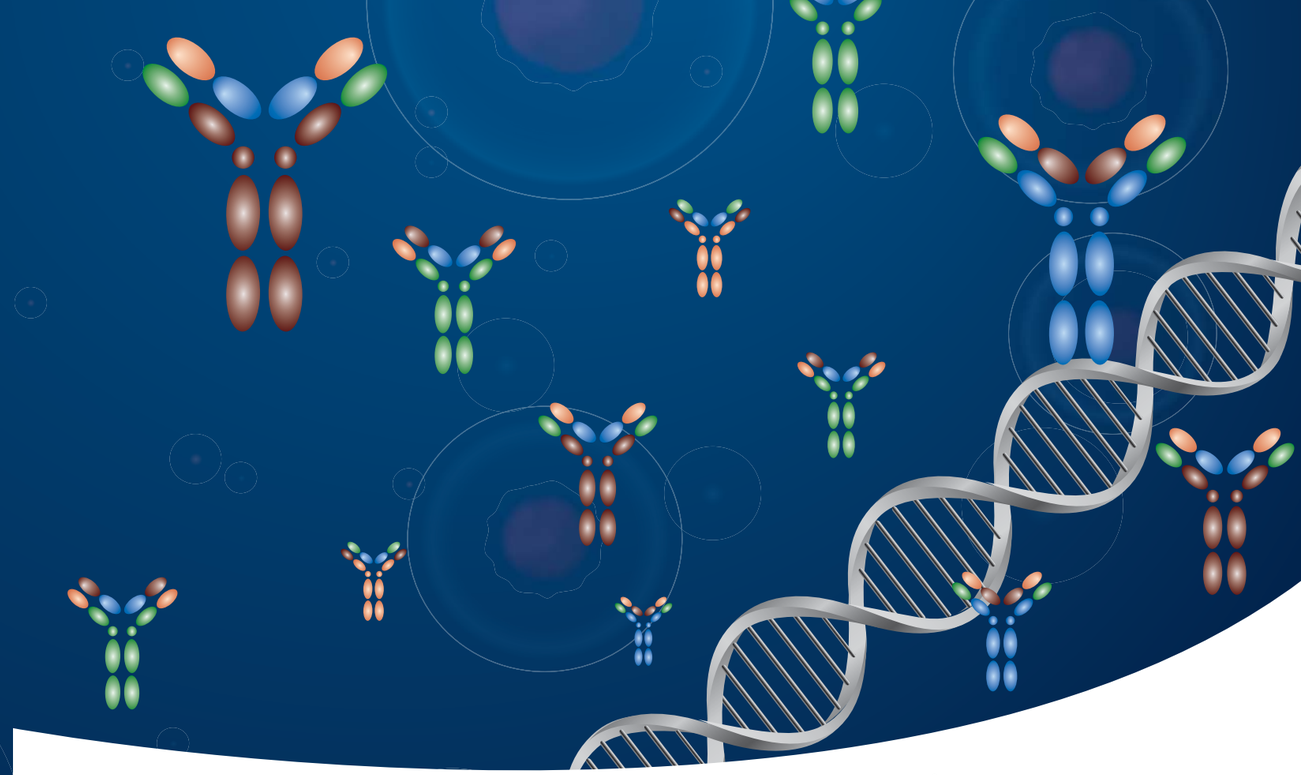
GenScript

Antibody Handbook

With Focus on Recombinant Antibodies and Antibody Drug Discovery

www.GenScript.com

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854 USA
Phone: 1-732-885-9188
Toll-Free: 1-877-436-7274
Fax: 1-732-885-5878



GenScript®
Make Research Easy

To download this handbook as a PDF, please visit

http://www.genscript.com/rAb_handbook.html

TABLE OF CONTENTS

This handbook provides a broad overview of the discovery process for generation of Antibody-based therapeutics, while discussing basic concepts around Abs and their recombinant production.

Antibodies produced from simple forms of inoculation are likely to have been used for quite some time before Edward Jenner pioneered vaccination in the late-eighteenth century.

GenScript is a leading Contract Research Organization (CRO) that is focused on providing high quality and reliable Ab drug discovery services. GenScript services include Antibody (Ab) production, Ab sequencing, humanization, affinity maturation, phage display and much more.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Introduction

Monoclonal antibodies (mAbs) were first described in the mid-1970s and there was great optimism around the clinical use of antibodies as “magic bullets” to target tumors. But this great hype gave way to pessimism as time went by. Antibodies derived from mouse induced unwanted immune responses in human subjects. Poor effector function in humans also limited murine Ab potency. Over the last 2-3 decades various technologies (display technologies, humanization, transgenic mice) have allowed the generation of Ab drugs that are better suited for the human immune system.

As such, Antibody (Ab)-based therapeutics is at the center stage of drug discovery with antibodies being the fastest growing class of drugs. Antibodies are ideal for therapeutic interventions in part owing to their:

- High specificity
- High tolerance
- Long half-life that allows infrequent dosing
- Amenability to manipulation in order to improve binding and effector functions

Given some of these factors there is a robust increase in the development of human mAbs. This interest is also fueled by advances in technologies to generate antibodies - transgenic mice, yeast display, phage display etc. As a result there has been a major shift in focus of many pharmaceutical companies as they started moving from exclusive small molecule drug discovery to a broader portfolio containing both, chemical entities [small molecules] as well as biotherapeutics [large molecules including antibodies].

This handbook serves to provide a broad overview of the discovery process for generation of Ab-based therapeutics, while discussing basic concepts around Ab production. Discovery topics will include target selection, target assessment & validation, screening, lead identification and optimization, leading up to candidate selection. GenScript’s service capabilities in recombinant antibody (rAb) production are provided where necessary. Glossary of important terms in alphabetical order, along with references and suggestions for further reading, are provided at the end.

The History of Antibodies

The earliest reference to antibodies came from Emil von Behring and Shibasabura Kitasato in 1890[1]. In a landmark publication they showed that the transfer of therapeutic serum from animals immunized against diphtheria to animals suffering from it could cure the infected animals[1,2]. The potential for treatment in humans was immediately apparent and Behring was later awarded the Nobel Prize for this work in 1901[3].

In 1900 Paul Ehrlich, proposed the side-chain theory, where he hypothesized that side chain receptors on cells bind to a given pathogen. He was the first to propose a model for an antibody molecule in which the antibody was branched and consisted of multiple sites for binding to foreign material, known as antigen, and for the activation of the complement pathway. This model agreed with the ‘lock and key’ hypothesis for enzymes proposed by Emil Fischer[4,5].

Astrid Fagraeus in 1948 described that plasma B cells are specifically involved in antibody generation and by 1957 Frank Burnet and David Talmage had developed the clonal selection theory[6]. This stated that a lymphocyte makes a single specific antibody molecule that is determined before it encounters an antigen, which was in contrast to the instructive theory developed by Linus Pauling in 1940 where the antigen acted as a template for the antibody[7].

By 1959 Gerald Edelman and Rodney Porter independently published the molecular structure of antibodies[8,9], for which they were later jointly awarded the Nobel Prize in 1972[10]. The first atomic resolution structure of an antibody fragment was published in 1973[11] and this was quickly followed by the invention of monoclonal antibodies[12] in 1975 by Georges Köhler and César Milstein signaling the start of the modern era of antibody research and discovery.

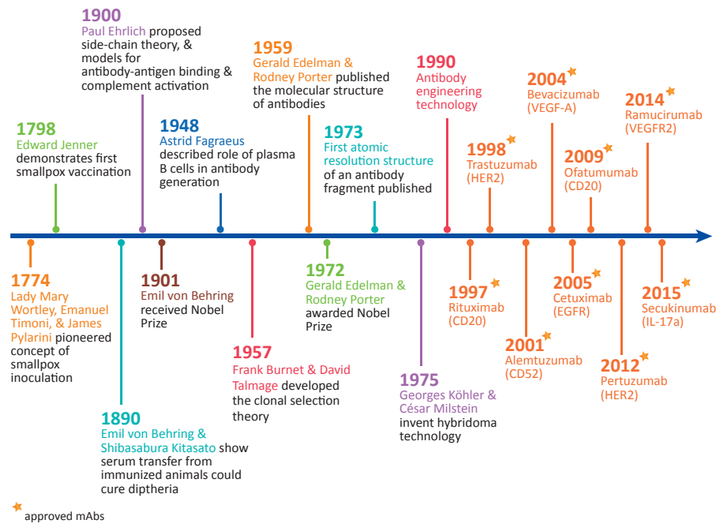


Figure 1: Antibody research has come a long way from its earliest reference by Emil Von Behring in 1890.

INTRODUCTION

The Nobel Prize in Physiology or Medicine, 1984 was awarded jointly to Niels K. Jerne, Georges J.F. Köhler and César Milstein "for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies".

GenScript offers a variety of services for the discovery of monoclonal antibodies and hybridomas to ensure that your project runs smoothly and in a cost-effective manner.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The IgG subclasses may have arisen from gene duplication during mammalian evolution.

GenScript can produce all IgG subclasses found in many species using recombinant expression technologies.

Antibody Basics

Humans have 5 classes of Abs (interchangeably used with Immunoglobulins or Igs). IgG, IgA, IgD, IgE and IgM. All 5 classes are secreted by activated B cells as glycoproteins. All human Igs possess a basic monomeric "H2L2" structure consisting of 2 Heavy (H) chains and 2 Light (L) chains. Each H chain is paired with one L chain. H chains define the class of Ig [symbolized by Greek letter γ, α, δ, ε, μ] and the L chains are comprised of either κ or λ isoforms. Each Ig possesses 2 defined regions. The upper-half antigen-binding regions (Fabs) and the lower-half crystallizable fragment (Fc). The Fc region is composed entirely of H chain whereas the Fab region consists of both H & L chain domains. In IgG, IgA, IgD classes the Fab is separated from the Fc by a flexible hinge region whereas in the IgE and IgM classes an extra constant domain replaces the hinge resulting in less flexibility of the Fab domains. Additionally, IgA and IgM possess the following

- Tailpiece region that confers ability to form multimers[13]
- J (Joining) chain region that confers ability to bind specific receptors

Antibody production in the body begins by the expression of IgM and IgD on the surface of naïve B cells in response to antigenic stimuli. Through the process of hyper mutation and class switching, high affinity IgGs are produced. Human IgG is further subdivided into IgG1, IgG2, IgG3 and IgG4 isotypes[14].

Since a vast majority of all therapeutic antibodies employ an IgG1 isotype, it has become the default isotype for many Ab candidates. And hence, more is known about the biology and functionality of IgG1 than any other isotype. As such, most of the references in the handbook will pertain to IgG1 isotype.

IgG overview

- Divalent Abs derived from gamma gene locus
- There are 4 isotypes (IgG1, 2, 3, 4)
- Half-life of IgG 1,2 and 4 is 2-3 weeks
- IgG3 half-life is 1 week
- IgGs are the most prevalent Abs found in serum
- Approximate MW is 150kDa

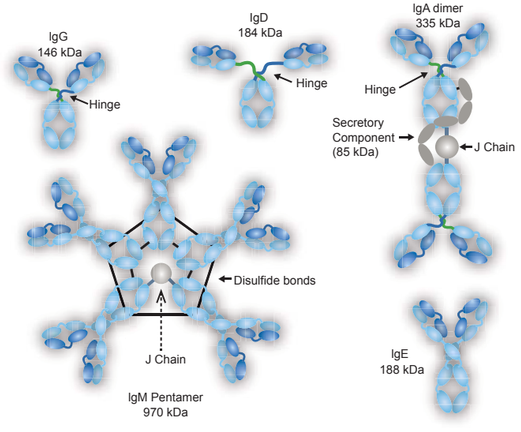


Figure 2: Structures of different human Immunoglobulin isotypes.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

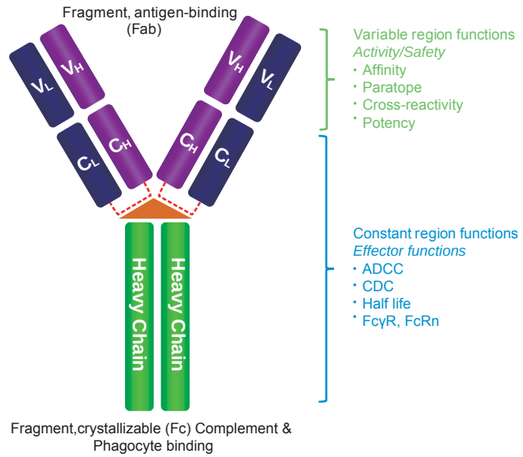


Figure 3: Basic Ab [IgG] structure - Ab is a Y shaped molecule consisting of 2 heavy (H) chains and 2 light (L) chains folded into constant (C) and variable (V) domains. The Fab domain consists of 2 variable and 2 constant domains with the 2 variable domains making up the variable fragment (Fv). The Fv provides antigen specificity of the Ab with the constant domains providing a structural framework. Each Fv contains 3 hypervariable loops known as Complementarity Determining Regions (CDRs). It is the hyper variability of the CDRs that allow an Ab, in theory, to recognize an unlimited number of Antigens. Approximate L chain amino acid residues are 220 and MW is ~25kDa. Approximate H chain amino acid residues are 455 and MW is ~50kDa. One L&H chain equals ~75kDa, thus full length IgG with both L&H chains is ~150kDa.

Antibody Formats

Trends in clinical development of monoclonal antibodies indicates that there is a nascent shift toward the study of antibody fragments and it is likely that antibody fragments are going to be the next important class of protein-based therapeutics after monoclonal antibodies. Antibody fragments [fragments], in particular the scFvs, Fabs and V_{HH}/V_H retain full antigen-binding capacity and superior properties for research, diagnostic and therapeutic applications. Fragments are particularly useful in applications where epitope binding is sufficient for the desired effect including therapeutic applications such as virus neutralization or receptor blocking.

The smallest antigen binding fragment that retains its complete antigen binding site is the Fv fragment, which consists entirely of variable (V) regions. A soluble and flexible amino acid peptide linker is used to connect the V regions to a scFv (single chain fragment variable) fragment for stabilization of the molecule, or the constant (C) domains are added to the V regions to generate a Fab fragment [fragment, antigen-binding]. scFv and Fab are widely used fragments that can be easily produced in prokaryotic hosts. Other Ab formats include disulfide-bond stabilized scFv (ds-scFv), single chain Fab (scFab), as well as di- and multimeric antibody formats like dia-, tria- and tetra-bodies, or minibodies (miniAbs) that comprise different formats consisting of scFvs linked to oligomerization domains. The smallest fragments are V_{HH}/V_H of camelid heavy chain Abs and single domain Abs (sdAb). For most therapeutic applications, however, the Fc portion of an Ig is essential as it is instrumental in mediating cytotoxic effector functions such as ADCC and CDC.

Domains found within IgG Fc regions are responsible for binding receptors or components of the complement system to elicit responses.

Using sdAbs for antibody drug discovery is advantageous because their high stability facilitates downstream engineering. GenScript offers comprehensive sdAb services for research and therapeutic applications.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

INTRODUCTION

When compared to traditional mAbs, V_{HH} antibodies are entirely devoid of the light chain and the constant C_{H1} domain of the heavy chain.

Using FragPower™ technology, GenScript can deliver 10mg of scFv or V_{HH}/V_H at ≥90% purity in 7 weeks.

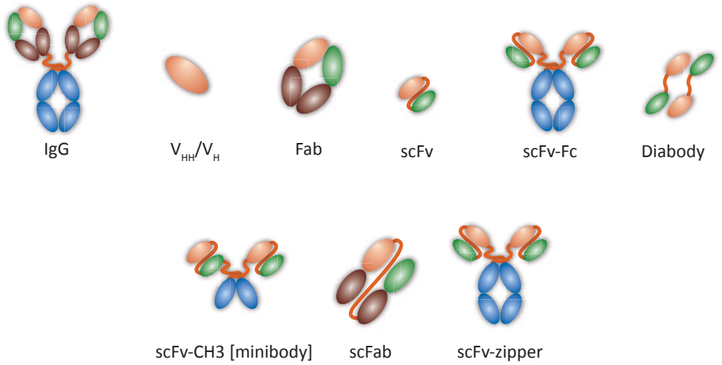


Figure 4: The modular domain architecture of Igs has been exploited to create a growing range of alternative antibody formats that spans a molecular-weight range of at least 12–150 kDa and a valency (n) range from monomeric (n = 1), dimeric (n = 2) and trimeric (n = 3) to tetrameric (n = 4) and potentially higher[15]. The building block that is most frequently used to create novel antibody formats is the single-chain variable (V)-domain antibody fragment (scFv), which comprises V domains from the heavy and light chain (V_H and V_L domain) joined by a peptide linker of ~15 amino acid residues.

Fab Antibody, scFv, V_{HH}/V_H Advantages

- Excellent tissue penetration due to small size[16,17]
- Better pharmacokinetic properties compared to parent mAbs
- Binding to cryptic epitopes not accessible to full-sized mAbs[18]
- Absence of cytotoxic effector function[19]
- Reduced systemic load
- Easy to express and produce in prokaryotic expression system[20,21]
- Genetically stable
- Less expensive, lowered production costs
- Easy to engineer and modify

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Antibody Drug Discovery Process

The overall discovery process for Ab therapeutics can be broadly divided into five stages:

- Target assessment & validation
- Screening preparation
- Hit generation, screening & lead selection
- Lead optimization & characterization
- Candidate selection

Often time, screening, hit generation & lead selection are also collectively referred to as "Lead Discovery". Activities leading up to the selection of the clinical candidate are referred to as "preclinical" work/research. While the language and choice of words changes from one publication to another, the concept remains the same.

Figure 5: Overview of discovery process for therapeutic antibodies leading to the selection of a clinical candidate is shown in the workflow above. Key activities at each of the five stages are listed in the text boxes.

How to decide whether to pursue Ab or small molecule?

It depends on the target. Small molecules penetrate the cell whereas large molecules (Ab therapeutics) typically target cell surface molecules like receptors or tumor antigens. And hence research will dictate what kind of intervention is required in a particular disease pathway. See major differences between small molecule and large molecule research ahead.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The Ab leads that emerge from discovery efforts usually need to meet established criteria regarding affinity and specificity.

GenScript offers comprehensive services for Ab drug discovery, including Ab humanization, affinity maturation, epitope mapping and more.

Large molecules such as antibodies can be used to carry toxins to cancer cells. Such combinations of antibodies and toxins are also known as antibody-drug conjugates.

GenScript has over 5 years' experience in developing sdAbs for therapeutic applications. Check out a case study developed by our scientists, in generating a set of sdAbs against a cytokine target. Learn more about GenScript's sdAb services.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

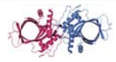
Small Molecule Drugs	Large Molecule Drugs
Differences	
 Small	Large 
Organic or metallic compounds that can bind proteins inside the body thereby altering their function in diseases	Biological molecules (proteins, monoclonal antibodies, vaccines etc.)
Produced using synthetic chemistry	Usually produced using recombinant DNA technology inside living cells
Small size <1,000 Daltons	Large size ~150,000 Daltons
Work inside the cell	Bind cell surface receptors
Less specific	Highly specific
Easier to deliver [often oral]	Delivery is difficult [usually thorough injection]
Relatively cheaper to manufacture	Manufacturing expensive
Easier to replicate [generics]	Difficult to replicate [biosimilars]
Example: Aspirin	Example: Somatropin, hGH, mAb

Table 1: Small molecule drugs vs large molecule drugs.

Target Identification

Steps involve identifying druggable targets (defined as the likelihood of being able to modulate a target with a therapeutic).

But how is a target selected?

- **Based on published data:** Literature target, patents etc.
- **"Omic" studies:** Transcriptional profiling and proteomics data that lead to the discovery of genes and proteins with aberrant expression patterns in disease states.

What are some of the considerations that go into target selection?

- **Target accessibility:** It should reside in physiological location accessible to Ab. Brain targets are notoriously difficult to treat using Ab therapeutics because the blood-brain barrier prevents their entry into brain. Single domain Abs/nanobodies can help in such cases owing to their small size (~13kDa).
- **Expression:** Should be expressed in pathological tissue.
- **Concentration:** Should be present at level that is detectable and can be stoichiometrically bound by Ab therapeutic.

What happens after a target is identified?

- **Therapeutic strategy is defined:** For example, a common molecular mechanism for an antibody-based therapeutic is the blockade of a ligand-receptor interaction, for which there are three conceivable targeting strategies: an anti-ligand antibody, an anti-receptor antibody, and a receptor-Fc fusion protein[22].

Target Assessment & Validation

The first step after a target has been identified is to validate it. Validation is simply defined as having gathered adequate scientific evidence for the target's disease association and its therapeutic potential. This is achievable through animal model studies, including mouse genetics. Examples listed below:

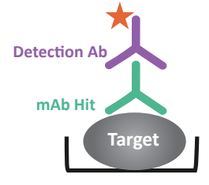
- Deletion of the mouse ortholog and/or overexpression of mouse protein should mimic human pathology[22].
- Ab-based intervention of target in an animal mimicking human disease should yield the desired therapeutic outcome[22].

Typically a monoclonal antibody (mAb) recognizing the mouse ortholog of the intended human protein is obtained as a surrogate Ab to facilitate target validation. Research materials/reagents at this stage typically include surrogate antibodies, transgenic animals and cell lines. Biological experiments also include knockout, transgenic animals or RNAi techniques. Majority of targets meet some but not all criteria and additional validation efforts are often conducted.

Screening Preparation

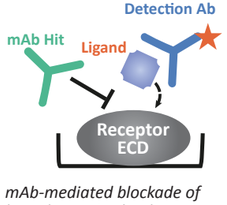
Please note that the word "screening" can be used very broadly. It can be loosely used to identify Abs with high titers, identify ideal expression conditions for Ab production etc. In Ab drug discovery, screening involves a lot of preparation and is typically the bottleneck of the entire process[22]. High quality reagents and optimized assays are key for successful screening outcome. Reagents include materials used to develop screening assays, antibody generation and for mechanistic studies. Examples are cDNA, expression plasmids, cell lines, purified proteins, reference antibodies, target orthologs etc. If companies are outsourcing their projects at this stage, they are very particular about the quality of reagents. For example proteins purified by CROs are typically validated in-house for purity, aggregation, endotoxin, bioactivity etc. Screening steps typically include screening assays [primary, secondary, tertiary assays], in vivo efficacy studies, PK/PD, toxicity studies. This comes after early Ab candidates (hits) have been generated. The screening library consists of cDNA or proteins. Researchers generate proof-of-principle efficacy data in relevant *in vitro/in vivo* models with surrogate agent[22].

Primary screening: Typically a binding assay - measures Ab binding to target antigen to identify "hits". ELISA in 96- or 384-well HTP format is most commonly used. Antigen is immobilized to ELISA plate and Ab is added to plate. Bound Ab is detected with secondary Ab. FACS is another primary screening option.
Figure 6: Schematic on right illustrates principle.



Binding of mAb to immobilized target by ELISA

Secondary screening: Designed to measure bioactivity in addition to binding. Typically it's a plate-based functional assay in HTP format. Example would be plate-based ligand/receptor binding ELISA to identify Abs that block ligand-receptor interactions.
Figure 7: Schematic on right illustrates principle.



mAb-mediated blockade of ligand-receptor binding in binding ELISA

For many target antigens, functional assays are not available. In such cases, secondary screening assays are designed to rank the affinities of Abs identified in a primary screen.

Purified Abs are often required for secondary screening assays. GenScript provides high quality rAb services that can deliver between microgram to gram quantities of purified Abs.

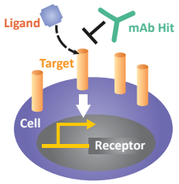
GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The word "hybridoma" is a portmanteau derived from hybrid and myeloma. Immunologist Leonard Arthur Herzenberg is credited for coining this term.

GenScript can sequence your hybridomas and produce rAbs for you.

Tertiary screening: Designed to measure bioactivity in addition to binding. Typically it's a low-throughput cell-based assay. Example would be cell-based ligand/receptor binding assay with a signaling readout.
Figure 8: Schematic on right illustrates principle.



mAb-mediated blockade of ligand-receptor binding in cell-based assay

What else is required during the screening step?

Positive control: Can be commercially available with function similar to the Ab lead. It can be a polyclonal Ab or it can be Ab reconstructed from sequences in public domain including competitor's patented Ab.
Negative control: Should be of same species and isotype as the lead Ab. Should not bind proteins expressed in the model system.
Animal models may also be initiated during this phase since this process may take years to complete.

Hit Generation, Screening & Lead Selection

Please note that in some publications, hit generation, lead selection, lead optimization and characterization are collectively referred to as "Lead Discovery", classified under "Late Stage Research" activities. Activities typically include generating early antibody candidates [hits]. Technologies include hybridoma, phage display and yeast display platforms.
Hybridoma technology: Classical technology to produce high-affinity rodent Abs[12]. Schematic below illustrates the principle.

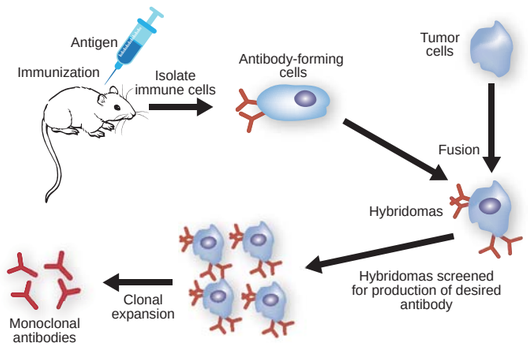


Figure 9: Technology involves forming hybrid cell lines (called hybridomas) by fusing an antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture, in order to produce monoclonal antibodies.

Phage display: It is an *in vitro* selection technique in which a peptide or protein is genetically fused to a coat protein of a bacteriophage, resulting in display of the fused protein on the exterior of the phage virion, while the DNA encoding the fusion resides within the virion[23-25]. This physical linkage between the displayed protein and the DNA encoding it allows screening of vast numbers of variants of the protein, each

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

linked to its corresponding DNA sequence, by a simple *in vitro* selection procedure called "biopanning."

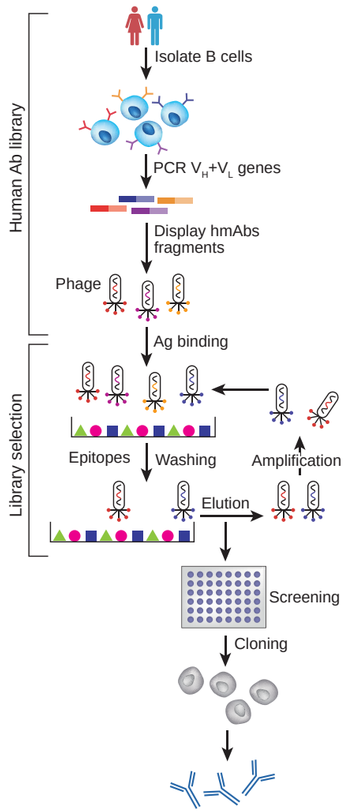


Figure 10: Three steps are included in this technique:
- **Antibody library construction and display onto the phage surface**
- **Selection by panning the library against antigen (Ag) targets**
- **Screening for desired specificity**

Description: Diverse human Ig-variable-region gene segments (such as scFv or Fab fragments) are amplified from human B cells of immune or non-immune sources to construct the antibody library. The library is then cloned for display on the surface of the phage. Selection against the desired target is then performed using the phage display library; antibodies that do not bind are washed away and the binders are eluted and amplified by infection of *E. coli*. After multiple rounds of such selection, desired specificity can be screened using ELISA [or FACS if a cell-membrane bound protein is the target]. Once the desired specificity is obtained, the genes of antibody variable regions can be cloned into whole human IgG expression vectors and transfected into cell lines to produce fully human mAbs[26].

In its simplest form, biopanning is carried out by incubating the pool of phage-displayed variants with a target of interest that has been immobilized on a plate or bead, washing away unbound phage, and eluting specifically bound phage by disrupting the binding interactions between the phage and target. The eluted phage is then amplified *in vivo* and the process repeated, resulting in stepwise enrichment of the phage pool in favor of the tightest binding sequences. After a few rounds of selection/amplification, individual clones are characterized by DNA sequencing[27,28]. Main advantage of this technology is the ease of screening and its HTP nature.

Yeast display: Similar to phage display. Protein of interest fused to yeast surface protein[29]. One of the most significant features of this approach is the use of eukaryotic host which possesses the secretory biosynthetic machinery to facilitate oxidative protein folding and glycosylation. And hence this technique is well suited to the display of mammalian-derived cell surface or secreted proteins[30,31]. In addition to Abs, yeast display has also been used to optimize proteins such as IL-2, T-cell receptors, integrins and epidermal growth factor receptors[32-35].

Ribosome display: Ribosome display is an *in vitro* method for display of peptides or proteins centered around the formation of a complex of mRNA, ribosome and translated peptide or protein of interest[36,37]. Conditions are optimized such that the

The wide use of phage display method is based on its advantages over other systems such as simplicity and stability of phage particles.

GenScript has its own phage display technology with diversity of 10^{10}.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

DID YOU KNOW

The most widely used method of humanization involves CDR grafting which involves grafting non-human Ab into human frameworks selected for their homology to the non-human framework.

QUICK TIP

GenScript's unique antibody humanization service combines CDR-grafting, library-based proprietary 'framework assembly' method and FASEBA screening (FAst Screening for Expression level, Biophysical properties, and Affinities).

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

complex is stabilized to the extent that libraries of variants can be screened against potential binding partners. The coupling of genotype to phenotype that is at the core of all display technologies exists in ribosome display. The gene that encodes the protein of interest is engineered with an efficient promoter and the ribosome binding site and the fusion of a tether that allows display of the folded protein outside of the ribosome tunnel. The construct lacks a stop codon causing translation to stall. High concentration of magnesium, combined with low temperature conditions increases the stability of the complex. Incubation with the antigen is performed, and elution of select clones is performed by dissociation of the complex with the addition of chelating agents. RT-PCR amplification is performed to enrich the gene that encodes the protein of interest.

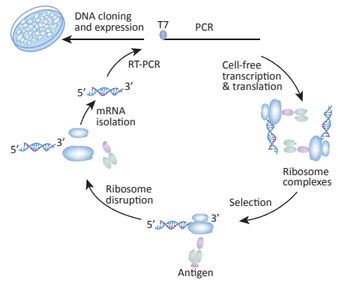


Figure 11: Ribosome display concept.

Basically, display technologies allow generation of human Abs without the need for humanization. Initial hits from this process may exhibit low affinity in which case a process called affinity maturation may be required [see description ahead for humanization and affinity maturation].

Lead selection refers to the process by which the early hits are interrogated in a vigorous, multi-step screening process to select lead molecules that meet pre-established criteria for progressing to the next stage. Screening via secondary and tertiary functional assays filters from hundreds of hits down to a few Ab molecules. These are then purified in milligram quantities for more detailed characterization[22].

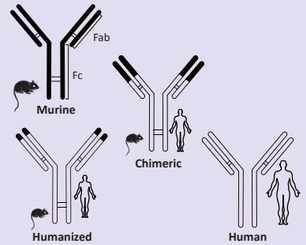
Characterization typically includes *in vitro* efficacy studies for confirmation of binding and functional activities as well as biochemical and biophysical analyses. Common molecular analysis includes determination of expression levels from mammalian expression systems, aggregation analysis by SEC, SDS-PAGE, Western blot analysis, determination of target protein binding affinity by Biacore and KinExA analysis, and crude epitope mapping[22]. Examples of *in vitro* studies include Fcγ, FcRn, C1q assays. Upon completion of *in vitro* characterization, selected hits are ready for expression and purification in sufficient quantity (typically few hundred milligrams to grams) for *in vivo* efficacy testing. Occasionally, a crude PK study is also conducted prior to *in vivo* efficacy study to help establish the dosing regimen. Typically the lead molecule is selected based on demonstrated *in vivo* efficacy, which is often a go/ no-go decision point for the program[22].

Lead Optimization & Characterization

Steps include Ab production, humanization (in case of rodent or other non-human antibody), affinity maturation, Fc engineering, characterization of biochemical properties, *in vitro* and *in vivo* pharmacology. Ab production is via transient expression or stable cell lines. *In vivo* pharmacology includes PoC efficacy, MOA, PK/PD and preliminary toxicological studies.

What is humanization and why is it performed?

Humanization is a technique to reduce the immunogenicity of a therapeutic antibody initially derived from rodents [non-humans]. The process refers to the replacement of more than 90% of rodent IgG sequence in the parental antibody molecule with human IgG sequence. **Figure 12: Schematic on right illustrates principle.**



What is affinity maturation and why is it performed?

Affinity maturation is usually applied to antibody leads selected from a naïve human library using a display technology. These leads may have relatively low (10–100nM)[22] target binding affinities but can be enhanced to reach a desired affinity range (normally 0.1–10nM). Note that high affinity does not always correlate with improved Ab efficacy.

What is Fc engineering and why is it performed?

The Fc region of an Ab is a functional molecular entity mediating

- ADCC via binding to Fcc receptors (FccR) on natural killer (NK) cells
- CDC via C1q binding
- Increase in the *in vivo* half-life via binding to the neonatal Fc receptor (FcRn)

Alteration of each of these activities has been explored to modulate the function of Ab in specific applications. For example, ADCC enhancement improves tumor cell killing, achieved by engineering site-directed mutations in the contact residues. You could also increase Ab half-life by Fc engineering. After the generation of an optimized lead, further functional and molecular characterization is carried out to confirm its *in vitro* and *in vivo* activity and favorable molecular attributes as a therapeutic candidate[22].

Candidate Selection

Clinical candidate selection is a milestone decision marking a commitment to advance a therapeutic antibody candidate into clinical trials in human patients. The optimized lead molecule(s) undergoes stringent assessments that constitute the candidate selection process. At the end of this process a critical decision is made regarding whether the antibody qualifies as a clinical candidate. Core criteria include:

- Efficacy demonstration in cellular and animal models
- Animal dose responses studies to guide dosing regimen in clinical development
- Satisfactory pharmacology and PK studies
- Acceptable safety risk
- Manufacturability

DID YOU KNOW

For therapeutic Ab programs, the affinity maturation of a single lead candidate to a target can take several months to achieve.

QUICK TIP

Antibodies generated with hybridoma technology usually have relatively high affinity but this affinity may still not suffice the need of a therapeutic antibody. GenScript's Antibody Affinity Maturation service can increase your Ab affinity to an ideal level.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Abs produced during HAMA response can neutralize or cause clearance of therapeutic Ab in immune complexes and can even sensitize the patient to allergic reaction on re-administration.

GenScript offers Custom Antibody Drug Discovery Services, using which our highly trained and experienced scientists provide high-quality deliverables in a timely and cost effective manner.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Candidate selection also represents transition from early discovery to clinical development phase. During transition, candidate(s) are assessed for optimization to facilitate process development and manufacturability. This usually involves an assessment of expression or titer based on data available from the discovery process that may include data from transient expression or pools derived from stable transfection in a CHO host cell line. In transient HEK-293 systems, titers below 50 mg/L may present challenges in supplying material to enable discovery research. While there does not appear to be a direct correlation between expression titer in a transient system and titer in the subsequent stable mammalian cell line, transient expression titers below 50 mg/L would be a potential concern[22]. Such expression levels would likely require close monitoring during development to ensure acceptable expression titers are achieved in stably transfected mammalian cell lines.

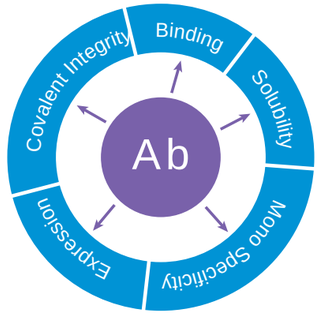


Figure 13: Important attributes of a good Ab candidate.

What else happens during this time? Stability studies are carried out that include evaluation of aggregation and degradation in a preferred formulation or set of formulations. Aggregation can occur during all phases of production and controlling the levels of aggregate in the final product can be challenging. In addition to aggregation, significant degradation pathways, such as oxidation, deamidation, isomerization, and peptide bond cleavage are also evaluated early, typically at multiple temperatures[38,39]. GenScript characterization services come handy in such cases.

Summary of Ab Drug Discovery

Candidate selection is the single most important discovery milestone marking the end of the discovery activities and the beginning of the clinical testing phase of an experimental drug. This decision point is reached after a comprehensive data package is assembled on the lead molecule and evaluated by a group of experts in various disciplines including discovery sciences, manufacturing, drug safety, drug metabolism, regulatory, legal, commercial, as well as clinical.

Note: Development activities include preclinical development, process development, clinical development, strategic planning, IND filing (not addressed in this handbook).

Important Challenges in Ab Drug Discovery

- Cost – takes more to develop than small molecule therapeutics[40]
- Human anti-mouse (HAMA) response[41,42]
- Non-specific targeting of antigens on healthy cells
- Toxicity caused by binding to surface antigens shed into circulation
- Limitations in biological activity due to location of binding and stimulation of immune response

Topics in rAb Production

There is a great need to develop expression systems with high production yields, fast turnaround times and improved process economics. Despite the fact that the majority of approved antibodies are full-length, there is increasing interest to produce smaller antibody fragments such as Fab, scFv, sdAbs as well as more complex bispecific antibodies.

Antibody Expression in Prokaryotic Hosts

Typically, if an antibody fragment is acceptable, expression in a bacterial host [*E. coli*] maybe the best choice.

E. coli: The simplicity and ease of fermentation has made *E. coli* an ideal host for antibody fragment production. But the absence of complex post-translational modifications and glycosylation has limited the use of *E. coli* for producing full length rAbs.

***E. coli* advantages include**

- Well characterized genetics
- Easy manipulation
- Short process development timeline
- Simple fermentation
- Scalability
- Less safety issues from viral contaminants

Cytoplasmic expression of antibody fragments in *E. coli* often results in the production of aggregates within inclusion bodies. Antigen binding activity can be reconstituted after polypeptide refolding[43,44] but recovery takes a hit in this process. Strategies to improve soluble expression include the removal of cysteine residues within rAb sequences.

Alternately, periplasmic expression can be attempted but yields can be an issue. In order to produce properly folded functional antibodies with disulfide bonds, the individual antibody chains must be transported to the oxidizing environment of the bacterial periplasm. The periplasm also contains proteins such as disulfide isomerases and chaperonins which aid in the refolding of the newly synthesized proteins. For periplasmic expression, the antibody is directed to the oxidizing environment of the periplasm by the use of a leader sequence (PelB, OmpA, PhoA)[45,46]. After expression, antibody can be recovered from the periplasmic space by osmotic shock. Yields have been reported in the range of 0.1mg/L to 100mg/L in shake flask cultures and up to 2g/L when using fermenters[45-48].

One can also try specialized *E. coli* strains that provide an oxidizing environment in the cytoplasm. These are usually trxB and gor mutants[49].

E. coli expression is used for therapeutic Ab fragment production of Lucentis® - a prescription medicine for the treatment of patients with wet age-related macular degeneration.

GenScript provides high quality Ab fragments at very competitive prices and timelines using its proprietary FragPower™ technology.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The structure of carbohydrate modifications can affect many biological properties of glycoproteins including bioactivity, pharmacokinetics, secretion, solubility, and antigenicity. The host glycosylation apparatus can thus have a major impact on the glycan content and potency of the rAb.

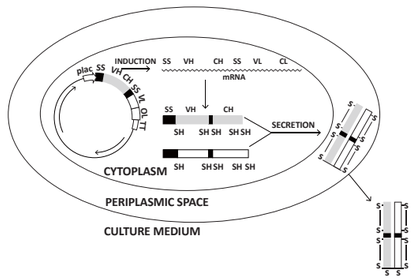


Figure 14: Expression of a soluble secreted Fab fragment in *E.coli*. After induction, dicistronic mRNA is synthesized and subsequently translated into preproteins in the cytoplasm. Secretion to the periplasm is facilitated by the use of signal peptides which are removed during the translocation process. The secreted chains fold and assemble in the oxidising environment of the periplasm and the active Fab fragment leaks into the culture medium.

Some of the factors that affect *E. coli* expression of rAbs are:

- Sequence[50]
- Secretion
- Transcription and translation efficiency[51]
- Protein folding rates[52]
- Aggregation and degradation[53]
- Ratio of HC and LC[54,55]
- Proteolysis

Antibody Expression in Eukaryotic Hosts

Since eukaryotic hosts possess complex protein folding and secretory pathways, they can carry out complex post-translational modifications and secretion of rAbs. Their ability to glycosylate [add sugar groups] is absolutely critical for antibody effector functions such as ADCC and CDC. And so they are the most ideal host choice for the production of full length rAbs.

Yeast

Pros	Cons
• Ability to fold, glycosylate and secrete rAbs • Simple fermentation requirements • Short generation time allows rapid scale up and high biomass production • Lack of endotoxin makes purification simpler • Low levels of endogenous secreted proteins	• Although high expression levels have been reported, typical yields are low • Mis-folding issues • Glycosylation different from mammalian cells • High amount of mannose sugars due to ineffective trimming • Excessive formation of O-linked carbohydrates[56,57] • Inability to add fucose residues

GenScript's ISO 9001 certified Antibody Sequencing Service can expedite and facilitate your patent application, database banking, and therapeutic antibody development.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Insect

Pros	Cons
• Expression levels can be as much as 50% of total cell protein • Stable insect cell lines possible (S2)[58]	• Low level of expression[59] • Long time to produce high titer virus stocks [baculovirus] • High incidence of intracellular accumulation of mis-folded and aggregated protein[60] • Protein recovery difficult since majority of rAb trapped within cells[60] • Glycosylation pattern different from mammalian cells although ADCC and CDC retained[61-63]

Alternative Hosts

The use of gram positive bacteria such as *Bacillus* has been reported for rAb expression[64,65]. Since Gram positive bacteria lack an outer cell wall and have no periplasmic space, secreted proteins accumulate into the culture media. Filamentous fungi such as Aspergillus have been known to express antibody fragments[66]. *Nicotianna tobaccum*[67], Alfalfa[68], rice, wheat[69], soya bean[70] have been engineered to produce rAbs. Transgenic animals are also known to produce antibodies[71]. But given its robust expression profile, suitable glycosylation pattern and wide usage, mammalian cells are the best choice for the production of rAbs.

Mammalian Cells

The production of antibodies was once limited to hybridomas. Hybridomas are unsuitable for large scale production due to low expression levels [<100mg/L] and low integrated viable cell count [IVCC] in bioreactors. These days, antibody genes are isolated from individual B-cells which are then cloned into mammalian expression vectors for rAb production. Mammalian cell lines typically used for rAb production include CHO, NS0, BHK, HEK293 and few others with HEK293 and CHO cells being the most popular choices for transient and stable expression respectively.

HEK293: Isolated from human embryonic kidney[72]. Used as host for small-scale recombinant protein production for over 2 decades. These cells can be efficiently transfected in suspension at large scale using cost-effective methods such as PEI or calcium-phosphate[73].

CHO cells: These cells are derived from the ovary of the Chinese hamster. They are the preferred mammalian host for biologics production worldwide, with over 70% approved mAbs expressed in these cells.

Some of the advantages of CHO cells include:

- Ease of cultivation at large scale
- Adaptability to suspension growth in serum-free media[75-77]
- Good safety profile with low risk of adventitious viral agent reproduction[78]
- Glycoforms that mimic human IgG glycoforms[79]

The first report of full-length Ab expression in plant cells was in 1989 by Hiatt *et al*. Since then there have been several reports and many companies are currently engaged in the production of Abs in plants.

GenScript uses its MamPower™ technology to deliver flexible-scale, rapid production of your target recombinant antibody in either CHO or HEK293 cells.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Genetically engineered CHO cell lines have been generated to improve productivity by enhancing post-translational processing of complex proteins, altering glycosylation profiles, delaying onset of apoptosis and altering proliferation rates.

If you are not sure if transient expression is right for you or if you are exploring a lower-cost mammalian protein expression evaluation package, GenScript offers PROTential™ standard service so you can test for soluble expression in CHO or HEK293 cells before deciding to scale up.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Factors that affect rAb production in mammalian cells

Expression vector technology is critical to achieve optimal stable or transient rAb expression. Key elements of a vector include the promoter and enhancer elements, kozak sequence, poly A signal, selectable markers, origin of replication and chromatin remodeling elements.

In order to achieve efficient IgG expression, it is essential to control the balance of heavy chain and light chain production. On entry into the endoplasmic reticulum as unfolded polypeptides, both chains are modified and assembled. Only completely assembled molecules can bind antigen and carry out effector functions. Light chains are synthesized 15%-25% faster than heavy chains[80] and the isotype of light chain has been shown to influence the kinetics of intracellular IgG assembly[81].

Most light chains can be secreted as free monomers or homodimers[82]. Whereas full length H chains are only exported from the cell when combined with the light chain to form complete antibody molecules[83]. The expression of both antibody chains is usually achieved either by co-transfecting cells with 2 independent monocistronic constructs or the use of a single vector with the light chain and heavy chain genes linked in series and transcription driven by 2 identical promoters.

Transient expression vs Stable expression

The choice of expression system used in different stages of antibody drug discovery is dependent on a number of factors, including the number of antibody variants, the quantity of material required, the quality of material required and the turnaround timeline.

If both Transient and Stable cell lines can be used for Recombinant antibody production, how do I pick the right one?

Good question! Unfortunately, there is no right answer.

It all depends on what your experimental needs are. Both these transfection methods involve getting the foreign (target) gene into the cells. In transiently transfected cells, the foreign DNA does not integrate into the host genome and as such it does not replicate and is eventually lost through cycles of cell division over several days. Transient transfection is good if you have relatively small requirements for rAb and if you need it fast. Transient gene expression results in short term recombinant Ab production, typically 6-10 days from the point of DNA transfection. HEK293 cells normally achieve higher transfection rates and higher yields with PEI which is inexpensive and resulting in lower overall production costs.
Stable transfection also begins transiently but through a process of careful selection and amplification, stable clones are generated. In stably transfected cells the foreign gene becomes part of the host genome and is therefore replicated. Descendants of these transfected cells, also express the foreign gene, resulting in a stably transfected cell line. Because the stable transfection of cells is a longer and more arduous process, it is practical for rAb production on larger scales. During initial selection process when research quantities of rAbs are sufficient (10-1000mg), rapid methods of rAb production are required and so large scale transient expression is routinely used. For large scale production of therapeutic antibodies, stable gene expression is the preferred option as it allows for greater process consistency and control of the final product quality.

TOPICS IN
rAb PRODUCTION

How are rAbs produced?

Recombinant antibodies are constructed *in vitro* using recombinant DNA technology. Antibody genes are isolated from the appropriate source or synthesized based on sequence information. They are then cloned into expression vectors for expression in a suitable expression system. Full length rAbs are produced in mammalian expression system owing to its superior post-translational modification capabilities. Another advantage of mammalian system is the low risk of immunogenicity that could arise from altered, non-human glycosylation patterns. rAbs are produced using Transient or Stable expression technology.

Transient Expression

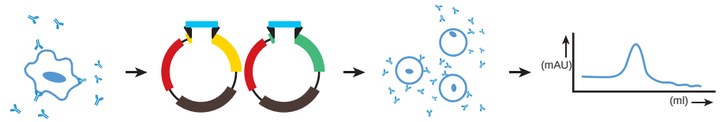


Figure 15: A typical transient expression workflow for rAb production would involve gene synthesis and/or subcloning the light chain and heavy chain genes into the appropriate expression vector followed by transient co-transfection into suspension (usually HEK293 cells). Once the cells start secreting Antibody, the cell culture broth is centrifuged and supernatant filtered (usually 5-6 days post-transfection). Filtered supernatant is then loaded onto protein A resin for purification by affinity chromatography.

Stable Expression

Two expression vector systems dominate antibody production in mammalian cell culture, one based on dihydrofolate reductase (DHFR) genes[84] and the other on glutamine synthetase (GS) genes[85].

DHFR Principle: In a DHFR expression system, the folate analog methotrexate (MTX) is used to inhibit the function of DHFR, an essential metabolic enzyme for purine and pyrimidine synthesis. Transfection with an expression vector containing the DHFR gene prevents MTX from poisoning transfected cells, whereas the cells without DHFR are selected against in the presence of MTX. Frequently, an antibiotic resistance gene is used in DHFR expression vectors to act as the selectable marker, turning the primary function of the DHFR gene to facilitating vector amplification. DHFR system is effective and has been used in conjunction with other aspects of cell line development to achieve mAb expression levels of multiple grams per liter.

GS Principle: GS synthesizes glutamine from glutamate and ammonium. Since glutamine is an essential amino acid, transfection of cells lacking endogenous GS, such as NS0, with the GS vector confers the ability to grow in glutamine-free media. Selection occurs in the absence of glutamine. Even for cells that do express functional levels of GS (CHO), including GS inhibitor methionine sulfoximine (MSX), enables use of the GS expression vectors.

Key attributes of GenScript's CHO-GS technology

- CHO K1 suspension cell line adapted to growth in serum-free media
- CHO-GS expression vector [double gene vector for mAbs]
- Guaranteed >g/L rAb expression

Neomycin, hygromycin, blasticidin, puromycin and zeomycin resistance genes are commonly used dominant selection markers that code for enzymes that confer antibiotic resistance to the cells and thus ensure cell survival in the presence of antibiotics.

GenScript offers comprehensive stable cell line development capabilities that either begin with DNA provided by our clients or that are powered by our fast, high efficiency gene synthesis service that seamlessly integrates all steps of stable cell line generation, delivering high quality stable clones of your choice.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

mAbs are among the most complex and expensive biopharmaceuticals to manufacture, in which the downstream purification process is known to represent >50% of the total manufacturing cost.

With a team of experienced scientists and a proprietary development platform, GenScript provides custom services for optimizing growth and production media for target cell lines, defining feeding strategies, studying cell line stability, and increasing production.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Stable Cell Line Service (DHFR expression)

Transfect host cells with expression vector coding for gene of interest (Total 26-29 weeks); single cell clones screening

4-5weeks
MTX amplification
15weeks
Subcloning
4-5weeks
Batch culture and stability test in shake flask
2-3weeks
Research cell bank generation
1week
Delivery of selected clones

Stable Cell Line Service (GS expression)

Transfect host cells with expression vector coding for gene of interest; single cell pool screening and optimization

4-5weeks
Single cell clone screening
4-5weeks
Fed-batch culture in shake flask
3-4weeks
Stability test
2-3weeks
Research cell bank generation
1week
Delivery of selected clones

Comparison of workflow between DHFR and GenScript GS systems shown above. GS expression system guarantees g/L rAb expression in ~4 months from the point of transfection.

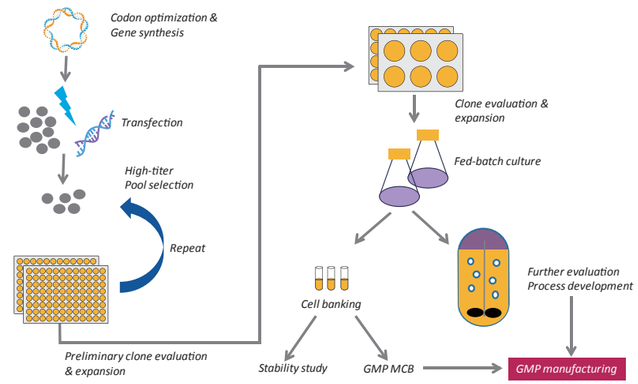


Figure 16: Schematic of GenScript GS technology workflow.

How are Antibodies purified?

No generic capture resin can purify all classes of Abs. Different resins may be required for different fragments. See schematic to understand which resins are used for Ab purification, based on what regions are present in the Ab being purified. Protein A is used for 1-step affinity purification in most cases. In addition, if required, Ion Exchange Chromatography and viral clearance procedures are usually sufficient to meet the purification demands for most therapeutic antibody candidates during the discovery phase[86]. Separation techniques such as Hydrophobic Interaction Chromatography and Mixed-Mode Chromatography are useful to reduce the formation of aggregates[87-89]. Note that resins have different names depending upon the supplier.

- Protein A or G: IgG sub classes 1, 2, 4 & Fabs containing V_{H3}
- Protein K or L: Fabs
- Protein L: Variable Light chains

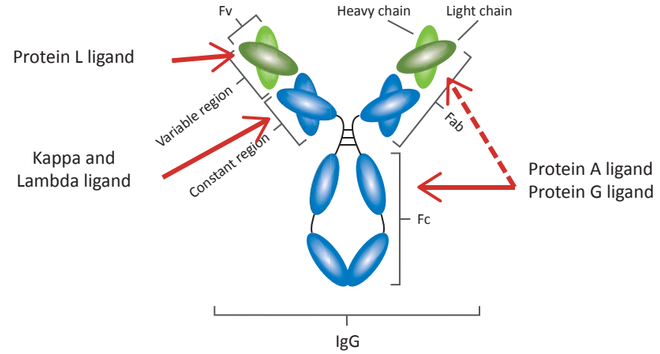


Figure 17: No generic resin can purify all classes of Abs. Schematic illustrates resin required based on the regions present in Ab being purified.

How are Antibodies characterized?

Attribute	Technique
Titer	ELISA
Quantitation	A280
MW	SDS-PAGE, MS
Purity	SDS-PAGE, HPLC
Homogeneity	SEC
Identity	Western blot, LC-MS/MS
Aggregation	SEC
Post Translational Modifications	MS
Disulfide bond analysis	MS
Deamidation, Oxidation, Peptide mapping	MS
N-terminal amino acid sequencing	Edman degradation
Endotoxin test	LAL chromogenic assay

LC-MS: What is it and why use it?

An LC-MS is a HPLC system with a mass spec detector. HPLC separates chemicals by conventional chromatography on a column (usually reverse phase chromatography). As the metabolites appear from the end of the column they enter the mass detector, where the solvent is removed and the metabolites are ionized. The metabolites must be ionized because the detector can only work with ions, not neutral molecules. The mass detector then scans the molecules it sees by mass and produces a full high-resolution spectrum, separating all ions that have different masses[90].

Why chromatography? Most biological molecules exist as isomers. Isomers have exactly the same mass and cannot normally be differentiated by a mass detector. Therefore it helps to be able to separate the isomers by chromatography[90].

Why Mass spectroscopy? HPLC separates things, but provides very little information about what a chemical might be. In fact, it is hard in HPLC to be certain that a particular peak is pure, and contains only a single molecule. Adding a mass spec tells us the masses of all molecules present in the peak, which helps to identify molecules and ensure their purity[90].

Protein A is a cell wall component of the gram positive bacteria *Staphylococcus aureus*. It is widely used as an affinity ligand for antibody purification because of its five highly homologous antibody-binding domains which can bind the Fc region of IgG through hydrophobic interactions.

GenScript has developed its own Protein A resin. Called Monofinity A Resin™, it is made of a novel recombinant protein A ligand that has a high specific binding capacity to the Fc region of Igs and enhances chemical stability under Cleaning-in-Place (CIP) conditions.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Most secondary screening methods require purified rAbs. And since these methods have become more adaptable to automated approaches, there is a great need for high throughput (HTP) expression and purification of rAbs.

GenScript leverages its expertise in high throughput gene synthesis and combines it with a proprietary transient antibody expression technology to produce high quality recombinant antibodies in either CHO or HEK293 cells in a HTP format.

GenScript Mammalian Expression Services Summary

#	Expression	Services	Target	Feature
1	Transient	MamPower™ Guaranteed Recombinant Antibody(rAb)	rAb	Flexible scale rapid production of target rAb up to 500mg in CHO or HEK293 cells
2	Transient	High Throughput Gene to Antibody	rAb	For transient recombinant antibody production of samples ≥24, up to 1mg
3	Transient	MamPower™ Guaranteed Protein	Protein	Rapid production of recombinant protein up to 10mg in CHO or HEK293 cells
4	Transient	Semi Standard Transient Expression	Secreted protein	Flexible and rapid production of secreted functional protein in CHO or HEK293 between 100ml-10L
5	Stable	Gram Level Stable Cell Line	rAb & Protein	Guaranteed gram level GS expression cell line in 4 months
6	Transient & Stable	Customized Production Services	rAb & Protein	Our customized transient and stable cell line services are for those targets that cannot be produced using our guaranteed services
7	-	Process Development	rAb & Protein	Upstream and Downstream process development
8	-	GLP-Compliant Bioprocess	rAb & Protein	For non-clinical laboratory studies. Smoothly integrate results for manufacturing and regulatory application

Table 2: Summary of GenScript mammalian expression services.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

GENSCRIPT
rAb SERVICES

GenScript Quick Reference rAb Service Selection Guide

Purpose	Service	Production Type	Volume	Antibody quantity	Timeline	Guiding Price per unit	QC standard
Screening	HTP Gene to Antibody Production	HTP Transient Expression	1ml-5ml	40μg-200μg	2-3 weeks	~$300 per variant*	· Titer: ELISA · Purity: SDS-PAGE -85% · Quantitation: UV A280
Candidate validation	MamPower™ guaranteed recombinant antibody production	Transient Expression in Shaking flask	0.1L-1L	4mg-100mg	3-4 weeks	$30-$500/mg antibody#	· Titer: ELISA · Purity: SDS-PAGE -90% · Quantitation: UV A280
In vitro efficacy	MamPower™ guaranteed recombinant antibody production	Transient Expression in Shaking flask	1L-10L	40mg-1g	4-5 weeks	$12-$75/mg antibody#	· Titer: ELISA · Purity: SDS-PAGE -95% HPLC -95% · Quantitation: UV A280 Endotoxin: <1EU/mg
	Large scale recombinant antibody production						
In vivo efficacy	Large scale recombinant antibody production	Transient Expression in Wavebag	10L-25L	400mg-2.5g	6-8 weeks	$10-$30/mg antibody#	· Titer: ELISA · Purity: SDS-PAGE -95%/99% HPLC -95%/99% · Quantitation: UV A280 · Endotoxin: <1EU/mg or <0.1EU/mg
	Custom Services	Wavebag cell pool	10L-25L	1g-12.5g	12-14 weeks	$3.2-$22/mg antibody#	
Safety test with GLP	Custom Services	Bioreactor cell pool	25L-100L	2.5g-50g	12-16 weeks	$2.4-$16/mg antibody#	· Titer: ELISA · Purity:SDS-PAGE -95%/99% HPLC -95%/99% · Quantitation: UV A280 · Endotoxin:<1EU/mg or <0.1EU/mg Identification: MS
	Gram Level Bioproduction cell line service	Bioreactor cell line	10L-100L	10g-100g	24-28 weeks	$1.5-$6/mg antibody#	

*Not including gene synthesis #Including gene synthesis

Table 3: Use chart to determine which GenScript packages are the best match for your application.

Immune checkpoints refer to the multitude of inhibitory pathways found in the immune system that are vital for maintaining self-tolerance and controlling the extent and amplitude of immune responses in peripheral tissues in order to reduce tissue damage. The discovery of immune-checkpoint protein involvement in controlling the immune response against cancer cells has led to the development of several immuno-modulatory antibody therapeutics.

GenScript has developed several cell-based immune-checkpoint functional assays to determine the immuno-modulatory profile of antibody leads.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Three core technologies that are used for identifying antibody leads include humanization of mouse mAbs, human Ig transgenic mice and *in vitro* display.

GenScript's express humanization service renders quick humanization in as little as 13 weeks.

Matching Client Needs to GenScript Services

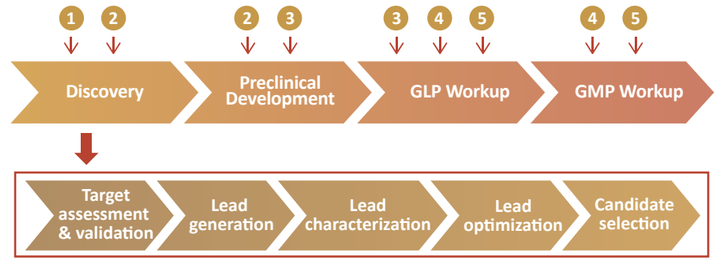
Biologics Discovery Step	Purpose	GenScript Service
Gene and cloning strategy optimization	Ensure high expression levels	Gene
Transient protein expression and purification	Druggability of the protein, initial yield evaluation	Transient protein expression
In vitro and cell based assay	Initial protein efficacy evaluation	*In vitro* pharmacology/APE
Large scale transient protein purification	More materials for protein evaluation	Transient protein expression/Stable cell pool
Cell based and *in vivo* assay	Deep evaluation on protein efficacy	*In vitro/In vivo* pharmacology
Stable cell line generation	Generate a stable cell line for GLP experiments and manufacturing	Stable cell line
Cell banking	Obtain Master Cell Bank (MCB) and Working Cell Bank (WCB)	Cell banking
Process development	Achieve SOP for production	BioProcess-Process Development
GLP protein production	Materials for safety/efficacy evaluation of the protein	BioProcess-GLP compliant service
Protein characterization	Characterization and establishment of QC standard of the protein	Protein characterization
GLP safety testing	Safety profile for the protein in primates	Not available at GenScript
GMP production of clinical materials	Clinical trials	In collaboration with business partner
Protein characterization	GMP material characterization	QC/release of the protein

Table 4: See how client needs are met by various services offered at GenScript.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

GenScript rAb Production Services



No.	Examples
1	HTP expression and purification of recombinant proteins & antibodies: Quantity: 1-5ml, purification: 96 well plates Protein A column
2	Lab scale transient expression in shaking flasks (100ml-10L): Transient expression in mammalian cells, mostly HEK293
3	Mid scale expression in wave bag (10L-25L): Transient (HEK293) or Stable pool production (CHO cells)
4	Large scale wave bag or bioreactor (varies): Transient or Stable pool production (CHO cells, >25L), or Stable cell line production (CHO GS, >10L, deliver cell line)
5	Characterization services with LC-MS as the core technology: Critical for verification and the release of bio products, more important in the latter stage of R&D, absolutely required in clinical stage

Figure 18: Match workflow with service examples in table to gain a perspective on our capabilities.

There are several analytical testing methods developed for Ab drug characterization including HPLC methods, capillary electrophoresis, analytical ultracentrifugation, mass spectrometry, Circular Dichroism (CD) spectroscopy and differential scanning calorimetry (DSC).

GenScript's one-stop-shop services provide end to end solutions taking your project from sequence to purified Ab, that include comprehensive characterization services.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The primary use of BsAb has been to redirect cytotoxic immune effector cells for enhanced killing of tumor cells by ADCC and other cytotoxic mechanisms mediated by the effector cells. Recombinant antibody fragments are also being generated as bispecific molecules to target human cytotoxic T cells or NK cells.

GenScript has launched a suite of recombinant antibody services for research surrounding cancer immunotherapy. Visit our website to choose from services that deliver between micrograms to gram quantities of pure recombinant antibody for each stage of your cancer immunotherapy, antibody drug discovery program.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Glossary

Ab humanization: *See humanization.*

ADC *Antibody Drug Conjugates (ADCs) are monoclonal antibodies (mAbs) attached to biologically active drugs by chemical linkers with labile bonds. By combining the unique targeting of mAbs with the cancer-killing ability of cytotoxic drugs, ADCs allow sensitive discrimination between healthy and diseased tissue.*

ADCC *Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies.*

Affinity maturation *In immunology, affinity maturation is the process by which T_{FH} cell-activated B cells produce antibodies with increased affinity for antigen during the course of an immune response. In Ab drug discovery, affinity maturation is often applied to antibody leads selected from a naïve human library using a display technology. These leads may have relatively low (10–100 nM) target binding affinities but can be enhanced using various affinity maturation technologies to reach a desired affinity range (normally 0.1–10 nM).*

Avidity *Refers to the accumulated strength of multiple affinities of individual non-covalent binding interactions, such as between a receptor and its ligand. It is commonly referred to as functional affinity.*

Biacore *Biacore is a GE Healthcare instrumentation that specializes in measuring protein-protein interaction and binding affinity. The technology is based on Surface Plasmon Resonance (SPR), an optical phenomenon that enables detection of unlabeled interactants in real time.*

Bioluminescence imaging (BLI) *Technology developed over the past decade that allows for the noninvasive study of ongoing biological processes in small laboratory animals.*

Bispecific Ab *A bispecific monoclonal antibody (BsMAb, BsAb) is an artificial protein that is composed of fragments of two different monoclonal antibodies and consequently binds to two different types of antigen.*

C1q assay *C1q receptor is the first component of the complement cascade; hence, it is very important in the mechanism of action for therapeutic MAbs. C1q is a very large, hexameric molecule, and its binding affinity in solution is extremely low, making it difficult to measure. Assay is typically in ELISA format.*

Camelid sdAb *Ab fragment consisting of single monomeric variable Ab domain that can selectively bind specific Ag.*

CAR-T cell therapy *Chimeric Antigen Receptor-T cell therapy. Technique in which T cells are genetically engineered to produce special receptors on their surface called chimeric antigen receptors (CARs). CARs are proteins that allow the T cells to recognize a specific protein (antigen) on tumor cells. scFv fusion used normally.*

CDC *Complement-dependent cytotoxicity (CDC) assays test the efficacy of antibodies or protein-based drugs to activate a multi-pathway attack mediated by the complement immune system to kill specific target cells. The general method for CDC is to mix target cells bound by the antibody being evaluated with serum that contains the components of the complement system, often human serum, and then measure cell death.*

Downstream process development *Important stage during bioprocess operations. Begins with harvest of material from a bioreactor, downstream processing removes or reduces contaminants to acceptable levels through several steps that typically include centrifugation, filtration, and/or chromatographic technologies.*

Effector-function enhancement *The effector functions of an antibody refer to antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).*

Efficacy *Ability to produce the desired or intended result.*

ELISA *Enzyme Linked Immunosorbent Assay.*

Epitope binning *A competitive immunoassay used to characterize and then sort a library of monoclonal antibodies against a target protein.*

Epitope mapping *Epitope mapping is the process of experimentally identifying the binding sites, or 'epitopes', of antibodies on their target antigens. Identification and characterization of the binding sites of antibodies can aid in the discovery and development of new therapeutics, vaccines, and diagnostics.*

Fab *The fragment antigen-binding (Fab fragment) is a region on an antibody that binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain.*

FACS *Fluorescence activated cell sorting (FACS) of live cells separates a population of cells into sub-populations based on fluorescent labeling.*

Fc engineering *Fc domain is critical to the functioning of an antibody and has been the focus of many engineering efforts. Broadly speaking these approaches can be broken down into three main areas:*

- *Increasing effector functions*
- *Decreasing effector functions*
- *Extending serum half-life*

Fc fusion protein *Fc-Fusion proteins (also known as Fc chimeric fusion protein, Fc-Ig, Ig-based chimeric fusion protein and Fc-tag protein) are composed of the Fc domain of IgG genetically linked to a peptide or protein of interest. Fc-Fusion proteins have become valuable reagents for in vivo and in vitro research.*

Fcγ receptor assay *Used to measure Fc binding response using SPR technique. Assays are developed by covering the surface of a laboratory chip with recombinant human Fcγ receptors. Independently prepared serum dilutions of test samples are then injected over two flow cells — a control cell and the Fc receptor–coupled cell — and the difference in binding response is then measured.*

FcRn assay *SPR binding assay. The Neonatal Fc Receptor (FcRn) transports Igs across the placenta between mother and fetus. It is also responsible for salvaging internalized immunoglobulin and albumin, thus making it responsible for the long in vivo serum half-life of mAbs.*

Half-life extension *Improving antibody half-life in vivo. Achievable by improving the affinity for the neonatal Fc receptor (FcRn) by Fc engineering.*

HAMA response *Human Anti Mouse Antibody. When patients react to mouse antibodies as if they were a foreign substance, and create a new set of antibodies against the mouse antibodies, such a response is termed the HAMA response.*

Small antibody fragments such as Fab, scFv, diabodies do not possess an intact Fc region and are below cut-off for filtration by kidney glomerulae, resulting in half-lives of less than an hour. Thus there are efforts to make antibody-like molecules that are larger than 60kDa yet retain Fc functionality.

GenScript can produce Fabs and scFvs for your research applications at competitive prices and with rapid turnaround time.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

GLOSSARY

There is increasing unmet medical need for patients with fibrotic diseases such as idiopathic pulmonary fibrosis and renal fibrosis. Therapeutics that target their underlying inflammatory pathways is becoming an emerging focus in fibrotic disease drug development.

GenScript has established expertise in fibrotic disease models for liver, lung and kidney.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

Humanization *Humanized antibodies are antibodies from non-human species whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans. The process refers to the replacement of more than 90% of rodent IgG sequence in the parental antibody molecule with human IgG sequence.*

Human knock-in/knock-out *Technique where gene encoding the human target protein is inserted into the locus encoding the mouse target ortholog within the mouse genome. These ''knock-in/knock-out'' mice will only express the human target protein but not the endogenous mouse ortholog.*

Hybridoma Technology *Technology of forming hybrid cell lines (called hybridomas) by fusing an antibody-producing B cell with a myeloma (B cell cancer) cell.*

Hybridoma stabilization *Antibody production by hybridoma cell lines is inherently unstable. Failure to maintain cell lines properly will lead to loss in antibody productivity and eventually the cell line itself. Stabilization involves Ab sequencing, vector construction and stable cell line generation for Ab production.*

IC_{50} *Half maximal inhibitory concentration. It is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. It is commonly used as a measure of antagonist drug potency in pharmacological research.*

In vitro pharmacology *Includes binding assays, cell culture based assays.*

In vitro screening *ELISA is an example of in vitro screening.*

In vivo efficacy services *Include ADME, whole animal assays, PK/TK/DMPK, full toxicology, IND enabling studies, small and large animal models, imaging.*

Isoforms *Functionally similar proteins that have a similar but not identical amino acid sequence and are either encoded by different genes or by RNA transcripts from the same gene which have had different exons removed.*

Isotype *refers to the phenotypic variations in the constant regions of the heavy and light chains. In humans, there are five heavy chain isotypes and two light chain isotypes.*

Isotype switching *Immunoglobulin class switching [or isotype switching, or isotypic commutation, or class switch recombination (CSR)] is a biological mechanism that changes a B cell's production of antibody from one class to another; for example, from IgM isotype to IgG isotype.*

KinExA *Stands for Kinetic Exclusion Assay. It is used to measure the concentration of a molecule. Advantageous when measure of free concentration is required in a mixture of both free and bound molecules.*

Knockout *technique in which an organism's genes are made inoperative.*

LC-MS *Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).*

Lead selection *Lead selection refers to the process by which the early hits are interrogated in a vigorous, multi-stepped screening process to select lead molecules that meet pre-established criteria for advancement into the next drug discovery stage.*

mAb sequencing *Knowing the sequence of a monoclonal antibody is not only the first step towards antibody engineering and function optimization, but also critical for patent application. Workflow involves mRNA isolation from hybridoma clone → reverse transcription of mRNA to cDNA → PCR amplification of H & L chain genes → cloning into sequencing vector → sequencing → analysis.*

Mechanistic study *Study or test designed to analyze the biological or chemical events responsible for, or associated with, an effect observed, and to provide information concerning the molecular, cellular or physiological mechanisms by which substances exert their effects on living cells and organisms.*

Monobody *Also known as Adnectins, are genetically engineered proteins that are able to bind to antigens. Despite their name, they are not parts of antibodies, which make them a type of antibody mimetic.*

Monovalent Ab *Monovalent antibody, an antibody with affinity for one epitope, antigen, or strain of microorganism.*

Multivalent Ab *Antibody with multiple Ag binding sites. Most Abs are at least bivalent since they have at least 2 Ag-binding sites.*

Naked mAb *Antibodies that work by themselves. There is no drug or radioactive material attached to them. These are the most common type of mAbs used to treat cancer.*

Ortholog *Genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes.*

Orthotopic *Relates to the grafting of tissue in a natural position.*

PD model *Pharmacodynamic model component into one set of mathematical expressions that allows the description of the time course of effect intensity in response to administration of a drug dose.*

Phage display *Technique for the production and screening of novel proteins and polypeptides by inserting a gene fragment into a gene responsible for the surface protein of a bacteriophage. The new protein appears in the surface coating of the phage, in which it can be manipulated and tested for biological activity.*

PK model *Pharmacokinetic model component into one set of mathematical expressions that allows the description of the time course of effect intensity in response to administration of a drug dose.*

Reference antibody *Often used in in vivo proof-of-concept studies either to validate the target or establish an efficacy model. Can be a commercially available monoclonal antibody with function similar to the intended therapeutic candidate, a polyclonal antibody functionally interacting with the target protein of interest, or an antibody reconstructed from sequences available in the public domain.*

RNAi *RNA interference is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Historically, it was known by other names, including co-suppression, post transcriptional gene silencing (PTGS), and quelling.*

SC xenograft *Subcutaneous xenograft tumor model is valuable for predicting cytotoxicity of cancer drugs and widely used in efficacy studies for its simplicity and easy access to tumor measurement.*

A major advantage of monobodies over conventional Abs is that monobodies can readily be used as genetically encoded intracellular inhibitors, that is one could express a monobody inhibitor in a cell of choice by simply transfecting the cell with a monobody containing expression vector.

With high quality recombinant antibody production, rapid turnaround time and competitive pricing, GenScript has been a reliable research partner to scientists in >86 countries around the world.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The camelid single domain Abs can simplify, stabilize and reduce the size of the therapeutic molecule. These unique antibodies are known to represent >50% of the total Ig population in camel sera.

GenScript offers comprehensive sdAb services that starts with either immunization of Llamas or isolation from naïve sdAb phage display library.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

scFv *It is a fusion of the variable regions of the heavy (V_H) and light chains(V_L) of immunoglobulins, connected with a short linker peptide of 10-25 amino acids.*

Single domain *See camelid sdAb.*

Single domain fragment *See camelid sdAb.*

Species cross-reactivity *It is a desirable feature for a candidate antibody and refers to the ability of the antibody to bind and functionally interact with the orthologous proteins from various animal species used as models for evaluation of in vivo efficacy, pharmacokinetic and pharmacodynamic (PK/PD), and safety. The animals routinely used for these purposes include but are not limited to mouse, rat, rabbit, and cynomolgus monkeys.*

SPR *Technique that enables the detection of unlabeled interactants in real time.*

Stable expression *Generating stably transfected cells begins with a transient transfection. But the hallmark of stably transfected cells is that the foreign gene becomes part of the genome and is therefore replicated. Descendants of these transfected cells, therefore, will also express the new gene, resulting in a stably transfected cell line. When developing a stable transfection, researchers use selectable markers to distinguish transient from stable transfections. Co-expressing the marker with the gene of interest enables researchers to identify and select for cells that have the new gene integrated into their genome while also selecting against the transiently transfected cells.*

Surrogate Ab *Surrogate Ab is a functionally equivalent antibody to the therapeutic antibody candidate which binds specifically to the target ortholog expressed in the intended animal species.*

Syngeneic model *Animal model to test efficacy of immuno-oncology antibody leads. Provides effective approach for studying how cancer therapies perform in presence of functional immune system.*

Target selection *Process of finding an agent with a particular biological action that is anticipated to have therapeutic utility.*

Tissue cross-reactivity *The unintended reaction of therapeutic monoclonal antibodies with tissues and proteins other than the target antigen.*

Tissue distribution study *Conducted to evaluate PK in several species. Distribution of drug between tissues is dependent on vascular permeability, regional blood flow, cardiac output and perfusion rate of tissue and ability of drug to bind tissue.*

Titer *A way of expressing concentration.*

Transgenic mouse model *Defined as a biological model that has been genetically modified by the introduction of a foreign DNA sequence/fragment into a mouse egg. The insertion of the foreign DNA usually results in a gain of function (expression of a new gene) or in the over-expression of endogenous genes.*

Transient expression *Transiently transfected cells express the foreign gene but do not integrate it into their genome. Thus the new gene will not be replicated. These cells express the transiently transfected gene for a finite period of time, usually several days, after which the foreign gene is lost through cell division or other factors.*

Upstream process development *Important stage during bioprocess operations. Begins most often with culturing of animal or microbial cells in a range of vessel types (such as bags or stirred tanks) using different controlled feeding, aerating, and process strategies.*

Xenograft model *Animal model typically used in cancer research. One of the most widely used models is the human tumor xenograft. In this model, human tumor cells are transplanted, either under the skin or into the organ type in which the tumor originated, into immunocompromised mice that do not reject human cells.*

Syngeneic mouse models serve as surrogates for human patients and allow researchers to study how cancer therapies perform in conjunction with a functional immune system. Few advantages of syngeneic models include the low cost of host animals, ease of validation, data reproducibility and the ability to monitor the immune system's response upon treatment.

GenScript provides various syngeneic tumor models in mouse to facilitate your drug discovery of cancer immunotherapeutics, including immune checkpoint blockers and cancer vaccines.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

REFERENCES

Creating a platform to produce human Abs in mice requires the introduction of human Ab genes into mouse genome in their germline configuration.

GenScript offers validated CRISPR products, services and resources to help you harness the power of CRISPR genome editing for your research.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

The references cited in this handbook are not intended to be exhaustive. We apologize for omitting, due to space constraints, many important contributions from other researchers.

1. Behring, E. a. K., S. Ueber das Zustandekommen der Diphtherie-Immunitat und der Tetanus-Immunitat bei thieren. *Deutsche medizinsche Wochenschrift* **16**, 1113-1114 (1890).

2. Behring, E. Untersuchungen ueber das Zustandekommen der Diphtherie-Immunitat bei Thieren. *Dt. Med. Wochenschr* **16**, 1145-1148 (1890).

3. Nobelprize.org. *The Nobel Prize in Physiology or Medicine 1908,* <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1908/ehrlich-bio.html >

4. Fischer, E. Einfluss der Configuration auf die Wirkung der Enzyme. *Berichte Der Deutschen Chemischen Gesellschaft* **27**, 2985-2993 (1894).

5. Lemieux, R. U., and Spohr, U. . How Emil Fischer was led to the lock and key concept for enzyme specificity. *Adv Carbohydr Chem Biochem* **50**, 1-20 (1994).

6. Burnet, F. M. A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA Cancer J Clin* **26**, 119-121 (1976).

7. Pauling, L. A Theory of the Structure and Process of Formation of Antibodies. *J. Am. Chem. Soc.* **62**, 2643-2657 (1940).

8. Porter, R. R. The hydrolysis of rabbit y-globulin and antibodies with crystalline papain. *Biochem J* **73**, 119-126 (1959).

9. Edelman, G. M., Benacerraf, B., Ovary, Z., Poulik, M.D. Structural differences among antibodies of different specificities. *Proc Natl Acad Sci USA* **47**, 1751-1758 (1961).

10. Nobelprize.org. *The Nobel Prize in Physiology or Medicine 1972,* <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1972/>

11. Inbar, D., Hochman, J., and Givol, D. . Localization of antibody-combining sites within the variable portions of heavy and light chains. *Proc. Natl. Acad. Sci. USA* **69**, 2659-2662 (1972).

12. Kohler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497 (1975).

13. Sorensen, V., Sundvold, V., Michaelsen, T. E. & Sandlie, I. Polymerization of IgA and IgM: roles of Cys309/Cys414 and the secretory tailpiece. *J Immunol* **162**, 3448-3455 (1999).

14. Brandtzaeg, P. & Prydz, H. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. *Nature* **311**, 71-73 (1984).

15. Carter, P. J. Potent antibody therapeutics by design. *Nat Rev Immunol* **6**, 343-357, doi:10.1038/nri1837 (2006).

16. Yokota, T., Milenic, D. E., Whitlow, M. & Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res* **52**, 3402-3408 (1992).

17. Jain, R. K. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res* **50**, 814s-819s (1990).

18. Ward, E. S., Gussow, D., Griffiths, A. D., Jones, P. T. & Winter, G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. *Nature* **341**, 544-546, doi:10.1038/341544a0 (1989).

19. Sanz, L., Cuesta, A. M., Compte, M. & Alvarez-Vallina, L. Antibody engineering: facing new challenges in cancer therapy. *Acta Pharmacol Sin* **26**, 641-648, doi:10.1111/j.1745-7254.2005.00135.x (2005).

REFERENCES

Since most mAbs are initially identified on the basis of their ability to bind antigen, it is important that mAb candidates are evaluated early using a functional assay dependent on target activity.

GenScript provides comprehensive assay services to support your Ab drug discovery programs.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

20. Holliger, P. & Hudson, P. J. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* **23**, 1126-1136, doi:10.1038/nbt1142 (2005).

21. Hudson, P. J. & Souriau, C. Engineered antibodies. *Nat Med* **9**, 129-134, doi:10.1038/nm0103-129 (2003).

22. Shih, H. H. in *Development of Antibody-Based Therapeutics* (ed M.A; Bornstein Tabrizi, G.G.; Klakamp, S.L.) Ch. 2, 9-32 (Springer, 2012).

23. Kehoe, J. W. & Kay, B. K. Filamentous phage display in the new millennium. *Chem Rev* **105**, 4056-4072, doi:10.1021/cr000261r (2005).

24. Smith, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317 (1985).

25. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552-554, doi:10.1038/348552a0 (1990).

26. Marasco, W. A. & Sui, J. The growth and potential of human antiviral monoclonal antibody therapeutics. *Nat Biotechnol* **25**, 1421-1434, doi:10.1038/nbt1363 (2007).

27. McGuire, M. J., Li, S. & Brown, K. C. Biopanning of phage displayed peptide libraries for the isolation of cell-specific ligands. *Methods Mol Biol* **504**, 291-321, doi:10.1007/978-1-60327-569-9_18 (2009).

28. Ehrlich, G. K., Berthold, W. & Bailon, P. Phage display technology. Affinity selection by biopanning. *Methods Mol Biol* **147**, 195-208, doi:10.1385/1-59259-041-1:195 (2000).

29. Boder, E. T. & Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotechnol* **15**, 553-557, doi:10.1038/nbt0697-553 (1997).

30. Pepper, L. R., Cho, Y. K., Boder, E. T. & Shusta, E. V. A decade of yeast surface display technology: where are we now? *Comb Chem High Throughput Screen* **11**, 127-134 (2008).

31. Levin, A. M. & Weiss, G. A. Optimizing the affinity and specificity of proteins with molecular display. *Mol Biosyst* **2**, 49-57, doi:10.1039/b511782h (2006).

32. Chao, G., Cochran, J. R. & Wittrup, K. D. Fine epitope mapping of anti-epidermal growth factor receptor antibodies through random mutagenesis and yeast surface display. *J Mol Biol* **342**, 539-550, doi:10.1016/j.jmb.2004.07.053 (2004).

33. Jin, M. *et al.* Directed evolution to probe protein allostery and integrin I domains of 200,000-fold higher affinity. *Proc Natl Acad Sci U S A* **103**, 5758-5763, doi:10.1073/pnas.0601164103 (2006).

34. Holler, P. D. *et al.* In vitro evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci U S A* **97**, 5387-5392, doi:10.1073/pnas.080078297 (2000).

35. Rao, B. M., Driver, I., Lauffenburger, D. A. & Wittrup, K. D. Interleukin 2 (IL-2) variants engineered for increased IL-2 receptor alpha-subunit affinity exhibit increased potency arising from a cell surface ligand reservoir effect. *Mol Pharmacol* **66**, 864-869, doi:10.1124/mol.66.4. (2004).

36. Lipovsek, D. & Pluckthun, A. In-vitro protein evolution by ribosome display and mRNA display. *J Immunol Methods* 290, 51-67, doi:10.1016/j.jim.2004.04.008 (2004).

37. Hanes, J. & Pluckthun, A. In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci U S A* **94**, 4937-4942 (1997).

38. Wang, W., Singh, S., Zeng, D. L., King, K. & Nema, S. Antibody structure, instability, and formulation. *J Pharm Sci* **96**, 1-26, doi:10.1002/jps.20727 (2007).

REFERENCES

The IgNAR is an Ig isotype discovered in the serum of nurse sharks in the 1990s. The IgNAR scaffold is an attractive alternative to the camelid V_{HH} and has similar potential as a biotherapeutic.

GenScipt can deliver up to 10mgs of V_{HH} at >90% purity as fast as 7 weeks.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

43. Cabilly, S. et al. Generation of antibody activity from immunoglobulin polypeptide chains produced in Escherichia coli. *Proc Natl Acad Sci U S A* **81**, 3273-3277 (1984).

44. Boss, M. A., Kenten, J. H., Wood, C. R. & Emtage, J. S. Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in E. coli. *Nucleic Acids Res* **12**, 3791-3806 (1984).

45. Skerra, A. & Pluckthun, A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science* **240**, 1038-1041 (1988).

46. Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. Escherichia coli secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043 (1988).

39. Daugherty, A. L. & Mrsny, R. J. Formulation and delivery issues for monoclonal antibody therapeutics. *Adv Drug Deliv Rev* **58**, 686-706, doi:10.1016/j.addr.2006.03.011 (2006).

40. Chames, P., Van Regenmortel, M., Weiss, E. & Baty, D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* **157**, 220-233, doi:10.1111/j.1476-5381.2009.00190.x (2009).

41. Schroff, R. W., Foon, K. A., Beatty, S. M., Oldham, R. K. & Morgan, A. C., Jr. Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res* **45**, 879-885 (1985).

42. Tjandra, J. J., Ramadi, L. & McKenzie, I. F. Development of human anti-murine antibody (HAMA) response in patients. *Immunol Cell Biol* **68 (Pt 6)**, 367-376, doi:10.1038/icb.1990.50 (1990).

47. Chen, C. *et al.* High-level accumulation of a recombinant antibody fragment in the periplasm of Escherichia coli requires a triple-mutant (degP prc spr) host strain. *Biotechnol Bioeng* **85**, 463-474, doi:10.1002/bit.20014 (2004).

48. Carter, P. *et al.* High level Escherichia coli expression and production of a bivalent humanized antibody fragment. *Biotechnology (N Y)* **10**, 163-167 (1992).

49. Levy, R., Weiss, R., Chen, G., Iverson, B. L. & Georgiou, G. Production of correctly folded Fab antibody fragment in the cytoplasm of Escherichia coli trxB gor mutants via the coexpression of molecular chaperones. *Protein Expr Purif* **23**, 338-347, doi:10.1006/prep.2001.1520 (2001).

50. Humphreys, D. P. *et al.* High-level periplasmic expression in Escherichia coli using a eukaryotic signal peptide: importance of codon usage at the 5' end of the coding sequence. *Protein Expr Purif* **20**, 252-264, doi:10.1006/prep.2000.1286 (2000).

51. Donzeau, M. & Knappik, A. Recombinant monoclonal antibodies. *Methods Mol Biol* **378**, 14-31 (2007).

52. Fernandez, L. A. Prokaryotic expression of antibodies and affibodies. *Curr Opin Biotechnol* **15**, 364-373, doi:10.1016/j.copbio.2004.06.004 (2004).

53. Sorensen, H. P. & Mortensen, K. K. Advanced genetic strategies for recombinant protein expression in Escherichia coli. *J Biotechnol* **115**, 113-128, doi:10.1016/j.jbiotec.2004.08.004 (2005).

54. Simmons, L. C. *et al.* Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. *J Immunol Methods* **263**, 133-147 (2002).

55. Humphreys, D. P. *et al.* A plasmid system for optimization of Fab' production in Escherichia coli: importance of balance of heavy chain and light chain synthesis. *Protein Expr Purif* **26**, 309-320 (2002).

56. Li, H. *et al.* Optimization of humanized IgGs in glycoengineered Pichia pastoris. *Nat Biotechnol* **24**, 210-215, doi:10.1038/nbt1178 (2006).

REFERENCES

57. Beck, A. *et al.* Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins. *Curr Pharm Biotechnol* **9**, 482-501 (2008).

58. Reavy, B. *et al.* Expression of functional recombinant antibody molecules in insect cell expression systems. *Protein Expr Purif* **18**, 221-228, doi:10.1006/prep.1999.1191 (2000).

59. Liang, M. *et al.* Baculovirus expression cassette vectors for rapid production of complete human IgG from phage display selected antibody fragments. *J Immunol Methods* **247**, 119-130 (2001).

60. Shuler M.L, H. D. A., Granados R.R., Wood, H.A. in *Baculovirus expression systems and biopesticides* 1-12 (Wiley-Liss, New York, 1995).

61. zu Putlitz, J. *et al.* Antibody production in baculovirus-infected insect cells. *Biotechnology (N Y)* **8**, 651-654 (1990).

62. Jin, B. R., Ryu, C. J., Kang, S. K., Han, M. H. & Hong, H. J. Characterization of a murine-human chimeric antibody with specificity for the pre-S2 surface antigen of hepatitis B virus expressed in baculovirus-infected insect cells. *Virus Res* **38**, 269-277 (1995).

63. Edelman, L. *et al.* Obtaining a functional recombinant anti-rhesus (D) antibody using the baculovirus-insect cell expression system. *Immunology* **91**, 13-19 (1997).

64. Jordan, E., Al-Halabi, L., Schirrmann, T. & Hust, M. Antibody production by the gram-positive bacterium Bacillus megaterium. *Methods Mol Biol* **525**, 509-516, xiv, doi:10.1007/978-1-59745-554-1_27 (2009).

65. Jordan, E. *et al.* Production of recombinant antibody fragments in Bacillus megaterium. *Microb Cell Fact* **6**, 2, doi:10.1186/1475-2859-6-2 (2007).

66. Frenken, L. G., Hessing, J. G., Van den Hondel, C. A. & Verrips, C. T. Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms. *Res Immunol* **149**, 589-599 (1998).

67. Valdes, R. **et al.** Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants. *Biochem Biophys Res Commun* **308**, 94-100 (2003).

68. Bardor, M. *et al.* Monoclonal C5-1 antibody produced in transgenic alfalfa plants exhibits a N-glycosylation that is homogenous and suitable for glyco-engineering into human-compatible structures. *Plant Biotechnol J* **1**, 451-462, doi:10.1046/j.1467-7652.2003.00041.x (2003).

69. Stoger, E. *et al.* Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol* **42**, 583-590 (2000).

70. Zeitlin, L. *et al.* A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat Biotechnol* **16**, 1361-1364, doi:10.1038/4344 (1998).

71. Kind, A. & Schnieke, A. Animal pharming, two decades on. *Transgenic Res* **17**, 1025-1033, doi:10.1007/s11248-008-9206-3 (2008).

72. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59-74, doi:10.1099/0022-1317-36-1-59 (1977).

73. Durocher, Y., Perret, S. & Kamen, A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* **30**, E9 (2002).

Radioimmunoconjugates (RICs) are ligand-targeted molecules for the systemic delivery of radionuclides to disease locations. RICs are used for diagnostic and therapeutic purposes and their use is referred to as radioimmunotherapy (RIT).

GenScript uses proprietary transient mammalian expression technology to produce flexible-throughput rAbs from microgram to gram quantities to support your Ab drug discovery programs.

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878

REFERENCES

Immunotherapy harnesses a patient's immune system to combat their disease. One approach involves engineering patients' own immune cells to attack their tumors. Called Adoptive Cell Transfer (ACT), this approach has generated some remarkable responses in patients with advanced cancer.

GenScript is committed to supporting groundbreaking research and accelerating new breakthroughs in CAR T-cell engineering for the treatment of cancer.

74. Kao, F. T. & Puck, T. T. Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc Natl Acad Sci U S A* **60**, 1275-1281 (1968).

75. Zang, M. **et al.** Production of recombinant proteins in Chinese hamster ovary cells using a protein-free cell culture medium. *Biotechnology (N Y)* **13**, 389-392 (1995).

76. Renner, W. A., Lee, K. H., Hatzimanikatis, V., Bailey, J. E. & Eppenberger, H. M. Recombinant cyclin E expression activates proliferation and obviates surface attachment of chinese hamster ovary (CHO) cells in protein-free medium. *Biotechnol Bioeng* **47**, 476-482, doi:10.1002/bit.260470409 (1995).

77. Lee, F. W. & Da Silva, N. A. Ty1-mediated integration of expression cassettes: host strain effects, stability, and product synthesis. *Biotechnol Prog* **12**, 548-554, doi:10.1021/bp9600288 (1996).

78. F.M., W. in *Mammalian Cell Biotech in protein production* 87-114 (W. de Gruyter, Berlin, New York., 1997).

79. Jefferis, R. Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* **21**, 11-16, doi:10.1021/bp040016j (2005).

80. Bergman, B., Holm, S. E., Holmlund, D. & Kaijser, B. Serum antibodies against Escherichia coli and Proteus mirabilis after different types of operations. *Acta Chir Scand* **147**, 93-97 (1981).

81. Montano, R. F. & Morrison, S. L. Influence of the isotype of the light chain on the properties of IgG. *J Immunol* **168**, 224-231 (2002).

82. Skvortsov, V. T. & Gurvich, A. E. Relative rates of synthesis of immunoglobulins and light chains in rabbit spleen cells during secondary response. *Nature* **218**, 377-378 (1968).

83. Leitzgen, K., Knittler, M. R. & Haas, I. G. Assembly of immunoglobulin light chains as a prerequisite for secretion. A model for oligomerization-dependent subunit folding. *J Biol Chem* **272**, 3117-3123 (1997).

84. Schimke, R. T., Roos, D. S. & Brown, P. C. Amplification of genes in somatic mammalian cells. *Methods Enzymol* **151**, 85-104 (1987).

85. Bebbington, C. R. et al. High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Biotechnology (N Y)* **10**, 169-175 (1992).

86. Shukla, A. A., Hubbard, B., Tressel, T., Guhan, S. & Low, D. Downstream processing of monoclonal antibodies--application of platform approaches. *J Chromatogr B Analyt Technol Biomed Life Sci* **848**, 28-39, doi:10.1016/j.jchromb.2006.09.026 (2007).

87. Chen, J., Tetrault, J. & Ley, A. Comparison of standard and new generation hydrophobic interaction chromatography resins in the monoclonal antibody purification process. *J Chromatogr A* **1177**, 272-281, doi:10.1016/j.chroma.2007.07.083 (2008).

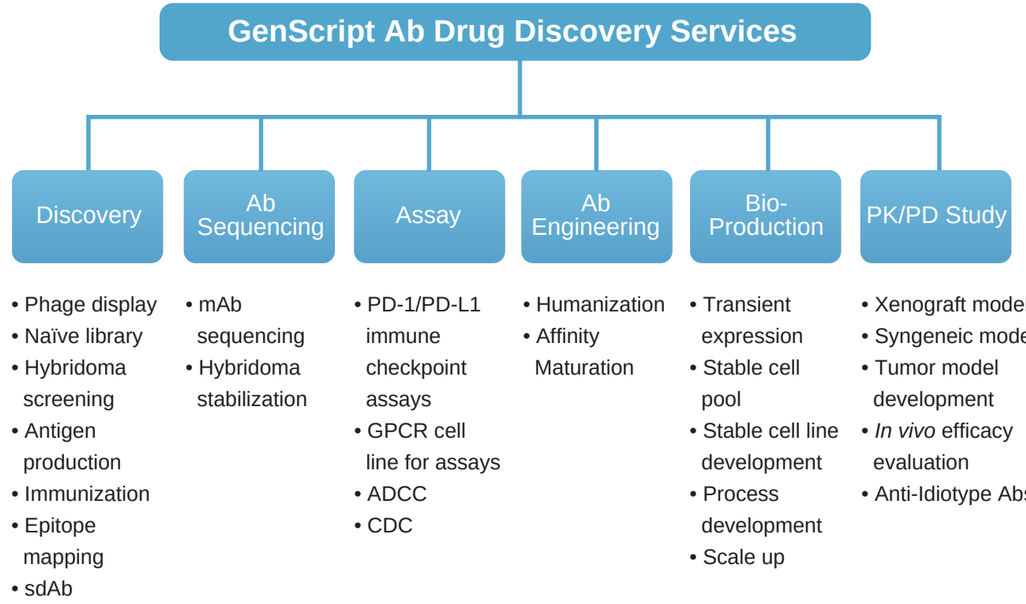
88. Gagnon, P. IgG aggregate removal by charged-hydrophobic mixed mode chromatography. *Curr Pharm Biotechnol* **10**, 434-439 (2009).

89. Chen, J., Nixon, A. in *Antibody Drug Discovery* Vol. 4 (ed C. R. Wood) Ch. 7, 271-314 (Imperial College Press, 2012).

90. Metabolite Services at John Innes Centre, N. R. P., Colney, Norwich, UK. *LC-MS: Why use it, and what is it?*, <https://www.jic.ac.uk/services/metabolomics/topics/lcms/why.htm>

GenScript USA Inc.
860 Centennial Ave.
Piscataway, NJ 08854
USA

Phone:
1-732-885-9188
Toll-Free:
1-877-436-7274
Fax:
1-732-885-5878



Above schematic highlights some of GenScript's Ab drug discovery service capabilities. For a complete list of Ab drug discovery services offered by GenScript, visit www.genscript.com.