pCMV-Script Vector

Instruction Manual

Catalog #212220

Revision C.0

For Research Use Only. Not for use in diagnostic procedures.

212220-12

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.genomics.agilent.com* and click **Contact Us**

pCMV-Script Vector

CONTENTS

pCMV-Script Vector

MATERIALS PROVIDED

Materials provided	Concentration	Quantity
pCMV-Script vector[a]	1 µg/µl	20 µg (20 µl)
XL1-Blue MRF´ host strain	---	0.5 ml

[a] The complete sequence and restriction sites for the pCMV-Script vector can be found at *www.genomics.agilent.com*.

STORAGE CONDITIONS

XL1-Blue MRF' Host Strain: –20° C for short term storage; –80°C for long term storage
pCMV-Script Vector: –20°C

ADDITIONAL MATERIALS REQUIRED

T4 DNA ligase
CIAP
TE buffer[§]
High efficiency competent cells (≥5 × 10^9 cfu/µg DNA)
LB-kanamycin agar[§] plates
LB-tetracycline agar[§] plates
Taq DNA polymerase
Taq DNA polymerase buffer

NOTICE TO PURCHASER

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

INTRODUCTION

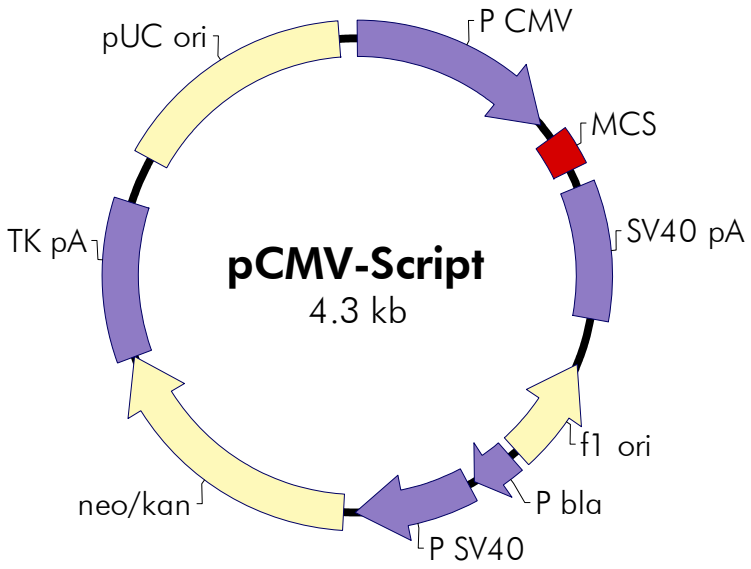
The pCMV-Script vector is derived from a high-copy-number pUC-based plasmid and is designed to allow protein expression in mammalian systems. Mammalian expression is driven by the human cytomegalovirus (CMV) immediate early promoter to promote constitutive expression of cloned inserts in a wide variety of cell lines. Selection is made possible in bacteria by the kanamycin-resistance gene under control of the prokaryotic β-lactamase promoter. The neomycin-resistance gene is driven by the SV40 early promoter, which provides stable selection with G418 in mammalian cells. [1]

The pCMV-Script vector does not contain an ATG initiation codon. A translation initiation sequence must be incorporated if the DNA fragment to be cloned does not have an initiating ATG codon or an optimal sequence for initiating translation, such as the Kozak sequence [GCC(A/G)CCATGG].[2]

The multiple cloning site (MCS) contains fifteen unique restriction enzyme recognition sites organized with alternating 5´ and 3´ overhangs to allow serial exonuclease III/mung bean nuclease deletions. T3 and T7 RNA polymerase promoters flank the polylinker for in vitro RNA synthesis. The choice of promoter used to initiate transcription determines which strand of the DNA insert will be transcribed.

The pCMV-Script vector can be rescued as single-stranded (ss) DNA. The plasmid contains a 454-nucleotide filamentous f1 phage intergenic region (M13-related) that includes the 307 bp origin of replication. The orientation of the f1 origin in pCMV-Script allows the rescue of antisense ssDNA by a helper phage. This ssDNA can be used for dideoxynucleotide sequencing (Sanger method) or site-directed mutagenesis.

pCMV-Script Vector



pCMV-Script Multiple Cloning Site Region
(sequence shown 620–799)

Sac I BstX I Sac II Not I
T3 promoter
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTA...

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

Xho I Apa I Kpn I
T7 promoter
...CTCGAGGGGGGGCCCGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site	620–639
multiple cloning site	651–758
T7 promoter and T7 primer binding site	778–799
SV40 polyA signal	811–1194
f1 origin of ss-DNA replication	1332–1638
bla promoter	1663–1787
SV40 promoter	1807–2145
neomycin/kanamycin resistance ORF	2180–2971
HSV-thymidine kinase (TK) polyA signal	2972–3421
pUC origin	3559–4226

Figure 1 Circular map and polylinker sequence of the pCMV-Script vector. The complete vector sequence is available at *www.genomics.agilent.com*.

HOST STRAIN AND GENOTYPE

XL1-Blue MRF´ Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^{r})]

The XL1-Blue MRF´ (Minus Restriction) strain is a restriction minus (McrA^{-}, McrCB^{-}, McrF^{-}, Mrr^{-}, HsdR^{-}) derivative of the XL1-Blue strain.[3]

For the appropriate media, please refer to the following table:

Bacterial strain	Agar plate for bacterial streak	Medium for bacterial glycerol stock
XL1-Blue MRF´	LB–tetracycline agar[a]	LB–tetracycline broth[a]

[a] 12.5 µg/ml.

On arrival, prepare the following from the glycerol stock:

Note *The host strain may thaw during shipment. The vial should be stored immediately at –20° or –80°C, but most strains remain 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-tetracycline agar plate.

3. Restreak the cells fresh each week.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of the LB-tetracycline liquid medium with one or two colonies from the plate. Grow the cells to late log phase.

2. Add 4.5 ml of a sterile glycerol–liquid medium solution (prepared by combining 5 ml of glycerol + 5 ml of liquid 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.

CLONING PROTOCOL FOR THE pCMV-SCRIPT VECTOR

The pCMV-Script vector features an MCS with fifteen unique, conveniently arranged restriction enzyme sites for cloning and subcloning the DNA sequence of interest. Expression is driven by the human CMV promoter, a strong promoter that allows high-level constitutive expression in a variety of mammalian cell lines. The vector has a neomycin-resistance gene for selection of stable cell lines (see Figure 1).

Preparing the pCMV-Script Vector

- A translation initiation sequence must be incorporated in the insert DNA if the DNA fragment to be cloned does not have an initiating ATG codon. Include a Kozak sequence for optimal translation. A complete Kozak sequence includes CC$\frac{A}{G}$CC**ATG**G, although CC**ATG**G, or the core **ATG**, is sufficient.

- Dephosphorylate the digested pCMV-Script 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 of the digested vector.

- 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

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

$$X\ \mu\text{g of insert} = \frac{(\text{Number of base pairs in insert})\ (0.1\ \mu\text{g of pCMV-Script vector})}{4278\ \text{bp of pCMV-Script vector}}$$

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 a separate sterile 1.5-ml microcentrifuge tube for each reaction:

Suggested Ligation Reactions

Ligation reaction components	Control			Experimental	
	1[a]	2[b]	3[c]	4[d]	5[d]
Prepared pCMV-Script 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 H_2O (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. We suggest using 1:1 and 2:1 ratios. 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

1. Transform competent bacteria with 1–5 µl of the ligation reactions.

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

2. Plate the transformed bacteria on LB-kanamycin agar plates.[§]

[§] See *Preparation of Media and Reagents*

VERIFICATION OF INSERT PERCENTAGE, SIZE, AND ORIENTATION

Individual colonies can be examined by PCR directly from the colony or by restriction analysis to identify the vectors with inserts and determine the insert size and orientation. T3 and T7 primers are recommended for use in PCR amplification and sequencing from the pCMV-Script vector.

Polymerase Chain Reaction Amplification of DNA from Individual Colonies

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

1. For each colony to be examined, 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-Script vector	T3	AATTAACCCTCACTAAAGGG
	T7	GTAATACGACTCACTATAGGGC

2. Stab a transformed colony with a sterile toothpick and swirl cells from the colony into the amplification reaction mixture. 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, then overlay the reactions 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 to determine insert sizes 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 180 bp plus the size of the insert**. Additional information can be obtained by restriction analysis of the PCR products.

TRANSFECTION INTO MAMMALIAN CELLS

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

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 time periods as tetracycline is light-sensitive

TE Buffer

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

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

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*

REFERENCES

1. Alting-Mees, M. A., Sorge, J. A. and Short, J. M. (1992) *Methods Enzymol* 216:483-95.
2. Kozak, M. (1991) *J Biol Chem* 266(30):19867-70.
3. Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987) *Biotechniques* 5(4):376–378.
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

Tween® is a registered trademark of ICI Americas, Inc.

MSDS INFORMATION

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