pCMV-Script XR Predigested Vector

Instruction Manual

Catalog #212224

Revision C.0

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212224-12

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pCMV-Script XR Predigested Vector

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity
pCMV-Script XR predigested vector[a,b]	30 ng/µl	55 µl (1650 ng)
XR LacZ test insert (600 bp)	10 ng/µl	3 µl (30 ng)

[a] The pCMV-Script XR predigested vector is digested with *Eco*R I/*Xho* I and CIAP-treated.

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

STORAGE CONDITIONS

pCMV-Script XR Predigested Vector: –20°C
XR LacZ Test Insert: –20°C

ADDITIONAL MATERIALS REQUIRED

T4 DNA ligase
TE buffer[§]
High efficiency competent cells (≥5 × 10^9 cfu/µg DNA)
LB-kanamycin agar plates[§]
X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)
IPTG (isopropyl-β-D-thio-galactopyranoside)
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

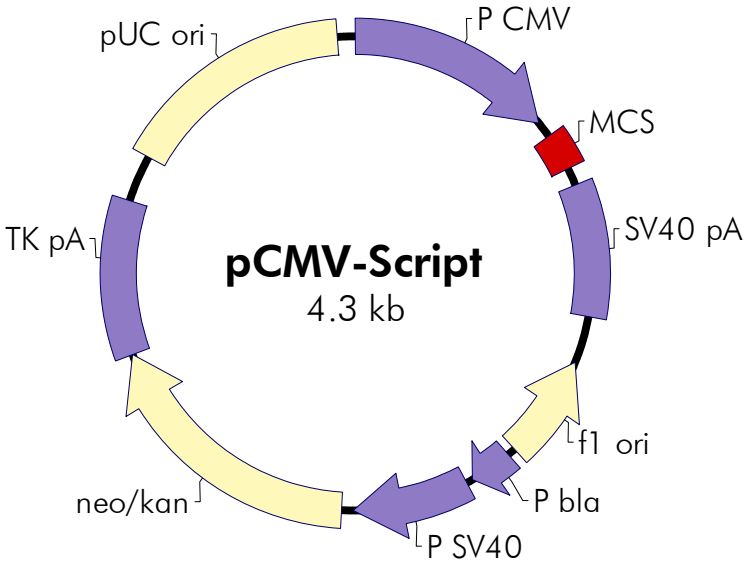
The pCMV-Script XR predigested vector is derived from a high-copy-number pUC-based plasmid and is designed to allow protein expression in mammalian systems. Mammalian expression is driven by the human cytomegalovirus (CMV) immediate early promoter to promote constitutive expression of cloned inserts 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 XR 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 pCMV-Script XR vector is predigested with *Eco*R I and *Xho* I restriction enzymes. The multiple cloning site (MCS) contains 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 XR 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*. The vector supplied in this kit has been digested with *Eco*R I and *Xho* I restriction enzymes, and does not contain the sequence between *Eco*R I and *Xho* I.

CLONING PROTOCOL FOR THE pCMV-SCRIPT XR VECTOR

The pCMV-Script XR vector is designed for the convenient insertion of DNA inserts compatible with the *Eco*R I/*Xho* I cloning site. This vector features an MCS with eleven unique, conveniently arranged restriction enzyme sites for 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).

Cloning Considerations

- 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. For optimal translation, include 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.

- The insert should have ends compatible with the *Eco*R I/*Xho* I ends of the pCMV-Script XR vector.

- The insert DNA should be suspended in a volume of TE buffer[§] that will allow the concentration of the insert DNA to be the same as the concentration of the vector DNA (0.03 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:

$$X\ \mu\text{g of insert} = \frac{(\text{Number of base pairs in insert})\ (\ 0.03\ \mu\text{g of pCMV - Script vector})}{4278\ \text{bp of pCMV - Script vector}}$$

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 one control and two experimental 5-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[b]
Prepared pCMV-Script XR vector (0.03 µg/µl)	1.0 µl	1.0 µl	1.0 µl
Prepared insert (0.03 µg/µl)	0.0 µl	*X* µl	*X* µl
XR LacZ test insert (0.01 µg/µl)	1.0 µl	0 µl	0 µl
rATP [10 mM (pH 7.0)]	0.5 µl	0.5 µl	0.5 µl
Ligase buffer (10×)[§]	0.5 µl	0.5 µl	0.5 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.5 µl	0.5 µl
Double-distilled H_2O (ddH_2O) to 10 µl	1.5 µl	*Y* µl	*Y* µl

[a] This control verifies the ability of the vector to ligate the test insert.

[b] These experimental ligation reactions vary the insert-to-vector ratio. We recommend preparing reactions with 1:1 and 2:1 ratios. Expect a majority of the transformant colonies to represent recombinants.

2. Incubate the reactions overnight at 4°C.

TRANSFORMATION

1. Transform competent bacteria with 1–5 µl of the ligation reactions. Refer to Hanahan (1983) for a protocol for producing competent cells.[3] When performing the control with the XR LacZ test insert, transform the ligation reaction into cells suitable for blue/white screening, such as the Agilent XL1-Blue strain.

   Note *Use competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg for preparing libraries. (Competent cells with transformation efficiencies of ≥5 × 10^9 cfu/µg are also available from Agilent.)*

2. Plate the experimental transformants on LB-kanamycin agar plates.[§] Plate the control transformants on LB-kanamycin agar plates containing X-gal and IPTG.[§] Blue colonies on the control plates contain the test insert.

[§] 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 XR vector.

Polymerase Chain Reaction Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in a pCMV-Script XR 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 150 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).[4]

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)

TE Buffer

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

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–Kanamycin Agar Plates Containing X-gal and IPTG (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin
Add X-gal [prepared in dimethyl-formamide (DMF)] to a final concentration of 80 µg/ml
Add IPTG (prepared in sterile distilled water) to a final concentration of 20 mM
Pour into petri dishes (~25 ml/100-mm plate)

Alternative Method

Spread 100 µl of 10 mM IPTG and 100 µl of 2% X-gal on LB-kanamycin agar plates 30 minutes prior to plating the transformations

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. Hanahan, D. (1983) *J Mol Biol* 166(4):557–80.
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

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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.