FINAL SCRIPT: APPROVED FOR FILMING

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Title: Extraction of Histones from Clinical Specimens for Epigenetic Profiling via Mass Spectrometry

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 25
Number of Shots: 55

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Tiziana Bonaldi:** The scope of our research is to analyze epigenetic changes in cancer samples, discover new diagnostic and prognostic biomarkers, and identify potential targets for therapy.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.4* File: A7sIII_9813

What are the current experimental challenges?

1.2. **Giulia Robusti:** A major challenge with clinical samples is the limited material and fixation-induced artifacts. However, with our optimized workflow and high-sensitivity MS, we can quantify up to 50 histone PTMs from just 1,000 cells.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1.* File: A7sIII_9811, A7sIII_981

What significant findings have you established in your field?

1.3. **Tiziana Bonaldi:** We showed that mass spectrometry can quantify PTM changes in clinical samples, offering insights into epigenetic mechanisms and enabling biomarker discovery and drug-target identification for precision epigenetic therapies.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.4.1* File: A7sIII_9814

What research gap are you addressing with your protocol?

1.4. **Paola Fulghieri:** Our protocol addresses the need for a robust, quantitative and sensitive profiling of histone PTM in various types of clinical samples.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.2* File: A7sIII_9815, A7sIII_9816

What advantage does your protocol offer compared to other techniques?

1.5. **Paola Fulghieri:** Mass spectrometry is ideal for histone PTM analysis, offering unbiased detection, simultaneous profiling of multiple modifications, and co-occurrence on the same peptide, thus overcoming the limitations of antibody-based methods.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.2.1* File: A7sIII_9817

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Ethical Committee at the European Institute of Oncology and the Istituto Neurologico Carlo Besta

NOTE: The filenames given by the videographer are added in red font against each shot

Protocol

2. Histone Extraction from Patient-Derived Tissues

Demonstrator: Giulia Robusti

2.1. To begin, thaw the fresh frozen tissue on ice **[1]** and, using a scalpel or blade, cut a piece of approximately 20 to 30 milligrams, corresponding to around 2 cubic millimeters of tissue **[2]**. Transfer the cut tissue sample to a 1.5 milliliter tube **[2]**. Using scissors, mince the tissue into small pieces **[4]**.

2.1.1. WIDE: Talent placing frozen tissue on crushed ice in a Petri dish. FILE: A7sIII_9819

2.1.2. Talent cutting a small piece of tissue with a scalpel, showing size reference. FILE: A7sIII_9821

2.1.3. Talent placing the tissue piece into a labeled 1.5 milliliter microcentrifuge tube. FILE: A7sIII_9822

2.1.4. Talent using scissors to mince the tissue thoroughly inside the tube. FILE: A7sIII_9823

2.2. Add 1 milliliter of freshly prepared Nuclei Isolation Buffer, supplemented with the specified protease inhibitors **[1]** and mince the tissue again **[2]**. After vortexing, transfer the sample to a Dounce homogenizer **[3]**. Using the loose pestle first and then the tight pestle, homogenize the tissue until no visible pieces remain to the naked eye **[4]**.

2.2.1. Talent adding buffer with a pipette into the tube containing minced tissue. FILE: A7sIII_9824

2.2.2. Talent using scissors to mince the tissue thoroughly inside the tube. FILE: A7sIII_9825

2.2.3. Talent pouring the minced tissue along with the buffer into the Dounce homogenizer. FILE: A7sIII_9827

2.2.4. Talent using the pestles to homogenize until the suspension appears uniform. FILE: A7sIII_9827

2.3. Filter the homogenized sample through a 100-micrometer cell strainer to eliminate

tissue debris **[1]**. Pipette the filtered sample vigorously up and down using a 200-microliter pipette to dissolve the plasma membrane **[2]**. Transfer the sample to a new 1.5 milliliter tube **[3]**.

2.3.1. Talent pouring the homogenized suspension onto a 100 micrometer cell strainer attached to a new container. FILE: A7sIII_9828

2.3.2. Talent pipetting the filtrate vigorously using a 200 microliter pipette. FILE: A7sIII_9831

2.3.3. Talent transferring the sample into a fresh labeled 1.5 milliliter tube. FILE: A7sIII_9831

2.4. Place the tube into a benchtop centrifuge and spin at 2,300 *g* for 15 minutes at 4 degrees Celsius to pellet the nuclei **[1]**. After discarding the supernatant, resuspend the nuclear pellet in 50 to 100 microliters of Nuclei Isolation Buffer supplemented with 0.1 percent SDS to dissolve the nuclear membrane **[2]**.

2.4.1. Talent placing the tube into a benchtop centrifuge. FILE: A7sIII_9832

2.4.2. Talent adding buffer containing sodium dodecyl sulfate and resuspending the pellet by gentle pipetting. FILE: A7sIII_9834

2.5. Then, add 250 units of Benzonase nuclease to the resuspended sample to digest nucleic acids **[1]**. Mix well and incubate for 2 minutes at 37 degrees Celsius **[2]**.

2.5.1. Talent pipetting Benzonase into the sample and mixing thoroughly. FILE: A7sIII_9835

2.5.2. Talent placing the tube into a 37 degrees Celsius heat block for incubation. FILE: A7sIII_9837

2.6. For optimal-cutting temperature frozen samples, wash the tissues with ethanol, water and PBS before mincing and histone extraction **[1]**. **NOTE**: The videographer has shot lot of scenes for this step. But use any one scene and put the text in front of the background.

2.6.1. TEXT ON PLAIN BACKGROUND:

- Place 4 - 5 tissue sections (10 µm thick; ~20 - 60 mg) into a 1.5 mL tube
- Wash with 1 mL ice-cold 70% ethanol (2 min on a rotating wheel at 4 °C)
- Centrifuge at 16,000 × *g* (2 min, 4 °C)
- Rehydrate in 1 mL ddH_20 (rotation for 2 min, 4 °C)
- Centrifuge again at 16,000 × *g* (2 min at 4 °C)

- Wash twice with 1× PBS
- Begin the tissue mincing process

3. Histone Extraction from Formalin-Fixed Paraffin-Embedded (FFPE) Samples

3.1. Add 1 milliliter of paraffin dissolving agent to the PFFE sample taken in a 1.5-milliliter tube **[1-TXT]** and vortex at maximum speed for 30 seconds until the paraffin is completely dissolved **[2]**. Centrifuge the sample at 16,000 *g* for 3 minutes at room temperature **[3]** and discard the supernatant carefully using a pipette fitted with a small tip, avoiding the pellet **[4]**.

3.1.1. Talent pipetting paraffin dissolving agent into the tube with formalin fixed paraffin embedded sections. **TXT: FFPE: Formalin-Fixed Paraffin-Embedded** FILE: A7sIII_9851

3.1.2. Talent vortexing the tube vigorously until the solution appears clear. FILE: A7sIII_9852, FILE: A7sIII_9853

3.1.3. Talent placing the tube into the benchtop centrifuge and starting the spin. FILE: A7sIII_9854

3.1.4. Talent aspirating the supernatant using a pipette fitted with a small tip while avoiding the pellet. FILE: A7sIII_9857

3.2. Now, add 1 milliliter of 95 percent ethanol to the pellet and vortex at maximum speed for 30 seconds **[1]**. Centrifuge the sample at 16,000 *g* for 3 minutes at room temperature and discard the supernatant **[2]**. After rehydrating the sample, add 200 microliters of Extraction Buffer to the pellet **[3]**.

3.2.1. Talent pipetting ethanol into the tube and vortexing thoroughly. FILE: A7sIII_9859

3.2.2. Talent centrifuging the sample and discarding the supernatant. FILE: A7sIII_9860

3.2.3. Talent adding Extraction Buffer into the tube using a pipette. FILE: A7sIII_9862

3.3. Homogenize the tissue in the Extraction Buffer by sonicating with a digital signifier using a 3-millimeter microtip **[1-TXT]**. After homogenization, incubate the sample in a thermomixer and briefly open the tube lid to let the formaldehyde evaporate **[2-TXT]**.

3.3.1. Talent performing sonication using a digital sonicator on the tube containing Extraction Buffer and tissue. **TXT: Sonication: >15 cycles; 5 s ON/2 min OFF;**

Power: 15 - 30% FILE: A7sIII_9864

3.3.2. Talent placing the tube into a thermomixer. **TXT: 95 °C, 45 min (open the tube every 15 min); 65 °C, 4 h (open the tube every 20 - 60 min)** FILE: A7sIII_9865

3.4. After centrifuging the sample, transfer the supernatant into a new labeled tube **[1-TXT]**.

3.4.1. Talent transferring the clear supernatant into a fresh tube using a pipette. **TXT: Load the extracted histone into a gel for SDS-PAGE** FILE: A7sIII_9866

4. Processing the Gels for Histone In-Gel Digestion and Derivatization with Propionic Anhydride

Demonstrator: Paola Fulghieri

4.1. Excise the gel bands corresponding to the molecular weight of core histone proteins, located between 10 and 18 kilodaltons, using a scalpel **[1]**. After cutting the gel bands, use the same scalpel to transfer the cut gel pieces into a 1.5-milliliter tube **[2]**.

4.1.1. Talent using a scalpel to identify and excise gel bands between 10 and 18 kilodaltons on a stained gel. FILE: A7sIII_9868

4.1.2. Talent carefully picking up gel pieces with the scalpel and placing them into a labeled microcentrifuge tube. FILE: A7sIII_9869, FILE: A7sIII_9870

4.2. To destain the gel, add a solution of 50 percent acetonitrile in double-distilled water **[3]** and incubate the tube in a thermomixer at 1,400 revolutions per minute for 10 minutes at room temperature **[4]**.

4.2.1. Talent pipetting 50 percent acetonitrile solution into the tube containing gel pieces. FILE: A7sIII_9871

4.2.2. Talent placing the tube in a thermomixer and later discarding the supernatant with a pipette. FILE: A7sIII_9872, FILE: A7sIII_9873

4.3. Dehydrate the gel pieces by adding 100 percent acetonitrile **[1]**. Mix in the thermomixer at 1,400 revolutions per minute for 10 minutes at room temperature **[2]**. After discarding the supernatant, dry the gel pieces for 5 minutes at room temperature in a vacuum centrifuge **[2]**.

4.3.1. Talent adding 100 percent acetonitrile into the tube. FILE: A7sIII_9874

4.3.2. Talent placing the sample in the thermomixer. FILE: A7sIII_9875

4.3.3. Talent placing the open tube with gel pieces into a vacuum centrifuge. FILE: A7sIII_9877

4.4. Next, add 15 microliters of 7.7 molar propionic anhydride solution and 26 microliters of 1 molar ammonium bicarbonate to each tube and incubate **[1-TXT]**. Then, incubate the sample with 80 microliters of 1 molar ammonium bicarbonate **[2-TXT]**.

4.4.1. Talent pipetting both reagents into the tube with dried gel pieces. **TXT: 350 rpm; 10 min; 37 °C** FILE: A7sIII_9878

4.4.2. Talent placing the sample in incubator. **TXT: 1,400 rpm; 4 h; 37°C** FILE: A7sIII_9878

4.5. Then, wash the gel pieces three times with double-distilled water **[1]** and discard the liquid after each incubation **[2]**.

4.5.1. Talent adding water into the tube. FILE: A7sIII_9880

4.5.2. Talent discarding the supernatant from the tube. FILE: A7sIII_9884

4.6. Next, add 50 percent acetonitrile to the gel pieces **[1]** and incubate at 1,400 revolutions per minute for 15 minutes at room temperature **[2]**.

4.6.1. Talent pipetting 50 percent acetonitrile into the tube. FILE: A7sIII_9885

4.6.2. Talent placing the sample in the incubator. FILE: A7sIII_9886

4.7. Then, add 100 percent acetonitrile to completely dehydrate the gel pieces **[1]**. Incubate in the thermomixer at 1,400 revolutions per minute for 15 minutes at room temperature **[2]**. After discarding the liquid, dry the gel pieces for 5 minutes at room temperature in a vacuum centrifuge **[3]**.

4.7.1. Talent pipetting 100 percent acetonitrile into the tube with gel pieces. FILE: A7sIII_9887

4.7.2. Talent removing the liquid after mixing in the thermomixer. FILE: A7sIII_9888, FILE: A7sIII_9889

4.7.3. Talent placing the tube into a vacuum centrifuge and running the drying cycle. FILE: A7sIII_9890

5. Digestion with Trypsin and Derivatization with Phenyl Isocyanate

5.1. Add 4 microliters of trypsin solution and 20 microliters of Digestion Buffer to each tube containing gel pieces **[1]**. Incubate the tubes on ice for 10 minutes to allow trypsin absorption into the gel **[2]**.

5.1.1. Talent pipetting trypsin solution and Digestion Buffer into each tube. FILE: A7sIII_9892

5.1.2. Talent placing the tubes on crushed ice for incubation. FILE: A7sIII_9892

5.2. Once the trypsin is fully absorbed, add 80 microliters of Digestion Buffer to completely cover the gel pieces **[1]** and incubate the tubes overnight at 37 degrees Celsius in a thermomixer **[2]**.

5.2.1. Talent topping up each sample with additional Digestion Buffer. FILE: A7sIII_9893

5.2.2. Talent placing the tubes into the thermomixer set to 37 degrees Celsius for overnight incubation. FILE: A7sIII_9894

5.3. To extract the digested peptides, add 100 microliters of 100 percent acetonitrile to each tube **[1]** and incubate at 1,400 revolutions per minute for 20 minutes at room temperature in a thermomixer **[2]**.

5.3.1. Talent pipetting 100 percent acetonitrile into the sample tubes. FILE: A7sIII_9895

5.3.2. Talent mixing the tubes in the thermomixer and setting the timer. FILE: A7sIII_9896, FILE: A7sIII_9897

5.4. Now, carefully collect the supernatant and transfer it into a new 1.5 milliliter tube **[1]**.

5.4.1. Talent pipetting the supernatant from the old tube into a fresh labeled microcentrifuge tube. FILE: A7sIII_9899

5.5. After repeating the acetonitrile extraction with the remaining gel, pool the supernatants **[1]**. Concentrate the pooled peptides in a vacuum centrifuge until the volume is between 1 and 5 microliters **[2]**.

5.5.1. Talent transferring the second extract into the same tube containing the first supernatant. FILE: A7sIII_9900

5.5.2. Talent placing the tube into a vacuum centrifuge and monitoring until the final volume is reduced. **TXT: Adjust the volume to 15 µL with ddH_2O** FILE: A7sIII_9901

5.6. Then, add 2 microliters of 1 molar triethylammonium bicarbonate and 3 microliters of phenyl isocyanate solution **[2]**. Incubate the sample at 350 revolutions per minute for 90 minutes at 37 degrees Celsius in a thermomixer **[3]**.

5.6.1. Talent pipetting the specified reagents into the sample. FILE: A7sIII_9902

5.6.2. Talent placing the tube into the thermomixer and setting the time and temperature. FILE: A7sIII_9903, FILE: A7sIII_9904

5.7. To stop the derivatization, add 8 microliters of 1 percent trifluoroacetic acid to each tube **[1]**.

5.7.1. Talent adding trifluoroacetic acid to each sample using a pipette. FILE: A7sIII_9905

5.8. Finally, dilute each sample by adding 100 microliters of Buffer A **[1]** and load the diluted samples onto C18 stage tips for elution **[2]**.

5.8.1. Talent pipetting Buffer A into each tube. FILE: A7sIII_9906

5.8.2. Talent loading each diluted sample onto labeled C18 stage tips. FILE: A7sIII_9908

Results

6. Results

6.1. Histone extraction was successfully achieved from fresh frozen, OCT frozen, and FFPE samples using differential processing workflows specific to each tissue type **[1]**.

6.1.1. LAB MEDIA: Figure 2B1. *Video editor: Highlight the dashed box*.

6.2. Despite lower enrichment and background protein presence, histone H3 was successfully extracted from FFPE samples, as shown by bands in lanes FFPE1 and FFPE2 aligning with approximately 1 microgram of the H3.1 standard **[1]**.

6.2.1. LAB MEDIA: Figure 1. *Video editor: Highlight the bands in FFPE1 and FFPE2 lanes corresponding to the row/line H3*

6.3. Nano-liquid chromatography and MS analysis of FFPE-derived histones showed successful separation of histone peptides over a 55-minute gradient, with clear retention time peaks shown in the total ion chromatogram **[1]**.

6.3.1. LAB MEDIA: Figure 3. *Video editor: Display panel A showing the chromatogram with labeled peaks*.

6.4. Extracted ion chromatograms confirmed the detection and quantification of unmodified and methylated forms of H3-K4, with separate elution profiles for each m/Z *(M-by-Zee)* value **[1]**, enabling the calculation of percentage relative abundance for each modification **[2]**.

6.4.1. LAB MEDIA: Figure 3. *Video editor: Highlight panel B.*

6.4.2. LAB MEDIA: Figure 3. *Video editor: Show panel C.*

6.5. Comparative analysis of histone modificatons between normal and tumor samples revealed significant increases in certain types **[1]** and a decrease in other types in tumor tissues **[2]**.

6.5.1. LAB MEDIA: Figure 4*. Video editor: In panel B, highlight the BLUE scatter plot for H3K27me2K36me2, H3K27me3K36me2, H3K9me3K14ac and showing the tumor group dots shifted higher*.

6.5.2. LAB MEDIA: Figure 4. *Video editor: Highlight the BLUE scatter plot for H3K9K14un, H3K27me1, H3K27me2 and H4K20me1 in panel B*

1. **nuclei**
   Pronunciation link: https://www.merriam-webster.com/dictionary/nuclei
   IPA: /ˈnuː.kli.aɪ/
   Phonetic Spelling: noo-klee-eye
2. **nucleic**
   Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/nucleic-acid Cambridge Dictionary
   IPA: /nuːˈkleɪ.ɪk/
   Phonetic Spelling: noo-KLAY-ik
3. **paraffin**
   Pronunciation link: https://www.merriam-webster.com/dictionary/paraffin
   IPA: /ˈpærəˌfɪn/
   Phonetic Spelling: PAIR-uh-fin
4. **derivatization**
   Pronunciation link: https://www.merriam-webster.com/dictionary/derivatization
   IPA: /dɪˌrɪvəˌtaɪˈzeɪʃən/
   Phonetic Spelling: dih-rih-vuh-tye-ZAY-shun
5. **isocyanate**
   Pronunciation link: https://www.merriam-webster.com/dictionary/isocyanate
   IPA: /aɪˈsoʊsaɪˌeɪt/
   Phonetic Spelling: eye-SOH-sye-ate

1. **Formaldehyde**
   Pronunciation link: https://www.merriam-webster.com/dictionary/formaldehyde Merriam-Webster
   IPA: /fɔːrˈmæl.də.haɪd/ Cambridge Dictionary+1
   Phonetic Spelling: for-MAL-duh-hyde
2. **Benzonase**
   Pronunciation link: https://www.howtopronounce.com/benzonase How To Pronounce
   IPA: /bɛn.zoʊ.neɪs/
   Phonetic Spelling: BEN-zoh-nays