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TITLE: The comet-based *in vitro* DNA repair assay: a standardized method to assess an individual's DNA repair activity.

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Abstract

This optimized protocol for the comet assay-based *in vitro* DNA repair assay (including links to instruction videos) is relatively simple, versatile, and inexpensive, allowing the detection of base and nucleotide excision repair activity. Protein extracts from samples are incubated with agarose-embedded substrate nucleoids ('naked' supercoiled DNA), containing specifically induced DNA lesions (e.g., through oxidation, UVC or benzo[a]pyrene-diolepoxide treatment). DNA incisions produced during the incubation reaction are quantified as strand breaks after electrophoresis, reflecting the extract's incision activity. An additional step, supplementing the extract with dNTPs, allows the measurement of ligation activity. Various innovations and optimizations have increased the assay's throughput and enabled the use of various samples (cell models, blood cells, tissues). Once extracts and substrates are prepared, the assay can be completed within two days. This method represents a unique functional measurement of DNA repair activity, with applications in human biomonitoring, *in vitro, in vivo*, and (clinical) intervention studies.

Key words: DNA repair, incision, comet assay, nucleotide excision repair, base excision repair, protein extract, single-cell gel electrophoresis

INTRODUCTION

The comet-based *in vitro* DNA repair assay is a modified version of the comet assay (also known as single-cell gel electrophoresis assay) to assess DNA repair activity: a cellular protein extract which contains repair enzymes is incubated with a DNA substrate containing induced lesions, and DNA incisions in the form of DNA strand breaks (SBs) accumulate. It is a relatively simple method for functional measurement of base excision repair (BER) and nucleotide excision repair (NER) activity of different types of samples, with many applications in human biomonitoring, in *in vitro* and *in vivo* studies, as well as in (clinical) intervention studies.

Development of the protocol

The comet assay is a versatile and sensitive method that detects, in its standard version, SBs and alkali-labile sites. The first paper on this single-cell gel electrophoresis assay was published in 1984 by Ostling and Johanson[1]. The protocol is simple: briefly, cells embedded in agarose on a microscope slide are lysed to remove membranes and soluble components (including histones) leaving nucleoids (i.e., supercoiled DNA attached at intervals to a nuclear matrix forming loops)[2]. Next, nucleoids undergo alkaline unwinding and electrophoresis. The presence of SBs in the DNA relaxes the supercoiled loops and enables the DNA to migrate towards the anode. The resulting comet-shaped figures, called comet(s), are visualised with a DNA fluorescent dye and fluorescence microscopy. In addition, the enzyme-modified comet assay includes an extra step between lysis and alkaline treatment; i.e. incubation with DNA repair enzymes from bacteria or human cells to gain further information on specific classes of DNA lesions[3]. For instance, among others, formamidopyrimidine DNA glycosylase (Fpg) detects oxidized purines, formamidopyrimidines (ring-opened adenine or guanine) and ring-opened N7 guanine adducts; human 8-oxo-guanine (8-oxoG) DNA glycosylase (hOGG1) detects oxidized purines and formamidopyrimidines; and T4 endonuclease V (T4endoV) detects dimerised pyrimidines. These enzymes are also used in combination with the comet-based *in vitro* DNA repair assay as 'incubation reaction controls '.

The comet assay (with and without the inclusion of lesion-specific enzymes) is widely used as a biomarker assay in human population studies and genotoxicity testing (including regulatory toxicology) - primarily to measure DNA damage, but increasingly also to assess the activity of cells for DNA repair. In the original publication, Ostling and Johanson also reported the first experiments to measure DNA repair by simply following the decrease of ionising radiation-induced SBs over time – referred to as a challenge assay or cellular repair assay. However, this approach merely measures the final step in the repair process (i.e., ligation). Still, useful information on the kinetics of NER and BER has been gained by following the removal of pyrimidine dimers or oxidised bases, respectively, using appropriate

enzymes[4,5]. Then again, this approach is time-consuming and laborious, and therefore not optimal for biomonitoring or intervention studies, which typically require high-throughput processing of many samples.

An alternative *in vitro* approach is based on assessing the ability of repair proteins in a sample extract to recognize and incise substrate DNA that contains induced lesions. The whole-cell extract can be prepared from blood cells, ground tissues or cultured cells, by 'snap-freezing' and subsequent lysis with Triton® X-100. The comet-based *in vitro* DNA repair assay was first devised in 1994 to measure NER and BER activity in a human cell extract[6]. However, over the past two decades, it has been modified and improved, as well as being applied to tissue samples in addition to cell suspensions. **Table 1** gives the comparison between the three main versions of the comet assay, i.e. the standard comet assay, the enzyme-modified comet assay, and the comet-based *in vitro* DNA repair assay.

Variations in the method

The nature of the lesions in the substrate nucleoids defines the repair pathway that is going to be studied. Early on, the comet-based *in vitro* DNA repair assay was applied to extracts from human lymphocytes to measure BER, using cells treated with a photosensitiser (Ro 19-8022) plus visible light to create substrate DNA containing 8-oxoG[7]. The use of the photosensitizer Ro 19-8022 has certain challenges; including that for a long time, it was only available on request from F. Hoffmann-La Roche, and that the irradiation with light may increase the level of DNA SBs. In the past 5 years, potassium bromate ($KBrO_3$) has been introduced as a novel, alternative DNA damage-inducing agent to prepare substrate cells containing oxidatively damaged DNA lesions. In addition, Fpg has a higher incision rate than hOGG1 on Ro 19-8022 treated cells, whereas these repair enzymes have the same incision rate in $KBrO_3$-generated substrate cells[8]. So far, $KBrO_3$-treated substrate cells have been used in only a few studies to assess DNA repair activity in cell cultures[9], animal tissues[10], and human blood cells[11].

The BER-specific *in vitro* DNA repair assay was modified in 2005 for evaluation of NER activity in human lymphocytes, using benzo[a]pyrene-diolepoxide (BPDE) – the active metabolite of the well-studied environmental mutagen benzo[a]pyrene (B[a]P) – to treat substrate cells[12]. This type of DNA-damaging agent induces BPDE-DNA adducts which are typically recognized by NER enzymes. The original version of this assay involved the treatment of nucleoids in the gel with BPDE after lysis[12]. Recent optimizations, performed by Working Group 5 of the hCOMET COST Action (CA15132), improved the standardization of the assay and successful creation of a batch of BPDE-exposed substrate cells (as explained in Step 1-D). In 2009 the *in vitro* DNA repair assay was further modified by using UVC-treated substrate cells to measure NER[13]. **Box 1** provides an overview of the various modifications of the assay,

including links to the corresponding steps in the protocol. An advantage of the assay is its versatility, illustrated by studies that have adopted alternative DNA damage-inducing agents, such as oxaliplatin, to study repair of DNA cross-links or H_2O_2 and methyl methanesulfonate (MMS) to induce various base modifications[14,15].

In most studies, the comet-based *in vitro* DNA repair assays have used protein extracts from cultured or isolated cells (e.g. blood cell fractions) to study DNA repair activity. Various attempts have been made by different laboratories to use the comet-based *in vitro* DNA repair assays with extracts from solid animals tissues, but only a few have succeeded[16,17], most being frustrated by low repair activity and/or low detection sensitivity due to the presence of non-specific nuclease activity[18]. However, from 2010 onwards, methods for assessing BER and NER activities were developed and optimized for their use with protein extracts from solid tissues. While Langie *et al.* modified both BER and NER assays to measure DNA repair activities from solid tissues of animal origin[16,18], human tissue samples were assayed for both repair pathways by Slyskova *et al.*[19]. It is our aim to optimize the assays further for use with non-invasively collectable tissues, such as buccal cells and saliva – which are currently used successfully in the standard alkaline comet assay to assess DNA damage levels.

BOX 1: OVERVIEW OF THE VARIOUS MODIFICATIONS OF THE COMET-BASED *IN VITRO* DNA REPAIR ASSAY

Schematic overview of the most used modifications of the comet-based *in vitro* DNA repair assay, including links to the corresponding steps in the protocol.

BER assay

- Substrate cells
  - Treatment with photosensitizer Ro 19-8022 + light
  - Treatment with $KBrO_3$
- Incubation step
  - To study incision activity – use extract + buffer B
  - To study synthesis & ligation – use extract + buffer B + dNTPs

NER assay

- Substrate cells
  - Treatment with UVC
  - Treatment with BPDE
- Incubation step
  - To study incision activity – use extract + buffer N
  - To study synthesis & ligation – use extract + buffer N + dNTPs

Protein extract preparation

- From frozen cells or PBMC/WBC in freezing medium
- From fresh or frozen cell pellets
- From snap frozen tissues

Abbreviations: BER – base excision repair, BPDE – benzo[a]pyrene-diolepoxide, dNTPs – deoxyribonucleotides, $KBrO_3$ – potassium bromate, NER – nucleotide excision repair, PBMC – peripheral blood mononuclear cells, UVC – ultraviolet (C), WBC – white blood cells.

Principle of the assay

Figure 1 provides a schematic overview of the principle behind the sample extract incubation reaction; it depicts substrate DNA from cells that were pre-treated, as an example, with the photosensitizer Ro 19-8022 plus light for the measurement of BER. In general, a protein lysate is extracted from cells or tissues and incubated with damage-containing substrate DNA, consisting of gel-embedded nucleoids from cells that were pre-treated with DNA damage-inducing agent. Incubation of these substrate nucleoids with cell or tissue extracts allows the initial steps of BER (or NER in the case of UVC- or BPDE-induced DNA lesions) to occur; repair enzymes present in the protein extracts will induce incisions at the site of the DNA lesions in the substrate. These incisions will result in single SBs that can then be determined by the standard alkaline comet assay. Thus, the increased migration of DNA into the tail is proportional to the DNA repair incision activity of the extracts.

This assay essentially assesses the DNA incision activity, measuring the accumulation of DNA SBs, and incision is generally regarded as the rate-limiting step of DNA repair. Therefore, by merely assessing the DNA repair incision activity, it is already possible to study the effect of external and internal factors on an organism's DNA repair activity. In real life, the SBs produced during the incision step are transient, being quickly followed by DNA repair synthesis (long patch synthesis in the case of NER, shorter patches or single nucleotides in BER). *In vitro*, the concentration of deoxyribonucleotides (dNTPs) is too low for this synthesis to occur – as is confirmed by the experimental addition of ATP and dNTPs, which prevented the increase in SBs after UVC irradiation in HeLa cells[6]. Thus, if it is also required to detect DNA synthesis & ligation activity of the extract, a parallel incubation of sample extracts supplemented with dNTPs can be performed (Box 1, and Optional step in parallel to Step 4 presented in detail in Box 5).

Applications of the method

The comet-based *in vitro* DNA repair assay has been used in some cell culture and animal studies – studying the effect of nutrition and ageing – but it is mostly used in human biomonitoring and intervention studies. We previously reviewed the different *in vitro*, *in vivo* animal and human studies where this technique has been applied to measure DNA repair activity[20]. The text below gives the main messages from this review. In the near future, we also plan to use the assay in genotoxicity testing to unravel the role of DNA repair in the Mode-of-Action (MoA) of potential (non)genotoxic carcinogens. In addition, DNA repair has recently been defined as a key event (KE) in an adverse outcome pathway (AOP) that was submitted to the OECD Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) for internal review[21] – which may also promote the use of the assay.

Cell culture studies

There are few studies in the literature where the comet-based *in vitro* DNA repair assay has been applied using cell cultures. Most of them studied the (beneficial) effects of nutrients, mainly polyphenols[22-24] and other antioxidants[5,25], on the DNA repair activity. In a few cases, the effects of therapeutic drugs[26,27] on DNA repair activity were tested, or the assay was used to unravel underlying disease mechanisms[15].

Animal studies

The first three reports of the use of the comet-based *in vitro* DNA repair assay on animal tissues (rodents and pigs) only came about 8-9 years after the first reports on the assay using human blood samples[16,17,28]. This slow start was due to the presence of high levels of non-specific incision activity when using protein extracts from tissues, making the measurement of DNA repair in mammalian tissues using the comet-based assay a challenge. The adapted and optimized assay[18] for quantification of BER-associated incision activity in rodent tissues opened opportunities for a wide range of *in vivo* studies, including effects of environmental exposures (such as toxins, dietary factors, and pharmaceutical agents) and physiological processes including growth, development, degenerative diseases, and ageing. The comet-based *in vitro* DNA repair assay has mainly been used to study the effect of ageing or dietary factors in animal tissues[17,29-31]. However, in recent work by Setayesh *et al.*, the effect of weight-loss strategies on the NER activity in obese mice was studied[32].

In 2014, the comet-based *in vitro* DNA repair assay was applied to *Drosophila melanogaster* to measure the DNA repair activity in extracts from different strains, proficient and deficient in DNA repair[33]. The *in vitro* approach can provide information about the genetic basis and regulation of specific repair enzymes.

Human studies

Individual DNA repair activity is a valuable biomarker since it has been regarded as a marker of susceptibility to mutation and cancer development. A high repair activity is related to a decrease in the chance of unrepaired damage when cells replicate and so to a decrease in potential mutations.

The comet-based *in vitro* DNA repair assay has been used mainly in human biomonitoring and nutritional intervention studies, but also in occupational and clinical studies[34-37]. In a recent review we give an overview of the use of the comet-based *in vitro* DNA repair assay in various human biomonitoring studies and describe how DNA repair activity can be affected by various external (e.g., chemicals, lifestyle, diet) and internal (e.g., genetics, age, sex) factors[38]. In occupational studies, while the harmful effect of exposure was clearly recognizable by high levels of various biomarkers of genotoxicity, the effect of exposure on DNA repair activity was not always that straightforward. For

instance, in workers exposed to stone wool, BER activity was unaffected by exposure but was negatively correlated with micronucleus frequency, implying that unrepaired 8-oxoG contributes to micronucleus formation[39]. However, a study on occupational exposure to asbestos showed that non-exposed women had higher mean BER activity compared with exposed women[40]. In styrene-exposed hand-lamination workers, an exposure-related increase in BER activity and a decrease in SBs was observed, suggesting possible induction of DNA repair enzymes in the course of chronic occupational exposure[41].

In studies investigating DNA repair activity in relation to human diseases, the comet-based *in vitro* DNA repair assay has been used only rarely, mainly on peripheral blood mononuclear cells (PBMC) of study subjects (e.g., patients suffering chronic renal failure[42], patients with lung[43] and colorectal cancer[44,45]), but also on biopsies from tumour and adjacent non-tumour tissue from colorectal cancer patients[19,46]. Since the comet-based *in vitro* DNA repair assay to study BER and NER in human solid tissues was optimized only recently[19,47], more clinical studies on DNA repair in relation to tissue-specific diseases might be expected to be published in the near future.

Results to date have demonstrated the range of repair activities in a healthy human population – a range far wider than can be explained by genetic polymorphisms. This emphasizes the importance of regulation of repair by environmental and/or intrinsic factors – about which we still know relatively little. Nonetheless, the assay allows the assessment of the intrinsic DNA repair activity, as observed from measurements for the same persons at different time points. The comet-based *in vitro* DNA repair assay is the perfect tool to phenotypically assess the activity of various DNA repair pathways and thereby to further unravel the effect of various modifying factors on the activity as well as investigating the DNA repair activity as an effect modifier in studies on exposures to genotoxic agents.

Comparison with other methods

Inducing DNA damage in cells and monitoring the rate of removal of the lesions over time is the most straightforward approach to measuring DNA repair activity (also known as cellular repair assay). The comet assay, in the standard version to measure DNA damage, has been used with this aim since the very beginning of the assay[1,48]. Moreover, the use of polymerase inhibitors (e.g., aphidicolin or cytosine arabinoside) increases the sensitivity of the assay[49-51]. Three *in vitro* studies have demonstrated an increase in the rate of removal of oxidized bases or DNA SBs in line with an increase in BER activity estimated by using the comet-based *in vitro* DNA repair assay[5,23,24]. However, from the logistical point of view, this approach is not very convenient when analysing a large batch of samples.

Several analytical techniques can be used to monitor the removal of the damage over time. A significant positive correlation was observed between the NER comet-based *in vitro* DNA repair assay

and BPDE-DNA adduct removal determined by the ^{32}P-post-labelling assay[12]. According to our knowledge, additional comparative studies between analytical techniques (e.g. HPLC or MS) and the comet-based *in vitro* DNA repair assay have not been carried out.

There are other methods to assess DNA repair activity. The unscheduled DNA synthesis (UDS) assay, used for many years, is based on the incorporation of [^{3}H] thymidine into the DNA after treatment with a genotoxic agent[52]; it is effective in measuring the repair of UVC-induced damage but less effective in measuring the smaller gaps produced during BER. In any case, this method has been widely criticised, and it is not recommended nowadays.

Different approaches using plasmids have been developed to measure DNA repair activity. In a host cell reactivation (HCR) assay[53], a plasmid containing a UVC- or BPDE-damaged reporter gene (e.g., luciferase gene) is introduced into the cells. The activity of the reporter gene gives an estimate of the extent to which the cells have been able to repair the lesion in the plasmid. *In vitro* plasmid-based assays are more common. Plasmids containing DNA lesions are incubated with cell extracts containing repair enzymes. Then, using a standard gel electrophoresis method, nicked plasmids (repaired) can be separated from closed, non-repaired ones[54]. Plasmids can also be incubated with the cell extract in the presence of ^{32}P-labelled dNTPs and the repair measured by their incorporation into the plasmid[55]. In this way, not only incision but the whole repair process is measured. An alternative to the use of plasmids is the use of oligonucleotides constructed with specific lesions and a terminal radioactive or fluorescent tag[56,57]. To date, no direct comparison of the comet-based *in vitro* DNA repair assay with plasmid or oligonucleotide-based techniques has been carried out. Although such methods have been applied in human biomonitoring studies, especially by Paz-Elizur *et al*.[58-60] and Leitner-Dagan *et al.*[61,62], the number of studies in which these techniques were applied is limited. The comet assay, on the other hand, has been used as an *in vitro* DNA repair assay more often[63].

DNA repair has also been measured in terms of the level of transcription of DNA repair-related genes. However, an important limitation of this approach is that post-transcriptional regulation and epigenetic changes are not taken into account. In fact, there seems to be little correlation between transcription levels and repair enzyme activities[60,64-66]. Thus, DNA repair phenotyping is a more direct measurement than genomic, transcriptomic and proteomic approaches.

The *in vitro* comet-based approach to measuring DNA repair activity became increasingly popular due to several positive aspects. Above all, it opens up the possibility to study DNA repair activity in diverse biological material, unlike cellular repair assays in which the removal of damage over time is monitored in cells in culture. The cell extract to be used in the *in vitro* assay can be prepared from virtually any

tissue. Moreover, frozen materials (cells in freezing medium, frozen cell pellets, or tissues) can be used to prepare protein extracts, which make it logistically a more attractive assay.

As compared to other DNA repair assays such as UDS, HCR, and plasmid- or oligonucleotide-based incision assays, the comet-based *in vitro* DNA repair assay detects the effect in nucleoids (condensed/supercoiled DNA, as occurs in cells), and it is not necessary to use radioactively-labelled material. Moreover, the density of lesions in the nucleoid is low, which may represent a theoretical advantage over other assay designs since it more closely resembles the environment that the repair enzymes encounter *in vivo*. However, it is still an artificial environment; 'naked' supercoiled DNA is not the natural substrate for repair.

Furthermore, the comet-based *in vitro* DNA repair assay is far less laborious and time-consuming compared to the cellular repair assay and can, therefore, be performed on many samples in parallel on a large scale. The practical advantages of the comet-based *in vitro* DNA repair assay are low-cost, simplicity, and versatility. It involves one simple incubation step, and the results on DNA incision activity are obtained within two days. It is therefore well suited for biomonitoring or intervention studies, or for the screening of new chemicals and therapeutics, which typically require high-throughput processing of many samples.

Experimental design

Overview of the assay

The whole procedure of the comet-based *in vitro* DNA repair assay can be divided into six major steps, as described in **Figure 2**. The most crucial steps are also demonstrated in the associated films (https://www.youtube.com/playlist?list=PLEVxCdaQpbj1GDqGUHgWiaBy9eVTUZOzX). Steps 1 and 2 have to be performed on day 0, Steps 3-5 on day 1, and Step 6 on day 2.

Step 1: Various types of cells can be used for preparing the substrate DNA – cultured cells or freshly isolated PBMC are convenient. The aim is to produce non-exposed cells with a low background level of DNA damage and exposed cells with a sufficiently high level of specific DNA damage for the enzymes in the extract to work on (obeying the biochemical principle that the lesions in the substrate DNA should be present in excess). (Video instructions: https://youtu.be/awtdmFBI1WA)

Checkpoint 1: DNA repair activity may vary between experiments for various reasons, including the amount of DNA lesions induced in the substrate cells. The ratio of specific DNA damage (8-oxoG or UVC-photoproducts/BPDE products) to non-specific damage (SBs and alkali-labile sites) should be verified in preliminary experiments. Such verifications involve the incubation of an endonuclease

specific for the type of DNA damage that is introduced in the substrate cells (Table 3). For expected results and recommended levels of DNA damage in both non-exposed and exposed cells, see Figure 9 (for Ro19-8022 and $KBrO_3$ exposure), Figure 10 (for UVC exposure), and Figure 11 (for BPDE exposure).

Step 2: To prepare protein extracts, various starting materials can be used: PBMC, cultured cells, animal and human tissues. It is advisable to prepare extracts from all the samples at roughly the same time, or at least in large batches, to reduce the risk of batch variations affecting results. (Video instructions: https://youtu.be/VHRHwkfFIDw)

Check point 2: Protein concentration should ideally be measured prior to the reaction so that all the extracts can be diluted to and used at the same concentration on the day of experiment. Retrospective normalization of the activity according to protein concentration (when extracts are used at different concentrations in experiments and results are adjusted for protein concentration afterwards) is not recommended because protein concentration and activity as measured in the assay are not proportionally related[67].

Step 3: When embedding the cells in agarose gel, the final concentration is essential as the migration of DNA and sensitivity of the assay depend on the density of the gel. There are several procedures for performing lysis of the cells in the comet assay. For the comet-based *in vitro* DNA repair assay, a lysis time of 1h is recommended. (Video instructions: https://youtu.be/T42JOvD2MnE)

Step 4: At this point in the protocol, substrate agarose-embedded nucleoids, both non-exposed and exposed, are going to be incubated with either reaction buffers or sample extracts (containing DNA repair enzymes). During the incubation, DNA repair enzymes contained in the sample extract induce DNA SBs at the sites of specific DNA lesions in the substrate nucleoids (8-oxoG for BER, or UVC- or BPDE-induced lesions for NER) (Figure 1).

This is one of the most critical steps of the assay and standardization is necessary regarding the time of incubation and concentration of the extracts (see Material setup). Several experimental controls should be included in the assay for the correct interpretation of the results (described below in section "Controls"). (Video instructions: https://youtu.be/GzghrROzD64)

Step 5: As a result of the incisions (i.e., SBs), the DNA will be drawn towards the anode forming a comet-like image. The proportion of total DNA in the comet tail reflects the DNA repair activity of the sample extract, which means more DNA incision activity will result in more DNA in the comet tail. The following steps comprise the neutralization and washing of the microscope slides. (Video instructions: https://youtu.be/kvgZ7O25kXo)

Step 6: For visualization of the comets, various dyes can be used. The use of tail intensity (TI, % tail DNA) is advised to express the results. However, other primary comet assay descriptors (e.g. tail moment or visual score) can be used to calculate the final DNA repair incision activity.

Controls

Positive and negative controls

It is essential to document the reliability of the comet-based *in vitro* DNA repair assay by analysing control samples in the validation process and on-going experiments. However, there is a lack of experimental controls - chemical or physical exposures - that consistently have been shown to alter the DNA repair activity without causing cytotoxicity or cell death. Instead, it is possible to use repair-deficient cells or tissue samples as negative controls. Ogg1 knockout fibroblasts and mouse tissues are useful sources of repair-deficient extract in the repair assay on Ro 19-8022- or $KBrO_3$-exposed substrate cells[7,68]. Fibroblast cell lines from xeroderma pigmentosum complementation group A and C can be used for the repair assay using UVC and BPDE exposure[12]. Heat inactivation of repair extracts is a simpler solution to generate a negative control if DNA repair-deficient cells or tissues cannot be obtained[17,18,69]. There is currently no "true" positive control in the sense that certain cells have higher than normal DNA repair activity. The development of knock-in cells is theoretically possible for at least some repair pathways, but it has not been common practice to do so.

Internal experimental controls

The comet-based *in vitro* DNA repair assay uses internal experimental controls, which are also used in the calculation of the repair-related DNA incision activity or simply to assess if the assay was performed well. These controls assess the incisions/cleavage in nucleoids from non-exposed or exposed substrate cells, incubated with incubation reaction buffer or sample protein extract:

i) the "**background control**" is non-exposed substrate cells incubated with the incubation reaction buffer to check the basal level of DNA damage in the substrate DNA (Figure 3 – yellow; or Figure 2, Step 4, Microscope slide 1);

ii) the "**treatment control**" is exposed cells incubated with the incubation reaction buffer to reveal the presence of non-specific DNA SBs or alkali-labile sites resulting from the exposure with the damaging agent (Figure 3 – green; or Figure 2, Step 4, Microscope slide 2);

iii) the "**specificity control**" is non-exposed substrate cells incubated with the sample's protein extract to check for non-specific incision or cleavage activity (Figure 3 – blue; or Figure 2, Step 4, Microscope slide 3);

iv) the "**incubation reaction control**" is exposed substrate cells incubated with a lesion-specific enzyme (Figure 3 – red; or Figure 2, used in Checkpoint 1 and Step 4).

Concerning the latter (iv), it has been common practice to consider the Fpg- or hOGG1- treatment as incubation reaction controls for the $KBrO_3$ and Ro 19-8022 + light exposed substrate cells[28,47,70]. T4 endonuclease V has been used as an incubation reaction control for UVC-irradiated substrate cells[13,47]. However, there are currently no enzymes or crude extracts available that can be used as incubation reaction control for BPDE-generated substrate cells or any other type of bulky DNA adducts that are used to assess NER activity.

Assay setup

Figure 3 illustrates an example of a potential assay setup in which protein extracts of 3 different samples are assessed for their BER and NER incision activity. When preparing the required number of slides, it is important to keep in mind to include the assay controls that were described above. In the scheme, the gels are randomized (e.g. the gels with UVC-exposed substrate cells are in different places on duplicate slides). Alternatively, one can simply put the duplicate gels on the same slide. An example of a setup when using a higher throughput 12-gel system can be found in Box 2.

Limitations

It is worth emphasizing that the repair pathway studied is defined by the kind of damage introduced in the substrate DNA, but there is 'cross-talk' between pathways. For instance, in the case of substrate cells containing $KBrO_3$-induced DNA lesions, it is not absolutely clear if the assay measures the overall BER activity or just the repair incision activity at oxidatively damaged DNA. Nonetheless, the various assays have been optimized by using knock-out cells or tissues for either BER or NER genes, confirming the specificity of the assays[7,12,18].

To prevent unreliable results, the presence of haemoglobin and bilirubin during extract preparation should be avoided as they interfere with quantification of the protein concentration, and so may lead to overestimation of the protein concentration of the extract[71,72]. Therefore, it is advisable to use a protein assay, such as the Lowry-based BIO-RAD DC Protein Assay Kit, which measures protein concentrations at 650–750 nm. At these wavelengths, the absorbance of haemoglobin (high absorption at ~250–600 nm) and bilirubin (high absorption at ~400–500 nm) is negligible, especially when samples are well diluted[18,73,74].

If the protein concentration of the cell/tissue extract and the incubation time are not optimized for each specific cell type, it is possible that only low repair rates are detected. Sometimes non-specific

401 incisions can even exceed the specific incisions produced by repair enzymes, leading to negative repair
402 rates. Therefore, it is important to optimize both the time of incubation and the protein concentration
403 of the extract (see Equipment setup and Checkpoint 2).

404 The limitation of any comet-based assay relates to the assessment of large numbers of samples.
405 Traditional practice involves processing of comets in relatively large gels on the microscope slides (with
406 one or two gels per slide), which limits the number of samples that can be run in one experiment. This
407 is ameliorated by the development of high throughput versions of the assay, where 12 mini-gels,
408 instead of 2, are run on one microscope slide (Box 2). However, even these gels require manual scoring
409 of the comets in the microscope, which sets a limit to the number of samples that can be processed
410 per working day. Automatic systems have been developed for the identification and scoring of comets,
411 but few researchers appear to use them.

412 Individual DNA repair activity is regarded as a marker of susceptibility to genotoxic agents, on the
413 assumption that a high intrinsic repair activity will be protective. However, it is still not clear whether
414 a high repair activity might be induced in response to, and therefore indicative of, exposure to DNA
415 damaging agents[75] - though this is a general limitation for all *in vitro* DNA repair assays. In any case, it
416 is undoubtedly of value to gather information about individual repair activity alongside DNA damage
417 measurements, since the two are intimately connected. The steady-state level of DNA lesions in a cell
418 is determined by the damage input and the capacity of the cell to repair the damage.

419

MATERIALS

Biological materials

- Cell cultures – using cultured cells (in monolayer or suspension culture) is the most straightforward way to create substrate cells. In addition, cultured cells can be used to prepare cellular protein extracts.
- Animal samples – blood (WBC, PBMC) and different tissues can be used for preparing protein extracts.
- Human samples – blood (WBC, PBMC) can be used to create substrate cells, while blood and different tissues (i.e. biopsies, potentially also buccal cells and saliva) can be used to prepare protein extracts.
- *Drosophila melanogaster* - Drosophila larvae cells (neuroblasts, haemocytes and anterior midgut cells) – can be used to prepare sample extracts (for details, see Box 3)

Δ CRITICAL Various cell types, used to create substrate cells, can show different levels of background DNA damage. Similarly, DNA repair activity and non-specific nuclease activity vary with cell or tissue type. Therefore, before each set of experiments with particular cells, it is essential to check the background levels of DNA damage in the substrate cells and titrate the protein concentration of the sample extracts (for details, see the Checkpoint 1 and 2).

Reagents

For all the reagents mentioned below the most commonly used provider is mentioned, though reagents purchased from other providers should perform equally well.

General reagents

- Agarose, normal melting point (NMP) (Sigma-Aldrich, cat. no. A4718)
- Agarose, low melting point (LMP) (Sigma-Aldrich, cat. no. A9414)
- Phosphate buffered saline (PBS) (Sigma-Aldrich, cat. no. P4417)
- Triton® X-100 (Sigma-Aldrich, cat. no. X100)
- 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (Sigma-Aldrich, cat. no. H3375)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2.2H_20$) (Sigma-Aldrich, cat. no. E5134)
- DL-Dithiothreitol (DTT) (Sigma-Aldrich, cat. no. D9163)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- Trizma® base (Sigma-Aldrich, cat. no. T1503)
- Potassium chloride (KCl) (Sigma-Aldrich, cat. no. P3911)

- Sodium chloride (NaCl) (Sigma-Aldrich, cat. no. S9888)
- Potassium hydrochloride (KOH) (Sigma-Aldrich, cat. no. P5958)
- Sodium hydroxide (NaOH) (Sigma-Aldrich, cat. no. 795429) **! CAUTION** NaOH is caustic
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat. no. A2153)
- Ethanol 96% (Merck Millipore, cat. no. 159010)
- Liquid nitrogen
- Adenosine 5'-triphosphate (ATP) (Sigma-Aldrich, cat. no. A1852)
- Deoxyribonucleotides (dNTPs) (ThermoFisher, cat. no. R0181)
- The Lowry-based BIO-RAD DC Protein Assay Kit using bovine serum albumin as a standard and controlling for the presence of Triton® X-100, DTT, and EDTA (BioRad, cat. no. 500-0116)

Reagents for cultivation, freezing and counting the substrate cells

- Cell culture medium (depending on the cells used)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. 41639)
- Foetal Bovine Serum (FBS) (Sigma-Aldrich, cat. no. TMS-016)
- Other; depending on the cells used, cell culture medium may need the addition of some complements (e.g., non-essential amino acids, glutamine, penicillin/streptavidin).

Reagents for preparation and checking of substrate cells

- Photosensitiser Ro 19-8022 - for preparing BER substrate (CAS 104604-66-2, can be obtained from e.g. Chiron – cat. no. C8504.19-1-DS, Pharmaffiliates – cat. no. PA 27 00232) **! CAUTION** Genotoxic, wear protective gloves.
- Potassium bromate ($KBrO_3$) - for preparing BER substrate (Merck, cat. no. 104912) **! CAUTION** Carcinogenic, toxic, wear protective gloves.
- Benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide(+/-) (anti) (BPDE) - for preparing NER substrate (Bio-connect BV., cat. no. MBS6101688). **! CAUTION** Carcinogenic, mutagenic, wear protective gloves.
- Formamidopyrimidine DNA glycosylase (Fpg) - Incubation reaction control for BER (New England Biolabs, cat. no. M0240S, or obtained from NorGenoTech)
- Human 8-oxoguanine DNA glycosylase (hOGG1) – alternative incubation reaction control for BER (Trevigen, cat. no. 4130-100-EB; or obtained from NorGenoTech)
- T4 Endonuclease V (T4endoV) - Incubation reaction control for NER (New England Biolabs, cat. no. M0308S)

Reagents for comet visualization

Several DNA fluorescence dyes are suitable, however, the most commonly used are:

- SYBR® Gold (ThermoFisher, cat. no. S11494) **! CAUTION** Potential mutagen, wear protective gloves.
- SYBR® Green (ThermoFisher, cat. no. S7567) **! CAUTION** Potential mutagen, wear protective gloves.
- Ethidium bromide (ThermoFisher, cat. no. 17898) **! CAUTION** Mutagenic activity, wear protective gloves.
- DAPI (ThermoFisher, cat. no. D1306) **! CAUTION** Mutagenic activity, wear protective gloves.

Other newly developed dyes, such as GelRed®, can be used as well.

Equipment

Common equipment and consumables to perform cell culture or to collect human/animal samples are needed. Moreover, general laboratory equipment and consumables are required (e.g., microwave oven, freezers, fridge, pH meter, cooled centrifuge, plastic tubes, vortex, plastic tips, pipettors, micropipettes). Special equipment and consumables needed for the comet-based *in vitro* DNA repair assay can be obtained from various providers. Although certain providers are recommended, the protocol works with most equipment.

- Microscope slides - standard microscope slides with frosted end are used
- GelBond® films (Lonza, cat. no. 53734) can be purchased for mounting of the gels instead of using microscope slides
- 20x20 mm coverslips, or 22x22 mm coverslips to mould gels
- 24x60 mm coverslips
- 500W tungsten halogen lamp - for activation of the photosensitiser Ro 19-8022
- Germicidal UVC lamp - for induction of UVC-induced damage in substrate DNA
- UVC-dosimeter
- Mr Frosty® (Nalgene, VWR cat. no. 479-3200) freezing container, or tick walled (min. 1 cm) polystyrene box – to slowly freeze substrate cells
- Pestle and mortar
- Hammer
- Nanodrop or plate reader – to quantify protein concentrations
- Microtube pestles - for homogenisation of tissues
- Water bath or thermoblock

- Staining (Coplin) jars - for cell lysis and slide washing
- 12-Gel Comet Assay Unit (NorGenoTech)
- Metal trays or plates – to keep slides cold and prevent enzyme reaction to start
- Incubator + moist box - for extract-substrate incubation (alternative is a heating plate or 'slide moat' purchased from Boekel Scientific)
- Large-bed horizontal gel electrophoresis chamber
- Power supply (one that reaches 1-2 Amp is advised, e.g. obtained from Consort)
- Epifluorescence microscope and filter set for green-light excitation, Charge-coupled device (CCD) camera (8-bit black-and-white camera is adequate); high sensitivity and high pixel density are preferred
- Optional: i) peristaltic pump to recirculate the electrophoresis solution (e.g., there are cheap peristaltic pumps made for aquariums); and ii) recirculating chiller to cool the platform of the electrophoresis tank.

Software

- For scoring comets, computer-assisted image analysis is recommended using commercially available software which gives the most reproducible results. Examples of scoring software: Comet assay IV (Instem), Comet Analysis software (Trevigen), Lucia Comet Assay™ software (Laboratory Imaging), Metafer (Metasystem).
- Several scoring programs are freely available, among which Casplab and CometScore showed a good agreement with the Comet assay IV Software (Instem), while OpenComet (plugin of ImageJ) showed the least agreement – especially when only samples with %Tail DNA<15% were analysed [unpublished data generated by Working Group 5 within the hCOMET COST Action CA15132].
- Alternatively, visual scoring – classifying comets into 5 classes based on the amount of DNA in the tail[76] – has shown good agreement with commercially offered software[77], but it needs proper training to classify the comets correctly and objectively.

Reagents setup

Solutions

- Cell freezing medium (for freezing cells): DMEM, 10% (vol/vol) foetal bovine serum (FBS), 10% (vol/vol) DMSO. Mix 8 mL of DMEM, with 1 mL foetal bovine serum and 1 mL DMSO. Prepare fresh on the day of use. If needed it can be stored at 4°C up to 24h. *Note*: The proportion of FBS in the freezing medium will depend on the cell type used.

- 1% (wt/vol) NMP agarose (for pre-coating slides): Dissolve 1 g NMP agarose in 100 mL distilled water (or proportional volume), microwave to dissolve, and cool to about 50-60°C in a water bath. One hundred millilitres are sufficient to coat about 75-100 microscope slides. 1% NMP agarose should always be made up fresh.
- 0.7% (wt/vol) LMP agarose in PBS (for embedding cells): Dissolve 0.35 g of LMP agarose in 50 mL PBS, microwave to dissolve, make aliquots of 2-5 mL and store at 4°C. Before use, microwave or submerge the aliquot in boiled water to melt the agarose and then cool to 37°C (in water bath or thermoblock).

  Δ CRITICAL It is best not to reheat LMP agarose aliquots (otherwise evaporation can cause a significant increase in concentration).

  Δ CRITICAL The final concentration of the LMP agarose gel, after mixing with the substrate cells, should be ≤0.8% (wt/vol), because higher concentrations reduce the sensitivity of the assay.
- Buffer A (extraction buffer): 45 mM HEPES, 0.4 M KCl, 1 mM EDTA-Na_2, 0.1 mM DTT, 10% (vol/vol) glycerol. Prepare 100 mL. Dissolve 1.07 g HEPES, 2.98 g KCl, 37.20 mg EDTA-$Na_2.2H_20$, 1.54 mg DTT into 90 mL of distilled water. Add 10 mL of glycerol. Adjust to pH 7.8 with 10 M KOH (dissolve 280.55 g in 0.5 L distilled water). Store frozen (-20°C) as 1-2 mL aliquots. Stable for at least 6 months.
- Buffer A/1% (vol/vol) Triton® X-100: Prepare 1% Triton® X-100 in buffer A: add 10 µL of Triton® X-100 to 990 µL of buffer A. Store frozen (-20°C) in 1 mL aliquots (for use in single experiment). Stable for at least 6 months.
- Buffer A/0.25% (vol/vol) Triton® X-100 (for background control incubation): Prepare 0.25% Triton® X-100 in extraction buffer A: add 2.5 µL of Triton® X-100 to 997.5 µL of buffer A. Store frozen (-20°C) in 0.5 mL aliquots (for use in single experiment). Stable for at least 6 months.
- Lysis solution: 2.5 M NaCl, 0.1 M EDTA-Na_2, 10.0 mM Trizma® base: Dissolve 146.10 g NaCl, 37.22 g EDTA-$Na_2.2H_20$, 1.21 g Trizma® base into 1 L of distilled water. Adjusted to pH 10 with 10 M NaOH (dissolve 200 g of NaOH in 0.5 L distilled water). Prepare 1 L. Will be stable for at least 6 months when stored at 4°C. Before use, add 1 mL of Triton® X-100 per 100 mL.
- Buffer B (washing buffer after lysis and incubation reaction buffer for BER): 40 mM HEPES, 0.5 mM EDTA-Na_2, 0.2 mg/ mL BSA, 0.1 M KCl: Dissolve 9.53 g HEPES, 7.45 g KCL, 0.19 g EDTA-$Na_2.2H_20$, 0.2 g BSA in 1 L distilled water. Adjusted to pH 8 with 10M KOH (dissolve 280.55 g in 0.5 L distilled water). We advise to prepare 500 mL of 10x concentrated stock and freeze (-20°C) in 50 mL tubes (to use for washing slides after lysis) and in 1 mL aliquots (to use as incubation reaction buffer). Stable for at least 6 months. Dilute 10x in distilled water on the

day of use. *Note:* The diluted buffer B could be stored at 4°C for use in a second assay within the same week.

- Buffer N (washing buffer after lysis and incubation reaction buffer for NER): 45 mM HEPES, 0.25 mM EDTA-Na_2, 0.3 mg/ mL BSA, 2% (vol/vol) glycerol. Dissolve 10.72g HEPES, 0.093 g EDTA-$Na_2.2H_20$, 0.3 g BSA into 980 mL distilled water. Add 20 mL of glycerol. Adjusted to pH 7.8 with 10 M KOH (dissolve 280.55 g in 0.5 L distilled water). We advise to prepare 500 mL of 10x concentrated stock and freeze (-20°C) in 50 mL tubes (to use for washing slides after lysis) and in 1 mL aliquots (to use as incubation reaction buffer). Will be stable for at least up to 6 months. Dilute 10x in distilled water on the day of use. *Note:* The diluted buffer N could be stored at 4°C for usage in a second assay within the same week.
- Electrophoresis solution: 0.3 M NaOH, 1 mM EDTA-Na_2: Mix 60 mL 10M NaOH (dissolve 200g of NaOH in 0.5 L of distilled water) and 10 mL 200mM EDTA-Na_2 (dissolve 74.45 g of EDTA-$Na_2.2H_20$ in 1 L distilled water) in 1930 mL of cold distilled water. Store at 4°C for up to one week.
- Neutralising solution: 1xPBS. Store at 4°C or according to manufacturer instructions.
- TE buffer (for SYBR® Gold and SYBR® Green) – Mix 10 mL of 1 M Trizma® base (dissolve 60.57 g in 0.5 L distilled water) and 2 mL of 0.5 M EDTA-Na_2 (dissolve 18.61 g EDTA-$Na_2.2H_20$ in 100 mL distilled water) in 988 mL of distilled water. Prepare 1L and store at room temperature (approx. 22°C). Will be stable for at least up to 6 months. Alternatively, it is possible to use TBE or TAE buffer as recommended by a provider.

Materials setup

Pre-coating microscope slides

1) Prepare 1% (see Reagents setup) NMP agarose solution in H_2O in microwave and keep it at 50-60°C in water bath.

   Δ CRITICAL To prevent boiling over, you can put the microwave at lowest power for longer time.
2) Dip the slides into the gel until the frosted part.
3) Wipe one side of the dipped slide and put the slide flat to dry on a heating plate or overnight on the bench. Remember to indicate with a mark on the frosted part, which side of the slide is the coated one.
4) Store them in boxes at room temperature. They can be used for at least up to 12 months.

? TROUBLESHOOTING.

Equipment setup

Most of the equipment does not require any special setup, apart from those mentioned below. For your information, particular setups are also demonstrated in the associated video protocol (video https://www.youtube.com/playlist?list=PLEVxCdaQpbj1GDqGUHgWiaBy9eVTUZOzX).

- ***Equipment setup for exposure of substrate cells***

The exposure of substrate cells to either Ro 19-8022 or UVC requires some specific instructions for the setup (see **Figure 4**, and https://youtu.be/awtdmFBI1WA).

1) To perform the exposure to Ro 19-8022 + light, a 500 Watt lamp needs to be mounted on a stand about 33cm above the cells on ice to expose the cells for 5min.
   Note: Alternatively, a 2000 Watt lamp at 33cm from the cells can be used for 2 min.
2) For the UVC exposure, you can use any UVC lamp (even those in a PCR hood or cell culture cabinet).
   (A) First measure the intensity of the lamp in mW/cm² with UVC Radiometer.
   (B) The time of exposure needed to achieve 1-2 J/m² can be calculated using the next formula: Time (seconds) = (E (mJ/cm²))/(I (mW/cm²)); with (I) for the intensity measured by the UVC Radiometer and the energy (E) is recommended to 0.1-0.2 mJ/cm².
   (C) If the measured dose is too high, prepare a box or other device with layers of gauze to reduce the intensity until you achieve a measurable timing. E.g. in our hands 6 layers of gauze gave an intensity of 0.0040 mW/cm², leading to an exposure time of 25 seconds.

- ***Electrophoresis setup***

Since the duration of electrophoresis and the electric potential (voltage drop across the electrophoresis tank platform) are the most important drivers of DNA migration[78], these should be measured and standardized for all your experiments. (Video instructions: https://youtu.be/kvgZ7O25kXo)

1) Measure the width of the platform in the electrophoresis tank.
2) Add sufficient volume of electrophoresis solution to cover the microscope slides with at least 5 mm of liquid.
3) Switch on the power supply and measure the voltage over the platform (holding the electrodes on either end of the platform).
   Δ **CRITICAL** Ensure that a power supply is used which can supply the output current at the constant voltage and with sufficient volume of liquid (a power supply that can reach 1-2 Amperes should do the job).

4) Calculate the Electric Potential * Time (EPT) value (dimension: V/cm*min) for your setup. *Note:* for comet-based *in vitro* DNA repair assays we recommend adopting a V/cm*min=30.
5) This EPT value (in this case 30 V/cm*min) should be used for all your experiments and reported in publications, to allow comparison between experiments and laboratories.

Optional:

- Use an external peristaltic pump to recirculate the electrophoresis solution. The advantages are: (1) stable conditions allowing more precise measurement of the electric potential; (2) more stable temperature during electrophoresis; and (3) (probably) reduced variations in the local electric potential.
- Use a recirculating chiller to cool the platform of the electrophoresis tank. Alternatively, the electrophoresis tank can be put in a cold room, dedicated fridge or even put on ice.

Before starting the experiment, it is essential to optimize/titrate the protein concentration of the extract as well as the lesion-specific enzymes, and to determine a suitable incubation time.

Optimization of incubation time

The optimal time of incubation should also be established; the incubation time is normally around 10-30 minutes. The preferred incubation time for the extracts should be the one that allows the detection of the DNA repair incision activity in the linear part of the activity-with-time curve (Figure 5). A typical curve shows an initial linear increase in DNA incision activity after which it reaches a plateau. Ideally an incubation time would be selected that is still on the linear part of the time-incisions curve, detecting a high enough number of incisions before reaching the plateau.

Titration of the lesion-specific enzyme

As indicated above, lesion-specific enzymes are used as “incubation reaction controls”, but also to check the levels of induced DNA lesions in the substrate cells (see Checkpoint 1). However, it has been shown that the enzymes from different producers differ both in their activity and specificity towards nucleobase lesions[8]. Therefore, it is important to perform titration curve experiments to achieve optimal conditions for the enzyme treatment. The optimal concentration elucidated from the titration experiments should detect the maximum enzyme-sensitive sites without inducing non-specific SBs. To do so, substrate nucleoids containing the correspondent lesions and substrate without lesions should

be used (as explained in Checkpoint 1). Muruzabal *et al.*[79] have described how to perform and interpret titration experiments.

Optimization/titration of the protein concentration of the extract

In addition to the incubation temperature, the incision activity of an extract is also dependent on its protein concentration. Although in the case of extracts from cells (i.e. either cell lines or PBMC/WBC) assay conditions are set according to cell concentration, cell counts are not always reliable (and some cells are invariably lost during centrifugation etc.). Therefore, we recommend measuring the concentration of protein in each extract. In the case of extracts from tissue, the protein estimation is essential. To identify an "optimal" protein concentration giving maximal discrimination between lesion-specific incision and non-specific nuclease action of the extract, we recommend running a titration experiment with different protein concentrations of extracts isolated from the cell type/tissue of interest, before starting the main experiments. In **Figure 6**, the general concept of selecting the "optimal" protein concentration (Figure 6 (B)) is shown; too low protein concentrations will yield low background, but also low repair specific incisions (Figure 6 (A)). At high concentrations, on the other hand, non-specific nucleases may increase the background (Figure 6 (C)) leading to suboptimal analysis of the repair rates. Although each lab should optimize their conditions, guidance can be given to the approximate concentration of protein needed as shown in Table 2.

Note: An option to reduce the non-specific activity of the extracts involves adding aphidicolin to the extract from a stock in DMSO, to a final concentration of 1.5 µM. Aphidicolin (a DNA polymerase inhibitor), when added to the protein extract has been shown to block non-specific nucleases in the BER assay[18].

PROCEDURE

Step 1: Preparation of substrate cells – day 0, ● Timing 2-6h (depending on the DNA damaging agent)

Exposure of substrate cells to DNA-damaging agents

Note: any cell type can be used, but it is advisable to use cells in suspension to avoid trypsinization and centrifugation steps (see Box 4 for advice on cell types).

1) Prepare the desired number of cells. Prepare enough flasks/dishes for both exposed and non-exposed cells (to serve as treatment controls).
   (A) Prepare cell suspension in cell culture medium *without* FBS.
   (i) PBMC are obtained from venous blood and isolated using a standard density gradient centrifugation method. Cell lines that grow in suspension can also be used.
   (ii) Count a sample of the cell suspension.
   (iii) Centrifuge cells at about 150-300xg, for 5 min.
   (iv) Wash cells with PBS and spin again.
   (B) Prepare adherent cell flasks.
   (i) Cells are grown in a flask or dish in culture medium to near confluence.
   (ii) Before exposure, remove medium and wash cells with PBS.
2) Treatment of the cells with DNA damage-inducing agent
   (A) Ro 19-8022 exposure - Induction of oxidative lesions to study BER
   (i) Resuspend the pellet with cold PBS containing photosensitiser Ro 19-8022 or add it to the flask with adherent cells. Generally, the final concentration of 1-2 µM.
   Δ **CRITICAL** Avoid excessive light by wrapping the tube containing Ro 19-8022 solution in aluminium foil.
   ! CAUTION Ro 19-8022 is a carcinogenic agent. Wear protective gloves.
   (i) Place cells on ice, 33 cm from a 500 W tungsten halogen lamp and irradiate for 5 min. *Note:* plastic is transparent to this visible light.
   (ii) Remove Ro 19-8022 solution and wash cells as described below.
   (iii) Also prepare control cells, with no photosensitizer in PBS, exposed to light only (non-exposed substrate cells).

   (B) $KBrO_3$ exposure - Induction of oxidative lesions to study BER

   (i) Resuspend the cells in culture medium and keep at 37°C.

(ii) Prepare a 10x stock solution of the final $KBrO_3$ concentration (e.g. 50 mM but may depend on the cell type) in 37°C warm cell culture medium (without FBS). *Note*: the stock solution is only ten-times lower than the water solubility limit of $KBrO_3$.

! CAUTION $KBrO_3$ is a carcinogenic agent. Wear protective gloves.

(iii) Mix the cell suspension with the $KBrO_3$ stock suspension in with 9:1 ratio (e.g. the final concentration of $KBrO_3$ in the cell suspension will be 5 mM).

Δ CRITICAL DNA damage induced by $KBrO_3$ is largely dependent on intracellular GSH. Therefore, it is advised always to run a dose-response experiment first.

(iv) Incubate the cells for 1 h at 37°C.

(v) Remove $KBrO_3$ solution and wash cells as described below.

(vi) Also prepare control, non-exposed cells that have been incubated only with cell culture medium.

(C) UVC exposure – induction of thymidine dimers to study NER

(i) Start with cells in cold PBS.

(ii) Place the dish under a measured UV source and irradiate with 1-2 J/m^2 of UVC.

Δ CRITICAL Irradiate without a dish lid, as plastic reduces UVC exposure.

? TROUBLESHOOTING

(iii) Also prepare control, non-exposed cells.

(D) BPDE exposure – induction of bulky BPDE-DNA adducts to study NER

(ii) Resuspend the pellet in cold PBS containing BPDE or add it to the flask with adherent cells. Generally, the final concentration is 1-3 µM.

Δ CRITICAL Substrate cells should be prepared with the active metabolite BPDE and not with the parent compound B[a]P, because during the metabolism of B[a]P reactive oxygen species may be formed leading to DNA damage that is typically repaired by BER enzymes instead of NER.

! CAUTION BPDE is a carcinogenic agent. Wear protective gloves.

(iii) In parallel non-exposed cells will be treated with vehicle control DMSO.

Δ CRITICAL The DMSO concentration should be kept as low as possible; <0.5% is advised to avoid toxic effects and to ensure low background DNA damage.

(iv) Incubate the cells for 30 min at 4°C.

(v) Remove BPDE or DMSO solution and wash cells as described below.

3) After treatment, adherent cells need to be washed with cold PBS and trypsinized. In case of cells in suspension go directly to the next step.

Δ **CRITICAL** Avoid long trypsin treatment since this can increase background damage.

4) Transfer the cells to tubes and centrifuge for 5 min at 4°C at 150-300xg (depending on cell line)

Δ **CRITICAL** Keep the temperature cold during all subsequent steps for the preparation of the substrate cells.

5) Remove the supernatant and resuspend each cell pellet in cold PBS.
6) Take an aliquot to count the cells and centrifuge the remainder again.
7) Remove supernatant and resuspend the cell pellet in cold freezing medium at ~1x10^6 cells/mL.
8) Prepare small vials. For instance, 0.3 mL (containing approximately 330,000 cells) in 1.5 mL microtubes. Each aliquot will have enough cells for 20 gels in 2 gels/slide format. Larger aliquots can be prepared in case you plan to run more gels or slides per assay.
9) Cryopreserve at -80°C (there is no special requirement for freezing procedure; the vials can be slowly frozen using Mr Frosty® containers with isopropanol or a thick-walled polystyrene box).
10) At the day of analysis, thaw the vial and embed the cells directly in agarose (i.e. washing steps and re-suspension in new medium are not necessary).

Δ **CRITICAL** It is essential to prepare a large batch of substrate cells (several million, depending on the number of experiments planned and the format used – see below) and they should be stored frozen in aliquots (-80°C), so that all extract samples in an experiment or a trial will be analysed on an identical substrate from the same batch. The doses of DNA-damaging agents given below are suggestions; they should be tested in dose-response experiments to confirm that the level of DNA damage is optimal for the assay (also see the Checkpoint 1).

■ **PAUSE POINT** Step 1 can be performed at any time before starting the experiment. The prepared substrate cells can be stored in aliquots at −80°C for at least 6 months.

Check point 1: Checking of substrate cells – day 0, ● Timing ~8 h (if scoring is performed on the same day)

11) Nucleoids from Ro 19-8022 and KBrO3 exposed cells can be checked via incubation with Fpg or hOGG1 enzymes.
12) Nucleoids from UVC-exposed cells are checked via incubation with T4 endo V enzyme.
13) Nucleoids from BPDE exposed cells can be checked with an extract of DNA repair proficient cell lines – there are currently no enzymes available that can be used to detect bulky DNA adducts.

Δ **CRITICAL** Ideally, the lesions in the substrate should be more than enough to saturate the assay, i.e. they should result in maximum % tail DNA when incubated with the control enzyme. But on the other hand, there should not be a substantial background of SBs seen without enzyme.

Δ **CRITICAL** Such control incubations (Steps 11-13) serve also as an *incubation reaction control* (Figure 2 – Step 4 and Figure 3), and are included in each experiment, to confirm that there really is an excess of damage for the extract to work on.

14) A sample of non-exposed substrate cells incubated with the corresponding enzyme – e.g. Fpg or T4endoV – will show the background level of endogenous lesions (normally insignificant in the case of T4endoV; but various cell types contain variable levels of oxidised bases) (Figure 2 – Step 4 and Figure 3).

? TROUBLESHOOTING

■ **PAUSE POINT** Step 11-14 can be performed at any time during the storage of substrate cells, but before starting the analysis of the extracts.

Step 2: Protein extract preparation – day 0, ● Timing ~4 h – 1 d (depending on the number of samples)

15) Collect biological material

(A) Peripheral blood mononuclear cells (PBMC): Approximately 5-10 mL of blood is needed for preparing extract. Isolate PBMC from whole blood according to standard procedure. Approximately 1x10^6 PBMC are isolated per 1 mL of whole blood.

(B) Cultured cells

(i) At least 5-10x10^6 cells are required.

(ii) Cells grown in suspension should be in log phase of growth.

(iii) Cells grown in monolayer in dishes or flasks should be collected by trypsinisation at sub-confluence. Centrifuge at 150-300xg for 5 min.

(C) Solid tissues (of animal or human origin) should be snap-frozen in liquid nitrogen as soon as possible after sampling. They can be further stored at -80°C.

16) Freeze and store biological material for extract preparation

(A) Freezing cells in medium

(i) Isolated cells can be stored in suspension in freezing medium.

(ii) Wash cells with PBS and count a sample. Centrifuge at 150-300xg for 5 min at 4°C.

(iii) Suspend cells in cold freezing medium at 5x10^6 cells/mL and prepare 1 mL aliquots in 1.5 mL microtubes.

(iv) Freeze slowly to -80°C using a Mr Frosty® freezing container, or in a thick-walled box of expanded polystyrene.

(B) Freezing cell pellets

(i) Isolated cells can be snap-frozen as pellets.

(ii) Suspend in PBS and count a sample. Centrifuge at 150-300xg for 5 min at 4°C.

(iii) Suspend cells in cold PBS at 5x10^6 cells/mL and prepare 1 mL aliquots in 1.5 mL microtubes.

(iv) Centrifuge at ~2,000xg for 5 min at 4°C.

(v) Carefully remove as much supernatant as possible, without disturbing the pellet.

(vi) Drop the tubes into liquid nitrogen. Store at -80°C. Alternatively the tubes can be placed directly at -80°C.

(C) Grinding of solid tissues

(i) At a later point, using a pestle and mortar pre-chilled in liquid nitrogen, grind the frozen tissue under liquid nitrogen, and divide into aliquots of about 30-50 mg using a chilled spatula.

! CAUTION Wear protective glasses and gloves when grinding tissues under liquid nitrogen.

(ii) Quickly weigh the frozen aliquots. Take care not to thaw them!

(iii) Store ground tissue aliquots at -80°C.

Δ CRITICAL Don't add any storage solution to the tissue for freezing.

Δ CRITICAL If amounts of tissue are already <50 mg - for example, small rodent organs such as hippocampus, and tissue biopsies - do not grind, since too much of the material will be lost in the mortar. Half a hippocampus or a biopsy is typically around 5 mg, which is enough to run two assays.

■ **PAUSE POINT** Steps 15-16 can be performed at any time before starting the experiment. The harvested biological material can be either stored at -80°C for several months or used immediately, i.e. proceed to Step 17.

17) Protein extraction

Δ CRITICAL Keep samples on ice during the whole procedure of extract preparation

(A) From frozen cells in freezing medium (either PBMC or cultured cells)

(i) Thaw the frozen cells (either at 37°C or at room temperature), and as soon as the ice has melted, centrifuge at 150-300xg for 5 min at 4°C.

(ii) Resuspend the pellet in cold PBS and spin again.

(iii) Resuspend once more in cold PBS and centrifuge at ~2,000xg for 5 min at 4°C.

(iv) Discard supernatant by tipping out, and carefully remove the last microlitres with a pipettor without disturbing the pellet. The pellet should be almost dry.

(v) Proceed to the next step.

(B) From fresh or frozen cell pellets

(i) Add 50 µl of buffer A to each pellet of $5x10^6$ cells.

(ii) Vortex vigorously, snap-freeze by dropping into liquid nitrogen and immediately thaw again.

(iii) To each 50 µL aliquot, add 15 µL of buffer A/1% Triton® X-100.

(iv) Vortex for 5 sec and leave for 10 min on ice.

(v) Centrifuge at ~15,000xg for 5 min at 4°C to remove cell debris.

(vi) Collect the supernatant in a new microtube.

(vii) Store at -80°C or proceed to Step 4.

(C) From snap frozen/frozen ground tissues

(i) Thaw tissue aliquots.

(ii) Add 100 µL of buffer A per 30 mg.

(iii) Vortex vigorously, snap-freeze by dropping into liquid nitrogen and immediately thaw again.

(iv) To each 100 µL aliquot, add 30 µL of buffer A/1% Triton® X-100.

(v) Vortex vigorously and incubate for 10 min on ice.

(vi) If necessary, larger particles of tissue can be homogenized with a microtube pestle.

(vii) Centrifuge at ~15,000xg for 5 min at 4°C to remove cell debris.

(viii) Collect the supernatant in a new microtube.

(ix) Store at -80°C or proceed to Step 4.

For preparation of protein extracts from *Drosophila melanogaster*, see the Box 3. A great advantage of using this model is that several efficient and deficient strains are available for most of the DNA repair pathways.

■ **PAUSE POINT** Step 17 can be performed at any time before starting the experiment. The prepared sample extracts can be either stored at -80°C for several months or used immediately, i.e. proceed to the Step 18.

Δ **CRITICAL** In case there is a large number of samples in one experiment/trial, from which the extracts are not possible to prepare in one day, it is essential to store all of them until later use. Tip: In case the protein concentration will be quantified later (step 18), store a small aliquot of the extract separately to avoid extra freeze-thaw cycles of the main extract.

? TROUBLESHOOTING

Checkpoint 2: Measure protein concentration – day 0, ● Timing ~2 h – 1 d (depending on the number of samples)

18) Quantify protein concentration

Although we advise the Lowry-based BIO-RAD DC Protein Assay Kit, using BSA as a standard, other assays might be as suitable to quantify the protein concentrations.

Δ **CRITICAL** When selecting a protein quantification assay, choose one that: i) controls for the presence of Triton® X-100, DTT, and EDTA (detergents) in the buffer; and ii) which allows you to measure the protein concentrations at 650–750 nm to avoid interference of haemoglobin and bilirubin.

Δ **CRITICAL** When storing the extracts for later use, it is advisable to measure protein concentration after the extract preparation, but to store it undiluted. Dilution with buffer should be performed just before the reaction.

■ **PAUSE POINT** Step 18 can be performed at any time before the evaluation of the DNA repair activity of the extracts.

Δ **CRITICAL** Protein extracts can be kept on ice for immediate use or stored as smaller aliquots (to reduce freezing/thawing cycles) at -80°C for later use in the incubation reaction. Extracts can be stored at -80°C. Extracts have been stored for 1.5 months at -80°C without losing enzyme activity[12,18]. Longer periods may also be appropriate but need to be tested.

Δ **CRITICAL** Tissue extracts with a protein concentration of ~20-30 mg/ml can be obtained from ~50 mg of tissue, which is sufficient material for several assays (approximately 20 assays when running samples in duplicate in a 2 gels/slide format).

Step 3: Embedding substrate cells in LMP agarose & cell lysis – day 1, ● Timing ~4 h

Prepare materials

19) Submerge the required number of LMP agarose aliquot in boiled water to melt the agarose and then cool to 37°C (in water bath or thermoblock).
20) Cool centrifuge to 4°C.
21) Prepare working lysis solution (100 mL is needed for Coplin jar of 16 slides): to 99 mL lysis stock solution (4°C) add 1 mL Triton® X-100, mix, put into a Coplin jar, store at 4°C until use.
22) Put metal plate on box with ice.
23) Label the required number of slides on the frosted end using a pencil, not a pen.

Embedding cells in agarose and lysis

24) Thaw an aliquot of frozen substrate cells, both non-exposed and exposed.
25) As soon as the aliquot is thawed, add 1 mL of cold PBS to the 1.5mL microtube and spin at 150-300xg for 5 min at 4°C to wash cells.
26) Suspend pellets in cold PBS and spin again.
27) Remove the supernatant, disperse the pelleted cells by tapping vigorously, and add required volume of 0.7% LMP agarose (dissolved in PBS) at 37°C to reach the concentration of $2x10^5$ cells/mL. For an aliquot of $3x10^5$ cells, simply add 1.5 mL of agarose. Mix by pipetting gently up and down once. *Note*: Alternatively, take 45 µL of cell suspension and mix it with 105 µL of 1% low melting point agarose at 37°C (see video https://youtu.be/T42JOvD2MnE). This option is often applied when working with a large number of samples, so that cells can be kept on ice until use.
28) From each LMP-cell suspension (containing non-exposed or exposed cells), transfer two 70 µL drops to each pre-coated microscope slide (the final number of cells per gel is ~14,000).
29) Cover gels with 20x20mm coverslips and keep for 5-10 min at 4°C.
30) Remove coverslips and place slides for 1 h in lysis solution in a Coplin jar at 4°C.

    Δ **CRITICAL** It is also critical to be quick, so the gels do not set before putting the coverslip.

■ **PAUSE POINT** The slides can be left in lysis solution for between 1 and 48 h but should be kept the same lysis duration for a whole set of experiments.

? TROUBLESHOOTING

Note: Alternatively, you can increase the throughput of the assay (for convenience in large epidemiological studies with many samples to be processed in a short time) using the assay with 12 gels/slide. The 12 mini-gels/slide format combined with the 12-Gel Comet Assay Unit[80] offers a perfect solution to process a large amount of samples. See Box 2 for instructions.

Step 4: Incubation reaction – day 1, ● Timing ~2 h

Prepare materials

31) Have ready a moist box in a 37°C incubator, containing suitable racks above water to ensure humidity without the slides getting wet. Alternatively, put the slide moat at 37°C.

32) Dilute all sample extracts to the same optimized protein concentration using Buffer A/0.25% Triton® X-100.

33) Dilute an aliquot of the 10x buffer B and/or buffer N stock in water to 1x working solution.

Detection of DNA incision activity of the extract

34) Wash slides in buffer B or N (depending on the repair pathway to be studied), 3 changes, 5 min each at 4°C (using Coplin jar).

35) Place slides on a metal plate on ice to prevent premature enzyme activity when the extract is added.

36) Preparing sample extracts and control enzymes for incubation reaction. For a 2 gels/slide format it is advised to prepare 250µL of extract mixed with incubation reaction buffer.

(A) To study BER

(i) Add to the extract 4 volumes of incubation reaction buffer B.

(ii) Prepare a control solution: buffer A/0.25% Triton® X-100, mixed with buffer B in a ratio 1:4.

(B) To study NER

(i) Add to the extract 4 volumes of buffer N.

Δ **CRITICAL** It is advised to add ATP to buffer N in a ratio 7:1, so the final working concentration of ATP in the extract is 2.5 mM. This is not so important for freshly prepared extract, but crucial for frozen extracts – since ATP degrades during long-term storage.

(ii) Prepare a control solution: buffer A/0.25% of Triton® X-100, mixed with buffer N+ATP in a ratio 1:4.

Δ **CRITICAL** Keep diluted extracts and control solution on ice until use.

37) Add 50 µL of diluted sample extract or control solution to each gel (containing nucleoids of either non-exposed or exposed cells; see Figure 3). Incubate duplicate aliquots of each sample (i.e. two gels).

38) Cover with coverslips (22 x 22 mm for each gel or 24 x 60 mm to cover both gels).

39) Incubate at 37°C in a moist box in the incubator or slide moat for the required time. *Note:* The incubation time is generally around 30 min but needs to be tested/optimized (see Material setup for instructions).

For incubation reactions using 12 gels/slide format, see the Box 2.

For the use of *Drosophila melanogaster* protein extracts, see the Box 3.

To study DNA synthesis and ligation activity, an optional parallel step can be included (see Box 5).

Step 5: Comet formation – day 1, ● Timing ~3 h (including washing steps)

Alkaline treatment & Electrophoresis

40) After the incubation of extract with substrate nucleoids, place slides immediately on ice to stop the enzyme reactions.
41) Remove the coverslips and keep on ice until alkaline treatment. *Note:* Alternatively, microscope slides can be transferred directly to the electrophoresis tank.
42) Incubate in cold electrophoresis solution for 40 min at 4°C.
43) Electrophoresis at ~1V/cm for 30 min at 4°C. *Note:* Alternatively use the EPT that you estimated.

Δ **CRITICAL** Voltage gradient should be measured across the platform carrying the slides. The time of electrophoresis should be tested before starting an experiment or a trial. Please see equipment setup.

Neutralization & Washing

44) Neutralise gels by washing slides for 10 min in the neutralising solution (ice-cold PBS) and 10 min in ice-cold dH_2O at 4°C (use Coplin jar, or lay slides flat in a dish).
Note: use EtOH for 12 gels/slide format (see the Box 2).
45) Allow gels to dry overnight.

■ **PAUSE POINT** The slides can be stored for a long time and stained and scored at any time point.

Step 6: Comet visualization & Analysis – day 2, ● Timing ~2 h – several days (depending on the number of samples)

Comet visualization

46) Slides can be stained with various dyes (see the section Reagents: Reagents for comet visualization).
47) For staining with ethidium bromide (0.01 µg/ mL in water), or DAPI (1µg/ mL in water) - add 20-40 µL of staining solution to each gel and cover with a coverslip.

48) For staining with SYBR® Gold or SYBR® Green, which give intense fluorescence, it is recommended to immerse slides in a bath of the dye at a dilution of 1:10,000 in TE buffer for 20 min, followed by two 10 min washes with dH_2O. Slides are left to dry, and for viewing, 20 µL of dH_2O is added to each gel and covered with a cover slip. Alternatively, SYBR® Gold can also be added as 50 µL of the 1:10,000 dilution on top of each gel and cover with a coverslip.

! CAUTION All dyes may be mutagenic or even carcinogenic.

49) Subsequently, comets are visualized with a fluorescence microscope.

Comet analysis

50) Computer-assisted image analysis, using commercially available software, gives the most reproducible results. We recommend the use of tail intensity (TI), representing % of DNA in the tail of the comet, as the parameter to describe the comets.

51) Score at least 50 comets per gel, i.e. 100 comets per sample when working in duplicates.

52) To obtain the TI value per sample or control, calculate first the median TI for each gel over the scored comets (i.e. the 50 comets in each gel) and then the mean TI over the replicate gels.

△ CRITICAL In an experiment or a trial, all comets should be scored by the same person to minimize inter-operator variation using the same software all the experiment/trial.

■ **PAUSE POINT** Slides can be stained and scored on the day of the experiment (day 1) or stored (un)stained.

BOX 2 - 12 GELS/SLIDE FORMAT

12 mini-gels/slide format: 12 mini-gels per slide (in 2 rows of 6 gels each), instead of two large gels, containing the substrate cells are placed in a microscope slide (see Figure 7). Each of the gels contains about 200-250 cells, from which 100 comets will be analysed (if not using an automated image analysis system).

12-Gel Comet Assay Unit: Each mini-gel can be incubated separately, with different extracts or control solutions, by isolating them using a silicon gasket, with the corresponding 12 holes, on top of the slide. A metal base with a guide on it is used to define the right position of the gels. The metal base, the slide containing the mini-gels, the silicon gasket and a top plate (with the corresponding 12 holes on it) are clamped together with metal clamps to isolate each mini-gel in a well and to avoid leakage between them (see Figure 7). A silicone seal/empty microscope slide on top is used to prevent evaporation of the solutions added to the wells.

Specification for the use of the 12 mini-gels/slide and 12-Gel Comet Assay Unit:

PROCEDURE:

Step 1: Preparation of substrate cells

- After treatment of substrate cells, suspend them at $2.5x10^3$ cells in 0.5 mL in cold freezing medium (This is enough for 20 slides containing 12 mini-gels each) and freeze them.

Step 3: Embedding substrate cells in LMP agarose gel on microscope slides & cell lysis

Embedding cells in agarose and lysis

- After thawing and washing the cells, remove the supernatant and add 0.7% low melting point agarose to a final concentration of $0.5x10^5$ cells/mL. Place an agarose-precoated slide in the ice-cold metal base and, using a multi-dispensing pipettor, place 12 drops of 5 µL of cell suspension (containing 250 cells approximately) following the guide (2 rows of 6 gels each). Keep for 2-3 min at 4°C.

Note: No coverslip is used; the mini-gels have domed shape.

Note: The two rows can contain exposed or non-exposed substrate cells, or alternatively, one row of exposed and one row of non-exposed substrate cells.

Step 4: Incubation reaction - Detection of DNA incision activity of the extract

-Place slide/s in the 12-gel comet assay unit/s, on ice. Add 30 µL of extracts or control solutions in the corresponding wells. Cover the chamber/ with the silicone seal/ empty microscope slide and place it/them in an incubator at 37°C for the required time. The incubation time is normally around 10-30 min. After then, put the chamber/s on ice, remove the slide/s containing the gel from it/them and immediately transfer it/them to cold electrophoresis solution (on the electrophoresis tank or in Coplin jars).

Note: results from enzyme titration are different when using 2 gels/slide or 12 mini-gel/slide plus the 12-Gel Comet Assay Unit[79]. Perform the titration experiments using the gel format and the equipment that is going to be used during the analysis of the samples.

Note: To stop the reaction, and even more important, to avoid cross-contamination, slides should be quickly removed from the 12-Gel Comet Assay Unit and rinsed in the electrophoresis solution before transferring slides to the electrophoresis tank (for the alkaline treatment & electrophoresis step).

Step 5: Comet formation (including washing steps)

Neutralization & Washing

- After neutralization and washing, mini-gels should be dehydrated for 15 min in 70% ethanol followed by 15 min in absolute ethanol.

? TROUBLESHOOTING

BOX 3 – APPLICATION OF THE COMET-BASED *IN VITRO* DNA REPAIR ASSAY ON *DROSOPHILA MELANOGASTER*

Specification for the use of *Drosophila melanogaster*[81]:

Step 2: Extract preparation and Step 4: Incubation reaction

- Place about 150 adult flies (between 7 and 12 days after eclosion/hatching), previously anesthetized, in a cold mortar on ice.

Note: the use of a mix of males and females avoids any effect of sex.

- Add 500 µL of buffer A adjusted at pH 8 and smash the flies with a pestle keeping the mortar on ice.

- Split the obtained solution in 10 aliquots of 50 µL in cryotubes (discard the solid part) and place them in liquid nitrogen immediately. Store the aliquots at -80 ºC until the day of the analysis.

On the day of the analysis:

- Place on ice an enough number of aliquots (extract obtained from one aliquot is enough for the treatment of 8 big gels, 4 slides) for thawing.

- Add 12 µL of 1% Triton® X-100 per aliquot, vortex for 5 s, and place on ice for 5 min.

- Centrifuge at about 15,000 x g for 10 min to separate cell debris.

- Remove 50 µL supernatant and add it to 200 µL of cold Buffer B (for BER) of Buffer N (for NER). Keep the tubes on ice.

Note: The protein concentration should be around 2.5 µg/µL.

- Add 30 µL of extracts or control solutions on top of each gel containing the substrate cells, cover them with coverslips (22x22 mm), place the slides on a humidity chamber and incubate them at 24°C for 30 min.

BOX 4: LIST OF CELL TYPES OR CELL LINES PREVIOUSLY USED AS SUBSTRATE CELLS BY THE AUTHORS

Note: Any cell type can be used, but it is advisable to use cells in suspension to avoid trypsinization and centrifugation steps.

- **WBC** fractions can be used, but keep in mind to isolate enough cells to be able to create a large enough batch of substrate cells for the whole series of experiments. **PBMC** and **lymphocytes** have both been used successfully to assess BER activity, using Ro 19-8022 + light to induce oxidized DNA bases[19,42,66,82].

Cell cultures growing in suspension:

- **THP-1** cells have been exposed to $KBrO_3$ to induce DNA oxidation damage[9-11,83] and UVC to assess NER (unpublished data).
- **TK6** cells have been exposed to UVC to assess NER activity, treated with MMS to assess repair of DNA alkylation lesions, and can also be treated with Ro19-8022 + light[19,84].

Attaching cells:

- **HeLa** cells: (i) treated with photosensitizer Ro 19-8022 + light, were used in several studies to assess BER activity; and (ii) exposed to UVC to study NER activity[7,13,18,23,34-36,39,40,64,69,85-89]
- **A549** cells were successfully used as substrate cells using BPDE[12,90], and UVC (unpublished data) as DNA damage inducing agents.
- **HepG2** cells have been used as substrate cells upon UVC-exposure and treated with Ro19-8022 + light[91].
- **HCT116** cells were treated by photosensitizer Ro 19-8022 + visible light to assess BER[46].
- **Caco-2** were exposed to paraquat to assess the repair of DNA oxidation lesions[92].

BOX 5: DETECTION OF DNA SYNTHESIS & LIGATION ACTIVITY OF THE EXTRACT

Optional step in parallel to Step 4:

As explained in the introduction, assessing the DNA repair incision activity is already sufficient to study the effect of external and internal factors on an organism's DNA repair activity. For instance, a significant correlation was observed between BPDE–DNA adduct removal as studied by ^{32}P-post-labelling (representing the whole DNA repair process from DNA incision to ligation) and the NER incision activity as measured by the comet-based *in vitro* DNA repair assay[12]. These data confirm that the DNA incision activity detected by the comet-based *in vitro* DNA repair assay is representative for an individual's DNA repair capacity.

Still, if one desires to show that DNA synthesis & ligation can take place, a parallel incubation of extracts supplemented with dNTPs can be performed[6]. Note that in this case a parallel set of slides should be prepared.

- First incubate this parallel set of slides with the same sample extract-reaction buffer mixture, as explained in step 35-39, for DNA incisions to occur and accumulate.
- After the first incubation (of 30 min – or as optimized), rinse the slides quickly in reaction buffer (B or N, depending on the type of assay).
- Add dATP, dCTP, dGTP and dTTP at 5 µM each and 2.5 mM ATP to the sample extract-reaction buffer mixtures, prepared as described in Step 36.
- Put 50µL of this mixture on each gel and cover with coverslips (22 x 22 mm for each gel or 24 x 60 mm to cover both gels).
- Incubate at 37°C in the incubator + moist box / slide moat for another 30 min (or as optimized). So, the total incubation time will then be 60 min: 30 min incubation with extract mixtures without dNTPs + ATP, followed by 30 min incubation with extract mixture including dNTPs + ATP.

In case the detected incisions/SBs are back to background levels, this indicates that DNA synthesis & ligation occurred efficiently.

TIMING

Day 0:

Step 1: Preparation of substrate cells: 1-10, 2-6 h (depending on the DNA damaging agent)

Checkpoint 1: Checking of substrate cells: 11-14, 8 h (if scoring is performed the same day)

Step 2: Protein extract preparation: 15-17, 4 h - 1 d (depending on the number of samples)

Checkpoint 2: Measure protein concentration: 18, 2 h – 1 d (depending on the number of samples)

Day 1:

Step 3: Embedding substrate cells in LMP agarose gel & cell lysis: 19-30, 4 h

Step 4: Incubation reaction: 31-39, 2 h

Step 5: Comet formation: 40-45, 3 h

Day 2:

Step 6: Comet visualization & Analysis: 46-52, 2 h - several days (depending on the number of samples)

TROUBLESHOOTING

Step	Problem	Possibly reason	Solution
Material setup: Pre-coating microscope slides; 1-4	Agarose does not hold on the slides	The presence of grease or dust on the slides	Degrease the slides by washing them with EtOH. Leave them to dry at room temperature or pass the slide through the flame of a Bunsen burner.
Step 1: Preparation of substrate cells; 2 (C)	Low levels of UVC-induced lesions (detected by the use of T4endoV)	Use of a cell line with low sensitivity to UVC (e.g., A549 cells are less sensitive than TK-6 and THP1 cells)	Increase the dose of UVC (e.g., for A549 cells 2 J/m² was needed instead of 1 J/m²).
Checkpoint 1: Checking of substrate cells; 11-14	Excessive level of background lesions	Use of old cells (i.e., with a high number of passages), problems in the exposure, or to high concentration of lesion-specific enzyme	Prepare another batch and/or use cells with a lower number of passages. Make sure to titrate the lesion-specific enzyme to determine the optimal conditions.
Step 3: Embedding substrate cells in LMP agarose & cell lysis; 24-30	Losing gels while removing the coverslip	Gels do not set properly due to air condensation in rooms with high temperature and/or humidity	Cool down the working room ideally to about 20°C. Embedding substrate cells in gels in an air-conditioned room is a good option.
Step 6: Comet visualization & Analysis; 50-51	Lack of incision activity of protein extracts	Presence of proteases in the sample extract	Use protease inhibitors during sample extract preparation or keep samples on ice during the whole procedure to minimise the activity of proteases in the extracts.
Box 2	Comet tails are going in all directions in the 12 mini-gels (referred to as “edge effect”)	Uneven drying of the mini-gels	Take care to dry the gels using EtOH immediately after the neutralisation. Dehydration is crucial to avoid the edge effect[93].

ANTICIPATED RESULTS:

Data analysis

Calculation of repair rates

There are 4 TI (%) values needed to calculate repair rate:

(1) The TI (%) for the background control, which represents background DNA breaks (Microscope slide 1 – in Figure 8);

(2) The TI (%) for the treatment control, which represents background DNA breaks plus breaks induced by the exposure to the DNA damage-inducing agent (Microscope slide 2 – in Figure 8);

(3) The TI (%) for the specificity control, which represents background DNA breaks plus non-specific incisions induced by the sample extract (Microscope slide 3 – in Figure 8);

(4) The TI (%) for the sample extract incubated with exposed substrate, which represents background DNA breaks, exposure-induced breaks, non-specific breaks plus specific extract incisions at lesion sites (Microscope slide 4 – in Figure 8).

To calculate the DNA repair activity, first subtract the background control value (1) from all other data (2, 3, 4), giving 'net' breaks. Subsequently, calculate repair rate by:

DNA repair incision activity =

net TI (%) for slide **(4) –** net TI (%) for slide **(3) –** net TI (%) for slide **(2)**

Example calculation (based on Figure 8)

The TI of the background control (1) is usually low (e.g. 2%), whereas the highest TI is usually found in the sample extract incubated with exposed substrate (4) (e.g., 23%). Reaction 2 and 3 usually yields slightly increased values when compared to incubation (1) (e.g., 5% and 3.5%). The repair rate is in this particular case: ΔTI = (23-2) - (5-2) - (3.5-2) = 21 – 3 – 1.5 = 16.5%. Results are normally reported as change in TI in a given time.

Negative values could be generated when calculating the 'net' breaks (or incisions). This can be due to the high levels of background DNA breaks in background controls. If values are close to 0 (between 0 and -2), they can be adjusted to 0 for the calculation of 'DNA repair incision activity'. However, if values are lower, the experiment should be repeated just in case technical problems have occurred. If results are the same, excessive level of background lesions can be present (see 'Troubleshooting table, Checkpoint 1: Checking of substrate cells; 11-14'). If the calculated DNA repair incision activity of an

extract is negative, but all the internal experimental controls give the expected results and the optimization of protein extract and time of incubation is correct, it can be assumed that the extract does not have DNA repair incision activity or that it is below the limit of detection of this assay.

Example data

Figure 9 depicts example data of a successful (Figure 9A) and a suboptimal (Figure 9B) assay that assessed the BER activity of piglet hippocampus samples, using substrate cells (HeLa) exposed to Ro 19-8022 + light or non-exposed substrate cells (PBS + light)[30]. The problem with the assay results shown in Figure 9B involves the high values observed for the non-exposed substrate cells. Since the incubation of exposed substrate cells with Fpg (incubation reaction control) showed expected results (% tail DNA = ~60-70%), it is suggested that there was not a technical error but possibly an error in the handling of the substrate cells or preparation of those slides.

Since $KBrO_3$ induces the same type of lesions as Ro 19-8022 + light, similar data can be expected when using $KBrO_3$-exposed substrate cells. The use of $KBrO_3$ in the comet-based *in vitro* DNA repair assay is increasing, and example data can be consulted in various reports showing DNA repair activity in cell cultures[9], animal tissues[10] and human PBMC[11].

Example data for NER assays are shown in **Figure 10** for the use of UVC-exposed substrate cells and **Figure 11** for the use of BPDE-exposed substrate cells. Figure 10A shows results from PBMC extracts from 7 volunteers and all assay controls indicate that the assay performed well. In contrast, Figure 10B shows results from saliva cell extracts from the same 7 volunteers. Here, the results of the incubation with T4endoV indicate that the incubation reaction performed well, but the background and treatment control are higher than expected, which could be due to the handling of the cells. Regardless, the specificity controls indicate that most extracts suffer from the presence of non-specific nuclease activity – which in the case of saliva could be due to the presence of bacterial enzymes. This assay would need further optimization to reduce this non-specific incision activity.

In **Figure 11** anticipated results from PBMC extracts incubated with BPDE-exposed substrate cells are shown, which illustrate the importance of checking the substrate cells (as described in checkpoint 1). When A549 cells were treated with 0.5% DMSO for 30 min at 37°C to create non-exposed substrate cells, increased background levels were observed – probably due to the toxicity of DMSO (Figure 11B). While treatment with 0.12% DMSO for 30 min at 4°C, in parallel to exposure to 1µM BPDE for 30min at 4°C, resulted in suitable substrate cells with low background levels (Figure 11A). Previously, PBMC samples from these 8 volunteers were tested, and the one showing the highest DNA repair incision activity was selected to serve as incubation reaction control (IC) for that series of experiments.

How to express results?

The incubation of the sample extract with substrate DNA should be performed for a set length of time within the same series of experiments (optimised as described in "Material setup"). So, the results can be expressed as a rate of accumulation of DNA SBs which are optimally expressed as % of DNA in tail. Comet assay results can be further converted to an actual DNA break frequency, using a calibration curve based on irradiated cells, so that the results can be expressed in terms of breaks per 10^9 Da[94,95]. Another way of expressing results is to calculate DNA SBs relative to protein concentration, e.g. amount/number of breaks per mg/mL protein. Since the same volume of the sample extract is added to each gel containing the approximately the same amount of substrate DNA, the incision activity can also be expressed as an amount/number of breaks per mg protein. It is, however, important to keep in mind that activity, as assayed with this method, is not linearly proportional to protein concentration but increases less than 2-fold for a doubling of concentration[18,67,96].

The latter point makes normalisation of the results difficult, but it is possible to allow correction for inter-assay variations. Including the "incubation reaction controls" in each assay allows for normalisation of the data. An alternative way to normalise the data is to include a sample extract from a pooled/reference sample in each assay that has a protein concentration similar to the tested samples and of which the detected incision activity is situated in the linear part of the titration curve. Based on the data for this pooled sample, or the incubation reaction control, it is possible to correct the values of the other samples as follows:

- First calculate the DNA repair incision activity, as indicated above.
- List the DNA repair incision activity for the pooled/reference sample (or the incubation reaction control) as detected in all the assays within one set of experiments.
- Calculate the median value, M
- The detected DNA repair incision activity for the pooled/reference sample (or the incubation reaction control) in a particular experiment X, defined as Q, will then serve in the correction factor - being M/Q.
- Multiply the values of DNA repair incision activity for each sample in experiment X by M/Q to normalize all values.
- Example data and calculation can be consulted in the supplementary file 1.

The variations to be expected in the levels of detected DNA repair activity, and the effect of intrinsic and external factors on DNA repair activity levels, have been described in a recent comprehensive review[38].

Reporting Summary

Further information on research design and ethical approvals is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the majority of the data shown here as examples or anticipated results are available in the original papers. Other supporting data are available upon reasonable request to the corresponding author. For instance, figures 10 and 11 are based on unpublished data, generated by WG5 within the hCOMET-COST CA15132 action].

FIGURES:

Figure 1. Principle of the sample extract incubation reaction (for BER acting against DNA oxidation damage as example).

Abbreviations: 8-oxoG – 8-oxoguanine, AP site – apurinic/apyrimidinic site.

Figure 2. Stepwise overview of the technique (using 2 gels/slide as example).

Abbreviations: dNTPs – deoxyribonucleotide triphosphates, WBC – white blood cells.

Figure 3. Overview of potential assay setup for the comet based *in vitro* DNA repair assay. This overview describes assay setup to assess BER and NER in 3 samples, using Ro 19-8022 + Light (Ro) or UVC (UV) exposed and corresponding non-exposed (noRo or nUV) substrate cells as example.

Figure 4. Equipment setup for exposure of substrate cells. Setup of the 500 Watt lamp at a distance of 33cm above the cells that are placed on an ice box, to perform the exposure to the photosensitiser Ro 19-8022 (left). Construction of a cardboard box (black) with cotton gauze to reduce the intensity during the UVC exposure.

Figure 5. Example data illustrating how to select the optimal incubation time. The red dotted line indicates that for experiments where a mouse hippocampus tissue extract of 3mg/mL is incubated with substrate cells (HeLa) exposed to 1µM Ro 19-8022 + light an incubation time of 25min will be the optimal timing, allowing the detection of the DNA repair incision activity in the linear part of the activity-with-time curve.

Figure 6. Principle of optimization of the extract's protein concentration. (A) A too low protein concentrations will yield low background levels, but also low DNA repair specific incisions; (B) the "optimal" protein concentration results in low background levels and high levels of DNA repair incisions; (C) at high concentrations, non-specific nucleases may increase the background.

Figure 7. The 12 gels/slide format. (A) 12-Gel Comet Assay Unit (NorGenoTech) to incubate the gels separately. From bottom: metal position guide, microscope slide, silicone gasket and top plate

tightened by metal clamps. Down right: twelve mini-gels set on a microscope slide; (B) Schematic overview of 12 gels/slide format. Gels containing non-exposed and exposed substrate cells on different slides. Incubation positions for buffer (B), sample extracts (1-10) and incubation reaction control (IC).

Figure 8. Overview of slides containing internal experimental controls and sample extract used to calculate the final DNA repair incision activity. 1, 2, and 3 represent internal experimental controls and 4 represents sample extract. The bars on the graph shows example data with SD for the internal experimental controls (1, 2 and 3) and a piglet (*Sus scrofa domesticus*) hippocampus protein extract (4)(3mg/mL). Data are shown as mean TI of two independent incubations (i.e., on 2 separate gels) within one experiment.

Figure 9. Examples of results obtained with a BER assay. Results were produced using substrate cells (HeLa) exposed to Ro 19-8022 + light (grey bars) or non-exposed substrate cells (white bars) incubated with various protein extracts from piglet hippocampus samples (n=5, *Sus scrofa domesticus*)[30]. (A) Example of a successful experiment and (B) example of a suboptimal experiment – showing too high background values. Data are shown as mean % tail DNA of two independent incubations with SD within one experiment.

Figure 10. Examples of results obtained with a NER assay. Results were produced using substrate cells (TK6) exposed to 1 J/m² UV (grey bars) or non-exposed substrate cells (white bars) incubated with various human protein extracts either (A) from PBMC, or (B) from saliva cells. Data are shown as mean % tail DNA of two independent incubations with SD within one experiment.

Figure 11. Examples of results obtained with a NER assay. Results were produced using substrate cells (A549) exposed to 1μM BPDE (grey bars) or non-exposed substrate cells (white bars; exposed to vehicle control DMSO) incubated with various protein extracts from human PBMC. (A) Non-exposed substrate cells were treated with 0.12% DMSO for 30 min at 4°C, while (B) treatment with 0.5% DMSO for 30 min at 37°C increased background levels. Data are shown as mean % tail DNA of two independent incubations with SD within one experiment. IC = Incubation Reaction Control.

TABLES:

Table 1. Overview of the various modifications of the comet assay.

	Standard comet assay	**Enzyme-modified comet assay**	**Cellular repair assay**	**Comet-based *in vitro* DNA repair assay**
Starting material to embed in the gel	Cells from samples	Cells from samples	Cells from samples	Substrate cells containing specific DNA lesions
Enzyme treatment during incubation step	No incubation step needed	DNA endonucleases isolated from bacteria or human cells	No incubation step needed, but cells are grown and harvested at various time points	DNA repair enzymes present in protein extracts from samples will incise at lesions in substrate DNA
Endpoint	Single and double SBs	Specific lesions; e.g. oxidized, alkylated, methylated, or dimerised bases	Induction and removal of DNA lesions over time.	DNA repair incision activity
Data interpretation	More migration of DNA to the tail indicates higher levels of DNA damage	More migration of DNA to the tail compared to controls (not incubated with enzyme) indicates higher levels of specific DNA lesions	More migration of DNA to the tail indicates higher levels of DNA damage. Over time, less DNA in the tail will indicate repair of SBs.	More migration of DNA to the tail compared to controls (not incubated with protein extract) indicates higher DNA repair activity

Table 2. Recommended protein concentrations for various cell types and tissues.

Cell type/Tissue	**Protein concentration (mg/mL)**	**References**
Lymphocytes	2	[12]
Fibroblasts	0.1	[12]
Human colon biopsies	3	[19,46,69]
Mouse brain & liver	3 - 5	[18]
Pig brain	3 - 5	[30]
Mouse & pig colon	0.3 - 0.5	[69]
Mouse lung	0.25-2.5	[69,97]

Table 3. Incubation reaction controls that can be used for checking the amount of DNA damage in substrate cells and to normalize between batches of experiments.

Type of exposure	**Type of DNA lesion**	**Potential Control enzyme**
Ro 19-8022	8-oxoG	Fpg, hOGG1
$KBrO_3$	8-oxoG	Fpg, hOGG1
UVC light	Thymidine dimers	T4endoV
BPDE	BPDE-G	Standard extract of DNA repair proficient cell line

SUPPLEMENTARY INFORMATION:

Supplementary Results 1 – Examples of data normalisation

AUTHOR CONTRIBUTIONS STATEMENTS:

S.V., A.A., R.G. and S.L. designed figures; S.L. provided anticipated results and managed the manuscript preparation; S.V., A.A. and S.L. drafted the paper, and A.C., M.D., P.M. A.O., I.G., and P.V. contributed to and revised the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS:

The authors declare no competing interests.

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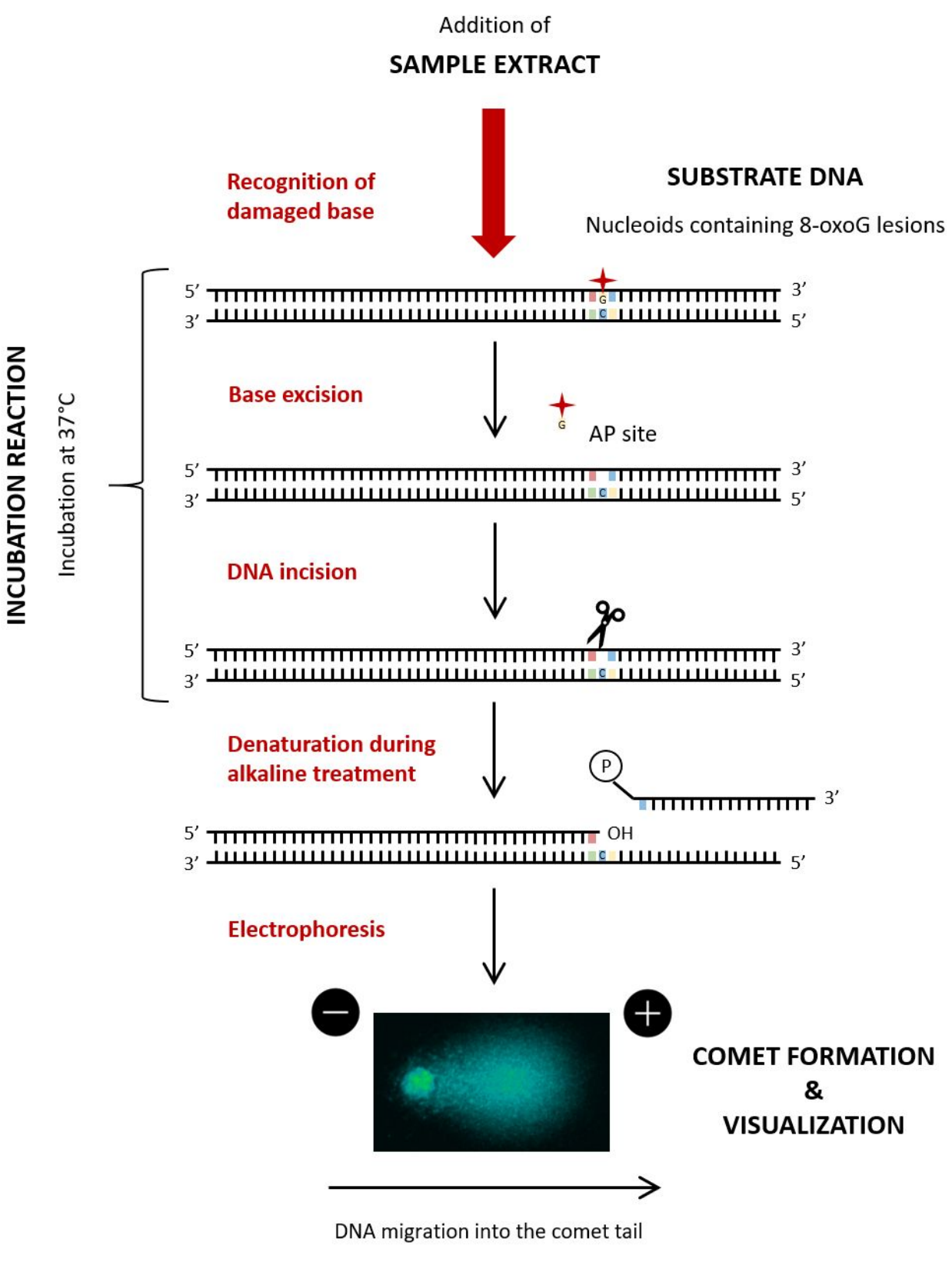

Addition of
SAMPLE EXTRACT
Recognition of damaged base
SUBSTRATE DNA
Nucleoids containing 8-oxoG lesions
INCUBATION REACTION
Incubation at 37°C
5′
3′
G
C
Base excision
AP site
DNA incision
Denaturation during alkaline treatment
P
OH
Electrophoresis
COMET FORMATION & VISUALIZATION
DNA migration into the comet tail

1

PREPARATION OF SUBSTRATE CELLS

Non-exposed cells

Cell culture or WBC

Exposed cells

DNA damaging agent

Cell culture or WBC

CHECKPOINT 1:
Assess levels of lesions in substrate cells

2

EXTRACT PREPARATION

Cell culture or WBC or Tissue

Protein/enzyme extraction

Extract

CHECKPOINT 2:
Measure protein concentration

3

EMBEDDING SUBSTRATE CELLS in LMP AGAROSE GEL & CELL LYSIS

Prepare 2 slides per sample and the buffer control

Set for Buffer: Non-exposed cells in gel (1) | Exposed cells in gel (2)

Set for Sample: Non-exposed cells in gel (3) | Exposed cells in gel (4)

+ 2 extra slides in each experiment to incubate with lesion-specific enzyme

Non-exposed cells in gel (X) | Exposed cells in gel (X)

Immersion of cells in lysis solution

Cell lysis 4°C

4

INCUBATION REACTION

Detection of DNA incision activity of the extract

Incubation with control buffer

Incubation with the sample's protein extract

1 Non-exposed substrate — **BACKGROUND CONTROL**

2 Exposed substrate — **TREATMENT CONTROL**

3 Non-exposed substrate — **SPECIFICITY CONTROL**

4 Exposed substrate — **SAMPLE EXTRACT**

Incubation with lesion-specific enzyme

X Non-exposed substrate

X Exposed substrate

INCUBATION REACTION CONTROL

Incubation 37°C

Optional: Detection of DNA synthesis/ligation activity of the extract

Addition of dNTPs to the extract (parallel incubation)

5

COMET FORMATION

Denaturation & Electrophoresis

4°C

NEUTRALIZATION

WASHING

6

COMET VISUALIZATION & ANALYSIS

STAINING & MICROSCOPIC EVALUATION

1 3

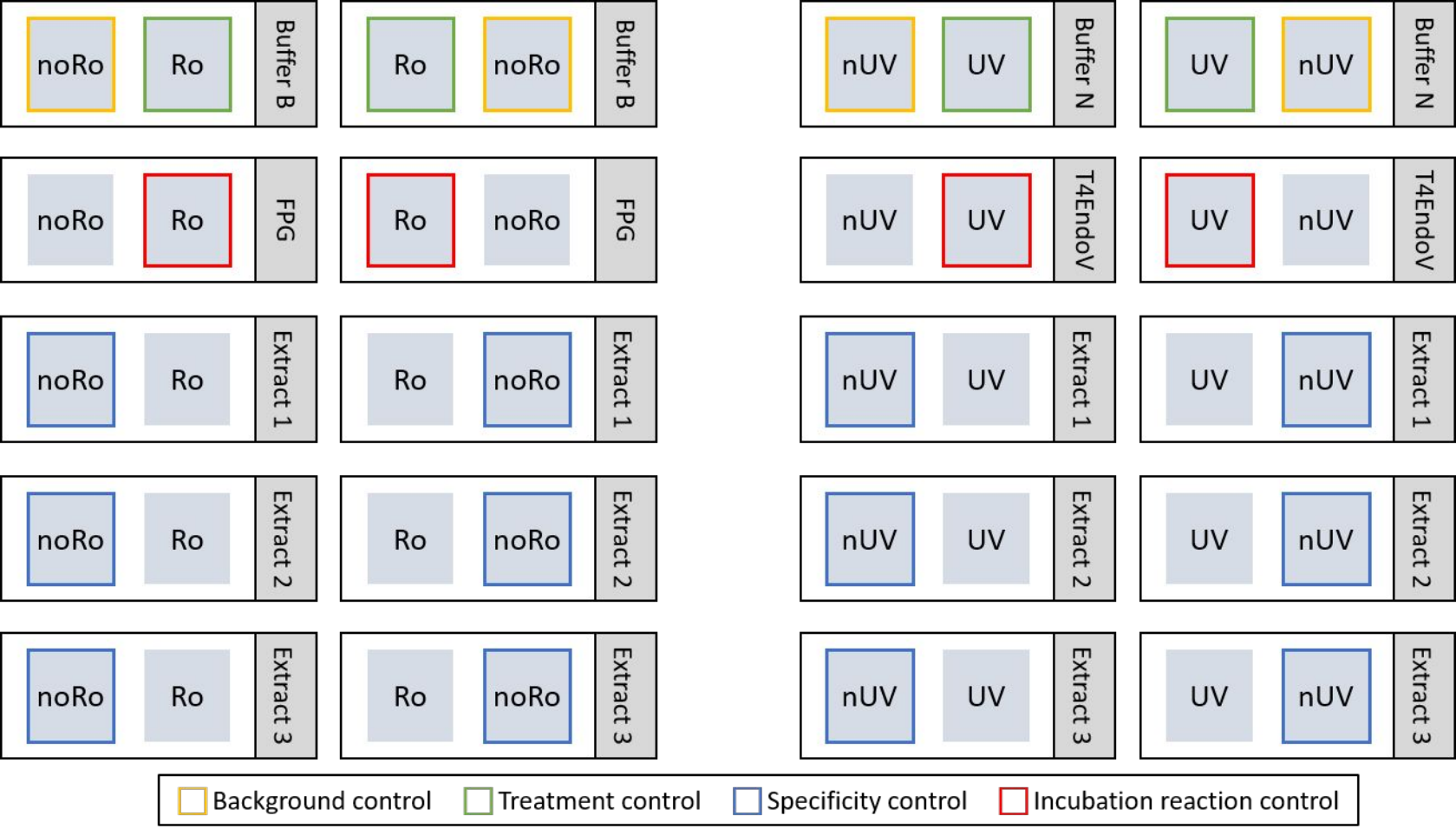
2 4

RESULTS

Formula for calculating repair-related DNA incision activity:

First subtract background value, i.e. (1) from all other mean values

DNA repair incision activity = net TI (4) – net TI (3) – net TI (2)

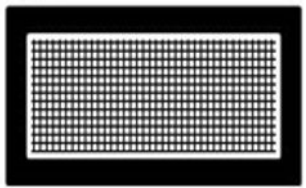

noRo
Ro
Buffer B
Ro
noRo
Buffer B
nUV
UV
Buffer N
UV
nUV
Buffer N
noRo
Ro
FPG
Ro
noRo
FPG
nUV
UV
T4EndoV
UV
nUV
T4EndoV
noRo
Ro
Extract 1
Ro
noRo
Extract 1
nUV
UV
Extract 1
UV
nUV
Extract 1
noRo
Ro
Extract 2
Ro
noRo
Extract 2
nUV
UV
Extract 2
UV
nUV
Extract 2
noRo
Ro
Extract 3
Ro
noRo
Extract 3
nUV
UV
Extract 3
UV
nUV
Extract 3
Background control
Treatment control
Specificity control
Incubation reaction control

Exposure to
Ro 19-8022 plus light

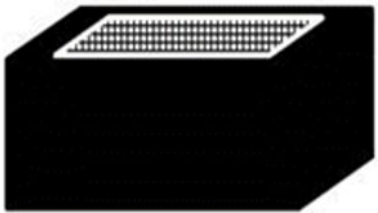
33cm

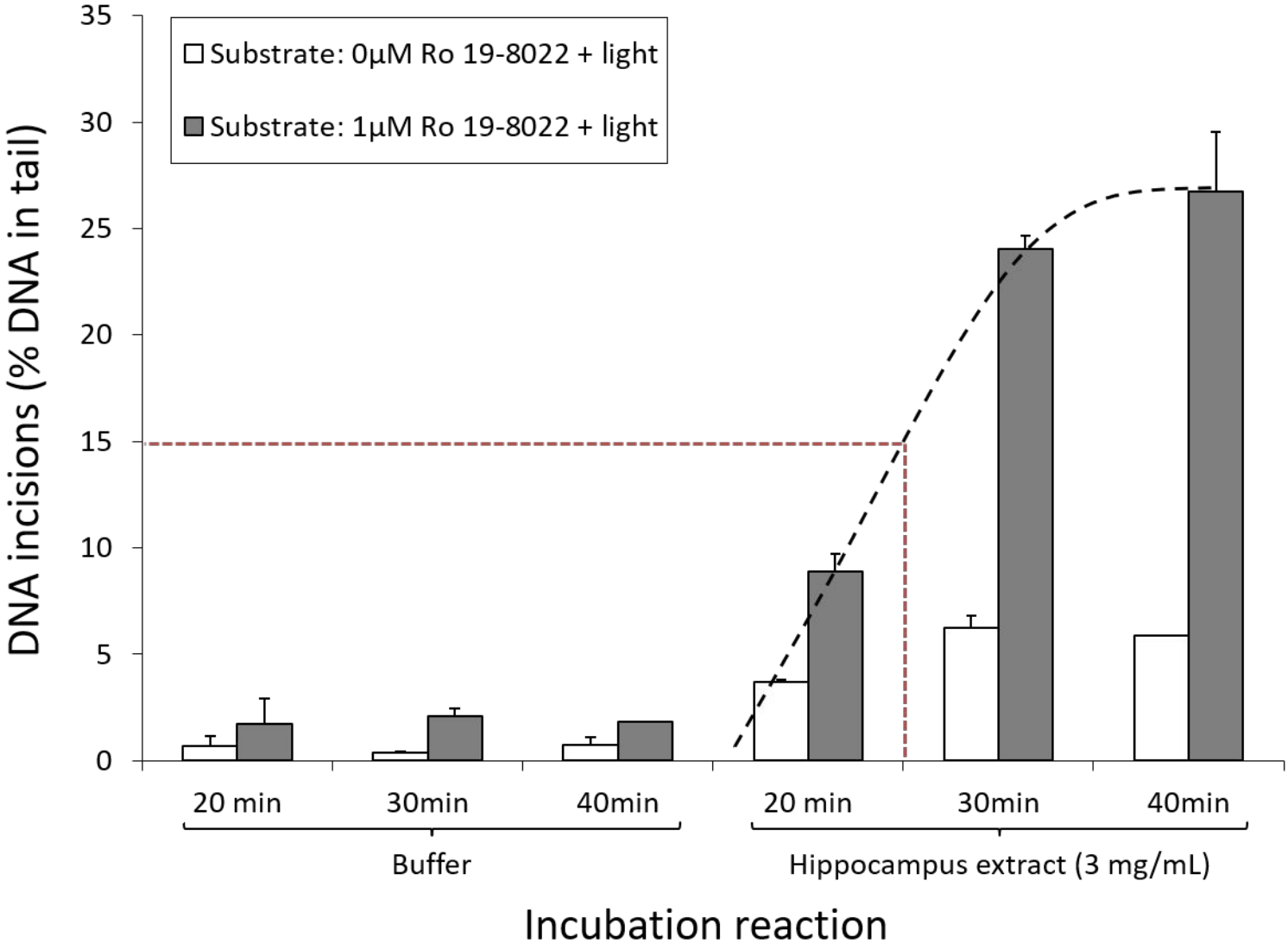
Ice box

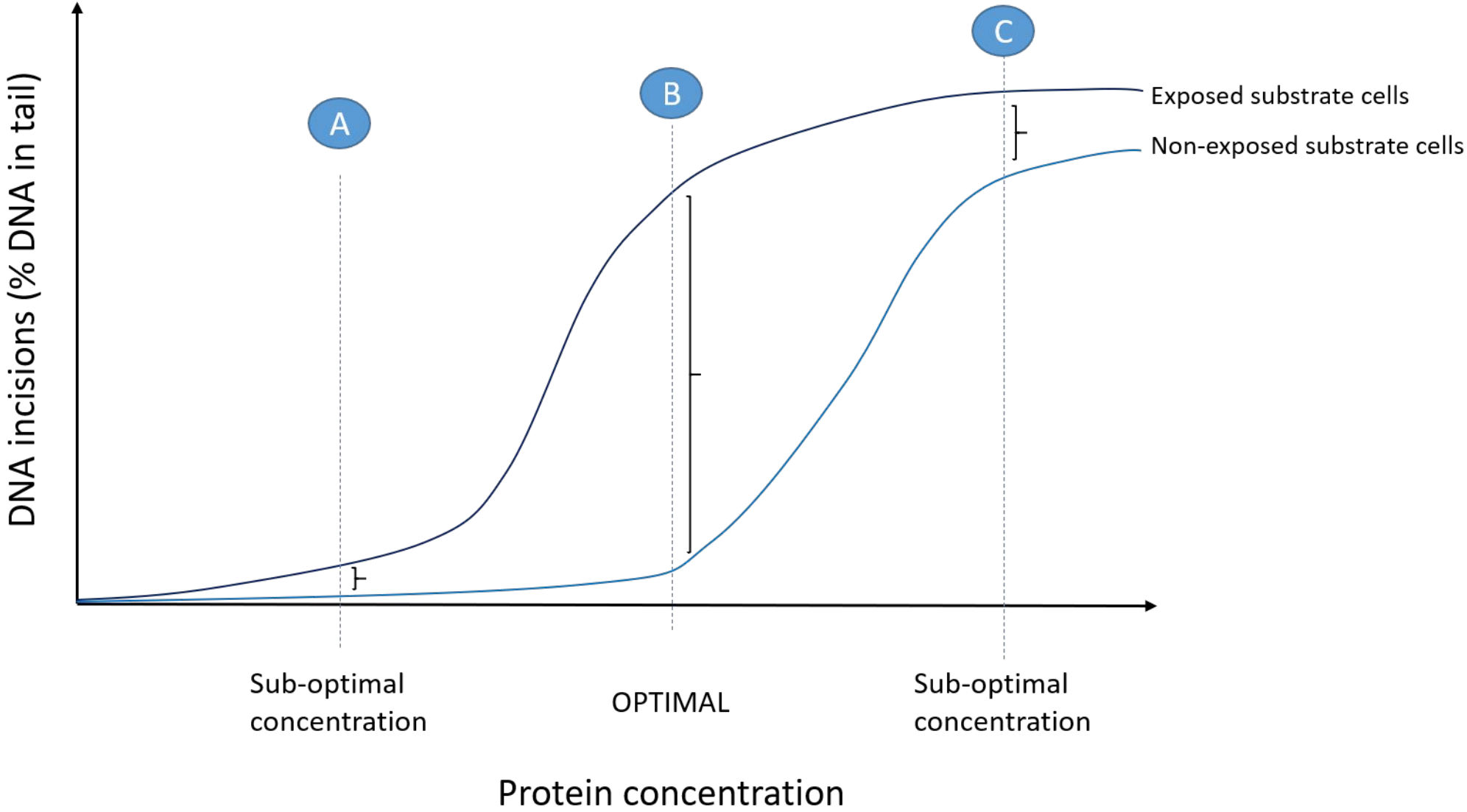
TOP



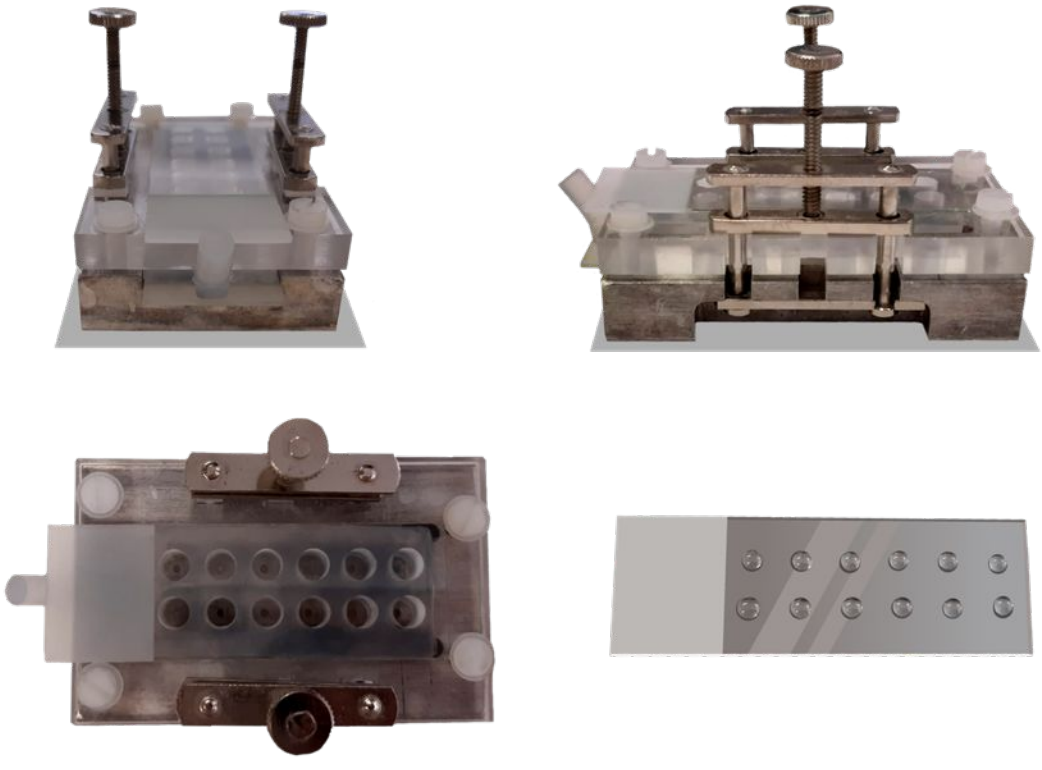
FRONT



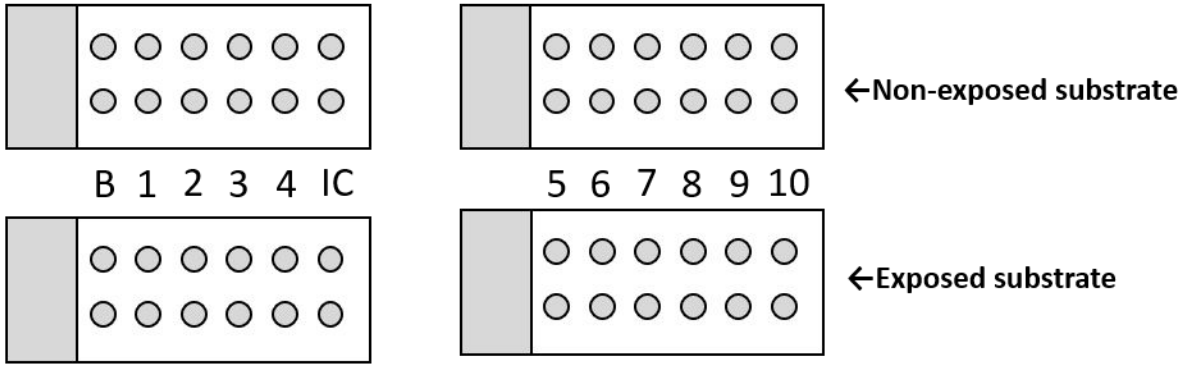

Substrate: 0µM Ro 19-8022 + light
Substrate: 1µM Ro 19-8022 + light
DNA incisions (% DNA in tail)
35
30
25
20
15
10
5
0
20 min
30min
40min
20 min
30min
40min
Buffer
Hippocampus extract (3 mg/mL)
Incubation reaction

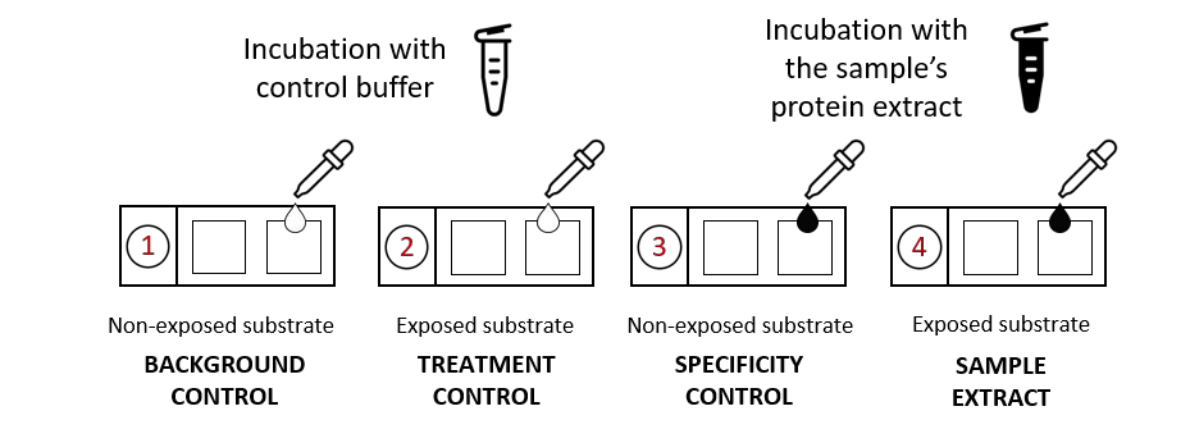

DNA incisions (% DNA in tail)
A
B
C
Exposed substrate cells
Non-exposed substrate cells
Sub-optimal concentration
OPTIMAL
Sub-optimal concentration
Protein concentration

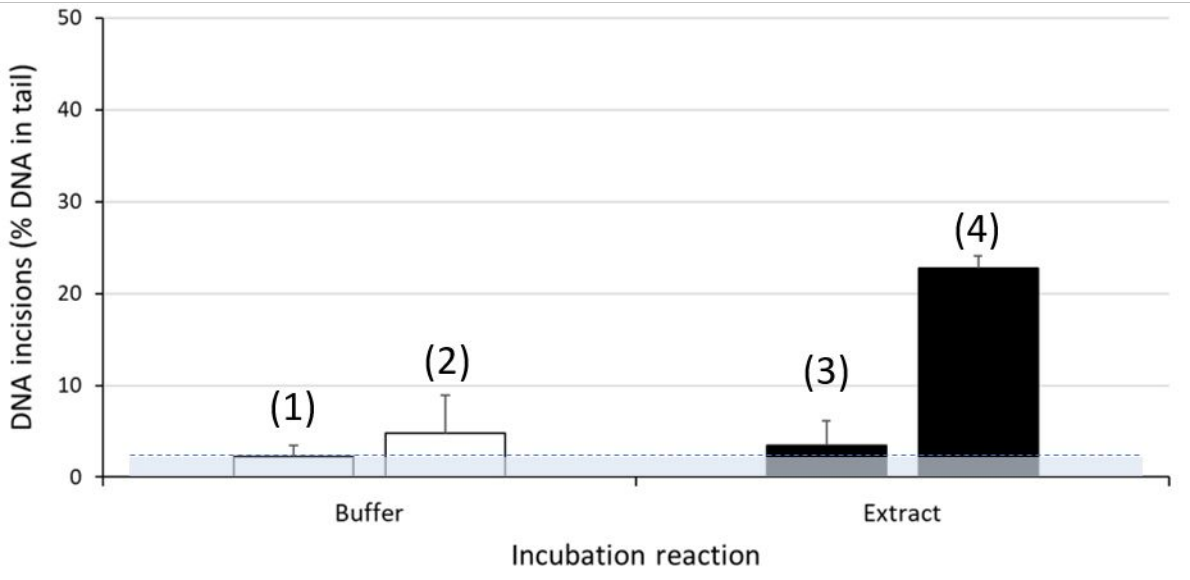
A)

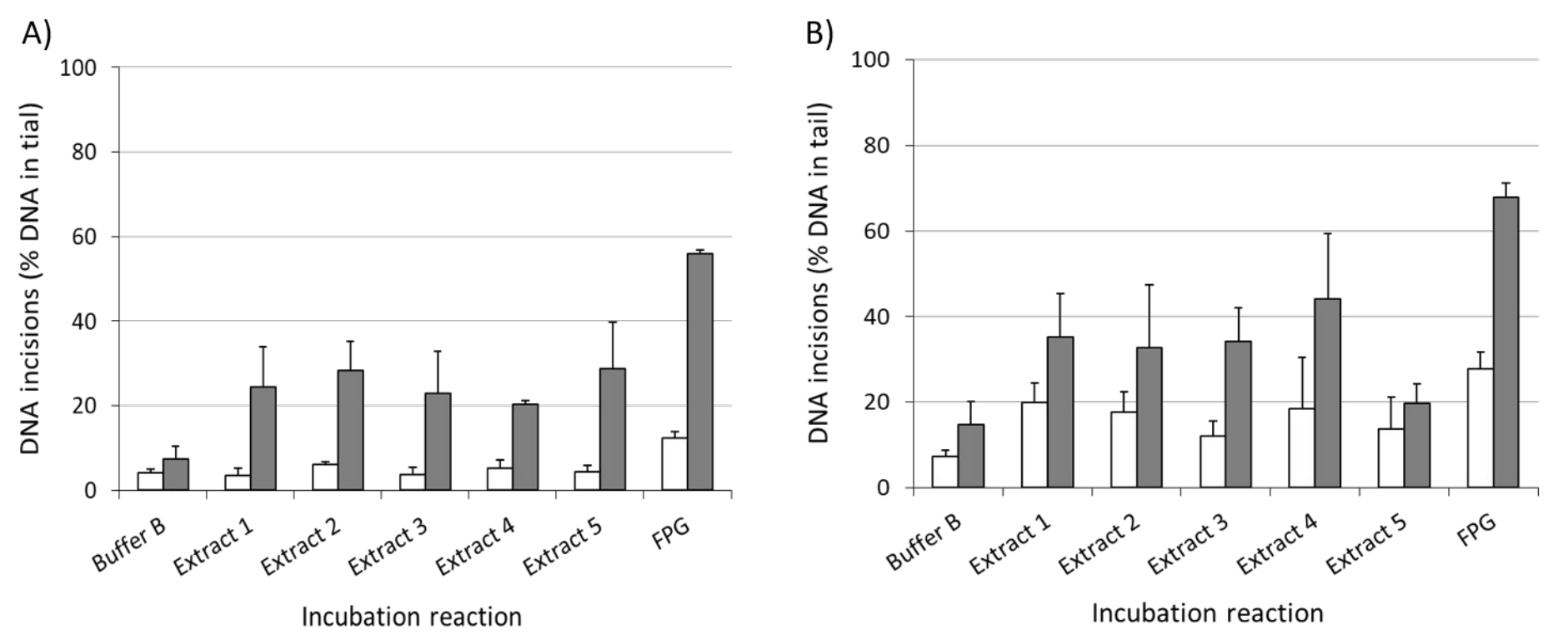


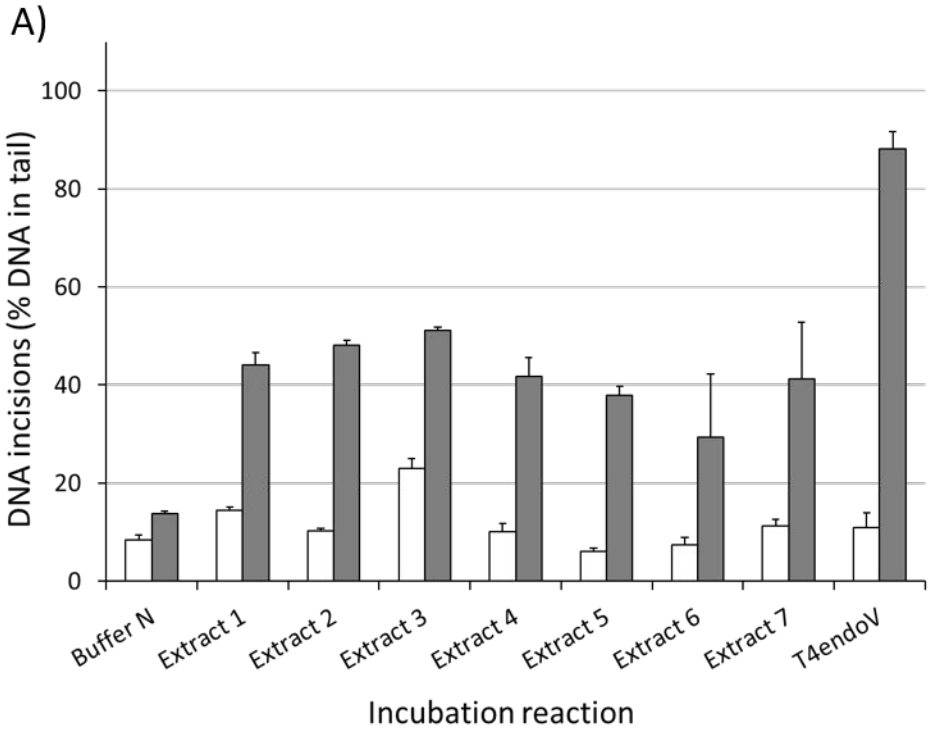
B)

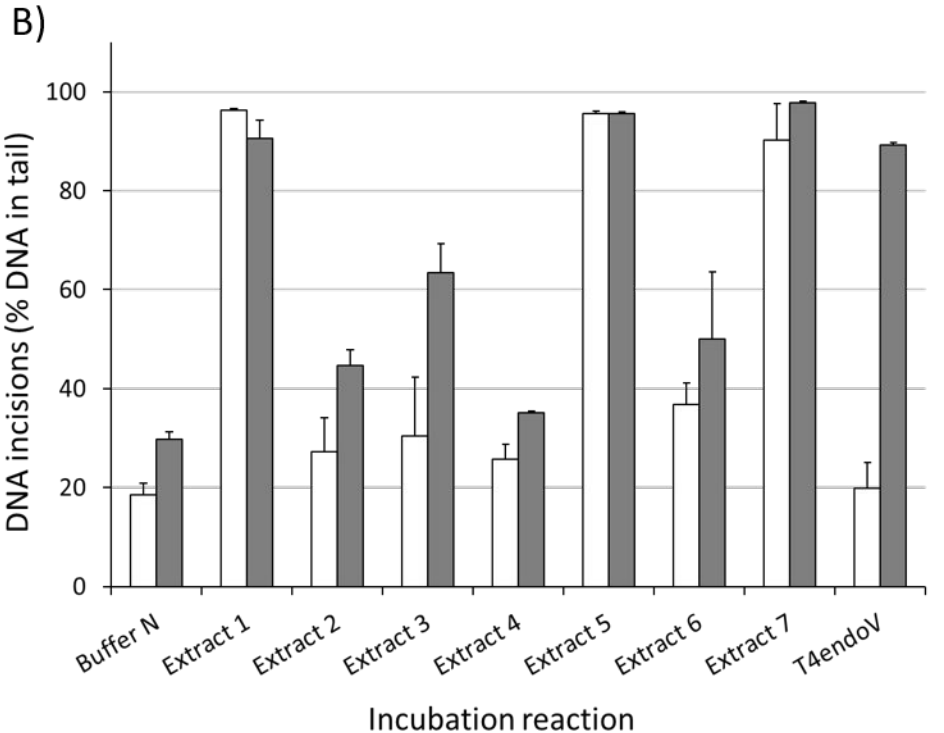


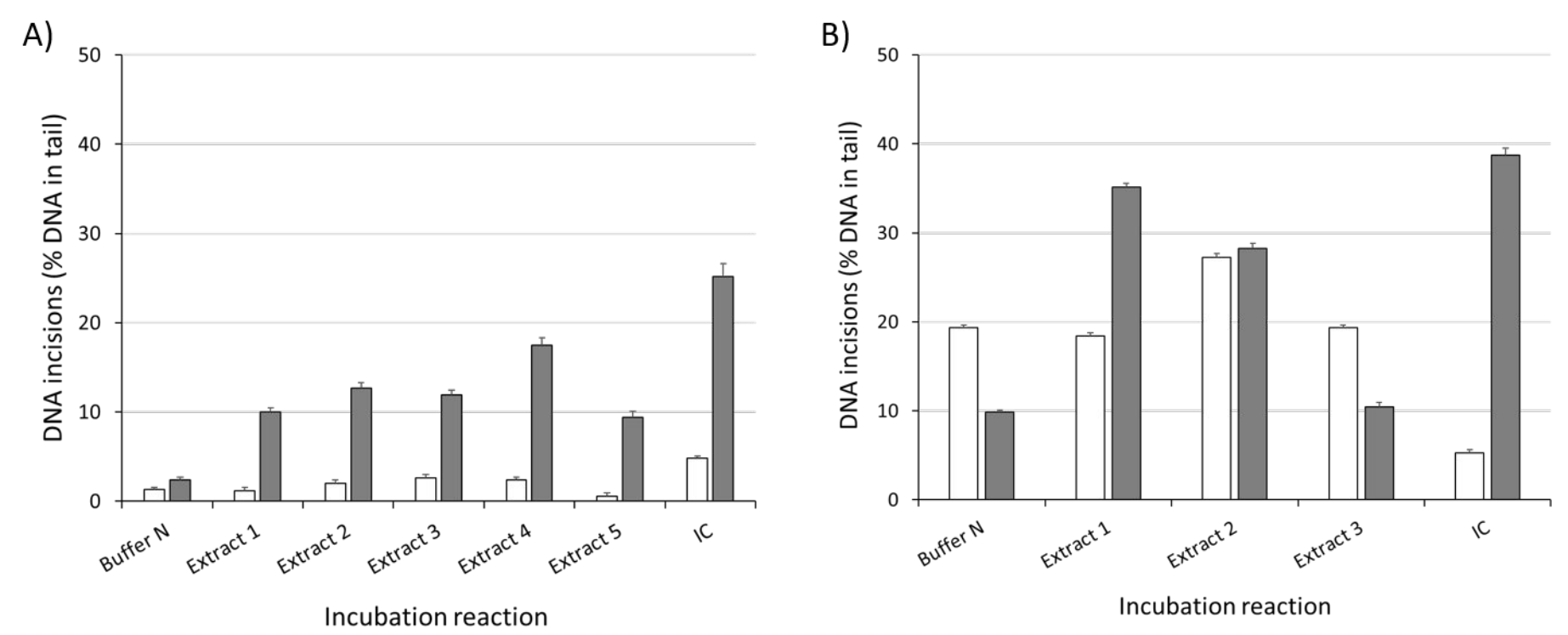

Incubation with control buffer
Incubation with the sample's protein extract
1
2
3
4
Non-exposed substrate
BACKGROUND CONTROL
Exposed substrate
TREATMENT CONTROL
Non-exposed substrate
SPECIFICITY CONTROL
Exposed substrate
SAMPLE EXTRACT


DNA incisions (% DNA in tail)
50
40
30
20
10
0
(1)
(2)
(3)
(4)
Buffer
Extract
Incubation reaction


A)
DNA incisions (% DNA in tial)
100
80
60
40
20
0
Buffer B
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
FPG
Incubation reaction
B)
DNA incisions (% DNA in tail)
100
80
60
40
20
0
Buffer B
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
FPG
Incubation reaction


A)
DNA incisions (% DNA in tail)
100
80
60
40
20
0
Buffer N
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
Extract 6
Extract 7
T4endoV
Incubation reaction


B)
DNA incisions (% DNA in tail)
100
80
60
40
20
0
Buffer N
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
Extract 6
Extract 7
T4endoV
Incubation reaction


A)
DNA incisions (% DNA in tail)
50
40
30
20
10
0
Buffer N
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
IC
Incubation reaction
B)
DNA incisions (% DNA in tail)
50
40
30
20
10
0
Buffer N
Extract 1
Extract 2
Extract 3
IC
Incubation reaction