Coupling of proteins to PureCube Carboxy Agarose using PureCube 1-step batch MidiPlus Columns

Overview

This protocol delineates a coupling procedure for proteins to PureCube Carboxy Agarose using the cross-linking reagents EDC and NHS. Proteins are coupled covalently and can be used for different applications, e.g. purification of interaction partners.

Amounts given in this protocol are for 2 mL of a 50% Carboxy Agarose suspension, corresponding to 1 mL agarose bed volume. The process can be linearly scaled up and down from 100 µL to several 100 mL bed volume. Separation of Agarose and supernatants can be done by centrifugation, or, more conveniently, using PureCube 1-step batch Columns. They are available in different sizes to accommodate volumes from several hundred microliters to 2 mL bed volume. See the separate protocol available for a combination of these products. For larger scales, use of special equipment like glass vaccum tank filters, is recommended. See also the protocol „Coupling of proteins to PureCube Carboxy Agarose".

Please contact us if you have questions or need assistance optimizing a protocol for your application (contact@www.cube-biotech.com); other protocols can also be found at www.cube-biotech/protocols.

Equipment

- ☐ Centrifuge for 50 mL tubes
- ☐ 50 mL centrifugation tubes (e.g. Falcon)
- ☐ End-over-end mixer or thermomixer
- ☐ Spectrophotometer

Materials

- ☐ PureCube Carboxy Agarose (10 mL, Cube Biotech #50303)
- ☐ PureCube 1-step batch MidiPlus Columns (Cube Biotech #63203)
- ☐ Sodium dihydrogen phosphate
- ☐ Sodium chloride
- ☐ Sodium acetate trihydrate
- ☐ Sodium hydroxide (NaOH)
- ☐ Acetic acid
- ☐ Ethanol
- ☐ Ethanolamine
- ☐ N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC)
- ☐ N-Hydroxysuccinimide (NHS)

Solutions and buffers

Phosphate buffer, pH 6.0, 250 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for buffer
NaH_2PO_4 dihydrate	25 mM	156.01	n.a.	975 mg
Instructions: Dissolve sodium phosphate in 200 mL water, adjust the pH to 6.0 with NaOH. Add water to a total volume of 250 mL.				

PBS Buffer, pH 7.2, 250 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for buffer
NaH_2PO_4 dihydrate	150 mM	156.01	n.a.	5.85 g
NaCl	100 mM	58.44	n.a.	1.463 g
Instructions: Dissolve components in 200 mL water, adjust the pH to 7.2 with NaOH. Add water to a total volume of 250 mL.				

Quenching Buffer, pH 7.4, 250 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for buffer
Ethanolamine	1 M	61.08	n.a.	15.27 g
Instructions: Dissolve component in 200 mL water, adjust the pH to 7.4 with HCl. Add water to a total volume of 250 mL.				

Agarose Storage Buffer, pH 6.5, 250 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for buffer
Sodium acetate trihydrate	20 mM	136.08	n.a.	674 mg
Ethanol	20 % (v/v)		100 % (v/v)	51 mL
Instructions: Dissolve sodium acetate in 150 mL water, adjust the pH to 6.5 with acetic acid. Add 48 mL water and 51 mL ethanol to yield a total volume of 250 mL.				

Procedure

1. Transfer 2 mL PureCube Carboxy Agarose suspension (corresponding to 1 mL bed volume) into the batch incubation chamber of the spin column barrel. Use the ***clear*** spin push cap to close the chamber and spin the resin at 400 x g for 5 min.
2. Empty the 50 mL centrifuge tube and place the spin column barrel containing the activated agarose matrix back into it. Resuspend the agarose in 3 mL Phosphate Buffer.
3. Dissolve 250 mg NHS in 1 mL Phosphate Buffer, add it to the agarose suspension, and mix.
4. Dissolve 250 mg EDC in 1 mL Phosphate Buffer, add it to the agarose suspension, and mix.
5. Incubate at room temperature for 1 h on an end-over-end shaker or in a thermoshaker.
6. Prepare a solution of 2.5 mL PBS containg the protein to be coupled to the agarose. The exact protein amount needs to be optimized, and 5 to 15 mg protein is a good starting point.
7. Add the protein solution to the agarose, close with the ***yellow*** cap and mix by inverting 2-3 times.
8. Depending on the temperature stability of the protein, incubate at room temperature or 4°C for 2 h on an end-over-end shaker or thermoshaker.
9. After batch incubation, replace the ***yellow*** cap with the ***clear*** spin push cap. Centrifuge at 400 x g for 5 min, or until the lysate has completely passed through. Collect the flow-through and analyze it in a spectrophotometer. Record absorption at 280 nm to monitor coupling efficiency.
10. Wash six times with 5 mL double distilled water each. Discard the flow-throughs.
11. Add 5 mL Quenching Buffer, close with the ***yellow*** cap and mix by inverting 2-3 times.
12. Incubate again for 1 h at room temperature or for 4 hours at 4°C.
13. Wash four times with 5 mL PBS each, and twice with 5 mL double distilled water each.
14. Resuspend the coupled Agarose from the spin column barrel in 2 mL Agarose Storage buffer, yielding a 50% suspension. Store at 4°C.

Note: NHS and EDC should always be prepared fresh. Equilibrate the two chemicals to room temperature before weighing, and store the powders under protective gas (nitrogen). Add the two chemicals immediately one after another to prevent hydrolysis of the NHS-activated matrix.

Tip: When coupling a particular protein for the first time, try 3-5 different protein concentrations to make sure you are offering enough protein in the reaction but not wasting any protein.

Tip: Monitoring absorbance at 280 nm tells you about the coupling efficiency of the protein (compare A280 of the original protein solution to the supernatant in step 6 to determine % coupling). It also helps you identify the optimal amount of protein required for efficient coupling.

Note: The quenching step ensures that no free NHS groups are left on the agarose matrix that might interfere with subsequent assays.