Preparation of Troponin-Tropomyosin (Native tropomyosin) and Reconstitution of Thin Filament (Regulated Actin)

Number:

I. Buffers, solutions

A. Extraction Buffer	**250 ml**
25 mM TRIS-HCl (pH 8.0)	0.757 g
1 M KCl	18.64 g
0.2 mM ATP	25.5 mg
0.1 mM $CaCl_2$	12.5 μl stock (2 M)
0.5 mm DTT	19.3 mg

*Note: prepare fresh

B. KCl-DTT	**100 ml**
1 M KCl	7.456 g
0.5 mM DTT	7.7 mg

*Note: prepare fresh

C. DTT-water	**100 ml**
0.5 mM DTT	7.7 mg

*Note: prepare fresh

D. Dialysis & Reconstitution (D & R) Buffer	**2000 ml**
25 mM imidazole-HCl (pH 7.4)	3.404 g
100 mM KCl	14.912 g
2 mM $MgCl_2$	4 ml stock (1 M)
1 mM EGTA	4 ml stock (1 M)
1 mM β-mercaptoethanol	156 μl

II. Steps: Preparation of Native Tropomyosin:

*Note: take SDS-PAGE sample at each step!!

1. Extract acetone powder (about 10 g) with 15 ml/g Extraction Buffer overnight (16 hours) at room temperature (20 ˚C), while gently stirring with overhead stirrer.
Weight of acetone powder:

Volume of Extraction Buffer:

2. Filter acetone powder through 4 layers of sterile cheesecloth. Set filtrate aside on ice.

3. Reextract residue with 7.5 ml/g Extraction buffer for 2 hours at room temperature (20 ˚C).

4. Filter acetone powder through 4 layers of sterile cheesecloth. Combine and cool filtrates on ice. Discard residue.

5. Clarify filtrate by centrifugation at 20,000g, 0˚C, for 10 minutes.

6. Isoelectric precipitation of tropomyosin from supernatant (For handling the pH electrode, see Note A.):
Add 1 N HCl drop-by-drop to the supernatant, while stirring. Keep on ice. Take care to make the temperature correction on the pH meter. *Bring pH down to 4.6.* White precipitate should start

forming on approaching pH 5, and dense, snowy precipitate should be obtained at pH 4.6.
Continue to stir on ice for 30 minutes.

7. Centrifugation: 20,000g, 0 ˚C, 10 minutes

8. Dissolve pellet in 10 ml/g-acetone powder KCl-DTT solution.
Volume of added KCl-DTT solution:

*Note: Supernatant may be set aside for further purification of troponin. Pellet is expected to contain sufficient quantities of troponin for obtaining "native tropomyosin." It is advised to check protein composition of pellet by electrophoresis before proceeding. In the meantime the pellet may be stored on ice.

9. To enhance dissolution and even distribution of the pellet, homogenize it in a cooled glass tissue grinder.

10. Adjust pH to 7.4 by adding 1 N KOH drop-by-drop.
Suspension might not clarify (!). Stir suspension on ice for 30 minutes.

11. Clarify solution by centrifugation: 20,000g, 0 ˚C, 10 minutes.

12. Repeat isoelectric precipitation of tropomyosin from supernatant. (see step 6 above)

13. Centrifugation: 20,000g. 0 ˚C, 10 minutes.

14. Dissolve pellet in 5 ml/g-acetone powder DTT-water.
Volume of DTT-water added:

*Note: To enhance dissolution, one may use tissue grinder. However, at this stage dissolution should be easier, faster, and more thorough.

15. Adjust pH of suspension to 7.4 by adding 1 N KOH drop-by-drop.

16. Clarify solution by centrifugation: 20,000g, 0 ˚C, 10 minutes.

17. Readjust pH of supernatant to 7.4.

18. $(NH_4)_2SO_4$ fractionation: 25 g/dl:
Add solid ammonium sulfate to the supernatant while stirring on ice. Keep stirring for 30 minutes after all the solid is dissolved.
Volume of supernatant:

Weight of added ammonium sulfate (25 * volume/100):

19. Centrifuge: 20,000g, 0 ˚C, 10 minutes.

20. $(NH_4)_2SO_4$ fractionation: 35 g/dl:
Add an extra 10 g solid ammonium sulfate to the supernatant while stirring on ice. Keep stirring for 30 minutes after all the solid is dissolved.
Volume of supernatant:

Weight of added ammonium sulfate (10 * volume/100):

21. Centrifuge: 20,000g, 0 ˚C, 10 minutes

22. Dissolve pellet in a minimal volume of D & R Buffer (begin with 1 ml; if pellet is not fully dissolved, add more buffer until the whole pellet is dissolved).

23. Dialyze sample against 1 liter of D & R Buffer: 3 x 2 hours

24. Keep "native tropomyosin" on ice until reconstitution with F-actin.

III. Steps: Reconstition of Thin Filament (Regulated actin):

1. Measure protein concentration of "native tropomyosin" sample (see Note B).

2. Mix "native tropomyosin, F-actin, and ATP in the appropriate ratios, in D & R Buffer:
Appropriate concentrations:
F-actin: 1 mg/ml
Native tropomyosin: 1-2 mg/ml
ATP: 1 mM
Mixing sequence: Tropomyosin-Buffer-ATP (vortex); then add actin.

3. Reconstitution:
Incubate Mixture at 40 ˚C for 10 minutes.
Subsequently, lower temperature gradually (0.2 ˚C/min) to 10 ˚C. (See Note C)

4. Centrifuge: 145,000g, 3 hours, 0 ˚C (to sediment thin filaments).

5. Soak pellet in D & R Buffer for at least 3 hours (preferably overnight) on ice.

6. Gently homogenize pellet:
First, vortex briefly at low setting, until pellet comes lose from the tube wall. Then, repeatedly draw contents of the centrifuge tube (typically 1.5 ml) into a 9" Pasteur pipette, until the solution becomes homogenous.

7. Label Reulated Actin with TRITC-Ph (See labeling protocoll; M.K.-methods handbook)

Notes:

A. Handling of pH electrode for measurement of pH in protein solutions:

*One should take utmost care to clean the electrode surface from protein, since protein forms a film on the electrode surface hampering its function.
Steps:
-Take electrode out of standard (pH 7.02) solution.
-Rinse electrode with distilled water.
-Drye electrode with Kimwipe (so that water does not get into the protein sample).
-Dip electrode into the protein solution. Wait until pH meter stabilizes to obtain accurate reading.
-Adjust pH of protein solution as desired. Wait for a few minutes for pH to stabilize, as proteins are buffers also.
-When done, pull electrode out of the protein solution and rinse with 0.1 % SDS from squirt bottle.
-Dip electrode into 1% SDS, and soak for a few minutes (4-5).
-Rinse electrode thoroughly with distilled water.
-Wipe electrode dry.
-Dip electrode into 1 M KCl solution. Soak for 4-5 minutes.
-Rinse electrode thoroughly with distilled water.
-Wipe electrode dry.
-Dip electrode into pH standard. Wait until pH reading readjusts to 7.02 (usually takes a few minutes).

B. Protein concentration measurement: Bio-Rad assay

Assay is based on Lowry's method. For details, see instruction manual.
Steps:
-Prepare 2 mg/ml BSA solution in Dialysis & Reconstitution (D & R) Buffer
-Make dilution series of BSA: mix buffer and 2 mg/ml BSA in the following ratios:

Concentration (mg/ml)	D & R Buffer (μl)	2 mg/ml BSA (μl)
0.2	900	100
0.5	750	250
0.8	600	400
1.2	400	600
1.5	250	750

-Prepare dilution series of "native tropomyosin" sample in D & R Buffer. Example:

Dilution	Sample volume (μl)	D & R Buffer (μl)
5 x	20	80
10 x	10	90
15 x	6	84
20 x	5	95

*Note: the final dilutions (at least one of them) should be in the linear range of the method. One can visually check for it: the color of at least one sample should be between those of the 0.2 and 1.5 mg/ml BSA.

The following steps are the same for the BSA standard and for the protein sample:
-Add 50 μl of protein solution to a clean test tube. Do not forget about the buffer control (add 50 μl of D & R Buffer to a clean test tube)!
-Add 250 μl of Solution A of the Bio-Rad Kit. Vortex.
-Add 2 ml of Solution B of the Bio-Rad Kit. Vortex.
-Wait for 15 minutes.
-Measure absorbance at 750 nm:
First, measure that of BSA solutions; then plot the standard curve
Second, measure that of "native tropomyosin" samples; calculate concentration of original sample based on the standard curve and the dilution factor.

C. Reconstitution step:

The following steps are a simple way of achieving gradual cooling of actin-tm mixture desired to get homogenos reconstitution.
Steps:
-Place a test tube rack in a 10-liter plastic deep tray (Nalgene).
-Place a thermometer in the rack.
-Fill the tray to half (5 liters) with 40-˚C water. Adjust temperature by mixing hot and cold tap water.
-Place Actin-Tm mixture (in a test tube) in the rack.
-Keep temperature at 40 ˚C for ten minutes by the mixing method (above).
-After 10 minutes place the tray in the refrigerator.
-Water in the tray should cool down to 10 ˚C in about 3 hours.

References:

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