Protocol for Reconstituting Tissue Factor (TF) into Phospholipid Vesicles Using Bio-Beads SM-2

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http://tf7.org/mlabprotocols.htm

Background: Tissue factor (TF), an integral membrane protein, must be incorporated into suitable phospholipid vesicles for optimal clotting activity. In this protocol, mixed detergent/phospholipid/TF vesicles are formed, and then the detergent is selectively removed by adsorption onto Bio-Beads. The phospholipids then spontaneously assemble into unilamellar vesicles, with TF embedded in them.

Preparation of Bio-Beads SM-2

Note: Bio-Beads must be washed before using. Perform these steps in the chemical hood.

A. Add 25 g dry beads to 175 ml methanol in a plastic bottle.
B. Rotate the bottle on the tube rotator for 15 min at about 20 rpm.
C. Pour the slurry of beads and methanol into a sintered glass funnel on a vacuum flask. Remove the methanol by suction.
D. Wash the beads while still in the sintered glass funnel with another 425 ml methanol.
E. Wash the beads with deionized water while still in the funnel. Use a total of 2500 ml water.
F. Suspend the washed beads in 50 ml HBS.
G. Place in a vacuum flask and degas (2 hr under house vacuum).
H. Remove any beads that are floating. Store the washed beads as a suspension in HBS at room temperature until needed. They are stable for months.
I. Just before adding beads to the relipidation mixture, weigh out an aliquot of beads as follows:
   - Dispense some slurried beads into a plastic weighing dish
   - Aspirate any excess liquid from the beads
   - Using a pipet tip or spatula, transfer damp beads to a second weighing dish on a balance until the weight of damp beads equals the desired amount (usually 50 or 350 mg damp weight)
   - Using a pipet tip or spatula, transfer the aliquot of weighed, damp beads to the relipidation mixture (step 7 or 9, below). Make sure the beads don't dry out before using them.

Relipidation

1. Dispense 2.6 µmol total phospholipids into a glass test tube as follows:

	PS (10 mg/ml)	PC (10 mg/ml)	PE (10 mg/ml)
For 20:80 PS:PC vesicles:	42 µl = 0.52 µmole	158 µl = 2.08 µmole	
For 20:40:40 PS:PC:PE vesicles:	42 µl = 0.52 µmole	79 µl = 1.04 µmole	80 µl = 1.04 µmole

2. Dry phospholipids using a stream of nitrogen until the chloroform has evaporated.
3. Put tube in speed vac under high vacuum for 60 minutes (to remove any residual chloroform).
4. To the tube of dried-down phospholipids, add the following (final volume will be 1 ml):
   250-*x* µl HBS (the value of *x* comes from step 6, below)
   750 µl detergent stock solution (to give desired final concentration – see Table 1)
5. Dissolve the phospholipids completely by gentle vortexing. Incubate 30 min at room temp.
6. Add *x* µl TF (i.e., add an amount TF stock solution that equals 0.3 nmol TF) and gently mix by vortexing. Incubate 10 min at room temperature, then transfer solution to an Eppendorf tube.
7. Add 50 mg damp beads to the 1 ml TF/PL/detergent solution in the Eppendorf tube from step 6.
8. Rotate for 90 min on tube rotator at about 20 rpm.
9. Add an additional 350 mg (damp weight) of Bio-Beads and rotate for another 90 min.
10. Allow beads to settle for about 2 min. Carefully pipet off the supernatant (= relipidated TF).

Final product: 300 nM TF, 2.6 mM phospholipid in HBS (molar ratio of phospholipid:TF is 8700:1)

Materials

- Phospholipids (stored in chloroform) are from Avanti Polar Lipids (Alabaster, AL). We typically use egg PC, bovine brain PS, and bovine liver PE.
- Bio-Beads SM-2 (20-50 mesh) are from Bio-Rad Laboratories (Hercules, CA)
- We us recombinant, membrane-anchored human TF expressed in the periplasmic space of *E. coli.* This version of TF consists of amino acids 1-244, plus a short peptide epitope on its amino terminus for affinity purification using immobilized HPC4 antibody. Its calculated M_r is 28,986. Our TF stock solutions are stored in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.02% NaN_3, 0.1% Triton X-100. Note that in step 6 of the protocol, 8.7 µg of our recombinant human TF equals 0.3 nmol.
- Detergents that can be used in this protocol are listed in Table 1. (Deoxycholate gives slightly higher specific activities than the other detergents.)

Table 1: Detergents suitable for use in this relipidation method

Detergent	Stock conc. (in HBS)	Final conc.	MW	Source
Sodium Deoxycholate	20 mM	15 mM	414.6	Calbiochem (La Jolla, CA)
$C_{12}E_8$	8 mM	6 mM	538.8	Fluka (Sigma-Aldrich)
Triton X-100	5.33 mM	4 mM	628 (average)	Fisher Scientific (Fair Lawn, NJ)
Octylglucoside	44 mM	33 mM	292.4	Calbiochem

Solutions

HBS: 20 mM HEPES-NaOH pH 7.5
100 mM NaCl
0.02% NaN_3

Notes

- To vary the TF:phospholipid molar ratio, vary the amount of TF added in step 6. *Do not* change the concentration of phospholipid.
- The 20 mM deoxycholate stock solution may gel; if so, warm briefly at 37° and it will liquefy.
- To make the $C_{12}E_8$ stock solution: melt $C_{12}E_8$ at 37°C, then add to pre-warmed beaker and pre-warmed HBS using pre-warmed pipets. We do all this in the 37°C room.
- To make the Triton X-100 stock, start with a 10% (w/v) Triton X-100 solution (which is 159 mM), and dilute with HBS accordingly.
- This method can be easily scaled up. Simply increase the volumes of solutions and the amount of beads proportionally.
- Depending on which detergent is chosen, this method can make relatively large vesicles. In some cases, the vesicles are large enough that they will settle out when stored. Be sure to resuspend the relipidated TF products by gentle vortexing before using them.

Abbreviations

$C_{12}E_8$	octaethylene glycol monododecyl ether
octylglucoside	*n*-octyl-β-D-glucopyranoside
PC	L-α-phosphatidylcholine
PE	L-α-phosphatidylethanolamine
PS	L-α-phosphatidylserine
TF	tissue factor