ProteoGenix

Transient expression handbook

Mammalian cell-based recombinant protein expression and purification technologies

EDUCATIONAL RESOURCES

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Content

Part A: Overview of recombinant protein expression technologies

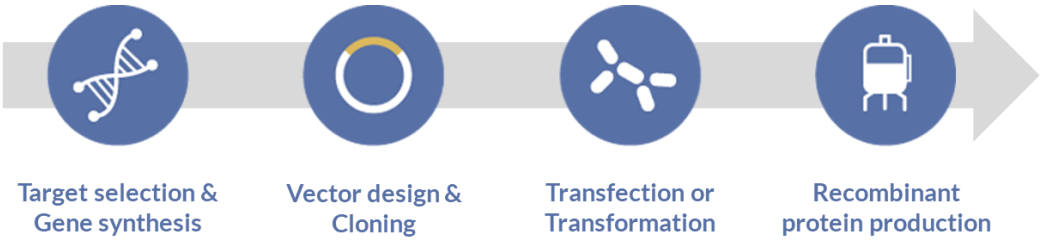
The expression of recombinant proteins represents one of the most significant breakthroughs in the life sciences. The technology was made possible by the advent of gene synthesis, genetic engineering, and the improvement of cell culture methods. Today, recombinant expression supports several applications including functional studies, bioanalysis, and biopharmaceutical production.

How are recombinant proteins produced?

Recombinant proteins are produced by cloning **one or several genes of interest** into an **expression vector** (i.e., plasmids or viral vectors). These synthetic constructs are subsequently introduced into an **expression host** via transformation, transfection, or transduction. In addition to the gene of interest, which typically encodes a target protein with a desired biological activity, expression vectors contain other elements such as **promoters** (inducers and modulators of expression), **selectable markers**, and **one or several expression tags** meant to improve purification, solubility, among other properties of the target protein.

The first successful expression of a recombinant protein took place in the 1970s when the hormone somatostatin was produced in *Escherichia coli*. Bacterial systems proved to be **amenable to engineering** and **easy to handle**. Plus, their **high turnover rates** and **inexpensive requirements** made them one of the most popular hosts for protein production.

Despite their cost-efficiency, these systems found limited applicability in the expression of **complex, glycosylated proteins**. As a result, eukaryotic expression systems arose as powerful alternatives. Today, prominent eukaryotic-based systems include cell-based mammalian, baculovirus/insect cells, and yeast expression systems such as *Streptomyces cerevisiae* and *Pichia pastoris*. In recent years, **mammalian systems have come to dominate the market**, especially in terms of drug screening and biopharmaceutical production.



Differences between recombinant and natural proteins

Before the onset of recombinant technologies, proteins were produced natively and extracted from the original organisms using time-consuming and inefficient processes. Today, very few applications still rely on native proteins. What makes recombinant proteins so advantageous for so many applications?

Higher purity	Most native organisms **grow very poorly in chemically defined media** and often require animal components to support *in vitro* conditions. These components are known to **interfere with purification** processes, leading to lower yields and purity. Moreover, extraction methods used for native proteins are often harsh, risking the integrity and stability of the target protein. This limitation is easily overcome by recombinant production.
High and consistent yields	Recombinant expression systems are continuously being engineered and optimized for protein production. In this way, most commercially available systems can achieve **moderate to high titers consistently and independently of the origin and structure of the target protein**.
Standardization of biological reagents	The lower purity of native proteins and inconsistent yields hinders attempts at the standardization of bioassays. In contrast, recombinant protein stocks not only possess higher purity, they are also better characterized and their **structure and amino acid composition** is widely known.
Intellectual property protection	Recombinant production requires the sequence of a specific protein, for this reason, it is easier to store the information and to protect it if desired.
Ease of engineering	The sequences of recombinant proteins are easily available, thus, they can be analyzed and engineered *in silico* with the intent of improving, enhancing, or minimizing specific properties such as affinity, stability, or biological activity. Moreover, these proteins are easily adapted to different formats.
Animal-free protein production	Recombinant protein production is carried out in chemically defined and animal-free media, thus, adhering to current recommendations for reducing the use of animals in scientific research.
Commercial solutions	As the field continues to push towards standardization, many commercial and accessible solutions for recombinant expression have become available.

Types of expression systems

Mammalian systems

Chinese hamster ovary (CHO), human embryonic kidney (HEK293), baby hamster kidney (BHK-21), Per.C6, and mouse myeloma (NS0) cells

Insect systems

- Order *Lepidoptera* (**moths and butterflies**): TN-368, BTI-Tn5B1-4, Tni PRO, IPLB-Sf21AE, Sf900+, Bm-N
- Order *Diptera* (**flies**): S2 and S2R+ from *Drosophila melanogaster*

Yeast systems

Saccharomyces cerevisiae and *Pichia Pastoris*

Bacterial systems

Escherichia coli and *Bacillus subtilis*

How to select an expression system

Bacteria, yeast, insect cells, and mammalian cells are all widely used systems for recombinant protein production. Each system has its unique advantages and limitations:

	ADVANTAGES	LIMITATIONS
Mammalian systems	• Enhanced protein folding • Human-like glycosylation • Efficient protein secretion and cost-effective purification • High batch-to-batch consistency • High purity • High production yields meeting important quality standards • Adapted to most types of proteins • Fast production possible (transient)	• Longer lead times (stable) • Time-consuming and laborious (stable) • Cell lines are harder to engineer due to the complexity of their genomes • Need for incubators with CO_2 gassing to maintain suitable pH values • Risk of viral infections or contamination with mycoplasma
Insect systems	• Good production yields • Adequate protein folding • Ability to carry out complex post-translational modifications – comparable to mammalian systems • Production is easily scaled up	• Time-consuming and laborious process due to the need to produce baculovirus (bacmid) for transduction • Risk of cell lysis upon infection – may reduce protein purity and quality
Yeast systems	• Efficient protein secretion – easier and cost-effective purification • Ease of genetic manipulation • Short turnaround times • Ability to carry out some post-translational modifications • No stable integration required for large-scale production • Production is easily scaled up	• Moderate risk of plasmid loss even in selective conditions • Inability to perform human-like glycosylation without extensive engineering • Yeast cells may be difficult to lyse resulting in protein denaturation during extraction and the need for *in vitro* refolding
Bacterial systems	• Extremely short turnaround times • Advantageous for antibody fragment (scFv, Fab, VHH) and small protein production • Ease of genetic manipulation • No stable integration required for large-scale production • Production is easily scaled up • Extremely cost-effective • Inexpensive medium and growth conditions required	• No post-translational modifications • Intracellular production – harsher purification methods required • Risks of forming inclusion bodies leading to low yields • Contamination with endotoxins (*E. coli*) requiring additional purification steps when proteins are meant for biopharmaceutical or cell culture use

Despite the different advantages and limitations of these systems, it is important to bear in mind that choosing an expression system depends, first and foremost of:

- **The origin of the target protein** – bacterial proteins are better expressed in bacterial systems, while mammalian proteins are better expressed in mammalian systems. For this reason, mammalian cell lines like CHO (Chinese Hamster Ovary) or HEK (Human Embryonic Kidney) are the systems of choice for human protein production
- **Required post-translational modifications** – proteins devoid of complex modifications (e.g., glycosylation, alkylation, phosphorylation, or specific proteolytic processing) are more easily produced in simpler systems with short turnaround times such as bacterial and yeast. In contrast, complex proteins with significant modifications should be produced either in insect or mammalian cells
- **Solubility of the recombinant protein** – some proteins are not properly folded in bacterial systems, as a result, they tend to form insoluble aggregates (inclusion bodies) that are difficult to extract. In these circumstances, higher eukaryotic systems should be preferred for the recombinant production of the target protein
- **Intended application of the recombinant protein** – even complex proteins such as IgG-like antibodies may be produced in simpler systems with high turnover systems. In these proteins, glycans are restricted to the Fc fragment (crystallizable fragment) and modulate the effector functions of these biomolecules. However, even devoid of glycans, antibodies can bind to target antigens. For this reason, antibodies for research (functional studies, medical, or basic research) and *in vitro* diagnostics ca be produced in bacteria or yeast, because effector functions are non-essential for these applications. In contrast, antibodies intended for therapeutic use are mostly produced in mammalian systems able to perform human-like glycosylation essential to modulate the immune response.

Differences between transient and stable gene expression

The terms transient and stable expression are used to describe two different gene expression strategies for protein production in higher eukaryotic systems, such as mammalian cells. Most higher organisms are naturally devoid of the genetic machinery necessary to replicate plasmids independently of their genome. Thus, exogenous DNA vectors are usually lost after some cycles of cell division. For this reason, when mammalian cells are transfected with conventional vectors, expression is only temporarily achieved. This is called transient expression and it is a widely used process due to its short lead times and cost-effective productivity.

Learn more about our transient expression services at proteogenix.science/protein-production/mammalian-cells-expression

To achieve ultra-high yields in the long term, another strategy must be employed. In contrast to transient expression, the exogenous DNA vectors must be stably integrated into the host's genome. This is achieved by a number of different strategies including random or transposon-mediated integration. Integration events are considered rare, for this reason, single clones need to be isolated from stable pools of cells in order to ensure consistent yields and avoid the loss of positive single clones via dilution or consecutive freeze-thawing cycles.

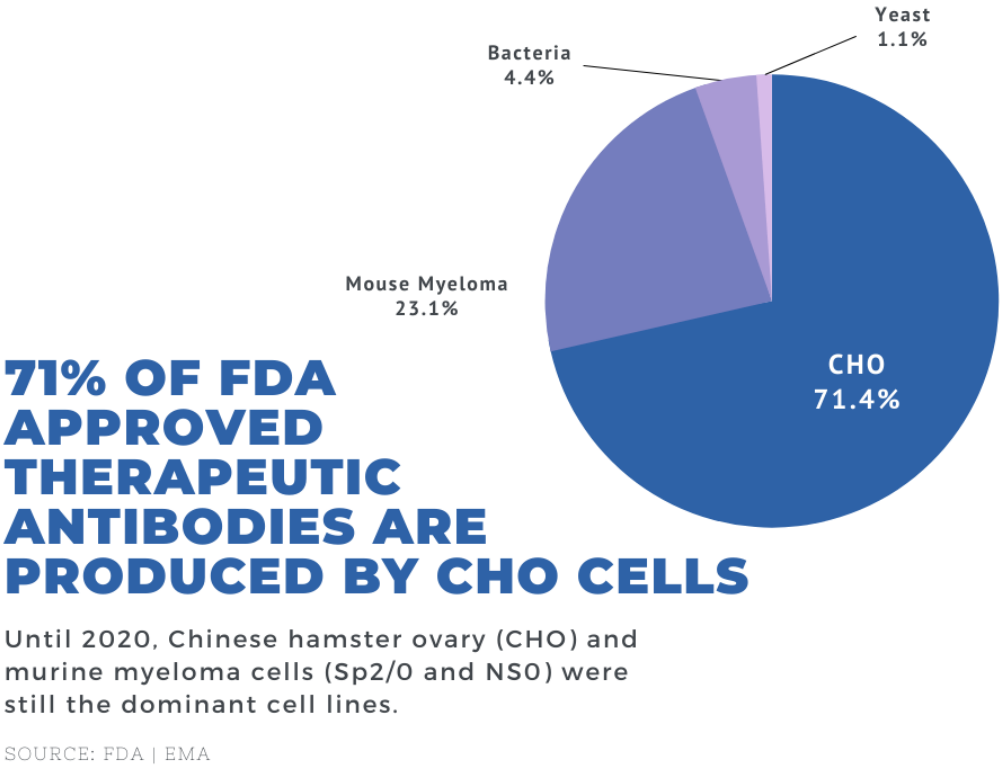
Learn more about our stable cell line generation services at proteogenix.science/bioprocess/stable-cell-line

Part B: Mammalian cell-based transient gene expression

Mammalian expression systems remain the system of choice for the production of eukaryotic and human-like proteins with complex structures and post-translational modifications. Despite the inherent complexity of their genomes, mammalian cell lines offer important advantages, namely, high yields and superior protein folding, which influence protein stability and activity. These two properties are highly prized when working with biopharmaceuticals and cell-based functional assays.

Mammalian cell lines used for high-yield production

Despite the vast diversity of mammalian cell lines available for recombinant production, **CHO and HEK** cells continue to be the industry's standard when it comes to complex protein expression. However, these two cell lines are used for widely different applications. CHO are prized for their robust and high yields. For this reason, about **60-70% of biopharmaceuticals approved by the Food and Drug Administration (FDA) are produced in these systems.** In contrast, the human HEK cell lines are typically reserved for research and diagnostics. Due to their origin, these cells can harbor human pathogens, resulting in an added risk for staff and the need to include additional viral inactivation and clearance steps.



Chinese Hamster Ovary (CHO) cells

CHO cells are today's industry most important workhorse for therapeutic protein production. Chinese hamsters were first used as laboratory specimens as early as 1919. However, cell cultures were only obtained in the late 1950s by Theodore Puck (University of Colorado, USA). Cultures containing cells isolated from an ovary of a female Chinese hamster soon revealed to be resilient, fast-growing, and quick to adapt to *in vitro* conditions.

Interestingly, the ease of genetic manipulation by mutagenesis has led to the establishment of many CHO-derived daughter cell lines with improved properties. Today, the most important CHO-derived cell lines include **CHO-K1, CHO-S, CHO-DXB11, and CHO-DG44**.

CHO's main variants were mostly obtained by random mutagenesis and selection of the original parental cell line. For instance, **CHO-S** was obtained when the parental CHO cell line was first adapted to growth in **suspension cultures**, essential for the large-scale production of biopharmaceuticals. While **K1** was cloned directly from the original cell line and later found to lack a gene necessary for **glycine synthesis**, a deficiency that paved the way for the creation of mutants with high value for recombinant transfection and **metabolic selection**.

The first approved therapeutic monoclonal antibody produced in a CHO cell line was Rituxan, an anti-CD20 antibody used for the treatment of non-Hodgkin's lymphoma (Genentech, 1997). Since then, researchers have generated impressive amounts of data on CHO genetics, which recently culminated with the determination of the CHO genome in the early 2010s. This discovery will certainly lead to a new generation of engineered CHO variants optimized for selection and gene amplification.

Human Embryonic Kidney (HEK) cells

As the name indicates, HEK cell lines were originally isolated from human embryonic kidney cells grown in tissue culture. The cell line was popularized by scientists from the University of Leiden (The Netherlands), who **transfected normal HEK cells with the DNA of sheared adenovirus 5.** This transfection resulted in the insertion of approximately **4.5 kb of the viral genome into chromosome 19.** The clonal subpopulation became known as HEK293 because it resulted from the 293rd attempt at transfecting the parental HEK cell line.

The incorporation of this region of the viral genome conferred HEK cells the **ability to recognize the CMV promoter region** and, thus, it can be used to increase expression levels of CMV-based vectors. The HEK293 variant was further used as the basis of subsequent transfection experiments. The two most relevant variants that resulted from these experiments are variant **HEK293T** and **HEK293E**.

The former resulted from transfection with the large antigen T of simian virus 40 (SV40). While the latter resulted from transfection with the nuclear antigen 1 (EBNA1) of the Epstein Barr virus (EBV). These transfected variants can recognize the origin of replication (*ori*) of the SV40 and EBV, respectively. Hence, they are capable of carrying out the semi-stable episomal propagation of vectors containing these *ori*. **HEK293-derived cell lines are thus easily transfected and can grow quite rapidly in suspension cultures.**

Most research on HEK293-based cell line centers around the search for better selectable markers and around strategies to improve the safety of these cells, thus making them more suited to biopharmaceutical production.

Expression vectors for transient mammalian systems

A typical expression vector used in mammalian systems may include the following elements:

- **One or several genes of interest (GOI)** associated with a strong **promoter** and a transcription terminator.
- **Bacterial origin of replication (ori)** – although mammalian systems are typically unable to recognize bacterial origins or naturally replicate plasmids, this element is used during the process of cloning the GOI into the vector and amplifying the construct. A step typically carried out in hosts that are easy to handle such as *E. coli*.
- **A selectable marker** – mammalian cell selection can be carried out via antibiotic or metabolic selection.
- **Reporter gene** – because transient systems rarely undergo a process of single clone selection, the efficiency of the transfection process can be assessed by measuring the expression of a specific reporter gene. This gene typically encodes fluorescent proteins such as GFP (green fluorescent protein) that can be easily be measured via non-destructive methods.
- **Tags** – affinity or solubility tags are typically included in mammalian expression vectors to improve the stability of the protein of interest and enhance the efficiency of the purification process, respectively.
- **Optional elements** – signal peptides can be used to improve translocate the protein of interest to the cell membrane and enhance protein secretion; protease recognition sites can be used to allow the cleavage of the peptide link between tags and the protein of interest; among others.

Commonly used promoters in mammalian systems

Promoters modulate the transcription process by controlling the binding of the RNA polymerase and associated factors to the DNA chain. This element dictates the timing and amount of protein that will be produced by a specific recombinant system. These elements are generally classified as **constitutive, inducible, or repressible**.

The first type of promoter is always active independently of the conditions. In contrast, inducible promoters are only activated by specific signals, acting like an **ON switch**. Repressible promoters have found limited applicability in recombinant protein production. However, they are substantially used in research. In their basal state, these promoters are always active, but they can be repressed by specific signals. Thus, these promoters act as **OFF switches.**

In the table below, you can find a list of the most commonly used promoters in mammalian recombinant expression:

Promoter	Origin	Promoter type
CMV	Human cytomegalovirus	Constitutive
EF1A	Human elongation factor alpha	Constitutive
SV40	Simian vacuolation virus 40	Constitutive
UBC	Human ubiquitin C gene	Constitutive
PGK	Human or mouse phosphoglycerate kinase gene	Constitutive
CAG	Synthetic promoter constructed from CMV, chicken beta-actin gene, and rabbit beta-globin gene	Constitutive
TET	*E. coli* Tn10-specified tetracycline-resistance operon	Inducible (tetracycline)
GAL4/UAS	Yeast transcriptional activator GAL4 and upstream activation sequence UAS	Inducible (heat shock)
cTA	*p-cmt* and *p-cym* operon in *Pseudomonas putida*	Inducible (cumate)

Constitutive promoters are dominant among mammalian systems. They can deliver high-level expression without the need to use additional signals (i.e., chemicals, heat, etc.), which are often toxic, expensive, or known to affect the integrity of the mammalian cell. This uncontrolled expression becomes disadvantageous only when expressing toxic proteins for long periods of time. In these specific circumstances, inducible or repressible promoters can be used to minimize the risks of early cell death and maintain cell viability for as long as possible.

Selectable markers for mammalian systems

The process of selection in mammalian expression systems can be carried out using metabolic (DHFR and GS) or antibiotic markers. Interestingly, most CHO-based systems rely on metabolic selection while HEK293-based systems tend to rely on antibiotic markers. Metabolic selection has a certain advantage over antibiotic markers for the following reasons:

- Unlike antibiotics, metabolites required for metabolic selection are usually **non-toxic** and **easily degradable**
- Metabolites tend to be **less expensive** than antibiotics – thus minimizing production costs at the large scale

Metabolic selection: DHFR system

The DHFR system was first established with the CHO-DXB11 cell line, obtained by chemical mutagenesis of the parental cell line with ethyl methanesulfonate. This process caused the alteration of the two alleles of the **dihydrofolate reductase gene (*dhfr*).** Hence, the resulting mutant became unable to synthesize several molecules including glycine, thymine, and purine.

This ability could be easily restored by transfecting the DXB11 with a functional copy of the *dhfr* gene, rendering the cells **susceptible to metabolic selection.** Selection in DXB11 could then be easily achieved by co-transfecting with both the **protein of interest** and the **selective element** – the *dhfr* gene. Only cells successfully transfected with this vector would be able to grow in **media devoid of glycine, hypoxanthine, and thymidine**, three essential factors for nucleotide synthesis in **DHFR- mutants**.

However, soon researchers found that DXB11 cells had limited usefulness, as they could spontaneously revert to a functional *dhfr* gene. This drawback led to the generation of the **CHO-DG44 cell line**. Unlike DXB11, **DG44 mutants were obtained by mutagenesis with gamma radiation** and this process caused the irreversible deletion of both alleles of the *dhfr* gene. For this reason, to this day DG44 is still one of the dominant CHO cell lines on the market.

Metabolic selection: GS system

The **glutamine synthetase (GS) selection process** arose as an alternative to the DHFR system. This system makes use of the activity of GS, which catalyzes the condensation of glutamate and ammonia to generate glutamine.

GS is the only enzyme able to perform *de novo* glutamine synthesis, moreover, since it uses ammonia for the process, it also prevents tissues from accumulating toxic levels of this metabolite. The GS gene is a dominant selectable marker. For this reason, without glutamine in the growth medium or GS activity, cells cannot survive. The system was initially designed for cell lines that don't express sufficient amounts of the GS enzyme such as mouse myeloma (NS0), but it was successfully adapted to CHO cell lines with basal GS expression by adding **methionine sulfoximine (MSX) to the medium, which is a powerful GS inhibitor.** In this case, positive transfection can be selected by culturing cells in **media with both MSX and glutamine.**

Compared to the DHFR system, the GS-based selection has a short turnaround time because it is not mandatory to knockout GS on the expression host. However, some mammalian cells may be extremely resistant to high MSX concentrations (5 mM), which may increase the abundance of negative clones.

Antibiotic selection

The following selectable markers are typically used for antibiotic-dependent selection in mammalian systems:

- **Blasticidin** – this antibiotic inhibits the termination of protein translation, and resistance to this drug can be conferred by the *bsd* gene
- **G418/Geneticin** – this antibiotic inhibits peptide chain elongation and resistance can be conferred by the *neo* gene
- **Hygromycin B** – like **G418**, this antibiotic inhibits chain elongation and resistance can be conferred by the *hygB* gene
- **Puromycin** – inhibits protein synthesis, resistance can be conferred by the *pac* gene
- **Zeocin** – it complexes with the DNA causing strand scissions, resistance can be conferred by transfection with the *Sh ble* gene

Fusion tags used in mammalian systems

Fusion tags can be short peptide sequences or large proteins. They are recurrently used to improve purification (**affinity or epitope tags**), solubility (**solubility tags**), or simply as a means to monitor or visualize protein expression (**fluorescent tags**). Tags can be removed by chemical or enzymatic methods. However, this is only done if the activity or structure of the recombinant protein are severely affected. In fact, many tags were found to improve stability during storage and extend the shelf-life of recombinant proteins.

Large tags are more commonly used to improve solubility, while shorter tags are more commonly used to mediate protein purification processes. In comparison, affinity tags are also larger than epitope tags. The first ones are fused to the protein of interest, allowing the purification via **affinity chromatography,** while the second (epitope) tags are typically engineered into the protein of interest, allowing **antibody-mediated purification**. Both affinity and antibody-mediated purification processes can be carried out by immobilizing antibodies or other compounds on resins (**affinity purification**) or on magnetic beads (**magnetic-based purification**). The former is suitable for large volumes, while the latter is more suitable for small-scale purification.

Tag name	Type	Sequence or Size	Matrix
Hemagglutinin (HA)	Epitope	YPYDVPDYA **(9)**	Anti-HA antibody
c-myc	Epitope	EQKLISEEDL **(10)**	Anti-Myc antibody
V5	Epitope	GKPIPNPLLGLDST **(14)**	Anti-V5 antibody
FLAG™	Epitope	DYKDDDDK **(8)**	Anti-FLAG antibody
Poly-His	Affinity	nH **(n=2-12, usually 6)**	Metal ions
Poly-Arg	Affinity	nR **(n=5-6, usually 5)**	Cation exchange resin
Strep-tag	Affinity	WRHPQFGG **(8)**	Streptavidin

Strep-tag II	Affinity	WSHPQFEK (**8**)	StrepTactin
Twin-Strep-tag	Affinity	3 kDa	StrepTactin
Biotin acceptor protein (BAP)	Affinity	GLNDIFEAQKIEWHE **(15)**	Avidin
Calmodulin-binding protein (CBP)	Affinity	3 kDa	Calmodulin
Chitin-binding tag	Affinity	6 kDa	Chitin
Maltose-binding (MBP) tag	Solubility	43 kDa	Transversely-linked amylose
Glutathione-S-transferase (GST) tag	Solubility	27 kDa	Glutathione
SUMO tag (small ubiquitin-like modifier)	Solubility	12 kDa	Accommodates any type of affinity tags such as 6xHis
NusA tag	Solubility	55 kDa	
TrxA	Solubility	24 kDa	
Fh8	Solubility	8 kDa	
Halo	Solubility	33 kDa	Chloroalkanes
Green Fluorescent Protein (GFP)	Fluorescence	27 kDa	Anti-GFP antibody or fusion with an affinity tag
Red Fluorescent Protein (RFP)	Fluorescence	26 kDa	Anti-RFP antibody or fusion with an affinity tag
mCherry	Fluorescence	28 kDa	Anti-mCherry antibody

Tag names shown in bold represent the most commonly used fusion tags.

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Of all tags mentioned, it is worth mentioning that **fluorescent tags** are extremely useful when working with **transient mammalian systems**. Because transient systems tend to lose their expression vectors with each cell division cycle, it is not recommended to select and isolate positive clones before protein production. In these systems, high transfection efficiencies directly correlate with high protein titers. For this reason, researchers use fluorescent tags to **measure and optimize transfection efficiency**.

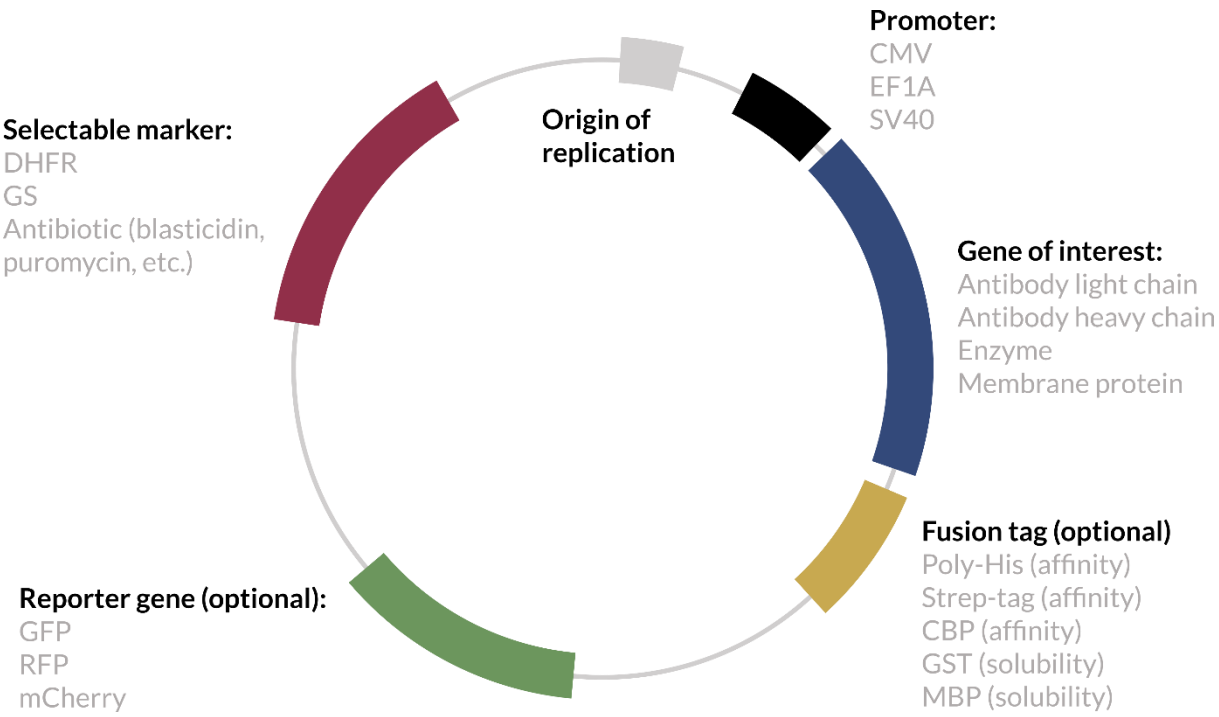
Affinity and epitope tags are recurrently used in mammalian systems for the purification of non-antibody proteins. In contrast, antibody molecules can be purified very easily without making use of these tags. A detailed overview of antibody purification methods can be found on Part C of this handbook.

Transfection approaches for transient systems

Transfection represents the process of **delivering a plasmid vector** to a recombinant system. Mammalian transient systems can be transfected using chemical or physical methods. However, chemical methods remain the most popular and widely used approach of the two due to the ease, low cost, and wide variety of commercially available transfection reagents.

The most common transfection methods used in transient systems include:

- **Chemical methods**
  - **Lipotransfection**: liposomes are synthetic analogs of the phospholipid bilayer, for this reason, they fuse easily with cell membranes. Liposomes encapsulate free DNA and RNA molecules quite efficiently, thus forming a simple delivery system able to introduce these nucleic acids into the cytoplasm of mammalian cells by endocytosis. These are considered first-generation chemical transfection methods and have only been sparsely used since the development of newer, more effective reagents.
  - **Cationic lipid transfection**: this method leverages the ease of fusion of liposomes with cationic polymers' natural ability to form strong complexes with negatively charged nucleic acids (DNA or RNA). Like first-generation reagents, these are taken up by the cell via endocytosis and subsequently release their cargo into the cytoplasm. Exogenous DNA is further translocated to the cell nucleus to be transcribed, while RNA-based vectors stay in the cytoplasm for direct translation.
- **Physical methods**
  - **Electroporation**: this method uses electrical pulses to create temporary pores in cell membranes. This process makes membranes permeable to negatively charged nucleic acids and allows them to migrate directly into the cytoplasm without the need for additional reagents. Electroporation typically results in higher transfection rates. However, it is more labor-intensive and harder to optimize than conventional chemical methods. One of the most important drawbacks of this method is the substantial cell death caused by high voltage pulses. For this reason, this method also requires a greater quantity of cells in comparison to chemical methods.



Typical expression vector used in mammalian systems

Part C: Recombinant antibody production in mammalian systems

Recombinant antibodies have become one of the most successful biological reagents for multiple applications. They are extremely versatile, highly specific, and stable. In this section, we provide an overview of their structure, function, therapeutic potential, use in immunoassays, and cover the principles of recombinant antibody production in transient mammalian systems.

Structure of monoclonal antibodies

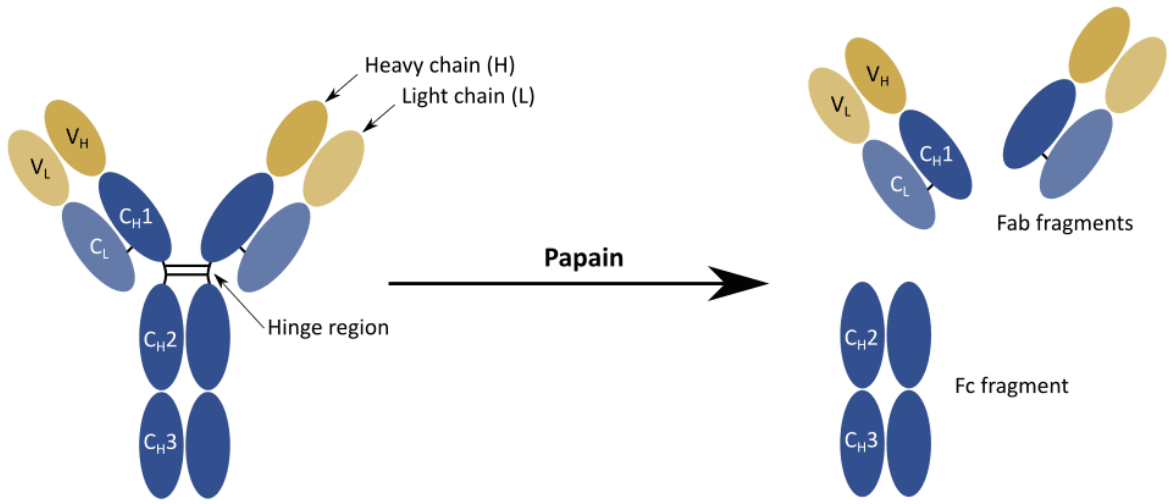
When we think of monoclonal antibodies, it is the IgG isotype that comes to mind. This isotype was the first to be fully elucidated, due to its high abundance and ease of isolation from serum. The IgG isoform is a monomeric immunoglobulin consisting of two heavy (H) chains and two light (L) chains. The interaction between these chains confers these molecules the canonical "Y" shaped structure that acts as a bridge between foreign antigens and our immune system.

The primary structure of V and H chains consist of an NH_2-terminal variable domain (V) and one or more COOH-terminal constant domains (C). L chains contain a single V domain (V_L) linked to a single C domain (C_L) by disulfide bonds. While the H chain contains a V_H and C_H1 regions linked by a flexible hinge sequence to two additional C domains (C_H2 and C_H3).

Early studies showed that the hinge region could be cleaved by papain, a protease that is still in use to produce antibody fragments for countless applications.

Papain can cleave immunoglobulins into:

- **Two identical fragments (Fab)** containing the V domains of both the L and H chain, V_L and V_H respectively, and the first C domains of these two chains, C_L and C_H1 respectively
- **One fragment (Fc)** containing the remaining C domains, C_H2 and C_H3



Structure of a monoclonal antibody

Interestingly, these fragments retained biological activity. The two identical fragments were found to be responsible for **a**ntigen **b**inding, thus they were named F**ab** fragments. In contrast, the third fragment could not bind to antigens, but it was readily **c**rystallizable, earning the name of F**c** fragment.

Despite not being fundamental for interacting with the antigen, the Fc region was soon found to be responsible **for vital effector functions** such as:

- Antibody-dependent cell-mediated cytotoxicity (**ADCC**)
- Antibody-dependent cellular phagocytosis (**ADCP**)
- Complement-dependent cytotoxicity (**CDC**)

Within the V domain, each chain contains three hypervariable loops with surface complementarity to a specific antigen. Thus, they were named **complementarity determining regions (CDRs)**. The four additional regions between the three CDRs loops were found to be more conserved and thus were termed **framework regions (FRs).** The identification of these precise regions propelled the development of many antibody engineering techniques.

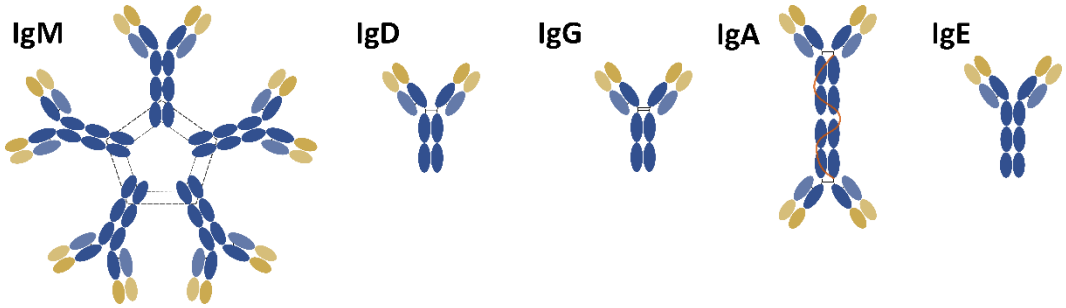
Isotypes and subtypes of antibodies

The IgG structure is the most well-known type of immunoglobulin because it is the most abundantly expressed and secreted by our organism. For this reason, molecules from the IgG isotype also became the preferred format used for the development of therapeutic antibodies.

Nevertheless, **IgG is only one of five isotypes** playing an important role in our immune system. The other four isotypes differ from IgG in terms of structure, sequence, number of constant domains, form, and role.

Isotype	IgM	IgD	IgG	IgA	IgE
Common structure	Pentamer	Monomer	Monomer	Monomer or Dimer	Monomer
Subtypes	None	None	4 (IgG1-4)	2 (IgA1-2)	None
H chain type	μ	δ	γ	α	ε
H chain weight (kDa)	70	60	50-60	55	70
L chain weight (kDa)	23	23	23	23	23
Total weight (kDa)	970	180	150-170	165 (monomer) 350 (dimer)	190
Relative abundance	5-10%	<1%	70-85%	5-15%	0.002%
Serum half-life	5 days	3 days	23 days	6 days	2.5 days
Complement activation	Yes	No	Yes (except IgG4)	No	No
Cross placenta	No	No	Yes	No	No
Function	Primary response	Homeostasis	Secondary response, and toxin and virus neutralization	Primary response	Mediators of the allergic reaction

Structurally, significant differences can be found among the different isotypes. For instance, the H chain of IgG, IgD, and IgA consists of 3 C domains (CH1-3) and a hinge region between the CH1 and CH2 domains. Interestingly, antibodies from the IgM and IgE isotypes lack a hinge region, but each H chain contains an additional C domain (C_H1-4).



The five isotypes of monoclonal antibodies

Therapeutic antibody design

Monoclonal antibodies are the dominant form of immunotherapy on the market. However, the effort to tackle new antigens and treat aggressive conditions has prompted the development of new forms of immunotherapy that go beyond the canonical structure of antibodies. Currently, many of these forms are gaining ground over conventional therapies for a multitude of applications. In this section, we will summarize the main new forms of immunotherapy.

Nanobodies

In the early 1990s, a group of researchers challenged our knowledge of the diversity of antibodies. They did this by describing the first naturally occurring antibodies devoid of L chains. These antibodies, named heavy-chain antibodies (HCAbs), first described in camelids and later in sharks, were found to have a simplified variable structure consisting of **a single V_H domain with three CDR**.

The variable domains of these antibodies were subsequently named **VHHs or nanobodies**. Interestingly, due to the lack of a V_L domain, VHHs are fully hydrophilic, resulting in an increased solubility and lower susceptibility to form aggregates in comparison to monoclonal antibodies and corresponding fragments.

Despite harboring only three CDR, these fragments were shown to have an antigen-affinity comparable to that of monoclonal antibodies, an ability attributed to the presence of a **very long third CDR (CDR3)** not found on their human counterparts. They also exhibit a high homology with human type 3 VH domains resulting in a low immunogenic burden and making them highly desirable for therapeutic applications.

For these applications, VHH antibodies present several advantages such as the ability to tackle **cryptic antigens, fast diffusion, ease of production (no glycosylation required), and engineering**. Their limitation stems from their naturally small size (15 kDa), leading to a short plasma half-life and fast clearance from the organism. To counter this limitation, researchers are now adapting VHH molecules to **multivalent formats** with longer plasma circulation and stronger antigen-binding affinity.

Bispecific antibodies

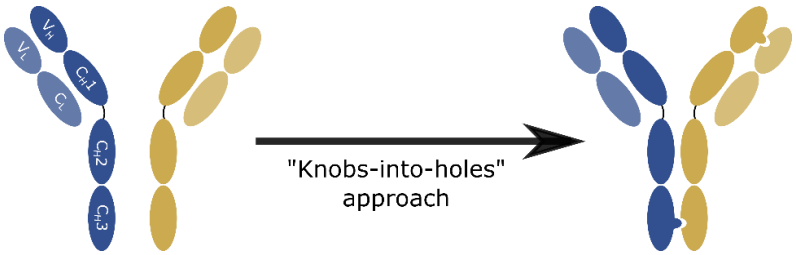
Bispecific antibodies have been around since the late 1960s when scientists first associated antigen-binding fragments (Fab) from different polyclonal sera into **bispecific F(ab')2 molecules**. Although bispecific antibody therapeutics are, for the most part, man-made molecules, bi-specificity is known to

occur in nature. The only reported case is that of **IgG4 antibodies**, known regulators of IgE and the allergy response, that undergo a mechanism known as **Fab-arm exchange** endowing them with anti-inflammatory activity and natural bi-specificity.

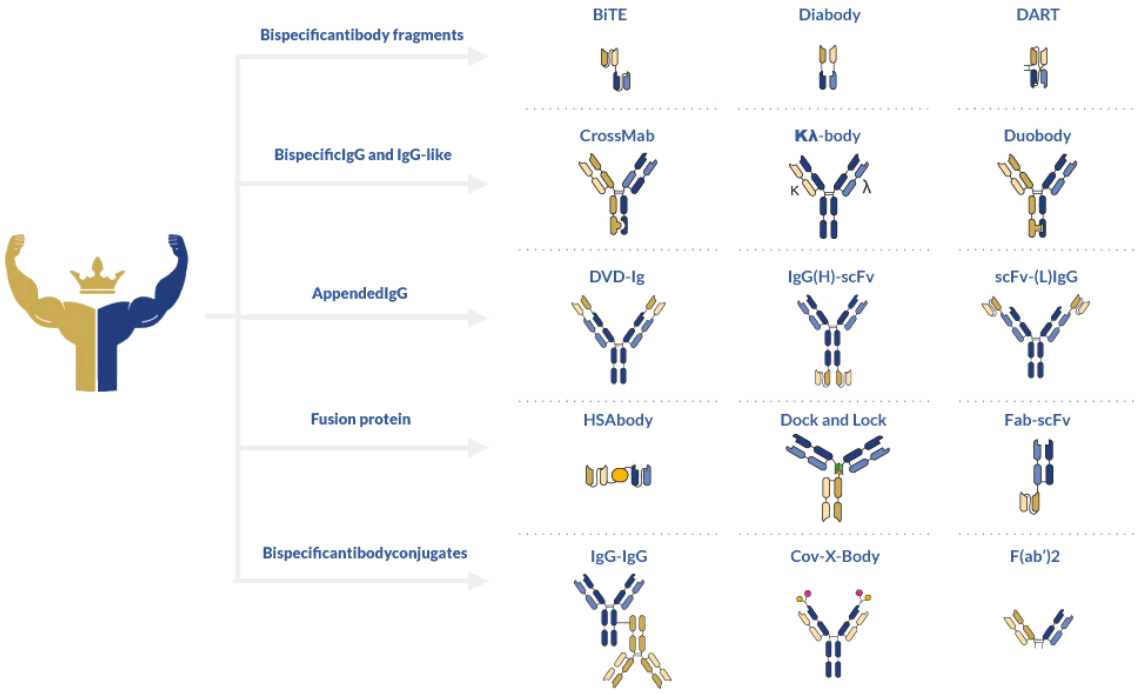
However, the full potential of bispecific antibodies and their usefulness for therapeutic applications have only begun to be exploited with the advent of hybridoma technology. Early attempts to produce bispecific antibody therapeutics *in vitro* started with the conjugation of hybridomas with different antigen-specificity into a single hybrid cell line (**quadroma technology**).

Despite the successful fusion of these cell lines, scientists soon realized the inherent difficulties of this process. Due to the presence of two different heavy and light chains, hybrid cell lines produced a mixture of correct and incorrectly assembled antibodies (mono- and bispecific). However, the correct bispecific assembly remained a minor component of the production batch and this issue has since been referred to as the **chain-association issue**.

This limitation has been overcome by the advent of recombinant technologies and the development of highly efficient antibody engineering methods. Currently, bispecific antibodies are produced in recombinant systems via the **"Knobs-into-holes"** method used to improve the affinity of each chain towards its proper pair and thus enhance adequate chain pairing during recombinant production.



Common antibody engineering approach used to reduce chain mispairing of bispecific antibodies



Common bispecific antibody formats used in therapeutic applications

Due to their multivalence, bispecific antibody therapies are better known for their ability **to retarget immune cells to tumors,** deliver payloads (e.g., small drugs) to cancer cells, and block important tumor signaling molecules.

T cell redirection is the most conventional application of bispecific antibody therapies. Using bispecific antibodies with a T cell-binding domain (CD3) and a tumor-binding domain helps physically linking the two cells and activating T cell's cytotoxic activity. **Blinatumomab** (generated with the BiTE® technology) was designed with this purpose in mind. Despite its short half-life (due to its small size) and need for continuous intravenous infusion, clinical studies have shown impressive treatment effectiveness.

However, CD3-based bispecific antibodies have been known to provoke toxicity by activating T cells indiscriminately. One strategy already being used to circumvent this issue is the development of antibodies to recruit only a subset of T cells with the greatest potential for positive therapeutic outcomes. Other approaches are also focused on retargeting and activating other immune cells such as NK.

Antibody-drug conjugates

Antibody-drug conjugates (ADC) are complex biomolecules consisting of a **monoclonal antibody and a payload connected by a linker**. Typical monoclonal antibodies used for **ADC generation belong to the IgG class.** These large glycoproteins (150 kDa) are used in conjugation with small cytotoxic drugs (e.g., calicheamicin at 1368 Da) that cannot be used as stand-alone chemotherapeutics. ADCs are one of the fastest-growing classes of biopharmaceuticals for oncology. For this reason, much of the work and research on these compounds has focused on the identification of highly specific **cancer markers** and antibody engineering for **enhanced surface-marker affinity**.

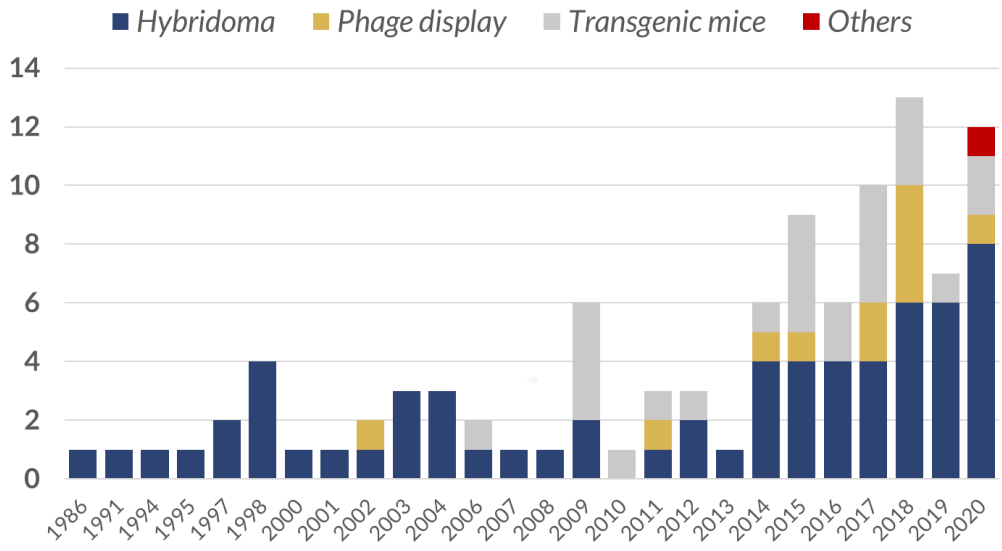
ADCs have different mechanisms of action according to the composition of the linker:

- **Cleavable ADCs – disulfides and dipeptides are the dominant motifs found in cleavable linkers.** These linkers are extremely versatile because they allow the controlled release of the payload intracellularly or in the tumors' proximity. Over the years, several methods have been developed to create linkers with inbuilt triggers allowing its catalysis in the presence of specific signals or enzymes. Within this vast class of linkers, ADCs can be engineered to cleave in response to a chemical signal (i.e., acid, glutathione, etc.) or to be degraded by a specific enzyme (e.g., cathepsin B – upregulated in cancer cells).
- **Non-cleavable ADCs** – Non-cleavable linkers offer the possibility to explore a completely different mechanism of action. These linkers are **independent of chemical triggers and directly integrate the payload**. For this reason, payload release only occurs upon **ADC internalization** and **lysosomal processing**. Once the antibody carrier is digested by lysosomal enzymes, the linker-payload pair is released into the intracellular environment and free to exert its cytotoxic activity. The advantage of these linkers is their enhanced safety. However, not all drugs can be conjugated via non-cleavable linkers because these molecules tend to alter the structure and activity of these drugs.

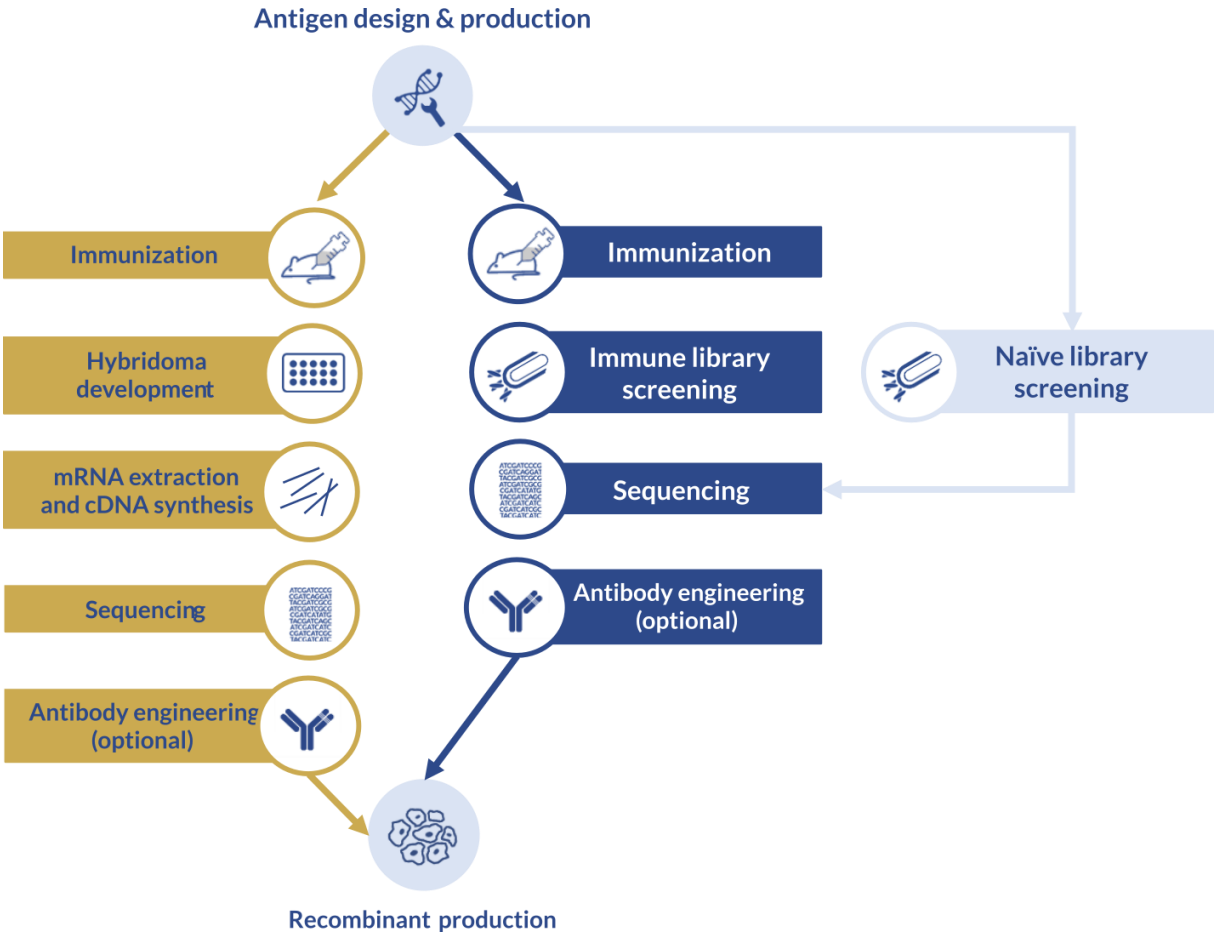
Technologies for antibody generation

There are currently four main technologies used for antibody generation: **(i) hybridoma; (ii) antibody display** (phage, yeast, ribosomal, etc.); **(iii) transgenic mice**; (iv) single-cell antibody screening; and (v) i*n silico* antibody design.

The first three dominate the market in terms of therapeutic antibody discovery. In contrast, hybridoma development continues to be the preferred technology for research applications.



FDA approved therapeutic antibodies per format and per year



Typical workflow of an antibody generation project via hybridoma or phage display

Principles of recombinant antibody production

Full-length IgG-like monoclonal or bispecific antibodies are glycosylated proteins with complex structures. For this reason, they are preferably produced in mammalian systems capable of performing **human-like glycosylation and correctly folding the individual light (L) and heavy (H) chains**. In fact, incorrect folding and chain misassembly are known to impact effector functions, hinder pharmacokinetics, and increase the risk of antibody aggregation resulting in low yields, poor effectiveness, and reduced stability.

The expression of antibodies in recombinant systems is not significantly different from the production of non-antibody proteins. Most of the elements discussed in Part A are used to drive antibody expression in mammalian systems. However, some considerations apply:

- **Co-transfection** – L and H chains of antibodies are typically produced using a **two-plasmid system**. Each chain is cloned into a different plasmid, associated with its own promoter, and transcribed separately. Generally, the two plasmids are transfected at an **equimolar ratio** to ensure the correct assembly of the two chains. Most vectors are controlled by the CMV constitutive promoter.

- **Simplified purification** – antibodies can be purified using alternative methods to conventional purification tags. The most common are:
  - **Immunoglobulin-specific purification:** a wide range of microbial proteins is known to bind strongly to antibody's Fc or Fab fragments. A few examples are protein A (*Staphylococcus aureus*), protein G (*Streptococcus* spp.), fusion protein A/G, and protein L (*Peptostroptococcus magnus*). These proteins can be immobilized into resins and easily used to capture antibodies from the culture medium.
  - **Antigen-specific purification:** alternatively, antibodies can be captured by immobilizing their specific antigen in a resin.

Interestingly, these purification methods are typically reserved for small and medium-scale production because they can become expensive at the large scale. Alternatively, antibodies can also be associated with a number of different affinity tags, allowing a more cost-effective purification.

Applications of recombinant antibodies produced in transient systems

Recombinant antibodies produced in transient systems are typically used for the following applications:

- **Drug candidate screening:** screening can help researchers identify the most promising lead candidates during early development. Candidate selection at this stage is known to improve the success rates of biopharmaceuticals by allowing the detecting of potential developmental issues.

- **Antibody variant screening:** after discovery, antibodies are often submitted to additional engineering. Whether they are modified to increase affinity or reduce immunogenicity (i.e., humanization), antibody variants need to be screened at a small scale to identify the most promising leads for further development.

- **Analytical and research applications:** transient antibody production is often used to supply small scale research applications. These will be discussed in detail in the next section.

Conventional immunoassays used for research and diagnostics

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

IHC and ICC are antibody-based methods used for staining specific cells (ICC) or tissues (IHC) samples. Together they represent a powerful microscopy-based technique providing a clear **visual and spatial** output at the tissue or cellular level of specific markers and how they correlate with different cell types, cellular compartments, or biological states.

Antibodies used in IHC **or ICC assays are labeled with chromogenic reagents such as horseradish peroxidase (HRP)** which then require a chemical substrate to produce a color change, making the samples easy to visualize under a light microscope. These assays are also amenable to multiplexing (the use of several enzyme reporter labels to produce different colors for different antigens).

Conventional IHC and ICC assays often use formalin-fixed paraffin-embedded (FFPE) tissues that preserve the histological morphology but can mask important epitopes. It is possible to partially revert **the chemical crosslinking in FFPE tissues** by using antigen retrieval protocols. However, these protocols need to be optimized for each specific tissue and antigen.

Alternatively, frozen tissues can be used to minimize this issue. But, in both cases, the conformation of target epitopes is expected to change during sample treatment, which makes **antibody validation in the specific cell or tissue type vital to ensure the success of IHC/ICC experiments.**

Western blot (WB)

WC is a widely used method to separate and detect proteins. It involves transferring (also known as blotting) proteins, previously separated by electrophoresis, from a polyacrylamide gel to a nitrocellulose membrane for visualization. The membrane is then blocked and marker-specific antibodies (labeled with chromogenic, fluorogenic, or chemiluminescent substrates) are added for protein imaging.

This immunoassay is typically performed in denaturing conditions meaning that the secondary and tertiary structures of the desired marker are lost. The development of antibodies for these applications needs to take this loss of native structure into account. More importantly, **it remains vital to** validate antibodies for WB in denaturing conditions **to ensure they detect only the marker of interest.**

Despite its enhanced sensitivity, WB assays remain complex and labor-intensive. For this reason, nowadays they are mostly used to confirm the results obtained by other techniques.

Immunoprecipitation (IP)

IP is a popular technique to capture and **concentrate proteins from complex mixtures**. It allows the enrichment of specific markers, which is particularly useful when dealing with low-abundance proteins.

Besides allowing the study of a protein outside its original environment, IP can be used to study the **interaction of the target protein with other molecules** (proteins, DNA, RNA, cells, etc.), as these complexes tend to co-precipitate.

Additional reagents may be used to stabilize complexes prior to precipitation, thus enhancing the usefulness of the assay for the study of important interactions in the context of disease.

This technique is often used in tandem with mass spectrometry providing a powerful **method for measuring and identifying low-abundance proteins in complex samples.** Thus, antibodies intended for IP use need to be carefully validated in the test conditions to ensure only the target protein or complex is efficiently recovered from specific samples.

Immunofluorescence (IF)

Fluorescent-labeled antibodies are the crucial reagents of IF methods, a specific type of immunostaining approach. In its essence, IF is similar to IHC/ICC, thus it can be used to detect and visualize a protein of interest on fixed (FFPE) or **frozen tissues** (immuno**histo**fluorescence) or **particular cell types** (immuno**cyto**fluorescence).

The difference lies in how the samples are visualized. For instance, IHC/ICC assays rely on an enzymatic reporter and a substrate to produce a color change, **while IF assays require only a fluorescent label and the proper lasers to produce a signal.** In both cases, multiplexing (detecting several antigens) is possible provided that the fluorochromes or enzymatic reporters **emit non-overlapping signals.**

Developing antibodies for this approach can be especially challenging since these reagents need to be highly specific to the target and, at the same time, **cause minimal background noise (minimal off-site binding).** Moreover, when different antibody conjugates are used in the same assay, it is even more important to validate the **panel or cocktail** of the different antibodies to ensure the data generated by IF experiments ca be easily interpreted.

Flow cytometry

These assays have been quickly gaining ground over classical immunoassays for the fast detection of disease markers in liquid samples (e.g., fluids, blood, plasma, etc.). Antibodies used in flow cytometry are typically used to detect low abundance markers (diagnostics) or determining the efficiency of specific treatments, substantially aiding the efforts of developing targeted or personalized therapeutic approaches.

These immunoassays can be performed with fixed or unfixed samples; moreover, **they typically employ fluorescence-labeled antibodies in multiplexing conditions.** Due to its enhanced sensitivity, off-target binding can become problematic in flow cytometry. For this reason, validation should foresee potential issues caused by different sample types, cell fixation protocols, and multiplexing conditions.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based immunoassay used for the detection of markers in complex samples. Several ELISA formats are currently employed in research and diagnostics depending on the abundance of the target. Generally, ELISA antibodies are tagged with enzymatic labels (similar to IHC/ICC applications) and these assays can be designed for antigen detection or quantification.

Of all ELISA formats, sandwich ELISA has become the most useful and, consequently, the most challenging to develop. Sandwich ELISA employs two primary antibodies binding non-overlapping epitopes of a given antigen. Similar to all other immunoassays, the validation of antibodies for ELISA should consider the type of samples and detection conditions to be used.

Learn more about our sandwich ELISA development services
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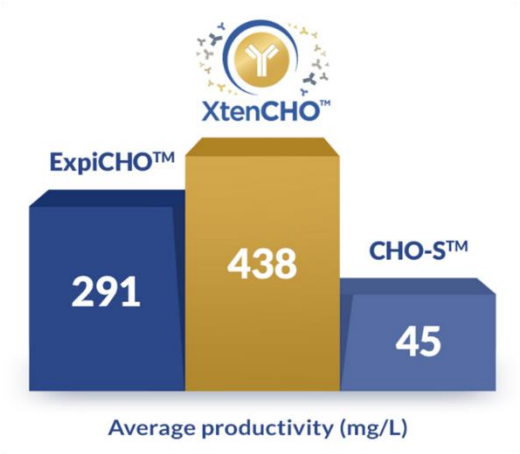


Average protein yields obtained in the XtenCHO™ system

IgG-like antibodies	1.3 g/L (record) 0.4 g/L (average)
Bispecific antibodies	> 100 mg/L
Humanized antibody variants	> 200 mg/L
Non-antibody proteins	> 100 mg/L

Proteogenix's XtenCHO™ mammalian expression system was designed to achieve **ultra-high yields with minimal hands-on time** without compromising on purity, protein folding, or vital post-translational modifications. This new system is ideal for rapid and cost-effective drug screening and small to medium-scale production for research applications.

This expression system combines the high-density growth and high expression levels of Chinese Hamster Ovary (CHO) cells with the optimal efficiency achieved by our proprietary transfection reagent – XtenFect. Drawing from 1,500+ successful protein expression projects and 28+ years of experience in antibody and hard-to-express proteins, XtenCHO™ offers:

- Highly efficient transfection designed to **maximize yields and extend the TGE** (transient gene expression) period (≥14 days) by maintaining high cell viability, enhancing plasmid stability, and boosting protein expression levels without the need for additional feeds
- **Smarter protocols** with fewer steps designed to simplify protocol integration into your workflows and make your experiments as effortless as possible without compromising on product quality or yields
- Time-tested expression system, shown to outperform other commercial systems and to result in a **1.5 to 9.7-fold increase** in productivity:
- High expression vector (pXen1) designed to minimize the need for case-by-case optimization
- Optimal protein folding and **human-like post-translational modifications**, comparable to stable systems used for clinical-grade biopharmaceutical production
- CHO cells provide a safer alternative to Human Embryonic Kidney (HEK) cell lines due to their enhanced resistance to viral infection
- Strong and responsive technical support

The XtenCHO™ mammalian expression system was designed to support high-density growth in chemically-defined and animal-free media (XtenCHO™ expression medium). The kit is also provided with an efficient expression booster (XtenCHO™ Enhancer) and control plasmids for maximum control and flexibility.

Ordering information

XtenCHO™ Starter kit Catalog no. PX-XTE-001	XtenCHO™ cells	2 cryotubes (2 x 1 mL)
	XtenCHO™ Expression vector (pXten1)	1 microtube (10 µL)
	XtenCHO™ Expression medium	1 bottle (1 L)
	XtenFect Reagent, Stock solution	1 microtube (0.51 mL)
	XtenFect Reagent, Working solution	3 microtubes (3 x 0.48 mL)
	Enhancer	3 microtubes (3 x 0.4 mL)
	Positive control	1 microtube (150 µL)
XtenCHO™ Transfection kit Catalog no. PX-XTE-003	XtenFect™ Reagent, Stock solution	1 microtube (0.51 mL)
	XtenFect™ Reagent, Working solution	3 microtubes (3 x 0.48 mL)
	Enhancer	3 microtubes (3 x 0.4 mL)
XtenCHO™ Expression medium Catalog no. PX-XTE-002	XtenCHO™ Expression medium	1 bottle (1 L)
XtenCHO™ Cells Catalog no. PX-XTE-004	XtenCHO™ cells	1 cryotube (1 mL)
XtenCHO™ Cell bank pack Catalog no. PX-XTE-005	XtenCHO™ cells	6 cryotubes (6 x 1 mL)

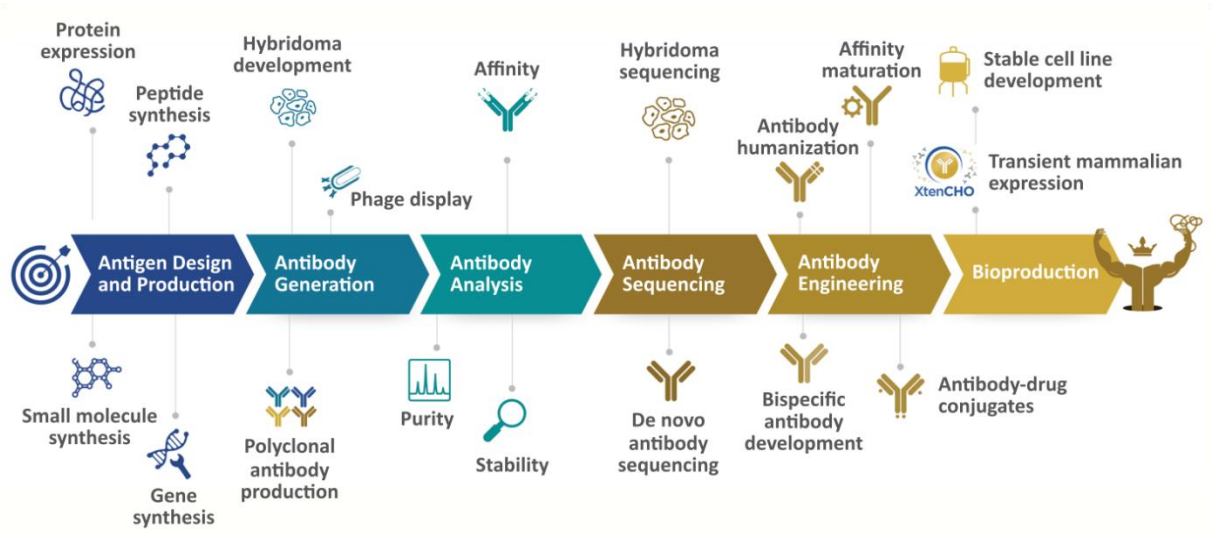
For more information, contact us at inquiry-products@proteogenix.fr

About **ProteoGenix**

Accelerating your way to the clinic

ProteoGenix is a biotech contract research organization (CRO) providing flexible end-to-end solutions for the discovery and development of life-saving biologics, precision diagnostic tools, and robust reagents for research. As a global leader in antibody, protein, peptide, and gene production, ProteoGenix aims at fostering the development of next-generation immunotechnologies.

THE MARKET'S BEST-INTEGRATED SOLUTIONS FOR THERAPEUTIC ANTIBODY DEVELOPMENT



ProteoGenix is dedicated to developing the best-integrated solutions with one goal in mind: "bring every new idea from the research bench to the clinic". In every project, you get to work side-by-side with a dedicated project manager and a team of experts committed to optimizing your chances to reach the market and providing the strongest guarantees.

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