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# Hydroxyapatite (CHT I, CHT II)

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## 1. Product Description

Hydroxyapatite Media (CHT) is a unique composite mode rigid filler in downstream purification. It has two combination modes:ion exchange and metal affinity, and has special separation capabilities that other fillers do not have. The protein of interest can be captured by calcium ions, or positively charged groups can be captured by phosphate. Hydroxyapatite medium is made by sintering inorganic substances at high temperatures and does not contain any animal or plant components. The medium can be applied to purify various biological macromolecules such as different types of monoclonal and polyclonal antibodies, double antibodies and antibody fragments, recombinant proteins, vaccines, enzymes, nucleic acids, virus particles, etc. Tiandiren and Company have two types of products, CHTI and CHTII. Each type provides three particle size options. The specific properties are shown in Table 1. CHT I is suitable for the purification of acidic proteins, and CHT II is more suitable for the purification of large particle biomolecules ( > 400KD) such as IgM, virus, VLP, plasmids, etc. due to its larger pore size. The powerful functions of hydroxyapatite media make it suitable for any stage of capture to purity.

Table 1. Characteristics of Hydroxyapatite

| Product model | CHT I | CHT II |
|---|---|---|
| Functional groups | $Ca^{2+}$, $PO_4^{3-}$,-OH | |
| Median particle size | 20 μm, 40 μm, 60 μm | |
| Aperture | 50-70 nm | 70-90 nm |
| Tap settled density | 0.71 g/ml | |
| Recommended flow rate | 50-1000 cm/h | |
| Observed dynamic binding capacity lysozyme(Lys) | 40-60 mg lysozyme/g CHT | 20-40 mg lysozyme/g CHT |
| Observed dynamic binding capacity IgG | > 40 mg IgG/ml | > 15 mg IgG/ml |
| pH stability | 6.5-14 , available in 1 M NaOH Medium storage for more than 1 year | |
| Reagent tolerance | 1 M NaOH, 6M Urea, 8M Guanidine Hydrochloride, Ethanol, Methanol, 100% Acetonitrile | |
| Sanitization | 1-2 M NaOH | |
| Regeneration | 0.4 M sodium phosphate buffer, pH 7-7.5; 1 M trisodium phosphate buffer, pH 11-12; For higher phosphate concentration, 0.4-1M potassium phosphate buffer can be used | |
| Autoclavability(bulk) | 121℃, 20 min | |
| Maximum pressure | 100 bar (1500 psi) | |
| Storage* | 0.1 M NaOH , Room temperature | |

* Before opening , please store it in the form of dry powder at sealed , dry and room temperature (4-20℃) . Here are the storage conditions after opening .

## 2. Purification Procedure

### 2.1 Chromatography column packing

#### 2.1.1 Column Packing Buffer

Phosphate-containing solutions of pH 6.5 or above, or high pH solutions, may be used. The recommended reagents are as follows:

- 20 mM phosphate, 150 mM NaCl, pH 6.8-8.0
- 0.1 M NaOH

#### 2.1.2 Weighing

Calculate the weight of chromatography medium required for packing:amount of CHT chromatography medium=column×swelling ratio,and weigh the required weight of medium;

Taking a filling volume of 20 ml as an example, the swelling ratio is 0.71, and the required amount of dry filler is: 20 ml×0.71 g/ml=14.2 g.

**2.1.3 Laboratory scale operation steps are as follows:**

1) suspending the required weight of the medium in 3.5 times the volume of the column packing buffer with a plastic stirring rod to fully swell the medium;
2) standing to separate the air in the packing;
3) clamping the empty chromatography column with a clamp, leveling the empty chromatography column;
4) infiltrate the bottom sieve plate, reserve 1-2 cm of column packing buffer, and close the outlet of the chromatography column;
5) Resuspend the packing with a plastic stir bar, and then pour it into the extension tube of the chromatography column along the column wall;
6) Open the bottom outlet of the chromatography column, turn on the pump and allow it to proceed at the set flow rate (600-1000 cm/h). The column should be packed at a flow rate of at least 2 times the expected operating flow rate;
7) Close the outlet of the chromatography column, remove the extension tube, and insert the adapter so that it just touches the surface of the medium;
8) connecting the chromatography system, opening the outlet of the chromatography column, and continuing to balance 3-5 column volumes with a flow rate of 250 cm/h;
9) adjusting the adapter to just contact the balanced medium surface;

**2.1.4 The production scale operation steps are as follows:**

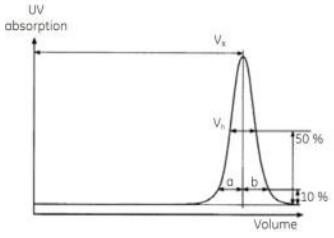
1) Weigh 710 g of medium dry powder per liter of column volume, suspend the medium in 1.82 L column buffer with a plastic stirring bar, and prepare a 50% (V/V) homogenate;
2) Use paddles or overhead impellers to stir and keep the filler suspended (stirring devices that generate shear forces cannot be used);
3) Close the drain outlet of the chromatography column, inject the homogenate into the chromatography column by decantation method or diaphragm pump, install the top adapter into the chromatography column, wait for the medium to settle after 5 minutes and the supernatant appears on the top, slowly lower the adapter to remove the top air, and clean the flow collector and inlet line on the adapter with column packing buffer;
4) Run 2 column volumes at a flow rate of 200-300 cm/h. After the medium surface no longer sinks, lower the adapter so that there is a distance of 1-5 mm between the medium fixed position plate and the top of the chromatography column. The column should be packed at a flow rate of at least 1.5 times the expected operating flow rate, and the column can be run at a lower flow rate (approximately 60 cm/h) for extended periods of time to remove air from the column bed. Do not lower the adapter into the media bed, causing Irreversible damage of CHT particles;

**2.1.5 Column efficiency evaluation**

1) The installed chromatographic column is tested for column efficiency according to the table below;

| | |
|---|---|
| Sample | 0. 1-1% acetone |
| Sample Load Amount | 1% Column Volume |
| El uent | Water |
| Linear flow rate | 60-100 cm/h |
| Detection | UV280 |

2) Column efficiency calculation;
3) Calculate theoretical tray height (H ETP), theoretical tray number (N) and asymmetry factor (As) according to UV or conductivity curve, formula: $HETP=L/N$, $N=5.54(V_R/Vh)^2$;
Where: $V_R$ = Retention volume
$V_h$=half peak width
L=column height
N=Number of theoretical plates
The units of Ve and Vh should be consistent;
As=a/b
Wherein: a=first half-width at 10% peak height, b=first half-width at 10% peak height;




4) Results evaluation
If the value of HETP calculated by the above formula is 2-4 times the average particle diameter of the medium, and the asymmetry factor is 0.8-1.8, it is judged as acceptable, and the value of HETP calculated by the above formula is 2-4 times the average particle diameter of the medium is 2-4 times, and the asymmetry factor is 0.8-1.8.

### 2.2 Chromatographic method

#### 2.2.1 Recommended purification reagents for different samples

| Procedure | Antibody purification reagent | Nucleic acid purification reagent |
|---|---|---|
| Rinse | Deionized water | Deionized water |
| Balance | 400 mM PB , pH 6.5<br>10 mM PB,4-8 ppm $Ca^{2+}$ ,pH 6.5 (can increase $Ca^{2+}$ concentration to improve media stability) | 400 mM PB, pH7.0<br>10 mM PB,1 mM EDTA , pH 7.0 (Note: EDTA can shorten the service life of the media) |
| Loading | 10 mM PB, 4-8 ppm $Ca^{2+}$, pH 6.5 | No non-acetate alkaline lysate in the sample |
| Rinse | 10 mM PB, 4-8 ppm $Ca^{2+}$, pH 6.5 | 10 mM PB, 1 mM EDTA, pH7 .0 |
| Elution | 10 mM PB , 15 ppm $Ca^{2+}$ , 0-2 M NaCl (linear elution) , pH 6.5 (increase phosphate concentration if no elution peak occurs) | 0-400 mM PB (linear elution) , 1 mM EDTA , pH 7.0 |
| Washing | 400 mM PB, pH 7.0-7.5<br>Deionized water | N/A (Note: This is a single cycle purification step , more cycles require method development) |

| Procedure | Virus purification reagent | Acidic protein purification reagent |
|---|---|---|
| Rinse | 600 mM PB, pH 7.2 | Deionized water |
| Balance | 10 mM PB, pH 7.2 | 400 mM PB , pH 6.7<br>5 mM PB, 12-20 ppm $Ca^{2+}$ , 50-100 mM NaCl , pH 6.7 (can increase Ca2 + concentration to improve media stability) |
| Loading | 10 mM PB, pH 7.2 | 5 mM PB, 12-20 ppm $Ca^{2+}$ , 50-100 mM NaCl , pH 6.7 |
| Rinse | 10 mM PB, pH 7.2 | 5 mM PB, 12-20 ppm $Ca^{2+}$ , 50-100 mM NaCl , pH 6.7 |
| Elution | 10-600 mM PB ( Linearity elution) | From 5 mM PB, 12-20 ppm $Ca^{2+}$, 50-100 mM NaCl, pH 6.7 to 120 mM PB, 50-100 mM NaCl, pH 6.7 |
| Washing | 600 mM PB, pH 7.2<br>800 mM potassium phosphate , pH 7-7.5 | 400 mM PB , pH 6.7 deionized water |

#### 2.2.2 Sample preparation

Before loading, ensure that the sample solution has the appropriate ionic strength and pH, the sample can be dialyzed or diluted with equilibrium or wash solution. It is recommended to centrifuge or filter the sample with a 0.22 μm or 0.45 μm membrane before loading to reduce impurities, increase protein purification efficiency and prevent column clogging.

#### 2.2.3 Purification by chromatography column

After the medium is filled, a variety of conventional medium and low pressure chromatography systems can be used.

1) Fill the pump pipe with deionized water. Connect the column to purification system, “drop to drop” to avoid introducing air into the column.

2) Rinse out the storage buffer with 3-5 times the column volume of deionized water.

3) Equilibrate the column with an equilibrium solution of at least 5 times the column bed volume.

4) Use a pump or sample ring to load the sample (the sample load should not exceed 80% of the medium load). Note: The increased viscosity of the sample causes even a small load volume to cause a large back pressure on the chromatography column, and a large sample volume may also cause a large back pressure, making the injector more difficult to use.

5) Rinse the column with the eluent until the UV absorption reaches a stable baseline (typically at least 10-15 column volumes).

6) Linear elution: at least 20 column volumes are eluted with 0-100% eluent, and the eluent, i.e. the protein component of interest, is collected.

Isocratic elution: The eluent elutes at least 15 column volumes. After the first test, the subsequent amplification purification can be carried out. The eluent with a selected salt concentration of 5-10 times the column volume can be used for elution. This method is beneficial for the antibody to elute at a relatively concentrated concentration, thereby reducing the use of eluent and the cycle purification time.

7) After the elution is completed, the medium is washed with 5-10 times the column volume of washing buffer to remove strongly bound impurities.

## 3. Cleaning-in-Place and storage

### 3.1 Cleaning and regeneration

After the chromatography medium is used for a period of time, the column efficiency may decrease, and the separation effect may decrease. The following process can be used for cleaning and regeneration.

1) Flush 2 column volumes with equilibrium solution;

2) Rinse 3-5 column volumes with 400 mM phosphate (if higher concentration of phosphate is required to remove tightly bound impurities, potassium phosphate can be used considering the solubility and dissolution rate of sodium phosphate; 1-2 M KCl/NaCl containing 5mM phosphate, 8 M urea or 6M guanidine hydrochloride, pH 6.5-7.5 can also be used for washing);

3) Rinse 1-2 column volumes with less than 20 mM phosphate;

4) Rinse 1-2 column volumes with 1.0 M NaOH;

5) Flush 4 column volumes with equilibration solution.

### 3.2 Sterilization

The medium can be treated with 1.0 M NaOH for 60 min or sterilized in phosphate buffer (pH7) at 121 ℃ for 20 min.

### 3.3 Long-term storage

Unopened dry powder-packed media can be stored at room temperature for five years under dry and sealed conditions. If the opened medium needs to be stored for a long time, it is recommended to use 0.1 M NaOH solution, and store it in a cool and closed shade at room temperature, avoiding direct sunlight.

It is not recommended to use azide-containing compounds or chlorhexidine-containing antibacterial agents to preserve fillers to prevent the growth of bacteria.

## 4. Related Products

| Name | Item Number | Specifications |
|---|---|---|
| Hydroxyapatite 60 (CHT I 60) | SA111005 | 5g |
| | SA111020 | 20g |
| | SA111100 | 100g |
| | SA111500 | 500g |
| | SA1111 KG | 1 KG |
| Hydroxyapatite 60 (CHT II 60) | SA112005 | 5g |
| | SA112020 | 20g |
| | SA112100 | 100g |
| | SA112500 | 500g |
| | SA1121 KG | 1 KG |
| Hydroxyapatite 40 (CHT I 40) | SA106005 | 5g |
| | SA106020 | 20g |
| | SA106100 | 100g |
| | SA106500 | 500g |
| | SA1061 KG | 1 KG |
| Hydroxyapatite 40 (CHT II 40) | SA107005 | 5g |
| | SA107020 | 20g |
| | SA107100 | 100g |
| | SA107500 | 500g |
| | SA1071 KG | 1 KG |
| Hydroxyapatite 20 (CHT I 20) | SA113005 | 5g |
| | SA113020 | 20g |
| | SA113100 | 100g |
| | SA113500 | 500g |
| | SA1131 KG | 1 KG |
| Hydroxyapatite 20 (CHT II 20) | SA114005 | 5g |
| | SA114020 | 20g |
| | SA114100 | 100g |
| | SA114500 | 500g |
| | SA1141 KG | 1 KG |