

# Applications of Promega's In Vitro Expression Systems



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**Editor's Note:** *The following is a summary of current applications as well as a preview of information to be included in our new "In Vitro Expression Guide." The guide will include complete descriptions of expression technologies and an extensive listing of references. Watch for the release of this new informative and instructive guide that will detail many applications using Promega's in vitro expression systems.*

## INTRODUCTION

In vitro expression has long been a valuable tool for the identification and characterization of genes. The development of PCR technology and the progress achieved in genome sequencing projects are creating new opportunities to understand genes and the proteins they encode. Promega's in vitro expression products continue to advance as well, and are being used in novel applications toward the identification of gene products and subsequent understanding of function, regulation and molecular partners.

PCR technology has made it possible to amplify a particular gene concurrent with incorporation of a phage polymerase promoter. Through in vitro transcription with SP6, T7 or T3 Phage RNA Polymerase, the resulting construct may be used to generate mRNA, which is subsequently used as a template for translation in rabbit reticulocyte lysates or wheat germ extracts. Alternatively, the amplified gene with phage promoter may be expressed directly in a coupled eukaryotic transcription/translation reaction or in an appropriate *E. coli* extract. Protein expressed in vitro may be analyzed in a variety of in vitro assays.

In vitro expression technologies are widely used by scientists across disciplines, including life science research, molecular diagnostics, drug development and high-throughput screening. [Table 1](#) contains examples of applications in which Promega's in vitro expression systems are used.

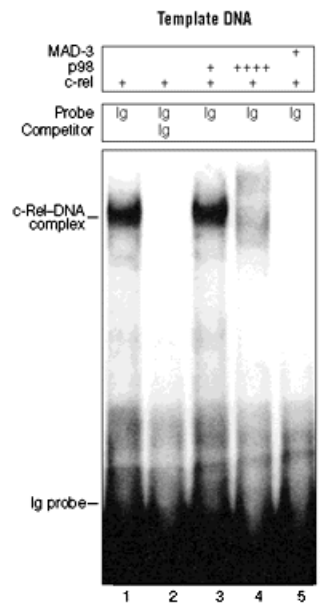
Verification of cloned genes	Large scale protein synthesis
Protein:protein interactions	Epitope/domain mapping
Protein:DNA and protein:RNA interactions	Co-expression of gene products Sensitive ELISA detection
Mutation detection analysis	Viral genome analysis
Screening of inhibitors of transcription/translation	Molecular environment characterization
In vitro expression cloning	Development of antiviral agents
Polysomal display	Rapid mutagenesis screening
Post-translational modification/analysis	

## VERIFICATION OF CLONED GENES

Verification of gene constructs continues to be a popular use of in vitro expression systems. An identified open reading frame may be tested for expression of a protein of expected size by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein products may be visualized by incorporation of either radiolabeled or non-natural amino acids (e.g., biotinylated lysine as with the Transcend<sup>TM</sup> Systems), or by antibody detection (e.g., Western analysis or immunoprecipitation).

## PROTEIN:PROTEIN, PROTEIN:DNA OR PROTEIN:RNA INTERACTIONS

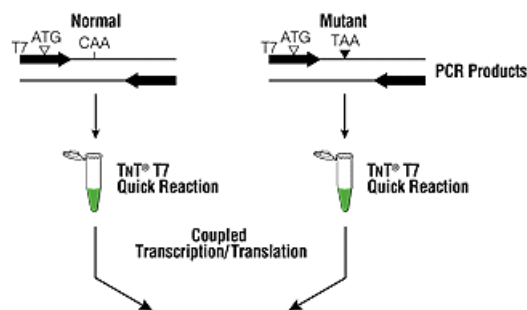
Identification of molecular interactions is an important step toward understanding a protein's function within the cell. When examining protein:protein interactions, an *in vitro* approach is often used to confirm *in vivo* results, such as those obtained using the yeast two-hybrid system (1). To detect interaction between potential protein partners, researchers employ a variety of approaches including the use of fusion tags, immunoprecipitation isolation of protein complexes with streptavidin-coated paramagnetic particles and Far-Western analysis. (A Far-Western is where a protein is immobilized on a solid support and is detected with a labeled protein probe.) Electromobility shift assays (EMSA) are commonly used to study transcription factors and other DNA binding proteins. A protein expressed *in vitro* is incubated with a specific oligonucleotide, and changes in DNA migration are detected by PAGE (see [Figure 1](#)). Either partner in the reaction (protein or oligonucleotide) may be radiolabeled. Protein:RNA interactions also may be studied using protein expressed *in vitro* and radiolabeled RNA. Proteins expressed *in vitro* may also be used to isolate targets of DNA or RNA binding (2,4). By incorporating biotinylated lysines in a TNT<sup>®</sup> System<sup>(a,b,c,d)</sup> reaction programmed with specific template DNA, a biotinylated binding protein or protein binding domain is produced, which can then be coupled to an avidin solid support matrix (such as SoftLink<sup>™</sup> Soft Release Avidin solid support<sup>(e)</sup>, Cat.# V2011), making an affinity column. Total RNA, poly(A)+ RNA or DNA may then be incubated with the affinity resin allowing the protein:nucleic acid interaction to occur. Unbound nucleic acid is washed through the column using non-denaturing conditions. Bound nucleic acid may then be identified using a variety of techniques.



**Figure 1. Electrophoretic mobility shift assays using proteins produced in the TNT<sup>®</sup> Wheat Germ Extract.** c-Rel, p98 and MAD-3 proteins were produced in unlabeled 25 $\mu$ l TNT<sup>®</sup> Wheat Germ Extract reactions containing 1  $\mu$ g of *c-rel* template DNA (lanes 1, 3 and 4, respectively), 0.25 $\mu$ g or 1 $\mu$ g of p98 template DNA (lanes 3 and 4, respectively), and 1  $\mu$ g of MAD-3 template DNA (lane 5). The relative expression levels of each protein were estimated using a parallel set of TNT<sup>®</sup> System reactions containing [<sup>35</sup>S]methionine. Gel shift assays were performed as described in Mercurio *et al.* (3). The volume of unlabeled TNT<sup>®</sup> reaction products added to each gel shift reaction was adjusted to contain a constant amount of c-Rel (from 2-8 $\mu$ l). Each reaction also contained 40pg of <sup>32</sup>P-labeled Ig enhancer probe DNA. In lane 2, the unlabeled competitor Ig probe was added in 50-fold excess (2ng). The gel shift reactions were analyzed by electrophoresis on a 5% polyacrylamide gel and detected by autoradiography for 24 hours at -70°C (4).

## MUTATION DETECTION ANALYSIS

A number of disease genes contain mutations that result in premature termination of translation. Coupled transcription/translation systems provide a rapid and efficient means of screening for the presence of truncation mutations. This screen has been designated the Protein Truncation Test (PTT) (5). There are three important steps (see [Figure 2](#)): 1) isolation of genomic DNA and amplification of the target gene using PCR, or alternatively, isolation of RNA and amplification of the target sequence using Reverse Transcription PCR (RT-PCR); 2) *in vitro* transcription and translation of the target sequence; and 3) SDS-PAGE analysis of the synthesized protein. Proteins may be detected by incorporation of radiolabeled amino acids and visualization by autoradiography or by incorporation of biotinylated lysines and visualization using chemiluminescence. A number of genes containing truncation mutations have been detected using this technique. Some examples include the genes for hereditary breast and ovarian cancer, Cystic Fibrosis, Duchenne Muscular Dystrophy and Neurofibromatosis types 1 and 2. The critical component of PTT is use of a forward primer in the PCR amplification step that contains a phage promoter sequence (T7, SP6 or T3) separated by 36bp from a eukaryotic translation initiation sequence (Kozak sequence), and a 3'-end containing the target gene sequence (1720bp) in-frame with the start codon from the Kozak sequence.



**Figure 2. Schematic diagram of the Protein Truncation Test.** This example illustrates normal and mutant forms of a gene segment amplified by PCR. The mutation of a glutamine codon (CAA) to a stop codon (TAA) is shown.

## SCREENING OF INHIBITORS OF TRANSCRIPTION/TRANSLATION

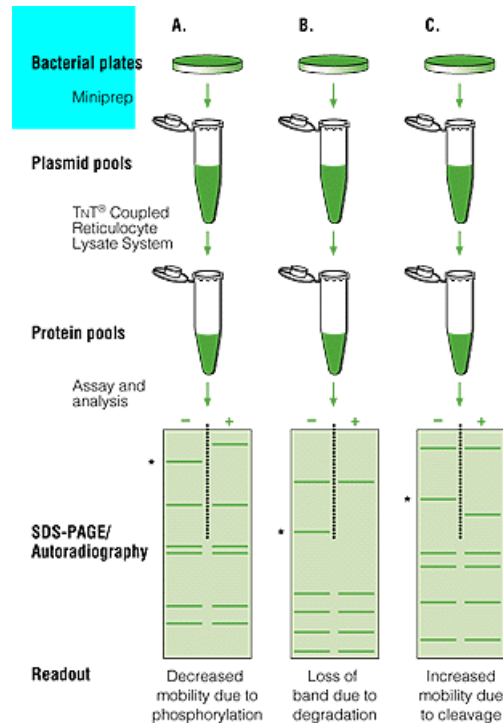
The emergence of antibiotic-resistant strains of bacteria has prompted a renewed effort to develop new antibiotics. Both prokaryotic and eukaryotic *in vitro* translation systems are valuable tools for screening organic compounds for inhibition of transcription and translation. These basic cellular functions are common targets for antibiotic compounds. *In vitro* systems have several advantages over *in vivo* systems, stemming from their relative simplicity when compared to the vast array of interconnected biochemical pathways present in a growing cell. Precise control over the compound concentration is much easier to achieve in an *in vitro* system. Furthermore, initial screens may be performed much faster because there is no need for cellular uptake to evaluate the effect of the test compound. It is also possible to separate effects and monitor individual processes such as transcription or translation. Investigation of organic compounds is generally done in a high throughput-screening format. This presents special challenges in terms of analyzing gene products. Since *in vitro* transcription/translation systems usually produce proteins that are correctly folded and retain their enzymatic activity, reporter systems are commonly used to simplify screening efforts. A number of reporter assays have been used in transcription/translation reactions, including beta-galactosidase, beta-lactamase, galactokinase, chloramphenicol acetyl transferase (CAT), luciferase and alkaline phosphatase.

## POLYSOMAL DISPLAY

Combinatorial display technologies are a tool for identifying ligands. They share an ability to combine a large degree of structural diversity with effective selection to isolate those individuals in a diverse population that interact with a specific target. The most commonly used of these technologies is phage display. Recombinant DNA techniques are used to introduce a randomized peptide sequence as a gene fusion to a surface protein on the phage. Each phage is a separate biological unit that carries both the displayed peptide and the genetic information encoding the peptide. Specific phages that are able to interact with the target (typically through interaction on a multiwell plate) are amplified by growth on the appropriate host strain. Following several rounds of enrichment, the randomized portion in the isolated phage clones is identified by DNA sequencing. Polysomal, or ribosomal, display is an example of a combinatorial display technology that utilizes *in vitro* expression. Polysomes are large complexes of ribosomes, mRNA and translated protein that form during the translation process. Polysomal display has the advantage of linking the genetic information encoded on the mRNA to the newly emerging translation product. This is based upon the finding that nascent polypeptides and their corresponding mRNAs form stable polypeptide-ribosome-mRNA complexes in the absence of a stop codon in cell-free systems (6,7). Other approaches to *in vitro* combinatorial display include the SELEX (systematic evolution of ligands by exponential enrichment) approach (8), and use of reverse micelles that act as reaction vessels and thus mimic *in vivo* cellular compartmentalization (9). The SELEX approach creates an RNA library from a degenerate DNA template. Diversity is derived from variations in both the primary sequence and secondary structure of the transcribed RNA.

## IVEC: IN VITRO EXPRESSION CLONING

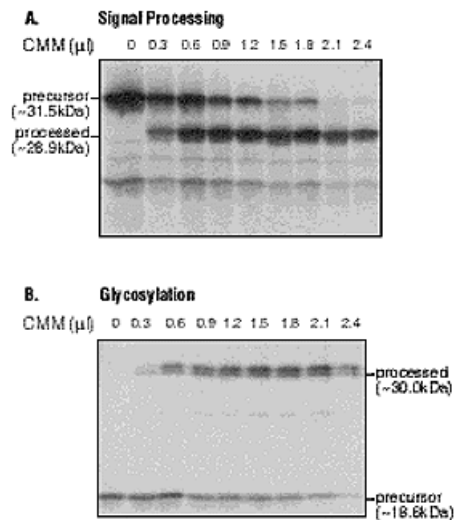
The *in vitro* expression cloning or IVEC technique was developed to expand the range of biochemical assays that can be used to characterize a gene product while circumventing the requirement for protein purification (1013). This technique uses the TNT® Coupled Reticulocyte Lysate System to express proteins from small pools of cDNA libraries, thus simplifying genetic characterization and biochemical screening of genes and the proteins they encode. A cDNA library may be constructed in a high-copy expression plasmid containing a T7, T3 or SP6 promoter. Plasmid DNA is used directly as a template in small-scale (e.g., 10µl) TNT® System reactions, and up to 3050 proteins may be produced in a single reaction depending upon the number of full-length cDNA clones in the library. Proteins may then be assayed for a number of different activities, including phosphorylation, proteolysis or cleavage (as illustrated in Figure 3). This technique may also be used to screen for interactions of the proteins produced from cDNA pools with bait molecules, such as antibodies, proteins (or protein complexes) and nucleic acids. Potential uses of IVEC technology will continue to expand. It may be possible to supplement TNT® System reactions with Canine Pancreatic Microsomal Membranes (Cat.# Y4041) and screen for secreted proteins using protease protection assays, or screen for cleavage of signal sequences using electrophoretic mobility screens. Furthermore, enzymatic activity may be screened in such a way as to identify mutant proteins with novel properties, such as activity in unusual conditions or insensitivity to specific inhibitors.



**Figure 3. The strategy of in vitro expression cloning (IVEC).** An unamplified cDNA expression library is plated at approximately 100 transformant colonies per plate. Pooled plasmid DNA is obtained by scraping colonies from each plate and performing a small-scale plasmid purification. Each plasmid pool is transcribed and translated using the TNT<sup>®</sup> Coupled Reticulocyte Lysate System. The resulting protein pool is assayed for functional activity. In the example shown, radioactive amino acids are incorporated during the translation reaction. The pool of proteins is then incubated with a modifying enzyme, such as a kinase or protease, and is assayed for modified mobility in SDS-PAGE analysis due to phosphorylation (**Panel A**), loss of band due to degradation (**Panel B**) or increased mobility due to cleavage (**Panel C**). In each case, the candidate protein band is marked with an asterisk (\*). Once a pool possessing a candidate activity is identified, the original cDNA pool is subdivided and retested until the single cDNA encoding the protein of interest is isolated. Reprinted with permission from "Expression Cloning in the Test Tube" (1997) *Science* 277, 973. ©1997 American Association for the Advancement of Science.

## POST-TRANSLATIONAL MODIFICATIONS

Systems for cell-free protein synthesis are a valuable tool to researchers for the investigation of co- and post-translational modifications. Rabbit reticulocyte lysates and wheat germ extracts are the most commonly used in vitro expression systems for this purpose, and studies are often performed in the presence and absence of Canine Pancreatic Microsomal Membranes (see [Figure 4](#)). Examples of modifications that have been observed in either rabbit reticulocyte lysates or wheat germ extracts, include signal peptide cleavage, glycosylation, acetylation, phosphorylation, isoprenylation, myristoylation, protein folding and proteolytic processing. The factors required for each type of modification may be present in varying levels from one preparation of lysate, extract and membrane to another due to the complexity of these systems. As a result, the co- and post-translational modifications observed in in vitro expression systems may vary among different preparations. Furthermore, some types of modifications may require the addition of microsomal membranes, or may be more efficient in one expression system than in another.



**Figure 4. Processing and glycosylation activity of Canine Pancreatic Microsomal Membranes.** Translation was performed using the Rabbit Reticulocyte Lysate System (Cat.# L4960) in a 25 $\mu$ l reaction for 60 minutes with 0.5 $\mu$ g of *E. coli* beta-lactamase (**Panel A**) or 0.5 $\mu$ g of *S. cerevisiae* alpha-factor (**Panel B**). Aliquots were then analyzed by gel electrophoresis and autoradiography of the <sup>35</sup>S-labeled proteins.

## CONCLUSION

Promega strives to assist researchers with their in vitro expression studies. An extensive bibliography of references using the TNT<sup>®</sup> Coupled Transcription/Translation Systems, as well as a reference list for PTT is available on our web site at [www.promega.com/expression/exrefr.html](http://www.promega.com/expression/exrefr.html). Promega's web site ([www.promega.com](http://www.promega.com)) has helpful links to technical resources (including protocols, an online seminar and *Promega Notes*) and online ordering. For further assistance please contact our Technical Services Department by phone (800-356-9526 or 608-274-4330) or e-mail ([techserv@promega.com](mailto:techserv@promega.com)).

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## Ordering Information

Product	Cat.#
TNT <sup>®</sup> T3 Coupled Reticulocyte Lysate System	<a href="#">L4950</a>
TNT <sup>®</sup> T7 Coupled Reticulocyte Lysate System	<a href="#">L4610</a>
TNT <sup>®</sup> SP6 Coupled Reticulocyte Lysate System	<a href="#">L4600</a>
TNT <sup>®</sup> T7/SP6 Coupled Reticulocyte Lysate System	<a href="#">L5020</a>
TNT <sup>®</sup> T7/T3 Coupled Reticulocyte Lysate System	<a href="#">L5010</a>
TNT <sup>®</sup> T7 Coupled Reticulocyte Lysate System, Trial Size	<a href="#">L4611</a>

TNT <sup>®</sup> SP6 Coupled Reticulocyte Lysate System, Trial Size	<a href="#">L4601</a>
TNT <sup>®</sup> T7 Coupled Wheat Germ Extract System	<a href="#">L4140</a>
TNT <sup>®</sup> SP6 Coupled Wheat Germ Extract System	<a href="#">L4130</a>
TNT <sup>®</sup> T3 Coupled Wheat Germ Extract System	<a href="#">L4120</a>
TNT <sup>®</sup> T7/SP6 Coupled Wheat Germ Extract System	<a href="#">L5030</a>
TNT <sup>®</sup> T7/T3 Coupled Wheat Germ Extract System	<a href="#">L5040</a>
Transcend <sup>™</sup> Non-Radioactive Translation Detection System (Colorimetric)	<a href="#">L5070</a>
Transcend <sup>™</sup> Non-Radioactive Translation Detection System (Chemiluminescent)	<a href="#">L5080</a>
Canine Pancreatic Microsomal Membranes	<a href="#">Y4041</a>

<sup>(a)</sup>U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, and Australian Pat. No. 649289, have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

<sup>(b)</sup>U.S. Pat. Nos. 5,324,637, 5,492,817, 5,665,563, European Pat. No. 0 566 714 B1, and Australian Pat. No. 660329, have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

<sup>(c)</sup>The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

<sup>(d)</sup>U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217 and Australian Pat. No. 646803 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). *Inhibitors of Angiogenin*, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

<sup>(e)</sup>For research purposes only. Not for diagnostic or therapeutic use. For bulk purchases of this product, contact TosoHaas, 156 Keystone Drive, Montgomeryville, PA 18936, 1-800-366-4875 or 215-283-5000.

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