Marc Fivaz 2004 methods

Binding of KRas-tail-CFP to CaM beads

I use Calmodulin Sepharose 4B (Amersham Biosciences 17-0529-01)

Preparation of the CaM-beads

- Beads are supplied swollen in EtOH. Mix (with a broken p1000 tip) and distribute 4 x 1ml in 4 eppendorf tubes
- Spin down beads in a microcentrifuge (12000 rpm; 30sec; 4C) and resuspend in BB (binding buffer; 50 mM Tris-HCl pH 7, 50 mM NaCl; 2 mM $CaCl_2$) in a ratio of 75% settled medium and 25% BB)
- Repeat last step twice.
- Alicot 100 μl of beads in BB (75% settled beads and 25% BB) in eppendorf tubes and store at 4C. Beads are ready to use.

Transfection of cells with KRas-tail-CFP

transfect 2x 10cm diameter Hela or R2 dishes (near confluent) with Fugene. Per dish: 5μg DNA, 15μl Fugene and 350 OM. Add to 7.5 ml cell medium (DMEM). Change Medium the next day.

Post-nuclear supernatant (PNS)

- ~36hrs after transfection, wash cells 2-3 times with cold PBS^{2+} (Ca^{2+};Mg^{2+}). Add 2.5 ml PBS^{-} and scrap cells. Spin down cells (4 min; 900 rpm) and resuspend the equivalent of 2 dishes in HB (3mM imidazole pH 7.4; 8.55% sucrose). Cells can be frozen at that point.
- Spin down cells and resuspend in 400μl HB^{CT}. Homogeneize cells (5-7 strokes with a $22G1^{1/2}$ needle) and spin down nuclear membranes (5000 rpm; 10 min). Take the PNS (milky appearance). Can be frozen at that point.

Quantify protein concentration (Bradford).

- Dilute Bradford (Bio-Rad) 1:5 in water.
- Prepare BSA standards. Adjust to 100 μl

BSA (μg/ml)	BSA vol (0.5mg/ml stock)	water
0	0	100
10	2	98
20	4	96
40	8	92
80	16	84

- Prepare an extra tube with 2 or 3 μl of PNS, adjusted to 100 μl with water.
- Add 1 ml of Bradford solution, vortex, and read immediately OD at 595 nm (don't forget the reference; 0 BSA!)

Binding the PNS to CaM Beads

- take 80-100μg PNS, and dilute it to 500μl in BB containing 0.15% TX-100 and protease inhibitors (C^{TM})
- devide sample in 2x 250μl (for input and unbound material)

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- add to 250μl to an alicot of CaM beads (previously spun down) and incubate 1hr at 4C on rotary shaker
- spin down beads and collect sup (unbound material!)
- $CHCl_3$/MeOH precipitate the input and unbound fractions
- Resuspend protein pellet in 30μl of SB 2x.