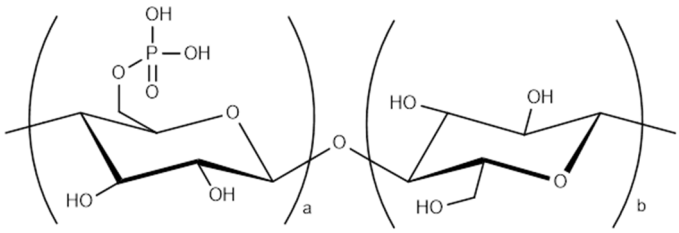
Three-step purification of T7 RNA Polymerase with Cellufine MAX DEAE, Cellufine Phosphate and Cellufine ET Clean L

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 hardly adsorbed.

Figure 1, Chemical Structure of Cellufine Phosphate

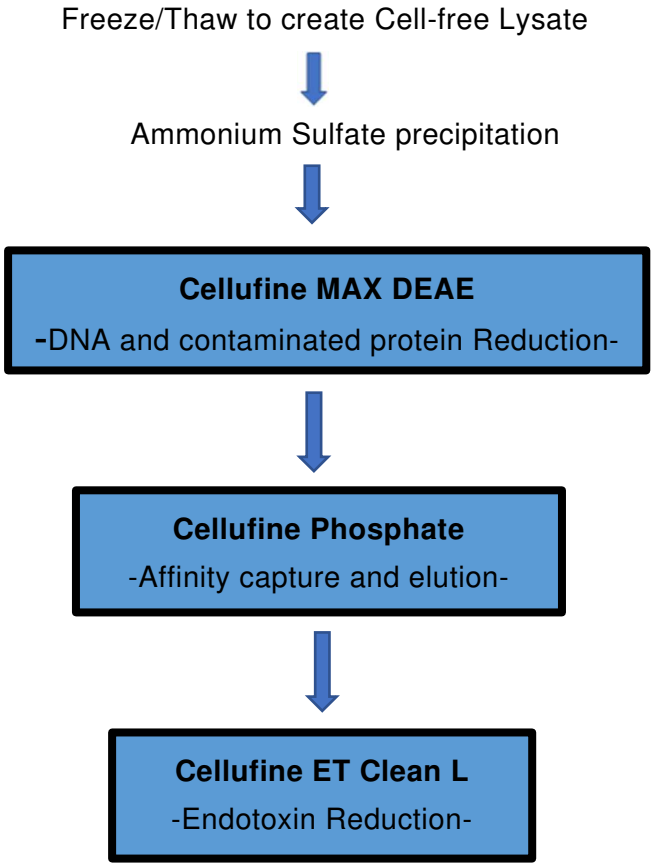


In this Technical Note, Cellufine MAX DEAE is first used after Ammonium sulfate precipitation to adsorb dsDNA present in the cell lysate. After elution from MAX DEAE at low ionic strength, direct loading onto Cellufine Phosphate and elution with high salt achieved a final product exhibiting a high degree of purity and an increase in T7 RNA polymerase enzyme specific activity over a commercially available purified enzyme. Furthermore, residual endotoxin could be further removed by Cellufine ET Clean L which adsorbs endotoxin specifically. 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 three-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. c) degrading enzymes such as protease and nuclease. d) host cell-derived endotoxin. To achieve efficient purification of the enzyme T7 RNA polymerase, a three-step chromatography purification workflow has been developed and will be described below. The combined multi-step precipitation, DNA removal, affinity capture and endotoxin removal protocol is summarized in Figure 2 below.

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



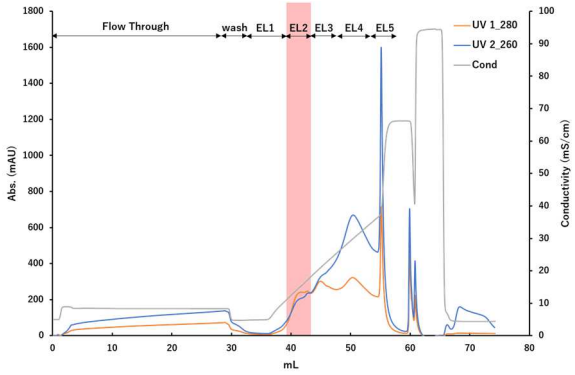
Step 1 – Ammonium Sulfate Precipitation

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 °C. 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 A280 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 of residual dsDNA. Enzyme activity, Protein and DNA elution data are summarized in Figure 4 and Table 1 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.

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

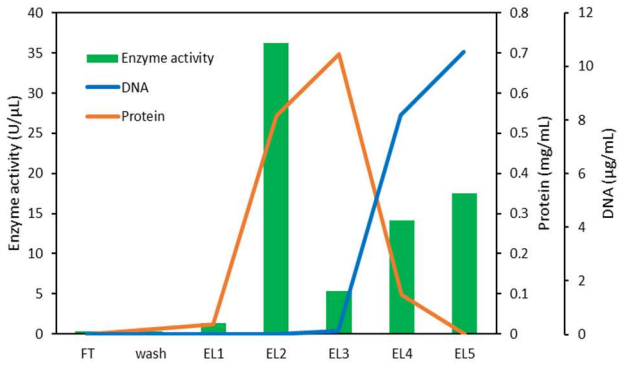


Table 1, DNA Recovery in MAX DEAE Step

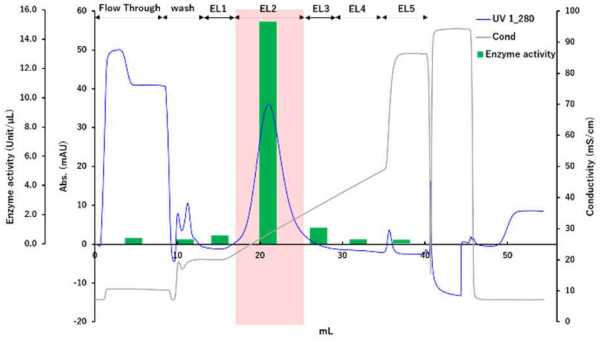
Fraction	DNA recovery (%)
Load	100
EL2	ND
EL3	0.50
EL4	50.8
EL5	54.5
Mass balance	105.8

MAX DEAE resin was able to remove dsDNA from T7 RNA Polymerase activity in elution fraction EL2 down to < 2 ng/mL (ND in Table 1). The bulk of the retained dsDNA was eluted by NaCl > 25 mS/cm in fractions EL4 and EL5. Since the contaminant DNA is negatively charged, it strongly interacts with the anion exchange group (DEAE). Therefore, it was eluted in fractions with high conductivity, and was separated from most protein. The activity of T7 RNAP is highest in the fraction EL2. It is also observed in EL4-5, but this is due to non-specific detection of nucleic acids in terms of the principle of measurement. It is considered that T7 RNAP and DNA could be separated because these fractions contain little protein.

Step 3-1 – 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 mm ID x 3.0 cm L 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. 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
Flow Through (FT)	2763	1.8	59.8
P-EL2	267034	70.2	24.7

Step 3-2 – Removal of Protease and Nuclease by Cellufine Phosphate

The activity of protease and nuclease (RNase and DNase) from T7 RNAP was evaluated by analyzing the eluted fraction after purification with Cellufine Phosphate.

First, Figure 6 shows the results of measurement of changes over time in protease activity before and after purification of Cellufine Phosphate. Activity was measured using a kit (Amplite Universal Fluorometric Protease Activity Assay Kit Green, AAT Bioquest). Before purification (Load), the activity increased with time, while after purification (Elution), the activity remained almost constant, indicating that the protease was removed.

Figure 6, Changes over time in protease activity before and after purification of Cellufine Phosphate

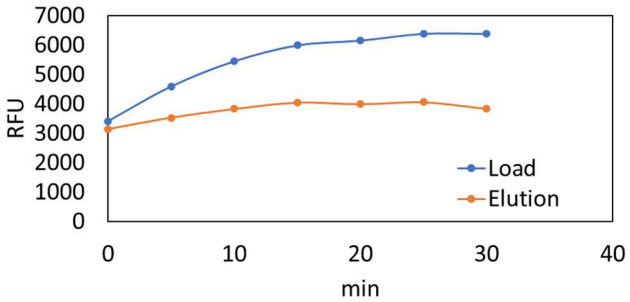
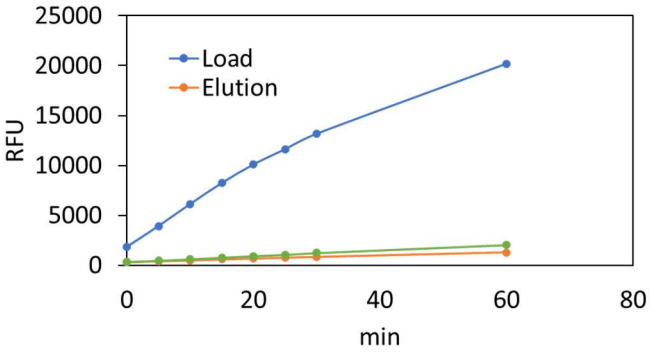


Figure 7 shows the results of measurement of changes over time in RNase activity before and after purification of Cellufine phosphate. Activity was measured using a kit (RNaseAlert Lab Test Kit, Thermo Fisher Scientific). Like the protease, the activity was compared before and after the purification. As a result, there was almost no change in the enzyme activity over time after

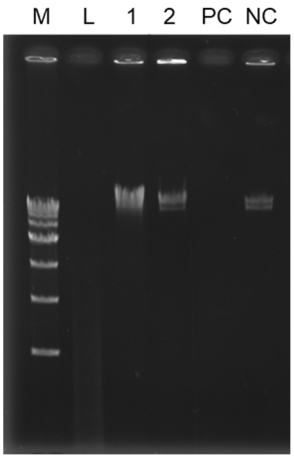
purification and the degree of activity changes was equivalent to the negative control, RNase-free water, therefore it was considered that RNase could be removed.

Figure 7, Changes over time in RNase activity before and after purification of Cellufine Phosphate



Finally, the DNase activity was evaluated by the following procedure. A volume of 500 µL was prepared containing 250 U of T7 RNAP and 10 µg of λDNA. A sample containing only λDNA was prepared as a negative control (NC) and containing λDNA and 0.2 U/µL of DNase I was prepared as positive control (PC). These solutions were incubated at 37 ° C for 16 hours. Then, DNA in the samples was detected by agarose gel electrophoresis. The results are shown in Figure 8. There were no bands in PC because λDNA was degraded by DNase. Before purification (Load), the λDNA band disappeared like PC, but after purification (Elution) the band remained like NC and commercial product, indicating that DNase was removed by Cellufine Phosphate.

Figure 8, Evaluation of DNase activity before and after purification of Cellufine Phosphate

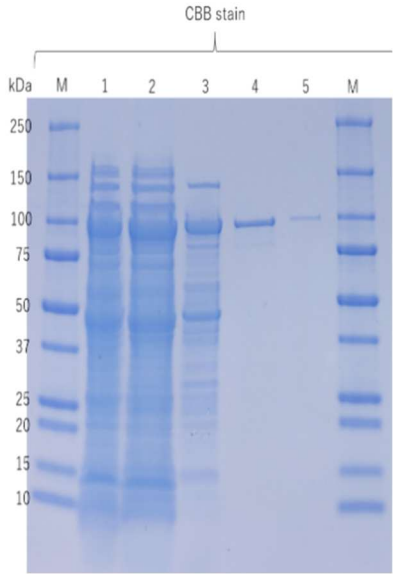


M: DNA ladder
L: Load
1: Elution
2: commercial product
PC: λDNA with DNase I
NC: λDNA

Step 3-3. 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 9 below.

Figure 9, 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. Cellufine Phosphate 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.

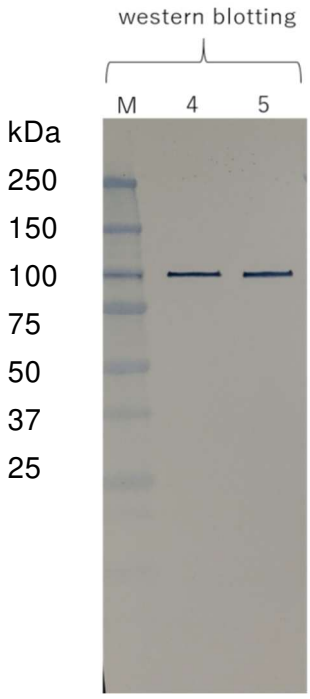
Table 3, T7 RNA polymerase activity at each step of the purification workflow

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

Step 3-4. Western Blot Analysis of Purification samples

Western blot analysis was used to confirm the purity of the 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 with Q Blot kit (ATTO, Tokyo, Japan) in a semi-dry blotting device (WSE-4025 HorizeBlot 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 3 x 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 10 below.

Figure 10, 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 elution fraction showed a single immunoreactive band at 100 kDa with the same mobility as the positive control sample.

Step 4 – Cellufine ET Clean L endotoxin reduction

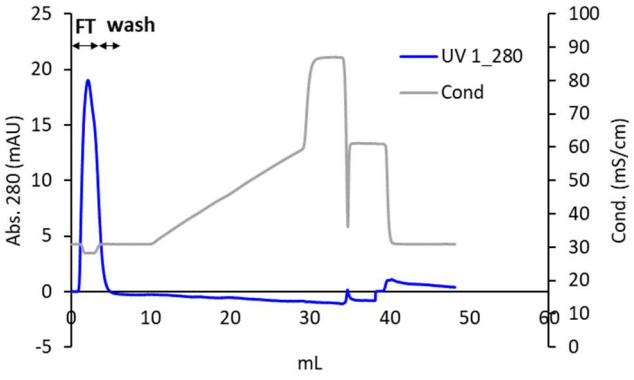
Since T7 RNAP is produced by recombinant *E. coli*, it is unavoidable that the extracted bacterial solution contains endotoxin. As a result of measuring the amount of endotoxin before and after purification with Cellufine Phosphate, it was found that nearly 97% of endotoxin could be removed, but a certain amount remained (Table 4). Therefore, we investigated the removal of endotoxin using Cellufine ET Clean L, which selectively adsorbs endotoxin. The amount of endotoxin was measured by Endosafe nexgen-PTS (Charles River).

Table 4, Amount of endotoxin before and after purification with Cellufine Phosphate

Fraction	Endotoxin (EU/mL)
Load	7691
Elution	222

First, we tried to remove endotoxin from T7 RNAP by flow through mode, in which T7 RNAP was collected in flow through fraction and endotoxin was removed by adsorption (Figure 11). It is considered that T7 RANP was eluted in the flow through (FT) fraction because of detection of UV peak. Table 5 shows the results of measurement of endotoxin removal properties and T7 RNAP activity. The recovery rate of T7 RNAP calculated from the enzymatic activity was 74.6% in fraction FT and wash, where UV peak was observed. The amount of endotoxin indicates concentration (EU/mL), unit amount (EU), amount per enzyme activity (mEU/unit), and amount per protein (EU/μg) measured by Bradford method. The amount of endotoxin contained in fraction FT and wash was significantly lower than before purification in all quantitative values, and as a result, 96.4% of endotoxin could be removed in this process.

Figure 11, Endotoxin removal from T7 RNAP by Cellufine ET Clean L (phosphate buffer)



Load sample: 2CV (Elution from Cellufine Phosphate)

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, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT

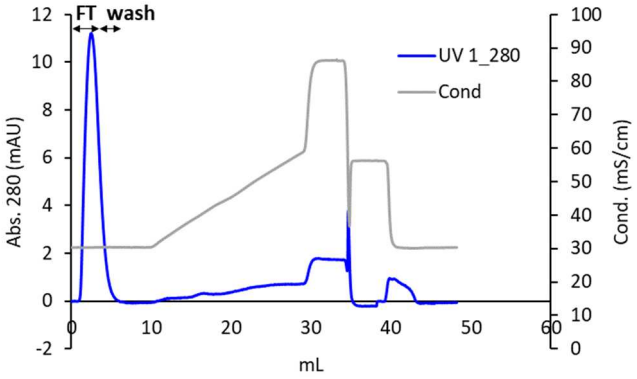
Elution buffer: Eq buffer + 1 M NaCl

Table 5, Results of measurement of endotoxin removal properties and T7 RNAP activity (phosphate buffer)

	Endotoxin					T7 RNAP
	EU/mL	EU	Reduction rate (%)	mEU/unit	EU/μg	Recovery rate (%)
Load	151	302	-	5.14	2.14	-
FT	2.89	7.23	-	0.32	0.10	40.7
wash	1.75	3.50	-	0.19	0.05	33.9
Total (FT+wash)	**2.38**	**10.7**	**96.4**	**0.26**	**0.07**	**74.6**

Next, we investigated purification under Tris buffer condition for the purpose of verifying the effect of the buffer type on purification with Cellufine ET Clean L (Figure 12). The eluted fraction after the Cellufine Phosphate purification was dialyzed with a membrane with permeation limit molecular weight of 3000 MWt. and was replaced with 100-fold volume of equilibration Tris buffer (Figure 12) at 4 °C for 1 hour twice and finally overnight to obtain a load sample. A UV peak was detected in the flow through fraction in Figure 12 similar to the result in Figure 11. The endotoxin removal property and T7 RNAP activity measurement results shown in Table 6 also showed that the total enzyme recovery rate for fraction FT and wash was a good result of 99.3%. The endotoxin removal property was higher than that of condition under phosphate buffer in all quantitative values, showing a removal property of more than 99%. The reason of such a difference in buffer type is that the phosphate ion buffer which has the opposite charge to the ion exchange group of Cellufine ET Clean L inhibits the adsorption of endotoxin by phosphate ions or the influence of disturbance of pH caused by ion exchange during adsorption. Even so, it showed a removal rate of 96.4% and it is advantage of phosphate buffer condition that the eluate from the previous step can be used.

Figure 12, Endotoxin removal from T7 RNAP by Cellufine ET Clean L (Tris buffer)



Load sample: 2CV (dialyzed 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 Tris-HCl pH7.5, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT

Elution buffer: Eq buffer + 1 M NaCl

Table 6, Results of measurement of endotoxin removal properties and T7 RNAP activity (Tris buffer)

	Endotoxin					T7 RNAP
	EU/mL	EU	Reduction rate (%)	mEU/unit	EU/μg	Recovery rate (%)
Load	134	268	-	11.0	3.46	85.8*
FT	0.30	0.75	-	0.07	0.02	46.5
wash	0.15	0.30	-	0.02	0.01	52.8
Total (FT+wash)	**0.23**	**1.05**	**99.6**	**0.04**	**0.01**	**99.3**

* recovery rate of dialysis

From the above results, it was shown that up to 99.6% of endotoxin could be removed by purifying Cellufine Phosphate eluate in flow through mode with Cellufine ET Clean L. A 96.4% removal rate can be achieved by using the eluate as it is under the same buffer condition as previous step (Cellufine Phosphate step). If a higher degree of removal is required, substitution with Tris buffer is recommended. In addition, it was confirmed that the enzyme activity of T7 RNAP obtained by both methods were equal to or higher than those of commercially product (data not shown).

Conclusion

In this Technical Note a three-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 next pseudo affinity step on Cellufine Phosphate incorporating, salt-based elution protein that showed a single band by SDS-PAGE gel analysis and was confirmed by western blotting. Finally remained endotoxin was selectively removed with Cellufine ET Clean L. The final product of the purification showed an increase in specific activity compared to a commercial control enzyme 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
Cellufine ET Clean L
https://www.jnc-corp.co.jp/fine/en/cellufine/grade/grade-1-etclean/

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/

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
Cellufine ET Clean L	1ml x 5 (Mini-Column)	20051
	5ml x 1 (Mini-Column)	20015
	10ml	681 984 324
	50ml	681 984 326
	500ml	681 984 328

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