PARP Activity Assay Kit

For radiometric detection of apoptosis

Catalog No. 30-1235, 50 tests

Instructions

Store at –20°C or lower.

For laboratory research use only. Not for human, clinical, or diagnostic use.

American Type Culture Collection
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Or contact your local distributor.

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Instruction Manual

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INTRODUCTION

Background

Poly (ADP-ribose) polymerase (PARP) is a nuclear protein of 116 kDa present at approximately one million copies in somatic cells. PARP has low activity in healthy cells but is active in DNA repair. During apoptosis, caspase activity cleaves PARP into two subunits, rendering it inactive and allowing complete fragmentation of all chromosomal DNA. The 85 kDa fragment contains the catalytic and automodification domains. It retains basal activity but it is not activated by exogenous nicked DNA.

ATCC's PARP Activity Assay Kit measures the incorporation of radiolabeled NAD (not included) by activated PARP into a trichloroacetic acid precipitate. Because PARP is cleaved in apoptosis and not activated by exogenous DNA, the assay can determine the relative loss of PARP activity.

Precautions

The 3-aminobenzamide reagent contains 200 mM 3-aminobenzamide, which is an irritant. Wear gloves, protective eye wear, and lab coat when handling this component. A Material Safety Data Sheet (MSDS) is available upon request.

KIT COMPONENTS

Store all components at –20ºC or colder.

Reagent	Quantity
PARP Enzyme	50 µl
10x PARP Buffer	700 µl
NAD (1 mM NAD)	700 µl
Histones (1 mg/ml histone H1)	700 µl
Distilled Water	3.75 ml
Activated DNA (1 mg/ml nuclease-treated salmon testes DNA)	500 µl
3-Aminobenzamide (200 mM 3-aminobenzamide)	60 µl

MATERIALS REQUIRED BUT NOT SUPPLIED

Bradford assay or similar reagent for determining protein concentration
^{32}P-NAD, 800 Ci/mmol, 10 µCi/µl or 3000 Ci/mmol, 2 µCi/µl (store at –80ºC)
10% and 20% trichloroacetic acid (TCA)
Phenylmethylsulfonyl fluoride (PMSF)
Tris-Cl, pH 8.0 (1 M solution)
$MgCl_2$ (250 mM solution)
Scintillation cocktail
Scintillation counter and vials
Timer
Thin-stem polyethylene transfer pipets

PREPARATION OF REAGENTS

Sonication buffer
Prepare fresh sonication buffer each time the assay is run. Up to 5 ml may be needed for each cell extract.

Step	Action
1	Prepare 100 mM stock solution of PMSF in ethanol or isopropanol and store at –20°C or below.
2	Combine PMSF solution, Tris-Cl (pH 8.0) stock solution, and magnesium chloride stock solution to this final concentration: 50 mM Tris-Cl 25 mM $MgCl_2$ 0.1 mM PMSF
3	Chill on ice before use.

10% and 20% Trichloroacetic Acid (TCA)
Approximately 1 ml of 20% TCA and 1 ml of 10% TCA are required per sample. Solutions can be prepared ahead of time and stored at 2 to 8°C.

Step	Action
1	Make a 100% (w/v) stock solution by adding deionized water directly to a 500-g bottle of solid TCA. Close bottle and invert repeatedly to dissolve.
2	Adjust the volume to 500 ml.
3	Prepare 10% and 20% TCA solutions from the 100% stock solution.

PREPARATION OF CELL EXTRACTS

Step	Action
1	Centrifuge 1 x 10^5 to 1 x 10^8 cells at 2000 x g for 5 minutes at room temperature. Resuspend in 5 to 10 pellet volumes of sonication buffer. Transfer to microcentrifuge tube chilled on ice. Sonicate on ice, for 10 seconds at a time, until sonicate becomes highly viscous. **Note:** Harvest adherent cells by scraping in a small volume of ice-cold sonication buffer. Cell suspensions from tissues can be disrupted using a gentle method such as a microcentrifuge tube pestle in ice-cold sonication buffer.
2	Microcentrifuge the disrupted cell suspension at 3,000 x g for 5 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. **Note:** If supernatant is cloudy, repeat centrifugation.
3	Determine the protein concentration of the supernatant spectrophotometrically using a Bradford assay or similar reagent. Dilute the sample with cold sonication buffer to 2 mg/ml. If the sample contains less than 2 mg/ml, use enough to contain 20 mg of protein and adjust the volume of distilled water in the reaction mix as necessary.

ASSAY PROTOCOL

Experimental overview and controls

Apoptosis is characterized by reduced PARP activity due to proteolytic cleavage by caspases. This reduction can be determined by evaluating a set of controls and test samples:

Reaction 1: Positive control (PARP + activated DNA). Generates the maximum number of counts for the standard assay using PARP of known activity.

Reaction 2: Experimental positive control (cell extract + activated DNA). Determines if the PARP present in the cell extract can be activated (compare to Reaction 4).

Reaction 3: Experimental negative control (cell extract +activated DNA + PARP inhibitor). Determines if incorporation of radiolabel can be attributed to components in the assay other than PARP.

Reaction 4: Experimental unknown (cell extract, no exogenous DNA). Determines PARP activity without addition of nicked DNA (i.e., reflects the degree of DNA damage present in the cell extract).

Reaction 5: Negative control (no PARP enzyme or cell extract). Determines the background level of counts and should be negligible (less than 1000 cpm). This reaction mix is also used to determine the total number of counts in the reaction mix, a value that is used to calculate PARP enzyme activity.

Recommendations

- Stagger the addition of PARP or cell extracts to avoid coincident start and stop times.
- Prepare the TCA solutions and hold on ice before beginning the reactions.
- ^{32}P-NAD **must** be stored at –80°C; it degrades rapidly at both room temperature and –20°C.
- The stated activity of 200 pmoles of NAD converted to acid-insoluble form in one minute in the PARP standard is based on initial rates and incubation for 1 minute at room temperature. Since the rate of NAD conversion varies with time, the estimated activity of the PARP standard may be less for longer reaction times that may be necessary for your experimental samples.

Step	Action
1	Set up a timed enzymatic reaction series in prelabeled microcentrifuge tubes. Add the PARP or cell extract last, then mix and start the timer. Allow the reaction to proceed for up to 10 minutes at room temperature. (All volumes are in µl.) **1 2 3 4 5** 10x PARP Buffer 10 10 10 10 10 1 mM NAD 10 10 10 10 10 1 mg/ml Histones 10 10 10 10 10 1 µCi/µl ^{32}P-NAD 2 2 2 2 2 Distilled Water 57 48 42 58 58 Activated DNA 10 10 10 - 10 3-Aminobenzamide - - 6 - - PARP Enzyme* 1 - - - - Cell extract (2 mg/ml) - 10 10 10 - To determine specific activity (cpm/nmol NAD) of your sample, remove 1 µl from the negative control tube (no enzyme added, Reaction 5), and place into a scintillation vial. Add liquid scintillation cocktail and count for ^{32}P in a liquid scintillation counter. Calculate the specific activity of the NAD and then estimate the enzyme activity in the sample (Reactions 1 through 4). See page 6 for calculations. **The PARP enzyme incorporates 200 pmol/µl poly (ADP-ribose) via histone ribosylation and autoribosylation in 1 minute at room temperature.
2	At the end of the incubation, precipitate ribosylated proteins by adding 900 µl of ice cold 20% TCA. Place each tube on ice until all samples have been processed.
3	Centrifuge at 12,000 x g for 10 min at room temp. Remove and discard the supernatant into a radioactive waste container using a thin-stem polyethylene transfer pipet. Centrifuge the tubes again for 10 seconds and then carefully remove any remaining supernatant. **Note:** When centrifuging, orient the tubes in the rotor so that you know on which side of the tube the protein pellet is located. When removing the supernatant, avoid this area so as not to inadvertently remove the pellet.
4	Add 1 ml cold 10% TCA to each tube. Vortex briefly and repeat step 3.
5	Add 1 ml of liquid scintillation cocktail to each tube. Vortex well over a period of 1 minute to solubilize the protein precipitate. Place the tube in a standard scintillation vial and count for ^{32}P in a liquid scintillation counter. Alternatively, transfer the liquid to a scintillation vial, wash the tube once with 1 ml of liquid scintillation cocktail, and add to the scintillation vial. Count for ^{32}P. **Note:** Use a liquid scintillation cocktail, such as 3a70 or Safety-Solve from Research Products International Corp. (1-800-323-9814; www.rpicorp.com) that can accommodate and dissolve aqueous samples. Similar products can be obtained from Beckman, Amersham, NEN, and ICN.

DATA INTERPRETATION

The counts obtained from the control reactions will indicate whether the reaction has been performed correctly. Counts in the negative control (Reaction 5) are typically less than 1000. The positive control (Reaction 1) will give counts that will typically ranging from 10,000 up to 100,000 depending upon the specific activity of the radiolabeled NAD. In the presence of an inhibitor (Reaction 3) counts should be close to the background (less than 20% of counts in the absence of inhibitor, or Reaction 2). If the counts in the different controls are not as expected refer to the troubleshooting guide below.

Calculate the enzyme activity in each of the samples:

The specific activity of the NAD is determined from the total counts per minute (cpm) present in 1 µl of Reaction 5 prior to TCA precipitation (recall that the final NAD concentration is 0.1 mM or 0.1 nmole/µl):

$$\text{specific activity (cpm/nmol NAD)} = \frac{\text{total cpm in 1 µl of Reaction 5}}{\text{0.1 nmoles of NAD (in 1 µl of Reaction 5)}}$$

$$= \text{Total cpm in 1 µl of Reaction 5} \times 10$$

The enzyme activity in the sample is determined from the specific activity of the NAD and the cpm in the experimental sample:

$$\text{activity (nmol/min/µl)} = \frac{\text{cpm for reaction}}{\text{incubation time (min)} \times \text{cell extract added (µl)} \times \text{specific activity of NAD (cpm/nmol)}}$$

To give the enzyme activity in pmol/min/µl multiply by 1000.

The values obtained in experimental samples indicate the relative activity of PARP in the reaction. The calculated values may be used to determine IC_{50} for inhibitors of PARP or may reflect the reduction in PARP activity due to proteolytic cleavage during apoptosis. PARP has an absolute requirement for DNA breaks for activity; therefore, in reactions containing cell extracts, the endogenous activity of PARP in the absence of activator DNA is an indirect measure of DNA damage in the cell at the time of cell extract preparation.

APPENDIX

Troubleshooting

Problem	Cause/Action
High background in negative control (Reaction 5, no enzyme)	Insufficient washing. Repeat Step 4 of the protocol. Remove as much supernatant as possible in Step 3.
No (or low) counts in the positive control (Reaction 1, PARP)	Enzyme inactive due to incorrect storage (store at –20°C in manual defrost freezer). Activated DNA or radiolabeled NAD was omitted from reaction. Radiolabeled material was not precipitated by TCA. The ^{32}P-NAD has degraded. **Store ^{32}P-NAD at –80°C.**
No counts in experimental sample when other controls give expected results.	Sample may have low or undetectable levels of active PARP.

Other Applications

The PARP Activity Assay can also be used to study PARP inhibitors. Use the standard assay control (Reaction 1) and a PARP inhibition control (PARP enzyme + 3-aminobenzamide). To test the PARP inhibitor compound of interest, replace the 3-aminobenzamide with the test compound, running multiple concentrations if desired.

REFERENCES

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RELATED PRODUCTS

Apoptosis Detection Products		
Annexin V Fluorescent Detection Kit	30-1236	100 tests
Anti-G3PDH Antibody	30-1255	100 µl
Anti-PARP Monoclonal Antibody	30-1253	50 µl
Anti-Phosphorylated Histone H2AX Polyclonal Antibody	30-1251	20 µl
Anti-Phosphorylated Histone H2AX Polyclonal Antibody	30-1252	100 µl
Apoptotic Cell DNA Laddering Kit	30-1231	20 tests
CV Caspase 3 & 7 Substrate Kit	30-1344	100 tests
FAM Caspase 3 & 7 Binding Kit	30-1304	100 tests
FAM Caspase 8 Binding Kit	30-1306	100 tests
FAM Caspase 9 Binding Kit	30-1308	100 tests
FAM Poly-Caspase Binding Kit	30-1302	100 tests
Mitochondrial Potential Assay Kit	30-1233	100 tests
PARP Activity Assay Kit	30-1235	50 tests
SR Caspase 3 & 7 Binding Kit	30-1324	100 tests
SR Poly Caspase Binding Kit	30-1322	100 tests
TdT Flow Cytometry Apoptosis Detection Kit	30-1207	60 tests
TdT In Situ Apoptosis Detection Blue Kit	30-1202	30 tests
TdT In Situ Apoptosis Detection DAB Kit	30-1201	30 tests
TdT In Situ Replenisher Kit	30-1205	30 tests
TdT Microplate Apoptosis Detection Kit	30-1206	96 tests
Stains and Reagents		
Erythrosin B Stain Solution	30-2404	40 ml
Trypan Blue Stain Solution	30-2402	40 ml
Dulbecco's Phosphate Buffered Saline (PBS)	30-2200	500 ml
Molecular Grade Water	60-2450	1 liter
Clone Sets		
Human Apoptosis Clone Set	MBA-89	
Mouse Apoptosis Clone Set	MBA-90	

ATCC also has apoptotic inducible cell lines, cell line model systems, and hybridomas, as well as a full line of products for cell culture. See our Web site at www.atcc.org for details.

Please refer to the Material Transfer Agreement and packing slip enclosed with the product for terms and conditions.

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