pCMV-Tag 1 Epitope Tagging Mammalian Expression Vector

Instruction Manual

Catalog #211170

Revision C.0

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211170-12

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MATERIALS PROVIDED

Material provided	Concentration	Quantity[a]
pCMV-Tag 1 mammalian expression vector	1.0 µg/µl	20 µg
pCMV-Tag 1 control vector	1.0 µg/µl	10 µg
XL1-Blue host strain[b]	—	500 µl

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

[b] Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]

STORAGE CONDITIONS

XL1-Blue Host Strain: Store immediately at –80°C

All Other Reagents: –20°C

ADDITIONAL MATERIALS REQUIRED

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

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.

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

Revision C.0

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INTRODUCTION

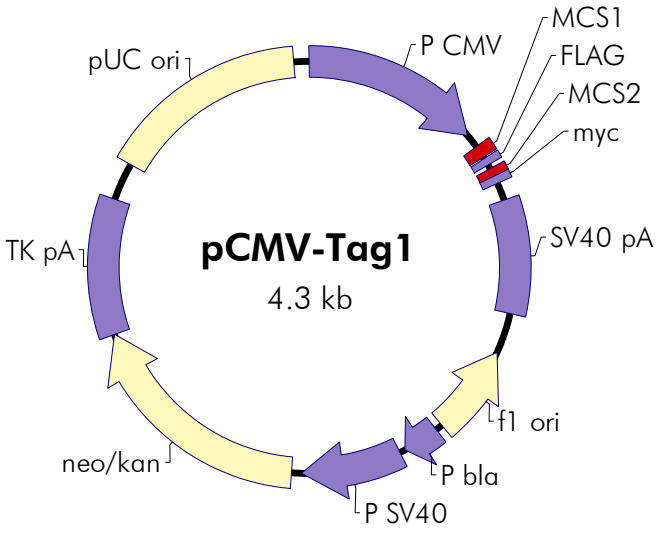
pCMV-Tag 1 is an epitope tagging vector designed for gene expression in mammalian cells. A target gene inserted into the pCMV-Tag1 vector can be tagged with the FLAG® epitope (N-terminal, C-terminal, or internal tagging), the c-myc epitope (C-terminal) or both the FLAG (N-terminal) and c-myc (C-terminal) epitopes. Tagged constructs generated in the pCMV-Tag 1 vector can be transfected into mammalian cells, and the tagged gene product can be easily characterized using commercially available tag-specific antibodies.

The epitope tagging technique involves fusion of a protein of interest to a peptide epitope that is recognized by a readily available antibody. In 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.

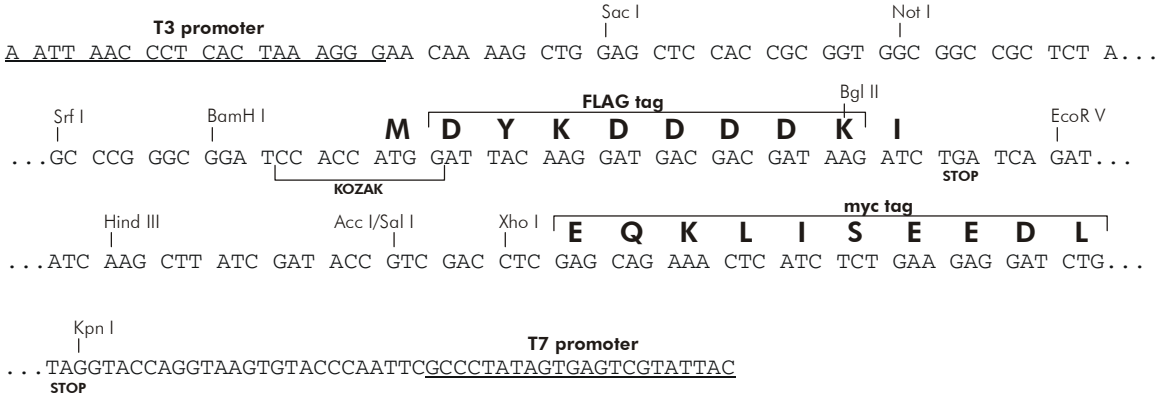
Description of the Vector

The pCMV-Tag 1 vector (see Figure 1) is derived from the pCMV-Script vector and contains sequences for the FLAG and c-myc epitopes. 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 comprises eight amino acid residues (DYKDDDDK)[1]. The c-myc epitope is derived from the human c-myc gene and comprises 10 amino acid residues (EQKLISEEDL).[2] In addition to the epitope tag sequences, the pCMV-Tag 1 vector contains 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 1 vector allows a variety of cloning strategies, resulting in C-terminal, N-terminal, or internal tagging (see Figure 2). 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.

pCMV-Tag 1 Vector Map



pCMV-Tag 1 Multiple Cloning Site Region
(sequence shown 620–840)



Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site 1 (Sac I to BamH I)	651–692
FLAG tag	699–722
multiple cloning site 2 (EcoR V to Xho I)	737–764
c-myc tag	762—791
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 1 Circular map and polylinker sequence of the pCMV-Tag 1 vector. The entire sequence of the pCMV-Tag 1 vector is available from *www.genomics.agilent.com* or from the GenBank® database (Accession #AF025668).

EPITOPE TAGGING STRATEGIES WITH PCMV-TAG 1

The gene of interest can be cloned into the pCMV-Tag 1 vector so that the protein can be terminally tagged with FLAG or c-myc. If, however, the protein function will be disrupted by a terminal tag, this vector also allows for internal tagging with FLAG (see the following table and Figure 2). For C-terminal tagging with FLAG or c-myc, a translation initiation sequence must be supplied by the insert. This may be supplied by the gene or engineered by PCR. A perfect Kozak sequence is CCACC**ATG**G, although CC**ATG**G, or the core **ATG**, is sufficient.

	Epitope tag	Tag location	Cloning site	Kozak sequence
A	FLAG	C terminus	MCS 1	Insert-supplied
B	FLAG	N terminus	Bgl II	Vector-supplied
C	FLAG and c-myc	N and C termini	Bgl II & MCS 2	Vector-supplied
D	c-Myc	C terminus	MCS 1 & 2	Insert-supplied
E	FLAG	Internal	MCS 1 & Bgl II	Insert-supplied
F	No tag	N/A	MCS 1 or MCS 1 & 2	Insert-supplied

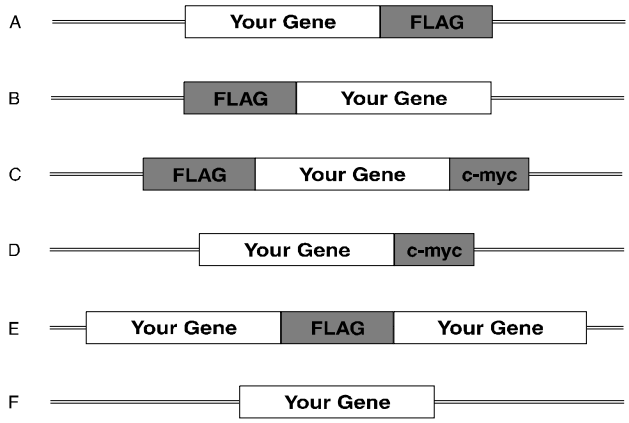


FIGURE 2 Possible cloning positions for gene of interest.

C-Terminal FLAG Tag

To achieve a C-terminal FLAG tag on the protein of interest, the gene must be cloned into MCS 1 (see Figure 3A). In this case, the Kozak sequence for the translational start site must be supplied by the insert. The FLAG tag will be picked up on the C terminus of the protein and the stop codon immediately downstream of the tag will cause translation to cease.

N-Terminal FLAG Tag

To achieve an N-terminal FLAG tag on the protein of interest, the gene should be cloned in at the Bgl II site (see Figure 3B). The Kozak sequence in the vector can be used and the FLAG sequence will be at the beginning of the protein. The Bgl II site is located before the stop codon, so translation may be terminated by a stop codon supplied either by the insert or by the vector.

Double Tagging with N-Terminal FLAG and C-Terminal c-Myc

To tag the protein of interest with FLAG at the N terminus and c-myc at the C terminus, the coding region of the gene should be cloned in between the Bgl II site and a restriction enzyme of choice in MCS 2 (see Figure 3C). The Kozak and stop codon sequences are supplied by the vector.

C-Terminal c-Myc Tag

To tag the protein of interest at the C terminus with the c-myc epitope tag, the gene should be cloned between MCS 1 and MCS 2 (see Figure 3D). This allows for the use of the stop codon after the c-myc tag. However, the Kozak sequence must be supplied with the insert.

Internal FLAG Tagging

To tag the protein of interest internally with the FLAG tag, the 5´ portion of the gene, including a Kozak sequence, must be cloned within MCS 1 (see Figure 3E). The 3´ portion of the gene must be cloned within the Bgl II site immediately after which the vector-supplied stop codon appears.

No Tag

To clone the gene of interest into the pCMV-Tag 1 vector without any tag, the gene, including its own initiation and termination codon, should be cloned into MCS 1 (see Figure 3F).

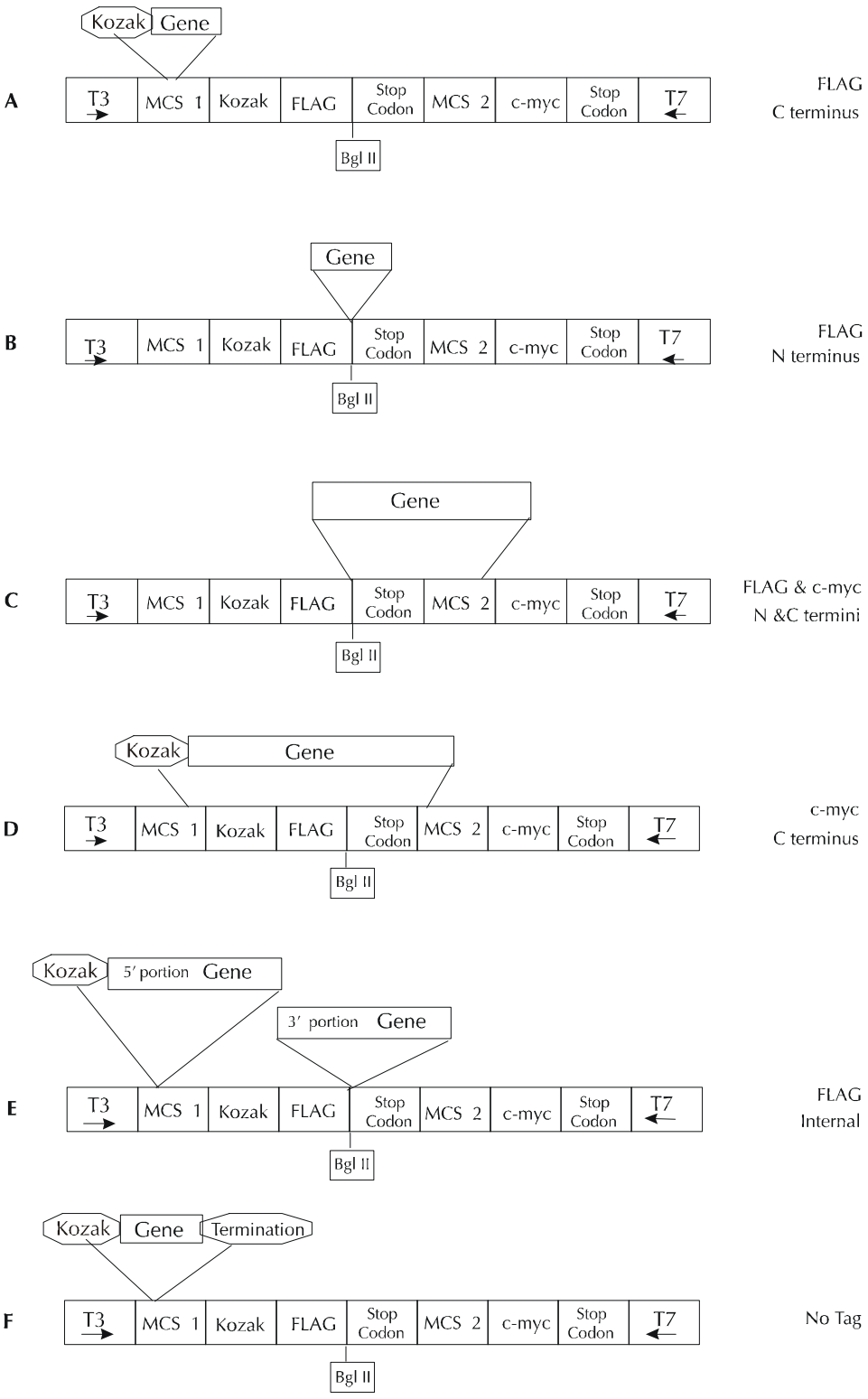


FIGURE 3 Cloning strategies for epitope tagging in the pCMV-Tag 1 vector.

PREPARATION OF HOST STRAINS

Bacterial Host Strain

The XL1-Blue host strain has been sent as a bacterial glycerol stock. For the appropriate media, please refer to the following table:

Agar for bacterial streak	Medium for bacterial glycerol stock
LB–tetracycline[a,b]	LB–tetracycline[a,b]

[a] 12.5 µg/ml.

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

On arrival, prepare the following from the bacterial glycerol stock using the appropriate medium as indicated in the previous table:

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. It is also best to avoid repeated thawing of the host strain in order 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 agar plate (see *Preparation of Media and Reagents*) containing the appropriate antibiotic, if one is necessary.

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.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of appropriate liquid medium containing antibiotic 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–liquid medium solution (5 ml of glycerol + 5 ml of appropriate medium) 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.

- Ensure that the coding sequence of the insert is in the correct reading frame.

- We suggest dephosphorylation of the digested pCMV-Tag 1 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 electrophoresing the DNA on an agarose gel and removing the desired vector band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.

- 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).

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:

$$X\ \mu\text{g of insert} = \frac{(\text{Number of base pairs of insert})\ (0.1\ \mu\text{g of pCMV-Tag1 vector})}{4576\ \text{bp of pCMV-Tag1 vector}}$$

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 sterile 1.5-ml microcentrifuge tubes:

	Control			Experimental	
Ligation reaction components	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[d]
Prepared pCMV-Tag 1 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×)[e]	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.

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

2. Incubate the reactions for 2 hours at room temperature (22°C) 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. Refer to reference 4 in *References* for a transformation protocol.

Note ***The XL1-Blue cells supplied with the pCMV-Tag 1 vector are not competent cells.*** *Refer to Hanahan (1983) for a protocol for producing competent cells.*[4] *Alternatively, Agilent competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg are also available.*

The control plasmid comprises the pCMV-Tag 1 vector and the luciferase coding sequence (1.65 kb). The firefly luciferase gene was cloned into the Bg1 *II/*Xho *I site of pCMV-Tag 1 so that the luciferase protein is tagged with the FLAG epitope at the N-terminus and with the c-myc epitope at the C terminus.*

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.

PCR Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in the pCMV-Tag 1 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	Binds to nucleotide (nt)	Nucleotide sequence (5′ to 3′)
pCMV-Tag 1 vector	T3	620–639	AATTAACCCTCACTAAAGGG
	T7	799–778	GTAATACGACTCACTATAGGGC

2. Stab the transformed colonies with a sterile toothpick and swirl the colony into reaction tubes. 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 167 bp plus the size of the insert**. 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	Suggestion
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
	Assay is not sufficiently sensitive or is being performed incorrectly. Use a positive control

PREPARATION OF MEDIA AND REAGENTS

10× *Taq* DNA Polymerase Buffer

100 mM Tris-HCl (pH 8.8)
15 mM $MgCl_2$
500 mM KCl
0.01% (w/v) gelatin

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

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–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 time periods as tetracycline is light-sensitive

TE Buffer

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

REFERENCES

1. Hopp, T., Prickett, K., Price, V., Libby, R., March, C. *et al.* (1988) *BioTechnology* 6:1204-1210.
2. Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985) *Mol Cell Biol* 5(12):3610-6.
3. Kozak, M. (1991) *J Biol Chem* 266(30):19867-70.
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

MSDS INFORMATION

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