# STANDARD OPERATING PROCEDURE

**Title: Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves**

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## Purpose

The purpose of this document is to describe the immunoaffinity enrichment of peptides as part of the characterization of immuno-MRM assays using response curves.

## Scope

This procedure may be used to generate immunoaffinity-enriched peptide response curve samples to be analyzed by MRM. It includes a description of i) generating the response curve samples, ii) setting up the immunoaffinity captures using the samples and a mix of antibodies that had previously been cross-linked to magnetic beads, and iii) the automated washing and elution of the target peptides using a robotic magnetic bead handling system. Last, the run order of the samples on the LC-MRM-MS system is given.

## 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 PCR magnet head
- LabQuake tube rotator (415110, Barnstead)
- Dynabeads® MPC®-S (Magnetic Particle Concentrator, A13346, Life Technologies) or DynaMag™-15 Magnet (12301D, Life Technologies)
- DynaMag™-96 Side Skirted Magnet (12027, Life Technologies)
- Centrifuge for use with 96 well plates (5810R, Eppendorf)

## Materials

- 1.7 mL microcentrifuge tubes (24-282, Genesee Scientific)
- 15 mL conical centrifuge tubes (352196, Corning Life Sciences)
- MColorpHast® pH Test Strips, 5-10 (1.09533.0001, EMD Millipore)
- KingFisher 96 KF microplate (200 µL) (97002540, Thermo Scientific)
- KingFisher 96 tip comb for PCR magnets (97002514, Thermo Scientific)
- Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates (HSP-9601, BioRad)
- Chemically resistant sealing foil (T-3025-8B, BioExpress)
- ThermalSeal® PCR Sealing Films (12-168, Genesee Scientific)
- 1 M Trizma hydrochloride buffer, pH 8.0 (Tris, T2694, Sigma)
- 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)
- Acetic acid, ACS reagent, ≥99.7% (242853, Sigma)
- Acetonitrile, Optima® LC/MS, suitable for UHPLC-UV (A955-4, Fisher Scientific)
- Sodium azide ($NaN_3$) (S8032, Sigma)
- Lima bean trypsin inhibitor (LS002829, Worthington Biochemical Corporation)
- Bovine 6 protein tryptic digest equal molar mix (PTD/00006/63, Bruker)

## Reagents

- Light and stable isotope-labeled heavy synthetic peptides for MRM analyses were purchased at >95% purity as measured by HPLC. Cysteine residues of the tryptic light and heavy peptides were carbamidomethylated, and the heavy peptides' C-terminal lysine (K) or arginine (R) residues were uniformly $^{13}C$ and $^{15}N$ labeled, yielding a mass increase of 8 Da (for K) or 10 Da (for R). For peptides at the C-terminus of proteins, other amino acids (such as valine) can be isotopically labeled. Amino acid analysis (AAA) was performed to determine peptide concentrations. Individual peptide stock aliquots were stored in 30% acetonitrile-0.1% formic acid at 100 pmol/µL at -80 °C.
- Rabbit-anti-human monoclonal antibodies (mAbs) were custom-generated to the tryptic and proteotypic peptide targets and covalently coupled to magnetic Protein G beads. For the coupling procedure, see the SOP titled "Antibody coupling and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform."
- Trypsinized human plasma was prepared according to the SOP titled "Trypsin Digestion of Human Plasma."

## Solutions

- Prepare approximately 30 µL of a 0.1 mg/µL solution of lima bean trypsin inhibitor in Optima® water.

- 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 bead transfer from plate to plate on the KingFisher.)
- Prepare 100 mL of a 1X PBS-0.01% CHAPS solution, pH 7 by adding 10.0 mL of 10X PBS and 200 µL of 5% CHAPS to Optima® water, for a total of 100 mL.
- Prepare 100 mL of a 1/10X PBS-0.01% CHAPS solution, pH 7 by adding 1.0 mL of 10X PBS and 200 µL of 5% CHAPS to Optima® water, for a total of 100 mL.
- Prepare 100 mL of 3% acetonitrile-5% acetic acid by adding 3.0 mL of Optima® acetonitrile and 5.0 mL of glacial acetic acid to Optima® water, for a total of 100 mL.
- Prepare a 10% (w/v) $NaN_3$ solution in water by dissolving e.g. 1.0 g of $NaN_3$ powder in 1.0 mL of Optima® water.
- Prepare 50 mL of a 1X PBS-0.03% CHAPS-0.1% $NaN_3$ solution by adding 5.0 mL of 10X PBS, 300 µL of 5% CHAPS, and 500 µL of 10% $NaN_3$ to Optima® water, for a total of 50 mL.
- Prepare a light peptide master mix with a 2 pmol/µL concentration per peptide from the individual light peptide stocks (at 100 pmol/µL, at -80 °C). For 40 individual peptides, for example, this would be done by adding 30 µL of Optima® water to a 1.7 mL microcentrifuge tube, and then adding 3 µL of each peptide stock to the tube. Aliquot this master mix into 1.7 mL microcentrifuge tubes, with 10 µL of the master mix in each aliquot, and store at -80 °C until use.
- Prepare, aliquot, and store a heavy peptide master mix with a 2 pmol/µL concentration per peptide as was done for the light peptide master mix.

## Procedure

The following describes the experiments for a 9-point response curve that has varying concentrations of the heavy (analyte) peptides and a constant concentration of the light (internal standard) peptides in a human plasma digest matrix, including a 0 fmol (blank) sample that does not contain heavy peptides. The 0 fmol sample is prepared for 5 replicate immuno-MRM experiments, and the other samples are prepared for triplicate experiments per curve point. The light peptides were not chosen as analytes since the endogenous detection of some of the peptides in human plasma would not have made it possible to determine the limits of detection and quantification.

The experiments encompass

- the preparation of a monoclonal antibody master mix (with each antibody having previously been covalently coupled (cross-linked) to magnetic Protein G beads, see the SOP titled “Antibody coupling and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform”),

- the preparation of the different curve point samples (with light and heavy peptides spiked into a diluted human plasma digest matrix, with 10 µL of original plasma used per immunoaffinity enrichment (capture) experiment),
- the immunoaffinity enrichment of the peptides from the plasma digest samples, and
- the automated washing of the beads+antibodies+peptides and the elution of the peptides from the antibodies using a KingFisher magnetic bead handling platform.

Each capture sample contains 200 µL of a mix of peptides and plasma digest, plus 50 µL of the antibody master mix. These volumes have been convenient to use for this procedure, yet could be changed to e.g. 150 µL and 25 µL, respectively. Of importance is, however, that the *concentrations* used for plotting the response curves in the end are defined as X fmol of heavy peptide per 10 µL of plasma, or X/10 fmol of peptide per 1 µL of plasma.

Preparation of the monoclonal antibody (mAb) master mix

1. Calculate the *total volume* of the antibody master mix needed based on the 50 µL of antibody-beads master mix wanted per capture and the number of captures for the response curves (allowing for a couple of extra captures for good measure).
2. Calculate the volume of *each individual* cross-linked antibody-to-beads stock needed for all captures, given an antibody concentration of 0.5 µg antibody/µL of the individual antibody-beads suspension (see the SOP titled "Antibody coupling and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform") and the number of captures (plus 2 extra captures for good measure).
   In the end, depending on which volume from these two calculations is larger, excess supernatant (1X PBS-0.03% CHAPS-0.1% $NaN_3$) will either need to be removed from the master mix, or extra 1X PBS-0.03% CHAPS-0.1% $NaN_3$ can be added to the master mix to arrive at the 50 µL of antibody master mix volume for each capture. (Alternatively, since the 50 µL per capture volume is rather arbitrary, 1X PBS-0.03% CHAPS-0.1% $NaN_3$ does not need to be added and a lower volume of the antibody master mix can be used per capture.)
3. Add the calculated volume of each antibody-beads suspension stock to either a 1.7 mL microcentrifuge tube or a 15 mL conical tube.
4. If excess supernatant needs to be removed, put the tube onto a magnetic particle concentrator for 2 minutes and remove the excess supernatant by pipetting.
5. Vortex the tube and spin it down briefly, then keep it on ice until use.

Preparation of the response curve samples

The levels of heavy peptides that were tested in these particular response curves were 5000, 1000, 200, 40, 8, 2, 0.5, 0.125, and 0 fmol per 10 µL of plasma digest. These levels were generated using serial dilutions, with the dilutions from 5000 to 8 fmol/10 µL plasma being covered by 1:5 serial dilutions, and from 8 to 0.125 fmol/10 µL plasma by 1:4 serial

dilutions (smaller intervals were chosen for the lower levels since this would allow more robust characterizations of the limits of quantification). The synthetic light peptides were spiked at a constant level of 100 fmol per 10 µL of plasma digest. Considering a) the 200 µL volume of peptides-in-plasma wanted per capture sample, b) the number of replicates per curve point, c) wanting to be able to keep the same pipettor volume setting during the serial dilution, and d) wanting comfortably enough volume at each step for pipetting purposes, the following procedure was followed to prepare the response curve samples.

6. Thaw a tube containing 1 mL of human plasma digest (see SOP titled "Trypsin Digestion of Human Plasma") on ice and add 15 µL of lima bean trypsin inhibitor solution at 0.1 mg/µL to yield a 1:2 trypsin inhibitor:trypsin ratio (w/w) (to avoid potential digestion of the antibodies during the enrichment).
7. Prepare a light peptide-in-plasma digest mix as follows (this yields a total volume of 8600 µL):
   7.1. Add 7738 µL of 1X PBS-0.01% CHAPS to a 15 mL conical centrifuge tube;
   7.2. add 430 µL of human plasma digest (equivalent to 430 µL of original, undigested plasma);
   7.3. add 430 µL of 1 M Tris, pH 8.0 (to ensure a pH between 7.0 and 8.0 since the plasma digest can be acidic after digestion and desalting; the antibodies that are added later might degrade under acidic conditions);
   7.4. add 2.15 µL of the 2 pmol/µL synthetic light peptide master mix (yielding a synthetic light peptide concentration of 100 fmol/10 µL of plasma).
   7.5. vortex and spin down the sample briefly.
8. Aliquot the light peptide-in-plasma digest mix, add heavy peptides, and perform the serial dilutions as follows:
   8.1. Aliquot the light peptide-in-plasma mix into eight 1.7 mL microcentrifuge tubes:
      a) 1100 µL into 1 tube (label the tube with 5000 (fmol/10 µL plasma))
      b) 880 µL into each of 4 tubes (label the tubes with 1000, 200, 40, and 8 (fmol/10 µL plasma))
      c) 660 µL into each of 3 tubes (label the tubes with 2, 0.5, and 0.125 (fmol/10 µL plasma)).
   8.2. Place the tube with the remaining light peptide-in-plasma digest mix on ice (5 x 200 µL of this mix will later be transferred to 5 wells of a 96 well plate, representing the 0 fmol/10 µL plasma replicate samples).
   8.3. Take 13.75 µL out of the first (5000) tube and discard, and add 13.75 µL of the 2 pmol/µL heavy peptide master mix to that tube; mix by centrifuging and spin down briefly. Since the heavy peptide mix contained formic acid, measure the pH of the peptide+plasma sample using e.g. 2 µL spotted onto a pH 5.0-10.0 pH test strip to ensure a pH above 7.0. The heavy peptide concentration in this sample is 5000 fmol/10 µL of plasma.

8.4. Perform the 1:5 and 1:4 serial dilutions using 220 µL from the first tube and diluting first in the tubes containing 880 µL of the light peptide-in-plasma mixes, and then into the tubes containing 660 µL. Ensure good mixing at each dilution step.

Overnight immunoaffinity enrichment (capture) of the peptides from the plasma digest samples

9. Add 3x 200 µL of each concentration's response curve sample to 3 wells of a KingFisher 96 KF microplate (cat. # 97002540; the product description calls it a 200 µL-per-well plate, but more than 200 µL of sample can be added if a KingFisher 96 tip comb for PCR magnets is used; here, the total volume will be 250 µL once the beads+antibodies are added).
10. Add 5x 200 µL of the light peptide-in-plasma digest mix to 5 wells of the microplate (representing the 0 fmol/10 µL plasma replicate samples).
11. Vortex the tube containing the antibody master mix until all beads are well suspended. Add 50 µL of the master mix to each well using a single-channel pipette, re-capping the tube after every ~5$^{th}$ addition and briefly vortexing it to continue to ensure a well-mixed bead suspension. Alternatively, the beads can also be added using a multi-channel pipette and a pipette tray.
12. If beads have settled to the bottom of the plate wells at the end of all additions, they can be briefly suspended again using a multi-channel pipette.
13. Immediately cover the plate using chemically resistant sealing foil, making sure that each well is completely sealed so no liquid can escape from any of the wells.
14. Quickly secure the plate onto a LabQuake rotator using lab tape, and move the rotator to a fridge or cold room and rotate the plate overnight at 4 °C.

Automated washing of the beads+antibodies(Abs)+peptides and elution of the peptides from the antibodies using a KingFisher magnetic bead handling platform

The following is an overview of the types of plates and contents that will be used with the KingFisher platform. Plate 1 should be left rotating on the LabQuake until the other plates have been prepared.

| Plate # | Plate type | KingFisher step | Contents | Volume per well (µL) |
|---|---|---|---|---|
| 1 | KingFisher 96 KF microplate | Bead+Ab+peptide mixing and pick-up | Response curve samples | 250 |
| 2 | KingFisher 96 KF microplate | Wash 1 | 1X PBS-0.01% CHAPS | 250 |
| 3 | KingFisher 96 KF microplate | Wash 2 | 1X PBS-0.01% CHAPS | 250 |
| 4 | KingFisher 96 KF microplate | Wash 3 | 1/10X PBS-0.01% CHAPS | 250 |
| 5 | Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates | Elution | 3% acetonitrile-5% acetic acid | 26 |
| 6 | KingFisher 96 KF microplate | Bead+Ab collection | 1X PBS-0.03% CHAPS-0.1% sodium azide | 100 |
| 7 | KingFisher 96 KF microplate | Tip comb | N/A | N/A |

The following shows the details of the KingFisher method employed for washing and eluting the samples. No precollection was employed at the beginning of any step, the samples were not heated during mixing, and no postmixing was employed at the end of any step. The method takes about 25 minutes.

| Plate # | KingFisher step | Beginning of step release time (hr:min:sec), speed | Mixing time (hr:min:sec), speed | End of step collect count | End of step collect time (sec) |
|---|---|---|---|---|---|
| 7 | Tip comb; Pick-up | N/A | N/A | N/A | N/A |
| 1 | Bead+Ab+peptide mixing and pick-up | 00:00:10, Slow | 00:05:00, Bottom slow | 5 | 1.5 |
| 2 | Wash 1 | 00:00:10, Slow | 00:01:30, Slow | 5 | 1.5 |
| 3 | Wash 2 | 00:00:10, Slow | 00:01:30, Slow | 5 | 1.5 |
| 4 | Wash 3 | 00:00:10, Slow | 00:01:30, Slow | 5 | 1.5 |
| 5 | Elution | 00:00:10, Bottom slow | 00:05:00, Bottom slow | 10 | 1.5 |
| 6 | Bead+Ab collection | 00:00:20, Fast | N/A | N/A | N/A |
| 7 | Tip comb; Leave | N/A | N/A | N/A | N/A |

15. Prepare Plate 5, cover it with a ThermalSeal® PCR Sealing Film, spin it down briefly, and place it on ice.
16. Prepare Plates 2, 3, 4, 6, and 7 and place them into the KingFisher.
17. Take the plate that contains the response curve samples (Plate 1) off the LabQuake and spin it down at 1000 rpm for 15 sec. This will spin down any liquid that might have contacted the foil cover.
18. Carefully take the foil cover off Plate 1, then resuspend the beads that have been compacted at the bottom of the wells using a multichannel pipette.
19. Right after the resuspension, place Plate 1 into the KingFisher and start the KingFisher method (this is done without delay to avoid having the beads settle again, which can cause them to not be transferred completely to Plate 2 (a small amount of beads might still be left behind in Plate 1, but this should be negligible compared to the total amount of beads).

20. Once the KingFisher starts the first wash step, take the PCR Sealing Film off Plate 5 and place the plate into the KingFisher.
21. Once the method has finished, take Plate 5 out of the KingFisher, cover the plate with PCR Sealing Film, and centrifuge it briefly.
22. Place Plate 5 onto a DynaMag™-96 Side Skirted Magnet for 5 minutes, then split the eluate samples into 2 new PCR plates, with 13 µL of each sample per plate (11 µL will be used per LC-MRM-MS run for a full-loop injection using a 10 µL sample loop). Spin down both plates and freeze them at -80 °C until use. One plate is a back-up plate in case a second injection should be needed; the back-up plate should not be thawed unless necessary.
23. The beads+Abs that were collected in plate 6 may be saved and stored at 4 °C.

Sample run order on the LC-MRM-MS system

The descriptions of LC and MRM system set-ups are given in the SOPs titled "Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments" and "MRM mass spectrometry for the analysis of immuno-MRM assay samples using a 5500 QTRAP," respectively.

The response curve samples are run in increasing order of heavy peptide level, with one injection per capture replicate. Before that, a system suitability standard peptide mix (unrelated peptides, e.g. a bovine 6 protein tryptic digest equal molar mix) is run to ascertain that the system is behaving as expected (see also Abbatiello, S. E., *et al.*[1]). An injection of the synthetic light peptide standards in buffer is then performed to determine the approximate retention times of the peptides, with which a scheduled LC-MRM method is generated using the Skyline software[2]. This is followed by the samples, with a buffer blank run (injecting 10 µL of just 3% acetonitrile-5% acetic acid) in between each sample run. The table below illustrates the run order. (Due to some peptides showing considerable carry-over from run to run, it is not practical to randomize the run order with respect to heavy peptide spike level.)

| Sample | Notes |
|---|---|
| System suitability standard peptide mix, run 1 | E.g. a 6 bovine protein tryptic digest equal molar mix |
| System suitability standard peptide mix, run 2 | Checking for consistency of the peptide profile; a third run of this mix might be needed if the system had been idle and the first run was used to equilibrate the system. |
| Synthetic light peptide mix in buffer | For retention time scheduling purposes. |
| Wash | 10 µL of 50% methanol-40% acetonitrile-10% Optima® water. |
| Buffer blank | 10 µL of 3% acetonitrile-5% acetic acid |
| 0 fmol / 10 µL plasma sample, repeat 1 | Adjust ret. time scheduling based on this run if needed. |
| Buffer blank | |
| Buffer blank | This run is used to characterize the light peptide carry-over signal that remains after the first buffer blank run; ideally, the light peptide intensity or peak area remaining in this run should be no more than 2% of the signal in the 0 fmol / 10 µL plasma sample, repeat 1. |
| 0 fmol / 10 µL plasma sample, repeat 2 | |
| Buffer blank | |
| 0 fmol / 10 µL plasma sample, repeat 3 | |
| Buffer blank | |
| 0.125 fmol / 10 µL plasma sample, repeat 1 | |
| Buffer blank | |
| 0.5 fmol / 10 µL plasma sample, repeat 1 | |
| Buffer blank | |
| 2 fmol / 10 µL plasma sample, repeat 1 | |
| Buffer blank | |
| etc. | |
| 5000 fmol / 10 µL plasma sample, repeat 1 | |
| Buffer blanks and washes | As many runs as needed until the heavy peptide signal has returned to baseline (i.e., no heavy peptide is detected). |
| 0 fmol / 10 µL plasma sample, repeat 4 | |
| Buffer blank | |
| 0.125 fmol / 10 µL plasma sample, repeat 2 | |
| Buffer blank | |
| 0.5 fmol / 10 µL plasma sample, repeat 2 | |
| etc. | |

## Referenced Documents

SOP “Antibody coupling and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform”

SOP “Trypsin Digestion of Human Plasma”

SOP "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves"

SOP "Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments"

1. Abbatiello, S. E., Mani, D. R., Schilling, B., Maclean, B., Zimmerman, L. J., Feng, X., Cusack, M. P., Sedransk, N., Hall, S. C., Addona, T., Allen, S., Dodder, N. G., Ghosh, M., Held, J. M., Hedrick, V., Inerowicz, H. D., Jackson, A., Keshishian, H., Kim, J. W., Lyssand, J. S., Riley, C. P., Rudnick, P., Sadowski, P., Shaddox, K., Smith, D., Tomazela, D., Wahlander, A., Waldemarson, S., Whitwell, C. A., You, J., Zhang, S., Kinsinger, C. R., Mesri, M., Rodriguez, H., Borchers, C. H., Buck, C., Fisher, S. J., Gibson, B. W., Liebler, D., MacCoss, M., Neubert, T. A., Paulovich, A., Regnier, F., Skates, S. J., Tempst, P., Wang, M., and Carr, S. A. (2013) Design, implementation and multisite evaluation of a system suitability protocol for the quantitative assessment of instrument performance in liquid chromatography-multiple reaction monitoring-MS (LC-MRM-MS). *Mol Cell Proteomics* 12, 2623-2639.

2. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., and MacCoss, M. J. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 26, 966-968.