TREVIGEN® Instructions

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interchim

HT F Homogeneous PARP Inhibition Assay Kit

96 Tests

Cat# 4690-096-K

HT Fluorescent Screening Assay for PARP Inhibitors

HT F Homogeneous PARP Inhibition Assay Kit

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Table of Contents

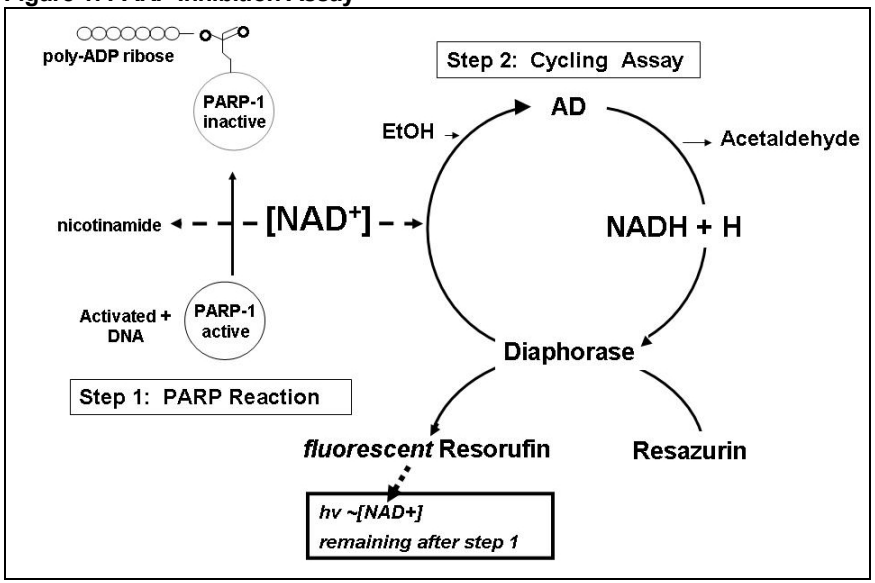
I. Introduction

Poly (ADP-ribose) polymerase 1 (PARP-1) plays an active role in various DNA repair processes by binding to DNA single-strand breaks and catalyzing the formation of polymers of ADP-ribose (PAR) onto itself and other proteins, resulting in NAD^+ depletion.[1-3] PARP inhibitors are potential chemotherapeutic agents for cancer treatment since they sensitize cells to DNA damaging agents, and recently, these inhibitors have been shown to have a high potential therapeutic index for cells homozygous mutant for the BRCA1 and BRCA2 genotypes.[4-6]

Trevigen's **Homogeneous PARP Inhibition Assay** is a highly sensitive fluorescent screening assay for the rapid identification of PARP-1 inhibitors in an *in vitro* system. This one hour endpoint assay is performed in two successive steps requiring only the consecutive addition of reaction components. A PARP reaction is first performed followed by a detection step (Figure 1). Inhibitors are identified by an increase in fluorescent signal when PARP mediated NAD^+ depletion is inhibited. The level of NAD^+ is coupled to a cycling assay involving alcohol dehydrogenase and diaphorase. Each time NAD^+ cycles through these coupled reactions, a molecule of highly fluorescent resorufin is generated. Alcohol dehydrogenase (AD) reduces NAD^+ to NADH, while the diaphorase cycles NADH back to NAD^+ with the generation of a highly fluorescent resorufin molecule (from the non-fluorescent substrate, resazurin). The number of cycles can be controlled by the time of incubation to adjust assay sensitivity, as needed, and the reaction is terminated by the addition of a stop solution. In addition, the assay can be used to determine relative IC_{50} values for PARP inhibitors and is capable of detecting as little as 10% inhibition of PARP-1 activity.

The Homogeneous PARP Inhibition Assay is designed for the screening of PARP inhibitors. Once potential inhibitors are identified, results can be confirmed using Trevigen's HT Universal PARP Assay kits (cat# 4676-096-K or 4677-096-K).

Figure 1: PARP Inhibition Assay



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 Homogeneous PARP Inhibition Assay 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. 10X Resazurin is light sensitive and 10X Cycling Enzymes are sensitive to oxidation.
4. Cycling reaction is sensitive to reducing agents. **Do not add DTT to reactions**.

III. Materials Supplied

Catalog #	Component	Quantity	Storage
4690-096-01	10X Cycling Enzymes (50% glycerol)	600 µl	-20 °C
4690-096-08	2 µM NAD	3.5 ml	-20 °C
4690-096-03	PARP I Enzyme, 200 ng/µl	100 µl	-20 °C
4690-096-04	Activated DNA, 200 ng/µl	600 µl	-20 °C
4690-096-05	10X Resazurin	600 µl	RT
4690-096-06	10X Buffer (1M Tris-HCl, pH 8)	3.5 ml	RT
4690-096-07	Stop Solution	6 ml	RT
4690-096-P	96-well Black Microplate	1 plate	RT

IV. Materials/Equipment Required But Not Supplied

1. PARP inhibitors to be tested.
2. Sterile distilled water (dH_2O)
3. 2 µl, 2 – 200 µl and 100-1000 µl pipettes and pipette tips
4. Multichannel pipettor for 25 µl and 50 µl volumes
5. 1.5 ml microtubes
6. 96-well fluorescent plate reader (544 nm excitation/ 590 nm emission filters) with gain adjustment
7. 95% ethanol (Reagent grade or higher)

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. 2X NAD Standards

2X NAD Standards are prepared using 2 µM NAD and 1X Buffer as described in Table 1. Standards maybe stored at -20°C for later use. Prepare 1X Buffer by adding 200 µl 10X Buffer to 1800 µl dH_20.

Table 1: Preparation of 2X NAD Standards

2X NAD Standard	Vol of 2 µM stock	Vol of 1X Buffer
2 µM	3.5 ml provided	X
1000 nM	125 µl	125 µl
500 nM	65 µl	195 µl
200 nM	25 µl	225 µl
100 nM	13 µl	247 µl
20 nM	3 µl	297 µl
0 nM	X	250 µl

2. Inhibitor Dilutions

It is preferred to dilute PARP inhibitors in 1X Buffer to 50X concentration. PARP inhibitors are added in a 1 microliter volume and duplicate reactions are recommended.

Dilution ranges for PARP inhibitors will vary. For example, significant inhibition of PARP activity is achieved with 100 nM PJ34.

If PARP inhibitors are diluted in DMSO, then DMSO should also be added to wells containing NAD standards as described in Section VI. Assay Design. DMSO concentrations of 1% will show slight inhibition of cycling assay.

To determine percent inhibition, an inhibitor control (see Table 4) should be run. The control is prepared by adding 1 µl of the high inhibitor concentration to a PARP minus reaction. This control provides the maximum NAD signal in the presence of the inhibitor.

Note: ~1.5 ml 10X Buffer is needed for preparation of NAD Standards, PARP Mix and Cycling Mix.

3. PARP Plus/Minus Mix (prepare directly before use).

PARP Minus Mix is dispensed using single channel pipette and PARP Plus Mix using multi channel pipette from Reagent Plate (Section VI. Assay Design and Table 3).

Make PARP Mix as follows (sufficient to aliquot 48 wells):

	Minus	x15	**Plus**	x44
10X Buffer	5 µl	75 µl	5 µl	220 µl
Activated DNA	5 µl	75 µl	5 µl	220 µl
dH_2O	15 µl	225 µl	14 µl	616 µl
PARP I Enzyme			1 µl	44 µl
Total volume of PARP Mix:	25 µl	375 µl	25 µl	1100 µl

4. 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. Sufficient reagents are provided if Cycling Mix is dispensed using a multichannel pipette from a Reagent Plate (Section VI. Assay Design and Table 3).

Order of addition: When preparing Cycling Mix, combine and mix dH_20, 10X Buffer and 95% EtOH first, **before** adding 10X Resazurin and 10X Cycling Enzymes, respectively.

Make Cycling Mix as follows (sufficient to aliquot 48 wells):

	1 well	x60
dH_2O	33.9 µl	2034 µl
10X Buffer	5.0 µl	300 µl
Reagent EtOH (95%)	1.1 µl	66 µl
10X Resazurin	5.0 µl	300 µl
10X Cycling Enzymes	5.0 µl	300 µl
Total volume:	50.0 µl	3.0 ml

5. Stop Solution

Stop Solution is ready for use. Sufficient reagent is provided if Stop Solution is dispensed using a multichannel pipette from a Reagent Plate (Section VI. Assay Design and Table 3). Do not refrigerate or freeze stop solution.

VI. Assay Design for PARP Inhibitor Testing

It is recommended that all reactions be performed in duplicate and a standard curve generated with each experiment. Reactions are assembled in a 96 well fluorescent plate to accommodate a final assay volume of 150 µl/well, and detection using a fluorescent plate reader. A 30 minute PARP reaction is performed followed by a cycling reaction to generate a fluorescent signal indicative of the level of PARP inhibition. The cycling reaction can be monitored in real-time to assure linearity of standard curve or at endpoint through the addition of a Stop Solution.

A black assay plate (4690-096-P) is provided. The use of separate reagent plate is recommended (example: non-treated Nunc U96 MicroWell™ Plate). A suggested assay plate setup for the screening of 16 inhibitors (columns 3 to 6) is shown in Table 2. Table 3 lists suggested volumes for a Reagent Plate for addition of reaction components to the assay plate using a multichannel pipette. In the initial screen of potential PARP inhibitors (Table 2), a background control (1A and 2A), standards (1B to 1G and 2B to 2G), and PARP control (1H and 2H) are recommended.

Once inhibitors are identified, titrations can be performed to determine the relative IC_{50} values. When determining IC_{50} values, an inhibitor control is performed to assure no inhibition of the cycling reaction. An inhibitor control is required for each inhibitor titration.

Table 4 lists the five types of reactions and the components present during the reaction.

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

	1	2	3	4	5	6
	(Standards)	(Standards)	(Inhibitors)			
A	0 nM NAD	0 nM NAD	I1	I1	I9	I9
B	10 nM NAD	10 nM NAD	I2	I2	I10	I10
C	50 nM NAD	50 nM NAD	I3	I3	I11	I11
D	100 nM NAD	100 nM NAD	I4	I4	I12	I12
E	250 nM NAD	250 nM NAD	I5	I5	I13	I13
F	500 nM NAD	500 nM NAD	I6	I6	I14	I14
G	1 µM NAD	1 µM NAD	I7	I7	I15	I15
H	1 µM NAD/ PARP	1 µM NAD/ PARP	I8	I8	I16	I16

Table 3: Reagent Plate for Screening 16 Inhibitors (48 wells):

	2 µM NAD	PARP Plus Mix	Cycling Mix	Cycling Mix	Stop Solution	Stop Solution
A	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
B	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
C	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
D	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
E	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
F	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
G	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl
H	125 µl	125 µl	175 µl	175 µl	175 µl	175 µl

Table 4: Reaction Type and Components Present

Reaction Type	NAD	PARP enzyme	Inhibitor	Cycling
1. Background control				X
2. Standards	x			X
3. PARP control	x	X		X
4. Inhibitor test	x	X	x	X
5. Inhibitor control	x		x	X

A. PARP Reaction for Screening Inhibitors

1. Aliquot 25 µl of 1X Buffer to wells containing 0 nM NAD (Table 2, 1A and 2A).

2. Aliquot 25 µl of appropriate 2X NAD Standards (V.1 Reagent Preparation) to wells in 1B to 1G and 2B to 2G (Table 2) for Standard Curve. For example, 25 µl of 20 nM NAD (2X Standard) is aliquoted into wells 1B and 2B to generate 10 nM NAD Standard.

3. Aliquot 25 µl of 2 µM NAD (2X Standard) to 1H and 2H (Table 2) for PARP Control.

4. Aliquot 25 µl of 2 µM NAD (2X Standard) to all wells in columns 3 to 6 (the Inhibitor Wells) (Table 2), using a multichannel pipette, from the reagent plate (Table 3).

5. Aliquot 1 µl of inhibitor concentrations (V.2 Reagent Preparation) to appropriate wells in columns 3 to 6 (see Table 2). If inhibitors were diluted in DMSO, add 1 µl DMSO to standards (1A to 1H,and 2A to 2H).

   Note: DMSO is inhibitory to the PARP and cycling reactions. DMSO concentrations greater than 2% in the PARP reaction are not recommended.

6. Aliquot 25 µl of PARP Mix minus Enzyme (V.3 Reagent Preparation) to 1A to 1G and 2A to 2G. Mix by gently pipetting up and down.

7. Aliquot 25 µl of PARP Mix plus Enzyme (V.3 Reagent Preparation) to PARP Control (1H and 2H) and inhibitor wells columns 3 to 6, using a multi-channel pipette, from the reagent plate (Table 3). Mix gently.

   Note: When determining IC_{50} values, an inhibitor control is required for each inhibitor titration. Adjust mixes as needed.

   Example:
   25 µl of 2 µM NAD
   1 µl of highest [inhibitor]
   25 µl of PARP Mix minus Enzyme

8. Incubate at 25°C for 30 min.

B. Cycling Reaction

1. Aliquot 50 µl of Cycling Mix (V.4 Reagent Preparation) to all wells using multichannel pipette from the reagent plate (Table 3). Mix gently.

2. Incubate at 25°C for 30-60 minutes. Shield plate from direct light.

3. Aliquot 50 µl Stop Solution to all wells, using a multichannel pipette, from the reagent plate using the same order as addition of Cycling Mix. Gently mix.

4. The increase in fluorescence (with excitation at 544 nm and emission at 590 nm) is measured using fluorescence plate reader.

Note: Recommend performing gain adjustment for entire plate. The purpose of the gain adjustment is to optimize the signal amplification so that the results have the maximum sensitivity and dynamic range. Maximal readings are expected from wells 1G and 2G and from some inhibitor wells.

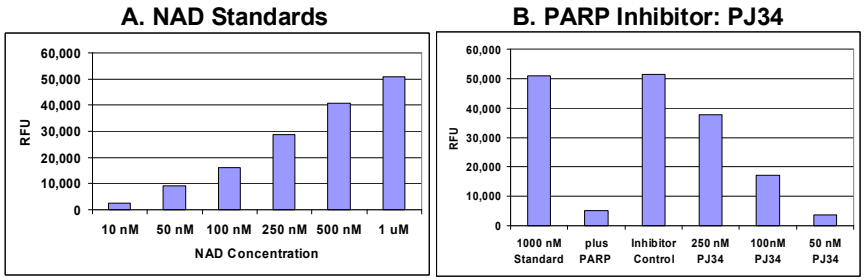
VII. Data Interpretation.

A. Screening

1. Calculate the average fluorescent value of all duplicates and subtract the value of "0 nM NAD Standard" to obtain relative fluorescent unit (RFU) values.
2. In EXCEL, use column chart plot to illustrate data (Figure 2).
   a. In Figure 2A, the NAD standards are on the X axis with their respective RFU values on the Y axis.
   b. In Figure 2B, the inhibitor concentrations present during the PARP reaction are on the X axis with their respective RFU values on Y axis. Control reactions containing 1 µM NAD minus and plus PARP are also plotted. In the absence of PARP activity maximal signal is observed, while minimal signal is observed when PARP activity is present.

c. At a given concentration of inhibitor, PARP inhibition can be monitored based on the final NAD concentration in the assay. In Figure 2B, 100 nM PJ34 showed significant inhibition of PARP activity (maximal signal).

Figure 2: Endpoint data.



B. Calculation of relative IC_{50} values

The Homogeneous PARP Inhibition Assay is designed for the screening of PARP inhibitors using a NAD concentration below the Km of PARP. Once potential inhibitors are identified, accurate Ki values using Michaelis-Menton kinetics can be determined using Trevigen's HT Universal PARP Assay kits (cat# 4676-096-K or 4677-096-K). The percent inhibition can be calculated using RFUs or the amount of NAD remaining as calculated from the standard curve. The instructions below are for converting to NAD remaining. In both cases, it is necessary to use the inhibitor control value to calculate percent inhibition.

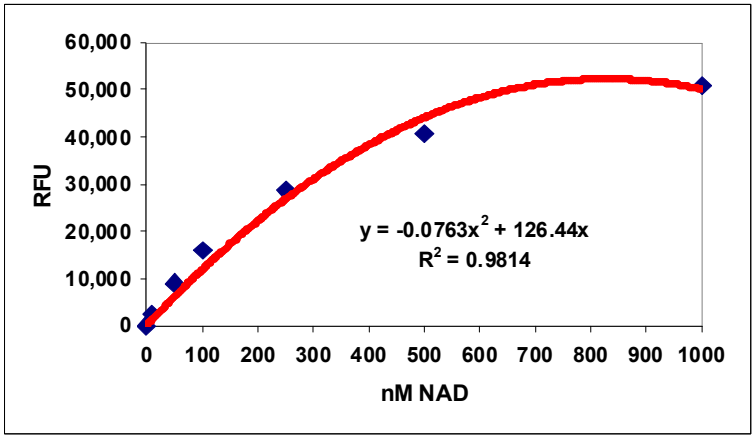
1. Calculate the average fluorescence value of all duplicates for each concentration of inhibitor and subtract value of "0 nM NAD Standard" to obtain RFU values (Table 5).

Table 5: PJ34 Data. Background (5169).

nM PJ34	Average Fluorescence	RFU
0	5136	0
10	5552	383
25	6051	882
50	8814	36458
100	22325	17156
250	42769	37600
500	49036	43867
1000	52321	47152
PJ34 Inhibitor Control	56657	51488
1000 nM Standard	55998	50829

2. In Excel, use XY (scatter) to plot "nM of NAD Standard" (X Axis) versus RFU (Y axis). Generate a trend line using a two order polynomial and force the Y intercept through zero (Figure 3). Display equation on graph.

Figure 3: NAD Standard Curve



3. Set up the quadratic equation from the Standard Curve in Excel to calculate the amount of NAD remaining at each inhibitor concentration and the inhibitor control using the observed RFU. This worksheet is available on the Trevigen web site (Figure 4).

a. The general quadratic equation is given by $ax^2 + bx + c = 0$.

b. Rearrange the equation generated from the Standard Curve.

$$-0.0763\ x^2 + 126.44x + -RFU_i = 0$$

c. Determine amount of NAD remaining at each inhibitor concentration by solving for the value of ***X*** that falls on the standard curve.

The solution of the quadratic equation is

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Where a=-0.0763, b=126.44 and c=-RFU_i observed at each inhibitor concentration.

Figure 4: Calculate amount of NAD remaining (Excel).

a	*b*	*c*	*X1*	*X2*
-0.0763	126.44	-17156	149.10	1508.04

PJ34=100 nM; RFU=17156

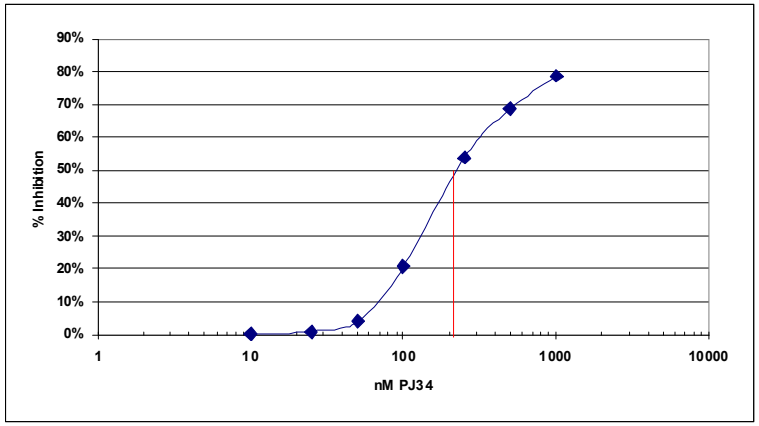
X1 (+) =	149.10	=(-B2 + SQRT(B2^(2)-4*A2*C2))/(2*A2)
X2 (-) =	1508.043	=(-B2 - SQRT(B2^(2)-4*A2*C2))/(2*A2)

4. Plot the % inhibition on the Y axis versus inhibitor concentration on the X axis using EXCEL. A log scale is used for the X axis.

   $$\% \text{ Inhibition} = \frac{100 \times [\text{NAD remaining}] \text{ at specific inhibitor conc.}}{[\text{NAD remaining}] \text{ for the inhibitor control}}$$

5. The IC_{50} is estimated from the curve at 50% inhibition. For example, the inhibitor PJ34 results in an estimated value of 110 nM at 50% inhibition (Figure 5).

Figure 5: Inhibition of PARP by PJ34.



6. Equivalent RFU values should be obtained from the 1 µM Standard and Inhibitor Control to assure no inhibition of the cycling reaction.

Table 6: Relative IC_{50} values for known PARP inhibitors*

PARP Inhibitors	Observed Ki*	Published Ki
3-aminobenzamide	51 ± 10 µM	33 µM
4-amino1,8-napthalimide	23 ± 7 nM	153-180 nM
6(5H)-phenanthridinone	408 ± 130 nM	305 nM
Benzamide	21 ± 5 µM	1-22 µM
PJ34	110 ± 3 nM	20 nM
EB47	25 ± 6 nM	45 nM

*Average of multiple experiments

VIII. References

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2. Hassa, P.O., et al., *Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going?* Microbiol Mol Biol Rev, 2006. **70**(3): p. 789-829.
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4. Tutt, A.N., et al., *Exploiting the DNA repair defect in BRCA mutant cells in the design of new therapeutic strategies for cancer.* Cold Spring Harb Symp Quant Biol, 2005. **70**: p. 139-48.
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IX. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No fluorescence in wells with NAD Standards	Low activity of Cycling Enzymes	Mix 10X Cycling Enzymes gently before adding to Cycling Mix.
High fluorescence in all wells	Resazurin is reduced.	Do not add reducing agents (ex. DTT). Prepare Cycling Mix just before use. Read fluorescence at completion of Cycling reaction.
	10X Resazurin light sensitive.	Minimize exposure to light.
	Plates	Purchase plates suitable for fluorescent assays
NAD Standard Curve is flat at higher concentrations.	Low [EtOH]	Use 190 proof EtOH (95%) or 200 proof EtOH (100%)
Poor sensitivity and dynamic range of NAD Standards	Fluorescent plate reader settings.	Perform gain adjustment for entire plate to optimize the signal amplification
High fluorescence with PARP Control	Low PARP activity	Lower DMSO levels if present Store PARP at -20°C. Mix gently before adding to PARP reaction
PARP inhibition expected but not observed.	Inhibition of Cycling Reaction.	Titrate inhibitor and perform inhibitor only controls with Cycling reaction.

X. Related Products Available From Trevigen

Kits:

Catalog #	Description	Size
4520-096-K	**PARP in vivo Pharmacodynamic Assay II**	**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**

Catalog #	Description	Size
4684-096-K	HT Colorimetric PARP/Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP/Apoptosis Assay	96 tests
4682-096-K	HT Chemiluminescent PARG Assay Kit	96 tests
4683-096-K	HT Colorimetric PARG Assay Kit	96 tests

Inhibitors and Antibodies:

Catalog #	Description	Size
4667-50-11	Benzamide PARP inhibitor (8 mM)	100 µl
4667-50-03	3-Aminobenzamide PARP inhibitor (200 mM)	100 µl
4667-50-10	6(5H)-Phenanthridinone PARP inhibitor (160 µM)	100 µl
4667-50-9	4-Amino-1,8-naphthalimide PARP inhibitor (800 µM)	100 µl
4335-AMC-50	PAR Monoclonal Antibody Affinity Purified	50 µl
4335-MC-100	Anti-PAR Monoclonal Antibody	100 µl
4336-APC-50	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

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

Trevigen, Inc.
8405 Helgerman Ct. Gaithersburg, MD 20877
Tel: 1-800-873-8443 • 301-216-2800
Fax: 301-560-4973
e-mail: info@trevigen.com
www.trevigen.com

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