Genomic 8-oxo-7, 8-dihydro-2'-deoxyguanosine Quantification

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[Abstract] 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) is among the most common reactive oxygen species-induced DNA lesions and can be used as a biomarker for oxidative stress. The lesion has been linked to several biological processes and diseases, including colorectal cancer, Huntington's disease, estrogen-induced gene expression, and thymine dimer repair (reviewed in Delaney *et al*., 2012). The following assay is used to quantify 8-oxo-dGuo levels in DNA as described in Sousa *et al*. (2013).

Materials and Reagents

1. NH_4HCO_3 (reagent grade ≥ 99% purity)
2. $MgCl_2$ (reagent grade ≥ 99% purity)
3. $CaCl_2$ (reagent grade ≥ 99% purity)
4. DNase I from bovine pancreas (F. Hoffmann-La Roche, catalog number: 04716728001)
5. Nuclease P1 from *P. citrinum* (Sigma-Aldrich, catalog number: N8630-1VL)
6. Phosphodiesterase I from *C. adamanteus* venom (Sigma-Aldrich, catalog number: P3242-1VL)
7. Alkaline phosphatase from *E. coli* (Sigma-Aldrich, catalog number: P5931-100UN)
8. 8-hydroxy-2'-deoxyguanosine (8-oxo-dGuo) (Sigma-Aldrich, catalog number: H5653-1MG)
9. [$^{15}N_5$]-8-hydroxy-2'-deoxyguanosine (Cambridge Isotope Laboratories, catalog number: NLM-6715-0) (this is the internal standard)
10. DNeasy® Blood & Tissue Kit (QIAGEN, catalog number: 69506)
11. LC/MS-grade methanol
12. Hydrolysis buffer (see Recipes)
13. Solvent A (see Recipes)
14. Solvent B (see Recipes)

Equipment

1. Vortexer

2. Microcentrifuge
3. Vacuum centrifuge
4. LC/MS/MS: We used an LC-20AD HPLC system (Shimadzu Corporation) coupled to an API 5000 triple-quadrupole mass spectrometer (Applied Biosystems)
5. Zorbax SB-C18 reverse phase chromatography column (2.1 x 150 mm, i.d., 3.5 µm) (Agilent Technologies)

Procedure

1. DNA isolation

   DNA can be isolated using a variety of methods. We used the DNeasy® Blood Tissue Kit from QIAGEN, but other analogous kits are likely to yield similar results so long as they are not phenol based. Phenol-based DNA isolation has been shown to oxidize DNA *in vitro* and therefore overestimate 8-oxo-dGuo (Hamilton *et al*., 2001). Moreover, the final DNA elution step should be performed with water because most elution buffers contain EDTA, which inhibit nucleases.
2. Enzymatic hydrolysis of DNA
   a. In 40 µl hydrolysis buffer, add
      i. 1 U DNase I
      ii. 0.2 mU phosphodiesterase I
      iii. 0.1 U alkaline phosphatase
      iv. 1.25 pmol [$^{15}N_5$]-8-hydroxy-2'-deoxyguanosine internal standard (this gives a final concentration of 10 nM on the LC/MS/MS)
      v. 0.5-5 µg DNA
   b. Incubate at 37 °C for 6 h to overnight.
   c. Add five volumes of ice-cold methanol to the samples and mix well by vortexing. This step precipitates enzymes and salts prior so that (1) they don't interfere with analyte ionization and (2) they don't precipitate upon exposure to organic mobile phase during chromatography, which can cause clogs.
   d. Centrifuge samples at 16,000 *x g* for 20 min at 4 °C.
   e. Transfer the supernatant to new tubes. The pellet contains precipitated enzymes and salts that can interfere with analysis and can be discarded.
   f. Vacuum centrifuge the supernatant until dry.
   g. Dissolve the resulting residue in 25 µl 5% methanol in water.
   h. Inject 20 µl sample into the LC/MS/MS for analysis.
3. A standard curve was made in 5% methanol in water containing 0.1-500 nM 8-oxo-dGuo, containing 10 nM internal standard.

4. HPLC program (flow rate 300 µl/min)
   a. 5% solvent A for 0.5 min.
   b. Ramp to 90% solvent B over 6 min.
   c. Hold at 90% solvent B for 1.5 min.
   d. Re-equilibrate with 5% solvent A for 5 min.
5. MS/MS program
   a. MS/MS acquisition should be used with positive electrospray ionization in multiple reaction monitoring mode.
      Note: The mass spectrometer settings are instrument specific and are therefore not included in this protocol.
   b. Mass transition for 8-hydroxy-2'-deoxyguanosine: 284.1 -> 168.2.
   c. Mass transition for [$^{15}N_5$]-8-hydroxy-2'-deoxyguanosine: 289.2 -> 173.1.
6. Quantification
   a. Integrate the area under the peaks.
   b. Divide the 8-oxo-dGuo peak area by the internal standard peak area for all samples.
   c. Make a standard curve from the standards of known concentrations.
   d. Use the slope and y-intercept from the standard curve to calculate the concentration of the unknowns.
7. (Optional) Deoxynucleoside quantification
   a. Dilute 1 µl of sample prior to MS injection 1:1,000 in 5% methanol in water. The reason for the dilution is that the canonical deoxynucleosides are much more abundant than 8-oxo-dGuo and would saturate the mass spectrometer's detector if injected undiluted.
   b. Inject 20 µl of diluted sample for LC/MS/MS analysis of deoxynucleosides.
   c. Use the same HPLC program as for 8-oxo-dGuo.
   d. Mass transitions for deoxyguanosine, deoxycytidine, deoxyadenosine, and thymidine: 252.1 ->136.1, 228.1 -> 112.1, 268.1 -> 152.0, and 243.1 -> 127.0, respectively.
   e. Use standard curves to quantify deoxynucleoside concentrations.
   f. Use the following formula to calculate 8-oxo-dGuo per 10^6 nucleosides:
      (mol 8-oxo-dGuo/[mol dAdo + dGuo + dCyd + Thd]) x 1,000,000

Recipes

1. Hydrolysis buffer
   100 mM NH_4HCO_3 (pH 7.6)
   10 mM $MgCl_2$
   1 mM $CaCl_2$

2. Solvent A
   0.1% formic acid in water
3. Solvent B
   0.1% formic acid in methanol

Notes

1. Hydrolysis procedure. The hydrolysis procedure used here is one of many viable alternatives. When choosing a hydrolysis method for nucleoside analysis one must consider the following:
   a. Does the method affect the bases? DNA can be chemically hydrolyzed, but this is more risky because the bases themselves are subject to damage. Some enzymes also have unintended activity. *For example*, commercial alkaline phosphatase has been shown to contain deaminase activity (or contamination by deaminases) (Dong and Dedon, 2006; Dong *et al*., 2003).
   b. How important is a short hydrolysis reaction time? Some nucleoside modifications can arise spontaneously in water and a short hydrolysis reaction time is therefore worth the extra cost and effort necessary. Our group has also measured genomic uracil, which can arise spontaneously from cytosine deamination in water. We therefore developed a method to hydrolyze DNA in 50 min instead of 6 h (Galashevskaya *et al*., 2013). Adding even more enzymes, one can lower the reaction time to 15-30 min at room temperature (using DNase I, SVPD, micrococcal nuclease, omnicleave, benzonase, alkaline phosphatase, and Antarctic phosphatase; unpublished results by Sarno, 2013). Note that adding more enzymes significantly increases the reaction cost.
2. Cleanliness. Mass spectrometry is a very sensitive technique, so great care should be taken to maintain a clean laboratory environment. Depending on the instrument and reagent quality, the assay can detect down to 0.1-0.5 fmol analyte. Thus, always ensure that all equipment and surfaces are clean and autoclaved if possible (*e.g.* pipettes, tips, tubes, centrifuges, *etc*.). Note that dust collects on surfaces over time, so even though a laboratory space may be contaminated even though it has not been used for some time. It is usually enough to wipe equipment and surfaces down with a laboratory wipe and deionized or milliQ water followed by either ethanol or isopropanol.
3. Yield. We have performed the assay with 0.5-5 µg DNA and have always measured 8-oxo-dGuo above the assay's limit of quantification. Nevertheless, one should always attempt to use as much DNA as possible (up to 5 µg) to ensure that there is enough

measureable 8-oxo-dGuo. Regarding DNA yield: We have obtained an average of ~3 µg DNA per 10^6 cells from a multiple myeloma cell line using the DNeasy kit.

4. Replicates. One should optimally have three technical replicates per sample. Thus, when analyzing 5 µg DNA, one should have at least 15 µg for three runs of 5 µg each. Additionally, one should always perform three independent experiments. Thus, one should have 3 x 15 µg per result.
5. Quantification. Although it is possible to normalize the amount of 8-oxo-dGuo measured to µg DNA used in the initial hydrolysis reaction, it is more accurate and reproducible to compare 8-oxo-dGuo per (10^6) deoxynucleoside. This involves a single additional step and no extra material.

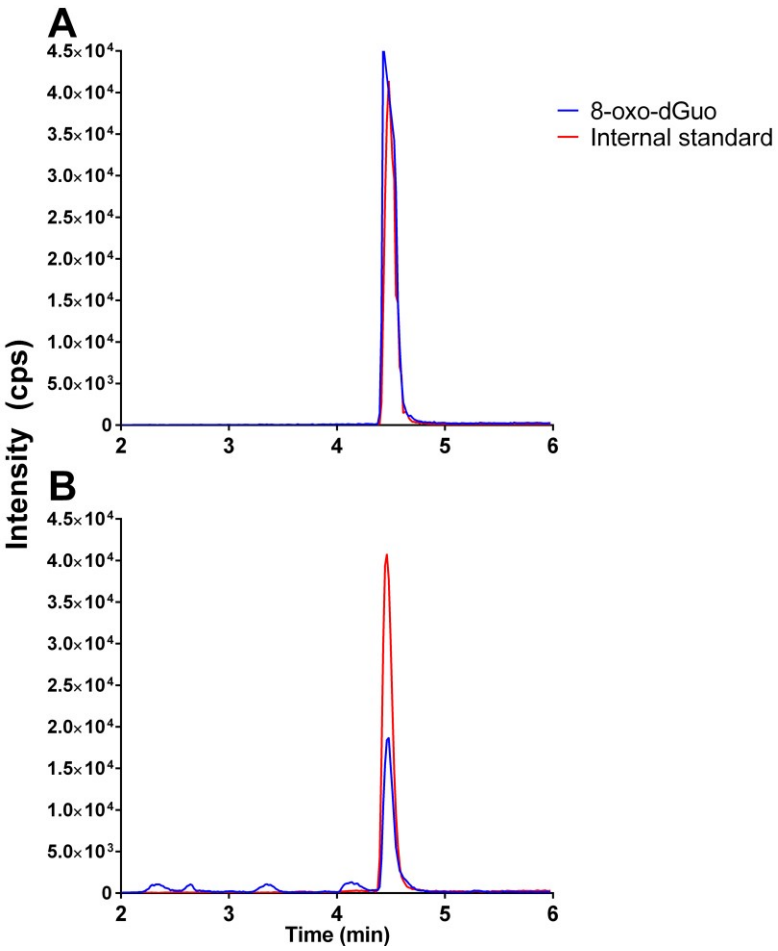

Figure 1. Typical chromatograms in 8-oxo-dGuo analysis. A. 50 nM 8-oxo-dGuo and 10 nM internal standard dissolved in 5 % methanol in water. B. 8-oxo-dGuo from 5 µg commercially obtained salmon sperm DNA containing 10 nM internal standard. *Note that the peaks that don't co-elute with the internal standard are discarded as contaminants.*

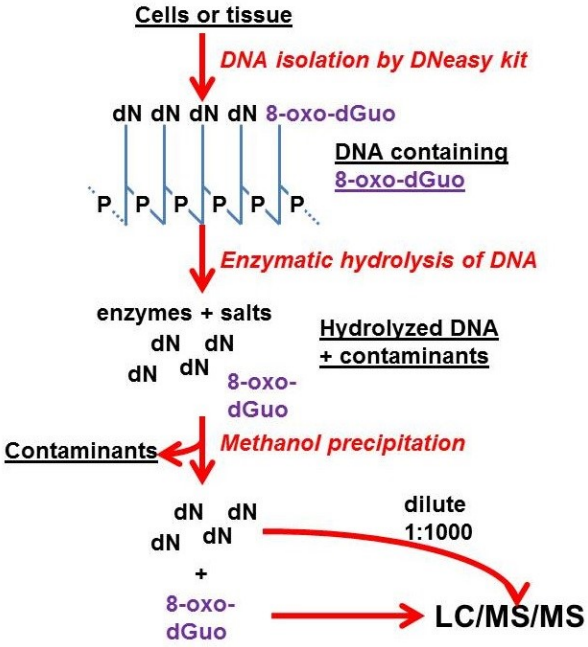

Figure 2. Visualized summary of the method

Acknowledgments

This protocol is adapted from Sousa *et al.* (2013).

References

1. Delaney, S., Jarem, D. A., Volle, C. B. and Yennie, C. J. (2012). Chemical and biological consequences of oxidatively damaged guanine in DNA. *Free Radic Res* 46(4): 420-441.
2. Dong, M. and Dedon, P. C. (2006). Relatively small increases in the steady-state levels of nucleobase deamination products in DNA from human TK6 cells exposed to toxic levels of nitric oxide. *Chem Res Toxicol* 19(1): 50-57.
3. Dong, M., Wang, C., Deen, W. M. and Dedon, P. C. (2003). Absence of 2'-deoxyoxanosine and presence of abasic sites in DNA exposed to nitric oxide at controlled physiological concentrations. *Chem Res Toxicol* 16(9): 1044-1055.
4. Galashevskaya, A., Sarno, A., Vagbo, C. B., Aas, P. A., Hagen, L., Slupphaug, G. and Krokan, H. E. (2013). A robust, sensitive assay for genomic uracil determination by LC/MS/MS reveals lower levels than previously reported. *DNA Repair (Amst)* 12(9): 699-706.
5. Hamilton, M. L., Guo, Z., Fuller, C. D., Van Remmen, H., Ward, W. F., Austad, S. N., Troyer, D. A., Thompson, I. and Richardson, A. (2001). A reliable assessment of 8-oxo-2-

deoxyguanosine levels in nuclear and mitochondrial DNA using the sodium iodide method to isolate DNA. *Nucleic acids Res* 29(10): 2117-2126.

6. Sousa, M. M., Zub, K. A., Aas, P. A., Hanssen-Bauer, A., Demirovic, A., Sarno, A., Tian, E., Liabakk, N. B. and Slupphaug, G. (2013). An inverse switch in DNA base excision and strand break repair contributes to melphalan resistance in multiple myeloma cells. *PLoS One* 8(2): e55493.