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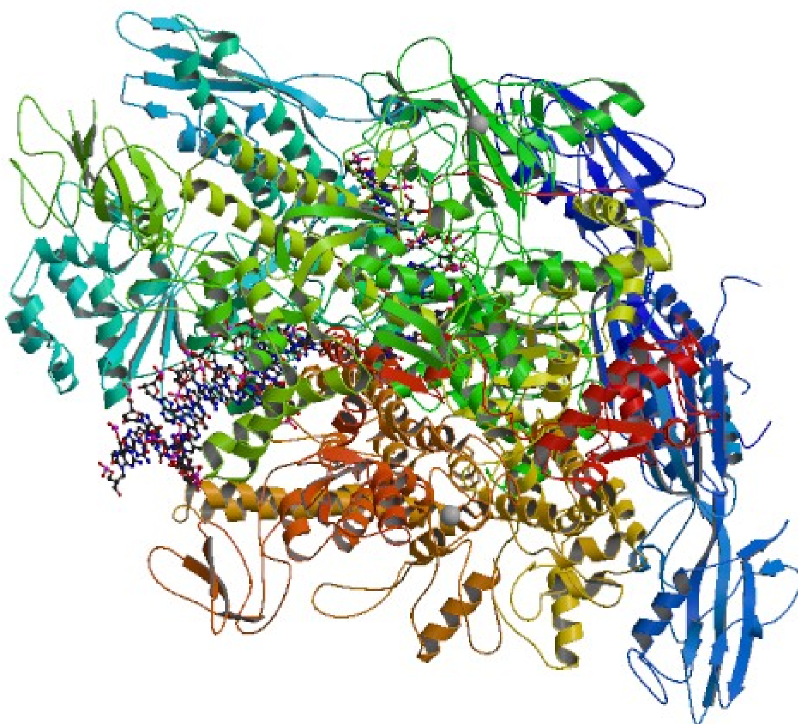
High Purity | High Activity | High Yield

CL7/Im7 Expression & Purification Protocol:

DNA-/RNA-Binding Proteins



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Introduction

TriAltus's proprietary high purity, high activity, and high yield (HHH) purification method is based on the ultrahigh affinity interaction between CL7 – a variant of Colicin E7 DNAse (CE7) that lacks DNAse and DNA-binding activity – and Immunity Protein 7 (Im7), which inhibits CE7. CL7 binds Im7 with a K_d of ~10^{-14} – 10^{-17} M.

With the TriAltus CL7/Im7 purification method, the target protein is expressed with an N- or C-terminal CL7 tag. The CL7-tagged protein is then loaded in high-salt buffer onto an Im7 column. CL7 stably binds the covalently crosslinked Im7 on the column's agarose beads with high affinity. Finally, the target protein ("T" in **Fig. 1**) is released from the CL7 tag via proteolytic cleavage

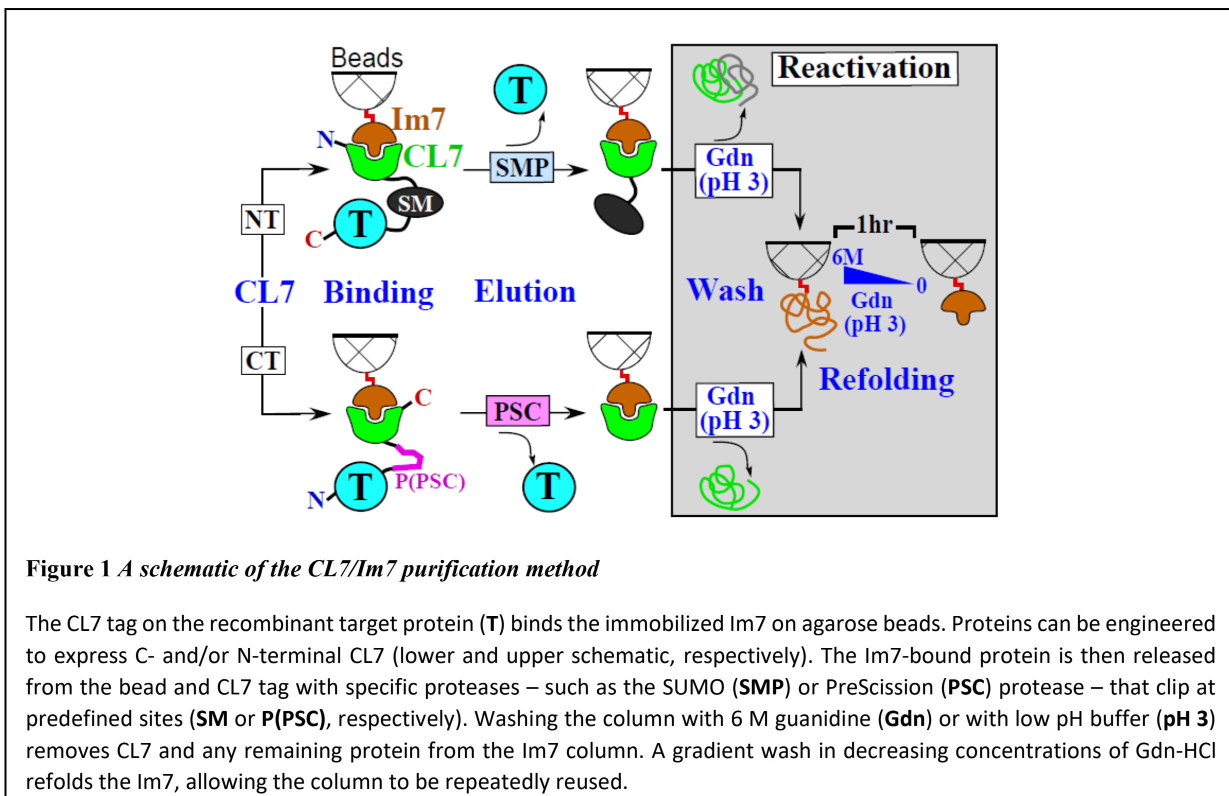


Figure 1 ***A schematic of the CL7/Im7 purification method***

The CL7 tag on the recombinant target protein (**T**) binds the immobilized Im7 on agarose beads. Proteins can be engineered to express C- and/or N-terminal CL7 (lower and upper schematic, respectively). The Im7-bound protein is then released from the bead and CL7 tag with specific proteases – such as the SUMO (**SMP**) or PreScission (**PSC**) protease – that clip at predefined sites (**SM** or **P(PSC)**, respectively). Washing the column with 6 M guanidine (**Gdn**) or with low pH buffer (**pH 3**) removes CL7 and any remaining protein from the Im7 column. A gradient wash in decreasing concentrations of Gdn-HCl refolds the Im7, allowing the column to be repeatedly reused.

The TriAltus system outperforms His-trap and other traditional purification methods in efficiency and purity.

1. **Efficiency**
   In a single-step, the CL7/Im7 method purifies many difficult-to-isolate proteins that elude traditional multistep methods.

2. **Purity**
   Compared with other tagging systems, the CL7/Im7 method significantly increases the purity level of the end product and maintains or improves the activity of the protein product. Protein purity improves because the Im7 interacts specifically with CL7; does not interact with untagged cellular components; and CL7/Im7 binding is not affected by widely used protein purification reagents, such as polyethyleneimine (PEI), detergents, reducing agents (β-mercatoethanol, DTT, TCEP) and metal chelating agents (EDTA).

1. Plasmid construction and preparation

TriAltus's CL7-tagged template vectors are derived from the commercial pET28a expression vector (Invitrogen). The CL7 vectors contain either an N- or C-terminal CL7 tag and may include additional tags, such as His.

1. To insert a coding nucleotide sequence into a TriAltus vector, simply use the appropriate restriction sites, according to the plasmid's respective Genebank file on the product page. Transform a cloning strain such as DH5α to make DNA preps and verify the insert sequence.
2. The resulting expression plasmids may be transformed into BL21 (DE3) (Invitrogen) competent cells or their derivatives.
3. Culture the cells according to the manufacturer's instructions.

Note 1.1 | The target nucleotide sequence may require optimization – such as reducing the codon G/C content to ~43-45% – for expression in *E. coli*. Original, non-optimized codons inserted into vectors – especially those that encode N-terminal CL7 tags – may significantly decrease the expression level of the full-length protein.

Note 1.2 | Compared with BL21 (DE3) cells, BL21 Star™ (DE3) (Invitrogen) competent cells generally increase target protein expression.

2. Cell culture and lysis

Use the BL21 (DE3) expression strain culture from section 1 with the following expression and lysis protocols.

Cell culture

1. Plate agar Petri plates with enough culture to produce 100-200 colonies on the plate.
2. Grow colonies overnight at 37°C (~17 h).
3. The next day, scrape the colonies from the plate into a 1L flask containing 0.5L TB media (for 2 plates, use 1L media, etc.).
4. Rinse the agar with a small amount of additional media and scrape any remaining culture into the flask.
5. Grow bacteria in a shaking incubator at 37˚C and 200 rpm until the OD_{560} of the culture reaches ~0.7-0.8 (~2-2.5 h).
6. Reduce the temperature to 18-20˚C and continue to incubate until the OD_{560} reaches ~1.
7. Add 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce overexpression.
8. Grow the cells overnight for ~20-24 h.
9. Centrifuge the culture at 4,000 *g* for ~30 min, decant the supernatant, and freeze the cell pellets at -80˚C.

Cell lysis

Note | *Different classes of proteins – namely DNA-/RNA-binding proteins and membrane proteins – require unique buffer modifications. Please refer to section 3 for specific lysis buffer recommendations.*

1. Suspend the frozen cell pellet from above in the appropriate lysis buffer (see table 3.1.1 and 3.1.2) at 1 g cells/10 mL buffer.
2. A. Use a high-pressure homogenizer at 4˚C and ~15,000 PSI for ~3 min (for ~3 g cells) to disrupt the cells.

   OR

   B. Use sonication to disrupt the cells. We recommend the following parameters with the Fisher Sonic Dismembrator Model 500 (500 W power):

   a. For 4 g of cells
      - Suspend cells in 40 mL of lysis buffer in a conical Falcon tube on ice
      - Use 58% power amplitude, 5 sec pulse/15 sec pause for 20 min

   b. For 100 g of cells
      - Suspend cells in 1 L of lysis buffer on ice

- Use 58% power amplitude, 5 sec pulse/15 sec pause for ~110 min
- Use a magnetic mixer to uniformly cool the lysate during sonication

3. Centrifuge the lysates at 40,000 g for 20 min.
4. Filter the lysate through a 45-µm filter.
5. Load the lysate onto an equilibrated Im7 column, according to the protocol in section 3.

Note 2.1 | In ~90% of cases, the above protocol resulted in the best cell growth and protein expression. If poor cell growth or expression is observed, consider modifying the protocol as necessary. For example, increase the IPTG concentration in step 7 to 1 mM and/or the temperature in step 6 to 30-37˚C.

3. Protein purification of DNA-/RNA-binding proteins

DNA-/RNA-binding proteins exhibit a strong nonspecific affinity for nucleic acids (NAs). If the CL7-tagged target is a DNA-/RNA-binding protein, the end eluate may be highly contaminated with NAs and NA-binding cellular proteins.

To avoid these potential impurities, consider one of the following lysate preparation/loading techniques:

(i) Use a high-salt loading buffer (for a sample protocol, see section 3.1.1 below)

(ii) Treat the lysate with DNAse or PEI in low-salt buffer and then use a high-salt loading buffer (for a sample protocol, see section 3.1.1 below)

3.1 Purification Option 1 – High-salt loading

Protocol

1. Cell lysis – Follow the protocol in section 2, using the appropriate lysis buffer (**Table 3.1.1**); then, dilute the filtered lysate 2-fold by adding an equal volume of Loading buffer A.

2. Load the lysate onto the equilibrated Im7 column (buffer A) – Load lysate onto the Im7 column that has been equilibrated with 5-10 column volumes (CV) of Loading buffer A, using a flow rate of 1/10 – 1/4 (CV).

For example, use

- 0.2-0.3 mL/min for a 1-mL column
- 0.5-1.2 mL/min for a 5-mL column
- 2-4 mL/min for a 20-mL column

To improve binding capacity in gravity mode, where it might be difficult to control the actual flow rate, load the lysate repeatedly 2-3 times. For large lysate volumes that require long loading times, alternate between loading and a high-salt wash (buffer A1) (e.g., after each 30-min of loading, wash with high-salt buffer) to remove impurities from the column.

3. Wash the Im7 column with high-salt wash buffer (buffer A1) – Wash the Im7 column with 6-8 CVs of **high-salt** buffer A1.

*4. Wash the Im7 column with **no-salt** wash buffer (buffer A2)* – Wash the Im7 column with 6-8 CVs of **no-salt** buffer A2.

5. Repeat the wash cycles – Repeat Steps 3 and 4 above two to three times. On a chromatography system, the flow rate may be as high as the column pressure limits allow.

6. Apply protease to elute the target protein (buffer A3) – Equilibrate the column with 2-3 CVs of the buffer A3. Add the elution protease to buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in section 4.

7. Clean the Im7 column (buffer A4) – Wash the Im7 column with 8-10 CVs of denaturing buffer A4 to remove the bound CL7-tag.

- When using guanidine (Gdn) on a chromatography system, the flow rate may be as high as the column pressure limits allow.

- The low-pH, Gly buffer elutes the bound CL7-tag slowly and nonuniformly. We recommend a flow rate of 0.5-1 mL/min; the elution may take 1-1.5 h. In gravity mode, follow the protocol described in section 5.

8. Regenerate the Im7 column (buffer A4 & buffer B) – On a chromatography system, exchange denaturing buffer A4 with refolding buffer B using a 1-h gradient (e.g., flow rate – 1 mL/min; gradient volume – 60 ml). In gravity mode, follow the protocol described in section 5.

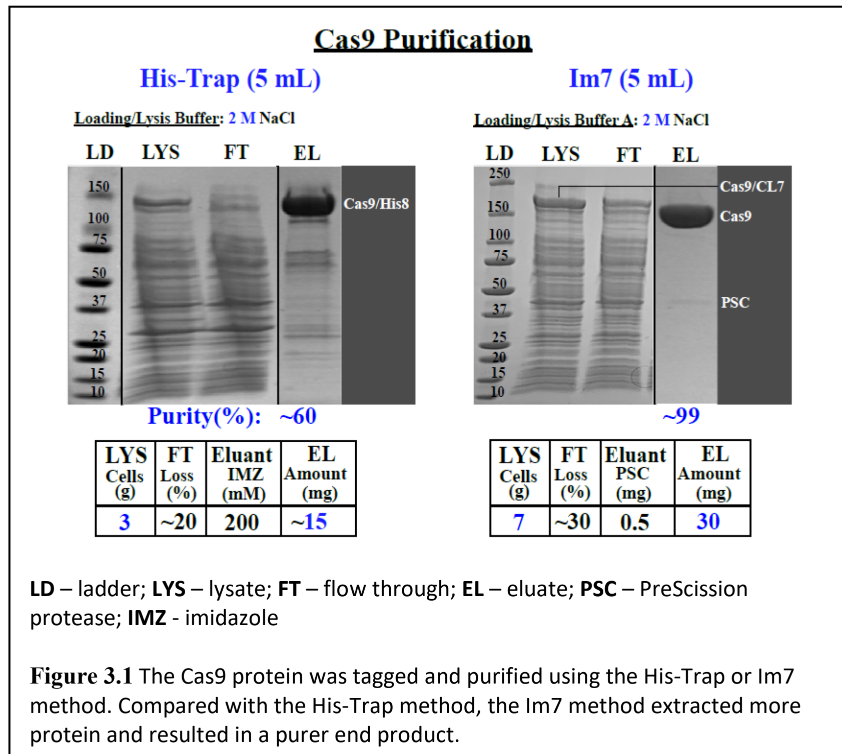
9. Final wash (buffer B) – Wash the regenerated Im7 column with 3-4 CVs of buffer B.

Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
1-2.5 M NaCl	1-2.5 M NaCl	2 M NaCl	20 mM Tris (pH 8)	0.2-0.5 M NaCl	6 M Gdn*** (pH 8)	0.5 M NaCl
20 mM Tris (pH 8)	20 mM Tris (pH 8)	20 mM Tris (pH 8)	5% Glycerol	20 mM Tris (pH 8)	OR	20 mM Tris (pH 8)
5% Glycerol	5% Glycerol	5% Glycerol		5% Glycerol	0.1 M Gly*** (pH 3)	5% Glycerol
0.1 mM PMSF				0.2 mM EDTA		
Inhibitory tablet(s)*				Elution Protease**		

Table 3.1.1 Buffers for high salt loading
* - add 1 tablet of inhibitory cocktail (Roche) for 30-40 ml of lysate
** - the details of the proteolytic elution are provided in Section 4
*** - Gdn - Guanidine Hydrochloride; Gly – Glycine

Example (Cas9)

The **Cas9** enzyme (MW ~144 kDa) is a key element of CRISPR gene editing technology, and its purity is directly correlated to CRISPR editing efficiency. Cas9 purity is especially important for trials that test the ability to edit genes in living human cells *in vivo*. Previously, extra-pure samples (99%) could only be obtained using a method with 4-5 chromatographic steps. With the CL7/Im7 technology, extra-pure samples of Cas9 were obtained in one step (**Fig. 3.1)** with activity that was practically identical to that of the samples purified by the conventional multistep techniques. For comparison, the His-Trap was run with essentially the same lysis/loading conditions and provided only ~60% sample purity (**Fig. 3.1**).



His-Trap (5 mL):

LYS Cells (g)	FT Loss (%)	Eluant IMZ (mM)	EL Amount (mg)
3	~20	200	~15

Im7 (5 mL):

LYS Cells (g)	FT Loss (%)	Eluant PSC (mg)	EL Amount (mg)
7	~30	0.5	30

LD – ladder; **LYS** – lysate; **FT** – flow through; **EL** – eluate; **PSC** – PreScission protease; **IMZ** - imidazole

Figure 3.1 The Cas9 protein was tagged and purified using the His-Trap or Im7 method. Compared with the His-Trap method, the Im7 method extracted more protein and resulted in a purer end product.

3.2 Purification Option 2 – DNAse treatment followed by high-salt loading

Protocol

1. Cell lysis – Follow the protocol in section 2, using the appropriate lysis buffer (**Table 3.1.2**). Then, continue according to the following protocol:

a. Incubate the filtered cell lysates for ~1.5-2 h at 4˚C in the DNAse-containing lysis buffer; add PMSF to 0.05 mM every 30 min.

b. Dilute the lysate 2 fold with 2x concentrated loading buffer so that the sample's final concentration matches that of high-salt buffer A.

2. Load the lysate onto the equilibrated Im7 column (buffer A) – Load lysate onto the Im7 column that has been equilibrated with 5-10 column volumes (CV) of Loading buffer A using a flow rate of 1/10 – 1/4 the column volume (CV).

For example, use

- 0.2-0.3 mL/min for a 1-mL column
- 0.5-1.2 mL/min for a 5-mL column
- 2-4 mL/min for a 20-mL column

To improve binding capacity in gravity mode, where it might be difficult to control the actual flow rate, load the lysate repeatedly 2-3 times. For large lysate volumes that require long loading times, alternate between loading (buffer A) and a high-salt wash (buffer A1) (e.g., after each 30-min of loading, wash with high-salt buffer) to remove impurities from the column.

*3. Wash the Im7 column with **high-salt** wash buffer (buffer A1)* – Wash the Im7 column with 6-8 CVs of high-salt buffer A1.

*4. Wash the Im7 column with **no-salt** wash buffer (buffer A2)* – Wash the Im7 column with 6-8 CVs of **no-salt** buffer A2.

5. Repeat the wash cycles – Repeat Steps 3 and 4 above two to three times. On a chromatography system, the flow rate may be as high as the column pressure limits allow.

6. Apply protease to elute the target protein (buffer A3) – Equilibrate the column with 2-3 CVs of the buffer A3. Add the elution protease to buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in section 4.

7. Clean the Im7 column (buffer A4) – Wash the Im7 column with 8-10 CVs of denaturing buffer A4 to remove the bound CL7-tag.

- When using guanidine (Gdn) on a chromatography system, the flow rate may be as high as the column pressure limits allow.
- The low-pH, Gly buffer elutes the bound CL7-tag slowly and nonuniformly. We recommend a flow rate of 0.5-1 mL/min; the elution may take 1-1.5 h. In gravity mode, follow the protocol described in section 5.

8. Regenerate the Im7 column (buffer A4 & buffer B) – On a chromatography system, exchange denaturing buffer A4 with refolding buffer B using a 1-h gradient (e.g., flow rate – 1 mL/min; gradient volume – 60 ml). In gravity mode, follow the protocol described in section 5.

9). Final Wash (buffer B) – Wash the regenerated Im7 column with 3-4 CVs of buffer B.

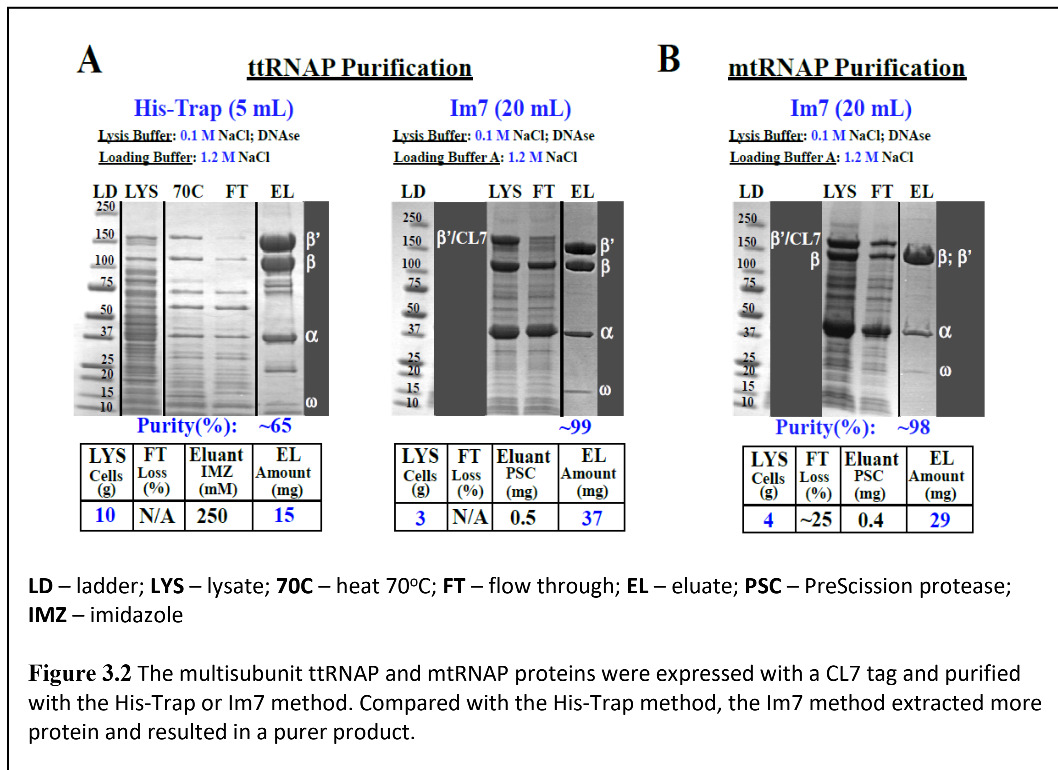
Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
0.1 M NaCl 20 mM Tris (pH 8) 5% Glycerol DNAse I* 10 mM $MgCl_2$ 0.5 mM $CaCl_2$ 0.1 mM PMSF Inhibitory tablet(s)*	1-2.5 M NaCl 20 mM Tris (pH 8) 5% Glycerol	2 M NaCl 20 mM Tris (pH 8) 5% Glycerol	20 mM Tris (pH 8) 5% Glycerol	0.2-0.5M NaCl 20 mM Tris (pH 8) 5% Glycerol 0.2 mM EDTA Elution Protease**	6 M Gdn (pH 8)*** OR 0.1 M Gly (pH 3)***	0.5 M NaCl 20 mM Tris (pH 8) 5% Glycerol

Table 3.1.2 Buffers for DNAse treatment with high salt loading
* - add 1 tablet of inhibitory cocktail (Roche) and ~120 mg DNAse Grade I (Roche) for 30-40 ml of lysate
** - the details of the proteolytic elution are provided in Section 3.4
*** - Gdn - Guanidine Hydrochloride; Gly – Glycine

Examples (ttRNAP and mtRNAP)

A. The core enzyme of RNA polymerase (RNAP) from T. thermophilus (**ttRNAP**) is a complex of five protein subunits (α_2, β, β', ω; MW ~400 kDa). Enzyme purity is essential for both activity and crystallization. Multisubunit RNAPs have at least 5 different DNA-/RNA-binding domains and therefore possess a strong nonspecific affinity for cellular NAs, resulting in significant NA and NA-binding protein contamination during purification. Traditionally, methods with 4-5 distinct chromatographic steps were required to obtain crystallization-quality RNAP samples. With the one-step CL7/Im7 method, an extra-pure (99%) sample of ttRNAP was purified (**Fig. 3.2A**) that was suitable for crystallization trials and exhibited high activity (Vassylyeva M.N. et al, (2017) Efficient, ultra-high-affinity chromatography in a one-step purification of complex proteins. *Proc Natl Acad Sci U S A, 114(26)*, E5138-E5147. doi: 10.1073/pnas.1704872114.). For comparison, the His-Trap method – with essentially the same lysis/loading conditions – provided only ~65% sample purity, even though the lysate was heated at 60°C prior to the His-Trap run to eliminate most of the *E. coli* proteins (**Fig. 3.2A**).

B. The core enzyme of RNAP from M. tuberculosis (**mtRNAP**) is a functional and structural analog of ttRNAP (α_2, β, β', ω; MW ~400 kDa). Despite the functional and overall structural similarity between mtRNAP and ttRNAP, these two multisubunit enzymes exhibit distinct structural and surface properties. To obtain high purity mtRNAP samples, methods used 4-5 distinct chromatographic steps that were unique from those used to purify ttRNAP. With CL7/Im7 technology, an extra-pure (~98%) sample of mtRNAP was obtained in one step using a protocol that is nearly identical to that of ttRNAP (**Fig. 3.2B**).



LD – ladder; **LYS** – lysate; **70C** – heat 70°C; **FT** – flow through; **EL** – eluate; **PSC** – PreScission protease; **IMZ** – imidazole

Figure 3.2 The multisubunit ttRNAP and mtRNAP proteins were expressed with a CL7 tag and purified with the His-Trap or Im7 method. Compared with the His-Trap method, the Im7 method extracted more protein and resulted in a purer product.

4. On-column proteolytic elution of a target protein

After loading and washing the Im7 column, the target protein is eluted with the appropriate protease according to the steps below.

Chromatography mode

1. Add elution protease (SUMO (SMP) or PreScission (PSC)) at ~1.5-3% of the expected amount of bound target protein (assuming a saturated column, or using an estimate from gel analysis) (e.g., 0.5 mg protease per 30-40 mg of target protein saturated on a 1 ml Im7 column).
2. Dilute the required amount of protease in 3-4 CVs of Elution buffer A3 plus the volume of the tubing through which the protease is loaded on a column.
3. Fill the loading tubing with the diluted protease at a fast flow rate (4-5 mL/min).
4. Reduce the flow rate to 0.05-0.1 CV/min.
5. Run the protease through the column slowly until the OD_{260} peak – which corresponds to the eluted target protein – returns to baseline.

   Note | Allowing the protease to incubate on the column for 20-30 min when the OD_{260} peaks may increase the concentration of the eluted protein. This may be repeated more than once per purification run.

Example

1. Use ~7 mL of tubing to load a 5-mL Im7 cartridge with the protease sample.
2. If the expected amount of bound protein is ~30-40 mg, use ~0.5 mg protease diluted in ~20 mL of elution buffer A3.
3. Load 7 mL protease at 5 mL/min to fill the tubing.
4. Reduce the speed to 0.2 mL/min.
5. Run the protease sample through the column (~1.5 – 2.5 hours) to elute the target protein.

   To improve the cleavage efficiency or to increase the concentration of the eluted target, consider interrupting protease loading for 20-30 min to give the protease more time to complete the on-column protein cleavage.

Gravity mode

1. Add elution protease (SUMO (SMP) or PreScission (PSC)) at ~1.5-3% of the expected amount of bound target protein as estimated above in the chromatography mode section (e.g., 0.5 mg protease per 30-40 mg of target protein).
2. After equilibrating the column with Elution buffer A3, drain the column but don't dry the beads entirely.

3. Dilute the required amount of protease in 0.5-1 CV of elution buffer.
4. Add the protease to the beads and incubate for ~1.5 – 2.5 h at 4°C, mixing the beads gently every 5-7 min.
5. To monitor protein elution, collect a 0.3-0.4 mL aliquot from the column every 20-30 min and check the sample concentration with a NanoDrop spectrophotometer. Return the aliquot to the column. When the protein concentration no longer significantly increases, the elution is complete.

   Columns typically have a "dead" volume (with no beads) – beneath the bottom frit – where cleavage will not occur. To avoid measuring the "dead" volume, return the first eluted aliquot to the column and collect and measure the next aliquot.

Note 4.1 | Several factors may reduce proteolytic efficiency, resulting in poor protein elution. Use the protocol below to determine the cause of low protein recovery and then modify the protocol according to the respective suggestions.

1. Strip the column with denaturing buffer A4 (~2CV).
2. Measure the eluate's $OD_{(260/280)}$ with a NanoDrop spectrophotometer
3. Evaluate a sample of the eluate on a gel using optimized conditions. (**Note /** Before loading the sample on a gel, Gdn should be exchanged for urea by dialysis, as Gdn precipitates when mixed with SDS).
   a. If the $OD_{(260/280)}$ ratio is 0.75 (or higher), or several protein bands are observed on the gel, then contamination ***(1)*** is the likely cause of poor elution efficiency.
   b. An $OD_{(260/280)}$ ratio of 0.7 (or less) AND a single dominant band on a gel corresponding to either uncleaved or cleaved target protein indicates that steric hindrance ***(2)*** or aggregation ***(3)***, respectively.

(1) Contaminants (in particular, NAs) may inhibit proteases during the on-column cleavage step. Increasing the salt concentration of Loading buffer A may reduce the number of contaminants and subsequently improve cleavage efficiency.

(***2***) The binding of target protein – especially large oligomeric proteins – to the column may create steric hindrance that impairs protease cleavage efficiency. Increasing the amount of protease (3-4 fold) in the reaction may improve protein elution. If additional protease is added, an additional purification step (for example, size exclusion, GST or His-Trap chromatography steps) may be required to remove excess protease from the purified, eluted target protein.

(3) An untagged (cleaved from a tag) target protein aggregates/precipitates. In this case, optimizing the Elution buffer conditions to improve solubility of the target protein may help. If it doesn't, the problem likely exists at, and should be resolved at, the upstream (expression) stage.

5. Cleaning & regenerating the Im7 column in gravity mode

Cleaning

The Im7 column can be cleaned with a guanidine- or glycine-based cleaning buffer (buffer A4) according to the following protocols.

Guanidine Cleaning Protocol

1. Remove Elution buffer A3 from the beads, but do not dry the beads entirely.
2. Add 4 CVs of denaturing buffer A4 (6 M Gdn) to the beads.
3. Equilibrate the column in buffer A4 for 4-5 min.
4. Remove the buffer.
5. Repeat steps 2 and 4 twice (skip the equilibration in step 3).

Glycine Cleaning Protocol

1. Remove Elution buffer A3 from the beads, but do not dry the beads entirely.
2. Add 4 CVs of denaturing buffer A4 (0.1 M Gly) to the beads.
3. Equilibrate the column in buffer A4 for 10-12 min.
4. Remove the buffer.
5. Repeat steps 2 and 4 five to six times (skip the equilibration in step 3).

Regeneration

The Gdn in the cleaning step denatures Im7. Before the column can be reused for another purification, the Im7 must be refolded. Regenerate the Im7 protein according to the following protocol.

1. Add 1-4 CVs of buffer A4 (in total – including the bead volume – the column will contain 2-3 CVs).
2. Dilute buffer A4 by 10% (0.6M) in 10 sequential steps (details below):
   a. In the first step, elute or exchange with a pipette 10% of the total volume of buffer A4. (use formula in d to calculate the volumes for the next steps).
   b. Add an equivalent volume of buffer B.
   c. Equilibrate the column for 5-6 min.
   d. Repeat steps a-c ten times, referencing the formula below.

$$\boldsymbol{V_{ra}(N) = V_{tot}\left(1 - \frac{1 - 0.1 * N}{1 - 0.1 * (N-1)}\right)}$$

N - Step # $\mathbf{V_{tot}}$ – Total buffer Volume; $\mathbf{V_{ra}(N)}$ – Volume to Remove/Add at Step# N

Note | The volume to remove/add will increase with each dilution.
Buffer A4 will be completely replaced with buffer B after ~50-60 min.

Example

If the bead volume is **2 mL** and the initial volume of buffer A4 in the column (**V_{tot}**) before regeneration is **4 mL** (bead volume + 1 CV buffer A4 on top of the bead bed), the volume of buffer to elute and add [**V_{ra}(N)**] for each step is provided in **Table 5**.

Dilution Step (N)	0	1	2	3	4	5	6	7	8	9	10
Volume to Add/Remove (mL) **V_{ra}(N)**	0.000	0.400	0.444	0.500	0.571	0.667	0.800	1.000	1.333	2.000	4.000
Gdn (%)	100	90	80	70	60	50	40	30	20	10	0

Table 5 Example case of buffer volumes to remove/add if total volume in step 1 of the regeneration protocol is 4 mL