

MULTI PRODUCT INFORMATION AND PROTOCOL

1. **Vecotr Name:** pTMU6-CMV Hygro

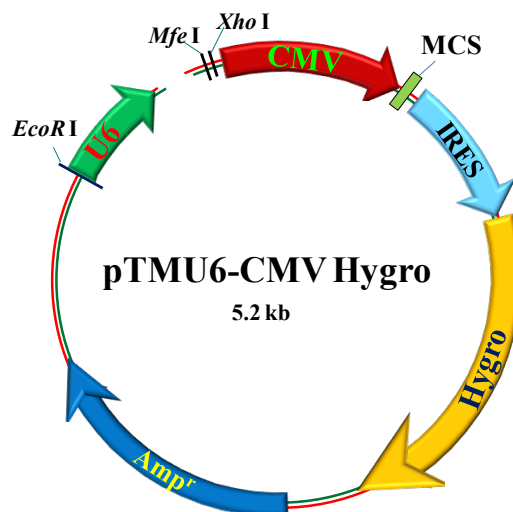
2. **Catalog #:** TMU6-007

3. **Main Application:**

The BIOGENOVA[™] pTMU6-CMV Hygro vector is designed for generating shRNA constructs. It can be used to insert single shRNA for single gene knockdowns or to facilitate the combination of multiple shRNA into a single vector for targeting several genes and/or multiple sites of one gene for maximizing knockdown efficiency. The multi-clone sites (MCS) following a CMV promoter allows inserting a cDNA for gene expression or gene replacement while the shRNAs knock down gene expression.

4. **Features:**

This vector is pre-digested and ready-to-use for directional insertion of shRNA oligos into the vector. The *EcoR* I, *Mfe* I and *Xho* I sites are designed to permit simple combination of multiple shRNAs in one vector, repeatedly. The *EcoR* I and *Mfe* I restriction sites have matched ends for ligation, but neither of them can be re-cut after ligation. After generating the shRNA constructs, a cDNA can be inserted into the multi-clone sites (MCS) following the CMV promoter in the same vector for gene expression and/or replacement. It uses a human U6 promoter to drive the shRNA expression and a CMV promoter to drive the gene expression, has the ampicillin resistant gene (*Amp^r*), functional in *E. coli.*, and the Hygromycin B resistant gene (*Hygro*), functional in mammalian cells. It can be used for either transient or stable transfection.



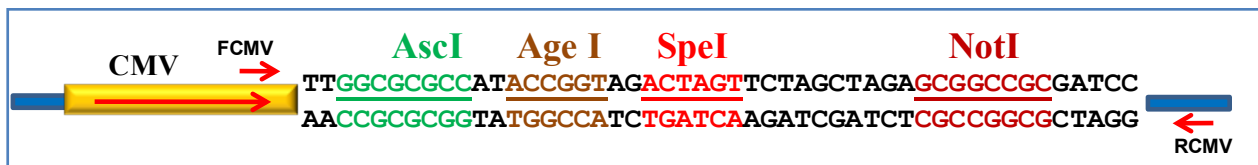
5. shRNA Clone Sites:



6. Synthesized shRNA Oligo Inserts (not included):

shRNA-F:	5'-	GATCAG _{X₁₉₋₂₁}	TTCAAGAGAN ₁₉₋₂₁	TTTTTTGGC	-3'
shRNA-R:	3'-	TCN ₁₉₋₂₁	AAGTTCTCT _{X₁₉₋₂₁}	AAAAAACCGGATC	-5'

7. CMV Multi Clone Sites (MSC):



8. Storage Condition: -20°C

9. Packaging Information:

- 1) Pre-digested vector (Amp^r): 1.0 µg (25ng/µl); for 20 ligations.
- 2) Sequencing primers: 50µl (5µM).
 - TM-For: 5'- GAGAGAGAATTACCCTCACT -3'
 - TM-Rev2: 5'-CCATTTACCGTAAGTTATGTA -3'
 - FCMV: 5'- CGTGTACGGTGGGAGGTCTA -3'
 - RCMV: 5'-GGCCTTATTCCAAGCGGCTT -3'

PROTOCOLS

Reagents (not included):

- shRNA oligos – user-defined for specific target genes:
 - Forward strand (shRNA-F): 5'- GATCAGX₁₉₋₂₁TTCAAGAGAN₁₉₋₂₁TTTTTTGGC -3'
 - Reverse strand (shRNA-R): 5'- CTAGGCCAAAAAAX₁₉₋₂₁TCTCTTGAAN₁₉₋₂₁CT -3'

Note: * The “X” (19-21 nucleotides) represent the sense sequence of the target site while the “N” (19-21 nucleotides) is the anti-sense sequence of the “X”. The “TTCAAGAGA” is a loop in shRNA;

** Since the “TTTT” is the stop signal of RNA Polymerase III, avoid selecting target sites with four or more replicate “T” or “A”. If the last two or three nucleotides of the target site are replicate “T”, adjust the loop sequence in the forward strand to “CTCAAGAGA”, and use “TCTCTTGAG” in the reverse strand.

- Quick Ligation™ Kit (New England BioLabs, cat # M2200)
- MAX Efficiency® DH5α™ Competent Cells (Invitrogen, cat # 18258-012)
- LB Broth Plates with 100 µg/ml Ampicillin
- LB Broth with 100 µg/ml Ampicillin
- QIAprep Spin Miniprep Kit (Qiagen cat # 27104)
- *EcoR* I, *Xho* I, *Mfe* I, and other restriction enzymes
- QIAquick Gel Extraction Kit (Qiagen cat # 28704)

Generation of single shRNA constructs.

1. For each shRNA, set up the reaction tabulated below to anneal the corresponding forward (shRNA-F) and reverse (shRNA-R) shRNA oligonucleotides. Heat the mix to 95°C for 5 min, and then let it cool slowly at room temperature (25°C).

Component	Volume per reaction	Final amount
shRNA-F (1.0 mg/ml)	1.0 µl	1.0 µg
shRNA-R (1.0 mg/ml)	1.0 µl	1.0 µg
1× TE buffer	18 µl	18 µl

2. Individually ligate each annealed oligonucleotide mix (1.0 µl) into the pre-digested pTM vector (2.0 µl) in 20 µl volume using a Quick Ligation™ Kit following the manufacturer’s instructions.
3. Use 2 µl of each ligation reaction to transform Max efficiency competent *E. coli* cells and plate on 50 µg/ml Ampicillin LB plates following the manufacturer’s instructions, and then incubate at 37°C overnight.

4. Choose 2-3 colonies for each ligation, and use them to individually inoculate 5 ml of LB broth supplemented with 100 µg/ml of Ampicillin. Grow overnight at 37°C in a shaking incubator at 250 rpm.
5. Extract each plasmid using a Qiagen QIAprep Spin Miniprep Kit, according to the manufacturer's instructions.
6. Sequence the plasmids using primers TM-For or TM-Rev (included in kit) to confirm that the generated shRNA vectors have the correct sequence.
7. The verified shRNA constructs can be used to transfect cells for gene knockdown if no multiple shRNA targeting is desired. Otherwise, go to next steps

Combining multiple U6-shRNA cassettes into one vector.

8. Digest one of the shRNA constructs (2-3 µg) with *Xho* I and *Mfe* I (this will provide the 'Vector') and digest another shRNA construct (3-5 µg) with *Xho* I and *EcoR* I (this will provide the shRNA 'Insert') in 50 µl volume, respectively.
9. Add 10 µl of 6× DNA loading buffer and load onto a 1% (wt/vol) agarose gel with 1 µg/ml of ethidium bromide and run in 1× TAE buffer at 100v about 30 min to allow the leading dye reach 2/3 of the gel length.
10. Excise the 4.9 kb vector band from *Xho* I and *Mfe* I digested reaction, and the 400 bp U6-shRNA fragment from *Xho* I and *EcoR* I digested reaction under a 360 nm UV light with a scalpel.
11. Use a Qiagen QIAquick Gel Extraction Kit to recover the digested plasmid from the gel and elute in 30 µl of elution buffer. Use 5 µl to measure the DNA concentration using a spectrophotometer.
12. Ligate the purified vector and the U6-shRNA fragment using a Quick Ligation™ Kit following the manufacturer's instructions.
13. Use 2 µl of ligated DNA from to transform high efficiency competent *E. coli* and plate on 50µg/ml Ampicillin LB plates, following the manufacturer's instructions, then incubate at 37°C overnight.
14. Choose 2-3 colonies, and use each to individually inoculate 5 ml of LB broth supplemented with 100 µg/ml of Ampicillin. Grow overnight at 37°C in a shaking incubator at 250 rpm.
15. Extract each plasmid using a Qiagen MiniPrep plasmid preparation kit (according to the manufacturer's instructions).
16. Sequence the plasmids using primer TM-Rev (included in kit) to confirm the U6-shRNA fragments have been inserted into the correct position of vectors.

17. If more genes are to be targeted, repeat steps 8-16 to combine more U6-shRNA cassettes into one vector. The plasmid created in step 16 should be used as the 'vector' in subsequent cloning rounds.

Clone cDNA for gene replacement.

18. Using PCR to amplify the cDNA of your gene without the 3'-UTR sequences. Design a restriction enzyme site on each primer end to facilitate the cloning. Insert the amplified cDNA into the multi-clone sites (MCS) of the shRNA vector generated in step 17,

19. Generate appropriate mutations (4-5 base changes on the wobble positions of the codons) in the shRNA target region of the cDNA using QuickChange Site-Directed Mutagenesis Kit (Stratagene) to prevent targeting by the shRNAs.

* If other genes not targeted by the shRNAs are expressed, the step 19 can be omitted.

20. Transfect mammalian cells gene knocking down and replacement studies

References:

- Yan, Y., Zhang, J., Guo, J.L., Huang, W., & Yang, Y.Z. (2009) Multiple shRNA-mediated knockdown of TACE reduces the malignancy of HeLa cells. *Cell Biol. Int.* 33, 158-164.
- Xu, X. M., Yoo, M. H., Carlson, B. A. and Hatfield, D. L. (2009) Simultaneous inhibition and subsequent re-expression of multiple genes. *Nature Protocols.* 4(9): 1338 - 1348.
- Gou, D. et al. (2007) A novel approach for the construction of multiple shRNA expression vectors. *J. Gene Med.* 9, 751-763.
- Dafny-Yelin, M., Chung, S.M., Frankman, E.L., & Tzfira, T. (2007) pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. *Plant Physiol* 145, 1272-1281.
- ter, B.O., Konstantinova, P., Ceylan, M., & Berkhout, B. (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol. Ther.* 14, 883-892.
- Wang, S., Shi, Z., Liu, W., Jules, J., & Feng, X. (2006) Development and validation of vectors containing multiple siRNA expression cassettes for maximizing the efficiency of gene silencing. *BMC. Biotechnol.* 6, 50.
- Jazag, A. et al. (2005) Single small-interfering RNA expression vector for silencing multiple transforming growth factor-beta pathway components. *Nucleic Acids Res.* 33, e131.

Schematic cloning steps:

