Purification of Recombinant Human PARG and Activity

Assays

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Abstract The purification of Poly(ADP-ribose) glycohydrolase (PARG) from over-expressing bacteria Escherichia coli is described here to a fast and reproducible one chromatographic step protocol. After cell lysis, GST-PARG-fusion proteins from the crude extract are affinity purified by a Glutathione 4B Sepharose chromatographic step. The PARG proteins are then freed from their GST-fusion by overnight enzymatic cleavage using the preScission protease. As described in the protocol, more than 500 µg of highly active human PARG can be obtained from 1.5 L of E. coli culture.

Key words: Poly(ADP-ribose) glycohydrolase, poly(ADP-ribose) metabolism, PARG purification

1 Introduction

Poly(ADP-ribosyl)ation is a transient modification of proteins, since poly(ADP-ribose) is efficiently and rapidly degraded by the endo- and exo-glycosidase activities of poly(ADP-ribose) glycohydrolase, (PARG). The unique PARG gene encodes a nuclear enzyme (PARG111) as well as smaller isoforms showing cytoplasmic and mitochondrial localisations (1, 2). Many studies revealed the importance of PARP and PARG to control poly(ADP-ribose) levels regulating the balance between life-and-death in response to DNA injury (3, 4, 5), pointing that PARG could also be a good therapeutic target with the advantage that PARG inhibition, by limiting the recycling of ADP-ribose to NAD⁺ provides an additional metabolic consequence that may affect cell survival (6). However, the currently available PARG inhibitors are questioned for their bioavailability or specificity, precluding from their evaluation in pre-clinical assays. Recents crystallographic studies have brought new insights into the structure-function relationships of the PARG catalytic domain and have opened the track to inhibitor design and optimisation (7, 8, 9, 10, 11, 12, 13). Therefore, the design of PARG inhibitors requires that pure and high amount of this enzyme is easily available for enzymatic inhibition evaluation and co-crystallographic studies. Here, we present a protocol specifically designed for the purification of the human full length PARG cloned in pGEX6P vectors in fusion with the GST tag and over-expressed in the bacterial system Escherichia coli BL21DE3. It results in a protein production of high purity suitable for studying the catalytic properties of the enzyme using biochemical or biophysical methods or for the induction of a specific immunogenic response for antibody production in the mouse or in the rabbit. We also present some tests to evaluate its activity in solution.
2 Materials

2.1 Materials for Bacterial Growth and Protein Expression

1. Sterile LB medium (10 g/L tryptone, 5 g/L Yeast Extract, 5 g/L NaCl (see Note 1).

2. 100 mg/mL Ampicillin stock solution in sterile water and stored in 1 mL aliquots at −20°C.

3. Sterile 2-L flasks containing 500 mL of LB medium supplemented with the appropriate antibiotic (Ampicillin 100 µg/mL).

4. 1 M IPTG stock solution prepared in sterile water and stored in 1 mL aliquots at −20°C.

5. A Infors HT Minitron Incubator Shaker or equivalent for the culture during IPTG induction at 20°C.

2.2 Materials for Purification of GST-hPARG

1. All the solutions are prepared using Milli-Q (18.2 MOhm.cm at 25°C, 4 ppb TOC) water purified with a Millipore Advantage A10 water purification system connected to a source of reverse osmosis water.

2. Cell lysis buffer stock solution: prepare 250 mL of 25 mM Tris-HCl pH 8.0 buffer containing 50 mM glucose, 10 mM EDTA, 1 mM PMSF. Store it at 4°C. Before use, prepare 20 mL aliquots containing two proteases inhibitor cocktail tablets.

3. Detergents: 100% Tween 20; 100% NP-40, 100% Triton® X100.

4. NaCl in powder and a 5 M stock solution (see Note 2).

5. Protamine sulfate 10 mg/mL in water.

6. Proteases inhibitor cocktail tablets (COMPLETE Mini, Roche Diagnostic GmbH, Mannheim, Germany).

7. Glutathione Sepharose 4B from GE Healthcare.

8. Purification buffer: 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM DTT, 0.1% Triton® X100.

2.3 Materials for PreScission Cleavage

1. PreScission protease (500 units, GE Healthcare).

2. PreScission cleavage buffer: 50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT.
2.4 Materials for Large Scale PAR Synthesis

1. Siliconised 10 mL Sarstedt tubes: to prevent unspecific binding of the poly(ADP-ribose) to the plastic, the 10 mL Sarstedt screw cap tubes (round base) are siliconised by vortexing 1 mL of Sigmacote, (Sigma-Aldrich, St-Louis, MO, USA). Remove the solution and let it air-dry under the chemical hood (Sorbonne).

2. PARP activity buffer (10 ×): 500 mM Tris-HCl pH 8.0, 40 mM MgCl$_2$, 2 mM DTT, 500 µg/mL BSA, 20 µg/mL DNase I activated DNA, 4 mM NAD$^+$. This buffer can be kept in 200 µL aliquots for more than a year at −20 °C.

3. Radioactive NAD$^+$: $^{[32P]}$NAD$^+$ (800 Ci/mmol, 5 µCi/µL, Perkin Elmer).

4. Purified PARP-1 (Enzo, Lausen, Switzerland).

5. 10 mg/mL solution of Proteinase K (Merk KGaA, Darmstadt, Germany), SDS 20%.

6. 0.2 M NaOH and 40 mM EDTA pH 8.0 solution. 0.2 M HCl solution.

7. Phenol/CHCl$_3$/isoamyl alcohol (25/24/1) mix (Roth GmbH, Karlsruhe, Germany).

8. 3 M potassium acetate pH 4.8 solution. 100% isopropyl alcohol.

9. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.

2.5 Materials for SDS-PAGE

1. Separating buffer (4 ×): 1.5 M Tris-HCl, pH 8.8, 0.4% SDS. Store at 4 °C.

2. Stacking buffer (4 ×): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS. Store at 4 °C.

3. Forty percent acrylamide/bis solution (37.5:1 with 2.6% C) (Bio-Rad) and N,N,N',N'-Tetramethylethylenediamine (TEMED) Store at 4 °C.

4. Ammonium persulfate: prepare 10% solution in water and freeze in aliquots at −20 °C.

5. Isopropanol.

6. Running buffer (5 ×): Tris-base 30 g/L, Glycine 144 g/L, SDS 5 g/L. The pH is not adjusted. Store at room temperature.
7. Prestained molecular weight markers (All blue Precision Plus Protein Prestained Standards, Bio-Rad).

8. Sample buffer: 50 mM Tris-HCl pH 6.8, Urea 6 M, SDS 3%, β-mercaptoethanol 6%. Add 3 mg of Bromophenol blue in 100 mL of sample buffer. Aliquot and store at −20 °C.

9. Coomassie blue staining solution: prepare 250 mL of 0.3% (w/v) Coomassie blue in 50% methanol, 10% acetic acid.

10. Coomassie blue destaining solution: Prepare 1 L of 50% methanol, 10% acetic acid in water.

2.6 Materials for Western Blot

1. Stock transfer buffer (10 ×): Tris base 30 g/L, glycine 144 g/L, SDS 10 g/L. The pH is not adjusted but should be 8.3. Store at room temperature.

2. Transfer buffer (1 ×): for a 1-L solution, mix in the following order 100 mL of 10 × stock transfer buffer, 700 mL of water, and 200 mL of 95% ethanol.

3. Nitrocellulose blotting membrane Amersham™ Protran™, 0.2 µm porosity (GE Healthcare), Whatman 3MM filter papers.

4. PBS buffer (10 ×): KH₂PO₄ 10.6 mM, Na₂HPO₄, 2H₂O 30 mM, NaCl 1.54 M. Prepared with water and filtered 0.2 µm. Store at room temperature.

5. PBS-Tween buffer (1 ×): in a cylinder to make a 1-L solution, dilute 100 mL of 10 × PBS buffer, 500 µL of Tween-20, and complete with water.

6. Anti-PARG mouse monoclonal antibody (Millipore).

7. Horseradish peroxydase (HRP)-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich) or Alexa680-conjugated goat anti-rabbit secondary antibody (ThermoFisher Scientific).

8. Blocking buffer: 5% (w/v) nonfat dry milk in PBS.

9. Enhanced chemiluminescent reagents, ECL Plus Western Blotting Detection System, Amersham, GE Healthcare and BioMax MR film (Kodak, Rochester, NY) or Amersham Hyperfilm™ ECL (GE Healthcare) or a ImageQuant LAS 4000 imager (GE Healthcare) or an Odyssey® Imaging System (Li-Cor Biosciences).
2.7 Detection of PARG activity in solution

1. $^32\text{P}$-labelled-PAR ($\approx 200,000$ dpm, see § 3.8.1)

2. PARG activity buffer (10 ×): 500 mM Tris-HCl pH 8.0, 20 mM MgCl$_2$, 10 mM DTT, 1 M NaCl. The buffer is stored at $-20^\circ$C in small aliquots.

3. Purified recombinant human PARG.

4. Stop/Loading solution (95% formamide, 20 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue).

2.8 Materials for Electrophoresis on Sequencing Gel

1. Model S2 Sequencing Gel Electrophoresis Apparatus from Gibco BRL® or equivalent.

2. One pair of 33-cm wide glass plates (one short of 39 cm, one long of 42 cm).

3. Sigmacote, (Sigma-Aldrich, St-Louis, MO, USA).

4. One pair of 0.35-mm thick mylar side spacers.

5. A 0.35-mm thick mylar 32-square-toothed comb.

6. Binder Clips.

7. One pair of 122-cm DC power cords.

8. High voltage power supply (3,000 V/150 A).

9. 10 × TBE (890 mM Tris, 890 mM boric acid, 20 mM EDTA) electrophoresis buffer.

10. Urea.

11. Forty percent acrylamide/bis solution (38:2 with 3.3% C) (Bio-Rad) and N,N,N,N’-Tetramethylethlenediamine (TEMED) Store at 4 °C.

12. Ammonium persulfate: prepare 10% solution in water and freeze in aliquots at $-20^\circ$C.

13. 250-mL Millipore Stericup® Millipore Express® Plus 0.22 μm PES.

14. Loading solution (95% formamide, 20 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue).
15. 35 × 3 cm band of Whatman 3MM filter paper.

16. Used autoradiographic Kodak acetate-film (minimum size 34 × 40 cm or an equivalent piece of rigid plastic that could accommodate the gel size after the electrophoresis).

17. Storage Phosphor ScreenImage and Typhoon™ FLA 9500 Biomolecular Imager (GE Healthcare).

2.9 Materials for Detection of PARG Activity on Dot Blot

1. Unlabelled purified poly(ADP-ribose) (200 ng/µL, determined by measuring the absorbance at 260 nm with a spectrophotometer and using the relationship $A_{260}=1 \Leftrightarrow 40 \mu g/mL$). Unlabelled PAR is obtained as in §3.8.1, but the reaction is done without radioactive NAD$^+$.

2. PARG activity buffer (10 ×): 500 mM Tris-HCl pH 8.0, 20 mM MgCl$_2$, 10 mM DTT, 1 M NaCl. The buffer is stored at −20°C in small aliquots.

3. Purified recombinant human PARG.

4. Nitrocellulose membrane.

5. PBS-Tween buffer (1 ×): in a cylinder to make a 1-L solution, dilute 100 mL of 10 × PBS buffer, 500 µL of Tween-20, and complete with water.

6. Anti-poly(ADP-ribose) antibody polyclonal or monoclonal 10H (Trevigen).

7. Horseradish peroxydase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody or Alexa680-conjugated goat anti-rabbit or anti-mouse secondary antibody (ThermoFisher Scientific, Molecular Probes).

8. Blocking buffer: 5% (w/v) nonfat dry milk in PBS.

9. Enhanced chemiluminescent reagents, ECL Plus Western Blotting Detection System, Amersham, GE Healthcare and BioMax MR film (Kodak, Rochester, NY) or Amersham Hyperfilm™ ECL (GE Healthcare) or a ImageQuant LAS 4000 imager (GE Healthcare) or an Odyssey® Imaging System (Li-Cor Biosciences).
3 Methods

3.1 Purification Protocol Overview

A schematic representation of the purification protocol of human PARG is presented Fig. 1. It summarises the various steps needed to obtain a fairly pure and active human PARG from a *E. coli* culture over-expressing the protein.

**Fig. 1:** Schematic representation of the purification protocol of human PARG

3.2 Bacterial Growth and Induction of Expression

The following protocol is suitable for the purification of the human recombinant PARG over-expressed in *Escherichia coli*. This system results in the expression of a GST-fusion protein facilitating it’s purification by a single affinity chromatographic step using the Glutathione Sepharose 4B matrix. The fusion-protein bound to the purification matrix is then later cleaved by enzymatic cleavage with the preScission protease to elute the full length PARG from it’s support. The expression of human PARG has been optimised by cloning into the expression plasmid pGEX6P3 a synthetic version of the human cDNA with codon usage adapted for expression in the bacteria *E. coli* (see Note 3).
1. The day before the culture a small pre-culture (3 mL) in LB containing the appropriate antibiotic (ampicillin at 100 µg/mL (LB-Amp)) is started with 30 µl from a stock of bacteria conserved at −80°C previously transformed with the expressing plasmid (pGEX6P3 containing the cDNA of human PARG), shake at 37°C.

2. After six hours, 100 µl of this culture is used to seed a 150 mL pre-culture (LB-Amp) in a 1-L flask, then shake at 200 rpm overnight at 37°C.

3. The next day, three 2-L flasks containing 500 mL of LB-Amp are seeded with 50 mL of the overnight pre-culture each. The bacteria are grown for 2 h at 37°C under agitation at 200 rpm.

4. The expression of the recombinant protein is induced by addition of 0.5 mM IPTG (275 µl of 1 M stock IPTG). The flasks are transferred to a Minitron shaker and cultivated for an additional 3 to 4 hours at 20°C and 200 rpm agitation.

5. Following the expression of the protein, harvest the bacteria by centrifugation (9,000 × g, 15 min at room temperature) in 500-mL bottles (JA-10 Rotor, Avanti J25 centrifuge, Beckman).

6. Wash once with 80 mL of cold PBS 1×, transfer in two 50-mL Falcon tubes and harvest at 4°C, 5,500 × g in a JA-12 rotor (Beckman). Discard the supernatant and dry freeze at −80°C.

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**Fig. 2: Details of the cloning region of hPARG expressing vector pGEX6P3-hPARGfl.** The N-terminal part of the GST is in green color and in a grey box. The preScission recognition site is in red and the cleavage position is depicted with the red arrow. The sequence of PARG is in bold and the purified protein is underlined. The PARG sequence was amplified by PCR using the forward and reverse oligonucleotides respectively: 5′ gctcacaagattcaatgacagttccggttgctg, 5′ ggtaccgggaatcttagctaccgggtacgt. The cloning sites are EcoRI.
3.3 Cell Lysate Preparation

All the steps are performed at 4 °C ideally in the cold room or on ice.

1. The cells are quickly defrosted and resuspended by vortexing in 5 mL of cold cell lysis buffer (keep working in the same 50-mL Falcon tubes).

2. Moderately sonicate (40% power, 1.2-cm diameter probe) 3 × 20 sec in ice. Do not stop agitating during the sonication process to ensure a good lysis. The viscosity of the solution should disappear.

3. Add 0.2% of Triton® X100, 0.2% of NP40, 4 mL of 5 M NaCl.

4. Gently rotate the cell suspension for 20 min on a rotating wheel in the cold room.

5. Add 30 mL of ice-cold Tris-HCl 20 mM pH 7.5, rotate for an additional 20 min in the cold room.

6. Clear lysate from cellular debris by centrifugation at 22,300 × g for 60 min at 4 °C (rotor JA-12, Avanti J25, Beckman).

7. Add to the supernatant 1 mg/mL final of a 10 mg/mL protamine sulfate solution in water to precipitate the nucleic acids, invert the tubes several times and centrifuge at 22,300 × g for 60 min at 4 °C (rotor JA-12 Beckman).

8. Optional step: the crude protein solution can be clarified by filtration through a 0.22-µm vacuum filter (Steriflip®).

9. The protein solution is then diluted with Purification buffer to final volume of 50 mL (see Note 6) in a 50-mL Falcon tube.

3.4 Glutathione Sepharose 4B Affinity Chromatography

This chromatographic step performed in batch mode allows the rapid purification of the GST-PARG fusion protein in less than two hours. It ensures minimal protein contamination as extensive washing of the specific bound GST-PARG can be achieved using high salt concentration buffers thanks to the high affinity of GST for the Glutathione Sepharose matrix.

1. Wash three times in PBS 600 µL of 50% slurry of Glutathione Sepharose 4B beads in a 2-mL Eppendorf tube (see Note 4).
2. After the last wash, suspend the beads in 1 mL of Purification buffer and transfer into the tube containing the protein solution. Agitate softly by rotating on a wheel at 4°C in a cold room for minimum 30 min.

3. Pellet the beads by centrifugation for 2 min at 500 × g at 4°C (rotor JA-12, Avanti J25, Beckman).

4. Carefully discard the supernatant, wash the hPARG-GST-Glutathione Sepharose 4B beads with 50 mL of Purification buffer. Repeat this washing step three times.

5. Transfer the beads in a 15-mL Falcon tube containing 12 mL of cold Purification buffer supplemented with NaCl to 1 M NaCl final. This step should eliminate any unspecific binding of protein.

6. Pellet the beads for 2 min at 500 × g at 4°C (Eppendorf centrifuge 5810R), carefully discard the supernatant. Suspend the beads in 14 mL of Purification buffer and pellet again the beads.

7. Transfer the beads in two 2.0-mL Eppendorf tubes, centrifuge for 1 min at 500 × g at 4°C (Eppendorf centrifuge 5415R), remove the supernatant.

8. Take off 5 µL of beads for further SDS-PAGE analysis of the bound proteins.

9. Tubes containing the hPARG-GST-Glutathione Sepharose 4B beads may be stored on ice or at 4°C in the fridge for a few hours.

3.5 PreScission Protease Cleavage

PreScission Protease is a genetically engineered fusion protein consisting of human rhinovirus 3C protease and GST. This protease was specifically designed to facilitate removal of the protease by allowing simultaneous protease immobilisation and cleavage of GST fusion proteins produced from the pGEX-6P vectors. PreScission Protease specifically cleaves between the Gln and Gly residues of the recognition sequence of LeuGluValLeuPheGln/GlyPro.

1. Wash the beads twice with 1.5 mL of cold PreScission cleavage buffer.

2. Suspend the beads in 1.3 mL of cold PreScission cleavage buffer.

3. Add 10 units of PreScission protease to the beads, mix well by inverting several times the tubes. The cleavage reaction is then carried out at 4°C by rotating the tubes on a wheel in the cold room overnight or for 10 to 12 h.
4. The GST-hPARG fusion cleavage should be complete after 10 to 12 h incubation. The supernatant should contain the free hPARG.

5. After centrifugation at $500 \times g$ at 4 °C for 2 min, collect as much of the supernatant as possible in a new 1.5 mL Eppendorf tube.

6. The beads are washed twice with 0.5 mL of cold PreScission buffer containing 0.2% Triton® X100 to recover most of the hPARG protein. The amount and the quality of the purified protein in each fraction is evaluated by SDS-PAGE, (see Fig. 3).

7. Add 10% glycerol final to the hPARG fractions. Aliquot and Store at −80 °C.

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**Fig. 3:** Purification of hPARG by affinity chromatography on Glutathione-Sepharose 4B. **Left:** 10% SDS-PAGE of purification fractions. MW : molecular weight markers; 1, clear lysate (10 µl); 2, clear lysate after protamine sulfate precipitation (10 µl); 3, proteins bound to Glutathione-Sepharose beads after washing (10 µl); 4, free hPARG following overnight cleavage using 10 U of PreScission protease (10 U, 16 h et 4 °C) (20 µl/700 µl); 5, Proteins bound to the Glutathione-Sepharose beads after cleavage (20 µl). SDS-PAGE 10%, stained with Coomassie blue and scanned using the Li-COR Odyssey imaging system. **Right:** Detection of PARG by Western Blot. Same deposit as for SDS-PAGE but only 1/10-volume of previous deposit. After transfer, the nitrocellulose membrane was incubated overnight at 4 °C with a 1:10,000-dilution of anti-PARG monoclonal antibody (clone D8B10, Millipore) in PBS-0.05%-tween 5% fat-free dry milk. After three washes the membrane was incubated 2 h at room temperature with a 1:20,000-dilution of GAM-Alexa680-secondary antibody in PBS-0.05%-tween 5% fat-free dry milk. The membrane was scanned on a Li-COR Odyssey imaging system.
3.6 Quality Control: SDS-PAGE

1. The following instructions assume the use of a 11 by 10 cm gel system. They are easily adaptable to other formats. It is important that the glass plates are cleaned before use with a soft detergent (Teepol) and rinsed extensively with distilled water then 95% ethanol and air-dried.

2. Prepare a 1-mm thick, 10% gel by mixing 5 mL water with 2.5 mL of 4 × separating buffer, 2.5 mL acrylamide/bis solution, 68 µL ammonium persulfate solution, and 14 µL TEMED. Pour the gel, leaving space for a stacking gel, and carefully overlay with isopropanol. The gel should polymerise in about 10-15 min.

3. Pour off the isopropanol and rinse the top of the gel twice with distilled water.

4. Prepare the stacking gel by mixing 3.2 mL water with 1.25 mL of 4 × stacking buffer, 500 µL acrylamide/bis solution, 40 µL ammonium persulfate solution, and 8 µL TEMED. Pour the stack and insert the comb. The stacking gel should polymerise in about 10 min.

5. Prepare the running buffer by mixing 100 mL of the 10 × running buffer with 900 mL of water.

6. After polymerisation of the stacking gel, the comb is carefully removed and the wells are washed with 1 × running buffer using a 3-mL syringe fitted with a 22-gauge needle.

7. Add the running buffer to the lower chamber first, (avoid any bubbles which could be trapped between the glass plates on the bottom of the gel), then to the upper gel unit and load the 10 to 25 µL of each sample in a well. Include one well for pre-stained molecular weight markers.

8. Complete the assembly of gel unit and connect the power supply. The gel is run at 12 V/cm for 90 min at room temperature or until the dye front reaches the bottom of the gel.

9. Coomassie blue staining of the proteins separated by SDS-PAGE is performed by incubating the gel one hour in the Coomassie blue staining solution under shaking at room temperature. The proteins are revealed by destaining by washing the gel under shaking in the Coomassie blue destaining solution. When this solution becomes blue, it must be replaced by a fresh one. This must be repeated until a gel with a clear background is obtained.

10. Rehydrate the gel in distilled water, change the water several times to remove the methanol/acetic acid solution.
3.7 Quality Control: Western Blot

1. The samples that have been separated by SDS-page are transferred to nitrocellulose membranes electrophoretically. Any transfer tank system can be used for this purpose. We use routinely a Mini Trans-Blot Electrophoretic Transfer Cell from Bio-Rad.

2. Prepare a tray of transfer buffer that is large enough to lay out a transfer cassette with its pieces of Scotch-brite® pads and with two sheets of Whatman® 3MM paper submerged on one side. Cut a sheet of the nitrocellulose just larger than the size of the gel (separating and stacking), wet and soak in the transfer buffer.

3. Disconnect the gel unit from the power supply and disassemble. Setup the sandwich from bottom to top: one side of the transfer cassette, a piece of Scotch-brite® pad, a sheet of Whatman® 3MM paper, the nitrocellulose membrane, the gel (ensuring that no bubbles are trapped between the membrane and the gel), a sheet of Whatman® 3MM paper, a piece of Scotch-brite® pad. The transfer cassette is then carefully closed.

4. Place the cassette into the transfer tank such as that the nitrocellulose membrane is between the gel and the anode (+). Fill up the tank with cold transfer buffer and add a magnetic stir bar. The electrophoretic transfer is performed in a cold chamber (4 °C) with the tank placed on top of a magnetic stirrer. Make sure that the magnetic stir bar rotates freely.

5. Put the lid on the tank and activate the power supply. The transfer is accomplished at 100 V (250 mA) for 1.5 h.

6. Once the transfer is completed, take out the cassette of the tank and carefully disassemble, with the top Scotch-brite® pad and sheets of 3MM paper removed. Do not forget to orientate the membrane. The gel can be discarded and the excess nitrocellulose cut off. The coloured molecular weight markers should be clearly visible on the membrane.

7. Incubate the nitrocellulose in 20 mL PBS-Tween for 5 min then in 20 mL blocking buffer for 20 min at room temperature on a rocking platform.

8. Discard the blocking buffer and replace by a minimum (10 mL) of blocking buffer containing an anti-PARG antibody (Millipore) at the working dilution (1:10,000). The incubation is performed at room temperature for 2 h or at 4 °C overnight on a rocking platform.
9. Remove the primary antibody. The dilution of antibody can be kept for further use and store at \(-20^\circ\C\).

Wash the membrane three times for 10 min each with 20 mL PBS-Tween.

10. Discard the blocking buffer and replace with a minimum (10 mL) of PBS-Tween buffer containing a Goat anti-mouse Alexa Fluor 680 conjugate at the working dilution (1:20,000). The incubation is performed at room temperature for 60 min on a rocking platform.

11. Remove the secondary antibody solution. Wash the membrane three times each with 20 mL PBS-Tween at room temperature for 5 min and once with PBS for 5 min.

12. The membrane is scanned with the LI-COR Odyssey infrared imaging system. An example of the results produced is shown in Fig. 3.

3.8 Detection of PARG Activity by Electrophoresis on Sequencing Gel

3.8.1 Purification of Radioactive Poly(ADP-ribose)

The poly(ADP-ribose) synthesised as described below allows to obtain pure radioactive ([\(^{32}\)P] labelled) poly(ADP-ribose) (PAR) that can be separated by electrophoresis on sequencing gels and detected by autoradiography. It is also the substrate for PARG that can be used to evaluate its activity in various ways (see Note 5).

1. The poly(ADP-ribosylation) reaction is performed in a siliconised 10-mL Sarstedt tubes that reduce unspecific binding of PAR to the plastic. For a 2-mL reaction add in the tube 1745 \(\mu\)L of water, 200 \(\mu\)L of 10 \(\times\) PARP activity buffer, 5 \(\mu\)Ci (1 \(\mu\)L) \([^{32}\)P]-NAD\(^+\) (800 Ci/mmol, 5 \(\mu\)Ci/\(\mu\)L, Perkin Elmer), 10 \(\mu\)g PARP-1, gently vortex and incubate 1 h at 30\(^\circ\)C.

2. DNase I digestion: add to the reaction mix 4 \(\mu\)L of CaCl\(_2\) 1 M and 4 \(\mu\)L of a 10 mg/mL solution of DNase I (Roche Diagnostics GmbH, Mannheim), mix and incubate 1 h at 37\(^\circ\)C.

3. Proteinase K digestion: add to the mix 935 \(\mu\)L of water, 15 \(\mu\)L of SDS 20\% (0.1\% final) and 50 \(\mu\)L of a 10 mg/mL solution of Proteinase K (Merck KGaA, Darmstadt, Germany). Mix and incubate 4 h or overnight at 37\(^\circ\)C.

4. Remove the amino acid residue covalently bound to the PAR by adding 2 mL of a 0.2 M NaOH and 40 mM EDTA pH 8.0 solution. Mix and incubate 1 h at 37\(^\circ\)C. Then, neutralise by adding 2 mL 0.2 M HCl.
Fig. 4: Examples of PARG activity and detection by electrophoresis on 20% Acrylamide-7 M Urea sequencing gel. Time course degradation of $^{32}$P-PAR by the human PARG purified from over-expressing *E. coli* BL21DE3. 2 $\times 10^5$ cpm of purified $^{32}$P-PAR were incubated with 10 ng of purified human PARG. At each indicated time point, 10 $\mu$L of the reaction were taken and mixed with 10 $\mu$L of loading solution (95% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) to stop the reaction. 6 $\mu$L of each mixture was loaded and separated on a 0.35-mm sick 20% Acrylamide, 7 M Urea 40 cm high sequencing gel. The gel was exposed for 24 h on a phosphor screen. The phosphor screen was scanned on a Typhoon™ FLA 9500 Biomolecular Imager.

5. *Phenol/CHCl$_3$ extraction*: the reaction mix is phenol/CHCl$_3$ extracted by adding 500 $\mu$L of Phenol/CHCl$_3$/isoamyl alcohol (25:24:1). Vortex and centrifuge at 3,000 $\times g$. Carefully transfer the upper phase to a new siliconised tube. Repeat the extraction until the organic/aqueous inter-phase is clean.

6. *PAR precipitation*: add to the aqueous phase 300 $\mu$L of 3 M potassium acetate pH 4.8 and 3.5 mL of
isopropyl alcohol, mix and let the PAR precipitate 1 h at −20 °C. Centrifuge 30 min at 6,000 × g at 4 °C. The pellet is carefully washed twice with 3 mL of ice cold 80% ethanol. After centrifugation 30 min at 6,000 × g at 4 °C, air dry the pellet by leaving the tube open on the bench. The pellet is resuspended in TE buffer (≃ 20,000 dpm/µL).

### 3.8.2 PARG Activity in Solution

1. In a 1.5-mL Eppendorf, incubate the following reaction mixture at room temperature: 10 µL of 10× PARG activity buffer, 10 µL of [³²P]-labelled-PAR (≃ 200,000 dpm), an appropriate dilution of purified PARG (10 ng), water to 100 µL.

2. For a kinetic experiment, in a 1.5-mL tube prepare the reaction mixture but without PARG, then at various time-points (T₀, T₁₀'', T₃₀'', T₁'', T₂'', T₄'', T₈'', T₁₆'', ...) after the addition of PARG, take off 10 µL of the mixture and stop the reaction in a new tube containing 10 µL of formamide Stop/Loading solution (see Note 7).

### 3.8.3 Electrophoresis on Sequencing Gel

#### Preparation of Glass Plates

1. Clean the glass plates thoroughly with a nonabrasive detergent and a plastic scouring pad. The cleaning solution should not leave a soap residue when rinsed thoroughly. Avoid scratching the surface of the glass plates.

2. Rinse the glass plates thoroughly with deionized water and wipe dry.

3. Treat the inside (acrylamide contact) surface of one or both glass plates (usually the short plate) with 2 mL of Sigmacote, wipe rapidly the entire surface with Kleenex tissue (see Note 8).

4. Before the assembly of plate, rinse the inside surfaces with ethanol and wipe them dry.

5. Assemble the glass plate the conventional manner: place the long glass plate inside-up on the bench, align the mylar side spacers at the sides such that you leave 1.5 to 2 cm free of spacer at the bottom of the plate (that will allow the insertion of the band of Whatman 3MM paper to seal the bottom of the plate assembly), and place the short glass plate inside-down on the side spacers. The spacers must be perfectly aligned with the glass plate edges.
6. Secure the assembly by clipping the plates with binder clips (5 or 6 on each sides).

7. Insert a band of Whatman 3MM filter paper at the bottom of the assembly, between the two plates. Make sure that the horizontal surface of the paper is perfectly in contact with the two side-spacers to avoid any leaks of acrylamide. Place binder clips at the bottom of the glass plate sandwich (see Note 9).

**Pouring the Gel**

1. Prepare 100 mL of 20% acrylamide-7 M Urea solution by mixing in a 250-mL beaker 42 g Urea, 10 mL TBE 10 × electrophoresis buffer and 50 mL acrylamide/bis-acrylamide 40% (38:2) stock solution (see Note 10).

2. Mix with a magnetic stirrer to complete dissolution, slightly heat the solution if necessary. Adjust the volume to 100 mL with water and vacuum filter using a 250-mL Millipore Stericup®. Keep the vacuum for a while to degas the solution as much as possible to reduce the possibility of bubbles formation during the polymerisation.

3. To pour the gel be ready with the following materials: a Pipetboy and a 35-mL or bigger disposable pipet, the ammonium persulfate 10% and TEMED solutions.

4. In the Stericup® add to the acrylamide solution, 600 µL ammonium persulfate 10% and 60 µL TEMED mix well by rotating the cup for a few seconds. Immediately, fill the pipet (using the Pipetboy) with the solution. Hold the assembled plate sandwich at a 45° angle on one bottom corner so that the gel solution flows evenly down along the lower side spacer. Maintain a constant, even flow to reduce the chance of forming bubbles in the solution.

5. As the gel sandwich fills, gradually lower the glass plates until they rest on the bottom edge, approximately at a 5° angle from the bench. The gel mold should be slightly overfilled to ensure complete polymerisation at the top (see Note 11).

6. Insert a well-forming comb into the top of the gel. Clip the top with Binder clips.

7. Leave the glass plates in their near-horizontal position until the acrylamide has polymerised. The polymerisation should take a few minutes only.
8. The gel could be prepared the evening before the electrophoresis. If so, after polymerisation, pour some water on top of the comb to keep the gel wet, then cover the comb with Saran Wrap.

**Pre-Electrophoresis**

1. Remove the clips. Carefully slide the comb from between the plates.

2. Rinse the top of the gel with water to remove any un-polymerised acrylamide. Rinse the comb.

3. Place the gel sandwich in the sequencing apparatus with the short glass plate inward, so that the foam blocks on the side spacers form a seal with the gray silicone gasket. Rest the bottom edge of the sandwich on the ribbed gel support blocks in the lower buffer tray.

4. Secure the gel sandwich with the integral gel clamps along the sides of the sequencing apparatus.

5. Verify that the upper buffer chamber drain valve is closed, and fill the upper buffer chamber with approximately 450 mL of electrophoresis buffer (TBE 1×). Make sure no buffer is leaking from the upper buffer chamber. Fill the front chamber of the lower buffer tray with approximately 450 mL of electrophoresis buffer. The Whatman 3MM paper on the bottom of the gel will ensure good contact between the gel and the buffer and it should not be removed.

6. Close the upper and lower safety lids and connect the DC power cords to the sequencing apparatus and the DC power supply.

7. Before loading samples, pre-electrophorese the gel for 30 to 45 min. Set the output power to 80 W.

**Electrophoresis**

1. Prepare the samples in the formamide Stop/Loading solution by heating to a temperature of 90°C to 100°C for 3 to 5 min and then chilling on ice.

2. At the end of the pre-electrophoresis period, turn off the power supply and disconnect both DC power cords, first from the power supply and then from the sequencing apparatus, before opening the upper safety lid.

3. Immediately prior to loading the samples, rinse the wells of the gel with electrophoresis buffer. Use a syringe and a fine needle to wash away urea that has diffused into the wells.
4. Load samples (4 to 8 µL) onto the bottom of the wells with capillary comb.

5. After loading the samples, close the upper and lower safety lids and connect the DC power cords to the sequencing apparatus and the DC power supply. Set the output power to 70 W.

6. Monitor the progress of electrophoresis by following the migration of the bromophenol blue marker dye. Stop the electrophoresis when it reaches 1/3 from the bottom of the gel to ensure that no radioactive signal is lost.

7. Disassemble the gel sandwich by prying off the short (or siliconised) glass plate, working gently from a bottom corner with a thin spatula.

8. For autoradiography, transfer the gel to a solid backing, such as used film and cover it with Saran Wrap film. Because of the high acrylamide concentration (above 15%) the gel cannot be dried before exposure as it may crack during the procedure.

9. The gel may be exposed on a traditional [32P]-type autoradiography film or on a Phosphor ScreenImage and revealed using a Typhoon™ FLA 9500 Biomolecular Imager (GE Healthcare). An example of such gel is shown Fig. 4.

3.8.4 PARG Activity Dot Blot and Immuno-Detection of PAR

1. In a 1.5-mL Eppendorf, incubate the following reaction mixture at room temperature: 5 µL of 10× PARG activity buffer, 400 ng of purified unlabelled-PAR, an appropriate dilution of purified PARG (24 ng), water to 50 µL.

2. For a kinetic experiment, in a 1.5-mL tube prepare the reaction mixture but without PARG, then at various time-points (T0, T3’, T6’, T9’, T12’, T15’, T18’, T21’, ... ) after the addition of PARG, take off 3 µL of the mixture and immediately deposit onto nitrocellulose to make regularly interspaced dots.

3. Dry the nitrocellulose, then crosslink 10 min under a 254 nm UV lamp at approximatively 100 millijoules/cm².

4. Incubate the membrane in 15 mL PBS-Tween buffer for 10 min at room temperature.
5. Incubate the membrane in 15 mL PBS-Tween buffer containing a 1:1,000 dilution of 10H monoclonal anti-PAR antibody for 60 min at room temperature.

6. Wash the membrane three times in 15 mL PBS-Tween buffer.

7. Incubate the membrane in 15 mL PBS-Tween buffer containing a 1:20,000 dilution of Alexa680-conjugated goat anti-mouse secondary antibody for 60 min at room temperature.

8. Wash the membrane three times in 15 mL PBS-Tween buffer.

9. Reveal the signal by scanning on a LI-COR Odyssey imaging system.

10. The signal can be quantified using the NIH ImageJ software.

![Image of Dot Blot and PAR Intensity Graph](image)

Fig. 5: **Time-course evaluation of PARG activity using Dot Blot and detection of PAR with an anti-PAR antibody (10H).** Kinetic of digestion of un-labelled poly(ADP-ribose) by PARG. After the indicated time, 3 µL of the reaction containing 8 ng/µL of purified PAR was spotted onto the nitrocellulose membrane. **Top row:** reaction without PARG, **Bottom row:** reaction containing 0.48 ng/µL of purified human PARG. PAR was detected by immuno-detection using the 10H anti-PAR monoclonal antibody as primary antibody (1:1,000) and the Alexa680-conjugated goat anti-mouse secondary antibody (1:20,000). The signal was reveal by scanning the membrane using the LI-COR Odyssey imaging system. Dots were quantified using NIH ImageJ software and values were plotted using R statistical package. **Blue curve:** Variation of PAR intensity from reaction without PARG. **Red curve:** Variation of PAR intensity from reaction incubated with PARG.
4 Notes

1. Alternative culture media as $2 \times$ TY medium (16 g/L tryptone, 5 g/L Yeast Extract, 5 g/L NaCl) or Terrific Broth (11.8 g/L tryptone, 23.6 g/L Yeast Extract, 9.4 g/L $K_2HPO_4$, 2.2 g/L $KH_2PO_4$, 4 mL/L glycerol) could also be used instead of LB medium for higher cell density and higher protein expression. However it may result in protein of less solubility and less activity.

2. When available all the Sigma-Aldrich products provided in powder should be of the bioXtra quality. Lower quality grade (of TRIS and NaCl particularly) may interfere with protein stability and activity but also with the chromatographic results as the compound may absorb at 280 nm.

3. Sequence available upon request.

4. The volume of beads to take depends on the expression level of the protein. The indicated amount should be sufficient for most cases as the typical GST-protein binding capacity indicated by the manufacturer is up to 25 mg per mL of beads.

5. Radioactive materials must be handled with care for yourself and others. Only authorised persons should manipulate radioactivity accordingly to local laboratory regulations.

6. To avoid any protein degradation this protein solution should not be kept but should be promptly loaded onto the first chromatographic column to start the purification.

7. The tubes can be kept in the fridge for a few days if necessary.

8. This procedure must be done under a fume hood for protection against the highly volatile vapours.

9. This will help maintain uniform gel thickness while pouring the gel. It is important that the binder clips be placed over the side spacers only. Clamping unsupported glass will distort the thickness of the gel.

10. Acrylamide is a neurotoxic. It must be manipulated with care.

11. If a bubble forms while the gel is being poured, raise the glass plates into a vertical position, and either tip the gel solution away from the bubble or carefully tap the plate sandwich at the bubble to make it rise to the surface. Once all bubbles have been removed, return the glass plates to their previous position.
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