TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Chemiluminescence Homogeneous PARG Inhibition Assay Kit

96 Tests

Cat# 4691-096-K

HT Chemiluminescence Screening Assay for PARG Inhibitors

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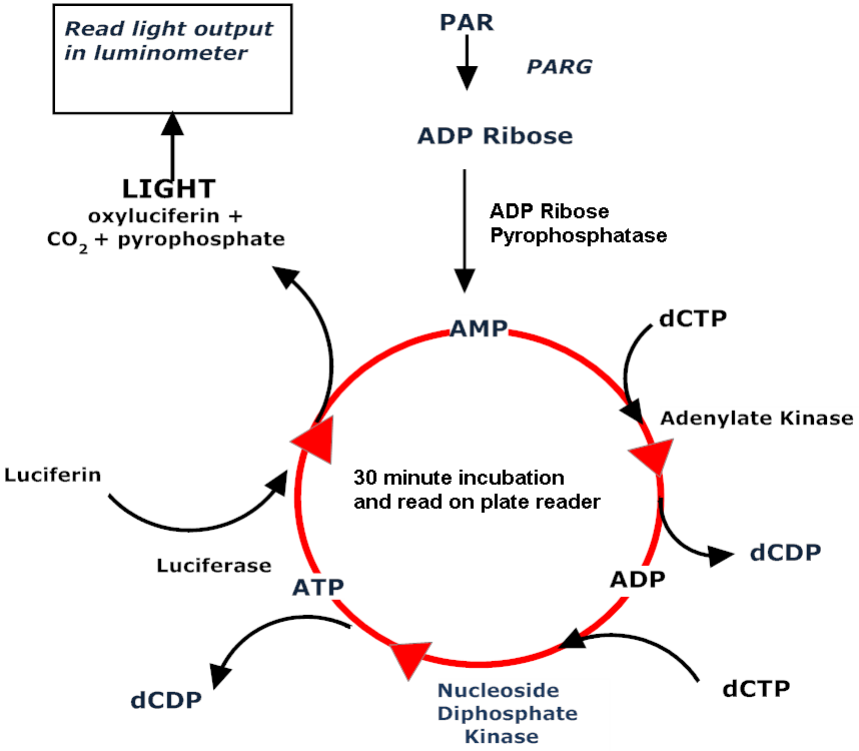
Table of Contents

I. Introduction

Poly(ADP-ribose) glycohydrolase (PARG) is a DNA repair protein which regulates the length of poly(ADP-ribose) (PAR) polymers synthesized by poly(ADP-ribose) polymerase (PARP) during the repair of DNA single-strand breaks[1]. PAR polymers are highly negatively charged molecules derived from NAD, whose acceptor proteins include histones, DNA polymerases, DNA ligases, p53, Fos, and PARP itself[2]. Increased polymer length, which is regulated by PARG's exo- and endoglycohydrolase activities, may have a negative effect on the activity of PARP[3]. Therefore, inhibition of PARG may indirectly reduce PARP activity. Lower PARP activity will reduce NAD depletion rates in cells[4]. It has also been shown that PAR metabolites hydrolyzed by PARG may stimulate PARP-mediated apoptosis[5]. Inhibition of PARG has led to positive outcomes in diseases such as ischemia and reperfusion injury, and cancer[6]. In addition, knockdown studies have shown that PARG inhibitors may sensitize cells to DNA damaging agents[7]. These studies have identified PARG as a potential therapeutic target.

Figure 1: PARG Inhibition Assay



Trevigen's Homogeneous PARG Inhibition Assay is a highly sensitive high-throughput chemiluminescence screening assay for PARG inhibitors *in vitro* (figure 1). This end point assay is performed in one step for rapid, easy use.

PARG hydrolyzes PAR polymer to ADP-Ribose (ADPR), which is enzymatically converted to AMP by ADP-Ribose pyrophosphatase. AMP enters a cycling reaction converting AMP into ATP. Luciferase cleaves ATP into AMP in the presence of the substrate luciferin, generating a quantifiable light signal. AMP re-enters the cycling reaction assay to sustain the light signal for consistent results. Inhibitors are identified by a decrease in chemiluminescence when PARG activity is inhibited. An example is provided to illustrate determination of IC_{50} values with the known PARG inhibitor DEA (please see Section VII. Data Interpretation).

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the **HT Chemiluminescence Homogeneous PARG Inhibition Assay Kit** may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.
3. **D-Luciferin is light sensitive.**
4. **Cycling reaction is sensitive to reducing agents. Do not add DTT to reactions.**
5. **Cycling reaction is inhibited by high salt concentrations (above 1 mM). Do not add salt to reactions.**
6. **Inhibitors that are ADPR analogs may act as a substrate for ADP-Ribose pyrophosphatase. Recommend testing potential inhibitors in absence of PARG (Inhibitor Control).**

III. Materials Supplied

Catalog Number	Component	Amount Provided	Storage Temperature
4691-096-01	PARG Enzyme, 100 mU/µl	50 µl	-80˚C
4691-096-02	10x PARG Dilution Buffer	1 ml	-20˚C
4691-096-03	D-Luciferin	900 µl	-80˚C
4691-096-04	Luciferase Cycling Enzymes	600 µl	-80˚C
4691-096-05	10x dCTP	900 µl	-20˚C
4691-096-06	PARG Substrate	600 µl	-80˚C
4691-096-07	Cycling Buffer	6 ml	RT
4691-096-P	96 Well White Round Bottom Plate	1 plate	RT

IV. Materials/Equipment Required But Not Supplied

Reagents/Disposables:

1. PARG inhibitors to be tested
2. 1 - 200 µl and 100-1000 µl pipette tips
3. Serological Pipettes

Equipment:

1. Micropipettors
2. Multichannel pipettor 10 µl - 100 µl
3. 96-well chemiluminescent plate reader or luminometer
4. Ice bucket
5. Microcentrifuge

6. 15 ml screw cap centrifuge tubes
7. 1.7 ml Eppendorf tubes
8. 25 ml solution reservoirs
9. Incubator set at 25 ^{o}C
10. Pipet Aid

V. Reagent Preparation

The preparation of reagents is based upon performance of a standard curve and the testing of inhibitors as discussed in Section VI. Assay Design.

1. PARG Standard Curve

PARG Standards are prepared using 100 mU/µl PARG and 1X PARG Dilution Buffer as described in Table 1. Standards must be discarded after use.

Prepare 1X PARG Dilution Buffer by adding 300 µl 10X PARG Dilution Buffer to 2700 µl dH_20.

All standards are 4X higher than their final concentrations

Table 1: Preparation of 4X PARG Standards (serial dilutions)

4X PARG Standards		PARG Stock	1X PARG Dilution Buffer
A	4 mU/µl	22 µl (100 mU/µl)	528 µl
B*	2 mU/µl	450 µl of A	450 µl
C	0.4 mU/µl	25 µl of B	100 µl
D	0.1 mU/µl	30 µl of C	90 µl
E	0.04 mU/µl	40 µl of D	60 µl
F	0.01 mU/µl	25 µl of E	75 µl
G	0.002 mU/µl	20 µl of F	80 µl

***For PARG standard curve and inhibitor wells. Dilution size is sufficient for addition to 32 inhibitor wells.**

2. Inhibitor Dilutions

It is preferred to dilute PARG inhibitors in 1X PARG Dilution Buffer to a 50X concentration. PARG inhibitors are added in a 2 µl volume and duplicate reactions are recommended.

Dilution ranges for PARG inhibitors will vary. For example, significant inhibition of PARG activity is achieved with 20 µM DEA.

If PARG inhibitors are diluted in DMSO, then DMSO should also be added to wells containing PARG standards as described in Section VI. Assay Design. Assay tolerance to DMSO is 1%.

Note: When determining IC_{50} values, an Inhibitor Control is recommended. The Inhibitor Control is prepared by adding 2 µl of the high inhibitor concentration in the absence of PARG. This control provides the minimal signal in the presence of inhibitor.

3. Cycling Mix (prepare directly before use).

The Cycling Mix is light sensitive and should be prepared just prior to use, and equilibrated to room temperature.

Note: ***Order of addition:*** When preparing Cycling Mix, add in the order listed into a 15 ml conical tube. **Add PARG Substrate and 10X dCTP *immediately* prior to use**.

Prepare Cycling Mix as follows (sufficient for 48 wells):

	1 well	x56
Cycling Buffer	50 µl	2800 µl
Cycling Enzymes	5 µl	280 µl
D-Luciferin	7.5 µl	420 µl
PARG Substrate	5 µl	280 µl
10X dCTP	7.5 µl	420 µl
Total volume:	75.0 µl	4.2 ml

VI. Assay Design for PARG Inhibitor Testing

It is recommended that all samples be run in duplicate along with a standard curve for each experiment. Reactions are performed in a 96 well white round bottom plate in a final reaction volume of 100 µl (102 µl in inhibitor wells). A chemiluminescence plate reader is used for detection following a reaction time of 30 minutes. The plate must be read immediately after 30 minutes for consistent results.

A 96 well white round bottom plate is provided (4691-096-P). A suggested assay plate setup for the screening of 16 inhibitors (columns 3 to 6) is shown in Table 2. In the screening of potential PARG inhibitors (Table 2), a Background Control (1H and 2H) and PARG Standards (1A to 1G and 2A to 2G) are recommended.

Please see Inhibitor Control note (previous page).

Table 2: Assay Plate Setup for Screening 16 Inhibitors (48 wells)

	1	2	3	4	5	6
	PARG Standards	**PARG Standards**	**Inhibitors Tested**			
A	100 mU	100 mU	I1	I1	I9	I9
B	50 mU	50 mU	I2	I2	I10	I10
C	10 mU	10 mU	I3	I3	I11	I11
D	2.5 mU	2.5 mU	I4	I4	I12	I12
E	1 mU	1 mU	I5	I5	I13	I13
F	0.25 mU	0.25 mU	I6	I6	I14	I14
G	0.05 mU	0.05 mU	I7	I7	I15	I15
H	0 mU (1x PARG Dil.Buffer)	0 mU (1x PARG Dil.Buffer)	I8	I8	I16	I16

Table 3 lists four types of reactions and the components present during the reaction:

Table 3: Reaction Type and Components Present

Reaction Type	PARG	Inhibitor	PAR Cycling Mix
1. Background Control			X
2. PARG Standards	X		X
3. Inhibitor Tests*	X	X	X
4. Inhibitor Controls**		X	X

***Inhibitors with a positive result must be investigated further to rule out the possibility of inhibiting the cycling enzymes.**

****Recommended for hydrolysable ADP-Ribose analogs (e.g. ADP-HPD).**

The final reaction volume in each well is 100 µL (102 µL in inhibitor wells):

	Volume	Order of Addition
PARG or 1X PARG Dilution Buffer	25 µL	1
50X Inhibitor (inhibitor wells only)	(2 µL)	2
Cycling Mix	75 µL	3
Total volume	100 µL (102 µL)	

A. One-Step Reaction for Screening Inhibitors

1. Aliquot 25 µl of appropriate PARG Standards into their respective wells for the Standard Curve (Rows A-G, Columns 1 and 2). Aliquot 25 µl of 1x PARG Dilution Buffer for Background Control wells (Row H, Columns 1 and 2).

2. Aliquot 25 µl of 2 mU/µl PARG into wells reserved for Inhibitor Tests.

3. Aliquot 2 µl of 50X Inhibitor into designated wells (Columns 3-6).

4. **Add PARG Substrate and 10X dCTP to Cycling Mix (see Section V3).** Immediately, add 75 µl of the Cycling Mix to each well using a multichannel pipette and mix by gently pipetting up and down.

5. Cover with plate lid and incubate at 25˚C for 30 minutes in the dark.

6. Chemiluminescence is measured using a luminescence plate reader. If necessary, perform a gain adjustment for the entire plate for maximum sensitivity and dynamic range. Maximal readings are expected from wells 1A and 2A and minimal signal from wells 1H and 2H.

VII. Data Interpretation

A. Screening

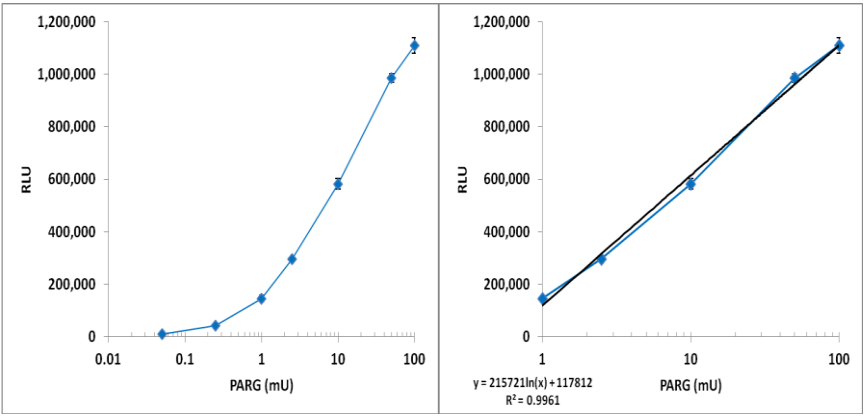
1. In EXCEL, calculate the average chemiluminescence value of all duplicates and subtract the average Background Control (average value of wells H1 and H2) to obtain relative light unit (RLU) values.

2. Use “XY Scatter Plot” and chart to illustrate data (Figure 2).
a. In Figure 2A, the PARG Standards (mUnits) are on the X axis and their respective RLU values on the Y axis. (A logarithmic scale is used on the X axis.) Figure 2B illustrates the linear portion of the standard curve (1 mU – 100 mU PARG). Note that inhibitor wells use 500 mU PARG to detect minimal changes in PARG activity.

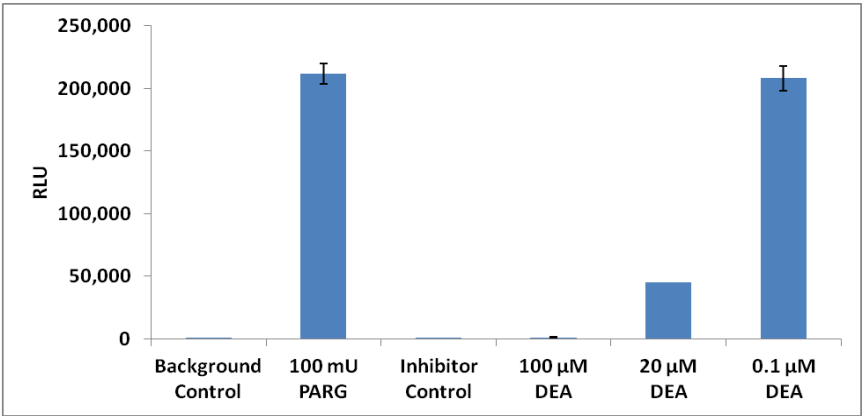
b. In Figure 2C, the chart plot illustrates PARG inhibition with DEA. Maximum signal is observed with ~50 mU PARG (from std. curve) and increasing concentrations of DEA show a decrease in signal. The Inhibitor Control demonstrates DEA is not a substrate for ADP-Ribose pyrophosphatase.

Figure 2. Sample Data

A. PARG Standard Curve **B. 1 mU – 100 mU PARG**



C. PARG Inhibitor: DEA



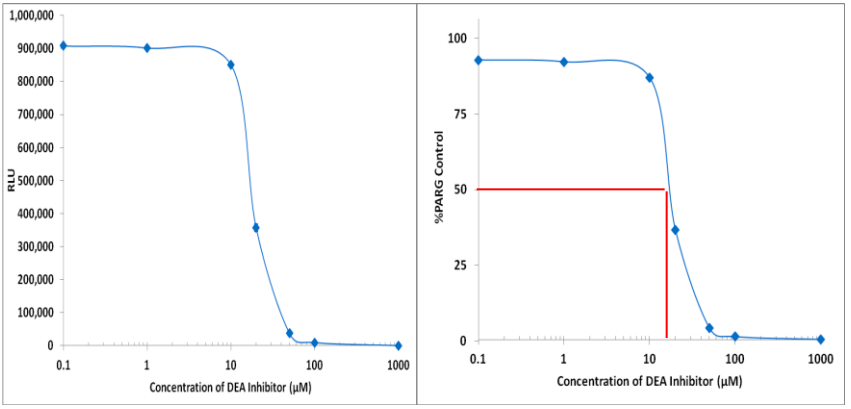
B. Calculation of Relative IC_{50} Values

Once potential PARG inhibitors are identified, IC_{50} values can be calculated.

1. In EXCEL, calculate the average luminescence value of all duplicates for each concentration of inhibitor and subtract the average Background Control (average value of wells H1 and H2) to obtain RLU values.

Figure 3. Inhibition of PARG by DEA

A. RLU Values **B. %PARG Control**



2. Use XY Scatter Plot to illustrate Inhibitor concentration versus RLU. In Figure 3A, the DEA inhibitor concentrations are on the X axis and their respective RLU values on the Y axis. PARG inhibition results in a decrease in signal. A logarithmic scale is used on the X axis.

3. In EXCEL, calculate the %PARG Control for each inhibitor concentration. %PARG Control is calculated by dividing the RLU values for a given inhibitor concentration by maximum signal (50 mU PARG from standard curve) and multiplying by 100.

4. Use "XY Scatter Plot" to illustrate inhibitor concentration versus %PARG Control. In Figure 3B, the DEA inhibitor concentrations are on the X axis and their respective %PARG Control values are on the Y axis. (A logarithmic scale is used on the X axis.)

5. IC_{50} values are estimated from the %PARG Control curve at 50% inhibition. The IC_{50} value for DEA, shown in Figure 3B, is 15 µM. The published IC_{50} value is 7 µM[8].

VIII. References

1. Brochu, G., G.M. Shah, and G.G. Poirier, *Purification of poly(ADP-ribose) glycohydrolase and detection of its isoforms by a zymogram following one- or twodimensional electrophoresis.* Anal Biochem, 1994. **218**(2): p. 265-72.

2. Gagné JP, et al., *Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes.* Nucleic Acids Res, 2008. **36**(22): p. 6959-76.

3. Brochu, G., et al., *Mode of action of poly(ADP-ribose) glycohydrolase.* Biochim BiophysActa, 1994. **1219**(2): p. 342-50.

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5. Andrabi, S.A., et al., *Poly(ADP-ribose) (PAR) polymer is a death signal.* Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18308-13.

6. Min, W., et al., *Deletion of the nuclear isoform of poly(ADP-ribose) glycohydrolase (PARG) reveals its function in DNA repair, genomic stability and tumorigenesis.* Carcinogenesis, 2010. **31**(12): p. 2058-65.
7. Cortes, U., et al., *Depletion of the 110-kilodalton isoform of poly(ADP-ribose) glycohydrolase increases sensitivity to genotoxic and endotoxic stress in mice.* Mol Cell Biol, 2004. **24**(16): p. 7163-78.
8. Blenn, C., P. Wyrsch, and F.R. Althaus, *The ups and downs of tannins as inhibitors of poly(ADP-ribose)glycohydrolase.* Molecules, 2011. **16**(2): p. 1854-77.

IX. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No luminescence in wells with PARG Standards	Low activity of Cycling Mix	Mix Cycling Enzymes gently before adding to Cycling Mix
	No PARG Enzyme in wells	Add defined amounts of PARG enzyme to wells
High luminescence in all wells (including background control)	Reducing agent added	Do not add reducing agents to the reaction
	PARG Substrate and dCTP not added *immediately* before use	Add substrate and dCTP to Cycling Mix after plate set-up
High luminescence in Inhibitor Controls	Inhibitor is substrate for ADP-Ribose Pyrophosphatase	Test inhibitor using HT PARG Assay Kit (cat# 4682-096-K or 4683-096-K)
	Reducing agent added	Do not add reducing agents to the reaction
Poor sensitivity in PARG Standards	Check plate reader settings	Perform gain adjustment for entire plate to optimize the signal amplification
	Loss of PARG activity	Improper storage of enzyme. Avoid freeze-thaws
	Salt added to reactions	Do not add salt to reactions
	D-Luciferin is light sensitive	Minimize exposure to light
Inconsistent signal between runs	Continuous PARG reaction	Read plate immediately after 30 minutes

X. Related Products Available From Trevigen

Kits:

Catalog #	Description	Size
4682-096-K	HT Chemiluminescent PARG Assay Kit	96 tests
4683-096-K	HT Colorimetric PARG Assay Kit	96 tests
4520-096-K	HT PARP *In Vivo* Pharmacodynamic Assay II	96 tests
4690-096-K	HT F Homogeneous PARP Inhibition Assay Kit	96 tests
4700-096-K	Tankyrase 1 Colorimetric Activity Assay	96 tests
4701-096-K	Tankyrase 1 Chemiluminescent Activity Assay	96 tests
4676-096-K	HT Universal Chemiluminescent PARP Assay Kit/w Histone Coated Strip Wells	96 tests
4677-096-K	HT Universal Colorimetric PARP Assay Kit/w Histone Coated Strip Wells	96 tests
4684-096-K	HT Colorimetric PARP/Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP/Apoptosis Assay	96 tests

Antibodies:

Catalog #	Description	Size
4335-AMC-050	PAR Monoclonal Antibody Affinity Purified	50 µl
4335-MC-100	Anti-PAR Monoclonal Antibody	100 µl
4336-APC-050	PAR Polyclonal Antibody Affinity Purified	50 µl
4336-BPC-100	Anti-PAR Polyclonal Antibody (rabbit)	100 µl
4338-MC-50	Anti-PARP Monoclonal Antibody (clone C2-10)	50 µg
2305-PC-100	Anti-Cleaved Caspase 3 Polyclonal	100 µl

Poly(ADP)-ribose:

Catalog #	Description	Size
4336-100-01	Poly(ADP) Ribose (PAR) Polymer	100 µl

Enzymes:

Catalog #	Description	Size
4668-100-01	PARP HSA (High Specific Activity)	1000 U
4680-096-01	PARG	100 µl
4700-02K-EB	Tankyrase 1 Enzyme and Buffer	20 U

PARG Inhibitor:

Catalog #	Description	Size
4680-096-03	100 mM DEA	200 µl

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