Clone-it™ Enzyme free Lentivectors

Cat. # LF520A-1: cDNA
Cat. # LF521A-1: cDNA
Cat. # LF522A-1: miRNA precursor
Cat. # LF522B-1: miRNA precursor
Cat. # LF523A-1: shRNA
Cat. # LF524A-1: shRNA
Cat. # LF527A-1: shRNA
Cat. # LF528A-1: Reporter

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 2-131008)

Contents

I. Introduction and Background

A. Purpose of this Manual

This manual provides detailed information on how to build lentiviral constructs to characterize your promoters, or to express your cDNA, precursor microRNA, or shRNA of interest using the easiest Heat'n'Mix™ Enzyme-free cloning technology. For new users of these vectors, please read the entire manual before starting. For packaging and transduction of your cloned constructs, please refer to the user manual "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells". For concentrating and titering your packaged lentivirus, please refer to the "PEG-*it*™ Virus Precipitation Solution", and "UltraRapid™ Lentiviral Titer Kit" user manuals, respectively. All these manuals are available on the SBI website (www.systembio.com).

B. Advantages of the Lentivector Expression System

Lentiviral expression vectors are the most effective vehicles for delivering your cDNA, microRNA or shRNA of interest into almost all types of mammalian model organisms, as well as both dividing and non-dividing cells (C.A. Machida, 2003; M. Federico, 2003; W. C. Heiser, 2004). As with standard plasmid vectors, it is also possible to introduce lentivector expression constructs in the plasmid form into cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lenti-construct into viral particles, you can obtain highly efficient transduction of your expression constructs, even with the most difficult-to-transfect cells, such as primary, stem, and differentiated cells. The transduced expression construct is integrated into the genome of target cells and provides stable and long-term expression of the transgene.

SBI is offering a third generation of the most popular HIV-1 based lentivector expression system, which consists of three main components:

1) The lentiviral expression vectors (*e.g.*, pPS-EF1-LCS-T2A-RFP)
2) The lentiviral packaging plasmids (*e.g.*, pPACKH1™ Packaging Plasmid mix)
3) A pseudoviral particle producer cell line (*e.g.*, 293TN cells)

The expression lentivector contains the genetic elements responsible for packaging, transduction & stable integration of the viral expression construct into genomic DNA with expression of the target gene sequence. The packaging vector provides in trans all of the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant viral particles. To produce a high titer of viral particles, expression and packaging vectors are transiently co-transfected into producer mammalian cells (*e.g.*, HEK 293 cells). For a detailed description of SBI's Lentivector expression system, please refer to the Lentivector Expression System user manual.

C. Features and Benefits of the Clone-it™ Cloning Vector

As outlined in Fig. 1, for cloning your promoter, cDNA, microRNA and shRNA of interest with the Clone-it™ Vectors, you can implement the following features and benefits:

- Clone any part of any gene into the vectors without the need for checking restricting cutting sites.
- Eliminate the need for restriction digestion[†], ligation or topoisomerase reactions for cloning.
- Totally enzyme-free when cloning shRNAs.
- Nearly 100% cloning efficiency.
- Directional and in frame with tag(s) and selection marker(s) built into the vectors.
- Simplest procedure just involving standardized heating and mixing.
- No optimization is needed if protocols in this manual are strictly followed.
- Short hands-on time and amenable to high-throughput cloning.
- No recombination errors.
- The constructs created are ready to be used directly for transfection or lentiviral packaging.

[†]Except for the use of DpnI to remove template DNA if plasmid is used in the PCR.

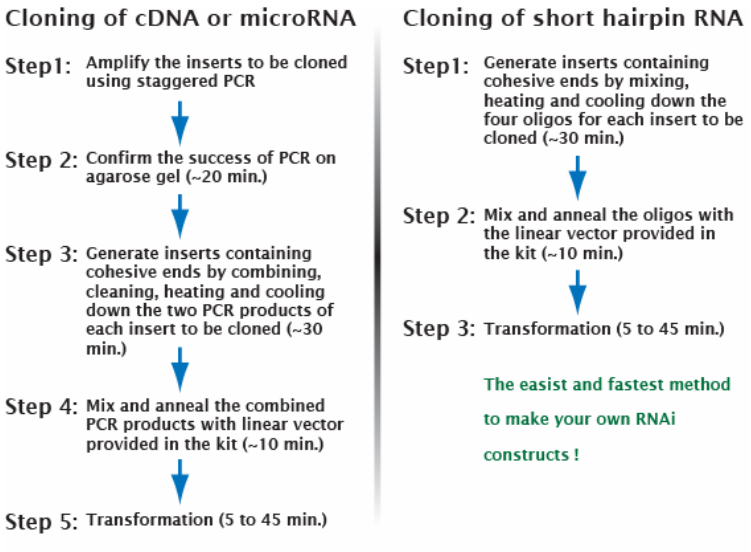


Fig. 1. Flow charts of the Clone-it™ Enzyme-free cloning systems.

D. Mechanism of the Clone-it™ Lentivectors

All the Clone-it™ Enzyme-free Cloning vectors in the kit are provided as linearized with the cohesive ends shown in Fig. 2a. The complementary ends (Fig. 2b) of the inserts to be cloned can be easily generated by users either through staggered PCR (for promoter, cDNA and miRNA expression vectors) or via traditional oligo annealing (for shRNA expression vectors), which will be described in detail in the protocol section.

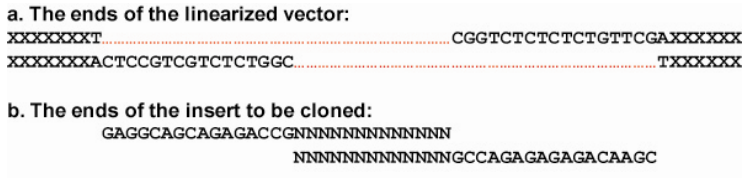


Fig. 2. The cohesive ends of the linearized Clone-it™ Enzyme-free lentivectors (a) and inserts (b). The dotted lines indicate the position of the "stuffer fragment" that was removed during linearization at SBI. Your insert sequence should be generated using the procedures described in the protocol section for directional insertion between the overhangs.

After mixing the linearized vector with the insert to be cloned, their cohesive ends anneal together due to complementary sequences, giving rise to nicked, hybrid plasmids. Following transformation of the mixture into competent *E. coli* cells, the nicks of the plasmids are then ligated by cellular enzymes.

1) Clone-it™ Promoter, cDNA and miRNA Lentivectors:

- **Promoter (cat# LF528A-1)**
- **cDNA (cat# LF520A-1; LF521A-1)**
- **miRNA (cat# LF522A-1; LF522B-1)**

The cohesive ends of either promoter, cDNA or miRNA sequences to be cloned are generated through staggered PCR (de Jong *et al*., 2006; Tillett and Neilan, 1999) as outlined in Fig. 3 and described in detail in the protocol section. Basically, two pairs of primers are designed for each template. The two pairs of primers are identical except that the second pair contains either the 16 or the 17 nt tail shown in Fig. 2b on their 5' ends. Two PCR reactions are performed separately either using the un-tailed forward primer and the tailed reverse primer, or using the tailed forward primer and the un-tailed reverse primer. These two PCR products then create fragments containing the overhangs shown in Fig. 2b after they are mixed, purified through PCR clean-up columns, denatured and re-annealed.

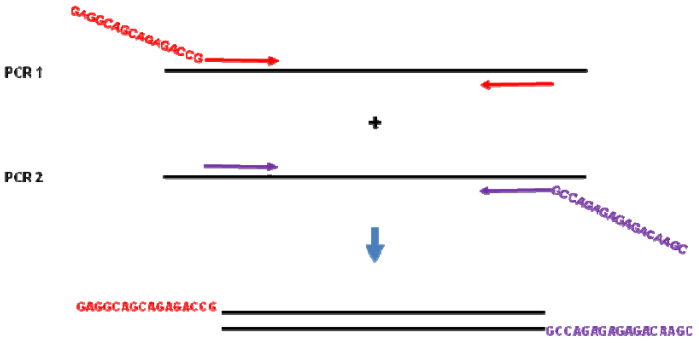


Fig. 3. Inserts with cohesive ends complementary to the ends of the linearized Clone-it™ vectors generated by staggered PCR.

2) Clone-it™ shRNA Cloning/Expression Vectors:

Two approaches have been developed for *in vivo* expression of siRNA from plasmid and viral vectors. In one approach, the sense and antisense strands are transcribed separately from two independent promoters and form the siRNA duplex (Lee 2002, Miyagishi 2002). The second approach, as employed with the Clone-it™ shRNA Cloning/Expression Vectors, uses a single-stranded shRNA sequence with a fold-back stem-loop structure (also known as a "hairpin") that is expressed from a single promoter (Abbas-Terki 2002, Qin 2003, Wiznerowicz 2003). This sequence is then converted into double-stranded siRNA after intracellular processing cleaves the loop (Brummelkamp 2002, Paddison 2002). In both approaches, the siRNA molecules are transcribed from constitutive RNA polymerase III promoters (*i.e.*, U6 and/or H1) and terminated with TTTTT (T_5) sequences (Tuschl 2002). The U6 and H1 promoters are different in size but contain the same conserved sequence elements (Myslinski 2001).

In the Clone-it™ shRNA Cloning and Expression Vectors, the RNA polymerase III H1 promoter is used to express the shRNA sequence. The shRNA template oligonucleotides are cloned into the Ligase-free Cloning Site (LCS) located just downstream of the H1 promoter (Figure 1). The linearized vectors provided contain two unique 5' overhangs to facilitate directional cloning of shRNA template oligos (Figure 4). When the shRNA construct is expressed from the constitutive H1 promoter and terminated with the TTTTT sequence, the shRNA transcript folds into the hairpin structure, which is recognized by the DICER enzyme, cleaved to form a functional double-stranded siRNA and transferred to a RISC complex for selective digestion of complementary target mRNAs (Figure 4).

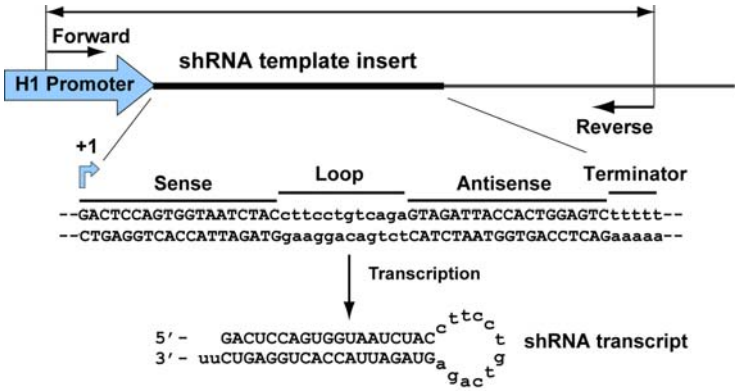


Fig. 4. Example shRNA construct targeting the p53 gene. The nucleotides for the specific siRNA sequence targeting the p53 gene are shown in capital letters. The shRNA sense and antisense sequences flank the region coding for the loop structure. In addition, the terminator sequence ttttt for the RNA polymerase III is included after the antisense portion. The Forward and Reverse arrows refer to the PCR primers contained in the vector to confirm positive clones. After transcription, a stem-loop-stem shRNA molecule is produced. This molecule is processed by the DICER enzyme to generate a double-stranded siRNA effector.

In order to clone such an shRNA expression cassette into the Clone-it™ shRNA expression vectors, instead of synthesizing two 65-nt oligos required for the conventional ligase-dependent method, we recommend the design and synthesis of four short oligos as depicted in Fig. 5. This approach is more cost-effective and is more accurate and efficient. The oligos are then mixed, heat and cooled to form the nicked, cohesive ended shRNA insert (Fig. 5).

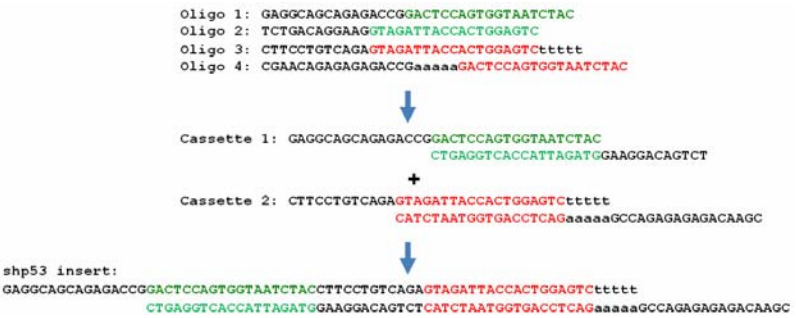


Fig. 5. Outline of the Clone-it™ procedure for generating the cohesive-ends of the p53 shRNA insert. Four desalted, non-phosphorylated oligos are synthesized, mixed, denatured and annealed by heating and cooling.

E. List of Components

All kits are shipped in dry ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 6 months from the date received.

1) Clone-it™ cDNA Cloning and Expression Lentivectors:

(1) pPS-EF1-LCS-T2A-RFP. Cat. #: LF520A-1
(2) pPS-EF1-LCS-T2A-Puro. Cat. #: LF521A-1

Component	Conc.	Amount
Clone-it™ cDNA Cloning/Expression Vector	4 ng/µl	20 µl
copGFP control primer1	10 µM	10 µl
copGFP control primer2	10 µM	10 µl
copGFP control primer3	10 µM	10 µl
copGFP control primer4	10 µM	10 µl
copGFP control template	10 ng/µl	10 µl
DpnI	20 units/µl	10 µl

2) Clone-it™ microRNA Cloning and Expression Lentivectors:

(1) pPS-EF1-copGFP-LCS. Cat. #: LF522A-1
(2) pPS-EF1-RFP-LCS Cat. #: LF522B-1

Component	Conc.	Amount
Clone-it™ microRNA Cloning/Expression Vector	4 ng/µl	20 µl
copGFP control primer1	10 µM	10 µl
copGFP control primer2	10 µM	10 µl
copGFP control primer3	10 µM	10 µl
copGFP control primer4	10 µM	10 µl
copGFP control template	10 ng/µl	10 µl
DpnI	20 units/µl	10 µl

3) Clone-it™ shRNA Cloning and Expression Lentivectors:

(1) pPS-H1-LCS-copGFP. Cat. #: LF523A-1
(2) pPS-H1-LCS-Puro. Cat. #: LF524A-1

Component	Conc.	Amount
Clone-it™ shRNA Cloning/Expression Vector	4 ng/µl	20 µl
shp53 control primer1	20 µM	10 µl
shp53 control primer2	20 µM	10 µl

shp53 control primer3	20 µM	10 µl
shp53 control primer4	20 µM	10 µl

4) Clone-it™ Reporter Lentivector:
(1) pPS-LCS-mCMV-RFP. Cat. #: LF528A-1

Component	Conc.	Amount
Clone-it™ Reporter Vector	4 ng/µl	20 µl
copGFP control primer1	10 µM	10 µl
copGFP control primer2	10 µM	10 µl
copGFP control primer3	10 µM	10 µl
copGFP control primer4	10 µM	10 µl
copGFP control template	10 ng/µl	10 µl
DpnI	20 units/µl	10 µl

F. Additional Required Materials for Cloning

- High Fidelity PCR Hot-Start enzyme (*e.g.* Phusion Hot Start from Finnzymes or Stratagene's *PfuUltra*® II Fusion HS DNA Polymerase)
- High efficiency competent *E. coli* cells (RecA$^-$) (Recommended: Invitrogen One Shot OmniMAX 2 competent cells, Cat. # C8540-03)
- Petri plates containing LB Agar media with 50 µg/ml Ampicillin

For Screening Inserts and Sequencing
- Taq DNA polymerase, reaction buffer, and dNTP mix (Recommended: Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- PCR machine
- 1-3% 1X TAE Agarose gel

For Purifying cDNA Constructs after Cloning

- Plasmid purification kit (Recommended: QIAGEN Endotoxin-free Plasmid Kit. The following kit combinations can be used for Midi scale (up to 200 µg of plasmid DNA) preparation of endotoxin-free DNA).

For Transfection of Clone-it™ Constructs into Target Cells
- Transfection Reagent (Recommended: Invitrogen Lipofectamine 2000, Cat. # 11668-027)

For Packaging of Clone-it™ Constructs in Pseudoviral Particles
- In order to package your Clone-it™ constructs into VSV-G pseudotyped viral particles, we recommend using the pPACKH1 Lentivector Packaging Kit (SBI, Cat. # LV500A-1). The protocols for packaging and transduction of packaged pseudoviral particles are provided in the Lentivector Expression System User Manual.
- 293 Producer Cell Line (Recommended: SBI 293TN Cell Line, Cat. # LV900A-1 or ATCC 293 Cells, Cat. # CRL-11268)
- Transfection Reagent (Recommended: Invitrogen Lipofectamine, Cat. # 18324-111 and Plus Reagent, Cat # 11514-015).

G. Safety Guidelines

SBI's Expression lentivectors together with the pPACK packaging plasmids comprise the third-generation lentiviral expression system. The HIV-based lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (US patent #5,665,577 and # 5,981,276).

Both FIV-based and HIV-based lentivector systems are designed to maximize their biosafety features, which include:
- A deletion in the enhancer of the U3 region of 3' LTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) and CMV promoter (in FIV-based vectors) upstream of 5' LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (*gag, pol, rev*), and the corresponding proteins are expressed from different plasmids (for HIV-based packaging plasmids) lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.

- Pseudoviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of SBI's lentivectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm. It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and always follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.
- Please keep in mind that Clone-it™ vectors can be integrated into genomic DNA and may have a risk of insertional mutagenesis.

II. Protocols

A. Clone-it™ cDNA/miRNA/Reporter Lentivectors:

1) Primer design and synthesis:

Four primers should be designed for each insert to be cloned. Desalted grade of the primers is adequate enough for the cloning, and there is no need for the primers to be phosphorylated. First of all, determine the region of the gene you want to clone. For cDNA, this usually encompasses the open reading frame or any domain of the protein you want to express; for miRNA precursors, it is normally the genomic sequences with 100 – 200 bp flanking each side of the precursor miRNA; for reporter, it can be the whole promoter, transcription responsive elements or any region of the promoter you want to characterize since the Clone-it™ Reporter vector contains the minimum CMV promoter. Once the cloning region is determined, design primers 1 (forward) and 2 (reverse) with a Tm of around 55°C for amplification of the region. **Remember to exclude the stop codon in the reverse primer for cDNA cloning to allow for translation read-through to include the C-terminal T2A tag.** Primers 3 and 4 will be adaptor sequences GAGGCAGCAGAGACCG and CGAACAGAGAGAGACCG plus the sequence of primers 1 and 2, respectively.

Example 1: To clone the copGFP open reading frame (Fig. 6) into the Clone-it™ cDNA cloning/expression vectors, the primers are as follows:

Primer 1: atggagagcgacgagagcgg
Primer 2: gcgagatccggtggagccggg
Primer 3: GAGGCAGCAGAGACCGatggagagcgacgagagcgg
Primer 4: CGAACAGAGAGAGACCGgcgagatccggtggagccggg

Fig. 6. Sequence of the copGFP open reading frame. Red bold letters: the forward primer binding site; green bold letters: the reverse primer binding site; dotted line: the other nucleotides of the open reading frame that are not shown in the figure.

Example 2: To clone the primary miRNA gene of the human miRNA hsa-mir-1-2, design the following primers to amplify the genomic sequence on chromosome 18, as shown in Fig. 7:

Primer 1: gcaaatgatttaccattgctctac
Primer 2: tctgttcatgactaggttaatttac
Primer 3: GAGGCAGCAGAGACCGgcaaatgatttaccattgctctac
Primer 4: CGAACAGAGAGAGACCGtctgttcatgactaggttaatttac

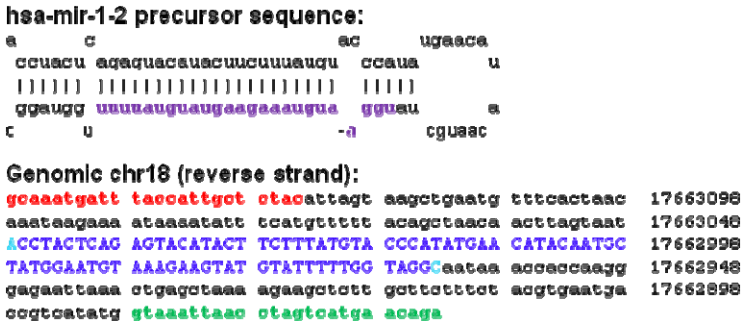


Fig. 7. Sequences of the precursor and primary miRNA of the human has-mir-1-2. Purple letters: the precursor miRNA; red letters: the forward primer binding site; green letters: the reverse primer binding site; blue letter in the genomic sequence: hsa-mir-1-2 precursor sequence.

Example 3: To clone the promoter region (Fig. 8) of the mouse gene for brain-derived neurotrophic factor (BDNF) into the reporter vector, design the following primers to amplify the genomic sequence shown in Fig. 8:

Primer 1: caaagcatgcaatgccctgg
Primer 2: cacctttttcagtcactacttgtc
Primer 3: GAGGCAGCAGAGACCGcaaagcatgcaatgccctgg
Primer 4: CGAACAGAGAGAGACCGcacctttttcagtcactacttgtc

Fig. 8. Sequences of the promoter region of the mouse gene for brain-derived neurotrophic factor (BDNF) to be cloned. Red letters: the forward primer binding site; green letters: the reverse primer binding site.

Example 4: To clone the transcription response element consensus sequence **cctttgatcttaccagctaac** that responds to transcriptional activation by TCF/LEF1 into the reporter vector, design the following oligos:

Oligo 1: GAGGCAGCAGAGACCGcctttgatcttaccagctaac
Oligo 2: CGAACAGAGAGAGACCGgttagctggtaagatcaaagg**,** where the red letters are the consensus TCF/LEF1 transcription response element sequence, and the green letters are its reverse complementary sequence.

2) Amplification of the insert to be cloned:

For transcription response element cloning, skip this step and proceed to step 6) – **Annealing** – of this protocol.

Set up two PCR reactions for each insert to be cloned using the conditions suitable for the high-fidelity PCR polymerases of your choice. IMPORTANT: Use only proof-reading enzymes which generate blunt end PCR products. The following sample conditions are optimized with the Stratagene *PfuUltra*® II Fusion HS DNA Polymerase to clone the control copGFP open reading frame into the vectors.

Master Mixture

	ul/reaction
Water	19.2
10x PfuUltra II buffer	2.5
DMSO	0.7
10 mM dNTP	0.6
PfuUltra II polymerase	0.5

PCR reaction 1
23.5 ul Master Mixture
0.5 ul 10 uM primer 1
0.5 ul 10 uM primer 4
0.5 ul template*
(* containing 10 ng of plasmid, 50 - 500 ng of cDNA, or 200 ng of genomic DNA)

PCR reaction 2
23.5 ul Master Mixture
0.5 ul 10 uM primer 2
0.5 ul 10 uM primer 3
0.5 ul of template**
(** the same template used in PCR reaction 1 containing 10 ng of plasmid, 50 - 500 ng of cDNA, or 200 of ng genomic DNA)

Program
1X 95°C, 2'
25 - 32 X 95°C, 20"; 55°C, 20"; 72°C, 2'

(Extension times may vary depending on the size of the insert and the PCR enzyme you use.)

1X 72°C, 3'

3) Degradation of template plasmid:
If the template used is a plasmid containing an ampicillin resistant gene, add 0.25 ul DpnI into each PCR reaction. Incubate at 37°C for 1 hour. Otherwise, if the template is either genomic DNA, cDNA or a plasmid lacking the ampicillin-resistance gene, skip this step.

4) Check the success of PCR amplification:
Run 5 ul of each PCR reaction on an agarose gel to ensure that your insert of interest is specifically amplified.

5) Cleaning the PCR products:
Combine the two successfully amplified PCR products for each insert and clean the mixture with a PCR Cleaning Kit. Elute with 30 ul of 10 mM Tris, pH 8.5.

6) Annealing:
For transcription response element cloning, mix 1 µl of 10 µM Oligo1 and Oligo2 in 18 µl of 10 mM Tris, pH 8.5. Heat the mixture at 95°C for 5' and let it cool down to room temperature in the PCR machine gradually over 20'.

For promoter/cDNA/miRNA cloning, heat the cleaned PCR mixture at 95°C for 5' and let it cool down to room temperature in the PCR machine gradually over 20'.

7) Mix the vector and insert to be cloned:
Mix 1 ul of the 4 ng/ul linear Clone-it™ cDNA, miRNA or reporter vector of your choice with 2 ul of the above annealed PCR products or oligo mixture from step 6). Heat in a PCR machine at 55°C for 2'. Incubate the tube at room temperature for 5', and then place on ice for at least 2'.

8) Transformation:
Transform competent cells (with a transformation efficiency of at least $1x10^9$ colonies/□g pUC19) with the whole mixture (3 µl) from step 7) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin agar plates.

9) Identify Clones with the cDNA or miRNA Insert
Since the Clone-it™ vectors have been optimized for low background cloning, a negative cloning control with the vector only is not necessary. Sometimes, due to non-specific amplified PCR products, you may encounter some clones with wrong inserts. To identify the correct clones containing your insert, you may pick 5 well-isolated colonies for plasmid purification, digestion and sequencing. You can digest the candidate cDNA expression clones with XbaI and EcoRI, and the miRNA expression clones with Acc65I and Not I, respectively. For sequencing, we recommend the use of the following primers, which are not provided with the kit:

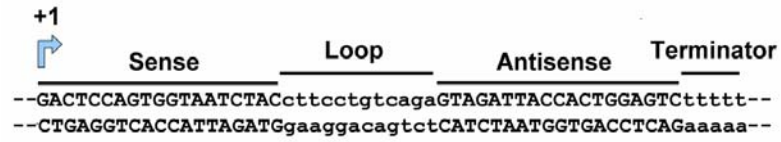
For cDNA clones: 5'- CTCCACGCTTTGCCTGACCCTGCTT - 3'
For miRNA clones: 5'- TGCTCGCCTGTGTTGCCACCTGG - 3'
For reporter clones: 5'- GATTGGGGGGTACAGTGCAG - 3'

B. Clone-it™ shRNA Cloning/Expression Lentivectors:

1) Primer design and synthesis:

Four short oligos should be designed for each shRNA insert to be cloned. Desalted grade is adequate enough, and phosphorylation of the oligos is not required. Oligo 1 is the 5' adaptor GAGGCAGCAGAGACCG followed by the sense strand of your shRNA. Oligo 2 is the reverse complementary loop followed by the anti-sense strand sequence. Oligo 3 is the loop sequence plus the anti-sense strand and the H1 terminator (five consecutive "Ts"). Oligo 4 is the 3' adaptor CGAACAGAGAGAGACCG plus five "As" and the sense strand. For example, the oligos to clone the shRNA targeting the human p53 gene are shown in Fig. 8.

A.



B.

Oligo 1: gaggcagcagagaccgGACTCCAGTGGTAATCTAC
Oligo 2: tctgacaggaagGTAGATTACCACTGGAGTC
Oligo 3: *cttcctgtcaga*GTAGATTACCACTGGAGTCttttt
Oligo 4: CGAACAGAGAGAGACCGaaaaaGACTCCAGTGGTAATCTAC

Fig. 9. Oligos for cloning the example shRNA targeting the human p53. Panel A. depicts the overall schematic of the shRNA design, with the sense, loop, antisense and terminator sequences labeled. **Panel B.** shows the individual oligos to be designed. Lower case black letters: 5' adaptor; green letters: sense strand; blue lower case letters: reverse complementary loop; red letters: antisense strand; lower case italic letters: loop sequence; five consecutive "t": H1 terminator; Capitalized balck letters: 3' adaptor.

2) Annealing of the oligos:

Dilute all oligos to 20 µM in 10 mM Tris, pH 8.5. In a thin walled PCR tube, mix 1 µl of oligo 1 with 1 µl of oligo 2 in 18 µl of 10 mM Tris, pH 8.5 buffer. In another thin walled PCR tube, mix 1 µl of oligo 3 with 1 µl of oligo 4 in 18 µl of 10 mM Tris, pH 8.5 buffer. Heat the mixtures at 95°C for 2' and then let the mixture cool down to room temperature in the PCR machine gradually over 20'.

3) Combining the vector and annealed oligo mixtures to be cloned:

Mix 1 ul of the 4 ng/ul linear Clone-it™ shRNA expression vector of your choice with 1 ul each of the above annealed oligo. Heat the vector-oligo mixture in a PCR machine at 40°C for 2'. Incubate the tube at room temperature for 5', and then on ice for at least 2'.

4) Transformation:

Transform competent cells (with a transformation efficiency of at least $1x10^9$ colonies/µg pUC19) with the whole mixture (3 µl) from step 3) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin agar plates.

5) Identifying Clones with the shRNA Insert

Since the Clone-it™ vectors have been optimized for low background cloning, a negative cloning control with the vector only is not necessary. To identify the correct clones containing your insert, you may pick 4 - 6 well-isolated colonies for plasmid purification, digestion and sequencing. For double digestion of the candidate shRNA expression clones, use BamHI and EcoR I. For sequencing, we recommend the following primer, which is not provided with the kit:

5'- TGCATGTCGCTATGTGTTCTGGGA – 3'

III. Troubleshooting

A. No or low number of colonies on plates

1) Wrong Primer/oligo Pairs were Used for PCR or Annealing:
Make sure use the right pairs of primers or oligos for staggered PCR or shRNA cassette formation.

2) Low Transformation Efficiency:
(a.) Low quality or poor handling of competent cells: Handle the competent cells gently. Many cells do not allow re-freezing cells after thawed. Quality of competent cells may be tested by transforming a circular plasmid to determine cells' competency. Use competent cells with a transformation efficiency of $1x10^9$ colonies/ µg of pUC19 plasmid.

(b.) Wrong antibiotic or too much antibiotic in the media: The plates used for cloning should contain 50-100 µg/ml ampicillin in the media.

3) Degradation of the Clone-it Vectors:
Avoid repeated freezing and thawing of the vectors. The vectors can be stable if stored at 4°C up to 2 weeks and at -20°C for 6 months.

B. No correct inserts

1) PCR Products Contain Non-specifically Amplified Artifacts:
Optimize your PCR reaction to improve the specificity. Screen more colonies for the correct clones.

C. The Insert contains mutations

1) Primers/oligos Contain Mutation:
Mutations can occur during primer/oligo synthesis. Screen more colonies for the correct clones.

2) The Insert outside the Primer Sites Contain Mutations:
Screen more colonies for correct clones. Alternatively, use a PCR enzyme with higher fidelity, or reduce the number of PCR cycles during amplification of the insert.

3) The shRNA Insert Contains Mutations:
Screen more colonies for correct clones. Always adhere to the recommended temperatures for denaturing/annealing the oligos and for incubation of the annealed oligo-vector mixture. If the problem cannot be solved by these measures, check the oligo design and re-synthesize the oligos.

IV. References

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Li MJ, Rossi JJ. Lentiviral vector delivery of recombinant small interfering RNA expression cassettes. Methods Enzymol. 2005;392:218-26.

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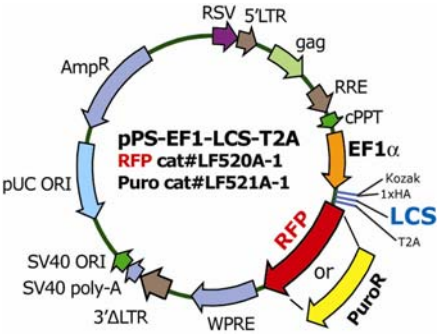
Methods Enzymol. 2005;392:145-73.

V. Appendix

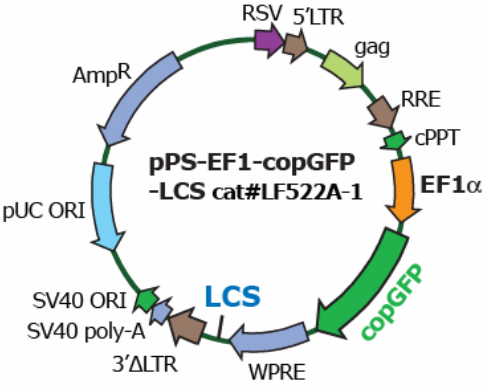
A. Maps and Features of the Vectors:

1) Clone-it™ cDNA Cloning/Expression Lentivectors:



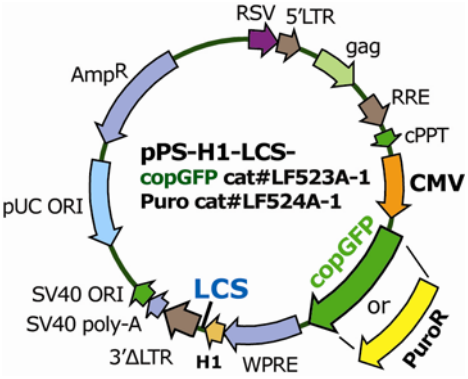
• **Elongation Factor 1 (EF1) promoter -** promotes a high level of co-expression of your gene of interest with either the RFP or the puromycin marker in a wide variety of cell lines.
• **Ligase-free Cloning Site (LCS) -** for cloning the gene of interest downstream of the EF1 promoter.
• **WPRE element -** enhances stability and translation of the CMV-driven transcripts.
• **SV40 polyadenylation signal -** enables efficient termination of transcription and processing of recombinant transcripts.
• **RFP or puromycin selection marker -** provides co-expression of aRFP or puromycin resistance gene reporter with your gene of interest under the control of the constitutive EF1 promoter for selection or FACS analysis of transduced cells.
• **Kozak sequence –** optimized bases and positions for efficient translation initiation.
• **HA tag -** allows for detection or purification of the protein encoded by your gene of interest.
• **T2A tag -** allows for translational cleavage of your gene of interest and the RFP (Puro) selection marker, and detection or purification of the protein encoded by your gene of interest.
• **Hybrid RSV-5LTR promoter -** provides a high level of expression of the full-length viral transcript in producer 293 cells.
• **Genetic elements (cPPT, GAG, LTRs) -** necessary for packaging, transducing, and stably integrating the viral expression construct into genomic DNA.
• **SV40 origin -** for stable propagation of the Clone-it™ plasmid in mammalian cells.
• **pUC origin -** for high copy replication and maintenance of the plasmid in *E.coli* cells.
• **Ampicillin resistance gene -** for selection in *E.coli* cells.

2) Clone-it™ miRNA Cloning/Expression Lentivector:



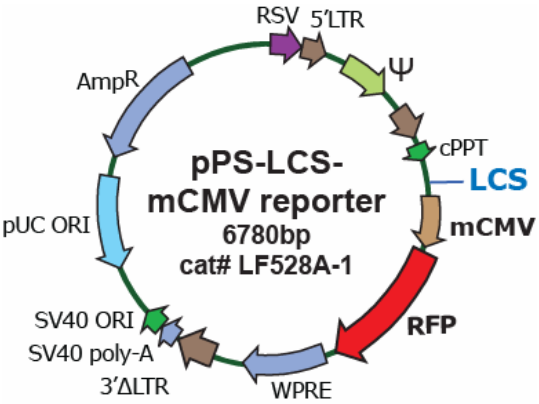
• **Elongation Factor 1 (EF1) promoter -** promotes a high level expression of a single transcript encoding copGFP, Zeocin resistence gene, WPRE and the precursor miRNA you have cloned in a wide variety of cell lines.
• **Ligase-free Cloning Site (LCS) -** for cloning the precursor miRNA of your choice downstream of WPRE.
• **WPRE element -** enhances stability and translation of the CMV-driven transcripts.
• **SV40 polyadenylation signal -** enables efficient termination of transcription and processing of recombinant transcripts.
• **copGFP selection marker -** provides co-expression of copGFP reporter with your precursor miRNA of interest under the control of the constitutive EF1 promoter for selection or FACS analysis of transduced cells.
• **Hybrid RSV-5LTR promoter -** provides a high level of expression of the full-length viral transcript in producer 293 cells.
• **Genetic elements (cPPT, GAG, LTRs) -** necessary for packaging, transducing, and stably integrating the viral expression construct into genomic DNA.
• **SV40 origin -** for stable propagation of the Clone-it™ plasmid in mammalian cells.
• **pUC origin -** for high copy replication and maintenance of the plasmid in *E.coli* cells.
• **Ampicillin resistance gene -** for selection in *E.coli* cells.

3) Clone-it™ shRNA Cloning/Expression Lentivectors:



• **H1 expression cassette -** provides constitutive and efficient RNA polymerase III-dependent transcription of shRNA transcripts in a wide range of cell lines.
• **CMV promoter -** promotes high level expression of copGFP, or puromycin-N-acetyl transferase (drug selectable marker) for detection and selection of transduced cells.
• **Hybrid RSV-5'LTR promoter -** provides a high level expression of the full-length viral construct in 293 cells.
• **Genetic elements (cPPT, GAG, LTRs) -** necessary for packaging, transducing, and stable integration of the viral expression construct into genomic DNA.
• **WPRE element -** enhances stability and translation of the CMV-driven transcripts.
• **Ligase-free Cloning Site (LCS) -** for cloning the shRNA of interest downstream of the human H1 promoter.
• **SV40 polyadenylation signal** - enables efficient termination of transcription and processing of recombinant transcripts.
• **copGFP or puromycin selection marker** - provides expression of copGFP or puromycin resistance gene reporter under the control of the constitutive CMV promoter for selection or FACS analysis of transduced cells.
• **SV40 origin -** for stable propagation of the Clone-it™ plasmid in mammalian cells.
• **pUC origin -** for high copy replication and maintenance of the plasmid in *E.coli* cells.
• **Ampicillin resistance gene -** for selection in *E.coli* cells.

4) Clone-it™ Reporter Lentivectors:



• **mCMV promoter –** minimum CMV promotes high level expression of RFP, for detection and selection of transduced cells only if a functional promoter or transcription response element is cloned at the LCS of the vector and the correspondent transcription factor is expressed in the cells.
• **Hybrid RSV-5'LTR promoter -** provides a high level expression of the full-length viral construct in 293 cells.
• **Genetic elements (cPPT, GAG, LTRs) -** necessary for packaging, transducing, and stable integration of the viral expression construct into genomic DNA.
• **WPRE element -** enhances stability and translation of the CMV-driven transcripts.
• **Ligase-free Cloning Site (LCS) -** for cloning the promoter or transcription response element of interest upstream of the mCMV promoter.
• **SV40 polyadenylation signal** - enables efficient termination of transcription and processing of recombinant transcripts.
• **RFP -** reporter for the functionality of your promoter or transcription response element of interest, as well as for selection or FACS analysis of transduced cells.
• **SV40 origin -** for stable propagation of the Clone-it™ plasmid in mammalian cells.
• **pUC origin -** for high copy replication and maintenance of the plasmid in *E.coli* cells.
• **Ampicillin resistance gene -** for selection in *E.coli* cells.

B. Related Products

- **pPACKH1™ Lentivector Packaging Kit (Cat. # LV500A-1)**

Unique lentiviral vectors that produce all the necessary HIV viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. 293TN cells (SBI, Cat. # LV900A-1) transiently transfected with the pPACKH1 and a pCDH cDNA expression construct produce packaged viral particles containing a pCDH cDNA construct.

- **UltraRapid Lentiviral Titering Kits (Cat. # LV960A-1, LV960B-1)**

The easiest kits that allow you to rapidly and accurately determine the titers of infectious pseudoviral particles that are generated with SBI's FIV and HIV lentiviral vectors or libraries. They are more accurate than all other titering kits on the market as they measure the copy numbers of integrated lentiviral constructs in the genomic DNA of each transduced target cell.

- **PEG-*it*™ Virus Precipitation Solution (LV810A-1, LV825A-1)**

To easily concentrate Lenti- and Retro-viruses without ultracentrifugation.

C. Technical Support

For more information about SBI products, to download manuals in PDF format, or to obtain vector sequences, please visit our web site:

http://www.systembio.com

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

System Biosciences (SBI)
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(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

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Use of the Clone-it Cloning Vectors (*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.
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The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.
This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

HIV Vector System

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CopGFP Reporter

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