GenTarget Inc

7930 Arjons Drive, Suite B
San Diego, CA 92126, USA
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Email: Orders@gentarget.com

Lentiviral CRISPR guild RNA Cloning Kit

for constructing CRISPR targeting gRNA lentivectors

Cat#	Product Name	Amount	Application
gRNA-H1-GB	pLenti-gRNA-H1-(**GFP**-**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **GFP**-Blasticidin dual selection marker
gRNA-H1-GP	pLenti-gRNA-H1-(**GFP**-**Puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **GFP**-Puromycin dual selection marker
gRNA-H1-RB	pLenti-gRNA-H1-(**RFP**-**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **RFP**-Blasticidin dual selection marker
gRNA-H1-RP	pLenti-gRNA-H1-(**RFP**-**puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **RFP**-Puromycin dual selection marker
gRNA-H1-Puro	pLenti-gRNA-H1-(**puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Puromycin** selection marker
gRNA-H1-Bsd	pLenti-gRNA-H1-(**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Blasticidin** selection marker
gRNA-H1-**Neo**	pLenti-gRNA-H1-(**Neo**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Neomycin** selection marker
gRNA-H1-**Zeo**	pLenti-gRNA-H1-(**Zeo**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Zeocin** selection marker
gRNA-U6-GB	pLenti-gRNA-U6-(**GFP**-**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with GFP-**Blasticidin** dual selection marker
gRNA-U6-GP	pLenti-gRNA-U6-(**GFP**-**Puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with GFP-Puromycin dual selection marker
gRNA-U6-RB	pLenti-gRNA-U6-(**RFP**-**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with RFP-**Blasticidin** dual selection marker

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gRNA-U6-RP	pLenti-gRNA-U6-(**RFP**-**puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **RFP**-Puromycin dual selection marker
gRNA-U6-Puro	pLenti-gRNA-U6-(**puro**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with Puromycin selection marker
gRNA-U6-Bsd	pLenti-gRNA-U6-(**Bsd**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with Blasticidin selection marker
gRNA-U6-Neo	pLenti-gRNA-U6-(**Neo**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Neomycin** selection marker
gRNA-U6-Zeo	pLenti-gRNA-U6-(**Zeo**) cloning kit	1 kit	Make CRISPR targeting gRNA lentivector with **Zeocin** selection marker

Each Kit Contents	**Amount**
One of the pre-cut, lniear gRNA lentivector: **pLenti-gRNA-H1 (Marker);** **or** **pLenti-U6-(Marker);** **(**dependent upon the product catalog #, the **Marker** is different).	10ul (10rxn)
10X gRNA oligo annealing solution	25 ul
10X ligation buffer	20 ul
T4 DNA ligase enzyme	10ul (10rxn)
Cloning control insert (ready to use): annealed Negative Control gRNA duplex : 10ul 5′- **AGCG**gtctccacgcgcagtacatt cagaggtgcgcgtcatgtaa**CAAA**	
Sequencing primer (forward reading): 5'- ggatccaatatttgcatgtcgctatg (for H1 promoter) Or 5'- ggactatcatatgcttaccg (for U6 promoter)	10ul (100 ng/ul, ~10 uM/L)
Chemical competent cells (DH5a)	10 vials (50 ul/vial x 10 tubes)

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Notes:

The clones generated from " **Cloning control insert**" will serve as universal negative control gRNA lentivector. The negative control gRNA sequence has minimal humongous to human and mouse transcripts by blast.

Storage:

The gRNA Cloning Kit is shipped on dry ice. Each kit contains all materials , enough for you to carry out **ten cloning reactions**. Upon received, stored at -20°C except, and stare the competent cells at -80°C freezer. Products stable for 6 months.

Protocol outlines:

1. Design target gRNA sequence (20bp in length) for Cas9 cutting sites in your desired genomic locus (select 2 or 3 target sequences for each locus);

2. Order two DNA oligonucleotides with the cloning overhung (top and bottom strand for each target sequence);
3. Anneal the two oligo to generate a duplex (cloning insert);
4. Clone the duplex into the provided linear **gRNA** lentivector by T4 ligation reaction;
5. transform into competent cells and grow in LB/ ampicillin plate;

6. Pick 2 colonies, mini prep the plasmid DNAs, confirm positive clone by sequencing; (**Note**: the generated gRNA plasmid can be used for transfection based CRISPR edition together with a Cas9 expression plasmid if you do not use lentivirus for CRISPR gene edition).

--The following protocol requires additional reagents (which are not included in this kit) to complete:

7. Produce the targeting gRNA lentiviurs using the gRNA lentivectors generated above. [you need 293T cell (CAT#: TLV-C), Packaging vector mixture (CAT#: HT-Pack)]

8. **For knock-Out:** apply premade Cas9 expression lentiviurs (can be purchased separately), and gRNA targeting lentivirus together into your desired cell culture or tissue, and select/sort the transduced positive cells

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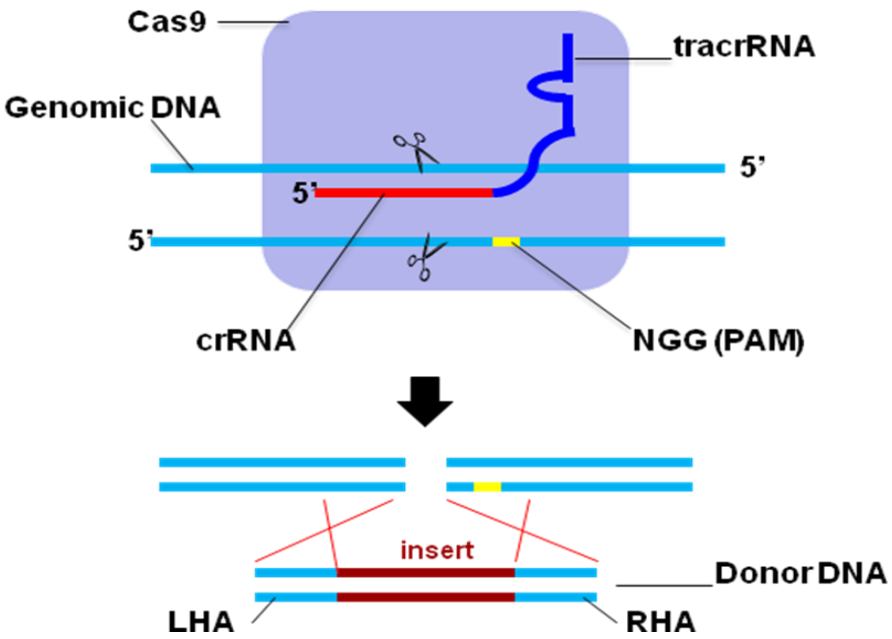
by antibiotic or fluorescent signal. The selected cells are ready for downstream gene knockout analyses (mRNA or protein detection or genomic sequencing analysis).

9. **For knock-In or sequence repair or changes:** you need deliver double stranded Donor DNA or single strand repair Oligo in addition to the Cas9 expression lentivirus and gRNA targeting lentivirus.

Introduction:

Targeted and precise genomic gene editing technologies are the tools for genomic correction, modification and gene therapy. The TALEN, ZFN and CRISPR/Cas are the three main genome editing technologies. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology was originally discovered as part of a prokaryotic adaptive immune system to protect against invading viruses and bacteriophageshas. The recent discovered CRISPR technology has many advantages: (1) higher targeting accuracy; (2) much more target sequence selection; (3) much less complexity; and (4) much less off-target cell toxicity, than the previous genome editing technologies TALEN (transcription activator-like effector nuclease) and ZEN (Zinc-finger nuclease).

Mechanism of CRISPR/Cas9 genomic editing:



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A target sequence-specific guide RNA molecule (gRNA) directs a Cas endonuclease to the genomic DNA target sequence. Then, the Cas enzyme creates a double-strand break (or single cut depends on Cas enzyme types) at the target sequence that can be repaired either by Non-Homologous End-Joining (NHEJ), which can result in insertion or deletions (InDels), or by Homology Directed Repair (HDR) when a repair Donor DNA template is present. InDels can disrupt expression of the target gene (So called, Knock-Out). While repaired by HDR, the genome then was modified with a foreign gene (So called, Knock-In), or corrected of the target gene (targeted gene therapy). Cas9 is the most frequently used cas endonuclease in CRISPR. See the illustration scheme below for the CRISPR's mechanism.

CRISPR/Cas9 technology requires three components:

1. **Target specific guild RNA (gRNA):** it comprises two segments: a targeting sequence (crRNA) containing the target complementary RNA, and an auxiliary trans-activating non-coding RNA sequence (tracrRNA or scaffold). The crRNA and tracrRNA are combined into a single guide RNA (gRNA) which directs the Cas9 nuclease to the target sequence.

   Gentarget developed **a set of gRNA lentivectors** (with different selection markers) for deliver the target specific gRNAs. You first design / select the target sequences, the crRNA region, and then synthesize the oligo and cloned into Gentarget's gRNA lentivector that contains the tracrRNA (the scaffold). The generated gRNA lentivectors can be used for transfection (Lipid) based CRISPR editing, or further produce gRNA lentivirus for virus based CRISPR editing. The gRNA is driven by either human U6 promoter, or by modified H1 promoter (an optional inducible promoter). A antibiotic marker or antibiotic-fluorescent fusion dual marker is expressed under the Rsv promoter. see the cloning scheme below.

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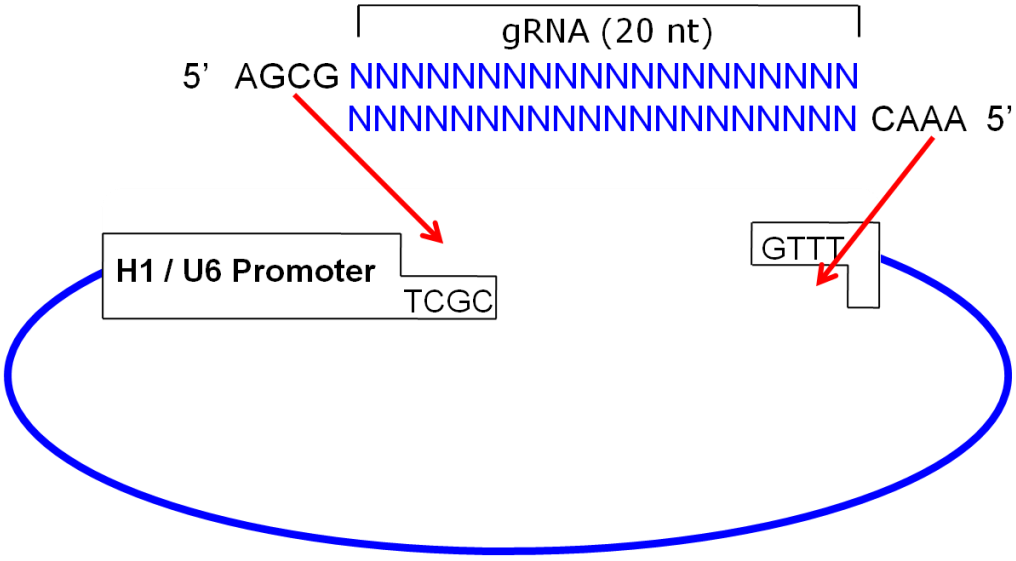
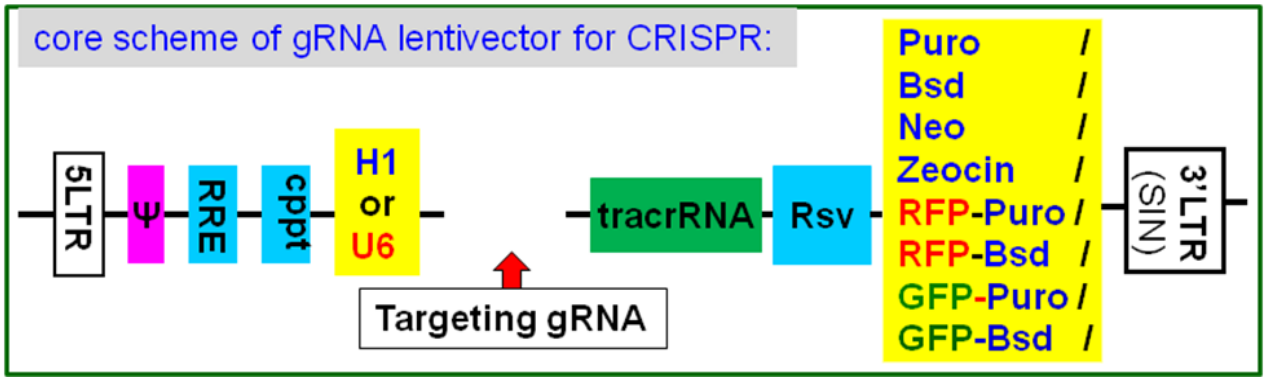
Cloning Scheme



gRNA lentivector schemes



2. **Cas9 endonuclease:** The co-existence of the gRNA sequence with Cas9 enzyme leads to the formation of a gRNA-Cas9 complex that will bind to and cleave the corresponding genomic DNA target sequence. Cas9 and gRNA can be constructed in one vector (so called All-in-One CRISPR vector). However, because of the vector's size, the All-in-one vector greatly increase the tranfection based delivery or greatly decrease the lentivirus titer for virus based delivery. Thus, the separating Cas9 expression from guild gRNA (the two vector system), has some advantages, such as,

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(1) the Cas9 can be pre-made as standalone products (like GenTargt's Cas9 expression lentivirus and Cas9 Adenovirus) which makes the delivery of Cas9 enzyme more efficient and more reliable;

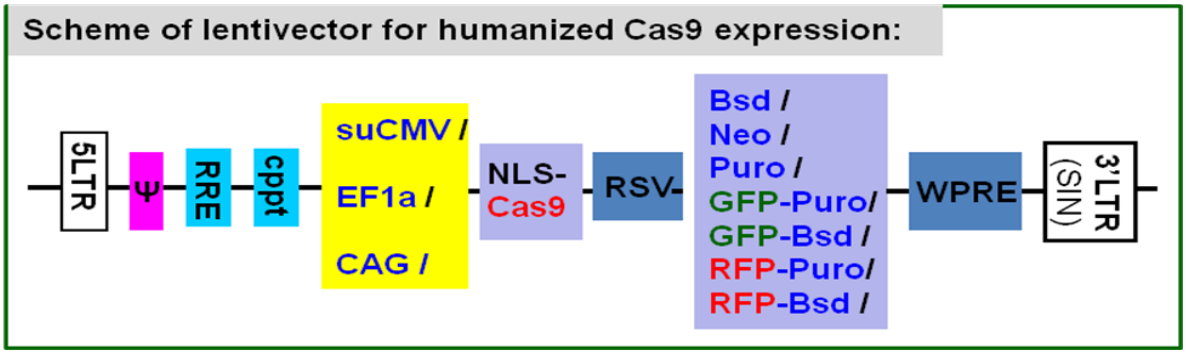
(2) One standalone Cas9 enzyme can used together with multiple gRNAs for editing multiple targets without over delivery Cas9 enzyme.

(3) it is much easier to construct and deliver the much smaller gRNA vector without the large Cas9 segment in the vector;

(4) the standalone gRNAs give you the flexibility to pair with different types of Cas enzymes (like the cas9 mutants with single cut/nicking in one strand of genome for better off-target effects) for optimized efficacy;

(5) the standalone Cas9 expression can make your cell line or animal as "CRISPR ready" which can used for multiplex gene editing and more flexible for any combination of "Editing on Demand").

Gentarget provide standalone Cas9 expression lentivirus with different antibiotic markers which provide great flexibility to combine a gRNA lentivirus with a different marker for double selection when desired. The humanized wild-type Cas9 enzyme is expressed under different promoters with two optimized nuclear localization signal leaders, demonstrating the highest CRISPR efficiency. See the Cas9 expression lentivirus core structure scheme below.



3. **The Donor DNA template ("knock In"):** For genomic modification application, a double strand or single stranded repair DNA is required after the Cas9 creates the double stranded breaks at desired genomic loci. (**Note**: when this Donor DNA is not present, the CRISPR only creates the knockOut via random non-humongous end jointing of the cleavage ends).

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The donor DNA provides the desired sequence insertion that flanked by the gene loci's homology sequences: left homologous arm (LHA) and right homologous arm (RHA), for the genomic editing via HDR mechanism. This Donor DNA can be single-strand or double stranded DNA which can be synthesize DNA or constructed in a vector and delivered by lipid based transfection.

gRNA lentivector cloning kits:

For CRISPR genomic editing, Gentarget provides the standalone premade cas9 expression lentivirus, and the gRNA lentivector cloning kits with which you can construct your desired target specific gRNA lentivectors (sequentially produce the gRNA lentivirus). You then apply both Cas9 lentivirus and gRNA lentivirus to achieve the target knockOut or modification /knockIn (when a donor DNA is also applied).

You can select the pLenti-gRNA lentivectors with a human **U6** or **H1** promoter to drive the gRNA, and a desired anionic / Fluorescent selection marker. see the sequence segment below:

```
                                human H1 promoter
1       GGATCCAATA TTTGCATGTC GCTATGTGTT CTGGGAAATC ACCATAAACG
        CCTAGGTTAT AAACGTACAG CGATACACAA GACCCTTTAG TGGTATTTGC
                    TRBS                              TRBS
              ~~~~~~~~~~~~~~~~~~~~~~~~          ~~~~~~~~~~~~~~~~~~~
51      TGAAATCCCT ATCAGTGATA GAGACTTATA AGTTCCCTAT CAGTGATAGA
        ACTTTAGGGA TAGTCACTAT CTCTGAATAT TCAAGGGATA GTCACTATCT

                 Transcription start
        ~~                              gRNA Scaffold
101     GA          GTTTTAGAGCTAGA AATAGCAAGT TAAAATAAGG CTAGTCCGTT
        CTTCGC            ATCTCGATCT TTATCGTTCA ATTTTATTCC GATCAGGCAA

         ATCAACTTGA AAAAGTGGCA CCGAGTCGGTGC
         TAGTTGAACT TTTTCACCGT GGCTCAGCCACG

TRBS: Tetracycline Repressor Binding Site.
```

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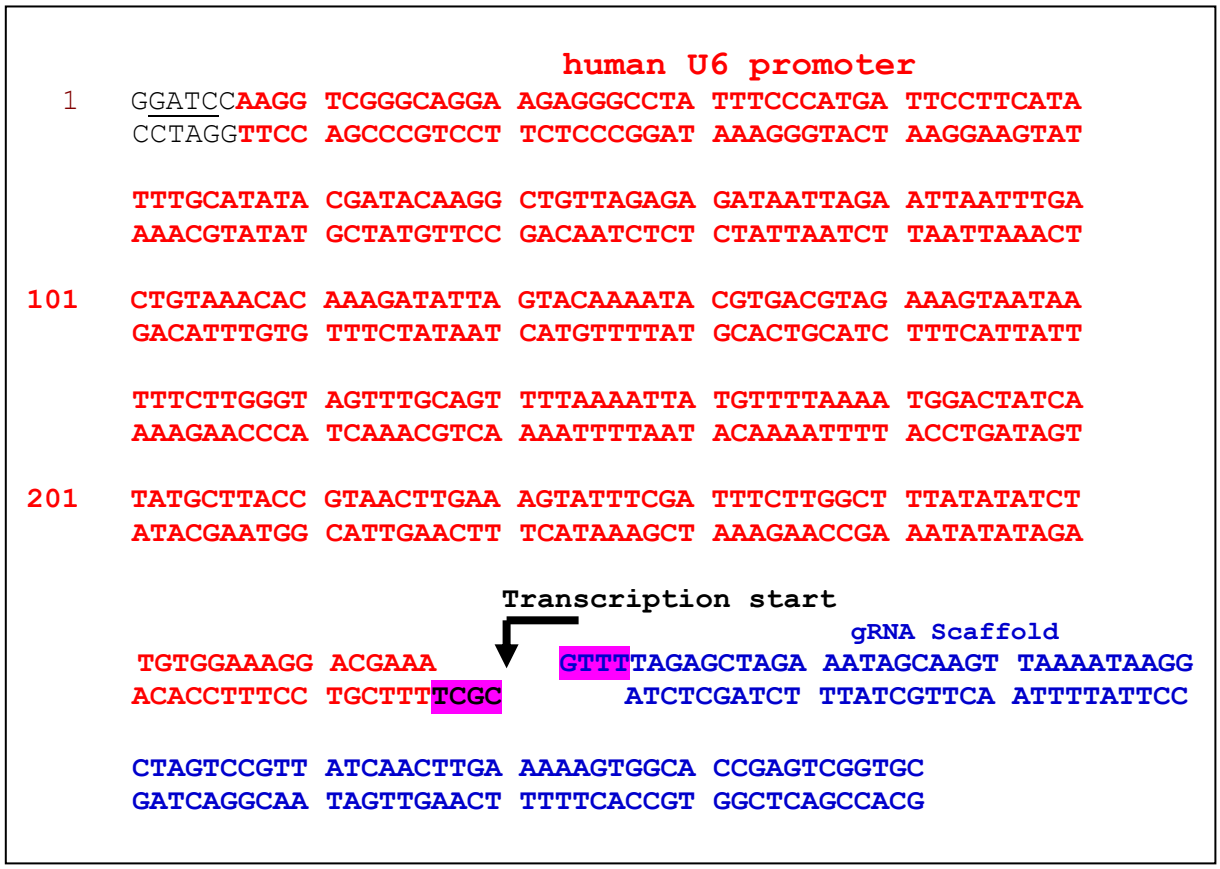

Note: The lentivector's backbone sequences can be **downloaded** from our website. The full lentivector sequence will be provided upon request after the purchasing. To make the final clone's map, simply paste gRNA target sequence (not include the 4 bp overhangs at both ends) at position between 106 and 107 for H1 promoter vector, or between 270 and 271 for U6 promoter vector.

Key features of gRNA lentivector cloning kit:

1. **Pre-linearized lentivector**--no need for tedious works at preparation of lentivector backbone,
2. **Precisely directional cloning** of your DNA duplex encoded gRNA structure,
3. **Rapid, highly efficient cloning** with very low background. Clones has >95% positive rate.
4. **Internal fluorescent reference (for some gRNA lentivectors)**: the vector encodes a fluorescent protein (**GFP** or **RFP**), allowing real-time monitoring of transfection or virus transduction efficiency.
5. **Desired selection**: the lentivector encodes an antibiotic marker or a **dual marker** (a fluorescent-antibiotic fusion marker), allowing to select the positive transduced cells via a desired

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antibiotic marker, like, Neomycin, Puromycin, Blasticidin, Zeocin, or sort via GFP / RFP fluorescent signal.

6. **Optional inducible knockdown (only for H1 promoter)**: The gRNA lentivectors driven by the optional inducible H1 promoter can be used for constitutive high expression without the need for induction. Optionally, this modified H1 promoter allows the inducible expression of gRNA when the tetracycline repressor protein (TetR) in present in advance (see inducible expression link for its mechanism).

Cloning procedure:

1. **Design gRNA sequences:**

   The gRNA sequence is 20 bp sequence complementary to the target. The effective target sequence region is 20bp target sequence upstream of a trinucleotide sequence (5'-NGG-3') called the protospacer adjacent motif (**PAM**). The wild-type Cas9 nuclease digests both strands of the genomic DNA at 3 to 4 nucleotides at 5' of the PAM sequence.

   The target sequence (20bp) can be in either the sense or anti-sense orientation with respect to the target gene. It is a good idea to create several target sequences for your gene of interest and to select sequences with minimal homology to other genes. There are many online CRISPR gRNA designing tool (see below links), for designing the target sequence: 5' "(20 bp target sequence) + PAM (NGG)"

   https://chopchop.rc.fas.harvard.edu/
   http://zifit.partners.org/ZiFiT/Introduction.aspx
   http://crispr.mit.edu/
   http://www.e-crisp.org/E-CRISP/designcrispr.html

   - the selected sequences are in front of the NGG in genomic sequence, but NGG should not be included in the synthesized gRNA oligo.
   - you can design multiple gRNA targeting sequence to lead Cas9 make specific cut in double-strand genomic DNA where the PAM is located. Thus, the break DNA ends will be repaired either by non-homologous end joining that create deletion, early stop

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(resulted in a target gene knockOut) or by homology-directed recombination with introduced donor DNAs (resulted in knockIn or correction).

2. **Anneal gRNA duplex:**
synthesize the top and bottom gRNA oligos with cloning overhung,
Top strand: 5'- **AGCG**-nnnnnnnnnnnnnnnnnnn
Bottom strand: 5'- **AAAC**-nnnnnnnnnnnnnnnnnnn

anneal the two oligo to generate a duplex, the cloning insert.

Set up the annealing reaction as follows:

100 µM Top strand oligo:	10 µl
100 µM Top strand oligo:	10 µl
10X oligo annealing buffer:	3 µl
DNase-free water:	7 µl
Total volume:	30 µl

Incubate the reaction mixture at 95°C for 5 minutes (can be done in PCR machine). Leave the mixture on the PCR machine to gradually cool down for **30 minutes**. The, put tube on ice. Make 1: 1000 dilution (first add 1µl annealed mixture in 99 µl Cold-1x annealing solution, and then take 2 µl, added into 18µl of 1x annealing solution on ice). Final diluted annealed duplex is ready for ligation. Save undiluted duplex at -20oC for long term storage. (**Note:** always put diluted, annealed duplex on ice / water to avoid double strand DNA melt.)

3. **Ligase the gRNA duplex into linearized lentivector:**
Set up the ligation reaction as follows:

pLenti-gRNA- linear vector (provided):	1 µl
Annealed duplex (1: 1000 diluted):	1 µl
5 X T4-ligase buffer:	2 µl
DNase free water:	5 µl
T4 ligase:	1 µl
Total volume:	10 µl

Mix reaction well and incubate for 30-60 minutes at room temperature (22°C to 25°C). Place reaction on ice and ready for transformation.

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Set up a **positive cloning control reaction** by using **1 µl** of annealed Control-gRNA duplex (provided). Set no-insert negative control reaction using pure water.

4. **Transformation:**
    1. Transfer all 10ul of the ligation reaction into a vial of DH5a chemically competent *E. Coli* cells, mix gently.
    2. Place cells on ice for 5 minutes, and then transfer cells into 42 °C water bath, incubate for 40 seconds without shaking. Immediately transfer cells to ice.
    3. Add 250 µl of SOC medium; incubate at 37 °C for 1 hour with shaking.
    4. Spread all 250~300 µl of transformation onto a pre-warmed LB plate containing 100 µg/ml **ampicillin** and incubate overnight at 37 °C

    Note: In general, 5-100 colonies will be produced from your reaction and 0 to 5 colonies from the no-insert control reaction, depends on your competent cell's efficiency.

5. **Confirm positive clones by direct sequencing:**
    - Pick 1-2 colonies, grow in LB/ampicillin medium, isolate plasmid DNAs by miniprep column, and sequence analysis using the sequencing primer provided. **Note:** The provided primer is at a ready-to-use concentration of [10 um]; simply use 1µl per reaction.

    - Purified lentivector plamsid DNAs are ready for transfection into cells, or for use in producing gRNA lentivirus in packaging cell lines. The generated gRNA lentivirus can be used to transduce cell lines for CRISPR assay (Need used with Cas9 expression lentivirus which can be purchased as off-shelf products).

6. **Production of gRNA lentivirus**:
    Note: GenTarget's pLenti-gRNA vectors are fully compatible with most commercially available lentiviral systems including ViraPower / Block-it (Thermofisher), MissionShRNA (Sigma), Lent-X (Clontech), etc. The following protocol is recommended for the highest virus titer using GenTarget's lentiviral reagents.

    - **Cells**: seed 293T packaging cells (cat# **TLV-C**) in plate or flask according virus production scale; incubate overnight in 5% CO2.

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- **Transfection**: at the time for transfection, cells should grow to 90% confluence. Use your favorite transfection protocol according to the transfection reagent manual to co-transfection of gRNA lentivector and **packaging plasmid mixture (CAT#: HT-pack)**.

- The next day, remove the medium and replace it with complete culture medium.

- Harvest viral supernatants at 48-72 hours after transfection.
- Centrifuge virus particles at 3000 rpm x 15 minutes at 4 ^{o}C to pellet cellular debris. Filter through a sterile 0.45 μm filter.

- Store virus at -80 ^{o}C.

7. **Apply both gRNA lentivirus and Cas9 lentivirus to the desired cell culture for CRISPR gene editing applications.**

Trouble shooting:

Problems	Solution
Few or no colonies	1. Make freshly annealed duplex and dilution for ligation reaction, 2. Use high efficient competent cells if available.

Lentiviral gRNA cloning service:

GenTarget offers a cost-effective **gRNA cloning services**. Simply tell us the target you want to editing or provide us with your own gRNA sequences, and we will design the gRNA for your target and clone the gRNA sequence into our gRNA lentivectors with the promoter and marker of your choice, and produce the gRNA lentivirus. We will deliver both sequence-verified gRNA plasmids and the pre-made gRNA lentivirus. For the cas9 part, you simply order them from our catalog products. Our service has the fastest turnaround time and lowest costs available. Please **contact us** for quote.

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Attachment: GenTarget's pre-made lentivirus product categories.

Product Category	Product Description (please click into each category's page)
Pathway Reporter	Lentivirus for all kinds of pathway assays
Cell Immortalization	Lentivirus for cell immortalization: Large T-antigen, hTERT, EBNA1/EBNA2, HpV16-E6/E7, Adenovial E1A, Kras_G12V, HOXA9, et al.
ImmunoOncology Research	Lentivirus products for immuno therapy research, CAR-T, TCR-T, Assay cell lines, and Cell Antigens & Receptors.
CRISPR Gene Editing	Preamde lentivirus express humanzied wild-type **Cas9** endonuclease, the **dCas9**, gRNAs, **CRISPR** gene editing research
Cell-Specific Reporter	a set of reporter lentiviruses to express a luminescence or fluorescent reporter (firefly Luciferase, Renilla luciferase, RFP or GFP fluorescent marker) under a tissue specific promoter
Infectious Antigens	Llentivirus that express all kinds of infectious antigens with C-term 6His-tag.
Virus Like Particles (VLP)	Lentiviral Like Particles, pseudo-typed with a different envelope proteins.
Non-integrating LV	Integration Defective Lentivirus, express different targets for transient expression without the unwanted insertional mutagenesis.
shRNA Knockdown	Knockdown verifeid and customized shRNA lentivirus for target knockdown,
microRNA lentivirus	Premade lentivirus expression human or mouse **precursor miRNA**. And **anti-miRNA** lentivector and virus for human and mouse miRNA.
Anti-miNA lentivirus	Pre-made lentivirus expression a specific anti-miRNA cassette.
Human and mouse ORFs	Premade lentivirus expressin a **human, mouse or rat** gene with RFP-Blastididin fusion dual markers.
Luciferase expression	Premade lentivirus for all kinds of luciferase protein expression**: firefly and Renilla, Red-Luc and more,** with different antibiotic selection markers.

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Fluorescent Markers	Lentivirus express all commonly used fluorescent proteins: GFP, RFP, CFP, BFP YFP, niRFP, unstable GFP and others.
Luminescent Imaging	Lentivirus express Nano-Latern as Bio-probes for in vivo imaging of sub-cellular structural organization and dynamic processes in living cells and organisms
Cytoskeleton Imaging	A fluorescent marker (GFP, RFP or CFP) fusion with a cellular structure protein, provides a convenient tool for visualization of cytoskeletal structure
Unstable GFP	Lentivirus express the the destabilized GFP (uGFP) which provides fast turnover responses in signal pathway assay and in knockdown / knockout detection
near-infrared RFP	The near-infrared Red fluorescent (niRFP) expression Lentiviurs provides the whole-body images with better contrast and brighter images
Fluorescent-ORF fusion	Pre-made lentivirus expression a "**GFP/RFP/CFP-ORF**" fusion target.
CRE recombinase	Premade lentivirus for expressing **nuclear permeant CRE** recombinase with different flurescent and antibiotic markers.
LoxP ColorSwitch	Premade lentivirus expressing "LoxP-GFP-Stop-LoxP-RFP" cassette, used to monitor the CRE recombination event in vivo.
SEAP Reporter	lentivirus expressing SEAP under different promoters (TetCMV, EF1a, CAG, Ubc, mPGK, Actin-beta or a signal pathway responsive promoter),
TetR Repressor	Premade lentivirus expressin TetR (tetracycline regulator) protein, the repressor protein for the inducible expression system.
rtTA Expression	rtTA binds to the tetracycline operator element (TetO) in the presence of doxycycline (Dox). Used for Tet-On /OFF inducible system.
iPS factors	Premde lentivirus for human and mouse iPS **(Myc, NANOG, OCT4, SOX2, FLF4**) factors with different fluorescent and antibitoic markers
LacZ expression	Express different full length **β- galactosidase (lacZ)** with different selection markers
Negative control lentiviruses	Premade **negative control lentivirus with different markers**: serves as the negative control of lentivurs treatment, for validation of the specificity of any

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	lentivirus target expression effects.
Other Enzyme expression	Ready-to-use lentivirus, expressing a specific enzymes with different selection markers.
Ultra titer lentivirus	Ultra-titer lentivirus used for the hard-to-transduced cells and for in vivo manipulation of sperm cells, or stem cells.