Chapter 27

Mitochondrial DNA as a Biosensor of UV Exposure in Human Skin

Amy Bowman and Mark A. Birch-Machin

Abstract

Mitochondrial DNA (mtDNA) has been demonstrated to be a reliable biomarker of UV-induced genetic damage in both animal and human skin. Properties of the mitochondrial genome which allow for its use as a biomarker of damage include its presence in multiple copies within a cell, its limited repair mechanisms, and its lack of protective histones. To measure UV-induced mtDNA damage (particularly in the form of strand breaks), real-time quantitative PCR (qPCR) is used, based on the observation that PCR amplification efficiency is decreased in the presence of high levels of damage. Here, we describe the measurement of UV-induced mtDNA damage, including the extraction of cellular DNA, qPCR to determine the relative amount of mtDNA, qPCR to determine UV-induced damage within a long strand of mtDNA, and the verification of the amplification process using gel electrophoresis.

Key words Mitochondrial DNA, Ultraviolet radiation, Skin, Real-time quantitative PCR, Genetic damage

1 Introduction

Mitochondrial DNA (mtDNA) has been utilized previously to reliably and sensitively detect UV-induced genetic damage in humans and laboratory animals, usually within a large mtDNA segment of up to 11 kb, as analyzed by real-time quantitative PCR (qPCR) [1–7]. The mitochondrial genome is a circular genome of 16,569 bp in length, and unlike nuclear DNA, is present in many copies within a cell and can therefore withstand high levels of mutation before cellular alterations occur, which means that damage is able to accumulate [8]. It also has a limited number of repair mechanisms; for example, it lacks the nucleotide excision repair pathway for the removal of some UV damage and also lacks protective histones [8]. Therefore, mtDNA is an excellent biomarker of UV-induced damage throughout the lifetime of an individual, as well as for the measurement of short-term changes in damage [8, 9],

Volkmar Weissig and Marvin Edeas (eds.), *Mitochondrial Medicine: Volume II, Manipulating Mitochondrial Function*, Methods in Molecular Biology, vol. 1265, DOI 10.1007/978-1-4939-2288-8_27, © Springer Science+Business Media New York 2015

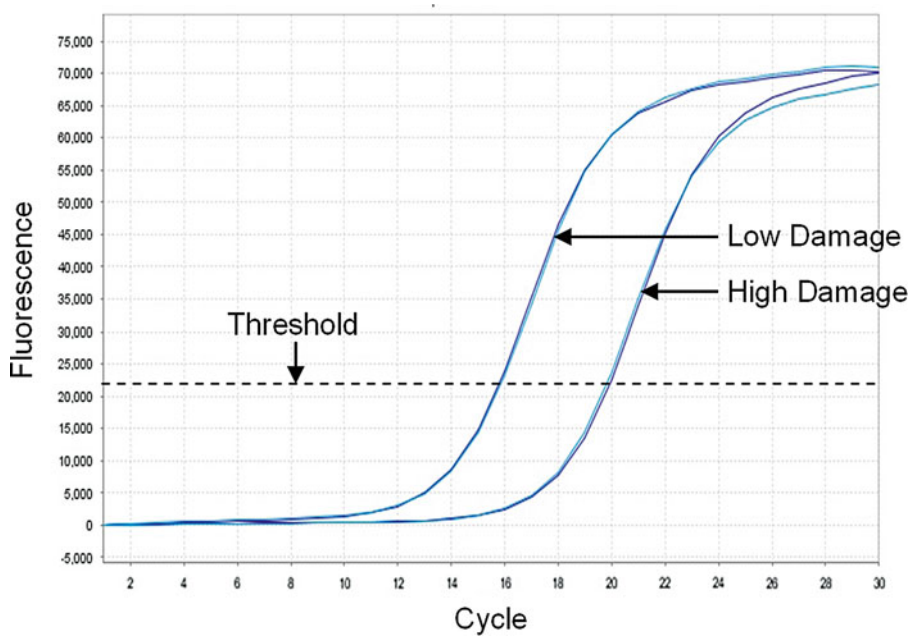

Fig. 1 The principle of real-time quantitative PCR. The qPCR amplification plot shows two samples run in triplicate, as shown by the *colored lines*, one with low mtDNA damage and one with high mtDNA damage. A threshold is set, and the number of cycles it takes before the level of amplified DNA crosses this threshold is known as the Ct, with a low value indicating low damage (or a high copy number) and a high value indicating high damage (or a low copy number)

and to investigate the potency of skin antioxidants [10]. In addition to studies using human skin, mtDNA has also been used as a biomarker of UV damage in animals as diverse as whales [11], for which it was found that the skin of three different whale species shows different levels of UV-induced damage, based on differences in UV exposure and UV defense mechanisms [12]. The detection of mtDNA damage in both human and whale skin uses qPCR and is based on the observation that genetic damage principally caused by UV can induce strand breaks in the mtDNA which block the amplifying ability of DNA polymerase [1, 13]. During this qPCR method, the level of amplified mtDNA product is detectable at each qPCR cycle via the binding of a dye which only displays fluorescence once bound to double-stranded DNA. The relative level of damage is determined by the number of qPCR cycles it takes before the level of amplified product reaches a certain threshold (the cycle threshold, Ct) (Fig. 1). Therefore, those samples with a higher number of intact mitochondrial genomes (i.e., less damage) will undergo amplification more efficiently and result in a lower Ct value. This paper describes the method for the detection of UV-induced damage in human skin mtDNA, which includes the

initial extraction of DNA from cells, qPCR to determine mtDNA damage in real time, and confirmation of DNA products of the correct length following separation by size using gel electrophoresis.

2 Materials

2.1 DNA Extraction Components

1. QIAamp DNA Mini Kit (Qiagen, UK).
2. NanoDrop ND2000 (Labtech, UK).

2.2 Real-Time qPCR Components

1. 5× Phusion High-Fidelity (HF) Buffer.
2. Phusion DNA Polymerase (Thermo Scientific, UK).
3. dNTP mix, 10 mM each dNTP.
4. 10 pmol/μl forward primer; 10 pmol/μl reverse primer. The primers for use in qPCR to amplify regions of either 500 base pairs (bp) or 83 bp are given in Table 1.
5. 5× SYBR Green dye.
6. JumpStart SYBR Green kit (Sigma Aldrich, UK).
7. MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, UK).
8. StepOnePlus Real-Time PCR System (Applied Biosystems, UK) with the results viewed using StepOne Software V2.1 (Applied Biosystems, UK).

2.3 Gel Electrophoresis Components

1. Agarose.
2. 50× TAE running buffer stock: 242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) up to 1 l in dH_2O. Final 1× working buffer concentration, 40 mM Tris acetate, and 1 mM EDTA, pH 8.3.
3. 6× DNA loading buffer; HyperLadder 1 (Bioline, UK).

Table 1
Primer sequences for qPCR

Primer set		Base sequence (5′ to 3′)	Length (bp)	Nucleotide numbers (bp)
500 bp human	F	ATC GGA GGA CAA CCA GTA AG	538	15,758–16,295
	R	CGT GGG TAG GTT TGT TGG TAT C		
83 bp human	F	GAT TTG GGT ACC ACC CAA GTA TTG	83	16,042–16,124
	R	AAT ATT CAT GGT GGC TGG CAG TA		

The primer sets specific for human mtDNA, with product sizes of approximately 500 bp and 83 bp are given. The base sequences from 5′ to 3′ are shown for the forward (F) and reverse (R) primers, as well as the exact product length in bp, and the nucleotide numbers in bp which give the positions of the final amplified products within the mtDNA

3 Methods

The technique for the determination of UV-induced mtDNA damage involves the initial extraction of total DNA (nuclear and mitochondrial) from cultured cells, after which, total DNA concentration can be determined by NanoDrop spectrophotometry (using a NanoDrop ND2000). The relative amount of mtDNA is determined using primers to amplify a region of 83 bp [14], which being a very small region of the mitochondrial genome is unlikely to contain high levels of damage. The results from 83 bp qPCR can therefore be used to ensure an equal amount of mtDNA is present per sample for the 500 bp strand-break qPCR assay. In order to determine damage levels, a region of 500 bp is amplified by qPCR. A gel electrophoresis reaction is performed following qPCR to confirm that a DNA segment size of the appropriate length is generated.

3.1 DNA Extraction

1. Total DNA is extracted from cells using a QIAamp DNA Mini Kit (*see* **Note 1**). For cells grown in culture flasks or dishes, wash cells twice with phosphate buffered saline (PBS) and remove cells from the flask using trypsin-ethylenediaminetetraacetic acid, which is neutralized after 5 min incubation at 37 °C using the appropriate media.
2. Place a maximum of 5×10^6 cells into a 1.5 ml Eppendorf tube and centrifuge at $6{,}000 \times g$ for 5 min, after which, discard the supernatant.
3. Resuspend the cell pellet in 200 μl PBS, and add 20 μl Proteinase K and 200 μl Buffer AL.
4. Vortex the sample for 15 s, and incubate at 56 °C for 10 min to allow cell lysis.
5. Add 200 μl 100 % ethanol to the sample to assist in DNA purification, and vortex the sample for 15 s.
6. Add the sample to a QIAamp spin column and centrifuge at $6{,}000 \times g$ for 1 min to allow DNA adsorption to the column.
7. Discard the flow-through, and add 500 μl Buffer AW1 to the column. Centrifuge at $6{,}000 \times g$ for 1 min.
8. Discard the flow-through, and add 500 μl Buffer AW2 to the column. Centrifuge at $20{,}000 \times g$ for 3 min.
9. Discard the flow-through, and centrifuge the column for 1 min at $20{,}000 \times g$ to completely remove any remaining Buffer AW2. Place the column into a 1.5 ml Eppendorf tube.
10. Add approximately 60 μl Buffer AE carefully to the center of the column membrane. Incubate the column at room temperature for 5 min, and centrifuge at $6{,}000 \times g$ for 1 min to elute the DNA from the membrane.

11. Repeat **step 10** using the eluted flow-through (*see* **Note 2**).
12. Store DNA samples at –4 °C for short-term use or at –20 °C for long-term use.
13. DNA concentration can be measured using a NanoDrop spectrophotometer at a wavelength of 260 nm (*see* **Note 3**).

3.2 Real-Time qPCR

1. To perform qPCR for the amplification of sections for the 500 bp strand-break qPCR assay (*see* **Note 4**), reagents were initially thawed and briefly vortexed (*see* **Note 5**). A master mix containing all PCR components (excluding the DNA) was assembled on ice in a PCR hood, in either a 1.5 ml Eppendorf tube or a 15 ml centrifuge tube depending on the volume required (*see* **Note 6**). For each well, use 11.8 μl deionized water (dH_2O), 4 μl 5× HF Buffer (final concentration 1×), 0.4 μl dNTP mix (final concentration 200 μM), 0.6 μl 500 bp forward primer (final concentration 0.5 μM), 0.6 μl 500 bp reverse primer (final concentration 0.5 μM), 0.4 μl SYBR Green dye (final concentration 0.1×), and 0.2 μl Phusion DNA Polymerase (final concentration 0.1×). Reagents were altered for the 83 bp DNA amplification (*see* **Note 7**).
2. Once the master mix is assembled, add 18 μl per well to a 96-Well Reaction Plate, in duplicate or in triplicate for each sample.
3. Add 2 μl DNA sample per well, at a concentration of 50 ng/μl (100 ng total) (*see* **Note 8**), to make a total volume per well of 20 μl. If the availability of DNA is limited, a lower concentration of DNA may be used (*see* **Note 9**).
4. Perform the qPCR reaction using the following conditions: 98 °C for 30 s, 30 cycles of 98 °C for 8 s, 60 °C for 20 s, and 72 °C for 135 s and a final stage of 72 °C for 8 min. The cycle conditions were altered for the 83 bp DNA amplification (*see* **Note 7**). A melt curve (*see* **Note 10**) was added immediately after the reaction with the conditions of 95 °C for 15 s and 60 °C for 1 min, followed by a plate read at every 0.3 °C temperature increase, ending at 95 °C for 15 s. Samples are held at 4 °C following the reaction (*see* **Note 11**).
5. Following the reaction, the threshold is manually altered to obtain the Ct (*see* **Notes 12–14**).

3.3 Gel Electrophoresis

1. Following qPCR, agarose gel electrophoresis may be performed (*see* **Note 15**). Agarose gels are made using agarose at 1.5 % weight/volume. To do this, add 1.5 g agarose to 100 ml 1× TAE running buffer (*see* **Note 16**) in a glass conical flask, and microwave heat for approximately 200 s or until the agarose is dissolved and the solution is bubbling. Hold the glass flask in cold water until it is cool enough to touch (but still hot)

while constantly swirling gently to avoid the mixture from setting. Add 10 μl ethidium bromide (final concentration 1 μg/ml), and swirl.

2. Pour the mixture into a gel template with the ends sealed using tape, and insert a 10-well gel comb. Allow to cool into a solid gel (*see* **Note 17**).
3. Add the gel to an electrophoresis chamber, and fill tank with 1× TAE running buffer. Remove the 10-well gel comb, and ensure no bubbles are present in the wells.
4. Add 2 μl 6× DNA loading buffer to 10 μl of sample, and add 10 μl sample plus loading dye per gel well. Load 5 μl HyperLadder 1 into 1 gel well as a reference for DNA lengths.
5. Run the gel electrophoresis at 120 V and 56 mA for 40 min.
6. Gel results are viewed using UV light.

4 Notes

1. The complete QIAamp DNA Mini Kit protocol for the extraction of DNA from cells or tissues as described here is also available online.
2. The QIAamp DNA Mini Kit protocol was amended to include a 5 min incubation with Buffer AE at room temperature, which was repeated following centrifugation using the eluted flow-through, as this was found to improve DNA yield.
3. When using the NanoDrop spectrophotometer, the ratio of the absorbance at 260 nm and 280 nm (260/280) should be approximately 1.8, and the absorbance at 260 nm to 230 nm (260/230) should be approximately 2.0–2.2, to ensure the extracted DNA is not contaminated with proteins or other impurities [15].
4. MtDNA sections of approximately 11 kb are most commonly used to determine mtDNA damage; however, sections of this size require large amounts of DNA and prolonged DNA polymerase extension times. To preserve DNA samples and reduce qPCR reaction lengths, shorter regions may be used; therefore, a region of 500 bp was validated and chosen to be used.
5. Real-time qPCR reagents are briefly vortexed following thawing to ensure homogeneity throughout the solution. Do not vortex the polymerase so as not to destroy this enzyme. Only remove the polymerase from the freezer when ready for use.
6. When preparing the master mix for qPCR, make up more than is required to account for slight losses during pipetting, due to the minute volumes required per well. For example, if the master mix is required for 9 wells, assemble enough master mix for 12 wells.

7. For the 83 bp qPCR reaction, a JumpStart SYBR Green Kit can be used, and the extension time shortened due to the decreased number of bases to be replicated. The master mix reagents to be used per well are 8.5 μl dH_2O, 12.5 μl JumpStart SYBR Green, 1 μl 83 bp forward primer (final concentration 0.5 μM), and 1 μl 83 bp reverse primer (final concentration 0.5 μM). Add 23 μl master mix per well, then add 2 μl DNA (50 ng total) per well, in duplicate or triplicate for each sample. The cycling conditions used are 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 60 °C for 45 s, and 72 °C for 45 s and a final stage of 72 °C for 2 min.
8. DNA is diluted to the appropriate concentration for qPCR in dH_2O. In order to dilute the DNA to the appropriate concentration, a dilution calculation is performed. If, for example, the DNA concentration is found to be 150 ng/μl and the concentration for use in the qPCR is to be 100 ng in 2 μl, the following equation would be used for the DNA dilution: $(100/150) \times$ number of wells = sample amount in μl. The amount of dH_2O to be added to the sample would be (number of wells × 2) – sample amount in μl. If the reaction is to be performed in triplicate, the "number of wells" would be a value of just over 3, such as 4, to allow excess DNA dilution solution to be made.
9. DNA concentrations of between 20 and 100 ng are recommended.
10. The melt curve is added to the real-time qPCR reaction to ensure that DNA products of a single length are being formed. During this process, the level of fluorescent dye (which fluoresces only when bound to double-stranded DNA) is measured at temperature increments of 0.3 °C. The melting temperature of DNA is dependent on its length; therefore, DNA strands of similar lengths melt (become separated) at similar temperatures, at which point the fluorescent dye becomes dissociated. The presence of a single peak indicates the generation of a single product length (Fig. 2).
11. Samples are held at 4 °C to preserve the DNA, for when gel electrophoresis is to be performed.
12. Following the qPCR reaction, the threshold is set manually to cross the amplification plot lines during the exponential phase (Fig. 1). A control sample should be used to determine where to set the threshold. Ct values are considered reliable when they are below approximately Ct 36, as above this value may be the result of background signal. A blank sample not containing DNA should be used to verify this qPCR cutoff value.
13. For the 500 bp strand-break qPCR assay, those samples with less UV-induced damage will cross the threshold in a lower number of cycles due to a higher number of intact strands and

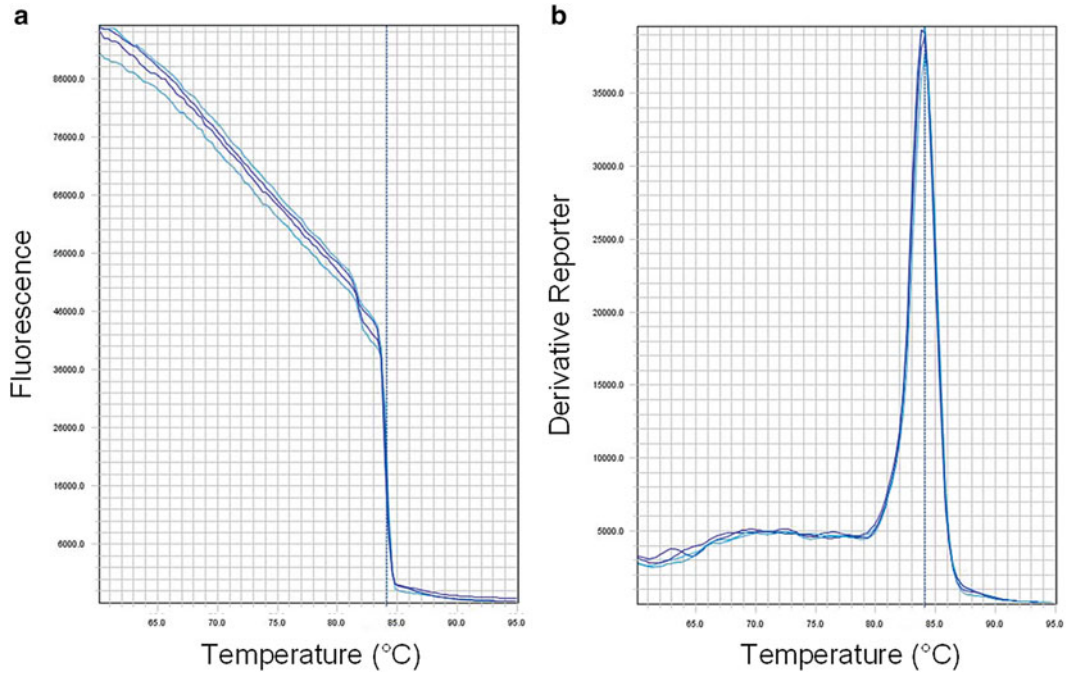

Fig. 2 The melt curve. The qPCR melt curve shows two samples run in triplicate, as shown by the *colored lines*. The fluorescence is measured over a period of temperature increments, with (**a**) showing the level of fluorescence at a range of temperatures and (**b**) showing the derivative plot of the melt curve showing a single peak at the temperature at which the DNA strands became separated

will therefore have a lower Ct value. For the 83 bp qPCR, those samples with a higher number of mitochondrial genomes will cross the threshold in a lower number of cycles and will therefore have a lower Ct value, as there are more strands available for amplification. If all of the 83 bp Ct values are within 1 Ct of the average, the concentration to add to the 500 bp strand-break qPCR reaction does not need to be altered [16–18].

14. As the amount of DNA should double at each qPCR cycle, each single Ct increase is equivalent to a twofold increase in DNA damage. For example, if there was a difference of 3 Cts between two samples, this would actually represent an eightfold difference in damage, as 2^3 is 8.
15. When first attempting the qPCR procedure, it is advisable to run an electrophoresis gel, to determine the size of the amplified mtDNA products to ensure they are of the expected size. During the gel electrophoresis procedure, an electrical charge is applied to a gel, which causes DNA movement from the

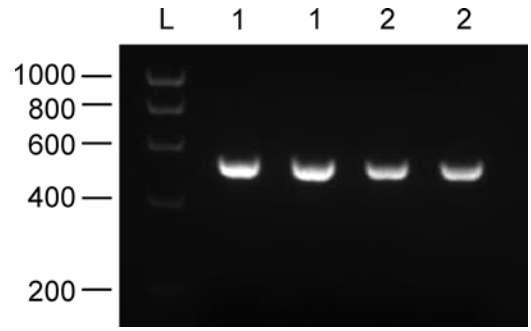

Fig. 3 Gel electrophoresis. The gel electrophoresis result shows two samples (samples 1 and 2) run in duplicate, with a band of the correct size between 400 and 600 bp generated for each sample. The DNA ladder sizes are shown to the *left* in bp

negative to the positive electrode (due to the negative charge of DNA). The agarose gel provides a matrix through which the differently sized DNA fragments pass at different rates [19]; larger fragments pass through this agarose matrix at a slower rate than shorter fragments, allowing DNA to be separated by size and visualized using UV via the incorporation of ethidium bromide into the DNA. This is performed in addition to the melt curve, to confirm that a single DNA product length of the correct size has been generated. An example is shown in Fig. 3.

16. TAE running buffer is composed at 50× and diluted to 1× in dH_2O when needed, due to the large volumes of 1× TAE required.
17. The gel used for electrophoresis takes approximately 15 min to set, ready for placement into the electrophoresis chamber. Following the addition of 1× TAE to the chamber, any bubbles should be removed from the wells using a pipette tip.

Acknowledgment

This work was supported by the Institute of Cellular Medicine, the Faculty of Medical Sciences (Newcastle University), the North Eastern Skin Research Fund (NESRF), and the UK National Institute for Health Research (NIHR) Newcastle Biomedical Research Centre based at Newcastle Hospitals Foundation Trust and Newcastle University. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health.

References

1. Kalinowski DP, Illenye S, Van Houten B (1992) Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. Nucleic Acids Res 20(13):3485–3494
2. Ray AJ et al (2000) The spectrum of mitochondrial DNA deletions is a ubiquitous marker of ultraviolet radiation exposure in human skin. J Invest Dermatol 115(4):674–679
3. Santos JH, Mandavilli BS, Van Houten B (2002) Measuring oxidative mtDNA damage and repair using quantitative PCR. Methods Mol Biol 197:159–176
4. Durham SE et al (2003) Mitochondrial DNA damage in non-melanoma skin cancer. Br J Cancer 88(1):90–95
5. Eischeid AC, Meyer JN, Linden KG (2009) UV disinfection of adenoviruses: molecular indications of DNA damage efficiency. Appl Environ Microbiol 75(1):23–28
6. Hunter SE et al (2010) The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. Methods 51(4): 444–451
7. Swalwell H et al (2012) Investigating the role of melanin in UVA/UVB- and hydrogen peroxide-induced cellular and mitochondrial ROS production and mitochondrial DNA damage in human melanoma cells. Free Radic Biol Med 52:626–634
8. Birch-Machin MA, Swalwell H (2010) How mitochondria record the effects of UV exposure and oxidative stress using human skin as a model tissue. Mutagenesis 25(2):101–107
9. Tulah AS, Birch-Machin MA (2013) Stressed out mitochondria: the role of mitochondria in ageing and cancer focussing on strategies and opportunities in human skin. Mitochondrion 13(5):444–453
10. Oyewole AO et al (2014) Comparing the effects of mitochondrial targeted and localized antioxidants with cellular antioxidants in human skin cells exposed to UVA and hydrogen peroxide. FASEB J 28(1):485–494
11. Bowman A et al (2013) The simultaneous detection of mitochondrial DNA damage from sun-exposed skin of three whale species and its association with UV-induced microscopic lesions and apoptosis. Mitochondrion 13(4): 342–349
12. Martinez-Levasseur LM et al (2013) Whales use distinct strategies to counteract solar ultraviolet radiation. Sci Rep 3:2386
13. Sikorsky JA et al (2007) DNA damage reduces Taq DNA polymerase fidelity and PCR amplification efficiency. Biochem Biophys Res Commun 355(2):431–437
14. Koch H, Wittern KP, Bergemann J (2001) In human keratinocytes the common deletion reflects donor variabilities rather than chronologic aging and can be induced by ultraviolet A irradiation. J Invest Dermatol 117(4): 892–897
15. Thermo Scientific UK (2013) 260/280 and 260/230 ratios. T009-Technical Bulletin Nano Drop 1000 & 8000. http://www.nanodrop.com/
16. Berdal KG, Holst-Jensen A (2001) Roundup Ready® soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. Eur Food Res Technol 213(6):432–438
17. Niemitz EL et al (2004) Microdeletion of LIT1 in familial Beckwith-Wiedemann syndrome. Am J Hum Genet 75(5):844–849
18. Mraz M et al (2009) MicroRNA isolation and stability in stored RNA samples. Biochem Biophys Res Commun 390(1):1–4
19. Lee PY et al (2012) Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp (62). doi:10.3791/3923