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Measuring of DNA Damage by Quantitative PCR

Ayse Gul Mutlu
Mehmet Akif Ersoy University, Arts and Sciences Faculty, Department of Biology, Burdur Turkey

1. Introduction

1.1 QPCR; principles and development

PCR is an in vitro method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primer that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. Successive cycles of amplification essentially double the amount of the target DNA synthesized in the previous cycle (1).

Recent advances of the fluorometric dyes allow the very sensitive and quick quantitation of DNA. Before the invention of fluorometric quantitative PCR (QPCR) method the researchers who measured a gene's amount, have used the different methods like competetive PCR, solid phase assays, HPLC, dot blot or immunoassay (2). Many of the applications of real-time Q-PCR include measuring mRNA expression levels, DNA copy number, transgene copy number and expression analysis, allelic discrimination, and measuring viral titers (3).

The detection of gene-spesific damage and repair has been studied in nuclear and mitochondrial DNA by the use of southern analysis. But this method requires knowledge of the restriction sites flanking the damaged site, the use of large quantities of DNA, and incision of DNA lesions with a spesific endonuclease (4). Govan and collegues has reported a new approach to measuring of DNA damage in 1990 (5). This PCR based quantitative technique has been improved by Kalinowski and collegues (6). Principle of this analysis is that lesions present in the DNA, block the progression of any thermostable polymerase on the template. So the DNA amplification decreases in the damaged template when compared to the undamaged DNA (4). QPCR is a suitable method for the measuring damage and repair in the subgene level functional units like promotor regions, exons and introns (7). Method also useful to determining DNA damage and repair that originated by the genotoxic agents and oxidative stress (8,9,10). The method capable of detect 1 lesion/10^5 nucleotides from as little as 5 ng of total genomic DNA (4).

DNA extraction, pre quantitation of DNA template, PCR amplification and quantitation of PCR products are crucial for success of the application (Figure 1). Quantity and quality of the DNA sample is important. We use mini column based kits for DNA extraction in our laboratory. These extraction kits and carefully pipetting, minimize the artificial DNA

damages. Pico Green dsDNA quantitation kit is used for both template DNA quantitation and the analysis of PCR products as fluorometrically 485 nm excitation, 530 nm emission. Pico Green and SYBR green are substantially more sensitive for quantifying DNA concentrations than ethidium bromide and some other fluorimetric dyes (11). Initial DNA template quantity in the all PCR tubes must be the same. mtDNA damage is quantified by comparing the relative efficiency of amplification of long fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller fragments, which have a statistically negligible likelihood of containing damaged bases. To calculate relative amplification, the long QPCR values are divided by the corresponding short QPCR results to account for potential copy number differences between samples. Decreased relative amplification is an indicator of the damaged DNA (4, 12).

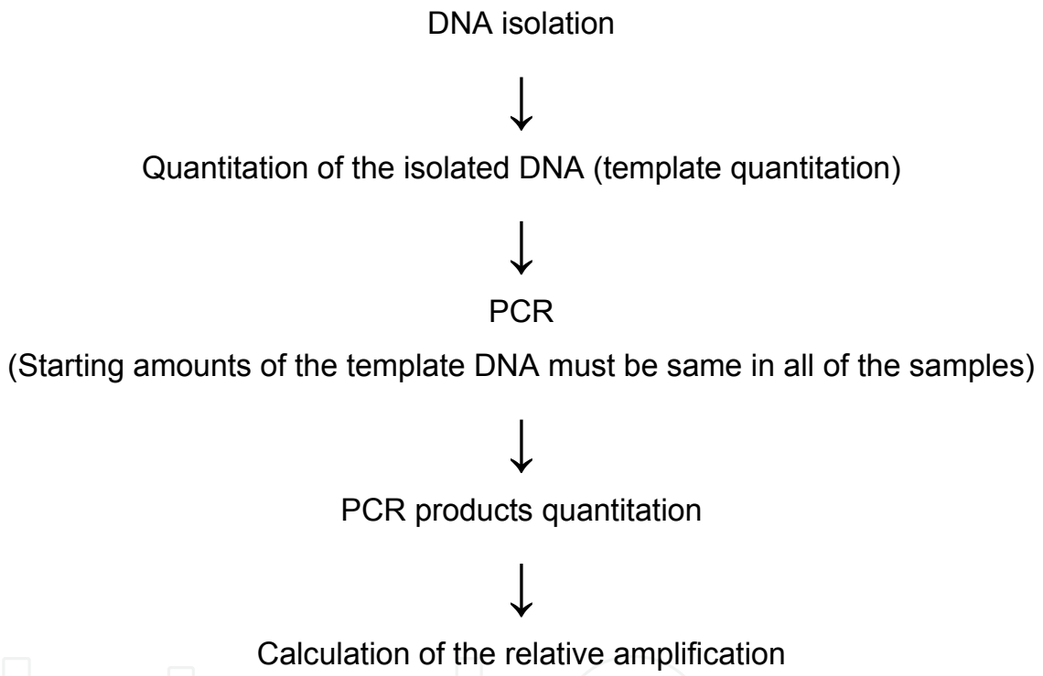

Fig. 1. Flowchart of the QPCR assay for measuring of DNA damage

2. Optimization of the assay; the crucial steps

Crucial step of the QPCR is PCR optimization. Thermal conditions, especially annealing temperature must be optimized. Extention temperature may be lower for long PCR amplifications. mtDNA amplification may needs some adjuvants. We use in our laboratory DMSO (%4) for improve the efficiency of the PCR reaction. Various authors recommend DMSO and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific products) of PCR, when used in concentrations varying between 5%–10% (vol/vol). However, in the multiplex reactions, these adjuvants give conflicting results. For example, 5% DMSO improve the amplification of some products and decrease the amount of others. There are similar results with 5% glycerol. Therefore, the usefulness of these adjuvants needs to be tested in each case. Also BSA may increase the efficiency of the PCR (13).

Hot start PCR improve specifity of PCR reaction. Hot start PCR is reported to minimize nontarget amplification and the formation of primer-dimer (14) .

Optimization might involve changes in nucleic acids preparation, in primer usage, in buffer usage and in cycling parameters. One of the recent developments in PCR optimization is to recognize the importance of eliminating some undesired hybridization events that often happen in the first cycle and can carry potentially devastating effects. Theoretically, if the amplification precedes with an efficiency of 100%, the amount of amplicons is doubling at each cycle. However, in most PCR procedures, the overall efficiency is less than 100% and a typical amplification runs with a constant efficiency of about 70-80% from the 15th cycle to the 30th cycle, depending on the amount of starting material. The increase in the amount of amplicons stays exponential only for a limited number of cycles, after which the amplification rate reaches a plateau. The factors that contribute to this plateau phenomenon include substrate saturation of enzyme, product strand reannealing, and incomplete product strand separation. In this latter phase, the quantitated amount of amplified product is no longer proportional to the starting amount of target molecules. Therefore, to make PCR suitable in quantitative settings, it is imperative that a balance be found between a constant efficiency and an exponential phase in the amplification process. This will ultimately depend on the number of cycles, on the amount of targets in the starting material, and on the system of detection and quantitation of the amplified product (15).

We run a 50% template control and a nontemplate control in PCR in our laboratory. 50% template control should given a 50% reduction of the amplification signal (values between 40%-60% reduction are acceptable). The nontemplate control would detect contamination with spurious DNA or PCR products (4).

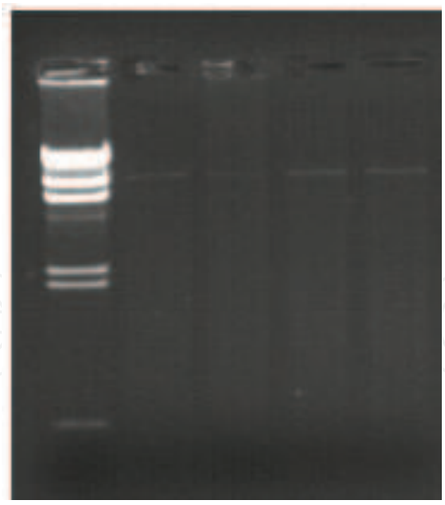

Fig. 2. PCR band of 10 kb mtDNA fragment of Mus musculus (Balb C). (Band 1: λ *Hind III* digest marker DNA)

3. Measuring of mtDNA damage on mice

We study mtDNA damage by QPCR method in different organisms like fruit flies, mice and snails in our laboratory. In our research that we used mice, oxidative mtDNA damage that

created by cigarette smoke and protective effects of VitE and selenium was investigated (Figure 3).

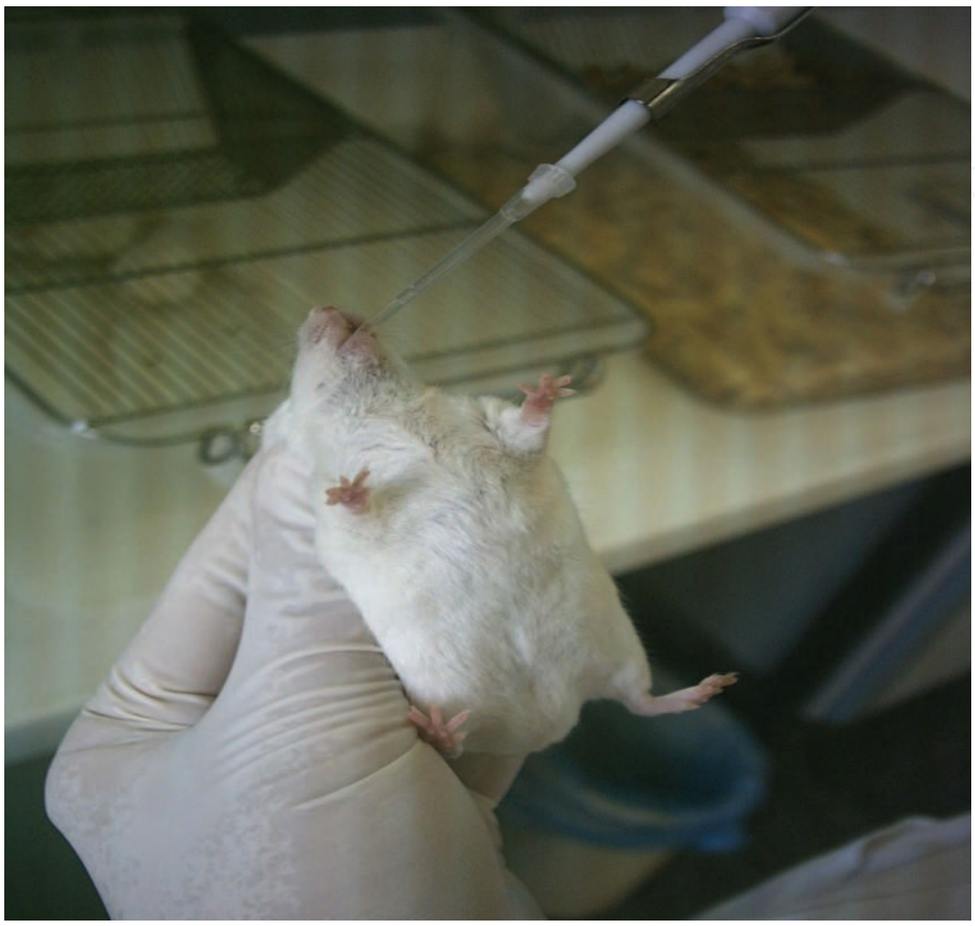

Fig. 3. Vitamin supplementation

DNA damage that is originated by cigarette smoke in various organs is declared by some research (16,17). Tobacco smoking contains many thousands of chemicals including a plethora of mutagens. Many carcinogens undergo metabolic activation in mammalian tissues to reactive intermediates that interact with and modify informational macromolecules, such as DNA with potentially mutagenic consequences (18). PAHs (Polycyclic aromatic hydrocarbons) cause irreversible DNA damage via covalent binding or oxidation (19). However genetic damage reflecting individual exposure and susceptibility to PAH may play a role in disease development (20). Tobacco smoke contains major classes of carcinogens that include PAHs, aromatic amines and tobaccospecific nitrosamines. In addition, toxic compounds such as formaldehyde, acetaldehyde, acrolein, short-lived radicals and reactive oxygen intermediates generated by redox cycling from catechol and hydroquinone and nitric oxide (NO) may also contribute to the toxic and carcinogenic effects of tobacco smoke. Direct DNAdamaging compounds that are present in cigarette smoke (CS) have previously been reported to include reactive oxygen intermediates, peroxynitrite, ethylating agents and unidentified compounds (21).

Many carcinogens in the cigarette smoke like PAHs, nitrosamine and cisplatin bind mitochondrial DNA (mtDNA) preferentially (22). The antioxidants are used frequently as food supplements may be effective to preventing cigarette smoke damage on mtDNA. Damages that are created by CS may be prevented by vitamin E (Vit E) and selenium (Se) which are powerful antioxidants.

Genomic DNA mini column kit (SIGMA) was used for total DNA isolation according to the technical bulletin. We used Pico Green dsDNA quantitation kit for both template DNA quantitation and the analysis of PCR products as fluorometrically 485 nm excitation, 530 nm emission (23). A crucial step of quantitative PCR is the concentration of the DNA sample. In fact, the accuracy of the assay relies on initial template quantity because all of the samples must have exactly the same amount of DNA. The Pico Green dye has not only proved efficient in regarding to template quantitation but also to PCR product analysis (10). Hot Start ready mix Taq (SIGMA) were used for PCR. In this mix, taq polymerase combines the performance enhancements of Taq antibody for hot start. When the temperature is raised above 70ºC in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active. DMSO as 4% of total volume and 20 ng of template total DNA were added into the each PCR tube. Mouse 117 bp Mouse 117 bp mtDNA fragment (small fragment) primers were:

13597 5′- CCC AGC TAC TAC CAT CAT TCA AGT- 3′

13688 5′- GAT GGT TTG GGA GAT TGG TTG ATG T- 3′ (Table 1)

Mus musculus primers for long fragment (10085 bp): 3278 5′- GCC AGC CTG ACC CAT AGC CAT AAT AT- 3′ 13337 5′- GAG AGA TTT TAT GGG TGT AAT GCG G- 3′ (4)
Mus musculus primers for small fragment (117 bp): 13597 5′- CCC AGC TAC TAC CAT CAT TCA AGT- 3′ 13688 5′- GAT GGT TTG GGA GAT TGG TTG ATG T- 3′ (4)
Drosophila primers for long fragment (10629 bp): 1880 5′- ATGGTGGAGCTTCAGTTGATTT - 3′ 12487 5′- CAACCTTTTTGTGATGCGATTA - 3′
Drosophila primers for small fragment (100 bp): 11426 5′- TAAGAAAATTCCGAGGGATTCA - 3′ 11504 5′- GGTCGAGCTCCAATTCAAGTTA - 3′

Table 1. QPCR primers for measuring of mtDNA damage in *Mus musculus* and *Drosophila melanogaster*

Thermal conditions for *Mus musculus* long fragment (10085 bp): 75ºC for 2 min 95ºC for 1 min **94ºC for 15 sec** **59ºC for 30 sec → 21 cycles** **65 ºC for 11 min** 72ºC for 10 min
Thermal conditions for *Mus musculus* small fragment (117 bp): 75 ºC for 2 min 95 ºC for 15 sec **94ºC for 30 sec** **50ºC for 45 sec → 19 cycles** **72 ºC for 45 sec.** 72ºC for 10 min
Thermal conditions for *Drosophila* long fragment (10629 bp): 75ºC for 1 min 95ºC for 1 min **94ºC for 15 sec** **52ºC for 45 sec → 21 cycles** **65 ºC for 11 min** 68ºC for 10 min.
Thermal conditions for *Drosophila* small fragment (100 bp): 75 ºC for 2 min 95 ºC for 15 sec **94ºC for 30 sec** **55ºC for 45 sec → 21 cycles** **72 ºC for 45 sec** 72ºC for 10 min

Table 2. Thermal conditions for QPCR in *Mus musculus* and *Drosophila melanogaster*

Mouse 10 kb mtDNA fragment (Figure 2) primers were:

3278 5′- GCC AGC CTG ACC CAT AGC CAT AAT AT- 3′

13337 5′- GAG AGA TTT TAT GGG TGT AAT GCG G- 3′

(4).

For long fragment PCR amplification, DNA was denatured initially at 75ºC for 2 min and 95ºC for 1 min, and then the reaction underwent 21 PCR cycles of 94ºC for 15 sec, 59ºC for 30 sec, and 65 ºC for 11 min. Final extension was allowed to proceed at 72ºC for 10 min (Table 2). For small fragment PCR amplification, DNA was denatured initially at 75 ºC for 2 min and 95 ºC for 15 sec, and then the reaction underwent 19 PCR cycles of 94ºC for 30 sec, 50ºC for 45 sec, and 72 ºC for 45 sec. Final extension was allowed to proceed at 72ºC for 10 min (23).

We were always run a 50% template control and a nontemplate control in PCR. To calculate relative amplification, the long QPCR values were divided by the corresponding short QPCR results to account for potential copy number differences between samples (mtDNA/total DNA value may be different in 20 ng template total DNA of each PCR tube) (3,4,10,23). The copy number results not indicate the damage.

We detected mtDNA damage in the mouse heart succesfully. According to these relative amplification results "cigarette smoke" application group was significantly different from all other groups. mtDNA damage was significantly higher in the cigarette smoke group than the other groups. However "Cigarette Smoke+Vitamin E+Selenium" group had lowest mean damage (23,24).

4. Measuring of oxidative mtDNA damage and copy number on Drosophila

The free radical theory of aging postulates that aging changes are caused by free radical reactions. Aging is the progressive accumulation of changes with time that are responsible for the ever-increasing likelihood of disease and death. These irreversible changes are attributed to the aging process. This process is now the major cause of death in the developed countries. The aging process may be due to free radical reactions (25). The free radical theory of aging posits that the accumulation of macromolecular damage induced by toxic reactive oxygen species (ROS) plays a central role in the aging process. The mitochondria are the principal generator of ROS during the conversion of molecular oxygen to energy production where approximately 0.4% to 4% of the molecular oxygen metabolized by the mitochondrial electron transport chain is converted to ROS (26). Cellular damage caused by radicals may induce cancer, neurodegeneration and autoimmun disease (27). Toxic materials may produce ROS and generate oxidative damage on mitochondrial DNA (mtDNA) (23). mtDNA damages may trigger mitochondrial dysfunction (28). Damage to mtDNA could be potentially more important than deletions in nDNA, because the entire mitochondrial genome codes for genes that are expressed while nDNA contains a large amount of non-transcribed sequences. Also, mtDNA, unlike nDNA, is continuously replicated, even in terminally differentiated cells, such as neurons and cardiomyocytes; hence, somatic mtDNA damage potentially causes more adverse effects on cellular functions than does somatic nDNA damage (29).

Cereals naturally contain a wide variety of polyphenols such as the hydroxycinnamic acids, ferulic, vanillic, and *p*-coumaric acids which show a strong antioxidant power and may help to protect from oxidative stress and, therefore, can decrease the risk of contracting many diseases. Flavonoids are present in small quantities, even though their numerous biological effects and their implications for inflammation and chronic diseases have been widely described. The mechanisms of action of polyphenols go beyond the modulation of oxidative stress-related pathways (30).

Wheat is an important component of the human diet. But the distribution of phytochemicals (total phenolics, flavonoids, ferulic acid, and carotenoids) and hydrophilic and lipophilic antioxidant activity in milled fractions (endosperm and bran/germ) are different each other. Different milled fractions of wheat have different profiles of both hydrophilic and lipophilic phytochemicals. Total phenolic content of bran/germ fractions is 15−18-fold higher than that of endosperm fractions. Hydrophilic antioxidant activity of bran/germ samples is 13−27-fold higher than that of the respective endosperm samples. Similarly, lipophilic antioxidant activity is 28−89-fold higher in the bran/germ fractions. In whole-wheat flour, the bran/germ fraction contribute 83% of the total phenolic content, 79% of the total flavonoid content, 51% of the total lutein, 78% of the total zeaxanthin, 42% of the total β-cryptoxanthin, 85% of the total hydrophilic antioxidant activity, and 94% of the total lipophilic antioxidant activity (31).

Aim of our study was investigate the effects of a wheat germ rich diet on oxidative mtDNA damage, mtDNA copy number and antioxidant enzyme activities in the aging process of *Drosophila* (32).

Genomic DNA kits (invitrogen) were used for total DNA isolation according to the technical bulletin. İnvitrogen (Molecular Probes) Pico Green dsDNA quantitation dye and QUBIT 2.0 fluorometer were used for both template DNA quantitation and the analysis of PCR products as fluorometrically (Figure 4). DMSO as 4% of total volume and 5 ng of template total DNA were added into the each PCR tube.

Primers for Drosophila mtDNA 100bp fragment were designed as;

11426 5′- TAAGAAAATTCCGAGGGATTCA - 3′

11525 5′- GGTCGAGCTCCAATTCAAGTTA - 3′

Primers for Drosophila mtDNA 10629 bp fragment were designed as;

1880 5′- ATGGTGGAGCTTCAGTTGATTT - 3′

12508 5′- CAACCTTTTTGTGATGCGATTA - 3′ (Table 1)

For long fragment PCR amplification, DNA was denatured initially at 75ºC for 1 min and 95ºC for 1 min, and then the reaction underwent 21 PCR cycles of 94ºC for 15 sec, 52ºC for 45 sec, and 65 ºC for 11 min. Final extension was allowed to proceed at 68ºC for 10 min (Table 2).

For small fragment PCR amplification, DNA was denatured initially at 75 ºC for 2 min and 95 ºC for 15 sec, and then the reaction underwent 21 PCR cycles of 94ºC for 30 sec, 55ºC for 45 sec, and 72 ºC for 45 sec. Final extension was allowed to proceed at 72ºC for 10 min.

Fig. 4. QUBIT 2.0 fluorometer were used for both template DNA quantitation and the analysis of PCR products as fluorometrically

5. Conclusions

QPCR is a suitable method for the measuring damage and repair in the subgene level functional units like promotor regions, exons and introns (7). Recent advances of the fluorometric dyes allow the very sensitive and quick quantitation of DNA. Before the invention of fluorometric quantitative PCR (QPCR) method, the researchers who measured a gene's amount, have used the different methods like competetive PCR, solid phase assays, HPLC, dot blot or immunoassay (2). Many of the applications of real-time Q-PCR include measuring mRNA expression levels, DNA copy number, transgene copy number and expression analysis, allelic discrimination, and measuring viral titers (3). Method also useful to determining DNA damage and repair that originated by the genotoxic agents and oxidative stress (8,9,10). Crucial step of the QPCR is PCR optimization. Thermal conditions, especially annealing temperature must be optimized. Important points of the optimization:

1. Determination of annealing temperature
2. Optimization of he extention temperature (Extention temperature may be lower for long PCR amplifications)
3. Adjuvants (if necessary)
4. Hot start PCR (minimize nontarget amplification and the formation of primer-dimer)
5. Determination of cycling number

6. Running of %50 template and nontemplate controls in PCR (50% template control should given a 50% reduction of the amplification signal -values between 40%-60% reduction are acceptable-. The nontemplate control would detect contamination with spurious DNA or PCR products)

We detected mtDNA damage that originated by the genotoxic agents, oxidative stress and age, above mentioned conditions in our various studies (23,24,32). Also, QPCR method is suitable for the nutritional studies and some cancer researches.

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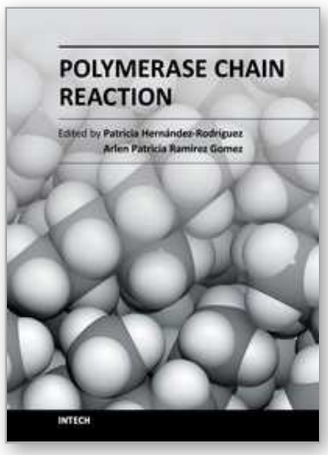

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