

PERSPECTIVE

THE COMET ASSAY AS A METHOD FOR ASSESSING DNA DAMAGE IN CRYOPRESERVED SAMPLES

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Abstract

The preservation of the nuclear genome's integrity is paramount for the viability and overall health of cells, tissues, and organisms. DNA, being susceptible to damage under physiological conditions and vulnerable to both endogenous and environmental factors, faces constant threats. To assess DNA damage and repair within individual eukaryotic cells, the comet assay presents itself as a versatile, gel electrophoresis-based, relatively simple, and highly sensitive method. Originally designed to monitor DNA damage and repair within populations of mammalian cells, the comet assay has now found applications across diverse domains, including yeast, protozoa, plants, and invertebrates. This technique has proven invaluable in cryopreservation studies, serving as a valuable adjunct for determining suitable cryopreservation protocols. These protocols encompass choices related to cryoprotectants, sample preparation, as well as storage conditions in terms of time and temperature. In the realm of animal cryopreservation research, the comet assay stands as a gold-standard method for assessing DNA integrity. Nevertheless, when applied in plant-oriented investigations, additional efforts are essential due to the distinct nature of plant cells and associated technical challenges. This review elucidates the fundamental principles underlying the comet assay, discusses its current iterations, and delineates its applications in the cryopreservation of both animal and plant specimens. Moreover, we delve into the primary challenges confronting the comet assay's utility as a monitoring tool in the context of plant sample cryopreservation.

Keywords: comet assay; cryopreservation; DNA strand breaks; 8-oxoG; single-cell gel electrophoresis.

INTRODUCTION

An organism's cells are in a perpetual struggle, facing two opposing forces: the common occurrence of DNA damage and the constant effort to safeguard genome integrity. DNA is exposed to continual threats, including spontaneous base loss (depurination), as well as assaults from endogenous and exogenous sources. Notably, reactive oxygen species (ROS), by-products of respiration, contribute to oxidative DNA damage. Also, external agents with DNA-damaging potential encompass ultraviolet (UV) and ionizing radiation, alongside a plethora of environmental stressors, such as temperature and desiccation (1). Exposure to these stressors can lead to various forms of DNA damage, such as single and double-strand breaks, abasic sites, point mismatches, and nucleobase modifications (e.g., alkylation) (2). Unlike other biomolecules, DNA does not undergo constant recycling but relies on a range of lesion-specific repair mechanisms to restore its integrity (3).

Cryopreservation, defined as the storage of biological material at ultra-low temperatures, stands as a crucial technique with broad applications in fundamental research, medicine, pharmaceuticals, animal and plant breeding, environmental sciences, space exploration, and plant sciences. Moreover, it holds promise for the long-term preservation of plant and animal genetic material, contributing to biodiversity conservation efforts (4, 5). This ability to extend the storage duration of biological materials by reducing temperature, thereby slowing degradation, has far-reaching implications. For example, in 2021, approximately 10,000 plant accessions were cryopreserved globally, with 20 out of 500 European banks using cryogenic temperatures for plant germplasm storage (6). Furthermore, nearly every biomedical research laboratory maintains frozen backup stocks of valuable or rare cells, including clinical samples and transformed cell cultures, to mitigate potential changes affecting cell traits due to continuous culture. Importantly, the cryostorage of oocytes, spermatozoa, and embryos has revolutionized *in vitro* fertility treatments, while the successful preservation of peripheral blood mononuclear cells (PBMC) has advanced adoptive cell therapies for cancer treatment (4, 5).

The techniques and applications developed for the cryopreservation of animal and plant

tissues and organs aim to store biological material for extended periods with minimal loss of function, integrity, or identity, necessitating continuous monitoring and an understanding of the impact of cryoprotectants, freezing, and thawing procedures (4). During cryopreservation, various physical damages and alterations in cell chemical composition may occur (7, 8, 9). Hence, the genetic integrity of cryopreserved biological material is of paramount importance for preserving true-to-type biological specimens (10, 11, 12). The sources of insults to genetic material are diverse, including mechanical stress caused by the transition of intracellular and extracellular water to ice crystals at freezing temperatures, and a significant biochemical contributor, the uncontrolled overproduction of toxic ROS (7, 8, 13, 14), which inflicts damage upon nucleic acids (13, 14, 15, 16).

Recognizing the central role of DNA integrity in cell biology and the critical importance of monitoring genetic material insults resulting from cryopreservation, this review examines the current state of the comet assay's application. Considered one of the gold-standard methods for assessing DNA strand breaks and modifications in eukaryotic cells (17), the comet assay is employed to evaluate DNA damage in cryopreserved animal and plant material.

THE PRINCIPLE OF THE COMET ASSAY

The single-cell gel electrophoresis (SCGE), commonly known as the comet assay, is a versatile technique that melds gel electrophoresis with fluorescent microscopy. It has gained widespread acceptance as a tool for detecting DNA damage and repair, applicable to the assessment of nuclear DNA integrity in virtually any type of eukaryotic cell (18, 19). Over the past four decades, the comet assay has found utility across diverse research domains, including genotoxicity testing, environmental toxicology employing plant and animal models, human biomonitoring encompassing nutritional factors and disease studies, and fundamental investigations into DNA damage mechanisms and repair processes (20, 21, 22, 23, 24, 25, 26, 27, 28). Researchers are drawn to this assay due to its simplicity, sensitivity, adaptability to various modifications, and cost-effectiveness.

Key advantages include its ability to work with a relatively small number of cells without the need for prior mitotic stimulation and the capacity to detect DNA damage at the individual cell level (18, 19). The assay's sensitivity allows the detection of damage within a range of 0.06 to 3 breaks per 10^9 bases, meaning it can measure a few hundred to a few thousand DNA strand breaks per cell. This range underscores the assay's sensitivity, as it can detect both background-level damage in control cells and experimentally induced damage that does not lead to cell death (1, 29).

The term "comet assay" was coined in 1990 (22, 30), though the method was initially described in 1984 as a technique for detecting radiation-induced DNA breaks in individual mammalian cells (31). Originally developed under neutral conditions, the comet assay later underwent modification by increasing the pH of the electrophoresis solution, resulting in the alkaline version of the assay (32). The alkaline comet assay assesses both single and double-

strand breaks (SSB and DSB) and alkali-labile sites (ALS) that arise from abasic sites, which convert to strand breaks under alkaline conditions. Unlike other methods for measuring DNA strand breaks, such as alkaline unwinding and alkaline elution, the comet assay does not necessitate DNA denaturation. Instead, migration in the comet assay depends on the relaxation of DNA supercoils, which occurs under both neutral and alkaline pH conditions. Consequently, some authors propose that both the neutral and alkaline versions of the comet assay provide information about the frequency of SSB and DSB, with the alkaline version exhibiting higher sensitivity (2, 29). Thus, while high pH is commonly associated with the alkaline comet assay, it may not be essential for detecting SSB (33). However, this interpretation is not universally accepted, as studies using antibodies specific to SSB have shown that the neutral comet assay is specific to DSB (34). Nevertheless, the debate continues, and traditionally, the neutral comet assay is

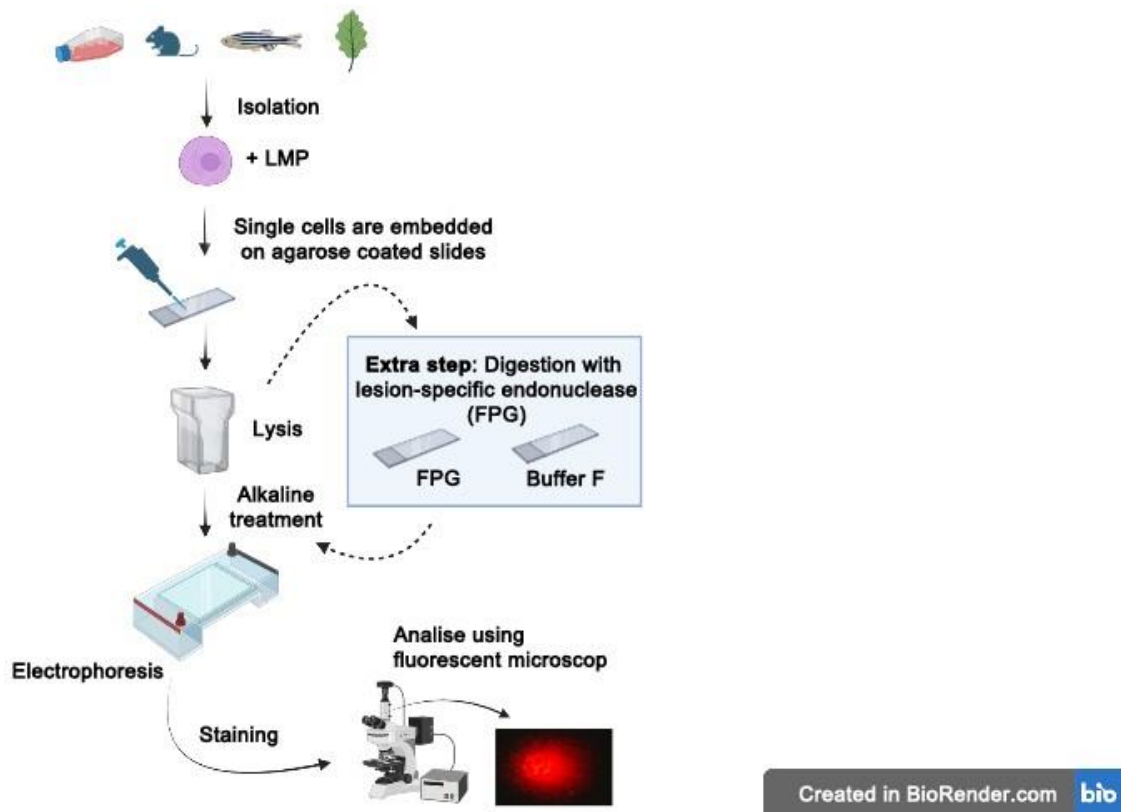


Figure 1. The general illustration depicts the alkaline comet assay protocol. An additional step involving treatment with lesion-specific endonucleases may be incorporated to identify specific nucleobase modifications of interest.

considered to provide data on DSB only (22, 34). Given that the alkaline comet assay is the most widely used, the following description of the procedure will primarily focus on this version, while acknowledging other variants when necessary.

In general, the comet assay procedure comprises several key steps (Fig. 1). It commences with 1) single cell isolation, followed by 2) immobilizing the cells in a layer of 0.5-1% low melting point agarose on either glass (microscope slide) or a plastic film (Gelbond®). Subsequently, 3) cell lysis is performed to remove cellular membranes and soluble components, while histones are removed by high-molarity NaCl. Next, in the alkaline version of the assay, 4) DNA unwinding occurs using an alkali solution to denature DNA and hydrolyze ALS, which result from damage or are generated as intermediates during the base excision repair of DNA base damage, ultimately converting ALS into strand breaks. Following this, 5) electrophoresis is carried out, and then 6) nucleoids are stained with a fluorescent DNA dye. Finally, 7) visualization is accomplished using a microscope with epifluorescence. It is crucial to note that whether employing standard horizontal or vertical electrophoresis or a high-throughput variant of the comet assay (20, 26), several protocol elements must be meticulously controlled. These include agarose concentration, the combined variables of lysis temperature and duration, alkaline unwinding time, and electrophoresis conditions, encompassing voltage strength, duration, and temperature. These conditions are pivotal for ensuring the reproducibility of the comet assay. Below, we delve into several important aspects regarding the comet assay protocol and the selection of buffers and chemicals.

The comet assay's fundamental principle hinges on the spatial arrangement of DNA within the nucleus. Specifically, DNA forms loops, tethered intermittently to the nuclear scaffold by residual nuclear proteins, and coils around protein cores to generate nucleosomes. Notably, the proteins constituting the nuclear scaffold remain unaffected by the lysis treatment employed in the assay (19, 22). This organization means that during the lysis step, where the nuclear membrane dissolves through detergent action and proteins (including histones and non-histone proteins) are removed by a high concentration of NaCl, the DNA remains compact and negatively supercoiled, akin to its

state when wound around nucleosomes. The persistence of a nuclear-like structure, termed the nucleoid, implies a lack of free DNA rotation (20, 22, 33, 36). Initially, it was estimated that 10^4 single-strand breaks (SSB) were necessary to unwind the DNA supercoils within a nucleoid, thus preserving the packaged DNA's integrity and resistance to shearing. Additionally, it remains stable even in the presence of non-ionic detergents and saturating salt concentrations (36). Importantly, certain DNA sequences relinquish histones only at NaCl concentrations exceeding 1.6–1.9 M, sufficient to disrupt DNA-histone electrostatic interactions within nucleosomes (37, 38).

The composition of lysis buffers can vary. However, the most commonly used lysis buffer for both alkaline and neutral comet assays typically comprises 2.5 M NaCl, 10 mM Tris-HCl at pH 10, 100 mM disodium EDTA, and 1% Triton-X (18, 24, 39, 40). Nevertheless, the concentration of specific compounds and the pH may differ, with variations in NaCl and Tris-HCl concentrations or the presence of additional detergents and ROS scavengers, such as N-lauryl-sarcosinate and dimethyl sulfoxide (DMSO) (41, 42). In the neutral version, 2.5% SDS is used to remove proteins in TBE buffer at pH ~8.3 (43), or alternatively, a solution of 10 mM Tris-HCl adjusted to pH 8.0, supplemented with 1% N-lauryl-sarcosinate, 1% Triton X-100, 0.5% DMSO, and a lower NaCl concentration (150 mM) (44). In essence, the composition of the lysis solution may vary, encompassing the type of detergent, presence of DMSO, or NaCl molarity. These variations acknowledge the heterogeneity of nuclear chromatin structure among different cell types. For example, cell types like keratinocytes and buccal cells may require extensive lysis, including proteinase K digestion, to remove residual proteins. In the case of sperm, which feature tightly packaged DNA, the use of both proteinase K and dithiothreitol is warranted (22, 40).

The duration of lysis can influence the results of the alkaline comet assay and can be manipulated to enhance or diminish assay sensitivity. However, a consistent lysis time should be maintained when comparing experiments (40). Typically, a lysis period of up to 1 h is considered non-critical. Nevertheless, prolonged incubation in the lysing solution has been reported to increase the detection of DNA strand breaks, as extended lysis allows for greater DNA unwinding, subsequently elevating

the assay's detection threshold (40). The effect of lysis duration is contingent on the damaging agent and the types of damage under investigation (40, 45). Consequently, a universally standardized "one-size-fits-all" protocol may not be feasible. Therefore, individual optimization of lysis conditions is recommended, considering factors like cell type and the anticipated DNA damage under investigation (18, 22).

The subsequent step in the comet assay is alkaline unwinding, where DNA loops relax due to strand breaks induced by exposure to a high-pH solution (>13). This high-pH solution disrupts the hydrogen bonds that hold the DNA strands together and converts abasic sites into DNA nicks, leading to a less compact nucleoid state (19, 33). Consequently, DNA loops are free to extend outward, forming a "halo" around the nucleoid core. However, nucleoids remain embedded in agarose, and negatively charged DNA loops, still connected to the nuclear scaffold, migrate towards the anode under the influence of the electric field during the subsequent assay step. Consequently, the cell takes on a "comet" appearance with a brightly fluorescent head and a tail region, symbolizing DNA loops relaxed by strand breaks. Some evidence suggests that in the alkaline comet assay, the tail primarily comprises single-stranded DNA fragments. In contrast, in the neutral comet assay, linear double-stranded fragments or extended loops dominate in tails depending on the level of damage (19). However, the relative amount of total DNA in the tail mirrors the frequency of breaks, indicating that more breaks lead to a higher relative tail intensity observed under a fluorescent microscope (18, 22, 46). Importantly, the duration of alkaline treatment can influence the level of DNA migration in electrophoresis by generating more breaks at alkali-labile sites (ALS) in the DNA. Consequently, it is imperative to report specific details regarding the alkaline solution's composition, pH, temperature, duration of treatment, and electrophoresis conditions (47). Typically, unwinding lasts for 20-40 min (46, 48), with authors providing details about the electrophoretic platform's voltage gradient (0.76-1.6 V/cm), current (260-300 mA), and electrophoresis duration, which is often 20-30 min (48).

The final step involves scoring the comets. This is commonly accomplished by visually

categorizing comets into five classes, with category 0 representing undamaged cells and categories 1-4 representing increasing relative intensities of damage. However, for precise quantitative analysis, specialized software is utilized to analyze digital comet images, providing percentages of DNA in the head and tail. Operators can manually select comet images, or automated systems can identify comets and carry out the analysis with minimal human intervention (29). To visualize comets, various fluorochrome dyes are employed, including ethidium bromide, DAPI, propidium iodide, SybrGold, and silver for non-fluorescent staining (29). It is recommended that approximately 100 to 150 nuclei are scored, with a minimum of 50 nuclei per slide in technical duplicates or triplicates (22, 47, 49, 50).

It is worth noting that among the various modifications, the enzyme-modified comet assay stands as one of the most popular techniques. DNA strand breaks often occur alongside other nucleobase modifications under stress conditions. For instance, oxidative stress is linked to the modification of DNA nucleobases, such as oxidation and alkylation. For example, 8-oxo-7,8-dihydroguanine (8-oxoG) is recognized as a marker of oxidative stress. Consequently, the comet assay, originally designed to measure DNA breaks, can be adapted with lesion-specific glycosylases/endonucleases to detect specific base alterations. These enzymes catalyze the conversion of base alterations into DNA breaks during enzymatic hydrolysis (1, 20, 22). Several enzymes are employed in this context, including endonuclease III to detect oxidized pyrimidines (10), formamidopyrimidine DNA glycosylase (Fpg) to digest 8-oxoG, along with other modified purines (11). Additionally, T4 endonuclease V recognizes UV-induced cyclobutane pyrimidine dimers (12), and Alk A incises DNA at 3-methyladenines (13). The enzyme-modified comet assay is applicable in both animal (1, 25, 51) and plant research (24, 52).

Plant-specific comet assay

Growing interest in the plant comet assay has emerged over recent decades. This technique has primarily been employed to evaluate plant stress levels induced by environmental factors (49), as well as to assess the effects of osmotic stress, ionizing irradiation, and chemical compounds (28, 43, 53, 54, 55). The plant comet

assay has been conducted on various plant vegetative tissues, including roots and leaves (49, 53, 56), and has also found application in seed research (24, 28, 57, 58, 59). However, it is essential to acknowledge that certain challenges in standardizing the protocol may arise. Herein, we discuss some of these challenges and offer guidance for key steps in the protocol.

In the first step of the protocol, rather than a single-cell solution, nuclei released from cells must be utilized. However, to obtain a sufficient quantity of nuclei, it is necessary to bypass the cellulose wall barrier through physical slicing of the plant tissue (60, 61, 62). Consequently, the mechanical isolation of nuclei from plant tissue represents a primary bottleneck that limits the throughput of the comet assay. To acquire nuclei with minimal basal DNA damage, it is recommended to employ short, vigorous chopping of the tissue, fully immersed in ice-cold isolation buffer (24, 49). It is worth noting that the selection of an appropriate isolation buffer should be tailored to the specific plant sample under investigation (24). The significance of the lysis step in the plant comet assay is a subject of discussion. While some research suggests that the lysis step may be redundant, many other protocols incorporate this step to obtain "clean" nucleoids and enable enzyme access to DNA (24, 28). It has also been noted that significant DNA repair processes may take place in plant cells during the time required for nuclei isolation and embedding (63). Therefore, these stages should be minimized to strike a balance between obtaining an adequate quantity of nuclei, a nuclei solution free from cellular debris and DNA-associated proteins, and minimizing the time from nuclei isolation to embedding and gel solidification.

CRYOPRESERVATION AS A TECHNIQUE FOR STORING BIOLOGICAL SAMPLES

Cryopreservation enables the stabilization of original cellular structures at extremely low temperatures, allowing for the preservation of a wide range of biological materials, including animal and plant vegetative tissues, cell cultures, embryos, gametes. Moreover, cryopreservation enables cell viability to be maintained long-term. The primary factor ensuring the preservation of these cells and tissues is the reduction in biological and chemical reactions

as a result of the low temperatures. Current cryopreservation methods involve the use of ultra-low temperature freezers (-80 °C) and liquid nitrogen (-196 °C) and its vapors (-130 °C). However, this approach presents a series of stresses, such as exposure to cryoprotectants, desiccation, detrimental ice formation during freezing and thawing, which pose the risk of devitrification and ice recrystallization. Decades of research on the preservation of plant and animal tissues have revealed that the sensitivity and response to cryopreservation stress vary depending on the species and tissue involved.

The use of the comet assay for cryopreserved samples serves a dual purpose. First, cryopreservation is often employed as an interim technique to postpone the analysis of interest. Long-term cryopreservation is not the primary objective, but rather relatively short-term cryostorage is utilized to safeguard the samples without causing any damage until further comparative research, including the comet assay or other analyses that require undamaged DNA, can be performed. From the perspective of animal and human biomonitoring studies, the ability to work with cryopreserved samples enhances their feasibility. When a large number of samples are collected simultaneously outside the laboratory or are biobanked for large cohort studies, immediate processing is often impractical. Additionally, many research projects utilizing the comet assay are constrained by equipment limitations, with typically only dozens of samples being processed per experiment. As a result, samples are cryopreserved and assayed at a later time (64).

The second intention is to use the comet assay to confirm the safety of long-term cryogenic storage of biologically authentic samples, suitable for future purposes such as tissue regeneration for conservation or DNA integrity monitoring in biobanked samples. However, it's important to note that both intentions, whether for short or long-term storage, raise questions about the suitability of different sample preparations and cryopreservation procedures and their impact on the basal level of DNA damage and DNA repair activity in the sample. Importantly, there is a distinction between cryopreserving samples for subsequent comet assay analysis and cryopreservation for long-term conservation. The former prioritizes minimizing cryo-induced DNA breakage, while the latter aims to enhance

Table 1. Comparison of Comet assay protocols used for cryopreserved animal samples. T, temperature; RT, room temperature; PBMC, peripheral blood mononuclear cell; WB, whole blood.

Cryopreservation treatment		Lysis conditions		Pre-electrophoresis treatment	Electrophoresis conditions	
Exp. Model / cell type, tissue (Reference)	T / thawing procedure	Lysis buffer composition	T / time (min)	Solution / time (min)	Solution	Voltage V and/or V/cm / time (min)
<i>Homo sapiens</i> / saliva leucocytes (21)	-80 °C / rapidly thawed at 37 °C	Buffer A: 10 mM Tris-HCl pH 10, 2.5 M NaCl, 100 mM Na ₂ EDTA; 250 mM NaOH; 1% Triton X-100	4 °C / overnight	Alkaline unwinding solution; 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13 / 20	Alkaline solution: 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13	0.83 V/cm / 20
<i>Homo sapiens</i> / WB, PBMC (66)	-80 °C / WB: on ice at RT PBMC: 37 °C	Buffer A; 1% Triton X-100; 1% DMSO	4 °C / 1	Alkaline unwinding solution / 40	Alkaline solution	1.1 V/cm / 20
<i>Homo sapiens</i> / lymphocytes (70)	-80 °C / rapidly thawed at 37 °C	Buffer A; 1% Triton X-100	4 °C / 1	Alkaline unwinding solution / 40	Alkaline solution	25V / 30
<i>Homo sapiens</i> / semen (74)	-196 °C / 1 min. at RT followed by 37 °C until completely thawed	Buffer A; 1% Triton-X; 10mM DTT	4 °C / 2	Neutral buffer: 100 mM Tris-HCl 500 mM NaCl 1mM Na ₂ EDTA 0.2% DMSO / 20	Neutral buffer: 100 mM Tris-HCl 500 mM NaCl 1mM Na ₂ EDTA 0.2% DMSO	10 V / 60
<i>Homo sapiens</i> / semen (77, 79)	-196 °C / 15-20 min. at RT	Buffer A; 1% Triton-X 100 followed by incubation with 10 mM DTT; followed by incubation with 4 mM lithium diiodosalicylate	4 °C / 1 4 °C / 30 20 °C / 90	Alkaline unwinding solution / 20	Alkaline solution	25 V / 0.714 V/cm / 10
<i>Homo sapiens</i> / semen (79)	-196 °C / n/a	1% Triton X-100 washed 3 times for 5 min by 0.9% NaCl followed by incubation with lysis solution Buffer B#; Buffer C#; Buffer D#	RT / 30 RT / 30 RT / 30	TBE buffer / 10	Neutral buffer: TBE	20 V / 1 V/cm / 10
<i>Homo sapiens</i> / semen (83)	-196 °C / 10 min. at 37 °C	3.65 M NaCl 50 mM EDTA	4 °C / 30	Alkaline unwinding solution / 50	Alkaline solution	15V / 1V /cm / 30
<i>Rattus norvegicus</i> / liver (88)	-80 °C / homogenization of deeply frozen tissue on sieve into ice cold Merchant's buffer*	Buffer A 1% Triton X-100	4 °C / overnight	Alkaline unwinding solution / 40	Alkaline solution	1.2 V/cm / 20

* Merchant's buffer: pH 7.4, 1.47 mM KH₂PO₄, 8.1 mM Na₂PO₄, 0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂EDTA. #Buffer B: 0.4 M Tris-HCl pH 7.5, 1% SDS, 0.8 M DTT; Buffer C: 0.4 M Tris-HCl pH 7.5, 2M NaCl, 50 mM Na₂EDTA, 0.4 M DTT; Buffer D: 0.4 M Tris-HCl pH 7.5, 1% SDS, 0.8 mM DTT.

the viability of thawed cells, even if it entails some degree of DNA damage. In this paper, we

review exemplary and commonly used applications of the comet assay as a technique

for monitoring DNA integrity to illustrate the versatility of this method.

Cryopreservation of blood cell samples

Biobanks have amassed thousands of human samples, constituting a significant resource for molecular epidemiology studies. Among the crucial biobanked samples are peripheral blood mononuclear cells (PBMCs, including lymphocytes and monocytes), whole blood (WB), and buffy coat (BC), which contains white blood cells and platelets. PBMCs are the most commonly utilized cells in human monitoring (21). In this context, the comet assay serves several purposes: 1) to assess the impact of dietary, drug, or chemical compound interventions on genotoxicity in both fresh and cryopreserved samples; 2) to test the suitability of cryopreserved biobank samples for other DNA-based analyses by preserving DNA integrity; and 3) to evaluate the cryopreservation procedure, including cryoprotection, aimed at reducing freezing artifacts. For instance, good concordance has been reported between fresh and frozen WB samples, whether frozen rapidly in small volumes without cryoprotection or in larger volumes with 10% DMSO. Detailed protocols for conducting the comet assay on fresh and cryopreserved blood samples have been published over the years (Table 1).

However, blood cells are typically stored at $-80\text{ }^{\circ}\text{C}$ (64, 65, 66, 67, 68). Nevertheless, comet assay analyses have shown either minor or no significant difference in DNA strand breaks and oxidative damage between fresh and cryopreserved cells when measured immediately after thawing (64, 65, 69). Notably, a comprehensive study based on extensive literature data comparing fresh and cryopreserved human blood samples revealed no difference in percentage tail DNA (64, 70). However, some contrasting reports indicated that improper cryopreservation procedures during longer storage periods (months) of cryoprotected PBMCs may induce a slight increase in DNA strand breaks (64, 68, 71). Cells cryopreserved for 4 or 12 months exhibited more DNA strand breaks and, importantly, Fpg-sensitive sites (66, 71). These results were attributed to the composition of the medium used for cryopreservation, as it may affect DNA integrity analyzed by the comet assay. Moreover, it was suggested that DNA stability of samples should be monitored over several days because the major effect of

cryopreservation, depending on the sample preparation method, may occur within the first few days of storage (64).

In addition to storage at $-80\text{ }^{\circ}\text{C}$, human blood samples are also stored long-term in liquid nitrogen (LN). Results based on samples collected by The European Prospective Investigation into Cancer and Nutrition (EPIC), one of the largest prospective cohort studies, showed that cryopreserved PBMC samples ($n = 299$) stored in LN for a decade were suitable for further comet assay analysis. This enabled an examination of relationships between individual factors such as weight or smoking habits and DNA damage. Given the large number of tested samples, this study confirms that cryopreservation of blood samples is a reliable storage method for future use. However, it appears that the method of cryopreservation, the type of cell culture medium used for freezing and storage, the thawing method, the type of cryopreserved blood fraction (WB vs. PBMC vs. BC), and even the storage temperature ($-80\text{ }^{\circ}\text{C}$ vs. LN) should be carefully selected for the purpose of long-term preservation (66). This will help prevent an increase in basal DNA damage that might be detected in the comet assay. Recent findings have shown that PBMCs cannot be used immediately after thawing; instead, they require 16 hours of recovery if the comet assay is to be used for testing DNA repair efficiency. Similar results were observed for salivary leukocytes stored at $-80\text{ }^{\circ}\text{C}$ after treatment with the genotoxic compound bleomycin. In this case, 24 h of regeneration and cell cycle stimulation were necessary after thawing to use them in DNA repair kinetics studies (21, 65).

Cryopreservation of semen samples

Cryopreservation is a widely used strategy for preserving fertility through the cryostorage of gametes and embryos. This is particularly valuable for individuals undergoing gonadotoxic medical treatments, those requiring gamete donations for infertility treatments, and in the fields of animal breeding and the preservation of endangered animal species (72, 73). Sperm DNA integrity is a critical parameter in assessing semen quality and serves as a diagnostic tool and biological marker for male reproductive health and infertility (74). The integrity of sperm DNA significantly influences offspring health and development, with DNA fragmentation being one of the most common

abnormalities encountered in male gametes (72). Despite advancements in semen cryopreservation techniques aimed at minimizing ice crystal formation and stabilizing the lipid bilayer, damage to sperm and subsequent impairment of its function continue to pose challenges for assisted reproduction procedures. As a result, it is essential to address ways to mitigate sperm damage caused by cryopreservation and identify potential markers of susceptibility to this damage. Strategies for improving semen cryopreservation outcomes have been extensively reviewed (72).

Human semen is comparatively less susceptible to cryoinjuries than semen from other animals due to its lower cytoplasmic content and lipid composition of the plasma membrane (75). Nonetheless, it can still suffer significant damage during the freezing and thawing process, with susceptibility varying depending on the initial quality of the semen. Most studies suggest that sperm DNA damage is primarily induced by the freezing and thawing process itself rather than prolonged storage in liquid nitrogen (LN), although at least one study has reported storage-dependent structural damages (72, 76).

The comet assay is a commonly employed technique for assessing DNA integrity in cryopreserved animal and human semen, including the evaluation of DNA fragmentation and an increase in 8-oxoG (73, 77, 78, 79). This assay allows for the quantification of DNA damage in individual spermatozoa, enabling the determination of the degree of heterogeneity in DNA quality within a population of mature sperm. Consequently, results indicating the proportion of sperm with low or high levels of percentage tail DNA provide valuable information for diagnosing male infertility and predicting outcomes in in vitro fertilization, intracytoplasmic sperm injection, and live births (80). The comet assay results can also be used to predict embryo development following assisted reproductive technologies, particularly in cases of unexplained infertility (81). Whilst the negative impact of cryopreservation on sperm DNA fragmentation is documented, there is no universal consensus about outcomes as some studies have not found significant differences in DNA fragmentation between fresh and cryopreserved semen (72). For example, one study reported only around a 4% increase in tail DNA as measured by the comet assay in cryopreserved sperm (n=157) (72). Another

study involving 498 samples demonstrated the suitability of the comet assay for assessing semen quality and found no differences between cryopreserved and non-cryopreserved samples from fertile donors (74). These discrepancies stem from the observation that the extent of damage varies individually; for instance, sperm DNA from infertile men or cancer patients tends to be more susceptible to freezing-induced damage than that from fertile men, exacerbating fertility challenges (72,77).

To enhance the safety of semen cryopreservation procedures, the comet assay has been employed to test various cooling methods in LN. This research has revealed that inter-individual variability plays a more significant role in viability after cryopreservation than the specific cryopreservation method employed (e.g., flash freezing vs. programmed freezing with or without cryoprotectants). Nevertheless, flash cooling without cryoprotectants has gained recommendation for use in epidemiological studies (74, 82). The comet assay has also proven valuable in evaluating cryoprotectants for semen cryopreservation, such as acetyl-L-carnitine (83), gangliosides (84), and genistein (85). Interestingly, due to the abundance of alkali-labile sites (ALS) in human sperm, the neutral comet assay is often preferred in these studies (74, 82). Furthermore, this technique has successfully assessed DNA damage in cryopreserved sperm from various animal species, including molluscs, cyclostomes, fishes, amphibians, reptiles, birds, and mammals (73).

Cryopreservation of animal solid tissues

The availability of viable human tissues has become critically important to support translational research, especially in the context of personalized cancer care. Presently, many molecular profiling studies and related analyses rely on fresh-frozen tissues sourced from biobanks. Cryopreserving viable solid tissues offers investigators the opportunity to evaluate assays in a time-independent manner (86).

Typically, small solid tissue samples are snap-frozen in cryotubes using liquid nitrogen (LN) and then stored at -80 °C. The comet assay can serve as a method for assessing the response of tested cell populations to pharmacologically relevant doses of drugs that impact DNA structure, such as DNA cross-linking agents used in cancer therapy (87). However, when dealing with cryopreserved solid tissues, it has

been demonstrated that the method of cell suspension preparation is critical to obtaining nucleoids without artificially induced damage resulting from improper sample processing. Therefore, the recommended method involves disaggregating deep-frozen tissue in ice-cold Merchant's medium using a metal sieve. This method yields very low levels of DNA strand breaks and is considered the safest approach. To emphasize, avoiding the thawing and preparation of single-cell suspensions in a cold environment appears to stabilize DNA integrity. Conversely, allowing the sample to thaw at room temperature has been associated with very

high levels of strand breaks (88). This procedure aligns with the criteria of threshold, namely a 6% tail DNA derived from strand breaks and alkali-labile sites (ALS) for liver samples from untreated animals (50). It's important to note that there is a significant difference in sample preparation for the comet assay between solid tissues and sperm or blood cells, for which rapid thawing at 37 °C is commonly reported (66, 74, 82). Additionally, a study demonstrated that storing frozen rodent liver samples at -80 °C for one year was adequate for the comet assay analysis of alkaline and Fpg-sensitive sites. However, in cryopreserved kidney and lung

Table 2. Comparison of comet assay protocols used for plant samples.

Material and preparation		Lysis conditions			Pre-electrophoresis treatment	Electrophoresis conditions	
Experimental Model / cell type, tissue (Reference)	Method of nuclei isolation	Nuclei isolation buffer	Lysis buffer composition	Time (min)	Solution / Time (min)	Solution	Voltage V and/or V/cm / Time (min)
<i>Acer pseudo-platanus</i> / embryonic axes (24)	Vigorous chopping, up to 30 s	Sørensen buffer: 50mM sodium phosphate pH 6.8 0.5 mM Na ₂ EDTA 0.5 % DMSO	Buffer A: 10 mM Tris-HCl pH 10, 2.5 M NaCl, 100 mM Na ₂ EDTA; 1% Triton X-100; 10 % DMSO	60	Alkaline unwinding solution: 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13 / 40	Alkaline solution: 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13	1V/cm / 20
<i>Lolium perenne</i> / <i>Vicia faba</i> / leaves, roots (49)	Chopping 5-30 s	400 mM Tris-HCl pH 7.5	Not recommended	n/a	Alkaline unwinding solution: 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13 / 15	Alkaline solution	26V / 0.72V/cm / 5
<i>Oryza sativa</i> / <i>Phaseolus vulgaris</i> / seeds, embryos (58, 59)	Maceration	PBS buffer	Neutral buffer: 0.5x TBE 2.5 % SDS	30	0.5x TBE / 5	Neutral buffer: 0.5x TBE	0.5 V/cm / 2
<i>Solanum Melongena</i> / seeds, embryos (90)	Chopping followed by 200 µm nylon filtration	Sörensön buffer; 0.1 mM EDTA; 0.5% DMSO	Neutral buffer: 0.5x TBE 2% sodium lauryl sulfate	30	-	Neutral buffer: 0.5x TBE	1 V/cm / 5
<i>Medicago truncatula</i> / radicles at protrusion and 4-days old seedlings (91)	Gentle slicing by razor blade	Sörensön buffer	100 mM Na ₂ EDTA, 2.5 M NaCl, 100 mM Tris-HCl pH 10) 2.5 M NaOH	20	Alkaline unwinding solution: 300 mM NaOH, 1 mM Na ₂ EDTA, pH >13 / 30	Alkaline solution	0.72 V/cm / 25

tissues, the heterogeneity of the organ appears to affect basal DNA damage, resulting in a shortened adequate storage time (88). Thus, it is evident that storage conditions preceding the comet assay, as well as the assay procedure itself, need to be tailored to the specific characteristics of each sample type.

Towards monitoring of DNA integrity in cryopreserved plant samples

The adaptation of the comet assay to plant models is relatively recent. Initially, it was employed to study the effects of irradiation on various plant-based products, including seeds, fruits, and spices. The neutral version of the comet assay became a recognized method for testing irradiated food, and later, the alkaline protocol was introduced to investigate roots and other tissues of terrestrial plants. Indeed, the alkaline comet assay is the most commonly used method in terrestrial plant research (Table 2). Presently, it is primarily applied to monitor the toxic effects of chemical compounds, nanoparticles, irradiation, and drought in species such as *Allium cepa* L., *Nicotiana tabacum* L., and *Vicia faba* L., which are among the most frequently studied (89). However, slight modifications in biomass sampling, buffer composition, or electrophoretic migration parameters are typically adequate to adapt the comet assay to other plant species (89). Previous applications of the comet assay include assessing DNA fragmentation in embryos or embryo axes isolated from *Solanum melongena* L. (90), *Medicago truncatula* L. (91), and *Acer pseudoplatanus* L. (24). Nevertheless, there are limited studies demonstrating the utility of the comet assay for monitoring DNA integrity in plant tissues and organs following long-term storage. For instance, Dantas et al. (92) employed the neutral comet assay to analyze DNA integrity in orthodox seeds of *Oryza sativa* L. and *Phaseolus vulgaris* L. stored at -20 °C for 36 and 25 years, respectively. Some research has also applied the assay to test the deleterious effects of accelerated aging on seeds of *Oryza sativa* L., *Phaseolus vulgaris* L., and *A. pseudoplatanus* (24, 59).

Successful cryopreservation has been achieved for various plant propagules, including calli (93), shoot tips (94), somatic embryos (95), pollen (96), embryonic axes (97), plumules (11), and seeds (98). However, there is currently a lack of data on monitoring the effect of cryopreservation on DNA integrity in plant

organs and tissues using the comet assay. Initial research has explored the feasibility of employing this technique to determine the optimal moisture content of seeds for safe cryopreservation (manuscript in preparation, Plitta-Michalak). Further investigations are warranted to fully elucidate the utility of the comet assay in supporting plant cryopreservation and other conservation efforts. It's important to note that when plant tissues are cryopreserved, additional biotechnological methods are required to restore the plants. The feasibility of the comet assay has been demonstrated in investigating the genotoxic effects of tissue culture parameters in sunflower calli tissues (99) or examining the relationship between plant growth regulators and DNA damage in in vitro cultures of *Crepis capillaris* (L.) Wallr. callus cells (100). This underscores the potential usefulness of this method in supporting plant cryobiotechnology procedures.

CONCLUSION

The comet assay is a well-established, user-friendly, and versatile technique that enables the assessment of DNA damage and the progression of DNA repair at the individual eukaryotic cell level. Historically, investigations into the impact of cryopreservation on DNA integrity have primarily focused on animal cells and tissues. However, there is growing evidence that this assay can also find utility in plant-related research. With the expanding body of work on DNA integrity in plant samples, we anticipate that the comet assay will increasingly be employed in cryopreservation studies. This is primarily due to its simplicity and adaptability, making it a valuable tool for selecting the most suitable storage protocols and monitoring the long-term safety of cryopreservation processes. In the future, we believe that the comet assay could play a particularly significant role in refining methods related to the extended storage of plant materials, such as vitrification or bead-based cryopreservation. In our view, this method holds immense potential in facilitating the search for new, non-toxic cryoprotectants, especially among recently synthesized compounds, including nanoparticles.

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