EpiCypher® Desthiobiotin Nucleosome Capture & Elution

For use in capture and recovery of nucleosomes and/or associated chromatin binding proteins

1. Overview

The following protocol describes the capture and elution of intact nucleosomes tagged with desthiobiotin (EpiCypher Catalog No. 16-0024) for the purpose of recovering the labeled target, either alone or bound to chromatin-binding protein(s) of interest, for downstream analysis. In the protocol, desthiobiotinylated nucleosomes are captured by streptavidin magnetic beads. Elution of intact, native nucleosomes is acheived under mild conditions utilizing biotin as a competing ligand. This is made possible by the fact that desthiobiotin, a modified form of biotin, exhibits a lower binding affinity for avidin-family proteins ($K_d = 10^{-11}$) compared to biotin ($K_d = 10^{-15}$). This protocol can be modified to isolate protein complexes from complex mixtures for downstream analyses such as mass spectrometry-based approaches. The user should note that such complexes may be sensitive to, or disrupted by, the choice of binding and/or wash buffer during the execution of this protocol. Therefore, this validated workflow can serve as a starting point and control condition that can be systematically modified to optimize user-defined endpoints.

2. Materials & Equipment Needed

Materials

Item	Vendor	Catalog No.
HCl	EMD Millipore	HX0607-2
NaCl	VWR	97061-268
Tris Base	VWR	JT4109-2
Tween-20	Amresco	0777-1L
Desthiobiotin rNuc	EpiCypher	16-0024
D(+)-biotin	VWR	0340-1G
Streptavidin Magnetic Beads	Pierce	88816
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418
5% Alkali-soluble Casein	Millipore	70955-3
60 mL Luer Lock Syringe(s)	BD	309653
PES Syringe Filter	Celltreat	229746
500 mL Bottle Top Filter	Celltreat	229717

Equipment

Item	Vendor	Catalog No.
DynaMag-2 Magnetic Rack	Thermo Fisher Scientific	12321D
NanoDrop Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific	Model: One^C
Magnetic stir plate	Thermo Fisher Scientific	Model: Cimarec$^+$
Pan balance	VWR	Model: 5002
Benchtop vortex	Scientific Industries	Model: Vortex Genie2
Nutating mixer	VWR	82007-202

3. Buffer Recipes

10X TBS (pH 7.6) – 1 L

24 g Tris base (FW: 121.1 g)
88 g NaCl (FW: 58.4 g)
Dissolve in 900 mL ultrapure water (18.2 MΩ·cm at 25°C)
pH to 7.6 with 12 N HCl
Add ultrapure water to a final volume of 1 L
Filter sterilize into autoclaved 1 L glass bottle with 0.22 µm cutoff PES filter top

10% (v/v) Tween-20 – 50 mL

5 mL of Tween-20
45 mL of ultrapure water
Add ultrapure water to 50 mL
Filter sterilize with 0.22 µm cutoff PES syringe filter into new sterile, 50 mL conical tube

1X TBST (Wash/Bind Buffer) – 50 mL

5 mL of 10X TBS (pH 7.6)
500 µL of 10% (v/v) Tween-20
Add ultrapure water to 50 mL
Filter sterilize with 0.22 µm cutoff PES syringe filter into new sterile, 50 mL conical tube

2X TBST – 50 mL

10 mL of 10X TBS (pH 7.6)
1.0 mL of 10% (v/v) Tween-20
Add ultrapure water to 50 mL
Filter sterilize with 0.22 µm cutoff PES syringe filter into new sterile, 50 mL conical tube

1X TBST w/ 0.5% casein (Blocking Buffer) – 5 mL

2.5 mL of 2X TBST (pH 7.6)
500 µL of 5% (w/v) akali-soluble casein
Add ultrapure water to 5.0 mL

200 mM D(+)-biotin – 1 mL

Mass 48.8 mg of D(+)-biotin
Add DMSO to 1 mL final volume

Elution Buffer (5 mM D(+)-biotin, 2.5% (v/v) DMSO) – 5 mL

2.5 mL of 2X TBST (pH 7.6)
125 µL 200 mM D(+)-biotin
Add ultrapure water to 5.0 mL

4. Protocol

Part I: Pre-washing/Blocking Streptavidin Magnetic Beads *(estimated time = 1 hour)*	
1. Add 30 µL (300 µg from 10 mg/mL stock) of Pierce Streptavidin Magnetic Beads into a 1.5 mL microcentrifuge tube(s) (see note a). 2. Place the tubes into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant. 3. Add 100 µL of Binding/Wash Buffer (1X TBST) to the tubes. Vortex gently to mix, taking care to avoid beads sticking to the cap of the tubes. Collect the beads with a magnetic stand, then remove and discard the supernatant. 4. Repeat this step an additional time (see note b). 5. Add 100 µL of Blocking Buffer (1X TBST, 0.5% (w/v) casein) to the tubes and vortex gently to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant. 6. Repeat this step an additional time. 7. Add 100 µL of Binding/Wash Buffer to the tube(s) and vortex gently to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant. 8. Repeat this step an additional two times for a total of three (**3**) washes. 9. Remove final wash supernatant just before adding desthiobiotinylated nucleosomes (**Part II**, next page).	**a.** To ensure homogeneity, mix the beads thoroughly before use by repeated inversion, gentle vortexing, or using a rotating/nutating platform. If running multiple samples simultaneously, it is recommended to remove sufficient volume of bead slurry for all samples and to scale the buffer volumes accordingly. For example, if nine samples are anticipated, remove 300 µL bead slurry (30 µL per sample x [9+1 extra]) and use 1.0 mL of buffer in the wash / blocking steps. **b.** In the Pierce Streptavidin Magnetic Beads protocol, it states that the user can deliver 1 mL of Binding/Wash buffer to 50 µg bead input to wash and equilibrate the beads to prepare them for binding. We anticipate that there would be no significant difference in results if the user choses to follow these parameters for bead preparation and equilibration.

Part II: Desthiobiotinylated Nucleosome Capture and Elution

(estimated time = 2 hours + O/N incubation)

10. Prepare 25 pmol (~5 – 6 µg) of desthiobiotinylated nucleosome (see note c) in 100 µL of Binding/Wash Buffer and add to the pre-washed/equilibrated magnetic beads (see note d) in 1.5 mL microcentrifuge tube(s) in which the final wash supernatant has been removed (see **Part I** above).

11. Allow to incubate at room temperature for 1 hour with gentle nutating mixing.

12. Collect the beads with a magnetic stand and remove the supernatant. Retain supernatant in a separate 1.5 mL microcentrifuge tube for analysis (see **Appendix I**). This fraction is denoted as 'flow-through' in subsequent steps.

13. Prepare a 200 mM D(+)-biotin stock solution in 100% DMSO (see **Section 3, Buffer Recipes**). Dilute the stock solution of D(+)-biotin to a final concentration of 5 mM in 1X TBST (see **Appendix II**).

14. Add 100 µL of Binding/Wash Buffer to the tube and gently mix to wash the beads of any partially bound input. Collect the beads with a magnetic stand and then discard the supernatant. Repeat this wash twice more.

15. Add 100 µL of the Elution Buffer to each tube containing the beads that are coated with the desthiobiotynylated rNuc and incubate at 4ºC overnight with gentle nutation (see note e).

16. The next day, magnetically separate the beads and remove the supernatant. Place supernatant in an appropriately labeled microcentrifuge tube for downstream analysis (see note f).

c. **Important note:** This protocol is a general guideline for desthiobiotinylated nucleosome capture and elution and will require optimization for each specific application. As written, the protocol is expected to result in the capture of >90% of the nucleosomal input (25 pmol starting material) during the binding step (1 hr room temperature incubation) and an effective elution of the captured desthiobiotinylated nucleosomes from the bead matrix during incubation in the presence of free-biotin.

d. The Pierce Streptavidin Magnetic Beads protocol states that the user should dilute the sample (10 µg of biotinylated IgG into 300 µL during the binding incubation step). This parameter was altered to the above 100 µL with a noted >90% capture efficiency.

e. The Pierce Streptavidin Magnetic Beads protocol states that the user may incubate the tube containing the magnetic beads and Elution Buffer at room temperature with mixing for 5 minutes; however, this is defined as either IgG Elution Buffer, pH 2.0 (Cat No. 21028) or 0.1 M glycine, pH 2.0. This may be another user identified parameter that will need to be independently verified.

f. The user will not be able to determine the nucleosome concentration of the eluate utilizing a UV-Vis spectrophotometer as the D(+)-biotin in the Elution Buffer obscures the spectral signal signature of the eluted nucleosomes (A260) in the sample. The user is to take this into consideration when trying to quantify the eluted nucleosome and/or associated complex. Quantification of the protein should be readily attainable using a protein assay compatible with up to 0.1% (v/v) Tween-20 present in the 1X TBST (*e.g.* Pierce™ Detergent Compatible Bradford Assay Kit, Thermo Fisher Scientific Cat No: 23246 or Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific Cat No: 23225). The user should note that the protein content of the nucleosome is approximately 55% (0.55 correction factor, desthiobiotynylated rNuc 147x601-desthiobiotin template DNA) of the total nucleosome weight and there is a detection limit associated with each protein assay kit.

Appendix I

Nucleosome concentration in the flow-through (unbound fraction) is easily measured via A260 utilizing a NanoDrop or equivalent UV-Vis spectrophotometer by giving the user a value with units ng/µL as a function of DNA concentration. The total nucleosome concentration can then be calculated by using the value obtained via these measurements and dividing through by a mass correction factor. In the case of the desthiobiotynylated rNuc (147x601-desthiobiotin template DNA), this correction factor is 0.45 (i.e. 45% of the rNuc molar mass of 199827.9 g/mol is comprised of DNA). Therefore, the user can take the concentration of DNA that is expressed in ng/µL (either calculated or determined via internal instrument settings) and transform the number into the total nucleosome concentration expressed in ng/µL.

Sample calculation:

20 ng/µL DNA as determined by A260 measurement

20/0.45 = 44.4 ng/µL nucleosome

Appendix II

When conducting the initial titrations of D(+)-biotin against the beads containing the captured desthiobiotynylated rNuc, 200, 100, 50, 25, and 12.5 mM solutions of D(+)-biotin where prepared via a 2-fold dilution series in 100% DMSO. In order to prepare the serially diluted Elution Buffers, these stock solutions were then added to 2X TBST and further diluted with dH_2O to result in buffers that contained 5.0, 2.5, 1.25, 0.63, and 0.31 mM D(+)-biotin in 1X TBST (2.5% (v/v) DMSO, final). It was noted that there was no statistical difference in the amount of desthiobiotynylated rNuc that was eluted from the magnetic beads when using concentrations within this range for this protocol. As a follow-up, D(+)-biotin was also tested at concentrations ranging from 375 µM to 0.12 µM in 1X TBST (2.5% (v/v) DMSO, final) in a 5-fold dilution series and it was noted that the floor for effective elution was between 300 and 60-fold molar excess of the ligand (75 to 15 µM D(+)-biotin, respectively) in comparison to the 25 pmol input of desthiobiotinylated nucleosomes. Additionally, there is no indication that the DMSO concentration in the buffer affects the integrity of the desthiobiotynylated rNucs as the eluted samples were still in a native and folded form with minimal noted free DNA as determined via Native PAGE (6% polyacrylamide, 0.4X TBE). However, the user may choose to lower the final concentration of the DMSO as to reduce the 'harshness' of the Elution Buffer to suit the needs of their experiment. The reduction of the DMSO content in the buffer is not expected to reduce the noted elution efficiency; however, this parameter will need to be independently verified.