Cellufine™ Phosphate HC

Cellufine™ Phosphate HC is an affinity chromatography media used to purify nucleic acid-related proteins such as protein kinases, restriction enzymes, nucleases, and polymerases. Compared to conventional Cellufine™ Phosphate, the pore size has been controlled to improve the adsorption capacity of large molecular weight proteins. This next-generation chromatography media is particularly effective for purifying high molecular weight enzymes such as T7 RNA polymerase.

Cellufine™ Phosphate HC is an affinity chromatography media with phosphate ester groups attached to spherical porous cellulose particles. The chemical structure of the cellulose particles with phosphate ester groups attached to the hydroxyl group at the 6-position is similar to that of nucleic acids (Figure 1). For this reason, it is known to have affinity activity for nucleic acid-binding proteins.

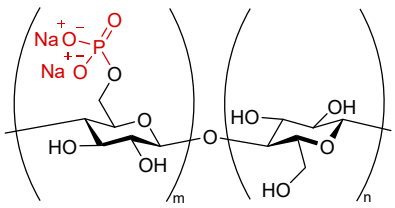
In recent years, RNA polymerases have been attracting attention due to the rise of mRNA drugs. T7 RNA polymerase (T7RNAP) is used to synthesize mRNA, i.e., in vitro transcription (IVT) RNA synthesis. T7RNAP has the characteristics of a nucleic acid-binding protein and exhibits affinity for Cellufine™ Phosphate HC.

Cellufine™ Phosphate HC is a new affinity chromatography media with phosphate ester groups. Compared to conventional meida, it has improved adsorption capacity for large molecular weight proteins by controlling the pore size (Table 1). In particular, relatively large soluble proteins with molecular weights of 45 kDa to 150 kDa are characterized by their large adsorption capacity, as they diffuse favorably inside the pores (Figure 2).

Table 1 Features of Cellufine™ Phosphate HC

	Cellufine™ Phosphate	Cellufine™ Phosphate HC
Ligand	Phosephate ester	
Base matrix	cellulose particles	
Particle size（μm）	40 - 130	
Exclusion Limit (kDa)	30～40	150
Ion exchange capacity (meq/mL-gel)	0.3 - 0.8	0.2 - 0.8
Lysozyme adsorption (mg/mL-gel)	140	-
BSA adsorption (mg/mL-gel)	-	100
Pressure（MPa）	＜0.2	
pH Stability	5 - 12	
Storage	2-8 ºC in 20 % ethanol	

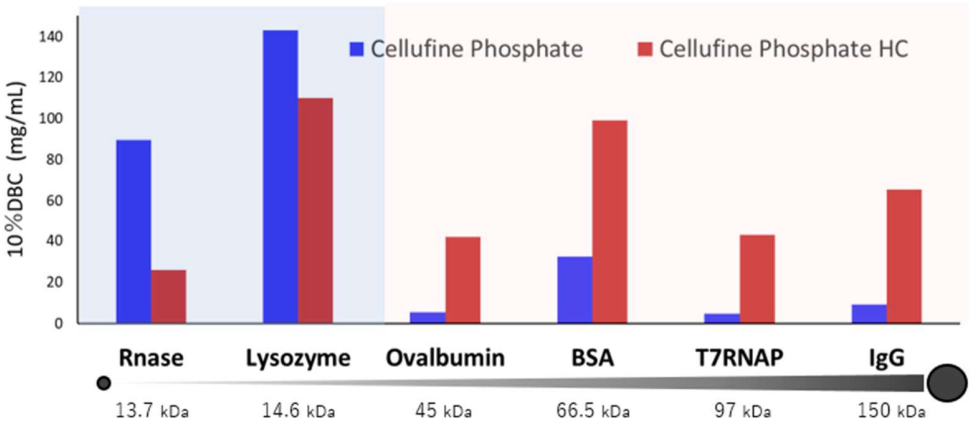
Fig. 1 Structure of Cellufine™ Phosphate HC



Adsorption performance of Cellufine™ Phosphate HC

Using a model protein, the protein adsorption capacity of Cellufine™ Phosphate HC and Cellufine™ Phosphate were compared (Figure 2).

Figure 2 Dynamic binding capacity with model proteins



Cellufine™ Phosphate has a high adsorption capacity for small molecular weight proteins such as lysozyme. This is because the pore size is optimized for proteins with relatively low molecular weights. On the other hand, Cellufine™ Phosphate HC has an extremely high adsorption capacity for proteins of 45 kDa or more. In particular, its adsorption capacity for T7RNAP, which is attracting attention in the synthesis of mRNA drugs, is nine times greater than that of conventional products.

In this way, the two phosphate media can be used depending on the molecular weight of the protein.

Affinity activity to other enzymes

Cellufine™ Phosphate HC exhibits strong affinity not only for T7RNAP but also for other enzymes such as pyrophosphatase (Figure 3) and vaccinia capping enzyme (Figure 4). After pyrophosphatase was adsorbed onto the column in 10 mM phosphate buffer, pH 7, the sodium chloride concentration was increased using a gradient, and the electrical conductivity was measured as the enzyme eluted from the column (Figure 3). The electrical conductivity at the elution peak was 74.5 mS/cm (equivalent to 0.9 M NaCl), indicating strong adsorption. Furthermore, the elution peak of vaccinia capping enzyme reached a maximum at 50 mS/cm (Figure 4).

At such a salt concentration, contaminating proteins adsorbed by electrostatic interactions do not remain in the column, allowing for effective purification of enzymes with affinity activity.

Figure 3 Electrical conductivity during pyrophosphatase elution

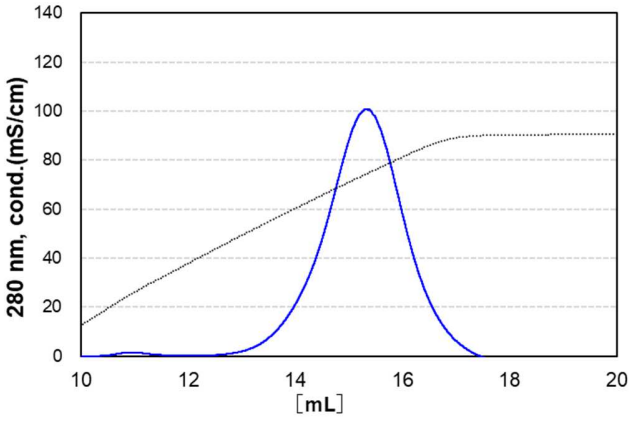
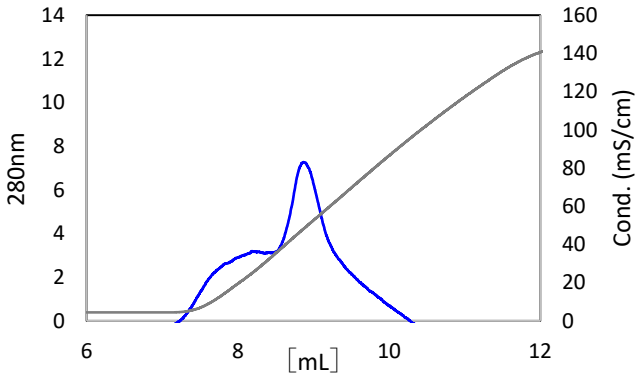


Figure 4 Electrical conductivity during elution of vaccinia capping enzyme



Purification example of T7 RNAP

T7RNAP can be highly purified using Cellufine™ Phosphate HC and the anion exchange chromatography media Cellufine™ MAX DEAE. Below are examples of T7RNAP purification using two Cellufine™ columns.

1. Protein expression

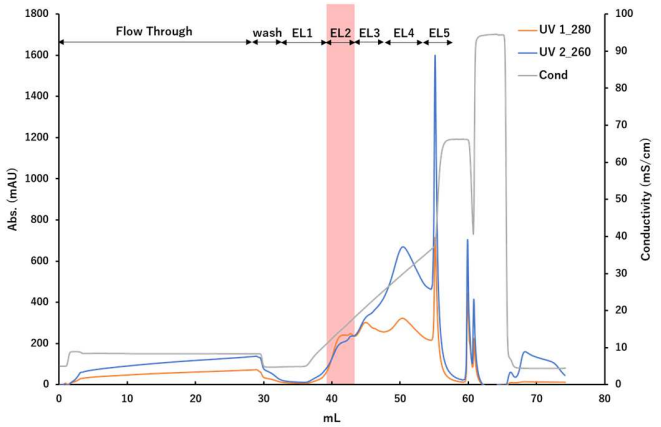
First, recombinant E. coli (pAR1219) expressing T7RNAP was cultured, and T7RNAP expression was induced with IPTG. The cells were harvested by centrifugation (5000 x g, 5 min) and washed with PBS. The pellet was suspended in lysis buffer, and lysed by adding lysozyme and freezing and thawing three times. Ammonium sulfate was added to the lysate at 35% (w/v) and stirred at 4°C for 60 min. The supernatant was discarded after centrifugation (4°C, 15000 x g, 60 min), and the precipitate was collected. These procedures prepared a crude extract containing T7RNAP.

2. Ion exchange chromatography

The ammonium sulfate precipitate was suspended in equilibration buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, protease inhibitor (+)) to reduce the conductivity to below 10 mS/cm.

As a first step, the enzyme was purified using Cellufine™ MAX DEAE, a weak anion exchange chromatography meida, and the enzyme activity and impurity behavior were analyzed.

Figure 5 Chromatogram with MAX DEAE



Column conditions

Load: Ammonium sulfate precipitate suspension (<10 mS/cm) filtered through a 0.22 µm PVDF syringe filter.

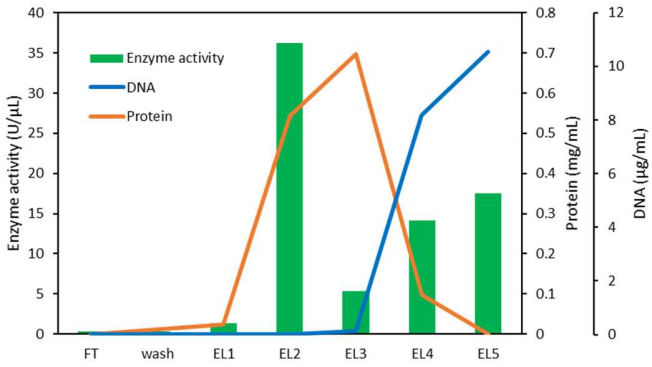
Column: ID 6.7 mm x 3.0 cm (1.0 mL)

Flow Rate: 0.5 mL/min (RT 2.0 min)

Equilibration: 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, protease inhibitor (+)

Elution: 10 mM Tris-HCl pH 7.5, 1.0 M NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, protease inhibitor (+)

Figure 6 Impurities in each fraction



FT: flow-through fraction, Wash: washing fraction, EL1-EL5: elution fractions in gradient elution

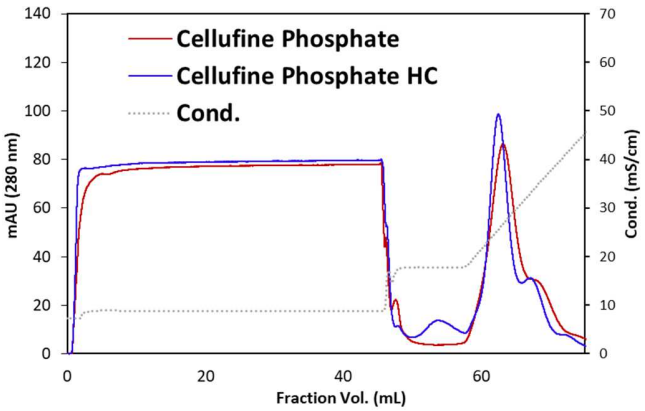
The chromatogram of the purified product chromatographically using Cellufine™ MAX DEAE is shown in Figure 5. The salt concentration was increased using a gradient during the elution step. The eluted fractions were divided into five samples (EL1-EL5) to confirm the impurity profile. The DNA content, protein content, and enzyme activity of each fraction were measured to analyze the impurities.

The elution fraction with high enzyme activity (EL2) and the elution fraction with a large amount of host-derived DNA (EL4-EL5) were separated, and the EL2 fraction was then purified by affinity chromatography.

3. Affinity chromatography

The eluted fractions of Cellufine™ MAX DEAE (EL2 fraction) were pooled, diluted approximately three-fold with equilibration buffer, and highly purified with Cellufine™ Phosphate HC or Cellufine™ Phosphate (Figure 7).

Figure 7 High-purity purification with Phosphate resins



Column conditions

Column volume: 1.06 mL

Flow rate: 0.53 mL/min (RT 2 min)

Equilibration: 10 mM potassium phosphate pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, protease inhibitor

Elution: Equilibration buffer + 1 M NaCl

After chromatography on Cellufine™ Phosphate HC, enzyme activity, protein content, and DNA content were measured (Table 2). Compared to the load sample, the amount of contaminating proteins was reduced by 32% and the amount of DNA by 42%.

Table 2 Purity analysis after Phosphate

	Enzyme activity (U)	Protein (ng/Unit)	DNA (pg/Unit)
Load Sample	-	5.27	1.14
Cellufine™ Phosphate	626,816	1.53	0.58
Cellufine™ Phosphate HC	603,621	1.69	0.49

4. Consideration

As shown in Figure 8, T7RNAP can be highly purified by undergoing a pretreatment process including lysis and ammonium sulfate precipitation followed by two stages of chromatography purification. The degree of purification at each stage was evaluated by SDS-PAGE (Figure 9). Impurities are removed at each chromatography step, and after purification with Cellufine™ Phosphate HC, almost a single band is obtained, demonstrating that T7RNAP has been purified to a high degree.

Figure 8: Overview of the T7RNAP purification process

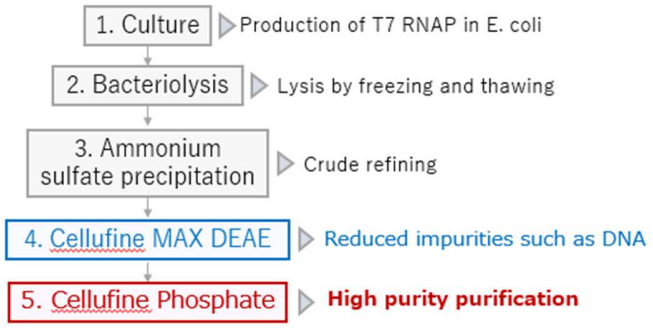
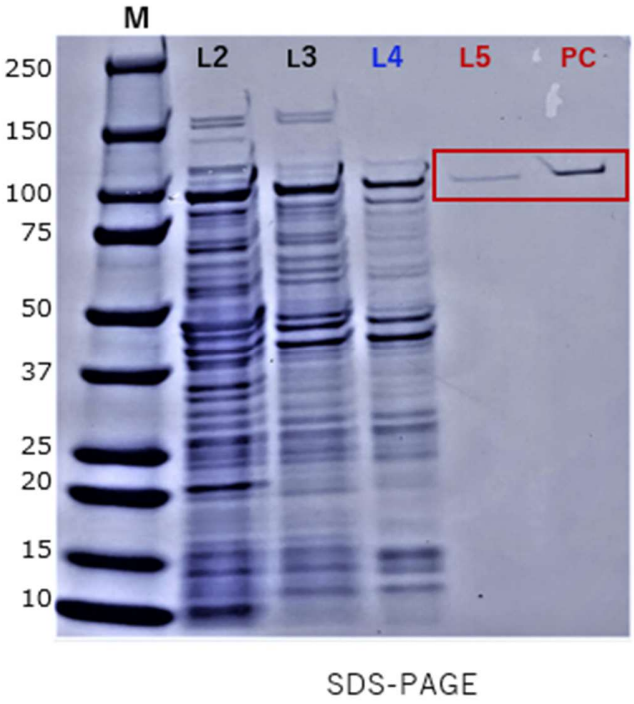


Figure 9 Purification of T7RNAP after each step (SDS-PAGE)



L2: After lysis

L3: After ammonium sulfate precipitation

L4: Cellufine™ MAX DEAE purification

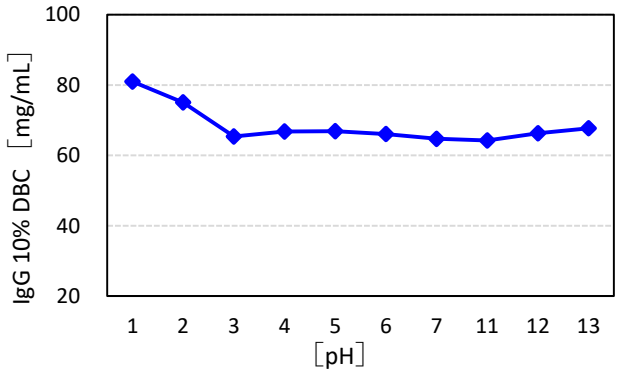
L5: Cellufine™ Phosphate HC purification

PC: Commercially available product

5. Clean-in-place and chemical stability

Cellufine™ Phosphate HC is able to consistently demonstrate high performance over a wide range of pH (Figure 10). It is particularly stable in the alkaline range above pH 3. The media was immersed in buffers adjusted to each pH for 7 days, after which the dynamic binding capacity was measured. Contrary to expectations, the dynamic binding capacity increased in the pH range below pH 3. The phosphate ester group, which serves as the ligand, is released at low pH. However, moderate release of the ligand resulted in an unintended improvement in intrapore diffusion. Because ligand release is observed in such low pH ranges, great care must be taken when using this media.

Figure 9 pH stability



Column Conditions

Load: IgG

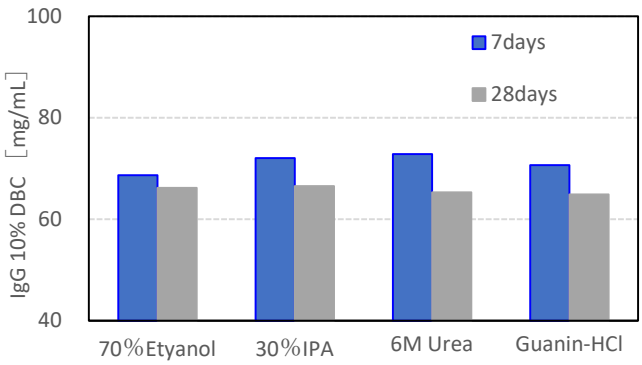
Column: 1.06 mL

Flow Rate: 0.265 mL/min (RT 4 min)

Soaking Time: 7 days

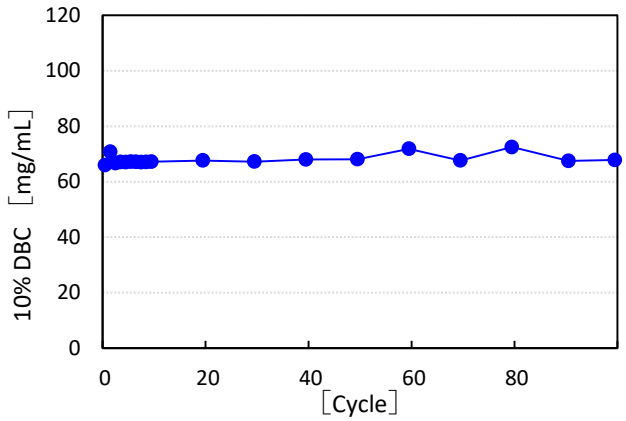
Cellufine™ Phosphate HC is chemically stable against 70% ethanol, 30% IPA, 6M urea, and guanine-HCl aqueous solutions (Figure 11).

Figure 11 Chemical stability



A repeated test was carried out using a 0.5M NaOH as the cleaning in place (Figure 12). No change in the dynamic adsorption amount was observed after 100 repeated tests, indicating stable use.

Figure 12 Cleaning in place with 0.5M NaOH



Column conditions

Load: IgG

Column: 1.06 mL

Flow Rate: 0.265 mL/min (RT 4 min)

CIP: 0.5M NaOH aqueous solution

Endotoxin removal using Cellufine™

When producing proteins using E. coli as a host, it is preferable to remove endotoxins derived from the host. For endotoxin removal, Cellufine™ ET Clean, an affinity chromatography media based on JNC's proprietary technology, is effective.

Cellufine™ ET Clean – An affinity chromatography media capable of removing endotoxins derived from Gram-negative bacteria under conditions of physiological saline pH and ionic strength (ionic strength μ = 0.02 – 1.0, temperature 0 – 25 °C).

ET Clean S

Pore exclusion limit is 2,000 Da, and many proteins cannot enter the pore.

ET Clean L

Pore exclusion limit molecular weight > 2 x 106 Da, most proteins can enter the pore

Ordering Information

Product	容量	カタログ No.
Cellufine™ Phosphate HC	5 x 1 ml Mini-Column	19400-15
	1 x 5 ml Mini-Column	19400-51
	10 ml	19400
	50 ml	19401
	500 ml	19402
	5 lt	19403
	10 lt	19405

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