PROTOCOL: Secondary Propionylation (K2)

Purpose

To propionylate N-terminal lysine residues after protein digestion for later mass spectrometric analysis.

Preparation

1. Prepare MIX01 (500mM Sodium Phosphate Buffer, pH 8) if not enough available
2. Prepare MIX02 (100mM Sodium Phosphate Buffer, pH 8) if not enough available
3. Prepare MIX03 (15% Hydroxylamine Solution) if not enough available
4. Prepare MIX04 (400nmol/uL NHS Propionate in Methanol)

Materials

- HPLC-grade water {K2-M01} [JT Baker, 4218-03]
- Anhydrous Methanol {K2-M02} [JT Baker, 9097-12]
- Hydroxylamine {K2-M03} [Sigma-Aldrich, 467804-10ML]
- NHS-Propionate {K2-M04} [SAI Chemicals]
- Sodium Phosphate, Monobasic {K2-M05} [Sigma-Aldrich, P0662-500G]
- Sodium Phosphate, Dibasic {K2-M06} [Sigma-Aldrich, P3786-500G]
- 500uL V-bottom plate {K2-M07} [VWR, 89005-016]
- 96-Well skirted PCR plate {K2-M08} [Bio-Rad, MSP-9601]
- Agilent 96LT-180uL Tips {K2-M09} [Agilent, 19477.002]
- Axygen -80°C Rated Foil Seal {K2-M10} [Axygen, PCR-AS-200]
- Breath-EASIER seal {K2-M11} [Diversified Biotech, BERM-2000]

Assets

- Agilent LT-Bravo Automated Liquid Handling Platform with VWorks4 {K2-A01}
- Thermo Scientific Savant SC210A Concentrator {K2-A02}

Reagent Mixes

NB: Prepare 400nmol/uL NHS Propionate in Methanol {MIX05} immediately before use to prevent the solution from hydrolyzing.

ID	Name	Step	Composition	Volume/Well	Use
MIX01	500mM Sodium Phosphate Buffer, pH 8	**K1/K2**	500mM Na_2HPO_4 in HPLC-grade water {K2-M01}	N/A	Creation of MIX02.
MIX02	100mM Sodium Phosphate	**K2**	100mM Na_2HPO_4 in HPLC-grade	3uL	To keep histone samples at pH 7 during propionylation

	Buffer, pH 8		water {K2-M01}		reaction.
MIX03	15% Hydroxylamine	**K2**	15% Hydroxylamine {K2-M03} in HPLC grade water {K2-M01}	~50mL	To quench the propionylation reaction.
MIX04	400nmol/uL NHS Propionate in Methanol	**K2**	68.4mg/mL of NHS propionate {K2-M04} in anhydrous methanol {K2-M02}	50uL	To derivatize lysine residues on histone peptides with propionyl groups.

Mix Preps and Mini-worksheets:

MIX02 – 100mM Sodium Phosphate Buffer, pH 8

1. Pipette 80mL of HPLC-grade water {K2-M01} into a 100mL bottle ❑
2. Pipette 20mL of 500mM sodium phosphate buffer, pH8 {MIX01} into the bottle. ❑
3. Invert bottle 7 times to mix ❑
4. Test the pH of the solution and ensure that it is at 8. ❑

MIX03 – 15% Hydroxylamine Solution

1. Measure 2333uL of HPLC-grade water {K2-M01} in a small vial. ❑
2. Pipette 1000uL of Hydroxylamine {K2-M03} into the vial. ❑

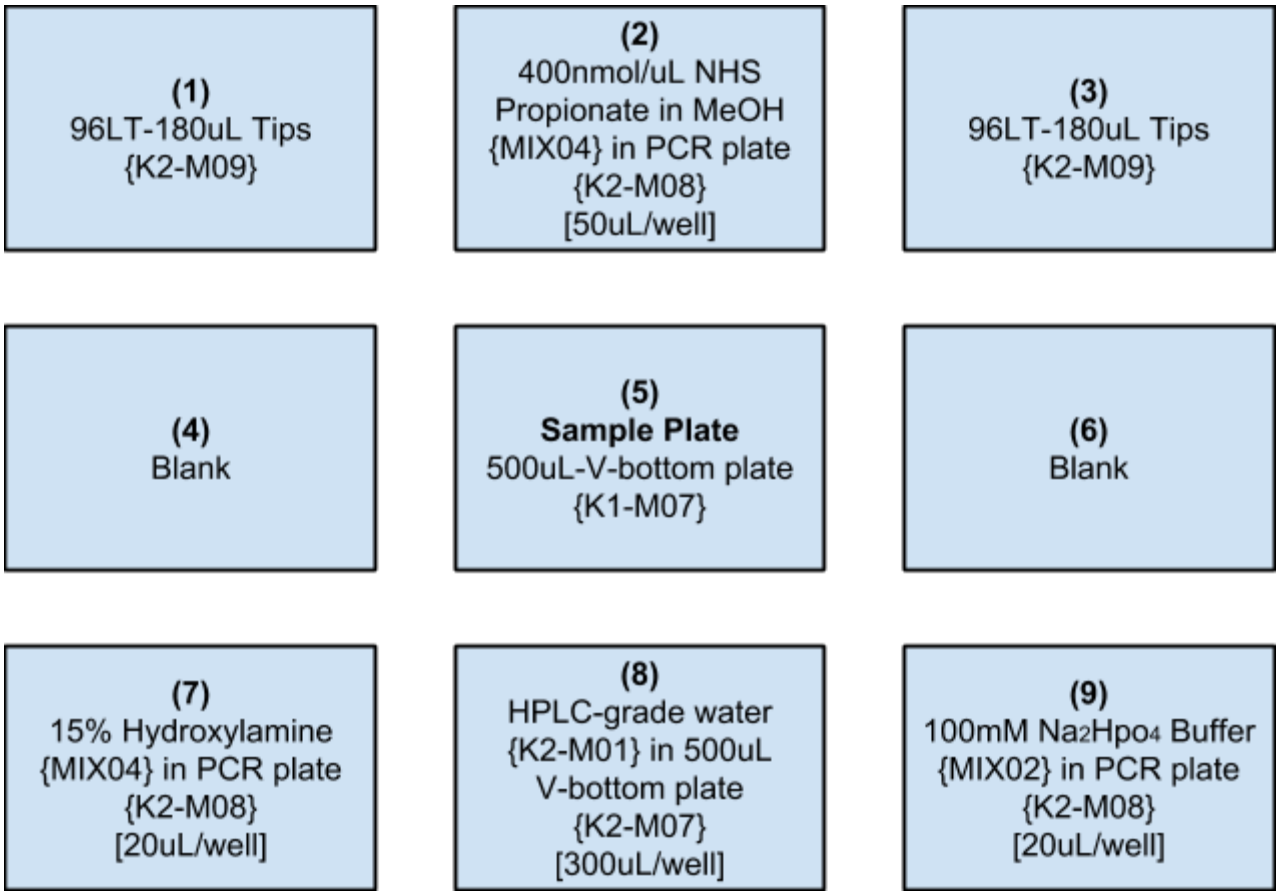
MIX04 – 400nmol/uL NHS Propionate in Methanol

1. Weigh out at least 470mg of NHS-propionate to make at least 7 mL
2. Calculate amount of methanol to add in mL by dividing amount weighed out by 68.4
   - ❑ Amount weighed: _____ mg
   - ❑ Divide by: 68.4
   - ❑ Methanol to add _____ mL.

Procedure

1. Fill each well of a 96-Well skirted PCR plate {K2-M08} with 20uL of 15% Hydroxylamine {MIX04}.
2. Fill each well of a 96-Well skirted PCR plate {K2-M08} with 20uL of 100mM sodium phosphate buffer, pH 8 {MIX03}.
3. Fill each well of a 96-Well 500uL V-bottom plate {K2-M07} with 300uL of HPLC-grade water {K2-M01}.
4. Fill each well of a 96-Well skirted PCR plate {K2-M08} with 50uL of 400nmol/uL NHS Propionate in Methanol {MIX05}.
5. On the LT-BRAVO Marvin {A01} load the device file “Bravo with Heated Shakers.dev”. This file is located at C:\\VWorks Workspace\Device Files\.
6. In the “Devices” page, click on “Agilent LT-BRAVO” and then “Marvin”. Select “Initialize all devices”.
7. Open the protocol file “SecondaryPropionylation_10ug_Peptides.pro”. This file is located at · C:\\VWorks Workspace\Protocol Files\CF\Histones\.

8. Assemble the deck of the LT-BRAVO according to the following layout:



9. On the LT-BRAVO, toggle to “Simulation is on” at the top of the screen from “Simulation is off”.
   9.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.
   9.2. A pop up entitled “Set Initial Values for Variables” will appear. Set the number of “Columns” to the appropriate amount of sample columns and press ok.
   9.3. 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 LT-BRAVO user and obtain help.
10. On the LT-BRAVO, toggle back to “Simulation is off”. Follow steps 9.1 through 9.3 to run the protocol.

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BRAVO Steps:
10.1) Resuspend samples in 40uL 400nmol/uL NHS Propionate {MIX04}
10.2) Add 10uL 100mM sodium phosphate buffer, pH 8 {MIX02}
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NB:  Protocol will pause and wait for user interaction
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11. After the protocol has paused, place a foil seal {K2-M10} on the sample plate and replace the plate in position 5 on the LT-BRAVO deck.
   11.1. Press GO.
   11.2. The LT-BRAVO will move the plate to the shaker where it will incubate at 25°C for 1 hour with shaking at 800rpm.

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NB: After 1 hour, the protocol will pause again. Remove the plate and spin it down.
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12. Remove the foil cover and replace the plate in position 4 at the heated shaker position.
   12.1. Press GO to continue the protocol.

12.2. The LT-BRAVO will add 10 uL 15% hydroxylamine solution to the sample plate and quench the reaction.

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NB: The protocol will pause after the addition of 15% hydroxylamine solution.
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13. After the protocol has paused, place a foil seal {K2-M10} on the sample plate and replace the plate in position 4 on the LT-BRAVO deck.
    13.1. Press GO.
    13.2. The sample plate will incubate at 25°C for 30 minutes with shaking at 800rpm.

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NB: After 30 minutes, the protocol will pause again. Remove the plate and spin it down.
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14. Remove the foil cover and replace the plate in positon 4 at the heated shaker position.
    14.1. Press GO to continue the protocol.
    14.2. The LT-BRAVO will add 200uL HPLC-grade water {K2-M01} to the sample plate and incubate for 30 minutes with tip mixing.
15. Clear the deck, cover the sample plate with a breathable seal {K2-M11} and then a foil seal {K2-M10}, and freeze at -80℃
16. Create balance plate with 260uL HPLC-grade water {K2-M01} in each well corresponding to a sample well. Cover with breathable seal {K2-M11} and foil seal {K2-M10} and freeze with sample plate.
17. Remove the foil seal and speedvac samples to dryness.
18. Continue on to **SepPak desalt**.