WorkBeads 40 TREN

WorkBeads™ 40 TREN resin for multimodal Ion Exchange Chromatography (IEX) has a ligand that is positively charged below approx. pH 9. The resin can be used for several different applications, e.g., for multimodal IEX, for sample cleanup in monoclonal antibody (MAb) purification processes in order to guard the Protein A column from chromatins and other host cell impurities, or as a polishing step in the MAb purification process.

- Improved selectivities through multimodal IEX separation
- Reduced fouling of Protein A resins by chromatin and host cell impurity removal
- High binding capacity and purity

Short protocol

This short protocol is for column packing of WorkBeads 40 TREN resin and general steps for purification. Detailed instructions and recommendations for optimization are given later in this instruction.

1. Make a slurry of the desired resin concentration.

2. Pour the slurry into the column.

3. Pack the resin with an appropriate flow rate.

4. Apply an axial compression of less than 2%.

5. Equilibrate the column with binding buffer.

6. Apply sample.

7. After sample application, remove unbound material by washing with, e.g., 20 - 30 CV (column volumes) washing buffer.

   Note: If the resin is used in “negative mode” the target molecule with elute in the wash step.

8. Elute the target protein with elution buffer.

9. Wash the column with deionized water.

10. Equilibrate the column with 20% ethanol for storage.

Principle

WorkBeads 40 TREN resins contain ligands based on Tris(2-aminoethyl)amine (TAEA). The structure of the ligand used in WorkBeads 40 TREN is shown in Figure 1.

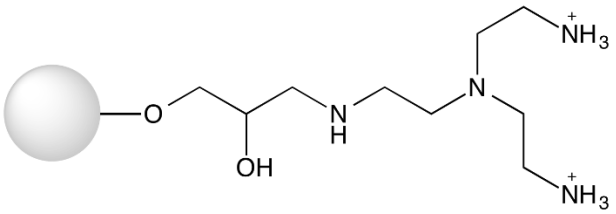

Figure 1. Structure of the ligand used in WorkBeads 40 TREN.

Multimodal ion exchange chromatography

Multimodal ion exchange chromatography separates protein, peptides and other biomolecules via a ligand acting with more than one interaction sites. The interaction utilizes two, or more, different properties for example charge and hydrophobicity. Depending on the chromatographic conditions the interactions differs, and work either together or separate in the purification procedure.

Using multimodal ion exchange chromatography can be an excellent alternative, when the common techniques (e.g., ion exchange chromatography, size exclusion chromatography or affinity chromatography) are insufficient. However, to reach the optimal purification using multimodal chromatography, the purification process need to be optimized according to the properties of the target molecule.

WorkBeads 40 TREN in MAb purification

Purification of monoclonal antibodies usually involves purification on Protein A chromatography resins followed by polishing steps based on anion- or cation exchange chromatography. The presence of chromatin fragments (fragments of chromosomes, composed of histone proteins and DNA) are a major cause for fouling of Protein A columns, and is also a key impurity in to be removed during MAb purification. Chromatin particles are heavily charged structures with massive negative net charges. Due to this, they can easily be adsorbed on WorkBeads 40 TREN, which has proved to be useful for removal of chromatin and other impurities such as nucleic acids, endotoxins and host cell proteins (Nian et al., J. Chromatogr. A, 1431 (2016) 1-7; Chen et al., J. Biotechnol., 236 (2016) 128-140.)

The use of WorkBeads 40 TREN in binding or flow through mode will also facilitate removal of nucleic acids, endotoxins, viruses, host cell proteins and other cell-derived impurities. As Protein A ligands may be cleaved by proteases, leached Protein A ligands can be removed by a polishing step using WorkBeads 40 TREN after the Protein A purification step. Notice that the majority of MAbs are basic, thus are mainly positively charged at neutral pH or low pH, and therefore do not bind to the resin.

The characteristics of WorkBeads 40 TREN can be exploited in several ways in MAb purification process:

1. As a precipitation agent added to the feed to induce chromatin precipitation for easy removal by continues centrifugation followed by depth filtration before the Protein A step. Only 0.5 - 5 g resin/ml feed is generally needed.

2. As a guard column for removal of chromatin and other impurities before the Protein A column.

3. In a polishing step after the Protein A purification step.

Column packing

WorkBeads resins are cross-linked using a proprietary method that results in a rigid resin that tolerate pressures of several bars and consequently can run at high flow rates. Follow both this general advice when packing a column and the column manufacturer's specific instructions. Preferably, use a column with an adjustable adaptor. In some instances, a packing reservoir or column extension may be needed.

Note: Always make sure that the maximum pressure of the column hardware is not exceeded. Backpressure caused by the chromatography system components connected downstream of the column may reduce the maximum flow that can be used. Wear eye protection.

1. Wash the resin

The resin is provided in 20% ethanol. To avoid undue backpressure when packing, wash the desired amount of resin with several column volumes of deionized water before packing, and use water for packing.

2. Make a slurry

Add deionized water to the washed resin to obtain a 40% to 60% slurry concentration. Make approximately 15% extra slurry to compensate for the compression during packing. The amount of slurry to be prepared can be calculated according to:

$$\text{Slurry volume} = \frac{\text{bed volume x } 100}{\text{\% slurry}} \text{ x } 1.15$$

3. Pour the slurry into the column

Pour the slurry slowly down the side of the column to avoid formation of air bubbles. Preferably, use a packing reservoir or an extra column tube to extend the column volume to accommodate the entire slurry volume during packing. If no packing adaptor is available packing can be done by stepwise additions and packing. Although not recommended this will give acceptable results for most applications.

4. Pack the bed

Pack the resin with a downward flow higher than the intended operational flow. We recommend 700 cm/h for columns up to 25 mm i.d. and with 200 mm bed height. Make sure the packing flow rate does not exceed the maximum pressure of the column hardware or the resin. The operational flow should not be more than 75% of the packing flow rate.

5. Close the column

When the bed height is constant mark the bed height on the column. Stop the flow and remove the packing reservoir or extra tube. Note that the bed height will increase temporarily when the flow is stopped. If needed, adjust the bed height by removing excess resin. Be careful not to remove too much resin. Gently fill the column with packing solution to its rim without disrupting the packed bed. Insert the adjustable adapter on top of the packed bed. Apply a small axial compression of less than 2% of the final bed height by lowering the adapter into the packed bed.

6. Apply a flow

Apply a flow of 450 cm/h (taking account of section 4) and check for any gap formation above the surface of the resin bed. If a gap is observed, stop the flow and adjust the adaptor to eliminate the gap.

Purification

Multimodal IEX

WorkBeads 40 TREN resin is positively charged below approx. pH 9. Choose a suitable pH and buffer for the binding of the target protein or the impurities (if the purification is done in “negative mode”). One pH unit above pI, for WorkBeads 40 TREN is a good starting point. The binding conditions should be optimized to achieve binding of the target protein or the impurities. When scouting for the best conditions it is important to start with sufficiently low ionic strength. Guideline for a starting point for designing the experiment is given in Table 1.

Table 1. Typical buffer composition for purification using WorkBeads 40 TREN.

Product	Buffer	Buffer composition
WorkBeads 40 TREN	Binding buffer	50 mM Tris-HCl, pH 7.4
	Elution buffer	50 mM Tris-HCl, 1 M NaCl, pH 7.4

When the target molecule is bound to the resin elution can be carried out by applying a linear gradient of increasing concentration of NaCl, by gradually increasing the proportion of elution buffer (high salt). A short step gradient to 1 or 3 M NaCl for 5 column volumes (CV) can be included after elution to ensure desorption of all interacting proteins. When suitable elution conditions are known it is common to apply step gradient elution. A Cleaning-in-place (CIP) step using 5 CV 1 M NaOH is recommended between all runs, and should be followed by a careful re-equilibration before the next run. Make sure that elution with neutral salt, e.g., NaCl, has been done before CIP to avoid precipitation of adsorbed proteins.

Sample preparation

After cell disruption or extraction, clarify the sample by centrifugation at 10 000 - 20 000 × *g* for 15 - 30 minutes. It is generally also recommended to pass the sample through a 0.22 - 0.45 µm filter, e.g., a syringe filter, to avoid transferring any remaining contaminating particles onto the column. Large sample volumes may be clarified by filtration through depth filters or by tangential flow filtration, which may be cheaper and more efficient than investing in a large-scale centrifuge. Application of a sample that has not been properly clarified may reduce the performance and lifetime of the column.

The sample should have a pH that confers a net charge to the target protein that is opposite to the charge of the column resin if the focus is to bind the target protein. The ionic strength should be low. The optimal binding conditions depends on the combination of the pH and the ionic strength. The sample solution may therefore need to be adjusted before applied to the column. It is generally recommended that the sample should have a similar pH and conductivity as the binding buffer. Sample adjustments can be done by dilution using the binding buffer, by chromatographic desalting or diafiltration, or through adjusting the pH by addition of an acid or base. When the resin is used a as a guard column for removal of chromatin and other impurities before the Protein A column in a MAb purification process the target protein is washed out with the wash buffer after sample application.

Optimization

The following paragraphs will give indications on some parameters that can be tuned to get the optimal conditions for purification of using WorkBeads 40 TREN resin.

Selection of buffer

Selecting a buffer with optimal conditions for the target protein will improve the result of the purification. The buffer should be chosen with a pK_a-value within 0.5 units from the intended pH to obtain a high enough buffering capacity. Table 1 shows one example of buffers which can be used for ion exchange chromatography, however the buffer choice will be depending on the target molecule and aim of the purification procedure. For other useful buffers and their pK_a-values at 25 °C see reference: Methods in Enzymology, Volume 463, pp 46-47, Burgess, R.R and Deutcher M.P.

The buffer substance should be selected to have the same charge as the resin. A buffer with opposite charge will interact with the charged groups in the resin and may cause local pH disturbances that destroys the separation. Usually, low conductivity in the binding buffer is preferred but optimization with regard to pH and conductivity can improve binding capacity. An increase in ionic strength may decrease the ability of contaminants to bind while the target protein remains bound. However, chromatographic conditions should be chosen so that the protein is stable during purification.

Optimization of binding conditions

The key conditions to be optimized is usually pH and conductivity (by addition of NaCl or other salts, or dilution). The conditions must also be selected to keep the protein in its native state.

The flow rate during sample loading affects the binding capacity and resolution during the elution. A low flow rate during sample application promotes binding capacity since more time is allowed for mass transport of the target substance into the pores of the resin. A small substance, e.g., a peptide, that has a high diffusion rate will have fast mass transport into the resin and can thus be adsorbed efficiently at high flow rates. A large target substance (e.g., a large protein) has a lower diffusion rate and is more hindered by the walls in the pores giving slow mass transport, causing reduced dynamic binding capacity. A high binding capacity of this substance may thus require a lowered flow rate. If only a part of the binding capacity of the column is used the sample application can be at a higher flow rate without loss of the target substance.

The residence time can be defined as the time between entering and exiting the column of specific part of the sample or buffer. The residence time depend on the flow rate and the dimensions of the column, and is typically 1 to 5 minutes in IEX. Typical linear flow rates are 150 - 300 cm/h. See further discussion about flow in the section *Scale-up*.

Optimization of washing

A continuously decreasing UV signal is an indication of unbound material still being washed out. The washing should continue until the UV signal is stable and the same as in the washing buffer, or at least not more than 20 mAU. The washing buffer can be the same as the binding buffer, but it may be useful to add an additional step with a dedicated washing buffer to improve purification.

Optimization of elution conditions

Elution can be carried out using a high salt concentration or by altering the pH to change the charge of the adsorbed protein. A stronger binding may require higher salt concentration for elution. The optimal salt concentration is dependent on the purity and recovery requirements as well as the properties of the target protein and the sample. Gradient elution often gives better purity than step elution, but step elution may be desired to obtain the highest possible concentration of the target protein, and to reduce the purification process time. In order to optimize the salt concentration for step elution an initial gradient test run can be carried out to obtain suitable step elution conditions for purification of the sample, see Figure 2.

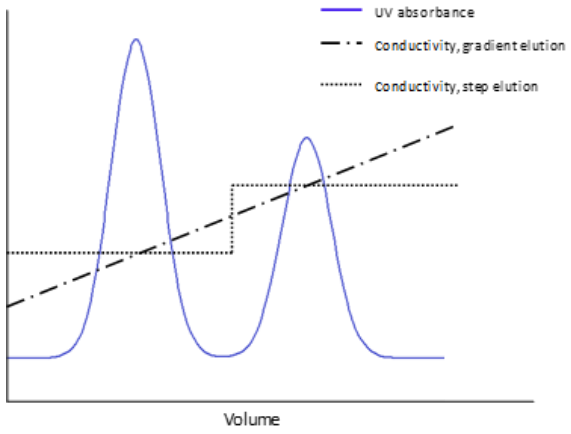

Figure 2. Optimization of step elution with salt. A test run with linear gradient elution gives information about suitable salt concentrations to be used in step elution. Note: Remember to take the system dead volume into account when comparing the print out of the gradient and the trace.

Scale-up

After developing a chromatographic procedure in a small scale column, e.g., 10 (i.d.) × 100 mm (bed height), WorkBeads resins can be packed into larger columns for scale-up. Large scale purification is often carried out in columns with bed heights of 200 - 300 mm.

Scale-up principles

During scale-up the ratio between sample volume and column volume should be kept constant. The column volume is scaled up by increasing the column diameter while keeping the bed height the same (e.g., 200 mm). The linear flow rate should remain the same while the volumetric flow rate increases. The volumetric flow rate for each column can be calculated according to:

$$\text{Volumetric flow rate (ml/min)} = \frac{\text{Linear flow rate (cm/h)} \times \text{Column cross sectional area (cm}^2\text{)}}{60}$$

Flow

The concepts of volumetric flow, linear flow rate and residence time is important when doing scale-up in chromatography. Volumetric flow is measured in ml/min or l/min, linear flow in cm/h and residence time in minutes. The relationship between these metrics are:

$$\text{Linear flow rate (cm/h)} = \frac{\text{Volumetric flow (ml/min)} \times 60}{\text{Column cross sectional area (cm}^2\text{)}}$$

$$\text{Residence time (minutes)} = \frac{\text{Column bed height (cm)} \times 60}{\text{Linear flow rate(cm/h)}}$$

In the initial process development work it is common to use a small column, e.g., 10 × 100 mm, to save sample, buffers and time. This column has a shorter bed height than the final column which may have a bed height of 200 mm or more. The flow rate for the larger column can be calculated from the flow that was established on the small column, using the equation above by keeping the residence time of the small column the same for the larger column. This will allow an increase of the linear flow in proportion to the increase in bed height between the columns see Table 2 for examples. If the column bed heights are kept constant during scale-up the linear flow rate should be kept constant (as well as the residence time).

Table 2. Example of scale-up parameters.

Column dimension	Residence time (minutes)	Linear flow rate (cm/h)	Volumetric flow rate (ml/min)
10 × 100	4	150	1.96
25 × 200	4	300	24.5
50 × 200	4	300	98.2

Additional purification steps

Optimization of the purification process by tuning the binding, washing and/or elution conditions of the IEX purification step may not be enough to obtain the required purity. Combining two or more purification step based on additional chromatography techniques is then recommended. For example, cation exchange chromatography and anion exchange chromatography can be combined in a purification process. Other techniques, such as size exclusion chromatography (gel filtration) and hydrophobic interaction chromatography (HIC) are commonly used alternatives. Each purification step should be thoroughly optimized, and preferably in the context of the other steps applied on the overall process.

Desalting and buffer exchange

Buffer exchange or desalting of a sample can be used before analysis and/or after purification by ion exchange chromatography. This can be carried out quickly and easily in lab-scale using BabyBio Dsalt 1 or 5 ml columns (see *Related products*). BabyBio Dsalt columns are also a useful alternative to dialysis for larger sample volumes or when samples need to be processed rapidly to avoid degradation. For large processes diafiltration is recommended.

To find out more about Bio-Works chromatography resins for additional purification, please visit www.bio-works.com

Chromatin precipitation process

During MAb purification impurities from the sample may gradually build-up in the resin and cause fouling. For example the presence of chromatin fragments (fragments of chromosomes, compoased of histone proteins and DNA) are a major cause for fouling of Protein A resins, and is also a key impurity to be removed during MAb purification.

Chromatin removal can successfully be carried out using WorkBeads 40 TREN as follows (see references Chen *et al.*, Journal of Biotechnology, 236(2016)128-140, Gagnon *et al.*, Journal of Chromatography A, 1408(2015)151-160 and Gagnon *et al.*, Journal of Chromatography A, 1374(2014)145-155):

1. Centrifuge the harvested cell culture at 4000 *x g* for 20 minutes in room temperature.
2. Collect supernatant and filtrate through 0.22 µm filter (e.g., Nalgen® Rapid-Flow Filters, Thermo Scientific).
3. Add 1.0% (w/v) allantoin and 0.4% (v/v) caprylic acid to the supernatant and adjust pH to 5.3 using 1.0 M acetic acid.
4. Agitate for 2 hours at room temperature.
5. Add 5% (v/v) pre-equilibrate WorkBeads 40 TREN (50 mM MES, 150 mM NaCl, pH 5.3) to the ~~cell culture~~ supernatant.
6. Agitate for 4 hours (or more if necessary) at room temperature.
7. Centrifuge the sample at 4000 *x g* for 20 minutes at room temperature for removal of solids.
8. If necessary, pass supernatant through a filter, for example, a SartoClear®PC1 Cap, from Sartorius.

Maintenance of the resin

Unpacking and inspection

Unpack the shipment as soon as it arrives and inspect it for damage. Promptly report any damage or discrepancies to your local supplier.

Cleaning and sanitization

During purification impurities such as cell debris, lipids, nucleic acids and protein precipitates from the samples may gradually build-up of contaminants in the resin, or fouling. The severity of this process depends on the type of sample applied to the column, and the pre-treatment of the sample. The impurities covering the resin may reduce the performance of the column over time. Regular cleaning (Cleaning-in-place, CIP) keeps the resin clean, reduces the rate of further fouling, and prolongs the capacity, resolution and flow properties of the column. Cleaning of a column using 1 M NaOH applied by a low reversed flow for 2 hours or overnight is often sufficient.

Sanitization (reduction of microorganisms) can be carried out using combinations of NaOH and ethanol, e.g., incubation with a mixture of 0.5 M NaOH and 40% ethanol for 3 hours. The sanitization procedure and its effectiveness will depend on the microorganisms to be removed, and needs to be evaluated for each case.

Storage

Store the resin at 2 to 25°C in 20% ethanol.

Additional information

Product description

	WorkBeads 40 TREN
Target substances	Proteins, peptides and oligonucleotides. Chromatin fragments.
Matrix	Rigid, highly cross-linked agarose
Average particle size[1] (D_{v50})	45 µm
Ligand	Tris(2-ethylaminoethyl)amine (TAEA)
Max flow rate (20 cm bed height and 5 bar)	600 cm/h
Chemical stability	Compatible with all standard aqueous buffers used for protein purification. Should not be stored at low pH for prolonged time.
pH stability	2 - 13
Storage	2 to 25 °C in 20% ethanol

1. The median particle size of the cumulative volume distribution.

Intended use

WorkBeads 40 TREN is intended for research, process development and industrial use only. The resin shall not be used for preparation of material for clinical or diagnostic purposes

Safety

Please read the associated Safety Data Sheets (SDS) for WorkBeads 40 TREN resin, and the safety instructions for any equipment to be used.

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
BabyBio TREN 1 ml	1 ml x 5	45 655 213
BabyBio TREN 5 ml	5 ml x 5	45 655 217
BabyBio Dsalt 5 ml	5 ml x 5	45 360 107
Bulk resins		
WorkBeads 40S	25 ml	40 200 001
WorkBeads 40Q	25 ml	40 100 001
WorkBeads 40 DEAE	25 ml	40 150 001

1. Other pack sizes can be found in the complete product list on our website www.bio-works.com

Ordering information

Product name	Pack size	Article number
WorkBeads 40 TREN	25 ml	40 603 001
	150 ml	40 603 003
	1 L	40 603 010

Orders: sales@bio-works.com or contact your local distributor.

For more information about local distributor and products please visit www.bio-works.com or contact us at info@bio-works.com

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