LmrR extraction and purification

**Materials**

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| **Name** | **Final volume** | **Ingredients** | **Storage** | **Note** |
| Sodium phosphate buffer (NaPi) | For 1 L  (with mQ) | Na2HPO4\*7 H2O  (50 mM) 🡪 13.4 g  NaCl (150 mM) 🡪 8.8 g  Adjust pH to 8.0 | Fridge 4 °C | *N.B.: In case proteolysis is observed, sometimes autoclaving the buffer prior use could solve it.* |
| Lysis buffer | 50 mL  (with NaPi pH 8) | PMSF (100 µM) 🡪 500 μL from stock solution  **OR**  1 tab of protease inhibitor cocktail  **AND / OR**  1 mM EDTA (from 200 mM stock, stored in -20 °C) | Always prepare fresh | *N.B.: PMSF and EDTA are cheaper alternatives to the protease inhibitor cocktail and work just as well. We work with BL21(DE3) cells which are also deficient of proteases, so in principle it could also be possible to avoid using them altogether* |
| Elution buffer (EB) | 20 mL for each Strep-column (with NaPi pH 8) | D-desthiobiotin  (5 mM, MW=214 mg/mmol)🡪 X mg  After adding, set pH to 8.0  (pH drops from 8.0 to ~7.3 after addition of D-desthiobiotin) | Always prepare fresh | *Example: 4 Strep columns of 3 mL each. Prepare 4 x 20 mL EB = 80 mL. To get to 5 mM D-desthiobiotin, dissolve 5 mmol / L x 0.08 L x 214 mg / mmol = 85.6 mg. Then set pH to 8.* |
| IF DIALYSIS:  Sodium phosphate buffer (NaPi)  pH 7.5 | 2 x 1L  (with mQ) | As above.  Adjust pH to 7.5 | Fridge 4 °C |  |
| IF REDUCTION:  TCEP 100 mM | 10 mL  (with NaPi pH 8) | TCEP (100 mM) 🡪 250 mg  Adjust pH to 8.0 | Freezer  -20°C | *N.B.: Avoid freeze-thawing cycles of aliquots. Always trash the leftovers of a used aliquot.* |

**Tip:** Remember to book in advance the Strep-tactin columns, sonicator and centrifuges! Make sure you have concentrators and desalting columns available.

1. **Protein extraction (sonication)**
2. Resuspend the cell pellet in lysis buffer, roughly 2 mL buffer for 50 mL cell culture;
3. From this moment on keep the tubes always on ice;
4. Sonication on ice-water for 7 min, pulse: 10 sec on and 15 sec off, at 70% amplitude ;

**Tip:** Use the 6 mm tip (the big one) if the resuspension volume is >10 mL, place it 1 cm above the bottom of the tube. Program lasts 17.5 min (7 min on, 10.5 min off).  
**Tip2**: Typically you’ll see the cell suspension turn from completely turbid and white/yellow, to a slightly more transparent yellow/brown after the sonication.

1. **Purification**
2. Centrifuge at 12 000 rpm, 45-60 min, 4 °C;

In the meantime, equilibrate a 3 mL Strep-Tactin column washing with 2x CV with NaPi pH 8 (usually it’s already done).

**Tip:** Use 2 columns of 3 mL each for 500 mL culture (column capacity is roughly 2 mg of protein/ml)

1. Filter supernatant using a 0.45 μm filter;
2. Pour it in the column and close its top and bottom (parafilm or cap);
3. Incubate for 30 min at 4 °C on mixing plate;
4. Collect the Flow Through Fraction;
5. Wash with 3x 2 CV with NaPi pH 8 (e.g. 3 x 4 mL if using 2 mL column);
6. Elute with 5x 1 CV with EB (e.g. 5 x 2 mL if using 2 mL column) and collect the 5 Elution Fractions (keep them always on ice);
   * Alternatively, make fractions of 2 mL (regardless of CV), using 2 mL eppendorf tubes and pipetting 2 mL of EB onto the column to collect. Repeat 5x for each column (10 mL EB / column).
7. Measure Absorbance of each fraction with nanodrop at 280 nm;
8. Save the fractions that have 260:280 ratio <1 and Abs280 ≥ 1;
9. Concentrate the eluate with filter units with 5 or 10 kDa cut-off at 4000 rpm 10 min (if filter unit is new, make a run with only mQ H2O to rinse the membrane prior your sample);
10. Discard the flowthrough and mix with the pipette the solution inside the filter;
11. Repeat from step 10 until volume is ~1-2 mL;
12. Transfer in Eppendorf and check final concentration with nanodrop;
13. If you need reduction go to section 4;
14. Wash the filter unit used with water, then put ethanol 20% inside and outside the membrane and store in fridge.
15. **Column regeneration**
16. Flush with 3 CV of EB;
17. Pour 5 CV of regeneration buffer (10X diluted orange HABA buffer), the column must show a homogeneous red/orange color;
18. Wash with 4 CV of NaPi **pH 10.5** until all the color fades out;
19. Wash with 5 CV of NaPi pH 8;
20. Fill the column with 3x CV of pH 8 NaPi;
21. Store the column in fridge with the buffer inside.
22. **Reduction of pAzF to pAF**
23. Add TCEP 100 mM pH 7-8 with 10x final dilution (10 μL for every 100 μL of solution);
24. Incubate at RT for 1.5 h;
25. Decide whether to do a desalting column (go to section 5) of dialysis (section 6);
26. **Desalting column (Cytiva PD10)**

**N.B.**: Large columns can accommodate 2.5 mL, the small ones 500 μL.

1. Equilibrate the column with NaPi pH 8 (at least 3x CV)
2. Add 2.5 mL (or 500 μL for small columns) of eluted LmrR and wait until it enters fully the column. If the volume is lower, top up with buffer.
3. Add 500 μL (or 100 μL for small columns) of new buffer (e.g. NaPi pH 7.5) and collect the fraction
4. Repeat 5 times step 30. Typically the protein elutes at the 2nd and 3rd fraction.
5. Measure protein concentration at nanodrop (ε280 = 53546 M−1 cm−1 for LmrR\_pAF);
6. Concentrate the protein until desired concentration is reached
7. Divide the protein in small aliquots and flash freeze in liquid nitrogen;
8. Store the aliquots in freezer (either -20°C or -70°C);
9. **Dialysis**
10. Place protein solution in dialysis bag (6-8 kDa cutoff);
11. Fill a bottle with 1000x protein volume with NaPi pH 7.5 (e.g. if 1 mL of protein, use 1 L of buffer) and soak the bag into it;
12. Incubate at 4 °C (cold room) while stirring;
13. Change the buffer after some hours (at least 2-3 h) with an identical volume as previously
14. Leave the second bottle ON;
15. Measure protein concentration (ε280 = 53546 M−1 cm−1 for LmrR\_pAF);
16. Concentrate the protein until desired concentration is reached
17. Divide the protein in small aliquots and flash freeze in liquid nitrogen;
18. Store the aliquots in freezer (either -20°C or -70°C).

**Notes**

1. In the rare case that you purify LmrR wild-type (without the lysine mutations K55D K59Q), for instance for crystallography, be sure to add DNAse + 1 mM MgCl2 or Denerase after the sonication step. LmrR (without K55D\_K59Q) binds a lot of DNA which would impair its activity.