pCMV-3Tag Epitope Tagging Mammalian Expression Vectors

Instruction Manual

Catalog #240195 (pCMV-3Tag-1), #240196 (pCMV-3Tag-2), #240197 (pCMV-3Tag-3), #240198 (pCMV-3Tag-4), #240200 (pCMV-3Tag-6), #240202 (pCMV-3Tag-7), #240203 (pCMV-3Tag-8), and #240204 (pCMV-3Tag-9)

Revision C.0

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240195-12

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pCMV-3Tag Epitope Tagging Mammalian Expression Vectors

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pCMV-3Tag Epitope Tagging Mammalian Expression Vectors

MATERIALS PROVIDED

Materials provided	Concentration	Quantity
Catalog #240195		
pCMV-3Tag-1A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-1B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-1C mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240196		
pCMV-3Tag-2A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-2B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-2C mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240197		
pCMV-3Tag-3A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-3B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-3C mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240198		
pCMV-3Tag-4A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-4B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-3Tag-4C mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240200		
pCMV-3Tag-6 mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240202		
pCMV-3Tag-7 mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240203		
pCMV-3Tag-8 mammalian expression vector	1.0 µg/µl	20 µg
Catalog #240204		
pCMV-3Tag-9 mammalian expression vector	1.0 µg/µl	20 µg

STORAGE CONDITIONS

All Vectors: –20°C

ADDITIONAL MATERIALS REQUIRED

T4 DNA ligase
Taq DNA polymerase
Taq DNA polymerase buffer
TE buffer[§]
CIAP

[§] See *Preparation of Media and Reagents*.

Revision C.0

NOTICES TO PURCHASER

CMV Promoter

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

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INTRODUCTION

The epitope tagging technique involves fusion of a protein of interest to a peptide epitope that is recognized by a readily available antibody. With this technique, expression of the fusion protein is monitored using a tag-specific antibody, allowing a new protein to be studied without generating a new antibody specific to the protein of interest. Epitope tagging can be used to localize gene products in living cells, identify associated proteins, track the movement of fusion proteins within the cell, or characterize new proteins by immunoprecipitation.

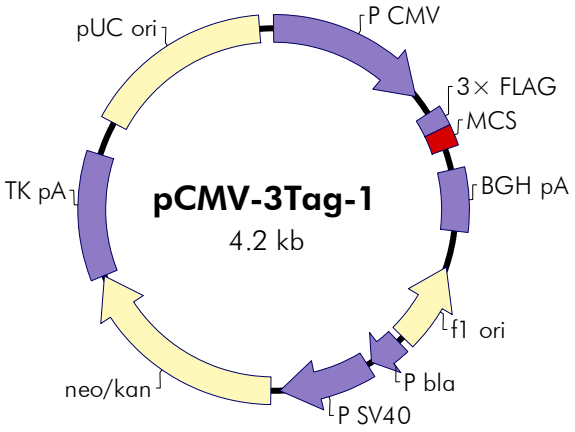
The Agilent pCMV-3Tag vectors are a series of epitope tagging mammalian expression vectors featuring three copies of the epitope tag in a variety of configurations. One group of vectors allows selection in mammalian cells using neomycin (G418) resistance. This group includes pCMV-3Tag-1 (Figure 1), an N-terminal FLAG® tagging vector, pCMV-3Tag-2 (Figure 2), an N-terminal c-myc tagging vector, pCMV-3Tag-3 (Figure 3), a C-terminal FLAG tagging vector, and pCMV-3Tag-4 (Figure 4), a C-terminal c-myc tagging vector. Each vector is available in three different reading frames to simplify subcloning. These reading frames, designated as A, B, and C, differ only by one or two bases.

For added flexibility, a second group of vectors allows selection in mammalian cells using hygromycin resistance. This group includes pCMV-3Tag-6 (Figure 5), an N-terminal FLAG® tagging vector, pCMV-3Tag-7 (Figure 6), an N-terminal c-myc tagging vector, pCMV-3Tag-8 (Figure 7), a C-terminal FLAG tagging vector and pCMV-3Tag-9 (Figure 8), a C-terminal c-myc tagging vector. Each of these vectors is available in a single reading frame.

The pCMV-3Tag vectors are derived from the pCMV-Script vector and contain sequences encoding three copies of either the FLAG or the c-myc epitope at either the N or C terminus. These specific epitope tags are small, highly immunoreactive, and are not likely to interfere with the function of the target protein. The synthetic FLAG epitope is composed of eight amino acid residues (DYKDDDDK).[1] The c-myc epitope is derived from the human c-myc gene and contains ten amino acid residues (EQKLISEEDL).[2] Tagged constructs generated in the pCMV-3Tag vectors can be transfected into mammalian cells and the fusion protein can be easily characterized using commercially available antibodies. The presence of three copies of the epitope in each vector enhances detection of the fusion protein in downstream applications.

In addition to the epitope tag sequences, the pCMV-3Tag vectors contain features for expression of fusion proteins in eukaryotic cells. The cytomegalovirus (CMV) promoter allows constitutive expression of the cloned DNA in a wide variety of mammalian cell lines. The vectors contain either a neomycin-resistance gene or a hygromycin-resistance gene under control of a mammalian promoter for selection in mammalian cells. The locations of the features for each vector are listed in Tables I and II.

pCMV-3Tag-1 Vector Map



pCMV-3Tag-1 Multiple Cloning Site Region
(sequence shown 620–893)

T3 promoter — FLAG tag

```
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCCACC ATG GAT TAC AAG GAT...
                                                              M   D   Y   K   D
```

FLAG tag — FLAG tag

```
...GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT GAC AAG GAC TAC AAA GAT GAT GAC GAT AAA * ...
   D   D   D   K   D   Y   K   D   D   D   D   K   D   Y   K   D   D   D   D   K
```

Srf I, BamH I, Pst I, EcoR I, EcoR V, Hind III, Acc I/Sal I

```
...GCC CGG GCG GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC...
```

Xho I, Apa I

```
...CTC GAG GGG GGG CCC GGT ACC TTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
```

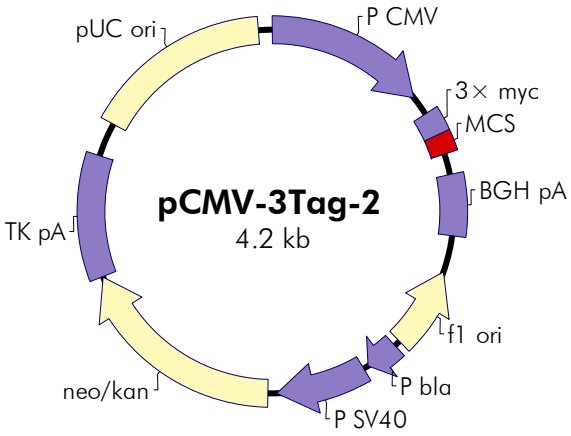
MULTIPLE STOP CODONS — T7 promoter

* In pCMV-3Tag-1A, no bases inserted; in pCMV-3Tag-1B, A inserted; in pCMV-3Tag-1C, AA inserted

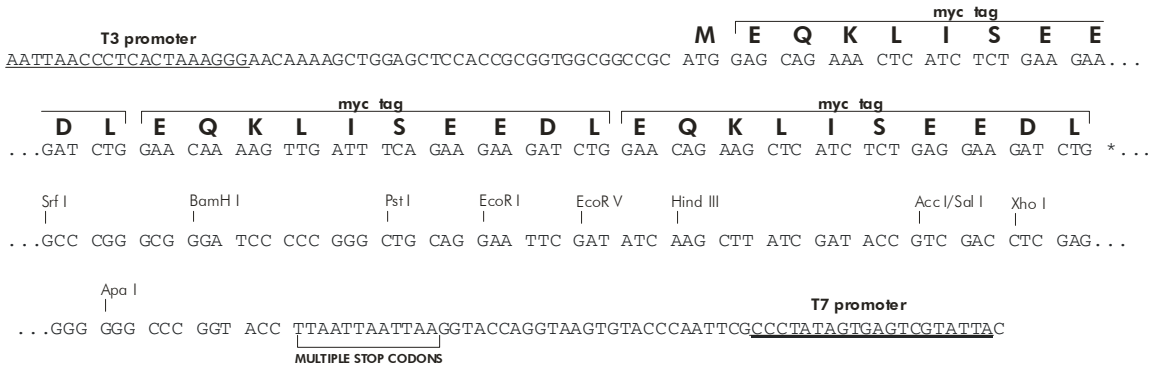
Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
3× FLAG tag	682–753
multiple cloning site	754–828
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	872–893
BGH polyA signal	908–1134
f1 origin of ss-DNA replication	1273–1579
bla promoter	1604–1728
SV40 promoter	1748–2086
neomycin/kanamycin resistance ORF	2121–2912
HSV-thymidine kinase (TK) polyA signal	2916–3371
pUC origin	3500–4167

FIGURE 1 Circular map of the pCMV-3Tag-1A–1C vectors, featuring eukaryotic expression, N-terminal FLAG epitope tagging, and neomycin and kanamycin resistance. The positions listed in the table above correspond to pCMV-3Tag-1A. See Table 1 for the complete list of feature positions for the pCMV-3Tag-1A–1C vectors.

pCMV-3Tag-2 Vector Map



pCMV-3Tag-2 Multiple Cloning Site Region
(sequence shown 620–907)

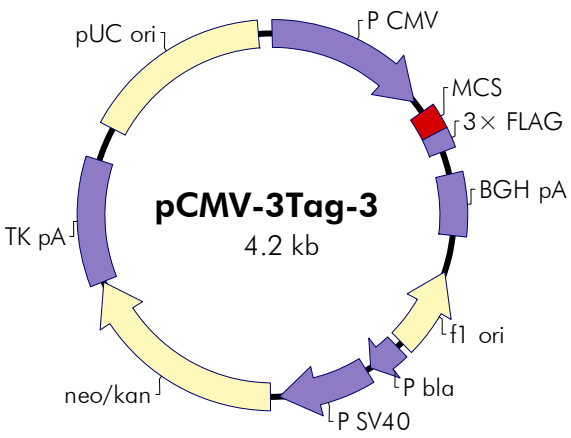


* In pCMV-3Tag-2A, no bases inserted; in pCMV-3Tag-2B, A inserted; in pCMV-3Tag-2C, AA inserted

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
3× c-myc tag	678–767
multiple cloning site	768–842
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	886–907
BGH polyA signal	922–1148
f1 origin of ss-DNA replication	1287–1593
bla promoter	1618–1742
SV40 promoter	1762–2100
neomycin/kanamycin resistance ORF	2135–2926
HSV-thymidine kinase (TK) polyA signal	2930–3385
pUC origin	3514–4181

FIGURE 2 Circular map of the pCMV-3Tag-2A–2C vectors, featuring eukaryotic expression, N-terminal c-myc epitope tagging, and neomycin and kanamycin resistance. The positions listed in the table above correspond to pCMV-3Tag-2A. See Table 1 for the complete list of feature positions for the pCMV-3Tag-2A–2C vectors.

pCMV-3Tag-3 Vector Map



pCMV-3Tag-3 Multiple Cloning Site Region
(sequence shown 620–888)

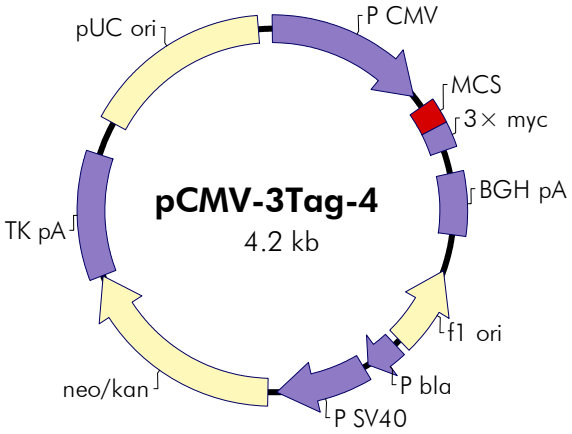
```
                                                  Sac I     BstX I  Sac II          Not I                 Srf I
          T3 promoter                               |         |       |              |                     |
A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG...

        BamH I              Pst I       EcoR I      EcoR V      Hind III                  Acc I/Sal I   Xho I
          |                   |           |           |           |                         |           |
...GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC * CTC GAG...

    FLAG tag                                FLAG tag                                FLAG tag
  D   Y   K   D   D   D   D   K   D   Y   K   D   D   D   D   K   D   Y   K   D   D
...GAT TAC AAG GAT GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT GAC AAG GAC TAC AAA GAT GAT...

  D   D   K                                                                  T7 promoter
...GAC GAT AAA TAG GGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
          STOP
```

* In pCMV-3Tag-3A, no bases inserted; in pCMV-3Tag-3B, A inserted; in pCMV-3Tag-3C, AA inserted. **When using the C reading frame version of this vector**, please note the presence of a stop codon (TAG) in frame with the 3× FLAG tag, positioned just upstream of the *Srf* I restriction site. Do not use the *Sac* I, *BstX* I, *Sac* II or *Not* I sites for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
3× FLAG tag	744–815
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	867–888
BGH polyA signal	903–1129
f1 origin of ss-DNA replication	1268–1574
bla promoter	1599–1723
SV40 promoter	1743–2081
neomycin/kanamycin resistance ORF	2116–2907
HSV-thymidine kinase (TK) polyA signal	2911–3366
pUC origin	3495–4162

FIGURE 3 Circular map of the pCMV-3Tag-3A–3C vectors, featuring eukaryotic expression, C-terminal FLAG epitope tagging, and neomycin and kanamycin resistance. The positions listed in the table above correspond to pCMV-3Tag-3A. See Table 1 for the complete list of feature positions for the pCMV-3Tag-3A–3C vectors.

pCMV-3Tag-4 Vector Map



pCMV-3Tag-4 Multiple Cloning Site Region
(sequence shown 620–907)

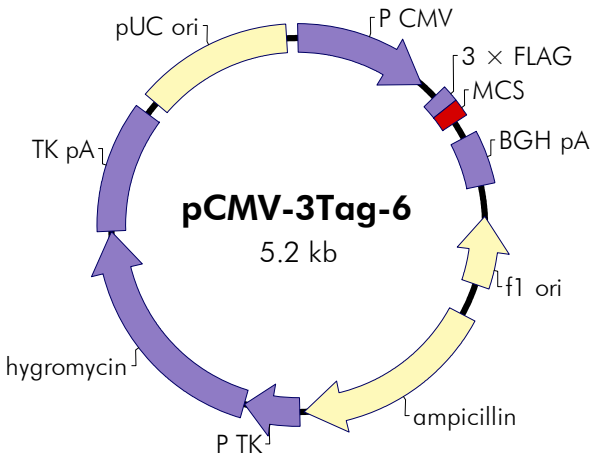
```
                                                  Sac I   BstX I  Sac II          Not I                   Srf I           BamH I
                T3 promoter                         |       |       |               |                       |               |
A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG GGC GGA TCC CCC...

          Pst I       EcoR I      EcoR V      Hind III                    Acc I/Sal I     Xho I                myc tag
            |           |           |           |                           |             |          E  Q  K  L  I  S  E
...GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC * CTC GAG GAG CAG AAA CTC ATC TCT GAA...

                                  myc tag                                   myc tag
   E  D  L  E  Q  K  L  I  S  E  E  D  L  E  Q  K  L  I  S  E  E  D  L
...GAA GAT CTG GAA CAA AAG TTG ATT TCA GAA GAA GAT CTG GAA CAG AAG CTC ATC TCT GAG GAA GAT CTG TAA ...
                                                                                                  STOP
                                                  T7 promoter
...GGGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
```

* In pCMV-3Tag-4A, no bases inserted; in pCMV-3Tag-4B, A inserted; in pCMV-3Tag-4C, AA inserted. **When using the C reading frame version of this vector**, please note the presence of a stop codon (TAG) in frame with the 3× c-myc tag, positioned just upstream of the *Srf* I restriction site. Do not use the *Sac* I, *BstX* I, *Sac* II or *Not* I sites for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
3× c-myc tag	744–833
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	886–907
BGH polyA signal	922–1148
f1 origin of ss-DNA replication	1287–1593
bla promoter	1618–1742
SV40 promoter	1762–2100
neomycin/kanamycin resistance ORF	2135–2926
HSV-thymidine kinase (TK) polyA signal	2930–3385
pUC origin	3514–4181

FIGURE 4 Circular map of the pCMV-3Tag-4A–4C vectors, featuring eukaryotic expression, C-terminal c-myc epitope tagging, and neomycin and kanamycin resistance. The positions listed in the table above correspond to pCMV-3Tag-4A. See Table 1 for the complete list of feature positions for the pCMV-3Tag-4A–4C vectors.

pCMV-3Tag-6 Vector Map



pCMV-3Tag-6 Multiple Cloning Site Region
(sequence shown 620–893)

T3 promoter — FLAG tag

```
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCCACC ATG GAT TAC AAG GAT...
                                                              M   D   Y   K   D
```

FLAG tag — FLAG tag

```
...GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT GAC AAG GAC TAC AAA GAT GAT GAC GAT AAA...
 D   D   D   K   D   Y   K   D   D   D   D   K   D   Y   K   D   D   D   D   K
```

Srf I, BamH I, EcoR V, Hind III, Cla I, Acc I/Sal I

```
...GCC CGG GCG GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC...
```

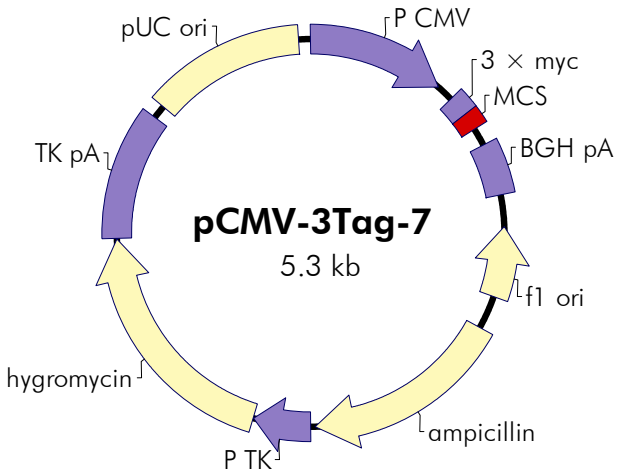
Xho I, Apa I — T7 promoter

```
...CTC GAG GGG GGG CCC GGT ACC TTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
                              MULTIPLE STOP CODONS
```

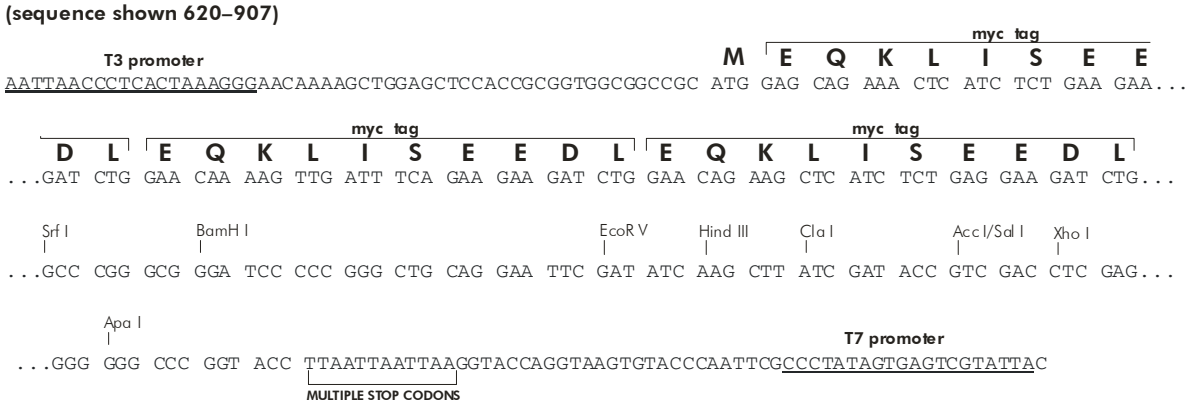
Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
3× FLAG tag	682–753
multiple cloning site	754–828
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	872–893
BGH polyA signal	908–1134
f1 origin of ss-DNA replication	1273–1579
ampicillin resistance (*bla*) ORF	1728–2594
HSV-thymidine kinase (TK) promoter	2615–2863
hygromycin resistance ORF	2867–3901
HSV-thymidine kinase (TK) polyA signal	3905–4461
pUC origin	4522–5189

FIGURE 5 Circular map of the pCMV-3Tag-6 vector, featuring eukaryotic expression, N-terminal FLAG epitope tagging, and ampicillin and hygromycin resistance.

pCMV-3Tag-7 Vector Map



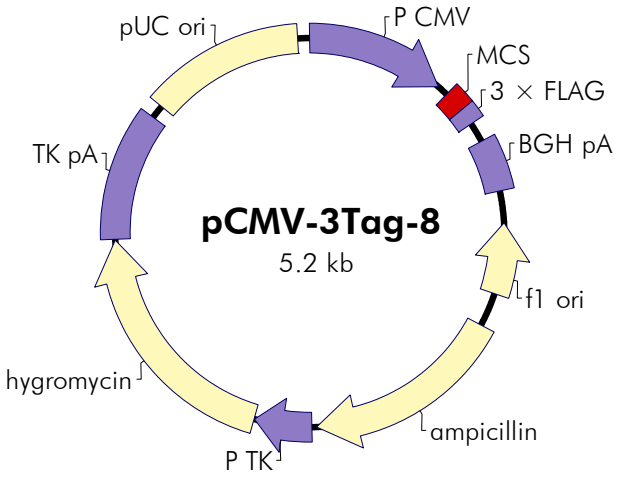
pCMV-3Tag-7 Multiple Cloning Site Region
(sequence shown 620–907)



Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
3× c-myc tag	678–767
multiple cloning site	768–842
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	886–907
BGH polyA signal	922–1148
f1 origin of ss-DNA replication	1287–1593
ampicillin resistance (*bla*) ORF	1742–2608
HSV-thymidine kinase (TK) promoter	2629–2877
hygromycin resistance ORF	2881–3915
HSV-thymidine kinase (TK) polyA signal	3919–4475
pUC origin	4536–5203

FIGURE 6 Circular map of the pCMV-3Tag-7 vector, featuring eukaryotic expression, N-terminal c-myc epitope tagging, and ampicillin and hygromycin resistance.

pCMV-3Tag-8 Vector Map



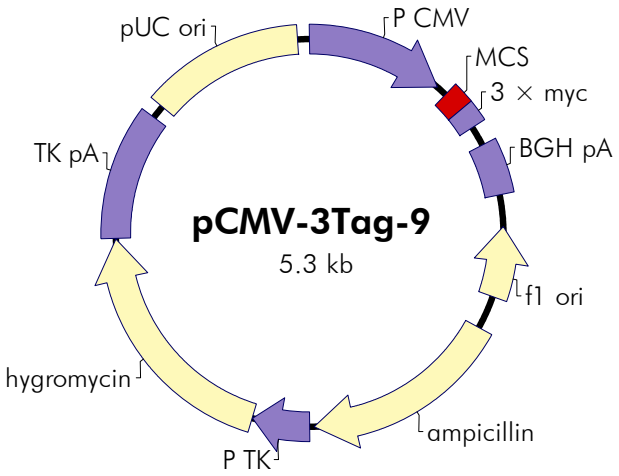
pCMV-3Tag-8 Multiple Cloning Site Region
(sequence shown 620–888)

```
                                                    Sac I     BstX I                Not I                 Srf I
                T3 promoter                           |         |                     |                     |
A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG...

          BamH I                                  EcoR V     Hind III    Cla I          Acc I/Sal I    Xho I
            |                                       |          |          |                |           |
...GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC CTC GAG...

      FLAG tag                                FLAG tag                                FLAG tag
  D   Y   K   D   D   D   D   K   D   Y   K   D   D   D   D   K   D   Y   K   D   D
...GAT TAC AAG GAT GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT GAC AAG GAC TAC AAA GAT GAT...

  D   D   K                                                                  T7 promoter
...GAC GAT AAA TAG GGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
          STOP
```

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
3× FLAG tag	744–815
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	867–888
BGH polyA signal	903–1129
f1 origin of ss-DNA replication	1268–1574
ampicillin resistance (*bla*) ORF	1723–2589
HSV-thymidine kinase (TK) promoter	2610–2858
hygromycin resistance ORF	2862–3895
HSV-thymidine kinase (TK) polyA signal	3900–4456
pUC origin	4517–5184

FIGURE 7 Circular map of the pCMV-3Tag-8 vector, featuring eukaryotic expression, C-terminal FLAG epitope tagging, and ampicillin and hygromycin resistance.

pCMV-3Tag-9 Vector Map



pCMV-3Tag-9 Multiple Cloning Site Region
(sequence shown 620–907)

T3 promoter
A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG GGC GGA TCC CCC...
Sac I BstX I Not I Srf I BamH I

...GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC CTC GAG GAG CAG AAA CTC ATC TCT GAA...
EcoR V Hind III Cla I Acc I/Sal I Xho I
myc tag E Q K L I S E

...GAA GAT CTG GAA CAA AAG TTG ATT TCA GAA GAA GAT CTG GAA CAG AAG CTC ATC TCT GAG GAA GAT CTG TAA ...
E D L
myc tag E Q K L I S E E D L
myc tag E Q K L I S E E D L
STOP

T7 promoter
...GGGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
3× c-myc tag	744–833
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	886–907
BGH polyA signal	922–1148
f1 origin of ss-DNA replication	1287–1593
ampicillin resistance (*bla*) ORF	1742–2608
HSV-thymidine kinase (TK) promoter	2629–2877
hygromycin resistance ORF	2881–3915
HSV-thymidine kinase (TK) polyA signal	3919–4475
pUC origin	4536–5203

FIGURE 8 Circular map of the pCMV-3Tag-9 vector, featuring eukaryotic expression, C-terminal c-myc epitope tagging, and ampicillin and hygromycin resistance.

TABLE I
LOCATIONS OF FEATURES FOR THE pCMV-3TAG-1–4 VECTORS

Vector	P CMV	MCS	3× FLAG	3× c-myc	BGH pA	f1 origin	P *bla*	P SV40	neo/kan ORF	pUC origin
1a	1–602	754–828	682–753	—	908–1134	1273–1579	1604–1728	1748–2086	2121–2912	3500–4167
1b	1–602	755–829	682–753	—	909–1135	1274–1580	1605–1729	1749–2087	2122–2913	3501–4168
1c	1–602	756–830	682–753	—	910–1136	1275–1581	1606–1730	1750–2088	2123–2914	3502–4169
2a	1–602	768–842	—	678–767	922–1148	1287–1593	1618–1742	1762–2100	2135–2926	3514–4181
2b	1–602	769–843	—	678–767	923–1149	1288–1594	1619–1743	1763–2101	2136–2927	3515–4182
2c	1–602	770–844	—	678–767	924–1150	1289–1595	1620–1744	1764–2102	2137–2928	3516–4183
3a	1–602	651–743	744–815	—	903–1129	1268–1574	1599–1723	1743–2081	2116–2907	3495–4162
3b	1–602	651–744	745–816	—	904–1130	1269–1575	1600–1724	1744–2082	2117–2908	3496–4163
3c	1–602	651–745	746–817	—	905–1131	1270–1576	1601–1725	1745–2083	2118–2909	3497–4164
4a	1–602	651–743	—	744–833	922–1148	1287–1593	1618–1742	1762–2100	2135–2926	3514–4181
4b	1–602	651–744	—	745–834	923–1149	1288–1594	1619–1743	1763–2101	2136–2927	3515–4182
4c	1–602	651–745	—	746–835	924–1150	1289–1595	1620–1744	1764–2102	2137–2928	3516–4183

TABLE II
LOCATIONS OF FEATURES FOR THE pCMV-3TAG-6–9 VECTORS

Vector	P CMV	MCS	3× FLAG	3× c-myc	BGH pA	f1 origin	amp ORF	P HSV-TK	hyg ORF	pUC origin
6	1–602	754–828	682–753	—	908–1134	1273–1579	1728–2594	2615–2863	2867–3901	4522–5189
7	1–602	768–842	—	678–767	922–1148	1287–1593	1742–2608	2629–2877	2881–3915	4563–5203
8	1–602	651–743	744–815	—	903–1129	1268–1574	1723–2589	2610–2858	2862–3895	4517–5184
9	1–602	651–743	—	744–833	922–1148	1287–1593	1742–2608	2629–2877	2881–3915	4536–5203

DESIGNING THE INSERT AND PREPARING THE pCMV-3TAG VECTORS

- When cloning into the C-terminal tagging vectors (pCMV-3Tag-3, pCMV-3Tag-4, pCMV-3Tag-8, or pCMV-3Tag-9), design the insert to contain a Kozak sequence and ensure that the insert contains a translation initiation codon. A complete Kozak sequence includes CC$\frac{A}{G}$CC**ATG**G, although CC**ATG**G, or the core **ATG**, is sufficient.

- In-frame stop codons are provided in each of the pCMV-3Tag-1–9 vectors. The N-terminal fusion vectors (pCMV-3Tag-1, pCMV-3Tag-2, pCMV-3Tag-6, and pCMV-3Tag-7) contain a multiple-stop-codon cassette that introduces a stop codon downstream of the insert in all three possible reading frames. The C-terminal fusion vectors (pCMV-3Tag-3, pCMV-3Tag-4, pCMV-3Tag-8, and pCMV-3Tag-9) contain in-frame stop codons immediately downstream of the epitope tags.

- We suggest dephosphorylation of the digested pCMV-3Tag vector with CIAP prior to ligation with the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by gel purification.

- After purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the plasmid DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

- The following are restriction enzymes which produce ends that are compatible with the cloning sites in the pCMV-3Tag vectors:

MCS Restriction Sites	Compatible Enzymes
BamH I	*Bcl* I, *Bgl* II, *Bst* I, *Mob* I, *Sau3*A I, *Xho* II
*Eco*R I	*Mun* I, *Mfe* I
EcoR V	Blunt ends
Not I	*Eae* I, *Eag* I, *Gdi* II, *Xma* III
Pst I	*Hgi*A I[a], *Nsi* I
Sal I	*Ava* I[a], *Pae*R71, *Xho* I
Srf I	Blunt ends
Xho I	*Ava* I[a], *Pae*R71, *Sal* I

[a] A subset of the sites apply.

LIGATING THE INSERT

The ideal insert-to-vector molar ratio of DNA is variable; however, a reasonable starting point is a 2:1 insert-to-vector ratio. The ratio is calculated using the following equation:

$$X\ \mu\text{g of insert} = \frac{(\text{Number of base pairs of insert})\,(\,0.1\,\mu\text{g of pCMV - 3Tag vector})}{\sim 4.2\,\text{kb of pCMV - 3Tag vector}}$$

where *X* is the quantity of insert (in micrograms) required for a 1:1 insert-to-vector molar ratio. Multiply *X* by 2 to get the quantity of insert required for a 2:1 ratio.

1. Prepare three control and two experimental 10-µl ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

Ligation reaction components	Control			Experimental	
	1[a]	2[b]	3[c]	4[d]	5[d]
Prepared pCMV-3Tag vector (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl	1.0 µl	1.0 µl
Prepared insert (0.1 µg/µl)	0.0 µl	0.0 µl	1.0 µl	*Y* µl	*Y* µl
rATP [10 mM (pH 7.0)]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Ligase buffer (10×)	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.0 µl	0.5 µl	0.5 µl	0.5 µl
Double-distilled (ddH_2O) to 10 µl	6.5 µl	7.0 µl	6.5 µl	*Z* µl	*Z* µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies if the digestion and CIAP treatment are effective.

[b] This control indicates whether the plasmid is cleaved completely or whether residual uncut plasmid remains. Expect an absence of transformant colonies if the digestion is complete.

[c] This control verifies that the insert is not contaminated with the original plasmid. Expect an absence of transformant colonies if the insert is pure.

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies to represent recombinants.

2. Incubate the reactions for 2 hours at room temperature or overnight at 4°C. For blunt-end ligation, reduce the rATP to 5 mM and incubate the reactions overnight at 12–14°C.

TRANSFORMATION

Transform competent bacteria with 1–2 µl of the ligation reaction, and plate the transformed bacteria on agar plates containing the appropriate antibiotic. For pCMV-3Tag-1–4 vectors, use LB-kanamycin agar plates. For pCMV-3Tag-6–9 vectors, use LB-ampicillin agar plates. (See *Preparation of Media and Reagents.*) Please see reference 3 for a transformation protocol.

VERIFICATION OF INSERT PERCENTAGE, SIZE, AND ORIENTATION

Individual colonies can be examined to determine the percentage of vectors with inserts and the insert size and orientation by PCR directly from the colony or by restriction analysis.

Polymerase Chain Reaction Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in a pCMV-3Tag vector may be determined by PCR amplification of DNA from individual colonies.

1. Prepare a PCR amplification reaction containing the following components:

   4.0 µl of 10× *Taq* DNA polymerase buffer
   0.4 µl of dNTP mix (25 mM each dNTP)
   40.0 ng of T3 primer
   40.0 ng of T7 primer
   0.4 µl of 10% (v/v) Tween 20
   1.0 U of *Taq* DNA polymerase
   dH_2O to a final volume of 40 µl

Vector	Primer	Nucleotide sequence (5´ to 3´)
pCMV-3Tag vector	T3	AATTAACCCTCACTAAAGGG
	T7	GTAATACGACTCACTATAGGGC

2. Stab a transformed colony with a sterile toothpick and swirl the colony into a reaction tube. Immediately following inoculation into the reaction mixture, remove the toothpick and streak onto antibiotic-containing patch plates for future reference.

3. Gently mix each reaction, overlay each reaction with 30 µl of mineral oil and perform PCR using the following cycling parameters:

Number of cycles	Temperature	Duration
1 cycle	94°C	4 minutes
	50°C	2 minutes
	72°C	2 minutes
30 cycles	94°C	1 minute
	56°C	2 minutes
	72°C	1 minute
1 cycle	72°C	5 minutes

4. Analyze the PCR products for the sizes of the genes inserted into the expression construct using standard 1% (w/v) agarose gel electrophoresis.

   When the recommended T3 and T7 primers are used for this analysis, the resulting PCR product spans the MCS and epitope tag regions. The expected sizes of the PCR products for plasmids lacking inserts are shown in the table below. (Plasmids containing inserts should produce PCR products of the insert size plus the size given below.) Additional information can be obtained by further restriction analysis of the PCR products.

Vector	Size of PCR Product (No Insert)
pCMV-3Tag -1	0.27 kb
pCMV-3Tag -2	0.29 kb
pCMV-3Tag -3	0.27 kb
pCMV-3Tag -4	0.29 kb
pCMV-3Tag -6	0.27 kb
pCMV-3Tag -7	0.29 kb
pCMV-3Tag -8	0.27 kb
pCMV-3Tag -9	0.29 kb

5. For protocols for transfection into mammalian cell lines please see Sambrook, *et al*. (1989).[4]

TROUBLESHOOTING

Observation	Suggestions
Western analysis does not detect fusion protein	Insert is cloned out of frame. Sequence the MCS region of the plasmid to verify the reading frame. Reclone if insert is out of frame.
	Transfer of proteins is poor. Repeat transfer and optimize time of transfer, current and gel concentration and/or use molecular weight markers that cover the range to be transferred.
	Membrane preparation is inadequate. Ensure proper membrane hydration.
	Primary or secondary antibody concentration is too low. Titrate antibody conjugates for optimum concentrations.
	Protein has degraded during storage of the membrane. Use fresh blots.
	Poor isolation of tagged protein. Use a different cell lysis procedure.
	Proteolytic cleavage may have occurred. Include protease inhibitors in the lysis buffer.
The membrane produces excessive background	Insufficient blocking solution may have been used or the membrane may not have been thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly.
	Too much protein was loaded on the gel. Load less protein.
	Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps when handling membranes.
	The concentration of the anti-FLAG, anti-c-myc, or secondary antibody is too high. Check the concentration of the antibodies and dilute if necessary.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

REFERENCES

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4. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

FLAG® is a registered trademark of Sigma-Aldrich Co.
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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.