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# Cell-Free Protein Synthesis Systems: Biotechnological Applications

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## Introduction

In recent years, cell-free protein synthesis, often referred to as *in vitro* protein synthesis, has emerged as an important tool that has been used widely by researchers across various disciplines. For example, applications are found in: 1) proteomics for the rapid identification and characterization of gene products; 2) molecular diagnostics for localization of chain-truncating mutations; and 3) structure–function studies by incorporating unnatural amino acids.

The use of cell-free translation systems has several distinct advantages over *in vivo* gene expression, especially in cases where the over-expressed gene product is toxic to the host cell, forms insoluble inclusion bodies, or the protein undergoes rapid proteolytic degradation by intracellular proteases. In addition, cell-free protein synthesis systems can be manipulated or controlled in ways that *cellular* systems cannot. For example, in a cell-free translation system, only a single DNA or RNA species is normally present to direct protein synthesis, there is no cellular membrane barrier to hinder reagent delivery, and cell growth or viability is not an issue. In principle, it should be possible to prepare a cell-free translation system from any cell type. However, only three cell-free translation systems – *Escherichia coli* extract, rabbit reticulocyte lysate, and wheat germ extract – are commonly used. Amongst them, rabbit reticulocyte lysate is often the cell-free translation system of choice because it is eukaryotic/mammalian based, can use either RNA or DNA as a template, and has the ability to carry out some post-translational protein modifications (Beckler *et al.*, 1995).

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Abbreviations: CFCF, continuous-flow cell-free; IVEC, *in vitro* expression cloning; IVSP, *in vitro* synthesized protein; PCR, polymerase chain reaction; PTT, protein truncation test; RTS, Rapid Translation System; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TRAMPE, tRNA-mediated protein engineering.

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The use of cell-free protein translation systems was, until recently, focused mainly on basic research. However, progress in several areas has made these systems attractive for biotechnology. This review, which is not intended to be comprehensive, summarizes some of this recent progress, with special emphasis on promising applications.

## Brief history of cell-free protein synthesis systems

Almost fifty years ago, researchers first realized that protein biosynthesis does not require an intact cell. The original cell-free protein synthesis system was made using extracts of rat liver cells (Littlefield *et al.*, 1955). Soon after, several *E. coli*-based systems were developed (Schachtschabel and Zillig, 1959; Lamborg and Zamecnik, 1960). A major breakthrough came when Nirenberg and Matthaei demonstrated that these systems were capable of translating exogenously introduced messages (Nirenberg and Matthaei, 1961). A few years later, cell-free systems for translation of mRNA were made from rabbit reticulocyte lysate (Pelham and Jackson, 1976) and wheat germ extracts (Roberts and Paterson, 1973). Around the same time, Zubay and colleagues reported a simple and reproducible method for preparation of an efficient cell-free protein synthesis system from *E. coli* that was capable of transcribing and translating DNA (Zubay, 1973). In recent years, all these systems have undergone major improvements, as discussed below.

## Basics of cell-free protein synthesis

*Figure 8.1* shows the basic steps in the transcription/translation reaction. The most frequently used cell-free protein synthesis systems (*E. coli*, wheat germ, and rabbit reticulocyte) are prepared as crude extracts containing all the macromolecular components required for translation of exogenous RNA/DNA (ribosomes, tRNAs, aminoacyl-tRNA synthetases, initiation, elongation and termination factors). In order to support transcription/translation, these extracts are supplemented with amino acids, energy sources (ATP/GTP), nucleotides, energy regenerating systems (creatine phosphate/phosphokinase, phosphoenol pyruvate, and pyruvate kinase), and other co-factors (e.g. $Mg^{2+}/K^{+}$).

There are two different classifications of cell-free translation systems. One is based on whether the system is derived from eukaryotic or prokaryotic cell types. *Table 8.1* lists the main differences between these systems. As seen, in addition to differences in the major components of the two systems (e.g. initiation factors), there are also differences in the template used for prokaryotic and eukaryotic transcription/translation. For example, at the DNA level, eukaryotic systems require a promoter sequence (e.g. T7) followed by a Kozak sequence (eukaryotic ribosome binding site: GCCACCATGG), which includes the initiation codon (ATG). In the case of the prokaryotic system, a promoter sequence (e.g. T7) is normally followed by a prokaryotic ribosome binding site (Shine–Dalgarno sequence: TAAGGAGGTGA) before the ATG start codon. A second classification relates to whether the system can synthesize proteins from just mRNA (translation only) or can perform coupled transcription/translation and thus can utilize DNA (see *Table 8.2*). For example, simple rabbit reticulocyte lysate and wheat germ extract typically require RNA as a template.

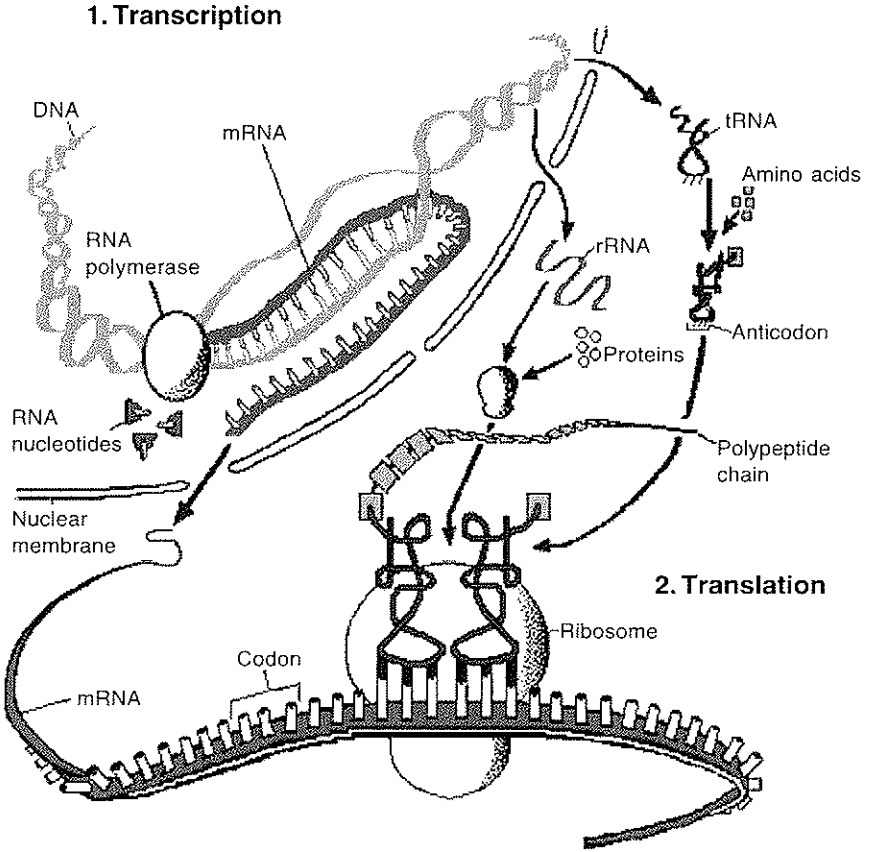


**Figure 8.1.** Schematic representation of transcription and translation (reprinted with permission from Access Excellence at the National Health Museum; http://www.accessexcellence.org/RC/VL/GG/protein_synthesis.html).

**Table 8.1.** Comparison of components in prokaryotic (*E. coli*) and eukaryotic (rabbit reticulocyte and wheat germ) cell-free protein synthesis systems

| Component | Prokaryotic cell-free system | Eukaryotic cell-free system |
|---|---|---|
| Ribosomes | 70S | 80S |
| mRNA | Shine–Dalgarno sequence | proper 5′- and 3′-UTRs |
| Elongator tRNAs | prokaryotic | eukaryotic |
| Initiator tRNA | $tRNA^{fMet}$ | $tRNAi^{met}$ |
| Formylase | *Met-*$tRNA^{fmet}$-formylase | none |
| 20 individual tRNA synthetases | prokaryotic | eukaryotic |
| Initiation factors | IF1, IF2, IF3 | eIFs: 1, 1A, 2, 2A, 2B, 3, 4A, 4B, 4F (4A + 4E + 4G), 5, 5A, 6 |
| Elongation factors | EF–Tu, EF–Ts, EF–G | eEF1A, eEF1B, eEF2 |
| Termination factors | RF1, RF2, RF3, RRF | eRF1, eRF3 |
| Amino acids | all 20 | all 20 |
| Nucleotides | rNTPS | ATP and GTP for mRNA translation and rNTPS for coupled system |
| $Mg^{2+}$ | 8–15 mM | 1–4 mM |
| $K^+$ and/or $NH_4$ | 100–250 mM | 100 mM |

However, coupled systems, such as Promega's TNT™ rabbit reticulocyte and TNT™ wheat germ (transcription and translation), or most commercially available *E. coli*-based extracts, can utilize DNA as a template. Recently, direct expression of PCR-generated DNA in such coupled cell-free protein synthesis systems has gained a considerable amount of attention since it circumvents cumbersome cloning steps (Sawasaki *et al.*, 2002).

## Improvements in cell-free protein synthesis systems

A major limitation of the cell-free protein synthesis systems discussed above is their inability to support translation or coupled transcription/translation for long periods, (e.g. >1 hour). As these systems run out of critical components, such as energy-producing molecules, NTPs, and amino acids, as well as accumulate by-products (waste molecules), protein production is ultimately halted, resulting in a low overall yield. For example, in the TNT™ rabbit reticulocyte cell-free translation system, typically 3–10 μg of nascent protein is produced per ml of reaction (http://www.promega.com/faq/tnt.html#q02). To avoid this problem, Spirin and co-workers have reported an alternative system based on a continuous-flow, cell-free (CFCF) translation (Spirin *et al.*, 1988). In this system, instead of performing a reaction in a closed vessel, sometimes referred to as a batch mode, the reaction is performed in a chamber where the by-products are removed and the consumable substrates are added continuously. This is achieved using a semi-permeable membrane that retains the high molecular weight components of the protein-synthesizing machinery within the reaction chamber, yet allows a continuous flow of the low molecular weight consumable substrates into the reaction chamber, such as amino acids, nucleotides, and phosphoenol pyruvate. Using a modification of this technology, Roche Molecular Biochemicals recently introduced the Rapid Translation System (RTS 500) for preparative-scale protein expression. According to the supplier, a single cell-free translation reaction is capable of producing more than 500 μg/ml of product, even for proteins greater than 120 kDa MW (www.proteinexpression.com). Although the initial system was based on prokaryotic *E. coli* lysate, which does not support post-translational modifications, an RTS version utilizing wheat germ cell-free protein synthesis has recently been introduced. In addition, Endo and colleagues (Endo and Sawasaki, 2003, 2004) have described a highly efficient and robust cell-free protein expression system derived from wheat germ that is capable of translating most eukaryotic proteins with significant yield (0.1 to 2.3 mg/ml). In this system, the translation extract was prepared by extensive washing of wheat embryos to eliminate endosperm contaminants, such as RNA N-glycosidase tritin, thionin, ribonucleases, deoxyribonucleases, and proteases, which inhibit translation.

Apart from the yield of proteins synthesized, an additional problem that relates to the use of whole-cell lysate is the presence of endogenous protease and/or nuclease activity. To overcome this problem, several researchers have attempted to develop reconstituted cell-free protein synthesis systems (e.g. systems which contain only the basic components needed to support protein synthesis). This is possible due to significant progress in understanding the mechanism of transcription and translation at the molecular level, especially in prokaryotic cells (Puglisi *et al.*, 2000;

Spirin, 2002; Zarivach *et al.*, 2002; Romby and Springer, 2003; Vanzi *et al.*, 2003). For example, as early as 1977, Weissbach's group reported the first partially reconstituted cell-free translation system using purified soluble components (Kung *et al.*, 1977, 1978). However, this system lacked a full set of aminoacyl-tRNA synthetases. More recently, Ueda and co-workers reported a completely reconstituted *E. coli* cell-free protein synthesis system from purified, individual components of the translation machinery (Shimizu *et al.*, 2001). This so-called PURE system is composed of recombinant $His_6$-tagged translation factors (10 proteins), aminoacyl-tRNA synthetases (20 proteins), methionyl-tRNA transformylase, T7 RNA polymerase, and the proteins of an ATP-regenerating subsystem. The system exhibits significantly reduced proteolytic activity and produced, in many cases, functional proteins. In addition, Algire and colleagues have reported a partially reconstituted eukaryotic cell-free translation system from yeast (Algire *et al.*, 2002). Importantly, this new generation of cell-free protein synthesis systems promises higher protein yields and tighter control of the translation reaction. For example, restricting the species of tRNA or aminoacyl-tRNA synthetase present in the mixture can facilitate tRNA mediated protein engineering (TRAMPE), as discussed later. Some of the reconstituted cell-free protein synthesis systems are listed in *Table 8.2*, along with other types of improved systems that are commercially available.

## APPLICATIONS OF CELL-FREE PROTEIN SYNTHESIS SYSTEMS

The use of cell-free protein synthesis is rapidly growing as more applications are found in basic research, proteomics, and molecular diagnostics. Some common applications are given in *Table 8.3*. A detailed description of many such applications is given in the valuable '*In Vitro* Expression Guide' from Promega Corporation (Beckler, 2000). We describe here a few of the latest and most significant applications, along with specific examples.

## APPLICATIONS IN BASIC RESEARCH

### *tRNA-mediated protein engineering (TRAMPE)*

Genetic engineering has been restricted largely to manipulating the 20 native amino acids normally found in proteins. In contrast, tRNA-mediated protein engineering (TRAMPE) allows expansion of the genetic code so that custom-designed, non-native amino acids can be randomly or selectively introduced into proteins. Novel methods of incorporating non-native amino acids and stable isotope labels into proteins using modified tRNAs open new opportunities for basic research and biotechnology that go beyond conventional site-directed mutagenesis. TRAMPE relies on the development of novel tRNAs and their aminoacylation with custom-designed amino acids, the recognition of special codons by the tRNAs, and the efficient expression of these modified proteins. For a more detailed description of TRAMPE, there are several reviews by various researchers (Thorson *et al.*, 1998; Rothschild and Gite, 1999; Hohsaka and Sisido, 2002; Rothschild *et al.*, 2004). An early example of TRAMPE is provided by the work of Johnson and co-workers, who

**Table 8.2.** Commonly used, commercially available, cell-free expression systems and their salient features

| System | Source | Supplier | Features |
|---|---|---|---|
| RTS-EC | *E. coli* | Roche Applied Sciences, US | High yield system (500 µg to 6 mg of protein/ml); uses RTS ProteoMaster instrument and two-chamber devices |
| RTS-WG | Wheat germ | Roche Applied Sciences, US | High yield system for eukaryotic proteins (up to 1 mg of protein/ml); uses RTS ProteoMaster instrument and two-chamber devices |
| TNT™ | Rabbit reticulocyte lysate | Promega, US | Very reliable; available in various formats (for T7/SP6 promoters); master mix to 96-well format; many post-translational activities are demonstrated in this extract |
| Expressway™ | *E. coli* | Invitrogen, US | Optimized for use with Gateway cloning technology for high throughput cell-free protein expression |
| Active-Pro™ | *E. coli* | Ambion, US | Shown to produce biologically active proteins with high yield (up to 500 µg/ml) |
| PURE | *E. coli* | Post Genome Institute, Japan | Completely reconstituted with purified factors; no nuclease or protease activity; easy target protein purification |

**Table 8.3.** Top 10 applications of cell-free protein synthesis systems

| | Application | References |
|---|---|---|
| 1 | Verification of cloned genes | (Beckler, 2000) |
| 2 | Functional analysis of synthesized proteins | (Beckler, 2000) |
| 3 | *In vitro* protein engineering/TRAMPE | (Rothschild and Gite, 1999) |
| 4 | Post-translational modification analysis | (Beckler, 2000) |
| 5 | Functional mapping of genomes through direct expression of genomic/cDNA libraries | (Endo and Sawasaki, 2004; Sawasaki *et al.*, 2004) |
| 6 | Mutation detection analysis | (Gite *et al.*, 2003) |
| 7 | Ribosome display | (He and Taussig, 2002) |
| 8 | *In vitro* expression cloning | (King *et al.*, 1997) |
| 9 | Protein–protein and protein–DNA interaction | (Beckler, 2000) |
| 10 | Screening inhibitors of protein synthesis | (Chen *et al.*, 1997; Gorokhovatsky *et al.*, 1998; Jefferson *et al.*, 2002) |

demonstrated that unnatural amino acids are accepted by the protein synthesis machinery and can be inserted randomly at lysine positions using an ε-amine modified lysine-tRNA$^{lys}$ (Johnson *et al.*, 1976). Subsequently, several researchers have developed methods for site-specific incorporation of non-native amino acids, including fluorescent, spin-labelled and isotope-labelled groups into nascent proteins utilizing an amber suppressor tRNA (Ellman *et al.*, 1991, 1992; Sonar *et al.*, 1994; Mendel *et al.*, 1995; Cload *et al.*, 1996). Some of the more recent examples of TRAMPE include site-specific incorporation of unnatural amino acids into HIV-1 reverse transcriptase (Klarmann *et al.*, 2004), site-specific incorporation of an unnatural amino acid using an engineered *E. coli* tyrosyl-tRNA synthetase (Kiga *et al.*, 2002), use of an unnatural base pair for incorporating amino acid analogues into proteins (Hirao *et al.*, 2004), and incorporation of selenomethionine into bacteriorhodopsin for FTIR studies (Bergo *et al.*, 2003).

## APPLICATIONS OF CELL-FREE TRANSLATION SYSTEMS IN PROTEOMICS

Recent advances in large-scale analysis of genes have led to the complete sequencing of all the genes in the human genome. While this accomplishment represents a major success for this first 'Manhattan-scale' project in biology, it is generally agreed that a much more ambitious goal is emerging for the post-genome era. This goal is to elucidate the function of the entire *prote*in complement of the gen*ome*, first referred to as the proteome in 1995 by Wilkins and Williams of the Macquarie University Centre for Analytical Biotechnology (MUCAB) in Sydney, Australia. The ability to synthesize large numbers of proteins in functional form is the primary bottleneck in proteomics. Due to several advantages associated with cell-free protein synthesis, including the speed, ease of protein expression, and compatibility with TRAMPE, this approach holds great potential in proteomics. Here, we discuss briefly a few areas of proteomics where cell-free translation systems have been exploited by researchers to provide distinct advantages.

### *For verification and functional analysis of cloned DNA sequence*

One of the most common uses of cell-free protein synthesis is to verify gene expression and characterize the resulting protein product. For example, known nucleotide sequences, plasmid DNA, or PCR amplicons with proper transcription/translation elements can be expressed and analysed for the expected protein product (Roberts and Paterson, 1973; Prives *et al.*, 1974; Melton *et al.*, 1984). Importantly, many cell-free expressed proteins are correctly folded, processed, and display their normal enzymatic or biological activity (Wood *et al.*, 1984; Kutuzova *et al.*, 1989; Kolb *et al.*, 1994; Lyford and Rosenberg, 1999; Miles *et al.*, 2002). The nascent protein can also be directly assayed for function, without further purification, provided the cell-free expression system does not also exhibit similar endogenous activity or otherwise interfere with the assay used (Warner *et al.*, 1995; Hempel *et al.*, 2001). It is also possible to quickly introduce a variety of mutations into the gene of interest and examine the effects on the activity of the expressed protein (Black and Loeb, 1993). This method can be used to identify functionally active domains or residues.

*For analysis of post-translational modification*

Post-translational modification of a protein, such as proteolytic cleavage or the addition of carbohydrate and/or phosphate groups, is often required for functional activity. However, cell-free protein synthesis systems differ in their ability to carry out post-translational modifications. Not surprisingly, cell-free protein synthesis systems derived from bacteria such as *E. coli* lack the ability to perform post-translational modifications. Conversely, various phosphorylation, myristoylation, farnesylation, isoprenylation, and proteolytic activities have been observed in cell-free protein synthesis systems derived from rabbit reticulocyte lysate (Wilson and Maltese, 1995; Wilson *et al.*, 1995). In the case of wheat germ-derived, cell-free protein synthesis systems, it has been shown that addition of microsomal membranes allows the study of glycosylation, methylation, and signal peptide cleavage (Humphries *et al.*, 2002). Since not all differences between the various cell-free systems are known, it may be desirable to try both reticulocyte lysate and wheat germ extract to determine which system can produce a functional gene product with the 'correct' post-translational modifications.

*For studying DNA–protein interactions*

Inside the cell, specific nucleic acid–protein interactions play an important role in controlling gene expression and ultimately cell physiology (von Hippel and McGhee, 1972; Sancar and Hearst, 1993; Guille and Kneale, 1997; Das *et al.*, 2004). Such interactions are relatively easy to determine using cell-free protein expression systems as compared to intact cellular systems. In the case of DNA binding proteins, such as transcription factors, protein–DNA interactions can be analysed based on the protein's ability to bind specific short nucleotide sequences. This specific binding can then be detected using a standard electrophoretic mobility shift assay (EMSA). In certain scenarios, wheat germ-based extracts are preferred since they lack endogenous mammalian transcription factors. Recently, a method has been reported that removes endogenous DNA-binding proteins from the reticulocyte system prior to the translation reaction (Ebel and Sippel, 1995).

*For studying protein–protein interactions*

Coupled cell-free protein synthesis systems provide a powerful and rapid means for analysis of protein–protein interactions. Examples of such interactions include antibody–antigen, macromolecular, or structural assemblies, and biologically functional protein interactions, such as formation of active transcription complexes. Generally, one partner (bait) is expressed *in vivo* or purified from cells/tissues in large quantities and the other partner (prey) is expressed in a cell-free protein synthesis system and can be readily labelled co-translationally with either radioactivity or fluorescence. Often, this technique is used to verify results obtained from other experimental screening systems, such as yeast two-hybrid (Song and Donner, 1995; Johnson *et al.*, 2001; Tyagi *et al.*, 2002; Gonzalez *et al.*, 2003). To define the interacting sequences, a series of deletion constructs are most commonly employed, or occasionally, specific point mutants are introduced into the cell-free

synthesized proteins and binding is compared to the wild-type proteins. Production of these variants is rapid (hours), simple (no transfection or cell culture), amenable to automation, and can be performed just as easily using eukaryotic, including mammalian, cell-free protein synthesis systems rather than prokaryotic.

In recent years, high-density DNA microarrays, or 'chips', have gained tremendous popularity due to their ultra high multiplexing capacity and small sample volume requirements. However, protein microarrays are a relatively new technology that will dramatically impact the biotechnology industry. According to a 2003 market research report from Front-Line Strategic Consulting Inc., 'The emergence of protein chips realizes the concept of high-throughput protein characterization and is a monumental advancement in proteomics' (Report, 2003). Protein microarrays have tremendous potential as a tool for the study of protein–protein, enzyme–substrate, and antibody–antigen interactions, among others. However, the manufacturing of validated protein microarrays with adequate numbers of proteins that are both correctly folded and fully functional still presents significant challenges. This is due mainly to the vast complexity of proteins when compared to DNA and RNA in terms of structural and biochemical diversity, and the difficulties of producing and purifying full-length proteins in sufficient amounts. Protein microarrays require a higher degree of sophistication in both assay design and data analysis. As mentioned earlier, the use of cell-free protein synthesis and the ability to generate such proteins directly from PCR amplicons opens a whole new chapter that will help rapidly advance the field of protein microarrays.

Several researchers have been at the forefront of developing cell-free expression-based protein microarrays and, to date, significant progress has been made. Initial work was primarily focused on developing a high throughput method for producing proteins and protein libraries in a streamlined fashion using cell-free synthesis. Rungpragayphan and co-workers have reported a high throughput method for construction and screening of protein libraries by directly using PCR-generated DNA templates (Rungpragayphan *et al.*, 2002, 2003). More recently, Sawasaki and co-workers (Sawasaki *et al.*, 2002, 2004) described a high throughput, genome-scale biochemical annotation method based on a cell-free protein synthesis system prepared from wheat germ. These authors converted a cDNA library to protein by selecting 439 cDNAs encoding kinases from *Arabidopsis thaliana*. Their results revealed that 207 members of the kinase library had autophosphorylation activity, and 7 out of 26 tested calcium-dependent protein kinases phosphorylated a synthetic peptide substrate in the presence of calcium, demonstrating the translation products retained their substrate specificity (Sawasaki *et al.*, 2004).

In an early attempt to create cell-free expressed protein microarrays, Kawahashi and co-workers used a fluorophore–puromycin conjugate in the cell-free protein synthesis reaction to selectively label the nascent proteins at their C-terminus (Kawahashi *et al.*, 2003). In this system, both the arrayed and probing proteins were prepared by cell-free translation and fluorescently labelled using the puromycin technology (Nemoto *et al.*, 1999; Tabuchi, 2003). The resulting fluorescently labelled proteins were found to be useful for probing protein–protein interactions on microarrays in model experiments. Similarly, Kinpara and co-workers attempted to make a highly integrated protein chip from a DNA library using cell-free protein synthesis on a micro-chamber array fabricated using PDMS (polydimethyl siloxane)

and a glass surface (Kinpara *et al.*, 2004). Expression of a model protein (GFP) was achieved using DNA immobilized on polymer beads that were added to the micro-chambers, and the produced nascent protein was detected by its fluorescence. Most recently, Ramachandran and co-workers designed self-assembling protein microarrays by printing cDNAs onto glass slides and subsequently bathing the slide in rabbit reticulocyte cell-free expression lysate in order to synthesize the proteins from the arrayed DNA (Ramachandran *et al.*, 2004). In this system, proteins are fused to an affinity tag (gluthathione-S-tranferase; GST), which facilitates *in situ* capture on the same DNA printed slide after translation. This *in situ* immobilization step simplifies the significant problem of purifying these proteins, eliminates the need for separate protein expression reactions, avoids microarray printing of the more fragile proteins themselves, and also avoids protein stability problems during storage. Using this microarray, the authors demonstrated several protein–protein interactions, including the pair-wise interactions among 29 human DNA replication initiation proteins, recapitulated the regulation of Cdt1 binding to select replication proteins, and mapped the geminin-binding domain. He and Taussig have described a similar method of making protein arrays termed PISA, which stands for 'protein *in situ* array' (He and Taussig, 2001). In this method, protein microarrays are fabricated directly from PCR-generated DNA fragments using cell-free protein synthesis and epitope tag-based *in situ* immobilization on the array surface. Using PISA, the authors demonstrate that the human single-chain antibody fragments (three domains, V(H)/K form) and luciferase can be functionally arrayed. Such a method is advantageous for arraying proteins and domains which cannot be functionally produced in heterologous expression systems, or for which the cloned DNA is not available (He and Taussig, 2003; He, 2004).

*For screening of translation inhibitors*

Cell-free protein synthesis systems are effective for screening various classes of translation inhibitors. For example, the rabbit reticulocyte translation system is used for identification of anti-sense nucleotides which inhibit expression of viral genes (Chen *et al.*, 1997). Recently, an *E. coli*-based, cell-free expression system was used for high throughput screening of new inhibitors of bacterial protein synthesis from a combinatorial library of macrocycles (Jefferson *et al.*, 2002). The *E. coli* system has also been used for characterization of novel antibacterial dipeptide agents that inhibit translation (Boddeker *et al.*, 2002). In addition, several other researchers are currently developing various translation inhibitor screens for particular compounds based on cell-free protein synthesis (Kuznetsov *et al.*, 1986; Citores *et al.*, 1993; Gorokhovatsky *et al.*, 1998; Humphries *et al.*, 2002; Abraham *et al.*, 2003).

*For functional genomics or* in vitro *expression cloning (IVEC)*

Generally, protein identification is based on function and the gene(s) that encode it, and requires both biochemical and genetic approaches. Typically, proteins are purified on the basis of a particular characteristic or function, and the genes that encode those proteins are identified and isolated through multiple steps, including protein purification, amino acid sequencing, and isolation of cDNA. This process is

quite cumbersome. Alternatively, one can clone the cDNA, translate those cDNAs, and assay the protein products for specific functions. In the past, *Xenopus* oocyte-derived cell-free expression systems have been used to identify genes that encode membrane proteins, secreted factors, and transmembrane channels (Masu *et al.*, 1987; Smith and Harland, 1992; Lemaire *et al.*, 1995). To avoid protein purification and ultimately streamline the assignment of function to a gene product, Kirschner and co-workers have developed a rapid *in vitro* approach termed '*in vitro* expression cloning' (IVEC) (King *et al.*, 1997). First, a cDNA library is constructed in a high copy expression plasmid and then cloned into pools of 50–100 genes each. After cell-free expression of the DNA pools, the results were analysed using SDS-PAGE followed by autoradiography, confirming the presence of approximately 30–50 nascent proteins in each reaction. These protein pools were then assayed for a specific function, such as kinase or protease activity. Subsequently, pools positive for the function of interest are subdivided (deconvolved) until the single cDNA that encodes the protein of interest is isolated. This method has been used successfully for discovering specific proteolysis of the protein kinase C-related kinase 2 by caspase-3 during apoptosis (Stukenberg *et al.*, 1997), for identification of a new uracil-DNA glycosylase family (Haushalter *et al.*, 1999), and for systematic identification of mitotic phosphoproteins (Cryns *et al.*, 1997).

*For ribosome display*

Ribosome display is a powerful technique for the *in vitro* selection of proteins and peptides from large libraries using cell-free protein synthesis. The technique is based on coupling individual nascent proteins (phenotypes) to their corresponding mRNA (genotypes) through the formation of stable protein–ribosome–mRNA (PRM) complexes. After cell-free translation of cDNA/mRNA libraries, the PRM complexes are screened for binding to a target and the retained mRNA is amplified using RT-PCR. The resulting cDNA is then used for another round of selection. Ribosome display has a number of advantages over cell-based systems, such as phage display. In particular, it can display very large libraries without the restriction of bacterial transformation. Furthermore, it is suitable for generating toxic, proteolytically sensitive and unstable proteins, and allows incorporation of modified amino acids at defined positions using TRAMPE. In combination with PCR, a variety of mutations can be introduced efficiently into the selected DNA pool, leading to continuous DNA diversification and protein selection (*in vitro* protein evolution). Both prokaryotic and eukaryotic ribosome display systems have been developed, and each has its own distinctive features. Initial ribosome display work was primarily done in an *E. coli* coupled transcription/translation system, which was used to generate large libraries of peptides for receptor ligand screening (Mattheakis *et al.*, 1994). Later improvements allowed folding of whole proteins into their native structure while still attached to the ribosome (Hanes and Pluckthun, 1997; Schaffitzel *et al.*, 1999).

Ribosome display and *in vitro* protein evolution can be used to produce novel proteins with desired catalytic properties. A cell-free system has been developed for performing evolution studies in which RNA amplification and the coupled reaction can be performed simultaneously at a given temperature (Joyce, 1993). In this system, 'protein evolution' was achieved by exerting selective pressure on

functional protein products necessary for RNA amplification. In one of the latest applications of ribosome display, Roberts and Szostak report a new method called PROfusion in which covalent fusions between an mRNA and the peptide or protein that it encodes is generated (Roberts and Szostak, 1997; Liu *et al.*, 2000). This is achieved by cell-free translation of modified mRNAs that carry puromycin, a peptidyl acceptor antibiotic, at their 3' end. The nascent peptide/protein was found to be covalently attached to its own message, and that specific mRNA could then be selected from a pool of random sequences by immunoprecipitation of the mRNA–peptide fusions. Using this technology, Weng and co-workers have generated addressable protein microarrays (Weng *et al.*, 2002). This was achieved by hybridizing the RNA–protein conjugate to surface-bound DNA capture probes. Such a protein microarray showed high sensitivity and specificity when visualized by autoradiography and antibody probing. Another application of ribosome display is so-called '*in vitro* pathway reconstruction'. Jung and Stephanopoulos report a novel method where mRNA–protein complexes, obtained by cell-free protein synthesis and ribosome display, are selectively immobilized by hybridization of capture probes with their mRNA component (Jung and Stephanopoulos, 2004). This method allows one to immobilize functional pathway enzymes in 'tunable' relative amounts, and has been used for studying the five-step pathway for trehalose synthesis.

## APPLICATIONS OF CELL-FREE TRANSLATION SYSTEMS IN DIAGNOSTICS

Another rapidly growing application of coupled cell-free transcription/translation, and in particular rabbit reticulocyte expression systems, is diagnosis of genetic diseases, a DNA-dominated technology field. The protein truncation test (PTT), also referred to as the *in vitro* synthesized protein (IVSP) assay, specifically detects mutations leading to the premature termination of protein translation. An increasing number of disease-related genes (primarily cancers) have been identified where the majority of occurring mutations result in premature termination of translation, thereby leading to an incomplete and non-functional protein product (Gite *et al.*, 2004). The PTT was first reported in 1993 for detecting truncating mutations in the APC and DMD genes (Powell *et al.*, 1993; Roest *et al.*, 1993), and has been used mainly for clinical research (www.genetests.org).

Typically, in the PTT, genomic DNA or mRNA is isolated from the patient's blood or tissue. This is followed by PCR/RT-PCR using specialized primers that incorporate a T7 promoter and optimal translation initiation sequences surrounding the desired start codon. The amplified DNA is then added directly to a coupled transcription/translation reaction, such as the rabbit reticulocyte cell-free system, and truncation mutations are detected in the nascent proteins. There are several ways to detect the newly synthesized proteins, with the most common being incorporation of radio-labelled amino acids (e.g. $^{35}$S-methionine or $^{13}$C-leucine) and separation by SDS-PAGE, followed by autoradiography. For separation of the translation products, appropriate SDS-PAGE conditions must be chosen for simultaneous detection of both full-length and truncated products. After electrophoresis, gels are dried and protein bands are detected using either X-ray film or a PhosphorImager screen. In general, a wild-type sample will have a strong band at the expected size of the full-length translation product and, ideally, any lower molecular weight bands would

correspond to the presence of mutant truncated proteins. However, contaminating lower molecular weight bands can also originate from internal weak translation initiation sites (AUG) and proteolytic degradation products. In some cases, these bands can obscure the analysis and/or detection of the true truncated fragments derived from a mutant template. In addition to radioactive labelling, methods have been reported which use non-isotopic detection. In one approach, biotin was incorporated during translation using biotin-lysyl-tRNA and was detected by Western blotting (Kirchgesser *et al.*, 1998). Similarly, Muller and co-workers used Western blotting to detect engineered epitope tags (Kahmann *et al.*, 2002). Radioisotope labelling can also be eliminated, and cumbersome Western blotting avoided by incorporating fluorescent labels using fluorescently derivatized tRNAs (Gite *et al.*, 2000; Traverso *et al.*, 2003).

Although non-isotopic methods have clear advantages, they still suffer from the intrinsic throughput problems associated with electrophoresis. In order to overcome these limitations, ELISA-PTT has been developed, which provides higher throughput and lower cost because it circumvents electrophoresis (Gite *et al.*, 2003). The basic ELISA-PTT approach is illustrated in *Figure 8.2*. Specially designed PCR primers, which incorporate N- and C-terminal epitopes, are used for amplification of target sequences. In addition to these epitopes, additional tags can be incorporated randomly along the protein chain for detection and capture purposes. This is accomplished using mis-aminoacylated tRNAs (e.g. biotin-lysyl-tRNA or/and BODIPY-lysyl-tRNA), which are added to the cell-free reaction mixture. A binding tag (B) can also be incorporated into the protein using a specially designed PCR primer (*Figure 8.2*). After translation, the test proteins are captured in a single well of a microtitre plate, and the N- and C-terminal epitope tags are detected using appropriate antibodies. The signals obtained are used to compare the total amount of target protein captured (N-terminal signal) versus the fraction that is full length (i.e. has a C-terminus). In addition, incorporation of a fluorescent label allows rapid non-isotopic detection of wild-type and truncated bands following SDS-PAGE. ELISA-PTT was tested using DNA isolated from familial adenomatous polyposis (FAP) patients, as well as cell lines containing defined truncating mutations in the APC gene. Results indicated that the ELISA-PTT is capable of detecting disease-related protein truncating mutations with high sensitivity and specificity (Gite *et al.*, 2003).

Though the ELISA-PTT does not require electrophoresis for the primary screen, it does require electrophoresis for sizing the proteins in order to locate and confirm the mutation in the amplicon. In contrast, Garvin and co-workers have reported a method to size *in vitro* synthesized proteins by mass spectrometry, thereby eliminating the need for electrophoresis (Garvin *et al.*, 2000). Experiments were carried out using a very short sequence (21 bases) of the *BRCA*1 gene. After cell-free protein synthesis, the test peptide was purified based on its incorporated FLAG-epitope and subjected to MALDI-TOF mass spectroscopy. Truncating mutations in heterozygotes are easily detected, and importantly, single amino acid substitutions are also detectable due to the high resolution of mass spectrometry. Although this process can be multiplexed and is amenable to automation, the small test sequence size reduces its effective throughput compared to an electrophoresis-based PTT or ELISA-PTT.

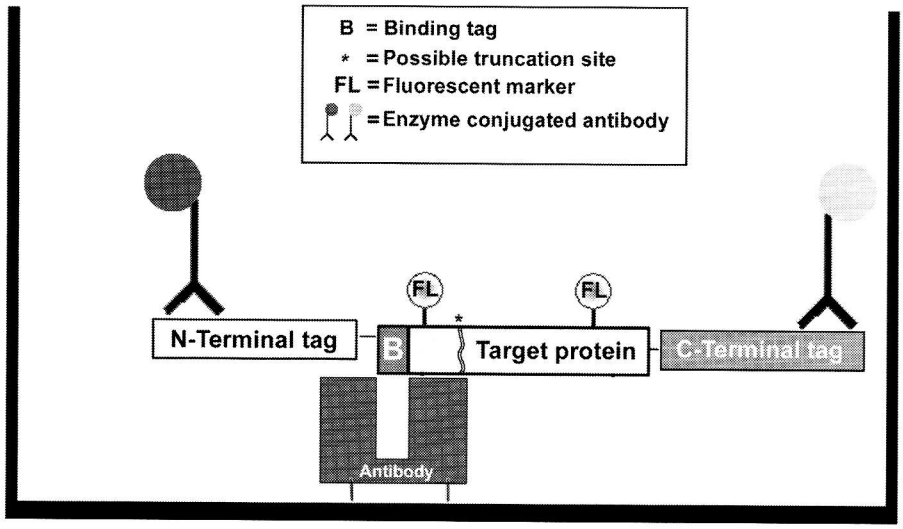


**Figure 8.2.** Schematic representation of an ELISA Protein Truncation Test (ELISA-PTT). Binding tag (B) incorporated at the N-terminal acts to immobilize the cell-free synthesized fragments on the ELISA well surface. N- and C-terminal tags are detected by corresponding antibodies, allowing estimation of the relative amount of truncated and total nascent protein. tRNA-mediated fluorescence labelling (FL) provides an independent validation of truncation and estimate of fragment size using SDS-PAGE and fluorescence readout (Gite *et al.*, 2003).

## Conclusions

Much of our knowledge about the molecular basis of protein synthesis was obtained using cell-free protein expression systems (including the genetic code, mRNA, ribosome functions, protein factors involved in translation, translation stages including initiation, translational control, and co-translational protein folding) (Nirenberg and Leder, 1964; Khorana, 1977). As described in this review, this same approach stands to contribute to several biotechnological applications. However, it is important to note that even with the significant advancements described, fundamental problems still remain. These include the need for improved methods to express various classes of proteins in a functional form, including membrane proteins, larger protein complexes, and proteins that require post-translational modifications. Nonetheless, the ease of use of cell-free translation systems, as well as improvements in yield of proteins expressed, make them attractive tools for many biotechnological applications, including protein engineering, molecular diagnostics, and the emerging field of protein microarrays.

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