Protocol

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Coupling Protocol for LodeStars Carboxyl Magnetic Beads with Co-Activators (Two Step)

Introduction

The following protocol provides users with guidance for the successful coupling of biomolecules to Agilent LodeStars magnetic beads with carboxyl surfaces. This general protocol describes steps for the coupling of biomolecules, but Agilent recommends further optimization to achieve maximum performance in your application.

The typical strategy for ligand coupling is the formation of an amide bond between a primary amino group of the ligand and the carboxylic acid groups on the surface of the magnetic particles, mediated by carbodiimide activation. The intermediate product of the reaction between the carboxylic acid and the carbodiimide is very labile and will hydrolyze quickly, regenerating the carboxyl group. To enhance coupling efficiency, Agilent recommends adding *N*-hydroxysulfosuccinimide (sulfo-NHS, Mw 217.1). The advantage of sulfo-NHS over other co-activators such as *N*-hydroxyl succinimide (NHS, Mw 115.1) is that, due to the sulfonate group, this ester improves water solubility and therefore the stability of the modified molecule. However, the use of a co-activator is not always used due to cost concerns or customer preference. To use only EDC as an activator, please see the Agilent EDC mediated coupling protocol.

EDC/Sulfo-NHS protein-binding protocol

Material required to perform the coupling protocol

- 0.1 M sodium hydroxide (NaOH) buffer
- 0.1 M MES (2-[morpholino]ethanesulfonic acid), pH 5
- EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide)
- sulfo-NHS (*N*-hydroxysulfosuccinimide). NHS (*N*-hydroxyl succinimide) can be used instead of sulfo-NHS. However, for higher efficiency, Agilent strongly recommends the use of sulfo-NHS as co-activator.
- Protein solution: 1 mL of protein prepared in MES pH 5 buffer at 3 mg/mL for LodeStars High Bind or 2 mg/mL for LodeStars Original beads
- 0.1 M Trizma-HCl for quenching EDC activation reaction
- Phosphate-buffered saline (PBS): 0.1 M sodium phosphate, pH 7.4 (for long storage periods, it is recommended to add 0.1w% of sodium azide to avoid microbial growth)
- PBS-Tween buffer: 0.1 M sodium phosphate, pH 7.4, 0.1w% Tween-20
- Vortex mixer
- Bottle roller
- Sonic probe to obtain desirable bead dispersion and size distribution (Agilent recommended, but optional).

Before starting with the binding protocol

- Check the expiration date of the buffers (6 months since preparation).
- Allow the buffers and reagents to reach room temperature.
- Disperse the beads using a bottle roller for a minimum of 2 hours for volumes above 250 mL or 1 hour for smaller volumes. Nonhomogeneous dispersion could result in an incorrect quantity of beads being used in conjugation or aggregations, causing incomplete coupling. For optimal bead dispersion, Agilent recommends rolling the beads at room temperature overnight (~16 hours).

This process is detailed in this tutorial video.

Procedure

1. Aliquot 50 mg of LodeStars beads into 10 mL test tubes. Place the tube in a magnet to separate the beads from solution. Once the separation has been completed, aspirate the supernatant, then remove the tube from the magnet.
2. Add 7.5 mL of reverse osmosis (RO) water to the tube and resuspend the beads using a vortex mixer. Place the tube into a magnet, allow magnetic separation, then aspirate the supernatant (washing step). Repeat this step twice with water.
3. To remove contaminants and precondition the available carboxyl groups, repeat the washing step (step 2) with NaOH buffer followed by three more washes in RO water to remove any traces of NaOH buffer. Resuspend in 7.5 mL of 0.1 M MES pH 5 buffer to precondition the beads before activation.
4. Prepare activation solution. In a new tube, weigh 200 mg of EDC and 100 mg of sulfo-NHS reagents in this order and ratio: 2:1 EDC:Sulfo-NHS. Then, add 2 mL of 0.1 M MES pH 5 buffer. **Note:** Activation solution must be added to the beads (Step 5) within 20 minutes of preparation to avoid negative impacts.
5. Place the beads in a magnet, allow magnetic separation, then aspirate 0.1 M MES pH 5 buffer (beads will adhere to the tube, but the aspirated solution should be clear). Remove the tube from the magnet and immediately add 2 mL of the activation solution. Resuspend the beads with a vortex mixer and roll the beads at room temperature for 1 hour.
6. Place the tube in a magnet and aspirate activation solution. Wash the beads twice with 0.1 M MES pH 5 buffer. Remove tube from the magnet and resuspend the beads in 1 mL of 0.1 M MES pH 5 buffer.
7. Add 1 mL of protein solution to the beads (for optimum coupling, steps 6 and 7 should be carried out in less than 30 minutes). Roll the beads at room temperature for 1 hour. **Note:** Titration of protein concentrations is recommended to achieve optimum performance in chosen applications.
   - For LodeStars High Bind (LSHB): saturation concentrations for Streptavidin are 50 to 60 µg of protein/mg of LSHB beads, whereas concentration for IgG is around 45 to 55 µg/mg.
   - For LodeStars Original (LS): saturation concentrations for streptavidin are 30 to 35 µg of protein/mg of LS beads, whereas IgG is around 20 to 30 µg/mg. Agilent recommends including saturated concentrations as part of a protein titration to determine optimal conditions.

8. Place the tube in the magnet and aspirate protein solution. If covalent blocking is to be used, addition of the blocker should be performed at this point. To deactivate the uncoupled EDC/Sulfo-NHS groups, add 7.5 mL of 0.1 M Trizma-HCl buffer and roll the beads at room temperature for 1 hour.
9. Separate the beads from solution with a magnet and aspirate 0.1 M Trizma-HCl buffer. Repeat the washing step (step 2) with PBS-Tween buffer, then twice with PBS buffer.
10. **Optional:** Measure the size distribution of the resulting beads by coulter. To obtain optimal dispersion and reduce nonspecific binding, sonicate the beads with a sonic probe for a short period, on low power and amplitude, to avoid warming and denaturing of proteins. Repeat this process until the desired size distribution is achieved. Repeat the washing step (step 2) once more to eliminate any type of free protein in the supernatant and resuspend the beads in 7.5 mL of PBS buffer. After this step, binding capacity characterization (BCA assay, B4F, labeled antibody) can be performed.
11. Store the beads at 2 to 8 °C. For long-term storage, it is recommended to add a preservative.

Characterization techniques for Agilent magnetic beads

BCA test

The bicinchonininc acid assay is a colorimetric assay that can be used to measure the total protein concentration successfully coupled to magnetic beads (nonspecifically bound and supernatant protein can contribute to the result).

This assay relies on two chemical reactions. The process starts when the peptide bonds of the bound protein reduce the Cu^{2+} ions from the copper(II) sulfate (reagent B) to Cu^{+}. This ion will then chelate with two molecules of bicinchonininc acid, resulting in a purple-colored complex that absorbs light at a 562 nm wavelength and is directly proportional to protein concentration.

Material and calculations required to perform the assay

- Pierce BCA Protein Assay Kit (Reagents A and B)
- 500 µL of protein prepared in PBS buffer at 2 mg/mL
- Water bath for carrying binding capacity characterization assay
- 96-well clear-bottom black microplate

To calculate the amount of reagent A and reagent B needed in mL, the following formulas should be applied:

a. **Reagent A (mL):** (number of samples + number of standards) × 4.2

b. **Reagent B (mL):** amount of reagent A/50

Assay protocol

1. Roll the recently coupled beads for at least 30 minutes before starting with this assay and prepare a water bath at 37 °C.
2. Prepare 500 µL of 2 mg/mL protein solution in the PBS buffer. This solution will be used to prepare the calibration standards:

Standard Concentration (ng/mL)	PBS Buffer Volume (µL)	Protein Solution Volume (µL)
0	250	0
200	225	25
400	200	50
600	175	75
800	150	100

3. In a Nalgene bottle, mix reagent A and reagent B reagents to obtain a green solution and aliquot 4 mL in different 10 mL tubes.
4. Add 200 µL of the standards and samples in different 10 mL tubes. Mix the samples and standards with the green solution using a vortex mixer and place the tubes in the water bath for 30 minutes.
5. Cool down the tubes for 1 minute by submerging them in cold water to slow the color change and place the tubes in a magnet for magnetic separation.
6. Pipette 300 µL of the colored solution into the microplate in triplicate and perform an absorbance reading at 562 nm wavelength. **Note:** Pipetting should be carried out with the minimum delay of time for an accurate reading of the binding capacity, as color will still develop with time.
7. Plot calibration curve and calculate binding capacity using the slope, the intercept, and the dilutions carried out in binding protocol (50 mg of beads in 7.5 mL).

Choose your options to achieve quality characterization

Technical information

Specifications	LodeStars Beads	LodeStars High Bind Beads
Diameter	2.7 µm	2.7 µm
Iron content	~ 20%	~ 20%
Carboxyl Binding Capabilities		
BCA assay*	~20 µg/mg bead	~40 µg/mg bead

* Assay details available on request.

Ordering information

Description	LodeStars beads (30 mg/mL)		LodeStars High Bind beads (50 mg/mL)	
LodeStars Carboxyl beads	2 mL	PL6727-0001	1 mL	PL6827-0001
	10 mL	PL6727-0003	10 mL	PL6827-0003
	100 mL	PL6727-0005	100 mL	PL6827-0005
	400 mL	PL6727-0006	400 mL	PL6827-0006
	800 mL	PL6727-0007		

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