Covalent coupling procedures for Sera-Mag™ and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles

Sera-Mag and Sera-Mag SpeedBeads provide a cost-effective magnetic bead separation technology for molecular biology applications, nucleic acid isolation, and immunoassays. The particles feature a large surface area, offering high sensitivity and physical stability. Our product range includes carboxylate-modified particles, as well as amine-blocked and protein A/G, oligo(dT), streptavidin, and neutravidin™ coated versions.

Introduction

The following protocols outline the suggested materials and process for coupling of Sera-Mag and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles to proteins and oligonucleotides. All protocols can be used with Sera-Mag and Sera-Mag SpeedBeads products. These recommended coupling protocols are designed for:

- Optimal covalent coupling of proteins and oligonucleotides to our magnetic particles
- Covalent coupling of proteins using a choice of two protocols
- Simplicity, efficiency, and confidence

Covalent coupling of proteins

Principles of protein binding

Proteins bind to carboxylate-modified particles by adsorption. Adsorption is mediated by hydrophobic and ionic interactions between the protein and the surface of the particles. Adsorption of proteins to particles occurs rapidly due to the free energy of the particle surface. In addition to being adsorbed, proteins may be covalently attached to the surface of carboxylate-modified particles. Carboxyl groups on the particles, activated by the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), react with free amino groups of the adsorbed protein to form amide bonds. We recommend covalent coupling of ligands. The one-step covalent coupling procedure with EDAC is recommended for covalent coupling of most proteins. However, if EDAC is found to damage the protein of interest, the two-step procedure, which includes a pre-activation (active ester) step prior to introducing the protein, can be used for covalent coupling.

Fig 1. SEM image of Sera-Mag particles showing the cauliflower-like surface that dramatically increases the overall surface area available for binding. Image shows Sera-Mag SpeedBeads, which have two layers of magnetite and encapsulation compared with one of each for the standard Sera-Mag particles. The second layer of magnetite within the SpeedBeads particle provides 2× faster speed in response to a magnetic field. Sera-Mag and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles feature carboxylic groups on the surface that permit easy covalent coupling using simple carbodiimide chemistry.

This document provides one- and two-step covalent coupling protocols for proteins that have been used successfully by many of our customers. It also includes a protocol for covalent coupling of oligonucleotides. These protocols are all written for 1.0 ml optimization reactions. For larger reactions, scale up all volumes proportionally.

Materials for both protein protocols

- **Magnetic particles**
  Sera-Mag and Sera-Mag SpeedBeads particles are available in a nominal 1 µm size. Both types of magnetic particles are encapsulated with a carboxylated polymer surface. The amount of acid on the particle surface is determined in milliequivalents (mEq) of carboxyl per gram of particles (mEq/g), with mEq measured in mmol. Our particles are provided with this value and the calculated parking area (area per carboxyl group).

  Note! Parking area (PA) is a parameter that allows comparison of carboxylate-modified particles of different diameters and titration values (mEq/g). PA is an area-normalized density of carboxyl groups, in units of A^2/COOH. If two particles have the same PA, a particular protein molecule will "park on" the same number of carboxyl groups on either particle's surface. The molecule will have an equivalent opportunity for covalent coupling to either particle, assuming that all the carboxyls are activated on both particles.
- **Bicinchoninic acid (BCA) protein assay**
- **Reaction buffer**
  2-(N-morpholino)-ethanesulfonic acid (MES) buffer. Prepare a 10× stock buffer at 500 mM, pH 6.1. Store the 10× stock at 4°C and discard if yellowed or contaminated.
- **1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)**
  Just before use, weigh approximately 10 mg of EDAC on an analytical balance. Add 1.0 ml of deionized water for each 10.0 mg to obtain a final concentration of approximately 52 µmol/ml.

  Note! EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. Therefore, EDAC should be stored in a desiccator at -20°C and brought to room temperature just before weighing.
- **For two-step coupling procedure only:**
  N-hydroxysuccinimide (NHS), 50 mg/ml in water.
- **Protein stock**
  The protein that will be used to coat the particles should be completely dissolved and not too concentrated. A concentration of 1–10 mg/ml in water is recommended for most proteins.
- **Distilled water**
- **Appropriate labware including:**
  - Pipettes and tips (10 µl–5 ml)
  - Mixing wheel or other mixing equipment
  - Appropriate magnetic separation device
  - Microcentrifuge tubes
  - Microcentrifuge
  - Immersible probe-type ultrasonicator. Use a microtip (1/8 inch or similar) to resuspend pellets during washing and to redisperse clumped particles. A few seconds of sonication should be sufficient for 1 ml reactions. Alternatively, pellets may be stirred or resuspended by repeated aspiration with a fine pipette tip.

    Note! Vortex mixing and bath-type sonicators are not effective for resuspending most pellets.

Considerations for both protein protocols

Optimizing the amount of EDAC and protein

1. Determine the optimal molar ratio of EDAC:COOH.
   a. **For one-step coupling:** Perform an EDAC titration while holding the amount of protein constant. We recommend EDAC:COOH ratios of 0, 0.5, 1, 2.5, 5 and 10:1 for optimization.
   b. **For two-step coupling:** Optimize the EDAC:COOH ratio, starting with a recommended 2.5:1 ratio. A molar ratio of 20:1 NHS:COOH is recommended for all reactions.
2. Determine the amount of protein to add.
   Perform a protein titration, holding the determined EDAC concentration fixed. The optimal amount of protein to use depends on several factors:
   a. Surface area available: surface area per mg of particles increases linearly with decreasing particle diameter.
   b. Colloidal stability: proteins can have stabilizing or destabilizing effects on the particles.
   c. Immunoreactivity: the optimal amount of bound sensitizing protein must ultimately be determined by functional assay. Performing a protein titration or binding isotherm is a good first experiment. We suggest starting with protein concentrations of 0, 25, 50, 75, 100, and 150 or 200 µg/mg of particle.

Other considerations

- When adding the protein to the particles, rapid mixing is critical for even coating. When working at a 1 ml scale, pipette the protein stock directly into the buffered particles. Use the same pipette tip to "syringe" the solution (mix up and down quickly.) When working at a larger scale, place the particles in a beaker with an overhead mixer. While mixing well, add the protein stock quickly into the middle of the vortex.
- For optimization scale (1 ml), it is convenient to run coupling reactions in microcentrifuge tubes. With conventional microcentrifuges, coated particles are pelleted at full speed in 10–30 min.

- The particles may clump during coupling due to the electrostatic effect of the positively charged EDAC molecules, the effect of the protein itself, or consumption of negative charge by amide bond formation. Washing into fresh buffer to remove EDAC and unbound protein, followed by sonication, generally reverses the clumping.
- To help prevent clumping during coupling, lower the percent solids in the coupling step to 0.5% instead of 1%.
- Long-term colloidal stability of coated particles requires development of an appropriate storage buffer.
  - The selection of storage buffer and pH is critical in achieving optimal particle performance. The following components have all been shown to be useful for stabilizing particle preparations while permitting specific agglutination reactions to occur: zwitterionic buffers (e.g., MOPSO), blocking proteins (e.g., bovine serum albumin (BSA) and fish skin gelatin [FSG]), higher pH, detergents, and sodium salicylate.
  - In addition to enhancing colloidal stability, blocking proteins with high negative charge, such as BSA and FSG, may be used to block the particle surface against nonspecific sample adsorption. FSG works especially well with antibody-coated particles.

Covalent coupling procedures for proteins

One-step coupling procedure

1. Use the following information to calculate the amount of EDAC required. See step 1a of *Optimizing the amount of EDAC and protein* for details. Reactions of 1 ml are recommended for optimization.

   The magnetic particle (MP) acid content, provided in mEq/g, is equivalent to µmole/mg.

   Note! 1 ml of 1% particles contains 10 mg particles.

   The µmol EDAC required = (acid content, in µmol/mg) × 10 mg particles × desired ratio. Use this value in the following equation to determine how much of the EDAC stock to add:

   $$\frac{\text{µmol EDAC required}}{52\ \text{µmol/ml}} = \text{ml of EDAC stock for a 1 ml reaction}$$

2. Set up each binding reaction by pipetting into microcentrifuge tubes in the following order:
   a. 50 µl of 10× MES buffer: 25 mM final
   b. Water to bring reaction up to 1.0 ml final volume
   c. 100 µl of 10.0% solids stock particles: 1.0% solids final
   d. Protein stock solution (add last). See step 2 of *Optimizing the amount of EDAC and protein for details*.
3. Mix the tubes for approximately 15 min on a mixing wheel at room temperature.

   Note! Gentle, constant mixing is important for particle reactions.
4. Prepare the EDAC solution immediately before use, and mix the calculated volume rapidly into the reaction by pipetting up and down repeatedly with the pipettor.
5. Mix tubes at room temperature on a mixing wheel or other device for 1 h. Particles may clump during this time, but this is not unusual or harmful.
6. To remove unbound protein, pellet particles by centrifugation (10–30 min in a standard microcentrifuge), and decant the supernatant.
7. Perform two washes with your buffer. You may use the MES buffer or a higher pH buffer of your choice. Pellet particles by centrifugation as in previous step, and decant the supernatant. Use ultrasonication to resuspend pellets between washes.
8. Resuspend the final pellet to the desired % solids with buffer that does not contain blocking proteins. You may use the MES buffer or a higher pH buffer of your choice. For example, if the target % solids is 1.0%, add 0.97 ml of the buffer, which accounts for a small amount of liquid that will remain after pellet formation.
9. Perform a BCA protein assay to determine the amount of protein bound on the particles.
10. For long-term colloidal stability, a stabilizing storage buffer will be needed. After performing the protein analysis, coated particles can be pelleted and resuspended in a variety of storage buffers, and the colloidal stability and reactivity can be optimized.

    Note! Covalently bound protein will not elute when subjected to detergent washes or buffer changes. Thus, covalently coupled reagents are compatible with a wide variety of buffer additives.

Active ester two-step coupling procedure

Step 1: Preactivation

1. Use the following information to calculate the amount of EDAC. See step 1b in *Optimizing the amount of EDAC and protein* for details. Reactions of 1 ml are recommended for optimization. MP is an abbreviation for magnetic particles.

   The magnetic particle (MP) acid content, provided in mEq/g, is equivalent to µmole/mg.

   Note! 1 ml of 1% particles contains 10 mg particles.

   The µmol EDAC required = (acid content, in µmol/mg) × 10 mg particles × desired ratio. Use this value in the following equation to determine how much of the EDAC stock to add:

   $$\frac{\text{µmol EDAC required}}{\text{52 µmol/ml}} = \text{ml of EDAC stock for a 1 ml reaction}$$

2. Pipette into microcentrifuge tubes in the following order:
   a. 100 µl of 10x MES buffer: 50 mM final
   b. Water to bring reaction up to 1.0 ml final volume
   c. 100 µl of 10.0% solids stock particles: 1.0% solids final
   d. 230 µl NHS solution: 100 mM final
   e. EDAC solution, calculated amount
3. Mix tubes at room temperature on a mixing wheel or similar equipment for 30 min.

   Note! Gentle, constant mixing is important for particle reactions.
4. Pellet particles by centrifugation, and decant the supernatant.
5. Resuspend particles with 1 ml of 50 mM MES buffer, pH 6.1. Pellet particles (10–30 min in a standard microcentrifuge), and decant the supernatant.
6. Resuspend the pellet by adding the following and sonicating:
   a. 100 µl of 500 mM MES buffer: 50 mM final
   b. Water to bring reaction up to 1.0 ml final volume

Step 2: Protein coupling

7. Add the protein stock solution, with rapid mixing.
8. Mix tubes at room temperature on a mixing wheel or similar equipment for 1 h.

   Note! Gentle, constant mixing is important for particle reactions.
9. To remove unbound protein, pellet particles as previously, and decant the supernatant.
10. Perform two washes with 50 mM buffer. The MES buffer or a higher pH buffer of your choice may be used. Pellet particles as previously, and decant the supernatant. Use ultrasonication to resuspend pellets between washes.
11. Resuspend the final pellet to the desired % solids with buffer that does not contain blocking proteins. The 50 mM MES buffer or a higher pH buffer of your choice may be used. For example, if the target % solids is 1.0%, add 0.97 ml of the buffer, which accounts for a small amount of liquid that will remain after pellet formation.
12. Perform a BCA protein assay to determine the amount of protein bound on the particles.
13. For long-term colloidal stability, a stabilizing storage buffer will be needed. After performing the protein analysis, coated particles can be pelleted and resuspended in a variety of storage buffers, and the colloidal stability and reactivity can be optimized.

    Note! Covalently bound protein will not elute when subjected to detergent washes or buffer changes. Thus, covalently coupled reagents are compatible with a wide variety of buffer additives.

Covalent coupling of oligonucleotides

Considerations

- It is good laboratory practice to perform this procedure in a DNase/RNase-free environment.
- The following protocol is for 1 ml reaction volumes. Larger reactions can be performed by scaling up all volumes proportionally to maintain the same % solids.
- We recommend starting with 2 nmol oligonucleotide/mg of particles. However, this value must be optimized for each application.

Materials for oligonucleotide coupling

- **Magnetic particles**
  Coupling reactions are typically performed at a particle concentration of 1% solids.
- **Coupling buffer**
  2-(N-morpholino)-ethanesulfonic acid (MES) buffer. Prepare a 10× stock buffer at 500 mM, pH 6.0. Store the 10× stock at 4°C and discard if yellowed or contaminated.
- **Amine-modified oligonucleotide of choice**
  Most oligonucleotides are provided as a lyophilized powder. Reconstitute in water before use.
- **Coupling reagent**
  N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). Within 5 min of adding to the reaction, weigh approximately 10 mg of EDAC on an analytical balance. Add DNase/RNase-free water to bring the final volume up to 1.0 ml. The final concentration will be approximately 10% (w/v).

  Note! EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. Therefore, EDAC should be stored in a desiccator at -20°C and brought to room temperature just before weighing.
- **DNase/RNase-free water**
- **Wash buffers:**
  - 0.1 M imidazole at pH 6.0
  - 0.1 M sodium bicarbonate buffer, do not need to adjust pH

Procedure for oligonucleotide coupling

1. Vortex the stock particle suspension before use to ensure that there is no visible pellet on the bottom or any particle clumps clinging to the wall of the storage container.

   Note! Upon storage, particles settle over time and must be completely resuspended before use.
2. In a suitable container (e.g., microcentrifuge tube, conical tube, or bottle) set up the coupling reaction by adding each of the components in the following order:
   a. DNase/RNase-free water: volume required to bring reaction up to desired final volume
   b. 100 µl of 10× Coupling buffer for each 1 ml reaction volume: 50 mM final
   c. Sera-Mag SpeedBeads; Use 200 µl of the supplied stock (at 5% solids) for each 1 ml reaction
   d. Amine-modified oligonucleotide
   e. Freshly prepared EDAC solution: 100 µl for a 1 ml reaction
3. Perform the coupling reaction at 37°C overnight with continuous mixing.

   Note! Use a roller or rocker. If using bottles, place them in a head-to-tail orientation. DO NOT use a magnetic stir bar.
4. Wash twice with a full reaction volume (e.g., 1 ml for a 1 ml reaction) of DNase/RNase-free water.

   Note! To perform a wash, magnetically separate the particles, aspirate the clear supernatant, and resuspend particles by vortexing them in an aliquot of wash buffer equivalent to the reaction volume.
5. Wash twice with a reaction volume of 0.1 M imidazole (pH 6.0). Incubate for 5 min at 37°C, agitating constantly.
6. Wash three times with a reaction volume of 0.1 M sodium bicarbonate. Incubate for 5 min at 37°C, agitating constantly.
7. Wash twice with a reaction volume of 0.1 M sodium bicarbonate. Incubate for 30 min at 65°C, agitating constantly.
8. Store the particles at 1% solids content in DNase/RNase-free water or an appropriate buffer for your downstream application.

Ordering information

Product	Quantity	Product code
More hydrophilic		
Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles	15 ml	45152105050250
	100 ml	45152105050350
Sera-Mag Carboxylate-Modified Magnetic Particles	15 ml	24152105050250
	100 ml	24152105050350
	1000 ml	24152105050450
Less hydrophilic		
Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles	15 ml	65152105050250
	100 ml	65152105050350
Sera-Mag Carboxylate-Modified Magnetic Particles	15 ml	44152105050250
	100 ml	44152105050350
	1000 ml	44152105050450

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