Non-Radioactive Detection of Proteins Expressed in Cell-Free Expression Systems

ABSTRACT

Cell-free expression is a convenient method for producing protein rapidly. Radioactive [^{35}S]methionine labeling is a traditional method for detecting and tracking proteins produced in cell-free expression systems. With heightened restrictions on the use, purchase and disposal of radioactive material and exposure of scientists to radioactivity, other detection methods with high specificity and sensitivity are becoming more common. In this article, we use the following non-radioactive techniques to detect five proteins produced in both rabbit reticulocyte lysate-based and wheat germ extract-based cell-free protein expression systems: FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System, Transcend™ Chemiluminescent Translation Detection System and Western blot analysis with both HRP- and Alexa Fluor® 647-conjugated secondary antibodies. All of these techniques yielded low background and sensitive detection, allowing researchers to distance themselves from traditional radioactive detection methods.

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Introduction

Cell-free expression systems generate protein from nucleic acid in as little as 60 minutes. Some systems require an mRNA template, but coupled transcription/translation systems can use plasmid DNA or a PCR product as a template, eliminating the need to transcribe DNA into RNA first. Promega offers cell-free protein expression systems based on lysates from mammalian, plant, bacterial and insect cells. Thus, the researcher has the flexibility to choose the type of template and cell-free expression system. The speed and ease of use of cell-free protein expression provide researchers options in addition to traditional cell-based expression.

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Detection of protein expressed using cell-free systems is required for most applications such as protein:protein interaction and protein:nucleic acid interaction studies. Traditionally, one adds radioactive [35S]methionine to cell-free expression reactions, and the methionine is incorporated into the expressed protein, allowing detection by autoradiography. Many researchers are moving away from radioactivity due to high costs, regulations, radioactive exposure, and waste and disposal issues. Traditional Western blot analysis provides the researcher a non-radioactive method for detection but, if performed improperly, can result in high background, which can mask expressed proteins and affect downstream applications. Western blots are performed using a primary antibody that recognizes the protein of interest directly or a tag attached to the protein of interest. The primary antibody then is detected using an appropriate secondary antibody. These secondary antibodies often are conjugated to horseradish peroxidase (HRP), fluorophores or alkaline phosphatase (AP) to allow visualization. In this article we performed Western blot analysis using HRP- and Alexa Fluor® 647-conjugated secondary antibodies (Figure 1, Panels A and B, respectively).

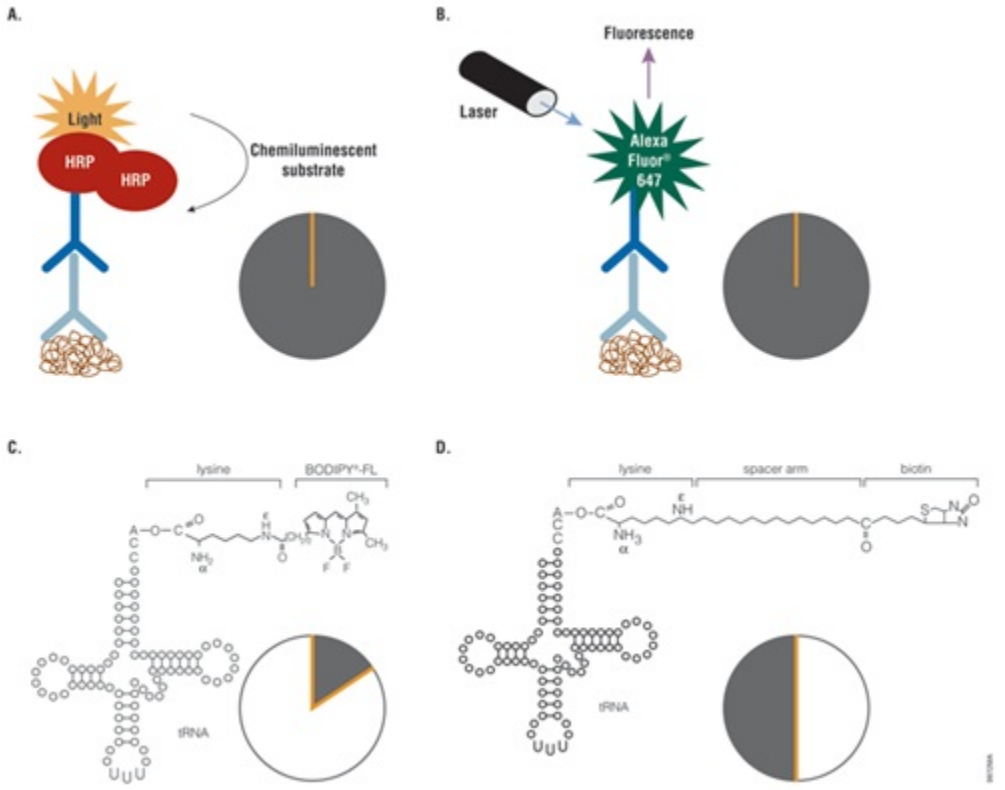

Figure 1. Non-radioactive detection methods for cell-free protein expression. Panel A. A schematic diagram showing detection by Western blot using an HRP-conjugated secondary antibody and chemiluminescent substrate. **Panel B.** A schematic diagram showing detection by Western blot using a fluorescently labeled secondary antibody. **Panel C.** The FluoroTect™ tRNA. The tRNA is charged with a BODIPY®-FL-labeled lysine residue. **Panel D.** The Transcend™ tRNA. The tRNA is charged with a biotin-labeled lysine residue. The black circles represent the relative time required for each detection method.

The FluoroTect™ GreenLys in vitro Translation Labeling System (Cat.# L5001) and Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080) are two methods for detecting proteins expressed in cell-free systems. The FluoroTect™ System employs a tRNA charged with a lysine that is labeled at the ε position with the BODIPY®-FL fluorophore (Figure 1, Panel C). These fluorescently labeled lysine residues are incorporated into synthesized proteins during in vitro translation. The Transcend™ System relies on incorporation of biotinylated lysine residues into nascent proteins during translation. The biotinylated lysine is added to the translation reaction as a charged ε-labeled biotinylated-lysine:tRNA complex (Transcend™ tRNA; Figure 1, Panel D) rather than a free amino acid. After SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) and electroblotting, biotinylated proteins can be visualized by binding Streptavidin-AP or Streptavidin-HRP, followed by colorimetric or chemiluminescent detection, respectively. Typically, these methods can detect 0.5–5ng of protein, with a sensitivity equivalent to that achieved with [^{35}S]methionine incorporation and autoradiographic detection.

Expression Constructs

Five proteins were expressed in both the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170; a rabbit reticulocyte lysate [RRL]-based system) and TNT® SP6 High-Yield Wheat Germ Protein Expression System (Cat.# L3260; a wheat germ extract [WGE]-based system). Table 1 provides the GenBank® Accession numbers of the protein-coding regions, species of origin and predicted protein sizes.

Green fluorescent protein (GFP) exhibits bright green fluorescence when exposed to blue light. In cell and molecular biology, the GFP gene is frequently used as a reporter of expression. In modified forms, GFP is used to make biosensors, and many animals that express GFP have been created to show that a gene can be expressed throughout a given organism.

Eukaryotic translation initiation factor 4E (eIF4E) is involved in directing ribosomes to the cap structure of eukaryotic mRNAs during protein synthesis and is the rate-limiting component of the translation apparatus. eIF4E binds the mRNA cap and ultimately brings the mRNA to the ribosome. eIF4E is part of the eIF4F pre-initiation complex, which is made up of eIF4E and eIF4G. Almost all cellular proteins require eIF4E for translation.

The human Nanos 1 gene encodes a $(CCHC)_2$ zinc finger protein that is homologous to Nanos orthologues in *Drosophila* and mouse, which have an evolutionarily conserved function in embryonic patterning and germ line development.

Protein phosphatase 1 alpha catalytic subunit (PPP1ca) is a serine/threonine-specific protein phosphatase involved in the regulation of various cellular processes such as cell division, glycogen metabolism, muscle contractility, protein synthesis and HIV-1 viral transcription. Increased PP1 activity has been observed in the end stage of heart failure. Studies in both humans and mice suggest that PP1 is an important regulator of cardiac function. Studies in mice suggest that PP1 also functions as a suppressor of learning and memory. Three alternatively spliced transcript variants encoding different isoforms have been identified for this gene.

Luciferase from the common North American firefly *Photinus pyralis* is one of the most extensively studied of the bioluminescent enzymes. *P. pyralis* luciferase requires luciferin, ATP and 0_2 as substrates.

Table 1. Proteins Expressed.

Protein	GenBank® Accession Number	Species	Predicted Size (kDa)
GFP	M62653	*Aequorea victoria*	27
eIF4E	NM_001968	Human	25

Nanos 1	NM_199461	Human	30
PPP1ca	NM_001008709	Human	38
Luciferase	AY738222.1	*Photinus pyralis*	61

The genes in Table 1 were amplified with PCR primers designed using the Flexi® Vector Primer Design Tool, then cloned into pF1A T7 Flexi® Vector (Cat.# C8441) and pF3A WG (BYDV) Flexi® Vector (Cat.# L5671) using the Flexi® Cloning System (Cat.# C8640). After verification of clones by sequencing, plasmids were transformed into Single Step (KRX) Competent Cells (Cat.# L3002). Plasmid DNA was prepared using the PureYield™ Plasmid Midiprep System (Cat.# A2492).

Cell-Free Protein Expression

Protein-coding regions in the pF1A Vector were expressed in a RRL-based expression system as described in the *TNT® T7 Quick Coupled Transcription/Translation System Technical Manual* #TM045. One microgram of circular plasmid DNA was added to each reaction, and reactions were incubated at 30°C for 90 minutes. Protein-coding regions in the pF3A Vector were expressed in a WGE-based expression system as described in the *TNT® SP6 High-Yield Wheat Germ Protein Expression System Technical Manual* #TM282. Three micrograms of circular plasmid DNA was added to each reaction, and reactions were incubated at 25°C for 120 minutes. The FluoroTect™ System was used to label proteins with BODIPY®-FL-labeled lysine residues (2µl of FluoroTect™ tRNA per reaction). The Transcend™ System was used to label proteins with biotinylated lysine residues (2µl of Transcend™ tRNA per reaction). Minus-DNA control reactions, which contained all components of the cell-free reactions except DNA template, were important negative controls for determining background from the expression and labeling systems.

Protein Detection Using FluoroTect™ GreenLys in vitro Translation Labeling System

Proteins were expressed using RRL-based and WGE-based cell-free expression systems and labeled using the FluoroTect™ tRNA. Five microliters of each reaction was added to 1µl of RNase ONE™ Ribonuclease (10 units/µl, Cat.# M4261) and incubated at 37°C for 5 minutes. One microliter of each reaction was added to 19µl of 1X SDS loading dye with 50mM DTT and incubated at 70°C for 2–3 minutes. After a brief centrifugation, 20µl was loaded onto a 4–20% gradient Tris-glycine SDS-PAGE gel. Five microliters of the Precision Plus Protein™ Dual Color Standards (BioRad Cat.# 161-0374) was used as a size marker. After running the gel at the appropriate voltage, the gel was removed, placed in water and imaged using a fluorescent scanner as described in the *FluoroTect™ GreenLys in vitro Translation Labeling System Technical Bulletin* #TB285. Figure 2, Panels A and B, shows expression of all five proteins in the RRL-based and WGE-based systems, respectively. Both systems had low background as seen in the minus-DNA control reactions. One observation found throughout this study is that the eIF4E protein was slightly larger than the predicted 25kDa. This same discrepancy is described by the antibody provider and can occur due to relative charge differences that cause the protein to migrate more slowly than expected.

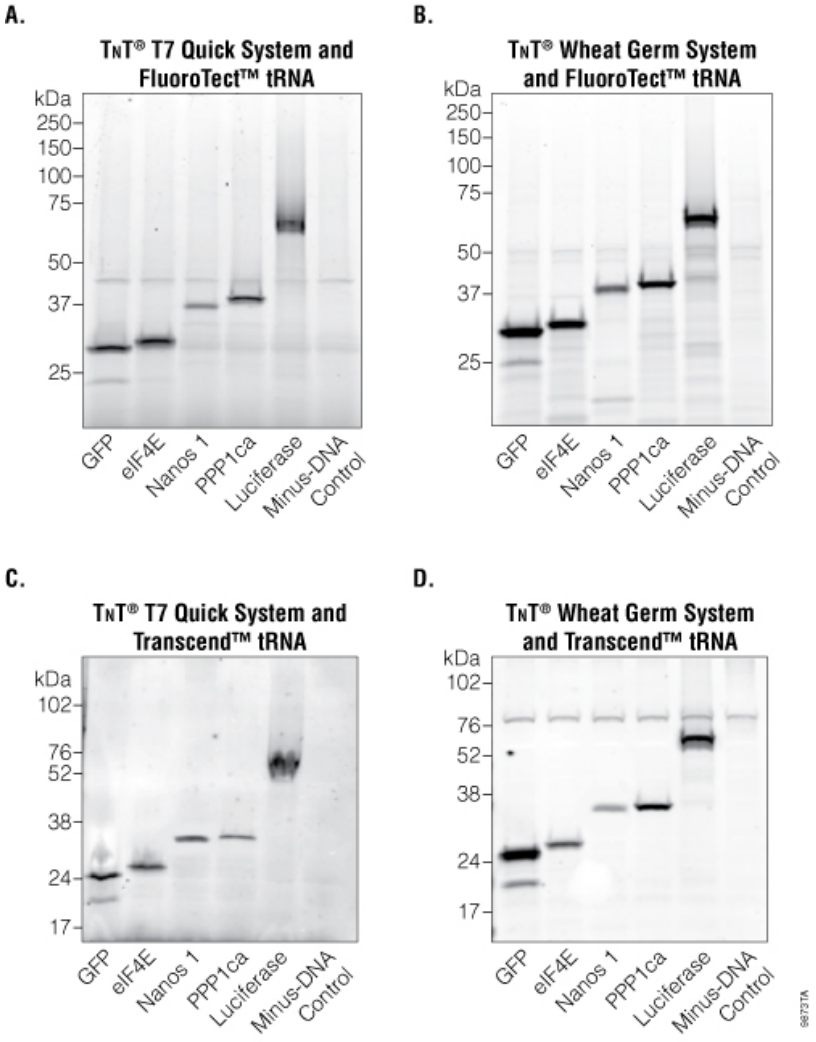

Figure 2. Detection of proteins using the FluoroTect™ and Transcend™ Systems. Proteins were expressed using a RRL-based expression system (TNT® T7 Quick System, **Panels A and C**) and a WGE-based expression system (TNT® SP6 High-

Yield Wheat Germ System, **Panels B and D**) in the presence of FluoroTect™ tRNA (**Panels A and B**) or Transcend™ tRNA (**Panels C and D**). Water was substituted for the DNA template in the minus-DNA control reactions.

Protein Detection Using Transcend™ Chemiluminescent Non-Radioactive Translation Detection System

Proteins were expressed using RRL-based and WGE-based cell-free expression systems and labeled using the Transcend™ tRNA. RNase treatment and SDS-PAGE were performed as described above for the FluoroTect™ System. After SDS-PAGE, proteins were transferred to a PVDF membrane using the Invitrogen iBlot® Dry Blotting System. The membrane was blocked with 15ml of 5% Blot-Qualified BSA (Cat.# W3841) in TBST (1X TBS + 0.1% Tween® 20) at room temperature for 1 hour with gentle shaking. The blocking solution was removed, and 15ml of a 1:7,500 dilution of Streptavidin HRP in 1X TBST was added to the membrane. After 1 hour at room temperature, the membrane was washed in 15ml of TBST for 5 minutes with gentle shaking. The wash was repeated two more times using TBST, then three times using 15ml of water. After blotting dry the membrane, 2ml of prepared ECL Western Blotting Substrate (Cat.# W1001) was added. After 1 minute, the membrane was blotted dry and exposed to Amersham Hyperfilm ECL to detect the expressed proteins. Figure 2, Panels C and D, shows expression of all five proteins in the RRL-based and WGE-based expression systems, respectively. The minus-DNA controls for both systems had low background. The wheat germ extract produces a significant background band at about 80kDa.

Western Blot Protocol

Unlabeled proteins were expressed using RRL-based and WGE-based expression systems. One microliter of each reaction was added to 19µl of 1X SDS loading dye with 50mM DTT and incubated at 95°C for 5 minutes. After a brief centrifugation, 20µl was loaded on a 4–20% gradient Tris-glycine SDS-PAGE gel. Five microliters of the Precision Plus Protein™ Dual Color Standards was used as a size marker for chemiluminescent Western blots, and 2µl of the ECL Plex Fluorescent Rainbow Markers IMPROVED (GE Healthcare Cat.# RPN850E) was used as a size marker for fluorescent Western blots. After running the gel at the appropriate voltage, the gel was removed and placed in water. Proteins were transferred to a PVDF membrane using the iBlot® System. The membrane was blocked with 15ml of 5% Blot-Qualified BSA in TBST (1X TBS + 0.1% Tween® 20) at room temperature for 1 hour with gentle shaking. The blocking solution was removed, and 15ml of the primary antibody, diluted in 1X TBST, was added to the membrane. Table 2 lists the primary antibodies used in this study. After 1 hour at room temperature, the membrane was washed in 15ml of TBST for 5 minutes with gentle shaking. This was repeated five more times using TBST. Fifteen microliters of the secondary antibody, diluted 1:2,500 in 1X TBST, was added to the membrane. Table 3 lists the secondary antibodies used in this study. After 1 hour at room temperature, the membrane was washed as described previously. For chemiluminescent detection, 2ml of prepared ECL Western Blotting Substrate was added to the membrane. After 1 minute, the membrane was blotted dry and exposed to Amersham Hyperfilm ECL to detect the expressed proteins. Identical exposure times were used. For fluorescent detection, the membrane was scanned directly on a fluorescent scanner.

Table 2. Primary Antibodies Used.

Antibody	Supplier	Cat.#	Source	Clonality
GFP antibody	Abcam	ab290	Rabbit	Polyclonal
eIF4E antibody	Abcam	ab1126	Rabbit	Polyclonal

Nanos Homologue 1 (NANOS1) antibody	Abcam	ab65203	Rabbit	Polyclonal
PPP1A antibody [EP1511Y]	Abcam	ab52619	Rabbit	Monoclonal
PPP1A antibody [4G3]	Abcam	ab66955	Mouse	Monoclonal
Anti-Luciferase pAb	Promega	G7451	Goat	Polyclonal

Table 3. Secondary Antibodies Used.

Antibody	Supplier	Cat.#	Label
goat Anti-Rabbit IgG (H+L), HRP Conjugate	Promega	W4011	HRP
goat Anti-Mouse IgG (H+L), HRP Conjugate	Promega	W4021	HRP
donkey Anti-Goat IgG, HRP	Promega	V8051	HRP
Alexa Fluor® 647 goat anti-rabbit IgG (H+L)	Invitrogen	A21244	Alexa Fluor® 647
Alexa Fluor® 647 goat anti-mouse IgG (H+L)	Invitrogen	A21235	Alexa Fluor® 647
Alexa Fluor® 647 donkey anti-goat IgG (H+L)	Invitrogen	A21447	Alexa Fluor® 647

Choosing the Correct Primary Antibody Dilution

One critical step in producing low-background, high-signal Western blots is choosing the correct dilution of the primary antibody. Figure 3 shows four Western blots to detect GFP. Figure 3, Panels A, B and C, are chemiluminescent Western blots with primary antibody dilutions of 1:5,000, 1:50,000 and 1:100,000, respectively. The manufacturer's recommended antibody dilution is 1:1,000 to 1:2,500. Figure 3, Panel A, shows significant background in the minus-DNA reactions for both the RRL- and WGE-based expression systems with the 1:5,000 antibody dilution. Positive signal was seen in control reactions that expressed GFP; however, the GFP signal was a small percentage of the total signal. When the antibody was diluted 1:50,000, background was decreased significantly, and the positive signal was a large percentage of the total signal (Figure 3, Panel B). Diluting the primary antibody to 1:100,000 did not reduce the background further (compare Figure 3, Panels B and C). Similar background and signal patterns were observed when the primary antibody was diluted to 1:100,000 and an Alexa Fluor® 647-conjugated secondary antibody was used (Figure 3, Panel D).

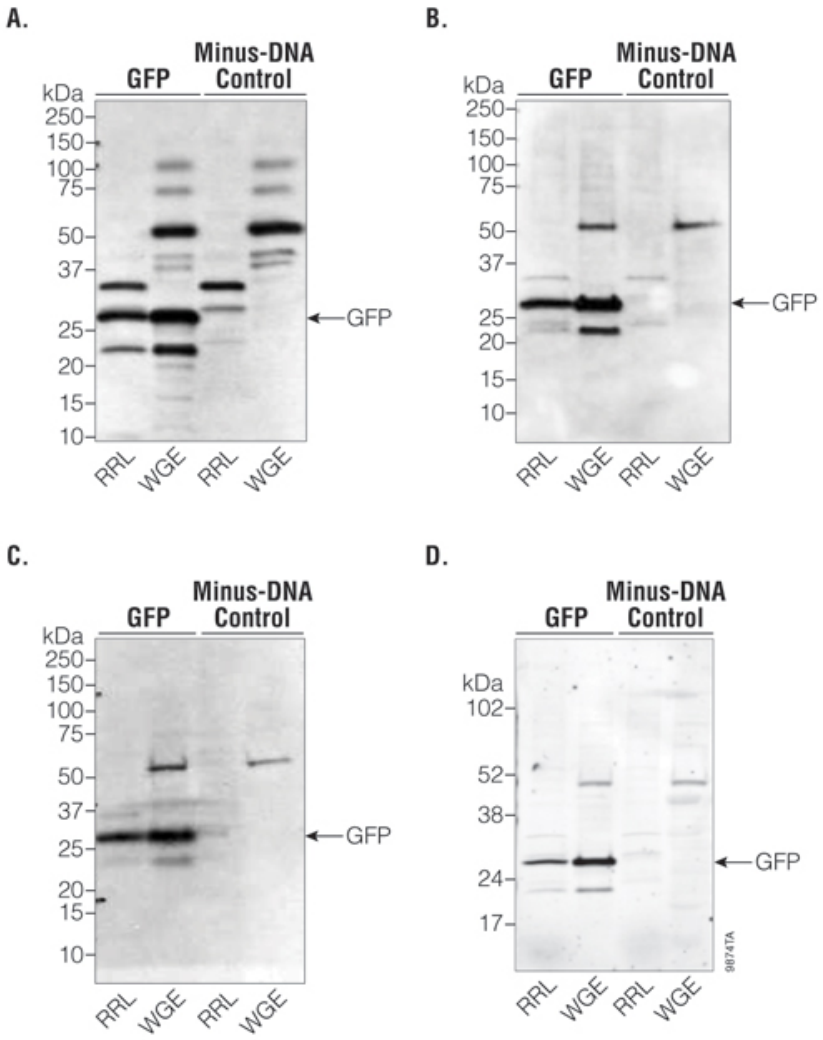

Figure 3. Detection of GFP by Western blot. GFP was expressed in a RRL-based system (TNT® T7 Quick System) and WGE-based system (TNT® SP6 High-Yield Wheat Germ Protein Expression System) and detected by Western blot using an anti-GFP rabbit primary antibody and a goat anti-rabbit HRP- or Alexa Fluor® 647-conjugated secondary antibody. Both secondary antibodies were used at a 1:2,500 dilution. **Panel A.** Detection using a 1:5,000 dilution of the primary antibody followed by a goat

anti-rabbit HRP-conjugated secondary antibody. **Panel B.** Detection using a 1:50,000 dilution of the primary antibody followed by a goat anti-rabbit HRP-conjugated secondary antibody. **Panel C.** Detection using a 1:100,000 dilution of the primary antibody followed by a goat anti-rabbit HRP-conjugated secondary antibody. **Panel D.** Detection using a 1:100,000 dilution of the primary antibody followed by a goat anti-rabbit Alexa Fluor® 647-conjugated secondary antibody.

Western blot analysis using an anti-PPP1ca mouse monoclonal antibody to detect PPP1ca showed a primary antibody dilution effect similar to that observed in Western blots to detect GFP. Figure 4, Panels A, B and C, show Western blots with primary antibody dilutions of 1:1,000, 1:5,000 and 1:10,000 respectively. The manufacturer's recommended antibody dilution is 1:500 to 1:2,000. Figure 4, Panel A, shows an intense background band in the minus-DNA control for the RRL-based system. Positive signal was seen in reactions expressing PPP1ca. When the primary antibody was diluted to 1:5,000, the background decreased only slightly (Figure 4, Panel B). Diluting the primary antibody to 1:10,000 reduced the background significantly, and only the PPP1ca signal was apparent (Figure 4, Panel C). Similar background and signal patterns were observed when a 1:5,000 dilution of the primary antibody and an Alexa Fluor® 647-conjugated secondary antibody were used (compare Figure 4, Panels B and D).

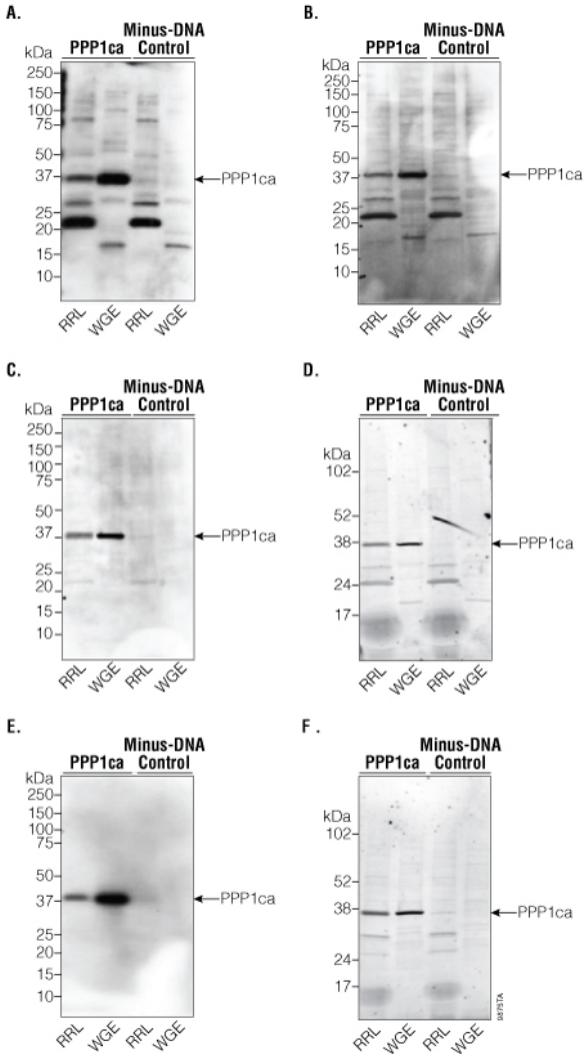

Figure 4. Western blot analysis of PPP1ca. PPP1ca was expressed using RRL-based and WGE-based expression systems as indicated and detected by Western blot using an anti-PPP1ca primary antibody and HRP- or Alexa Fluor® 647-conjugated secondary antibody. Both secondary antibodies were used at a 1:2,500 dilution. For chemiluminescent detection, exposure times were identical. **Panels A–C.** Detection of PPP1ca using a 1:1,000 (**Panel A**), 1:5,000 (**Panel B**) or 1:10,000 (**Panel C**) dilution of anti-PPP1ca mouse primary antibody followed by a goat anti-mouse HRP-conjugated secondary antibody. **Panel D.** Detection of PPP1ca using a 1:5,000 dilution of the mouse primary antibody followed by a goat anti-mouse Alexa Fluor® 647-conjugated secondary antibody. **Panel E.** Detection of PPP1ca using a 1:200,000 dilution of the rabbit primary antibody followed by a goat anti-rabbit HRP-conjugated secondary antibody. **Panel F.** Detection of PPP1ca using a 1:200,000 dilution of the rabbit primary antibody followed by a goat anti-rabbit Alexa Fluor® 647-conjugated secondary antibody.

When performing Western blot analysis of proteins expressed in cell-free systems, one must experimentally determine the optimal dilution of the primary antibody. In the Western blots performed in this study, primary antibodies were diluted ~50-fold more than the provider's recommended dilution. In these experiments, only 1µl of the completed reaction was loaded on SDS-PAGE gels. Increasing the volume can increase signal but also will increase background, so the researcher must adjust the volume based on the level of expressed protein.

When using proteins expressed in cell-free systems for downstream applications such as protein:protein interaction assays, the intensity of the protein-specific signal will be much lower than that from an equivalent volume of the original cell-free

expression reaction. Western blot procedures must be altered to detect the lower amount of protein. One can add more protein, use a lower dilution of primary antibody and increase the incubation time with the primary antibody to overnight. These variables must be experimentally optimized for each experiment.

Choosing Between Rabbit and Mouse Primary Antibodies

For some proteins, antibodies raised in both mice and rabbits are available. Some researchers choose the mouse antibody rather than the rabbit antibody due to fear of cross-reactivity with the rabbit reticulocyte lysate. To help clear up this common misconception, PPP1ca expressed in RRL- and WGE-based cell-free expression systems was detected by Western blot using either a mouse monoclonal (Figure 4, Panels A, B, C and D) or rabbit monoclonal antibody (Figure 4, Panels E and F). No significant differences were seen, and both antibodies yielded clear, sensitive detection of PPP1ca with little background. The rabbit antibody did not produce more background than the mouse antibody. Likewise, the Western blots in Figure 3 showed clean detection of GFP using rabbit polyclonal primary antibodies. We have performed many other blots with rabbit primary antibodies to recognize proteins produced in rabbit reticulocyte lysates with clean results (data not shown).

Using HRP-Labeled and Fluorescent Secondary Antibodies

Secondary antibodies used to detect proteins by Western blot analysis often are labeled with HRP, which is detected by enhanced chemiluminescence (ECL), or fluorophores. We detected three proteins (luciferase, eIF4E and Nanos 1) expressed in both RRL- and WGE-based systems using Western blot analysis with both HRP- and Alexa Fluor® 647-conjugated secondary antibodies (Figure 5). For all blots, the primary antibody dilutions were optimized, and all antibodies were significantly (~50-fold) more dilute than the provider suggested. When comparing HRP to Alexa Fluor® 647 detection, no background difference was seen (compare Figure 5, Panels A and B; Panels C and D; and Panels E and F). Detection of Alexa Fluor® 647 was slightly less sensitive than that of HRP but still yielded detectable signal. Both secondary antibodies performed well in the Western blots shown in Figures 3, 4 and 5.

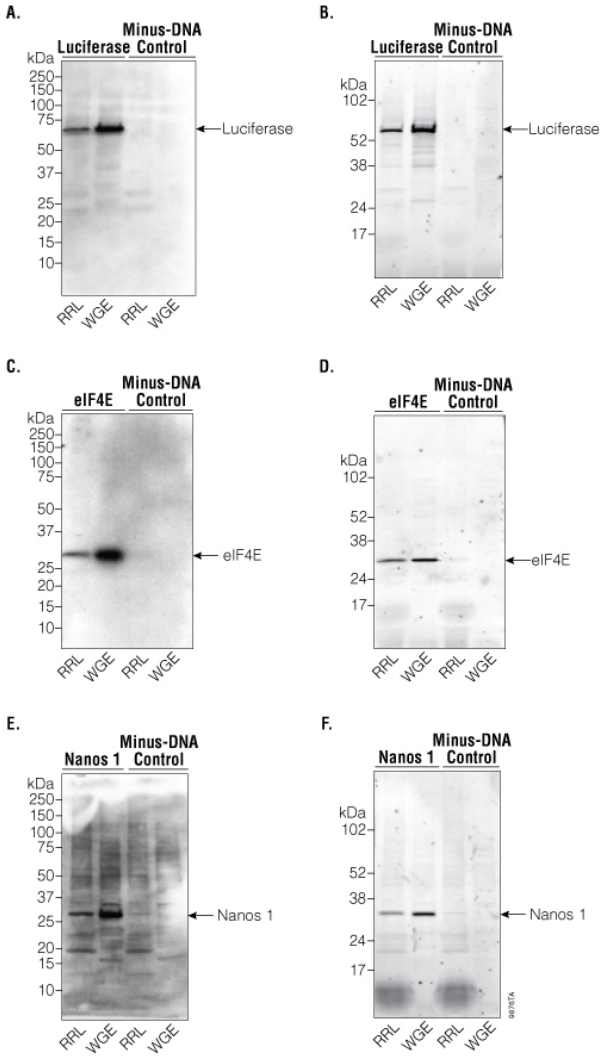

Figure 5. Detection of firefly luciferase, eIF4E and Nanos 1 by Western blot. Firefly luciferase, eIF4E and Nanos 1 were expressed using RRL-based and WGE-based expression systems as indicated and detected using the appropriate primary antibody and HRP- or Alexa Fluor® 647-conjugated secondary antibody. Both secondary antibodies were used at a 1:2,500 dilution. **Panel A.** Detection of luciferase using a 1:10,000 dilution of the goat anti-luciferase primary antibody followed by a donkey anti-goat HRP-conjugated secondary antibody. **Panel B.** Detection of luciferase using a 1:10,000 dilution of goat anti-luciferase primary antibody followed by a donkey anti-goat Alexa Fluor® 647-conjugated secondary antibody. **Panel C.** Detection of eIF4E using a 1:10,000 dilution of the rabbit anti-eIF4E primary antibody followed by a goat anti-rabbit HRP-conjugated

secondary antibody. **Panel D.** Detection of eIF4E using a 1:10,000 dilution of the rabbit anti-eIF4E primary antibody followed by a goat anti-rabbit Alexa Fluor® 647-conjugated secondary antibody. **Panel E.** Detection of Nanos 1 using a 1:1,000 dilution of the rabbit anti-Nanos 1 primary antibody followed by a goat anti-rabbit HRP-conjugated secondary antibody. **Panel F.** Detection of Nanos 1 using a 1:1,000 dilution of the rabbit anti-Nanos 1 primary antibody followed by a goat anti-rabbit Alexa Fluor® 647-conjugated secondary antibody.

Protein Expression Using RRL- and WGE-Based Systems

The correct detection method is essential to make a fair comparison between protein expression levels in two different cell-free expression systems. The FluoroTect™ and Transcend™ Systems cannot be used to compare expression levels between two different systems, for instance between mammalian and plant systems. These detection systems are based on incorporation of modified lysines during translation, and the amount of endogenous lysyl tRNA is different in different cell-free systems. Therefore, the ratio of modified and native lysyl tRNA will affect incorporation levels and signal intensity. The best way to compare expression of proteins from cell-free systems is using Western blot analysis. In this report, we used two different cell-free systems: RRL- and WGE-based systems. Both systems expressed all five proteins tested; however, the expression levels were different between the systems. The WGE-based system consistently produced more protein than the RRL-based system (Figures 3, 4 and 5). Even though expression levels were lower in RRL-based systems, there was enough protein to perform many downstream assays such as protein:protein interaction assays.

Conclusions

The FluoroTect™ and Transcend™ Systems are easy, sensitive and fast methods to detect proteins expressed in cell-free expression systems. They require little optimization and eliminate the need for radioactivity. For Western blot analysis the primary antibody concentration must be optimized to allow clean, sensitive detection of expressed proteins. Fluorescently conjugated secondary antibodies were less sensitive than HRP-conjugated secondary antibodies in our studies. However, both can be used to detect proteins expressed in cell-free systems. We found that primary antibodies raised in rabbits did not produce higher background due to cross-reactivity to RRL than primary antibodies raised in mice. The WGE-based system produced more protein than the RRL-based system; however, RRL-based system produced enough material for common assays such as protein:protein and protein:nucleic acid interaction assays.

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