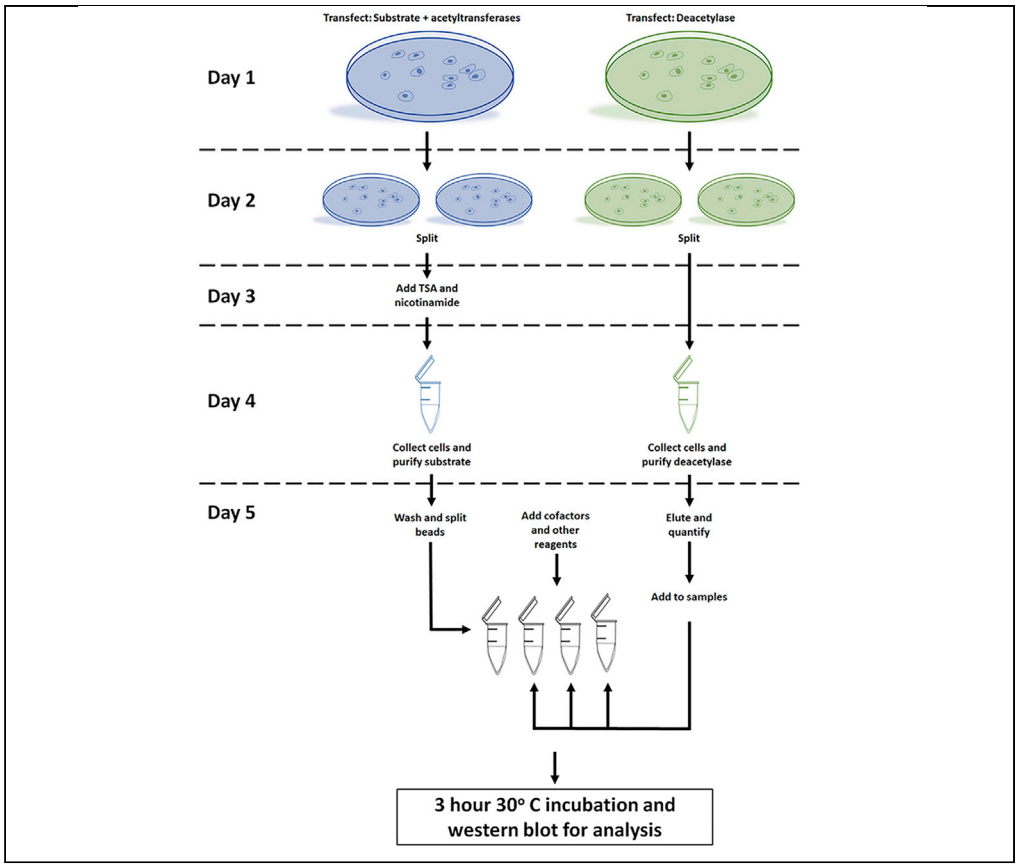
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for *in vitro* lysine deacetylation to test putative substrates of class III deacetylases



Lysine acetylation is an important post-translational modification that is used in multiple cellular pathways, such as the regulation of gene expression at the histone level. The purpose of this assay is to test for putative substrates of class III deacetylases using an *in vitro* method. The *in vitro* analysis helps circumvent confounding variables when assessing for a direct relationship between deacetylase and substrate, such as the effects of other cellular deacetylases or acetyltransferases that modify the substrate *in vivo*.

Elizabeth V. Minten, David S. Yu

eminten@hms.harvard.edu (E.V.M.)
dsyu@emory.edu (D.S.Y.)

Highlights

A protocol to aid in the identification of substrates of class III deacetylases

Steps can be modified for use for versatile substrates

In vitro analysis allows for screening of direct deacetylase-substrate interactions

Minten & Yu, STAR Protocols *3*, 101313
June 17, 2022 © 2022 The Author(s).
https://doi.org/10.1016/j.xpro.2022.101313

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for *in vitro* lysine deacetylation to test putative substrates of class III deacetylases

Elizabeth V. Minten[1,2,3,*] and David S. Yu[1,4,*]

[1]Department of Radiation Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

[2]Department of Radiation Oncology and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322, USA

[3]Technical contact

[4]Lead contact

*Correspondence: eminten@hms.harvard.edu (E.V.M.), dsyu@emory.edu (D.S.Y.)
https://doi.org/10.1016/j.xpro.2022.101313

SUMMARY

Lysine acetylation is an important post-translational modification that is used in multiple cellular pathways, such as the regulation of gene expression at the histone level. The purpose of this assay is to test for putative substrates of class III deacetylases using an *in vitro* method. The *in vitro* analysis helps circumvent confounding variables when assessing for a direct relationship between deacetylase and substrate, such as the effects of other cellular deacetylases or acetyltransferases that modify the substrate *in vivo*.

For complete details on the use and execution of this protocol, please refer to Minten et al. (2021).

BEFORE YOU BEGIN

The deacetylase and putative substrate should be purified using separate tags or antibodies. The deacetylase should also be elutable for purification. We recommend FLAG-tagged deacetylases. Purification as described in this protocol and subsequent quantification (through SDS-PAGE and subsequent Coomassie stain) of the deacetylase should be done before preparation of the substrate. Finally, HEK 293T cells (ATCC CRL-3216) are suggested for this protocol due to their ease for transfection and growth, but other easily transfectable cells, such as HeLa (ATCC CCL-2) are expected to also work.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Acteyl-lysine antibody (1:500)	ImmuneChem	Cat#ICP0380
FLAG antibody (1:1000)	Cell Signaling Technology	Cat#2368S
Acetyl Lysine antibody (1:500)	ImmuneChem	Cat#ICP0380
Alexa Fluor® Plus 488 anti-mouse secondary antibody (1:10000)	Fisher Scientific	Cat#A32723
Alexa Fluor® Plus 594 anti-rabbit secondary antibody (1:10000)	Fisher Scientific	Cat#A32740
Chemicals, peptides, and recombinant proteins		
3× FLAG Peptide	Sigma	Cat#MFCD01863911
Lipofectamine 2000	Invitrogen	Cat#11668019
KCl	Sigma	Cat#7447-40-7

(*Continued on next page*)

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Continued

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
$MgCl_2$	Sigma	Cat#7791-18-6
Glycerol	Sigma	Cat#56-81-5
NP-40	Fisher Scientific	Cat#85124
PMSF	Sigma	Cat#329-98-6
Aprotinin	VWR	Cat#9087-70-1
Leupeptin	Sigma	Cat#103476-89-7
NaF	Sigma	Cat#7681-49-4
β-Glycerophosphate	Sigma	Cat#154804-51-0
Na_2VO_3	Sigma	Cat#13721-39-6
DTT	Bio-Rad	Cat#27565-41-9
NaCl	Fisher Scientific	Cat#7647-14-5
Tris-Base	Sigma	Cat#77-86-1
Tricostatin A (TSA)	Sigma	Cat#58880-19-6
Nicotinamide	Sigma	Cat#98-92-0
SDS (sodium dodecyl sulfate)	Sigma	Cat#151-21-3
Bromophenol blue	Sigma	Cat#34725-61-6
Dulbecco's Modified Eagle Medium (DMEM), high Glucose	Gibco	Cat#11965092
Regular Fetal Bovine Serum (FBS)	Corning	Cat#MT35010CV
Opti-MEM™ I Reduced Serum Medium	Fisher Scientific	Cat#31985062
Experimental models: Cell lines		
HEK 293T	ATCC	CRL-3216
Recombinant DNA		
P300 (expression ready plasmid)	Addgene	23252
CBP (expression ready plasmid)	Addgene	32908
pCAF (expression ready plasmid)	Addgene	8941
Software and algorithms		
Image Studio™	LI-COR	https://www.licor.com/bio/image-studio/
Other		
60 mm TC-treated Culture Dish	Corning	Cat#430166
Pierce™ Protein G Agarose	Fisher Scientific	Cat#20397
Pierce™ Protein A Agarose	Fisher Scientific	Cat#20333
Sepharose® CL-4B	Sigma	Cat#61970-08-9
Odyssey® M	LI-COR	https://www.licor.com/bio/odyssey-m/

Alternatives: Other cell lines can be substituted for HEK 293T, such as HeLA (ATCC CCL-2). See troubleshooting: Problem 3 if purification of the deacetylase is low. Different acetyltransferases than P300, CPB, and pCAF may be needed for optimal acetylation signal. See troubleshooting: Problem 1. The secondary antibodies needed for dual labeling may also differ depending on the species of any primary antibodies used.

MATERIALS AND EQUIPMENT

⚠ CRITICAL: Many of the chemicals listed in this protocol may be hazardous or toxic. For all chemicals, safety guidelines should be consulted before use or disposal.

HEK 293T medium	
Reagent	Final concentration
DMEM, high glucose	90%
FBS	10%

Lysis Buffer		
Reagent	Final concentration	Amount
KCl	180 mM	9 mL (1 M stock)
HEPES pH 7.4	20 mM	1 mL (1 M stock)
$MgCl_2$	1.5 mM	75 µL (1 M stock)
Glycerol	20% V/V	10 mL
NP-40	1% V/V	500 µL
ddH_2O	n/a	Fill to 50 mL (~29.425 mL)

Note: The lysis buffer should be stored a 4°C, for up to one year. Protease inhibitors should be added fresh before each lysis.

Protease Inhibitors		
Reagent	Final concentration	For every 1 mL lysis buffer
PMSF (100 mM)	1 mM	10 µL
Aprotinin (5 mg/mL)	5 µg/mL	1 µL
Leupeptin (5 mg/mL)	5 µg/mL	1 µL
NaF (750 mM)	1 mM	1.3 µL
β-Glycerophosphate (1 M)	1 mM	1 µL
Na_2VO_3 (200 mM)	1 mM	5 µL
DTT (1 M)	1 mM	1 µL

Note: Inhibitors should be added fresh before each lysis due to their short half-lives.

TBS		
Reagent	Final concentration	Amount
Tris pH 7.5	50 mM	2.5 mL uL (1 M stock)
NaCl	150 mM	438 mg
ddH_2O	n/a	Fill to 50 mL (~47.5 mL)

Note: Store at room temperature (23°C) for up to one year.

Deacetylation buffer 5×		
Reagent	Final concentration	Amount
Tris pH 7.5	250 mM	12.5 mL (1 M stock)
NaCl	750 mM	2.2 g
$MgCl_2$	5 mM	250 mL (1 M stock)
ddH_2O	n/a	Fill to 50 mL (~37.25 mL)

Note: Store at 4°C. The buffer can be kept for up to one year.

Master mix (10 reactions)	
Reagent	Amount
TSA (100 mM)	2.4 µL
$MgCl_2$ (150 mM)	1 µL
Deacetylation buffer 5×	18 µL
dH_2O	17.6 µL
Total	39 µL

Note: This master mix should be made fresh each time.

4× SDS		
Reagent	Final concentration	Amount
Tris pH 6.8	250 mM	12.5 mL (1 M stock)
DTT (dithiothreitol)	5%	2.5 mL
SDS (sodium dodecyl sulfate)	8% W/V	4 g
Bromophenol blue	0.4% W/V	0.2 g
Glycerol	40% V/V	20 mL
ddH_2O	n/a	Fill to 50 mL (~15 mL)

Note: Store aliquots at −20°C for up to a year. For longer-term storage, store at −80°C for up to two years.

⚠ CRITICAL: SDS causes respiratory irritation. Weigh in a fume hood and use personal protective equipment, including a face mask.

Alternatives: The same concentration of β-mercaptoethanol can be used in place of DTT.

STEP-BY-STEP METHOD DETAILS

Enzyme and substrate preparation: Day 1

Timing: 30 min

Transfection of cells should be done in the hood.

1. Transfecting of the putative substrate and the deacetylase 10 μg of DNA of the substrate with 250 μL of Opti-MEM. To increase baseline substrate acetylation levels, it's recommended to co-transfect 2–3 μg of DNA of the acetyltransferases known to target the substrate.

   Note: We recommend, as a starting point if the specific acetyltransferases are unknown, using a combination of p300, pCAF, and CBP (see troubleshooting: Problem 1 for further details). Separately, mix gently by tube inversion 18 μL of Lipofectamine 2000 in 250 μL of Opti-MEM. Let both mixtures stand for 5 min, then combine in one tube. Let sit at room temperature (23°C) for 20 min, then add to 5–6 million HEK 293T cells in one 60 mm dish. Add drop-wise, then gently mix the cells.

2. Mix 5 μg of DNA of the deacetylase with 250 μL of Opti-MEM.
   a. Separately, mix 10 μL of Lipofectamine 2000 in 250 μL of Opti-MEM.
   b. Let both mixtures stand for 5 min, then combine together in one tube.
   c. Let sit at room temperature (23°C) for 20 min.
   d. Add in a drop-wise fashion to 5–6 million HEK 293T cells in one 60 mm dish.
   e. Gently mix the cells.
3. Incubate the cells with the transfection reagents and DNA for about 16 h overnight. HEK 293T cells should be incubated at 37°C with 5% CO_2 content, but if other cell lines are used, other conditions may be required. We recommend using HEK 293T cells with a passage number less than 30.

   Alternatives: Different acetyltransferases will optimally target different substrates. This step can be optimized for the substrate using a different combination of acetyltransferases. Refer to troubleshooting: Problem 1.

Enzyme and substrate preparation: Day 2

Timing: 15 min

4. Check the cell confluency and viability of the transfected cells from day 1.

   Note: The cells should be about 90%–95% confluent for both the cells transfected with the deacetylase and the cells transfected with the substrate. The number of dead cells should be minimal, but will depend on the substrate being transfected.

5. Trpysinize the cells and split each dish equally into two 10 cm dishes for a total of four 10 cm dishes.

Enzyme and substrate preparation: Day 3

Timing: 15 min

6. Add to the cells with the substrate transfection ONLY: Tricostatin A (TSA) to a final concentration of 0.5 μM, and nicotinamide to a final concentration of 1 μM to inhibit class I and II, and class III acetyltransferases, respectively. Let cells incubate overnight for 12–16 h to maximize acetylation.

Deacetylase and substrate preparation: Day 4

Timing: 2 h

7. Trypsinize the cells and collect cells in an Eppendorf tube, combining duplicate dishes into one tube. Add an equal amount of media to the amount of trypsin used to neutralize the trypsin.
8. Centrifuge the cells at 3,500 × *g* – 5,000 × *g* for 5 min and discard the supernatant, being careful not to discard the cell pellet.
9. For each tube, wash cells once with 1 mL of PBS and spin for another 5 min at 3,500 × *g* – 5,000 × *g*. Remove the supernatant after the wash, leaving behind only the cell pellet.
10. Lyse each tube of cells with about 400 μL–500 μL cell lysis buffer (described under materials and equipment). Gently rotate the tubes or place on a rocker at 4°C for 30–40 min.
11. Spin the cells down at maximum speed (~17,000 × *g*) for 15 min at 4°C.
12. While the cells are spinning, prepare the beads that will be used for pre-clearing the lysate. 30 μL of a slurry of 50% beads will be used for every 500 μL of lysate. Spin the beads down, then wash the beads 2 times with 500 μL lysis buffer, then resuspend the beads in lysis buffer using a volume equal to $^1/_2$ the initial volume of the bead slurry, to bring back up to the original volume.

    Note: To spin and wash the beads, spin the beads for 30 s at 100 × *g*, then turn the tubes 180 degrees in the centrifuge and spin again for another 30 s at 100 × *g*. Remove supernatant, leaving only the beads behind. Add 500 μL lysis buffer and repeat the spin using the same conditions as just described. Remove buffer leaving only the beads behind.

13. Keep the beads on ice and centrifuge at 4°C. For FLAG-tagged substrates, CL-4B Sepharose beads can be used for pre-clearing.

    Note: Protein G agarose beads can also be used if the antibody that will be used for the IP will be mouse, while protein A agarose beads can be used for preclearing if the IP antibody will be rabbit.

14. After the spin is complete from step 13, carefully transfer the supernatant to a clean tube; discard the cell debris. To preclear the lysate, add 30 μL of the cleaned pre-clearing beads per every 500 μL of lysate. Rotate the lysate and beads at 4°C for 15 min.

15. While the lysate is pre-clearing, prepare the beads for immunoprecipitation for the tags that will be used. 30 μL of a 50% slurry of conjugated beads will be used for every 500 μL of lysate. Prepare the beads as described in step 12.

Note: Protein G agarose beads should be used if the antibody that will be used for the IP will be mouse, while protein A agarose beads should be used if the IP antibody will be rabbit.

16. After the pre-clear has completed, spin the lysate and beads down using the same conditions used in step 12 for washing the beads. Move the supernatant to their respective beads for immunoprecipitation using an anti-FLAG antibody (or antibody against the tag).
17. Allow tubes to rotate at 4°C overnight (12–16 h). The amount of antibody used for the immunoprecipitation will depend on manufacturer's instructions, but a 1 μg antibody:1 mg of lysate is generally suggested as a starting point for optimization.

Deacetylase preparation: Day 5

Timing: 45 min

18. To collect the deacetylase, wash beads 4 times with lysis buffer using the same conditions as described in step 12.
19. Wash the beads 3 more times with TBS using the same conditions as described in step 12 with. At the end, remove the supernatant, only leaving the beads. Add 100 μL of TBS for every two dishes of cells to the beads (this volume can be adjusted but should be 2 times the volume of the packed beads at minimum).
20. For elution using a FLAG-tagged deacetylase, add 3 μL of 3× FLAG Peptide stock (5 mg/mL) for every 100 μL TBS.
21. To elute, either manually agitate the beads by gentle tapping every 5 min for 30 min on ice or rotate at 4°C for 30 min, if the volume is sufficient (we recommend about no more than 2/3 of the volume of the tube be empty; e.g., for a 1.5 mL Eppendorf tube, the minimum volume would be about 500 μL).
22. After 30 min, spin down the beads as described in step 12. Transfer the supernatant, which contains the eluted deacetylase, into small aliquots for storage at −80°C, for up to 1 year.
23. To quantify the concentration of the deacetylase, run a sample of the elutant on an SDS-PAGE gel against a BSA standard curve and stain with Coomassie blue.

Note: Measurement using a Bradford protein assay can also be used, but may be less precise depending on the amount of non-specific proteins pulled down during purification. Roughly, the concentration of the deacetylase should be around 1 μg of protein/15 μL. About 1 μg of deacetylase will be needed for each reaction where the deacetylase is used.

Deacetylase assay: Day 5

Timing: 4 h

24. Wash the beads bound to the substrate 4 times with lysis buffer.
25. After the wash with lysis buffer, wash the beads 2 times with 1× deacetylation buffer. Resuspend the beads in 1 mL of deacetylation buffer and equally split the beads into N tubes, where N is the number of treatments/conditions. We recommend four reactions below to demonstrate deacetylation of the substrate of interest.
26. Spin the beads down as described in step 12 and carefully aspirate away all liquid using a vacuum or pipette, ensuring even distribution of the beads in each tube.
27. The reactions for the conditions should be set up as follows:

Reagent	Reaction 1	Reaction 2	Reaction 3	Reaction 4*
Master Mix	3.9 μL	3.9 μL	3.9 μL	3.9 μL
Deacetylase*	0	~15 μL**	~15 μL**	~15 μL**
NAD^+ (50 mM)	0†	0	4.8 μL	4.8 μL
Nicotinamide (2 M)	0	0	0	.3 μL*
TBS	15 μL	0	0	0
dH_2O	5.1 μL†	5.1 μL	0.3 μL	0*
Total	24 μL	24 μL	24 μL	24 μL

Note: The deacetylase should be added LAST to each tube.

*: A deacetylase-dead mutant may be used instead of the wild-type deacetylase in place of using nicotinamide to inhibit the enzymatic reaction. If this is done, omit the addition of nicotinamide and instead add 0.3 μL of dH_2O.

**: Volume may vary, refer to step 23.

†: Alternatively, 4.8 μL of NAD^+ and 5.1 μL of dH_2O can be added instead as a control for contamination of the pull down of the substrate with class III deacetylases.

a. Reaction 1: No deacetylase.
   i. This reaction should show the baseline levels of substrate acetylation.
b. Reaction 2: Deacetylase only.
   i. This reaction should not show any change in acetylation levels compared to reaction 1 due to lack of necessary cofactor NAD^+ for class III deacetylases. NAD^+ can be added if contamination with other class III deacetylases is expected.
c. Reaction 3: Deacetylase + NAD^+
   i. A stock of 50 mM of NAD^+ is used for class III deacetylases for the cofactor. If the deacetylase directly targets the substrate, a decrease in acetylation should be seen.
d. Reaction 4: Deacetylase + cofactor + deacetylase inhibitor.
   i. A stock of 50 mM of NAD^+ is used for class III deacetylases for the cofactor.
   ii. A stock of 2 M nicotinamide is used to inhibit class III deacetylases. No change in acetylation levels of the substrate should be seen compared to Reaction 1 and 2 due to deacetylase inhibition.

   The volume of the deacetylase may vary depending on the concentration after purification (see step 23). 1 μg of deacetylase is suggested for use in Reactions 2–4.

28. Incubate the reactions in a 30°C water bath with gentle tapping to agitate the beads every 15 min for 3 h. Alternatively, a 30°C shaker can be used.
29. After 3 h of incubation, add ~8 μL of 4× sodium dodecyl sulfate (SDS) loading buffer to stop the reaction. Boil the samples for 5 min at 100°C and run on western blot for analysis, or store at −20°C for later analysis.

Note: Transfer conditions may depend on the substrate, but 1 h at 100 V or overnight (12–16 h) at 45 V should give sufficient transfer. Blocking can be done with 5% BSA or 5% milk but may need to be optimized.

30. For the best acetyl-lysine signal, it is suggested to incubate the membrane overnight (12–16 h) on a rocker at 4°C. The deacetylase should also be probed for to ensure there was even addition to the correct samples.

⚠ CRITICAL: Dual channel labeling is most ideal to show the overlap of the acetylated protein and total protein. We suggest using differently colored fluorescent probes, which can be imaged using a LI-COR Odyssey system (LI-COR), which allows for dual channel labeling detection. We also suggest probing for the deacetylase to ensure even levels of the enzyme were added to Reactions 2–4. During the primary antibody step, for dual-labeling, the antibody to the substrate should be a different species to the acetyl-lysine antibody (see reagents) without species cross-reactivity.

EXPECTED OUTCOMES

A successful experiment should show on the final western blot clear and even inputs of the substrate being tested for deacetylation. In addition to reactions 1–4, a negative control lane can be added to ensure specific staining of the substrate and acetylation signal. For tagged substrates, the negative control can be obtained using lysate from cells that were not transfected with the tagged substrate. For endogenous substrates, the negative control can be obtained using a control antibody during the immunoprecipitation step. Reaction 1, where no deacetylase is added, should show an acetylation signal that overlaps the signal that corresponds to the protein. Reaction 2, where the deacetylase but not NAD^+ is added, should show comparable acetylation to reaction 1 due to the lack of necessary cofactor (if acetylation is not comparable, see troubleshooting: Problem 4). However, when NAD+ is added to the reaction, the deacetylase should be activated. If the deacetylase targets the potential substrate, a decrease in acetylation level should be seen in the putative substrate compared to Reaction 1, 2, and 4. If no decrease in acetylation is seen, this may suggest the substrate is not a direct substrate of the deacetylase (see troubleshooting: Problem 5). Reaction 4 should show comparable acetylation to reactions 1 and 2 due to inhibition of the deacetylase. The final western blot should also show even inputs of the deacetylase in the reactions it was added.

An example of a representative blot showing the outcome of the four reactions with successful deacetylation can be seen in Figure 1. Also see (Head et al., 2017; Minten et al., 2021; Zhang et al., 2013, 2016).

LIMITATIONS

This protocol relies on immunoprecipitation from cells. Immunoprecipitation from cells has its own pitfalls and limitations that are outlined in depth in other protocols (Takahashi, 2015). Briefly, proteins that are transiently overexpressed can lead to artifacts, such as misfolding or aggregation. Furthermore, immunoprecipitation from cells in conditions that attempt to preserve enzymatic function can lead to the pulldown and interaction of the deacetylase with other proteins or cofactors that may affect function. In this protocol, the deacetylase has a FLAG tag, which can potentially affect function under certain circumstances.

This protocol also measures acetylation levels using western blotting. Though in the protocol, we suggest over-expressing acetyltransferases and treating the cells with deacetylase inhibitors to maximize acetylation signal, some proteins will nevertheless have low levels of acetylation. This can make signal detection difficult using a pan acetyl-lysine antibody, especially if the acetyltransferases listed in this protocol are not optimal for the tested substrate.

In *in vitro* experiments, the forced interaction between enzyme and substrate may also produce non-specific results. Deacetylation may not occur in normal cellular environments, but *in vitro* conditions may show non-specific deacetylation. Furthermore, *in vitro* experiments use purified proteins and force interactions between enzyme and substrate, which may not reflect what endogenously happens in cells due to due to compartmentalization. The results should be verified in cells under more physiological-like settings.

Finally, this protocol does not necessarily identify the site of acetylation and deacetylation on a protein and is a semi-quantitative measure of acetylation levels.

TROUBLESHOOTING

Problem 1

Low acetylation signal/low substrate expression.

Potential solution

Increase the dilution of the primary antibody (see steps 24–25). We suggest a 1:500 dilution for the acetyl-lysine antibody, but higher concentrations can be used. The membrane should also be

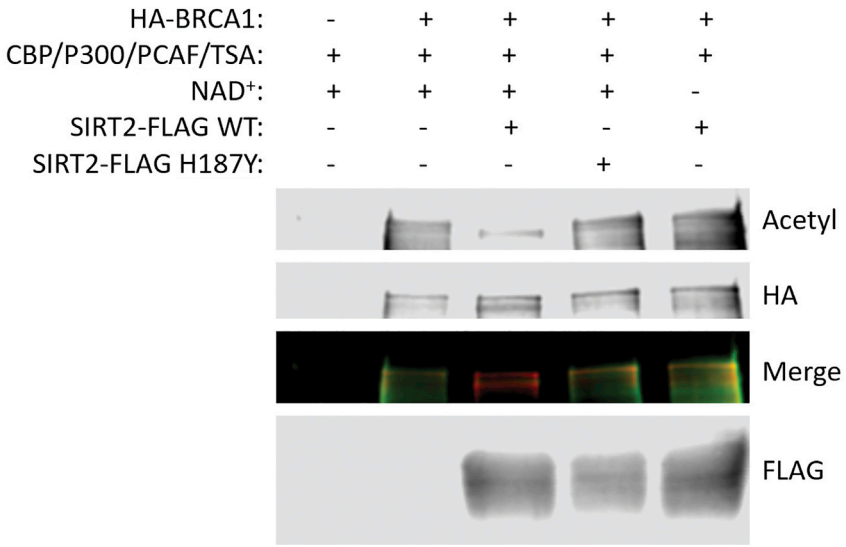


Figure 1. An example western blot showing the deacetylation of the substrate BRCA1 by the class III deacetylase SIRT2

Lane 1 shows a negative control with no transfection of BRCA1 or addition of the deacetylase. Lane 2 shows the addition of BRCA1 and NAD^+, and represents Reaction 1. Lane 3 shows the addition of NAD^+ and SIRT2 to BRCA1, and represents Reaction 3. Lane 4 shows the addition NAD^+ and a deacetylase-dead SIRT2 mutant to BRCA1, and represents Reaction 4. Lane 5 shows the addition of SIRT2 to BRCA1 without NAD^+, and represents Reaction 2. Dual channel labeling of the acetylation signal and BRCA1 are shown.

incubated in antibody overnight (12–16 h) at 4°C vs. for a few hours. Either a PVDF or nitrocellulose membrane can be used, but optimization may be required depending on the substrate being tested.

The acetyltransferases listed in this protocol (P300, CBP, and pCAF) are a broad suggestion for a starting point if an increase in acetylation signal is needed, but should be optimized to each substrate used (see step 1). A low acetylation signal may be due to a non-optimized choice of acetyltransferase.

Large proteins (> 200 kDa) can be difficult to transfect or tend to overexpress at lower levels than smaller proteins (see step 24). Different transfection methods may be required, or even stable expression by lentiviral transduction with selection. Larger proteins can also take a longer transfer time after SDS-PAGE has been performed, so an overnight transfer of about 16 h at 45 V is suggested as a starting point.

The immunoprecipitation of the substrate may need optimizing. Ensuring the beads used for pull-down are able to immunoprecipitate the substrate before doing the deacetylase reaction will aid in this protocol (see step 13).

Problem 2

Reaction 2 shows lower acetylation levels than Reaction 1.

Potential solution

This may suggest there is contamination in the reaction that is allowing the enzyme to function without purposeful addition of the necessary cofactor. Ensure that NAD^+ has not been added to this reaction and that the substrate input is even between Reaction 1 and Reaction 2 (see step 22).

Problem 3

Reaction 4 shows decreased acetylation compared to Reaction 1.

Potential solution

If Reaction 4 shows decreased acetylation, this may suggest nicotinamide is not effectively inhibiting the deacetylase (see step 27). Make sure the stock is not degraded and that the proper concentration has been added to Reaction 4.

Problem 4

Low yield of deacetylase after purification or contamination.

Potential solution

Check that the deacetylase is transfecting properly and try increased transfection time/increased DNA used to transfect cells. As mentioned previously, larger proteins tend to overexpress at lower levels (see step 23).

Make sure the elutant is functioning properly. After eluting, boil the beads and run on a western blot next to a sample taken from the elutant to ensure the enzyme is dissociating from the beads or is being properly immunoprecipitated (see step 23).

Purification of recombinant deacetylase and/or substrate can also be performed from *E. coli* and/or Sf9 insect cells if contaminants are co-purifying when using mammalian cells.

Problem 5

In Reaction 3, the substrate shows no change in acetylation levels.

Potential solution

Probe for the deacetylase on the final western blot to ensure that the deacetylase was correctly added to Reactions 2–4 (see step 27, and steps 29–30). Even amounts of the deacetylase should be seen in the lanes corresponding to Reactions 2–4, and about 1 mg should be added to Reactions 2–4 to ensure a sufficient amount of enzyme.

If not stored correctly, the deacetylase may denature and become non-functional (see step 22). Ensure that the eluted deacetylase has been stored properly, or reprep the deacetylase to get a new batch for use in the assay.

No acetylation may be the result of failure to properly agitate the beads during the 3 h incubation of the deacetylase with the substrate (see step 28). Be careful to ensure that the beads are not sticking to the walls of the tube above the solution that contains the deacetylase, or failing to properly mix. Improper mixing of the beads can lead to failure of the deacetylase to reach the substrate.

The substrate being tested may not be a direct substrate of the deacetylase.

RESOURCE AVAILABILITY

Lead contact

Requests for further information or resources should be directed to the lead contact, D.S. Yu (dsyu@emory.edu).

Materials availability

No new or unique regents were generated. Questions relating to any of the regents should be directed to the lead contact, D.S. Yu (dsyu@emory.edu).

Data and code availability

No data sets or code were generated.

ACKNOWLEDGEMENTS

We thank members of the Yu lab for their helpful discussion and technical expertise. This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) (5T32GM008367-29), the National Cancer Institute (NCI) of the NIH (F31CA225124 to E.V.M. and R01CA178999 and R01CA254403 to D.S.Y.), the U.S. Department of Defense (BC180883 and OC160540 to D.S.Y.), the Basser Center for BRCA (32356 to D.S.Y.), and the Winship Cancer Institute/Brenda Nease Breast Cancer Research Fund (53237 to D.S.Y.). Research reported in this publication was supported in part by the Emory Integrated Genomics Core (EIGC) Shared Resource of Winship Cancer Institute of Emory University and the NIH/NCI under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

AUTHOR CONTRIBUTIONS

E.V.M. and D.S.Y. wrote the draft of the manuscript and contributed to editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Head, P.E., Zhang, H., Bastien, A.J., Koyen, A.E., Withers, A.E., Daddacha, W.B., Cheng, X., and Yu, D.S. (2017). Sirtuin 2 mutations in human cancers impair its function in genome maintenance. J. Biol. Chem. *292*, 9919–9931.

Minten, E.V., Kapoor-Vazirani, P., Li, C., Zhang, H., Balakrishnan, K., and Yu, D.S. (2021). SIRT2 promotes BRCA1-BARD1 heterodimerization through deacetylation. Cell Rep. *34*, 108921.

Takahashi, Y. (2015). Co-immunoprecipitation from transfected cells. Methods Mol. Biol. *1278*, 381–389.

Zhang, H., Head, P.E., Daddacha, W., Park, S.H., Li, X., Pan, Y., Madden, M.Z., Duong, D.M., Xie, M., Yu, B., et al. (2016). ATRIP deacetylation by SIRT2 drives ATR checkpoint activation by promoting binding to RPA-ssDNA. Cell Rep. *14*, 1435–1447.

Zhang, H., Park, S.H., Pantazides, B.G., Karpiuk, O., Warren, M.D., Hardy, C.W., Duong, D.M., Park, S.J., Kim, H.S., Vassilopoulos, A., et al. (2013). SIRT2 directs the replication stress response through CDK9 deacetylation. Proc. Natl. Acad. Sci. U S A *110*, 13546–13551.