



Knock-in/out, Conditional Knockout Mouse / Rat Model

Applied StemCell, Inc. provides high quality, one-stop services for the generation of gene-targeted mouse/rat models.

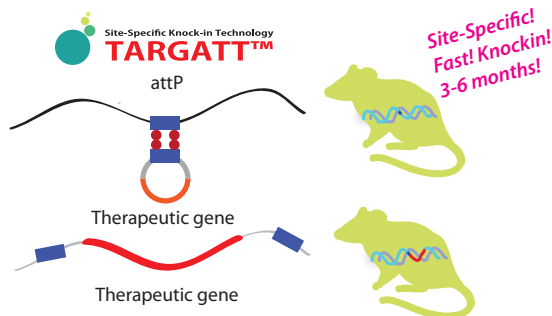
Why select ASC among other companies and core facilities?

- ASC is a leading transgenic service company recognized in Nature Biotechnology*
- CRISPR technology licensed from Broad Institute
- Unique IP only available at ASC
- Over 400 clients worldwide
- All works is done in the USA and all mice/rats are born in ASC's own AAALAC facility in California
- Fast turnaround - F1 breeding pair delivered in as fast as 4-6 months
- Immediate start - no more waiting in queue!
- Stress-free, from plasmid design to founders / embryo banking
- Dedicated project managers
- Project reports at each milestone

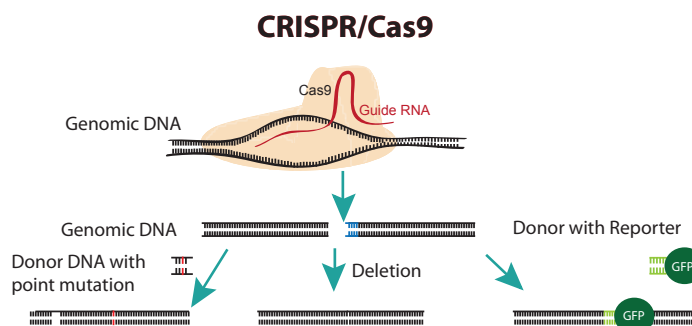


*Nature Biotechnology: "CRISPR mouse model boom, rat model renaissance" Nature Biotechnology 34, 893-894, September (2016) and "Gene editing at CRISPR speed", Nature Biotechnology 32, 309-312, April (2014).

ASC's Genome Editing Technologies Overview



- Site-Specific Knockin for mouse models (FVB, B6)
- Site-Specific Knockin for rat models (SD)
- High efficiency for long DNA Insertion (up to 22kb)
- Full service or do-it-yourself



- Knockout, Point Mutation, Conditional Knockout
- Precise deletion

Comparison

Methods	Technical Analysis
TARGATT™ phiC31 integrase	Site-specific integration in "Safe harbor locus" (Rosa26, H11) High efficiency (up to 40%) Works for large fragment knock-in (~ 20 kb) Insert promoter of choice for gene: overexpression and inducible expression
CRISPR / Cas9	High specificity High efficiency for knockout, point mutation Large DNA knock-in up to 5 kb

2. Region-Specific Knockout

Goal: To delete a 587 bp fragment from a specific site in C57Bl/6 mouse genome using CRISPR.

How? The knockout mouse model was generated using an optimized three step procedure: (1) a mixture of active guide RNA molecules (gRNAs) and qualified Cas9 mRNA was injected into the cytoplasm of C57BL/6 embryos; (2) The new mice born from the microinjection were screened using PCR; (3) the positive animals were confirmed to have deletion by sequencing the modified region in the desired mouse locus.

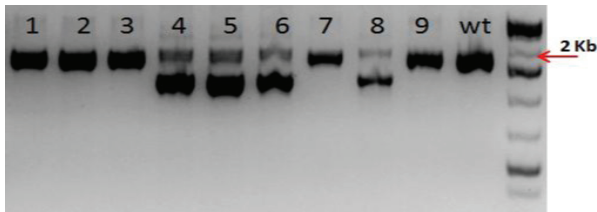


Figure 2. Mice# 4, 5, 6, 8 were identified as F1 germline transmitted animals carrying the ~ 600 bp deletion. The lower band was extracted, purified and sequenced. The sequence results showed that the deletion removed the entire targeted exon.

3. Constitutive Knockout

Goal: To generate a constitute knockout (KO) of a gene of interest in C57Bl/6 mouse genome using CRISPR.

How? The knockout mouse model was generated by microinjection of a cocktail of CRISPR elements into cytoplasm of C57BL/6 mouse embryos. Ten mice born from the microinjection were screened using PCR and sequencing. Four out of the ten mice were identified to have a constitutive KO in the gene of interest with a deletion in exons 6 to 8: mice #4,5 and 7 were identified to have heterozygous deletion genotype while mouse #10 was a homozygous deletion mutant.

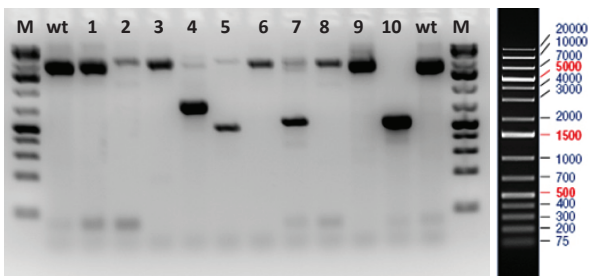


Figure 3a. Genotype screening for 10 mice born after microinjection of a CRISPR cocktail designed to generate a constitutive KO mouse model. (a) PCR products for mice #1-10 and a wild type (wt) control; GeneRuler 1kb DNA marker used, lane M.

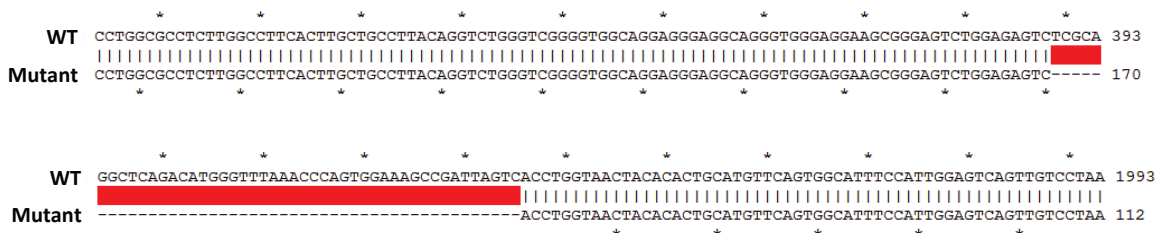


Figure 3b. Sequence alignment for mouse #10 (bottom) showing deletion in gene of interest when compared to wild type (top)

4. Large Fragment Knock-in

Goal: To insert a 2 kb DNA fragment (gene of interest) at a specified locus in the mouse genome using CRISPR/Cas9.

How? An optimized mixture of Cas9 gRNA mRNA and donor vector was microinjected into embryos of C57BL/6 mouse background. Using a panel of genotyping primer pairs, three out of 31 pups born after microinjection (#15, 19, and 26) were identified as founders (F0), with the gene of interest inserted at the desired locus.

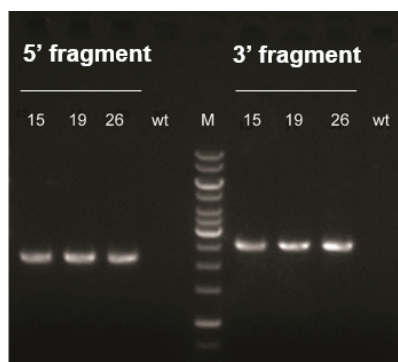


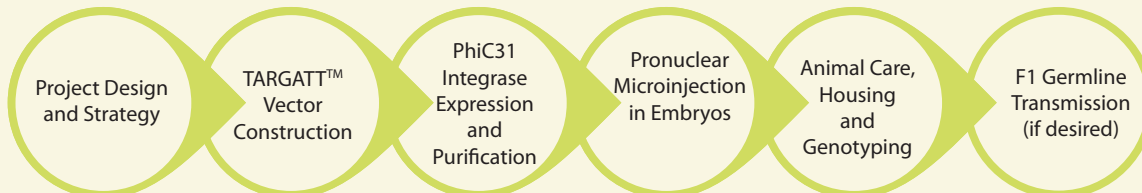
Figure 4. Agarose gel electrophoresis of PCR amplicons in F0 mice (#15, 19, and 26) with site-specific gene knock-in. The left part of the gel shows the 5' junction fragment (2,191 bp), and the right part of the gel shows the 3' junction fragment (2,557 bp). [wt: wildtype control; M: 1 kb DNA ladder].



Fast & Site-Specific Knock-in Mouse Service

With our proprietary technology we will generate your site-specific (Rosa26 or H11) knock-in mouse in as fast as 3 months. Reliable: Any gene of interest can be specifically inserted at a defined, transcriptionally active locus. We guarantee transgene expression without any interruption in internal gene expression.

Step:



Deliverable:

- Founders with desired mutation or genomic modification.
- Milestone reports and a final report on the TARGATT™ project, including the original targeting strategy, microinjection details, and genotyping results.



Case Studies

1. Site-specific knock-in of a coat color gene in mice



Figure 1a. Left: H11;C57BL/6 mice used for donor embryos. Center: Site-specific gene knockin founders. Right: Germline transmission in F1 mouse. Ref: Guenther et al., "A molecular basis for blond hair color in Europeans", Nature Genetics, doi:10.1038/ng.2991.

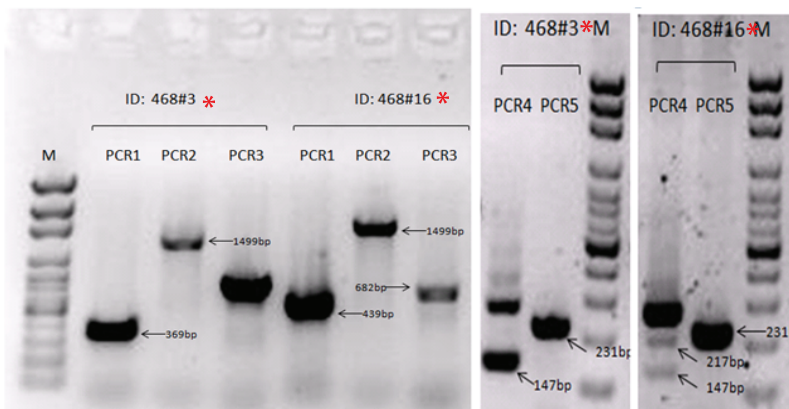


Figure 1b. Twenty-three pups were born from one microinjection experiment. *Two positive founders were identified by using PCR primers (PCR1, 2, 3, 4, 5). To confirm the insertion is site specific at H11.

More sample studies are available upon request.

References:

Description of the technology:

Zhu, F., et al. (2014). Nucleic acids research, 42(5), e34-e34.
Tasic, B., et al. (2011). PNAS USA, 108(19), 7902-7907.

Commentary, comparison with other transgenic methods:

Rossant, J., et al. (2011). PNAS USA 108(19), 7659-7660.

Tet inducible mice generated by TARGATT™:

Fan, X., et al. (2012). Endocrinology, 153(11), 5637-5644.

Advantage of Hipp11 (H11) locus:

Hippenmeyer, S., et al. (2010). Neuron, 68(4), 695-709.

Application for mice generated by TARGATT™:

Booze, M. L., et al. (2016). Free Radical Biology and Medicine, 99: 533-543.

Feng, D., et al. (2016). The Journal of Clinical Investigation, 126(6).

Park, K. E., et al. (2016). International journal of molecular sciences, 17(6), 810.

Sun, N., et al. (2015). Measuring in vivo mitophagy. Molecular cell, 60(4), 685-696

Guenther, C.A., et al. (2014). Nature genetics, 46(7), 748-752

Devine, W.P., et al. (2014). eLife, 3, e03848.

Villamizar, C. A. (2014) UT GSBS Dissertations and These (Open Access). Paper 508 (2014)

Fogg, P.C., et al. (2014). Journal of molecular biology, 426(15), 2703-2716

2. TARGATT™ Large Fragment Knock-in

Goal: To insert a large (8.5kb) DNA fragment into the H11 safe harbor locus in FVB mice using TARGATT™ integrase based technology.

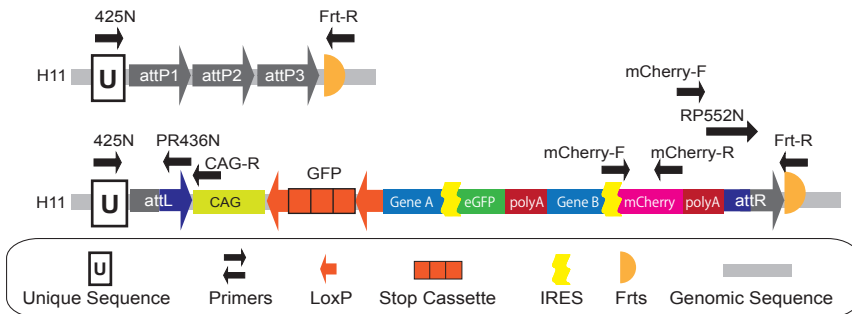


Figure 2a. Transgene integration and genotyping scheme for gene A transgenic Model. Upper panel: genomic sequence with H11P3 landing pad; lower panel: founder with gene A transgene inserted at the 1, 3-attP site

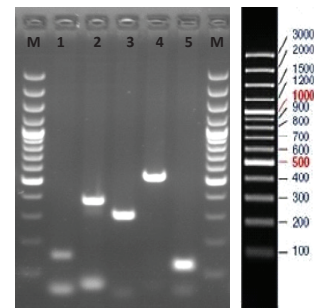


Figure 2b. Genotyping of gene A founder 1614#1 (a) PCRs 1 to 5 as described in Method (Lanes 1 to 5); (b) 100bp ladder DNA marker used in lanes M.

Fast & Site-Specific Knock-in Mouse Do-it-yourself Products

Using our novel TARGATT™ system, a gene of interest can be inserted at a well-characterized, transcriptionally active locus in the mouse genome with guaranteed transgene expression. Make your own TARGATT™ knock-in mouse by purchasing the TARGATT™ Mouse from Charles River, available in FVB and C57BL/6 backgrounds. Essential components, including TARGATT™ Plasmids and Transgenic kits, are available directly from us.

Ask us for a list of core facilities currently using this system, including UCSF, NIH, Max Planck, Harvard, Columbia and more!

Step 1. Purchase Plasmids from ASC TARGATT™ Plasmids

- AST-3042 TARGATT™ 2 (CAG + Poly A)
- AST-3043 TARGATT™ 3 (no promoter + MCS)
- AST-3050 TARGATT™ 6.1 (CAG-L4SL-MCS-PolyA)
- AST-3047 TARGATT™ 7 (PGK-MCS-PolyA)
- AST-3048 TARGATT™ 8 (PCA-MCS-PolyA)
- AST-3051 TARGATT™ 9 .1 (PCA-L4SL-MCS-PolyA)

Step 2. Purchase attP mice from Charles River TARGATT™ Mouse (Charles River)

- STRAIN CODE #537 (FVB), #549 (C57BL/6)
- Ordering: 1.800.LABRATS

Step 3. Purchase Transgenic Kits from ASC for injection TARGATT™ Transgenic Kit

- AST-1003 Transgenic Kit (5 rounds of microinjections)
- AST-1004 Transgenic Kit (2 rounds of microinjections)

Step 4. Confirm sequence with Genotyping Kit

Site-Specific!
Fast! Knockin!



- AST-2005 H11 Mouse Genotyping Kit
- AST-2006 Rosa26 Mouse Genotyping Kit

FAQ for TARGATT™ System (Selected)

1. Can I create models to over-express a gene of interest?

Yes, TARGATT™ system is ideal for gene over-expression. Different promoters, e.g., tissue-specific promoters or ubiquitous promoters, and inducible systems (Tet On/Off, loxP-stop-loxP) can be used for tissue-specific, ubiquitous, or inducible gene expression.

2. Can I use TARGATT™ system to create transgenic mice with tissue-specific gene expression?

Yes, TARGATT™ system can be used to generate tissue-specific transgenic mouse models. Just use a tissue-specific promoter to drive the transgene expression. Alternatively, a loxP-stop-loxP cassette can be placed between a ubiquitous promoter and the transgene. Upon crossing with tissue-specific Cre mice, the transgene will be expressed in that particular tissue.

3. What promoters are used to drive gene expression?

Any defined promoters provided by the customer or published in the literature can be used.

4. Can I integrate a reporter gene? What kind of reporter genes do you recommend?

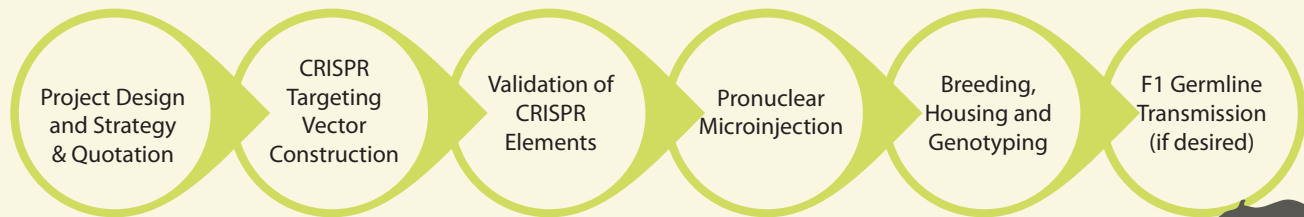
Yes, you can express any reporter genes such as GFP, DsRed, mCherry, LacZ, Luciferase, etc.

5. What is the maximum size of a gene you can insert? Will the efficiency of your system be affected if the gene is too large?

To date, the largest DNA fragment we have inserted 22 kb. Insertion efficiency appears to decrease with increasing DNA fragment size. Larger fragments (>10 kb) require additional embryo injections to obtain positive animals.

Bacterial Artificial Chromosome (BAC) Knock-in Mouse/Rat Models

BAC = ultra-low copy vectors that can hold up to 300 kb of DNA: Ideal vectors for **introduction of entire genes** into the rat genome. BAC transgenic mice/rats are similar to other knock-in models in the expression pattern of the murine/rattus gene of interest, except that the BAC is randomly integrated into the genome as a transgene. Applied StemCell can create BAC knock-in and conditional knock-in animal models for you.



Deliverables

- Germline transmission guaranteed F1 rat in 6-8 months
- Milestone reports and a final report



Case Study

1. Reporter Gene Insertion

Goal: The purpose of this project was to generate a transgenic rat model with GFP insertion at a specific locus.

How? A BAC vector containing GFP inserted at the desired locus was prepared and microinjected into the pronucleus of embryos from the Sprague Dawley rat strain. After F1 breeding, 6 out of 10 rats were identified as containing the BAC-GFP sequence at the desired locus sequence by PCR.

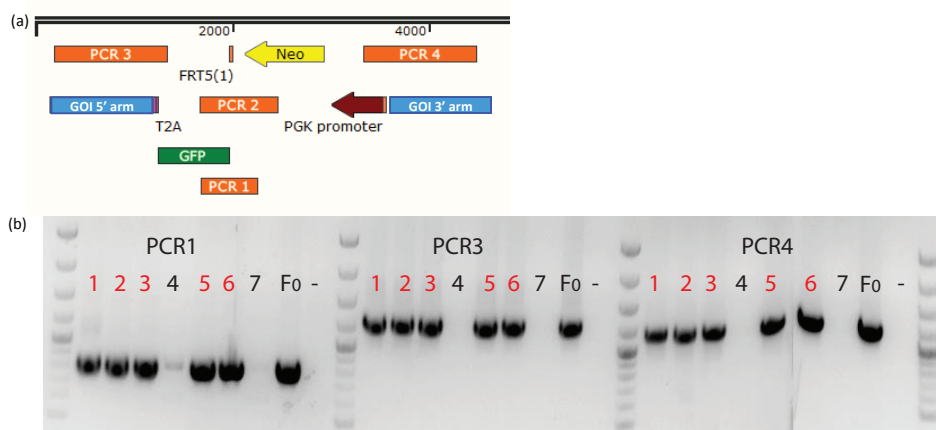


Figure 1. (a) Schematic illustration of BAC-GFP construct and genotyping scheme; PCR reactions 1 to 4 are labeled in orange. (b) F1 rats from litter B (#2-6) were tested for PCRs 1, 3 and 4 and compared to F0 founder; GeneRuler 100bp marker was used as DNA standard.



Fast & Site-Specific Knock-in Rat Service

With our proprietary technology we will generate your site-specific (SD) knock-in rat in as fast as 4 months. Reliable: Any gene of interest can be specifically inserted at a defined, transcriptionally active locus, with guaranteed transgene expression. Ask for details.

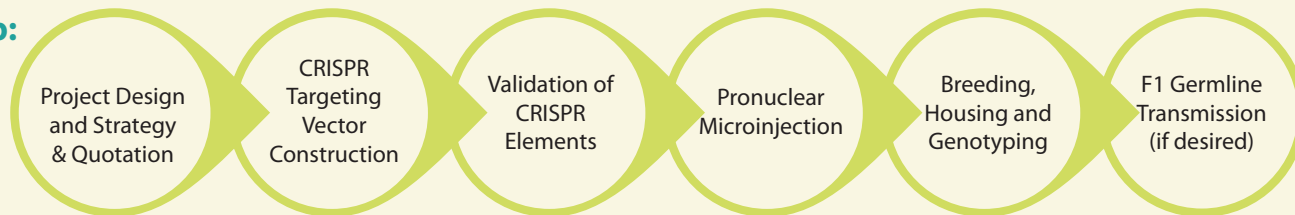
Deliverables

- Germline transmission guaranteed, F1 rats in 6-8 months
- Milestone reports and a final report

CRISPR Rat Model Generation Service

The successful isolation of rat embryonic stem cells (rES) and complementary advances in site-directed mutagenesis using techniques such as TARGATT™ and CRISPR/Cas9 have made the generation of transgenic rat models possible.

Step:



Deliverable:

- Founder(s) with desired mutation or genomic modification.
- Milestone reports and a final report on the CRISPR project, including the original targeting strategy, microinjection details, and genotyping results.



Case Studies

1. Constitutive Knockout

Goal: To generate a knockout rat model by using CRISPR technology to create a deletion within the gene of interest in Sprague Dawley (SD) rat strain.

How? Sixteen pups were born after microinjection of CRISPR elements targeting the gene of interest, screened for deletions by PCR, and confirmed by sequence analysis. Three pups (#3, 6 and 16) were confirmed to have homozygous deletions and one (#13) was a heterozygous knockout.

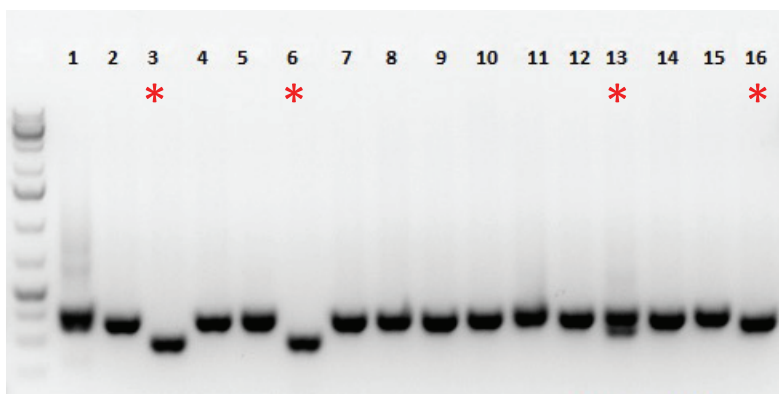


Figure 1. Genotype screening for knockout founders by PCR. (a) Four rats were tested by PCR using primer sets for gene of interest and confirmed to have deletion in gene of interest (marked by *); GeneRuler 1kb DNA Marker used in unlabeled lanes.

2. Point Mutation

Goal: To generate a mutant rat model by knocking in an R > H (CGC > CAC) point mutation in the gene of interest in the dark agouti rat strain.

How? Two out of 31 rats were positive for the desired point mutation following microinjection of a CRISPR cocktail into rat embryos.

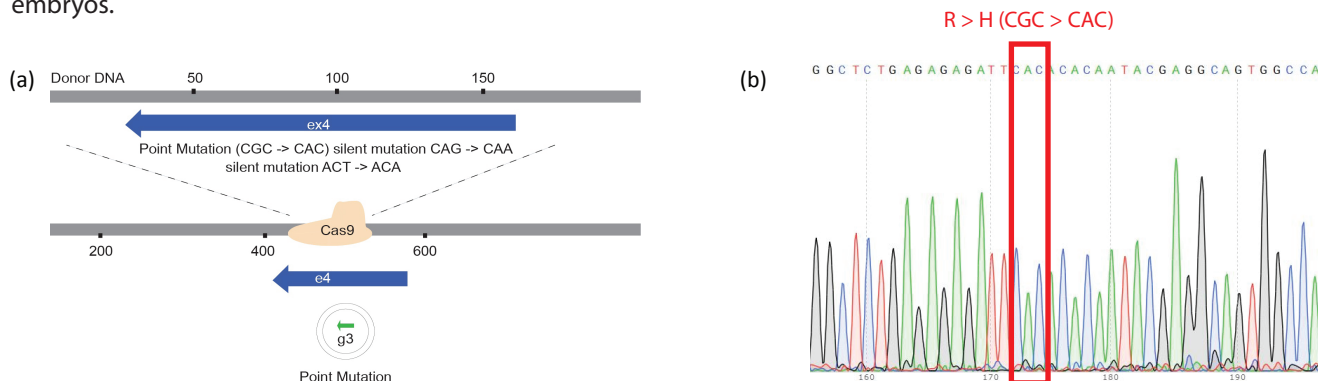


Figure 2. Sequence analyses of point mutation rats. (a) Schematic representation of targeting strategy; (b) Representative chromatogram of a founder rat.

Products for CDX/PDX Model Research

Ready-to-Use, Substrate-Independent Autobioluminescent Technology.

Applied StemCell, Inc. (ASC) has developed a genetically encoded synthetic luciferase system based on the bacterial luciferase gene cassette (Figure 1). Unlike traditional bioluminescent systems that encode only the luciferase enzyme, and therefore require the application of a chemical substrate to induce light output, ASC's synthetic luciferase system encodes all of the components required for signal initiation and maintenance. This allows cells to continuously and autonomously produce a bioluminescent signal without the need for chemical stimulation, and without sample destruction. This revolutionary new approach to optical imaging provides you with increased data acquisition in both tissues and small animal models. By taking advantage of our continuously Luminescence human cell lines it has finally become possible to break free from the expensive and time consuming introduction of substrate for bio-imaging purposes.

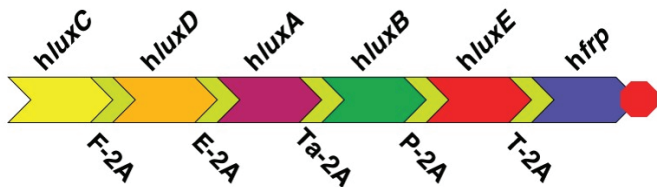


Figure 1. ASC's synthetic bacterial luciferase is capable of generating a bioluminescent phenotype without sample destruction by utilizing substrates found naturally within the host cell.

ASC's ready-to-use bioluminescent cell lines provides users with:

- Decreased costs – no need to purchase separate luciferin substrate
- Increased imaging flexibility – image on your own schedule
- Ability to image the same samples repeatedly – no cellular destruction required
- Reduced hands on time – simply plate and image, no additional steps required
- Non-invasive, *in vivo* tumor tracking in small animal models - imaging possible directly through tissue

in vivo imaging

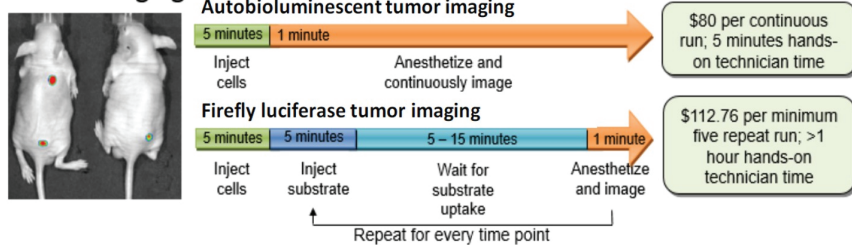


Figure 2. Comparison between substrate-dependent tumor imaging vs. substrate-independent tumor imaging techniques highlights the advantages of using ASC's autobioluminescent cell lines. Advantages include lower cost, reduced hand-on time, non-invasive tumor tracking in animals, and straightforward protocols.

ASE-5901	Autobioluminescent Human Colorectal Cancer Cells (HCT116)
ASE-5902	Autobioluminescent Human Kidney Cells (HEK293)
ASE-5903	Autobioluminescent Human Breast Cancer Cells (MCF7)
ASE-5904	Autobioluminescent Human Breast Cancer Cells (T47D)
ASV-1001	Substrate-free Autobioluminescent Vector (pCMVlux)
ASV-1002	Substrate-free Autobioluminescent Vector (pEF1alux)

Custom autobioluminescent cell line development

Do you have a specialty or proprietary tissue that can benefit from autobioluminescent expression? Contact us today to speak with one of our technology specialists and find out how ASC can modify your existing cell lines to produce data continuously without costly substrate addition or sample destruction. We can quickly modify your samples to express an autobioluminescent phenotype and return them to your laboratory for in-house testing at a fraction of the cost of purchasing and maintaining an existing, substrate-requiring cell line.