phCMV Expression Vectors
Instruction Manual

Catalog Numbers

P003100
P003200
P003300

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Table of Contents

OVERVIEW

Purchaser Notification

Limited License

The purchase price paid for the phCMV™ Vectors and Kits by end users grants them a non-transferable, non-exclusive license to use the Vectors and associated components (as listed in the Contents section). These Vectors are intended for ***internal research only*** by the purchaser. Such use is limited to the cloning of genes into the Vectors for sequencing or subcloning, and for *in vitro* protein expression in cells or *in vivo* protein expression in animals conducted by licensed facilities. Furthermore, ***internal research only*** use means that these Vector kits and all of their contents are excluded, without limitation, from resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of Genlantis, a division of Gene Therapy Systems, Inc ("GTS").

Separate licenses are available from GTS for the express purpose of non-research use or applications of the phCMV Vectors. To inquire about such licenses, or to obtain permission to transfer or use the enclosed material, contact the Director of Licensing at GTS.

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Product Use Limitations

The phCMV Vectors and all of the Kits components are developed, designed, intended, and sold for research use only. They are not to be used for human diagnostic or included/used in any drug intended for human use. All care and attention should be exercised in the handling of the kit components by following appropriate research lab practices.

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Director of Licensing
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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 USA.

Available Kits and Contents

The following phCMV vectors are currently available from Gene Therapy Systems, Inc.

Product Name	Description	Kit Contents	Catalog Number
phCMV1 Vector Kit	For encoding recombinant proteins in their ***native*** form, without any tags	1. 25 µg supercoiled phCMV1 Vector, 1 µg/µl solution. 2. 10 µg phCMV1/CAT positive control vector, 1 µg/µl solution.	P003100
phCMV2 Vector Kit	For encoding recombinant proteins with an ***N-terminal HA*** fusion tag	1. 25 µg supercoiled phCMV2 Vector, 1 µg/µl solution. 2. 10 µg phCMV2/CAT positive control vector, 1 µg/µl solution.	P003200
phCMV3 Vector Kit	For encoding recombinant proteins with a ***C-terminal HA*** fusion tag	1. 25 µg supercoiled phCMV3 Vector, 1 µg/µl solution. 2. 10 µg phCMV3/CAT positive control vector, 1 µg/µl solution.	P003300

Accessory Products

GTS offers the following products for use in conjunction with the phCMV vectors.

3-minute transformation and efficient propagation of phCMV vectors.

Product Name	Cat. No.	Quantity
TurboCells™ Chemically Competent *E. coli*	C300020	20 x 50 µl
TurboCells™ F′ Chemically Competent *E. coli*	C301020	20 x 50 µl

For sensitive and highly-specific detection of HA-tagged proteins

Product Name	Catalog Number	Quantity
Anti-HA Polyclonal Antibody	ABC025	500 µl
Anti-HA-HRP Polyclonal Antibody	ABC125	250 µl

For efficient transfection and high-level expression

Product Name	Catalog Number	Quantity
GenePORTER™ 2 Transfection Reagent	T202007	75 reactions (0.75 ml)
GenePORTER™ 2 Transfection Reagent	T202015	150 reactions (1.5 ml)
GenePORTER™ 2 Transfection Reagent	T202075	75 reactions (5 x 1.5 ml)
BoosterExpress™ Reagent Kit	T20100B	3 boosters (1.5 ml each)

Shipping and Storage

The phCMV Vector Kits are shipped frozen. Upon receipt, we recommend that you store the Vectors or Kits at –20°C.

METHODS AND PROCEDURES

Introduction

The phCMV series of vectors are designed to achieve significantly higher expression levels than traditional human cytomegalovirus (CMV) promoter-based constitutive expression vectors. Through their maximized CMV promoter activity, the maximized levels of protein expressed from the phCMV vectors will allow you to easily proceed to subsequent protein detection and functional studies.

In order to achieve maximum expression levels, the CMV promoter/enhancer sequence in the phCMV vectors have been systematically modified so that only the necessary sequences that confer high transcriptional activity are retained. In addition to the optimized CMV promoter, the phCMV vector includes the intron A from the human CMV immediate-early (IE) gene and an efficient artificial terminator to ensure the highest transcription levels possible. The backbones of the phCMV vectors have also been rigorously engineered to provide both high plasmid yield in *E. coli* and enhanced expression levels *in vivo*. This makes the phCMV vectors ideal tools for routine protein expression studies as well as animal injection experiments.

The phCMV vectors consistently deliver superior expression levels when compared with other commercially available CMV promoter-based expression vectors. It is not unusual to obtain protein expression levels from phCMV that are several fold higher than those obtained from other popularly used vectors. In addition, because phCMV vectors use the *Kan/Neo* gene for selection in both bacteria and cultured cell lines, they offer smaller vector sizes and improved transfection efficiency.

In summary, the three phCMV vectors offer the following benefits:

- Maximized high-level expression with optimized CMV promoter.
- G418 resistance gene for the selection of stable cell lines.
- Optional N- or C-terminal HA fusion tags for simplified protein detection and purification with anti-HA antibodies and affinity resins.
- Extensive multiple cloning region for convenient and easy cloning.
- Small vector sizes for efficient transfection

phCMV Vector Features

Feature	Function
Modified Human cytomegalovirus (CMV) immediate-early promoter/enhancer/intron.	High-level expression promoter *in vitro* and *in vivo*.
T7 promoter priming site in phCMV, phCMV3	For sequencing of the inserted DNA fragment.
SV40 polyadenylation signal.	Efficient transcription termination and polyadenylation of mRNA
SV40 promoter.	High-level expression of the neomycin resistance gene for selection of stably transfected cells.
Kanamycin resistance gene.	Efficienct selection of vector in *E. coli cells*.
pUC origin.	High copy number replication of vector in *E. coli* cells.
Multiple cloning site and HA tags.	For cloning a gene into vector with either wild type (phCMV), N-terminal HA tag (phCMV2), and C-terminal HA tag (phCMV3).

1. Cloning

1.1. Design gene specific PCR primers that contain the appropriate restriction enzyme sites that need to be added to the 5' and 3' ends of your gene of interest, OR

1.2. Digest your gene of interest out of a cloning vector with carefully chosen restriction enzymes that are compatible with the restriction enzyme sites available in the multiple cloning region of the phCMV vector of choice.

1.3. If no restriction enzyme sites are conveniently available for cutting your gene of interest out of a cloning vector, then perform a PCR reaction to amplify your gene of interest out of your cloning vector using the technique described in Section 1.1 above.

1.4. Digest the phCMV vector with the chosen restriction enzyme sites following the manufacturer's protocol. Be aware of the following factors:

1.4.1. Restriction enzyme site that are too close to each other might interfere with each other's digestion. Consult your restriction enzyme supplier's manual or catalog for additional instructions on restriction enzyme footprint requirements.

1.4.2. Some restriction enzymes might be blocked by methylation while the plasmid DNA id prepared in E. coli. Check the vector sequence carefully and consult your restriction enzyme supplier's manual for additional instructions on restriction enzyme site methylation requirements.

1.5. Mix your gene of interest with the linearized phCMV vector in the appropriate buffer for ligation. Use insert:vector ratios that work best for you or that are recommended in standard laboratory manuals[i].

1.6. Add ligase enzyme and allow reaction to incubate according to supplier's instructions.

2. Transformation

The following protocol for the propagation of the phCMV vectors are optimized for use with the following SmartCells™ competent *E. coli* cells

Product Name	Cat. No.	Quantity
SmartCells™ Chemically Competent *E. coli*	C101020	20 x 50 µl
SmartCells™ F′ Chemically Competent *E. coli*	C101120	20 x 50 µl

SmartCells™ competent *E. coli* offers superior transformation efficiency under diverse transformation conditions. They are especially useful when the quality of your plasmid prep is not satisfactory or certain, or if you would like to use more of the ligation mix into the transformation reaction.

NOTE *Alternatively, you can propagate the phCMV vectors with many other commercially available strains by following the appropriate supplier's protocol.*

2.1. Thaw one tube of the SmartCells™ competent cells on ice (10-15 minutes).

2.2. Add 1-10 µl of the ligation mix from Step 1.6 above to SmartCells™ *E. coli*; mix gently and incubate on ice for 15 to 30 minutes.

2.3. Heat the mix at 42° C for 45 seconds.

2.4. Add 250 µl SOC medium and incubate at 37° C for 1 hour in an air incubator. Shake tubes horizontally at 225 rpm.

2.5. Dilution transformation mixture if necessary and spread 100 µl of the transformation mix on LB/Agar plates containing 50µg/ml kanamycin.

2.6. Incubate overnight at 37° C.

2.7. Pick colonies and analyze positive recombinant plasmids by digesting miniprep DNA and agarose gel electrophoresis.

3. Transfection

Plasmid DNA for transfection into mammalian cells must be clean and free of phenol and sodium chloride. Methods for trasfection include calcium phosphate, cationic lipid, and electroporation. We recommend the use of GenePORTER 2 Transfection Reagent for maximized transfection efficiency and high expression levels.

Product Name	Cat. No.	Quantity
GenePORTER™ 2 Transfection Reagent	T202007	75 reactions (0.75 ml)
GenePORTER™ 2 Transfection Reagent	T202015	150 reactions (1.5 ml)
GenePORTER™ 2 Transfection Reagent	T202075	750 reactions (5 x 1.5 ml)

IMPORTANT *We recommend that you include a positive expression control vector and a mock transfection as negative control. A positive control vector expressing the Chloramphenicol (CAT) gene is included in each vector kit. We recommend using a CAT ELISA Assay Kit (Roche Molecular Biochemicals, Cat. No. 1363727) for measuring CAT expression.*

4. Stable Cell Line Selection

The phCMV vectors contain the neomycin resistance gene for selection of stable cell lines using neomycin (G418). Neomycin (G418) is an aminoglycoside that blocks protein synthesis in mammalian cells by interfering with ribosomal function. Expression of the bacterial aminoglycoside phosphotransferase gene (APH) in mammalian cells results in the detoxification of neomycin (G418).

4.1. Determining G418 Sensitivity

In order to generate a stable cell line expressing your gene of interest from a cloned phCMV vector, you need to determine the minimum concentration of G418 required to kill your untransfected host cell line. Because natural resistance varies among cell lines, we recommend that you test a range of concentrations using the following protocol:

4.1.1. Split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.

4.1.2. The next day, substitute culture medium with medium containing varying concentrations of G418 (0, 50, 100, 200, 400, 600, 800 μg/ml G418).

4.1.3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.

4.1.4. Count the number of viable cells at regular intervals to determine the appropriate concentration of G418 that prevents growth within 2-3 weeks after addition ofG418.

4.2. Creating Stable Integrants.

Once you have determined the appropriate G418 concentration to use for selection in the host cell line, you can generate a stable cell line expressing your gene of interest.

4.2.1. Transfect your mammalian host cell line with linearized phCMV construct. Include a plate of untransfected cells as a negative control and The phCMV/CAT plasmid as a positive control.

4.2.2. 24 hours post transfection, wash the cells and add fresh medium.

4.2.3. 48 hours post transfection, split the cells into fresh medium containing G418 at the pre-determined concentration. Split the cells so that they are no more than 25% confluent.

4.2.4. Feed the cells with selective medium every 3-4 days until G418 -resistant foci can be identified.

4.2.5. Pick and expand colonies in 96- or 48-well plates.

APPENDIX

Quality Control

The phCMV1, phCMV2 and phCMV3 vectors are verified for identity and quality by restriction enzyme digestion analysis. Furthermore, the polylinker regions of each vector have been verified by sequencing. Purchased vectors can be verified for identity by users according to the restriction digest patterns listed in the table below.

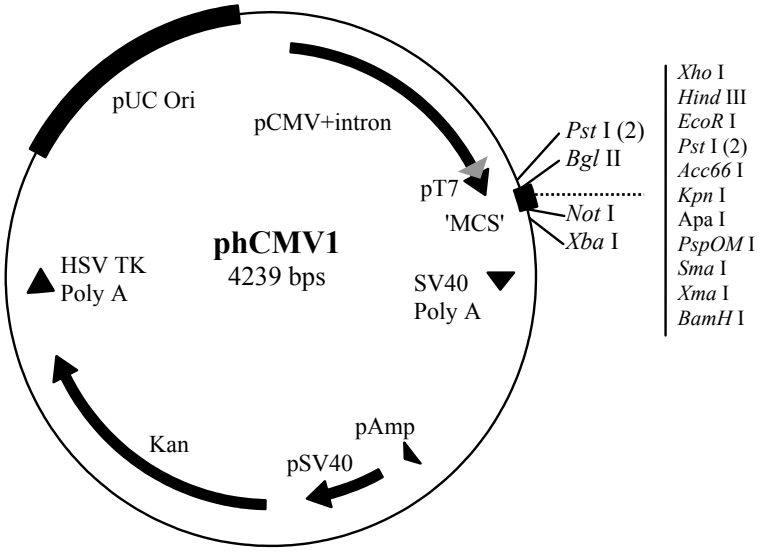
Vector	Restriction Enzyme	Expected Fragments (size in base pairs)
phCMV1	Acc III or *BspE* I	No site
	Nhe I	No site
	Pvu II	3631, 608
	Nco I	1904, 1632, 703
phCMV2	Acc III	4261
	Nhe I	4261
	Pvu II	3653, 608
	Nco I	1904, 1654, 703
phCMV3	Acc III	4251
	Nhe I	No site
	Pvu II	3643, 608
	Nco I	1904. 1644, 703
phCMV1/CAT	Acc III	4895
	Msc I	3227, 1668
phCMV2/CAT	Acc III	4677, 222
	Msc I	3222, 1677
phCMV3/CAT	Acc III	4404, 462
	Msc I	3222, 1644

Product Support

Telephone	858-457-1919 OR 888-428-0558 (US Toll Free)
Fax	858-623-9494
Email	tech1@genlantis.com
Web Site	http://www.genlantis.com

Vector Information

Map of phCMV1: 4239 bps, Circular DNA



Vector Elements

Element	Start-End	Description
pCMV + intron	59-808	Human CMV promoter/enhancer and intron sequence.
pT7	759-774	T7 promoter priming for sequencing.
MCS	834-890	Multiple cloning sites.
SV40 Poly A	1058-1108	SV40 polyadenylation signal sequence.
pAmp	1672-1700	Ampicillin resistance gene promoter sequence.
pSV40	1784-2013	SV40 promoter sequence
Kan	2135-2929	Kanamycin resistance gene sequence.
HSV TK Poly A	3165-3183	HSV Thymidine Kinase polyadenylation signal sequence.
pUC Ori	3514-4157	pUC origin of replication sequence.

MCS sequence of phCMV1 (801-960)

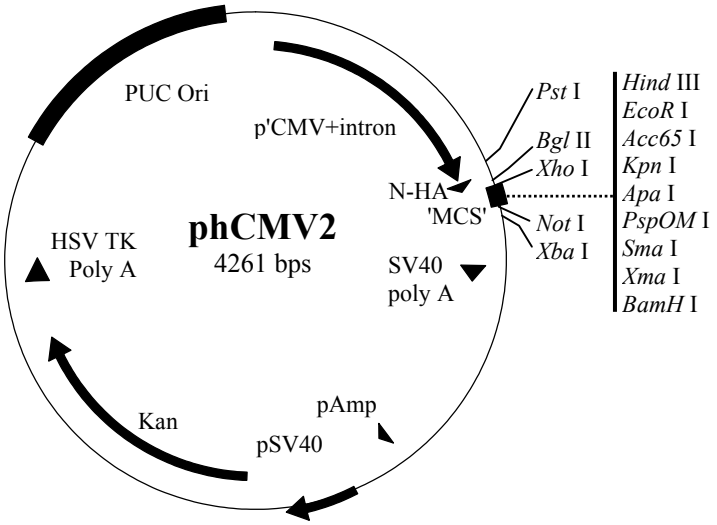
T7 promoter priming site

```
TAGGCACACCCCTTTGGCTCTTATGCATGAATTAATACGACTCACTATAGGGAGACAGACTGTTCCTTTCCTGG

End of CMV Promoter
                Pst I (2)                    Bgl II  Xho I     Hind III   EcoR I        Pst I (2)   Acc65 I/Kpn I
GTCTTTTCTGCAGGCACCGTCGACTTAACAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGC

          Apa I  Sma I  BamH I                  Not I          Xba I*
GGGCCCGGGATCCACCGGGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTA
```

* Enzyme site is methylated by Adenine Methylase.

For complete vector sequence and a comprehensive list of enzyme cut sites, visit our web site at http://www.genlantis.com

Map of phCMV2: 4261 bps, Circular DNA



Vector Elements

Element	Start-End	Description
pCMV + intron	59-808	Human CMV promoter/enhancer and intron sequence.
N-HA	820-846	N-terminal Hemagglutinin tag sequence.
MCS	856-912	Multiple cloning sites.
SV40 poly A	1080-1130	SV40 polyadenylation signal sequence.
pAmp	1694-1722	Ampicillin resistance gene promoter sequence.
pSV40	1806-2035	SV40 promoter sequence.
Kan	2157-2951	Kanamycin resistance gene sequence.
HSV TK poly A	3187-3205	HSV Thymidine Kinase polyadenylation sequence.
pUC Ori	3536-4179	pUC origin of replication sequence.

MCS sequence of the phCMV2 (805-949)

End of CMV Promoter — *Pst* I

```
AGGCACACCCCTTTGGCTCTTATGCATGCTGACAGACTAACAGACTGTTCCTTTCCTGGGTCTTTTCTGCAGG
```

HA tag — *Bgl* II — *Xho* I — *Hind* III

```
CACCGTCGACTTAACAATGTACCCATACGATGTTCCGGATTACGCTAGCCTCCCAGATCTCGAGCTCAAGCTT
```

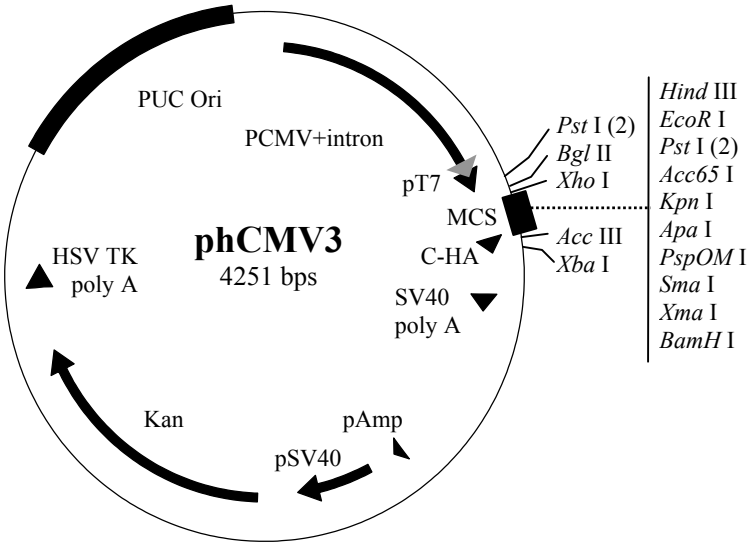
EcoR I — *Kpn* I — *Apa* I — *Sma* I — *BamH* I — *Not* I — *Xba* I*

```
CGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGGTACAAGTAAAGCGGCCGCGACTCTAGA
```

* Enzyme site is methylated by Adenine Methylase.

For complete vector sequence and a comprehensive list of enzyme cut sites, visit our web site at http://www.genlantis.com

Map of phCMV3: 4251 bps, Circular DNA



Vector Elements

Element	Start-End	Description
pCMV + intron	59-808	Human CMV promoter/enhancer and intron sequence.
pT7	759-774	T7 promoter priming site for sequencing.
MCS	843-870	Multiple cloning sites.
C-HA	871-916	C-terminal Hemagglutinin tag sequence.
SV40 poly A	1070-1120	SV40 polyadenylation signal sequence.
pAmp	1684-1712	Ampicillin resistance gene promoter sequence.
pSV40	1796-2025	SV40 promoter sequence.
Kan	2147-2941	Kanamycin resistance gene sequence.
HSV TK poly A	3177-3195	HSV Thymidine Kinase polyadenylation sequence.
pUC Ori	3526-4169	pUC origin of replication sequence.

MCS sequence of the phCMV3 (796-920)

T7 promoter priming site

ACCGCCTATAGACTCTATAGGCACACCCCTTTGGCTCTTATGCATGAATTAATACGACTCACTATAGGGAGA

End of CMV Promoter; *Pst* I (2); *Bgl* II; *Xho* I; *Hind* III

CAGACTGTTCCTTTCCTGGGTCTTTTCTGCAGGCACCGTCGACTTAACAGATCTCGAGCTCAAGCTTC

EcoR I; *Pst* I (2); *Acc65* I/*Kpn* I; *Apa* I; *Sma* I; *BamH* I; HA tag

GAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCTACCCATACGATGTTCCGGATTACGCT**TAA**GGCCG

For complete vector sequence and a comprehensive list of enzyme cut sites, visit our web site at http://www.genlantis.com

References

[i]Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).