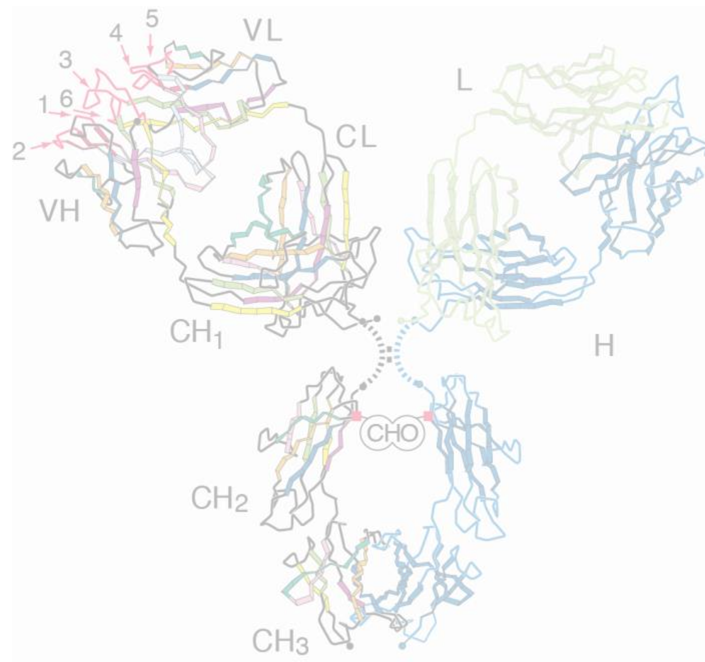
Understanding ANIMAL RESEARCH

lasa Laboratory Animal Science Association

The Physiological Society

# *in vitro vs in vivo* Antibodies Factsheet

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**Definitions as applied in this factsheet**

*in vivo* = in an animal
*in vitro* = in a test tube
B-cells = cells in the body that make antibodies
recombinantly = produced by genetic engineering
phage = a virus carrying antibody gene(s)
*E.coli* = the bacterium used in phage display
antigen = the target (protein) to which an antibody binds
transgenic = an animal with human antibody genes
hybridoma = a single immortalised B-cell which grows and makes antibody
affinity = the strength of binding of an antibody to its antigen
antibody library = a collection of billions of recombinant antibodies
diversity = the range of different antibody sequences

**Summary**

The bioscience sector is strongly supportive of the policy of using animal replacements in medical research where this is achievable. This document will focus upon the scientific and practical reasons that alternatives to animal antibodies are not yet achievable for all applications. It includes quotes throughout from specialist antibody scientists working in new medicines discovery.

**Context**

In May 2020, the European Commission's Joint Research Centre (EC-JRC) released a recommendation that non-animal-derived antibodies (ie derived *in vitro*) should be favoured over those produced by animals (*in vivo*). It made the assertion that non-animal methods were ready and able to replace animal-derived products. This assertion was in turn based on the scientific opinion of the EURL ECVAM Scientific Advisory Committee (ESAC), which has repeated this claim for more than 20 years. Several members of this committee have since gone on record to state that they disagree with this conclusion.

Now, as then, it is too strong a statement to claim that all applications can be undertaken to the same standard using *in vitro* methods, for a range of scientific and practical reasons; not least that animal antibodies have features that the non-animal antibodies do not.

**History of this issue at EVCAM**

The EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) defers to the scientific opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) about specific issues related to alternative methods. In 1998, 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 (which is no longer used in the UK) was no longer scientifically necessary, except in rare cases. This has been implemented in the UK in the area of bulk production of antibodies, which, importantly, differs from the generation of novel antibodies.

In 2018, the ESAC focused on antibody generation, highlighting the availability of non-animal-derived antibodies generated by phage-display, whereby a bacteriophage is genetically modified to display an antibody. This is the most mature *in vitro* technology, though there are others including yeast, ribosome and mammalian.

*"It's easy to make an antibody. It's incredibly difficult to make a therapeutic antibody."*

**Antibodies in brief**

Antibodies are molecules produced by the immune system to fight infection. They bind strongly to a unique target molecule (antigen), usually on the surface of a virus or bacterium. In this way they allow the body to kill and remove the infection. This specific strong binding also makes them crucial tools for biological research, diagnostics for disease, therapeutics to treat disease and regulatory procedures.

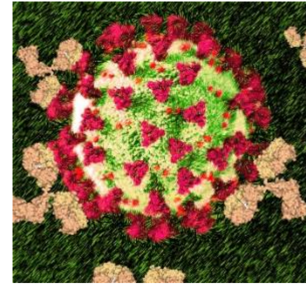

**Figure 1: Antibodies binding to a target virion**
Image adapted from: *Carbon Arc*/Wellcome

Animal-derived antibodies have unique properties, being based on biological processes that have evolved over millions of years that can't yet be matched by current technology. In practice this can make animal antibodies far better at binding to disease molecules than those produced *in vitro*. This has been clearly illustrated in the COVID pandemic, where the first therapeutic antibodies to be licensed to treat patients were isolated from infected humans or immunised transgenic mice.

In the animal-based or *in vivo* technique, animals are immunized with an antigen produced by a specific disease organism and antibodies are made in their B-cells. The spleen, and often lymph nodes, are removed from smaller animals such as mice, while a blood sample is sufficient for larger animals such as rabbits. The B-cells are then either fused with immortal cells to make hybridomas and grown in a cellular antibody 'factory'; or screened for binding, DNA-sequenced and expressed recombinantly. Recent advances in the techniques of B-cell isolation and single-cell sequencing, means animal use is decreasing whilst delivering an ever-larger diversity of antibodies, meaning more antibodies can be sampled from a single animal.

Producing antibodies in this way takes advantage of a number of naturally-occurring processes that influence the qualities of the antibody to make it more likely to bind to disease molecules and otherwise make it more fit for purpose. It also means that, whereas phage libraries are physically limited to 10 billion sequences (and yeast libraries, 0.1 billion sequences), an animal is naturally able to select the strongest binding antibody out of a possible 1,000 billion antibodies.

On the other hand, *in vitro* generation encodes antibodies that are different to those naturally made by animals. The diversity of these sequences can be increased by making many libraries.

However, realising the potential of artificially-created antibodies requires advanced technology for testing target molecules against these large libraries. In addition, a great deal of downstream work needs to be done in areas such as improving the developability of computer-designed antibodies, or in poorly understood areas such as how the immune system reacts to the antibody and how the antibody reacts in return.

It has long been the dream of antibody scientists to replace the use of animals in antibody generation. There has been much investment in *in vitro* platforms over the last 30 years, and there are some areas where an *in vitro* platform has been successful. However, this technology alone cannot always satisfy all of the complex properties required, especially when using antibodies to treat disease in patients.

To deliver the full therapeutic promise of biologics drugs to patients, both *in vivo* and *in vitro* antibody discovery approaches are required. Each of these methods has unique, and occasionally shared, advantages and disadvantages; however, using them in combination provides the best chance of finding new medicines that address almost any drug target across a range of diseases.

*"We are comparing 450 million years of evolution with a few decades of technology."*

**Phage display for animal replacement**

The ESAC focused on *in vitro* phage display technologies as animal replacements, which use bacteria to generate the antibody, instead of animals. This is an elegant technology, whereby synthetic antibody genes encoding antibodies are combined on a strand of DNA; this is then placed inside a virus that only infects *E.coli* bacteria. This is called a 'phage virus' or 'bacteriophage'. Each phage carries one antibody gene and expresses the antibody fragment on its surface. Many phage antibodies with different binding sequences can be produced in *E.coli,* to create a 'library' of phages that carry the antibody fragments on their surface. When the phage library is mixed with the disease target, the phage carrying the specific antibody fragment binds to it, and the genetic sequence to produce that antibody is determined from the phage DNA.

Phage-derived antibodies have been successful in a number of applications; these include therapeutics, as binding reagents for *in vitro* assays and as crystallisation partners. Phage technology is useful in identifying novel binders and in driving the field of multi-specific antibody formats.

**Areas where *in vitro* technologies alone may be advantageous**

| | |
|---|---|
| **Target:**<br>When the target or medium is toxic to animals | **Binding site selection:**<br>When the epitope (part of antigen molecule that binds to antibody) to be targeted is known |
| **Application:**<br>Proven use of the technology for applications including binding assays and crystallisation partners | **Biology:**<br>When the animal is likely to be unresponsive to the target and greater control over sequence |

There is currently a lack of *in vitro* libraries for species other than humans, which is an issue for both the human and veterinary research field. There are also not yet any universally accessible phage, yeast, ribosome or mRNA display technologies.

*"It will take at least 10 years of further investment in in vitro platforms before we know whether in vivo methods can be replaced."*

**What phage displays do not do**

Antibodies created by *in vitro* methods can be more difficult to refine to be suitable to use. When a binder is found (an antibody that binds to its antigen successfully) , further libraries may be required to select an antibody with the right binding qualities, with no guarantee of success. The diversity of *in vitro* libraries is limited by the number of variants that can be physically created and tested. The availability of suitable purified target protein for use in the phage display process, particularly for certain target classes that required a cell membrane for appropriate conformation.

Animal biology is thought to be able to select the strongest binding antibody out of a possible one trillion antibodies. This is because of a complex process *in vivo* known as affinity maturation/somatic hypermutation, which occurs at the onset of the immune response in the animal; it continues under the bodily conditions created by the presence of the disease. This gives the *in vivo* response a greater chance of providing clinical-quality antibodies in the shortest timeframe.

Phage particles express antibody fragments, and there is no guarantee that these fragments will retain all the required properties, nor that in the final therapeutic format they will still function as a whole antibody. When antibodies are raised in animals, they are naturally stable *in vivo*. These limitations apply to all current *in vitro* antibody generation platforms, and computer modelling has not yet been able to replace the natural antibody evolution in an animal.

These factors can extend the timelines in both the research and the development stages of an antibody therapeutic . In the Covid Pandemic, out of more than 150 therapeutic antibody products in pre-clinical development, the vast majority and fastest to clinical approval were derived from *in vivo* sources.

**Areas where *in vivo* antibodies outperform *in vitro***

| | |
|---|---|
| **Target:**<br>A broad range of target formats can be used to immunise animals, such as protein, DNA, RNA and cells | **Affinity:**<br>During infection, the animal constantly improves the strength of binding of the antibody to the target |
| **Application:**<br>Animal-derived antibodies are highly specific, so widely used in diagnosis and therapeutics | **Biology:**<br>Naturally stable in animals, so good record of safety and developability |

*"A therapeutic antibody, with potential to treat the global population, can be generated by one mouse in 2 months."*

**The combined approach**

Antibodies generated by *in vitro* methods are different to those arising *in vivo*; using both platforms increases the chance of finding the best antibody. While the phage method is dependent on a limited format of target, the *in vivo* process can use many formats. Recent advances in the antibody field use a combination of *in vivo* and *in vitro* methods: following *in vivo* immunisation, *in vitro* selection methods can be used. This combined *in vitro/in vivo* approach results in highly tailored antibodies that can be more potent than those produced by either *in vivo* or *in vitro* techniques alone.

**What is a Tool Antibody?**

Most of this document refers to therapeutic antibodies. The term "tool antibodies" describes antibodies that are not administered as therapeutics to humans. They are used in a broad range of applications such as target detection, disease models, product quality and as indicators of protection to disease.

Tool antibodies are an essential step on the path to a creating a new medicine. When a target for a medicine is proposed, the first step is to demonstrate the presence and role of the target in that disease. This is done by checking patients' samples and establishing a research path. Tool antibodies must be of high quality and specificity, or the project will fail. The tool antibody also lays down the qualities that need to be present to make a successful therapeutic antibody for that disease.

Tool antibodies are generated in the same way as therapeutic antibodies, but often in different species. Different species of animal provide antibodies of specialised function, such as the exquisite sensitivities of rabbit antibodies, which are used to probe diseased tissue. Many companies sell large collections of such antibodies for use in research and development. These catalogues are extensively searched, and the antibodies are tested for suitability, before any new antibody discovery project is initiated. Many tool antibodies can be made *in vitro* and this is always considered the first choice of strategy.

**Commercial considerations**

The therapeutic antibody market is worth $125Bn annually and is expected to reach twice this in five years' time. Currently, out of 80 US FDA-approved monoclonal antibodies, 89% are from hybridoma or transgenic mouse origin (Lu et al, 2020). This number of successful treatments is reflected by the sales they generate, which are almost double those of phage library origin.

In addition, the apparent success of drugs created using phage libraries is distorted by the success of a single drug, Humira, which was approved almost 20 years ago. This was a huge step for phage display technologies, and its discovery was predicated on a pre-existing murine antibody. Since then, only a handful of antibodies derived from phage display have won approval.

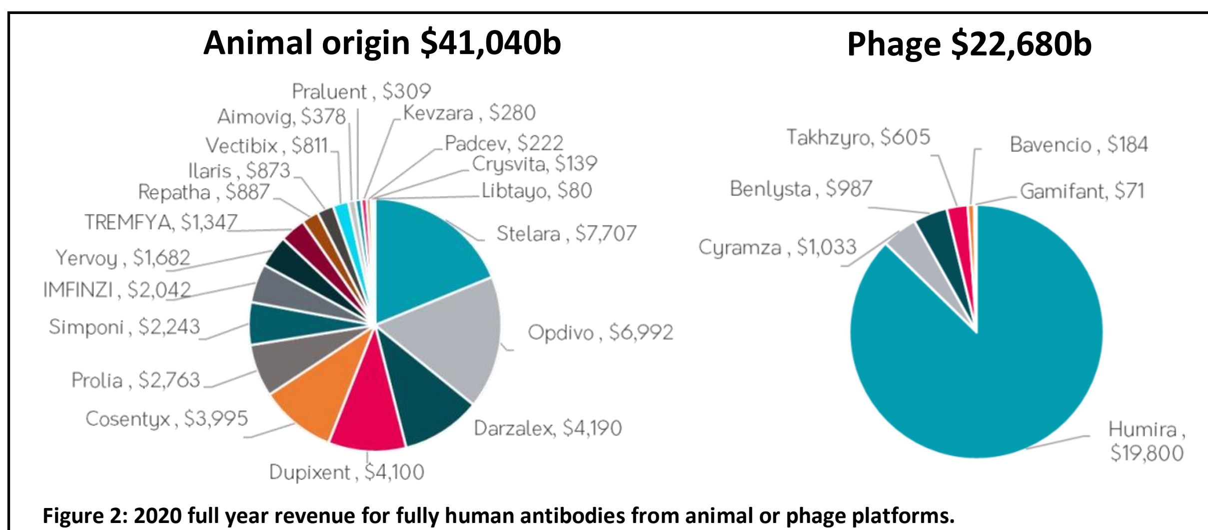


**Figure 2: 2020 full year revenue for fully human antibodies from animal or phage platforms.**

### *In vitro* and *In vivo* antibody comparison

| ***In vivo* advantages** | ***In vitro* advantages** |
|---|---|
| 1. A broad choice of antigen format; including native - enables generation of antibodies against cDNA encoded targets or in forms not suitable for *in vitro* technologies<br>2. High specificity and affinity due to natural selection and affinity maturation process<br>3. High solubility and stability, naturally selected for secretion and *in vivo* performance<br>4. Transgenic mice with human antibodies allow isolation of human variable regions to prevent immunogenic reactions in the patient<br>5. More successful in reaching approved status<br>6. Large numbers of top-quality antibodies can be identified from one animal<br>7. Integrates mammalian *in vivo* posttranslational modifications; such as glycosylation<br>8. Highly available in the antibody market (89%)<br>9. Technically easy and quick to generate (typically 2-3 months)<br>10. Technology well-established in academic and applied research<br>11. Can synergise with *in vitro* technologies to maximise success of antibody recovery | 1. No animal use (for synthetic libraries)<br>2. A greater theoretical diversity can be achieved<br>3. Not limited to the germline of the strain of the animal<br>*4.* Potentially larger output than *in vivo*<br>5. Large range of human germlines/individuals can be used in the starting library<br>6. Fast process (once libraries have been established) if affinity maturation of the clones is not required<br>7. Possible to directly screen human libraries<br>8. Possible to screen non-immunogenic antigens<br>9. Possible to screen toxic antigens<br>10. Can synergise with *in vivo* technologies to maximise success of antibody recovery |
| ***In vivo* drawbacks** | ***In vitro* drawbacks** |
| 1. Can take up to 8 months generation time (although rapid immunisation protocols can be 1 month)<br>2. May require humanisation or use of proprietary transgenic animal strains for therapeutic antibodies<br>3. Limitations with non-immunogenic antigens<br>4. Limitations with toxic antigens<br>5. Requires immunisation of animals<br>6. Requires application of Animal Use regulations | 1. It is technically challenging to create large phage libraries that deliver diverse panel of top-quality antibodies.<br>2. Limited antigen formats when panning. Restricted to purified protein and/or peptides and high-expressing cell lines. Some challenging target classes, e.g. integral membrane proteins are likely to be incompatible.<br>3. The antigen format may also prevent affinity maturation of isolated binders.<br>4. For some of the in vitro antibodies an optimisation process is required. Without it, they might have lower affinity, specificity and immunogenicity issues. Many of these processes are underdeveloped and add time and cost to production.<br>5. Binders often have properties that are non-optimal for manufacturing and clinical use.<br>6. Phage display antibodies may not have the same binding properties when they are reformatted back to whole IgG<br>7. Lack mammalian post-translational modifications during the panning process that can then adversely affect the whole IgG properties<br>8. Low availability in the antibody market (11% of approved antibodies)<br>9. Technically more difficult to generate and requires extensive automation to screen full diversity<br>10. Difficult and expensive to implement in academia, start-ups and small-medium sized companies<br>11. 6-7 months generation time for one-off development of a library. Currently a lack of libraries for species other than humans, rabbits & camelids (issue for the veterinary research field)<br>12. Can require animal usage for immune and naive libraries. |

*Table expanded from "EARA/EFPIA response to EURL ECVAM Recommendation on Non-Animal-Derived antibodies."*