

## Protein Expression Application Note No. 1

# Workflow for Protein Production at the 1–3 Liter Scale in Cultured Animal Cells

Linda Jacobsen<sup>1</sup>, Susan Calvin<sup>2</sup>, Jay Wang<sup>3</sup>, and Brigitte Hloch<sup>3</sup>.

1. Berit Biotech LLC, Groveland FL

2. Ball State University, Muncie IN

3. Roche Diagnostics, Indianapolis IN and Penzberg, Germany

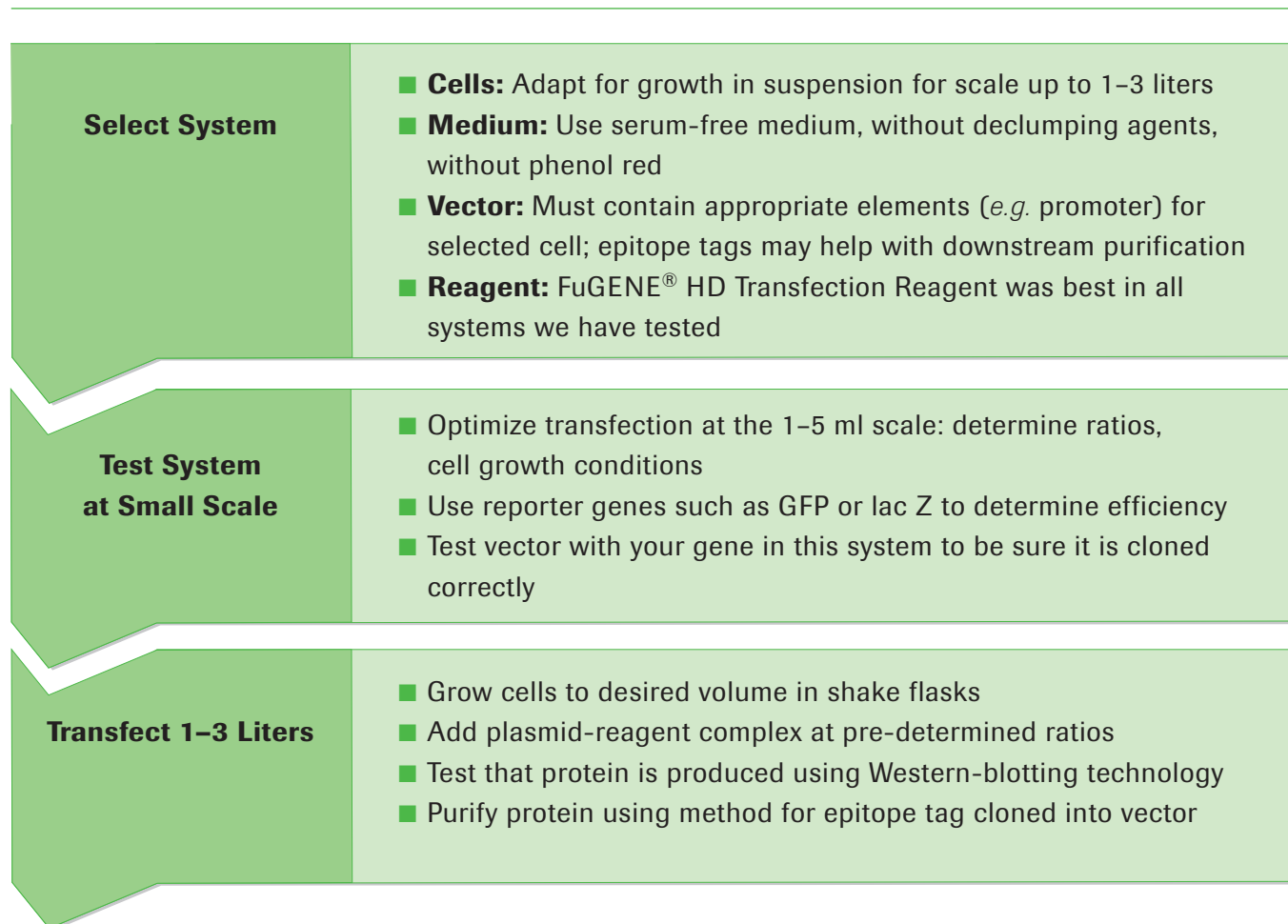


## 1 Introduction

Proteins can be produced from either transiently transfected cells or from stable cell lines that express the protein of interest. Transient transfection of cultured animal cells offers rapid production of heterologous protein at a medium level in a week, as compared to the weeks or months required to express a similar level of protein in either a Baculovirus system or a prepared stable cell line. We believe that transient transfection is the method of choice for applications where 100 mg or less of cloned protein is needed, and when development time is limited. Also, at the 100 milligram to gram level, transient transfection may still be the method of choice in a highly expressing system.

We tested three different systems for production of milligram quantities of protein: two mammalian and one insect. In this article we will describe the details of how we verified that we could transfect insect cells, and then produce a sufficient amount of protein for further experiments. Similar experiments were also done using mammalian cells; we will detail information we learned during our experiments that may be beneficial to other researchers. The major purpose of this application note is to provide a general description of the steps that could be taken to scale up for production of milligram quantities in transiently transfected cells.

When undertaking our experiments to express proteins in insect cells we had to first learn the best conditions to express protein using our selected cells, vectors, medium, and transfection reagent before we could scale up to the 1–3 liters. We will explain the steps we took specifically for insect cells, in detail as shown in Figure 1, and provide some examples of what we found using similar methods in mammalian cell lines.



**Figure 1: Workflow to produce protein at the 1–3 liter scale**

Note: These same procedures can be used up to the 10–100 liter scale by using appropriate bioreactors or disposable bags.

## 2 Decide on Cell System

The first thing that needs to be decided is the cell system, *i.e.* mammalian or insect, then which cell line to use. For medium to large scale protein expression, cells are usually grown in suspension. Most mammalian cells used in protein expression grow as monolayers and need to be adapted for growth in serum-free medium. Several suppliers offer such medium, some already proven to contain the correct components to permit transfection and increase production levels.

In this note, we describe the steps we followed for transient transfection of insect cells, a cell type that was not as familiar to us as CHO and HEK cell lines. Some results from our experiments on mammalian cells are also shown in this note.

We wanted to know if insect cells could be transiently transfected and yield sufficient levels of protein, thus eliminating the need for using Baculovirus systems. This would be for proteins needed at some nominal level; for very large scale production of proteins (*e.g.* when over 10 grams are needed), Baculovirus production has advantages. For the workflow described here, we show the processes we used to evaluate insect cells for transient transfection. We read publications and cell line information from suppliers that sold cells. We concluded that High Five insect cells produced the highest level of heterologous protein in transient transfections, while not necessarily the best for protein production using Baculovirus system; thus the cells, vectors, and medium were ordered from Invitrogen.

### Mammalian Cells

Both HEK and CHO cells in suspension are frequently used for protein production at the multi-liter scale. We have previously done numerous experiments using HEK variants and CHO both as monolayers and as suspension cells. We have used both, commercially available media and custom made media, designed to increase protein expression in cells grown in suspension. For HEK cells, we worked closely with a medium supplier to prepare a medium that did not contain declumping agents like dextran sulfate or heparin, as they can interfere with transfection. We also found that the hydrolysate source and amount was critical for high levels of protein production.

### HEK Cell Variants

HEK cells are commonly used for protein expression. We have used both the HEK EBNA cell line (293c18 ATCC® CRL-10852™) and the HEK cell line (293 ATCC® CRL-1573™) from which the EBNA variant was derived. We found, as have other researchers (1, 2, 3), that the HEK EBNA cells resulted in two- to three-fold higher levels of protein expression than the parental HEK-293 cells, depending upon vector. The HEK EBNA cell line expresses a nuclear antigen Epstein-Barr Virus protein (EBNA-1), which supports the episomal replication of plasmids containing the oriP to help maintain the multiple copies of the plasmid as the cells divide (3).

Vectors such as pCEP4 and pTT were successful yielding similar percentages of the HEK cells transfected, while total protein yield was found to be more dependent upon the selected vector (3). Vectors already containing an epitope tag and cloning sites such as the pM1 plasmid series and phCMV may be advantageous. We found high levels of expression in both HEK and HEK EBNA cells using the pM1 plasmid series which did not contain the oriP.

## 3 Prepare Epitope Tagged Vector

For both ease of tracking and protein purification, epitope tagging may be a useful technology (see paragraph “Epitope Tagging”). There are two ways to prepare an epitope tagged vector, as shown in Figure 2:

1. Add the epitope tag to the desired gene sequence, then insert into selected vector.
2. Clone the gene sequence into a vector already containing all of the appropriate elements, including the desired epitope tag.

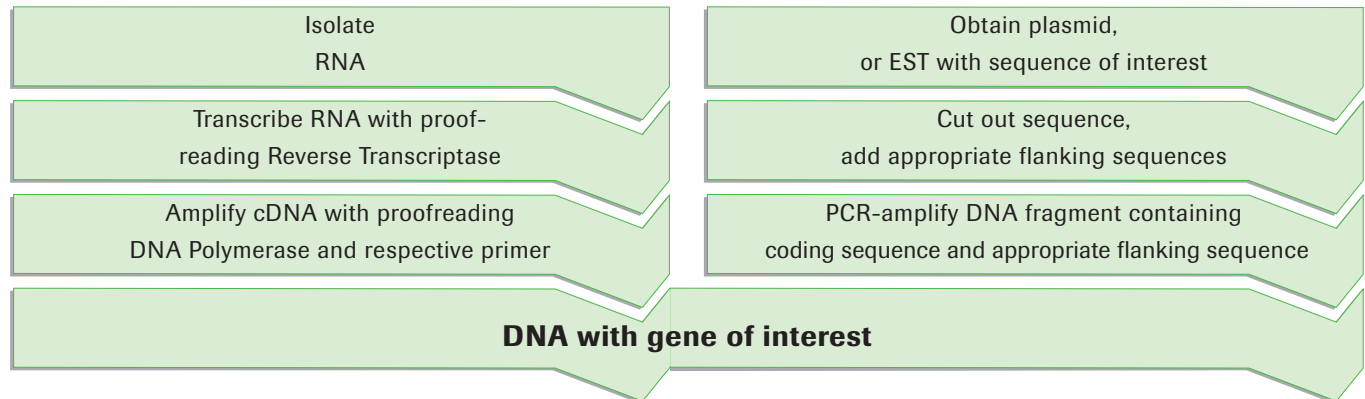
Our model system was to express reporter genes and known protein kinases. We started with an insect vector that already contained an epitope tag. Our starting insect vectors were pIB/His for internal proteins and pMIB/V5-His for secreted proteins. Since the vectors already had the His epitope tag, we decided to use the His tag rather than switch to a different epitope tag such as the HA tag (see paragraph “Epitope Tagging”).

We used the path on the right side of Figure 2 for cloning our gene sequences into the vectors. Briefly, the reporter genes and two protein kinases (hAkt 1 and MKK6) were amplified using Roche PCR Master. The PCR-amplified DNA fragments containing the coding sequence and appropriate flanking sequence were inserted into pre-digested cloning sites of either the pIB/His or pMIB/V5-His vector, digested with the respective restriction enzymes. To speed up ligation commercially available kits like the Roche Rapid DNA Dephos & Ligation Kit could be used.

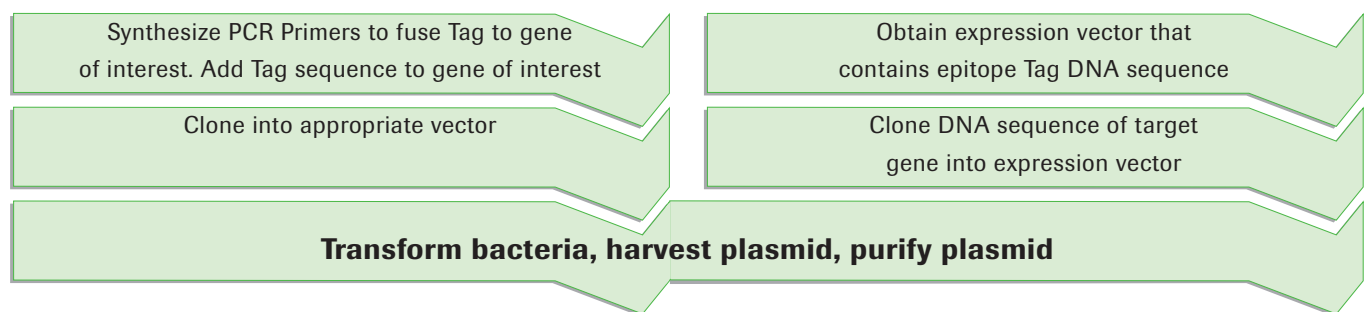
The vector was transformed into *E. coli*. Transformants were selected, and the vector was isolated and purified for use in transfection of the insect cells.

**Figure 2. Two ways to add the epitope tag to the gene of interest**

A. Prepare DNA of gene of interest either from mRNA, EST, or another vector containing the gene



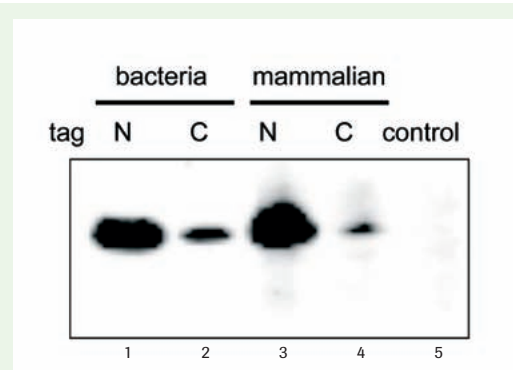
B. Clone gene sequence into selected vector



### Epitope Tagging

Epitope tagging eliminates the laborious, cost intensive and time-consuming task of producing a new antibody every time a different protein is studied. However, the “best” epitope tag for a particular experimental system is the one that does not interfere with the function or cellular processing of the tagged protein, yet provides a strong signal on western blots or immunofluorescence microscopy. No one can reliably predict how a particular tag will behave in a particular protein. The location of the tag (N- or C-terminal or within the reading frame) may affect both function and expression levels.

Note: placing the tag at the C-terminus demonstrates the complete translation of the tagged protein; however, total expression may be dependent upon tag location, as shown in Figure 3. The histidine tag was not our first choice in the insect cell experiments because mammalian cells often contain proteins with runs/repeats of histidine, or endogenous myc protein, and the baseline level of either in insect cells is unknown. We usually prefer HA, VSV-G, or Protein C when we add the tag ourselves.



**Figure 3: Effect of tag location (N- or C-terminal on amount of protein expressed)**

The VSV-G epitope tag was cloned onto either the N- or the C-terminus of a protein and expressed either in bacteria or mammalian cells. The proteins were detected by western blot using Anti-VSV-G. Proteins expressed in bacteria are in lanes 1 and 2 and proteins expressed in mammalian cells are in lanes 3 and 4. The proteins in lanes 1 and 3 were epitope tagged on the N-terminus, while lanes 2 and 4 were epitope tagged on the C-terminus. Lane 5 is an untransfected control.

## 4 Transfect at Low Scale with a Model Protein

Low-scale transfection was performed with a model protein to select the correct ratio and verify the system and the cloned gene. Since insect cells, like the High Five cells, were loosely attached and grown at room temperature, the standard protocol in the package insert for FuGENE® HD Transfection Reagent was followed with minor modifications (shown in italics).

1. Plated cells at 400,000 cells per well (200,000 cells per ml in 2 ml *serum-free medium*)
2. Incubated overnight, *then changed medium to stimulate cell growth prior to transfection. Depth of medium was found to be important in non-aerated cultures. At liter scale, shaker flasks provided sufficient aeration.*
3. Transfection complex formed in *sterile water* at ratios ( $\mu\text{l}$  FuGENE® HD Reagent:  $\mu\text{g}$  DNA) between 8:1 and 3:1.

4. Added complexes dropwise to cells, and swirled to mix. Returned the cells to *room temperature* incubator.

Once a system has been established for a particular vector backbone, that same backbone should be used for the protein of interest. This protocol was followed first with the reporter gene constructs to determine transfection efficiency (%), then with each of the protein kinase gene constructs. We expressed GFP in the pMIB/V5-His vector, and lacZ in pIB/His. The protein kinase genes were then cloned into the same constructs we used for reporter gene testing and were tested at low scale to verify the correct cloning prior to scale up. Since the vectors had a His tag, we could easily demonstrate protein expression.

### Protocol for HEK EBNA and CHO Cells in Suspension; Importance of Vector

We followed the protocol for 6-well trays in the pack insert protocol, but changed to a 12-well plate using 2 ml of medium. Green Fluorescent Protein (gWIZ GFP vector) vector was selected as a reporter gene to measure both % transfected and protein expressed.

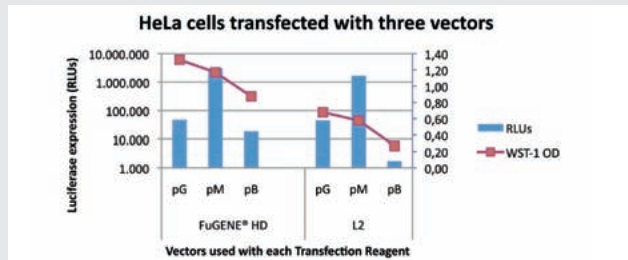
- Placed cells in 12-well plate (non-tissue culture treated) *in serum-free medium on platform shaker*. Incubated overnight or transfected immediately upon plating. (*With good medium, cells plated at  $0.5$  to  $1 \times 10^6$  cells/ml.*)
- Prepared transfection complex at several ratios and added to cells.
- Returned plates with cells to incubator *placing them on shaking platform to maintain cells in suspension*.
- Cells were examined microscopically for signs of cytotoxicity.
- Measured expression to determine best ratio for scale up.

#### Selection of vector is critical.

When cells do not grow as well following transfection, researchers sometimes think that the heterologous protein is having deleterious effects on the cells, or that the transfection reagent is to blame.

This is not necessarily the case. Some vectors contain toxic sequences that are unnoticed unless they are actually tested for. We tested three luciferase expression vectors for detection of any interference with cell proliferation that might be attributable to the vector rather than the transfection reagent used. These three luciferase expres-

sion vectors were transfected into HeLa cells using two different reagents. Protein expression was measured by relative luminescence units (RLUs) (Luciferase Reporter Gene Assay, high sensitivity), and cell proliferation was measured by the WST-1 assay (Cell Proliferation Reagent WST-1). This experiment clearly shows that luciferase gene expression did not correlate with cell growth. Cell proliferation did not correlate with protein expression or reagent used, but with elements within the vector itself (Figure 4). Gene Expression Profiling has also been used to examine the effects of the vector backbone (4, 5, 6). This further emphasizes the importance of vector selection experimental systems.



**Figure 4: Protein expression does not correlate with cell growth when different vectors are used**

Cell proliferation (WST-1) and protein expression (luciferase) were measured following transfection of the luciferase gene using two different reagents (FuGENE® HD Transfection Reagent and L2). Gene expression had no correlation with proliferation using any of the three luciferase vectors with different backbones (pG, pM, and pB).

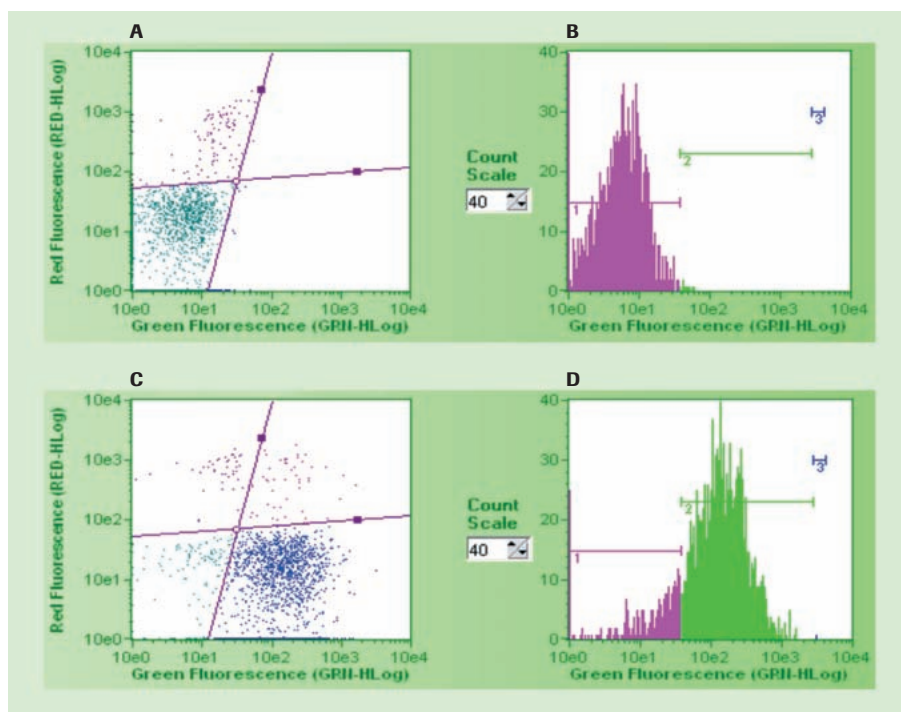
## 5 Detect Transfection using Histochemical Staining, Flow Cytometry, and Western Blot

### Transfection Efficiency with Reporter Genes

We needed to demonstrate that we were able to deliver genes to High Five cells using FuGENE® HD Transfection Reagent such that a high proportion of the cells was transfected and that the cells remained viable. We determined that nearly 100% of the cells expressed beta-galactosidase when transfected with the pIB/His vector containing the lacZ gene using the  $\beta$ -Gal Staining Set (data not shown).

GFP was cloned into the pMIB/V5-His plasmid so that we could measure both secreted and protein retained in the cells. We used flow cytometry (Guava® PCA-96) to assess cell viability and determine the percentage of cells transfected. Most of the cells were viable by propidium iodide exclusion following transfection and over 80% of the cells were GFP positive, as shown in Figure 5.

Transfection efficiency with GFP was not as high as the percentage detected with the beta-galactosidase staining results, which may be explained by two related reasons. First, histochemical staining is more sensitive; it is based on enzymatic detection, and cells with low amounts of protein are more readily detected. Second, more GFP protein within the cell is required to detect a cell as positive using flow cytometry. Since the GFP was cloned into a vector that allowed the GFP to be secreted into the medium, GFP did not accumulate within the cells further, making detection of all transfected cells difficult (as shown in the histogram of the GFP-expressing cells in Figure 5). We had originally hoped that we could detect the amount of GFP secreted into the medium using a spectrofluorimeter, but found that inherent fluorescence in the media we used made that impossible.

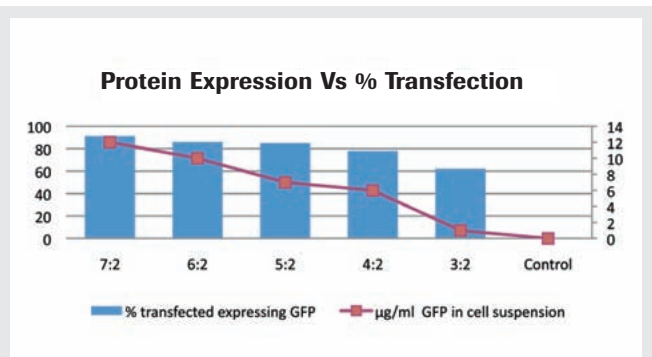


**Figure 5: Viability and expression in High Five cells transfected with FuGENE® HD Transfection Reagent**

Control cells are in the upper panels (A, B), and cells transfected with GFP are in the lower panels (C, D). Viable cells (exclude propidium iodide) are in the lower two quadrants of panels A and C (turquoise and blue dots), and transfected cells (express GFP) are in the right two quadrants of panels A and C (blue and pink dots; blue dots represent live transfected cells). The transfection efficiency was calculated from the histograms in panels B and D.

## Results using HEK EBNA Cells

Transfection with the reporter gene GFP is frequently used as a method to detect the percentage of cells transfected. It is sometimes assumed that cultures containing similar percentages of cells transfected will result in roughly similar amounts of protein expressed. Using the 12-well plate protocol described above, we measured the transfection efficiency by the percentage of cells expressing GFP, and the total amount of GFP produced. As shown in Figure 6, although the percentage of cells transfected at ratios between 7:2 and 4:2 remained relatively constant, the protein yields from the cultures decreased by 50%. This demonstrates the importance of not depending upon a single method to determine optimal ratios for scale up to produce large amounts of protein in a transient transfection system.

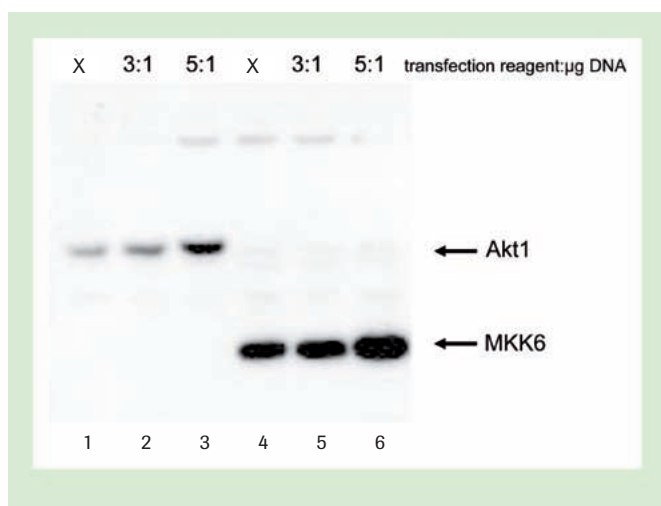


**Figure 6: Comparisons of percentage transfected cells and protein expression**

The percentage of HEK EBNA cells expressing GFP following transfection with FuGENE® HD Transfection Reagent was compared to the amount of protein produced by the same culture.

## Specific Expression of Protein Kinases

Once we demonstrated the high efficiency of transfection using reporter genes, the cells were transfected with the vectors containing the protein kinases. The expression of the kinases in the High Five cells was confirmed using western blot analysis.



Transfected cell samples were transferred to Roche PVDF Western Blotting Membranes. Since we were using a His tagged protein, we probed the blot with a peroxidase-labeled antibody that detects His tagged proteins using the Roche Lumi-Light<sup>PLUS</sup> Western Blotting Substrate.

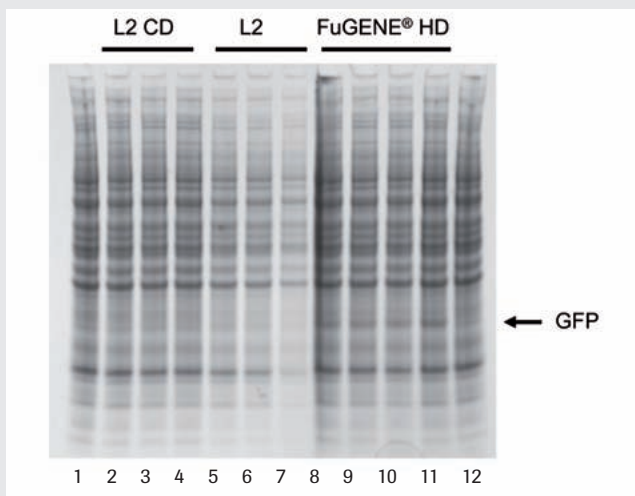
**Figure 7: Kinase expression in High Five cells**

Samples from cells transfected with Akt1 were loaded onto lanes 1, 2, and 3, and samples from cells transfected with MKK6 were loaded onto lanes 4, 5, and 6. Reagent X was used for transfection in samples in lanes 1 and 4 following manufacture's instructions. In lanes 2 and 5 FuGENE HD® Transfection Reagent was used at a ratio of 3 µl Reagent:1 µg DNA while lanes 3 and 6 were transfected with a ratio of 5 µl Reagent:1 µg DNA.

## Results using CHO Suspension Cells

Using the 12-well plate protocol described above, we transfected CHO-S cells with GFP using three different transfection reagents at several ratios. Four wells were transfected using FuGENE® HD Transfection Reagent. The other wells were used to test two other commercially available reagents: Reagent L2 (3 wells), and Reagent L2-CD (3 wells). Two wells were used for untransfected control cells.

Following incubation on a shaking platform, samples were taken to determine protein expression. Samples were

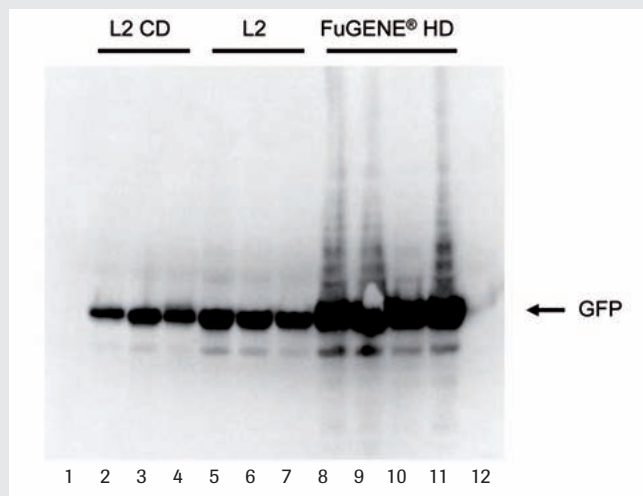


**Figure 8: Coomassie stained SDS-PAGE gel**

GFP bands are visible only in the 4 samples where FuGENE® HD Transfection Reagent was used (lanes 8–11). Lanes 1 and 12 contain control cell lysates. Lanes 2–7 contain the lysates of cultures transfected with other reagents. It is not possible to see the GFP in the samples from the other reagents.

prepared by centrifuging 1 mL of media with suspended cells. The cell pellet was resuspended in 400  $\mu$ L of reducing sample buffer. In the results shown in Figure 8, 10  $\mu$ L of sample was loaded on the gel.

The above samples were transferred to a PVDF Western Blotting Membrane. The western blot was probed with Anti-Green Fluorescent Protein as primary antibody and anti-mouse HRP secondary antibody using the Lumi-Light<sup>PLUS</sup> Western Blotting Kit (Mouse/Rabbit).



**Figure 9: Specific expression of GFP was confirmed using indirect western blot analysis**

GFP bands are only visible in all transfected samples, but is clearly higher in lanes 8–11 where FuGENE® HD Transfection Reagent was used.

## 6 Scale Up to 1–2 Liter Size (High Five cells)

Akt1 was selected as the more challenging protein for expression at 2 liter scale in suspension culture, because lower expression was detected at the 6-well plate scale as shown in Figure 7.

1. High Five cells were grown in two liters of serum-free medium in a shake flask.
2. The cells were transfected with the Akt1 expression plasmid at a 5:1 ratio ( $\mu$ l FuGENE® HD Transfection Reagent:  $\mu$ g DNA). This ratio yielded the best expression in the 6-well plate experiment.

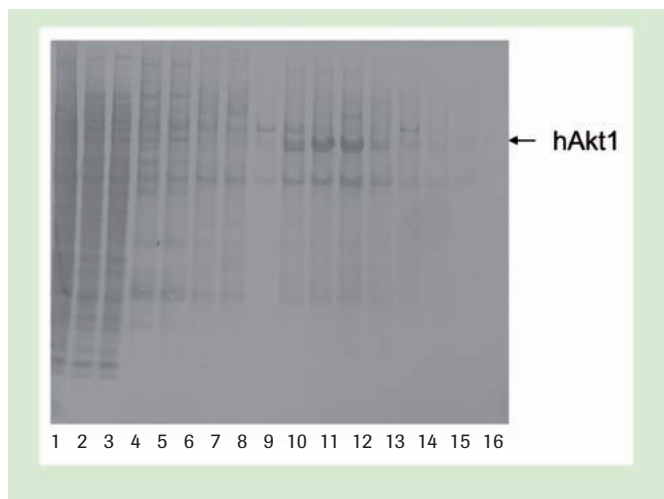
3. The cells were harvested 72 hours post transfection, lysed, and sonicated.

### Scale up using HEK and CHO Cells

HEK and CHO cells are often scaled up to 3 liters in 5 liter shake flasks, leaving room for aeration. We have also used disposable bags on gently rocking platforms (*i.e.*, WAVE bags) for transfection at the 10 liter scale.

## 7 Purify Protein

The soluble fraction of the sonicated cell sample was clarified by centrifugation. To prevent the target protein from degradation by proteases, cOmplete EDTA-free Protease Inhibitor Cocktail Tablets were added to the clarified supernatant. The His-tagged target protein was purified by Ni-NTA affinity chromatography by gravity flow following the recommendations of the manufacturer



of the affinity gel. Analysis was done using SDS-PAGE followed by Coomassie Blue staining, as shown in Figure 10, the His-tagged protein was purified from the imidazole eluates.

The eluates containing the His-tagged protein were pooled; 5 mg/L purified protein was obtained from the transient transfection culture. Thus a total of 10 mg protein was purified from the 2 liter culture, which is comparable to that reported for this protein in Baculovirus systems. This was a low-expressing protein in cell culture and in the Baculovirus system. Other proteins would have yielded much higher amounts in transient transfections.

**Figure 10: Purification of His-tagged hAkt and detection by Coomassie Blue staining**

Lanes: 1: total lysates; 2: soluble; 3: flow through; 4-7: wash 8-16: imidazole eluates containing hAkt protein

### Protein Purification and Protein Degradation

When proteins are tagged with HA or Protein C we use an Affinity Matrix for purification:

#### Anti-HA Affinity Matrix

- Uses HA peptide to gently purify biological active HA-tagged proteins from immunoaffinity supports by competitive elution with the HA peptide.

#### Anti-Protein C Affinity Matrix

- Uses an elution buffer with a calcium-chelating agent to gently recover the Protein-C tagged proteins.
- This purification method offers significant advantages over other epitope-tag antibodies. The binding of Anti-Protein C is dependent on the presence of calcium ions. The calcium-binding site is associated with the antibody rather than the epitope-tagged protein. This feature eliminates the possibility of calcium ions altering the structure or function of expressed proteins.

When cells are lysed, the intracellular proteases are also released. These proteases can immediately begin to degrade the protein just produced. We always use a protease inhibitor to prevent this. Our choice of which cOmplete product to use depends upon the cells used for production and the protein produced. For example we used one of the EDTA-free cOmplete products when purifying a metal-dependent protein to maintain protein stability.

For more information about cOmplete, please visit [www.roche-applied-science.com/proteaseinhibitor](http://www.roche-applied-science.com/proteaseinhibitor)

## 8 Conclusion

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Production of proteins using transient transfection at the liter scale is relatively easy using FuGENE® HD Transfection Reagent. Use of the appropriate cells and vector is critical for protein expression as with any transient transfection system. Equally important is selection of medium that will support cell growth at high density, or appropriate means of supplying nutrients and removal of cellular wastes. Also protection of the expressed protein is important as, uninhibited proteolytic activity can degrade the protein in just minutes.

Once the decision is made to produce protein using transient transfection, it is important to determine the best vector backbone, cells, and medium for this task. In this report we discussed what we considered when selecting medium, cells, and vector. When the proper combinations are identified, upscaling from the milliliter level to the multi-liter level is relatively simple. We also showed that different proteins can be produced using the same conditions when the desired gene is cloned into the

same vector. Once the appropriate amount of DNA and reagent is determined at the milliliter scale, all components (cell density, amounts of DNA and reagent) are directly scaled up. For example, if an optimal expression was found when 6 µl FuGENE® HD Transfection Reagent complexed with 2 µg plasmid DNA was added to 2 ml of cells at a density of 1 million cells per ml, then the cells would be placed in 2 liters of medium at the same density (1 million cells per ml). The transfection complex, consisting of 6 ml of reagent and 2 mg of plasmid, would then be added to the cells.

With the proper combination of cells, medium, and vector, we have seen reports of 50-100 mg/liter of monoclonal antibody routinely produced in HEK cells using FuGENE® HD Transfection Reagent. This protein is produced within a week; 10-fold more protein (500 mg to 1 g) could easily be produced from 10 liters of transiently transfected cells.

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Product	Cat. No.	Pack Size
<b>PCR Master</b>	11 636 103 001	1 kit (200 reactions)
<b>Restriction Enzymes</b>	For all restriction enzymes please visit our special interest site at <a href="http://www.restriction-enzymes.com">www.restriction-enzymes.com</a>	
<b>Rapid DNA Dephos &amp; Ligation Kit</b>	04 898 117 001	40 reactions
	04 898 125 001	160 reactions
<b>FuGENE® HD Transfection Reagent</b>	04 883 560 001	1 trial pack
	04 709 691 001	0.4 ml
	04 709 705 001	1.0 ml
	04 709 713 001	5 x 1 ml
	05 061 369 001	10 ml
<b>Luciferase Reporter Gene Assay, high sensitivity</b>	11 669 893 001	200 assays
	11 814 036 001	1,000 assays
<b>Cell Proliferation Reagent WST-1</b>	05 015 944 001	8 ml
	11 644 807 001	25 ml
<b>β-Gal Staining Set</b>	11 828 673 001	1 set (100 tests)
<b>Propidium Iodide</b>	11 348 639 001	20 ml (available in the US)
<b>PVDF Western Blotting Membrane</b>	03 010 040 001	30 cm x 3.00 m
<b>cOplete, EDTA-free Protease Inhibitor Cocktail</b> Tablets supplied in <i>EASYpacks</i>	04 693 132 001	20 tablets
<b>Lumi-Light<sup>PLUS</sup> Western Blotting Substrate</b>	12 015 196 001	100 ml
<b>Lumi-Light<sup>PLUS</sup> Western Blotting Kit (Mouse/Rabbit)</b>	12 015 218 001	1 kit
<b>Anti-VSV-G</b>	11 667 351 001	200 µg
<b>Anti-Green Fluorescent Protein</b>	11 814 460 001	200 µg
<b>Anti-HA Affinity Matrix</b>	11 815 016 001	1 ml
<b>HA peptide</b>	11 666 975 001	5 mg
<b>Anti-Protein C Affinity Matrix</b>	11 815 024 001	1 ml

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