pDual GC Expression Vector

Instruction Manual

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Revision C.0

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pDUAL GC EXPRESSION VECTOR

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pDual GC Expression Vector

MATERIALS PROVIDED

Materials provided	Quantity
pDual GC expression vector (1 μg/μl in TE buffer)	20 μg
XL1-Blue MRF´ host strain, glycerol stock	500 μl

STORAGE CONDITIONS

pDual GC expression vector: –20°C
XL1-Blue MRF´ host strain: Store glycerol stock immediately at –80°C. Process according to the protocol in *Host Strains and Genotypes*.

ADDITIONAL MATERIALS REQUIRED

*Eam*1104 I
alkaline phosphatase, molecular biology grade
5-methyldeoxycytosine (^{m5}dCTP)
DNA polymerase, high fidelity
T4 DNA ligase

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.

T7 Promoter

U.S. Patent No. 4,952,496. For academic and non-profit laboratories, an assurance letter accompanies the sale of the products. For commercial laboratories, a research use license agreement must be entered into prior to purchase of the products.

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INTRODUCTION

The pDual GC vector, which is based on Agilent's pDual expression vector, is designed for high-level protein expression in mammalian and bacterial cells (see Figure 1). The vector contains the promoter and enhancer region of the human cytomegalovirus (CMV) immediate early gene‡ for constitutive expression of the clones in either transiently or stably transfected mammalian cells. Inducible gene expression in prokaryotes is directed from the hybrid T7/*lacO* promoter; the vector carries a copy of the *lac* repressor gene (*lacI^q^*), which mediates tight repression in the absence of isopropyl-β-D-thio-galactopyranoside (IPTG). Expression is therefore regulated using IPTG in bacteria that contain the T7 RNA polymerase, for example, BL21(DE3) bacterial cells. A tandem arrangement of the bacterial Shine-Dalgarno[1] and mammalian Kozak[2] ribosomal binding sites (RBS) allows for efficient expression of the ORF in both bacterial and mammalian systems.

The unique cloning region of the pDual GC expression vector is characterized by the presence of two *Eam*1104 I recognition sequences (CTCTTC) directed in opposite orientations and separated by a spacer region encoding the β-lactamase gene with a prokaryotic promoter. Digesting the vector with the *Eam*1104 I restriction enzyme creates a 3-nucleotide 5´ overhang that is complementary to the translation initiation codon (ATG) of the DNA insert.

Inserts must be generated by PCR amplification with primers that contain *Eam*1104 I recognition sites and a minimal flanking sequence at their 5´ termini. The ability of *Eam*1104 I to cleave several bases downstream of its recognition site allows the removal of superfluous, terminal sequences from the amplified DNA insert. The elimination of extraneous nucleotides and the generation of unique, nonpalindromic sticky ends permit the formation of directional seamless junctions during the subsequent ligation to the pDual GC expression vector.[3]

In both bacterial and mammalian cells, the dominant selectable marker is the neomycin phosphotransferase gene, which is under the control of the β-lactamase promoter in bacterial cells and the SV40 promoter in mammalian cells. Expression of the neomycin phosphotransferase gene in mammalian cells allows stable clone selection with G418, whereas in bacteria the gene confers resistance to kanamycin selection.

‡ See *Notices to Purchaser*.

All pDual GC clones express a fusion protein consisting of the cDNA, a thrombin cleavage site, three copies of the c-myc epitope tag, and a single copy of the 6×His purification tag. The c-myc epitope is derived from the human *c-myc* gene and contains 10 amino acid residues (EQKLISEEDL).[4] This allows for convenient and sensitive detection of expressed proteins with anti–c-myc antibody. The 6×His purification tag consists of six histidine residues and allows for quick and easy purification of the fusion protein from bacterial cells.[5] A thrombin cleavage site between the protein encoded by the cDNA and the c-myc and 6×His tags allows the removal of both tags when desired, for example, following protein purification.

Features of the pDual GC Expression Vector[a]

Feature	Nucleotide Position
bla promoter	2–126
SV40 promoter	146–484
neomycin/kanamycin resistance ORF	519–1310
HSV-thymidine kinase (TK) polyA signal	1487–1759
pUC origin of replication	1898–2565
lacI^q repressor ORF	2652–3608
CMV promoter	3810–4394
T7 promoter with *lac* operator	4399–4444
ribosome binding site	4460–4466
Kozak sequence	4467–4472
Eam1104 I site (reverse complement)	4477
ampicillin resistance (*bla*) ORF	4596–5453
Eam1104 I site	5498
thrombin target	5532–5549
3x c-myc tag	5556–5645
6x His tag	5676–5693
5´-splice site	5745–5760
lac operator	5767–5792
SV40 3´-splice site	5840–5899
SV40 polyA signal	5909–6121
T7 terminator	6130–6173
f1 origin of ss-DNA replication	6311–6617

[a] The complete sequence and list of restriction sites for the vector pDual GC are available at *www.genomics.agilent.com*.

pDual GC Vector Map

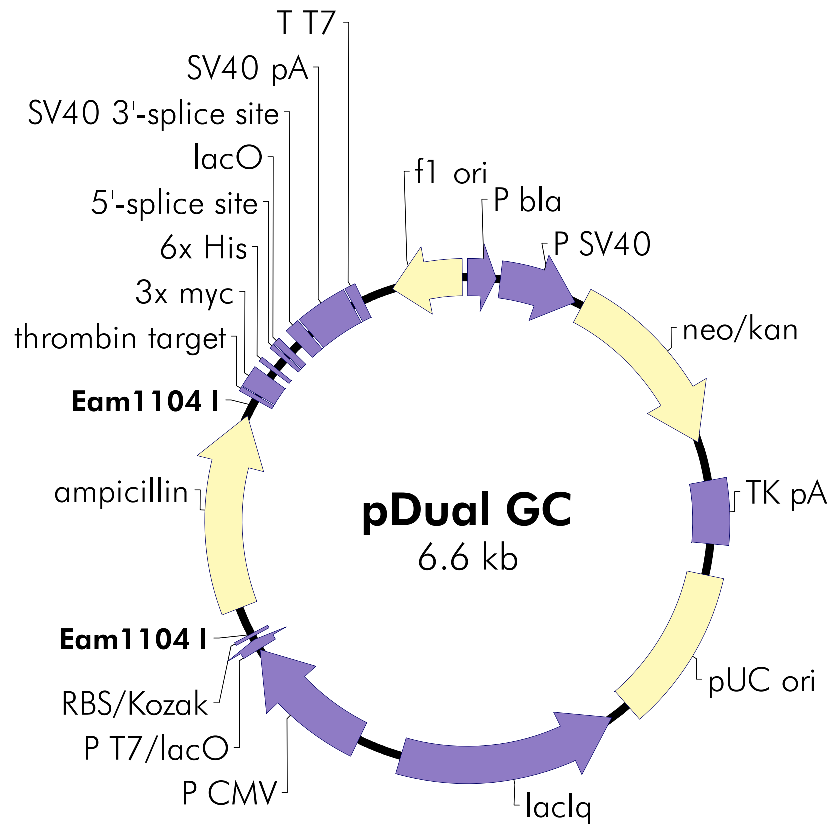


Figure 1 The pDual GC expression vector

Host Strains and Genotypes

XL1-Blue MRF´: Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F´ *pro*AB *lac*I^qZΔM15 Tn*10* (Tet^r)]

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

Bacterial strain	Plates for bacterial streak	Media for glycerol stock
XL1-Blue MRF´	LB–tetracycline[a]	LB–tetracycline[a]

[a] 12.5 µg/ml; see *Preparation of Media and Reagents*.

On arrival, prepare the following from the glycerol stock:

Note *Do not allow the contents of the vial to thaw. Store the vial at –80°C.*

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.

   Restreak the cells onto fresh plates each week.

Preparation of 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.
2. Add 4.5 ml of a sterile glycerol–liquid media solution (prepared by mixing 5 ml of glycerol + 5 ml of liquid media) to the bacterial culture from step 1. Mix well.
3. Aliquot into sterile centrifuge tubes (1 ml/ tube). This preparation can be stored at–80°C for more than 2 years.

Note *This strain can be obtained as high-efficiency frozen competent cells from Agilent (XL-1 Blue MRF´ supercompetent cells >1 × 10^9 colonies/µg of pUC 18).*

PROTOCOL CONSIDERATIONS

Insert Primer Design

1. The 5´ terminus of the primers must contain an *Eam*1104 I recognition site. *Eam*1104 I is a type IIS restriction enzyme that cuts outside its recognition sequence (5´-CTCTTC-3´). The cleavage site extends one nucleotide on the upper strand in the 3´ direction and four nucleotides on the lower strand in the 5´ direction (see Figure 2). Digestion with *Eam*1104 I generates termini with three nucleotides in their 5´ overhangs.

2. A minimum of two extra nucleotides (N_{-1} and N_{-2} in Figure 2) must precede the 5´-CTCTTC-3´ recognition sequence in order to ensure efficient cleavage of the termini. The bases preceding the recognition site can be any of the four nucleotides.

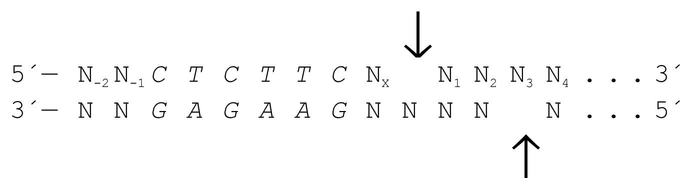


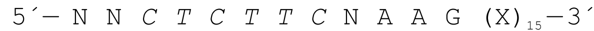
FIGURE 2 Restriction recognition sequence for *Eam*1104 I

3. The forward primer must be designed with one extra nucleotide (N) located between the *Eam*1104 I recognition sequence and the gene's translation initiation codon, in order to generate the necessary 5´-ATG overhang that is homologous to the vector sequence. The forward primer should be designed as follows:

where N denotes any of the four nucleotides and X represents gene-specific nucleotides.

4. The reverse primer must be designed with one nucleotide (N) located between the *Eam*1104 I recognition sequence and the AAG triplet which comprises the 5´ overhang that is homologous to the vector sequence. Depending on whether or not the c-myc and 6×His affinity tags are desired as fusion partners, the reverse primer should be designed as follows:

   a. Reverse primer design that allows the expression of the affinity tags:

      
      5´— N N *C T C T T C* N A A G (X)$_{15}$—3´

      where N denotes any of the four nucleotides and X represents the gene-specific nucleotides.

   b. Reverse primer design that does not allow expression of the affinity tags:

      5´— N N *C T C T T C* N A A G **C T A** (X)$_{12-15}$ —3´

      where N denotes any of the four nucleotides and X represents the gene-specific nucleotides. The necessary stop codon is shown in bold.

5. Agilent suggests that the primer include a perfect template match of at least 15 nucleotides on the 3´ end of the PCR primer in addition to the *Eam*1104 I recognition sequence. The estimated T_m of the homologous portion of the primer should be 55°C or higher, with a G-C ratio of 60% or more. To calculate T_m use this formula:

   $$[T_m \approx 2°C\ (A+T) + 4°C\ (G+C)]$$

pDual GC Expression Vector Protocol

Digesting the Vector with *Eam*1104 I

To generate a ligation-ready vector for PCR cloning, Agilent recommends the following for digesting the pDual GC expression vector.

Note *For optimal cleavage of the vector and to reduce the amount of background in the ligation reaction, it is important to include 100 μg/ml BSA in the* Eam*1104 I restriction digestion reaction, to treat the vector with alkaline phosphatase following digestion, and to isolate the cut vector from the uncut vector on an agarose gel.*

1. Digest 1 μg of the pDual GC expression vector using at least 24 units of the *Eam*1104 I restriction endonuclease. Include 100 μg/ml BSA in the reaction.

2. Incubate the reaction at 37°C for 2 hours.

3. Treat the cut vector with alkaline phosphatase, using the manufacturer's protocol.

4. Electrophorese the *Eam*1104 I digested, alkaline phosphatase treated vector on an agarose gel. It is important to fully separate the doubly cut vector band (~5.7 kb) from the linearized vector band (~6.7 kb) to reduce background carried over into the ligation reaction.

5. Gel purify the digested vector and resuspend in TE buffer to a final concentration of 0.04 µg/µl (see *Preparation of Media and Reagents*).

Preparing PCR Amplified Insert

PCR Amplification of Insert

Agilent suggests using a high fidelity polymerase, such as *Pfu* DNA polymerase, in the amplification reaction to eliminate mutations that could be introduced during the PCR.

To generate PCR products with vector-compatible termini, *Eam*1104 I recognition sequences need to be present at the ends of each primer (see *Primer Design*).

If the insert contains an internal *Eam*1104 I recognition site, the amplification reaction should be performed in the presence of 5-methyldeoxycytosine (^{m5}dCTP) for the last five cycles of the PCR.[3] Incorporation of ^{m5}dCTP during the PCR amplification protects already-existing internal *Eam*1104 I sites from subsequent cleavage by the endonuclease. The primer-encoded *Eam*1104 I sites are not affected by the modified nucleotide because the newly synthesized strand does not contain cytosine residues in the recognition sequence.

Note *The addition of the ^{m5}dCTP is delayed until the final five cycles of amplification to avoid the possible deamination of the ^{m5}dCTP due to extended exposure to heating and cooling cycles.*

Insert Purification

Before proceeding with the cloning protocol, carefully transfer the PCR products (from **below** the layer of mineral oil) to a fresh microcentrifuge tube. The insert may be purified following several different methods [phenol:chloroform extraction, selective precipitation (see *Appendix: Purifying the PCR Product by Selective Precipitation*), gel purification, spin-cup purification, or any other method of purification].

Note *Once the amplified insert has been purified, run an aliquot on an agarose gel to verify the success of the PCR amplification. (For PCR products <1 kb use 2% agarose. For PCR products >1 kb, use ≤1% agarose.)*

To prepare the insert for ligation, treat the insert with *Eam*1104 I (≥24 units for ≤ 1µg of insert.)

Ligating Vector and 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:

$$X\ \mu\text{g of insert} = \frac{(\text{Number of base pairs of insert})\,(0.04\ \mu\text{g of pDUAL GC vector})}{5627\ \text{bp of pDUAL GC vector}}$$

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, multiply X by 3 to get the quantity of insert required for a 3:1 ratio, etc….

1. Prepare one 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**[b]
Double-distilled water (ddH_2O)	6 µl	To a final volume of 10 µl	To a final volume of 10 µl
*Eam*1104 I digested pDual GC vector (0.04 µg/µl)	1.0 µl	1.0 µl	1.0 µl
*Eam*1104 I digested insert (0.04 µg/µl)	0 µl	1 µl	3 µl
rATP [10 mM (pH 7.0)]	1 µl	1 µl	1 µl
Ligase buffer (10×)[§]	1 µl	1 µl	1 µl
T4 DNA ligase (4 U/µl)	1 µl	1 µl	1 µl

[a] This control verifies the inability of the vector to religate.
[b] These experimental ligation reactions are insert-to-vector ratios of 1:1 and 3:1.

Note In some cases the inclusion of 6 units of *Eam*1104 I in the ligation reaction is beneficial to reduce background. If *Eam*1104 I is included in the ligation reaction, the total volume of the ligation of the reaction may have to be increased to ensure that the final glycerol concentration is ≤ 10% (glycerol is introduced when the restriction enzyme is added). Adjust the amount of the other components in the reaction proportionally. **If there are no internal *Eam*1104 I sites in the insert, *Eam*1104 I may be included in the ligation reaction. If *Eam*1104 I sites are present in the insert, it is necessary to prepare the insert by performing PCR in the presence of ^{m5}dCTP (see *PCR Amplification of Insert*) . This will prevent the undesired cleavage of *Eam*1104 I sites within the insert.**

2. Incubate the reactions overnight at 4°C.

Transforming/Transfecting Ligated Vector/Insert

Note *For the initial transformation of the ligated product, we recommend using XL1-Blue MRF´ cells, provided. These cells are* end*A*$^{-}$ *and are deficient in the restriction systems which cleave methylated DNA. It will be necessary to prepare competent cells from the XL1-Blue MRF´ glycerol stock provided; see reference 6 for protocols.*

Because the vector confers kanamycin resistance, a long transformation protocol should be followed to reach an optimal expression level prior to selection with kanamycin (incubate transformations in appropriate medium for 1 hour at 37°C before plating). Plate 5–10% of the transformed product on LB-kanamycin agar§ plates. Incubate the plates overnight at 37°C.

Note *For bacterial expression, use the miniprep DNA to transform an* end*A*$^{-}$ *host strain capable of supporting expression from a T7 promoter (e.g. a DE3 lysogen). Agilent offers BL21-Gold(DE3) competent cells. Following transformation, plate 5–10% of the transformed cells on LB-kanamycin agar§ plates. Select a colony and perform induction studies with and without Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (50–200 μM final concentration).*

For bacterial expression of genes containing rare codons, Agilent offers a series of BL21-CodonPlus cells, which express extra copies of rare tRNA.[7, 8]

For mammalian expression, use the miniprep DNA to transform the appropriate mammalian cells. For a transfection protocol, see reference 6.

§ See *Preparation of Media and Reagents*.

Appendix: Purifying the PCR Product by Selective Precipitation

Selective precipitation purifies the insert by removing excess PCR primers from the PCR product. In order to improve the overall cloning efficiency, Agilent recommends selectively precipitating the PCR product regardless of the PCR enzyme used to generate the inserts as indicated in the following protocol.

1. Add an equal volume of 4 M ammonium acetate.
2. Add 2.5 volumes of 100% (v/v) ethanol equilibrated at room temperature.
3. Immediately spin the reaction tube in a microcentrifuge at 10,000 × *g* for 20 minutes at room temperature to pellet the DNA.
4. Carefully remove and discard the supernatant.
5. Wash the DNA pellet with 200 μl of 70% (v/v) ethanol.
6. Spin the reaction tube in a microcentrifuge at 10,000 × *g* for 10 minutes at room temperature. **Carefully** remove the ethanol with a pipet.
7. Dry the DNA pellet under vacuum.
8. Resuspend the DNA to the original volume using TE buffer (see *Preparation of Media and Reagents*).
9. Measure the optical density of the sample at an absorbance of 260 nm (OD_{260}) to determine the concentration of the PCR product.
10. Store the purified PCR product at 4°C until ready for further use.

Preparation of Media and Reagents

LB Broth (per Liter)	TE Buffer
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	10 mM Tris-HCl (pH 7.5) 1 mM EDTA
LB–Kanamycin Agar (per Liter)	**LB–Kanamycin Broth (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)	Prepare 1 liter of LB broth Autoclave Cool to 55°C Add 5 ml of 10-mg/ml filter-sterilized kanamycin
LB–Tetracycline Agar (per Liter)	**LB–Tetracycline Broth (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	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

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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.