Preparation of native titin from rabbit *longissimus dorsi*

(Based on Soteriou, A., Gamage, M, and Trinick, J. *J. Cell Sci.* **104**, 119-123, 1993)

Number:

I. Solutions

A. Homogenization buffer	**500 ml**	**1000 ml**	**2000 ml**
50 mM KCl	1.864 g or 10 ml stock (2.5 M)	3.728 g	7.456 g
5 mM EGTA	5 ml stock (0.5 M)	10 ml (0.5 M)	20 ml (0.5 M)
1 mM $NaHCO_3$, pH 7.0	1 ml stock (0.5 M)	2 ml (0.5 M)	4 ml (0.5 M)
0.01% NaN_3	0.5 ml stock (10%)	1 ml (10%)	0.2 g or 2 ml (10%)
Add fresh:			
1 mM DTT	0.5 ml stock (1 M)	1 ml (1M)	2 ml stock (1 M)
1 mM PMSF	2.5 ml stock (0.2 M in EtOH)	5 ml (0.2 M)	10 ml stock
20 μg/ml Trypsin inhibitor	10 mg	20 mg	40 mg
40 μg/ml Leupeptin	1 ml stock (20 mg/ml) =10 aliq.	(10 aliq.)	
20 μM E-64	1 ml stock (10 mM) =10 aliq.	(10 aliq.)	

*Note: prepare 2000 ml's. Take out 500, and complete with E-64 and Leupeptin. To the remaining 1500 ml add 6 aliquots Leupeptin (8 μg/ml) and 6 aliquots E-64 (4 μM).

B. Extraction Buffer	**250 ml**
10 mM Imidazole-HCl, pH 7.0	2.5 ml stock (1 M stock solution)
0.9 M KCl	16.776 g
2 mM $MgCl_2$	0.5 ml stock (1 M)
2 mM EGTA	1 ml stock (0.5 M)
0.01% NaN_3	0.25 ml stock (10%)
Add fresh:	
1 mM DTT	250 μl stock (1 M)
1 mM PMSF	2 ml
40 μg/ml leupeptin	500 μl stock (20 mg/ml) -> 5 aliquots
30 μM E64 (MW=357.4)	500 μl stock (10 mM) -> 5 aliquots
40 μg/ml Trypsin inhibitor	10 mg

C. Precipitation buffer (fresh)	**3000 ml**	**1500 ml**
0.1 mM $NaHCO_3$	0.6 ml stock (0.5 M)	0.3 ml (0.5 M)
0.3 mM DTT	0.9 ml stock (1 M)	0.45 ml (1 M)
0.1 mM EGTA	0.6 ml stock (0.5 M)	0.3 ml (0.5 M)
2 μg/ml Leupeptin	0.3 ml stock (20 mg/ml) -> 3 aliquots	1.5 aliq.
1 μM E-64	0.3 ml stock (10 mM) -> 3 aliquots	1.5 aliq.

D. Chromatography Buffer	**2000 ml**	**1000 ml**	**500 ml**	**400 ml**
30 mM K-phosphate, pH 7				
0.6 M KCl	89.472 g	44.736 g	22.368 g	17.894 g

Preparation:

1. Prepare 30 mM KH_2PO_4 and K_2HPO_4 buffers separately:	**2000 ml**	**1000 ml**
a. 30 mM KH_2PO_4	8.165 g	4.0825 g
b. 30 mM K_2HPO_4	10.451 g	5.2255 g

2. Mix the two so that a pH of 7 is reached. (Lot of dibasic will be needed!).
3. Add KCl to the pH-7 phosphate buffer.

Add fresh:

1. For elution:	**500 ml**
0.1 mM EGTA	0.1 ml (0.5 M)
0.3 mM DTT	0.15 ml stock (1 M)
2 μg/ml Leupeptin	50 μl stock (20 mg/ml) -> 0.5 aliq.
1 μM E-64	50 μl stock(10 mM) -> 0.5 aliq.
0.01% NaN3	0.5 ml stock (10%)
0.05% Tween-20	2.5 ml stock (10%)

2. For solubilization of titin pellet:	**10 ml**
1 mM EGTA	20 μl stock (0.5 M)
1 mM DTT	10 μl stock (1 M)
20 μg/ml Leupeptin	10 μl stock (20 mg/ml)
10 μM E-64	10 μl stock (10 mM)

0.01% NaN3	10 μl
0.05% Tween-20	50 μl

E. Distilled water: **4000 ml** Chill overnight!

F. Grinder & Homogenizer soaking solution **2000 ml**

1 mM EDTA (pH 7.4)	4 ml (0.5 M stock)
0.1 mM PMSF (add fresh)	1 ml (0.2 M stock)

G. Inhibitor Cocktail for titin storage (fresh)

for 0.1 % NaN_3	400 μl stock (10 %)
for 40 μg/ml Leupeptin	80 μl stock (20 mg/ml)
for 20 μM E-64	80 μl stock (10 mM)

(add 14 μl to each 1-ml fraction)

II. Preparation steps: Check:

1. Very quickly excise rabbit back muscle. _____

*Rabbit anaesthesia:

Rabbit weight:	Volume of added anaesthetics
35 mg/kg Ketamin (Calypsol)	(3 ml typ)
7.5 mg/kg Xylozine	(0.5 ml typ)
0.02 mg/kg Atropin	(0 typ)

In deep anaesthesia of the rabbit, incise carotids and bleed rabbit. Soak fur on back of rabbit with cold water. Make incision along spine. Expose back muscles, remove fascia, peel off longissimus dorsi. Put muscle immediately in pre-chilled, pre-washed/soaked electric grinder.

2. Grind and weigh muscle quickly. Weight of muscle (typ. 111 g): _____
Homogenization buffer volume (3 x g-muscle) (typ. 333 ml):
Homogenize in 1-l beaker, on ice (ULTRA-TURRAX). _____

3. Wash-centrifuge myofibrils in Homogenization buffer 3 times (2000g) _____
(three tubes in Sorvall GSA rotor, 4500 rpm, 10 min, 0 ˚C)
Buffer volume (3 x g-muscle) [typ. 111 ml/each of three tubes]:

4. Suspend final pellet in Extraction solution (2 x g-muscle) _____
(Do not remove pellet from centrifuge tube.)
Buffer volume [typ. 74 ml/each of three tubes]:

5. Extract for 5 minutes while stirring. Do procedure in Centrifuge tube! _____
6. Centrifuge 20,000g, 30 min.(Sorvall GSA rotor, 12,000 rpm, 30 min, 0 ˚C) _____
7. Supernatant diluted 3 times (to achieve 0.2 M ionic strength) _____
Supernatant volume (typ ~90 ml):
Volume of added Precipitation solution (typ ~180 ml):
Total volume (typ ~270 ml):
*Note: optionally, filter supernatant through gauze to get rid of fat particles.

8. Incubate on ice for 1 hour _____
9. Centrifuge, 20,000g, 30 min (sediment myosin) _____
(Sorvall GSA rotor, 12,000 rpm, 30 min, 0 ˚C)
10. Supernatant diluted 5 times (final ionic strength 0.05 M) _____
Supernatant volume (typ ~245 ml):
Volume of added Precipitation solution (typ ~980 ml):
Total volume (typ ~1225 ml):

11. Incubate on ice for 40 minutes _____
12. Centrifuge, 10,000g, 30 min (Sorvall GSA rotor, 8000 rpm, 30 min, 0 ˚C) _____

13. Titin pellet solubilized in Chromatography buffer

Volume of Chromatography buffer (typ ~700 μl/tube+2x700 μl wash):
Use plastic transfer pipet to softly dislodge pellet. Pellet is on side of tube(!)
Add as much chromarography buffer so that OD is about 15.

14. Centrifuge, 25,000g, 30 min (Sorvall UC, 17,000 rpm, 30 min, 0°C) _____
Supernatant volume (typ ~4 ml):

15. Supernatant loaded onto Sepharose CL-2B column. _____
Column: Bio-Rad 1x120 cm, max. vol. 103 ml
Chromatography Conditions:

a. Sample loaded onto column (typ in two 2.0-ml turns, labelled as #a and #b):
-Volume (typ 2.0): #a: #b:
-OD (typ ~18/ml):
b. Flow rate(Typ 0.2 ml/min; 80 setting on Gilson pump using narrowest tube):
c. Fraction size (Typ 1ml):
d. Elution/fraction collection:
-Void volume (Typ 20 ml): #a: #b:
-Paper speed: (Typ 0.2 mm/min):
-AUFS (Typ 0.2):
e. Elution profile:
Abs_{280}:
-UV-Monitor: attach records
-Spectrophotometer (260 nm, 280 nm): attach records

16. SDS-PAGE:
Gel type: Fairbanks (Typ 2.5-12%). Note: FBX denotes Fairbanks solubilization sol.
Sample preparation:
a. Standard: Rabbit LD
10 μl LD
+90 μl 1xFBX (incubate on 65 °C for 45 s)
Load 3-5 μl on gel.
b. Titin fractions:
-10 μl sample + 5 μl 3xFBX + 35 μl 1xFBX (incubate on 65 °C for 45 s).
-10 μl of the above mixture + 90 μl 1xFBX (incubate on 65 °C for 45 s).
Load 20 μl on gel.

[17. Optionally, pool titin peak. Use in *in vitro* motility assay**]**

18. Store titin:
To the chromatography fractions, add:
-0.1% NaN3
-40 μg/ml Leupeptin
-20 μM E-64
Then, a. Quick-freeze in liquid nitrogen, store at -80 °C
or, b. Store some on ice for a few days.

III. Notes, Remarks, Modifications: