MULTI-I PRODUCT INFORMATION AND PROTOCOL

1. **Vector Name:** pTMU6-GFP Puro

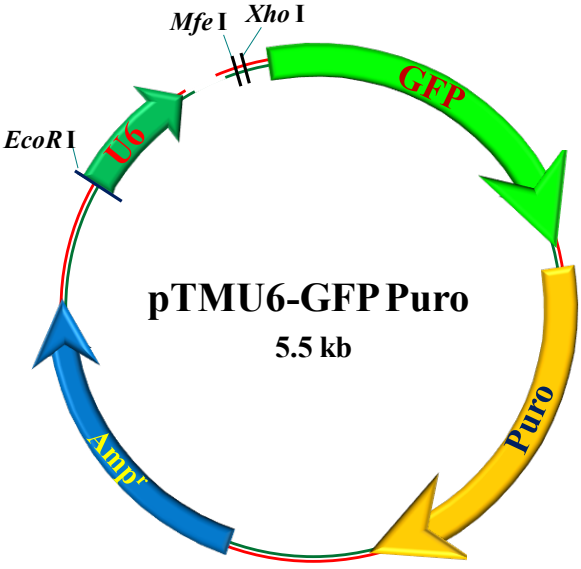
2. **Catalog #:** TMU6-006

3. **Main Application:**

   The BIOGENOVA™ pTMU6-GFP Puro vector is designed for generating shRNA constructs. It can be used to insert single shRNA for single gene knockdowns and to facilitate the combination of multiple shRNAs into a single vector for targeting several genes and/or multiple sites of one gene for maximizing knockdown efficiency.

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. It uses the human U6 promoter to drive shRNA expression, has the ampicillin resistant gene (Amp^r), functional in *E. coli*., and the Puromycin resistant gene (Puro), functional in mammalian cells. It can be used for either transient or stable transfection. This vector also contains a GFP protein which can be used to track the vector in the cells.



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5. shRNA Clone Sites:

6. Synthesized shRNA Oligo Inserts (not included):

shRNA-F:	5'-	GATCAGX_{19-21}TTCAAGAGAN_{19-21}TTTTTTGGC -3'
shRNA-R:	3'-	TCN_{19-21}AAGTTCTCTX_{19-21}AAAAAACCGGATC -5'

7. Storage Condition: -20ºC

8. Packaging Information:

1) Pre-digested vector (Amp^r): 1.0 µg (25ng/µl); for 20 ligations.

2) Sequencing primers: 50µl (5µM).

1). TM-For: 5'- GAGAGAGAATTACCCTCACT -3'

2). TM-Rev2: 5'-CCATTTACCGTAAGTTATGTA -3'

PROTOCOLS

Reagents (not included):

- shRNA oligos – user-defined for specific target genes:
  - Forward strand (shRNA-F): 5'- GATCAGX_{19-21}TTCAAGAGAN_{19-21}TTTTTTGGC -3'
  - Reverse strand (shRNA-R): 5'- CTAGGCCAAAAAAX_{19-21}TCTCTTGAAN_{19-21}CT -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-F (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 ug/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.

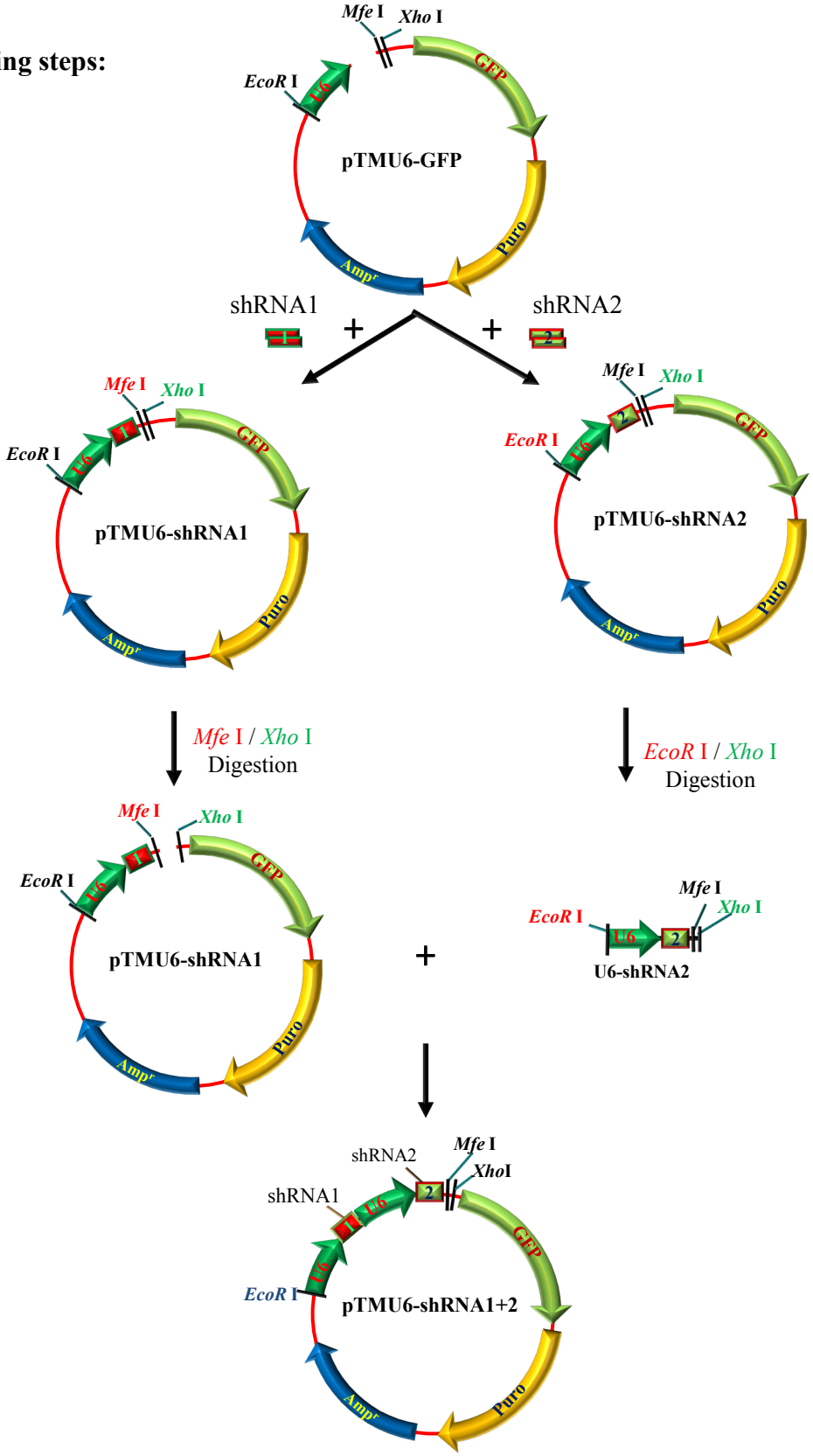
8. Digest one of the shRNA constructs (2-3 µg) with *Xho* I and *Mfe* I (this will provide the 'Vector') and digest another shRNA construct (3-5 µg) with *Xho* I and *EcoR* I (this will provide the shRNA 'Insert') in 50 ul volume, respectively.
9. Add 10 µl of 6× DNA loading buffer and load onto a 1% (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 ul of ligated DNA from to transform high efficiency competent *E. coli* and plate on 50ug/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.

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:



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