# Recombinant Antibody Handbook

## Protocols and Tips

**CHAPTER**

# 5

GenScript
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# CHAPTER 5

## PROTOCOLS & TIPS FOR
### RECOMBINANT ANTIBODY EXPRESSION AND PURIFICATION

The process of recombinant antibody (rAb) expression and purification can be generalized into three major steps:

**01** Plasmid DNA encoding the rAb is transfected into cells of the target

**02** Transfected cells expressing the rAb are grown in selective culture media

**03** The target rAb is harvested and purified

Under ideal conditions, rAb generation would begin with the efficient transfection of the antibody expression plasmid(s) into a fast-growing cell line with high specific productivity for the rAb. Expression of the target rAb would be robust, and the subsequent harvest and purification steps would be straightforward and high yielding processes that result in pure, functional antibody. The steps listed above are, of course, an oversimplification of rAb production. In reality, the options for establishing an optimal rAb production protocol are vast, demanding that every aspect of the rAb expression and purification operation be subject to rigorous optimization.

GenScript acknowledges that one size does not fit all when it comes to developing a strategy for expressing and purifying your target rAb. In this chapter, we provide you with some general tips for what to consider as you design your rAb purification scheme. We suggest ways to increase your chances of achieving successful rAb expression, as well as how to establish optimal transfection, cell culture conditions, and protein purification methods. We also equip you with some of GenScript's bioinformatics resources, and supply you with two general rAb production protocols, one outlining the expression and purification of IgM and the other, a bispecific antibody, to help you get started in your rAb generation endeavors.

# 1

# Optimizing recombinant antibody expression

Selecting an expression system depends greatly on the characteristics of the target rAb, what it will be used for, and how much antibody is needed. For example, bacterial systems are best suited for the expression of smaller rAbs that do not require glycosylation like single chain fragment variable (scFv), Fab (fragment antigen binding), and single domain (VH-VL) antibody fragments[1–4]. The production of functional antibody fragments in Gram-negative bacteria, like E. coli, typically occurs in the periplasmic space, which is located between the inner cytoplasmic membrane and outer cell membrane. The periplasm is the location of choice because it is an oxidizing chaperone-containing environment that facilitates the proper folding of rAb fragments[2,5]. Insect and mammalian systems, on the other hand, are preferred for the expression and secretion of larger, more complex rAbs. Generating full-length rAbs often requires the advanced protein folding, assembly, post-translational modification (PTM) and secretion machinery available in insect and mammalian cells. Mammalian cells, in particular, are favored for the production of therapeutic rAbs because of their capacity to produce rAbs that are similar to human antibodies, and therefore, less likely to be immunogenic.

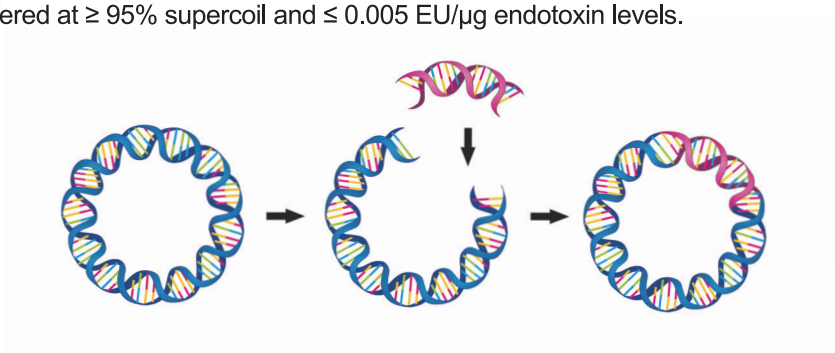
Once the expression system best suited for production of the target rAb has been selected, the next major decision involves engineering the rAb plasmid expression vector. In this section, we go over several aspects that are important to consider when designing the antibody DNA construct for optimal rAb production in your expression system of choice.

## DNA expression vector elements

There are a variety of plasmid vectors that have been optimized for DNA cloning and rAb/protein production within each expression system. Hence, it is worthwhile reviewing the different types of plasmids available, as well as their corresponding expression elements, to determine which cloning vector is most suitable for the expression of your rAb. Selecting cloning vectors that possess robust promoters to drive rAb gene transcription is very important. For example, some constitutively active promoters that are commonly used for mammalian rAb expression in a wide variety of cells include human cytomegalovirus (CMV), simian vacuolating virus 40 (SV40), and cellular elongation factor 1-alpha promoter (EF1α)[1,6]. Enhancers and splicing-competent introns may also be included to accompany some promoters in order to enhance mRNA transcription. Additionally, elements that promote transcription termination such as the SV40 polyadenylation signal sequence, as well as those that improve mRNA transcript stability and translation efficiency like bovine growth hormone (BGH) are also important to consider having in a mammalian expression vector[1,6]. Other key vector characteristics to review include the kozak consensus sequence for translation initiation, selectable markers, origin of replication and chromatin remodeling elements.

Bicistronic vectors are another useful plasmid format to consider using when expressing heavy (H) and light (L) immunoglobin chains (Ig) in prokaryotic and mammalian systems. Antibodies, such as IgG, are normally made up of paired IgH and IgL chains, and only completely assembled IgG molecules can bind antigen and carry out effector functions. However, light chains are synthesized 15% – 25% faster than heavy chains, and the isotype of light chain has been shown to influence the kinetics of intracellular IgG assembly[6–8]. As such, it can be inefficient and challenging to obtain balanced expression of these Ig chains if each chain is encoded by separate, monocistronic DNA plasmids. As a solution, bicistronic vectors can be used to co-express both the heavy and light chain antibody genes from the same RNA transcript via an internal ribosome entry site (IRES)[6].

GenScript's renowned Plasmid DNA Preparation service can provide customizable yields of DNA suitable for any experiment. Plasmid Preps are offered at ≥ 95% supercoil and ≤ 0.005 EU/μg endotoxin levels.



## Codon optimization

It is well-known that due to the degeneracy of the genetic code, 18 out of 20 amino acids can be encoded by more than 1 of the 61 available DNA triplet codons. This codon redundancy is known as a synonymous mutation because the translated amino acid remains the same[9]. However, while the translated amino acid remains unchanged, synonymous codons can still affect protein expression levels because in a cell, certain codons are translated more efficiently than others, creating the phenomenon known as codon bias[9,10]. A synonymous mutation in a codon with a limited availability of corresponding tRNA anticodons results in significantly lower protein expression due to ribosome stalling. Many organisms display biased codon usage and it is generally accepted that codon biases reflect a balance between mutational biases and natural selection for translational optimization[9].

There are a variety of tools that use the power of bioinformatics to help with rAb gene design by determining and optimizing codon usage within the target rAb expression system, and reducing codon bias[11,12]. However, codon optimization tools can differ greatly and newer design algorithms can analyze much more than

organism-specific codon usage, such as transcriptional efficiency (GC content, splicing efficiency, promoter and TATA box sequences, termination signals, CpG-methylated sequences), as well as translational efficiency and stability (codon usage bias, mRNA secondary structure, premature polyA sites, Shine-Delgarno sequences, AU-rich 3' mRNA elements, protein folding etc.). To help with designing and optimizing your rAb gene sequence, we recommend getting started with GenScript's new user-friendly and accessible codon optimization tool, GenSmart™ Codon Optimization (more details available in section IV of this chapter).

## Cell line engineering

In addition to optimizing rAb expression plasmids, cell lines have also been genetically engineered to enhance rAb production across all expression systems and conditions. Transformed versions of the HEK293 cell line are popular choices for transient rAb expression because they can be transfected at high efficiency and highly productive cell suspensions, with antibody yields ranging in the milligram to gram range in just a few days[1,13,14]. For example, the HEK293-6E cell line which expresses a truncated form of the Epstein-Barr Virus, has been shown to improve recombinant protein titers[14].

While CHO cells are more challenging to transiently transfect, their importance in the production of biopharmaceutical products have led to the engineering of several CHO cell lines that are better adapted for transient rAb production[15,16]. Many CHO cell lines have both alleles of the dihydrofolate reductase (DHFR) gene deleted and depend on screening conditions that select only for cells successfully transfected with plasmids expressing both the rAb gene and DHFR. One such DHFR-deficient cell line is CHO-DG44, which also stably overexpresses the anti-apoptotic protein bcl-xL[15]. As a result, apoptosis-resistant CHO-DG44 cells exhibit increased longevity and also higher recombinant protein production yields in cell culture.

In stably transfected cell lines, the use of inducible homologous recombination methods such as the Flp/FRT or the Cre-lox system, have afforded researchers the ability to better control site-specific integration of rAb transgenes6. Use of the DHFR and glutamyl synthetase selection systems are also effective for increasing the number of rAb gene copies in the genomes of stably transfected dhfr- CHO and GS-NS0 (non-secreting murine myeloma) cells[1,6,17].

In insect cells, protein secretion can be improved through the use of protease-deficient baculovirus strains and engineered cell lines that can carry out complex glycosylation, resulting in a protein product with glycosylation patterns that closely mimic mammalian proteins. Antibody glycosylation is an important process to monitor because glycan modifications can impact a rAb's bioactivity and clearance rate, and the ultimate quality of the rAb[18–20].

While we provide just a few examples of cell lines engineered to improve rAb production in stably and transiently transfected calls, new cell lines are constantly being developed and tested to improve rAb expression and production. For your rAb production project, it is certainly worthwhile reviewing available expression cell lines that can help you achieve the highest possible rAb titers. It is also possible to tailor cell lines for the specific production of your rAb using revolutionary CRISPR/Cas9 gene editing technology. GenScript's GenCRISPR™ Mammalian Cell Line Service is led by scientists who are experts in CRISPR gene editing and can guide you in the engineering a cell line that maximizes the yield of your desired rAb.

## Chaperone expression

Chaperone proteins that are co- or overexpressed with the rAb can assist with antibody folding and solubility. One of the major benefits of rAb expression in mammalian systems is that cells already possess the advanced cellular machinery and relevant

chaperones to promote protein folding, PTMs, solubility, assembly, and secretion. In fact, most of the chaperones used to improve rAb expression in bacteria and insect cells are derived from mammalian proteins.

In bacteria, some of the common chaperones that are used to help with the folding and solubility of antibody fragments such as ScFv and Fab include GroES/L, peptidyl prolyl cis-trans isomerases, DnaKJE and FkPa[21,22]. In the insect-baculovirus expression system, co-expression of the human chaperone Hsp70 together with its co-factor Hsp40, can improve solubility of recombinant target proteins and reduce aggregation[23]. Additionally, overexpression of endoplasmic reticulum (ER) resident chaperone binding proteins, such as immunoglobin heavy chain binding protein (BiP) and protein disulfide isomerase (PDI), have also been shown to enhance soluble and secreted IgG[20,24]. Co-expression of calreticulin, another ER chaperone, together with the translation initiation factor eIF4E was also found to increase protein secretion[24].

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# Optimizing transfection and growth conditions

Once rAb plasmid DNA has been generated, the next step involves introducing the DNA into the relevant cellular expression system, and establishing growth conditions for rAb production. For bacterial systems, plasmids are normally introduced into cells via a process known as transformation. In bacterial transformation, cells are treated to make them competent so that they can take up exogenous plasmid DNA. Common treatments to make cells competent include electroporation, or the use of chemicals like calcium chloride together with a brief heat shock step. Since bacterial transformation is a relatively simple method, we will focus on the process of introducing plasmid DNA into eukaryotic cells, a process known as transfection, in the rest of this section.

## Transfection

There are several different ways to transfect plasmids into eukaryotic cells. Transient transfection is especially favored for rAb expression because it is fast and high-yielding, with rAb harvest possible just 6 days post-transfection. Being a relatively straightforward process that does not require integration of rAb DNA into the host genome, standardized protocols using transiently transfected cells to establish parallel rAb production processes can be easily accomplished[13]. Forming a stably-transfected cell line, on the other hand, relies on the random integration of the recombinant gene cassette and a selectable marker, such as an antibiotic resistance gene, into the host cell's genome. Since just ~1 in $10^4$ cells successfully integrate

the gene and marker into the host genome, generating a cell line stably expressing the rAb gene takes a longer time and requires several rounds of careful selection and amplification. The use of stable cell lines, however, is required for the production of GMP compliant, clinical-grade therapeutic antibodies to guarantee a stable, long lasting supply of the rAb[1].

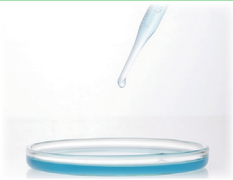
Protocols for optimal transfection, be it transient or stable, will differ from one project to the next depending on the expression system. Parameters such as the amount of DNA used, the type of transfection reagent, the transfection method, cell density, and target cell line all need to be considered and optimized.

Across all transfection methods, however, there are some common experimental procedures that are critical to transfection success.

### DNA quality & quantity

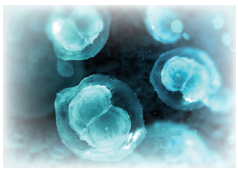
Ensure that the plasmid DNA you have is

- of high, transfection-grade quality
- in a sterile buffer (water or TE)
- free of protein, RNA, chemical contaminants, with endotoxins removed
- sufficient for the scale and design of your experiment



### Type of transfection reagent

The combination of DNA and certain types of transfection agents and enhancers can be toxic to cells. Optimize transfection conditions to limit cell cytotoxicity by reducing excessive, unnecessary use of plasmid DNA and transfection reagents.



### Cell viability & vitality

Use cells of a lower passage and ensure viability and vitality by passaging cells twice before transfection. Transfect healthy cells that are 40-80% confluent and in log growth phase.

### Cell culture conditions

Cells should also be grown at recommended temperatures, $CO_2$ and humidity levels.

Ensure that the cell culture media is uncontaminated and optimized for the serum and supplement types that encourage post-transfection recovery, cell growth, and protein expression.

## Cell culture conditions

It goes without saying that appropriate, sterile cell culture and growth conditions are essential factors that contribute to a high rAb quality and yield. For bacteria, smaller scale preparations of antibody purification can be accomplished by growing cells using a straightforward shaker flask production method. Large scale bacterial rAb preparations can be accomplished through high-cell density fermentation in bioreactors where production volumes can exceed 10,000 L[25].

Like bacteria, mammalian rAbs can also be prepared at a smaller scale in suspension cultures using shaker flasks. Large scale production of mammalian rAbs produced by transient gene expression is usually accomplished using batch or fed-batch cell culture processes in a bioreactor at volumes up to 150 L[1,26]. In some cases, inducible expression of cell cycle regulators like p18 and p21, acidic fibroblast growth factor, as well as additives like valproic acid, hydroxyurea, and histone deacetylase inhibitors (NaBut and Trichostatin A) can all potentially improve rAb production levels[27].

Protein production is a complex process with a multitude of variables that can impact the quality and yield of the final rAb product. In general, for small scale preparations of rAb, GenScript recommends assessing the parameters for optimization for each project, and carefully monitoring biological parameters such as cell density, viability and growth rate of the rAb culture throughout the production process. Maintaining cell growth in optimal, well-controlled conditions are the best guarantee for a high rAb yield.

## The cost vs. quality vs. yield conundrum

When in comes to recombinant antibody production, it is often difficult to determine how the costs of producing a custom antibody justify the quality and yield of the final product.

GenScript has developed a proprietary cocktail reagent that leverages the costs of production with the quality and yield of the antibody, ensuring that you are always getting the best quality product at the lowest possible price.

# III

# Optimizing recombinant antibody purification and characterization

The recovery of rAbs from cell culture involves several critical purification steps that will need to be rigorously optimized in order to maximize rAb yield, purity, and quality. In this section, we offer some general suggestions on how to approach the process of rAb purification, and provide you with some advice on optimizing your rAb purification and characterization protocol.

## Recombinant antibody purification

The purification of rAbs is a very structured and systematic process that usually involves the use of affinity chromatography. Depending on the final application and purity requirements of the rAb, a purification strategy should be developed. In order to do so, several basic questions should be addressed prior to beginning rAb purification, including –

- Where is the expressed rAb located?
- What are the contaminants that need to be removed?
- What is the scale of purification and degree of rAb purity required (Table 1)?
- What is the end application of the antibody being purified?
- What are the resources and equipment that are available?

| Purity | Applications |
|---|---|
| **>99%** | Therapeutic use, *in vivo* studies |
| **>95%** | Research use as reagents (e.g. WB, IF, IHC, FACS etc.) |
| | Antibody engineering (e.g. combinatorial display libraries) |

**Table 1:** General antibody purity requirements and their respective applications.

Affinity chromatography (AC) is a highly selective, indispensable technique for rAb purification because it separates and resolves proteins from complex mixtures by taking advantage of their different physical and chemical properties[28]. Some of the more common AC methods are described briefly ahead. However, the key to successful protein and antibody purification using AC is selecting the most appropriate affinity column for your rAb, and optimizing the purification strategy to suit your purity requirements and to maximize yield. In this regard, most chromatographic protein purification procedures involve a 4-step process.

1. **Binding:** Clarified and filtered rAb-containing cell lysate is bound to the capture resin
2. **Washing:** Bound rAb is made pure by washing away contaminants
3. **Refining:** If necessary, the rAb can be further purified by eliminating trace impurities
4. **Elution:** The purified rAb is eluted from the purification resin

For example, Protein A has a high affinity for the Fc region of IgG-type antibodies. Thus, if the expression of an IgG-type rAb antibody is sufficiently robust, a single Protein A-based AC step can sometimes yield sufficient antibody of adequate purity for a variety of applications. However, if the rAb needs to be further purified, it can be followed up with other purification methods using different types of resins such as size exclusion chromatography (SEC) and/or ion exchange (IEX) chromatography[28,29]. Different types of antibodies will have varying affinities to different AC resins, and no one type of AC resin can purify all classes of antibodies. As a result, it is important to carefully evaluate which AC resin is best suited for purifying your target rAb (Table 2).

| Affinity resin | Target antibody class or fragment |
|---|---|
| Protein A | IgG subclasses 1, 2, 4 |
| Protein G | IgG subclasses 1 – 4 |
| Protein L* | ScFv, Fab, Dab |
| 2-Mercaptopyridine | IgM, IgY |

**Table 2:** Common affinity resins used for antibody purification. *Protein L is particularly useful for capturing antibody fragments, but can bind any class of antibody that has the appropriate variable region of kappa light chains

In general, every resin has specifications that define its optimal operating conditions, and depending on the column supplier that you go with, be sure to carefully review basic column operation parameters such as –

- Loading volume
- Flow rate and pressure limit
- Type of buffers (wash & elution) that the column can tolerate
- Column washing
- Column storage

Following rAb elution, antibody storage practices are similar to conventional protein storage methods.

| Storage Duration | Recommended Temperature | Best Practices |
|---|---|---|
| 1-2 days | 4°C | Filter sterilize using a 0.22µm filter |
| Longer than 2 days | 4°C | Add 0.1% sodium azide to avoid bacterial growth |
| A few months | -20°C | Add 50% glycerol to avoid freezing |
| Months-Years | -80°C | Adding 5%-50% glycerol helps keep the protein stable |

Extreme storage conditions such as extreme pH values or pH values that are close to pI of the protein should be avoided. It may also be a good idea to avoid any additives that could interfere with any future applications, or ensure these additives are removed prior to use. For longer-term storage, it is important to freeze the protein sample rapidly in liquid nitrogen or a dry ice/ethanol mixture to avoid denaturation and avoid multiple freeze-thaw cycles to prevent loss of rAb stability and activity.

## Recombinant antibody characterization

Protein characterization is important when it comes to determining the identity, purity, concentration, and activity of your rAb. This evaluation process should be conducted after each purification step. There are many ways to go about assessing the integrity and quality of your purified rAb. In this section, we suggest a few methods and provide some tips.

### Purity and identity

*SDS-PAGE with colorimetric or immunostaining (Western blot)*

- Under non-reducing conditions, SDS-PAGE of rAbs should yield one band corresponding to the full sized immunoglobulin. Additional bands of higher and lower than expected rAb molecular weights may correspond to purification-related impurities, as well as rAb fragments and aggregates. Additional lower molecular weight bands may also be due to rAb inter-chain disulfide bond breakage during sample preparation for SDS-PAGE[30].
- Under reducing conditions, the heavy and light antibody chains are fully denatured and reduced. Normally two bands, one at 23 kDa corresponding to the light chain, and one at 50 kDa corresponding to the heavy chain, can be visualized.

*Gel filtration, IEX, HPLC*

- Usually, when the rAb is eluted by using an AC, an elution chromatogram is generated. Symmetric elution profiles with a single absorbance peak are characteristic of homogeneous proteins, whereas asymmetric profiles are often reflective of non-homogeneous or partially aggregated proteins. Elution profiles can also reveal the primary oligomerization state of the purified protein.

*Mass spectrometry (LC-MS, MALDI-TOF)*

- The mass spectra of a rAb can verify protein identity by providing the protein sequence and an accurate molecular weight estimate (within a few Daltons). Proteomics can also be used to reveal whether proteolysis may have occurred, as well as identify contaminants that may have co-purified with the target rAb.

## Concentration

*Absorbance readings*

- Measuring protein absorbance using a UV-Vis spectrophotometer is one of the easiest methods for rAb quantification. A popular technique involves using a Nanodrop™ (Thermo) to measure the absorbance of proteins containing Trp and/or Tyr residues as well as disulfide bonds which display absorbance at 280 nm. This method does not require the generation of a standard curve but blank buffer measurements without protein are essential.
- The Bradford and BCA (bicinchoninic acid) assays are also useful for the detection and quantitation of proteins. These techniques are centered on the binding of proteins to the assay reagent to elicit a measurable color change at 595 nm (Bradford) or between 550 – 570 nm (BCA). While all proteins >3 residues long can be measured using these assays, reference standard curves using bovine serum albumin (BSA) must be established. Additionally, as assay reagents can be incompatible with certain buffer components and properties (detergents, pH level etc.), it is important to review the assay reagent requirements and sample properties.

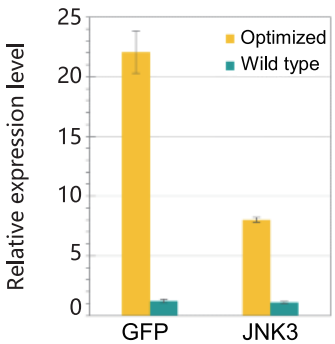
# IV

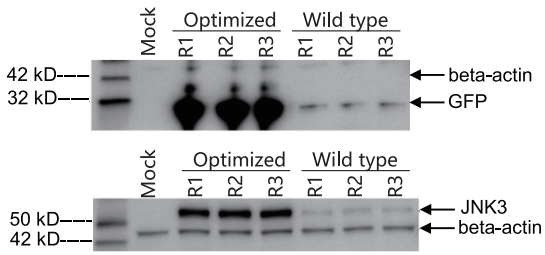
# Bioinformatics Tools

GenScript has developed and licensed a number of comprehensive bioinformatics tools to assist with the design of your recombinant antibody expression experiment.

## Key reasons to use GenSmart™ Codon Optimization

**Accessibility:** Free online tool for gene sequence optimization
**Comprehensive Factor Analysis:** >200 factors influencing gene expression are screened & validated
**Advanced Computing:** Patented Population Immune Algorithm generates the best gene sequence
**Individual Sequence-based:** Highly customized algorithm avoids weight allocation bias on key factors




GenSmart-optimized gene sequences for GFP and JNK3 result in a 22- and 8-fold increase in protein expression respectively when compared to unoptimized sequences.

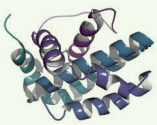
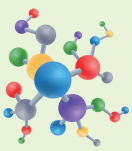
**a. Codon Optimization:** GenScript offers two main codon optimization tools for gene design. **GenSmart™** is GenScript's free, user-friendly codon optimization platform based on the "Population Immune Algorithm" that takes advantage of both population genetics and immunology theories. In this approach, over 200 factors involved in gene expression, including GC-content, codon usage and content index, RNase splicing sites, and cis-acting mRNA destabilizing motifs, are screened and validated. A multifactor approach is employed to ensure that all key factors in a certain target gene sequence carry weight. As a result, each gene optimization run is fully customized to maximize the chance of obtaining a functional and active protein. GenScript's other gene design system, **OptimumGene™**, is a proprietary PSO-based optimization technology that can alter both naturally-occurring and recombinant gene sequences to achieve the highest possible levels of productivity in any given expression system.

**b. WoLF PSORT II Advanced Protein Subcellular Localization Prediction Tool:** WoLF PSORT is an extension of the PSORT II program for protein subcellular localization prediction, which is based on the PSORT principle. WoLF PSORT converts a protein's amino acid sequences into numerical localization features based on sorting signals, amino acid composition and functional motifs. After conversion, a simple k-nearest neighbor classifier is used for prediction.

**c. ProtBank™ Protein Database: ProtBank™** is an integrated online database that contains the comprehensive information of >2 million proteins from 186 different species. The database provides in-depth information on protein nomenclature, sequence, structure, function, domains, post-translational modifications, sub-cellular localization, protein-protein interactions, and much more.

**d. Antigen Design Tool:** Peptides created through GenScript's **OptimumAntigen™** Design Program are optimized using the industry's most advanced antigen design algorithm. Each peptide is measured against several protein databases to confirm the desired epitope specificity. Additional benefits of using

**OptimumAntigen™** include the avoidance of unexposed epitopes, the ability of users to specify desired cross-reactivity, the assurance of strong antigenicity, identification of the best conjugation and presentation options for desired assay(s), the use of a built-in peptide synthesis and solubility tutorial, as well as a guaranteed immune response for the designed antigen.

| GenScript's Bioinformatics Tools | |
|---|---|
|  | GenSmart ™ Codon Optimization<br>GenScript's advanced, user-friendly gene design and codon optimization technology. |
|  | WoLF PSORT<br>Advanced protein subcellular localization prediction tool. |
|  | ProtBank ™<br>Comprehensive online protein database. |
|  | OptimumAntigen™<br>Peptide antigens are optimized using the industry's most advanced antigen design algorithm. |

# V

# Recombinant Antibody Purification Protocols

In this section, we provide you with general protocols for the purification of two different types of secreted rAbs produced in a mammalian expression system – recombinant IgM and a bispecific monoclonal antibody (bsAb). Conducting the whole purification process at 4°C using an AKTA brand chromatography system (GE Healthcare) is highly recommended.

## General protocol for IgM production in HEK293-6E cells

### A) Gene synthesis and plasmid preparation

1. Design the IgM DNA constructs encoding the light, heavy and J chains of the IgM immunoglobulin with codon optimization for HEK293 cell line expression to maximize the protein yield.
2. Synthesize the target DNA fragments designed in step A1.
3. Separately sub-clone each target DNA fragment into an expression vector such as pTT5.
4. Depending on the expression scale, mini-, midi-, or maxi-prep high quality transfection-grade IgM plasmids for downstream transfection of the HEK293-6E cell line.

## B) Tissue culture and transient transfection of HEK293-6E cells

1. Grow HEK293-6E cells in suspension using serum-free FreeStyle™ 293 Expression Medium (Thermo, Cat. No. 12338018) by maintaining the culture in Erlenmeyer flasks (Corning Inc.) at 37°C with 5% $CO_2$ in an orbital shaker (VWR Scientific).
2. One day before transfection, seed cells at an approximate density of 1 – 1.5 x $10^6$ cells/ml.
3. On the day of transfection, mix plasmids with a transient transfection reagent such as polyethylenimine (PEI; Polysciences, Inc., Cat. No. 23966-1), and transfect cells with the DNA-PEI complex.
    - GenScript recommends mixing heavy chain, light chain, and J chain DNA at a ratio of 10:10:1 respectively because the relative expression level of each chain is closely related to IgM assembly and will affect the secretion of functional IgM.
4. Regularly monitor cell viability and density on days 1 and 5 post-transfection during the expression process.
    - Cell viability should exceed 50% on day 5 post-transfection.
    - To establish the downstream IgM purification strategy, evaluate preliminary IgM expression by conducting SDS-PAGE on samples of the cell culture supernatant from days 1 and 5. Stain the gel using standard colorimetric protein staining techniques to visually determine the purity and abundance of IgM.
5. Harvest cell culture broth containing the IgM on day 6, centrifuge and follow up with filtration through a 0.22 µM filter to clarify the antibody sample.

## C) IgM purification and QC analysis

1. Equilibrate POROS™ CaptureSelect™ IgM Affinity Matrix (Thermo, Cat. No. 195289010) with PBS, pH 7.2 (GE, Cat. No. SH30256.01).
2. Load the filtered antibody sample onto the column.
3. Wash the column with PBS, pH 7.2.
4. Elute IgM from column using 0.1 M glycine-HCl, pH 3.0, and neutralize eluate directly with 1 M Tris, pH 9.0 by adding 75 – 100 µl of the neutralization buffer per 1 ml of eluate.

5. Pool eluted IgM fractions and buffer exchange into your desired formulation buffer such as PBS, pH 7.2 containing 5-7% sucrose or 10-20% glycerol.

- If precipitation is observed, remove the precipitation first via centrifugation before buffer exchanging IgM into the final formulation buffer.

6. Assess IgM molecular weight and purity by SDS-PAGE, Western blot and/or via SEC-HPLC (TSKgel G3000SWxl column, 7.8 mm × 300 mm; TOSHO, Cat. No. 0008541), and determine IgM concentration by using standard techniques such as the Bradford protein assay.

- If the purity of IgM is unsatisfactory, a further purification step such as SEC can be conducted to increase recombinant antibody purity.

## Achieve robust protein yields using GenScript's High Density Transient Expression service!

GenScript's HD Transient Expression service can help you achieve high-titer production of recombinant antibodies and proteins in mammalian cells.

### HD Transient Expression Service Key Features

**Choose** between HD-HEK and HD-CHO cell-lines for choice of HEK293 or CHO expression

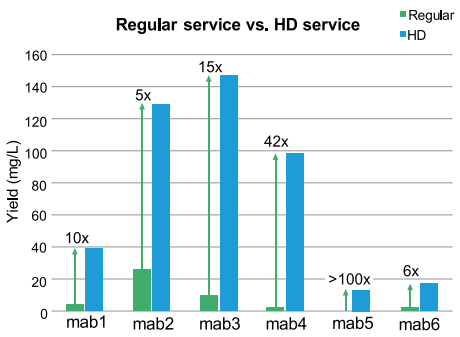
**Compare** expression between regular and HD transient expression cell-lines

**Alternative approach** for difficult and low-yielding proteins

**Yield improvements** of up to 100-fold

**Quick turnaround time** from sequence to protein/antibody in as little as 10 weeks

**Up to 3 g/L** recombinant antibody titer possible




HD Transient Expression service resulted in yield improvements of 5- to >100-fold for 6 different monoclonal antibodies compared to the regular service

# General protocol for bispecific antibody production in CHO-3E7 cells

## A) Plasmid preparation

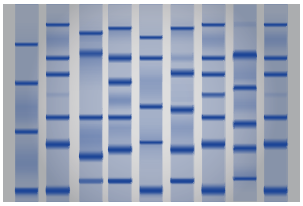
1. Design your bispecific antibody (bsAb) DNA constructs with codon optimization for CHO-3E7 cell line expression to maximize protein yield.
    - To avoid an immunoglobulin chain pairing mismatch, we suggest trying the CrossMAb approach (Roche) of heavy and light chain domain swapping, or the 'knobs-into-holes' method that introduces mutations to force heavy chain pairing.
2. Synthesize the target DNA fragments designed in step A1.
3. Separately sub-clone each target DNA fragment into an expression vector such as pTT5.
4. Depending on the expression scale, mini-, midi-, or maxi-prep high quality transfection-grade plasmids for downstream transfection of the CHO-3E7 cell line.

## B) Cell culture and transient transfection of CHO-3E7 cells

1. Maintain CHO-3E7 cells in serum-free FreestyleTM CHO expression medium (Thermo, Cat. No. 12651014) in Erlenmeyer flasks at 37°C with 5% $CO_2$ (Corning Inc.) on an orbital shaker (VWR Scientific).
2. One day before transfection, seed cells at an approximate density 1 – 1.5 x 106 cells/ml in Erlenmeyer flasks.
3. On the day of transfection, mix the bsAb expression plasmids with a transient transfection agent such as polyethylenimine (PEI, Polysciences, Inc., Cat. No. 23966-1) and transfect CHO-3E7 cells with the DNA-PEI complex.
4. Regularly monitor cell viability and density on days 1 and 5 post-transfection during the expression process.
    - Cell viability should exceed 50% on day 5 post-transfection.
    - To establish the downstream bsAb purification strategy, evaluate

preliminary antibody expression by conducting SDS-PAGE on samples of the cell culture supernatant from day 1 and day 5. Stain the gel using standard colorimetric protein staining techniques to visually determine antibody purity and abundance.
5. Harvest cell culture broth containing the bsAb on day 6, centrifuge, and follow up with filtration through a 0.22 μM filter to clarify the antibody sample.

GenScript's precast gel series include **SurePAGE™** Bis-Tris Gels which are our premium, higher resolution polyacrylamide gels, and **ExpressPlus™** Gels, which are good quality and very cost-effective gels.



## C) Bispecific antibody purification and analysis

1. Equilibrate Monofinity A Resin 5 ml pre-packed column (GenScript, Cat. No. L00433) with PBS pH 7.2 (GE, Cat. No. SH30256.01).
2. Load the filtered antibody sample onto the equilibriated column.
    - If required, adjust the pH of the filtrate to 7.0 – 7.4 using either 0.2 M $Na_2HPO_4$ or 0.2 M $NaH_2PO_4$ (1 M NaOH or 1 M HCl may also be used for pH adjustment).
3. Wash column with PBS pH 7.2.
4. Elute protein using 0.05 M sodium citrate, pH 3.0, and neutralize eluate directly with 1 M Tris, pH 9.0 by adding 75 – 100 μl of the neutralization buffer per 1 ml of eluate
5. Pool eluted bsAb fractions and buffer exchange into the final formulation buffer such as PBS pH 7.2 containing 5-7% sucrose or 10-20% glycerol.
    - If precipitation is observed, remove precipitation by centrifugation before buffer exchanging bsAb to the final formulation buffer.

6. Assess bsAb molecular weight and purity by SDS-PAGE, Western blot and/or via SEC-HPLC (TSKgel G3000SWxl column, 7.8 mm × 300 mm; TOSHO, Cat. No. 0008541), and determine concentration using standard techniques such as the Bradford protein assay. Mass spectrometry can be also used to identify the target bsAb if it cannot be distinguished by SDS-PAGE.

- If sample purity is unsatisfactory, SEC (explained below) can be used to remove protein aggregates and impurities. Normally at this point in the purification process, the molecular weight of impurities are distinct from target bsAb and can be easily separated by SEC. In rare circumstances, IEX can also be used.

## D) Size exclusion purification

1. Concentrate the target bsAb obtained from step C5 to ≤5 ml and load onto a pre-equilibrated HiLoad 16/600 Superdex 200 pg 120 ml column (GE, Cat. No. 28-9893-35)

- Select the proper SEC column according to the scale of your purification process. For <5 mg sample, concentrate protein to ≤ 0.5 ml and load onto a Superdex 200 Increa se 10/300 GL 24 ml column (GE, Cat. No. 28-9909-44); For >40 mg sample, concentrate protein to ≤13 ml and load onto a HiLoad 26/600 Superdex 200 pg 320 ml (GE, Cat. No. 28-9893-36).

2. Combine fractions containing the bsAb and concentrate the antibody if desired.

3. The final bsAb product can be analyzed as described in Section C (step 6) of this protocol.

- If your protein yield is low, you can redesign your DNA constructs by screening a variety of bispecific platforms, or explore switching the orientation of variable domains and modifying linker regions of the bsAb. Alternatively, GenScript's High Density Transient Expression service can help you achieve your research goal for high-titer production of your rAb.

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