ACES™

ACES™ ■ Promoter Selection Kit

AthenaES™ Complete Expression System

The ACES™ Promoter Selection Kit applies the principle that expression rate can determine the amount of soluble protein made in addition to total accumulation. By titering promoter activity, the expression level can be fine-tune to meet the specific needs of target protein production levels.

AthenaES®

Athena Enzyme Systems™
888-892-8408
www.athenaes.com

Application Manual
V 1.0

ACES™

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Promoter Selection Kit

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V. 1.0

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ACES™ Promoter Selection Kit
Application Manual

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Introduction

In the early days of producing proteins in *E. coli* as the host, limitations to transcription initiation were believed to lower expression levels.[1] Often this was true and the historical result was the almost exclusive construction and deployment of expression vectors which carried strong promoters. These strong promoters include the phage promoters T7 and T5, the synthetic promoters **tac** and *trc*, and the arabinose inducible *ara*. The T7 IPTG-inducible system has become the dominant expression system currently employed. However, the use of a strong promoter, which leads to hyper-expression levels, is not without potential adverse consequences.

The most frequent adverse consequence of hyper-expressing proteins is that they accumulate as inclusion bodies. These insoluble particles require that the target protein be denatured and then refolded in order to recover soluble protein. The process of refolding proteins can be a daunting task because the optimum conditions for recovering active protein requires the evaluation of several variables, is not always possible and, when successful, often results in poor yields. The reasons for proteins accumulating as inclusion bodies are varied, involving both the intrinsic properties of the protein as well as interactions with host proteins. One approach of limiting the accumulation of inclusion bodies is to lower the expression level. This is done by lowering the culture temperature during expression, decreasing the amount of inducer (if a titratable promoter is used), employing alternative strains (with higher levels of chaperone proteins or other accessory proteins), or using weaker promoters. None of the approaches by themselves will give the best solution and maximum production of soluble protein requires the use of matrix analyses to identify the optimum expression conditions. The first three alternatives are relatively easy to control experimentally. The latter, however, currently requires the subcloning of the target gene sequences into a restricted set of alternative expression vectors which carry different inducible promoters. Thus, the ability to examine a range of promoter strengths to identify the best one suited to the expression of a particular protein is limited. The intent of this kit is to simplify the process of selecting alternative promoters thus allowing for the more rapid identification of an optimal promoter.

Principle of the Kit

This kit is designed to facilitate the construction of promoter variants that yield the maximum level of soluble active target protein. Promoters that direct the expression of a given target protein with different strengths are made using a set of specifically designed PCR primers, in an inverse PCR technique, that introduces random sequences within the promoter region. The promoter variant that yields maximum levels of soluble, active protein is identified by screening the library. Since the promoter selection is performed in the expression vector, no further subcloning is needed to express and purify the target protein.

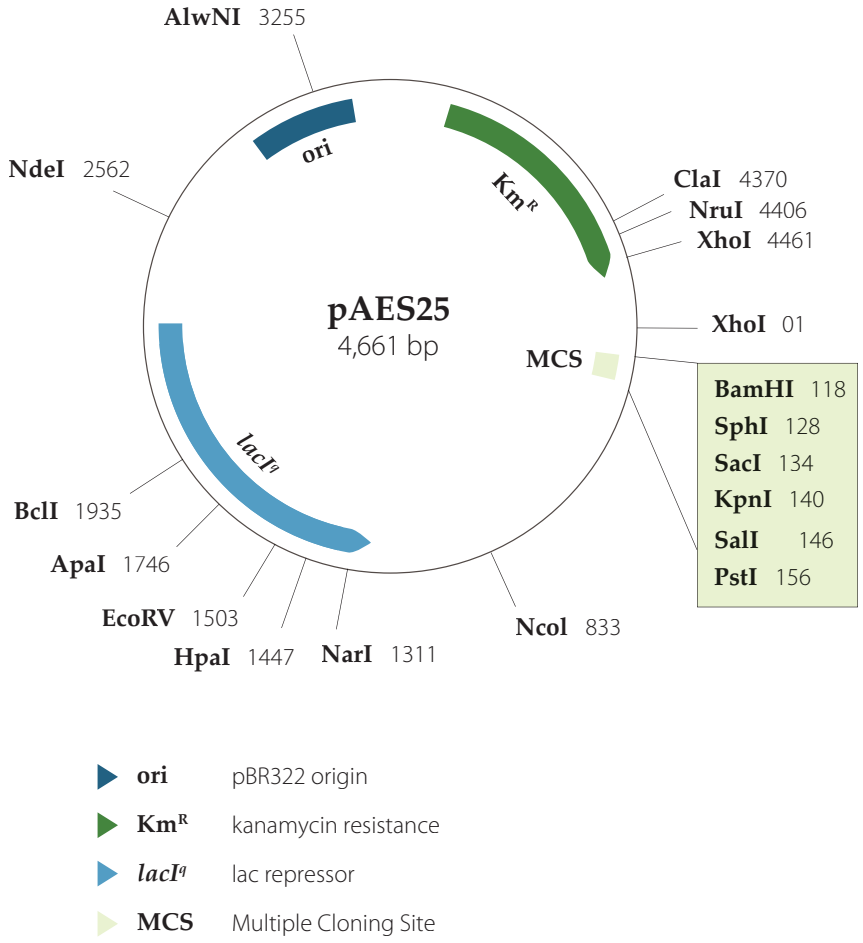

Figure 1. Plasmid map of pAES25

Kit Components

ACES™ Promoter Selection Kit Components		
Component	**Amount**	**Catalog Number**
pAES25	10 µg	0149-25
JM109 Competent Cells	2 x 200 µL	0151-JM109-C
Primer A	250 pmoles	0150-1
Primer B	250 pmoles	0150-2
LB Broth (Miller)	1 L Mix	0103
Turbo Broth™	1 L Mix	0104
Superior Broth™	1 L Mix	0105
Power Broth™	1 L Mix	0106
Hyper Broth™	1 L Mix	0107
Glucose M9Y	1 L Mix	0108
Glucose Nutrient Mix	21 g	0109
Augmedium™	25 mL Mix	0123
Inducer Solution A	1mL	0152
Reagents needed but not provided: Glycerol, Buffer, nucleotides and polymerase for amplification reactions, LB Plates supplemented with 50 µg/ml kanamycin		

Solution Information	
Component	**Reagent**
Primer A	10 µM in 10 mM Tris-Cl, pH 8.5
Primer B	10 µM in 10 mM Tris-Cl, pH 8.5
Inducer Solution A	1 M IPTG

Protocols

Preparation: Media

1. Dissolve the contents of each of the media packets in deionized water as directed on the individual packets.

2. Add 4 mL of glycerol to the Turbo Broth™ and Power Broth™ solutions.

3. Dispense desired volume into appropriate bottles or flasks. (We recommend 2 x 500 mL glass bottles.)

4. Autoclave at 121°C for 20 min. The autoclaved media without antibiotics are stable for at least 6 months at 4°C.

5. Dissolve the contents of the Glucose Nutrient Mix in 100mL deionized water and filter sterilize using a 0.2 µm filter.

6. Add 50 mL of the sterile Glucose Nutrient Mix to 1 liter of Hyper Broth™ and 20 mL to 1 liter of Glucose M9Y using aseptic technique.

7. Add sterile antibiotics as needed.

Preparation: Medium Supplements

1. **Augmedium**

    1.1. Dissolve the contents of the Augmedium™ container in 25 mL deionized water.

    1.2. Filter sterilize using a 0.2 µm filter.

    1.3. Aliquot 5 mL portions into sterile 15 mL conical tubes and store at -20°C. The stock will be a 50x solution.

    1.4. For use, snap thaw at 37°C and store at 4°C for no more than five (5) days.

2. **Inducer Solution A** (1M IPTG)

    2.1. Use to a final concntration of 1mM.

    2.2. Store at -20°C.

Task 1: Subclone the Coding Sequence for the Target Protein into plasmid pAES25

1. Insert the DNA fragment carrying the target sequences such that the AUG start codon carried on pAES25 is the first amino acid of the protein sequence.

    1.1. Note: There are several restriction sites in the MCS of pAES25 that are suitable for subcloning (see Fig. 1). However, it is recommended that if possible the BamHI site be used as the 5′ terminal restriction site to minimize the number of non-native amino acids at the amino-terminus of the protein. Inserting the target sequence at the BamHI site will yield a protein with the N-terminal sequence of Met-Gly-Ile. (For convenience this new construct will be referred to as "pAES25-Target Gene.")

Task 2: Prepare a Promoter Library

The promoter library is generated using the set of primers (A and B) provided. Each primer was designed to insert randomized sequences at the -35 and -10 regions of the promoter.

1. Prepare a PCR amplification using 10 ng of the plasmid constructed in Task 1 along with 20 pmoles of each of the two primers (final concentration of 0.2 µM) in a standard 100 µl reaction mixture.

2. Amplify for 20 cycles of 95°C, 30s; 60°C, 30s with -0.5°C per cycle; 72°C, 1 min per kb followed by 10 cycles except holding the annealing temperature at 50°C.

    2.1. Use a 15 min hot start if prescribed by the vendor of the PCR kit.

    2.2. Incubate at 72°C for an additional 10 min to flush the end before chilling to 4°C.

        2.2.1. Analyze a 5 µl aliquot of the reaction on an agarose gel. If the correct sized product is present and in sufficient quantities, proceed to Task 3. Otherwise, repeat the amplification adjusting the following parameters:

            2.2.1.1. Increase the number of cycles of the second amplification.

            2.2.1.2. Increase the extension reaction incubation time.

            2.2.1.3. Follow the PCR kit vendor instructions for improving the amplification.

3. Purify the PCR product by fractionating on an agarose gel and recovering the linear DNA by any number of commercially-available "DNA gel-extraction" kits.

4. Phosphorylate the linear DNA, ligate and introduce into a suitable *E. coli* host strain (JM109, DH5α, NovaBlue, XL1-Blue or similar strains are recommended).

    4.1. Select kanamycin-resistant (Km^R) transformants.

        4.1.1. Note: It is recommended that two parent strains be prepared by introducing pAES25 and pAES25-Target Gene into the same host strain as the promoter library.

Task 3: Screen for Target Protein Expression

1. Select 90 colonies and inoculate 1 ml of medium in a 96-deep well sterile culture dish. Use a rich medium such as Turbo Broth™ (AthenaES™) or Terrific Broth, rather than LB medium.

    1.1. Inoculate 2 wells each with a colony of the pAES25 parent strain and 2 wells with the pAES25-Target Gene parent strain.

2. Incubate the plate at 37°C for 3 hours with shaking at 250-300 rpm.

3. Add IPTG to a final concentration of 1 mM and incubate at 30°C for 3 hours.

4. Screen for expression of the Target Protein by one of several approaches. A functional assay is the recommended approach.

    4.1. Remove a 500 µl aliquot (do not collect the entire culture) to a 1.5 ml microfuge tube and store the culture plate at 4°C.

    4.2. Prepare a soluble extract by harvesting the cells for 2 min in a micro-centrifuge and re-suspending the pellet in an appropriate volume of extraction buffer. The buffer composition will depend on the type of extraction process to be employed (sonication, lysozyme/freeze-thaw or chemical extraction).

        4.2.1. Prepare the extract according to the selected method and remove the insoluble material by microfuging for 10 min. (>12,000 xg).

        4.2.2. Collect the supernatant and assay for active protein.

    4.3. If a functional assay is not available, then perform a screen for the construct yielding the maximum amount of soluble protein. This is done by SDS-PAGE or immunoblot.

        4.3.1. Prepare a soluble extract as described above using 500 µl of the desired buffer.

4.3.2. Prepare a whole cell extract sample by removing 100 µl from the original culture, harvest the cells and suspend in 100 µl of the buffer used to prepare the soluble extract.

4.3.3. Mix a 10 µl aliquot from each sample with 30 µl water and 10 µl 5x SDS-PAGE loading dye.

4.3.4. Heat at 100°C for 5 min. and load an appropriate volume in each well (e.g. use 5 µl for 3-5 mm wide wells) loading the soluble and whole cell samples side-by-side.

4.3.5. After staining the gel or detection by immunoblot, determine the construct that produces the highest amount of soluble protein (compare the soluble to whole cell extracts).

4.4. To recover the strain with the highest production level of functional or soluble protein, streak a portion of the culture corresponding to the selected strain on solid medium containing kanamycin and preserve accordingly.

For more technical information and an example of use, see our Technical Brief: ACES™ Promoter Selection Vector at www.athenaes.com

Supplemental Protocols

Supplemental Protocol 1: Rapid Transformation Protocol (Kit available: AthenaES™ No. 0156)

1. Materials:

1.1. 2x TSS (DO NOT store or use near flame, DMSO is flammable).

1.2. *E. coli* strains to be transformed.

1.3. Tryptic Soy Agar (TSA) plates or comparable non-antibiotic containing plate medium.

1.4. Sterile microcentrifuge tubes, 1.5mL (or other convenient tube).

1.5. LB medium.

1.6. SOC medium.

1.7. Antibiotic-containing plate medium.

2. Methods:

2.1. Streak strain(s) on non-antibiotic plate medium and incubate at 37°C overnight. (This protocol MUST be performed with fresh, overnight bacterial colonies.)

2.2. Dispense 0.1 mL LB medium into microfuge tubes using sterile technique.

2.3. Pick 4 colonies using a 1µL loop or sterile toothpick and resuspend in the 0.1 mL LB using sterile technique.

2.4. Add 0.1 mL 2x TSS (DO NOT FLAME) and mix well.

2.5. Incubate on ice for 15 minutes. Once chilled, do not allow the cells to warm above 14°C.

2.6. Add 100 ng plasmid DNA and incubate on ice for 20 min.

2.7. Heat shock in a 42°C water bath for 1 min.

2.8. Add 1 mL SOC medium using sterile technique.

2.9. Incubate at 37°C for 30 min.

2.10. Plate 0.1 mL of transformation mix on antibiotic-containing plate medium and incubate overnight at 37°C.

2.11. Streak purify 2 or 3 colonies on appropriate antibiotic plates.

2.12. Prepare master cell bank(s) or cryostock(s) of new strain(s).

Supplemental Protocol 2: Media Optimization Protocol

1. Materials

1.1. Strain selected from Task 2: Prepare a Promoter Library, step 4 - 4.1.1.

1.2. 25 mL of each of the six culture media provided in 250 mL baffle bottomed flasks.

2. Methods

2.1. Inoculate a single colony of the selected recombinant strain into 25 mL of LB Broth and incubate at 37°C overnight.

2.2. Inoculate 25 mL of each of the six media with 2 mL of the overnight culture. Incubate the cultures at 37°C until the OD_{600} reaches 0.8 ± 0.2.

2.3. Remove a 1mL sample ("pre-induction") from each of the six flasks, harvest the cells in a microfuge tube, and process as in step 2.7.

2.4. Add inducer *(see Tip 1)* and continue incubating for 3 hours *(see Tip 2)*.

2.5. Remove 1mL samples ("post-induction"), record the OD_{600}, harvest the cells in microfuge tubes, and process as in step 2.7.

2.6. Harvest the remainder of the culture, determine the mass of the cell pellet, and store the cell pellets at –80°C. *(See Tip 3.)*

2.7. Analyze for expression of the target protein as follows:

2.7.1. To determine protein production per mL of culture:

2.7.1.1. Resuspend the cell pellets from the pre- and post- induction samples in 500 µL water.

2.7.1.2. Mix 5 µL of each cell suspension with 15 µL water and 5 µL 5x SDS-PAGE loading buffer. Heat at 100°C for 5 min and load 10 µL per lane.

2.7.2. To determine the relative level of expression:

2.7.2.1. Resuspend the cell pellets from the pre- and post-induction samples in water to a density of 10 OD_{600}/ml.

2.7.2.2. Mix 5 µL of each cell suspension with 35 µL water and 10 µL 5x SDS-PAGE loading buffer. Heat at 100°C for 5 min. and load 10 µL per lane.

2.7.3. To determine the relative level of soluble protein production:

2.7.3.1. Prepare whole cell, soluble and insoluble extract as described in Task 3: Screen for Target Protein Expression, step 4 or by sonication.

2.7.3.2. Load 5µg of total protein per lane of a 10cm gel (4-20% Tris-Glycine gradients are recommended).

2.7.4. Stain the gel with Coomassie Blue, colloidal Coomassie Blue or silver stain. *(See Tips 4 and 5.)*

2.7.5. Use functional and immunoblot assays to complement the SDS-PAGE analysis and to verify the identity of the protein.

3. Interpretation

3.1. After staining the gel, observe each lane and compare the "pre-induction" sample with the "post-induction" sample from each medium. Elevated expression is indicated by the presence of a unique polypeptide band corresponding to the molecular mass of the target protein in the "post-induction" sample.

3.2. Compare the level of target protein obtained from cells grown in each of the six media. ***Select the medium which produces the highest level of target protein per ml of culture.***

3.3. If two or more media give the same level of production per ml, then use the analysis of 2.7.2 to select the medium which gives the highest relative level of expression.

3.4. Compare the level of target protein obtained in the soluble fractions for each of the six media. ***Select the medium which produces the highest level of target protein in the soluble fraction.*** *(See Tip 2.)*

Supplemental Protocol 3: Determine the Critical Factors for Expression.

1. Materials

1.1. Expression strain

1.2. Medium – identified in Step 2

1.3. 50x Augmedium™

1.4. Two shaking incubators set at 37°C and 27°C.

1.5. 1 M IPTG

2. Methods

2.1. Inoculate a 50 mL culture with a single colony of the expression strain and incubate at 37°C overnight.

2.2. Inoculate 500 mL of fresh medium with the overnight culture.

2.3. Incubate at 37°C until the absorbance at 600nm reaches 1.0 ± 0.2.

2.4. Remove a 1mL sample, harvest the cells by microfuging and suspend the cells in water to give 10 OD/ml. Store at -20°C.

2.5. Dispense 25mL of the culture into each of 16 baffle bottomed flasks (250mL).

2.5.1. Alternative Protocol 1: This experiment can be done in sets of eight cultures. Reduce the culture volume to 250mL.

2.5.2. Alternative Protocol 2: If an incubator that can shake microtiter plates is available, this experiment can be done using 24-well or 6-well plates. Adjust the volume of medium accordingly.

2.6. Add the requisite volume of 50 x Augmedium™ to give the prescribed final amount according to Table 2.

2.7. Transfer the cultures to the indicated temperatures and incubate for 20 min.

2.8. Add IPTG to the final concentrations shown in Table 2.

2.9. Incubate for 3 hours at the temperatures indicated.

2.10. Remove 1mL samples and process as in step 2.4.

Critical Factors that Affect Accumulation of a Target Protein			
Culture Number	**IPTG (mM)**	**Temperature (˚C)**	**Augmedium™ (x)**
1	1	37	0.1
2	1	27	0.1
3	0.05	37	0.1
4	1	37	1
5	1	27	0.1
6	0.05	37	0.1
7	0.05	37	1
8	0.05	27	1
9	1	37	1
10	1	37	0.1
11	0.05	37	1
12	0.05	27	1
13	1	27	1
14	1	27	1
15	0.05	27	0.1
16	0.05	27	0.1

Table 2. Experimental design matrix for determining critical factors that affect accumulation of a target protein.

2.11. Harvest the remainder of the cultures and store the cells at -20°C.

2.12. Prepare cell-free extracts as described in Task 3 , step 4.

2.13. Analyze the whole cell, soluble and insoluble fractions as in Task 3, step 4., by SDS-PAGE and if available functional or immunoblot assays.

3. Interpretation

3.1. Determine the amount of target protein accumulated in each of the different culture conditions.

3.1.1. To determine the approximate total amount of protein produced, analyze the whole cell extracts by SDS-PAGE and calculate the amount of protein using densitometry scans of the stained gel.

3.1.2. To determine the amount of target protein in the soluble fraction, prepare cell-free extracts and measure the amount of activity (by functional assay) or amount of protein (by SDS-PAGE). (Alternatively, a quantitative immunoblot could be used to determine the relative accumulation levels.)

3.2. Determine the factors which are having a major effect on protein accumulation.

Critical Factors that Affect Accumulation of a Target Protein				
Culture Number	**IPTG**	**Temperature**	**Augmedium™**	
1				
2				
3				
...				
...				
16				
	0	0	0	Sum_{High}
	0	0	0	Sum_{Low}
	0	0	0	Relative Effect

Table 3. Template for determining critical factors.

3.2.1. Prepare a spreadsheet with 16 rows corresponding to cultures numbered 1 to 16 and 4 columns corresponding to each of the factors tested (inducer concentration, temperature, Augmedium™ concentration) as shown in Table 3.

3.2.2. Enter the value (i.e., enzyme activity, mass, pixel density etc.) obtained for each culture into each cell in the row. For any given culture each factor will have the same value entered. (Note: numeric descriptors for qualitative assessments will also work, but with less accuracy.)

3.2.3. Calculate the sum of protein recovered for each factor when the factor was high in the solution. Sum_{High} (For example, for IPTG sum cultures 1,2, 4, 5, 9, 10, 13 and 14.)

3.2.4. Calculate the sum of protein recovered for each factor when the factor was low in the solution. Sum_{Low}. (For example, for IPTG sum cultures 3, 6, 7, 8, 11, 12, 15 and 16.)

3.2.5. Calculate the difference between the High and Low and divide by 8 for each factor. Relative Effect = $[Sum_{High} - Sum_{Low}] / 8$.

3.2.6. Compare the Relative Effect numbers obtained.

3.2.6.1. A positive number indicates a positive effect on protein production.

3.2.6.2. A negative number indicates no or an adverse effect on protein production.

3.2.6.3. The larger the positive number the greater the effect of the given factor.

3.2.7. Once the critical factor or factors have been identified, the culture conditions can be further optimized by examining the effect on protein accumulation of the identified factor. Supplemental Protocol No. 2 provides a suggested method.

3.2.8. Alternatively, if the production level is sufficient for the intended use of the target protein, the culture conditions yielding the highest level of production can be used for further work.

3.2.9. Other factors that can affect the level of protein accumulation, particularly soluble protein, include i) induction duration, ii) glycine levels, iii) non-ionic detergent levels, and iv) lactose versus IPTG (in $LacY^+$ strains only).

4. Optimization of the Culture Conditions: For the optimization of one factor only.

4.1. Prepare a 150 mL final volume culture as described in Task 3, steps 2.1-2.4.

4.2. Divide the culture into five 25 mL portions and treat as described for the factor being tested, using two levels above and two below the level found to give higher relative accumulation in Task 3.

4.3. Determine the conditions that yield the highest level of target protein using a functional assay, SDS-PAGE or immunoblot.

4.4. Refine as needed for each factor.

Tips of the Trade

Tip 1: Media Optimization Inducer

The inducer used will depend on the expression system employed. The concentration of inducer is strain-dependent and the optimum concentration should be determined empirically. For *lac*P-based expression systems, 1 mM IPTG is good for the media optimization.

Tip 2: Inclusion Bodies

Some recombinant proteins are expressed in *E. coli* as insoluble particles known as inclusion bodies. The formation of inclusion bodies can not be predicted, but are indicated by the presence of intracellular refractive objects when viewed under oil immersion microscopy.[3] The formation of inclusion bodies will not affect the results of the media screen, because the analysis is done on whole cell extracts prepared by boiling the cells in sodium dodecylsulfate. This procedure completely denatures inclusion bodies as well as membrane and cytoplasmic proteins. (In some instances lowering the temperature after induction can increase the amount of soluble protein recovered. This should be determined experimentally.)

Tip 3: Cell Paste

Once the medium yielding the highest level of expression has been determined, the cell paste can be used to prepare a small-scale extract.

Tip 4: SDS-PAGE gels

Coomassie Blue stain should be sufficient to visualize the expression of a recombinant protein. Silver stain, while allowing detection of smaller amounts of protein, is more difficult to interpret and should only be used for examining whole cell extracts which are separated by SDS-polyacrylamide gels that are 20 cm in length or longer. The long gel will give better resolution of individual polypeptide bands.

Tip 5: Alternative Techniques

Alternative techniques can be applied to the media screen analysis. Immunoblot or functional assays can be employed as appropriate. Care should be taken when using functional assays by first demonstrating that there is no interfering activity contributed by the host. In most cases, the SDS-PAGE analysis is the method of choice during the early stages of developing the expression system. Immunoblots should be used when the Coomassie blue stain does not reveal any expressed protein.

References

1. Gralla, J., D. 1990. Promoter Recognition and mRNA Initiation by *Escherichia coli* $E\sigma^{70}$. Methods in Enzymology 185:37-54.

2. Broedel, Jr., S. E. and Papciak, S. M. 2007. ACES™ Promoter Expression Vector. Athena Environmental Sciences, Inc., Technical Brief, December 2007, http://athenaes.com/osc/TechBrief_ACESPromoter_Web.pdf.

3. Broedel, Jr., S. E., S. M. Papciak, and W. R. Jones. 2001. The selection of optimum medium formulations for improved expression of recombinant proteins in *E. coli.* Athena Enzyme Systems Technical Bulletin , January 2001.

Technical Assistance

The scientific staff of the Athena Enzyme Systems™ are specialists in the expression of recombinant proteins in microbial systems. They have extensive expertise in all aspects of protein expression from the construction of expression vectors to the commercial production of recombinant proteins. No matter what your question, please feel free to ask them for help. A technical support scientist can be reached at support@athenaes.com.

Product Use Limitations

The ACES™ Promoter Selection Kit was designed and is sold for research use only. It should not be used for human diagnosis or drug use or administered to humans unless expressly cleared for that purpose by the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials contained in the kit.

Product Warranty

AthenaES™ guarantees the quality and performance of the media and reagents contained in this kit for the cultivation of *E. coli*. The suitability of a medium formulation or additive for a particular use is the responsibility of the end user. No guarantee is made that a given protein will be expressed when applying this kit. AthenaES™ will replace the product free of charge if it does not conform to the stated specifications. Notice for replacement must be received within 60 days of opening the product.

Ordering Information

To place an order:

Phone: 1-888-892-8408 Email: media@athenaes.com
Fax: 410-455-1155 Website: www.athenaes.com

Or visit our website to order through one of our international distributors.

When placing an order, please provide the following:

- Institution name and customer service account
- Purchase order number
- Catalog number(s) or names of products and quantity of item(s)
- Billing and shipping address
- Contact name and telephone number

Delivery:

Telephone orders received Monday through Friday before 12 noon will be shipped that day. All other orders will be shipped the next business day, unless otherwise stipulated.

ACES™ Accessory Products Information

Catalog Number	Product	Size
0149-25	pAES25	10µg
0149-30	pAES30	10µg
0149-31	pAES31	10µg
0149-32	pAES32	10µg
0149-33	pAES33	10µg
0149-34	pAES34	10µg
0149-35	pAES35	10µg
0149-40	pAES40	10µg
0150-1	Primer A	250pmoles
0150-2	Primer B	250pmoles
0151-JM109-C	JM109 Competent Cells	2 x 200µL
0151-	Strain Stab	Stab
0152-1	Inducer Solution A	1mL
0152-5	Inducer Solution A	5 x 1mL
0153	Inducer Solution B	500mL
0154	Secretion Enhancer A	500mL
0155	Secretion Enhancer B	500mL
0156	Rapid Transformation Kit	1 kit
0157	2x TSS	5 x 1 mL
0313-1	Anti-YebF Antisera	0.5mL