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Global level quantification of histone post-translational modifications in a 3D cell culture model of hepatic tissue

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Abstract

Flat cultures of mammalian cells are a widely used in vitro approach for understanding cell physiology, but this system is limited in modeling solid tissues due to unnaturally rapid cell replication. This is particularly challenging when modeling mature chromatin, as fast replicating cells are frequently involved in DNA replication and have a heterogeneous polyploid population. Presented below is a workflow for modeling, treating, and analyzing quiescent chromatin modifications using a three-dimensional cell culture system. Using this protocol, hepatocellular carcinoma cell lines are grown as reproducible 3D spheroids in an incubator providing active nutrient diffusion and low shearing forces. Treatment with sodium butyrate and sodium succinate induced an increase in histone acetylation and succinylation, respectively. Increases in levels of histone acetylation and succinylation are associated with a more open chromatin state. Spheroids are then collected for isolation of cell nuclei, from which histone proteins are extracted for the analysis of their post-translational modifications. Histone analysis is performed via liquid chromatography coupled online with tandem mass spectrometry, followed by an in-house computational pipeline. Finally, examples of data representation to investigate the frequency and occurrence of combinatorial histone marks are shown.

Summary

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This protocol outlines how a three-dimensional cell culture system can be used to model, treat, and analyze chromatin modifications in a close to physiological state.

Keywords

3D culture; histones; mass spectrometry; direct injection; post-translational modifications

Introduction

Since the late 19th century, cell culture systems have been used as a model to study the growth and development of cells outside of the human body[1,2]. Their use has also extended to studying how tissues and organs function in both healthy and diseased contexts[1,3]. Suspension cells, e.g. blood cells, grow in petri dishes or flasks seamlessly and interchangeably as they do not assemble in 3D structures in vivo. Cells derived from solid organs can grow as either two-dimensional (2D) or three-dimensional (3D) culture systems. In 2D culture, cells are grown in a monolayer that adheres to a flat surface[2,4]. 2D cell culture systems are characterized by exponential growth and a fast doubling time, typically 24 hours to a few days[5]. Cells in 3D systems grow forming intricate cell-cell interactions modeling tissue-like conglomerates more closely, and they are characterized by their ability to reach a dynamic equilibrium where their doubling time can reach one month or even longer[5].

Presented in this paper is an innovative methodology to grow 3D spheroids in rotating cell culture systems that simulate reduced gravity[6]. This is a simplified derivative of a cell culture system introduced by NASA in the 1990s[7]. This approach minimizes shearing forces, which occurs in existing methods like spinning flasks, and increases spheroid reproducibility[6]. In addition, the rotating bioreactor increases active nutrient diffusion, minimizing necrotic formation that occurs in systems like hanging drop cell culture where media exchange is impractical[6]. This way, cells grow mostly undisturbed, allowing for formation of structural and physiological characteristics associated with cells growing in tissue. C3A hepatocytes (HepG2/C3A) cultured in this manner not only had ultrastructural organelles, but also produced amounts of ATP, adenylate kinase, urea, and cholesterol comparable to levels observed *in vivo*[1,2]. In addition, cells grown in 2D vs 3D cell culture systems exhibit different gene expression patterns[8]. Gene expression analysis of C3A hepatocytes grown as 3D spheroids showed that these cells expressed a wide range of liver-specific proteins as well as genes involved in key pathways that regulate liver function[8]. Prior publications demonstrated that the proteomes of exponentially growing cells in 2D culture vs cells at dynamic equilibrium in 3D spheroid cultures are different[5]. These differences include cellular metabolism, which in turn affects the structure, function, and physiology of the cell[5]. The proteome of cells grown in 2D culture was more enriched in proteins involved in cell replication, while the proteome of 3D spheroids was more enriched in liver functionality[5].

The slower replication rate of cells grown as 3D spheroids more accurately models specific phenomena associated with chromatin state and modifications, e.g. histone clipping[9]. Histone clipping is an irreversible histone post-translational modification (PTM) that causes

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proteolytic cleavage of part of the histone N-terminal tail. While its biological function is still under discussion[10-13], it is clear that its presence in primary cells and liver tissue is modeled by HepG2/C3A cells grown as spheroids but not as flat cells[9]. This is critical, as chromatin state and modifications regulate DNA readout mostly by modulating accessibility to genes and thus their expression[14]. Histone PTMs either influence chromatin state directly by modulating the net charge of the nucleosomes where histones are assembled, or indirectly by recruiting chromatin writers, readers, and erasers[14]. Hundreds of histone PTMs have been identified to date[15], reinforcing the hypothesis that chromatin hosts a "histone code" used by the cell to interpret DNA[16]. However, the identification of a myriad of PTM combinations[15] and the discovery that combinations of histone PTMs frequently have different biological functions from PTMs present in isolation (e.g. Fischle, et al.[17]) highlights that more work is required to decrypt the "histone code".

Currently, histone PTM analysis is either based on (1) techniques utilizing antibodies, [e.g. western blots, immunofluorescence, or chromatin immunoprecipitation followed by sequencing (ChIP-seq)] and (2) mass spectrometry (MS). Antibody-based techniques have high sensitivity and can provide detailed information about the genome-wide localization of histone marks, but are frequently limited in studying rare PTMs or PTMs present in combinations[18-20]. MS is more suitable for high-throughput and unbiased identification and quantification of single and co-existing protein modifications, in particular histone proteins[18-20]. For these reasons, this and several other laboratories have optimized the MS pipeline for the analysis of histone peptides (bottom-up MS), intact histone tails (middle-down MS), and full-length histone proteins (top-down MS)[21-23].

Detailed below is a workflow for growing HepG2/C3A spheroids and preparing them for histone peptide analysis (bottom-up MS) via nano-liquid chromatography coupled online with tandem mass spectrometry (nLC-MS/MS). To start, a 2D cell culture is grown; afterwards the cells are harvested and transferred to a bioreactor where they will start to form spheroids (Figure 1). After 18 days in culture, spheroids are treated with sodium butyrate or sodium succinate to increase the relative abundances of histone acetylation and succinylation. Notably, 3D cultures can be treated with genotoxic compounds just as well as their flat culture equivalents; in fact, recent publications highlight that the toxicology response of cells in 3D culture is more similar to primary tissues than those in 2D flat culture (e.g.[24,25]). Cells are then collected at specified time points and nuclear isolation is performed. Afterwards, histones are extracted and derivatized with propionic anhydride before and after trypsin digestion according to a protocol first developed by Garcia et al.[26]. This procedure generates peptides of an appropriate size for online separation with reversed-phase chromatography (C_{18}) and detection with MS. Finally, histone peptides are identified and quantified, and the generated data is represented in multiple ways for a more complete biological interpretation.

Protocol

1. Prepared buffers and reagents

1.1 Cell growth media (for HepG2/C3A cells)

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1.1.1 To prepare, add fetal bovine serum (FBS) (10% v/v), non-essential amino acids (1% v/v), L-glutamine supplement (1% v/v) and penicillin/streptomycin (0.5% v/v) to Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g/L glucose). Growth media is stored at 4 °C for a maximum of 2 weeks.

1.2 200 mM sodium butyrate (NaBut) solution

1.2.1 To prepare 10 mL, resuspend 220.18 mg NaBut in 10 mL ddH_2O for a concentration of 20 mM. Store 1 mL aliquots at −20 °C

1.2.2 Prior to cell treatment, filter solution using a 0.45 mm syringe filter. Add 1 mL filtered solution to 9 mL cell growth media for a working concentration of 10 mM

1.3 100 mM sodium succinate (NaSuc) solution

1.3.1 To prepare 10 mL, resuspend 162.05 mg NaSuc in 10 mL ddH_2O. Store 1 mL aliquots at −20 °C

1.3.2 Prior to cell treatment, filter solution using a 0.45 mm syringe filter. Add 1 mL filtered solution to 9 mL cell growth media for a working concentration of 10 mM

1.4 Cold 0.2 M H_2SO_4

1.4.1 To prepare 1L, add 10 mL concentrated H_2SO_4 to 990 mL HPLC grade water. Store at 4 °C

1.5 Cold acetone + 0.1% hydrochloric acid (HCl)

1.5.1 To prepare, add concentrated HCl (0.1% v/v) to acetone. Store at 4 °C

1.6 100 mM NH_4HCO_3 solution, pH 8.0

1.6.1 To prepare 1 L, resuspend 7.91 g NH_4HCO_3 in 1 L HPLC grade water. Store 50 mL aliquots at −20 °C

1.7 0.1% Trifluoroacetic acid (TFA) solution

1.7.1 To prepare, add concentrated TFA (0.1% v/v) to HPLC grade water. Store at 4 °C

1.8 60% acetonitrile/0.1% TFA solution

1.8.1 To prepare, add HPLC grade acetonitrile (60% v/v) to HPLC grade water. Afterwards, add concentrated TFA (0.1% v/v) to solution. Store at 4 °C

1.9 2% HPLC-grade acetonitrile + 0.1% formic acid

1.9.1 To prepare, add HPLC-grade acetonitrile (2% v/v) to HPLC-grade water. Afterwards, add concentrated formic acid (0.1% v/v) to solution

1.10 80% HPLC-grade acetonitrile + 0.1% formic acid

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1.10.1 To prepare, add HPLC-grade acetonitrile (80% v/v) to HPLC-grade water. Afterwards, add concentrated formic acid (0.1% v/v) to solution

2. Preparation of the 3D culture system

Different cells, primary or immortalized, have different culture properties, so the formation of spheroids may differ among cell types. This protocol has been established for HepG2/C3A spheroid formation using bioreactors and an innovative 3D cell culture system.

2.1 Grow cells as a monolayer, using standard growth media, until 80% confluent

2.2 Wash cells with HBSS (5 mL for a 75 cm^2 flask), then incubate cells with 5 mL of 0.05% trypsin-EDTA diluted in HBSS (1:2 dilution) for 5 min at 37 °C with 5% CO_2

2.3 Check cell detachment under a microscope and neutralize trypsin by adding 3 mL of fetal bovine serum (FBS) or growth media (containing 5-10% FBS)

2.4 Count the number of cells and dilute the cell suspension to obtain 1 x 10^6 cells in a maximum volume of 1.5 mL

2.5 Equilibrate an ultra-low attachment 24-well round bottom plate (containing multiple microwells per well) by washing the wells with 0.5 mL of growth media. Centrifuge the plate for 5 min at 3,000 x g to remove air bubbles from the well surface

2.6 Transfer the cell suspension into the plate and centrifuge for 3 min at 120 x g

2.7 Incubate the plate for 24 h at 37 °C with 5% CO_2 for spheroid formation. Meanwhile, equilibrate the bioreactor by filling the humidity chamber with 25 mL of sterile water and the cell chamber with 9 mL of growth media

2.8 Incubate the bioreactor, rotating in the clinostat incubator, for 24 h at 37 °C with 5% CO_2

3. Growth of spheroids in bioreactors

NOTE: To preserve the structure of spheroids, wide bore tips are used for handling the 3D structures.

3.1 Detach spheroids from the ultra-low attachment plate by gently pipetting up and down with 1 mL wide bore tips and transfer to a tissue culture-treated dish

3.2 Wash the plate with 0.5 mL pre-warmed growth media and transfer to the same dish

3.3 Evaluate the quality of spheroids by microscopy and select sufficiently formed spheroids. Good quality spheroids have uniform size, compactness, and roundness

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3.4 Transfer spheroids into equilibrated bioreactors filled with 5 mL of fresh growth media. After transferring the spheroids, completely fill the bioreactor with fresh growth media

3.5 Place bioreactor in the clinostat incubator and adjust the rotation speed to 10-11 rpm

3.6 Exchange growth media every 2-3 days by removing 10 mL of old media and replacing with 10 mL of fresh media

3.7 Adjust rotation speed according to spheroid growth, increasing as spheroids grow in size and number

3.8 After 18 days in culture, spheroids are ready for treatment and/or collection

4. Spheroid treatment and collection

NOTE: In this protocol, HepG2/C3A spheroids are treated with sodium butyrate (NaBut) and sodium succinate (NaSuc) to evaluate the levels of histone marks containing acetylation and succinylation, respectively.

4.1 Prepare growth media with the appropriate working concentration of compound (e.g. 20 mM of NaBut or 10 mM NaSuc). Exchange the media in the bioreactor with the treated media.

NOTE: To establish a control condition, either collect enough spheroids for experiments before adding the treatment or designate a bioreactor for untreated spheroids.

4.2 Collect spheroids for proteomic analysis following sufficient treatment time (e.g. 48 h-1 wk for NaSuc treatment and 48-72 h for NaBut treatment)

NOTE: For histone extraction using this protocol, 6-8 spheroids containing approximately $1x10^6$ cells were collected.

4.2.1 Remove 3-5 mL of media from the bioreactor through the top port

4.2.2 Open front port and use a 1 mL wide bore tip to remove spheroids and place into microcentrifuge tubes

4.2.3 Centrifuge the spheroids at 100 x g for 5 min and discard media. NOTE: The media in the bioreactor can be changed and the bioreactor returned to the incubator for additional treatment time or recovery

4.2.4 Wash spheroids with 200 μL of HBSS to remove the FBS. Centrifuge at 100 x g for 5 min and remove the supernatant

4.3 Spheroids can be stored at −80 °C until processing

5. Histone extraction

The many basic amino acid residues present in histones allow them to closely interact with DNA, which has a phosphoric acid backbone. Because histones are some of the most basic proteins in the nucleus, when they are extracted with ice-cold sulfuric acid (0.2 M H_2SO_4) there is minimal contamination. The non-histone proteins will precipitate in strong acid.

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Highly concentrated TCA (diluted to a final concentration of 33%) is used afterwards to precipitate the histones from the sulfuric acid.

NOTE: Keep all samples, tubes and reagents on ice for the entire histone extraction.

5.1 Add 5 volumes (~100 µL) of cold 0.2 M H_2SO_4 to cell pellet (~10-20 µL), then pipette up and down to disrupt the pellet and release histones

5.2 Incubate tubes for up to 4 h at constant rotation or shaking at 4 °C.

NOTE: For resuspended samples that have a volume greater than 500 µL, a 2 h incubation is sufficient to extract histones (a longer incubation may result in extraction of other basic nuclear proteins). For resuspended samples with a volume less than 200 µL, a 4 h incubation is required for a better yield.

5.3 Centrifuge at 3,400 x g for 5 min at 4°C. Collect supernatant in a new tube and discard the pellet later

5.4 Add cold concentrated TCA such that it makes up a final 25-33% v/v (e.g. 40-60 µL cold TCA: 120 µL supernatant) and mix by inverting the tube a few times

5.5 Incubate tubes for at least 1 h at constant rotation or shaking at 4 °C. NOTE: For smaller starting pellet sizes, overnight incubation is recommended

5.6 Centrifuge at 3,400 x g for 5 min at 4 °C

5.7 Discard supernatant by pipetting. Aspirate the supernatant carefully; do not touch the sides of the tube or the pellet. NOTE: Histones deposit at both the sides and the bottom of the tube. The white insoluble pellet formed at the very bottom of the tube contains mostly non-histone proteins and other biomolecules.

5.8 Wash the tube (walls and pellet) with cold acetone + 0.1% HCl using a glass Pasteur pipette (~500 µL/tube)

5.9 Centrifuge at 3,400 x g for 5 min at 4 °C. Discard supernatant by flipping the tube

5.10 Wash the tube (walls and pellet) with 100% cold acetone using glass Pasteur pipette (~500 µL/tube). Centrifuge at 3,400 x g for 5 min at 4 °C

5.11 Discard supernatant by flipping the tube and pipet out remaining acetone. Open lid and air dry the sample on the bench for ~20 min

5.12 Proceed to propionylation or store samples in −80 °C until use

6. First round of derivatization

The use of trypsin to digest histone proteins leads to excessively small peptides that are hardly identified using traditional proteomics setups. For this reason, propionic anhydride is used to chemically derivatize the ε-amino groups of unmodified and monomethyl lysine residues. This restricts trypsin proteolysis to C-terminal arginine residues. For samples in 96-well plates, the use of multi-channel pipettes and reservoirs for reagent pick up are recommended (Figure 2A). Derivatization is also performed after digestion to label the

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free N-termini of the peptides increasing peptide hydrophobicity and thus reversed-phase chromatographic retention.

6.1 Resuspend samples in 20 µL of 15-20% acetonitrile in 100 mM ammonium bicarbonate pH 8.0. Vortex for 15 s, then spin down at 1000 x g for 30 s

6.2 If there are 8 or more samples, transfer each re-suspended sample into a 96-well plate

NOTE: If the samples have not been transferred to a 96-well plate, a single channel pipette can be used in steps 6.3-6.7, 7.1-7.5, and 8.1-8.5.

6.3 Under the hood, add 2 µL of propionic anhydride using a multichannel pipette. Pipette up and down five times to mix

6.4 Quickly add 10 µL of ammonium hydroxide using a multichannel pipette. Mix by pipetting up and down five times.

NOTE: Propionic acid is a product of the reaction between propionic anhydride and free amines from peptides and can decrease sample pH. pH 8 can be re-established by adding ammonium hydroxide to the sample at a ratio of 1:5 (v/v).

6.5 Check the pH using pH test paper. It should be 8. If pH < 8, adjust by adding 1 µL of ammonium hydroxide. When pH > 10, labeling of other amino acid residues with higher pKa is possible

6.6 Incubate at room temperature for 10 min

6.7 Repeat steps 6.3-6.6. A double round of histone propionylation ensures nearly complete reaction efficiency

6.8 Dry plate in speed vacuum until all wells are completely dried (~9 h)

6.9 Proceed to trypsin digestion or store samples in −80 °C until use

7. Histone digestion

Histones are digested into peptides using trypsin, which cuts at the carboxyl side of arginine and lysine residues. However, since propionylation modifies lysine residues, only arginine residues are cleaved (Figure 2B).

7.1 Prepare trypsin solution (25 ng/µL in 50 mM NH_4HCO_3, pH 8.0). Add 20 µL of trypsin (500 ng) to each sample

7.1.1 To prepare 50 mM NH_4HCO_3 solution, dilute 100 mM NH_4HCO_3 solution 1:1 v/v with HPLC grade water.

7.2 Check pH using pH test paper. It should be around 8. If pH < 8, adjust by adding 1 µL of ammonium hydroxide. If pH > 8, it can be adjusted with formic or acetic acid

7.3 Digest at room temperature overnight or incubate at 37 °C for 6-8 h

7.4 If feasible, check the pH after ~3 h of digestion, as it may have decreased. If pH < 8, adjust by adding 1 µL of ammonium hydroxide.

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7.5 Add an additional 5 μL of 50 ng/μL trypsin solution (250 ng) and continue digestion

7.6 Proceed to the second round of propionylation or store samples in −80 °C until use

8. Derivatization of peptide N-termini

The propionylation of histone peptides at their N-terminus improves retention of the shortest peptides by reversed phase liquid chromatography (e.g. amino acids 3-8 of histone H3), as the propionyl group increases peptide hydrophobicity.

8.1 Under the hood, add 2 μL of propionic anhydride using the multichannel pipette. Pipette up and down five times to mix

8.2 Quickly add 10 μL of ammonium hydroxide using the multichannel pipette. Mix by pipetting up and down five times.

NOTE: Propionic acid is a product of the reaction between propionic anhydride and free amines from peptides and can decrease sample pH. pH 8 can be re-established by adding ammonium hydroxide to the sample at a ratio of 1:5 (v/v)

8.3 Check the pH using pH test paper. It Should be 8. If pH < 8, adjust by adding 1 μL of ammonium hydroxide. When pH > 10, labeling of other amino acid residues with higher pKa is possible

8.4 Incubate at room temperature for 10 min

8.5 Repeat steps 8.1-8.4. A double round of histone propionylation ensures nearly complete reaction efficiency

8.6 Dry plate in speed vacuum until all wells are completely dried (~9 h)

8.7 Proceed to desalting or store samples in −80 °C until use

9. Desalting and sample cleanup

Salts that are present in the sample interfere with mass spectrometry analysis. Salts are also ionized during electrospray and can suppress signals from peptides. Salts can form ionic adducts on peptides, which causes the adducted peptide to have a different mass. This reduces the signal intensity for the normal peptide and prevents proper identification and quantification. The setup for desalting is illustrated in Figure 2C.

9.1 Start mixing the HLB resin (50 mg/mL in 100% acetonitrile) on a magnetic stir plate

9.2 Make sure there is a 96-well collection plate placed underneath the 96-well polypropylene filter plate to collect the flow through

9.3 Add 70 μL of HLB suspension per well to the filter plate. Gently turn on vacuum to prevent splashing. Discard the flow through

9.4 Wash the resin with 100 μL 0.1% TFA. Gently turn on vacuum to prevent splashing. Discard the flow through

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9.5 Re-suspend each sample in 100 µL 0.1% TFA. Check the pH; it should be ~2-3

9.6 Load each sample into each well. Turn on vacuum gently to prevent splashing. Discard the flow through

9.7 Wash with 100 µL 0.1% TFA. Turn on vacuum gently to prevent splashing. Discard the flow through

9.8 Replace the collection plate with a new 96-well collection plate

9.9 Add 60 µL 60% acetonitrile/0.1% TFA per well. Turn on vacuum gently to prevent splashing. Collect the flow through and dry in speed vacuum

9.10 Proceed to LC-MS/MS or store samples in −80 °C until use

10. Histone peptide analysis via liquid chromatography coupled online with mass spectrometry

10.1 Prepare the mobile phases to run on the HPLC

10.1.1 Mobile Phase A (MPA): 2% HPLC-grade acetonitrile + 0.1% formic acid. Mobile Phase B (MPB): 80% HPLC-grade acetonitrile + 0.1% formic acid

10.2 Program the HPLC method

10.2.1 (1) 4-34% MPB over 30 min; (2) 34-90% MPB over 5 min; (3) isocratic 90% MPB for 5 min. Recommendations for column properties are as follows: C_{18} 3 µm packing material, 75 µm internal diameter, 20-25 cm length. The flow rate should be set to 250-300 nL/min for nano-columns with 75 µm internal diameter.

10.2.2 In the case that the HPLC is not programmed to automate column equilibration before sample loading, include: (4) 90-4% MPB over 1 min; (5) isocratic 4% MPB over 10 min

10.3 Program the MS acquisition method

10.3.1 First, the instrument should perform one full MS scan at the beginning of each duty cycle. High resolution instrumentation (e.g. orbitrap or time-of-flight analyzers) is recommended, due to the mass accuracy that can be utilized during signal extraction. However, low resolution instrumentation can also be utilized as previously described[28]

10.3.2 The full MS scan should be followed by 16 MS/MS scan events, each one with an isolation width of 50 m/z, spanning the m/z range 300-1100. E.g. the first scan should isolate signals at 300-350 m/z, the second at 350-400 m/z, etc. If possible, MS/MS scans should also be acquired in high resolution, but lower resolution compared to the full MS scan is sufficient (due to the smaller masses of fragment ions compared to intact ions).

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10.3.3 The HPLC method will generate chromatographic signals with peak widths of approx. 3-40 secs; to ensure proper signal quantification the mass spectrometer should perform at least 10 duty cycles per chromatographic peak, i.e. a duty cycle of 3 secs or faster.

10.3.4 If using a trapping-style mass analyzer (orbitrap, ion trap) ensure that the ion injection time limit is set <200 msec; for other analyzers (quadrupole, time-of-flight) this is not an issue due to their faster scan time. A preliminary test might be required. More details on MS methods for histone peptide analysis can be found in the following references[28,29]

Resuspend sample in 10 µl of MPA, which corresponds to approx. 1 µg/µl of digested histone sample. Exact loading amount is not critical (also not trivial to assess), if all samples in the batch are loaded using similar dilutions and volumes.

10.4 Load 1 µl of sample onto the HPLC column

10.5 Run the LC-MS/MS method programmed in steps 10.1-10.3

11. Data analysis

11.1 Import the MS raw data files into software designed to perform peak area integration

11.1.1 EpiProfile[30,31] is recommended, as it is optimized for reliable peak area extraction of known histone peptides. However, other freely available software for extracted ion chromatography such as Skyline[32,33] are suitable

11.1.1 The relative abundance of a given (un)modified peptide is calculated as follows: area of single peptide ÷ total area of the peptide in all its modified forms. Software such as EpiProfile[30,31] already contain libraries of peptides for signal extraction. Otherwise, a library of peptides of interest should be generated either manually or via peptide identification using routine proteomics pipelines.

Representative results

In this protocol, HepG2/C3A spheroids were treated with 20 mM NaBut and 10 mM NaSuc, both of which affected global levels of histone PTMs (Figure 3A). Histone PTMs were then identified and quantified at the single residue level via MS/MS acquisition (Figure 3B).

When samples are run in replicates, statistical analysis can be performed to assess the fold change enrichment of a PTM between samples, as well as the reproducibility of the observation. The data shown demonstrate that peptides modified with acetylations are enriched in spheroids treated with sodium butyrate vs control (Figure 3C), while samples treated with sodium succinate have a higher relative abundance of histone peptides modified with lysine succinylation (Figure 3D). These calculations are done in Microsoft Excel and are detailed in a separate publication[34]. An overall increase of a given histone modification

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can be better represented in radar plots, where observing a higher global abundance of a certain modification becomes more intuitive, even maintaining detailed information about the modification sites analyzed (Figure 3E-F).

This protocol generates a peptide from histone H3 amino acid residues 9 to 17, which includes the frequently modified residues K9, S10 and K14. The data shown indicate that treatment with sodium butyrate increases the levels of H3K14ac, but only on histones co-modified with H3K9me2 and not H3K9me3 (Figure 4A). The co-existence frequency between two modifications can be represented more intuitively as a ring graph, where the nodes represent individual modifications while the thickness of connector lines represents the co-existence frequency between the two PTMs (Figure 4B). Sometimes, the co-existence frequency is unaffected, but data represented as bar plots might be misleading. For instance, the data represented in Figure 4A indicate that the combination H3K9me2K14ac is more abundant in sodium butyrate treatment than control. This is correct, but this given combination is the most frequent regardless of the treatment. Figure 4B clearly shows that H3K9me2K14ac and H3K9me3K14ac are the most frequent combinatorial patterns regardless of the treatment (line thickness), but that global levels of H3K14ac (node) are what is truly changing in the experiment.

This protocol generates a peptide from histone H4 residues 4 to 17, which includes modifiable residues at positions K5, K8, K12 and K16 (mostly by acetylations). When comparing control and sodium butyrate treatment, it is possible to observe an increase of combinations of acetylations by representing data as, e.g., word clouds (Figure 4C). This representation clearly highlights that the unmodified version of histone H4 is most abundant in the control sample, while spheroids treated with sodium butyrate are enriched in doubly, triply and quadruply acetylated histone H4 proteoforms. However, word clouds are limited in displaying exact values; the relative abundance of a histone code should be deconvoluted by the size of the text, which may be inaccurately estimated. Therefore, Venn diagrams or more modern equivalents such as the UpSetR representation[35] can be used to show exact quantification of co-existing histone PTMs (Figure 4D-E). The data shown highlight once again that selected combinations of acetylations on histone H4 are relatively more abundant in sodium butyrate treatment compared to control.

Discussion

The analysis of histone PTMs is fundamentally different from the typical proteomics analysis pipeline. Most histone PTMs still have enigmatic biological functions; as a result, annotations such as Gene Ontology or pathway databases are not available. Several resources exist that associate histone modifications with the enzyme responsible for their catalysis or proteins containing domains that bind these PTMs (e.g. HISTome[36]). As well, it is possible to speculate on the overall state of the chromatin when global levels of histone PTMs are regulated. For instance, an overall increase of histone acetylation or other acylations like succinylation is normally associated with chromatin decondensation[37,38].

MS analysis provides more detailed information about these modifications, such as their exact localization on the amino acid sequence. In this protocol, MS/MS acquisition is used

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to identify and quantify histone PTMs, which can be critical for biological interpretation. For example, trimethylation on lysine 4 of histone H3 (H3K4me3) is enriched on promoters of actively transcribed genes[39], while the same modification on lysine 9 (H3K9me3) benchmarks constitutive heterochromatin[40]. Histone modifications are currently being used as biomarkers in specific diseases; as such, histone analysis can be used to study disease pathology in addition to response to treatment (e.g. with epigenetic drugs)[41,42].

It is more challenging to visually represent interactions between multiple PTMs as opposed to single PTMs. While existing charts such as ring graphs can show the co-existence frequency of two PTMs, they cannot represent the co-existence frequency between more than two PTMs at a time since this would require a three-dimensional representation of the network. For this reason, other representations could be more appropriate to highlight changes of histone codes when three or more PTMs are considered. In general, diversifying data representation offers higher chances to observe significant changes between samples. This protocol presents examples of different illustrations for displaying regulations of histone PTMs and co-existing PTMs.

Though this protocol generates relatively small histone peptides due to trypsin digestion (approx. 4-20 amino acids), selected peptides contain multiple modifiable residues. The analysis of these peptides allows for the quantification of co-existence frequencies of PTMs, which could reveal important information about which combinatorial histone marks are regulated in a given dataset. Notably, there are no steps during sample preparation where quantification of histones is performed. There are four reasons for this: (1) trypsin has a wide range of activity and can be used at a broad range of enzyme:sample ratios (1:10-1:200). Even when the experimental yield of extracted histones differs from the expected one, issues with digestion have not been encountered using this protocol. (2) This protocol is intended for minute amounts of material, where histone quantification might be difficult to perform due to lack of sensitivity. (3) By using a constant trypsin concentration regardless of the amount of histone material, we can use tryptic peptides (as trypsin autolyses) to benchmark chromatography performance. Slight variations in sample yield will be normalized by EpiProfile, which uses all (un)modified signals for a given peptide as the denominator during the normalization process. (4) Finally, dramatic underestimation of amount of starting material might create problems of overloading the nanoLC chromatographic column. However, performing the desalting step as indicated in this protocol prevents this issue from taking place. In case of excessive amounts of starting material, e.g. >100s µg, the limit of capacity of the desalting resin will be exceeded and any excess sample will be washed away during the loading step.

It is important to note that not all of the peptides detected by this analysis have been highlighted in Figures 3 and 4. As well, not all histone modifications are detectable using these specific sample preparation and acquisition methods. Supplementary Table 1 is provided to list all the peptide signals that are extracted with EpiProfile using the described pipeline. A few well-known modifications are not listed in the table, as the described sample preparation is not suitable for their detection. Notable examples are ubiquitinylated peptides from histone H2A and H2B, and phosphorylation of histone H2A.X (general marker of DNA damage). This is because the propionylation of the

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peptides associated with these PTMs protocol leads to excessively long peptides, which are not suitable for C18 chromatography and the described MS detection method. Other modifications that are present in the literature but not present in Supplementary Table 1 are very low abundance modifications (currently only detectable using MS after enrichment strategies such as immunoprecipitation or specific cell treatment) such as lactylation[43] or serotonylation[44]. Histone modifications with unpredictable mass shift caused by polymerization or heterogeneous covalent binding to the histone sequence are also not considered, e.g. poly-ADP-ribosylation[45] and glycation[46].

This protocol specifically uses NaSuc and NaBut to treat HepG2/C3A spheroids, but it can be modified for use with other drugs/epigenetic modifiers and 2D/3D cell culture types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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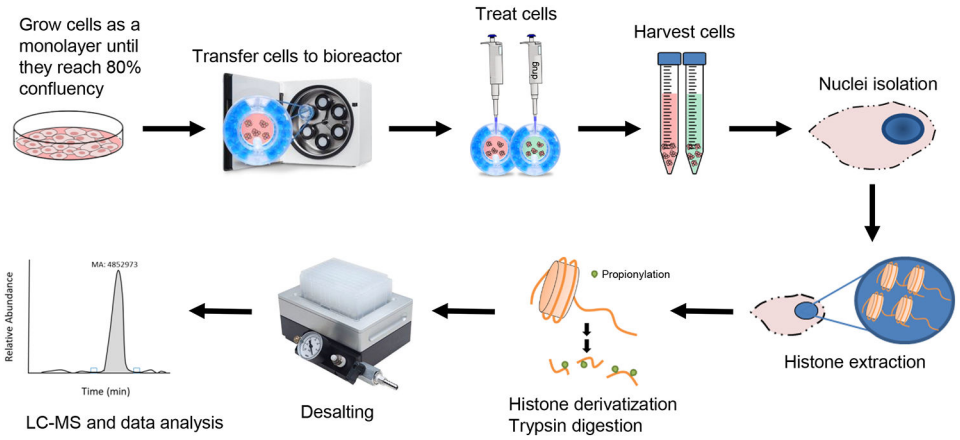


Figure 1: Workflow for histone peptide analysis of 3D spheroids.

HepG2/C3A cells are first grown in 2D culture until they reach 80% confluency. The cells are then transferred to an equilibrated bioreactor and placed within the clinostat incubator where they will rotate at 10-11 rpm to form spheroids. After 18 days, the spheroids are treated with either 20 mM NaBut or 10 mM NaSuc and are harvested after their corresponding time points. Nuclei are isolated from the cells and histone extraction is performed with 0.2 M H_2SO_4. Histone derivatization is then performed with propionic anhydride before and after trypsin digestion to ensure retention of the resulting short peptides by liquid chromatography. Samples are desalted and then run using the LC-MS/MS method mentioned in Section 10 and the resulting data is analyzed as described in Section 11.

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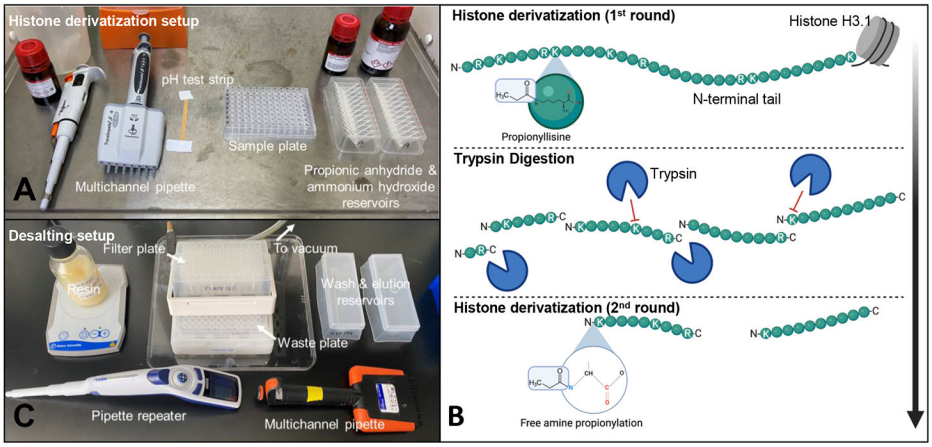


Figure 2: Setup for propionylation and desalting steps.

(A) Propionylation is performed in a fume hood and all components are laid out so that the steps can be performed in quick succession. **(B)** Schematic of 1st round of propionylation, trypsin digestion, and 2nd round of propionylation on histone H3.1 tail. **(C)** Desalting is performed on the bench using a 96-well vacuum manifold and a 96-well polypropylene filter plate.

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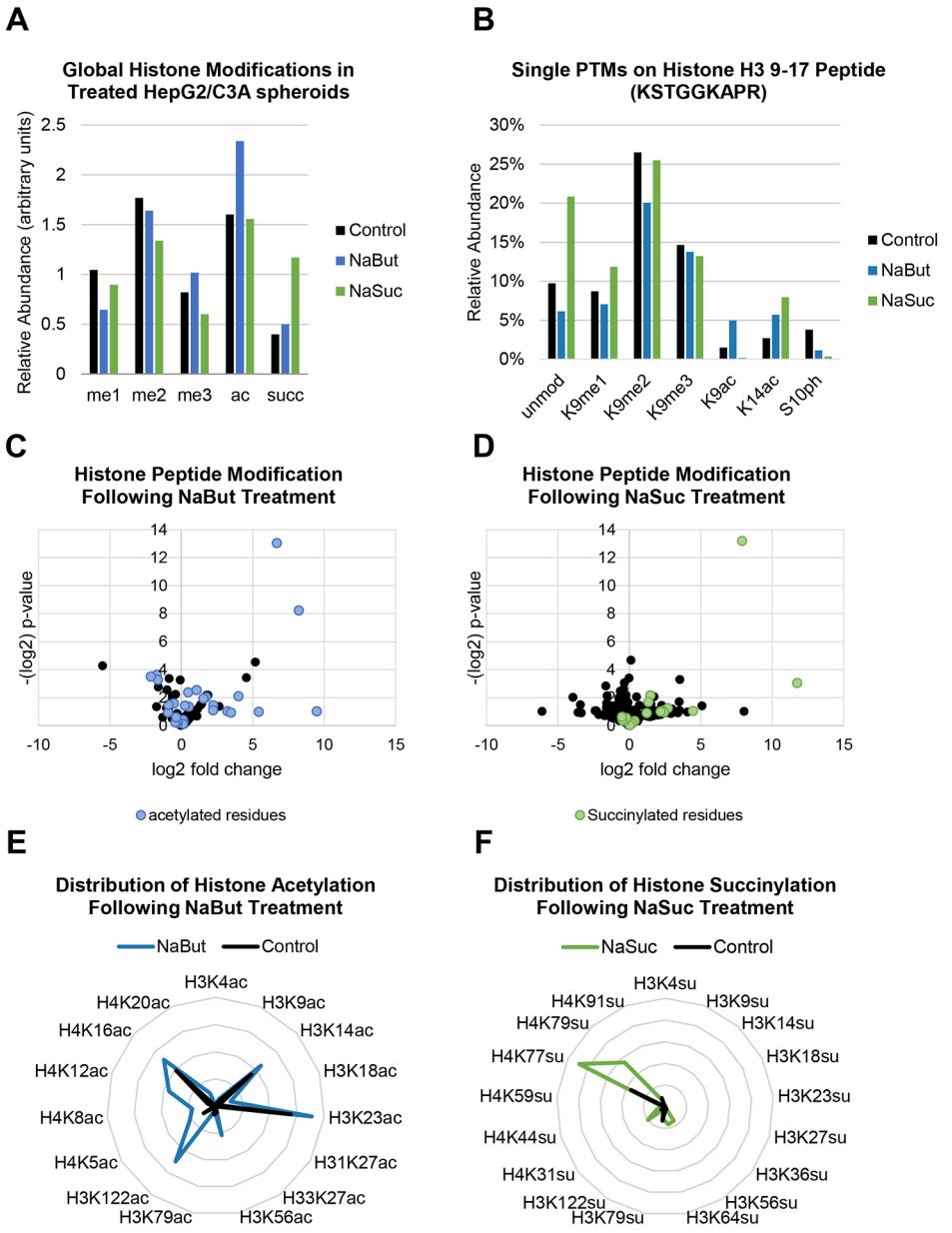


Figure 3: Representation of individual histone modifications

(A) Bar graph showing the relative abundance of common global histone modifications in control and treated (20 mM NaBut or 10 mM NaSuc) HepG2/C3A spheroids. **(B)** Bar graph showing the abundance of single histone PTMs occurring on residues 9-17 of histone H3 peptide (KSTGGKAPR) in control and treated (20 mM NaBut or 10 mM NaSuc) HepG2/C3A spheroids. **(C,D)** Volcano plots showing the fold change and significance of differential expression of histone peptide PTMs following treatment with 20 mM NaBut (C) or 10 mM NaSuc (D). Highlighted blue and green points represent acetylated and succinylated residues, respectively. **(E,F)** Radar plots showing the abundance of single histone peptide acetylation (E) or succinylation (F) following treatment with 20 mM NaBut or 10 mM NaSuc respectively as compared to control.

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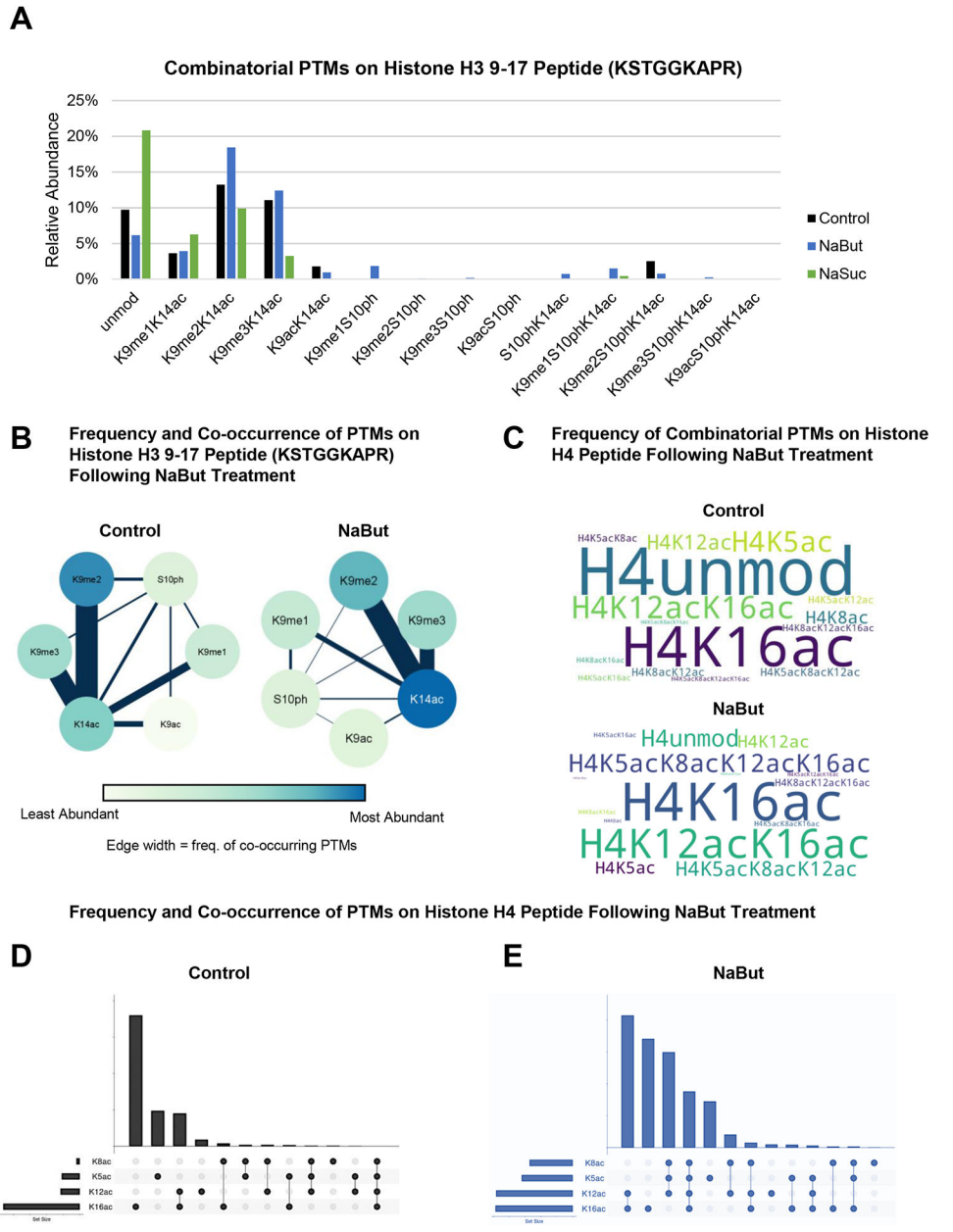


Figure 4: Representation of co-existing histone modifications.

(A) Bar graph showing the abundance of combinatorial histone PTMs occurring on residues 9-17 of histone H3 (KSTGGKAPR) in control and treated (20 mM NaBut or 10 mM NaSuc) HepG2/C3A spheroids. **(B)** Ring graphs showing the relationship between combinatorial histone PTMs on residues 9-17 of histone H3 (KSTGGKAPR) in control and treated (20 mM NaBut) HepG2/C3A spheroids. The intensity of the node color corresponds to the abundance of a single PTM within its treatment group, while the line thickness corresponds to the frequency of PTM co-occurrence. **(C)** Word clouds showing the frequency of combinatorial histone PTMs on histone H4 residues in in control and treated (20 mM NaBut) HepG2/C3A spheroids. The size of the text corresponds with the abundance of the specified combinatorial PTM. **(D,E)** Venn diagram representing the frequency of co-existing

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modifications on histone H4 peptide 4-17 in control and 20 mM NaBut treated samples. Data are displayed using the ShinyApp UpSetR[35].

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Table_of_Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.05% trypsin-EDTA solution	Gibco	25300054	
0.5-20 µL pipet tips	BRAND	13-889-172 (Fisher Scientific)	
1.5 ml microcentrifuge tubes	Bio-Rad	2239480	
10 µL multi-channel pipette	BRAND	BR7059000 (Millipore Sigma)	
10 mL syringe	Henke Sass Wolf	14-817-31 (Fisher Scientific)	Luer lock tip, graduated to 12 mL
10, 20, 200, and 1000 µL single-channel pipettes	Eppendorf	14-285-904 (Fisher Scientific)	
1000 µL pipet tips	Rainin	30389164	
18G syringe needle	Air-Tite	14-817-100 (Fisher Scientific)	3" length, 0.05" diameter
200 µL multi-channel pipette	Corning	4082	
2-200 µL pipet tips	BRAND	Z740118 (Millipore Sigma)	
24-well ultra-low attachment microplate	Corning	07-200-602	
75 cm^2 U-shaped cell culture flask	Corning	461464U	Untreated, with vent cap
96-well skirted plate	Axygen	PCR-96-FS-C (Corning)	
Acetone	Fisher Scientific	A949-1	Acetone should be used cold
Ammonium bicarbonate (NH_4HCO_3)	Sigma-Aldrich	A6141-25G	
Ammonium hydroxide solution	Fisher Scientific	AC423300250	
Cell culture grade water	Corning	25-055-CV	
ClinoReactor	CelVivo	10004-12	Bioreactor for 3D cell culture
ClinoStar	CelVivo	N/A	Clinostat CO_2 incubator for 3D cell culture
Control unit	CelVivo	N/A	Tablet for ClinoStar settings
Dulbecco's Modified Eagle's Medium (DMEM)	Corning	17-205-CV	1X solution with 4.5 g/L glucose and sodium pyruvate, without L-glutamine and phenol red
Fetal bovine serum (FBS)	Corning	35-010-CV	
Formic acid	Thermo Scientific	28905	
Fume hood	Mott	N/A	Model 7121000
Glass Pasteur pipette	Fisher Scientific	13-678-8B	9", cotton-plugged, borosilicate glass, non-sterile
Glutagro supplement	Corning	25-015-CI	200 mM L-ananyl-L-glutamine

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Hank's Balanced Salt Solution (HBSS)	Corning	21-022-CV	1X solution without calcium, magnesium, and phenol red
HPLC grade acetonitrile	Fisher Scientific	A955-4	
HPLC grade water	Fisher Scientific	W6-1	
Hydrochloric acid (HCl)	Fisher Scientific	A481-212	
Ice	N/A	N/A	
MEM non-essential amino acids	Corning	25-025-CI	100X solution
Oasis HLB resin	Waters	186007549	Hydrophilic-Lipophilic-Balanced (HLB) Resin with 30µm particle size
Orbitrap Fusion Lumos Tribrid mass spectrometer	Thermo Fisher Scientific	IQLAAEGAAPFADBMBHQ	High resolution mass spectrometer
Oro-Flex I polypropylene filter plate	Orochem	OF1100	96-well polypropylene filter plate w/ 10 µM PE frit
Penicillin-Streptomycin	Corning	30-002-CI	100X solution
pH paper	Hydrion	Z111848 (Sigma-Aldrich)	0-13 pH test paper
Pipette gun	Eppendorf	Z666467 (Millipore Sigma)	
Polymicro capillary	Molex	50-110-7740 (Fisher Scientific)	Flexible fused silica capillary tubing with polymide coating, 75 µM ID x 363 µM OD
Polystyrene 10ml serological pipets, sterile	Fisher Scientific	1367549	
Propionic anhydride	Sigma-Aldrich	240311-50G	
Refrigerated centrifuge	Thermo Scientific	75-217-420	
Reprosil-Pur resin	MSWIL	R13.AQ.0003	120 Å pore size, C18-AQ phase, 3 µM bead size
Rotator	Clay Adams	25477 (American Laboratory Trading)	Nutator Mixer 1105
Sequencing grade modified trypsin	Promega	V5111	
Sodium butyrate	Thermo Scientific	A11079	
Sodium succinate dibasic	Sigma-Aldrich	14160-100G	
SpeedVac vacuum concentrator (1.5 mL microcentrifuge tubes)	Savant	20249 (American Laboratory Trading)	
SpeedVac vacuum concentrator (96-well)	Thermo Scientific	15308325	Savant SPD1010
Sterile hood	Thermo Scientific	1375	Class II, Type A2
Sulfuric acid (H_2SO_4)	Fisher Scientific	02-004-375	Baker Analyzed ACS reagent
Tissue-culture treated 100 mm x 20 mm dish	Fisher Scientific	08-772-23	
Trichloroacetic acid (TCA)	Thermo Scientific	AC421451000	Resuspend 100% w/v in HPLC grade water
Trifluoroacetic acid (TFA)	Fisher Scientific	PI28904	Sequencing grade

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Vacuum manifold 96-well	Millipore	MAVM0960R	
Vortex	Sigma-Aldrich	Z258415	
Water bath	Fisher Scientific	FSGPD10	
Wide bore pipet tips 1000 µL	Axygen	14-222-703 (Fisher Scientific)	
Wide bore pipet tips 200 µL	Axygen	14-222-730 (Fisher Scientific)	