

Skeletal Muscle Thin Filament Complex (Rabbit Skeletal Muscle from psoas tissue)

CS-TFC02

V. 1.0

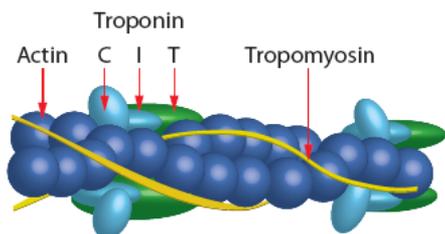
Upon arrival store at 4°C (desiccated)

See datasheet for storage after reconstitution

Material

The skeletal muscle thin filament complex (SMTFC) has been assembled from purified F-actin (Cat. # AKL99) and Tropomyosin / Troponin protein (TT) complex (Cat. # TT06). Thus, SMTFC is composed of six proteins: Actin : Tropomyosin α : Tropomyosin β : Troponin C : Troponin I : Troponin T in a stoichiometric ratio of 7:1:1:1:1:1; see Figure 1. After assembly, the thin filaments were centrifuged at 100,000 x g and the pellets resuspended in reaction buffer which purifies the calcium sensitive complex. The complex has been determined to be biologically active in a calcium activated myosin ATPase assay (see biological activity assay). The complex is supplied as a white lyophilized powder.

Figure 1—Schematic diagram of muscle thin filament.



Storage and Reconstitution

Briefly centrifuge to collect the product to the bottom of the tube. The protein should be reconstituted to 2 mg/ml by the addition of room temperature (RT) PM12 buffer. Do not pipette vigorously as this will denature the filaments. A white solution will appear first. Leave this for 10 min, then centrifuge at 500 x g to remove air bubbles. The solution should now look nearly clear. Do not centrifuge at high speed because the filaments will sediment.

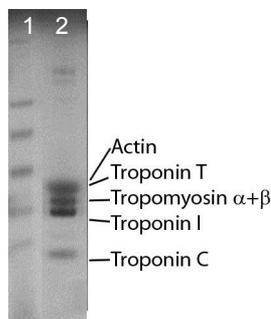
The protein will be in the following buffer: 16.8 mM PIPES pH 6.8, 2.8 mM MgCl₂, and 2% (w/v) sucrose.

The protein solution can be frozen by aliquoting into "experiment sized" amounts, snap frozen in liquid nitrogen and stored at -70°C. The protein is stable for 6 months if stored at -70°C. The protein should not be exposed to repeated freeze-thaw cycles. The lyophilized protein is stable at 4°C desiccated (<10% humidity) for 1 year.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient polyacrylamide gel. The purity of the SMTFC is >95% (Figure 2, lane 2).

Figure 2. Thin filament protein analysis.



Legend: A 20 μ g sample of CFTC protein was separated by electrophoresis in a 4-20% SDS-PAGE system (Lane 2) and stained with Coomassie Blue. Molecular weights are 43 kDa actin, 38 kDa Troponin T, 32 kDa Tropomyosin (α + β), 24 kDa Troponin I, and 18 kDa Troponin C. Protein concentration was measured with the Precision Red™ Protein Assay Reagent (Cat.# ADV02). SeeBlue molecular weight markers (Lane 1, Life Tech Inc.)

Biological Activity Assay

The biological activity of the SMTFC can be determined from its ability to regulate myosin ATPase activity. The SMTFC re-creates coated filaments which are analogous to the thin filaments of muscle fibers. Myosin is added in substoichiometric amounts and the reaction is initiated with ATP and calcium. There is a small amount of calcium present which must be complexed with 2.5 μ M EGTA prior to starting the reaction, then in the absence of exogenous calcium, SMTFC completely inhibits myosin ATPase. On addition of 10 μ M calcium, myosin ATPase will be restored. Calcium binds to Troponin C which dissociates from F-actin, allowing myosin to bind.

Reagents

1. Skeletal Muscle Thin Filament Complex (1 x 1 mg, # TFC02)
2. Skeletal Muscle Myosin S1 (0.25 mg, # MYS04)
3. ATPase Assay Biochem Kit (Cat. # BK051)
4. 100 mM ATP in 50 mM Tris-HCl pH 7.5 (100 μ l)
5. PM12 Reaction buffer (12 mM Pipes-NaOH, pH 6.8, 2 mM MgCl₂).
6. 250 μ M EGTA solution in nanopure water.

Equipment

1. Spectrophotometer capable of measuring absorbance at 360 nm (+/- 5 nm bandwidth). We recommend a Spectra-Max M2 (Molecular Devices). Filter based machines are

not suitable.

- Half area 96 well microtiter plate (Corning Cat.# 3696 or 3697)
- Multi-channel pipette

Method

The following major steps are recognized:

- Step 1. Assemble required reagents and compounds (30 min).
- Step 2. Prepare Thin Filament stock (15 min)
- Step 3. Prepare Motor Mix and plate reader (15 min).

Step 4. Pipette Motor Mix into wells and start reaction/plate reader (10 min).

Thin Filament stock

- Gently resuspend 1 x 1 mg TFC02 with room temp PM12 buffer to 2 mg/ml; it will be a white solution (500 ul per vial for 1 mg vial). Do not pipette vigorously.
- Incubate at RT for 10 min.
- Centrifuge at 500 x g for 30s; now it is a semi-clear solution.
- Store at room temperature for up to 20 min.

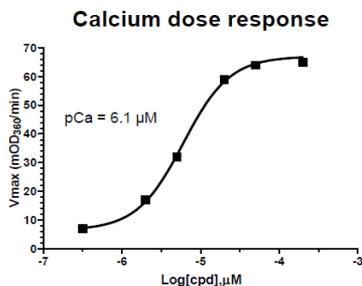
Myosin reaction stock

- Dilute S1 myosin to 1.0 mg/ml with ice cold PM12 buffer.
- Mix the following in the stated order at RT, to make 4.0 ml of Myosin/Thin Filament control mixture:
 - 2610 μ l of PM12
 - 800 μ l of 5x MSEG (this is a BK051 component)
 - 40 μ l of 250 μ M EGTA solution.
 - 500 μ l of TFC02
 - 30 μ l of Myosin S1 solution
 - 20 μ l of 100 mM ATP
 - 40 μ l of 100x PNP (this is a BK051 component)
 - Shelf life 20 min at RT.

- Using the pre-warmed half area 96-well plate, pipette the Following for Enhancer detection:
 - Pipette 10 μ l of 100 μ M calcium chloride into "activated" wells.
 - Pipette 10 μ l of nanopure water into "non-activated" wells.
 - Pipette 10 μ l of 10 x [test compound] into appropriate wells.
 - Incubate at 37°C for 2 min to warm the mixture.
 - Pipette 100 μ l of Myosin/Thin Filament mixture into all wells.
 - Start protocol, 41 readings, 30 seconds apart, 37°C, OD 360 +/- 5 nm.
 - Calculate Vmax and compare non-activated to calcium activated samples.

Note: For Inhibitor detection add 10 or 20 μ M calcium chloride to every well apart from the nanopure water control wells.

Figure 3: Calcium Dose Response Curve



Legend: The ATPase assay was set up as described in the protocol above. Calcium was titrated between 2 and 200 μ M and the results plotted on this dose response graph. pCa = 6.1 μ M is similar to published pCa values for reconstituted cardiac sarcomeres (Holroyde et al. 1980, Fig.6). The pCa of different batches will vary between 3.0 and 7.0 μ M.

Product Uses

- Measurement of calcium activated myosin ATPase activity when bound to thin filaments.
- Identification/characterization of proteins or small molecules that affect the SMTFC regulation and myosin ATPase activity
- Identification/characterization of proteins or small molecules that affect myosin / F-actin interaction

References

- J.M Murray. 1982. Hybridization and reconstitution of the thin filament. *Methods Enzymol.* 85: 15-17.
- M.J. Holroyde et al. 1980. The calcium and magnesium binding sites on cardiac troponin their role in the regulation of myofibrillar adenosine triphosphatase. *J. Biol. Chem.* 255: 11688-11693.

Product Citations/Related Products

For the latest citations and related products please visit www.cytoskeleton.com.