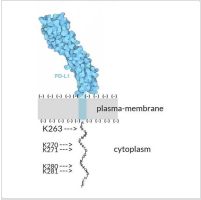

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Method for detecting acetylated PD-L1 in cell lysates

PLOS One Peer-reviewed method

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ABSTRACT

Here we present a method that allows detection of acetylated PD-L1 and is applicable to a wide range of cell lines. The method captures >90% of acetylated PD-L1 species, is semi-quantitative and simple to perform in any lab equipped with tissue culture and western blot equipment. The method involves processing cells in a lysis buffer that has been optimized for efficient immunoprecipitation (IP) of acetylated species, an IP enrichment step utilizing an acetyl-lysine affinity matrix and western blot detection of both total and acetylated PD-L1 on the same blot. This technique compliments the alternative IP approach utilizing a PD-L1 antibody as the IP reagent and an anti-acetyl lysine antibody as the detection reagent. However, because the protocol described here enables the detection of both total and acetylated PD-L1 on the same blot, this method has the advantage of allowing quantitation of the percent of PD-L1 that is acetylated, an important parameter for mechanistic interpretation.

The method described here utilizes beads that are covalently linked to the affinity antibody, resulting in extremely clean IP results. Western blots can be re-probed with a pan anti-acetyl lysine antibody to visualize the total protein acetylation profile in any given lysate, a property that is useful when examining PD-L1 acetylation in the presence of HDAC inhibitors or other treatments affecting global acetylation.

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KEYWORDS

PD-L1, Acetylation, Immunoprecipitation, Western Blot, Post-translational Modification, PTM, Posttranslational Modification, PD-L1 Modification

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MATERIALS TEXT

SECTION 1: Lysate Preparation Raw Materials

Material	Vendor	Catalog #/ Part #	Reconstitution	Storage After Reconstitution
BlastR Dilution Buffer	Cytoskeleton Inc	BDB01	Resuspend in 130 mls sterile water for a 1X stock solution.	4°C
BlastR Lysis Buffer	Cytoskeleton Inc	BLST01	Resuspend in 15 mls sterile water for a 1X stock solution.	4°C
Halt Protease Inhibitor Cocktail (100X)	ThermoFisher	78429	supplied as 100x stock in DMSO	4ºC
Trichostatin A (Class I & II HDACs inhibitor)	Cayman Chemical	89730	Dissolve 1mg in 660 µl DMSO for a 5 mM stock. Make 50 µl aliquots.	-20°C
Nicotinamide (Class III HDACs inhibitor)	Millipore Sigma	N0636	Dissolve 0.24g in 1 ml of sterile water for a 2M stock. Make 50 µl aliquots.	-20°C
N-Ethylmaleimide	Sigma	E3876	Dissolve 125 mg in 1 ml DMSO for a 1M stock. Make 50 µl aliquots.	-20°C
Precision Red Protein Assay	Cytoskeleton Inc	ADV02	na	room temp.
BlastR Filters	Cytoskeleton Inc.	BLR02	na	room temp.

We always include the deubiquitinase (DUB) inhibitor N-ethylmaleimide in our buffer as we are looking at acetylation and ubiquitination in PD-L1. We have not tested this buffer in the absence of the DUB inhibitor.

SECTION 2: Acetyl-Lysine Immunoprecipitation Assay: Raw Materials

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Material	Distributor	Catalog #	Reconstitution	Storage After Reconstitution
Trizma Base	Millipore Sigma	T1503	1M solution: Dissolve 121g Trizma base in 1L deionized water. Add hydrochloric acid to required pH. Filter sterilize	room temp.
Sodium chloride	Millipore Sigma	S7653	1M solution: Dissolve 58.4g sodium chloride in 1L deionized water. Filter sterilize.	room temp.
IGEPAL CA-630	Millipore Sigma	I3021	na	room temp.
Acetyl-Lysine Beads	Cytoskeleton Inc	AAC04-Beads	Resuspend in 500ul of water with 50% glycerol for a 1X stock solution.	-20°C
Control Beads for Acetyl-Lysine IP	Cytoskeleton Inc	CIG02-Beads	Resuspend in 500ul of water with 50% glycerol for a 1X stock solution.	-20°C
Glycerol	Millipore Sigma	G7893	na	room temp.
Sodium dodecyl sulfate	Millipore Sigma	L4509	na	room temp.
Bromophenol Blue	Millipore Sigma	B7021	0.2%: Dissolve 20 mg in 10 ml of water.	room temp.
beta mercaptoethanol	Millipore Sigma	M6250	na	room temp.
Multipurpose Mini Spin Columns	BioVision	6572-50	na	room temp.

SECTION 3: SDS-PAGE

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Material	Distributor	Catalog #	Reconstitution	Storage After Reconstitution
Novex WedgeWell 4-20%, Tris-Glycine, 1.0 mm, mini protein gel, 15 well	Thermo Fisher	XP04205BOX	na	4°C
Novex WedgeWell 4-20%, Tris-Glycine, 1.0 mm, mini protein gel, 10 well	Thermo Fisher	XP04200BOX	na	4°C

SECTION 4: WESTERN BLOT DEVELOPMENT/ANALYSIS

Material	Distributor	Catalog #	Reconstitution	Storage After Recinstitution
Immobilon-P PVDF membrane	Millipore Sigma	IPVH304F0	na	room temp.
Anti-PD-L1 Primary Antibody (rabbit polyclonal)	GeneTex	GTX104763	na	-20°C
Donkey anti-rabbit-HRP secondary antibody	Jackson Immunoresearch	711-035-152	0.8 ml deionized water	4°C
Anti-acetyl-lysine antibody (mouse monoclonal Ab)	Cytoskeleton Inc	AAC02	100 µl 50% glycerol in water	4°C
Goat anti-mouse-HRP secondary antibody	Jacksom Immunoresearch	115-035-068	0.8 ml deionized water	4°C
Super Signal West Dura chemilluminescence detection reagents	Thermo Fisher	34075A/34075B	na	4°C

PD-L1 Antibodies Tested in this study

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Vendor	Catalog #	Ab Type	Dilution Used	Recommended for this Protocol
GeneTex	GTX104763	rabbit polyclonal	1:1500	YES
GeneTex	GTX52553	mouse monoclonal	1:500	NO
Cell Signaling Technologies	mAb 13684	rabbit monoclonal	1:1000	NO*
AbCam	ab279292	mouse monoclonal	1:1000	NO
AbCam	ab213524	rabbit monoclonal	1:1000	NO*
Proteintech	17952-1-AP	rabbit polyclonal	1:600	NO
Proteintech	66248-1-Ig	mouse monoclonal	1:2000	NO

- * AbCam Cat# 213524 and Cell Signaling Tech Cat# 13684 could detect signal if used at lower dilutions (1:200) but were less sensitive than the GeneTex Ab at 1:1500 dilution.

BEFORE STARTING

The IP assay takes 48h. Extensive tests were performed to confirm that the lysis buffer in this protocol maintains the stability of PD-L1 over at least a 72h time course, however, it is recommended to determine lysate stability with your specific cell lysate prior to carrying out the IP assay.

Each IP requires 0.5-1.0 mg of lysate protein. If the lysate protein yield from your tissue culture is not known, we recommend processing a "test plate" following the CELL LYSATE PREPARATION section below. We recommend using 150cm^2 plates for cell growth. As a rough guide, 50% confluent cell culture will generally yield 0.3 mg (e.g. Swiss 3T3) to 2 mg (e.g. HeLa) from this size growth vessel.

CELL LYSATE PREPARATION 1h 5m

1 30m

LYSATE PREPARATION: BUFFERS AND EQUIPMENT

Determine the volume of lysis and dilution buffer needed to process your cells.

For each 1 mg of lysate protein you will need:

300 µl supplemented Lysis Buffer
1.2 ml supplemented Dilution Buffer

Make buffers as detailed below just prior to processing cell lysates. Buffer volumes should be scaled according to needs, the recipes are for 1 ml of each buffer. Each 1ml of Lysis buffer requires 4 ml of Dilution buffer.

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Supplemented Lysis Buffer (1 ml): Store at room temperature, use within 3h

Material	Stock Concentration	Volume	Final composition
BlastR Lysis Buffer	1X	1 ml	1X
Halt Protease Inhibitor Cocktail*	100X	10 µl	1X
Trichostatin A	5 mM	0.4 µl	2 µM
Nicotinamide	2 M	20 µl	40 µM
N-ethylmaleimide**	1 M	10 µl	10 µM

*This inhibitor cocktail can be replaced with the following mix of protease inhibitors; pepstatin A (10 µM final), leupeptin (50 µM final), aprotinin (2 µg/ml final), bestatin (40 µM final)
**N-ethylmaleimide is included as a deubiquitinase (DUB) inhibitor as we are studying acetylation/ubiquitination of PD-L1, we have not tested this assay in the absence of N-ethymaleimide.

Supplemented Dilution Buffer (1 ml): Store on ice, use within 3h

Material	Stock Concentration	Volume	Final composition
BlastR Lysis Buffer	1X	1 ml	1X
Halt Protease Inhibitor Cocktail*	100X	10 µl	1X
Trichostatin A	5 mM	0.4 µl	2 µM
Nicotinamide	2 M	20 µl	40 µM
N-ethylmaleimide	1 M	10 µl	10 µM

*This inhibitor cocktail can be replaced with the following mix of protease inhibitors; pepstatin A (10 µM final), leupeptin (50 µM final), aprotinin (2 µg/ml final), bestatin (40 µM final)

1:4 Dilution Buffer (1 ml): Store on ice, use within 3h

Supplemented Lysis Buffer	Supplemented Dilution Buffer
200 µl	800 µl

Reagents and Equipment

Phosphate Buffered Saline (PBS) pH 7.0
Microfuge tubes
Cell lifter
BlastR filters (each filter can process up to 1 ml of lysed cells)
Liquid nitrogen (to snap freeze lysates)

1.1 CELL LYSATE PREPARATION: PROCESSING CELL LYSATES

The required number of cells should be grown and treated as required (see Before Starting section above).

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Times given in each step assume 5 plates are being processed. It is convenient, and highly recommended, to process only one experimental condition at a time (from cell harvest to freezing lysate).

1.2 ⚠ 5m

Remove culture media and wash cells twice with 10 ml each of room temperature PBS buffer.

A vacuum aspirator is recommended for media removal. It is critical to remove all PBS in the final wash as dilution of lysis buffer with PBS will be detrimental to efficient cell lysis and will alter the final bead binding buffer composition. The plates should be left at an angle to collect all PBS and allow efficient aspiration.

1.3 Add the appropriate volume of Supplemented Lysis Buffer determined in STEP 1 and harvest cells using a cell lifter. The lysate will become highly viscous, due to nuclear lysis. If processing multiple plates for a given condition, the lysates can be pooled at this point (use a snipped 1 ml pipette tip). 5m

1.4 Use a snipped 1 ml pipette to transfer up to 1 ml of crude lysate per BlastR filter and harvest the flow through into a 15 ml tube on ice. The filter step is necessary to reduce sample viscosity (by removing DNA from the lysate). 3m

1.5 Record the volume of the lysate and add 4 volumes of Supplemented Dilution Buffer. Mix gently. 2m

1.6 Immediately take the protein concentration. 5m

We use Precision Red Advance Protein Assay to take protein concentrations, however any reagent that is not affected by 0.05% (w/v) SDS.

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1.7 Dilute the lysate to 1 mg/ml using 1:4 Dilution Buffer and immediately dispense the lysate into 1 mg aliquots. 5m

1.8 Snap freeze the lysate aliquots in liquid nitrogen and store at -80°C. 10m

Lysates are stable for at least 3 months under these conditions. Avoid freeze thaws.

ACETYL-LYSINE IMMUNOPRECIPITATION (IP)

2 ACETYL-LYSINE IP: BUFFERS AND EQUIPMENT 1h

Bead Wash Buffer: 100 ml, store at room temp.

Material	Amount	Final Concentration
1M Tris pH8.0	3ml	30 mM
1M sodium chloride	20 ml	200 mM
IGEPAL	1 ml	1%
De-ionized water	76 ml	

Bead Elution Buffer: 10 ml, store at room temp.

Material	Amount	Final Concentration
1M Tris pH6.8	1.3 ml	130 mM
Glycerol	1.6 ml	16%
SDS	0.4 g	4%
0.2% Bromophenol Blue	0.5 ml	0.01%
De-ionized water	to 10 ml	

Reagents and Equipment
Acetyl-Lysine Affinity beads
Mouse IgG Control beads
beta mercaptoethanol
Microfuge at 4°C
Rotator at 4°C equipped to hold microfuge tubes
Multipurpose mini spin columns (optional)

2.1 ACETYL-LYSINE IP: IMMUNOPRECIPITATION STEP 2h

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The handling time for the IP step is approximately 2h focused time. IP incubations take 2 x 24h

Aliquot 50 µl of Acetyl-lysine affinity bead slurry (approx. 50 µg conjugated antibody) for the required number of IP reactions.

It is recommended to resuspend the affinity beads (see Methods) at least 1h prior to use to allow complete rehydration of beads as this helps reduce non specific binding of lysate proteins to beads.
In all cases when handling beads use a snipped pipette tip to avoid damage to beads.

2.2 Aliquot 50 µl of mouse IgG control bead slurry (approx. 50 µg of conjugated antibody) for the required number of IP control reactions.

2.3 Wash the beads once with 1 ml each of PBS. Collect beads by centrifugation at 2000 x g for 1 minute at 4°C and remove PBS.

2.4 Add 1 mg of appropriate lysate to corresponding beads and incubate with rotation (approx. 25-30 rpm) at 4°C for 24h. 1d

24:00:00

2.5 Collect beads by centrifugation at 2000 x g, 1 minute, 4°C . Save the supernatants in clearly labeled tubes on ice

Saved lysates will be used later in STEP 2.12 and processed for a further 24h IP.

2.6 Wash the beads by adding 1 ml of Bead Wash Buffer and rotating (25-30 rpm) for 10 minutes at 4°C. 10m

00:10:00

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2.7 Collect beads as above and repeat the bead wash step 4 more times. 40m

00:10:00
00:10:00
00:10:00
00:10:00

2.8 ⚠

After the final wash, make sure that the wash buffer is completely removed from the beads. A fine bore pipette tip can be used for this step. Failure to remove all wash buffer will reduce effectiveness of the elution buffer and will result in volumes too large for gel analysis.

2.9 Elute the acetylated proteins by adding 27 µl of Bead Elution Buffer and incubating at room temp. for exactly 5 minutes. The tubes can be gently flicked to mix the beads and elution buffer. Do not use a pipette for mixing. 5m

2.10 Collect beads by centrifugation at 5000 x g for 1 minute and remove supernatant containing acetylated proteins.

It is convenient to collect supernatants by passing the bead elution reaction through a microspin column containing a polyethylene FRIT. BioVision sell multipurpose mini spin columns suitable for separating IP beads from eluted protein supernatants (see Materials section)

2.11 Add 2 µl of beta mercaptoethanol to each tube and place at -80°C. These samples are the 24h IP elutions.

2.12 Take the supernatants saved in step 2.5 and add a fresh 50 µl aliquot of appropriate beads to each.

2.13 Incubate for a further 24h with rotation at 4°C. These will be the 48h IP samples. 1d

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24:00:00

2.14 Collect the 48h IP beads as above. Lysate supernatants can be discarded. 2h

2.15 The 48h IP elutions should be pooled with the corresponding 24h elutions. Samples can now be processed for western blot analysis.

SDS-PAGE 2h 30m

3 2h 30m

1. Boil samples for 5 minutes before running on SDS-PAGE.

SDS-PAGE was performed on Thermo Fisher pre-cast 4-20% Tris-Glycine acrylamide gels at 120V for 2h. Any standard lab SDS-PAGE system capable of resolving a 50 kD protein should be acceptable for this step.

WESTERN BLOT PROCESSING AND ANALYSIS 23h 40m

4 WESTERN BLOT: BUFFERS AND EQUIPMENT 1h

Western Transfer Buffer (1L per gel), store at room temp.

Material	Amount	Final Concentration
Glycine	14.4g	192 mM
1M Tris-HCl pH8.0	25 ml	25 mM
Methanol	150 ml	15%
Deionized water	to 1L final volume	

Tris Buffered Saline/Tween (TBST Buffer) (10L). store at room temp.

Material	Amount	Final Concentration
1M Tris-HCl pH8.0	100 ml	100 mM
5M sodium chloride	300 ml	750 mM
Tween-20	5 ml	0.05%
Deionized water	to 10L final volume	

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Antibody Block Buffer: 50ml, store at room temp.

Material	Amount	Final Concentration
TBST pH8.0	50 ml	1X
powdered skimmed milk	2.5 g	5%

Antibody Dilution Buffer: 50ml, store at room temp.

Material	Amount	Final Concentration
TBST pH8.0	50 ml	1X
powdered skimmed milk	1.5 g	3%

4.1 ⚠ 17h

WESTERN BLOT: PROCESS & ANALYSIS

Transfer the proteins to PVDF membrane using the Western Blot Transfer Buffer. Transfer at 20V for 17h.

17:00:00

The transfer process is critical to obtaining reproducibly sensitive detection of acetylated PD-L1.
We have failed to get reproducible signals using semi-dry blotting techniques. The voltage and transfer times have been optimized and should be adhered to.

4.2 Block the membrane for 2h in Antibody Block Buffer. 2h

02:00:00

4.3 ⚠ 1h 30m

Dilute primary antibody 1:1,500 in Antibody Dilution Buffer and incubate with shaking for 1.5h at room temperature. We use 10 ml of diluted primary antibody for a mini-gel sized blot.

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01:30:00

Overnight incubation at 4°C is not recommended as it results in higher background signal.

The GeneTex rabbit polyclonal anti-PD-L1 primary antibody is critical to obtain the sensitive detection needed for this assay. As we have not exhaustively tested all antibodies, we have listed those tested in the Materials section.

4.4 Wash blot 2 x for 5 minutes each in TBST. 10m

00:05:00

00:05:00

4.5 Dilute secondary-HRP antibody 1: 25,000 in Antibody Dilution Buffer and incubate with shaking for 40 minutes at room temperature. We use 10 ml of diluted primary antibody for a mini-gel sized blot. 40m

00:40:00

4.6 Wash the blot in 50-100 ml of TBST for 10 minutes. 10m

00:10:00

4.7 Repeat wash 5 more times. 50m

00:10:00

00:10:00

00:10:00

00:10:00

00:10:00

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4.8 Develop the blot using Thermo Fisher Super Signal West Dura Chemiluminescent reagent, 10 minute incubation at room temp. in 4ml of reagent per mini gel blot is recommended.

4.9 Develop the blot and save the image as a tiff file. Analyze the PD-L1 signal (approximately 50 kD) using ImageJ software.

The blot can be re-probed with a pan anti-acetyl antibody AAC02 (1:1000 dilution in Antibody Dilution Buffer) and goat anti mouse-HRP secondary antibody (1:25,000 dilution in Antibody Dilution Buffer) to analyze the total protein acetylation profile.

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