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Title: Assays for Validating Histone Acetyltransferase Inhibitors

Authors and Affiliations:

Aaron R. Waddell[1], Daiqing Liao[1]

[1]Department of Anatomy and Cell Biology, and UF Health Cancer Center, University of Florida College of Medicine, Gainesville, Florida

Corresponding Authors:

Daiqing Liao (dliao@ufl.edu)

Email Addresses for All Authors:

aawaddell@ufl.edu
dliao@ufl.edu

Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **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: 14
Number of Shots: 44

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Aaron Waddell:** These protocols are significant because they provide detailed steps for validation of the potency and selectivity of novel HAT inhibitors, which are important research tools and potential therapeutics.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Aaron Waddell:** The techniques demonstrated in this video are easy to perform and provide information about the effects of HAT inhibitors on global and regional histone acetylation. They make it possible to understand epigenetic regulation of gene expression.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Aaron Waddell:** When attempting this procedure, attention to the details is critical. It is important to follow the protocols step by step.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. In Vitro HAT Assay

2.1. Begin by preparing the enzymatic reaction in a 10-microliter volume inside a 0.2-milliliter PCR tube according to manuscript directions **[1]**, then incubate the complete reaction mixture at 30 degrees Celsius for 1 hour in a PCR thermal cycler **[2]**. *Videographer: This step is important!*

2.1.1. WIDE: Establishing shot of talent at the lab bench mixing the reagents.

2.1.2. Talent putting the reaction mixture into the thermocycler.

2.2. Meanwhile, add 2-mercaptoethanol at a 1 to 10 ratio to the 6 X SDS sample buffer **[1]**. Remove samples from the PCR thermal cycler **[2]** and add 2 microliters of the prepared SDS sample buffer to each reaction mix **[3]**.

2.2.1. Talent adding 2-mercaptoethanol to the SDS sample buffer buffer.

2.2.2. Talent taking the samples out of the thermal cycler.

2.2.3. Talent adding the SDS sample buffer to a sample.

Added shot 2.2.3.1, videographer didn't mention what's in the shot.

2.3. Heat the samples to 95 degrees Celsius for 5 minutes on a heat block **[1]**, then cool them on ice. Store the samples at -20 or -80 degrees Celsius or proceed with gel electrophoresis and immunoblotting **[2]**.

2.3.1. Talent putting the samples on the heating block.

2.3.2. Talent taking the samples out and putting them on ice.

3. ChHAI Assay

3.1. Seed 100,000 MCF-7 cells in 1 milliliter of cell culture medium into each well of a 12-well plate and allow the cells to grow to 80 to 90% confluency **[1]**.

3.1.1. Talent adding cells to a few wells.

3.2. When the cells reach the desired confluency, aspirate the cell culture medium from wells 4, 5, and 6 **[1]** and pipette 1 milliliter of 3 micromolar MS-275 in medium into each well **[2]**. Then, aspirate the cell culture medium from wells 1, 2, and 3 **[3]** and pipette 1 milliliter of diluted DMSO into each well **[4]**. *Videographer: This step is important!*

3.2.1. Talent aspirating the medium from wells 4, 5, and 6.

3.2.2. Talent adding MS-275 in medium into a few wells, with the MS-275 container in the shot and labeled.

3.2.3. Talent aspirating the medium from wells 1, 2, and 3.

3.2.4. Talent adding DMSO into a few wells, with the DMSO container in the shot and labeled.

3.3. Return the cells to the incubator for 4 hours to allow for accumulation of acetylated histones in cells exposed to MS-275 **[1]**. While the cells are incubating, prepare dilutions of A-485 in DMSO according to manuscript directions **[2]**. When the incubation is finished, aspirate the medium from the wells and add the dilutions **[3]**. *Videographer: This step is important!*

3.3.1. Talent putting the plate in the incubator and closing the door. *Videographer: Obtain multiple usable takes, this will be reused in 3.4.1.*

3.3.2. Talent putting a tube with a dilution of A-485 into a rack with a few other dilutions, with the tubes labeled. NOTE: 3.3.2 and 3.3.3 combined

3.3.3. Talent adding a dilution to a well.

3.4. Return the cells to the incubator and culture them for 20 hours **[1]**. Then, aspirate the cell culture medium from the wells **[2]** and wash the cells with 1 milliliter of PBS **[3]**. Aspirate the PBS and add 100 microliters of passive lysis buffer **[4]**. Store the plate at −80 degrees Celsius overnight **[5]**.

3.4.1. *Use 3.3.1.*

3.4.2. Talent aspirating the medium from a few wells.

3.4.3. Talent adding PBS to a well and washing the cells.

3.4.4. Talent adding the passive lysis buffer to a few wells.

3.4.5. Talent putting the plate in a freezer and closing the door.

4. Chromatin Immunoprecipitation (ChIP)

4.1. Pipette 100 microliters of sonicated chromatin from cells treated with DMSO into two 1.5-milliliter tubes **[1]**, then pipette 400 microliters of ChIP *(pronounce 'chip')* dilution buffer to each tube to bring the total volume up to 500 microliters **[2]**. Remove 5 microliters of the solution from one of the tubes **[3]** and store it at -20 degrees Celsius as DMSO Input **[4]**. *Videographer: This step is important!*

4.1.1. Talent pipetting the sonicated chromatin from cells treated with DMSO into the 2 tubes.

4.1.2. Talent adding the ChIP dilution buffer to the tubes.

4.1.3. Talent removing 5 microliters from a tube.

4.1.4. Talent storing the 5-microliter aliquots in the freezer.

4.2. Prepare 2 tubes with sonicated chromatin from cells treated with A-485 in the same way **[1]**. Use a pipette to add the IgG and H3K27ac antibodies to the DMSO and A-485 samples **[2-TXT]**, then add 20 microliters of protein A magnetic beads to each tube, making sure that the beads are well resuspended **[3]**. Rotate the samples overnight at 4 degrees Celsius **[4]**. *Videographer: This step is difficult and important!*

4.2.1. Rack of tubes with the prepared sonicated chromatin samples from cells treated with DMSO and A-485.

4.2.2. Talent adding antibodies to the tubes. **TEXT: DMSO chromatin with IgG; DMSO chromatin with H3K27ac; A-485 chromatin with IgG; A-485 chromatin with H3K27ac**

4.2.3. Talent adding beads to one of the IP samples and mixing them.

4.2.4. Samples rotating at 4 degrees Celsius.

4.3. Pellet the protein A magnetic beads using a magnetic separator **[1]** and remove the supernatant without disturbing the beads **[2]**. Wash the beads with 500 microliters to 1 milliliter of low salt wash buffer **[3]** and rotate them for 5 minutes at 4 degrees Celsius **[4]**. *Videographer: This step is difficult and important!*

4.3.1. Talent putting the sample tubes on the magnetic separator.

4.3.2. Talent removing the supernatant.

4.3.3. Talent adding the low salt buffer to the samples, with the buffer container in the shot.

4.3.4. Talent putting the samples to rotate.

4.4. Perform a quick spin down **[1]**, pellet the beads with the magnetic separator, and remove the supernatant **[2]**. Repeat the wash procedure with high salt wash buffer, lithium chloride wash buffer, and TE buffer **[3]**.

4.4.1. Talent spinning down the samples.

4.4.2. Talent removing supernatant from the samples on the magnetic separator.

4.4.3. The three buffer containers, labeled.

4.5. After the wash with TE buffer, remove the input samples from the freezer and put them on ice **[1]**. Thaw an aliquot of Proteinase K **[2]**, then pellet the beads using a magnetic separator and remove the TE buffer from the beads **[3]**.

4.5.1. Talent removing the Input samples from the freezer and placing them on ice. NOTE: This one split into 2 shots

4.5.2. Talent taking the Proteinase K out of the freezer.

4.5.3. Talent putting the sample tubes on the magnetic separator.

4.6. Add 100 microliters of ChIP elution buffer and 1 microliter of Proteinase K to every sample, including the Input samples **[1]**, and incubate them with shaking at 62 degrees Celsius for 2 hours using a thermocycler **[2]**.

4.6.1. Talent adding the elution buffer and Proteinase K to one sample tube.

4.6.2. Talent putting the samples in the thermocycler and programming it to 62 degrees. NOTE: This one split into 2 shots

4.7. After the incubation, heat the samples to 95 degrees Celsius for 10 minutes **[1]**, then cool them to room temperature **[2]**. Pellet the magnetic beads with the magnetic separator and transfer the DNA-containing supernatant to a new 1.5-milliliter tube **[3]**. Purify the DNA using a standard PCR cleanup kit and run qPCR **[4-TXT]**.

4.7.1. Talent programming the thermocycler to heat to 95 degrees.

4.7.2. Talent taking the samples out of the thermocycler.

4.7.3. Talent transferring the supernatant from the samples in the magnetic separator.

4.7.4. Talent at the lab bench with the samples and the PCR cleanup kit. **TEXT: Purified DNA can be stored at -20 ˚C**

Results

5. Results: Inhibitors of Histone Acetyltransferases

5.1. The in vitro histone acetyltransferase assay was used to investigate the effect of anacardic acid on p300 HAT *(pronounce 'hat')* activity towards a histone substrate **[1]**. A concentration range was tested **[2]** and Acetyl-CoA *(pronounce 'acetyl-co-A')* was not added to the negative control reaction **[3]**.

5.1.1. LAB MEDIA: Figure 1 B.

5.1.2. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize lanes 2 – 5.*

5.1.3. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize lane 6.*

5.2. The immunoblot results were quantified with imageJ **[1]**, showing a clear reduction in acetyl H3K18 and acetyl H3K9 at 100 micromolar anacardic acid compared to the DMSO control **[2]**.

5.2.1. LAB MEDIA: Figure 1 C.

5.2.2. LAB MEDIA: Figure 1 C. *Video Editor: Emphasize the 100 µM Anacardic Acid column.*

5.3. In the Chromatin Hyperacetylation Inhibition assay, treatment of MCF-7 cells with HDACi *(pronounce 'H-dac-eye')* MS-275 strongly upregulated acetylation of Histone 3 on several lysine residues **[1]**. The basal levels of acetyl H3K18 and acetyl H3K27 were low, showing the benefits of adding an HDACi in the ChHAI *(pronounce 'chai')* assay **[2]**.

5.3.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize lanes 1 and 4.*

5.3.2. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize lanes 1 – 3.*

5.4. The addition of A-485 in cells pretreated with MS-275 attenuates the increased histone acetylation at H3K18 and H3K27, but not H3K9 **[1]**. The immunoblot results were also quantified with ImageJ **[2]**.

5.4.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize lanes 4 – 6.*

5.4.2. LAB MEDIA: Figure 2 B and C.

5.5. ChIP-qPCR *('chip-Q-P-C-R')* was used to investigate the effects of HAT inhibitors at gene regulatory elements that control oncogene expression **[1]**.

5.5.1. LAB MEDIA: Figure 3 B, C, and D.

5.6. In the DMSO sample, the DNA precipitated by the IgG *('I-G-G')* control antibody produced a higher Ct value than the acetyl H3K27 antibody in the qPCR reaction for the Cyclin D1 promoter, indicating that the non-specific IgG control precipitated less DNA-histone complexes than the acetyl H3K27 specific antibody at the promoter **[1]**.

5.6.1. LAB MEDIA: Figure 3 B.

5.7. Compared with the DMSO control, A-485 reduced acetyl H3K27 enrichment at the Cyclin D1 promoter. Importantly, A-485 is known to significantly reduce acetyl H3K27 in cell culture **[1]**.

5.7.1. LAB MEDIA: Figure 3 C.

Conclusion

6. Conclusion Interview Statements

6.1. **Aaron Waddell:** When attempting this protocol, keep in mind that the pre-incubation steps of the in vitro HAT and ChHai assays are essential and should not be forgotten. It is also important to remember to save the Input samples and to avoid loss of the beads during ChIP.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.2, 3.3.1, 4.3.2.*

6.2. **Aaron Waddell:** After performing these procedures, ChIP-seq is an additional method that can be executed to gain global information about histone acetylation at regulatory elements for the entire genome.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

6.3. **Aaron Waddell:** These protocols are helpful for scientists to carefully validate novel HAT inhibitors and to avoid publishing low quality chemical probes in the literature. The validated HAT inhibitors can undergo further development as potential therapeutics.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This statement is optional. If you don't have time, don't film it.*