Molecular analysis of \textit{hprt} mutation in B6C3F1 mice exposed to ozone alone and combined treatment of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butaneone and/or dibutyl phthalate for 32 and 52 weeks

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Potential toxicological interactions of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butaneone (NNK) and/or dibutyl phthalate (DBP) on ozone were investigated after 32- and 52-wk exposures using \textit{hprt} mutation assay. Male and female B6C3F1 mice exposed to ozone (0.5 ppm), NNK (1.0 mg/kg), DBP (5,000 ppm), and two or three combinations of these toxicants 6 h per day for 32- and 52-wk showed increases in the frequencies of TG\textsubscript{\textit{\textgamma}} lymphocytes compared to the control groups. Additive interactions were noted from two combination groups compared to the ozone alone in both sexes of 32- and 52-wk studies. The most common specific mutation type in the \textit{hprt} genes of test materials-treated male and female mice was transversion with very few transition. The results indicate that such dominant transversion may be responsible for toxicity and combined exposure to ozone, NNK, and DBP induces additive genotoxocities compared to ozone alone.

**Key words:** Ozone, NNK, DBP, \textit{hprt} mutation

**Introduction**

Ozone is the major irritating oxidant gas found in photochemical smog, and, among the air pollutants for which National Ambient Air Quality Standards (NAAQS) has been designated under the Clean Air Act, currently emerges as the most pervasive problem [31]. Repeated exposures to high sporadic concentrations of ozone in large metropolitan areas such as Los Angeles, and Mexico City, pose significant threats to the health of the inhabitants. Like many other developing countries in Asia, Korea has witnessed rapid increases in urbanization and industrialization over the past few decades. Korean ambient air quality standards (KAAQS) for ozone is currently set at 1-h/0.12-ppm and 8-h/0.06-ppm. There are concerns, however, that exposure to ozone even at comparatively low concentrations may produce signs of acute and perhaps also of chronic lung injuries in human [22].

The tobacco-specific nitrosamine of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butaneone (NNK) is formed by nitrosation of [-]1-methyl-2-[3-pyridyl]-pyrrolidine (nicotine) during maturation, air-curing, and storage of tobacco, as well as during combustion of cigarettes [13,14]. NNK induces lung tumors in rodents independent of the route of administration and has been suggested as a causative factor in human lung cancer [13,15].

Dibutyl phthalate (DBP) attracted attention as a potential endocrine disruptor because cell-based-assays revealed it to be a weak estrogen receptor agonist [12,18]. It is presently used as a plasticizer for nitrocellulose, polyvinyl chloride, and polyvinyl acetate and in adhesives, plastic coatings, and cosmetic formulations. DBP is also contained in a variety of consumer products including plastic food wrap and other plastic products, perfumes, skin emollients, hair spray, nail polish, and insect repellents. Furthermore, DBP is ubiquitous in the environment. The principal source of human exposure to DBP appears to be through dietary intake [17]. Upon ingestion, DBP is rapidly absorbed through the gastrointestinal tract, mainly as a monosubstituted phthalate ester mono (\(n\)-butyl) phthlate (MBP). In the rat, MBP has a half-life in blood of less than 24 h [27]. DBP is...
toxic to the Sertoli cell of the testis [10,11], and acute or subacute high doses (greater than 1g/kg/day) impair spermatogenesis in rats by inducing widespread exfoliation of the seminiferous epithelium in the rat. Neonatal and pubertal rats are more sensitive than sexually mature animals to the testicular toxicity of DBP, which is mediated by the monosubstituted phthalate ester metabolite [10], and other phthalate esters [8,10].

Mutation at the hypoxanthine-guanine phosphoribosyltransferase (hpert) locus can provide information on the mechanisms of in vivo mutation in population exposed to exogenous carcinogens and in individual with inherent susceptibility to cancer and other disease [7]. The hpert gene is located on the long arm of the X chromosome and consists of nine [28,29]. The complete sequence totaling 57 kb nucleotide was determined by Edwards et al. [9]. Transcription of these genes produces an mRNA of 1.6 kb, which contains a protein-encoding region of 654 nucleotide [19]. The X-chromosomal gene for hpert, first recognized through its human germinal mutations, quickly became a useful target for studies of somatic mutations in vitro and in vivo in humans and animals. In this role, hpert serves as a simple reporter gene. The distributions of hpert mutants among T cell receptor (TCR) gene-defined T cell clones in vivo in humans and animals. We, thus, determined the genotoxic effects of ozone, NNK, DBP, and two or three combination of these toxicants on splenic T-cells of male and female mice following in vivo 32- and 52-wk exposures.

Materials and Methods

Chemicals

NNK (CAS NO. 64091-91-4) was obtained from Chemsyn Science laboratories (Lenexa, USA), with over 99% purity as revealed through HPLC analysis (data not shown). Trioctanoin, obtained from Wako (Japan), was redistilled before use. DBP (CAS NO. 84-74-2) was acquired from Sigma (USA). Diet containing DBP was freshly prepared each week. A predetermined amount of DBP was added to a small aliquot of ground basal diet, and handblended. This premix was then added to a preweighed ground basal diet and blended in a mill for 30 min.

Animals

Male and female B6C3F1 mice, 4- to 5-wk-old, were purchased from Laboratory Animal Facility, Seoul National University and were acclimated for about 7 days prior to the initiation of chemical exposure. Food and water were provided ad libitum except during the period of ozone exposures. Rooms were maintained at 23 ± 2°C, with a relative humidity of 50 ± 20% and a 12-h light/dark cycle.

All methods used in this study were approved by the Animal Care and Use Committee at SNU and conform to the NIH guidelines (NIH publication No.86-23, revised 1985).

The experimental groups were as follows: (a) unexposed group (control); (b) group exposed to 0.5 ppm ozone (ozone group); (c) group exposed to 1.0 mg NNK/kg body weight (NNK group); (d) group exposed to 5,000 ppm DBP (DBP group); (e) group exposed to 0.5 ppm ozone + 1.0 mg/kg NNK (ozone + NNK group); (f) group exposed to 0.5 ppm ozone + 5,000 ppm DBP (ozone + DBP group); (g) group exposed to 0.5 ppm ozone + 1.0 mg/kg NNK + 5,000 ppm DBP (three-combination group).

Exposures

Mice (5 male and 5 female mice per each group) were exposed to ozone (0.50 ± 0.02 ppm) for 6 h per day (between 9:00 AM and 3:00 PM), 5 days per week for 32- and 52-wk in 1.5 m³ whole-body inhalation exposure chambers (Air-Dynamics, USA). Ozone (CAS NO. 10028-15-6) was generated from pure oxygen using a silent electric arc discharge ozonator (Model KDA-8, Sam-II Environment Technology, Korea) and was mixed with the main stream of filtered air before entering the exposure chamber. Ozone concentrations in the chambers were monitored through a gas detection system with O3 gas sensor (Analytical Technology, USA). Ozone concentrations in the chambers were monitored through a gas detection system with O3 gas sensor (Analytical Technology, USA). O3 gas sensor probes were placed within the breathing zone of the mice in the middle cage rack. Measurements were taken from 12 locations in each chamber to ensure the uniformity of ozone distribution, which was enhanced through a recirculation device. Airflow in the chambers was maintained at 15 changes per hour. During exposure, the wire cage allowed visual observation of all individually housed animals. Before and after ozone exposures, the mice were housed five per cage in polycarbonate cages with bottom wire nets. During the test periods, mice were subcutaneously injected with 1.0 mg NNK per kg body weight in trioctanoin three times per week. They also received diets containing DBP at a concentration of 5,000 ppm for 32- and 52-wk. The concentration of each test material was determined based on the National Toxicology Program, carcinogenesis study [26,27].

Isolation and culture of mutatant lymphocytes

In this study, the T-cell cloning assay was performed for measuring mutant frequencies (MFs) at the hypoxanthine-guanine phosphoribosyltransferase (hpert) locus of lymphocytes isolated from spleens of mice following exposure to ozone, NNK, and DBP, and combined treatments of NNK and DBP on ozone for 32- and 52-wk. The procedures for isolating lymphocytes from spleen and culturing hpert mutant T-cell colonies, modified in detail previously, were used [30]. Briefly, T-cells were isolated by macerating spleens individually in 12-well plates, layering the cells on a histopaque 1077 and washing the recovered
cells with RPMI 1640 medium. The cells were then resuspended in primary culture medium for mitogenic stimulation for 36-40 hours. Both primary culture and mutant plating media were modified by the addition of a conditioned medium from concanavalin A-stimulated mouse splenocyte and blood cultures for the stimulation and growth of mouse T-cells [30]. After primary culture, cells were then enumerated using a haemocytometer and cultured in 96-well U-bottom microtiter plates with supplemented medium to determine the clonal efficiency (CE) and to identify hprr mutants. For determining the cloning efficiencies of T-cells from mice, aliquots of primed cultures were diluted in cloning medium to culture 5 cells/well in the presence of 1×10³ lethally irradiated mouse splenic lymphocytes (feeder cells)/well. Excess lymphocytes isolated from untreated mice were used as a source of feeder cells. To isolate hprr mutants, primary cultures were diluted to 1×10³ cells/ml using mutant plating medium supplemented with 1 μg 6-thioguanine (TG)/ml, and were then seeded in 96-well plates at 100 μl per well for incubation. Plates were scored for colony growth at 40× magnification (and confirmed at higher magnification as necessary) on days 10-15. hprr mutant frequencies (MFs) were calculated as described previously [1] using the and following equations: (a) P (0) = P0 = number of negative wells/total number of wells; (b) mutant fraction (Mf) = (−ln P0 in TG-plates)/(1×10⁶); (c) clonal efficiency (CE) = (−ln P0 in CE-plates)/(5 cells/well); (d) mutant frequency (MF) = mutant fraction (Mf)/clonal efficiency (CE).

Molecular analysis of mouse T-cell clones for mutations in the hprr mutation

6-Thioguanine-resistant T-cell colonies from the control and treated mice after 32- and 52-wk exposure were used to evaluate the effect of concanavalin A stimulation on T-cell colony expansion. Mutant colonies were taken from unexposed and test materials exposed mice, respectively. Mutant colonies were propagated sufficiently for molecular analysis by RT-PCR using the propagation procedure for mouse clones described elsewhere [24]. Propagated mutant T-cell clones from the control and test materials exposed mice were evaluated for mutations in hprr cDNA of the mouse gene using RT-PCR procedure. Mutant clones that produced hprr cDNA were further analyzed by DNA sequencing. As an internal control to check the methodology used for preparing mRNA to generate hprr cDNA, RT-PCR of the β-actin gene was performed on clones that did not yield hprr cDNA to ascertain the successful or unsuccessful isolation of mRNA from these clones. For preparation of total RNA, frozen pellets of expanded clones were thawed on ice, and 1×10¹⁰ cells were transferred into 500 μl Eppendorf tubes containing 10 μl RNase-free H₂O (Promega, USA), 0.4% Rnasin (Promega, USA), and 2.5% Non-ident P-40 (Sigma, USA). The cells were mixed with a pipette tip to assist in cell lysis and incubated for 20 min on ice. The cell lysate was then used as the source of total RNA for RT-PCR reactions. For the initial RT-PCR amplification of hprr mRNA, 4 μl of the cell lysate was used in a final volume of 20 μl RT-PCR containing 2 μl of 10×VM buffer 10 mM MgCl₂ (Promega, USA), 0.4 μl of each dNTP 25 mM (Promega, USA), 0.4 μl of oligo dT (0.5 μg/μl), 1.0 μl of hprr-specific 5’ primer (10 μM; 5-TTA CCT CAC TGC TTT CG GA-3) and 3’ primer (10 mM; 5-GAT GGC CAC AGG ACT AGA AC-3), 0.4 μl of AMV reverse transcriptase (5 U/μl) (Promega, USA), 0.4 μl of Tfl DNA polymerase (5 U/μl) (Promega, USA), and 10.4 μl of sterile dd H₂O. The reaction mixture was overlaid with mineral oil and placed in a Robocycler gradient 96 (Stratagene, USA) for 45 min at 48°C and for 2 min at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 68°C, with the last cycle containing 7 min extension at 68°C. The product from this reaction was diluted 1:100 in sterile H₂O, and 1 ml of this dilution was used as cDNA template in a nested PCR. Thirty microliter nested PCR reaction contained 3 μl of 10×VM buffer (27.5 mM MgCl₂), 1 μl of hprr-specific 5’ primer (10 mM: 5-GGC TTC CTC CTC AGA CCG CT-3) and 3’ primer (10 mM: 5-GGC AAC A TC AAC AGG ACT CC-3), 0.3 μl of Taq DNA polymerase (5 U/μl) (Takara, Japan), and 22.7 μl of sterile H₂O. The reaction mixture was overlaid with mineral oil, and incubated for 4 min at 94°C, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with the last cycle containing a 7 min extension at 72°C. An aliquot of 5 ml of the nested PCR product was analyzed on an 8% polyacrylamide gel to evaluate the PCR efficiency. For direct sequencing of hprr PCR products, the remainder of the nested PCR products was filtered using PCR product purification kit (Roche, Germany), and aliquots of these PCR products were then sequenced.

Statistical analysis

Mann-Whitney U-statistic was used to evaluate the statistical difference between mutation frequency data from control versus various treated groups. The statistical analysis for hprr mutation spectra was performed using Cariello’s method [6].

Results

Test material-associated mutagenicity

Ozone, NNK, DBP, and combined treatment of NNK and DBP on ozone were assayed for the mutant frequency of 6-thioguanine-resistant (TG) spleen lymphocytes in male and female mice after 32- and 52-wk exposures. All treated groups showed higher frequencies of TG lymphocytes compared to the control groups in both mice sexes. Additive interactions were noted from ozone + NNK and ozone + DBP groups compared to the ozone alone group in both
sexes in the 32-wk study (Figs. 1 and 2). The frequencies of TG lymphocytes in ozone + NNK and ozone + DBP groups were higher than that of ozone alone group in male and female mice, respectively. All of which except ozone treated female mice showed statistically significant increase of hprt mutation frequency. Higher frequencies of TG lymphocytes were observed in all treated groups in both sexes compared to the control group after 32-wk exposure. However, NNK and DBP alone group did not show any significant changes in 32-wk exposure (Figs. 1 and 2). In contrast to 32-wk data, clear significant changes were observed in 52-wk group. Ozone, NNK, and DBP groups showed high significant increase of hprt mutation frequencies except NNK treated female mice. All combination group indicated that combined treatment caused additive effects. Especially, all treated groups exhibited dramatic additive effects (Figs. 1 and 2).

**Analysis of hprt mutations in T-cells from spleens of control and test materials-exposed B6C3F1 mice**

Analysis of the spontaneous hprt mutant clones yielding cDNAs revealed that transversion was the most frequent mutations (Tables 1 and 2). We were interested in testing whether two mutational spectra of control group and each treatment group were derived from the same underlying population. For this purpose we used Cariello et al. [5] code which we downloaded from http://www.ibiblio.org/dnam/mainpage.html, which was the pc version of Adams and Skopek’s [30] algorithm. The number of iterations that we requested was 10,000 for each run. We observed that the unadjusted p-values for DBP and ozone + DBP groups for male mice were 0.0147 and 0.0423, respectively. Therefore, even after correcting for multiplicity via a Bonferroni adjustment, DBP group has a significant difference with the control for alpha = 0.1.

**Discussion**

The toxicologic actions of ozone, NNK, and DBP have been extensively studied. However, relatively little is known on the significant toxicologic interactions among these toxicants. Studies examining the effects of air pollutants often use a single compound. However, because actual exposures involve more than one chemical, it is necessary to assess responses following the exposures to various combinations of chemicals. The effects of simultaneous exposure to two or more chemicals produce a response that may simply be additive of their individual responses or may be greater or less than that expected by addition of their individual responses. The study of these interactions can lead to a better understanding of the toxic mechanism of the chemicals involved. A number of terms have been used to describe pharmacological and toxicological interactions. An additive effect occurs when the combined effect of two or more chemicals is equal to the sum of the effects of each agent given alone (example: 2 + 3 = 5). A synergistic effect occurs when the combined effect of two or more chemicals are much greater than the sum of the effects of each agent given alone (example: 2 + 2 = 20). Potentiation occurs when one substance does not have a toxic effect on a certain organ or system, but, when added to another chemical, makes that chemical much more toxic (example: 0 + 2 = 10). Antagonism occurs when two or more chemicals administered together interfere with others actions or one interferes with the action of the other (example: 4 + 0 = 1). Thus, the potential additive effects of NNK, DBP, and NNK/DBP-coexposure on the genotoxic capacity of ozone were determined.

In our study, all treated groups showed increases in the frequencies of TG lymphocytes compared to the control groups in both sexes of mice. Additive interactions were noted from two combination groups compared to the ozone alone group in both sexes of the 32-wk study. In addition, the increases in the frequency of TG lymphocytes were observed in all treated groups in both sexes compared to the control group for 52-wk exposure. Furthermore, all
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combination groups in both sexes showed additive effects on ozone alone in the 52-wk study. Interestingly, *hprt* mutation spectra did not match with *hprt* mutation frequency except that DBP and DBP+ozone showed significant changes. This finding strongly suggests that *hprt* mutation frequency rather than *hprt* mutation spectra may be useful for biomarker of exposure. In fact, the pattern of *hprt* mutation spectra could appear to vary by different chemicals, *i.e.*, ozone, DBP, NNK in our experiment. In fact, the mutation spectra could be the results of different mutagenic process as well as varying selectivity. Therefore, this may be why the discrepancy between mutation frequency and spectra is present in our study. Meng *et al.* [25] found that both exposure duration and exposure concentration were important in determining the magnitude of mutagenic response to butadiene. Therefore, *hprt* mutation spectra in our study could be variable upon to exposure duration and concentration as well.

### Table 1. DNA sequence analysis of *hprt* mutant in splenic cells of B6C3F1 male mice in 52-wk study

| Type of mutation | Control | Ozone | NNK | DBP** | Ozone+NNK | Ozone+DBP* | Ozone+NNK +DBP |
|------------------|---------|-------|-----|-------|-----------|------------|----------------|
| Base substitution |         |       |     |       |           |            |                |
| GC to AT         | 2 (12)  | 4 (17) | 1 (5) | 1 (4) | 2 (6)     | 5 (20)     | 3 (9)         |
| TA               | 0 (0)   | 3 (13) | 3 (14) | 7 (27) | 6 (17)    | 4 (16)     | 2 (6)         |
| CG               | 2 (12)  | 6 (25) | 0 (0) | 3 (12) | 4 (11)    | 6 (24)     | 4 (12)        |
| AT to GC         | 3 (18)  | 1 (4)  | 3 (14) | 5 (19) | 3 (9)     | 2 (8)      | 6 (18)        |
| CG               | 0 (0)   | 3 (13) | 5 (24) | 3 (12) | 3 (9)     | 0 (0)      | 7 (21)        |
| TA               | 7 (41)  | 2 (8)  | 5 (24) | 1 (4)  | 6 (17)    | 1 (4)      | 4 (12)        |

**Insertions**

1 (6) 3 (13) 2 (10) 4 (15) 5 (14) 3 (12) 3 (9)

**Deletions**

2 (12) 2 (8) 2 (10) 2 (8) 6 (17) 4 (16) 5 (15)

**Total Clones**

17 (100) 24 (100) 21 (100) 26 (100) 35 (100) 25 (100) 34 (100)

*unadjusted* p<0.05

**adjusted** p<0.1

The number in the parenthesis indicate percentage versus the number of total clones.

### Table 2. DNA sequence analysis of *hprt* mutant in splenic cells of B6C3F1 female mice in 52-wk study

| Type of mutation | Control | Ozone | NNK | DBP** | Ozone+NNK | Ozone+DBP* | Ozone+NNK +DBP |
|------------------|---------|-------|-----|-------|-----------|------------|----------------|
| Base substitution |         |       |     |       |           |            |                |
| GC to AT         | 3 (21)  | 3 (14) | 3 (14) | 5 (30) | 6 (23)    | 4 (15)     | 2 (6)         |
| TA               | 2 (14)  | 2 (10) | 3 (14) | 0 (0)  | 2 (8)     | 3 (12)     | 3 (9)         |
| CG               | 1 (7)   | 2 (10) | 2 (9)  | 2 (11) | 1 (4)     | 4 (15)     | 3 (9)         |
| AT to GC         | 3 (21)  | 4 (19) | 1 (5)  | 0 (0)  | 4 (15)    | 4 (15)     | 7 (21)        |
| CG               | 2 (14)  | 5 (24) | 3 (14) | 4 (22) | 5 (19)    | 2 (8)      | 3 (9)         |
| TA               | 1 (7)   | 2 (10) | 4 (18) | 3 (17) | 1 (4)     | 3 (12)     | 5 (15)        |

**Insertions**

1 (7) 2 (10) 3 (14) 3 (17) 4 (15) 3 (12) 5 (15)

**Deletions**

1 (7) 1 (5) 3 (14) 1 (6) 3 (12) 3 (12) 5 (15)

**Total Clones**

14 (100) 21 (100) 22 (100) 18 (100) 26 (100) 26 (100) 33 (100)

*unadjusted* p<0.05

**adjusted** p<0.1

The number in the parenthesis indicate percentage versus the number of total clones.
distinct nucleotide sequences, which carry hereditary information. Alteration to any of these sequences resulting in base-pair substitutions, deletions, insertions or frameshifts may lead to mutation. Mutation induction has been implicated in several other debilitating disorders, suggesting the importance of this biological phenomenon to human health and disease. Mutation is thought to arise from three major sources: endogenous DNA damage, errors of DNA replication, and unknown exogenous factors [4]. Other important elements in mutagenesis include the various DNA repair and damage tolerance pathways, which may be responsible for and mitigate against the formation of mutations [2]. The characterization of induced mutations might provide clues to their origin and has, therefore, been pursued at different levels. Base substitution types often can be explained by known mechanisms of mutagenesis and may be examined at unique sites in specially designed bacterial reversion assays. For forward mutations, which are known to be non-randomly distributed, the location, strand bias, and sequence context of the mutations may be assessed additionally. The resulting distributions of alterations along known reference sequences, known as mutation spectra, are compiled in databases [5]. The concept of mutation spectra was originally developed in connection with the tumor suppressor gene p53, which has been found to be frequently and diversely mutated in tumor biopsies. Comparison of such mutation spectra from specific cancer types, thus, can provide clear clues to unravel the mechanisms of carcinogenesis [16]. To strengthen the linkage with chemical exposure, the observation of mutated cell cycle regulation genes in tumors ought to be accompanied by in vitro and biomonitoring studies of mutational specificity, which may be carried out using endogenous selectable markers such as hprt or artificially introduced reporter genes. All of these systems require the selection and DNA sequence analysis of numerous mutant clones. An important component in the application of lymphocyte hprt assays for the study of in vivo mutation is the characterization of DNA sequence changes responsible for the mutant phenotype. The generation of a mutant spectrum, i.e. the relative frequency of the different types of DNA sequence alterations and their distribution over the sequence of the target gene, is generally considered to be mutagen-specific. This specificity is related to the types of DNA lesions induced, the sites where lesions are formed, the mutagenic potency of the lesion, and the rate at which the lesions are repaired. In this study, mutants from treated and control B6C3F1 mice were examined for mutations in the hprt gene to determine if the test material treatment resulted in an agent-specific mutation profile.

Our study revealed that the most common type of mutations in treated male and female mice was transversion with few transitions. Such dominant transversion may be responsible for mixture-induced genotoxicity in our study. In fact, Masumura et al. [23] found that long term treatment of 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline in gdp delta transgenic mice caused the increase of G: C to T: A transversion in both time- and dose-dependent manner. Their findings support our results that mixture-induced genotoxicity is associated with transversion. In fact, large accumulation of transversion is known to be related to aging-dependent mutations [3]. Taken together, large portion of transversion may be responsible for mixture-induced genotoxicity in our study.

In conclusion, this study examined the potential additive effects of genotoxities of NNK, DBP, ozone, and their various combinations. The results indicate that, under our experimental conditions, combined exposure to ozone, NNK, and DBP induces additive effects of genotoxities compared to exposure to ozone alone. Furthermore, mutational responses, as revealed by the lymphocyte hprt assay, are capable of producing mutation profiles that reflect the DNA damage-induced mutation.

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