

KAMIYA BIOMEDICAL COMPANY

Oxidative DNA Damage Kit Quantitative

For the quantification of abasic sites in genomic DNA

Cat. No. DN-004

For research use only, not for use in diagnostic procedures.

GENERAL INFORMATION

Oxidative damage to DNA is a result of the interaction of DNA with reactive oxygen species (ROS), in particular, the hydroxy radical which is converted from superoxide and hydrogen peroxide by the Fenton reaction. Hydroxy radicals produce a multiplicity of modifications in DNA. Oxidative attack by hydroxy radical on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites (AP sites). In fact, AP sites are one of the major types of damage generated by ROS. It has been estimated that endogeneous ROS can result in about 2×10^5 base lesions per cell per day.

Aldehyde Reactive Probe (ARP) reagent (N'-aminooxymethylcarbonylhydrazino-D-biotin, Fig. 1) reacts specifically with an aldehyde group which is the open ring form of the AP sites. This reaction makes it possible to detect DNA modifications that result in the formation of an aldehyde group. After treating DNA containing AP sites with ARP reagent, AP sites are tagged with biotin residues. By using an excess amount of ARP, all AP sites can be converted to biotin-tagged AP sites. Therefore, AP sites can be quantified using avidin-biotin assay followed by a colorimetric detection of peroxidase or alkaline phosphatase conjugated to the avidin (Fig. 2). **DNA Damage Quantification Kit** contains all the necessary solutions, enabling the determination of 1 to 40 AP sites per 1×10^5 bp.

DESCRIPTION OF THE KIT

DNA Damage Quantification Kit -AP Site Counting- is for the determination of abasic sites in sample DNA, which correspond to colorimetric 96-well microplate assay. ARP standard DNA solutions in this kit are prepared by heat/acid depurination (Lindahl & Nyberg, 1972, Kubo, *et al.*, 1992) of calf thymus DNA to control the number of abasic sites from 0 to 40 per 1×10^5 bp. These DNA solutions are treated with ARP and purified. The Standard 0 ARP-DNA is prepared using methoxyamine-treated calf thymus DNA without heat/acid depurination. Since this kit contains centrifugal filtration tubes for the purification of ARP-labeled sample DNA, there is no need to determine the DNA concentration after the ARP reaction.

Standard ARP DNA and purified ARP-treated sample DNA are fixed on the 96 well plate with DNA Binding Solution. Then, the number of AP sites in the sample DNA can be determined by the biotin-avidin-peroxidase assay. Since the standard ARP DNA are double

stranded DNA, this kit is not applicable to abasic sites detection of single stranded DNA. The O.D. value of a single stranded DNA sample will be nearly twice as high as that of a double stranded DNA sample under the same assay condition. For the isolation of genomic DNA from samples, guanidine/detergent-based DNA isolation methods are recommended.

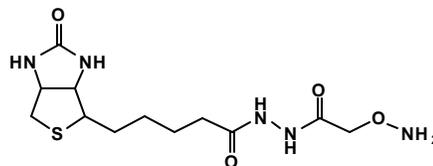


Figure 1. Chemical structure of ARP reagent

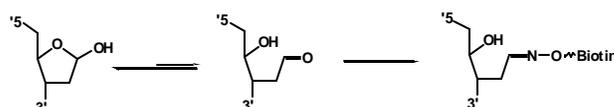


Figure 2. Reaction of AP site with ARP

CONTENTS

ARP Solution (10mM ARP)	100 μ l	x 1
0 ARP-DNA Standard Soln.* (0 ARP/100,000 bp)	250 μ l	x 1
2.5 ARP-DNA Standard Soln.* (2.5 ARP/100,000 bp)	250 μ l	x 1
5 ARP-DNA Standard Soln.* (5 ARP/100,000 bp)	250 μ l	x 1
10 ARP-DNA Standard Soln.* (10 ARP/100,000 bp)	250 μ l	x 1
20 ARP-DNA Standard Soln.* (20 ARP/100,000 bp)	250 μ l	x 1
40 ARP-DNA Standard Soln.* (40 ARP/100,000 bp)	250 μ l	x 1
DNA Binding Solution	10 ml	x 1
Substrate Solution	10 ml	x 1
TE Buffer	15 ml	x 1
HRP-Streptavidin	25 μl	x 1
Washing Buffer (powder for 1 L)	1 packet	
Filtration Tube	5 tubes	
96-well Microplate/ U bottom	1 plate	
Manual	1 booklet	

* ARP-DNA Standard Solutions: 0.5 μ g DNA / ml

HOW TO USE DNA DAMAGE QUANTIFICATION KIT

1. Required Equipments and Materials for Abasic Sites Determination using This Kit

Microplate reader with 650 nm filter
0.5 ml and 1.5 ml tube
10 μ l and 100-200 μ l pipettes
50-250 μ l multi-channel pipette
Centrifuge: for ARP-labeled DNA purification using filtration tubes
Incubator: for incubation at 37 °C

2. Protocol

Purification of genomic DNA

Several different methods and products are available for the isolation of genomic DNA from samples such as membrane binding method, guanidine/detergent lysis method, and polyelectrolyte precipitation method. Among these methods, the guanidine/detergent lysis method is simple, and it gives highly purified genomic DNA for the ARP-based abasic sites detection. During the purification process, avoid heating of the DNA solution. Determine the concentration and purity of the purified genomic DNA using the spectrophotometer* and agarose gel electrophoresis. Dissolve the genomic DNA in TE at the concentration of 100 μ g/ml. It is important for an accurate assay that the DNA concentration is adjusted exactly to 100 μ g/ml.

* 1 OD_{260nm} = 50 μ g/ml. The ratio of OD_{260nm}/OD_{280nm} of highly purified DNA solution is 1.8 or higher. Protein contamination in the sample solution may cause a positive error.

ARP reaction (Preparation of ARP-labeled DNA)

- 1) Mix 10 μ l of purified genomic DNA solution (100 μ g/ml) and 10 μ l of ARP Solution in a 0.5 ml tube, and incubate at 37 °C for 1 hour.
- 2) Wash the inside of the Filtration Tube cup with 100 μ l of TE twice (see Fig. 3).
- 3) Add 380 μ l of TE to the reaction solution, and transfer the solution to the Filtration Tube (see Fig. 3).¹⁾
- 4) Centrifuge the Filtration Tube at 2500 g for 15 min, and discard the filtrate solution.
- 5) Add 400 μ l of TE to the Filtration Tube and resuspend the DNA on the filter with a pipette.
- 6) Centrifuge the Filtration Tube at 2500 g for 15 min.²⁾
- 7) Add 200 μ l of TE to the Filtration Tube to resuspend the DNA on the filter with a pipette.
- 8) Transfer the DNA solution to the 1.5 ml tube, and add 200 μ l of TE again to the Filtration Tube to transfer the ARP-labeled DNA on the filter completely to the 1.5 ml tube.³⁾
- 9) Store the ARP-labeled genomic DNA solution at 0 - 5 °C.

- 1) Ethanol precipitation can be used for the purification of the ARP-labeled DNA instead of purification by Filtration Tube. After ethanol precipitation, dissolve the DNA pellet in 100 μ l of TE, and determine the DNA concentration.
- 2) If the DNA solution still remains on the filter after the centrifugation, spin for another 5 min, then proceed to step 7.
- 3) Recovery rate of DNA using the filtration tube is 90 %, so the concentration of the ARP-labeled DNA is 2.25 μ g/ml. For more accurate determination of the number of abasic sites in the sample DNA, we recommend measuring the DNA concentration.

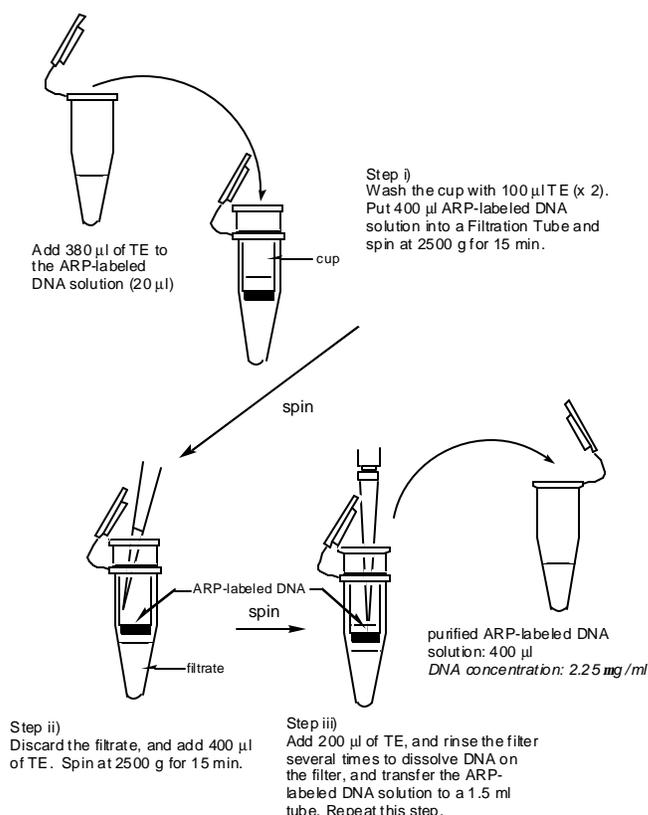


Figure 3. ARP-labeled genomic DNA purification Procedure

Determination of the number of abasic sites in DNA

Day 1:

- 1) Dilute 90 μ l of the ARP-labeled genomic DNA with 310 μ l of TE.
- 2) Add 60 μ l of Standard ARP-DNA Solution per well. Use three wells per 1 standard solution (please see "DNA Solution Arrangement on the Plate" on page 3).
- 3) Add 60 μ l of the diluted ARP-labeled genomic DNA solution per well. Use at least three wells per 1 sample.
- 4) Add 100 μ l of the DNA Binding Solution to each well¹⁾, then allow the plate to remain at room temperature overnight.

¹⁾ Alternatively, mix 200 μ l of DNA solution and 333 μ l of DNA Binding Solution in a tube, and put 160 μ l of the mixture to each well (see notes 4).

Day 2:

Preparation of the solutions

Washing Buffer: Dissolve the contents of the Washing Buffer packet in 1 L of deionized or distilled water. Store this Washing Buffer solution at room temperature.

HRP-Streptavidin solution: Dilute HRP-Streptavidin with Washing Buffer to prepare 1/4000 diluted working solution.*

1/4000 dilution preparation: Centrifuge HRP-Streptavidin tube for 30 sec. Add 10ml of HRP-Streptavidin into 40 ml of Washing Buffer solution, and mix well.

* Since this working solution is not stable, always use freshly prepared solution.

- 6) Add 150 µl of diluted HRP-avidin solution to each well, and incubate the plate at 37 °C for 1 hour.
- 7) Discard the solution in the well, and wash the well with 250 µl Washing Buffer 5 times.²⁾
- 8) Add 100 µl of Substrate Solution to each well, and incubate at 37 °C for 1 hour.
- 9) Measure the O.D. at 650 nm within 1 hour after the incubation is finished, and prepare a calibration curve using the data obtained with standard ARP-DNA solutions.
- 10) Determine the number of abasic sites in the genomic DNA using the calibration curve.

²⁾ After discarding the solution, invert the plate and tap it on a paper towel several times to remove the solution completely.

* *Typical standard curve is shown in the Certificate of Analysis of this kit.*

- 5) Discard the DNA Binding Solution in the wells, and wash the well with 250 µl Washing Buffer 5 times.²⁾

DNA Solution Arrangement on the Plate

Standard ARP-DNA (60 ml/well) or sample DNA (60 ml/well)
+ DNA Binding Solution (100 ml/well)

	1	2	3	4	5	6	7	8	9	10	11	12
A		0 ARP DNA std.					sample 5			sample 13		
B		2.5 ARP DNA std.					sample 6			sample 14		
C		5 ARP DNA std.					sample 7			sample 15		
D		10 ARP DNA std.					sample 8			sample 16		
E		20 ARP DNA std.					sample 9			sample 17		
F		40 ARP DNA std.					sample 10			sample 18		
G			sample 3				sample 11			sample 19		
H		blank	sample 4				sample 12			sample 20		

blank: 60 µl TE buffer+ 100 µl DNA Binding Solution/well
Do not use column 1 and 12

Washing (250 ml/well)
HRP -Streptavidin (150 ml/well)
Washing (250 ml/well)
Substrate (100 ml/well)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES

- 1) Please store the kit at 0-5 °C. Do not freeze. Store Washing Buffer solution at room temperature.
- 2) AP-DNA is not stable. Please treat it with ARP and purify with Filtration Tube after the isolation of genomic DNA from a sample. Purified ARP-DNA solution in TE buffer is stable over one year at 0-5 °C storage.
- 3) After the spinning of Filtration Tube for ARP-labeled DNA purification, add 200 µl TE immediately. If the DNA stays in Filtration Tube for more than 30 minutes after the spinning, the DNA recovery ratio may decline.
- 4) γ-Ray-sterilized tubes may cause DNA binding on the surface of the tube during the mixing of the DNA solution with DNA Binding Solution. If you prefer to mix ARP-DNA solution with DNA Binding Solution in a tube rather than mixing them in a well, please avoid using γ-ray-sterilized tubes.
- 5) For accurate determination of the number of abasic sites in sample DNA, multiple measurements are recommended.
- 6) If the volume of the sample DNA solution is smaller than 10 µl, use an equal volume of ARP Solution, and determine the concentration of DNA after the purification by Filtration Tubes.
- 7) If the 650 nm filter is not available for the measurement of O.D. after the color development, transfer 50 µl of the solution in each well to a well of a new plate (not provided). Then, add 50 µl of 1 M sulfuric acid, and measure the O.D. at 450 nm.
- 8) Remaining solution in a well may cause error, so please remove the solution thoroughly by tapping the plate on a paper towel in each step.

TROUBLE SHOOTING

- at 0-5 °C? - Storage at room temperature or at freezing temperature may cause significant decrease in the enzyme activity.
- B. No coloring in sample DNA
1. Did you adjust the original DNA concentration of your sample exactly to 100 µg/ml?
 2. Did you add ARP solution to DNA solution?
 3. Did you purify the DNA solution after the ARP reaction with a Filtration Tube or ethanol precipitation?
 4. The number of AP sites in the sample DNA may be smaller than 1/100000 bp (the detection limit).
- C. High coloring in all wells
1. Did you dilute HRP-Streptavidin to 1/4000 with Washing Buffer solution? - High concentration of HRP-Streptavidin causes high background.
 2. Did you wash each well 5 times with Washing Buffer solution?
- D. Calibration curve is not linear and/or high CV (over 20%)
1. Did you take the same volume of ARP-DNA Standard Solution from each vial and mix them with DNA Binding Solution?
 2. Did you wash each well 5 times with Washing Buffer solution?
 3. Did you remove Washing Buffer solution thoroughly? - After discarding the solution, invert the plate and tap it on a paper towel several times to remove the solution completely.
 4. Did you use the Washing Buffer in this kit and dissolved it in 1 liter of deionized or distilled water? - Lower or higher concentration of Washing Buffer causes serious error.
 5. Did you leave the plate overnight at room temperature in order to allow the DNA to bind on the plate surface? - At least 4 hours of incubation time is necessary to bind DNA to the plate surface in the right consistency.
 6. Did you follow the direction of the DNA purification protocol using Filtration Tube after the ARP reaction? - ARP contamination in the ARP-labeled DNA solution causes serious error.
- E. Signal of the sample DNA is out of range
1. Dilute the sample DNA with 0 ARP Standard DNA Solution, and determine the number of AP sites in the diluted sample DNA. Then, multiply the number by the dilution ratio.
 2. Did you dilute the DNA solution with TE after the purification by Filtration Tube?
 3. Is your sample DNA pure enough? - Please measure the ratio of $O.D._{260} / O.D._{280}$. The ratio should be 1.8 or higher.
- A. No coloring or very low O.D. in all wells
1. Did you add DNA Binding Solution to the DNA solution? - Insufficient amount of DNA Binding Solution may cause no DNA binding on a plate.
 2. Did you add 1/4000 diluted HRP-Streptavidin solution?
 3. Did you add Substrate Solution?
 4. Is the O.D. measurement in the right wavelength?
 5. Did you prepare 1/4000 diluted HRP-Streptavidin solution just prior to use?
 6. Is the HRP-Streptavidin solution properly stored



*If you have any questions regarding this kit,
please contact us at 206-575-8068 or send e-mail:
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- NOTES -

General Protocol at a Glance

Read Technical Information carefully prior to using this General Protocol

Day 1

Step 1)

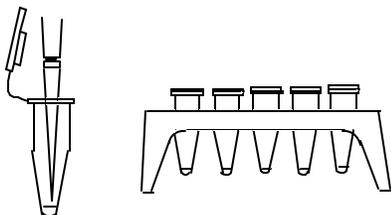
Add 10 μl^{a} of DNA solution and 10 μl^{a} of ARP Solution, and incubate at 37 °C for 1 hour.



1 hour



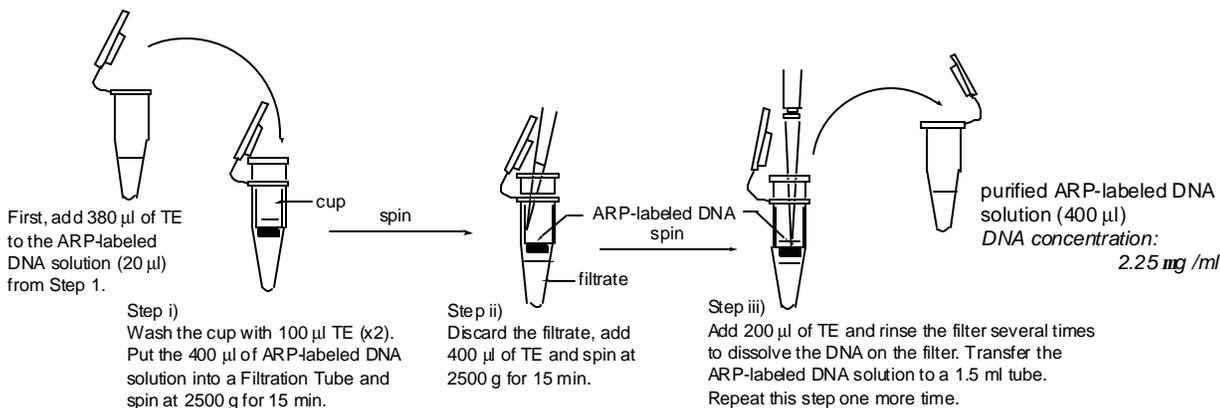
37 °C



a) if the volume of the DNA solution is smaller than 10 μl , add the same volume of ARP Solution.

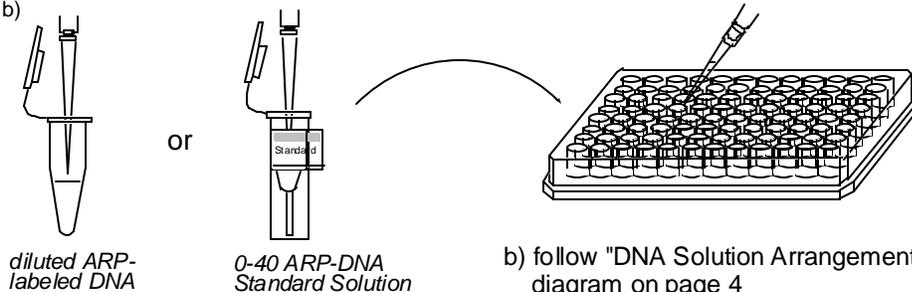
Step 2)

Purify the ARP-labeled DNA with a Filtration tube.



Step 3)

Take 90 μl of the ARP-labeled DNA solution, and dilute it with 310 μl of TE. Add 60 μl of the diluted solution or ARP-DNA Standard Solutions to each well.^{b)}

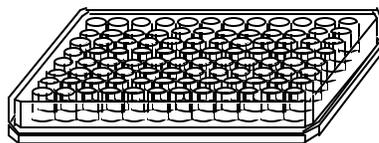


Step 4)

Add 100 μl of DNA Binding Solution to each well and incubate the plate at room temperature overnight.



overnight



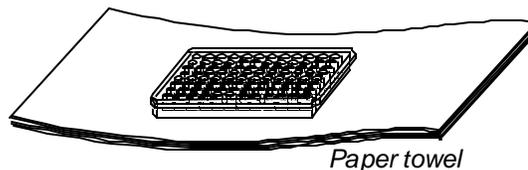
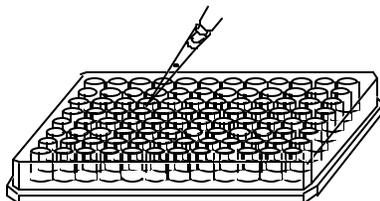
General Protocol at a Glance

Read Technical Information carefully prior to using this General Protocol

Day 2

Step 5)

Discard the solution from each well, and wash the well with 250 μ l of Washing Buffer solution 5 times. Tap the plate on a paper towel several times to remove the Washing Buffer solution thoroughly from each well.



Step 6)

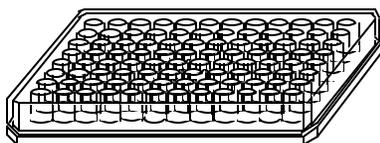


1 hour



37 °C

Add 150 μ l of the diluted HRP-Streptavidin solution^{c)} to each well, and incubate at 37 °C for 1 hour.



c) Use 1/4000 diluted HRP-Streptavidin solution. Centrifuge tube for 30 sec. Add 10 μ l HRP-Streptavidin in 40 ml of washing buffer solution, and mix well.

Step 7)

Discard the solution from each well, and wash the well with 250 μ l of Washing Buffer solution 5 times. Tap the plate on a paper towel several times to remove the Washing Buffer solution thoroughly from each well.

Step 8)

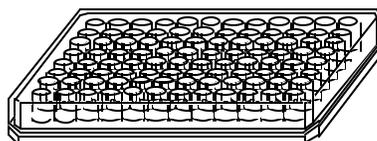


1 hour



37 °C

Add 100 μ l of Substrate Solution to each well, and incubate at 37 °C for 1 hour.



Step 9)



Read the O.D. of each well at 650 nm^{d)}, and determine the number of abasic sites in the sample DNA using a calibration curve.

d) If 650 nm filter is not available, transfer 50 μ l from each well to a well of a new plate, and add 50 μ l of 1 M sulfuric acid. Then, measure the O.D. at 450 nm.