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Title: Capturing Common Fragile Site Breaks by Native γH2A.X ChIP

Landing Page Title (not for video use): Efficient Detection of Fragile Site Breaks via Native γH2A.X ChIP Assay

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

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Current Protocol Length
Number of Steps: 25
Number of Shots: 57

Introduction

1.1. **Qian Xie:** Our laboratory focuses on elucidating the role of common fragile site instability in driving tumorigenesis. Try to identify vulnerabilities in oncogene-driven cancer cells that can be exploited therapeutically.

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

What technologies are currently used to advance research in your field?

1.2. **Qian Xie:** Advanced imaging and sequencing technologies, such as single-cell sequencing and live-cell imaging, provide high-resolution mapping of genome instability events. Leveraging in vivo models, including organoids, genetically engineered mouse models (GEMMs), and CRISPR-based systems, allows us to investigate the mechanisms of oncogene-driven genome instability in detail.

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

What research questions will your laboratory focus on in the future?

1.3. **Linling Ke:** The long-term interest of our lab lies in identifying specific weaknesses in cancer cells that can be strategically targeted for effective treatments, with a focus on minimizing harm to normal cells. This includes exploring genetic and epigenetic vulnerabilities to uncover novel therapeutic opportunities.

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

Protocol

2. Subcellular Fractionation and Chromatin Fragmentation Optimization in Native ChIP Assays

Demonstrator: Xiaoman Wang

2.1. To begin, treat the HEK 293T *(H-E-K-two-nine-3-T)* cells with DMSO *(D-M-S-O)*, Aphidicolin *(AY-fid-ih-koh-lin)*, and hydroxyurea in 3 different plates for 24 hours **[1]**.

2.1.1. WIDE: Talent placing the labeled dishes containing treated HEK 293T cells on a working platform.

2.2. After washing the cells twice with PBS, centrifuge them at 500 *g* for 5 minutes at 4 degrees Celsius **[1].** Resuspend the pellet in 500 microliters of freshly prepared cold Buffer A and incubate on ice for 5 to 10 minutes **[2]**. Observe the lysis progression under a light microscope to confirm complete cell lysis **[3]**.

2.2.1. Talent placing the tubes in a centrifuge.

2.2.2. Talent resuspending the cell pellet in cold Buffer A using a pipette and placing the tube on ice.

2.2.3. Talent observing the cell lysis under the microscope with an attached computer screen visible in the frame.

2.3. Once lysed, centrifuge the cell suspension at 500 *g* for 5 minutes at 4 degrees Celsius **[1]** and resuspend the nuclei pellet in 500 microliters of cold Buffer A **[2]**.

2.3.1. Talent placing the tubes in the centrifuge.

2.3.2. Talent resuspending the nuclei pellet in cold Buffer A.

2.4. Centrifuge again **[1]** and resuspend the intact nuclei with 100 microliters of micrococcal nuclease Buffer by pipetting 5 to 10 times **[2]**. Immediately add the pre-determined amount of micrococcal nuclease to the samples **[3-TXT]**.

2.4.1. Talent placing the tubes in the centrifuge.

2.4.2. Nuclei being resuspended in MNase Buffer.

2.4.3. Talent adding MNase to the sample tubes. **TXT: Add 1.25 U MNase/100 µL MNase Buffer**

2.5. Place the tubes on a rotator for 5 minutes at 37 degrees Celsius **[1]**. Immediately return the tubes to ice and add EDTA *(E-D-T-A)* to terminate the digestion **[2]**.

2.5.1. Talent placing the tubes on a rotator.

2.5.2. Talent placing the tubes on ice and adding EDTA.

2.6. After vortexing, add 500 microliters of Buffer B and pipette up and down for mixing **[1]**. Incubate the tube on ice for 5 minutes to solubilize the proteins **[2]**.

2.6.1. Talent adding Buffer B to the samples and mixing by pipetting up and down.

2.6.2. Talent placing the tubes on ice for protein solubilization.

2.7. To pellet the insoluble material, centrifuge the samples at maximum speed for 5 minutes at 4 degrees Celsius **[1]**. Transfer the clear supernatant to new 1.5 milliliter tubes labeled as the native chromatin fraction **[2].**

2.7.1. Talent placing the tubes in the centrifuge.

2.7.2. Talent transferring the clear supernatant to labeled 1.5-milliliter tubes.

2.8. To verify chromatin fragmentation, aliquot 10 microliters of the supernatant into a new 1.5-milliliter tube **[1]**. Mix the supernatant with 20 microliters of distilled water and 30 microliters of phenol-chloroform-isoamyl alcohol **[2-TXT]**.

2.8.1. Talent pipetting 10 microliters of supernatant into a new 1.5-milliliter tube.

2.8.2. Talent transferring distilled water and phenol-chloroform-isoamyl alcohol mixture from the labeled container into the supernatant-containing tube. **TXT: Phenol:Chloroform:Isoamyl alcohol:25:24:1** *Video Editor: This is a ratio*

2.9. After vigorous vortexing, centrifuge the tubes at 20,000 *g* for 10 minutes at 4 degrees Celsius **[1]** and observe three distinct layers: a clear top layer, a white middle layer, and a yellow bottom layer **[2]**. Carefully transfer 20 microliters of the upper aqueous phase containing DNA *(D-N-A)* to a fresh tube **[3]**.

2.9.1. Talent placing the tubes in the centrifuge.

2.9.2. Talent showing 3 separated layers from the tube.

2.9.3. Talent transferring the upper aqueous phase to a new tube.

2.10. Separate the purified DNA in a 1.5% agarose gel for 30 minutes at 100 volts **[1]**. Visualize the digestion patterns, ensuring the size of chromatin fragments is primarily between 200 and 1,000 base pairs **[2]**.

2.10.1. Talent loading purified DNA sample on an agarose gel.

2.10.2. Talent observing the gel bands for digestion patterns on a computer.

3. Immunoprecipitation and Validation of γH2A.X Enrichment for Assessing Replication Stress in ChIP Assays

Demonstrator: Min Li

3.1. Transfer 20 microliters of digested chromatin into each tube containing 180 microliters of Elution Buffer **[1]**. Label the tubes as Input samples and store them at minus 20 degrees Celsius **[2]**.

3.1.1. WIDE: Talent pipetting 20 microliters of digested chromatin into a 1.5 mL tube.

3.1.2. Talent placing the labeled Input sample tubes in a freezer.

3.2. Add 400 microliters of digested chromatin into another 1.5-milliliter tube for chromatin immunoprecipitation or ChIP *(chip)* **[1]**. Transfer γH2A.X *(gamma H-two-A-X)* antibody to DMSO *(D-M-S-O)*-treated, Aphidicolin *(AY-fid-ih-koh-lin)*, -treated, and hydroxyurea-treated sample **[2-TXT]**. Place the tubes on a rotator at 4 degrees Celsius for 5 hours, or preferably overnight **[3]**.

3.2.1. Talent transferring 400 microliters of chromatin into a new tube labeled for ChIP.

3.2.2. Talent pipetting γH2A.X antibody into the designated ChIP sample tubes. **TXT: Prepare negative control with normal IgG and DMSO-treated sample**

3.2.3. Talent placing ChIP tubes on the rotator at 4 degrees Celsius.

3.3. Meanwhile, using wide-orifice tips, aliquot 100 microliters of ChIP-grade magnetic Protein A/G *(A-G)* beads into a new 1.5-milliliter tube **[1]**. Place the tube on a magnetic stand for 1 minute **[2]**. Then, carefully discard the liquid **[3]**.

3.3.1. Talent pipetting magnetic beads into a new tube.

3.3.2. Talent placing the tube on a magnetic stand.

3.3.3. Talent removing supernatant from the tube.

3.4. Next, resuspend the beads in 1 milliliter of PBS containing 0.5% BSA *(B-S-A)* **[1]**. Rotate the tube at 4 degrees Celsius for 4 hours **[2]**. Place the tube back on the magnetic stand for 1 minute and discard the supernatant **[3-TXT]**.

3.4.1. Talent pipetting 1 milliliter of PBS with 0.5% BSA into the tube with beads.

3.4.2. Talent placing the tube on a rotator.

3.4.3. Shot of the tube on a magnetic stand with the beads pelleted and the supernatant being discarded. **TXT: Repeat washing 1x**

3.5. After the second wash, resuspend the pre-coated beads in 100 microliters of Buffer B **[1]**.

3.5.1. Talent pipetting 100 microliters of Buffer B to resuspend the beads.

3.6. Add 25 microliters of the pre-coated magnetic bead suspension to each ChIP sample tube **[1]**. Rotate the tubes at 4 degrees Celsius for 2 hours **[2]**. Then, place the ChIP tubes on a magnetic stand and wait until the beads attach to the side of the tube and the solution becomes clear **[3]**.

3.6.1. Talent adding 25 microliters of bead suspension to each ChIP sample tube.

3.6.2. Talent placing the tubes on a rotator.

3.6.3. Shot of the tube on a magnetic stand and beads attached to the side of the tube.

3.7. Discard the clear supernatant without disturbing the magnetic beads **[1]**. Resuspend the beads in 1 milliliter of Wash Buffer **[2]** and rotate at 4 degrees Celsius for 10 minutes **[3]**. Place the tubes back on the magnetic stand and wait until the solution becomes clear before discarding the wash buffer **[4-TXT]**.

3.7.1. Talent carefully discarding the supernatant from the magnetic tubes.

3.7.2. Talent adding 1 milliliter of Wash Buffer to resuspend the beads.

3.7.3. Talent transferring the tubes on a rotator.

3.7.4. Tube on the magnetic stand with the solution clear and wash buffer is discarded. **TXT: Repeat washing 4X**

3.8. After the final wash, briefly centrifuge the tubes at 400 *g* for 30 seconds at 4 degrees Celsius to remove residual liquid **[1]**. Place the tubes back on the magnetic stand and carefully remove any remaining liquid from the bottom of the tube **[2]**.

3.8.1. Talent placing the tubes in the centrifuge.

3.8.2. Tube on the magnetic stand showing residual liquid being carefully removed.

3.9. After verifying the ChIP antibody pull-down efficiency using Western blot, add 50 microliters of Elution Buffer to each of the remaining ChIP samples **[1]**. Place the tubes on a thermomixer and shake for 15 minutes at room temperature **[2]**.

3.9.1. Talent pipetting 50 microliters of Elution Buffer into ChIP sample tubes.

3.9.2. Talent transferring the tubes on a thermomixer.

3.10. Place the tubes on a magnetic holder for 1 minute and collect the elute into new tubes **[1-TXT]**. Add 100 microliters of Elution Buffer to each ChIP elution sample and 180 microliters of Elution Buffer to each Input sample **[2]**.

3.10.1. Talent placing tubes on the magnetic holder and transferring the elute into new tubes using a pipette. **TXT: Repeat elution 1x**

3.10.2. Talent pipetting Elution Buffer into ChIP and Input samples.

3.11. Then, add 200 microliters of phenol-chloroform-isoamyl alcohol to each sample **[1]** and vortex the tubes vigorously to mix **[2]**.

3.11.1. Talent pipetting phenol-chloroform-isoamyl alcohol mixture into the tubes.

3.11.2. Talent vortexing the tubes.

3.12. After centrifuging the samples, carefully transfer the upper aqueous layer to the tubes containing 19 microliters of 3 molar sodium acetate and 2 microliters of glycogen solution. Mix by vortexing the tubes **[1-TXT]**. NOTE: The VO is edited for the deleted shot ans the information is added as an on screen text

~~3.12.1. Talent placing the tubes in a centrifuge.~~ NOTE: Not filmed

3.12.2. Talent transferring the aqueous layer into the tubes containing sodium acetate and glycogen solution. **TXT: Centrifugation: 20,000g, 4 ºC, 10 min**

3.13. Add 500 microliters of 100% ethanol to each sample and vortex thoroughly **[1]**. Place the tubes at minus 20 degrees Celsius for 2 hours or overnight to precipitate the DNA **[2]**.

3.13.1. WIDE: Talent adding ethanol to the samples.

3.13.2. Talent placing the tubes in a freezer.

3.14. Then, centrifuge the tubes before discarding the supernatant and resuspending the pellet in 1 milliliter of 70% ethanol **[1-TXT]**. NOTE: The VO is edited for the deleted shot

3.14.1. Talent placing the tubes in the centrifuge

~~3.14.2. Talent removing supernatant from the tube.~~ NOTE: Not filmed

~~3.14.3. Talent adding 70% ethanol and vortexing the tubes. **TXT: Repeat centrifugation to remove ethanol**~~

3.15. Once residual ethanol is removed by centrifugation, air-dry the DNA pellets for 2 to 3 minutes before resuspending them in 400 µL of TE *(T-E)* Buffer **[1-TXT]**.

3.15.1. Talent resuspending the ChIP in TE Buffer. **TXT: Resuspend the input DNA in 1 mL of TE buffer**

Results

4. Results

4.1. Higher concentrations of micrococcal nuclease led to more extensive digestion of chromatin, resulting in a predominance of mono-nucleosome fragments **[1]**. In contrast, at lower micrococcal nuclease concentrations, the majority of chromatin fragments were larger, often exceeding 1 kilobase **[2]**.

4.1.1. LAB MEDIA: Figure 2 *Video Editor: Please emphasize lanes 1, 2, and 3*

4.1.2. LAB MEDIA: Figure 2 *Video Editor: Please emphasize lanes 6, 7, and 8*

4.2. γH2A.X levels were significantly elevated in replication-stressed cells treated with aphidicolin **[1]** compared to DMSO-treated controls **[2]**, as shown in the input samples for both native ChIP and crosslinked ChIP **[3]**.

4.2.1. LAB MEDIA: Figure 4A *Video editor: Highlight the γH2A.X bands from APH lanes of Input (N-ChIP) and Input (X-ChIP)*

4.2.2. LAB MEDIA: Figure 4A *Video editor: Highlight the γH2A.X bands from DMSO lanes of Input (N-ChIP) and Input (X-ChIP)*

4.2.3. LAB MEDIA: Figure 4B *Video editor: Highlight the γH2A.X bands (top and bottom) from APH lanes*

1. HEK 293T

Pronunciation link:
No confirmed pronunciation link found in Merriam-Webster, OED, or HowToPronounce.com specifically for "HEK 293T"—though general resources explain the name and usage (Wikipedia).

IPA: /eɪtʃ-i-keɪ tuː-naɪn-θri tiː/
Phonetic Spelling: H-E-K two-nine-three-T

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2. DMSO

Pronunciation link:
No confirmed link found (acronym; common lab shorthand).
IPA: /ˌdiː ɛm ɛs ˈoʊ/
Phonetic Spelling: DEE-EM-ESS-OH

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3. Aphidicolin

Pronunciation link:
Collins Dictionary provides pronunciation details (Wiktionary).
IPA: /ˌæfɪdɪˈkoʊlɪn/
Phonetic Spelling: af-i-DI-koh-lin

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4. Hydroxyurea

Pronunciation link:
No confirmed link found.
IPA: /haɪˌdrɒksiˈjʊəriə/
Phonetic Spelling: hy-DROK-see-YOO-ree-uh

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5. MNase (micrococcal nuclease)

Pronunciation link:
No confirmed link found for the abbreviation.
IPA: /ɛm-ɛn-eɪz/
Phonetic Spelling: M-N-A-Z

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6. EDTA

Pronunciation link:
No confirmed link found for the acronym.
IPA: /ˌiː di-ti ˈeɪ/
Phonetic Spelling: EE-DEE-TEE-A

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7. γH2A.X (*gamma H-two-A-X*)

Pronunciation link:
No confirmed link found; represents the phosphorylated histone variant.
IPA: /ˈgæmə eɪtʃ tuː eɪ ɛks/
Phonetic Spelling: GAM-uh H-two-A-X