# PROTOCOL: Automated IMAC Enrichment (IMAC)

## Purpose

To enrich phosphopeptides via immobilized metal affinity chromatography (IMAC) so that P100 peptide probes can be easily quantified via mass spectrometric analysis.

## Preparation

1. Prepare IMACMIX01 (0.1% TFA)
2. Prepare IMACMIX02 (80% ACN/0.1% TFA)
3. Prepare IMACMIX03 (1M Potassium Phosphate, Monobasic)
4. Prepare IMACMIX04 (1M Potassium Phosphate, Dibasic)
5. Prepare IMACMIX05 (500mM Potassium Phosphate Buffer, pH 7)
6. Prepare IMACMIX06 (1:1:1 Priming Buffer)

## Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {IMAC-M01}
- Acetonitrile, EMD Millipore, Cat. No. AX0156-1 {IMAC-M02}
- Methanol, Fisher Scientific, Cat. No. A456-1 {IMAC-M03}
- 0.01% Acetic Acid, diluted from EMD Millipore, Cat. No. AX0074-6 {IMAC-M04}
- Potassium Phosphate Monobasic, Sigma-Aldrich, Cat. No. P0662-500G {IMAC-M05}
- Potassium Phosphate Dibasic, Sigma-Aldrich, Cat. No. P3786-500G {IMAC-M06}
- Trifluoroacetic Acid, Sigma-Aldrich, Cat. No. T6508-25ML {IMAC-M07}
- 500uL V-bottom plate, VWR, Cat. No. 89005-016 {IMAC-M08}
- 96-Well Half-Area Flat Bottom Microplate, Greiner Bio-One, Cat. No. 675101 {IMAC-M09}
- 96-Well skirted PCR plate, Bio-rad, Cat. No. MSP9601 {IMAC-M10}
- 96-Well Round Bottom Microplate, Greiner Bio-One, Cat. No. 650101 {IMAC-M11}
- Fe-NTA Polymeric AssayMap Bravo Cartridges, Agilent Technologies, Cat. No. G5496-60085 {IMAC-M12}
- Axygen -80°C Rated Foil Seal, Axygen, Cat. No. PCRAS200 {IMAC-M13}
- 1-Well Low Profile Reagent Reservoir, Axygen, Cat. No. RES-SW1-LP {IMAC-M14}

## Assets

- Agilent AssayMap-BRAVO Automated Liquid Handling Platform with VWorks4 {IMAC-A01}

## Reagent Mixes

| ID | Name | Step | Composition | Vol/ Well | Use |
|---|---|---|---|---|---|
| IMACMIX01 | 0.1% TFA | **IMAC** | 0.1% TFA {IMAC-M07} in HPLC grade water {IMAC-M01} | N/A | To wash internal and external AssayMap parts |

| | | | | | during protocol; CAUTION ACID |
|---|---|---|---|---|---|
| IMACMIX02 | 80% ACN/0.1% TFA (Binding Buffer) | **IMAC** | 80% acetonitrile {IMAC-M02}/0.1% TFA {IMAC-M07} in HPLC-grade water | 240uL | To equilibrate IMAC cartridges prior to sample loading. To wash IMAC cartridges prior to elution. |
| IMACMIX03 | 1M Potassium Phosphate, Monobasic | **IMAC** | | N/A | Creation of IMACMIX05. |
| IMACMIX04 | 1M Potassium Phosphate, Dibasic | **IMAC** | | N/A | Creation of IMACMIX05. |
| IMACMIX05 | 500mM Potassium Phosphate Buffer, pH 7 (Elution Buffer) | **IMAC** | 500mM $K_2HPO_4$ in HPLC-grade water {IMAC-M01} | 240uL | To elute phosphopeptides from IMAC cartridges. |
| IMACMIX06 | 1:1:1 Priming Buffer | **IMAC** | Acetonitrile {IMAC-M02}/Methanol {IMAC-M03}/0.01% Acetic Acid {IMAC-M04} | 150uL | To activate IMAC cartridges prior to sample loading. |

**Mix Preps and Mini-worksheets:**

IMACMIX01 – 0.1% TFA
1. Measure 999 mL of HPLC-grade water {IMAC-M01} in a graduated cylinder and add to a 1L bottle. ❏
2. Pipette 1mL of TFA {IMAC-M07} into the bottle. ❏

IMACMIX02 – 80% ACN/0.1% TFA
1. Measure 20mL of HPLC-grade water {IMAC-M01} in a graduated cylinder and add to a 1L bottle. ❏
2. Measure 80mL of acetonitrile {IMAC-M02} in a graduated cylinder and add to the bottle. ❏
3. Pipette 100uL Trifluoroacetic acid {IMAC-M07} into the bottle. ❏

IMACMIX03 – 1M Potassium Phosphate, Monobasic
1. Weigh out at least 13.609g of potassium phosphate monobasic to make at least 100 mL
2. Calculate amount of water to add in mL by dividing amount weighed out by 0.13609
   ❏ Amount weighed: _____ g
   ❏ Divide by: 0.13609
   ❏ Water to add _____ mL
3. Test the pH of the solution, it should be around 4.

IMACMIX04 – 1M Potassium Phosphate, Dibasic
1. Weigh out at least 17.42g of potassium phosphate dibasic to make at least 100 mL
2. Calculate amount of water to add in mL by dividing amount weighed out by 0.1742
   - ❏ Amount weighed:      _____ g
   - ❏ Divide by:                  0.1742
   - ❏ Water to add              _____ mL
3. Test the pH of the solution, it should be around 10.

IMACMIX05 – 500mM Potassium Phosphate Buffer,  pH 7
1. Pipette 9.625mL of 1M potassium phosphate monobasic {IMACMIX03} into
   a 100mL bottle.                                                                                                          ❏
2. Pipette 15.375mL of 1M potassium phosphate dibasic {IMACMIX04} into the bottle. ❏
3. Pipette 25mL of HPLC-grade water {IMAC-M01} into the bottle, cap, and mix.      ❏
4. Test the pH of the solution and ensure that it is at 7.                                                 ❏
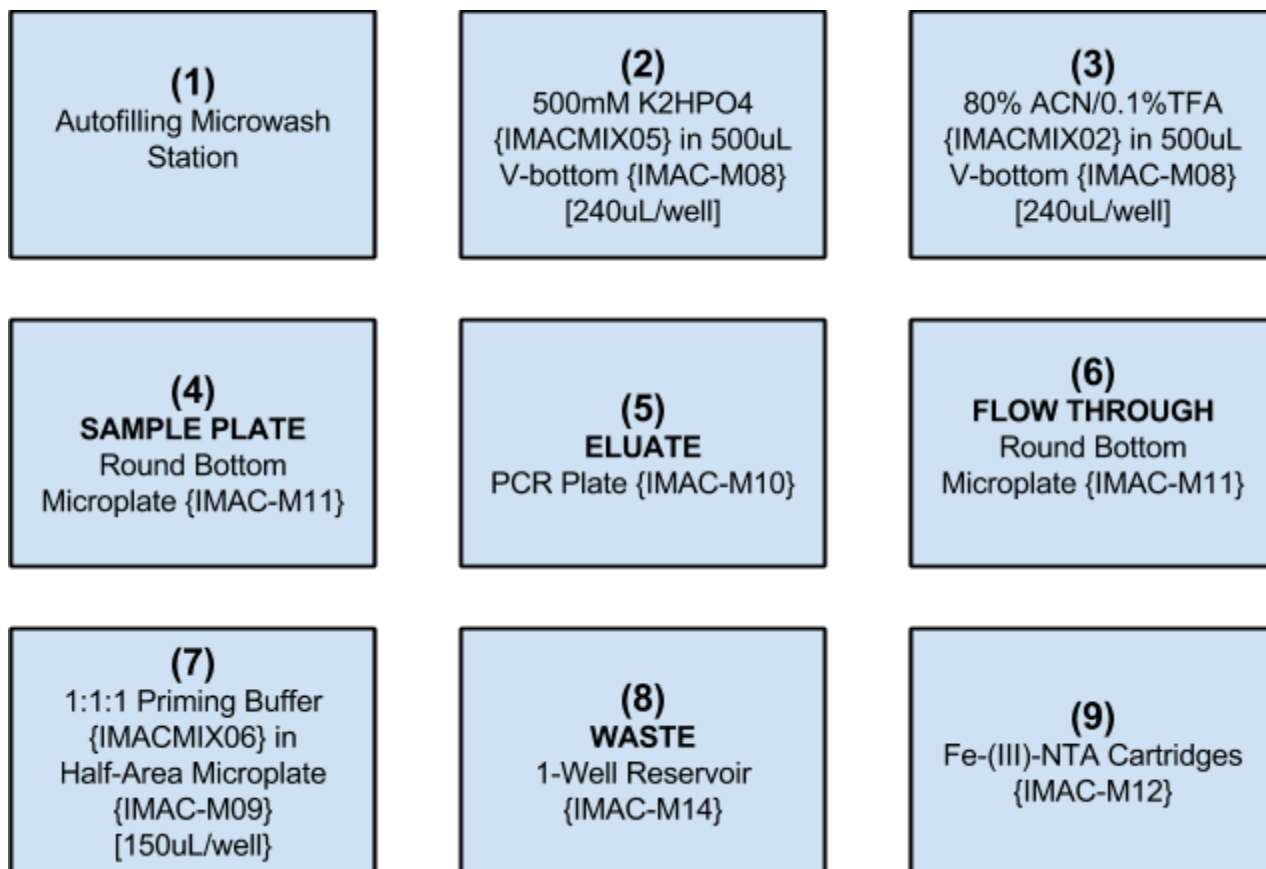
IMACMIX06 – ACN/MeOH/0.01%AcOH
1. Pipette 6mL of 100% acetonitrile {IMAC-M02} into a conical vial.                          ❏
2. Pipette 6mL of methanol {IMAC-M03} into the vial.                                                 ❏
3. Pipette 6mL of 0.01% acetic acid {IMAC-M04} into the vial.                                    ❏

**Procedure**

1. Fill the Wash Station reservoir with 0.1% TFA {IMACMIX01} and ensure that the tubing is fully submerged. Check the waste container to see if the tubing leading into it is still above liquid level. If the container is full, dispose of the waste in the correct satellite waste container
2. Aliquot 150uL of 1:1:1 Priming Buffer {IMACMIX06} into each well of a 96-well half-area flat bottom microplate {TIP-M07} with a multichannel pipette.
3. Prepare AM-BRAVO for operation:
   3.1. On the AM-BRAVO Eva {IMAC-A01} load the device file "AssayMap Bravo_CF.dev". This file is located at C:\\VWorks Workspace\Device Files\.
   3.2. In the "Devices" page, click on "Agilent Bravo" and then "AM-EVA". Select "Initialize all devices".
   3.3. Open the protocol file "2_IMAC_Bind Wash Elute_CF_AgilentIMAC_V2.pro". This file is located at C:\\VWorks Workspace\Protocol Files\AM-Bravo\IMAC\.
4. Assemble the deck of the LT-BRAVO according to the following layout:

NB: Wait until the "Sample Loading" step has completed and the protocol has paused to fill and place the 80%ACN/0.1%TFA {IMACMIX02} and 500mM potassium phosphate buffer {IMACMIX05} on the deck.

| (1) | (2) | (3) |
|---|---|---|
| Autofilling Microwash Station | 500mM K2HPO4 {IMACMIX05} in 500uL V-bottom {IMAC-M08} [240uL/well] | 80% ACN/0.1%TFA {IMACMIX02} in 500uL V-bottom {IMAC-M08} [240uL/well] |

| (4) | (5) | (6) |
|---|---|---|
| SAMPLE PLATE Round Bottom Microplate {IMAC-M11} | ELUATE PCR Plate {IMAC-M10} | FLOW THROUGH Round Bottom Microplate {IMAC-M11} |

| (7) | (8) | (9) |
|---|---|---|
| 1:1:1 Priming Buffer {IMACMIX06} in Half-Area Microplate {IMAC-M09} [150uL/well} | WASTE 1-Well Reservoir {IMAC-M14} | Fe-(III)-NTA Cartridges {IMAC-M12} |

5. On the AM-BRAVO, toggle to "Simulation is on" at the top of the screen from "Simulation is off".

 5.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.

 5.2. A pop up entitled "Set Initial Values for Variables" will appear. Set the number of "CartridgeColumns" to the appropriate amount of sample columns. (IMAC Automation Protocol Step 1).

 5.3. Change the values for the other parameters listed if necessary and press ok.

 5.4. The simulation will run and provide feedback on any warnings or errors that the protocol may encounter. If there are any **unknown** errors that come up, notify the key AM-BRAVO user and obtain help.

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NB: There will always be errors and warnings when running this protocol. A list of the expected errors and warnings can be found below.

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Main Log

| Timestamp | Class | Device | Location | Process | Task | Description |
|---|---|---|---|---|---|---|
| 11/30/2015 10:55:03 AM | Info | | | | | File loaded |
| 11/30/2015 10:57:21 AM | Info | | | | | Compile protocol |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Cup Washing | 6 | Attempting to use cartridges-off while there are no cartridges on the head. |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Cup Washing | 6 | 75 µL in the tips when the tips are being pushed off. |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Cup Washing | 6 | Attempting to use cartridges-off while there are no cartridges on the head. |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Cup Washing | 6 | 75 µL in the tips when the tips are being pushed off. |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Cup Washing | | A Cartridges-On task should be paired by Cartridges-Off inside a loop. |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 2 | The distance from well bottom of -3 for this labware may cause crash. A negative value should be used only for a cartri |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 5 | The distance from well bottom of -1 may cause crash. A negative value should be used only with bare probes |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 5 | The distance from well bottom of -1 for this labware may cause crash. A negative value should be used only for a cartri |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 5 | The distance from well bottom of -1 may cause crash. A negative value should be used only with bare probes |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 5 | The distance from well bottom of -1 for this labware may cause crash. A negative value should be used only for a cartri |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 6 | The distance from well bottom of -2 may cause crash. A negative value should be used only with bare probes |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 6 | The distance from well bottom of -2 for this labware may cause crash. A negative value should be used only for a cartri |
| 11/30/2015 10:57:25 AM | Error | AM-EVA | | Sample Loading | 6 | The distance from well bottom of -2 may cause crash. A negative value should be used only with bare probes |
| 11/30/2015 10:57:26 AM | Error | AM-EVA | | Sample Loading | 6 | The distance from well bottom of -2 for this labware may cause crash. A negative value should be used only for a cartri |
| 11/30/2015 10:57:26 AM | Info | | | | | Compile complete with 14 errors and 0 warnings |

6.   On the AM-BRAVO, toggle back to "Simulation is off". Follow steps 7.1 to 7.3 in order to run the protocol. (IMAC Automation Protocol Steps 2-7).

**7.   The protocol will pause after "Sample Loading" is complete.**

   7.1.   Fill a 500uL V-bottom plate {IMAC-M08} with 240uL per well of 80%ACN/0.1%TFA {IMACMIX02} and place the plate in position 3.

   7.2.   Fill a 500uL V-bottom plate {IMAC-M08} with 240uL per well of 500mM potassium

   phosphate buffer {IMACMIX05} and place the plate in position 2.

8.   Upon completion of the protocol, note the condition of samples and seal plates to be saved.

   8.1.   There will be residual volume in the **SAMPLE PLATE** in position 4.        Place foil seal {IMAC-M13} on the plate and transfer to -80C.
   ❏

   8.2.   Place foil seal {IMAC-M13} on **FLOW THROUGH** plate in position 4 and transfer to -80C.                                                                                     ❏

   8.3.   Carefully move the **ELUATE** plate from position 5  to position 4 and start with next protocol **Reversed Phase Peptide Desalt (RPS).**
   ❏

9.   Clear the deck.

   9.1.   Dispose of waste in the "Waste" reservoir at the appropriate satellite accumulation station.

   9.2.   Retain any labware that can be reused. Empty appropriately, rinse with water, and leave to dry.

   9.3.   Label remaining IMAC Cartridges with date and project {IMAC-M12} and store at 4°C.

**IMAC Automation Steps (BRAVO-AssayMAP)**

1.   Define Variables

   1.1.   CartridgeColumns = 12

   1.2.   ElutionRate = 0.083

   1.3.   ElutionVolume = 50

   1.4.   EquilibrationRate = 0.417

   1.5.   EquilibrationVolume = 100

   1.6.   LoadLoops = 4

   1.7.   NumberOfCupWashes = 2

   1.8.   PrimingRate = 1.67

   1.9.   SampleRate = 0.333

   1.10.   SampleVolume = 175

   1.11.   WashRate = 0.417

2.   Priming

   2.1.   Set head mode to all barrels

   2.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

   2.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

  2.2. Wash tips with 240uL at Postion 1. (Wash Station)

   2.2.1. Liquid class = AM_50uLperSec

   2.2.2. Mix cycles = 1

  2.3. AM Aspirate 100uL from Position 7. (Priming)

   2.3.1. Volume = EquilibrationVolume

   2.3.2. Liquid class = AM_100uLperSec

   2.3.3. Distance from well bottom = 0.5

  2.4. AM Cartridges on at Position 9.

  2.5. AM Dispense contents of tips to Position 8. (Waste)

   2.5.1. Liquid class = AM_25uLperSec

   2.5.2. Dispense flow rate = PrimingRate

   2.5.3. Distance from well bottom = 2

  2.6. AM Cartridges off at Position 9.

3. Sample Loading

  3.1. Set head mode to all barrels

   3.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

   3.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

  3.2. AM Aspirate 175uL from Position 4. (Sample Plate)

   3.2.1. Volume = SampleVolume

   3.2.2. Liquid class = CF_200uLperMin_10secDelay

   3.2.3. Distance from well bottom = -3

  3.3. AM Cartridges on from Position 9.

  3.4. Loop 4 times changing tips every 1 time.

  3.5. AM Dispense contents of tips to Position 4. (Sample Plate)

   3.5.1. Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay

   3.5.2. Dispense flow rate = SampleRate

   3.5.3. Distance from well bottom = -1

  3.6. AM Aspirate 150uL from Position 4. (Sample Plate)

   3.6.1. Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay

   3.6.2. Aspirate flow rate = SampleRate

   3.6.3. Distance from well bottom = -2

  3.7. Loop End.

  3.8. AM Dispense contents of tips to Position 6. (Flow Through)

   3.8.1. Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay

   3.8.2. Dispense flow rate = SampleRate

   3.8.3. Distance from well bottom = 3

  3.9. AM Cartridges off at Position 9.

  3.10. Wash tips with 240uL at Position 1. (Wash Station)

   3.10.1. Liquid class = AM_50uLperSec

   3.10.2. Mix cycles = 3

   3.10.3. Distance from well bottom = 20

  3.11. AM Dispense contents of tips to Position 1. (Wash Station)

   3.11.1.1. Liquid class = CF_200uLperMin_10secDelay

3.11.1.2. Dispense flow rate = EquilibrationRate

3.11.1.3. Distance from well bottom = 20

3.12. Wait for the user to press GO.

    3.12.1. Text: Put 80%ACN/0.1%TFA in Pos 3 and 500mM K2HPO4 at Pos 2, then press GO.

4. Cup Washing

4.1. Set head mode to all barrels

4.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

4.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

4.2. Loop 2 times changing tips every 1 time.

4.2.1. Number of times to loop =NumberOfCupWashes

4.3. AM Aspirate 50uL from Position 3. (Binding Buffer)

4.3.1. Volume = ElutionVolume

4.3.2. Liquid class = CF_200uLperMin_10secDelay

4.3.3. Distance from well bottom = 2

4.4. AM Dispense contents of tips to Position 9. (IMAC Cartridges)

4.4.1. Liquid class = CF_200uLperMin_10secDelay

4.4.2. DIstance from well bottom = -13

4.5. AM Aspirate 75uL from Position 9. (IMAC Cartridges)

4.5.1. Liquid class = CF_200uLperMin_10secDelay

4.5.2. Distance from well bottom = -17

4.6. AM Cartridges off at Position 9.

4.7. AM Dispense contents of tips to Position 6. (Flow Through)

4.7.1. Liquid class = CF_200uLperMin_10secDelay

4.7.2. Distance from well bottom = 3

4.8. Wash tips with 240uL at Position 1. (Wash Station)

4.8.1. Liquid class = AM_50uLperSec

4.8.2. Mix cycles = 1

4.8.3. Distance from well bottom = 20

4.9. Loop End.

5. Internal Cartridge Washing

5.1. Set head mode to all barrels

5.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

5.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

5.2. AM Aspirate 50uL from Position 1. (Binding Buffer)

5.2.1. Liquid class = CF_200uLperMin_10secDelay

5.2.2. Aspirate flow rate =ElutionRate

5.2.3. Distance from well bottom = 2

5.3. AM Cartridges on from Position 9.

5.4. AM Dispense contents of tips to Position 4. (Flow Through)

5.4.1. Liquid class = CF_20uLperMin_0.333uLperSec_1MinDelay

5.4.2. Distance from well bottom = 3

5.5. AM Cartridges off at Position 9.

5.6. Wash tips with 240uL at Position 1. (Wash Station)

5.6.1. Liquid class = AM_50uLperSec

        5.6.2.     Mix cycles = 2

        5.6.3.     Distance from well bottom = 20

6. Stringent Syringe Washing
   - 6.1. Set head mode to all barrels
     - 6.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
     - 6.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
   - 6.2. Loop 2 times changing tips every 1 time.
     - 6.2.1. Number of times to loop =NumberOfCupWashes
   - 6.3. AM Aspirate 50uL from Position 2. (Elution Buffer)
     - 6.3.1. Liquid class = CF_200uLperMin_10secDelay
     - 6.3.2. Distance from well bottom = 2
   - 6.4. AM Dispense contents of tips to Position 8. (Waste)
     - 6.4.1. Liquid class = CF_200uLperMin_10secDelay
     - 6.4.2. Dispense flow rate = PrimingRate
     - 6.4.3. Distance from well bottom = 7
   - 6.5. Wash Tips with 0uL at Position 3. (Wash Station)
     - 6.5.1. Liquid class = AM_10uLperSec
     - 6.5.2. Mix cycles = 0
     - 6.5.3. Distance from well bottom = 20
   - 6.6. Loop End.

7. Elution
   - 7.1. Set head mode to all barrels
     - 7.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
     - 7.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
   - 7.2. AM Aspirate 50uL from Position 2. (Elution Buffer)
     - 7.2.1. Volume =ElutionVolume
     - 7.2.2. Liquid class = CF_200uLperMin_10SecDelay
     - 7.2.3. Distance from well bottom = 1
   - 7.3. Wash tips with 0uL at Position 1. (Wash Station)
     - 7.3.1. Liquid class = AM_10uLperSec
     - 7.3.2. Distance from well bottom = 20
   - 7.4. AM Cartridges on at Position 9.
   - 7.5. AM Dispense contents of tips to Position 5. (Eluate)
     - 7.5.1. Liquid class = CF_20uLperMin_0.333uLperSec_1MinDelay
     - 7.5.2. Dispense flow rate =ElutionRate
     - 7.5.3. DIstance from well bottom = 2
   - 7.6. Wash tips with 0uL at Position 1. (Wash Station)
     - 7.6.1. Liquid class = AM_2_5sec Delay
     - 7.6.2. Mix cycles = 0
     - 7.6.3. Distance from well bottom = 22
   - 7.7. AM Cartridges off at Position 9.
   - 7.8. Wash tips with 240uL at Position 3. (Wash Station)
     - 7.8.1. Liquid class = AM_50uLperSec
     - 7.8.2. Mix cycles = 1

       7.8.3.     Distance from well bottom = 20

  7.9.     Move above location 1.

7.10.     Dispense to waste contents of tips at Position 1. (Wash Station)

       7.10.1.     Distance from well bottom = 25