Technical Note:
TN-027.02

Particle Reagent Optimization - Recommended Adsorption and Covalent Coupling Procedures

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Key Words:

- Polystyrene
- Magnetic
- Protein Binding
- Covalent Coupling
- Parking Area
- Oligonucleotides
- Carboxyl
- Amino

Our strength is in offering you a complete particle technology. We give you simple protocols for working with particles. We provide you concrete data, backed by years of applications research in our own labs.

Introduction

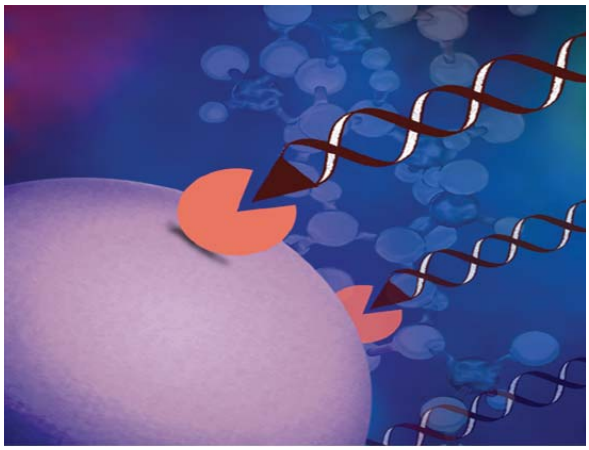
The following procedure outlines the suggested materials and process for the coupling of Thermo Scientific polymer particles to proteins. These recommended coupling procedures are designed for:

- Optimal adsorption of proteins to particles
- Optimal covalent coupling of proteins to particles
- Choice of two protocols for covalent coupling
- Simplicity, efficiency, and confidence

Principles of Protein Binding

Proteins bind to polystyrene (PS) or carboxylate-modified (CM) particles (magnetic and nonmagnetic) 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 particle surface free energy. Proteins may also 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. For magnetic particles, we recommend following a covalent coupling procedure. Performing covalent coupling with the direct EDAC procedure is universally useful. If exposure of a protein to EDAC is discovered to be harmful to the protein, then a pre-activation (active ester) step prior to introducing the protein is an alternative for successful covalent coupling.

Here we give the protocols for both adsorption and covalent coupling which have proven useful for many of our customers. These protocols are written for 1.0 mL "optimization series" reactions. For larger reactions, all volumes may be scaled up proportionally.



Materials and Methods

1. Particles

- Polystyrene Particles

Thermo Scientific Polystyrene Particles for immunoassays are available in standard sizes ranging from 0.05 µm to 2.0 µm. Larger particles are also available. These polystyrene particles are manufactured by emulsion polymerization using an anionic surfactant and have surface sulfate groups which arise from the polymerization initiator. Thermo Scientific polystyrene particles are formulated to have low free surfactant and, generally, the surfactant used does not interfere with protein binding. For this reason, it is recommended that Thermo Scientific polystyrene particles be used without any preliminary clean-up.

- Carboxylate Modified Particles

Nonmagnetic and Magnetic

Thermo Scientific carboxylate modified particles (nonmagnetic) are available in sizes ranging from 0.05 µm to 3 µm. These carboxylate modified particles are manufactured by the co-polymerization of styrene and acrylic acid using emulsion polymerization methods. Carboxylate modified particles are available in a wide range of carboxyl densities. Titration values in milliequivalents of carboxyl per gram of particles (mmoles/g, or µmoles/mg) are provided with each lot. In addition, the calculated parking area (area per carboxyl group) is provided with each lot. Thermo Scientific carboxylate modified particles are formulated to have low free detergent. The detergent used does not generally interfere with protein binding. Carboxylate modified

particles may be rigorously cleaned by ion exchange with mixed bed resin or by tangential flow filtration (TFF). Such cleaning removes various ionic byproducts, including detergent, soluble polymers, and buffer salts, which may affect coupling chemistry. The need for preliminary clean-up of carboxylate modified particles must be established on a case-by-case basis.

Thermo Scientific Sera-Mag particles are available in a nominal 1 µm size. Our magnetic particles are encapsulated with a carboxylated polymer surface. The amount of acid on the surface of Sera-Mag particles is typically higher than our nonmagnetic particles.

Note: Parking area (PA) is a parameter that allows comparison of carboxylate modified particles of different diameters and titration values (mEq/g). It is an area-normalized density of carboxyl groups, given in Å²/COOH. If two particles have the same PA, a particular protein molecule will "park on" the same number of carboxyl groups on the surface of either particle, and have an equivalent opportunity for covalent coupling (assuming all the carboxyls are activated.)

2. BCA (Bicinchoninic Acid) Protein Assay for Particles

See our Technical Supplement, BCA Assay for Particles, for materials & methods. The information can be found on www.thermo.com/particletechnology under Technical documents in the Technical Notes folder titled TN-038 - Reagent Optimization BCA Assay Quick Elution Technique.

3. Reaction Buffer: MES Buffer 2-(N-morpholino)-ethanesulfonic acid

Prepare 10X stock buffer at 500 mM, pH 6.1. The pH will not change significantly on dilution. Store at 4°C and discard if yellowed or contaminated.

4. EDAC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride 52 µmol/mL:

Just before use, weigh approximately 10 mg of EDAC on an analytical balance: for each 10.0 mg weighed add 1.0 mL deionized water.

Note: EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. EDAC should be stored in a desiccator at -5°C and brought to room temperature before weighing.

5. NHS, N-hydroxysuccinimide

(Active Ester-Two-step Coupling Procedure only)

50 mg/mL in water (very soluble)

6. Protein Stocks

Typically, a protein stock in the range of 1-10 mg/mL is recommended.

Note: The protein to be coated onto the particles should be completely dissolved and not too concentrated.

7. DI water

Appropriate labware including:

- Pipettes and Tips (10 µL – 5 mL)
- Mixing Wheel or Other Device
- Appropriate Magnetic separation device if using Sera-Mag particles
- Microcentrifuge tubes
- Microcentrifuge
- Tangential flow filtration: Smaller particles may require tangential flow filtration or ultracentrifugation for washing.

Note: Tangential flow membrane devices are available from several suppliers such as Spectrum Labs in sizes suitable for processing particles in milliliter to liter quantities. Particles as small as 0.05 µm may be reliably processed with TFF membranes.

- Probe-type Ultrasonicator

Probe-type ultrasonicator with microtip should be used for resuspending pellets during washing. Sonication is also helpful for redispersing clumped particles in a stabilizing buffer. An immersible ultrasonic probe is the ideal tool for efficient resuspension of particle pellets. For 1.0 mL reactions a few seconds of sonication is sufficient. 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.

Adsorption

Before You Begin:

- The optimal amount of protein to use depends on several factors:

(1) Surface area available: surface area per mg of particles increases linearly with decreasing particle diameter.

(2) Colloidal stability: proteins can have stabilizing or destabilizing effects on particles.

(3) Immunoreactivity: the optimal amount of bound sensitizing protein must ultimately be determined by functional assay.

- When the protein is added 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, and using the same pipette tip, "syringe" the solution (mix up and down quickly.)

When working at a larger scale, have the particles in a beaker with a stir bar for nonmagnetic particles or an overhead mixer for magnetic particles, mixing well, and add the protein stock quickly into the middle of the vortex.

- Performing a protein titration or binding isotherm is a good first experiment.
- For a 0.3 µm diameter particle (nonmagnetic), a reasonable starting range would be 10-200 µg protein/mg of particles.
- Adsorbed proteins may elute from the particle surface if the wash/storage buffers are different from the adsorption buffer.
- Many detergents will elute adsorbed proteins and should not be used with the adsorption protocol.

Procedure:

1. Calculate the amount of each component needed.

Note: The Coupling Procedure MS Excel Calculation Sheet may be utilized by placing "0" in the fields for EDAC:COOH. The Calculation sheet can be found on www.thermo.com/particletechnology under Technical documents in the Technical Notes folder titled TN-041 - Direct EDAC Coupling Procedure.

2. Prepare and check all stock components required.

3. Once the amount of each component is prepared, set up the binding reaction by pipetting the following into microcentrifuge tubes in the order given:

a. 50 µL 500 mM stock MES buffer: 25 mM final

b. DI Water to make 1.0 mL final volume

c. 100 µL of 10.0% solids stock particles:
1.0%final solids

d. Protein stock solution

(the protein should be added last and mixed very rapidly into the reaction mixture by syringing repeatedly with the pipettor)

Note: Improper mixing can yield unevenly coated particles.

4. Mix tubes at room temperature on a mixing wheel or other device for one hour.

Note: Gentle, constant mixing is important for particle reactions.

5. Remove unbound protein: pellet particles by centrifugation and decant the supernatant.

6. Perform *two* washes with your buffer (this may be the MES buffer). Pellet particles by centrifugation and decant the supernatant. Resuspend pellets between washes using ultrasonication.

7. Resuspend final pellet to desired % solids with the same buffer.

For example: If the target % solids is 1.0%, then one would add 0.97 mL of the same buffer, given that some liquid remains after pellet formation.

8. Perform the BCA Assay for Particles procedure as an analytical tool to assess the amount of protein bound on the particles.

Covalent Coupling

Before You Begin:

• To determine the optimal amount of EDAC Concentration (EDAC:COOH) in one step covalent coupling, an EDAC titration (holding the protein constant) is performed.

Note: The Coupling Procedure MS Excel Calculation Sheet may be utilized by placing ranges of concentrations in the "EDAC:COOH" fields and a constant value for the "Protein added" fields.

It is recommended that approximately a 0.5 to 2.5 fold molar excess over particle carboxyl concentration be used.

For Sera-Mag particles, the following ratios of EDAC: COOH – 0, 0.5, 1, 2.5, 5 and 10 are suggested for optimization.

• For Active Ester (two step coupling) the concentration of EDAC:COOH may be varied, however the recommended molar ratio is 2.5 to 1 and for NHS: COOH the recommended molar ratio is 20 to 1.

• Once an optimal EDAC concentration is determined, the optimal amount of protein to be added for meeting the application performance criteria is to be determined. To determine the optimal protein amount to be added, perform a protein titration holding the determined EDAC concentration fixed.

Note: The Coupling Procedure MS Excel Calculation Sheet may be utilized by placing ranges of concentrations in the "Protein added" field and the determined optimal EDAC: COOH concentration in the "EDAC:COOH" fields.

• The optimal amount of protein to use depends on several factors:

(1) Surface area available: surface area per mg of particles increases linearly with decreasing particle diameter.

(2) Colloidal stability: proteins can have stabilizing or destabilizing effects on the particles.

(3) 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.

For a 0.3 µm diameter particle (nonmagnetic), a reasonable starting range would be 10-200 µg protein/mg MP.

For Sera-Mag particles, we would suggest starting with protein concentrations of 0, 25, 50, 75, 100 and 150 or 200 (µg/mg of particle.)

• When the protein is added 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, and using the same pipette tip, "syringe" the solution (mix up and down quickly.)

When working at a larger scale, have the particles in a beaker with a stir bar for nonmagnetic particles or an overhead mixer for magnetic particles, mixing well, and add the protein stock quickly into the middle of the vortex.

• For optimization scale, it is convenient to run coupling reactions in microcentrifuge tubes. With conventional microcentrifuges such as Eppendorf, coated particles of 0.150 µm or greater diameter are pelleted in 10-30 minutes.

For smaller particles (<0.150 µm diameter), longer centrifugation times are needed and the pellets are more difficult to resuspend.

• Smaller particles may require tangential flow filtration or ultracentrifugation for washing.

• Colloidal stability problems increase with decreasing

particle diameter. Lowering the percent solids in the coupling step to 0.5% instead of 1% will help prevent clumping during coupling.

- 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. Long term colloidal stability of coated particles requires development of the correct storage buffer.
- The selection of storage buffer and pH is critical in achieving optimum particle performance. Zwitterionic buffers, such as MOPSO, blocking proteins, such as bovine serum albumin (BSA), and fish skin gelatin (FSG), higher pH, detergents and sodium salicylate have all proven to be useful for stabilizing particle preparations while permitting specific agglutination reactions to occur.
- Blocking proteins with high negative charge, such as BSA and FSG, may be used to add colloidal stability, as well as block the surface against nonspecific sample adsorption. FSG works especially well with antibody-coated particles.

One Step Coupling Procedure

1. Calculate the amount of EDAC required.

Note: The Coupling Procedure MS Excel Calculation Sheet may be utilized to perform the calculations.

Given Equations:

(MP acid content) meq/g is equivalent to µmole/mg

Note: 1 mL of 1% particles contains 10 mg particles

(Acid content, µmol/mg) (10 mg particles) (desired ratio) = µmol EDAC required

(µmol EDAC required) / (52 µmol/mL) = mL EDAC stock per mL of reaction

2. Set up binding reaction by pipetting into microcentrifuge tubes in the order given:

   a. 500 mM stock MES buffer: 25 mM final

   b. Water to make 1.0 mL final volume

   c. 10.0% solids stock particles: 1.0% solids final

   d. Protein stock solution (add last)

3. Mix the tubes for approximately 15 minutes 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 syringing repeatedly with the pipettor.

5. Mix tubes at room temperature on a mixing wheel or other device for one hour. Particles may clump during this time, but this is not unusual or harmful.

6. Remove unbound protein: pellet particles by centrifugation for carboxylate modified particles, or magnet for Sera-Mag particles, and decant the supernatant.

7. Perform two washes with your buffer. (This may be the MES buffer or a higher pH buffer of your choice.) Pellet particles by use of a centrifuge for carboxylate modified particles, or magnet for Sera-Mag particles, and decant the supernatant. Resuspend pellets between washes by ultrasonication.

8. Resuspend final pellet to desired % solids with buffer that does not contain blocking proteins. (This may be the MES buffer or a higher pH buffer of your choice.)

For example: If the target % solids is 1.0%, then one would add 0.97 mL of the same buffer, given that some liquid remains after pellet formation.

9. Perform the BCA Assay for Particles procedure as an analytical tool to assess 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 optimized.

Note: Covalently bound protein will not elute when subjected to detergent washes or buffer changes; thus, covalently coupled reagents are compatible with a wider variety of buffer additives than reagents where the proteins are only adsorbed to the particles.

Active Ester Two Step Coupling Procedure

Step One: Preactivation

1. Pipette into microcentrifuge tubes in the order given:

   a. 100 µL of 500 mM MES buffer: 50mM final

   b. Water to make 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

2. Mix tubes at room temperature on a mixing wheel or other device for 30 minutes.

Note: Gentle, constant mixing is important for particle reactions.

3. Pellet particles by centrifugation for carboxylate modified particles, or magnet for Sera-Mag MP, and decant the supernatant.

4. Resuspend particles with 1 mL 50 mM MES buffer, pH 6.1. Pellet particles by centrifugation for carboxylate modified particles, or magnet for Sera-Mag particles, and decant the supernatant.

5. Resuspend the pellet by adding the following and sonicating:

   a. 100 µL 500 mM MES buffer: 50mM final

   b. Water to make 1.0 mL final volume

Step Two: Protein Coupling

6. Add the protein stock solution.

7. Mix tubes at room temperature on a mixing wheel or other device for 1 hour.

Note: Gentle, constant mixing is important for particle reactions.

8. Remove unbound protein: pellet particles by centrifugation for carboxylate modified particles or magnet for Sera-Mag-particles and decant the supernatant.

9. Perform two washes with your 50mM buffer. (This may be the MES buffer or a higher pH buffer of your choice.) Pellet particle by centrifugation for carboxylate modified particles or magnet for Sera-Mag particles and decant the supernatant. Resuspend pellets between washes by ultrasonication.

10. Resuspend final pellet to desired % solids with buffer that does not contain blocking proteins. (This may be the MES buffer or a higher pH buffer of your choice.)

For example: If the target % solids is 1.0%, then one would add 0.97 mL of the same buffer, given that some liquid remains after pellet formation.

11. Perform the BCA Assay for Microparticles procedure as an analytical tool to assess the amount of protein bound on the particles.

12. For long-term colloidal stability, a stabilizing storage buffer will be needed. After performing the protein analysis, coated particles can be centrifuged and resuspended in a variety of storage buffers, and the colloidal stability and reactivity optimized.

Note: Covalently bound protein will not elute when subjected to detergent washes or buffer changes; thus, covalently coupled reagents are compatible with a wider variety of buffer additives than reagents where the proteins are merely adsorbed to the particles.

Coupling Oligonucleotides Procedure

Before You Begin:

- It is good lab practice to perform this procedure in an RNAse/DNAse free lab environmet.
- If one selects a 5ml reaction volume: one is to adjust to maintain % solids & concentrations accordingly. Another option for the scale is an 1ml build and one could then multiply by 10 for a larger 10ml scale.
- An oligo input loading of 2 nmol/mg (2 nmol of oligo per mg of particle) is recommended as a starting point for experimentation, but this value MUST be optimized to fit each particular application.

Materials

1. Sera-Mag magnetic or Sera-Mag SpeedBeads: Coupling reactions are typically performed at a particle concentration of 1 % solids.
2. Coupling Buffer: 50 mM MES Buffer at pH 6.0
3. Amine-Modified Oligo:
4. Coupling Reagent:

N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC). The final EDAC concentration should be 1 % (w/v) of the final reaction volume. For example, add 0.5 mL of a freshly prepared 10% (w/v) EDAC solution for a 5 mL total reaction volume.

Note: EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. EDAC should be stored in a desiccator at -5°C and brought to room temperature before weighing.

5. Wash Buffers:
   a. 0.1 M Imidazole at pH 6.0
   b. 0.1 M Sodium bicarbonate buffer, native pH

Coupling Procedure:

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

Note: Upon storage, Sera-Mag SpeedBeads settle over time and must be completely resuspended before use.

2. In a suitable container, i.e Falcon tube, microcentrifuge tube, HDPE bottle, etc., set up the coupling reaction by adding each of the following components in order.

   a. RNAse/DNAse free water – Amount necessary to bring the reaction to desired final volume

   b. Coupling Buffer

Note: Typically, a 10x buffer stock is prepared and an aliquot of 1/10th the final reaction volume is added. For example: add 0.5 mL of 500 mM MES Buffer at pH 6.0 for a 5 mL total reaction volume.

   c. Sera-Mag SpeedBeads

Note: Sera-Mag Speedbeads are provided as a 5 % stock particle suspension. To use at a 1 % final particle concentration, perform a 5x dilution. For example, add 1 mL of the stock particle suspension at 5 % solids to 4 mL of coupling buffer for a total reaction volume of 5 mL.

   d. Amine-Modified Oligo of choice in H_2O

Note: Most oligo comes as a lyophilized powder. Reconstitute oligo in water before starting experiment.

   e. Freshly prepared EDAC solution

Note: Prepare a fresh 10 % (w/v) EDAC stock solution in RNAse/DNAse free water LESS THAN 5 minutes before use.

3. Perform the coupling reaction at 37 °C overnight with continuous mixing.

Note: Use a roller or rocker {as long as the bottle has a head-to-tail orientation on the rocker}. DO NOT use a magnetic stir bar.

4. Wash twice with 1x reaction volume of RNAse/DNAse Free water

Note: To perform a wash, magnetically separate the particles, aspirate the clear supernatant liquid, remove magnet and resuspend particles by vortexing in an aliquot of wash buffer equivalent to the reaction volume.

5. Wash twice with 1x reaction volume of 0.1 M Imidazole (pH 6.0) at 37 °C. Incubate for 5 min.*

6. Wash three times with 1x reaction volume of 0.1 M sodium bicarbonate at 37 °C. Incubate for 5 min.*

7. Wash twice with 1x reaction volume of 0.1 M sodium bicarbonate at 65 °C. Incubate for 30 min.*

8. Store at 1 % solids in RNAse/DNAse free water or an appropriate buffer for your downstream application

*** Note: For particles less than 1 µm no agitation is required during incubation. For particles greater than 1µm incubation with continuous agitation is preferred.**

Reference: Benjamin B Stone and W. G. Weisburg, Mol. and Cell. Probes, 1996, 10, 359-370.

Coupling Procedure for SeraMag Blocked Amino Particles

Before You Begin

- Coupling efficiency and particle performance is application specific. As a result, this procedure should be optimized to produce best results. Refer to MSDS for reagents used and follow appropriate handling precautions.
- Each wash consists of magnetic separation, aspiration of supernatant, addition of buffer, and vortexing for 15 seconds or a time sufficient to resuspend pellet. A wash step begins with each addition of buffer.

Materials

1. Sera-Mag SpeedBeads Streptavidin-Blocked or blocked amino particles: Coupling reactions are typically performed at a particle concentration of 1 % solids.

2. Coupling Reagents
25% Glutaraldehyde Solution, in water, electron microscope grade
Sodium Cyanoborohydride

3. Buffer A
10 mM Pyridine, pH 6.0

4. Buffer B
Option 1: 10 mM Pyridine, pH 6.0
Option 2: 50 mM Bicarbonate Buffer at pH 10.0

5. Buffer C
Option 1: 0.1 M Ethanolamine, pH 8.0
Option 2: 0.1 M Glycine, pH 8.0

6. Storage Solution
Option 1: 0.05 % Sodium Azide
Option 2: Buffer of Choice

Coupling Procedure

1. Place desired amount of particle into a suitable container. SeraMag Blocked particles are supplied at 1 % solids. For a 5 mL coupling reaction volume (50 mg particle solid), place 5 mL of mixed particle suspension into the container.

2. Wash the particles 2 times with Buffer A. For a 5 mL reaction, add 5 mL of Buffer A to the pellet for each wash.

3. Resuspend particle pellet to 2.5 % solids with Buffer A. For a 5 mL reaction, resuspend pellet to 2 mL final volume.

4. Add 10 µL of 25 % glutaraldehyde solution per mg of particle (should bring particle concentration to 2 % solids, and glutaraldehyde concentration to 5 % during activation). For a 5 mL reaction, add 500 µL of 25% glutaraldehyde.

5. Mix (roll or mechanically stir) for 3 hours

6. Wash particles 4 times with Buffer B. For a 5 mL reaction, each wash consists of 5 mL of Buffer B.

7. Resuspend final pellet with Buffer B to a concentration of 2 % solids. For a 5 mL reaction, resuspend pellet with 2.5 mL of Buffer B.

8. Add protein stock solution to achieve desired input loading (20 µg/mg to 100 µg/mg typical input range). For Example, a 5 mL (50 mg particle solid) reaction with a desired protein input of 40 µg/mg and a protein stock solution concentration of 20 mg/mL would require 100 µL of protein stock solution.

9. Dilute with Buffer B to achieve a final particle concentration of 1 % solids. For a 5 mL reaction, dilute with Buffer B to a total final reaction volume of 5 mL.

10. Mix (roll or mechanically stir) overnight

11. Magnetically separate and remove supernatant

12. Resuspend pellet to 2 % solids with Buffer B. For a 5 mL reaction, resuspend pellet in 2.5 mL of Buffer B.

13. Prepare a 2.5 % sodium cyanoborohydride stock solution in Buffer B. For a 5 mL reaction, prepare 2.5 mL of stock solution by dissolving 0.063 g of sodium cyanoborohydride in 2.44 mL of Buffer B. Mix until completely dissolved. Dispose of excess solution in accordance with local regulations.

14. Add sodium cyanoborohydride stock solution and additional Buffer B to produce 1 % sodium cyanoborohydride and 1 % particle solid final. For a 5 mL reaction, add 2.0 mL of sodium cyanoborohydride stock solution and 0.5 mL of Buffer B.

15. Mix (roll or mechanically stir) for 1 hour

16. Add 250 µL of Buffer C per each 1 mL of reaction volume. For a 5 mL reaction, add 1.25 mL of Buffer C.

17. Continue to mix (roll or mechanically stir) for 1 hour

18. Wash 4 times with Storage Solution. For a 5 mL reaction, use 5 mL of Storage Solution (typically 0.05 % sodium azide or any buffer of choice) for each wash.

19. Resuspend to final desired particle concentration with Storage Solution. For a 5 mL reaction, resuspended to 5 mL to achieve 1 % solids.

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