



SCARABGENOMICS
LLC
CLEAN GENOME® E. coli

Clean Genome® *E. Coli*
ScarabXpress® T7lac Chemically
Competent Cells
(Cat. No. C-1709-05, C-1709-10, C-1709-20)

FOR RESEARCH USE ONLY

Quick Protocol for Experienced Users

PRE-TRANSFORMATION STEPS

1. Equilibrate a water bath to 42°C.
2. Thaw the provided SOC medium and warm to 37°C to dissolve any visible precipitate.
3. Warm selective antibiotic plates to room temperature or 37°C.
4. If you are transforming a plasmid with antibiotic resistance other than ampicillin, you will need at least one LB agar plate containing 100 µg/ml ampicillin (or 50 µg/ml carbenicillin) for plating the pUC19 Control DNA transformation.

TRANSFORMATION PROCEDURE

1. Place required number of 17x100mm culture tubes (14 ml) or 1.5 ml Eppendorf tubes on ice.
2. Thaw ScarabXpress T7lac Chemically Competent Cells on ice.
3. Flick the competent cell tube gently 2-3 times to evenly suspend cells. Add 50 µl cells to each pre-chilled tube.
4. Add controls and DNA samples to culture tubes.
 - For no DNA Control, add 1 µl Ultrapure water.
 - For pUC19 Control DNA, add 1 µl DNA
 - For ligation products, add 2-5 µl (2-10 ng DNA) of heat inactivated ligation reaction.
 - Gently flick the cells/DNA mix 2-3 times.
5. Incubate tubes on ice for 30 min.
6. Heat shock cells for 30 s in a 42°C water bath without agitation.
7. Place tubes on ice for 2 min.
8. Add 450 µl pre-warmed SOC medium to each tube.
9. Incubate at 37°C with shaking at 250–275 rpm for 1 h.
10. For cells transformed with pUC19 Control DNA, spread 100 µl of the outgrowth culture onto pre-warmed LB agar plates containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin.
11. For cells transformed with experimental DNA, spread up to 150 µl of the outgrowth culture onto selective plates.
12. Store the remaining cultures at 4°C for future plating, if desired. Expect reduced efficiency.
13. Incubate plates overnight at 37°C.



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COMPONENTS	C-1709-5	C1709-10	C-1709-20
ScarabXpress® T7lac Chemically Competent Cells	1 x 0.25 ml	2 x 0.25 ml	4 x 0.25 ml
pUC19 Control DNA (10 pg/μl)	1 x 50 μl	1 x 50 μl	1 x 50 μl
SOC Medium	1 x 10 ml	1 x 10 ml	2 x 10 ml

STORAGE CONDITIONS

Store components at –80°C. **Do not store cells in liquid nitrogen.**

GENOTYPE

ScarabXpress T7lac = MDS™42 multiple-deletion strain (1) with a chromosomal copy of the T7 RNA Polymerase gene.

BACKGROUND

Using synthetic biology methods, the *Escherichia coli* K-12 genome was reduced by making a series of planned, precise deletions. The multiple-deletion series (MDS) platform exemplifies the “Clean Genome” concept, providing bacterial strains with ideal characteristics for regulated biopharmaceutical applications:

- Scarab Genomics has engineered its Clean Genome *E. coli* hosts for robust growth in minimal salts media, allowing production under strictly defined conditions.
- Plasmid and genomic stability are enhanced because transposable insertion sequences (I.S. elements) have been eliminated.
- Cultures are more stable because cryptic prophage were deleted, eliminating spontaneous cell lysis.
- Genes for toxins, virulence factors, flagella and fimbriae have also been removed to improve product purity and safety.
- Cells can continue to grow during protein expression because over 700 non-essential genes are eliminated and no longer compete for cellular resources, increasing metabolic efficiency
- High yields of recombinant protein drives down post fermentation processing costs.

The ScarabXpress T7lac host strain carries the gene for T7 RNA polymerase on its chromosome under the control of a modified lac promoter and operator and is designed for use with T7 promoter based expression vectors. The dynamics of recombinant protein induction are significantly different in this host than in those experienced with the commonly used expression host BL21(DE3). In minimal medium lacking lactose, the ScarabXpress® T7lac strain has more tightly regulated protein expression than BL21(DE3), providing the ability to reproducibly and precisely control induction. ScarabXpress T7lac utilizes the wild-type lac promoter, which is of lower strength relative to the lacUV5 variant present in BL21(DE3). Moreover, the wild-type lac promoter is subject to regulation by the catabolite activator protein, CAP, whereas BL21(DE3) lacUV5 activity is largely CAP-independent due to a change in the CAP binding site. The properties of the wild-type promoter render it more sensitive to the effects of catabolite repression and promoter activation occurs gradually post-induction. This is in contrast to the rather abrupt expression of lacUV5 upon induction that is a reflection of its strength and CAP-independence.

The ScarabXpress® T7lac strain is also more sensitive to repression; the promoter is more tightly regulated by LacI due to an alteration in a lac operator. The net result is a lowering of the background levels of expression relative to BL21(DE3). As a consequence of this tighter regulation, the ScarabXpress T7lac host often works



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optimally with expression vectors that do NOT supply extra lac repressor from a plasmid encoded copy of the lacI gene. In multiple cases, significantly higher expression yields of a target protein have been observed by using a vintage pET vector i.e. one that carries neither the lacI gene nor a lac operator on its backbone. lacI-based pET plasmids were created to address the inherent leakiness of BL21(DE3). The additional lac repressor generated from this type of vector prevents full induction of the ScarabXpress® T7lac host and may only yield optimal results when attempting to express proteins that substantially inhibit the growth of the Scarab host strain.

Critical Factors for Success

The Scarab Clean Genome strains represent a major advance for the production of biotherapeutics in *E. coli*. The strains are fundamentally different from and when used in the proper context, superior to any other *E. coli* strain. In order to utilize these unique strains optimally, there are a few critical parameters that require attention and optimization.

Minimal Medium

The Clean Genome strains were specifically designed for the production of biotherapeutic protein and DNA. The “cleanest” medium to use for biotherapeutic production is a chemically defined, minimal medium. Accordingly, the Clean Genome strains have been designed and tested for their capacity to grow and function well in minimal, chemically defined medium. As a consequence, Scarab Genomics has not performed an extensive characterization of the strains in complex, rich media. In fact, the use of rich medium may cause the strains to substantially underperform relative to their capacity in minimal medium.

Scarab Genomics has performed extensive testing of the MDS strains in Modified Korz Medium. This minimal medium was originally designed for high density fed-batch fermentation of *E. coli* (Korz et al. 1995). The medium consists of phosphate buffer, magnesium, ferric citrate, trace elements, and uses glucose as the carbon source. The medium can also be used for optimizing expression in shake flasks thereby providing continuity between the shake flask and fermentation processes. In fed-batch fermentations, the same medium is simply supplemented with higher glucose content. A separate Korz Feed Medium supplies glucose, magnesium, iron, and trace elements for the feeding stage of fed-batch fermentation.

To simplify the use of minimal medium, Scarab Genomics now offers a two component kit consisting of 10X Modified Korz Medium with 2% Glucose and separate Magnesium Sulfate solutions (Cat. No. D-0710-1L2). The concentrated minimal medium is designed for dilution to 1X and needs to be supplemented with the supplied MgSO₄ solution. The final 1X medium containing 0.2% glucose is used for expression optimization in shake flasks. The same medium (supplemented with additional glucose to a final concentration of 0.5%) also serves as the “batch” phase medium in fed-batch fermentations.

For the feeding stage of fed-batch fermentations, Scarab Genomics also offers Korz Feed Medium (Cat. No. D-0710-1L5).

Optimal Inducer Concentration

To achieve optimal recombinant protein expression and yield, it is essential to define the optimal concentration of inducer. The optimal inducer concentration will be compatible with both good protein expression per cell and high cell density per ml of culture. The concentration of inducer required will be dependent on the type of expression vector used (previously noted) and the nature of the recombinant protein.

Optimal Feed Rates

When performing fed-batch fermentations, it is imperative that cultures not be over fed with glucose. Overfeeding will result in acetate accumulation and subsequent inhibition of culture growth. The Clean Genome strains may require slower feed rates than those used with common *E. coli* strains.



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TRANSFORMATION DETAILS

CHEMICALLY COMPETENT CELL QUALITY CONTROL

Transformation efficiency is tested using pUC19 control DNA, performed in duplicate. Transformed cells are plated on LB plates containing 50 µg/ml carbenicillin. Transformation efficiency is $\geq 1 \times 10^8$ cfu/µg DNA.

BEFORE YOU BEGIN

- Please note, Clean Genome® strains do not remain viable for extended periods (greater than 2 weeks) when stored at 4°C. We recommend preparing glycerol stock cultures of clones and storing at -80°C, or keeping plates at room temperature for up to 5 days. **Do not store in liquid nitrogen.**
- For protein expression, Scarab Hosts perform best at temperatures $\geq 25^\circ\text{C}$.
- To ensure that the cells grow on minimal media and to prevent a significant lag when transferring to liquid culture, streak from glycerol stocks onto minimal plates with 0.2% glucose and grow at 37°C for 24 h, at 30°C or 48 h, or at room temperature (RT) for several days (e.g., over the weekend). Colonies picked from these plates are used for cultures.
- Clean Genome strains do not have flagella and tend to aggregate and drop fairly quickly from solution. To obtain accurate OD readings, cells should be mixed just before taking an aliquot for dilution, and dilution samples should be mixed just before taking an OD reading.

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4. If you are transforming a plasmid with antibiotic resistance other than ampicillin, you will need at least one LB agar plate containing 100 µg/ml ampicillin (or 50 µg/ml carbenicillin) for plating the pUC19 Control DNA transformation.

TRANSFORMATION PROCEDURE

1. Place required number of 17x100mm culture tubes (14 ml) or 1.5 ml Eppendorf tubes on ice.
2. Thaw ScarabXpress T7lac Chemically Competent Cells on ice.
3. Flick the competent cell tube gently 2-3 times to evenly suspend cells. Add 50 µl cells to each pre-chilled tube.
4. Add controls and DNA samples to culture tubes.
 - For no DNA Control, add 1 µl Ultrapure water.
 - For pUC19 Control DNA, add 1 µl DNA
 - For ligation products, add 2-5 µl (2-10 ng DNA) of heat inactivated ligation reaction.
 - Gently flick the cells/DNA mix 2-3 times.



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NOTES:

Transformation efficiency of ligation products can be increased several fold by diluting the ligation reaction 5-fold with TE or water prior to adding DNA to the cells.

Unused cells may be re-frozen in dry ice/ethanol bath for 5 min before returning to the -80°C freezer. Expect re-freezing cells to decrease their transformation efficiency.

5. Incubate tubes on ice for 30 min.
6. Heat shock cells for 30 s in a 42°C water bath without agitation.
7. Place tubes on ice for 2 min.
8. Add 450 µl pre-warmed SOC medium to each tube.
9. Incubate at 37°C with shaking at 250–275 rpm for 1 h.
10. For cells transformed with pUC19 Control DNA, spread 100 µl of the outgrowth culture onto pre-warmed LB agar plates containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin.
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SHAKE FLASK INDUCTION PROCEDURES

Two distinct protein induction protocols are provided for the evaluation of recombinant protein expression.

A) High OD₆₀₀ – Traditional Induction Procedure (Late Induction)

The High OD₆₀₀– Traditional Induction Procedure involves growing a shake-flask culture to an OD₆₀₀ between 0.4 – 0.6, inducing with a range of inducer concentrations and harvesting 2-5 hrs post-induction or overnight. The optimal inducer concentration for maximal yield is typically one that results in good induction while still allowing a significant increase in cell density.

B) Low OD₆₀₀ – Inducer Titration Induction Procedure (Early Induction)

The Low OD₆₀₀ – Inducer Titration Induction Procedure involves inducing a culture at an OD₆₀₀ of 0.01 with a range of inducer concentrations, monitoring culture growth rates, and sampling multiple times during the growth of the culture. In this procedure, the optimal inducer concentration is typically one that does not significantly inhibit culture growth (relative to an un-induced control) while still allowing good recombinant protein production.

Empirical testing is often required to elucidate the optimal procedure for any given recombinant protein. In general, the High OD₆₀₀ procedure may prove optimal for proteins that significantly inhibit the growth of *E. coli* i.e. “toxic” proteins. In contrast, the Low OD₆₀₀ procedure may work best for proteins that are well tolerated by *E. coli*. Initial inoculums for both procedures are prepared in the same manner.

1. Prepare a 30 ml overnight culture in minimal medium and selective antibiotic(s) in a 250 ml Erlenmeyer Flask (1X Modified Korz Medium with 0.2% Glucose recommended). Inoculate with a single isolated colony representing the strain + plasmid combination of interest. Incubate the culture overnight at 37°C, 250-300 rpm.
2. Measure the OD₆₀₀ of the overnight culture.
3. The following table provides recommendations of culture volumes and vessel types for optimal aeration when performing inductions.

For optimal growth and aeration, use the following volume per vessel.

Culture volume (ml)	Vessel type
0.5	12 mm x 75 mm sterile snap-cap tube
2	17mm x 100 mm sterile snap-cap tube
15	125 ml Erlenmeyer flask
30	250 ml Erlenmeyer flask
100	500 ml baffled flask
200	1-L baffled flask
500	2.8-L baffled flask

The following protocols assume that one large common culture is grown to an OD₆₀₀ at or near the recommended density for induction and then split into multiple smaller flasks prior to adding varying concentrations of inducer. The examples provided assume an initial culture volume of 150 mls (1-L Erlenmeyer baffled flask) which is then split into multiple 15 ml cultures (125 ml Erlenmeyer baffled flask).



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A) High OD₆₀₀ Induction – Traditional Procedure IPTG Induction

1. Prepare 150 mls of medium with antibiotics in a 1-L Erlenmeyer baffled flask. Inoculate the common culture with enough overnight culture (assume the overnight culture has an OD₆₀₀ = 2.1) to give a starting OD₆₀₀ of approximately 0.05.

EXAMPLE: 0.05 OD/ml x 150 ml ÷ 2.1 OD/ml = 3.57 ml of overnight culture to inoculate the 150 ml common culture

common culture vol. overnight culture OD

2. Incubate the culture at 37°C, 250 -300 rpm until the OD₆₀₀ is between 0.4-0.6 (typically 3-5 hrs).
3. The following IPTG levels are recommended to initiate the characterization. Split the common culture into separate cultures by transferring 15 ml aliquots into 125 ml Erlenmeyer baffled flasks. Add the indicated amounts of IPTG to the various flasks to give the range of final IPTG concentrations indicated below. Do not add IPTG to one culture in order to have an un-induced control.

EXAMPLE

		<u>Final [IPTG]</u>
Flask 1:	NO IPTG addition	0 μM
Flask 2:	7.5 ul of 10 mM IPTG	5 μM
Flask 2:	15 ul of 10 mM IPTG	10 μM
Flask 3:	37.5 ul of 10 mM IPTG	25 μM
Flask 4:	75 ul of 10 mM IPTG	50 μM
Flask 5:	11.25 ul of 100 mM IPTG	75 μM
Flask 6:	15 ul of 100 mM IPTG	100 μM
Flask 7:	30 ul of 100 mM IPTG	200 μM
Flask 8:	60 ul of 100 mM IPTG	400 μM
Flask 9:	150 ul of 100 mM IPTG	1000 μM

4. Incubate the induced cultures at the desired induction temperature while shaking at 250 -300 rpm. In general, both 25°C and 37°C should be tested to determine which gives better yields. This is especially important when attempting to optimize the production of soluble protein and/or when targeting proteins to the periplasmic space.
5. After 2-5 hours of incubation at 37°C, remove aliquots for harvest by centrifugation and prepare soluble, insoluble and periplasmic fractions as desired. In the case of 25°C incubation, the time of post-induction incubation should be extended several fold (5 hrs to overnight) prior to harvest. For all harvested aliquots, create lysates, fractionate as desired, and analyze by SDS-PAGE.

B) Low OD₆₀₀ Induction – IPTG Titration Induction Procedure

1. Prepare 150 mls of medium with antibiotics in a 1-L Erlenmeyer baffled flask. Inoculate the common culture with enough overnight culture (assume the overnight culture has an OD₆₀₀ = 2.1) to give a starting OD₆₀₀ of approximately 0.005.

EXAMPLE: 0.005 OD/ml x 150 ml ÷ 2.1 OD/ml = 357 ul of overnight culture to inoculate the 150 ml common culture

common culture vol. overnight culture OD



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- Incubate the culture at 37°C, 250 -300 rpm for approximately 90 minutes (OD₆₀₀ will be in the range of 0.01 to 0.015).
- The following IPTG levels are recommended to initiate the characterization. Split the common culture into separate cultures by transferring 15 ml aliquots into 125 ml Erlenmayer baffled flasks. Add the indicated amounts of IPTG to the various flasks to give the range of final IPTG concentrations indicated below. Do not add IPTG to one culture in order to have an un-induced control.

EXAMPLE A – Induction Profile for pET vectors without a lacI gene on their backbone

		<u>Final [IPTG]</u>
Flask 1:	NO IPTG addition	0 µM
Flask 2:	1.5 µl of 10 mM IPTG	1 µM
Flask 3:	3.75 µl of 10 mM IPTG	2.5 µM
Flask 4:	7.5 µl of 10 mM IPTG	5 µM
Flask 5:	15 µl of 10 mM IPTG	10 µM
Flask 6:	22.5 µl of 10 mM IPTG	15 µM
Flask 7:	30 µl of 10 mM IPTG	20 µM
Flask 8:	37.5 µl of 10 mM IPTG	25 µM
Flask 9:	75 µl of 10 mM IPTG	50 µM

EXAMPLE B – Induction Profile for pET vectors with a lacI gene on their backbone

		<u>Final [IPTG]</u>
Flask 1:	NO IPTG addition	0 µM
Flask 2:	15 µl of 10 mM IPTG	10 µM
Flask 3:	30 µl of 10 mM IPTG	20 µM
Flask 4:	45 µl of 10 mM IPTG	30 µM
Flask 5:	60 µl of 10 mM IPTG	40 µM
Flask 6:	75 µl of 10 mM IPTG	50 µM
Flask 7:	112.5 µl of 10 mM IPTG	75 µM
Flask 8:	15 µl of 100 mM IPTG	100 µM
Flask 9:	37.5 µl of 100 mM IPTG	250 µM
Flask 10:	75 µl of 100 mM IPTG	500 µM

- Incubate the induced cultures at the desired induction temperature while shaking at 250 -300 rpm. In general, both 25°C and 37°C should be tested to determine which gives better yields. This is especially important when attempting to optimize the production of soluble protein and/or when targeting proteins to the periplasmic space.
- If incubating at 37°C, determine the OD₆₀₀ of each flask every hour. Late in the afternoon, remove a sufficient aliquot of each culture for harvesting and SDS-PAGE analysis the following day. Harvest the culture aliquots by centrifugation and store the pellets at -20°C. Allow the flasks to continue shaking at 37°C overnight.

If incubating at 25°C, determine the OD₆₀₀ of each flask every 2-3 hours. If sufficient growth has occurred by late in the afternoon to allow for SDS-PAGE analysis, remove a sufficient aliquot, harvest by centrifugation and store the pellets at -20°C. Allow the flasks to continue shaking at 25°C overnight.

- The following morning determine the OD₆₀₀ of the overnight cultures. Plot the various culture OD₆₀₀s (y-axis) vs. time (x-axis) to obtain approximate growth rates for each culture.



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- Note: Growth rates are expected to decline at higher inducer levels because the induction process and/or the production of the recombinant protein becomes inhibitory to the cells. An example of this type of analysis is provided in Figure 1. The growth curves depicted were obtained with an apparatus that continuously measures the OD₆₀₀ of a culture.

Harvest aliquots of the overnight cultures by centrifugation, create soluble, insoluble, and periplasmic fractions as desired and analyze the samples by SDS-PAGE. The example given in Figure 1 demonstrates the induction of a soluble 85 kDa recombinant human protein in a pET9 vector (pET vector lacking *lacI* gene).

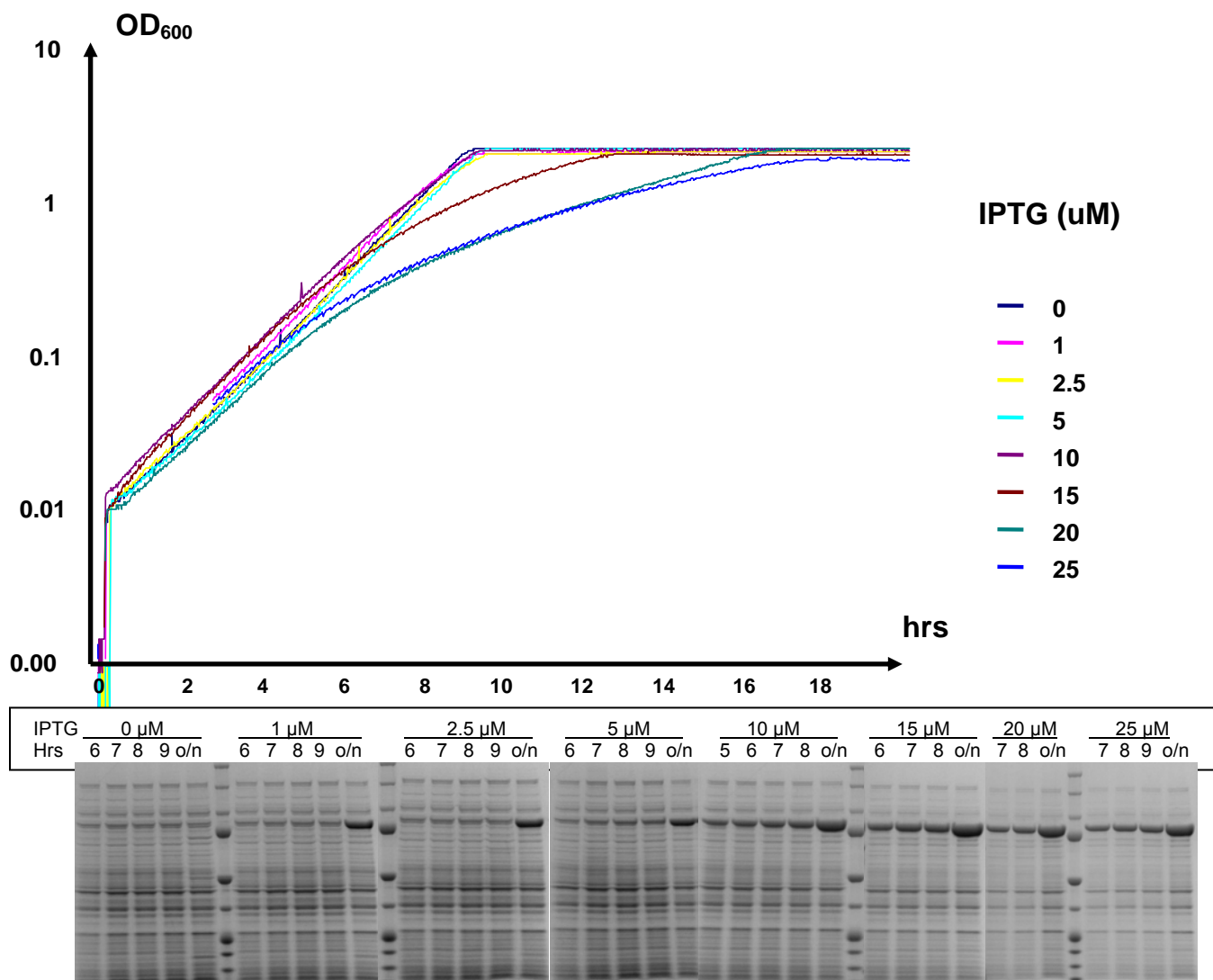


Figure 1: Shake Flask Culture Growth Rates

Shake flask cultures were grown in 50 ml Korb Minimal Medium with 0.2% glucose at 37°C, with monitoring of OD₆₀₀ at 1-minute intervals. The indicated concentrations of IPTG were added to the cultures at an OD₆₀₀ of 0.01.



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Optimal Inducer Concentration = the concentration of inducer that gives the highest level of protein expression **WITHOUT** decreasing the growth rate of the culture relative to that of the un-induced control. Results from the shake flask experiment should be directly transferable to a fed-batch fermentation procedure using the same Modified Korz Medium supplemented with glucose to a final concentration of 0.5%. To mimic the Low OD₆₀₀ induction of the shake flask, the fed-batch fermentation would be induced while still in the batch phase (OD₆₀₀ of approximately 2).

Notes:

Optimal expression conditions will vary depending on the protein expressed and the expression vector. Maximum protein yield may be achieved by optimizing medium, IPTG concentration, post-induction growth temperature and harvest time.

When using nondenaturing, detergent-based lysis reagents with ScarabXpress Cells, we recommend increasing the incubation time. For example, for 1 ml of a 4 OD culture lysed with 200 µl of EMD's BugBuster® Protein Extraction Reagent, increase the incubation time from 20 to 30 minutes on a platform rocker at room temperature. We recommend testing for complete cell lysis, with any cell cracking method, by streaking a small amount onto an LB plate and incubating overnight at 37°C.



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2. Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. *J Biotechnol.* 1995 Feb 21;39(1):59-65. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*.

TROUBLESHOOTING

The pUC19 Control DNA plasmid provided should be used as a positive control.

Problem	Possible Solution
No colonies or low number of colonies	<ol style="list-style-type: none"> 1. Incorrect drug selection or drug concentration. Verify that LB agar plates contained the appropriate selective antibiotic. Repeat transformation or plate an aliquot of the remaining out growth culture onto new plates. 2. Incorrect incubation temperature. Be sure to incubate at 37°C. Verify the temperature setting. Incubate plates at 37° for the remainder of one day. If no/low number of colonies is still observed, repeat transformation or plate an aliquot of the remaining out growth culture onto new plates. 3. Cells were not handled correctly. Cells must be handled very gently. Do not pipette vigorously or vortex. Always gently pipette or gently flick cells to resuspend. Thaw cells and keep on ice until ready to transform. Repeat transformation.
Lawn or confluent cell growth or satellite colonies	<ol style="list-style-type: none"> 1. Incorrect drug concentration. Verify that LB agar plates contain the appropriate selective antibiotic concentration. Repeat transformation or plate an aliquot of the remaining out growth culture onto new plates. 2. Antibiotic has degraded. Plates are too old, antibiotic stock(s) have degraded, or antibiotic was added when medium was too hot. Prepare fresh antibiotic stock(s) and fresh plates. Repeat transformation or plate an aliquot of the remaining out growth culture onto new plates. 3. Incubated at 37°C too long. Plates should incubate for 16-18 h. Amp^R cells secrete β-lactamase that creates a drug-free zone in the surrounding medium, allowing small amp^S colonies to grow. Carbenicillin (an ampicillin analog) appears to be less vulnerable to degradation. Repeat transformation, or plate an aliquot of remaining out growth culture, and incubate plates for 16-18 h.



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Scarab Genomics' technology is covered by U.S. Pat. No. 6,989,265 and related foreign applications.

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