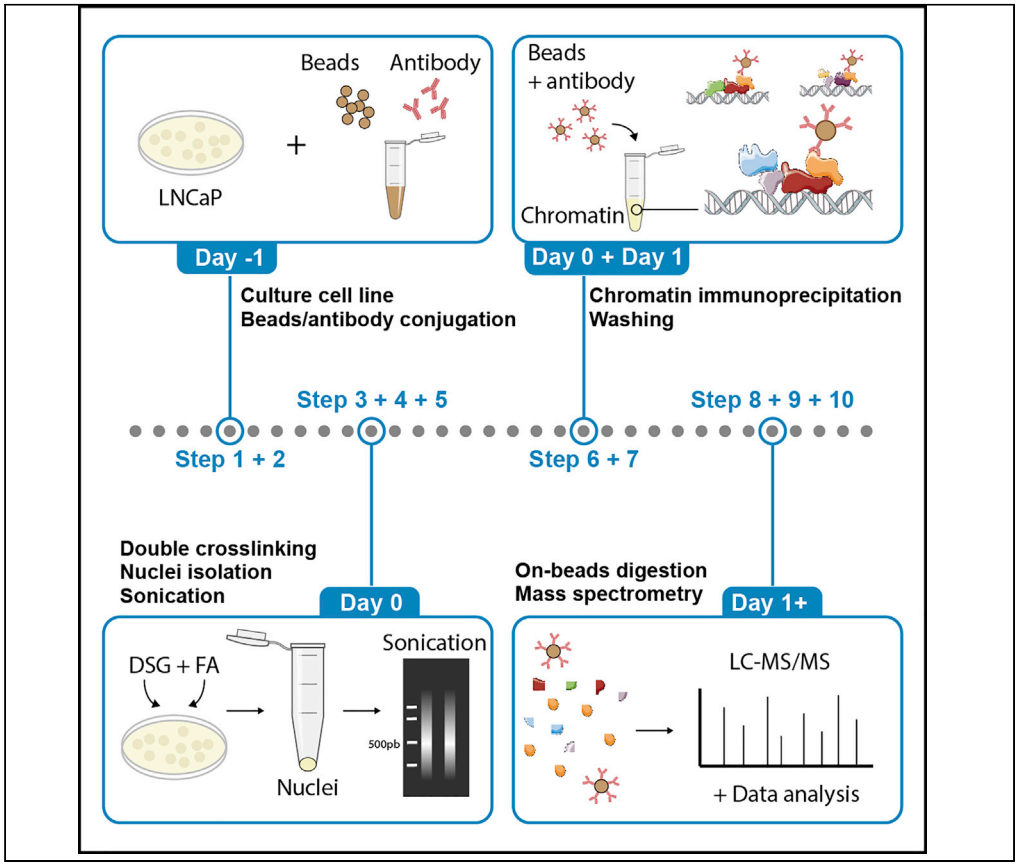
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) to identify chromatin-interactome in prostate cancer cells



Rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) is a technique to study protein complexes on chromatin. The protocol below describes specific steps for RIME analysis of the male human-derived prostate cancer cell line LNCaP. This approach can also be applied to other prostate cancer cell lines such as 22Rv1, DU145, and PC3. For other cell types, we recommend optimizing the number of cell culture plates to ensure adequate sample for mass spectrometry protein detection.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Charlotte Scholtes, Catherine R. Dufour, Vincent Giguère

charlotte.scholtes@mcgill.ca (C.S.)
vincent.giguere@mcgill.ca (V.G.)

Highlights

Protocol for RIME-based chromatin interactome discovery in prostate cancer cells

Double crosslinking chromatin immunoprecipitation of prostate cancer cells

Protocol is validated for mTOR in distinct prostate cancer cell lines

Scholtes et al., STAR Protocols 3, 101434
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101434

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) to identify chromatin-interactome in prostate cancer cells

Charlotte Scholtes,[1,3,4,*] Catherine R. Dufour,[1,3] and Vincent Giguère[1,2,5,*]

[1]Goodman Cancer Institute, McGill University, Montréal, QC H3A 1A3, Canada

[2]Department of Biochemistry, Faculty of Medicine, McGill University, Montréal, QC H3G 1Y6, Canada

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: charlotte.scholtes@mcgill.ca (C.S.), vincent.giguere@mcgill.ca (V.G.)
https://doi.org/10.1016/j.xpro.2022.101434

SUMMARY

Rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) is a technique to study protein complexes on chromatin. The protocol below describes specific steps for RIME analysis of the male human-derived prostate cancer cell line LNCaP. This approach can also be applied to other prostate cancer cell lines such as 22Rv1, DU145, and PC3. For other cell types, we recommend optimizing the number of cell culture plates to ensure adequate sample for mass spectrometry protein detection.

For complete details on the use and execution of this protocol, please refer to Mohammed et al. (2016) and Dufour et al. (2022).

BEFORE YOU BEGIN

Chromatin-bound protein complexes can be identified using rapid immunoprecipitation mass spectrometry of endogenous protein (RIME). Briefly, a bait protein is enriched from chromatin-associated protein complexes by chromatin immunoprecipitation (ChIP) followed by mass spectrometry (MS) identification of its interacting partners. RIME provides valuable information regarding chromatin protein-protein interactions, a key element of transcriptional regulation, although it cannot resolve precise transcriptional modules at a given loci. Thus, this protocol can therefore be used for proteins that bind directly or indirectly to DNA.

We have found that freezing samples significantly reduces the efficiency of the RIME by greatly diminishing MS peptide signal intensities, thus compromising the quality and usability of the data. This is particularly evident if cells are frozen prior to performing the chromatin-immunoprecipitation (ChIP) step. We therefore advise against freezing cells before on-beads digestion. To ensure reproducibility between independent experiments, carry all steps carefully under the same conditions. We recommend using at least three independent biological replicates per RIME experiment.

Due to the high associated costs with the RIME method (beads, antibodies, MS), we advise to perform a preliminary experiment with limited conditions as optimization may be required and suggest following our guidelines described in the troubleshooting subsection to ensure the quality of the results.

1. Prepare 2% charcoal-stripped serum (CSS).
2. Grow two 15 cm plates of LNCaP cells per condition (2 × 15 cm confluent plates ≈ 2 × 10^7 cells). Cells should be around 90% confluent.

3. Prepare all the buffers as indicated. Prepare enough of each solution for all your conditions. Filter-sterilize buffers as indicated with 0.22 μm filter units.
4. Add protease inhibitors to the buffers where indicated.
5. Prepare 1 M disuccinimidyl glutarate (DSG) stock solution in DMSO, aliquot and store at −20°C.

Note: If one of the conditions involves a cell treatment that will decrease cell numbers, three 15 cm plates of LNCaP cells should be used instead of two (e.g., mTOR inhibitor Torin 1).

⚠ CRITICAL: As MS is very sensitive, it is important to not personally contaminate the samples. During the experiments, tie your hair back, wear a mask, avoid speaking over the samples, wear a clean lab coat, clean the bench area used for sample preparation, and change gloves often to avoid keratin contamination of your samples. (troubleshooting 1).

Preparation of charcoal-stripped serum

Timing: 2 days

Charcoal treatment of serum removes a wide range of small molecules such as lipophilic molecules like steroid hormones. It is indispensable to use this serum for steroid hormone treatments of cells.

6. In a large plastic container, add 50 g of resin (AG® 1-X8).
7. Add enough milli-Q water to cover the resin.
8. Stir 10 min at 22°C–24°C.
9. Let settle and discard supernatant.
10. Wash one more time (repeat steps 7–9).
11. Add 25 g of prepared resin above to 500 mL fetal bovine serum (FBS).
12. Stir at 22°C–24°C for 5 h.
13. Transfer the FBS using decantation to a clean glass bottle with a flat bottom for good mixing.
14. In a 15 mL conical tube, mix 4 mL charcoal powder with 1 mL dextran T70 powder.
15. Add 5 mL of milli-Q water to the 5 mL dextran/charcoal powder mix.

Note: Always prepare fresh charcoal/dextran solution. You can store this solution at 4°C on the day of the experiment.

16. Add the remaining 25 g of prepared resin and the 10 mL dextran/charcoal solution to the glass bottle containing 500 mL FBS.
17. Stir at 4°C for 16–24 h.
18. The next day, aliquot the mix into 50 mL conical tubes and spin at 95 × *g* (1,000 rpm) for 10 min at 4°C.
19. Pool the supernatants into a clean glass bottle and add another freshly prepared 10 mL dextran/charcoal solution following steps 14 and 15.
20. Incubate at 55°C in a water bath for 1 h with occasional shaking.
21. Aliquot the mix into 50 mL conical tubes and spin at 95 × *g* (1,000 rpm) 10 min at 4°C.
22. Filter the supernatant twice through Whatman paper into a new clean glass bottle at 22°C–24°C.

Note: This step is very long because the FBS flows very slowly. Plan a lot of time for this step.

23. Then, filter the FBS further using a bottle top filter and vacuum system.

Note: There is a good chance that the filter will clog when it reaches the halfway point. Simply transfer out the FBS that didn't pass the bottle top filter into a clean bottle. Then, clean the filter clogged with charcoal with distilled water to remove the charcoal, place back the filter, transfer back the remaining FBS to filter it.

24. Finally, further filter the FBS through a syringe with a 0.22 μm filter in a tissue culture (TC) hood. Filter directly into 50 mL conical tubes and store at −20°C (TC ready).

Note: This step can also be time consuming and requires strength even though the FBS has undergone previous filtrations.

⚠ CRITICAL: It is possible to use uncoated charcoal. However, dextran-coated charcoal is preferable because there is less unintentional loss of non-steroid serum proteins compared to using uncoated charcoal.

Cell culture

Timing: 5–7 days

25. Culture and maintain LNCaP cells at 37°C and 5% CO_2 in 15 cm dishes in Phenol Red-free RPMI-1640 medium supplemented with 10% FBS, 2 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin.

⚠ CRITICAL: Phenol Red-free RPMI medium is important for androgen-dependent cells because phenol red shares structural similarity with steroid hormones, thus avoids interference with androgen stimulation.

If you need to treat your cells in your experiment, please consider the additional steps and processing time. See below for treatments used in the study by (Dufour et al., 2022) to modulate our mTOR bait protein involving androgen (R1881)-mediated induction of nuclear mTOR accumulation, Torin 1-mediated inhibition of mTOR activity, R1881 + Torin 1 co-treatment, and DMSO as the vehicle treatment.

26. Androgen-responsive LNCaP cells are left for 48 h in Phenol Red-free RPMI-1640 medium containing 2% CSS.

⚠ CRITICAL: It is important to use medium supplemented with 2% CSS for a steroid-deprived medium, otherwise the androgen stimulation will be hindered.

27. Then, the media is changed for fresh medium supplemented with 2% CSS to which the drug treatments are added: either vehicle (1% DMSO) or 10 nM R1881 and/or 100 nM Torin 1 and the cells are left for another 48 h.

Note: Seed your cells to achieve 90% confluence after your treatments on the day of the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-mTOR (Y391)	Abcam	Cat#ab32028; RRID: AB_881283
Rabbit IgG isotype control	Thermo Fisher Scientific	Cat#10500C; RRID: AB_2532981
Chemicals, peptides, and recombinant proteins		
Acetonitrile (Optima™ LC/MS grade)	Fisher Scientific	Cat#A9554
Ammonium hydrogen carbonate (AMBIC)	Alfa Aesar	Cat#14249
Ammonium hydroxide (NH_4OH) solution 28%	MilliporeSigma	Cat#338818-100ML
Bovine serum albumin (BSA)	Multicell	Cat#800-095-CG
Charcoal, activated	MilliporeSigma	Cat#C9157

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dextran T70	Pharmacia	Cat#17-0280-01
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	Cat#BP231100
Disuccinimidyl glutarate (DSG)	Santa Cruz Biotechnology	Cat#sc-285455
Dithiothreitol (DTT)	MilliporeSigma	Cat#43815-1G
Dynabeads® Protein A	Thermo Fisher Scientific	Cat#10008D
Dynabeads® Protein G	Thermo Fisher Scientific	Cat#10009D
Ethylenediaminetetraacetic acid (EDTA)	BioShop	Cat#EDT001.1
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	Bio Basic	Cat#ED0077
Fetal bovine serum (FBS)	Gibco	Cat#12483-020
Filter 0.2 µm (Filtropur BT 50, bottle top filter, 500 mL)	Sarstedt	Cat#83.3941.101
Filter 0.22 µm (syringe filter)	Fisher Scientific	Cat#09-720-004
Formaldehyde 37%	JT Baker	Cat#2106-01
Formic acid (Optima™ LC/MS grade)	Fisher Scientific	Cat#A11750
Glycerol	VWR	Cat#BDH1172-4LG
Glycine	VWR	Cat#0167-1KG
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Fisher Scientific	Cat#BP310-500
Iodoacetamide (IAA)	MilliporeSigma	Cat#I1149-5G
Lithium chloride (LiCl)	MilliporeSigma	Cat#310468-100G
Methanol (HPLC Grade)	Fisher Scientific	Cat#A452-4
Sodium chloride (NaCl)	Fisher Scientific	Cat#S671-3
N-Lauroylsarcosine	Acros Organics	Cat#434371000
Nonidet P-40 (NP-40)	Roche	Cat#11754599001
Penicillin-Streptomycin solution	Wisent Bioproducts	Cat#450-200-EL
Protease inhibitors	Thermo Fisher Scientific	Cat#A32965
Proteinase K	BioShop	Cat#PRK403.250
R1881	Steraloids	Cat#E3164-000
Resin AG® 1-X8	Bio-Rad	Cat#140-1441
RPMI-1640	Wisent Bioproducts	Cat#350-046-CL
Sodium dodecyl sulfate (SDS)	BioShop	Cat#SDS001.1
Sequencing Grade Modified Trypsin	Promega Corporation	Cat#V5111
Sodium bicarbonate ($NaHCO_3$)	Fisher Scientific	Cat#BP328-1
Sodium desoxycholate	Fisher Scientific	Cat#S285-100
Sodium pyruvate	Wisent Bioproducts	Cat#600-110-EL
Torin 1	Toronto Research Chemicals	Cat#T548700
Trifluoroacetic acid (TFA, Optima™ LC/MS grade)	Fisher Scientific	Cat#A11650
Tris (UltraPure)	Invitrogen	Cat#15504-020
Triton X-100	BioShop	Cat#TRX506.500
Urea	MilliporeSigma	Cat#U5378-100G
Water (Optima™ HPLC grade)	Fisher Scientific	Cat#W71
Whatman Filter paper (8 µm)	Fisher Scientific	Cat#05-714-4
Critical commercial assays		
QIAquick PCR purification kit	QIAGEN	Cat#28106
Oasis MCX 96-well µelution plate	Waters	Cat#186001830BA
Deposited data		
mTOR RIME(LNCaP +/- R1881 and/or Torin1; 22Rv1 +/- R1881 and/or Torin1; DU145; PC3; RWPE1 +/- R1881 and/or Torin1)	Dufour et al. (2022)	ProteomeXchange Consortium via the PRIDE partner repository: PXD026101 and DOI: http://dx.doi.org/10.6019/PXD026101
Experimental models: Cell lines		
Human LNCaP cells (clone FGC)	ATCC	Cat#CRL-1740
Human 22Rv1 cells	ATCC	Cat#CRL-2505
Human DU145 cells	ATCC	Cat#HTB-81
Human PC3 cells	ATCC	Cat#CRL-1435
Software and algorithms		
Proteome Discoverer	Thermo Scientific	Cat#OPTON-20141
Mascot	Matrix Science	http://www.matrixscience.com/
Scaffold	Proteome Software	https://www.proteomesoftware.com/

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Easy-nLC II system	Thermo Fisher Scientific (Proxeon Biosystems)	https://www.thermofisher.com/
LTQ Orbitrap Velos spectrometer	Thermo Fisher Scientific	https://www.thermofisher.com/

MATERIALS AND EQUIPMENT

Lysis buffer 1 (LB1)		
Reagent	Final concentration	Amount
HEPES-KOH 1 M (pH 7.5)	10 mM	2.5 mL
NaCl 5 M	140 mM	1.4 mL
EDTA 0.5 M	1 mM	100 µL
Glycerol	10%	5 mL
NP-40	0.5%	250 µL
Triton X-100	0.25%	125 µL
Water (HPLC grade)	n/a	40.625 mL
Total	n/a	**50 mL**

Add protease inhibitors only before use. We noted that preparing fresh buffer is more efficient, but you can keep it 1 month at 4°C with less efficiency. Check pH before use. Filter buffer with 0.22 µm filter unit.

Lysis buffer 2 (LB2)		
Reagent	Final concentration	Amount
Tris-HCl 1 M (pH 8)	10 mM	500 µL
NaCl 5 M	200 mM	2 mL
EDTA 0.5 M	1 mM	100 µL
EGTA 0.5 M	0.5 mM	50 µL
Water (HPLC grade)	n/a	47.35 mL
Total	n/a	**50 mL**

Add protease inhibitors only before use. We noted that preparing fresh buffer is more efficient, but you can keep it 1 month at 4°C with less efficiency. Check pH before use. Filter buffer with 0.22 µm filter unit.

Lysis buffer (LB3)		
Reagent	Final concentration	Amount
Tris-HCl 1 M (pH 8)	10 mM	500 µL
NaCl 5 M	100 mM	1 mL
EDTA 0.5 M	1 mM	100 µL
EGTA 0.5 M	0.5 mM	50 µL
Sodium desoxycholate	0.1%	0.05 g
N-Lauroylsarcosine 10%	0.5%	2.5 mL
Water (HPLC grade)	n/a	Adjust to 50 mL
Total	n/a	**50 mL**

Add protease inhibitors only before use. We noted that preparing fresh buffer is more efficient, but you can keep it 1 month at 4°C with less efficiency. Check pH before use. Filter buffer with 0.22 µm filter unit.

Fast de-crosslink buffer		
Reagent	Final concentration	Amount
SDS 10%	1%	100 µL
$NaHCO_3$ 1 M	0.1 M	100 µL
NaCl 5 M	0.2 M	40 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Proteinase K (10 mg/mL)	0.5 mg/mL	50 µL
Water (HPLC grade)	n/a	710 µL
Total	n/a	**1 mL**

Prepare freshly, do not keep. There is no need to filter this buffer as it is used to test an aliquot of the samples for sonication efficiency.

RIPA buffer		
Reagent	Final concentration	Amount
HEPES (pH 7.6)	50 mM	0.596 g in 25 mL water Adjust pH to 7.6
EDTA 0.5 M	1 mM	100 µL
NP-40	1%	500 µL
Sodium desoxycholate	0.7%	0.35 g
LiCl	0.5 M	1.05 g
Water (HPLC grade)	n/a	Adjust to 50 mL
Total	n/a	**50 mL**

Add protease inhibitors only before use. Prepare freshly, do not keep. Filter buffer with 0.22 µm filter unit.
Keep cold at 4°C prior to use.

AMBIC 100 mM		
Reagent	Final concentration	Amount
AMBIC	100 mM	0.395 g
Water (HPLC grade)	n/a	Adjust to 50 mL
Total	n/a	**50 mL**

Prepare freshly, do not keep. Keep cold at 4°C prior to use. Filter buffer with 0.22 µm filter unit.

PBS 1×/0.5% BSA		
Reagent	Final concentration	Amount
BSA	0.5%	0.25 g
PBS 1×	n/a	Adjust to 50 mL
Total	n/a	**50 mL**

Prepare freshly. This solution can be kept 1 week at 4°C. Filter buffer with 0.22 µm filter unit.

Glycine 2.5 M		
Reagent	Final concentration	Amount
Glycine	2.5 M	18.76 g Adjust pH to 3.5
Water (HPLC grade)	n/a	Adjust to 100 mL
Total	n/a	**100 mL**

This solution can be kept several months at 22°C–24°C. Filter solution with 0.22 µm filter unit.

⚠ CRITICAL: Using HPLC grade water helps to obtain better results during mass spectrometry.

Alternatives: This protocol uses a manual sonicator, Fisher Scientific model 100. However, other sonicators can be used while keeping in mind that the sonication needs to be optimized to obtain a chromatin concentration around 500 base pairs (bp) while avoiding overheating the samples.

STEP-BY-STEP METHOD DETAILS

ChIP bead preparation (day -1)

Timing: 1 h + 16–24 h incubation

This step allows conjugation of the target protein directed antibody to magnetic beads for ChIP the next day.

1. For each ChIP, place an Eppendorf tube containing 1 mL of ice-cold PBS 1×/0.5% BSA on a magnetic rack. Add 50 µL of well resuspended magnetic Dynabeads® Protein A or G (mix by gentle inversions), taking care to pipet up and down repeatedly to not leave any beads in the tips.

   ⚠ CRITICAL: Avoid vortexing the magnetic beads as this may damage them and affect the quality of the MS results (troubleshooting 2).

   Note: The choice of Dynabeads® Protein A or G depends on the antibody species and isotypes. Please refer to the manufacturer's instructions.

2. Wash four times with 1 mL of ice-cold PBS 1×/0.5% BSA. For each wash, resuspend the beads by tube inversions, spin quickly and use the magnet to remove the liquid before the next wash to avoid losing beads.
3. Add 500 µL ice-cold PBS 1×/0.5% BSA and 5 µg of antibody against your protein of interest (In Dufour et al., 2022, we used an mTOR-specific antibody) or non-specific IgG of the species targeted by your specific antibody (e.g., IgG Rabbit in our case). Resuspend by tube inversions.

   Note: The quantity of antibody for each ChIP needs to be optimized as it depends on the antibody and the genomic abundance of the target protein on chromatin (troubleshooting 3).

   Note: To ensure reproducibility between independent experiments, keep enough antibody from the same lot for all replicates to avoid differences in antibody concentration and/or affinity for the target protein.

4. The antibody-bead mixture is left 16–24 h on a rotator at 4°C.

Double crosslinking (day 0)

Timing: 1.5 h

In this step, protein-protein crosslinking is achieved with disuccinimidyl glutarate (DSG) and protein-DNA crosslinking with formaldehyde.

5. Remove media from the cell culture plate and wash with 20 mL of PBS 1× containing protease inhibitors.
6. For double crosslinking:
   a. Incubate cells with mild agitation for 20 min in 20 mL of PBS 1× containing 2 mM DSG (40 µL of DSG 1 M for 20 mL of PBS 1×).
   b. Without removing the PBS/DSG, add formaldehyde directly to 1% final (541 µL of 37% formaldehyde) for an additional 10 min at 22°C–24°C with mild agitation.

   Note: Add formaldehyde inside the plate with PBS/DSG, so DSG stays 30 min and formaldehyde 10 min.

7. Crosslinking is quenched by the addition of 0.1 M glycine final, pH 3.5 (824 µL of 2.5 M glycine, pH 3.5).
8. Wash twice with 20 mL of ice-cold PBS 1× quickly.

Note: To quickly stop the double-crosslinking, simply remove the PBS + DGS/formaldehyde by inverting the plate into a waste container. Do not waste time aspirating the media which could also dislodge adherent cells. When cold PBS is added to the plates during the washes, add it gently near the sides of the plates to avoid detaching cells.

9. Scrape cells using a silicone scraper and collect cells from two plates into a 15 mL conical tube.
10. Centrifuge at 2,000 × *g* (3,170 rpm) for 5 min at 4°C to obtain the crosslinked cell pellet.
11. Remove the supernatant and go to the next step.

⚠ CRITICAL: Crosslink all plates for the exact same time. To avoid variations, label the plates from 1, 2,… etc. and start the timer after addition of the first crosslinker to plate #1 and space the addition of PBS/DSG every 30 s for subsequent plates. Do the same for the second crosslinker in the same order of the numbered plates.

Nucleus isolation (day 0)

Timing: 1 h

This step allows isolation of nuclear pellets without cytosolic protein contamination.

12. Add 10 mL of cold lysis buffer 1 (LB1) containing protease inhibitors to crosslinked cell pellets, resuspend by pipetting up and down and rotate at 4°C for 10 min.
13. Centrifuge at 2,000 × *g* (3,170 rpm) for 5 min at 4°C and remove the supernatant. The pellet is constituted of nuclei.
14. Add 10 mL of cold LB2 with protease inhibitors to the nuclear pellets, resuspend by pipetting up and down and rotate at 4°C for 10 min to wash the nuclei and remove contamination from cytosolic proteins.
15. Centrifuge at 2,000 × *g* (3,170 rpm) for 5 min at 4°C and remove the supernatant. See below for an optional pause point after this step, although we do not recommend it.
16. Add 500 µL of cold LB3 with protease inhibitors per number of plates used (e.g., if you collected cells from two plates, put 1 mL of LB3) and resuspend nuclei by pipetting up and down.
17. Aliquot resuspended nuclei in 500 µL volumes in Eppendorf tubes and continue to sonication.

Pause point: Freezing samples decreases the efficiency of RIME. If you wish to freeze at this point, freeze the nuclear pellets without supernatant (after completing step 15) using a dry ice/ethanol slurry, then store the samples at −80°C. We strongly recommend to not freeze samples until reaching step 27 after completing the ChIP. Ideally, if possible, do not freeze samples until after the entire on-beads digestion protocol is completed.

Sonication (day 0)

Timing: 2 h

This step allows for the generation of chromatin fragments enriched ~500 bp in size prior to performing ChIP for isolation of bait protein-containing complexes bound to chromatin.

18. Sonicate nuclei on an ice bath 8–10 times each for 15 s by pulse using a manual sonicator, Fisher Scientific model 100, set to power 11. Additional rounds of sonication may be necessary. See Notes below.

Note: To prevent the samples from overheating, we recommend using an ice bath rather than ice only and rotate between tubes during sonication pulses. Adding water to ice helps keep the samples colder during sonication with the ice-cold water forming a more direct contact with the tubes. Do not add too much water, otherwise the tubes may flip over and risk sample loss and contamination. Tubes should be firmly placed in the ice bath. Remember to clean the probe between tubes from different conditions.

Note: During sonication, the sonicator probe should be inserted enough into the liquid (middle is recommended) to avoid sample bubbling (foaming) causing likely sample loss and protein degradation. Avoid touching the bottom of the tubes with the sonicator probe to prevent plastic contamination and tube piercing from overheating.

Note: As an indicator that the sonication is working, the liquid should become more clear (less opaque) and consequently the graduated lines of the Eppendorf tube will be more visible. The only way to ensure that the sonication efficiency is reached is to validate this by analyzing the samples by agarose gel electrophoresis (see steps 19–21 below). A double crosslinked sample is always harder to sonicate than a single crosslinked sample (troubleshooting 4).

19. Add 30 μL of 10% Triton X-100 to each sample tube.
20. Centrifuge at 13,684 × *g* (12,000 rpm) for 15 min at 4°C and pool supernatants from the same condition into the same new Eppendorf tube. Keep cold on ice.
21. Verify the sonication efficiency by agarose gel electrophoresis to ensure that DNA was fragmented to 200–1,000 bp with a concentration at ~500 bp:
    a. Transfer 5 μL of sonicated nuclear lysate in a new Eppendorf tube for each condition.
    b. Add 95 μL of chromatin fast de-crosslinking buffer and incubate at 65°C for 1 h.

Note: Vortexing the samples prior to the start of the incubation, at one point during the incubation, and at the end will aid in the efficiency of the de-crosslink.

    c. Purify de-crosslinked samples using a QIAquick PCR purification kit (Refer to manufacturer's instructions) and elute in 30 μL elution buffer from the kit.
    d. Load 15 μL of eluate with DNA loading dye on a 1% agarose gel with ethidium bromide to detect sonication efficiency. Examples of chromatin efficiency are shown in Figure 1.
    e. If the desired sonication is achieved, continue to step 22, otherwise sonicate more, and repeat steps 17, 18, and 21.

⚠ CRITICAL: Always sonicate on ice and avoid foaming to not denature and precipitate proteins.

Chromatin immunoprecipitation (day 0)

Timing: 1 h + 16–24 h incubation

This step allows to keep only the protein complexes that contain your protein of interest.

22. Wash the antibody-bound beads (step 4) three times with 1 mL ice-cold PBS 1×/0.5% BSA to remove any unbound antibody. Refer to step 2 for washing guidelines.
23. Add the sonicated nuclear lysate (~1 mL) from step 20 per ChIP (two 15 cm plates / ChIP).
24. Tubes are left to rotate at 4°C for 16–24 h.

Washing (day 1)

Timing: 1 h

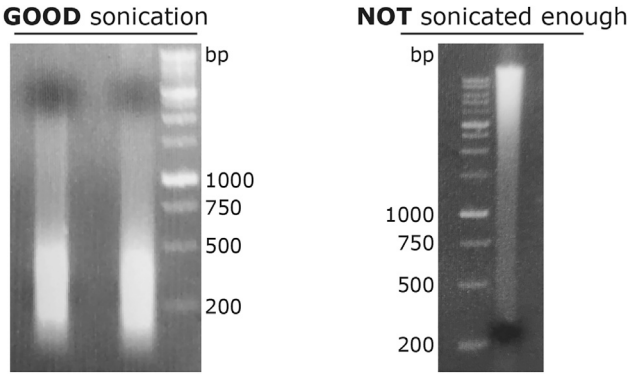


Figure 1. Example of good and bad sonicated chromatin smears
Bp, base pairs.

This step removes non-specific protein complexes with RIPA buffer washes and removes detergents and salts for peptide ionization and trypsin digestion of proteins with ammonium bicarbonate (AMBIC) buffer washes (troubleshooting 5).

25. The next day, wash beads five times with 1 mL of fresh ice-cold RIPA buffer with protease inhibitors. Refer to step 2 for washing guidelines.
26. Wash 4 times with 500 µL of cold 100 mM AMBIC freshly made. For the second AMBIC wash, transfer the beads to a new Eppendorf tube to avoid non-specific protein attachment to the tube.
27. Resuspend beads in 50 µL of cold fresh 100 mM AMBIC and keep on ice. Your samples are now ready to be digested by trypsin and subsequent MS analysis.

Pause point: Freezing samples decreases the efficiency of RIME, but if you wish to freeze at this point, freeze the resuspended beads prior to on-beads digestion using a dry ice/ethanol slurry and store the samples −80°C. If possible, do not freeze until after completing all steps of the on-beads digestion protocol.

On-beads digestion

Timing: 3 h + 16–24 h incubation

The on-beads digestion used in the study by Dufour et al. (2022) was performed as follows:

28. For 16–24 h at 37°C with a ratio trypsin enzyme:protein of 1:20 in 2 M urea/≈50 mM AMBIC performed as follows (troubleshooting 6):
    a. Add 100 µL of 6 M urea/100 mM AMBIC directly onto the washed beads that were resuspended with 50 µL 100 mM AMBIC from step 27, for a final concentration of 4 M urea.
    b. Shake the samples for 10 min at 1,200 rpm on an Eppendorf MixMate®.
    c. Add 200 µL of 50 mM AMBIC to decrease the urea concentration to less than 2 M.
    d. For trypsin, 5 µg protein were considered. Thus, for a ratio 1:20 enzyme:protein, 0.25 µg of trypsin was added to the sample (e.g., 5 µL of trypsin at 0.05 µg/µL).
    e. Incubate at 37°C for 16–24 h.
29. Reduce samples with dithiothreitol (DTT) at a final concentration of ≈13 mM (e.g., add 150 µL of 45 mM DTT/100 mM AMBIC) at 37°C for 30 min.
30. After cooling for 10 min, alkylate the samples with iodoacetamide (IAA) at a final concentration of ≈23 mM (e.g., add 150 µL of a 100 mM IAA/100 mM AMBIC solution) at 22°C–24°C for 20 min in the dark.

31. Acidify the supernatants with 4.5 µL of trifluoroacetic acid (TFA) 100% and clean from residual detergents and reagents with MCX cartridges (Waters Oasis MCX 96-well µElution Plate) following these instructions:
    a. Wet cartridge with 500 µL of methanol.
    b. Equilibrate with 500 µL of 0.1% TFA.
    c. Resuspend sample with a minimal amount (200–400 µL) of 0.1% TFA or acidify sample with 10% TFA so that the pH is < 3.
    d. Load sample slowly to cartridge. Can load repeatedly to ensure binding (recuperate the flow through in the original tube).
    e. Wash with 500 µL of 0.1% TFA.
    f. Wash with 500 µL of 80% acetonitrile/0.1% TFA.
    g. Wash with 500 µL of water (HPLC grade).
    h. Elute with 350 µL of fresh 1:9 ratio of ammonium hydroxide (NH_4OH):methanol (or with 250 µL if using LowBind tubes).

Note: Use a vacuum manifold to pull solvents through the plate. Be sure to elute into glass tubes. MCX cartridges should be able to be run dry without loss of recovery. Cover plate with aluminum tape and cut holes for each sample. Re-cover used holes with aluminum tape and mark as used.

Note: For the 1:9 ratio of NH_4OH:methanol solution: add 200 µL of 28% NH_4OH to 1 mL of methanol, then bring the volume up to 2 mL with methanol.

32. Dry samples with a Speed-vac and store them at −20°C for a few days prior to MS analysis.

Mass spectrometry

Timing: 1 day

This MS method is intended to serve as an example. When using other LC or MS instruments, users are recommended to adjust the MS method to their setup. The liquid chromatography tandem mass spectrometry (LC-MS/MS) setup used in the study by (Dufour et al., 2022) consisted of:

33. Samples were reconstituted under agitation for 15 min in 12 µL of 2% acetonitrile/1% formic acid and loaded into a 75 µm i.d. × 150 mm Self-Pack C18 column installed in the Easy-nLC II system. The HPLC system was coupled to a LTQ Orbitrap Velos spectrometer through a Nanospray Flex Ion Source.

Note: The buffers used for chromatography were 0.2% formic acid (solvent A) and 90% acetonitrile/0.2% formic acid (solvent B). Peptides were eluted with a three-slope gradient at a flow-rate of 250 L/min. Solvent B first increased from 2 to 29% in 70 min, then from 29 to 44% in 50 min and finally from 44 to 95% B in 6 min.

34. LC-MS/MS data acquisition was accomplished using a seventeen-scan event cycle comprised of a full scan MS for scan event 1 acquired in the Orbitrap.

Note: The mass resolution for MS was set to 60,000 (at m/z 400) and used to trigger the sixteen additional MS/MS events acquired in parallel in the linear ion trap for the top sixteen most intense ions. Mass over charge ratio range was from 360 to 1700 for MS scanning with a target value of 1,000,000 charges and from ~1/3 of parent m/z ratio to 2000 for MS/MS scanning with a target value of 10,000 charges. The data dependent scan events used a maximum ion fill time of 100 ms and 1 microscan. Target ions already selected for MS/MS were dynamically excluded for 31 s after 2 counts. Nanospray and S-lens voltages were set to 1.3–1.7 kV and

50 V, respectively. Capillary temperature was set to 250°C. MS/MS conditions were the following: normalized collision energy, 35 V; activation q, 0.25; activation time, 10 ms.

Alternatives: Other HPLC and mass spectrometry instrumentation represent valid options for this analysis. For other examples, refer to: (Mohammed et al., 2013), (Mohammed et al., 2016), (D'Santos et al., 2015), and (Serandour et al., 2018).

RIME protein identification

Timing: 1 day

The method for RIME protein identification used in (Dufour et al., 2022) consisted of:

35. The peak list files were generated with Proteome Discoverer version 2.3 using the following parameters:
    a. Minimum mass set to 500 Da.
    b. Maximum mass set to 6000 Da.
    c. No grouping of MS/MS spectra.
    d. Precursor charge set to auto.
    e. Minimum number of fragment ions set to 5.
36. Protein database searching was performed with Mascot version 2.6 against the human Uniprot complete protein database using the following parameters:
    a. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.6 Da, respectively.
    b. Trypsin was used as the enzyme allowing for up to 2 missed cleavages.
    c. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation and phospho of serine, threonine and tyrosine were variable modifications.
37. Data interpretation was performed using Scaffold software version 4.10.0 (See quantification and statistical analysis).

EXPECTED OUTCOMES

A successful RIME should show a good coverage of your protein of interest and be identified among top proteins with the most peptides identified. The actual value of the coverage varies between proteins. This is dependent on the size of the proteins: the larger the protein, the more peptides are needed to cover it properly. On the contrary, when a protein is small, few peptides are needed to have a good coverage, but these peptides must come back often in the case of a good RIME.

For example, in the study by (Dufour et al., 2022), we performed RIME against mTOR, a large protein of 289 kDa. From our mTOR RIME study in LNCaP cells, mTOR was ranked 15th, 9th, 13th, and 10th among the total unique proteins identified in vehicle (263 proteins), R1881 (androgen)-stimulated (289 proteins), Torin 1-mediated mTOR inhibition (240 proteins), and R1881+Torin 1-treated (248 proteins) conditions, respectively (Figure 2A). The coverage of mTOR was around 30% for all the conditions (Figure 2B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 0.5–1 day

- For MS quantification, the first part was performed using the proteome software tool Scaffold (Figure 3).
  - Open all your conditions in the same window.

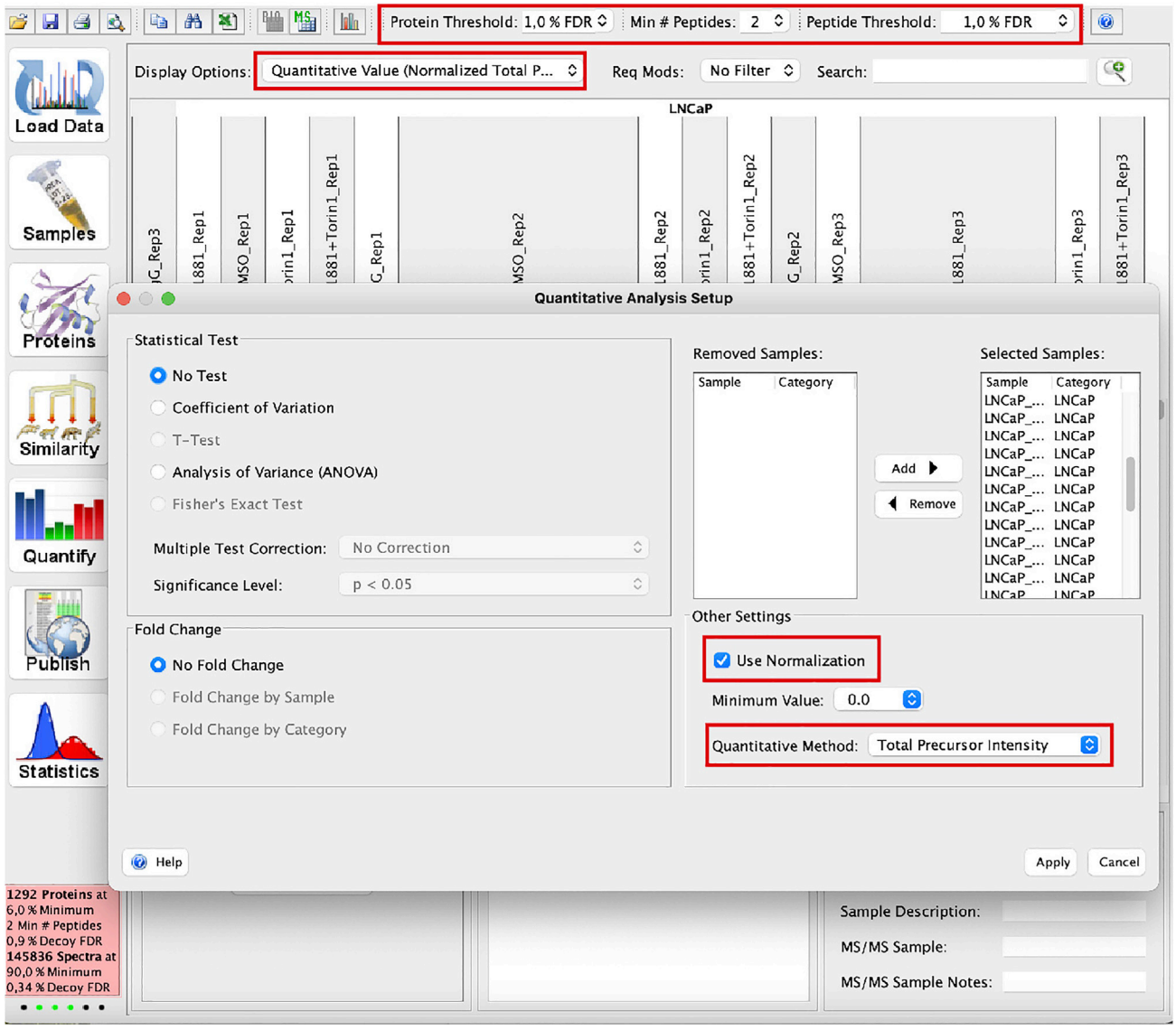


Figure 2. Example of Scaffold software parameter windows

- Select a 1% protein and 1% peptide FDR threshold with a minimum number of detected peptides set to 2.
- Select total precursor intensity and normalization of the samples for the quantitative method.
- Export the data in a table sheet format.

- Further computational analyses are performed outside of Scaffold in a table software (e.g., Microsoft excel).
  - Quantitative values are log2-transformed.
  - Missing values are designated as "NaN".
  - Protein clusters and DECOYs are removed.
- For each condition, proteins are only retained if peptides are detected in at least 2 of 3 replicates and for which we could validate the presence of 2 unique peptides in at least 1 replicate.
- For the identification of interacting proteins in a specific condition, only proteins with no peptides found in matching IgG controls or having a mean TPI fold-change $\geq$ 5 over IgG were retained.

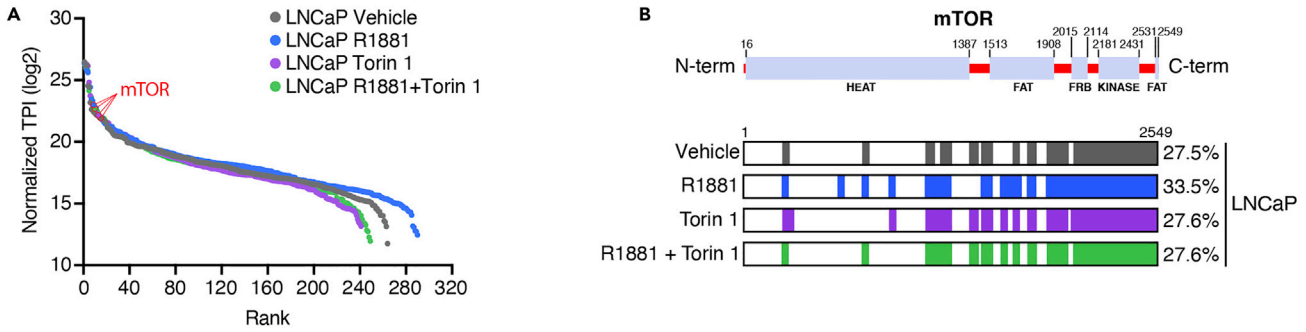


Figure 3. Example of results of an mTOR RIME experiment in LNCaP cells

(A) Ranking of the mTOR bait protein among all the proteins identified in LNCaP cells treated with either vehicle (DMSO), R1881, Torin 1, or R1881 + Torin 1.

(B) Coverage of mTOR by RIME in LNCaP cells treated with different conditions. Data in (A and B) were adapted from Dufour et al. (2022).

- For comparison of groups, a gain or loss in associations was denoted as significant if one of the following criteria were met:
  - Proteins found in at least 2 of 3 replicates in both conditions and display a fold-change ≥ 5 or ≤ -5 with a significant associated p-value < 0.05 for comparison of mean TPI.
  - Proteins found in at least 2 of 3 replicates in one condition and 1 replicate in the second group and display a fold-change ≥ 5 or ≤ -5 between the two groups (no p-value can be calculated).
  - Detected in at least 2 of 3 replicates from one group and completely absent in the other (no statistical values can be calculated).

LIMITATIONS

The use of RIME for the identification of chromatin-associated interactors of a protein of interest has its limitations including false positives via non-specific antibody affinities or non-chromatin bound interactions as well as inaccuracies in quantitative measures as not all peptides are detected by MS and the use of label-free quantification remains a semi-quantitative analysis. Other issues can affect the outcome of the RIME method and are addressed in the troubleshooting section.

TROUBLESHOOTING

Problem 1

Keratin is one of the most abundant proteins in your sample, hindering identification by MS of other proteins of interest (refer to subsection before you begin).

Potential solution

Keratin is found everywhere, on our skin, hair, etc., thus, we must handle the samples with the greatest possible care. Tie your hair, wear a clean gown, mask, and gloves, clean the bench area used for sample preparation, and do not talk over the samples as this will greatly avoid keratin sample contamination.

Problem 2

Foreign product is detected in abundance during mass spectrometry (refer to subsection ChIP bead preparation (day -1), step 1).

Potential solution

We had encountered an issue with contamination from the coating of the magnetic beads when beads were taken from a bottle that was almost finished. Indeed, it seems that if the beads are too vortexed or old, the coating of the beads can contaminate the results of MS. Thus, we recommend not to vortex your beads, resuspend them gently by inversion. For complete assurance, use a new bottle.

Problem 3

Peptides of the antibody are too abundant and hide the signal of proteins of interest (refer to subsection ChIP bead preparation (day -1), step 3).

Potential solution

This is a delicate point in the optimization of the protocol. We advise starting with 5 μg of antibody or the amount you are already using if you are accustomed to performing ChIPs with this antibody. It is recommended to perform a test RIME experiment with the bait protein and IgG control in one condition to verify that the ratio of peptides of the antibody to peptides of the protein of interest is correct. Keep in mind that the more antibodies you put in, the more background due to antibody peptides as well as possible non-specific interactions.

Problem 4

Sonication is not effective (refer to subsection sonication (day 0), step 18).

Potential solution

Double crosslinked samples are always quite hard to sonicate. Do not hesitate to increase the number of sonication cycles compared to single crosslinked samples. Also, sonicating in small volumes is always more efficient than sonicating in one large volume.

Problem 5

Detergents or salts contaminate your sample (refer to subsection washing (day 1)).

Potential solution

Detergents and salts are removed by washing in AMBIC solution. Do not hesitate to increase the number of these washes if you still have contamination.

Problem 6

Incomplete protein digestion (refer to subsection on-beads digestion, step 28).

Potential solution

If your MS data is limited (low peptide coverage) due to insufficient protein digestion, it is possible to use an enzyme other than trypsin, or to perform sequential digestions with multiple enzymes for a more complete digestion of proteins. Alternative proteolytic enzymes include AspN, GluC, chymotrypsin, and clostripain.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vincent Giguère (vincent.giguere@mcgill.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article (Dufour et al., 2022) includes all datasets generated or analyzed during this study. mTOR RIME data reported in this paper were deposited to the ProteomeXchange Consortium via the PRIDE partner repository under accession number PXD026101 and DOI: http://dx.doi.org/10.6019/PXD026101.

ACKNOWLEDGMENTS

We thank Dr. Jason Carroll for his guidance and help in setting up this protocol in our laboratory. We also thank members of the V.G. laboratory as well as Denis Faubert and Josée Champagne from the

proteomic facility at the Institut de Recherches Cliniques de Montréal for helpful discussions. This work was supported by a Terry Fox Research Institute Team Grant (PPG-1091), a Foundation Grant from the Canadian Institutes of Health Research (CIHR) to V.G. (FDT-156254), and an operating grant from the Cancer Research Society and Génome Québec (CRS-25085). C.S. is a recipient of a Canderel Scholarship.

AUTHOR CONTRIBUTIONS

Development of protocol: C.S. and C.R.D. Data analysis: C.R.D. and C.S. Writing of the manuscript: C.S., C.R.D., and V.G. Study supervision: V.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

D'Santos, C., Taylor, C., Carroll, J.S., and Mohammed, H. (2015). RIME proteomics of estrogen and progesterone receptors in breast cancer. Data Brief *5*, 276–280. https://doi.org/10.1016/j.dib.2015.08.019.

Dufour, C.R., Scholtes, C., Yan, M., Chen, Y., Han, L., Li, T., Xia, H., Deng, Q., Vernier, M., and Giguere, V. (2022). The mTOR chromatin-bound interactome in prostate cancer. Cell Rep. *38*, 110534. https://doi.org/10.1016/j.celrep.2022.110534.

Mohammed, H., D'Santos, C., Serandour, A.A., Ali, H.R., Brown, G.D., Atkins, A., Rueda, O.M., Holmes, K.A., Theodorou, V., Robinson, J.L., et al. (2013). Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. Cell Rep. *3*, 342–349. https://doi.org/10.1016/j.celrep.2013.01.010.

Mohammed, H., Taylor, C., Brown, G.D., Papachristou, E.K., Carroll, J.S., and D'Santos, C.S. (2016). Rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) for analysis of chromatin complexes. Nat. Protoc. *11*, 316–326. https://doi.org/10.1038/nprot.2016.020.

Serandour, A.A., Mohammed, H., Miremadi, A., Mulder, K.W., and Carroll, J.S. (2018). TRPS1 regulates oestrogen receptor binding and histone acetylation at enhancers. Oncogene *37*, 5281–5291. https://doi.org/10.1038/s41388-018-0312-2.