

Expresso[®] CMV Cloning and Expression System

IMPORTANT!
-80°C and -20°C Storage Required
Immediately Upon Receipt

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Expresso[®] CMV Cloning and Expression System

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Expresso[®] CMV Cloning and Expression System

System Designations

The Expresso[®] CMV Cloning and Expression System contains pre-processed pME-HA vector DNA and *E. coli* 10G Chemically Competent Cells. The catalog numbers are listed below.

Expresso CMV Cloning and Expression Kits

	5 Reactions	10 Reactions
Expresso [®] CMV Cloning and Expression System	49031-1	49031-2

Components & Storage Conditions

The Expresso CMV Cloning and Expression Kit consists of two separate containers. Container 1 includes the pME-HA Expression Vector, Positive Control Insert DNA, and DNA primers for screening inserts by PCR and sequencing. This container should be stored at **-20°C**. Container 2 includes *E. coli* 10G Chemically Competent Cells, which must be stored at **-80°C**.

Expresso CMV Cloning Kit, N-His Container Store at -20°C

	Concentration	Volume
pME-HA Vector DNA (5 reactions)	25 ng/μl	10 μl
β-gal Positive Control Insert DNA	50 ng/μl	10 μl
Primers for PCR screening and sequencing		
pME Forward Primer	50 pmol/μl	100 μl
pME Reverse Primer	50 pmol/μl	100 μl

E. coli 10G Chemically Competent Cells Container

Store at **-80°C**

	Cap Color	5 Reaction Kit	10 Reaction Kit
<i>E. coli</i> 10G Chemically Competent Cells	Yellow	6 x 40 μl	12 x 40 μl
Transformation Control pUC19 DNA (10 pg/μl)	Clear with Red Insert	20 μl	20 μl
Recovery Medium (Store at -20°C or -80°C)	N/A	1 x 12 ml	2 x 12 ml

System Description

The Expresso CMV Cloning and Expression System is a simple method for rapid cloning of genes for expression in mammalian cell culture.

The pME-HA vector facilitates instant cloning of target genes under control of the CMV promoter. It is provided with *E. coli* 10G Chemically Competent Cells for high-efficiency cloning in *E. coli*.

The pME-HA vector is provided in a pre-processed, linearized format for rapid enzyme-free cloning. After amplification of the target gene with appropriate primers, the PCR product is simply mixed with the pME-HA vector and transformed directly into chemically competent *E. coli* 10G cells. Recombination within the host cells seamlessly joins the insert to the vector (Figure 1).

Unlike other ligation-independent cloning systems, no enzymatic treatment or purification of the PCR product is required. No restriction enzymes are used, so there are no limitations on sequence junctions. Open reading frames are directionally cloned into the pME-HA vector behind the CMV promoter and optimal Kozak sequence. The C-terminal HA tag provides for fast and easy affinity purification of proteins under native or denaturing conditions. The HA tag can also be used as an additional tool for immunodetection of the expressed protein.

E. coli 10G cells are used for construction of clones in the pME-HA vectors. Their *recA*⁻ *endA*⁻ genotype allows recovery of high quality plasmid DNA.

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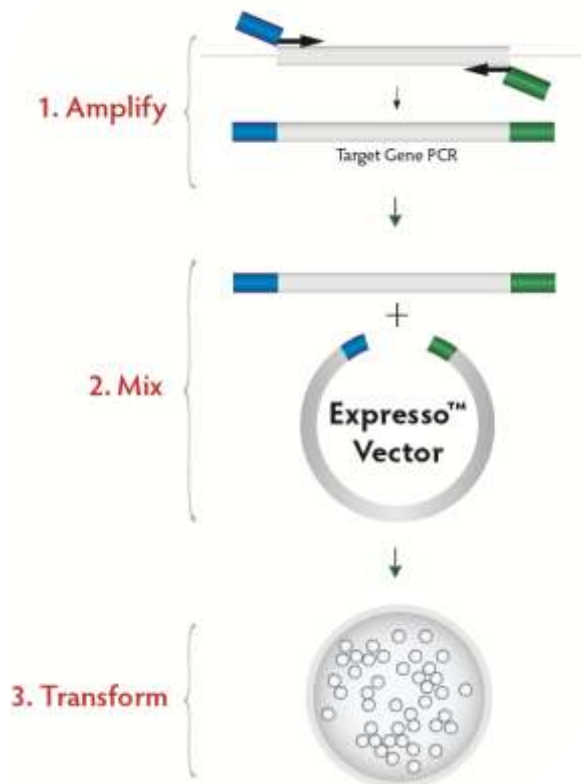


Figure 1. Expresso cloning. A target gene is amplified with primers that contain short homology to the ends of the pME-HA vector. The PCR product is then mixed with the pre-processed vector and transformed directly into *E. coli* 10G cells.

pME-HA Vector

The pME-HA vector is a direct replacement for most CMV-driven expression vectors. The small size of the pME-HA vector (3.4 kb) facilitates cloning of larger inserts and performing DNA manipulations, such as site-directed mutagenesis. The high-copy number pME HA vector is similar to that of pUC plasmids (~300 copies/ bacterial cell), yielding up to 20 µg of plasmid DNA per ml of culture. The background of empty vector is typically <5%, so minimal colony screening is necessary.

The pME-HA vector is pre-linearized for instant, directional cloning of inserts (Figure 2). The vector includes signals for high levels of protein production in mammalian cell lines, including a CMV promoter and Kozak sequence. The vector also encodes a C-terminal HA tag.

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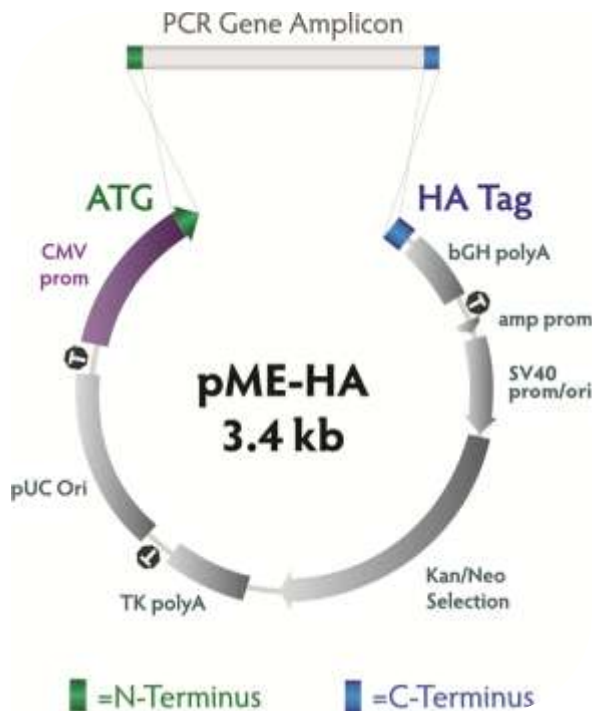


Figure 2. pME-HA expression vector.

ATG, translation start site; bGH polyA, polyadenylation site for recombinant gene; amp prom, promoter for bacterial expression of kanamycin resistance gene; SV40 prom/ori, promoter for mammalian expression of neomycin resistance gene (includes SV40 origin of replication); Kan/Neo, kanamycin and neomycin resistance gene; TK polyA, polyadenylation site for Neo resistance gene; pUC Ori, bacterial origin of replication. CloneSmart[®] transcription terminators (T) prevent transcription into or out of the vector backbone, and another bacterial terminator follows cloning region. If desired, the HA affinity tag can be fused to the carboxy terminus of the expressed protein.

***E. coli*® 10G Chemically Competent Cells**

E. coli 10G cells are an *E. coli* strain optimized for high efficiency transformation. The *E. coli* 10G cells are ideal for cloning and propagation of plasmid clones. They give high yield and high quality plasmid DNA due to the *endA1* mutation.

***E. coli* 10G Genotype:**

mcrA Δ (*mrr-hsdRMS-mcrBC*) *endA1* *recA1* ϕ 80*dlacZ* Δ M15 Δ *lacX74* *araD139* Δ (*ara,leu*)7697 *galU galK rpsL nupG* $\lambda^- tonA$

As a control for transformation, *E. coli* 10G Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/ μ l. Use 1 μ l (10 pg) for transformation. Select pUC19 transformants on plates containing ampicillin or carbenicillin (100 μ g/ml). *E. coli* 10G Chemically Competent Cells yield $\geq 1 \times 10^9$ cfu/ μ g supercoiled pUC19 DNA.

Cloning Strategy

The pME-HA vector preparation enables a simple strategy for precise, directional cloning. The vector is provided in a linear form, ready for recombinational cloning. Simply co-transform the vector with a PCR product containing the gene of interest.

The desired insert is amplified with primers that include 15-18 nt of overlap with the ends of the vector (Figure 3). Recombination between the vector and insert occurs within the *E. coli* 10G host strain, seamlessly fusing the gene of interest to the vector. No restriction digestion, enzymatic treatment, or ligation is necessary for efficient recombination. The method is similar to cloning by homologous recombination (1), and does not require single-stranded ends on the vector or the insert, as in "PIPE" cloning (2).

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pME-HA Vector:

Vector Prep:

CMV Promoter Kozak Start

```
ATAAGAAGGAGATA CCACC ATG
TATTCTTCTCTAT GGTGG TAC
```

HA Tag

Stop

```
Y P Y D V P D Y A *
TAT CCG TAT GAC GTG CCC GAC TAT GCC TAA...
ATA GGC ATA CTG CAC GGG CTG ATA CGG ATT...
```

PCR product:

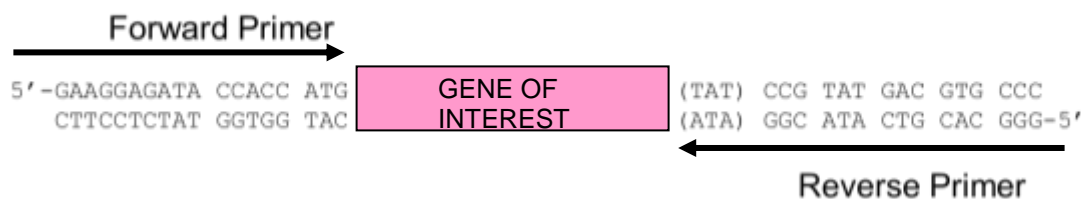


Figure 3. Insertion of a gene into the pME-HA vector for expression. PCR primers are designed to amplify the gene of interest and add 18 bp of flanking sequence identical to the vector. The reverse primer can encode an optional stop codon (shown in parentheses) to avoid addition of the HA tag. Recombination within the host cell recombines the blunt PCR product to the vector. See Detailed Protocol.

Positive Control Insert

The Control Insert included with the Kit encodes the entire 3.1 kb β -galactosidase gene from *E. coli*, flanked by sequences for enzyme-free cloning into the pME-HA vector. This insert serves as a control for cloning efficiency in bacteria, as well as for expression in bacterial and mammalian cells. The β -galactosidase protein migrates at ~116 kD on SDS PAGE.

Colony Screening

Background with the pME-HA vector is typically very low (<5%), so minimal screening is necessary. Colony PCR, size analysis of uncut plasmid, or restriction digestion may be used to verify the presence of inserts. Primers included with the kit are suitable for screening by colony PCR and for sequencing of plasmid DNA. We strongly recommend sequence analysis to confirm the protein coding sequence as well as the junctions of the insert with the vector.

Protein Expression

Recombinant plasmids should be constructed and verified in the *E. coli* 10G host strain before being transfected into mammalian cells for expression. Bacterial transformants are selected on plates containing kanamycin (30 μ g/mL); mammalian cells are selected in medium containing neomycin or geneticin (G418, 100 -1,000 μ g/mL). Expression of HA-tagged proteins can be evaluated by SDS-PAGE or Western blot analysis.

Protein Purification

HA-tagged proteins can be detected and purified by specific antibodies. Materials for purification are not provided with the Expresso CMV System. These reagents may be obtained from any of several suppliers, including: Covance, QED Bioscience, Sigma, and others.

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Materials and Equipment Needed

The Expresso CMV Cloning and Protein Expression Kit supplies many of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting. The following items are required for this protocol:

- Custom Primers for target gene amplification.
- Thermal cycler and PCR reagents
- Sterile polypropylene 17 x 100 mm culture tubes.
- Water bath at 42°C.
- LB Broth or YT Broth.
- YT agar plates containing kanamycin (see Appendix A for recipes).
- Agarose gel electrophoresis equipment.

Detailed Protocol

Preparation of Insert DNA

To perform enzyme-free cloning with the pME-HA vectors, the DNA to be inserted must be amplified with primers that append appropriate flanking sequences to the gene of interest (See below, and Figure 3). These flanking sequences must be identical to the vector sequences flanking the cloning site. Rules for correctly designing primer pairs are presented below.

1) Primer design for target gene amplification

PCR primers for enzyme-free cloning into the pME-HA vectors consist of two segments: 18 nt at their 5' ends that match the sequences of the pME-HA vector, and 18-24 nt at their 3' ends that anneal to the target gene. Factors affecting the length of the target-specific portion of the primer include GC content, T_m , and potential for formation of hairpins or primer-dimers.

Forward primer (defined vector sequence includes Start codon):

5'-GAAGGAGATACCACC ATG XXX₂ XXX₃ XXX₄ XXX₅ XXX₆ XXX₇ XXX₈

(XXX₂-XXX₈ represents codons 2 through 8 of the target coding region).

Reverse primer (defined vector sequence includes 6 HA anticodons):

5'-GGG CAC GTC ATA CGG ATA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}

(XXX_n - XXX_{n-6} represents the **reverse complement** of the last 21 bases of the target coding region. See Figure 3.)

The defined vector portion of the Forward primer includes the ATG codon for translation initiation. The gene-specific portion of the Forward primer should include the sequence beginning from codon 2 (or other desired internal codon) of the protein coding region of interest.

An in-frame stop codon immediately follows the HA tag in the pME-HA vector. A Stop anti-codon should NOT be included in the Reverse primer, unless expression of an untagged form of the protein is desired (see below).

Primer design for untagged target protein:

The pME-HA vector can also be used to construct untagged expression clones. In this case, include sequence complementary to a termination codon before the defined vector-specific sequence. This sequence will cause translation to terminate before the HA tag.

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Reverse primer for untagged target gene:

5'-GGG CAC GTC ATA CGG ATA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}

(The reverse complement of a TAA termination codon is underlined. XXX_n - XXX_{n-6} represents sequence complementary to the last 7 codons of the target coding region.)

2) Amplification of target gene

Amplify the desired coding sequence by PCR, using primers designed as described above. Amplification with a proofreading PCR polymerase is strongly recommended to minimize sequence errors in the product. The performance of the Expresso CMV system has been verified with PCR products from various proofreading polymerases, including Vent[®] (NEB) and Pfu (Agilent) DNA polymerases, and Taq non-proofreading polymerase. Sequence errors are quite common with Taq polymerase, especially for larger inserts, so this enzyme is not recommended. Complete sequencing of several candidate clones is strongly recommended.

A typical amplification protocol is presented below. Adjustments may be made for the particular polymerase, primers, or template used.

Example amplification protocol:

For a 50 µl reaction, assemble the following on ice:

5 µl 10X reaction buffer
4 µl dNTPs (at 2.5 mM each)
1 µl 50 µM Forward primer
1 µl 50 µM Reverse primer
X µl DNA polymerase (follow manufacturer's recommendations)
Y µl DNA template (~5 ng plasmid DNA, or ~50-200 ng genomic DNA)
Z µl H₂O (bring total volume to 50 µl)

50 µl

Cycling conditions:

94°C, 2'
94°C, 15"
55°C, 15"
72°C, 1' per kb } 25 cycles
72°C, 10'
4°C, Hold

Analyze the size and amount of amplified DNA by agarose gel electrophoresis. If the reaction yields a single product at a concentration of 10 ng/µl or higher, you can proceed directly to **Expresso[®] Enzyme-free cloning**. If the desired product is weak or contains spurious bands, it can be purified by agarose gel fractionation prior to use.

IMPORTANT: If the template DNA is an intact circular plasmid encoding kanamycin resistance, it can very efficiently transform the *E. coli* 10G cells, creating a high background of parental clones on kanamycin agarose plates. Therefore, we strongly recommend restriction digestion of kanamycin-resistant plasmid templates and gel purification of the linearized fragment prior to using it as a template for PCR. Alternatively, the PCR product can be gel purified to isolate it from the circular plasmid DNA.

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Short Wavelength UV Light Severely Damages DNA

During gel fractionation, use of a short-wavelength UV light box (e.g., 254, 302, or 312 nm) **must** be avoided. Most UV transilluminators, including those sold for DNA visualization, use shortwave UV light, which can rapidly reduce cloning efficiencies by several orders of magnitude (Figure 4).

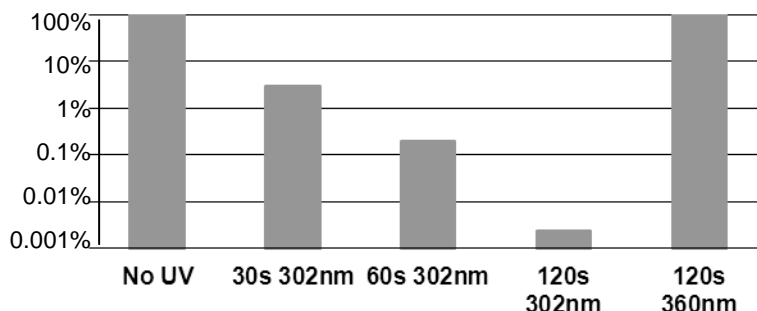


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 120 seconds (“30s 302nm, 60s 302nm, 120s 302nm”) or to 360 nm UV light for 120 seconds (“120s 360nm”). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

A hand-held lamp with a wavelength of 360 nm is very strongly recommended. After electrophoresis, DNA may be isolated using your method of choice.

Use a 360-nm UV lamp when isolating DNA fragments from agarose gels.

Expresso[®] Enzyme-free cloning with the pME-HA Vector

In the Expresso enzyme-free cloning strategy, the pre-processed pME-HA Vector is co-transformed with insert DNA having ends complementary to the vector. After verification of PCR product by agarose gel electrophoresis, the unpurified PCR product (1-3 μ l) is mixed with 50 ng of pME-HA vector and transformed directly into competent *E. coli* 10G cells. If desired, the PCR products can be purified before cloning into pME-HA vector.

We recommend using 25-100 ng of insert DNA with 50 ng of pME-HA vector preparation per transformation.

Optional Control Reactions include the following:

β -gal Positive Control Insert DNA	To determine the transformation efficiency with a known insert, use 1 μ l (50 ng) of Positive Control Insert DNA and 2 μ l (50 ng) of pME-HA vector.
Vector Background	To determine the background of empty vector, omit insert from the above reaction.

To ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. coli* 10G chemically competent cells, which are included with the kit. These cells yield $\geq 1 \times 10^9$ cfu/ μ g of pUC19. The following protocol is provided for transformation.

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Heat Shock Transformation of *E. coli* 10G Chemically Competent Cells

E. coli 10G Chemically Competent Cells are provided in 40- μ l aliquots, sufficient for a single transformation. Transformation is performed by incubation on ice followed by heat shock at 42°C. For maximal transformation efficiency, the heat shock is performed in 15-ml disposable polypropylene culture tubes (17 x 100 mm). The use of other types of tubes may dramatically reduce the transformation efficiency. To ensure successful transformation results, the following precautions must be taken:

- All culture tubes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.

Transformation of *E. coli* 10G Chemically Competent cells

1. Remove Recovery Medium from the freezer and bring to room temperature.
2. Remove *E. coli* 10G cells from the -80°C freezer and thaw completely on wet ice (10-15 minutes).
3. Thaw the tube of pME-HA vector DNA. Vortex briefly and microcentrifuge to collect the solution in the bottom of the tube.
4. Add 2 μ l (50 ng) of the pME-HA vector DNA and 1 - 3 μ l (25 to 100 ng) of insert PCR product to the cells. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
5. Transfer the cells and DNA to a pre-chilled disposable polypropylene 15-ml culture tube (17 x 100 mm).
6. Incubate culture tube containing cells and DNA on ice for 30 minutes.
7. Heat shock cells by placing the tube in a 42°C water bath for 45 seconds.
8. Return the tube of cells to ice for 2 minutes.
9. Add 960 μ l of room temperature Recovery Medium to the cells in the culture tube.
10. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
11. Plate 100 μ l of transformed cells on YT (LB) agar plates containing 30 μ g/ml kanamycin.
12. Incubate the plates overnight at 37°C.

Transformed clones can be grown in LB, TB, or any other rich culture medium for preparation of plasmid DNA. Growth in TB medium gives the highest culture density and plasmid yield. Use kanamycin (30 μ g/ml) to maintain selection for transformants.

EXPECTED RESULTS USING *E. coli* 10G CHEMICALLY COMPETENT CELLS

Plating chemically transformed cells and expected results.

Reaction Plate	μ l/Plate	CFU/Plate	Efficiency
Experimental Insert (~25-100 ng per transformation)	100	variable	NA
β -gal Positive Control Insert (50 ng)	100	> 30	> 95% inserts
No-Insert Control (Vector Background)	100	< 5	< 5% background
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Ampicillin ^R)	2 (ampicillin plate)	> 200	> 1 x 10 ⁹ cfu/ μ g plasmid

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The results presented above are expected when transforming 50 ng of intact, purified control insert DNA along with 50 ng of pME-HA vector using Lucigen's *E. coli* 10G Chemically Competent Cells. The background number of empty vector is constant (< 5 colonies per 100 µl of cells plated). Cloning AT-rich DNA and other recalcitrant sequences may lead to fewer colonies. With relatively few recombinant clones, the number of "empty vector" colonies becomes more significant. For example, if the Experimental Insert reaction produces only 20 colonies from 100 µl of cells plated, then 5 colonies obtained from 100 µl of the No-Insert Control transformation will represent a background of 25%.

Getting More Recombinants

Certain genes can prove recalcitrant to cloning due to a large size, toxic gene products, secondary structures, extremely biased base composition, or other unknown reasons. For highest transformation efficiencies, we recommend performing the heat-shock transformation in pre-chilled 15 ml culture tubes as specified in the Transformation Protocol. If necessary, the entire 1-ml transformation mix for can be pelleted in a microfuge (10,000 rpm, 30 seconds), resuspended in 100 µl of recovery media, and plated. See Appendix C for troubleshooting suggestions.

Colony PCR Screening for Recombinants

Because the background of empty vector transformants is low, colonies can be picked at random for growth and plasmid purification. If desired, colonies can first be screened for inserts by colony PCR. Lucigen's EconoTaq[®] PLUS GREEN 2X Master Mix (available separately, Cat. No. 30033-1) is a convenient premix of Taq DNA polymerase, reaction buffer, and dNTPs that provides everything needed for colony PCR, except primers and template DNA. Screening by colony PCR with EconoTaq PLUS GREEN 2X Master Mix is performed as follows:

Colony PCR with EconoTaq PLUS GREEN 2X Master Mix

Per 25 µl reaction:

12.5 µl EconoTaq PLUS GREEN 2X Master Mix
0.5 µl pME Forward primer (50 µM)
0.5 µl pME Reverse primer (50 µM)
11.5 µl water
<hr/>
25 µl

Using a pipet tip, transfer part of a colony to the PCR reaction mix. Disperse the cells by pipetting up and down several times.

Cycling conditions:

94°C 5'	
94°C 15"	} 25 cycles
55°C 15"	
72°C 1' per kb	
72°C 10'	
4°C Hold	

The EconoTaq PLUS GREEN reactions can be loaded directly onto an agarose gel for analysis. The Master Mix contains blue and yellow tracking dyes that will separate upon electrophoresis. Empty vector clones will yield a product of ~180 base-pairs.

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DNA Isolation & Sequencing

Grow transformants in LB or TB medium plus 30 µg/ml kanamycin. Use standard methods to isolate plasmid DNA. The pME-HA plasmids contain the high copy number pUC origin of replication and produce DNA yields of up to 20 µg/ml of culture. *E. coli* 10G cells are *recA* and *endA* deficient to provide high quality plasmid DNA. The pME Forward and pME Reverse Sequencing Primers are provided with the Kit at a concentration of 50 µM; they must be diluted before use in sequencing. Their sequences and orientations are shown in Appendix B.

Affinity Detection and Purification of HA-tagged proteins

Many protocols are available for detection and purification of HA-tagged proteins under native or denaturing conditions. For best results, follow the procedures recommended by the manufacturer of your antibody.

Transfection of Mammalian Tissue Culture Cells

The pME-HA vector can be transfected into mammalian tissue culture cells using standard techniques. Commercial transfection reagents may be used to obtain the highest transfection efficiency. Lucigen has observed best results with TransIT[®]-2020 Transfection Reagent (Mirus Bio). Other reagents include Xtreme GENE[®] (Roche) or Lipofectamine[™] (Invitrogen). Selection can be performed with neomycin or geneticin (G418) at 100 – 1,000 µg/mL.

Measuring Positive Control Expression

The β-gal positive control insert DNA can be expressed and visually detected in both *E. coli* cells and mammalian tissue culture cells. For bacterial cells, plate cells on appropriate media containing X-gal and look for blue colonies. For mammalian cells, β-gal protein expression can be detected and measured either quantitatively by using the Mammalian β-Galactosidase Assay Kit (ThermoScientific, catalog # 75707). A simple, qualitative staining assay is also available from Mirus Bio (catalog # MIR2600), which allows staining and direct visualization of cells expressing the β-Galactosidase control protein.

References

1. Bubeck, P., Winkler, M. and Bautsch, W. (1993). Rapid cloning by homologous recombination *in vivo*. *Nuc. Acids Res.* **21**, 3601.
2. Klock, H.E., Koesema, E.J., Knuth, M.W. and Lesley, S.A. (2008). Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts. *Proteins***71**, 982.

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Appendix A: Media Recipes

YT + kan30 Agar Medium for Plating of Transformants

Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Mix components, autoclave and cool to 55°C. Add kanamycin to a final concentration of 30 µg/ml. Pour into petri plates.

LB Culture Medium

Per liter: 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl. Mix components and autoclave.

Appendix B: Vector Map and Sequencing Primers

The sequences of the pME Forward and pME Reverse primers are:

pME Forward: 5'–ACCCACTGCTTACTGGCTTATC–3'

pME Reverse: 5'–TGGCTGGCAACTAGAAGGCACA –3'

The pME-HA vector is linearized after the start codon and immediately before the HA tag coding sequence.

The region surrounding the cloning site in the pME-HA vector is shown below.

pME-HA Vector:

```
AACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGA  
TTGAGGCGGGGTAAC TGC GTTTACCCGCCATCCGCACATGCCACCCCTCCAGATATATTCGTCTCGAGAGACCGATTGATCTCT
```

pME Forward

```
ACCCACTGCTTACTGGCTTATCGAAAACACCCAAGCTGGCTAGCGACACCTATAAGAAGGAGATACCACCATG Cloned  
TGGGTGACGAATGACCGAATAGCTTTGTGGGTCGACCGATCGCTGTGGATATTTCTCTCTATGGTGGTAC Gene
```

HA Tag

Y P Y D V P D Y A STOP

```
Cloned Gene TAT CCG TAT GAC GTG CCC GAC TAT GCC TAA GACATGGCTCGAGAAAATCAGCCTCGAC  
ATA GGC ATA CTG CAC GGG CTG ATA CGG ATT CTGTACCGAGCTCTTTTAGTCGGAGCTG
```

```
TGTGCCTTCTAGTTGCCAGCCATCTGTT  
ACACGGAAGATCAACGGTCGGTAGACAA
```

pME Reverse

A link to the complete sequence of the pME-HA Vector (3416bp) can be found on Lucigen's Expresso CMV Cloning and Expression product page or in the Vector Sequences section of the Technical Information Page (See www.lucigen.com).

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Appendix C: Cloning Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	No DNA, degraded DNA, or insufficient amount of DNA.	Check insert DNA by gel electrophoresis. Determine concentration of insert and add the correct amount. Use the supplied control insert to test the system. Avoid exposure of the insert DNA to short-wavelength UV light.
	Incorrect primer sequences.	Be sure the 5' ends of the primer sequences match the ends of the pME-HA vector.
	Wrong antibiotic used.	Add 30 mg/L of kanamycin to molten agar at 55°C before pouring plates.
	Incorrect amounts of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain inserts.	Transformants are due to intact plasmid used as the PCR template.	Linearize the plasmid DNA used as a template for PCR. Gel-isolate template DNA fragment.
	Inserts are too small to detect.	Analyze colonies by sequencing to confirm the presence of inserts.
	Incorrect amount of antibiotic in agar plates.	Add 30 mg/L of kanamycin to molten agar at 55°C before pouring plates. DO NOT spread antibiotic onto the surface of agar plates.
Recombinant protein not detected in mammalian culture	Recombinant protein not expressed	Confirm junction between CMV promoter and recombinant gene. Confirm expression of recombinant protein by SDS-PAGE and/or Western blot.
	HA tag not expressed	Confirm junction between recombinant protein and HA tag.
	Recombinant proteins may be cleaved during expression or lysate preparation.	Check lysate and column flow through by SDS-PAGE and Western blot to confirm HA tag is attached to the overexpressed protein of the expected molecular weight. Use protease inhibitors during purification to prevent cleavage.

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is critical that the reagents supplied by the user, especially the DNA targets to be cloned, are of the highest quality. Please follow the manual carefully or contact our technical service representatives for information on preparation and testing of the target DNA. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

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