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).

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. 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 "T7lac" 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.,
supplementing standard media such as LB with glucose to keep cAMP levels low. Apparently, although the LB-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% 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 (OD600 = 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 SDSPolyacrylamide 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 3–4). 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 λD E3 lysogens grown to stationary phase with glucose.

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 lysozyme 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 would be 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-
Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. It is almost obligatory to assess the purity of a protein through an electrophoretic method. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix (1). The technique is also a powerful tool for estimating the molecular weights of proteins (2, 3). The success of SDS-PAGE as an indispensable tool in protein analysis has been attributed to three innovations that permitted the correlation of electrophoretic mobility with a protein's molecular mass (4). First was the introduction of discontinuous buffer systems where the sample and gel running systems differ in both composition, Tris-glycine (11) and pH, 6.8/8.3, respectively (5, 6). Discontinuous buffer systems allow larger sample volumes to be loaded while maintaining good resolution of sample components because the proteins are focused, or “stacked,” as thin bands prior to entering the resolving gel. Second was the use of the detergent sodium dodecyl sulfate (SDS) and reducing agents to denature proteins (7). SDS binds strongly to proteins at an approximate ratio of 1 dodecyl sulfate molecule per 2 amino acid residues (8). Therefore, the negative charge/unit mass ratio when SDS is bound to the polypeptide chain is similar for all proteins. Third was the combination of the first two discoveries employing a simple Tris-glycine buffer system (9). More recently, buffer combinations such as Tris-borate (10) and Tris-tricine (11) have improved the resolving power of the original methods. Modern SDS-PAGE has evolved to use microslab precast gels (12). Precast and packaged gels in a wide variety of gel formulations, acrylamide percentages, thicknesses, well formats, and buffer systems are now commercially available from several manufacturers. Therefore, successful SDS-PAGE analysis of protein samples no longer depends on tedious gel casting, buffer preparation and apparatus set-up, but on careful sample preparation and treatment prior to loading the gel. This article describes techniques and procedures as a guide for preparation of protein samples for SDS-PAGE analysis.

Sample buffer preparation

To ensure consistent and successful PAGE analysis, the highest purity reagents should be used to prepare sample buffer stock solutions. After a reliable source of electrophoresis reagents has been identified, the vendor and buffer component chemicals should be maintained. High purity electrophoresis Ultrapure grade, and molecular biology grade reagents are available through Novagen’s partner brand, Calbiochem. Solutions must be carefully and safely prepared, dated, and chemical lot numbers recorded. Concentrated stock solutions should not be stored for long periods of time. Tris base, rather than Tris-Cl, should be used for buffer preparation and pH adjustment made with HCl. Use of Tris-Cl should...