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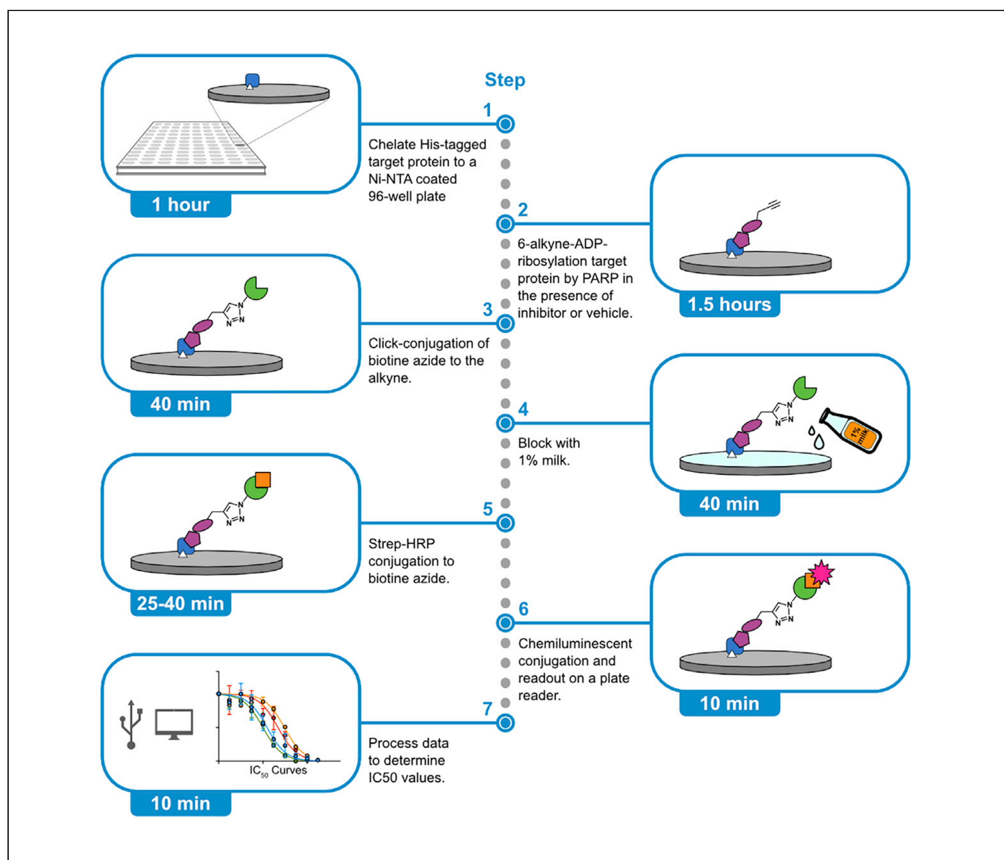
Protocol

PASTA: PARP activity screening and inhibitor testing assay



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HIGHLIGHTS

Semi high-throughput *in vitro* screening of PARP inhibitors in 96-well format

Simple, single-day experimental process producing easily quantifiable data

Detailed methods from protein purification to assay protocol and troubleshooting

Small-molecule inhibitors have been instrumental in uncovering the biological importance of poly-ADP-ribose polymerases (PARPs), a family of enzymes involved in wide-ranging aspects of cell biology. However, few PARP inhibitors are tested against the entire family of PARPs. This makes it impossible to confidently assess the role of a single PARP in cellular processes using small molecules. Here, we detail a PARP activity screening and inhibitor testing assay (PASTA) for determining relative selectivity of PARP inhibitors.

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Protocol

PASTA: PARP activity screening and inhibitor testing assay

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SUMMARY

Small-molecule inhibitors have been instrumental in uncovering the biological importance of poly-ADP-ribose polymerases (PARPs), a family of enzymes involved in wide-ranging aspects of cell biology. However, few PARP inhibitors are tested against the entire family of PARPs. This makes it impossible to confidently assess the role of a single PARP in cellular processes using small molecules. Here, we detail a PARP activity screening and inhibitor testing assay (PASTA) for determining relative selectivity of PARP inhibitors.

For complete details on the use and execution of this protocol, please refer to Kirby et al. (2018).

BEFORE YOU BEGIN

- All proteins should be in hand with concentration determined.
- 800× DMSO stocks of compounds should be made up.
- All buffers (unless noted that fresh is need) should be prepared.
- QuantaRed reagents should be warmed to 22°C–25°C.

Cloning

Timing: 3–10 days

To begin, identify which PARPs you wish to test in your screen, and whether you would like to measure ADP-ribosylation of a target protein (i.e., SRPK2, histones, etc.) or auto-ADP-ribosylation, as this will dictate whether an activating cofactor needs to be added at step 6 of the assay, and whether buffer (auto-ADP-ribosylation) or PARP enzyme (ADP-ribosylation of a target protein) is added in step 8 of the protocol. Once determined, the recombinant proteins and reagents for the assay must be obtained.

Some recombinant PARPs are commercially available, but for best results we recommend expressing and purifying the enzymes with a His-SUMO tag. cDNA of full-length or truncated PARP enzymes can be obtained from various commercial suppliers (Genscript, DNASU plasmid repository, Addgene, etc.) and cloned into a pET-His6-SUMO-TEV LIC cloning vector (Addgene plasmid # 29653) by a standard isothermal assembly protocol using the Gibson assembly mix (NEB).

Note: Full-length PARPs are preferable for inhibitor testing as they most accurately reflect selectivity in cells, however some PARP family members are prohibitively difficult to work with as full-length constructs, for example, PARP14 and PARP4 (Kirby and Cohen, 2019). In

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these instances, we recommend using a truncated construct comprising the catalytic domain and the immediately adjacent domains. In developing this assay, we found that constructs comprising only the catalytic domain of some PARPs led to higher signal to noise compared to the full-length proteins in this assay; additionally, some compounds that potently inhibited the catalytic domain variant did not inhibit the full-length PARP (Morgan et al., 2018).

General expression and purification of His-SUMO-tagged PARP enzymes

Timing: ~5 days

Note: SRPK2, a generalizable pseudo-target for most PARPs that can be extensively ADP-ribosylated by all active PARPs *in vitro*, can be expressed and purified using this same protocol. This assay was optimized to assess either ADP-ribosylation of a target using SRPK2 or auto-ADP-ribosylation using an active PARP bound to the plate. SRPK2 has been previously established as a target for several PARPs and used as a substrate for inhibitor screens for PARP10 and PARP15 (Venkannagari et al., 2013). In developing this assay, we have found that this holds true for every member of the PARP family.

1. Transform BL21(DE3) Rosetta cells (Millipore) with the desired plasmid and grow colonies at 37°C for 24 h on LB plates with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol.

Pause point: LB plates with colonies can be stored at 4°C for ~1 week.

CRITICAL: All the following cultures need to contain 50 μg/mL kanamycin and 34 μg/mL chloramphenicol.

2. Inoculate a swath of colonies into 10 mL of LB medium (10 g tryptone, 10 g sodium chloride, 5 g yeast extract for 1 L) with 50 μg/mL kanamycin, 34 μg/mL chloramphenicol and grow the cell cultures at 37°C and 225 rpm orbital rotation for 16–18 h.
3. Dilute the 10 mL culture into 1 L of TB medium (12 g Bacto Tryptone, 24 g yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 1% glucose, 50 μg/mL kanamycin, 34 μg/mL chloramphenicol) and grow until OD_{600} is between 0.6 and 0.8.
4. Cool the 1 L cultures in ice for 20 min then add IPTG to 0.4 mM to induce protein expression at 16°C and 225 rpm orbital rotation for 18–24 h.
5. Pellet cells by centrifugation at 4,000 rcf at 4°C and carefully remove supernatant.

Pause point: Cell pellets can be stored at −80°C for ~1 year.

Optional: To verify the expression of our desired proteins, take a 1 mL aliquot of the 1 L cultures immediately before induction (store at −20°C) and after overnight expression. Use 5–15 μL of sample to run a western blot analysis using primary His-tag antibodies, if expression has been successful there will be a clear band at the appropriate molecular weight for your desired protein in the post-overnight induction sample, but none in the pre-induction sample.

6. Resuspend cell pellet in 25 mL lysis buffer (20 mM HEPES, pH 7.5, 1 mM β-mercaptoethanol, 1 mM benzamidine, 0.2% NP-40, 0.2% Tween 20, 500 mM NaCl, 8.3 mg/L DNase I (Roche)) and homogenized using a Dounce tissue homogenizer.
7. Add phenylmethylsulfonyl fluoride (PMSF) to 1 mM then lyse by the homogenized solution by sonication on ice (Branson sonifier450).
   Alternate 30 s of sonication at 80% amplitude with 2 min rest periods six times for a total of 180 s (3 min) sonication with 10 min total rest time. The rest periods are crucial as they allow the lysates to cool between sonication pulses and prevent protein denaturation.

⚠ CRITICAL: Add PMSF to lysis solution within 30 s of beginning sonication.

8. Centrifuge the lysates at 12,000 rcf for 30 min at 4°C.
9. For every 1 L of culture, equilibrate 4–5 mL of Ni-NTA (50% slurry in storage solution, QIAGEN) in lysis buffer.
   a. Centrifuge 4–5 mL of Ni-NTA (50% slurry in storage solution) in a 15 mL conical at 1,000 rcf for 1 min at 4°C, remove supernatant, and resuspend in 5 mL sterilized water. Repeat wash step twice with sterile water, once with lysis buffer, then resuspend in lysis buffer.
10. Pass lysate supernatant through a 0.45 μm syringe filter into a sterile a 50 mL conical tube.
11. Add 4–5 mL of 50% Ni-NTA resin in lysis buffer to the clarified lysate and incubate at 4°C for 1 h with gentle agitation.

Optional: Add imidazole to 25 mM to the lysate and resin slurry before 1 h incubation to reduce non-specific binding of bacterial proteins.

12. Transfer the lysate and resin slurry to a reusable gravity chromatography column and collect the eluate.
13. Flow 40 mL of 50 mM imidazole wash buffer (20 mM HEPES, pH 7.5, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 500 mM NaCl, 50 mM imidazole).
14. Elute desired protein with 10 mL of 100 mM imidazole elution buffer (20 mM HEPES, pH 7.5, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 500 mM NaCl, 100 mM imidazole), followed by 10 mL each of 200 mM, 300 mM, and 400 mM imidazole elution buffers.
15. Use 15 μL of each elution (loading eluate, 50 mM imidazole wash eluate, 100 mM imidazole eluate, etc.) to run an SDS-PAGE and total protein stain analysis to identify fractions containing sufficiently pure PARP of interest.
16. Use a centrifugal filter unit to concentrate the PARP containing fraction(s) to ~5 mL.
17. Transfer solution to a dialysis cassette and stir *very gently* in 1 L dialysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF) for 12–16 h at 4°C, then transfer to 1 L fresh dialysis buffer and stir *very gently* for 3–5 h at 4°C.

Alternatives: Dilute protein to 5 mL with dialysis buffer and reconcentrate to minimal volume. Repeat 3–5 times and proceed to step 19.

18. Collect the dialyzed protein solution and use a centrifugal filter unit to concentrate the protein solution to ≤500 μL.
19. Gently add glycerol to 10% (v/v) and snap freeze small aliquots (50–100 μL) in liquid nitrogen and store at −80°C.

⚠ CRITICAL: Add 10% glycerol to the dialyzed protein solution and snap freeze as quickly as possible. Use a small needle to add a vent to the caps of aliquot tubes to prevent bursting as liquid nitrogen evaporates.

20. Determine protein concentration by comparison to a standard curve of a protein with a known concentration using the method of your choice: dye-based protein detection (Bradford, Lowry, or bicinchoninic acid assay), Tyrosine/Tryptophan fluorescence on a NanoDrop instrument, or direct band quantification on a Coomassie stained SDS-PAGE gel or western blot.

Note: For very pure protein solutions (>98%) a dye-based method or a NanoDrop analysis is an efficient and effective method to assess protein concentration, however if your protein solution is not very pure (90%–97%) we recommend using an in-gel quantification method that will allow you to specifically measure the concentration of your protein of interest and not the total protein in the sample. If your protein solution is less than 90% pure, we recommend adding an additional size-exclusion purification step, though this has never been necessary.

21. To test PARP activity, 1 μM of purified proteins can be incubated in PBR with 100 μM NAD+ and 3 μM of SRPK2 for 1 h at 30°C. Labeling can be detecting via western blot using pan-ADP-ribose antibody E6F6A, 1:1,000 (Cell Signaling Technology) and compared to previous batches to ensure relative consistency in activity.

Stock solutions for assay

Timing: 30 min

22. Make up PARP Buffer for the ADP-ribosylation reaction (PBR), 1× PBS with 0.02% Tween (PBST), and 1× PBS

Note: PBR can be stored at RT indefinitely so long as the TCEP is not added until you begin the assay.

Prepare DMSO stock solutions of small-molecule inhibitors

Timing: 5 min per compound

23. Prepare 800× stock solutions in DMSO of the small-molecule inhibitors to be tested.

Note: To save time later, make a dilution series of 800× stocks for a full dose response curve (DRC), i.e., to test a compound at 100, 30, 10, 3, 1 μM prepare DMSO stocks at 80, 24, 8, 2.4, 0.8 mM. Based on a 96-well plate format we have found an 8-point DRC (including a DMSO control and a no-enzyme control) effective for determining IC50s, but the DRC can be truncated or extended depending on the needs of the user.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal anti-His produced in mouse	Invitrogen	Cat# MA1135A647
Poly/Mono-ADP ribose (E6F6A) rabbit (Ab)	Cell Signaling Technology	Cat# 83732
Bacterial and virus strains		
Escherichia coli BL21 (DE3) competent cells	Millipore	Cat# 69450
Chemicals, peptides, and recombinant proteins		
6-Alkyne-nicotinamide adenine dinucleotide (N-6 alkyne-NAD^+)	Synthesized in house for this project (Jiang et al., 2010, Carter-O'Connell et al., 2014), also commercially available from Biolog Life Science Institute	N/A; Biolog Cat# N051
Ampicillin	Fisher Scientific	Cat# BP1760-5
Bacto Tryptone	BD Biosciences	Cat# 211699
Biotin-PEG3-azide	Click chemistry tools	Cat# AZ104
Chloramphenicol	Fisher Scientific	Cat# BP904-100
Deoxyribonucleic acid from calf thymus	Sigma-Aldrich	Cat# D4522
Dextrose	Fisher Scientific	Cat# D16500
DNase I	Roche	Cat# 10104159001
Dnick 5′P for PARP3 activation (Langelier et al., 2014)	IDT	Custom
Glycine	Fisher Scientific	Cat# BP381-1
HEPES	Fisher Scientific	Cat# BP310-1
Kanamycin	Fisher Scientific	Cat# BP9065
NP-40 Substitute	Fisher Scientific	Cat# 507517565

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phosphatase inhibitor cocktails 2 and 3	Sigma	Cat# P5726-1ML, Cat# P0044-1ML
Potassium phosphate dibasic	Sigma-Aldrich	Cat# P3786-500g
Potassium phosphate monobasic	Sigma-Aldrich	Cat# P9791-100G
Strep-HRP	Fisher Scientific	Cat# NC9705430
TCEP	Fisher Scientific	Cat# PI-20490
Tris[(1-benzyl-1H-1,2, 3-triazol-4-yl)methyl]amine (TBTA)	Click Chemistry Tools	Cat# 1061-100
Triton X-100	Fisher Scientific	Cat# BP151-500
Yeast extract	Fisher Scientific	Cat# DF0127-07-1
β-Mercaptoethanol	Sigma-Aldrich	Cat# M3148-100mL
β-Nicotinamide adenine dinucleotide	Fisher Scientific	Cat# AAJ6163803
Critical commercial assays		
QuantaRed enhanced chemifluorescent HRP Substrate	Thermo Fisher	Cat# 15159
Other		
Ni-NTA agarose	QIAGEN	Cat# 30210
96-well Ni-NTA coated plate	Thermo Scientific	Cat# 15242

MATERIALS AND EQUIPMENT

We recommend using a multichannel pipet and reagent reservoirs when conducting this assay, results can be measured on any standard plate reader equipped to measure luminescence.

PARP buffer for ribosylation reaction (PBR)				
Reagent	Stock concentration	Dilution	Final concentration	Volume
HEPES, pH 7.5	1 M	20×	50 mM	2.5 mL
NaCl	4 M	40×	100 mM	1.25 mL
$MgCl_2$	1 M	250×	4 mM	200 μL
TCEP	0.1 M	500×	0.2 mM	100 μL
ddH_2O	-	-	-	45.95 mL
Total				**50 mL**

Click reaction solution				
Reagent	Stock concentration	Dilution	Final concentration	Volume
TBTA	5 mM	5×	100 μM	40 μL
$CuSO_4$	50 mM	5×	1 mM	40 μL
Biotin-N_3	5 mM	5×	100 μM	40 μL
TCEP	0.1 M	5×	2 mM	40 μL
PBS	10×	10×	1×	200 μL
ddH_2O	-	-	-	1640 μL
Total				**2 mL**

Strep-HRP solution				
Reagent	Stock concentration	Dilution	Final concentration	Volume
BSA	100 μg/μL	500×	200 ng/μL	20 μL
Strep-HRP	0.5 μg/μL	1,000×	0.05 ng/μL	1 μL
PBS	1×	-	-	9979 μL
Total				**10 mL**

STEP-BY-STEP METHOD DETAILS

96-well plate preparation

Timing: 1 h

Bind your His_6-tagged target protein onto a 96-well Ni-NTA plate for subsequent ADP-ribosylation.

CRITICAL: This assay was developed using His_6-tagged target proteins chelated to Ni-NTA plates (QIAGEN), but could likely be easily adapted to other protein-tag and plate pairs.

1. Prepare a 1 μM solution of the target protein in PBR.
2. Add 50 μL of the target protein solution to a Ni-NTA coated 96-well plate and incubate at RT for 1 h.

Note: All incubation times, temperatures, and protein concentrations should be optimized for each target protein. The conditions given throughout this protocol have been effective for all target proteins tested to date but are not necessarily the optimal conditions for each PARP.

Enzymatic reaction

Timing: 1.5 h

PARP enzymatic reaction with 6-alkyne-NAD^+ in the presence of small-molecule inhibitors.

3. Prepare a 4× solution of 6-alkyne-NAD^+ at 0.4 mM. The final concentration of 6-alkyne-NAD^+ is set at 100 μM to most accurately reflect the cellular concentration of NAD^+, but can be adjusted to increase or decrease PARP activity or reflect a low NAD^+ environment (Cambronne, et al. 2016).

Note: While 6-alkyne-NAD^+ was synthesized for this protocol, it is commercially available from Biolog Life Science Institute (Cat. No.: N 051-05).

4. Prepare 4× solutions of inhibitors from 800× DMSO stocks, including a DMSO control, by adding 1 μL of the DMSO stock to 199 μL PBR.
5. Prepare a 2× compound/6-alkyne-NAD^+ solution by combining equal volumes of each 4× compound dilution and 4× 6-alkyne-NAD^+ solution.
6. Prepare a 2× solution of the PARP at 20 nM.

CRITICAL: Some PARPs require an activating cofactor, i.e., damaged DNA for PARP1 and PARP2 and 5′-nicked-dumbbell RNA for PARP3. These cofactors should be incorporated into the 2× protein stock.

Note: Active PARPs show robust enzymatic activity at a final concentration of 10 nM, but we recommend measuring enzymatic activity at a range of concentrations before testing compounds to ensure consistency across different batches of protein.

Alternatives: If conducting an auto-ADP-ribosylation assay where the PARP is being adhered to the plate, prepare a 2× stock of cofactors (if necessary) in PBR here instead of 2× PARP mix.

7. Remove the target protein solution and wash plate 3× with 100 μL 1× PBST, 1× with 100 μL 1× PBS, and 1× with 100 μL PBR leaving each wash for ≥2 min at RT.

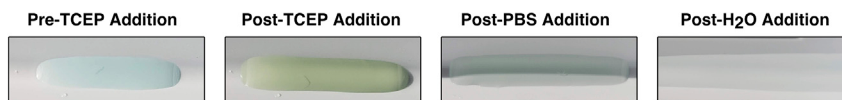

Figure 1. Expected color progression of click reaction solution

Pause point: The plate can be left with 1× PBS for up to 1 h at RT, and up to 3 h at 4°C at any wash step throughout the assay.

8. Add 25 μL of 2× PARP solution to the 96-well plate followed by 25 μL of 2× compound/6-alkyne-NAD+.

   Alternatives: If an active PARP was chelated to the 96-well plate to assess auto-modification then replace the 2× PARP solution with an equal volume of PBR, being sure to include any activating cofactors.

9. Incubate the plate at 30°C for 1 h.

Click reaction

Timing: 40 min

React biotin-N_3 with the alkyne handle on 6-alkyne-ADP-ribose moieties now appended to the target protein on the plate.

10. Remove the enzymatic reaction solution and wash the plate 3× with 100 μL 1× PBST and 1× with 100 μL 1× PBS, leaving each wash for ≥2 min at RT.
11. Add 50 μL click reaction solution (100 μM TBTA, 1 mM $CuSO_4$, 100 μM biotin-N_3, 2 mM TCEP, 1× PBS) to the 96-well plate.

   CRITICAL: The click reaction solution must be prepared within 10 min of use and the TCEP must be added from an aqueous stock solution that is no more than 2 h old and has been kept on ice. The order of addition of the click reaction solution is critical, and must be: 1. TBTA, 2. $CuSO_4$, 3. biotin-N_3, 4. TCEP, 5. 1× PBS, and 6. DI water to the desired volume. Do not proceed with this step unless the complete click reaction solution progresses through the color changes depicted in Figure 1. The solution should be light blue prior to TCEP addition, yellow-green post addition, and revert to a faint blue after PBS addition.

Troubleshooting: if the complete click reaction solution is not faintly blue it is most likely due to an error in order of addition, insufficient mixing after each addition, or insufficiently potent TCEP solution.

12. Incubate at 30°C for 30 min.

Block with 1% milk

Timing: 40 min

Block with 1% milk to prevent non-specific Strep-HRP binding in the following step.

13. Remove the click reaction solution and wash the plate 3× with 100 μL 1× PBST and 1× with 100 μL 1× PBS, leaving each wash for ≥2 min at RT.
14. Add 50 μL 1% milk in PBST to the 96-well plate.
15. Incubate at RT for 30 min.

Strep-HRP reaction

Timing: 25–40 min

Append Strep-HRP to biotin for subsequent chemiluminescent visualization.

16. Remove the milk block and wash the plate 3× with 100 μL 1× PBST and 1× with 100 μL 1× PBS, leaving each wash for ≥2 min at RT.
17. Add 50 μL Strep-HRP (300 ng/μL BSA, 0.5 ng/μL Strep-HRP, 1× PBS).
18. Incubate at RT for 15–30 min.

Chemiluminescent reaction and readout

Timing: 10 min

Visualize the extent of ADP-ribosylation using QuantaRed Enhanced Chemifluorescent HRP substrate kit (Thermo).

19. Remove the Strep-HRP and wash the plate 3× with 100 μL 1× PBST and 1× with 100 μL 1× PBS, leaving each wash for ≥2 min at RT.
20. Prepare the QuantaRed solution per the instructions in the kit.

CRITICAL: Prepare the QuantaRed solution immediately before adding it to the plate.

21. Add 100 μL QuantaRed solution to each well and incubate for 30 s to 1 min then quench with the QuantaRed stop solution.

Note: The solution will quickly turn pink in any wells where ADP-ribosylation has occurred and can over develop if left un-quenched for too long.

Pause point: We recommend measuring luminescence on a plate reader directly after quenching the QuantaRed reaction, but we have compared readings at 5 min, 1 h, and 18 h and as long as the plate is covered to prevent evaporation the results remained consistent.

EXPECTED OUTCOMES

The luminescence data collected should have a signal to noise ratio of 5- to 50-fold and there should not be significant variance between biological replicates. If you are assessing the IC50 of an active compound there should be a clear dose response curve as shown in Figure 2. See following section for a detailed description and example of how to analyze this data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Depending on the activity of the PARP being tested, the signal to noise (STN) can vary from 5- to 50-fold; in our experience, any results with a STN ratio less than 5 should be considered suspect. No issues of evaporation, signal loss in wells at the edge of the plate, or signal interference from adjacent wells have been observed.

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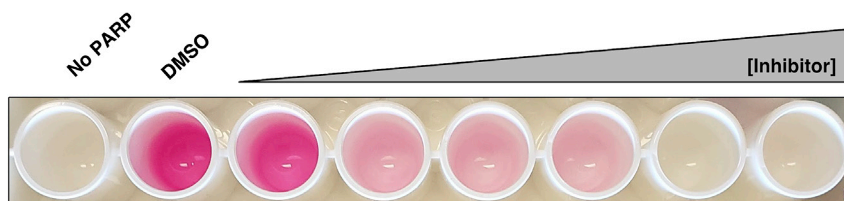

Figure 2. Example of inhibition curve after development with QuantaRed solution

Raw luminescence data can be processed in a simple spreadsheet or graphing program such as Prism GraphPad. To process raw data from the plate reader, use the following equation on each data point:

$$\frac{Lumi_{sample} - Lumi_{background}}{Lumi_{Max}}$$

where $Lumi_{sample}$ is the signal for an individual compound concentration (column 2, Table 1; Figure 3), $Lumi_{background}$ is the signal from a well with no PARP enzyme, and $Lumi_{Max}$ the signal from a well with DMSO (or other compound vehicle). We recommend normalizing data to the DMSO control treatment to account for different activity levels of each PARP when comparing relative efficacy of a compound across multiple PARP family members.

LIMITATIONS

It is important to note that *in vitro* screening does not always accurately reflect cellular activity of given PARP enzyme. The compounds tested in this assay have been taken forward to cellular studies were shown to have comparable selectivity and potency in cells, with the notable exception of compounds tested *in vitro* against a truncated PARP10 construct consisting of the catalytic domain compared with full-length PARP10 in cells (Morgan et al., 2018). This assay is most appropriate for determining relative selectivity and potency of small-molecule inhibitors of PARPs, and for assessing ADP-ribosylation levels by individual PARPs under various conditions of time, temperature, concentration, cofactors, etc.

TROUBLESHOOTING

Problem 1

Insufficiently active PARP is most commonly the result of low purity protein preps with inaccurately calculated concentrations, PARP enzyme that has degraded over time or due to mishandling, or forgetting to include activating cofactors.

Table 1. Example data and data processing from a dose response curve of a pan-PARP small-molecule inhibitor (Olaparib) with $PARP10_{cat}$ modifying SRPK2 bound to a Ni-NTA 96 well plate

Olaparib (μM)	Luminescence	$Lumi_{sample} - Lumi_{background}$	$(Lumi_{sample} - Lumi_{background})/Lumi_{Max}$
100	70,962,736	36,454,948	0.062569988
30	117,916,304	83,408,516	0.143159437
10	188,675,248	154,167,460	0.264607594
3	317,602,400	283,094,612	0.485893613
1	386,552,960	352,045,172	0.604237924
0.3	514,838,368	480,330,580	0.824422477
0.1	533,599,712	499,091,924	0.856623786
0.03	571,378,688	536,870,900	0.921466289
DMSO ($Luminescence_{Max}$)	617,134,528	582,626,740	1
No enzyme ($Luminescence_{Background}$)	34,507,788	0	0

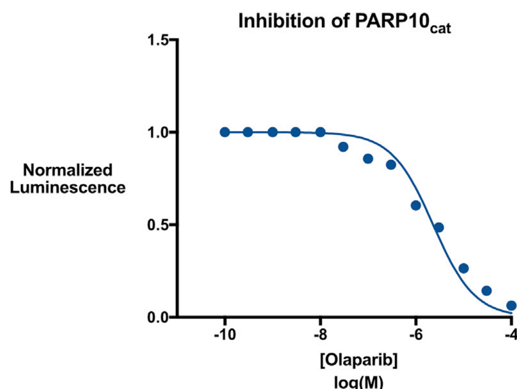

Figure 3. Inhibition curve of normalized data shown in Table 1

Potential solution

We recommend determining the purity and concentration of all proteins using western blots and in-gel concentration curves with a known standard rather than relying on Bradford reagent or similar. It is important to remember that PARP1 and PARP2 require damaged DNA for full activity and PARP3 requires an activating 5′-nick dumbbell RNA (Langelier et al., 2014). If a particular PARP still has low ADP-ribosylating activity we suggest testing activity with higher concentrations of 6-alkyne-NAD$^+$ (up to 400 μM). If you are testing auto-ADP-ribosylation with a PARP bound directly to the Ni-NTA plate low activity could also be the result of low binding of the enzyme. In this instance we recommend extending the chelation step to 2 h at RT.

Problem 2

The preparation of the click reaction solution, though simple, is arguably the most sensitive step in this assay. Improper order of addition, insufficient mixing, or insufficiently fresh and potent reducing agent can all lead to failure of this step, which cannot be detected until the end of the assay when the QuantaRed chemiluminescent reagent will produce little to no signal.

Potential solution

It is crucial that you use freshly dissolved TCEP and follow the order of addition given in the protocol, mixing well after each step. The best indicator that you have made the click reaction solution correctly is a pale blue tinge to the final solution as it is added to the plate. If there is no blue tinge, remake the click reaction solution with fresh TCEP, or a slightly increased concentration of TCEP (up to 3 mM), will usually solve the problem.

Problem 3

The QuantaRed chemiluminescent solution is extremely sensitive and fast acting, which makes it ideal for detecting low levels of ADP-ribosylation. However, with more active PARPs the solution can easily oversaturate if left too long without quenching.

Potential solution

When adding the QuantaRed chemiluminescent solution be sure to have the quenching solution ready to add within 1 min, before any wells become bright magenta.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Michael Cohen (cohenmic@ohsu.edu).

Materials availability

Plasmids for bacterial PARP expression are available upon request. 6-alkyne-NAD^+ is commercially available from Biolog Life Science Institute (Cat. No.: N 051-05).

Data and code availability

This study did not generate any code or datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.T.K. and M.S.C.; Methodology, I.T.K.; Validation, I.T.K. and D.S.; Writing – Original Draft, I.T.K.; Writing – Review & Editing, I.T.K., D.S., and M.S.C.; Funding Acquisition, M.S.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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