Activation, coupling and purification of proteins using PureCube Thiol-Activated Agarose

Overview

NOTE: This protocol has been updated effective June 2016. Please ensure to follow this protocol and to discard previous versions.

This protocol delineates several procedures required for

A. Activation of lyophilized PureCube Thiol-Activated Agarose prior to use

B. Coupling of proteins to PureCube Thiol-Activated Agarose via free thiol groups (e.g. cysteine residues) in the protein. Proteins are coupled covalently and can be used for different applications, such as pull-down of interaction partners. Upon coupling of the protein moiety, a 2-thiopyridone group is released from the matrix. Because of the nature of the disulfide bond, the coupled proteins can also be eluted from the Agarose using reducing agents such as DTT or beta-mercaptoethanol. Different thiol containing proteins can be eluted by using a combination or concentration gradient of weaker (e.g. L-cysteine, reduced glutathione) and stronger (DTT or mercaptoethanol) reducing agents.

C. Elution of proteins after coupling to PureCube Thiol-Activated Agarose.

D. Storage of PureCube Thiol-Activated Agarose.

E. Regeneration of PureCube Thiol-Activated Agarose for reuse.

Amounts given in this protocol are for 1 mL Thiol-Activated Agarose suspension (corresponding to 500 µL Agarose). This reaction can be linearly scaled up or down.

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

- ☐ Microcentrifuge or Centrifuge for 15 mL tubes
- ☐ Microtubes (2 mL) or Centrifuge tubes (15 mL)
- ☐ End-over-end mixer or thermomixer
- ☐ Spectrophotometer

Materials

- ☐ PureCube Thiol-Activated Agarose (10 mL, Cube Biotech #50503)
- ☐ Sodium phosphate
- ☐ Sodium chloride
- ☐ Sodium hydroxide
- ☐ beta-mercaptoethanol or DTT for elution
- ☐ Optional: L-cysteine or glutatione for modified elution
- ☐ Optional: EDTA and / or protease inhibitor

<u>For activation:</u>

- ☐ Sodium borate
- ☐ 2,2`-dipyridyl disulphide (e.g. Merck Milipore cat. no. 841109) - 40 mg are provided with the product.

<u>For storage:</u>

- ☐ Sodium acetate
- ☐ Ethanol

Solutions and buffers

PBS Buffer pH 7.4, 100 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaH_2PO_4	20 mM	119.98	0.5 M	29.99 g/ 500 mL	4 mL
NaCl*	150 mM	58.44	5 M	146.1 g / 500 mL	3 mL
Instructions: Mix components in 80 mL water. Adjust the pH to 7.4 using NaOH and then add water to a total volume of 100 mL. **Important: Degas the buffer prior to use.** Alternatively, 0.1 M Tris-HCL, or acetate buffers at pH 7.0-8.0 with 0.1-0.5 M NaCl can be used. 1 mM EDTA may be added to remove traces of metal ions which may catalyze thiol oxidation.					

Elution Buffer, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for buffer
Binding buffer	1x	n.a.	n.a.	50 mL
DTT	10-25 mM	154.25		77-193 mg
Alternative to DTT: beta-mercaptoethanol	20-50 mM	78.13	(100%=14.3 M)	70-175 µL
Alternative to DTT: reduced glutathione	50 mM	307.32		770 mg
Alternative to DTT: L-cysteine	5-25 mM	121,16		30.5-152 mg
Instructions: Dissolve reducing agent in binding buffer. Only use one reducing agent at a time.				

Activation Buffer, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Required amount	Volume
Sodium borate, pH 8.0	50 mM	381.37	953 mg	50 mL
2,2`-dipyridyl disulphide	saturated	220.3		40 mg
Instructions: Dissolve sodium borate in 40 mL ddH_2O, set pH to 8.0 and fill up to 50 mL. Stir overnight to dissolve. The next morning, add dipyridyl disulphide and incubate on an end over end shaker for 2-3 h to dissolve. Pass through a 0.45 µm filter to remove insoluble particles. **Note:** Depending on the pack size, aliquot(s) of 2,2`-dipyridyl disulphide are provided with the Thiol-Activated Agarose. **Important: Always prepare fresh. Degas the buffer prior to use.**				

Agarose Storage Buffer, 100 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Sodium acetate, pH 6.5	20 mM	82.03	0.5 M	20.5 g/ 500 mL	4 mL
Ethanol	20 % v/v		100 %		20 mL
Instructions: Dissolve sodium acetate in 60 mL water. Adjust the pH to 6.5 using NaOH. Add 20 mL ethanol and add water to a total volume of 100 mL.					

Procedure

A. Activation

1. Pipet 1 mL PureCube Thiol-Activated Agarose in a 2 mL microcentrifuge tube.
2. Spin for 5 min at 500 x g. Remove the supernatant. Resuspend with 1 mL PBS Buffer.
3. Repeat step 2.
4. Spin for 5 min at 500 x g. Resuspend with 1.5 mL Activation Buffer. Mix by vortexing. Incubate at room temperature for 30 min on an end-over-end shaker or thermoshaker.
5. Spin for 5 min at 500 x g. Remove the supernatant.
6. Wash twice with 1 mL PBS Buffer each.
7. Proceed directly to a coupling reaction (Procedure B).

Note: For optimal results, Thiol-Activated agarose should be activated directly before use in the coupling reaction.

B. Protein coupling

1. Spin for 5 min at 500 x g. Remove the supernatant and add 1 mL PBS containing your protein of interest. Mix by vortexing.
2. Depending on the temperature stability of the protein, incubate at room temperature or 4°C for 30 min on an end-over-end shaker or thermoshaker.
3. Spin for 5 min at 500 x g. Remove the supernatant and resuspend in 1 mL PBS Buffer.
4. Repeat step 3 twice.

Note: Ensure that Thiol-Activated agarose is freshly activated before use.

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.

Note: Incubation time may be extended to 60 min if required.

Tip: To monitor protein coupling efficiency, analyze the supernatant in a spectrophotometer at 280 nm. Note that upon protein coupling, 2-thiopyridone is eluted which absorbs at 280 and 343 nm with similar extinction coefficents of 8.08 x 10^3 M^{-1} cm^{-1}. Alternatively, 2-thiopyridone can be washed off the Agarose with a low concentration of reducing agent (e.g. 5 mM DTT or mercaptoethanol) before eluting the protein of interest. However, please check this wash fraction by SDS-PAGE to ensure your protein of interest is not eluted under these conditions.

Procedure, continued

C. Protein elution

1. After protein coupling, resuspend the agarose with 750 µL Elution Buffer. Mix by vortexing.
2. Spin for 5 min at 500 x g. Remove the supernatant.
3. Repeat steps 1+2, and analyze the elution fractions by SDS-PAGE.
4. Optional: Repeat steps 1+2 with different types and/or concentrations of reducing agents in the elution buffer.

Note: The supernatant contains the eluted protein. Depending on the type of protein, and available number of thiol groups, the type and concentration of reducing agents may vary.

D. Storage

1. After elution, wash twice with PBS Buffer.
2. Add 1 mL Agarose Storage Buffer. The agarose can be stored at 4°C for several months.

E. Regeneration

1. Add 1 mL PBS Buffer. Spin for 5 min at 500 x g. Remove the supernatant.
2. Repeat step 2.
3. Resuspend with 1 mL of Elution Buffer. Incubate for 1 h at room temperature on an end-over-end shaker.
4. Spin for 5 min at 500 x g. Remove the supernatant.
5. Wash twice with 1 mL PBS Buffer each.
6. Resuspend with 1.5 mL Activation Buffer. Mix by vortexing. Incubate at room temperature for 30 min on an end-over-end shaker or thermoshaker.
7. Spin for 5 min at 500 x g. Remove the supernatant.
8. Wash twice with 1 mL PBS Buffer each.
9. Proceed directly to a coupling reaction (Procedure B).

Note: Regenerate Thiol-Activated Agarose immediately before the next use in a coupling reaction.