Non-muscle actin polymerization assay using muscle actin polymerization kit (cat# BK003) or pyrene-labeled muscle actin (cat# AP05) with non-muscle actin (cat# APHL99).

The non-muscle actin polymerization assay is performed by using a mixture of pyrene-labeled muscle actin (cat# AP05 or actin polymerization kit, cat# BK003) plus a majority of unlabeled non-muscle actin (cat# APHL99). The pyrene muscle actin will not polymerize efficiently on its own at the concentration used in this assay, so the reaction is dependent on non-muscle actin polymerization for F-actin formation. Pyrene non-muscle actin has been shown to be unstable at the normal storage conditions and so was discontinued.

2) Equipment required

1) Fluorescence spectrophotometer with 4-10 nm bandwidth at 365 nm excitation wavelength, and 4-10 nm bandwidth at 407 nm emission wavelength.
2) Small capacity (100-1000 ul) fluorescence spectrophotometer cuvette.
3) Pipettors 20, 200 and 1000 ul capacity.
4) 5-15 ml disposable tubes.
5) Ice supply, and 4 and -70°C storage areas.

3) Materials required

1) 5 x 1 mg Pyrene-labeled actin (cat# AP05).
2) 5 x 1 mg Non-muscle actin (cat# APHL99)
3) 1 x 100 ml General actin buffer (cat# BSA01-010). **STORE AT 4°C**. Resuspend in 100 ml of Milli-Q water.
4) 2 x 2 ml Actin polymerization buffer (cat# BSA02-001).
5) 1 x 1 ml ATP 100 mM (cat# BSA04-010).
6) 3 x 2 ml Tris-HCl pH 7.5 100 mM
7) One Manual

NB: Store General Actin Buffer at 4°C and store all other components at -70°C. Components are stable for at least one year.

4) Methods

1) Set the machine to 365 nm excitation and 407 nm emission for reading pyrene-actin.

2) To prepare Actin Polymerization Buffer (cat# BSA02) and ATP solution (cat# BSA04), do the following. Resuspend one vial of BSA02 in 1.8 ml of 100 mM Tris-HCl pH 7.5 (provided). Immediately aliquot the buffer into microfuge tubes as 10 x 200 ul. Store in -20 or -70°C freezer where the aliquots are stable for up to one year. Resuspend one vial of BSA04 in 1.0 ml of 100 mM Tris-HCl pH 7.5 (provided). Immediately aliquot the ATP solution into microfuge tubes as 10 x 100 ul. Store in -20 or -70°C freezer where they are stable for up to one year.

3) Take one 1 mg aliquot of AP05 and resuspend with 50 ul of Milli-Q water ($<10^{-14}$ ohm) at room temperature. This solution will be thick and viscous, so use a wide bore pipette tip for ease of pipetting. Aliquot 5.0 ul aliquots into 1.5 ml microfuge tubes and drop freeze into liquid nitrogen. These aliquots will be 20% less active than the unfrozen sample, so if you want them to be similar, either perform all polymerizations without freezing by using a 96-well fluorescence plate reader, or freeze all samples and then defrost one to perform the test at hand.

4) Make 10 ml of G-buffer which contains 20 ul of BSA04 for every 10 ml of General Actin Buffer. The components in this solution are 0.2 mM $CaCl_2$, 5 mM Tris-HCl pH 8.0 and 0.2 mM ATP.

5) Resuspend 1 mg of APHL99 with 50 ul of Milli-Q water. Now defrost one vial of AP05 quickly in a beaker of room temperature water and mix this with the APHL99 solution in the AP05 tube.

6) Transfer the actin mixture to a 15 ml tube and add 2.40 ml of G-buffer, pipette up and down and leave at room temperature for exactly 60 min (do not leave longer than this because the protein will denature). This is your monomer actin stock.

5) Experimental Procedure

We recommend using the stocks described above from Step 4 as a starting point for polymerization/depolymerization assays. As you become familiar with the experimental system the final actin stock solutions can be varied as required. Simple polymerization studies are described below. A good reference for more detailed analysis can be found in Chapter 3 of *The Cytoskeleton: A Practical Approach* (J. Cooper, Eds Carraway and Carraway, IRL Press, 1992).

Turn on fluorescence spectrophotometer and adjust to settings derived in "Preliminaries" described above.

Actin Polymerization Studies

Make enough of pyrene actin monomer as appropriate for your assay. For polymerization studies, perform a control lacking your test substance. Place monomer stock into the cuvette and set the reference to zero. Start the timing device and leave sample for 10 min. It should not produce any difference in fluorescence signal (reference phase, see Figure 1 legend). Then add $1/10^{th}$ volume of Actin Polymerization Buffer (BSA02 [at 10x strength BSA02 contains 500 mM KCl, 20 mM

MgCl2 and 10 mM ATP] should be resuspended in 100 mM Tris-HCl pH 7.5 for stability), mix well and immediately start readings again with time intervals of 30 or 60 seconds. Continue for 1h or until a plateau in signal strength is achieved. This is your control reaction (see Line 1, Figure 1). The samples should be shuttered when not being measured because the excitation beam can bleach the sample leading to erroneous results. We suggest opening the light beam for less than 3 seconds every minute.

Figure 1: Actin Polymerization with reference phase

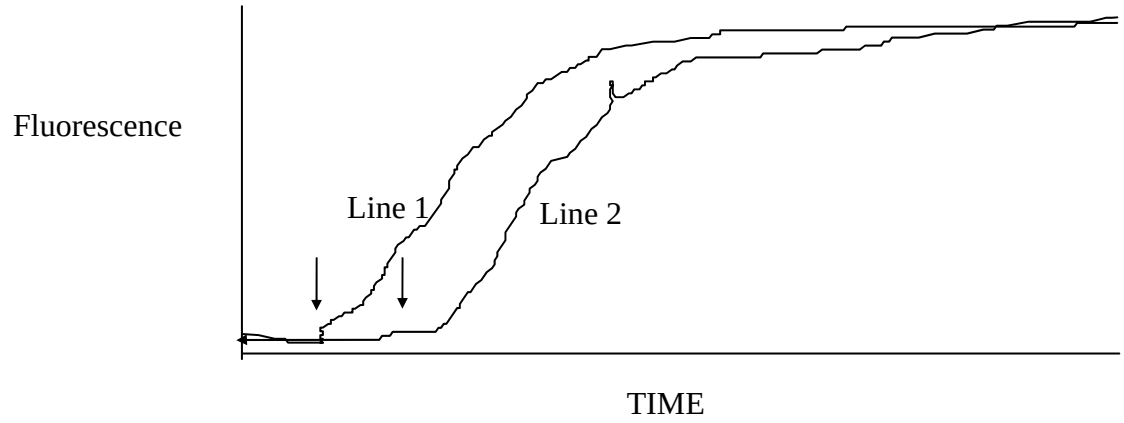

Figure legend: Native data from single cuvette monochromatic fluorescence spectrophotometer. Reference phase is ended at the arrow (10min intervals), where polymerization inducer is added. See text for description of lines 1 and 2.

The test solution should be added to a similar sample at T=10 min without Actin Polymerization Buffer. The mixture should be returned to the fluorescence spectrophotometer and observed for a further 20 min. This will indicate if any component of the test solution is enhancing polymerization (the profile in figure 1, line 2 indicates there is no effect from the test solution). Next add $1/10^{th}$ volume of Actin Polymerization Buffer, mix by vortex and record the increase in fluorescence over time. Compare the final profiles to identify activities associated with your test samples. In the example shown in figure 1 the test solution (Line 2) does not appear to affect polymerization. A good reference for more detailed analysis can be found in Chapter 3 of *The Cytoskeleton: A Practical Approach* (J. Cooper, Eds Carraway and Carraway, IRL Press, 1992).

If you are investigating a possible F-actin nucleating activity, then you should first reduce the endogenous nucleation centers (these form during the freeze/thaw step of actin storage) by centrifugation at 150 000 x g for 2h at 4°C. Aspirate out the top 80% of the supernatant and use this for polymerization studies. This method will also increase the sensitivity of the polymerization assay by reducing the starting background level of fluorescence.

Some actin binding proteins require alternative conditions for different activities. For example, cofilin requires pH 8.0 for F-actin binding and full severing activity and pH 6.8 for F-actin binding activity only. So several parameters may have to be tested in this assay in order to characterize a particular protein or compound. A particularly useful parameter to vary is the amount of actin polymerization buffer added. At 0.25x strength polymerization buffer and pH 7.0, cofilin is clearly seen to alter actin polymerization. Whereas the reaction performed with 1.0x polymerization buffer would not show a difference. Cofilin should be used at 20 ug/ml final concentration.

5) Trouble-shooting

Observation	Possible cause	Remedy
1. No increase in fluorescence between G-actin control and F-actin test.	1. Sensitivity of fluorescence spectrophotometer set too low. 2. Incorrect labeling of tubes	1. Increase sensitivity by: increasing emission gain or increasing bandwidth of emission channel or increase intensity of excitation.
2. During polymerization, increase in fluorescence is too slow.	1. Actin concentration is too low 2. Excitation light is too intense	1. Increase actin concentration. 2. Reduce light intensity reaching the sample.
3. During polymerization, increase in fluorescence is too quick.	1. Actin concentration is too high 2. Nucleation is too great	1. Use less Actin Polymerization Buffer 2. Decrease actin concentration. 3. Centrifuge samples (100,000 x g for 1h at 4°C) prior to adding test solution or Actin Polymerization Buffer.
4. During polymerization increase in fluorescence is not reproducible.	1. Inconsistent preparation of pyrene actin solutions. 2. Fluorescence spectrophotometer is sensitive to changes in cuvette. 3. Actin filaments are sheared differentially during pipetting.	1. More consistent technique. 2. Change from 96-well plate reader to single cuvette monochromatic machine. 3. Use equal force and mixing technique to all samples, especially important for assays requiring multiple pipetting steps.
5. During polymerization increase in fluorescence is low.	1. Concentration of actin too low (i.e. below 100 ug/ml). 2. Too much of pyrene actin in the form of small oligomers.	1. Increase actin concentration to greater than 0.2 mg/ml. 2. Centrifuge diluted actin at 150 000 x g before polymerization.
6. Buffer components of the reaction interfere with the	1. $CaCl_2$ 2. ATP	1. $CaCl_2$: Add EGTA 2. ATP: Do not add ATP to

activity of the test protein or compound.	3. NaN_3 4. Tris-HCl 5. pH 6. $MgCl_2$	A-buffer or Actin Polymerization Buffer, this is OK for up to 1h after actin dilution, after this point actin denaturation will be significant. 3. NaN_3: Make new General Actin Buffer each new day that you perform the experiment. 4. Tris-HCl: Make new Actin Buffer with a different "GOOD" Buffer. 5. pH: actin can be polymerized at pH 6.0-8.5. 6. $MgCl_2$: Omit from the Actin Polymerization Buffer.
7. Test solution has no effect on polymerization	1) Protein binds abnormally to pyrene-actin 2) Test substance does not affect actin polymerization.	1) Use different ratios of unlabeled to pyrene-labeled actin to see the effect.