GE Healthcare

Technical Manual

Dharmacon™ RNAi Consortium (TRC) Lentiviral shRNA

Product Description:

The TRC Lentiviral shRNA Library is the result of a collaborative research effort based at the Broad Institute of MIT and Harvard, and included six MIT and Harvard associated research institutions and five international life sciences organizations. The goal of TRC was to create lentiviral shRNA libraries targeting 15,000 human and 15,000 mouse annotated genes with multiple constructs per gene. We have partnered with the TRC to make these shRNA libraries available to researchers worldwide.

Shipping And Storage:

All constructs are shipped as bacterial cultures of *E. coli* (DH5α) in 2x LB-Lennox broth (low salt) medium with 8% glycerol, 100 µg/mL carbenicillin. Individual constructs are shipped on wet ice and collections are shipped on dry ice in a 96-well plate format. All constructs should be stored at -80 ˚C. All cultures are checked for growth prior to shipment. **To allow any CO_2 that may have dissolved into the medium from the dry ice during shipping to dissipate, please store plates at -80 °C for at least 48 hours before thawing.**

Important Safety Note:

Please follow the safety guidelines for use and production of vector-based lentivirus as set by your institution's biosafety committee.

- For glycerol stocks of *E. coli* containing lentiviral plasmids, BSL1 guidelines should be followed
- For handling and use of lentiviral products to produce lentiviral particles, BSL2 or BSL2+ guidelines should be followed
- For handling and use of lentiviral particle products, BSL2 or BSL2+ guidelines should be followed

Design Information:

Dharmacon TRC Lentiviral shRNA Library Design:

The shRNA constructs were designed to include a hairpin consisting of a 21 base paired stem (sense and antisense strands) separated by a 6 base loop. Each hairpin sequence was cloned into the lentiviral vector (pLKO.1) and sequence verified. Multiple constructs (4-5) were created per gene to ensure adequate coverage of the target gene. The TRC predicts that 1 or 2 out of the 4-5 constructs offered per gene are expected to give at least 70% knockdown.

Features of TRC Lentiviral shRNA Library include

- Rules-based shRNA design for efficient gene knockdown
- Already cloned into lentiviral vectors
- Amenable to in vitro and in vivo applications such as the creation of stable cell lines
- Lentiviral vector enables transduction of primary and non-dividing cell lines
- Broad coverage: 4-5 constructs per gene

TRC Hairpin Design

Stem: 21 base pairs

Loop: 6 bases, XhoI restriction site: CTCGAG

Flanking = 5' CCGG overhang for AgeI

3' TTTTT termination for Pol III and AATT overhang for EcoRI

Vector Information:

The pLKO.1 HIV-based lentiviral vector (Figures 1-2, Table 1) allows for transient and stable transfection of shRNA and also the production of viral particles using lentiviral packaging systems. Stable cell lines can be selected using the puromycin selectable marker.

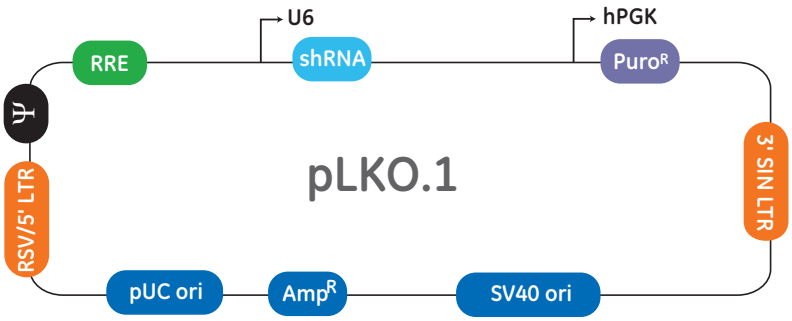


Figure 1. The pLKO.1 vector.

Table 1. Features of the pLKO.1 vector.

Vector Element	Utility
U6	Human U6 (RNA polymerase III) promoter provides high level of expression in the target cells
shRNA	Simple stem-loop shRNA for gene knockdown
hPGK	Human phosphoglycerate kinase promoter drives expression of the puromycin resistance gene
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
RSV/5' LTR	RSV promoter/5' long terminal repeat promotes strong lentiviral transcription in the packaging cells in the absence of Tat
3' SIN LTR	3' self-inactivating long terminal repeat for increased lentivirus safety
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev response element enhances titer by increasing packaging efficiency of full-length viral genomes

Vector Map:

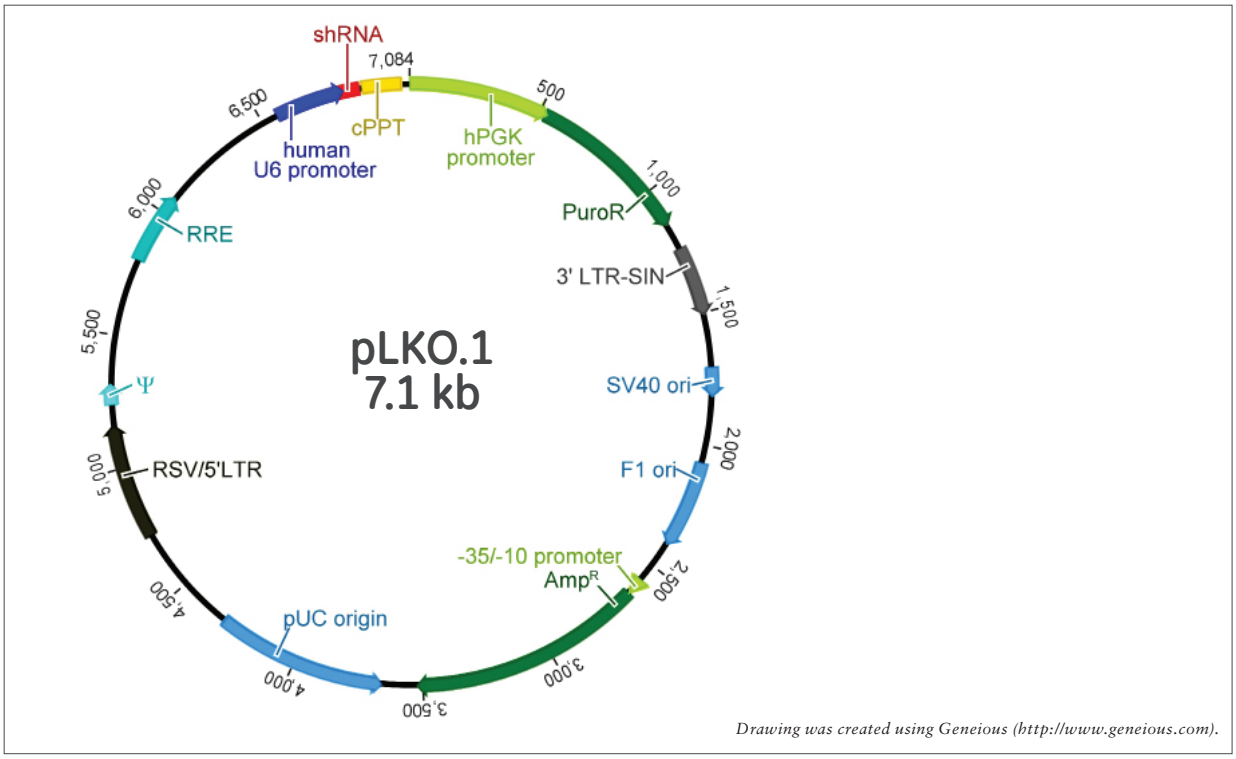


Figure 2. Map of the pLKO.1 vector.

Antibiotic Resistance:

pLKO.1 contains two antibiotic resistance markers (Table 2). TRC recommends the use of carbenicillin instead of ampicillin for the growth and maintenance of pLKO.1 in bacterial cells.

Table 2. Antibiotic resistances conveyed by pLKO.1.

Antibiotic	Concentration	Utility
Carbenicillin	100 µg/mL	Bacterial selection marker (outside LTRs)
Puromycin	Variable	Mammalian selection marker

Culturing Protocols and Maintenance of pLKO.1:

TRC shRNA Library is constructed in the pLKO.1 vector. This vector allows for both transient and stable gene silencing through the mechanism of RNA interference (RNAi). The vector is capable of producing self-inactivating lentiviral particles when used in conjunction with an appropriate lentiviral packaging system.

In order to obtain a good yield of cells in a short period of incubation, rich medium containing carbenicillin should be used to culture pLKO.1 constructs. An incubation period of 14-20 hours at 37 °C with aeration is sufficient. It is recommended that the glycerol stocks remain frozen at -80 °C when not in use. Freeze/thaw cycles do not seem to have any detrimental effects, provided the glycerol stocks are not incubated at room temperature or higher for long periods of time.

Protocol I – Replication:

Table 3. Materials and reagents for plate replication.

Item	Fisher Scientific Cat #
LB-Lennox Broth (low salt)	BP1427500
Peptone, granulated, 2 kg – Difco	BP9725-2
Yeast Extract, 500 g, granulated	BP1422-500
NaCl	BP3581
Glycerol	BP2291
Carbenicillin	BP2648-250
Puromycin	BP2956-100
96-well microplates	12-565-363
Aluminum seals	12-565-475
Disposable 96 pin replicators	NC9584102

2x LB broth (low-salt) medium preparation*

LB-Lennox Broth	20 g/L
Peptone	10 g/L
Yeast Extract	5 g/L

Appropriate antibiotic(s) at recommended concentration(s). Glycerol 8% for long term storage.**

**1x LB medium can be used instead of 2x LB medium.*

***Glycerol can be omitted from the medium if you are culturing for plasmid preparation. If making copies of the constructs for long-term storage at -80 °C, 8% glycerol is required.*

Replication of Plates

Prepare target plates by dispensing ~ 160 µL of 2x LB broth supplemented with 8% glycerol and appropriate antibiotic (100 µg/mL of carbenicillin).

Prepare Source Plates

Carefully remove foil seals while the source plates are still frozen. This minimizes cross-contamination. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.

Replicate

Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the plate of the well.

1. Gently remove the replicator from the source plate, carefully place in the target plate and mix in the same manner to transfer cells.
2. Dispose of the replicator.
3. Place the lids back on the source plates and target plates.
4. Repeat steps 1-4 until all plates have been replicated.
5. Return the source plates to the -80 °C freezer.
6. Place the inoculated target plates in a 37 °C incubator for 14-20 hours.
   Note: Due to the tendency of viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your glycerol stock for each plasmid preparation.

Protocol II – Plasmid Preparation:

Culture Conditions For Individual Plasmid Preparations

Most plasmid mini-prep kits recommend a culture volume of 1-10 mL for good yield. For shRNA constructs, 5 mL of culture can be used for one mini-prep generally producing from 5-20 µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNA of interest, store at -80 °C until ready to begin.
2. To prepare plasmid DNA, first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Using a sterile loop or a pipette tip, streak the shRNA culture onto a LB agar plate containing 100 µg/mL carbenicillin. Return the glycerol stock(s) to -80 °C. Incubate the plate overnight at 37 °C.
4. The following day, pick 3 to 5 colonies from the agar plate and inoculate 6 mL of the 2x LB broth medium. Incubate at 37 °C for 16-20 hours with vigorous shaking (300 rpm).

5. The following day remove 1 mL of the culture and place in a sterile 2 mL sterile microcentrifuge tube. Place this tube at 4 °C until the plasmid DNA from the remaining culture has been analyzed. Pellet the remaining 5 mL culture and begin preparation of plasmid DNA. We recommend preparing ultra-pure DNA to ensure both high-purity and low endotoxin levels as required for transfection into eukaryotic cells.
   Note: If you wish to continue at a later time, cell pellets can be kept frozen at -20 °C overnight.
6. Run 3-5 µL of the plasmid DNA on a 1% agarose gel. The uncut pLKO.1 shRNA constructs run at about ~ 7-10 kb (Figure 3). Prepare an 8% glycerol stock culture using the 1 mL of culture you removed prior to plasmid preparation. This culture can be used for future plasmid preparations, but it is still recommended you streak the isolate and work from a fresh colony. Store the glycerol stocks at -80 °C.
   Note: Due to the tendency of viral vectors to recombine we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock or the colony glycerol stock for each plasmid preparation.

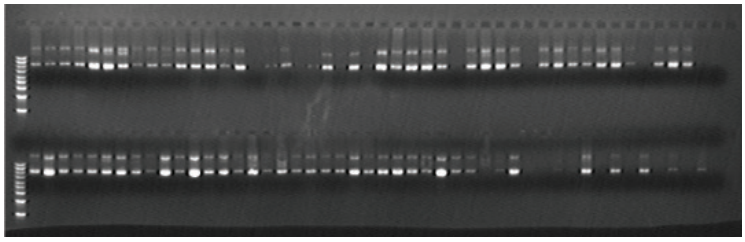


Figure 3. Gel image of 1.5 mL cultures of 92 different shRNA constructs after 20 hours of incubation at 37 °C with shaking. 2x LB broth (low-salt) medium with 8% glycerol was used for culturing

Protocol III – Restriction Digest:

You may wish to perform a restriction digest with the plasmid DNA following plasmid DNA preparation. Figure 4 displays expected results for a dual restriction enzyme digestion using BamHI and NdeI restriction enzymes for quality control of pLKO.1 vectors.

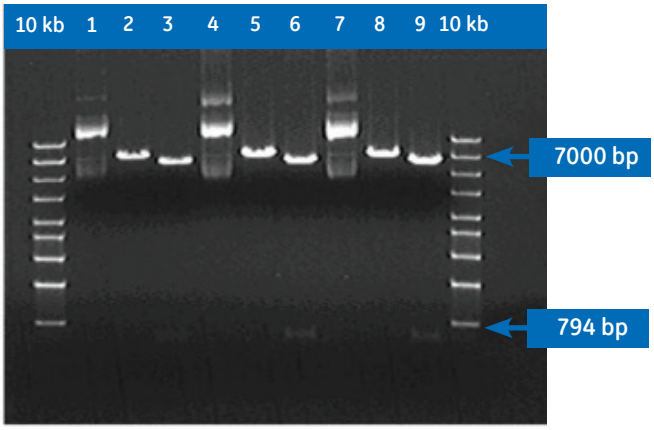


Figure 4. The 1% agarose gel above contains a 10 kb ladder, undigested sample, and restriction digests of three TRC shRNA clones.

The lanes are loaded as follows:
1 - Clone 1 Uncut plasmid.
2 - Clone 1 Cut with BamHI. Expected to linearize at 7032 bp.
3 - Clone 1 Cut with BamHI and NdeI. Band sizes of 6238 bp and 794 bp expected.
4-6 - Repeat of 1-3 only with clone 2.
7-9 - Repeat of 1-3 only with clone 1.

Protocol IV – General Protocol for Transfection of Adherent and Suspension Cells in a 24-well Plate:

Quantities and volumes should be scaled up according to the number of cells/wells to be transfected (Table 4).

1. In each well, seed ~ 5×10^4 adherent cells or ~ 5×10^5 suspension cells in 1 mL of growth medium 24 hours prior to transfection.
   Note: The recommended confluency for adherent cells on the day of transfection is 70-90%. Suspension cells should be in logarithmic growth phase at the time of transfection.
2. Dilute 1 µg of DNA in 100 µL of DMEM or other growth medium.
   Note: The transfection efficiency with Dharmacon™ TurboFect™ Transfection Reagent is equally high in the presence of serum. This is not the case with many other transfection reagents.
3. Briefly vortex TurboFect™ Transfection Reagent (Cat #R0531) and add 2 µL of it to the diluted DNA. Mix immediately by pipetting or vortexing.
4. Incubate 15-20 minutes at room temperature.
   Note: Prepare immediately prior to transfection. We recommend starting with 1 µg of DNA and 2 µL of TurboFect™ reagent per well in a 24-well plate (Table 4). Subsequent optimization may further increase transfection efficiency depending on the cell line and transgene used.
5. Add 100 µL of the TurboFect/DNA mixture drop-wise to each well. Do not remove the growth medium from the cells.
6. Gently rock the plate to achieve even distribution of the complexes.
7. Incubate at 37 °C in a CO_2 incubator.
8. Analyze transgene expression 24-48 hours later (Figure 5). Puromycin selection is recommended for optimal target silencing, and should be continued for 10-15 days if stable transfection is desired.
   Note: If puromycin selection is desired, it is recommended to perform a dose response curve experiment prior to beginning transfection to determine optimal antibiotic concentration conditions.

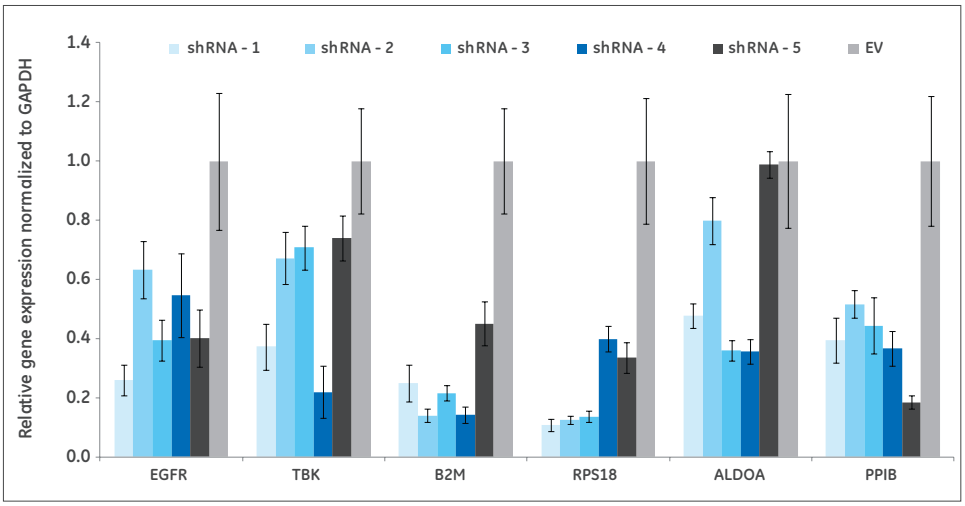


Figure 5. Gene silencing by TRC constructs in HeLa cells. HeLa cells were plated at 5,000 cells/well in 96-well format. The following day, TRC plasmids targeting six genes and five constructs per gene were transfected using the Dharmacon TurboFect Transfection Reagent (0.4 µL per well), and 24 hours later were puromycin selected (0.9 µg/mL) for five days. RT-qPCR (Thermo Scientific™ Maxima™ First Strand cDNA Synthesis Kit and Thermo Scientific™ Solaris™ Assays and Master Mix) was used to determine the level of gene silencing, normalized to empty vector (EV) and GAPDH as reference gene.

Cells Successfully Transfected with Dharmacon TurboFect Transfection Reagents include:

Permanently growing cell lines

Cos-7	african green monkey kidney cells
HeLa	human cervix adenocarcinoma cells
CHO	chinese hamster ovary cells
HEK293	human embryonic kidney cells
B50	rat nervous tissue neuronal cells
Calu1	human lung epidermoid carcinoma cells
RAW264	mouse leukaemic monocyte-macrophage cells
WEHI	mouse B cell lymphoma cells
MDCK	Madin Darby Canine Kidney cells
Raji	human Burkitt's lymphoma cells
COLO	human colon adenocarcinoma cells
Jurkat	human leukaemic T cells
Sp2/Ag14	mouse myeloma cells
HeLa S3	human cervix carcinoma cells
Hep2C	human larynx carcinoma cells
L929	mouse connective tissue fibroblasts
NIH/3T3	mouse embryo fibroblasts

Primary cell cultures

Rat fibroblasts
Mouse bone marrow derived dendritic cells
Mouse bone marrow derived macrophages
HLF human lung fibroblasts

Transfection Optimization Using Dharmacon TurboFect Transfection Reagent:

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells.

The most important parameters for optimization are DNA to transfection reagent ratio, DNA concentrations, and cell confluency. We recommend that you initially begin with the TurboFect reagent and DNA amount indicated in Table 4 and extrapolate the number of cells needed for your vessel size from the number of cells used in a well of a 24-well plate.

Additional Factors Influencing Successful Transfection:

1. Concentration and purity of nucleic acids — Determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. Transfection in serum containing or serum-free medium — Transfection using TurboFect Transfection Reagent can be performed in the presence or absence of serum. The transfection efficiency with TurboFect reagent is equally high in the presence of serum. This is not the case with many other transfection reagents.
3. Presence of antibiotics in transfection medium — Antibiotics do not interfere with either the DNA/TurboFect Reagent complex formation or cell transfection. This is not the case for other transfection reagents.
4. Cell history, density, and passage number — It is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before. However, adequate time should be given to allow the cells to recover from the passaging (generally ≥ 12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.
5. If the TurboFect reagent seems to be toxic to a particular cell line, try reducing the DNA : TurboFect reagent ratio.

Additional Protocols:

Further protocols recommended by TRC for culturing, plasmid prep, virus production, and transduction of TRC lentiviral shRNA constructs are available on the Broad Institute website, and can be accessed from the following link. See the following links for the protocols for titering virus by crystal violet, and performing a antibiotic dose response curve.

Validated Controls:

TRC eGFP shRNA is a positive control (Cat #RHS4459) designed against the enhanced GFP reporter (BD Biosciences Clontech Cat #6085-1; GenBank Accession #pEGFP U476561). This construct has been validated by TRC to produce knockdown of GFP fluorescence. TRC eGFP shRNA sequence is provided in pLKO.1, an HIV-based lentiviral vector and is expressed under the control of the U6 promoter. The GFP control sequence is as follows: 5'-TACAACAGCCACAACGTCTAT-3'.

The pLKO.1 empty vector (Cat #RHS4080) contains a 18 bp stuffer sequence between the AgeI and EcoRI restriction sites. The stuffer sequence is as follows: 5'-ACCGGACACTCGAGCACTTTTTGAATTC-3'

Packaging Lentivirus:

For packaging our lentiviral shRNA constructs, we recommend the Trans-Lentiviral shRNA Packaging System (Cat #TLP5912, TLP5917). The Trans-Lentiviral Packaging System allows generation of a replication-incompetent HIV-1-based lentivirus that can be used to deliver your shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging with our Trans-Lentiviral shRNA/ORF Packaging System, please see the product manual available on our website.

Reference:

Kappes J.C., Wu X. Safety considerations in vector development. *Somat. Cell Mol. Genet.* 26 (1-6), 147-58 (2001).

FAQs/Troubleshooting:

What clones are part of my collection?

A USB drive containing the data for this collection will be shipped with each collection. This file contains the location and accession number for each construct in the collection.

Where can I find the sequence of an individual shRNA construct?

If you are looking for the sequence of an individual shRNA construct, you can use the gene search. Just enter the catalog number or clone ID of the TRC clone into the search field, hit submit and then click on the query result. If you click on the "+" sign, you will find annotation of the target sequence listed in the "Targets" tab and the mature antisense sequence for each shRNA hairpin listed in the "Sequence" tab. For additional assistance, please contact Technical Support at ts.dharmacon@ge.com.

Can I use ampicillin instead of carbenicillin?

No. The TRC and the Broad Institute suggest that carbenicillin be used with the pLKO.1 vector. Constructs grown in ampicillin tend to not produce high plasmid yield.

Should I use a second or third generation packaging cell line for packaging TRC constructs?

The pLKO.1 vector contains a chimeric 5′ LTR, so this vector can be packaged using either second or third generation packaging systems. However, second generation packaging is recommended and will result in higher titers than third generation. The Broad Institute and the TRC recommend use second generation packaging to make viral particles.

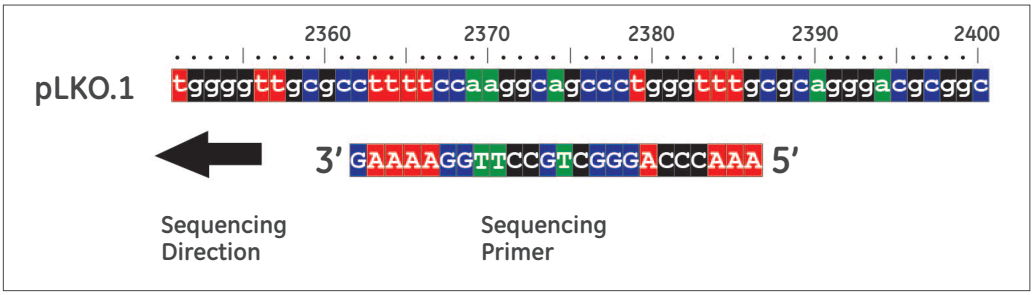
What restriction sites were used to clone the hairpin clone into the pLKO.1 vector?

The hairpins were cloned in at AgeI and EcoRI, but the AgeI site is usually destroyed upon ligation.

What is the sequencing primer for the pLKO.1 vector?

The pLKO.1 sequencing primer is: 5′-AAACCCAGGGCTGCCTTGGAAAAG-3′ 1540 R.

Using this primer the hairpin will be found approximately 180-260 bp into the read. Notice it is reading in the reverse orientation.



For help with transfection or transduction of your lentiviral constructs, please email technical support at ts.dharmacon@ge.com with the answers to the questions below, your sales order or purchase order number, and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What did the uncut and restriction digested DNA look like on a gel?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (the empty vector or the eGFP shRNA positive control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (such as RT-qPCR or western blot analysis)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using infection rather than transfection?
9. What was your viral titer?
10. What was your MOI?
11. Did you maintain the cells on puromycin after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

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OXFORD TECHNOLOGY

PCT application (filing date)	Priority Application(s) (filing date)	National Applications	Issued Patents
	US 07/586,603 (21 Sept 1990)	US 08/361,839	5,817,491
PCT/US91/05699 (9 Aug 1991) WO 92/14829	US 07/658,632 (19 Feb 1991)	EP 91915104.3 JP 3-514518 AU 84302/91 CA 2,104,396	0572401 3547129 663470 2,104,396
	US 07/1 70,515 (21 March 1988) US 07/1 70,515 (21 March 1988)	US Continuation 08/156,789 US Continuation 08/462,492 US Continuation 10/205,179	5,591,624 5,716,832 7,070,994
PCT/US91/06852 (20 Aug 1991) WO 92/05266	US 07/586,603 (21 Sept 1990)	AU 88424/91 AU Divisional 47984/96	665176 690427
PCT/GB97/02857 (17 Oct 1997) WO 98/1 7815	GB 9621680.9 (17 Oct 1996) GB 9624457.9 (25 Nov 1996)	EP 97909436.4 EP Divisional 00202432.1 US 09/224,014 US Divisional 09/91 5,169 US CIP 10/661,761 US Continuation 11/646,041 JP 10-519086 AU 47122/97 CN 97198767.X NZ 334860	0904392 6,312,682 6,669,936 7,198,784 725143 ZL97198767.X 334860
PCT/GB97/02858 (17 Oct 1997) WO 98/17816	GB 9621680.9 (17 Oct 1996)	GB 9903117.1 EP 97909437.2 US 09/284,011 JP 10-519087 AU 47123/97 CN 97198883.8 NZ 334522	2331522 6,235,522 737801 ZL971 98883.8 334522
PCT/GB97/02969 28 Oct 1997) WO 98/1 8934	GB 9622500.8 (29 Oct 1996)	US CIP 10/324,616 US Continuation 11/155,043 US Continuation 11/726,679 JP 10-520197	6,924,123 7,056,699

BENITEC TECHNOLOGY

LICENSED PATENTS

NAMED INVENTOR	TITLE	PATENT NO. APPLICATION NO	
Graham / Rice	Control of Gene Express	ZA 2000/4507*.	HK 01105904.3*
		AU2001100608*	HU PO101225*
		SG75542*	IN 2000/00 169/DEL*
		US 6,573,099*	JP P2000-537990*
		GB2353282*	KR 7010419/2000*
		AU 743316*	MX 008631*
		NZ 506648*	PL P.343064*
		US 10/346853*	SK PV1372-2000*
		PCT/AU/99/00195*	AU 35647/02*
		BR PI9908967-0*	NZ 525941*
		CA 2323726*	SG200205122-5*
		CB 99804255-2*	US 09/646807*
		CZ PB2000-3346*	PP2492/98*
		EP 99910039.9*	AUPP2499/98*
			US 10/646,807*
		US 10/646,070	US 10/821,710*
		US 10/759,841*	US 10/821,726*
		EP 04015041.9*	AU 2005209648
		AU 2005211538	
Graham / Rice / M / R	Genetic Silencing	WO 01/70949	SG 91678
		GB 237722	ZA 2002/7428
		AU PQ6363	AU 2001240375
			AU PR2700
Grahm, et al.	Double-Stranded Nucleic Acid	US 60/475,827	AU 2003906281
		US 60/550,504	AU 2004902279
		US 60/479,616	AU 2003906281
		US 60/553,920	PCT/AU04/000759
		US 10/861,191	
Kolvykhalov and Schroeder	Improved Method for Detecting and Characterization of Short. Nucleic Acids	US 60/647,317	
Roelvink, et al.	RNAi Expression Constructs	US 60/649,641	US 60/653,580
Roelvink, et al.	RNAi Expression Constructs with Ribozyme	US 60/678,389	

*Indicates a "Co-Owned Patent". "Co-Owned Patents" are jointly owned by Benitec and CSIRO.

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