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MARCC (Matrix-Assisted Readers Chromatin Capture): an antibody-free method to enrich and analyze combinatorial nucleosome modifications

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Abstract

Combinatorial patterns of histone modifications are key indicators of different chromatin states. Most of the current approaches rely on the usage of antibodies to analyze combinatorial histone modifications. Here we detail an antibody-free method named MARCC (Matrix-Assisted Readers Chromatin Capture) to enrich combinatorial histone modifications. The combinatorial patterns are enriched on native nucleosomes extracted from cultured mammalian cells and prepared by micrococcal nuclease digestion. Such enrichment is achieved by recombinant chromatin-interacting protein modules, or so-called reader domains, which can bind in a combinatorial modification-dependent manner. The enriched chromatin can be quantified by western blotting or mass spectrometry for the co-existence of histone modifications, while the associated DNA content can be analyzed by qPCR or next-generation sequencing. Altogether, MARCC provides a reproducible, efficient and customizable solution to enrich and analyze combinatorial histone modifications.

Keywords

Affinity enrichment; Antibody-free; Chromatin; Histone; PTM; Reader domain; MARCC

INTRODUCTION

Histone modifications are key regulators in chromatin-based processes (Strahl and Allis, 2000). Crosstalk among different histone modifications generates a dynamic and complex histone language (Phanstiel et al., 2008; Young et al., 2009). These combinatorial histone modification patterns can be used to define chromatin states computationally (Ernst and Kellis, 2010; Ernst et al., 2011; Kharchenko et al., 2011; Mikkelsen et al., 2007; Zhu et al.,

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INTERNET RESOURCES

PlasmID (<http://plasmid.med.harvard.edu/PLASMID/faces/Home.jsp>).

NEB Tm calculator (<https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator>).

A detailed protocol about nucleosome extraction from Ruthenburg Lab at University of Chicago (http://www.ruthenlab.org/web/Protocols_files/MNase%20mononucleosome%20preparation.pdf).

2013). The physical enrichment of combinatorial histone modifications on a nucleosome basis allows quantitative analysis of coexisting modifications and genomic mapping of a particular combination. Traditionally this kind of enrichment employs chromatin immunoprecipitation by histone antibodies, which are not always reliable and available (Bock et al., 2011; Egelhofer et al., 2011; Heubach et al., 2013; Nishikori et al., 2012). Great promises have come from engineering-friendly recombinant alternatives of histone antibodies (Hattori et al., 2013). Recently the authors have developed an antibody-free approach (Matrix-Assisted Reader Chromatin Capture or MARCC) for PTM-specific chromatin enrichment. MARCC uses recombinant interaction-modules (or reader domains) occurring in natural proteins that target combinatorial modifications (Su et al., 2014). This ChIP-less approach has been applied to enrich different chromatin states with three reader domains and successfully utilized for downstream analysis including qPCR, western blotting and mass spectrometry (Su et al., 2014).

Figure 1 summarizes the overall procedures of MARCC. First, the reader domain of interest is cloned into HaloTag expression vector (Steps 1–13) and expressed recombinantly in *E.coli*. HaloTag is a modified haloalkane dehalogenase developed as a versatile fusion tag, which features the covalent bond formation with its substrate (Gallo et al., 2011; Hata and Nakayama, 2007; Kovalenko et al., 2011; Locatelli-Hoops et al., 2013; Los et al., 2008; Martincova et al., 2012). Efficient immobilization of reader domains is achieved by this covalent linkage of HaloTagged protein with the solid support while enabling extensive washes to reduce non-specific bindings. An additional His-tag is introduced to purify the reader domain by nickel resin (Steps 14–36). At the same time, native chromatin can be isolated from cultured mammalian cells and digested by micrococcal nuclease (MNase) to desired resolution (Steps 47–60). The digested chromatin is then enriched by resin-bound reader domains. The enriched chromatin can be released by TEV protease cleavage (Steps 37–43). This mild elution ensures the intact nucleosome structure and maintains post-translational modifications for downstream analysis.

The Basic Protocol details the procedures from cloning, expression and batch purification of the reader domain to MARCC (Matrix-Assisted Reader Chromatin Capture). The Support Protocol includes standard procedures for chromatin extraction and micrococcal nuclease digestion to generate a native nucleosome pool from mammalian cell cultures.

BASIC PROTOCOL

MATRIX-ASSISTED READER CHROMATIN CAPTURE (MARCC)

This protocol starts with cloning the reader domain of interest into His-HaloTag bacterial expression system (Step 1–13) and purification of the reader domain by HisTag (Step 14–36). Native chromatin purified from cell cultures is then incubated with reader domain covalently immobilized on resin via HaloTag. Enriched chromatin with combinatorial histone modifications can be analyzed after TEV protease elution (Step 37–46). Downstream analysis includes but is not limited to western blotting, mass spectrometry, qPCR and next-generation sequencing. It is recommended to use reader domains that have shown relatively strong interactions ($K_d = 1\sim 10\ \mu\text{M}$) to their specific histone target peptide, but weaker binding or unknown reader domains could also be adapted.

Materials

Reagents and solutions: 2X YT liquid medium containing 50 µg/ml kanamycin

LB agar plate containing 50 µg/ml kanamycin

DNA polymerase (Q5 Hot Start High-Fidelity 2X Master Mix, New England Biolabs, cat no. M0494)

Nuclease-free Water (UltraPure DNase/RNase-Free Distilled Water, Life Technologies, cat no. 10977-015)

50X TAE Buffer

DNA gel stain (SYBR Safe DNA Gel Stain, Life Technologies, cat no. S33102)

Gel purification kit (GeneJet Gel Extraction Kit, Thermo Scientific, cat no. K0692)

Restriction enzymes (10X Flexi Enzyme Blend, Promega, cat no. R1851) containing:

5X Flexi Digestion Buffer

Flexi Enzyme Blend (SgfI and PmeI)

Carboxy Flexi Enzyme Blend (PmeI and EcoICRI)

Ligation-independent cloning kit (In-Fusion HD Cloning System, Clontech, cat no. 649646)

Plasmid miniprep kit (GeneJet Plasmid Miniprep Kit, Thermo Scientific, cat no. K0503)

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Zinc sulfate (2.0 M Zinc sulfate solution in water, Sigma-aldrich, cat no. 83265)

Nickel resin (Ni Sepharose 6 Fast Flow, GE Life Sciences, cat no. 17-5318)

Resuspension Buffer (see recipe)

Lysozyme from chicken egg white (Sigma-Aldrich, cat no. L6876)

HBS (see recipe)

Wash Buffer (see recipe)

Elution Buffer (see recipe)

Dialysis tubing (SnakeSkin Dialysis Tubing, 10K MWCO, Thermo Scientific, cat no. PI-68100)

Dialysis Buffer 1 (see recipe)

Dialysis Buffer 2 (see recipe)

Dialysis Buffer 3 (see recipe)

Centrifugal Filters for protein concentration (Amicon 10K Ultra-15, Millipore, cat no. UFC901024)

Nucleosome Binding Buffer (see recipe)

HaloLink Resin (Promega, cat no. G1914)

MARCC Elution Buffer 1 (see recipe)

MARCC Elution Buffer 2 (see recipe)

TEV protease (HaloTEV, Promega, cat no. G6602)

PCR purification kit (GeneJet PCR Purification Kit, Thermo Scientific, cat no. K0702)

Isopropanol

SsoFast EvaGreen Supermix (Bio-Rad, cat no. 172-5200)

Real-time PCR machine (CFX96, Bio-Rad)

Equipment: 37°C floor shaker/incubator

PCR machine

NanoVue Plus Spectrophotometer (GE Life Sciences)

42°C water bath

DNA sequencing facility

Spectrophotometer (UV1800, Shimadzu)

Floor centrifuge (Sorvall Evolution RC with rotor F8-6x1000y and F21-8x50y, Thermo Scientific)

Tabletop centrifuge (Eppendorf 5810R)

Sonicator (Sonic Dismembrator Model 505 with ¼” probe, Fisher Scientific)

Microcentrifuge with cooler (Sorvall Legend Micro 21R, Thermo Scientific)

Tube rotators

Sample material: Stella competent cells (Clontech)

BL21 (DE3) competent cells (lab made)

HaloTag *E.coli* expression vector (pFN29K N-term His₆HaloTag T7 Flexi Vector, Promega, G8331; or pFC30K C-term His₆HaloTag T7 Flexi Vector, Promega, G8381)

HaloTag expression control vector (pH6HTN His₆HaloTag T7 Vector, Promega, G7971)

Plasmid containing cDNA sequence of reader domain (PlasmID, DF/HCC DNA Resource Core)

DNA oligos (Integrated DNA Technologies)

Native nucleosomes extracted from cell culture (see Support Protocol)

Prepare reader domain expression plasmid

- 1 Obtain plasmid that contains cDNA for reader protein of interest from PlasmID.
- 2 Design primers for amplifying reader domain from cDNA. Append the primer with an additional 15-nt flanking sequences to the target vector (pFN29K or pFC30K) for ligation-independent cloning.
- 3 In a PCR tube, combine the following component:

Final concentration/amount	Component
1 pg - 1 ng	Template Plasmid
10 μM	Forward Primer
10 μM	Reverse Primer
25 μl	NEB Q5 Hot Start High-Fidelity 2X Master Mix
to 50 μl	Nuclease-free Water

- 4 Pipette up and down gently to mix the reaction and do a quick spin. Transfer the tube to a PCR machine and follow the thermocycling conditions:

Temperature	Time	Cycle
98°C	30 seconds	
98°C	10 seconds	
*50–72°C	20 seconds	25–35 cycles
72°C	20 – 30 seconds/kb	
72°C	2 minutes	
4°C	∞	

* Annealing temperature can be estimated by online NEB T_m calculator. Remember only input the sequences that anneal with the template and amplify the reader domain instead of the whole primer sequence.

- 5 Make 1% agarose DNA gel in 1X TAE with 1:10,000 SYBR Safe DNA Gel Stain. Run PCR reaction on gel.
- 6 Examine the amplification products on gel. Cut the band with expected size and purify with GeneJet Gel Purification Kit. Elute with 30 – 50 μl Nuclease-free

Water and determine the nucleotide concentration by NanoVue. The purified products can be stored in -20°C at this point.

- 7 Combine the following component and digest target vector (pFN29K or pFC30K) in a PCR tube:

Volume	Component
2 μl	5X Flexi Digestion Buffer (from Promega kit)
2 μl	Target vector (pFN29K or pFC30K) (200 ng)
0.5 μl	Flexi Enzyme Mix or Carboxy Enzyme Mix (from Promega kit)
to 10 μl	Nuclease-free Water

- 8 Pipette up and down gently to mix and do a quick spin. Transfer the tube to a PCR machine and digest as follows:

Temperature	Time
37°C	15 minutes
65°C	20 minutes
4°C	∞

There is no need to purify this reaction - pFN29K and pFC30K plasmids encode a suicide gene and thus will not grow if undigested.

- 9 Add 200 ng purified PCR product from Step 6 into the digested mixture from Step 8. Add in Clontech 5X Infusion Enzyme Mix. React as follows:

Temperature	Time
37°C	15 minutes
50°C	15 minutes
4°C	∞

- 10 Take 5 μl reaction from Step 9 and mix with 50 μl Stella Competent cells. Incubate on ice for 20 minutes before heat shock at 42°C water bath for 45 seconds. Incubate on ice for 2 minutes and recover the cells with 450 μl 2X YT medium at 37°C . Plate 5 μl and 50 μl mixture on LB agar plates containing 50 $\mu\text{g}/\text{ml}$ kanamycin. Incubate at 37°C incubator for 12 – 16 hours.
- 11 Once the colonies form, use a sterile pipet tip to inoculate 2–5 individual colonies into 5 ml 2X YT liquid medium containing 50 $\mu\text{g}/\text{ml}$ kanamycin and grow at 220 rpm, 37°C for 8 – 12 hours.
- 12 Extract plasmids with GeneJet Plasmid Miniprep Kit.
- 13 Validate the insert sequence by restriction enzyme digestion and sequencing.

Express and purify recombinant reader domain

- 14 Transform the sequence-verified plasmid with reader insert from Step 13 and the HaloTag control plasmid pH6HTN into BL21 (DE3) competent cells. Draw lines on LB agar plate containing 50 µg/ml kanamycin. Incubate at 37°C incubator for 12 – 16 hours.
- 15 Inoculate single colonies into individual 10 ml 2X YT liquid medium containing 50 µg/ml kanamycin and grow overnight at 37°C at 220 rpm.
- 16 Transfer each 10 ml starter culture into a 1L 2X YT liquid medium containing 50 µg/ml kanamycin.

Save 1 ml 2X YT liquid medium as blank control in spectrophotometer

- 17 Grow at 37°C at 220 rpm. Monitor optical density (OD) at 600 nm by spectrophotometer every 10 – 30 minutes.
- 18 When OD_{600nm} reaches 0.6 – 0.8, transfer the flasks to a shaker at 25 °C. Induce with 0.5 mM (final concentration) IPTG for 8 – 16 hours at 220 rpm, 25 °C.

Induction efficiency can be checked by collecting 1 ml cells at different time points after induction and running the boiled cell pellets on 5–12% SDS-PAGE. Induction temperature and time can be optimized for each protein. When expressing zinc-containing proteins (for example, PHD fingers), 150 µM (final concentration) zinc sulfate can be supplemented into the medium along with IPTG.

- 19 Harvest cells for 20 minutes at 5000 rpm, 4°C in F8-6x1000y rotor. Decant supernatant. Scoop pellets into a 50 ml falcon tube. Cell pellets can be stored at –80°C.
- 20 Equilibrate nickel resin (use 0.5 – 1 ml bed volume per liter culture, or 1 – 2 ml slurry per liter culture) with 50 ml Resuspension Buffer or 50X bed volume in a 50 ml falcon tube. Hand mix buffer into resin briefly before centrifuging at 2000 rpm for 5 minutes on tabletop centrifuge. Discard buffer between washes. Repeat the washes for a total of three times.
- 21 Resuspend bacterial pellet in Resuspension Buffer. Use 10 – 30 ml buffer per liter culture grown. Add lysozyme to 1 mg/ml final and mix for 30 – 60 minutes at 4°C.
- 22 Sonicate lysate with ¼” probe of FB-505 Sonic Dismembrator. Settings: amplitude of 20 – 25% (power of 10 – 20 watts), 5 seconds on / 10 seconds off, total on time of 3 – 5 minutes. Keep lysate on ice.

After sonication, the resuspension will become more homogenous and less viscous. If not, repeat the sonication.

- 23 Clarify lysate for 30 minutes at 15,000 rpm in F21-8x50y rotor, 4°C.
- 24 Add supernatant from clarified lysate to equilibrated resin from Step 20. Mix for at least 2 hours at 4°C with rotation to bind protein to resin.

- 25 Centrifuge resin for 5 minutes at 2000 rpm in tabletop centrifuge. Discard the supernatant.
- 26 Wash resin once with 50 ml HBS or 50X bed volume, mixing briefly by hand inversion. Centrifuge and discard flow-through.
- 27 Wash resin twice with 50 ml Wash Buffer or 50X bed volume, mixing for 5 minutes at 4°C with rotation. Centrifuge resin and discard wash buffer.
- 28 Elute three times with 10 – 15 ml Elution Buffer or 10–15X bed volume, mixing for 10 minutes at 4°C with rotation. Centrifuge resin and set aside eluent. After last spin, pool eluents.
- 29 Dialyze protein in 10K MWCO dialysis tubing for 3 hours or longer at 4°C in 4 liter or 1000X total eluent volume of Dialysis Buffer 1.
- 30 Transfer protein to 4 liter or 1000X total eluent volume of Dialysis Buffer 2 and dialyze for 3 hours or longer at 4°C.
- 31 Transfer protein to 4 liter or 1000X total eluent volume of Dialysis Buffer 3 and dialyze for 3 hours or longer at 4°C.
- 32 Retrieve protein from dialysis. Centrifuge for 5 minutes at 4000 rpm in tabletop centrifuge to pellet any precipitation.
- 33 Concentrate the supernatant in 10K MWCO 50 ml centrifugal concentrator at 2000 – 4000 rpm in tabletop centrifuge to desired volume (500 µl to 1 ml).
- 34 Transfer the concentrated protein to 1.5 ml tube. Spin at maximum speed at 4°C for 15 minutes in a microcentrifuge to remove any precipitation.
- 35 Aliquot protein and snap freeze in liquid nitrogen. Store the proteins at –80°C.
- 36 Determine protein concentration (by UV_{280nm} or Bradford) and analyze protein purity by SDS-PAGE followed by coomassie staining. If a histone modification target is known for the reader domain, reader domain should be verified with peptide binding assays as well.

Generate customized affinity resin to enrich specific chromatin

- 37 Equilibrate 200 µl HaloLink resin slurry (50 µl bed volume) with 1 ml (or 20X bed volume) Nucleosome Binding Buffer in a 1.5 ml tube. Decant supernatant after spinning at 800 g for 1 minute. Repeat for a total of three washes.
- 38 After last wash, add saturating amount (more than 10 nmole) of HaloTagged reader protein and HaloTag-alone control protein to the resin in two separate tubes and bring to a total volume of 500 µl (100X bed volume). Bind the HaloTag proteins to the resin with rotation at 4°C for 30 – 60 minutes.
- 39 Wash the resin with 1 ml (or 20X bed volume) Nucleosome Binding Buffer by brief hand inversion. Decant supernatant that contains unbound HaloTag proteins after spinning at 800 g for 1 minute. Repeat for a total of five washes.

The capture efficiency can be estimated by quantifying input protein and flow-through on a protein gel.

- 40** After washes, add purified nucleosomes to the reader affinity resin and HaloTag control resin, in a total volume of 1 ml. Save 100 µl as input chromatin for qPCR analysis. Rotate at 4°C for 12 – 16 hours.

The amount of input nucleosomes can be optimized but 1 nmole can be used as a starting point.

- 41** Wash the resin with 1 ml (or 20X bed volume) Nucleosome Binding Buffer by brief hand inversion. Decant supernatant by spinning at 800 g for 1 minute. Repeat for a total of five washes.
- 42** After washes, mix resin with elution buffer to elute bound nucleosomes. Mild TEV protease cleavage can be used for most analysis including mass spectrometry. Rotate resin with 200 µl (or 4X bed volume) MARCC Elution Buffer 1 and 4 µl (20 units) HaloTEV protease at room temperature for at least 1 hour or at 4°C overnight.

Alternatively, SDS-containing MARCC Elution Buffer 2 can be used but is not recommended for mass spectrometry analysis.

- 43** Spin down resin and transfer supernatant to another tube. Recover the resin with an additional 200 µl (or 4X bed volume) MARCC Elution Buffer 1 at room temperature for 1 hour with constant rotation. Spin down resin and pool eluents.
- 44** The eluent can be stored at –20°C for histone or DNA analysis. For histones, specific PTM enrichment of MARCC-ed samples can be probed with different histone antibodies by western blotting or mass spectrometry quantitation (Garcia et al., 2007; Young et al., 2009).
- 45** For DNA analysis, DNA was isolated from the MARCC-ed samples (reader and HaloTag control from Step 44) and the input chromatin (from Step 40) using GeneJet PCR purification kit in the presence of isopropanol.
- 46** Quantify the DNA amount by NanoVue and run the samples on 1.2% agarose gel to verify the DNA quantify. Perform qPCR analysis or next-generation sequencing to quantify associated DNA fragments.

Real-time PCR can be performed in replicates. For each specific PCR primer set, a standard curve should be created from a serial dilution covering 0.2 – 5% of input DNA. For a linear regression, plot Ct (cycle threshold) values with log starting quantity. Nuclease-free water can be used as NTC (no template control) to monitor contaminants interfering with the reactions. To quantify a specific DNA fragment in enriched chromatin samples, convert the individual Ct values into starting DNA quantity using corresponding standard curve. Primer sets need to be validated with R² over 0.99 and amplification efficiency over 90% (efficiency = 10^(–1/slope) – 1). The specificity of primer sets can also be checked by running the PCR products on an agarose gel.

SUPPORT PROTOCOL

NATIVE NUCLEOSOME PREPARATION FROM CELL CULTURE

This protocol is modified on multiple protocols (Brand et al., 2008; Ruthenburg et al., 2011). It is used for preparing nucleosomes from native (non-crosslinked) chromatin of mammalian cell cultures. The procedure starts with a detergent-based cell lysis and isolation of nucleis. After washing the nucleis, the chromatin is then digested by micrococcal nuclease to desired resolution.

Materials

Reagents and solutions: Buffer A (see recipe)

2X Lysis Buffer (see recipe)

Sucrose Cushion Buffer (see recipe)

Nuclei Storage Buffer (see recipe)

1M Calcium chloride, CaCl₂

0.5M EDTA

Chromatin Recover Buffer (see recipe)

Micrococcal nuclease (New England Biolabs, cat no. M0247S)

2M NaCl

Dialysis cassette (Slide-A-Lyzer Dialysis cassette 10K MWCO, Thermo Scientific, cat no. 66385 or 66382)

Equipment: Tabletop centrifuge (Eppendorf 5810R)

Microcentrifuge with cooler (Sorvall Legend Micro 21R, Thermo Scientific)

Spectrophotometer (UV1800, Shimadzu)

Tube rotators

Vortex mixers

37°C water bath

Sample material: Cell pellets of 2×10^7 MCF-7 cells

- 47 In 15 ml falcon tube, resuspend the cell pellet with 1 ml Buffer A. Mix with another 9ml Buffer A, spin down at 800g, 4°C for 5 minutes in a tabletop centrifuge. Repeat the washes for three times total.

- 48** Resuspend the cell pellet in 2.5 packed cell volumes (about 1 ml) of Buffer A. While vortexing the tube at low speed, add 1:1 volume of 2X Lysis Buffer drop-wise.
- 49** Incubate the tube on ice for 10 minutes to complete cell lysis. Spin down at 1300g, 4°C for 5 minutes.
- The nuclei pellet should be white. If not, repeat steps 4 with higher concentration of detergent. The percentage of lysis can be examined by looking under microscope with Trypan dye.
- 50** (Optional) Resuspend the nuclei in 6 packed cell volumes (about 3 ml) of Buffer A and lay on top of 7.5 ml Sucrose Cushion Buffer in a 50 ml tube. Centrifuge at 1300g, 4°C for 12 minutes.
- This step is to further purify the nuclei, especially getting rid of the detergent. The nuclei will remain at the bottom of the tube. This step can be substituted by washing the nuclei in Buffer A if cell number is limited.*
- 51** Resuspend the nuclei in 500 µl Buffer A and transfer to a 1.5 ml tube. Wash the 50 ml tube with another 500 µl Buffer A and combine into the 1.5 ml tube. Spin down at 1300g for 5 minutes in microcentrifuge at 4°C.
- 52** Wash the nuclei in 1 ml Buffer A and spin down. Resuspend the nuclei in 100 µl or less Buffer A.
- 53** Determine the approximate nucleic acid content by absorbance.
- 54** At this point, you can store nuclei with equal volume of Nuclei Storage Buffer (incubate on ice for 15 minutes before aliquoting) in -80°C.
- 55** Based on the DNA concentration, dilute nuclei resuspension with Buffer A to 1.2 – 1.6 µg/µl DNA. Add CaCl₂ to final concentration of 1 mM.
- Make sure nuclei are well suspended.*
- 56** Warm the nuclei at 37°C for 5 minutes. Add 1 µl MNase (2000 gel unit) and mix well. Incubate at 37°C water bath for 12 minutes. Stop the reaction with 10mM EDTA.
- Time points need to be pre-determined by stopping the reaction at different times and run on 1.2% agarose gel to check the size of digested chromatin. 12 minute was selected to yield mostly mononucleosomes. The amount of MNase can also be optimized.*
- 57** Spin down the reaction at highest speed, 5min at 4°C in a microcentrifuge. Save supernatant as S1 and keep at 4°C. Resuspend the pellet in 50 µl Chromatin Recover Buffer to recover more heterochromatin. Rotate at 4°C for 1 hour to overnight and spin down. Save supernatant as S2 and pellet as P.
- 58** Combine S1 and S2 as the final nucleosome preparation. Concentrate to desired volume if needed and dialyze against Dialysis Buffer 3 or other desired buffer.

- 59** Determine the approximate nucleic acid content by absorbance. Check the size of chromatin by 1.2% agarose gel.

Purify the DNA from the nucleosome preparation and run the gel, or supplement nucleosome preparation with 0.1% SDS (to dissociate DNA from histones) before running. The second option also requires a low voltage and after-run stain. Run with a gradient of known amount of 146 bp DNA standards to help quantification.

- 60** Check the histone by running on 18% SDS-PAGE and follow by coomassie staining.

REAGENTS AND SOLUTIONS

Resuspension buffer

30 mM HEPES

500 mM sodium chloride (NaCl)

pH 7.4, filter.

Right before use, add in:

1 mM phenylmethylsulfonyl fluoride (PMSF)

10 µg/ml leupeptin

10 µg/ml aprotinin

1 mM dithiothreitol (DTT) or other preferred reducing agents

HBS (HEPES Base Saline)

30 mM HEPES

150 mM NaCl

pH 7.4, filter

Wash Buffer

30 mM HEPES

150 mM NaCl

20 mM imidazole

pH 7.4, filter and keep at 4°C

Elution Buffer

30 mM HEPES

150 mM NaCl

0.3 M imidazole

pH 7.4, filter and keep at 4°C

Dialysis Buffer 1

30 mM HEPES

150 mM NaCl

pH 7.4 and keep at 4°C

Before use, add in 3 mM DTT or other preferred reducing agents

Dialysis Buffer 2

30 mM HEPES

150 mM NaCl

pH 7.4

5% (v/v) glycerol and keep at 4°C

Before use, add in 3 mM DTT or other preferred reducing agents

Dialysis Buffer 3

30 mM HEPES

150 mM NaCl

pH 7.4

10% (v/v) glycerol (or higher if needed) and keep at 4°C

Before use, add in 3 mM DTT or other preferred reducing agents

Nucleosome Binding Buffer

30 mM HEPES

150 mM NaCl

0.01% (v/v) NP-40

10% (v/v) glycerol

pH 7.4, filter and keep at 4°C.

MARCC Elution Buffer 1

10 mM Tris-HCl

pH 7.4, filter

MARCC Elution Buffer 2

30 mM HEPES

150 mM NaCl

5 mM ethylenediaminetetraacetic acid (EDTA)

1% (w/v) sodium dodecyl sulfate (SDS)

pH 7.4, filter

Buffer A

10 mM HEPES

10 mM potassium chloride (KCl)

1.5 mM magnesium chloride (MgCl₂)

340 mM sucrose

pH 7.9

10% (v/v) glycerol, filter and keep at 4°C.

Right before use, add in:

1 µg/ml trichostatin A (TSA, HDAC inhibitor)

10 mM β-Glycerophosphate (phosphatase inhibitor)

1 mM DTT

0.5 mM PMSF

10 µg/ml leupeptin

10 µg/ml aprotinin

2X Lysis Buffer

Buffer A plus 0.2% (v/v) Triton X-100

Sucrose Cushion Buffer

10 mM HEPES

30% (w/v) sucrose

1.5 mM MgCl₂

pH 7.9

Filter and keep at 4°C

Nuclei Storage Buffer

10 mM HEPES

10 mM KCl

1.5 mM MgCl₂

340 mM sucrose

pH 7.9

80% (v/v) glycerol, filter and keep at 4°C

Chromatin Recover Buffer

5 mM HEPES

0.2 mM EDTA

pH 7.9

Filter and keep at 4°C

COMMENTARY

Background Information

Reader domains are diverse collections of histone-interacting modules that can interpret histone language in cells (Musselman et al., 2012). Systematic profiling has revealed multisite interaction between some reader domains and targeted histone region (Fuchs et al., 2011; Garske et al., 2010).

Comparison with traditional histone antibodies—The authors have demonstrated three recombinant reader domains with great specificity and affinity for nucleosome enrichment (Su et al., 2014). Tested by combinatorial histone peptide microarray, these reader domains display better sequence specificity and can distinguish better among different combinatorial modification patterns than their corresponding histone antibodies. A MBT domain has been developed into pan-methylation affinity reagent towards lysine mono- and di-methylation (Moore et al., 2013).

Critical Parameters

Reader domain and targeted chromatin—The affinity reagent in this protocol, the recombinant reader domain, is the most critical parameter in the enrichment process. Not every reader domain is suitable as being an enrichment tool. An ideal candidate should have good affinity and also specificity towards the targeted PTM state. Good examples include ATRX-ADD domain, ING2-PHD domain and AIRE-PHD domain (Su et al., 2014). If prior knowledge is lacking about the reader of interest, histone peptide microarray can be employed to help identify potential binding targets in an unbiased fashion. The amount of enriched chromatin also depends on the natural abundance of the targeted chromatin. If a chromatin state of low abundance is of interest, optimization of starting chromatin amount is recommended. A modified MARCC can be performed by skipping Steps 24–36 and directly attaching HaloTag reader domain to the HaloLink resin.

Chromatin isolation and digestion—The condition, purity and size of input chromatin should be considered based on the downstream analysis. For mass spectrometry analysis, we used native chromatin and a mild non-SDS elution method for MARCC. This elution method also ensures the intactness of enriched nucleosomes for referring co-existence of PTMs and associated DNA. The *SUPPORT PROTOCOL* describes determination of MNase time point to achieve a population of >90% mononucleosomes. If higher percentage of mononucleosomes is required, a sucrose gradient ultracentrifugation can be employed (Ruthenburg et al., 2011). Furthermore, the nucleosome purity can be improved by removing chromatin-binding proteins via hydroxyapatite resin (Brand et al., 2008).

Replicates and controls—Same as antibody-based immunoprecipitations, MARCC should be done in proper biological and technical replicates. A HaloTag control should be included to measure background binding. Input chromatin should be saved for all analysis to normalize for fold enrichment.

Troubleshooting

Step	Problem	Possible reason	Solution
6	No amplification.	1) Annealing temperature is too high. 2) Secondary DNA structure in primers.	1) Optimize annealing temperature. 2) Re-design primers.
6	Non-specific amplification.	Annealing temperature is too low.	1) Optimize annealing temperature. 2) Gel purify the band at expected size.
11	No colonies.	1) Target vector is not digested. 2) InFusion reaction is unsuccessful.	1) Check the digestion by running on agarose gel. 2) Increase the amount in transformation or run a control reaction included in InFusion kit.
36	Low protein yield.	1) Protein is not soluble. 2) Selected protein truncation is not stable. 3) Protein is not folded properly.	Protein expression is very case-dependent and so there is no universal solutions. 1) Optimize the following parameters: <i>E.coli</i> expression strains, induction temperature, induction time and IPTG concentration. 2) Switch the tag to a different terminal (N-term or C-term). 3) Try a different tag (for example, Strep tag) for purification instead of HisTag. 4) If a domain is cloned, try a

Step	Problem	Possible reason	Solution
			different truncation of the full-length protein.
43	Low yield of eluted chromatin.	1) Targeted chromatin with combinatorial PTM state is of low abundance. 2) Reader capture is not efficient.	Check fold enrichment by western blotting or qPCR. If enrichment is successful, try increasing the amount of input chromatin in the enrichment. If enrichment fails, optimize protein expression as above.
43 or 46	High HaloTag background binding.	Inefficient washings.	1) Increase washing time and remove the supernatant during washes as much as possible. 2) Increase binding / washing stringency (salt concentration or detergent concentration).
60	Low chromatin extraction yield.	1) Nuclei pellet gets lost during washes. 2) Chromatin is not digested by MNase.	1). If cell number is limited, substitute sucrose cushion wash with washing the nuclei in Buffer A. 2) Increase enzyme amount or/and digestion time of MNase treatment. If starting with large amount of cells, divide them into multiple tubes for nucleosome extraction and make sure nuclei are well resuspended. An optimal cell number for each tube is between 5×10^6 to 5×10^7 .

Anticipated Results

Step	Anticipated results
36	As shown in Figure 2A–B, purified His-HaloTagged reader domain displays at least 80% purity at expected molecular weight. If targeted histone modification is known, reader binding can be verified by peptide binding assays (Figure 2C–D).
39	5 – 10 µg HaloTag protein is immobilized on 1 µl HaloLink resin (Figure 3A).
42	TEV protease cleavage releases about 90% of the bound reader domain and the associated chromatin (Figure 3A–B). Chromatin-associated DNA is readily quantifiable for downstream analysis (Figure 3C–D).
43	MARCC-ed chromatin displays specific enrichment with certain histone modifications, as shown by western blotting in Figure 4A–B.
46	MARCC-ed chromatin is enriched of different DNA fragments by qPCR quantification (Figure 4C–D).
56	Upon MNase treatment, the size of chromatin decreases as ladders and gets to most mononucleosomes over time (Figure 5A–B).
59	S1 and S2 have most soluble chromatin, while P has larger size of chromatin (Figure 5C). The protein-DNA complex will create a shift centered at around 750bp. In the end, around 2 nmole nucleosomes (or 200 µg DNA) can be purified from 2×10^7 cells.
60	Core histones and some other chromatin-associated proteins are visible when the nucleosome preparation was examined on SDS-PAGE (Figure 5D).

Time Considerations

Step	Time	Stopping point
1	Varies	
2–10	1 day	Step 6 and Step 9
11–12	1 – 2 days	Step 11
13	Varies	
14–20	3 days	Step 20

Step	Time	Stopping point
21–36	2 – 3 days	Step 35
37–43	2 days	Step 43
47–60	2 – 3 days	Step 54 and Step 58

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Figure 1. Overall workflow of MARCC

Reader domain is expressed and purified in His-HaloTag bacterial expression vector. Reader domain is immobilized on HaloLink resin and incubated with MNase-digested native mononucleosomes. Enriched nucleosomes are released by TEV protease cleavage and analyzed for coexisting histone PTMs and associated DNA.

Figure 2. Preparation of recombinant reader domains

Part of this figure is adapted from Figure S1 (Su et al., 2014). (A) Construction of recombinant reader domains. All readers (ING2-PHD, AIRE-PHD1 and ATRX-ADD) were expressed with N-terminal (His)₆-HaloTag (HH for short) with a TEV protease cleavage site. (B) Purification of recombinant reader domains. Purified proteins were separated on 12% SDS-PAGE before stained with coomassie blue. (C) Fluorescence polarization to determine binding affinity of ING2-PHD and H3K4me3 peptide. Titration of ING2 PHD protein (0 – 100 μM) was mixed with 3 nM fluorescein-labeled histone peptide. Fraction

bound (Y-axis) was calculated from polarization values (measured by BioTek microplate reader Synergy H4, excitation 428/20 nm, emission 528/20 nm and sensitivity 60) and plotted against protein concentration (X-axis). The binding affinity is derived from fit curve in KaleidaGraph v4.1.3. (D) Biotinylated peptide pull-down of ING2-PHD. Biotinylated peptide was immobilized on streptavidin resin and then incubated with HaloTag ligand TMR-labeled proteins. After washes, the resin was boiled in SDS sample buffer and the bound proteins were separated on 12% SDS-PAGE. TMR-labeled HaloTagged proteins were included as input and HaloTag alone was immobilized as negative control.

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Figure 3. Preparation, capture and elution of customized MARCC resin

This figure is reproduced from Figure S7 (Su et al., 2014). (A) Release of reader domains by TEV cleavage on resin. ATRX ADD domain was cleaved off the resin by incubating immobilized HH-ATRAX ADD with HaloTEV protease. FT (flow-through), E1 (elution1), E2 (elution2), bead (resin after elution).

(B, C) Elution by TEV cleavage yields intact mononucleosomes for downstream analysis. Histones (B) and DNA (C) were resolved on gel. Glycine elution did not achieve similar elution efficiency as TEV cleavage.

(D) DNA purified from AIRE-PHD, ING2-PHD and ATRX-ADD MARCC enrichment was run on 1% agarose gel. The DNA size was enriched at 146bp.

Figure 4. Reader modules display specific binding in MARCC

This figure is adapted from Figure 4 and Figure 6 (Su et al., 2014). (A, B) AIRE-PHD, ING2-PHD and ATRX-ADD were immobilized on resin and incubated with native mononucleosomes. Bound nucleosomes were boiled on beads and separated on 12% SDS-PAGE and probed with corresponding histone antibodies. HaloTag protein was included as a negative control. Bound nucleosomes were quantified as % input.

(C, D) Relative enrichment of active chromatin by ING2-PHD and heterochromatic DNA (SAT-2) by ATRX-ADD measured by qPCR after MARCC. qPCR quantification for active

chromatin marker GAPDH and heterochromatin marker SAT-2 were performed for DNA extracted from MARCC by ING2-PHD or ATRX-ADD and ChIP by H3K4me3 antibody (ab8580) or H3K9me3 antibody (ab8898). IgG and HaloTag were used as negative control for MARCC and ChIP. The DNA was quantified by input DNA standard curve and plotted as % input.

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Figure 5. Preparation of native nucleosomes from mammalian cell cultures

This figure is reproduced from Figure S6 (Su et al., 2014). (A) Schematic illustration of native mononucleosome library preparation. Cells were pelleted and lysed to isolate nuclei. Nuclei were then digested by micrococcal nuclease to generate mononucleosomes in the soluble fractions.

(B, C) Preparation of mononucleosome library by MNase digestion. Nuclei were digested with MNase and the reaction was stopped at different time points (0, 1, 3, 6, 9 and 12 min) by addition of EDTA. The digested chromatin was supplemented with 0.1% SDS (w/v, final) and run on 1.2% agarose gel at 2V/cm for 6 hours before staining with ethidium bromide. S1: soluble fraction 1; S2: soluble fraction 2; P: precipitation. After 12min, the pooled soluble fractions (S1+S2) were mostly mononucleosomes (>95%).

(D) Protein purity and reproducibility of native mononucleosome library preparations. Two independent preparations of native MCF-7 mononucleosome library were run on 18% SDS-PAGE gel and stained with coomassie blue.