CELLECTA

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Cloning of shRNA Templates into shRNA Expression Vector
User Manual

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A. Background

The protocols below provide the instructions on how to phosphorylate shRNA template oligos, ligate them to shRNA cloning vectors, transform competent cells, and grow plasmid DNA to generate plasmid shRNA expression constructs. For packaging of constructs into pseudovirus, please refer to the User Manual for Packaging and Transduction of Lentiviral Constructs. Please read the entire user manual before proceeding with your experiment.

B. Required Materials

For Phosphorylation and Annealing of shRNA Template Oligonucleotides

- T4 Polynucleotide Kinase and 10X reaction buffer
  (Recommended: New England BioLabs T4 Polynucleotide Kinase, 10 U/µl, Cat. # M0201S)
- rATP (Recommended: GE/Amersham, Cat. # 27-2056-01)

For Ligating and Transforming shRNA Constructs

- *Linearized* shRNA Expression Vector
  NOTE: For DECIPHER™ pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro Empty Vector (undigested), Cellecta recommends digestion with Fermentas BpiI (Bbsl), [10 U/µl], Cat.# ER1012, and gel-purification using QIAGEN QIAquick Gel Extraction Kit, Cat.# 28706.
- T4 DNA Ligase and 10X ligation reaction buffer
  (Recommended: New England BioLabs, T4 DNA Ligase, 400 U/µl. Cat. # M0202S)
  Before using, dilute T4 DNA-ligase 10-fold with 1X T4 DNA ligase buffer to 40 U/µl.)
- Competent *E. coli* cells (RecA$^-$)
  (Recommended: Invitrogen, OmniMAX™ 2 T1 cells, Cat. # C8540-03)
- Petri plates containing LB Agar media with 100 µg/ml Ampicillin or Carbenicillin

For Screening shRNA Inserts

- Taq DNA polymerase, and 10X reaction buffer
  (Recommended: BDB Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- dNTP mix (Recommended: GE/Amersham, dNTP set, Cat. # 27-2035-01)
- Thermal Cycler

- 3% 1X TAE Agarose gel

For Purifying shRNA Constructs after Cloning

- Plasmid purification kit

  (Recommended: QIAGEN Endotoxin-free Plasmid Kit. The following kit combinations can be used for Midi scale preparation of endotoxin-free DNA:

  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Maxi Kit, Cat. # 12362
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the user manual:

  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

C. Cloning of shRNA Template Oligonucleotides into shRNA Expression Vector

1. Phosphorylate and Anneal the shRNA Template Oligonucleotides

Note: This protocol was developed for regular non-phosphorylated oligos. If your oligonucleotides are already phosphorylated, dilute them to 10 μM in 1X T4 polynucleotide kinase buffer, heat at 95°C for 2 min and anneal as in steps 1.d-1.e below.

a. Dissolve the shRNA template oligonucleotides in an appropriate amount of deionized water to a final concentration of 20 μM.

b. Set up 20 μl phosphorylation/annealing reactions for each shRNA template:

1 μl	Top Strand shRNA template oligo (20 μM) *
1 μl	Bottom Strand shRNA template oligo (20 μM) *
2 μl	10X T4 Polynucleotide Kinase Buffer
2 μl	5 mM ATP
13 μl	Deionized water
1 μl	T4 Polynucleotide Kinase (10 U/μl)
20 μl	Total volume

* For the insert-minus control, use 2 μl deionized water in place of the top and bottom strands.

c. Incubate the phosphorylation reaction at 37°C for 30 minutes in a thermocycler.

d. Heat the reaction mix to 95°C for 2 min in a thermocycler.

e. Turn off the thermocycler and let it cool to room temperature.

f. Take a 2 μl aliquot from the reaction (1 μM shRNA template), and make a 1:5 dilution by adding 8 μl of 1X T4 Kinase buffer. Mix well.

g. Use 0.5 μl of the diluted shRNA template (0.2 μM) for the following ligation reaction.

2. Ligate the shRNA Template into Linearized shRNA Lentivector

a. Set up 10 μl ligation reactions for each phosphorylated shRNA template as follows:

1.0 μl	Linearized shRNA Expression Vector (10 ng/μl) *(see Required Materials)*
0.5 μl	Phosphorylated ds shRNA template (step 1; 0.2 μM) *
1.0 μl	10X T4 DNA Ligase Buffer
6.5 μl	Deionized water
1.0 μl	T4 DNA ligase (40 U/μl) **
10.0 μl	Total volume

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* For negative control use insert-minus.

** Dilute T4 DNA ligase (400 U/μl) 10-fold to 40 U/μl with 1X T4 DNA ligase buffer if you are using New England Biolabs enzyme.

b. Incubate the ligation reaction at 16°C for 1-2 hrs.

3. Transform *E. coli* with the ligation product

a. For each shRNA template, use the whole volume of ligation product for transformation.
b. Follow the manufacturer's protocol for transforming the competent cells.
c. Plate an appropriate amount of cells on LB plates with 100 μg/ml ampicillin or carbenicillin and grow overnight at 37°C.
d. You could expect to get at least 10-fold more colonies in experimental samples in comparison with negative control (vector-only ligation reaction).

D. Identify clones with the target shRNA template

1. Prepare colony cultures

a. Randomly pick up 10 well-separated colonies from each plate, and grow each clone in 100 μl of LB Broth with 100 μg/ml ampicillin or carbenicillin at 37°C for 2 hours with shaking.
b. Take 1 μl of each bacteria culture for PCR screening (see B.2) and continue to grow the culture for another 6 hours.
c. Store the bacterial culture at 4°C.

2. Screen for shRNA template inserts

a. Prepare a PCR master mix for each clone you would like to screen for the presence of an shRNA template insert as follows:

1 rxn	10 rxn	Composition
0.5 μl	5 μl	Forward PCR Primer (10 μM)
0.5 μl	5 μl	Reverse PCR Primer (10 μM)
0.5 μl	5 μl	50X dNTP mix (10 mM of each)
2.5 μl	25 μl	10X PCR Reaction Buffer
19.5 μl	195 μl	Deionized water
0.5 μl	5 μl	Taq DNA Polymerase (5 U/μl)
24.0 μl	240 μl	Total volume

b. Mix the master mix very well and aliquot 24 μl into each well of a 96-well PCR plate or individual tubes.
c. Add 1 μl of each bacterial culture from B.1 into each well or tube from B.2.b. Mix.
d. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min	25 cycles
68°C, 2 min	1 cycle

e. Take 5 μl of PCR product from step d and run it on a 3% agarose/EtBr gel in 1X TAE buffer.
f. Confirm identity of shRNA template inserts by sequence analysis of positive PCR products using the Forward PCR primer.

E. Purify Plasmid shRNA Construct

a. Take 15 μl of each positive bacteria culture from Step B.1.c, inoculate each clone in 10 ml of LB broth media with 100 μg/ml ampicillin or carbenicillin, and grow overnight at 37°C with shaking.
b. Purify shRNA lentivector construct plasmid DNA in Midi or Maxi scale using an Endotoxin-free plasmid purification kit.

F. Technical Support

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

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For more information about Cellecta's products and services, please visit our web site at http://www.cellecta.com.

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G. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter upstream of 5'LTR in the lentivector allows 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). The corresponding proteins are expressed from different 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 pseudovirus.
- None of the HIV-1 genes (gag, pol, rev) will be present in the packaged pseudoviral 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 HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating pseudovirus 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 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.

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H. References

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