# STANDARD OPERATING PROCEDURE

**Title: Antibody coupling and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform**

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## Purpose

The purpose of this document is to describe the coupling and covalent immobilization of IgG antibodies to Protein G magnetic beads for subsequent peptide immunoaffinity enrichment and mass spectrometry analysis of eluted peptides. The procedure is similar to a previously reported procedure by Schoenherr *et al.*[1]

## Scope

This procedure may be used to couple and covalently link IgG antibodies to Protein G magnetic beads using dimethyl pimelimidate dihydrochloride (DMP), triethanolamine, and monoethanolamine.

## Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

## Equipment

- KingFisher 96 magnetic bead handling platform (5400500, Thermo Scientific), equipped with a deep well magnet head. The KingFisher should be placed into a fume hood for this work, since triethanolamine, monoethanolamine, and DMP (see below) are toxic.
- Dynabeads® MPC®-S (Magnetic Particle Concentrator, A13346, Life Technologies) or DynaMag™-15 Magnet (12301D, Life Technologies)
- LabQuake tube rotator (415110, Barnstead)

## Materials

- KingFisher Microtiter deep well 96 plates (95040450, Thermo Scientific)
- KingFisher 96 tip comb for deep well magnets (97002534, Thermo Scientific)
- 1.7 mL microcentrifuge tubes (24-282, Genesee Scientific)
- 1.5 mL screw-cap tubes (72.692, Sarstedt, Nümbrecht, Germany)
- 1.0 mL filter pipette tips (e.g. RT-L1000F, Rainin)
- Dynabeads® MyOne™ Protein G, 1 μm in diameter, supplied as 30 mg/mL in PBS with 0.02% $NaN_3$ (custom-made, Life Technologies)
- 10X Phosphate buffered saline (PBS) (BP39920, Fisher Scientific)
- CHAPS detergent (28300, Thermo Scientific)
- Water, Optima® LC/MS, suitable for UHPLC-UV (W6-4, Fisher Scientific)
- DMP (dimethyl pimelimidate dihydrochloride, D8388, Sigma)
- Triethanolamine (T1377, Sigma)
- Monoethanolamine (411000, Sigma)
- Hydrochloric acid (A144-500, Fisher Scientific)
- Acetic acid (ACS reagent, ≥99.7%, 242853, Sigma)
- Sodium azide (S8032, Sigma)

## Reagents

- Purified antibody.

## Solutions

- Prepare a 5% (w/v) CHAPS solution in Optima® water by dissolving 1.0 g of CHAPS powder in 20 mL of Optima® water. It will take some time to dissolve the powder completely. (CHAPS aids in the beads transfer from plate to plate on the KingFisher.)
- Prepare 100 mL of a 1X PBS solution, pH 7 by adding 10.0 mL of 10X PBS, pH 7 to distilled and deionized water, for a total of 100 mL.
- Prepare 100 mL of a 1X PBS-0.03% CHAPS solution, pH 7 by adding 10.0 mL of 10X PBS, pH 7 and 600 μL of 5% CHAPS to Optima® water, for a total of 100 mL.
- The following should be prepared in a fume hood, due to the toxicity of triethanolamine. Prepare 125 mL of a 200 mM triethanolamine, pH 8.5 solution from neat triethanolamine by adding 3.32 mL of neat triethanolamine to 120.0 mL of distilled and deionized water, adjusting the pH to 8.5 using 6 M HCl (approximately 900 μL will be needed), then adding distilled and deionized water to arrive at 125 mL.
- The following should be prepared in a fume hood, due to the toxicity of triethanolamine and DMP. This solution (20 mM DMP in 200 mM triethanolamine) should be prepared fresh each day. For e.g. 40 antibodies to be cross-linked, at least 40 mL of 20 mM DMP in 200 mM triethanolamine is needed (1 mL per antibody). Therefore, using the molecular weight for DMP (259.17 g/mol), for e.g. 43 mL of 20

mM DMP in 200 mM triethanolamine, 222.9 mg of DMP needs to be dissolved in 43 mL of 200 mM triethanolamine, pH 8.5.

- The following should be prepared in a fume hood, due to the toxicity of monoethanolamine. Prepare 50 mL of 150 mM monoethanolamine (using a density of 1.015 g/mL and a molecular weight of 61.08 g/mol for monoethanolamine) by adding 451 µL of neat monoethanolamine to distilled and deionized water, for a total of 50 mL. The pH of this solution should be 9.0 without needing adjustment.
- Prepare 100 mL of 5% acetic acid-0.03% CHAPS by adding 5 mL of glacial acetic acid and 600 µL of 5% CHAPS to Optima® water, for a total of 100 mL.
- Prepare a 10% (w/v) sodium azide in water by dissolving e.g. 0.1 g of sodium azide powder in 1 mL of Optima® water.
- Prepare 50 mL of a 1X PBS-0.03% CHAPS-0.1% sodium azide solution by adding 5.0 mL of 10X PBS, 300 µL of 5% CHAPS, and 500 µL of 10% sodium azide to Optima® water, for a total of 50 mL.

## Procedure

The following procedure describes the coupling and immobilization of 100 µg of each antibody to magnetic beads. The antibody-beads ratio used here is 1:1.5 (w:v), with the volume corresponding to the beads suspension volume in the original vendor bottle. The procedure here describes washing the beads in individual tubes, but they can also be washed in bulk first, and then aliquoted into separate tubes for each antibody.

Overnight coupling of antibodies to Protein G magnetic beads, performed individually per antibody in 1.7 mL or 15 mL centrifuge tubes.

1. Calculate the volume of each antibody needed for 100 µg of antibody, and the volume of 1X PBS and 5% CHAPS to arrive at a final volume of 1000 µL per 1.7 mL microcentrifuge tube, with a final CHAPS concentration of 0.03%. The volume of the actual beads (once the supernatant is taken off) is neglected in the calculation here. In case the antibody solution is very dilute, the incubation might need to be performed in a 15 mL centrifuge tube; in this case a 5000 µL total volume should be used to ensure good mixing.
2. Vortex the MyOne beads in the original vendor bottle to obtain a well-mixed suspension.
3. Add 150 µL of MyOne beads to a 1.7 or 15 mL tube for each antibody to be coupled. Vortex the bead bottle after each 3rd or 5th tube since the beads will settle to the bottom of the bottle.
4. Place the tubes into a Dynabeads® MPC®-S or DynaMag™-15 magnetic particle concentrator and let the beads migrate to the magnet for 1-2 minutes. Rotating the tubes from side to side aids in moving the beads from the bottom towards the middle of the tubes, which makes it easier to pipette off the supernatant.
5. Pipette off the supernatant from each tube and discard.

6. Take the tubes out of the Concentrator and add 500 µL of 1X PBS, pH 7; vortex the tubes briefly to dislodge the beads from the side of the tubes.
7. Repeat steps 4-6 once more, for a total of two PBS washes.
8. Repeat steps 4-5 after the second 1X PBS addition.
9. Depending on the volume of each antibody needed (see step 1), add a corresponding volume of 1X PBS to each tube.
10. Add 5% CHAPS to each tube, for a final concentration of 0.03% CHAPS in 1000 or 5000 µL.
11. Add the calculated volume of antibody needed for 100 µg of antibody.
12. Vortex each tube thoroughly to ensure that the beads are well suspended, then put the tubes onto a LabQuake tube rotator and tumble them overnight at 4 °C.

Configuring the KingFisher 96 software method

Eight 96 deep well plates are used for the procedure on the KingFisher, along with a tip comb for a deep well magnet. The following is an overview of each plate's contents per well, along with the mixing time for each step. A bead collection count of 5 was used for each plate, except for plate 7, since the beads are left in plate 7 at the end of the procedure. A medium mixing speed was used for each step, and the procedure was performed at room temperature.

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| Plate 1: | 1.0 mL of antibody-coupled beads suspension, 5 minutes |
| Plate 2: | 1.0 mL of 20 mM DMP in 200 mM triethanolamine (the antibodies are covalently linked to Protein G during this step), 30 minutes |
| Plate 3: | 1.0 mL of 150 mM monoethanolamine (quenching of the cross-linking reaction), 30 minutes |
| Plates 4 and 5: | 1.0 mL of 5% acetic acid-0.03% CHAPS (washing any unbound antibody from the beads), 5 minutes |
| Plate 6: | 1.0 mL of 1X PBS-0.03% CHAPS, 5 minutes |
| Plate 7: | 200 µL of 1X PBS-0.03% CHAPS-0.1% sodium azide (to arrive at a final antibody concentration of 0.5 µg/µL), 5 minutes |
| Plate 8: | Tip comb |

Immobilization of the antibodies on the Protein G magnetic beads using a KingFisher 96

The KingFisher was moved into a fume hood for the procedure, due to the toxicity of DMP, triethanolamine, and monoethanolamine. Of note is that small amounts of the beads will be left behind in some of the deep-well plates (coating the wells), especially in plates 2 and 3 that do not contain CHAPS, yet the amounts are usually negligible compared to the total amount of beads. Whether adding CHAPS to plates 2 and 3 would have negative effects on the cross-linking and quenching reactions has not been tried.

13. Prepare plate 2 by adding 1.0 mL of freshly-prepared 20 mM DMP in 200 mM triethanolamine to each well to be used using filter pipette tips, since the volatile

triethanolamine can damage pipettes. (Plate 2 is prepared before plate 1, to avoid too much settling of the beads before the KingFisher procedure is started.) Place plate 2 into the KingFisher.

14. Take the tubes containing the bead-coupled antibodies off the LabQuake, and centrifuge briefly.
15. Mix each bead-antibody suspension in the 1.7 mL microcentrifuge tubes by pipetting, then transfer to a pre-defined well on plate 1. In the case of the 5 mL antibody-beads suspensions in the 15 mL tubes, the tubes can be placed onto a magnet and 4 mL of the supernatants can be taken out. The remaining antibody-beads suspensions can then be transferred to the deep-well plate.
16. If a large number of antibodies are being immobilized, the beads in some of the wells might have settled to the point at which it is advisable to mix them again using a multi-channel pipette before placing plate 1 onto the KingFisher. This aids in the beads transfer from plate 1 to plate 2.
17. Start the KingFisher method.
18. Prepare plate 3 by adding 1.0 mL of 150 mM monoethanol to each designated well using filter pipette tips and place it into the KingFisher ~5 minutes before the end of the incubation time of plate 2.
19. Prepare plates 4 and 5 by adding 1.0 mL of 5% acetic acid-0.03% CHAPS to each designated well and place them into the KingFisher ~5 minutes before the end of the incubation time of plate 3.
20. Prepare plate 6 by adding 1.0 mL of 1X PBS-0.03% CHAPS to each designated well and place it into the KingFisher.
21. Prepare plate 7 by adding 200 µL of 1X PBS-0.03% CHAPS-0.1% sodium azide to each designated well and place it into the KingFisher.
22. At the end of the method, inspect the tip comb for any beads left on the comb. If beads are stuck to the comb, it can be manually reinserted into plate 7 and manually agitated to dislodge the beads into the solutions.
23. Transfer each antibody solution to separate, labeled 1.5 mL screw cap tubes and store them at 4 °C until use.

## Referenced Documents

1. Schoenherr, R. M., Whiteaker, J. R., Zhao, L., Ivey, R. G., Trute, M., Kennedy, J., Voytovich, U. J., Yan, P., Lin, C., and Paulovich, A. G. (2012) Multiplexed quantification of estrogen receptor and HER2/Neu in tissue and cell lysates by peptide immunoaffinity enrichment mass spectrometry. *Proteomics* 12, 1253-1260.