Applied StemCell

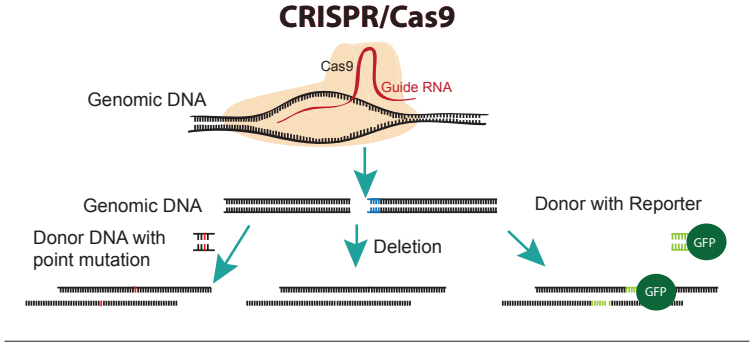
Genome editing *in vitro* and *in vivo*

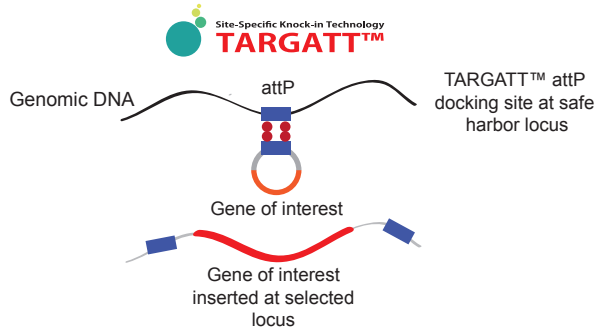
Genome Editing for Cell Line Model Generation

CRISPR and TARGATT™ technologies for versatile gene editing in cell lines

Why work with ASC?

- ASC is a global leader in cell line engineering with a clientele of over 400 companies worldwide and has been published in peer-reviewed journal such as *Nature, PNAS* and more.
- ASC is one of the earliest licensees and service provider of the CRISPR/Cas9 Technology.
- Seamless workflow with dedicated project managers, and detailed milestone reports.
- Full service cell line engineering portfolio includes customizable deliverables such as choice of zygosity and choice to use silent mutations.
- Affordable, budget-friendly custom engineered cell line service and gene editing toolkits.





Hassle-free gene editing!

We have engineered more than 100 major cell lines[#] for clients using CRISPR & TARGATT™ gene editing technologies.

Human				Other Species	
Cancer Cells	786-O (Renal carcinoma) A-375 (Melanoma) Breast cancer cells CHLA-10 (Neuro-ectodermal) DLD-1 (Colon cancer) Gist-T1 (Gastrointestinal tumor) HCT 116 (Colon cancer) HEK293 (Kidney) HEK293Braf HEK293T (Kidney) HeLa (Cervical cancer) HepG2 (Liver sarcoma) HT1080 (Fibrosarcoma) * HT29 (Colon cancer) KBM-7 (Myeloid leukemia) KM12-Luc (Colon carcinoma) LnCaP (Prostrate cancer) MDA-MB231 (Breast cancer) NCI-H2228 (Adenocarcinoma0 RKO (Colon cancer) TC32 (Neuro-ectodermal cancer) SCC35 (Head and neck tumor) * SH-SY5Y (Glioblastoma)	**Epithelial Cells**	A-549 (Lung) BEAS-2B (Bronchial) BT-474 (Mammary gland epithelium) Follicular thyroid cell line HaCaT (Keratinocyte) HBE (Bronchial) Huh7 (Liver) MCF-10A (Mammary gland) OCCM-30 (Cementoblats) RPE-1 (Retinal epithelium) SK-MEL-31 (Skin epithelium) Tert-RPE (Retinal epithelium) * U-2 OS (Bone)	**Mouse**	3617 (Adenocarcinoma) 4T1 (Mammary cancer) C2C12 (Myoblast) cTEC (Cortical thymic epithelial) Embryonic fibroblasts (MEF; primary cells) * Embryonic stem cells (mESCs; C57BL/6) GD25 (Fibroblasts) Induced pluripotent cells (miPSC) MWCL-1 (Lymphoma) * OCCM (Cementoblasts) Tonsil epithelial cells
		Blood-Derived Cells	BCWM-1 (Bone marrow) H929 (Bone marrow) Jurkat (T lymphocyte) K562 (Erythroleukemia) KHYG-1 (NK cell leukemia) LAD2 (Mast cells) * MM.1s (B lymphocyte) MWCL-1 (Lymphoma) NCI-H929 (Bone marrow) T2 cells (Lymphocyte) TF-1 (Leukemia)	**Rat**	CWSV-1 Cells (Hepatocytes) DAC8 embryonic stem cells (Dark agouti male) Immortalized Keratinocytes L77 embryonic stem cells (Fisher) PCC13 (Follicular thyroid cells) Chondrosarcoma cells
Stem Cells	Human embryonic stem cells Human multipotent adult progenitor stem cells iPSC (Healthy and various disease models) Limbal stem cells Neural stem cells (Adult and fetal)	**Fibroblasts & Adipocytes**	Alzheimer's fibroblasts * Immortalized fibroblasts SGBS (Preadipocyte/adipocyte) * UCI 162	**Primate**	COS-7 (Kidney cells) GL37 (Kidney cells) Macaque ES cells
				Hamster	CHO-K1 (Ovarian) CHO-S (Ovarian)
				Insect & Others	Mosquito cells Wolf keratinocytes

** Ask for details* *[#]Cell lines provided by clients or purchased by ASC*

CRISPR/Cas9

Knock-in Knockout Cell Line Model Generation Service

ASC's cell line engineering service portfolio includes optimized cell culture, CRISPR transfection conditions for high efficiency editing of cell lines with short turnaround times and customized deliverables:

- Gene knockout, insertion, correction, or replacement
- Site-specific large fragment knockout (10 kb and more)
- Custom heterozygous and homozygous clones
- CRISPR genome editing without silent mutations (for critical research areas)

Service Includes:
- Targeting vector construction and validation
- Cell transfection and selection
- Single cell cloning
- Expansion and cryopreservation

Deliverables and Timeline:
- Genetically engineered cells with confirmed mutation(s)
- Dedicated project managers; milestone reports

Knockout	3 - 5 months
Knock-in	3 - 6 months
Point Mutations	4 - 6 months

Applied StemCell also provides CRISPR / Cas9 services with gRNA validation. We use two methods to perform functional tests:

1) Cell Based Deep Sequencing Assay: Using mouse N2A cells or human K562 cells, the selected gRNAs are first transfected. Then, the PCR amplicons of the targeted region are subjected to Next Generation Sequencing (NGS) to assess frequency of the NHEJ events that result from Cas9/ gRNA cleavage.

2) Cell-Free System is used for gRNA validation in cell lines other than mouse and human origin and is based on a sequence-specific cleavage assay of episomal plasmids. The efficiency of cleavage is detected by PCR.

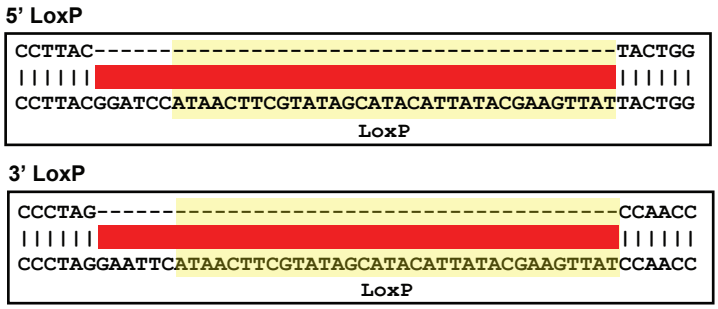
Case Studies

1. Conditional Knockout in HCT116 cells

Goal: To create a conditional knockout cell line model for the gene of interest in HCT116 cells.

How? The CRISPR/Cas9 system was used to insert two LoxP sites, flanking exons 4-6 of the gene of interest. A set of two gRNAs was used for this approach.

Result: Two homozygous conditional knockout clones containing the floxed allele were identified and confirmed by PCR and sequencing.

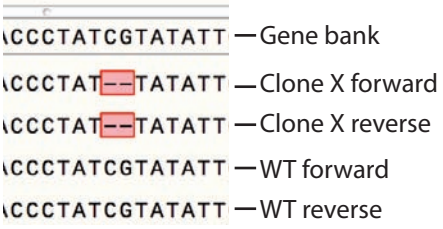


2. Multi-copy gene knockout in Human Cancer Cells

Goal: To create a gene knockout in a cancer cell line that contains four copies of the gene of interest.

How? Optimized CRISPR/Cas9 protocols were used to introduce a two basepair deletion in all four copies of the gene of interest causing frame shift mutations, and thus generating a functional gene knockout.

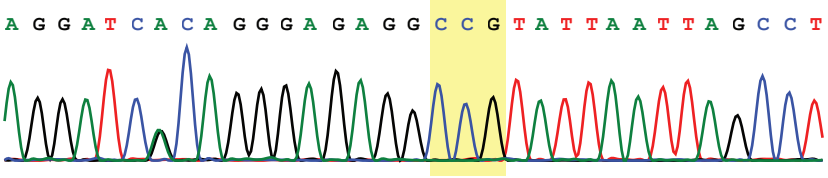
Result: Sequencing results show successful engineering of a stable homozygous mutant cell line (Clone X) with a two base pair deletion in all four copies of the targeted gene.



3. Point Mutation Correction in Human iPS Cells

Goal: To correct a point mutation in human iPSCs.

How? A mutant allele was corrected using CRISPR/Cas9 technology in human iPSC.



Result: Two clones were identified by Sanger Sequencing to carry the corrected point mutation (yellow box; CTG -> CCG).

CRISPR/Cas9

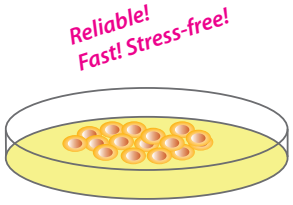
Blood Lineage Cell Line Gene Editing Service

ASC offers full service to genetically modify "hard-to-transfect" blood lineage cell line models (such as Jurkat, TF-1, B Lymphocytes) using CRISPR/Cas9 technology. Our custom gene modification services include:

- Knock-in, knockout and point mutations
- Custom homozygous and heterozygous clones
- gRNA validation assays in Jurkat cells

Service Includes:

- Targeting vector construction and validation
- Cell transfection and selection
- Single cell cloning
- Expansion and cryopreservation



Deliverables and Timeline:

- Genetically engineered cells with confirmed mutation(s).
- Dedicated project managers; milestone reports

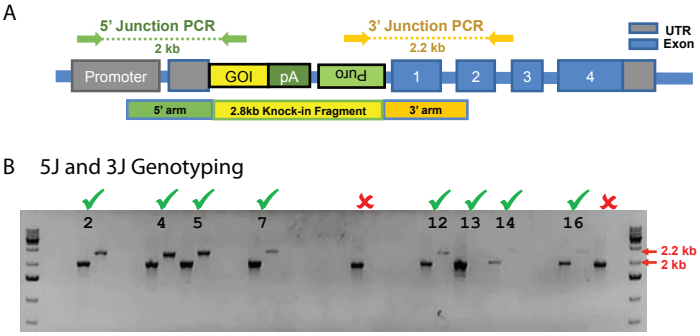
Knockout	3 - 5 months
Knock-in	3 - 6 months
Point Mutations	4 - 6 months

Case Studies

1. Knock-in Modification in Jurkat Cells

Goal: To genetically engineer Jurkat cells to express a specific isoform of the gene of interest while preventing the expression of other isoforms.

How? The 2.8 kb gene of interest (GOI) was designed to be inserted at the 5' UTR, followed by a transcription terminator sequence (polyA; pA) to ensure that only the specific isoform of the GOI would be expressed. The entire knock-in fragment was inserted into Jurkat-C9m cell lines (Jurkat-expressing Cas9 cells) using optimized CRISPR protocols.

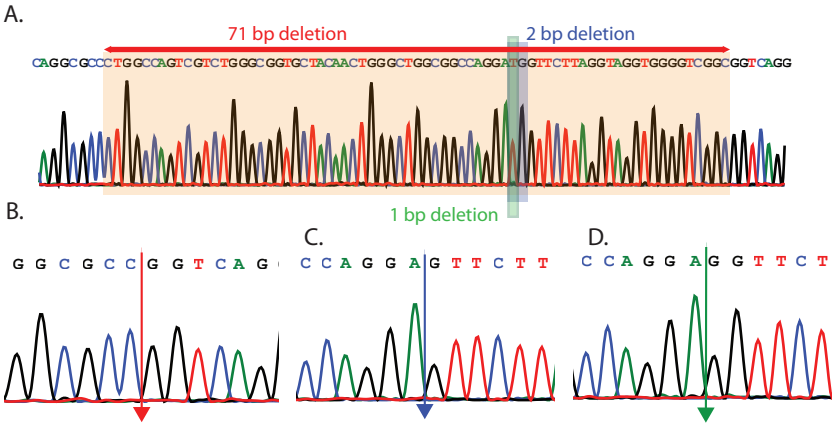


Result: (A) Targeting strategy to express a specific isoform of the gene of interest (GOI). (B) Fifteen out of 34 clones sequenced were identified with the desired knock-in fragment. Junction PCR for clones #1-17; 5' junction (5J; 2 kb band) and 3' junction (3J; 2.2 kb band). Note: "✓" indicates clones with correct insertion of gene; "X" indicates clone does not have insertion.

2. Gene Knock-out – KHYG-1 (NK) cells

Goal: To introduce deletions in 3 alleles (71 bp, 2 bp and 1 bp, respectively) of the gene of interest in a natural killer (NK) cell line to generate a knockout model.

How? The gene of interest in the NK cells was genetically engineered using optimized CRISPR protocols and transfection conditions to ensure high cell viability and efficiency of modification.

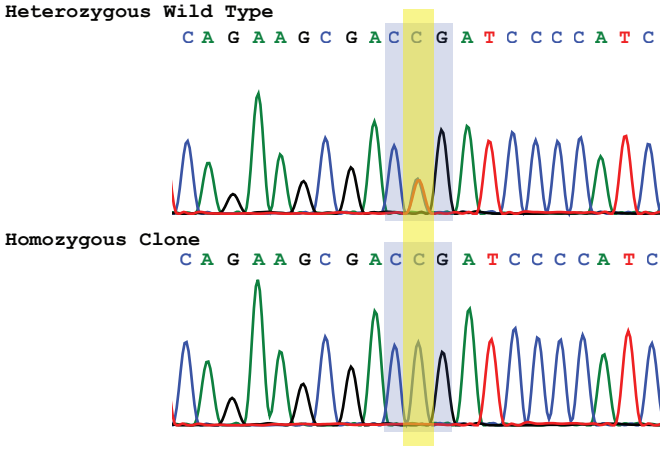


Result: (A) Chromatogram of wild type sequence of the gene of interest. (B-D) Deletion of 71 bp, 2 bp and 3 bp was engineered in the 3 alleles, respectively as confirmed by Sanger Sequencing.

3. Point Mutation– BCWM-1 (B Cells) Cells

Goal: To convert a heterozygous allele to a homozygous form with a single nucleotide point mutation in bone marrow-derived B cells using CRISPR.

How? The gene of interest in the NK cells was genetically modified using optimized CRISPR protocols and transfection conditions to ensure high transfection efficiency and optimal cell viability.



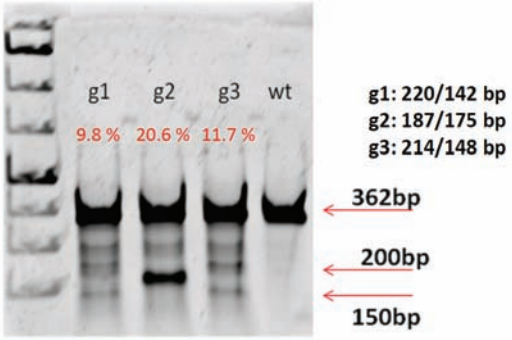
Result: Chromatogram comparing Wild type and B cell clone confirms single nucleotide point mutation (T > G) to convert a heterozygous wild type (CCG/CTG; red and blue peaks highlighted in yellow) to a homozygous allele (CCG/CCG; only one blue peak highlighted in yellow).

CRISPR Cas9 Expressing Cell Lines

For reliable gRNA validation in cell lines and for engineering stable isogenic cell lines, use our Cas-9 expressing Jurkat, K562 and mESC (mouse embryonic stem cells) cell lines. These cell lines stably express S. pyogens Cas9 nuclease and eliminate the inconsistencies observed because of unsynchronized expression of gRNA and Cas9 plasmids.

- ASE-9300 Jurkat-C9m cells (Cas9-expressing Jurkat)
- ASE-9311 K562_LC9 cells (Cas9-expressing K562)
- ASE-9312 mESC-C9m (Cas9-expressing mESC; C57/BL6)

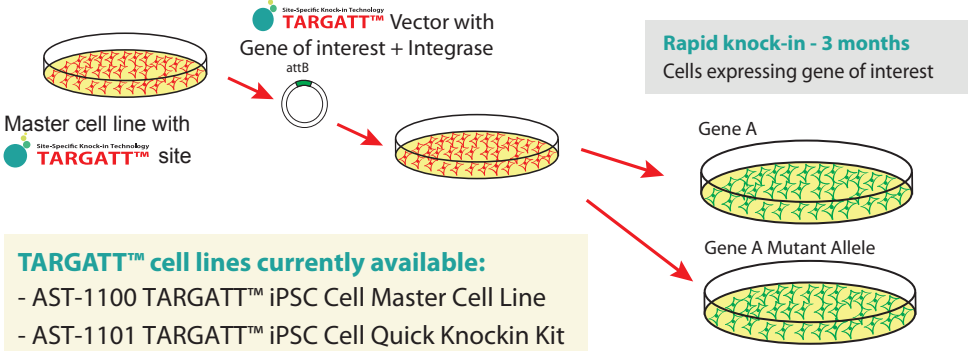
Result: Validation result of oligo-synthesized gRNAs that were transfected into Jurkat-C9m show that all three gRNAs to be active in the mismatch assay.



Site-Specific Knock-in Technology
TARGATT™

Fast & Site-Specific TARGATT™ Knock-in Cell Model Generation Service

ASC's proprietary TARGATT™ technology enables highly efficient site-specific gene integration of large DNA fragment (up to 20 kb) in mammalian cells and animals. This technology uses ΦC31 integrase to insert any gene of interest into a pre-engineered docking site at a safe harbor locus.



Rapid knock-in - 3 months
Cells expressing gene of interest

TARGATT™ cell lines currently available:
- AST-1100 TARGATT™ iPSC Cell Master Cell Line
- AST-1101 TARGATT™ iPSC Cell Quick Knockin Kit
- AST-1102 TARGATT™ iPSC Genotyping Kit

Interested in Bioproduction? Inquire about our TARGATT™-CHO cells for high yield expression of your recombinant proteins

CRISPRCLEAR™

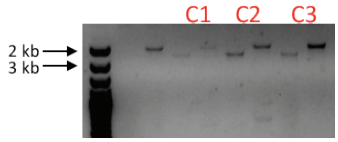
CRISPRCLEAR™ Safe Harbor Locus Kit

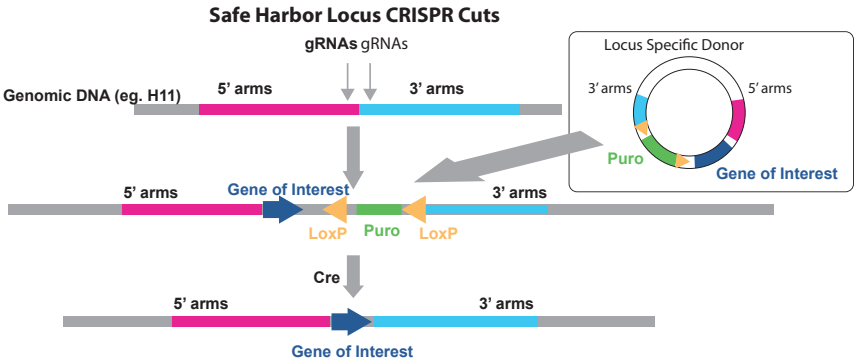
CRISPRCLEAR™ Safe Harbor Locus Knock-in Kit for Human Cell Lines is a do-it-yourself genome editing toolkit for generating CRISPR knock-in cell line models at a safe harbor locus (hH11, hROSA26 or hHAAVS1) in your own lab. A CRISPRCLEAR™ Safe Harbor Locus mROSA26 Knock-in Kit is also available for editing mouse cell lines.

Kit contains:

hAAVS1, hH11 or hROSA26 locus specific targeting vectors and primers:

- gRNA plasmid
- Donor plasmid
- Cas9 puro plasmid
- 5' HR primer pair
- 3' HR primer pair



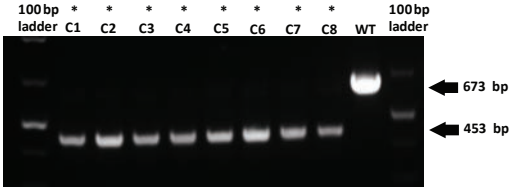


Figures: Schematic: The strategy used to design the CRISPR kits. Gel image: A 4.3 kb sized gene of interest was inserted into the hH11 locus using the CRISPRCLEAR™ Safe Harbor Locus kit for human cell lines. Junction PCR at hH11 locus showed a 2.3 kb 5' arm fragment and a 2.8 kb 3' arm fragment indicative of transgene knock-in at the locus.

CRISPRCLEAR™ Knock-in, Knockout, Point Mutation Kit

The CRISPRCLEAR™ Knock-in, Knockout or Point Mutation Kit is designed to target a specific gene in your cell line of interest.

- Order by gene ID#
- Deep sequencing validated gRNA
- Customized for your request – Ex. double knockout, targeting specific region, avoid homologues
- Primer design service optional



Kit contains:

- Validated gRNA plasmids
- Customized donor DNA
- Cas9 expression plasmids

Figure: The CRISPRCLEAR™ Knockout kit was used to generate a 220 bp deletion in the gene of interest in HeLa cells; WT (wild type) - 673 bp and clones with deletion (C1-C8) - 453 bp.

CRISPRCLEAR™ Kits for Editing Blood-derived Cell Lines

ASC's Blood-derived Cells Knock-in, Knockout or Point Mutation kit is designed with gRNA plasmid(s) to target a gene of interest in your blood-derived cell line (T Lymphocytes, B lymphocytes, NK cells, etc.) as a do-it-yourself option. The kit is designed with the most efficient CRISPR elements and optimized protocols to ensure high modification efficiency and cell viability. The gRNA and donor plasmids will be customized to match the needs of your projects after detailed discussions between our scientists and you. Our kits ensure fast turnaround and quality deliverables for your disease-specific studies in these hard-to-transfect cell lines.

Kit contains:

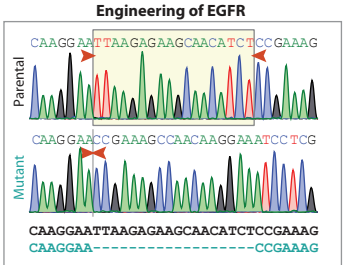
- Three (3) Customized gRNAs
- Three customized gRNA-matched donor plasmids for Knock-in and Point Mutation Kits
- CRISPR Master Mix

ONCOREF™
Molecular Reference Standards

MAPK Mutation Series (EGFR, KRAS, BRAF)

ASC's series of isogenically matched cancer cell lines, FFPE slides/ Scrolls and Tissue Blocks represent ideal materials for companion diagnostics, assay development and routine quality control. The matched cell lines will focus on mutations in the MAPK Pathway, and particularly on mutations in the EGFR, KRAS and BRAF genes. Our comprehensive, 51-cell line mutation panel will serve an unmet need as pharmacogenomic reference standards and as precision tools for drug discovery.

Engineering of EGFR



An 18 bp deletion in exon 19 of EGFR, corresponding to COSMIC mutation 6255 (c.2239_2256del18), was engineered into the RKO cell line. The homozygous positive clone, above, was identied, expanded, and confirmed by Sanger sequencing.

Engineering of KRAS

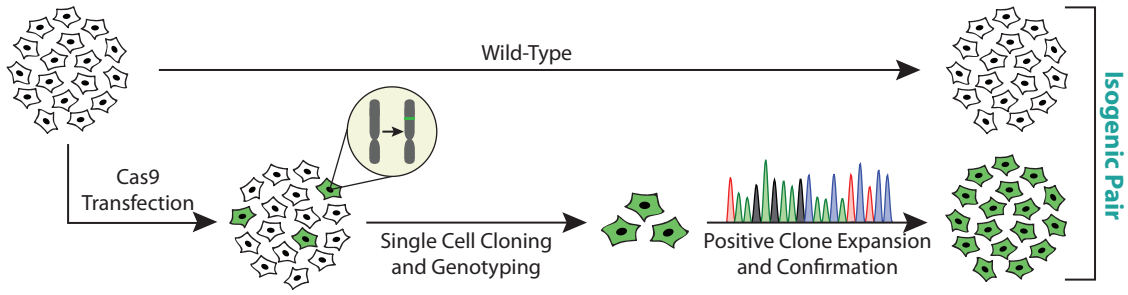
A G>T mutation at codon 12 of KRAS exon 2 (c.35G>T), corresponding to COSMIC mutation 520, was engineered into the RKO cell line. The homozygous positive clone, above, was identied, expanded, and confirmed by Sanger sequencing.

Key Features:

- Most comprehensive MAPK mutation panel on the market: 51 mutations in the EGFR, KRAS, and BRAF genes
- Footprint-free genome editing using CRISPR/Cas9
- Homozygous knock-in of the desired mutation
- Isogenically paired positive and negative controls
- Available in well-established colorectal cancer cell lines: EGFR (RKO), KRAS (RKO), BRAF (HCT116)
- Biorelevant samples: Eliminates the reliance on plasmids and synthetic DNA reference materials

MAPK Isogenic Cell Lines

Isogenic cell lines are a unique toolset for studying cellular biology, such as the impact of genotype on cellular phenotype, as well as for parallel, high throughput screening to enable the discovery of therapeutic compounds that exhibit genotype-specific toxicity.



MAPK Isogenic FFPE Slides, Scrolls, and Cell Blocks

Cell line-based FFPE reference standards represent ideal materials for companion diagnostics assay development and routine quality control.

Benefits and Applications:

- Optimize your DNA extraction protocols prior to using valuable patient samples
- Monitor the impact of workflow changes on downstream results
- Establish limits of detection (LODs) using mixtures of isogenically-paired, mutant and wild-type DNA
- Easily transfer workflows across labs or institutions using validated reference standards
- Accurately evaluate batch-to-batch variability to streamline your quality control process

COMING SOON!
PI3K/AKT/mTOR Pathway Panel & Expanded RAS Panel

- Most comprehensive RAS and PI3K/AKT/mTOR mutation panels
- Development of NRAS and HRAS reference materials

Custom Isogenic Cell Line Engineering and Reference Standards Service:

Service Includes:

- ASC can help you engineer your own screening panel by engineering your desired mutations into a cell line of your choosing
- Leverage our genome editing expertise: ASC has generated hundreds of engineered cell lines in more than 100 different human and mammalian cell line

Custom FFPE Service

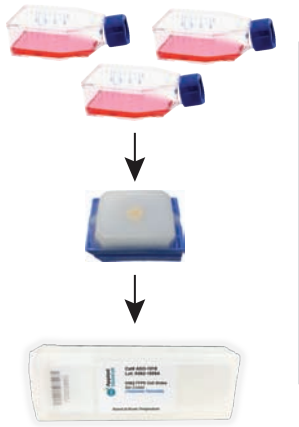
Ship us your cell lines, and ASC can generate custom FFPE blocks, slides, or scrolls. For slide generation, both single-spotting and multi-spotting services are available.

Applications:

- Controls for IHC, ISH, qPCR, sequencing
- Reference materials for in vitro diagnostics development
- Perfect for pairing drug discovery with companion diagnostics development

Key Features:

- Generation of FFPE reference materials from your own lab's cell lines
- Utilize our multi-spotting capacity to mix and match different mutations or cell lines on a single slide
- Homogenous and consistent FFPE blocks



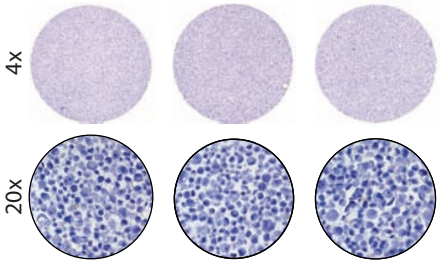


Figure 1. Three separate batches of FFPE blocks were generated using the KRAS G12V mutant cell line. Blocks were sectioned at 5 um thickness, mounted on microscopy slides, stained with haematoxylin, and imaged by optical microscopy. All blocks showed very high uniformity in both cross-sectional coverage and cell packing density.

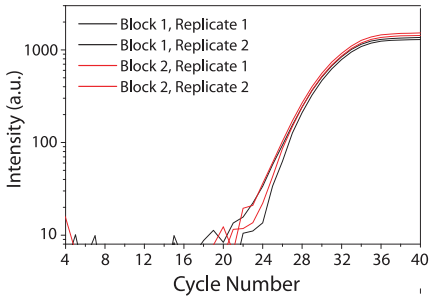


Figure 2. Two separate batches of FFPE blocks were sectioned and used for qPCR to detect BRAF V600E. The two separate batches showed very high reproducibility.

Custom Virus Packaging Service

Applied StemCell provides lentiviral and retroviral services to help advance your research without the struggle of designing and preparing your own viruses. We also provide custom virus packaging for efficient delivery of CRISPR components into in vivo and in vitro models.

Applications of recombinant lenti- and retroviruses:

- Transgene expression in both dividing and non-dividing cells
- Useful for enabling long-term transgene expression in infected cells
- Suitable for hard-to-transfect cell lines
- Enables direct local injection to targeted tissue in animals

Features:

- Ready-to-transduce viral particles
- High quality and high titer viral particles in as little as 10 days
- VSV-G pseudotyped viruses that exhibit broad tropism across a range of cell types
- High titer amplification of viruses (up to 10^9 IFU/mL)
- qRT-PCR quantified viral titers

Cell Line Immortalization Service

Simian virus 40 (SV40) T antigen has been shown to be the simplest and most reliable agent for the immortalization of many different cell types and the mechanism of SV40 T antigen in cell immortalization is relatively well understood. Recent studies have also shown that SV40 T antigen can induce telomerase activity in the infected cells. Applied StemCell's fibroblast immortalization techniques and service can provide you immortalized cell lines from your valuable patient samples for an ideal cell line model for your research.

Service Includes:

- Lentivirus vector(s) expressing SV40 large and small T antigens
- Viral transduction
- Colony selection (up to 10 passages)
- Characterization (transgene expression by RT-PCR)
- Culture, cryopreservation

Also available is a do-it yourself-kit, with reagents and protocols optimized by our scientists for efficient cell immortalization in your own lab.

ASK-3501 Cell Immortalization Kit

Deliverables and Timeline:

- 2 vials of 1 x 10^6 cells/vial of immortalized cells
- 2-5 months depending on immortalization method, source cells and species

Primary Cell Lines

Primary cells are an excellent biomedical research model and are particularly valuable for clinically relevant studies. Our cells have been used for many in vivo and in vitro applications including iPSC-reprogramming, cell-based assays, tissue regeneration, tissue engineering, transplantation studies and as feeder cells in co-cultures. ASC's primary cell catalog also has mouse primary cells for researchers seeking murine cellular models.

Key features:

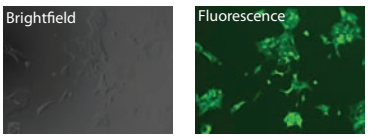
- Isolated from single donors using strict IRB-approved guidelines
- Cryopreserved at an early passage
- Quality tested for viability and growth potential
- Cells identified and characterized by biomarker staining
- Pathogen and mycoplasma –free cultures

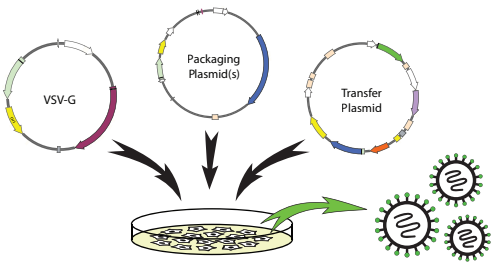
Vector Design and Cloning Service

Applied StemCell has decades of expertise in anything DNA-related. We can help you develop the best strategies to tackle your cloning projects, and find solutions to your technical problems.

Service include:

- Services for Genome Engineering, *in vivo* and *in vitro* applications
- Restriction fragment cloning
- Design and validation of CRISPR/Cas9 components; gRNA design and *in vitro* validation by Next Generation Sequencing (NGS)
- Design and construction of TARGATT™ vectors for use with hiPSC and C57BL6 master lines
- Gene targeting vectors for homologous recombination
- Design and construction of vectors for transient mammalian transgene expression
- Bacterial Artificial Chromosome (BAC) recombineering for large fragment insertion
- Design of RNAi and inducible vectors, gene tagging, and site-directed mutagenesis
- Vector design for generation of random transgenic cell line and animal models





Select Scientific Publications Citing Applied StemCell's Cell Line Service and Products

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- Peng, L., et al. (2016). Reprogramming macrophage orientation by microRNA 146b targeting transcription factor IRF5. EBioMedicine, 14, 83-96.
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- Besschetnova, T. Y., et al. (2015). Regulatory mechanisms of anthrax toxin receptor 1-dependent vascular and connective tissue homeostasis. Matrix Biology, 42, 56-73.

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