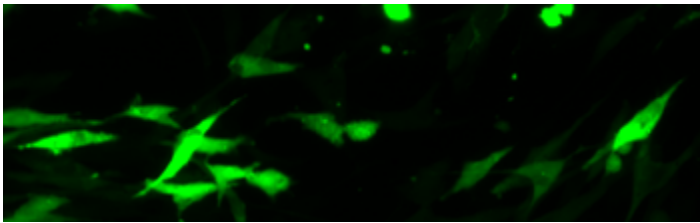


Important Vector Factors for Gene Expression

Technical Reference Guide



vectors expressing the same luciferase gene in different backbones and expression cassettes and obtained highly discriminative expression levels. In this guideline, we show the most important aspects to be considered when choosing an expression vector and their impact on your experimental results.

Introduction

Our Scientific Support Team is commonly asked: “Why don’t I get the same expression level of my gene if I use various vector backbones?” There are many components to a vector that can have an effect on the level of gene expression. Below you will find the 10 most important factors to consider when looking at vectors. The selection of an appropriate expression vector is crucial for efficient gene expression. Just take a look at Figure 1. We tried 10 different

1. Promoter Strength

Is your promoter appropriate for the cell type that you are working with? Table 1 describes the promoter strengths as a relative percent of the strength of the CMV promoter for various cells for which Lonza has Optimized Protocols. The CMV promoter activity is set to 100% based on their CAT assay values from the referenced publications. Although CMV is a strong promoter in many mammalian cells, another promoter may give stronger expression in your cells (e.g., promoter SV40 in BHK-21 cells).

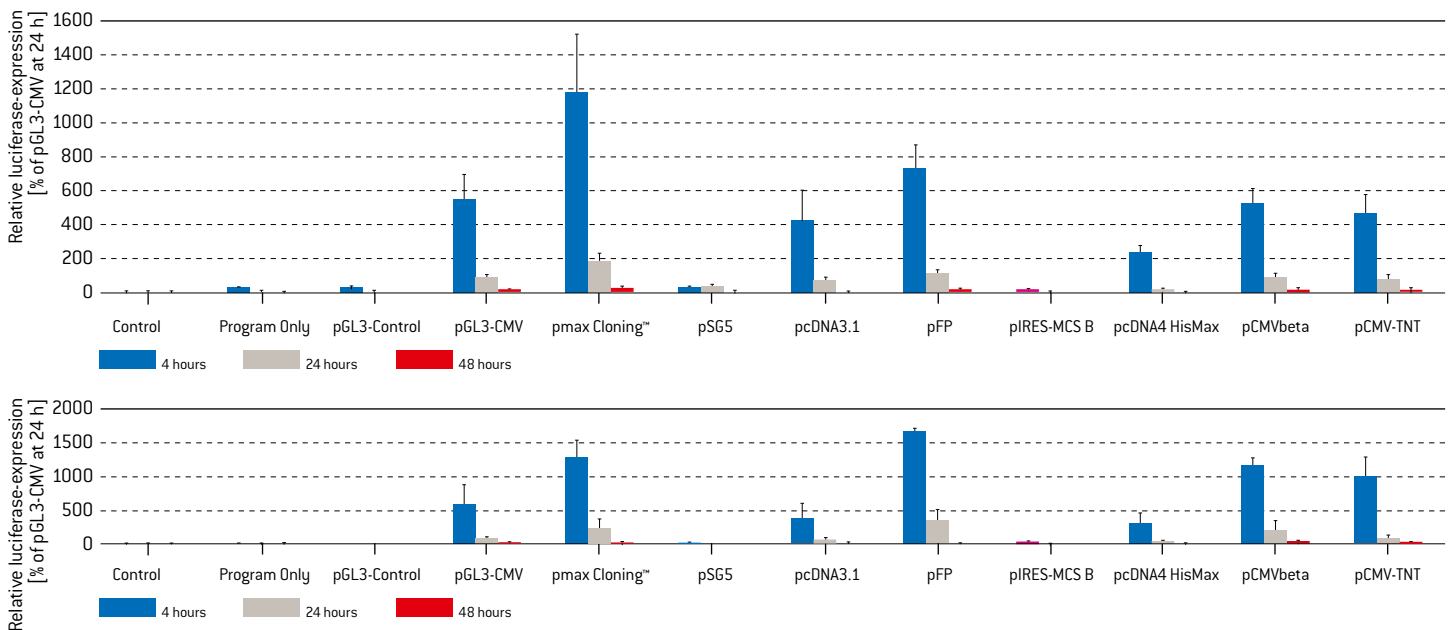


Figure 1: Luciferase expression levels depend on vector backbones. We looked at luciferase expression at 4, 24 and 48 hours in THP-1 and HUVEC cells. The amount of DNA was held at equimolar amounts based on plasmid size. For THP-1, the DNA amount ranged from 0.3 – 0.5 µg per reaction. For HUVECs, we used 2.5 – 4.4 µg of plasmid.

Cell Line	Source of Cells	SV40	EF1a	RSV	CMV	References
293	Human embryonic kidney	5		74	100	4, 6
BHK-21	Hamster kidney	200		200	100	4
C6	Rat glioma	44			100	5
CHO-K1	Chinese hamster ovary	16		11	100	2, 4, 5
Cos-7	African green monkey kidney	7	15	7	100	1, 2, 4, 5, 8
HeLa	Human cervical carcinoma	43	73	29	100	2, 3, 4, 5, 6, 8
N2A (Neuro-2A)	Murine neuroblastoma	50			100	5
NIH-3T3	Murine fibroblast	67	143	107	100	2, 3, 7, 8

Table 1: Promoter strengths in different cell types.

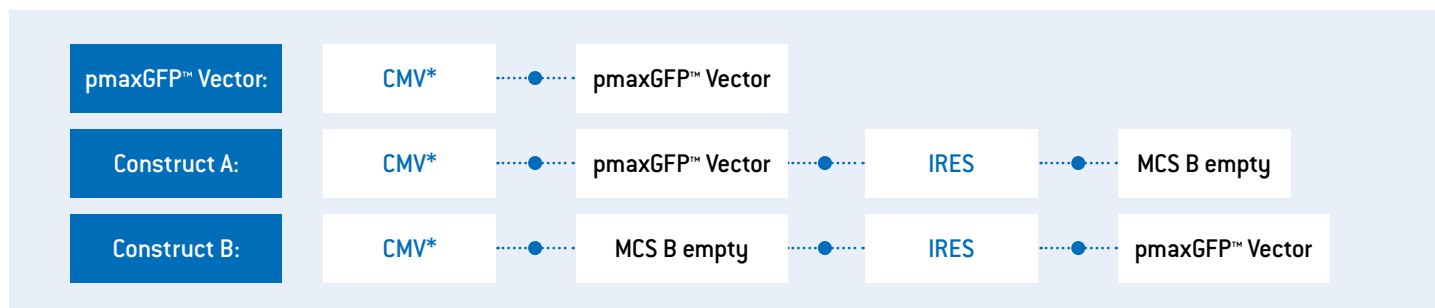
2. Introns

Many researchers consider constitutively spliced introns to be required for optimal gene expression; however, this point is not always agreed upon. The intron position and strength can affect transcription, mRNA export and polyadenylation. Thus, depending on its position, an intron can even lead to decreased gene expression.

3. IRES

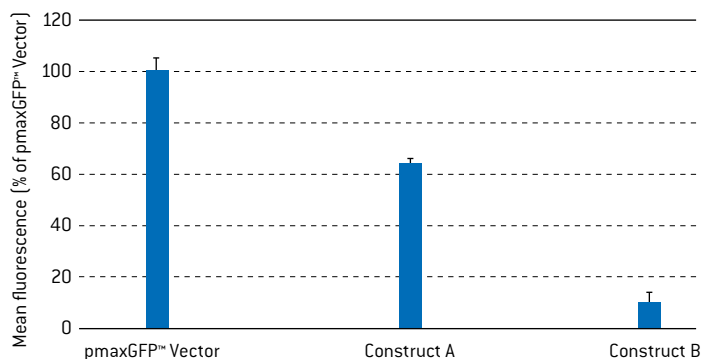
With IRES plasmids, the promoter drives the expression of two genes, the cloned gene of interest (usually the upstream gene), and a reporter gene, often encoding a GFP protein (the downstream gene). The mRNA expressed from an IRES plasmid is a bicistronic message, meaning that both genes are present on the same mRNA molecule. Equal amounts of the messages

2A



2B

Expression in HL-60



Expression in HUVE

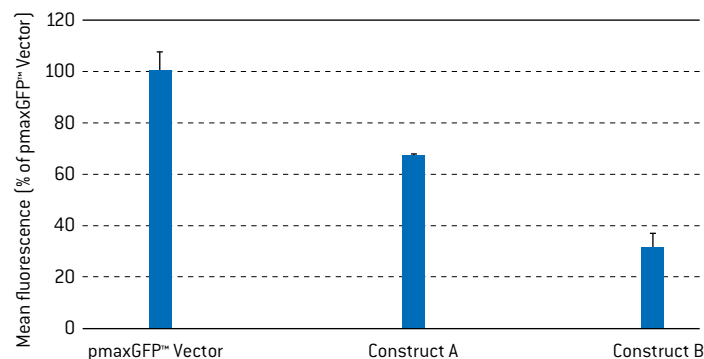


Figure 2: Reporter gene expression is dependent on the position in an IRES expression vector. HL-60 and HUVEC cells were transfected by Nucleofection™ with either pmaxGFP™ Vector or pIRES variants containing maxGFP™ Reporter Protein cloned in either the Multiple Cloning Site (MCS) upstream (construct A) or downstream of the IRES sequence (construct B; Figure 2A). Figure 2B shows reduced GFP expression using IRES plasmids especially if GFP is located downstream of the IRES sequence.

which encode each gene are present in the mRNA population. However, the translation initiation efficiency of the two genes differs significantly. Ribosome binding to the initiation region of the upstream gene is very efficient, while the IRES allows ribosome binding and translation initiation for the downstream gene often at a significantly lower level. Since the downstream gene is usually GFP, the expression level of GFP will be lower than it would be normally seen when compared to plasmids without an IRES-sequence. Equal amounts of the protein of interest and GFP can be obtained using a fusion of both proteins, constructs containing two expression cassettes, or co-transfection.

We looked at pIRES expression vectors (Clontech) with a reporter (GFP) cloned in either the Multiple Cloning Site (MCS) upstream of the IRES (construct A) or downstream of the IRES (construct B; Figure 2A). Figure 2B demonstrates that GFP expression is drastically reduced if GFP is located downstream of the IRES. This study is shown for the GFP reporter gene, but was also done using luciferase with similar results.

Does your plasmid contain an IRES sequence? If so, where is it located? Keep in mind that the stability of the bicistronic mRNA can be influenced by either of the inserted genes. The levels of expressed protein for the first and second genes will not be identical, and this can create problems with analysis and interpretation. As a result, the true efficiency of the plasmid can be underestimated due to the lower expression level of your reporter. Be sure to use a very sensitive detection method for the reporter gene downstream of the IRES.

4. LTR (Viral Long Terminal Repeats)

Some expression plasmids utilize promoters and enhancers obtained from the Long Terminal Repeats (LTRs) of retroviruses, and when these expression plasmids are transfected by Nucleofection™ into certain cells, the expression of the cloned genes is suppressed by the cell. Although the mechanism of suppression is not completely understood, it is likely

that plasmids containing promoters or enhancers derived from retroviral LTRs will not function well in primary cells and some cell lines. The only effective alternative can be to reclone the gene of interest into a different expression plasmid that uses conventional promoters such as CMV (e.g., pmaxCloning™ Vector), EF1a or SV-40.

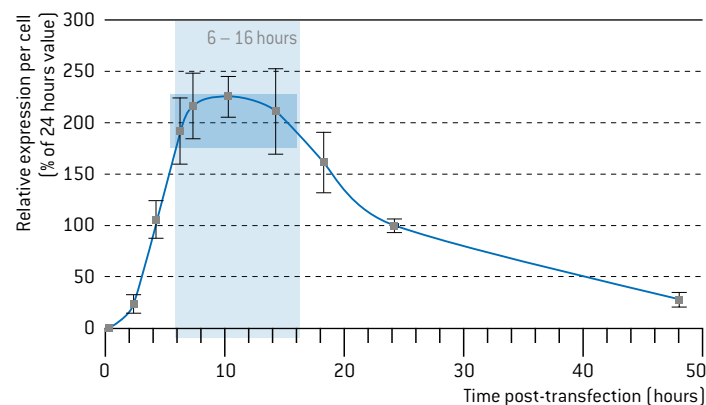
5. Size of Vector

We routinely use plasmids of 4–7 kb in our laboratories and Nucleofection™ of plasmids up to approximately 20 kb is achieved. Using plasmids larger than this will most likely result in lower transfection efficiency. The general rule is that the larger a plasmid, the more difficult it becomes to get it inside the cell. This is true for electroporation or lipid-mediated transfections. Some preliminary results using Nucleofection™ also indicate that BAC's can be transfected as well but also with low transfection efficiency.^{9,10}

6. Reporter

What kind of reporter are you using? It needs to be safe, reproducible, quantitative, and sensitive. Your reporter should not be expressed by the cell endogenously at high levels and should function well with your downstream assays. When you change reporters, or if you change transfection methods, the kinetics of that reporter's expression using the current transfection method need to be evaluated to make sure that you are analyzing at the optimal time point. Luciferase, for example, has very different expression kinetics, depending on whether the transfections are being done by Nucleofection™ (maximum of expression at 6–16 hours post-transfection) or lipids (maximum of expression at 24 hours post-transfection). We found that luciferase kinetics are related to the transfection method and not to the vector backbone or cell type tested. Since kinetics of reporter expression also depend on mRNA and protein stability, we then compared the kinetics

Kinetics of luciferase expression in HUVEC (protein/well, 24 hours value = 100%)



Kinetics of β -gal expression in HUVEC (protein/well, 24 hours value = 100%)

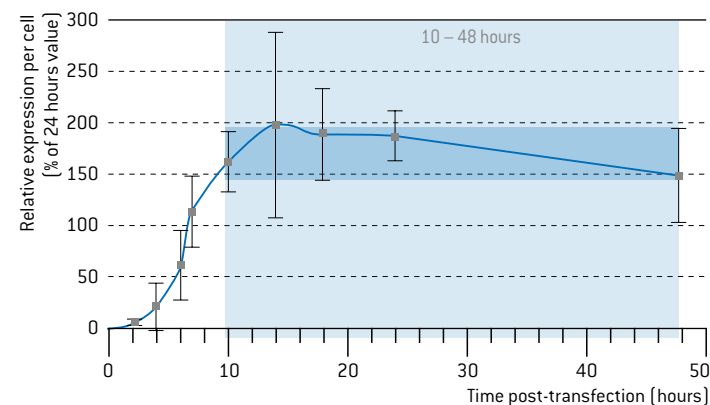


Figure 3: Expression kinetics are reporter gene dependent. HUVEC cells were transfected by Nucleofection™ with either a luciferase or a β -gal expression vector. While luciferase expression shows a maximum 6–16 hours post-transfection, β -gal expression is sustained for several days after transfection.

of luciferase expression to that of β -gal expression following Nucleofection™ (Figure 3). These data are from a co-transfection of HUVEC cells with a luciferase vector and a β -gal vector. Both reporters can be underrepresented at very high levels. However, the kinetics of expression for each reporter appear very differently. Luciferase has a very pronounced drop-off of expression after 16 hours. However, the β -gal expression reaches a maximum at 10 hours post-transfection and levels off. If a single time point for analysis is chosen, such as 24 hours, the maximum expression of luciferase will be missed and again can underrepresent the efficiency of that vector. As a consequence, we recommend to perform luciferase analysis 6 – 16 hours post Nucleofection™, whereas the optimal analysis time point for β -gal or GFP expression is after 10 – 48 hours post Nucleofection™.

7. Detection Methods

The detection method is predetermined by the reporter. Some reporters can be measured in multiple ways. GFP, for example, can be read by a fluorescent microscope, flow cytometer or a fluorescent plate reader. If only a qualitative picture is needed, the fluorescent microscope can provide a cost effective option. When looking for quantitative data, a flow cytometer or fluorescent plate reader should give more accurate data.

8. Fusion Vectors vs. Co-Transfections

Expression of a fusion protein depends on the localization of the protein, transcription and translation, as well as the folding and stability of the fusion protein. To improve expression, it may also be advisable to change the terminus to which the protein is fused. Co-transfections can be used instead of a fusion vector. One plasmid would contain the reporter gene (i.e., GFP) and the second plasmid would contain the gene of interest to be expressed. Depending on differences in promoter strength and vector size, the ratio of the 2 vectors needs to be optimized.

9. Hairpin Structures in Gene Product

A hairpin structure that forms in the RNA can affect translation of the gene. This should be considered, for example, when introducing mutations into the gene to be expressed.

10. Kozak Sequence

The Kozak sequence can slow down the rate of scanning by the ribosome and improve the chance of it recognizing the start of translation at the ATG start codon. If the Kozak sequence is contiguous with the ATG start codon, it can greatly increase the efficiency of translation and the overall expression of the gene of interest.

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