Cell Culture Considerations

While *Escherichia coli* systems are more commonly selected than eukaryotic systems due to their cost and speed, both have their pros and cons. Some factors should be considered when deciding which method to use

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Recombinant protein technologies are clearly essential for the manufacture of biological therapeutics. Additionally, they are equally important in the discovery of novel protein inhibitors where they are used to validate targets, pan, and screen libraries in the optimisation of binding affinities, crystallographic studies, and the selection of final candidates for properties such as specificity. Although the underlying technologies between the generation of proteins as drugs and as reagents are similar, the practice is different. Generating proteins as reagents usually only requires, at maximum, a few tens of milligrams and sometimes only micrograms, but these reagents are quickly required in a matter of weeks. Furthermore, multiple proteins are frequently needed to support a single project. These can be variants of the target itself as well as a number of homologous proteins or protein complexes.

The first choice to express any protein, if it can be achieved, would always be a microbial host, in particular *Escherichia coli*, as the cells are easy, fast, and cheap to culture. However, many targets for biological drugs will be post-translationally modified, and *E coli* is very limited in this respect. Additionally, many proteins will not express in a soluble form in *E coli*. As a consequence, higher eukaryotic hosts are frequently used, the two most common being baculovirus infected insect cells and various different mammalian cell hosts – most commonly Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells. Both insect and mammalian systems are used most simply when the cells are maintained in suspension culture in protein-free medium. Both insect and mammalian cells are able to generate a full range of post-translational modification, with the one exception being that N-linked glycosylation in insect cells is less complicated, thereby restricted to simpler, mostly mannose structures unless the cell has been modified with additional enzymes to create mammalian-type structures in a system known as SweetBac. Culturing cells in suspension enables a much greater cell density to be achieved and, therefore, higher expression per volume of cell culture medium. The use of protein-free medium greatly facilitates purification if the protein is secreted.

The focus of this article is on the provision of proteins as reagents using eukaryotic expression systems, in particular, transient expression in mammalian cell lines and baculovirus infection of insect cells, and has recently been reviewed (1).

Transient Expression in Mammalian Cells

Transient expression of recombinant proteins in mammalian cells has traditionally suffered from very low yields in comparison to stable cell lines. Over the past 10-15 years, a number of developments have been described with transient systems that enable the generation of tens to hundreds of milligram quantities of a wide range of proteins very rapidly (2). The process is simple, involving the generation of plasmid, transfection in log phase, optional feeds from 24 hours onwards, and then harvest from 48 hours to 14 days, depending on the particular protein, cell line, and conditions used. The most important factors contributing to these developments have been:

- The development of cell lines that grow to high cell density in suspension culture
- Combinations of recombinant cell lines and vectors that dramatically increase expression
- The development of transfection-compatible, chemically defined, serum-free media and feed that allows transfection at higher cell density while prolonging cell culture viability

Additionally, a range of other culture parameters and additives have been analysed for their ability to boost expression of recombinant proteins.

Recombinant Cell Lines, Vectors, and Media

Two cell lines dominate in terms of their useage in this technology: HEK293 and CHO. Both have been adapted by multiple laboratories to grow in suspension, which greatly facilitates the routine culture of the cells as well as being able to generate high cell densities to maximise expression on a per volume basis. Productivity from the two lines has been dramatically increased by the use of viral elements. Thus, the cells are stably transfected with Epstein-Barr virus (EBV) nuclear antigen (EBNA-1) or SV40-T antigen and, when used in conjunction with cis-acting elements in a plasmid (EBV oriP or SV40 ori), produce a large increase in expression. This is thought to enable episomal retention and replication of the plasmid, as well as increased transcription and translation over a prolonged period of time. However, although many high-yielding systems use these combinations of viral elements, not all do so. Two studies describe a system that relies on a combination of cell line and transfection reagent to give efficient transfection combined with media and supplements that generate high cell densities (3).

Both CHO and HEK lines have been adapted to grow in chemically defined, serum-free medium that greatly facilitates purification of proteins when secreted in contrast to cells that have been grown in serum-containing medium. A few examples of harvested culture supernatant are shown in Figure 1 for a range of different proteins expressed in HEK cells, demonstrating the very low background of proteins compared to the recombinant protein even before any purification.

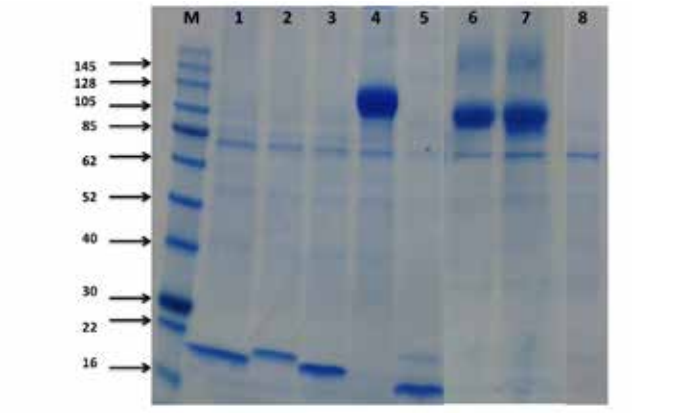

Figure 1: Expression of a range of different His- and Fc-tagged proteins in HEK293-6E cells. The samples are all culture supernatants harvested five to six days after transfection and before any purification. M is molecular weight markers; lanes 1-3 are three variants of 6His-tagged TIMP2 expressing at ~20mg/L; lanes 4, 6, and 7 are a single imuunoglobulin domain containing receptor fused at the C-terminus with Fc; the expression level was ~100mg/L; lane 5 is the same immunoglobulin domain containing protein without a tag expressed at ~20mg/L; and lane 8 is a 6His-tagged secreted protease with expression at 50-100µg/L and was undetectable prior to Ni purification. Expression levels were determined after purification using one or two chromatographic steps

A number of other parameters that can influence yield can be considered. A variety of transfection reagents have been reported, although the most common is polyethyleneimine (PEI) due to its low cost and ease of use when purchased as a hydrochloride salt, eg, PEI MAX. It is often necessary to optimise a particular system, especially with respect to cell density at transfection, the ratio of transfection agent to DNA, and the concentration of DNA. This is most simply performed using a statistical design of experiment approach to evaluate a number of parameters at the same time.

Difficult-to-Express Proteins

Many reports on different expression systems in the literature use proteins such as antibodies, Fc fusions, or cytokines that are often straightforward to express. However, the range of proteins that many labs need to express is far more diverse than this, and a common occurrence is that expression levels can be very low (<100µg/L) or not detectable at all. Under these circumstances, validating the cells and methods at the same time using a protein known to express well is essential. Resolving this type of problem is probably one of the most time-consuming and frustrating tasks in a protein expression and purification lab with no simple generically applicable solution. Manipulating the transfection and cell culture conditions, the methods for purification, the design of the expression plasmid, and the sequence of the protein itself is frequently necessary. Two recent reports have sought to understand these problems in much more detail with a family of related proteins whose expression is quite different (4-5). The authors identified that, in this case, the problem was a post-translational block. They used a computational approach, allied with making a series of chimeras, to identify that, in the poorly expressed proteins, certain unfavourable regions and amino acids were on the surface of the proteins that caused the blockage. This could be overcome by mutating out these regions.

Baculovirus-Infected Insect Cells

The insect cell baculovirus expression vector system (BEVS) was first developed in 1983 by Smith, Summers, and Fraser in their landmark publication on the production of IFN-β protein (6). Initially, the potential of the system was unknown, but it has become a 'workhorse' system to generate protein reagents. The overall process is shown in Figure 2 and is a more complicated process than the transient mammalian system described earlier. The process starts with a gene of interest cloned into a transfer vector that includes one of the strong, very late promoters, frequently polyhedrin. This is then recombined into a baculoviral genome that is used to generate a recombinant infectious virus that can be amplified to infect large scale cultures to generate the protein of interest. Over the years, many different systems have evolved and diverged, and, now, a multitude of different approaches can be used that are often not compatible with each other.

The most commonly used insect cell lines in research are Sf9 and Sf21cells, both derived from the *Spodoptera frugiperda* fall army worm pupal ovary and Hi5 derived from the *Trichoplusia ni* cabbage looper embryo. Hundreds of different transfer vectors are compatible with a wide range of different methods to generate recombinant baculovirus, although they essentially use just two methods: one that uses homologous recombination using the insect cell's own machinery, and one that uses bacterial-based transposition. A recent study has been published comparing the expression of four proteins in the most commonly used systems in different cell lines and across 15 different labs (7). One notable observation in the study is the very large diversity of techniques used between the various labs in either homologous recombination versus *E coli* transposition-based bacmid technology, cell lines and media, cell and virus concentrations at infection, and time before harvest. Differences that are more difficult to quantify are inevitable. Due to the many different methodologies used, definitively concluding that any particular system, cell line, or method is better than

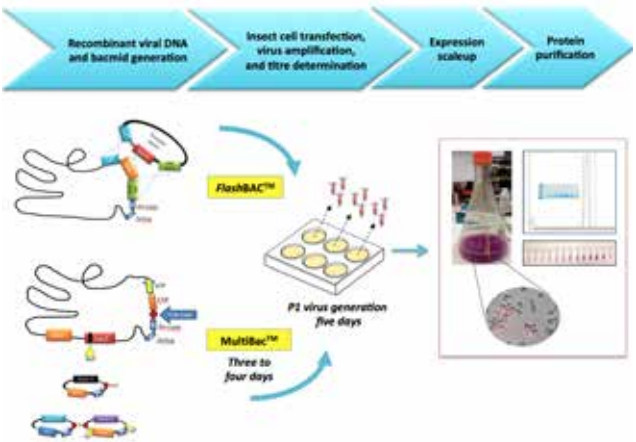

Figure 2. Comparative workplan for recombinant baculovirus generation using the FlashBACTM and MultiBacTM methods. The molecular differences between the FlashBACTM recombination and MultiBacTM transposition based methods are shown. The approximate timelines for each method from cloning to protein production are depicted. The example shows the production of a His-tagged mCherry red fluorescent control protein

any other is not possible, as the better-performing labs use a range of different approaches. However, the authors did tentatively suggest that the transposition method, in particular EMBacY, may give better and more robust expression.

A vast range of improvements are now available compared to the basic systems. Thus, the baculoviral genome has been modified to knock out non-essential genes. Vectors and systems (MultiBac) are available that enable the co-expression of many proteins either to form a multi-protein complex or to enable co-expression of chaperones to improve stability. As already mentioned, SweetBac enables the production of mammalian-type glycosylation. Additionally, improvements in the media can lead to prolonged cultures and increased expression. Eukaryotic expression systems are an essential tool to generate protein reagents to support drug discovery activities by enabling the rapid supply of many different proteins with correct post-translational modifications. Transient expression in mammalian cells and baculoviral infection of insect cells are the two most commonly used methods and both are continuing to evolve to enable the generation of an even broader range of protein targets.

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About the author

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