MonoRab™
GenScript’s Premier Rabbit Monoclonal Antibody Generation Platform
White Paper
MonoRab™

A Proprietary Rabbit Monoclonal Antibody Generation Platform for Developing High Sensitivity, Specificity, and Affinity Antibodies

Abstract

Distinct advantages of rabbit monoclonal antibodies (mAbs) have led to their increasing applications in all areas of life sciences, from basic research to diagnostics and therapeutics. To address the growing demand for access to reliable rabbit mAbs, GenScript has developed MonoRab™, a proprietary rabbit antibody generation platform that can generate antibodies with high specificity, affinity, and sensitivity against a diverse plethora of antigens. This white paper describes the process of generating reliable rabbit mAbs with the MonoRab™ platform, and highlights the unique features and diverse applications of rabbit mAbs in biomedical sciences.

Advantages of Rabbit Monoclonal Antibodies

Monoclonal antibodies are expressed by a single B lymphocyte that has raised an immune response against one specific antigen. As a result, mAbs offer distinct advantages over polyclonal antibodies, which are produced by multiple B-cells and can recognize multiple epitopes on an antigen. Among the top advantages of mAbs are their high specificity and affinity for a single epitope, homogeneity, and reproducibility. These features render mAbs suitable as excellent reagents for sensitive, quantification-based assays, and pharmaceutical or clinical applications. Over the last 100 years, a variety of methods have been developed to generate mAbs in mice and rabbits. However, distinct natural features of rabbits render this animal the preferred species for generating mAbs.

The following is a list of these characteristics:

Unique B-Cell Ontogeny: The rabbit’s immune system is able to generate stronger mAbs than those generated in mice or humans due to the rabbit’s enhanced means of B-cell maturation. Rather than relying on common mechanisms of gene conversion and somatic hyper mutation, immature rabbit B-cells also go through a complex gene conversion-like mechanism for sequence development. This enhanced maturation process allows for the development of longer and more heterogeneous complementarity-determining regions (CDRs) than those commonly produced in mice or humans. Therefore, rabbits are able to generate a much more diverse and distinctive set of mAbs against targets non-immunogenic in other model species.

Strong Immune Response: The rabbit’s immune system, unlike mouse’s, is capable of eliciting a strong immune response against small, non-amino acid antigens, which are either non-immunogenic in mice or mount a weaker response in mice than in rabbits. As a result, rabbit mAbs can easily recognize phospho-peptides, carbohydrates, haptens, and small molecules. Since a variety of advanced experimental or diagnostic assays as well as antibody-based therapeutics are based on the detection or blockage of these molecules, access to rabbit mAbs is key in the success of such applications.
High Genetic Heterogeneity: Compared to the inbred strains of mice, inbred strains of rabbit are less ubiquitous. Presence of genetic heterogeneity in outbred strains lends itself to a more diverse immune response in rabbits. Therefore, mAbs raised in rabbits offer larger diversity and sensitivity, allowing them to be used for a wider range of applications.

Different Evolutionary Distance: The evolutionary distance between human and rabbit is farther than the one between human and mouse. As a result, rabbits can recognize a larger number of targetable epitopes compared with mice and, therefore, can generate antibodies capable of cross-reacting with mouse orthologs of human antigens. This in turn avoids the issue of self-antigen recognition occurring in the application of an antibody.

Unique IgG Structure: Compared to human and mouse, rabbit immunoglobulins have a simpler and more stable structure. Human and mouse IgGs have five main classes (IgG, IgM, IgE, IgA, and IgD) and several subclasses, whereas rabbits lack IgD and have no subclasses of IgG. In addition, rabbit IgGs contain fewer amino acids at their N terminal and D-E loop as well as extra disulfide bonds in the variable region of their heavy chain. These characteristics are suggested to render rabbit Abs more stable and, therefore, easier to work with.

Large Body Size: Recovery of B-cells from lymphoid tissues, such as the thymus and the spleen, and from the blood, is a key part of the process of generating a mAb. Therefore, the larger body size of a rabbit compared to a mouse facilitates the collection of immune cells and blood in larger quantities, improving the process of antibody production. This advantage is specifically useful in antibody drug development, since access to a greater number of bioactive mAbs increases the success rate and turnaround time of lead identification.

All of the above features of rabbits contribute to the generation of mAbs with higher specificity, affinity, and diversity than mAbs generated in mice. Therefore, rabbit mAbs are considered the best reagent of choice for numerous applications, including biochemistry, molecular biology, and medicine.

Generation of Rabbit Monoclonal Antibodies Using the MonoRab™ Platform

The MonoRab™ platform generates high quality mAbs through Genscript’s proprietary rabbit-mouse hetero-hybridoma technology. A hybridoma is generated from the fusion of a mature B-cell with an immortal myeloma cell line. This fusion ensures the immortality of the mature B-cell and in turn, the indefinite generation of identical mAbs. Because hybridomas (homo or hetero) are generated using one specific B-cell clone, impure immunogens can be used to generate highly specific antibodies. Moreover, all generated mAbs will have maintained their natural heavy and light variable chain (VH and VL) pairing. This hybridoma technology is historically known to have the strongest success record for generating well-trusted mAbs, which is why Genscript has turned to the hetero-hybridoma technology in order to generate the highest quality mAbs for biomedical scientists.

The process of generating a MonoRab™ custom rabbit mAb (Fig. 1) begins by designing and generating an antigen tailored to a projects intended downstream application, antigens range from DNA, peptides, proteins, and whole cells. Once the antigen is generated, four New Zealand white rabbits are immunized with the antigen using an optimized strategy developed by GenScript’s antibody experts. The immunized rabbits will go through multiple rounds of additional immunogen boosts, test bleeds, and ELISA screenings until one rabbit is identified as having a high titer of ELISA and application specific pAb binders. After immunization and screening, individual B-cells are isolated from the rabbit and electro-fused to mouse myeloma cells to generate hybridoma cells. These parental clones go through additional rounds of screening until 10 clones are identified which meet GenScript’s stringent ELISA screening thresholds. These clones will be used immediately for hybridoma antibody sequencing in order to generate 10 different variable chain sequences which all bind to the original antigen. These sequences will then be used for small recombinant expression, using our high efficiency recombinant antibody (rAb) methodology. The resulting rAbs will be sent directly to the customer for an additional round of in-house application screening. Once application specific binders are identified, GenScript will deliver up to 10 customer selected sequences, and perform large scale rAb expression on selected clones.
Alternative Methods for Generating Rabbit Monoclonal Antibodies

In addition to hybridoma technology, current methods for the generation of rabbit mAbs include phage display and single B-cell technologies. The following summarizes the key features of each strategy:

**Phage Display Technology**: This method uses a library of pre-designed antibody fragments (usually ScFv) with unknown binding partners to identify which antibody sequences bind to a specific antigen. The principle behind this technology is that a specific ScFv gene is sub-cloned into a plasmid encoding the bacteriophage coat protein. This plasmid is transfected into a bacterial culture and allowed to translate into a fully functional bacteriophage. However, since the phage DNA now contains a specific ScFv DNA, the bacteriophage coat contains multiple identical ScFv regions on its surface. This process is repeated multiple times to generate a phage display library, which is able to display thousands of different ScFv regions simultaneously. Once the library is exposed to an antigen, the ScFv with the highest specificity, affinity, and sensitivity is selected, sequenced and recombinantly expressed as a full mAb. Advantages of this methodology include high speed, easy control of various selection parameters, in vitro high affinity maturation, and the capability to generate synthetic antibody libraries. However, the dependency of this technology on prokaryotic transcription and translation machinery can interfere with proper display of eukaryotic IgG chains. Another limitation of this technology is the loss of natural cognate pairing information of antibodies. The manual and random construction of libraries cannot replace the natural maturation and selection of antibodies that occur during an in vivo immune response. As a result, specific diversity of antibodies are lost and antibodies generated by this technology require an additional step of in vitro maturation to impart increased affinity.

**Single B-Cell Technology**: As a recently-developed technology, this approach is based on direct sampling of single B-cells from rabbit blood followed by cloning, sequencing, and recombinant expression of the antibody variable region from antigen specific B-cells. Since this technology takes advantage of the natural process of affinity, specificity, and stability profiles of mAbs, it preserves the natural heavy and light chain pairing and, therefore, enables a more thorough interrogation of the B-cell population. On the other hand, the current status of this methodology has limitations in lack of access to useful B-cell surface markers in rabbits, sub-optimal PCR and cloning steps, a low throughput output due to reliance on manual micromanipulation, and low IgG amount in the supernatant of single B-cells; all restricting the number of assays necessary for the antibody development and validation processes.
Given that advantages of the hybridoma technology in generating high quality rabbit mAbs exceeds the advantages of alternative methodologies, GenScript developed the MonoRab™ hybridoma technology in order to generate reliable rabbit mAbs which can be used in a wide range of applications.

**Key Features of MonoRab™ Platform**

Capable of generating an immune response against a variety of difficult antigens, such as

- small molecules,
- nanobodies,
- partial or full-length recombinant proteins,
- highly similar epitopes,
- antigens that are not immunogenic in mice,
- post-translationally modified antigens.

Capable of generating mAbs with superior features, such as

- high-affinity: antibodies generated by the MonoRab™ platform have $K_D$ values at picomolar range, compared to mouse-generated antibodies with nano or micromolar $K_D$ values. This allows for the preservation of antibody-antigen interaction for a robust detection and function;
- high-sensitivity: MonoRab™-generated antibodies enable the detection of less abundant antigens with a minimum amount of antibody;
- high-specificity: antibodies generated by the MonoRab™ platform reduce background signals and allow for the precise targeting of desired antigens;
- high working antibody dilutions: this feature makes it feasible to use a low titer of an antibody in each assay, making the MonoRab™ antibody a cost-effective reagent.

**Applications of Rabbit Monoclonal Antibodies**

The following highlights major applications of rabbit mAbs:

**A. Discovery Science:** Classical applications of mAbs in research laboratories include immunoblotting, immunohistochemistry, pull-down assays, and *in vivo* studies. These applications for long were reliant on non-rabbit mAbs, which although helpful, are associated with limitations impeding scientific discovery. Non-rabbit mAbs often fail to detect difficult antigens such as small molecules, antigens with highly similar epitopes, post-transcriptionally-modified antigens, and antigens with no immunogenicity in mice. They also may necessitate optimization of parts of an experimental procedure that they are employed in, with no guarantee of giving optimal results. For example, in the most widely-used immunohistochemistry (IHC) procedure, use of mouse mAbs may require a stringent antigen retrieval procedure or use of a higher titer antibody. Although these steps are taken to help obtain “a” or “any” signal, they can distort tissue architecture and increase the noise to signal ratio, ultimately affecting the interpretation of the results. In addition, non-rabbit mAbs may not be able to have high enough affinity to ensure preservation of the antigen-antibody interaction throughout the long duration of a pull-down assay. Rabbit mAbs have a high level of diversity, affinity, and specificity; overcoming the limitations of non-rabbit mAbs and increasing the range of research capabilities.

**B. Diagnostics:** Rabbit mAbs enable the development of a wide range of sensitive and precise diagnostic assays and reagents for *in vivo* and *in vitro* applications. The following describes two major areas of such applications:

**B.1. In vitro Diagnostics (IVD):** IVD can be defined as the analysis of patient samples in order to detect
cell/tissue biomarkers for identifying a disease or predicting change in disease status, help determine the optimum dose of a drug for patient treatment, and assess the effectiveness of a therapeutic intervention over short and long time periods. A variety of IVD assays, such as IHC, ELISA, and radio immunoassays (RI), are based on the principles of antigen-antibody binding. Given the critical role of these assays in patient or animal care, it is extremely important that IVD assays are carried out using the highest sensitivity and affinity mAbs available.

**B.2. In Vivo Imaging:** mAbs used for in vivo imaging are able to localize diseased tissues, assess disease progression, and monitor therapeutic progress in patients. Visualization of mAbs can be accomplished in numerous means; however, the most common method is through labelling of mAbs with radioactive or fluorescent molecules to help track them in the body and aid in quantifying the results. The superior features of rabbit mAbs, such as high diversity, sensitivity, specificity, and affinity, are therefore very appealing for imaging applications.

**C. Anti-Idiotype (anti-ID) Antibodies:** Antibodies that can detect and bind to an idiotope of another antibody with high specificity are called anti-ID antibodies. Based on their properties and binding mode, these antibodies can be: (i) antigen-blocking (neutralizing) in which the anti-ID antibody directly competes with the target antigen of an antibody drug; therefore, making this type of antibody useful in identifying free drug in a sample, (ii) drug-target complex-specific in which the anti-ID antibody can bind to an idiotope outside the antigen binding site of the antibody drug as well as an epitope on the antigen; therefore, making it suitable for detecting bound drug, and (iii) non-blocking in which the anti-ID antibody binds only to an idiotope outside the antigen binding site of the antibody drug; therefore, making it useful for detecting bound, partially bound, and free drug in a sample. The scope and range of anti-ID antibody applications in medicine are numerous and include monitoring disease progression, drug and vaccine development, and cancer therapy. Unique characteristics of rabbit mAbs make them excellent candidates as anti-ID antibodies in the following applications:

**C.1. Pharmacokinetic (PK) Studies:** In drug development, PK assays are used to measure the absorption and excretion rates, distribution, and half-life of candidate small molecules or mAb therapeutics. Results from these assays help identify the optimal dose and toxicity risks of a therapeutic molecule. Robust PK assays are, therefore, key in the selection and development of therapeutics and their future success in clinical applications. In the case of mAb therapeutics, anti-ID antibodies need to be sensitive enough to capture trace amounts of their antibody target in free, bound, and total forms in biological fluid samples. High sensitivity and specificity rabbit mAbs are, hence, useful reagents in PK studies for obtaining accurate quantitative measurements of a drug’s concentration with no worries about false positive or negative results.

**C.2. Immunogenicity Assays:** Successful use of biotherapeutic products, such as therapeutic mAbs, requires the development of sensitive assays for detecting anti-drug antibodies (ADA) raised by a patient’s own immune system against a therapeutic agent. Similar to polyclonal antibodies, rabbit mAbs are employed as positive controls in highly sensitive ADA assays to avoid or minimize false results. This in turn leads to increased efficacy and decreased risk of adverse reactions of therapeutic mAbs.

**C.3. Vaccine Development:** Passive immunotherapy by means of immune-based reagents, such as mAbs, is the basis of therapeutic vaccines as a treatment option for cancer or Alzheimer’s disease. Similar to the dependency of small molecule drug development on rabbit anti-ID mAbs, successful development of therapeutic mAb vaccines relies on these highly specific and sensitive antibodies. The concept of anti-ID mAb vaccines is based on their ability to break the immune tolerance associated with tumor/tissue associated antigens (TAA), which the immune system cannot recognize as foreign. Rabbit anti-ID mAb vaccines accomplish this by acting as...
antigen surrogates to induce efficient humoral and/or cellular immune responses.

**E. Development of Therapeutics:** Advancements in the development of high quality rabbit mAbs against a wide range of disease-associated antigens, which include non-protein antigens, is fast advancing the frontiers of antibody-based therapeutics. Due to the nature of these therapies, i.e. modulating the immune system that involves an auto-immune response, this category of therapeutics require a very high level of safety and the least level of toxicity\(^7,\)\(^8\). Specificity and sensitivity of an antibody towards its target antigen ensure both safety and toxicity concerns: highly specific antibodies avoid off-target binding and hence, offer safety; highly sensitive antibodies require low dosage administration and hence, cause a minimum level of toxicity. Given that rabbit mAbs have both of these sought-after characteristics, they are considered as excellent therapeutic candidates. Therapeutic rabbit mAbs can directly target disease-specific cell surface antigens or can be used as cytotoxic drug delivery agents\(^9\).

**F. Development of Chimeric Antigen T-cell (CAR-T) Therapy:** As one of the most effective forms of personalized therapy, CAR-T cell therapy is dependent on reliable rabbit mAbs. In CAR-T therapy, T-cells are removed from patient’s body and subjected to genetic engineering in order to express chimeric tumor-specific antigen receptors (CARs) on their surface. Then these engineered cells are transferred back into the patient’s body to serve as “living drugs” to help fight cancer\(^20\). Platforms for generating rabbit mAbs, such as MonoRab™, help with both the development and monitoring of CAR-T cell therapy: (i) they help construct the scFv chain of the chimeric antigen receptor, and (ii) they generate sensitive and specific anti-ID mAbs that help with monitoring the efficacy of the therapy.

**Case Studies**

The following list showcases the successful application of high specificity, affinity, and sensitivity rabbit mAbs generated by the MonoRab™ platform in several key experimental settings.

**1. MonoRab™ Antibody Detecting Small Molecule Tags**

Small molecule tags are widely used in a variety of laboratory procedures for protein purification and detection. Application of reliable antibodies that can specifically detect such tags are, therefore, key in the successful execution of associated assays and experiments. To investigate whether the MonoRab™ platform could generate a rabbit mAb with characteristics equal or superior to its mouse mAb counterpart, mouse and rabbit mAbs were raised against two widely-used tags. Specifically, mouse and rabbit mAbs’s were raised against the mid-region, C and N terminals of a Flag tagged protein (X) (Fig. 2A-C) as well as a polyhistidine (His) fusion protein tag (Fig. 2C,D). Using immunoblotting, the quality of rabbit mAbs was compared to mAbs raised in mice. The resulting analysis showed that MonoRab™ antibodies had higher affinity and specificity in detecting and binding to the tagged proteins compared to their mouse mAb counterparts.

![Fig 2. Detection of small molecule tags by MonoRab™ rabbit mAbs.](image-url)

Western Blot results of rabbit and mouse mAbs targeting (A-C) different regions of Flag tag, and (B) a His tag fusion protein showed superior affinity and specificity of the MonoRab™ rabbit mAb with a \(K_D\) value of \(10^{-11}\) M for each.
2. Anti-Idiotype MonoRab™ Antibody Binding to An Antibody Drug

Successful development, characterization, and monitoring of therapeutic antibodies is dependent on high affinity and specificity reagent mAbs that can recognize an antibody drug and/or antibody-target complex. To demonstrate the capability of the MonoRab™ platform, GenScript developed a neutralizing (antigen blocking) anti-ID mAb against the humanized mAb drug Pembrolizumab (Keytruda®, Pem). To compare the affinity and specificity of the rabbit anti-Pem MonoRab™ mAb to similar anti-Pem antibodies raised in mouse, a series of ELISA assays were performed. Comparison of the EC50 values of all tested mAbs showed that the neutralizing rabbit anti-Pam MonoRab™ mAb was capable of binding to its drug target with an affinity greater than or equal to almost every mouse mAb analyzed (Fig. 3A). Moreover, the rabbit mAb was found to be stronger in blocking the antibody drug, Pem (Fig. 3B). Results from a binding affinity test with one of the anti-Pem mouse mAbs and the rabbit MonoRab™ mAb also displayed very similar K_D values between the two antibodies (Fig. 3C-D), further confirming the high affinity of the rabbit mAb to its target. We then analyzed the capability of rabbit anti-Pem MonoRab™ mAb in binding to Pem. Results revealed that the anti-Pem MonoRab™ mAb bound to Pem with a K_D value of 2.32E-12 (Fig 3E), significantly higher than K_D values normally generated from mouse anti-IDs. The inhibitory anti-ID was also subjected to a direct binding ELISA as well as a competitive ELISA assay against Pem as its drug target. The resulting analysis of these experiments showed that the anti-ID antibody bound to Pem with an EC50 of 5.001 ng/ml (Fig. 3F) and showed an inhibitory effect (IC50) on its drug target at 18.8 ng/ml (Fig. 3G). These results indicated that the neutralizing anti-ID mAb that GenScript generated was able to bind to the antibody drug Pem with high affinity and specificity.

![Figure 3. Characterization of anti-Pembrolizumab MonoRab™ mAb.](image)

Results from (A) direct binding ELISA assay, (B) competitive ELISA assay, and (C-D) binding affinity assays showed an equal affinity and higher blocking functionality of the rabbit mAb compared to mouse mAbs raised against the antibody drug target, Pem. Results from (E) affinity measurement, (F) direct binding ELISA, and (G) competitive ELISA assays revealed the high affinity and specificity of anti-Pem MonoRab™ mAb.

3. MonoRab™ Antibody Detecting A Small Molecule Drug

Small molecule drugs comprise a large category of therapeutic and industrial agents for clinical and veterinary applications. Monoclonal antibodies are increasingly being used for the in vivo monitoring and in vitro pharmacological assays of small molecule drugs. Due to the nature of these applications, employed mAb reagents need to (i) bind to their small molecule target with high specificity, (ii) high affinity, and (iii) at very low titer to avoid toxicity in vivo and non-specific binding in vitro. Therefore, the best mAbs for detecting small molecule drugs should have low EC50 and IC50 values. To demonstrate the capability of the MonoRab™ platform in generating mAbs as reliable reagents for binding to small molecule drugs, an antibody against the small molecule drug Ractopamine...
(Rac) was developed. Results from a direct binding ELISA assay showed a very low EC50, indicating the high affinity and specificity of this antibody binding to its drug target (Fig. 4A). Similarly, a low IC50 value obtained from a competitive ELISA assay showed the strong inhibitory binding of the generated antibody to Rac (Fig. 4B). Next, to test whether the anti-Rac rabbit MonoRab™ mAb was specific enough to only detect its target, a competitive ELISA assays with two drugs of very similar structure to Rac were performed. Comparison of the results from similar drugs, Salbutamol (Sal) and Clenbuterol (Cle), proved the lack of cross-reactivity of anti-Rac MonoRab™ mAb as well as its high potency in competitively binding to Rac (Fig. 4C-E). Overall, these results clearly demonstrate that the MonoRab™ platform is robust in generating anti-small molecule rabbit mAbs in applications where highly potent, specific, and sensitive reagents are required.

![Graphs showing binding affinities and IC50 values](image)

Figure 4. Anti-Ractopamine MonoRab™ mAb characterization. The anti-Rac MonoRab™ mAb showed (A) EC50=1.156 ng/ml and (B) IC=0.05 ng/ml values in direct binding and competitive ELISA assays, respectively. The anti-Rac rabbit MonoRab™ mAb showed (A) very high affinity (IC50=0.05) against Rac, but very low affinity against (B) Salbutamol (Sal; IC50=173.7; cross reactivity rate=0.0304%), and (C) Clenbuterol (Cle; IC50=160.7; cross reactivity rate=0.0329%).

Summary

The comprehensive set of data presented in this paper demonstrate that the MonoRab™ rabbit mAb generation platform is a versatile and robust technology for the development of custom mAbs, capable of targeting a plethora of antigens. Antibodies produced by MonoRab™ provide unique and superior features, such as high specificity, sensitivity, and affinity, which are otherwise not attainable by mouse mAbs. Characteristics of MonoRab™ rabbit mAbs are key in obtaining reliable, effective, and reproducible results in a wide range of applications for both therapeutic and research purposes.

Awards

Recipient of the "2018 CiteAb Most Popular Custom Antibody Supplier Award" by CiteAb, which celebrates the very best suppliers and individuals in the research reagent sector worldwide.

Resources

https://www.genscript.com
https://www.genscript.com/anti-idiotype-antibody.html
https://www.genscript.com/antibody-blog-what-is-car-t.html
References


