TARGATT™ FOR TARGETED GENE INSERTION IN MOUSE MODELS

Site-specific, large fragment knock-in in a single step

OVERVIEW

Recent advances in gene editing technology have greatly increased our ability to make precise changes in the genome of cells or embryos and have eliminated major stumbling blocks in the generation of better models mimicking human diseases. As a result, site-specific transgenic animal models can be produced in an efficient and timely fashion. To enable large DNA fragment knock-in, we at Applied StemCell, have refined the sequence specific bacteriophage φC31 (PhiC31) integrase-mediated integration, called TARGATT™, as our proprietary technology. TARGATT™ yields up to 40% insertion efficiency and enables insertion of DNA fragments up to 22 kb at defined safe harbor sites in the mouse genome. The turnaround time for gene knock-in modification is as short as three months. Here, we present data showing the accuracy, sensitivity, versatility and consistency of our TARGATT™ technology in generating site-specific knock-in mouse models.

INTRODUCTION

Engineering mouse models of human diseases

Genomic engineering is in the midst of a technological revolution supporting the hope that it will pave the way in the future for truly personalized therapy for human diseases (Doudna, 2015). Transgenic mouse model, which has benefited greatly from advances in genetic research and engineering, is a powerful and essential tool for preclinical research and drug screening. Traditionally, transgenic mice were generated by methods such as direct pronuclear microinjection of DNA, use of transposons or homologous recombination in embryonic stem cells. These methods entailed a milieu of problems including but not limited to low efficiency, high incidence of undesirable recombination outcomes; random insertion of gene of interest, multiple copies of the gene, ectopic expression, gene silencing and insertional mutations (Rocha-Martins, 2015). Such methods of generating mouse models are very labor intensive, require large colonies of animals and have unpredictable deliverables or long turnaround time. To that end, the development of targeted gene editing technologies has greatly improved site-directed mutagenesis and thereby generation of better mouse models (knock-in, knock-out, conditional expression models) with increased efficiency and faster turnaround times.

Advances in gene modification technologies

The new millennium witnessed the development and rise in site-specific gene modification techniques such as ZFNs, TALENs and more recently, the revolutionary CRISPR/Cas9 technology (Ishida K et al., 2015). In all three technologies, designer endonucleases introduce double stranded DNA breaks (DSB) at specific sites in the genome, which might be repaired by non-homologous end joining repair (NHEJ) or homologous directed repair (HDR). ZFNs and TALENs methods are highly site-specific and efficient. But, the design and delivery of the protein probes is complicated and requires knowledge of advanced techniques, thereby increasing the cost and turnaround time. The CRISPR/Cas9 system is based on simple site-specific guide RNAs (gRNA) as probes and
is very easy to use. It is by far the more efficient, user-friendly and least expensive nuclease-based gene editing system. However, all three techniques are inefficient for site-specific DNA knock-in with insert size > 5 kb.

**TARGATT™ integrase-based method for gene knock-in mouse models**

Another avenue of site-specific gene editing technique uses the φC31 (PhiC31) bacteriophage integrase which allows site-specific integration with higher efficiency because it catalyzes an irreversible reaction between two appropriate attP and attB sites (Figure 1) (Tasic et al., 2011; Zhu et al., 2014).

In mice, the φC31 integrase catalyzes insertion of the gene of interest into an attP site that has been pre-engineered into specific loci (Rosa26 or H11) in the mouse genome (Tasic et al., 2011; Rossant and Gertsenstein, 2011). The preselected loci are at transcriptionally active and well-characterized regions on the chromosomes, which guarantees transgene expression without causing position effects or insertional mutations. The Rosa26 locus is widely used for generalized expression of a single copy knock-in transgene (Zambrowicz, 1997) and supports stable gene insertion and expression with very high efficiency (Srinivas S et al., 2001). Our proprietary H11 locus is another well-characterized, intergenic locus that supports higher level expression (Tasic et al., 2011).

The highpoint of this system is that it supports insertion of large fragment DNA (up to 22 kb) with high efficiency. We successfully inserted a 22 kb large fragment DNA into the H11 locus in FVB mice. Of the 17 pups born, one founder was identified (6% efficiency). The TARGATT™ system also allows inducible/conditional gene expression in a tissue and/or time specific manner. In this communication, we will provide details of Applied StemCell’s proprietary TARGATT™ integrase system for site-specific gene integration in mouse models, including data showing its site-specificity, efficiency, and ability for large fragment gene knock-in.

![Figure 1. Scheme of the TARGATT™ technology.](image)

**Strategy for integrase mediated site-specific transgenic mouse models**

**Mouse model generation:** Mouse models were generated by microinjection of an integration cocktail into the pronuclei of zygotes from heterozygous H11 or Rosa26 mice (each locus contains three tandem attP sites). The integration cocktail consisted of the targeting vector (gene of interest + attB sequence) and in vitro transcribed φC31 mRNA. The vector was either designed at Applied StemCell’s Molecular Biology facility or was provided by clients. Zygotes injected with the integration cocktail were implanted into pseudo pregnant CD1 recipient mice to carry to term. The founders were identified by PCR-based genotyping of genomic DNA extracted from tail snips, using a panel of primers to confirm insertion/integration of gene of interest at the correct locus.

**Mouse background:** We used zygotes from two strains of mice: C57BL/6 and FVB in our projects. Both strains have attP docking sites in either the H11 or R26 locus.
Knock-in Loci: Mice were engineered with three tandem attP sequences at either the Rosa26 or H11 locus.

Calculations and Statistics: In all analyses, the knock-in efficiency was calculated as the percentage of founders over total number of F0 mice born. The insert size was calculated as the size of gene of interest including the attB recognition sequences. Statistical analysis was performed using GraphPad Prism 7 for Windows.

RESULTS

Comparing the size of insert (gene of interest) and the efficiency of insertion/ integration (knock-in efficiency)

A frequent issue with gene editing technology is that increasing the size of the gene to be inserted lowers the efficiency of insertion. When we compared the insert size (kb) versus the knock-in efficiency (%), there was no direct correlation ($r = 0.026$) as analyzed by Pearson’s correlation coefficient. Site-specific insertion efficiency of DNA fragments ranging from 2kb to 10kb varied with no significant correlation between insert size versus knock-in efficiency (Figure 2).

Figure 2. Insert size vs. Insertion Efficiency: The size of insert does not influence efficiency of site-specific insertion (knock-in efficiency) in mice using the TARGATT™ integrase-mediated recombination. Values in the X-axis indicate insert size (length of inserted sequence; kb) and values in the Y-axis indicate knock-in efficiency (% of correct transgenic founders among total number of pups born) in TARGATT™ mice. Correlation coefficient ($r$) was found to be 0.026 indicative of a weak correlation between the insert size and efficiency.

Efficiency of knock-in at Rosa26 and H11 loci in mice

We also compared the knock-in efficiency between the Rosa26 and H11 loci, two commonly used loci, to see if the choice of genomic locus affected insertional efficiency (Figure 3). Three tandem attP were pre-engineered at either the Rosa26 or H11 locus. When using the TARGATT™ method, there was no significant effect of locus on the efficiency of gene insertion, assessed using unpaired t test ($p = 0.29$). Both loci are equally suitable for gene knock-in using TARGATT™.

Figure 3. Efficiency of knock-in between 2 commonly used loci: Rosa26 and H11. Efficiency of insertion (knock-in) was not significantly different between the two different loci with the TARGATT™ integrase method for generating transgenic mice, as calculated by t-test (p>0.05). Values in the Y-axis indicate knock-in efficiency (% of founders to total number of pups born) in TARGATT™ mice.

Knock-in efficiency between C57BL/6 and FVB mouse strain background

Next, we compared efficiency of knock-in between the two strains of TARGATT™ mice: C57BL/6 and the FVB strain (Figure 4). Both strains have 3 tandem attP, pre-engineered at either the Rosa26 or H11 locus. Again, there was no effect of mouse strain background on the insertion efficiency of TARGATT™, as assessed by unpaired t test ($p = 0.33$). The C57BL/6 and FVB strains are equally permissible for gene knock-in using TARGATT™.
Comparing knock-in efficiency across strain and insertional loci

When we separated projects based on the insertional site (Figure 3) and mouse strain (Figure 4), and analyzed effect of strain and loci on efficiency using a two-way ANOVA, there was no significant effect of strain (p = 0.37) or locus (p = 0.26). However, when combining data for both the locus and strain, there was a marginally significant difference (p = 0.05) of efficiency at Rosa26 vs H11 in the FVB strain of TARGATT™ mice.

DISCUSSION

The data summarized in this white paper explains the advantages of using the TARGATT™ technology for site-specific knock-in of the gene of interest. The TARGATT™ method of gene insertion allows for gene insertion up to 22 kb at a preselected locus in the mouse genome. We observed that there was no significant effect of insert size on site-specific knock-in efficiency up to 10 kb which is far better than the capabilities of other technologies such as CRISPR, TALENs, ZFNs, and homologous recombination. With insert size of >10 kb, there might be an effect on insertion efficiency which has not been explored in this study. In our project with the 22 kb large DNA knock-in, we had an efficiency of 6%. The TARGATT™ integrase recognition sequence, attP has been engineered into one of two loci in the mouse genome: Rosa26 and H11 in either the C57BL/6 or FVB strains. There is no effect of either strain or locus on the insertional efficiency of the TARGATT™ system. However, there is a slightly higher efficiency at the Rosa26 locus in the FVB strain of TARGATT™ mouse for which the mechanism is not yet clear. These data demonstrate that the TARGATT™ technology is highly efficient and site-specific for knock-in of large DNA fragments, with an average efficiency of 12%. The high efficiency is due to the irreversible reaction catalyzed by the integrase. This technology also allows a single copy or defined copy number of the gene to be inserted, with guaranteed stable transgene expression at the specified locus.

In addition, there is no position effect or gene silencing with the TARGATT™ method and off-target events are very low because the effect of integrase catalyzed integration at pseudo-attP sites in the mouse genome is...
negligible. This system bypasses the ES cell homologous recombination step with a fast turnaround time for generating founders in as short as 3 months.

While CRISPR and other nuclease based editing technologies are a great platform for knock-out, point mutation and small fragment knock-in, we conclude that the TARGATT™ system is a better method for site-specific insertion, especially for large fragment DNA in mouse models and for developing both conventional knock-in or inducible knock-in mouse models.

Why choose ASC’s proprietary TARGATT™ knock-in mouse model and service?

Applied StemCell’s co-founder and CSO, Dr. Ruby Yanru Chen-Tsai is one of the co-inventors of the TARGATT™ technology. Our team of scientists and associates are well trained and very experienced with details in each step during the model generation. If you would like to generate the model in your own facility, you can purchase the TARGATT™ mice in either C57BL/6 or FVB strain from Charles River (strain code 537 for FVB and 549 for C57BL6), and the TARGATT™ knock-in kit with a corresponding genotyping kit from Applied StemCell. Contact our experienced staff at info@appliedstemcell.com to learn more about this site-specific, versatile, high efficiency technology and to find out how we can help you be on target with our TARGATT™ platform.

REFERENCES