INSTRUCTIONS

CarboxyLink™ Immobilization Kit

Pub. No. MAN0011418
Rev. C
Pub. Part No. 2160480

44899

Number	Description
44899	**CarboxyLink Immobilization Kit**, sufficient reagents to prepare five affinity columns

Kit Contents:

CarboxyLink Columns, 5 × 2mL prepacked columns containing diaminodipropylamine (DADPA) immobilized to crosslinked 4% beaded agarose in a 50% slurry of 0.02% sodium azide (NaN_3); Activation Level: 16-20µmol amine/mL of settled resin

EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide], 5 × 60mg

Conjugation Buffer: BupH™ MES Buffered Saline Pack, 0.1M MES [2-(N-morpholino) ethanesulfonic acid], 0.9% NaCl, pH 4.7 when reconstituted, 1 pack

Wash Solution, 120mL of 1M NaCl

Column Accessories, porous discs (6) and resin separator

Storage: Upon receipt store kit at 4°C. EDC is best stored desiccated at -20°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific CarboxyLink Immobilization Kit contains a crosslinked beaded agarose derivatized to contain diaminodipropyl-amine (DADPA). The resulting resin contains reactive primary amines at the end of a long spacer arm (Figure 1) to which molecules may be conjugated by various means. The CarboxyLink Kit includes a crosslinker (EDC) to covalently couple molecules containing carboxyl (-COOH) groups to the agarose resin. The kit is ideal for immobilizing peptides for use in affinity purification of antibodies or other binding partners.

Although other amine-reactive methods can be used for attachment of molecules to the DADPA agarose resin, the carbodiimide EDC is unique among crosslinkers in enabling conjugation through carboxylates. In near-neutral to acidic pH buffer, EDC effectively reacts with carboxylates to form an intermediate ester that is reactive with nucleophiles such as the primary amines on DADPA. The resulting amide bond between a molecule and the resin is stable and practically leak-proof in most affinity purification methods. This kit is also suitable for immobilization of oligonucleotides at 5′-phosphate groups (see Additional Information section). In the presence of imidazole, EDC reacts effectively with phosphate groups in much the same manner as with carboxylates.

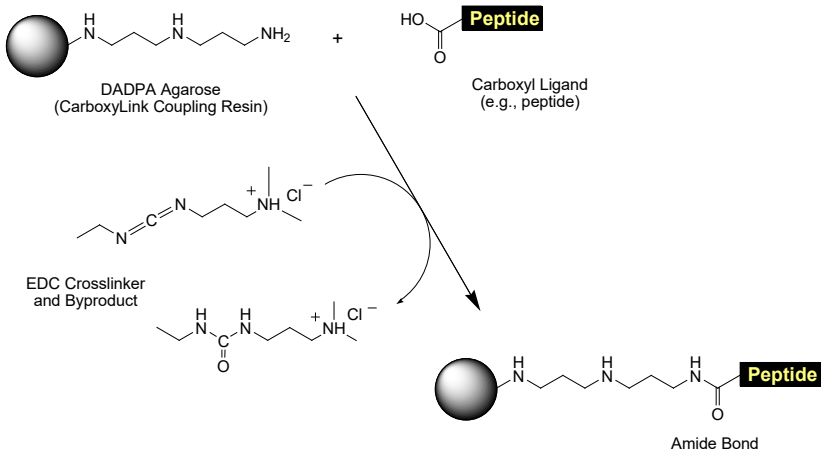

Figure 1. Structure and reaction scheme of CarboxyLink Coupling Resin.

Important Product Information

- When using EDC, the Resin will couple peptides at their C-termini and side chains of aspartic and glutamic acid residues. Because peptides also contain primary amines (the N-terminus and the side chain of lysine residues), coupling using EDC will result in polymerization of peptides as well as their immobilization to the agarose resin. Usually such polymerization is not detrimental for subsequent affinity purification methods.
- When coupling water-insoluble peptides or other molecules, use water-miscible solvents such as ethanol, methanol, DMSO or DMF. Dissolve the peptide in the water-miscible solvent first, then add this solution to the conjugation buffer. Organic solvent concentrations up to 50% in the coupling reaction are compatible unless the peptide is known to denature in this concentration.
- EDC is moisture sensitive and hydrolyzes quickly when dissolved in aqueous buffers. For best results, store the dry powder reagent sealed tightly in its original vial in desiccant at -20°C. Equilibrate the vial to room temperature before opening to avoid moisture condensation into the vial. Dissolve the required amount of reagent quickly and immediately before use, and discard any unused solution.
- Traditionally, MES buffer is used for EDC reactions because it does not contain competing amines or phosphates, and it effectively maintains optimal acid conditions for coupling. However, other buffers may be substituted if necessary, and coupling will proceed effectively up to pH 7.2. Although phosphate buffer can react with EDC reducing conjugation efficiency, it may be used if compensated by a greater excess of crosslinker. Acetate, Tris and glycine buffers react with EDC or the *O*-acylisourea intermediate and are not appropriate conjugation buffers. Also, avoid using buffers containing thiols, which irreversibly bind and inactivate EDC. Halides such as iodide, chloride and bromide have minimal effect on EDC at pH 5-7.
- For additional information about EDC (Product No. 22980), consult the instructions for the product.
- The EDC/DADPA method can also be used to immobilize oligonucleotides through their 5′-phosphate groups; for a generalized protocol, see Additional Information section at the end of these instructions.

Procedure for Peptide Immobilization

A. Reagent Preparation

Coupling Buffer	Dissolve contents of the BupH MES Buffered Saline Pack in 500mL ultrapure water. **Note:** For buffer storage longer than 2 weeks, add sodium azide (NaN_3) to a final concentration of 0.02% and store at 4°C.
Peptide Sample	Dissolve 1-10mg of peptide in 2mL of Coupling Buffer. For estimation of coupling efficiency following peptide coupling, measure the absorbance of this sample at 280nm. (This method assumes that the peptide absorbs at 280nm; other wavelengths that detect the presence of the peptide vs. Coupling Buffer alone may be used).

B. Coupling Procedure

1. Equilibrate a CarboxyLink Column, a vial of EDC and the bottle of Wash Solution to room temperature.
2. Place the column into a 16 × 150mm test tube (in a test tube rack) or clamp the column in a laboratory stand. Remove top and twist off reusable bottom closure from the column (SAVE for later use), and allow the storage buffer to drain.

   Note: Throughout the entire procedure, do not allow the resin bed to become dry; add additional solution or replace the bottom cap (white tips supplied) whenever the buffer drains down to the top of the resin bed.
3. Equilibrate resin by adding 10mL of Coupling Buffer to the column and allowing it to drain.
4. Reseal the column by inverting the snap-off closure from the column bottom, and with a slight twisting motion, press it firmly to the bottom tip of the column, then add the 2mL Peptide Sample to the column.
5. Replace top cap on the column and mix the Sample/Resin slurry gently end over end for several minutes.
6. Add 0.5mL of the Coupling Buffer to the vial of EDC that has been equilibrated to room temperature.
7. Immediately after the EDC is dissolved, add the 0.5mL of reagent to the Sample/Resin slurry from step 5.
8. Place the top cap on the column and mix the reaction slurry gently end-over-end for 3 hours at room temperature.
9. Place the column upright. Allow several minutes for the resin to settle, then remove the top and bottom caps and drain the reaction solution into a clean collection tube.

10. Without changing collection tubes, gently add 2mL of Wash Solution to the column and collect the 2mL of solution that drains from the column. Replace the bottom column cap.

    Note: The collected sample (~4mL) contains the non-bound peptide. To measure coupling efficiency, compare the absorbance of this solution to the starting Peptide Sample, accounting for the two-fold dilution effect.
11. With the bottom cap in place, gently add 2mL of Wash Solution to the column so as not to disturb the settled resin bed.
12. Insert a porous polyethylene disc into the column and use the open end of the Resin Separator (or reverse end of a Pasteur pipette) to push it down to 1mm above the settled resin bed.
13. Remove the bottom cap and allow the column to drain into a new collection tube. Column flow will stop automatically when the solution drains down to the top porous disc.
14. If desired, add more Wash Solution (5-10mL) to the column to more thoroughly wash the column of non-bound peptide.
15. Equilibrate the column in an appropriate storage buffer or binding buffer by passing 6mL through the column.
16. For immediate use of the column, equilibrate with a buffer appropriate for the binding interaction. For storage of the column, equilibrate with phosphate buffered saline (PBS) or other suitable buffer containing 0.05% sodium azide, and store the capped column upright at 4°C.

General Protocol for Affinity Purification of Protein

Note: This protocol uses a gravity-flow column with a resin-bed volume of 2mL. The amount of protein sample needed and incubation time are dependent upon the affinity system involved (e.g., antibody-antigen interaction) and must be optimized. For a centrifuge column procedure, see instructions for Product No. 44894.

A. Additional Materials Required

- Binding/Wash Buffer: Phosphate Buffered Saline (PBS, Product No. 28372), Tris Buffered Saline (TBS, Product No. 28379) or other buffer that is compatible with the intended affinity interaction.
- Sample: Prepare antigen or other molecule in Binding/Wash Buffer, or dilute sample 1:1 in Binding/Wash Buffer
- Elution Buffer: IgG Elution Buffer (Product No. 21004) or 0.1-0.2M glycine•HCl, pH 2.5-3.0
- Neutralization Buffer (optional): 1mL of high-ionic strength alkaline buffer such as 1M phosphate or 1M Tris; pH 9

B. Procedure

Note: Degas all buffers to avoid introducing air bubbles into the column. Throughout the procedure, do not allow the resin bed to become dry; replace bottom cap as soon as buffer drains down to the top of resin bed.

1. Equilibrate the prepared affinity column to room temperature.
2. Remove top cap and bottom closure and allow excess storage solution to drain from column.
3. Equilibrate column by adding 6mL of Binding/Wash Buffer and allowing it to drain from column. SAVE reusable bottom closure for later use.
4. Add Sample to column and allow it to flow into the resin bed. For samples < 2mL, extend binding time by replacing the bottom cap to stop flow for a time (e.g., 1 hour) after the sample has entered resin bed. For samples > 2mL, extend binding time by resealing column bottom to stop column flow after each 2mL volume of sample has passed into/through the resin bed. To reseal the column, invert the original snap-off closure from the column bottom, and with a slight twisting motion, press it firmly to the bottom tip of the column.
5. Remove top cap and bottom closure from column, place column in new collection tube, and wash the column with 12mL of Binding/Wash Buffer.
6. Elute the bound protein by applying 8mL of Elution Buffer. Collect 1mL (or 0.5mL) fractions. The pH of each fraction can be adjusted to neutral by adding 50µL of Neutralization Buffer per 1mL of collected eluate.
7. Monitor elution by absorbance at 280nm. Pool fractions of interest and exchange into an appropriate storage buffer by desalting or dialysis.

C. Column Regeneration and Storage

Note: Regenerate the column soon after elution to prevent damage to the immobilized molecule by the low pH elution buffer.

1. Wash column with 16mL of Binding/Wash Buffer to remove any residual protein and reactivate the resin.
2. Equilibrate column with 8mL of Binding/Wash Buffer containing 0.05% sodium azide.
3. Reseal the column by inverting the original snap-off closure from the column bottom, and with a slight twisting motion, press it firmly to the bottom tip of the column, then add 2mL of Binding/Wash Buffer to the column and cap the top. Store column upright at 4°C.

Troubleshooting

Problem	Possible Cause	Solution
Peptide not soluble in Coupling Buffer	Peptide was hydrophobic or not soluble at low pH	Use water miscible solvent (see Important Product Information) or use phosphate-buffered saline, pH 7.2
Coupling efficiency poor	EDC reagent was degraded (hydrolyzed)	Use only previously unopened vials of EDC and use reagent immediately after dissolving vial contents or purchase new EDC
Poor flow rate in packed column	Air bubbles (large and visible or too small to see) formed in resin bed	Use procedure for removing air bubbles, see Tech Tip procedure from our website - prevent air bubble formation by using only degassed solutions (e.g., solutions that have been subjected to vacuum to remove excess dissolved air)

Appendix

Procedure for Nucleic Acid or Oligonucleotide Immobilization Through 5´-Phosphate Groups

Nucleic acid affinity purification methods are more commonly performed on a smaller scale than that suited to a 2mL column. Accordingly, the following protocol is presented as a batch method, and requires that a small portion of CarboxyLink Resin be removed from the supplied 2mL column. Use 1µL of resin (2µL of mixed slurry) for each 10µg of oligonucleotide to be coupled.

A. Additional Materials Required

- Pipettors and pipette tips, including one wide-orifice (cut-tip) pipette tip for dispensing resin slurry
- Microcentrifuge tubes and microcentrifuge
- 0.1M imidazole, pH 6
- Ultrapure DNase- and/or RNase-free water

B. Procedure

1. Mix the column by end-over-end rotation to achieve a uniform resin suspension. Use a wide-orifice pipette tip to transfer an appropriate volume of CarboxyLink Resin to a microcentrifuge tube.
2. Centrifuge the tube for 2 minutes at low speed (e.g., 1,000 × *g*), then carefully remove and discard the supernatant.
3. Wash the resin 3-5 times with two bed volumes of ultrapure water, centrifuging and removing the supernatant each time.
4. For each microliter of resin used, dissolve up to 10µg of DNA or RNA in 1µL of 0.1M imidazole, pH 6. Add the nucleic acid solution to the resin and mix well.
5. Weigh and dissolve 1mg of EDC in 67µL of 0.1M imidazole, pH 6. For each microliter of resin used, add 2µL of the EDC solution.
6. Mix the resin reaction by shaking or rotating for 3 hours at room temperature.
7. Centrifuge the tube and remove the supernatant, which contains the non-bound nucleic acid.
8. Wash the resin 3-5 times with two bed volumes of water or an appropriate wash buffer (e.g., Tris-EDTA), centrifuging and discarding the supernatant each time.

Related Thermo Scientific Products

28390	**BupH MES Buffered Saline Packs**, 10 packs
28372	**BupH Phosphate Buffered Saline (PBS),** 40 packs
22980	**EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride)]**, 5g
53154	**UltraLink™ EDC/DADPA Immobilization Kit**
20266	**CarboxyLink Coupling Resin (diaminodipropylamine agarose),** 25mL
21004	**Pierce™ IgG Elution Buffer,** 1L
89896	**Pierce Centrifuge Columns, 2mL,** 25/pkg
89897	**Pierce Centrifuge Columns, 5mL,** 25/pkg
89898	**Pierce Centrifuge Columns, 10mL,** 25/pkg

General References (for EDC coupling chemistry)

Gilles, M.A. *et.al*. (1990). Stability of water-soluble carbodiimides in aqueous solution. *Anal Biochem* **184**:244.

Grabarek, Z. and Gergely, J. (1990). Zero-length crosslinking procedure with the use of active esters. *Anal Biochem* **185**:244-8.

Williams, A. and Ibrahim, I.A. (1981). A mechanism involving cyclic tautomers for the reaction with nucleophiles of the water-soluble peptide coupling reagent 1-ethyl-3-(dimethyl amino)propyl)carbodiimide (EDC). *J Am Chem Soc* **103**:7090-5.

Product References

Li, L. *et.al*. (2000). Retroviral cDNA integration: stimulation by HMG I family proteins. *J Virol* **74(23):**10965-74.

Morrissette, J. M. *et.al*. (2003). Characterization of ryanodine receptor and Ca2+ -ATPase isoforms in the thermogenic heater organ of blue marlin (makaira nigricans). *J Exp Biol* **206:**805-12.

Xainli, J. *et.al*. (2003). Epitope-specific humoral immunity to plasmodium vivax duffy binding protein. *Infection and Immunity* **71(5):**2508-15.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition. The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0011235 B

Revision	Date	Description
C	31 July 2024	Correcting spin column usage.
B	17 August 2017	Corrected inconsistent wash/buffer solutions.
A	17 October 2015	New document for CarboxyLink™ Immobilization Kit.