We have recently developed two mouse monoclonal anti-acetyl lysine (Ac-K) antibodies that have demonstrated excellent ability in enriching acetylated proteins from cell and tissue extracts. The antibodies were raised against a proprietary mixture of acetylated proteins designed to optimize acetyl lysine recognition for a wide range of sequence motifs. Our validation data showed that both 7B5A1 and 19C4B2.1 immunoprecipitated a broad range of acetylated proteins, with unique specificity, from cells treated with trichostatin A (TSA) and nicotinamide. A more complete enrichment profile was observed when both clones were used together in immunoprecipitation validation experiments. For western blot applications, clone 7B5A1 detected more acetylated proteins than clone 19C4B2.1. Both clones showed an enhanced ability to detect acetylated proteins when conjugated to horseradish peroxidase (HRP). Compared to other commercial Ac-K reagents, clones 7B5A1 and 19C4B2.1 were superior at immunoprecipitating acetylated proteins. They also detected a slightly different acetylated protein profile in western blot analysis. Currently, both clones have also been validated for immunofluorescence staining applications (see separate white paper for this data).

**Introduction**

Acetylation of proteins can occur as a co-translational or post-translational modification (PTM) (1). Co-translational acetylation occurs at the N-terminus of approximately 85% of mammalian proteins, is irreversible, and is thought to be important in protein stability, localization, and synthesis (1). Post-translational acetylation occurs on the epsilon amino group of lysine residues as a reversible and highly dynamic PTM that is known to be a key regulator in multiple cellular events, including chromatin structure, transcription, metabolism, signal transduction, and cytoskeletal regulation (2-3).

Identification and enrichment of acetylated proteins have been hindered by a lack of high affinity Ac-K antibodies. Here, we present validation data for two newly developed Ac-K antibodies, 7B5A1 and 19C4B2.1, and their usefulness in enriching and detecting acetylated proteins in both cell and tissue lysates.

**Results**

Two newly developed Ac-K antibodies were compared to AAC01 (Cytoskeleton), an anti-acetyl lysine antibody, that has been validated for a variety of applications such as western blot, immunoprecipitation (IP), immunofluorescence staining and ChIP. Figure 1a shows the detection of acetylated proteins by western blot from Cos cells treated with 1mM TSA and 1mM nicotinamide.

For IP experiments, 7B5A1 and 19C4B2.1 were separately crosslinked to protein G beads (hereafter termed affinity beads) to minimize the co-elution of IP antibodies (heavy and light chain) during the final protein elution step. Interestingly, IP with 7B5A1 and 19C4B2.1 affinity beads from Cos cell lysates treated with TSA and nicotinamide resulted in slightly different acetylated protein isolation patterns (Figure 1b). Still, either antibody showed a more robust and higher enrichment of acetylated proteins relative to AAC01 affinity bead acetyl lysine enrichment. These data suggest that both 7B5A1 and 19C4B2.1 performed better than AAC01 in detecting and enriching acetylated proteins.

Next, 7B5A1 and 19C4B2.1 were tested against other commercial anti-acetyl lysine reagents from Cell Signaling and ImmuneChem to compare their detection abilities. Western blot analysis, using the various antibodies showed that both 7B5A1 and the rabbit antibody from cell signaling detected the most robust acetylation profile; however, the profiles differed slightly different. Of note, both 7B5A1 and 19C4B2.1 showed enhanced detection signals when they were conjugated to HRP directly (Figure 2).

Both 7B5A1 and 19C4B2.1 showed slightly different enrichment patterns of acetylated proteins; thus, when combined the mixture of these affinity beads isolated a more complete acetylation profile (figure 3). We then took these beads and performed side-by-side IP comparisons with different Ac-K reagents. 1mg of cell lysate from Cos cells treated or untreated with TSA and nicotinamide
were used per IP reaction. 7B5A1-19C4B2.1 bead mixture was used at 20 and 40ug per IP reaction. Ac-K affinity bead from ImmuneChem was used at 40 and 80ug. Ac-K rabbit monoclonal mix from Cell Signaling was used at 1:100 per manufacturer’s instruction. We didn’t include the Cell Signaling Ac-K mouse monoclonal antibody in IP experiment because it is not recommended for IP applications. 60ug of normal mouse IgG bead was included as a non-specific proteins binding control. The results suggested that our antibodies bead mixture performed exceptionally well in enriching a wide range of acetylated proteins whereas the other commercial Ac-K reagents could only enrich the most abundance acetylated proteins (e.g. acetylated tubulin and histones) (Figure 3).

Isolation of a robust and comprehensive acetylation profile is critical, but having the ability to identify acetylated target proteins is paramount for endogenous studies, validation studies, and for new target protein identification. Figure 4a demonstrates the detection of changes in the acetylation profile of four different proteins (EGFR, Hsp90, P53 and RhoGDI) from the enriched acetylated protein pool from A431 cells treated with TSA and nicotinamide.

Utilization of these antibodies and affinity beads are not limited to cell culture studies. Data provided here show IP of acetylated proteins from mouse liver and heart tissue that was lysed with BlastR buffer. IP was performed using 7B5A1-19C4B2.1 bead mixture (60ug). Total cell lysate (Input) and immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blot with antibodies against EGFR (Millipore, 1:1000), Hsp90 (Abcam, 1:20,000), P53 (Sigma, 1:2000), and RhoGDI (Millipore, 1:1000).

**Summary**

Two high-affinity pan-Ac-K antibodies, clone 7B5A1 and 19C4B2.1, were developed and compared to established Ac-K reagents. These data show that these highly optimized reagents perform as well as other for detecting acetylated proteins, and are superior for enrichment of endogenous, acetylated proteins in cell and tissue lysates. Importantly, the enrichment bead formulation was optimal for endogenous studies, validation studies, and for new target protein identification.
mized to minimize heavy and light chain interference, while simulta-
neously providing the most robust acetylated protein profile. This
was achieved in part by accounting for the existence of high-
abundance acetylated histones that can often saturate other Ac-K
enrichment tools. In doing so, this allowed for the enrichment of a
representative pool of acetylated proteins without using subcellular
fractionation. Of importance, these data demonstrated that detection
of physiologic changes in acetylation of multiple target proteins was
possible using the 7B5A1-19C4B2.1 bead mixture. The enrichment,
isolement, and detection of these acetylated target proteins occurred
in the same enriched protein pool, instead of conducting IP for each
protein as would need to be done using protein specific antibody
enrichment. This level of enrichment speaks to the quality of the IP
capabilities that these antibodies posses. In conclusion, 7B5A1 and
19C4B2.1 antibodies performed exceptionally well compared to other
commercial Ac-K reagents; especially, in IP applications. We be-
lieve that our Ac-K antibodies and enrichment affinity beads are a
great discovery tool for identifying post-translational modification of
proteins by acetylation.

Materials and Methods

Cell Culture and Reagents

Cos and A431 cells were grown in DMEM media (ATCC, VA) sup-
plemented with 10% FBS (Atlas Biologicals, CO) and penicillin/
streptomycin (ThermoFisher, MA). Trypsin/EDTA was obtained from
Gibco (ThermoFisher, MA). For treatment, cells (either Cos or A431)
were grown to about 70% confluent and then treated with 1 mM TSA
(Cayman Chemical, MI) and 1 mM nicotinamide (Sigma, MO) for 6
hours. BlastR lysis buffer and filter system (Cytoskeleton, CO) was used
to obtain a complete whole cell lysates for western blot analysis and
enrichment of acetylated proteins. Anti-acetyl lysine mouse mono-
clonal antibody and rabbit monoclonal mix antibodies were from Cell
Signaling. Anti-acetyl lysine rabbit polyclonal antibodies and bead
conjugates were from ImmuneChem. Antibodies to EGFR and RhoGDI were from Millipore. Antibody to P53 was from Sigma. Anti-
body to Hsp90 was from Abcam.

Western blotting

Cos cells were lysed with ice-cold BlastR lysis buffer (Cytoskeleton,
CO) containing a cocktail of TSA (1 mM), nicotinamide (16.5 mM)
and protease inhibitors (PIC02) (Cytoskeleton, CO). DNA was removed
by passing the lysate through the BlastR filter system (Cytoskeleton,
CO). After dilution with BlastR dilution buffer, protein concentrations
were determined with protein reagent, ADV02 (Cytoskeleton, CO),
and measured at 600 nm OD. Protein lysate samples were separat-
ed using Tris-glycine SDS-polyacrylamide gel electrophoresis
(ThermoFisher, MA) and transferred to Immobilon-P membranes
(Millipore, MA). Membranes were blocked for 1 hr at room tempera-
ture in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl)
containing 0.05% Tween-20 (TTBS) and 5% milk (Thrive Life, UT),
and then incubated with 3% milk in TTBS solution containing prima-
ry antibodies for 1 hr at room temperature (RT). Membranes were
washed in TTBS 3 x 10 minutes, prior to secondary antibody for 1 hr at
RT. Bound antibodies were visualized with horseradish peroxidase-
coupled secondary antibodies and chemiluminescent reagent
(Cytoskeleton, CO) according to the manufacturer’s directions.

Immunoprecipitation assay

Cos and A431 cells were lysed with ice-cold BlastR lysis buffer con-
taining a cocktail TSA (1 mM), nicotinamide (16.5 mM), and protease
inhibitors (PIC02). DNA was removed by passing the lysate through
the BlastR filter system (Cytoskeleton, CO). After dilution with
BlastR dilution buffer, protein concentrations were determined with
ADV02 and measured at 600 nm OD. For tissue lysate preparation,
mouse liver or heart was lysed at 1 mL of BlastR lysis buffer per
100 mg of tissue. For these experiments 100 mg of tissue was pro-
cessed. After addition of 1 mL of BlastR lysis buffer to 100 mg of
fresh tissue, the sample was placed into a homogenizer and 10-12
strokes were applied. The viscous tissue lysate was first passed
through the BlastR filter to remove genomic DNA. Equal volume of
BlastR dilution buffer was then added to the filtered lysate and gen-
tly mixed. Lysate was then spun in a microcentrifuge at 14 k rpm for
15 min at 4°C to pellet any remaining tissue debris. Additional BlastR
dilution buffer was added to the recovered supernatant for a final
dilution of 1:5. Protein concentrations were determined with ADV02
and measured at 600 nm OD.

The appropriate amount of Ac-K reagents or mlgG control beads
were added to 1 mg of lysate for 1-2 hr at 4°C on an end-over-end
tumbler. After incubation, the affinity beads from each sample were
pelleted, and washed 3 X with BlastR wash buffer. Bound proteins
were eluted using bead elution buffer (Cytoskeleton, CO) and de-
ected by western immunoblotting.

References

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2. Lundby A. et al. 2012. Proteomic analysis of lysine acetylation sites in rat

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