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A General Non-Radioactive ATPase Assay for Chromatin Remodeling Complexes

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Abstract

Chromatin remodeling complexes couple the energy released from ATP hydrolysis to facilitate transcription, recombination and repair mechanisms essential for a wide variety of biologic responses. While recombinant expression of the regulatory subunits of these enzymes is possible, measuring catalytic (ATPase) activity of the intact complexes recovered from normal or mutant cells is critical for understanding their mechanisms. SWI/SNF-like remodeling complexes can be megadaltons in size and include many regulatory subunits, making reconstitution of purified subunits challenging for recapitulating *in vivo* function. The protocol in this article defines the first highly quantitative ATPase assay for intact remodeling complexes that does not require radiation or reconstitution of recombinantly expressed subunits. This protocol is specifically useful for defining the catalytic role of active-site mutations in the context of other regulatory subunits and quantitatively rank-ordering inactivating catalytic-site mutations.

Keywords

Chromatin remodeling; epigenetic regulators; ATPase activity; luciferase; catalysis

INTRODUCTION

The need for a quantitative highly reproducible ATPase assay for intact chromatin remodeling complexes

A radioactive method for measuring ATPase activity of chromatin remodeling complexes has been previously reported by Bultman and co-workers (Bultman et al., 2005). The authors characterized a Brg hypomorph allele from a mutagenesis screen, and co-immunoprecipitated the BAF complex to run an on-bead assay measuring the conversion of gamma-labeled ³²P-ATP to inorganic ³²P_i and ADP. This method was modified from previous methodologies for catalysis assays for epigenetic regulatory enzymes (Cote et al.,

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Conflicts of Interest

The authors declare they have no competing financial interests relating to the research herein.

1994; Laurent et al., 1993; Zhao et al., 1998). It has been used widely (Dykhuizen et al., 2013) since, and is an effective method for determining relative hydrolytic capacities of remodelers. Unfortunately, this method is prone to certain limitations for running many different conditions in parallel which is difficult for sensitive radiometric experiments, day-to-day variability related to the batch of gamma-labeled ^{32}P -ATP, and limited by the sensitivity of quantification related to determining saturation. While we acknowledge the utility of previously reported methodologies in measuring hydrolytic rate of chromatin remodelers, we have developed a non-radioactive luciferase-coupled ATPase assay for remodeling enzymes that addresses certain limitations.

Initial efforts were inspired by readily available luminescence-based assays for purified kinase enzymes. Many assays are available for monitoring activities of kinases and specifically provide platforms for asking questions about the initial step in kinase activation, liberation of inorganic phosphate from ATP. Chromatin-remodeling enzymes can be studied in vitro by capturing the same information about relative rates of production of inorganic phosphate, but because of experimental limitations on studying purified intact chromatin remodeling enzymes (see above), it has been difficult. We therefore developed and optimized a general method for coupling purification of intact chromatin-remodeling complexes with luciferase-based measurements of production of ADP from ATP.

This method is useful for SWI/SNF-like remodelers that convert energy from ATP-hydrolysis into various cellular activities including altering the chromatin-DNA interface through several potential pathways (Figure 1). The enzymes intended for use in our assay are assembled into large complexes that cannot be studied in vitro through recombinant expression of certain subunits, but rather must be measured in the context of intact complexes. For example, the mammalian SWI/SNF-like BAF remodeling complex is approximately ~2 MDa, and it is difficult to study the cloned and bacterially expressed fragments of the ATPase domain alone. Therefore, remodeling complexes can be purified by co-immunoprecipitation and analyzed for hydrolytic activity on beads. Our method couples the purification of intact remodeling complexes with immunoprecipitations with a luciferase-based quantitative assay for making many measurements in parallel. This method is well suited for study of complexes containing mutant subunits recovered from human tissues or cell lines and can also be used to measure the effects of protein or small molecule modulators.

STRATEGIC PLANNING

Adapting our ATPase assay method should not require extensive implementation for most labs equipped for standard biochemistry. A cold room for overnight incubation of immunoprecipitations is necessary, as are magnetic racks for washing Dynabeads and purifying chromatin remodeling complexes. A luminescence plate reader should be available, but our protocol has luminescence levels that are stable for over an hour at room temperature, so a plate reader is not necessarily required in the same lab space as long as it is accessible nearby. Most investigators with a basic knowledge of biochemistry should be able to perform these experiments. The main limitations of our protocol are the cost of reagents,

availability of specific antibodies for immunoprecipitations and background levels of luminescence.

Basic Protocol 1. MEASURING ENZYMATIC ACTIVITY OF INTACT ATP-DEPENDENT CHROMATIN REMODELING COMPLEXES

This method is especially useful for making standardized measurements for related mutations of remodeling enzymes that can be efficiently immunoprecipitated with intact complexes and tested side-by-side. Because of limitations in making large numbers of side-by-side measurements with radioactivity-based ATPase assays, this method is preferable. Traditionally, TLC-plate based measurements of radioactive ^{32}P require overnight incubation with phosphoimager screen. While x-ray film based measurements of ^{32}P can also be used for traditional ATPase assays of chromatin remodelers, the data are frequently out of the linear range of detection, and it is difficult to determine appropriate linear range of detection, making precise quantification problematic. Our method involves same-day immunoprecipitation and detection, allowing for more streamlined experimental design.

Materials

REAGENTS

1. 293T cells
2. DNA encoding desired chromatin remodeler (e.g., Brg [Mut] V5)
3. PEI (*Polysciences* Cat # 24765-2)
4. Dynabeads Protein A (*Life Technologies*, Cat#10001D)
5. Brg (G7) Antibody (*Santa Cruz Biotechnology*, Cat.# sc-17796)
6. Opti-MEM (ThermoFisher, Cat# 51985091)
7. DMEM 1X (*Life Technologies*, Cat# 11960-077)
8. Fetal Bovine Serum (*Omega Scientific*, Cat# FB-01)
9. ADP-Glo MAX Assay (*Promega* Cat# V7001)
10. Ultra-Pure Glycerol (*Thermo Fisher* Cat# 15514-011)
11. Opaque-bottom non-treated round bottom 96-well plates (*Corning* Cat# CLS3789-100EA)
12. Chymostatin, protease inhibitor (EMD Millipore, Cat# 230790)
13. Leupeptin, protease inhibitor (EMD Millipore, Cat# 108975)
14. Pepstatin, protease inhibitor (EMD Millipore, Cat# 516481)
15. ssDNA (for use in 1X ATPase Reaction Buffer):
CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
AGATGT (IDT, www.idtdna.com)
16. 10N Sodium Hydroxide solution (VWR, Cat# VW3247-1)

EQUIPMENT

1. EnVision Plate Reader (*Perkin Elmer* Cat# 2104-0010)
2. Eppendorf Thermomixer (*Fisher Scientific* Cat# 05-400-201)

STEPS AND ANNOTATIONS

1. Plate 293T cells in a 10 cm tissue-culture dish (6 million cells, the evening before PEI transfection).
2. Prepare 12 µg DNA encoding desired chromatin remodeler (Brg [Mut] V5) and mix with 2 mL Opti-MEM. Mix well and incubate at room temperature for 2 minutes (Figure 2A).
3. Add 45 µL 1mg/mL buffered PEI (pH 7.0) to the DNA/Opti-MEM mixture and mix well. Incubate the DNA/PEI/Opti-MEM mixture at room temperature for 10 minutes.
4. Change the media on the 293T cells during the 10 minute incubation of DNA/PEI/Opti-MEM. Add the DNA/PEI/Opti-MEM mixture dropwise to the 293Ts after changing the media, swirling the dish to equilibrate the transfection solution.
5. Change media on the transfected 293T cells 24h after transfection (Figure 2A).
6. Wait 48h after media change, then trypsin digest and dissociate and prepare nuclear extracts by incubation with 10 mL *Buffer A* on ice 7 min.
7. After centrifugation at $500 \times g$ for 5 minutes at 4° C, repeat *Buffer A* incubation on ice for 3 min and centrifuge again.
8. For each 50 million cell equivalent, incubate with 200–300 µL *Lysis Buffer* and incubate at 4° C with end-over-end rotation for 30 min.
9. Spin microfuge tubes at $16,000 \times g$ for 10 minutes at 4° C. Collect supernatant and use for subsequent immunoprecipitations.
10. Perform immunoprecipitations of Brg with 18 µL G7 antibody per 50 million cell equivalent. Incubate at 4° C with end-over-end rotation overnight.
11. Immediately before use, resuspend a slurry of protein A Dynabeads. Prepare 33 µL for each immunoprecipitation, and wash twice with *Lysis Buffer* (including 1 mM DTT and 10 µg/mL PI). For each immunoprecipitation add 30 µL protein A Dynabeads suspended in *Lysis Buffer* to immobilize complexes (Figure 2B) by incubating at 4° C for 4 hours with end-over-end rotation.
12. Wash beads twice with 1 mL *Wash Buffer* and buffer exchange into 25 µL reactions in 1X *ATPase Assay Reaction Buffer*. Incubate at 37°C for 1 hour.
13. Immobilize beads by magnet and use the 25 µL supernatant directly in the ATP-coupled luciferase reaction by adding 25 µL ADP-Glo reagent. Incubate at room temperature for 1 h with gentle agitation.

14. Add 50 μ L *ADP-Glo Detection Substrate* and incubate at room temperature for 1 hour. Measure luciferase activity with EnVision Plate Reader using 1 second integration time (Figure 2C). 1X ATPase Assay Reaction Buffer should be used as a negative control for subsequent normalization of luciferase activity.

REAGENTS AND SOLUTIONS

1. PEI, 1 mg/ml
 - a. Resuspend in distilled water at a concentration of 2 mg/mL and adjust with 10N Sodium Hydroxide solution to pH 7.0. As the pH approaches 7.0, a diluted 1N Sodium Hydroxide solution may be optimal for accuracy.
 - b. Dilute the pH 7.0 solution with distilled water to 1 mg/mL PEI.
 - c. Filter the PEI solution using 0.2 μ m pore size. The PEI can be stored at -20°C for up to two years, and stored at 4°C for two weeks.
2. Protease Inhibitors (PI)
 - a. Using a balance, measure 100 mg Leupeptin, 100 mg Chymostatin, and 100 mg Pepstatin.
 - b. Mix the Leupeptin, Chymostatin, and Pepstatin together and dissolve in 10 mL DMSO to make a 10 mg/mL (1000X) stock.
 - c. Aliquot and freeze at -20°C for subsequent use. The protease inhibitor aliquots can be stored for up to two years at -20°C .
3. Buffer A for harvest of nuclei
 - a. Prepare Buffer A for nuclear isolation (25 mM HEPES pH 8.0, 5 mM MgCl_2 , 25 mM KCl, 0.05 mM EDTA pH 8.0, 10% Glycerol, 0.1% NP-40).
 - b. Sterile Filter Buffer A through 0.2 μ m pore filter apparatus.
 - c. Store Buffer A at 4°C , add 1 mM DTT and 10 μ g/mL PI immediately before use. Buffer A can be stored without DTT or PI at 4°C for up to one year.
4. Lysis Buffer for immunoprecipitation
 - a. Prepare Low-Salt Lysis Buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP-40).
 - b. Sterile Filter the Lysis Buffer through 0.2 μ m pore filter apparatus.
 - c. Store Lysis Buffer at 4°C , add 1 mM DTT and 10 μ g/mL PI immediately before use.
5. ATPase Assay Wash Buffer
 - a. Prepare Wash Buffer (10 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl_2).

- a.** Prepare 2X Reaction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 40% Glycerol).
- b.** Do not sterile filter this solution as it will not easily pass through a 0.2 µm membrane.
- c.** Store 2X ATPase Reaction Buffer at 4 °C for up to one year.

- a. It is important to never let Dynabeads dry during wash steps.
- b. Avoid centrifugation of Dynabeads.

- a.** Thaw ADP-Glo Reagent at room temperature and aliquot (store 500 μ L aliquots at -20°C for up to two years).
- b.** Reconstitute ADP-Glo Detection Substrate and aliquot (store 250 μ L aliquots at -20°C for up to two years).

during the transfection step should be performed such that the DNA/Opti-MEM is mixed by pipetting half the mixture volume (1 mL) at least five times before the introduction of PEI. After PEI is added to the DNA/Opti-MEM mixture, mix again by pipetting 1 mL five times. It is critical that the DNA/Opti-MEM/PEI mixture is incubated at room temperature for 10 minutes before addition to the 293T cells. Toxicity may be observed after transfection if the DNA/Opti-MEM/PEI mixture is allowed to incubate for longer periods of time. If the transfection mixture is preincubated for different periods of time across conditions, variability may result from inconsistent expression levels. Additionally, the 293T media should be changed within 24 hours after transfection or toxicity may result. PEI should not be left at room temperature after use in transfection experiments and should be stored at 4 °C for up to two weeks or at –20 °C for long-term storage.

Immunoprecipitations with Dynabeads—To ensure consistent results from independent immunoprecipitation experiments it is critical that Dynabeads are not allowed to dry. Thus, it is important that if independent immunoprecipitation reactions are washed in parallel, that the immobilizations on the magnetic rack are carried out in small batches of no more than six samples. If more than six immunoprecipitations are washed in parallel, drying may result during the aspiration steps while the beads are immobilized. If drying results during wash steps, this may reduce signal in the luciferase assay and cause variability or false negatives. When measuring catalytic activity it is critical that the association between Dynabeads and immunopurified chromatin-remodeling complexes is not perturbed by centrifugation or drying of the beads during any step of the protocol.

Assay Buffer Preparation & Reagent Preparation—Immediately before starting the ATPase reactions, it is critical to adjust the 2X Reaction Buffer composition to include BSA, ATP, DTT, PI, and ssDNA for a final *1X Reaction Buffer composition* (10 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 20 % Glycerol, 1 mg/mL BSA, 4 mM ATP, 0.5 µg/µL ssDNA, 1 mM DTT, and PI). For long-term storage, the 2X Reaction Buffer should not include BSA, ATP, DTT, PI or ssDNA but rather should be adjusted to the 1X composition immediately before use. Other buffers used in immunoprecipitation reactions should be sterile-filtered for long-term storage at 4 °C. There may be some precipitate in the ATPase assay Detection Substrate even after equilibration to room temperature before use. It is critical to mix the Detection Substrate very well before use in the ATPase assay. After addition of the Detection Substrate to the individual assay reactions in the 96-well plate, it is critical to cover and spin the plate for 30 seconds at 300 × g to remove buffer on well edges.

Troubleshooting

STEP4—*Problem:* Observation of significant cell death

Possible Reason: PEI incubation was over 10 minutes

Solution: Repeat transfections with shorter PEI:DNA:OptiMEM incubation (can optimize with GFP-positive control plasmid)

STEP 14—*Problem:* Low signal after Detection Substrate added

Possible Reason: insufficient mixing of ADP-Glo substrate in Step 13

Solution: Cover, and briefly vortex assay plate after completing Step 13, then spin 30 seconds at $300 \times g$.

STEP 14—Problem: Low signal after Detection Substrate added

Possible Reason: did not thaw Detection Substrate sufficiently

Solution: Thaw ADP-Glo and Detection Substrate at room temperature for at least 30 min before Step 13.

STEP 14—Problem: High variability between replicates

Possible Reason: Batch-variability in antibody used in Step 10.

Solution: Always use the same batch number and lot number for co-immunoprecipitations so luminescence values in in Step 13 can be directly compared across experiments.

STEP 14—Problem: High variability between replicates

Possible Reason: Different DNA used in 1X ATPase Assay Reaction Buffer

Solution: Use a defined ssDNA PCR ~50-mer primer for each set of experiments. ATP is a substrate of chromatin remodelers, but DNA is also a substrate and can affect enzyme activity.

STEP 14—Problem: High variability between replicates

Possible Reason: Different promoters used to drive expression of chromatin remodeler in Step 2.

Solution: When comparisons in catalytic activity are desired, across a certain class of mutations in a defined chromatin remodeler, always compare experimental conditions when remodeler ATPase are expressed from the same vector backbone.

Anticipated Results

Results from the remodeler ATPase assay will be quantitative when in the linear range (Figure 3A,B). In our testing, statistical tests are generally independent of whether data are expressed in raw luminescence activity (Figure 3C,E) or instead normalized to buffer (Figure 3D,F). The sensitivity in measurements afforded by this assay allowed us to make the observation that many mutations tiled across the Brg ATPase domain were associated with catalytic defects that clustered with highly conserved motifs. The on-bead ATPase assay provides enough sensitivity to measure the hydrolytic capacities for weak enzymes, or enzymes that may have catalytic dysfunction as a result of mutations, and also provides a platform for comparison of point mutants in chromatin-remodeling ATPases. The assay provides a rigorous quantitative method to compare active-site mutants of the Brg1 remodeler ATPase enzyme, which often appear in human cancers. Because the transfected

cells will express the protein complexes at high levels, background ATPase activity will be negligible, as can be evidenced by comparing ATPase activity of many of the Brg catalytic mutants to wild type and buffer control.

Time Considerations

Step 1, 24h

Step 2, 1h;

Step 3, 24h;

Step 4+5, 10 min;

Step 6, 48h;

Step 7, 3 min;

Step 8, 40 min;

Step 10, 16h;

Step 11, 60min;

Step 12+13, 2h.

Total: 4.5 days from plating cells for transfection to quantification of luciferase signal.

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Figure 1. The BAF complex hydrolyzes ATP and alters chromatin structure

Recent studies have revealed that SWI/SNF complexes are capable of altering chromatin accessibility, nucleosome positioning, and Polycomb activity. It is presently unknown whether these chromatin-remodeling activities rely strongly on the ATPase activity of the complexes or whether catalytic mutations may alter the ability of SWI/SNF complexes to regulate chromatin structure with unique downstream effects.

Figure 2. Experimental design and workflow for measuring ATPase activity

(A) Cells for use in the ATPase assay protocol are plated and prepared for expression of unique alterations of remodeling enzymes, which will be incorporated into intact ATP-dependent chromatin remodeling complexes for subsequent isolation. (B) The soluble fraction is isolated from harvested nuclei for immunoprecipitation reactions. (C) The isolated chromatin remodeling complexes are found to retain catalytic activity while bound to the beads used in the immunoprecipitations. This allows catalytic activities to be measured for native, intact remodeling complexes that remain bound to other subunits. (D) The luciferase assay is performed in small volumes in 96-well opaque-bottom plates for sensitive quantitation of catalytic activity.

Figure 3. Quantification of ATPase assay results and normalization

(A) The linear range was determined for a range of ADP concentrations, designed to simulate complete efficiency of enzymatic hydrolysis of ATP in order to define limiting conditions for the linear range at high and low (B) substrate concentrations. (C) BrgWT-expressing cells were harvested, and nuclei were extracted in order to immunopurify Brg-containing SWI/SNF complexes. It was found that concentrations 200 μ M ATP and 2 mM ATP produced on-bead hydrolysis levels that could be readily differentiated by the assay conditions. Raw luciferase values (obtained from 1-second exposures per well) directly from the luminometer and (D) buffer-normalized values are shown. Raw (E) and buffer-normalized (F) ATPase assay data were obtained for Brg catalytic-site mutations. The highly quantitative ATPase assay allows for direct comparison and differentiation of unique point mutations based on either raw or normalized luciferase-coupled ATPase activity.