Bio-Works

INSTRUCTION

WorkBeads 40/1000 ACT
WorkBeads 40/10 000 ACT

WorkBeads™ 40/1000 ACT and WorkBeads 40/10 000 ACT are pre-activated resins that enable simple and reliable coupling of proteins, peptides and low-molecular weight substances for the preparation of customized chromatography resins or enzyme reactors. The bromohydrin active group reacts with thiol, amino and hydroxyl groups. Two different resin porosities are available to facilitate optimized coupling of ligands of different sizes, and to optimize the prepared affinity resin for target molecules of different sizes.

- Simple, reliable coupling procedure
- Stable covalent linkage
- Suitable for coupling of ligands containing thiol, amino and hydroxyl groups

Intended use

WorkBeads resins are developed and supported for both research and production-scale chromatography. WorkBeads resins are produced according to ISO 9001:2015, and Regulatory Support Files (RSF) are available to assist the process validation and submissions to regulatory authorities.

GoBio™ prepacked column family is developed for convenient, reproducible, and fast results and can be used from small scale purification through process development to full-scale manufacturing.

Safety

Please read the associated Safety Data Sheets (SDS) for WorkBeads 40/1000 ACT and WorkBeads 40/10 000 ACT and the safety instructions for any equipment to be used.

Unpacking and inspection

Unpack the shipment as soon as it arrives and inspect it for damage. Promptly report any damage or discrepancies to complaints@bio-works.com

Short protocol

This short protocol is for coupling ligand onto WorkBeads ACT resins. Detailed instructions and recommendations are provided later in this instruction. Recommended coupling buffers are listed in Table 1.

1. Wash the resin with deionized water on a glass filter, suction dry the resin.
2. Dissolve the substance to be coupled (the ligand) in suitable coupling buffer
3. Add the ligand-solution to the resin.
4. Incubate overnight with agitation.
5. Wash with buffer or deionized water to remove unreacted ligand. Suction the resin dry.
6. Block the remaining reactive groups by incubation overnight under agitation with a suitable blocking reagent, for example 1 M ethanolamine-HCl, pH 9.5.
7. Wash with buffer or deionized water to remove excess blocking reagent.
8. Use the resin for the intended application, or transfer to 20% ethanol for storage.

The resin may be packed in a chromatography column or be used as a suspension in a batch process.

Principle

The development of customized chromatography resins requires methods for covalent attachment of a functional ligand to the matrix. The ligand can be a protein, peptide, carbohydrate, or an organic substance. The WorkBeads 40/1000 ACT and WorkBeads 40/10 000 ACT resins contain bromohydrin groups that are reactive towards the nucleophilic N, S, or O atoms in primary amines (sulfhydryl, hydroxyl, aldehyde, carboxylic or histidyl groups), in the ligands to be coupled. The nucleophilic displacement reaction occurs at ambient temperature in aqueous solution under mildly alkaline or alkaline conditions to create a stable covalent bond between the resin and the ligand (Figure 1). There is no need for any additional reagent and the coupling does not create any additional charged groups. After ligand coupling, remaining active groups must be blocked. This is carried out by adding a blocking agent which reacts with the remaining bromohydrin groups. Ethanolamine or mercaptoethanol are often used as blocking agent, since the reaction introduce a $-CH_2CH_2OH$ group. For alkali-stable ligand/resins constructs NaOH can be used instead of a blocking agent to hydrolyse the remaining bromohydrin groups.

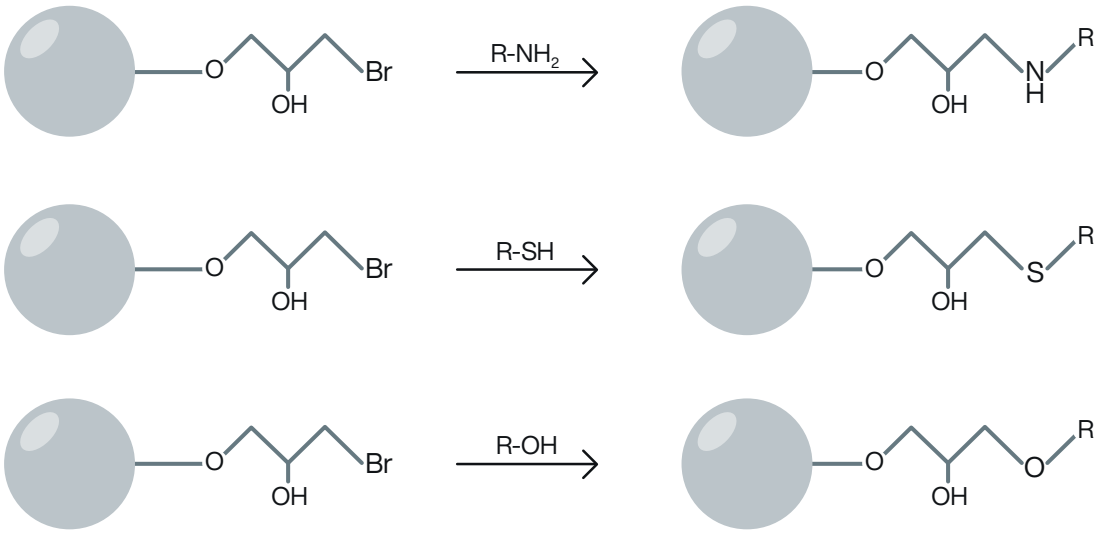

Figure 1. Reaction scheme for coupling a, from top to bottom, primary amine, thiol and alcohol to bromohydrin activated resin.

Instructions

This is a general protocol for ligand coupling of WorkBeads 40/1000 ACT or WorkBeads 40/10 000 ACT. Successful coupling may require further optimization of the conditions for coupling, blocking and washing conditions.

1. Prepare the resin

Wash the required volume of resin 3 times with 3 volumes (resin bed volumes) of deionized water on a glass filter with vacuum suction. Between washes, re-suspend the resin by careful stirring using a soft spatula. After the third wash, maintain the vacuum until the resin cake cracks (suction dry). Weigh the resin and transfer it to a suitable reaction vessel. The reactions can be carried out in a test tube, flask or in a reaction vessel with overhead stirring.

2. Prepare coupling solution with the ligand

Prepare 1 to 2 mL coupling solution per gram of suction dried resin. This corresponds to a slurry concentration in the final reaction mixture of approx. 33 to 50%. Dissolve the required amount of ligand in 100 mM sodium carbonate buffer, pH 8.5 or other suitable solution, see Table 1 and “Optimization”.

General recommendations: For protein and peptide, coupling it is typical to use 0.1 to 20 mg polypeptide per mL suction dried resin. For organic substances with low molecular weight, use 0.1 to 5 equivalents of ligand per single equivalent bromohydrin groups on the resin.

3. Prepare the coupling mixture

Add the entire volume of the prepared coupling solution (containing the dissolved ligand) to the reaction vessel, containing the prewashed, suction dried resin, and start agitation. Avoid magnetic stirrer since the magnet may grind the resin particles against the bottom of reaction vessel destroying the beads and cause fines.

4. Incubate

Leave the reaction to take place overnight at room temperature (approximately 16 hours). A different time may be required, “Optimization”.

5. Remove free ligand by washing

Transfer the ligand-coupled resin to a glass filter and wash using 3 – 10 volumes of coupling solution (not containing ligand), deionized water or other suitable solution (for buffers, etc., see “Optimization“). Suction dry and transfer the washed ligand-coupled resin back to the reaction vessel.

6. Block the remaining active groups

Add 1 mL blocking reagent solution, for example 1 M ethanolamine-HCl, pH 9.5, per 1 g suction dried ligand-coupled resin. Allow the blocking reaction to take place at room temperature overnight (16 hours) with agitation. Avoid magnetic stirring.

7. Wash to remove the blocking solution

Transfer the blocked resin to a glass filter. Wash with coupling buffer or deionized water until all excess of blocking agent is removed.

8. Transfer to storage solution

Wash the blocked resin with 3 volumes storage solution (e.g., 20% ethanol) or a suitable buffer, see “Maintenance” for suggestions regarding storage.

The ligand-coupled resin is now ready for use.

Table 1. Suggested coupling buffers. Other buffers may be used.

Type of ligand	Functional group of ligands	Coupling conditions
Proteins and peptides	Primary amino (-NH_2)	100 mM sodium carbonate buffer, pH 8 – 8.5[1]
	Sulphydryl (-SH)	200 mM sodium phosphate, pH 8
		Higher pH (within the protein stability range)
Organic molecules	Amino (-NH_2, -NH, -N)	Coupling pH determined by the ligand basicity[2]
	Sulphydryl (-SH)	pH 7 and higher
Carbohydrates	Hydroxyl (-OH)	pH > 12[3, 4]

1 Sufficient coupling without denaturation of sensitive polypeptides and proteins. Coupling reaction at lower temperature is also possible.
2 When the ligand is used in excess, dissolve it in deionized water and let the basicity of the ligand determine the coupling pH.
3 High pH is required due to the low nucleophilicity of the hydroxyl group.
4 Note: At this pH, cross-linking and hydrolysis will compete with the coupling reaction.

Column packing

When packing a column follow both this general advice and the column manufacturer's instructions. Preferably, use a column with an adjustable adaptor to allow a final minimal axial compression. 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 before coupling 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.

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:

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

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 600 cm/h for columns up to 26 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.

Evaluation of the packed column

Test the column efficiency to check the quality of packing. Testing should be done after packing, at regular intervals during the usage of the column or when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_s). These values are easily determined by applying a sample such as 1% acetone solution to the column.

For optimal results, the sample volume should be 2.5% of the column volume (CV) and the flow rate 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Note: The calculated number of plates will vary according to the test conditions and should only be used as a reference value. Keep test conditions and equipment constant so that results are comparable. Changes of for example solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.

Measuring HETP and A_s

Calculate HETP and A_S from the UV curve (or conductivity curve).

$$HETP = \frac{L}{N}$$

$$N = 5.54 \times \left(\frac{V_R}{W_h}\right)^2$$

L = bed height (cm)

N = number of theoretical plates

V_R = volume eluted from the start of sample application to the peak maximum

W_h = peak width measured as the width of the recorded peak at half of the peak height

V_R and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance. The reduced plate height, h, is calculated:

$$h = \frac{HETP}{d_{50v}}$$

d_{50v} = Median particle size of the cumulative volume distribution (cm)

As a guideline, a value of < 3 is very good.

The peak must be symmetrical, and the asymmetry factor as close to 1 as possible. (A typical acceptable range is $0.7 < A_S < 1.3$). A change in the shape of the peak is usually the first indication of bed deterioration.

Peak asymmetry factor calculation:

$$A_s = \frac{a}{b}$$

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

Figure 2 on next page shows a UV trace for acetone in a typical test chromatogram from which the HETP and A_s values are calculated.

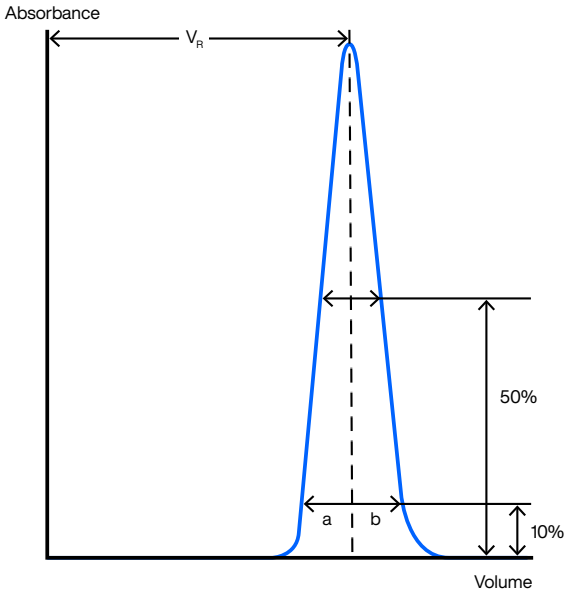

Fig 2. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

Optimization

Users should develop a specific coupling procedure suitable for the nature and stability of the specific ligand and the requirements of the intended application. There are several factors that can be optimise for the coupling protocol.

Selection of resin

The porosity of the resin used for ligand coupling may affect the amount of ligand that can be immobilized. A high porosity can accommodate larger ligands. On the other hand, high porosity resins have lower available pore wall surface area, which may limit the amount of ligand that can be attached to the resin. The optimum porosity for coupling a high amount of ligand may therefore differ between ligands of different sizes. It should also be noted that the porosity of the final resin (resin with attached ligand) may also affect the binding capacity of the target substance when the ligand-resin is used for affinity chromatography. WorkBeads 40/1000 ACT and WorkBeads 40/10 000 ACT has a cut-off of approximately 1×10^6 and 1×10^7 Da, respectively. WorkBeads 40/1000 ACT is recommended to be used for the immobilization of most proteins, peptides and low-M_r substances, and WorkBeads 40/10 000 ACT for larger proteins and protein complexes.

Optimization of coupling conditions

Coupling solution

Coupling should be carried out in aqueous, mild alkaline buffered solutions (e.g., using carbonate, borate or phosphate buffers) or in strongly alkaline solutions (e.g., high concentration of NaOH). The buffer substance should not contain any nucleophilic functional groups (e.g., Tris, glycine or Good's buffers) since these compounds will react with the resin and compete with the coupling of the desired ligand. Suggested coupling buffers are provided in Table 1. Due to possible pH reduction, as a result of the release of HBr during the reaction, it is generally recommended to use a high buffer concentration or a high enough concentration of NaOH to neutralize the released HBr.

Always check, and if needed, adjust the pH after dissolving the ligand since it may change the pH upon dissolution. Sodium hydroxide and hydrochloric acid may be used to adjust the pH of solutions, but precautions should be taken to avoid denaturation when working with protein ligands.

The ligand to be coupled should be fully soluble in the coupling solution. The addition of organic solvents may be needed to dissolve the ligand. Dimethylformamide and dioxane may be used to up to 50% of the final mixture. If the ligand is a protein make sure that it is stable in the coupling solution.

pH

The coupling reaction can be carried out in the pH range 7 to 14. Ligands carrying amine or sulfhydryl (thiol) groups can often be coupled in the pH range 7 to 10, whereas coupling via hydroxyl groups requires higher pH (pH > 12) to deprotonate the hydroxyl group. Although the coupling yield will increase at higher pH, the cross-linking and hydrolysis will compete with the coupling reaction at pH higher than 12. The chemical stability and the solubility of the ligand limits the maximum pH that can be used.

Temperature

Coupling can be carried out from 4 to 40 °C. The coupling decreasing at higher temperatures. Direct heating should be avoided. The stability of the ligand limits the maximum temperature that can be used. For protein coupling it is recommended to use room temperature. Lower temperatures may be required, but will reduce mass transfer and reaction rate, thus require longer reaction times.

Time

The time for the reaction depends on the properties of the ligand, the pH and the temperature of the coupling reaction. A reaction time of 16 hours at ambient temperature (20 to 25°C) is a general recommendation. Optimum reaction times may vary from 2 to 48 hours. The determination of a suitable reaction time can be made by following the progress of the reaction by analyzing the change in ligand concentration of the free solution:

1. Take out a small volume of the reaction mixture (resin suspension).
2. Centrifuge at 100 × g for 2 minutes.
3. Analyze the presence of the ligand in the supernatant. The reaction is complete when there is no ligand left in the supernatant, or when the ligand concentration has stabilized. (When no more ligands can be coupled).

Removal of free ligand after coupling

After coupling and blocking, it is important to remove any remaining ligand and blocking agent that has not been coupled to the resin. This will avoid subsequent leakage of these substances from the ligand-coupled resin. Multiple washes with alternating high and low pH, and high and low ionic strengths are recommended, especially when the ligand is a biomolecule such as a protein or a peptide. Organic solvent washes should be considered if the ligand is a low molecular weight organic substance with limited solubility in aqueous solutions.

To find out more about Bio-Works chromatography resins visit www.bio-works.com

Storage

WorkBeads 40/1000 ACT and WorkBeads 40/10 000 ACT resins are supplied as aqueous suspensions containing 20% ethanol as preservative.

The activated forms of the resins are stable at pH 4 to 7 at 2 to 25°C.

After ligand coupling the stability of the ligand-coupled resin will usually be dependent on the chemical stability of the ligand. The ligand is often more stable when coupled compared to when in solution. Although it is often possible to store the ligand-coupled resin in 20% ethanol, alternative storage solutions may need to be selected to optimize stability. If 20% ethanol cannot be used then addition of antimicrobial agents may be useful. If the coupled ligand is a peptide or protein, addition of proteolytic enzyme inhibitors should be considered. Sensitive ligand-coupled resins should be stored at 2 to 4 °C.

Product information

	WorkBeads 40/1000 ACT	WorkBeads 40/10 000 ACT
Target substance	Small molecules and peptides	Small molecules, peptides, proteins, e.g., immunoglobulins
Target groups	Thiol, amino, and hydroxyl groups	Thiol, amino, and hydroxyl groups
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Average particle size[1] (D_{v50})	45 µm	45 µm
Reactive groups	Bromohydrin	Bromohydrin
Exclusion limit	1×10^6 Da (globular proteins)	1×10^7 Da (globular proteins)
Max flow rate[2]	600 cm/h	600 cm/h
Reactive-groups content	200 µmol/mL	200 µmol/mL
Chemical stability (before coupling[3])	Buffers pH < 8.5	Buffers pH < 8.5
Chemical stability (after coupling[4])	Compatible with all standard aqueous buffers used for protein purification, 1 M NaOH, 30% isopropanol or 70% ethanol. Should not be stored at < pH 3 for prolonged time.	
pH stability[4]	2 – 13 (after coupling)	2 – 13 (after coupling)
Storage[5]	2 to 25 °C in 20% ethanol	2 to 25 °C in 20% ethanol

1 The median particle size of the cumulative volume distribution.
2 Determined in water using a 10 × 300 mm column
3 Avoid substances containing thiol and amino groups. Substances containing hydroxyl groups will only react if deprotonated. The unreacted resin is stable in alcohols at neutral pH.
4 Agarose matrix and linker. Stability of the coupled substance may differ.
5 The choice of storage conditions for the coupled resin depends on the nature of the ligand.

GoBio prepacked column family

GoBio prepacked column family is developed for convenient, reproducible and fast results and includes columns with different sizes and formats.

GoBio Mini 1 mL and GoBio Mini 5 mL for small scale purification and screening using a shorter packed bed.

GoBio Screen 7x100 (3.8 mL) for reproducible process development including fast and easy optimization of methods and parameters.

GoBio Prep 16x100 (20 mL) and GoBio Prep 26x100 (53 mL) for lab-scale purifications and scaling up.

GoBio Prep 16x600 (120 mL) and GoBio Prep 26x600 (320 mL) for preparative lab-scale size exclusion chromatography.

GoBio Prod 80x200 (1 L), GoBio Prod 130x200 (2.7 L), GoBio Prod 200x200 (6 L), GoBio Prod 240x200 (9 L) and GoBio Prod 330x250 (21.4 L) for production-scale purifications.

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
GoBio Mini ACT 1 mL[2]	1 mL × 1 1 mL × 5 1 mL × 10	45 400 001 45 400 003 45 400 004
GoBio Mini ACT 5 mL[2]	5 mL × 1 5 mL × 5 5 mL × 10	45 400 005 45 400 007 45 400 008
GoBio Mini Dsalt 1 mL	1 mL × 5	45 360 103
GoBio Mini Dsalt 5 mL	5 mL × 5	45 360 107
GoBio Prep 16x100 Dsalt[3]	20 mL × 1	55 700 021
GoBio Prep 26x100 Dsalt	53 mL × 1	55 700 031
GoBio Prod 80x200 Dsalt[3]	1 L	55 700 42
GoBio Prod 130x200 Dsalt[3]	2.7 L	55 700 62
GoBio Prod 200x200 Dsalt[3]	6 L	55 700 72
GoBio Prod 240x200 Dsalt[3]	9 L	55 700 82
GoBio Prod 330x250 Dsalt[3]	21.4 L	55 700 93
Bulk resins		
WorkBeads Dsalt	300 mL 1 L	40 360 003 40 360 010

[1] Other pack sizes can be found in the complete product list on www.bio-works.com
[2] GoBio Mini ACT columns are prepacked with WorkBeads 40/1000 ACT.
[3] Packed on request.

Ordering information

Product name	Pack size	Article number
WorkBeads 40/1000 ACT	25 mL 300 mL 1 L 5 L	40 400 001 40 400 003 40 400 010 40 400 050
WorkBeads 40/10 000 ACT	25 mL 300 mL 1 L 5 L	40 450 001 40 450 003 40 450 010 40 450 050

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

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

bio-works.com

Bio-Works, Virdings allé 18, 754 50 Uppsala, Sweden. For local office contact information, visit bio-works.com/contact.

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