

Post-translational processing: Use of the TNT™ Lysate Systems with Canine Microsomal Membranes

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Canine Pancreatic Microsomal Membranes effectively perform signal peptide cleavage and core glycosylation of several polypeptides synthesized with Promega's TNT Lysate Coupled Transcription/Translation Systems. Processing efficiencies averaged between 70 and 90%.

Introduction

Promega's TNT Lysate Systems allow coupled transcription and translation in rabbit reticulocyte lysate of genes cloned downstream of SP6, T3 or T7 RNA polymerase promoters (1). Typically, much higher levels of proteins are synthesized than in separate transcription and translation reactions. Many customers have asked if the TNT Systems can be used in conjunction with Promega's Canine Pancreatic Microsomal Membranes.

Canine Pancreatic Microsomal Membranes are useful for studies of eukaryotic co-translational and initial post-translational processing of proteins (2). When Microsomal Membranes are included in a rabbit reticulocyte *in vitro* translation reaction, many proteins are co-translationally transported into the microsomal vesicles and processed. Depending on the protein, the processing may consist of signal peptide cleavage, membrane insertion, core glycosylation or other modifications.

To study the effectiveness of Microsomal Membrane processing with the three TNT Coupled Transcription/Translation Systems, we constructed six test plasmids containing either the *E. coli* beta-lactamase or the yeast alpha-factor gene, each downstream from either the SP6, T3 or the T7 RNA polymerase promoter. The *E. coli* beta-lactamase (ampicillin resistance) precursor gene product is co-translationally processed *in vivo* from an apparent molecular weight of 31.5kDa to 28.9kDa. The *S. cerevisiae* alpha-mating factor is glycosylated *in vivo*, which is detectable as a shift in apparent molecular weight from 18.6kDa to 32kDa.

Results

For each experiment, 0.5µg of supercoiled plasmid DNA from a given construct was added to the appropriate TNT Lysate reaction (3) containing [³⁵S]methionine as a label. Microsomal Membranes (2.5µl) were added and the reaction (25µl final volume) was incubated at 30°C for 90 minutes. Expression and processing levels were measured by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by quantitation with a Molecular Dynamics PhosphorImager™.

Signal processing and glycosylation in TNT Lysate

Figure 1, panel A shows that signal processing of the beta-lactamase precursor occurred in the SP6, T3, and T7 TNT Lysate Systems. In panel B, glycosylation of alpha-factor could be demonstrated in the T3 and SP6 TNT Lysate Systems. To date, we have not been able to demonstrate glycosylation of the alpha-factor gene product in the T7 TNT Lysate System. The T7/alpha-factor construct is expressed poorly in T7 TNT Lysate *without* added Microsomal Membranes, and consequently we believe this has contributed to our difficulty in demonstrating processing.

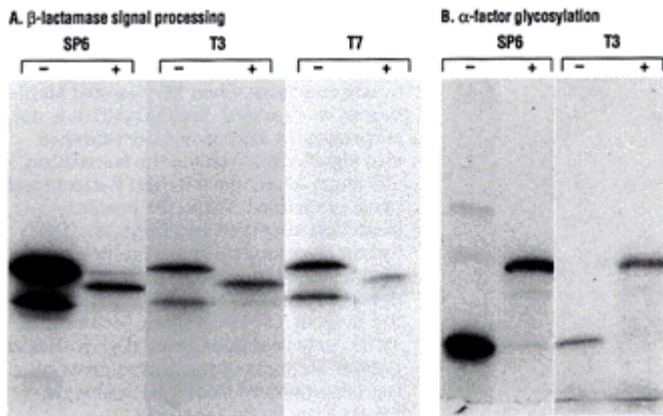


Figure 1. Signal peptide processing and glycosylation activity of Canine Microsomal Membranes in TNT Lysates. SP6, T7 or T3 promoter constructs of the beta-lactamase (panel A) or alpha-factor (panel B) genes were expressed in the appropriate TNT Lysate reactions performed as described above, either with (+) or without (-) added Canine Microsomal Membranes. The [³⁵S]methionine-labeled translation products were resolved on 12% SDS polyacrylamide gels. Autoradiography was performed for 2 hours at -70°C or overnight at room temperature (panel A), or for 48 hours at room temperature (panel B).

Quantitation of post-translational processing

The amounts of processed and unprocessed polypeptides were quantitated using a Molecular Dynamics PhosphorImagerTM. [Table 1](#) and [Table 2](#) show quantitation of signal cleavage and glycosylation, respectively, each using data from two independent experiments. Signal peptide was processed from 65-95% of the beta-lactamase precursor expressed in the presence of Microsomal Membranes. Similarly, 70-91% of the expressed alpha-factor was glycosylated in the presence of the membranes.

We observed a significant (50-80%) decrease in total gene expression in TNT Lysate reactions when Microsomal Membranes were added. This inhibition is not surprising, as Microsomal Membranes also significantly reduce the translation efficiency of standard Rabbit Reticulocyte Lysate reactions. While the *percent* inhibition observed was large, the TNT Lysate reactions produced sufficiently high levels of translation product to easily detect processing and glycosylation. In the presence of Microsomal Membranes, TNT Lysate reactions generally synthesize greater amounts of processed proteins than do standard Rabbit Reticulocyte Lysate translation reactions.

Table 1. Signal Peptide Processing of beta-Lactamase in TNT Lysate containing Microsomal Membranes.

TNT Lysate	Processed*	Unprocessed*	% Processed	Average % processed
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SP6 (a)	318,000	70,000	82%	
SP6 (b)	132,000	34,000	80%	81%
T3 (a)	39,000	5,600	87%	
T3 (b)	15,000	8,500	64%	76%
T7 (a)	167,000	10,000	94%	
T7 (b)	79,000	12,000	87%	91%

* The radioactivity measured in the processed and unprocessed bands is expressed in arbitrary PhosphorImager units. The results of two experiments, (a) and (b), are shown.

Table 2. Glycosylation of alpha-Factor in TNT Lysate containing Microsomal Membranes.

TNT Lysate	Glycosylated*	Unglycosylated*	% Processed	Average % processed
SP6 (a)	98,000	14,000	87%	
SP6 (b)	37,000	16,000	70%	79%
T3 (a)	102,000	9,600	91%	
T3 (b)	151,000	19,000	89%	90%

*The radioactivity measured in the processed and unprocessed bands is expressed in arbitrary PhosphorImager units. The results of two experiments, (a) and (b), are shown. T7 data is not shown because of low expression with the T7/alpha-factor construct tested.

Protocol for using TNT Lysate with Microsomal Membranes

The reaction conditions given below should be suitable for most applications. These conditions may need to be adjusted slightly for different genes because the efficiency of processing may vary with the gene being expressed.

1. Remove the reagents from the freezer and allow them to thaw on ice.
2. In a sterile 1.5ml microcentrifuge tube, mix the following components on ice, in the order given.

TNT Lysate	12.5µl
TNT Reaction Buffer	0.5µl

Each order of Canine Microsomal Membranes contains the specified amount of membranes along with 5µg of Signal Sequence Control mRNA, 1µg of Core Glycosylation Control mRNA and a Technical Manual.

Product	Cat.#
TNT™ T3 Coupled Reticulocyte Lysate System	L4950
TNT™ T7 Coupled Reticulocyte Lysate System	L4610
TNT™ SP6 Coupled Reticulocyte Lysate System	L4600

Each system contains sufficient reagents to perform 30 x 50µl reactions. TNT™ Lysate is provided in 200µl aliquots.

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