Absolute Mag™ Oriented Magnetic Particles Conjugation Kit, 200 nm

Conjugation Protocol

Cat# WHM-K045

1. Formats

WHM-K045 – Absolute Mag™ Oriented Magnetic Particles Conjugation Kit, 200 nm - 1mL

2. Introduction

Absolute Mag™ Oriented Magnetic Particles Conjugation Kit, 200 nm is an easy-to-use magnetic nanoparticle conjugation kit for antibodies. The KIT guarantees the oriented covalent immobilization of IgG antibodies of your own choice to 200 nm magnetic nanoparticles (MNPs). Unlike other antibody-nanoparticle binding Kits, it is not necessary a chemical modification step of the antibody previous to its coupling to ensure an oriented binding. You just need to activate MNPs and mix them with your IgG antibody to have your Ab-MNP conjugate. The final conjugates are ready to be used in a broad range of applications such as magnetic separation, immunoprecipitation, biosensing, exosome analysis, etc.

Typically the maximum amount of antibody that could be bound to the nanoparticles is 5-7µg IgG/mg MNP. The immobilization yield could vary depending on the specific antibody. Final conjugate concentration is 10 mg of MNPs/mL, although it can be possible to have the conjugate at higher/lower MNPs concentration depending on the final application.

The overall conjugation process that takes approx. 6 h (1 h hands on time) and consists in four steps: ***i) reagents, buffers and material preparation*** (hands on time approx. 15 min)**,** ***ii) magnetic nanoparticles activation step*** (hands on time approx. 15 min) ***iii) antibody conjugation step*** (hands on time approx. 10 min) and ***iv) blocking step*** (hands on time approx. 20 min).

3. Kit contents

- 1 vial of Magnetic Nanoparticles at 25mg/mL.
- 1 vial of Activation Reagent A.
- 1 vial of Activation Reagent B.
- 30 mL of 5x Conjugation Buffer
- 2 mL of Blocking Solution (Bovine Serum Albumin solution).
- 10 mL of Storage Buffer (PBS 1x)
- Bovine Gamma Globulin 2 mg/mL, 50 µL

Material needed but not included on this kit:

- 1.5 mL tubes.
- Custom antibody (polyclonal or monoclonal IgG, 120 µg)
- ddH_2O
- Magnet

4. Amount of antibody - magnetic nanoparticles

Each kit is thought to conjugate a maximum of 5-7 µg of polyclonal or monoclonal IgGs per milligram of magnetic nanoparticle. Initial antibody solution must be at 1mg/mL. If the concentration of your Ab solution is higher, the antibody must be diluted using the 1x Conjugation Buffer. (Please see section 7 to prepare 1x Conjugation Buffer). If concentration is lower, you can concentrate the antibody by Protein Purify&Concentrate kit.

5. Shipping and storing conditons

Kit is shipped at 4ºC and each component must be stored properly. Activation Reagent A must be placed at -20ºC. Magnetic nanoparticles solution and, Blocking Solution must be placed at 4ºC; Rest of components can be kept at 22-25ºC.

6. Antibody buffer considerations

Please see below a summary table for compatible buffer conditions for antibodies starting solution.

Ab's BUFFER COMPOSITION	Is it OK?
pH	Around 5.5-8.5
Amine free buffers (MES, MOPS, PBS, Hepes, Conjugation Buffer)	YES
Amine containing buffers (Tris, Glycine …)	NO. Must be removed using a purification system (not provided)
Glycerol	NO. We recommend to remove it using a purification column (not provided)
Thimerosal, Sodium Azide, Merthiolate, Thiomersal	NO. Must be removed using a purification system (not provided)
BSA, Gelatin	NO. Must be removed using a purification system (not provided)

7. Conjugation protocol

The maximum Ab loading capacity that could be achieved is of 5-7 µg Ab/mg of MNP. This correspond to approximately 91-128 molecules of antibody per nanoparticle (**See Section 9-** How to calculate molecules of antibody per nanoparticle?). The following protocol refers to the conjugation of 50-70µg IgG on 10mg of 200nm magnetic nanoparticles (5-7 µg/mg of NP). If lower amounts of conjugated antibody per mg of MNPs are needed, decrease the antibody concentration solution in Step 2 (**See Section 10**- How to change the amount of antibody conjugated to the MNPs?).

Allow kit components to reach room temperature prior to use them. All incubation steps must be carried out at 37ºC, as indicated. Mixing steps are crucial and must be properly performed.

Step1. Reagents, buffers, and material preparation (hands on time approx. 15 min)

Step 1.1. 1 x Conjugation Buffer

1. Mix x 5 ml of 5 x Conjugation Buffer, with 20 mL of Water Type I.

Step 1.2. Wash magnetic nanoparticles

1. Re-suspend the magnetic nanoparticles (vortex for 30 seconds)
2. Transfer 400 µL of magnetic beads to a new 1.5mL tube.
3. Add 600 µL of 1x Conjugation Buffer and re-suspend.
4. Place the tube on a suitable magnetic rack for 5 minutes (or until the supernatant is clear).
5. Carefully and slowly pipette off the supernatant leaving the beads undisturbed. Discard the supernatant.
6. Remove the tube from the magnetic rack and re-suspend gently the nanoparticles adding 1 mL of 1x Conjugation Buffer.
7. Repeat steps 5 and 6, three times.
8. Finally, remove all the supernatant.

Step 1.3. Initial Antibody solution

Immobilization requires a minimum mass of 120 µg of antibody at a concentration of 1 mg/ml for each 10 mg of MNPs. Antibody preparations could be as solids or liquids. Prepare the Initial Antibody solution according to your particular antibody preparation

Solid Antibody preparation

If your antibody preparation to be immobilized is lyophilized (solid form), then simply re-suspend the antibody in sufficient volume to obtain a 1.0 mg/ml solution. Use the buffer recommend by the antibody producer for re-suspension.

Antibody Solution preparation

1. Transfer 120µL of 1mg/mL IgG solution to a new 1.5 mL tube.
2. Add 1080 µL of 1x Conjugation Buffer and mix carefully.
3. Store this 100µg/mL antibody solution at 4ºC (hereafter named as Initial Antibodysolution) until use.
4. Antibody concentration should be determined using Bradford or similar method. BovineGamma Globulin 2 mg/mL is included to be used as standard. Use 1x Conjugation Buffer1x, as blank.

Step 2. Magnetic nanoparticles activation step (hands on time approx. 15 min)

1. Immediately before using, dissolve Activation Reagent A in 500μL of 1x Conjugation Buffer.
2. Immediately before using, dissolve Activation Reagent B in 500μL of 1x Conjugation Buffer.
3. Re-suspend the previously washed magnetic nanoparticles with 500μL of Activation Reagent A and 500μL of Activation Reagent B. (Final volume: 1.0mL).
4. Incubate for 30 min at 37ºC with gentle stirring.

   Note: Agitation has to be employed to insure efficient activation of MNPs and to prevent the MNPs from settling during the activation step.
5. Place the tube on the magnetic rack for 5 minutes (or until the supernatant is clear).
6. Carefully and slowly pipette off the supernatant leaving the beads undisturbed. Discard the supernatant.
7. Remove the tube from the magnetic rack and re-suspend gently the nanoparticles adding1.0 mL of 1x Conjugation Buffer.
8. Place the tube in a magnet for 5 minutes (or until the supernatant is clear).
9. Carefully discard the supernatant and use the activated nanoparticles immediately to conjugate your antibody.

Step 3. Antibody conjugation step (hands on time approx. 10 min)

1. Immediately after MNPs activation step, re-suspend the activated magnetic nanoparticles with 1.0mL of the IgG solution prepared in Step 1.3.
2. Incubate for 2 h at 37ºC with gentle stirring.

   Note- Agitation has to be employed to insure efficient immobilization of the antibody and to prevent MNPs from settling during the coupling conjugation step.
3. Place the tube on the magnetic rack for 5 minutes (or until the supernatant is clear).
4. Carefully and slowly pipette of the supernatant (hereafter named as Immobilization Supernatant) leaving the beads undisturbed.
5. Store the Immobilization Supernatant for later determination of the IgG immobilization yield.
6. Remove the tube from the magnetic rack and re-suspend gently the nanoparticles adding1.0 mL of 1 x Conjugation Buffer.
7. Place the tube on the magnetic rack for 5 minutes (or until the supernatant is clear).
8. Carefully and slowly pipette the supernatant leaving the beads undisturbed. Discard the supernatant and block the Ab-MNP conjugate immediately.

Step 4. Blocking step (hands on time approx. 20 min)

1. Re-suspend the IgG conjugated MNPs with 1.0 mL of Blocking Solution.
2. Incubate for 2 h at 37ºC with gentle stirring or overnight at 4ºC.
3. Centrifuge the Ab-MNP conjugates at 12000 g and 4ºC for 10 min.
4. Carefully and slowly pipette off the supernatant, leaving the beads undisturbed. Discard the supernatant.
5. Re-suspend the conjugates with 1.0 mL of Storage Buffer.
6. Repeat steps 3-5, three times.
7. Re-suspend the conjugates with 1.0 mL of Storage Buffer.
8. Antibody functionalized MNPs are now ready for your application.

8. Determination of the amount of Ab conjugated to MNPs

1. An aliquot of supernatant obtained after the immobilization process (ImmobilizationSupernatant, See Step 3, point 4) can be measured using Bradford assay. BovineGamma Globulin 2 mg/mL is included to be used as standard. Use 1x Conjugation Buffer 1x, as blank.

   Note: any magnetic nanoparticle that could remain in the supernatant must be removedbefore quantifying its protein content with Bradford assay. Before doing the proteinquantification, centrifuge the supernatant at 12000 g for 10 min at 4ºC.

2. Insert the result of Bradford assay (µg/mL immobilization supernatant) in this equation:

$$\mu g\ immobilized\ IgG/mgMNP = \frac{\mu g/ml\ antibody\ initial\ solution - \mu g/ml\ immobilization\ supernatant}{10}$$

9. How to calculate molecules of Ab per MNP?

The following equation is used to determine the molecules of immobilized antibody per nanoparticle:

$$IgG/MNP = \frac{\frac{mol\ of\ immobilized\ Ab}{mg\ MNP} \cdot Na}{\frac{2.2 \cdot 10^{11}\ particles}{mg\ MNP}}$$

Where:

$$mol\ of\ immobilized\ Ab = \frac{g\ of\ immobilized\ Ab}{Molecular\ Weight\ (g/mol)}$$

Na (Avogadro constant): $6.023*10^{23}$ mol^{-1}

Note: Typical IgG molecular weight: 150000 g/mol

10. How to change the amount of antibody conjugated to the MNPs?

If a lower amount of immobilized antibody than 5-7 µg per mg of MNPs is needed, dilute the initial antibody solution. Although the immobilization yield could vary depending on the specific antibody, typical immobilization yields obtained with mouse anti human CD3 (table below) may be used as guidance.

Initial Ab solution (µg/mL)	Dilution Factor*	Immobilization Yield (%)	IgG/MNP**
100	-	50-70	91-128
100	2	100	91
100	3	100	55
100	5	100	37

*Dilution factor to be applied to the initial Ab solution.
**Molecules of immobilized antibody per nanoparticle.

11. Storage of Ab-MNPs conjugate

Store the IgG conjugated MNPs at 4ºC until use. Do not freeze the MNP-bioconjugate. Since, the binding between magnetic nanoparticles and antibodies is covalent, the bioconjugate stability will depend on the long-term stability of your antibody.

If nanoparticles are settle to the bottom of the storage container, shake (vortex) the container for 10-30 seconds or sonicate it employing an ultrasonic bath for 30 -60 seconds, until the nanoparticles have re-dispersed into the solution.

12. Colloidal stability of Ab-MNPs conjugates

The obtained Ab functionalized MNPs are stable within a broad range of pH and ionic strength. We recommend their storage in PBS (Conjugation Storage buffer provided with the Kit), but you could also use bicarbonate pH 8-9 and biological buffers such as MES pH 5-6, HEPES pH 6-7.

13. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
MNPs stock are not attracted by the magnet	The magnet is not strong enough	- Use a stronger magnet. - Centrifuge the conjugates at 12000 g, 4ºC, 10 min
Supernatants are not clear	The magnet is not strong enough	- Use a stronger magnet. - Centrifuge the conjugates at 12000 g, 4ºC, 5 min
MNPs aggregates during the antibody immobilization process	Antibody purity is not adequate	- If the purity of your antibody is undetermined, check its purity on an SDS-PAGE gel PAGE prior to **Step 3: *Antibody conjugation***.
Low or undetectable IgG immobilization yield	Activation Reagents A and B are not well dissolved	- Make sure that both reagents are well dissolved. - Dissolve them immediately before using.
	Improper storage of Activation Reagent A	- Keep and store the Activation Reagent A sealed in the vial provided at -20ºC.
	Proteins such as BSA or gelatin may be present in your antibody solution	- Remove and purify the antibody sample of all protein carriers such as BSA or gelatin prior to **Step 3: *Antibody conjugation***.
	Presence of non-protein amine contaminants	- Remove all non-protein amine contaminants such as glycine or Tris prior to **Step 3: *Antibody conjugation*** using an ultra-centrifugal filter or Protein Purify&Concentrate kit
More IgGs molecules are bound to the MNPs than the IgG amount incubated	Initial concentration of IgG is not correctly determined	- Make sure that the concentration of offered IgG is correct.
	Presence of magnetic nanoparticles in the immobilization supernatant	- Remove the magnetic nanoparticles by centrifugation of the supernatant at 12000 g, 4ºC, 10 min

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