An optimized comet-based in vitro DNA repair assay to assess base and nucleotide excision repair activity

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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

61 # INTRODUCTION

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

69 ## Development of the protocol

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

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

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

97 An alternative *in vitro* approach is based on assessing the ability of repair proteins in a sample extract
98 to recognize and incise substrate DNA that contains induced lesions. The whole-cell extract can be
99 prepared from blood cells, ground tissues or cultured cells, by 'snap-freezing' and subsequent lysis
100 with Triton® X-100. The comet-based *in vitro* DNA repair assay was first devised in 1994 to measure
101 NER and BER activity in a human cell extract[6]. However, over the past two decades, it has been
102 modified and improved, as well as being applied to tissue samples in addition to cell suspensions. **Table**
103 **1** gives the comparison between the three main versions of the comet assay, i.e. the standard comet
104 assay, the enzyme-modified comet assay, and the comet-based *in vitro* DNA repair assay.
105

106 ***Variations in the method***

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

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

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

131 In most studies, the comet-based *in vitro* DNA repair assays have used protein extracts from cultured
132 or isolated cells (e.g. blood cell fractions) to study DNA repair activity. Various attempts have been
133 made by different laboratories to use the comet-based *in vitro* DNA repair assays with extracts from
134 solid animals tissues, but only a few have succeeded[16,17], most being frustrated by low repair activity
135 and/or low detection sensitivity due to the presence of non-specific nuclease activity[18]. However, from
136 2010 onwards, methods for assessing BER and NER activities were developed and optimized for their
137 use with protein extracts from solid tissues. While Langie *et al.* modified both BER and NER assays to
138 measure DNA repair activities from solid tissues of animal origin[16,18], human tissue samples were
139 assayed for both repair pathways by Slyskova *et al.*[19]. It is our aim to optimize the assays further for
140 use with non-invasively collectable tissues, such as buccal cells and saliva – which are currently used
141 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.

142

143 ***Principle of the assay***

144 **Figure 1** provides a schematic overview of the principle behind the sample extract incubation reaction;
145 it depicts substrate DNA from cells that were pre-treated, as an example, with the photosensitizer Ro
146 19-8022 plus light for the measurement of BER. In general, a protein lysate is extracted from cells or
147 tissues and incubated with damage-containing substrate DNA, consisting of gel-embedded nucleoids
148 from cells that were pre-treated with DNA damage-inducing agent. Incubation of these substrate
149 nucleoids with cell or tissue extracts allows the initial steps of BER (or NER in the case of UVC- or BPDE-
150 induced DNA lesions) to occur; repair enzymes present in the protein extracts will induce incisions at
151 the site of the DNA lesions in the substrate. These incisions will result in single SBs that can then be
152 determined by the standard alkaline comet assay. Thus, the increased migration of DNA into the tail is
153 proportional to the DNA repair incision activity of the extracts.

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

165

166 Applications of the method

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

176 ***Cell culture studies***

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

182 ***Animal studies***

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

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

198 ***Human studies***

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

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

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

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

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

231 Comparison with other methods

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

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

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

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

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

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

270 The *in vitro* comet-based approach to measuring DNA repair activity became increasingly popular due
271 to several positive aspects. Above all, it opens up the possibility to study DNA repair activity in diverse
272 biological material, unlike cellular repair assays in which the removal of damage over time is monitored
273 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)

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

340

341 ***Controls***

342 *Positive and negative controls*

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

355 *Internal experimental controls*

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

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

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

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

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

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

377 ***Assay setup***

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

384

385 ## Limitations

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

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

399 If the protein concentration of the cell/tissue extract and the incubation time are not optimized for
400 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

420 # MATERIALS

421 ## Biological materials

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

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

437 ## Reagents

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

440 ### *General reagents*

441 • Agarose, normal melting point (NMP) (Sigma-Aldrich, cat. no. A4718)
442 • Agarose, low melting point (LMP) (Sigma-Aldrich, cat. no. A9414)
443 • Phosphate buffered saline (PBS) (Sigma-Aldrich, cat. no. P4417)
444 • Triton® X-100 (Sigma-Aldrich, cat. no. X100)
445 • 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (Sigma-Aldrich, cat. no. H3375)
446 • Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2.2H_20$) (Sigma-Aldrich, cat.
447 no. E5134)
448 • DL-Dithiothreitol (DTT) (Sigma-Aldrich, cat. no. D9163)
449 • Glycerol (Sigma-Aldrich, cat. no. G5516)
450 • Trizma® base (Sigma-Aldrich, cat. no. T1503)
451 • Potassium chloride (KCl) (Sigma-Aldrich, cat. no. P3911)

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

462 ***Reagents for cultivation, freezing and counting the substrate cells***

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

468 ***Reagents for preparation and checking of substrate cells***

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

483 ***Reagents for comet visualization***

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

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

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

493 **Equipment**

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

500 - Microscope slides - standard microscope slides with frosted end are used
501 - GelBond® films (Lonza, cat. no. 53734) can be purchased for mounting of the gels instead of
502 using microscope slides
503 - 20x20 mm coverslips, or 22x22 mm coverslips to mould gels
504 - 24x60 mm coverslips
505 - 500W tungsten halogen lamp - for activation of the photosensitiser Ro 19-8022
506 - Germicidal UVC lamp - for induction of UVC-induced damage in substrate DNA
507 - UVC-dosimeter
508 - Mr Frosty® (Nalgene, VWR cat. no. 479-3200) freezing container, or tick walled (min. 1 cm)
509 polystyrene box – to slowly freeze substrate cells
510 - Pestle and mortar
511 - Hammer
512 - Nanodrop or plate reader – to quantify protein concentrations
513 - Microtube pestles - for homogenisation of tissues
514 - Water bath or thermoblock

515 • Staining (Coplin) jars - for cell lysis and slide washing
516 • 12-Gel Comet Assay Unit (NorGenoTech)
517 • Metal trays or plates – to keep slides cold and prevent enzyme reaction to start
518 • Incubator + moist box - for extract-substrate incubation (alternative is a heating plate or 'slide
519 moat' purchased from Boekel Scientific)
520 • Large-bed horizontal gel electrophoresis chamber
521 • Power supply (one that reaches 1-2 Amp is advised, e.g. obtained from Consort)
522 • Epifluorescence microscope and filter set for green-light excitation, Charge-coupled device
523 (CCD) camera (8-bit black-and-white camera is adequate); high sensitivity and high pixel
524 density are preferred
525 • Optional: i) peristaltic pump to recirculate the electrophoresis solution (e.g., there are cheap
526 peristaltic pumps made for aquariums); and ii) recirculating chiller to cool the platform of the
527 electrophoresis tank.

528 ## Software

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

541 ## Reagents setup

542 **Solutions**

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

547 • 1% (wt/vol) NMP agarose (for pre-coating slides): Dissolve 1 g NMP agarose in 100 mL distilled
548 water (or proportional volume), microwave to dissolve, and cool to about 50-60°C in a water
549 bath. One hundred millilitres are sufficient to coat about 75-100 microscope slides. 1% NMP
550 agarose should always be made up fresh.
551 • 0.7% (wt/vol) LMP agarose in PBS (for embedding cells): Dissolve 0.35 g of LMP agarose in 50
552 mL PBS, microwave to dissolve, make aliquots of 2-5 mL and store at 4°C. Before use,
553 microwave or submerge the aliquot in boiled water to melt the agarose and then cool to 37°C
554 (in water bath or thermoblock).
555 Δ **CRITICAL** It is best not to reheat LMP agarose aliquots (otherwise evaporation can cause a
556 significant increase in concentration).
557 Δ **CRITICAL** The final concentration of the LMP agarose gel, after mixing with the substrate
558 cells, should be ≤0.8% (wt/vol), because higher concentrations reduce the sensitivity of the
559 assay.
560 • Buffer A (extraction buffer): 45 mM HEPES, 0.4 M KCl, 1 mM EDTA-Na_2, 0.1 mM DTT, 10%
561 (vol/vol) glycerol. Prepare 100 mL. Dissolve 1.07 g HEPES, 2.98 g KCl, 37.20 mg EDTA-$Na_2.2H_20$,
562 1.54 mg DTT into 90 mL of distilled water. Add 10 mL of glycerol. Adjust to pH 7.8 with 10 M
563 KOH (dissolve 280.55 g in 0.5 L distilled water). Store frozen (-20°C) as 1-2 mL aliquots. Stable
564 for at least 6 months.
565 • Buffer A/1% (vol/vol) Triton® X-100: Prepare 1% Triton® X-100 in buffer A: add 10 µL of Triton®
566 X-100 to 990 µL of buffer A. Store frozen (-20°C) in 1 mL aliquots (for use in single experiment).
567 Stable for at least 6 months.
568 • Buffer A/0.25% (vol/vol) Triton® X-100 (for background control incubation): Prepare 0.25%
569 Triton® X-100 in extraction buffer A: add 2.5 µL of Triton® X-100 to 997.5 µL of buffer A. Store
570 frozen (-20°C) in 0.5 mL aliquots (for use in single experiment). Stable for at least 6 months.
571 • Lysis solution: 2.5 M NaCl, 0.1 M EDTA-Na_2, 10.0 mM Trizma® base: Dissolve 146.10 g NaCl,
572 37.22 g EDTA-$Na_2.2H_20$, 1.21 g Trizma® base into 1 L of distilled water. Adjusted to pH 10 with
573 10 M NaOH (dissolve 200 g of NaOH in 0.5 L distilled water). Prepare 1 L. Will be stable for at
574 least 6 months when stored at 4°C. Before use, add 1 mL of Triton® X-100 per 100 mL.
575 • Buffer B (washing buffer after lysis and incubation reaction buffer for BER): 40 mM HEPES, 0.5
576 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-
577 $Na_2.2H_20$, 0.2 g BSA in 1 L distilled water. Adjusted to pH 8 with 10M KOH (dissolve 280.55 g in
578 0.5 L distilled water). We advise to prepare 500 mL of 10x concentrated stock and freeze (-
579 20°C) in 50 mL tubes (to use for washing slides after lysis) and in 1 mL aliquots (to use as
580 incubation reaction buffer). Stable for at least 6 months. Dilute 10x in distilled water on the

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

583 • Buffer N (washing buffer after lysis and incubation reaction buffer for NER): 45 mM HEPES,
584 0.25 mM EDTA-Na_2, 0.3 mg/ mL BSA, 2% (vol/vol) glycerol. Dissolve 10.72g HEPES, 0.093 g
585 EDTA-$Na_2.2H_20$, 0.3 g BSA into 980 mL distilled water. Add 20 mL of glycerol. Adjusted to pH
586 7.8 with 10 M KOH (dissolve 280.55 g in 0.5 L distilled water). We advise to prepare 500 mL of
587 10x concentrated stock and freeze (-20°C) in 50 mL tubes (to use for washing slides after lysis)
588 and in 1 mL aliquots (to use as incubation reaction buffer). Will be stable for at least up to 6
589 months. Dilute 10x in distilled water on the day of use. *Note:* The diluted buffer N could be
590 stored at 4°C for usage in a second assay within the same week.

591 • Electrophoresis solution: 0.3 M NaOH, 1 mM EDTA-Na_2: Mix 60 mL 10M NaOH (dissolve 200g
592 of NaOH in 0.5 L of distilled water) and 10 mL 200mM EDTA-Na_2 (dissolve 74.45 g of EDTA-
593 $Na_2.2H_20$ in 1 L distilled water) in 1930 mL of cold distilled water. Store at 4°C for up to one
594 week.

595 • Neutralising solution: 1xPBS. Store at 4°C or according to manufacturer instructions.

596 • TE buffer (for SYBR® Gold and SYBR® Green) – Mix 10 mL of 1 M Trizma® base (dissolve 60.57
597 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
598 mL distilled water) in 988 mL of distilled water. Prepare 1L and store at room temperature
599 (approx. 22°C). Will be stable for at least up to 6 months. Alternatively, it is possible to use TBE
600 or TAE buffer as recommended by a provider.

601 ## Materials setup

602 ### *Pre-coating microscope slides*

603 1) Prepare 1% (see Reagents setup) NMP agarose solution in H_2O in microwave and keep it at 50-60°C
604 in water bath.
605 **Δ CRITICAL** To prevent boiling over, you can put the microwave at lowest power for longer time.
606 2) Dip the slides into the gel until the frosted part.
607 3) Wipe one side of the dipped slide and put the slide flat to dry on a heating plate or overnight on
608 the bench. Remember to indicate with a mark on the frosted part, which side of the slide is the
609 coated one.
610 4) Store them in boxes at room temperature. They can be used for at least up to 12 months.

611 **? TROUBLESHOOTING**.

612

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).

645 4) Calculate the Electric Potential * Time (EPT) value (dimension: V/cm*min) for your setup. *Note:* for
646 comet-based *in vitro* DNA repair assays we recommend adopting a V/cm*min=30.
647 5) This EPT value (in this case 30 V/cm*min) should be used for all your experiments and reported in
648 publications, to allow comparison between experiments and laboratories.
649
650 Optional:
651 • Use an external peristaltic pump to recirculate the electrophoresis solution. The advantages are:
652 (1) stable conditions allowing more precise measurement of the electric potential; (2) more stable
653 temperature during electrophoresis; and (3) (probably) reduced variations in the local electric
654 potential.
655 • Use a recirculating chiller to cool the platform of the electrophoresis tank. Alternatively, the
656 electrophoresis tank can be put in a cold room, dedicated fridge or even put on ice.

657

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

660 ***Optimization of incubation time***

661 The optimal time of incubation should also be established; the incubation time is normally around 10-
662 30 minutes. The preferred incubation time for the extracts should be the one that allows the detection
663 of the DNA repair incision activity in the linear part of the activity-with-time curve (Figure 5). A typical
664 curve shows an initial linear increase in DNA incision activity after which it reaches a plateau. Ideally
665 an incubation time would be selected that is still on the linear part of the time-incisions curve,
666 detecting a high enough number of incisions before reaching the plateau.
667

668 ***Titration of the lesion-specific enzyme***

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

676 be used (as explained in Checkpoint 1). Muruzabal *et al.*[79] have described how to perform and interpret
677 titration experiments.
678

679 ***Optimization/titration of the protein concentration of the extract***

680 In addition to the incubation temperature, the incision activity of an extract is also dependent on its
681 protein concentration. Although in the case of extracts from cells (i.e. either cell lines or PBMC/WBC)
682 assay conditions are set according to cell concentration, cell counts are not always reliable (and some
683 cells are invariably lost during centrifugation etc.). Therefore, we recommend measuring the
684 concentration of protein in each extract. In the case of extracts from tissue, the protein estimation is
685 essential. To identify an "optimal" protein concentration giving maximal discrimination between
686 lesion-specific incision and non-specific nuclease action of the extract, we recommend running a
687 titration experiment with different protein concentrations of extracts isolated from the cell type/tissue
688 of interest, before starting the main experiments. In **Figure 6**, the general concept of selecting the
689 "optimal" protein concentration (Figure 6 (B)) is shown; too low protein concentrations will yield low
690 background, but also low repair specific incisions (Figure 6 (A)). At high concentrations, on the other
691 hand, non-specific nucleases may increase the background (Figure 6 (C)) leading to suboptimal analysis
692 of the repair rates. Although each lab should optimize their conditions, guidance can be given to the
693 approximate concentration of protein needed as shown in Table 2.
694 *Note:* An option to reduce the non-specific activity of the extracts involves adding aphidicolin to the
695 extract from a stock in DMSO, to a final concentration of 1.5 µM. Aphidicolin (a DNA polymerase
696 inhibitor), when added to the protein extract has been shown to block non-specific nucleases in the
697 BER assay[18].
698
699
700
701

702 **PROCEDURE**

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

705 ***Exposure of substrate cells to DNA-damaging agents***

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

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

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

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

733 (ii) Prepare a 10x stock solution of the final $KBrO_3$ concentration (e.g. 50 mM but may depend
734 on the cell type) in 37°C warm cell culture medium (without FBS). *Note*: the stock solution is
735 only ten-times lower than the water solubility limit of $KBrO_3$.
736 **! CAUTION** $KBrO_3$ is a carcinogenic agent. Wear protective gloves.
737 (iii) Mix the cell suspension with the $KBrO_3$ stock suspension in with 9:1 ratio (e.g. the final
738 concentration of $KBrO_3$ in the cell suspension will be 5 mM).
739 **Δ CRITICAL** DNA damage induced by $KBrO_3$ is largely dependent on intracellular GSH.
740 Therefore, it is advised always to run a dose-response experiment first.
741 (iv) Incubate the cells for 1 h at 37°C.
742 (v) Remove $KBrO_3$ solution and wash cells as described below.
743 (vi) Also prepare control, non-exposed cells that have been incubated only with cell culture
744 medium.

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

746 (i) Start with cells in cold PBS.
747 (ii) Place the dish under a measured UV source and irradiate with 1-2 J/m^2 of UVC.
748 **Δ CRITICAL** Irradiate without a dish lid, as plastic reduces UVC exposure.
749 **? TROUBLESHOOTING**
750 (iii) Also prepare control, non-exposed cells.

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

752 (ii) Resuspend the pellet in cold PBS containing BPDE or add it to the flask with adherent cells.
753 Generally, the final concentration is 1-3 µM.
754 **Δ CRITICAL** Substrate cells should be prepared with the active metabolite BPDE and not
755 with the parent compound B[a]P, because during the metabolism of B[a]P reactive oxygen
756 species may be formed leading to DNA damage that is typically repaired by BER enzymes
757 instead of NER.
758 **! CAUTION** BPDE is a carcinogenic agent. Wear protective gloves.
759 (iii) In parallel non-exposed cells will be treated with vehicle control DMSO.
760 **Δ CRITICAL** The DMSO concentration should be kept as low as possible; <0.5% is advised to
761 avoid toxic effects and to ensure low background DNA damage.
762 (iv) Incubate the cells for 30 min at 4°C.
763 (v) Remove BPDE or DMSO solution and wash cells as described below.
764

765 3) After treatment, adherent cells need to be washed with cold PBS and trypsinized. In case of cells
766 in suspension go directly to the next step.
767 Δ **CRITICAL** Avoid long trypsin treatment since this can increase background damage.
768 4) Transfer the cells to tubes and centrifuge for 5 min at 4°C at 150-300xg (depending on cell line)
769 Δ **CRITICAL** Keep the temperature cold during all subsequent steps for the preparation of the
770 substrate cells.
771 5) Remove the supernatant and resuspend each cell pellet in cold PBS.
772 6) Take an aliquot to count the cells and centrifuge the remainder again.
773 7) Remove supernatant and resuspend the cell pellet in cold freezing medium at ~$1x10^6$ cells/mL.
774 8) Prepare small vials. For instance, 0.3 mL (containing approximately 330,000 cells) in 1.5 mL
775 microtubes. Each aliquot will have enough cells for 20 gels in 2 gels/slide format. Larger aliquots
776 can be prepared in case you plan to run more gels or slides per assay.
777 9) Cryopreserve at -80°C (there is no special requirement for freezing procedure; the vials can be
778 slowly frozen using Mr Frosty® containers with isopropanol or a thick-walled polystyrene box).
779 10) At the day of analysis, thaw the vial and embed the cells directly in agarose (i.e. washing steps
780 and re-suspension in new medium are not necessary).
781
782 Δ **CRITICAL** It is essential to prepare a large batch of substrate cells (several million, depending on the
783 number of experiments planned and the format used – see below) and they should be stored frozen
784 in aliquots (-80°C), so that all extract samples in an experiment or a trial will be analysed on an identical
785 substrate from the same batch. The doses of DNA-damaging agents given below are suggestions; they
786 should be tested in dose-response experiments to confirm that the level of DNA damage is optimal for
787 the assay (also see the Checkpoint 1).
788 ■ **PAUSE POINT** Step 1 can be performed at any time before starting the experiment. The prepared
789 substrate cells can be stored in aliquots at −80°C for at least 6 months.
790
791 **Check point 1: Checking of substrate cells – day 0, ● Timing ~8 h (if scoring is**
792 **performed on the same day)**
793 11) Nucleoids from Ro 19-8022 and KBrO3 exposed cells can be checked via incubation with Fpg or
794 hOGG1 enzymes.
795 12) Nucleoids from UVC-exposed cells are checked via incubation with T4 endo V enzyme.
796 13) Nucleoids from BPDE exposed cells can be checked with an extract of DNA repair proficient cell
797 lines – there are currently no enzymes available that can be used to detect bulky DNA adducts.

798 Δ **CRITICAL** Ideally, the lesions in the substrate should be more than enough to saturate the assay,
799 i.e. they should result in maximum % tail DNA when incubated with the control enzyme. But on the
800 other hand, there should not be a substantial background of SBs seen without enzyme.
801 Δ **CRITICAL** Such control incubations (Steps 11-13) serve also as an *incubation reaction control* (Figure
802 2 – Step 4 and Figure 3), and are included in each experiment, to confirm that there really is an excess
803 of damage for the extract to work on.
804 14) A sample of non-exposed substrate cells incubated with the corresponding enzyme – e.g. Fpg or
805 T4endoV – will show the background level of endogenous lesions (normally insignificant in the
806 case of T4endoV; but various cell types contain variable levels of oxidised bases) (Figure 2 – Step
807 4 and Figure 3).
808
809 **? TROUBLESHOOTING**
810 ■ **PAUSE POINT** Step 11-14 can be performed at any time during the storage of substrate cells, but
811 before starting the analysis of the extracts.
812

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

815 15) Collect biological material
816 *(A) Peripheral blood mononuclear cells (PBMC)*: Approximately 5-10 mL of blood is needed for
817 preparing extract. Isolate PBMC from whole blood according to standard procedure.
818 Approximately $1x10^6$ PBMC are isolated per 1 mL of whole blood.
819 *(B) Cultured cells*
820 (i) At least $5\text{-}10x10^6$ cells are required.
821 (ii) Cells grown in suspension should be in log phase of growth.
822 (iii) Cells grown in monolayer in dishes or flasks should be collected by trypsinisation at sub-
823 confluence. Centrifuge at 150-300xg for 5 min.
824 *(C) Solid tissues* (of animal or human origin) should be snap-frozen in liquid nitrogen as soon as
825 possible after sampling. They can be further stored at -80°C.

826 16) Freeze and store biological material for extract preparation
827 *(A) Freezing cells in medium*
828 (i) Isolated cells can be stored in suspension in freezing medium.
829 (ii) Wash cells with PBS and count a sample. Centrifuge at 150-300xg for 5 min at 4°C.

830 (iii) Suspend cells in cold freezing medium at $5x10^6$ cells/mL and prepare 1 mL aliquots in 1.5
831 mL microtubes.
832 (iv) Freeze slowly to -80°C using a Mr Frosty® freezing container, or in a thick-walled box of
833 expanded polystyrene.
834 *(B) Freezing cell pellets*
835 (i) Isolated cells can be snap-frozen as pellets.
836 (ii) Suspend in PBS and count a sample. Centrifuge at 150-300xg for 5 min at 4°C.
837 (iii) Suspend cells in cold PBS at $5x10^6$ cells/mL and prepare 1 mL aliquots in 1.5 mL microtubes.
838 (iv) Centrifuge at ~2,000xg for 5 min at 4°C.
839 (v) Carefully remove as much supernatant as possible, without disturbing the pellet.
840 (vi) Drop the tubes into liquid nitrogen. Store at -80°C. Alternatively the tubes can be placed
841 directly at -80°C.
842 *(C) Grinding of solid tissues*
843 (i) At a later point, using a pestle and mortar pre-chilled in liquid nitrogen, grind the frozen
844 tissue under liquid nitrogen, and divide into aliquots of about 30-50 mg using a chilled
845 spatula.
846 **! CAUTION** Wear protective glasses and gloves when grinding tissues under liquid nitrogen.
847 (ii) Quickly weigh the frozen aliquots. Take care not to thaw them!
848 (iii) Store ground tissue aliquots at -80°C.
849 **Δ CRITICAL** Don't add any storage solution to the tissue for freezing.
850 **Δ CRITICAL** If amounts of tissue are already <50 mg - for example, small rodent organs such as
851 hippocampus, and tissue biopsies - do not grind, since too much of the material will be lost in
852 the mortar. Half a hippocampus or a biopsy is typically around 5 mg, which is enough to run
853 two assays.
854
855 ■ **PAUSE POINT** Steps 15-16 can be performed at any time before starting the experiment. The
856 harvested biological material can be either stored at -80°C for several months or used immediately,
857 i.e. proceed to Step 17.
858
859 17) Protein extraction
860 **Δ CRITICAL** Keep samples on ice during the whole procedure of extract preparation
861 *(A) From frozen cells in freezing medium (either PBMC or cultured cells)*
862 (i) Thaw the frozen cells (either at 37°C or at room temperature), and as soon as the ice has
863 melted, centrifuge at 150-300xg for 5 min at 4°C.

864 (ii) Resuspend the pellet in cold PBS and spin again.
865 (iii) Resuspend once more in cold PBS and centrifuge at ~2,000xg for 5 min at 4°C.
866 (iv) Discard supernatant by tipping out, and carefully remove the last microlitres with a pipettor
867 without disturbing the pellet. The pellet should be almost dry.
868 (v) Proceed to the next step.

869 *(B) From fresh or frozen cell pellets*
870 (i) Add 50 µl of buffer A to each pellet of $5x10^6$ cells.
871 (ii) Vortex vigorously, snap-freeze by dropping into liquid nitrogen and immediately thaw
872 again.
873 (iii) To each 50 µL aliquot, add 15 µL of buffer A/1% Triton® X-100.
874 (iv) Vortex for 5 sec and leave for 10 min on ice.
875 (v) Centrifuge at ~15,000xg for 5 min at 4°C to remove cell debris.
876 (vi) Collect the supernatant in a new microtube.
877 (vii) Store at -80°C or proceed to Step 4.

878 *(C)* From snap frozen/frozen ground tissues
879 (i) Thaw tissue aliquots.
880 (ii) Add 100 µL of buffer A per 30 mg.
881 (iii) Vortex vigorously, snap-freeze by dropping into liquid nitrogen and immediately thaw
882 again.
883 (iv) To each 100 µL aliquot, add 30 µL of buffer A/1% Triton® X-100.
884 (v) Vortex vigorously and incubate for 10 min on ice.
885 (vi) If necessary, larger particles of tissue can be homogenized with a microtube pestle.
886 (vii) Centrifuge at ~15,000xg for 5 min at 4°C to remove cell debris.
887 (viii) Collect the supernatant in a new microtube.
888 (ix) Store at -80°C or proceed to Step 4.
889

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

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

896 Δ **CRITICAL** In case there is a large number of samples in one experiment/trial, from which the extracts
897 are not possible to prepare in one day, it is essential to store all of them until later use. Tip: In case the
898 protein concentration will be quantified later (step 18), store a small aliquot of the extract separately
899 to avoid extra freeze-thaw cycles of the main extract.
900
901 **? TROUBLESHOOTING**
902

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

905 18) Quantify protein concentration
906 Although we advise the Lowry-based BIO-RAD DC Protein Assay Kit, using BSA as a standard, other
907 assays might be as suitable to quantify the protein concentrations.
908 Δ **CRITICAL** When selecting a protein quantification assay, choose one that: i) controls for the
909 presence of Triton® X-100, DTT, and EDTA (detergents) in the buffer; and ii) which allows you to
910 measure the protein concentrations at 650–750 nm to avoid interference of haemoglobin and
911 bilirubin.
912 Δ **CRITICAL** When storing the extracts for later use, it is advisable to measure protein
913 concentration after the extract preparation, but to store it undiluted. Dilution with buffer should
914 be performed just before the reaction.
915
916 ■ **PAUSE POINT** Step 18 can be performed at any time before the evaluation of the DNA repair
917 activity of the extracts.
918

919 Δ **CRITICAL** Protein extracts can be kept on ice for immediate use or stored as smaller aliquots (to
920 reduce freezing/thawing cycles) at -80°C for later use in the incubation reaction. Extracts can be stored
921 at -80°C. Extracts have been stored for 1.5 months at -80°C without losing enzyme activity[12,18]. Longer
922 periods may also be appropriate but need to be tested.
923 Δ **CRITICAL** Tissue extracts with a protein concentration of ~20-30 mg/ml can be obtained from ~50
924 mg of tissue, which is sufficient material for several assays (approximately 20 assays when running
925 samples in duplicate in a 2 gels/slide format).
926
927

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

929 ***Prepare materials***

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

937 ***Embedding cells in agarose and lysis***

938 24) Thaw an aliquot of frozen substrate cells, both non-exposed and exposed.
939 25) As soon as the aliquot is thawed, add 1 mL of cold PBS to the 1.5mL microtube and spin at 150-
940 300xg for 5 min at 4°C to wash cells.
941 26) Suspend pellets in cold PBS and spin again.
942 27) Remove the supernatant, disperse the pelleted cells by tapping vigorously, and add required
943 volume of 0.7% LMP agarose (dissolved in PBS) at 37°C to reach the concentration of $2x10^5$
944 cells/mL. For an aliquot of $3x10^5$ cells, simply add 1.5 mL of agarose. Mix by pipetting gently up
945 and down once. *Note*: Alternatively, take 45 µL of cell suspension and mix it with 105 µL of 1%
946 low melting point agarose at 37°C (see video https://youtu.be/T42JOvD2MnE). This option is often
947 applied when working with a large number of samples, so that cells can be kept on ice until use.
948 28) From each LMP-cell suspension (containing non-exposed or exposed cells), transfer two 70 µL
949 drops to each pre-coated microscope slide (the final number of cells per gel is ~14,000).
950 29) Cover gels with 20x20mm coverslips and keep for 5-10 min at 4°C.
951 30) Remove coverslips and place slides for 1 h in lysis solution in a Coplin jar at 4°C.
952 Δ **CRITICAL** It is also critical to be quick, so the gels do not set before putting the coverslip.

953

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

956 **? TROUBLESHOOTING**

957

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

962

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

964 ***Prepare materials***

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

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

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

970 ***Detection of DNA incision activity of the extract***

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

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

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

977 *(A) To study BER*

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

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

980 *(B) To study NER*

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

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

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

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

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

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

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

995

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

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

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

999

1000 **Step 5: Comet formation – day 1, ● Timing ~3 h (including washing steps)**

1001 ***Alkaline treatment & Electrophoresis***

1002 40) After the incubation of extract with substrate nucleoids, place slides immediately on ice to stop
1003 the enzyme reactions.

1004 41) Remove the coverslips and keep on ice until alkaline treatment. *Note:* Alternatively, microscope
1005 slides can be transferred directly to the electrophoresis tank.

1006 42) Incubate in cold electrophoresis solution for 40 min at 4°C.

1007 43) Electrophoresis at ~1V/cm for 30 min at 4°C. *Note:* Alternatively use the EPT that you estimated.

1008 Δ **CRITICAL** Voltage gradient should be measured across the platform carrying the slides. The time
1009 of electrophoresis should be tested before starting an experiment or a trial. Please see equipment
1010 setup.

1011 ***Neutralization & Washing***

1012 44) Neutralise gels by washing slides for 10 min in the neutralising solution (ice-cold PBS) and 10 min
1013 in ice-cold dH_2O at 4°C (use Coplin jar, or lay slides flat in a dish).
1014 *Note:* use EtOH for 12 gels/slide format (see the Box 2).

1015 45) Allow gels to dry overnight.

1016 ■ **PAUSE POINT** The slides can be stored for a long time and stained and scored at any time point.

1017

1018 **Step 6: Comet visualization & Analysis – day 2, ● Timing ~2 h – several days**
1019 **(depending on the number of samples)**

1020 ***Comet visualization***

1021 46) Slides can be stained with various dyes (see the section Reagents: Reagents for comet
1022 visualization).

1023 47) For staining with ethidium bromide (0.01 µg/ mL in water), or DAPI (1µg/ mL in water) - add 20-
1024 40 µL of staining solution to each gel and cover with a coverslip.

1025 48) For staining with SYBR® Gold or SYBR® Green, which give intense fluorescence, it is
1026 recommended to immerse slides in a bath of the dye at a dilution of 1:10,000 in TE buffer for 20
1027 min, followed by two 10 min washes with dH_2O. Slides are left to dry, and for viewing, 20 µL of
1028 dH_2O is added to each gel and covered with a cover slip. Alternatively, SYBR® Gold can also be
1029 added as 50 µL of the 1:10,000 dilution on top of each gel and cover with a coverslip.
1030 **! CAUTION** All dyes may be mutagenic or even carcinogenic.
1031 49) Subsequently, comets are visualized with a fluorescence microscope.

1032 ***Comet analysis***

1033 50) Computer-assisted image analysis, using commercially available software, gives the most
1034 reproducible results. We recommend the use of tail intensity (TI), representing % of DNA in the
1035 tail of the comet, as the parameter to describe the comets.
1036 51) Score at least 50 comets per gel, i.e. 100 comets per sample when working in duplicates.
1037 52) To obtain the TI value per sample or control, calculate first the median TI for each gel over the
1038 scored comets (i.e. the 50 comets in each gel) and then the mean TI over the replicate gels.
1039 △ **CRITICAL** In an experiment or a trial, all comets should be scored by the same person to minimize
1040 inter-operator variation using the same software all the experiment/trial.
1041
1042 ■ **PAUSE POINT** Slides can be stained and scored on the day of the experiment (day 1) or stored
1043 (un)stained.

1044

1045 ## BOX 2 - 12 GELS/SLIDE FORMAT

1046 **12 mini-gels/slide format:** 12 mini-gels per slide (in 2 rows of 6 gels each), instead of two large gels,
1047 containing the substrate cells are placed in a microscope slide (see Figure 7). Each of the gels contains
1048 about 200-250 cells, from which 100 comets will be analysed (if not using an automated image analysis
1049 system).

1050 **12-Gel Comet Assay Unit:** Each mini-gel can be incubated separately, with different extracts or control
1051 solutions, by isolating them using a silicon gasket, with the corresponding 12 holes, on top of the slide.
1052 A metal base with a guide on it is used to define the right position of the gels. The metal base, the slide
1053 containing the mini-gels, the silicon gasket and a top plate (with the corresponding 12 holes on it) are
1054 clamped together with metal clamps to isolate each mini-gel in a well and to avoid leakage between
1055 them (see Figure 7). A silicone seal/empty microscope slide on top is used to prevent evaporation of
1056 the solutions added to the wells.

1057 Specification for the use of the 12 mini-gels/slide and 12-Gel Comet Assay Unit:

1058 **PROCEDURE:**

1059 ***Step 1: Preparation of substrate cells***

1060 - After treatment of substrate cells, suspend them at $2.5x10^3$ cells in 0.5 mL in cold freezing medium
1061 (This is enough for 20 slides containing 12 mini-gels each) and freeze them.

1062 ***Step 3: Embedding substrate cells in LMP agarose gel on microscope slides & cell lysis***

1063 ***Embedding cells in agarose and lysis***

1064 - After thawing and washing the cells, remove the supernatant and add 0.7% low melting point agarose
1065 to a final concentration of $0.5x10^5$ cells/mL. Place an agarose-precoated slide in the ice-cold metal base
1066 and, using a multi-dispensing pipettor, place 12 drops of 5 µL of cell suspension (containing 250 cells
1067 approximately) following the guide (2 rows of 6 gels each). Keep for 2-3 min at 4°C.

1068 *Note:* No coverslip is used; the mini-gels have domed shape.

1069 *Note:* The two rows can contain exposed or non-exposed substrate cells, or alternatively, one row of
1070 exposed and one row of non-exposed substrate cells.

1071 ***Step 4: Incubation reaction - Detection of DNA incision activity of the extract***

1072 -Place slide/s in the 12-gel comet assay unit/s, on ice. Add 30 µL of extracts or control solutions in the
1073 corresponding wells. Cover the chamber/ with the silicone seal/ empty microscope slide and place
1074 it/them in an incubator at 37°C for the required time. The incubation time is normally around 10-30
1075 min. After then, put the chamber/s on ice, remove the slide/s containing the gel from it/them and
1076 immediately transfer it/them to cold electrophoresis solution (on the electrophoresis tank or in Coplin
1077 jars).

1078 *Note:* results from enzyme titration are different when using 2 gels/slide or 12 mini-gel/slide plus the
1079 12-Gel Comet Assay Unit[79]. Perform the titration experiments using the gel format and the equipment
1080 that is going to be used during the analysis of the samples.

1081 *Note:* To stop the reaction, and even more important, to avoid cross-contamination, slides should be
1082 quickly removed from the 12-Gel Comet Assay Unit and rinsed in the electrophoresis solution before
1083 transferring slides to the electrophoresis tank (for the alkaline treatment & electrophoresis step).

1084 ***Step 5: Comet formation (including washing steps)***

1085 ***Neutralization & Washing***

1086 - After neutralization and washing, mini-gels should be dehydrated for 15 min in 70% ethanol followed
1087 by 15 min in absolute ethanol.

1088 **? TROUBLESHOOTING**

1089

1090

1091

1092 **BOX 3 – APPLICATION OF THE COMET-BASED *IN VITRO* DNA REPAIR ASSAY ON**

1093 ***DROSOPHILA MELANOGASTER***

1094 Specification for the use of *Drosophila melanogaster*[81]:

1095 **Step 2: Extract preparation and Step 4: Incubation reaction**

1096 - Place about 150 adult flies (between 7 and 12 days after eclosion/hatching), previously
1097 anesthetized, in a cold mortar on ice.

1098 *Note:* the use of a mix of males and females avoids any effect of sex.

1099 - Add 500 µL of buffer A adjusted at pH 8 and smash the flies with a pestle keeping the mortar on ice.

1100 - Split the obtained solution in 10 aliquots of 50 µL in cryotubes (discard the solid part) and place
1101 them in liquid nitrogen immediately. Store the aliquots at -80 ºC until the day of the analysis.

1102 On the day of the analysis:

1103 - Place on ice an enough number of aliquots (extract obtained from one aliquot is enough for the
1104 treatment of 8 big gels, 4 slides) for thawing.

1105 - Add 12 µL of 1% Triton® X-100 per aliquot, vortex for 5 s, and place on ice for 5 min.

1106 - Centrifuge at about 15,000 x g for 10 min to separate cell debris.

1107 - Remove 50 µL supernatant and add it to 200 µL of cold Buffer B (for BER) of Buffer N (for NER).
1108 Keep the tubes on ice.

1109 *Note:* The protein concentration should be around 2.5 µg/µL.

1110 - Add 30 µL of extracts or control solutions on top of each gel containing the substrate cells, cover
1111 them with coverslips (22x22 mm), place the slides on a humidity chamber and incubate them at 24°C
1112 for 30 min.

1113

1114

1115

1116 **BOX 4: LIST OF CELL TYPES OR CELL LINES PREVIOUSLY USED AS SUBSTRATE**
1117 **CELLS BY THE AUTHORS**

1118 *Note:* Any cell type can be used, but it is advisable to use cells in suspension to avoid trypsinization
1119 and centrifugation steps.

1120 • **WBC** fractions can be used, but keep in mind to isolate enough cells to be able to create a large
1121 enough batch of substrate cells for the whole series of experiments. **PBMC** and **lymphocytes** have
1122 both been used successfully to assess BER activity, using Ro 19-8022 + light to induce oxidized DNA
1123 bases[19,42,66,82].

1124

1125 ***Cell cultures growing in suspension:***

1126 • **THP-1** cells have been exposed to $KBrO_3$ to induce DNA oxidation damage[9-11,83] and UVC to assess
1127 NER (unpublished data).

1128 • **TK6** cells have been exposed to UVC to assess NER activity, treated with MMS to assess repair of
1129 DNA alkylation lesions, and can also be treated with Ro19-8022 + light[19,84].

1130

1131 ***Attaching cells:***

1132 • **HeLa** cells: (i) treated with photosensitizer Ro 19-8022 + light, were used in several studies to
1133 assess BER activity; and (ii) exposed to UVC to study NER activity[7,13,18,23,34-36,39,40,64,69,85-89]

1134 • **A549** cells were successfully used as substrate cells using BPDE[12,90], and UVC (unpublished data) as
1135 DNA damage inducing agents.

1136 • **HepG2** cells have been used as substrate cells upon UVC-exposure and treated with Ro19-8022 +
1137 light[91].

1138 • **HCT116** cells were treated by photosensitizer Ro 19-8022 + visible light to assess BER[46].

1139 • **Caco-2** were exposed to paraquat to assess the repair of DNA oxidation lesions[92].

1140

1141

1142

1143

1144 **BOX 5: DETECTION OF DNA SYNTHESIS & LIGATION ACTIVITY OF THE EXTRACT**

1145 **Optional step in parallel to Step 4:**

1146 As explained in the introduction, assessing the DNA repair incision activity is already sufficient to study
1147 the effect of external and internal factors on an organism's DNA repair activity. For instance, a
1148 significant correlation was observed between BPDE–DNA adduct removal as studied by ^{32}P-post-
1149 labelling (representing the whole DNA repair process from DNA incision to ligation) and the NER
1150 incision activity as measured by the comet-based *in vitro* DNA repair assay[12]. These data confirm that
1151 the DNA incision activity detected by the comet-based *in vitro* DNA repair assay is representative for
1152 an individual's DNA repair capacity.

1153 Still, if one desires to show that DNA synthesis & ligation can take place, a parallel incubation of extracts
1154 supplemented with dNTPs can be performed[6]. Note that in this case a parallel set of slides should be
1155 prepared.

1156 - First incubate this parallel set of slides with the same sample extract-reaction buffer mixture, as
1157 explained in step 35-39, for DNA incisions to occur and accumulate.

1158 - After the first incubation (of 30 min – or as optimized), rinse the slides quickly in reaction buffer (B or
1159 N, depending on the type of assay).

1160 - Add dATP, dCTP, dGTP and dTTP at 5 µM each and 2.5 mM ATP to the sample extract-reaction buffer
1161 mixtures, prepared as described in Step 36.

1162 - Put 50µL of this mixture on each gel and cover with coverslips (22 x 22 mm for each gel or 24 x 60
1163 mm to cover both gels).

1164 - Incubate at 37°C in the incubator + moist box / slide moat for another 30 min (or as optimized). So,
1165 the total incubation time will then be 60 min: 30 min incubation with extract mixtures without dNTPs
1166 + ATP, followed by 30 min incubation with extract mixture including dNTPs + ATP.

1167

1168 In case the detected incisions/SBs are back to background levels, this indicates that DNA synthesis &
1169 ligation occurred efficiently.

1170

1171 **TIMING**

1172 **Day 0:**

1173 Step 1: Preparation of substrate cells: 1-10, 2-6 h (depending on the DNA damaging agent)

1174 Checkpoint 1: Checking of substrate cells: 11-14, 8 h (if scoring is performed the same day)

1175 Step 2: Protein extract preparation: 15-17, 4 h - 1 d (depending on the number of samples)

1176 Checkpoint 2: Measure protein concentration: 18, 2 h – 1 d (depending on the number of samples)

1177

1178 **Day 1:**

1179 Step 3: Embedding substrate cells in LMP agarose gel & cell lysis: 19-30, 4 h

1180 Step 4: Incubation reaction: 31-39, 2 h

1181 Step 5: Comet formation: 40-45, 3 h

1182

1183 **Day 2:**

1184 Step 6: Comet visualization & Analysis: 46-52, 2 h - several days (depending on the number of
1185 samples)

1186

1187

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/m^2 was needed instead of 1 J/m^2).
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

33 extract is negative, but all the internal experimental controls give the expected results and the
34 optimization of protein extract and time of incubation is correct, it can be assumed that the extract
35 does not have DNA repair incision activity or that it is below the limit of detection of this assay.
36

37 ***Example data***

38 **Figure 9** depicts example data of a successful (Figure 9A) and a suboptimal (Figure 9B) assay that
39 assessed the BER activity of piglet hippocampus samples, using substrate cells (HeLa) exposed to Ro
40 19-8022 + light or non-exposed substrate cells (PBS + light)[30]. The problem with the assay results shown
41 in Figure 9B involves the high values observed for the non-exposed substrate cells. Since the incubation
42 of exposed substrate cells with Fpg (incubation reaction control) showed expected results (% tail DNA
43 = ~60-70%), it is suggested that there was not a technical error but possibly an error in the handling of
44 the substrate cells or preparation of those slides.

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

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

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

66

67 ***How to express results?***

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

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

86 • First calculate the DNA repair incision activity, as indicated above.
87 • List the DNA repair incision activity for the pooled/reference sample (or the incubation reaction
88 control) as detected in all the assays within one set of experiments.
89 • Calculate the median value, M
90 • The detected DNA repair incision activity for the pooled/reference sample (or the incubation reaction
91 control) in a particular experiment X, defined as Q, will then serve in the correction factor - being M/Q.
92 • Multiply the values of DNA repair incision activity for each sample in experiment X by M/Q to
93 normalize all values.
94 • Example data and calculation can be consulted in the supplementary file 1.
95

96 The variations to be expected in the levels of detected DNA repair activity, and the effect of intrinsic
97 and external factors on DNA repair activity levels, have been described in a recent comprehensive
98 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].

111 **FIGURES:**

112

113 **Figure 1. Principle of the sample extract incubation reaction (for BER acting against DNA oxidation**
114 **damage as example).**
115 Abbreviations: 8-oxoG – 8-oxoguanine, AP site – apurinic/apyrimidinic site.
116

117 **Figure 2. Stepwise overview of the technique (using 2 gels/slide as example).**
118 Abbreviations: dNTPs – deoxyribonucleotide triphosphates, WBC – white blood cells.
119

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

123

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

128

129 **Figure 5. Example data illustrating how to select the optimal incubation time.** The red dotted line
130 indicates that for experiments where a mouse hippocampus tissue extract of 3mg/mL is incubated
131 with substrate cells (HeLa) exposed to 1µM Ro 19-8022 + light an incubation time of 25min will be the
132 optimal timing, allowing the detection of the DNA repair incision activity in the linear part of the
133 activity-with-time curve.
134

135 **Figure 6. Principle of optimization of the extract's protein concentration.** (A) A too low protein
136 concentrations will yield low background levels, but also low DNA repair specific incisions; (B) the
137 "optimal" protein concentration results in low background levels and high levels of DNA repair
138 incisions; (C) at high concentrations, non-specific nucleases may increase the background.
139

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

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

145

146 **Figure 8. Overview of slides containing internal experimental controls and sample extract used to**
147 **calculate the final DNA repair incision activity.** 1, 2, and 3 represent internal experimental controls
148 and 4 represents sample extract. The bars on the graph shows example data with SD for the internal
149 experimental controls (1, 2 and 3) and a piglet (*Sus scrofa domesticus*) hippocampus protein extract
150 (4)(3mg/mL). Data are shown as mean TI of two independent incubations (i.e., on 2 separate gels)
151 within one experiment.

152

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

159

160 **Figure 10. Examples of results obtained with a NER assay.** Results were produced using substrate cells
161 (TK6) exposed to 1 J/m² UV (grey bars) or non-exposed substrate cells (white bars) incubated with
162 various human protein extracts either (A) from PBMC, or (B) from saliva cells. Data are shown as mean
163 % tail DNA of two independent incubations with SD within one experiment.

164

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

171

172

173 **TABLES:**

174 **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

175

176 **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

177

178 **Table 3.** Incubation reaction controls that can be used for checking the amount of DNA damage in
179 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

180

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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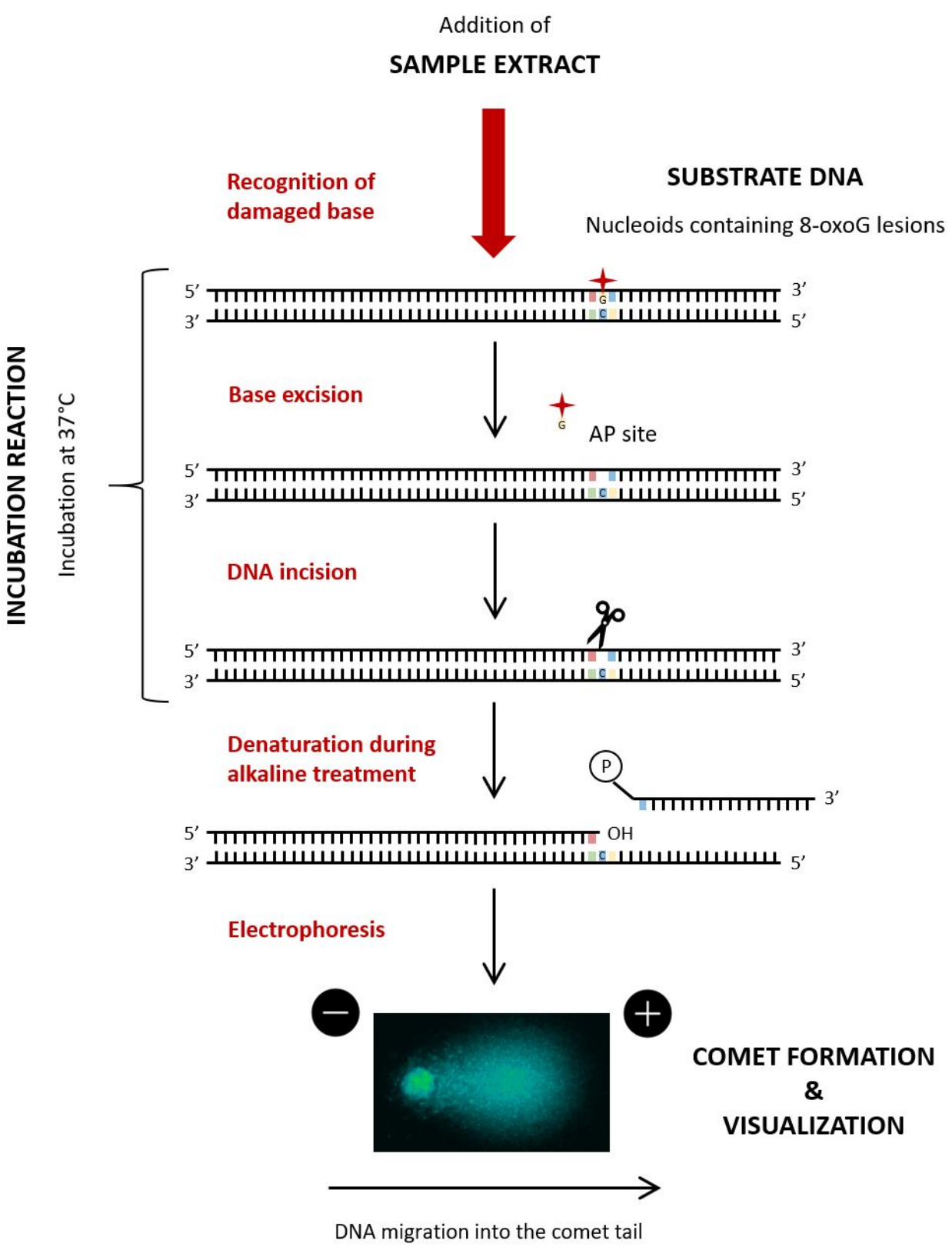
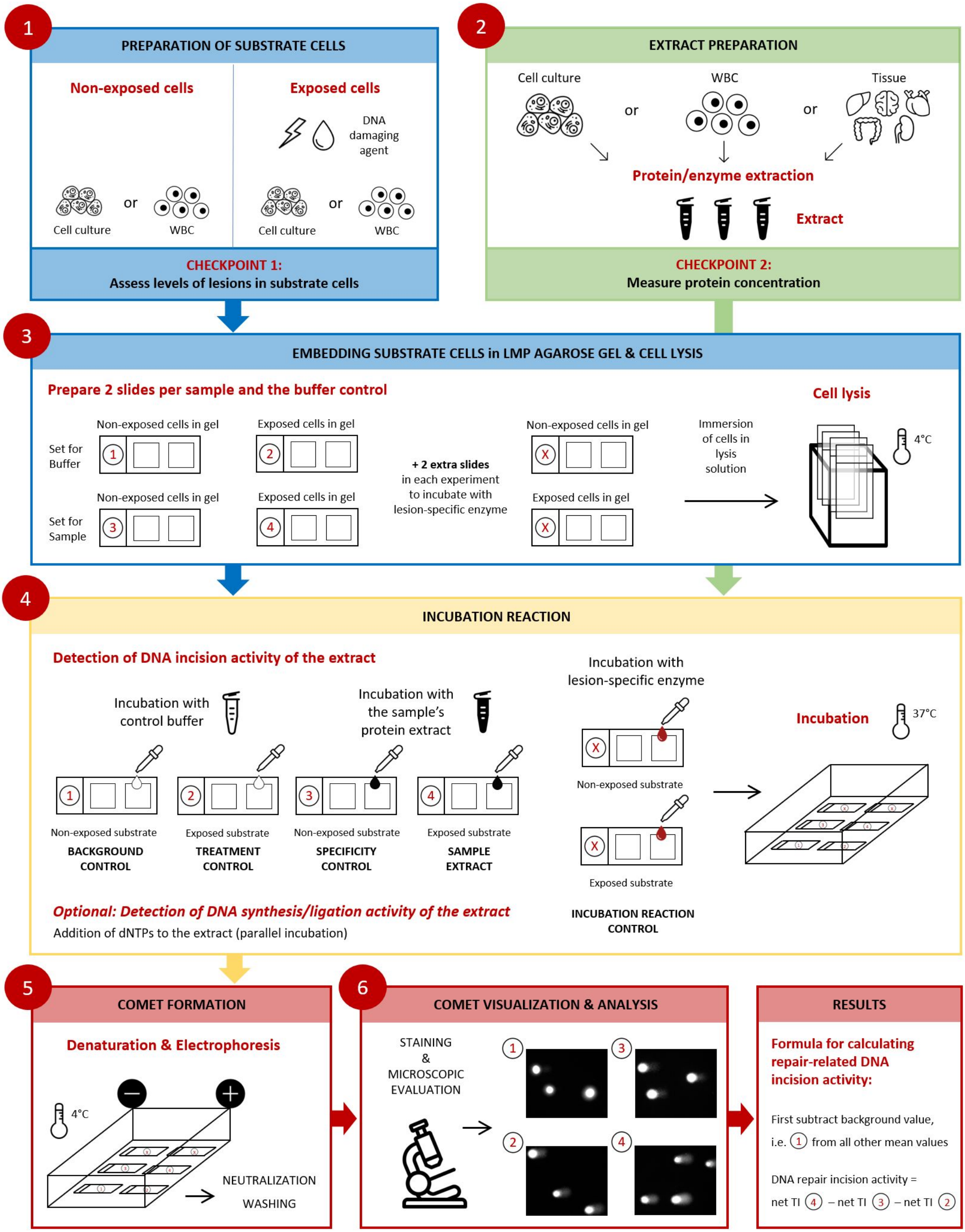
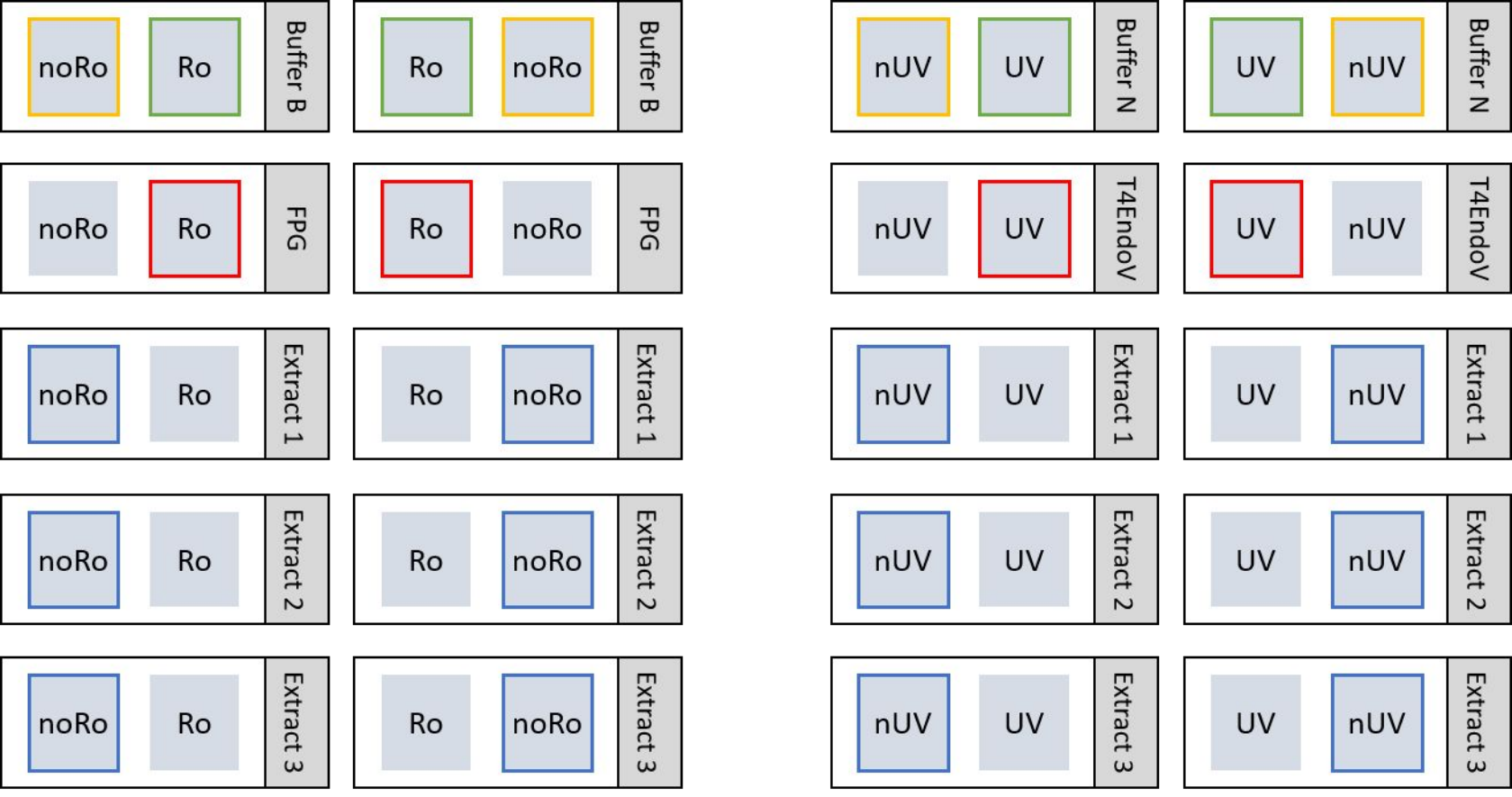
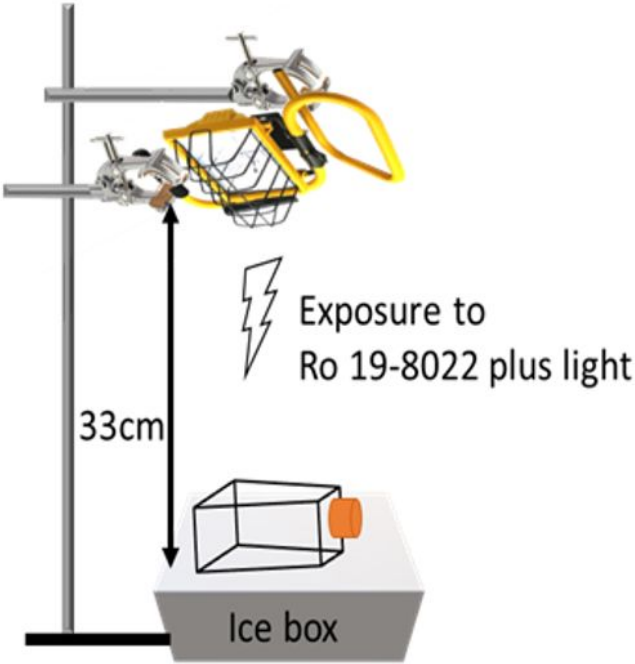
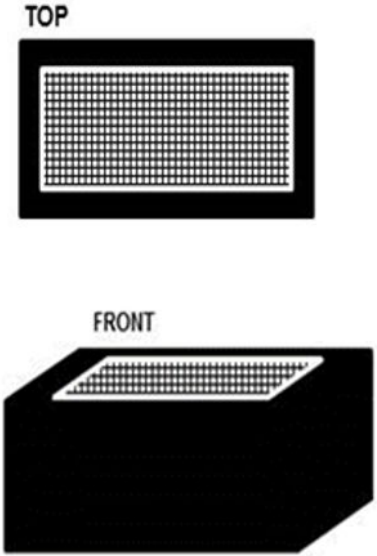
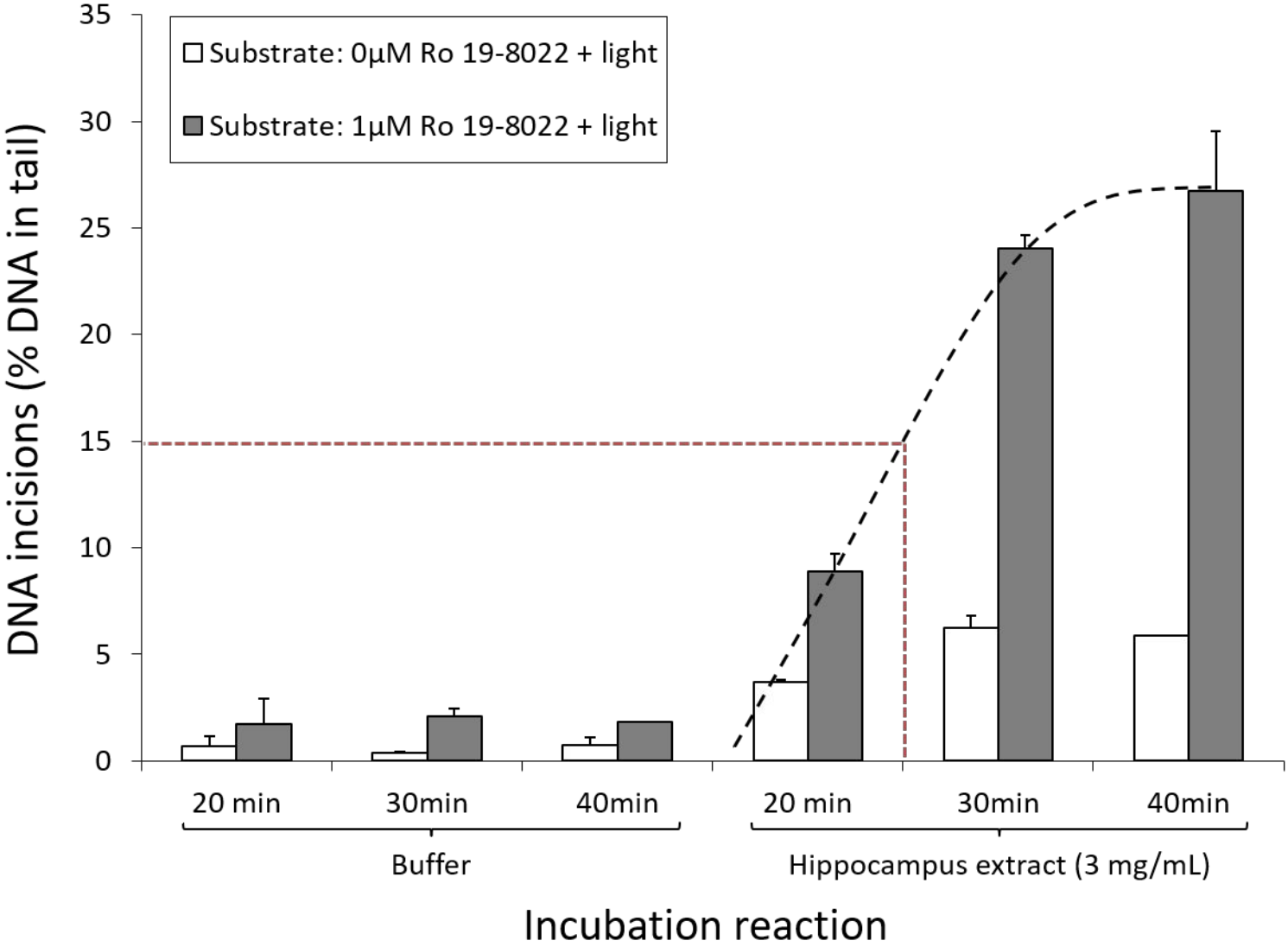

Addition of
SAMPLE EXTRACT
Recognition of damaged base
SUBSTRATE DNA
Nucleoids containing 8-oxoG lesions
INCUBATION REACTION
Incubation at 37°C
5'
3'
G
C
Base excision
AP site
DNA incision
Denaturation during alkaline treatment
P
OH
Electrophoresis
COMET FORMATION & VISUALIZATION
DNA migration into the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

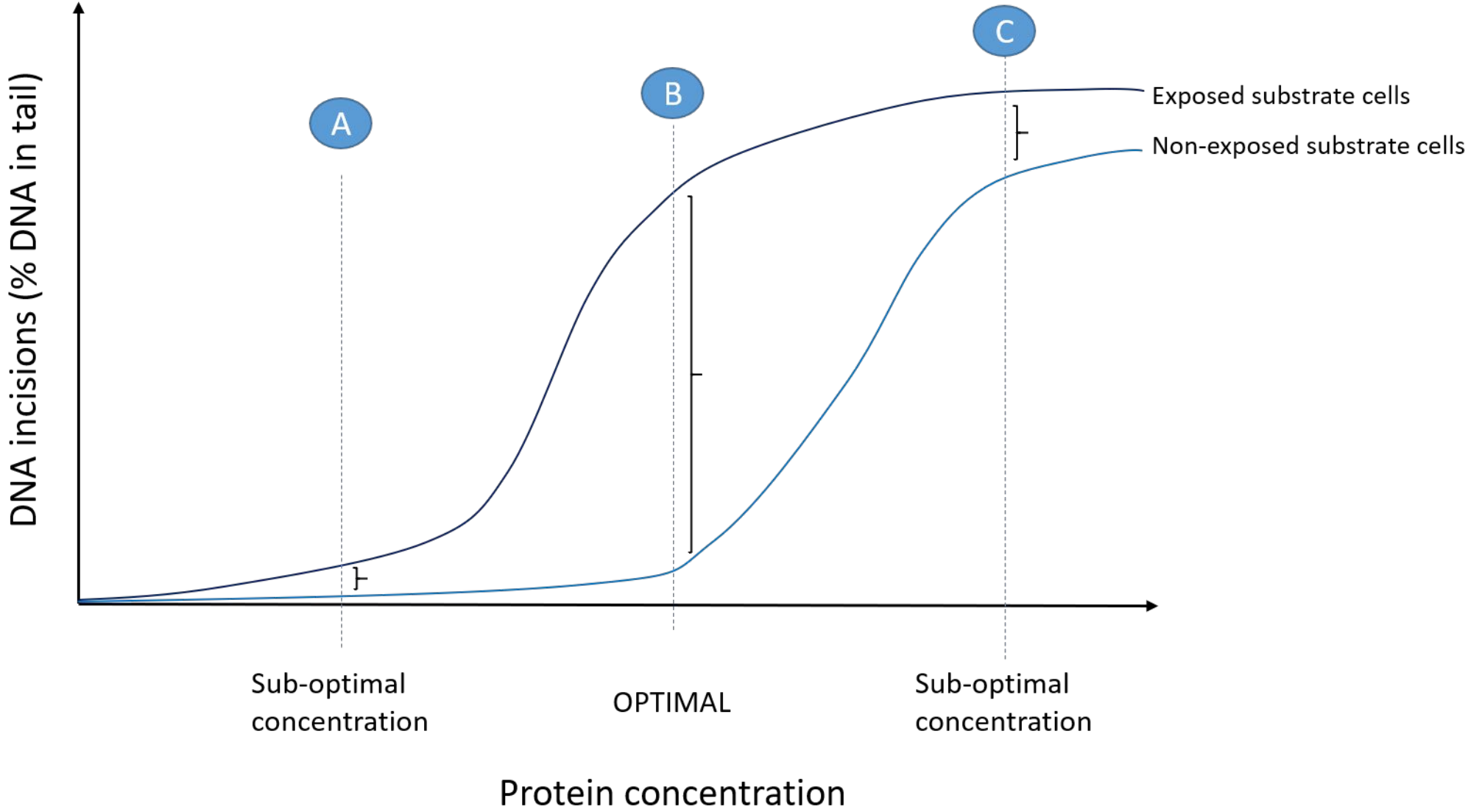

1
PREPARATION OF SUBSTRATE CELLS
Non-exposed cells
Cell culture
or
WBC
Exposed cells
DNA damaging agent
Cell culture
or
WBC
CHECKPOINT 1:
Assess levels of lesions in substrate cells
2
EXTRACT PREPARATION
Cell culture
or
WBC
or
Tissue
Protein/enzyme extraction
Extract
CHECKPOINT 2:
Measure protein concentration
3
EMBEDDING SUBSTRATE CELLS in LMP AGAROSE GEL & CELL LYSIS
Prepare 2 slides per sample and the buffer control
Non-exposed cells in gel
Exposed cells in gel
Set for Buffer
Non-exposed cells in gel
Exposed cells in gel
Set for Sample
+ 2 extra slides
in each experiment
to incubate with
lesion-specific enzyme
Non-exposed cells in gel
Exposed cells in gel
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
Non-exposed substrate
BACKGROUND CONTROL
Exposed substrate
TREATMENT CONTROL
Non-exposed substrate
SPECIFICITY CONTROL
Exposed substrate
SAMPLE EXTRACT
Incubation with lesion-specific enzyme
Non-exposed substrate
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
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

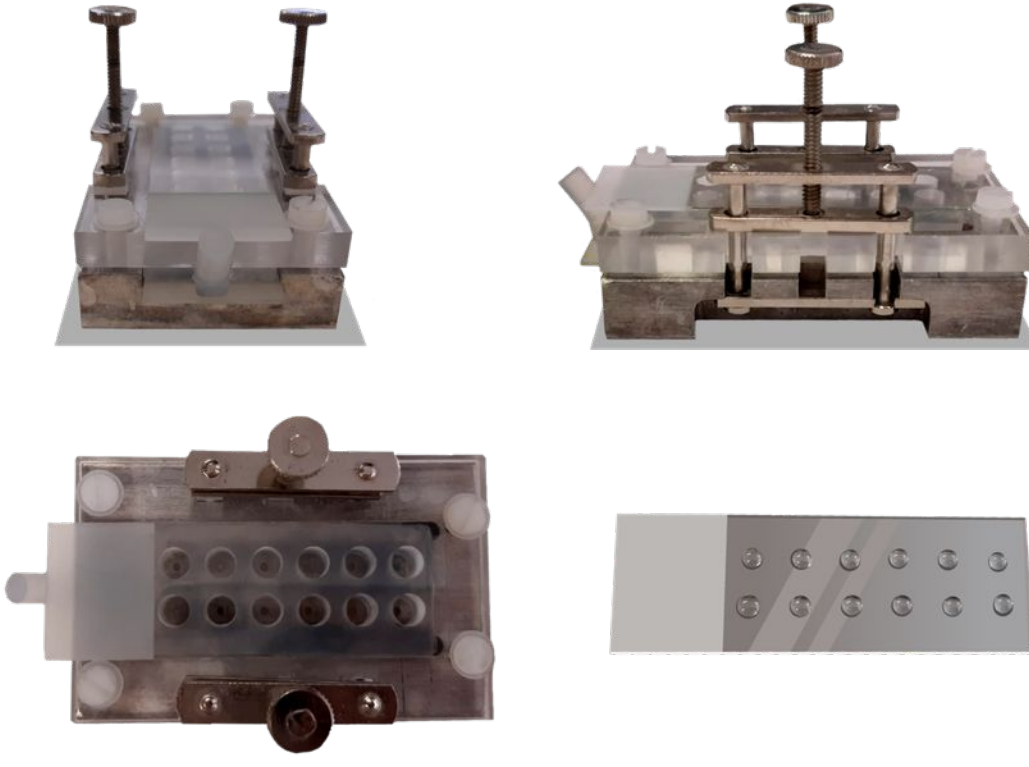

Exposure to
Ro 19-8022 plus light
33cm
Ice box


TOP
FRONT

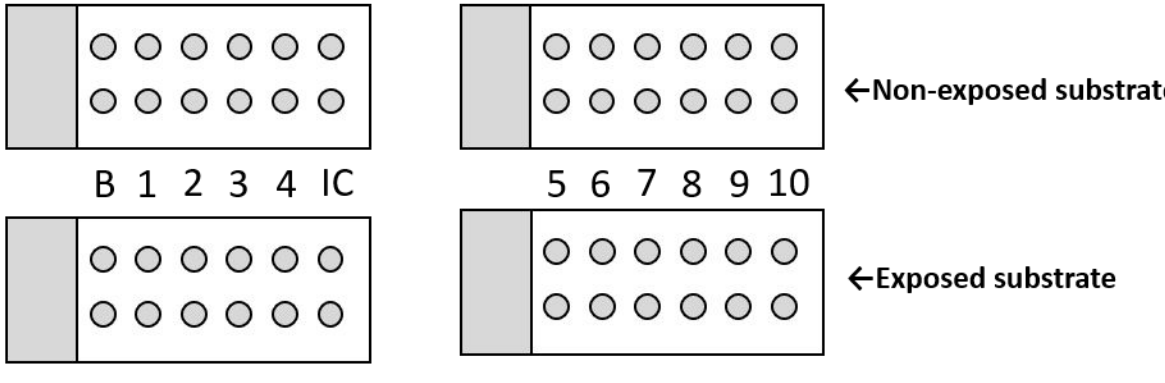

Substrate: 0µM Ro 19-8022 + light
Substrate: 1µM Ro 19-8022 + light
DNA incisions (% DNA in tail)
35
30
25
20
15
10
5
0
20 min
30min
40min
20 min
30min
40min
Buffer
Hippocampus extract (3 mg/mL)
Incubation reaction

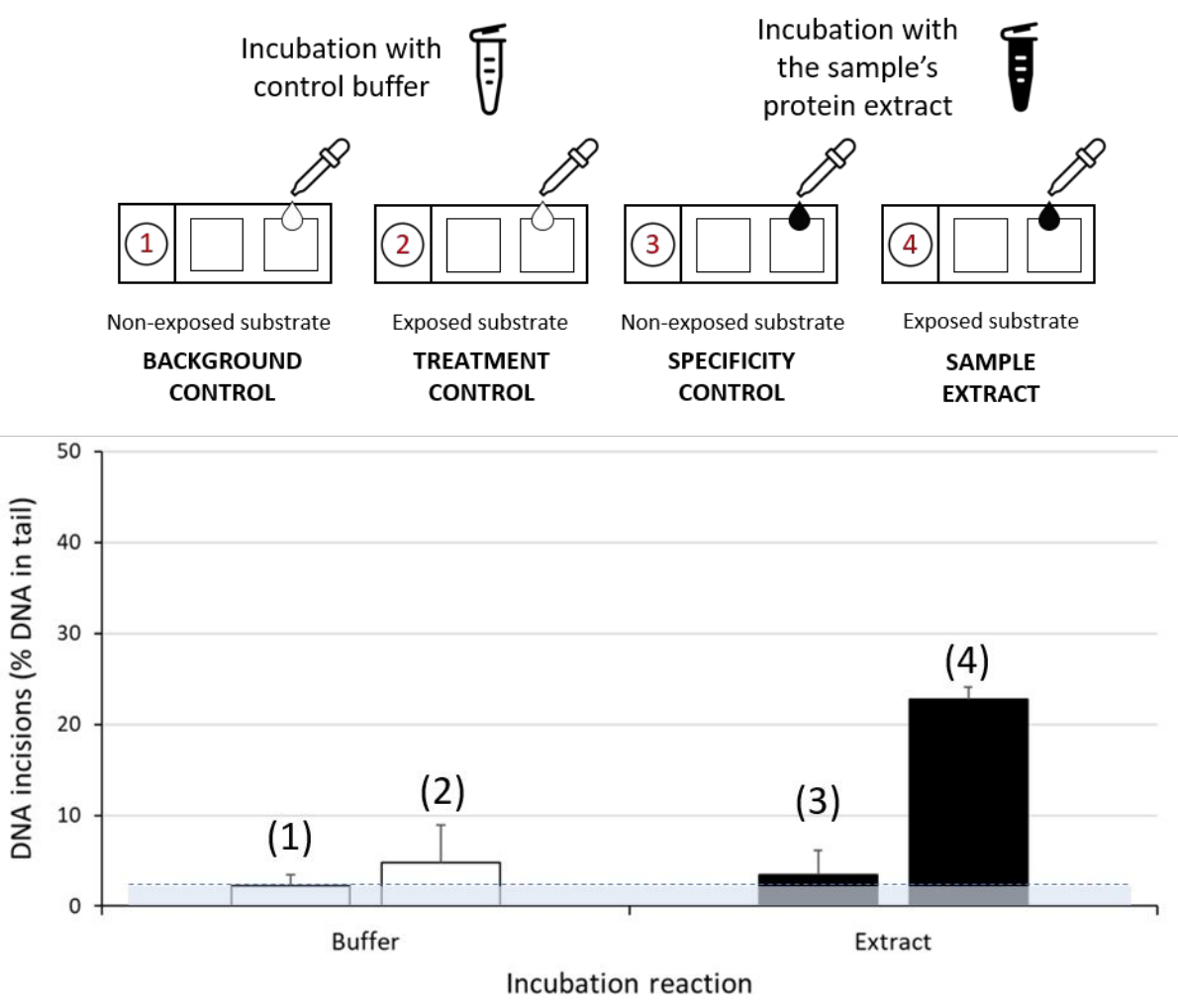

DNA incisions (% DNA in tail)
A
B
C
Exposed substrate cells
Non-exposed substrate cells
Sub-optimal concentration
OPTIMAL
Sub-optimal concentration
Protein concentration

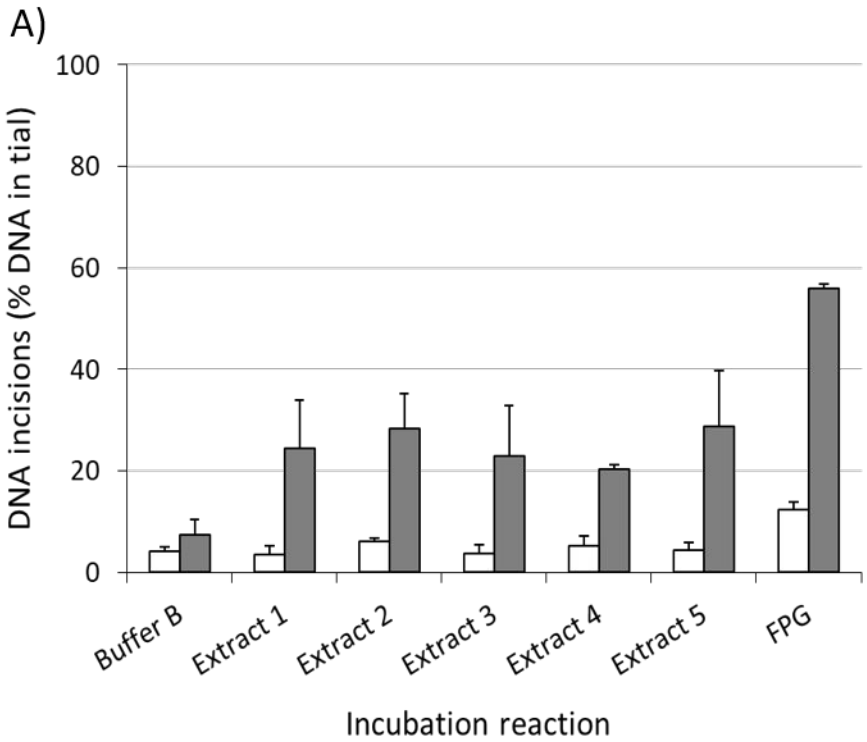
A)

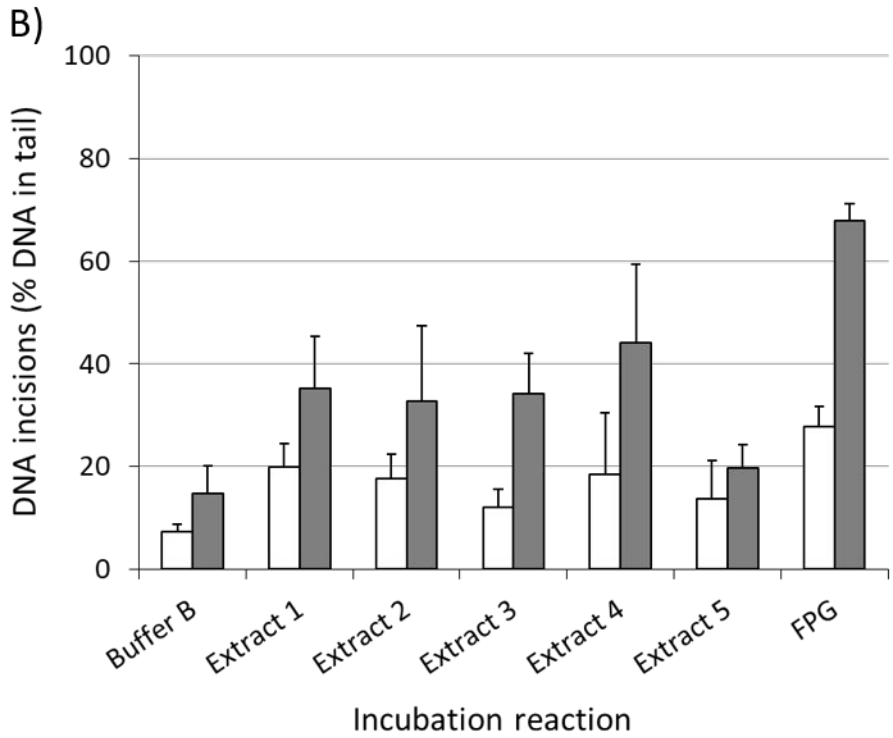


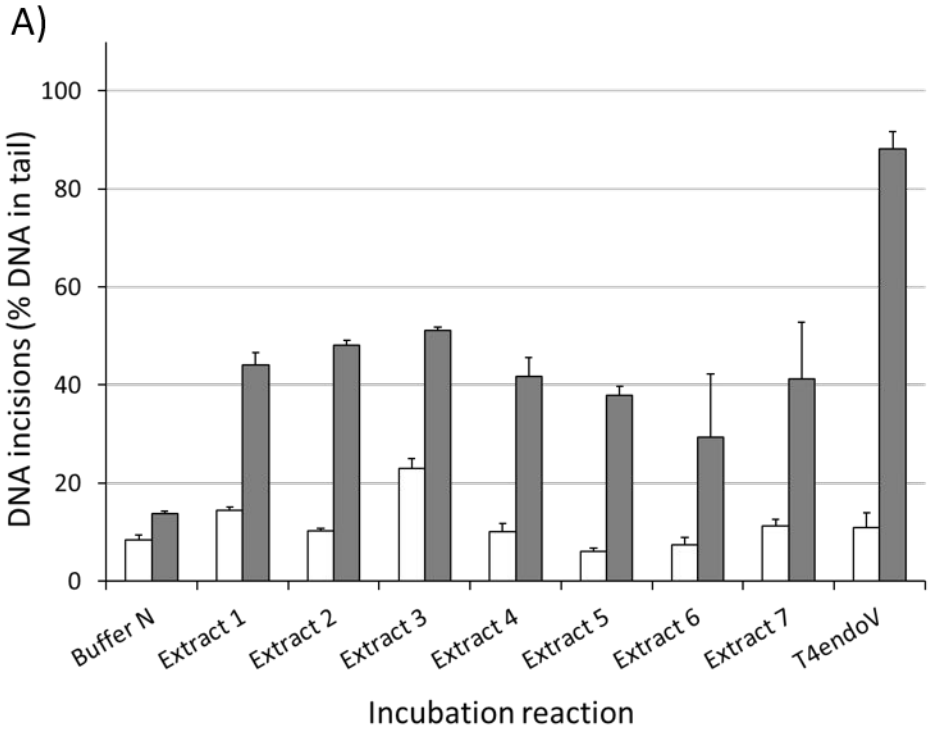
B)

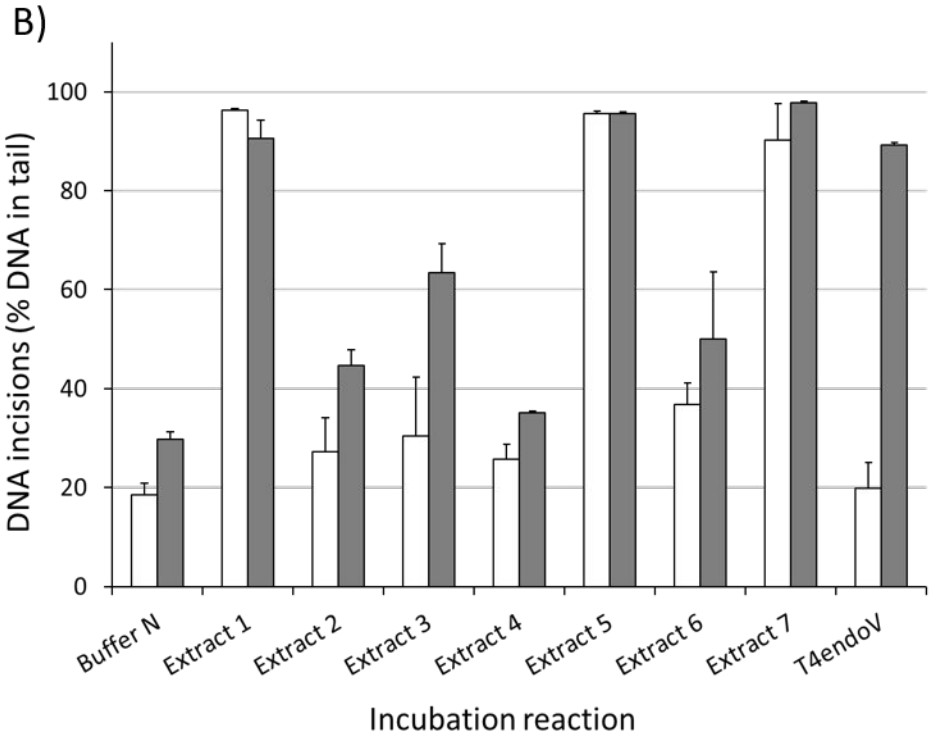


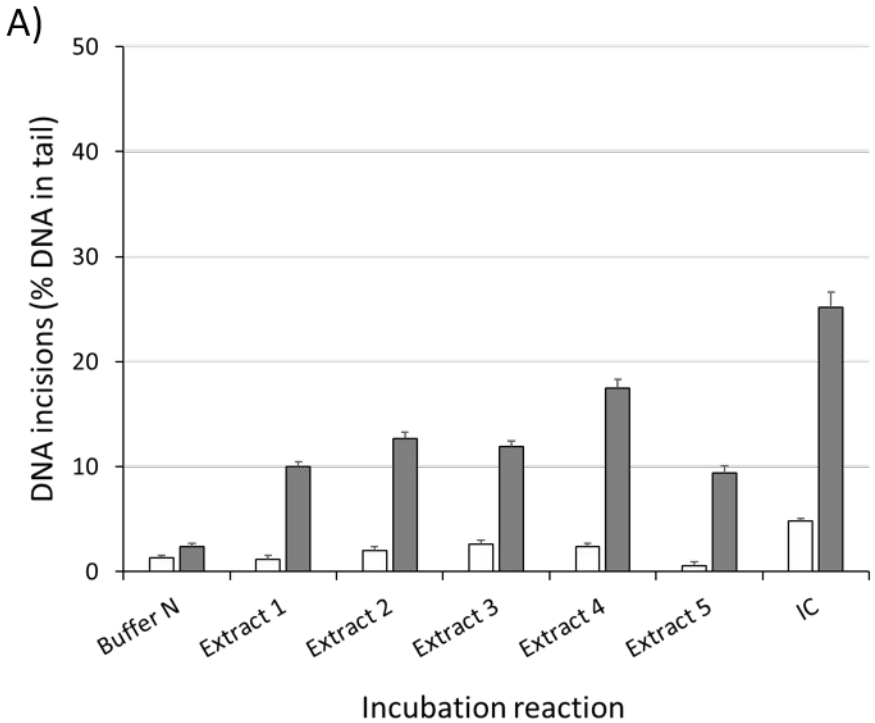

Incubation with control buffer
Incubation with the sample's protein extract
1
2
3
4
Non-exposed substrate
BACKGROUND CONTROL
Exposed substrate
TREATMENT CONTROL
Non-exposed substrate
SPECIFICITY CONTROL
Exposed substrate
SAMPLE EXTRACT
DNA incisions (% DNA in tail)
50
40
30
20
10
0
(1)
(2)
(3)
(4)
Buffer
Extract
Incubation reaction

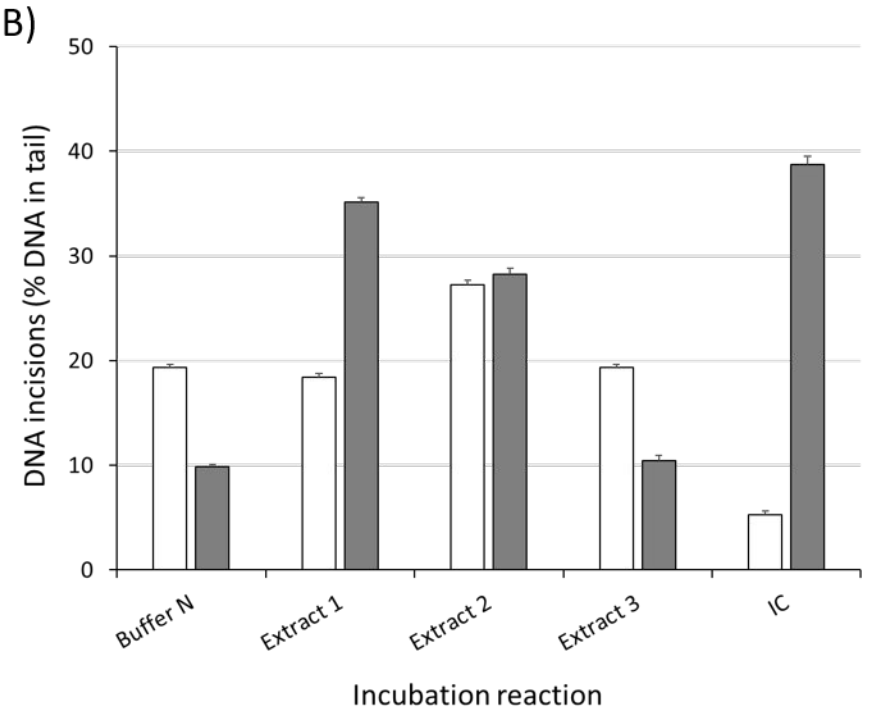

A)
DNA incisions (% DNA in tial)
100
80
60
40
20
0
Buffer B
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5
FPG
Incubation reaction


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