Published in final edited form as:

Methods Enzymol. 2019 ; 626: 587–620. doi:10.1016/bs.mie.2019.06.030.

Methods for characterizing protein acetylation during viral infection

Laura A. Murray, Ashton N. Combs, Pranav Rekapalli, Ileana M. Cristea*

Department of Molecular Biology, Princeton University, Lewis Thomas Laboratory, Princeton, NJ, United States

Abstract

Lysine acetylation is a prevalent posttranslational modification that acts as a regulator of protein function, subcellular localization, and interactions. A growing body of work has highlighted the importance of temporal alterations in protein acetylation during infection with a range of human viruses. It has become clear that both cellular and viral proteins are decorated by lysine acetylations, and that these modifications contribute to core host defense and virus replication processes. Further defining the extent and dynamics of protein acetylation events during the progression of an infection can provide an important new perspective on the intricate mechanisms underlying the biology and pathogenesis of virus infections. Here, we provide protocols for identifying, quantifying, and probing the regulation of lysine acetylations during viral infection. We describe the use of acetyl-lysine immunoaffinity purification and quantitative mass spectrometry for assessing the cellular acetylome at different stages of an infection. As an alternative to traditional antibody-mediated western blotting, we discuss the benefits of targeted mass spectrometry approaches for detecting and quantifying site-specific acetylations on proteins of interest. Specifically, we provide a protocol using parallel reaction monitoring (PRM). We further discuss experimental considerations that are specific to studying viral infections. Finally, we provide a brief overview of the types of assays that can be employed to characterize the function of an acetylation event in the context of infection. As a method to interrogate the regulation of acetylation, we describe the *Fluor de Lys* assay for monitoring the enzymatic activities of deacetylases.

1. Introduction

Protein acetylation on lysine residues, although initially thought to be a rare posttranslational modification (PTM), is now recognized not only as prevalent, but also as a key regulator of diverse cellular processes in both eukaryotes and prokaryotes. Cellular functions and processes shown to be regulated by acetylation include enzyme activity, chromatin structure, transcription, protein localization, protein-protein interactions, and metabolism (Carabetta, Greco, Cristea, & Dubnau, 2019; Choudhary et al., 2009; Choudhary, Weinert, Nishida, Verdin, & Mann, 2014; Kim et al., 2006; Svinkina et al., 2015). Acetylation can be both enzymatically and non-enzymatically driven. Enzymatic acetylation is modulated in eukaryotes by an array of acetyltransferases (HATs) and deacetylases (HDACs) (Table 1).

*Corresponding author: icristea@princeton.edu.

Non-enzymatic acetylation, which is better understood in bacteria, can derive from environmental or stress factors that induce acetyl-coA levels and/or increase local pH (Kosono et al., 2015; Wagner & Payne, 2013; Weinert et al., 2014).

Emerging evidence has pointed to acetylation as an important regulatory event during a range of infections with human viruses, with acetylation being implicated in both proviral and antiviral processes (Fig. 1). Some of the early work on acetylation in the context of infection focused on viral protein acetylation. One of the most well studied acetylations is that of the human immunodeficiency virus type 1 (HIV-1) protein Tat, for which acetylation was shown to be necessary for its transcriptional activity (Berro et al., 2006; Brès, Kiernan, Emiliani, & Benkirane, 2002; Deng et al., 2000; Dorr et al., 2002; He et al., 2013; Kiernan et al., 1999; Ott et al., 1999). More recently, Giese et al. found that acetylation of the Influenza A protein NP can have either inhibitory or beneficial effects for viral replication depending on the modified site (Giese et al., 2017), while Murray et al. demonstrated that acetylation of the human cytomegalovirus (HCMV) transcriptional activator pUL26 inhibits virus production (Murray, Sheng, & Cristea, 2018).

Other early work on acetylation during viral infection focused on the regulation of viral gene expression. HDAC1-mediated deacetylation of cellular histones was shown to act in host defense by repressing viral reactivation from latency for both HIV-1, whose genome is integrated into the host genome, and herpes simplex virus type 1 (HSV-1), whose genome becomes chromatinized separately from the host genome (Roizman, 2011; Shirakawa, Chavez, Hakre, Calvanese, & Verdin, 2013). During lytic replication of the DNA viruses HCMV and HSV-1, viral gene expression is mediated by both host and viral transcription factors that modulate the acetylation status of the chromatinized viral genomes (Cuevas-Bennett & Shenk, 2008; Guise, Budayeva, Diner, & Cristea, 2013).

Additional work has shown that regulation of acetylation on non-histone proteins is crucial for activating host immune responses. The nuclear localization of NF- κ B, a transcriptional activator of cytokine production, is promoted by the p300/CBP-mediated acetylation of its RelA subunit (Chen & Greene, 2004; Tummers et al., 2015). Another well-characterized example is the acetylation of p53, whose acetylation at K373, K379, and K382 is required for its activation of interferon-inducible genes and apoptotic pathways (Muñoz-Fontela et al., 2011). The importance of p53 acetylation is highlighted by the fact that several viruses, including HCMV, human T-cell leukemia virus, the small DNA tumor viruses human papillomavirus and adenovirus type 5, and HIV-1, have acquired mechanisms to block cellular apoptosis by specifically inducing the deacetylation of p53 (Harrod et al., 2003; Hebner, Beglin, & Laimins, 2007; Hsu et al., 2004; Savelyeva & Dobbelsstein, 2011; Wright et al., 2016). Acetylation can also serve as a modulator of the subcellular localization and function of other immune response factors. For example, acetylation of the DNA sensor gamma-interferon-inducible protein 16 (IFI16) within its nuclear localization motif dictates its subcellular localization (Li, Diner, Chen, & Cristea, 2012). Recent work has also shown that the HDAC6-mediated deacetylation of RIG-I, a cytoplasmic RNA sensor, is necessary to facilitate its binding to pathogenic RNA (Choi et al., 2016).

Beyond transcriptional regulation, emerging evidence points to the contribution of acetylation to either promoting or inhibiting different steps in the virus replication cycle, including virus assembly, maturation, and trafficking events. Murray et al. determined that an increase in K134 acetylation on Lamin B1 late in HCMV infection inhibits the virus-induced formation of nuclear laminar infoldings, thereby blocking viral capsid nuclear egress (Murray et al., 2018). Additional work by several groups has established that the stabilization of microtubules via α -tubulin acetylation is induced by a broad spectrum of viruses. Specifically, the acetylation of microtubules during replication of noroviruses and rotavirus, both RNA viruses, stabilizes their cytoplasmic replication compartments (Naghavi & Walsh, 2017). HCMV induces acetylation of microtubules to facilitate the formation of the assembly complex, a cytoplasmic nuclear-adjacent compartment critical for viral particle maturation (Procter et al., 2018). Finally, some viruses, such as influenza A, employ acetylated microtubules for trafficking virion components to the plasma membrane, and interference with this process by activating HDAC6 reduces virus production (Husain & Cheung, 2014).

Altogether, these findings provide a growing perspective of acetylation as an important molecular toggle within diverse host defense and viral replication processes during viral infection. This is supported by the discovery that the seven human sirtuins can act as broad antiviral factors against several DNA and RNA viruses, such as HCMV, HSV-1, adenovirus 5, and influenza A (Koyuncu et al., 2014). As the progression through virus replication cycles relies on the temporal modulation of cellular transcription and metabolism processes among other organelle functions, studies that will further explore the regulation and functions of acetylation during infection are needed for a better understanding of the biology of viral infections and for uncovering novel targets for antiviral treatments.

Here, we provide protocols for several robust methods that can be used to study protein acetylation in the context of infection. The methods presented address a range of biological questions. In order to discover protein acetylation sites and determine their possible temporal changes during the progression of an infection, we present an approach that uses anti-acetyl-lysine immunoaffinity purification and quantitative mass spectrometry. As the generation of anti-acetyl-lysine antibodies is not trivial, can be time-consuming, expensive, and prone to cross-reactivity issues, we provide an alternative method for quantifying site-specific acetylations on proteins of interest. This method uses targeted mass spectrometry, and we specifically provide a protocol for parallel reaction monitoring (PRM). We discuss experimental aspects that should be considered when focusing on viral infection studies, and provide a brief overview of approaches that could be used to interrogate the biological function and regulation of an acetylated site of interest. We describe the *Fluor de Lys* assay, which provides a method for monitoring the enzymatic activities of deacetylases during viral infection.

2. Assessing global acetylome changes during the course of viral infection using anti-acetyl-lysine immunoaffinity purification and mass spectrometry

The method established in recent years as the most effective and commonly used for characterizing global lysine acetylomes in complex biological samples involves the immunoaffinity enrichment of acetylated peptides using anti-acetyl-lysine antibodies (Ac-K IP), followed by the identification of site-specific acetylations using mass spectrometry. The enrichment facilitates the analysis of acetylation on low abundance proteins, such as transcription factors. While the protocol for Ac-K IP is similar to that for protein immunoaffinity purification, the Ac-K IP is conducted at the peptide level, not at the protein level. Consequently, the proteins must be digested prior to the Ac-K IP. In addition, for optimal IP efficiency, several sample cleanup steps are necessary, as described in the protocol below. The procedure outlined here takes advantage of the PTMScan® Acetyl-Lysine Motif [Ac-K] Kit #13416 from Cell Signaling and uses trypsin as the enzyme for protein digestion. This 5-day protocol can be applied to discovering acetylation events and their temporal regulation during the progression of an infection (Fig. 2), as it was reported during HCMV infection (Murray et al., 2018).

The users should be aware of several limitations and experimental considerations when using this Ac-K IP approach. Based on current literature, the acetyl enrichments obtained with this workflow seem to range from 15% observed in the less effective isolations to 40% in the more successful enrichments. The effectiveness of an enrichment is influenced by the solubilization efficiency, the number and types of antibodies used, and the efficiency of the protein digestion. All these aspects should be carefully considered and monitored when designing and performing this experiment. An important choice is whether to use a single anti-acetyl-lysine antibody or a mixture of different anti-acetyl-lysine antibodies for the enrichment. One concern when using a single antibody is the possible presence of a bias for the identification of certain acetyl motifs. On the other hand, the use of antibody mixtures may enhance non-specific associations, as well as result in some cross-inhibitory interactions between the antibodies. In our hands, we have had success with using a combination of two anti-acetyl-lysine antibodies (Carabetta, Greco, Tanner, Cristea, & Dubnau, 2016), as well as the larger mixture of acetyl antibodies that is reported here (Murray et al., 2018). We strongly encourage the users to monitor and report the percentage of enrichment in their studies, as this provides an indication of the effectiveness of the IP and the level of non-acetyl background.

Additional experimental design considerations pertain specifically to studies focused on viral infections. First, depending on the type of viral infection studied and the biological questions tackled, it may be necessary to synchronize the infection to ensure that the infected cells represent equivalent stages of the virus replication cycle. A well-established method for doing this is infecting the cells at a low temperature (Burkard et al., 2014; Hazrati et al., 2014; Lakadamyali, Rust, Babcock, & Zhuang, 2003; Lin, Greco, Döhner, Sodeik, & Cristea, 2013). This will allow the virus to attach to the cells, but not to enter the cells. Shifting to a higher temperature will enable all the viruses to enter simultaneously.

Second, the infection time points of interest will need to be determined, and the number of plates of cells necessary for each time point will need to be optimized for the cell type of interest. The experimental procedure outlined here lends it self to multiple time point collections, even if those time points are relatively close in time, as the collection of each cell pellet should take no more than 30min. If the time points are closer to each other, then the infections for the different time points may need to be started sequentially. Third, one of the advantages of the Ac-K IP is that it can enrich for acetylations on low abundant proteins. In the context of infection, such modifications can be identified on both cellular and viral proteins, and the database searching performed should take this into account. Fourth, it is important to consider what enzyme or combination of enzymes will be used for protein digestion. For example, trypsin, which typically cleaves C-terminally of lysine and arginine, will not cleave after a modified lysine; therefore, there will be numerous missed cleavages in the acetylome data from tryptic peptides. In contrast, GluC, which cleaves C-terminally to glutamic acid and aspartic acid, will not be predominantly biased by acetylated lysines. However, the efficiency of this enzyme for digestion needs to be carefully optimized.

2.1 Equipment

*For all pipetting, LoBind pipette tips should be used (fit P20 and P200 pipettes) unless the volume does not permit. In this case, filter tips should be used.

Day 1: Sample collection

1. Cell scraper
2. 5-mL LoBind Eppendorf tubes (Fisher Scientific)
3. 4 °C centrifuge that can handle 15- and 50-mL conical tubes

Day 2: Cell lysis and methanol-chloroform extraction

1. 70 °C heatblock
2. 95/100 °C heatblock
3. Cup horn sonicator
4. Table top centrifuge at room temperature that fits the size tubes used during the methanol/chloroform extraction
5. Table top centrifuge at 4 °C that fits the size tubes used during the methanol/chloroform extraction

Day 3: Overnight enzyme digestion

1. Cup horn sonicator
2. Tip sonicator
3. Rocker at 37 °C

Day 4: Column desalting and lyophilization

1. Table top centrifuge at 20 °C

2. Oasis 3 cc columns (Waters)
3. 10-mL syringes
4. Lyophilizer

Day 6: Acetyl-lysine IP and StageTip desalting

1. Gel loading tips
2. 1.5-mL Eppendorf tubes
3. Microcentrifuge at 4 °C
4. pH paper
5. Spectrophotometer
6. Microresico[®] Low Bind Tube, 1.5 mL (Amuza, Inc. (Eicom USA))
7. Rotating rocker at 4 °C
8. Hamilton syringe plunger (#1162-02) (Fisher Scientific)
9. 3M Empore SDB-RPS membrane (#2241) (Phenomenex)
10. Hamilton 14 gauge needle (#90514) (Fisher Scientific)
11. 3 mL Combitip (Eppendorf)
12. Thermo Scientific autosampler vials (Fisher Scientific)
13. SpeedVac

2.2 Reagents

Day 1: Sample collection

1. Sterile 1× PBS (cold)

Day 2: Cell lysis and methanol-chloroform extraction

1. 1 × cell lysis buffer (50mM Tris-HCl pH 8, 100mM NaCl, 0.5mM EDTA, 4% SDS)
2. BCA assay reagents (BSA, Solution A, Solution B from Thermo Fisher Scientific)
3. 500mM TCEP (Thermo Scientific[™] Bond-Breaker[™] Neutral pH TCEP Solution)
4. 500 mM chloroacetamide (Fisher Scientific)
5. MS-grade methanol (Optima[™] LC/MS Grade) (room temperature) (Fisher Scientific)
6. HPLC-grade chloroform (Thermo Fisher Scientific)
7. HPLC-grade water (Fisher Scientific)
8. MS-grade methanol (−20 °C)

Day 3: Overnight enzyme digestion (all buffers and reagent dilutions should be made in HPLC water)

1. 25 mM HEPES ~pH 8.2
2. MS-grade/sequencing grade trypsin (0.5mg/mL) stock (Thermo Scientific™ Pierce™ MS Grade Trypsin Protease)

Day 4: Column desalting and lyophilization (all buffers and reagent dilutions should be made in HPLC water)

1. 10% TFA (Thermo Scientific™ Pierce™ Trifluoroacetic Acid (TFA))
2. 100% acetonitrile (ACN) (Fisher Scientific)
3. HPLC water
4. 50% ACN in HPLC water

Day 6: Acetyl-lysine IP and StageTip desalting (all buffers and reagent dilutions should be made in HPLC water)

1. PTMScan® Acetyl-Lysine Motif [Ac-K] Kit #13416 from Cell Signaling
2. 1 × PBS (chilled)
3. 1 × IAP buffer (chilled) (from PTMScan® Kit)
4. 1M Tris base (pH 8)
5. HPLC water (chilled)
6. 0.15% TFA
7. 10% TFA
8. Wash buffer: 0.2% TFA
9. Elution buffer: 5% ammonium hydroxide/80% ACN; ammonium hydroxide (Fisher Scientific)
10. Dilution solution: 1% formic acid/1% ACN; formic acid (Fisher Scientific)

2.3. Method

Procedure Overview (Note 1):

Day 1: Sample collection

Day 2: Cell lysis, methanol-chloroform extraction, freeze at -80 °C

Day 3: Sample resuspension and overnight trypsinization

Day 4: Oasis column desalting, setup lyophilization

Day 5: Lyophilization continued (no active work)

Day 6: Ac-K IP, StageTip desalting

2.3.1 Day 1: Sample collection

1. Wash cells (six 15 cm plates/condition) (Note 2) two times with cold $1 \times$ PBS, then scrape the cells from each plate into 1 mL cold $1 \times$ PBS. Combine three plates per 5mL Eppendorf tube.
2. Spin down at $250 \times g$ for 5min at 4°C .
3. Aspirate the PBS and replace with another 3mL cold PBS. Repeat the centrifugation.
4. Aspirate the PBS and flash freeze the pellet in liquid nitrogen.
5. Store at -80°C until ready to proceed (Note 3).

2.3.2 Day 2: Cell lysis and methanol-chloroform extraction

1. Preheat $1 \times$ lysis buffer to 70°C .
2. Thaw the cell pellet on ice for 5 min. Resuspend the cell pellet in $1 \times$ lysis buffer (2.7mL for three plates worth of cells), and heat at 100°C for 3 min (Note 4).
3. Sonicate the suspension with a cup horn sonicator for 30 1s pulses two times.
4. Repeat heating and sonication until all the sample is solubilized (three to five times).
5. Take a 1:10 dilution to estimate protein concentration via a standard BCA assay.
6. Reduce and alkylate with 1:20 of 500mM TCEP (final concentration 25 mM) and 1:10 of 500 mM chloroacetamide (final concentration 50 mM). Heat at 95°C for 5min.
7. Conduct methanol-chloroform extraction (Note 5). *In all methanol-chloroform steps, “ $\times$ ” refers to the sample volume.*
 - a. Add the sample(s) to each tube.
 - b. Add $4 \times$ methanol.
 - c. Vortex 5—10 s to ensure complete mixing.
 - d. Add $1 \times$ chloroform (Chloroform should be added in a chemical hood, and the tubes should only be opened inside a chemical hood until the chloroform is removed in Step i).
 - e. Vortex 5–10s to ensure complete mixing.
 - f. Add $3 \times$ HPLC-water.
 - g. Vortex 5–10 s to ensure complete mixing.
 - h. Centrifuge for 10min at $2000 \times g$ at RT.
 - i. Discard supernatant (portion above the white protein disc layer) (see Fig. 2).
 - j. Add $3 \times -20^\circ\text{C}$ methanol.

- k. Centrifuge for 2min at $9000 \times g$ at 4 °C.
- l. Discard the supernatant.
- m. Add 5×-20 °C methanol.
- n. Centrifuge for 2min at $9000 \times g$ at 4°C.
- o. Discard the supernatant.
- p. Air dry for 5–10 min.
- q. Sample may be frozen at -80 °C for up to 1 month. Alternatively, proceed directly to Day 3.

2.3.3 Day 3: Trypsin digestion

1. Add 25 mM HEPES ~pH 8.2 to the sample and sonicate until resuspended. Resuspend so there is a protein concentration of 0.5mg/mL for each sample (Note 6).
2. Trypsin digestion (Note 7): Add trypsin at a 1:200 trypsin:protein (w:w) ratio. Incubate at 37 °C on a rocker for 3–6h. Parafilm around the caps of the tubes to ensure no leakage.
3. Add a second round of trypsin at a 1:200 trypsin:protein (w:w) ratio (final trypsin concentration in each sample is 1:100). Incubate at 37 °C on a rocker O/N. Parafilm around the caps of the tubes to ensure no leakage.

2.3.4 Day 4: Desalting and lyophilization

1. Acidify each sample to a final concentration of 1% TFA. Incubate on ice for 15min.
2. Spin samples at $3700 \times g$ for 10min at 20 °C, and transfer the supernatant (soluble fraction) to a new tube.
3. Conduct an Oasis Column desalting using 3cc cartridges per manufacturer's instructions (Note 8).
 - a. Wash the column with 1.5 mL 100% ACN.
 - b. Wash the column with 3mL HPLC water.
 - c. Load sample. If there is more than 10 mL of sample, load in multiple iterations.
 - d. Elute into a 15-mL conical tube with 1.5mL 50% ACN. Repeat the elution for a total elution volume of 3 mL.
4. Parafilm over the top of the tube and poke two holes in the top to allow for gas release (Note 9). Flash freeze the samples in liquid nitrogen, taking care not to get liquid nitrogen into the tube.
5. Lyophilize the sample until the sample is completely dry (1.5–2 days).

2.3.5 Day 6: Immunoaffinity purification of acetylated peptides and StageTip desalting

A. Acetyl lysine immunoaffinity purification

1. Follow the Cell Signaling protocol for Ac-K IP per manufacturer's instructions (see below for protocol overview). Keep samples, buffers, and beads on ice or at 4 °C as much as possible until step 6.

For bead preparation:

- a. Transfer the beads from the Cell Signaling tube to a 1.5-mL Eppendorf tube by adding ice cold PBS and gently pipetting up and down to resuspend. Wash antibody-bead slurry sequentially four times with 1 mL of cold 1 × PBS. One wash consists of adding 1 mL cold PBS, inverting the tube five times to suspend the beads, spinning down at $2000 \times g$ for 30s at 4 °C, and removing the supernatant. Gel loading tips should be used for removing the supernatant without disturbing the beads (Notes 10 and 11). After the last wash, store the beads on ice until ready to apply the sample.

For sample preparation:

- a. After resuspending the sample in 1.4 mL cold 1 × IAP buffer by pipetting, test the pH with pH paper and adjust to approximately pH 7 if necessary with 1M Tris base pH 8.
 - b. Transfer the solution to 1.7 mL tubes. Clear solution by centrifugation for 5min at $10,000 \times g$ at 4 °C.
 - c. Make a 1:500 dilution of each sample and of 1 × IAP buffer (for determining the signal of a blank sample). Use a spectrophotometer to determine the peptide concentration of the supernatant. Put 50 µg of peptides in a LoBind microcentrifuge tube for whole proteome analysis and store at 4 °C.
2. Add the same number of µg of peptides (as determined in step c of sample preparation) for each sample to the beads and incubate, rotating at 4 °C for 2 h.
 3. Centrifuge at $2000 \times g$ for 30 s at 4 °C and transfer the supernatant to a new tube to save as flow through (unbound fraction).
 4. Wash (as described in Section 2.3.5 A.1a) two times with 1 mL cold 1 × IAP Buffer.
 5. Wash (as described in Section 2.3.5 A.1a) three times with 1 mL cold HPLC water.
 6. To elute, add 50 µL 0.15% TFA to the beads and incubate at RT for 10min, gently flicking to mix every 2—3min. Do NOT vortex. Spin

down at $2000 \times g$ for 30 s at 4 °C, and transfer elution to a LoBind tube.

Repeat the elution and combine with the first elution.

B. StageTip desalting

For additional details, please see Rappsilber, Ishihama, and Mann (2003), Rappsilber, Mann, and Ishihama (2007).

1. Adjust both IP and input samples to 1% TFA with a final volume of 150 μ L.
2. Make StageTips using a Hamilton 14 Gauge Needle, Hamilton Plunger, and 3M 47mm SDB-RPS Empore Discs. Use P200 LoBind tips. Layer 5 discs per StageTip to ensure that there is enough material to capture all the peptides. Tap down with the plunger to ensure that the discs form a packed column near the base of the tip. Put each StageTip in a StageTip holder and place in a 1.5- or 2-mL microcentrifuge tube with the cap removed (Note 12).
3. Add sample to the top of a StageTip. Flick the StageTip to ensure that there are no air bubbles separating the sample from the discs.
4. Place tips into collection tube and centrifuge at $1000\text{--}2000 \times g$ at RT until *all the sample* has passed through the tip. Choose a centrifuge speed that gives a flow rate over the disc of $\sim 50 \mu\text{L}/\text{min}$. Centrifuge until all the sample has passed through the discs. Empty the collection tubes if needed.
5. Apply 100 μ L wash buffer to each StageTip and centrifuge as above until all wash solution has passed through the discs. Empty collection tubes if needed. Repeat this step.
6. Add 50 μ L elution solution to each StageTip. Elute into an autosampler vial using a 3 mL Combitip. Each elution should take $\sim 15\text{--}20$ s.
7. Concentrate samples to approximately 1 μ L using a SpeedVac.
8. Resuspend in dilution solution: Resuspend IP samples in 6 μ L and whole proteome/input samples in 25 μ L (Note 13).

2.3.6 Data-dependent acquisition (DDA) mass spectrometry analysis—

We have used the protocol provided, with the parameters listed, on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). However, a similar analysis can be performed using a different mass spectrometer, likely with some adjustments to the parameters to fit the specific instrument configuration.

1. Using a Q Exactive HF mass spectrometer, set up two distinct sample runs for the acetyl-IP and the input (i.e., proteome) samples. For both the acetyl-IP and the input samples, peptides should be separated over a 150 min continuous

gradient from 3% to 35% of 0.1% formic acid in 97% acetonitrile at a flow rate of 250nL/min.

2. In the Xcalibur method control software, configure the following method settings. For each acetyl-lysine IP sample, we recommend a MS¹ survey scan from 350 to 1800 *m/z* at 120,000 resolution with an automatic gain control (AGC) setting of 3e6 and a maximum inject time (MIT) of 30 ms. MS² scans should be acquired at a resolution of 30,000 with an AGC setting of 1e5, a MIT of 150 ms, an isolation window of 1.6 *m/z*, a fixed first mass of 100 *m/z*, a minimum intensity threshold of 1e5, peptide matching set to preferred, a loop count of 10, dynamic exclusion of 45.0s, acquired in centroid. For each input (whole proteome) sample, we recommend the same MS¹ acquisition settings. For the MS² acquisition settings, we recommend the following adjustments: resolution of 15,000, MIT of 25 ms, loop count of 20, and isolation window of 1.2 *m/z*.
3. Raw instrument files can be processed in Proteome Discoverer (Thermo Fisher Scientific). Mass accuracy is recalibrated using the spectrum files RC node. The Minora feature detection node is used for quantifying isotope features.
4. The SEQUEST HT node is used to obtain peptide spectrum matches by searching fragmentation spectra against the appropriate protein sequence database (*e.g.*, human) appended with common contaminants and containing reversed protein sequences, the latter of which is used by Percolator to filter peptide-to-spectrum matches (PSMs) to 1% false discovery rate (FDR) within each replicate. In SEQUEST HT, it is important to set the acetyl-lysine (+42 Da) as a dynamic modification and to allow for a sufficient number of missed cleavages so as not to exclude acetylated peptides. We recommend setting this to a maximum of two missed cleavages.
5. The ptmRS node in Proteome Discoverer can be used to calculate the probability that the acetyl modification is present on each potential lysine within the peptide. Based on the observed fragment ions, each potential site is assigned a localization probability, which is translated into a percent confidence score. We recommend using a minimum score threshold of 75% confidence so that non-confidently localized modifications are not considered. We also recommend manual inspection of some of the acetyl lysine spectra to verify the correct assignment of the acetylation modification.
6. In the PD consensus workflow, the label-free peptide and protein quantification (LFQ) analysis Feature Mapper node can be used to map peptide features between runs, thereby reducing missing values.

2.4 Notes

1. Samples can be stored at -80 °C after cell collection (end of Day 1) and after methanol-chloroform extraction (end of Day 2). Starting from the trypsin digestion, all steps must be conducted sequentially.

2. This procedure is commonly carried out with relatively large amounts of starting material. Approximately 20 mg of protein is frequently used. This amount helps to capture acetylations of varying abundances. The current reported average enrichment of acetylated peptides is approximately 30%. For primary human fibroblasts, six confluent 15 cm plates yield approximately 20 mg of protein.
3. Cell pellets can be stored at -80°C for a few weeks. This means that cell pellets can be collected from different infection time points and stored until all time points have been taken.
4. Once heated, do not put the sample back on ice as this will cause the SDS in the lysis buffer to precipitate.
5. It is important to calculate the largest volume that will be present during the methanol-chloroform extraction so that the correct size tube (1.7, 2, 5, 15, 50mL) is used.
6. We recommend resuspending by adding the buffer in incremental volumes and sonicating after each volume addition until the desired volume is reached, and all of the sample is resuspended. For example, first add 5 mL and use a cup horn sonicator with 30 s of 1 s pulses two times. Then, add another 5 mL and use a tip sonicator for 10–15 s. Then add the rest of the volume and use a tip sonicator to sonicate in rounds of 10 s until all the pellet is in solution.
7. Trypsin was selected for use in this protocol as it cleaves C-terminally of arginine and lysine; this will result in a missed cleavage after an acetylated lysine. Another enzyme, such as GluC could also be used, but optimizations must be performed to ensure effective digestion.
8. We recommend using 15-mL conical tubes to collect the flow through from the washes and the sample addition. It is important that the base of the column does not touch the flow through collected, so the liquid in these tubes needs to be discarded periodically throughout the procedure. The tubes of 10-mL syringes can be used to provide more volume space on top of the columns. Ideally, a liquid flow rate of 1 drop per second should be maintained. If there is a large amount of protein, it may be necessary to apply gentle pressure with the plunger to these syringes to help the sample, washes, and elution to flow through the column. This step typically takes 4–5h.
9. The parafilm will ensure that your sample remains in the tube during lyophilization.
10. It is essential not to exceed $2000 \times g$ because doing so will crush the beads.
11. All washes throughout the protocol are conducted in this manner unless otherwise stated.
12. Each disc binds ~20–30 μg peptides.
13. For optimal acetylation quantification, samples should be placed at 4°C upon elution and analyzed by mass spectrometry immediately. Do not freeze samples.

3. Quantify changes in site-specific acetylation levels using targeted mass spectrometry

Whether focusing on known acetylation sites of interest or on newly discovered acetylation sites (*e.g.*, from acetylome studies as described in Section 2), monitoring the regulation of these modifications during a biological process requires the ability to detect and quantify a specific site of interest. A traditional method for detecting PTMs is to use antibodies that have been raised against an acetylated peptide or protein. However, antibodies are not available for the majority of the reported acetylation sites, and the number of identified protein acetylations continues to increase. Furthermore, for the few antibodies that are commercially available, there are concerns regarding their cross-reactivity as well as their limited ability for accurate quantification across a broad dynamic range. In recent years, targeted mass spectrometry methods, such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM), have become established as powerful tools for the detection and quantification of PTMs (Lange, Picotti, Domon, & Aebersold, 2008; Peterson, Russell, Bailey, Westphall, & Coon, 2012). In contrast to data-dependent acquisition (DDA) methods, which involve sampling of precursor ions based on signal intensity, targeted methods scan for a set of signature parameters that uniquely identify a certain modified peptide of interest. These parameters include retention time on the liquid chromatography gradient, the m/z of the modified peptide, and the m/z of prominent fragment ions. Therefore, this method can be used once a certain modification site has been detected (*e.g.*, from broader acetylome studies or within the literature), and these signature parameters have been obtained. However, once these parameters are known, this information is transferable, and different groups that have mass spectrometry technology can use this knowledge to monitor specific PTM sites. In a nutshell, this method can be described as finding a “needle in a haystack,” as PTMs present at relative low abundances can be targeted and detected in complex samples, while the rest of the proteins present in the mixture are ignored. These targeted mass spectrometry assays can be designed to search not only for one acetylation, but rather for a list of modifications of interest. This method can be used to either validate newly identified acetylations (*e.g.*, information from the acetylome study in Section 2) or to monitor the changes in acetylation at sites previously determined to be acetylated (Fig. 3A).

In this section, we describe a protocol for the quantification of changes in site-specific acetylations over the course of a viral infection using PRM (Fig. 3B). It is important to consider that distinct acetylation sites on the same protein may play different roles, and thereby display distinct abundance trends during infection. This method enables the precise quantification of multiple sites on the same protein and facilitates distinguishing site-specific regulation (Fig. 3C). We also explain how this method can be adapted to use heavy-labeled peptides for absolute quantification of a particular acetylation (Fig. 3A).

3.1 Equipment and reagents

3.1.1 Lysis

1. See reagents and buffers required in Section 2.1 Day 2 and Section 2.2 Day 2.

3.1.2 Methanol-chloroform extraction and sample digestion

1. See reagents and buffers required in Section 2.1 Days 2 and 3 and Section 2.2 Days 2 and 3.
2. Light and heavy-labeled peptides matching the acetylated peptide of interest (Note 1).

3.1.3 Sample cleanup (StageTip)

1. 10% trifluoroacetic acid (TFA) (diluted in HPLC water)
2. 1% trifluoroacetic acid (TFA) (diluted in HPLC water)
3. SpeedVac
4. Elution buffer: 80% LC-MS grade acetonitrile, 15% LC-MS grade water, 5% (v/v) ammonium hydroxide.
5. 3M Empore SDB-RPS membrane (#2241)
6. 200 μ L LoBind pipette tips (Eppendorf)
7. Hamilton 14 gauge needle (#90514) and syringe plunger (#1162-02)
8. 3mL Combitip (Eppendorf)
9. Autosampler vials
10. Dilution solution: 98% LC-MS grade water, 1% LC-MS grade formic acid, 1% LC-MS grade acetonitrile

3.1.4 Parallel reaction monitoring (PRM)

1. Inclusion list(s) of all the peptides to be measured
2. Q Exactive HF mass spectrometer (Thermo Fisher Scientific)
3. Windows computer with at least 24GB RAM and a 1TB hard drive
4. Proteome Discoverer v2.2 software (Thermo Fisher Scientific)
5. Skyline software (<https://skyline.ms>)

3.2 Method

3.2.1. Lysis

1. Preheat lysis buffer at 70 °C.
2. Thaw the cell pellet on ice for 5min. Resuspend cells in lysis buffer so that there is a ~1—2 μ g/ μ L protein concentration. Usually, ~100,000 cells = 20 μ g of protein.
3. Heat samples at 95 °C for 5min.
4. Sonicate in a cup horn sonicator using 1 s pulses for 12s.
5. Repeat heating and sonication until sample is completely resuspended.

6. Perform a BCA assay to determine protein concentration.

3.2.2 Methanol-chloroform extraction and sample digestion

1. Transfer 50–100 µg of lysate to a new tube (Note 2). Reduce and alkylate by adding TCEP to each sample to a final concentration of 25 mM and chloroacetamide to a final concentration of 50mM.
2. Incubate at 70°C for 20min.
3. Conduct a methanol-chloroform extraction as in Section 2.3.2.
4. Resuspend the pellet at a concentration of 0.5 µg/µL in 25 mM HEPES pH 8.2 using sonication. Sonicate until the pellet is fully dispersed and the solution appears cloudy. If conducting absolute quantification, the heavy labeled peptide may be added once the pellet is fully resuspended. Adding 2.5fmol of heavy peptide/µg is recommended (Note 3).
5. Add 1:50 (w:w) sequencing-grade trypsin to each sample and incubate overnight, rocking at 37 °C.

3.2.3 Sample cleanup (StageTip)

1. Conduct a StageTip desalting as in Section 2.3.5 (section B). We recommend using a 14-gauge needle and three discs inserted into a P200 LoBind tip. Elution should be conducted using a 3 mL Combitip directly into an autosampler vial.
2. After SpeedVac drying, resuspend the peptides in dilution solution at a concentration of 0.5 µg/µL.

3.2.4. Parallel reaction monitoring (PRM)

1. Using Skyline software, perform proteotypic peptide selection for protein(s) and acetyl peptide(s) of interest (Notes 4 and 5). Once the list is complete, export a standard inclusion list for the Q Exactive HF (Notes 6 and 7).
2. For PRM analysis, a 60min LC separation time with peptides separated over a linear gradient from 3% to 30% mobile phase B (0.1% formic acid, 97% LC-MS acetonitrile) at a flow rate of 250nL/min is sufficient for most applications.
3. In the Xcalibur method control software, configure the settings. For targeting acetylated peptides, we recommend a resolution of 30,000 (Note 8), AGC target of 2e5, max inject time of 300ms, isolation window of 0.8, and fixed first mass 125. For targeting unmodified peptides, we recommend a resolution of 15,000, AGC target of 1e5, max inject time of 30–50ms, isolation window of 0.8, and fixed first mass 125. Import the peptide inclusion list(s) generated previously.
4. Inject 2 µL of sample (1 µg peptides loaded on column) per sample.
5. Once complete, import all generated data into the Skyline .skyfile that was generated to create the inclusion list. Match the transition settings to the settings used for acquisition. Import results as single injection replicates in files.

6. Imported results are automatically integrated, but a manual review is recommended.
7. Once a peptide has been identified, the integration boundaries can be manually adjusted to correct errors introduced by automated peak sampling.
8. If using PRM for validation without heavy labeled peptides:
 - a. Verify that all of the monitored unmodified peptides display the same trend across all infection time points measured.
 - b. Select the most abundant 3–5 transitions from the acetylated peptide and the most abundant 3–5 transitions from the most abundant unmodified peptide.
 - c. Export the data from Skyline to Microsoft Excel.
 - d. Conduct the following set of normalizations:
 - i. Normalize the unmodified peptide abundance by dividing the abundance value from each time point by the median abundance value.
 - ii. Normalize the abundance of the acetylated peptide by dividing the abundance at each time point by the normalized protein abundance at the corresponding time point.

3.3. Absolute quantification of acetylation abundance using PRM

In order to conduct an absolute quantification of an acetylation of interest, it is necessary to generate a calibration curve in advance. To do so, prepare a sample (*e.g.*, matrix) that most closely mimics the sample that will be used to conduct the infection time course experiment. Process the matrix in the same manner in which the actual samples will be processed. At the end of the preparation, split the matrix into 6–10 aliquots. Then, add the same number of fmol of light acetylated peptide to each sample. Next, add varying fmol dilutions of the heavy labeled acetylated peptide to each sample in a range from 1 to 200 fmol. From the mass spectrometry data, generate a heavy/light ratio curve that will establish the linear range and the upper and lower limits of quantification. This curve can then be used to determine the fmol of acetylated peptide present in each time course sample by calculating the number of fmol that correspond to the heavy (spiked in peptide of known concentration) to light (endogenous acetylated peptide) ratio.

3.4. Notes

1. Highly purified light and heavy peptides that match the sequence of the acetylated peptide of interest (with the acetyl modification added) can be ordered from several suppliers, including New England Peptide, LLC (Gardner, MA).
2. Freeze the remaining lysate at -80°C for potential future use.

3. Alternatively, the heavy labeled peptide may be spiked into each sample immediately prior to injection. One caveat to this approach is that it will not account for losses during sample preparation.
4. Several factors should be taken into consideration when selecting proteotypic peptides for PRM analysis. Because acetylation induces a missed trypsin cleavage, both the modified and unmodified version of the same peptide cannot be monitored (as can be done for phospho-peptides) unless a different digestion enzyme is used. Instead, if using a trypsin digestion, it is advisable to monitor 3–5 peptides from the protein of interest that do not contain any modifications. These peptides should be 8–25 amino acids in length and be unique to the protein of interest. If possible, it is recommended to exclude amino acids that frequently undergo chemical modification (*e.g.*, methionine/tryptophan oxidation, asparagine/glutamine deamidation). For the acetylated peptide of interest, it is critical to ensure that the peptide being targeted is the acetylated form. The modification may need to be manually annotated in Skyline as the peptide results from a missed cleavage.
5. If the initial detection of the peptides results from a run of a different time gradient (*e.g.*, 150 min instead of 60min), then an unscheduled PRM needs to be run in order to determine the retention time windows to be used during the scheduled PRM.
6. We recommend exporting the unmodified peptides in one inclusion list and the acetylated peptide(s) in a separate inclusion list since these two types of peptides may require different parameters on the instrument.
7. For more detail on optimizing PRM methods, we recommend (Gallien, Bourmaud, Kim, & Domon, 2014). If PRM is conducted on a mass spectrometer with an Orbitrap, all transitions for each peptide will be acquired concurrently, allowing the user to select the transitions that will be used for quantification post-acquisition. Since acetyl-lysine peptides are likely to have relatively low abundance in an unfractionated whole cell lysate sample, in a single PRM run we recommend targeting 5–10 acetylated peptides plus unmodified peptides for each protein of interest. One caveat is that if too many of the peptides fall within the same retention time window (*e.g.*, 5–8 min), then the targeted peptides may need to be split between two or more runs.
8. While 30,000 resolution is the recommended starting resolution, the resolution can be increased without penalty because the injection time is so long. Additionally, if the peptides are very low in abundance, the injection time may need to be increased.

4. Perspective on characterizing the biological function of specific acetylation sites

After identifying proteins with varying acetylation states during viral infection, the next step is to elucidate the function of these acetylations and how they may benefit host defense or

viral replication processes. Often, a combination of techniques, such as the ones discussed below, enables profiling the function of a specific acetylation site.

First, site-directed mutagenesis using standard cloning techniques can be utilized to generate plasmids expressing mutant proteins in which the lysine of interest is either replaced with a glutamine (acetyl mimic) or an arginine (charge mimic that cannot be acetylated). These plasmids can then be transfected into cells or used to generate stable cell lines, either in a wild type background or a background in which the expression of the endogenous protein of interest has been knocked out. The cells expressing the wild type protein, acetyl mimic, or charge mimic mutant protein can be infected. Measurements of viral gene expression, viral protein levels, viral genome copy numbers, and viral titers can be used to determine how the acetylation may impact viral replication. Second, as previous work has shown, the acetylation status of a protein can influence its subcellular localization (Chen & Greene, 2004; Li et al., 2012). Therefore, confocal microscopy can be used to visualize the subcellular localization of acetyl-mimic or charge-mimic proteins throughout the course of a viral infection. If the identified acetylations are on viral proteins, the same point mutation strategy can be used to generate mutant viral strains, which can then be investigated using similar functional assays. These techniques have been applied in several studies focused on both cellular and viral proteins (Bres et al., 2002; Giese et al., 2017; Choi et al., 2016; Murray et al., 2018).

In addition to understanding the function of an acetylation, it is also important to determine the mechanisms underlying its regulation by identifying the acetyltransferase and the deacetylase that act on the site of interest. Certain online tools, such as the Cuckoo Workgroup, have databases that allow the prediction of which acetyltransferases are likely to act at a particular site, as in Deng et al. (2016). These predictions can then be assessed via both *in vitro* and cell culture experiments. For example, an acetylated peptide from the protein of interest can be synthesized and incubated with the predicted acetyltransferase or deacetylase; the degree of addition or removal of the acetyl modification can be determined by mass spectrometry or western blotting (if an antibody against the acetylated site of interest is available), as in (Chen et al., 2012; Choudhary et al., 2009; Mathias et al., 2014). If there are specific drugs that inhibit or activate the predicted modifying enzymes, treatments with these drugs can be performed in cell culture to then determine the effect on the site of interest both in uninfected cells and during viral infection. Alternatively, CRISPR-Cas9-mediated knockouts or shRNA- or siRNA-mediated knockdowns of the predicted modifying enzyme can be used in combination with mass spectrometry to assess the impact of these enzymes on the site of interest. One consideration when conducting these experiments is that several deacetylases and acetyltransferases have overlapping substrates.

5. Using the *Fluor de Lys* assay to measure *in vitro* the enzymatic activities of deacetylases

The *Fluor de Lys* assay is a commonly employed fluorometric method for assessing the *in vitro* enzymatic activity of deacetylases (Gurvich, Tsygankova, Meinkoth, & Klein, 2004; Joshi et al., 2013; Li et al., 2015; Miteva & Cristea, 2014; Zhang, Patel, Xu, & Veenstra,

2018; Zhou, Marks, Rifkind, & Richon, 2001), including HDACs and sirtuins (Fig. 4). The assay can be used to test the activity of purified enzymes or enzymes isolated as part of a complex. Alternatively, the assay can test the activity within a cell lysate. In this section, we provide a protocol for the application of this method to assess the enzymatic activity of a particular HDAC of interest following its immunoaffinity purification (IP). In a previous global HDAC interactome study (Joshi et al., 2013), we have applied this *IP-Fluor de Lys* workflow to determine the enzymatic activities of the 11 human HDACs in T cells, and we started each IP with approximately 0.1 g of T cells. However, we found this approach also to be effective at studying deacetylase activities in different human cell types, including the commonly used HEK293, U2OS, and HFF (fibroblast) cells. For an example of a detailed protocol for the IP of HDACs, please see Guise and Cristea (2016). It should be noted that different types of magnetic beads conjugated with an antibody against either an endogenous HDAC or a tagged form of the HDAC may be used. At the end of the IP, the HDAC still bound to the beads is incubated with the acetylated substrate, which can be either a single acetylated lysine amino acid or a peptide containing an acetylated lysine residue. The use of a single acetylated lysine can help assess whether an enzyme of interest has enzymatic activity or whether a mutation in an enzyme abolishes or enhances this activity. The use of modified peptides can help test potential substrates and the ability of an enzyme to preferentially deacetylate a certain sequence. If the enzyme is capable of deacetylation, the removal of the acetyl group sensitizes the tested substrate. Upon the addition of a developer reagent, a fluorophore is produced, and the relative fluorescence level is indicative of the relative degree of enzyme activity. The protocol outlined below can be applied to testing samples from different time points of a viral infection in order to assess how individual HDAC activities change at different stages of infection.

5.1 Equipment and reagents

1. Fluorometric HDAC activity assay kit (Fluor-de-Lys[®]) (Enzo Life Sciences or other similar kit)
 - a. This kit includes (1) assay buffer (50mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂), (2) acetyl substrate, (3) inhibitor (Trichostatin A = TSA), and (4) detection reagent.
2. 96-well plate with optical bottom
3. 2 mL round bottom tubes
4. Magnetic tube holder rack (Fisher Scientific)
5. Microplate-reading fluorimeter with excitation at 350–380nm and detection at 450–480 nm
6. 1 × TES buffer (2% SDS, 0.5mM EDTA, 53mM Tris-HCl, 70mM Tris base)
7. 70 °C heatblock

5.2. Method (Note 1)

This protocol starts after the purification of an enzyme of interest using immunoaffinity purification. Specifically, the steps outlined here occur after the enzyme was bound to the

resin used (*e.g.*, magnetic beads) and after the washes of the beads (according to the IP protocol used). Importantly, this protocol is carried out while the enzyme is still bound to the resin; therefore, an elution step is not performed at the end of the enzyme IP.

1. Once the enzyme is bound to the beads, wash the beads three times with assay buffer with sufficient volume to properly cover and suspend the beads (usually 500 μ L).
2. Resuspend each sample in 300 μ L assay buffer and aliquot 60 μ L into each of three round bottom tubes (tubes #1, 2, 3).
3. The first tube (tube #1) is used for western blotting to assess variability between the isolation of the bait in the IPs at different time points.
 - a. The protein can be eluted from the beads in 50 μ L 1 $\times$ TES buffer by incubating the beads with the elution buffer for 10 min at 70 $^{\circ}$ C. Each elution should be transferred to a new tube for protein concentration analysis and western blot sample preparation.
4. The contents of tubes #2 and #3 should be divided into three additional round bottom tubes (tubes a-c) for three technical replicates (a-c). This will result in the set of tubes that will be used in the following experimental setup:

Tubes	Assay condition
#2a—c	90 min, master mix without TSA
#3a—c	90 in, master mix with TSA

5. Make two master mixes of 400 μ M substrate (*i.e.*, acetyl-Lys or acetylated peptide) $\pm$ 2 μ M TSA (HDAC inhibitor) in assay buffer pre-warmed to 37 $^{\circ}$ C (Notes 2 and 3).
6. Use a magnetic rack to hold the tubes while aspirating the assay buffer from all the technical replicate tubes.
7. Remove tubes from the magnetic rack. Add 20 μ L of respective master mix to tubes #2a-c and #3a-c. Incubate all tubes at 37 $^{\circ}$ C for 1.5 h. Flick tubes every 10 min to ensure mixing.
8. Quench the reaction with 40 μ L 1 $\times$ Developer diluted in assay buffer. Incubate with the developer for 15 min at 37 $^{\circ}$ C.
9. Place tubes on magnetic rack and transfer supernatants to a 96-well plate with optical bottom.
10. Use a microplate-reading fluorimeter to read the fluorescence. To ensure full development of the signal, take readings every 5 min until all the fluorescence signals plateau.
11. Determine consistency of isolation by western blot from tube #1. Additionally, the proteins bound to the beads in the tubes used for the *Fluor de Lys* assay can

be eluted as in step 3a and can be analyzed by mass spectrometry to determine how protein interactions with the HDAC of interest change during viral infection.

5.3 Notes

1. This protocol is applicable to all HDACs (HDACs and SIRT).
2. The common HDAC inhibitor TSA will not inhibit class III HDACs (SIRT). To inhibit a SIRT, we recommend protocol optimization in the presence of nicotinamide and the inclusion of NAD⁺, the required cofactor as in Miteva and Cristea (2014).
3. The HDAC inhibitor (*e.g.*, TSA or your inhibitor of choice) serves as a control for no HDAC activity.

6. Conclusion

Modulation of protein acetylation has emerged as a critical regulatory toggle during viral infection. An accumulating body of evidence has shown that the acetylation of both cellular and viral proteins can be finely-tuned during the progression of an infection, and these events can contribute to either host immune responses or different stages of the virus life cycle. In this chapter, we have provided protocols for identifying protein acetylation sites and characterizing their regulation during viral infection. Enrichment of acetylated peptides via anti-acetyl lysine immunoaffinity purification, followed by quantitative mass spectrometry analyses, provides an effective way to determine global changes in the cellular acetylome at different time points of infection. As viruses are known to manipulate organelle-specific functions (Jean Beltran, Cook, & Cristea, 2017), we anticipate that this method can be combined with organelle fractionation techniques to further increase the depth of detection of changes in acetylation within a specific subcellular compartment. In recent years, there has been a transition from using the more conventional antibody-based western blotting detection methods to using targeted mass spectrometry for the specific and accurate quantification of site-specific posttranslational modifications. In this chapter, we provide a protocol for using PRM-MS to monitor lysine acetylation during infection. We also indicate that heavy-labeled acetylated peptides can be used for absolute quantification and information about stoichiometry. Although numerous protein acetylations have been identified in human cells, few studies have interrogated infection-induced alterations in acetylation. We expect that many acetylation sites and their dynamic regulation during infection remain to be discovered. Furthermore, the functions of the majority of the reported protein acetylation sites either during or outside the context of infection remain unknown. Future studies will be needed to further characterize the temporal roles and regulation of acetylation events. We provide a brief overview of the types of assays, including site-directed mutagenesis, microscopy, and viral titer measurements, that can be conducted to start to understand the impact of an acetylation event during viral infection. We also describe the protocol for the *Fluor de Lys* assay, which is one of the most commonly used assays for determining the activity of a deacetylase. We expect that the application of these techniques to viral infection will provide greater insight into acetylation-driven mechanisms at the interface between host defense and virus replication. We further hope that these methods

will promote the use of mass spectrometry-based methods in virology studies, which will offer biological insights that can only be reached by the integration of these two fields of science, virology and proteomics.

Acknowledgments

We thank past and present members of the Cristea lab for their work in developing the methods presented in this chapter. We are grateful for funding from the NIH (GM114141) and a Mallinckrodt Scholar Award to I.M.C., a National Science Foundation Graduate Research Fellowship (NSF-GRFP DGE-1656466) to L.A.M., and the National Institutes of Health NIGMS (T32GM007388).

References

- Berro R, Kehn K, de la Fuente C, Pumfery A, Adair R, Wade J, et al. (2006). Acetylated tat regulates human immunodeficiency virus type 1 splicing through its interaction with the splicing regulator p32. *Journal of Virology*, 80(7), 3189–3204. 10.1128/jvi.807.3189-3204.2006. [PubMed: 16537587]
- Brès V, Kiernan R, Emiliani S, & Benkirane M (2002). Tat acetyl-acceptor lysines are important for human immunodeficiency virus type-1 replication. *The Journal of Biological Chemistry*, 277(25), 22215–22221. 10.1074/jbc.M201895200.
- Burkard C, Bloyet LM, Wicht O, Van Kuppeveld FJ, Rottier PJM, De Haan CAM, et al. (2014). Dissecting virus entry: Replication-independent analysis of virus binding, internalization, and penetration using minimal complementation of p-galactosidase. *PLoS One*, 9(7), 13–16. 10.1371/journal.pone.0101762.
- Carabetta VJ, Greco TM, Cristea IM, & Dubnau D (2019). YfmK is an N^e-lysine acetyltransferase that directly acetylates the histone-like protein HBSu in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, 116, 3752–3757. 10.1073/pnas.1815511116. [PubMed: 30808761]
- Carabetta VJ, Greco TM, Tanner AW, Cristea IM, & Dubnau D (2016). Temporal regulation of the *Bacillus subtilis* acetylome and evidence for a role of MreB acetylation in cell wall growth. *MSystems*, 1(3), e00005–16. 10.1128/mSystems.00005-16.
- Chen LF, & Greene WC (2004). Shaping the nuclear action of NF-κB. *Nature Reviews. Molecular Cell Biology*, 5(5), 392–401. 10.1038/nrm1368. [PubMed: 15122352]
- Chen Y, Zhao W, Yang JS, Cheng Z, Luo H, Lu Z, et al. (2012). Quantitative acetylome analysis reveals the roles of SIRT1 in regulating diverse substrates and cellular pathways. *Molecular & Cellular Proteomics*, 11(10), 1048–1062. 10.1074/mcp.M112.019547. [PubMed: 22826441]
- Choi SJ, Lee H-C, Kim J-H, Park SY, Kim T-H, Lee W-K, et al. (2016). HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I. *The EMBO Journal*, 35(4), 429–442. 10.15252/embj.201592586. [PubMed: 26746851]
- Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, et al. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*, 325(5942), 834–840. 10.1126/science.1175371. [PubMed: 19608861]
- Choudhary C, Weinert BT, Nishida Y, Verdin E, & Mann M (2014). The growing landscape of lysine acetylation links metabolism and cell signalling. *Nature Reviews. Molecular Cell Biology*, 15(8), 536–550. 10.1038/nrm3841. [PubMed: 25053359]
- Cuevas-Bennett C, & Shenk T (2008). Dynamic histone H3 acetylation and methylation at human cytomegalovirus promoters during replication in fibroblasts. *Journal of Virology*, 82(19), 9525–9536. <https://doi.org/10.1128/JVI.00946-08>. [PubMed: 18653451]
- Deng L, De la Fuente C, Fu P, Wang L, Donnelly R, Wade JD, et al. (2000). Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones. *Virology*, 277(2), 278–295. 10.1006/viro.2000.0593. [PubMed: 11080476]
- Deng W, Wang C, Zhang Y, Xu Y, Zhang S, Liu Z, et al. (2016). GPS-PAIL: prediction of lysine acetyltransferase-specific modification sites from protein sequences. *Scientific Reports*, 6(39787). 10.1038/srep39787.

- Dorr A, Kiermer V, Pedal A, Rackwitz HR, Henklein P, Schubert U, et al. (2002). Transcriptional synergy between Tat and PCAF is dependent on the binding of acetylated Tat to the PCAF bromodomain. *The EMBO Journal*, 21(11), 2715–2723. 10.1093/emboj/21.11.2715. [PubMed: 12032084]
- Gallien S, Bourmaud A, Kim SY, & Domon B (2014). Technical considerations for large-scale parallel reaction monitoring analysis. *Journal of Proteomics*, 100, 147–159. [PubMed: 24200835]
- Giese S, Ciminski K, Bolte H, Moreira EA, Lakdawala S, Hu Z, et al. (2017). Role of influenza A virus NP acetylation on viral growth and replication. *Nature Communications*, 8(1), 1259 10.1038/s41467-017-01112-3.
- Guise AJ, Budayeva HG, Diner BA, & Cristea IM (2013). Histone deacetylases in herpesvirus replication and virus-stimulated host defense. *Viruses*, 5(7), 1607–1632. 10.3390/v5071607. [PubMed: 23807710]
- Guise AJ, & Cristea IM (2016). Approaches for studying the subcellular localization, interactions, and regulation of histone deacetylase 5 (HDAC5). *Methods in Molecular Biology*, 1436, 47–84. 10.1007/978-1-4939-3667-0. [PubMed: 27246208]
- Gurvich N, Tsygankova OM, Meinkoth JL, & Klein PS (2004). Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Research*, 64(3), 1079–1086. 10.1158/0008-5472.CAN-03-0799. [PubMed: 14871841]
- Harrod R, Nacs J, Van Lint C, Hansen J, Karpova T, McNally J, et al. (2003). Human immunodeficiency virus type-1 Tat/co-activator acetyltransferase interactions inhibit p53 Lys-320 acetylation and p53-responsive transcription. *The Journal of Biological Chemistry*, 278(14), 12310–12318. 10.1074/jbc.M211167200.
- Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Herold BC, et al. (2014). Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. *Journal of Immunology*, 177(12), 8658–8666. 10.4049/jimmunol.177.12.8658.
- He M, Zhang L, Wang X, Huo L, Sun L, Feng C, et al. (2013). Systematic analysis of the functions of lysine acetylation in the regulation of tat activity. *PLoS One*, 8(6), e67186. 10.1371/journal.pone.0067186.
- Hebner C, Beglin M, & Laimins LA (2007). Human papillomavirus E6 proteins mediate resistance to interferon-induced growth arrest through inhibition of p53 acetylation. *Journal of Virology*, 81(23), 12740–12747. 10.1128/JVI.00987-07. [PubMed: 17898049]
- Hsu CH, Chang MDT, Tai KY, Yang YT, Wang PS, Chen CJ, et al. (2004). HCMV IE2-mediated inhibition of HAT activity downregulates p53 function. *The EMBO Journal*, 23(11), 2269–2280. 10.1038/sj.emboj.7600239. [PubMed: 15141169]
- Husain M, & Cheung C-Y (2014). Histone deacetylase 6 inhibits influenza A virus release by downregulating the trafficking of viral components to the plasma membrane via its substrate, acetylated microtubules. *Journal of Virology*, 88(19), 11229–11239. 10.1128/jvi.00727-14.
- Jean Beltran PM, Cook KC, & Cristea IM (2017). Exploring and exploiting proteome organization during viral infection. *Journal of Virology*, 91(18), 1–9. 10.1128/jvi.00268-17.
- Joshi P, Greco TM, Guise AJ, Luo Y, Yu F, Nesvizhskii AI, et al. (2013). The functional interactome landscape of the human histone deacetylase family. *Molecular Systems Biology*, 9(1), 1–21. 10.1038/msb.2013.26.
- Kiernan RE, Vanhulle C, Schiltz L, Adam E, Xiao H, Maudoux F, et al. (1999). HIV-1 tat transcriptional activity is regulated by acetylation. *The EMBO Journal*, 18(21), 6106–6118. 10.1093/emboj/18.21.6106. [PubMed: 10545121]
- Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, et al. (2006). Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Molecular Cell*, 23(4), 607–618. 10.1016/j.molcel.2006.06.026. [PubMed: 16916647]
- Kosono S, Tamura M, Suzuki S, Kawamura Y, Yoshida A, Nishiyama M, et al. (2015). Changes in the acetylome and succinylome of *Bacillus subtilis* in response to carbon source. *PLoS One*, 10(6), 1–24. 10.1371/journal.pone.0131169.
- Koyuncu E, Budayeva HG, Miteva YV, Ricci DP, Silhavy TJ, Shenk T, et al. (2014). Sirtuins are evolutionarily conserved viral restriction factors. *MBio*, 5(6). 10.1128/mBio.02249-14. <https://doi.org/ARTNe02249-14>.

- Lakadamyali M, Rust MJ, Babcock HP, & Zhuang X (2003). Visualizing infection of individual influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 100(16), 9280–9285. 10.1073/pnas.0832269100. [PubMed: 12883000]
- Lange V, Picotti P, Domon B, & Aebersold R (2008). Selected reaction monitoring for quantitative proteomics: a tutorial. *Molecular Systems Biology*, 4, 222. 10.1038/msb.2008.61. [PubMed: 18854821]
- Li T, Diner BA, Chen J, & Cristea IM (2012). Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. *Proceedings of the National Academy of Sciences of the United States of America*, 109(26), 10558–10563. 10.1073/pnas.1203447109.
- Li S, Fossati G, Marchetti C, Modena D, Pozzi P, Reznikov LL, et al. (2015). Specific inhibition of histone deacetylase 8 reduces gene expression and production of proinflammatory cytokines in vitro and in vivo. *The Journal of Biological Chemistry*, 290(4), 2368–2378. 10.1074/jbc.M114.618454. [PubMed: 25451941]
- Lin AE, Greco TM, Dohner K, Sodeik B, & Cristea IM (2013). A proteomic perspective of inbuilt viral protein regulation: pUL46 tegument protein is targeted for degradation by icp0 during herpes simplex virus type 1 infection. *Molecular & Cellular Proteomics*, 12(11), 3237–3252. 10.1074/mcp.m113.030866. [PubMed: 23938468]
- Mathias RA, Greco TM, Oberstein A, Budayeva HG, Chakrabarti R, Rowland EA, et al. (2014). Sirtuin 4 is a lipamidase regulating pyruvate dehydrogenase complex activity. *Cell*, 159(7), 1615–1625. 10.1016/j.xell.2014.11.046. [PubMed: 25525879]
- Miteva YV, & Cristea IM (2014). A proteomic perspective of sirtuin 6 (SIRT6) phosphorylation and interactions and their dependence on its catalytic activity. *Molecular & Cellular Proteomics*, 13(1), 168–183. 10.1074/mcp.M113.032847.
- Muñoz-Fontela C, Gonzalez D, Marcos-Villar L, Campagna M, Gallego P, Gonzalez-Santamaria J, et al. (2011). Acetylation is indispensable for p53 antiviral activity. *Cell Cycle*, 10(21), 3701–3705. 10.4161/cc.10.21.17899. [PubMed: 22033337]
- Murray LA, Sheng X, & Cristea IM (2018). Orchestration of protein acetylation as a toggle for cellular defense and virus replication. *Nature Communications*, 9(1), 4967. 10.1038/s41467-018-07179-w.
- Naghavi MH, & Walsh D (2017). Microtubule regulation and function during virus infection. *Journal of Virology*, 91(16), 1–16. 10.1128/jvi.00538-17.
- Ott M, Schnölzer M, Garnica J, Fischle W, Emiliani S, Rackwitz HR, et al. (1999). Acetylation of the HIV-1 tat protein by p300 is important for its transcriptional activity. *Current Biology*, 9(24), 1489–1493. 10.1016/S0960-9822(00)80120-7. [PubMed: 10607594]
- Peterson AC, Russell JD, Bailey DJ, Westphall MS, & Coon JJ (2012). Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Molecular & Cellular Proteomics*, 11(11), 1475–1488. 10.1074/mcp.o112.020131. [PubMed: 22865924]
- Procter DJ, Banerjee A, Nukui M, Kruse K, Gaponenko V, Murphy EA, et al. (2018). The HCMV assembly compartment is a dynamic golgi-derived MTOC that controls nuclear rotation and virus spread. *Developmental Cell*, 45(1), 83–100. 10.1016/j.devcel.2018.03.010. [PubMed: 29634939]
- Rappsilber J, Ishihama Y, & Mann M (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical Chemistry*, 75(3), 663–670. 10.1021/ac026117i. [PubMed: 12585499]
- Rappsilber J, Mann M, & Ishihama Y (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature Protocols*, 2(8), 1896–1906. 10.1038/nprot.2007.261. [PubMed: 17703201]
- Roizman B (2011). The checkpoints of viral gene expression in productive and latent infection: the role of the HDAC/CoREST/LSD1/REST repressor complex. *Journal of Virology*, 85(15), 7474–7482. 10.1128/jvi.00180-11. [PubMed: 21450817]
- Savelyeva I, & Dobbelstein M (2011). Infection with E1B-mutant adenovirus stabilizes p53 but blocks p53 acetylation and activity through E1A. *Oncogene*, 30(7), 865–875. 10.1038/onc.2010.461. [PubMed: 20935676]
- Shirakawa K, Chavez L, Hakre S, Calvanese V, & Verdin E (2013). Reactivation of latent HIV by histone deacetylase inhibitors. *Trends in Microbiology*, 21(6), 277–285. 10.1016/j.tim.2013.02.005. [PubMed: 23517573]

- Svinkina T, Gu HB, Silva JC, Mertins P, Qiao J, Fereshetian S, et al. (2015). Deep, quantitative coverage of the lysine acetylome using novel anti-acetyl-lysine antibodies and an optimized proteomic workflow. *Molecular & Cellular Proteomics*, 14(9), 2429–2440. 10.1074/mcp.0114.047555. [PubMed: 25953088]
- Tummers B, Goedemans R, Pelascini LPL, Jordanova ES, van Esch EMG, Meyers C, et al. (2015). The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NF κ B activation. *Nature Communications*, 6 10.1038/ncomms7537.
- Wagner GR, & Payne RM (2013). Widespread and enzyme-independent N-is an element of-acetylation and N-is an element of-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *The Journal of Biological Chemistry*, 288(40), 29036–29045. 10.1074/jbc.M113.486753.
- Weinert BT, Iesmantavicius V, Moustafa T, Scholz C, Wagner SA, Magnes C, et al. (2014). Acetylation dynamics and stoichiometry in *Saccharomyces cerevisiae*. *Molecular Systems Biology*, 10(10), 716 10.1002/msb.134766. [PubMed: 24489116]
- Wright DG, Marchal C, Hoang K, Ankney JA, Nguyen ST, Rushing AW, et al.(2016). Human T-cell leukemia virus type-1-encoded protein HBZ represses p53 function by inhibiting the acetyltransferase activity of p300/CBP and HB01. *Oncotarget*, 7(2), 1687–1706. 10.18632/oncotarget.6424. [PubMed: 26625199]
- Zhang X, Patel D, Xu Q, & Veenstra R (2018). Differences in functional expression of connexin43 and NaV1.5 by pan- and class-selective histone deacetylase inhibition in heart. *International Journal of Molecular Sciences*, 19(8), 2288 10.3390/ijms19082288.
- Zhou X, Marks PA, Rifkind RA, & Richon VM (2001). Cloning and characterization of a histone deacetylase, HDAC9. *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), 10572–10577. 10.1073/pnas.191375098.

Fig. 1.

Acetylation of cellular and viral proteins contributes to critical regulatory mechanisms during infection. Host proteins (green), viral proteins (purple), host genome (black), viral genome (blue). Acetylation of histones (1) associated with integrated retroviral genomes, such as that of HIV, or (2) associated with viral genomes that become chromatinized upon entry into the nucleus, such as those of HCMV and HSV-1, modulates viral gene expression. (3) Deacetylation of NF-κB induces shuttling to the cytoplasm. (4) Acetylation of p53 activates its antiviral transcriptional activity. (5) Deacetylation of IFI16 induces shuttling into the nucleus. (6) Deacetylation of the RNA binding protein RIG-I activates its binding to

pathogenic RNA. (7) Acetylation of Lamin B1 stabilizes the lamina during HCMV infection. Acetylation of microtubules (8) stabilizes the cytoplasmic replication centers of RNA viruses, (9) stabilizes the assembly complex formed during HCMV infection, and (10) facilitates transport of viral proteins to the cell membrane for assembly. (11) Acetylation of the HIV protein Tat activates its transcriptional activity. (12) Acetylation of the Influenza A protein NP impacts viral replication. (13) Acetylation of the HCMV protein UL26 inhibits viral replication.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Defining and quantifying the temporal cellular acetylome during viral infection. This figure outlines the workflow for the anti-acetyl lysine immunoaffinity purification protocol and highlights the key steps on each day.

Fig. 3.

Detection and quantification of site-specific acetylation by targeted mass spectrometry using parallel reaction monitoring (PRM). (A) This workflow highlights considerations when selecting acetylated peptides for PRM analysis and highlights the differences between label-free relative quantification and absolute quantification. (B) The upper two graphs illustrate how the order of transitions detected for an acetyl peptide of interest in uninfected cells versus infected cells is the same, but the abundance of these transitions may vary. In this panel, an increase in acetylation during infection is depicted. A representative integration

graph comparing uninfected cells to cells from an infection time point is shown in the lower panel. (C) This schematic represents how different acetylation sites on the same protein may display distinct changes in abundance during infection (increased abundance shown in yellow, decreased abundance shown in blue, no change in abundance shown in gray).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Assessing deacetylase activity of HDACs and sirtuins using the *Fluor de Lys* assay. After conducting an immunoaffinity purification of the deacetylase of interest (HDAC or sirtuin) from the infection time point(s), the deacetylase remains bound to the beads. The *Fluor de Lys* acetyl substrate is added to the sample in the presence or absence of a deacetylase inhibitor (such as TSA). After incubation, during which the substrate is deacetylated by the enzyme, a detection reagent is added. The supernatant is transferred to a 96-well plate in which a fluorometric readout indicates the activity of the immunoaffinity purified deacetylase.

Table 1

Human deacetylases (HDACs) and acetyltransferases (HATs).

Deacetylases		
Enzyme	Class	Subcellular localization
HDAC1	I	Nucleus
HDAC2	I	Nucleus
HDAC3	I	Nucleus/cytoplasm
HDAC8	I	Nucleus
HDAC4	IIa	Nucleus/cytoplasm
HDAC5	IIa	Nucleus/cytoplasm
HDAC7	IIa	Nucleus/cytoplasm
HDAC9	IIa	Nucleus/cytoplasm
HDAC6	IIb	Cytoplasm
HDAC10	IIb	Nucleus/cytoplasm
HDAC11	IV	Nucleus/cytoplasm
SIRT1	III	Nucleus
SIRT2	III	Cytoplasm
SIRT3	III	Mitochondria
SIRT4	III	Mitochondria
SIRT5	III	Mitochondria
SIRT6	III	Nucleus
SIRT7	III	Nucleolus/nucleus

Acetyltransferases		
Enzyme	Family	Subcellular localization
HAT1/KAT1	GNAT	Nucleus/cytoplasm
GCN5/KAT2A	GNAT	Nucleus
PCAF/KAT2B	GNAT	Nucleus
ELP3	GNAT	Nucleus/cytoplasm
Tip60/KAT5	MYST	Nucleus
KAT6A	MYST	Nucleus
MORF/KAT6B	MYST	Nucleus
HBO1/KAT7	MYST	Nucleus/cytoplasm
KAT8	MYST	Nucleus
p300	p300/CBP	Nucleus/cytoplasm
CBP	p300/CBP	Nucleus/cytoplasm