# Preface

When setting out to decide on the content of *DNA Repair Protocols: Prokaryotic Systems*, I was conscious of the need to portray the vast array of pathways and enzymatic activities that are part of the discipline of DNA repair. In addition to the classical DNA repair activities, I wanted to convey the significant interest that has been generated in recent years in the use of the proteins and repair systems as research tools, much like the use of restriction enzymes over the last few decades. Therefore, in addition to chapters detailing protocols for investigating specific repair activities, I have included several chapters in this book on the applied use of DNA repair proteins and systems.

The many years of research on bacterial DNA repair systems have allowed us to really understand the majority of DNA repair pathways in bacterial cells. Building on this knowledge, research has lead to major advances in understanding mammalian DNA repair and uncovered its links to human disease, such as DNA mismatch repair and colon cancer, nucleotide excision repair and xeroderma pigmentosum, DNA helicase function in Bloom's syndrome, and so on. Such have been the advances that *Science* magazine identified the collective DNA repair systems as its "Molecule of the Year" in 1994. Because interest in DNA repair continues today, our hope is that new researchers in the field will find this book useful to bring themselves up to date on the basis of cellular DNA repair activities, be their interest in prokaryotic or eukaryotic systems. Also included here is the investigation of DNA repair in thermophilic bacteria. It is interesting to note that in these cells, repair protein needs to sustain activity at high temperatures in an environment where DNA is inherently more susceptible to damage. Therefore it is clear that more researchers will be attracted to the investigation of the classical DNA repair activities in these organisms, both because of the novel nature of the molecular environment and of the possible biotechnological use of these thermostable enzymes.

As stated earlier, I have included many protocols detailing the study of various repair activities found in cells, and contrasted this with a number of chapters where researchers have taken these same DNA repair enzymes and systems and used them as tools or reagents in various techniques. Several chapters deal with the use of repair proteins in mutation detection. Mutation and polymorphism detection is a growing field of research. It is central to the

frantic race to uncover the multitude of genetic variations impacting on human and animal health, both in the search for disease genes, drug response and drug metabolism genes, and to breeding and trait selection in animals and plants. The use of DNA repair proteins as biotechnological tools is invaluable in this effort owing to the specificity of these proteins and our in-depth understanding of their action.

I thank all the authors of this volume for their diligence and cooperation in putting together this book and wish them continued success in their research. I hope you the reader find our collection interesting, informative, and practical.

***Pat Vaughan***

# 2

# Assays for the Repair of Oxidative Damage by Formamidopyrimidine Glycosylase (Fpg) and 8-Oxoguanine DNA Glycosylase (OGG-1)

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## 1. Introduction

Oxidative damage produced by endogenously and exogenously generated reactive oxygen species (ROS) has been implicated in mutagenesis and carcinogenesis and may play an important role in the pathogenesis of aging ***(1)***. Among ROS, the hydroxyl radical is highly reactive, producing a variety of purine- and pyrimidine-derived lesions in DNA ***(2,3)***. A major pathway of hydroxyl radical-induced DNA damage involves attack on the C8 position of purines to produce 8-oxoG (7,8-dihydro-8-oxoguanine), 8-oxoA (7,8,-dihydro-8-oxoadenine) and imidazole ring fragmented lesions (formamidopyrimidines ***[2,4]***). There is strong evidence to suggest that the 8-oxoG lesion, which is produced in abundance, is highly mutagenic in vitro and in vivo ***(5,6)***. Such oxidized purines are primarily repaired by the base excision repair pathway, the initial step of which is excision of the modified base by DNA glycosylases ***(7,8)***.

The Fpg (MutM) protein of *Escherichia coli* is a DNA glycosylase/AP lyase that efficiently releases modified purines such as 8-oxoG (when paired with cytosine in duplex DNA) and 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (me-Fapy-G ***[9,10]***). The cDNA encoding the eukaryotic homolog of Fpg, OGG-1 has now been isolated by a number of laboratories ***(11–15)***. OGG-1 demonstrates a similar substrate specificity to Fpg, excising 8-oxoG, preferentially when it is paired with cytosine (but being inactive when paired with adenine), and me-Fapy-G ***(11,14,16)***. More recently, a second mammalian 8-oxoG-DNA glycosylase, OGG-2 has been isolated ***(12,17)***, which prefers 8-oxoG paired with adenine and guanine and it has been proposed ***(17)*** that

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OGG-1 and OGG-2 have distinct anti-mutagenic functions in vivo. OGG-1 prevents mutation by removing 8-oxoG formed in DNA *in situ* and paired with cytosine, whereas OGG-2 removes 8-oxoG that is incorporated opposite adenine in DNA from ROS-induced 8-oxodGTP. Hazra et al. ***(17)*** also report, in HeLa cell extract, the presence of a protein that is an 8-oxoG- specific binding protein or an inhibitor specific for both OGG-1 and OGG-2. This protein, if found to be ubiquitously present in mammalian cells and tissue would obviously have an impact on measurement of OGG activity in crude mammalian extracts.

Two methods have been developed to assay Fpg and OGG-1 activity based on the substrate specificities of these enzymes. The method employed for several years in a number of laboratories, including ours, involves measuring the release of [$^{3}$H]-me-Fapy-G from a suitably treated methylated calf thymus DNA or poly (dG-dC) substrate ***(10)***. Briefly, substrate is incubated with cell-free extract for 15–60 min at 37°C, substrate DNA is ethanol precipitated and ethanol-soluble radioactivity, released by the enzyme into the supernatant, is measured by scintillation counting.

More recently, evidence suggesting that 8-oxoG plays an important role in a number of biological processes (*see* above) has generated a great deal of interest in developing assays that would specifically measure repair of this particular adduct. We describe here such an assay, which is based on the ability of Fpg and OGG-1 to remove the damaged base and to subsequently cleave at the resulting apurinic (AP) site via the AP lyase activity causing strand cleavage. Essentially, an oligo containing one 8-oxoguanine residue is labeled to high specific activity (SA) with [$^{32}$P], annealed to its oligo complement, incubated with cell-free extract, and the resulting cleavage products are analyzed by denaturing polyacrylamide gel electrophoresis (PAGE).

## 2. Materials

### 2.1. Preparation of Cell/Tissue-Free Extracts

1. FPG assay buffer 5X stock : 350 m*M* potassium-HEPES, 500 m*M* potassium chloride, 10 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* dithiothreitol (DTT), 25% v/v glycerol, pH 7.6. Aliquot and store at –20°C until use. Before use, dilute to 1 in 5 in dd$H_2O$. Dispose of excess, once thawed.
2. PBS (phosphate-buffered saline) : 0.8% NaCl, 0.02% KCl, 0.15% $Na_2HPO_4$, 0.02% $KH_2PO_4$, pH 7.4.
3. PMSF (phenylmethylsulphonyl fluoride; Sigma), 50 m*M* in 100% ethanol. Store at –20°C. Stable for at least 3 mo.
4. Leupeptin (Sigma), 10 mg/mL in dd$H_2O$. Store at 4°C. Stable for at least 1 mo.
5. Sonicator fitted with microtip probe suitable for ultrasonic disruption of cells in 1.5 mL Eppendorf tubes (*see* **Note 1**).

## 2.2 Protein Estimation

1. CBG (Coomassie brilliant blue G250 ) dye reagent concentrated (5X) stock: 780 m*M* CBG (Sigma, 75% dye content), 25% (v/v) ethanol (BDH analar), 7.4 *M* orthophosphoric acid, 0.01% Triton X-100 (v/v), 0.01% SDS (w/v). Store at 4°C in the dark for up to 1 yr.
   Before use, dilute to 1 in 5 in dd$H_2O$, leave at 4°C overnight then filter through 3MM chromatography paper (Whatman). Store at 4°C in the dark for up to 3 mo. Commercial reagents are available (*see* **Note 2**).
2. IBSA: 1mg/mL bovine serum albumin (BSA) in buffer I. Store at 4°C for up to 3 mo.
3. BSA (Sigma) protein standards: standards of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 mg/mL BSA in buffer I are prepared from IBSA. Standards are filtered (0.2 microns) and stored for up to 6 mo at 4°C.
4. Scintillation minivials.
5. Plastic spectrophotometer cuvets.
6. Multidispense pipet (*see* **Note 3** ).
7. Spectrophotometer set to read at 595 nm.

## 2.3. DNA Estimation

1. TNE Buffer 10X stock: 100 m*M* Tris base, 10 m*M* EDTA, 2 m*M* NaCl, pH 7.4. Store at 4°C for up to 6 mo. Before use, dilute to 1 in 10 with dd$H_2O$ and filter (0.45 microns). Store at 4°C for up to 3 mo.
2. Calf thymus DNA (Pharmacia Biotech, ultrapure): standards of 100, 200, 300, 400, 500 µg/mL in 1X TNE. Store at 4°C for up to 3 mo.
3. Hoechst 33258 (*bis*-benzamide) stock dye solution, 1mg/mL in dd$H_2O$. Store at 4°C in the dark for up to 6 mo.
4. TKO 100 minifluorometer and fluorometer cuvet (Hoefer, *see* **Note 4**) .

## 2.4. 8-Oxoguanine-DNA Glycosylase (OGG) Assay

### 2.4.1. Preparation of Oligo Substrate

#### 2.4.1.1. $^{32}$[P] Labeling and G25 Sephadex Purification of Oligo Substrate

1. Oligo-5' CGT TGT CAG AAG TAA OTT GGC CGC AGT GT 3'
   O = 8-oxoguanine (*see* **Note 5**).
   Prepare a 2.5 pmol/µL stock and store at –20°C for up to 3 mo.
2. T4 polynucleotide kinase (PNK) 10X buffer: 0.5 *M* Tris-HCl, pH 7.6, 100 m*M* $MgCl_2$, 100 m*M* 2-mercaptoethanol (supplied with enzyme *see* **item 3**).
3. T4 PNK ($10^4$ U/mL; Boehringer Mannheim, *see* **Note 6**).
4. Oligo sizing markers (8–32 bases; Pharmacia Biotech).
5. $^{32}$[P]-γATP. Preferably 6000 Ci/mmol (NEN Life Science Products, 10 mCi/mL).
6. TE (STE) buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA pH 8.0 (+0.1 *M* NaCl).
7. Sephadex G25 slurry: Mix 5 g Sephadex G25 (Sigma (G2550) with approx 50 mL TE buffer. Leave to swell overnight, then add fresh TE to make the slurry. Autoclave (20 min at 15 lb psi on liquid cycle) before use and store at 4°C.
8. 15-mL Falcon tubes (Falcon 2096).

9. Sterile glass wool.
10. Liquid scintillation counter.

#### 2.4.1.2. Annealing of Labeled Oligo to Complement

1. Complementary oligo: 5' AC ACT GCG GCC AAC TTA CTT CTG ACA AC 3' (*see* **Note 5**). Prepare a stock solution (4 pmol/µL) and store at –20°C for up to 3 mo.
2. 0.5 *M* NaCl.
3. Heating block set at 94°C (*see* **Note 7**).

### *2.4.2. OGG Assay*

1. Oligo substrate (*see* **Subheading 3.4.1.2.**).
2. Cleavage buffer 4X stock: 100 m*M* Tris-HCl pH 7.6, 200 m*M* KCl, 20 m*M* EDTA.
3. Denaturing loading buffer (LB): 80% formamide (v/v), 50 m*M* Tris-borate, 1 m*M* EDTA pH 8.0, 0.1% xylene cyanol (w/v), 0.1% bromophenol blue (w/v). Aliquot and store at 4°C.

### *2.4.3. PAGE Analysis of Cleavage Products*

1. SequaGel Concentrate: 8.3 *M* Urea containing 25% (w/v) acrylamide: bis-acrylamide (19:1; National Diagnostics, Mensura).
2. SequaGel buffer: 8.3 *M* urea in 1.0 *M* Tris-Borate–20 m*M* EDTA Buffer, pH 8.3 (10X TBE).
3. SequaGel Diluent : 8.3 *M* Urea.
4. TEMED (N, N, N', N, N', N- Tetramethylethylenediamine).
5. 10% Ammonium persulphate (APS; w/v). 10% APS may be aliquoted and stored at –20°C until use. Dispose of excess once thawed.
6. Vertical minigel apparatus (*see* **Note 8**).
7. TBE 10X stock: 1.0 *M* Tris-Borate–20 m*M* EDTA, pH 8.3. Before use, dilute to 1 in 10 in dd $H_2O$.
8. Hamilton syringe (25 µL or 50 µL).
9. Disposable gel loading tips (Bio-Rad 223-9917).
10. Saran Wrap™ or similar.
11. Phosphor imager and image analysis software (*see* **Note 9**).

## *2.5. Fapy–DNA Glycosylase (FPG) Assay*

### *2.5.1. Preparation of FPG Substrate DNA (*see *Note 10)*

#### 2.5.1.1. Deproteinization of DNA

1. Calf thymus DNA (*see* **Note 11**).
2. TE (*see* **Subheading 2.4.1.1.**).
3. Duran (or other wide-necked glass) bottles. Because of the hazards associated with this procedure (*see* **Note 12**) minimize the possibility of leakage by ensuring that the bottles have a good seal.

4. Proteinase K (Sigma).
5. Phenol equilibrated with 1 *M* Tris-HCl, pH 8.0. Prepare fresh as required. Add an equal volume of 1 *M* Tris, pH 8.0 to the phenol. Shake, allow to settle, and aspirate off as much of aqueous phase as possible. Extreme caution must be exercised when handling and disposing of phenol (*see* **Note 12**).
6. 3 *M* NaAc, pH 4.0.
7. Absolute ethanol.
8. Ether.
9. $N_2$ gas.
10. Water bath set at 55°C.
11. 5-mL plastic syringe.
12. Water vacuum pump aspirator.
13. 50-mL Falcon tubes (conical bottom).

#### 2.5.1.2. Methylation of DNA

1. Duran (or other wide-necked glass) bottles.
2. 0.02 *M* Ammediol (2-Amino-2-methyl-1,3-propanediol), pH 10.0 (Sigma).
3. 5 mCi [$^3$H] MNU. Preferably approx 20 Ci/mmol (Amersham International, 1 mCi/mL in ethanol). Use immediately on delivery.
4. Absolute ethanol.
5. Pasteur pipets.
6. Water vacuum pump aspirator.
7. Ether.
8. Ethanol: Ether (1:1 v/v).
9. $N_2$ gas.
10. Chemical fume cupboard.

#### 2.5.1.3. Imidazole Ring Opening of 7-Methylguanine to Generate FPG Substrate DNA

1. Sodium phosphate buffer: 50 m*M*, pH 11.4.
2. Absolute ethanol.
3. 3 *M* NaAc, pH 4.8.
4. FPG assay buffer (*see* **Subheading 2.1.**).

### *2.5.2. FPG Assay*

1. Substrate DNA.
2. FPG assay buffer (*see* **Subheading 2.1.**).
3. FPG assay stop solution: 2 *M* sodium chloride, 1 mg/mL BSA, 0.5 mg/mL salmon sperm DNA (sonicated; *see* **Note 13**).
4. Ice-cold ethanol.
5. Scintillation minivials.
6. Aqueous scintillation cocktail (e.g., Ecoscint; National Diagnostics/Mensura).
7. Liquid scintillation counter.

## 3. Methods

### 3.1. Preparation of Extracts

**Samples (tissues, cells, and extracts) must be stored on ice throughout the procedure to conserve Fpg/OGG-1 activity.**

1. Preparation and storage of cells/tissues/lymphocytes.
   Cells (bacterial or mammalian): harvest, wash with PBS, store cell pellet at –20°C.
   Tissue: snap-freeze (dry ice or liquid nitrogen) and store at –20°C (<1 mo) or –70°C (>1 mo).
   Lymphocytes: collect whole blood into universal containing EDTA (final concentration 25 m*M*). Isolate lymphocytes by density centrifugation ***(18)***, wash with PBS, and store cell pellet at –20°C.
   The number of cells or weight of tissue required for the assay will depend on the level of activity (e.g., *see* **Note 14***)*.
2. Transfer tissue or cell pellet to 1.5-mL Eppendorf tube in ice and add cold buffer I (500–1000 µL) containing 5 µg/mL leupeptin (*see* **Note 15**).
3. Sonicate sample (*see* **Note 1**) within a MSC class I cabinet to minimize exposure to aerosols. It may be necessary to mince with fine scissors or add glass beads to the sample to aid ultrasonic disruption (*see* **Note 16**).
4. Add PMSF ( 50 m*M* solution in alcohol) to the sample immediately following sonication so that the final concentration is 0.5 m*M* (i.e.*,* 1/100 of volume).
5. Centrifuge at 15,000–20,000*g* for 10 min at 4°C (*see* **Note 17**).
6. Transfer supernatant to a clean Eppendorf tube in ice. Extracts are now ready for use. For short-term storage (≤ = 48 h), in ice preferably in cold room/cabinet, is recommended. If for longer periods, extracts should be snap-frozen (dry ice or liquid nitrogen) and stored at –20°C. Activity may be lost on freeze-thawing, but we have not systematically investigated this.

### 3.2. Protein Estimation (19) (see *Note 18)*

1. Switch on the spectrophotometer at least 10 min before taking readings so that the lamp is ready.
2. Add, in duplicate, 40 µL of each BSA standard or unknown (*see* **Note 19**) to the bottom of a scintillation minivial. Blank tubes contain buffer I only.
3. Add 2 mL of CBG/ Bio-Rad reagent to each tube and gently mix (*see* **Note 3**).
4. Transfer 1 mL of blank sample to the cuvet and zero the machine at 595 nm. Repeat with duplicate blank sample to check reproducibility and stability of readings (*see* **Note 20**).
5. Transfer 1 mL of the lowest standard to the cuvet, record the reading, empty, and drain the cuvet thoroughly by blotting upside down on a paper towel and repeat with the next standard sample.
6. After reading all standard samples, transfer 1 mL of the unknown sample to the cuvet, record the reading, empty, and drain the cuvet thoroughly by blotting

upside down on a paper towel and repeat with the next unknown sample (*see* **Note 21**).

7. Construct a standard curve by plotting absorbance of standards versus protein concentration (mg/mL). Calculate mean protein concentration (mg/mL) of duplicate samples (*see* **Note 21**) by reference to the standard curve.

### 3.3. DNA Estimation (20,21)(see Note 4)

1. Switch on the TKO 100 at least 15 min before taking measurements so that the lamp is ready and the temperature in the chamber stabilizes.
2. Freshly prepare working solution of Hoechst 33258 by diluting the stock solution to 1 μg/mL in 1X TNE, wrap in foil to protect from light, and allow to warm to room temperature before use.
3. Set the sensitivity of the detector monitor to about 50% by turning the scale knob approx 5 full clockwise turns from the fully counter position.
4. Add 2 mL of Hoechst working solution to the cuvet, if necessary wipe the sides of the cuvet with a low-lint tissue and place in the sample chamber.
5. Zero the reading.
6. Deliver 2 μL of lowest DNA standard into the 2-mL dye solution and mix by pipetting the solution into and out of a disposable pipet several times without introducing bubbles.
7. Close the sample chamber and record the reading (*see* **Note 22**).
8. Remove the cuvet from the sample chamber. Empty and drain the cuvet thoroughly by blotting it upside down on a paper towel between readings.
9. Repeat **steps 4–8** at least once to verify that results are reproducible (*see* **Note 21**).
10. Repeat **steps 4–9** with rest of DNA standards.
11. Finally, repeat steps 4-9 with unknown sample (*see* **Note 23**).
12. Construct a standard curve by plotting standard readings vs DNA concentration (μg/mL) of standards. Calculate mean DNA concentration (μg/mL) of replicate unknown samples by reference to the standard curve.

### 3.4. 8-Oxoguanine-DNA Glycosylase (OGG) Assay

Before starting work, ensure that appropriate shielding is in place and that a radiation monitor is at hand to monitor for possible contamination. Handling and disposal of [$^{32}$P] must be performed in accordance with local rules pertaining to radioactive substances.

#### 3.4.1. Preparation of Oligo Substrate

##### 3.4.1.1 [$^{32}$P] Labeling and Sephadex G25 Purification of Oligo Substrate

1. Set up the following reaction in an Eppendorf tube:
   1 μL oligo (2.5 pmol) or 1 μL oligo sizing marker
   1 μL 10X PNK buffer
   3.8 μL dd$H_2O$

Working behind a perspex screen, add:
4 µL $^{32}$[P]- γATP
0.2 µL PNK

2. Incubate reactions at 37°C for 30 min. During this time, prepare the Sephadex G25 columns.
3. At the end of the incubation, add 40 µL TE to the mix, transfer 1 µL to a scintillation minivial, add 2 mL scintillation cocktail and count (*see* **Note 24**). Proceed to purify the rest by passing through a G25 column.
4. Pack the tip of a 1mL plastic disposable syringe with sterile glass wool. Use the plunger to pack the wool tightly to approx 50µL.
5. Pipet 1 mL of the Sephadex/TE slurry to the syringe, place the syringe in a 15-mL Falcon tube (to act as a carrier in the rotor), and centrifuge at 1700*g* for 4 min at room temperature.
6. Repeat **step 2** until a packed column bed of 0.8–0.9 mL is obtained.
7. Wash and equilibrate the column by applying 100 µL STE to the top of the column and centrifuging at 1700*g* for 4 min at room temperature.
8. Place column in a fresh Falcon tube and apply the oligo (50 µL) to the top of the column.
9. Centrifuge at 1700*g* for 4 min at room temperature and transfer the eluate to a 1.5-mL Eppendorf. Dispose of column in accordance with local rules pertaining to radioactive substances.
10. Transfer 1 µL of eluate to a scintillation minivial, add 2 mL scintillation cocktail and count.
11. Calculate percent incorporation of label (*see* **Note 24**).

#### 3.4.1.2. Annealing of Labeled Oligo to Complement

1. Remove 40 µL of the column purified oligo (approx 2 pmol) into an Eppendorf tube, add twofold excess of complementary oligo (i.e., 4 pmol), 5 µL 0.5 *M* NaCl and ddH$_2$O to a total volume of 50 µL (*see* **Note 25**).
2. Place tube in a heating block (*see* **Note 7**) set at 94°C, incubate for 2–3 min then switch off block and allow tube to cool to room temperature slowly.
3. Efficiency of annealing may be checked by PAGE (*see* **Note 26**).

### 3.4.2. OGG Assay

1. Set up the following reaction (*see* **Note 27**) in an Eppendorf tube in ice:
   1 µL labeled, annealed oligo
   2.5 µL 4X cleavage buffer
   0–6.5 µL cell/tissue extract
   and add an appropriate volume of ddH$_2$O to give final volume of 10 µL.
2. Vortex mix and centrifuge briefly to collect tube contents.
3. Incubate at 37°C for 15–60 mins (*see* **Note 28**).
4. Add 20 µL denaturing LB and either proceed to next stage (analysis of cleavage products) or store at –20°C until required (*see* **Note 29**).

### 3.4.3. PAGE Analysis of Cleavage Products

1. Wash all minigel apparatus carefully with $ddH_2O$ and dry thoroughly (*see* **Note 30**).
2. Assemble minigel apparatus according to manufacturer's instructions.
3. Prepare 20% SequaGel by mixing 8 mL SequaGel Concentrate (25%) with 1 mL SequaGel Buffer and 1 mL SequaGel Diluent. Just prior to pouring add 100 µL 10% APS, mix well, then immediately add 10 µL TEMED and mix. Pipet into apparatus avoiding air bubbles.
4. Following polymerization, remove comb and, using a Hamilton syringe, rinse wells thoroughly (*see* **Note 31**) with running buffer (i.e., TBE).
5. Heat samples for 5 min at 95°C and cool on ice for 10 min.
6. Rinse each well with 1X TBE just before loading 6 µL of each sample, preferably using disposable tips. Load labeled size marker (*see* **Subheading 3.4.1.1.**) in order to confirm size of cleaved products.
7. Run gel in 1X TBE at 100–200 V until bromophenol blue dye front is approx 1 cm from the bottom of the gel.
8. At the end of the run remove one plate and wrap the gel (still supported on the other plate) with Saran Wrap. Detect bands using phosphorimager or autoradiography, confirming size of products by reference to the size marker. Calculate percent oligo cleaved by image analysis (*see* **Note 9**).

### 3.4.4. Calculation of Enzyme Activity

1. Calculate nmoles oligo cleaved in each reaction by multiplying % oligo cleaved (determined by image analysis) by amount of oligo (in nmoles) in reaction.
2. Plot nmoles oligonucleotide cleaved vs mg protein/ µg DNA in extract and from the linear part of the curve calculate nmoles oligo cleaved/mg protein or /µg DNA, respectively.
3. Divide nmoles oligo cleaved/mg protein or /µg DNA by incubation time in hours to give specific activity in nmoles oligo cleaved /mg protein/h or /µg DNA/h.

## 3.5. Fapy-DNA Glycosylase (FPG) Assay

### 3.5.1. Preparation of FPG Substrate DNA

#### 3.5.1.1. Deproteinization of Substrate DNA

1. Dissolve CT DNA at 2 mg/mL in TE (up to 300 mL) on a stirrer overnight in a IL Duran bottle. There will be some insoluble bits, but it is not necessary to remove them.
2. Place bottle in 55°C water bath for 5 min then add solid proteinase K (1 mg/10 mL DNA solution). The bits should disappear quickly, but leave for 1 h swirling occasionally before adding another 1 mg of proteinase K per 10-mL solution.
3. After a further 1 h at 55°C, move to fume cupboard on tray, cool under running tap water, and add equal volume of phenol equilibrated to pH 8.0 using 1 *M* Tris (*see* **Subheading 2.5.1.1.**). Cap and shake vigorously for 5 min—be aware of the possibility of leakage.

4. Allow to stand for about 1.5 h at room temperature: the phenol should settle out and can be almost completely removed by aspiration through the upper aqueous layer using a glass pipet.
5. Decant supernatant into 50-mL Falcon tubes (conical bottom) and spin at 1000*g*, room temperature, 10 min.
6. Observe interface carefully: if clear, re-extraction is not necessary (*see* **Note 32**). Remove all traces of phenol from bottom of tube using glass 5-mL pipet or Pasteur pipet and rubber pipet bulb or pipet pump. Do not worry about taking some of the aqueous layer. Pour off supernatants into Duran bottle of appropriate capacity.
7. Add 1/10 vol of 3*M* NaAc pH 4.0 to pooled aqueous phases, mix well, and add 2 vol cold ethanol. Cap and mix by inversion.
8. Lift out DNA on glass pipet and transfer to smaller Duran. Wash three times with ethanol at room temperature by vigorous shaking and water vacuum pump aspiration of the ethanol. Make sure DNA spreads out in ethanol to ensure complete penetration of ethanol.
9. Wash at room temperature three times with ethanol:ether (1:1) and then three times with ether alone. Each time, pour off the washes into a tray in a fume cupboard for evaporation (no naked flames/electrical appliances) or alternatively dispose of according to local rules.
10. Dry DNA in stream of $N_2$ to remove the ether, teasing apart fibrous DNA with Pasteur pipets. Dry to constant weight.

#### 3.5.1.2. Methylation of DNA

**Because of the radiochemical hazard involved, the following procedure should be carried out in a fume cupboard with an appropriate airflow rate. Handling and disposal of [3H] must be performed in accordance with local rules pertaining to radioactive substances. We advise monitoring for [3H] contamination of the work area before starting and, of course, on completion.**

1. For 5 mCi [$^3$H]-MNU in 1 mL ethanol: dissolve 40 mg DNA on a stirrer plate overnight at 8 mg/mL in 0.02 *M* Ammediol, pH 10.0 in a 25 mL Duran bottle. Transfer 2 mL of this solution to a separate container (This is to be used for rinsing [$^3$H] vial—*see* **step 3**).
2. In tray in fume cupboard, CAREFULLY remove seal from [$^3$H]MNU vial using blunt forceps. Use a twisting rather than pulling action and put aluminium ring and sealing disk directly into beaker in tray. Recap vial with black plastic cap provided—avoid shaking.
3. Put Duran on stirrer in tray, and using 5-mL plastic syringe, carefully transfer MNU solution into stirring DNA solution. Rinse out vial with two 1-mL aliquots of DNA solution by serial transfer. Put syringe and empty vial in sink for careful rinsing.
4. Continue stirring DNA for 5 h at room temperature. Carefully remove stirrer bar, then add 1/10 volume of 3 *M* NaAc, pH 4.0 and 2 vol of cold ethanol. Form DNA precipitate by swirling and inversion being very careful of leakage—any spills will contain [$^3$H]-methanol, which will blow off rapidly.

5. When DNA has formed a tight ball, carefully remove supernatant using a Pasteur pipet attached to a water vacuum pump, the outflow of which is piped directly into the sink drain hole to avoid splashing. Do not be concerned about tiny fragments of DNA being sucked down the sink, but do avoid the bulk of the DNA! It is possible to remove all of the supernatant safely in this way.
6. Wash the DNA with ethanol (about 20–30 mL per wash) making sure pellet is "fluffed" out each time. After 10 washes, check [$^3$H] radioactivity counts in 500 µL of wash (+3 mL scintillant) to monitor the washing efficiency. Ideally, the last wash should be close to background, but as small fragments of DNA may be present, two consecutive washes with similar cpm is acceptable.
7. Dry DNA by washing in ethanol:ether (1:1) twice and ether twice. DO NOT aspirate supernatants down the sink! Pour supernatants carefully into a stainless steel tray for evaporation and thorough washing down the sink.
8. Blow off residual ether in a slow stream of $N_2$ gas, teasing apart DNA with Pasteur pipets if necessary. Transfer to preweighed clean glass vial and dry to constant weight.

#### 3.5.1.3. Imidazole Ring Opening of 7-Methylguanine to Generate FPG Substrate DNA

1. Dissolve DNA (2 mg/mL) in 50 m*M* sodium phosphate buffer (pH 11.4) and leave at room temperature for 24 h to open the imidazole rings of the 7 meG residues.
2. Add 0.1 vol 3 *M* NaAc (pH 4.8) and 2 vol of cold ethanol.
3. Form DNA precipitate by swirling and inversion.
4. Spool DNA, transfer to a fresh tube, and wash extensively with 70% ethanol.
5. Resuspend DNA at 2 mg/mL in 1X FPG assay buffer (without DTT) and store at 4°C.

### *3.5.2. FPG Assay*

1. Dilute substrate with FPG assay buffer (containing DTT) to give approx $10^5$ cpm/mL.
2. Into 1.5-mL Eppendorf tubes in ice, add 1–20 µL of tissue or cell extract (*see* **Subheading 3.1.**), 10 µL of 5X FPG assay buffer and make volume up to 50 µL with dd$H_2O$ ( *see* **Note 33**).
3. Start the assay reaction by adding 50 µL of diluted substrate (approx $5 \times 10^3$ cpm).
4. Vortex briefly, and centrifuge to collect contents in the bottom of the tubes.
5. Incubate tubes at 37°C for 15–60 min (*see* **Note 28**).
6. At the end of this time, add 25 µL of FPG assay stop solution with vortex mixing, followed immediately by 250 µL of ice-cold ethanol.
7. After cooling the tubes for 20 min on dry ice (or at –80°C), centrifuge at 25,000*g* for15 min at 4°C (see **Note 34**).
8. Carefully remove 300 µL of supernatant into scintillation minivials (*see* **Note 35**), mix with 3 mL Ecoscint and count for 5 min in a suitable scintillation counter.
9. Calculate the cpm by averaging two 5-min counts. Unusually high counts, particularly in the first few tubes, may indicate chemiluminescence and these tubes should be recounted to constant cpm.

### 3.5.3. Calculation of Enzyme Activity

1. Calculate total cpm released from each test sample by subtracting mean of "blank tubes" (*see* **Note 33**) and multiplying by 380/300 (*see* **Note 36**).
2. Plot total cpm vs µg protein or ng DNA and from the linear part of the curve calculate cpm/µg protein of cpm/ng DNA.
3. Multiply cpm/µg protein or cpm/ng DNA by the conversion factor (*see* **Note 37**) to give FPG specific activity in pmoles Fapy released/g protein/h or pmoles Fapy released/mg DNA/h, respectively.

## 4. Notes

1. We routinely use a Heatmaster Sonicator fitted with a microtip probe (standard tapered with 3.2-mm diameter) and sonicate for 10 s at 216 µm (peak-to-peak amplitude). Appropriate sonication conditions will depend upon the particular machine and probe used and should be established by experiment.
2. Bio-Rad dye reagent (Bio-Rad) supplied as a 5X stock may be used as an alternative to CBG. Standard curves using CBG are linear up to 1 mg/mL compared to those using Bio-Rad, which plateau around 0.6–0.8 mg/mL. We consider absorbances less than half that of the lowest standard as inaccurate, and absorbances between 0.1 and 0.6 to be ideal.
3. We recommend using an Eppendorf multipet plus (Eppendorf) or similar positive displacement machine to repeat dispense quickly and accurately.
4. Hoechst 33258 binds to the minor groove of DNA. When 365 nm light (long UV) excites this dye, fluorescence results and thus can be measured by minifluorometer (as described here), fluorescence spectrophotometer ***(21)*** or fluorescence microtiter plate-reader. We have also used a microtiter (96-well) based assay;10 µL sample or standard (range 0–50 µL/mL DNA) and 100 µL Hoechst (1 µL/mL) per well.
5. We purchase our 8-oxoguanine-containing oligos (synthesized according to standard phosphoramidite chemistry) from either Alta Bioscience, School of Biochemistry, University of Birmingham, UK or Genset Oligos, Paris, France. Nonmodified oligos are now readily available at a reasonable price from a number of commercial sources (e.g., Life Technologies and Cruachem).
6. We purchase our T4 polynucleotide kinase (PNK) from either Boehringer Mannheim or United States Biochemical (USB).
7. The annealing reactions may be heated in a PCR machine, however, it is important to allow the reactions to cool slowly to room temperature.
8. We use the Bio-Rad mini-PROTEAN II electrophoresis system.
9. We use the STORM phoshorimager (Molecular Dynamics) in combination with Imagequant software to analyze our results. Alternatively, autoradiography in combination with a suitable densitometer may be used.
10. An alternative substrate for this assay is poly (dG-dC) treated with [$^3$H]-DMS ***(10)***.
11. We routinely use Sigma calf thymus DNA (<3% protein).
12. Phenol is a powerful systemic poison, which is readily absorbed through the skin and causes chemical burns on contact. Phenol should be neutralized with an equal

volume of 4 *M* NaOH, before disposal down the sink in high dilution. Alternatively, follow local rules governing disposal of phenol.

13. Prepare this solution by mixing 8 parts 5 *M* NaCl, 2 parts 10 mg/mL BSA, 1 part 10 mg/mL salmon sperm DNA (sonicated) with 9 parts $ddH_2O$.
14. Cells and tissues vary in Fpg/OGG-1 activity and therefore the amount of extract required to detect activity will also vary. The following may be used as a starting guide if Fpg/OGG-1 activity is unknown.

| | | |
|---|---|---|
| Cells: | Bacterial | Pelleted from 1.5 mL of stationary culture |
| | Mammalian | $10^7$ cells |
| | Lymphocytes | 10 mLs whole blood |
| Tissue: | 1–10 mg | |

More (or less) cells or tissue may be used providing sonication is effective without compromising enzyme activity.

Inclusion of a positive control (i.e., Fpg/OGG-1 expressing cell line or tissue) in both extraction and assay procedures is strongly advised, particularly when assaying samples with very low or unknown activity.

If substrate-limiting conditions are attained at the lowest volume of extract used, repeat the assay using appropriate dilutions of extract .

15. A minimum buffer volume of 500 µL is needed to avoid heating (>40°C) of the sample during sonication. If smaller extract volumes (300–500 µL) are required, it may be necessary to sonicate the sample for shorter times or on ice. Establish appropriate conditions by experiment. We find that a thermocouple thermometer is optimal for monitoring temperature during procedures.
16. Some tissues (e.g., gut and tumor) are more difficult to disrupt and therefore require the addition of 0.1–0.2 *g* glass beads (100 mesh, BDH) and/or mincing before sonication.
17. It may be possible to omit this step if very few cells are used but this should be checked by experiment.
18. If a platereader with appropriate filter is available this procedure may be adapted as a microtiter (96-well) plate assay. We have successfully used, in triplicate, 40 µL of sample or standard (linear range 0–0.1 mg/mL protein) and 200 µL of dye reagent per well.
19. Samples (e.g., liver or tumor tissue) may need to be diluted in buffer I to achieve absorbance within linear range of the standard curve.
20. Rezero the machine where necessary; frequency will depend on the particular machine.
21. Replicate protein and DNA estimations should be within 5%.
22. The observed fluorescence stabilizes after a few seconds and then begins to drop as the chamber warms.
23. Samples (e.g., tumor tissue) may need to be diluted in FPG assay buffer to achieve fluorescence within the range of the standard curve. If the reading is lower than the lowest standard, use a larger volume of extract (up to 10 µL) and apply a correction factor.

24. Percent incorporation of label = (total postcolumn cpm) / (total precolumn cpm) × 100.
    To ensure that the labeled oligo has a high specific activity, the number of pmoles of [$^{32}$P]-γATP in the labeling reaction should be at least twice the number of pmol of oligo 5' ends. In this reaction we use 2.5 pmol oligo and 6.7 pmol of [$^{32}$P]-γATP. Therefore, if all the oligo is labeled, 37% of the [$^{32}$P] would be incorporated.
    The specific activity (SA; in Ci/mmol) of the labeled oligonucleotide may be calculated as follows:

$$dpm = cpm/CE$$

    where CE = counting efficiency of scintillation counter. dpm/pmol oligo = total cpm (i.e., in 50 µL) / (2.5 × CE) dpm/mmol oligo = (cpm × $10^9$) / (2.5 × CE) 1 Ci = 2.22 × $10^{12}$ dpm therefore, SA (Ci/mmol) = (cpm × $10^{9)}$ ) / (2.5 × CE × 2.22 × $10^{12}$).
25. To ensure efficient annealing it is important to use small reaction volumes and incubate with excess complement. We have found twofold excess of complement to be sufficient and greater excess should be avoided as this may cause problems when trying to denature the annealed oligo.
26. Annealed oligo may be run on a 20% nondenaturing gel to check efficiency of annealing. Follow the procedure described in **Subheading 3.4.3.** with three exceptions. (1) Use a nondenaturing acrylamide gel mix (e.g., Accugel, National Diagnostics) to prepare the gel, (2) Use a nondenaturing loading buffer : 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, adding 2 µL loading buffer to 4 µL sample, and, (3) do not heat the samples prior to loading.
27. It is advisable to include OGG-1/Fpg expressing and nonexpressing extracts as controls in this assay. It may be necessary to increase substrate concentration when assaying OGG activity in human/mammalian cell/tissue extracts because of the presence of an 8-oxoG specific-binding protein (***17***).
28. Incubation time may be reduced for highly active extracts, however incubation times of > 1 h are not recommended: in our hands, loss of activity becomes a problem.
29. The $T_{1/2}$ for decay of $^{32}$[P] is 2 wk. Therefore, it is advisable to use the substrate quickly (i.e., within 2 wk) so as not to compromise the specific activity.
30. It is very important to use clean, dry apparatus. Remains of polymerized gel can cause the plates to crack and may compromise the running of the gel. Also examine the edges of the plates carefully for chips and cracks because if an inadequate seal is formed between the bottom of the plates and the gasket the gel will leak.
31. It is essential to rinse the wells thoroughly as soon as the comb is removed so as to remove small amounts of acrylamide solution trapped by the comb, which will polymerize in the wells, and produce irregular wells that give rise to distorted bands. It is equally important to wash away excess urea, a component of the denaturing gel mix, which leaches out of the gel into the wells and affects loading if not dispersed.
32. If the interface is not clear, reextraction is necessary. Remove upper aqueous layer from each tube and combine in a Duran bottle. Repeat **steps 3–5**.

33. It is necessary to include a number of "blank" tubes to assess the counts released from the substrate in the absence of protein. The mean value of these is deducted from the counts released from each test sample. It is also advisable to include OGG-1/Fpg expressing and nonexpressing extracts.
34. The samples should be centrifuged with the brake off to avoid disruption of the pellet.
35. If it is sometimes difficult to remove 300 µL supernatant without disturbing the pellet, a smaller aliquot can be removed, but remember to take this into account when calculating the total cpm released (*see* **Note 37**).
36. Because only 300 µL out of total reaction of 380 µL are counted, cpm must be multiplied by 380/300 to give total cpm released per sample. If less supernatant is removed, total counts = cpm × 380/vol counted.
37. Conversion factor (CF) is calculated from the counting efficiency and substrate specific activity.

    (a) dpm = cpm/CE.

    where CE = counting efficiency of scintillation counting.

    (b) Substrate specific activity (SA) in Ci/mmol (= nCi/pmol). Recalculate monthly by reference to decay chart.

    (c) 1 Ci = $2.22 \times 10^{12}$ dpm, therefore, 1 nCi = 2220 dpm.

    From (a), (b), and (c):

    FPG activity (in pmol Fapy released /µg /h) = cpm/µg × 1/ (SA × 2220 × CE × h).

    (Where h = assay incubation time in hours).

    Therefore,

    FPG activity (in pmol Fapy released/g/h) = cpm/µg × $10^6$/(SA × 2220 × CE × h
    = cpm/µg × CF

    therefore, CF = $10^6$/(SA × 2220 × CE × h).

## References


1. Halliwell, B. and Gutteridge, J. M. C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **186,** 1–85.
2. Dizdaroglu, M. (1991). Chemical determination of free radical-induced DNA damage to DNA. *Free Radical Biol. Med.* **10,** 225–242.
3. Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992) Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry* **31,** 106–110.
4. Cadet, J., Berger, M., Douki, T., and Ravanat, J.L. (1997) Oxidative damage to DNA: formation, measurement and biological significance. *Rev. Physiol. Biochem. Pharmacol.* **131,** 1–87.
5. Michaels, L. M. and Miller, J. H. (1992) The GO system protects organisms from the mutagenic effects of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). *J. Bacteriol.* **1,** 6321–6325.
6. Grollman, A. P. and Moriya, M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.* **9,** 246-–249.

7. Demple, B. and Harrison, L. (1994). Repair of oxidative damage to DNA: enzymology and biology. *Annu. Rev. Biochem.* **63,** 915–948.
8. Boiteux, S. and Laval, J. (1997) Repair of oxidised purines in DNA, in *Base Excision Repair of DNA Damage* (Hickson, I. D., ed.), Landes Bioscience-Springer, Austin, TX, pp. 31–44.
9. Karakaya, A., Jaruga, P., Bohr, V. A., et al. (1997) Kinetics of excision of purine lesions from DNA by *Escherichia coli* FPG protein. *Nucleic Acids Res.* **25,** 474–479.
10. Bjørås, M., Luna, L., Johnsen, B., et al. (1997) Opposite base-dependent reactions of a human base excision repair enzyme on DNA containing 7,8-dihydro-8-oxoguanine and abasic sites. *Embo J.* **16,** 6314–6322.
11. Van der Kemp, P. A., Thomas, D., Barbey, R., et al. (1996) Cloning and expression in *Escherichia coli* of the *OGG1* gene of *Saccaromyces cerevisiae*, which coded for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine and 2,6-diamino-4-hydroxy-5-methylformamidopyrimidine. *Proc. Natl. Acad. Sci. USA* **93,** 5197–5202.
12. Nash, H. M, Bruner, S. D., Scharer, O. D., et al. (1996) Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. *Curr. Biol.* **6,** 969–980.
13. Roldan-Arjona, T., Wei, Y.F, Carter-K. C., et al. (1997) Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase. *Proc. Natl. Acad. Sci. USA* **94,** 8016–8020.
14. Rosenquist, T. A., Zharkov, D. O., and Grollman A. P. (1997). Cloning and characterisation of a mammalian 8-oxoguanine DNA glycosylase. *Proc. Natl. Acad. Sci. USA* **94,** 7429–7434.
15. Tani, M., Shinmura, K., Kohno, T., et al. (1998) Genomic structure and chromosomal localisation of the mouse *Ogg1* gene that is involved in the repair of 8-hydroxyguanine in DNA damage. *Mamm. Genome* **9,** 32–37.
16. Girard, P. M., D'Ham, C., Cadet, J., and Boiteux S. (1998). Opposite base-dependent excision of 7,8-dihydro-8-oxoadenine by the ogg1 protein of *Saccharomyces cerevisiae. Carcinogenesis* **19,** 1299–1305.
17. Hazra, T. K., Izumi, T., Maidt, L., et al. (1998) The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. *Nucleic Acids Res.* **26,** 5116–5122.
18. Coutinho, L. H., Gilleece, M. H., DeWynter, E. A., Will, A. and Testa, N. G. (1993). Clonal and long-term culture using human bone-marrow. in *Haemopoesis, A Practical Approach* (Testa, N. G. and Molyneux, G. M., eds.) Oxford University Press, Oxford, UK, p. 288.
19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–272.
20. Instructions—TKO 102 Standards Kit (Hoefer Scientific Instruments UK, Newcastle-under-Lyme, UK.
21. Cesarone, C. F., Bolognesi L., and Santi L. (1979) Improved microfluorimetric DNA determination in biological material using 33258 Hoechst. *Anal. Biol.* **100,** 188.