**Updated schedule (7.26.16)**

**The new schedule is made based on the discussion of 7.25.2016 meeting**

**(Assuming honokiol is prioritized for HPLC experiments)**

**PMC-XG1**: **Deacetylated FdL2 peptide to check the detection limitation of old HPLC** *(7.21.2016 and 7.26.2016) ----- Done, will prepare report.*



Peptide-3 (P53 317-320-AMC) is the deacetylation product of FdL2 peptide.

[Peptide 3]= 0.25, 0.5, 1, 1.5 uM (10, 20, 40, 60 pmole)

The samples will be run three times for inter-day variation, and another day for intra-day variation.

|  |  |  |  |
| --- | --- | --- | --- |
|   | Assuming 8% product formation | KmFdL2 peptide = 32 uM | **v'/v** |
| [Pep-3], uM | **[FdL2 peptide], uM** | **y**Fraction of km (FdL2 pepide) | **x=0.7** | **x=0.8** | **x=0.9** |
| 0.25 | **3.125** | 0.0977 | 1.3761 | 1.2228 | 1.1002 |
| 0.5 | **6.250** | 0.1953 | 1.3351 | 1.2009 | 1.0913 |
| 1 | **12.500** | 0.3906 | 1.2751 | 1.1680 | 1.0775 |
| 1.5 | **18.750** | 0.5859 | 1.2333 | 1.1443 | 1.0673 |

**Reason:** Based on the previous experimental results (DHP1c – in-house SIRT3 Endpoint and repeat), 30pmoles product provided small peak. Therefore, little lower concentrations are designed for measure the minimum peak area that old HPLC can detect.

**PMC-XG2: DHP1c solution preparation** (*7.27.2016 – 7.29.2016*)

To validate how accurate the DHP1c solution concentration made different day.

1. Weigh different mass of DHP1c sample, then prepare the solution, run on HPLC, find out the linear relationship between peak area and [DHP1c]
2. In terms of solubility of the DHP1c, it will be necessary to use CRO to find out the maximum DHP1c concentration at 5 % DMSO. CRO Reach out follow up:
* Quote request was sent to 3 companies. Candidate compounds are Honokiol, and DHP1c. Two companies have been replied. Briefly,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Company name** | **Measurement** | **Cost, $** | **Lead time** | **Sample needed** |
| Pion Inc.(Billerica, MA 01821) | SolubilitySupersaturation & kinetic solubility | 4379.00 | 10 days | 25mg sample60ml buffer |
| Ascendex Scientific, LLC (Bristol, PA 19007) | Solubility | 2000.00 | 2-3 days | 25mg sample100ml buffer |

**PMC-XG3**: **Deacetylation reactions condition modification within HPLC detection limitation**-**FdL2 peptide.** (*8.01.2016 – 8.04.2016)*

[FdL2 peptide] = the lowest [peptide-3] to convert to [FdL2 peptide]

[NAD+] = 3000, 100 uM

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 5, 30 min

|  |  |  |
| --- | --- | --- |
| **Assuming**  **[FdL2 peptide]= 18.75uM** | KmFdL2 peptide = 32 uM | **v'/v** |
| **y**, Fraction of km (FdL2 pepide) | **x=0.7** | **x=0.8** | **x=0.9** |
| 0.5859 | 1.2333 | 1.1443 | 1.0673 |

|  |  |  |
| --- | --- | --- |
| [NAD+], uM | Km,NAD+ = 2000 uM | **v'/v** |
| **y**, Fraction of km (NAD+) | **x=0.7** | **x=0.8** | **x=0.9** |
| 100 | 0.0500 | 1.4000 | 1.2353 | 1.1053 |
| 3000 | 1.5000 | 0.7727 | 0.7826 | 0.7917 |

**Reason:** Based on previous experimental results (DHP1c – in-house SIRT3 Endpoint and repeat), the % product formed was fell into the range of 2.799-8.513. From **Exp. P1** we will figure out the lowest peptide-3 concentration. Then using the % product formed data, we can roughly calculate [FdL2 peptide] used for reaction (Table below).

|  |  |  |
| --- | --- | --- |
| **[Peptide-3], pmole / uM** | **Product formed, %** | **[FdL2 peptide], uM** |
| 10/0.25 | 2.799 | 8.932 |
| 10/0.25 | 8.513 | 2.937 |
| 20/0.5 | 2.799 | 17.864 |
| 20/0.5 | 8.513 | 5.873 |
| 40/1 | 2.799 | 35.727 |
| 40/1 | 8.513 | 11.747 |
| 60/1.5 | 2.799 | 53.591 |
| 60/1.5 | 8.513 | 17.620 |

This experiment is aim for the successful of deacetylation reaction under such condition. In other words, under lowest [FdL2 peptide], at different [NAD+] (250 and 3000uM are the low/high end of the NAD+ concentration chosen), at short time period (5 min and 30 min), the deacetylation reaction can proceed successfully and the quantitativable peak can also be achieved at the same time. If the peak cannot be detected, we need to increase the FdL2 peptide concentration.

The same reaction can be run (1) same day use same master mix for intra-day variation; (2) in different day to evaluate inter-day variation.

Also to test out if Enzo bulk SIRT3 and in-house SIRT3 have similar behavior, the reactions will be carried out using both Enzo and in-house SIRT3.

**PMC-XG4**: **Deacetylation reactions condition modification within HPLC detection limitation- NAD+.** (8.05.2016 -8.10.2016)

[FdL2 peptide] = 250uM

[NAD+] = 10, 25, 50, 75, 100 uM

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 5, 30 min

|  |  |  |
| --- | --- | --- |
|  [NAD+], uM | Km,NAD+ = 2000 uM | **v'/v** |
| **y**, Fraction of km (NAD+) | **x=0.7** | **x=0.8** | **x=0.9** |
| 10 | 0.0050 | 1.425531915 | 1.2484472 | 1.1104972 |
| 25 | 0.0125 | 1.421052632 | 1.2461538 | 1.109589 |
| 50 | 0.0250 | 1.413793103 | 1.2424242 | 1.1081081 |
| 75 | 0.0375 | 1.406779661 | 1.238806 | 1.1066667 |

**PMC-XG5 and PMC-XG6 are designed for refuting purpose, three conditions are applied. Condition 1 and 2 are for saturating peptide or NAD. Condition 3 is the JMC condition. We want to show that under different condition, activation of SIRT3 by DHP1c is not detected. The experiments will be done on TeCan and HPLC for both Enzo and in-house SIRT3. The result will indicate that DHP1c is not an activator for SIRT3.**

**PMC-XG5**: **DHP1c endpoint experiment-HPLC** (*8.10.2016 – 8.17.2016)*

***Condition 1:*** [FdL2 peptide] = the lowest [peptide 3] to convert to [FdL2 peptide]

[NAD+] = 3000uM

***Condition 2:***[FdL2 peptide] = 250 uM

[NAD+] = the lowest [NAD+] from PMC-XG4.

***Condition 3:***[FdL2 peptide] = 250 uM

[NAD+] = 500uM

%DMSO = 5%

[DHP1c]=0, 50, 75, 100, 200, the maximum DHP1c concentration at 5 % DMSO determined by CRO.

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 60 min

**PMC-XG6**: **DHP1c endpoint experiment-FdL assay on TeCan** (*8.18.2016 – 8.19.2016)*

***Condition 1:*** [FdL2 peptide] = the lowest [peptide 3] to convert to [FdL2 peptide]

[NAD+] = 3000uM

***Condition 2:***[FdL2 peptide] = 250 uM

[NAD+] = the lowest [NAD+] from PMC-XG4.

***Condition 3:***[FdL2 peptide] = 250 uM

[NAD+] = 500uM

%DMSO = 5%

[DHP1c]=0, 50, 75, 100, 200, the maximum DHP1c concentration at 5 % DMSO determined by CRO.

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 60 min

**PMC-XG7 is designed for refuting purpose, a SIRTainty kit will be used to repeat PMC-XG6. The synthesized acetylated peptide without AMC will be used for this assay. Outcome of PMC-XG7 (1) No activation is detected ----- DHP1c is not an activator for SIRT3 with p53 317-320 without AMC**

**(2) If activation is detected, and then HPLC (PMC-XG8) will be carried out. If we do not see activation by HPLC method, then it will indicate that SIRTainty assay provide false positive results and most important: DHP1c is not an activator for SIRT3.**

**PMC-XG7**: **DHP1c endpoint experiment-SIRTainty assay** (*8.22.2016-8.24.2016*)

***Condition 1:*** [Peptide] = the lowest [peptide]

[NAD+] = 3000uM

***Condition 2:***[Peptide] = 250 uM

[NAD+] = the lowest [NAD+] from PMC-XG4.

***Condition 3:***[Peptide] = 250 uM

[NAD+] = 500uM

%DMSO = 5%

[DHP1c]=0, 50, 75, 100, 200, the maximum DHP1c concentration at 5 % DMSO determined by CRO.

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 60 min

**PMC-XG8**: **DHP1c endpoint experiment-SIRTainty-HPLC** (*8.25.2016-8.31.2016*)

***Condition 1:*** [Peptide] = the lowest [peptide]

[NAD+] = 3000uM

***Condition 2:***[Peptide] = 250 uM

[NAD+] = the lowest [NAD+] from PMC-XG4.

***Condition 3:***[Peptide] = 250 uM

[NAD+] = 500uM

%DMSO = 5%

[DHP1c]=0, 50, 75, 100, 200, the maximum DHP1c concentration at 5 % DMSO determined by CRO.

[Enzo SIRT3 and in-house SIRT3] = 10 U

Time point = 60 min

**PMC-XG9 - PMC-XG12 are designed for replacing biorxiv paper data.**

**PMC-XG 9: Km NAD+ for in-house SIRT3** (8.22.2016-8.26.2016)

[in-house SIRT3]=10 U

[FdL2 peptide] =250 uM

[NAD+]= 0, 100, 250, 750, 1500, 3000uM

Time point=0, 5, 10, 20, 30, 60 min

Total: 30 reactions (0.5 day for deacetylation reaction, 3.75 days for running HPLC by 8 reactions per day, 0.5 day for data analysis)

|  |  |  |
| --- | --- | --- |
| **[NAD+], uM** | Km,NAD+ = 2000 uM |  **v'/v** |
| y, Fraction of km (NAD+) | x=0.7 | x=0.8 | x=0.9 |
| 100 | 0.0500 | 1.4000 | 1.2353 | 1.1053 |
| 250 | 0.1250 | 1.3636 | 1.2162 | 1.0976 |
| 750 | 0.3750 | 1.2791 | 1.1702 | 1.0784 |
| 1500 | 0.7500 | 1.2069 | 1.1290 | 1.0606 |
| 3000 | 1.5000 | 1.1364 | 1.0870 | 1.0417 |

**PMC-XG 10: Km FdL2 peptide for in-house SIRT3** (9.5.2016-9.9.2016)

[In-house SIRT3]=10ul

[FdL2 peptide]=0, 10, 50, 100, 250uM

[NAD+]= 3000uM

Time point=0, 5, 10, 20, 30, 60 min

Total: 30 reactions (0.5 day for deacetylation reaction, 3.75 days for running HPLC by 8 reactions per day, 0.5 day for data analysis)

|  |  |  |
| --- | --- | --- |
| **[FdL2 peptide], uM** | Km,FdL2 peptide = 32 uM |  **v'/v** |
| y, Fraction of km (FdL2 pepide) | x=0.7 | x=0.8 | x=0.9 |
| 10 | 0.3125 | 1.2963 | 1.1798 | 1.0825 |
| 50 | 1.5625 | 1.1326 | 1.0847 | 1.0406 |
| 100 | 3.1250 | 1.0784 | 1.0510 | 1.0248 |
| 250 | 7.8125 | 1.0352 | 1.0232 | 1.0115 |

**Reason:** Exp. P5 and P6 is aim for obtaining the Km NAD and FdL peptide using in-house SIRT3. Then we can get more accurate “y” value for the plot discussion. Also later on for the initial rate experiments, Exp. P5 and P6 will be one set repeat for control experiments for intra-day variation calculation.

**PMC-XG 11: Initial rate DHP1c-in-house SIRT3- Km, Vmax vs. FdL2 peptide**

\*For 1 [DHP1c], 2x5x2x7 = 140 reactions (18 days) ---- *(9.12.2016 - 9.30.2016)*[In-house SIRT3]=10 U

[FdL2 peptide]=250 uM

[NAD+]= 0, 100, 500, 1500, 3000uM

[DHP1c]=0, ? to be determined from **Exp. P4**

%DMSO=0, 5 %

Time point=0, 5, 10, 20, 30, 45, 60 min

**PMC-XG 12: Initial rate DHP1c-in-house SIRT3-Km, Vmax vs. FdL2 peptide**\*For 1 [DHP1c], 2x5x2x7 =140 reactions (18 days) --- (*10.3.2016 – 10.26.16)*[In-house SIRT3]=10 U

[FdL2 peptide]=0, 10, 50, 100, 250 uM

[NAD+]= 3000uM

[DHP1c]=0, ? to be determined from **PMC-XG5**

% DMSO= 0, 5%

Time point=0, 5, 10, 20, 30, 45, 60 min

**Notes:**

* **Since we are refuting the JMC paper, binding affinity experiments (MST/ITC) for SIRT3-DHP1c and SIRT1-DHP1c are important. If no interaction is detected, then it is convincing to support our claims. The SIRT3 can be both Enzo and in-house, and SIRT1 will be Enzo. SIRT1 will be optional.**
* **XG can help out AU for running the second HPLC if time is permitted.**
* **PMC-XG7 and 8 are optional (depends on time availablility).**
* **Duplicate of Exp. PMC-XG11 and PMC-XG12, the second HPLC is needed.**