BioResearch

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Guideline for Generation of Stable Cell Lines
Technical Reference Guide

1. Background

Stable, long-term expression of a gene of interest can be either achieved by eukaryotic vectors that harbor elements for episomal maintenance in the nucleus of a transfected cell or via direct integration of the transfected plasmid into the target cells genome. Episomal stability is often limited, resulting in gradual loss of transfected vectors that can only be prevented by sustained antibiotic selection eliminating cells that lost the plasmid. Furthermore, the functionality of episomal plasmid elements is often restricted to certain species. Although integration into the host cell chromosome is a rare event and, for most purposes, clonal events have to be isolated, stability of the intended genetic modification usually is much higher.

Initially, the gene of interest has to be introduced into the cell (A), subsequently into the nucleus (B), and finally, it has to be integrated into chromosomal DNA (C) (Figure 1).

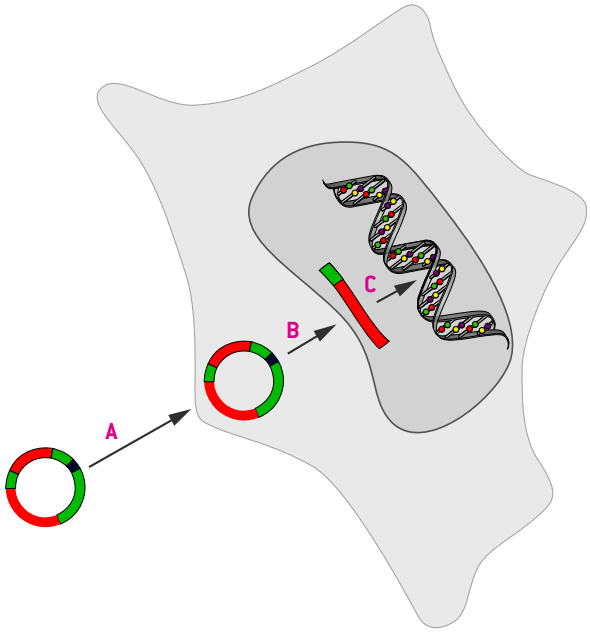


Figure 1. The general path of stable integration.

Since chromosomal integration into host chromosomes is a rare event, stably-transfected cells usually have to be selected and cultured in various ways. For the selection of stably-transfected cells, a selection marker is co-expressed on either the same construct or on a second, co-transfected vector. A variety of systems for selecting transfected cells exists, including resistance to antibiotics such as neomycin phosphotransferase, conferring resistance to G418, dihydrofolate reductase (DHFR), or glutamine synthetase (Southern and Berg, 1982). After gene transfer, cells are cultivated in medium containing the selective agent. Only those cells which have integrated the plasmid survive, containing the drug resistant gene.

Several options are used for the generation of a stable cell line, depending on the scope of the experiment. A mixed population of drug resistant cells can be used directly for experimental analysis (batch culture) with the advantage of generating fast results, but also the disadvantage of dealing with an undefined and genetically mixed cell population. To generate clonal cells, it is necessary to dilute the resistant cells in such a way that culture as single, isolated cells is achieved e.g., by plating in 96-well plates or other methods. Subsequently, the selection process is applied to the single cell cultures. The procedure of single cell cloning may be repeated several times to obtain 100% clonal purity. This culture method allows for conduction of the study or the screening using a defined and homogenous cell system. So far, generation of stable cell lines has been a major challenge for many cell types (e.g., Jurkat, MCF7 or U937) since overall transfection efficiencies and/or integration frequencies have been low. While common transfection methods, such as lipofection, can be used for the stable expression in easy-to-transfect cell lines (e.g., HeLa, COS-7 or CHO), Nucleofection™ is the method of choice for stable expression in difficult-to-transfect cell types.

The generation of stably-transfected cell lines is essential for a wide range of applications, such as gene function studies (Grimm, 2004), drug discovery assays or the production of recombinant proteins (Wurm, 2004). In contrast to transient expression, stable expression allows long term, as well as defined and reproducible, expression of the gene of interest.

Culture System	Advantage	Application
Batch culture – polyclonal	Fast, useful for cells which do not grow in single cell culture	Overexpression, protein expression systems (e.g., for basic research)
Limiting dilution – monoclonal	Defined cell clonest	Studies of gene function, protein production (e.g., for therapeutic applications)

2. Culture Conditions for Generation of Stable Cell Lines

As for transient transfection experiments, culture conditions (passage number, split rhythm, etc.) of your selected cell type are very important for the generation of stably-transfected cell lines. For optimal results, we recommend following the cell culture recommendations of the supplier for the respective cell type. In general, the cell line should be passaged two days before the experiment to promote good proliferation and cell physiology. Cell passage should not be higher than 30. Interference of higher passage numbers with integration efficiency is possible and may be cell-type dependent.

Depending on the scope of your experiment, cells can be cultivated as polyclonal batches or monoclonal single cell clones post-transfection.

3. Transfection Method

Stable expression can be influenced by the transfection method used. The choice of transfection method determines which cell type can be targeted for stable integration. While biochemical transfection reagents (e.g., HiFect™ Transfection Reagent) can be used to transfer DNA into standard cell lines, efficient delivery of DNA into notoriously difficult-to-transfect suspension cell lines or even primary cells is only possible with viral methods or Nucleofection™ (Figure 2). Unfortunately, viral methods suffer from several limitations, such as time consuming production of vectors and safety concerns (Hacein-Bey-Abina et al., 2003).

We recommend using the following transfection methods:
- Nucleofector™ Technology for transfection of difficult-to-transfect cell lines
- HiFect™ Transfection Reagent for transfection of adherent standard cell lines

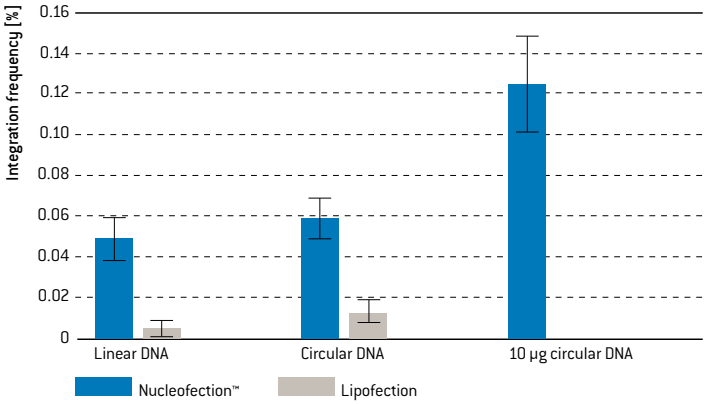
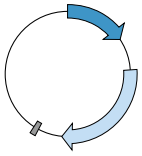
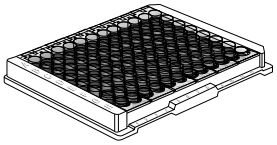
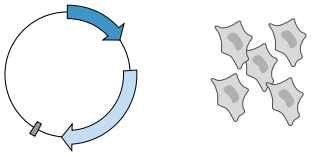
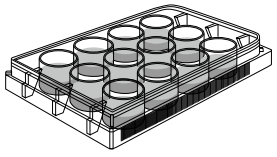
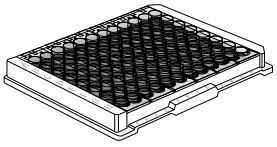
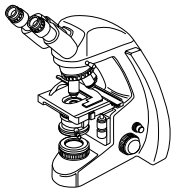


Figure 2. Higher integration rates in difficult-to-transfect cell lines using Nucleofection™. Jurkat cells were transfected using either Nucleofection™ (2 µg DNA) or lipofection Reagent L (0.7 µg DNA) according to the respective manufacturers instructions. 24 hours after transfection, cells were plated on a 96-well plate containing culture medium supplemented with G418 for selection of stably-transfected cells. 30 days after plating, cells were analyzed for clonal outgrowth (Integration frequency = number of resistant clones per number of living cells seeded). Due to toxic effects, lipofection with 10 µg circular DNA could not be performed.

4. Experimental Outline

	Procedure Outline	Important Information
Expression plasmid 	Design experiment and choose cell type, expression vector and transfection method.	Make sure that transfection method and expression vector are suitable for your cell type.
	Determine appropriate cell number per well (only for limiting dilution) and G418 concentration.	Cells diff er in their susceptibility to G418. The active concentration of stock G418 can vary from batch to batch.
Expression plasmid + cells 	Transfect expression vector into cells.	Amount of expression vector per experiment is dependent on transfection method and cell type.
	Plate transfected cells and cultivate cells in medium without G418.	Do not add G418 to culture medium immediately after transfection as this may drastically increase mortality.
	Dilute cells into culture plates and start selection 24 – 48 hours post-transfection. Feed every 2 – 3 days (for batch culture) or 10 days (for limiting dilution) with selection medium.	Choose culture conditions (batch culture, limiting dilution) depending on your experimental design. Refreshed selection medium is important to avoid false positive cells.
	Analyze stably-transfected cells.	Make sure that the chosen assay is suitable for your application.

5. Protocol for Batch Culture

5.1 Determination of G418 Concentration Using Batch Culture

Stably-transfected cells can be selected by the addition of drugs to the culture medium, if the expression plasmid carries a drug resistance gene. Here we describe the neomycin resistance system, which uses resistance to G418 as a selection marker. Cells differ in their susceptibility to G418, which may even vary with cell passage numbers. Cells that are cultured in serum-free media may require much lower G418 concentrations as compared to cells in media containing sera. The selection condition for your specific cell type needs to be established experimentally. Determine the minimum level of G418 to be added to the culture medium to prevent cell growth. Note that the active concentration of stock G418 can vary considerably from batch to batch. We therefore recommend testing G418 sensitivity for every new batch or to buy a large amount of one lot to standardize selection conditions.

1. Split cells into 12-well plates containing culture medium without G418 in plating densities according to cell supplier instructions.
2. The next day, aspirate growth medium and feed cells with medium containing increasing concentrations of G418 (e.g., titrate G418 in a range of 0.1 mg/ml to 1.5 mg/ml; in serum-free culture expand range down to 20 µg/ml).
3. Feed cells every 2 – 3 days with selection medium.
4. Check cell death after 7 – 14 days by light microscopy.
5. Choose the concentration which is 0.1 or 0.2 mg/ml above the one which shows complete cell death as the appropriate G418 concentration for selection. If the lowest concentration used shows complete cell death at day 7, the titration should be repeated with a lower concentration range.

5.2 Transfection

For transfection, please follow the respective manufacturer's instructions of your transfection system and transfect the expression plasmid, containing the gene of interest and the sequence for a drug resistance gene, into your cell type.

After transfection, plate cells according to the instructions from the supplier of your transfection system on tissue culture plates. Usually 6-well plates are used for 10^6 adherent cells and 12-well plates for 10^6 suspension cells.

Important Controls

We suggest including a sample of untransfected cells as a negative control for selection. We also strongly recommend checking the transfection efficiency and integration frequency of your experiment with a GFP-control plasmid, such as pmaxFP™-Green Vector. pmaxFP™-Green Vector encodes the green fluorescent protein (maxFP™-Green Reporter Protein) from Pontellina p. maxFP™-Green Reporter Protein expressing cells can easily be analyzed by fluorescence microscopy or flow cytometry to monitor the efficiency of your experiment.

5.3 Cell Culture Post-Transfection

Under selective conditions, resistant cells outgrow non-resistant cells, resulting in a polyclonal population of stably-expressing cells. This heterogeneously expressing population of resistant cells can then be used for experimental analysis.

1. After transfection, allow cells to grow and to express the protein for G418 resistance under non-selective conditions for at least 24 hours (for sensitive cells, G418 selection may begin after 48 hours).
2. Trypsinize adherent cells by standard procedures or use suspension cells directly for analysis. If possible, analyze for transfection efficiency 24 – 48 hours post-transfection on an aliquot of the positive control sample and your gene of interest (transient transfection control).
3. For the selection of stably expressing cells, cultivate cells in standard medium with supplements and the appropriate amount of G418, pre-tested for your cell type.
4. Plate cells on culture plates or flasks according to cell supplier instructions and incubate cells under standard conditions.
5. Feed and if necessary split cells until outgrowth of resistant cells.
6. Harvest cells of batch culture.

Usually, cells which have not integrated the resistance gene die during the first days of selection. Outgrowth of resistant cells can be observed normally after 2 weeks of selection. For some cells this may take up to 4 weeks. G418 is labile at 37°C, therefore it is recommended to change medium containing G418 every 2 – 3 days to compensate for loss of selection pressure. In some cases, it might be feasible to lower the G418 concentration after 1 – 2 weeks. Cells should be grown for at least 3 weeks under selection pressure to avoid contamination with non-resistant cells. Negative control wells (e.g., sample without expression plasmid) should be inspected by light microscopy and should not contain any signs of cell growth.Dependent on cell type and cell growth, selection can be extended up to 4 – 5 weeks or longer in total.

5.4 Analysis of Batch Culture

Once you have obtained resistant cell batches or clones, expand the cells and assay for your gene of interest. For the analysis of maxFP™-Green Reporter Protein expressing cells (positive control), use fluorescence microscopy or flow cytometry.

6. Protocol for Limiting Dilution

6.1 Determination of G418 Concentration and Plating Density

Stably-transfected cells can be selected by the addition of drugs to the culture medium, if the expression plasmid carries a drug resistance gene. Here we describe the neomycin resistance system, which uses resistance to G418 as a selection marker. Cells differ in their susceptibility to G418, which may even vary with cell passage numbers. Cells that are cultured in serum-free media may require much lower G418 concentrations as compared to cells in media containing sera. The selection condition for your specific cell type needs to be established experimentally. Determine the minimum level of G418 to be added to the culture medium to prevent cell growth. Note that the active concentration of stock G418 can vary considerably from batch to batch. We therefore recommend testing G418 sensitivity for every new batch or buying a large amount of one lot to standardize selection conditions. The final plating density after transfection depends on the culture conditions of the specific cell type and the G418 concentration. We therefore recommend combining the titration of G418 with the titration of cell numbers for determination of plating density in a matrix (Figure 3).

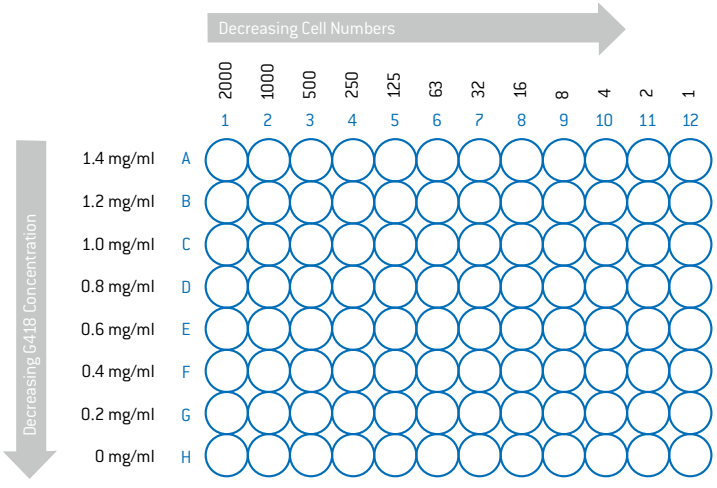


Figure 3. Matrix titration of G418 and titration of cell number for determination of plating density in one 96-well plate.

1. Pre-plate 100 µl medium in each well of the plate.
2. Add 100 µl of cell suspension containing 4000 cells per well to the first column (#1).
3. Carry over 100 µl to the next column after gentle up and down pipetting, thereby diluting in a ratio of 1:2. Repeat this procedure for each consecutive column.
4. After completing, discard 100 µl from the last column (#12). The first column should then contain about 2000 cells, the last less than one cell on average.
5. Add 100 µl of G418 containing medium (2.8 mg G418 per ml) to the first row (A) for a final G418 concentration of 1.4 mg/ml.
6. Add G418 to the following rows in decreasing concentrations of G418 in steps of 0.2 mg/ml. For the last row (H) add medium without G418.
7. Incubate cells at standard conditions.
8. Analyze cell growth by microscope. In some cases, cell growth can also be observed by change of medium color.
9. If you observe cell growth (after > 10 days) in the wells without G418 containing less than 4 cells, it is reasonable to assume that those cells can grow out starting as single cells.
10. Choose the G418 concentration which is just above the one which shows complete cell death as the appropriate G418 concentration for selection.

Certain cell types might need a critical number of neighboring cells to grow appropriately. If this is the case, limiting dilution experiments can only be done at higher cell concentrations, making it more difficult to obtain pure clones. In these cases, clones should be generated by plating an appropriate number of cells, selecting and diluting the resistant clones together with non-transfected cells of the same type as feeder cells. Other possibilities are culturing cells in 96-well plates with communicating channels, on soft-agar or methylcellulose.

6.2 Transfection

For transfection, please follow the respective manufacturer's instructions of your transfection system and transfect the expression plasmid containing the gene of interest and the sequence for a drug resistance gene into your cell type.

After transfection, plate cells according to the instructions from the supplier of your transfection system (e.g., on 96-well tissue culture well plates).

Important Controls

We suggest including a sample of untransfected cells as a negative control for selection. We also strongly recommend checking the transfection efficiency and integration frequency of your experiment with a GFP-control plasmid, such as pmaxFP™-Green Vector. pmaxFP™-Green Vector encodes the green fluorescent protein (maxFP™-Green) from Pontellina p. maxFP™-Green Reporter Protein expressing cells can easily be analyzed by fluorescence microscopy or flow cytometry to monitor the efficiency of your experiment.

6.3 Cell Culture Post-Transfection

Single cell clones from adherent and suspension cell types can be generated by diluting cells in a 96-well plate or other methods that allow the outgrowth of isolated cell clones under selective pressure.

1. After transfection, allow cells to grow and to express the protein for G418 resistance under non-selective conditions for at least 24 hours (for sensitive cells, G418 selection may begin after 48 hours).
2. Trypsinize adherent cells by standard procedures or use suspension cells directly for analysis. If possible, analyze for transfection efficiency 24 – 48 hours post-transfection on an aliquot of the positive control sample and your gene of interest (transient transfection control).
3. Count living cells via trypan blue staining or other appropriate methods.
4. Using standard medium with supplements and the appropriate amount of G418 pretested for your cell type, plate cells in a 96-well plate with different cell numbers per well (e.g., 10, 100, 1000) in a volume of at least 100 µl per well. Depending on cell concentration determined before, conduct several serial dilution steps as applicable. It is important to thoroughly suspend cells before seeding, but avoid harsh treatment by frequent pipetting. Use the lower limit determined before as the minimum number of cells per well (for generation of single cell clones, choose the dilution which statistically yields between 5 and 20 clones per 96-well plate, thereby minimizing the probability of wells with more than one clone).
5. Incubate cells under standard conditions and feed cells after 10 – 14 days with fresh selection medium.
6. Cell clones can be analyzed or further expanded as soon as cells in the non-transfected control wells have completely died.
7. In order to help assuring that selected cell populations are clones derived from a single cell, another round of limiting dilution under selection is recommended.

Usually, cells which have not integrated the resistance gene die during the first days of selection. Outgrowth of resistant cells can be observed normally after 2 weeks of selection. For some cells this may take up to 4 weeks. G418 is labile at 37°C, therefore, it is recommended to add fresh medium containing G418 after 10 – 14 days to compensate for loss of selection pressure. In some cases, it might be feasible to lower the G418 concentration after 1 – 2 weeks. Cells should be grown for at least 3 weeks under selection pressure to avoid contamination with non-resistant cells. Negative control wells (e.g., sample without expression plasmid) should be inspected by light microscopy and should not contain any signs of cell growth. Dependent on cell type and cell growth, selection can be extended up to 4 – 5 weeks or longer in total.

6.4 Analysis of Stable Clones

Once you have identified resistant clones, expand the cells and assay for your gene of interest by using an appropriate analysis method (e.g., microscopy, flow cytometry, ELISA). For the analysis of the positive control cells, use fluorescence microscopy to screen the 96-well plate.

7. Troubleshooting

Symptom	Suggestion
Transient transfection effi ciency is low	Optimal cell density should be determined for each cell type. For adherent cells, the optimal confluency at the time of transfection is normally 60-80%. Higher, as well as substantially lower, cell densities may cause lower transfection efficiencies. Suspension cells must be in their logarithmic growth phase. Choose appropriate transfection method (e.g., Nucleofection™ for difficult-to-transfect cell lines or HiFect™ Transfection Reagent for standard cell lines).
Viability is low 24 hours post-transfection	Try lower DNA amounts when using cells known to be DNA sensitive. Check passage number, split rhythm and medium in cell supplier instructions for your cell type. Choose appropriate transfection method (e.g., Nucleofection™ for difficult-to-transfect cell lines or HiFect™ Transfection Reagent for standard cell lines).
Transfected cells do not grow in 96-well plates, even without G418	Re-titrate plating densitiy for optimal cell growth. Don't go below minimal cell number for single cell growth even without selection. Check passage number, split rhythm and medium in cell supplier instructions for your cell type. Use flat-bottomed plates for adherent cells and round-bottomed plates for suspension cells.
Number of resistant clones in 96-well plates is low after selection	Check transient transfection effi ciency of your transfection method (e.g., using maxGFP™ Reporter Protein as a control). Try higher DNA amounts. Re-evaluate G418 amount for optimal cell growth in single cell cultures. Try lower G418 concentration. Re-check the optimal plating density in 96-well plates. If correct, increase cell numbers per well. Control passage number of cells and confluency of cells before transfection. Choose appropriate transfection method (e.g., Nucleofection™ for difficult-to-transfect cell lines or HiFect™ Transfection Reagent for standard cell lines).
After selection, too many resistant clones mixed with non-resistant cell clones in 96-well plates	Feed cells with fresh G418 selection medium at least 14 days after transfection. Use the same batch of G418 you used for initial G418 titration.
Clones are growing on negative control plate	Re-check the optimal plating density in 96-well plates. If correct, decrease cell numbers per well. Use a similar passage number (difference not more than 10) of cells for titration of G418 and for transfection and selection.
Clone does not grow out after selection	Wait 4 – 5 weeks before picking a resistant clone to obtain a sufficient number of cells for culture expansion. Use the same batch (and concentration) of G418 you used for initial G418 titration.
Clone is resistant but gene of interest does not show expression in several clones checked	Check expression of gene of interest in a transient expression assay, if possible. Try linearizing the plasmid before transfection, this prevents disruption of gene of interest during integration. Check the sequence of gene of interest for ATG and Stop-Codon.
Positive control provides high number of resistant clones, but gene of interest does not	Reverify correct insertion of gene of interest and the resistance marker into plasmid by sequencing or restriction digest. Check whether the expressed recombinant protein is toxic for the cells.

References

1. Grimm S. (2004). The art and design of genetic screens: mammalian culture cells. Nature Rev Gen, 5: 179-189.
2. Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulff raat,N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T. H., Le Deist, F., Fischer, A., Cavazzana-Calvo, M. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science, 302: 415-419.
3. Southern, P. J., Berg, P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genet, 1: 327-341.
4. Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnol, 22: 1393-1398.

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