

## High-Quality In Vitro Expression Cloning Using the Gold TNT® T7 Express 96 System

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### Abstract

The Gold TNT® T7 Express 96 System is a recent enhancement to Promega's line of in vitro expression products. The system is composed of components similar to the original TNT® Coupled Transcription/Translation Systems, with the Gold TNT® System components meeting a higher specification for luciferase activity. In addition, the Gold System components allow for large-scale screening assays through the convenience of a 96 well plate configuration. The authors used the Gold TNT® T7 Express 96 System for in vitro expression cloning (IVEC) to identify a gene that produces a substrate of separase.

**IVEC is a powerful technique for identifying new genes based solely on the biochemical properties of the gene products.**

### Introduction

The Gold TNT® Express 96 Systems<sup>(a,b,c,d,e)</sup>, available in either SP6 or T7 versions, are designed for transcription and translation of genes cloned downstream of either the T7 or SP6 RNA polymerase promoter. To use these systems, plasmid DNA containing the appropriate promoter is added, with either unlabeled or [<sup>35</sup>S]-labeled methionine and Nuclease-Free Water (Cat.# P1193), to 96 well plate wells containing lysate. The proteins synthesized are then analyzed by functional testing or by SDS-polyacrylamide gel electrophoresis with subsequent detection by fluorescent imager, colorimetric/chemiluminescent methods or autoradiography.

In classical biochemistry proteins are purified based on a particular activity, and the derived amino acid sequence information is then used to isolate the corresponding genes. However, as these traditional biochemical methods reach their limits of detection, "reverse" biochemistry has gained importance as a tool for the identification of novel genes and their expressed proteins. IVEC is a powerful technique for identifying new genes (in a given process) based solely on the biochemical properties of the gene products (1,2). This functional genomics approach is not dependent on the purification of the respective proteins.

The strategy of IVEC is as follows: A cDNA library is subdivided into small pools of 50–100 clones each, which are then expressed in vitro. The resulting small protein pools are used in substrate, interaction or activity assays. Once a pool that contains a protein with the desired

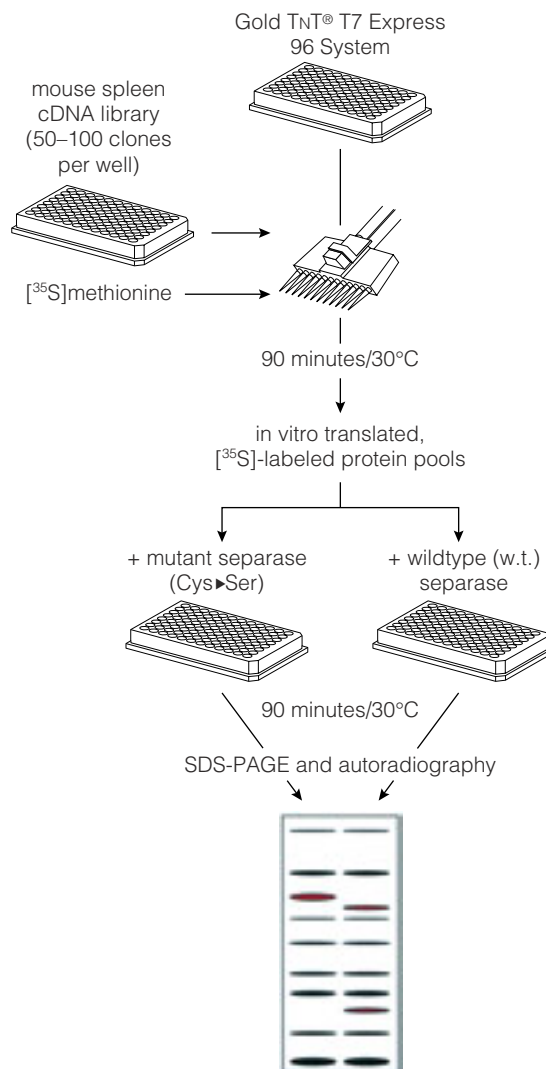


Figure 1. Schematic diagram of the IVEC screen for separase substrates.

property is identified, the corresponding gene is easily identified by systematic subdivision of the respective pool, a technique called sib selection (1–4).

### Performance of an IVEC Screen in a 96 Well Format

Recently, both in vitro expression cDNA libraries and coupled transcription/translation systems have been adapted to 96 well formats. This innovation greatly reduces the hands-on time necessary to perform an IVEC screen, making it a true high-throughput method amenable even to small research laboratories.

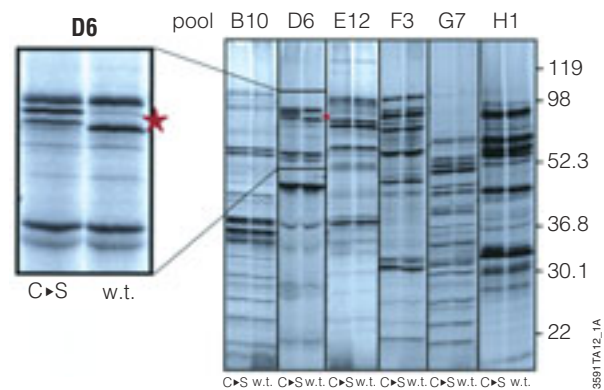
We used an IVEC approach in combination with the new Gold TNT<sup>®</sup> T7 Express 96 System to look for substrates of separase (Figure 1). Separase is a site-specific cysteine endopeptidase that governs sister chromatid separation (5). It does so by cleavage of Scc1, a component of the multisubunit protein complex, cohesin. The cohesin complex mediates sister chromatid cohesion until the beginning of anaphase. In mitotically dividing vertebrate cells Scc1 is the only known separase substrate. We attempted to unambiguously identify this particular target protein by IVEC. To this end we first generated active human separase (6). A mutant separase, with its active site cysteine replaced with a serine, served as a negative control. In vitro expressed human Scc1 is indeed cleaved by the wildtype separase but not by the catalytically inactive separase mutant or by incubation in TNT<sup>®</sup> Lysate alone (6). To perform the screen we used a mouse spleen cDNA library and the Gold TNT<sup>®</sup> T7 Express 96 System. The library consisted of five 96 well plates, each containing 50–100 clones per well, which corresponded to roughly 36,000 clones total.

Five Gold TNT<sup>®</sup> T7 Express 96 well plates, each containing 20µl of predispensed translation-optimized reticulocyte lysate per well, were thawed on ice for 10 minutes. Using a multichannel pipettor, we added 0.6µg of DNA from each well of the library and 1µl of [<sup>35</sup>S]methionine (1,000 Ci/mmol at 10mCi/ml) to each reaction to give a total volume of 25µl. After incubation of the Gold TNT<sup>®</sup> T7 Express 96 System plates at 30°C for 90 minutes, we transferred 4µl from each well to a new 96 well plate and added 2µl per well of either wildtype or mutant separase. The reactions were incubated at 37°C for 90 minutes, then 20µl of SDS sample buffer was added to each well. After heating to 70°C for 3 minutes, wildtype and mutant samples of each pool were quantitatively loaded and run side-by-side on 10–15% SDS-polyacrylamide gels. Following electrophoresis, unincorporated methionine was removed by fixing the gels in 25% methanol/7.5% acetic acid with subsequent rinsing in water. The dried gels were exposed to Kodak BioMax<sup>®</sup> MR film for 48 hours.

## Results

Of the 480 protein pools analyzed, only 1 displayed a difference in banding patterns upon incubation with wildtype versus mutant separase: In pool D6 an ~80kDa band was converted to a ~75kDa band upon incubation with active (wildtype; w.t.) separase (Figure 2). The corresponding plasmid was isolated from this pool by sib selection as described (1–4). The insert of this clone was then partially sequenced. A BLAST search against the mouse database unambiguously identified the respective insert as a fragment of the mouse Scc1 (mScc1) gene (6).

The fact that the IVEC approach allowed the identification of the mScc1 gene from a total of approximately



**Figure 2. Autoradiograph of a selection of in vitro translated pools after incubation with either wildtype or mutant separase with subsequent SDS-PAGE.** The protein pool numbers are given at the top; the numbers on the right indicate molecular weight in kDa. A red star indicates the mScc1 that has been cleaved by wildtype but not by mutant separase. w.t. = wildtype separase, C>S = mutant separase.

36,000 clones exemplifies the sensitivity of this method. Furthermore, the fact that no false positives were seen demonstrates the specificity of separase for its substrate and the potential of this assay for the identification of an as yet unknown substrate of vertebrate separase—assuming that more than one substrate exists.

We observed an average 25–30 [<sup>35</sup>S]-labeled protein bands per translated cDNA pool containing 50–100 clones (Figure 2). This discrepancy has been previously reported (1,2). The difference in numbers of bands versus clones might reflect the presence of noncoding sequences and/or partial genes lacking efficient start codons. The bands are usually of different intensity, probably reflecting variations in the amount of encoded methionines and the efficiency with which a given mRNA is recruited by the translation machinery. Protein bands of 150kDa and above were readily observed and more frequently so than in former IVEC screens performed in our lab (Figure 2 and data not shown). This demonstrates that the library contained many large and potentially full-length cDNAs. During sib selection performed with ordinary TNT<sup>®</sup> Lysate, we noticed that the signals were weaker than those from the Gold TNT<sup>®</sup> T7 Express 96 System (data not shown). The reason for this is probably the more stringent specification for luciferase activity applied to the Gold TNT<sup>®</sup> T7 Express 96 System versus that for the TNT<sup>®</sup> Quick Coupled Transcription/Translation Systems<sup>(a,b,c,d,e)</sup> (7).

## Conclusions

The Gold TNT<sup>®</sup> T7 Express 96 System realizes the adaptation of a high-quality coupled in vitro transcription/translation system to a 96 well plate format. Using an IVEC approach we tested this system and found it extremely useful. The lysates are already

supplemented with amino acids (except methionine) and T7 RNA polymerase and, therefore, require only the addition of [<sup>35</sup>S]methionine and DNA. This reduction to just two pipetting steps, together with the convenient format, greatly reduce the manipulations that are required to start the expression reactions. When the cDNA library is similarly formatted in 96 well plates and a multichannel pipettor is used, 480 [<sup>35</sup>S]-labeled protein pools representing an entire expression library can easily be generated with a hands-on time of just one hour. We found the Gold TNT<sup>®</sup> T7 Express 96 System to perform well when used with IVEC in large-scale screening. This system increases the speed and ease of high-throughput analyses.

### References

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7. Promega Product Information #9PIL560, Promega Corporation.

### Protocols

- ◆ Gold TNT<sup>®</sup> T7 Express 96 System and Gold TNT<sup>®</sup> SP6 Express 96 System Technical Manual #TM054, Promega Corporation. ([www.promega.com/tbs/tm054/tm054.html](http://www.promega.com/tbs/tm054/tm054.html))



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### Ordering Information

Product	Size	Cat.#
Gold TNT <sup>®</sup> T7 Express 96 System	96 wells	L5600
Gold TNT <sup>®</sup> SP6 Express 96 System	96 wells	L5800

### Related Products

Product	Size	Cat.#
ProteoLink <sup>™</sup> In Vitro Expression Cloning System (Human Adult Brain) <sup>(f)</sup>	10 × 96 wells*	L6500
ProteoLink <sup>™</sup> In Vitro Expression Cloning System, Set 1 (Human Adult Brain) <sup>(f)</sup>	5 × 96 wells*	L6501
ProteoLink <sup>™</sup> In Vitro Expression Cloning System, Set 2 (Human Adult Brain) <sup>(f)</sup>	5 × 69 wells*	L6502

\*Each system contains the indicated number of Gold TNT<sup>®</sup> SP6 Express 96 System plates as well as either 10 plates cDNA Library, plates A–J (Cat.# L6500); 5 plates cDNA Library, plates A–E (Cat.# L6501); or 5 plates cDNA Library, plates F–J (Cat.# L6502).

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BioMax is a registered trademark of Eastman Kodak Company.

<sup>(a)</sup> U.S. Pat. No. 5,283,179, 5,641,641, 5,650,289, 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

<sup>(b)</sup> U.S. Pat. Nos. 5,324,637, 5,492,817, 5,665,563, Australian Pat. No. 660329 and other patents.

<sup>(c)</sup> U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

<sup>(d)</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>(e)</sup> U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

<sup>(f)</sup> Exclusively licensed under U.S. Pat. No. 5,654,150.