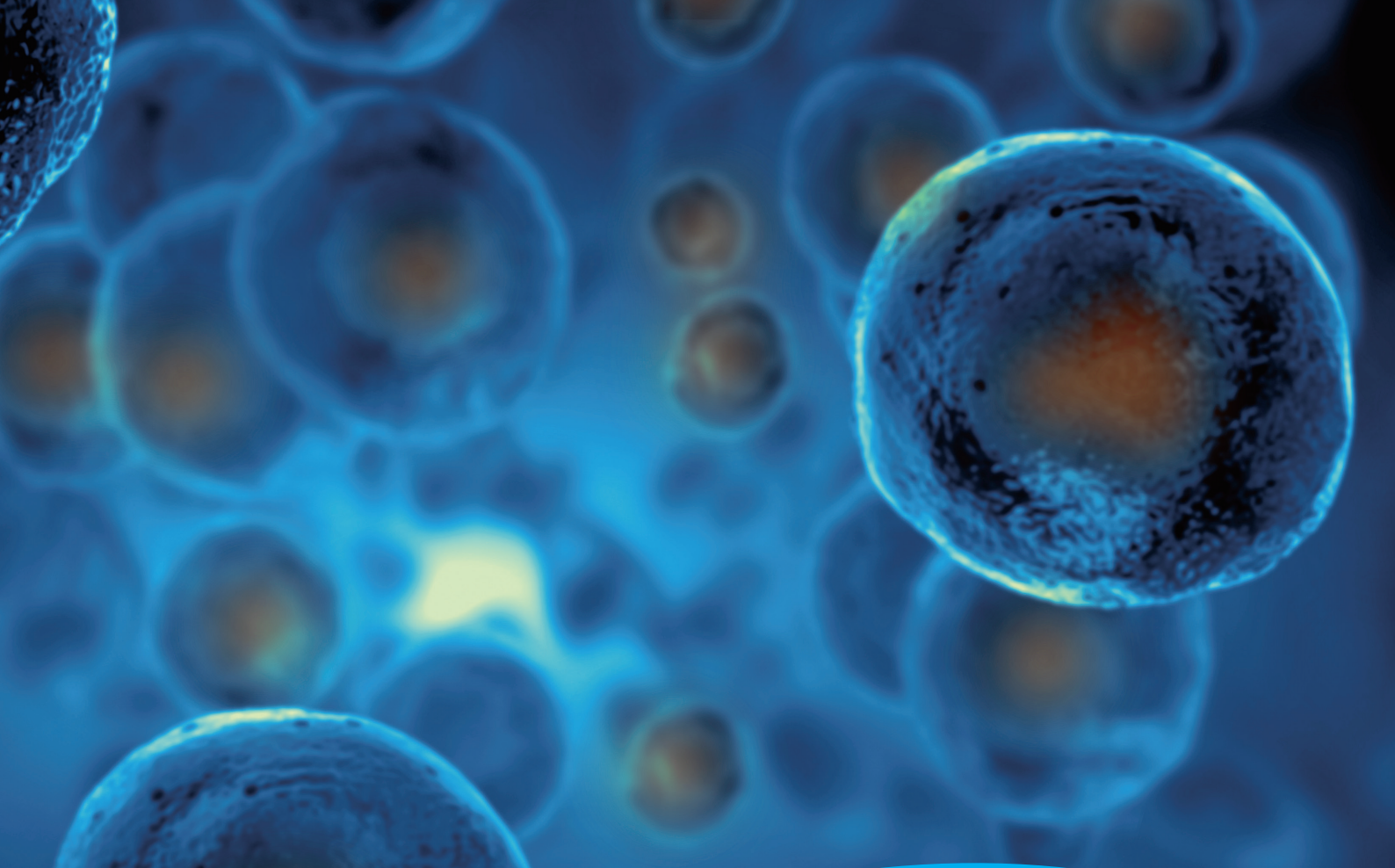

GenScript
Make Research Easy

GENE AND CELL THERAPY

GenScript's viral and
non-viral therapeutic total solutions

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01 Introduction

Gene and cell therapy (GCT), represent overlapping fields of biomedical research with similar therapeutic goals, i.e. to treat and cure diseases by altering and modifying the genetic make-up. Despite the commonality in both treatment, they work in different manner.

Specifically, human gene therapy seek to repair or reconstruct defective genetic material modify in the cell to produce a therapeutic effect or a resultant treatment of the disease. This technique can work by several mechanisms, including

- Replacing a disease-causing gene with a healthy copy of the gene,
- Inactivating a disease-causing gene that is not functioning properly,
- Introducing a new or modified gene into the body to help treat a disease

Currently genetic material into the cells physically, chemically, biologically or via a viral vector, with viral vectors being the most common gene delivery method.

Cell therapy is the introduction of new cells into a patient's body to grow, replace or repair damaged tissue in order to treat a disease. A variety of different types of cells can be used in cell therapy, including stem cells, T-lymphocytes, dendritic cells, pancreatic islet cells and thymus tissues.

Cell therapies can use cells from the patients' own body (autologous) or from a donor (allogenic). Many cell therapies use adult cells that have been genetically reprogrammed and capable of becoming one of many types of cells inside a patient's body. This technology may enable the development of an unlimited types of specific human cells needed for therapeutic purposes.

02 Development and timeline of FDA approved GCT

To date, there are twenty two FDA approved GCT (Figure 1). Among the twenty two approved treatments, only two gene therapies are approved, namely i) Luxturna, to treat inherited retinal diseasae due to mutations to both copies of the RPE65 gene and ii) Zolgensma, that targets the genetic root cause of spinal muscular astrophy (SMA) through the introduction of a functional SMN1 gene. The number of gene therapies approved are due to the complexities of the targeted disease for gene therapies and also lack of technologies and standards to efficiently delivery such genes treatment.

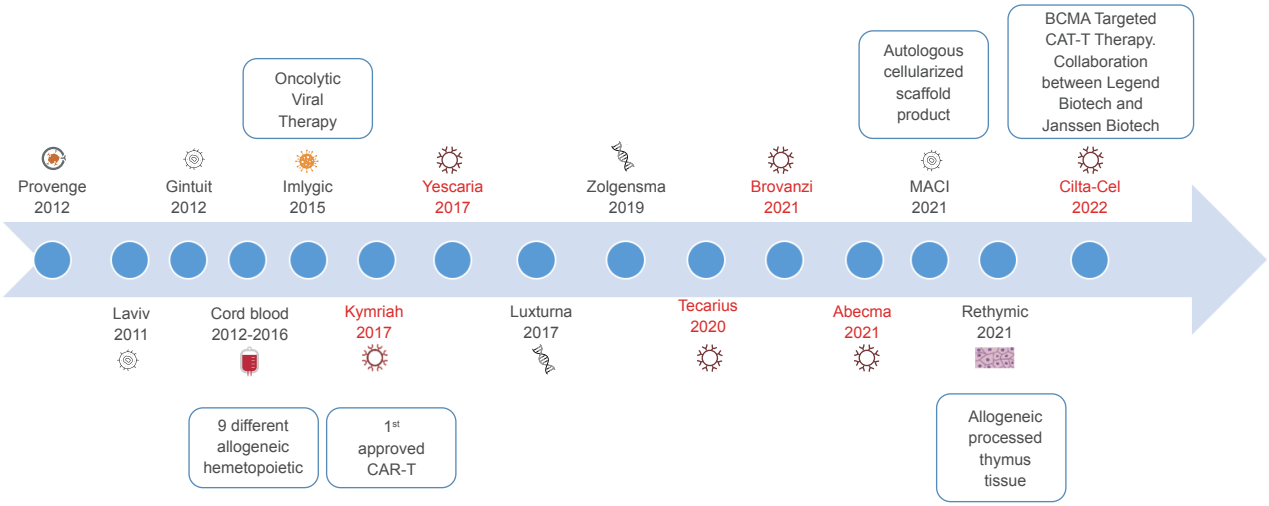


Figure 1. Timeline showing the different GCT approved by the FDA.

***Icon Glossary:** indicates antigen presenting cell (APC) treatment. indicates fibroblasts or fibroblast-like cell treatment. indicates allogeneic cord blood hematopoietic progenitor cell therapy. indicates oncolytic viral therapy. indicates chimeric antigen receptor (CAR)-T cell therapy. indicates gene therapy. indicates tissue slice therapy.

The rest of the approved GCT are cell therapies that involved various types of cell treatment (Figure 1). The majority of the treatment involves the introduction of allogeneic hematopoietic cells to patients with indications and disorders affecting the hematopoietic system. Such treatment, derived from cord blood and produced in the bone marrow, will be able to renew and differentiate into specialized cells. When transplanted, these treatments are life-saving for people suffering from lymophoma, immune disorders or other related genetic conditions. However, in recent years there has been increasing interests in CAR-T cell therapy. Within four years from 2017 to 2021, there had been five approved CAR-T cell therapy. All five CAR-T cell therapies provide subsequent line of treatment for refractory/ relapsed B cell lymphoblastic leukemia.

It is no coincidental that five CAR-T cell therapy are approved within a four years span. But rather, through much development and progress starting in the 1939 when the first bone human bone marrow transfusion was given to a patient with aplastic anemia to raise her leukocyte and platelet counts (Osgood et al., 1939). In 1956, Barnes et al. demonstrated in mice that allogeneic marrow transplantation successfully prevented a leukemia relapse, illustrating the activity of the engrafted immune system against the recipient (Barnes et al., 1956). This is then followed by the work of E. Donnall Thomas, who first reported in 1957, the first allogeneic hematopoietic stem cell treatment (HSCT) in patients but resulted in patients dying after 100 days (Thomas et al., 1957). Through a better understanding on human leukocyte antigens (HLA) and advancing in methods in identifying and typing them, in 1979, he reported a cure rate of 50% in acute myeloid leukemia (AML) (Thomas et al., 1979). But most importantly, he found the power of the immune system to eradicate cancer.

With such discoveries, new forms of cancer treatment evolved, with Steven Rosenberg and colleagues, in 1986, treating patients with tumor-infiltrating lymphocytes (TIL therapy) which are removed from a tumor and expanded in a lab before giving back to patients, with a few cured of advanced cancer from the treatment, showing the immune cells can fight cancer. With a better understanding in various types of cancer antigens, work on introducing gene into T cells to engineer such immune cells as anti-cancer therapy was started by Michel Sadelain (1992) (TCR therapy). Due to limitations in the TIL and TCR therapy, in terms of number of T cells and major histocompatibility complex (MHC) incompatibility, efforts had been made to overcome this via synthetic receptors. The first CAR (first generation), which is a fusion of an antibody to the T cell receptor, was developed by Zelig Eshhar in 1993 (Figure 2). Such early CARs do not persist in the body and are not clinically effective. This was optimized by Michel Sadelain, who introduced a co-stimulatory molecule, e.g. CD28, for persistent T cell activation and survival, creating the second generation of CARs. A third generation that include different co-stimulatory domains, such as CD28 with 4-1BB and a recent fourth generation or armored CAR to reduce immunosuppression and further mediate anti-tumor efficacy (Figure

2). There is also increase development efforts for CAR in natural killer (NK) cells. Within a short time span, cell therapy have evolved much with exploding development and interests in CAR. As such, in this technical handbook, a focus will also be made on the development of CAR in cell therapy.

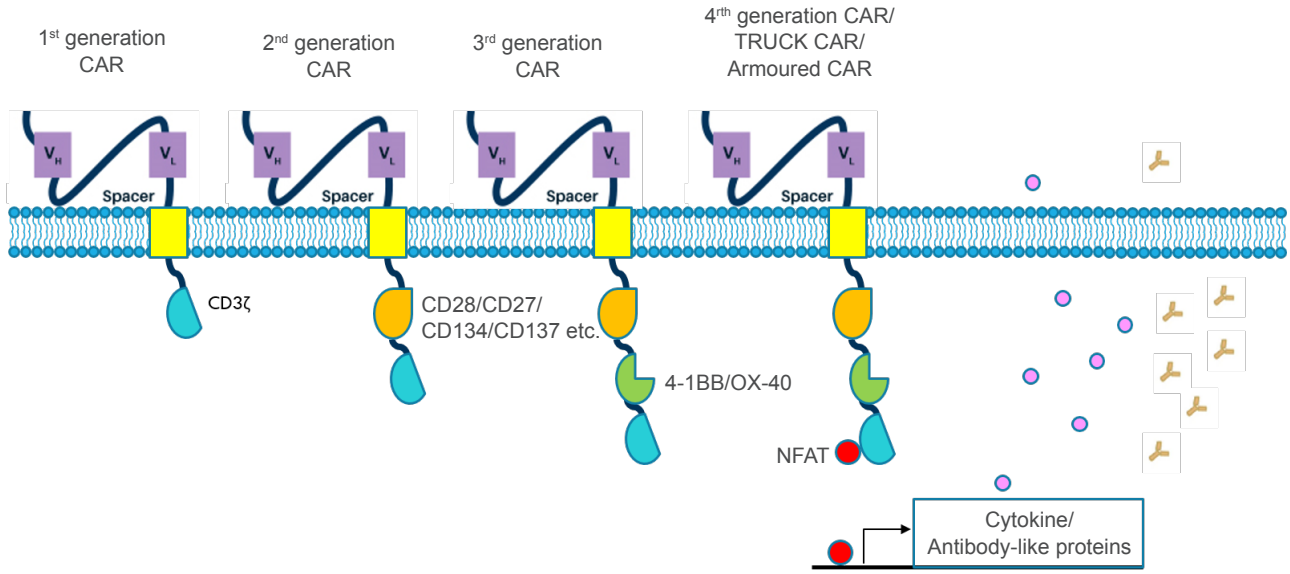


Figure 2. Development of CAR.

03 Gene and Cell Therapy Workflow

To simplify the discussion, we have provided an overall view of in the gene and cell therapy workflow, divided into 4 main steps of i) target identification, ii) lead optimization iii) validation in pre-clinical settings and iv) the production of the GCT (Figure 3). In gene and CAR-T cell therapy, the introduction of the gene could either be in vivo, for gene therapy and ex vivo for CAR-T cell therapy. But most importantly, the target responsible for the genetic disease (gene therapy) or the cancer antigen for the CAR-T cell therapy has to be identified. Subsequently, such leads has to be optimized, either in their gene delivery or for CAR-T cell therapy, better affinity to the cancer antigen or more localized therapy or better cytotoxic effects. Finally, with validation and success in pre-clinical trials, many of such GCT will require the GMP production of genes or vectors or gene engineering. In this handbook, we have simplified the processes into lead identification, target optimization and production (**Figure 3**). Work involved will be elaborated in subsequent sections.

Figure 3. Workflow overview for GCT.

3.1 Target Identification in Gene Therapy

In gene therapy, the identification of the disease-causing target genes is through genomics and proteomics approach to uncover novel functional pathway and therapeutic targets. With the advancement in bioinformatics approach, such identification have been made easier in highlighting key targets and functional networks through a vast array of in silico resources and data of high throughput profiling available. Tools such as TARGETgene can efficiently identify mutation drivers and possible therapeutic targets through intuitive mapping, analysis at the systems level and validate such predictions using user-defined benchmark genes. In fact, validating such potential drug targets is one of the most critical step in target identification and discovery. A high throughput means of producing such DNA targets, its related mutants and the different combinations of gene expression regulations will be crucial and can be achieved through the approach such as guide RNA (gRNA) libraries.

High-Throughput DNA Library assembly allows the accurate and efficient assembly and cloning of various predefined DNA fragments or parts into a vector of customer choice to generate a library of cloned constructs strategically assembled in a specific arrangement. These predefined DNA fragments or parts may be of promoters, enhancers/repressors, specific binding sites, localization signals, genes, terminators, and barcodes. Such libraries are powerful tools often utilized by scientists for a variety of combinatorial optimization applications, such as identification of an optimal genetic circuit design for the best possible output. However many times, production of such DNA libraries are limited by the size of the inserts. Recombinant plasmids greater than 10 kb transform bacteria very inefficiently.

In GenScript, we have developed our proprietary system such that the arrayed format developed that hold up to 1 x 10^8 construct, containing 4 variable slots for 15 kb inserts.

3.1.1 Validation of targets through CRISPR

To confidently proceed with the gene candidate that have the highest likelihood of being the ideal therapeutic target, target validation step is crucial in narrowing to a few critical and promising genes from a larger target identification screen as described above.

Traditionally, the commonly used methods were RNA interference (RNAi) or small molecule strategies to cause gene disruption to result in loss of function in order to validate potential gene targets. However, such methods have some limitations such as non-specific gene disruption or at times, suffer from incomplete knockdown. This could result in false positives or false negatives, wasting downstream effort, time and money.

Gene editing using Clustered Regularly Interspaced Short Palindomic Repeats (CRISPR) offers several advantages with its simplicity, versatility and specificity. A brief look at the basic mechanism of CRISPR indicates how specificity and versatility can be achieved (**Figure 4**).

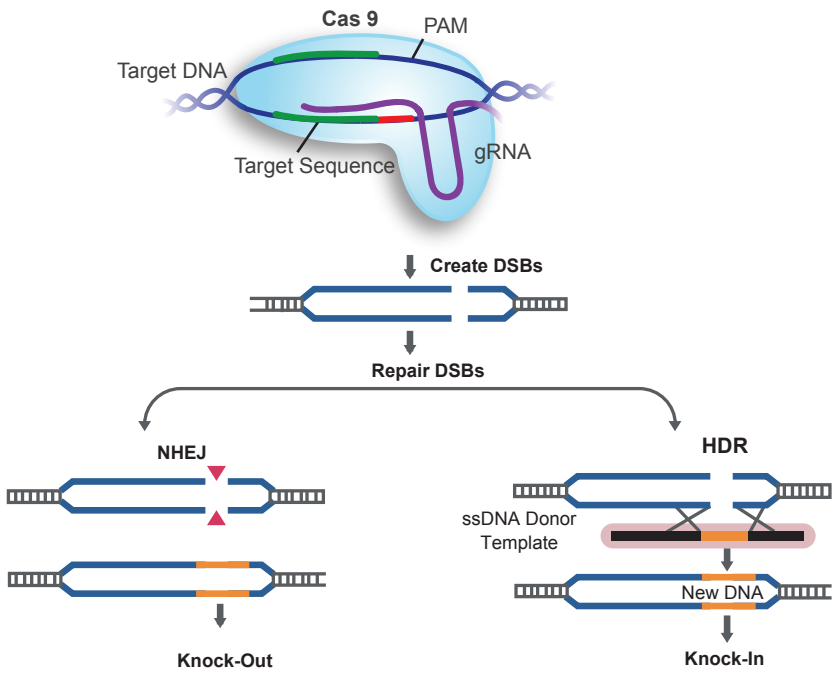


Figure 4. Mechanism of CRISPR as a genome editing tool.

The guide RNA (gRNA) recognizes specific regions on the host RNA and complexes with Cas9, which recognizes the protospacer adjacent motif (PAM) on the target and exerts its endonuclease function to cause double stranded breaks (DSBs). This triggers two mechanisms for repair: one is non-homologous end-joining (NHEJ, which introduces mutations in the DSB site. The other mechanism is homology directed repair (HDR) which enables the donor DNA information to be inserted at the break site.

CRISPR and the CRISPR-associated proteins (Cas) were originally identified in the Escherichia coli (E.coli) genome and are part of an RNA-based adaptive immune system. Upon complexing with a guide RNA (gRNA), Cas, e.g. Cas9, recognizes the protospacer adjacent motif (PAM) on the target, allowing specificity. Cas then exert its endonuclease function to cause a double stranded breaks (DSBs) to trigger a repair mechanism, via the non-homologous end-joining (NHEJ) to introduce mutation at the DSB site or homology directed repair (HDR) to enable donor DNA information to be inserted (Figure 7). As such, CRISPR is great for gene deletion, insertion and correction in target identification studies for gene therapies.

The design of these gRNA will be one of the important criteria in determining the successfulness of CRISPR in genome editing. This is greatly dependent on the type of genomic editing that is required. For gene knockout via indels, it is important that the gRNA is highly active and is able to reduce off targets. The location for such gRNA is of secondary importance. However, for gene knock down, the target location should be close to the transcriptional start site (TSS) and the sequence selection of the gRNA will be based mostly on the sequences found around the region. For knock in via HDR, the location is very important and that one have to target within ~30 nt of the proposed edit. At times, due to this limitations, there are only few gRNAs that one can choose from at the desired site. In view of the challenges in obtaining the optimal gRNAs for best function, in GenScript, we have exclusive licenses for the CRISPR technology from Feng Zhang's laboratory in Broad Institute of MIT and Harvard. The algorithms for the design of gRNA and their optimization are also created at the Broad institute. The gRNA design tool will identify single guide RNAs for use with wild-type S. pyrogenes Cas9 for any DNA sequence developed (Figure 5).

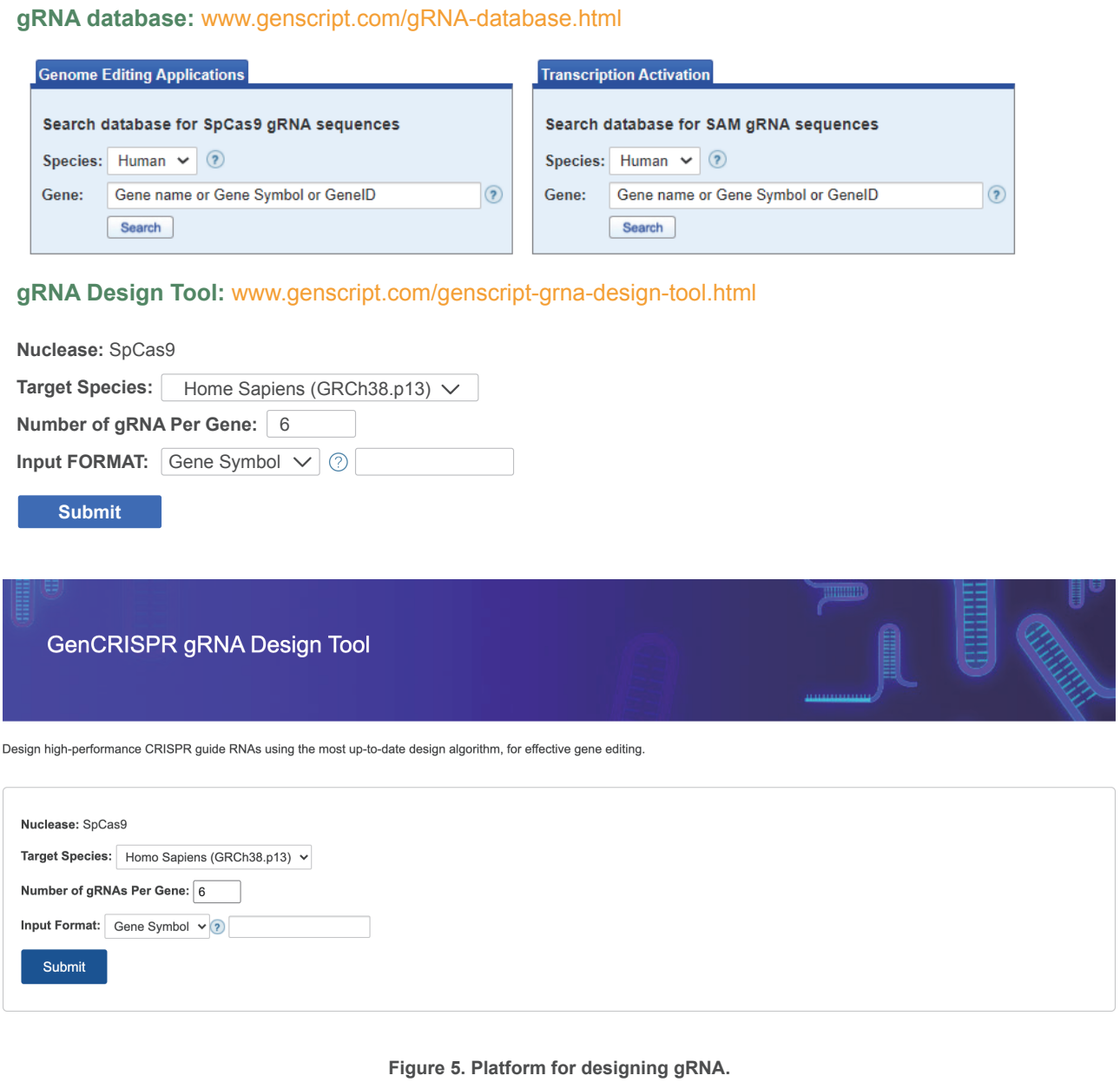


Figure 5. Platform for designing gRNA.

Processes can also be eased out by generating the cell line with us (Table 1).

Service Name	Service Details	Cell Line Options*	Deliverables	Price	Delivery Time
EZ Knockout Cell Line Service	• Single gene knockout cell line • Transfection based	Available for 120+ popular transfection-suitable cell lines, including A549, CHO-K1, HEK293, HEK293T, HT-29, MDA-MD-231, 4T1, A20, HCT116, MCF7, MDCK, U937, RPMI 8866, etc	• Two full-allelic knockout cell lines validated by sequencing • One negative knockout cell line as control	Starting from $7,000	10-19 weeks depending on project complexity and cell line difficulty
Customized Knockout Cell Line Service	• ingle or multiplex gene editing knockout cell line. • Transfection-based or Lentivirus-based or Ribonucleoprotein-based	Any cancer cell line	• 1-2 full allelic knockout cell line(s) validated by sequencing. • one negative knockout cell line as a control	Starting from $15,000	16-24 weeks depending on project complexity and cell line difficulty
Knockout Cell Pool Service	• Single gene knockout stable cell pool • Lentivirus based	Any cell line	• Stable cell pool containing knockout clones validated by sequencing	Starting from $4,800	5-11 weeks depending on cell line difficulty

Table 1. Cell line services available in GenScript for generating knock outs.

In additional to the design of gRNA, the Cas protein is also a crucial determinant. Over the years, efforts and discoveries have been made on the Cas proteins to lower the probability of off-target editing. Table 2 shows the various Cas9 variants available for different research applications and the characteristics of each of them.

Cas9 Name	Applications	Characteristics
SpCas9	Research Standard	• Create site-specific double strand breaks (DSBs) in the genome.
eSpCas9 (Enhanced Specificity SpCas9)	Less Off-target Effects	• Mutation vs SpCas9: K848A, K1003A & R1060A; • Reduce off-target effects by up to 10-fold compared to wild-type SpCas9 • Maintaining robust on-target cleavage efficiency.
SpCas9 Nickase (Cas9nD10A)	Creating Single Strand Nicks	• Contains a mutation allowing the endonuclease to create single-strand nicks, as opposed to DSBs. • Pairing two opposite facing gRNA sequences with SpCas9 nickase can efficiently prevent unwanted indels from forming.
SaCas9	Adeno-Associated Virus (AAV) applications	• SaCas9 is approximately 1kb shorter than SpCas9 and offers additional flexibility around AAV packaging constraints.
Transcription Activation (SAM) Plasmids	Transcription Activation	• Enable transcriptional activation of both coding and non-coding genetic elements. • Includes three components: ‣ A gRNA incorporating two MS2 RNA aptamers ‣ A catalytically inactive dCas9-VP64 fusion protein ‣ A MS2-P65-HSF1 activator fusion protein

Table 2. Different Cas proteins and their characteristics for various applications.

Besides having variants engineered to overcome off-target cleavage at unintended genome, other Cas systems such as the Cas12 and Cas13 are also discovered for different needs. Cas12 is a compact enzyme that, unlike Cas9, create staggered cuts in dsDNA. It also has its ability to process its own guide RNA, leading to multiplexing ability. Not only so, Cas12 can indiscriminantly chop up single-stranded DNA once activated by a target DNA molecule matching its spacer sequence, making them a powerful tool in detecting small amounts of target DNA. Unlike the Cas9 and Cas12, Cas13 targets RNA and its non specific RNase activity will be activiated by a ssRNA sequence bearing complementarity to its crRNA spacer. As such, Cas13 are good for RNA knockdown or RNA sequence editing, without altering the genome sequence. Bellowing Table 3 summarizes the characteristics of the different Cas proteins. These Cas nucleases are available in RUO or GMP quality.

GenCRISPR™ Nucleases			
	Cas9	**Cas12a**	**Cas13a**
Targeted Nucleic Acid	ds DNA	ds or ss DNA	ss RNA
Size of the protein	~1,000 - 1,600 AA	~1,300 AA	~1,400 AA
Guide	tracrRNA + crRNA	crRNA	crRNA
Type of cut	Double-strand, blunt cut	Double-strand, staggered cut	ss RNA
Cleavage	Cis-cleavage	Cis-cleavage Trans-cleavage	Cis-cleavage Trans-cleavage
Targeting restriction	GC-rich sequences	AT-rich sequences	-
PAM/PFS	5' - NGG - 3'	5' - TTTV - 3' upstream	A or U
GenScript variants	eSp, eGFP, Nickase, WT	WT	WT
Compliance	Research GMP	RUO	RUO

Table 3. GenScript's solutions to DNA and RNA editing.

The availability of GMP grade is in consideration for gene editing for GCT applications. Having both research grade and GMP grade available will allow the smooth transition from research and development towards therapy production in the future. Our GMP grade, known as GenCRISPR™ Ultra nucleases are also tested to have superior efficiency for T-cell engineering (Figure 6).

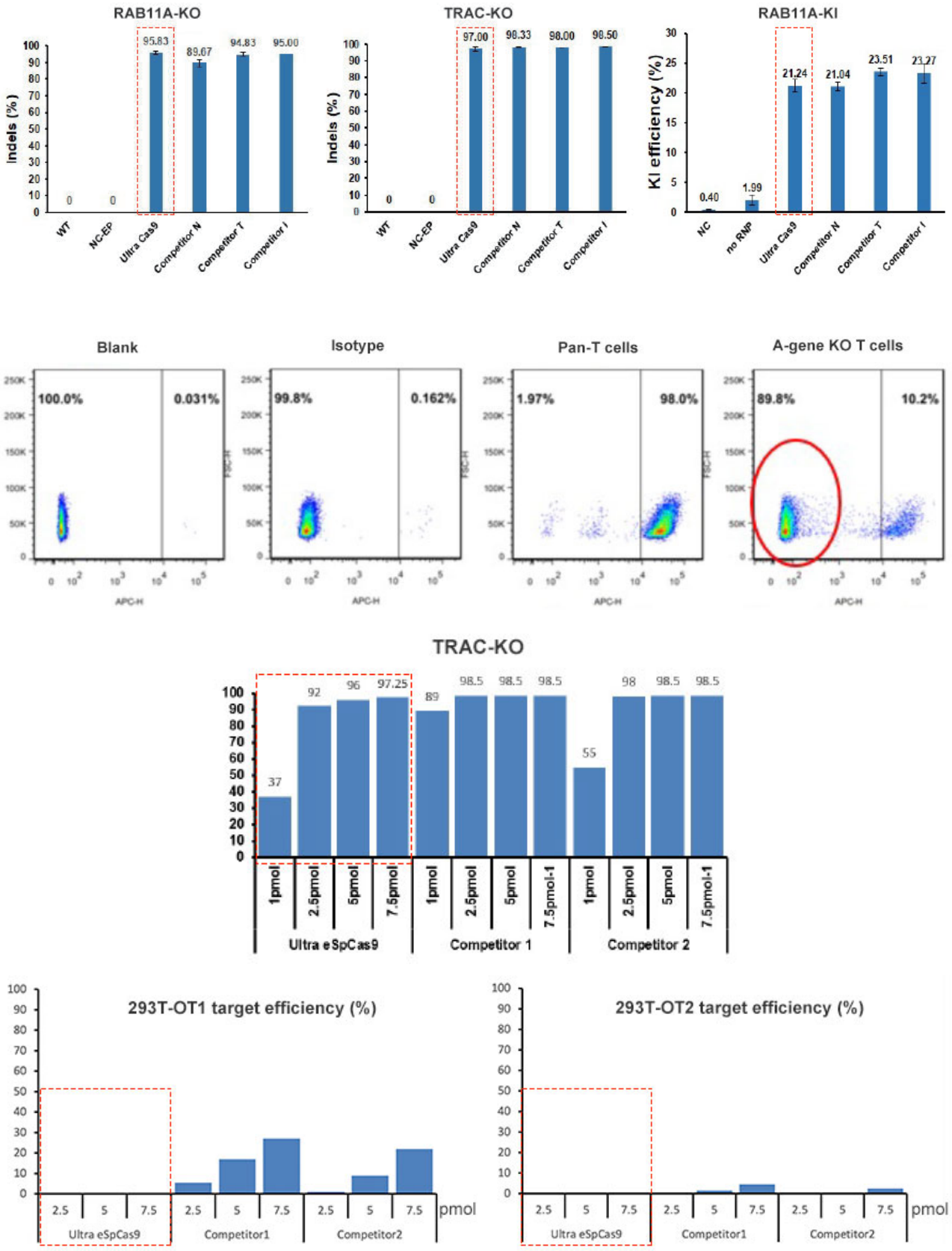


Figure 6. High efficiency for gene editing for T-cell engineering.

From the Figure 6, (Top panel, left and middle) RAB11A and TRAC genes knock-out in 293T cells by electroporation and (Top panel, right) dsDNA knock-in at the RAB11A site in Jurkat cells by electroporation. (Middle panel) Gene knock-out in T cells. (Bottom panel, left) TRAC knock-out in 293T cells by electroporation. (Bottom panel, middle and right) Off-target efficiency at the OT1 and OT2 sites of 293T cells.

3.1.2 Genome wide and Pathway-specific Screens Using CRISPR libraries

CRISPR has been adapted for genome-wide screening to discover genes whose inhibition or aberrant activation can drive phenotypes implicated in disease, development, and other biological processes.

Genome-scale CRISPR knock out libraries (GeCKO v2 libraries) in mouse and human enable rapid screening of loss-of-function mutations. GeCKO v2 libraries express a mixed pool of CRISPR gRNAs that target every gene and miRNA in the genome. Each gRNA is cloned into a lentiviral vector optimized for high-titer virus production and high efficient transduction of primary cells or cultured cell lines. After transduction, deep sequencing can be performed to assess gRNA representation in the cell pool prior to screening. After selection, a second round of sequencing is performed to identify the gRNAs that were lost or enriched represent positive hits. A detailed GeCKO screening protocol may be found on the GeCKO Genome Engineering website: http://genome-engineering.org/gecko/

Molecular pathway-focused gRNA libraries have also been developed for targeted screening of specific molecular pathways. Pathway-specific gRNA libraries were designed using gene targets identified through the Drug Gene Interaction Database by the McDonnell Genome Institute at Washington University in St. Louis. All gRNA sequences have been pre-designed and validated by the Broad Institute.

Alternatively, built upon our proprietary semiconductor-based electrochemical DNA synthesis platform, GenScript can also offers fully customized, ready-to-package CRISPR gRNA libraries to help maximize the screening efficiency.

3.1.2.1 Advantages

- No sequence restriction
- Top-notch library converge and uniform distribution
- Next-generation sequencing report upon request
- Deliver up to 100μg of DNA per library
- Up to 90,000 sgRNAs per library

3.2 Target Identification in CAR-T Cell Therapy

In CAR-T cell therapy, the identification is of the appropriate tumor antigen will be crucial in the development of CAR. There are different types of tumor antigen currently in focus (**Table 4**).

Cancer antigens	Characteristics	Examples
Tumor associated antigens	1) Processed fragment of proteins 2) Normally low expression in other cells but high expression in cancer cells	1) Erbb2 (Breast cancer) 2) CD19 (B-cell malignancies) 3) yrosinzse (mealnomas)
Cancer/ Testis (CT) antigens	1) Usually present in reproductive tissues and not other adult tissues 2) Appear in significant subset of malignant tumors	1) MAGE2 2) NY-ESO-1
Viral antigens	1) Associated with viral infections 2) Cancer cells expressing proteins encoding viral open reading frames	1) Human papilloma virus 2) Cervical or oropharyngeal cancers
Neoantigen	1) Mutation in protein coding region in non-germline cell after birth 2) Somatic mutations accumulate in cancer cell during the initiation, development and metastasis of tumor	

Table 4. Type of cancer antigen, their characteristics and corresponding examples.

There are currently several ways for their identification, e.g. through i) in silico prediction, ii) LC-MS/MS based immunopeptidomics and iii) whole exome sequencing data. Each have their pros and cons and these are briefly summarized in the **Table 5**.

Cancer antigens	Characteristics	Examples
In silico prediction	1) narrow down number of candidates 2) Identify minimal epitopes	1) Polymorphism in major histochemistry complex II (MHC II), thus not optimal 2) Variations in epitope length 3) Influences from peptide flanking regions 4) Challenges in identifying correct binding core 5) Failure to respond in vivo 5) Depends on accuracy of prediction algorithms 7) Less accurate predictions from low frequent human leukocyte antigen (HLA) clonotypes
LC-MS/MS based immunopeptidomics	1) High throughput 2) Detect mutant peptides and non-canonical peptides 3) Direct identification of naturally presented HLA binding peptides	1) Limited sensitivity of mass spectrometry 2) Biased towards detecting the more abundant peptides 3) Relies on efficient peptide ionization and fragmentation 4) Depends on HLA expression of tumor cells 5) High amount of tumor tissue needed
Whole-exome sequencing data	1) Identification of all candidate neoantigens	1) Minimal epitope not defined 2) Limited feasibility for tumors with high mutation burden

Table 5. Pros and cons of each identification method.

Particularly for the identification of neoantigen associated with MHC, deep sequencing analysis is performed to identify such MHC associated neoantigens. Human or mouse tumor cell are lysed under optimal conditions for releasing intact MHC complexes. Using immunoaffinity chromatography, MHC-specific antibodies enrich the pool of MHC class I or class II from lysates. MHC associated peptides are released under gentle eluting conditions and separated from the captured MHC molecules. The peptides are sequenced by LC-MS/MS and then matched to protein sequence databases or RNA-seq data, as well as analyzed for mutations and post-translational modifications to facilitate the identification of potential neoantigens. Validation of neoantigens using peptides on T cell cytotoxicity assays or cytokine release assays to determine the potential of these neoantigens as tumor antigens that could be useful in immunotherapy.

However, such neoantigen peptides are typically difficult to synthesize due to the following as illustrated in **Table 6:**

Characteristic	Description	Difficulty
Length	The isoelectric point (pI) of a peptide chain	Neoantigen peptides range from 8 - 50 amino acids. Peptides over 20 AA are difficult to be synthesized
Charge	The isoelectric point (pI) of a peptide chain	Many purification steps only work with specific charges, the further from neutral a peptide is, the synthesis will be more difficult
Hydrophobicity	Amino acids which naturally repel water due to their charge	The more hydrophobic a peptide is, the more difficult synthesis and purification will be due to its insolubility in water
Yield	The amount of one peptide in weight (mg)	It is very difficult to find a provider who can generate a range of yields for difficult peptides
Purity	The percentage of an individual peptide in a solution of peptides	The charge and hydrophobicity (solubility & aggregation) make standard purification difficult

Table 6. Challenges in synthesizing neoantigen peptides during validation as tumor antigens.

To circumvent this bottleneck, GenScript has launched their neoantigen peptide synthesis platform featuring their proprietary NeoPre™ algorithm that identifies synthesis difficulties and recommends the best synthetic technology based on each individual peptides intrinsic characteristics. Based on machine learning (LinearSVM), NeoPre™ analyzes six intrinsic characteristics of each peptide to predict synthesis difficulty. These include i) length, ii) yield, iii) charge (pI), iv) aggregation, v) hydrophobicity and vi) purity. NeoPre™ can then recommend the most efficient approach to successfully synthesize peptides using one of GenScript's many synthesis platform **(Table 7)**.

Length	Yield	Purity	pI	Aggregation	Hydrophobicity	Prediction	Protocol
27	20mg	>98%	7.4	24.4	-0.25	Difficult	Automatic synthesis
25	4mg	>98%	10.2	52.2	0.65	Difficult	Mankind handling with improved coupling reagent, monitored under all process, involving Mid-control process to detect MS results
25	24mg	>98%	8.6	48.3	0.15	Difficult	Semi-automatic synthesis, monitores under all process to ensure coupling efficiency. "O-Acyl isopeptide method" for peptide synthesis
25	4mg	>98%	6.4	39.3	0.05	Difficult	R&D

Table 7. Example read out of NeoPre™.

GenScript also offer two synthetic technologies specific for hydrophobic peptide production on top of our standard five synthetic methodologies to reach a 95% success rate for difficult peptides. One of them is our HiSyn technology which utilizes an optimized reaction temperature and condensation reagents to increase coupling efficiency. We also have our advanced coupling technology that uses coupling reagents from our HiSyn technology in optimized reaction conditions for our microwave synthesis method. We are the first CRO in the field to apply such optimized reaction technology utilizing microwave technology.
Also rather than relying on traditional peptide purification, we have optimized standard methods to enhance peptide purity through the use of numerous different reagents and purification column. This allows us to reach peptide purities of even the most difficult neoantigen peptides of up to 98% (**Figure 7**).

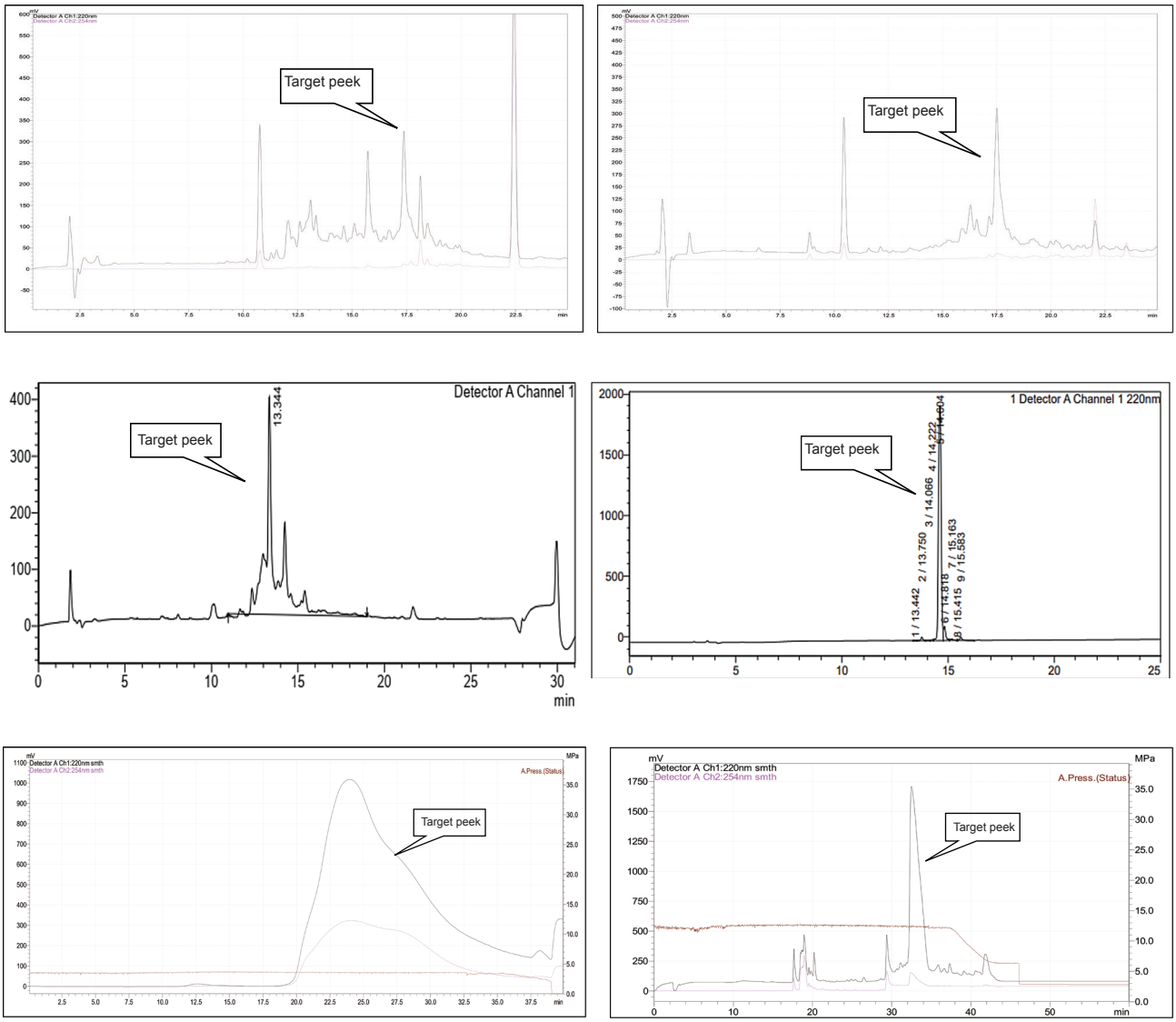
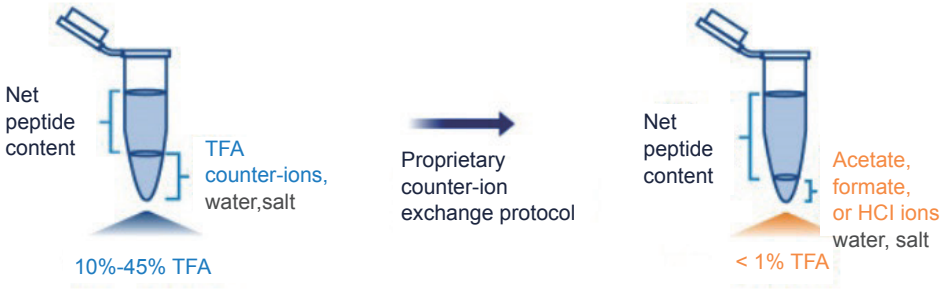


Figure 7. Improved purity of peptides using GenScript's technology.

(Top) HiSyn technology, (middle) advanced coupling and (bottom) optimized purification procedures.

From. In addition, our peptides are ISO9001 certified with Total Qualityity Management (TQM) platform to ensure each cusomt peptide is triple checked for quality via mass spectrometry (MS) and high performance liquid chromatography (HPLC) analyses after each step during peptide purification and quality control (QC) procedures. In view of many of these peptides to be used in T-cell cytotoxicity assays or cytokine relases assays, our AccuPept+QC that includes many additional customized QC such as Trifluoroacetic acid (TFA) ion removal (**Figure 8**) and endotoxin control.



Service TYPE	TFA Residue	Salt Type	Report
Guaranteed TFA removal	<1%	HCl, Formate, Acetate	Included
Standard TFA removal	<10%	HCl, Formate, Acetate, Phosphate	Upon Request*

In general, the TAT for Guaranteed TFA removal service is 8 days, and 2 days for Standard TFA removal service.

*If Report is selected for Standard TFA removal service, extra cost and 1day will be needed

Figure 8. TFA ion removal service options.

3.3 Lead Optimization CAR-T Cell Therapy

Upon the identification tumor antigens, the antibody recognizing the tumor antigens is produced as part of the module in the CAR. Currently, the antibody portion of the CAR exist as a single chain fragment variable (scFv) harboring only the variable region of the heavy chain linked to the variable region of the light chain via a linker (Figure 9). In recent years, progresses to improve the recognition of the tumor antigen had been investigated. Having a greater affinity and selectivity towards the tumor antigen is beneficial in reducing the side effects and to have better efficacy. One way is through the use of improved through the single variable domain on the heavy chain (VHH), harbouring only the heavy variable chain. VHH are typically produced by camelid. Due to a smaller and more compact size, they are more soluble, more stable and allow for dimerization and even multimerization to increase the binding strength and specificity towards the tumor antigen. In addition, with its small and compact size, they are able to bind hidden epitopes that are not accessible to whole antibodies. Indeed, the function of CAR could be greatly improved and toxicity can be reduced if the recognition towards the tumor antigen by the antibody module could be optimized and made more specific with greater binding affinity.

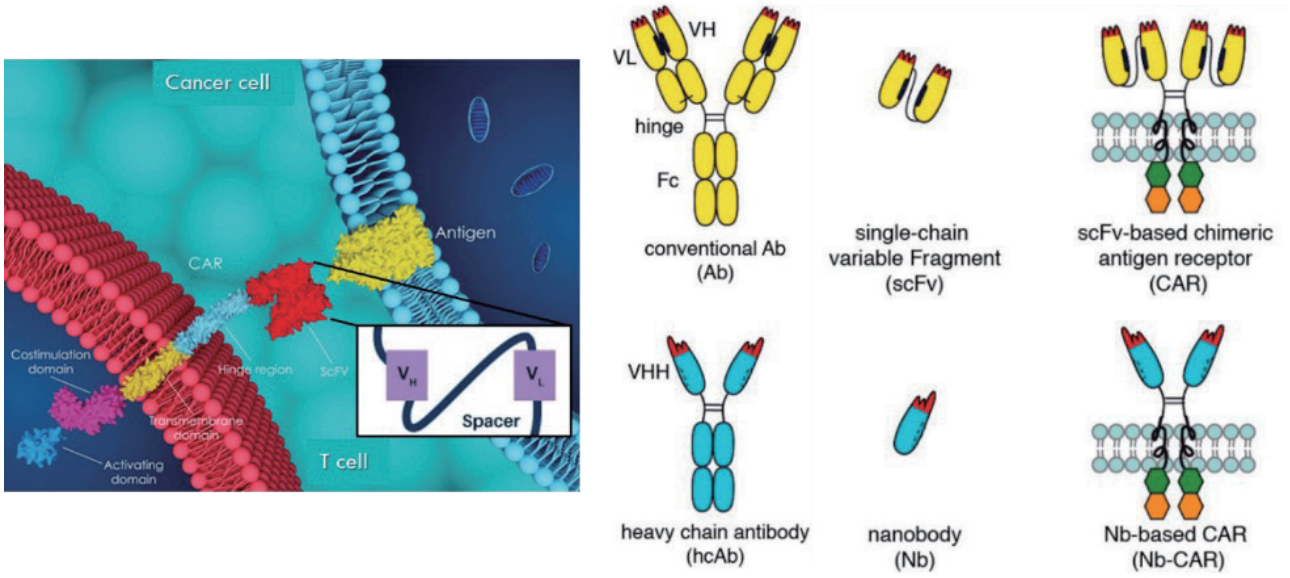


Figure 9. Optimization of CAR binding towards tumor antigen.

On the **Figure 9**, (Left panel) Interaction between CAR and antigen via scFv. (Right panel) Differences in structure between scFv and VHH. (*Note that this right panel is adapted from Hambach et al., 2020.)
In GenScript, our CDMO business, ProBio, streamlines such optimization processes and even the downstream production/ engineering of these CARs into T-cells (Figure 10). We provide services for single domain antibody discovery (SdAb) for producing an optimized CAR lead. The binding affinity, specificity and stability of these CARs can be further optimized in an in vitro manner, akin to an affinity maturation of the antibodies, through the Precision Mutant Library (PML) in which the each position of the region of interest, e.g. the variable region of the antibody, is mutated to all other 20 amino acids, to identify the best scFv or VHH. ProBio also provide developability assessment and affinity measurement of such CARs. The functionality of such CARs can also be demonstrated in vitro through cytotoxicity assay, cytokine release and proliferation assays. For downstream production, cell line development, cell banking and GMP manufacturing of lentivirus are available.

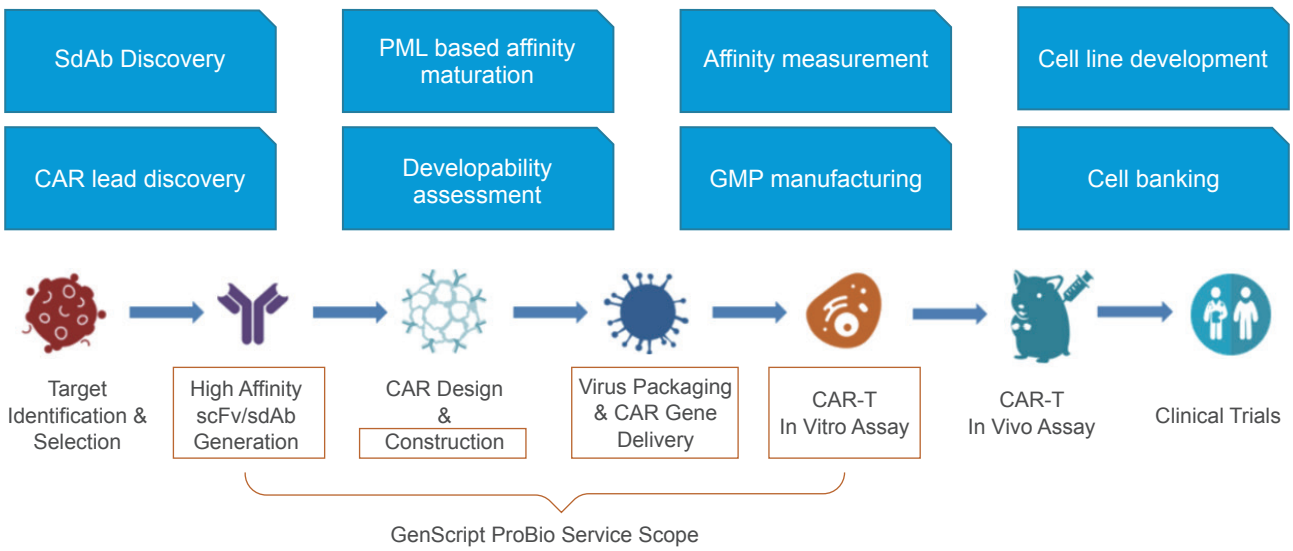


Figure 10. Streamlined CAR development and discovery services available in ProBio.

A brief summary of the service offerings is demonstrated in **Figure 15**. We provide either discoveries of scFv through antibody generation through hybridoma technology or single B cell screening and sdAb libraries (naïve or immunized library) for VHH generation. While the naïve library is able to produce a larger number of clones within a shorter timeline, immunized libraries usually produce antibodies with greater affinity. As such, ProBio provides the options of naïve and immunized library to cater to various project needs. Upon choosing the right starting point for the ideal antibody discovery, antibodies obtained will undergo a series of affinity ranking using surface plasmon resonance (SPR) to identify the highest affinity leads. Generation of antibodies from mice or llama may lead to issues of immunogenicity. Immunogenicity can potentially lead to reduced efficacy through rapid clearance or neutralization of the antibody or toxicity due to cross-reation with proteins that could lead to catastrophic consequences for the patient. Understanding this, in the CAR lead discovery, we also provide antibody humanization to reduce the immunogenicity of the monoclonal antibodies generated. After which, the highest affinity leads, with reduced immunogenicity, can be further optimized on our our antibody optimization platforms such as PML and FASEBA. Upon the identification of such leads, we will work on the construction of a CAR and the production of the CAR-T cell. The function of the CAR-T cells will then be further validated in CAR=-T developmental project through in vitro CAR-T cells functional assays, such as cytotoxicity assays, cytokine release and proliferaction assays. Such tests can be performed according to project requirements.

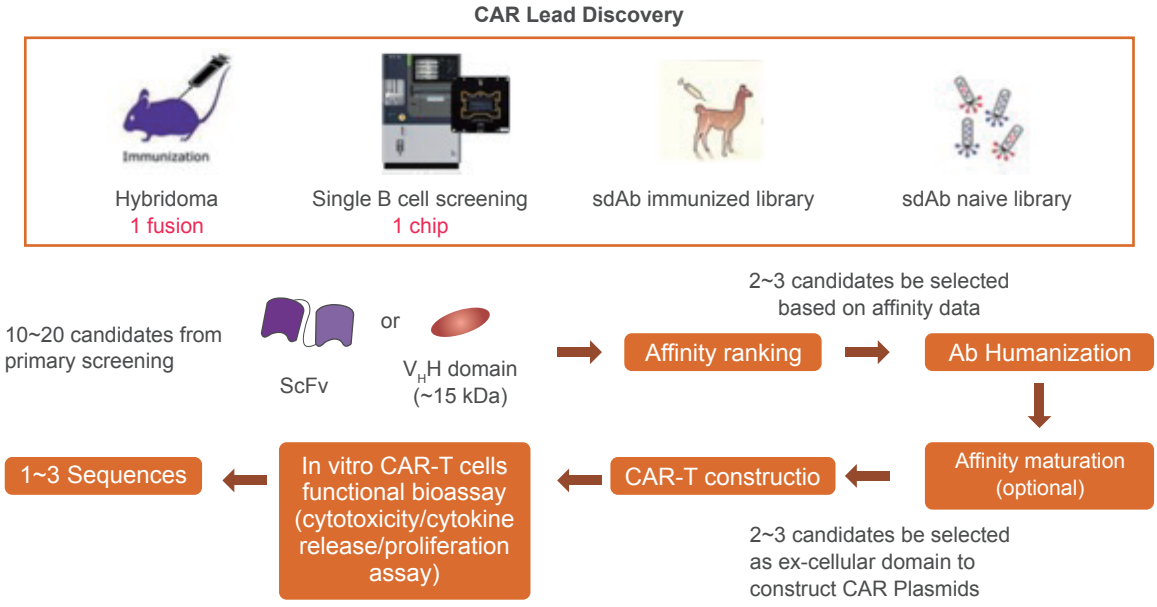


Figure 11. Workflow of CAR development.

3.3.1 Affinity Maturation of Antibodies Through Precision Mutant Library (PML)

If optimization of the antibody is desired on your end, GenScript could provide standalone PML libraries for affinity maturation in vitro for the antibodies discovered. 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 (**Figure 12**).

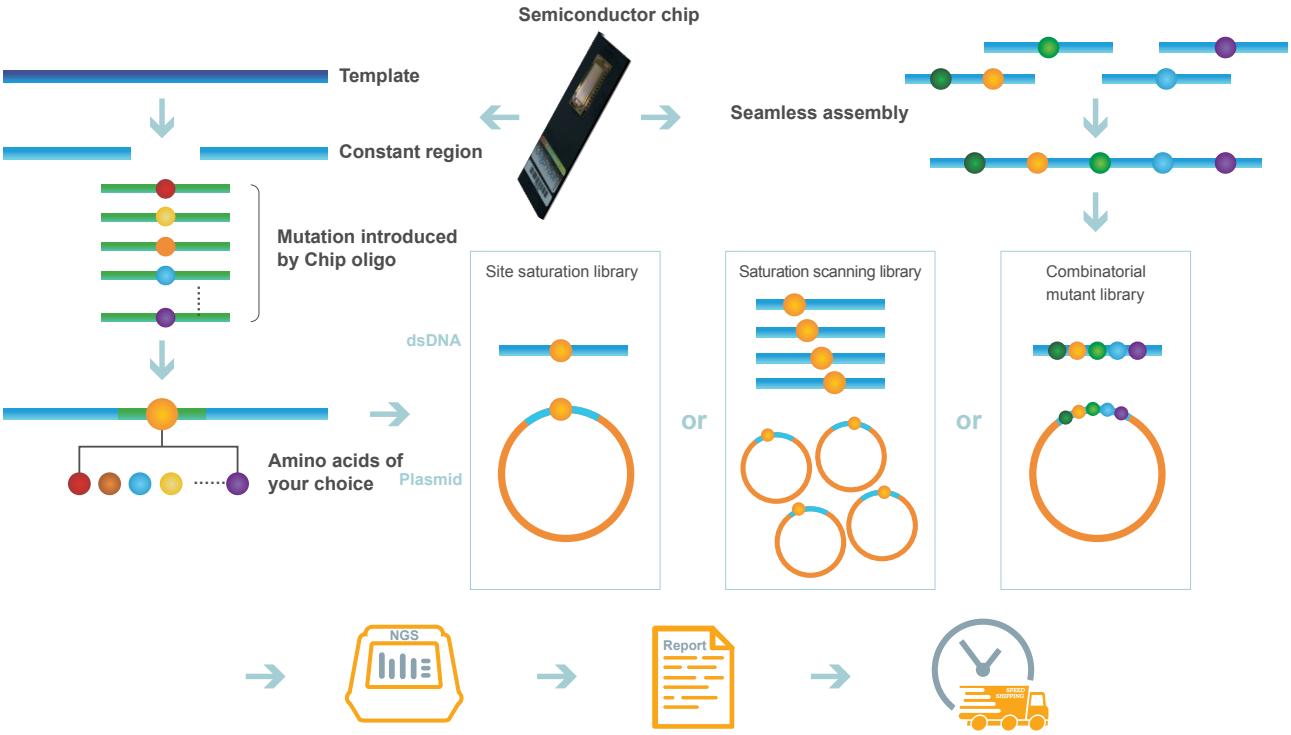
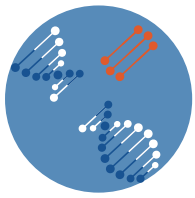


Figure 12. Workflow for constructing mutant libraries using arrayed, semiconductor-based oligonucleotides.

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.

This technology 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.

3.3.1.1 Advantages



Precise control of codon usage

Superior library diversity

Minimize screening bueden

3.3.1.2 Outcome and Case Study:

Case 1: Saturation Mutagenesis Library, generated by PML, to improve antibody affinity

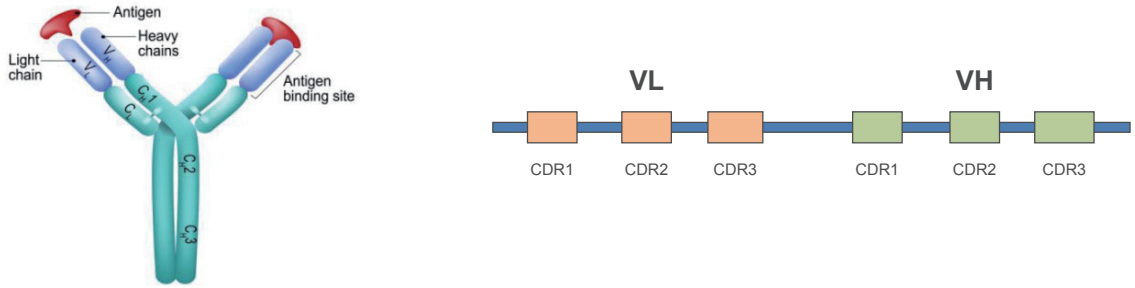


Figure 13. A schematic illustration of the light chain (VL) and the heavy chain (VH) of anantibody of interest to be engineered for affinity improvement using a saturation mutagenesis library targeting mutations in CDR regions.

A total of 63 sites across 6 CDR regions within the heavy and light chains were mutated, replacing each wildtype amino acid with all other 19 amino acids, via saturation mutagenesis.

Key Highlight 1: Equal Representation of Amino Acids at Each Site with 100% Coverage

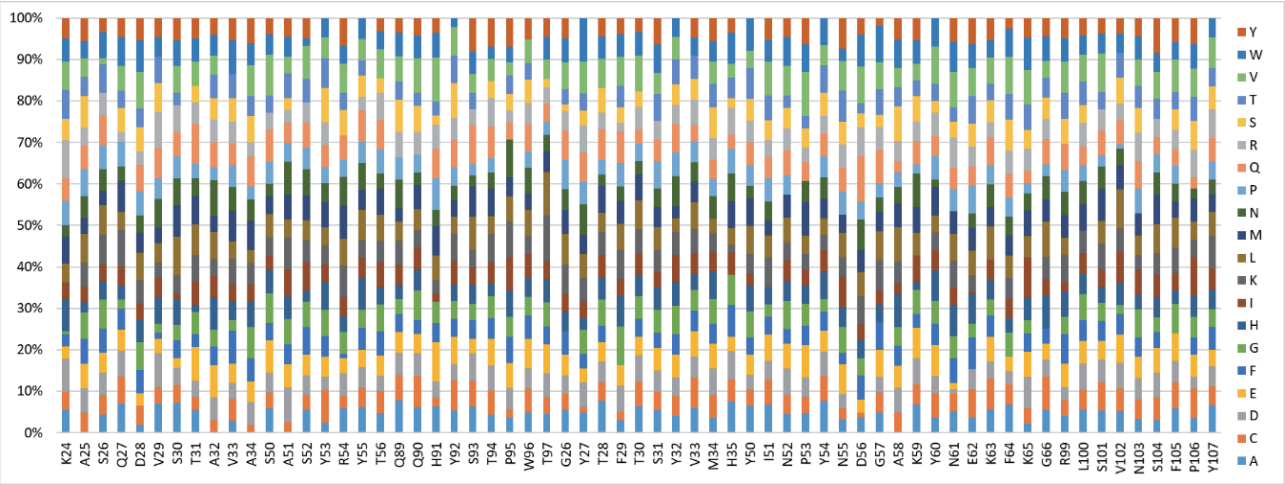


Figure 14. The amino acid distribution of the saturation mutagenesis library determined by the next generation sequencing (NGS) technology.

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.

Key Highlight 2: Generation of Candidates, through PML, with Improved Affinities

Figure 15. Mutant clones with higher affinities relative to that of the wildtype antibody.

The library was screened with FASEBA to identify candidates with improved affinities. A value above 1 represents a higher affinity than the wildtype. 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.

Mutant/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

Figure 16. Binding affinities of 5 improved clones with beneficial mutations.

Key Highlight 3: The affinity of the variant can be further improved through combinatorial library

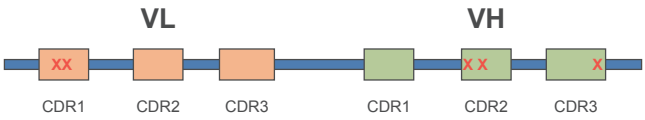


Figure 17. A schematic illustration of the 5 beneficial mutations in the specific CDR regions of the antibody of interest. Each of the 5 beneficial mutation sites is marked "x".

Mutant	KD(M)	Rmax
AA38/AA93/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

Figure 18. Binding affinities of improved mutants harboring specific combinations of beneficial mutations.

Furthermore, with the proprietary gene synthesis technology in GenScript, we can help with the generation of different types of libraries to meet different demand, including

- Site-directed Mutagenesis libraries
- Scanning Point Mutation Libraries
- Randomized and Degenerated Libraries

3.3.2 High-Throughput Combinatorial DNA Assembly

The flexibility of a customized CARs design for different oncological applications have made them an attractive anticancer treatment option in the recent years. The general design of a CAR construct consists of an extracellular ligand-binding domain, a spacer domain, a transmembrane domain, and one or more signaling endodomains.

Even though the future of CAR-T cell therapy may seem promising, several challenges of this treatment is still yet to be overcome prior to full adoption, clinically. These challenges include toxicity, responses to CAR-T therapy could be varied, individually, due to both heterogeneity in target-antigen expression in malignant cells, high rates of antigen escape and downregulation of target cancer markers. All these factors may result in affecting their function in the neoplastic microenvironment. Several attempts have been made in order to improve the off-target effects and enhancing the tumor eradication and persistence, including the use of appropriate compositional and structural design of CARs.

High-Throughput DNA Library assembly allows the accurate and efficient assembly and cloning of various predefined DNA fragments or parts into a vector of customer choice to generate a library of cloned constructs strategically assembled in a specific arrangement. These predefined DNA fragments or parts may be of promoters, enhancers/repressors, specific binding sites, localization signals, genes, terminators, and barcodes. Such libraries are powerful tools often utilized by scientists for a variety of combinatorial optimization applications, such as identification of an optimal genetic circuit design for the best possible output. However many times, production of such DNA libraries are limited by the size of the inserts. Recombinant plasmids greater than 10 kb transform bacteria very inefficiently.

In GenScript, we have developed our proprietary system such that the arrayed format developed that hold up to 1 x 10^8 construct, containing 4 variable slots for 15 kb inserts (**Figure 19**).

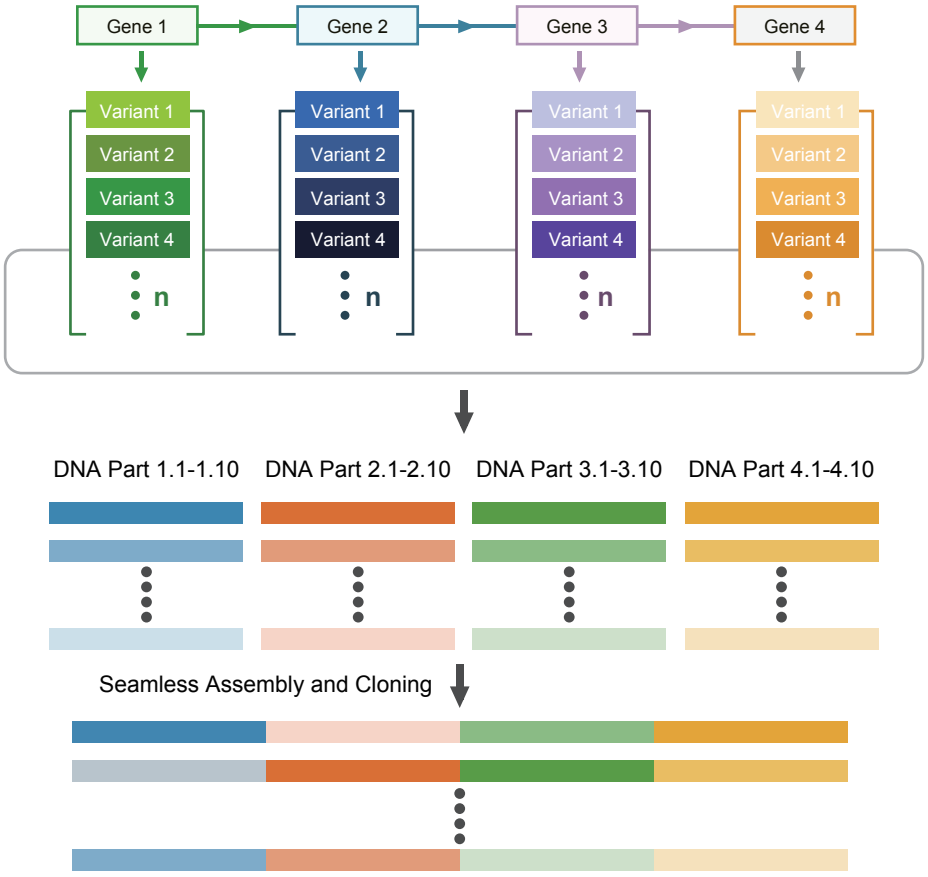


Figure 19. Construction of an arrayed library with 4 variable slots.

Different libraries can be constructed for investigating different applications, e.g. genetic circuit and pathway construction, to identify potential gene targets involved in diseases and how such gene targets can be modulated. (Bottom) Various DNA parts are seamlessly and combinatorially assembled and cloned into a specific vector to generate a large library of variant constructs, which can be delivered in a pooled or arrayed format. Instead of combinatorial assembly, the service is also customizable for assembling and cloning specific DNA parts to generate specific constructs as desired by customers.

Such arrayed library would be ideal for the testing of every single design of the library in every combination of up to 10,000 individual. A typical production workflow in producing such library is shown in **Figure 20**. Each sequence-verified individual construct is cloned into pUC57 or custom vectors, deliverable at 4 µg.

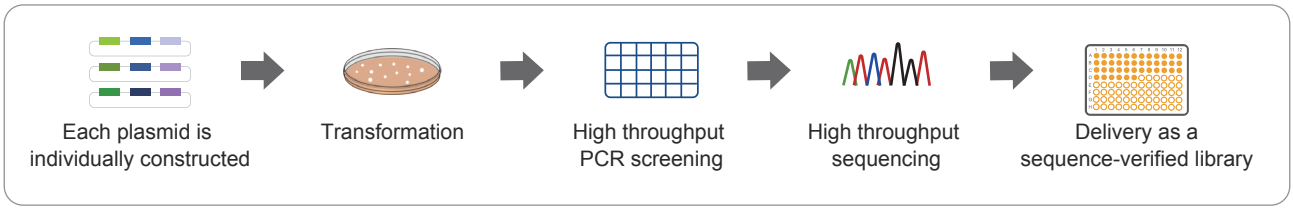


Figure 20. Workflow of arrayed DNA library construction and respective deliverables.

In addition, such arrayed library can be delivered as a pooled library format ideal for > 10^4 throughput screening and for screening platforms that are not sensitive to the presence of negative clones. Such pooled library will be PCR verified for more than 48 clones to determine positive rate. To ease out screening burdens, representative libraries can also be constructed for a first-hand sense of potential targets. This avails 10^2-10^4 throughput screening good for screening a pool of individual construct with no concern for the presence of exact sequence. Constructs are randomly picked and PCR verified individual constructs will be delivered. Such constructs are also guaranteed to contain all designed parts or modules (**Figure 21**).

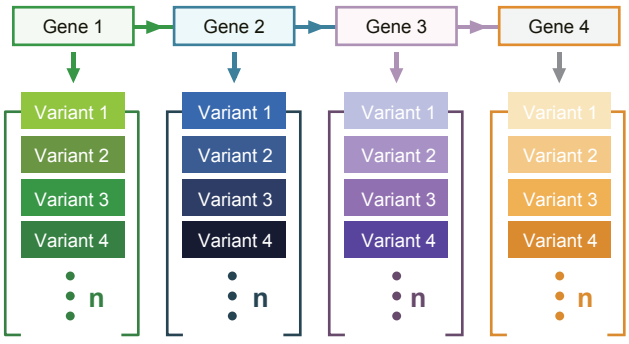
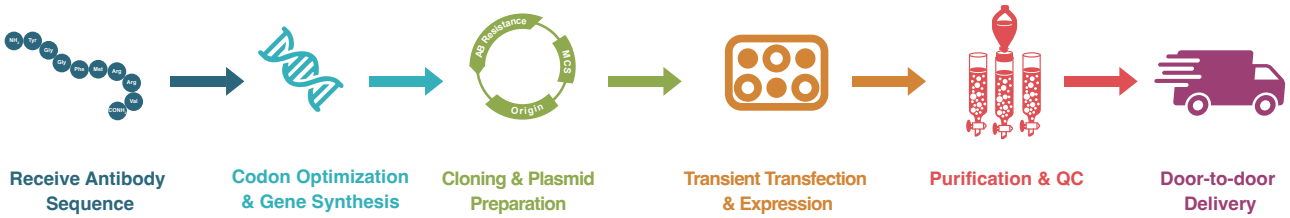
Target	Service items	Platform
scFc	VH/VL order（or mutation） GS linker optimization SSoptimization Extra AA introduction	
Hinge region	CD8a CD28 IgG1/IgG4(hinge-Fc part)	
Transmembrane domain	CD8a CD28 CD3zeta	
co-stimulator	4-1BB CD28 OX40	
CD3 zeta	immunoreceptor tyrosine-based activation motif (ITAM) mutagenesis	

Figure 21. The use of High-Throughput Combinatorial DNA Assembly Service to accurately and efficiently assembly and cloning of various predefined DNA fragments or parts into a vector of customer choice to generate a library of cloned constructs strategically assembled in a specific arrangement

3.3.3 High throughput expression of antibody targets

To screen for these various antibody leads, a high throughput expression of recombinant antibodies will be required. Due to the large number of potential targets to be tested and screened, a cost effective means of such expression is needed. In addition, to minimize the time required for screening, a fast turnaround time for will increase the efficacy of finding the optimized target.

In **GenScript**, we understand the needs for cost effective and fast protein expression and we have developed our proprietary CHO expression platform for the expression and production of these leads (**Figure 22 and Table 8**).



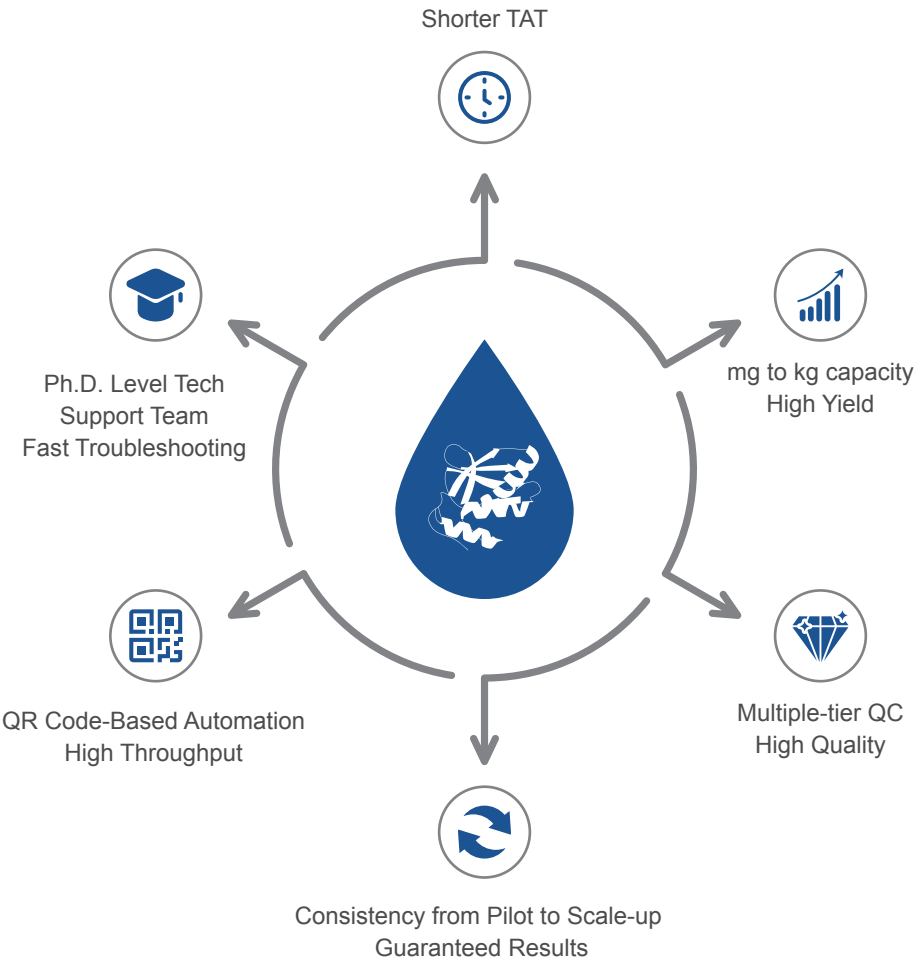


Figure 22. Workflow and highlights for recombinant antibody expression in GenScript.

	TurboCHO™ HT (High Throughput)	TurboCHO™ Express	TurboCHO™ HP (High Performance)
Requested Protein Amount	Expression screening or protein delivery (Target amount* 0.1-5mg)	500mg or less	500mg to kilograms
Production Time Starting from (gene synthesis included)	2 weeks	3 weeks	6 weeks
Average yield (antibody)	300mg/L	400mg/L	1.5g/L
Deliverables	Purity: ≥90% Endotoxin control*: ≤1EU/mg	Purity: ≥95% for antibody; >90% for protein Endotoxin level: ≤1EU/mg Conc.: ≥1.0mg/mL	
Default QC	SDS-PAGE, A280	SDS-PAGE, SEC-HPLC, endotoxin	
Additional QC and Characterization	Endotoxin*, LC-MS* SEC-HPLC	LC-MS	
	Affinity test, cross reactivity, epitope mapping, in vitro and in vivo efficacy, stability test by freeze-thaw cycles, sterility test and more		
Purification	Standard 1-step purification (optional*: 2 or 3 steps)	Multiple purification steps included	

Table 8. Various expression volume with high throughput expression TAT as short as 2 weeks and endotoxin control at < 1 EU/mg good for preclinical testing.

3.4 Preclinical Validation and Production of Gene Therapy and CAR-T cell therapy

Upon the target identification and lead optimization, identified targets will be ready for preclinical validation and even downstream production if results in the preclinical and clinical validation are promising. Genes can be introduced in vivo, by directly introducing into the human body, or ex vivo, where specific cells, e.g. T cells are collected, and gene engineered in vitro into cells, expanded before its reintroduction into the patient, in an autologous manner (**Figure 23**).

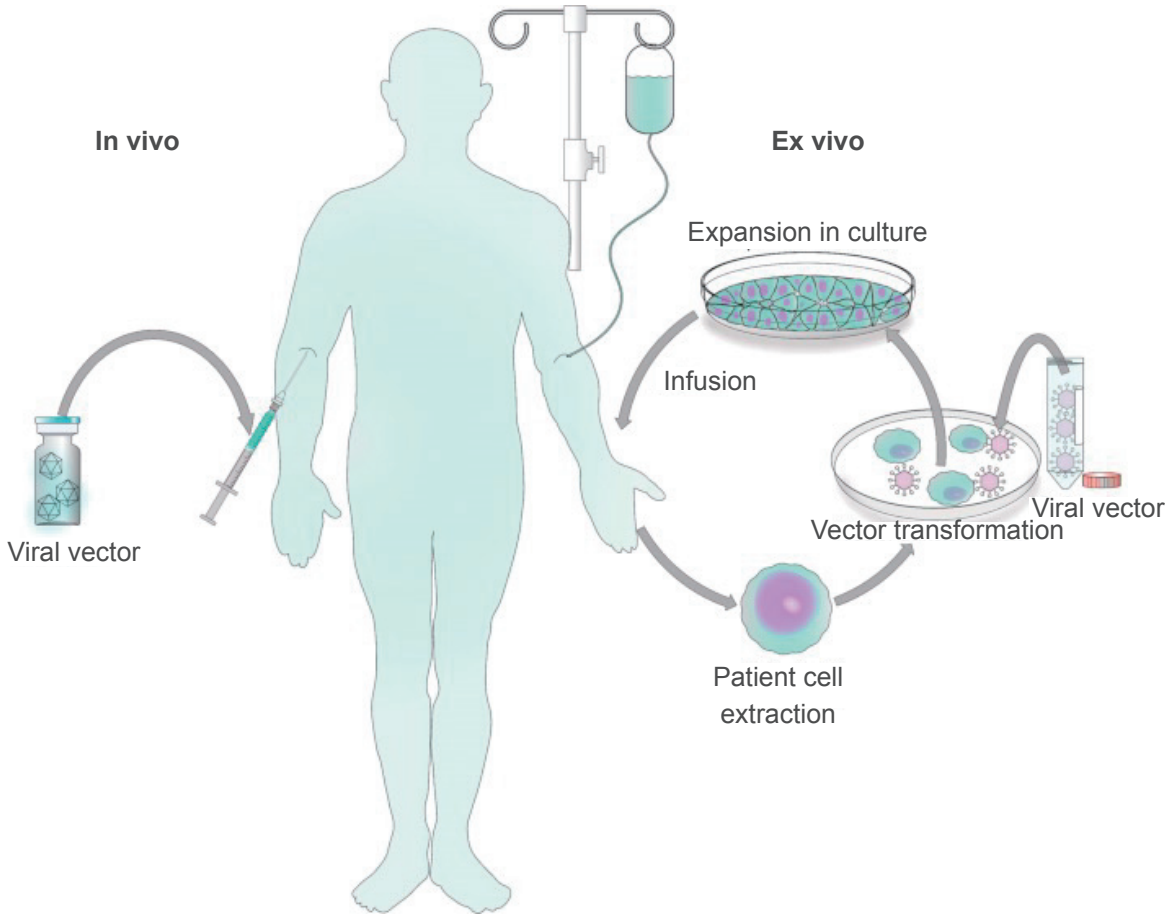


Figure 23. Different ways for introduction of genes into patient. Diagram adapted from Bucha et al., 2021.

One of the major limiting factor is how to effectively introduce these genes or CARs into the cells in a cost effective manner. Due to the nature of the treatment, such production of these therapies are required to be performed under Good Manufacturing Practice (GMP) manner and it could be costly.

Currently, there are several modes of gene delivery, i.e. via i) viral vectors or ii) non-viral vectors such as liposomes, cationic polymers, dendrimers, CP peptides, transposons or RNA transfection. Genes can also be transferred via physical means by electroporation, gene gun, microinjection, ultrasound or hydrodynamic delivery. Despite potential issues with toxicity, viral vectors are still preferred due to their long term expression, stability and integrity. The permanent transgene expression is much required to ensure a durable clinical response in the gene or CAR-T cell therapy. In fact, the preference to virus is also due to the fact it has long been practiced. Considerable clinical evidence shows that retroviral vectors are safe in human T cells, however they are considered less safe when used in human stem cells. In contrast, lentiviral vectors, especially, the third generation self-inactivating vectors have a lower risk of insertional mutagenesis due to the absence of strong enhancer element unlike that in retroviruses. Besides, lentivirus, adeno-associated virus is also used for gene transfer due to their high stability and safety and efficacy. Non-viral methods commonly used are transposons method and RNA transfection. Each of the methods have their pros and cons and are chosen based on the needs of the project. **Table 9** illustrate the advantages and disadvantages of using each system.

Viral or non-viral vectors	Method	Advantages	Disadvantages
Viral	Retroviral	1) Long experience in use 2) High expression 3) Large lots available	1) In-vivo safety concerns 2) Long term monitoring of recipient 3) Complex manufacturing 4) Possibility of replication competent virus
	Lentiviral vector	1) Transduce non-dividing cells 2) Safety 3) High efficiency 4) Stable expression 5) Can be pseudotyped to broaden tropism 6) High vector titer 7) Less risky integration pattern by stably integrating transgene into target cell without transferring sequence from packaging virus	1) Limited lot sizes 2) Long term monitoring of recipients 3) Complex manufacturing and testing
Non-viral	Adeno-associated virus	1) Long lasting expression 2) Wide host range 3) High stability, safety and efficacy	1) Small packaging capacity
	Transposon/ Transposase system	1) Simpler manufacturing 2) Simpler testing 3) Lower cost than viral vectors	1) Potential oncogenic risk
	mRNA transfer	1) Simple endocytosis/ electroporation 2) No genomic integration 3) No risk of replication competent virus	1) Transient expression

Table 9. Advantages and disadvantages of different gene transfer method.

3.4.1 Lentivirus packaging

As mentioned, the use of viral method is still preferred due to the long history of usage and the ability to have a more stable expression. Due to its ability to integrate a significant amount of viral cDNA into the DNA of the host cells and also can efficiently infect non-dividing cells, they are one of the most efficient methods of gene delivery.

The lentivirus genome usually comprises three open reading frames (ORFs):
(1)Group-specific antigen (gag): Encodes structural proteins that form the viral capsid
(2)Polymerase (pol): Encodes the reverse transcriptase, protease and integrase enzymes
(3)Envelope (env): Encodes the viral envelope (surface and transmembrane glycoprotein) proteins

Lentiviruses that have been developed as gene transfer vectors include mainly HIV-1 and HIV-2, but HIV-1 is the one that is very well-studied. Most of the others have not yet reached the clinical study stage.

Considering the safety issue of the pathogenicity of HIV-1 in humans, different generations of LVV systems have been developed by modification of the helper plasmids and the existing genes. The first generation contains some HIV accessory components and have the risk of generating replication competent lentivirus. To improve on this, both the second and third generation have the unnecessary accessory genes of HIV-1 (vif, vpr, vpu and nef) removed. Different from the second generation, the third generation is made even safer by placing the rev gene on a independent helper plasmid. The regulatory tat gene is also eliminated as its transacting function is deem dispensable (**Figure 24**).

	2nd Generation	3rd Generation
Deletion of U3 region of 3' LTR Deletion of 5' LTR	NO	YES
tat and rev genes	On a single packaging plasmid	rev on a single plasmid; tat is removed
gag and pol genes		gag and pol on a single plasmid
Expression of transgene	Driven by tat dependent WT promoter	tat independent Driven by other viral promoter (i.e.CMV)
Total number of plasmids	3	4

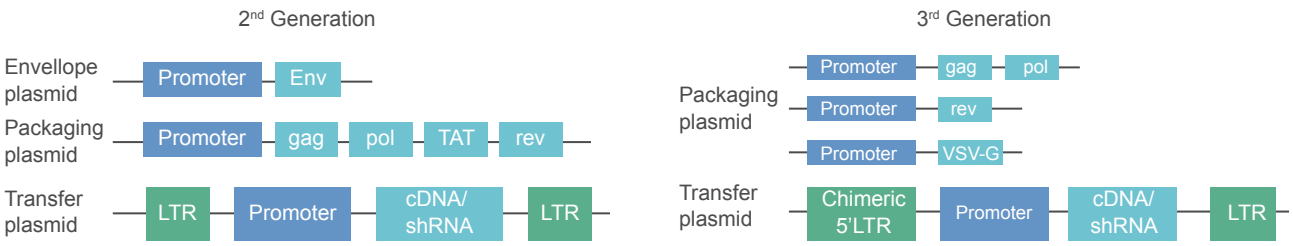


Figure 24. Differences between 2nd and 3rd generation lentivirus system.

In GenScript, we provide lentivirus packaging of the 3rd generation to ensure the downstream safety issues required by GCT. We provide both research grade, pro-GMP grade for non-clinical usage and also GMP grade lentivirus. The lentivirus produced are good for transducing hard to transduce cells and also T cells (**Figure 25**).

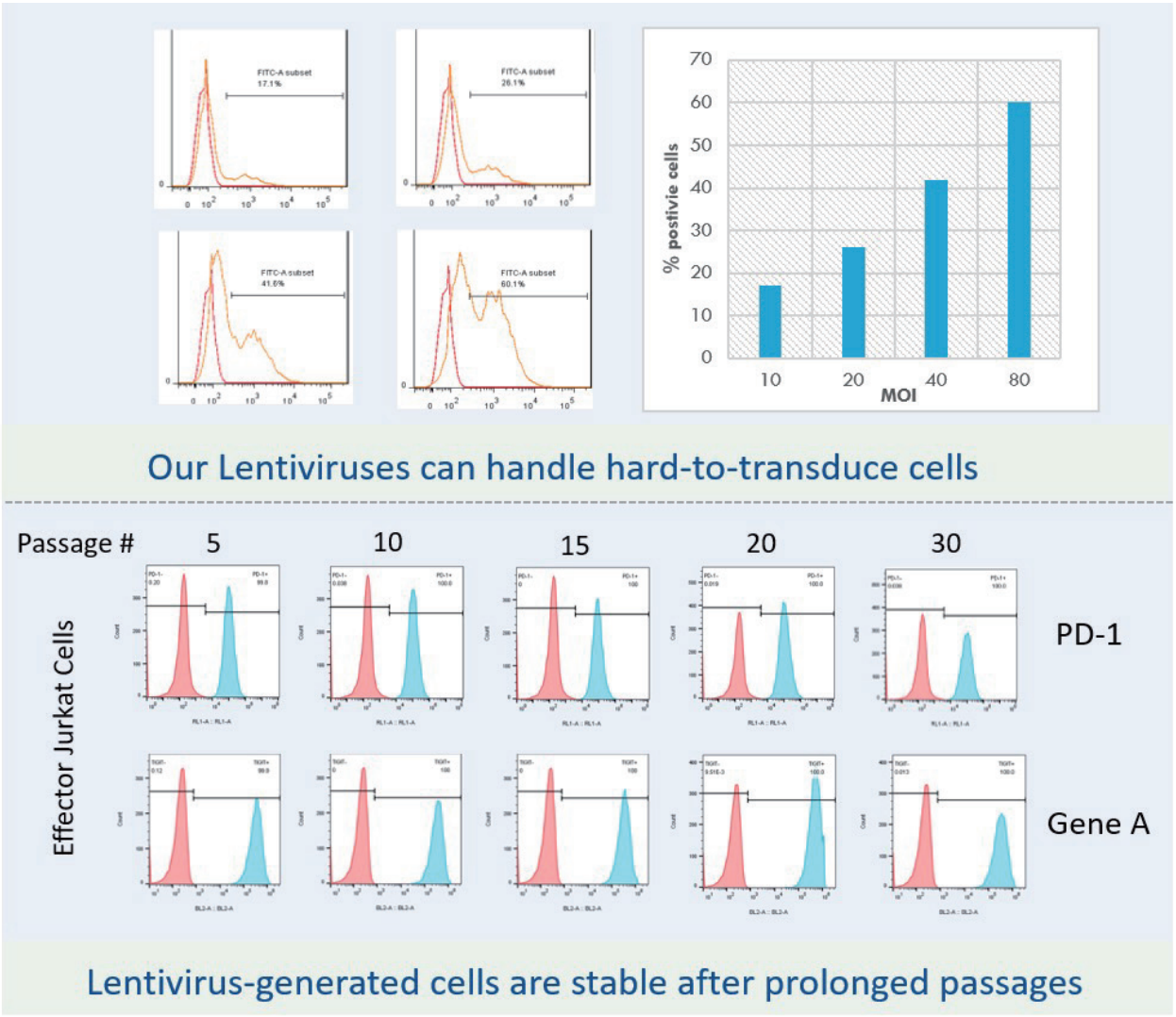


Figure 25. Efficiency of transduction from lentivirus produced in GenScript.

In GenScript Probio, we have years of experience in lentiviral vectors production, especially those with better T cell transduction efficiency. We have a manufacturing scale of up to 200 L. Due to the difficulties in establishing stable lentivirus production cell lines, transient transfection of human embryonic kidney 293 (HEK293) or HEK293-derived cells with vector and helper or packaging plasmids is the most widely used method for generating lentiviral vectors. This is what GenScript ProBio lentiviral vectors services are based on. We have also both the adherent and suspension cell types to cater to different manufacturing scales and needs. **Figure 26** and **Table 10** shows the differences in adherent and suspension cell culture production.

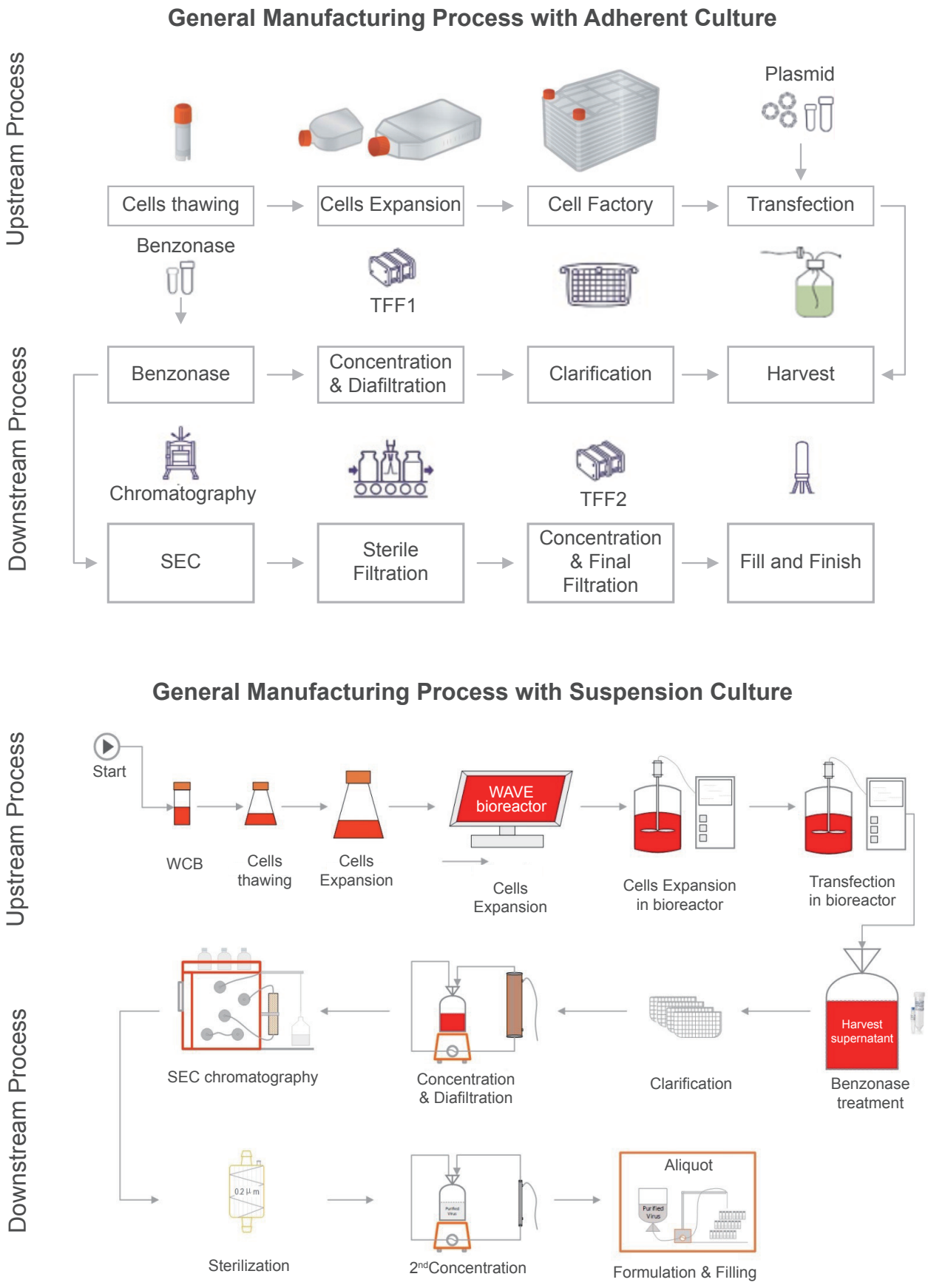


Figure 26 Workflow in generating lentivirus using adherent cell culture (*Top*) and suspension culture (*Bottom*).

Items	Adherent	Suspension
Definition	A type of culture in which cells are grown in a single layer on a flask or Petri dish containing the culture medium	A type of culture in which single cells or small aggregates of cells multiply while suspended in an agitated liquid medium.
Culture Medium	The cells require a substrate for attachment	The cells **do not require a substrate for attachment.**
Passaging	Requires periodic passaging; Detach the adherent cells from a surface and plating onto a new surface in a fresh medium.	Easy to passage; Dilute into the fresh medium.
Scale Up	Difficult in scaling up	**Easy to scale up**

Table 10. Differences between using adherent cell culture and suspension cell culture.

Although adherent cells has a mature methodology in producing lentivirus, it is limited in scalability. In addition, suspension cell culture also uses serum-free media, which is more ideal for clinical manufacturing as this decreases the risk of contamination by adventitious agents. As such, for large-scale production of lentivirus, suspension cells would be in inevitable tendency in the near future. As such, in ProBio, we have developed our PowerS™-293T system to have a superior yield improvement with 50% unit cost reduction (**Figure 27-29**). Our PowerS™-293T can be licensed for IND, clinical and commercial application. It is royalty free. Cell lines with a clear traceability will be a critical issue for regulatory compliance. We have also established processes for cell bank management under GMP for adherent processes. This is good for adeno-associated virus generation and such service of AAV generation is also available.

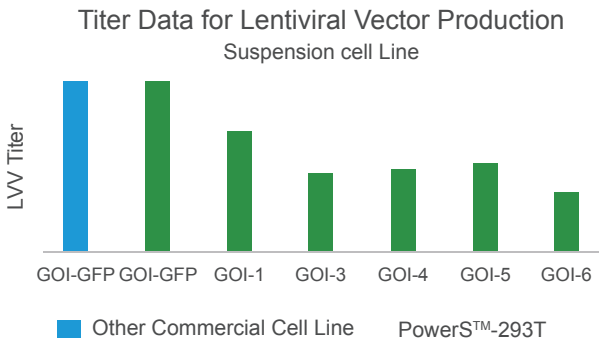


Figure 27. Lentiviral vector titer comparison with different GOI in PoweSTM-293T relative to commercially available suspension cell line.

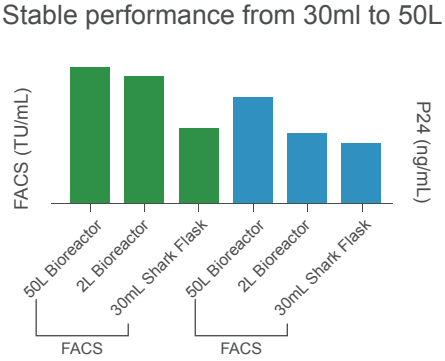


Figure 28. Titer data of lentiviral vector in 30 ml shaker flask, 2 L bioreactor and 50 L bioreactor.

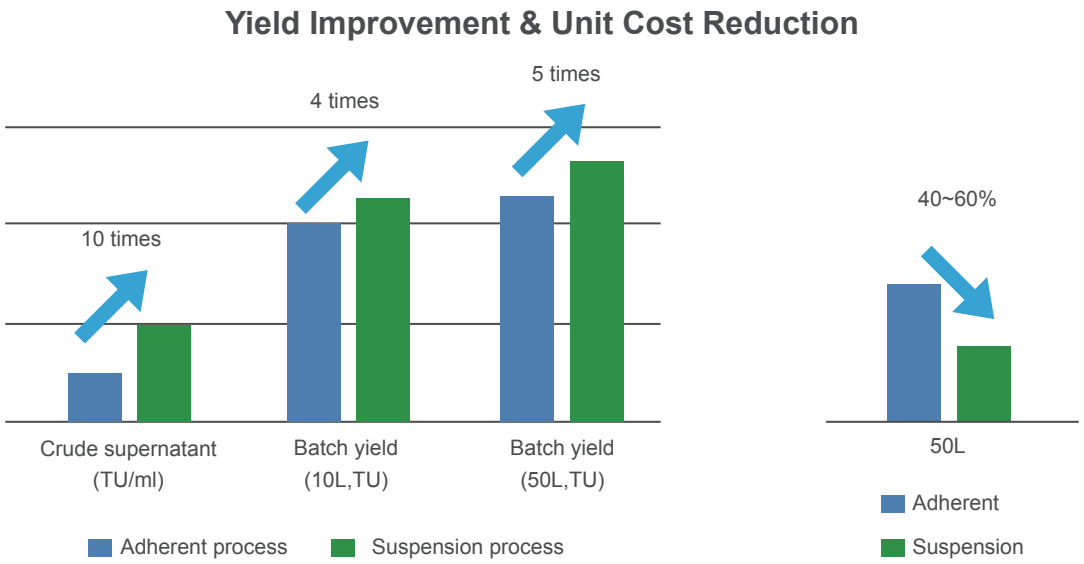


Figure 29. Comparison of titer, yield and cost of adherent and suspension processes.Left: crude titer improved 10 times from adherent to suspension process while the batch yield of 10 L and 50 L also improved 4-5 times. Right: the unit cost of suspension process reduced 40-60% compared to adherent process.

3.4.2 AAV packaging

AAVs are small single stranded DNA virus. They are low immunogenicity and lacking of pathogenicity. AAVs infect specific tissue and cell types based on different serotypes used which reduce the chance of non-target cell type infection. AAV delivered transgenes remain episomal and avoid the random insertional mutagenesis to occur. Therefore, AAVs are highly efficient gene delivery vehicles used in gene and cell therapy/engineering application. GenScript provides RUO AAV packaging service, we have optimized the production workflow to achieve higher viral purity AAV packaging for different serotypes (**Figure 30**).

In addition to producing AAV packaging, we also provide plasmids for AAV packaging. Of concern, the sequence accuracy of the inverted terminal repeats (ITR). The ITR regions consists of the elements for virus replication and encapsidation. In a AAV plasmid, there are two ITR sequences of 145 bases each. The DNA sequence between the ITRs is what gets packaged into the AAV molecules. Unfortunately, studies have shown that the ITRs are inherently unstable and prone to the formation of highly-stable secondary structure as a result of palindromic nature, and high GC content. This instability can cause partial loss of an ITR during routine plasmid propagation, with complete ITR loss abolishing AAV packaging. Thereby, several approaches are used in order to prevent the ITR loss or to ensure the quality, including the use of recombination-deficient bacterial strain, such as StbI3s, the use of restriction enzyme, like SmaI, that cut the RE site within the ITR region or Sanger sequencing. But, even so, challenge occurs particularly sequencing fails to sequence through ITR due to their secondary structure and high GC content.To overcome this issue, GenScript developed a Sanger-dependent ITR specific sequencing kit (**Figure 31**) that can sequence through the whole ITRs to determine its integrity with good signal providing good quality of AAV plasmids (**Figure 32**).

3.4.2.1 Advantages

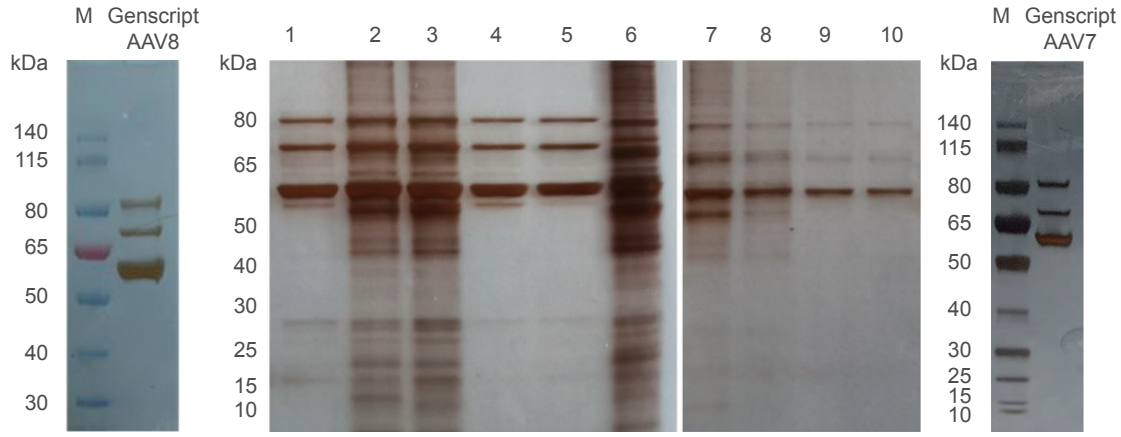
Special protocol for ITR Sanger

High purity and quality of AAV packaging

High success rate via GenITRv1 strain

Super stringent QC for guaranteed quality

3.4.2.2 Outcomes



	AAV 8 Produced by Competitor		AAV 7 Producced by Competitor
1	pAAV-CAG-CAN5 WT-P2A-EGFP-polyA-AAV8	1	pAAV-CAG-CAN5 WT-P2A-EGFP-polyA-AAV7m8
2	AAV-CAG-EGFP-polyA-AAV8	2	AAV-CAG-EGFP-polyA-AAV7m8
3	AAV-CAG-CAN5 R289W-P2A-EGFP-polyA-AAV8	3	AAV-CAG-CAN5 R289W-P2A-EGFP-polyA-AAV7m8
4	pAAV-CAG-CAN5 R243L-P2A-EGFP-polyA-AAV8	4	pAAV-CAG-CAN5 R243L-P2A-EGFP-polyA-AAV7m8
5	pAAV-CAG-CAN5 G267S-P2A-EGFP-polyA-AAV8	5	pAAV-CAG-CAN5 G267S-P2A-EGFP-polyA-AAV7m8

Figure 30. Quality comparison between GenScript synthesized AAV packaging with competitor. From silver staining results, GenScript synthesized AAVs have higher purity and quality.

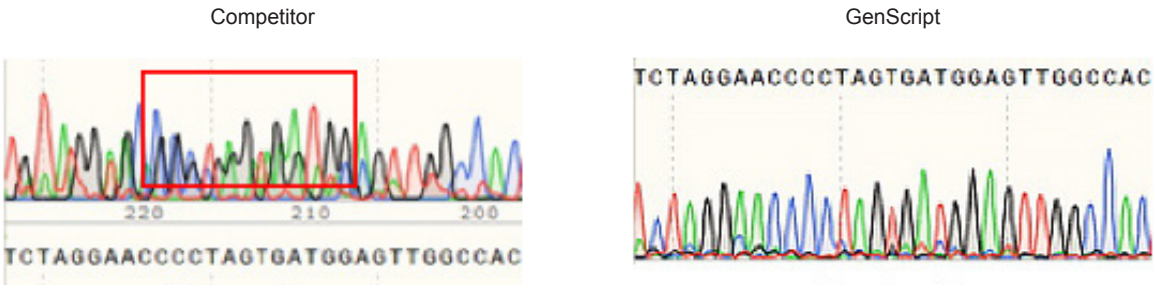


Figure 31. High stringency of GenScript's ITR sequencing compare to competitor.

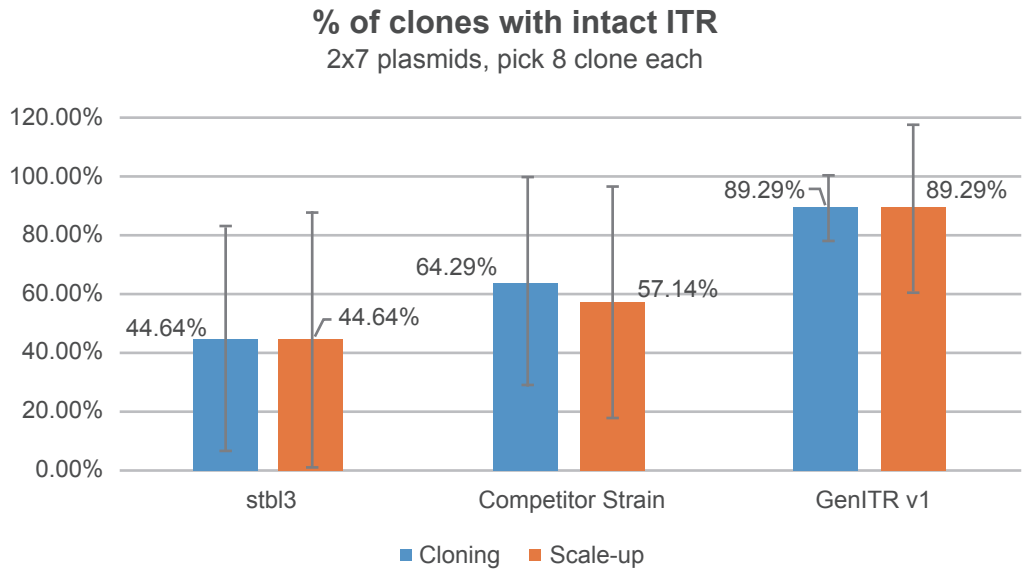


Figure 32. High quality of AAV plasmids.

3.4.3 T cell engineering using CRISPR and homology-directed repair (HDR)

In addition to providing more traditional way of CAR introduction into T cell via lentiviral vectors, GenScript also strive to overcome some of the concerns using viral vectors, such as increase risk of insertional mutagenesis, high cost and long lead time for manufacturing. As such, GenScript had recently worked with Dr Shy et al. to produce a non-viral delivery of CRISPR/Cas9 editing tools and payload template DNA to enable site-specific transgene insertion via homology-directed repair (HDR) with minimized toxicity to reduce regulatory concerns. One of the limitations in introducing double-stranded DNA HDR templates is the toxicity at higher dsDNA concentrations, thus reducing yield and efficiency.

As such, Dr. Shy *et al.*, had developed a hybrid single –stranded DNA ssDNA HDRT with Cas9 target sequences (ssCTS) to boost knock-in efficiency by > 5 fold and live cell yields by > 7 fold. This takes the advantages of using ssCTS's low toxicity at high concentration. However, the challenge is in the production of long ssDNA sequences. Through collaboration with GenScript, we developed these long ssDNA sequences and were able to perform therapeutic gene editing in primary T cells (**Figure 33-36**).

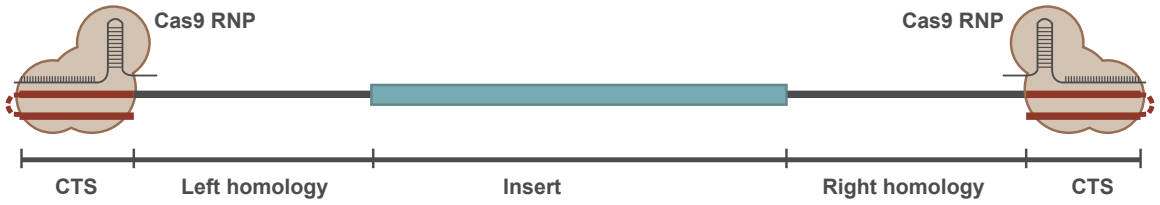


Figure 33. Hybrid ssDNA HDR template (HDRT) structure comprising small stretches of dsDNA incorporating the Cas9 target sites (CTS), through annealed complementary oligonucleotides, in addition to the homology arms on each end of the gene insert.The added CTS can interact with Cas9 ribonucleoproteins (RNPs) to shuttle the template to the nucleus and enhance HDR efficiency.

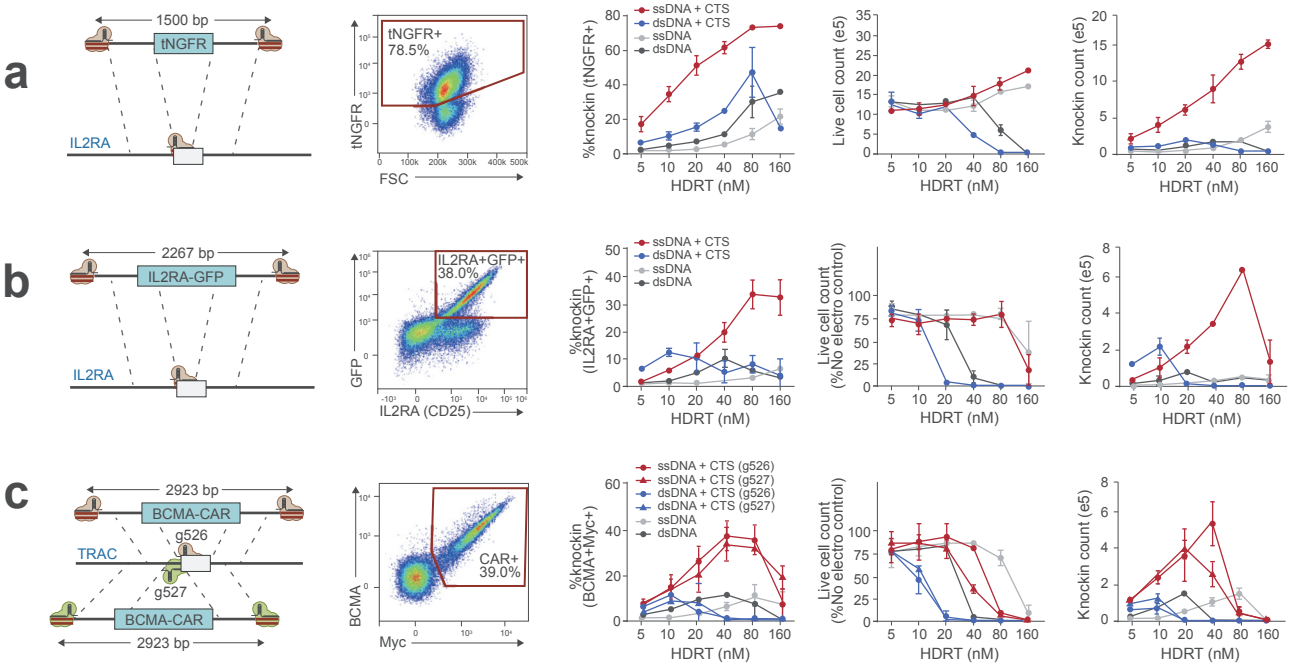


Figure 34. Development of large ssCTS templates for different gene knock-in across different target loci for high-efficiency and low-toxicity HDR in primary human T cells.

On the above Figure, (a) tNGFR knock-in at the IL2RA locus at 78.5% knock-in efficiency with a ~1.5 kb tNGFR construct. (b) IL2RA-GFP fusion protein knock-in at the IL2RA locus at 38% knock-in efficiency with a ~2.3 kb IL2RA-GFP construct. (c) Two different HDRTs inserting a BCMA-CAR construct at TRAC locus via two different gRNAs (g526 and g527) at 39% knock-in efficiency with a ~2.9 kb BCMA-specific CAR construct. The use of ssCTS with large ssDNA constructs outperformed all other templates with higher knock-in efficiencies and lower toxcitiy, including dsCTS templates.

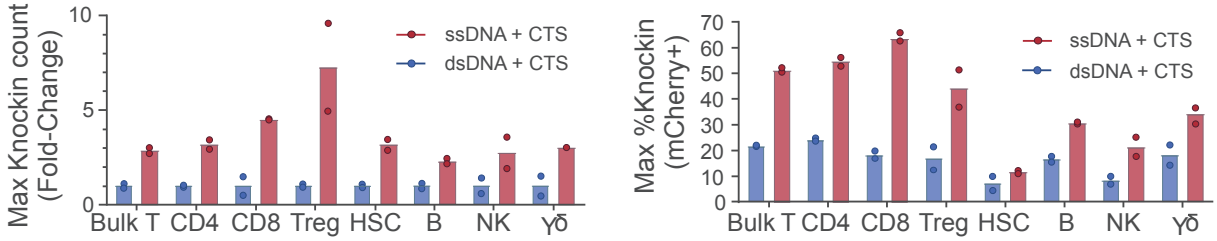


Figure 35. Evaluation of performance of ssCTS templates across a variety of clinically relevant primary cell types including CD4+ T cells, CD8+ T cells, regulatory T cells (Treg), NK cells, B cells, hematopoietic stem cells (HSC) and gamma-delta T cells using a mCherry knock-in construct targeting the clathrin light chain A (CLTA) gene.

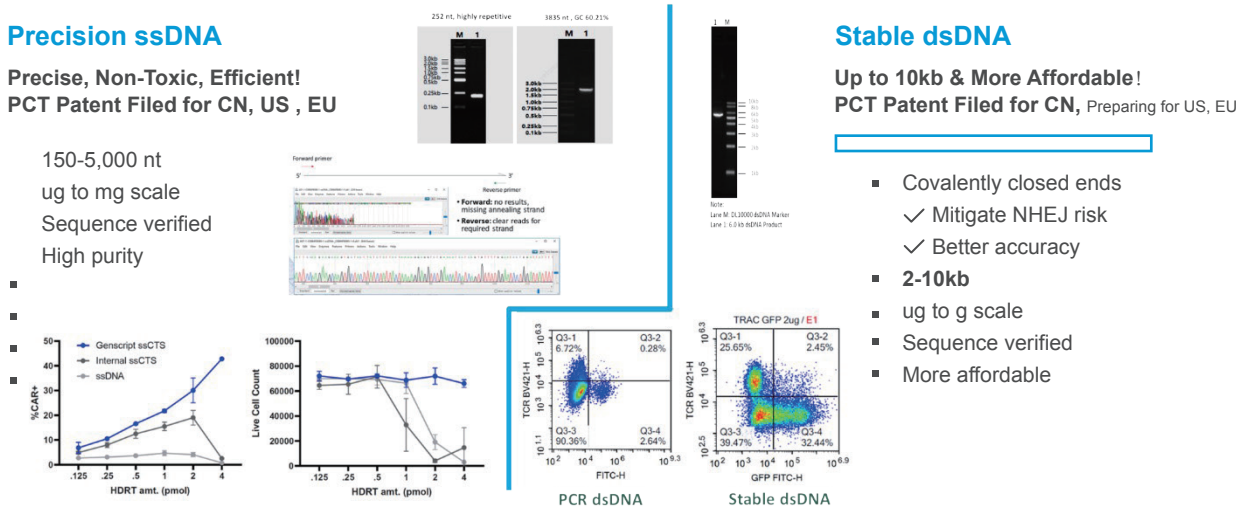


Figure 36. Highlights of ssDNA and dsDNA production in GenScript.

3.4.4 Plasmids production

The most commonly used material in gene therapies development is plasmid. It is also the key building blocks for viral vectors production. Furthermore, plasmids are one of the crucial elements in cell therapies development, in which it is used for genetic modification of cells, such as CAR-T cell development. Truly, the demand for plasmids has greatly increased as a result of the booming of gene and cell therapy.

Nonetheless, the production of plasmids faces several challenges. A high-quality of plasmid DNA is required for gene and cell therapy. This has led to the need to optimize manufacturing in order to meet the demand for volume and quality. Parameters include the size, shearing sensitivity, viscosity and impurities need to be well taken into accounts during the production, which may lead to a longer lead time.

Another major concern is the regulatory for plasmid production. Currently, the framework is confusing for the industry. Despite the fact that the main guidelines refer to the quality of the product, more clarity is definitely required to define the level of plasmid quality required for drug development.

GenScript's Plasmid DNA Preparation Service is accommodating to both small research labs as well as large-scale manufacturing biotech and pharmaceutical companies, providing you with high quality plasmids with 100% full insert sequence accuracy (**Figure 37-39**).

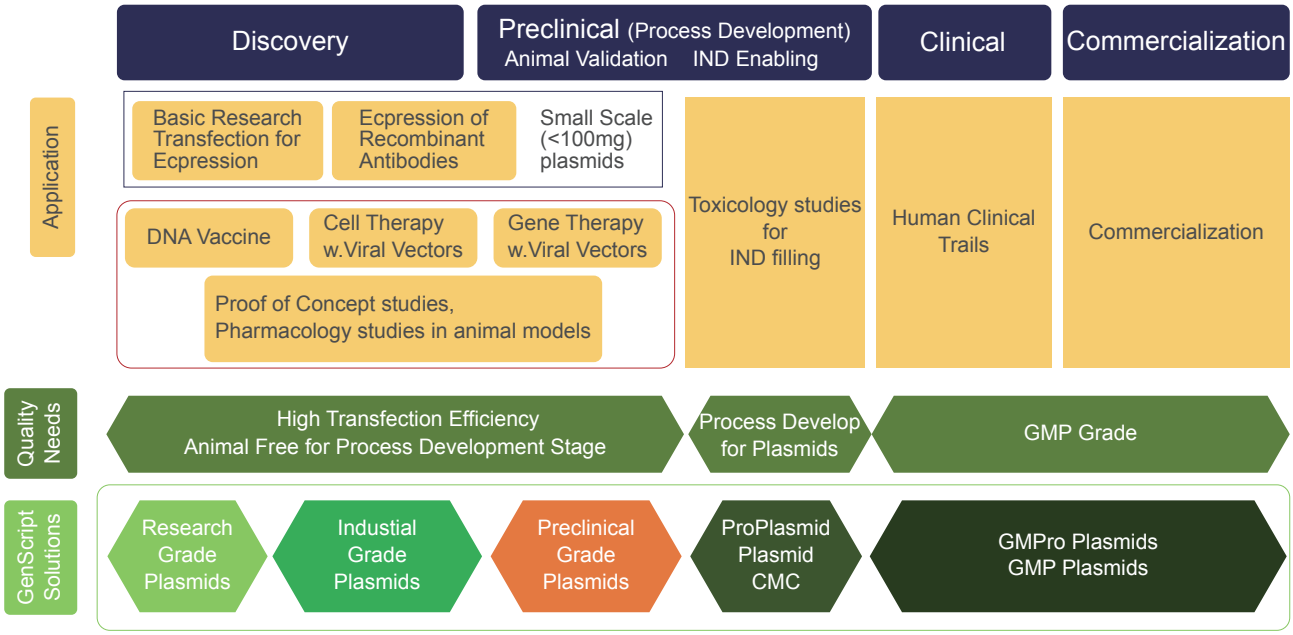


Figure 37. Different grades of plasmid production available catering to different applications and needs.

Research Grade

Industrial Grade

Pre-clinical Grade

Basic Research	Early Drug Discovery	Pre-clinical Studies
Target identification *In-vitro and In-vivo testing*	**Lead optimization** *In-vitro and In-vivo testing*	**Efficacy and Safety** *In-vitro and In-vivo testing*
Customer needs · Price · Speed	**Customer needs** · Accuracy · Quality	**Customer needs** · Accuracy · Quantity · Quality
GS offers · Fast TAT starting from 3 days,>98%OTD · Predominantly supercoiled	**GS offers** · High quality plasmid prep · Endotoxin free, ≥90 supercoiled	**GS offers** · Stringent QC with advanced add-on · Animal-free production · Large fermenter for speedy production
Hit discovery Assay development and screening High throughput screening Hit to lead	Lead discovery Lead validation High throughput screening Assay development and screening	Proof of concept Pharmacology and toxicology Animal models and cell lines Evaluate for clinical trials
Research grade HT plasmids	Industry grade Endotoxin free, high supercoil	Pre-Clinical grade Contamination ctrl, ITR sequencing

Figure 38. Highlights of each grade of plasmids and their respective usage.

Dvelopment Cycle	① Pilot Study	② IND	③ Early stage Clinical trials	Late stage clinical trials ④ Commercialization
GenScript ProBio's Offerings	**Pro Grade** • ProPlasmid	**CMC** • Plasmid CMC	**GMP Compliant** • GMPro Plasmid	**cGMP** • GMP plasmid
	• Manufacturing Environment • Antibiotic free • QC • Documentation	• Process Development	• Early stage clinical Phase 1 & 2	• Genopis • Phsae 3/DNA vaccine
	• TAT 5-6 weeks	• ~6-7-weeks	• 8-10 weeks	• 12-14 weeks

Figure 39. Production of GMP grade plasmids and their expected TAT.

3.4.4 T-cell isolation for CAR-T cell therapy

One of the most important component in CAR-T cell therapy is the isolation of T-cells. Work is still in progress to determine the best collection methods to optimize T cell yield. Many considerations had to be taken to ensure the availability of good quality T cells. Such considerations in includes i) care to be taken to prevent contamination, ii) medical conditions of donor that could affect yield and purity, iii) disease status, iv) contaminants such as red blood cells and v) ability to enrich T cell population.

It is generally believed that the efficacy of CAR-T cell therapy is often attributed to CD8 T cells. However, CD4 T cells are also known for their helper function and to evoke cytolytic™ activities by enhancing CD8 T cells activity through cytokine™ production. Moreover, CD4 CAR-T cells also show initial slower granzyme B secretion and tumor killing but are less prone to AICD and exhaustion compared to CD8 counterparts. As such, CD4 CAR-T cells have better persistence following antigen exposure. Having a 1:1 ratio of CD4:CD8 CAR-T cells also confers better antitumor reactivity.

As such in GenScript, we have developed our proprietary CytoSinct™ Nanobeads to enrich the CD4 and CD8 T cells. The CytoSinct™ Nanobeads are nanometer-sized and biodegradable making them compatible to most downstream application, e,g, cell culture, activation, expansion, flow cytometry analysis and translational research. Starting materials could be either PBMCs or leukapheresis products. Based on immunomagnetic separation technology to combine specificity and sensitivity of antibody-based purification with the gentleness of nanoparticles and ease and speed of magnetic separation. To make it flexible for researchers, the CytoSinct™ NanoBeads and Columns are compatible with other column-based cell separation platforms, simplifying cell separation procedures. The Cytosinct™ beads are efficient means of CD4 and CD8 viable and activatable cell separation (**Figure 40**).

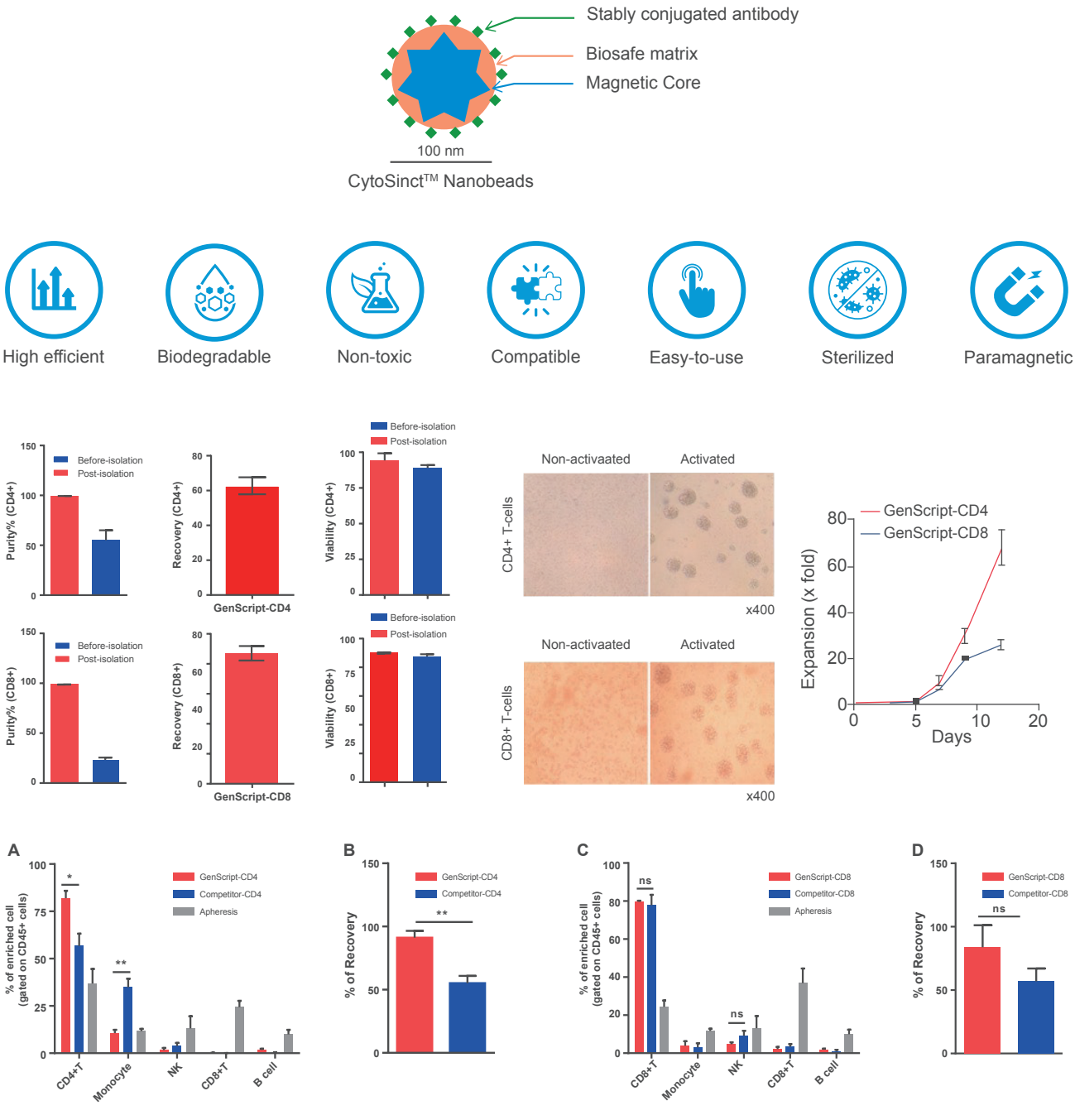


Figure 40. Capabilities of Cytosinct™ Nanobeads in CD4 and CD8 cell separation.

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TCR-T/CAR-T Proliferation

Cytokines & Growth Factors

IL-2, IL7, IL15, IL21

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PD-1, PDL1, 4-1BB
Claudin 18.2

LVV Lentiviral Vector IND enabling (CMC)

LVV Lentiviral Vector Manufacturing

Plasmid Manufacturing

Plasmid IND enabling (CMC) services

Cytokines & Growth Factors

IL-7, IL15, IL21, IL2 (RUO)

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