



Early Endosome Fusion Assay

Authors: Roger Lippé, written by Heidi McBride and Marta Miaczynska

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Reference: Ohya et al., Nature 2009

1. Solutions and reagents preparation

Energy:

800 mM Creatine Phosphate in water
4 mg/ml Creatine Kinase in 50 % glycerol
5 mM NaH₂PO₄ pH 7.0
100 mM ATP in water pH 7.0

Fusion Buffer (1x):

12.5 mM Hepes pH 7.4
1.5 mM MgOAc
3 mM Imidazole
1 mM DTT
75 mM KOAc
- to prepare 50x solution, the pH should be around 8.8 and check that when diluted to 1X it will be 7.4.

Wash buffer (for assay):

100 mM NaCl
50 mM Tris-HCl pH 7.4
mg/ml BSA
2% Triton X-100

SIM (for Early endosome prep):

mM Sucrose
3 mM Imidazole
1 mM MgCl₂
pH 7.4

KEHM (for cytosol prep):

50 mM KCl
50 mM Hepes, pH 7.4
10 mM EGTA
mM MgCl₂

62 % Sucrose (for density gradient):

80.49 g sucrose
3 mM Imidazole
Fill up to 100 ml with water

**35 % Sucrose (for density gradient):**

40.3 g sucrose

3 mM Imidazole

Fill up to 100 ml with water

25 % Sucrose (for density gradient):

27.59 g sucrose

3 mM Imidazole

Fill up to 100 ml with water

Protease inhibitor mix (CLAAP)

		Sigma	for 5ml of 1000x (mg)
chymostatin	6 μ g/ml	C-7268	25
leupeptin	0.5 μ g/ml	L-2884	2.5
antipain-HCL	10 μ g/ml	A-6191	50
aprotinin	2 μ g/ml	A-1153	10
pepstatin	0.7 μ g/ml	P-4265	3.5
APMSF	10 μ g/ml	A-6664	50

Prepare as 1000x stock in dry DMSO, stor- 100ul aliquots at -20C.

2. Procedure

S-Hela growth for 2.5 | Spinner Culture Solutions and Media

S-MEM (Sigma M0518-10L), containing:

- L-glutamine
- Non-essential amino acids
- Fetal Calf Serum (5%)
- Penicillin/Streptomycin
- Trypsin-EDTA

Cells

Prepare 4 confluent medium sized flasks (175 cm²) of S-Hela in S-MEM.

Trypsinize them and add to 400 ml of S-MEM media in a 1L spinner flask. Place on a stirrer in the 37 °C room for 24 hours.

The next day, transfer these spinning cells into a 3 L spinner flask containing 2 L of fresh media.

Grow at 37 degrees until cells are 0.8-1.2 x 10⁶ cells/ml. This should take 4 days. If the cells are too dense, they will not internalize, so dilute them and grow into log phase again.

Harvest

Collect cells in GS-3 bottles and spin at 3K for 10 minutes at room temperature (Beckmann Coulter JLA-8.1000).

Wash in PBS, room temp.

Spin 3K for 10 min.

Transfer washed pellet into 50 ml falcon tube and wash again with PBS.

Spin in tabletop fuge 3 K for 10 minutes, r.t.

If preparing cytosol, resuspend cells in 1 cell volumes of ice cold cytosol buffer (KEHM) containing freshly added 1mM DTT, 1X CLAAAP, then proceed to break the cells. The half-life of most protease inhibitors is within the 10-20 minute range once in water so break the cells immediately after adding the cocktails.

Acceptor internalization

- Anti-transferrin antibodies are dialyzed against CO₂-independent media (+ 0.2 % BSA) beforehand and prewarmed just prior to use.
- Resuspend cells in prewarmed internalization media (containing antibodies) to a final volume of 10-15 ml (2-3 cell volumes).
- Incubate (internalize) at 37 °C in a waterbath for 5 minutes.
- Transfer immediately to an ice water bath and spin out the cells at 4 degrees in the tabletop 3K for 10 minutes (Heraeus Varifuge 3.0 R).
- Keep the antibody-containing media for re-use (filter sterilize and adjust pH).
- Resuspend the cells in cold homogenization buffer (SIM) and wash 2 times.
- Resuspend final cell pellet in 2 cell volumes of ice cold SIM containing freshly added CLAAP, 1 mM DTT and APMSF (see above).
- Proceed to "cracking".

Donor internalization

- Resuspend cell pellet in 10-15 ml prewarmed CO₂-independent media + 0.2 % BSA, which contains 20 µg/ml biotinylated transferrin (stock is 10 mg/ml in PBS).
- Incubate 5 minutes at 37 °C in a waterbath.
- Dilute up to 50 ml with ice cold SIM and transfer to ice bath.
- Spin in tabletop fuge at 3K, 10 minutes and wash 2 times with SIM.
- Resuspend final cell pellet in 2 cell volumes of ice cold SIM containing freshly added CLAAP, 1mM DTT and APMSF.
- Proceed to “cracking”.

Cracking

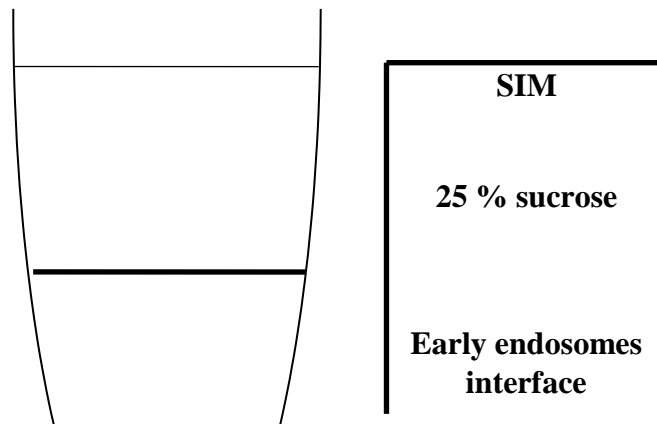
- Assemble the cell cracker (made at the EMBL) using ball bearing size 8.004 and two syringes 5 or 10 ml, depending on the cell volumes. Pre-cool the cracker in the cold room.
- From here on, all steps must be at 4 °C to avoid protein degradation and keep label at early endosome level.
- Passage the cells through the cracker 6 or 7 times before you check under the microscope to see the breakage. This takes much strength and patience, since there is extreme pressure in the chamber. Wear goggles or glasses in case the syringe pops out and the homogenate flies around.
- Compare the broken cells (10µl on a coverslip) with unbroken. It is probable that you will need another passage or two depending on your strength.
- When all the cells are broken, if preparing cytosol, continue to cytosol protocol. If preparing endosomes, spin the cell homogenate in the tabletop at 4K for 20 min at 4 °C.
- Collect the post nuclear supernatant (PNS). Sometimes it is difficult to distinguish the interface between the nuclei/unbroken cells and the supernatant so look through the light. If it is a problem, you can dilute the homogenate with SIM and re-spin. The PNS can be snap frozen at -80 for a later date if you need to, otherwise continue with the sucrose density gradient.

Sucrose Density Gradient

- Adjust the PNS sucrose concentration to 40.6 % sucrose using the refractometer and an ice-cold 62 % sucrose stock. Begin by adding 600 µl of 62 % sucrose to 500 µl PNS.
- One pot of 2.4 L should be divided among 6 tubes to fill one rotor.
- In the cold room, use a syringe to load this amount at the bottom of a SW40 tube (Beckman Coulter Ultra Clear, No: 344060) . Carefully cover this with 4 ml of cold 35 % sucrose. Overlay this with another 4 ml of 25 % sucrose. Fill the tube with SIM.
- Spin the gradients at 35K for 6 hours at 4 °C (Beckman Coulter SW40 Ti).
- Collect the early endosome band at the 25-35 % interface using the peristaltic pump and aliquot in 200 µl fractions. Take 40 µl for a Biorad protein assay and snap freeze the rest for storage at -80 °C.

Cytosol

Take cell homogenate and spin in TLA 100.4 (about 3 ml per tube) at 80K for 30 minutes at 4 °C. Remove the supernatant (cytosol) and aliquot. Take a 10 µl aliquot for Biorad assay and snap freeze the rest at -80 °C



Fusion Assay

Each fusion assay is carried out in 20 μ l and contains:

0.4 μ l 50X fusion buffer.

1.0 μ l cold holotransferrin (40 mg/ml in PBS)

1.3 μ l energy mix (freshly mixed 1:1:1 each creatine kinase, creatine phosphate and ATP) 1-2 μ l donor endosomes or donor clathrin-coated vesicles

2-4 μ l acceptor endosomes 3 mg/ml HeLa cytosol

remainder of 20 μ l filled with water and/or reagents to be tested.

Basal experiment consists of

- +/- energy
- +/- cytosol
- +/- Rab-GDI
- 100 % input (5 % total solubilized membranes)

In order to avoid pipetting mistakes it is advisable to prepare a premix of reagents present in every reaction such as fusion buffer, transferrin, donor and acceptor membranes, as well as energy mix and cytosol when required. Never mix donor and acceptor membranes in the absence of cold holotransferrin even for short times, as accidental breaking of vesicles will result in content mixing and false positive signal.

The recommended order of addition of reaction components (reactions have to be set up on ice):

- water
- reagents to be tested, e.g. Rab-GDI
- cytosol (if not present in the membrane premix)
- energy (if not present in the membrane premix)
- membrane premix containing also fusion buffer and cold transferrin.

Do reactions at least in duplicate, better in triplicate.

Fusion reactions are initiated by transfer of tubes from ice to 37 °C for 30 minutes. During this time block and wash MSD plate with Wash Buffer.



- Arrest reaction by adding 60 μ l of ice cold wash buffer and vortexing.
- Stop reaction by adding 50 μ L of ice cold Wash Buffer. Vortex gently.
- Add 60 μ l samples to 96-well streptavidin coated MSD plate (L15SA-1)
- Mix for 1 h at RT (600 rpm) on shaker
- Wash plate 3x with Wash Buffer
- Add 50 μ L of 1.5 μ g/mL 2° Ab (Anti-Goat MSD Sulfo Tag) diluted in 10 % wash buffer
- Mix 1 h RT, protected from light
- Wash plate 3x with Wash Buffer
- Add 150 μ L 1x MSD Read Buffer
- Read on MSD plate reader