Canine Pancreatic Microsomal Membranes

INSTRUCTIONS FOR USE OF PRODUCT Y4041.

Quick PROTOCOL

Cotranslational Processing and Post-Translational Analysis

To test the processing efficiencies of the Canine Microsomal Membranes, the following procedure can be performed using the positive control mRNAs supplied. The efficiency of processing using alternate translation systems or mRNAs may vary and may affect the amount of Canine Pancreatic Microsomal Membranes required.

1. Before you begin, remove the Canine Microsomal Membranes and control mRNAs from storage at –70°C. Thaw slowly on ice.
2. Heat the mRNA at 65°C for 3 minutes to denature. Cool immediately on ice.
3. Assemble the reaction components, appropriate for the label being used, in a 0.5ml polypropylene microcentrifuge tube, in the order listed below. Mix gently by pipetting and stirring the reaction with a pipette tip. If necessary, centrifuge briefly to collect the sample on the bottom of the tube.

Component	Amount per Reaction
Nuclease-Treated Rabbit Reticulocyte Lysate	17.5µl
Amino Acid Mix, Minus Methionine, 1mM	0.5µl
Canine Pancreatic Microsomal Membranes (see Notes 1 and 2 on back)	1.5–2.4µl
RNA substrate in water (e.g., pre-β-lactamase or α-factor mRNA at 0.1µg/µl; see Notes 3 and 4 on back)	1.0µl
[^{35}S]methionine (1,200Ci/mmol) at 10mCi/ml (see Note 8 on back)	2.0µl
Nuclease-Free Water to a final volume of	25.0µl

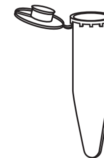
Note: RNasin® Ribonuclease Inhibitor (approximately 40 units) may be added to the above reaction.

Small-scale reactions may be performed by reducing volumes proportionally. To prepare 12.5µl reactions, use one-half of the stated volumes for all reaction components listed above.

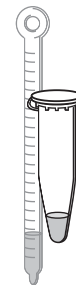
4. Incubate at 30°C for 60 minutes.
5. Analyze the results of translation and processing by SDS-polyacrylamide gel electrophoresis. Procedures are provided in the Technical Manual #TM231 (Section 4.B) for gel analysis of translation products. For information on analysis of cotranslational processing, see Notes 4 and 7 in Section 4.A of TM231 and on the back of this card.

Thaw reaction components on ice.

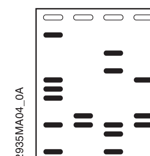
Heat mRNA at 65°C for 3 minutes, then cool on ice.



Assemble reaction components. Return unused components to –70°C.



Incubate at 30°C.

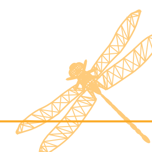


Analyze translation and processing results.

Control Translation Reactions

We recommend including a control reaction without added mRNA. This allows measurement of any background incorporation of labeled amino acids.

See complete protocol information in Technical Manual #TM231, available online at: **www.promega.com**



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Additional Information for Cotranslational and Post-Translational Analysis

Notes

1. The amount of Canine Pancreatic Microsomal Membranes used in the reaction may need to be titrated. While these reaction conditions will be suitable for most applications, the efficiency of processing using membranes will vary. Thus, reaction parameters may need to be altered to suit individual requirements. In general, increasing the amount of membranes in the reaction increases the proportion of polypeptides that are processed but reduces the total amount of polypeptides synthesized.
2. We do not recommend exceeding 1.8µl of Canine Microsomal Membranes in the TNT® SP6 Coupled Reactions. The SP6 Polymerase is more sensitive to the presence of Canine Microsomal Membranes than are the T7 and T3 Polymerases. Thus, synthesis of polypeptides in SP6 reactions is inhibited by higher concentrations of membranes.
3. Addition of 0.3–2.4µl of Canine Microsomal Membranes per 25µl of translation mix will process ≥50% of pre-β-lactamase to β-lactamase. The same amount of membranes will process ≥50% of α-factor to core glycosylated forms of α-factor.
4. When analyzed by SDS gel electrophoresis, the precursor for β-lactamase migrates at 31.5kDa and the processed β-lactamase at 28.9kDa. The precursor for α-factor migrates at 18.6kDa, and the core-glycosylated α-factor has a molecular weight of 32kDa but will migrate faster than the β-lactamase precursor. Figure 1 in TM231 is an example of SDS gel analysis of these translation products.
5. The amount of protein produced in lysates using Canine Microsomal Membranes will be less than the amount of protein produced in lysates alone. Depending on the construct used, translation efficiency can be expected to drop 10–50% in the presence of Canine Microsomal Membranes.
6. The storage buffer for the Canine Microsomal Membranes consists of 50mM triethanolamine, 2mM DTT and 250mM sucrose.
7. In some cases, it is difficult to determine by gel analysis alone if efficient processing or glycosylation has occurred. The following references provide information on assays for detecting cotranslational and post-translational processing events.

   A general assay for cotranslational processing makes use of the protection afforded the translocated protein domain by the lipid bilayer of the microsomal membrane. In this assay, protein domains are judged to be translocated if they are observed to be protected from exogenously added protease (see TM231, references 4–7 for details).

   In a separate assay, endoglycosidase H can be used to determine the extent of glycosylation of translation products. In cell-free systems, N-linked glycosylation occurs only within intact microsomes. Endoglycosidase H cleaves the internal N-acetylglucosamine residues of high mannose carbohydrates, resulting in a shift in apparent molecular weight on SDS gels to a position very close to that of the nonglycosylated species.

   Endoglycosidase F also may be used in a protease protection assay. Consult references 8 and 9 of TM231 for more information on the uses of endoglycosidase F for monitoring glycosylation or membrane insertion of translation products.
8. The use of a grade of [^{35}S]methionine, such as EasyTag™ L-[^{35}S]-Methionine (Perkin Elmer Cat.# NEG709A) will help prevent background labeling of the rabbit reticulocyte lysate 42kDa protein, which can occur using other grades of label. This [^{35}S]methionine may be stored at 4°C without dispensing into aliquots. Other ^{35}S-labeled amino acids may be easily stored in aliquots at –70°C in the presence of 1mM dithiothreitol or β-mercaptoethanol.

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