FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System

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Abstract

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System is the first commercially available fluorescent technology for labeling in vitro translation products. The FluoroTect™ System uses a modified charged lysine transfer RNA (tRNA) labeled with the fluorophore, BODIPY®-FL. The modified lysine tRNA is used efficiently in both prokaryotic and eukaryotic in vitro translation systems. Data is obtained in a few minutes by the detection of the fluorescently labeled proteins "in-gel" with laser-based fluorescent gel scanning. This feature circumvents the multiple manipulations associated with traditional isotopic and non-isotopic incorporation and detection methodologies.

Introduction

Cell-free expression systems have been a standard tool for the rapid characterization of gene products of interest for almost two decades. Interestingly, the use of cell-free systems for the in vitro expression of proteins continues to rapidly expand with applications in basic research, molecular diagnostics and high-throughput target/drug discovery. Increasingly, extract-based expression tools are being used in functional genomic applications as a "bridge" between traditional genomic and proteomic approaches (1). Research is moving toward the systematic characterization of gene expression and protein function in living cells. Currently, however, researchers must use multiple methods to determine the many levels of function, including: predictive "*in silico*" bioinformatics, expression profiling, and extract-based, cell-based and whole animal assays. In vitro expression technologies offer significant time savings over cellular and whole animal approaches and are generally easy to perform. Examples of these technologies include detection of open reading frame expression (2), mutation analysis (3), post-translational modification analysis (4), protein-protein interaction (5) and high-throughput screening (6). Typically, experimental results are obtained through the detection of [^{35}S]methionine incorporation into the nascent protein chain.

Promega's new FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System offers a simplified alternative method that dramatically reduces the time (2–5 minutes vs. 24 hours) required to obtain data and eliminates manipulations associated with radioactivity (i.e., gel fixing and drying or transferring to a membrane). The FluoroTect™ System also eliminates the requirement for electroblotting associated with other non-isotopic technologies.

> The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System is the most sensitive non-radioactive protein labeling system available.

Conventional methods

One of the most common methods used in the detection of proteins synthesized or characterized using in vitro translation systems is the incorporation of radioactively labeled amino acids. Typically [^{35}S]methionine is the label of choice, but other labels such as [^{35}S]cysteine, [^{14}C]leucine and [^{3}H]leucine could be used as well. These standard reactions are resolved by SDS-PAGE, which is fluorographically enhanced, dried and then exposed to X-ray film for 6–18 hours or transferred to membranes and scanned by phosphorimaging (7).

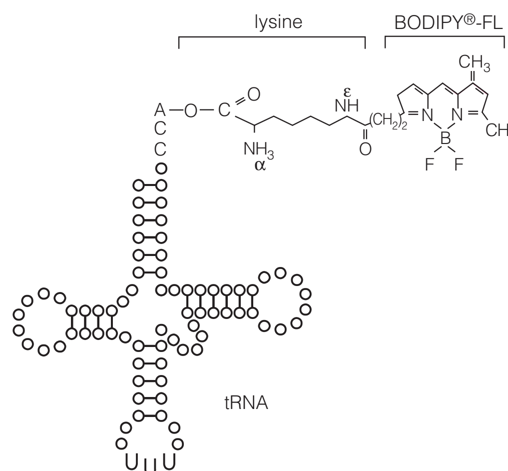


Figure 1. Structure of FluoroTect™ Green$_{Lys}$ tRNA.

Safety, regulatory issues, waste disposal and lengthy exposure times are the primary drawbacks associated with the use of radioactivity. The additional steps of fluorographic enhancement, fixing and drying of protein gels can also delay results and, in some cases (i.e., gel cracking), result in complete failure.

Current commercially available non-isotopic detection systems rely on modified lysine tRNAs, with the lysine labeled at the epsilon position with biotin. These modified lysine tRNAs can be added to the translation reactions, and the modified lysines will be incorporated into the synthesized proteins. As with radioactive incorporation, reactions containing biotinylated proteins are resolved by SDS PAGE and the proteins must be electroblotted to a solid matrix such as nitrocellulose or PVDF membranes. Biotinylated proteins can be detected nonisotopically using streptavidin-alkaline phosphatase or horseradish peroxidase conjugates and either colorimetric or chemiluminescent detection reagents (8).

However, depending on the type of translation system used, the presence of background bands due to endogenous biotinylated proteins may interfere with the detection of specific translation products (8).

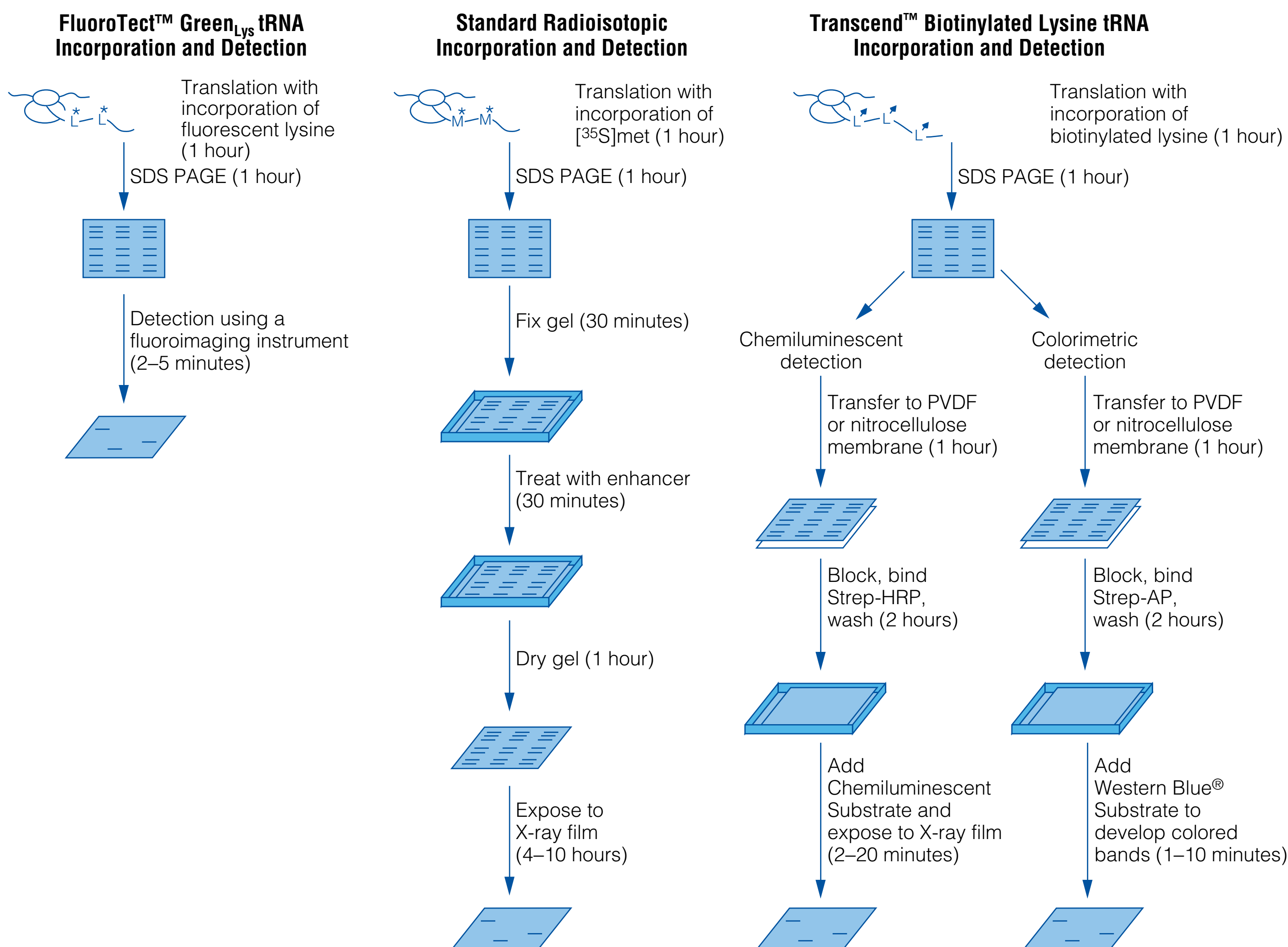


Figure 2. Comparison of incorporation and detection protocols using FluoroTect™ Green$_{Lys}$ tRNA, Transcend™ tRNA and radiolabeled amino acids.

Characteristics of the new FluoroTect™ System

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System uses a charged lysine tRNA molecule labeled with the fluorophore BODIPY®-FL at the epsilon (ε) amino acid position of lysine (Figure 1). The BODIPY®-FL fluorophore, with an absorbance at 502nm and an emission at 510nm, was developed to be compatible with widely used excitation sources and common optical filter sets (9). The BODIPY®-FL fluorophore is applied to a charged tRNA using the method described by Johnson *et al.* (10). The labeled FluoroTect™ tRNA is added directly to the translation reaction, and the labeled lysine is incorporated into the synthesized protein.

Recently, a method using BODIPY®-FL-labeled methionine was shown to be able to detect nanogram levels of protein using laser-based fluorescent gel scanning (11). For the FluoroTect™ System, lysine was chosen as the labeled amino acid because it is one of the more frequently used amino acids, comprising, on average, 6.6% of a protein's amino acids. This compares to 1.7% for methionine (12).

Detection of the labeled proteins is accomplished in 2–5 minutes directly "in-gel" by use of a laser-based fluorescent gel scanner. This eliminates any requirements for protein gel manipulation such as fixing/drying or any safety, regulatory or waste disposal issues such as those associated with the use of radioactively labeled amino acids. The convenience of non-isotopic "in-gel" detection also avoids the time-consuming electroblotting and detection steps of conventional non-isotopic systems. Comparison of the FluoroTect™ System to standard autoradiographic detection of radiolabeled translation products and chemiluminescent/colorimetric detection of biotin-containing translation products is illustrated in Figure 2.

Flexibility of the FluoroTect™ System

Two basic approaches exist for in vitro translation: programming with mRNA templates or programming systems with DNA templates (i.e. coupled transcription/translation). Selection of the correct system is based on several criteria including template (mRNA or DNA), promoter elements and possible secondary protein modifications present in the translation cell lysate/extract. No matter which system is used, radioactive incorporation has always been an option. But with biotin-based systems, due to the level of background generated by endogenous biotinylated proteins, Wheat Germ Extract is not recommended (7). The FluoroTect™ System is compatible with all in vitro translation systems and lacks the presence of multiple endogenous fluorescent proteins.

We next determined how well the FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System functioned when used in conjunction with several different systems. Figure 3 shows the results using the TNT® Coupled Reticulocyte Lysate System, the TNT® Coupled Wheat Germ Extract System[a,b,d,e], the Rabbit Reticulocyte System[a,d,e] (Cat.# L4960) or the Wheat Germ Extract System (Cat.# L4380) to express the Luciferase T7 Control DNA[d] (Cat.# L4821) or the Luciferase Control RNA[d,e] (Cat.# L4561). All systems expressed the luciferase template well and the Fluorescent Luciferase tRNA was easily detected.

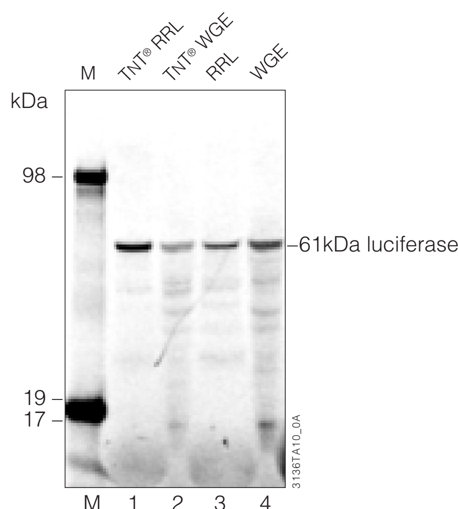


Figure 3. Comparison of FluoroTect™ Green$_{Lys}$ tRNA in different in vitro translation systems. Luciferase T7 Control DNA or Luciferase Control RNA was expressed in the following systems: lane 1, TNT® Coupled Reticulocyte Lysate System (TNT® RRL); lane 2, TNT® Coupled Wheat Germ System (TNT® WGE); lane 3, Rabbit Reticulocyte Lysate System (RRL); and lane 4, Wheat Germ Extract System (WGE). For the coupled systems, 200ng of linearized Luciferase T7 Control Plasmid was expressed. For the RRL or WGE systems, 1µg of Luciferase Control RNA was used as a template. Aliquots (5µl) of each reaction were treated with 1µl of a 1:10 dilution of RNase ONE™ Ribonuclease for 5 minutes at 37°C and run on a 4–12% Novex NuPAGE™ Bis-Tris gel in MES running buffer using LDS sample buffer (Invitrogen). The gel was visualized using a Hitachi FMBIO® II instrument set on the 505 channel.

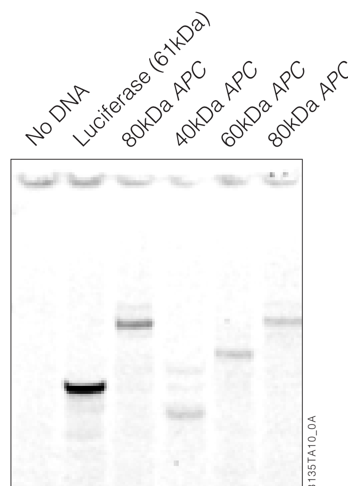
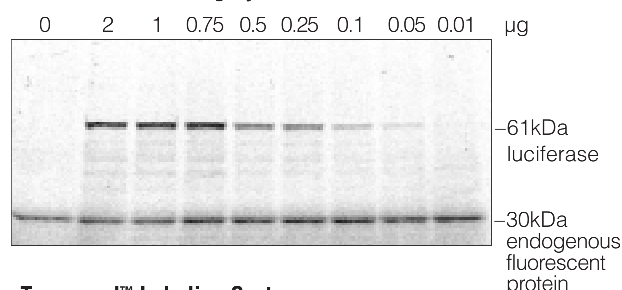


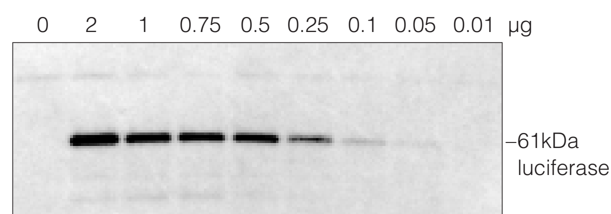
Figure 4. Expression of various sizes of products using the FluoroTect™ System. *APC* gene products of the size indicated were expressed using the TNT® T7 Quick for PCR DNA system and the FluoroTect™ System. Electrophoresis and fluoroimaging were performed as described for Figure 3.

The ability to express and detect different size protein products was also evaluated. Different-sized products were expressed using the recommended TNT® T7 Quick for PCR DNA[b,c,e] system (Cat.# L5540) and the FluoroTect™ tRNA. A cloned *APC* (adenomatous polyposis coli) locus or Luciferase T7 Control DNA was used as the template. The results are shown in Figure 4. APC products ranging in size from 20kDa to 85kDa and the 61kDa luciferase product expressed well in this system, demonstrating the utility of the FluoroTect™ fluorescent tRNA label. These different protein products contained between 8 to 45 lysine residues.

A. FluoroTect™ Labeling System



B. Transcend™ Labeling System



C. [35S]Methione Labeling

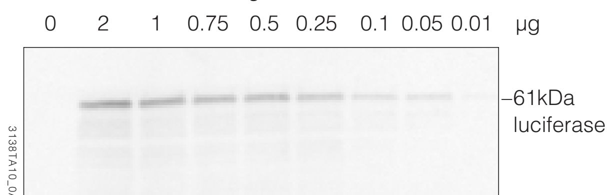


Figure 5. Comparison of Detection Sensitivity with FluoroTect™ tRNA, Transcend™ tRNA and [35S]methionine labeling. Luciferase Control RNA in the amounts indicated was expressed using the Rabbit Reticulocyte Lysate System, Nuclease Treated (Cat.# L4960), labeled by three different labeling techniques. **Panel A**. Labeling reactions were performed using 1µl of the FluoroTect™ $Green_{Lys}$ tRNA. Five microliters of each reaction were loaded on the gel and visualized on a FluorImager™ SI (Molecular Dynamics) using the 530 DF 30 emission filter. **Panel B.** Labeling reactions were performed using the Transcend™ Biotinylated tRNA (Cat.# L5061). One microliter of each reaction was loaded on the gel, which was electroblotted to a PVDF membrane. The products were detected using the Transcend™ Chemiluminescent Detection System (Cat.# L5080). **Panel C.** Labeling reactions were performed using 20µCi of [35S]methionine. One microliter was loaded on the gel and electroblotting was done as in Panel B. The membrane was exposed overnight using a Molecular Dynamics PhosphorImager® instrument.

Sensitivity of the FluoroTect™ System

A comparison of Promega's FluoroTect™ System with traditional labeling techniques (i.e., radioactivity and biotin) was performed to evaluate the sensitivity levels of each method (Figure 5). The results show that the FluoroTect™ System produces in 2–5 minutes results that are as sensitive as chemiluminescence methods (and equivalent to [35S]methionine labeling). The FluoroTect™ System is the most sensitive non-radioactive labeling system currently available.

Removal of endogenous background bands

When using the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System in conjunction with translation systems based on Rabbit Reticulocyte Lysate, two minor background bands appear without the addition of template DNA or mRNA. They migrate at approximately 18kDa and at 30kDa. Additionally, a major background band from hemoglobin migrates at around 12kDa. As seen in Figure 6, the ~18kDa fluorescent background band can be removed by treatment of the lysate with RNase ONE™ Ribonuclease. The background band present at approximately 30kDa was not removed with RNase ONE™ Ribonuclease digestion. It should be noted that the intensity of bands can vary dramatically depending on the gel system used. Bands may appear more intense with a Tris-glycine system as compared to the bis-tris system.

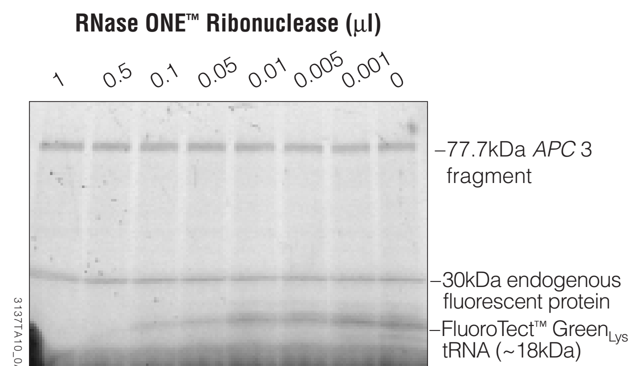


Figure 6. Removal of endogenous background band with RNase ONE™ Ribonuclease treatment. Transcription/Translation reactions were performed using a PCR-generated fragment of Segment 3 of the *APC* gene in the TNT® T7 Quick for PCR DNA System with 1µl of the FluoroTect™ $Green_{Lys}$ tRNA. Five microliters of the reactions were treated with varying amounts of RNase ONE™ Ribonuclease for 5 minutes at 37°C. Samples were loaded onto a gel as described in Figure 5 and visualized as described in Figure 5, Panel A.

Conclusions

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System offers clear advantages over standard protein labeling methods. Benefits of this system include i) efficient fluorescent labeling of both prokaryotic and eukaryotic proteins in in vitro translation systems; ii) simple, rapid "in-gel" detection of fluorescently labeled proteins; and iii) a non-radioactive procedure, which means no isotope handling and waste disposal concerns.

References

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Protocols

- *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System Technical Bulletin* #TB285, Promega Corporation. (www.promega.com/tbs/tb285/tb285.html)
- *TNT® Quick Coupled Transcription/Translation Systems Technical Manual* #TM045, Promega Corporation. (www.promega.com/tbs/tm045/tm045.html)
- *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182, Promega Corporation. (www.promega.com/tbs/tb182/tb182.html)

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Ordering Information

Product	Size	Cat.#
FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System	20–40 reactions	L5001

Related Products

Product	Size	Cat.#
TNT® T7 Coupled Reticulocyte Lysate System	1 system	L4610
TNT® T7 Coupled Wheat Germ Extract System	1 system	L4140
Transcend™ Chemiluminescent Detection System	1 system	L5080
Transcend™ Biotinylated tRNA	30µl	L5061
RNase ONE™ Ribonuclease	1,000u	M4261

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[a] U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

[b] U.S. Pat. Nos. 5,324,637, 5,492,817, 5,665,563, Australian Pat. No. 660329 and other patents.

[c] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[d] 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.

[e] 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.

Product Bibiolgraphy

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