PrecisionX™ Multiplex gRNA Cloning Kit

Cat. # CAS9-GRNA-KIT

User Manual

Store at -20°C upon receipt

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I. Introduction

The recent discovery of the CRISPR/Cas9 system has provided researchers an invaluable tool to target and modify any genomic sequence with high levels of efficacy and specificity. The system, consisting of a nuclease (Cas9) and a DNA-directed guide RNA (gRNA), allows for sequence-specific cleavage of target sequences containing a protospacer adaptor motif "NGG". By changing the gRNA target sequence, virtually any gene sequence upstream of a PAM motif can be targeted by the CRISPR/Cas9 system, enabling the possibility of systematic targeting of sequences on a genomic scale.

In order to facilitate the cloning of gRNAs into Cas9 expression vectors, SBI has developed a revolutionary cloning kit, the PrecisionX™ Multiplex gRNA Cloning Kit (Cat# CAS9-GRNA-KIT). This system allows for the cloning of **multiple gRNAs** into any Cas9/gRNA "all-in-one" expression vector or gRNA cloning vector of the customer's choice, including SBI's PrecisionX Cas9/gRNA SmartNuclease plasmids and lentivectors. The Multiplex gRNA Cloning Kit is also compatible with the most popular Cas9/gRNA cloning vectors developed in labs across the world, such as pX330, pX335, pX458, and pX459. The broad applicability of this kit across different formats of Cas9/gRNA vectors will appeal to both beginners and advanced users of CRISPR/Cas9 technology alike by simplifying the process and lead time required to generate powerful CRISPR/Cas9 constructs for their experiments.

A. Key Features

The PrecisionX Multiplex gRNA Cloning Kit provides the following advantages to the researcher:

- **Saves time and reagents - No need to clone separate constructs bearing different gRNAs**
- **Simple two-step PCR and "fusion" reaction to generate multi-cistronic constructs**
- **Compatible with all types of Cas9/gRNA expression vectors containing H1 or U6 promoters**
- **Ideal for Cas9 nickase applications requiring expression of two gRNAs simultaneously for precise targeting**
- **Single plasmid system reduces transfection variability associated with multi-plasmid transfection**
- **Facilitates the use of more advanced Cas9 targeting applications, including tandem gRNA cutting and tandem double-nicking**
- **Enables precise deletion of defined genomic segments with a single vector**

The Multiplex gRNA Cloning Kit is based on SBI's proven Cold Fusion cloning technology, which has been the standard for restriction and ligase-free cloning technology for many years, freeing customers from laborious and time-consuming traditional cloning approaches.

B. Overview of the Multiplex gRNA Cloning Kit

Addition of a gRNA cassette (H1-gRNA) into U6 vector

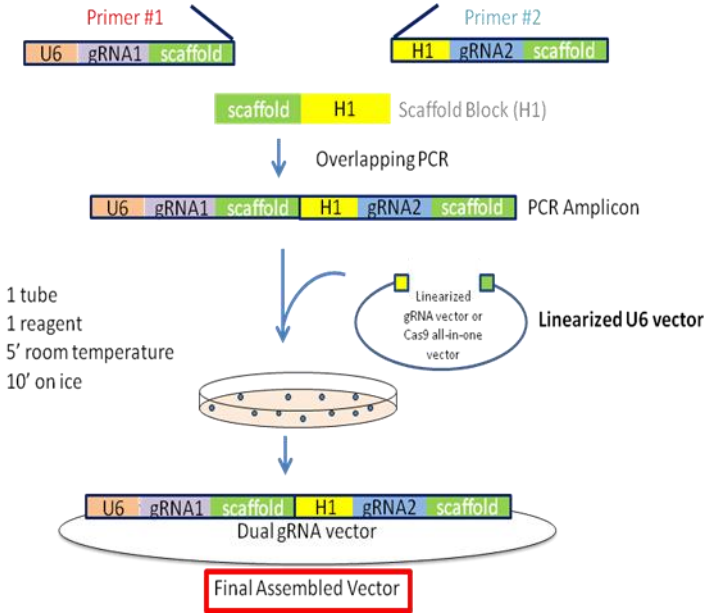


Fig. 1: General workflow of Multiplex gRNA Cloning Kit to insert H1-gRNA cassette into existing vector with U6 promoter

Addition of a gRNA cassette (U6-gRNA) into H1 vector

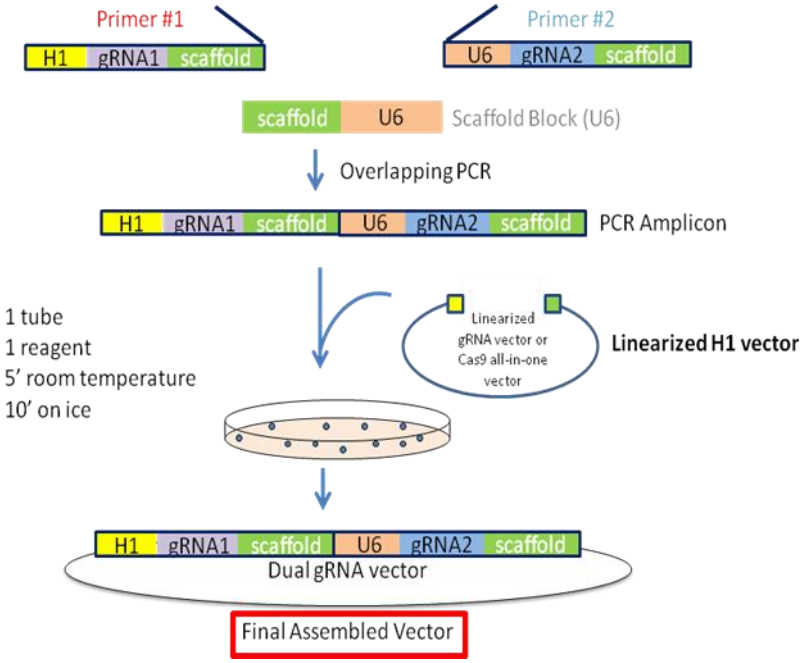


Fig. 2: General workflow of Multiplex gRNA Cloning Kit to insert U6-gRNA cassette into existing vector with H1 promoter

The multiplex cloning reaction involves two steps:

Step 1:
Overlapping PCR of the two primers (designed by the user) with a scaffold-promoter block (provided in the kit) to create a PCR product containing the desired amplicon containing both gRNAs.

Step 2:
Using the PCR amplicon created in Step 1, fuse the fragment into linearized destination vector.

This cloning process is extremely efficient, and has been demonstrated to be useful in applications such as targeting non-coding RNAs (Ho *et al.* 2014). In addition, the kit can be adapted to more than two gRNAs in single reaction. SBI has validated the cloning and fusion of 4 separate guide RNAs (Fig. 3 below) in a two-step PCR and fusion reaction into a linearized destination vector (Section II.B). The level of flexibility, ease of use, and efficiency of the Multiplex gRNA Cloning Kit is an industry-first – designed with the researcher in mind who needs freedom from limitations imposed by traditional gRNA cloning methods.

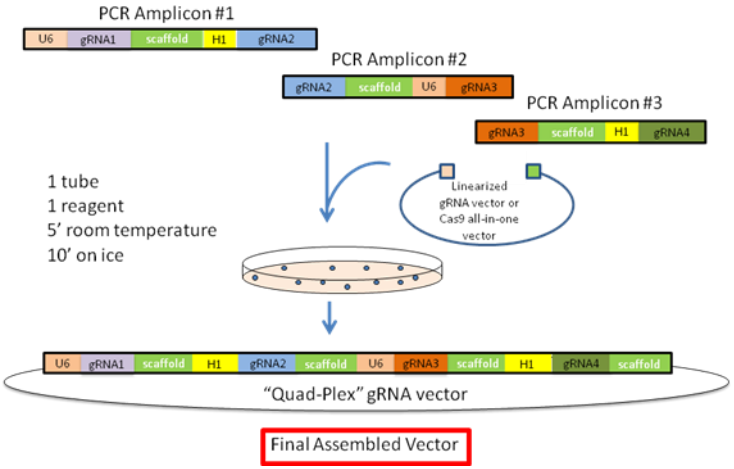


Fig. 3: Example diagram of generating a "quad-plex" multi-cistronic gRNA cassette using the Multiplex gRNA Cloning Kit

C. List of Components

Cat #CAS9-GRNA-KIT (10 reactions)

Component	Volume
5X Master mix	20ul
Linearized vector (positive control)	5ul
PCR product (positive control)	5ul
H1 Block	5ul
U6 Block	5ul

Note: Primers for the PCR reaction are sequence-dependent and will need to be designed by the end-user per guidelines in the user manual (See Section II).

D. Storage

Store the kit at -20˚C.

E. Other Reagents Needed

- Linearized Cas9/gRNA destination vector
- Vector/gRNA-specific primers (standard desalted, non-phosphorylated oligos)
- dNTPs, Phusion (recommended) or other high fidelity polymerase, and corresponding buffers for PCR
- PCR-Grade Dimethyl Sulfoxide (Cat #D9170, Sigma)
- QIAquick PCR Purification Kit (Cat # 28106, Qiagen)
- QIAquick Gel Extraction kit (Cat # 28704, Qiagen)
- SOC or LB Broth for transformation of bacteria
- LB + 50 µg/ml Ampicillin or Kanamycin plates
- High-quality competent cells (>1 x 10^9 cfus/ug DNA)

II. Protocol

A. Design of Primers for PCR reaction (For Dual gRNA cloning)

Prior to the experiment, complete forward and reverse primers for generating the desired multiplex gRNA PCR amplicon will need to be designed. Once the correct sized amplicon is generated and gel-purified, it can be used as the insert in the subsequent fusion reaction with a suitable linearized destination vector.

The general format of the PCR primers for selected Cas9/gRNA vectors is shown below for generating constructs containing H1 and U6 promoters to drive gRNA expression.

Note: For those researchers who want to use two copies of the same promoter for both gRNAs, please see the Appendix (Section IV) for primer design details.

For vectors with existing U6 promoter (e.g. Addgene pX series and SBI's CASLV U6-based gRNA cloning vectors):

Forward Primer Sequence:

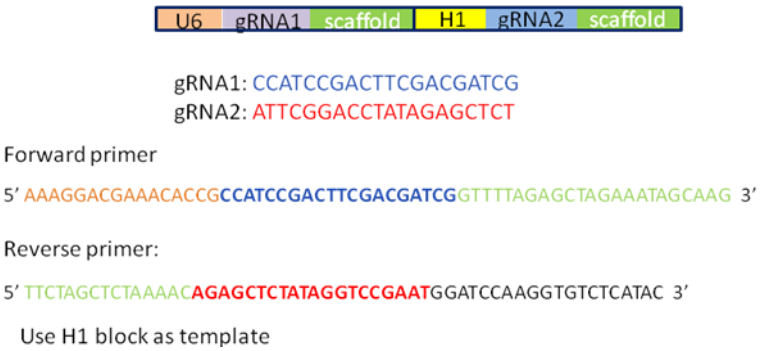
5'AAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAACXXXXXXXXXXXXXXXXXXXXGGATCCAAGGTGTCTCATAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

Here is an example of two gRNAs (gRNA1 and gRNA2) that need to be cloned in to an U6-gRNA vector to make a dual gRNA vector (U6-gRNA1-H1-gRNA2)



The **H1 scaffold block** will need to be used for the PCR reaction

For SBI's CAS8xx and 9xx vectors with H1 promoter

Forward Primer Sequence:

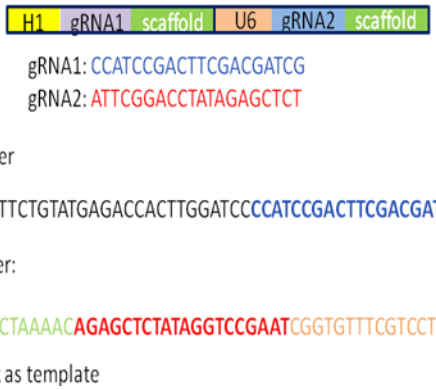
5'CTTATAAGTTCTGTATGAGACCACTTGGATCCNNNNNNNNN
NNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAACXXXXXXXXXXXXXXXXXXXXCGGTGTT
TCGTCCTTTCCAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

Here is an example of two gRNAs (gRNA1 and gRNA2) that need to be cloned into these vectors to make a dual gRNA vector (H1-gRNA1-U6-gRNA2)



The **U6 scaffold block** will need to be used for the PCR reaction

For SBI's CAS7xx and CASLVxxx vectors with H1 promoter

Forward Primer Sequence:

5'AGACACCTTGGATCCNNNNNNNNNNNNNNNNNNNGTTTT AGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAACXXXXXXXXXXXXXXXXXXXCGGTGTT TCGTCCTTTCCAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

Here is an example of two gRNAs (gRNA1 and gRNA2) that need to be cloned into these vectors to make a dual gRNA vector (H1-gRNA1-U6-gRNA2)

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT

Forward primer

5' AGACACCTTGGATCCCCATCCGACTTCGACGATCGGTTTTAGAGCTAGAAATAGCAAG 3'

Reverse primer:

5' TTCTAGCTCTAAAACAGAGCTCTATAGGTCCGAATCGGTGTTTCGTCCTTTCCAC 3'

Use U6 block as template

The **U6 scaffold block** will need to be used for the PCR reaction

B. Design of Primers for PCR reaction (For "Quad-Plex" gRNA cloning)

The scalability of the Multiplex gRNA cloning kit allows for simultaneous cloning of more than 2 gRNAs at once into a single vector. This allows researchers to perform more advanced CRISPR/Cas9 techniques such as tandem double-nicking (4 gRNAs total) which allow removal of defined genomic segments using Cas9 nickase with significantly decreased chance of off-target cutting.

The cloning of four gRNAs will require the researcher to perform three separate PCR reactions with separate primer pairs and blocks. Once the correct size amplicons are generated and gel-purified, they can be mixed at equimolar ratios (1:1:1) based on their concentrations, and used as the insert in the subsequent fusion reaction with a suitable linearized destination vector.

The general format of the PCR primers for selected Cas9/gRNA vectors for this application is the following:

For vectors with existing U6 promoter (e.g. Addgene pX series and SBI's CASLV U6-based gRNA cloning vectors):

Amplicon #1 (gRNA1 and gRNA2)

Forward Primer Sequence:

5'AAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNNGTTT TAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXGGATCCAAGGTGTCTCATAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

The **H1 scaffold block** will need to be used for the PCR reaction for generating amplicon #1.

Amplicon #2 (gRNA2 and gRNA3)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXCGGTGTTTCGTCCTTTCCAC3'

N = Denotes 15bp of gRNA2 sequence upstream of PAM X= Denotes Reverse Complement of gRNA3 sequence

The **U6 scaffold block** will need to be used for the PCR reaction for generating amplicon #2.

Amplicon #3 (gRNA3 and gRNA4)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAAC**XXXXXXXXXXXXXXXXXXX**GGATCCA
AGGTGTCTCATAC3'

N = Denotes 15bp of gRNA3 sequence upstream of PAM X= Denotes Reverse Complement of gRNA4 sequence

The **H1 scaffold block** will need to be used for the PCR reaction for generating amplicon #3.

For example, given 4 gRNAs below which need to be cloned into a single destination vector, the following primers will need to be designed to generate three separate PCR amplicons:

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT
gRNA3: TACGGCAATGCAGCATTCGA
gRNA4: CTACTGGCTAACCGTTAGCT

Forward primer-1
5' AAAGGACGAAACACCG**CCATCCGACTTCGACGATCG**GTTTTAGAGCTAGAAATAGCAAG 3'
Reverse primer-1
5' **AGAGCTCTATAGGTCCGAAT**GGATCCAAGGTGTCTCATAC3'
H1 block as template

Forward primer-2
5' **GACCTATAGAGCTCT**GTTTTAGAGCTAGAAATAGCAAG 3'
Reverse primer-2
5' **TCGAATGCTGCATTGCCGTA**CGGTGTTTCGTCCTTTCCAC 3'
U6 block as template

Forward primer-3
5' **CAATGCAGCATTCGA**GTTTTAGAGCTAGAAATAGCAAG 3'
Reverse primer-3
5' TTCTAGCTCTAAAAC**AGCTAACGGTTAGCCAGTAG**GGATCCAAGGTGTCTCATAC 3'
H1 block as template

For SBI's CAS8xx and 9xx vectors with H1 promoter

Amplicon #1 (gRNA1 and gRNA2)

Forward Primer Sequence:

5'CTTATAAGTTCTGTATGAGACCACTTGGATCCNNNNNNNNN
NNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXXCGGTGTTTCGTCCTTTCCAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

The **U6 scaffold block** will need to be used for the PCR reaction for generating amplicon #1.

Amplicon #2 (gRNA2 and gRNA3)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXXGGATCCAAGGTGTCTCATAC3'

N = Denotes 15bp of gRNA2 sequence upstream of PAM X= Denotes Reverse Complement of gRNA3 sequence

The **H1 scaffold block** will need to be used for the PCR reaction for generating amplicon #2.

Amplicon #3 (gRNA3 and gRNA4)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAACXXXXXXXXXXXXXXXXXXXXCGGTGTT
TCGTCCTTTCCAC3'

N = Denotes 15bp of gRNA3 sequence upstream of PAM X= Denotes Reverse Complement of gRNA4 sequence

The **U6 scaffold block** will need to be used for the PCR reaction for generating amplicon #3.

For example, given 4 gRNAs below which need to be cloned into a single destination vector, the following primers will need to be designed to generate three separate PCR amplicons:

For SBI's CAS7xx and CASLVxxx vectors with H1 promoter

Amplicon #1 (gRNA1 and gRNA2)

Forward Primer Sequence:

5'AGACACCTTGGATCCNNNNNNNNNNNNNNNNNNNGTTTT AGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXCGGTGTTTCGTCCTTTCCAC3'

N = Denotes gRNA1 sequence X= Denotes Reverse Complement of gRNA2 sequence

The **U6 scaffold block** will need to be used for the PCR reaction for generating amplicon #1.

Amplicon #2 (gRNA2 and gRNA3)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'XXXXXXXXXXXXXXXXXXXXGGATCCAAGGTGTCTCATAC3'

N = Denotes 15bp of gRNA2 sequence upstream of PAM X= Denotes Reverse Complement of gRNA3 sequence

The **H1 scaffold block** will need to be used for the PCR reaction for generating amplicon #2.

Amplicon #3 (gRNA3 and gRNA4)

Forward Primer Sequence:

5'NNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence:

5'TTCTAGCTCTAAAACXXXXXXXXXXXXXXXXXXXXCGGTGTT TCGTCCTTTCCAC3'

N = Denotes 15bp of gRNA3 sequence upstream of PAM X= Denotes Reverse Complement of gRNA4 sequence

The **U6 scaffold block** will need to be used for the PCR reaction for generating amplicon #3.

For example, given 4 gRNAs below which need to be cloned into a single destination vector, the following primers will need to be designed to generate three separate PCR amplicons:

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT
gRNA3: TACGGCAATGCAGCATTCGA
gRNA4: CTACTGGCTAACCGTTAGCT

Forward primer-1
5′ AGACACCTTGGATCC**CCATCCGACTTCGACGATCG**GTTTTAGAGCTAGAAATAGCAAG 3′
Reverse primer-1
5′ **AGAGCTCTATAGGTCCGAAT**CGGTGTTTCGTCCTTTCCAC 3′
U6 block as template

Forward primer-2
5′ **GACCTATAGAGCTCT**GTTTTAGAGCTAGAAATAGCAAG 3′
Reverse primer-2
5′ **TCGAATGCTGCATTGCCGTA**GGATCCAAGGTGTCTCATAC 3′
H1 block as template

Forward primer-3
5′ **CAATGCAGCATTCGA**GTTTTAGAGCTAGAAATAGCAAG 3′
U6 block as template
Reverse primer-3
5′ TTCTAGCTCTAAAAC**AGCTAACGGTTAGCCAGTAG**CGGTGTTTCGTCCTTTCCAC 3′

C. Setting up the PCR Reaction

1. Once the primer sets and the appropriate blocks needed for the reactions have been determined, set up the PCR reaction according to the table below (for 1 reaction):

Component	Volume
5X Phusion HF buffer	10 µl
10 mM dNTPs	1 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
H1 block or U6 block	0.5 µl
DMSO	1.5 µl
Phusion DNA Polymerase	0.5 µl
Nuclease-free water	To 50 µl

Note: SBI recommends the use of Phusion DNA polymerase and buffer for the PCR reaction as this combination was utilized successfully for in-house validation.

2. Run the PCR reaction according to the cycling conditions below.

Step	Temperature	Time
Initial Denaturation	98°C	3 min
30 cycles	98°C	5 seconds
	51°C	15 seconds
	72°C	15 seconds
Final Extension	72°C	10 minutes
Hold	4°C	

3. After completion of the PCR reaction, run out 2-5 µl of the PCR product on an 2% agarose gel to determine specificity of the PCR

reaction. If there are no additional bands (e.g. primer dimer, other non-specific bands) visible, the PCR reaction may be cleaned up using the QIAquick PCR Purification Kit (Cat # 28106, Qiagen), concentration measured by UV-Vis spectrophotometry, and directly used for the fusion reaction.

If primer dimers or non-specific products are seen, we highly recommend gel-purification of the correct band to avoid their carryover into the fusion reaction as these products will inhibit the efficiency of the reaction.

D. Fusion Reaction

IMPORTANT!

Complete linearization of the destination vector is critical to achieve a successful fusion reaction. Incomplete linearization of the vector will result in high background. The linearized vector can be generated by PCR (e.g. inverse PCR) or restriction enzyme digest (single or double digest) and should be purified using either a gel or PCR purification kit.

Due to the digestion efficiency, different restriction enzymes will generate different levels of background. In general, two enzyme digestion is better than a single enzyme digestion. The further the restriction sites are apart, the better the digestion efficiency. Increasing the enzyme digestion time and the digestion reaction volume will also help reduce the background. For many enzymes, we recommend incubate the digestion reaction between 3 hours and overnight in order to increase linearization and reduce background (w/exception of certain high-fidelity “HF” enzymes).

Check the background of your vector by transforming 1μl of 10-100ng/μl linearized vector into competent cells. If the background is high, continue digesting the remaining vector for a longer time after addition of more restriction enzyme(s).

We recommend digesting 2μg vector in 50μl reaction overnight. Use QIAGEN's QIAquick Spin Gel Extraction kit for gel purification and elute the DNA with 30μl dH_2O.

Set up the following reaction in a 1.5 ml sterile reaction tube by mixing the following reagents gently and then spin down briefly to collect the reagents at the bottom of the tube.

Fusion reaction

Linearized destination vector (10-100ng/μl)	1μl*
PCR insert(s) (20-200ng/μl) (for each PCR Product)	1μl*
dH_2O	_μl
5x master mix	2μl
Total	10μl

Positive control reaction

Linearized vector (positive control)	1μl
500bp PCR insert (positive control)	1μl
dH2O	6μl
5x master mix	2μl
Total	10μl

Negative control reaction

Linearized destination vector (10-100ng/μl)	1μl*
dH_2O	7μl
5x master mix	2μl
Total	10μl

* 2:1 or 1:1 molar ratio of insert: vector works well in the fusion reaction.

When using the kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your fusion reaction. The positive control 500bp PCR insert and linearized vector provided in the kit have already been

purified. There is no treatment (e.g. phosphatase, etc.) needed prior to the cloning reaction.

Fusion Reaction Incubation

1. 5 minutes at room temperature
2. 10 minutes on ice

E. Transformation of Cells

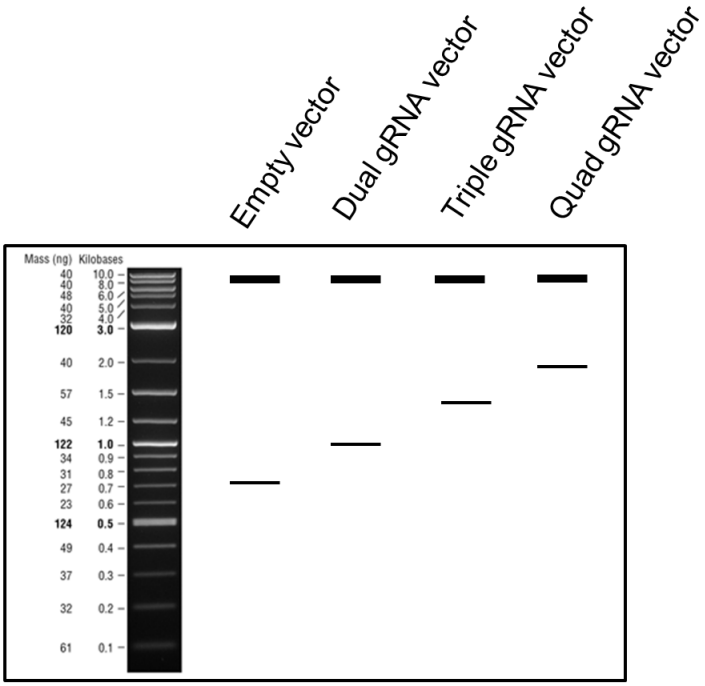
1. Add 30-50μl of competent cells (> 1 x10^9 cfus/ug DNA) to the cloning mixture per instructions provided by manufacturer of competent cells.
2. Incubate on ice for 20 minutes
3. Heat shock at 42˚C for 50 seconds
4. Transfer on ice for 2 minutes
5. Add 250μl S.O.C medium or LB broth
6. Incubate at 37˚C for an hour
7. Take 100μl culture spread on pre-warmed (37˚C) culture plate containing 50μg/ml Ampicillin or Kanamycin
8. Incubate the plate at 37˚C overnight

F. Confirmation of Positive Clones

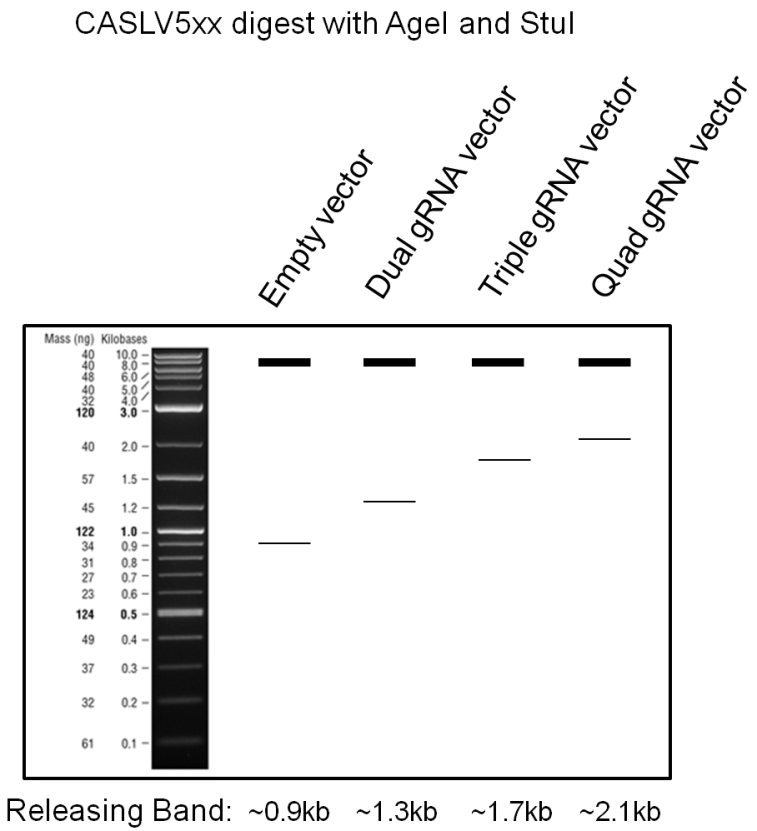
1. Pick 1 to 2 colonies, grow in LB/Kanamycin or Ampicillin medium overnight at 37°C with shaking

2. Next day, miniprep plasmid DNAs and check for proper ligation via restriction digestion using AgeI and EcoRV (for SBI's CAS7xx, CAS8xx, CAS9xx vectors), or AgeI and StuI (for SBI's CASLV5xx vectors). For non-SBI vectors, please find suitable unique restriction sites flanking gRNA cloning sites.

CAS7xx, CAS800, CAS900 digest with AgeI and EcoRV



Releasing Band: ~0.7kb ~1kb ~1.4kb ~1.8kb



The correct sized restriction product should indicate successful cloning of the multi-gRNA construct into the destination vector.

3. Send out finished construct for sequencing using appropriate sequencing primer for the construct (see Appendix for primer information).

III. Validation Data

A. Gene repair by double-nicking and HR

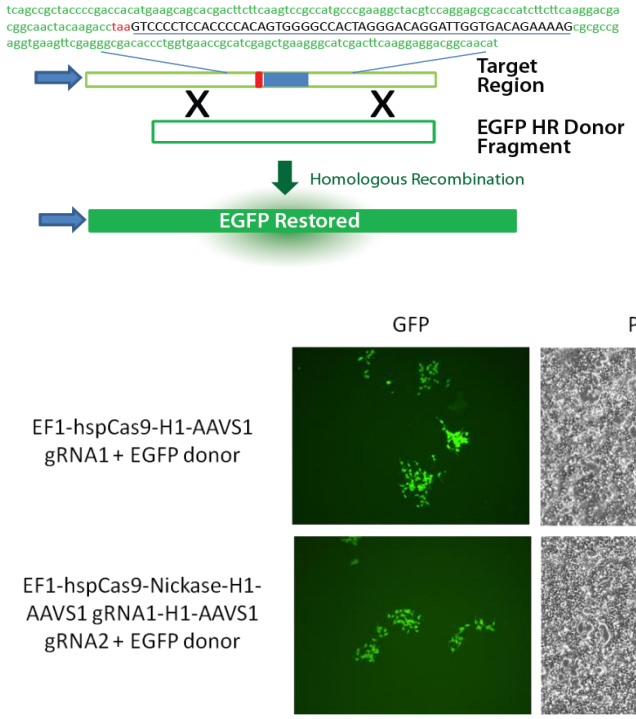
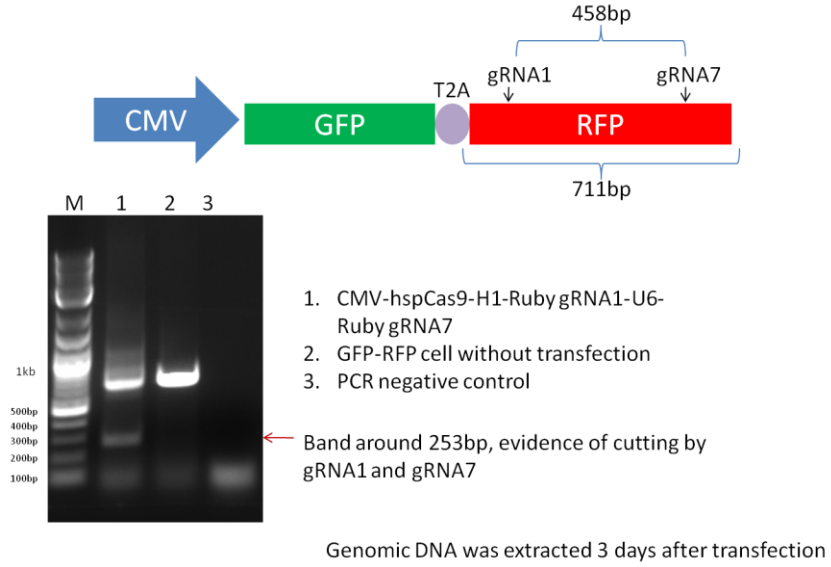


Fig. 4: Functional validation of dual gRNAs generated by the Multiplex gRNA Cloning Kit for combined double-nickase and homologous recombination to rescue EGFP in the EGIP control cell line. (EGIP cell line courtesy of Dr. Jizhong Zou, NIH CRM.)

B. Gene Knockout by dual gRNAs targeting RFP



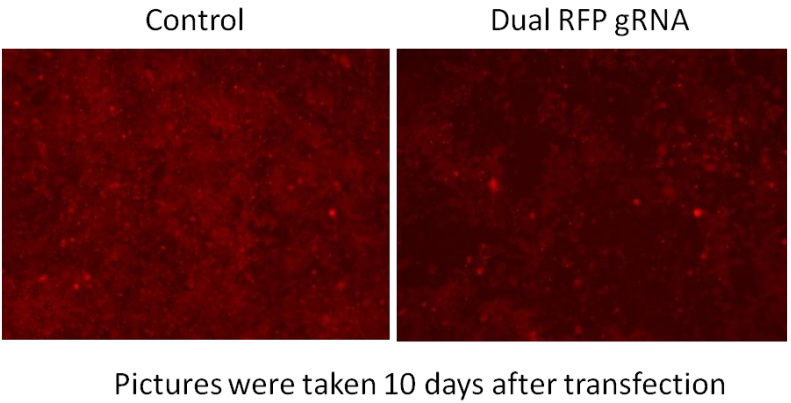


Fig. 5: Functional validation of dual gRNAs cloned into SBI's CAS940A-1 vector (CMV-hspCas9-H1-gRNA) using the Multiplex gRNA Cloning Kit to targeting RFP in a cell line stably expressing a CMV-GFP-T2A-RFP cassette. PCR assay with primers flanking

RFP indicate evidence of cutting by gRNAs, and fluorescence images of targeted cells vs control show reduction in RFP expression.

C. Tandem paired-nicking (4 gRNAs in nickase vector)

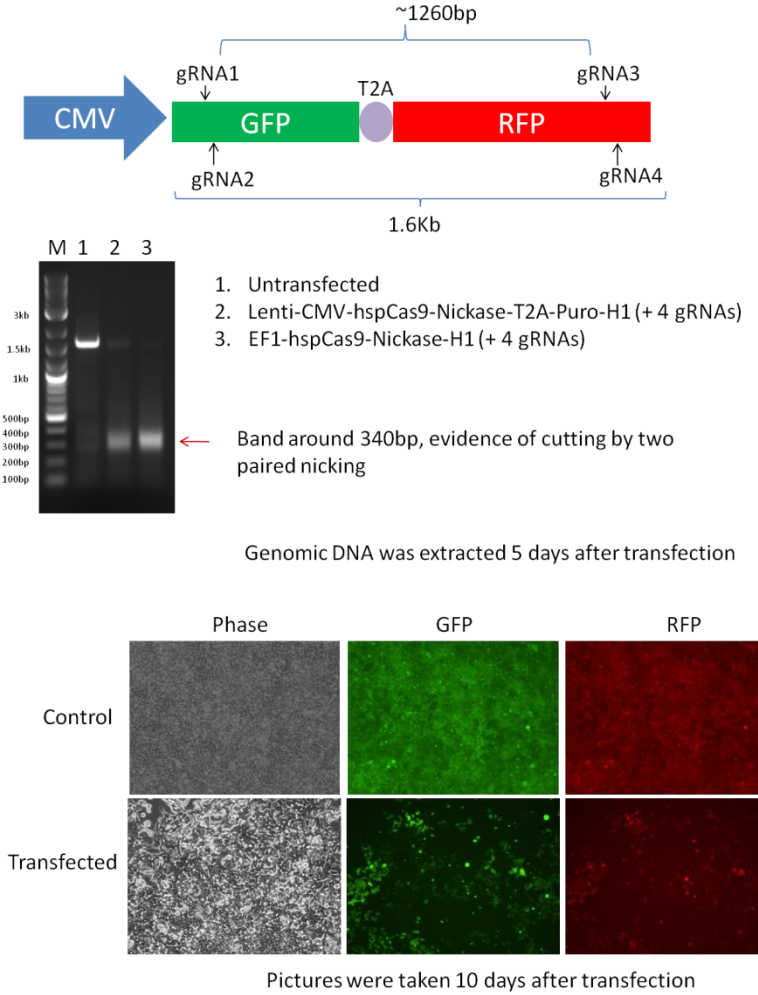


Fig. 6: Functional validation of "quad-plex" gRNAs cloned into CAS840A-1 nickase vector (EF1-Nickase-H1-gRNA) using the

Multiplex gRNA Cloning Kit for targeting GFP and RFP in a cell line stably expressing a CMV-GFP-T2A-RFP cassette. PCR assay with primers (one at 5' end of GFP and other at 3' end of RFP) indicate evidence of cutting by gRNAs. Fluorescence images of targeted cells vs control show significant reduction in both RFP and GFP expression.

IV. Appendix

A. Primer design for dual gRNA/single promoter constructs

For customers interested in having two copies of the same promoter driving both gRNAs, the following sets of PCR primers can be used to generate U6-U6 or H1-H1 constructs for a wide variety of different CRISPR/Cas9 expression vectors.

For U6-U6 constructs (e.g. Addgene pX series and SBI's CASLV U6-based gRNA cloning vectors)

Sample gRNAs to be cloned:

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT

Forward Primer Sequence (gRNA1 sequence in light blue)

5'AAAGGACGAAACACCGCCATCCGACTTCGACGATCGGTTTT
AGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence (Reverse complement of gRNA2 sequence in red)

5'TTCTAGCTCTAAAACAGAGCTCTATAGGTCCGAATCGGTGT
TTCGTCCTTTCCAC3'

Note: Use U6 Block for PCR reaction

For H1-H1 constructs (e.g. SBI's CAS8xx and 9xx vectors)

Sample gRNAs to be cloned:

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT

Forward Primer Sequence (gRNA1 sequence in light blue)

5'CTTATAAGTTCTGTATGAGACCACTTGGATCCCCATCCGACT
TCGACGATCGGTTTTAGAGCTAGAAATAGCAAG3'

Reverse Primer Sequence (Reverse complement of gRNA2 sequence in red)

5'TTCTAGCTCTAAAACAGAGCTCTATAGGTCCGAAT
GGATCCAAGGTGTCTCATAC3'

Note: Use H1 Block for PCR reaction

For H1-H1 constructs (e.g. SBI's CAS7xx and CASLVxxx H1-based vectors)

Sample gRNAs to be cloned:

gRNA1: CCATCCGACTTCGACGATCG
gRNA2: ATTCGGACCTATAGAGCTCT

Forward Primer Sequence (gRNA1 sequence in light blue)

5'AGACACCTTGGATCCCCATCCGACTTCGACGATCGGTTTTA
GAGCTAGAAATAGCAAG3'

Reverse Primer Sequence (Reverse complement of gRNA2 sequence in red)

5'TTCTAGCTCTAAAACAGAGCTCTATAGGTCCGAAT
GGATCCAAGGTGTCTCATAC3'

Note: Use H1 Block for PCR reaction

B. Sequencing primer information

The following primer sequences can be used for sequence-verification of inserted gRNAs using the Kit.

For all U6-H1 constructs:

U6 primer: 5' GGACTATCATATGCTTACCG 3'

For all H1-U6 constructs:

H1 primer: 5' GTCATCAACCCGCTCCAAGG 3'

For non-SBI U6-U6 and H1-H1 constructs:

Please find a suitable primer sequence upstream of 5' most U6 or H1 promoter based on vector sequence.

For SBI U6-U6 or H1-H1 constructs:

GRNA-PR-F: 5' GACGAGTCGGATCTCCCTTTG 3'
GRNA-PR-R: 5' CAAGTTCCTCTCACTCTCTGAT 3'

NOTE: The primer set above (GRNA-PR-F/R) is available from SBI (Cat# CAS9-GRNA-PR)

V. Troubleshooting

Problem	Probable cause	Solution
1. No or few colonies obtained from the transformation	Primer sequences are incorrect	Check primer sequences to ensure that the gRNA sequence is correct and primer ends are homologous to vector and block, respectively
	Suboptimal PCR product	Optimize your PCR amplification reactions so that you generate pure PCR products. Use a different method to purify your PCR product.
	Low DNA concentration in reaction	It is imperative to obtain as high a DNA concentration as possible in your fusion reaction.
	Inhibitory contaminants from PCR product or linearized vector	Both the PCR product and the linearized vector should be purified.
	Transform with too much reaction mixture	Do not add more than 10μl of reaction mixture to 50μl of competent cells. Too much reaction mixture inhibits the transformation.

Problem	Probable cause	Solution
	Low quality or poor handling of competent cells	Handle the competent cells gently. Do not re-freeze cells after thawing. Quality of competent cells may be tested by transforming a circular plasmid to determine cells' competency. Competent cells with a transformation efficiency of $1x10^9$ cfu/ µg are recommended.
	Wrong antibiotic or too much antibiotic in the media	Choose plates with the appropriate concentration of the right antibiotic.
2. Large numbers of colonies contain no insert	Incomplete linearization of your vector	It is critical to remove any uncut vector prior to use in the fusion reaction. If necessary, re-digest your vector and gel purify.

Problem	Probable cause	Solution
2. Large numbers of colonies contain no insert	Plates are too old or contained incorrect antibiotic	Make sure that your antibiotic plates are fresh. Check the antibiotic resistance of your fragment.
3. Clones contain incorrect insert	PCR products contain non-specifically amplified artifacts	Optimize your PCR reaction to improve the specificity. Screen more colonies for the correct clones.

VI. References

Ho *et al.* Targeting non-coding RNAs with the CRISPR/Cas9 system in human cell lines. Nucleic Acids Research 2014; Nov 20 [Epub ahead of print]

VII. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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Fax: (650) 968-2277
E-mail: General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com
Address: System Biosciences (SBI)

265 North Whisman Rd.
Mountain View, CA 94043

VIII. Licensing and Warranty

Limited Use License

Use of the PrecisionX™ Multiplex gRNA Cloning Kit (i.e., the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms. Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein. SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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