APPLICATION NOTE

Optimization of Protein Expression in an *E. coli* System using Thermo Scientific MaxQ 8000 Refrigerated Stackable Shakers

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Introduction

Recombinant proteins are an invaluable part of the life scientist's tool-kit and are increasingly being used as therapeutics. Arguably, the most commonly used expression host is *Escherichia coli* (*E. coli*)[1], a relatively simple and well characterized system capable of producing large quantities of soluble protein in a short amount of time, without the need for extensive equipment or skills. *E. coli* does have a couple of key drawbacks: it does not support Post Translational Modifications (PTMs); and it is common for foreign genes to be poorly expressed, or for their protein products to become insoluble, forming inclusion bodies. This application note looks at the effect of varying temperature of induction, length of induction and type of media used, using the Thermo Scientific™ MaxQ™ 8000 refrigerated stackable shaker.

Thermo Scientific MaxQ Refrigerated Stackable Shakers

Overcoming expression and solubility obstacles

Low yields of soluble protein from *E. coli* have been tackled on multiple fronts. The use of expression and solubility tags has enabled the expression of many recombinant proteins; however, it is common for a recombinant protein to become insoluble once removed from its fusion-tag. Another focus of improving expression yield in *E. coli* has been codon usage, as there is a large difference in the codons most commonly used in Eukaryotes compared to those preferred by *E. coli*. This has been tackled by two approaches, either through the use of *E. coli* strains that encode tRNAs

that are rare in *E. coli*, but are used frequently in other organisms, or through the use of synthetic genes that have been codon optimized via complex algorithms that take into account the codon bias of *E. coli*.

Another distinct approach has been to empirically determine the best expression conditions for each individual recombinant. Variables that often have a large effect on the amount of soluble recombinant protein include temperature of induction, length of induction and type of media used. It is not directly clear how, or why, these factors are important. It is postulated that lower temperatures, or general slower growth conditions, increase the time that proteins have to fold, although this is not always borne out.

Troubleshooting the best expression conditions for each individual recombinant protein often requires brute force efforts to try multiple sets of variables. These solubility efforts can take weeks as variables are tried one by one.

This application note describes the expression screening experiment used to optimize the expression of a target protein using MaxQ 8000 refrigerated stackable shakers to simultaneously test eight expression conditions. The target His-tagged recombinant protein is a 10.5 kDa, multi-stranded β barrel with an intervening helix insert region. This protein is commercially significant to the Thermo Scientific Pierce product line and will be named Protein X for this study. By using the MaxQ 8000 refrigerated stackable shakers we were able to test growth temperatures, length of induction, and effect of media in hours, rather than the many days normally needed to perform these studies.

The investigation was planned for maximum efficiency using a Design of Experiments (DoE) process, to ensure that both the individual and combinatorial effects of each of the multiple experimental factors could be statistically analyzed.

Procedure

Protein X was expressed in *E. coli* expression strain BL21 DE3 (Stratagene), from a plasmid under the control of a T7 promoter and the gene of interest.

A 100 ml overnight culture was grown at 37° C. To scale up, 16 hours later a 10 ml aliquot of the overnight culture was added to each of eight, 2 L baffled flasks. Four flasks contained 1 L of (Luria Bertani) LB media and four flasks contained 1 L of a super rich media that, anecdotal data had shown, improves expression of Protein X (50 mM Tris pH 7.5, 10 g yeast extract, 25 g tryptone, 5 g glucose, 5 g $MgSO_4$).

All eight cultures were grown at 30° C and were shaken at 200 rpm until O.D.600 reached 0.5 (approximately 3 hours). At this point Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each flask to a final concentration of 200 µM. IPTG is a gratuitous inducer of the *lac* promoter, which is used to drive T7 polymerase expression in DE3 cells. The Protein X gene has a T7 promoter and is transcribed by T7 polymerase, allowing it to be overexpressed in the cell.

At this point, as detailed in Table 1, two LB and two super rich flasks were moved to a second shaker running at 15° C and 200 rpm. The remaining four flasks were kept in the original shaker, at 200 rpm, but its temperature was lowered to 25° C. (Flasks equilibrated to their new

Table 1. 3-factor 2-level experimental design

	A	B	C
Flask	**Temp**	**Time of Induction**	**Media**
1	15 °C	3 Hours	S rich
2	25 °C	3 Hours	S rich
3	15 °C	16 Hours	S rich
4	25 °C	16 Hours	S rich
5	15 °C	3 Hours	LB
6	25 °C	3 Hours	LB
7	15 °C	16 Hours	LB
8	25 °C	16 Hours	LB

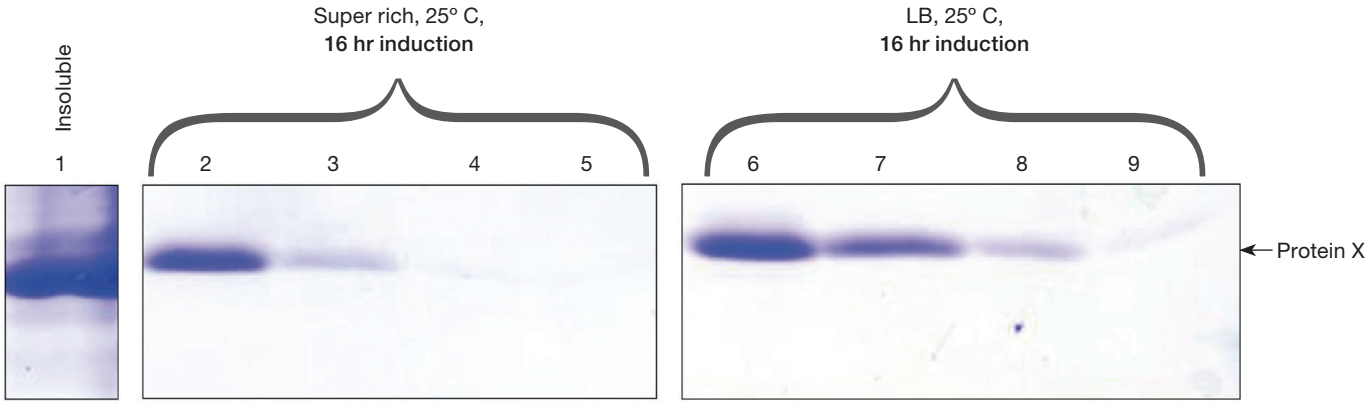

Figure 1: SDS page gels that represent the findings of the study. The gels were all scanned in and the bands evaluated. The insoluble fraction contains the majority of the expressed protein. Legend for lanes: 1- Insoluble protein; 2, 3, 4 & 5 - sequential elutions with 250 mM imidazole for sample incubated in super rich media at 25° C with a 16 hour induction (flask 4); 6, 7, 8 & 9 - sequential elutions with 250 mM imidazole for sample incubated in LB media at 25° C with a 16 hour induction (flask 8).

temperatures in less than 30 minutes, at this time-point protein expression of our gene of interest in undetectable).

At 3 hours, the cells from 1 flask at each condition (15° C LB; 15° C super rich, 25° C LB; 25° C super rich) were harvested by centrifugation (4 min @ 12,000 *xg*). The remaining four flasks were harvested the same way 16 hours after induction. Cell pellets were all frozen at -80° C to aid lysis.

Cells were lysed in Thermo Scientific™ B-PER Bacterial Protein Extraction Reagent + enzymes and the lysate cleared by centrifugation (20 min @ 48,000 *xg*). Each lysate was then purified on a fresh 1 mL Thermo Scientific™ HisPur™ Cobalt Resin column using fast protein liquid chromatography (FPLC) at a flow rate of 2 mL/min. The column was washed with 20 mM Tris (pH 8.0 500 mM NaCl). Increasing concentrations of imidazole (10 mM, 20 mM and 250 mM) were used to wash and then elute Protein X.

Fractions were analyzed by SDS gel electrophoresis (Figure 1) and quantitated by densitometry to determine the amount of Protein X that had been purified. Measurements were performed in duplicate to assess error. The data were then analyzed statistically using the Design-Expert™ 7 Workstation.

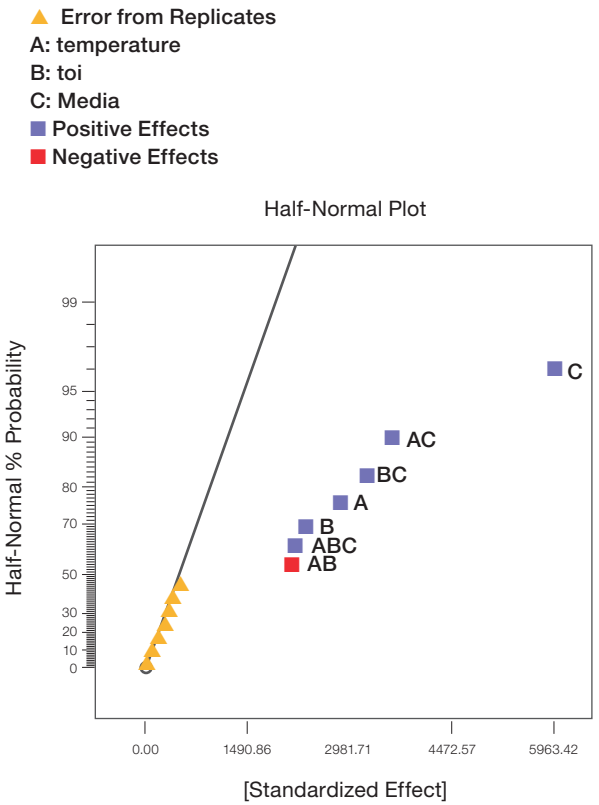

Figure 2: Half normal plot showing statistical relevance of the three experimental factors.

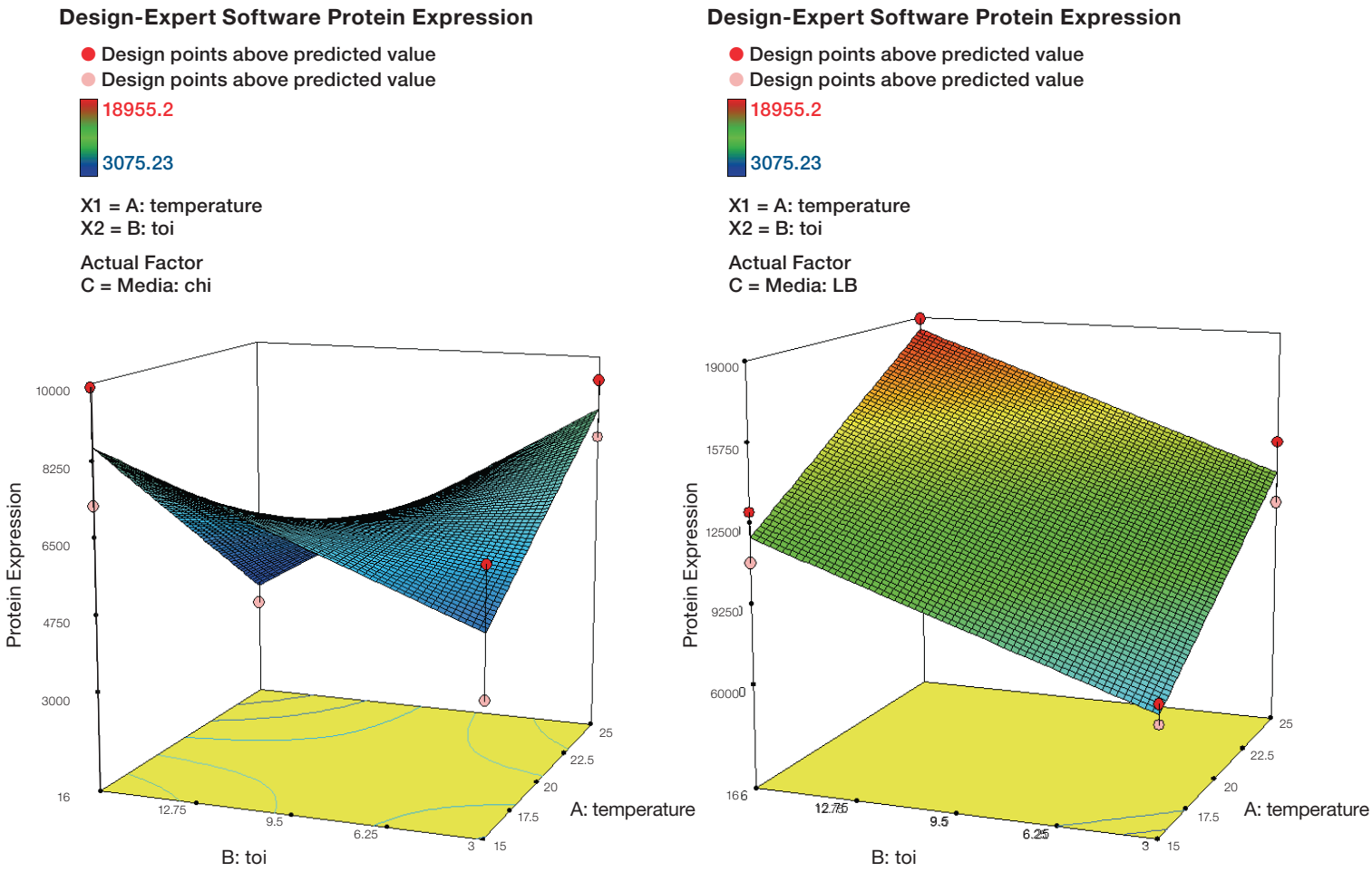

Figure 3. Plot of the effects of temperature, time of induction (TOI) using either Super Broth (left) or LB (right).

Results

Using Design-Expert 7 Workstation, all three factors (temperature, time of induction [TOI] & media) were shown to have statistically important effects on the level of soluble Protein X expression, as judged from the half-normal plot (Figure 2). Half normal plots compare the absolute values of ordered residuals from the data, to the expected values of ordered observations from a normal distribution to establish which experimental effects are important and which unimportant.

The screening identified conditions of expression that resulted in a greater than two-fold improvement in the yield of Protein X (Figure 3). Notice in the top plot, the super rich media had an overall lower expression yield.

Temperature and time of induction (TOI) both had a positive effect on the soluble expression of the protein, but the effect was not cumulative. In fact, when both temperature and time of induction were increased, the solubility of the protein dropped. The bottom plot, shows the effects of the variables when the cultures was grown in LB. Here again, both temperature and time of induction increased the yield of soluble protein. In combination, increased temperature and time of induction had an additive effect, increasing the solubility of the protein.

Conclusion

The procedure above describes a method for the optimization of soluble protein expression in *E. coli* using the MaxQ 8000 refrigerated stackable shakers. Using the shakers cultures were grown at different temperatures and the effect of temperature, time of induction (TOI) and media on the amount of soluble Protein X produced was studied. All three factors were seen to have statistically important effects on the level of soluble Protein X expression as judged from the halfnormal plot. Interestingly, the effect of increasing both temperature and time of induction (TOI) was different for the two media. This experiment not only shows the importance of optimizing incubation parameters for recombinant protein expression in *E. coli*, but also that it can be achieved over hours rather than days when using good experimental design, coupled with superior equipment.

Key to the success of this investigation was the ability to design the experiment to run all flasks simultaneously using only two separate, stackable refrigerated shakers – the MaxQ 8000. The MaxQ 8000 shaker range includes both refrigerated and incubated models and can be stacked (up to 3 units high), providing excellent use of any available floor space. In addition, the slide out platform provides easy and rapid access to all the samples, during loading, unloading and induction, for example. As a result, multiparameter, multi-level studies, such as the one demonstrated here, can be conducted very efficiently.

References

1. Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol. 1999 10(5):411-21.

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