Amplification Using CHO Cell Expression Vectors

The ability to select for integration of plasmid DNA into the host chromosome allows the generation of stably transfected cell lines. With transfection of a selectable marker linked to a nonselectable target gene (or by cotransfection of the two unlinked genes), high-level expression of the desired gene is obtained by selecting for amplification of the selectable marker.

This unit presents two systems for gene amplification and expression. The first (see Basic Protocol 1) describes the dihydrofolate reductase (DHFR) selection system while the second (see Basic Protocol 2) is based on selection of the glutamine synthetase (GS) gene. The DHFR system is probably more widely used, and results in very high levels of amplification (up to 1000 copies per cell in some cases) and expression; however, the DHFR amplification process is lengthy and may require several months to isolate and characterize a stable, amplified line. In contrast, the GS system typically requires only a single round of selection for amplification to achieve maximal expression levels. In this system the length of time necessary to isolate stably amplified clones from the primary tranfectants is dramatically reduced to 2 months or less.

AMPLIFICATION USING DIHYDROFOLATE REDUCTASE

BASIC PROTOCOL 1

The pED series of dicistronic vectors (Fig. 16.23.1) can be used to obtain high-level expression of a targeted gene in stably transfected cells. These vectors carry a cloning sequence for insertion of the target gene followed by the selectable and amplifiable marker gene, dihydrofolate reductase (DHFR). Alternatively, a plasmid expressing the gene of interest and a plasmid expressing DHFR can be cotransfected. DHFR-deficient CHO cells transformed with the appropriate vector(s) are selected by ability to grow in nucleoside-free medium. Subsequent selective cycling in the presence of increasing concentrations of methotrexate (MTX)—a potent inhibitor of DHFR function—results in amplification of the integrated DNA and increased expression of the desired gene product.

Materials

pED (Kaufman et al., 1991) expressing appropriate cDNA; *or* pCVSVEII-DHFR or pAd26SV(A) (Kingston et al., 1984; Kaufman and Sharp, 1982a) and a separate vector expressing appropriate cDNA
CHO DXB11 or CHO DG44 cell lines (available from Lawrence Chasin, Columbia University) or CHO GRA (available from Randal Kaufman, University of Michigan)
Complete ADT medium (see recipe)
10% glycerol
Dialyzed fetal bovine serum (FBS; see recipe)
Complete α^- medium (Life Technologies) with 10% dialyzed FBS
Sterile vacuum grease
0.05% trypsin/0.6 mM EDTA in PBS (see *APPENDIX 2* for PBS), 37°C
2% methylene blue in 50% ethanol (optional)
5 mM methotrexate (see recipe)
Cloning cylinders (see recipe)

Additional reagents and equipment for subcloning (*UNIT 3.16*), and either $CaPO_4$-mediated transfection (*UNIT 9.1*), electroporation (*UNIT 9.3*), or liposome-mediated transfection (*UNIT 9.4*)

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise indicated.

Contributed by Robert E. Kingston, Randal J. Kaufman, C.R. Bebbington and M.R. Rolfe
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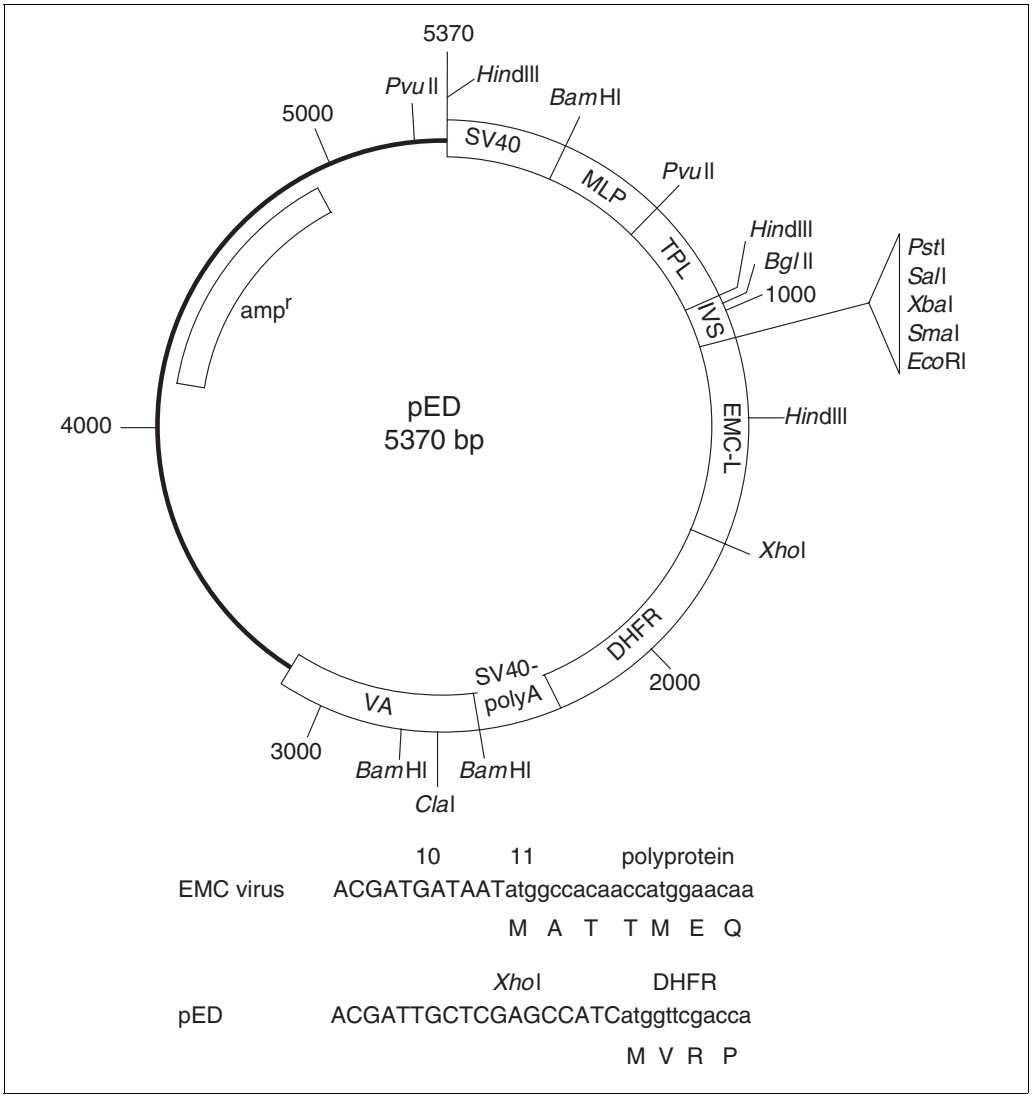


Figure 16.23.1 Map of dicistronic mRNA expression vector pED. The components of the 5360-bp pED expression vector in the pUC18 background are indicated as follows: SV40, *Hin*dIII-*Pvu*II fragment containing the SV40 origin of replication and enhancer element; MLP, adenovirus major late promoter fron the *Xho*I site (15.83 map units, m.u.) to the 5′ cap site (16.55 m.u.); TPL, 180 bp of the first two and $^{2}/_{3}$ of the third leaders from adenovirus major late mRNAs; IVS, a hybrid intron composed of the 5′ splice site from the first leader of adenovirus major late mRNAs and a 3′ splice site from an immunoglobin gene; *Pst*I and *Eco*RI unique cloning sites; EMC-L, the 5′ untranslated leader from EMC virus (nucleotides 260-827); DHFR, a murine DHFR coding region; SV40-polyA, the SV40 early polyadenylation signal; VA, the adenovirus VAI RNA gene from the *Hpa*I (28.02 m.u.) to the *Bal*I (29.62 m.u.); and β-lactamase, a selectable gene for propagation in *E. coli*. Below is indicated the sequence junction of the EMC-L and DHFR as compared to the context of the AUG 11 which is initiation codon for the EMC virus polyprotein. A unique *Xho*I restriction site is available for insertion of other coding regions to be translated from the EMC virus leader. Adapted with permission from IRL Press.

Transfect CHO cells

1. The day before transfection, split a confluent dish of CHO cells 1:15 in complete ADT medium.

2. Transfect the cells with 5 to 10 µg plasmid DNA (per dish) from step 1, using either electroporation (*UNIT 9.3*), the calcium phosphate technique (*UNIT 9.1*), or liposome-mediated transfection (*UNIT 9.4*).

   The calcium phosphate treatment followed by glycerol shock works well for CHO DXB11 cells. A 3-min shock with 10% glycerol should be performed 4 to 6 hr after the DNA precipitate is placed on the cells. The gene can then be introduced into pED that also contains a DHFR gene. Alternatively, if a pED vector is unavailable, it is possible to introduce the desired gene by cotransfection using two separate plasmids. In this latter case, transfected DNA should contain the plasmid whose amplification is desired and a plasmid expressing the DHFR gene from a strong promoter [e.g., pCVSVEII-DHFR or pAdD26SV(A)]. Use a 5:1 molar ratio of the gene of interest to the DHFR gene.

   It is not necessary to physically link the DHFR gene to the gene whose amplification is desired. The two genes will integrate in the same region of the chromosome and will coamplify (Kaufman and Sharp, 1982b). Using one-fifth the molar amount of the DHFR gene makes it likely that most of the selected cell lines will contain the gene of interest in an intact form as well as the DHFR gene.

3a. *For cells transfected by electroporation or calcium phosphate:* Allow the cells to reach confluence after transfection (this should occur after 2 to 3 doublings in 2 days.) Split each dish 1:15 into α^- medium containing 10% dialyzed FBS (complete α^- medium).

3b. *For cells transfected using liposomes:* Add 5 ml complete α^- medium and incubate overnight. Remove medium, wash twice with 37°C PBS, add 5 ml complete α^- medium, and incubate 2 days. Dilute cells 1:10 or 1:15 into complete α^- medium without ADT.

   Complete α^- medium (containing no added nucleosides) is a selective medium, as cells need DHFR to synthesize necessary nucleosides. Use of dialyzed serum is necessary to avoid addition of nucleosides present in normal FBS. Note that methotrexate is not needed for selection.

4. Incubate cells 10 to 12 days if proceeding from step 3a or 14 days if proceeding from step 3b. Move the dishes as infrequently as possible during this time to prevent formation of sibling colonies.

   Cells can float away from their original colonies, land elsewhere on the dish, and produce a colony of their own. This should be minimized, as picking and analyzing two such sibling colonies is inefficient (they presumably are identical).

Pick stable colonies

5. At a time point 10 to 14 days after placing the cells in selective medium, check the dishes for colonies by holding the dish above one's head at an angle with respect to the overhead lights and looking for opaque patches. Circle such patches with a laboratory marker so that they can easily be located and examined in the phase-contrast microscope.

   In order to determine how well the transfection worked, one of the transfected dishes can be stained with methylene blue. To stain, first aspirate off the medium, then place ~2 ml of a 2% methylene blue solution (made up in 50% ethanol) on each dish. Wait 2 min, then pour the dye solution off and wash off the residual methylene blue by dipping the dish in a bucket of cold water. In order to have good success picking colonies for stable lines, each dish should have several heavily staining colonies as well as 10 more smaller colonies.

6. Select the colonies to be picked. Circle the chosen colonies with a laboratory marker to determine where to place the cloning cylinders.

   Choose only large, healthy colonies. Colonies should have ~500 cells, and the cells should appear to be compact and polygonal. Colonies with many flat and spread-out cells should be avoided, as this morphology indicates that they are not making very much DHFR. Pick ~20 colonies so that in the end there will be many stably transfected cell lines available for amplification. Keep track of which dish a colony comes from, as colonies from the same dish may be siblings. This is most easily done by numbering the dishes and using that number in the name of the colony that has been picked.

   It is also possible at this point to pool large numbers of transformants that have integrated the vector into different sites. Because different integration sites have quite different potentials for amplification, one can use sequential increases in MTX resistance to select cells rapidly that have amplified the gene to high copy number.

7. Coat one end of a cloning cylinder with sterile vacuum grease by touching the cylinder to grease that has been autoclaved in a glass petri dish. Gently place the cylinder around the colony to be picked (Fig. 16.23.2).

   Make certain that there is not too much grease on the end of the cloning cylinder—use a sufficient amount of grease to form a thin film between the cloning cylinder and the tissue culture dish, but do not allow the grease to cover any of the colony. The cloning cylinder is most easily positioned using bent forceps sterilized by flaming in ethanol immediately prior to use.

8. Using a sterile Pasteur pipet, rinse the colony with 37°C 0.05% trypsin/0.6 mM EDTA by filling and emptying the cloning cylinder (Fig. 16.23.2).

   Care must be taken to avoid knocking the cylinder over, and also to avoid scraping cells off the colony with the end of the Pasteur pipet. Hold the Pasteur pipet as close to vertically as possible in order to avoid knocking over the cloning cylinder. A little trypsin can be left in the well after rinsing.

9. Add 3 drops of 37°C trypsin/EDTA to the cloning cylinder. Wait 1 min. Fill the cloning cylinder with medium and repeatedly run the contents of the cylinder in and out through a Pasteur pipet in order to remove the trypsinized cells from the dish and disperse them. Plate the cells in a 40-mm dish.

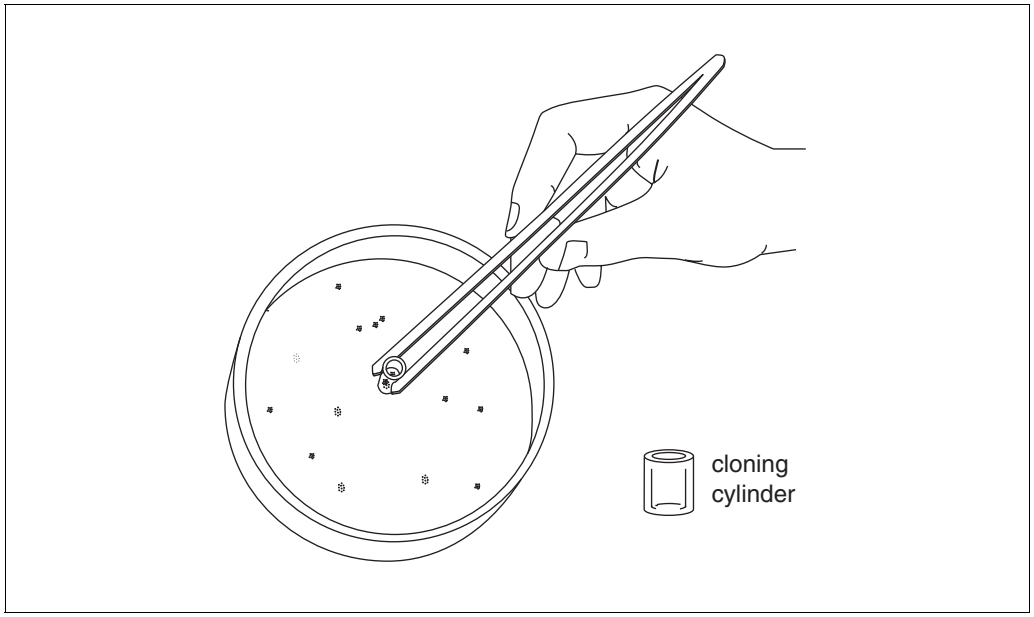


Figure 16.23.2 Placement of cloning cylinder around CHO colony.

10. As the cells grow out, split them frequently (every 4 to 5 days or so) so that they do not form large colonies.

    The central cells in large colonies do not fare well.

Amplify stable transfectant

Amplification is a long process. Before amplifying a stable transfectant, one should be sure that the gene of interest has indeed been integrated into the cell in a functional form. This can be done by examining the cellular DNA by Southern analysis (*UNIT 2.9*), by examining the cellular RNA (Chapter 4), or most easily in many cases by using a functional assay for the introduced protein. Some cell lines amplify more readily than others (chromosome location of the introduced DNA appears to play a role), and the gene of interest can rearrange or mutate during the process. These considerations make it prudent to amplify six or more stable transfectants or pools of cells containing the gene of interest at the same time.

11. Split a confluent dish of cells growing in complete α^- medium 1:6 into two dishes complete α^- medium supplemented with 0.005 μM methotrexate (added from 5mM methotrexate stock).

    CAUTION: *Use gloves when handling methotrexate as it is carcinogenic.*

    The addition of methotrexate to the medium increases the level of selection, as methotrexate is a potent inhibitor of DHFR. Empirically, 0.005 μM methotrexate requires ~4-fold more DHFR to be made in the cell than does complete α^- medium with no methotrexate. By splitting the cells into this medium, one is selecting for cells making elevated levels of DHFR. This is generally accomplished by increasing the copy number of the transfected DHFR gene.

12. The cells should grow to confluence fairly readily. When they do, split them 1:6 again.

    The cells will probably grow more slowly and take on a flat, spread-out morphology. This indicates that they are starved for DHFR.

    Cells grow well immediately after splitting into a higher level of methotrexate because they have an endogenous reserve of nucleosides that needs to be depleted before the selection takes place.

13. Keep splitting the cells 1:6 into complete α^- medium supplemented with 0.005 μM methotrexate. When their rate of growth increases and when they begin to take on a more normal morphology, increase the degree of the split to 1:8, then 1:10, then 1:15. When the cells grow to confluence in 3 days from a 1:15 split and have regained a polygonal morphology, perform the next amplification step.

    It is possible that cells will immediately grow well in 0.005 μM methotrexate, indicating that they are already making enough DHFR to survive at this level of selection. If so, switch immediately to 0.02 μM methotrexate. Many researchers start out by placing the DHFR-containing cell line in 0.005, 0.02, and 0.05 μM methotrexate (at step 11) to see at what level the initial recombinant lines can survive.

14. Repeat the above process (steps 12 and 13) using complete α^- medium supplemented with 0.02 μM methotrexate.

15. Continue amplifying by increasing the level of methotrexate in the medium by 4-fold increments. Continue until the cells are growing in 20 to 80 μM methotrexate (the cells should now contain 500 to 2000 copies of the transfected gene).

    Be sure to freeze samples of the cells at each amplification step to avoid having to go back to the beginning in the event of contamination. Each amplification step should take 3 to 4 weeks. Use of methotrexate levels above 80 μM does not result in much further amplification, as the ability of the cell to transport the drug becomes limiting.

ALTERNATE PROTOCOL

AMPLIFICATION BY CLONING AT EACH SELECTIVE STEP

The above procedure is straightforward and does not require very much hands-on time. It does not, however, necessarily result in a clonal cell line at the end of each passage. The protocol requires a long time (9 to 12 months) to generate an amplified line. One may clone lines from the final amplified line to see whether some clones express more of a desired protein than others. Alternatively, one may amplify by cloning cells at every step and selecting for those that retain high levels of production of the protein of interest. This is a particularly attractive approach if the desired protein is secreted and easily assayed from the medium. This second approach may result in an overproducing line more rapidly, but it requires substantially more effort. If this approach is desired, substitute the following for steps 11 to 14 of Basic Protocol 1.

11a. Split a confluent dish of cells growing in complete α^- medium 1:15 into complete α^- supplemented with 0.02 μM methotrexate (8 dishes) and into complete α^- medium supplemented with 0.08 μM methotrexate (7 dishes). Feed every 4 days with the appropriate selective medium.

The goal of this step is to find individual cells that have amplified the DHFR gene enough to grow in a significantly higher level of methotrexate. These cells will expand into colonies during the 10- to 12-day incubation. As described in step 4, sibling colonies must be avoided, so dishes should be disturbed as little as possible.

12a. Check the dishes for colonies after 10 to 12 days and pick healthy colonies, as in steps 5 to 10 of the basic protocol.

13a. Expand the colonies and check for the level of expression of the desired product. Choose a colony that is producing good levels of the desired product, and repeat steps 11a and 12a using levels of methotrexate 16- and 64-fold higher than the level of methotrexate in which the colony is growing.

Each of these rounds will take ~1 month, and after three to four rounds, the cells should be growing in 80 μM methotrexate and contain highly amplified sequences.

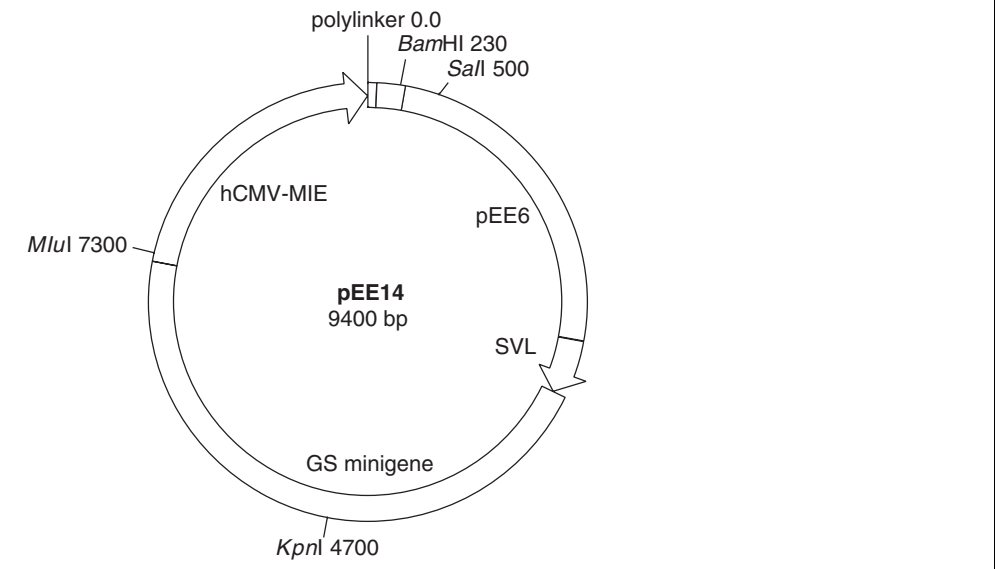
BASIC PROTOCOL 2

AMPLIFICATION USING GLUTAMINE SYNTHETASE

In the glutamine synthetase (GS) gene amplification system, a cDNA or genomic coding sequence is inserted into the multilinker cloning site of the plasmid pEE14 (Fig. 16.23.3) such that it is expressed from the powerful hCMV promoter-enhancer. pEE14 also contains a glutamine synthetase gene that can be used as a dominant selectable marker in a variety of cell lines including CHO K1. The GS gene expressed from the plasmid confers resistance to a low level of the GS inhibitor methionine sulfoximine (MSX). CHO cells transformed with the vector are selected for lines containing increased numbers of copies of the vector using increased levels of MSX in a single round of amplification.

Materials

Plasmid vector pEE14 (Celltech)
Complete Glasgow modified Eagle medium containing 10% dialyzed FBS (complete GMEM-10; see recipe)
CHO K1 cell line (ATCC #CCL61)
100 mM L-methionine sulfoximine (MSX; Sigma) prepared in PBS (see *APPENDIX 2* for PBS; filter sterilize MSX solution and store in aliquots at −20°C; handle carefully)



polylinker

*Hin*dIII *Xba*I *Xma*I *Sma*I *Eco*RI *Bcl*I
AAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCG ATCCCCGGGCGAGCTCGAATTCATTGATCA

Figure 16.23.3 Map of pEE14 GS expression vector. pEE14 (~9.4 kb in length) contains a GS minigene as the selectable marker which has a single intron and GS polyadenylation signals and is driven from an SV40 late promoter. The hCMV-MIE promoter-enhancer and 5′ untranslated region are used to express the gene of interest and the remainder of the plasmid contains an ampicillin-resistance gene and replication origin for replication in *E. coli*. The plasmid was constructed as follows. A 900-bp *Eco*RI fragment from the cDNA clone λgs1.1 (Hayward et al., 1986) was assembled with a 3.4-kb *Eco*RI-*Sac*I hamster GS genomic fragment from pGS1 (Sanders and Wilson, 1984), which provides the 3′ end of the minigene. (The *Sac*I site was converted to a *Bam*HI site to facilitate vector construction.) The *Eco*RI site within the GS coding sequence was destroyed by site-directed mutagenesis without altering the amino acid sequence and a *Hin*dIII site in GS 3′-flanking DNA was destroyed by digestion with *Hin*dIII, filling in the single-stranded ends, and religation. A 340-bp SV40 late promoter (Cockett et al., 1990) was added to the 5′ end as a *Bam*HI-*Eco*RI fragment and the *Eco*RI site between the promoter and the GS sequences was destroyed by filling in. The resulting 4.5-kb *Bam*HI fragment was inserted into pEE6hCMV (Stephens and Cockett, 1989) at a single *Bgl*II site upstream of the hCMV enhancer (hence destroying the *Bgl*II and *Bam*HI sites) to form pEE14. The resulting SV40-GS minigene in pEE14 is functionally equivalent to that in pSVLGS.1 (Bebbington and Hentschel, 1987) but has been deleted of *Eco*RI and *Hin*dIII sites. Polylinker sequence of pEE14 is shown below.

Additional reagents and equipment for subcloning (*UNIT 3.16*), $CaPO_4$-mediated transfection and glycerol shock (*UNIT 9.1*), and cloning by limiting dilution (*UNIT 11.8*)

NOTE: All tissue culture incubations are performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise indicated.

1. Subclone the target gene into the appropriate site within the polylinker of plasmid pEE14 (Fig. 16.23.3).
2. Maintain CHO K1 cells growing exponentially in complete GMEM-10. The day

before transfection, trypsinize the cells and seed several 9-cm petri dishes at 10^6 cells per dish.

3. Introduce 10 μg circular plasmid DNA (per dish) from step 1 into the cells using calcium phosphate–mediated transfection followed by glycerol shock. "Mock"-transfect several plates without added DNA.
4. After 24 hr, replace the medium with fresh complete GMEM-10 containing MSX at a final concentration of 25 μM (selective medium).

   CAUTION: *MSX is toxic and should be handled carefully.*
5. After 4 to 5 days, refeed the plates with fresh selective medium and wait for MSX-resistant colonies to appear, typically two weeks after infection.
6. Score the number of MSX-resistant colonies on transfected and "mock"-transfected plates.

   There should be 20 to 30 colonies per plate on transfected dishes and <5 colonies per "mock"-transfected plate.
7. Isolate several independent transfected cell lines producing significant amounts of the desired product (see Basic Protocol 1, steps 6 to 11) for amplification using DHFR. Plate out each cell line on several petri dishes at a density of ~10^6 cells per dish in complete GMEM-10. Incubate 24 hr.

   Whenever trypsinizing GS-selected cells, leave the cells for 24 hr to recover before reapplying MSX. In our experience, independent transfectants amplify more efficiently than pools of transfectants.
8. Replace the medium with fresh selective medium containing various concentrations of MSX, ranging between 100 μM and 1 mM.
9. Incubate the dishes 10 to 14 days, changing the medium once during this time.

   After this time considerable cell death should have occurred and colonies resistant to the higher levels of MSX should have appeared. The maximum concentration of MSX at which colonies survive will depend on the particular initial transfectant, but is typically between 250 μM and 500 μM.
10. Isolate the colonies at the highest MSX concentration yielding several discrete colonies. The colonies can either be picked and assayed individually or all colonies from one initial cell line can be pooled and assayed together.

    The increased production rate can be up to 10-fold in this first round of amplification. It is not normally appropriate to select for subsequent rounds of amplification because the production rate does not usually increase significantly at higher levels of MSX.
11. Clone the amplified cells with high production rates by limiting-dilution cloning.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Cloning cylinders

Glass or metal cloning cylinders can be purchased (e.g., stainless steel Penicylinders, Fisher). Place clean cylinders well rinsed in distilled water in 95% ethanol. Sterilize by flaming immediately prior to use. Alternatively, disposable cloning cylinders can be prepared by cutting off the fat end of a 200-μl pipettor tip ~1 cm from the large opening with a razor blade. Sterilize by autoclaving.

Complete ADT medium

α^- medium (Life Technologies) supplemented with:
10 µg/ml adenosine (Sigma)
10 µg/ml deoxyadenosine (Sigma)
10 µg/ml thymidine (Sigma)
10% FBS

It is convenient to prepare stocks of the nucleosides for addition to the medium. Adenosine (1 mg/ml), deoxyadenosine (1 mg/ml), and thymidine (4 mg/ml) stocks are prepared using distilled water, and are filter sterilized.

Complete GMEM-10

Add the following in order given, using aseptic technique:
500 ml Glasgow modified Eagle medium (GMEM) without tryptose phosphate broth (Life Technologies, but made *without* glutamine; must be made to order as it is not a stock item)
5 ml 100× nonessential amino acids (Life Technologies)
5 ml G+A (see recipe)
5 ml 100 mM sodium pyruvate (Life Technologies)
10 ml 50× nucleoside mix (see recipe)
50 ml dialyzed FBS (see recipe)
5 ml of 5000 U/ml penicillin/streptomycin (Life Technologies)

It is essential to use dialyzed FBS when performing GS selection because serum contains significant amounts of glutamine.

Dialyzed fetal bovine serum (FBS)

Purchase dialyzed FBS from commercial supplier (e.g., Life Technologies or J.R.H. Biosciences) or prepare as follows:

1. Heat inactivate FBS at 56°C for 60 min.
2. Soak Spectrapor dialysis tubing (MWCO 6000 to 8000) in PBS (*APPENDIX 2*). Remove, rinse tubing, clip one end closed, and fill with the heat-inactivated FBS.
3. Dialyze (also see *APPENDIX 3C*) 6 to 8 hr in cold room against PBS. Change dialysis solution at least once.
4. Filter sterilize using a 0.02-mm filter and store frozen (–20°C) in 50-ml aliquots.

Glutamate + asparagine (G+A)

600 mg L-glutamic acid (Sigma)
600 mg L-asparagine (Sigma)
H_2O to 100 ml
Filter sterilize using a 2-µm filter and store at 4°C

Methotrexate, 5 mM stock

Dissolve methotrexate to 5 mM in α^- medium (Life Technologies) and filter sterilize. Store at –20°C in a foil-wrapped container.

It is important to make a large 5 mM stock solution before starting amplification, then dilute that stock solution in α^- medium for the various levels of selective media. The potency of methotrexate can vary somewhat from lot to lot, making it desirable to use one stock throughout the amplification process.

CAUTION: *Methotrexate is a carcinogen and should be handled only with gloves and in a fume hood.*

Nucleoside mix, 50×

35 mg adenosine (Sigma)
35 mg guanosine (Sigma)
35 mg cytidine (Sigma)
35 mg uridine (Sigma)
35 mg thymidine (Sigma)
H_2O to 100 ml
Filter sterilize and store at −20° in 10-ml aliquots

COMMENTARY

Background Information

Gene amplification

When mammalian cells are placed in an environment that requires an increase in a normally constitutive gene product, cells that survive in many cases do so because of an increase in copy number of the gene. This process has been termed gene amplification and involves large regions of the chromosome, so that not only the selected gene becomes amplified, but the surrounding regions as well. Amplification was first detected when cells were treated with increasing concentrations of methotrexate, and the copy number of the resident DHFR gene was analyzed in the surviving cells (Alt et al.,1978). This observation, combined with the observation that cotransfected segments of DNA tend to integrate in the same chromosomal location (Wigler et al., 1978), has resulted in the ability to amplify any desired gene. Notable among the many reports of amplified genes in mammalian cells are *E. coli* XGPRT (Ringold et al., 1981), hepatitis B surface antigen (Christman et al., 1982), mouse *c-myc* (Wurm et al., 1986), tissue inhibitor of metalloproteinases using glutamine synthetase (GS) selection (Cockett et al., 1990), CD4 T lymphocyte glycoprotein (Davis et al., 1990), and human initiation factor 2α using DHFR selection (eIF-2α; Kaufman et al., 1991).

Until recently, gene amplification was achieved by cotransfection of a vector carrying a selectable marker gene with another vector carrying the nonselectable gene of interest. In this process, called cotransformation, separate DNA molecules become ligated and cointegrate as a unit by nonhomologous recombination into the host chromosome (Wigler et al., 1978). Amplification of the region of DNA containing the selectable gene and the target gene is accomplished by incubating the cells in increasing amounts of a specific inhibitory drug. However, varying DNA transfection methods and cell lines can yield dramatically different frequencies of cotransformation with different plasmids. Consequently, expression vectors have been developed that contain both the selectable gene and a transcription unit for the inserted target gene linked on the same plasmid. Various approaches in constructing these plasmids have been used to ensure adequate expression of both the selectable and nonselectable marker.

CHO cells

Many amplifiable selection markers are now available for use in mammalian cells (see Table 16.23.1; Kaufman, 1989, 1990). Although various cell lines (including monkey COS-1 and NIH 3T3) can be used for gene amplification, many protocols rely upon the use of Chinese hamster ovary (CHO) cells. The advantages of CHO cells for heterologous gene expression are (1) amplified genes that are integrated into host chromosome may be stably maintained, even in the absence of continued drug selection; (2) a variety of proteins have been expressed at high levels (Ringold et al., 1981; Cockett et al., 1990; Davis et al., 1990; Kaufman et al., 1991); and (3) volumes of CHO cells have been scaled up to >5000 liters. Two of the most successful strategies employing CHO cells—the dihydrofolate reductase (DHFR) and glutamine synthetase (GS) gene amplification systems—are described below.

Amplification using dihydrofolate reductase vectors

DHFR catalyzes the conversion of folate to tetrahydrofolate, which is required for purine, amino acid, and nucleoside biosynthesis. The folic acid analog methotrexate (MTX) binds and inhibits DHFR, causing cell death. Surviving populations of cells exposed to sequentially increasing concentrations of MTX contain increased levels of DHFR that result from gene amplification. The pED (DHFR) vector produces a transcript containing the target gene in the 5′ position and the selectable marker in the

Table 16.23.1 Amplifiable Markers for Mammalian Cells[a]

Selection	Gene
Adenosine, alanosine, and 2′-deoxycoformycin	Adenosine deaminase
Adenine, azaserine, and coformycin	Adenylate deaminase
β-aspartyl hydroxamate or albizzin	Asparagine synthetase
PALA	Aspartate transcarbamolyase
Methotrexate	Dihydrofolate reductase
Methionine sulfoximine	Glutamine synthetase
Cadmium	Metallothionein
α-difluoromethylornithine	Ornithine decarboxylase
Multiple drugs	P-glycoprotein 170
6-azauridine or pyrazofuran	UMP synthetase
Mycophenolic acid	Xanthine-guanine phosphoribosyltransferase

[a]Adapted with permission from Academic Press.

3′ position of the transcript. This vector has been optimized for translation of the selectable gene in the 3′ position by use of specific sequences from the encephalomyocarditis (EMC) virus that promote internal ribosome binding and translation initiation (Kaufman et al., 1991). The development of CHO cell lines (e.g., CHO DG44) that are deleted for the endogenous DHFR genes greatly increases the ease of amplification using DHFR (Urlaub and Chasin, 1980; Urlaub et al., 1983). Amplification of exogenous genes has been accomplished either by using cells and vectors that express a normal DHFR gene (Ringold et al., 1981; Kaufman and Sharp, 1982a; Kaufman et al., 1991), or by using vectors that encode a DHFR gene partially resistant to methotrexate and normal CHO cells (Christman et al., 1982; Kaufman et al., 1991).

Amplification using glutamine synthetase vectors

GS provides the only pathway for synthesis of glutamine in mammalian cells (using glutamate and ammonia as substrates); thus, in a glutamine-free medium, GS is an essential enzyme. CHO cells contain endogenous GS enzyme, but concentrations of methionine sulfoximine (MSX) in excess of 20 to 25 μM are sufficient not only to inhibit wild-type levels of GS but also to prevent the growth of the majority of natural variants that arise by amplification of the endogenous GS genes. Hence, essentially all nontransfected cells are killed when grown in media containing MSX at these levels. The GS vector pEE14 carries both the selectable GS marker and the nonselectable target gene, each transcribed from a separate promoter. The target gene is cloned into the multilinker cloning site with transcription initiating from the powerful human cytomegalovirus (hCMV-MIE) promoter-enhancer sequence, while transcription of the GS gene is driven from an SV40 late promoter (Cockett et al., 1989). The GS "minigene" in pEE14 permits sufficient GS expression to allow transformants to survive at low levels of MSX, and increased levels of MSX select cells that have undergone amplification of vector sequences integrated into the cell genome. The appropriate choice of MSX concentration for initial selection together with the use of a weakly expressed GS gene typically selects cell lines containing multiple copies of the vector. The combination of the hCMV promoter to drive the gene of interest and the GS-selection system usually provides relatively high levels of expression after only a single round of selection following gene amplification.

Critical Parameters

For this approach to be successful, the gene of interest must be integrated in a functional form in the original cell line. It is also crucial that the gene of interest not rearrange during amplification. These two criteria may be difficult to achieve if the expressed gene is cytotoxic when overproduced. This problem can be circumvented by expressing the gene of interest from an inducible promoter and thus amplify-

ing the gene in an "off" state (Wurm et al., 1986).

The key to obtaining high-level expression in primary transfectants is to screen as many as possible (typically ~100 lines) since the site of integration of the vector in the host cell genome has a profound influence on expression levels. To get good results on amplification, it is also important to screen a number of lines (e.g., 5 to 10 high producers), as again the integration site influences the frequency of amplification. Once high-producing lines have been isolated, it is often useful to reclone these to ensure a homogeneous population of cells, in order to increase the likelihood that the productivity will be maintained over long periods of culture. The selective agent should be present throughout these procedures and only when frozen stocks of recloned amplified cells have been secured may selection be relaxed, if it is important to choose lines that are stable without selective agent. Finally, it may again be necessary to screen a number of lines to identify ones that are stable without selective agent.

Anticipated Results

Dihydrofolate reductase. Cell lines containing >100 copies of an exogenous gene can be produced. The levels of mRNA and protein obtained depend upon the target gene to be expressed, but can constitute up to 5% of total protein synthesis (Kaufman, 1991).

Glutamine synthetase. Introduction of pEE14-based vectors using the $CaPO_4$-mediated transfection usually leads to multiple copies of the vector becoming integrated in the genome (up to 200 copies). The copy number can increase up to 30-fold in one round of selection for amplification (Cockett et al., 1989). The amount of product made depends both on the individual transfectant and on the protein being expressed, but can be up to 10 μg protein/10^6 cells per 24-hr period from primary transfectants for some proteins. On amplification, protein expression can parallel the increase in copy number but is likely to reach a plateau, usually after a single round of amplification for many secreted proteins, probably because the secretion apparatus is saturated. Final yields of secreted proteins from overgrown cultures have been 180 mg/ liter for tissue inhibitor of metalloproteinases (TIMP), and up to 120 mg/liter for secreted variants of the rat CD4 protein (Davis et al., 1990). pEE14 has also been used successfully to express an integral membrane protein (Harfst et al., 1992).

Time Considerations

DHFR. Transfection and detection of stably transformed colonies takes 2 weeks. It will take ~2 additional weeks to expand the colonies and analyze them to ensure that they contain the gene of interest. Amplification of the resultant stable lines may take up to 6 months by the standard protocol. If cells are cloned at every step, as described in the alternate protocol, the amplification process can take as little as 3 months.

GS. Transfection and analysis of initial cell lines takes ~4 weeks (as for DHFR selection). Selection for vector amplification and screening of resultant cell lines typically takes an additional 4 to 6 weeks; there is usually no advantage in undergoing further rounds of selection.

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Key References

Kaufman et al., 1991. See above.

Describes the construction and application of dicistronic DHFR vectors that allow stable, high-level expression of inserted cDNAs by selection for methotrexate resistance in both DHFR-containing and DHFR-deficient cells.

Cockett et al., 1990. See above.

Describes the construction of vectors that provide for high-level expression using the GS gene-amplification system.

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