ACES™

ACES™ ■ YebF Protein Export Kit

AthenaES™ Complete Expression System

The ACES™ YebF Protein Export Kit uses the YebF protein to chaperone target proteins through the inner and outer membranes of *E. coli* cells to the extracellular medium in active form, allowing for the expression of toxic and difficult-to-fold proteins.

AthenaES®

Athena Enzyme Systems™
888-892-8408
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Application Manual
V 1.1

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YebF Protein Export Kit

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AthenaES®

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ACES™ YebF Protein Export Kit

Application Manual

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Introduction

E. coli is often the host strain of first choice for the production of recombinant proteins. Despite its long and successful history, several limitations remain when the protein desired can not be expressed in a functional state. This is often due to the inherent properties of expressing heterologous proteins in the cytoplasm. One means of overcoming these impediments is to express the protein such that it is secreted into an environment that is more conducive to correct folding and where proteolytic degradation is minimized such as the periplasm or extracellular matrix. Periplasmic or extracellular protein export has been exploited for the production of a number of recombinant proteins.[1] The extracellular accumulation of a target protein provides several advantages. These include:

- **A Simplified Downstream Purification Scheme**

E. coli does not naturally export a significant number or amount of proteins to the extracelluar matrix. Therefore, the contamination level of host cell proteins as well as endotoxin and nucleic acids, contaminants that present considerable challenges to the purification of proteins destined for pharmaceutical use, would be significantly reduced.

- **Enhanced Biological Activity**

Export through the periplasmic space exposes proteins to a set of disulfide isomerases and foldases which facility correct protein folding as well as into an oxidizing environment which favors disulfide bridge formation.

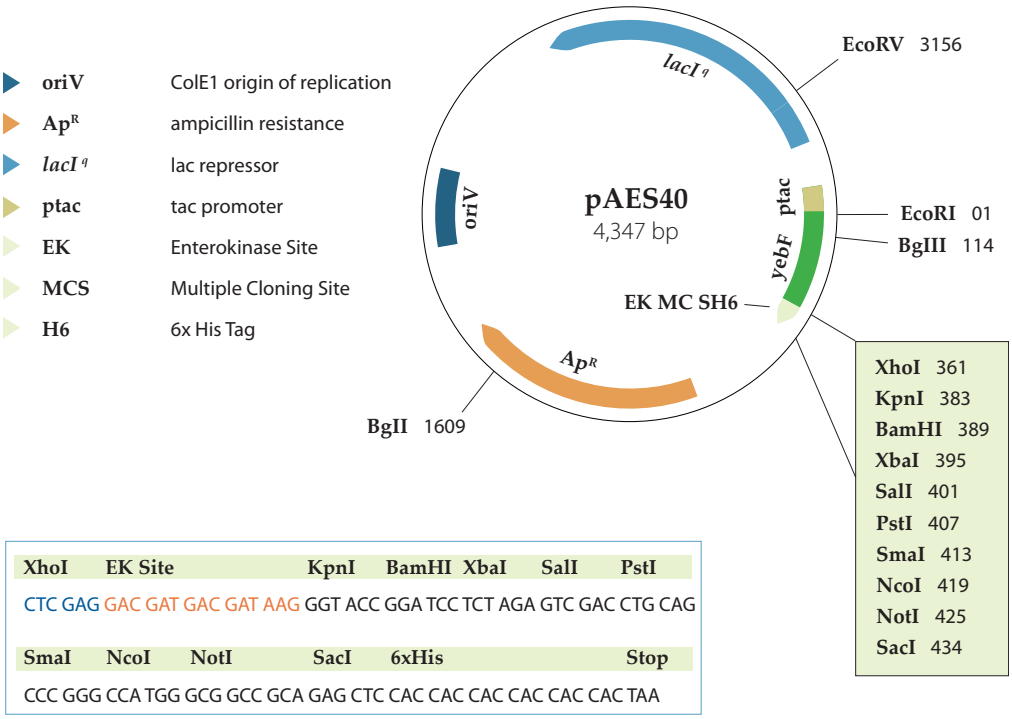
- **Higher Product Stability and Solubility**

In addition to chaperones, there are fewer proteases in the periplasmic space and even fewer extracellular proteases.

However, *E. coli* does not normally secrete large numbers or quantities of proteins to the extracellular environment. Most secreted proteins reside in the periplasmic space. In order to export recombinant proteins to the culture medium a carrier protein is needed to effect transport across the outer membrane. One such protein shown to support protein export to the culture medium is YebF.[2] YebF has an unknown function, but is an actual extracellular protein. It also effectively transports both small and large prokaryotic and eukaryotic proteins to the extracellular medium in an active form. In this study, the *E. coli* expression vector pMS119 (Ap^R, *tac*p),[3] was used to construct pYebFH6/MS. This plasmid expresses wild-type YebF protein under the control of a IPTG-inducible promoter, *tac*p, and with a C-terminal hexa-His affinity tag. Analysis of the subcellular localization of the YebFH6 protein after induction showed that the protein accumulated in the culture medium. To demonstrate that YebF could facilitate the export of other proteins, C-terminal fusions were made by inserting the coding sequences for mature alkaline phosphatase (*E. coli pho*A), α-amylase (*Bacillus subtilis X-23, amy*) and the human IL-2 between the C-terminal residue of YebF and the His tag. After induction all three proteins were found to accumulate in the culture medium, indicating that the YebF protein could effect extracellular transport of the fused protein. Importantly, cytoplasmic proteins did not leak into the medium. Therefore, YebF can be used to facilitate the extracellular export of recombinant proteins. Athena scientists have extended the work of Zhang *et al*.[2] by demonstrating that the YebF export function works in several commonly strains of *E. coli* that are used for the expression of heterologous proteins including HB101, HMS174, BLR and TOP10 as well as with other proteins.[4] The basis for the ACES YebF Protein Export kit is a more user friendly vector along with the accessory reagents needed for the successful production of a recombinant protein.

Principle of the Kit

YebF is used as a carrier protein to direct the extracellular export of other recombinant proteins. This is achieved by fusing the desired protein to the C-terminus of YebF. After induction of expression, the fusion protein is exported to the culture medium where it accumulates. The fusion protein is recovered and the YebF portion removed using an endoprotease.



XhoI	EK Site	KpnI	BamHI	XbaI	SalI	PstI
CTC GAG	GAC GAT GAC GAT AAG	GGT ACC	GGA TCC	TCT AGA	GTC GAC	CTG CAG

SmaI	NcoI	NotI	SacI	6xHis	Stop
CCC GGG	CCA TGG	GCG GCC	GCA GAG CTC	CAC CAC CAC CAC CAC CAC	TAA

Blue - C-terminal amino acids of YebF
Orange - Enterokinase cleavage site

Figure 1. Plasmid map and MCS of pAES40

To use YebF as a carrier protein for the extracellular production of recombinant proteins, the gene encoding the desired protein is subcloned into the plasmid pAES40 (Fig. 1). The multiple cloning site for constructing the YebF-Target Protein fusion is shown in the vector drawing. The C-terminal two amino acids of YebF are the XhoI site with the remainder of the sequence written in the reading frame of YebF. An enterokinase proteolytic cleavage site (GAC GAT GAC GAT AAG) is between the multiple restriction sites used for subcloning and the end of YebF to permit removal of the YebF sequences after export. A hexa-His sequence is at the end of the MCS to provide an affinity tag if desired.

Kit Components

ACES™ YebF Protein Export Kit Components		
Component	**Amount**	**Catalog Number**
pAES40	10 µg	0149-40
Anti-YebF Antisera	0.5 mL	0313-1
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
Inducer Solution B	100mL	0153
Secretion Enhancer Solution A	60mL	0154
Secretion Enhancer Solution B	60mL	0155
Reagents needed but not provided: Glycerol		

Protocols

Preparation: Media

1. Dissolve the contents of each of the media packets in deionized water as directed on the individual packets.
2. Add 4mL of glycerol to the Turbo Broth™ and Power Broth™ solutions.
3. Dispense desired volume into appropriate bottles or flasks. (We recommend 2 x 500mL glass bottles.)
4. Autoclave at 121°C for 20 min. The autoclaved media without antibiotics are stable for 6 months at 4°C.
5. Dissolve the contents of the Glucose Nutrient Mix in 100mL deionized water and filter sterilize using a 0.2µm filter.
6. Add 50mL of the sterile Glucose Nutrient Mix to 1 liter of Hyper Broth™ and 20mL to 1 liter of Glucose M9Y using aseptic technique.
7. Add sterile antibiotics as needed.

Preparation: Medium Supplements

1. Dissolve the contents of the Augmedium™ container in 25mL deionized water and filter sterilize using a 0.2µm filter. Aliquot 5mL portions into sterile 15mL conical tubes and store at -20°C. The stock will be a 50x solution. For use, snap thaw at 37°C and store at 4°C for no more than five (5) days.

Step 1: Subclone the Desired Gene Sequences into pAES40

Use any of the restriction sites located downstream of the enterokinase cleavage site to insert the coding sequence for the target protein. Be sure to design the subcloning such that the reading frame of the target protein matches that of YebF.

Step 2: Express the YebF-Target Protein Fusion

The protocol presented below is the basic method for producing and recovering proteins exported using the YebF Export System. Suggested alternative methods to this basic scheme are noted at the respective step. Briefly, a Lac operon-based induction protocol is employed. IPTG or Lactose can be used as the inducer. IPTG is used initially at 50 µM and can be increased to 1 mM depending on the viability of the host strain and production levels of the target protein. Lactose is used at 2%. For the YebF Export System, induction times should be greater than 16 hours to allow the exported protein to accumulate in the culture medium. Any rich medium can be employed.

Basic Protocol: (For 1 Liter, Adjust Volumes as Needed)

1. Introduce the plasmid construct from Step 1 into the desired production strain.

    1.1. Supplemental Protocol 1 gives a protocol for the rapid transformation of any *E. coli* host.

    1.2. Streak purify two to eight transformants and verify the integrity of the plasmid construct.

    1.3. Store the strains accordingly.

2. Use a single colony to inoculate a 50 ml starter culture supplemented with 100 µg/ml ampicillin.

    2.1. Turbo Broth™ or Turbo Prime Broth™ (AthenaES™ Cat. Nos. 0104 and 0110, respectively) are recommended but any rich medium will suffice.

    2.2. Incubate at 37°C overnight.

3. Inoculate 1 liter of medium supplemented with 100 µg/ml ampicillin the overnight culture and incubate at 30°C until the A600 reaches 0.8-1.0.

   3.1. The use of baffle bottomed flasks is strongly recommended to provide sufficient aeration.

   3.2. Turbo Broth™ or Turbo Prime Broth™ are recommended but any rich medium will suffice.

4. Induce expression by adding IPTG to 50 µM (50 µl Induction Solution A) or lactose to 2% (100 ml Induction Solution B).

   4.1. Note: Lactose can only be used if the host strain is wild-type for lacZ and lacY.

5. Incubate at 30°C for 20-24 hours.

6. Remove the cells by centrifugation at 5,000 xg for 30 min.

7. Add 472 g ammonium sulfate to the medium and dissolve thoroughly.

8. Chill on ice for 2 hours with gentle stirring.

9. Collect the precipitated protein by centrifugation at 15,000 xg for 30 min. at 4°C.

10. Suspend the pellet in 25 ml of the desired buffer and proceed with subsequent purification steps as needed.

Optimization Protocol:

1. Introduce the plasmid construct from Step 1 into each of three to six host strains.

   1.1. Most commonly used strains for the production of recombinant proteins can be used. See Supplemental Protocol 1 for a simple rapid transformation protocol. It is recommended that the parent pAES40 plasmid be introduced into each strain used to serve as a control in expression experiments.

2. Select the strains with the highest level of export of the fusion protein.

   2.1. Inoculate 5 ml of Turbo Broth™ (AthenaES™ Cat. No. 0104) supplemented with 100 µg/ml ampicillin with a single colony of each strain harboring the pAES40 derivative. Incubate the culture at 37°C overnight.

   2.2. Use the overnight culture to inoculate duplicate cultures of 25 ml fresh Turbo Broth™ supplemented with 100 µg/ml. Incubate the cultures at 30°C until the absorbance at 600 nm reaches 0.8. Remove a 1 ml sample, remove the cells and store the culture supernatant at 4°C. (Note: The cells can reserved by storing at -20°C.)

2.3. Induce expression by adding IPTG to 50 µM or lactose to 2%.

2.3.1. Dilute Inducer Solution A by adding 5 µl to 495 µl sterile water. Add 125 µl of the diluted solution to one culture for each strain. Store the remainder of the diluted solution at -20°C for use in later experiments.

2.3.2. To the duplicate culture, add 2.5 ml of Inducer Solution B. (Note: In some cases expression may be enhanced by using 2% lactose in place of IPTG as the inducer. This is suitable only for strains which are wild-type for lacZY.)

2.4. At 3-6 hours post-induction remove a 1 ml sample and process as in step 2.2.

2.5. At 20-24 hours post-induction remove a 1 ml sample and process as in step 2.2.

2.6. Remove the cells by centrifugation and store the culture supernatant at 4°C.

2.7. Analyze the culture supernatants by immunoblot, ELISA of functional assay. Do not use a stained gel to visualize the expression of the YebF fusion as the accumulation levels in a non-optimized system may be below the detection limits of SDS-PAGE. The immunoassays can be done with anti-Target Protein, anti-YebF (AthenaES Cat. No. 0313), or anti-His tag antibodies.

2.8. Select the strain that produced the most YebF-Target Protein per ml of culture.

3. Determine the optimum medium for production.

3.1. Perform a medium screen as described in Supplemental Protocol 2 and select the medium that yields the highest level of Target Protein per ml.

4. Optimize the culture conditions for production of the fusion protein.

4.1. Determining the optimal culture conditions is an iterative process. First, a two-level fractional factorial experimental design is used to identify the critical factors affecting accumulation of the Target Protein.1 Table 1 lists the conditions to be tested. Use an immunoassay or functional assay to quantify the level of expression and select the conditions that yield the highest level of production. Supplemental Protocol 3 provides a method for interpreting the data. Further optimization is then done by modifying those parameters identified as critical to accumulation of the target protein.

Critical Factors that Affect Accumulation of the Target Protein						
Culture	IPTG (mM)	Lactose (%)	Glycine (%)	Triton X-100 (%)	Augmedium™ (x)	Temp.
1	1	0	1	0	0	37°C
2	0.05	2	1	0	0	27°C
3	1	2	1	1	1	37°C
4	1	0	0	0	1	27°C
5	1	2	0	0	0	37°C
6	1	2	1	0	1	27°C
7	0.05	0	0	0	0	27°C
8	1	0	0	1	1	37°C
9	0.05	2	1	1	0	37°C
10	1	0	1	1	0	27°C
11	0.05	2	0	1	1	27°C
12	1	2	0	1	0	27°C
13	0.05	0	0	1	0	37°C
14	0.05	0	1	0	1	37°C
15	0.05	0	1	1	1	27°C
16	0.05	2	0	0	1	37°C

Table 1. Experimental design matrix for determining critical factors that affect accumulation of a target protein.

Step 3: Purification of the YebF-Target Protein

Using the strain and culture conditions identified in Step 2, perform a production using 0.5 to 1 liter sized cultures. It is strongly recommended that baffle bottomed flasks be used to ensure adequate aeration. The YebF-Target Protein is recovered from the culture medium as follows:

1. After 20-24 hours post-induction, remove the cells by centrifugation at 5,000 xg for 20 min.

2. For each liter of medium, add 472 g of ammonium sulfate.

3. Allow the salt to completely dissolve and then chill the medium on ice for 2 hours with gentle stirring.

4. Dispense the mixture into centrifuge bottles and collect the precipitated protein at 15,000 xg for 30 min. at 4ºC.

5. Decant the liquid and suspend the pellets in 25 ml of a buffer suitable for the next purification step.

    5.1. To endure complete removal of any residual cells or debris, filter the solution using a 0.45 µm cartridge or depth filter.

6. Dialyze against the desired buffer to ensure removal of the ammonium sulfate and process as needed.

Supplemental Protocols

Supplemental Protocol 1: Rapid Transformation Protocol

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 micro(centri)fuge 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 for 30 min.

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. 25 mL of each culture medium in 250 mL baffle bottomed flasks.

1.2. Wash Buffer: 40 mM sodium phosphate pH 7.5, 150 mM NaCl

1.3. 2x SDS-PAGE Loading Dye: 125 mM Tris-Cl pH 6.8, 4% SDS (w/v), 0.005% bromophenol blue (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v)

1.4. Tris-Glycine SDS-polyacrylamide gel of appropriate composition.

2. Methods

2.1. Inoculate a single colony of the recombinant strain into 10 mL of LB Broth in a shake flask with baffle bottom. Incubate at 37°C overnight.

2.2. Inoculate 25 mL of each of the six media with 1 mL of the overnight culture. Incubate the cultures at 37° C until the OD_{600} reaches 0.6.

2.3. Remove a 1 mL sample ("pre-induction"), harvest the cells in a pre-weighed microfuge tube, and store on ice.

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

2.5. Remove a 1 mL sample ("post-induction") and process as in step 2.3

2.6. Harvest the remainder of the culture, wash with 10 mL of wash buffer, 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. Suspend the cell pellets from the pre- and post-induction samples in 0.5 mL of water.

2.7.1.2. Mix 5 µL of each cell suspension with 7.5 µL water and 12.5 µL 2x SDS-PAGE loading buffer. Heat at 100°C for 5 minutes and load 10 µL per lane of acrylamide gel.

2.7.2. To determine the relative level of expression:

2.7.2.1. Suspend the cell pellets from the pre- and post- induction samples in water to a density of 10 OD/mL

2.7.2.2. Mix 5 µL of each cell suspension with 7.5µL water and 12.5µL 2x SDS-PAGE loading buffer. Heat at 100°C for 5 minutes and load 10 µL per lane of acrylamide gel.

2.7.3. Stain the gel with Coomassie Blue, colloidal Coomassie Blue or Silver stain. (see Tips 4 and 5).

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 from cells grown in each of the six media. Select the medium which produces the highest level of target protein per mL of culture. Figure 1 shows the results of a media screening experiment.

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 with the highest relative level of expression.

Supplemental Protocol 3: Determining the Critical Factors for Expression.

1. Prepare a spreadsheet with 16 rows corresponding to culture conditions 1 to 16 and 6 columns corresponding to each of the factors tested and the solutions as shown in the figure below.

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

3. Calculate the sum of protein produced for each factor when the factor was present in the solution. $Sum_{Present}$

    3.1. Note for IPTG consider the 1 mM level "Present."

4. Calculate the sum of protein recovered for each factor when the factor was absent from the solution. Sum_{Absent}

    4.1. Note: For IPTG consider the 0.05 mM level "Absent."

5. Calculate the difference between the Present and Absent and divide by 8 for each factor. Relative Effect = $Sum_{Present}$ - Sum_{Absent} / 8.

6. Compare the Relative Effect numbers obtained.

    6.1. A positive number indicates a positive effect on accumulation.

    6.2. A negative number indicates no effect on accumulation.

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

Critical Factors that Affect Accumulation of the Target Protein						
Culture	**IPTG (mM)**	**Lactose (%)**	**Glycine (%)**	**Triton X-100 (%)**	**Augmedium™ (x)**	**Temp. (°C)**
1						
2						
.						
.						
.						
15						
16						
$Sum_{Present}$						
Sum_{Absent}						
Rel. Effect						

Table 2. Example analysis table used to determine the critical factors to protein accumulation.

Tips ??

References

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5. Montgomery, D. C. 2001. Design and Analysis of Experiments. John Wiley & Sons, Hoboken, NJ, ISBN No. 0-471-31649-0.

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™ YebF Protein Export Kit and Vector were designed and are sold for research use only. None of the kit components should 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.

The plasmids and other components contained in the ACES™ YebF Protein Export Kit are covered under international patents and patent applications exclusively licensed by the University of Alberta to Athena Environmental Sciences, Inc. The use of these reagents requires a license.

- Academic and Not-for-Profit entities are required to execute a non-commercial use license. This agreement is a non-fee bearing license which grants the user the rights to use the plasmid and other kit components for research purposes only and restricts the user from dissemination the plasmids to other researchers without the expressed written consent of AthenaES.

- Commercial users are required to execute a commercial evaluation license agreement. The agreement is a non-fee bearing license which grants to the user the right to use the plasmid and other kit components for research purposes only for the period of one (1) year after which a commercial use license is required.

Copies are both types of license agreements are available at <insert website>.

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 many 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