STANDARD OPERATING PROCEDURE

Title: Peptide immunoaffinity enrichment for the characterization of intra- and inter-day repeatability of immuno-MRM assays (Validation Samples)

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Purpose

The purpose of this document is to describe the immunoaffinity enrichment of peptides as part of the characterization of the intra-day and inter-day repeatability of immuno-MRM assays over 5 days.

Scope

This procedure may be used to generate immunoaffinity-enriched peptide repeatability (validation) samples to be analyzed by MRM. It includes a description of i) generating the repeatability 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)
- Formic acid (FA) (1.11670.1000, EMD Millipore)
- Sodium azide (NaN_3) (S8032, Sigma)

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.
- A synthetic light peptide master mix with a 2 pmol/µL concentration per peptide was prepared from the individual light peptide stocks (at 100 pmol/µL, at -80 °C). See the SOP titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves."
- 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 beads 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 in water by dissolving e.g. 1.0 g of NaN_3 powder in 10 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 1 L of 3% acetonitrile-0.1% formic acid by adding 30.0 mL of Optima® acetonitrile and 1.0 mL of neat formic acid to Optima® water, for a total of 1 L.
- Prepare a light peptide master mix with a 500 fmol/µL concentration per peptide from the 2 pmol/µL light peptide master mix (see above under Reagents) using 3% acetonitrile-0.1% formic acid as the diluent. Aliquot this diluted master mix into at least five 1.7 mL microcentrifuge tubes (more than 5 tubes is preferable, in case some days' experiments need to be repeated), with 10 µL of the master mix in each aliquot, and store at -80 °C until use.

Procedure

The following describes experiments for determining the intra- and inter-day repeatabilities of a multiplexed set of immuno-MRM assays whose lower limits of quantification (LLOQs) and linear ranges have previously been determined using response curves (see the SOP titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves"). The repeatability experiments are performed on 5 different days, and each assay is tested at three different analyte levels (low, med, high) per day (see below), with three replicates performed at each analyte level per day (i.e., for a total of 45 experiments). See also the CPTAC assay portal's guidance document for more information (https://assays.cancer.gov/guidance-document/).

The spread of the three analyte levels (e.g. 3x, 10x, and 100x of the LLOQs or 3x, 50x, and 500x) depends on the range of the LLOQs and the linear ranges determined via the response curves for a set of multiplexed assays. For example, for the experiments described here, the assays' LLOQs ranged from 0.0125 to 0.8 fmol of analyte/μL of plasma and the upper limits of quantification (ULOQs) ranged from 20 to ≥500 fmol of analyte/μL of plasma, and hence it was possible to define the three analyte levels as 3x (low), 50x (medium), and 500x (high) of the LLOQs of the assays; a narrower range (e.g. 3x, 10x, 100x) might need to be defined for a set of assays whose LLOQs have a wider range.

As was done for the response curve experiments, the stable isotope-labeled heavy synthetic peptides are used as analytes in these experiments, and the light peptides are used as internal standards, since the endogenous detection of some of the peptides in human plasma does not make it possible to use the light peptides as the analyte peptides.

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 repeatability samples for each day's experiments (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* are defined in terms of X fmol of heavy peptide per 10 μL of plasma for each capture, which are in the end back-calculated to 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 repeatability experiments over the 5 days (allowing for a few 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-bead suspensions (based on the SOP titled "Antibody coupling

and immobilization to Protein G magnetic beads using a 96-well plate KingFisher automated magnetic bead handling platform").
In the end, depending on which volume from these 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 volume per capture 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 repeatability samples for one day's experiments

Briefly, the following considerations were taken into account when preparing the samples:

- The assays will be tested in a multiplexed fashion, which in turn requires that a heavy peptide master mix is generated that contains different concentrations of the peptides, depending on LLOQ,
- the volume of peptides+plasma sample per capture is 200 µL, and
- it is convenient to perform the dilutions from the 500x to the lower level samples in bulk first, and only after the dilutions have been made are the samples aliquoted to arrive at 3 replicate samples per concentration level.

6. A heavy peptide master mix was generated at high enough concentrations so that e.g. 10 µL could be spiked into the light peptide-in-plasma digest mix (see below) for the high (500x) concentration point. This master mix was aliquoted into at least 5 microcentrifuge tubes and stored at -80 °C, with one tube to be thawed for a day's experiments. (At least 5 tubes are prepared to have more than enough of the master mix in case any day's experiments need to be repeated.)
7. 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). Aliquot the plasma into 8 microcentrifuge tubes (125 µL each) and store at -80 °C, with one tube to be thawed for a day's experiments. (Eight tubes are prepared to have more than enough of the plasma in case any day's experiments need to be repeated.)
8. Prepare a light peptide-in-plasma digest mix for one day's experiments as follows, with a concentration of 100 fmol peptide per 10 µL of plasma, which is the amount of light peptide and volume of plasma aimed for per capture (the following yields a

total volume of 2200 µL, which is more than enough for the 9 samples needed for one day):

8.1. Add 1978 µL of 1X PBS-0.01% CHAPS to a 15 mL conical centrifuge tube;

8.2. add 110 µL of human plasma digest (equivalent to 110 µL of original, undigested plasma);

8.3. add 110 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);

8.4. add 2.2 µL of the 500 fmol/µL synthetic light peptide master mix (yielding a synthetic light peptide concentration of 100 fmol/10 µL of plasma for each capture).

8.5. vortex and spin down the sample briefly.

9. Distribute the light peptide-in-plasma digest mix, add heavy peptides, and perform the dilutions as follows:

9.1. Distribute the light peptide-in-plasma digest mix into three 1.7 mL microcentrifuge tubes using volumes of 800 µL, 630 µL, and 627 µL (representing the high (500x), medium (50x), and low (3x) samples, respectively).

9.2. Take 10 µL out of the tube with 800 µL and discard the 10 µL, and add 10 µL of the heavy peptide master mix (see step 6 above) to the 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.

9.3. Dilute the 500x sample to 50x by taking 70 µL of the 500x sample and adding the 70 µL to the tube containing the 630 µL of the light peptide-in-plasma digest mix; vortex the latter tube briefly and spin it down.

9.4 Dilute the 50x sample to 3x by taking 40 µL of the 50x sample and adding the 40 µL to the tube containing the 627 µL of the light peptide-in-plasma digest mix; vortex the latter tube briefly and spin it down.

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

10. Add 3x 200 µL of each of the samples 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).
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 the ~5th addition and briefly vortexing it to continue to ensure a well-mixed bead suspension.
12. If beads have settled 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 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	Repeatability (validation) 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 repeatability 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 repeatability 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
LOW capture 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.
LOW capture sample, repeat 2	
Buffer blank	
LOW capture sample, repeat 3	
Buffer blank	
MED capture sample, repeat 1	
Buffer blank	
MED capture sample, repeat 2	
Buffer blank	
MED capture sample, repeat 3	
Buffer blank	
HIGH capture sample, repeat 1	
Buffer blank	
HIGH capture sample, repeat 2	
Buffer blank	
HIGH capture sample, repeat 3	

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"

SOP "MRM mass spectrometry for the analysis of immuno-MRM assay samples using a 5500 QTRAP"

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.