
pCDH cDNA Cloning and Expression Lentivectors

Cat. #s CD500 – CD800 series

User Manual

**Check Package Contents for Storage
Temperatures**

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 License and Warranty Statement contained in this user manual.

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I. Introduction and Background

A. Purpose of this Manual

This manual provides details and information necessary to generate expression constructs of your gene of interest in the pCDH cDNA Cloning and Expression Lentivectors. Specifically, it provides critical instructions on amplification and cloning cDNA into the pCDH vectors, and verification of the final expression constructs. This manual does not include information on packaging the pCDH expression constructs into pseudotyped viral particles or transducing your target cells of choice with these particles. This information is available in the user manual Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells which is available on SBI's website (<http://www.systembio.com/lentiviral-technology/delivery-systems/ppack/literature>). Before using the reagents and material supplied with this system, please read the entire manual.

B. Advantages of the Lentivector Expression System

Lentiviral expression vectors are the most effective vehicles for the delivery and expression of a gene of interest to almost any mammalian cell—including non-dividing cells and model organisms (C.A. Machida, 2003; M. Federico, 2003; W. C. Heiser, 2004). As with standard plasmid vectors, it is possible to introduce lentivector expression constructs in plasmid form into the cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lentivector construct into viral particles, you can obtain highly efficient transduction of expression constructs—even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. The expression construct transduced in target cells is integrated into genomic DNA and provides stable, long-term expression of the target gene.

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

- (1) The lentiviral expression vector (e.g., pCDH-EF1-MCS-T2A-Puro)
- (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, and expression of the target gene sequence. The packaging vector provides all 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. pCDH Cloning and Expression Lentivectors

SBI provides a collection of cDNA cloning and expression vectors for various applications (Tables 1-4). For optimal results, we highly recommend packaging the lentiviral vectors into pseudoviral particles for transduction into target cells. Transfection of plasmid into cells, while functional, may not be indicative of actual gene expression as there is an upstream constitutive promoter (RSV) that drives expression of all transgenes downstream.

For all pCDH lentivectors, there is a limit for the size of the insert(s) that can be cloned into the vectors for efficient packaging into pseudoviral particles. Typically, a pCDH lentivector can hold up to 3-5kb of insert (depending on the vector) such that the total 5' LTR to 3' LTR size including the insert is ~9.5kb.

1. Single Promoter Vectors

These lentivectors are characterized by the presence of a single mammalian promoter driving the gene of interest cloned into the MCS, either expressed by itself or co-expressed with a marker gene of interest (e.g. copGFP or Puro) in a T2A format (please see Section I.D). Vector maps and additional information can be found here:

	cDNA vectors	Catalog #	target gene promoter	Expression level	Application
Single Promoter	pCDH-EF1-MCS	CD502A-1	EF1	Medium	robust in most cell types, including primary, differentiated, stem cells
	pCDH-EF1-MCS-T2A-Puro	CD527A-1	EF1		
	pCDH-EF1-MCS-T2A-copGFP	CD526A-1	EF1		
	pCDH-MCS-T2A-Puro-MSCV	CD522A-1	MSCV	High	Hematopoietic/ stem cells
	pCDH-MCS-T2A-copGFP-	CD523A-1	MSCV		
	pCDH-CMV-MCS	CD500B-1	CMV	High	commonly used cell lines (e.g.

pCDH-CMV-MCS2	CD501A-1	CMV		HeLa, HEK293, HT1080) H1299)
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<http://www.systembio.com/lentiviral-technology/expression-vectors/cdna/vector-maps-single>

2. Dual Promoter Vectors

These lentivectors are characterized by the presence of two independent mammalian promoters, one driving the gene of interest cloned into the MCS and the other driving the expression of a marker (e.g. copGFP, Puro, or GFP-T2A-Puro). Since the gene of interest and the marker(s) are driven independently, their expression may differ depending on promoter activity. These vectors are typically used to assess transduction efficiency or generate stably selected cell lines independent of the gene of interest. Vector maps and additional information can be found here:

<http://www.systembio.com/lentiviral-technology/expression-vectors/cdna/vector-maps>

	cDNA vectors	Catalog #	target gene promoter	Expression level	Application
Dual Promoter	pCDH-CMV-MCS-EF1-Puro	CD510B-1	CMV	High	commonly used cell lines (e.g. HeLa, HEK293, HT1080, H1299)
	pCDH-CMV-MCS-EF1-copGFP	CD511B-1			
	pCDH-CMV-MCS-EF1-RFP	CD512B-1			
	pCDH-CMV-MCS-EF1-copGFP-T2A-Puro	CD513B-1			
	pCDH-CMV-MCS-EF1-Neo	CD514B-1			
	pCDH-CMV-MCS-EF1-Hygro	CD515B-1			
	pCDH-CMV-MCS-EF1-RFP-T2A-Puro	CD516B-2			
	pCDH-UbC-MCS-EF1-Hygro	CD615B-1	UbC	Low	Long-term stable expression of transgene
	pCDH-MSCV-MCS-EF1-Puro	CD710B-1	MSCV	High	Hematopoietic/stem cells
	pCDH-MSCV-MCS-EF1-copGFP	CD711B-1			
	pCDH-MSCV-MCS-EF1-copGFP-T2A-Puro	CD713B-1			
	pCDH-EF1-MCS-pA-PGK-copGFP-T2A-Puro	CD550A-1	EF1	Medium	robust in most cell types, including primary, differentiated, stem cells

3. Bicistronic Vectors

Bicistronic vectors are vectors that generate multiple, native proteins from a single mRNA transcript. It is useful for applications where coupled transcription of a gene of interest with a marker (e.g. GFP) under the control of a single promoter is desired. One well-characterized approach for bicistronic vectors is the use of an Internal Ribosomal Entry Site (IRES) element (Jang 1988) which allows for translation of a marker gene of interest anywhere in the mRNA transcript by recruiting ribosomes to IRES sites to initiate translation in a cap-independent manner.

Note: The translation of the ORF downstream of the IRES element may show significant differences in expression depending on the cell type where the construct is being expressed.

	cDNA vectors	Catalog #	target gene promoter	Expression level	Application
Bicistronic	pCDH-EF1-MCS-IRES-copGFP	CD530A-2	EF1	Medium (EF1) Low to High (IRES)	robust in most cell types, including primary, differentiated, stem cells

	pCDH-EF1-MCS-IRES-RFP	CD531A-2			
	pCDH-EF1-MCS-IRES-Puro	CD532A-2			
	pCDH-EF1-MCS-IRES-Neo	CD533A-2			
	pCDH-MSCV-MCS-IRES-copGFP	CD731A-1	MSCV	High (MSCV) Low to High (IRES)	hematopoietic/ stem cells
	pCDH-UbC-MCS-IRES-copGFP	CD630A-1	UbC	Low (UbC) Low to High (IRES)	Long-term stable expression of transgene

4. Bidirectional Promoter Vectors

Divergently transcribed genes contribute to more than 10% of the human genome. This signifies that the bidirectional promoters are very prevalent and these promoters are responsible for coordinated expression of genes in the cell (Adachi et al. 2002, Golding et al. 2011). Coordinated expression of more than one transgene is very important for studying gene or non-coding RNA function and regulation or in gene therapy (Kay et al. 2001). IRES based vectors or bicistronic vectors offer that opportunity, however it has been shown that the second gene in the IRES based vector gets compromised by the expression of the first gene (Mizuguchi et al. 2000) and promoter interference is often a factor in bicistronic vectors (Curtin et al. 2008, Callen et al. 2004). Self cleaving peptides are routinely used to express two or more transgene but it has been shown that these peptides can sometimes affect protein function and stability.

SBI offers a synthetic bi-directional promoter with EF1 and PGK in a divergent configuration coordinating the regulation of two or more different transgenes. This promoter configuration takes advantage of the natural bidirectional activity of the PGK promoter and the divergent configuration is stable and resistant to silencing in embryonic stem (ES), trophectoderm stem (TS) and extraembryonic endoderm (XEN) stem cells (Shin et al. 2006, Stegmeier et al. 2005, Golding et al. 2011).

The multiple cloning site (MCS) in the positive orientation of the bi-directional promoter which allows for convenient cloning of your gene or non-coding RNA of interest. The SV40 and BGH polyadenylation signals in both end of the vector enables efficient termination of transcription, resulting in high levels of steady-state expression. In the negative orientation of your expression cassette is the expression of the Puro, copGFP or GFP-T2A-Puromycin marker genes.

	cDNA vectors	Catalog #	target gene promoter	Expression level	Application
Bidirectional Promoter	pCDH-EF1-MCS-(PGK-Puro)	CD810A-1	EF1	Medium/High (with PGK)	robust in most cell types, including primary, differentiated, stem cells
	pCDH-EF1-MCS-(PGK-copGFP)	CD811A-1			
	pCDH-EF1-MCS-(PGK-copGFP-T2A-Puro)	CD813A-1			
	pCDH-EF1-MCS-T2A-RFP-(PGK-Puro)	CD822A-1			
	pCDH-EF1-MCS-T2A-copGFP-(PGK-Puro)	CD823A-1			

Benefits and Features

- Coordinated expression of two genes or reporters
- Combining the expression of cDNAs or non-coding RNAs along with selectable markers
- Reduced promoter interference
- Lentivirus-based expression system ensures efficient expression of genes or non-coding RNA in wide range of cell lines including nondividing or difficult-to-transfect cell lines.
- Replication incompetent HIV and FIV-based lentiviral expression systems are biologically safe.

Validation Data

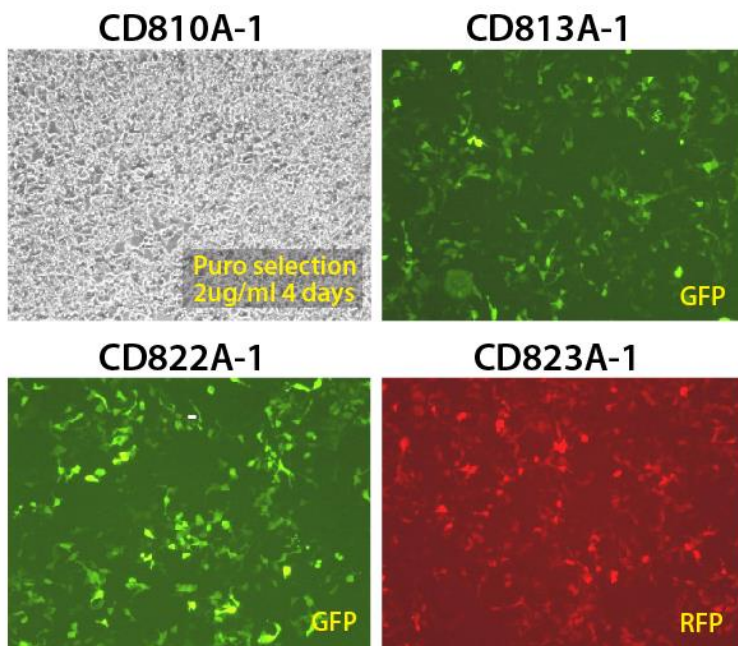


Figure 1 – Representative images for GFP/RFP/Puro marker expression in HT1080 cells transduced with pseudoviral particles containing individual bidirectional constructs

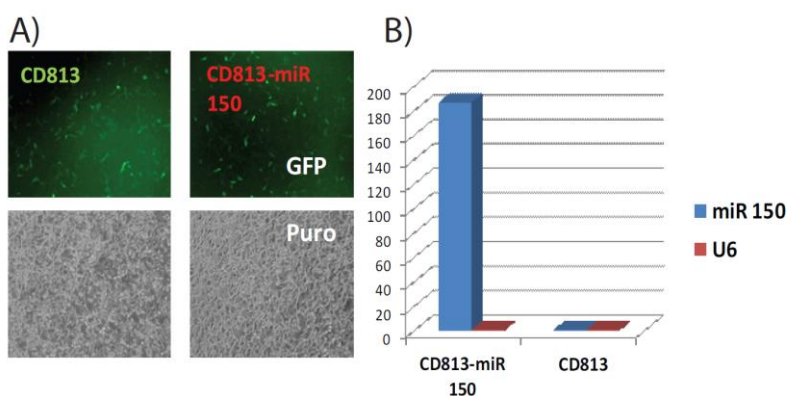


Figure 2 – Data for expression of human microRNA-150 cloned into CD813 bidirectional lentivector construct. A) Representative images for marker expression in HT1080 cells transduced with pseudoviral particles B) qPCR data showing overexpression of miR-150 normalized to U6 internal control in CD813A-1 bidirectional vector

D. Additional Considerations & Features

Choice of Promoter

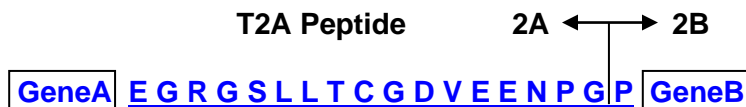
The major concern of cDNA expression in lentivectors is the efficiency level and stability of expression in target cell lines. The Cytomegalovirus (CMV) promoter is a strong and most commonly used viral promoter that constitutively expresses downstream genes. While the CMV promoter works perfectly in the most common cell lines, it shows poor expression in some stem cell lines and hematopoietic cell lines (R.F. Doll, 1996; E.D. Papadakis, 2004). The housekeeping elongation factor 1 α (EF1) promoter has been shown to exceed and outlast CMV-mediated expression in retroviral, lentiviral, and adenoviral vectors, in hematopoietic cell lines (K. Tokushige 1997; H. Nakai, 1998; C. Teschendorf, 2002). EF1 also performs well in most common cell lines.

MSCV promoter is the 5'-LTR promoter of murine stem cell virus. When a portion of the U3 region of the 3' HIV LTR was replaced with the U3 region of MSCV LTR, the resulted hybrid HIV/MSCV LTR has dramatically increased the transgene expression level in human CD34+ hematopoietic cells (J.K. Choi, 2001). After integration into genomic DNA, this promoter transcribes a long transcript with an intron in the 5'UTR flanked with splice donor and acceptor sites derived from the lentiviral vector. Further studies found that additional CpG mutations in the MSCV LTR reduced transcriptional silencing in embryonic stem cells (C.S. Swindle, 2004). We constructed cDNA expression vectors with the CpG-deficient MSCV incorporated into the 3' HIV LTR. After integration into genomic DNA, 3'MSCV/LTR will replace the 5'LTR and provide a high level of expression of the target gene and reporter gene downstream.

2A Peptide-enabled dual expression system

Coexpression of a reporter gene together with a gene of interest is a useful approach for selecting transfected or transduced cells. This is commonly achieved by using two independent internal promoters, such as CMV and EF1 in pCDH-CMV-MCS-EF1-copGFP, or by linking two transgenes with an internal ribosomal entry site (IRES) element in a single bicistronic transcript. Many dual promoter pairs have shown a high level of expression of both transgenes in standard cell lines—however, promoter interference often occurs in some cell lines. There are also two main problems that limit the use of IRES: the large size and the imbalanced expression between the first and second cistrons (H. Mizuguchi, 2000; X.Yu, 2003).

The “self-cleaving” 2A peptides have been used successfully to generate multiple proteins from a single promoter in many applications (P. de Felipe, 2004; M.J. Osborn, 2005; P. de Felipe, 2006). The 2A-like sequences exist in several viruses and are used to mediate protein cleavage from a single open reading frame. Through a ribosomal skip mechanism, the 2A peptide prevents normal peptide bond formation between the 2A glycine and the 2B proline without affecting the translation of 2B (M.L. Donnelly, 2001):



SBI's cDNA expression vectors incorporate the 2A-like sequence (T2A) from the insect virus *Thosea asigna* to mediate the coexpression of a reporter gene with the target cDNA. Reporter genes have been cloned at either the first or second positions, and we achieved high expression levels at both locations (see Figure 1).

A.

B.

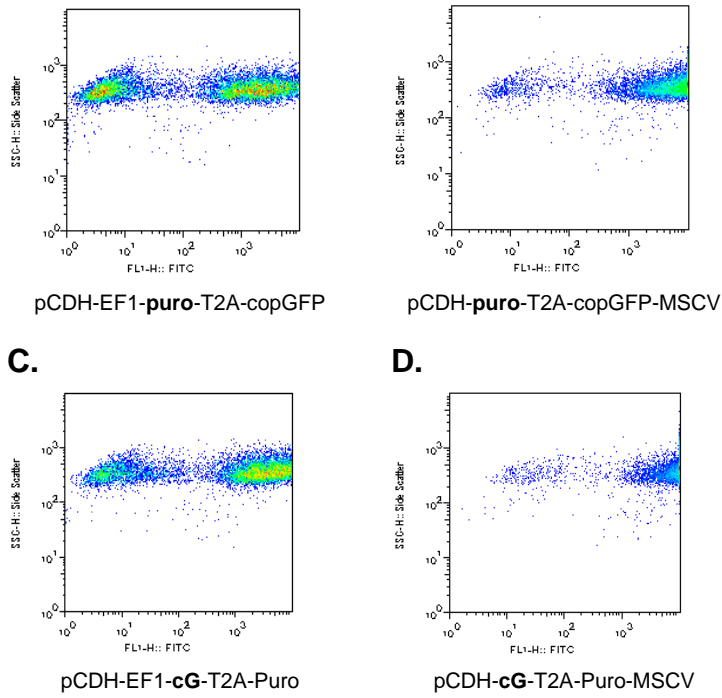


Fig. 1. Flow cytometry analysis of HT1080 cells transduced with dual reporter constructs. The puromycin-resistance gene (puro) was cloned into pCDH-EF1-MCS-T2A-copGFP (**A**) and pCDH-MCS-T2A-copGFP-MSCV (**B**); and the copGFP gene (cG) was cloned into pCDH-EF1-MCS-T2A-Puro (**C**) and pCDH-MCS-T2A-Puro-MSCV (**D**). The resulting dual reporter constructs were packaged into pseudoviral particles followed by transduction into HT1080 cells. All constructs were also puromycin resistant (data not shown).

The HIV-1 derived pCDH vectors contain the following common features:

- **Multiple Cloning Site (MCS)**—for cloning the gene of interest in the MCS located downstream of the CMV 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.
- **Hybrid RSV/5LTR promoter**—provides a high level of expression of the full-length viral transcript in producer 293 cells.
- **Genetic elements (cPPT, gag, env, LTRs)**—necessary for packaging, transducing, and stably integrating the viral expression construct into genomic DNA.
- **SV40 origin**—for stable propagation of the pCDH 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.

E. List of Components

Component

pCDH cDNA Expression Vector

Please refer to Product Analysis Certificate accompanying the product for exact concentration and the total amount. All plasmids are shipped in dry ice or blue ice and should be stored at -20°C upon receipt. Properly stored plasmids are stable for 12 months from the date received.

F. Additional Required Materials

For Cloning

- Restriction enzymes for digestion of the vectors and/or inserts
(Recommended: New England BioLabs enzymes)
- High Fidelity Long-distance PCR enzymes
- T4 DNA Ligase and ligation reaction buffer
(Recommended: New England BioLabs T4 DNA Ligase (400 U/μl), Cat. # M0202S. Dilute to 40 U/μl in 1X ligation buffer with the provided 10X buffer just before use)
- High efficiency competent *E. coli* cells (RecA⁻)
(Recommended: MaxEfficiency Stbl2 competent cells, Life Tech (Cat # 10268-019) or One Shot OmniMAX 2 T1R competent cells, Cat. # C854003)
- 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
- 2-3% 1X TAE Agarose gel

For Purifying cDNA Constructs after Cloning

- Plasmid purification kit
(Recommended: QIAGEN Endofree Plasmid Maxi Kit, Cat. # 12362. The following kit combination can be used for Midi scale (up to 200 μg of plasmid DNA) preparation of endotoxin-free DNA:
 - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048Please visit the QIAGEN website to download the specialized protocol that is not contained in the current user manual:
 - <http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf>

For Transfection of pCDH Constructs into Target Cells

- Transfection Reagent
(Recommended: Invitrogen Lipofectamine 2000, Cat. # 11668-027)

For Packaging of pCDH Constructs in Pseudoviral Particles

- In order to package your pCDH cDNA constructs into VSV-G pseudotyped viral particles, you will need to purchase the pPACKH1 Lentivector Packaging Kit (Cat. # LV500A-1). The protocol for packaging and transduction of packaged pseudoviral particles is provided in the Lentivector Expression Systems 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 (U.S. patents # 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'ΔLTR 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 all the time 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 pCDH vectors are integrated into genomic DNA and could have a risk of insertional mutagenesis.

II. Protocol

The following section provides general guidelines for the cloning of cDNA, amplified by PCR, into pCDH vectors.

A. cDNA Amplification

Full length cDNA fragments can be recloned from another plasmid or amplified by PCR. PCR-based cloning is the most convenient way for full-length cDNA cloning in pCDH vectors. The cDNA lentivector does not contain an ATG initiation codon. A translation initiation sequence must be incorporated in the insert cDNA if the cDNA fragment to be cloned does not already have an ATG codon. We also recommend including a Kozak sequence (*i.e.* GCCACC) before the ATG for optimal translation. For amplification of the target cDNA fragment, design a 5'-primer (containing a Kozak sequence and ATG codon) and 3'-primer with unique restriction sites present in the MCS of the pCDH vector but not present in the cDNA sequence. Amplify the cDNA fragment by high fidelity long-distance PCR using about 200 ng of plasmid template DNA and a minimum number of cycles (usually 12-15 cycles), purify, digest the amplified product with end-specific restriction enzyme(s) and purify the digested PCR product in a 1.2% agarose gel to prevent contamination with the original plasmid used for amplification.

B. Primer Design for Cloning into Vectors with T2A Sequence

Since the gene of interest and the reporter gene in cDNA expression vectors containing a T2A peptide sequence will form one open-reading frame, extra attention should be paid when designing the 3' primer for amplifying the target sequence. First of all, do not include a stop codon at the 3' end of target sequence—this would prevent the expression of the reporter gene; secondly, place the target sequence in-frame with the 2A peptide. The ATG start site of the insert that cloned into the MCS must be in-frame with the GAG of the T2A sequence. Please verify the cloning strategy to ensure that this will occur prior to cloning.

C. Preparation of Digested pCDH Vector

Digest the pCDH vector with the corresponding restriction enzymes used in the preparation of the cDNA fragments, and then verify complete digestion of the vector by agarose gel electrophoresis. We suggest that you perform only preparative gel purification of the digested vector if more than one restriction enzyme is used. If you use a single restriction enzyme, dephosphorylation as well as gel purification of the vector is necessary to reduce the background in the vector ligation step.

D. Cloning of cDNA into pCDH Vector

The optimal insert-to-vector molar ratio may be different for different inserts. Always try at least two different ratios (e.g., 10:1 and 30:1) for each experiment. Also make sure to include one negative control reaction, which contains only the digested vector.

1. Ligation of cDNA to Vector

- a. Dilute the gel-purified, digested vector to 10 ng/ μ l.
- b. Set up 10 μ l ligation reactions for each sample and control as follows:

1.0 μ l	Digested pCDH Vector (10 ng/ μ l)
7.0 μ l	cDNA insert (usually 30-50 ng) or Nuclease-free water
1.0 μ l	10X T4 DNA Ligase Buffer
1.0 μ l	T4 DNA ligase (40 U/ μ l)
<hr/>	
10.0 μ l	Total volume

- c. Incubate the ligation reactions at 16°C for 1-2 hrs if it is sticky-end ligation. For blunt-end ligation, use an overnight incubation.

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

Transform competent cells** (with a transformation efficiency of at least 1×10^9 colonies/ μ g pUC19) with the whole ligation reaction (10 μ l) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin agar plates.

****Note: We recommend using Stbl2 or OmniMax 2 T1R competent cells for transformation and propagation of the lentivector construct to avoid unwanted lentivector recombination events.**

3. Identify Clones with the cDNA Insert

- a. Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly pick 5 or more well-isolated colonies and grow each clone in 100 μ l of LB Broth with 75 μ g/ml ampicillin at 30°C for 2 hours with shaking.
- b. Use 1 μ l of each bacterial culture for screening cDNA inserts by PCR and continue to grow the culture for another 4 hours. Store the culture at 4°C.

Prepare a PCR Master Mix with PCR primers flanking the cDNA insert:

<u>1 rxn</u>	<u>10 rxn</u>	<u>Composition</u>
0.5 μ l	5 μ l	PCR primer 1 (10 μ M)
0.5 μ l	5 μ l	PCR primer 2 (10 μ M)
0.5 μ l	5 μ l	50X dNTP mix (10 mM of each)
2.5 μ l	25 μ l	10X PCR Reaction Buffer
19.5 μ l	195 μ l	Nuclease-free water
0.5 μ l	5 μ l	Taq DNA polymerase (approx. 5 U/ μ l)
24.0 μ l	240 μ l	Total volume

- c. Mix the master mix very well and aliquot 24 μ l into each well of 96-well PCR plate or individual tubes.
- d. Add 1 μ l of each bacterial culture from step (b) into each well (or tube).
- e. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min/1 kb*	25 cycles
68°C, 3 min	1 cycle

* depending on the size of final PCR product, use a shorter or longer time.
- f. Take 5 μ l of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer to identify clones with correct insert.

Grow a positive clone with the cDNA insert in an appropriate amount of LB-Amp Broth, and purify the construct using an endotoxin-free plasmid purification kit (see Section I.E).

Confirm identity of the cDNA insert by sequence analysis of the construct using the one of the PCR primers. Alternatively, you may use one of the following sequencing primers which are located upstream of the MCS:

Vectors with CMV: 5'-CACGCTGTTTTGACCTCCATAGA-3'
 Vectors with EF1: 5'-CTCCACGCTTTGCCTGACCCTGCTT-3'
 Vectors with MSCV: 5'-GGGGTACAGTGCAGGGGAAAGAAT-3'

E. Packaging of the pCDH Expression Constructs into Pseudoviral Particles

If you are planning to create a stably transduced cell line expressing your gene of interest, you first need to package the cDNA lentiviral construct into lenti pseudoviral particles. For this purpose, you will need to purchase the pPACKH1 Lentivector Packaging Kit from SBI (see Appendix). Figure 3 schematically shows all steps which need to be performed in order to generate pseudoviral packaged cDNA expression constructs.

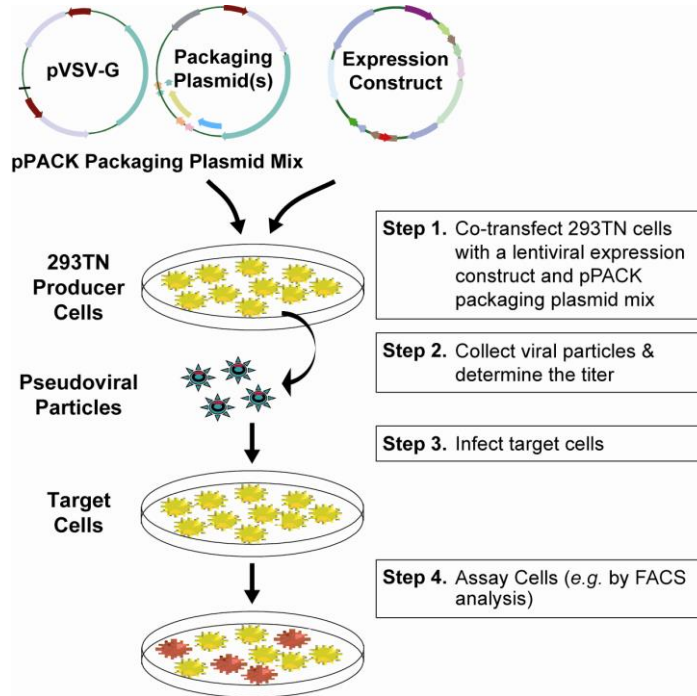


Fig. 2. Schematic presentation of the packaging procedure for lentivector expression constructs and making of stable cell lines.

The Lentivector Expression System User Manual includes the procedural information for packaging and transducing the expression constructs. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the lentiviral construct using standard transfection and selection protocols, transduction of the lentiviral cDNA construct using packaged pseudoviral particles is the most efficient way to deliver cDNA constructs in a wide range of cells, including dividing, non-dividing, and hard-to-transfect cells.

III. Troubleshooting

A. Large number of colonies on negative control plate

If you see that the colony number on the negative control plates (with no insert) is equal or more than on the plate with the cDNA sample, there is probably undigested plasmid contamination. Check your digestion conditions, and repeat digestion with an increased concentration of restriction enzyme(s) or use a longer reaction time. For best results, gel-purify and dephosphorylate the vector after single enzyme digestion. Also, check the sequences of the PCR primers in order to be sure that the necessary restriction sites are present.

B. No or low number of colonies on plate with cDNA sample

The efficiency of cDNA cloning into the pCDH vector depends on many factors, including size, purity, integrity, modification of insert, selection of restriction sites, etc. If your cDNA sample ligation resulted in only a few colonies, please continue with PCR screening first. If none of these few colonies has the right insert, or you did not get any colonies at all, it may be caused by:

1. Inappropriate ratio of insert-to-vector

Not enough or too much insert could inhibit the ligation reaction. Try a different ratio of insert-to-vector to optimize the ligation reaction. Sometimes, the yield of the ligation reaction may also be improved by increasing both the insert and vector amounts.

2. Low ligation efficiency

- | | | |
|---|---|--------------------------------|
| a. Inactive ligase and /or ligase reaction buffer | Test your ligase and reaction buffer for activity using different reagents if they are proven inactive. | vector and insert. Replace the |
| b. Ligation inhibitors are present | EDTA and high salt may inhibit the ligation reaction. | |

3. Low transformation efficiency

- | | | |
|--|---|-----------|
| a. Low quality or poor handling of competent be tested by transforming | 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 cell competency. Use competent cells with a transformation efficiency of at least 1×10^9 colonies/ μg of pUC19 plasmid. | cells may |
| b. Wrong antibiotic or too much antibiotic in the media | The plates used for cloning should contain 50-100 $\mu\text{g}/\text{ml}$ ampicillin in the media. | |

C. No correct cDNA inserts

If the colony number for the cDNA sample is more than for the negative control sample (*i.e.* vector only), but you failed to amplify cDNA insert, it could be that:

1. Inactive Taq polymerase

Test the activity of the PCR master mix by amplifying cDNA from the original template. Replace the PCR reagents if they are proven inactive.

2. Wrong primer was used

Make sure you are using the correct primers for the specific orientation of cDNA insert.

3. Not enough clones were screened

Pick more colonies for screening.

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V. Appendix

A. Maps and Features for pCDH Vectors

For the latest annotated vector maps and features for all of the pCDH vectors, please visit our website at

<http://www.systembio.com/lentiviral-technology/expression-vectors/cdna/overview>

Additionally, the latest vector sequence files for all of the pCDH vectors can be requested by emailing tech@systembio.com.

B. Descriptions of Features in pCDH Vectors

Feature	Function
3' ΔLTR (ΔU3)	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
Amp^R	Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>
CMV promoter	Constitutive Human cytomegalovirus (CMV) promoter for transcription of reporter and/or cloned cDNA insert
copGFP	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
cPPT	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
EF1 promoter	Constitutive Elongation factor 1α promoter for transcription of reporter and/or cloned cDNA insert
env	Packaging signal
gag	Packaging signal
MSCV	Constitutive LTR enhancer/promoter of murine stem cell virus (MSCV) for transcription of reporter and/or cloned cDNA insert
pUC ORI	Allows for high-copy replication in <i>E. coli</i>
Puro^R	Puromycin-resistant marker for selection of the transfected/transduced cells
PGK promoter	Phosphoglycerate Kinase promoter for transcription of reporter genes in pCDH lentivectors
RRE	Rev response element binds gag and involved in packaging of viral transcripts
RSV / 5'LTR	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
SV40 ORI	Allows for episomal replication of plasmid in eukaryotic cells
SV40 Poly-A	Transcription termination and polyadenylation
T2A Peptide	The “self-cleaving” 2A peptide mediates protein cleavage from a single open reading frame to generate multiple proteins from a single promoter
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element—enhances the stability of the viral transcripts

C. Properties of the copGFP Fluorescent Protein

The pCDH copGFP Vectors contain the full-length copGFP gene with optimized human codons for high level of expression of the fluorescent protein from the CMV, EF1, or MSCV promoter in mammalian cells. The copGFP marker is a novel natural green monomeric GFP-like protein from copepod (*Pontellina sp.*). The copGFP protein is a non-toxic, non-aggregating protein with fast protein maturation, high stability at a wide range of pH (pH 4-12), and does not require any additional cofactors or substrates. The copGFP protein has very bright fluorescence that exceeds at least 1.3 times the brightness of EGFP, the widely used *Aequorea victoria* GFP mutant. The copGFP protein emits green fluorescence with the following characteristics:

emission wavelength max – 502 nm;
excitation wavelength max – 482 nm;
quantum yield – 0.6;
extinction coefficient – 70,000 M⁻¹ cm⁻¹

Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of lentivector constructs into cells.

D. 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.
- **FIV-Based pCDF cDNA Cloning and Expression Vectors**
 - **pCDF1-MCS1** (Cat. # CD100A-1)
 - **pCDF1-MCS2-EF1-Puro** (Cat. # CD110B-1)
 - **pCDF1-MCS2-EF1-copGFP** (Cat. # CD111B-1)
- **RNAi Cloning and Expression Lentivectors**
These FIV and HIV-based single- and double-promoter shRNA and siRNA cloning vectors allow you to clone siRNA templates and efficiently transduce these siRNA constructs in a wide range of cells. For a list of currently available vectors, please visit our website at <http://www.systembio.com>.

- **MicroRNA Precursor Construct Collection**

FIV-based microRNA Precursor Constructs allow you to express pre-miRNA, consisting of the stem loop structure and upstream and downstream flanking genomic sequence. For a list of currently available vectors, please visit our website at <http://www.systembio.com>.

- **PathNet™ Transcriptional Reporter Lentivectors**

FIV and HIV-based transcriptional reporter vectors, allow detection of the activation of transcriptional factors (TFs) in a natural environment (nuclei). For a list of currently available vectors, please visit our website at <http://www.systembio.com>.

E. 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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General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the pCDH cDNA Cloning and Expression Vector (*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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This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

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CMV Promoter

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

CopGFP Reporter

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