

TrueTag™ Donor DNA Kit

USER GUIDE

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Product information

Product description

The Invitrogen™ TrueTag™ Donor DNA Kits enable generation and enrichment of cell lines with knock-in fluorescent or epitope-tagged genes, or functional knockout of a target gene. The kits enable knock-in DNA donors to be prepared in just a few hours using one-step PCR and are compatible with various gene editing tools, including CRISPR- and TALEN-based methods. All kits include donor template options for either puromycin or blasticidin selection markers that allow enrichment of the edited cell population.

Contents and storage

The TrueTag™ Donor DNA Kit consists of an Assembly Kit (with control primers) and a Cleanup Kit. Each kit provides sufficient reagents to make 10 donor molecules (produces >5 µg donor/prep).

Product	Catalog Number
TrueTag™ Donor DNA Kit, GFP	A42992
TrueTag™ Donor DNA Kit, RFP	A42993
TrueTag™ Donor DNA Kit, BFP	A53806
TrueTag™ Donor DNA Kit, YFP	A53807
TrueTag™ Donor DNA Kit, DDK ^[1]	A53808
TrueTag™ Donor DNA Kit, HA	A53809
TrueTag™ Donor DNA Kit, Myc	A53810
TrueTag™ Donor DNA Kit, 6xHis	A53811
TrueTag™ Donor DNA Kit, GFP Stem	A53812
TrueTag™ Donor DNA Kit, RFP Stem	A53813
TrueTag™ Knockout Enrichment Donor DNA Kit	A53815

^[1] – DYKDDDDK Tag (FLAG® tag, FLAG® octapeptide)

Assembly Kits

The Assembly Kit contains Phusion™ Flash High Fidelity PCR Master Mix, donor templates for fluorescent reporters or epitope tags with selection markers, and pre-mixed positive control primers.

Note: Maintain DNase- and RNase-free conditions by using RNase-free reagents, tubes, and barrier pipette tips when setting up experiments.

Note: Store all kits and components at –20°C.

Kit	TrueTag™ Donor Templates (400 ng (20 ng/μL) each)				Phusion™ Flash High Fidelity PCR Master Mix (500 μL)	Positive Control Primers (Forward and Reverse) (100 μL)
Cat. No. A42992	N-Puro-GFP	N-Blast-GFP	C-Puro(Cre- Lox)-GFP	C-Blast(Cre- Lox)-GFP	✓	Human ACTB
Cat. No. A42993	N-Puro-RFP	N-Blast-RFP	C-Puro(Cre- Lox)-RFP	C-Blast(Cre- Lox)-RFP	✓	Human ACTB
Cat. No. A53806	N-Puro-BFP	N-Blast-BFP	C-Puro(Cre- Lox)-BFP	C-Blast(Cre- Lox)-BFP	✓	Human ACTB
Cat. No. A53807	N-Puro-YFP	N-Blast-YFP	C-Puro(Cre- Lox)-YFP	C-Blast(Cre- Lox)-YFP	✓	Human ACTB
Cat. No. A53808	N-Puro-DDK	N-Blast-DDK	C-Puro(Cre- Lox)-DDK	C-Blast(Cre- Lox)-DDK	✓	Human ACTB
Cat. No. A53809	N-Puro-HA	N-Blast-HA	C-Puro(Cre- Lox)-HA	C-Blast(Cre- Lox)-HA	✓	Human ACTB
Cat. No. A53810	N-Puro-Myc	N-Blast-Myc	C-Puro(Cre- Lox)-Myc	C-Blast(Cre- Lox)-Myc	✓	Human ACTB
Cat. No. A53811	N-Puro-His	N-Blast-His	C-Puro(Cre- Lox)-His	C-Blast(Cre- Lox)-His	✓	Human ACTB
Cat. No. A53812	C-GFP- pPuro(Cre-Lox)	C-GFP- pBlast(Cre- Lox)	C-2A-GFP- pPuro(Cre-Lox)	C-2A-GFP- pBlast(Cre- Lox)	✓	Human GFAP

(continued)

Kit	TrueTag™ Donor Templates (400 ng (20 ng/μL) each)				Phusion™ Flash High Fidelity PCR Master Mix (500 μL)	Positive Control Primers (Forward and Reverse) (100 μL)
Cat. No. A53813	C-RFP- pPuro(Cre-Lox)	C-RFP- pBlast(Cre- Lox)	C-2A-RFP- pPuro(Cre-Lox)	C-2A-RFP- pBlast(Cre- Lox)	✓	Human GFAP
Cat. No. A53815	2A-Puro- pGFP(Cre-Lox)	2A-Blast- pGFP(Cre-Lox)	2A-Puro- pRFP(Cre-Lox)	2A-Blast- pRFP(Cre-Lox)	✓	Human HPRT

Control Primers

Control Primers are provided to create donor molecules for tagging ACTB, GFAP, or HPRT (depending on kit type). The forward and reverse N-terminal human positive controls are supplied at a concentration of 10 μM each, and come pre-mixed in a single tube.

Sequence for human ACTB control primers ^[1]
Forward primer
5'-CFOAGCGCGCCCGGCTATTCTCGCAGCTCACCATGGGAGGTAAGCCCTTGCATTGCG-3'
Reverse primer
5'-COETTGTGACGACGAGCGCGGCGATATCATCATCACCGCTTCCACTACCTGAACC-3'

Human HPRT control primers to make a Knockout Enrichment donor ^[1]
Forward primer
5'-GOZGAGGATTTGGAAAGGGTGTATTCTCATGGAAGTGGCTCAGGTTCTGGA-3'
Reverse primer
5'-CFZTTTAAGATCTTACTTACCTGTCCATAATTAGTCCCTTGGCCGATCGCATAACAGAG-3'

Human GFAP control primers to make a Stem donor ^[1]
Forward primer
5'-CFZTAAGGAGTCCAAGCAGGAGCACAAGGATGTGATGGGAAGTGGCTCAGGTTCTGG-3'
Reverse primer
5'-GFOGGGGCAGAGGCCACCAGGTGGGTCTGCCTCACTTGGCCGATCGCATAACAGAG-3'

^[1] The primers have 2X phosphorothioate modifications at the 5' ends. F = Phosphorothioate-A; O = Phosphorothioate-C; E = Phosphorothioate-G; Z = Phosphorothioate-T

Clean Up Kit

The Clean Up Kit is designed for rapid and efficient purification of DNA from PCR reaction mixtures.

Note: Maintain DNase- and RNase-free conditions by using RNase-free reagents, tubes, and barrier pipette tips when setting up experiments.

Component	Amount	Storage
Binding Buffer	4.8 mL	Room temperature
Wash Buffer (concentrated)	1.8 mL	
Elution Buffer	3 mL	
Purification columns/spin tubes	10 each	

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Item	Source
Primers	
Custom DNA oligos to add homology arms to the TrueTag™ Donor DNA (for details see "Design" on page 15)	10629095 Custom DNA Oligos
Instruments	
A fluorescence microscope with appropriate filters for emGFP, tagRFP, tagBFP, or tagYFP	MLS
A programmable thermal cycler	MLS
Genome editing nuclease (one of the following)	
TrueCut™ Cas9 protein v2 or HiFi protein and a TrueGuide™ sgRNA	A36498 or A50574 and A35514
GeneArt™ CRISPR Nuclease mRNA	A29378
GeneArt™ CRISPR Nuclease Vector with OFF or CD4 Reporter Kit	A21174 or A21175
TALEN mRNA pair	A43763
Consumables	
Cell line-appropriate plastics and media	MLS

(continued)

Item	Source
Reagents	
Genomic Cleavage Detection Kit	A24372
Ethanol 95–99%, molecular biology grade	MLS
Transfection reagent or electroporation system	
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	CMAX00003
Lipofectamine™ MessengerMAX™ Transfection Reagent	LMRNA001
Neon™ Transfection System	MPK1025
Reagents and instruments for tag detection	
Attune™ NxT Flow Cytometer	A29003
iBlot™ 2 Gel Transfer Device	IB21001
iBlot™ 2 Transfer Stacks, nitrocellulose, regular size	IB23001
DDK Tag Monoclonal Antibody (FG4R)	MA1-91878
DDK Tag Monoclonal Antibody (FG4R), DyLight™ 488	MA1-91878-D488
HA Tag Monoclonal Antibody (2-2.2.14)	26183
HA Tag Monoclonal Antibody (2-2.2.14), DyLight™ 488	26183-D488
c-Myc Monoclonal Antibody (9E10)	MA1-980
c-Myc Monoclonal Antibody (9E10), DyLight™ 488	MA1-980-D488
6x-His Tag Monoclonal Antibody (HIS.H8)	MA1-21315
6x-His Tag Monoclonal Antibody (HIS.H8), DyLight™ 488	MA1-21315-D488
Beta Actin Loading Control Monoclonal Antibody (BA3R)	MA5-15739-HRP
GAPDH Loading Control Monoclonal Antibody (GA1R), HRP	MA5-15738-HRP
WesternBreeze™ Chemiluminescent Kit, anti-rabbit	WB7106
WesternBreeze™ Chemiluminescent Kit, anti-mouse	WB7104
SuperSignal™ West Dura Extended Duration Substrate	34075

(continued)

Item	Source
Puromycin Dihydrochloride	A1113803
Blasticidin S HCl	A1113903

Ordering CRISPR gRNA and custom oligos

To assist in designing and ordering the gene-specific reagents required for knock-in of your TrueTag™ DNA donor, TrueDesign Genome Editor software can be accessed at <http://thermofisher.com/truedesign>. This free online tool will walk you through the steps to identify your gene of interest, choose an optimal CRISPR-Cas9 gRNA (or TALEN mRNA pair), and design the locus-specific adaptor primers to add homology arms to the DNA donor from your TrueTag™ kit. All necessary components may be ordered directly from the software or a results file may be downloaded for ordering from the Thermo Fisher Scientific website.

Required products available for design and ordering from the TrueDesign Genome Editor software:

- CRISPR guide RNA (synthetic sgRNA) or TALEN mRNA pairs
- TrueTag™ primers (for amplification and addition of homology arms to the donor DNA template provided in the kit)
- TrueTag™ verification primers (to validate proper insertion of the DNA donor template)
- TrueCut™ Cas9 protein or mRNA
- Lipofectamine CRISPRMAX™ Transfection Reagent
- GCD/sequencing primers (to verify and analyze CRISPR-Cas9 cutting efficiency)

If you are unable to order through the TrueDesign software, you may download the experimental details and use the following guidelines:

- Order custom DNA oligos from Thermo Fisher Scientific by visiting thermofisher.com/oligos. We recommend adding 2 phosphorothioate linkages to the 2 terminal bases at the 5' ends of the oligos to inhibit nuclease digestion of the donor. Standard 50 nM scale with desalted purification is recommended.
- Order custom CRISPR gRNA from Thermo Fisher Scientific by visiting <http://thermofisher.com/order/custom-oligo/crispr>. We recommend ordering modified sgRNA for best performance and stability.
- Order custom TALEN mRNA pairs by visiting thermofisher.com/order/custom-oligo/talens.

Workflow

1. Design and order target specific oligos to add the homology arms to the supplied template.
2. Use the custom homology arms oligos to amplify the desired TrueTag™ template.
3. Purify the PCR reaction with the included purification spin columns.
4. Co-transfect the donor DNA with the TrueCut™ Cas9 protein and TrueGuide™ gRNA or a FlexCut™ TALEN mRNA pair. Additional reagent types are also acceptable. See the Methods section on page 15 for more details.
5. Enrich the edited cells using antibiotic resistance.
6. *(Optional)* Remove antibiotic selection cassette by Cre/Lox.
7. Confirm successful tagging and clonal cell line generation.

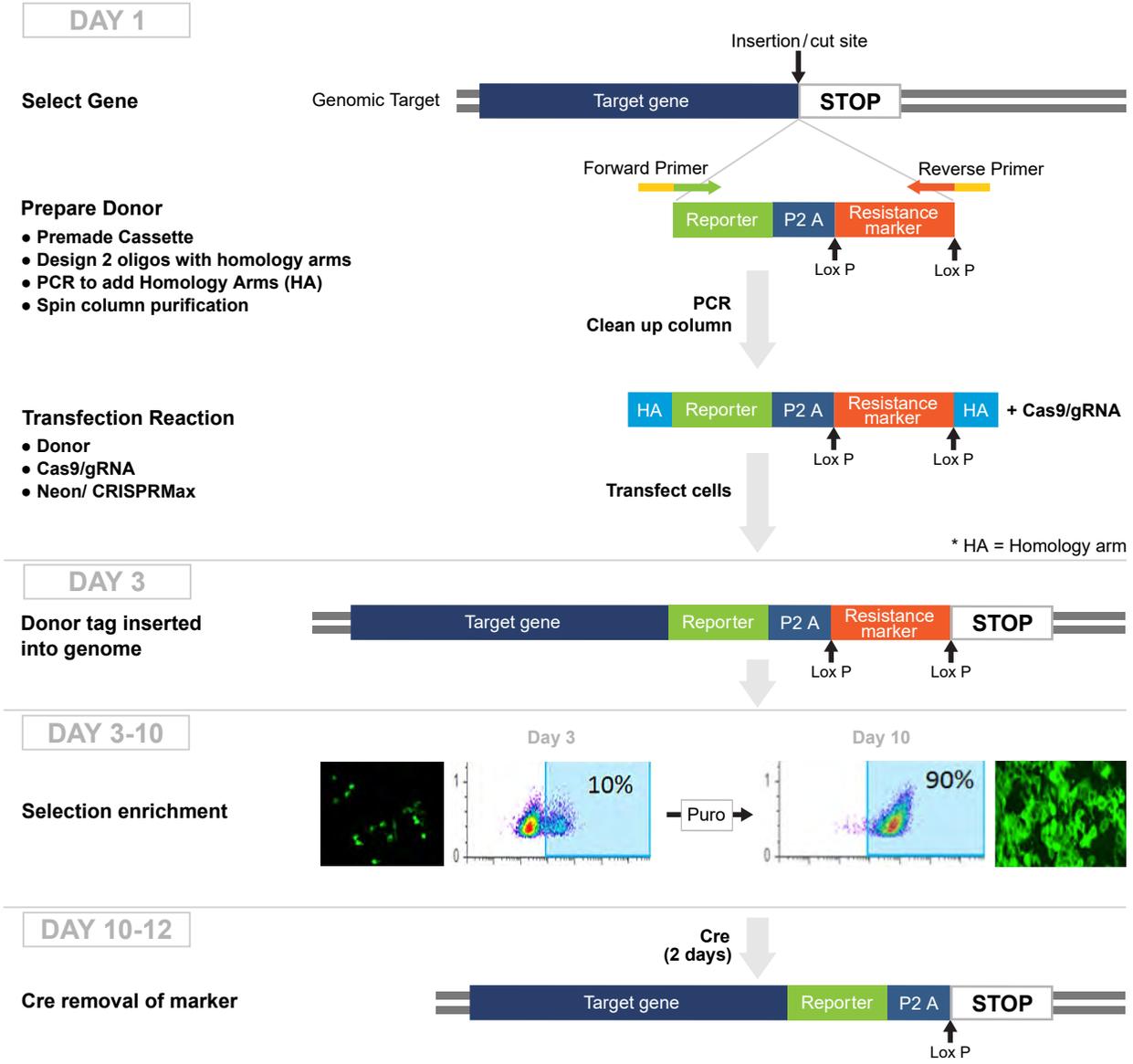


Figure 1 TrueTag™ workflow.

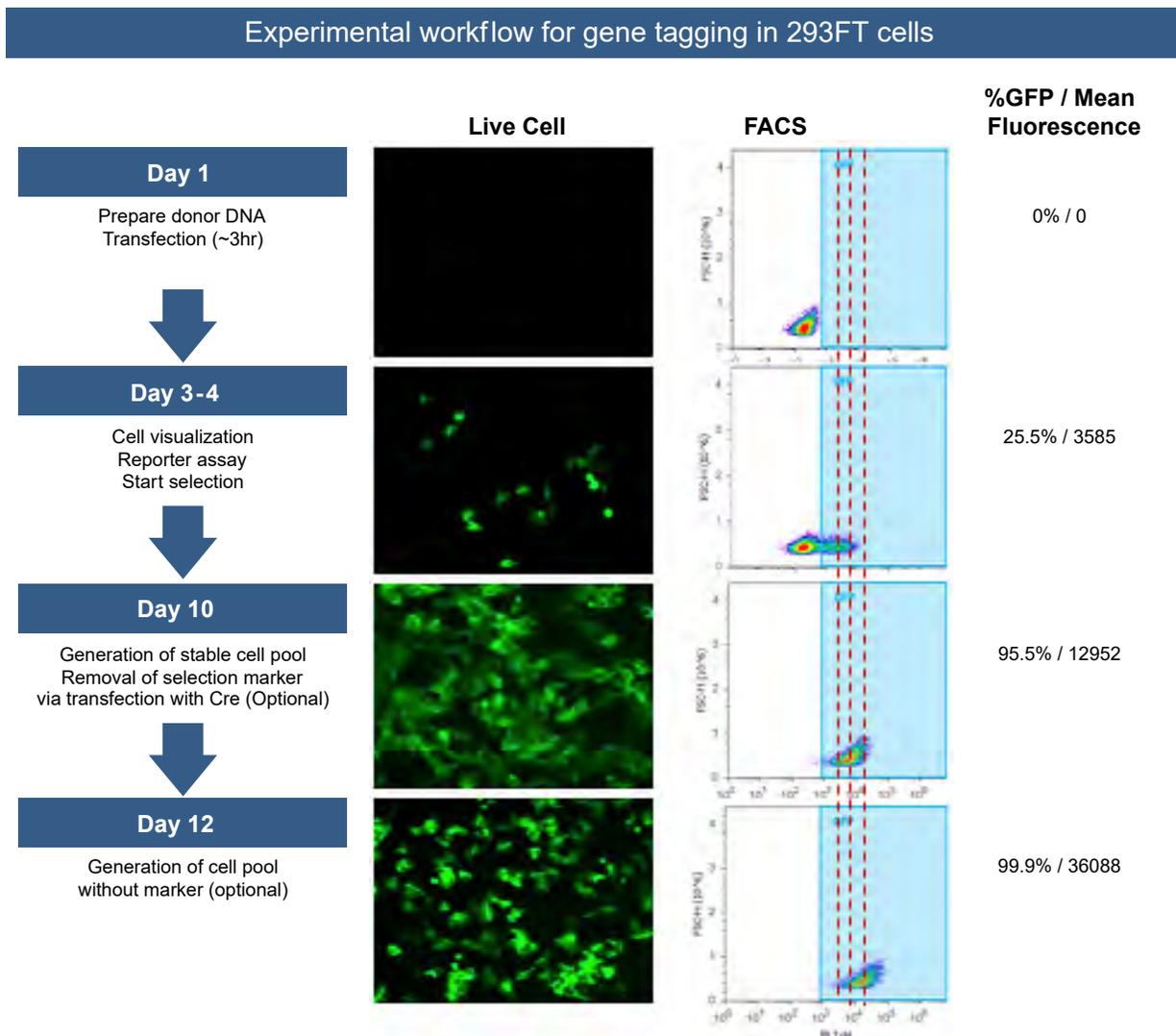


Figure 2 Workflow example targeting ACTB with carboxyl GFP tag showing enrichment by microscope and flow cytometry.

Overview

Invitrogen™ TrueDesign Genome Editor software is a free online tool for fast, simple design of all components needed for use with TrueTag™ Donor DNA Kits. The software assists in avoiding the time and effort of manual design by searching for your gene, configuring your gene tagging or knockout enrichment insert, and ordering the required components. Additionally, workflow design results can be downloaded for offline ordering or keeping for your records. The software is available at thermofisher.com/truedesign.

Design

To add a tag to a gene of interest, the natural homology-directed repair (HDR) pathway is used to repair the genomic DNA at the site of a double-stranded break caused by an endonuclease. This double-stranded break can be generated by CRISPR-Cas9 nucleases or transcription activator-like effector nucleases (TALEN). Repair and insertion of the tagging DNA is accomplished by designing a donor template that has matching homology arms on each side of the tag (Figure 3).

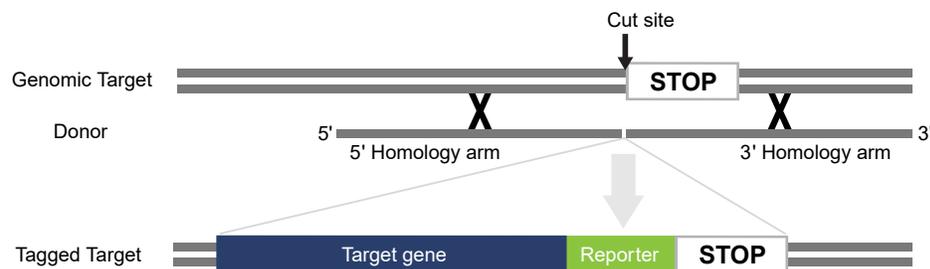


Figure 3 Overview of HDR using a donor template for insertion of a carboxy gene tag.

It has been shown that short homology arms (30–35 bases each) are sufficient for efficient insertion of TrueTag™ donors, and allows for easy construction of the donor molecule where the homology arms are added by PCR to a premade tagging template (Liang et al., 2017). Compared to plasmid donor constructs having an extensive vector backbone, using a PCR product without unnecessary components minimizes the size of the donor, which then eliminates the risk of toxic *E. coli* elements being integrated into the cell and results in overall higher editing efficiency.

For amino termini (N-terminal) tagging, the TrueTag™ donor should be inserted immediately after the start ATG with the homology arms located to the left and right of the start ATG. For carboxy (C-terminal) tagging, the TrueTag™ donor should be inserted immediately before the stop codon with the homology

arms located to the left and right of the stop codon. When choosing between N- and C-terminal tagging, we recommend C-terminal tagging for the following reasons:

- Placement of the tag is at the end of the gene transcript will have less impact on the regulation of the gene.
- Insertion of the selection marker requires successful in-frame insertion of the tag resulting in lower background.

For the TrueTag™ Knockout Enrichment kits, the donor template may be inserted anywhere into the protein coding region while remaining in-frame with protein translation (Figure 4). The 5' and 3' short homology arms (30-35 bases each) are added to the donor template by PCR amplification. The endogenous promoter will drive the expression of puromycin or blasticidin antibiotic selection marker and the exogenous CMV promoter will drive the expression of an EmGFP or RFP reporter gene.

Take into consideration that alternative splicing often occurs in eukaryotes to produce different transcript variants. To knock out a specific transcript variant, a unique gRNA specific to this transcript variant should be designed. To knock out all of the transcript variants, a common gRNA among all the transcript variants should be used.

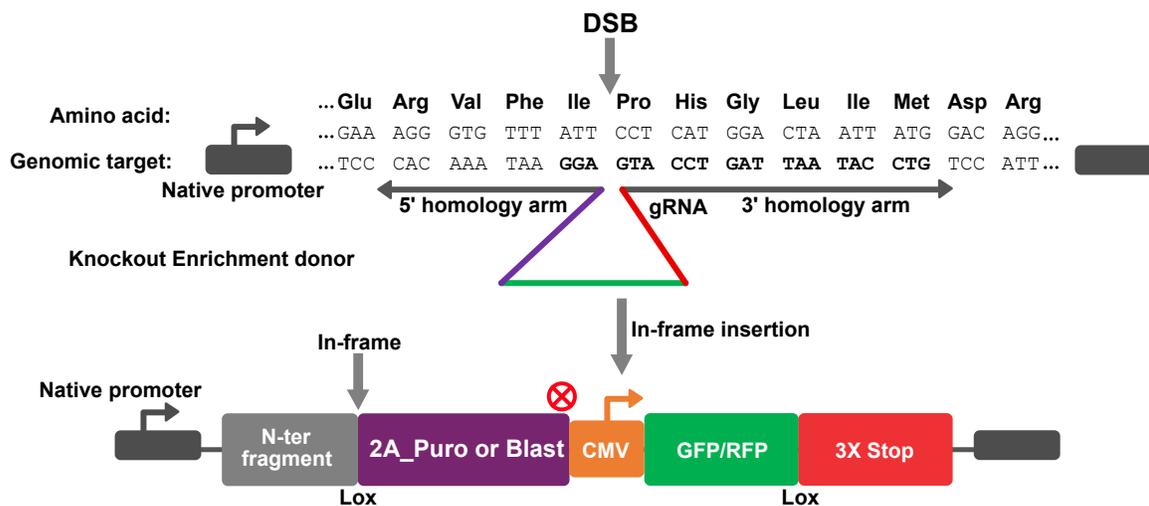


Figure 4 Diagram of TrueTag™ Knockout Enrichment donor insertion strategy.

Donor features

TrueTag™ Donor DNA kits – Fluorescent tags

The fluorescence donor templates (Figure 5) are designed to make a fusion between the target gene and either GFP, RFP, BFP, or YFP linked to puromycin or blasticidin by a 2A self-cleaving peptide so both markers can be expressed off the endogenous promoter. The fusion includes a GSGSGSG linker to minimize steric hindrance of the fusion protein. The amino donor templates and carboxy donor templates have separate universal Forward and Reverse priming sequences to ease the design of the PCR primers for generating the donor. In the C-terminal tag versions, the antibiotic marker is flanked by loxP sites so that the marker can be easily removed by Cre to allow for iterative tagging or minimizing the size of the insertion.

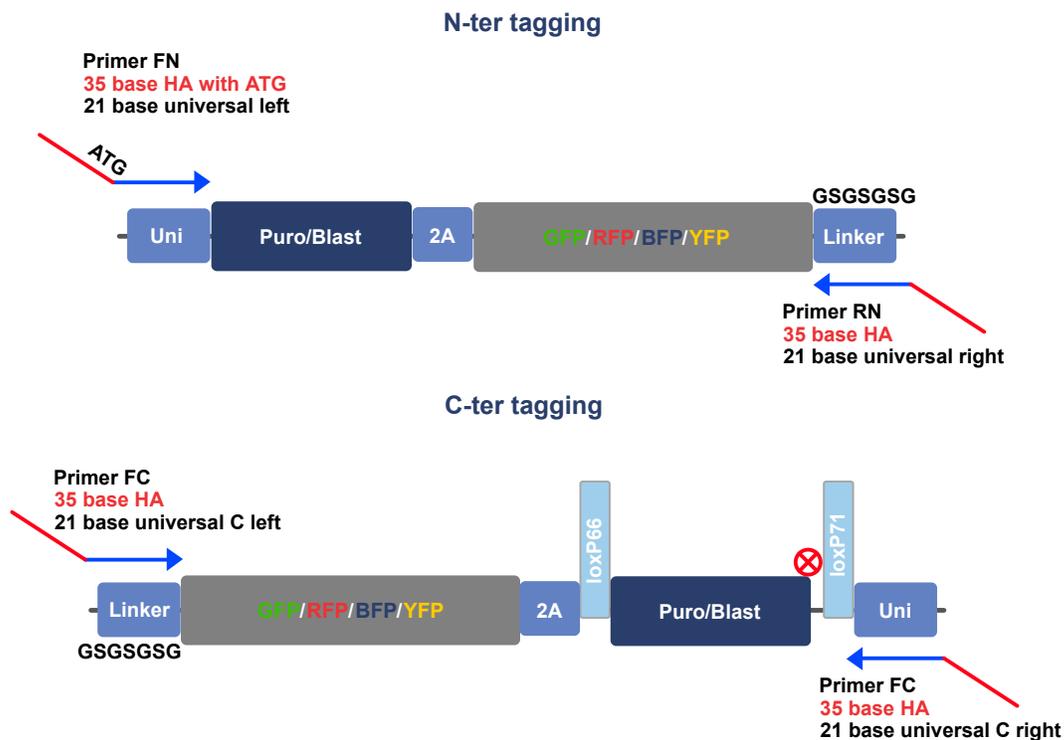


Figure 5 Amino and Carboxyl Tag designs. Add the gene-specific 35-base homology arms to 5' ends.

- | | |
|--|---|
| <p>① Primer FN</p> <ul style="list-style-type: none"> • 35-base homology arms with endogenous ATG • 21-base UniF-N | <p>③ Primer FC</p> <ul style="list-style-type: none"> • 35-base homology arms • 21-base UniF-C |
| <p>② Primer RN</p> <ul style="list-style-type: none"> • 35-base homology arms • 21-base UniR-N | <p>④ Primer RC</p> <ul style="list-style-type: none"> • 35-base homology arms with endogenous STOP codon • 21-base UniR-C |

The available tags are monomeric proteins and include Emerald GFP (EmGFP), tagRFP, tagBFP, and tagYFP.

Table 1 Fluorescent tags in the TrueTag™ Donor DNA kits.

Tag	Color	Excitation Wavelength (nm)	Emission Wavelength (nm)
EmGFP	Green	487	509
tagRFP	Red	555	584
tagBFP	Blue	402	457
tagYFP	Yellow	508	524

Table 2 Universal primers for standard tags.

These primers are not included in the TrueTag™ Donor DNA kits. Design and order primers using the TrueDesign Genome Editor Software. The tool will add the gene-specific, 35-base homology arms to 5' ends of the primers.

Amino donor	UniF-N	5' GGAGGTAAGCCCTTGCATTCG 3'
	UniR-N	5' ACCGCTTCCACTACCTGAACC 3'
Carboxy donor	UniF-C	5' GGAAGTGGCTCAGGTTCTGGA 3'
	UniR-C	5' CTTGGCCGATCGCATAACAGAG 3'

To design your fluorescent TrueTag™ Donor DNA, the TrueDesign Genome Editor Software will take you through the procedure:

1. Select a protein-coding gene transcript to be tagged.
2. Choose amino (N-terminus; at ATG start codon) or carboxy fusion (C-terminus; before stop codon).
3. Choose a fluorophore for your tag (EmGFP, tagRFP, tagBFP, or tagYFP).
4. Choose a selection marker (puromycin, blasticidin).

The TrueDesign software will:

- Identify and score RNA reagents to make a nuclease cut (CRISPR-Cas9 or TALEN) near the insertion site.
- Design gene-specific PCR oligos to add homology arms to donor templates provided in the TrueTag™ Donor DNA kits.

All items may be added to your Thermo Fisher Scientific cart or downloaded as an Excel file for subsequent ordering.

TrueTag™ Donor DNA Kits – Epitope tags

The epitope donor templates (Figure 6) are designed to create a fusion between the target gene and DDK (DYKDDDDK; FLAG octapeptide), HA, c-Myc, or 6xHis tag linked to puromycin or blasticidin by a 2A self-cleaving peptide. This fusion, including a GSGSG linker to minimize steric hindrance of the fusion protein, allows for both markers to be expressed off of the endogenous promoter. The amino- and carboxy-donor templates have separate universal Forward and Reverse priming sequences to ease the design of the PCR primers for generating the donor. In the C-terminal tag versions, the antibiotic marker is flanked by loxP sites so the marker can be easily removed by Cre to allow for iterative tagging or minimizing the size of the insertion.

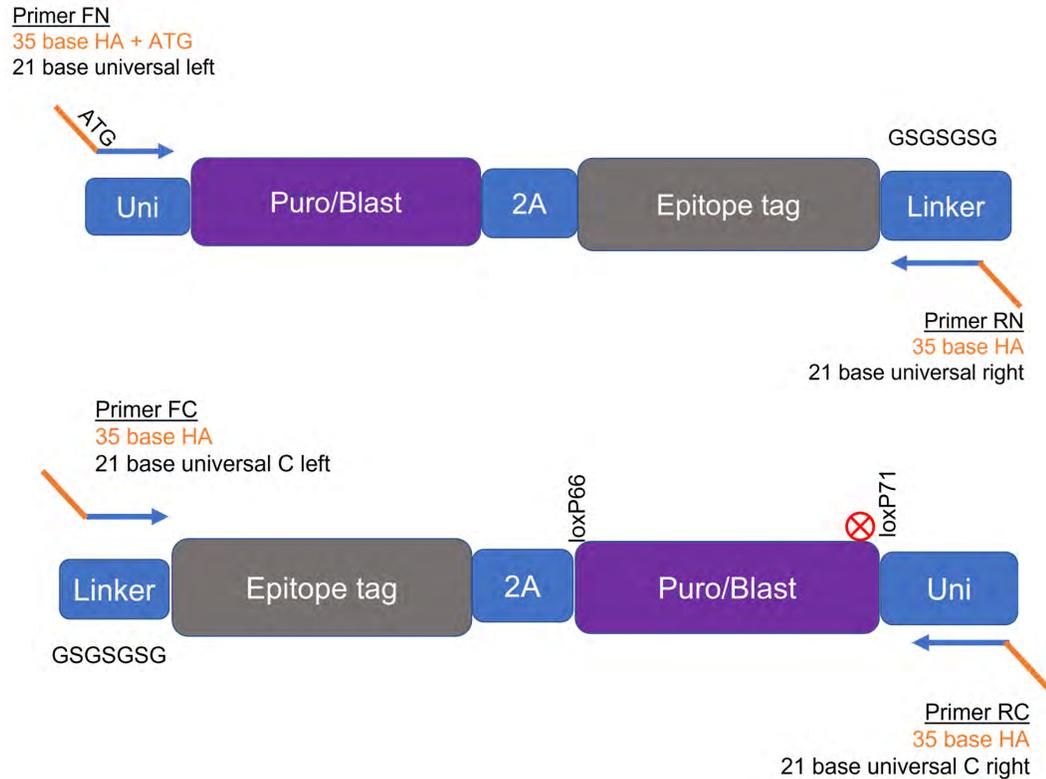


Figure 6 Amino and carboxyl epitope tag designs. Add the gene-specific, 35-base homology arms to 5' ends of primers.

To design your epitope TrueTag™ Donor DNA, the TrueDesign Genome Editor Software will take you through the procedure:

1. Select a protein-coding gene to be tagged.
2. Choose amino (N-terminus; at ATG start codon) or carboxy fusion (C-terminus; before stop codon).
3. Choose an epitope tag (6xHis, HA, DDK, or Myc).
4. Choose selection marker (puromycin, blasticidin).

The TrueDesign software will:

- Identify and score RNA reagents to make a nuclease cut (CRISPR-Cas9 or TALEN) near the insertion site.
- Design gene-specific PCR oligos to add homology arms to donor templates provided in the TrueTag™ Donor DNA kits.

All items may be added to your Thermo Fisher Scientific cart or downloaded as an Excel file for subsequent ordering.

TrueTag™ DNA Donor Kits – Stem donor kit

The stem donor templates (Figure 7) are designed to create a fusion (Figure 7, upper diagram) or non-fusion (Figure 7, lower diagram) between the target gene and GFP or RFP linked to puromycin or blasticidin by a 2A self-cleaving peptide. This fusion, including a GSGSGSG linker to minimize steric hindrance of the fusion protein, is driven by an exogenous EFS promoter. This design allows users to tag endogenous genes while they are at silent stages but become expressed during cell differentiation. The donor templates have separate universal Forward and Reverse priming sequences to ease the design of the PCR primers for generating the donor. The antibiotic marker is flanked by loxP sites so that the marker can be easily removed by Cre to allow for iterative tagging or minimizing the size of the insertion.

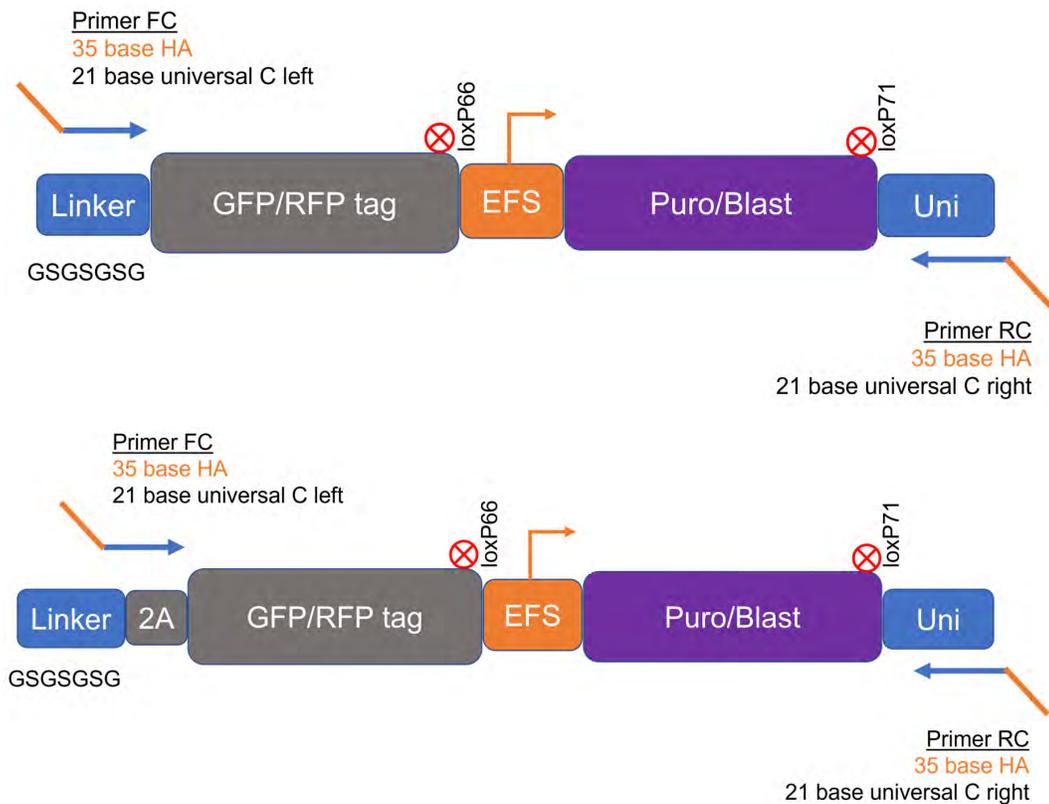


Figure 7 TrueTag™ stem donor designs for fusion (upper diagram) and non-fusion (lower diagram) fluorescent tagging. Add the gene-specific, 35-base homology arms to 5' ends of primers.

To design your TrueTag™ Donor DNA, the TrueDesign Genome Editor Software will take you through the procedure:

1. Select a protein-coding gene to be tagged.
2. Select Expressed or Non-expressed gene. The TrueTag™ Stem kits are specially designed to tag non-expressed genes.
3. Choose a fluorophore for your tag (EmGFP or tagRFP).
4. Choose selection marker (puromycin, blasticidin).
5. Choose fusion or co-expressed fluorescent tag expression. If the co-expressed option is selected, the tag will be cleaved from the endogenous protein following translation.

The TrueDesign software will:

- Identify and score RNA reagents to make a nuclease cut (CRISPR-Cas9 or TALEN) near the insertion site.
- Design gene-specific PCR oligos to add homology arms to donor templates provided in the TrueTag™ Donor DNA kits.

All items may be added to your Thermo Fisher Scientific cart or downloaded as an Excel file for subsequent ordering.

TrueTag™ Knockout Enrichment DNA Donor kit

The Knockout Enrichment donor templates (Figure 8) are designed to enrich knockout cell lines by antibiotic selection and/or cell sorting. The donor template contains puromycin or blasticidin selection marker expressed via a native promoter as a non-fusion protein, a GFP or RFP reporter gene expressed through a CMV promoter, and a 3X stop sequence for termination of protein translation in three possible reading frames. The homology arm needs to be in-frame with the universal linker sequence. The donor templates have separate universal Forward and Reverse priming sequences to ease the design of the PCR primers for generating the donor. The antibiotic marker and fluorescence reporter are flanked by loxP sites so that they can be easily removed by Cre to allow for iterative tagging or minimizing the size of the insertion.

For bi-allelic gene disruption, a donor DNA containing GFP/puromycin and a donor DNA containing RFP/blasticidin are prepared separately by PCR amplification using the corresponding donor templates, and then co-delivered into cells along with Cas9 nuclease or TALEN mRNA. Alternatively, a donor DNA pair of GFP/blasticidin and RFP/puromycin can be used instead. At 48–72 hours post transfection, the cells are selected with both puromycin and blasticidin at a concentration that is determined by their kill curves.

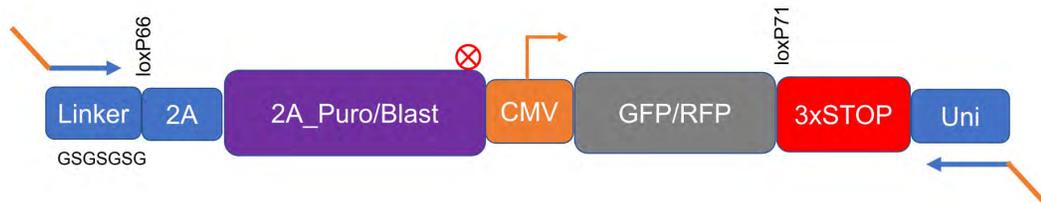


Figure 8 TrueTag™ Knockout Enrichment template.

Add the gene-specific, 35-base homology arm to the 5' ends.

Bi-allelic gene disruption

As a strategy for knockout of each allele with a different fluorophore and selection marker, a donor DNA containing GFP/puromycin and a donor DNA containing RFP/blasticidin are prepared separately by PCR amplification using the corresponding Knockout Enrichment donor templates, and then co-delivered into cells along with Cas9 nuclease (or TALEN mRNA). Typically, 250 ng of GFP/puromycin donor DNA and 250 ng of RFP/blasticidin are used for transfection of $1-2 \times 10^5$ cells. Alternatively, a donor DNA pair of GFP/blasticidin and RFP/puromycin can be used. At 48–72 hours post-transfection, the cells are selected with both puromycin and blasticidin at a concentration that is determined by their kill curves. Upon selection and expansion for 7–14 days, aliquots of cells can be subjected to flow cytometric analysis to determine the percentage of dual GFP/RFP-positive cells. The GFP/RFP double-positive cells can be further enriched by cell sorting. The bi-allelic disruption of gene can be verified by junction PCR and sequencing analysis.

Editing nucleases

TrueDesign Genome Editor Software will identify, score, and rank CRISPR gRNA and/or TALENs for your indicated protein-coding transcript of interest. For knockout enrichment, the nuclease cut site only needs to be within the coding region since there is no consideration for ongoing endogenous protein expression.

CRISPR-Cas9

Editing can be done using TrueCut™ Cas9 Protein v2 (Cat. No. [A36496](#), 10 µg), a *Streptococcus pyogenes* wild-type Cas9 with optimized NLSs. Cas9 mRNA (Cat. No. [A29378](#)) is a human codon optimized *Streptococcus pyogenes* Cas9 with NLSs, 5' cap and polyadenylation for efficient nuclear localization of the translated protein. TrueCut™ HiFi Cas9 protein (Cat. No. [A50574](#), 10 µg) is modified for greater specificity and is recommended in cases where the available guide RNA might have a high number of predicted off-targets.

Invitrogen™ TrueGuide™ Synthetic gRNAs are a ready-to-use, chemically modified single-guide RNA (sgRNA) format to drive maximum editing efficiency when working with difficult-to-edit and valuable cells such as primary cells, immune cells, and stem cells.

TALEN

TAL effector proteins consist of constant N and C terminal domains (containing translocation and nuclear localization/activation signals respectively) flanking a central repeat domain. Each repeat is 34–35 amino acids in length, with two centrally located residues that make up a repeat variable domain (RVD) that dictates the affinity of the repeat for different nucleotide targets. Combination and order of various repeat types define the genomic target site specificity of a particular TAL effector. With TAL effector nucleases (TALEN) exact DNA loci can be cleaved, and the design does not depend on context sequence (i.e., protospacer adjacent motifs, PAM sites.) If the desired tag site does not have suitable PAM sites or gRNA sequences, TALENs are good alternatives to CRISPR-Cas9.

PCR

PCR is done using the custom-designed homology arm primers, the donor template, and the included Phusion™ Flash High Fidelity PCR Master Mix. The Phusion™ master mix has very high fidelity to minimize the chance of PCR introduced errors. The template is provided in a linear format for more robust PCR amplification. For more information, see the Phusion™ Flash High Fidelity PCR Master Mix User Guide (Pub. No. MAN0012774).

Transfection

The donor and nuclease can be delivered by Neon™ electroporation or Lipofectamine™ transfection (Lipofectamine™ CRISPRMAX™ for Cas9/gRNA; ribonucleic proteins; Lipofectamine™ MessengerMAX™ for Cas9 mRNA or TALEN mRNA pairs; or Lipofectamine™ 3000 for Cas9 plasmids). See “General Cas9:gRNA transfection guidelines” on page 40 for more details and 7 different transfection protocols.

Selection/kill curve

Puromycin or blasticidin selection can be used to enrich for edited cells. For your cell line, a kill curve needs to be conducted to determine the concentration of antibiotic required to kill the unedited population. Efficient killing of the unedited and recovery of the edited populations generally takes 5–10 days.

Analysis

Edited cells can be analyzed in multiple ways. We recommend visual confirmation if using EmGFP, tagRFP, tagBFP, or tagYFP. For well-expressed genes, the fluorescent tag can become visible as early as 1–2 days after transfection. We also recommend sequencing at the pool and clonal level using junction PCR of TOPO™ cloned amplicons. The Genomic Cleavage Detection Kit (Cat. No. A24372) can be used with a no-donor control to confirm that the nuclease is cutting efficiently. For troubleshooting it is important to understand how well your nuclease is causing double-stranded DNA breaks. Inefficient CRISPR-Cas9 or TALEN designs will make HDR and protein tagging very difficult.

For gene tagging using an epitope, such as DDK tag, HA tag, Myc tag or His tag, we recommend using immunostaining or western blotting to confirm a successful edit. See “Required materials not supplied” on page 9 for recommended antibodies. Junction PCR and sequencing analysis are also highly recommended.

To tag unexpressed genes in iPSC or stem cells using a TrueTag™ Stem donor kit, it is important to maintain the cells in pluripotent stages and analyze the clones by junction PCR and sequencing. Successfully edited clones are then induced to differentiate toward a specific cell type or organoid of interest, including neurons, cardiomyocytes, astrocytes, and hepatocytes, etc. The target protein must be expressed at high enough levels for the fluorescent tag to be detected. Ensure you have validated your differentiation strategy and target gene expression prior to initiating your tagging experiment.

To enrich knockout cell lines using a TrueTag™ Knockout Enrichment donor kit, it is important to determine the kill curves of puromycin and blasticidin for your specific cell line. You must also make sure the targeted gene is not essential to cell survival. When cells are transfected with Knockout Enrichment DNA donors containing GFP- and RFP-expression cassettes, the transfected cells glow transiently and diminish over time. A small percentage of cells may still be bright after 5 to 10 days because the donor DNA gets stably inserted into the genome due to random integration. However, un-transfected cells or cells with random integration are expected to die upon antibiotic selection with puromycin and blasticidin because the expression of the selection marker is driven by the endogenous promoter of your targeted gene. To achieve a high percentage of knockout cells in your pool, you must optimize the doses of puromycin and blasticidin for enrichment of bi-allelic knock cell lines. Note that the effective range of blasticidin is broader than puromycin. A few dose combinations of puromycin and blasticidin are recommended for dual selection. After antibiotic selection for approximately 7 days, a significant population of cells should be both GFP- and RFP-positive. The actual percentage of dual GFP- and RFP-positive cells can be determined by flow cytometric analysis. The integration of Knockout Enrichment cassettes into edited loci can be verified by junction PCR and sequencing. To achieve more than 90% knockout in a cell pool, sorting of dual GFP- and RFP-positive cells with a cell sorter is highly recommended. The knockout efficiency of targeted gene can be determined by western blot analysis or immunostaining followed by flow cytometric analysis.

Donor design

Design TrueTag™ Donor DNA

To begin the design of your TrueTag™ Donor DNA, go to thermofisher.com/truedesign, then follow these general steps using the TrueDesign Genome Editor Software.

1. Choose gene to be tagged.
2. Choose amino (at ATG start codon) or carboxy fusion (before stop codon).
3. Choose fluorophore or epitope tag.
4. Choose selection marker (puromycin, blasticidin).
5. Select a locus-specific nuclease strategy (CRISPR gRNA or TALEN) to cut near insertion site.
6. Select and order the gene-specific PCR oligos to add homology arms to premade donor templates.

Choosing your donor template

The first step is to choose the appropriate tag for your downstream analysis. Each kit includes templates for making amino or carboxy gene fusions with either puromycin or blasticidin antibiotic selection.

For gene tagging, we offer fluorescence tags (EmGFP, tagRFP, tagBFP, or tagYFP) as well as small epitope tags (DDK tag, HA tag, Myc tag, or His tag). Each kit includes templates for making amino or carboxy gene fusions with either puromycin or blasticidin antibiotic selection. The carboxy donor template contains a pair of LoxP sites, which allow removal of a selection cassette upon gene tagging.

Next, choose the location for the tag. For gene fusions, the C-terminal is less likely to impact on the upstream regulatory elements of the gene. In addition, our C-terminal constructs include the option to remove the selection cassette using Cre/lox. Some proteins can be inhibited by having more amino acids on one terminus than the other due to steric hindrance, blocking an active site, or preventing protein interactions. You may need to test both termini to determine which works best for your protein of interest and cell type.

The TrueTag™ Stem donor kit allows user to tag an unexpressed gene in stem cell stage, which becomes expressed during cell differentiation. To minimize the interference of transcription regulation of an endogenous gene, we offer EmGFP or tagRFP fusion tags or non-fusion tags at carboxy terminus with either puromycin or blasticidin antibiotic selection. The selection cassette is flanked by a pair of LoxP sites.

Besides gene tagging, we also offer the TrueTag™ Knockout Enrichment Donor DNA kit, which allows users to enrich mono-allelic or bi-allelic knockout cell lines. The TrueTag™ Knockout Enrichment donor template consists of a puromycin or blasticidin antibiotic selection marker driven by an endogenous promoter, an EmGFP or tagRFP fluorescence reporter gene driven by an exogenous CMV promoter, and a 3X STOP sequence for termination of protein translation in 3 possible reading frames. The knockout cell lines can be enriched by antibiotic selection and fluorescence cell sorting. The antibiotic selection marker and fluorescence reporter can be removed by Cre recombinase.

For gene fusion with fluorescence reporter or epitope tag, you will need to choose the location of the tag. For gene fusions, a C-terminal knock-in is less likely to impact the upstream regulatory elements of the gene. In addition, our C-terminal constructs include the option to remove the selection cassette using Cre/lox. Some proteins can be inhibited by having more amino acids on one terminus than the other due to steric hindrance, blocking an active site, or preventing protein interactions. You may need to test both termini to determine which works best for your protein of interest and cell type.

For N-terminal fusions, the ideal insertion site is immediately after the start ATG. For C-terminal fusions, the ideal insertion site is immediately before the STOP codon (TAA, TAG, TGA). The TrueDesign Genome Editor Software is recommended to carry out this design step to identify CRISPR and TALEN cut sites and analyze potential off-target cut sites.

Selecting a CRISPR-Cas9 site

Once you have determined where you want to add the tag, you need to identify the closest CRISPR Cas9 cut site. Proximity of the cut site to the insert position strongly influences insertion efficiency and we recommend selecting a gRNA that cuts within 10 bases of the insertion site. The TrueDesign Genome Editor Software (thermofisher.com/truedesign) is recommended to carry out this design step to identify CRISPR and TALEN cut sites and analyze potential off-target cut sites.

A second consideration when using CRISPR Cas9 is to ideally select a PAM/gRNA sequence that lies across the insertion site to prevent the Cas9:gRNA complex from targeting your donor molecule or re-cutting the edited genome. When choosing a gRNA that lies across the insertion site, the donor will disrupt the gRNA site (Figure 9). If gRNAs are only available completely to the left or right of the insertion site, the TrueDesign Genome Editor Software will automatically add a mutation to the PAM site or within the first 10 bases of the gRNA recognition region. If this is within a coding region, changing at a codon wobble position will give you the most flexibility to disrupt the gRNA without changing the amino acid sequence.

The final consideration for gRNA choice is to select one with low off-target potential to minimize unwanted changes to the genome. The TrueDesign Genome Editor Software will analyze and report potential off-target cut sites for predicted off-target regions. If a gRNA with low predicted off-targets is not available in the appropriate genomic space, it is recommended to use a high-fidelity Cas9 enzyme like TrueCut™ HiFi Cas9 protein to reduce off-target cleavage events.

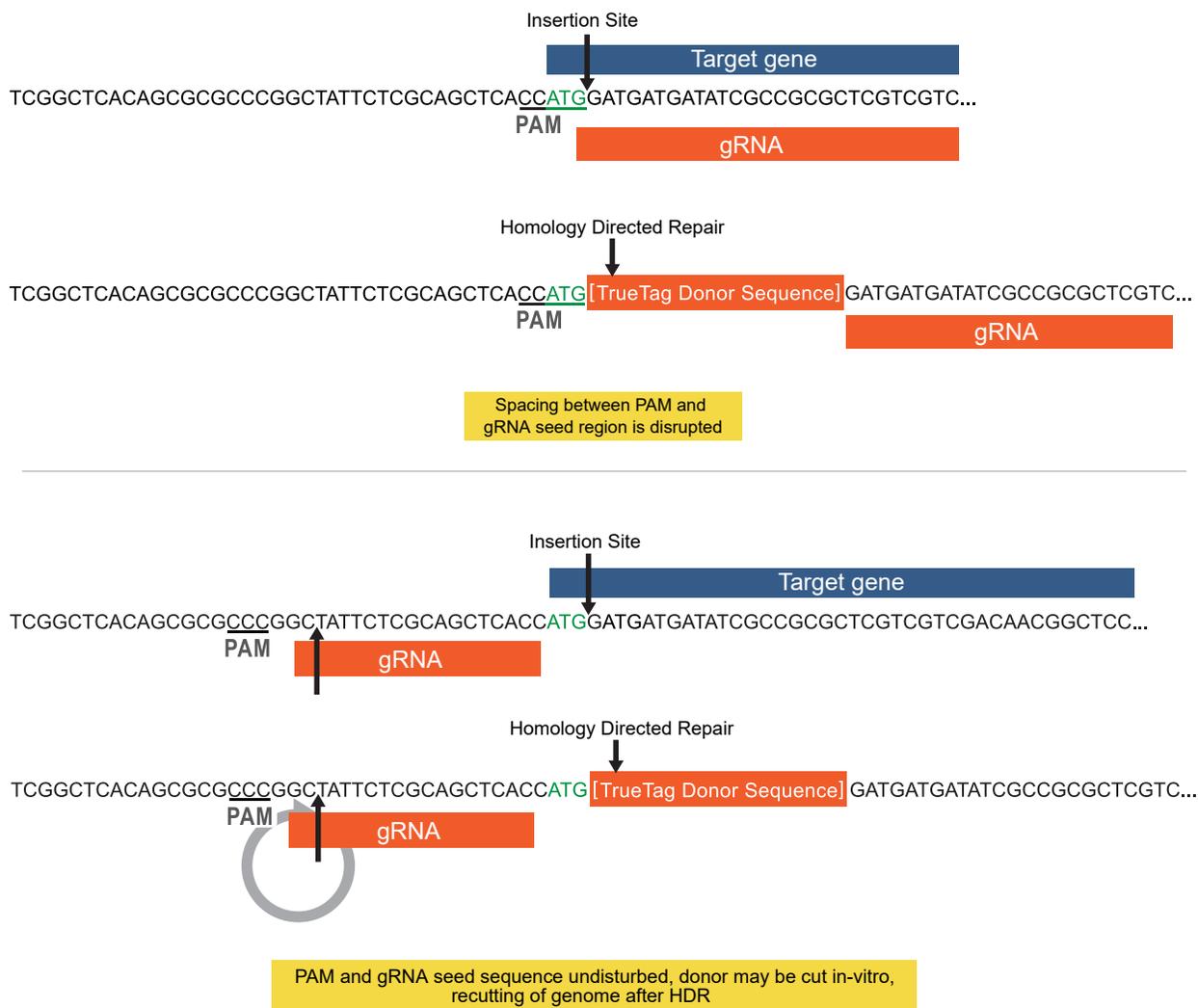


Figure 9 Choosing a gRNA.

For TrueTag™ Knockout Enrichment, the selection of CRISPR-Cas9 cut site depends on which transcript variant(s) of a target gene you intend to knock out. If all transcript variants of a target gene are required to be knocked out, then a common gRNA among all transcript variants should be selected. On the other hand, if knockout of a specific transcript variant is desired (e.g. a disease-related isoform), then a more specific gRNA needs to be selected that only targets the transcript of interest. For the Knockout Enrichment workflow, the insertion of the TrueTag™ donor should be within the protein coding region. Furthermore, the design of 5' homology arm needs to be in-frame with the donor template so that the endogenous promoter will drive the expression of 2A-puromycin or 2A-blasticidin selection marker. As with the gene tagging workflows, the TrueDesign Genome Editor software will generate gRNA targeting the selected region of an indicated transcript. We recommend viewing transcript alignment details on NCBI to determine the appropriate insertion region for your Knockout Enrichment workflow.

Selecting a TALEN site

If a good PAM site is not available to design a CRISPR gRNA, TALENs are an excellent alternative. The predictability with which Invitrogen™ TAL effector nucleases bind to exact DNA sequences makes it possible to position the cut site directly at the ATG or STOP codon position without sequence limitations. The TrueDesign Genome Editor Software will assist in the design of FlexCut TALEN mRNA pairs that are appropriate for the required edit.

Design of donor homology arm PCR primers

Once you have decided on your genome editing method and your proposed cut site, you need to design custom DNA oligos to add homology arms to the TrueTag™ DNA Donor template. This is done in a single design step when using the TrueDesign Genome Editor Software (thermofisher.com/truedesign). If you are manually designing, the N-terminal and C-terminal donors have universal priming sites that need to be incorporated into the oligo design.

The universal donor PCR oligo sequences are:

N-terminal Fluorescence or Epitope donor	UniF-N	5' GGAGGTAAGCCCTTGCATTCTG 3'
	UniR-N	5' ACCGCTTCCACTACCTGAACC 3'
C-terminal Fluorescence, Epitope, Stem, or Knockout Enrichment donor	UniF-C	5' GGAAGTGGCTCAGGTTCTGGA 3'
	UniR-C	5' CTTGGCCGATCGCATAACAGAG 3'

Use the TrueDesign Genome Editor Software appropriate for your DNA Donor template and genomic locus of interest. To learn more about the process, the following examples walk through how to design the donor molecule using CRISPR and the standard *Streptococcus pyogenes* Cas9 with an NGG PAM.

We recommend adding 2 phosphorothioate modifications to the 5' ends of the oligos to inhibit nuclease digestion of the donor. Standard 50-nmol scale with desalted purification is recommended.

Example 2: N-terminal, cut site downstream of ATG start

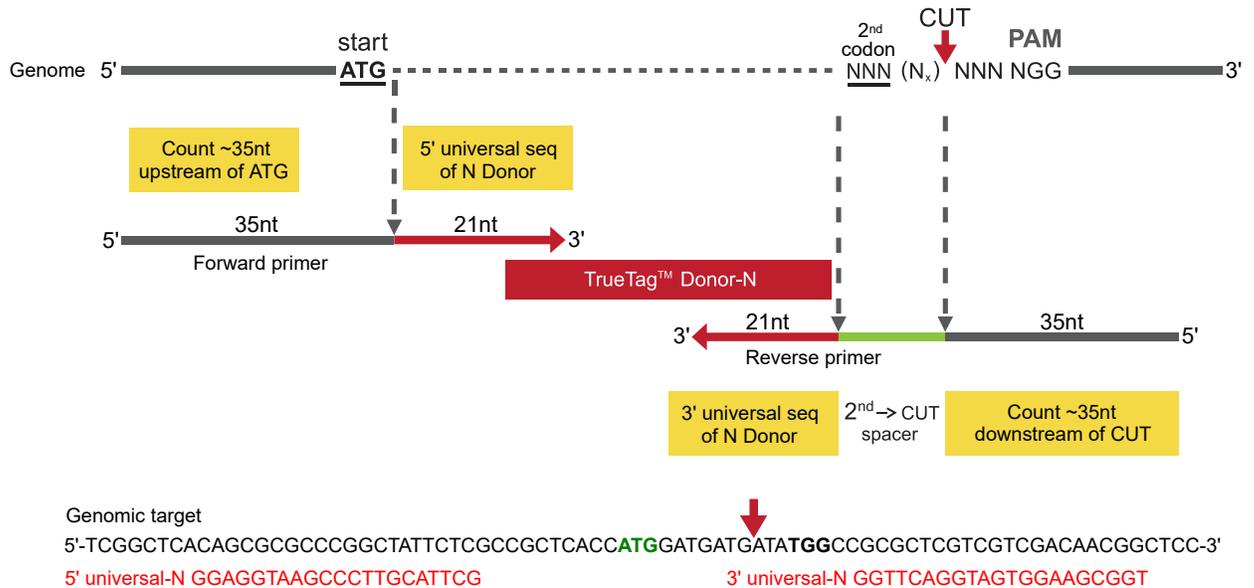


Figure 11 Example 2: N-terminal, cut site downstream of ATG start

Forward primer

1. Forward primer

CACAGCGCGCCCGGCTATTCTCGCCGCTCACCATG

2. Add 5' universal-N sequence (UniF-N):

CACAGCGCGCCCGGCTATTCTCGCCGCTCACC**ATGGAGGTAAGCCCTTGCAATTCG**

Reverse primer

1. 3' universal-N sequence (UniR-N):

GGTTCAGGTAGTGGAAGCGGT

2. Add region from second codon to CUT:

GGTTCAGGTAGTGGAAGCGGTGATGATG

3. Add 35 bases downstream from ATG:

GGTTCAGGTAGTGGAAGCGGTGATGATGATATGGCCGCGCTCGTCGTCGACAACGGCTCCGA

4. Reverse complement:

TCGGAGCCGTTGTCGACGACGAGCGCGGCCATAT**CATCATACCGCTTCCACTACCTGAACC**

Example 3: C-Terminal Tag, cut located upstream of STOP codon

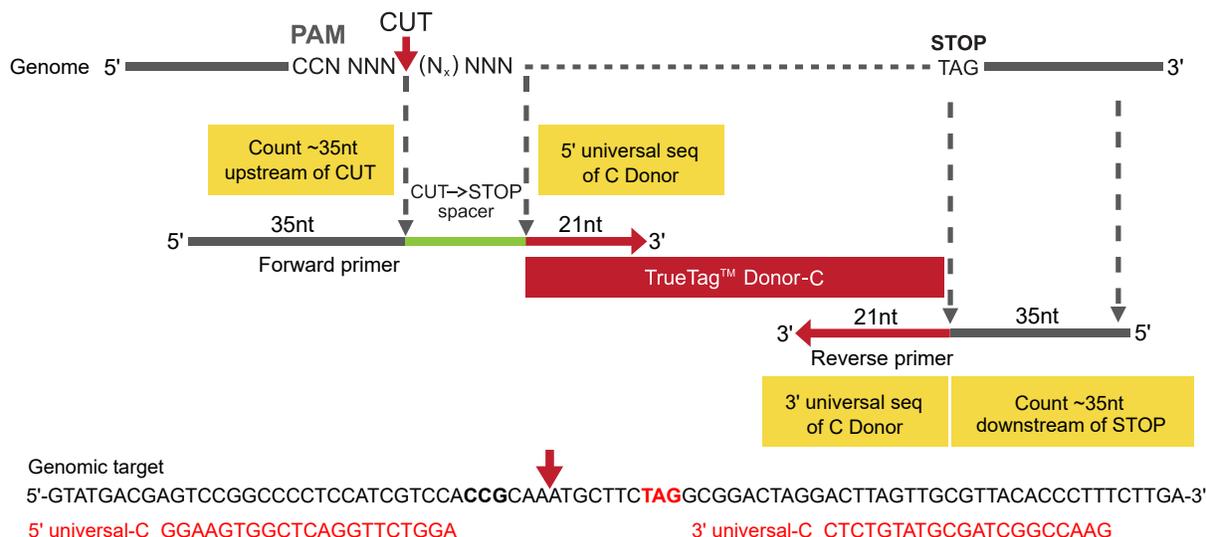


Figure 12 Example 3: C-Terminal Tag, cut located upstream of STOP codon

Forward primer

1. Forward primer

TATGACGAGTCCGGCCCCCTCCATCGTCCACCGCAA

2. Add region between CUT and STOP:

TATGACGAGTCCGGCCCCCTCCATCGTCCACCGCAAATGCTTC

3. Add 5 universal-C sequence (UniF-C):

TATGACGAGTCCGGCCCCCTCCATCGTCCACCGCAAATGCTTCGGAAGTGGCTCAGGTTCTGGA

Reverse primer

1. 3' universal-N sequence (UniR-C):

CTCTGTATGCGATCGGCCAAG

2. Add 35 bases downstream from STOP:

CTCTGTATGCGATCGGCCAAGTAGGCGGACTAGGACTTAGTTGCGTTACACCCTTT

3. Reverse complement:

AAAGGGTGTAAACGCAACTAAGTCCTAGTCCGCCTACTTGGCCATCGCATAACAGAG

Example 4: C-Terminal Tag, Cut located downstream of STOP codon

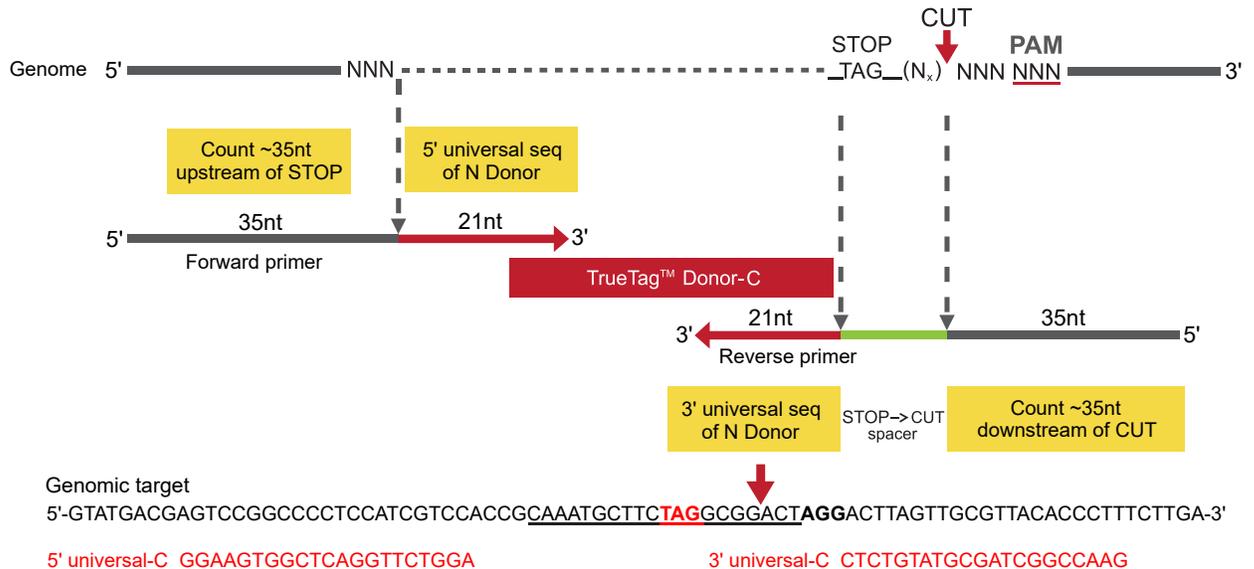


Figure 13 Example 4: C-Terminal Tag, Cut located downstream of STOP codon

Forward primer

1. Count 35 bases upstream from STOP position:

TATGACGAGTCCGGCCCCTCCATCGTCCACCGCAA

2. Add 5' universal-N sequence:

TATGACGAGTCCGGCCCCTCCATCGTCCACCGCAAATGCTTCGGAAGTGGCTCAGGTTCTGGA

Reverse primer

1. Add 3' universal-N sequence:

CTCTGTATGCGATCGGCCAAG

2. Add region from STOP to CUT:

CTCTGTATGCGATCGGCCAAGTAGGCGG

3. Add 35 bases downstream from CUT:

CTCTGTATGCGATCGGCCAAGTAGGCGGCGGACTAGGACTTAGTTGCGTTACACCCTTTCTT

4. Reverse complement:

AAGAAAGGGTGTAAACGCAACTAAGTCCTAGTCCGCCGCTACTTGCCGATCGCATAACAGAG

Example 5: gRNA does not cross the insertion site

In this case, the gRNA design does not cross the insertion site. This may result in the Cas9 complex cutting the donor directly or recutting a successful genomic edit thereby lowering editing efficiency. We recommend avoiding gRNAs like this if possible. In order to avoid this possibility, a mutation that disrupts the PAM site in the donor can be made. You may minimize the impact of the mutation in coding regions by changing the PAM at a codon wobble position for a silent change (same amino acid is still made).

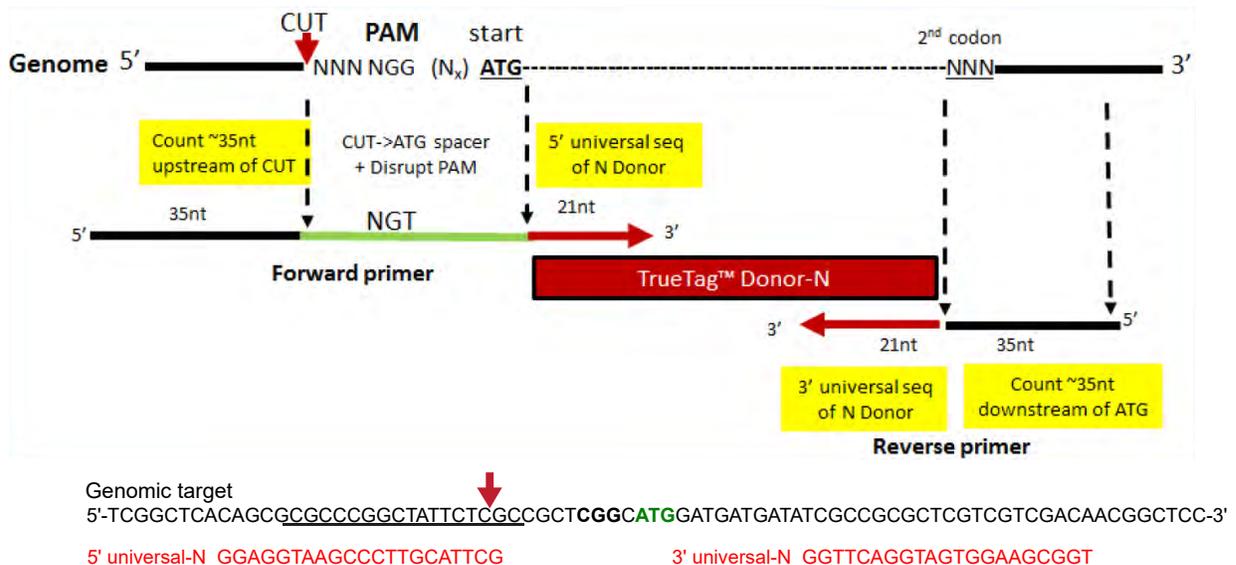


Figure 14 Example 5: gRNA does not cross the insertion site

Forward primer

1. Count 35 bases upstream from the CUT position:

TGAATCGGCTCACAGCGCGCCCGGCTATTCTCGCC

2. Add region between the CUT position and ATG:

TGAATCGGCTCACAGCGCGCCCGGCTATTCTCGCCGCTCGGCATG

3. Disrupt PAM with mutation:

TGAATCGGCTCACAGCGCGCCCGGCTATTCTCGCCGCTCGGCATG

4. Add 5' universal-N sequence (UniF-N):

TGAATCGGCTCACAGCGCGCCCGGCTATTCTCGCCGCTCGGCATGGAGGTAAGCCCTTGCAATCG

Reverse primer

1. 3' universal-N sequence (UniR-N):

GGTTCAGGTAGTGGAAGCGGT

2. Add 35 bases downstream from ATG:

GGTTCAGGTAGTGGAAGCGGTGATGATGATATCGCCGCGCTCGTCGTCGACAACGG

3. Reverse complement:

CCGTTGTCGACGACGAGCGCGGCGATATCATCATCACCGTTCCACTACCTGAACC

Example 6: Donor homology arm PCR primer design for TrueTag™ Knockout Enrichment Kits

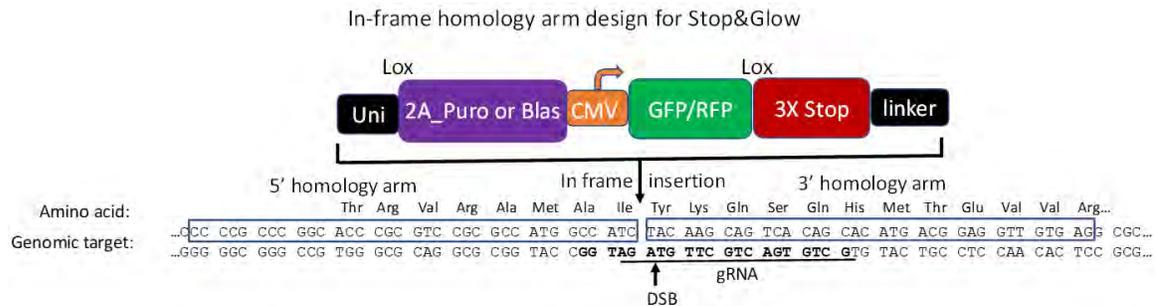
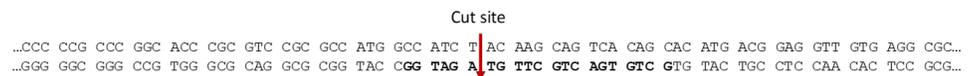


Figure 15 Example 6: Donor homology arm PCR primer design for TrueTag™ Knockout Enrichment Kits

The donor homology arm primer design for Knockout Enrichment Kits is slightly different. Determine which exon to edit based on all of the transcript variants of a target gene. Then design a few gRNAs with a high score and low predicted off-targets. If the Cas9 cleavage site (double-stranded break) happens to be out of frame in the protein coding region, the homology arm needs to shift either one or two bases to make it in-frame. If you are using the TrueDesign Genome Editor Software (thermofisher.com/truedesign), the tool shifts the homology arm for you. In this example, the gRNA is located at the antisense strand and the cut site is three bases upstream of PAM GGT, which causes one base frameshift.



Therefore, the donor homology arm primer needs to shift one base correspondingly to make it in-frame.

Forward primer

1. Count 35 bases upstream from the CUT position but shift to the left by one base:

CCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATC

2. Add 5' universal-C sequence (UniF-C):

CCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATC**GGAAGTGGCTCAGGTTCTGGA**

Reverse primer

1. 3' linker sequence (UniR-C):

CTCTGTATGCGATCGGCCAAG

2. Add 35 bases downstream from CUT position, but shift to the left by one base:

CTCTGTATGCGATCGGCCAAGTACAAGCAGTCACAGCACATGACGGAGGTTGTGAG

3. Reverse complement:

CTCACAACTCCGTCATGTGCTGTGACTGCTTGTACT**TTGGCCGATCGCATAACAGAG**

Design of verification primers

Once you have edited your genomic target, PCR and sequencing can be used to confirm that the tag is in the correct location and that the junctions are accurate.

If you are not using the TrueDesign Genome Editor Software for verification primer design, we recommend designing a genomic target-specific forward primer (F1) that is 100–300 bases upstream of the left donor junction and a reverse primer (R1) that is 100–300 bases after the left junction. For the internal primers, F2 and R2, you can design your own, again 100–300 bases internal of the junctions or order the ones needed from the following list. The internal primers, F2 and R2, are identical among all of the epitope tags. Overall, each amplicon should be 200–600 bases. See the following five diagrams for further guidance. For more discussion of verification, see “Confirmation of Cas9 or TALEN cutting activity by GCD or sequencing” on page 68.



Figure 16 Verification primer design for gene tagging with a fluorescence reporter at amino termini.

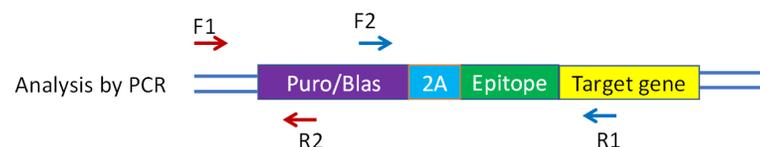


Figure 17 Verification primer design for gene tagging with an epitope at amino termini.

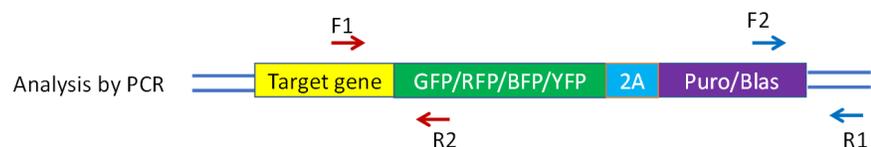


Figure 18 Verification primer design for gene tagging with a fluorescence reporter at carboxy termini.

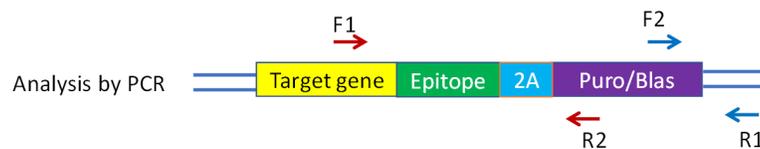


Figure 19 Verification primer design for gene tagging with an epitope at carboxy termini.

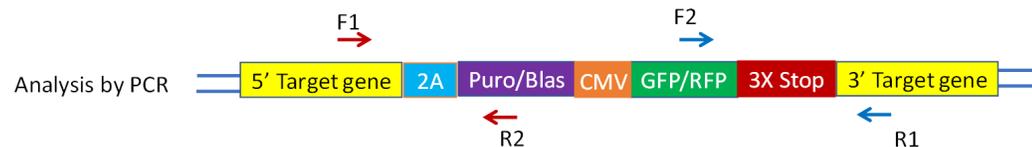


Figure 20 Verification primer design for Knockout Enrichment.

Table 3 Internal verification primers for epitope tags

Tag	Donor	Donor size (bp)	Verification Primer R2 (not provided)	Verification Primer F2 (not provided)
GFP	N-Puro-GFP	1416	GGGTAATCGGCGAAGGCAGCGG	GTCCGCCCTGAGCAAAGACCCC
	N-Blast-GFP	1215	CCACGAGTTCTGCACAAGGTCC	GTCCGCCCTGAGCAAAGACCCC
	C-Puro(Cre-Lox)-GFP	1494	CTTGTGGCCGTTTACGTGCGCG	GCCCTAGAACCTGGTGCATGAC
	C-Blast(Cre-Lox)-GFP	1293	CTTGTGGCCGTTTACGTGCGCG	TGCGGACGGTGCCGACAGGTGC
RFP	N-Puro-RFP	1410	GGGTAATCGGCGAAGGCAGCGG	GCTAAGAACCTCAAGATGCCCG
	N-Blast-RFP	1209	CCACGAGTTCTGCACAAGGTCC	GCTAAGAACCTCAAGATGCCCG
	C-Puro(Cre-Lox)-RFP	1488	GGCTTGCCTTCGCCCTCGGATG	GCCCTAGAACCTGGTGCATGAC
	C-Blast(Cre-Lox)-RFP	1287	GGCTTGCCTTCGCCCTCGGATG	TGCGGACGGTGCCGACAGGTGC
BFP	N-Puro-BFP	1410	GGGTAATCGGCGAAGGCAGCGG	CTGATCGCAAACGCCAAGACCAC
	N-Blast-BFP	1209	CCACGAGTTCTGCACAAGGTCC	CTGATCGCAAACGCCAAGACCAC
	C-Puro(Cre-Lox)-BFP	1488	CTCGACCACCTTGATTCTCATG	GCCCTAGAACCTGGTGCATGAC
	C-Blast(Cre-Lox)-BFP	1287	CTCGACCACCTTGATTCTCATG	TGCGGACGGTGCCGACAGGTGC
YFP	N-Puro-YFP	1416	GGGTAATCGGCGAAGGCAGCGG	CAACCACTACCTGAGCTACC
	N-Blast-YFP	1215	CCACGAGTTCTGCACAAGGTCC	CAACCACTACCTGAGCTACC
	C-Puro(Cre-Lox)-YFP	1494	GCAGCTTGCCGGTGGTGCAG	GCCCTAGAACCTGGTGCATGAC
	C-Blast(Cre-Lox)-YFP	1293	GCAGCTTGCCGGTGGTGCAG	TGCGGACGGTGCCGACAGGTGC
Epitope DDK HA Myc 6xHis	N-Puro-Epitope	729-801	GGGTAATCGGCGAAGGCAGCGG	GCCCTAGAACCTGGTGCATGAC
	N-Blast-Epitope	528-600	CCACGAGTTCTGCACAAGGTCC	GTGCTTCTCGATCTGCATCCTGG
	C-Puro(Cre-Lox)-Epitope	807-879	GGGTAATCGGCGAAGGCAGCGG	GCCCTAGAACCTGGTGCATGAC
	C-Blast(Cre-Lox)-Epitope	606-678	CCACGAGTTCTGCACAAGGTCC	GTGCTTCTCGATCTGCATCCTGG

Table 3 Internal verification primers for epitope tags (continued)

Tag	Donor	Donor size (bp)	Verification Primer R2 (not provided)	Verification Primer F2 (not provided)
Stem GFP	C-GFP-pPuro(Cre-Lox)	1680	CTTGTGGCCGTTTACGTGCGCCG	GCCCTAGAACCTGGTGCATGAC
	C-GFP-pBlast(Cre-Lox)	1479	CTTGTGGCCGTTTACGTGCGCCG	TGCGGACGGTGCCGACAGGTGC
	C-2A-GFP-pPuro(Cre-Lox)	1746	CTTGTGGCCGTTTACGTGCGCCG	GCCCTAGAACCTGGTGCATGAC
	C-2A-GFP-pBlast(Cre-Lox)	1545	CTTGTGGCCGTTTACGTGCGCCG	TGCGGACGGTGCCGACAGGTGC
Stem RFP	C-RFP-pPuro(Cre-Lox)	1674	GGCTTGCCTTCGCCCTCGGATG	GCCCTAGAACCTGGTGCATGAC
	C-RFP-pBlast(Cre-Lox)	1473	GGCTTGCCTTCGCCCTCGGATG	TGCGGACGGTGCCGACAGGTGC
	C-2A-RFP-pPuro(Cre-Lox)	1740	GGCTTGCCTTCGCCCTCGGATG	GCCCTAGAACCTGGTGCATGAC
	C-2A-RFP-pBlast(Cre-Lox)	1539	GGCTTGCCTTCGCCCTCGGATG	TGCGGACGGTGCCGACAGGTGC
Knockout Enrichment	2A-puro pGFP(Cre-Lox)	2341	GGGTAATCGGCGAAGGCAGCGG	GTCCGCCCTGAGCAAAGACCCC
	2A-Blast pGFP(Cre-Lox)	2140	CCACGAGTTCTGCACAAGGTCC	GTCCGCCCTGAGCAAAGACCCC
	2A-puro pRFP(Cre-Lox)	2335	GGGTAATCGGCGAAGGCAGCGG	GCTAAGAACCTCAAGATGCCCCG
	2A-Blast pRFP(Cre-Lox)	2134	CCACGAGTTCTGCACAAGGTCC	GCTAAGAACCTCAAGATGCCCCG

Generating your TrueTag™ donor DNA

This protocol will generate >5 µg of linear dsDNA donor with the desired gene specific homology arms.

1. Set up two 50-µL PCR reactions:

Component	Amount
2X Phusion™ Flash High Fidelity PCR Master Mix	25 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Donor template (20 ng/µL)	1 µL
Water	22 µL
Total	50 µL

2. PCR parameters:

Step	Cycles	Temperature	Time
Denature	1	98°C	10 seconds
Amplification	30	98°C	1 seconds
		55°C	5 seconds
		72°C	15–30 seconds/kb
Final extension	1	72°C	2 minutes
Final hold	1	4°C	HOLD

3. Confirm successful PCR reaction by running 2 µL of PCR on 1–2% agarose gel. A successful PCR will have the following band sizes:

TrueTag™ DNA donor type	Band Size (kb)
Fluorescent	1.3–1.5
Epitope	0.5–0.9
Stem	1.4–1.8
Knockout Enrichment	2.1–2.4

Note: The adaptor primers for PCR amplification are ~56 bases, so occasionally the PCR reaction may fail. If this happens, breaking down the forward and reverse adaptor primers into two 21-nt overlapping inner and outer forward primers and two 21-nt inner and outer overlapping reverse primers will generally resolve this issue. See the following example.

TrueTag™ Forward primer:

G*C*ACTTTGGGAGGCCGAGGCCGGCAGATCACGAGGGGAAGTGGCTCAGGTTCTGGA

Breakdown into two 21nt overlapping inner and outside Forward primers:

Inner Forward Primer:

CGAGGCCGGCAGATCACGAGGGGAAGTGGCTCAGGTTCTGGA

Outer Forward primer:

G*C*ACTTTGGGAGGCCGAGGCCGGCAGATCACGAGG

Note: * Indicates the position of phosphorothioate linkages at the 5' ends. Bolded text indicates universal-C sequence.

TrueTag™ Reverse primer:

A*G*AGACGGGGTTTCACCGTGGTCTCGATCTCCTGACTTGGCCGATCGCATAACAGAG

Breakdown into two 21nt overlapping inner and outside Reverse primers:

Inner Reverse Primer:

ACCGTGGTCTCGATCTCCTGACTTGGCCGATCGCATAACAGAG

Outer Reverse primer:

A*G*AGACGGGGTTTCACCGTGGTCTCGATCTCCTGA

Note: * Indicates the position of phosphorothioate linkages at the 5' ends. Bolded text indicates universal-C sequence.

To generate donor DNA using overlapping primers, perform sequential PCR reactions. In this case, perform the first PCR reaction using a pair of inner Forward and inner Reverse primers. Use 1 µL of the first PCR product as a template to perform a second PCR reaction using a pair of outer Forward and outer Reverse primers.

Alternatively, set up a one-step overlapping PCR reaction to prepare the donor DNA with an outer/inner primer ratio of 20:1.

Set up two 50-µL PCR reactions:

Component	Amount
2X Phusion™ Flash High Fidelity PCR Master Mix	25 µL
Inner Forward primer (0.5 µM)	1 µL
Outer Forward primer (10 µM)	1 µL
Inner Reverse primer (0.5 µM)	1 µL
Outer Reverse primer (10 µM)	1 µL
Donor template (20 ng/µL)	1 µL
Water	20 µL
Total	50 µL

4. PCR Cleanup using the TrueTag™ PCR Purification module. All steps should be carried out at room temperature. All centrifugations should be carried out in a table-top microcentrifuge at $>12,000 \times g$ (10,000–14,000 rpm, depending on the rotor type).
- Prior to the initial use of the purification module, dilute the Wash Buffer (concentrated) with 9 mL of ethanol (96–100%)
 - Examine the Binding Buffer for precipitate before each use. Re-dissolve any precipitate by warming the solution to 37°C and cooling to 25°C.
- a. Combine the two completed 50- μ L PCR reactions for each donor into 1 tube
 - b. Add a 100 μ L of Binding Buffer to completed PCR mixture (1:1). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 μ L of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
 - c. Transfer the 200 μ L of the solution from step b to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through.
 - d. Add 700 μ L of Wash Buffer (diluted with ethanol) to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through and place the purification column back into the collection tube.
 - e. Centrifuge the empty GeneJET™ purification column for an additional 1 minute to completely remove any residual wash buffer.
-
- Note:** This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
-
- f. Transfer the GeneJET™ purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 μ L of Elution Buffer (10 mM Tris-HCl, pH 8.5) or nuclease-free water to the center of the GeneJET™ purification column membrane and centrifuge for 1 minute.
 - g. Discard the GeneJET™ purification column and store the purified DNA at -20°C .
 - h. Concentration can be measured by A_{260} . If higher concentration is required a SpeedVac™ vacuum concentrator can be used.

Delivery of the TrueTag™ DNA donor and editing reagents into target cells

General Cas9:gRNA transfection guidelines

- The efficiency with which mammalian cells are transfected with gRNA varies according to cell type and the transfection reagent used. See Table 4 for delivery reagent recommendations.
- For gene editing, the highest editing efficiency is achieved with a 1:1 molar ratio of gRNA to TrueCut™ Cas9 Protein v2. In some cell types such as iPSC and THP1, it may be necessary to use up to 2 µg of TrueCut™ Cas9 Protein v2 and 400 ng of gRNA per well in a 24-well format.
- Use 200 ng (0.2 pmol) to 500 ng (0.5 pmol) of TrueTag™ donor DNA (average size is ~1500 bp) per well for best results in a 24-well format. Higher amounts often results in decrease in cell viability and editing efficiency.
- The optimal cell density for transfection varies depending on cell size and growth characteristics. In general, 30–70% confluence on the day of transfection when using lipid-mediated delivery, or 70–90% confluence for electroporation using the Neon™ Transfection System is recommended.
- After the optimal cell number and dosage of TrueCut™ Cas9 Protein gRNA that provides maximal gene editing efficiency is determined, do not vary these conditions across experiments for a given cell type, to ensure consistency.

For an overview of the factors that influence transfection efficiency, refer to the “Transfection Basics” chapter of the Gibco™ Cell Culture Basic Handbook, available at [thermofisher.com/cellculturebasics](https://www.thermofisher.com/cellculturebasics).

- Use the TrueGuide™ Positive Controls (human AAVS1, CDK4, HPRT1, or mouse Rosa 26; available at [thermofisher.com/us/en/home/life-science/genome-editing/geneart-crispr/crispr-libraries/crispr-controls.html](https://www.thermofisher.com/us/en/home/life-science/genome-editing/geneart-crispr/crispr-libraries/crispr-controls.html)) and negative control gRNA (non-targeting) to determine gRNA amount and transfection conditions that give the optimal gene editing efficiency with highest cell viability. The TrueGuide™ Positive and Negative sgRNA Controls are available separately from Thermo Fisher Scientific.
- The cell number and other recommendations provided in the following procedures are initial guidelines based on cell types that have been tested in-house. For multiple wells, prepare a master mix of components to minimize pipetting error, then dispense the appropriate volumes into each reaction well. When making a master mix for replicate wells, we recommend preparing extra volume to account for any pipetting variations.

Recommended delivery options

- Choosing the correct delivery reagent is critical for transfection and gene editing efficiency. See our recommendations in Table 4. For more information on transfection reagents, see [thermofisher.com/transfection](https://www.thermofisher.com/transfection).

Table 4 Recommended delivery options for Cas9:gRNA and TALENs

Cas9 Format	Transfection reagent	Electroporation
TrueCut™ Cas9 Protein v2 + gRNA or TrueCut™ HiFi Cas9 Protein + TrueGuide™ sgRNA	Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Cat. No. CMAX00001)	For maximum efficiency in difficult-to-transfect cell types, use the Neon™ Transfection System (Cat. Nos. MPK5000, MPK1025).
Cas9 mRNA + gRNA	Lipofectamine™ MessengerMAX™ (Cat. No. LMRNA003)	
Cas9 plasmid + gRNA	Lipofectamine™ 3000 (Cat. No. L3000015)	
TALEN mRNA	Lipofectamine™ MessengerMAX™ (Cat. No. LMRNA003)	

- To optimize the electroporation conditions for a particular cell line, follow the Neon™ transfection 24-well optimization protocol described in the Neon™ Transfection System user guide. Each of the 24 optimized conditions are varied in pulse voltage, pulse width, and the number of pulses to determine which condition works best with your cell line. Alternative electroporation systems may be used, but optimal conditions will vary.
- For cell-line specific transfection conditions using the Lipofectamine™ CRISPRMAX™ Transfection Reagent or the Neon™ Transfection System, see Appendix C, “Transfection tables”.

Preparing your gRNA

Prepare working stock of TrueGuide™ synthetic sgRNA

Resuspend TrueGuide™ Synthetic gRNA in 1X TE buffer to prepare 100 μM (100 pmol/μL) stock solutions using this procedure.

1. Before opening, centrifuge each TrueGuide™ Synthetic gRNA tube at low speed (maximum RCF 4,000 × g) to collect the contents at the bottom of the tube, then remove the cap from the tube carefully.
2. Using a pipette and sterile tips, add the required volume of 1X TE buffer to prepare 100 μM (100 pmol/μL) stock solutions. For 1.5 nmol of sgRNA, this volume equals 15 μL.
3. Vortex the tube to resuspend the oligos, briefly centrifuge to collect the contents at the bottom of the tube, then incubate at room temperature for 15–30 minutes to allow the sgRNA oligos to dissolve.
4. Vortex the tube again to ensure that all the contents of the tube are resuspended, then briefly centrifuge to collect the contents at the bottom of the tube.

5. Optional: Check the concentration of the resuspended oligos using the NanoDrop™ Spectrophotometer (or equivalent) or a UV-base plate reader.
6. Optional: Create aliquots of the working stock for storage.
7. Use working stocks immediately or freeze at -20°C until use.

(Optional) Generate gRNA by in vitro transcription

If you wish to use in vitro transcribed gRNA for use with TrueCut™ Cas9 Protein v2 in CRISPR-Cas9-mediated genome editing, we recommend that you use the GeneArt™ Precision gRNA Synthesis Kit to synthesize your gRNA. For detailed instructions, refer to the Precision gRNA Synthesis Kit User Guide (Pub. No. MAN0014538), available for download at thermofisher.com.

Transfection methods

Transfect cells with TrueTag™ donor DNA, TrueCut™ Cas9 protein v2 and TrueGuide™ sgRNA using Lipofectamine™ CRISPRMAX™ Transfection Reagent

The following protocol is provided as a starting point for transfecting cells with TrueTag™ donor DNA template, TrueCut™ Cas9 Protein v2, and TrueGuide™ sgRNA using the Lipofectamine™ CRISPRMAX™ Transfection Reagent. The same protocol may be used with TrueCut™ HiFi Cas9 protein.

IMPORTANT! Add the reagents in the order indicated. Prepare TrueTag™ donor DNA, TrueCut™ Cas9 Protein v2:sgRNA:Cas9 PLUS™ reagent solution (Tube 1) before diluting the Lipofectamine™ CRISPRMAX™ Reagent (Tube 2).

Day 0 - seed cells

The day before transfection, seed your adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	96-well	24-well	6-well
Cell density per well	8,000–18,000 cells	40,000–90,000 cells	250,000–450,000 cells
Final volume of media per well	100 μL	0.5 mL	2 mL

Day 1 – transfect cells

1. Prepare Tube 1: TrueTag™ donor DNA + TrueCut™ Cas9 Protein v2 + gRNA solution with Cas9 PLUS™ Reagent in Opti-MEM™ I Medium

IMPORTANT! Always prepare the TrueCut™ Cas9 Protein v2:gRNA:Cas9 PLUS™ reagent solution (Tube 1) before diluting the CRISPRMAX™ Transfection Reagent (Tube 2).

- a. Mix the TrueTag™ donor DNA, TrueCut™ Cas9 Protein v2, TrueGuide™ sgRNA, Lipofectamine™ Cas9 PLUS™ Reagent, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNase-free microcentrifuge tube according to the appropriate table below. Mix well.

IMPORTANT! Add the Lipofectamine™ Cas9 PLUS™ Reagent last.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
TrueCut™ Cas9 Protein v2	250 ng (1.5 pmol)	1250 ng (7.5 pmol)	6250 ng (37.5 pmol)
TrueGuide™ sgRNA	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1200 ng (37.5 pmol)
TrueTag™ donor DNA	100 ng	500 ng	2500 ng
Lipofectamine™ Cas9 PLUS™ Reagent	0.5 µL	2.5 µL	12.5 µL

2. Prepare Tube 2: Dilute Lipofectamine™ CRISPRMAX™ reagent in Opti-MEM™ I Medium

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Lipofectamine™ CRISPRMAX™ Reagent	0.3 µL	1.5 µL	7.5 µL

3. Incubate Tube 2 for 1 minute at room temperature

Incubate the Lipofectamine™ CRISPRMAX™ Reagent diluted in Opti-MEM™ I Medium (Tube 2) at room temperature for 1 minute. Do not leave Tube 2 at room temperature for longer than 5 minutes.

Note: You can incubate the gRNA/ Opti-MEM™ I solution at room temperature for longer than 1 minute. We have observed no change in transfection efficiency when Tube 1 was left at room temperature for up to 30 minutes.

4. Mix Tube 1 + Tube 2

Add the diluted Lipofectamine™ CRISPRMAX™ Reagent (Tube 2) to the TrueTag™ donor DNA+RNP/ Opti-MEM™ I solution (Tube 1) and mix well by pipetting.

Note: For high-throughput setup (e.g., 96-well format or others), always add the contents of Tube 2 into Tube 1 because you can prepare Tube 2 as a bulk master mix.

5. Incubate for 10–15 minutes at room temperature

Incubate the Tube 1 + Tube 2 mixture (i.e., transfection complex) for 10–15 minutes at room temperature.

6. Add the transfection complex to cells and incubate at 37°C

- a. Add the transfection complex (from step 5) to your adherent cells at 30–70% confluence according to the following table:

Reagent	96-well	24-well	6-well
TrueTag™ donor DNA:RNP:transfection reagent complex	10 µL	50 µL	250 µL

- b. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Day 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA, TrueCut™ Cas9 protein v2 and TrueGuide™ sgRNA using the Neon™ transfection system

The following protocol is provided as a starting point for transfecting wild-type cells with TrueCut™ s9 Protein v2 and gRNA using the Neon™ Transfection System. The same protocol may be used for TrueCut™ HiFi Cas9 protein. For cell type-specific transfection conditions using TrueCut™ Cas9 Protein v2 and TrueGuide™ Synthetic gRNA, see Appendix C, “Transfection tables”.

IMPORTANT! The following recommendations are for a single well in 24-well format using the 10 µL Neon™ tip. For multiple wells, prepare a master mix of components with extra volume to minimize pipetting errors, and then dispense the appropriate volumes into each well. Avoid creating bubbles while mixing and dispensing. For details on optimizing the Neon™ electroporation conditions and scaling down/up for different plate formats, refer to the Neon™ Transfection System User Guide (Pub. No. MAN0001557), available for download at thermofisher.com.

Note: To optimize the electroporation conditions for a particular cell line, follow the Neon™ transfection 24-well optimization protocol described in the Neon™ Transfection System user guide. Each of the 24 optimized conditions are varied in pulse voltage, pulse width, and the number of pulses to determine which condition works best with your cell line. Alternative electroporation systems may be used, but optimal conditions will vary.

Day 0 – seed cells

1–2 days before transfection, transfer the adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection.

Day 1 – transfect cells**1. Prepare 24-well plate with media**

Add 1000 μL of cell type-specific growth medium into each well of the 24-well plate and place it in the 37°C incubator to pre-warm.

2. Prepare Tube 1: TrueTag™ donor DNA + TrueCut™ Cas9 Protein v2 + TrueGuide™ sgRNA in Resuspension Buffer.

- a. Mix the TrueCut™ Cas9 Protein v2, TrueGuide™ sgRNA, and Resuspension Buffer R in a fresh, RNase-free microcentrifuge tube according to the appropriate table below. Mix well.

IMPORTANT! Maintain TrueCut™ Cas9 Protein v2:sgRNA at a 1:1 molar ratio. Use high concentration TrueCut™ Cas9 Protein v2 and ensure that the total volume of the RNP complex (TrueCut™ Cas9 Protein v2 + gRNA) does not exceed 1/10th of the total reaction volume (e.g., 1 μL of Cas9 protein + gRNA in 10 μL total reaction volume).

Reagent	Amount per well of 24-well plate
TrueCut™ Cas9 Protein v2	1250 ng (7.5 pmol)
TrueGuide™ sgRNA	240 ng (7.5 pmol)
TrueTag™ donor DNA	500 ng (0.5 pmol)
Resuspension Buffer R	to 10 μL

For Knockout Enrichment, two TrueTag™ donors with different fluorophores and antibiotic markers are co-delivered into cells along with Cas9 nuclease and guide RNA.

Reagent	Amount per well of 24-well plate
TrueCut™ Cas9 Protein v2	1250 ng (7.5 pmol)
TrueGuide™ sgRNA	240 ng (7.5 pmol)
Knockout Enrichment GFP-Puromycin	250 ng (0.25 pmol)
Knockout Enrichment RFP-Blasticidin	250 ng (0.25 pmol)
Resuspension Buffer R	to 10 μL

Reagent	Amount per well of 24-well plate
TrueCut™ Cas9 Protein v2	1250 ng (7.5 pmol)
TrueGuide™ sgRNA	240 ng (7.5 pmol)
Knockout Enrichment Donor RFP-Puromycin	250 ng (0.25 pmol)
Knockout Enrichment Donor GFP-Blasticidin	250 ng (0.25 pmol)
Resuspension Buffer R	to 10 μL

- b. Incubate the TrueTag™ donor DNA + TrueCut™ Cas9 Protein v2 + gRNA in Resuspension Buffer R at room temperature for 5–20 minutes.

3. Prepare Tube 2: Cells in Resuspension Buffer (Buffer R)

Note: Prepare extra amount (2X) of cells needed.

- a. If you are using suspension cells, remove an aliquot and determine viable cell count. If you are using adherent cells, detach the cells from the culture flask using Gibco™ TrypLE™ Dissociation Reagent, resuspend the cells in an appropriate volume of growth medium, then determine viable cell count.
- b. Transfer the appropriate amount of cells into a 15-mL centrifuge tube, then pellet the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.

Note: Optimal cell number used for electroporation varies depending on the cell type. For example, in the case of iPSC and THP1, we had best results with 80,000 and 200,000 cells per electroporation respectively.

- c. Wash the cells with PBS without Ca²⁺ or Mg²⁺ using the same volume as original cell volume, then pellet the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.
 - d. Aspirate the PBS and resuspend the cell pellet in Resuspension Buffer R or T (depending on cell type) at the desired concentration. For example, to use 100,000 cells/reaction, resuspend the cells at 2.0 × 10⁷ cells/mL, then use 5 μL of the resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.
- ### 4. Add Tube 2 (cells) to Tube 1
- a. Pipette the cells in Resuspension Buffer (from step 3) up and down to resuspend any cells that might have settled at the bottom of the tube.
 - b. Add 5 μL of the cell suspension to tube 1 from Step 2.
- ### 5. Electroporate using the cell type-specific Neon™ condition
- a. Using the 10 μL Neon™ tip, aspirate 10 μL of the cell + editing mix in Resuspension Buffer, then electroporate using your cell type-specific Neon™ condition (see Appendix B, “Additional procedures”).

IMPORTANT! Avoid creating bubbles that can hinder electroporation.

- b. After electroporation, transfer the contents of the Neon™ tip immediately into one well of the 24-well culture plate containing 1000 μL of pre-warmed growth medium (from step 1).
- c. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Days 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications

1. When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA, GeneArt™ CRISPR nuclease mRNA, and TrueGuide™ sgRNA using the Neon™ transfection system

The following protocol is provided as a starting point for transfecting wild-type cells with TrueTag™ donor DNA, GeneArt™ CRISPR Nuclease mRNA and gRNA using the Neon™ Transfection System.

IMPORTANT! The following recommendations are for a single well in 24-well format using the 10- μ L Neon™ tip. For multiple wells, prepare a master mix of components with extra volume to minimize pipetting errors, and then dispense the appropriate volumes into each well. Avoid creating bubbles while mixing and dispensing. For details on optimizing the Neon™ electroporation conditions and scaling down/up for different plate formats, refer to the *Neon™ Transfection System User Guide* (Pub. No. MAN0001557), available for download at thermofisher.com.

Day 0 – seed cells

1–2 days before transfection, transfer your adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection.

Day 1 – transfect cells

1. Prepare 24-well plate with media.

Add 1000 μ L of cell type-specific growth medium into each well of the 24-well plate and place it in the 37°C incubator to pre-warm.

2. Prepare Tube 1: TrueTag™ donor DNA + GeneArt™ CRISPR Nuclease mRNA + TrueGuide™ sgRNA in Resuspension Buffer.
 - a. Mix the TrueTag™ donor DNA, GeneArt™ CRISPR Nuclease mRNA, TrueGuide™ sgRNA, and Resuspension Buffer R in a fresh, RNase-free microcentrifuge tube according to the appropriate table below. Mix well.

Reagent	Amount per well of 24-well plate
TrueTag™ donor DNA	500 ng
GeneArt™ CRISPR Nuclease mRNA	500 ng
TrueGuide™ sgRNA	100 ng
Resuspension Buffer R	to 10 μ L

3. Prepare Tube 2: Cells in Resuspension Buffer (Buffer R).

Note: Prepare an extra amount (2X) of cells needed.

- a. If you are using suspension cells, remove an aliquot and determine viable cell count. If you are using adherent cells, detach the cells from the culture flask using Gibco™ TrypLE™ Dissociation Reagent, resuspend the cells in an appropriate volume of growth medium, then determine viable cell count.

- b. Transfer the appropriate amount of cells into a 15-mL centrifuge tube, then pellet the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.

Note: Optimal cell number used for electroporation varies depending on the cell type. For example, in case of iPSC and THP1, we had best results with 80,000 and 200,000 cells per electroporation respectively.

- c. Wash the cells with PBS without Ca²⁺ or Mg²⁺ using the same volume as the original cell volume, then sediment the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.
 - d. Aspirate the PBS and resuspend the cell pellet in Resuspension Buffer R at the desired concentration. For example, to use 100,000 cells/reaction, resuspend the cells at 2.0 × 10⁷ cells/mL, then use 5 μL of the resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.
4. Add Tube 2 to Tube 1 : Cells to Editing Mix.
 - a. Pipette the cells in Resuspension Buffer (from step 3) up and down to resuspend any cells that might have settled at the bottom of the tube.
 - b. Add 5 μL of the cell suspension to Tube 1 from step 2.
 5. Electroporate using the cell type-specific Neon™ condition.
 - a. Using the 10-μL Neon™ tip, aspirate 10 μL of the cell + Editing Mix in Resuspension Buffer, then electroporate using your cell type-specific Neon™ condition.

IMPORTANT! Avoid creating bubbles that can hinder electroporation.

IF these conditions have not yet been determined, review our <https://www.thermofisher.com/us/en/home/life-science/cell-culture/transfection/neon-transfection-system/neon-transfection-system-cell-line-data.html> for initial guidelines.

- b. After electroporation, transfer the contents of the Neon™ tip immediately into one well of the 24-well culture plate containing 1000 μL of pre-warmed growth medium (from step 1).
- c. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Days 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications.

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA, GeneArt™ CRISPR nuclease mRNA, and TrueGuide™ sgRNA using the Lipofectamine™ MessengerMAX™ Transfection Reagent

The following protocol is provided as a starting point for transfecting cells with TrueTag™ donor DNA, GeneArt™ CRISPR nuclease mRNA, and gRNA using the Lipofectamine™ MessengerMAX™ Transfection Reagent.

Day 0 – seed cells

The day before transfection, seed the adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	96-well	24-well	6-well
Cell density per well	8,000–18,000 cells	40,000–90,000 cells	250,000–450,000 cells
Final volume of media per well	100 µL	0.5 mL	2 mL

Day 1 – transfect cells

1. Prepare Tube 1: TrueTag™ donor DNA + GeneArt™ CRISPR nuclease mRNA + TrueGuide™ sgRNA solution in Opti-MEM™ I Medium
 - a. Mix the TrueTag™ donor DNA, GeneArt™ CRISPR Nuclease mRNA, gRNA, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNase-free microcentrifuge tube according to the appropriate table below. Mix gently.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
GeneArt™ CRISPR Nuclease mRNA	250 ng (1.5 pmol)	250 ng (7.5 pmol)	6,250 ng (37.5 pmol)
TrueGuide™ sgRNA	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1,200 ng (37.5 pmol)
TrueTag™ donor DNA	100 ng	500 ng (0.5 pmol)	2,500 ng

For Knockout Enrichment, two TrueTag™ donors with different fluorophores and antibiotic resistance markers are co-delivered into cells along with Cas9 nuclease and guide RNA. Use one of the following two options to ensure the correct combination of antibiotic resistance markers.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
GeneArt™ CRISPR Nuclease mRNA	250 ng (1.5 pmol)	1,250 ng (7.5 pmol)	6,250 ng (37.5 pmol)
TrueGuide™ sgRNA	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1,200 ng (37.5 pmol)
Knockout Enrichment Donor GFP-Puromycin	50 ng	250 ng (0.25 pmol)	1,250 ng
Knockout Enrichment Donor RFP-Blasticidin	50 ng	250 ng (0.25 pmol)	1,250 ng

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
GeneArt™ CRISPR Nuclease mRNA	250 ng (1.5 pmol)	1,250 ng (7.5 pmol)	6,250 ng (37.5 pmol)
TrueGuide™ sgRNA	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1,200 ng (37.5 pmol)
Knockout Enrichment Donor RFP-Puromycin	50 ng	250 ng (0.25 pmol)	1,250 ng
Knockout Enrichment Donor GFP-Blasticidin	50 ng	250 ng (0.25 pmol)	1,250 ng

2. Prepare Tube 2: Dilute Lipofectamine™ MessengerMAX™ reagent in Opti-MEM™ I Medium, then mix gently.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Lipofectamine™ MessengerMAX™ Reagent	0.3 µL	1.5 µL	7.5 µL

3. Mix Tube 1 + Tube 2

Add the diluted Lipofectamine™ MessengerMAX™ Reagent (Tube 2) to the Editing Mix (Tube 1) and mix well by pipetting.

Note: For high-throughput setup (e.g., 96-well format or others), always add the contents of Tube 2 into Tube 1, because you can prepare Tube 2 as a bulk master mix.

4. Incubate for 5 minutes at room temperature.
5. Add the transfection complex to cells and incubate at 37°C.
- Add the transfection complex (from step 4) to the adherent cells at 30–70% confluence according to the following table:

Reagent	96-well	24-well	6-well
TrueTag™ donor DNA:RNP:transfection reagent complex	10 µL	50 µL	250 µL

- b. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Day 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications.

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA and TALEN pair, using Lipofectamine™ MessengerMAX™ Transfection Reagent

The following protocol is provided as a starting point for transfecting cells with TrueTag™ donor DNA and an mRNA TALEN pair using Lipofectamine™ MessengerMAX™ Transfection Reagent.

Day 0 – seed cells

The day before transfection, seed the adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	96-well	24-well	6-well
Cell density per well	8,000–18,000 cells	40,000–90,000 cells	250,000–450,000 cells
Final volume of media per well	100 µL	0.5 mL	2 mL

Day 1 – transfect cells

- Prepare Tube 1: TrueTag™ donor DNA + mRNA TALEN pair in Opti-MEM™ I Medium.
 - Mix the TrueTag™ donor DNA, mRNA TALEN pair, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNase-free microcentrifuge tube according to the appropriate table below, then mix gently.

Table 5 One TrueTag™ donor DNA

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Left TALEN mRNA	50 ng	200 ng	1000 ng
Right TALEN mRNA	50 ng	200 ng	1000 ng
TrueTag™ donor DNA	100 ng	500 ng	2500 ng

For Knockout Enrichment, two TrueTag™ donor DNAs with differential fluorophores and antibiotic markers are co-delivered into cells along with Cas9 nuclease and TALEN mRNAs.

Table 6 Knockout Enrichment with Donor GFP-Puromycin and Donor RFP-Blasticidin

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Left TALEN mRNA	50 ng	200 ng	1000 ng
Right TALEN mRNA	50 ng	200 ng	1000 ng
Knockout Enrichment Donor GFP-Puromycin	50 ng	250 ng	1250 ng
Knockout Enrichment Donor RFP-Blasticidin	50 ng	250 ng	1250 ng

Table 7 Knockout Enrichment with Donor GFP-Blasticidin and Donor RFP-Puromycin

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Left TALEN mRNA	50 ng	200 ng	1000 ng
Right TALEN mRNA	50 ng	200 ng	1000 ng
Knockout Enrichment Donor RFP-Puromycin	50 ng	250 ng	1250 ng
Knockout Enrichment Donor GFP-Blasticidin	50 ng	250 ng	1250 ng

2. Prepare Tube 2: Dilute Lipofectamine™ MessengerMAX™ reagent in Opti-MEM™ I Medium, then mix gently.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Lipofectamine™ MessengerMAX™ reagent	0.3 µL	1.5 µL	7.5 µL

3. Mix Tube 1 + Tube 2.

Add the diluted Lipofectamine™ MessengerMAX™ reagent (Tube 2) to the Editing Mix (Tube 1), then mix gently by pipetting.

Note: For high-throughput setup (e.g., 96-well format or others), always add the contents of Tube 2 to Tube 1 because you can prepare Tube 2 as a bulk master mix.

4. Incubate for 5 minutes at room temperature.

5. Add the transfection complex to cells and incubate at 37°C.
 - a. Add the transfection complex (from step 4) to the adherent cells at 30–70% confluence according to the following table.

Reagent	96-well	24-well	6-well
TrueTag™ donor DNA:TALEN/transfection reagent complex	10 µL	50 µL	250 µL

- b. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Day 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications.

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA and TALEN mRNA pairs using the Neon™ transfection system

The following protocol is provided as a starting point for transfecting wild-type cells with TrueTag™ donor DNA and TALEN mRNA pairs using the Neon™ Transfection System.

IMPORTANT! The following recommendations are for a single well in 24-well format using the 10-µL Neon™ tip. For multiple wells, prepare a master mix of components with extra volume to minimize pipetting errors, and then dispense the appropriate volumes into each well. Avoid creating bubbles while mixing and dispensing. For details on optimizing the Neon™ electroporation conditions and scaling down/up for different plate formats, refer to the *Neon™ Transfection System User Guide* (Pub. No. MAN0001557), available for download at thermofisher.com.

Day 0 – seed cells

1–2 days before transfection, transfer the adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection.

Day 1 – transfect cells

1. Prepare 24-well plate with media.

Add 1000 μ L of cell type-specific growth medium into each well of the 24-well plate and place it in the 37°C incubator to pre-warm.

2. Prepare Tube 1: TrueTag™ donor DNA + TALEN mRNA pair in Resuspension Buffer.

- a. Mix the TrueTag™ donor DNA, TALEN mRNA pair and Resuspension Buffer R in a fresh, RNase-free microcentrifuge tube according to the following table. Mix well.

Reagent	Amount per well of 24-well plate
TrueTag™ donor DNA	500 ng
Left TALEN mRNA	100 ng
Right TALEN mRNA	100 ng
Resuspension Buffer R	to 10 μ L

3. Prepare Tube 2: Cells in Resuspension Buffer (Buffer R).

Note: Prepare an extra amount (2X) of cells needed.

- a. If you are using suspension cells, remove an aliquot and determine viable cell count. If you are using adherent cells, detach the cells from the culture flask using Gibco™ TrypLE™ Dissociation Reagent, resuspend the cells in an appropriate volume of growth medium, then determine viable cell count.
- b. Transfer the appropriate amount of cells to a 15-mL centrifuge tube, then pellet the cells by centrifugation at 100–400 $\times g$ for 5 minutes at room temperature.

Note: The optimal cell number used for electroporation varies depending on the cell type. For example, in the case of iPSC and THP1, we had best results with 80,000 and 200,000 cells per electroporation, respectively.

- c. Wash the cells with PBS without Ca^{2+} or Mg^{2+} using the same volume, then pellet the cells by centrifugation at 100–400 $\times g$ for 5 minutes at room temperature.
 - d. Aspirate the PBS, then resuspend the cell pellet in Resuspension Buffer R at the desired concentration. For example, to use 100,000 cells/reaction, resuspend the cells at 2.0×10^7 cells/mL, then use 5 μ L of the resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.
4. Add Tube 2 to Tube 1 : Cells to Editing Mix.
 - a. Pipette the cells in Resuspension Buffer (from step 3) up and down to resuspend any cells that might have settled at the bottom of the tube.
 - b. Add 5 μ L of the cell suspension to Tube 1 from step 2.

5. Electroporate using the cell type-specific Neon™ condition.
 - a. Using the 10- μ L Neon™ tip, aspirate 10 μ L of the cell + Editing Mix in Resuspension Buffer, then electroporate using your cell type-specific Neon™ condition.

IMPORTANT! Avoid creating bubbles that can hinder electroporation.

- b. After electroporation, transfer the contents of the Neon™ tip immediately to one well of the 24-well culture plate containing 1000 μ L of pre-warmed growth medium (from step 1).
 - c. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Days 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications.

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Transfect cells with TrueTag™ donor DNA and GeneArt™ CRISPR nuclease vector using Lipofectamine™ 3000 Transfection Reagent

The following protocol is provided as a starting point for transfecting cells with TrueTag™ donor DNA and a CRISPR nuclease vector using the Lipofectamine™ 3000 Transfection Reagent.

Day 0 – seed cells

The day before transfection, seed the adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	96-well	24-well	6-well
Cell density per well	8,000–18,000 cells	40,000–90,000 cells	250,000–450,000 cells
Final volume of media per well	100 μ L	0.5 mL	2 mL

Day 1 – transfect cells

GeneArt™ CRISPR Nuclease Vector with OFF (Cat. No. A21174) or CD4 (Cat. No. A21175) is an all-in-one vector that expresses Cas9 nuclease and custom gRNA along with an orange fluorescent protein (OFF) or CD4 surface marker which can be used for sorting the transfected cell population. This vector requires cloning the gRNA sequence into the vector prior to use. Alternatively, other plasmids expressing the Cas9 and gRNA components may be used.

The correct dose of Lipofectamine™ 3000 Transfection Reagent varies amongst cell lines; the following protocol tests two common concentrations. For repeat use in the same cell line use whichever works best, or further titrate the dose.

1. Prepare Tube 1: TrueTag™ donor DNA + CRISPR nuclease vector in Opti-MEM™ I Medium
 - a. Mix the TrueTag™ donor DNA, CRISPR nuclease vector, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNase-free microcentrifuge tube according to the appropriate table, then mix gently.

Table 8 One TrueTag™ donor DNA

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	10 µL	50 µL	250 µL
GeneArt™ CRISPR Nuclease Vector	100 ng	500 ng	1000 ng
TrueTag™ donor DNA	100 ng	500 ng	2500 ng
P3000 Reagent (2 µL/µg DNA)	0.4 µL	2 µL	7 µL

For Knockout Enrichment, two TrueTag™ donor DNAs with differential fluorophores and antibiotic markers are co-delivered into cells along with Cas9 nuclease and guide RNA

Table 9 Knockout Enrichment with Donor GFP-Blasticidin and Donor RFP-Puromycin

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	10 µL	50 µL	250 µL
GeneArt™ CRISPR Nuclease Vector	100 ng	500 ng	1000 ng
Knockout Enrichment Donor GFP-Blasticidin	50 ng	250 ng	1250 ng
Knockout Enrichment Donor RFP-Puromycin	50 ng	250 ng	1250 ng
P3000 Reagent (2 µL/µg DNA)	0.4 µL	2 µL	7 µL

Table 10 Knockout Enrichment with Donor GFP-Puromycin and Donor RFP-Blasticidin

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	10 µL	50 µL	250 µL
GeneArt™ CRISPR Nuclease Vector	100 ng	500 ng	1000 ng
Knockout Enrichment Donor RFP-Blasticidin	50 ng	250 ng	1250 ng
Knockout Enrichment Donor GFP-Puromycin	50 ng	250 ng	1250 ng
P3000 Reagent (2 µL/µg DNA)	0.4 µL	2 µL	7 µL

2. Prepare Tube 2: Set up 2 Lipofectamine™ 3000 Transfection Reagent dilutions in Opti-MEM™ I Medium.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL × 2	25 µL × 2	125 µL × 2
Lipofectamine™ 3000 Transfection Reagent	0.15 µL and 0.3 µL	0.75 µL and 1.5 µL	3.75 µL and 7.5 µL

3. Mix Tube 1 + Tube 2.
Add half of Tube 1 (donor DNA and CRISPR nuclease vector) to each dilution of Lipofectamine™ 3000 Transfection Reagent (Tube 2) and mix gently by pipetting.
4. Incubate for 10–15 minutes at room temperature.
5. Add the transfection complex to cells and incubate at 37°C.
- a. Add the transfection complex to the adherent cells at 30–70% confluence according to the following table.

Reagent	96-well	24-well	6-well
Donor DNA + nuclease vector/transfection reagent complex	10 µL	50 µL	250 µL

- b. Incubate the cells in a humidified 37°C, 5% CO₂ incubator for 48–72 hours.

Day 2 to 4 – verify editing

Verify editing efficiency and proceed to downstream applications.

When the cells have reached 80–100% confluence, you are ready to proceed to the next steps.

Verify editing

Once the cells reach confluency, usually 2-4 days post-transfection, they are ready to screen for the assessment of the edited population. Alternatively, if you wish to move to highly enriched populations directly, you can begin antibiotic selection. The following assays can be done before and after selection to monitor the population.

We recommend that a portion of the edited cells be maintained while the various tests are conducted. If more cells are needed for the tests, the cells can be split and grown up for a larger population.

For TrueTag™ GFP, RFP, BFP and YFP Donor DNA Kits

- Visually confirm the presence of fluorescent tag (for GFP, RFP, BFP and YFP) by observation with a fluorescent microscope with the proper filter set. The appropriate excitation and emission filters for these proteins can be found in Table 1 on page 18.
- Flow cytometry can be used to measure the populations of tagged and untagged cells.
 - Flow cytometry generally needs greater than 10^4 cells for an accurate count.
 - FACS™ can be used to enrich the GFP+, RFP+, BFP+, or YFP+ cell population, and it generally needs $>10^6$ cells as a starting population.
 - For more information on flow cytometry, go to thermofisher.com/attune

For TrueTag™ Epitope Donor DNA Kits

- Immunostaining and flow cytometric analysis, to measure the populations of tagged and untagged cells

Depending on the subcellular localization of epitope-tagged proteins, the cells can be stained with the corresponding fluorescence-labeled epitope antibody (see “Required materials not supplied” on page 9, then visualized under fluorescence microscopy. The percentage of cells with positive staining can be determined by flow cytometry. For more information, go to [Immunofluorescent staining of intracellular antigens on cultured cells protocol](#).

- Western blot analysis

Upon antibiotic selection with puromycin or blasticidin for 5 to 7 days, the cells are expanded in a 6-well plate. When reaching approximately 80-90% confluency, the cells can be detached from the culture plate using TrypLE™ Express Enzyme (Cat. No. [12604021](#)) and then washed once with 1 mL dPBS. Untransfected cells and unselected cells are also harvested and served as controls. The cell pellets are lysed on ice for approximately 15 minutes with 80 to 100 μ L RIPA Lysis and Extraction Buffer (Cat. No. [89900](#)) or Pierce™ IP Lysis Buffer (Cat. No. [87788](#)) supplemented with Halt™ Protease Inhibitor Cocktail (100X) (Cat. No. [78429](#)). The samples are centrifuged at 14,000 rpm for 10 minutes. The resulting supernatant is then transferred to a clean Eppendorf tube. An aliquot of 20 μ L supernatant is added to 8 μ L of 4X NuPAGE™ LDS Sample Buffer (Cat. No. [NP0007](#)) containing 100 mM to 200 mM DTT. The samples are heated at 70°C for 5 minutes and then loaded onto a 1.0-mm NuPAGE™ 4 to 12%, Bis-Tris, Mini Protein Gel (Cat. No. [NP0321BOX](#)). The fractionated proteins are transferred to a nitrocellulose membrane using iBlot™ 2 Gel Transfer Device (Cat. No. [IB21001](#)). The protocol for Western blotting is described in WesternBreeze™ Chemiluminescent Kit (Cat. No. [WB7104](#)). The membrane is developed with SuperSignal™ West Dura Extended Duration Substrate (Cat. No. 34076) and imaged with iBright™ Imaging System (Thermo Fisher Scientific).

For TrueTag™ Stem Donor DNA Kits

- Junction PCR and sequencing analysis
Although the TrueTag™ stem donor DNAs can be used to tag an expressed gene, the tag cannot be visualized if it is used to tag an unexpressed gene in stem cell stage. However, junction PCR and sequencing analysis can be used to determine the correctness of tag insertion.
- Stem cell differentiation
Stem cells, such as human pluripotent stem cells, can be differentiated into various cell lineages or organoids. A unique cell type-specific marker can be used to track the differentiation process. The general cell differentiation medium and protocol can be found at [thermofisher.com](https://www.thermofisher.com).
 - PSC Neural Induction Medium (Cat. No. [A1647801](#))
 - PSC Cardiomyocyte Differentiation Kit (Cat. No. [A2921201](#))
 - PSC Dopaminergic Neuron Differentiation Kit (Cat. No. [A3147701](#))

For TrueTag™ Knockout Enrichment Donor DNA Kit

- Flow cytometric analysis and cell sorting
 - Although the GFP+ and RFP+ cells can be easily detected by fluorescence microscopy, the actual percentage of GFP+, RFP+, or dual GFP+/RFP+ cells needs to be determined by flow cytometry. For more information, go to [thermofisher.com/attune](https://www.thermofisher.com/attune).
 - A fluorescence cell sorter can be used to enrich GFP+, RFP+, or dual GFP+/RFP+ cells. We recommend sorting greater than 10³ cells for downstream applications.
- Western blot analysis
The knockout efficiency of the target gene can be verified by Western blot analysis using the corresponding antibody. For more information, go to [thermofisher.com/westernblot](https://www.thermofisher.com/westernblot). Following is the general protocol.
 - a. Transfer proteins to a PVDF or nitrocellulose membrane using iBlot™ 2 Dry Blotting System.
 - b. Incubate the membrane with a sufficient volume of blocking buffer for 30–60 minutes at room temperature with agitation.
 - c. Dilute the primary antibody per supplier recommendations in the blocking buffer.
 - d. Incubate the membrane protein-side up in the primary antibody solution with agitation, for 1 hour at room temperature or overnight at 2–8°C. Ensure the volume of the antibody solution is enough to fully cover the membrane.
 - e. Wash the membrane 3 times with agitation for 10 minutes each in wash buffer.
 - f. Prepare dilutions of the conjugated secondary antibody in the appropriate volume of wash buffer or alternatively in blocking buffer.
 - g. Incubate the membrane protein-side up in the secondary antibody solution for 1 hour with agitation at room temperature. Ensure the volume of the antibody solution is enough to fully cover the membrane.
 - h. Wash the membrane 6 times with agitation for 5 minutes each in wash buffer to remove any unbound secondary antibodies. It is crucial to thoroughly wash the membrane at this step.
 - i. Prepare a working solution of chemiluminescent substrate based upon manufacturer instruction. Suggested volume of approximately 8–10 mL for mini blots and 15 mL for midi blots (0.1 mL working solution per cm² of membrane).
 - j. Incubate the blot with the working solution for 1 minute when using standard ECL substrates or 5 minutes when using high-performance substrates, such as SuperSignal™ substrates.

- k. Remove the blot from the working solution and drain excess reagent.
 - l. Place the blot in clear plastic wrap or sheet protector and remove bubbles by rolling with a blot roller or glass pipette.
 - m. Image the blot using film or an appropriate imaging system.
- Immunostaining and flow cytometric analysis
Immunostaining and flow cytometric analysis can be used to determine the percentage of knockout upon enrichment with antibiotic selection and cell sorting. For more information, visit Epitope tag section.

Verify correct integration of tag by junction PCR and sequencing

- We recommend confirmation of the correct insertion of the TrueTag™ cassette. This can be done by using junction primers on the pooled or clonal populations.
- On pooled populations, junction PCR (one primer on the genome and one in the cassette) only can tell you that the junction exists but is not accurate for the percentages. We recommend junction PCR over amplifying the full tag because PCR will be biased towards the smallest amplicon and will heavily enrich the short indel amplicons over the long tag amplicons.
- On clonal populations, both methods can work and we recommend doing both. You may be able to determine homozygous from heterozygous populations by using Copy Number Variation Analysis TaqMan™ assays. For more information, go to [thermofisher.com/taqmancnv](https://www.thermofisher.com/taqmancnv).
- The junction PCR amplicon can be TOPO™ cloned and then sequenced to give a representation of the accuracy of the junctions. On clonal populations, this can also be used to confirm the tagging of the individual alleles. For more information, go to [thermofisher.com/us/en/home/life-science/cloning/topo/topo-ta-cloning/topo-ta-for-sequencing](https://www.thermofisher.com/us/en/home/life-science/cloning/topo/topo-ta-cloning/topo-ta-for-sequencing).

Enrich with antibiotic selection

Once the cells have recovered and are reaching confluency, antibiotic selection can be used to further enrich the edited population. With antibiotic selection, a population of >99% positive cells can be obtained and used for downstream applications

1. Split the cells into 5 aliquots for the following treatments.
 - a. Junction PCR or other assays
 - b. Maintenance (no selection)
 - c. Antibiotic dose 1
 - d. Dose 2
 - e. Dose 3
2. Maintain the no-selection cells as normal in case the selection fails.

3. For the antibiotic selection we recommend using the optimal antibiotic killing dosage as determined by kill curve and also 1 dose above and 1 dose below the optimal killing dosage. Some endogenous promoters are weak and may produce only a low level of the antibiotic resistance gene so the lower dosage may still provide adequate selection.
4. Maintaining the cells at these 3 doses for 5–10 days is usually sufficient for selection. Enrichment can be monitored by microscope with GFP, RFP, BFP, or YFP tags.
5. Once selection is completed, the cells can be assayed again to characterize the population.



Troubleshooting

Observation	Possible cause	Recommended action
Cells not expressing tag	There was poor cleavage efficiency of the gRNA or TALEN mRNA pair.	Validate the cleavage efficiency of the gRNA or TALEN mRNA pair with a Genomic Cleavage Detection Assay. For successful HDR, we strongly recommend using gRNA or TALEN that cleave >50%.
		Use validated gRNA sequences that have demonstrated high level editing efficiency with up to 90% editing efficiency or greater in several cell types.
	There was poor transfection efficiency.	Check if the transfection reagent used was suitable for the cell line (refer to the transfection reagent recommendations at thermofisher.com/transfection).
		Use validated gRNA sequences that have demonstrated high level editing efficiency with up to 90% editing efficiency or greater in several cell types
		Perform a positive control reaction with the human ACTB primers and the N-term donor template if working in a human cell line.
	The donor design was out of frame.	Check donor oligos to ensure that the reading frame is kept intact for both N-terminal and C-terminal tags. It is also important to ensure that any bases between the cut site and the insertion site are added back to the forward or reverse primer design, to ensure frame continuity and produce a scarless insertion.
	The target protein was not expressed.	TrueTag™ fusion tags are expressed off of the endogenous promoter. If the tagged protein is expressed at low levels, it may be difficult to visualize the florescent tag, or perform antibiotic selection. However, HDR may have occurred and can be observed via PCR or sequencing.

Observation	Possible cause	Recommended action
Low DNA yield	There was inefficient DNA binding.	Verify that a 1:1 volume of Binding Buffer is added to the reaction mixture.
		Ensure the solutions are mixed well.
		Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the solution color is orange or violet, add 10 μ L of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
	There were inefficient membrane washes.	Ensure that the recommended volume of ethanol has been added to the Wash Buffer (concentrated) before first use.
	There was inefficient DNA elution from the membrane.	Add Elution Buffer directly to the center of the membrane and not to the side of the GeneJET™ purification column.
		Use 20–50 μ L of Elution Buffer and ensure that the volume completely covers the surface of the membrane.
Increase the Elution Buffer volume two-fold or perform two elution cycles when purifying larger amounts of DNA (e.g., >15 μ g).		
In step 5 of Protocol A (step 7 of Protocol B), ensure that all residual wash buffer is removed from the membrane. A longer centrifugation time (extra minute) can aid in removal of wash buffer.		
Poor A260:A230 ratio	There was inefficient DNA elution from the membrane.	Add Elution Buffer directly to the center of the membrane and not to the side of the GeneJET™ purification column.
		A low A260:A230 ratio is a sign of guanidinium thiocyanate carry over. We have observed that trace amounts of guanidinium thiocyanate do not affect transfection or cell viability, but are observable via spectrophotometry.



Additional procedures

Antibiotic toxicity assay (kill curve) with PrestoBlue™ cell viability reagent

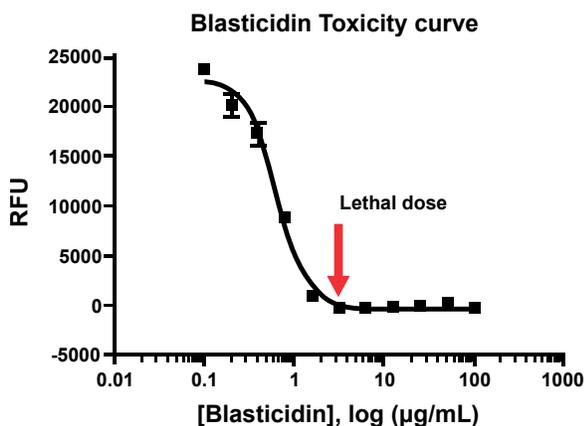
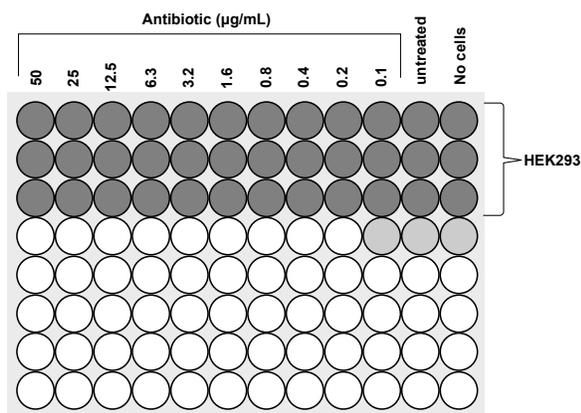
This assay will determine the level of antibiotic (Puromycin or Blasticidin) that is toxic for your cell line. You may need to vary the starting cell amounts to avoid overgrowth. For your cell line we recommend you use the starting density used during passaging your cell line. This example uses HEK293 cells.

1. Harvest the HEK293 cells by trypsinization
2. Prepare a cell suspension at a concentration of 40,000 cells/mL in Growth Medium.
3. Plate 100 μ L of the cell suspension (~4000 cells) in three rows (3 \times 11 wells) of a 96-well Plate. Add 100 μ L growth medium to the 12th well (background control). Incubate in a 37°C incubator with a humidified atmosphere of 5% CO₂ for 18 to 24 hours.

Note: It is important to start with a relatively small number of cells because the experiment requires 7–8 days of continuous cultivation. Using more cells will require a replating step.

4. Prepare 1 mL of a 100 μ g/mL Blasticidin or Puromycin stock solution in Growth Medium in a 1.5-mL microcentrifuge tube. This is “Tube 1” of the serial dilution.
5. Prepare 9 additional 1.5-mL microcentrifuge tubes (numbered 2 to 10) containing 0.5 mL growth medium.
6. Prepare a 10-point two-fold serial dilution by transferring 0.5 mL of the Blasticidin or Puromycin stock solution from tube 1 to tube 2 (mix by pipetting), followed by transferring 0.5 mL from tube 2 to tube 3 and so on until you reached tube 10. Use a new pipette tip for each dilution.
7. Add 100 μ L of each dilution to one column of the 96-well plate containing the cells to get the following series (50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0 μ g/mL). Add 100 μ L of growth medium to column 11 (untreated control) and 12 (background control)
8. Incubate the cells for ~72 hours in a 37°C incubator with a humidified atmosphere of 5% CO₂.
9. After the incubation, remove the medium by aspiration and add 100 μ L of fresh Growth Medium.
10. Prepare and add the new serial dilution as described in step 4.
11. Repeat media exchange cycle (step 8) up to 3 times (for up to 14 days total). The cells can be tested as early as 3 days post selection, but the curve is more accurate for slow growing cells at 7–14 days.

12. Prepare PrestoBlue™ reagent by diluting it 1:10 in Growth Medium 1:10 (refer to the PrestoBlue™ Cell Viability Reagent User Guide (Pub. No. [MAN0018370](#)). Prepare enough PrestoBlue™ reagent for 40 wells (33 wells for antibiotic serial dilution and 3 wells for no cell control wells).
13. Remove the medium by aspiration and add 100 μ L of PrestoBlue™ reagent
14. Incubate the cells for at least 10 minutes at 37°C. Longer incubation time increases the sensitivity of detection.
15. Read the fluorescence or absorbance. Fluorescence is more sensitive than absorbance and is the preferred detection method (excitation 540–570 nm, emission 580–610 nm; for more information, refer to the instructions provided with the PrestoBlue™ Cell Viability Reagent, which are also available at [thermofisher.com](#)).
16. Calculate and plot the results (the no-cell control values can be used to determine background fluorescence). Higher values correlate with higher metabolic activity. The target dose for selection is the dilution that kills all cells.



Clonal isolation

Limiting dilution cloning (LDC)

- The number of single clones needed to obtain a desired tagged clonal cell line is estimated based on the editing efficiency and estimated cell viability.

For example, if you desire a homozygous knock-in (KI) with tags in both copies of a gene, and 10% of your cells are positive for GFP then the probability of having both alleles knocked out in any cell is 1% ($0.1 \times 0.1 = 0.01$).

Some tags will disrupt the natural function of the gene and would be lethal if homozygous so you cannot always get biallelic tagging (e.g., ACTB). Of the tagged clones, usually >70% have perfect junctions.

- Perform limiting dilution by targeting 0.8 cells/well, which requires you to resuspend the transfected cells (post-counting) at a density of 8 cells/mL in complete growth medium, then transferring 100 μ L of this to each well of a 96-well plate.

If you plate at least ten 96-well plates in this manner and expect ~20% of cells to survive, the probability of having homozygous KI clones in the 192 surviving cells is ~1–2 cells ($192 \times 1\%$). The following formula can be used to determine minimum number of wells.

$$\text{Minimum no. wells} = \left(100 \times \frac{C}{A}\right) \times \left(\frac{100}{D}\right)^B$$

A = Estimated cell recovery percentage after dilution

B = Minimum number of edited copies of the gene (1, for single copy; 2, for bi-allelic; more if there are multiple copies of the gene)

C = Number of desired successful clones

D = Editing percentage

By doing antibiotic selection you can significantly increase your chances and lower the workload. After selection we usually see >50% tagged and often >90% if the selection is stringent. Even at 50% tagged, the probability of biallelic is now 25% and of the 192 cells, now ~134 are likely biallelic. In this case, instead of screening 10 plates, 1 plate may be sufficient.

We recommend using at least five 96-well plates as it is much more time (3–6 weeks) to repeat the clonal dilution if you do not get your clone. If the editing frequency is high, we may just process one plate initially and freeze the other plates as backups.

- Single cell clone survivability varies by cell type. Some cells that do not like to remain as single cells need to be plated at a low density to get well separated colonies, which then have to be manually picked for further screening.

Example LDC procedure using 293FT cells

1. Wash the transfected cells in each well of the 24-well plate with 500 μ L of PBS. Carefully aspirate the PBS and discard.
2. Add 500 μ L of TrypLE™ cell dissociation reagent to the cells and incubate for 2–5 minutes at 37°C.
3. Add 500 μ L of complete growth medium to the cells to neutralize the dissociation reagent. Pipette the cells up and down several times to break up cell aggregates. Make sure the cells are well separated and not clumped together.
4. Centrifuge the cells at 300 $\times g$ for 5 minutes to pellet.
5. Aspirate the supernatant, resuspend the cells in an appropriate volume of pre-warmed (37°C) growth medium, then perform a cell count.
6. After counting, dilute the cells to a density of 8 cells/mL of complete growth medium. Prepare a total of 50 mL of cell suspension at this cell density and transfer to a sterile reservoir.

Note: A serial dilution can be performed to get a better estimate of cell density.

7. Using a multichannel pipettor, transfer 100 μ L of the cell suspension into each well of 96-well tissue culture plates until the desired number of plates is seeded. Make sure to mix the cells in between seeding the plates to avoid the formation of cell aggregates.

Note: In general, we seed ten 96-well plates to achieve a large number of clones. Number of plates to seed depends on the editing efficiency of pooled cell population and viability of cells post single cell isolation.

8. Incubate the plates in a 37°C, 5% CO₂ incubator.
9. Scan the plates for single cell colonies as soon as small aggregates of cells are visible under a 4X microscope (usually after first week, depending on the growth rate of the cell line).
10. Continue incubating the plates for an additional 2–3 weeks to expand the clonal populations for further analysis and characterization.

Example single cell sorting procedure in a 96-well plate using flow cytometer

You can sort single cells per well into a 96-well plate format using a flow cytometer with single or dual fluorescence cell sorting capability. After sorting and expanding the single cell clones, analyze and characterize the clonal populations using suitable assays. The following is an example of single- or dual-fluorescence cell sorting procedure with 293FT cells.

1. Wash the transfected 293FT cells in each well of the 24-well plate with 500 μ L of PBS. Carefully aspirate the PBS and discard.
2. Add 500 μ L of TrypLE™ cell dissociation reagent to the cells and incubate for 2–5 minutes at 37°C.
3. Add 500 μ L of complete growth medium to the cells to neutralize the dissociation reagent. Pipette the cells up and down several times to break up cell aggregates. Make sure the cells are well separated and not clumped together.
4. Centrifuge the cells at $300 \times g$ for 5 minutes to pellet.
5. Aspirate the supernatant, then wash the cell pellet once with 500 μ L of PBS.
6. Resuspend 1×10^6 cells in 1 mL of FACS™ buffer, then add propidium iodide (PI) to the cells at a final concentration of 1 μ g/mL. Keep the resuspended cells on ice.
7. Filter the cells using suitable filters before analyzing them on a flow cytometer with single- or dual-fluorescence cell sorting capability.
8. Sort PI-negative cells into a 96-well plate containing 100 μ L of complete growth medium. If desired, use 1X antibiotics with the complete growth medium.
9. Incubate the plates in a 37°C, 5% CO₂ incubator.
10. Scan the plates for single cell colonies as soon as small cell aggregates are visible under a 4X microscope. Colonies should be large enough to see as soon as 7–14 days (usually after first week, depending on the growth rate of the cell line). Perform image analysis to ensure that the colonies are derived from single cells.
11. After image analysis, continue incubating the plates for an additional 2–3 weeks to expand the clonal populations for further analysis and characterization.

Confirmation of Cas9 or TALEN cutting activity by GCD or sequencing

Tagging efficiency is dependent on efficient nuclease cutting of the target site. Generally, 10–40% of the cut population is repaired by HDR, dependent on cell line. We recommend screening multiple gRNAs or TALENs that cut near the desired edit site to confirm the cutting efficiency.

Genomic Cleavage Detection Kit

- After transfections, use the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. [A24372](#)) to estimate the CRISPR-Cas9-mediated cleavage efficiency in a pooled cell population.
- You can design and order target-specific primer sets for the GCD assay using the TrueDesign Genome Editor Software, available at [thermofisher.com/truedesign](https://www.thermofisher.com/truedesign). The tool automatically generates GCD (or sequencing) primers for every gRNA that is designed.
- To perform the GCD assay for the positive control, you need the primers listed in “Control Primers” on page 8. Target specific primer sets needed for the GCD assay can be custom ordered from <https://www.thermofisher.com/order/custom-standard-oligo>, for target-specific primer sets needed for the GCD assay.
- You can set up the GCD assay in a 96-well plate format and analyze multiple gRNA-treated samples in parallel on a 2% E-Gel™ 48 agarose gel (48-well).
- Pick the clones that show the highest cleavage efficiency to use in your experiments. Note that the clone that shows the highest cleavage efficiency may not always be the clone with the highest expression.
- For more information and detailed protocols, see *GeneArt™ Genomic Cleavage Detection Kit User Guide* (Pub. No. MAN0009849)

Sequence analysis

- For Sanger sequencing-based editing efficiency analysis, refer to our application note referenced at [thermofisher.com/sangercrispr](https://www.thermofisher.com/sangercrispr)
- If you are experienced in next generation sequencing (NGS) and analysis, you can use barcoded target-specific amplicon primers and perform multiplex analysis using several gRNA-treated samples in parallel. Multiplex analysis using NGS is especially useful when using the custom arrayed plate format for TrueGuide™ Synthetic gRNA transfections. For more information on NGS analysis, refer to Ion Torrent™ targeted sequencing solutions at [thermofisher.com/ionapliseqsolutions](https://www.thermofisher.com/ionapliseqsolutions)

Removal of antibiotic selection by Cre/Lox

For cells in a 24 well plate, transfect in 500 ng of Cre mRNA (Trilink Cat. No. L-7211) following the recommendations for Lipofectamine™ MessengerMAX™ reagent. After 3 days, proceed to clonal isolation and screening by sequencing to verify successful removal of the selection marker.

Positive control ACTB donor reaction

For users that are new to the TrueTag™ system, we recommend performing the reaction with the control primers, N-terminal human ACTB, and N- Puro donor DNA template for fluorescence or an epitope in a human cell line. This design works with a cleavage site between the G and A of the second codon of ACTB using the following gRNA target sequence. See the following tables for gRNA and target gene recommendations for TrueTag™ Stem and Knockout Enrichment Kits.

ACTB gRNA = 5' GCGGCGATATCATCATCCA 3' (TGG=PAM)

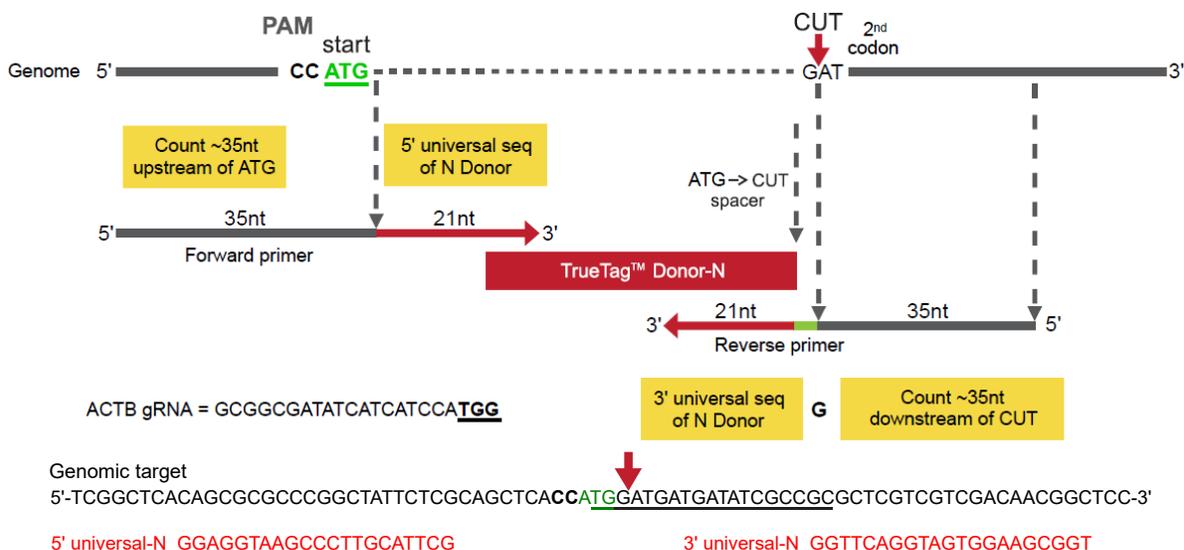


Figure 21

For the following primer sequences, bases in bold are the UniF-N and UniR-N sequences, respectively. The non-bolded bases are the homologous sequences for human ACTB. The start codon, which needs to be added back in the forward primer in addition to 35 bases of homology, is underlined.

N-terminal human ACTB donor PCR primers for N-GFP/Puro tag
Forward primer
5'-CFOAGCGCGCCCGGCTATTCTCGCAGCTCACC ATGG AGGTAAGCCCTTGCATTG-3'
Reverse primer
5'-COETTGTGACGACGAGCGCGCGATATCATCATC ACCGCTT CCACTACCTGAACC-3'

The following primers can be used to verify successful knocking in of the tag. This example is for the GFP donor.

Table 11 ACTB N-GFP/Puro tag verification junction PCR primers

Primer type	Primer name	Sequence
Gene-specific	ACTB-N F1	GACGCCTCCGACCAGTGTTTGCC
Tag-specific	N-Puro-GFP R2	GGGTAATCGGCGAAGGCAGCGG
Tag-specific	N-Puro-GFP F2	GTCCGCCCTGAGCAAAGACCCC
Gene-specific	ACTB N R1	GATGGAGGGGAAGACGGCCCGG

For Lipofectamine™ transfection in 293FT cells, follow the standard protocols.

For Neon™ transfection with Cas9 RNPs in 293FT cells, follow the standard Neon™ RNP protocol, and we recommend using the following setting: 1150 V, 20 ms, 2 pulses.

Table 12 Positive control gRNAs and primers for other TrueTag™ kits

TrueTag™ kit type	Target gene	gRNA
Stem GFP, RFP	GFAP	5'-GAGCACAAGGATGTGATGTG-3'
Knockout Enrichment	HPRT	5'-CTGTCCATAATTAGTCCATG-3'

Tagging without the selection marker

If the intended application cannot use antibiotic selection, alternative primers may be used to generate TrueTag™ donor DNAs that will still tag the protein with GFP/RFP/BFP/YFP fusions, but will not include the sequence of the antibiotic selection marker.

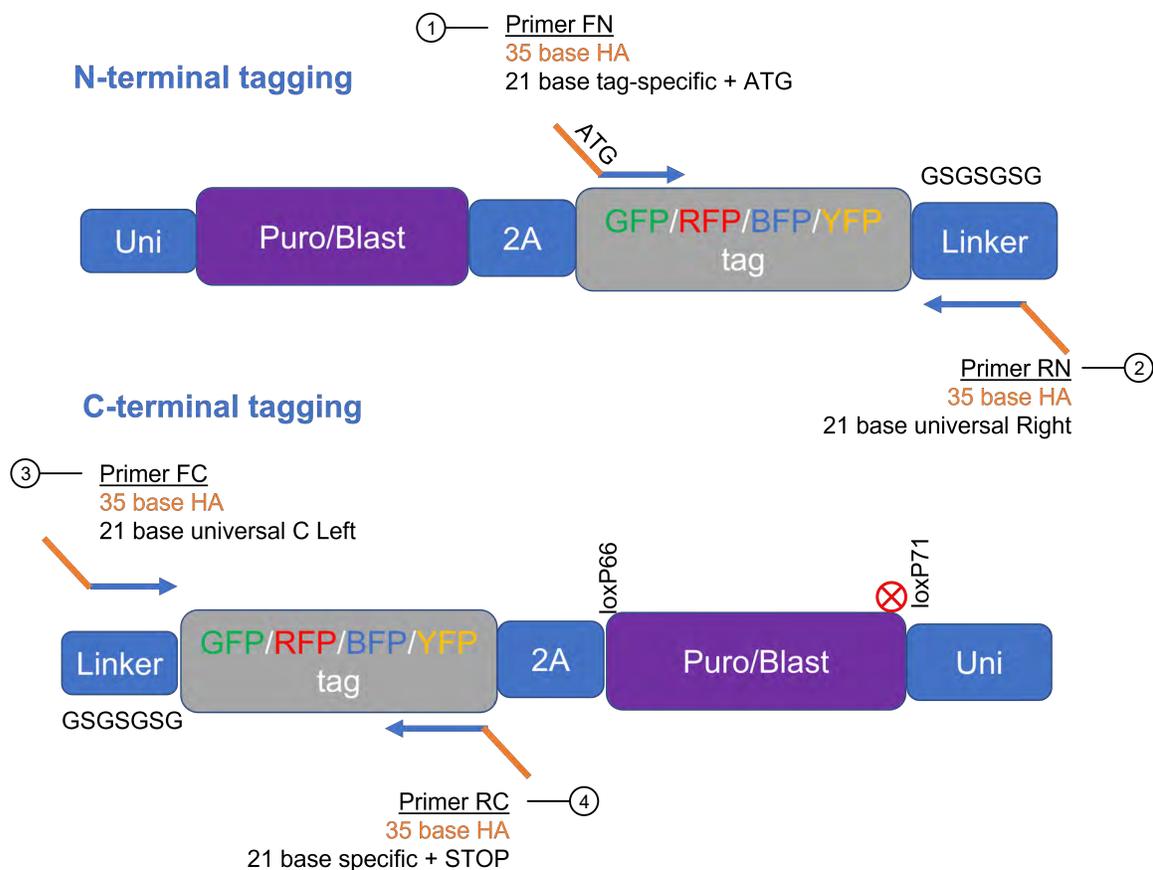
Table 13 Universal primers for tagging without the selection marker.

Donor DNA type	Primer type	Primer sequence (not included ^[1])
Amino donor	UniF-N GFP	5' ATGGTGAGCAAGGGCGAGGAGCTG 3'
	UniF-N RFP	5' ATGGTGTCTAAGGGCGAAGAGCTG 3'
	UniF-N BFP	5' ATGGTGTCTAAGGGCGAAGAGCTG 3'
	UniF-N YFP	5' ATGGTTAGCAAAGGGCGAGGAGCTG 3'
	UniR-N	5' ACCGCTTCCACTACCTGAACC 3'
Amino 2A donor	UniF-N2A	5' ATGGGAAGTGGCTCAGGTTCTGGA 3'
	UniR-N2A	5' AGGTCCAGGGTTCTCCTCCAC 3'
Carboxy donor	UniF-C	5' GGAAGTGGCTCAGGTTCTGGA 3'
	UniR-C GFP	5' TTAATTGACAGCTCGTCCATGCC 3'
	UniR-C RFP	5' TTAATTAAGTTTGTGCCCCAGTTTGC 3'
	UniR-C BFP	5' TTAATTAAGCTTGTGCCCCAGTTTGC 3'

Table 13 Universal primers for tagging without the selection marker. (continued)

Donor DNA type	Primer type	Primer sequence (not included ^[1])
Carboxy donor	UniR-C YFP	5' TTAGCGGTACAGCTCGTCCATGCC 3'
Carboxy 2A donor	UniF-C2A	5' GGAAGCGGAGCTACTAACTTC 3'
	UniF-C2A	5' TTAACCGCTTCCACTACCTGAACC 3'

^[1] These primers are not included in the TrueTag™ Donor DNA kits and must be adapted with homology arm sequences before use.

**Figure 22 Fluorescent tag fusions without selection markers.**

The universal primers and linker sequences for epitope tags are identical to those for fluorescence tags.

- | | |
|--|--|
| ① Forward primer (N-terminal) | ③ Forward primer (C-terminal) |
| <ul style="list-style-type: none"> • 35-base homology arms with ATG • 21-base UniF-N tag | <ul style="list-style-type: none"> • 35-base homology arms • 21-base UniF-C |
| ② Reverse primer (N-terminal) | ④ Reverse primer (C-terminal) |
| <ul style="list-style-type: none"> • 35-base homology arms • 21-base UniR-N | <ul style="list-style-type: none"> • 35-base homology arms with endogenous STOP codon • 21-base UniR-C tag |

Table 14 Universal primers for epitope tagging without the selection marker.

Donor DNA type	Primer type	Primer sequence (not included ^[1])
Amino donor	UniF-N DDK	5' ATGGACTACAAGGACCACGACGGT 3'
	UniF-N HA	5' ATGGCCTACCCATACGATGTTCT 3'
	UniF-N Myc	5' ATGCAGAAGCTGATCTCAGAGGAG 3'
	UniF-N His	5' ATGCACACCGGTCACCACCACCAC 3'
	UniR-N	5' GGTCAGGTAGTGAAGCGGT 3'
Carboxy donor	UniF-C	5' GGAAGTGGCTCAGGTTCTGGA 3'
	UniR-C DDK	5' TACAAGGACGACGACGACAAGTAA 3'
	UniR-C HA	5' GTTCCAGATTACGCTCCGGCCTAA 3'
	UniR-C Myc	5' CTGATCTCAGAGGAGGACCTGTAA 3'
	UniR-C His	5' GGTCACCACCACCACCACCTAA 3'

^[1] These primers are not included in the TrueTag™ Donor DNA kits and must be adapted with homology arm sequences before use.

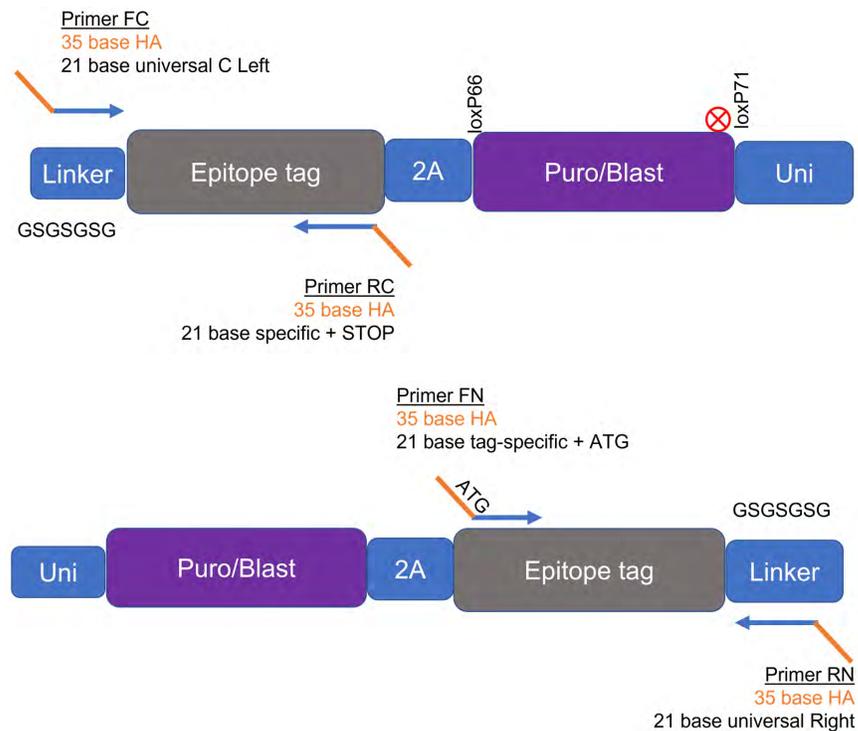


Figure 23 Epitope tag fusions without selection markers.

Upper diagram: C-terminal epitope tagging template. Lower diagram: N-terminal epitope tagging donor template.

If antibiotic selection is not desirable, alternative primers may be used to generate TrueTag™ donor DNAs that will still tag the endogenous gene with the epitope of choice.

Table 15 Universal primers for stem donor tagging without the selection marker.

Donor DNA type	Primer type	Primer sequence (not included ^[1])
GFP/RFP fusion or non-fusion	UniF-C	5' GGAAGTGGCTCAGGTTCTGGA 3'
	UniR-C GFP	5' TTACTTGTACAGCTCGTCCATGCC 3'
	UniR-C RFP	5' TTAATTAAGTTTGTGCCCCAGTTTGC 3'

[1] These primers are not included in the TrueTag™ Donor DNA kits and must be adapted with homology arm sequences before use.

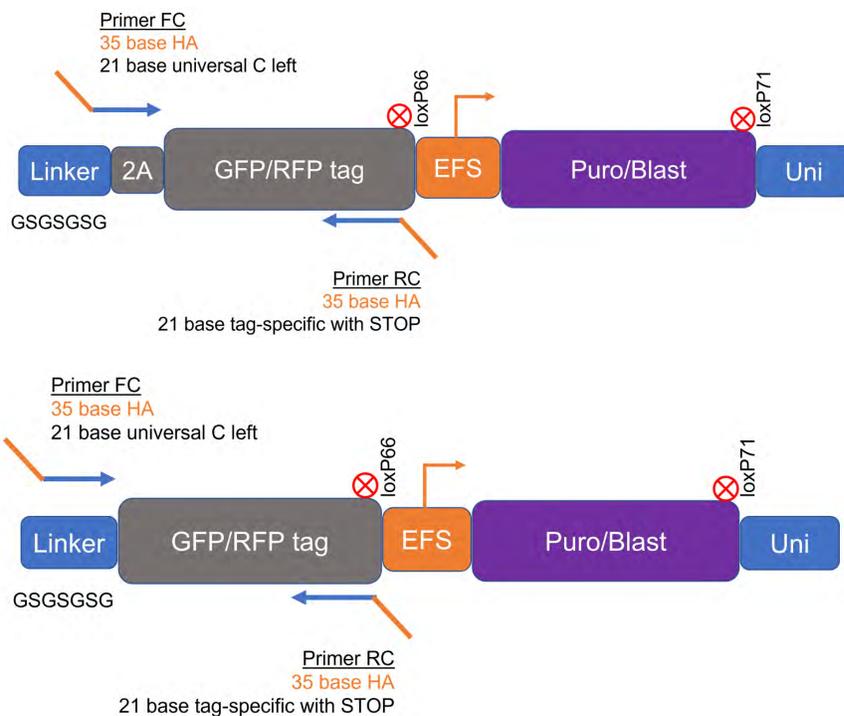


Figure 24 Stem tag without selection markers.

Upper diagram: Non GFP/RFP fusion. Lower diagram: GFP/RFP fusion.



Transfection tables

Cell line-specific transfection conditions using Lipofectamine™ CRISPRMAX™ Transfection Reagent

These conditions are a recommended starting point for transfection optimization in your cell line of interest when using Cas9 protein + sgRNA. Further optimization of the transfection conditions may be necessary for best results.

Cell type	Media	Cell seeding density/well (× 10 ³) one day before transfection			TrueCut™ Cas9 Protein v2/gRNA (ng/pmol)			Lipofectamine™ Cas9 Plus™ Reagent/well (μL)			Lipofectamine™ CRISPRMAX™ Reagent/well (μL)			
		Well format	—	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well
HEK293 [1]	DMEM		18	90	450	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.4	2	10
U2OS [2]	McCoy5A		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5
A549 [3]	DMEM		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5
THP1 [4]	RPMI		10	50	250	400/ 2.4	2000/ 12	10000/ 60	0.8	4	20	0.3	1.5	7.5
K562 [5,6]	RPMI		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5
iPSC [5,7]	Essential 8™ Medium		8	40	200	300/ 2	1500/ 10	7500/ 50	0.6	3	15	0.3	1.5	7.5
HepG2 [8]	DMEM		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5



(continued)

Cell type	Media	Cell seeding density/well (× 10 ³) one day before transfection			TrueCut™ Cas9 Protein v2/gRNA (ng/pmol)			Lipofectamine™ Cas9 Plus™ Reagent/well (μL)			Lipofectamine™ CRISPRMAX™ Reagent/well (μL)			
		Well format	—	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well
MDA- MB231 ^[9]	DMEM		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5
N2A ^[10]	DMEM		10	50	250	250/ 1.5	1250/ 7.5	6250/ 37.5	0.5	2.5	12.5	0.3	1.5	7.5

^[1] Source: Human embryonic kidney^[2] Source: Human osteosarcoma^[3] Source: Human epithelial lung carcinoma^[4] Source: Human peripheral blood monocyte leukemia^[5] Use the Neon™ Transfection System for higher editing efficiency.^[6] Source: Human leukemia bone marrow^[7] Source: Human induced pluripotent stem cells^[8] Source: Human hepatocellular carcinoma^[9] Source: Human epithelial (breast) adenocarcinoma^[10] Source: Mouse brain neuroblastoma

Neon™ transfection table

We recommend the Neon™ protocol optimization to find the best conditions for delivering donor DNA. We usually observe that the higher voltage conditions work better for delivery of the donor DNA but have lower viability.

Cell type	Media	Number of cells/10- μ L reaction ($\times 10^3$)	TrueCut™ Cas9 Protein v2/gRNA (ng/pmoles)	Neon™ electroporation conditions ^[1]
Well format	—	24-well		
HEK293	DMEM	150	1250/7.5	1150 V/20 ms/2 pulses
U2OS	McCoy5A	150	1250/7.5	1400 V/15 ms/4 pulses
A549	DMEM	120	1250/7.5	1200 V/20 ms/4 pulses
THP1	RPMI	200	2000/12	1700 V/20 ms/1 pulse (#5)
K562	RPMI	200	1250/7.5	1700 V/20 ms/1 pulse (#5)
iPSC	Essential 8™ Medium	80	1500/10	1200 V/20 ms/2 pulses (#14)
iPSC	StemFlex™ Medium	80	1500/10	1200 V/30 ms/1 pulse (#7)
Human primary T cell	OpTmizer™ + 2% human serum	200	1250/7.5	1600 V/10 ms/3 pulses (#24)
Jurkat T cell	RPMI	200	1250/7.5	1700 V/20 ms/1 pulse (#5)
HepG2	DMEM	120	1250/7.5	1300 V/30 ms/1 pulse (#8)
N2A	DMEM	100	1250/7.5	1400 V/30 ms/1 pulse (#9)

^[1] Recommendations for the Neon™ electroporation settings are based on the culture conditions tested.



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit [thermofisher.com/support](https://www.thermofisher.com/support).

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
<https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf>
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
www.who.int/publications/i/item/9789240011311



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References

Liang X, Potter J, Kumar S, Ravinder N, Chesnut JD. "Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA." *PubMed*, 2017, <https://pubmed.ncbi.nlm.nih.gov/27845164>.

