Promega

Technical Manual

Wheat Germ Extract Plus

INSTRUCTIONS FOR USE OF PRODUCTS L3250 AND L3251.

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Wheat Germ Extract Plus

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1. Description

Wheat Germ Extract Plus is a highly efficient, single-tube protein expression system designed to give yields in the range of 10–80µg/ml. The extract contains all the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, and initiation, elongation and termination factors). Potassium acetate and magnesium acetate are also included at concentrations that enhance translation of a wide range of mRNAs. Protein synthesis is initiated by addition of the appropriate mRNA template, and the reaction is incubated for 2 hours at 25°C (Figure 1). Synthesized proteins can be analyzed by SDS-PAGE or used directly in numerous applications. There is no requirement for dialysis or specific incubation equipment.

In general, wheat germ extracts provide some co/post-translational modifications such as phosphorylation, farseylation and myristoylation (1). Signal sequence recognition and targeting require addition of the signal recognition particle and microsomes to the extract (2–4). Glycosylation can occur with the addition of canine microsomal membranes to wheat germ extract but is less efficient than that achieved using reticulocyte lysate systems (5,6). Cell-free extracts containing DTT or other reducing reagents are unable to provide an oxidizing environment that promotes disulfide modifications.

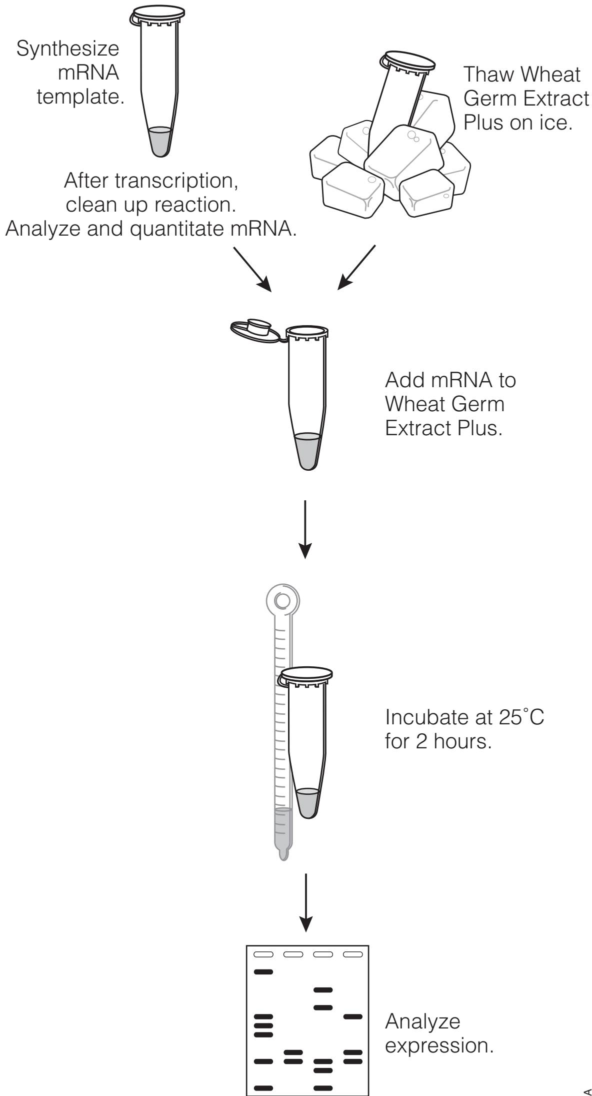

Figure 1. Wheat Germ Extract Plus protocol overview.

2. Product Components and Storage Conditions

Product	Size	Cat. #
Wheat Germ Extract Plus	40 × 50µl reactions	L3250

Includes:

- 1.2ml Wheat Germ Extract Plus (4 × 300µl)
- 1.25ml Nuclease-Free Water

Product	Size	Cat. #
Wheat Germ Extract Plus	10 × 50µl reactions	L3251

Includes:

- 300µl Wheat Germ Extract Plus
- 1.25ml Nuclease-Free Water

Storage Conditions: Store all components at –70°C. Product is sensitive to CO_2 (avoid prolonged exposure), and multiple freeze-thaw cycles, which may have an adverse affect on activity/performance. Do not freeze-thaw more than 3 times.

3. Template RNA Preparation and Purification

Wheat Germ Extract Plus is optimized for translation of mRNAs with 5´ ends that are uncapped, capped or contain viral leader enhancers. The 3´ end can have a poly(A) tail or 3´ viral enhancer, either of which significantly increases protein expression (7). Incorporation of these 5´ and 3´ elements into the mRNA template can be achieved by cloning the protein-coding regions into one of the Wheat Germ Flexi® Vectors (Cat.# L5671, L5681; Figure 2). These vectors incorporate sequences from the barley yellow dwarf virus (BYDV), an RNA plant virus, upstream and downstream of the protein-coding region of interest. The BYDV elements interact with each other, form a closed loop and act synergistically to stimulate translation in wheat germ extracts, bypassing mRNA cap and polyadenylation dependencies (8–11). If using the Flexi® Vectors, we recommend linearizing the vector with *Sal* I, *Xba* I or *Nru* I and performing a phenol:chloroform extraction before in vitro transcription. Alternatively, supercoiled DNA templates can be used for transcription, although protein expression from these RNAs may be lower.

Typically 6-12µg mRNA is required for a standard translation reaction. Therefore, the initial transcription reaction should be performed using systems that generate sufficient mRNA, such as the RiboMAX™ or RiboMAX™ Express Systems (Cat.# P1300, P1320, P1280). These systems typically yield ~100µg mRNA from 10µg linearized template DNA.

mRNA must be purified from transcription reactions to ensure efficient translation in Wheat Germ Extract Plus. Column purification or phenol:chloroform extraction is required. **Ethanol precipitation alone is not recommended**. There is no need to DNase treat the sample after transcription as DNA will not interfere with translation.

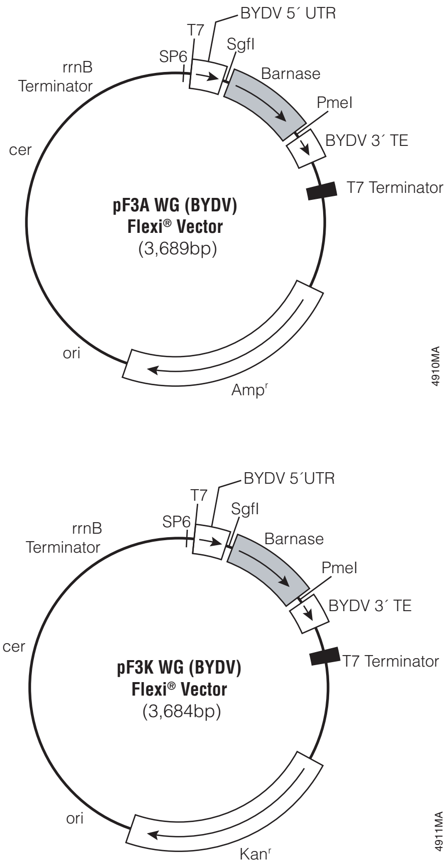

Figure 2. Wheat Germ Flexi® Vectors. The pF3A WG (BYDV) and pF3K WG (BYDV) Flexi® Vectors (Cat.# L5671 and L5681, respectively) are designed for expression of proteins in wheat germ extract. These vectors incorporate sequences from the barley yellow dwarf virus (BYDV), upstream and downstream of the protein coding region of interest. The vectors contain *Sgf* I and *Pme* I sites to facilitate directional cloning and transfer of protein-coding sequences to other Flexi® Vectors with different expression options. The lethal barnase gene allows positive selection of vectors containing insert. Ampicillin (pF3A WG Vector) and kanamycin (pF3K WG Vector) resistance genes allow selection in *E. coli*. Please refer to the *Flexi® Vector Systems Technical Manual* #TM254 for further details on the Flexi® Vector technology.

3.A. Small-Scale Spin Column Purification

MicroSpin™ G-25 columns (Amersham Biosciences Cat.# 27-5325-01), which allow purification of 25–50µl of transcription reaction per column, are recommended for purification of mRNA prior to use in Wheat Germ Extract Plus translation reactions. For transcription reactions less than 50µl, better results can be obtained by adding water to bring the load volume up to 50µl. Dilution of T7 RiboMAX™ Express Large Scale RNA Production System reactions 1:2 with water before purification may increase RNA recovery, however, the 50µl loading limit per column should still be observed.

3.B. Large-Scale Column Purification

NAP™-5 (Amersham Biosciences Cat.# 17-0853-01) and NAP™-10 columns (Amersham Biosciences Cat.# 17-0854-01) are recommended for purification of mRNA from large-scale transcription reactions. NAP™-5 Columns should be used for transcription reaction volumes of 0.1–0.5ml, and NAP™-10 Columns should be used for transcription reaction volumes of 0.5–1ml. These columns should be equilibrated with water and sample application and elution performed according to the manufacturer's instructions.

3.C. Phenol Extraction

1. Extract RNA with 1 volume of sodium acetate-saturated (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 minute and then spin the sample at top speed in a microcentrifuge for 2 minutes.

2. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as described in Step 1. At this point, unincorporated nucleotides may be removed or the RNA may be precipitated directly (Step 3, below).

3. Transfer the upper, aqueous phase to a fresh tube. Any transferred chloroform can be removed by performing a quick spin (10 seconds) in a microcentrifuge followed by removal of the bottom phase with a micropipette. Add 0.1 volume of 3M sodium acetate (pH 5.2), and 1 volume of isopropanol or 2.5 volumes of 95% ethanol. Mix and place on ice for 2–5 minutes. Spin at top speed in a microcentrifuge for 10 minutes.

4. Carefully pour off or aspirate the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and suspend the RNA sample in TE buffer or Nuclease-Free Water to a volume identical to that of the transcription reaction. Store at –70°C.

4. Translation Protocol

The following is a general guideline for setting up a translation reaction. For optimal results, the mRNA template should be purified using a G-25 column or by phenol extraction (see Section 3). For convenient detection of the synthesized protein [^{35}S]methionine, or a non-radioactive alternative such Transcend™ tRNA (Cat.# L5061) or FluoroTect™ $Green_{Lys}$ tRNA (Cat.# L5001), is recommended.

Materials to Be Supplied by the User

- G-25-purified or phenol extracted mRNA template
- [^{35}S]methionine (>1,000Ci/mmol, e.g., Redivue™ [^{35}S]methionine, Amersham Biosciences Cat.# AG1094), **or** Transcend™ tRNA (Cat.# L5061), **or** FluoroTect™ $Green_{Lys}$ tRNA (Cat.# L5001)

1. Thaw the Wheat Germ Extract Plus on ice.
2. After the extract has thawed, gently mix several times by pipetting.
3. Following the example below, assemble the reaction components in the appropriate 0.5–1.5ml microcentrifuge tube.

Standard Reaction

Component	Volume
Wheat Germ Extract Plus	30µl
Purified RNA substrate in Nuclease-Free Water	6–12µg
[^{35}S]methionine, Transcend™ or FluoroTect™ $Green_{Lys}$ tRNA	1–2µl
Nuclease-Free Water	to final volume **50µl**

Note: It is important to include a negative control reaction containing no mRNA. This allows measurement of any background incorporation of labeled amino acids. Also, when using Transcend™ tRNA, the negative control reaction allows identification of any endogenously biotinylated proteins in the Wheat Germ Extract Plus.

4. Incubate the translation reaction at 25°C for 2 hours.
5. Analyze the results of the translation by SDS-PAGE (see Section 5).

Table 1. Final Concentration of Wheat Germ Extract Plus Components in a 50µl Translation Reaction.

Component	Concentration
Creatine phosphate	10mM
Creatine phosphokinase	50µg/ml
DTT	5mM
Magnesium acetate	1.5mM
Potassium acetate	90mM
Spermidine	0.5mM
ATP	1.2mM
GTP	0.1mM
Complete amino acids	80µM

5. Post-Translation Analysis

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.A.)

- 1M NaOH/2% H_2O_2
- 25% TCA/2% casamino acids (Difco® brand, Vitamin Assay Grade)
- 5% TCA
- Whatman® GF/A glass fiber filter (Whatman® Cat.# 1820 021)
- acetone
- Whatman® 3MM filter paper
- SDS sample buffer
- SDS-PAGE running buffer
- SDS polyacrylamide gels*
- Gel fixing solution

*Precast gels are available from a number of manufacturers. (e.g., NOVEX® 14% Tris-Glycine [Invitrogen Cat.# EC64852], and 4-20% Tris-Glycine gradient gels [Invitrogen Cat.# EC60252]). In addition to convenience and safety, precast gels provide consistent results.

5.A. Determination of Percent Incorporation of Radioactive Label

1. Remove 2µl from the completed translation reaction and add it to 98µl 1M NaOH/2% H_2O_2.
2. Vortex briefly and incubate at 37°C for 10 minutes.
3. At the end of the incubation, add 900µl of ice-cold 25% TCA/2% casamino acids to precipitate the translation product. Incubate on ice for 30 minutes.
4. Wet a Whatman® GF/A glass fiber filter with a small amount of ice-cold 5% TCA. Collect the precipitated translation product by vacuum filtering 250µl of the TCA reaction mix. Rinse the filter 3 times with 1–3ml ice-cold 5% TCA. Rinse once with 1–3ml acetone. Allow the filter to dry at room temperature or under a heat lamp for at least 10 minutes.
5. To determine ^{35}S incorporation, put the filter in the appropriate scintillation cocktail, invert to mix and count in a liquid scintillation counter.
6. To determine total counts, spot a 5µl aliquot of the TCA reaction mix directly onto a filter. Dry the filter for 10 minutes. Count in a liquid scintillation counter as in Step 5.
7. To determine background counts, remove 2µl from a 50µl translation reaction containing no RNA and proceed as described in Steps 1–5.
8. Determine percent incorporation as follows:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of unwashed filter (Step 6)} \times 50} \times 100 = \text{percent incorporation}$$

9. Determine fold stimulation over background as follows:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of “no RNA control reaction” filter (Step 7)}} = \text{fold stimulation}$$

5.B. Denaturing Gel Analysis of Labeled Translation Products

A protocol for gel analysis of radiolabeled proteins is given below. For fluorescent detection of proteins using FluoroTect™ $Green_{Lys}$ tRNA, refer to the *FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System Technical Bulletin* #TB285. For colorimetric or chemiluminescent detection using Transcend™ tRNA refer to the *Transcend™ Systems Technical Bulletin* #TB182. These Technical Bulletins are provided with the FluoroTect™ and Transcend™ products, and are also available on our Web site at: **www.promega.com/tbs**

1. Once the 50µl translation reaction is complete (or at any desired timepoint), remove a 1µl aliquot and add to 20µl SDS sample buffer. The remainder of the reaction may be stored at –20°C, or at –70°C for long-term storage.
2. Cap the tube and heat at 100°C for 2 minutes to denature the proteins. This may cause protein aggregation. If this is a problem, incubation at a lower temperature (e.g., 20 minutes at 60°C, 10 minutes at 70°C or 3–4 minutes at 80–85°C) may be more appropriate.
3. The denatured sample can then be loaded onto an SDS-polyacrylamide gel or stored at –20°C. It is not necessary to separate labeled polypeptides from free amino acids by acetone precipitation.
4. Perform electrophoresis according to the gel manufacturer's instructions. Electrophoresis is usually performed until the bromophenol blue dye has run off the bottom of the gel. Disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel, as the dye front also contains the unincorporated labeled amino acids. If transferring the gel to a membrane filter for Western blotting, proceed to Step 7.
5. Fix the gel by soaking in 50% methanol, 7% acetic acid for 15 minutes followed by soaking in 7% glycerol, 7% methanol, 7% acetic acid for 5–10 minutes.
6. Dry the gel before exposure to film as follows: Soak the gel in 10% glycerol for 5 minutes to prevent cracking during drying. Place the gel on a sheet of Whatman® 3MM filter paper, cover with plastic wrap and dry at 80°C for 30–90 minutes under vacuum using a conventional gel dryer. Dry completely. The gel may also be dried overnight using the Gel Drying Kit (Cat.# V7120). To decrease the likelihood of cracking gradient gels, dry them with the wells pointing down. Expose the gel on Kodak X-OMAT® AR film for 6–15 hours at room temperature (with autoradiography).

   Alternatively, the fixed gel can be exposed to a phosphorimaging screen. Phosphorimaging systems provide greater sensitivity, greater speed and the ability to quantitate radioactive bands.
7. For Western blot analysis, transfer (immobilize) the protein from the gel onto a nitrocellulose or PVDF membrane. Detailed procedures for electrophoretic blotting are usually included with commercial devices.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com.** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low translation efficiency or no protein	mRNA integrity may be an issue. Verify that the mRNA is intact by gel analysis.
	mRNA may be contaminated. Mix the mRNA with Luciferase Control RNA (Cat.# L4561) and determine if translation of the control RNA is inhibited compared to a reaction containing Luciferase Control RNA alone.
	Possible cloning error. Verify the sequence of the DNA clone used in the transcription reaction.
	Determine the optimal mRNA concentration for translation. Serially dilute your mRNA template first and then add the same volume of mRNA to each translation reaction to ensure that other variables are kept constant.
	If phenol:chloroform extraction was used to purify the mRNA, ethanol may be present in the translation reaction. Remove residual ethanol from mRNA preparations.
Unexpected bands present on the gel	Possible internal initiation site. Verify by sequencing the cDNA clone. Alter any internal initiation codon by mutagenesis.
	Possible premature termination. Verify sequence for secondary structure of mRNA.

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8. Appendix

8.A. Composition of Buffers and Solutions

1X SDS sample buffer

50mM	Tris-HCl (pH 6.8)
2%	SDS
0.1%	bromophenol blue
10%	glycerol
100mM	dithiothreitol

1X SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

SDS polyacrylamide running 10X buffer

30g	Tris base
144g	glycine
100ml	10% SDS

Add water to a final volume of 1L. Store at room temperature.

Gel fixing solution

Gel Solution 1:

50%	methanol
7%	acetic acid

Gel Solution 2:

7%	glycerol
7%	methanol
7%	acetic acid

Soak gel in Gel Solution 1 for 15 minutes, then transfer to Gel Solution 2 for 5–10 minutes.

8.B. Related Products

Product	Size	Cat#
pF3A WG (BYDV) Flexi® Vector	20µg	L5671
pF3K WG (BYDV) Flexi® Vector	20µg	L5681
RiboMAX™ Large Scale RNA Production System—T7*	1 system	P1300
RiboMAX™ Large Scale RNA Production System—SP6*	1 system	P1280
T7 RiboMAX™ Express Large Scale RNA Production System*	1 system	P1320
Luciferase Control RNA	20µg	L4561
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System*	40 reactions	L5001
Transcend™ Colorimetric Non-Radioactive Translation Detection System*	30 reactions	L5070
Transcend™ Chemiluminsecent Non-Radioactive Translation Detection System*	30 reactions	L5080
Transcend™ Biotinylated tRNA*	30µl	L5061

*For Laboratory Use.

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