RAPID Purification of Rabbit Muscle Actin from Acetone Powder

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last modified 98/09/23 (note steps 4-7)
Modified from Pollard lab modifications of Pardee & Spudich (Meth. Enzymol. **85**, 164).

Day before

1. Reserve the table-top ultracentrifuge and Sorvall centrifuge.

Solution

2. Use the following recipe for buffer A:

Buffer A (4 liter)	
8 ml 1 M Tris pH 8 (at room temp)	**final 2 mM**
8 ml 100 mM ATP pH ~7	**final 0.2 mM**
2 ml 1 M DTT	**final 0.5 mM**
0.4 ml 1 M $CaCl_2$	**final 0.1 mM**
0.26 g NaN_3	**final 1 mM**
cold water to 4 liters	

Small-Scale Prep

Extraction

3. Weigh out 0.5 g of muscle acetone powder (-80°C freezer).

4. Mix with 10 ml of buffer A in a capped scintillation vial. Stir in an ice-water bath for **30 minutes** with gentle stirring.

5. Pre-wet some cheesecloth with buffer A and squeeze out excess buffer before using it. Filter the mixture through two layers of cheesecloth; wearing gloves, squeeze the muscle residue to get the last bit of solution through. Save on ice in a 50-ml conical tube.

6. Scrape the muscle residue from the cheesecloth and extract it with an additional 10 ml of buffer A, repeating steps 4-5. Pool supernatants.

7. Spin the combined extracts in the SS-34 at 15,000 rpm for 20 minutes.

> The supe is **S1+S2**; save a 30 µl aliquot and add it to 10 µl of 4× SB for later SDS-PAGE analysis. Discard the pellet.

Polymerization

8. Measure the volume of the pooled supes (should be 16-18 ml) and polymerize to F-actin by adding KCl to 50 mM final and $MgCl_2$ to 2 mM final. Allow polymerization for **1 hour** at 4°C (with stirring). Chill 6 ultracentrifuge tubes and a homogenizer.

9. Add solid KCl with gentle stirring to give a final concentration of 800 mM (about 1 g of dry KCl). Gently stir the solution for **30 minutes**.

High-speed spin

10. Pellet the actin by spinning for **30 minutes** at 4°C at 75,000 rpm in the TLA-100 rotor. You will need six tubes.

11. Pour off the supernatant. Rinse the pellets with ~1 ml each buffer A (just pour in a little buffer A, then pour it back out).

> Actin that is capable of polymerizing will have sedimented to the bottom of the tube and hence will be in the pellet (**P3**). The supernatant is **S3**; save a 30 µl aliquot and add it to 10 µl of 4× SB for later SDS-PAGE analysis.

Resuspend pellets

12. Using a metal spatula (I have one with a bent tip that works well), remove as much of the pellet as possible and place it in a chilled teflon/glass homogenizer (4 ml size).

13. Add 1 ml of buffer A to one of the tubes. Use a 1-ml pipette to triturate the solution. NO BUBBLES! Set the pipetteman on 0.5 ml so that not all the solution is taken up at once. When the pellet residue is broken up enough, transfer the 1 ml to another tube and repeat the resuspension. Be carefully to get all of the traces of the pellet, which contains the actin. Use another 1 ml to repeat the resuspension on all of the tubes.

14. Gently homogenize with a chilled teflon/glass homogenizer (move the pestle up and down about ten times by hand; avoid foaming by keeping the pestle immersed at all times). A total of 2-3 ml of buffer A is required to successfully get the pellet into solution.

Dialysis

15. Dialyze using Pierce slide dialyzers against buffer A. You will probably need to use a 3-ml syringe with a 20 or 22 g needle to work with the viscous solution; be careful not to puncture the dialyzer. Use three to four changes of buffer (1 liter per change) in 48 hours.

> This dialysis step depolymerizes the actin filaments, leaving G-actin. This solution can then be centrifuged at high speed, removing insoluble material not capable of cyclical polymerization-depolymerization.

16. Centrifuge the G-actin at 75,000 rpm for 30 minutes in the TLA-100. Actin that is capable of depolymerizing will be in the supernatant and is now called G-actin (**S4**).

> Save a 15 µl aliquot of the supe and add it to 5 µl of 4× SB for later SDS-PAGE analysis. Resuspend the pellet in 1 ml of 1× SB.

Medium-Scale Prep

Extraction: medium scale

17. Weigh out **4 g** of **Pel-Freez** muscle acetone powder (-80°C freezer).

18. Mix with **80 ml** of buffer A in a Erlenmeyer flask. Stir in an ice-water bath for **30 minutes** with gentle stirring.

19. Pre-wet some cheesecloth with buffer A and squeeze out excess buffer before using it. Filter the mixture through two layers of cheesecloth; wearing gloves, squeeze the muscle residue to get the last bit of solution through. Save on ice.

20. Scrape the muscle residue from the cheesecloth and extract it with an additional **50 ml** of buffer A, repeating steps 4-5. Pool supernatants.

21. Spin the combined extracts in the SS-34 at 15,000 rpm for 20 minutes.

> The supe is **S1+S2**; save a 30 µl aliquot and add it to 10 µl of 4× SB for later SDS-PAGE analysis. Discard the pellet.

Polymerization

22. Measure the volume of the pooled supes (should be **~100 ml**) and polymerize to F-actin by adding KCl to 50 mM final and $MgCl_2$ to 2 mM final. Allow polymerization for **1 hour** at 4°C (with stirring). Chill **10-12 T-1270 ultracentrifuge tubes** and a homogenizer.

23. Add solid KCl with gentle stirring to give a final concentration of 800 mM. Gently stir the solution for **30 minutes**.

High-speed spin

24. Pellet the actin by spinning for **90 minutes** at 4°C at **65,000 rpm** in the **T-1270 rotor**.

25. Pour off the supernatant. Rinse the pellets with ~1 ml each buffer A (just pour in a little buffer A, then pour it back out).

> Actin that is capable of polymerizing will have sedimented to the bottom of the tube and hence will be in the pellet (**P3**). The supernatant is **S3**; save a 30 µl aliquot and add it to 10 µl of 4× SB for later SDS-PAGE analysis.

Resuspend pellets

26. Using a metal spatula (I have one with a bent tip that works well), remove as much of the pellet as possible and place it in a chilled teflon/glass homogenizer (10 ml size).

27. Add 1 ml of buffer A to one of the tubes. Use a 1-ml pipette to triturate the solution. NO BUBBLES! Set the pipetteman on 0.5 ml so that not all the solution is taken up at once. When the pellet residue is broken up enough, transfer the 1 ml to another tube and repeat the resuspension. Be carefully to get all of the traces of the pellet, which contains the actin. Use another 1 ml to repeat the resuspension on all of the tubes.

28. Gently homogenize with a chilled teflon/glass homogenizer (move the pestle up and down about ten times by hand; avoid foaming by keeping the pestle immersed at all times). A total of 4-5 ml of buffer A is required to successfully get the pellet into solution.

Dialysis

29. Dialyze using **two** Pierce slide dialyzers against buffer A. You will probably need to use a 3-ml syringe with a 20 or 22 g needle to work with the viscous solution; be careful not to puncture the dialyzer. Use three to four changes of buffer (1 liter per change) in 48 hours.

> This dialysis step depolymerizes the actin filaments, leaving G-actin. This solution can then be centrifuged at high speed, removing insoluble material not capable of cyclical polymerization-depolymerization.

30. Centrifuge the G-actin at 75,000 rpm for 30 minutes in the TLA-100. Actin that is capable of depolymerizing will be in the supernatant and is now called G-actin (**S4**).

> Save a 15 µl aliquot of the supe and add it to 5 µl of 4× SB for later SDS-PAGE analysis. Resuspend the pellet in 1 ml of 1× SB.

Purification by polymerization

31. For most purposes (e.g., ATPase assays, actin pelleting, myosin purification), this is the best way to prepare the actin.

32. Adjust the G-actin solution to final concentrations of 50 mM KCl and 1 mM $MgCl_2$ using stock solutions. The actin should increase in viscosity rapidly as it polymerizes into F-actin.

33. Dialyze in a Pierce slide dialyzer against ATPase assay buffer (15 mM HEPES pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.005% NaN_3) overnight at 4°C.

34. Centrifuge the F-actin at 75,000 rpm for 30 minutes in the TLA-100. Discard the supernatant (**S5**) after saving an aliquot for SDS-PAGE.

35. Resuspend the pellet (**P5**) with 1-2 ml of ATPase assay buffer (with NaN_3); use a glass/teflon homogenizer. If you are using the acetone powder from Pel-Freez, resuspend with 0.5 ml or less (the recovery is lower).

    Only that actin capable of polymerization-depolymerization (and those proteins that bind to actin filaments) will be purified.

36. Measure the OD of a 1:10 dilution at **290 nm** (note longer wavelength). A 38.5 μM solution has an OD_{290} of 1.000. Store at 4°C as filaments. Use within 2-3 weeks.

Purification by FPLC

37. This method is favored if you need to get rid of the last bits of contaminating actin-binding proteins.

38. Skip steps (17)-(22); instead use G-actin from step (16).

39. Inject 0.5 – 1.0 ml at a time of the supernatant on to Superdex 200 equilibrated in buffer A. The baseline OD will be higher because of the ATP in buffer A. Collect 0.5 ml fractions (peak probably in fractions 21-25). You can collect three successive separations in the same collection tubes.

    The actin peak will be somewhat sawtooth in shape, as the dialysis step probably left some oligomers in addition to the monomers.

40. Using a quick Bradford test with 5 μl of fractions 15-30 to locate the protein peak. Pool the fractions with protein.

    The protein peak contains the purified G-actin. Keep 30 μl of each tube and add to 10 μl 4× SB for SDS-PAGE analysis.

41. Make the G-actin solution 50 mM KCl and 1 mM $MgCl_2$.

42. Dialyze in several Pierce slide dialyzers against ATPase assay buffer (presently 15 mM HEPES pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA) overnight at 4°C.

43. Centrifuge the polymerized actin at 75,000 rpm for 30 minutes in the TLA-100. Discard the supernatant (**S4**) after saving an aliquot for SDS-PAGE.

44. Resuspend the pellet with 1-2 ml of ATPase assay buffer; use a glass/teflon homogenizer.

45. Measure the OD of a 1:10 dilution at **290 nm** (note longer wavelength). A 38.5 μM solution has an OD_{290} of 1.000. Store at 4°C as filaments.