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Technical recommendations to perform the alkaline standard and enzyme-modified comet assay in human biomonitoring studies

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Highlights:

- The comet assay is widely used in human biomonitoring to measure DNA damage
- Variation in comet assay results between laboratories has been a problem
- Specific steps in the assay have been identified as causes of this variation
- Including reference standards in experiments helps to control variation
- We give recommendations for improving the reliability of the assay

Abstract

The comet assay (single cell gel electrophoresis) is widely used as a biomonitoring tool to assess DNA damage - strand breaks, as well as oxidised bases; it can also be adapted to measure DNA repair. It is based on the ability of breaks in the DNA to relax supercoiling, allowing DNA loops to extend from the nuclear core (nucleoid) under an electric field to form a comet-like tail. Most commonly, it is applied to white blood cells. The range of detection is between a few hundred breaks per cell and a few thousand, encompassing levels of damage that can be repaired and tolerated by human cells. Its applications include monitoring various diseases, studying the influence of nutrition on DNA stability, and investigating effects of environmental and occupational mutagens. Here we address the issue of inter-laboratory variation in comet assay results. This variation is largely due to differences in methods. Imposing a standard protocol is not practical, but users should be aware of the crucial parameters that affect performance of the assay. These include the concentration of agarose in which the cells are embedded; the duration of cell lysis, and of enzyme incubation when oxidised bases are being measured; the duration of alkaline unwinding; the duration of electrophoresis and the voltage gradient applied; and the method used to score the comets. Including reference standards in each experiment allows experimental variability to be monitored - and if variation is not extreme, results can be normalised using reference standard values. Reference standards are also essential for inter-laboratory comparison. Finally, we offer recommendations which, we believe, will limit variability and increase the usefulness of this assay in molecular epidemiology.

Keywords: standard comet assay; Fpg-modified comet assay; DNA damage; protocol; controls; recommendations

1. Introduction

DNA damage is unavoidable. In addition to spontaneous loss of bases (depurination, described by Lindahl [1]), cellular DNA is subject to attack by endogenous and exogenous agents. Reactive oxygen species (ROS) are released as a by-product of respiration, and while most are inactivated by the cell's antioxidant defences, some oxidation of DNA bases does occur. Exogenous damaging agents include ultraviolet (UV) and ionising radiation, and a multitude of environmental chemicals, causing a variety of lesions, from single and double strand breaks, to altered bases, bulky adducts and cross-links within or between strands or between DNA and protein. DNA repair pathways have evolved that deal efficiently with the damage, so the steady state level of damage is low, but measurable.

The comet assay (single cell gel electrophoresis) was developed in the 1980s as a way of detecting DNA damage at the level of individual cells [2, 3]. Essentially, cells are embedded in agarose on a glass or plastic substrate, lysed to remove membranes and soluble cell components, and the DNA stripped of histones by high molarity NaCl. This leaves the DNA attached at intervals to a nuclear matrix, as supercoiled loops - a structure known as a nucleoid. Electrophoresis (normally at high pH) draws the DNA towards the anode, but only those loops containing breaks, which allow release of supercoiling, are able to move significantly; they form an image resembling the tail of a comet when stained with a fluorescent dye and viewed under fluorescence microscopy. (Alternative staining methods exist - for example, silver staining - but are not often used.) The relative intensity of tail fluorescence reflects the proportion of relaxed loops and therefore the frequency of breaks. The actual number of breaks can be estimated by calibration against ionising radiation, which is known to introduce 0.31 breaks per 10^9 Da per Gy [4]. The range of detection with this assay is between a few hundred breaks per cell (i.e. a few breaks per chromosome) to a few thousand, which conveniently encompasses levels of damage that can be repaired and tolerated by normal healthy cells.

In addition to its simplicity and sensitivity, it has the advantage of being applicable to non-dividing cells, and peripheral white blood cells (WBCs) from humans were seen as an appropriate test material. Early clinical applications included a study of WBCs from infected and malnourished children [5], bladder cancer (using cells from bladder washings), male infertility (testing sperm) [6], and diabetes [7]. Studies of nutrition and lifestyle with the comet assay have generally involved intervention with antioxidants or antioxidant-rich foods. Green et al. [8] found that dosing healthy subjects with vitamin C protected WBCs from the damaging effects of X-rays *ex vivo*, while Hartmann et al. [9] showed that vitamin E prevented exercise-induced DNA damage. At the same time, effects of environmental or occupational mutagens were beginning to be studied with the comet assay; Vodicka et al. [10] found increased DNA damage in WBCs of lamination workers exposed to styrene.

A new era was opened up by the introduction of an additional step in the comet assay - digestion of the nucleoid DNA with an enzyme that converts particular lesions to DNA breaks. The first enzyme to be used was endonuclease III, which recognises oxidised pyrimidines [11]; it was applied in a nutritional intervention study with antioxidants (vitamin C, vitamin E and β-carotene) [12], and showed a significant decrease in base oxidation after supplementation. Subsequently, formamidopyrimidine DNA glycosylase (Fpg) was introduced to detect oxidised purines [13]; various other enzymes have been used, but Fpg is now the most widely used, at least for human biomonitoring purposes.

DNA damage should be regarded as a marker of exposure - whether to harmful agents, or to beneficial substances such as antioxidant micronutrients. Although mutations are initiated by DNA damage, and may represent an early stage in the carcinogenic process, the link between DNA damage as measured with the comet assay and cancer incidence is extremely

tenuous; almost all DNA lesions are removed before the DNA is replicated (the stage at which DNA damage may be fixed as a permanent change in the genome), and even unrepaired lesions are unlikely to have any effect unless they occur in oncogenes, tumour suppressor genes or other genes associated with genome stability and cell division. The capacity of cells to repair DNA damage is likely to affect the probability of mutagenesis, and individual repair capacity is regarded as a marker of cancer susceptibility - a high intrinsic repair capacity supposedly protecting against cancer. However, while various polymorphisms in repair genes affect repair capacity, only very few have shown significant links with cancer risk [14]. DNA repair is not an unambiguous guide to susceptibility; it could be elevated in certain individuals as a result of induction by exposure to a carcinogen, in which case it might indicate increased rather than decreased risk.

Inter-laboratory variation in comet assay results has been a worrying factor over the years; in this article we describe ways to reduce it. The COST Action hCOMET was set up in 2016 to bring together researchers involved in human biomonitoring, and to collect the many thousands of individual sets of comet assay data that now exist, in order to carry out a pooled analysis. This should identify causes of variability, and - using statistical techniques - give definitive answers to questions such as the effect of age on DNA damage, possible differences between men and women, effects of smoking, etc.

As implied above, it is not clear whether DNA damage measured in an individual bears any relation to cancer risk. The necessary prospective study has yet to be carried out, to determine whether DNA damage is a predictive marker of cancer risk or mortality. This will involve large numbers of subjects, efficient recording of cancer incidence and deaths, and a long wait for disease to occur. An attractive alternative approach, the feasibility of which is currently under investigation in hCOMET, is to carry out a retrospective trial, making use of stored blood samples in a nested case-control study.

The purpose of this paper is to describe the causes of variation in comet assay results, and to explain how to limit or control the variation. When the variation is known to be due to the use of different experimental conditions, it may be possible to compensate for the variation and make comparisons between different laboratories' results. We refer here in particular to applications of the assay in human biomonitoring, but do not deal with how to obtain, store or handle samples; rather we concentrate on the performance of the assay.

2. Differences in parameters and experimental conditions

Different parameters (composition of solutions, buffers and/or duration of different steps of the comet assays protocol) have been supposed or demonstrated to critically influence comet assays results. The range of different conditions used in the assay is enormous, as illustrated by the fact that among 33 hCOMET laboratories, there are 55 different methods in use.

First, the format of gels varies, with more than 80 % of protocols using a 1, 2 or 3 gel format on glass microscope slides, while other laboratories use 12 mini-gels on glass slides or up to 96 gels on GelBond® films.

Most protocols include pre-coating of glass slides with a 1 % agarose layer to improve gel adhesion. The % of the low melting point (LMP) agarose layer containing the cells is 0.5-0.7 %, for 60 % of the users. About 1 in 3 protocols include a third layer of agarose.

The standard lysis solution composition is 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, supplemented with dimethyl sulfoxide (DMSO) in 60 % of the protocols

and/or supplemented with sodium sarcosinate (10 % of the protocols). The duration of lysis is very variable, as depicted in table 1.

Twelve of the 33 laboratories perform the enzyme-modified assay, with 15 different protocols; the most used enzyme is Fpg. Incubation of nucleoids with the enzyme takes place after lysis, and a parallel incubation with reaction buffer provides a control.

The standard unwinding and electrophoresis solution is 0.3 M NaOH, 1 mM EDTA, pH > 13. The durations of unwinding and of electrophoresis differ somewhat between protocols (see table 1 for details).

After electrophoresis, the neutralization step involves between 1 and 3 washes of 5 or 10 min, with either a Tris buffer, phosphate-buffered saline (PBS) or H_2O. After neutralization, staining can be performed with different dyes (e.g. 4,6-diamidino- 2-phenylindole (DAPI), ethidium bromide, SYBR Gold, silver nitrate, Gelred, SYBR Green or Yoyo-1, the most used being DAPI and ethidium bromide (42/55 protocols).

Forty two % of protocols use reference standards, such as cells treated with radiation (X-rays or ɣ-rays), chemical alkylating agents (ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS)) or oxidising agents (e.g. H_2O_2 or photosensitiser Ro 19-8022 plus light).

Among 55 protocols, most advise that some steps of the assay are performed in the dark.

3. Effects of differences in experimental conditions

A high inter-, and even intra-, laboratory variation is often reported when using the alkaline comet assay [15-20]. The inter-laboratory variation is most likely due to the many different protocols that are in use, as illustrated in the previous section and in table 1. Most protocols are based on the standard alkaline version of this technique as first described by Singh et al. in 1988 [3]. In this paper, untreated, X-ray- or H_2O_2-treated human lymphocytes were embedded in 0.5% LMP agarose on a glass microscope slide and lysed for 1h in a lysis buffer (2.5M NaCl, 100mM Na_2-EDTA, 10mM Tris, 1% sodium sarcosinate and 1% Triton X-100, pH 10). The DNA was then unwound for 20 min in an alkaline solution (1mM Na_2 -EDTA and 0.3M NaOH, pH > 13) and electrophoresis was carried out in the same solution at 25 V for 20 min (they did not specify the V/cm). Slides were then stained with ethidium bromide and visualized using a fluorescence microscope. Deviations from this protocol, however, are common; some are widely accepted, such as the pre-coating of slides with agarose to improve adhesion, while others are laboratory-specific. But small variations in the protocol may have a substantial impact on the outcome of the assay.

In this section, we are going to describe the different factors that influence the outcome of the comet assay and some others that should be taken into account. Moreover, we are going to discuss some factors that have not been thoroughly studied yet but may also have an impact on the results.

The term standard comet assay will be used for the assay in which enzymes are not used.

It is worth mentioning that the value of the comet assay descriptor (e.g. % DNA in tail) can be influenced by particular features of the assay protocol in use, whereas the “true” level of DNA lesions is independent of the protocol. The general approach to remove the influence of the assay conditions is to calibrate the test results against DNA breakage generated by ionizing radiation [21-23].

3. 1. Factors known to influence the results

Several studies have been carried out in order to assess the impact of different comet assay conditions on the outcome of the assay. Most of the studies have been performed using established cell lines or human lymphocytes treated with different genotoxic agents. However, results are also relevant for the use of the comet assay in human biomonitoring.

Some of the comet assay conditions affect the DNA migration (notably, agarose concentration, and strength and duration of electrophoresis) while some others may influence the kinds of DNA lesions detected. In this section, studies of the effect of the different conditions will be assessed following the chronological order of the protocol.

Concentration of agarose for embedding the cells

Final agarose concentration in gels, i.e. after mixing the agarose with the cell suspension, has a marked effect on the comet assay outcome; it is inversely proportional to % DNA in tail in treated cells [24, 25]. This value increased from 23.1% in 0.95% agarose to 40.4% in 0.4% agarose in H_2O_2-treated human lymphocytes, and from 28.3% in 0.95% agarose to 50.5% in 0.4% agarose in H_2O_2-treated TK6 cells [24]. This increase was not so clear in non-treated cells, which is expected since such cells have low levels of DNA damage. Ersson and Møller found similar results in ɣ-irradiated THP1 cells, though they also reported a slight increase in the % tail DNA of non-irradiated cells [25].

Higher agarose concentrations reduce the mobility of molecules during electrophoresis, so mechanistically the importance of agarose is well understood. To a certain extent the agarose concentration may be varied to increase or reduce the sensitivity of the assay. It is worth mentioning, however, that the lowest concentration of agarose tested, 0.4%, is not recommended for general use, as such gels are very fragile [24].

Duration of the lysis step

Lysis time has no or very small effect on the % DNA in tail of untreated cells [26, 27]. However, it has a marked effect in cells containing certain types of lesions such as H_2O_2- or MMS-induced damage; a longer period of lysis allows the detection of a considerably greater amount of lesions (Table 2). The increase in the % tail DNA observed with longer times of lysis may correspond to spontaneously formed apurinic/apyrimidinic site (AP) sites from both alkylated and oxidized bases during the lysis step at pH 10 [26]. Regarding X-ray-induced lesions, the duration of the lysis step does not seem to have a pronounced impact [27].

The absence of lysis gave similar results to lysing the cells for one hour, since the alkaline treatment that follows the lysis step is enough to lyse the cells. Actually, some researchers carry out the standard alkaline comet assay by performing the lysis and the unwinding of the DNA at the same time [28-31]. In this regard, a good correlation was observed in control and ɣ-irradiated lymphocytes, whether the lysis step was performed or not [32].

Regarding the Fpg-modified assay, Enciso et al. showed that lysis is essential and that the duration of lysis also has an impact on the DNA lesions detected [26]. In this case, a brief lysis (i.e., 5 min) is sufficient to allow the enzyme to reach the DNA. Moreover, it has also been shown, in the particular case of glycidamide-exposed cells, that extended lysis at pH 10 leads to chemical modification of the induced DNA base adducts, making them more prone to detection with Fpg [33].

Measuring base oxidation: Fpg concentration and duration of incubation

Fpg concentration and incubation time are critically important in the Fpg-modified comet assay. The concentration of Fpg should be high enough to detect the maximum amount of lesions present in the cells (ideally all of them) without exhibiting non-specific breakage activity. To determine this concentration, a titration experiments must be carried out using cells that contain 8-oxo-guanine (e.g., cells treated with Ro 19-8022 plus visible light, or with potassium bromate) and cells with intact DNA. These experiments should be carried out using the same conditions that are going to be used in the planned experiments; Fpg concentration and time of incubation vary depending on the procedure and the equipment [34, this issue].

The time of incubation is also critical; ideally, a plateau is reached after a certain time. Ersson and Møller observed that the net Fpg-sensitive sites increase from 35% to 50% DNA in tail in A549 cells treated with Ro 19-8022 (plus visible light) when increasing the time of Fpg incubation from 10 to 30 minutes, while longer times of incubation (i.e., 45 minutes) did not substantially increase the level of Fpg-sensitive sites detected [25].

Duration and temperature of the alkaline treatment

The duration of the alkaline treatment, also referred as unwinding period, has a clear effect on the extent of DNA damage detected as has been shown by different authors [24, 25, 35-38]. All of them demonstrated how increasing the duration of the alkaline treatment induces an increase in the DNA strand breaks detected. During the alkaline treatment, alkali labile sites (ALS) are converted into breaks; the kinetics of this conversion has not been thoroughly studied but, judging by the results cited above, appears to be time-dependent.

The % tail DNA of H_2O_2- treated human lymphocytes increases from 19.6 when using 10 min of alkaline incubation to 41.0% when using 60 min. In H_2O_2-treated TK6 cells the increase was from 20.7% after 10 min to 35.8 after 60 min. In both cases there was time-dependence; however, results after 40 and 60 min were similar [24]. Untreated cells did not show a clear time-dependence response. Similar effects were observed by Ersson and Moller using ɣ-irradiated THP1 cells and H_2O_2-treated A549 cells [25]. However, results after 40 and 60 min of alkaline treatment were significantly different in the ɣ-irradiated THP1 cells but not in the H_2O_2-treated A549 cells. They also showed that the duration of the alkaline treatment affects the detection of Fpg-sensitive sites; longer alkaline incubation (from 20 to 60 min) gave slightly but significantly higher Fpg-sensitive sites in A549 cells treated with Ro 19-8022 plus visible light (from approximately 47% DNA in tail to 52%).

A study by Forchhammer et al. showed higher levels of DNA migration for the determination of DNA strand breaks and Fpg-sensitive sites in peripheral blood mononuclear cells (PBMCs) from healthy volunteers using comet assay protocols with long alkaline unwinding time (40 as compared to 20 min) and electrophoresis time (30 as compared to 20 min) [37]. The difference in Fpg-sensitive sites attributed to alkaline incubation and electrophoresis times was removed by standardizing the DNA migration levels, using a calibration curve. On the other hand, the PBMC samples had low level of DNA strand breaks (less than 15 arbitrary units, evaluated by visual scoring) and the difference between protocols only disappeared by using a reference standard to standardize the results.

Speit et al. showed that not only the duration of the alkaline incubation (20 or 40 min) but also the temperature affects the standard comet assay results [38]. They performed both alkaline treatment and electrophoresis at 4 or 20°C and showed an increase in DNA migration, in the background level and the MMS- and ɣ ray-induced lesions, in V79 and human blood cells when the steps were carried out at 20°C. Sirota et al. showed that results obtained using alkaline incubation temperature between 8 and 20°C in X-irradiated human

leukocytes were statistically similar (though a slight increase with the temperature was observed). However, increasing the temperature to 25°C produced statistically significant differences [39].

The increase in the DNA damage detected when the alkaline treatment and/or the electrophoresis are performed at higher temperatures can be due to a higher rate of transformation of ALS into breaks. Moreover, changes in the agarose gel at high temperatures can also affect the DNA migration [39].

Voltages, duration, temperature and recirculation of the solution of the electrophoresis

Electrophoresis is a key step in the alkaline comet assay. Variations in this step have been clearly shown in several reports to affect the results. Furthermore, the mechanisms behind the importance of electric potential and time of electrophoresis are well understood [40].

The voltage applied in the electrophoresis tank has a great impact on the % DNA in tail; the higher the voltage, the higher are the electrophoretic forces, and the higher is the % DNA in tail [24, 25]. The voltage in this context denotes the local electric potential, i.e. V/cm, where the samples reside in the tank during electrophoresis. Table 3 shows the results obtained by Azqueta et al [24]. Ersson and Moller observed similar results using ɣ-irradiated THP1 cells and H_2O_2-treated A549 cells [25].

Similar to V/cm, the duration of electrophoresis also has great impact on the % DNA in tail; increasing the duration of electrophoresis enhanced the DNA migration in both controls and ɣ-irradiated human lymphocytes [35], whole blood and V79 cells [38], control and H_2O_2-treated human lymphocytes and TK6 cells [24], and control and ɣ-irradiated THP1 cells and H_2O_2-treated A549 cells [25].

To illustrate the increase, here we describe the results obtained by Azqueta et al [24]. A steady increase in % tail DNA was observed in H_2O_2-treated cells and in untreated cells - both human lymphocytes and TK6 cells. The % DNA in tail of untreated human lymphocytes increased from 0.9 after 5 min of electrophoresis to 5.9 after 60 min, while the increase in H_2O_2-treated lymphocytes was from 9.5 after 5 min to 51.5 after 60 min. The same pattern of results was observed in TK6 cells. All these experiments were performed using 0.83 V/cm for electrophoresis.

It is important to note that the % tail DNA is linearly related to both V/cm and to duration (minutes) of electrophoresis. This has been observed experimentally and the mechanism is also well understood. These interdependences could be used as a basis for introducing correction factors when comparing results obtained in laboratories with electrophoresis conditions deviating significantly from standard ones [40].

As was mentioned in the previous section, Speit et al. performed both alkaline treatment and electrophoresis at 4 and 20°C and showed an increase in the DNA migration when both steps were carried out at 20°C [38]. Sirota et al. presented results along these lines [39]. It seems that temperature is also important.

Recirculation of the electrophoresis solution during electrophoresis also has an impact on the comet assay outcome. Gutzkow et al. showed how the recirculation of the electrophoresis solution not only decreased the variability of replicated samples within minigels (i.e., 96-minigels/Geldbond® film format) but also increased the DNA migration detected in X-ray irradiated human lymphocytes [41].

Staining comets

Olive et al. demonstrated that the concentration of dye can also affect the level of DNA damage detected [28]. They tested different concentrations of propidium iodide (0.1-10 µg/mL) for staining X-irradiated V79 cells and controls. They concluded that at low concentration the image detection was compromised, but at concentrations higher than 5 µg/mL the compound affected the DNA and increased the background fluorescence (they used 2.5 µg/mL to perform their experiments). The same group tested 3 different staining methods - namely propidium iodide as an intercalating DNA-binding dye, and Hoechst 33342 and DAPI as non-intercalating DNA-binding dyes [29]. All approaches showed similar sensitivity. Sirota et al. also reported the same results using SYBR green and ethidium bromide [39].

Scoring comets

Scoring comets can be a potential cause of variation; microscope quality and adjustments, aging of the fluorescence microscopy light source, in particular the traditional mercury lamp, method use for scoring, and even settings within the image analysis software may affect the results.

Different ways of scoring are actually used. They can be classified in 3 main groups: visual scoring (classifying the comets according to the relative amount of DNA in the tail and the head), conventional computerised image analysis systems (also called semiautomatic image analysis systems), and automated image analysis systems. Both the latter express results as a variety of descriptors; the most used are % DNA in tail, and tail moment, which integrates the % DNA in tail and the tail length [22]. In a comparative study, visual scoring using five categories, from 0 (no tail) to 4 (almost all the DNA in tail), the semiautomatic image analysis system 'Comet Assay IV' (Perceptive) and the automated image analysis system 'Pathfinder_Cellscan Comet' (IMSTAR), gave valid and interchangeable results [42]. The three approaches showed similar sensitivity in the ability to give quantitative estimates of damage caused by different concentrations of MMS or H_2O_2. However, visual scoring over-estimates low levels of DNA damage while the Pathfinder-Cellscan Comet system had problems to detect some of the heavily damaged comets.

Sirota et al. also reported no significant effect on the results when using two different image analysis software (namely, CASP, from CaspLab, and a homemade software) [39].

Aging of the fluorescence microscope lamp is a parameter that should always be checked. The effect of aging can be clearly seen by analysing the same slides before and after changing a lamp.

3.2. Other factors

A number of other experimental factors have been suggested to be of importance for comet assay results. These factors might not influence the results directly but might be indirectly of relevance. This is the case with the number of cells per gel. Having very few cells can complicate the scoring or invalidate the assay if there are not enough comets to evaluate. On the other hand, too many cells can also be a serious problem; it is not possible to evaluate overlapping comets when using an image analysis system, and in particular heavily damaged cells may be systematically and erroneously excluded. Therefore, getting the right concentration of cells is really important.

The electric current (mA) during electrophoresis was in the past claimed to be important for the comet assay outcome, and the current was, in the early protocols, set at a specific level [3]. This is, however, now known to be unnecessary. The irrelevance of the current has been

supported by both experimental and theoretical evidence. Even so, the level of the electrophoresis solution, usually adjusted to set up a certain current, may in some tanks have an indirect although moderate impact on the voltage gradient [24, 40]. Therefore, it is recommended to use a constant volume of alkaline solution, selected so that the power supply can provide an optimal voltage gradient.

It should also be taken into account that a high current could increase the temperature of the electrophoresis buffer, in which case cooling will be necessary.

4. Published guidelines

The literature contains a number of "recommendations", "guidelines" and specific "protocols" for the comet assay (listed in table 4). The terms seem to be used synonymously in the literature. However, a "guideline" is a stricter procedure than a "recommendation" as instructions are required to be followed in a guideline. Similarly, a "procedure" (or "standard operating procedure, SOP") is a very specific description of conditions that have to be followed. As an historic overview of the eagerness to standardize the comet assay, Table 4 lists protocols that have been published since 2000. Certain authors have focused on applications of the comet assay in genotoxicity testing rather than biomonitoring, although the instructions are applicable to samples collected from humans too. The protocols for biomonitoring studies have focused mainly on easily isolated cells such as WBCs. Importantly, protocols used in genotoxicity studies of adherent tissue cells (*in vitro* studies) or animal models (*in vivo* studies) have relevant information for researchers who will use the comet assay to measure DNA damage in tissue samples (biopsies) or various types of exfoliated cells from humans. Biopsy samples and exfoliated cells have much higher heterogeneity in DNA damage levels than WBCs; and the use of a non-optimal procedure may lead to high baseline levels of DNA damage. These limitations have been ameliorated in genetic toxicology through modifications in various steps of the comet assay protocol. With the exception of the OECD guideline from 2016 [54], they have been published by researchers from only one laboratory and they are predominantly protocols for the standard alkaline comet assay in cell culture studies or animal models. The OECD Test Guideline 489, *In Vivo* Mammalian Alkaline Comet Assay, was developed for analysis of DNA strand breaks in tissues from animals [54]. Thus, it is restricted to only the standard comet assay and contains recommendations rather than well-defined operating procedures. That being said, the OECD document contains a useful list of positive controls for the standard comet assay, which are also applicable as reference samples in biomonitoring studies, although they may not be suitable reference samples for the enzyme-modified comet assay for measurement of oxidatively damaged DNA.

Table 5 summarises the previously published assay protocols for the standard and enzyme-modified comet assay, which have been tested in multi-laboratory studies. The enzyme-modified protocol was developed by the European Standards Committee on Oxidative DNA Damage (ESCODD) for the purpose of determining true levels of oxidatively damaged DNA in cells from humans [15, 56, 57]. The comet assay results in this trial were compared with results from alternative methods to determine Fpg-sensitive sites, such as alkaline elution or alkaline unwinding, and measurements of 8-oxodG by chromatographic assays. In principle, the former protocol was identical to the protocol that had been developed by Collins and co-workers [23]. In retrospect, it was realized that the participating laboratories had not strictly followed the recommended protocol.

The ESCODD protocol was essentially adopted in the NewGeneris project, which also involved a number of the same researchers as took part in ESCODD. The European Comet Assay Validation Group (ECVAG) developed their validation studies on the notion that it is

not necessary to adhere to an SOP if primary comet assay descriptors are standardized by use of laboratory-specific calibration curve samples [16]. It was shown that the inter-laboratory variation would be reduced by this procedure for both the standard and Fpg-modified comet assay [17, 18, 20, 58]. Nevertheless, in an attempt to reduce the inter-laboratory variation in DNA damage levels, ECVAG developed a standard comet assay protocol [19]. Surprisingly, half of the participating laboratories experienced technical problems when using the standard procedure. The experiences in ESCODD and ECVAG indicate that standardising the comet assay procedure is not straightforward. Researchers are reluctant to change a procedure that works in their laboratory.

5. Controls

5.1. Positive and negative controls

Møller et al. defined positive control as 'an exposure that will generate DNA damage in cells or tissues (typically a separate exposure group)' [59]. Together with negative controls, they are used not only to demonstrate the correct performance of the assay but also to ensure that the biological system has a reasonable background level of DNA damage and is sensitive to external exposure. Obviously, for ethical reasons, positive controls cannot be included in human biomonitoring studies. However, a control group, matched with respect to sex, age, smoking habit, alcohol consumption, nutrition and lifestyle to the exposed group should be included in any study. The control group is formed by a group of subjects who are unexposed, untreated or take a placebo, depending on the type of study. Theoretically, they should exhibit a low level of DNA damage (% DNA in tail). The background level of DNA damage in human PBMCs (commonly referred to as lymphocytes) may be around 8-10% [60, 61].

5.2. Assay controls or reference standards

In human biomonitoring studies, when a long series of human cell samples are being analysed over a period of months or years, routine inclusion of assay controls or reference standards (terms used interchangeably) has been proposed [61, 62]. An assay control has been defined as **'**Samples that are included in every comet assay experiment within the same laboratory (preferably cryopreserved samples that have been exposed to a DNA damaging agent)' [59].

Assay controls are prepared from a single batch of cells (or pooled samples), either untreated (negative standard) or treated with an appropriate damaging agent (positive standard), frozen slowly as a large number of aliquots in freezing medium and stored at - 80° C. Freshly isolated or cryopreserved human PBMCs or mammalian cell lines with background level of DNA damage have been used as a reference standard as well as cells exposed to genotoxic agents [60, 62-64]. As examples, for strand breaks, cells exposed to a known DNA-damaging agent such as H_2O_2, ionising radiation or MMS provide a good positive reference standard, while if oxidised bases are of interest, cells treated with a photosensitiser plus visible light or with potassium bromate can be used. However, it is very important to establish the long-term stability of the assay controls at -80°C.

Assay controls should be included in each electrophoresis run, during the whole study. Even better, reference cells may be included in the same samples if they can be distinguished from (e.g.) PBMCs during scoring; such methods have recently been described based on the use of blood cells from fish with DNA content much lower than that in humans [65]. Assay controls or internal reference cells will guarantee that intra-laboratory variability is properly

controlled; if variation exceeds a certain limit, test results should not be trusted, but if variation is modest, test results can be normalised on the basis of the reference standard value [66]. Møller et al. demonstrated the great value of assay controls many years ago [67]. They included assay controls in a study on sunlight exposure where blood samples were obtained from volunteers over a period of 15 months. The unexplained (i.e. residual) variation in the full multivariate statistical analysis was identical to the variation in the reference samples, which meant that the residual variation in the dataset was indeed assay variation. There was no need for further statistical analysis.

Reference standards are also essential for inter-laboratory comparison. Results from those laboratories that share the same reference standards can be directly compared. Otherwise, calibration against X-irradiated cells with defined amounts of damage can control for inter-laboratory variation.

5.3. Historical controls

It is standard practice in genotoxicity testing to keep records of results of tests of positive and negative controls, as a 'historical control' dataset. This is important for quality control, defining an acceptable (limited) level of variation, and assessing assay reliability. In the case of human biomonitoring, it is also important to build a database of background damage levels in PBMCs (or other cell types) from control populations. This allows the consistency of the assay to be monitored over time. 'Background damage' refers to DNA strand breaks (and ALS) with the standard assay, as well as to base oxidation with the enzyme-modified assay.

6. Observations and recommendations

Below we present some observations and specific recommendations, mostly based on ideas that have been thoroughly explored above. Recommendations are never completely comprehensive; for some applications, special conditions are needed. But in general, the following advice can be followed. Most of these recommendations are applicable also to genotoxicity studies, whether *in vitro* or *in vivo*.

A top layer of agarose, covering the layer containing the cells, is quite unnecessary (and may delay or prevent access of enzymes).

The lysis solution does not need to contain sodium sarcosinate. Triton X-100 is a perfectly adequate detergent, effectively lysing cell and nuclear membranes. Sperm are an exception to this; they need more severe treatment to 'unwrap' the DNA. Epithelial cells, such as from the buccal lining, are also refractory.

DMSO was not present in the lysis solution formulated by Singh et al. [3] and is generally not necessary (Nor was it include in the solution used by Ostling and Johanson [2] to lyse the cells.). It was added later on for the use of whole blood in order to prevent the DNA induced by the iron released from erythrocytes during lysis and tissue samples [68, 43]. However, satisfactory results haven been obtained without including it [69-71].

With human (or animal) cells, it is normally not necessary to perform any stages of the comet assay in the dark [only in plant cells, where illuminated chloroplasts will produce damaging free radicals, is this important]. *In vitro* experiments with chemicals or particles known to be photoreactive are an exception.

Electrophoresis solution can be used more than once; as a rule of thumb it can be used 3 times but it must be stored sealed (don't store in an open vessel as it can absorb CO_2 which will reduce the pH).

A deeper layer of electrophoresis solution above the gels reduces the problem associated with tanks not being completely level.

Within reason, the current can be as high as your power supply allows. There is no rationale for aiming at 300 mA (as many people do).

Lowering the pH after electrophoresis is essential - but this doesn't need 3 washes with Tris buffer. PBS, or another simple neutral pH buffer, or even water will do this.

Mercury lamps in microscopes used for scoring need to be changed frequently, as aging can affect detection of comets. Consider using a LED light source, which is more stable with time.

When writing a paper, it is not necessary to describe the whole comet assay procedure (refer to a published paper instead). But certain variables should be mentioned: gel format, final agarose concentration, lysis time, period of alkaline unwinding, voltage gradient, electrophoresis time, staining and scoring method. These are the factors that most affect reproducibility of the assay.

Voltage gradient and electrophoresis time are worth considering together, as in each case there is a positive - and over a limited range linear - relationship with observed % tail DNA. In theory, therefore, the product of voltage gradient and time ('time-integrated electrophoretic field strength') could be used as a descriptor in publications, as advocated by Brunborg et al. [40]. This would allow a correction factor to be applied when comparing results from laboratories employing differing voltage gradients and electrophoresis times. Agarose concentration is another example, showing - over a certain range - an inverse relationship with % tail DNA, and so a correction factor could be devised. Practically, there are some difficulties; for instance, the range over which linearity applies may vary according to cell type or other conditions; and - in the case of voltage gradient - visual scoring is much less affected by voltage gradient than is image analysis [42]. Further studies are needed to validate the correction factor approach.

We recommend the following:

- Keep a constant time of lysis, to reduce variability between experiments.
- Use a constant volume of electrophoresis solution, giving a reasonable depth of solution above the gels.
- Apply cells at optimal densities of around 12000 in a 20x20 gel or 200 in a mini-gel.
- If possible, use a voltmeter to measure the voltage gradient across the platform. Calculating voltage gradient by dividing the voltage applied by the distance between the electrodes or the distance across the platform will give an incorrect answer. Voltage gradient depends on the tank dimensions. Most of the voltage drop occurs across the platform, where electrophoresis solution is shallowest and the resistance highest.
- Consider using mini-gels to maximise the number of samples that can be processed in one experiment - especially useful in human studies where many samples are to be analysed and minimising variation is important.
- When using the enzyme-modified assay, perform a titration experiment to determine enzyme concentration and incubation time sufficient to detect all lesions present, without causing non-specific damage in untreated nucleoids.
- Include reference standards in (all) experiments - and include the results in published accounts of the experiments.

- Code samples so that scoring can be done 'blind'.
- Calibrate the assay against DNA breakage generated by ionizing radiation, to allow calculation of 'real' break frequencies; this can help when evaluating the biological significance of DNA damage levels in human studies.

Conflict of Interest statement

The authors declare that there are no conflicts of interest

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Table

Table 1: Parameters for comet assay protocols: most frequently described, and range. From hCOMET database.

	% LMP agarose gels including cells	Duration of lysis	Duration of Unwinding	V/cm of electrophoresis	Duration of electrophoresis
Described range of parameters	0.5-1.5%	1 h-weeks	15-40 min	0.7-8 V/cm	20-30 min
Most frequently used	0.7%	1 h	20 min	1 V/cm	20 min

Table 2: % tail DNA obtained using different duration of lysis in HeLa cells. Redrawn from data in [26] (data: Mean (SD) from 3 independent experiments).

Damaging agents (time of treatment)	Time of lysis			
	No lysis	1 h	24 h	1 week
Untreated	3.3 (2.2)	3.1 (3.0)	1.4 (1.1)	2.5 (2.4)
90 μM MMS (3 h)	14.4 (2.6)	12.2 (4.7)	22.2 (2.7)	55.0 (14.4)
180 μM MMS (3 h)	38.5 (14.6)	23.7 (2.2)	44.7 (6.0)	81.5 (8.59)
Untreated	5.7 (2.4)	2.4 (1.8)	1.9 (1.5)	3.4 (0.1)
10 μM H_2O_2 (5 min, on ice)	13.3 (7.1)	8.8 (0.4)	16.6 (6.7)	33.4 (0.2)
40 μM H_2O_2 (5 min, on ice)	42.4 (3.1)	40.1 (1.6)	63.5 (4.9)	80.7 (6.8)

Table 2

Table 3: % DNA in tail obtained using different electrophoresis voltage (V/cm). Redrawn from data in [24] (data: Mean (range of values) from 2 independent experiments).

Table 3

Cell type	**Damaging agents (time of treatment)**	**V/cm applied**				
		0.16	**0.49**	**0.83**	**1.5**	**1.48**
Human lymphocytes	Untreated	0.5 (0.5)	0.6 (0.7)	1.4 (1.6)	4.7 (2.5)	4.5 (0.4)
	70 μM H_2O_2 (5 min, on ice)	3.5 (0.2)	11.6 (8.7)	31.2 (12.8)	36.4 (13.8)	52.4 (13.2)
TK6	Untreated	0.9 (0.1)	1.1 (1.0)	2.5 (0.8)	5.2 (3.5)	3.8 (1.4)
	70 μM H_2O_2 (5 min, on ice)	5.0 (1.8)	14.5 (5.8)	31.7 (4.0)	50.1 (0.9)	50.1 (3.3)

Table 4: Previous protocols on the comet assay published by researchers from only one laboratory.

Endpoint	**Experimental model**	**Ref.**
DNA strand breaks	*In vitro*, *in vivo* (tissues)	[43]
DNA strand breaks (+ other endpoints)	Not specified	[44]
DNA strand breaks (+ enzyme sites)	*In vitro*, biomonitoring	[45]
DNA strand breaks	*In vitro*	[31]
DNA strand breaks	*In vitro* (cultured cells and WBC)	[46]
Enzyme-sensitive sites	Not specified	[47]
DNA strand breaks (+ enzyme sites)	Not specified (adaptation to 12-gels format)	[48]
DNA strand breaks	*In vitro*, *in vivo* (tissues)	[49]
DNA strand breaks	*In vitro* (cultured cells and WBC)	[50]
DNA strand breaks	*In vitro*, *in vivo* (tissues)	[51]
DNA strand breaks (+ enzyme sites)	Biomonitoring (lymphocytes, sperm cells)	[52]
DNA strand breaks (+ enzyme sites)	*In vitro*, *in vivo* (bronchoalveolar lavage and lung tissue) (evaluation of nanoparticles, adaptation to 12-gels format)	[53]
DNA strand breaks	*In vivo* (tissues)	[54]
DNA strand breaks (+ enzyme sites)	Not specified (adaptation to 12-gels format)	[55]

Table 5. Comet assay steps in various protocols that have been used in multi-laboratory trials.

Step	ESCODD (2001)	NewGeneris (2008)	ECVAG (2012) [19]	OECD (2016) [54]
LMP (with cells)	0.5%	0.5%	1%	Not less than 0.45%
Lysis time	1 h	1 h	≥1h, ≤24h	1 h or overnight
Enzyme treatment	30 min (Fpg) 45 min (EndoIII)	30 min (Fpg and EndoIII)	30 min (Fpg)	Not included
Alkaline time	40 min	40 min	20 min	≥20 min
Electrophoresis time	30 min	30 min	20 min	30 or 40 min
Electrophoresis (V)	25 V	25 V	1.2 V/cm (platform)	0.7 V/cm
Electrophoresis (mA)	Highest level	300 mA (not crucial)	Not specified	Not specified (“starting current of 300 mA")
Neutralisation	3 x 5 min (Tris)	10 min (PBS)	2 x 5 min (Tris) and 5 min (water)	5 min
Additional treatment	Not reported	Not reported	Dried and fixed	Absolute EtOH (5 min)

				and air dried
Staining	DAPI, PI, Hoechst 33258 or EtBr	Own choice	Own choice	SYBR, Green I, PI or EtBr
Scoring	Visual scoring (5-class system) or software (%T)	Visual scoring (5-class system) or software	Visual scoring (5-class system) or software	Software ("should be coded slides")
Reporting	AU, %DNA in tail, Gray-equivalents or breaks per unit length of DNA	%DNA in tail, Gray-equivalents or breaks per unit length of DNA	Lesions/10^6 bp (using calibration curve samples)	%DNA in tail (recommended), tail length or tail moment