

***In vitro* AMPylation Assays Using Purified, Recombinant Proteins**

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[Abstract] Post-translational protein modifications (PTMs) orchestrate the activity of individual proteins and ensure their proper function. While modifications such as phosphorylation or glycosylation are well understood, more unusual modifications, including nitrosylation or AMPylation remain comparatively poorly characterized. Research on protein AMPylation—which refers to the covalent addition of an AMP moiety to the side chains of serine, threonine or tyrosine—has undergone a renaissance (Yarbrough *et al.*, 2009; Engel *et al.*, 2012; Ham *et al.*, 2014; Woolery *et al.*, 2014; Preissler *et al.*, 2015; Sanyal *et al.*, 2015; Truttmann *et al.*, 2016; Truttmann *et al.*, 2017). The identification and characterization of filamentation (fic) domain-containing AMPylases sparked new interest in this PTM (Kinch *et al.*, 2009; Yarbrough *et al.*, 2009). Based on recent *in vivo* and *in vitro* studies, we now know that secreted bacterial AMPylases covalently attach AMP to members of the Rho family of GTPases, while metazoan AMPylases modify HSP70 family proteins in the cytoplasm and the endoplasmic reticulum (ER) (Itzen *et al.*, 2011; Hedberg and Itzen, 2015; Truttmann and Ploegh, 2017). AMPylation is thought to trap HSP70 in a primed yet transiently disabled state that cannot participate in protein refolding reactions (Preissler *et al.*, 2015). *In vitro* AMPylation experiments are key to assess the activity, kinetics and specificity of protein AMPylation catalyzed by pro- and eukaryotic enzymes. These simple assays require recombinant AMPylases, target proteins (Rho GTPases, HSP70s), as well as ATP as a nucleotide source. Here, we describe strategies to qualitatively and quantitatively study protein AMPylation *in vitro*.

Keywords: AMPylation, Adenylation, Grp78/BiP, HSP70, GTPase, Proteostasis

[Background] Metazoan cell signaling is complex and requires tight control. Aberrations in this well-balanced system threaten cellular homeostasis and induce several maintenance systems aimed at restoring the balance (Kim *et al.*, 2013). Protein AMPylation is directly linked to cellular stress: AMPylation of Rho GTPases by bacterial toxins rewires GTPase-dependent signaling, eventually leading to a collapse of the actin cytoskeleton and cell death (Yarbrough *et al.*, 2009; Mattoo *et al.*, 2011). In contrast, AMPylation of Grp78/BiP in the ER keeps this chaperone in a primed, yet silent conformation to be awoken and set in motion once the burden of unfolded protein in the ER surpasses a certain threshold (Preissler *et al.*, 2015; Sanyal *et al.*, 2015). We and others have extensively used *in vitro* AMPylation assays to study general properties, target selectivity as well as reaction kinetics of Fic domain-containing AMPylases. We used a combination of distinct *in vitro* AMPylation assays to i) identify novel targets in complex cell lysates, ii) validate suspected targets and iii) approach the role of

AMPyase dimerization and auto-modification as prerequisites for enzymatic activity (Truttmann *et al.*, 2015; 2016 and 2017). Our efforts aim at understanding the scope and impact of the AMPylome on cellular signaling. The *in vitro* AMPylation assays described herein present methods to achieve this goal.

Materials and Reagents

1. 1.5 ml tubes (1.5 ml Snaplock Microcentrifuge Tube) (Corning, Axygen®, catalog number: MCT-150-C-S)
2. Pipette tips (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: 9400327, 9401255, 9401410)
3. Microcentrifuge Tube Locks (Sorenson BioScience, catalog number: 11870)
4. Autoradiography film (Carestream Health X-Omat™ LS Film) (Eastman Kodak, catalog number: 05-728-45)
5. Saran wrap (generic)
6. Whatman 3MM filter paper (GE Healthcare, catalog number: 3030-6185)
7. Sterile, deionized water (generic)
8. Ice in isolated containment (generic)
9. Ethanol (Sigma-Aldrich, catalog number: 362808)
10. Purified recombinant AMPylase at 1.0 µg/µl or higher (HIS₆-HYPE_{aa187-437}; homemade; see Truttmann *et al.*, 2015)
11. Purified recombinant target proteins at 1.0 µg/µl or higher (*i.e.*, HIS₆-Histone H3; homemade; see Truttmann *et al.*, 2015)
12. Appropriate TRIS-glycine gels (Criterion™ TGX™ Precast Midi Protein Gel) (Bio-Rad Laboratories, catalog number: 5671023)
13. Molecular weight marker (Precision Plus™ Protein Dual Color Standard) (Bio-Rad Laboratories, catalog number: 1610374)
14. 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base) (Sigma-Aldrich, catalog number: 252859)
15. 11.8 M hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: 258148)
16. DL-dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 43815)
17. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: M8266)
18. [Alpha-³³P]ATP, 10 mCi/ml; 3,000 Ci/mmol (Hartman Analytic, catalog number: SRF-207)
Note: It is of uttermost importance to use [Alpha-³³P]ATP and not [Gamma-³³P]ATP, which is used to study kinase-dependent phosphorylation events.
19. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S3014)
20. Potassium chloride (KCl) (EMD Millipore, catalog number: PX1405)
21. Glycerol (Sigma-Aldrich, catalog number: G5516)
22. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: 74255)

23. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
24. Bromophenol blue (Sigma-Aldrich, catalog number: B0126)
25. Poly-Phenyl-Oxazole (PPO) (Sigma-Aldrich, catalog number: 216984)
26. Dimethyl sulfoxide (DMSO) Sigma-Aldrich, catalog number: 276855)
27. 1 M Tris-HCl (pH 7.5) (see Recipes)
28. 1 M DTT (see Recipes)
29. 1 M MgCl₂ (see Recipes)
30. 100 mM ATP (see Recipes)
31. 5 M NaCl (see Recipes)
32. Protein storage buffer (see Recipes)
33. Reaction buffer (see Recipes)
34. SDS-PAGE 6x sample buffer (see Recipes)
35. DMSO/PPO solution (see Recipes)

Equipment

1. Pipettes (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: 4600170, 4600240 and 4600250)
2. -20 °C freezer (generic)
3. 4 °C refrigerator (generic)
4. Geiger-counter (generic)
5. Refrigerated tabletop centrifuge for 1.5 ml Eppendorf tubes (Eppendorf, model: 5810 R)
6. 10 µl Hamilton syringe (Hamilton, catalog number: 80075)
7. Radiation safety gear and personal protection equipment (generic)
8. Vacuum gel dryer (Bio-Rad Laboratories, model: Model 583)
9. Glass tray (generic; 5 x 10 inches at least)
10. Timer (Alarm Timer) (Grainger, catalog number: 8RLR2)
11. pH meter (Thermo Fisher Scientific, Thermo Scientific™, model: Orion Star™ A111)
12. Balance (Sartorius, model: Cubis® Precision Balance)
13. Vortex (Vortex-Genie 2 Vortexer) (VWR, catalog number: VWR-VG3)
14. SDS-PAGE system (Bio-Rad Laboratories, model: Criterion™ Cell and PowerPac™ Basic Power Supply, catalog number: 1656019)
15. Autoradiography cassettes (FisherBiotech Electrophoresis Systems Autoradiography Cassette, 8 x 10 in) (Fisher Scientific, model: FBAC 810)

Note: This product is not available anymore.

Software

1. Fiji/ImageJ image analysis software (<https://fiji.sc/>)

Procedure

Note: ^{33}P -ATP is radioactive. Please counsel with your radio safety officer regarding your institute's radio waste disposal and radio protection routine and ensure to obtain all required protective equipment—that should include but may not be limited to a lab coat, double-gloves and safety goggles—before starting the experiment. Continuously monitor work surfaces using a Geiger-counter and decontaminate if required following your institute's decontamination protocol.

A. *In vitro* AMPylation reaction

1. Mix 5 μg of recombinant AMPylase enzyme in 10 μl protein storage buffer (see Recipes) with 20 μl reaction buffer (see Recipes) supplemented with 0.5 μl /sample ^{33}P -ATP.

Notes:

- a. *The amount of AMPylase to use in an *in vitro* reaction depends on the relative activity of the individual enzymes; very potent enzymes (i.e., VopS, IbpA) will *in vitro* modify a molar excess of target protein (i.e., Rac1, Cdc42); thus, 1 μg /reaction is sufficient. In contrast, wild-type versions of metazoan AMPylases in general perform poorly *in vitro*; thus, 5-10 μg /reaction is preferred.*
- b. *Wear proper protective equipment and monitor work surfaces during this step with a Geiger-counter.*

2. Incubate reaction for 60 min at 20 °C.

Notes:

- a. *This step pre-loads or primes the enzyme, which will maximize target AMPylation.*
- b. *Throughout the protocol, suggested 20 °C incubation steps can also be performed at room temperature (approximately 20 °C).*

3. Centrifuge sample for 30 sec at 7,000 x g.
4. Add 2 μg of target protein in 10 μl protein storage buffer to the primed enzyme (final reaction volume: 40 μl).
5. Incubate reaction for 60 min at appropriate temperature (VopS, IbpA: 20 °C; FIC-1: 30 °C; HYPE: 37 °C).

Note: Reactions can be performed at 20 °C; however, incubation at higher temperatures (37 °C) enhances target AMPylation.

6. Quench the reaction by adding 8 μl of 6x SDS-PAGE loading buffer (see Recipes).
7. Properly close 1.5 ml tubes and put on tube locks to prevent lids from opening inadvertently during step A9.
8. Boil samples for 10 min at 95 °C.

Note: Tube locks are essential, as they will minimize the chance for tubes to pop open while boiling (see next step); should accidental opening of a tube have occurred, identify likely [^{33}P]-contamination using a Geiger-counter and decontaminate according to your radiation safety office's guidelines.

9. Proceed with gel electrophoresis or store samples at -80 °C.

B. Sample analysis by gel electrophoresis and autoradiography

1. Equilibrate samples on ice for 10 min.
2. In the meantime, choose appropriate gel: if available, a TRIS-glycine gradient gel (*i.e.*, 4-16%) should be used; alternatively, select the polyacrylamide percentage according to your target protein's mass: *i.e.*, 12% for HSP70 (approximately 70 kDa), 15% for Histones (approximately 15 kDa) (acrylamide: Bis-acrylamide = 30:0.8); prepare gel running buffer according to gel manufacturer's instructions.
3. Centrifuge samples for 30 sec at 7,000 x g.
4. Load gel: add molecular weight marker and individual samples to each well of the SDS-PAGE gel.

Note: The use of gel loading tips is recommended to minimize cross-well spilling. Alternatively, use a 10 µl Hamilton syringe for loading. Load only ~50% of your total sample; store remaining half in case you need to re-run the gel.

5. Run the gel at 60-100 V during the stacking stage, then adjust to 120 V (constant voltage) for ~2 h.

Note: The running time depends on the gel system used.

*Note: Steps B6-B17 are referred to as fluorography, a method developed by Bonner and Laskey (Bonner and Laskey, 1974) to be able to detect ³H in autoradiograms of SDS-PAGE gels. The method also improves sensitivity for other soft emitters, less so for higher energy isotopes such as ³²P. An added advantage is that the procedure confers mechanical stability to low-percentage gels, owing to the precipitation of PPO. Gels with acrylamide percentages lower than 5% should not be subjected to fluorography, as the mechanical properties of these gels can yield an irregular final product. Commercially available solutions for impregnation of gels with fluorophores are available, often at higher cost than home-made DMSO-PPO solutions. **Wear gloves at all times, since the DMSO-PPO solution can penetrate the skin and cause local precipitation of PPO in tissues.***

6. Carefully transfer the gel into a metal or glass tray (a baking dish will serve the purpose).
7. Add 500 ml of DMSO for a large gel of 100 ml volume, smaller gels can be handled with less.

Notes:

- a. Add ~5 times the volume of the gel of DMSO to cover the gel completely.
- b. Incubate at room temperature for 60 min with agitation.

8. Discard DMSO

Note: This DMSO must be considered as potentially radioactive; dispose accordingly.

9. Add a fresh volume (see step B7) of DMSO and incubate for 60 min at room temperature.

10. Discard DMSO.

Note: This DMSO must be considered as potentially radioactive; dispose accordingly.

11. Cover the gel in DMSO-PPO solution (22.2 g of PPO and 80 ml of DMSO; scale volume according to need) (see Recipes).

12. Incubate for 2 h at room temperature with agitation.

13. Remove DMSO-PPO as completely as possible.

Note: The DMSO-PPO solution can be recycled and reused for up to 3 gels/experiments. Replenish PPO in the DMSO-PPO solution after each cycle with an amount of PPO approximately equal to that now precipitated in the gel.

14. Cover the gel in the glass tray with 2 cm of ddH₂O and incubate for 60 min at room temperature (Figure 1A and Video 1).

Notes:

- a. Adding ddH₂O to the dehydrated, PPO-soaked gel will result in immediate precipitation of PPO and will rapidly turn the transparent gel into a milky-white gel.
- b. Use a paper towel to remove any precipitated PPO from the tray.

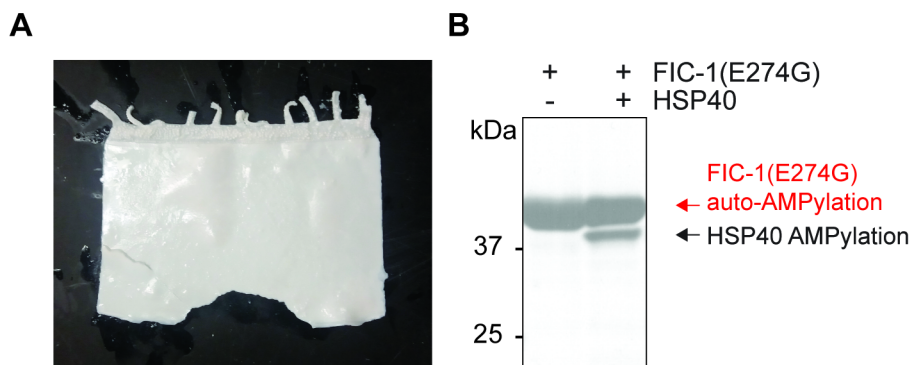


Figure 1. PPO treatment and final autoradiography plot. A. Typical appearance of SDS-PAGE gel after successful dehydration and PPO loading (see Video 1 for more details); B. Representative autoradiography plot depicting auto-AMPylation of the conferring enzyme (FIC-1(E274G), a constitutive-active FIC-1 version), as well as target modification (HSP40).

Video 1. PPO precipitation in dehydrated SDS-PAGE gel. Dehydrated and PPO-loaded SDS-PAGE gel is kept in a glass tray and distilled water is added. Upon contact with water, PPO precipitates within the gel. Excess PPO still present in the glass tray precipitates, too. To achieve best results, incubate SDS-PAGE gel for 60 min at room temperature (step B14) before proceeding with washing (step B15).



15. Discard ddH₂O.
16. Add fresh ddH₂O.
17. Incubate gel in ddH₂O for 10 min.
18. Discard ddH₂O.
19. Repeat previous step 5 times.
20. Pre-soak filter paper (Whatman 3MM) with ddH₂O.
21. Transfer gel face-up onto soaked filter paper.
22. Transfer filter-gel stack to gel drying unit.
23. Cover the filter-gel stack with Saran wrap.

Note: Adding a single layer of Saran wrap or similar prevents the gel from sticking the gel dryer's rubber cover and facilitates the removal of the gel once dried onto the filter.

24. Dry gel for 90 min at 65 °C. It is essential that a strong vacuum be maintained to avoid cracking of the gel. If necessary, use a lyophilizer-style set-up with a cold trap.
25. Transfer gel into autoradiography cassette.

Note: At this stage, it is sometimes worthwhile to use a Geiger-counter to get a feeling for the signal intensity attained; if audible with a sensitive Geiger-counter, an overnight exposure of the film will usually yield an interpretable signal.

26. In a dark room, expose to film.
27. Expose autoradiogram for 10 min to 10 days, depending on the expected signal intensity.
Note: This is a rather arbitrary factor and often must be evaluated empirically; ³³P has a half-life time of ~30 days.
28. If the film is exposed for longer than 12 h, store the autoradiography cassette at -80 °C to enhance sensitivity of detection.
29. Develop film according to manufacturer's instructions (Figure 1B).

Data analysis

1. *In vitro* AMPylation assays as presented in this protocol will primarily result in a qualitative assessment of target AMPylation. It is strongly recommended to repeat experiments and to verify target AMPylation in three independent replica.
2. If required, protein AMPylation can be quantified as follows:
 - a. Scan develop autoradiography film.
 - b. Open scan image in Fiji/ImageJ image analysis software (<https://fiji.sc/>).
 - c. Use the integrated analyze => gels plugin and redeem the intensities of individual bands depicting AMPylated proteins.
 - d. Determine average and standard deviation combining data from at least 3 independent replica.
 - e. Use appropriate statistical methods to evaluate significance of observed AMPylation levels (e.g., Student's *t*-test).

Notes:

- i. *The appropriate statistical test will depend on the study design*
- ii. *Users are strongly encouraged to consult available online tutorials prior to using this tool for the first time (<https://www.youtube.com/watch?v=JIR5v-DsTds>).*

Notes

1. If performed under identical experimental conditions, *in vitro* AMPylation assays are highly reproducible and should provide a similar qualitative assessment of target modification.
2. [³³P] has a half-life time of 25.3 days; thus, signal intensity of particles emitted from AMPylated targets will decrease with daily increments. If quantification of signal intensities is desired, it might be necessary to use relative (intensity as compared to an internal control) rather than absolute quantification strategies.

Recipes

1. 1 M Tris-HCl (pH 7.5)
For 100 ml:
12.1 g Tris-base
90 ml ddH₂O
Adjust to pH 7.5 with HCl
Adjust volume with ddH₂O to 100 ml
Store at room temperature
2. 1 M DTT
For 100 ml:
15 g DTT in 100 ml ddH₂O
Store in aliquots at -20 °C
3. 1 M MgCl₂
For 100 ml:
47.6 g MgCl₂
Adjust volume with ddH₂O to 100 ml
Store in aliquots at -20 °C
4. 100 mM ATP
For 100 ml:
5.51 g ATP
Adjust volume with ddH₂O to 100 ml
Store in aliquots at -20 °C

5. 5 M NaCl
For 100 ml:
29.22g NaCl in 100 ml ddH₂O
6. Protein storage buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 10% glycerol [v/v])
For 10 ml:
500 µl 1 M Tris-HCl pH 7.5
300 µl 5 M NaCl
1 ml glycerol
Adjust volume with ddH₂O to 10 ml
7. Reaction buffer (50 mM TRIS-HCl pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 2 mM DTT)
For 10 ml:
500 µl 1 M Tris-HCl pH 7.5
100 µl 1 M MgCl₂
300 µl 5 M NaCl
20 µl 1 M DTT
9.08 ml ddH₂O
8. SDS-PAGE 6x sample buffer (375 mM Tris-HCl pH 6.8, 6% SDS [w/v], 48% glycerol [v/v], 9% 2-mercaptoethanol [v/v], 0.03% bromophenol blue [w/v])
For 100 ml:
5.91 g Tris-HCl
6 g SDS
48 ml 100% glycerol
9 ml 14.7 M 2-mercaptoethanol
30 mg bromophenol blue
9. DMSO/PPO solution
30 g PPO in 120 ml DMSO

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