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Expression and purification of CENP-T proteins

Expression

Day 1: Transform pET28a plasmid with CENP-T construct into ArcticExpress (DE3) *Escherichia coli* (Agilent Technologies, 230192). Grow culture in 150 mL LB/Kan media (LB + 50 μg/mL Kanamycin). Grow culture overnight at 37 ºC / 240 rpm.

Day 2:

1) Split 150 mL of overnight culture into 2 x 1L of LB/Kan.
2) Incubate the cultures at 37 ºC / 240 rpm.
3) Measure changes in OD600 over time.
4) Induction of protein growth:
    a) When OD600 is about 0.3-0.4, transfer the culture to the cool shaker to 10 ºC.
    b) After 15-20 minutes, check OD600. When OD600 = 0.5-0.6 induce using 0.5 mM IPTG.
    c) Incubate at 10 ºC / 240 rpm for 22 hours.

Day 3:

1) Spin the cultures at 4,000 *g* / 4 ºC / 30 minutes / (1L bottles, rotor Beckman JLA-9.1) and collect pellets.
2) Freeze the pellets in liquid nitrogen, and store them at -80 ºC.

Purification

Protocol A. This version of the protocol was used to purify CENP-T^{6D}, CENP-T^{WT}, CENP-T^{2D}, CENP-T^{T11A}, CENP-TT^{T85A}, and CENP-$T^{2D,short}$ proteins.

For purification of all CENP-T proteins, we use the fast protein liquid chromatography (FPLC) system AKTA Pure (GE Healthcare) at 4 °C.

1. **Prepare buffers:**

1) **Buffer A** (1L)**:** 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol. Use this buffer as a base for Lysis and Elution buffers.
2) **Lysis Buffer** (100 mL; prepare on the day of the experiment): Buffer A supplemented with 0.15 mg/mL lysozyme (Sigma-Aldrich, L6876), cOmplete EDTA-free protease inhibitors cocktail (Roche, 11873580001) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, P7626).
3) **Elution buffer** (250 mL): Buffer A supplemented with 500 mM imidazole; adjust pH after Imidazole dissolving.

2. Cell Lysis. All manipulations should be done on ice and/or in a cold room.

1) Thaw cell pellets on ice for 15 min (alternatively use fresh culture).
2) Resuspend cells in Lysis buffer (25 mL per 1 L of culture).
3) Sonicate on ice bath 30 sec ON, 30 sec OFF, 40% power (60 W), 2.5 min total time, ½ in probe.
4) Spin the crude lysate by ultracentrifugation at 50,000 *g* for 30 min, 4 °C (Beckman 50.2Ti rotor). Collect supernatant.

3. Ni-NTA column. All manipulations should be done in a cold room.

1) Equilibrate 5 mL HisTrap column (Cytiva, 17524801) with 5 column volumes of Buffer A.
2) Transfer the cleared supernatant to the 100 mL super loop and attach it to AKTA.
3) Load the protein to the column at the flow rate of 2.5 mL/min.
4) Wash the column with ten volumes of Buffer A, five volumes of Buffer A supplemented with 25 mM imidazole, and five volumes of Buffer A with 50 mM imidazole at the flow rate of 2.5 mL/min.
5) Elute the protein with ten-column volumes of Buffer A containing the gradient of imidazole in the range 50-500 mM. The flow rate at this step is 2.5 mL/min. Collect 2 mL fractions.
6) Monitor the protein elution by absorbance at 280 nM.
7) Run the peak fractions on the SDS-PAGE to select those that contain clean CENP-T protein.

4. Size-exclusion chromatography. All manipulations should be done in a cold room.

1) Equilibrate to HiLoad Superdex 200 pg column (Cytiva, 28-9893-35) with 200 mL of Buffer A.

2) Combine the peak fractions containing CENP-T protein (~ 5 mL total), and concentrate them to 2-2.5 mL using 10 kDa Amicon Ultra Centrifugal Filter Units (EMD Millipore, UFC901008).
3) Centrifuged protein to remove aggregates at 30,000 *g* for 15 min, 4 °C.
4) Apply protein to the equilibrated HiLoad Superdex 200 pg column using a 5 mL loop and wash the loop with three loop volumes at the flow rate of 0.5 mL/min.
5) Elute the protein with Buffer A applied at a flow rate of 0.5 mL/min.
6) Monitor the protein elution by absorbance at 280 nM.
7) Run the peak fractions on the SDS-PAGE to select those that contain clean CENP-T protein. Based on the SDS-PAGE analysis, the fractions containing CENP-T proteins are in the 70-80 mL range.
8) Purified proteins were supplemented with 20% glycerol, aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C.

Protocol B. This version of the protocol was used to purify CENP-T^{site1}, CENP-T^{site2}, and CENP-T^{TR} proteins.

The protocol is similar to Protocol A with the following modifications:

1. Prepare 500 mL Buffer A with 0.5 mM EDTA.
2. Lysis Buffer had a different composition: Buffer A was supplemented with cOmplete protease inhibitors cocktail (Roche, 11697498001) and 0.5 mM EDTA.
3. Instead of step "**Ni-NTA column**" perform "**GST Sepharose purification**" described below.
4. All other steps of Protocol B are the same, as in Protocol A.

GST Sepharose purification. All manipulations should be done on ice and/or in a cold room.

1) Pack the gravity column with 3 mL of GST Sepharose 4B (Cytiva, 17-0756-01).
2) Equilibrate the column by washing with 20 mL of Buffer A with 0.5 mM EDTA.
3) Apply clear lysate using funnel extension to the funnel, let the column drop for 2 hours, and collect flowthrough.
4) After lysate has been applied to the column wash the column with 50 mL of wash buffer Buffer A with 0.5 mM EDTA.
5) Do an additional two washes by applying 50 mL of Buffer A with 0.5 mM EDTA each.
6) Close the outlet of the column with a cap.

7) Add to the column 2 mL of Buffer A with 0.5 mM EDTA supplemented with 200 µL of 2 mg/mL of homemade TEV protease.
8) Close tightly the inlet of the column with a cap, additionally inlet and outlet of the column can be sealed with Parafilm.
9) Secure the column on the rotator and incubate overnight in a cold room (4 °C; 10-15 rpm).
10) Elute protein from the column by applying 2 mL of ice-cold Buffer A with 0.5 mM EDTA.
11) *If TEV cleavage reaction was not effective, close the cap of the column and add 2 more mL of Buffer A with 0.5 mM EDTA. Add 200 µl of 2 mg/mL of homemade TEV protease. Incubate the column additional 1-1.5 hours at room temperature on the rotator (10-15 rpm). Elute protein by gravity followed by the addition of 2 mL of Buffer A with 0.5 mM EDTA.*