

In vitro Expression Cloning Using the TNT[®] Coupled Reticulocyte Lysate System



By Greg Beckler
Promega Corporation

Email to gbeckler@promega.com

Author's Note: This article summarizes information previously published in peer-reviewed journal articles that featured the use of Promega's reagents and systems in novel experiments in the life sciences. The publisher of the original journal article has granted Promega permission to reproduce the figure shown here.

The *in vitro* expression cloning^(a) or IVEC technique is a systematic and broadly applicable method for the genetic characterization and biochemical screening of genes and cognate proteins. IVEC uses the TNT[®] Coupled Reticulocyte Lysate System^(b) to express proteins from small pools of cDNA libraries, greatly simplifying the process of isolating genes that encode proteins identified by a particular biochemical function or characteristic. Here, we summarize the experimental procedure and suggest possible uses for the technology.

INTRODUCTION

The systematic identification of proteins based upon function and the identification of the genes that encode them requires both biochemical and genetic approaches. In traditional biochemical techniques, proteins are purified on the basis of a particular characteristic or function, and the genes that encode those proteins are isolated through multiple steps, including protein purification, peptide sequencing and isolation of complementary DNA (cDNA). This process is labor-intensive. Earlier, alternative methods, such as expression cloning, use "reverse" biochemistry to first clone cDNAs, translate those cDNAs in prokaryotic or eukaryotic cells, and then assay the protein products for specific functions or attributes. These cell-based expression systems have been used to identify the genes that encode membrane proteins, secreted factors and transmembrane channels (1-7). To expand the range of biochemical assays that can be used to identify a particular gene and its protein product while still circumventing the strict requirement for protein purification, researchers in the Kirschner lab developed an *in vitro* approach, IVEC (8).

THE IVEC METHOD

The IVEC protocol first expresses small pools (50-100 clones) of cDNAs, as plasmids, in the TNT[®] Coupled *in vitro* Transcription/Translation System. This is in contrast to the whole cell approach, which limits the accessibility of the expressed proteins. Briefly, an oligo(dT)-primed cDNA library is constructed in a high copy expression plasmid containing a T3, T7 or SP6 promoter. (In theory, the library could also be constructed in an appropriate phage expression vector.) The plasmid library is then transformed into *E. coli* and stored as glycerol stocks at 70°C. Approximately 10⁵ independent transformants are spread on bacterial plates containing antibiotic. The bacterial colonies are grown to a specific size (e.g., 1mm in diameter), collected and pooled. An aliquot of the pool is stored as a glycerol stock, and the remainder is purified using a plasmid miniprep technique.

Plasmid DNA is used directly as template in small-scale (e.g., 10µl) TNT[®] System reactions in the presence of [³⁵S]methionine following the protocol in the TNT[®] Coupled Reticulocyte Lysate Systems Technical Bulletin (9). Depending upon the number of the full-length cDNA clones in the library, the TNT[®] System allows approximately 30-50 proteins to be produced in a single reaction. Protein pools are then stored at 70°C until assayed. Proteins can be assayed for any number of activities. Examples include phosphorylation, proteolysis or cleavage (Figure 1). Positive pools are subdivided until the single cDNA that encodes the protein of interest is isolated. References 8, 10 and 11 contain specific details on the IVEC technique.

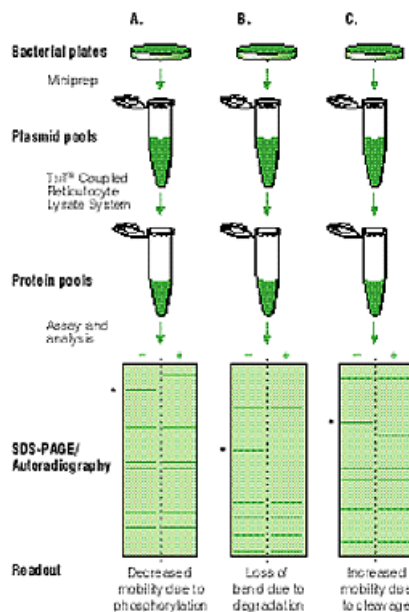


Figure 1. The strategy of *in vitro* expression cloning (IVEC). An unamplified cDNA expression library is plated at approximately 100 transformant colonies per plate. Pooled plasmid DNA is obtained by scraping colonies from each plate and performing a small-scale plasmid purification. Each plasmid pool is transcribed and translated using a TNT[®] 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 decreased 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.*

EXAMPLES OF IVEC SCREENING

IVEC has been used to identify substrates of particular enzymes and DNA binding proteins. Kirschner and colleagues have screened for substrates of kinases and proteases using small pools of radiolabeled proteins (11,12). Typically, the kinase or protease (either present as a crude fraction or purified protein) is incubated with the radiolabeled products from the TNT[®] System reaction. The proteins are then resolved by SDS-PAGE analysis, and candidate substrates are identified by changes in electrophoretic mobility.

This approach was used to identify proteins that are phosphorylated or degraded during mitosis (10,11). Pools of radiolabeled proteins expressed in the TNT[®] System were incubated with interphase or mitotic extracts from *Xenopus* embryos. Phosphorylated, radiolabeled proteins were identified by their reduced mobility, compared to control proteins, by SDS-PAGE. In addition, a monoclonal antibody to mitotic phosphoproteins was used to immunoprecipitate substrates after treatment with the embryo extracts. This screen identified 20 distinct, mitotically phosphorylated proteins, 15 of which have sequence similarity to other known proteins. Five of the 15 proteins have been shown previously to be phosphorylated during mitosis, five are related to proteins with putative roles during mitosis, and nine are related to known transcription factors (11). Interestingly, five proteins were identified with genetic sequences that did not match any known gene. This demonstrates the power of this technology to identify novel genes encoding proteins with particular functions. Most of the identified substrates could be directly phosphorylated by the cyclin-dependent kinase, Cdc2, although physiologically these proteins may be substrates for other mitotic kinases. A similar screen was used to identify proteins degraded by proteases during mitosis (10). In this case, substrates of mitotic proteases were identified using SDS-PAGE by their presence when incubated with interphase extract, and their absence when incubated with mitotic extract.

A secondary screen is used to verify potential positive substrates. For example, to verify protein kinase substrates, alkaline phosphatase treatment demonstrates that the post-translational change in electrophoretic mobility is due to phosphorylation. For proteolytic degradation experiments, substrate modifications are tested for dependence on the ubiquitin system by incubation with inhibitors of the ubiquitin-mediated system. In addition, these *in vitro* studies can be followed up by *in vivo* studies to determine whether candidate proteins behave similarly during the regular cell-cycle.

In a separate study, Kothakota and colleagues sought to identify substrates of caspase-3, a protein involved in apoptosis. Screening of cDNAs using the IVEC technique, gelsolin was identified as an *in vitro* target of caspase-3 (13). *In vivo* studies confirmed gelsolin as a substrate for the caspase-3 enzyme. In addition, the cleaved gelsolin protein appears to have a role in morphology of apoptotic cells: The protein severs actin filaments after cleavage by caspase-3.

IVEC can also screen for interactions of the proteins produced from cDNA pools expressed in the TNT[®] System with bait molecules, such as antibodies, proteins (or protein complexes) and nucleic acids. For identification of DNA-binding proteins, the unlabeled protein

pools are incubated with labeled DNA fragments. Specific interactions are detected as gel mobility shifts of the radiolabeled DNA fragment on non-denaturing polyacrylamide gels (10). Two negative controls are performed: A probe without protein and a probe with lysate from the TNT[®] Coupled Reticulocyte Lysate System to test for no shift and possible DNA-binding activities in the reticulocyte lysate, respectively. If available, a positive control containing a protein known to bind the DNA fragment is also conducted.

The potential uses of IVEC are far-reaching. The technique could be used to detect modified versions of proteins using a specific antibody. In addition, the bait molecule may be a large complex, such as a multimeric enzyme, ribosome, virus or cellular organelle. Also, TNT[®] System reactions can be supplemented with Canine Pancreatic Microsomal Membranes (Cat.# Y4041), and screens for secreted proteins could be developed using protease protection assays, or electrophoretic mobility screens could be performed to detect cleavage of signal sequences. By incorporating a chemical or epitope tag into the synthesized proteins, assays could be designed to identify proteins that are localized to specific structures. Theoretically, biotinylated lysines, such as with the Transcend[™] Systems, can be incorporated to facilitate capture of the protein pools in an arrayed fashion on a solid support, such as SAM^{2™} Biotin Capture Membrane^(c) (Cat.# V2861) or the TetraLink[™] Tetrameric (Cat.# V2591) or SoftLink^{™(d)} Soft Release (Cat.# V2011) Avidin Resins. Enzyme activity screens could also be used to identify proteins with activity in unusual conditions (such as extremes of pH, temperature or ionic strength). Also, enzyme activity screens could be used to identify mutant proteins with novel properties, such as insensitivity to an inhibitor.

SUMMARY

IVEC provides a powerful tool for biochemical and genetic investigations that is both simple and feasible, even in small laboratories. IVEC does hold some limitations that should be considered in experimental design. Current IVEC technology will not succeed in activity assays where multiple proteins are required, unless putative binding partners are known and supplemented in the reaction. In addition, the success of activity-based approaches depends on the TNT[®] System not containing the activity being assayed. For some cases, the TNT[®] Wheat Germ Extract System may circumvent this problem because it lacks some of the activities present in the rabbit reticulocyte lysate, such as certain transcription factors (e.g., NF-kappaB family members).

Finally, the success of the IVEC screen depends directly on the quality of the cDNA library used (i.e., a high percentage of full-length clones). "Normalized" cDNA libraries, where the frequency of each cDNA clone is made nearly equivalent, or UniGene libraries (National Center for Biotechnology Information, reference 14) developed from sequenced genomes (e.g., *Saccharomyces cerevisiae*) will greatly expand the vitality of IVEC. The IVEC technique may have widespread use with the multiple gene clones becoming available from genome sequencing projects. IVEC promises to be an important method for identifying gene function and is only limited by the creativity of researchers to develop appropriate functional assays.

ACKNOWLEDGEMENTS

I would like to acknowledge the expert advice and assistance of Drs. Randall King and Marc Kirschner during the preparation of this article.

REFERENCES

1. Masu, Y. *et al.* (1987) *Nature* **329**, 836.
2. Wong, G.C. *et al.* (1985) *Science* **228**, 810.
3. Lustig, K.D. *et al.* (1996) *Development* **122**, 3275.
4. Smith, W.C. and Harland, R.M. (1992) *Cell* **70**, 829.
5. Lemaire, P., Garrett, N. and Gurdon, J.B. (1995) *Cell* **81**, 85.
6. Lustig, K.D. *et al.* (1996) *Development* **122**, 4001.
7. Simonsen, H. and Lodish, H.F. (1994) *Trends Pharmacol. Sci.* **15**, 437.
8. King, R.W. *et al.* (1997) *Science* **277**, 973.
9. TNT[®] Coupled Reticulocyte Lysate Systems Technical Bulletin #TB126, Promega Corporation.
10. Lustig, K.D. *et al.* (1997) *Meth. Enzymol.* **283**, 83.
11. Stukenberg, P.T. *et al.* (1997) *Curr. Biol.* **7**, 338.
12. McGarry, T.J. and Kirschner, M.W. (1998) *Cell*, in press.
13. Kothakota, S. *et al.* (1997) *Science* **278**, 294.
14. Boguski, M.S. and Schuler, G.D. (1995) *Nat. Genet.* **10**, 369.

Ordering Information

Product	Cat.#

TNT [®] T7 Quick Coupled Transcription/Translation System	L1170
TNT [®] T7 Quick Coupled Transcription/Translation System, Trial Size	L1171
TNT [®] T3 Coupled Reticulocyte Lysate System	L4950
TNT [®] T7 Coupled Reticulocyte Lysate System	L4610
TNT [®] T7 Coupled Reticulocyte Lysate System, Trial Size	L4611
TNT [®] SP6 Coupled Reticulocyte Lysate System	L4600
TNT [®] SP6 Coupled Reticulocyte Lysate System, Trial Size	L4601
TNT [®] T7 Coupled Wheat Germ Extract System	L4140
TNT [®] SP6 Coupled Wheat Germ Extract System	L4130
TNT [®] T3 Coupled Wheat Germ Extract System	L4120
Canine Pancreatic Microsomal Membranes	Y4041

Related Products

Product	Cat.#
Transcend [™] Non-Radioactive Translation Detection System (Colorimetric)	L5070
Transcend [™] Non-Radioactive Translation Detection System (Chemiluminescent)	L5080

^(a)The method of in vitro expression cloning is covered by U.S. Pat. No. 5,654,150 assigned to The President and Fellows of Harvard College.

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

^(c)Patent Pending.

^(d)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.

**Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher.*

© 1998 Promega Corporation. All Rights Reserved.

TNT is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office. SAM², SoftLink, TetraLink and Transcend are trademarks of Promega Corporation.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.