pCMV-Tag Epitope Tagging Mammalian Expression Vectors

Instruction Manual

Catalog #211172 (pCMV-Tag 2), #211173 (pCMV-Tag 3), #211174 (pCMV-Tag 4), and #211175 (pCMV-Tag 5)

Revision C.0

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211172-12

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MATERIALS PROVIDED

Material provided	Concentration	Quantity[a]
Catalog #211172		
pCMV-Tag 2A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 2B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 2C mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 2 expression control plasmid[b]	1.0 µg/µl	20 µg
XL1-Blue host strain[c,d]	—	500 µl
Catalog #211173		
pCMV-Tag 3A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 3B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 3C mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 3 expression control plasmid[b]	1.0 µg/µl	20 µg
XL1-Blue host strain[c,d]	—	500 µl
Catalog #211174		
pCMV-Tag 4A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 4B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 4C mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 4 expression control plasmid[b]	1.0 µg/µl	20 µg
XL1-Blue host strain[c,d]	—	500 µl
Catalog #211175		
pCMV-Tag 5A mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 5B mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 5C mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 5 expression control plasmid[b]	1.0 µg/µl	20 µg
XL1-Blue host strain[c,d]	—	500 µl

[a] Sufficient reagents are provided for 25 reactions and 1 control reaction.

[b] Each control plasmid contains the firefly luciferase gene fused, in frame, with either the FLAG or c-myc tag.

[c] XL1-Blue Genotype: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)].

[d] **The XL1-Blue cells supplied with the pCMV-Tag vectors are not competent cells.** Refer to Hanahan (1983) for a protocol for producing competent cells.

STORAGE CONDITIONS

XL1-Blue Host Strain: Store immediately at –80°C
All Other Reagents: –20°C

Revision C.0

ADDITIONAL MATERIALS REQUIRED

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

NOTICES TO PURCHASER

CMV Promoter

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INTRODUCTION

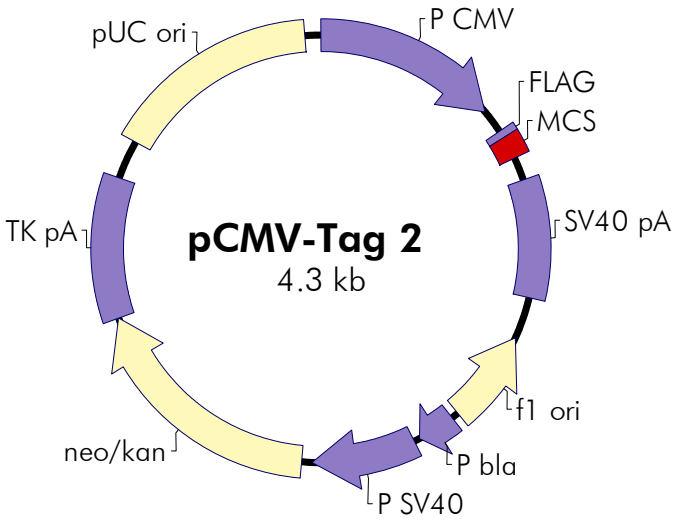
The epitope tagging technique involves fusion of a protein of interest to a peptide epitope that is recognized by a readily available antibody. With this technique, expression of the fusion protein is monitored using a tag-specific antibody, allowing a new protein to be studied without generating a new, specific antibody to that protein. 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 vectors pCMV-Tag 2, pCMV-Tag 3, pCMV-Tag 4, and pCMV-Tag 5 are a series of epitope tagging mammalian expression vectors. pCMV-Tag 2 (Figure 1) is an N-terminal FLAG® tagging vector, pCMV-Tag 3 (Figure 2) is an N-terminal c-myc tagging vector, pCMV-Tag 4 (Figure 3) is a C-terminal FLAG tagging vector and pCMV-Tag 5 (Figure 4) is 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. Thus, each pCMV-Tag vector has a reading frame that will allow cloning a gene of interest so that it is fused correctly with the epitope tag. Tagged constructs generated in the pCMV-Tag vectors can be transfected into mammalian cells and the fusion protein can be easily characterized using commercially available antibodies.

The pCMV-Tag vectors are derived from the pCMV-Script vector and contain sequences for either the FLAG or 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] In addition to the epitope tag sequences, the pCMV-Tag 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 neomycin-resistance gene is under control of both the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells. The multiple cloning site (MCS) of the pCMV-Tag vectors allows for a variety of cloning strategies, resulting in either C-terminal or N-terminal fusions with either FLAG or c-myc. A Kozak consensus sequence provides optimal expression of the fusion protein when the N-terminal FLAG epitope is used.[3] Other cloning options, which require fusion proteins to include their own translational start sequence, are also possible. The relative locations of the features for each vector are listed in Table 1.

The pCMV-Tag 2, pCMV-Tag 3, pCMV-Tag 4, and pCMV-Tag 5 expression control plasmids are included for use as positive controls to confirm that the CMV promoter is active in the cell line used for transfection. Each control plasmid contains the firefly luciferase gene fused, in reading frame, with either the FLAG or c-myc tag.

pCMV-Tag 2 Vector Map



pCMV-Tag 2 Multiple Cloning Site Region
(sequence shown 620–843)

T3 promoter — M

AA TTA ACC CTC ACT AAA GGG AAC AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCC ACC ATG...

KOZAK

FLAG tag

D Y K D D D D K

Srf I — BamH I — Pst I — EcoR I

...GAT TAC AAG GAT GAC GAC GAT AAG * GCC CGG GCG GAT CCC CCG GGC TGC AGG AAT TC...

EcoR V — Hind III — Acc I/Sal I — Xho I — Apa I

...G ATA TCA AGC TTA TCG ATA CCG TCG ACC TCG AGG GGG GGC CCG GTA CCT...

T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

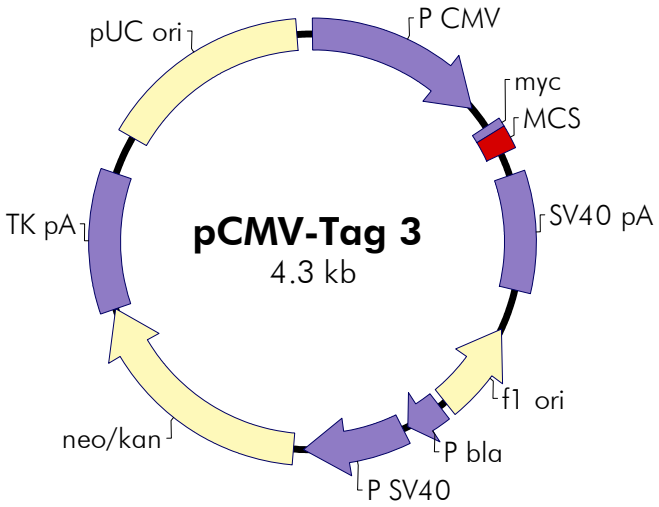
MULTIPLE STOP CODONS

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

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
FLAG tag	682–705
multiple cloning site	706–779
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	823–844
SV40 polyA signal	856–1239
f1 origin of ss-DNA replication	1377–1683
bla promoter	1708–1832
SV40 promoter	1852–2190
neomycin/kanamycin resistance ORF	2225–3016
HSV-thymidine kinase (TK) polyA signal	3017–3475
pUC origin	3604–4271

FIGURE 1 Circular map of the pCMV-Tag 2A–2C vectors, featuring eukaryotic expression, FLAG epitope tagging, neomycin and kanamycin resistance, T3 and T7 RNA promoters, and single-stranded rescue. The positions listed in the table above correspond to pCMV-Tag 2A. See Table 1 for the complete list of feature positions for the pCMV-Tag 2A–2C vectors.

pCMV-Tag 3 Vector Map



pCMV-Tag 3 Multiple Cloning Site Region
(sequence shown 620–846)

T3 promoter

AA TTA ACC CTC ACT AAA GGG AAC AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCC ATG...

M

KOZAK

myc tag

E Q K L I S E E D L

Srf I BamH I Pst I

...GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG * GCC CGG GCG GAT CCC CCG GGC TGC ...

EcoR I EcoR V Hind III Acc I/Sal I Xho I Apa I

...AGG AAT TCG ATA TCA AGC TTA TCG ATA CCG TCG ACC TCG AGG GGG GGC CCG GTA CCT...

T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

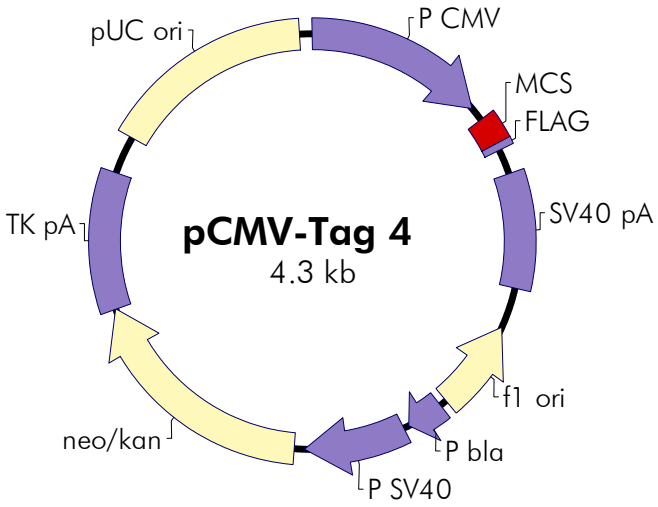
MULTIPLE STOP CODONS

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

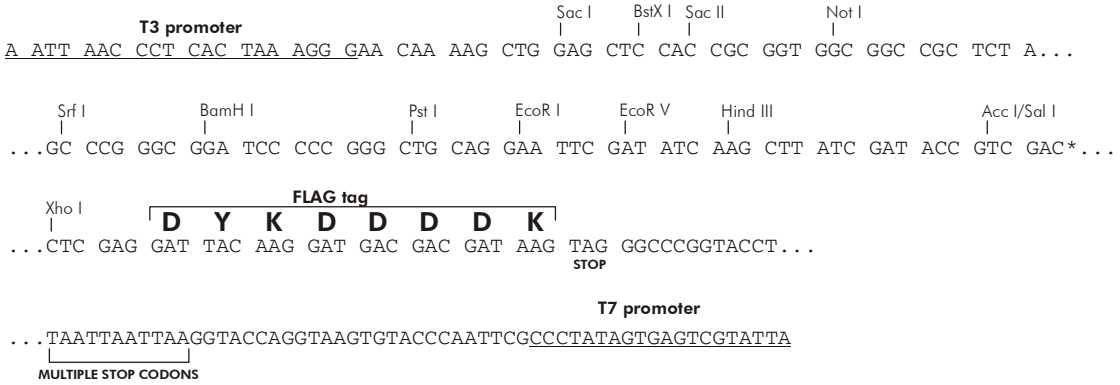
Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
c-myc tag	679–708
multiple cloning site	709–782
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	826–847
SV40 polyA signal	859–1242
f1 origin of ss-DNA replication	1380–1686
bla promoter	1711–1835
SV40 promoter	1855–2193
neomycin/kanamycin resistance ORF	2228–3019
HSV-thymidine kinase (TK) polyA signal	3020–3478
pUC origin	3607–4274

FIGURE 2 Circular map of the pCMV-Tag 3A–3C vectors, featuring eukaryotic expression, myc epitope tagging, neomycin and kanamycin resistance, T3 and T7 RNA promoters, and single-stranded rescue. The positions listed in the table above correspond to pCMV-Tag 3A. See Table 1 for the complete list of feature positions for the pCMV-Tag 3A–3C vectors.

pCMV-Tag 4 Vector Map



pCMV-Tag 4 Multiple Cloning Site Region
(sequence shown 620–839)

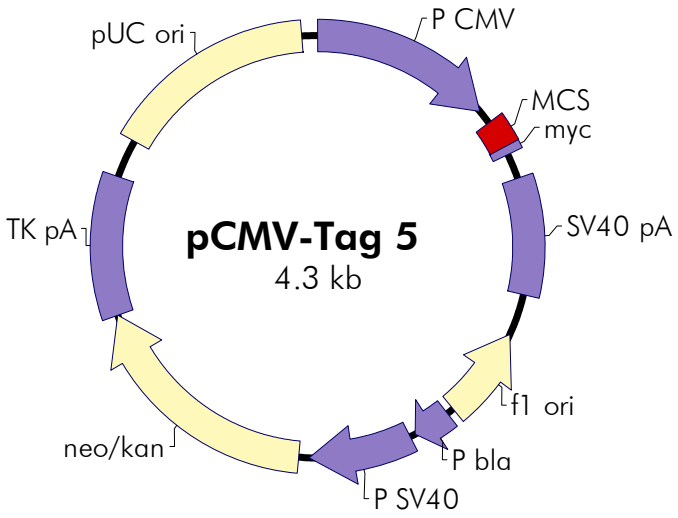


* In pCMV-Tag 4A, no bases inserted; in pCMV-Tag 4B, A inserted; in pCMV-Tag 4C, AA inserted

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
FLAG tag	744–767
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	819–840
SV40 polyA signal	852–1235
f1 origin of ss-DNA replication	1373–1679
bla promoter	1704–1828
SV40 promoter	1848–2186
neomycin/kanamycin resistance ORF	2221–3012
HSV-thymidine kinase (TK) polyA signal	3013–3471
pUC origin	3600–4267

FIGURE 3 Circular map of the pCMV-Tag 4A–4C vectors, featuring eukaryotic expression, FLAG epitope tagging, neomycin and kanamycin resistance, T3 and T7 RNA promoters, and single-stranded rescue. The positions listed in the table above correspond to pCMV-Tag 4A. See Table 1 for the complete list of feature positions for the pCMV-Tag 4A–4C vectors.

pCMV-Tag 5 Vector Map



pCMV-Tag 5 Multiple Cloning Site Region
(sequence shown 620–842)

T3 promoter

Sac I BstX I Sac II Not I

A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT A...

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

...GC CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC*...

Xho I myc tag

E Q K L I S E E D L

...CTC GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG TAG GGCCCGGTACCT...

STOP

T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

MULTIPLE STOP CODONS

* In pCMV-Tag 5A, no bases inserted; in pCMV-Tag 5B, A inserted; in pCMV-Tag 5C, AA inserted

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
c-myc tag	741–770
T7 promoter and T7 primer binding site [3′ CGGGATATCACTCAGCATAATG 5′]	822–843
SV40 polyA signal	855–1238
f1 origin of ss-DNA replication	1376–1682
bla promoter	1707–1831
SV40 promoter	1851–2189
neomycin/kanamycin resistance ORF	2224–3015
HSV-thymidine kinase (TK) polyA signal	3016–3474
pUC origin	3603–4270

FIGURE 4 Circular map of the pCMV-Tag 5A–5C vectors, featuring eukaryotic expression, myc epitope tagging, neomycin and kanamycin resistance, T3 and T7 RNA promoters, and single-stranded rescue. The positions listed in the table above correspond to pCMV-Tag 5A. See Table 1 for the complete list of feature positions for the pCMV-Tag 5A–5C vectors.

TABLE I
LOCATIONS OF FEATURES FOR THE pCMV-TAG 2–5 VECTORS

Vector	P CMV	MCS	FLAG	myc	SV40pA	f1 origin	P *bla*	P SV40	neo/kan ORF	pUC origin
2a	1–602	706–779	682–705	—	856–1239	1239–1700	1708–1832	1852–2190	2225–3016	3604–4271
2b	1–602	707–780	682–705	—	857–1240	1240–1701	1709–1833	1853–2191	2226–3017	3605–4272
2c	1–602	708–781	682–705	—	858–1241	1241–1702	1710–1834	1854–2192	2227–3018	3606–4273
3a	1–602	709–782	—	679–708	859–1242	1242–1703	1711–1835	1855–2193	2228–3019	3607–4274
3b	1–602	710–783	—	679–708	860–1243	1243–1704	1712–1836	1856–2194	2229–3020	3608–4275
3c	1–602	711–784	—	679–708	861–1244	1244–1705	1713–1837	1857–2195	2230–3021	3609–4276
4a	1–602	651–743	744–767	—	852–1235	1235–1696	1704–1828	1848–2186	2221–3012	3600–4267
4b	1–602	651–744	745–768	—	853–1236	1236–1697	1705–1829	1849–2187	2222–3013	3601–4268
4c	1–602	651–745	746–769	—	854–1237	1237–1698	1706–1830	1850–2188	2223–3014	3602–4269
5a	1–602	651–743	—	741–770	855–1238	1238–1699	1707–1831	1851–2189	2222–3013	3603–4270
5b	1–602	651–744	—	742–771	856–1239	1239–1700	1708–1832	1852–2190	2223–3014	3604–4271
5c	1–602	651–745	—	743–772	857–1240	1240–1701	1709–1833	1853–2191	2224–3015	3605–4272

Note *Complete sequences of the pCMV-Tag vectors are available from* www.genomics.agilent.com *or from the GenBank® database under the following accession numbers:*

pCMV-Tag vector	GenBank database accession number
pCMV-Tag 2A	AF072538
pCMV-Tag 2B	AF072539
pCMV-Tag 2C	AF072540
pCMV-Tag 3A	AF072997
pCMV-Tag 3B	AF072998
pCMV-Tag 3C	AF072999
pCMV-Tag 4A	AF073000
pCMV-Tag 4B	AF076310
pCMV-Tag 4C	AF076311
pCMV-Tag 5A	AF076312
pCMV-Tag 5B	AF076777
pCMV-Tag 5C	AF076778

PREPARATION OF HOST STRAINS

Reviving the Host Strain

Note *The host strain may thaw during shipment. The vial should be stored immediately at –20° or –80°C, but the strain remains viable longer if stored at –80°C. Avoid repeated freeze–thaw cycles to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.

2. Streak the splinters onto an LB-tetracycline agar plate.[§]

3. Incubate the plate overnight at 37°C.

4. Seal the plate with Parafilm® laboratory film and store the plate at 4°C for up to 1 week.

5. Restreak the colonies onto a fresh plate every week.

Preparing a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of LB-tetracycline broth[§] with one or two colonies from the plate. Grow the cells to late log phase (OD_{600} = 0.8–1.0).

2. Add 4.5 ml of a sterile glycerol–LB solution (5 ml of glycerol + 5 ml of LB broth) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/tube).

This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

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

PREPARING THE pCMV-TAG VECTORS

- When cloning into the pCMV-Tag 4 or pCMV-Tag 5 vectors, design the insert to contain a Kozak sequence. 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-Tag 2–5 vectors. The N-terminal fusion vectors pCMV-Tag 2 and pCMV-Tag 3 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-Tag 4 and pCMV-Tag 5 contain in-frame stop codons immediately downstream of the epitope tags.

- To reduce background, dephosphorylate the digested pCMV-Tag 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 with compatible ends to cloning sites in the pCMV-Tag vectors:

Enzyme	Compatible enzymes
BamH I	*Bcl* I, *Bgl* II, *Bst* I, *Mob* I, *Sau3*A I, *Xho* II
*Eco*R I	*Mun* I, *Mfe* I
*Eco*R 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:

$$X\,\mu g \text{ of insert} = \frac{(\text{Number of base pairs of insert})\,(0.1\,\mu g \text{ of pCMV - Tag vector})}{\sim 4.3\,kb \text{ of pCMV - Tag vector}}$$

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-Tag 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 LB-kanamycin agar plates (see *Preparation of Media and Reagents*). Please see reference 4 for a transformation protocol.

Note ***The XL1-Blue cells supplied with the pCMV-Tag vectors are not competent cells.*** *Refer to Hanahan (1983) for a protocol for producing competent cells.*[4] *(Competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg are available from Agilent.)*

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-Tag 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-Tag 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	Length of time
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 gene inserted into the expression construct using standard 1% (w/v) agarose gel electrophoresis. Because the forward and reverse PCR/sequencing primers are located on both sides of the MCS, **the expected size of the PCR product should be 0.23 kb plus the size of the insert (for pCMV-Tag2, the expected size is 204 bp)**. Additional information can be obtained by further restriction analysis of the PCR products.

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

TROUBLESHOOTING

Observation	Suggestions
Western analysis does not detect fusion protein	Insert is cloned out of frame. Sequence to ensure correct reading frame. Reclone if insert is out of frame
	The promoter is not active in the cell line used. Use the provided control plasmid
	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 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. Include protease inhibitors in lysis buffer
The membrane produces excessive background	Insufficient blocking solution may have been used or the membrane was not thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly
	Too much protein was loaded on gel. Load less protein on gel
	Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps
	The concentration of the anti-FLAG, anti-c-myc, or secondary anti-mouse 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 Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

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–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 1.25 ml of 10 mg/ml-filter-sterilized tetracycline
Pour into petri dishes (~25 ml/100-mm plate)
Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended periods as tetracycline is light-sensitive

LB–Tetracycline Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 1.25 ml of 10 mg/ml-filter-sterilized tetracycline
Store broth in a dark, cool place as tetracycline is light-sensitive

TE Buffer

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

REFERENCES

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4. Hanahan, D. (1983) *J Mol Biol* 166(4):557-80.
5. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

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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.