Assessing the Fast Micromethod® as a tool in DNA integrity analysis

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Abstract

Over the past decade the relatively new Fast Micromethod® for detecting DNA damage (strand breaks, alkali-labile sites and incomplete excision repair) and related DNA integrity in cell suspensions or tissue homogenates in single microplates has been developed. The technique is based on the ability of the specific fluorophore PicoGreen® to preferentially interact with high integrity DNA molecules, dsDNA, allowing direct fluorimetric measurement of fluorophore-dsDNA complex decay in alkaline conditions. This analytical technique allows simple and fast simultaneous analysis of DNA integrity in numerous, low DNA content samples. Research has confirmed that the Fast Micromethod® is applicable for the measurement of DNA integrity in not only human biological material for medical purpose, but also in marine invertebrates for genotoxicity assessment (biomonitoring) and the estimation of harmful effects in the ecosystem. This article focuses on a comprehensive description of the method and its uses, and offers detailed descriptions and insights into samples and chemicals preparation, fluorescence analysis and calculation of DNA integrity rate. The results and features of many provided studies using this method are discussed, in order to elucidate advantages and imperfections of this analytical technique and to highlight approaches that may allow its further development.

Keywords: DNA alkali-labile sites, DNA cross-links, DNA integrity, DNA strand breaks, Fast Micromethod®
I. INTRODUCTION

In the chromatin of the majority of living organisms, a functionally stable DNA is a double-stranded chain of nucleotides (dsDNA), devoid of discontinuities or abnormal structural modifications, associated in protein complex. Such a high integrity DNA molecule is a frequent subject of different environmental xenobiotic agents (ionizing and UV radiation, genotoxic chemicals) and endogenous (free radicals, imperfect DNA repair mechanisms) physiological factors which caused the several different types of DNA damages and its integrity disruptions (Friedberg et al., 1995). The living world creates the whole variety of mechanisms to repair those lesions and maintain the dynamic equilibrium between occurring DNA damage and its repair during the cells lifetime. The permanent inefficiency of these processes undoubtedly leads to mutations, genetic instability, sometimes, neoplastic evolution, and final cell death (Kaufmann and Paules, 1996).

The primary DNA lesions, which significantly reduce the DNA integrity, are alkali-labile sites and DNA single or double-strand breaks. The DNA molecule is insensitive to alkaline hydrolysis, but regular physiological processes results with alkaline labile sites, which are prone to specific endonuclease activity and single-strand (nicks) and, in some cases, double-strand breaks formation (Teebor and
Brent, 1981; Tomei et al., 1993). Therefore, the most frequent spontaneous DNA damage, abasic sites usually arise during the normal physiological processes or may be a consequence of N-glycosylase activity (Gupta and Siover, 1981). The other type of alkali-labile sites, alkyl phosphate triesters, are result of xenobiotic diol-epoxide binding on free hydroxyl group of phosphate molecule in DNA backbone, and may lead to hydrolysis of alkyl phosphate triesters (Gamper et al., 1977).

Furthermore, the single-strand breaks may also appear as a consequence of normal cell metabolism such as imperfect DNA repair mechanisms (Cleaver, 1984) and apoptotic process (Barry and Eastman, 1993). The physical agents like ionizing (γ- and X-ray) radiation predominately leads to single and, much less frequently, to double-strand breaks. The UV-radiation and other genotoxic agents cause the several different types of DNA damage that leads to activation of DNA repair mechanisms and temporal single-strand breaks formation (Shugart al, 1992; Shugart 1998). Thereby, the single-strand breaks are result of direct cleavage of DNA backbone, alkaline hydrolysis or enzymatic cleavage of phosphodiester bonds between nucleotides, or actions of DNA hydrolases, girases and topoisomerases (Bradly and Sina, 1984). The ionizing radiation may produce the detached sugar derivates or loss of bases and formation
of nucleotide gaps on DNA chain (Obe et al., 1992). The mechanism of double-strand breaks formation is not fully elucidated but it is a quite clear that they arise upon the free radicals actions (Ward, 1990), and the repair of those is a much more complex than any other type of DNA damage.

The DNA-protein and DNA-DNA cross-linking agents caused the covalent binding of exogenous or endogenous agents with DNA, and the consequences of their action may disturb the normal cell physiology and processes of replication, recombination and transcription of DNA. The UV-irradiation is known inducer of DNA-protein cross-links formation, while formaldehyde, frequent environmental pollutant, induces both, DNA-DNA and DNA-protein cross-links. Furthermore, the natural agent mitomicine C, laboratory synthesised cisplatin or alkylating agents, such as nitrogen and sulphur mustard, and UV induced psoralenes, forms inter and intra-strand DNA cross-links (DNA-protein cross-links formation by cisplatin is also described), and have been extensively used in various types of cancer treatment as chemotherapy drugs (Friedberg et al, 1995).

Up to date, there are several different methods to measure the DNA damage and its integrity. The most popular are centrifugation in alkaline gradient of saharose (Dean et al, 1966; Lett 1981), alkaline filter elution (Kohn and Grimek-Ewig, 1973; Kohn et al., 1976;
Kohn, 1991), alkaline unwinding assay (Birnboim and Jevack, 1981; Kanter and Schwartz, 1982), agarose gel electrophoresis of DNA (Seawell and Ganesan 1981; Theodorakis et al., 1994), and single cell gel electrophoresis or comet assay (Rydberg and Johansen, 1978; Fairbarin et al., 1995). All those methods are based on the rate at which the ssDNA is obtained from high integrity dsDNA under denaturing conditions of high pH and ionic strength. This rate is proportional to the number of strand breaks and alkali-labile sites and possible cross-links in observed DNA sample (Rydberg, 1975). The alkaline unwinding method measures the partial alkaline unwinding of DNA and determinate the remained dsDNA fraction by fluorescence of bounded ethidium-bromide but does not allow the continuous measurement of kinetics of DNA unwinding (it can not stain DNA in the presence of alkali and the alkaline unwinding has to be stopped by neutralization), and requires at least 1-2 ×10^6 cells. Efficiency of sensitive alkaline filter elution technique is dependent upon the source of DNA and their deproteination ability. The DNA with numerous single-strand breaks and alkali-labile sites are eluted from the filter faster than undamaged high integrity ds-DNA, and the comparison of those elution rates gives us the relative measure of DNA integrity in examined samples. In a spite of their sensitivity, the method is time consuming, and difficult to apply on huge number of samples.
simultaneously. The very popular Comet assay enables detection of DNA strand breaks after the alkaline denaturation at the individual cell level, but not in a tissue sample.

To assure the simple and quick assessment of DNA integrity in routine analysis, and overcame the imperfections of existed techniques, the new, denaturation based, technique for DNA single-strand breaks estimation and alkali-labile sites, called the Fast Micromethod® has been developed (Müller et al., 1997; Batel et al, 1999). This procedure utilizes the ability of fluorophore PicoGreen® to, in the presence of other macromolecules such as proteins, single-stranded DNA (ssDNA) and RNA, and at high pH and ionic strength, preferentially interact with double-stranded DNA (dsDNA) and enhance the fluorimetric quantum yields over a 1000-fold (Haugland, 1996). The employment of fluorescence microplate reader allow following the decay of high integrity DNA, and its interaction with PicoGreen® dye, over a time in alkaline conditions in analysis of large sets of samples simultaneously. Beside the simplicity of this technique, the minute amount of samples and the sensitivity and relatively short time consuming are the main preferences and advantages of this analytical technique (Batel et al., 1999).
II. ANALITICAL PROCEDURE

The Fast Micromethod® analytical procedure, as the DNA integrity assay to detect changes in the pattern of DNA denaturation, was described for the first time in the German patent application (Müller et al, 1997). Its improvements and applicability were well described in the papers published later, in 1999 (Batel et al) and 2003 (Jakšić and Batel), and the recent technique overview were published in Methods in Molecular Biology Series (Schroder et al., 2005).

A. Assumptions of Theory

The hydrogen bonds in DNA double helix are destabilized in high alkaline (pH above 11.0) and high ionic strength solutions. The unwinding process may be followed by continuous fluorescence measurement of specific dsDNA-PicoGreen® complex decay. The low integrity DNA unwinds faster than high integrity DNA and that kinetics depends not only on the DNA molecule length itself, but also on the frequency of present alkali-labile sites and single-strand brakes. The measured fluorescence of that dsDNA-PicoGreen® complex is directly proportional to those failures, as well as may be a consequence of present DNA-DNA or DNA-protein cross-links.
**B. Fluorophore Dyes**

This analytical technique employs two cyanine dyes with very high affinity to nucleic acid detection (Haugland, 2005). Both fluorophores were invented and trademarks of Molecular Probes Inc., Eugene, Oregon, USA, which is today, product family member of Invitrogen Corp., Carlsbad, California, USA. In the spite of no available data addressing the toxicity or mutagenicity, but according to their ability to bind to nucleic acids both dyes should be treated as a potential mutagen and handled with care. The cyanine dyes shows differences in some physical characteristics, particularly differences in permeability to cell membranes and nucleic acid specificity.

The unsymmetrical oxazole yellow homodimer, YOYO®-1 iodide (C\(_{49}\)H\(_{58}\)I\(_4\)N\(_6\)O\(_2\); MW=1270.65), has a very high affinity for nucleic acids. Compared to the free form in solution exhibits approximately 400 and 250-fold increase in fluorescence quantum yield (the ratio of the number of fluorescence photons emitted to the number of photons absorbed) upon binding to dsDNA and ssDNA respectively (Cosa et al., 2001). These complexes (intercalation and external binding model) are very stable under the high alkaline and ionic strength conditions, and do not succumb under the influence of proteins, sodium dodecyl sulphate (C\(_{12}\)H\(_{25}\)SO\(_4\)Na; SDS), or urea (N\(_2\)H\(_4\)CO) and results in very high sensitivity for a wide range of nucleic acid concentrations (Rye et
al., 1993). The YOYO®-1 iodide is characterized by high molar extinction coefficient of 118.600 M\(^{-1}\)cm\(^{-1}\) at 482 nm, and show 3200-fold fluorescence enhancement upon binding to dsDNA (Rye et al., 1992). Upon the intercalation of YOYO®-1 iodide and formation of complexes with both ssDNA and dsDNA the dimmer disappears and its decay is best treated as biexponential, and the maximum absorbance and fluorescence emission of dye complexed with ssDNA and dsDNA were recorded at 490/510 and 490/507 nm respectively. (Cosa et al., 2001).

The PicoGreen® dsDNA quantitation reagent is derivate of unsymmetrical cyanine dye, and it is ultrasensitive for dsDNA fluorescence quantization (about 10.000 and 400-fold more sensitive then UV measurements at 260 nm and Hoechst 33285 dye-based assay respectively) (Haugland, 2005). It exhibit the unique and outstanding properties like high molar absorptivity with extinction coefficient of about 70.000 cm\(^{-1}\)M\(^{-1}\) at visible wavelengths, very low intrinsic fluorescence (background signal), quantum yields of 0.0006, 0.66 and 0.30 of free dye or bounded to dsDNA and ssDNA respectively (Cosa et al., 2001). This high increase of fluorescence quantum yield or fluorescent enhancement, of about 1000 and 500-fold, upon binding to dsDNA and ssDNA respectively, as compared to free in solution, makes it sensitive probe for the dsDNA determination.
even in the presence of equimolar amounts of ssDNA, RNA and proteins. The free rotation about its central methane bridge is blocked by intercalation between the DNA base pairs and the complex with ss-DNA remains less rotationally restricted. The PicoGreen® showed significantly lower affinity to ssDNA than to dsDNA molecules. Even tough complexes with ssDNA are more prone to deactivation and the different lifetime characteristics for each of the complexes allow a simple approach to quantifying their relative amount in solution containing both types of DNA. All of this makes the PicoGreen® a suitable tool to determine the ssDNA/dsDNA ratio and sensitive probe for the high integrity dsDNA determination. The maximum absorbance and fluorescence emission of dsDNA-PicoGreen® and ssDNA-PicoGreen® complexes were recorded at 501/522 and 503/525 nm respectively (Cosa et al., 2000; 2001). This selectivity behaviour is utilized to follow DNA denaturation with decreasing fluorimetric signal intensity proportionate to increasing ssDNA and mononucleotide content. The PicoGreen® dynamic range, sensitivity and linear detection range extends over more than four orders of magnitude in DNA concentration from 25 pg ml^{-1} to 1 μg ml^{-1} and maintained in the presence of salts, urea, proteins and other contaminants (Singer et al., 1997; Haugland, 2005). In our laboratory experiment the fluorescence values of calf thymus ssDNA-PicoGreen® and dsDNA-PicoGreen®
complex showed the large difference between the two over a wide range of DNA concentrations (1-33 ng DNA) and alkaline conditions (pH 7.5-12.6). Furthermore, the denaturation of dsDNA-PicoGreen® complex starts at about pH 12.1, continuing very fast up to pH value of 12.6 where the difference between fluorescence of dsDNA and ssDNA disappears (Batel et al., 1999).

**C. Total DNA determination by YOYO®-1-iodide**

The application of equal amount of DNA to the microplate and measurement of its fluorescence decay is one of the critical points of DNA integrity determination by Fast Micromethod®. Therefore, the total DNA content measurement in tissue homogenates or counting the cells number in cell culture samples and their standardization (e.g. 100 pg DNA ml\(^{-1}\) or \(10^5\) cells) and application in microplates is not only recommended but also even mandatory step. The total DNA content in samples may be achieved by UV measurement of 260 nm / 280 nm ratio, but the fluorochromatic dye YOYO®-1-iodide provide a much sensitive and precise determination of total DNA content in the presence of other macromolecules in crude tissue homogenate or cell suspension. The original supplied stock of 1mM YOYO®-1-iodide in DMSO should be stored at -20 °C in 5 μl aliquots (expected shelf life is 6 to 12 months), and diluted 100-fold in 25% DMSO just before analysis. This 10 μM YOYO®-1-iodide solution has to be diluted 25-fold
in 10% DMSO in TE buffer (pH 7.4) to prepare the $4 \times 10^{-7}$ M YOYO®-1-iodide working solution. All YOYO®-1-iodide solutions have to be maintained in the plastic containers and protected from light. Mixing the equal volume (at least 50 µl) of YOYO®-1-iodide working solution and sample homogenates or cell suspensions the recommended final YOYO®-1-iodide concentration of $2 \times 10^{-7}$ M will be provided (Rye et al., 1993). After 10-15 minutes, the fluorescence, measured as close to excitation and emission maximum of DNA-fluorochrome (e.g. 485/520 nm), should be recorded, data compared to the DNA standard curve (50 – 500 ng ml$^{-1}$) and the total DNA in samples calculated. Prior the Fast Micromethod® analysis we used to dilute homogenate samples and apply 100 ng DNA ml$^{-1}$ 10 % DMSO in TE buffer (pH 7.4) for DNA integrity determination.

**D. Cell lines and tissues**

The Fast Micromethod® is able do deal with kinetic fluorimetric measurement and dsDNA determination in minute amount of samples from different sources: native DNA, cell cultures and cell suspensions, lymphocytes and tissue homogenates. Zahn and coworkers (1996) used liquid nitrogen precooled mortar and pestle and homogenize the frozen stored tissues (-80 °C) in buffer with DMSO, as a preservation component, and recorded no evidence of significant influence on the Fast Micromethod® sensitivity. The detailed descriptions,
recommendations, and tips for DNA sample handling and use of the proper alkaline unwinding solution are presented in the works of Batel et al. (1999), Jakšić and Batel (2003) and Schröder et al. (2005).

**E. Sample lysing**

The tissue homogenates or cell suspensions have to be lysed first upon which the interactions between fluorochrome dye PicoGreen® and DNA occurs. This step has to provide the complete lyses of the cell walls and make the high integrity dsDNA prone to interact with PicoGreen®. This dye is originally supplied diluted in dimethyl sulfoxide (CH₃CH₃SO; DMSO) and should be protected from light and stored at -20 °C before use. The temperature, buffer component and time play important factors in this process. The composition (9 M urea, 0.2 M EDTA, 0.1 % SDS) and related ionic strength and the alkality (pH 10.0) of lysing solution equipped with PicoGreen® (20 µl of stock dye in 1 ml of lysing solution) assure the efficiency of this processes. The initial procedure deal with “lysing on ice” (suppression of DNA repair mechanisms), which may lead to precipitation of lysing buffer components (e.g. urea). Later, in our laboratory research, we reduce the urea to 4.5 M in lysing buffer and provide the lysing step on RT (up to 30 °C) for only 30 minutes (Jakšić and Batel, 2003).
**F. Alkaline unwinding**

The working alkaline NaOH solution should be prepared daily in order to assure the proper final pH of reaction mixture. It should be prepared by mixing the 0.1 M NaOH in 20 mM EDTA with 20 mM EDTA (ethylenediaminetetraacetic acid) solution. Five volumes (e.g. 2.5 ml) of such working alkaline solution is added to one volume of lysed sample (0.25 ml 10% DMSO in TE buffer pH 7.4 and 0.25 ml Lysing solution with PicoGreen® pH 10.0) and the pH is checked. If necessary, add few more drops of 0.1 M NaOH in 20 mM EDTA or 20 mM EDTA solution to alkaline working solution, make a new reaction mixture and check the pH. The procedure is repeated until the proper pH is reached. Addition of alkaline NaOH-EDTA solution immediately initiate the alkaline denaturation of DNA, and as soon as possible reduction of dsDNA-PicoGreen® complex should be started to follow by fluorimeter (e.g. 485/520 nm, depending on fluorimeter characteristics). Depending on DNA source (complexity of DNA organization, size of dsDNA), the optimal final pH should be reached by addition of alkaline lysing solution. Thereby, the human lymphocytes, RTG-2 cell lines and marine mussel gills DNA showed the optimal time-dependent DNA denaturation kinetics at the pH of 12.4, 11.6 and 11.5 respectively (Batel et al., 1999; Jakšić and Batel 2003; Sánchez-Fortún et al., 2005).
**G. Fluorescence Analysis and Calculation**

The extent of DNA denaturation was followed directly in the microplate by measuring the decline in fluorescence of the dsDNA-PicoGreen® complex for 20 to 30 min by microplate fluorescence reader. In our measurements we used the Fluoroscan II reader or Fluoroscan Ascent, both product of the Labsystems, Finland. Each sample should were analysed in quadruplicate. That approach allowed to calculate the average values of dsDNA-PicoGreen® fluorescence and drew the denaturation curves. Furthermore, according to Meyen and Jenkins (1983), after several minutes of denaturation, the strand scission factors (SSFs) may be calculated:

\[
SSF = \log_{10} \left( \frac{\text{% dsDNA}_{\text{sample}}}{\text{% dsDNA}_{\text{reference}}} \right)
\]

where recorded fluorescence values, after correction for blank readings, where used as a measure of dsDNA amount in both sample thus giving the equation:

\[
SSF = \log_{10} \left( \frac{\text{Fluorescence Units}_{\text{sample}}}{\text{Fluorescence Units}_{\text{reference}}} \right)
\]

Thus SSF=0 assumes an absence of DNA strand breaks and alkali-labile sites, while SSF<0 indicates increasing frequencies of strand breaks and alkali-labile sites (loss of DNA integrity) in samples.

The SSF is a measure of quantity of DNA damage excess. This logarithmic relationship between SSF values and relative amount of
damaged DNA (lowest integrity) in exanimate samples in the order of reference shows that SSF×(-1) a value from 0.1 to 1.0 indicates decrease of damaged DNA (lowest integrity) from 20.6 to 90.0 % respectively.

The slopes of denaturation curve describes the rate of the DNA unwinding and decay of dsDNA-PicoGreen® complex in alkaline conditions. It may be calculated as the difference in fluorescence values divided by denaturation time:

$$\text{Slope} = \frac{\Delta \text{Fluorescence Units}}{\text{time (min)}}$$

The time range where the slopes of the denaturation curves between reference and other samples showed evident but constant differences are suitable for SSF calculations and DNA integrity differences determination.

**H. Expression of Results**

The results are usually expressed as a strand scission factors (SSFs) with corresponding standard deviations in histograms or in scatter plot diagrams. The widely used a box-and-whisker plot is recommended to display a set of monitoring analysis data where high number of samples and corresponding replicates when the median, quartile, minimum, maximum, and outlier-extreme values should be taken into consideration. For practical reasons, and with the
expectation of lower DNA integrity in assessment than reference samples, the SSFs may be multiplied by (-1) and used in graphical presentations. On that way, with the expectation of lower DNA integrity in any other than reference sample, the bars on the histogram will be higher than bar representing the control – reference sample. The reference SSF×(-1) value remains equals about zero with corresponding standard deviation. Just in cases of present DNA cross-links that reduce rate of DNA unwinding the fluorescence achieved and calculated SSF values in samples will be higher than in reference, and corresponding SSF×(-1) values will be negative.
III. METHOD APPLICATION OVERVIEW

In the last decade, the Fast Micromethod® has been used in several research laboratories and studies. Their continuous development and application maintain from its German patent recognition in 1997 (Müller et al.). It has been employed in DNA integrity determination of several target organisms and their tissues and cell lines. Those research were founded by the joint research team of German and Croatian scientist who started to publish the results obtained by the Fast Micromethod® of DNA single-strand breaks and alkali-labile sites in marine sponge *Suberites domuncula* and human peripheral blood mononuclear cells. Furthermore, the method has been validated by its application on mouse tissues and hamster cells, marine mussel and fish cells and tissues.

In detailed description of this microplate assay by Batel et al. (1999) the authors confirmed the PicoGreen® specific characteristics to bind preferentially to dsDNA and explained the optimum alkalinity of the assay buffer. The calf thymus DNA was used and the fluorescence of dsDNA and ssDNA (prepared by 10 min. heating in a boiling water bath) in alkaline buffer assay were measured. The fluorescence of the dsDNA-PicoGreen® complex was higher than ssDNA-PicoGreen® and at the pH about 12.1 to 12.2 the dsDNA starts slowly to denaturate and at the pH of 12.6 the process became very fast and the fluorescence
difference between dsDNA and ssDNA disappears (Fig. 1). This transition region of the optimal pH, where the denaturation extends in optimum time, should be defined for different DNA samples from different sources, tissues and organisms. In the same work, the method sensitivity on cell numbers (cellular density) and DNA content used in measurement were demonstrated, and the matrix protein influence on DNA denaturation despite the high denaturating conditions postulated. Those findings lead to recommendation and mandatory use of same amount of cells or DNA per sample in the analysis.

The sensitivity and precision of the Fast Micromethod® has been confirmed by its comparison with the widely used Comet assay by measuring the DNA damage in HeLa cells (Bihari et al., 2002) induced by \( \gamma \)-radiation (8 to 500 cGy), UV-C light (10 to 1000 Jm\(^{-2}\)) and chemical agent 4-nitroquinolone-N-oxide (0.0026 to 0.26 \( \mu \)M NQO). The Fast Micromethod® results showed the slightly better sensitivity and wider linear dose response to UV-C and \( \gamma \)-radiation, although the similar sensitivity were achieved by up to 1 \( \mu \)M NQO by both techniques. However, the Fast Micromethod® results obtained the lowest standard deviations in a whole range of all three DNA damaging agents (Fig. 2).
Further adaptation, adjustment and application of this technique on DNA integrity research in marine invertebrates was presented in the work of Jakšić and Batel (2003). We clearly demonstrate the influence of DNA amount and denaturation media pH value on denaturation curve slopes and suggest the optimal 100 ng ml\(^{-1}\) of DNA and the denaturation media pH 11.5 value as a optimal parameters in DNA integrity analyses by Fast Micromethod\(^{\circledR}\) in mussel *Mytilus galloprovincialis* gills homogenate samples. The clear dose responses and DNA deterioration effects were achieved with benzo[a]pyrene (B[a]P) and 4-nitroquinoline-N-oxide (NQO) in our laboratory *in vivo* mussels exposition. *In vitro* treatment of cotton-spinner *Holothuria tubulosa* native DNA (Jakšić, 2002) and mussel *Mytilus galloprovincialis* gills DNA homogenate (Jakšić and Batel, 2003) with bleomycin-Fe(II) complex showed the decrease of the DNA integrity and the induction of DNA strand breaks and alkali-labile sites. The cotton-spinner native DNA showed the higher level of DNA damage by smaller amount of bleomycin-Fe(II) complex then DNA in mussel gills homogenate (Fig. 3). This finding confirmed the influence of DNA size and complexity with proteins in different organisms on DNA denaturation curves.

The Sánchez-Fortún research group (2005) optimized the cell density and alkaline unwinding conditions to RTG-2 cell lines. The
optimal output for its DNA denaturation was achieved at pH of 11.6
and with the respect to cell density and DNA amount the 3,000 to
30,000 cells per assay (attached or suspended) may be used in order
to get comparable fluorescence response.

Recently, Schröder and coworkers (2005) published the
comprehensive method instruction overview in Methods in molecular
biology series. This work describes the instrumentation, material and
chemicals needed for this technique performance. They gave the full
instructions and technical explanations of several important steps in
the procedure. The article deals with recommendations for work with
cells, cell lines and tissue preparation and explains the fluorimetric
measurement and calculations of strand scission factor values. They
enlisted several important notes for better understanding of the
procedure and tips to avoid possible technical problems.

A. Human peripheral blood mononuclear cells (PBMC)

The peripheral blood mononuclear cells (PBMC) were one of the
first cell types used by the Fast Micromethod® technique in order to
determinate its DNA integrity. Those cell samples were taken from
healthy donors as well as from cancer and X-ray treated patients, and
their DNA deterioration was compared. A strong increase of SSF×(-1)
values were found in PBMC irradiated with 0.5 to 2.5 Gy. Due to DNA
repair, after the 2 hours of incubation of the cells at 37 °C, the amount
of DNA single-strand breaks and alkali-labile sites decreased for 56 to 86% for lower and higher applied doses respectively (Schröder et al., 1998). Despite the variations between different individuals, the significant decrease in DNA integrity was measured in isolated PBMC from cancer patients after the radiotherapy (Elmendorff-Dreikorn et al., 1998). The age related increase of DNA strand breaks after the ex-vivo γ-ray treatment of isolated PBMC from healthy donors was observed. The basal level of DNA lesions in PBMC from donors aged >70 years was about 2-fold higher than in donors aged 20-30 and 40-60 years and the levels of γ-ray induced DNA damage were much higher in the oldest group by 1.8-fold and just 1.3-fold higher in other two groups of individuals. In the same laboratory, the correlation between apoptosis and DNA single-stand breaks in γ-ray irradiated human PBMC were found only for the individuals below 60 years, but the lack of correlation in oldest group of individuals remains unknown (Chauvin et al., 1998). All those findings recommend the Fast Micromethod® as a method of choice in pretherapeutic sensitivity test for the assessment of the individual radiosensitivity prior the radiation therapy (Elmendorff-Dreikorn et al., 1999). The Fast Micromethod® has been employed as a tool for the estimation of DNA repair efficiency in human lymphocytes after in vitro γ-ray (0-250 rad) irradiation and two hours of incubation at room temperature. The results showed the
significant decrease of DNA strand breaks in the entire dose range (Batel et al., 1999).

**B. Cell lines**

The induction of DNA strand breaks and alkali-labile sites in cultivated human HeLa cells by physical and chemical agents were investigated by the Fast Micromethod® application. The increased SSF×(-1) values and clear dose doses response were found after \( \gamma \)-radiation doses of 1 Gy and higher (Hassanein et al., 2002). The similar results of DNA damage induction in HeLa cells by \( \gamma \)-rays, UV-C light and NQO were indicated above (Bihari et al., 2002). The Fast Micromethod® results of activated bleomycin *in vitro* HeLa cell culture treatment showed the clear dose response to bleomycin-Fe(II) complex in cell culture (Jakšić, 2002). The level of DNA deterioration was similar to those achieved by the same doses bleomycin-Fe(II) in a marine mussel *Mytilus galloprovincialis* gills homogenate and up to 10-fold lower than SSF values achieved with native DNA from cotton-spinner sample (Jakšić, 2002).

**C. Rodents**

Above mentioned method sensitivity on DNA size and amount and the influence of cellular density in microplate wells has been studied on mouse lymphoblasts. Those cells have been exposed to \( \gamma \)-ray
irradiation of 0-500 rad, and different amount of cells were taken into the wells. The liner dose response for doses up to 500 rad was obtained in experiments with 5000 cells per well, while the higher sensitivity for doses up to 15 rad were achieved in experiments with only 2000 cells per well. Furthermore, the mouse lymphoblasts exposed to a 0-3 \( \mu \text{M} \) NQO and incubated for a 90 min, showed a clear dose response and higher level of DNA damage in entire range of applied genotoxic agent. The mouse muscle and liver tissues homogenate were exposed to \( \gamma \)-rays (0-200 rad) and the level of induced DNA damage were significantly higher in mouse muscle than liver tissue homogenate for all applied radiation doses (Batel et al., 1999). The clear dose response on \( \gamma \)-ray radiation doses of 0-1500 rad were found in experiment with Chinese hamster ovary cells (Jakšić, 2002).

**D. Marine organisms**

The Fast Micromethod® has been developed and applied on marine invertebrates since its beginnings. The marine sponge *Suberites domuncula* has been exposed to polychlorinated biphenyls up to six days and the extent of DNA damage in the sponge tissue were determinated after 12, 24, 72 and 144 hours of exposure. The time dependent increase of the DNA strand breaks and alkali-labile sites frequency, achieved by the Fast Micromethod® and expressed as a
strand scission factor, with the 25 μg of PCB118 and PCB153 per gram wet mass of sponges has been recorded. The DNA deterioration by exposition to PCB153 has been observed just after the 12 hours exposition period, but the strong reduction rate of DNA unwinding was recorded following the exposition to same amount of PCB77 that is indicative to DNA cross-link formation (Schröder et al., 1998; 1999a). Furthermore, those sponges were used as a bioindicator species in natural field study, in the Rovinj area of the northern Adriatic Sea, Croatia, for the assessment of potential cadmium genotoxic risk. The sponges collected from heavy metal nearly unpolluted pelagic area and polluted harbour sites, showed the positive correlation among the DNA damage, heat shock protein induction and heavy metal load. Following the additional laboratory exposure, of sponges from those sites, to single cadmium dose of 5 mg l⁻¹ for 5 days, the significant increase of heat shock protein and DNA damage level were recorded in all samples (Müller et al., 1998). The following year Schröder and coworkers (1999b) published the results of their research of cadmium induction of DNA strand brakes in the marine sponge Suberites domuncula too. They findings confirmed the inducibility of DNA damage by cadmium chloride exposition for up to six days. The similar time and dose dependent induction rates were found for both, heat shock protein and DNA damage levels. The highest SSF×(-1) values, following exposure
to 0.01, 0.1 and 1 mg l⁻¹ cadmium chloride, were observed after 72, 24 and 12 hours respectively. The maximum increase of SSF value was achieved with the highest cadmium chloride amount, and most likely due to DNA repair processes the ratio of DNA damage increased by the time for all applied doses. The Fast Micromethod® has been employed to determinate the induction of DNA damage in *Suberites domuncula* cells by γ-ray irradiation. The cells irradiated with 0-500 rad showed a slight increase of single-strand breaks, and the same or even lower γ-ray doses induced the significantly higher frequency of strand breaks and alkali-labile sites in mouse lymphocytes, muscle and liver tissue homogenates, Chinese hamster ovary cells, human lymphocytes and HeLa cell lines (Jakšić, 2002).

This technique was applied to quantify the single-strand breaks in DNA from tissue samples of marine sponge *Geodya cydonium* irradiated with 0-3000 J m⁻² UV-B light in controlled experimental conditions and from samples collected in the field (Batel et al., 1998). The expected and achieved induction of DNA damage and corresponding highest SSF×(-1) values in samples exposed to highest doses of UV-B irradiation confirmed the applicability of the Fast Micromethod® as a suitable assay for DNA damage estimation in sponge cells.
A few years later, the assay has been used to estimate the induction of DNA damage in Baikalian sponge *Baikalospongia intermedia* by heavy metals. The increased frequency of DNA single-strand breaks and alkali-labile sites were founded following the exposure to Pb, Zn and Cu (Efremova et al., 2002), and exposure to waste water from the final refinement and aeration pond of Baikalsk Pulp and Paper Plant as a known source of those xenobiotics (Schröder et al., 2006).

In the last decade, the sustainable process of Croatian Adriatic Sea monitoring programme employes the mussel *Mytilus galloprovincialis* as a bioindicator organism for estimation of genotoxic risk in marine ecosystem. The Fast Micromethod® carried out the DNA integrity deterioration measurement caused by the presence and effect of unknown mixture of genotoxic xenobiotics and their metabolites at 24 sampling sites along the eastern Adriatic coast. The results obtained in our biomonitoring campaign shoved the significant differences among the DNA integrity SSF values of mussel *Mytilus galloprovincialis* gills from different sampling sites, confirmed the presence of genotoxic compounds, their influence and effect on mussel gills DNA integrity, at “hot spots” areas near the industrial facilities and inhabited places (Jakšić et al., 2005). Furthermore, the assay has been successfully used in several spatial genotoxicity biomonitoring research of mussel
Mytilus galloprovincialis DNA integrity from the Mljet Island and Istrian peninsula at the Adriatic Sea (Bihari et al., 2005; Jakšić et al., 2008).

Furthermore, the Fast Micromethod® has been used to estimate the DNA strand breaks and alkali-labile sites in marine fish cell lines and tissues. In the monitoring of the North Sea and English Channel marine environment the dab, Limanda limanda, liver homogenates has been subject of the DNA strand breaks and alkali-labile sites determination. It was found the parallel extent of DNA damage and heat-shock protein levels, the low amount of both of the HSP70 forms and DNA damage at the Heligoland sampling site, but significantly higher HSP73 and HSP75 levels as well as DNA deteriorations at the North Sea and English Channel sampling sites (Schröder et al., 2000). The achieved correlation between selected biomarkers of exposure and effect of environmental xenobiotics confirmed not only the suitability of chosen bioindicator species and tissues, but also the convenience of the Fast Micromethod® as a method of choice in DNA damage determination in fishes and its adequacy in application in environmental monitoring studies.

The DNA damage in fibroblastic-like cell lines derived from rainbow trout, Oncorhynchus mykiss, gonadal tissue (RTG-2 cell line) exposed to THPC (tetrakis(hydroxymethyl) phosphonium (THPC) and benzalkonium chloride (BC) as biocides used in disinfection of cooling
towers (Sánchez-Fortún et al., 2005). The attached and RTG-2 cells in suspension were exposed for 24 hours to 1/10-, 1/25-, 1/50- and 1/100-IC50(48) value of each biocide and the achieved denaturation curves and corresponding SSF values showed significant dose dependent genotoxic effects of THPC and BC. The Fast Micromethod® has been used in the validation of primary culture of the rainbow trout, *Oncorhynchus mykiss*, hepatocytes for the estimation of DNA damage as the biomarker of exposure to genotoxic compounds of Slovenian rivers sediment genotoxic potential (Tollefsen et al., 2006). The significant increase of DNA strand breaks and alkali-labile sites was achieved by the cell exposition to 15-150 mg l⁻¹ NQO for 2 hours, but no increase of the DNA damage was observed above background at any sampling site.

All those research and achieved results indicates the Fast Micromethod®, as a fast and sensitive assay for DNA damage detection in a different cell types and tissues form several species, and gave the additional weight on method implementation. It recommends the Fast Micromethod® for the measurement of DNA integrity not only in human biological material for medical purpose, but also in marine species for genotoxicity assessment (biomonitoring) and for the estimation of harmful effects in the ecosystem.
IV. CONCLUSION

The Fast Micromethod®, as any other techniques based on DNA unwinding under alkaline conditions, cannot distinguish alkaline labile sites from single-strand breaks, because those lesions are converted to single-strand breaks by alkali. Furthermore, the DNA cross-links remains undetected and unresolved from primary DNA lesions, like in other assays. However, despite the enlisted shortcomings the Fast Micromethod® deal with some of the other method drawbacks.

The important issue performing all known analytical procedures is the generation of artificial strand breaks into the observed DNA sample. The tissue collected by biopsy may be frozen at -80 °C and homogenized under liquid with only negligible amounts of DNA damage without recorded evidence of significant influence on the Fast Micromethod® sensitivity. Moreover, the described assay allows the fast and simple approach for a huge number and series of samples. It utilizes the finest characteristics of newly synthesised fluorochrome, PicoGreen® to preferentially bind and give the stable complex with dsDNA. The decay of those high integrity DNA in alkaline medium may be continuously followed by fluorimetry and allow the high sensitivity up to picogram scale.

For the further development of this technique, the estimation of DNA-DNA and DNA-protein cross-links should be worked out. The
proteolytic enzymes, e.g. proteinase-K, or dispase should be able to destroy all the DNA-protein cross-links, and similarly, the appropriate agent should be able to deal with DNA-DNA cross-links. On that way, the estimation of amount of those DNA damage types presented in the sample and their resolving from the DNA strand breaks and alkali-labile sites of DNA damage will be possibly allowed. Moreover, the different denaturation media alkalinity and ionic strength might be able resolving the single to double-strand DNA breaks in observed samples.

The presented portrait of the Fast Micromethod®, with all respect to some shortcomings and its inchoateness, affirms as a good and additional, to currently existing techniques, tool and method of choice for DNA integrity estimation. The data collected in several described studies with different kind of tissue and cell types approve its applicability in a wide range of genotoxicity research and applications from human medical purpose to biomonitoring assessment studies.

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V. REFERENCES


Tollefsen, K. E., Bratsberg, E., Boyum, O., Finne, E. F., Gregersen, I.


**Figure captions**

Fig. 1. Effect of alkaline NaOH-EDTA assay buffer pH value on the fluorescence of dsDNA-PicoGreen® and ssDNA-PicoGreen® complexes. The ssDNA was prepared by temperature denaturation of calf thymus dsDNA - 10 minutes heating in a boiling bath (adapted from Batel et al, 1999).

Fig. 2. DNA deterioration induced by A) $\gamma$-radiation, B) UV-C light and C) NQO in HeLa cells measured by Fast Micromethod® and Comet assay (adapted from Bihari et. al., 2002).

Fig. 3. *In vitro* effect of bleomycin–Fe(II) complex on the induction of strand brakes in A) *Holothuria tubulosa* native DNA and B) mussel *Mytilus galloprovincialis* gills homogenate DNA (adapted from Jakšić and Batel, 2003).
A)

![Graph A: X-ray dose vs. Extent tail moment for Fast Micromethod and Comet assay. The graphs show an increase in Extent tail moment with increasing X-ray dose.](image)

B)

![Graph B: UV dose vs. Extent tail moment for Fast Micromethod and Comet assay. The graphs show an increase in Extent tail moment with increasing UV dose.](image)

C)

![Graph C: NQO concentration vs. Extent tail moment for Fast Micromethod and Comet assay. The graphs show an increase in Extent tail moment with increasing NQO concentration.](image)