Use of glucose to control basal expression in the pET System

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Many popular bacterial expression systems, including the pET system, contain components of the *lac* operon. For example, in the pET system transcription of the target gene is controlled by a bacteriophage T7 promoter, and the production of the T7 RNA polymerase in expression hosts (λDE3 lysogens) is regulated by a *lac* promoter derivative, the *E. coli* L8-UV5 *lac* promoter (see Figure 1).

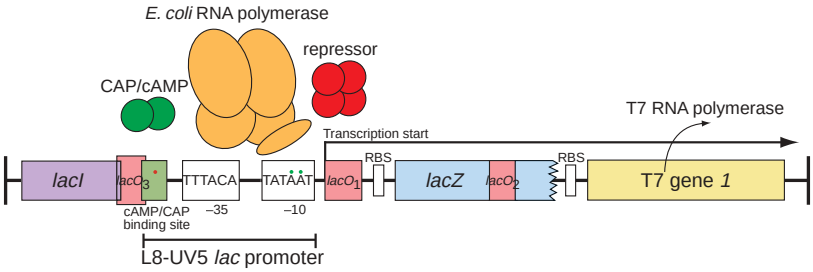

Figure 1. Transcriptional control of T7 gene *1* in λDE3 lysogens

Transcription of T7 gene *1* (encoding T7 RNA polymerase) in pET System expression hosts (λDE3 lysogens) is controlled by the L8-UV5 *lac* promoter. T7 gene *1* is transcribed as the second gene in a bicistronic mRNA (the first gene contains an N-terminal fragment of *lacZ* that includes the α-peptide coding region). Positions of the three mutations of the wild type *lac* promoter region are indicated by colored circles. The *lac* repressor (*lacI* gene product) binds to $lacO_1$, and then interacts with pseudo-operators $lacO_2$ and $lacO_3$ to prevent transcription by *E. coli* RNA polymerase. The inducer IPTG binds to the repressor, reducing its affinity for $lacO_1$ and thus enabling transcription to occur. When cAMP levels are sufficiently high (e.g., in the absence of glucose) the CAP/cAMP complex is formed and binds immediately upstream from the promoter to fully stimulate transcription. In the presence of glucose, CAP/cAMP is not formed and transcription is decreased. This is called the glucose effect, or catabolite repression.

Negative regulation by *lac* repressor

The wild type *lac* operon has two distinct mechanisms of regulation; one is negative (decreases transcription), and the other is positive (stimulates transcription). Negative regulation is mediated by the *lac* repressor. Transcription initiation begins with the binding of *E. coli* RNA polymerase to the promoter; however, the successful transition from transcription initiation to transcription elongation can be influenced by downstream elements. Between the promoter and the coding regions in the operon is the *lac* operator, which is a specific DNA sequence to which *lac* repressor binds. The binding of repressor to the operator greatly decreases the frequency of successful transcription elongation events by the RNA polymerase. Inducers of the *lac* operon (e.g., IPTG) permit transcription because they bind to the *lac* repressor and substantially decrease its binding affinity to the *lac* operator.

Positive regulation by CAP + cAMP and the glucose effect

It would seem that there should be little to no expression in cells in the absence of inducer and expression should proceed when an inducer is added. However, efficient transcription initiation also requires the presence of cyclic AMP (cAMP) and cyclic AMP receptor protein, called CRP or CAP. The CAP/cAMP complex binds just upstream of the *lac* promoter and directly stimulates transcription by RNA polymerase. Because the binding of CAP to DNA requires cAMP, induction of transcription depends on the level of cAMP in the cell. cAMP levels are strongly influenced by the carbon source present in the medium. In the presence of glucose (an easily metabolized monosaccharide), cAMP levels are low, so transcription from the *lac* promoter is low. This phenomenon is called the glucose effect or catabolite repression and is shared by a number of *E. coli* operons. When glucose is absent and the cell is forced to use an alternative carbon source, such as glycerol, cAMP levels rise. The resulting formation of the CAP/cAMP complex stimulates transcription from the *lac* promoter. Therefore, full induction of the *lac* operon is achieved only in the presence of both inducer and elevated cAMP levels.

lac elements and other transcriptional controls in the pET System

The lambda DE3 prophage encoding T7 RNA polymerase in pET expression hosts carries the L8-UV5 promoter, which has three point mutations that distinguish it from the wild type *lac* promoter (Figure 1). Two point mutations in the –10 region increase promoter strength and decrease its dependence on CAP/cAMP stimulation for full activation. The third-point mutation is located in the CAP/cAMP binding site and decreases the affinity for CAP/cAMP. This mutation reduces, but does not eliminate, sensitivity to catabolite repression. The net effect of the three-point mutations is the creation of a stronger promoter that is less sensitive to the glucose effect. This allows strong IPTG induction of T7 RNA polymerase expression even in the presence of glucose.

Although the *lac* and L8-UV5 promoters are well repressed in the absence of inducer, both exhibit detectable basal activity. In the case of λDE3 lysogens, basal expression of even a small amount of T7 RNA polymerase can lead to problems if the target gene in the pET vector produces a protein toxic to the host cell. Therefore, additional levels of control are built into the pET vectors and hosts. Vectors with a "T7*lac*" promoter have a T7 promoter followed by a *lac* operator sequence. The operator in these plasmids provides a place for *lac* repressor to bind, reducing transcription by any T7 RNA polymerase that may be expressed in the absence of inducer. Another level of control is provided in expression hosts containing the pLysS plasmid, which expresses T7 lysozyme, a protein that binds to and inhibits T7 RNA polymerase. The need for these additional sources of regulation depends on the target protein being expressed; the more damaging the protein is to bacterial cells, the more regulation is required.

As first described by Grossman et al. (1), yet another level of regulation can be employed with the pET System by exploiting the glucose effect described above, i.e.,

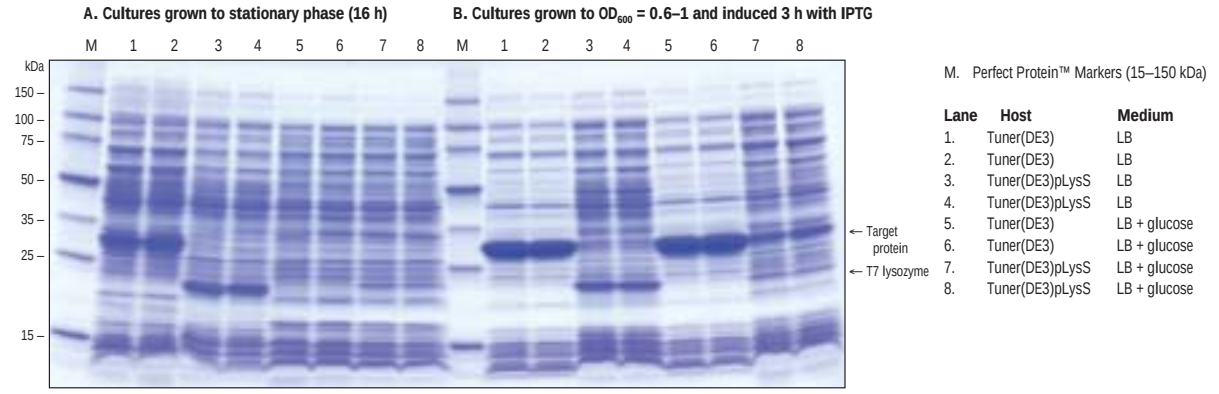

Figure 2. Expression of GFP from a pET-30 construct under different conditions

Tuner(DE3) and Tuner(DE3)pLysS hosts carrying a pET-30 Ek/LIC GFPuv recombinant were grown under various conditions and analyzed for target protein expression by SDS-PAGE of total cell extracts. In Panel A, 3-ml cultures were grown to stationary phase by overnight incubation (16 h) at 37°C with shaking at 300 rpm. In Panel B, 3-ml cultures were grown to an OD_{600} between 0.6 and 1.0 and then induced by the addition of 1 mM IPTG for 3 h at 37°C with shaking at 300 rpm. For gel analysis, cells were harvested by centrifugation and the pellets resuspended in BugBuster™ HT Protein Extraction Reagent. After the addition of 4X SDS Sample Buffer, samples corresponding to equivalent numbers of cells (based on harvest OD_{600}) were loaded on a 10–20% gradient gel. In both panels, lanes 1–4 represent cultures grown in standard LB broth and lanes 5–8 represent cultures grown in LB broth supplemented with 1% glucose. The respective hosts are indicated. Pairs of lanes represent duplicate samples derived from independent clones.

supplementing standard media such as LB with glucose to keep cAMP levels low. Apparently, although the L8-UV5 promoter is less dependent on CAP/cAMP stimulation than the wild type *lac* promoter, in practice there is still a significant reduction in basal transcription in the presence of glucose. This can be particularly important for pET vector expression when hosts that do not carry the pLysS plasmid are allowed to grow to stationary phase, where uninduced expression is maximal (1, 4). Others have also reported that supplementing LB media with glucose to a final concentration of 0.5–1.0% prevents the increased basal activity observed in cultures grown to stationary phase (1, 2).

Example of the glucose effect on pET expression

Figure 2 demonstrates the dramatic difference that glucose can make when cultures are grown to stationary phase by overnight incubation at 37°C. pET-30 recombinants expressing green fluorescent protein (GFP) in Tuner™(DE3) and Tuner(DE3)pLysS hosts were tested under different growth conditions. Cultures in LB medium lacking or containing 1% glucose were grown to stationary phase (16 hours) or to log phase (OD_{600} = 0.6 and 1.0). The log phase cultures were then induced with IPTG for 3 hours at 37°C. Each condition was carried out in duplicate with two independent recombinants. Cells were harvested at the end of the culture period and total cell protein (TCP) samples were analyzed by SDS-polyacrylamide gel electrophoresis.

Figure 2, panel A shows the gel profiles of the overnight cultures grown without added inducer. The overnight Tuner(DE3) cultures lacking glucose (lanes 1–2) exhibited easily detectable levels of target protein production, whereas target protein was undetectable in the same cultures supplemented with 1% glucose (lanes 5–6). In contrast, the Tuner(DE3)pLysS cultures grown to stationary phase did not require glucose to prevent uninduced expression (lanes 3–4 without glucose vs. lanes 7–8 with glucose).

Supplementing LB medium with 1% glucose prevents increased basal expression in λDE3 lysogens grown to stationary phase.

Figure 2, panel B demonstrates that glucose addition did not interfere with IPTG induction of the target protein. In fact, IPTG induction from the pLysS host appeared to be enhanced in the presence of glucose. High induction was observed from the Tuner(DE3) cultures regardless of glucose addition (panel B, lanes 1–2 vs. lanes 5–6). Much lower expression of the target protein was observed from the pLysS-based host grown in glucose (lanes 7–8), but it was barely detectable in the host grown without glucose (lanes 3–4).

One possible explanation for the low IPTG induction results observed in the pLysS host is that in the absence of glucose the expression of T7 lysozyme from pLysS may be substantially elevated. In uninduced pLysS host cultures, some transcription of the LysS gene is probably achieved via read-through transcription from the upstream chloramphenicol acetyltransferase (CAT) promoter. In the absence of glucose, cAMP levels would be expected to rise during the later stages of the growth cycle. Because the CAT promoter is also stimulated by the CAP/cAMP complex (3), elevated transcription of the lysozyme gene from the CAT promoter would occur. In Figure 2, a unique protein band was observed between the 15 and 25 kDa protein markers in pLysS cultures, which corresponds to the predicted size of T7 lysozyme (17 kDa). This band was significantly more intense in pLysS cultures grown without glucose relative to those grown in the presence of glucose. A sufficiently high level of T7 lysozyme may saturate all of the available T7 RNA polymerase and thereby block target gene transcription. This may account for the variability that is sometimes observed when attempting to induce target proteins in pLysS hosts.

Summary

In conclusion, supplementing culture media with glucose provides a simple, inex-

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pensive method to maintain very low basal expression levels of T7 RNA polymerase in the λDE3 lysogenic expression hosts used in the pET System. This is especially true when λDE3 hosts carrying pET plasmids are grown to stationary phase. A disadvantage with glucose addition is that after an initial rapid growth phase the metabolic breakdown products of glucose will lead to acidic culture conditions and lower cell density at stationary phase. The data presented in Figure 2 demonstrate that strong induction can be achieved from λDE3 lysogens in the presence of glucose for some target proteins. Note, however, that theoretically the strongest induction of T7 RNA polymerase would be expected when glucose is absent and cAMP levels are elevated. Accordingly, in some cases (see preceding article), higher target protein expression may be observed in the absence of glucose. Overall, the optimal combination of stringent uninduced repression and high induced expression may be achieved by initial growth in the presence of glucose, followed by switching to medium without glucose for induction.

Novagen's recommendations for growth and induction of pET constructs in expression hosts are based on the information presented above. For innocuous proteins, any pET vector and λDE3 lysogen are suitable in a variety of media. But for proteins that are potentially toxic to the bacterial cell, we recommend using either a pET vector with a T7*lac* promoter or expression hosts that carry the pLysS plasmid. In addition, our general advice is to avoid growing a λDE3 lysogen carrying a pET plasmid to stationary phase. If the cells must be grown to stationary phase, we recommend the addition of 0.5 to 1.0% glucose to the medium, so that the glucose effect can be exploited to reduce basal expression.

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