Technical Brief:
Dream Nutrient Mix™
Sheldon E. Broedel, Jr., Ph.D.
Athena Environmental Sciences, Inc., Baltimore, MD
AthenaES
Athena Enzyme Systems™
1450 South Rolling Road
Baltimore, MD 21227
www.atheneas.com

Dream Nutrient Mix™
For the Overnight Expression of Recombinant Proteins

By Sheldon E. Broedel, Jr., Ph.D.
Chief Science Officer, Athena Enzyme Systems
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E. coli has been the primary host system for the expression of recombinant proteins for over 40 years. A number of different expression vectors have been developed which permit the controlled production of the target protein. For historical and practical reasons, the *lac* operator is the most widely used control element to repress transcription until induced with the gratuitous inducer IPTG. For optimum results, the IPTG must be added to a culture when a specific cell density is reached and the cells harvested after a defined period of time post-induction, which often varies from protein to protein. This requires close monitoring of the culture growth. Dream Nutrient Mix™ is designed to simplify production in *E. coli* using overnight protein expression. The medium makes use of the diauxic growth properties of *E. coli* that permit the auto-induction of recombinant proteins, a technique that eliminates the need to add IPTG and will often yield higher levels of protein. The medium is suitable for the cultivation of any *E. coli* host strain that is phenotypically Lac^+.

The Basis for Dream Nutrient Mix™

The bacterium *Escherichia coli* has been used as the host organism for the production of recombinant proteins since the late 1970's. Plasmid-based vectors, which permit the controlled expression of the target protein, have become highly refined and several different variations are routinely used. A typical expression vector is designed with a promoter, a section of DNA where RNA polymerase binds and initiates transcription, which yields high transcription rates. Transcription, and ultimately protein production, is controlled by genetic elements that serve to prevent expression under certain conditions (i.e., during the biomass production phase) and permit protein production when desired.[1]

By far the most popular expression vectors are those that employ the genetic control elements of the lactose operon. The lactose operon was one of the first genetic control systems described and the early principles of gene expression were developed by studying this system.[2] The lactose operon is composed of three genes, *lacZ*, *lacY* and *lacA* that encode three proteins, β-galactosidase, lac permease and a transacylase, that allow the microbe to utilize lactose as a carbon source. In the absence of lactose, the proteins are not needed and the bacterium has evolved systems to conserve energy and resources by not producing unnecessary proteins. In terms of genetic control, this means that in absence of lactose the lactose operon is repressed or in the off position. Repression is achieved by blocking transcription from initiating at the promoter. This is accomplished by preventing the RNA polymerase from binding to the promoter when another DNA binding protein is physically obstructing the promoter. This

second protein is the lac repressor. The lac repressor binds to a DNA sequence known as the lac operator which is located adjacent to the promoter sequence but before the transcription initiation site. When the organism needs the proteins encoded in the operon (i.e., when lactose is available as a carbon source), transcription of operon, and consequently protein synthesis, is initiated by inducing the repressor to releases from the operator sequence. This release is accomplished when lactose binds to the repressor. The binding alters the repressor's DNA binding properties and the repressor disengages from the operator allowing transcription and the synthesis of the proteins encoded in the operon to proceed.

This genetic switch has been exploited to create a vast array of expression vectors for the production of a wide variety of proteins. By combining different promoters, such as the strong phage T7 and T5 promoters, with the lac operator sequences and building these constructions into plasmid vectors which simplifies the insertion of gene sequences encoding a desired protein adjacent to the control elements, a means of producing proteins in large quantities under controlled conditions was made possible.[3] These so called expression vectors are introduced into an *E. coli* host where the desired proteins can be made under controlled conditions yielding μg to kg.

Induction, or turning the genetic switch on to synthesize the proteins, is most often done using a lactose analog, isopropyl-thio-β-galactoside (IPTG). IPTG is not cleaved by β-galactosidease and, therefore, cannot be used by the bacterium as a carbon source and has chemical additional properties that make it a more potent inducer than lactose. To produce the desired protein, the *E. coli* strain harboring the expression vector is cultured in a suitable medium and synthesis induced by adding IPTG typically when the culture is in the transition phase between exponential growth and stationary phase. This requires monitoring of the cultures and an aseptic transfer. For large scale production, adding IPTG is expensive both to add to the fermentation and to validate its removal from process streams and requires development work to determine the optimal conditions for induction. For high throughput, small-scaled cultures this requires a means of monitoring potentially hundreds of cultures and aseptically adding the IPTG to many cultures most of which may not be growing synchronously. Further, with the modern expression systems commonly used, induction with IPTG is a binary type of switch; it is off or on. This is beneficial when the protein of interest is easily synthesized and accumulates as a soluble protein in the host cytoplasm. However, if the protein needs to be exported or has a tendency to form insoluble inclusion bodies due to misfolding, one approach to increasing the recovery of soluble protein is to decrease the expression levels. Titration of the inducer, which is often difficult with IPTG, or the use of a weaker inducer is one such means of reducing overall expression levels. Therefore, an alternative approaches for inducing protein production have been devised.

One approach to address these expression challenges is to make use of the diauxic growth properties of *E. coli*. Diauxic growth is the sequential use of carbon sources. When *E. coli* is cultured in the presence of two carbon sources, for example glucose and lactose, the bacterium utilizes the glucose first and then the lactose. This diauxic growth was first described by Monod in 1941[4] and has since been shown to involve genetic control known as catabolite repression.[5,6] Thus, a recombinant strain expressing a target gene under the control of the *lac*

operon will exhibit production of the encoded protein only when the glucose is exhausted and the bacterium begins to consume the lactose.[7] By modulating the carbon source levels, the host strain can be cultured in a medium that contains all of the ingredients needed to promote rapid growth and induce expression of the target protein at an optimal time in the growth of the host bacterium. Several different variations on this technique, known as auto-induction or overnight expression, have been developed each employing slightly different media compositions.[8,9]

The Dream Nutrient Mix™ is designed for the production of recombinant proteins that are expressed in *E. coli*. The nutrient mix is designed to work with any standard medium including LB Broths as well as specialty media formulations designed to increase protein production such as Hyper Broth™, Power Broth™, Superior Broth™ and Turbo Broth™. The Dream Nutrient Mix™ is reformulated for lactose-based induction of the target protein by including low amounts of glucose, which supplement the carbon sources present in the medium base, with higher amounts of lactose (the inducer) as the alternative carbon sources. The nutrient mix also contains additional micronutrients which are known to improve *E. coli* growth and protein expression. When the mix is added to any medium, the Dream Nutrient Mix™ provides the carbon sources and additional nutrients needed for diauxic growth and lactose induction of the target protein which is achieved using an overnight incubation of the culture.

Protocol:

1. Introduce the expression vector, which has the promoter controlling expression under the control of *lacO* into an *E. coli* host strain that is genotypically wild-type for *lacZYA*.
2. Prepare the medium of choice as per the manufacturer's directions and sterilize.
3. Dissolve the contents of the Dream Nutrient Mix™ packet in 100 ml water and filter sterilized.
4. Aseptically add 100 ml of Dream Nutrient Mix™ to 1 liter of medium.
5. Aseptically add the required antibiotic (typically a 1,000x stock solution).
6. Inoculate with the host strain using a 1:100 dilution of a fresh overnight or cryogenically preserved working cell bank.
7. Incubate at 27ºC to 37ºC for 16 to 22 hours with shaking.
    a. For shake flask cultures, the best results are obtained using baffle bottomed flasks containing medium that is 1/5 the total volume of the flask.
    b. For fermentation vessels, maintain a dissolved oxygen level of 30% saturation.
8. Harvest the culture.

<u>Example</u>:

E. coli strain HMS174 harboring the plasmid pAES25-Pt-GFP was used to demonstrate the use of Dream Mix for overnight induction. Plasmid pAES25 is a pBR322 derivative modified to carry the *npt*II gene encoding kanamycin-resistance. Expression of recombinant proteins is directed by the T5 promoter under the control of the *lac* operator sequence which is 5′ to a multiple cloning site. pAES25-Pt-GFP was constructed by inserting the coding sequence for the *Ptilosarcus* green fluorescence protein (Prolume, Ltd., Pinetop, AZ) into pAES25. To demonstrate expression of the GFP protein, 20 µl of a working cell bank[10] was inoculated into 0.5 ml cultures of medium composed of SelenoMet Base Medium (Molecular Dimensions,

Altamonte Springs FL) supplemented with either standard nutrient mix or the Dream Nutrient Mix™ and kanamycin. Each medium was prepared as replicates of four in a 24-well culture dish. The cultures were incubated with shaking at 37ºC for three hours at which time IPTG was added to one set of four wells to a final concentration of 1 mM. The cultures were then incubated for an additional 17 hours. Accumulation of GFP was measured in each culture by diluting the culture 1:10 in buffered saline and recording the fluorescence at 535 nm (485 ex). The relative fluorescence for each set of cultures is shown in Figure 1. These results show that the Dream Nutrient Mix yielded as much fluorescent protein as IPTG with less intervention using a minimal medium of employed for selenomethionine labeling of proteins.

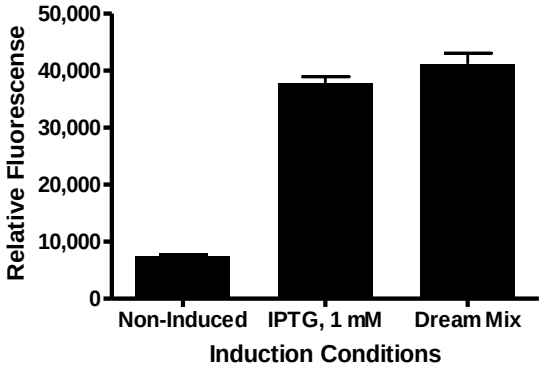

Figure 1. Induction of Pt-GFP using Dream Mix yielded the same level of protein as induction with IPTG.

It is well known that a number of factors can influence the expression and accumulation of a recombinant protein. Beyond the gene and vector design and construction, the host, medium composition and culture conditions can influence expression and whether the protein accumulates as a soluble or insoluble product. As the optimum conditions cannot be known *a priori*, it is often best to perform a screen of different host strains, media types, incubation temperatures and induction regimens. For proteins that are exported, reducing the accumulation rate can increase production. As an example, a *Vibrio* hexosaminidase enzyme[11] was expressed using pET22a with and without a His-tag in three strains, grown at two temperatures in eight different media formulations using not inducer, IPTG or Dream Nutrient Mix. In all, 240 different conditions in duplicate were evaluated for production of the hexosaminidase (Table 1).

Table 1. Conditions used to express the hexosaminidase.

Vector	Strains	Media[a]	T	Inducers
pVAL-CB-pelB	HMS174(DE3)	LB (Miller) Broth	37°C	None
(w/ tag)	BLR(DE3)	Hyper Broth™	27°C	1 mM IPTG
pVAL-CB-ompT	RosettaBlue(DE3)	Power Broth™		1x Dream Nutrient
(w/out tag)		Superior Broth™		Mix™
		Turbo Broth™		
		Power *Prime-olate* Broth™		
		Superior *Prime-olate* Broth™		
		Turbo *Prime-olate* Broth™		

[a] Media were manufactured by Athena Enzyme Systems (Baltimore, MD). BLR(DE3)/pVAL-CB-ompT was not viable.

Briefly, 0.5 ml of each medium supplemented with 50 μg/ml kanamycin was dispensed in duplicate wells of ten 48-well culture dishes (two plates for each strain). Each well was inoculated with 5 μl of an overnight culture of the respective strain harboring one or the other plasmid. To the cultures to be induced using the Dream Nutrient Mix, 50 μl of a 10x stock was

added. The cultures were incubated in a HiGro four tower incubator (Digilab, Framingham, MA) with shaking at 250 rpm at infused with 10 l/m air. One sent of plates was incubated at 25°C (tower 1) and the second set at 37°C (tower 2). After three hours, the plates were removed and IPTG added to 1 mM to the respective wells. The plates were returned to the incubator and after an additional 3 hours incubation, the IPTG induced cultures harvested. The remaining cultures were incubated for an additional 12 hours. The culture supernatant was collected and the hexosaminidase activity determined using p-nitrophenyl-N-acetyl-β-D-glucosaminide substrate. The initial rate of change in absorbance at 405 nm over the first 2 min of the reaction was used to calculate the enzyme activity.

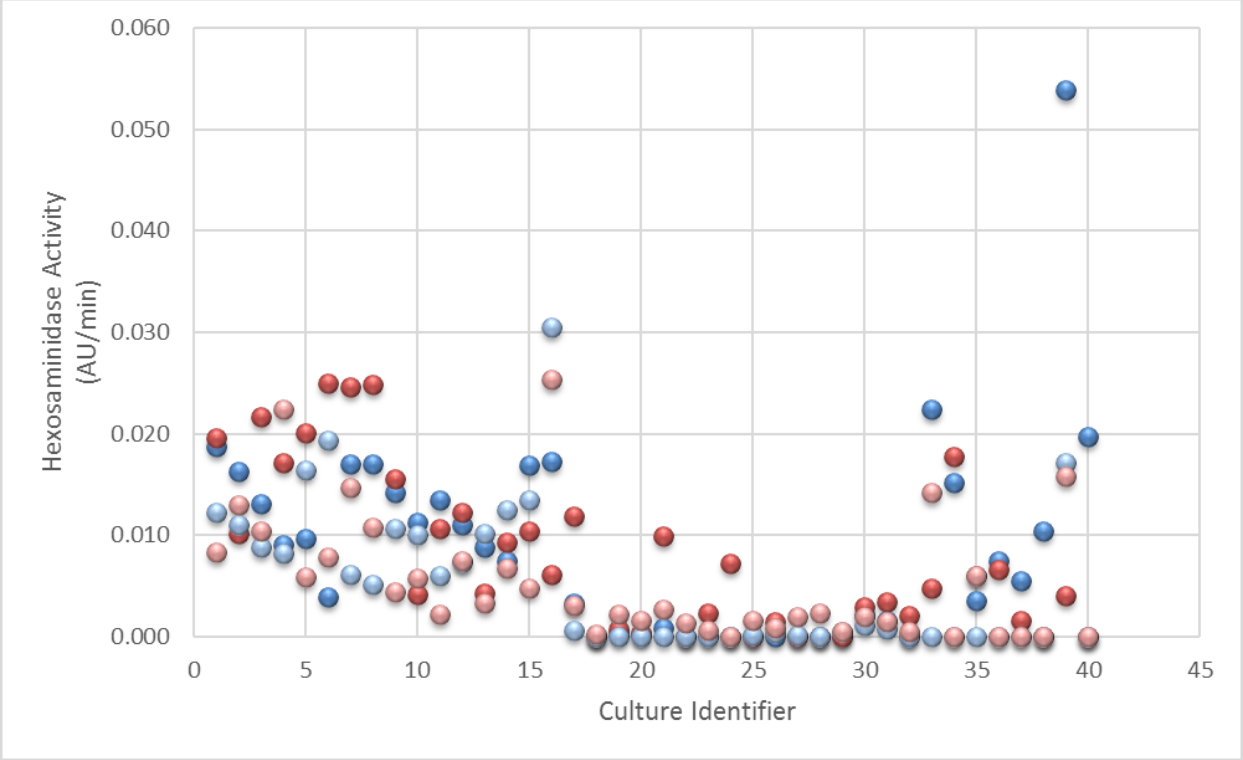

Figure 2. Hexosaminidase activity in cultures induced with IPTG or Dream Nutrient Mix™. Culture were cultivated as described in the text. Enzyme activity was measured in the culture supernatant using a Tecan Genios microplate reader in 96-well plates. Duplicate reactions of each sample contained 10 µl of culture supernatant, 90 µl of 1x PBS pH 7.4 and 100 µl of 200 µM p-nitrophenyl-N-acetyl-β-D-glucosaminide. The absorbance at 405 nm was measured at 10 sec intervals over 5 min and the rate calculated from the initial 2 min and the rate from non-induced cultures subtracted from corresponding induced culture. Red dots were cultures induced with Dream Nutrient Mix, blue dots were IPTG induce cultures. Light colored dots were cultures at 25°C while the darker colored dots were cultures incubated at 37°C. Cultures #1 to #8 were HMS174(DE3)/pVAL-CB-pelB; #9 to #16 were strain HMS174/pVAL-CB-ompT; #17 to #24 RosettaBlue(DE3)/pVAL-CB-pelB; #25 to #32 RosettaBlue(DE3)/pVAL-CB-ompT; #33 to #40 BLR(DE3)/ pVAL-CB-pelB in in LB Broth, Hyper Broth, Power Broth, Power *Prime-olate* Broth, Superior Broth, Superior *Prime-olate* Broth, Turbo Broth and Turbo *Prime-olate* Broth, respectively.

The level of hexosaminidase activity ranged from a low of zero activity to 53 mAU/min. As is often observed, the level of enzyme activity differed between strains, media composition, incubation temperature and induction method. All of the cultures with the HMS174 strains yielded enzyme activity above that of the corresponding non-induced cultures whereas most of

the RosettaBlue and some of the BLR strains did not show activity much above the non-induced cultures. HMS174(DE3)/pVAL-CB-pelB gave the highest levels of enzyme when cultured in Dream Nutrient Mix at 37°C. In contrast, the HMS174(DE3)/pVAL-CB-ompT strain gave only slightly higher levels of enzyme activity with IPTG induction as compared to the Dream Nutrient Mix and overall lower than the HMS174(DE3)/pVAL-CB-pelB strain induced with Dream Nutrient Mix. While both RosettaBlue strains yielded lower levels of activity than the HMS174 or BLR strains, higher levels of enzyme activity were observed when induced with Dream Nutrient Mix as compared to IPTG. The BLR/pVAL-CB-pelB strain yielded the highest level of enzyme activity when grown at 37°C in Turbo Broth and induced with IPTG. The other BLR cultures yielded enzyme activity levels comparable but collectively lower than the HMS174 strains with IPTG induction at 37°C and Dream Nutrient Mix at 25°C yielding the higher levels of activity amongst the BLR set.

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[10] The working cell bank was prepared by growing the strain overnight in Turbo Broth™ medium (Athena Enzyme Systems, Baltimore, MD) supplemented with 50 μg/ml kanamycin, adding sterile glycerol to 20%, dispensing 1 ml of the mixture into 1.5 ml sterile vials and storing the vials at -80ºC.

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