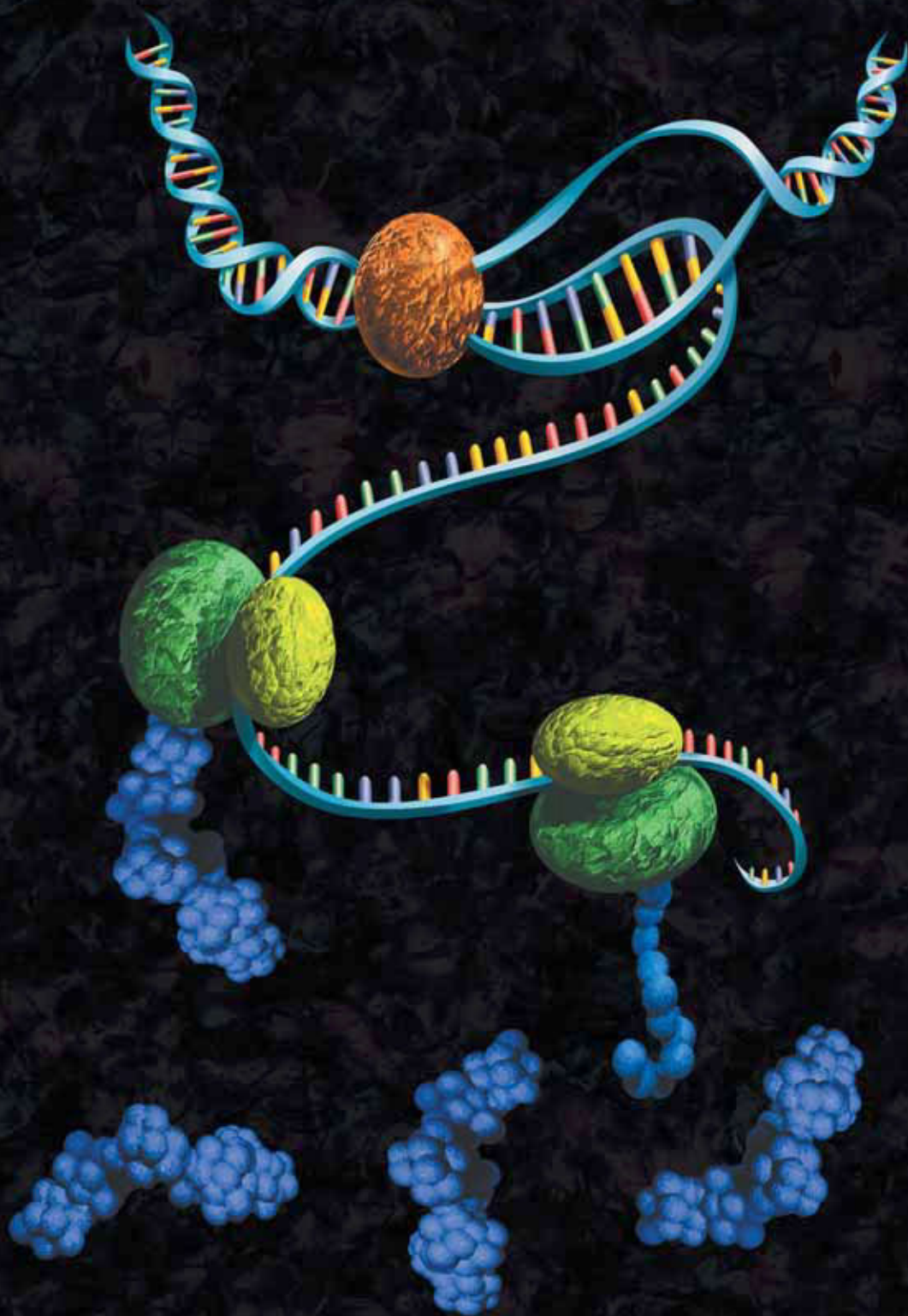


CHAPTER

7

About the Image:

A number of large-scale protein synthesis methods have been developed recently to increase the yield of cell-free systems to a preparative scale. Some of these systems are prokaryotic and utilize DNA templates containing either prokaryotic promoters or a phage RNA polymerase promoter and eukaryotic extracts. Such systems are utilized to generate micro- to milligram protein yields per milliliter of reaction.



Chapter Seven: Large-Scale Protein Synthesis

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Introduction

Cell-free systems for in vitro gene expression and protein synthesis have been described for many different prokaryotic and eukaryotic systems (1). Eukaryotic cell-free systems, such as rabbit reticulocyte lysate and wheat germ extract, are prepared from crude extract containing all of the components required for translation of either natural or in vitro transcribed RNA templates. Prokaryotic systems, however, are typically coupled in that they contain RNA polymerase, which transcribes mRNA from an exogenous DNA template. During transcription, the 5'-end of the mRNA becomes available for ribosome binding and translation initiation, allowing transcription and translation to occur simultaneously. Prokaryotic systems are available that utilize DNA templates containing either prokaryotic promoters (such as *lac* or *tac*; *E. coli* T7 S30 Extract System for Circular DNA^(a,b) [Cat.# L1130] or a phage RNA polymerase promoter. Coupled eukaryotic cell-free systems have been developed that combine a prokaryotic phage RNA polymerase/promoter with eukaryotic extracts and utilize an exogenous DNA template for in vitro protein synthesis (TNT® Coupled Reticulocyte Lysate^(a,b,c,e) [Cat.# L4600, L4610, L4950, L5010, L5020] and TNT® Wheat Germ Extract Systems^(a,b,c,e) [Cat.# L4380]).

Cell-free expression systems offer several advantages over in vivo expression systems, including the ability to express toxic, proteolytically sensitive, or unstable gene products. Cell-free systems are often used to verify that the appropriately sized gene product(s) is synthesized from a cloned gene. Other applications of in vitro expression systems include analysis of protein-protein and protein-nucleic acid interactions, mutational analysis, epitope mapping and in vitro evolutionary studies. In addition, the ability to incorporate unnatural amino acids containing photoactivatable, fluorescent, or biotin groups allows for product analysis by new methods (2-5).

Typically, standard in vitro systems produce picomole (or nanogram) amounts of proteins per 50µl reaction. This yield is sufficient for most types of analyses, such as polyacrylamide gel separation, Western blotting, immunoprecipitation and, depending on the protein of interest, enzymatic or biological activity assays. More recently, a number of methods have been developed to increase this yield to a preparative scale, which would allow alternative types of analyses and applications for the in vitro synthesized proteins.

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Improving Yield

Some investigators have improved the yield from a standard wheat germ batch translation system by optimizing the temperature, tRNA concentration and, most importantly, ATP regeneration system (6). These modifications increased yields 4- to 8-fold, resulting in the synthesis of 30µg of protein (*E. coli* dihydrofolate reductase; DHFR) per milliliter of reaction mixture. Protein synthesis was prolonged for up to 10 hours with various templates, and both capped and uncapped mRNA templates were used. Protein synthesis directed by uncapped dihydrofolate reductase mRNA containing a viral cap-independent translation initiation sequence

resulted in the synthesis of 18µg DHFR per milliliter of reaction mixture. The highest yields were obtained with capped mRNA containing the viral cap-independent translation initiation sequence (30µg/ml reaction). Protein synthesis in this improved batch wheat germ system can sustain a translation reaction for half as long as the continuous-flow cell free (CFCF) systems (see below), but it is superior to the CFCF system in that no special apparatus is required, and it is more convenient and more reproducible. Large-scale S30 batch reactions synthesizing T4 lysozyme with site-specific incorporated spin label (fluorescent or photoactivatable amino acids) have produced protein product yields of 20–40µg/ml (3,7).

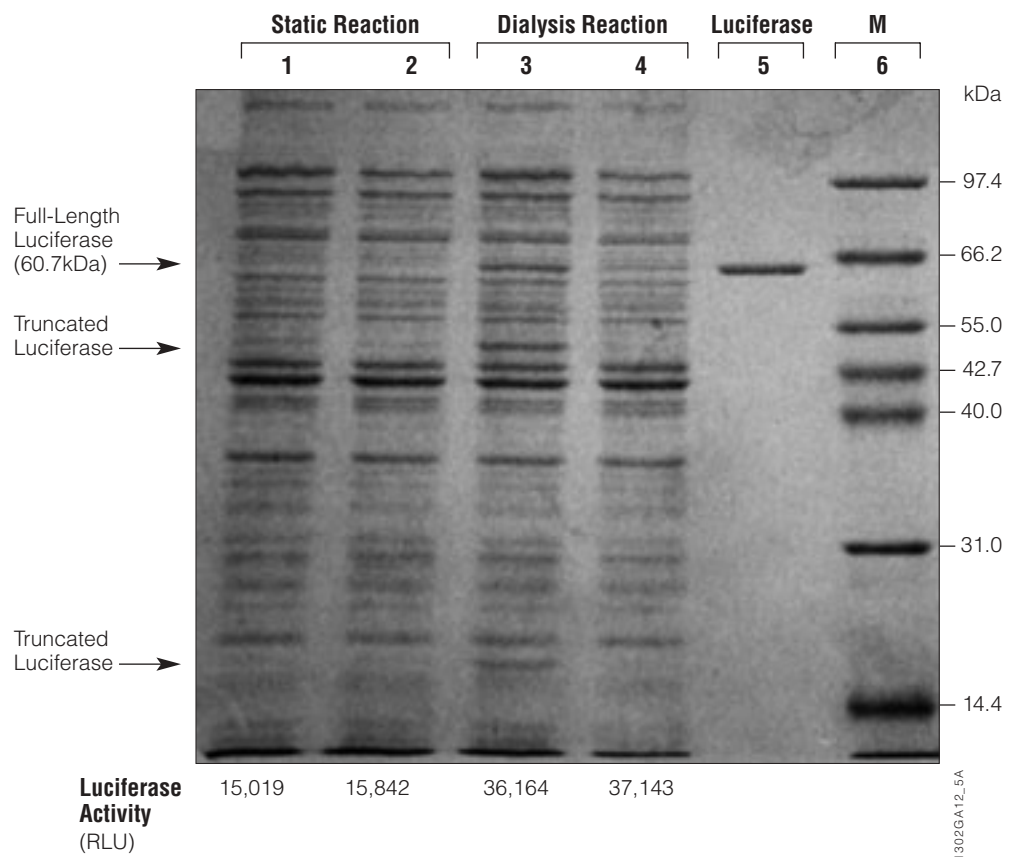


Figure 1. Coomassie® stain detection of SDS-PAGE-separated luciferase gene products synthesized in an *E. coli* S30 Extract equilibrium dialysis cell reaction. A 1.5ml *E. coli* S30 Extract reaction (containing 60µg pBEST[™] *luc*[™] DNA^(b), 600µl of Premix 2.5X Solution, 450µl of Promega's *E. coli* S30 Extract for Circular DNA and 390µl of Nuclease-Free Water as described in reference 8) was separated by a 300kDa MWCO cellulose ester membrane (Amicon, Cat.# XM300) from a constantly stirred dialysis solution (Premix 1X Solution). The dialysis reaction cell (similar to Fisher Cat.# 08-666-15), the stir bar in the lower chamber and membrane were sterilized by soaking in 5% formalin for 1 hour, followed by 3 rinses with distilled water and air-drying in a sterile hood. The reaction proceeded at room temperature for 20 hours. An identical 1.5ml standard static reaction (1) served as a control reaction. The samples were analyzed on a 10% SDS-polyacrylamide gel as described (9). Lane 1, 5µl of control reaction; lane 2, 5µl of control reaction supernatant (after reaction was centrifuged at 12,000 x *g* for 5 minutes); lane 3, 5µl of dialysis reaction; lane 4, 5µl of dialysis reaction (after reaction was spun at 12,000 x *g* for 5 minutes); lane 5, purified firefly luciferase (1µg); lane 6, 2µl of Promega's Mid-Range Protein Molecular Weight Markers (no longer available). For determination of functional luciferase activity, 5µl from each reaction were assayed using the Promega Luciferase Assay Reagent^(a) as described (10).

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A disposable dialyzer used in conjunction with Promega's *E. coli* S30 Extract System^(a,b) allowed synthesis of several hundred micrograms of protein in a single coupled transcription/translation reaction. Using dialysis with this cell-free system can increase protein synthesis by 10- to 20-fold over standard batch reactions (180–360 μ g luciferase per 1.5ml reaction; Figure 1). For these experiments, a DispoDialyzer[®] (Spectrum[®]) was used (Figure 2). It is important to select a membrane with a molecular weight cut-off below the size of the expressed protein. In addition, the promoter utilized, position of ribosome binding site, DNA template and preparation, reaction temperature and dialysis solution components may need to be optimized. In particular, the proper magnesium concentration is critical for optimal protein yields. Such a dialysis system is simple, convenient and economical.

Continuous-Flow Systems

While the "static" cell-free systems are extremely useful, they are still limited in the amount of total protein produced. Spirin and coworkers have reported several continuous-flow cell-free (CFCF) systems in which the protein products are removed through a membrane by pumping a feeding solution (containing amino acids, ribonucleotides and energy source) through the reaction vessel during the course of the 20–30-hour reaction (11). These continuous-flow systems can produce hundreds of micrograms of protein from a 1ml reaction. For example, total protein yields included 0.2mg BMV coat protein

in a wheat germ system, 2.0mg globin in a RRL system and 0.2mg β -lactamase or DHFR in an *E. coli* S30 system. In general, yield from 1ml of incubation mixture can vary from 50 μ g to 4mg of protein depending on the size of the protein, its solubility, the expressibility of the template and the type and quality of the cell extract used.

These CFCF systems, however, have several problems, such as clogging of the ultrafiltration membrane, protein aggregation, unexplained translation disruption, high running cost and low reproducibility. These problems have been addressed through additional modifications to the centrifugation steps used when preparing the *E. coli* S30 Extract^(a,b) (12) and the combination of the Q β replicase reaction with the *E. coli* S30 Extract System^(a,b) (13,14). The phage Q β contains an RNA-directed RNA polymerase, which can efficiently amplify RNA in vitro for large-scale protein synthesis. Significant stimulation of RNA synthesis by the addition of the Q β replicase enzyme is only observed in the presence of a completely functioning translation system. For this type of system, the template of interest must be cloned into an efficient, naturally occurring Q β replicase template (such as RQ135₁ RNA). In this system, approximately 0.2pmol DHFR was synthesized per 30 μ l reaction mixture. The continuous production of sense strand RNA by Q β replicase could compensate for the mRNA losses due to degradation in the CFCF systems, thereby extending the reaction life and increasing protein yield.

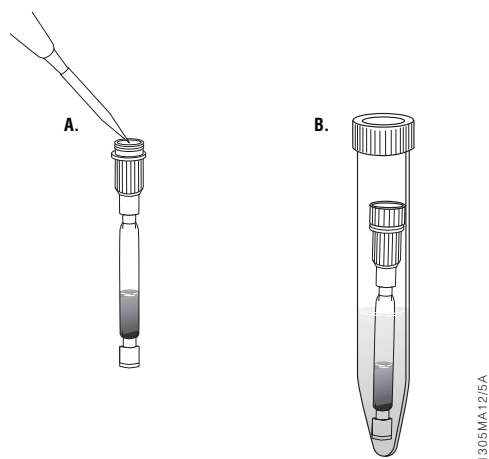


Figure 2. Diagram of the DispoDialyzer[®]. Panel A: Loading the dialyzer with *E. coli* S30 Extract. Panel B: The dialysis reaction consisting of the dialyzer in a sterile 15ml conical tube containing 3.5–7ml of dialysis solution.

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Modifications of the Technique

Studies by Tohda *et al.* suggest that increased protein yield in an *E. coli* CFCF system may be obtained using phosphorothioate-containing mRNA as a template. The thio-mRNA for DHFR showed higher translational activities than the corresponding unsubstituted mRNA (probably due to increased mRNA stability) and the single substitution of adenosine residues was most effective in translational activity (15). The thio-mRNA for DHFR was able to produce the intact protein possessing catalytic activity.

A reactor for cell-free protein synthesis was developed by Kim and Choi (16). This system is similar but simpler than CFCF, in that it enables extremely high productivity without using any complex apparatus. The continuous supply of substrates and removal of by-products was performed by in- and outcome diffusion through a dialysis membrane, which separates the reaction mixture from the feeding solution. By use of this system, protein synthesis occurred for at least 14 hours, yielding 1.2mg chloramphenicol acetyltransferase (CAT) protein per milliliter of reaction mixture.

In a GATT (gene amplification with transcription/translation) system developed by Resto *et al.* (17), greater than 10^9 copies of DHFR can be produced from each plasmid DNA molecule employed. This system involves sequential coupling of DNA amplification by PCR and in vitro transcription, followed by in vitro translation in rabbit reticulocyte lysate. Another group has

reported the use of affinity ligands to continuously remove the synthesized protein products, thereby improving translation efficiency of the dialysis-based systems (18).

For those laboratories that require intermediate amounts of protein for analysis, Alimov *et al.* (19) recently described a system utilizing *E. coli* S30 extract in conjunction with expression vectors that encode viral structural elements known to enhance translation in vivo and to protect mRNA from ribonuclease action. The viral elements include: i) the 133 nucleotide-long cDNA sequence of an RQ RNA that can be replicated by Q β replicase; and ii) the epsilon (ϵ) sequence, a powerful translational enhancer of the phage T7 gene coat protein. The designed vectors also include a Strep-tag oligopeptide at the C-terminus, which allows affinity purification of the expressed protein using streptavidin ($K_d = 10^{-5}$ M). The reaction can yield up to 40 μ g/ml, or about 1nmol, of a standard protein.

For the rapid in vitro production of proteins on the preparative scale, continuous-flow systems currently have more promise than utility, although their predictability appears to be improving. For yields in the hundreds of microgram to low milligram range, the dialysis-based systems seem most applicable. The development of coupled transcription/translation systems from the hyperthermophilic archaeobacterial strains may improve CFCF techniques and allow for more stable and longer lasting systems than those derived from mesophilic organisms.

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