GO BIGGER, STAY ON TARGET, AND DEVELOP THE CELL LINES THAT GET THE JOB DONE

TARGATT™ Large Knock-in Technology

Build the most relevant cell line for your project with the ability to insert 50+ kb of DNA site specifically, in single copy, and at efficiencies approaching 100% after selection.

Controlled knock-in for speedy mammalian cell line development

While CRISPR has revolutionized the life science industry, it's not the right tool for every gene editing project. With a dependence on host DNA repair factors, low knock-in efficiency for DNA fragments above 3-5 kb, and the risk of off-target events related to induction of double-strand breaks, CRISPR can be challenging and time-consuming to use for mammalian cell applications requiring large knock-ins and high safety.

These applications include building mammalian cell lines for:

Protein Expression & Co-expression

- Expression/over-expression of proteins, including large proteins
- Expression of challenging proteins such as membrane proteins
- Co-expression of multiple proteins
- Co-expression of proteins at strict stoichiometries

Screening & Display

- Consistent protein expression across clones for pooled screening
- Mammalian display and antibody screening
- Promoter screens

Therapeutic Development

- Cell therapy development

As a company dedicated to enabling allogeneic cell therapies, we knew we needed a better tool for safely and efficiently inserting DNA into mammalian cells, which is why we created TARGATT™ gene knock-in technology.

ACCELERATE APPLICATIONS

Express large mammalian proteins that are challenging for bacterial and yeast systems

Use pool-based screening approaches with the confidence of equal expression from each clone

Co-express proteins at defined stoichiometries

Screen in human cells for more relevant hits

	TARGATT™	CRISPR	Lentivirus	Transposon
High Efficiency	✓		✓	✓
50+ kb Insert	✓			✓
Site Specific	✓	✓		
Single Copy	✓	✓		
Negligible Off-Target Events	✓			
Clear IP for Commercialization	✓			

The TARGATT™ technology system

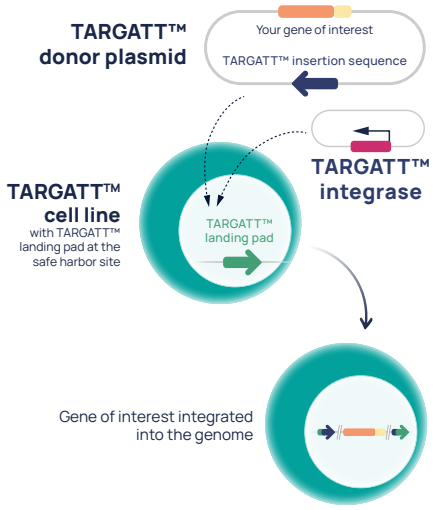


Figure 1. TARGATT™ technology consists of a donor plasmid, TARGATT™ landing pad, and TARGATT™ integrase.

TARGATT™ large DNA knock-in technology is a three-component system that consists of the TARGATT™ integrase, the TARGATT™ landing pad at the H11 safe harbor site, and a donor plasmid that contains your gene of interest and a TARGATT™ att site (Figure 1).

The TARGATT™ integrase enables rapid, efficient, and site-specific insertion of large DNA fragments into the genome—up to 50 kb in a single reaction and much larger insert sizes with nested reactions. Because integration is precise, single copy, and targeted to a defined site, we can avoid the challenges encountered with random integration mechanisms such as position effects, gene silencing, and gene instability due to the integration of multiple transgene copies.

An added advantage of TARGATT™ technology is its unidirectionality, which makes it more stable and efficient than Flp- and Cre-based integration systems.

Located in an intergenic region of human chromosome 22, the H11 safe harbor site provides a convenient locus to insert DNA without disrupting existing genes or their transcription. We've completed numerous projects that show robust and reliable expression at the H11 site for constructs with demonstrated expression.

How TARGATT™ technology works

TARGATT™ integrase binds the att sites located in the landing pad and the donor plasmid, and mediates a site-specific recombination event that results in the stable insertion of a single copy of the donor DNA into the genome at the landing pad (Figure 1).

We also offer recombinase-mediated cassette exchange (RMCE) configurations to avoid inserting bacterial sequences from your cloning plasmid into the genome (Figure 2).

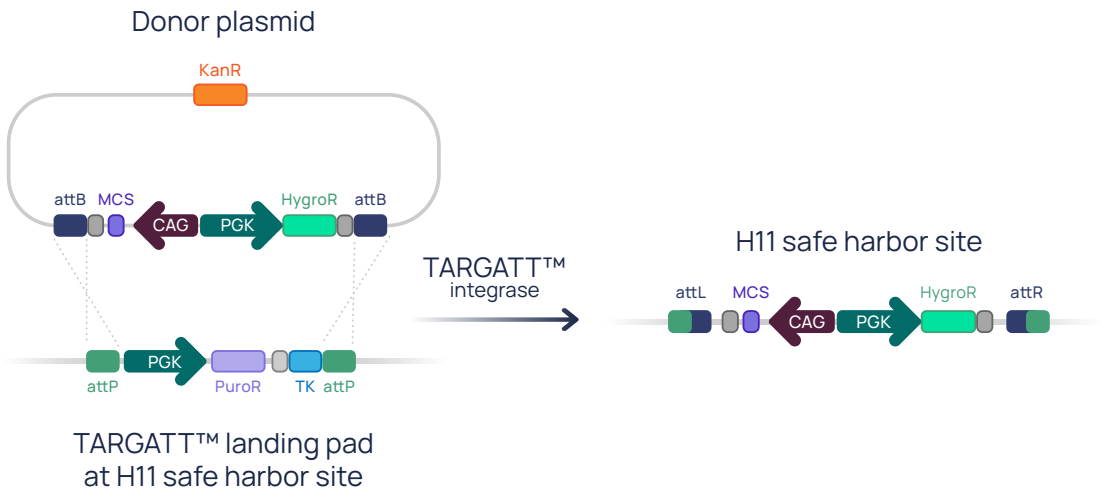


Figure 2 TARGATT™ technology configured for RMCE

Ready-to-use TARGATT™ kits enable single-copy gene insertion at the H11 safe harbor site for stable and reliable expression

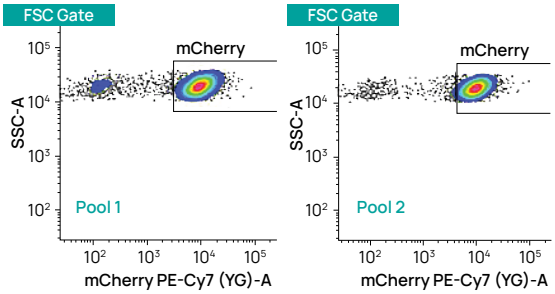
While our service team is happy to create a custom cell line with the TARGATT™ landing pad at the location of your choice in the cells of your choice, we offer proven, ready-to-use TARGATT™ cell lines with the landing pad at the H11 safe harbor site in iPSCs, CHO K1, and HEK293 cell lines. Expression from the H11 locus is consistently robust (Figure 3), single copy (Figure 4), and efficient for large DNA insert sizes even without selection (Figure 5).

Figure 3. TARGATT™ technology delivers efficient integration with consistent expression.

We inserted a construct expressing mCherry into the TARGATT™ landing pad at the H11 safe harbor site in HEK293 cells and used flow cytometry to assess mCherry expression in two pools of integrants.

The high integration efficiency after drug selection can be seen in the large number of mCherry-expressing cells.

The reproducibly tight clustering of the mCherry signal demonstrates the consistency of expression from the H11 locus.



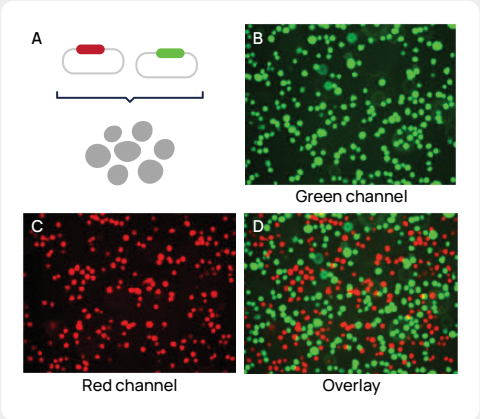


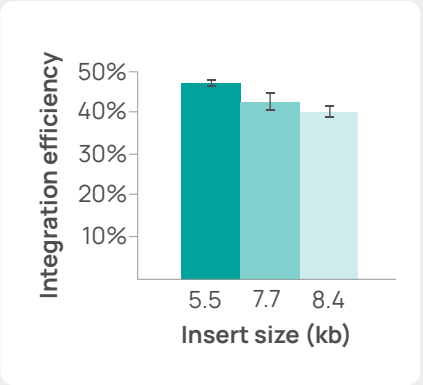
Figure 4. TARGATT™ knock-in is a single copy.

(A) We co-transfected GFP and mCherry TARGATT™ donor plasmids into CHO cells and imaged the resulting cells using the green channel (B), the red channel (C), and as an overlay (D). The absence of yellow cells in the overlay indicates that cells expressing both GFP and mCherry are not present, demonstrating that genomic insertion occurs only in a single copy.

Figure 5. TARGATT™ technology efficiently inserts large DNA cargo

Integration is highly efficient even with inserts of 8 kb and 50 kb (see the data at appliedstemcell.com/ knock-in-of-a-50-kb-construct/). Larger insertions can be accommodated with multiple, nested integrations.

This opens the door to expressing multiple proteins while maintaining desired stoichiometric ratios.



TARGATT™ technology is automation-ready and pool-friendly

Highly efficient integration into the robust, reliably-expressing H11 locus means you can be confident that your inserted genes will be expressed* and you don't need to hunt for the optimal clone. These features make TARGATT™ technology compatible with streamlined pool-based studies and automated workflows.

*For constructs that have demonstrated expression in mammalian cells.

	TARGATT-Based Mammalian Display	Virus-Based Mammalian Display	Phage Display (bacteria, yeast)
Library Size	10^7 - 10^9	10^3 - 10^6	10^9 - 10^{10}
Protein Levels	Consistent	Variable	Variable
Antibody Formats	ScFv, Fab, IgG	ScFv, Fab, IgG	ScFv or Fab
Native Post-Translational Modifications	Yes	Yes	No

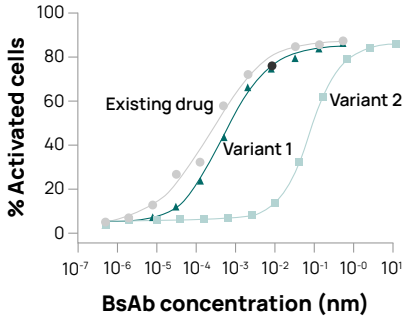
TARGATT™ technology enables library creation in mammalian cells

Run variant and discovery screens directly in mammalian cells to achieve more biologically relevant results, with library sizes comparable to those of phage display systems (Figure 6).

Figure 6. TARGATT™ technology enables automated library screening in mammalian cells.

We used TARGATT™ technology to create a bispecific antibody library in HEK293 cells with 20,000 variants (Segaliny, et al .) This complexity was sufficient for the authors to identify a high-performing variant that matched an existing therapeutic in a single round of screening. (Figure adapted from Figure 6 in Segaliny, et al.)

Segaliny AI, Jayaraman J, Chen X, et al. A high-throughput bispecific antibody discovery pipeline. *Communications Biology*. 2023;6(1). doi:10.1038/s42003-023-04746-w



	hiPSCs	HEK293	CHO K1
iPSC-based cell therapy development	**AST-9480** (GMP) **AST-9450** (GMP-matching research line) **AST-9650** (hypoimmunogenic)		
General DNA insertion and protein expression	**AST-1602** (Female line)	**AST-1305**	**AST-1405** (A2 site) **AST-1420**
Screening libraries—FACS selection		**AST-1307** **AST-1308** (High-efficiency)	**AST-1409**
Screening libraries—drug selection		**AST-1306**	**AST-1410**

We also offer donor plasmids that support Cre-inducible expression—request more information at **appliedstemcell.com/contact**

TARGATT™ kits speed your cell line development

To accelerate your ability to engineer cell lines and libraries with large DNA insertions, we offer TARGATT™ technology in a variety of cell lines, including human induced pluripotent stem cells (hiPSCs), which can be differentiated into a range of cell types after genome editing.

About Applied StemCell

Founded in 2008 to give industry and academic researchers the ability to leverage the power of genome engineering and induced pluripotent stem cell (iPSC) technologies, we continue to use our innovation and expertise to power basic research, drug discovery, and GMP-compliant development.

Our 17+ years at the frontiers of applied biology are the bedrock of our optimized reagents and protocols, and ensure speed, consistency, and quality in everything we do for you.

CELL LINE ENGINEERING

Strategies for engineering mammalian cells to express difficult proteins—4-6 weeks from start to final clone or pool

Alfonso Farruggio and Ruby Yanru Tsai

appliedstemcell.com • Milpitas, CA • (408) 773-8007

Watch poster presentation

ABSTRACT

Engineering transgenic mammalian cell lines for protein production can be time-consuming and labor-intensive. CRISPR/Cas9 is inefficient at knocking-in fragments greater than 5 kb. Lentivirus and piggyBac can handle larger insertions but integrate randomly, resulting in time spent isolating and identifying a clone that provides the desired expression levels rather than moving quickly with pooled integrants.

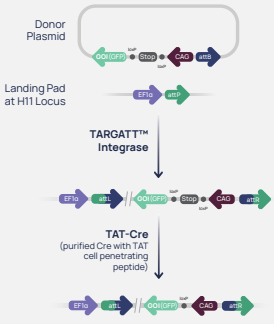
Expression is even more challenging when your protein is toxic, a membrane protein, or interferes with cell culture.

Here we compare several methods for generating clonal and pooled protein expression lines that deliver inducible expression in just 4-6 weeks after donor plasmid creation and using cells with an existing TARGATT™ landing pad.

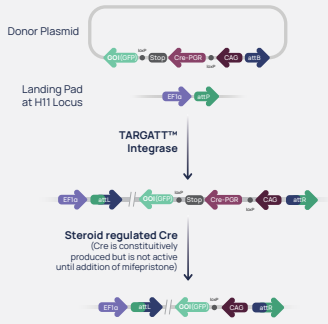
METHODS

We explored three different approaches for generating HEK293 cell lines that use Cre induction and assessed percent induced cells after either clonal or pool-based enrichment. For approaches A and B, we used GFP as our "gene-of-interest" (GOI). Approach C uses mCherry as the GOI.

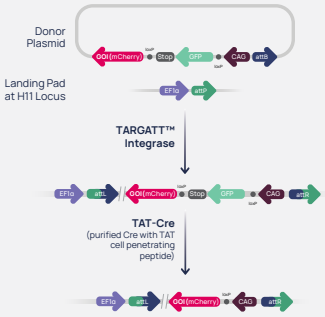
A. TAT-Cre approach



B. Steroid-regulated Cre approach



C. GFP-off approach



Pros and cons of each approach

A. TAT-Cre
Pros: Fast, tight control
Cons: TAT-Cre costs, no detection during the process if your GOI is not fluorescent

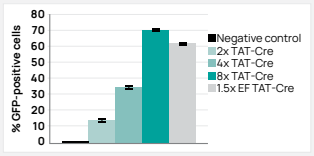
B. Steroid-regulated Cre
Pros: Easier scale-up, cost-effective
Cons: Leaky, no detection during the process if your GOI is not fluorescent

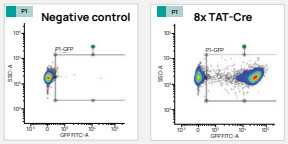
C. GFP-off
Pros: Tight control, GFP detection during process
Cons: Slightly longer, TAT-Cre costs

RESULTS

Approach A1: TAT-Cre with enriched pools delivers a max of 70% GOI (GFP)-positive cells

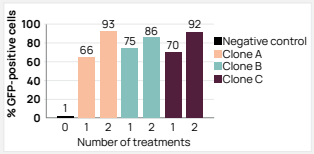
We knocked-in the donor plasmid and created enriched cell pools via drug selection for 2 weeks. We then added increasing amounts of TAT-Cre to remove the stop element and turn on GOI (GFP) expression. We also tested endotoxin-free TAT-Cre (EF TAT-Cre). After 60 minutes incubation with TAT-Cre, we found that 8x TAT-Cre resulted in the highest percentage (70%) of GFP-positive cells in the pool.





Approach A2: TAT-Cre with clonal enrichment delivered >90% GFP-positive cells

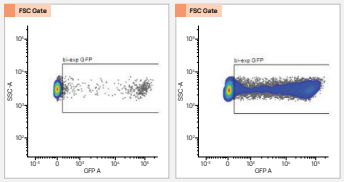
We knocked-in the donor plasmid and isolated clonal lines to ensure removal of parental cells, growing clonal lines for 2 weeks after isolation. We then added 4x TAT-Cre to remove the stop element and turn on GFP expression, testing double treatment with TAT-Cre.



Single treatment with TAT-Cre resulted in a larger percentage of GFP-positive cells than the pooled enrichment (66-75% vs 35%). Double treatment with TAT-Cre resulted in >90% GFP-positive cells.

Approach B: Compared to TAT-Cre, steroid-regulated Cre is more cost-efective and scalable but has reduced control, efficiency and induction speed

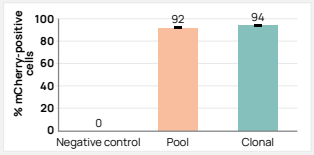
We only tested the pooled approach for the steroid-regulated Cre approach. As previously reported, this approach is leaky but still inducible. It takes more time to do than the TAT-Cre approach (5-7 days vs. 2-3 days) and induction efficiency only reaches 40-60%.



Approach C: GFP-off approach takes longer but is robust and reliable

Pools: We created drug-enriched pools to select for integrants and used FACS to remove parental cells. We treated with TAT-Cre and grew for 1 week before analyzing GFP-negative cells for mCherry fluorescence.

Clonal lines: We used the same process as for the pooled approach up to the FACS step, where we isolated single cells for outgrowth in selective medium. We then treated with TAT-Cre and grew for 1 week before analysis.



Both pool enrichment and clonal enrichment resulted in >90% mCherry-positive cells.

SUMMARY

Using TARGATT™ knock-in technology and Cre induction, we can achieve robust, inducible expression in HEK293 cells in just over a month with pooled or clonal enrichment.

Approach	Enrichment	Inducer	Max induction	Weeks*
A. TAT-Cre	Pool	TAT-Cre	70%	4
A. TAT-Cre	Clonal	TAT-Cre	90%	6
B. Steroid-regulated Cre	Pool	Mifepristone	60%	5
C. GFP-off	Pool	TAT-Cre	92%	6
C. GFP-off	Clonal	TAT-Cre	94%	6
Lentivirus	Clonal	TAT-Cre		11-29**
Non-viral (transposon, etc.)	Clonal	TAT-Cre		10-27**

*After donor plasmid construction **Time range found on different CRO websites

CONCLUSION

This fast timeline can accelerate the development of mammalian cell lines that produce difficult-to-express proteins. Compared to lentivirus and other random-integration methods (e.g. transposons, plain plasmid transfection, etc.), our approaches support pool-based expression from a fixed, well-characterized locus, which is faster and less labor-intensive than isolation and characterization of clones that express from random locations in the genome.

