# pCMV6-Neo Vector – Application Guide

## Contents

# Package Contents and Storage Conditions

Each kit comes with the following components:

1 vial of pCMV6-Neo vector, undigested (10 ug dried DNA)
1 vial of pCMV6-Neo vector, Not I digested, dephosphorylated (100 ng, dried)
1 vial of primer v1.5, 5' vector DNA primer (100 picomoles, dried)
1 vial of primer XL39, 3' vector DNA primer (100 picomoles, dried)
1 copy of Application Guide

Products are shipped at room temperature, but lyophilized or solubilized DNA (vectors and primers) should be stored at – 20ºC. Add 10 uL deionized water to the tube; incubate for 10 minutes at room temperature or overnight at 4°C, and vortex briefly to resuspend DNA before use.

# Product Description

This pCMV6-Neo vector has a neomycin resistance gene added to OriGene's industry-proven mammalian expression vector, pCMV6-XL5. Following the protocol provided, the cDNA insert from any TrueClone can be easily transferred to this vector to select for transformed cells or to enrich gene expression by establishing stable clones using neomycin selection. The pCMV6-Neo vector retains the ampicillin resistance gene for the selection of *E. coli* transformants and for plasmid DNA propagation in *E. coli*.

The vector is supplied both uncut and precut with the Not I restriction enzyme. The uncut version provides nine unique restriction sites for flexible subcloning, while the pre-cut/dephosphorylated version is optimized for subcloning directly from any TrueClone. Vector primers are included to aid in subclone screening.

# Introduction

This vector combines the features the cytomegalovirus (CMV) immediate-early promoter (suitable for protein over-expression) with the selectable marker, neomycin, for mammalian cell selection. Neomycin is a natural product that binds to ribosomal subunits thereby inhibiting protein synthesis/elongation and causing cell death. G418 is a synthetic analog that mimics this inhibition, but whose action on mammalian cells is blocked by the presence of the neomycin resistance gene product, a bacterial protein, aminoglycoside 3'-phosphotransferase, which phosphorylates/inactivates G418. Culturing post-transfected cells in the presence of G418 effectively permits only those containing the pCMV6-Neo gene construct to survive. This produces a growing plate of cells at an effective 100% transfection efficiency, and continued growth of these cells under antibiotic pressure permits clonal selection of stable cell lines perpetually over-expressing the protein of interest. Optimization of the transfection protocol and G418 concentration with the employed cell line must precede any attempt at positive selection. Established stable cell lines are useful in studying long term effects of the expressed protein and producing enriched extracts for antibody validation or protein purification.

# Production and Quality Assurance

The circular plasmid DNA has been purified from an *E. coli* host strain using a commercial plasmid purification kit. The DNA is suitable for transformation into *E. coli*, transfection into mammalian cells, and for restriction enzyme digestion or other molecular manipulations. It has been tested to be free of nuclease activity. Each batch of Not I digested, dephosphorylated vector has been tested to successfully religate to Not I generated DNA fragments and produce sufficient colonies when transformed into *E. coli* competent cells. The self-ligation background (vector religating to itself without an insert) is less than 5% of transformants. The amount of digested DNA provided in the kit is sufficient for ten ligation reactions.

# Methods

Maintenance of pCMV-Neo:
When transforming the DNA, a recA- and endA- E*. coli* host strain should be used to avoid recombination and endonuclease activities. Transformants should be selected on LB ampicillin-agar plates as described in the manufacturer's protocol. Single colonies can be cultured for larger scale plasmid vector purification. For downstream applications, DNA must be clean and in some cases free of endotoxin. (Several commercial purification kits are available which provide transfection-grade purities and achievable endotoxin levels.) A portion of the bacterial culture should be brought to 15% v/v glycerol and stored at –80ºC for long-term maintenance. Resuspended DNA should be stored at –20ºC.

Subcloning Procedure:

Strategy

The full-length cDNA inserts in the TrueClone vectors (pCMV6-XL4, pCMV6-XL5 and pCMV6-XL6) are flanked by two Not I sites. Not I specifically recognizes an uncommon eight base sequence; therefore, the majority of the TrueClone inserts can be released through Not I digestion without internal cutting of the insert. If an internal Not I site exists in a TrueClone insert, a complete Not I digestion will result in multiple fragments, unsuitable for subcloning directly. In this case, one can take one of the following two approaches to generate a full-length fragment. The first method employs partial digestion of the TrueClone with Not I by limiting the amount of enzyme used and digestion time. In this way, a full-length insert can be released with the internal Not I sites uncut. The second method is to select other restriction sites. The selected sites should flank the full-length insert and also be present in the multiple cloning sites (MCS) of pCMV-Neo. If the latter approach is adopted, the uncut vector included in the kit must also be digested using the select enzyme or enzymes.

Preparation of restriction enzyme digested insert:

1. Digest 0.5 - 1 ug donor plasmid DNA (TrueClone) using the conditions recommended by the restriction enzyme provider.
2. Perform agarose gel electrophoresis to separate the insert and vector fragments.
3. Use a gel purification kit to purify the released insert.
4. Estimate the recovered DNA quantity by spectrophotometric analysis or by running an agarose gel and by comparing to a reference DNA such as DNA Quanti-Ladder (cat # QLD100).

Preparation of restriction enzyme digested vector from uncut pCMV6-Neo (if necessary):

1. Digest 1 ug DNA with selected enzyme or enzymes using conditions recommended by the restriction enzyme provider.
    a. Note: If two restriction enzymes are used, steps 2 to 4 are optional.
2. Add 10% volume of 1M Tris. HCl, pH 8.3 to the digestion tube.
3. Add 5 units of Calf intestine alkaline phosphatase (CIP).
4. Incubate at 37 ºC for 30 min, or as recommended by the manufacturer.
5. Perform agarose gel electrophoresis to separate the insert and vector fragments.
6. Use a gel purification kit to purify the vector fragment.
7. Estimate the recovered DNA quantity by spectrophotometric analysis or by running an agarose gel and by comparing to a reference DNA such as DNA Quanti-Ladder (cat #QLD200).

Ligation and transformation

1. Prepare a ligation according to the following protocol

| | |
|---|---|
| 10x Ligation buffer | 1 uL |
| Purified digested pCMV-Neo vector (10 ng/uL) | 1 uL |
| Purified digested insert from donor plasmid | 2 uL * |
| Ligase (0.5 u/uL, Weiss unit) | 0.5 uL |
| $H_2O$ to 10 uL | |

* The optimal insert:vector molar ratio is about 2:1. Adjust the insert concentration accordingly.

2. Incubate the tube at 22 to 37ºC or room temperature for two hours (according to the manufacturer's recommendation).
3. Add 3 to 5 uL of the ligation mixture to 50 uL of competent cells (efficiency rated > $10^6$ cfu/ug DNA) on ice.

Transform the mixture according to the directions of the manufacturer of the competent cells. For example, for chemically competent cells, follow steps 4-5.

4. Mix the tube gently and keep it on ice for 25 minutes.
5. Heat shock the tube for 30 seconds at 42ºC.
6. Move the tube to room temperature and add 500 uL LB or SOC medium.
7. Incubate the tube at 37 ºC for 1 hour.
8. Spread 50 uL of the cells on an LB ampicillin- agar plate.
9. Centrifuge the remaining medium at 5K for 5 minutes.
10. Discard the supernatant and resuspend cell pellet in the remaining liquid.
11. Spread all the cells in an LB ampicillin- agar plate.
12. Keep the plates at 37ºC for 16 hour to allow colony formation.

Screening for correct subclone

In a typical subcloning ligation, at least 95% of colonies should harbor a plasmid containing a desired insert. If the subcloning is done by single enzyme cut, the insert can be cloned in both orientations. Only the correct orientation will be transcribed *in vivo*. One can either use sequencing with the primers provided in the kit or restriction enzyme digestion to determine if the insert has been successfully cloned and whether it was cloned in the desired orientation. The correct orientation should be the sequence encoding the N terminus (the 5' end) positioned closer to the CMV promoter. To identify the clones with the correct orientation, pick 6 colonies, grow 5 mL cultures in LB-ampicillin, and perform DNA purification using a mini-prep kit. Select restriction enzymes which cut both internally and in the vector to generate fragments that allow one to distinguish the insert orientation. Run the digestion on an agarose gel to determine if the restriction pattern reveals the correct orientation.

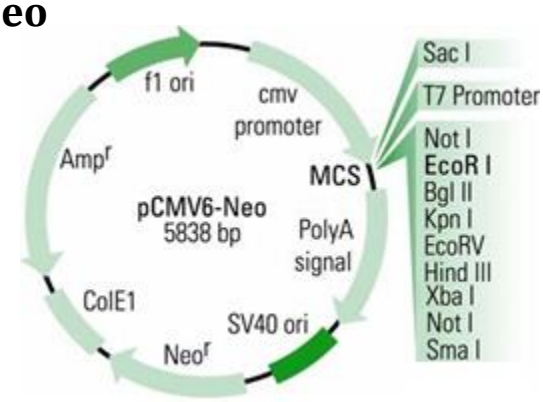
## *Other required reagents*

Restriction enzymes
Ligase
Competent cells
*E. coli* growing media
DNA plasmid purification kit or reagents

# Troubleshooting

| | |
|---|---|
| Problem: | Few or no colonies obtained |
| Cause/Solution: | The ligase may not work properly./Perform troubleshooting as recommended by the manufacturer of the ligase. |
| Cause/Solution: | Competent cells have lost efficiency./Evaluate efficiency of cells using control DNA. |
| Cause/Solution: | Insert:vector concentrations are not optimal./Verify quantitation of fragments, and double-check calculation of molar ratios before repeating ligation. |
| Cause/Solution: | The expression of the insert cDNA may be toxic to the host cells./Try picking colonies which may grow slower than others (appear as smaller colonies on transformation plates). Alternatively, change host cells to find a more receptive strain. |
| Problem: | Too high self-ligation background (no insert) with self-digested vector |
| Cause/Solution: | The plasmid is not completely digested./Allow reaction to digest to completion. Verify by comparing size of digested plasmid to undigested plasmid on an agarose gel. |
| Cause/Solution: | Dephosphorylation is not completed./Allow reaction to dephosphorylate to completion. Use excess CIP if reaction conditions include high salt concentrations. |
| Problem: | The donor vector insert religates to the donor plasmid instead of the pCMV6-Neo plasmid. |
| Cause/Solution: | The donor vector is co-purified with the insert due to inefficient separation./Run the agarose gel longer to well separate the fragments before purification. Because the pCMV6-Neo vector is 1 kb larger (5.8 kb) than the donor vector, this can be incorporated into the orientation screening strategy. |
| Problem: | Inserts are all in reversed orientation. |
| Solution: | Use cloning adaptors or adopt alternate digestion strategy to utilize directional subcloning. |

# Physical Map of pCMV6-Neo




**pCMV-Neo (5838 nucleotides)**

| Feature | Function | Location |
|---|---|---|
| CMV Promoter | protein expression promoter | bases 201-926 |
| T7 promoter | in vitro transcription promoter | bases 953-971 |
| Multiple Cloning Sites | restriction sites for subcloning gene of interest | bases 972-1030 |
| Poly-A signal | terminates/ stabilizes cRNA | bases 1063-1648 |
| SV40 origin | allows replication in mammalian cells | bases 1740-2047 |
| Neomycin resistance gene | allows positive selection in mammalian cells | bases 2120-2904 |
| ColE1 origin | allows replication in bacterial cells | bases 3588-4206 |
| Ampicillin resistance gene | allows positive selection in bacterial cells | bases 4405-5256 |

# Polylinker Sequence of pCMV6-Neo

```
                        Vector Primer v1.5 >
TTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACG
AAACCGTGGTTTTAGTTGCCCTGAAAGGTTTTACAGCATTATTGGGGCGGGGCAACTGCGTTTACCCGCCATCCGCACATGC

                          Sac I                                  T7 Promoter              Not I EcoRI
GTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGAATTTTGTAATACGACTCACTATAGGGCGGCCGCGAATT
CACCCTCCAGATATATTCGTCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGTGATATCCCGCCGGCGCTTAA

 Bgl II  KpnI    EcoRV   Hind III        Xba I     Not I                                        Sma I
CAGATCTGGTACCGATATCAAGCTTGTCGACTCTAGATTGCGGCCGCGGTCATAGCTGTTTCCTGAACATGTGATCCCGGGT
GTCTAGACCATGGCTATAGTTCGAACAGCTGAGATCTAACGCCGGCGCCAGTATCGACAAAGGACTTGTACACTAGGGCCCA

GGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAA
CCGTAGGGACACTGGGGAGGGGTCACGGAGAGGACCGGGACCTTCAACGGTGAGGTCACGGGTGGTCGGAACAGGATTATTT
                                                                    < Vector Primer XL39
```

## *Polylinker Sequence of pCMV6-Neo (pre-cut and dephosphorylated)*

```
                        Vector Primer v1.5 >
TTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGG
AAACCGTGGTTTTAGTTGCCCTGAAAGGTTTTACAGCATTATTGGGGCGGGGCAACTGCGTTTACCCGCC

                                    Sac I                                  T7 Promoter
TAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGAATTTTGTAATACGACTCA
ATCCGCACATGCCACCCTCCAGATATATTCGTCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGT

          Not I (cut)          Not I (cut)                                    Sma I
CTATAGGGC        dephos   GGCCGCGGTCATAGCTGTTTCCTGAACATGTGATCCCGGGTGG
GATATCCCGCCGG    ends         CGCCAGTATCGACAAAGGACTTGTACACTAGGGCCCACC
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CATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGT
GTAGGGACACTGGGGAGGGGTCACGGAGAGGACCGGGACCTTCAACGGTGAGGTCACGGGTGGTCGGAACA
                                                    <Vector Primer XL39
```

## *Suggested Sequencing Primers (included)*

**Vector Primer v1.5** (forward seq primer) 5' GGACTTTCCAAAATGTCG 3'
**Vector Primer XL39** (reverse seq primer) 5' ATTAGGACAAGGCTGGTGGG 3'

Nucleotide Sequence of pCMV6-Neo
All sequences are available electronically at the following URL
http://www.origene.com/assets/Documents/NucleotideSequenceofpCMV6-NEO.doc

# Limited Warranty and Licensing

For more information on the use of G 418, see www.g418.com. Use of this compound is covered by US patents 3,959,254 and 3,997,403 from the Schering Corporation.
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.