Protocol for Back Exchange UPLC HDX System Suitability Test v 1.1

1) Material

Stock solutions:

- Bradikinin (BK) 10 uM (stored in the -80 °C freezer)
- Angiotensin II (ATII) 100 uM (stored in the -80 °C freezer)
- 2M Tris buffer
- Q0 (quench buffer) (All details can be found in protocol nr: SP_03, titel: HDX-MS Sample Prep, version: 1.1)

Other:

- Eppendorf tubes
- Deuterated and undeuterated HXMS buffers
  (for all details see protocol nr: SP_03, titel: HDX-MS Sample Prep, version: 1.1)
- Box with ice
- Two 20-200 pipettes
- Pipette tips
- A timer
- A vortex (make sure the vortex mixer is set to position I)
- Snow-covered tube rack in the -80 °C freezer
- A clear mind

2) Sample Preparation

The following tubes are required for the experiment:

Content	**Additional**
50 uL quench buffer	Properly labeled* Keep on ice-water**
Protein samples	Keep at 25 °C thermo-shaker (no shaking)
HXMS and $HXMS^+$ buffer (undeuterated and deuterated)	Keep at 25 °C thermo-shaker (no shaking)
Empty	Mix required volumes of protein and HXMS/$HXMS^+$ buffer Keep at 25 °C thermo-shaker (no shaking)

Those are the tubes that will contain the* ***final samples* *after labeling and quenching, thus label properly. Remember to both label the side and the lid of all Eppendorf tubes.*
***Make sure that the tubes containing the quench buffer should be submerged in slurry ice-water to reach a temperature as close to 0 °C as possible; they should be on ice for at least 30 min prior to quenching.*

1. Freshly prepare deuterated 1x $HXMS^+$ buffer with 20 mM Tris buffer in 99.99% D_2O and undeuterated 1x HXMS buffer with 20 mM Tris buffer in 99.99% H_2O
2. Label Eppendorf tubes: time point, replicate, state, initials
3. Have a pipette ready with a tip on and set to 50 uL withdraw volume (for labeling)
4. Add 50 uL of quench buffer into each Eppendorf tube and place them on the ice box
5. Dilute ATII and BK to 5 uM from the stock solution (50 uL each)
6. Mix ATII and BK 1:1 (45 uL each) to obtain a 2.5uM combined solution (final volume: 90 uL)

3) Labeling Experiment

Make sure everything is in place since when the labeling starts you WILL NOT be able to change things around.
For this training, samples will be labeled for at least 2 hours in duplicates. Voluntary, a 15 s time point can be practiced (recommended).

1. The maximum labeled control needs to be prepared the day before the actual experiment. Therefore samples are left in deuterated 1x $HXMS^{+}$ buffer overnight in the thermo-shaker at 25 °C with constant slight shaking.
2. Dilute and mix the ATII+BK solution 1:10 with the HDX buffer and simultaneously start the timer. The concentration is now 0.25 uM. Keep sample protein and labeling buffers at 25 °C using the thermo-shaker.
3. 5s before the due time point withdraw 50 uL of the labeling solution and place the pipette tip into the corresponding Eppendorf tube in the ice box BUT DO NOT PRESS YET. Press the pipette at the exact time point and immediately close the tube, vortex it instantly and place it in the -80 °C freezer as fast as possible. The concentration of the sample in now 0.125 uM, thus a 100 uL injection contains 12.5 pmol of each peptide.
4. Prepare everything for the next time point (exchange pipette tip, open the Eppendorf tube and look at the timer) and clear your mind again ;)
5. Prepare the unlabeled (0%) control likewise, but with undeuterated 1 x HXMS buffer.

4) HDX MS Analysis

Run samples on the G2Si according to the protocol (nr: Si01, title: How to use Synapt G2Si for HXMS, version: 1.1)

The injection volume is 100 uL and the following Tune page was used in MassLynx:

Uea007_hdx_peptideIPR (standard tune page)

The following methods were chosen in MassLynx:

1) Sample

MS: STD_MS_pep_MT12min

Inlet: STD_LC_BSM(7min_8to40_0C_ASM(200_3min)_MT12min

2) Wash

MS: STD_MS_Pep_MT8min(wash)

Inlet: STD_LC_BMS(wash_0C)_ASM(200_1min)_MT8min

After the wash, a blank needs to be injected, in order to check for carry-over (CO). Determine how many washes are necessary to minimize the CO in the blank to a maximum of 10 %.

5) Data Processing

For this training, data analysis was performed only in MassLynx. The following data should be summarized in the specific excel sheet for back-exchange UPLC HDX system suitability data:

- Uptake
- Standard Deviation (STD and STE)
- Retention time
- Full Width at Half Maximum (FWHM)
- Back-exchange (see excel template)

For the data analysis, a template excel sheet is available (see google drive).

Note: This is a simplified example of an HDX experiment. For larger protein analysis set-ups it is recommended to prepare an accurate labeling time schedule. Test the timing of the schedule with water, before putting hands on the real samples. Also keep in mind that extra steps within the sample preparation and analysis part need to be conducted (see PAG protocols).

Protocol Log:

Date	Version	Comments/Changes	Responsible
2018-04-25	1.0	new protocol	SS
2018-05-14	1.1	Time interval from 30s to at least 2 hours (requested by KDR)	IRM