Transfected COS cells will tend to clump when lifted from the dish. Pipetting the cells up and down will tend to disrupt these clumps. More effective dispersion of the clumps can be obtained by forcing the cells through a 100-μM nylon mesh.

Reference: Warren and Shields, 1984.

Contributor: Alejandro Aruffo

UNIT 16.14

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 cotransfection of the two unlinked genes), high-level expression of the desired gene is obtained by selecting for amplification of the selectable marker. The first basic protocol describes the dihydrofolate reductase (DHFR) selection system while the second 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 amplification process is lengthy and can require from 9 to 12 months to isolate a stable, amplified line. In contrast, the GS typically requires only a single round of selection for amplification to achieve maximal expression levels; in this system the time required to isolate stably amplified clones from primary transfectants is dramatically reduced to 2 months or less.

Cell lines containing at least 1000 copies of an exogenous gene can be produced using the DHFR selection system. 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. Introduction of pEE14-based vectors using $CaPO_4$-mediated transfection usually leads to multiple copies of the vector becoming integrated in the genome in the GS selection system. The copy number can increase up to 30-fold in one round of selection for amplification. The amount of product depends both on the individual transfectant and on the protein being expresssed, but can be up to 10 μg protein/10^6 cells per 24-hr period 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.

BASIC PROTOCOL

AMPLIFICATION USING DIHYDROFOLATE REDUCTASE

The pED series of dicistronic vectors (Fig. 16.14.1) 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 their 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.

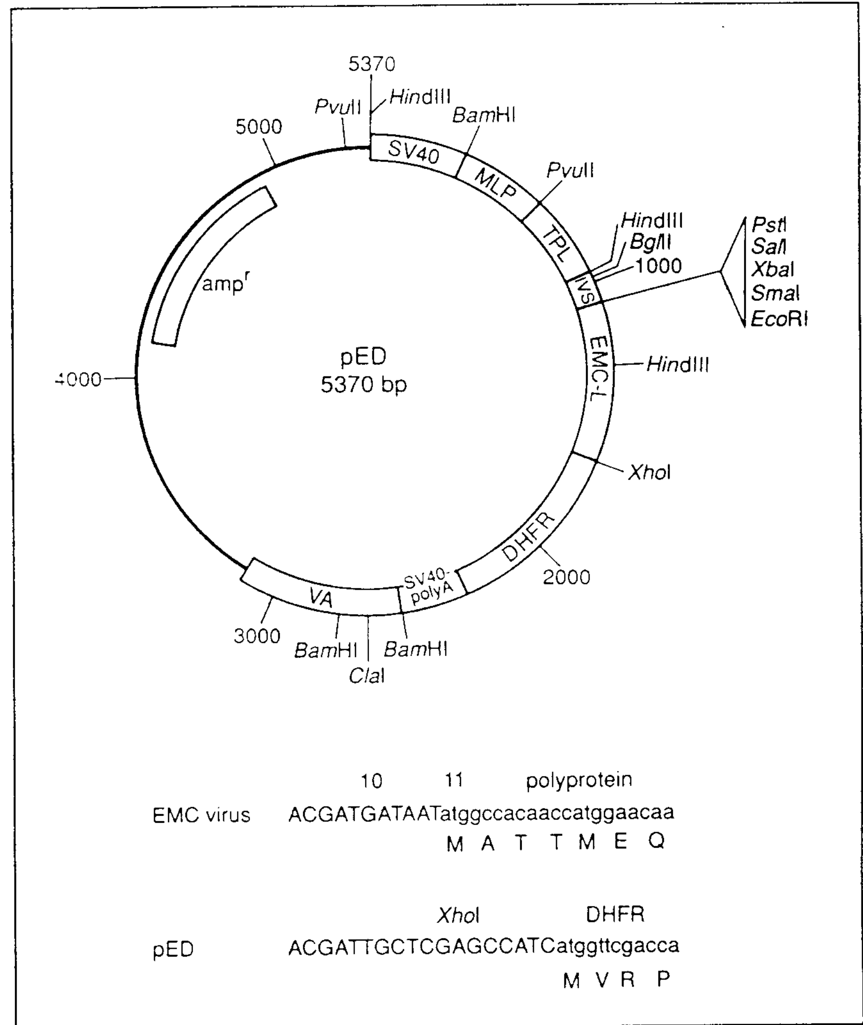

Figure 16.14.1 Map of dicistronic mRNA expression vector pED. The components of the 5360-bp pED expression vector in the pUC18 background are indicated as follows: SV40, *Hin*dIII-*Pvu*II fragment containing the SV40 origin of replication and enhancer element; MLP, adenovirus major late promoter from the *Xho*I site (15.83 m.u.) to the 5′ cap site (16.55 m.u.); TPL, 180 bp of the first two and ⅔ 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 late 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 the 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.

Materials (see APPENDIX 1 for items with ✓)

- Vector from pED series (Kaufman et al., 1991); *or* pCVSVEII-DHFR or pAd26SV(A) (Kingston et al., 1984; Kaufman and Sharp, 1982) 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 R.J. Kaufman)
- ✓ Complete ADT medium
- 10% glycerol
- ✓ Dialyzed fetal calf serum (FCS)
- Complete α^- medium (α^- medium with 10% dialyzed FCS)
- Sterile vacuum grease
- 0.05% trypsin/0.6 mM EDTA in PBS, 37°C
- ✓ Methotrexate
- ✓ Cloning cylinders

Transfect CHO cells

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

2. Transfect cells with 5-10 µg plasmid DNA/dish from step 1, using electroporation (*UNIT 9.3*), liposome-mediated transfection (*UNIT 9.4*), or the calcium phosphate technique (*UNIT 9.1*; the latter treatment followed by glycerol shock works well for CHO DXB11 cells). Perform a 3-min shock with 10% glycerol 4 to 6 hr after DNA precipitate is placed on cells. The gene can then be introduced in 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 gene of interest to DHFR gene.

   It is not necessary to link physically the DHFR gene to the gene whose amplification is desired.

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

3b. *For cells transfected using liposomes.* Add 10 ml complete α^- medium and incubate overnight. Remove medium, wash twice with 37°C PBS, add 10 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. Methotrexate is not needed for selection.

4. Incubate cells 10 to 12 days (14 days for liposome-mediated transfection). Move dishes as infrequently as possible to prevent formation of sibling colonies.

Pick stable colonies

5. Ten to twelve days (14 days for liposome-mediated transfection) after placing cells in selective medium, check dishes for colonies by holding dish above one's head at an angle to the overhead lights and looking for opaque patches. Circle such patches with a laboratory marker so they can easily be located and examined in phase contrast microscope.

   To determine how well the transfection worked, one of the transfected dishes can be stained with methylene blue. To stain, aspirate off medium, then place ~2 ml of 2% methylene blue solution (made up in 50% ethanol) on each dish. Wait 2 min, pour dye solution off and wash off residual methylene blue by dipping the dish in a bucket of cold water. 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 colonies to be picked. Circle the chosen colonies with a laboratory marker to determine where to place cloning cylinders.

   Choose only large, healthy colonies. Colonies should have ~500 cells, and cells should appear 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 and 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.

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 cylinder around colony to be picked (Fig. 16.14.2).

   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.

8. Using a sterile Pasteur pipet, rinse colony with 37°C trypsin/EDTA by filling and emptying cloning cylinder (Fig. 16.14.2).

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

10. As cells grow out, split frequently (every 4 to 5 days) so 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, be sure that the gene of interest has been functionally integrated into the cell. 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 and the gene of interest can rearrange or mutate during the process. It is 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 complete α^- supplemented with 0.005 µM methotrexate (2 dishes).

    CAUTION: *Methotrexate is carcinogenic; use gloves to handle.*

12. The cells should grow to confluence fairly readily. When they do, split 1:6 again. The cells will probably grow more slowly and take on a flat, spread-out morphology, indicating that they are starved for DHFR.

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

14. Repeat steps 13 and 14 using complete α^- medium supplemented with 0.02 µM methotrexate.

15. Continue amplifying by increasing methotrexate level in medium by 4-fold increments, until 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.

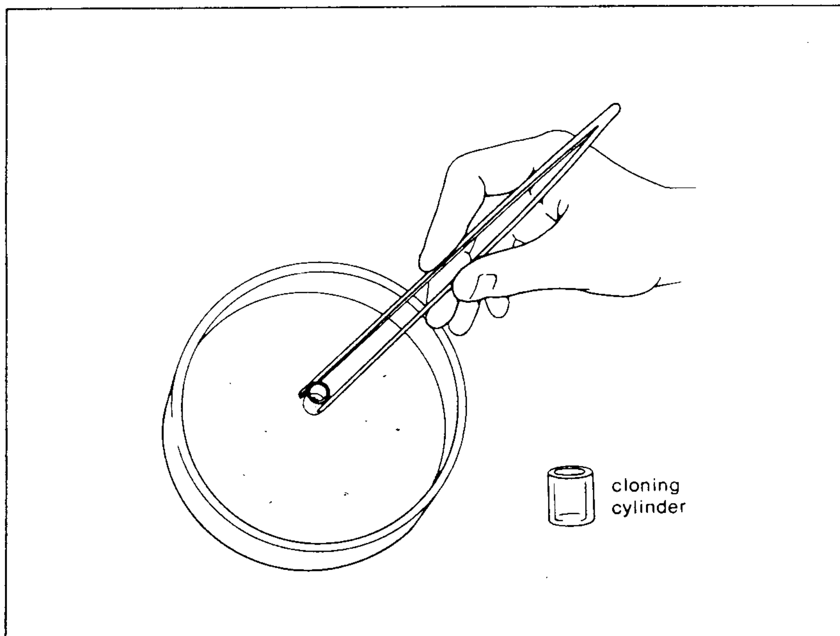

Figure 16.14.2 Placement of cloning cylinder around CHO colony.

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 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 much more effort. If this approach is desired, substitute the following for steps 11 to 14 of the basic protocol.

11a. Split 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^- supplemented with 0.08 µM methotrexate (7 dishes). Feed every 4 days with appropriate selective medium.

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.

12a. Check dishes for colonies after 10 to 12 days and pick healthy colonies, as in steps 6 to 11 of basic protocol.

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

16.14

BASIC PROTOCOL

AMPLIFICATION USING GLUTAMINE SYNTHETASE

This protocol describes 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. 16.14.3) 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 sulfoxamine (MSX). CHO cells transformed with the vector are selected for lines containing increased numbers of the vector using increased levels of MSX in a single round of amplification.

Materials (*see* APPENDIX 1 *for items with* ✓)

Plasmid vector pEE14 (Celltech)
✓ Complete Glasgow modified Eagles medium containing 10% dialyzed FCS (GMEM-10)
CHO-K1 cell line (ATCC #CCL61)
100 mM L-methionine sulfoximine (MSX) prepared in PBS (filter sterilized and stored in aliquots at −20°C)

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator.

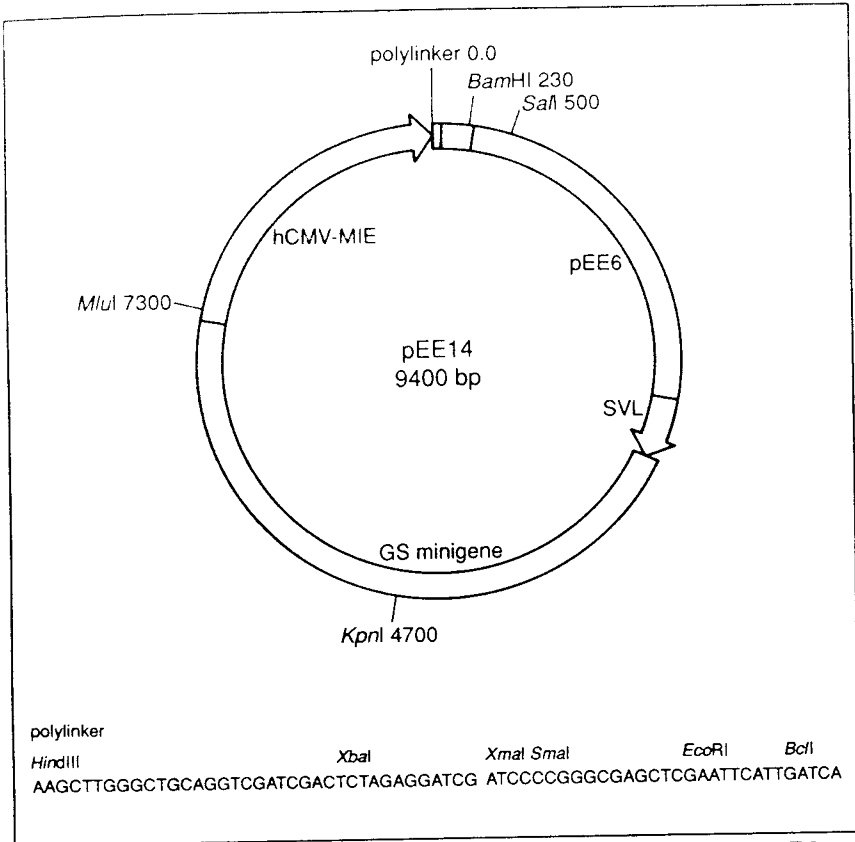

Figure 16.14.3 Map of pEE14 GS expression vector. pEE14 (~9.4 kb in length) contains a GS minigene as the selectable marker which has a single intron and GS polyadenylation signals and is driven from an SV40 late promoter. The hCMV-MIE promoter-enhancer and 5′ untranslated region are used to express 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 was assembled with a 3.4-kb *Eco*RI-*Sac*I hamster GS genomic fragment from pGS1, 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 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 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 but has been deleted of *Eco*RI and *Hin*dIII sites. Polylinker sequence of pEE14 is shown below.

1. Subclone target gene into appropriate site within polylinker of plasmid pEE14 (Fig. 16.14.3; *UNIT 3.16*).
2. Maintain CHO-K1 cells growing exponentially in complete GMEM-10. The day before transfection, trypsinize cells and seed several 9-cm petri dishes at 10^6 cells/dish.

3. Introduce 10 µg circular plasmid DNA per dish from step 1 into cells using calcium phosphate–mediated transfection followed by glycerol shock (UNIT 9.1). "Mock"-transfect several plates without added DNA.
4. After 24 hr, replace medium with fresh GMEM-10 containing MSX at 25 µM final (selective medium).

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

   There should be 20 to 30 colonies/plate on transfected dishes and <5 colonies/"mock"-transfected plate.
6. Isolate several independent transfected cell lines producing significant amounts of desired product as in steps 5 to 10 of the first basic protocol and plate out each cell line on several petri dishes at $\sim 10^6$ cells/dish in complete GMEM-10. Incubate 24 hr.

   Whenever trypsinizing GS-selected cells, leave cells for 24 hr to recover before reapplying MSX.
7. Replace medium with fresh selective medium containing various concentrations of MSX, ranging between 100 µM and 1 mM. Incubate dishes 10 to 14 days, changing medium once.

   Considerable cell death should have occurred and colonies resistant to higher levels of MSX should have appeared. The maximum concentration of MSX at which colonies survive depends on the particular initial transfectant, but is typically between 250 µM and 500 µM.
8. Isolate 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.
9. Clone amplified cells with high production rates by limiting-dilution cloning (UNIT 11.8).

References: Kaufman and Sharp, 1982; Kaufman et al., 1991; Kingston et al., 1984.

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