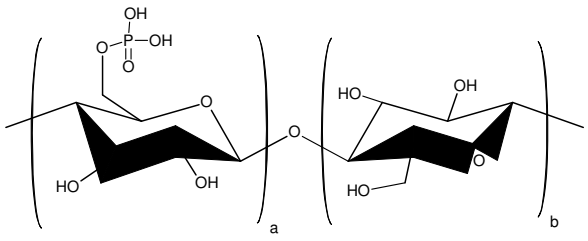
Two-step purification of T7 RNA Polymerase with Cellufine MAX DEAE and Phosphate Affinity Chromatography Resins

Cellufine resins are spherical porous cellulose particles that have been surface modified with a range of chromatography chemistries. They are widely used in the manufacture of biologics such as antibodies, vaccines, and therapeutic proteins. T7 RNA polymerase is an enzyme derived from T7 phage used to produce mRNA transcripts from the template DNA. In the manufacture of COVID-19 mRNA vaccines. T7-RNA polymerase is generally produced by expression using *E.coli* as a host. Therefore, it is necessary to purify T7-RNA polymerase to remove contaminants such as endotoxin, dsDNA derived from host and vector, and host cell proteins (HCP).

Cellufine Phosphate is a pseudo affinity chromatography resin with an affinity for nucleic acid binding proteins and is used to purify this important enzyme for production of mRNA-based vaccines. The structure of Cellufine Phosphate has a phosphate group ester-bonded to the hydroxyl group at the 6-position of cellulose (Figure 1). Since this structure is like that of nucleic acid, a nucleic acid-binding protein such as T7-RNA polymerase are strongly adsorbed. Further, since the phosphate group acts as a negatively charged cation exchange resin, negatively charged endotoxin is not adsorbed.

Figure 1, Chemical Structure of Cellufine Phosphate

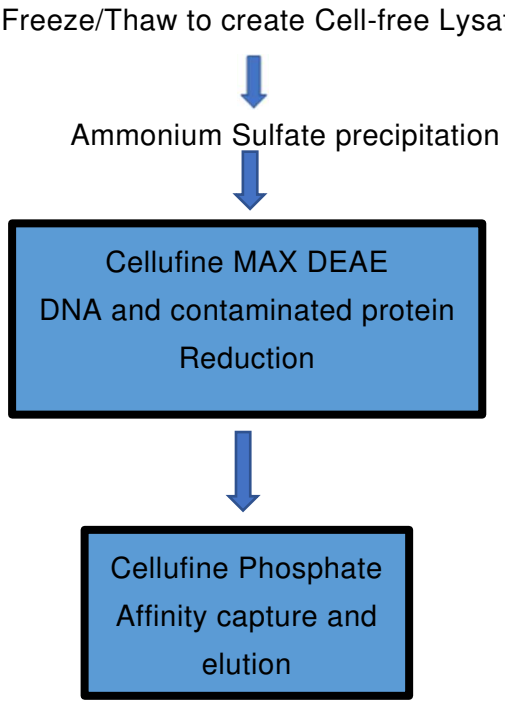


In this Technical Note, Cellufine MAX DEAE is first used after Ammonium sulfate precipitation to adsorb dsDNA present in the cell lysate. After elution from MAX DEAE at low ionic strength, direct loading onto Cellufine Phosphate and elution with high salt achieved a final product exhibiting a high degree of purity and an increase in T7 RNA polymerase enzyme specific activity over a commercially available purified enzyme. In addition to T7-RNA polymerase, other nucleic acid-binding proteins such as pyrophosphatase, 2-O-methyltransferase, and poly A polymerase are used for *in vitro* transcription of mRNA. The two-step purification described in this study could also be applied to the purification of these other enzymes as well. The robust purification process of these enzymes, which are important for the manufacture of mRNA drugs, will contribute to reducing manufacturing costs, shortening of development lead time and stable production.

1. Purification Outline

Cell culture and recovery of an expressed enzyme is a purification challenge. In addition to the target enzyme there are the following contaminants that must be removed from the final product; a) host cell proteins, and b) host cell-derived dsDNA fragments. To achieve efficient purification of the enzyme T7 RNA polymerase, a two-step chromatography purification workflow has been developed and will be described below. The combined multi-step precipitation, DNA removal and final affinity capture protocol is summarized in Figure 2 below.

Figure.2 Multi-Step T7 RNA polymerase Enzyme Purification Workflow



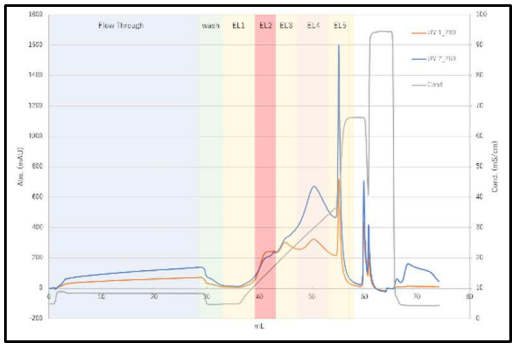
Step 1 – Ammonium Sulfate Precipitation

Recombinant Escherichia coli (derived from pAR1219) expressing T7 RNA polymerase (T7 RNAP) was cultured, and expression was induced by adding IPTG. After washing the cells with PBS, 0.2 mg / mL of lysozyme was added, and 3 cycles of freeze-thaw were used to lyse the cells. Then Ammonium sulfate was added to the lysate to a final concentration of 35% (w / v), and the mixture was stirred at 4 ℃. for 1 hour and then centrifuged to recover the precipitate.

Step 2 – Cellufine MAX DEAE DNA and contaminated protein reduction

Precipitate from Step 1 above was resuspended into 28.5 mL of load buffer (10 mM Tris HCl pH 7.5, 50 mM NaCl, 0.1mM EDTA, 0.5mM DTT, 10% (v/v) glycerol + Proteinase inhibitors [10 µg/mL PMSF, 100µM Benzamidine and 10µM Bacitracin]). The sample was then diluted with the above buffer for a final conductivity of 8 mS/cm. This sample was then loaded onto a 1 mL (CV) pre-packed Cellufine MAX DEAE cartridge equilibrated in the above loading buffer at 0.5 mL/min (2 min. residence time). After sample loading the column was washed with 5CV of the load buffer. Retained fractions were eluted from the cartridge with a linear NaCl gradient up to 35 mS/cm conductivity. Followed by step elution to 65 and 95 mS/cm. See Figure 3, MAX DEAE chromatogram. monitoring at A260 A 280 nm. Protein recovered was estimated by the Bradford assay. DNA eluted was measured by Pico green, fluorescent assay (Thermo Fisher).

Figure 3, MAX DEAE chromatography after Step 1Ammonium Sulfate Precipitation



Load sample: Ammonium sulfate precipitate, 28.5 CV diluted with equilibration buffer to 10.0 mS / cm

Column: 1mL Mini column（6.7mm ID x 30 mm L, JNC）

Flow rate: 0.5 mL/min（85 cm/h, residence time 2 min）

Equilibration buffer (Eq.): 10 mM Tris-HCl pH7.5, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10 % glycerol, protease inhibitor

Elution buffer : Eq. + 1 M NaCl

Elution fraction EL2 was found to contain the T7 RNA polymerase enzyme activity and showed a A260/280 ratio typical of a protein with a low level < 1 µg/mL of residual dsDNA. Protein and DNA elution data are summarized in Figure 3 and Table 1 below.

Figure 4, Summary of Protein and DNA data from MAX DEAE chromatography

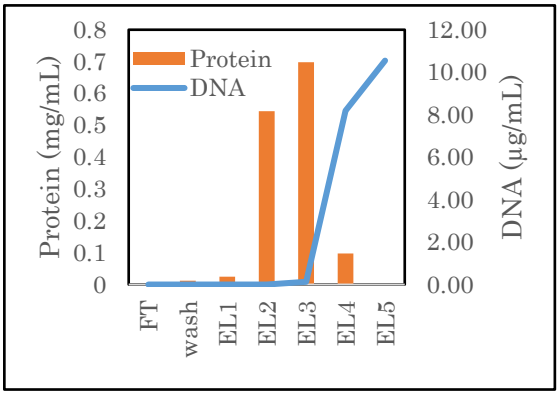


Table 1, DNA Recovery in MAX DEAE Step

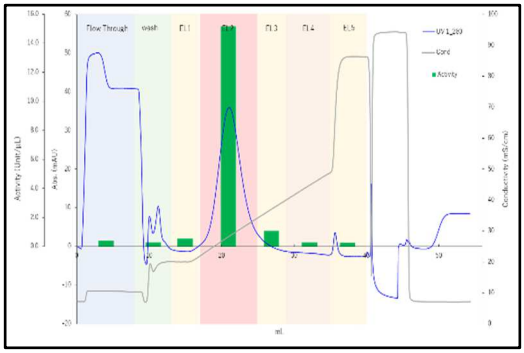
Fraction	DNA recovery (%)
Load	100
EL3	0.48
EL4	49.1
EL5	54.5
Mass balance	105.8

MAX DEAE resin was able to remove dsDNA from T7 RNA Polymerase activity in elution fraction EL2 down to < 1 µg/mL. The bulk of the retained dsDNA was eluted by NaCl > 25 mS/cm in fractions EL4 and EL5.

Step 3 – Cellufine Phosphate Affinity capture and high salt elution

Elution fraction EL2 from the above step was diluted 3-fold with equilibration buffer (10 mM K Phosphate pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.1mM DTT and Proteinase inhibitors [10 µg/mL PMSF, 100µM Benzamidine and 10µM Bacitracin]). This sample was then loaded onto a 1 mL (CV) Cellufine Phosphate 6.7 mmID x 3.0 cmL cartridge at 0.5 mL/min flow rate pre-equilibrated in the above buffer. After loading and a 5 CV wash with the same buffer, the retained fractions were eluted with a linear gradient up to 1M NaCl. Fractions were collected across the chromatogram shown in Figure 5 below. T7 RNA polymerase activity in the fractions was measured with a commercial kit (Catalog number T7RPA100KE, ProFoldin, Hudson, MA). One unit is defined as the amount of enzyme capable of incorporating 1 nmol ATP into an acidic insoluble matter at 37 ° C for 1 hour. Fraction EL2 (P-EL2) was found to contain the T7 RNA Polymerase enzyme activity.

Figure 5, Cellufine Phosphate Chromatography of T7 RNA Polymerase.



Load sample: 8 CV (3-fold dilution with equilibration buffer)

Column: 1mLpre-packed column（6.7mm ID x 30 mm L, JNC）

Flow rate: 0.5 mL/min（85 cm/h、residence time 2 min）

Equilibration buffer (Eq.): 10 mM potassium phosphate pH7.5, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, protease inhibitor

Elution buffer: Eq buffer + 1 M NaCl

It was found that the activity of T7-RNA polymerase enzyme in the elution fraction P-EL2 after adsorption to Cellufine Phosphate showed 70.2% recovery. The amount of protein in this fraction was reduced to 24.7%, indicating that contaminants were efficiently removed. The enzyme activity and protein recovery after column purification with Cellufine Phosphate are shown in Table 2 below

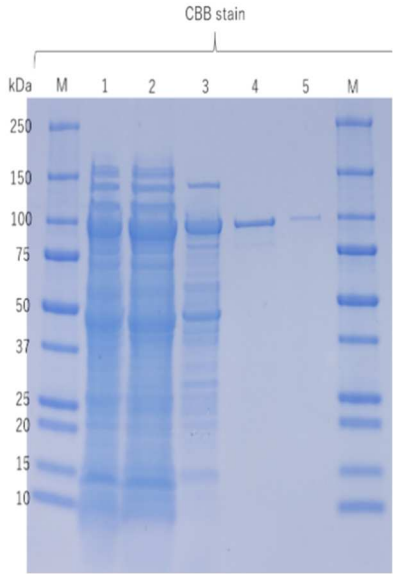
Table 2, Recovery of Enzyme Activity in Cellufine Phosphate Purification Fractions

Fraction	Enzyme activity (Unit/protein)	Enzyme Recovery (%)	Protein Recovery (%)
Load	94043	100	100
FT	2763	1.8	59.8
P-EL2	267034	70.2	24.7

2. SDS-PAGE Analysis of Purification Samples

Samples of fractions representing the above steps in the purification workflow were analyzed by SDS-PAGE analysis (Mini-PROTEAN TGX Precast gel 4-20% (Bio-Rad)) and stained with Coomassie Blue (Bio-Rad) and destained in water. Results are summarized in Figure 6 below.

Figure 6, SDS-PAGE Analysis of Purification Fractions



M: marker
1. Lysate
2. Ammonium Sulfate precipitation (AP)
3. Cellufine MAX DEAE EL2
4. Cellufine Phosphate EL2
5. Positive control (PC)

The results showed that the initial Ammonium Sulfate precipitation step reduced the volume of the sample followed by MAX DEAE fraction which showed an enrichment of the 100 kDa protein band. The final Cellufine Phosphate pseudo affinity step led to the elution of a fraction with > 95% of the protein in a single band of high purity. The overall recovery of enzyme activity in the two-step workflow is summarized in Table 3 below and shows that the final protein elution from Cellufine Phosphate showed a higher specific enzyme activity that in the commercial positive control.

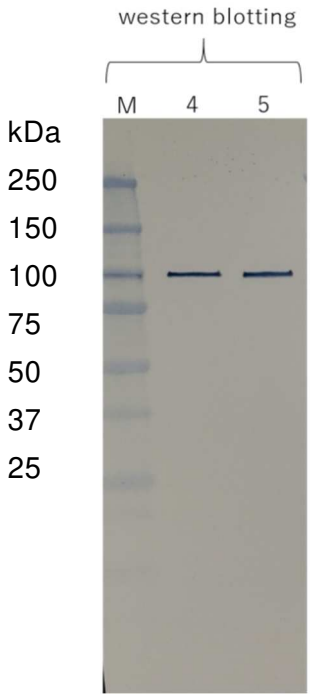
Table 3, T7 RNA polymerase activity at each step of the purification workflow

Fraction	Enzyme activity (Unit/protein)
Lysate	24,011
Ammonium sulphate precipitate	34,296
MAX DEAE elution (D-EL2)	66,741
Phosphate elution (P-EL2)	267,034
Control	208,535

3. Western Blot Analysis of Purification samples

Western blot analysis was used to confirm the purity of the final Cellufine Phosphate elution fraction EL2 compared to a commercial source of the T7 RNA Polymerase (Takara Bio, Inc. Shiga, Japan) enzyme. After SDS-PAGE, protein transfer was carried out in a semi-dry blotting device (WSE-4025HorizeBlot 2M from ATTO, Tokyo, Japan). The western blot was then washed with 0.05% (v/v) Tween 20 in Phosphate buffered saline (PBS) and blocked with Ezblock chemi (ATTO, Tokyo, Japan) for 1 h at room temperature. After blocking the membrane was washed 3 times for 5 min with above buffer in PBS. The detection of the blotted protein was then carried out with a 1/2000 dilution of anti-T7 RNAP polyclonal rabbit IgG (Creative Diagnostics, Shirley, NY) in the above blocking solution for 1h at RT. After washing 3 x 5 minutes in above PBS buffer the western blot was then incubated with a secondary antibody goat anti-rabbit IgG (H+L) – HRP (Zymax, Thermo-Fisher, Waltham, MA) as a 1/2000 dilution in blocking solution for 1 h at RT. After this step the membrane was washed 3x 5 minutes with the above buffer in PBS. The western blotted bands were then visualized with an HRP chromogenic substrate (EzWestBlue, ATTO, Tokyo, Japan) for 30 min at RT and finally washed with water and stored in the absence of light to stoop the band signal fading. Western blot data is summarized in Figure 7 below.

Figure 7, Western Blot Analysis of final Cellufine Phosphate elution peak EL2



M: marker
4. Cellufine Phosphate EL2
5. Positive control (PC)

Western blot analysis of the Cellufine Phosphate final elution fraction showed a single immunoreactive band at 100 kDa with the same mobility as the positive control sample.

Conclusion

In this Technical Note a two-step purification workflow is described for purification of T7 RNA polymerase expressed in an *E. coli* cell line. After initial volume reduction by Ammonium Sulfate precipitation, dsDNA and contaminated protein are removed by adsorption to an anion exchange IEX resin Cellufine MAX DEAE. In the final pseudo affinity step on Cellufine Phosphate incorporating salt-based elution, enzyme protein was eluted and showed a single band by SDS-PAGE gel analysis and was confirmed by western blotting. The final product of the purification showed an increase in specific activity compared to a commercial control enzyme with an overall recovery of 70.2% of the activity from the crude lysate sample.

Product information

Cellufine MAX DEAE
https://www.jnc-corp.co.jp/fine/en/cellufine/guide/pdf/ion_max/TD_MAX_IEX_N1_V5_E.pdf
Cellufine Phosphate
https://www.jnc-corp.co.jp/fine/en/cellufine/guide/pdf/affinity/TD_Phosphate_N1_V2_E.pdf
Operating instructions manual and technical data can be downloaded as pdf from the following website.https://www.jnc-corp.co.jp/fine/en/cellufine/guide/index.html

Purchase information

Product	Quantity	Catalogue No.
Cellufine MAX DEAE	1ml x 5 (Mini-Column)	21000-51
	5ml x 1 (Mini-Column)	21000-55
	100ml	21000
	500ml	21001
	5 lt	21002
	10 lt	21003
Cellufine Phosphate	1ml x 5 (Mini-Column)	19551
	5ml x 1 (Mini-Column)	19515
	10ml	19524
	50ml	19545
	500ml	19546
	5 lt	684 987 330
	10 lt	684 944 324

Inquiry & technical support

(North America & Europe))
JNC America, Inc.
555 Theodore Fremd Ave,
Rye, NY 10580

Tel: 914-921-5400
Fax: 914-921-8822
Email: cellufine@jncamericany.com

(Japan, Asia, Other)
JNC Corporation
Life Chemical Division

Shin Otemach Bldg.,2-1 Otemachi 2-Chome,
Chiyoda-ku, Tokyo 100-8105 Japan
Tel: 03 3243 6150
Fax: 03 3243 6219
Email: cellufine@jnc-corp.co.jp