PARP1 Enzyme Activity Assay (Fluorometric)

Catalog No. 17-10149

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Poly(ADP-ribose) (PAR) is a polymer of ADP-ribose present in the nucleus in a free state or as a post-translational modification on glutamate residues on a number of substrates. Synthesis and degradation of PAR are highly regulated, and its presence mediates DNA repair, chromatin structure, cell cycle, and cell death (1). A family of poly(ADP-ribose) polymerases (PARPs) catalyzes the addition of ADP-ribose moieties onto substrate proteins via cleavage of NAD+ and liberation of nicotinamide. In humans the PARP family is comprised of 17 members, and the members vary significantly in structure and biological function. PARP1 plays a key role in DNA damage repair, particularly base excision repair. It is activated by DNA strand breaks to add PAR to itself and to histones, which results in the recruitment of PAR-binding DNA repair factors, such as XRCC1, to the site of damage. PARP2 overlaps functionally with PARP1 in DNA damage repair, but PARP2 plays a distinct role from PARP1 in development of spermatogonia, adipose tissue, and thymus tissue (3). Other members of the PARP family are less characterized, and some may function as mono(ADP-ribosyl) transferases rather than polymerases. Consequently, a new nomenclature has been proposed that refers to the common ADP-ribosyltransferase activity of the members (5).

A number of PARP1 inhibitors have been developed to block repair of chemotherapeutic agent-induced genotoxic damage, and thereby potentiate the cytotoxic effect of chemotherapeutics in cancer patients (2). Inhibition of PARP1 appears to be particularly efficacious in conjunction with chemotherapeutics on cancer cells harboring mutations in components of homologous recombination DNA repair pathways, primarily BRCA1, and BRCA2 (4). PARP inhibitors are also being evaluated in clinical trials.

The most commonly used assays for PARP activity employ biotinylated NAD+ as a substrate for PARP to incorporate biotinyl-ADP-ribose into the PAR chain on an immobilized histone substrate. The quantity of incorporated biotinyl-ADP-ribose is determined with streptavidin-HRP.

Disadvantages of this technique include:

1) Non-native NAD+ analogs as a substrate may yield different kinetics than unmodified NAD+.
2) A lack of flexibility in protein/peptide substrates
3) Multiple time-consuming wash steps.

To overcome these obstacles, EMD Millipore's PARP1 Enzyme Activity Assay utilizes a fluorescently-coupled enzyme assay for Nicotinamide degradation to permit the use of native NAD+ substrate and flexibility with peptide/protein acceptor substrates.

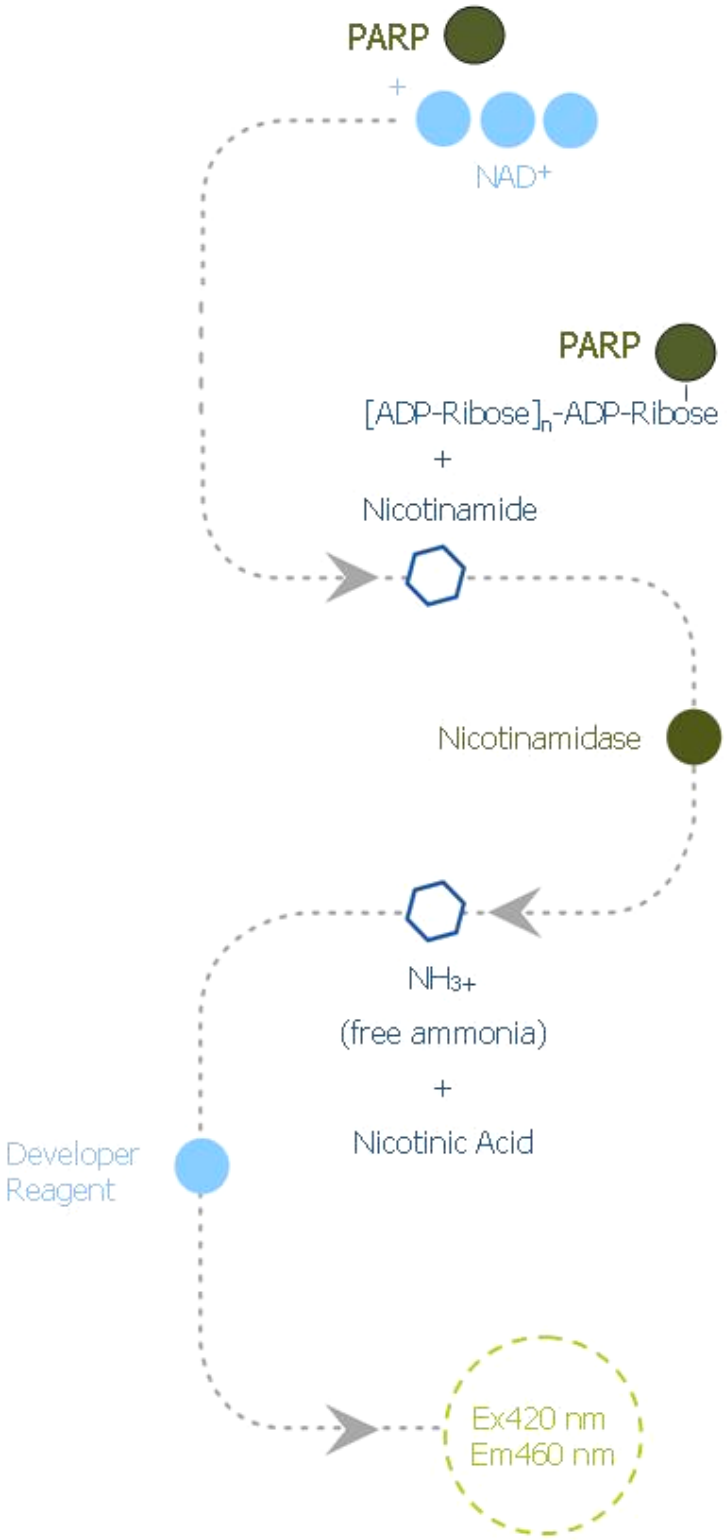
PARP1 Assay Kit Overview

The PARP1 Enzyme Assay (Cat. # 17-10149) is a flexible and reliable, homogeneous, no-wash assay for quantifying PARP1 & PARP2 activity. Based upon a novel patent-pending technology, this easy-to-perform assay employs nicotinamidase to measure nicotinamide generated upon cleavage of NAD^+ during PARP-mediated poly-ADP-ribosylation of a substrate (see **figure 1**), thereby providing a direct, positive signal assessment of the activity of PARP1 & PARP2.

To perform the assay, a PARP enzyme, β-NAD, activated DNA, test compound, and recombinant nicotinamidase enzyme are combined and incubated for 30 minutes. During the incubation, the activated DNA triggers PARP1 or PARP2 to produce poly(ADP-ribose) and nicotinamide. In a secondary reaction, the nicotinamidase enzyme converts the nicotinamide into nicotinic acid and NH_3^+ (free ammonia). To generate a signal for readout, a proprietary developer reagent is added and the signal is read using a fluorescent plate reader.

The robust performance of this assay makes it appropriate for measuring PARP1 and PARP2 activity as well as for screening of activators and inhibitors of PARP enzymes.

Figure 1. PARP Assay Flow Chart



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Kit Components

PARP1 Assay (Cat. # 17-10149) Store components at -80º C Plates and covers can be stored at room temperature (18-25º C)		
Component	**Component Part # ***	**Quantity**
Black 96 well plate	R5013	1 plate
PARP1 Assay Buffer	CS207771	4 mL
Plate Covers	3006581	2 plate covers
Activated DNA	CS207768	1 vial (100 µg at 10 mg/mL)
β-NAD	CS207788	1 vial (30 µL at 50 mM)
Nicotinamide (NAM):	CS207786	1 vial (10 µL at 10 mM)
Recombinant PARP1 Enzyme	CS207770	1 vial (2 µg at 0.2 µg/µL)
Recombinant Nicotinamidase Enzyme with 50% glycerol.	CS207784	1 vial (60 µg at 1 mg/mL)
3-ABA (PARP Inhibitor) in DMSO	CS207769	1 vial (300 µL at 100 mM)
Developer Reagent	CS207780	4 mL at 10 mM.

***Individual component part#s are not available of sale**

Additional Materials Required But Not Provided

- Multi-channel or repeating pipettes
- Plate shaker
- Pipettors and tips capable of accurately measuring 1-1000 µL
- Graduated serological pipettes
- 96-well microtiter plate reader with fluorescence readout.
- Graphing software for plotting data or graph paper for manual plotting of data
- Microcentrifuge tubes for standard and sample dilutions
- Mechanical vortex
- 1 liter container
- Distilled or deionized water
- Appropriate solvents (i.e. DMSO) to dissolve inhibitor compounds.

Calbiochem® Related Inhibitors

Catalog No.	Product	Catalog No.	Product
165350	PARP Inhibitor I, 3-ABA	493800	PARP Inhibitor VI, NU1025
407850	PARP Inhibitor II, INH_2BP	528820	PARP Inhibitor Set
300270	PARP Inhibitor III, DPQ	For a full list of PARP inhibitors, please visit www.emdchemicals.com and www.millipore.com.	
419800	PARP Inhibitor IV, IQD		
164585	PARP Inhibitor V, 4-ANI		

Storage and Stability

Maintain the unopened kit (Cat. # 17-10149) at -80ºC. Unused components should be stored at -80ºC. When stored properly this kit is stable for up to 4 months after date of receipt. Avoid repeated freeze/thaw cycles by aliquoting, if components are needed after first use.

Hazards and Precautions

- This kit is designed for research use only. It is not intended for use in humans or animals.
- All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- Wear gloves and use PPE (personal protective equipment) as appropriate.
- **Important:** Developer Reagent is light sensitive. Protect from exposure to bright light.

Important Technical Notes

- Please read the entire manual before using this kit for the first time. Important information on reagent preparation and handling as well as guidelines for customizing and optimizing the reaction for user-provided peptide substrates, enzymes, and test compounds are outlined below and in the appendix. It is important to review this information and carefully design your experiment before running your assay.
- The protocol provided has been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and inaccurate data.
- All reagents should be rapidly thawed just prior to use and kept on ice until ready to use.
- Promptly return unused reagents to appropriate storage condition.
- Do not mix or interchange reagents from various kit lots.
- **Note:** Vials containing less than 150 µL of material should be spun briefly in a microcentrifuge to settle the contents and ensure that no droplets are present on the cap.

Preparation of Reagents – Required before starting the assay

1. **3-ABA and Other Test compounds.** For the provided 3-ABA (PARP inhibitor) use PARP Assay Buffer to generate 10-fold serial dilutions that will yield final concentrations between 0 and 1 mM. For a greater than 75% inhibition of PARP1 for example, use >100 uM of 3-ABA. For other compounds, determine a suitable solvent to dissolve the test compounds to create a concentrated stock. For most compounds, DMSO is an appropriate solvent, and DMSO at concentrations up to 2% have no effect on assay performance (**Figure 3**, appendix). If other organic solvents are used, the user must determine the most appropriate solvent and effective concentrations that do not interfere with assay performance. After creating the stock solution, make further dilutions with PARP Assay Buffer.

2. **Activated DNA solution.** Briefly spin the vial to settle contents prior to opening. The reagent is provided as a 10 mg/mL concentrated stock. The final amount recommended in the standard protocol is 50 ng per reaction. Prepare a fresh working solution using the PARP Assay Buffer (part # CS2207771) to dilute the concentrated activated DNA solution provided with the kit. Keep the diluted solution on ice until ready to use.

   Example: For a final 50 ng in 5 µL, prepare a 10 ng/µL working solution by diluting the stock solution (part # CS207768) 1:1000 in PARP Assay Buffer. Vortex for 10 seconds at medium speed to mix well and keep on ice until use.

3. **Nicotinamide (NAM) standard.** Briefly spin vial to settle contents prior to opening. To create a standard curve for quantitative analysis of your assay, prepare a NAM 2-fold dilution series from 25 µM to 0 µM.
   i) To prepare the1st point, a 5X solution at 125 µL, dilute stock NAM (part # CS207786) 1:80 in PARP Assay Buffer (1 µL NAM stock solution in 79 µL assay buffer), and vortex for 10 seconds at medium speed to mix well.
   ii) To prepare the 2nd point, a 5X solution at 62.5 µM, make a 2-fold serial dilution from 1st point (i.e. add 40 µL of 1st point to 40 µL assay buffer, and mix well).
   iii) Repeat step 2 to create a 7 point standard curve as shown in **Table 1** below.
   iv) Use 40 µL of assay buffer to create an 8th point for a background control.

Table 1. NAM Standard Curve

Sample	5X solution concentration (µM)	Final Concentration (µM)
1	125	25
2	62.5	12.5
3	31.25	6.25
4	15.63	3.123
5	7.81	1.56
6	3.91	0.78
7	1.95	0.39
8	0	0

4. **Recombinant Nicotinamidase**. Briefly spin the vial to settle contents prior to opening. Just prior to starting the assay, dilute the 1 mg/mL Recombinant Nicotinamidase stock solution (part # CS207784) to 0.1 mg/mL with PARP Assay Buffer (part # CS207792). For 100 reactions, prepare 500 µL Nicotinamidase working solution by adding 50 µL stock Nicotinamidase to 450 µL assay buffer and vortexing for 10 seconds at medium speed to mix well. Keep on ice until ready to use.

5. **β-NAD solution.** Briefly spin the vial to settle contents prior to opening. Prepare a fresh dilution of the 50 mM stock β-NAD solution (part # CS207788) to 5X of the desired final concentration (0.5 mM for standard protocol) with PARP Assay Buffer (part # CS207792), and keep on ice until ready to use.

   Example: For a final concentration of 0.5 mM β-NAD, prepare a 2.5 mM (5X) working solution by diluting the β-NAD stock solution 1:20 in PARP Assay Buffer. Vortex for 10 seconds at medium speed to mix well, and keep on ice until ready to use.

6. **Recombinant PARP1 Enzyme.** Briefly spin the vial to settle contents prior to opening. Refer to the label of the provided Recombinant PARP1 Enzyme (part # CS207770) and determine the units per µL. Use PARP Assay buffer (part # CS207771) to dilute this enzyme to create a 5X stock (typically 10 ng/µL).

   Example: To use 50 ng of PARP1 per reaction, prepare a 10 ng/µL working solution by diluting the enzyme stock in PARP assay buffer. Vortex for 10 seconds at medium speed to mix well. Keep on ice until ready to use.

   Note: 2 µg of PARP1 enzyme is provided with this kit. This enzyme can be used as a positive control or as an enzyme for compound or substrate activity screening. The amount of enzyme provided allows the analysis of **40 samples** plus 24 standards and 2 negative controls, each containing 50 ng of enzyme per reaction. This assay is designed to work with PARP1 & PARP2.

7. **Developer reagent.** Warm the ready-to-use developer reagent (part # CS207780) at 37ºC for about 15 minutes prior to adding to the reaction samples. If a precipitate is present, continue to incubate and swirl the tube until reagent completely solubilizes. Once the reagent has been solublized it can remain at room temperature, protected from light, until ready for use.

Standard Assay Protocol

Important: Before starting this assay, please read and follow instructions in the preceding sections. The reagents provided in this kit are designed and optimized to run the standard assay protocol. This protocol is ideal for testing of inhibitors/activators of PARP1 and PARP2 activity. If alternate enzymes or substrates are to be used, reagent titration may be required. If using alternate sources of reagents, for optimal results please see page 13 "Guidelines for Assay Optimization".

I. Setup a NAM standard curve.

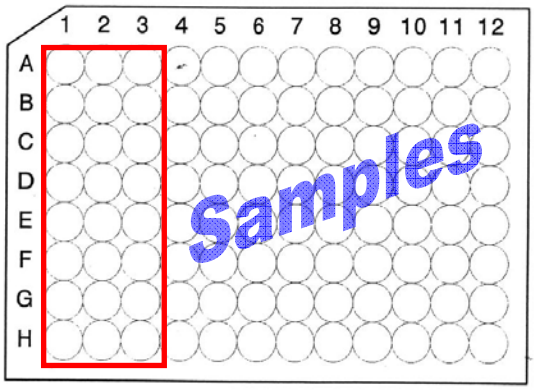
As shown in the figure below, set-up 3 columns of NAM standard (in triplicate) using the NAM serial dilutions prepared in the previous section. Cover unused wells with plate sealer.

1. Label 8 test tubes #1-8 and mark #8 as "0 dose". Prepare as described in "Reagents Preparations" section – see page 7, Step 3.
2. Dispense 5 µL/well of the standard solutions made above.

Standard Curve Layout:

NAM Standard *(µM)*

1) 25
2) 12.5
3) 6.25
4) 3.125
5) 1.56
6) 0.78
7) 0.391
8) 0



Example

II. Setup PARP1 activity samples. Compounds can be added to the PARP1 reactions in a dose-response curve to determine IC_{50} values.

3. Setup PARP1 reactions for controls, test sample, standards and buffer only background control as outlined in **Table 2**.

Table 2.

PARP Reaction	Negative	Positive	Inhibitor/ Compounds	NAM Standard	Buffer only
Inhibitor/Compounds	-	-	5 µL	-	-
Activated DNA (10 ng/µL)	5 µL	5 µL	5 µL	-	-
PARP1 (10 ng/µL)	-	5 µL	5 µL	-	-
β-NAD (2.5 mM)	5 µL	5 µL	5 µL	-	-
Nicotinamidase (0.1 µg/µL)	5 µL	5 µL	5 µL	5 µL	-
NAM standard as in table 1.	-	-	-	5 µL	-
PARP Assay Buffer	10 µL	5 µL	-	15 µL	25 µL

4. For reactions with test compounds, combine PARP enzyme and test compounds, and incubate for 10-15 minutes at room temperature. Then add the other reagents to the final reaction mix to obtain a total volume of 25 µL.

5. Gently tap the plate to mix the solutions, and cover with one plate sealer. Incubate for 30 minutes at 30-37°C on a plate shaker with gentle agitation (e.g. Labnet, Vortemp 56™ EVC temperature controlled plate shaker at 45 rpm).

6. At least 20 minutes prior to completion of the reaction in step 5, prepare the developer reagent as described in "Reagents Preparation" section page 8, step 7.

   Remember: Developer Reagent is light sensitive; protect from bright light.

7. Pipet 25 µL developer reagent to each well under dimmed light. Gently tap to mix the solutions, cover wells with plate sealer and wrap the 96-well plate in aluminum foil to protect from light. Incubate for 30 minutes at room temperature on a plate shaker with gentle agitation (e.g. Lab-line Instruments, Titer plate shaker at set at speed 2).

8. Read the fluorescence on a fluorometer with filters set to excitation ~420 nm +/- 20, and emission ~450 nm (+/- 20). A 0.1s or 1s read is generally recommended.

   Guideline Instrument Parameters:

   A) Molecular Devices Analyst® Plate Reader. For complete details please refer to parameters recommended in the instrument instruction manual:

Excitation	405 nm +/- 20
Emission	460 nm +/- 40
Integration time	50 µsec

   B) Perkin-Elmer VICTOR2™. For complete details please refer to parameters recommended in the instrument instruction manual:

Excitation	405 nm
Emission	460nm
Measurement time	1 sec
CW-lamp energy	10000

9. Data can be interpreted by GraphPad PRISM® nonlinear fit, sigmoidal dose-response (variable slope). Alternatively, similar data analysis software such as SigmaPlot®, Origen®, etc. can be used.

Appendix

Section I. Examples of Assay Results

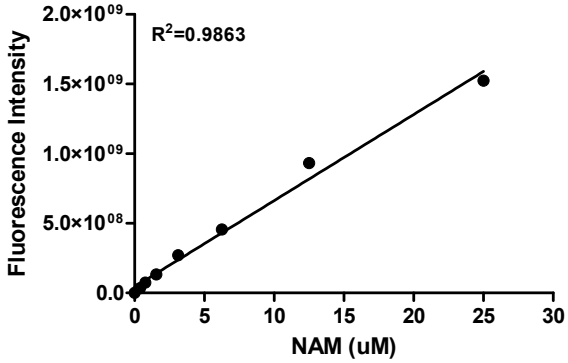


Figure 1. Standard curve of Nicotinamide (NAM).
Nicotinamide from 0-25 µM was incubated with 0.5 µg Nicotinamidase for 30 minutes. Fluorescent signal was determined after incubation with 10 mM developer reagent for 30 minutes at room temperature, and a linear curve fit was applied.

NOTE: Standard curve data presented above for reference use only. This data should not be used to interpret your assay results. A standard curve must be generated for each assay.

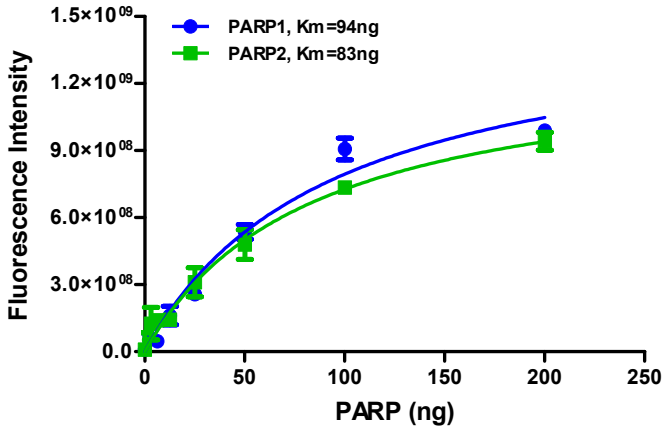


Figure 2. Determination of PARP K_m values.
PARP1 and 2 at a range of concentrations were incubated with 50 ng activated DNA and 0.5 mM β-NAD using the PARP assay. The K_m value was determined by non-linear curve fit of Michaelis-Menten. This value may be referenced when planning assays with test compounds.

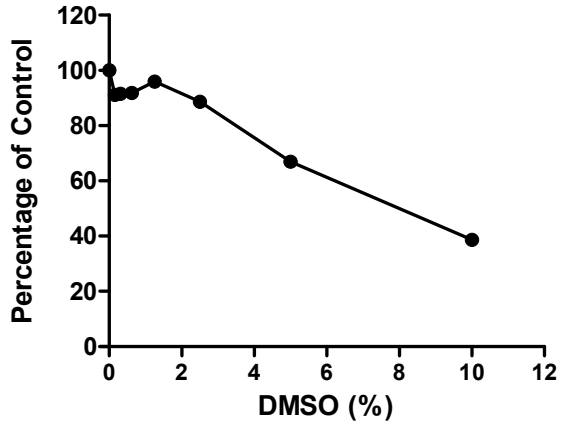


Figure 3. DMSO Effects on PARPs.
Recombinant PARP1 (50 ng) were incubated with 50 ng Activated DNA and 0.5 mM β-NAD using PARP assay. A range of DMSO (0-10%) was tested along with the PARP reactions. Up to 2% final DMSO was tolerated and thus represents the maximum that should be present during analysis of test compounds.

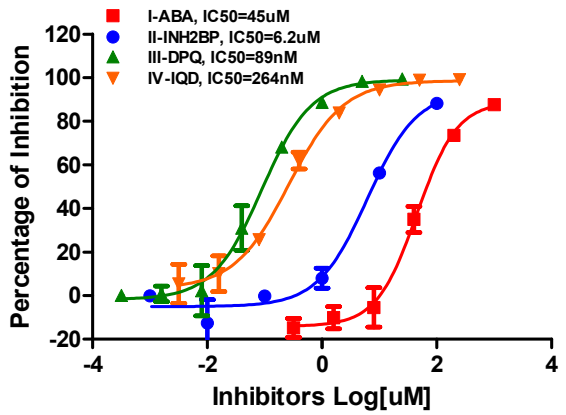


Figure 4. Determination of PARP1 Inhibitor IC_{50} values
Recombinant PARP1 (50 ng) was incubated with 50 ng Activated DNA, 0.5 mM β-NAD, and PARP inhibitors I (3-ABA, included) to IV (available separately, Calbiochem). Data was analyzed by non-linear curve fit with sigmoidal dose response with variable slope.

Guidelines for Assay Optimization Using Other Substrates, Test Compounds, and Enzymes

To ensure reliable results using user provided enzymes, substrates and test compounds, it is important to establish appropriate working concentrations for the different components. As appropriate for your assay, perform the different titrations and studies in order to establish specific assay parameter for your reagents.

- **PARP Titration**
  The optimal PARP concentration depends on whether inhibitors or activators are being tested. For inhibitors, a signal of 80~90% of the maximum is recommended, and for activators a signal ~30% of the maximum is recommended. To determine optimal PARP concentration, test a range of PARP concentrations (1.56-200 ng/reaction using a two fold serial dilutions) with a fixed concentration of the activated DNA (50 ng) and β-NAD (0.5 mM). Follow the Assay Protocol starting at step 3. Graph the fluorescence observed vs. units of enzyme added. An example of PARP titration data is shown in **figure 2** on page 10.

- **Kinetic Study**
  Using the optimal PARP concentration and conditions established above starting at step 3 of the assay protocol, perform a time-course experiment to establish the optimal reaction time. Continue the Assay Protocol as indicated, omitting the addition of any test compounds. At step 5, the reaction is stopped at different time points by the addition of the detection reagents (0, 5, 10, 15, 30, 60, 90 minutes). Read the fluorescence and graph the fluorescence reading for each time point. The optimal reaction time occurs when the fluorescent signal reaches around 80-90% of the maximum. This optimal reaction time may be used for the remaining experiments.

- **PARP Specific Substrate Titration**
  To determine the K_m for the same PARP specific substrate, test a range of substrate (8-200 µM using 5-fold serial dilutions) with the optimized PARP concentration and other reagents starting at step 3 of the Assay Protocol. Continue the Assay Protocol as indicated. Graph the fluorescence observed for each substrate concentration. Michaelis-Menten kinetics is applied to acquire the substrate K_m value. Typically, a substrate concentration with less than the K_m value that still gives sufficient fluorescence is chosen when setting up compound screening of PARPs.

- **β-NAD Titration**
  To determine of the K_m for β-NAD, test a range of β-NAD concentrations (8-1000 µM in a 5-fold serial dilutions) with the optimized PARP and acetylated peptide concentrations, and other reagents starting at step 3 of the Assay Protocol. Continue with the Assay Protocol as indicated. Graph the fluorescence observed for each concentration of β-NAD used. Michaelis-Menten kinetics are applied to acquire the K_m value. Typically, a β-NAD concentration with less than the K_m value that still gives sufficient fluorescence is chosen when setting up the compounds screening of PARPs.

- **Inhibitor compounds IC_{50}**
  The PARP activity is tested over a broad range of inhibitor concentrations to generate a dose response curve. The test is generally run using the previously determined optimal assay conditions. A proper β-NAD concentration (reference the K_m value determined at the β-NAD titration step) should be considered. To create the IC_{50} curve, graph the percent inhibition relative to the log of the inhibitor concentration used. An example of IC_{50} data is shown in **figure 4** on page 12.

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal in all wells	Missing components or key steps	Check to make sure all components were added in the appropriate steps and amounts.
	Recombinant PARP1 or Nicotinamidase is no longer active or has reduced activity	Make sure all components are stored at the recommended temperature and minimize the freeze/thaw cycle as manual recommends. Make aliquots of components when first thawed if planning more than one assay.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures Incorrect assay temperature	Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.
No detectable signal in test samples	Incomplete PARP1 enzyme reaction or missing key components.	Check the enzyme reaction setup procedure, and ensure that activated DNA, β-NAD and Nicotinamidase are added to the reaction buffer.

Low PARP1 activity signal during inhibitor screening	High DMSO concentration or PARP1 enzyme degraded.	Ensure that the final DMSO concentration in the enzyme reaction does not exceed 2%. Prepare a more concentrated compound stock in DMSO, then further dilute to the desired concentration with PARP assay buffer to minimize the DMSO effects.
High background signal	Improper activated DNA usage. Use of cell or tissue lysate	Test a range of activated DNA amount in the final reaction to ensure a proper usage. Activated DNA at high concentrations may cause non-specific high assay background. The assay is not recommended for use with cell or tissue lysates, as free ammonia in cell lysates interferes with the assay.

Cited References

1. Hakmé A *et al.* (2008) The expanding field of poly(ADP-ribosyl)ation reactions. *EMBO Rep.* 9: 1094-1100.
2. Megnin-Chanet F *et al.* (2010) Targeting poly(ADP-ribose) polymerase activity for cancer therapy. *Cell. Mol. Life Sci.* 67: 3649-3662.
3. Yélamos J *et al.* (2008) Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends Mol. Med.* 14: 169-178.
4. Rouleau M *et al.* (2010) PARP inhibition: PARP1 and beyond. *Nat. Rev. Cancer* 10: 293-301.
5. Hottiger MO *et al.* (2010) Toward a unified nomenclature for mammalian ADP-ribosyltransferases. *Trends Biochem. Sci.* 35: 208-219.

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