The Journal of Young Investigators
The premier undergraduate research journal

In vitro Method to Quantify UV mediated DNA Damage

Kimberly Lynch[1,2] and Robert G. Pergolizzi[1]

[1]Bergen County Academies, Hackensack, New Jersey, 07601, USA
[2]Columbia University, New York, New York, 10027, USA

Volume 20, Issue 2
August 2010

In vitro Method to Quantify UV mediated DNA Damage

Kimberly Lynch[1,2] and Robert G. Pergolizzi[1]
[1]Bergen County Academies, Hackensack, New Jersey, 07601, USA
[2]Columbia University, New York, New York, 10027, USA

ABSTRACT
The gel-based electrophoretic assay described in this study measures the levels of DNA damage caused by ultraviolet (UV) light, particularly UVB radiation, directly, in the absence of any possibility for DNA repair. The correlation between exposure to UV light, particularly UVB radiation, and increased skin cancer risk has necessitated the ability to easily assess DNA damage and the protective effects of certain UV absorbing chemicals. UV exposure followed by alkali treatment and gel electrophoresis reveals the extent of DNA Damage to be directly correlated with gel mobility. To enhance the sensitivity of the assay, T4 Endonuclease V was used to induce strand breaks at positions of cyclobutane dimmer formation. The potential utility of this assay in assessing the ability of sunscreen reagents to protect against DNA damage was demonstrated by examining the impact of UV-absorbing chemicals PABA and HMBS at different doses of UVB light. When these chemicals were included during irradiation of DNA protection was demonstrated in a dose-dependent manner. The assay described in this study provides a simple and rapid measure of DNA damage with the potential application to quantify the level of protection afforded by various chemicals.

INTRODUCTION

UV Light

Exposure of DNA to ultraviolet (UV) radiation, including UVB (280-315nm) and UVA (315-400nm), is known to have structural and chemical effects. These reactions occur within picoseconds of exposure (Schreier 2007). Absorption of UVB by cellular DNA gives the highest degree of DNA damage, with both cytotoxic and genotoxic effects (Sinha 2002). UV exposure has been linked to increased occurrence of several important conditions including skin cancer, cataracts, premature aging of the skin, and impaired immune function (Ambach 1993).

Depletion of the stratospheric ozone layer, the protective layer extending upward from about 6 to 30 miles above the Earth's surface that shields the Earth from harmful solar radiation, has been clearly evidenced (Elkins 1993, Toon 1991, Tabazadeh 2000). Chemicals in the environment, including chlorofluorocarbons, chlorocarbons and organo-bromides are gradually degrading the stratospheric ozone layer resulting in an increase in the solar UVB radiation that reaches the Earth's surface (Blumthaler 1990, Crutzen 1992, Kerr 1993, Lubin 1995). Studies have shown that exposure to UV correlates to an increased risk of skin cancer (Armstrong 2001, English 1997). This relationship, with its affects on public health, specifically skin cancer incidence, raises concerns about exposure to ultraviolet radiation.

DNA Damage

Absorption of UV radiation by DNA produces specific pyrimidine photoproducts, which cause distortion of the DNA helix (Sinha 2002, Cleaver 2002). Among the products of the

interaction between DNA and UV radiation are the cyclobutane pyrimidine dimers (CPDs), and 6-4 photoproducts (6-4PPs) (Sinha 2002). Both of these lesions have been shown to be mutagenic and cytotoxic, and to cause alterations in the sequence of nucleotide bases, DNA-protein interactions, and secondary structure of the DNA (Cleaver 2002). Formation of pyrimidine dimers, involving the covalent bonding of adjacent thymine and cytosine bases, is a common occurrence resulting from the interaction of DNA with UV radiation (Heikkinen 2006). These lesions alter transcription and replication processes, and can be fatal to the cell if uncorrected (Sinha 2002, Altieri 2008).

The relationship between UV radiation and skin cancer has been firmly established (Armstrong 2001, English 1997). With increasing exposure to UV radiation at sea level, as the protective ozone layer of the atmosphere diminishes, public concern about protection from UV radiation has increased (Elkins 1993, Toon 1991, Tabazadeh 2000). UVB light is known to produce covalent bonds between adjacent thymidines and less frequently between other neighboring pyrimidine pairs. These structures are known to induce kinks in the structure of DNA and must be repaired prior to DNA replication. Failure to repair these defects is known to contribute to the development of skin cancer.

Sunscreen Agents HMBS and PABA

Sunscreens have been shown to reduce the risk of certain cancers, and have become increasingly popular in recent years (Cummings 1997, Forestier 2008, Villalobos-Hernández 2006, Westerdahl 2000). It has been shown that regular use of sunscreen products promotes positive public health as part of a strategy to reduce overall UV exposure (Gasparro 1998). Although the level of protection afforded by these products is commonly listed as the “SPF” level (sun protection factor), an index of the amount of UV light absorbed by the cream, it does not indicate the relative toxicity of the various components, nor does it indicate how rapidly the protective effect is exhausted by exposure to UV (Maier 2005).

In an effort to slow the increase in the number of skin cancer cases reported, the public has been urged to use sunscreen products, reduce skin exposure during hours of intense sunlight, wear protective clothing, and be aware of the effects of UV radiation on health (Cummings 1997). Increasing importance has been put upon the application of sunscreens containing UV-absorbing chemicals, which work to protect against cellular damage (Bissonauth 2000). Consequently, selection and concentration of active ingredients for use in sunscreens has become an important public health issue. Chemicals commonly employed for this purpose include benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (HMBS) and 4-aminobenzoic acid (PABA), among others. These compounds should be able to intercept and/or scatter UV radiation before it can penetrate the skin and cause DNA damage. Attempts have been made to identify the optimal combination of compounds to maximize protection while limiting toxicity (Tanner 2006). However, the degree of protection provided by various chemicals, alone and in combination, has been difficult to assess.

Other Established Studies and Results

Other methods have been described that attempt to measure the type and level of DNA damage caused by UV radiation, but each such assay has limitations. The current gold standard, known as the comet assay, is widely used by pharmaceutical companies to assess DNA damage. In this cell-based method one suspends and lyses cells in low-melting-point agarose and subsequently electrophoreses the samples (Singh 1988). Visualization of the DNA using fluorescence and imaging software proved difficult to quantify and was less precise due to the potential for DNA repair in a cell mediated environment. Another technique for measuring DNA single strand breaks is the alkaline elution procedure. Although this technique is sensitive to DNA damage it is prone to variability and inconsistency due to incomplete exchanges of solutions between cell rinses and elution portions of the procedure (Koch 1994). Supercoiled circular DNA has also been used to detect DNA single-strand breaks (Cahová-Kucharíková 2005). T4 Endonuclease V enzyme has been used to detect the breaks with alternating current voltammetry and can be used to detect base damage in DNA and living bacterial cells (Cahová-Kucharíková 2005, Audic 1993). This technique is used with plasmid DNA and so the procedure described in this paper provides an alternate for use with different DNA structures.

Gel Electrophoresis

In this assay, high molecular weight DNA exposed to UV radiation for varying periods of time was treated with alkali, electrophoresed on agarose gels and stained with ethidium bromide. Smaller pieces of DNA migrate faster through a gel matrix due to the charge/size ratio of the DNA molecule and the enhanced ability of small molecules to navigate the network of channels and spikes that structurally characterize the topography of the gel (Bissonauth 2000). Double-stranded DNA molecules behave as rigid rods in solution and migrate in a manner that is logarithmically related to their size (Zhu 2005).

The goal of the research presented here was to develop an assay to directly measure the impact of UV radiation on DNA in the absence of cellular repair mechanisms. This assay is superior to the comet assay in that it can be scaled up as needed, and does not permit any DNA repair to occur. In this research we demonstrate that this method provides a means to compare the efficacy and potency of potential sunscreen ingredients, and should permit the development of better and safer protective agents.

MATERIALS AND METHODS

The assay described in this section is an improved gel electrophoretic method for the detection of UV induced DNA damage. The specific materials used are described below.

Reagents. Chemicals and reagents were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise specified. HMBS was obtained from Riedel-de Haën (Steinheim, Germany). PABA was obtained from Fluka (Steinheim, Germany). T4 Endonuclease V was obtained from

Epicentre Biotechnologies (Madison, Wisconsin). All experiments were conducted within the Stem Cell Research Laboratory at the Bergen County Academies, Hackensack, NJ.

Analytic Methods. High molecular weight, double stranded salmon sperm DNA was dissolved in 0.04M Tris-acetate (pH 7.4), 1mM EDTA and 0.5% Triton-X-100 at a concentration of 100µg/ml. Reactions were performed using 1.5µg of this DNA solution in clear 1.5ml micro-centrifuge tubes (Eppendorf AG, Hamburg, Germany). Following addition of other components for testing, tubes were placed flat on the surface of a UV transilluminator (Gel Logic 1500 Imaging System, Carestream Health, Inc, Rochester, NY) and exposed to UV light for varying periods of time. A UVA/UVB meter was obtained from Solar Light Co. (Glenside, PA). Using this device, the spectral power distribution of UV from the transilluminator was found to provide 16.9W/m^2 and 1.0 Minimal Erythema Dose (MED) per hour. A MED of 1.0 will produce a minimum erythema (reddening) for fair Caucasians (type II skin) on skin areas not ordinarily subjected to UVR. A dose of 21 +/- 3mj/cm^2 at 297nm is a minimal erythema dose (Parrish 1982). The exposure times used in this study, which ranged from 0 to 35 minutes at this dose level, span a dose range from levels encountered under normal physiologic conditions to more extreme conditions. The UV transilluminator provided UVB radiation of 306nm wavelength, and permitted identical exposure of up to 20 reactions at once.

The extent of damage to the DNA was assessed by gel electrophoresis on a 1% agarose gel containing TAE buffer (0.04M Tris acetate, pH 7.4 and 1mM EDTA) (Sambrook 2001). Agarose-TAE gels (1%) were cast in a 7.5cm x 7cm gel box (Edvotek, West Bethesda, MD). To assess the impact on UV damage to DNA, chemicals such as HMBS and PABA, commonly found in commercial sunscreen products, were added at varying concentrations to the tubes containing DNA prior to UV exposure, and subjected to UV radiation for 0 to 35 minutes. It was determined that a minority of DNA molecules showed complete double strand breakage, and that to correctly assess the amount of damage it was necessary to denature the DNA molecules prior to electrophoresis. Following exposure, 5ml of 10% glycerol in TAE was added to the samples, which were then loaded into their respective lanes on the gel and electrophoresis was performed for 50 to 75 minutes at 86V. The gels were stained with ethidium bromide (2.5µg/ml in TAE for 15 minutes) and photographed on the Gel Logic 1500 Imaging System. The general flow of the experiment is detailed in Figure 1. All experiments were repeated a minimum of five times and results reported as the mean and standard deviation of all measurements.

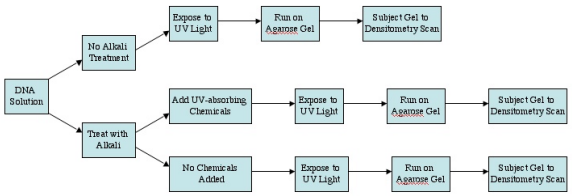


Figure 1: Flow Diagram of Methodology for Assessing DNA Damage. This diagram illustrates the basic steps in performance of the assay.

T4 Endonuclease V, an enzyme which cleaves DNA at sites of CPD damage, was used to compare the gel analysis of UV damaged DNA to determine what percent of the total damage was of the CPD category. To examine specific cyclobutane pyrimidine dimer formation, UV exposed DNA was treated with 40 units of T4 Endonuclease V in a 50ml reaction of activity assay buffer containing 50mM Tris-HCl (pH 7.5) and 5mM EDTA. The samples were then incubated for 5 hours at 37ºC and electrophoresed as described above. The outline of this experiment is detailed in Figure 2.

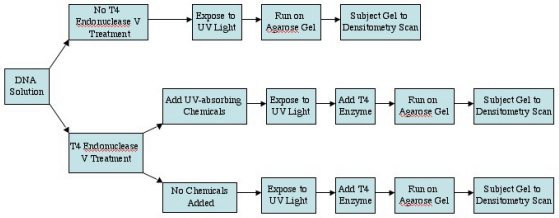


Figure 2: Flow Diagram for an Experiment Demonstrating the Ability of the Assay to detect Damage at CPDs and 6-4PPs.

Statistics and Analysis. Photographs of DNA after electrophoresis were subjected to densitometric analysis using Molecular Imaging software (Carestream Health, Inc, Rochester, NY; version 4). Each lane was imaged to analyze the migration of DNA. The extent of DNA damage was determined by the distance from the well to the midpoint of the stained DNA. All determinations were done in triplicate, and results were reported as mean +/- standard deviation. For assays that were performed as a function of time, the best fit line was determined using Excel (Microsoft Corporation, Redmond, Washington, U.S.A.). For experiments where the impact of UV-absorbing chemicals were measured, the differences in the slopes of the best-fit lines were compared, and taken to be a measure of the effectiveness of protection of DNA from damage. Since the greatest change in migration was always seen between 0 and 15 minutes of exposure, comparisons between different UV-absorbing chemicals were made using the 0, 5, and 15 minute time points to derive the slope of the best-fit line.

RESULTS

An agarose gel assay measuring UV-induced damage

DNA damage resulting from exposure to UV light was quantified by a gel electrophoresis assay. The distance from the loading well to the midpoint of the region of maximum intensity after alkali treatment and staining was used as a measure of strand breakage. To determine optimal alkali treatment several methods of denaturation were compared. This led to the conclusion that damage could be best observed by adding 15ml of 0.3M NaOH, 1mM EDTA (subsequently referred to as alkaline buffer) to each tube. The distance migrated was found to be directly proportional to the degree of UV exposure. An example of this analysis is shown in Figure 3. Using UV exposure times of 0 to 20 minutes (lanes 1-5) without alkali treatment, no change in migration was noted. However, the same exposure times followed by alkali treatment (lanes 6-10) clearly showed migration patterns directly proportional to exposure. The implication is that, for this range of UV exposures, single strand DNA damage does not cause double strand breakage. Despite the damage inflicted by UV

exposure the strands are held together by hydrogen bonding. Alkali treatment, which denatures the DNA strands, reveals the underlying damage clearly. This was most apparent when alkali was added to the post-exposure loading solution. Inclusion of alkali in the gel electrophoresis buffer did not improve the overall result (data not shown).

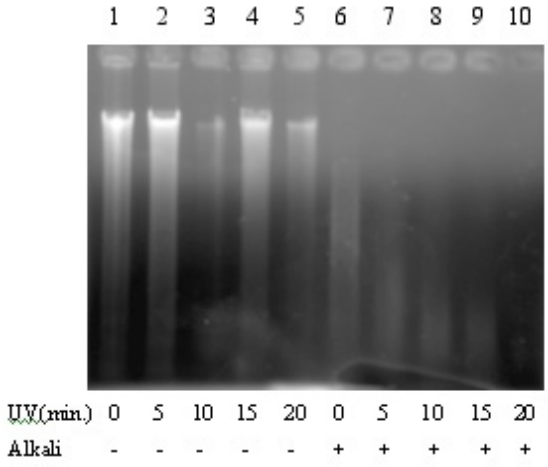


Figure 3: Effect of alkali on UV-induced DNA strand breakage Double-stranded salmon sperm DNA was exposed to UV light, treated with alkali, electrophoresed, stained with ethidium bromide, and visualized by UV excitation as described in Materials and Methods.

UV - dose dependent fragmentation patterns

Experiments to determine the optimal times of exposure, as a prelude to measuring the impact of UV-absorbing chemicals on DNA damage, were performed. DNA exposed to UV light for intervals from 0 to 35 minutes, revealed a dose dependent fragmentation pattern, as illustrated in Figure 4. In Figure 4a, the impact of UV exposure ranging from 0 to 35 minutes, followed by alkali treatment are shown. No UV, no alkali and no UV, plus alkali controls were always included on the gel assays as an index of initial DNA conditions, and are shown in lanes 1 and 2, respectively. Based on the amount of fragmentation that occurred when DNA was exposed to UV light in this experiment, an average exposure time of 15 minutes was chosen for most subsequent experiments. The assay detected that even very brief UV exposures produced damage.

Gel densitometry was performed on all experiments to determine the point of greatest DNA density following UV exposure and alkali treatment. These maxima were used in all subsequent calculations and comparisons. Examples of densitometric analysis of selected lanes (lane 2, 4 and 6) are shown in Figure 4b. Having determined these maxima, results of three analyses were averaged, and plotted as UV exposure time vs. migration (in centimeters), with each data point illustrating the mean +/- standard deviation. The analysis of the gel shown in Figure 4a is represented in Figure 4c. The best straight line from these data points was determined in each assay, and the slope of the line was taken to be an index of UV damage to DNA per unit time. This formed the basis for all subsequent comparisons. The impact of UV exposure on DNA in the absence of any other UV absorbers was quantified as described in Materials and Methods, using the 0, 5 and 15 minute time points. The best fit line using these points was found to be $y = 0.0643x + 5.1214$.

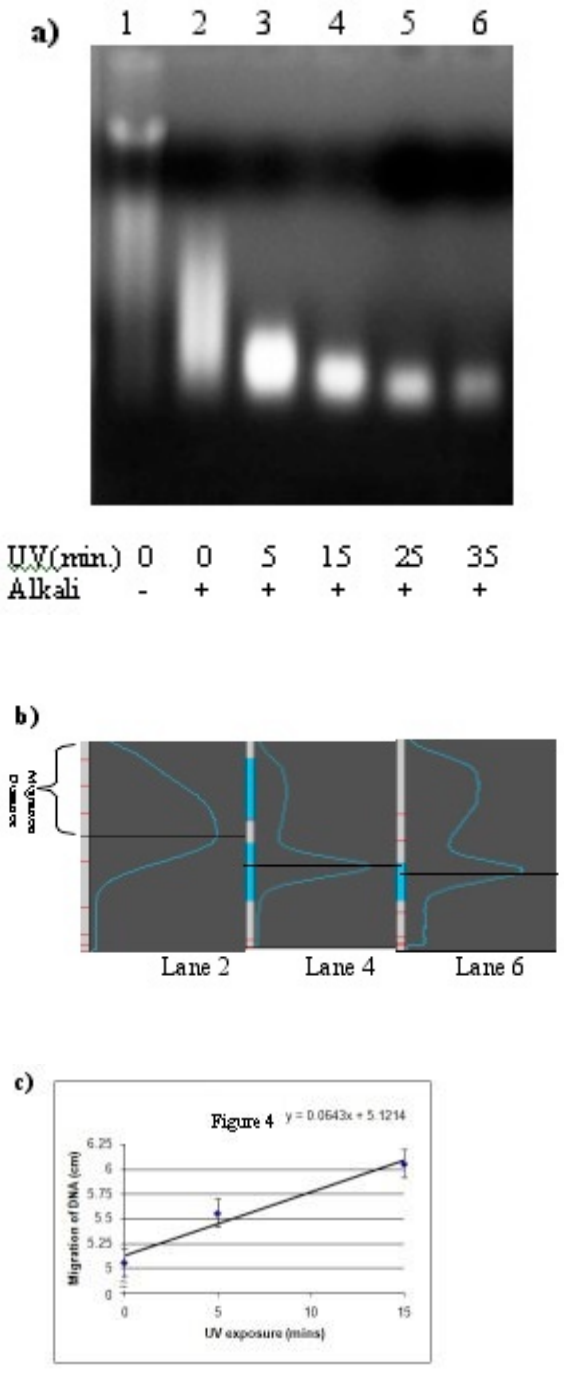


Figure 4: Impact of increased UV exposure on DNA damage. a) A photograph of the gel assay. **b)** Graphical densitometry of selected lanes from Figure 2a, illustrating the decision making process for maximum intensity of DNA staining. **c)** Graphical representation of UV-induced strand breakage with increasing exposure.

UV-dose dependence with the addition of T4 Endonuclease V

Cyclobutane pyrimidine dimers (CPDs) and 6-4 PPs are the predominant UV-induced lesions formed in DNA (Cahová-Kucharíková 2005). It has been shown that 6-4 PPs are more efficiently repaired in human cells than CPDs, consequently CPDs hold a higher risk for genetic consequences of DNA damage (Garinis 2005). CPDs have been shown to be more heritable than 6-4 PPs and have been shown to be involved in the development of non-melanoma skin cancer (Tung 1996). The enzyme, T4 Endonuclease V, recognizes and removes cyclobutane pyrimidine dimers. During subsequent electrophoresis, enzyme-induced strand breakage is seen in the more rapid migration of DNA fragments. The increased sensitivity of the T4 Endonuclease V enzyme to detect specific UV-induced DNA lesions allows for the use of much lower doses of UVB thus making the assay more physiologically meaningful.

Based on the demonstrated ability of the repair enzyme, T4-Endonuclease V, to identify DNA lesions induced by UV (specifically CPDs), we assessed the impact of CPD damage to DNA resulting from UV exposure in aqueous solution. DNA was exposed to UVB light for intervals from 0 to 5 minutes at a dose rate of 16.9 W/m^2, with the subsequent addition of the T4-Endonuclease V enzyme. DNA migration following this treatment was compared to migration of UV damaged DNA without the enzyme. The results of this experiment are shown in Figure 5. Activity Assay buffer was added to every lane in this figure. No UV, no enzyme and no UV, plus enzyme controls were

included as an index of initial DNA conditions and are shown in lanes 1 and 2, respectively. Lanes 3 through 8 show the relative effects of increased UV exposure to the DNA with and without the enzyme allowing for a direct comparison between the amount of CPD lesions present and the amount of total lesions induced by UV exposure. Lane 9 showed a control of DNA, activity assay, alkali, and 5 minutes of UV to show the comparative abilities between the use of alkali in the assay and the use of the enzyme.

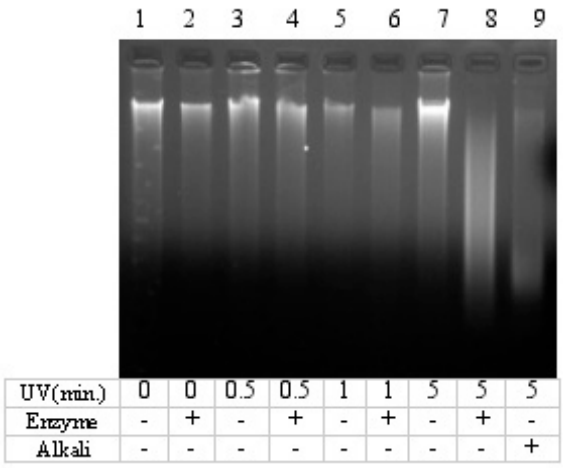


Figure 5: Formation of UV-induced CPDs and 6-4 PPs in DNA. This figure shows the impact of increasing UV time exposure with and without the enzyme (T4 Endonuclease V).

Impact of UV-absorbing chemicals on DNA damage

Using the standard exposure time of 15 minutes (determined by the analysis in Figure 4a), this assay was employed in an attempt to compare the relative abilities of UV-absorbing chemicals commonly used in commercial sunscreen formulations to protect DNA from UV-induced damage. In preliminary analyses, increasing concentrations of UV-absorbers were added to solutions of DNA, exposed to UV light for varying amounts of time, and studied for their ability to protect DNA from damage (data not shown). These analyses determined the optimal concentrations for comparison. Final concentrations of 12 mM HMBS and PABA, two chemicals commonly used in commercial sunscreen products, were chosen as a basis of comparison, and the results compared as described above. The UV-absorption spectra of these compounds are shown in Figure 6. Although PABA has stronger absorption in the UVB range, HMBS has an absorption maximum at around 285nm. HMBS also absorbs more strongly than PABA in the UVA range.

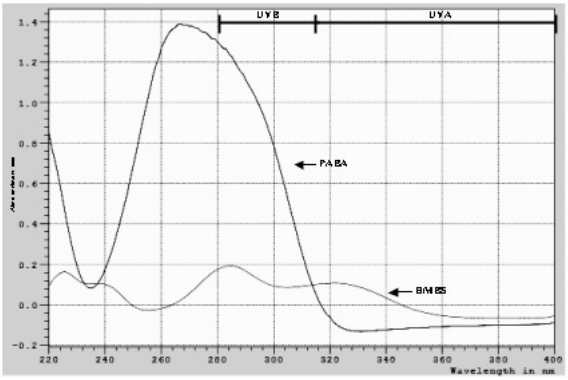


Figure 6: UV absorption spectra of PABA and HMBS. This figure shows a comparison of the absorption spectra of aqueous solutions of 3.6mM HMBS and .09mM PABA. UVA and UVB ranges are illustrated on the figure.

PABA was shown to effectively protect DNA in this assay. The results of this study are shown in Figure 7. The protective effect of 12 mM PABA was not exhausted after the maximum exposure to UV light, affording high levels of protection. The gel analysis is shown in Figure 7a. The usual controls of no UV, no alkali and no UV, plus alkali are shown in lanes 1 and 2, respectively. Additional controls included PABA, plus alkali, without UV

(lane 3), and the standard 15 minute exposure, plus alkali, without PABA (lane 4). Lanes 5 through 8 illustrate the impact of PABA on DNA damage as UV exposure increases.

Not only is the protection significant, but it is apparent that the protective effect of PABA is not exhausted over time. A graphical representation of this analysis in triplicate is shown in Figure 7b. Importantly, the slope of the line representing DNA damage in the presence of PABA is 0.0307. This value, when compared to the slope (0.0643) of UV-induced strand breakage with increasing exposure in Figure 4c, indicates that the UV light is causing less damage to DNA per unit time with the addition of PABA.

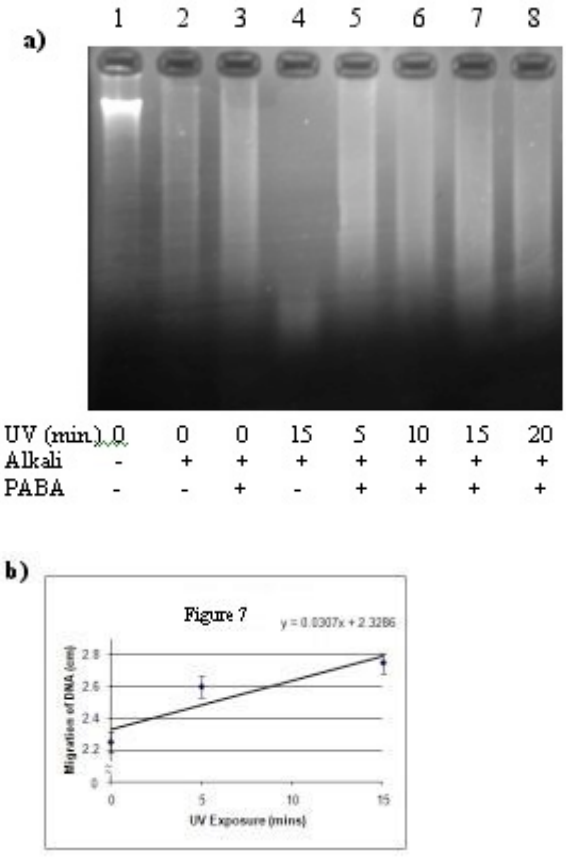
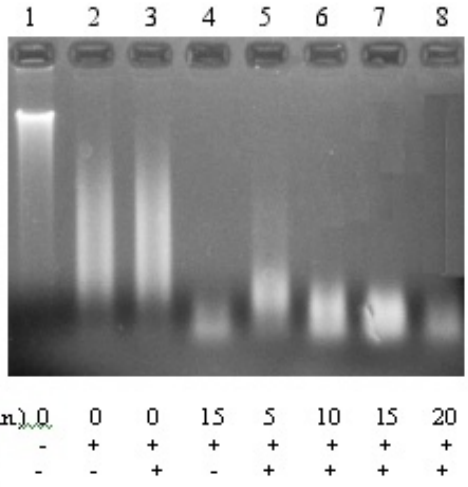


Figure 7: Effect of PABA on UV-induced DNA strand breakage. a) A photograph of the gel assay. **b)** Graphical representation of the impact of PABA on UV-induced strand breakage with increasing exposure.

In contrast, a similar experiment performed with the UV protecting chemical HMBS at the same concentration (12 mM) revealed not only that the molecule provided less protection to the DNA than PABA, but also that this UV protection was easily overwhelmed by prolonged exposure. The results of this analysis are shown in Figure 8. The gel image in Figure 8a shows the controls of no UV, no alkali and no UV plus alkali in lanes 1 and 2; HMBS, plus alkali, without UV (lane 3) and the standard 15 minute exposure, plus alkali, without HMBS (lane 4). Lanes 5 through 8 show the impact of HMBS on DNA damage as UV exposure increases. Although some protective effect is evident, HMBS is relatively ineffective as a protective agent compared to PABA. A graphical representation of this analysis in triplicate is shown in Figure 8b. It is significant that the slope of the line representing DNA damage in the presence of HMBS is 0.0521, indicating that the UV light is causing less damage to DNA per unit time than the original solutions of DNA without UV-absorbing chemicals. The decrease in size of the DNA product over time demonstrated the limited ability of HMBS to protect DNA.

a)



b)

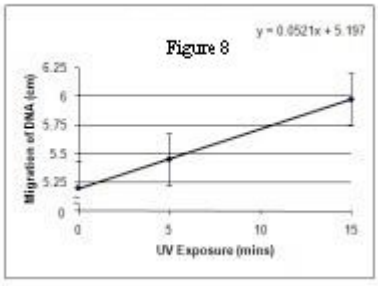


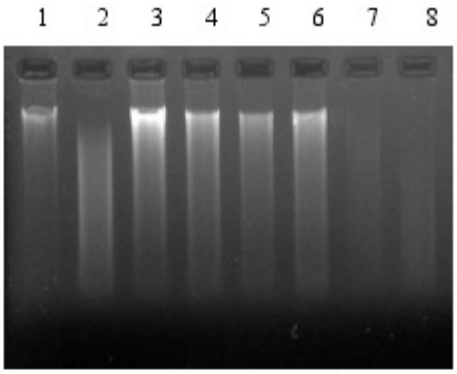
Figure 8: Effect of HMBS on UV-induced DNA strand breakage. a) A photograph of the gel assay. **b)** Graphical representation of the impact of HMBS on UV-induced strand breakage with increasing exposure.

Since there was a direct relationship between the dose of UV radiation and the average migration distance of the resulting DNA fragments, it was possible to mathematically relate dose and size in this assay. The UV dose-dependent fragmentation patterns with and without UV absorbing chemicals were analyzed for the quantitative assessment of DNA damage by fitting the data to the best straight line. In this manner, the slope of the line can be used as an index of the degree of protection afforded by various agents. This analysis not only illustrates the level of protection, but also the effective lifetime of the absorbing chemicals. DNA in the presence or absence of protective chemicals was exposed to UVR for 0, 5, or 15 minutes, fragmentation ranges were measured and used in the calculation of the corresponding slope for each. The resulting graphs are shown in Figure 4c, 7b, and 8b. In the absence of protective chemicals the slope of the resulting line was 0.0643 whereas with the addition of PABA the slope was changed to 0.0307 indicating a smaller change in migration distance over the same time periods. This shallower slope relates to the protection of PABA even at higher UV time exposures. The slope of the line relating HMBS to time exposure was 0.0521. This value is higher than that of PABA but lower than that of the original alkali experiment without any UV absorbing chemicals. HMBS provided some protection from UVR but not as much as PABA.

UV-absorbing chemicals in the presence of T4 Endonuclease V

T4 Endonuclease V was employed in an attempt to compare the relative ability of PABA and HMBS to protect DNA from UV-induced CPD lesions. Figure 9 clearly depicts the protective abilities of 12mM PABA in the presence of T4 Endonuclease V to protect DNA from damage after exposure to UV light. The control of no enzyme, no UV, is shown in lane 1. Lanes 2 through 6 show the impact of PABA on DNA damage as UV exposure increases. Lanes 7 and 8 show the limited protective ability of 12 mM HMBS with the addition of enzyme under increasing UV exposure. Figure 9 gives a comparison between the relative protective abilities of PABA and HMBS with the addition of enzyme. The data illustrate that PABA protects specifically

against CPD lesions as well as generally against other UV-induced lesions in DNA. This can be seen by comparing the data in Figures 6 and 8, in which the damage assessed by alkali fragmentation of the UV-damaged DNA is more pronounced than the damage detected by the enzyme.



UV(min.)	0	5	5	10	15	20	5	10
Enzyme	-	+	+	+	+	+	+	+
PABA	-	-	+	+	+	+	-	-
HMBS	-	-	-	-	-	-	+	+

Figure 9: Protective Effect of HMBS + PABA on the formation of UV-induced CPD lesions. This figure shows the impact of the UV-absorbing chemicals PABA and HMBS on UV-induced DNA damage in the presence of the enzyme (T4 Endonuclease V).

DISCUSSION:

The assay described in this study permits a rapid and quantitative assessment of the impact of UV-absorbing chemicals and other chemical modulators on the extent of UVB induced DNA damage. Although conceptually similar to the comet assay; a cell-based gel electrophoresis method to measure DNA damage (Singh 1988), the assay described here employs the combination of gel electrophoresis and digital imaging to allow quantitative assessment of DNA damage in relation to the samples' migration on the gel. The comet assay not only was more difficult to quantify, but also was less precise due to the potential for DNA repair, which can vary among cell types. Variations on the basic theme of this assay include exposing DNA to UV light at different intensities for a fixed time or at a fixed UV- intensity for different periods of time. Mixing DNA with chemicals that have varying characteristics prior to exposure to UV radiation allowed a determination of their ability to protect DNA from damage, in the absence of any cellular repair activity. Substances used in this study included PABA and HMBS, but other chemicals that absorb light in the UVB range could also be used.

It was determined that UV damage was most frequently single-stranded in nature, with the result that only extreme exposure to UV caused a shortening of the average DNA length at neutral pH. To enhance the sensitivity of the assay, alkali denaturation of the exposed DNA was employed. Although this restricted the ability to precisely size the DNA fragments, since single stranded DNA molecules cannot be accurately sized on non-denaturing gels, it enabled the visualization of UV damage after very short exposure times. Although it is conceivable that the extent of damage observed might vary dependent on the DNA concentration it is anticipated that the overall results would be the same.

The extent of damage to the DNA induced by UV light was directly related to the increased migration of the median intensity band of the DNA. In contrast to previous assays, including the comet assay, the amount of DNA and UV light exposure can be controlled by the experimenter. In addition, since the assay is completely extra-cellular, there is no opportunity for DNA repair and the

degree of damage can be directly assessed. Since the measurement of DNA damage is taken directly on DNA in solution, the impact of UV-absorbing chemicals can be measured without any cell-mediated interference. Several kinds of damage to DNA are known to occur as a result of UV radiation (intrastrand breakages, thymine dimer formation, and CPD formation) (Sinha 2002); many of these remain obscured in the context of double strand migration in previous assays. In this assay, the exposure of DNA to alkali allows for these types of damage to be evaluated by single strand migration.

The addition of the T4-endonuclease V enzyme validates the assay at low UV exposures and gives more physiological meaning to the assay. This enzyme cleaves DNA at the sites of CPD damage, specifically the glycosyl bond of the 5'-pyrimidine of the dimer and the 3' phosphodiester bond, resulting in breakage of the DNA strand (Friedberg 1995, Schrock 1993). It is thus particularly well adapted to the objective of the present study. The use of this enzyme allows for further examination of specific lesions other than single strand breaks that were easily seen with the alkali method. A recent study found that cyclobutane pyrimidine dimers appeared as the primary lesion responsible for responses in UV-exposed skin such as sunburn, hyperplasia, and apoptosis (Jans 2005). In that study CPDs were also verified as the principal lesions seen in non-melanoma skin cancer (Jans 2005).

The enzyme is useful in that UV-absorbing chemicals can be tested at physiologically meaningful UV doses thus giving a direct comparison to test the efficacy of each individual chemical in regard to DNA damage and the specific lesions as well. Experiments with HMBS showed that it did not protect DNA to a significant degree with respect to all lesions (Figure 8) and with respect to the CPD lesions studied by the T4 Endonuclease V enzyme (Figure 9). These experiments clearly show the greater efficacy of PABA than HMBS in protecting against all types of UV-induced DNA lesions.

Other methods have been described that attempt to measure the type and level of DNA damage caused by UV radiation, but each such assay has limitations. Supercoiled plasmid DNA is exquisitely sensitive to DNA damage but since a single lesion causes relaxation of the entire molecule the assay is limited in the sense that any lesion results in the same pattern (Cahová-Kucharíková 2005). With regard to the comet assay (Singh 1988), the results of this assay are highly variable even within the same lab and it has been notoriously difficult to quantify DNA damage based on this assay. Again, it is impossible to determine which type of damage has occurred to DNA. Other tests, such as the 3T3 NRU test serve to test the photoxic potential of a substance (Spielmann 1994, Anon 1998). Although DNA damage and phototoxicity can be directly related, the current assay is not designed to measure phototoxicity. Thus, it would be appropriate to run the 3T3 NRU test in addition to the one proposed here as they both provide different but complementary information sets.

The results shown in this report illustrate the difference in the protective lifetimes of two commonly used sunscreen ingredients, HMBS and PABA. A better index of protectiveness would be a great asset in making comparisons between products. From

the data included here it is apparent that PABA is able to absorb UV radiation more efficiently and for a longer time than HMBS and is therefore able to provide sunscreen protection at lower doses. Since the assay described in this paper can assess damage caused by all mechanisms in the absence of cellular repair processes and can assess the protective lifetimes of sunscreen ingredients, it may be a useful tool in developing more meaningful comparisons. Determining the contribution of different lesions to the overall level of damage observed and to the effectiveness of UV-absorbing chemicals on each type of damage is currently under investigation in our laboratory.

Acknowledgements- This research was supported by funds from the Bergen County Executive, the Bergen County Board of Freeholders, and Board of Education of the Vocational Schools in the County of Bergen. We are grateful for their generous support.

REFERENCES

1. Schreier, W. J., T. E. Schrader, F. O. Koller, P. Gilch, C. E. Crespo-Hernandez, V. N. Swaminathan, T. Carell, W. Zinth, B. Kohler (2007) Thymine dimerization in DNA is an ultrafast photoreaction. Science 315, 625-629.
2. Sinha, R. P. and D. P. Hader (2002) UV-induced DNA damage and repair: a review. Photochem Photobiol Sci. 4, 225-236.
3. Ambach, W. and M. Blumthaler (1993) Biological effectiveness of solar UV radiation in humans. Experientia 49(9), 747-753.
4. Elkins, J. W., T. M. Thompson, T. H. Swanson, J. H. Butler, B. D. Hall, S. O. Cummings, D. A. Fisher, and A. G. Raffo (1993) Decrease in the growth rates of atmospheric chlorofluorocarbons 11 and 12. Nature 364, 780–783.
5. Toon, O. B. and R. P. Turco (1991) Polar stratospheric clouds and ozone depletion. Sci. Am. 264, 68–74.
6. Tabazadeh, A., M. L. Santee, M. Y. Danilin, H. C. Pumphrey, P. A. Newman, P. J. Hamill, and J. L. Mergenthaler (2000) Quantifying denitrification and its effect on ozone recovery. Science 288, 1407–1411.
7. Blumthaler, M. and W. Ambach (1990) Indication of increasing solar ultraviolet-B radiation flux in alpine regions. Science 248, 206–208.
8. Crutzen, P. J. (1992) Ultraviolet on the increase. Nature 356, 104–105.
9. Kerr, J. B. and C. T. McElroy (1993) Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. Science 262, 1032–1034.
10. Lubin, D. and E. H. Jensen (1995) Effects of clouds and stratospheric ozone depletion on ultraviolet radiation trends. Nature 377, 710–713.
11. Armstrong, B. K. and A. Kricker (2001) Epidemiology of skin cancer. Photochem. Photobiol. 63, 8-18.
12. English, D. R., B. K. Armstrong, A. Kricker, and C. Fleming (1997) Sunlight and cancer. Cancer Causes Control 8(3), 271-283.

13. Cleaver, J. E. and E. Crowley (2002) UV Damage, DNA Repair and Skin Carcinogenesis. Front Biosci. 7, 1024-1043.
14. Heikkinen, K., K. Rapakko, S. M. Karppinen, H. Erkko, S. Knuutila, T. Lundán, A. Mannermaa, A. L. Børresen-Dale, A. Borg, R. B. Barkardottir, J. Petrini, and R. Winqvist (2006) RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability. Carcinogenesis 27, 1593–1959.
15. Altieri, F., C. Grillo, M. Maceroni, and S. Chichiarelli (2008) DNA Damage and Repair: From Molecular Mechanisms to Health Implications. Antioxidants & Redox Signaling. 10(5), 891-938.
16. Cummings, S. R., M. K. Tripp, and N. B. Herrmann (1997) Approaches to the prevention and control of skin cancer. Cancer Metastasis Rev. 16(3-4), 309-327.
17. Forestier, S. (2008) Rationale for sunscreen development. J. Am. Acad. Dermatol. 58, 133-138.
18. Villalobos-Hernández, J. R. and C. C. Müller-Goymann (2006) Artificial sun protection: sunscreens and their carrier systems. Curr. Drug Deliv. 3(4), 405-415.
19. Westerdahl, J. et al. (2000) Sunscreen use and malignant melanoma. International Journal of Cancer 87, 145–150.
20. Gasparro, F.P., M. Mitchnick, and J. F. Nash (1998) A review of sunscreen safety and efficacy. Photochem Photobiol. 68(3), 243-256.
21. Maier, T. and H. C. Korting (2005) Sunscreens – which and what for? Skin Pharmacol. Physiol. 18(6), 253-62.
22. Bissonauth, V., R. Drouin, D. L. Mitchell, M. Rhainds, J. Claveau, and M. Rouabhia (2000) The efficacy of a broad-spectrum sunscreen to protect engineered human skin from tissue and DNA damage induced by solar ultraviolet exposure. Clin. Cancer Res. 6(10), 4128-4135.
23. Tanner, P. R. (2006) Sunscreen Product Formulation. Dermatol. Clin. 24, 53–62.
24. Singh, N. P., M. T. McCoy, R. R. Tice, and E. L. Schneider (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175(1), 184-191.
25. Koch, C. J. and A. R. Giandomenico (1994) The alkaline elution technique for measuring DNA single strand breaks: increased reliability and sensitivity. Anal Biochem. 220(1), 58-65.
26. Cahová-Kucharíková, K., M. Fojta, T. Mozga, and E. Palecek (2005) Use of DNA repair enzymes in electrochemical detection of damage to DNA bases in vitro and in cells. Anal Chem. 77(9), 2920-2927.
27. Audic, A. and P. U. Giacomoni (1993) DNA nicking by ultraviolet radiation is enhanced in the presence of iron and of oxygen. Photochem Photobiol. 57(3), 508-512.
28. Zhu, J. and Y. L. Feng (2005) Size exclusive capillary electrophoresis separation of

DNA oligonucleotides in small size linear polyacrylamide polymer solution. Journal of Chromatography A 1081(1), 19-23.
29. Parrish, J.A., K.F. Jaenicke, and R.R. Anderson (1982) Erythema and melanogenesis action spectra of normal human skin. Photochem Photobiol. 36(2), 187-191.
30. Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual 3rd Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
31. Garinis,. G.A., et al. (2005) Transcriptome analysis reveals cyclobutane pyrimidine dimers as a major source of UV-induced DNA breaks. Embo J. 24, 3952–3962.
32. Tung, B. S. et al. (1996) Comparison of the rate of excision of major UV photoproducts in the strands of the human HPRT gene of normal and xeroderma pigmentosum variant cells. Mutat Res. 362, 65–74.
33. Friedberg, E.C. et al. (1995) DNA Repair and Mutagenesis. ASM Press, Washington D.C., 164.
34. Schrock, R.D. and R. S. Lloyd (1993) J. Biol. Chem. 268, 880.
35. Jans, J. et al. (2005) Powerful skin cancer protection by a CPD-photolyase transgene. Curr Biol 15, 105–115.
36. Spielmann, H. et al. (1994) EEC/COLIPA project on in vitro phototoxicity testing: First results obtained with a Balb/c 3T3 cell phototoxicity assay. Toxicology in Vitro 8, 793-796.
37. Anon (1998) Statement on the scientific validity of the 3T3 NRU PT test (an in vitro test for phototoxicity), European Commission, Joint Research Centre: ECVAM and DGXI/E/2, 3 November 1997, ATLA 26, 7-8.