C. Carbonate extraction

A final assay for a subclass of translocation phenomena is carbonate extraction. This is useful for determining if an intergral membrane protein has achieved stable insertion into the bilayer. Basically, it involves treatment of the translation reaction with sodium carbonate at pH 11.5. Under these conditions, vesicles are converted to sheets and protein-protein interactions are disrupted, but protein-lipid interactions remain and the bilayer is otherwise intact. Thus cytosolic, cysternal and peripheral membrane proteins are retained to sediment with the membrane remnants. It is critical to do a parallel tube treating with sucrose buffer pH 7.5, conditions which should not disrupt the vesicle and to do, in parallel, carbonate and sucrose controls with a protein known to be extracted (e.g. a bona fide secretory or cytosolic protein) and a protein known to be extraction resistant to carbonate (e.g. a bona fide integral membrane protein). With these controls, it is possible to determine if, and to what extent, your protein achieved stable integration into the bilayer.

Traditional Procedure: Ten ul aliquots of translation X (and in parallel, of known secretory and integral membrane controls) adjusted to 1 ml with either ice cold 0.1 M sodium carbonate pH 11.5 or 0.25 M sucrose 0.1 M Tris pH 7.5. Be sure to save an aliquot (1 ul)of total products to run on the gel separately. Incubate for half an hour on ice, then centrifuge in TLA100 in polycarbonate tubes for 2 hours at 50,000 rpm for 15 min. Carefully aspirate supernates and save both supernates and pellets. Neutralize supernates with glacial acetic acid, precipitate with TCA to 15% (either with or without 50 ug carrier BSA added after neutralization). Spin out TCA precipitates full speed in microfuge. Wash with ethanol:ether. Dissolve both precipitate of supernate and original pellets in 100 ul 0.1 M Tris pH 8.9, 1% SDS., and either take an aliquot to mix with SDS gel sample buffer directly or dilute sample up in TX-SWB for standard immunoprecipitation. When running supernate and pellet be sure to compare to total products (or to immunoprecipitates of total products, as the case may be) to provide a reference for the relative intensities of the band in supernate and pellet. Densitometry will allow a balance sheet of recoveries and relative efficiencies of extraction of the unknown compared to the secretory and integral membrane controls.

Updated Methods: We have found that certain secretory proteins such as preprolactin are not extracted well by the above conditions. In general, supplementing the carbonate extraction with 1 M NaSCN and 10 mM DTT provides clearer results. However, this is not the case for all proteins and you should be prepared to test other conditions if needed. Pelleting of the extracted membranes can be performed in the airfuge, the TLA-100 or TLA-100.2 rotors in 15 to 30 min. We have also found that gel filtration using Sepharose CL-2B instead of centrifugation helps distinguish between membrane binding and aggregation of cell-free translation products.

Sample small scale carbonate extraction protocol:

1. Spin out membranes from a 10 ml translation reaction over a physiological cushion (20 ml) at 10 psi for 5 minutes in the airfuge, or 35 000 rpm for 15 min in the TLA100). Resuspend to the original volume with 1x retic buffer.
2. Add resuspended membrane to 800 ml of extraction buffer for 30 minutes on ice.
3. Overlay 750ml of the extraction onto a 250ml cushion. Spin at 80 000 rpm for 30 minutes

at 4 degrees in the TLA 100.2 rotor using 1ml pollyallomer tubes.

4. Remove 700 ml of the supernatant and add 450ml of 50% trichloroacetic acid (TCA) and 13 ml of glacial acetic acid (GAA).
5. To the pellet and cushion add 200ml of 50% TCA and 5ml of GAA.
6. Spin full speed in a microfuge for 15 minutes.
7. Wash with 300ml of ethanol:ether; spin again for 15 minutes full speed in a microfuge.
8. Dry pellets; resuspend in 10 or 20ml of SDS-PAGE loading buffer.

Buffers:

	Physiological Cushion	**Physiological Buffer**
1X Retic	50mL of 20X	1X retic
1mM DTT	1mL of 1M	1mM DTT
2X PIN	10mL of 200X	2X PIN
0.5M sucrose	250mL of 2M	

Extraction buffer:

	10 mL buffer	**5 mL cushion**
200mM Na2Co3	0.21g	0.105g
10mM DTT	100mL of 1M	50mL of 1M
2X PIN	100mL of 200X	50mL of 200X
0.5M sucrose	0	1.25mL of 2M
2% glycerol	200mL	100mL
1M urea*	0.6g	0.3g

*urea is optional; depending on the protein, it may not be required for complete extraction.