Methods for Purification of Proteins Associated with Cellular Poly(ADP-Ribose) and PARP-Specific Poly(ADP-Ribose)


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Methods for purification of proteins associated with cellular poly(ADP-ribose) and PARP-specific poly(ADP-ribose)

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Summary
Poly(ADP-ribose) (pADPr) is a post-translational modification that regulates protein function through two major mechanisms: covalent modification of acceptor proteins and non-covalent binding of proteins to pADPr. pADPr is synthesized by a family of enzymes called poly(ADP-ribose) polymerases (PARPs) that are themselves major targets of pADPr modification. Here, we outline two methods for the purification of pADPr binding proteins via pADPr purification under native conditions: purification of cellular pADPr and purification of pADPr covalently linked to specific PARPs. Together, these methods provide complementary approaches to the identification of non-covalent pADPr-protein interactions in the cell.

Keywords
Poly(ADP-ribose); poly(ADP-ribose) polymerase; PARP; immunoprecipitation; poly(ADP-ribose) purification; poly(ADP-ribose) associated protein; poly(ADP-ribose) acceptor protein; cis-diol; boronate affinity

1. Introduction
Poly(ADP-ribose) (pADPr) is a large protein modification consisting of up to 200 ADP-ribose residues (1). In addition to its significant size, pADPr is also a structurally complex molecule due to the presence of both linear and branched ribose-ribose linkages in the polymer (Fig. 1). It is essential for life in multicellular eukaryotes, where it plays critical roles in the regulation of transcription, cell division, innate immune responses, and DNA damage responses (2, 3, 4, 5). One of the primary mechanisms of pADPr function is to recruit proteins to specific sites in the cell through non-covalent protein-pADPr binding interactions (5, 6). To better understand this mechanism of function, our laboratory has developed several methods for the purification of proteins associated with pADPr.

Each of these techniques uses HeLa S3 suspension cells to generate cytoplasmic lysate. Suspension cells are used due to their superior cell volume-to-media volume ratio relative to adherent cells, thus providing greater amounts of cytoplasmic lysate per ml of media. Of the human suspension cell lines, we specifically chose HeLa S3 cells since these cells are well characterized, easily transfected with exogenous DNA, have retained critical cell cycle checkpoints and react predictably to small molecule treatments such as cell cycle arrests, stressors, and inhibitors (7). Thus, by treating HeLa S3 cells with inhibitors prior to generating cytoplasmic lysates, we can identify pADPr binding proteins under distinct...
cellular conditions. Finally, HeLa S3 cells can rapidly convert between suspension and adherent growth, providing convenient methods for the removal of dead cells during adherent growth as well as the generation of monoclonal cell lines via manual isolation of monoclonal colonies on plates (8).

The first pADPr purification method, *cellular pADPr purification*, modifies an existing pADPr purification technique that uses boronate affinity to isolate *in vitro* synthesized pADPr under denaturing conditions (9). Our approach also utilizes boronate affinity, but facilitates pADPr purification directly from cytoplasmic lysate. pADPr contains two cis-diols per ADP-ribose moiety, each of which binds covalently to the cis-diol residues found on boronate affinity matrices. Since cis-diols are also found in some small molecules, RNA, and polysaccharides, these contaminants must be depleted from cytoplasmic lysates prior to incubation with the boronate affinity matrix. We remove small molecules from the cytoplasmic lysates using Sephadex G-25 gel filtration, RNA by treating with RNases, and polysaccharides via preadsorption on lectin columns. Once these contaminants are removed, boronate affinity chromatography is used to purify pADPr and its associated binding proteins from the cytoplasmic lysate. pADPr binding proteins are then eluted from the boronate affinity matrix via incubation with ARH3, a soluble pADPr glycohydrolase that hydrolyzes pADPr and thereby releases pADPr binding proteins (Fig. 2, left). These proteins are then resolved by gel electrophoresis, visualized by Coomassie or silver stain, and identified by mass spectrometry analyses (Fig. 3, left).

The *cellular pADPr purification* method isolates the cellular pool of pADPr generated by all active pADPr polymerases (PARPs). Seventeen PARPs, defined as such by the presence of a PARP domain, have been identified in humans by bioinformatic analysis (10). Currently the polymerization activity of these PARPs is not entirely known. PARP-1, PARP-2, PARP-3, PARP-4, PARP-5a and PARP-5b have been shown experimentally to exhibit polymerase activity, while PARP-10 and PARP-14 have been shown to catalyze mono(ADP-ribose) modification of acceptor proteins. The polymerization activity of the remaining PARPs has not been conclusively determined, although bioinformatic analyses suggest many of them do not exhibit pADPr polymerization activity (11, Vyas, S., et al, submitted). Importantly, PARPs active for polymerization have been shown to modify other PARP isoforms (trans-modify) as well as automodify, making it likely that non-enzymatically active PARP proteins also have pADPr attached (10, 12).

Data suggests that pADPr polymerized by specific PARPs can be structurally distinct. For example, pADPr polymerized by PARP-1 is highly branched, while pADPr synthesized by PARP-5a is largely linear (10, 13). PARP-1 and PARP-5a are involved in unrelated cellular pathways, and although both proteins share the cytoplasm during mitosis, each binds distinct sets of proteins (5). This evidence suggests that the regulation of pADPr structure by the PARPs that polymerize it may in turn regulate the recruitment of specific pADPr binding proteins. Thus, the structure of pADPr attached to each PARP might determine the identity of the pADPr binding proteins. A comprehensive mechanistic understanding of pADPr-protein interactions can be attained by identifying proteins non-covalently bound to the pADPr generated by specific PARPs.

To identify PARP-specific pADPr binding proteins, we have developed two related protocols based on the immunoprecipitation of epitope-tagged PARPs expressed in HeLa S3 cells: a *lysate modification assay* (Fig. 2, middle) and a *pure PARP modification assay* (Fig. 2, right). These were previously described in Chang et al 2009 (5). In the *lysate modification assay*, exogenous β-NAD+, the substrate for PARP catalysis, is added to the cytoplasmic lysate prior to immunoprecipitation to increase the concentration of pADPr linked to the immunoprecipitated PARP. Since this reaction occurs prior to purification of the PARP, the
pADPr attached to the PARP is due to the activity of the purified PARP along with other PARPs that modify it in trans. In this assay, the PARPs containing covalently linked pADPr and any pADPr binding proteins are purified from cytoplasmic lysate and then analyzed without further incubations. In the pure PARP modification assay, epitope-tagged PARP is purified without addition of exogenous β-NAD⁺ and washed in high concentrations of salt to remove binding proteins. The purified PARP is then incubated with exogenous β-NAD⁺ in vitro to stimulate automodification. Thus, the pADPr structures linked to PARPs in this assay are mainly due to the intrinsic activity of the purified PARP. The modified PARP is then incubated with fresh cytoplasmic lysate to recruit PARP-specific pADPr binding proteins.

2. Materials

2.1 Cell culture

1. Dulbecco’s Modified Eagle’s Medium (DMEM) (Cellgro/Mediatech, Manassas, VA), supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA) and 1% 100× penicillin-streptomycin solution (Cellgro/Mediatech)
2. 0.05% trypsin/0.53 mM EDTA in HBS (Cellgro/Mediatech)
3. Phosphate-Buffered Saline (Cellgro/Mediatech)
4. Disposable tissue culture flasks: 125 ml, 250 ml, 500 ml and 1 L vented and sterile flat base polycarbonate Erlenmeyer flasks (VWR, West Chester, PA)
6. Bright-Line Haemocytometer (Hausser Scientific, Horsham, PA)

2.2 Mitotic arrest

1. (+)-S-Trityl-L-cysteine (Fluka/Sigma-Aldrich, Buchs, Switzerland)
2. Hoechst 33342, trihydrochloride, trihydrate, 10 mg/ml solution in water (Invitrogen, Carlsbad, CA)

2.3 Transfection

1. Opti-MEM I reduced serum media (Gibco, Grand Island, NY)
2. 293fectin transfection reagent (Invitrogen)
3. GFP-PARP DNA (characterized in Vyas, S., et al, submitted)

2.4 Cytoplasmic lysate preparation

1. Cell lysis buffer (CLB): 150 mM NaCl, 50 mM HEPES, pH 7.4, 1 mM MgCl₂, 0.5% Triton-X 100, 1 mM DTT, 1 mM EGTA (see note 1)
2. EDTA-free protease inhibitor cocktail tablets (Roche, Indianapolis, IN)
3. Latrunculin B (Enzo Life Sciences, Plymouth Meeting, PA)

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¹Cell lysis buffer should be made fresh on the day of the experiment. 50 ml of CLB is prepared in the following order from stock solutions: 1.5 ml 5 M NaCl, 25 ml 100 mM HEPES, pH 7.4, 500 µl MgCl₂, pH 7.4, and 500 µl Triton X-100. To resuspend the Triton X-100, use a 10 ml pipet to pipet gently up and down. The addition of air bubbles to the CLB should be avoided as much as possible. After the Triton X-100 has been completely resuspended, the CLB should be chilled on ice. Then the remaining reagents can be added: 50 µl 1 M DTT (which should be made fresh on the day of the experiment), 100 µl of 500 mM EGTA, and 21.25 ml ddH₂O. Add protease inhibitor tablet.
4. Cytochalasin D (Sigma-Aldrich, St. Louis, MO)
5. Nocodazole (Tocris, Ellisville, MO)
6. Adenosine 5'-diphosphate (Hydroxymethyl)pyrrolidinediol, dihydrate, ammonium salt (ADP-HPD) (Calbiochem, La Jolla, CA)

2.5 Cellular pADPr purification
1. RNase Cocktail (Ambion, Austin, TX)
2. PD-10 columns (GE Healthcare, Piscataway, NJ)
3. Concanavalin A (ConA) lectin resin, 50% slurry (Pierce/Thermo Fisher, Rockford, IL)
4. Wheat germ agglutinin (WGA) lectin resin, 50% slurry (Pierce/Thermo Fisher)
5. Affi-Gel Boronate Gel (Bio-Rad Laboratories, Hercules, CA)
6. Kontes Flex-Columns (Kimble Chase, Vineland, NJ)

2.6 Immunoprecipitation
1. Anti-GFP, clone 3E6, stored as dry powder at −20°C, then reconstituted in PBS to 1 mg/ml, stored at 4°C (Invitrogen, Carlsbad, CA)
2. Magnetic beads: Dynabeads Protein A (Invitrogen)
3. Magnetic bead rack: DynaMag-2 Magnet (Invitrogen)

2.7 PARP activation (Pure PARP modification assay)
1. beta nicotinamide adenine dinucleotide (β-NAD+), made in 50 mM stock solutions in ddH2O stored at −20°C (Sigma-Aldrich)
2. Purified ARH3 (14)

3. Methods
3.1 Growth conditions of HeLa S3 cells
1. All procedures are performed under sterile conditions. HeLa S3 cells grown in suspension are passaged in vented sterile polycarbonate Erlenmeyer flasks. Media volume should not exceed 20% of the total flask volume; for example, no more than 50 ml of media should be used in a 250 ml flask. This ensures proper aeration and minimizes clumping during shaking. HeLa S3 cells are grown in DMEM (10% FBS, 1% pen/strep) at 37°C, 5% CO₂, 80% humidity in an Infors Multitron rotation shaker (Appropriate Technical Resources) set to 120 rpm.
2. Cells are passaged every 48 h and are diluted to a density of 1×10⁶ cells/ml during every passaging. Cell density is determined using a haemocytometer or Coulter counter.
3. To passage cells, pellet in a table-top centrifuge at 25°C for 3 min at 100 × g. Aspirate or decant the old media, removing air bubbles in the process. Using a sterile serological pipette, resuspend the cells in fresh DMEM warmed to 37°C, pipetting vigorously to disrupt clumped cells while avoiding introducing air bubbles into the cell mixture. Transfer the desired volume of resuspended cells to a new culture flask containing fresh DMEM warmed to 37°C. Place into Infors Multitron rotation shaker.
4. To maintain high cell viability, dead cells must be removed periodically. Transfer 5 × 10^7 HeLa S3 cells (at 1 × 10^6 cells/ml) from the suspension flask to a 150 mm tissue culture plate. Allow cells to adhere for at least 4–6 hours, then replace old media containing dead cells with fresh media warmed to 37°C. 24 h post adherence, wash cells once in 10 ml sterile PBS warmed to 37°C, treat with 2 ml trypsin for 5 min, then transfer to a flask containing 50 ml of DMEM warmed to 37°C.

3.2 Mitotic cell cycle arrest

We provide this protocol as one example of a cell cycle-specific enrichment. Wild type or HeLa S3 cells engineered to express PARP fusion proteins are arrested in mitosis of the cell cycle, and are used to purify pADPr or PARP fusions containing covalently modified pADPr.

1. The evening prior to purification, add 10 µM (+)-S-trityl-L-cysteine (STLC) to the cells. Incubate for a maximum of 12 hours. The mitotic index may be assayed by staining cells with Hoechst 33342 and visualizing under a fluorescent microscope.

2. Proceed to cytoplasmic lysate preparation (section 3.4).

3.3 Transfection

1. HeLa S3 cells are passaged at 1 × 10^6 cells/ ml the day prior to transfection. For GFP-PARP expression, a typical transfection requires 50 ml of confluent cells to generate 1 µg of protein, although this varies with transfection efficiency of various constructs.

2. On the day of transfection, mix 50 µg of pure maxiprep GFP-PARP plasmid DNA (260/280 ratio of at least 1.8) with 1 ml Opti-MEM media. Simultaneously, mix 50 µl of 293fectin with 950 µl Opti-MEM media. Incubate for 5 min.

3. Combine DNA and 293fectin solutions and incubate for 20 min.

4. During the 20 min incubation, count cells and collect 5 × 10^7 cells via centrifugation at 100 × g for 3 min at 25°C. Resuspend cell pellet in 48 ml of DMEM plus 10% FBS, without penicillin/streptomycin. At the end of the incubation in step 3, add transfection mixture to cells and return to Infors.

5. 6 h post transfection, pellet cells at 100 × g for 3 min and resuspend in 50 ml DMEM, 10% FBS, containing 1% penicillin/streptomycin.

3.4 Cytoplasmic lysate preparation

Maintaining sterile conditions is no longer critical.

1. Latrunculin B is added to cells to a final concentration of 1.26 µM. Incubate for 1 h under normal culturing conditions (see note 2).

2. Collect cells via centrifugation at 400 × g for 3 min at 4°C. Wash 3x in ice-cold PBS (need not be sterile). During the pelleting and all subsequent steps, it is critical to maintain the samples at 4°C (on ice) to maximize protein stability. Transfer cell pellet to a 1.7 ml microcentrifuge tube for the final wash.

3. Remove excess PBS and dilute cell pellet in 3 × volume of CLB (for example, if the pellet size is 200 µl, add 600 µl CLB). If the cytoplasmic lysate is for use in the cellular pADPr purification protocol (3.5), the lysate modification assay (3.6.3), or

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2Latrunculin B is an actin depolymerizing agent. Actin and actin binding proteins are common contaminants in HeLa S3 preparations.
assembly of pADPr-dependent complexes (3.8), add 1 µM ADP-HPD to the CLB. ADP-HPD is a small molecule inhibitor of pADPr glycohydrolase (PARG), an enzyme that degrades pADPr and is highly active in cytoplasmic lysates. For immunoprecipitation of PARPs for the pure PARP modification assay (3.6), ADP-HPD addition is optional because the downstream formation of polymer is of greater interest.

4. Incubate cells on ice for 10 min.

5. Spin the cell mixture in a microcentrifuge at 14,000 × g, 4°C, for 10 min. This step removes cell membranes and nuclei from the cytoplasmic lysate.

6. Transfer the supernatant that contains the cytoplasmic lysate to a new tube, then add 1 µg/ml cytochalasin D and 25 µM nocodazole. Cytochalasin D inhibits actin polymerization while nocodazole depolymerizes microtubules. Both treatments minimize contamination by aggregated and precipitated cytoskeletal components.

7. The resulting supernatant is centrifuged again at 14,000 × g for 1 min to remove remaining cytoskeletal contaminants. The supernatant comprises the cytoplasmic lysate used for cellular pADPr purification (3.5) or GFP-PARP immunoprecipitation (3.6).

### 3.5 Cellular pADPr purification (see note 3)

1. Treat cytoplasmic lysate with 2 units/ml Ambion RNase cocktail for 45 min, then resolve through PD-10 column. Collect flow through.

2. Mix equal volumes of Concanavalin A (ConA) and wheat germ agglutinin (WGA) bead slurry in an appropriately-sized centrifuge tube (usually 1.7 ml). The binding capacity of each matrix is 10 mg of protein per ml of resin. Per 500 µl of cytoplasmic lysate use a mixture of 100 µl of WGA bead slurry and 100 µl ConA bead slurry. Wash the ConA/WGA bead mixture twice in CLB.

3. Save 5 µl of each cytoplasmic lysate as an “input” sample. Add the remaining cytoplasmic lysate to the ConA/WGA bead mixture and rotate for 90 min at 4°C.

4. Generate the boronate column during the incubation of cytoplasmic lysate with the ConA/WGA bead mixture. The binding capacity of Boronate Affi-Gel is 130 µmol sorbitol/ml gel. Boronate Affi-Gel is stored as a dry powder that must be rehydrated. Rehydrate in CLB for at least 60 min. The ratio of boronate bead to cytoplasmic lysate should be 1:3 (that is, 100 µl boronate column to 300 µl cytoplasmic lysate).

5. Transfer the rehydrated boronate beads to a Kontes column, then wash in 10 column volumes of CLB.

6. Spin the cytoplasmic lysate/ConA/WGA bead mixture gently (~100 × g) to remove the ConA/WGA beads.

7. Save a “post-lectin” sample of 5 µl, then add the remaining supernatant to the boronate column.

8. Cap both ends of the column and rotate for 90 min at 4°C.

9. Collect the flow through.

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3Cellular pADPr purification is normally performed without transfection (3.3) but can be combined with cell cycle arrests to enrich for proteins that bind to pADPr during specific cell cycle stages.
10. Save 5 µl of flow through as “boronate remainder.” pADPr and associated proteins are bound to the column.

11. To remove non-specifically bound components as well as remaining ADP-HPD, wash with 20 column volumes CLB.

12. Cap both ends of the column, then add 1 column volume of 100 µg/ml ARH3 and incubate 30 min on ice and 10 min at room temperature. ARH3 is a pADPr glycohydrolase that hydrolyzes pADPr and thus specifically elutes proteins bound to pADPr. ARH3 is used instead of PARG because it is not inhibited by ADP-HPD (in case there is residual inhibitor in the prep) and because it is easily purified as recombinant protein.

13. Collect the flow through, then wash the boronate column in three column volumes CLB and collect each column volume.

14. Collect the beads from the column via resuspension in CLB, then transfer to a fresh tube. Allow beads to settle. Add 1 column volume of 1× Laemmli sample buffer in CLB containing 2% beta-mercaptoethanol to the beads and all samples. Heat to 95°C for 5 min and resolve on SDS-PAGE gel. Amount of protein bound may be quantitated by Coomassie staining and whole samples or specific protein bands may be analyzed by mass spectrometry.

3.6 Immunoprecipitation (lysate modification assay and pure PARP modification assay)

1. Cytoplasmic lysates are generated as described in section 3.4 at 24 h post transfection. Maximum expression of recombinant DNA is generally achieved 48 h post transfection in HeLa S3 cells. Due to the toxicity of PARP overexpression, we perform PARP purifications 24 h post transfection. This provides a good balance between toxicity and expression. Period of expression may also be optimized in each laboratory.

2. During generation of cytoplasmic lysate, conjugate precipitating antibody to protein A magnetic beads (binding capacity= 250 µg IgG/ml of beads). Add 10 µl of magnetic bead slurry per reaction to a 1.7 ml microcentrifuge tube. Place the tube in a magnetic tube rack to pellet the beads. Remove the supernatant. Wash beads once in 1 ml ice-cold PBS. Calculate the amount of antibody required to precipitate the predicted amount of fusion protein expressed. Fusion protein expression should be determined prior to the purification. Ideally the engineered protein should be at a four-fold molar excess to antibody. 50 ml of transfected cells is normally sufficient to saturate 0.5 µg of antibody. Dilute antibody in ice cold PBS to 1 µg/ml, then incubate with beads on a rotating incubator for 1 h at 4°C.

3. Add each cytoplasmic lysate to one bead reaction and rotate for 90 min at 4°C. To perform the lysate modification assay on the PARP being immunoprecipitated, 100 µM – 1 mM β-NAD+ is added to the cytoplasmic lysate during this bead-binding step. Optimal β-NAD+ concentration may be determined experimentally.

4. Remove the cytoplasmic lysate by placing the tubes in a magnetic tube rack to pellet the magnetic beads.

5. Wash 1 × 10 min in 1 ml CLB. ADP-HPD is not necessary at this step since preservation of polymer is not critical.

6. Wash 2 × 10 min in 1 ml CLB containing 300 mM NaCl (for the lysate modification assay) or 450 mM NaCl (for the pure PARP modification assay). This high salt wash reduces non-specific binding.
7. Wash 1 × 2 min in 1 ml CLB + 1 µM ADP-HPD.
8. Transfer to new tube. If the sample is being used for the *lysate modification assay*, elute the sample in sample buffer (proceed to step 3.8.5). If performing the *pure PARP modification assay*, proceed to section 3.7.

### 3.7 Pure PARP modification assay: PARP Activation

1. Incubate PARP-conjugated magnetic beads with desired concentration of β-NAD⁺ (100 µM – 1 mM) in 50 µl CLB + 1 µM ADP-HPD for 30 min on ice.
2. Using magnet, wash 3 × 2 min in CLB + 0.1 µM ADP-HPD to remove unincorporated β-NAD⁺.

### 3.8 Assembly of pADPr-interacting complexes

1. Incubate magnetic beads containing purified PARP and polymerized pADPr with 500 µl – 1 ml of fresh cytoplasmic lysate (as generated in 3.4) and rotate at 4°C for 1 h in a 1.7 ml microcentrifuge tube. Save a portion of the magnetic beads as a “no lysate added” control.
2. Wash 3 × in 1 ml CLB containing 450 mM NaCl for 10 min.
3. Wash 1 × in 1 ml CLB for 10 min.
4. Pellet beads. Incubate with 100 µg/ml ARH3 in CLB for 30 min on ice followed by 10 min at room temperature. The volume of ARH3 in CLB should equal the elution volume of sample buffer in no lysate controls. ARH3 treatment elutes proteins bound solely through pADPr interactions. Remove the supernatant and add sample buffer to the supernatant, which now contains pADPr binding proteins.
5. Add sample buffer to the magnetic bead-conjugated purified PARP and its protein interactors. Heat to 95°C for 5 min (see note 5). Run samples from steps 1, 4 and 5 on an SDS-PAGE gel. Protein can be quantified by Coomassie or silver stain followed by protein identification by mass spectrometry, or alternatively by immunoblot.

### References


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5 If the sample will be immunoblotted for pADPr, we recommend heating the sample to 70°C for 10 min as boiling the sample at 95°C will degrade polymer.


Figure 1. Poly(ADP-ribose) structure
Poly(ADP-ribose) (pADPr) is covalently attached to proteins through ester linkages. Two types of glycosidic ribose-ribose linkages connect subunits of ADP-ribose into pADPr: linear (1'→2') and branched (1''→2'').
Figure 2. Overview of purification strategies

The cellular pADPr purification method is illustrated on the left (white arrows) and is used to purify cellular pADPr and its associated proteins. The PARP immunoprecipitation method is shown on the right and summarizes two methods, the lysate modification assay (gray arrows) and the pure PARP modification assay (black arrows). Asterisk indicates optional step.
Figure 3. Examples of purification strategies
Both images show steps of each purification resolved on SDS PAGE-gels. **Left.** Results of cellular pADPr purification experiment. Letters above each lane refer to steps in the purification as marked in Figure 2. **Right.** Purified epitope-tagged PARPs prior to automodification and incubation with fresh cell lysate.