a range of densities (18,000, 57,000, 459,000 antigens/bead). To help summarise and present the results for all 90 antibodies, the median fluorescent intensity for the bead coated with 459,000 target molecules was calculated (Table V).

Efna2		Efna4		Plaur		Alcam	
Antibody	**Score**	**Antibody**	**Score**	**Antibody**	**Score**	**Antibody**	**Score**
ant14_19 - D10	182.0	ant16_21 - D02	402	ant552_360 - D03	165	ant567_733 -C10	296
ant14_19 - E12	162.0	ant16_21 - E09	384	ant552_360 - D01	117	ant567_733 - F10	271
ant14_19 - C03	151.0	ant16_21 - G10	361	ant552_360 - B12	106	ant567_733 -C01	216
ant14_19 - G10	84.8	ant16_21 - B03	357	ant552_360 - F08	54.1	ant567_733 - F11	205
ant14_19 - D09	84.0	ant16_21 - A06	271	ant552_360 - C12	44	ant567_733 -A02	194
ant14_19 - H11	70.8	ant16_21 - E02	254	ant552_360 - A07	39.4	ant567_733 -G01	149
ant14_19 - B09	60.8	ant16_21 - G01	232	ant552_360 - H04	36.1	ant567_733 -B08	111
ant14_19 - A11	14.9	ant16_21 - B01	176	ant552_360 - B06	UN	ant567_733 - F01	101
ant14_19 - G06	2.3	ant16_21 - A02	125	ant552_360 - A05	UN	ant567_733 - F09	85.5
ant14_19 - A08	1.5	ant16_21 - D10	84	ant552_360 - C09	UN	ant567_733 -E06	UN

IL3Rra1		Sigrr		Ngfr		CD22	
Antibody	**Score**	**Antibody**	**Score**	**Antibody**	**Score**	**Antibody**	**Score**
ant568_734 - B08	273	ant575_741 - G05	199	ant54_71 - E06	111	ant582_777-B09	304
ant568_734 - F11	220	ant575_741 - C01	135	ant54_71 - C09	105	ant582_777 -C08	252
ant568_734 - B04	192	ant575_741 - C09	102	ant54_71 - G02	101	ant582_777 -A08	222
ant568_734 - H11	131	ant575_741 - B02	76.8	ant54_71 - C07	86.3	ant582_777 -A03	216
ant568_734 - B09	117	ant575_741 - C12	55.1	ant54_71 - H02	53.6	ant582_777 -C12	182
ant568_734 - F02	101	ant575_741 - G12	44.4	ant54_71 - D03	44.8	ant582_777 -B06	159
ant568_734 - F10	91.9	ant575_741 - A01	34.5	ant54_71 - B07	29.6	ant582_777 -C10	72
ant568_734 - B06	75.4	ant575_741 - D07	26.1	ant54_71 - F09	18.7	ant582_777 -E03	19
ant568_734 - E01	48.5	ant575_741 - D01	24.3	ant54_71 - B06	9.4	ant582_777 -H08	4.8
ant568_734 - E12	21.6	ant575_741 - C04	8.4	ant54_71 - B03	3.1	ant582_777 -B05	1.7

VCAM		Jagged-1	
Antibody	**Score**	**Antibody**	**Score**
ant588_783 - D03	117	Jag-A06	182
ant588_783 - E11	63	Jag-D05	177
ant588_783 - D08	59.2	Jag-D07	158
ant588_783 - C09	53.6	Jag-A01	153
ant588_783 - D04	40.2	Jag-C07.1	109
ant588_783 - E02	10.3	Jag-F08	99.7
ant588_783 - C11	9.44	Jag-C05	33.6
ant588_783 - B04	7.96	Jag-A01.2	22.6
ant588_783 - D01	2.07	Jag-E03	21.8
ant588_783 - A01	UN	Jag-A06	182

Table V: (Reprinted under CC BY 4.0 from Schofield *et al.*, 2007, *Genome Biology*, 8, additional data file 2, © 2007, Springer Nature) Flow cytometry calibration beads were coated with various antigen densities/bead, analysed by flow cytometry and the relative median fluorescent intensity of the bead with 459,000 copies/bead was calculated. Shown are the actual values and the clone identifications associated with them. At the flow cytometry settings used, the uncoated bead gave a score of 0.1-0.2.

V Non-animal-derived antibody resources

V.a Catalogue antibodies

Some non-animal-derived antibody catalogue companies (defined here as non-animal-derived affinity ligand) can be exemplified:

- Abcalis (exclusively offers non-animal-derived antibodies, all from human phage display libraries)
- Abcam (14 non-animal-derived antibodies, all affibodies)
- Adipogen (21 non-animal-derived antibodies, all from human phage display libraries)
- Affibody (~ 40 non-animal-derived antibodies, all affibodies)
- Amsbio (~ 80 non-animal-derived antibodies, all aptamers)
- Avacta (23 non-animal-derived antibodies, all affimers)
- Bio-Rad Antibodies (~ 300 non-animal-derived antibodies in catalogue, all from a human synthetic library)
- Recombinant Antibody Network (RAN - 850 scFv-Fc antibodies) *https://proteincapture.org/about/ran/*
- Source Bioscience, ATLAS (2,400 scFv antibodies) *https://www.sourcebioscience.com/media/1261/proteomic-resources-atlas_db_new.xls*

V.b Custom generation of non-animal-derived antibodies

- University of Geneva (non-commercial, limited to research purposes): *https://www.unige.ch/medecine/antibodies/*
- Centre for the Commercialisation of Antibodies and Biologics (CCAB): *https://ccabcanada.com/*
- Bio-Rad: *https://www.bio-rad-antibodies.com/custom-monoclonal-antibody-generation.htm*
- Yumab: *https://yumab.com/*
- University of Zurich (DARPin platform, limited to academic users): *http://www.bioc.uzh.ch/research/core-facilities/high-throughput-binder-selection/*

V.c Phage display antibody library construction

The following is a non-exhaustive list of organisations offering phage display antibody library construction from non-animal sources, although not necessarily exclusively restricted to non-animal-derived sources. Libraries may be limited to research purposes or available for out-licensing to the biotechnology and pharmaceutical community,

- Source Bioscience, Human Domain Antibody Library (DAb): *https://www.sourcebioscience.com/products/life-sciences-research/clones/artificial-antibody-libraries/human-domain-antibody-library-dab/*
- Iontas: *https://www.iontas.co.uk/IONTAS-services/phage-display-library-construction/*
- Creative Biolabs: *https://www.creative-biolabs.com/phage-display-library-construction.html?gclid=cjwkcajwstfkbrboeiwadtmnecopc3uweashyemjm_4jmdo4zxpskz0utwostqp9ol7iwwrcnfu-yhocnyuqavd_bwe*
- Biologics International corp: *https://www.biologicscorp.com/phage-display-antibody-library-construction.html#.XJZr4iL7TIU*
- Oak Biosciences *https://www.oakbiosciences.com/services/antibodyengineering/antibody-library-construction/*
- Yumab: *https://www.yumab.com/contract-research/libraries/*

Appendix references

Blackshaw, S., Venkataraman, A., Irizarry, J., Yang, K., Anderson, S., Campbell, E., Gatlin, C.L., Freeman, N.L., Basavappa, R., Stewart, R., Loss, M.A., Pino, I., Zhu, H. and Bader, J.S. (2016). The NIH Protein Capture Reagents Program (PCRP): a standardized protein affinity reagent toolbox. *Nature Methods, 1*(10):805-806. doi:*10.1038/nmeth.4013*

Colwill, K., Renewable Protein Binder Working Group and Gräslund, S. (2011). A roadmap to generate renewable protein binders to the human proteome. *Nature Methods, 8*(7):551-558. doi:*10.1038/nmeth.1607*

European Commission (2008). Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). *Official Journal of the European Union* L142:1-739. Available at: *http://data.europa.eu/eli/reg/2008/440/oj*

Frenzel, A., Schirrmann, T. and Hust, M. (2016). Phage display-derived human antibodies in clinical development and therapy. *MAbs, 8*(7):1177-1194. doi:*10.1080/19420862.2016.1212149*

Hornsby, M., Paduch, M., Miersch, S., Sääf, A., Matsuguchi, T., Lee, B., Wypisniak, K., Doak, A., King, D., Usatyuk, S., Perry, K., Lu, V., Thomas, W., Luke, J., Goodman, J., Hoey, R.J., Lai, D., Griffin, C., Li, Z., Vizeacoumar, F.J., Dong, D., Campbell, E., Anderson, S., Zhong, N., Gräslund, S., Koide, S., Moffat, J., Sidhu, S., Kossiakoff, A. and Wells, J. (2015). A high through-put platform for recombinant antibodies to folded proteins. *Molecular & Cellular Proteomics, 14*(10):2833-2847. doi:*10.1074/mcp.O115.052209*

Huang, H., Economopoulos, N.O., Liu, B.A., Uetrecht, A., Gu, J., Jarvik, N., Nadeem, V., Pawson, T., Moffat, J., Miersch, S. and Sidhu, S.S. (2015). Selection of recombinant anti-SH3 domain antibodies by high-throughput phage display. *Protein Science, 24*(11):1890-1900. doi:*10.1002/pro.2799*

Mersmann, M., Meier, D., Mersmann, J., Helmsing, S., Nilsson, P., Gräslund, S., Structural Genomics Consortium, Colwill, K., Hust, M. and Dübel, S. (2010). Towards proteome scale antibody selections using phage display. *New Biotechnology, 27*(2):118-128. doi:*10.1016/j.nbt.2009.10.007*

Nelson, A., Dhimolea, E. and Reichert, J.M. (2010). Development trends for human monoclonal antibody therapeutics. *Nature Reviews. Drug Discovery, 9*(10):767-774. doi:*10.1038/nrd3229*

OECD (1992). Test No. 406: Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264070660-en*

OECD (2010a). Test No. 429: Skin Sensitisation: Local Lymph Node Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264071100-en*

OECD (2010b). Test No. 442A: Skin Sensitisation: Local Lymph Node Assay: DA, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264090972-en*

OECD (2015). Test No. 442C: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA), OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264229709-en*

OECD (2018a). Test No. 442B: Skin Sensitisation: Local Lymph Node Assay: BrdU-ELISA or –FCM, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264090996-en*

OECD (2018b). Test No. 442D: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264229822-en*

OECD (2018c). Test No. 442E: *In Vitro* Skin Sensitisation: *In Vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:*10.1787/9789264264359-en*

Schofield, D., Pope, A., Clementel, V., Buckell, J., Chapple, S., Clarke, K., Conquer, J., Crofts, A., Crowther, S., Dyson, M., Flack, G., Griffin, G., Hooks, Y., Howat, W., Kolb-Kokocinski, A., Kunze, S., Martin, C., Maslen, G., Mitchell, J., O'Sullivan, M., Perera, R., Roake, W., Shadbolt, S., Vincent, K., Warford, A., Wilson, W., Xie, J., Young, J. and McCafferty, J. (2007). Application of phage display to high throughput antibody generation and characterisation. *Genome Biolology, 8*(11):R254. doi:*10.1186/gb-2007-8-11-r254*

Taussig, M., Stoevesandt, O., Borrebaeck, C., Bradbury, A., Cahill, D., Cambillau, C., de Daruvar, A., Dübel, S., Eichler, J., Frank, R., Gibson, T., Gloriam, D., Gold, L., Herberg, F., Hermjakob, H., Hoheisel, J., Joos, T., Kallioniemi, O., Koegl, M., Konthur, Z., Korn, B., Kremmer, E., Krobitsch, S., Landegren, U., van der Maarel, S., McCafferty, J., Muyldermans, S., Nygren, P., Palcy, S., Plückthun, A., Polic, B., Przybylski, M., Saviranta, P., Sawyer, A., Sherman, D., Skerra, A., Templin, M., Ueffing, M. and Uhlén, M. (2007). ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. *Nature Methods, 4*:13–17. doi:*10.1038/nmeth0107-13*

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