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Maranhao Polymerase/Protein Purification Protocol

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1 *Works for me* This protocol may be deleted by the owner

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ABSTRACT

I present this as a somewhat universal/standard/basic purification protocol that has been successfully used to purify polymerases (e.g. RTX and Bst-LF) as well as other accessory proteins. Although this protocol was developed and codified for the purification of RTX (1), it has and can be used to purify a range of proteins of differing stabilities, solubilities, etc.

GUIDELINES

There is a key optional heat-treatment step prior to IMAC (Ni-NTA) purification. The use of this heat-treatment step is meant enrich for a thermostable target protein. The temperature and duration of a heat-treatment step should only be implemented when the stability of a target protein is known.

Furthermore, there are two buffers used in this protocol: Tris and phosphate. One should avoid buffers containing primary amines (like Tris) when purifying over a Ni-NTA column. Tris and other primary amine containing buffers can behave like EDTA and strip coordinated metal ions.

So that's why this purification protocol/strategy has two phases in a buffer sense. The first phase (cell lysis, lysate prep, purification over a Ni-NTA column) uses phosphate buffer to avoid Ni2+ stripping from the IMAC column. This would affect overall yield and maybe purity. The second phase (FPLC purification and dialysis into storage buffer) uses Tris buffer as that buffer is compatible with PCR.

Should one want a simplified protocol, I would suggest standardizing purification buffers to Tris. This may affect your overall expression yield. However, phosphate in storage buffer (and especially in the PCR buffer itself) could/would negatively impact PCR.

MATERIALS TEXT

Resuspension Buffer	50 mM Phosphate Buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent), 5 mM MgSO4
Equilibration Buffer	50 mM Phosphate Buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole
Lysis Buffer	30 mL Resuspension Buffer + 1x EDTA-free protease inhibitor tablet + 30-60 mg HEW (hen egg white) Lysozyme
Wash Buffer	50 mM Phosphate Buffer, pH 7.5, 300 mM NaCl, 50 mM imidazole
Elution Buffer	50 mM Phosphate Buffer, pH 7.5, 300 mM NaCl, 250 mM imidazole
Heparin Buffer A	40 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)
Heparin Buffer B	40 mM Tris-HCl pH 7.5, 2 M NaCl, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

Table 1: Purification Buffers

Ni-NTA Dialysis Buffer	40 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent), 10 mM beta-mercapto ethanol (BME) or 1 mM DTT
Heparin Dialysis Buffer	50 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Tween-20, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

Final Dialysis Buffer	50% glycerol, 50 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Tween-20, 1 mM DTT, 0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

Table 2: Dialysis Buffers

Culture Centrifugation 30m

1 Spin down **1 L** culture **5000 x g, 4°C 00:20:00** . Discard the supernatant (i.e. spent media). Keep cell pellet on ice or in a cold room enivronment (**4 °C**).

If working from a frozen pellet, simply thaw cell pellet on ice.

Lysis

2 Resuspend cell pellet in **30 ml** **Lysis Buffer** and transfer to a clean 50 mL beaker with stir bar. Securely place the beaker setup into an ice bath.

Sonicate with moderate spinning on ice at:
- 40% amplitude
- 1 sec ON / 4 sec OFF cycle
- 4 min total ON time

Transfer sonicated samples to clean, cold Nalgene Oak Ridge ultracentrifugation tubes.

Generating cleared lysate

3 **First Ultracentrifugation:**

Carefully balance all Oak Ridge tubes containing sonicated cell pellet.

Centrifuge lysate **35000 x g 00:30:00** .

Following first ultracentrifugation, transfer supernatant to a new Nalgene Oak Ridge ultracentrifugation tubes and carefully rebalance all tubes.

3.1 **Optional Heat-treament Step:**

NOTE: Skip this optional step if target protein is not thermostable or is not known to be thermostable.

Otherwise, following first ultracentrifugation, place Nalgene Oak Ridge ultracentrifugation tubes into a thermomixer and shake at 400-800 rpm for **00:10:00** :

Set thermomixer to the appropriate temperature depending on target protein properties.

KOD/RTX: 85°C
Bst-LF: 65°C

After shaking at temperature, place Nalgene Oak Ridge tubes on ice for **00:10:00** prior to the second ultracentrifugation.

3.2 **Second Ultracentrifugation:**

Centrifuge lysate at **35000 x g, 4°C 00:30:00** .

Following this second ultracentrifugation, transfer supernatant to a 50 mL Falcon tubes and keep cold.

3.3 **Lysate Filtration:**

Filter resulting supernatant using a 0.2 µm filter. The resulting lysate is "Cleared Lysate" (CL).

Ni-NTA Column Purification

4 Apply Cleared Lysate (CL) to preassembled gravity flow columns containing properly equilibrated Ni-NTA. Collect all Flow-through (FT).

Wash column with 20 column volumes (CVs) of **Equilibration Buffer**. Collect Wash 1 (W1).

Wash column with 5 CVs of **Wash Buffer**. Collect Wash 2 (W2).

Elute target protein with 5 CVs **Elution Buffer**.

Ni-NTA Dialysis

5 Perform all setup and the sequential dialysis in a cold room environment (4°C). Use a dialysis cassette of the appropriate volume and molecular weight cut-off (MWCO). Select a MWCO equal to or less than half the size of target protein.

5.1 First, hydrate the dialysis cassette and its membrane in 2 L **Ni-NTA Dialysis Buffer** for at least 5 min. Next, fill the now hydrated dialysis cassette with eluate from Step 4, carefully remove air from the cassette, and allow to dialyze for 3-4 hours.

5.2 Following initial dialysis, transfer cassettes from the first 2 L **Ni-NTA Dialysis Buffer** to a second volume of dialysis buffer and allow to dialyze overnight.

FPLC Polishing

6 Equilibrate a 5 mL Heparin column with **Heparin Buffer A**. Apply the overnight dialyzed eluate to equilibrated Heparin column. Elute along an NaCl gradient from 100 mM NaCl (**Heparin Buffer A**) to 2 M NaCl (**Heparin Buffer B**) collecting fractions along the salt gradient.

Run a PAGE gel to verify the identity and relative purity of the peak presumed to be target protein.

Final Dialysis

7 Perform all setup and the sequential dialysis in a cold room environment (4°C). Use the same dialysis cassette selected for Setp 5.

7.1 First, hydrate the dialysis cassette and its membrane in 2 L **Heparin Dialysis Buffer** for at least 5 min. Next, fill the now hydrated dialysis cassette with the desired fractions from Step 6, carefully remove air from the cassette, and allow to dialyze for 3-4 hours.

7.2 Following initial dialysis, save an aliquot (~40 mL) of **Final Heparin Dialysis Buffer** to be used in dilutions of the final dialysis product. Transfer cassettes from the first 2 L **Final Heparin Dialysis Buffer** to a second volume of dialysis buffer and allow to dialyze overnight.

Quantification

8 Quantify protein concentration using the saved aliquot of **Final Heparin Dialysis Buffer** as a blank. Use that same aliquot of **Final Heparin Dialysis Buffer** to perform dilutions from the final/stock protein.