Purification and Refolding of mini-TGF-b2-7m (7m)

All steps are scaled for a 6L prep!

**1. Cell culture**

1. On the day before culturing the cells:

a) Prepare 6L 2X LB medium (20 g tryptone, 10 g yeast extract, 10 g NaCl, adjusted to pH 7.4).

b) 7m plasmid (gBlock 3 clone) is transformed into EMD BL21(DE3) cells and plated onto LB plates with 50 ug/mL carbenicillin (at around 5 – 6 PM).

2. Next morning, carbenicillin is added to the six 1 L LB cultures to 50 ug/mL and after mixing, 12 mL of medium is pipetted onto the plate of transformed cells. The colonies are then suspended by using an EtOH-sterilized glass spreader and the suspended cells are taken up in the same pipet and 1.5 mL of the suspended cells are added to each culture.

3. Cells are cultured at 37 °C at 250 rpm (non-baffled) or 190 rpm (baffled).

4. Protein production is initiated when the A600 reaches 0.6 by adding 200 mg solid IPTG per 1L of cells.

5. Cells are cultured an additional 6 h (or overnight) at 37 °C and then pelleted by centrifuging at 6 krpm in the JLA 8.1 rotor. Supernatant is removed and the pellets are stored at -20 °C (can store at 4 °C IF you will be proceeding to isolate the inclusion bodies the following morning).

**2. Recover non-soluble, monomeric mmTGF-b2-7M**

1. Resuspend cell pellet (use tissue grinder) in 200 ml disruption buffer: 100 mM Tris, 10 mM EDTA, pH 8.0.

2. Sonicate in 30 ml batches for 5.0 min at power level 8 at 50% duty cycle (20 s on, 20 sec off) two times, then centrifuge in 250 ml bottle for 20 min at 15,000 rpm (JLA 16.25 rotor). Note: supernatant will be very cloudy … this is from suspended lipids, which are difficult to pellet (so don’t worry about cloudiness or the rather slimy interface on the pellet .. these are all lipids and can be safely removed … protein inclusion bodies are very dense and will be at the bottom of the pellet).

3. Resuspend pellet (using tissue grinder) in 200 ml disruption buffer containing 1M NaCl, then centrifuge as before.

4. Resuspend pellet (using tissue grinder) in 200 mL disruption buffer containing 1% Triton X-100 (v/v), then centrifuge as before.

5. Resuspend pellet (using tissue grinder) in 150 mL solubilization buffer: 8M Urea, 1 mM EDTA, 25 mM Tris, pH 8.0. Stir in centrifuge bottle with cap screwed tight overnight at room temperature.

6. In the morning, reduce the protein by adding 50 mM DTT and stirring at room temperature for one hour. Then add 20 mM NaOAc and lower the pH to 4.2 using acetic acid. You will notice the formation of a slimy precipitate in addition to the unsolubilized cell debris. Centrifuge in 250 ml bottles at 13,000 rpm for 20 minutes to remove this junk. The supernatant is purified by SP Sepharose.

**2. SP sepharose (cation exchange) purification**

1. Equilibrate a 35 – 40 mL SP sepharose column in 8M urea, 20mM NaOAc, 1 mM EDTA, pH 4.2.

2. Load the solubilized protein and then follow with equilibration buffer until it returns close to the original baseline (save load and wash in case some of the protein does not bind).

3. Elute the bound 7m by running a linear gradient to 100%B over 190 mL (collecting 1.5 mL fractions); buffer B = 8 M urea, 20 mM sodium acetate, 0.5 M NaCl, pH 4.0.

4. Pool fractions corresponding to the eluted 7m and measure the protein concentration via the A280 (see below for extinction coefficient and MW).

5. Dialyze 3X versus 4L 100 mM Acetic acid. It is critical to maintain low pH so that the protein remains reduced before folding.

**3. Refolding**

1. Add concentrated fractions dropwise to enough 100mM Tris, 30mM CHAPS, 1M NaCl, 5 mM reduced glutathione and 20% (v/v) DMSO pH 9.5 that has been pre-chilled (and pH adjusted) at 4 °C. Stir gently during the addition and ensure that the final 7m concentration is 0.1 mg/ml or lower. The pH should be monitored during addition so that its kept between 9.65 and 9.75. Once the addition is complete lower the pH to 9.50.

2. Continue stirring for 24-48 hours uncovered at 4°C

3. Concentrate using the Millipore TFF concentrator to bring the volume down to about 150 mL; remove the protein from the tubing and then rinse 2 X w/ 150 mL of eluate.

4. Transfer to multiple 400 mL Amicons and concentrate the folding mixture to approximately 150 mL total.

5. Dialyze 3X versus 4L 100 mM HOAc @4°C

6. Centrifuge 15 min. at 15,000 rpm in the JLA 16.25 rotor and pass through 0.45 uM syringe filter.

**4. HPLC Purification of the Dimer**

1. Equilibrate a Source 15S HR 10/10 column with 20mM NaOAc, 30 % isopropyl alcohol, pH 4.0 (Buffer A) at a flow rate of 2.0 mL/min.

2. Load 50 mL of protein and wash column with 25 mL of Buffer A.

3. Elute with a linear gradient over 150 mL from 0% to 40% BufferB (Buffer A + 1M NaCl). Collect 1.5 mL fractions.

4. Collect peak fractions and check by non-reducing SDS-gel

5. Pool fractions corresponding to purified 7m and dialyze 3X versus 4L 100 mM HOAc @4°C.

CLUSTAL O(1.2.1) multiple sequence alignment

7m MALDAAYCFRNVQDNCCLRPLYIDFRKDLGWKWIHEPKGYNANFCAGACPYRASKSPSCV

wt MALDAAYCFRNVQDNCCLRPLYIDFKRDLGWKWIHEPKGYNANFCAGACPYRASKSPSCV

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7m SQDLEPLTIVYYVGRKPKVEQLSNMIVKSCKCS

wt SQDLEPLTILYYIGNTPKIEQLSNMIVKSCKCS

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7m:

MALDAAYCFR NVQDNCCLRP LYIDFRKDLG WKWIHEPKGY NANFCAGACP YRASKSPSCV 60

SQDLEPLTIV YYVGRKPKVE QLSNMIVKSC KCS 93

MW 10618.326 g/mol

Extinction coefficient estimated by the method of Gill and von Hippel (*Analytical Biochemistry*, **182**: 319-326, 1989)  
where lyophilized proteins were used to establish an absorbance curve based on the number of tyrptophans, tyrosines, and disulfide bonds. Units are in M-1 cm-1.

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| Wavelength | Molar Extinction w/o Disulfides | Molar Extinction w/ All Disulfides |
| 278 | 19600 | 20108 |
| 279 | 19390 | 19870 |
| 280 | 19060 | 19540 |
| 282 | 18400 | 18800 |

SP-sepharose pre-purification in 8 M urea (Run on Akta Prime):



Source S final purification (Run on Akta HPLC):

