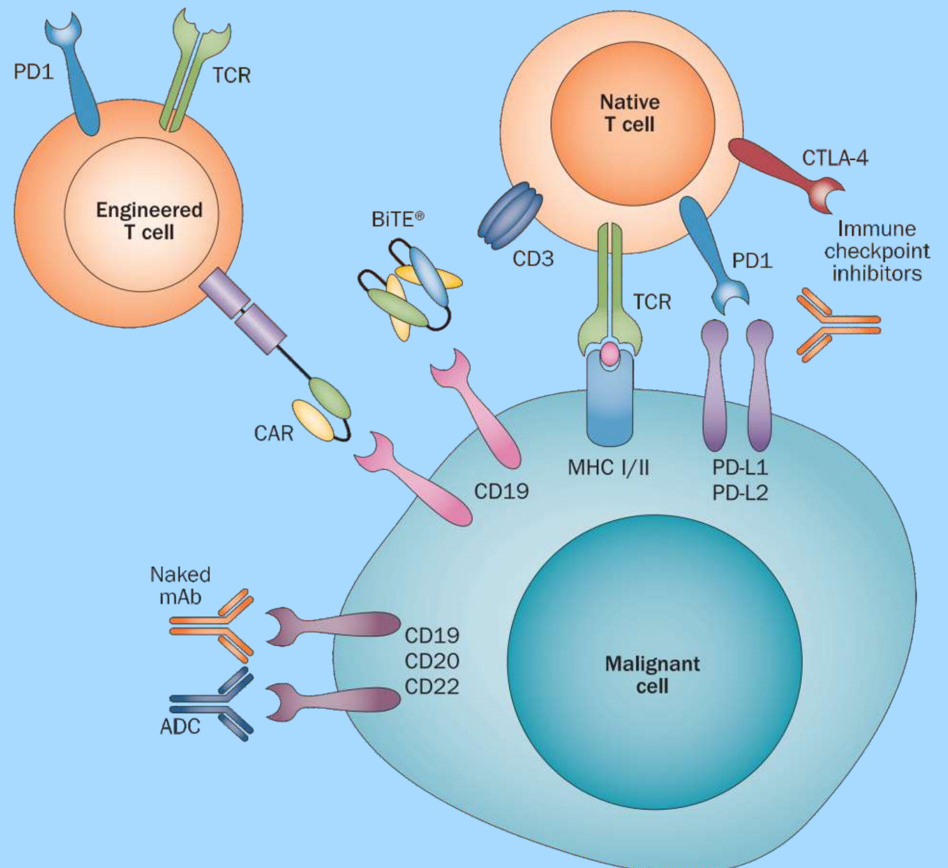
AtaGenix
From Gene to Antibody

AtaGenix Custom Service Manual

Antibody Discovery



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Mammalian Expression System: Expression of Active Target Proteins

Introduction

Mammalian expression system features with protein folding and post-translational modifications, whose expressed proteins are much closer to natural proteins in terms of spatial structure, protein post-translational modifications, and biological activities. Therefore, it is widely used in the expression and production of active proteins and therapeutic antibody drugs.

1.Vector Construction

(1) Source of Vector: AtaGenix provides proprietary vectors or customers- specified commercial vectors.

(2) Protocol Design: AtaGenix recommends selecting signal peptides, tags, and expression fragments.

2.Transient Gene Expression (TGE)

(1) Compared with stable gene expression, TGE is mainly suitable for the preparation of recombinant protein in a short period of time which is usually obtained within 10 days.

(2) The transient expression of AtaGenix uses suspension cells with volumes ranging from milliliters to 100L.

3.Stable Gene Expression.

To meet long -term production needs, the entire stable transfection and screening process provides long -term stability and scale-adjustable protein production.

4.Process Development and Mass Production.

(1) Fed-batch optimizations.

(2) Culture medium screening.

(3) Downs tream purification process development.

Expression Methods	Cell Lines
Transient Expression	HEK293: HEK293 cells are derived from the 293 cell line and adapted in suspension with serum-free media. CHO-S: CHO-S cells are clonal isolates from Chinese hamster ovary cells adapted to serum-free suspension culture
Stable Expression	CHO-K1: CHO-K1 is derived from CHO, a cell line with simple culture. conditions and moderate adherence strength, which is relatively easy to transfect.
Stable Cell Line Construction	DG44: DG44 cells (dihydrofolate reductase deficient, DHFR-) are derived from Chinese hamster ovary (CHO) cells and are commonly used to. construct cell lines suitable for recombinant protein production. DG44 cell selection and co-amplification marker is the DHFR gene.

Expression Vectors: pcDNA3.1, pIRES, pTT3, pCEP4, and pATX1, etc.

Advantages

1. Experienced Team: 10 years+ experience in protein expression,5000+ mammalian expression projects and 500+ stable cell line construction projects.

2. Transient Expression: To meet the need for high expression, several choices of mammalian cells adapted in suspension serum free media are available, combined with proprietary expression vectors that can greatly increase yield by 3-6 times higher than conventional systems.

3. Recombinant Antibody Expression: With a set of proven vector-cell transfection high-efficiency expression system, short-term, large-scale production of recombinant antibodies above the g level.

4. Construction of Stable Cell Lines: CHO-K1 modified cell lines with independent intellectual property rights, ideal for mass production of protein, antibody drugs and other industrial-grade raw materials.

5.Class 100,000 Clean Room for Cell Culture : endotoxin level <0.1EU/ml.

6. Large Scale Productions: 200L+ production per batch of serum-free suspension cell culture.

7. One-stop Service: Gene optimization, protein structure analysis and expression design, transient transfection, stable cell line construction, recombinant antibody production, and data analysis.

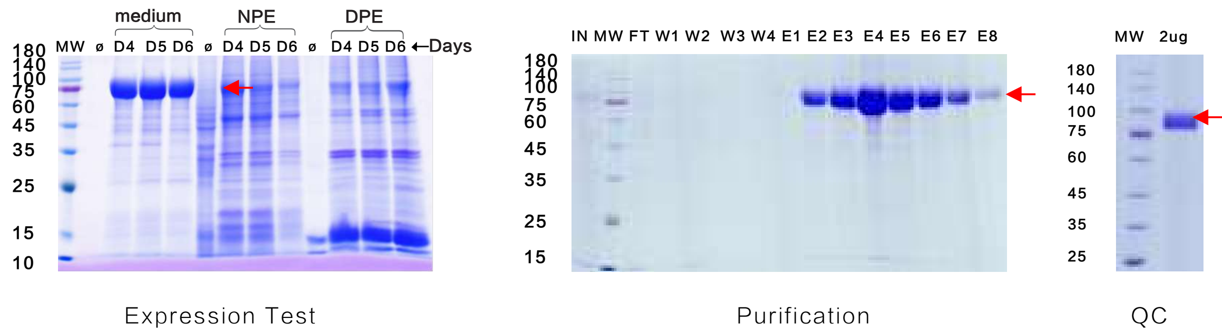
Content

Content	Receivables	Process	Time Frame	Deliverables
Mammalian expression system	Gene sequence or vector	Gene synthesis	2-4 weeks	
		Pilot expression & purification test	2 weeks	Expression and purification test reports to decide whether to proceed to the next step of scale up.
		1L expression & purification	2 weeks	Protein samples & reports
		Shake flask or bioreactor scale up	2-4 weeks	Protein samples & reports

Cases

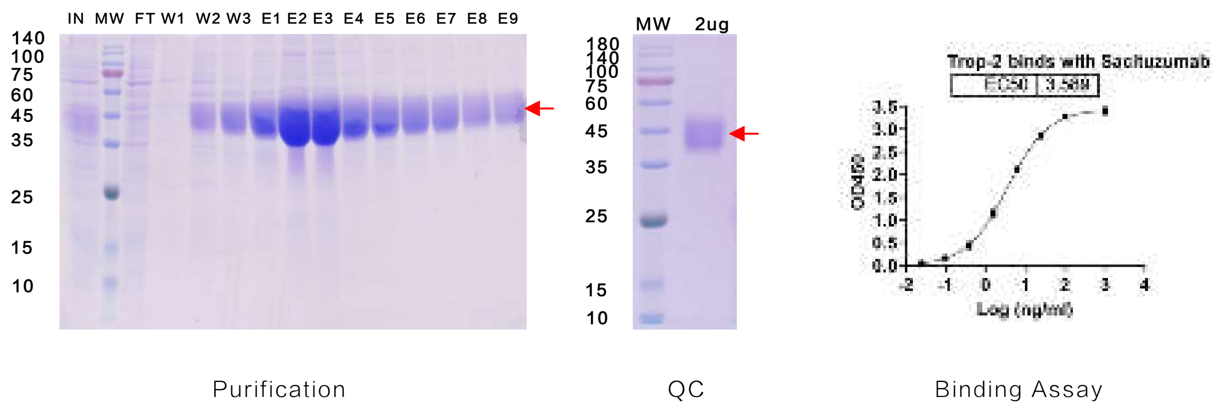
Transient Expression

1.A viral hemagglutinin (HA) Trimer Expressed in HEK293.



Expression Test　　Purification　　QC

2.Protein Trop2 Expressed in CHO.

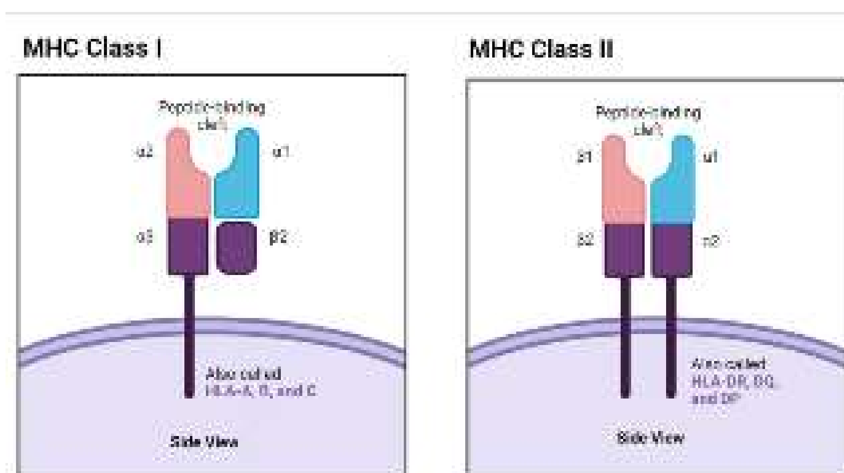


Purification　　QC　　Binding Assay

MHC Complex Custom Service

Introduction

Human MHC is known as Human Leukocyte Antigen (HLA). Human MHC can be divided into MHC I, MHC II and MHC III. MHC I is composed of an alpha chain that spans the cell membrane and an extracellular β2-microglobulin (B2M) attached to this chain. The entire molecule consists of four regions, three of which are in the α chain (α1, α2, α3) and the β2-microglobulin forms the fourth region. The α1 and α2 located outside the cell form a groove in which the polypeptide can bind. Most T lymphocytes express a single, highly specific antigen receptor, TCR, on their surface, which, in the presence of an antigen-presenting cell (APC), can bind directly to the MHC I- peptide complex and initiate a CD8-specific immune response. The TCR has a relatively low affinity for monomeric MHC I- polypeptide complexes and a rapid mismatch rate, while its affinity for MHC I-polypeptide tetramers is greatly enhanced.



Schematic Representation for human MHC I and MHC II Molecular Structure

Advantages

1. Three mature expression systems can be freely selected .(E. coli, mammalian cells, insect baculovirus)

2.Robust MHC-peptide complex preparation process. (Optimized folding ratio and binding ratio, more stable process)

3.SA active protein self-developed by AtaGenix . ((Forms tetramers of biotinylated MHC- peptide monomers)

Content

Content	Provided by Customer	Service Period	Deliverables
MHCI- Peptide complex protein preparation	Peptide, MHC restriction information	About 2-3 weeks	Protein samples & Technical service report

Phage Display Antibody Discovery Platform

Fully Human Antibody Screening Platform

In 1986, the first mouse monoclonal antibody, muromonab-CD3 (muromonab-CD3, OKT3), was approved by the US FDA for immunosuppressive therapy after organ transplantation. In 2002, the first fully humanized monoclonal antibody, adalimumab (Adalimumab, Humira) was approved for clinical use. Since then, humanized antibodies and fully human antibodies have become mainstream technology for antibody drug development. Phage display antibody technology unifies genotype and phenotype in one, combines selection ability with amplification ability, and features with powerful screening ability, which is an important technical tool for fully humanized antibody development.

There are variety of fully human antibody libraries available, which can be divided into naive antibody libraries,semi-synthetic and fully synthetic antibody libraries. However, there are many challenges to build a qualified phage library. For example, the number of donors, capacity and diversity of the library, affinity, and matching rate. AtaGenix has constructed a 100 billion-capacity, high-affinity, and fully humanized recombinant antibody library. With proven record, the library shows great diversity and specificity in binding with various target antigens.

Fully Human Antibody Approved by FDA

Fully Human Recombinant Antibody Drugs (9 from Phage Display, 21 from Transgenic Mice) Launched

Humanized Recombinant Antibody Library with 10^{11} Capacity

Advantages:

1. High sequence accuracy and capacity (10^{11}pfu).

2. Two-step construction protocol to avoid mismatches introduced by PCR. The light chain library is built in advance and then heavy chains are cloned by enzymatic reactions.

3. Different from most commercially available libraries, the single-chain antibody library (scFv) is built into two light chain subtypes, κ and λ. Both libraries can be used separately.

4. All sequences are HIS tagged,expressed directly by using phagemid and purified by affinity chromatography.

QC Testing:

1. Total Insertion Rate and Accuracy

The fragment insertion rate is 100%, and the antibody sequence accuracy is higher than 90%.

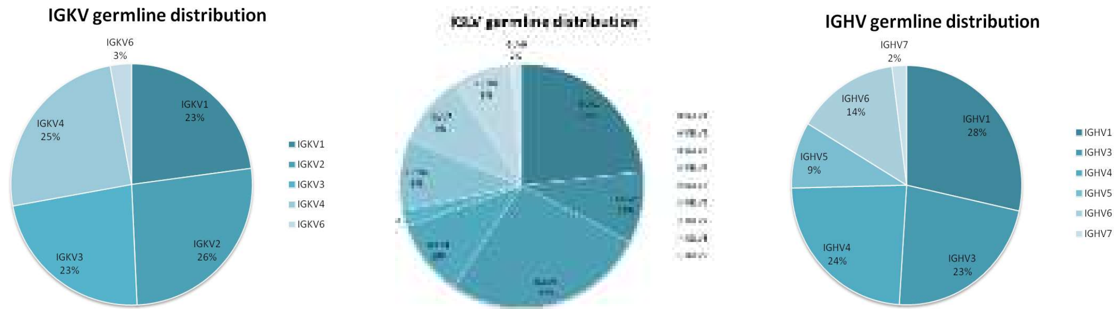
2. Antibody Sequence Diversity Analysis

Germline Analysis: Germline genes mainly refer to all genes contained in haploid germ cells and stem.cells without rearrangement. Usually, the germline distribution of V genes in antibody sequences is analyzed.

Complementarity Determining Region (CDR) Analysis: Antibody consists of 2 regions, constant region, and variable region. A small part of the amino acid residues in the variable region are highly variable between antibodies, and this region is called the hypervariable region (HVR). There are three hypervariable regions in the V regions of the L chain and H chain, which are complementarity determining regions because they can form precise complementarity with antigenic determinants in terms of spatial structure.

2.1 Germline Gene Distribution Characteristics

Humanized recombinant antibody library is divided into two light chain subtypes, κ and λ. The distribution ratio of light chain germline genes of the two subtypes and the distribution ratio of heavy chain germline genes are analyzed, indicating that the library germline gene coverage is high. The distribution characteristics are in line with the germline gene distribution law of antibody drugs.

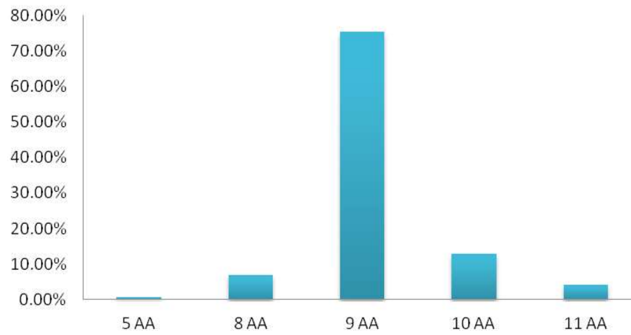


K-type light chain germline gene distribution ratio λ -type light chain germline gene distribution ratio Heavy chain germline gene distribution ratio

2.2 CDR3 Length Distribution Analysis

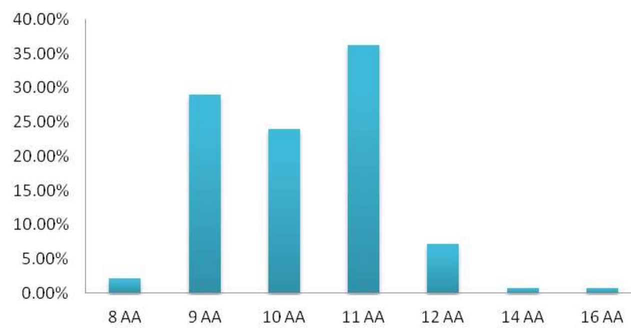
Sequencing analysis was performed by randomly selecting 500 clones from each of the K and λ subtype libraries with no repeats. Its CDR3 length distribution conforms to the normal distribution, which is consistent with the CDR3 length distribution law of the fully human recombinant antibody drug.

Length of VK-CDR3



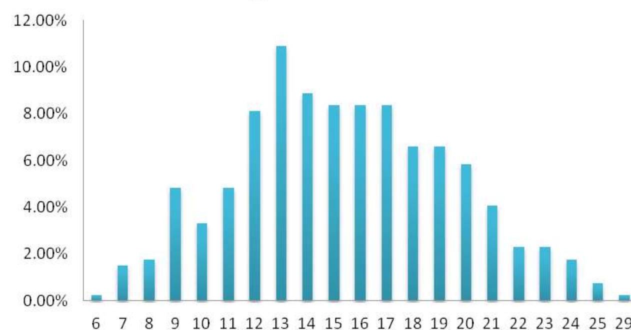
K-type Light Chain Antibody CDR3 Length Distribution Ratio

Length of VL-CDR3



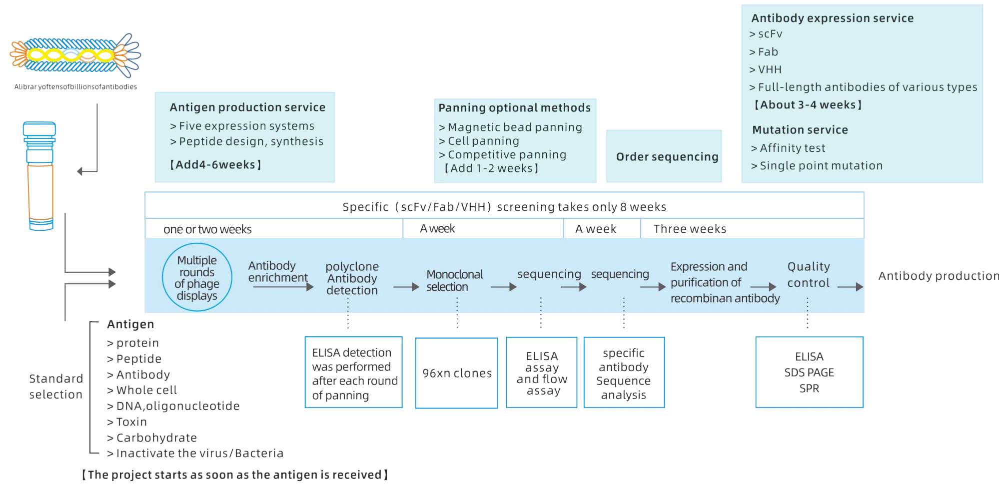
λ -type Light Chain CDR3 Length Distribution Ratio

Length of VH-CDR3



Heavy Chain CDR3 Length Distribution Ratio

Process



1.10^{11}capacity library antigen production service/ Five protein expression systems / Peptides design and synthesis / Additional 4-6 weeks

2. Panning Method: Magnetic beads / Cell panning / Competitive panning / Additional 1-2 weeks

3. Antibody Expression: >Full length antibod

4. Mutagenesis Service: Affinity test; Point mutation

5. Specific screening in only 8 weeks (scFv/Fab/VHH)

6. Multiple rounds of phage display / Antibody enrichment / pAb validations / Monoclone selection / Sequencing / Recombinant antibody expression and purification / QC / Antibody production

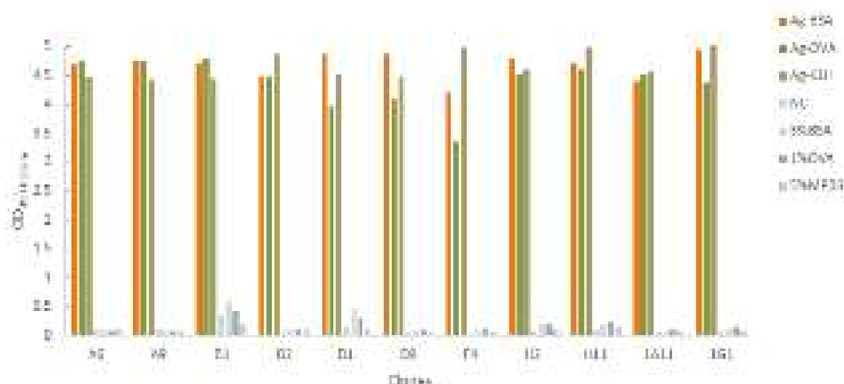
7. ELISA tests after each panning / 96* number of clones / ELISA/FCM validations / Specific antibody sequence analysis / Specificity (ELISA) Purity (SDS-PAGE) Affinity test (SPR)

8. Standard Choices: Antigen protein / Peptide / Antibody / Whole cells / DNA / Oligonucleotides / Toxin Carbohydrate / Inactivated viruses /Bacteria

9. Project initiates upon receiving antigens.

Cases

Phage ELISA of 11 Clones



Screening Results for the Fully Human Recombinant Phage Library of AtaGenix

Nanobody Discovery

In recent years, single domain antibodies (SdAb) have been increasingly concerned. They are special antibodies consisting of only two heavy chains naturally found in camelids and cartilaginous fish, containing only one variable domain of heavy chain antibodies (VHH) and two conventional CH2 and CH3 regions. Single domain antibodies bind antigen through a variable region of the heavy chain (VHH), which can be stable in vitro on its own, called camel-like single domain antibodies (SdAb) or nanobodies. Nanobody crystals are 2.5 nm wide and 4 nm long, and their molecular weighs only 1/10 of the traditional intact antibodies (about 15 kD), but they still have intact antigen recognition ability. Thanks to their tiny structure, intact antigen recognition ability, and phage screening technology, nanobodies feature with high affinity, high specificity, strong penetration, as well as easy modification and expression. Moreover, because the complete sequence of antibodies can be obtained, it allows stable production by in vitro recombinant expression and effectively avoids the batch-to-batch variation problem of conventional antibodies.

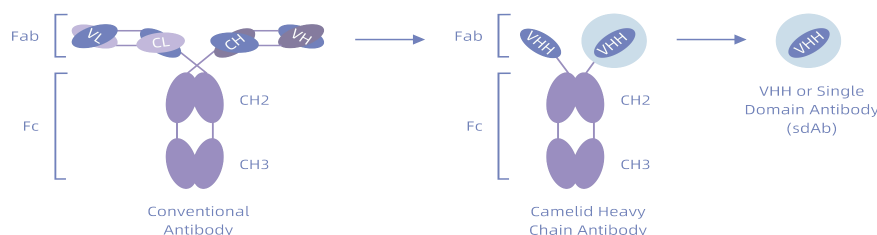
Nanobody Features

1. Longer CDR3 region (nanobody CDR3 is usually composed of 16-24 amino acids, while the traditional antibody CDR3 has only 7-12 amino acids), stronger antigen-specific binding ability.

2. Simple but stable structure.

3. Easy to produce from small to industrial scale-ups.

4. Easy to humanize.



Schematic Structure of Single Domain Antibody

AtaGenix has built a naive nanobody library with 10^{11} capacities. Rapid screening of specific nano bodies against various target proteins can be completed within 2-3 weeks, and the affinity reaches 10^{-9} M level.

Advantages

1. Sample Diversity: Derived from over 100 samples from different species of camelid – Alpaca, Camel, Llama, providing more antibody samples.

2. High Capacity: The total library capacity is nearly 10^{11} cfu and will be optimized to the level of over 100 billion.

3. High Quality: 1100% correct insertion rate, 97% sequence accuracy, 200 clones are randomly selected for sequencing, no repeated sequences. Customized immune library constructions and panning services are also available, including antigen preparation, alpaca immunization, blood collection, titer detection, nanobody library construction and panning, nanobody expression, and quality control.

Cases

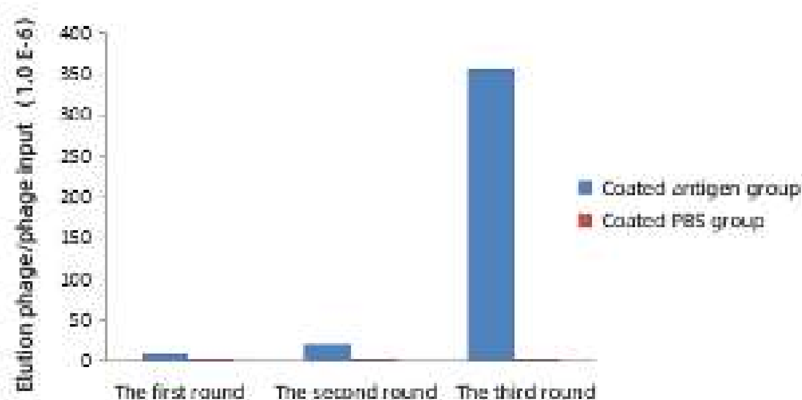
1. Target B is immunized in alpacas. After four rounds of immunization, blood is collected to extract PBMCs.After RNA extraction, cDNA is obtained by reverse transcription and then amplified to obtain VHH variable region fragments.

2. Construction of a nano-antibody library for immunized alpacas. VHH variable region fragment is linked into the phage display vector. The host strain is electroporated and transformed to obtain a nanobody library with a library capacity of 8.76×10^9. The insertion accuracy rate is 100%.

3. Panning and screening of immunized alpaca nanobody library

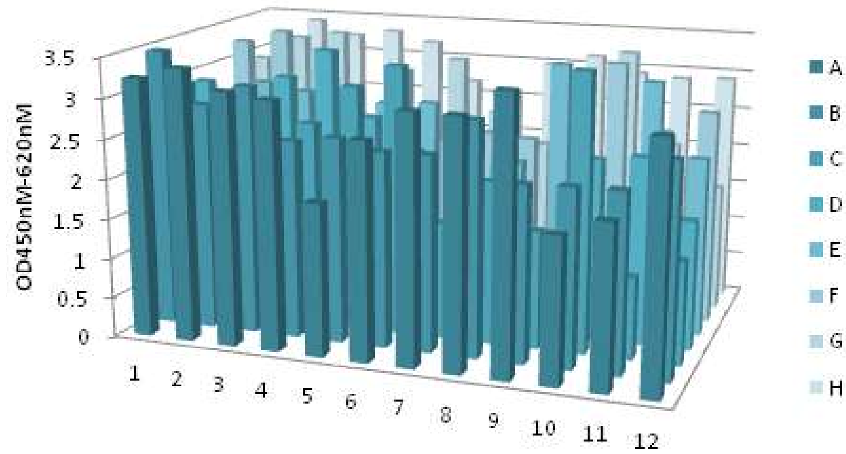
Using the constructed nanobody library for immunized alpacas, three rounds of panning are completed against the target B protein. The panning enrichment is shown as below:

Panning Enrichment



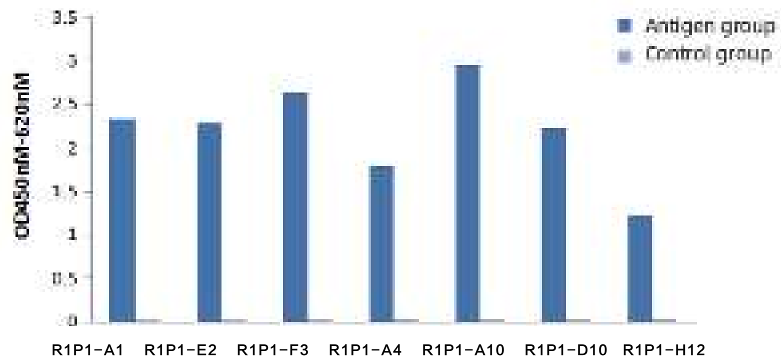
96 clones were screened, of which 94 are positive clones ,with a positive rate of 97.9% .

Eluted Phage Monoclonal Screening (Antigen Group - Background Group)



Repeated sequences are excluded, resulting in 7 different antibody sequences. These antibody sequences are expressed as soluble VHH antibodies for further validations. The results are as follows:

Antibody Protein Validation

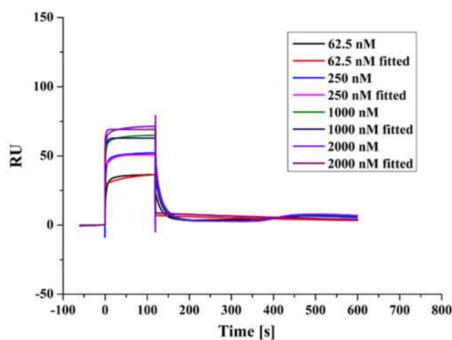


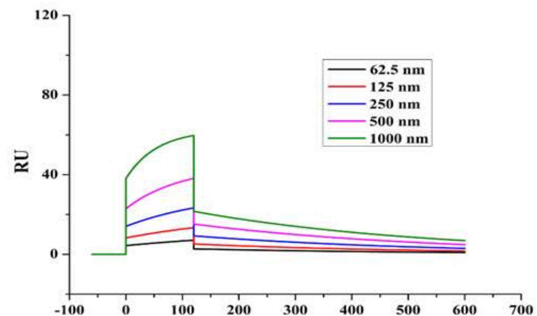
R1P1-A1 R1P1-E2 R1P1-F3 R1P1-A4 R1P1-A10 R1P1-D10 R1P1-H12

Two antibodies are randomly selected for affinity determination, and the results are as follows:

R1P1-A10：KD：6.49E-09M

R1P1-H12：KD：1.52E-07M





Phage Antibody Screening Service Platform for Rare Species,Dogs and Cats

With the development of society, pets have played an increasingly important role in human life, and the treatment market of pets' disease has also received extensive attention. On January 13, 2022, the US Food and Drug Administration (FDA) approved the first antibody drug for feline arthritis pain relief: Solensia (frunevetmab injection). This is a cat-derived NGF (nerve growth factor) monoclonal antibody that can improve cat mobility. In cooperation with scientific research institutions and pharmaceutical companies, AtaGenix has constructed phage display naïve antibody libraries for dogs and cats, validated by using standard control projects. Features of the libraries are shown below.

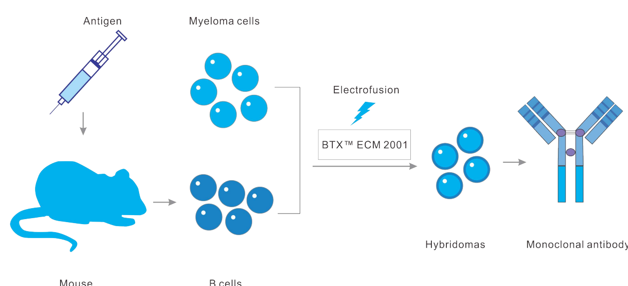
Library	Display Technology	Library Format	Species	Library Size	Insert Rate	Accuracy
CT-ScFv	Phage Display	scFv	Cat	2.19×10^9	99%	>95%
DN-ScFv	Phage Display	scFv	Dog	3.98×10^9	100%	>95%

Hybridoma antibody Discovery & Sequencing

Introduction

Hybridoma technology was invented in 1975 and awarded the Nobel Prize in Physiology and Medicine in 1984. It is an important milestone in the history of the whole life science research, as mouse monoclonal antibodies were introduced as powerful tool. Due to the advantages of high specificity, strong affinity, stable passage and easy handling and large-scale productions, monoclonal antibodies generated by hybridoma technology are widely used in fundamental research, in-vitro diagnostics and therapeutics.

AtaGenix provides quality hybridoma cell development services, including antigen design and production, multiple immunization protocols, and electric cell fusion, screening, detection, strain determination, subtype identification, sequencing, and antibody recombinant production in mammalian cells.



Advantages

1.Various Antigens Available: proteins (five expression systems), peptides, small molecules, and DNA.

2.One-stop Service: offering total solutions from gene sequence to hybridoma development and identifications.

3.High Success Rate: experience of 2000+ mAb projects, with a success rate of 95.4%.

4.Customized Screening: design customized screening protocol according to final applications, such as WB/IHC/IF/FC

5.Extensive Validations: WB/IHC/IF/FC and more detection methods

6.Professional Team: 10+ years' experience in antibody research and development.

7.Downstream Engineering: Hybridoma cell sequencing, antibody humanization, recombinant antibody expression, sandwich pair screening for chemiluminescence applications, and mass production of antibodies.

Content

Content	Receivables	Time frame	Deliverables
Antigen preparation (antigen expression & purification) Host immunization Hybridoma cell line construction (fusion cells, selective culture,and Screening) Antibody production & identification (Protein A/G purification, SDS-PAGE and UV analysis, ELISA detection)	Antigen	4-6 months	Purified monoclonal antibody (customized amount) Hybridoma cell line (customized quantity) Antigen sample Technical service report

Cases

Interleukin 6 (IL6) Diagnostic Antigen Development and Sandwich Antibody Pair Development

IL6 Antigen – expressed by mammalian expression system, high activity, and good stability.

（1）Activity Study:

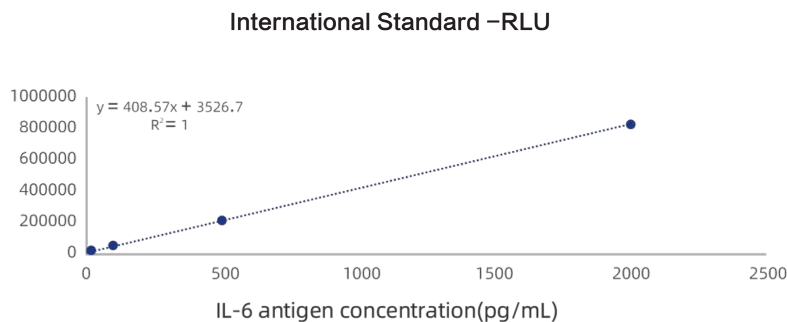
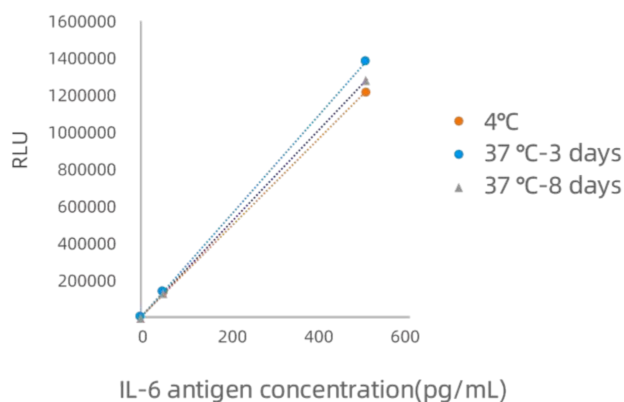


Table 1. Comparison of AtaGenix IL6 with the International Standard

AtaGenix IL6 Antigen (pg/ml)	RLU Mean	Converted concentration (pg/ml) (Corresponding to IL6 international standard concentration)	Ratio	Ratio Mean
5	15603	29.56	5.91	6.06
50	130250	310.16	6.20	

AtaGenix IL6 antigen activity is higher, 1pg/ml IL6 antigen and 6pg/ml international standard reactivity are equivalent. (IL6 International Standard: NIBSC 1st IL6 89/548)

（2）IL6 Antigen Stability Study:



AtaGenix IL6 antigen shows a good stability, with no distinctive differences in RLU values between 3 days at 37° C and 8 days at 37° C and long-term storage at 4° C, and still maintains high activity.

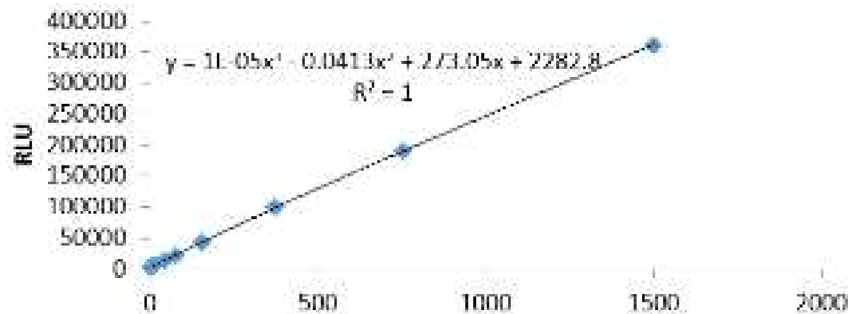
Rabbit Immune Antigen Preparation: Protein expression in proprietary mammalian system.

Screening Antigen: WHO international standard of NIBSC (IL−6, human rDNA derived, NIBSC code 89/548)

Monoclonal Antibody Preparation: A total of 44 IL6−positive cell lines are screened by using mouse hybridoma technology.

A total of 1892 antibody pairs are screened by the chemiluminescence platform.

IL6 International Standard − RLU

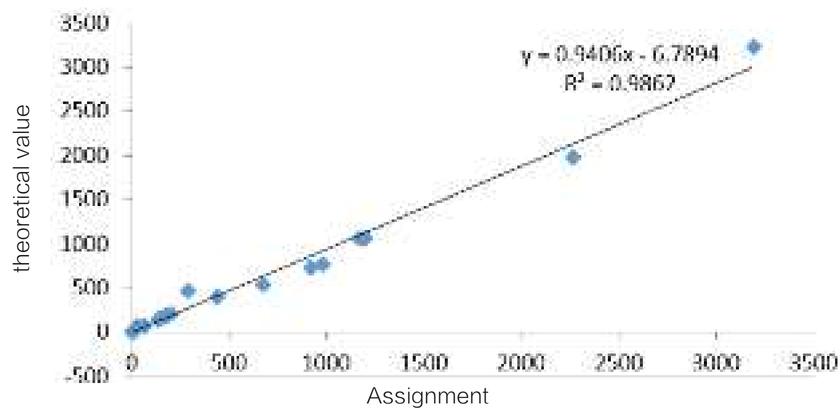


In the chemiluminescence platform, Roche measured samples are used for screening. One pair of antibodies is obtained with desirable specificity for serum samples and international standards. The sensitivity is 1pg/ml, and R^2=1.

Parallel Comparison:

Comparing the theoretical value from Roche, the correlation with the result from AtaGenix is R^2=0.9862.

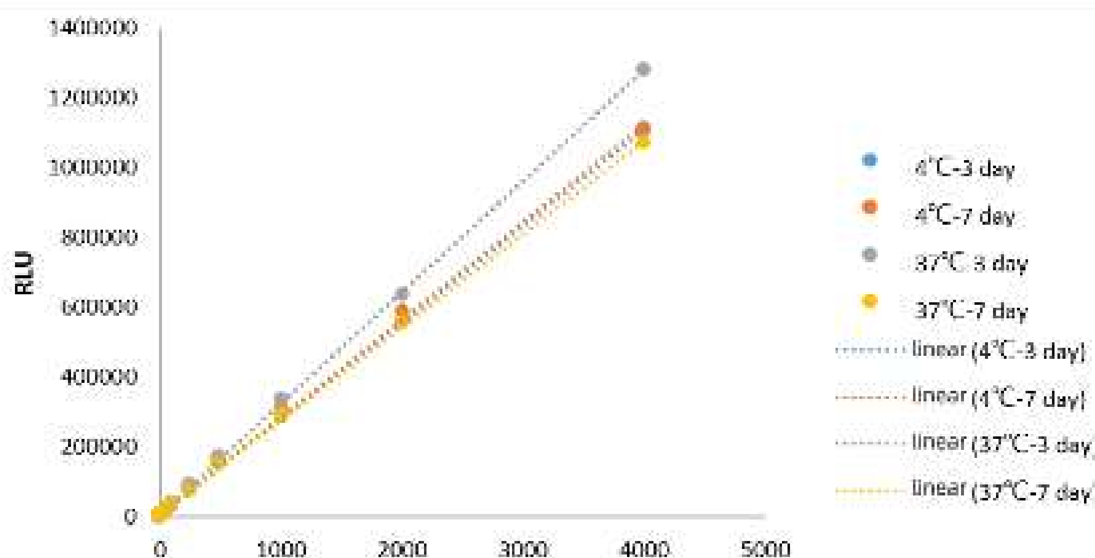
Correlation



Stability Test:

AtaGenix IL6 Antibody (pg/ml)	4℃ -3 day-RLU	4℃ -7 day-RLU	37℃ -3 day-RLU	37℃ -7 day-RLU
0	1886	3162	2790	2972
1	2022	3341	3172	3504
2.5	2586	3948	3775	3985
5	3442	4924	4815	4932
10	5110	6539	6483	6569
25	9541	11216	11938	11049
50	17791	19547	20801	18950
100	32047	36028	37825	33702
250	76150	83036	90499	78330
500	150229	157355	171096	149662
1000	289376	305072	336157	291227
2000	560092	587798	636806	553922
4000	1106263	1112355	1280067	1070342

The working solution was placed at 4° C and 37° C for 3 days and 7 days, respectively before test. The results show that the IL6 antibody was very stable.

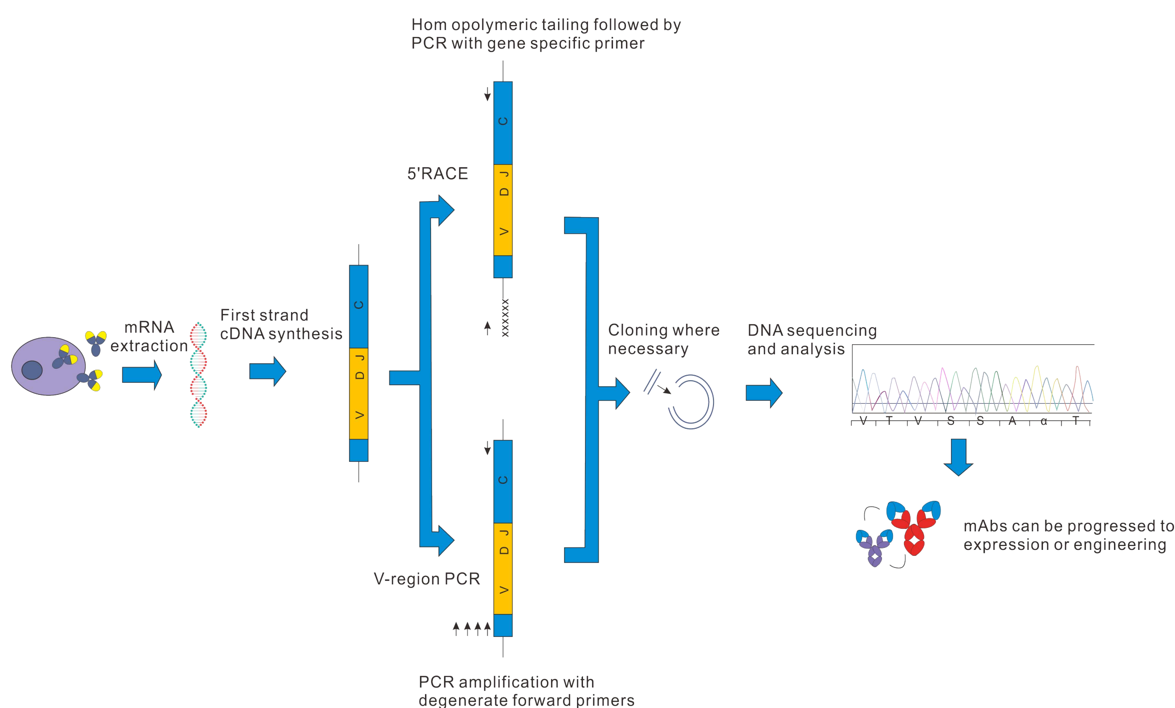


AtaGenix IL6 antibody stored at 37° C for 3 days/7 days and 4° C for 3 days/7 days – RLU Comparison

Hybridoma Antibody Sequencing

Even though hybridoma cell lines are stable, there are still many other factors leading to the loss of the cell lines. For example, mishandling on cell culture passages, cell bank contaminations and even the force majeure should be considered. Antibody sequencing on hybridoma cell guarantees continuous recombinant antibody production without risks, provides valuable data and foundation for further engineering modifications. For instance, humanization, sequence optimizations and bispecific antibodies or ADCs.

AtaGenix provides an overall solution from hybridoma sequencing to recombinant antibody expression: high sequencing accuracy, 2 weeks turnaround time. We could reach up to 200-500mg/L in transient expression by using the third-generation proprietary vector pATX3.0 and mammalian cells.



Advantages

1.Short Turnaround Time: ~ 2 weeks

2.Variable region/Full-length sequencing available

3.Complete Lab Report (CDR analysis)

Content

Content	Receivables	Steps	Time Frame	Deliverables
Antibody variable region/ full-length sequencing	$1x10^6$ Hybridoma cells cultured cells or cryopreserved cells and isotype information	RNA extraction mRNA reverse transcription cDNA PCR amplification of antibody heavy and light chain genes Cloning and sequencing of heavy and light chain genes Bioinformatics analysis of sequencing results to determine functional antibody genes and antibody CDR regions Antibody sequencing results report	2-3weeks	Sequencing report Sequence original data Heavy/Light chain analysis Cloning vector containing antibody fragments

Xten^{TM}Mab SingleB Rabbit monoclonal antibody development

Introduction

Compared to the conventional hybridoma mouse monoclonal antibody development, the rabbit monoclonal antibodies can provide better antigen recognition. Since the rabbit myeloma cell lines are protected by patents and cannot be used to develop the rabbit monoclonal antibodies by using hybridoma technology, AtaGenix has established the phage-display antibody library technology platform and the novel XtenTM Mab Single B rabbit monoclonal antibody development platform.

The single B cell antibody preparation technology is a new type of rapid monoclonal antibody preparation technology developed in recent years. It is based on that each B cell contains only one functional heavy chain variable region DNA sequence and one light chain variable region DNA sequence, and each B cell produces only one specific antibody. The IgG heavy chain and light chain variable region genes are amplified from the individual antibody-secreting B cells by single-cell PCR, and then expressed in mammalian cells to obtain the active monoclonal antibodies.

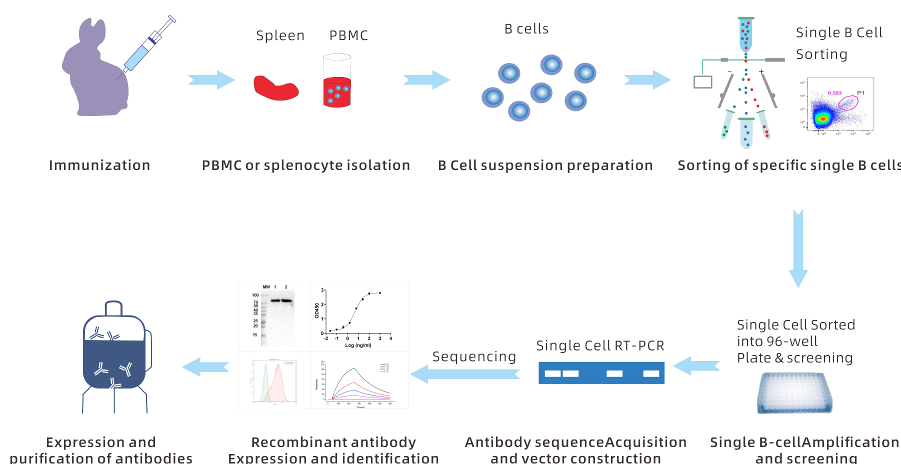


Fig 1. Xten^{TM} Mab Rabbit Monoclonal Antibody Development Procedure

Advantages

1.High Specificity: Compared to mice and other rodents, rabbits have a superior immune system, producing antibodies with greater specificity and higher affinity

2.High Stability: High structural stability of rabbit IgG compared to the conventional mouse monoclonal antibodies

3.High Affinity: Rabbit B Cell maturation process generates 10-100 times higher affinity than it in rodents

4.High Diversity: Rabbits have a rich B cellular repertoire that produces antibodies with significant advantages in terms of epitope variants, mutations, conformational changes, and high diversity

Content

Content	Receivables	Steps	Time Frame	Deliverables
Xten™ Mab rabbit monoclonal antibody development	Antigen sequence	Antigen preparation Animal immunization Splenocyte isolation and positive B-cell screening Positive clone antibody variable region amplification and sequencing analysis Recombinant antibody expression and purification	20-24 weeks	100 μ g/strain monoclonal antibody pUC plasmid 3 strains of the optimal antibody sequences Service report

Cases

The B cell sorting enrichment strategy, supported by FCM sorting equipment, It can effectively enrich antigen-specific B cells, improve the viability of B cells in vitro culture, preserve the diversity of B cells, and develop antibodies for different applications.

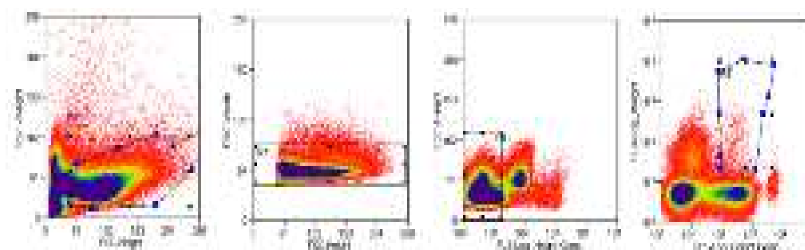


Fig 2. Xten™ Mab Rabbit Monoclonal Antibody Development for B-cell Screening

Using the XtenCHO™ high concentration transient expression system, some positive antibodies recombinantly expressed in vitro and purified for functional validation.

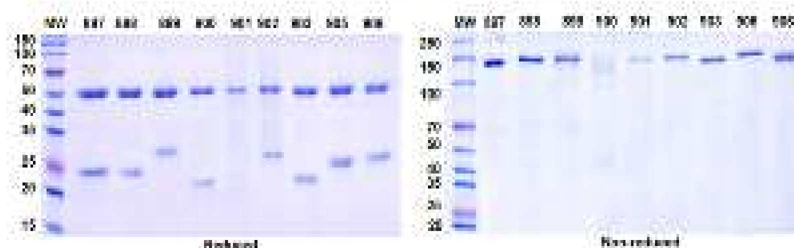


Fig 3. Xten™ Mab Rabbit Monoclonal Antibody Development for Recombinant Antibody Expression

	a	b	c	d
EC50(ng/ml)	6.897	145	15.48	23.43

The recombinantly expressed purified antibody can be subjected to functional analysis by ELISA, WB, and flow-through, etc.

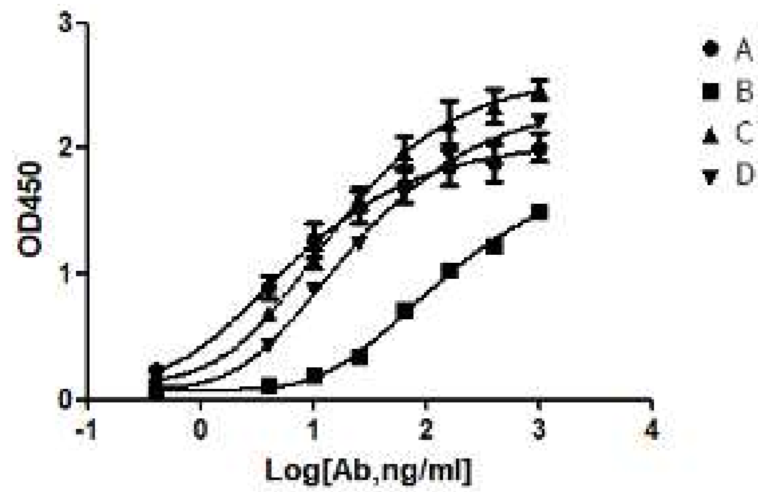
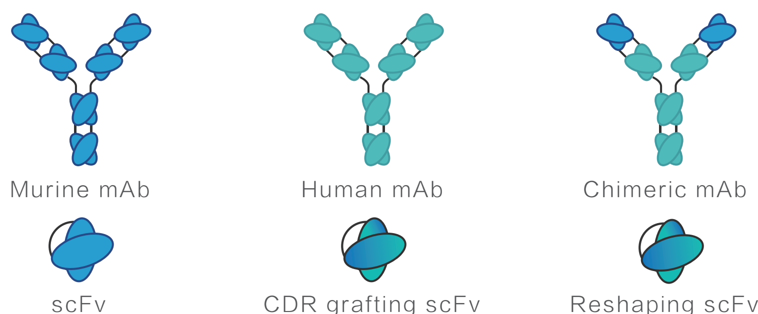


Fig 4. Recombinant Antibody ELISA Assay Results for Xten™ Mab Rabbit Monoclonal Antibody Development

Antibody Humanization

Introduction

Non-human antibodies have been demonstrated to induce human immune responses, which result in neutralization of administered antibody and limits the application of such antibodies in treatment of human diseases. To overcome this problem, the technology of antibody humanization has been developed. It refers to the humanization of most amino acids of mouse monoclonal antibodies through DNA recombination technology and protein engineering technology to reduce its heterogeneity (immunogenicity) while retaining the affinity and specificity of parental mouse monoclonal antibodies. Antibodies expressed by recombinant genes have both murine and human components, so they are called humanized antibodies.



AtaGenix combines its unrivalled know-how in antibody engineering with experts boasting more than 25 years of experience and a strong track record in antibody humanization. Our humanization process encompasses CDR grafting, molecular modeling and sequence optimization.

Molecular modeling is an important part of this process as it allows the analysis of the contributions of individual amino-acids localized in the murine CDR loops and in the framework regions. This step is essential for identifying residues eligible for back mutations and those which can be mutated for further properties optimization. Amino-acids involved in the variable regions can be classified as follows:

1.Residues involved in the antibody-antigen interaction,

2.Residues playing a structural role, for instance by maintaining the CDR loop conformation or stabilizing the VH-VL interaction,

3.Residues interacting with the solvent.

Residues from the murine framework regions considered as critical to maintain the conformation of the CDR loops and the antibody bioactivity will be back mutated in the selected human germlines. The human germline selection is based on sequence homology, but other humanization variants, demonstrating lower sequence homology but presenting good physicochemical properties, can be tested. In a humanization project, AtaGenix selects several heavy chains and light chains in order to generate between 9 and 18 combinations.

The different antibodies generated are further characterized and compared to the reference parental and/or chimeric antibody in order to assess their:

1.Immunogenicity,

2.Physicochemical properties (stability, aggregation rate…),

3.Pharmacological properties (affinity, specificity…),

4.Manufacturability.

Following successful testing, lead candidates can be selected for further humanization and/or CDRs sequence optimization for improvement of biophysical properties (heterogeneity, fragmentation, aggregation…) and manufacturability (bioproduction).

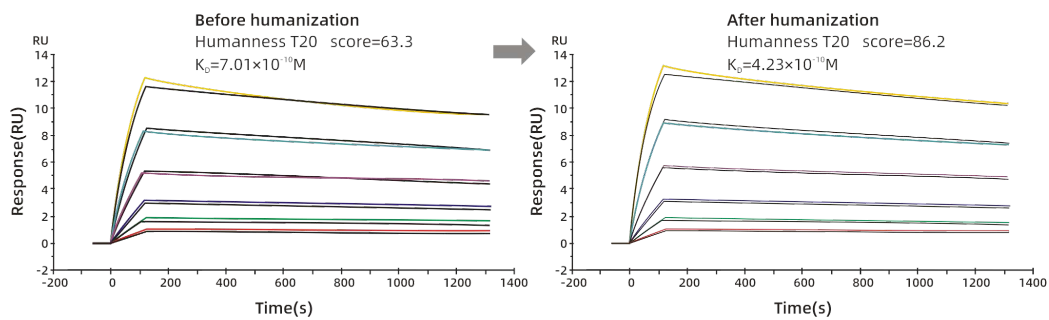
Content

Steps	Content	Timeline	Deliverables
Parental antibody sequencing from hybridoma	Hybridoma cell line provided by customer or developed by AtaGenix. RNA extraction and purification Reverse transcription cDNA amplification Sequencing analysis	2-3 weeks	Detailed report Antibody sequence
Chimeric antibody expression and purification	Codon optimization for Mammalian expression system Gene synthesis Subcloning into expression vectors Transient expression Purification QC analysis: SDS-PAGE, WB, UV280	~7-9 weeks	Detailed report Chimeric antibody sample for testing
Design of humanized antibody	Bioinformatics analysis 3D structure modeling and identification of back mutations Human germlines selection In silico CDR-grafting Sequence optimization	~2 weeks	Detailed report Discussion with one of our therapeutic antibody expert
Transient recombinant production of 9 to 18 humanized antibody variants	Codon optimization for Mammalian expression system Gene synthesis Subcloning into expression vectors Transient expression Purification QC analysis: SDS-PAGE, WB, UV280	~7-9 weeks	Detailed report Purified humanized antibody samples for testing
Characterization of humanized monoclonal antibody variants	Affinity analysis by ELISA against the antigen Analysis of antibody aggregates (SEC-HPLC) Affinity determination (Kd) against soluble antigen (SPR/Biacore X100) Affinity Determination (Kd) against antigen expressed on cell surface (SPRi/ Horiba XelPlex)	To be determined	Detailed report Discussion with one of our therapeutic antibody expert

Advantages

1. Recognized experts with proven records of reaching clinics.
2. 3D modelling platform guarantees biological function conservation.
3. Customer gets the full ownership of the humanized antibodies.
4. Full set of charaterization services (ELISA, Kd, thermostability, aggregation rate, IC50...).
5. Efficient production by XtenCHO transient system.
6. Optimize broad range of antibody formats including full length IgG, scFv, Fab, nanobodies...

Cases



Bispecific Antibody

Introduction

Bispecific antibodies (hetero-conjugated antibodies) are a class of bifunctional hybrid antibody molecules with two different antigen-specific binding Fab segments, which can bind to different ligands and simultaneously bind to tumor antigens by specific binding. Different effector cells and molecules achieve targeted killing of tumor cells.

1.1 Bispecific Antibodies with Fc Region

The biologic al functions of the Fc domain of antibodies include antibody -dependent c ell-mediate

cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC). There are several technical means for the preparation of bispecific antibodies:

Triomabs: Triomabs bind to tumor cells and T cells respectively through the Fc functional region, and through Fc functional region, they recruit Fc R-expressing functional cells, such as NK cells, monocytes,macrophages, granulocytes, and dendritic cells to form complex that stimulates T cells to secrete cytokines to eliminate tumor cells. Triomabs are also called trifunctional antibodies. The Triomabs bispecific antibody technology platform was jointly developed by the German company Fresenius and Trion Pharma.

Knobs-into-holes: This technology was developed by Genentech. The specific method is to mutate the smaller threonine (T) at position 366 of the heavy chain CH3 region of one of the antibodies to the larger tyrosine (Y) to form a prominent "Knobs" type structure (T366Y); The larger tyrosine (Y) residue at position 407 of the CH3 region of another antibody heavy chain was mutated to a smaller threonine (T) to form a recessed “holes" type structure (Y407T); using "Knobs - The steric hindrance effect of the "into-holes" structure enables the correct assembly of two different antibody heavy chains. After mutation, the correct assembly rate of the product is increased from 57% of wild type to 92%, which can meet the requirements. of large-scale production. However, this modification of the heavy chain CH3 reduces the stability of the antibody structure. To overcome the shortcoming, the researchers conducted random mutation screening. through phage display technology and constructed a more stable "3+1" model, Knobs-holes Structure: T366W mutation forms a prominent "Knobs" type, and 3 amino acid mutations (T366S, L368A and Y407V) form a recessed "holes” type. Knobs-holes structural design facilitates the assembly of 2 hetero logous antibody heavy chains.

Crossmab: The representative products of Crossmab technology are Roche's RG7221 and RG7716, both of which are anti-Ang- 2/VEG Fbi specific antibodies. Its structure is based on the “knobs -holes” structure, through chain exchange technology, the CL and CH1 in the Fab domain of the Ang-2 antibody are exchanged, while the Fab structure of the VEGF antibody remains unchanged. The engineered Ang- 2 antibody light chain is not prone to mismatch with the heavy chain of vascular endothelial growth factor (VEGF) antibody, and the “knobs-holes “structure promotes heterodimerization of the two heavy chains.

Ortho-Fab: This technique is a design strategy to overcome light chain mismatches reported by Lewis et al. Lewis et al. carried out orthogonal complementary mutation design of VH/VL and CH1/ CL through computer modeling combined with X-crystal diffraction technology, thereby reducing the phenomenon of light chain mismatch. This technology combined with heavy chain heterodimerization method can achieve efficient expression of bispecific antibodies in single cells. Recently, electrostatic steering has also been used to construct Orthogonal Fab bispecific antibodies.

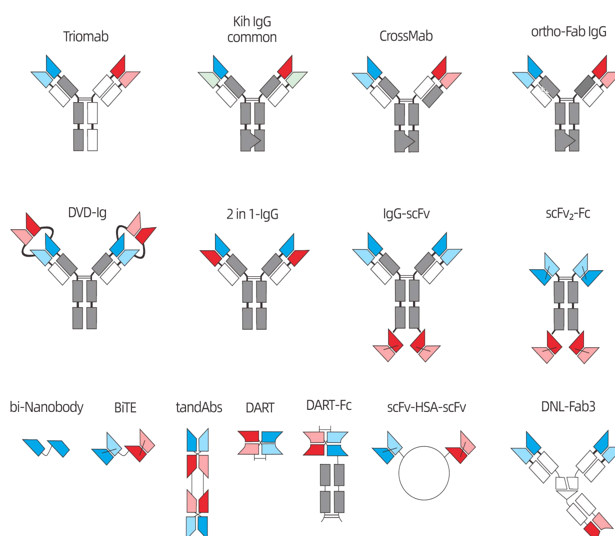
IgG-scFv and scFv2-Fc: IgG-sc Fv bispecific antibody is a single-chain antibody (sc Fv) linked to the C -terminus of a normal IgG antibody molecule, and the CDR regions at both ends of the molecule are combined with the target molecule to achieve dual functions. The sc Fv2-Fc molecule is similar in structure to IgG -sc Fv, and two sc Fv molecules are connected at both ends of the Fc functional domain to form a bifunctional domain.

1.2 Bispecific Antibodies without Fc Region

Another type of bispecific antibody does not contain an Fc region, which has the advantage of a small relative molecular mass, can be expressed in prokaryotic cells and can more easily pass through tissues and tumor cells to reach the target; the disadvantage is that the absence of the antibody Fc region does not mediate the corresponding biological function and the half-life is usually short. For example, the blood half-life of the marketed blinatumomab is only 2.11 h, and it needs to be continuously administered by a syringe pump for 28 days. At present, such bispecific antibodies mainly include BiTE, DART, TandAbs, and bi-Nanobody, etc.

BiTE Bispecific Antibody: BiTE series products developed by Micromet company in Germany are typical representatives of bispecific antibodies without Fc region structure. BiTE is obtained by linking anti-CD3 single-chain antibody (sc Fv) with different anti-tumor cell surface antigen single-chain antibodies through peptide fragments, which can simultaneously bind CD3-positive T cells and tumor cells. BiTE recruits T cells to the surface of tumor cells by binding to CD3 on the surface of T cells, thereby activating T cells for tumor killing. BiTE technology researchers overcame the production problems of poor sc Fv stability, low expression, and low solubility, and successfully commercialized BiTE products.

Bi-Nanobody Bispecific Antibody: Nanobody is Ablynx's reference camel and llama single-domain antibody structure(without light chain and CH1 region) , a patented platform technology developed by simplifying the structure and retaining only the VH region. In practical applications, Nanobodies link the VH regions of two or more antibody molecules to achieve multispecific binding. The main advantages of this type of products are small molecules, high stability, easy humanization,easy connection, and can be administered through a variety of routes. In addition, a functional domain that binds to human album in can be optionally added during molecular design to extend the half-life to 2 to 3 weeks, and the drug can be transported to the target site through album in.

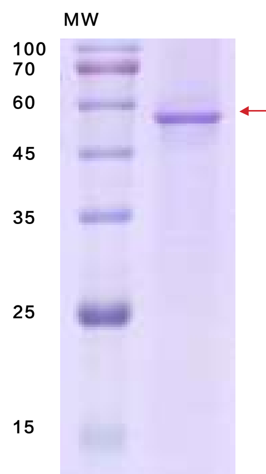


AtaGenix has an experienced bispecific antibody development team that can provide the design of the bispecific antibody types above.

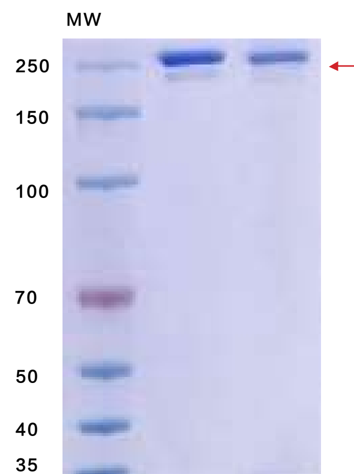
Content

Content	Customer Provides	Steps	Time Frame	Deliverables
Bispecific antibody	Antibody sequence	Gene synthesis and codon optimization Vector construction Expression & purification QC analysis	5 weeks	Purified antibody Technical service report

Cases



Tandem scFv



Crossmab

Anti-idiotype Antibody Development

Introduction

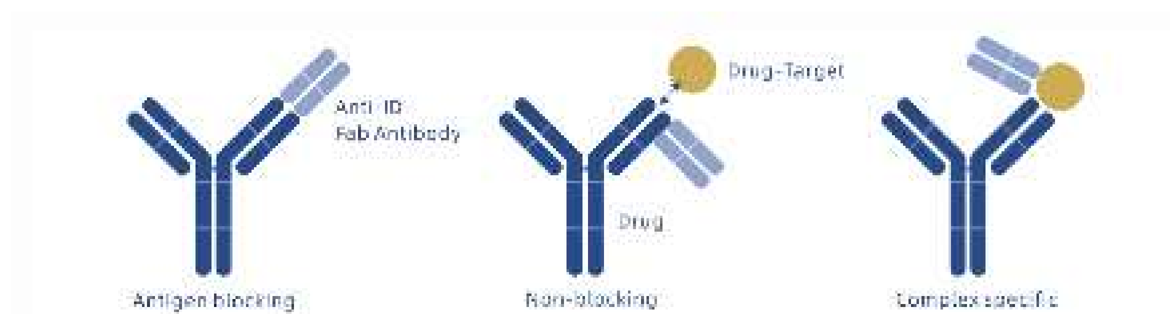
Anti-idiotype antibody refers to an antibody that can specifically bind to the idiotype (Idiotype) of the variable region of the antibody to be tested. In the process of antibody drug development, it can specifically detect antibody drugs in vivo and is a key reagent for pharmacokinetic research. Main application is pharmacokinetic analysis of antibody drugs, immunogenicity analysis of antibody drugs and clinical development of anti-drug antibodies. With years of experience in anti-idiotype antibody development, AtaGenix can provide a full range of technical services from antigen design and preparation, antibody development and assay development.

Types of Anti-idiotypic Antibodies

1.Antigen Blocking Anti-idiotype Antibodies: This type of anti-idiotypic antibodies is paratope specific meaning that antibodies idiotope and paratope overlap with one another. Therefore, the target antigen and the anti-idiotypic antibody compete for the same binding site. These antibodies are commonly used for free antibody drug measurement.

2.Non-blocking Anti-idiotypic Antibodies: This type of anti-idiotypic antibodies is characterized by an antibody drug binding outside of the antigen binding site (antibodies idiotype and paratope do not overlap). These antibodies are used for total antibody drug detection and quantification.

3.Complex Specific Anti-idiotypic Antibodies: Complex specific anti-idiotypic antibodies bind only to the antibody-drug antigen complex. Therefore, they are typically used to quantify bound antibody drug in a sample.



Advantages

1.Experienced in both polyclonal and monoclonal anti-idiotypic antibody development, covering blocking, non-blocking and complex specific antibodies.

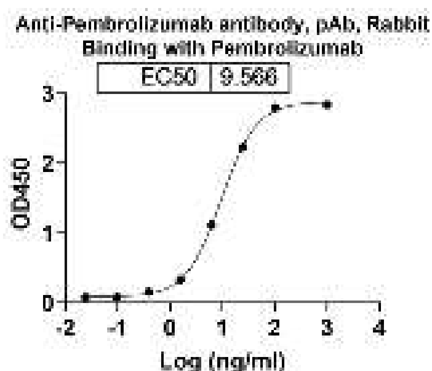
2.Hybridoma, single B sorting and phage display technologies are all available.

3.ELISA assay development for PK/PD, immunogenicity assays etc.

Content

Content	Customer Provides	Steps	Time Frame	Deliverables
Anti-idiotype rabbit polyclonal antibody preparation service	Target antibody drug with >85% purity isotype control	Antigen analysis Antigen preparation Immune and serum titer detection and purification QC analysis	4~6 months	Serum before and after immunization Purified antibody Technical service report
Anti-idiotype mouse monoclonal antibody preparation service		Antigen review antigen preparation Immune and serum titer detection fusion & screening Antibody production and purification QC analysis ELISA paired assay	4~6 months	Hybridoma cell culture supernatant of positive clone Purified antibody Hybridoma cell line Technical service report
Anti-idiotype rabbit monoclonal Antibody preparation service		Antigen analysis Antigen preparation Immune and serum titer detection Library construction and panning Antibody production and QC analysis ELISA paired assay	2~3 months	Purified antibody Complete antibody sequence (including heavy and light chains) cloned in XXX vector Technical service report

Cases



Elisa binding of anti-pembrolizumab antibody, pAb, rabbit with pembrolizumab coating antigen:

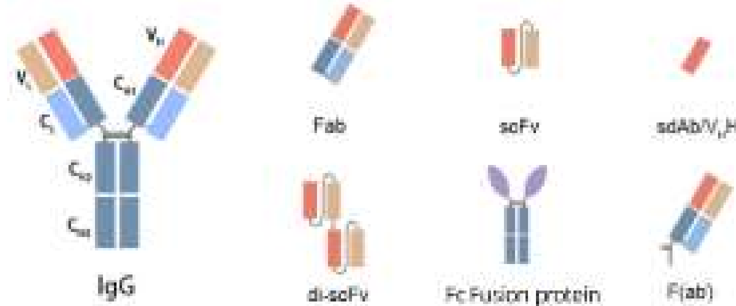
Pembrolizumab, 1ug/mL

Anti-pembrolizumab antibody, pAb, rabbit dilution from 1000ng/mL EC50=9.566ng/mL

Recombinant Antibody Production and Affinity Test

Introduction

Recombinant antibody production has been a breakthrough in the therapeutic antibody development context as it leads to several advantages over “traditional” antibody production in hybridoma cell lines.Because crucial parameters such as DNA and protein sequence are chemically defined, recombinant antibody production allows creating a standardized process for monoclonal antibody production with very low batch-to-batch variations. Obtaining the sequence of antibody also provides access to the antibody structure at the genetic level, which is particularly important when modulating the pharmacological properties of an antibody.



Advantages

1.The proprietary XtenCHO™ protein expression system includes a genetically modified CHO cell line, an expression vector, transfection reagent and feed supplement, all developed in-house by AtaGenix. The system is designed for high cell density growth and longer transient expression period which is extended from 6-7 days to 10-14 days. As a result, it provides an economic solution for antibodies with high yield and purity.

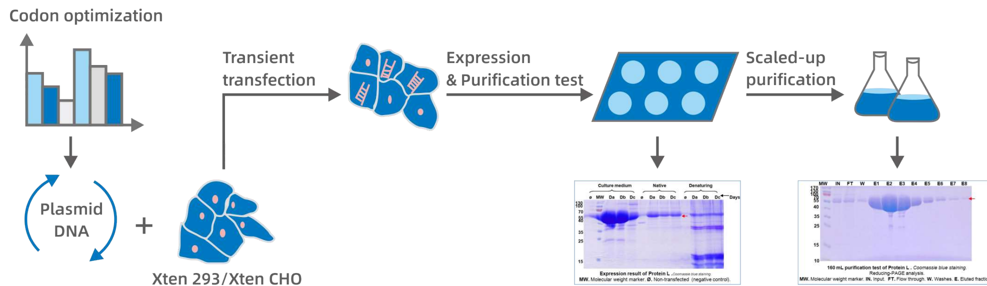
2.Various formats are available, including full-length IgG, chimerics, scFv, Fab, VHH and diverse Fc fusion proteins.

3. Increasing high-throughput capacity. >200 antibodies can be transfected in parallel.

4. Low endotoxin process: 0.1 EU/ml

Content	Steps	Time Frame	Deliverables
High–throughput low–endotoxicity antibody expression	Sequence optimization and gene synthesis	2~3 weeks	Purified antibody and technical service report
	Endotoxin free plasmid preparation		
	Cell transfection and culture		
	Low endotoxin antibody purification		
	Endotoxin assay		

Process



Transient Transfection Example

1.Comparison of XtenCHO™ with Oher Commercial Expression Systems

Using XtenCHO™ high-density transient expression system and two commercial CHO transient expression systems, CHO Expression system 1 and CHO Expression system 2. Expressions are performed in parallel. Transfected cell density, viability and antibody yields are shown in Figures 1 and 2:

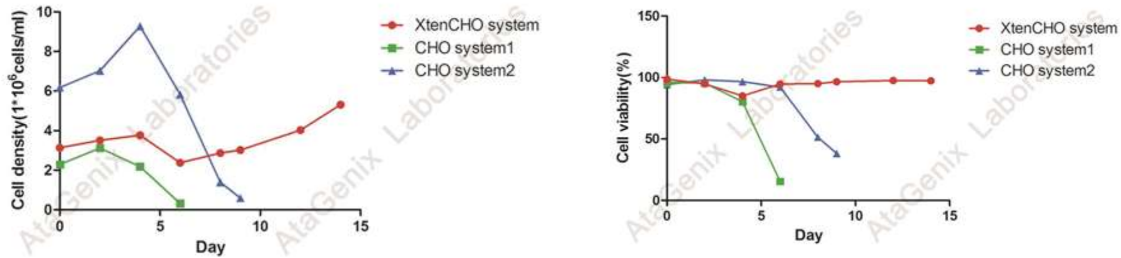


Figure 1. Monitoring of Cell Density and Viability after Transfection with Different Expression Systems

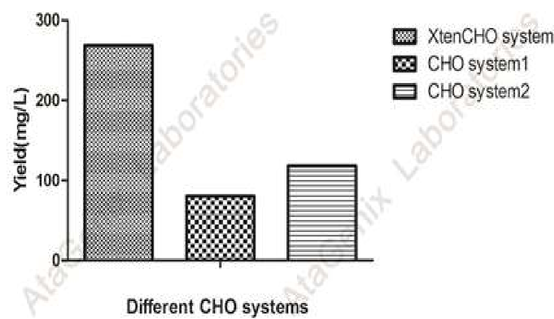


Figure 2 .Comparison of the Yields of the Same Antibody Expressed by Different Expression Systems

2.Different Therapeutic Recombinant Antibody Expression Tests

Four typical therapeutic recombinant antibody sequences were selected and tested for expression using the XtenCHO^{TM} high-density transient expression system. Antibody yields are shown in Figure 3:

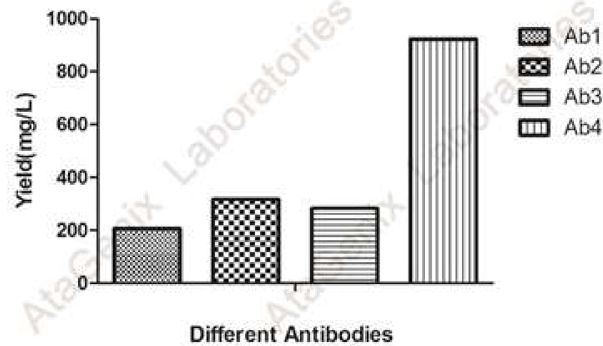
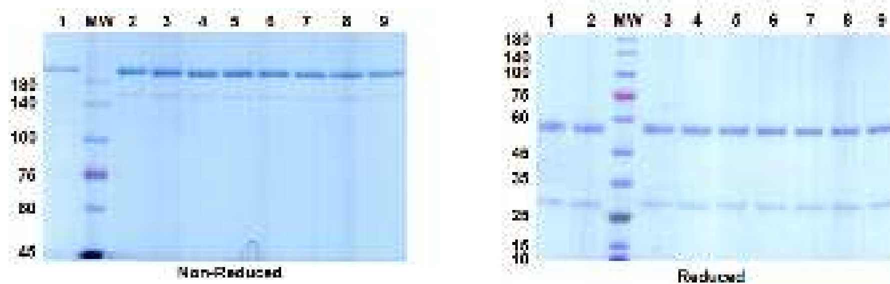


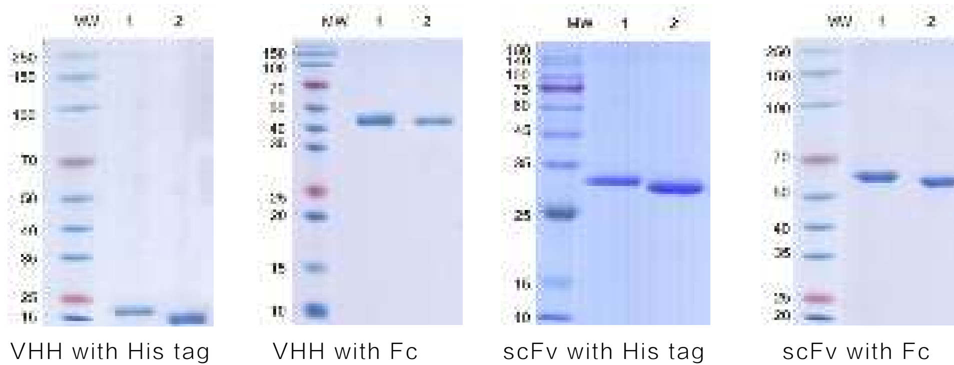
Figure 3. Yield Comparison of Different Antibodies Expressed by the XtenCHO^{TM}system

Ab1: Pembrolizumad, Ab2: Utomilumab, Ab3: Trastuzumab, Ab4: Claudiximab

Full-length Antibody Production



Antibody Fragment Production



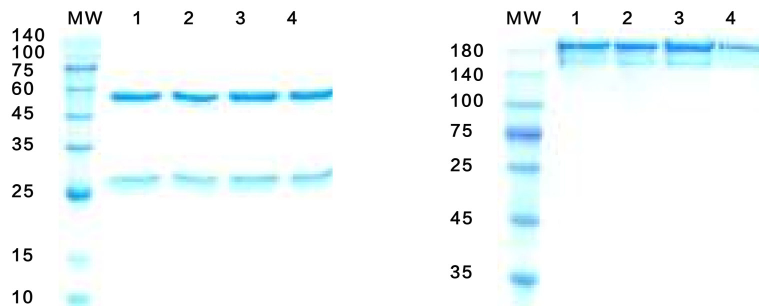
VHH with His tag VHH with Fc scFv with His tag scFv with Fc

Stable Transfection Case Study

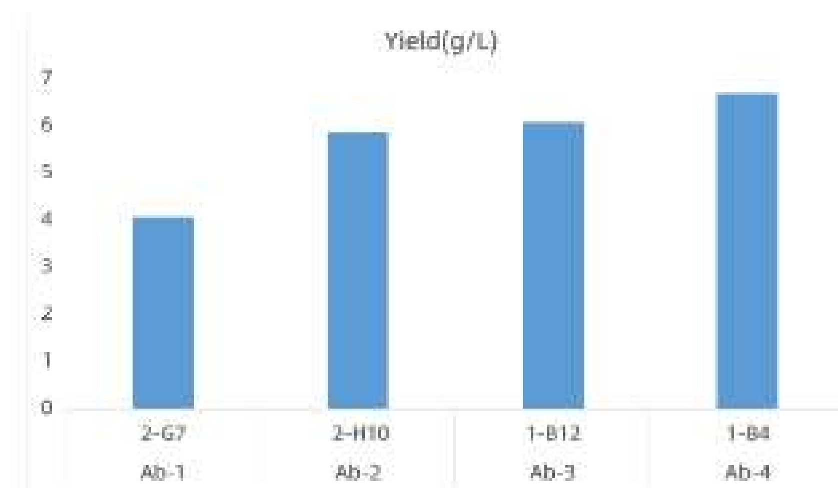
Construction of Stable Cell Line for Recombinant Antibody Production

Protein Name	Cell Line Name	Yield(g/L)
Ab-1	2-G7	4.05
Ab-2	2-H10	5.86
Ab-3	1-B12	6.07
Ab-4	1-B4	6.68

SDS-PAGE Test

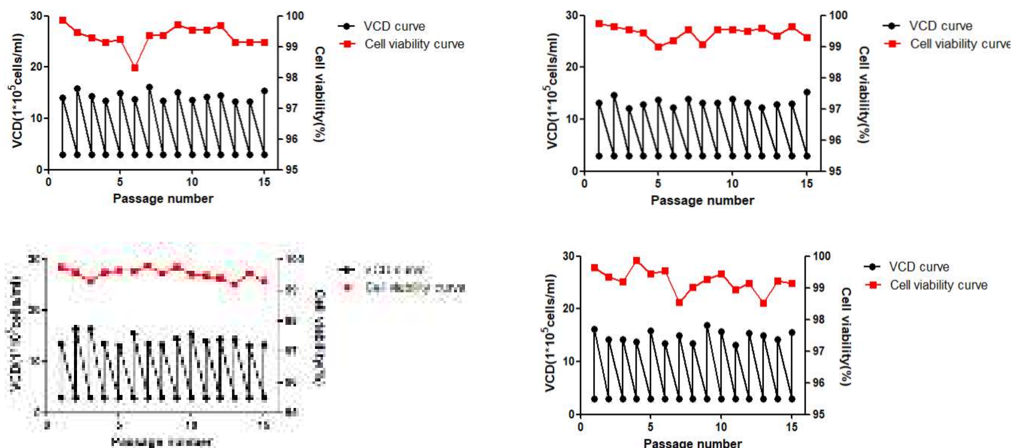


Yield Analysis



Different Cell Lines

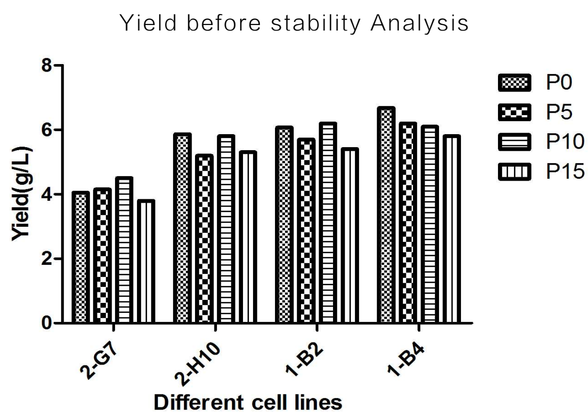
Growth Stability Analysis



Cell Line	CV% of VCD	CV% of Viability
2-G7	6.59	0.37
2-H10	6.67	0.22
1-B12	7.98	0.18
1-B4	7.71	0.37

Yield Stability Analysis

Yield before stability Analysis



Cell Lines	Yield before stability analysis(g/L)	Yield of P5(g/L)	Yield of P10(g/L)	Yield of P15(g/L)	CV%
2-G7	4.05	4.15	4.5	3.8	7.03
2-H10	5.86	5.2	5.8	5.3	6.1
1-B12	6.07	5.7	6.2	5.4	6.21
1-B4	6.68	6.2	6.1	5.8	5.9

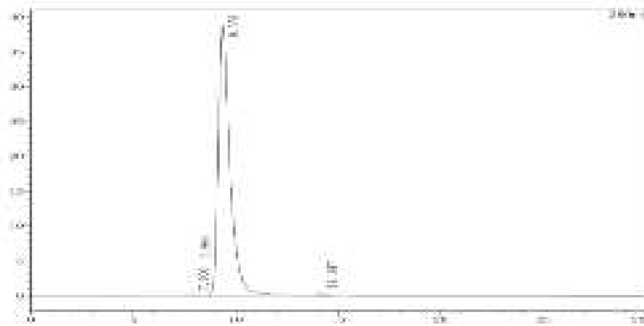
QC (Mycoplasma Detection + SEC-HPLC)

Figure 28. PCR results of mycoplasma test.

MW.DL2000 marker.

+. Positive control (290 bp).

−. Negative control



Affinity Measurement

Affinity is an important parameter for measuring intermolecular interactions and an important indicator for understanding molecular recognition, biological processes, drug discovery, and screening. In the evaluation of drug efficacy, the stability evaluation of macromolecules and their complexes requires comprehensive research in terms of thermodynamics, kinetics and thermal stability, including intermolecular binding, binding speed, binding strength, and the mechanism of binding, etc.

Based on the Biacore, ForteBio Octet and Open−SPR platforms, AtaGenix provides corresponding intermolecular interaction detection services,including proteins, antibodies, antibody fragments, and antibody fusion proteins.

Biacore T200

Octet® R4

Open−SPR

Content

1.Affinity measurement between macromolecules.

2.Small molecule affinity test.

3.Fc receptor protein/complement and antibody affinity detection.

4.Antibody screening and affinity ranking.

Proof-of-Concept Tools for Drug Development

Average R&D cost per novel drugs increased from 1.3 billion in 2013 to 2.4 billion USD in 2020 while success rate remains low. Therefore, to efficiently validate “new ideas”, it is vital to conduct proof-of-concept work in the earliest possible stage.

Once the structure or sequence of the candidate molecule is determined, the cost of the subsequent developments (animal safety assessment, process development, clinical phases) will dramatically increase. Hence, to avoid failures in such late-stage development, it is cost effective to expose problems and discover risks as early as possible, to achieve low-cost but high throughput trials.

AtaGenix provides a series of biologically active drug target proteins and research grade biosimilars and isotope controls that are corresponding to the your NEW CONCEPT.

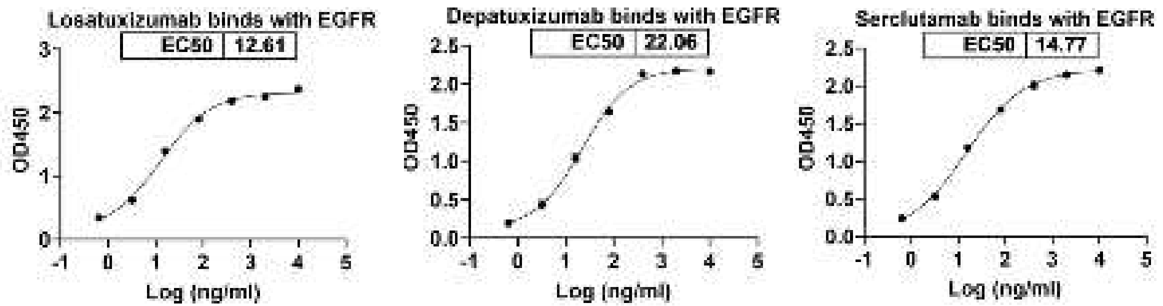
EGFR

Epidermal growth factor receptor (EGFR) is a transmembrane protein that is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α (TGFα). Upon activation by its growth factor ligands, EGFR undergoes a transition from an inactive monomeric form to an active homodimer.

SDS-PAGE Test

Human EGFR/HER1 Protein , C-His Tag

Activity Test

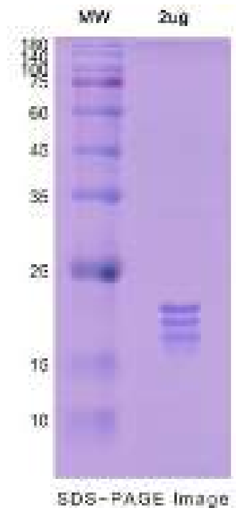


In addition to the three analogs of Losatuxizumab, Depatuxizumab and Serclutamab, marketed and clinical biosimilars targeting EGFR include: Olinvacimab, Alacizumab, Tanibirumab, Vulinacimab, Modotuximab,Tomuzotuximab, Matuzumab, Demupitamab, Zatuximab, Imgatuzumab, Laprituximab, Nimotuzumab, Zalutumumab, Pimurutamab, Petosemtamab, Icrucumab, Cetuximab, Panitumumab, Ramucirumab, Necitumumab, Amivantamab, Pulocimab, Bafisontamab, etc. Atagenix has also done some related verification on its affinity and activity.

IL17A

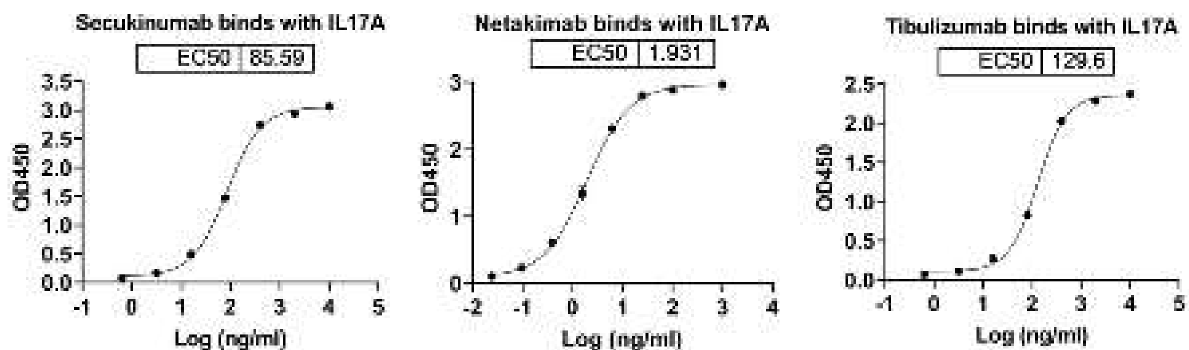
Interleukin-17A is a proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases and stimulates the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide.

SDS-PAGE Test



Human IL-17A / CTLA-8 Protein, No Tag

Activity Test

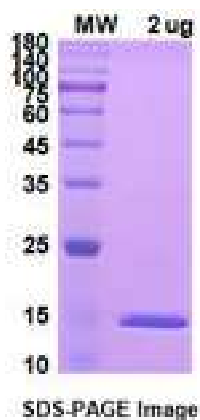


In addition to Netakimab, Tibulizumab, Secukinumab, the three analogs, listed and clinical analogs targeting IL17A include: Bimekizumab, Vunakizumab, Perakizumab, Afasevikumab, Sonelokimab, Remtolumab, Ixekizumab, Xeligekimab, Gumokimab, etc. Some related verifications were also done on its affinity and activity.

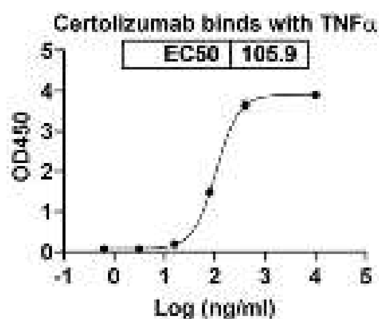
TNF α

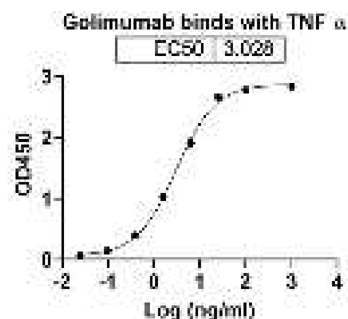
Tumor necrosis factor (TNF, cachexin, or cachectin; often called tumor necrosis factor alpha or TNF-α) is an adipokine and a cytokine. TNF is a member of the TNF superfamily, which consists of various transmembrane proteins with a homologous TNF domain.

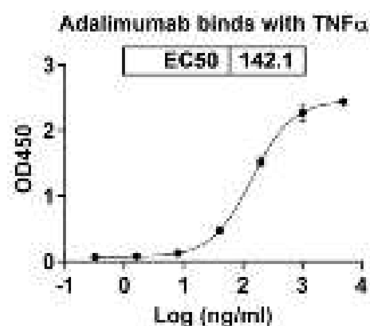
SDS-PAGE Test

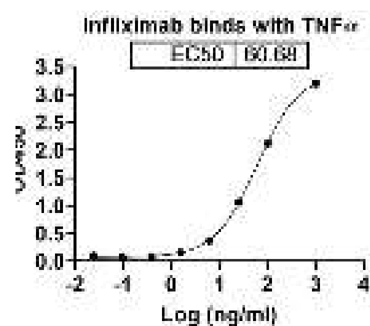


Activity Test







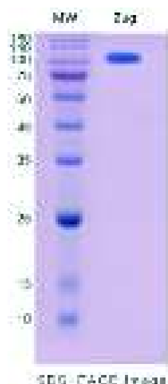


In addition to the analogs of Certolizumab, Golimumab, Adalimumab, and Infliximab, there are also Remtolumab, Afelimomab, Nerelimomab, Placulumab, Ozoralizumab, Etanercept, and Licaminlimab, etc., which target TNF α in the market and clinical analogs. Activity has also done some related verification.

ERBB2

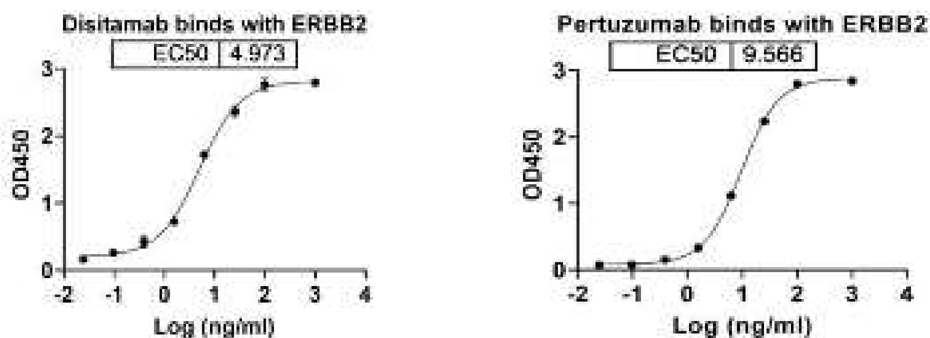
HER2 is a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. Amplification or over-expression of this oncogene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer. The protein has become an important biomarker and target of therapy for approximately 30% of breast cancer patients

SDS-PAGE Test



Recombinant Human ERBB2 Protein ,C-His Tag

Activity Test



In addition to Disitamab and Pertuzumab, the marketed and clinical analogs targeting TNFα include Timigutuzumab, Gancotamab, Zanidatamab, Ertumaxomab, Zenocutuzumab, Anbenitamab, Coprelotamab Runimotamab, Trastuzumab, Margetuximab, M802, Fidasimtamab, etc., and AtaGenix have also done some related verification on their affinity and activity.

All the target proteins, such as CD3E, CD269, CD262, CTLA4, CD115, FOLR1, Trop2, etc., as well as their corresponding research grade biosimilars, have been produced and verified by AtaGenix; Those tools provide a complete P-O-C verification to reduce the development risks of novel drugs.

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