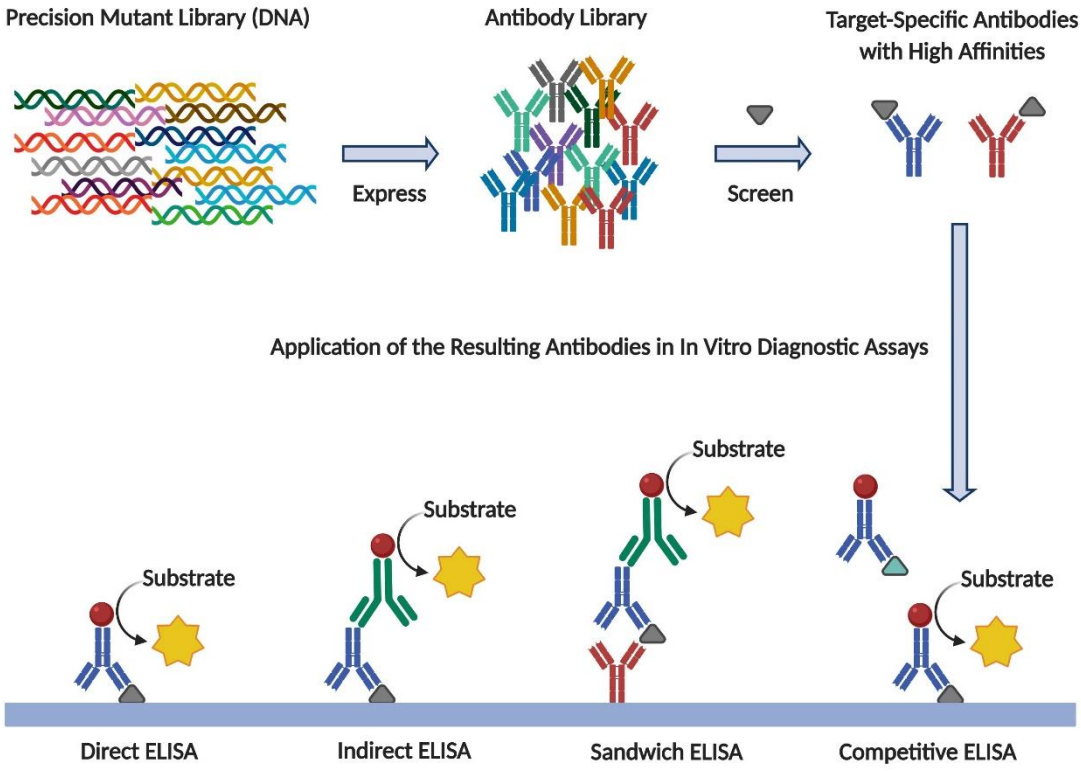
Reducing Screening Burden in Antibody Engineering through GenScript's Synthetic Precision Libraries

Department of Reagent Services and ProBio, GenScript, 860 Centennial Ave, Piscataway, NJ 08854

GenScript has recently developed an advanced, arrayed **semiconductor-based DNA synthesis platform** to provide high-quality **precision mutant libraries** for antibody engineering applications. The platform allows mutant library construction with **precise control over codon usage**, enabling the generation of diverse mutant libraries with **well-distributed codons and minimized biases**, unlike the traditional NNN or NNK degenerate codon libraries. **Custom codon optimization** capabilities are also built into the platform, which allows researchers to not only define expression optimization for a particular host organism but also **eliminate unwanted stop codons** or specific codons in their mutant libraries. This **smart and user-defined DNA synthesis** platform resembles a semi-rational design by minimizing or eliminating the unwanted mutants during the construction of individual libraries without requiring much pre-existing information or knowledge about the antibody of interest to be engineered, thereby **saving valuable time and effort during the screening and characterization process, speeding up the engineering workflow, and reducing the overall cost of downstream expenses**. Here, we showcase a case study for application of our precision mutant libraries in affinity maturation of a monoclonal antibody with an initial affinity of 0.539 nM to **a mutant with a significantly improved affinity in the femtomolar range** (33.1 fM), screened from a saturation mutagenesis library consisting of only ~1200 mutants arising from targeting 63 sites within the 6 CDR regions of the antibody sequence, highlighting the capability of these libraries to generate such a high-affinity binder.



1. Engineering Antibodies

Antibodies are large, Y-shaped glycoproteins mostly produced by B cells to neutralize pathogens through their specific recognition and binding interaction with certain parts of the pathogens to protect the host organism from pathogenic growth by its adaptive immune system. The high affinities and specificities displayed by antibodies in their target binding and recognition make them very attractive for their applications as therapeutics for various diseases and reagents for in vitro diagnostics[1]. Consequently, antibody engineering has gained a major interest in recent years in efforts to develop new therapeutic and reagent products. Accordingly, substantial technological advances have been made in numerous areas of antibody engineering encompassing discovery and library design, selection and screening, characterization, analysis, host expression, and large-scale production.

GenScript offers high-quality mutant libraries to academic researchers and industrial scientists regardless of their strategies used to engineer antibodies of interest (See Sections 3 and 4).

2. Design Strategies

Engineering proteins, including antibodies and enzymes, to improve specific properties, such as affinity, specificity, and functional activity is founded on two fundamental strategies: (1) rational design and (2) directed evolution[2]. The rational design strategy requires preconceived knowledge of protein structure and function attained experimentally or predicted computationally, and targeted mutations are introduced at specific sites based on such knowledge, thus often resulting in smaller libraries of mutants[2, 3]. On the other hand, the directed evolution strategy generally involves large libraries of mutants to diversify the mutant coverage with mutations randomly introduced within different regions due to little knowledge available around protein structure and function[2, 4]. However, if partial knowledge or information about the protein of interest is available, a semi-rational design strategy can be employed that combines the benefits of rational design and directed evolution strategies; this strategy often renders a mid-size mutant library and thus can help mitigate the screening challenges associated with large libraries, as the demand for high-throughput screening increases as the library size increases (**Figure 1**). Libraries are then screened for mutants with improved properties via an appropriate platform and can be further improved through multiple rounds of mutation and screening.

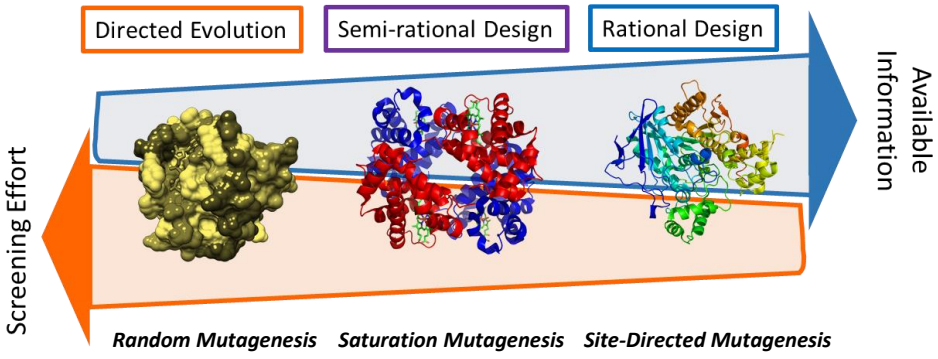
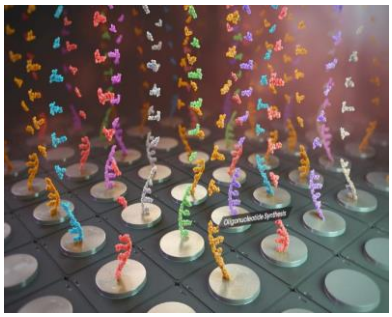


Figure 1. The correlation between the engineering strategies, the information/knowledge available, and the corresponding screening efforts needed. The less information/knowledge about an antibody of interest is available, the harder it can be to engineer rationally and thus the larger the library size becomes; the larger the library size, the greater the screening effort.

3. GenScript's Advanced Semiconductor-based DNA Synthesis Platform

Regardless of the strategy used to create a mutant library or the size of the library, the success of the engineering effort on a given antibody relies on two critical factors: (1) the availability of a **high-quality mutant library** devoid of unwanted mutants and (2) the availability of a **robust screening method** to effectively and accurately access the mutant library (note that high-throughput capabilities are required for large mutant libraries to efficiently screen and identify rare top candidates).

GenScript has recently developed an advanced, arrayed semiconductor-based DNA synthesis platform to address the first critical factor for a successful outcome – the availability of a high-quality mutant library. Traditional methods for library construction, such as using error prone polymerase chain reaction (PCR) or degenerate codons (such as NNN or NNK), introduce mutations across the sequence space; however, there is limited control over the codons introduced in these methods, rendering major disadvantages in the resulting mutant libraries: (1) uneven amino acid distributions due to significant codon biases, (2) compromised expression of individual mutants in a target host due to the inability to incorporate codon optimization, and (3) truncated products due to the inability to eliminate stop codons. All of these disadvantages result in a large library with poor mutant representation with missing candidates due to unoptimized expression, thereby exerting a large screening effort on the researchers to identify top candidates and posing them a huge opportunity cost by missing an ideal candidate.



The GenScript semiconductor-based DNA synthesis platform allows the mutant library construction with **precise control over codon usage**, thus enabling the generation of high-quality mutant libraries that are diverse **yet well-distributed among amino acids** with little bias. Custom **codon optimization capabilities** are also built into the platform, which allows researchers to not only define expression optimization for a particular host organism but also **incorporate specific codons** and **eliminate stop or unwanted codons** in their mutant libraries.

In addition, unlike other chip-based technologies such as inkjet printing, the DNA synthesis in our semiconductor-based technology is electronically controlled by the chip electrodes in a closed chamber with regulated temperature and humidity to ensure minimal variations and exposure to outside elements, thereby providing high-quality DNA with superior sequence fidelity.

Our smart and user-defined DNA synthesis platform resembles a semi-rational design by minimizing or eliminating the unwanted mutants during the construction of individual libraries without requiring much pre-existing information or knowledge about the antibody of interest to be engineered, thereby saving valuable time and effort during the screening and characterization process, speeding up the engineering workflow, and reducing the overall cost of downstream expenses.

4. Precision Mutant Libraries

Figure 2 illustrates the three types of precision mutant libraries (PMLs) that are offered by GenScript to fit various customer needs and interests: **(1) site-saturation mutagenesis, (2) saturation-scanning mutagenesis, and (3) combinatorial mutagenesis.** A site-saturation mutagenesis library or a saturation-scanning mutagenesis library involves mutating an amino acid at a specific position or consecutive amino acids within a region or different regions of an antibody sequence, with one mutation at a time to all other 19 amino acids, respectively, whereas a combinatorial mutagenesis library involves mutating multiple positions simultaneously within an antibody sequence, with each mutant containing multiple mutations.

In all three types of libraries, oligonucleotides harboring specified mutations are **synthesized through our arrayed semiconductor-based DNA synthesis platform in a well-controlled and precise manner** and used in the construction of mutant variants. In the context of engineering applications, saturation mutagenesis libraries are suitable for targeted engineering efforts with smaller library sizes, whereas combinatorial mutagenesis is favored for engineering efforts with limited rational design inputs and thus requiring large libraries for mutant diversification.

All three types of libraries are synthesized using our advanced semiconductor-based DNA synthesis platform to ensure high quality and precise control.

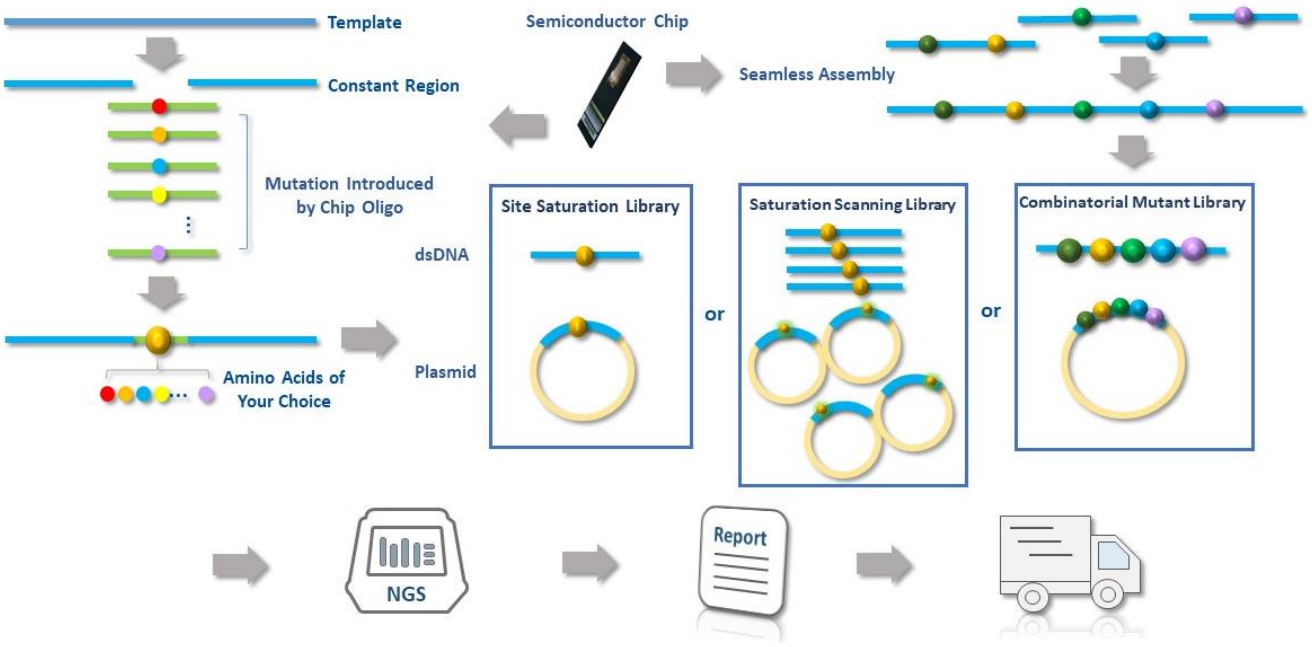


Figure 2. Workflow for constructing mutant libraries using arrayed, semiconductor-based oligonucleotides. Oligonucleotides are synthesized using our advanced semiconductor-based DNA synthesis technology to create individual mutants that are either PCR amplified or cloned into a vector of choice to produce various types of mutant libraries. The libraries are then sequenced using NGS, and the corresponding reports are provided when delivered to the customers. The libraries can be delivered as double-stranded DNA fragments or cloned plasmids, which can be pooled, sub-pooled, or individually arrayed to meet different customer needs.

Once a desired precision mutant library is constructed, it can be subject to any appropriate type of screening method, such as phage display, mRNA display, or cell-surface display, for selection of mutants exhibiting a specific property of interest[4] (**Figure 3**).

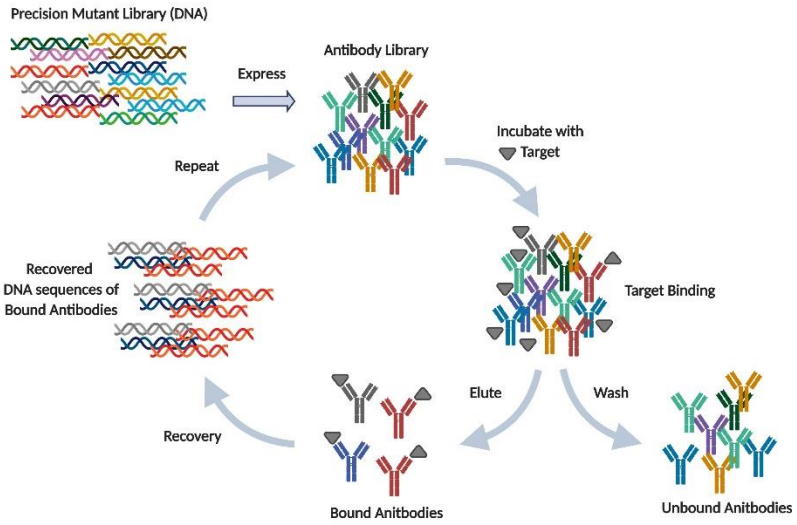


Figure 3. A schematic illustration of a precision mutant library serves as an initial pool of DNA mutants in an antibody screening process via an appropriate method. The initial antibody mutant library is expressed from the corresponding precision DNA mutant library for screening antibody mutants exhibiting a specific property, for instance, binding affinity towards a target antigen. Bound antibodies are then eluted and recovered for their respective DNA sequences, which can subsequently serve as an initial pool for the next round of screening. The screening cycle can be repeated as many times as needed.

5. Improving Antibody Affinity Using Precision Mutant Libraries

5.1. Saturation Mutagenesis Library

In this application note, we provide a case study to demonstrate an example of the application of a saturation mutagenesis library for antibody engineering to improve target-binding affinity. In this study, we created a saturation mutagenesis library consisting of mutants in 63 sites across 6 CDR regions in the light chain and the heavy chain of an antibody (**Figure 4**); each targeted site was mutated to all other 19 non-wild type amino acids, following the library construction workflow shown in **Figure 2**.

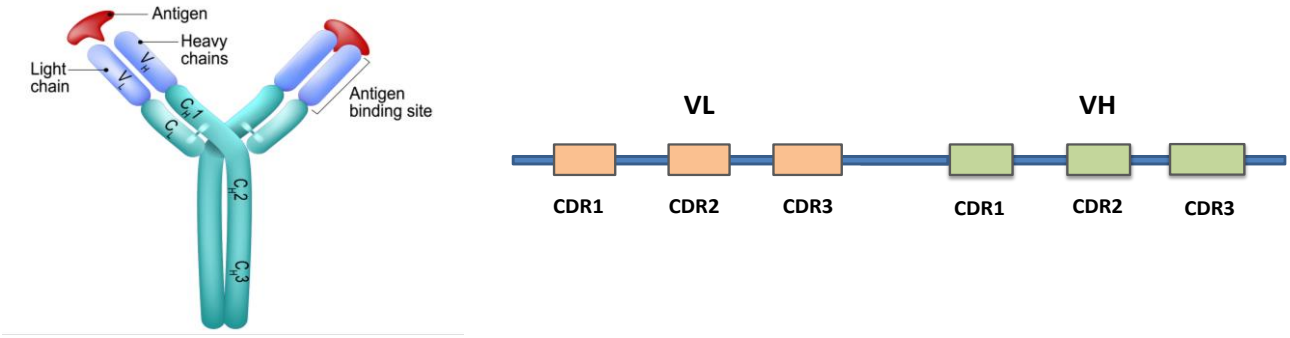


Figure 4. A schematic illustration of the light chain (VL) and the heavy chain (VH) of an antibody of interest to be engineered for affinity improvement using a saturation mutagenesis library targeting mutations in CDR regions. A total of 63 sites across 6 CDR regions within the heavy and light chains were mutated, replacing each wild type amino acid with all other 19 amino acids, via saturation mutagenesis.

5.2. Equal Representation of Amino Acids at Each Site with 100% Coverage

The quality of the saturation mutagenesis library was analyzed using next generation sequencing (NGS) to determine the distribution and the coverage of amino acids at each mutated site. The sequencing results showed a relatively even distribution of amino acids with mutants representing all 19 non-wild type amino acids at each of the 63 corresponding sites, rendering a mutant library of 100% coverage (**Figure 5**). This distribution and coverage profile demonstrated that the utilization of the arrayed, semiconductor-based DNA synthesis platform in the construction of mutant libraries provides precise control over the codons used in each mutant, thereby avoiding poor representation of mutants and providing high-quality libraries with tremendous benefits in the subsequent screening and characterization process.

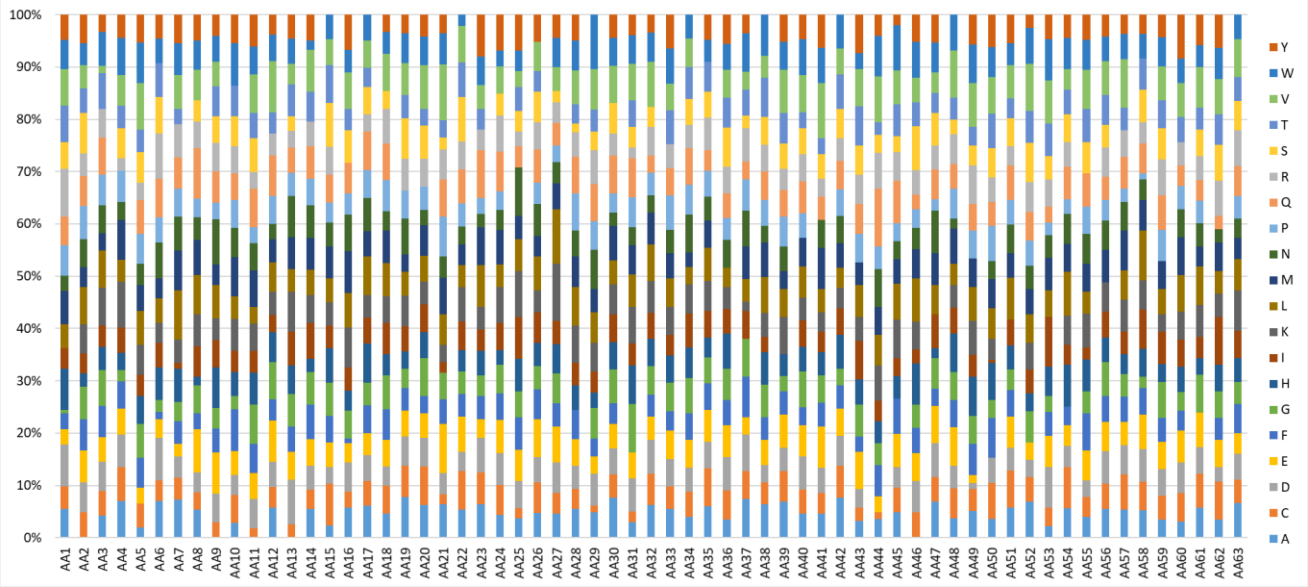


Figure 5. The amino acid distribution of the saturation mutagenesis library determined by the next generation sequencing (NGS) technology. Each multi-colored bar is an individual site, and each color represents an amino acid (AA). The thickness of each colored band is the % of each mutant with that particular amino acid at that site.

5.3. Screening of the Precision Mutant Library for Candidates with Improved Affinities

A total of ~3,000 clones arising from the precision mutant library containing the 63 mutated sites were screened for candidates with improved affinities against the target antigen via a screening method called FASEBA (Fast Screening of Expression, Biophysical-properties, and Affinity). It is a licensed patented technology for high-throughput screening of the best antigen binders, based on expression level, thermostability, and binding affinity without actually purifying the candidate antibodies. Using this screening method, several mutants with significantly improved affinities were identified, as depicted in the blue circles in **Figure 6**.

5.4. Characterization of Top Candidates via Surface Plasmon Resonance Assays

A number of mutant clones with significantly improved affinities from the FASEBA screening were characterized for their binding affinities via surface plasmon resonance (SPR) binding assays using Biacore. The SPR assay results revealed 5 mutants with affinities higher than that of the wild type (**Table 1**). One of these mutants exhibited an affinity in the femtomolar range (33.1 fM), 4 orders of magnitude better than the wild type in target binding, highlighting the capability of our precision mutant library to generate such a high-affinity mutant sequence.

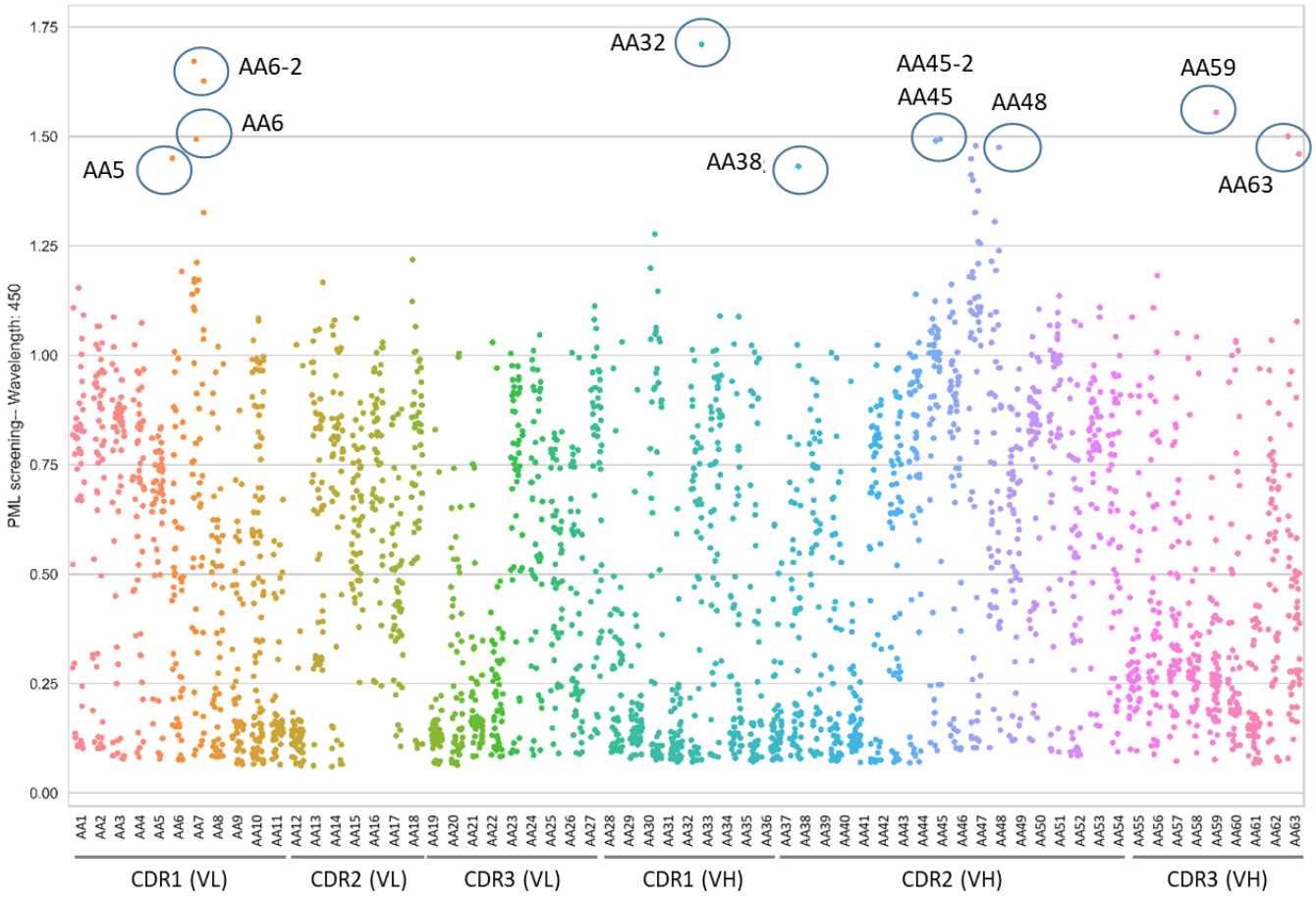


Figure 6. Mutant clones with affinities relative to that of the wild type antibody. The library was screened with FASEBA to identify candidates with improved affinities. A value above 1 represents a higher affinity than the wild type. The x axis shows each targeted mutated site and each dot is a mutant at that site. The blue circles are examples of clones with significantly improved affinities.

Table 1. Binding affinities of 5 improved clones with beneficial mutations.

Mutation/Site	KD (M)	Rmax
AA45	3.31E-14	112.5
AA5	1.44E-12	76.5
AA6	9.71E-12	59.5
AA63	2.67E-10	167.6
AA38	2.63E-10	50.3
WT	5.39E-10	107.2

Our precision mutant libraries are high-quality sources of diverse sequences, from which antibody mutants with exceptional target-binding affinities can be screened and achieved.

5.5. Further Improvement through a Combinatorial Library of Beneficial Mutations

In order to assess the combination effects of the 5 beneficial mutations and potentially further improve binding affinity, a combinatorial library enabling simultaneous mutations within each single mutant was constructed using the same semiconductor-based DNA synthesis technology (**Figure 7**). A total of 96 clones from the library transformation were subsequently screened via FASEBA. Characterization of a number of top clones from the FASEBA screening via SPR binding assays revealed a few combination mutants with improved affinities compared to those of their corresponding parent mutants as shown in **Table 2**. These results highlighted the ability to utilize different types of precision mutant libraries to enable the generation of improved high affinity binders.

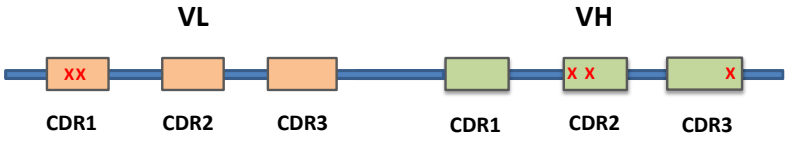


Figure 7. A schematic illustration of the 5 beneficial mutations in the specific CDR regions of the antibody of interest. Each of the 5 beneficial mutation sites is marked "x".

Table 2. Binding affinities of improved mutants harboring specific combinations of beneficial mutations.

Mutant Combination	KD (M)	Rmax
AA38/AA63/AA6	1.23E-13	13.7
AA63/AA6	1.91E-13	143.5
AA38/AA63	8.63E-11	33.7
WT	8.22E-10	45.8

6. Comparison of Precision Mutant Libraries to Traditional Libraries

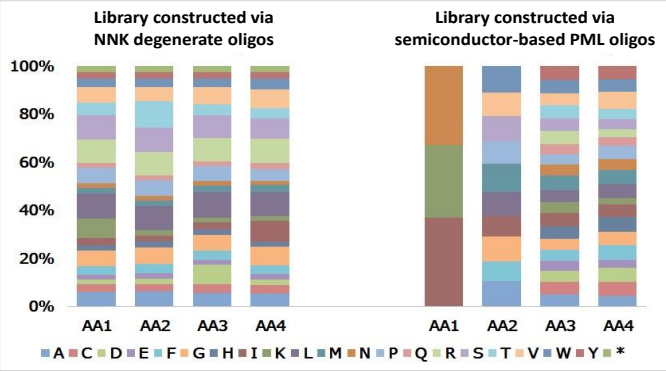
Our precision mutant libraries (PMLs), as described above, are constructed with precise control of codon usage via an arrayed, semiconductor-based DNA synthesis platform whereas such control is not feasible for libraries constructed via traditional methods using error prone PCR or degenerate codons. Consequently, our PML method can offer even distribution of amino acids with little bias and incorporation of user-defined codons, including codon optimization and elimination of unwanted or stop codons, whereas the traditional methods fail to offer any of these desired properties (**Table 3**).

Table 3. Comparison of different DNA mutant library synthesis methods.

DNA Mutant Libraries Synthesis Techniques			
	Error Prone PCR	**Degenerate (NNK)**	**GenScript (PML)**
Sequence Bias	YES	YES	NO
Number of Codons Available	Unknown	32	All 64
Prevents Undesirable Motifs	NO	NO	YES
Allows Codon Optimization	NO	NO	YES
Avoids Stop Codons	NO	NO	YES
Expedite Downstream Screening	NO	NO	YES

The advantages of GenScript's mutant libraries constructed with precise control of codon usage over traditional libraries constructed with degenerate codons are validated by experimental data provided by our collaborators.

(A)



(B)

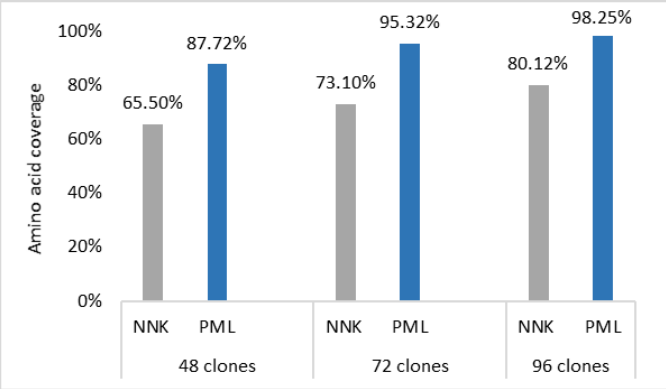


Figure 8. Comparison between a PML and an NNK library. (A) Profiles of amino acid distributions of the mutant libraries constructed via a semiconductor-based DNA synthesis platform (PML) (**right**) and via NNK degenerate codons (**left**), respectively. The PML method enabled a user-defined amino acid profile with evenly distributed amino acids at all 4 mutated sites whereas the NNK library method resulted in an uneven distribution profile of amino acids with failure to incorporate user-defined codons only. **(B)** Screening efficiencies of the two different libraries. The PML method provides higher screening efficiencies due to its higher diversity coverage with unbiased amino acid distribution.

In order to experimentally validate these advantageous properties, we conducted a comparison study, in which combinatorial mutagenesis libraries with 4 mutation sites were constructed via the two different methods, yielding the respective PML and NNK library. In this study, a user specification was also considered for amino acid incorporation at these sites: 3 specific amino acids in the 1st position, 10 specific amino acids in the 2nd position, and 20 amino acids each in the 3rd and 4th positions. The amino acid distribution profiles of the two libraries are shown in **Figure 8**. As seen in **Figure 8A**, the PML not only exhibited evenly distributed amino acids but also followed the user specification for amino acid incorporation at all 4 sites whereas the NNK library lacked both features, illustrating the much higher quality of the PML over the NNK library. In addition, as shown in **Figure 8B**, the screening efficiencies in achieving a full mutation coverage at each mutated site were also found to be substantially higher for the PML than the NNK library in all three experiments (48, 72, and 96 clones screened); this result was expected, as the PML contained only the desired mutant sequences in addition to its even distribution of amino acids whereas the NNK library contained both desired and unwanted mutant sequences due to its failure to incorporate user-defined codons only, thus resulting in a larger library size for screening and thus reducing the screening efficiency. This result, now experimentally validated, highlighted the utilization of PMLs in antibody engineering requiring less effort and saving valuable time in screening downstream.

Our precision mutant libraries are smart libraries that can be utilized to improve various properties such as specificity, solubility, and stability, in addition to affinity maturation shown here as an example. Furthermore, these libraries can be utilized to engineer various biomolecules in addition to antibodies.

7. Summary

Mutant libraries can be used to identify critical residues within an antibody or to optimize its specific function or property, for instance, binding affinity for a target antigen. Traditional methods, such as error prone PCR or degenerate codons, for constructing these libraries suffer from limited control over the codons introduced, and thus the resulting libraries can incur significant codon biases, poor expression, and early termination. Consequently, these disadvantages lead to large libraries with over- or under-represented mutants and truncated products, exerting a large screening effort on the researchers to identify top candidates and posing them a huge opportunity cost by missing an ideal candidate.

To address these issues, **GenScript has developed a method for creating diverse, well-designed mutant libraries using our advanced DNA synthesis platform**. The platform uses our proprietary semiconductor-based electrochemical DNA synthesis technology, which allows **customizable codon usage** at individual mutated sites, thereby **enabling the minimization of codon bias and uneven distribution of amino acids and preventing the introduction of unwanted or stop codons**. These capabilities together render mutant libraries **far superior than the traditional NNN or NNK degenerate codon libraries**. Mostly importantly, mutant antibody sequences with significantly higher affinities than their parent counterparts can be generated from our smart and user-defined libraries, as evidenced in the case study provided above, demonstrating **the highly attractive utility of these libraries in engineering antibodies for therapeutic and in vitro diagnostic applications**.

GenScript's precision mutant libraries can be utilized not only for antibody engineering but also for other types of engineering such as promoter engineering, enzyme engineering, protein engineering (non-enzyme or non-antibody), and viral vector engineering for various biotechnological and biopharmaceutical applications.

8. References

1. Maynard, J. and Georgiou, G. Antibody Engineering. *Annual Review of Biomedical Engineering*. 2: 339-376 (2000).
2. Chen, R. Enzyme Engineering: Rational Redesign versus Direction Evolution. *Trends in Biotechnology*. 19: 13-14 (2001).
3. Kuhlman, B. and Bradley, P. Advances in protein structure prediction and design. *Nature Reviews Molecular Cell Biology*. 20: 681-697 (2019).
4. Davis, A.M. et al. Directing evolution: the next revolution in drug discovery? *Nature Reviews Drug Discovery*, 16:681-698 (2017).
5. Hoogenboom, H. R. Selecting and screening recombinant antibody libraries. *Nature Biotechnology*. 23: 1105-1116 (2005).

9. Notes

GenScript's Precision Mutant Library Service

GenScript's precision mutant libraries, powered by our strong expertise in de novo gene synthesis and advanced semiconductor-based electrochemical DNA synthesis technology, provide precise control over codon usage and amino acid distribution, ensuring a well-designed library containing all requested mutations, preventing poor representation of mutants, eliminating unwanted or stop codons, and minimizing screening burden:

- Precise control of codon usage: desired amino acid ratios at all mutated positions, no introduction of stop or unwanted codons, and incorporation of user-defined codons
- Customizable libraries: flexible design and deliverables
- Stringent QC: guaranteed >90% coverage of desired variants and superior diversity

Contact us: gene@genscript.com
Visit us: https://www.genscript.com
For Precision Mutant Libraries: https://www.genscript.com/precision-mutant-library.html