

Modification of the ^{32}P -Postlabeling Method to Detect a Single Adduct Species as a Single Spot

Masako Ochiai, Takashi Sugimura, and Minako Nagao

Summary

The original ^{32}P -postlabeling method developed by Randerath and his colleagues has been modified to detect a single type of adduct as a single spot in thin-layer chromatography (TLC), because some types of adducts gave multiple adduct spots by the original method. In the remodified methods, DNA is first digested with micrococcal nuclease and phosphodiesterase II and then labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under standard or adduct-intensification conditions. Since the labeled digest includes adducted mono-, di-, and/or oligo-deoxynucleotides, it is further treated with phosphatase and phosphodiesterase prior to TLC. The labeled digest is treated with nuclease P1 (NP1) in method I, and with T4 polynucleotide kinase and NP1 in method II, and then with phosphodiesterase I in both cases, and subjected to TLC. The advantage of these methods is that the number of adduct species formed can be estimated by TLC.

Key Words: Adducted deoxynucleoside 5'-phosphate; nuclease P1; T4 polynucleotide kinase; phosphatases; phosphodiesterase I; phosphodiesterase II; micrococcal nuclease; intensification method; standard method; method I; method II.

1. Introduction

The ^{32}P -postlabeling method devised by Randerath and his group (1,2) is widely used to detect DNA adducts formed in vitro and in vivo with various mutagens and carcinogens. The advantages of this method (and simple modifications like butanol extraction and use of nuclease P1 [NP1] to detect adducts with high efficiency) are introduced in Chapter 1 of this volume. The principle is to detect DNA lesions as adducted deoxynucleoside 3', 5'-diphosphate elements. However, with the established methodology, a single type of adduct is not necessarily detected as a single spot owing to incomplete digestion of adducted DNA.

In this chapter, approaches are introduced that allow for the detection of single adducted forms as single spots, with modifications to the original Randerath method. In these methods, DNA is first digested with micrococcal nuclease (MN) and phosphodiesterase II (PDE II), and labeled with [γ - ^{32}P]ATP under standard (2) or adduct-intensification conditions (3), the labeled digests obtained are further treated with NP1, T4 polynucleotide kinase (PNK), and phosphodiesterase I (PDE I), before analysis of adducted deoxynucleoside 5'-phosphate formation by thin-layer chromatography (TLC).

In some cases, the labeled DNA digests include mono-, di-, and/or oligo-deoxynucleotides: [^{32}P]pX(pN)_np, where X is an adducted deoxynucleoside, and N is a normal deoxynucleoside. By treatment with NP1, the 3'-phosphate of [^{32}P]pX(pN)_np can be removed to yield [^{32}P]pX(pN)_n (4,5), and further treatment with PDE I may then produce [^{32}P]pX and n(pN). Some types of adducted deoxynucleoside 3', 5'-diphosphate are, however, resistant to the phosphatase activity of NP1, although they are sensitive to that of PNK. Thus, in method I, labeled digests are treated with NP1, and in method II, with PNK and NP1 and then treated with PDE I in both cases, as shown in **Fig. 1**. It is known that the optimum pH for the 3'-phosphatase activity of PNK is 5.9, whereas that for its kinase activity is 6.5–8.5 (6).

To give a concrete example, when DNA from rats treated with the food-borne mutagenic/carcinogenic heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was analyzed by the ^{32}P -postlabeling method under standard or intensification conditions, several adduct spots were detected on TLC, and the spot for authentic *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine 3', 5'-diphosphate (3', 5'-pdGp-C8-PhIP) coincided with a minor adduct spot, although it has been demonstrated to be the sole adduct of PhIP by high performance liquid chromatography (HPLC) analysis using [^3H]PhIP (7,8). The additional spots were demonstrated to be owing to incomplete digestion of DNA (8). A similar result was also obtained with a second heterocyclic amine, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) (9). Single spots thus were generated with DNA from animals treated with PhIP or MeIQ by method I (8,9).

In the case of the another heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), five spots were detected on TLC by the standard method (10), and for two of them their structures were tentatively identified as *N*-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline 3',5'-diphosphate (pdGp-C8-IQ) (11) and 5-(deoxyguanosin- N^2 -yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline (pdGp- N^2 -IQ) (12). However, relatively large amounts of radioactivity were also present in the remaining three spots. When method I was applied for the analysis of IQ-DNA adducts, four spots were detected, and pdGp- N^2 -IQ was demonstrated to be resistant to the phosphatase activity of NP1. However, it was converted to pdG- N^2 -IQ with PNK, with or without NP1 (10). Thus, for IQ-DNA adducts, method II is appropriate, and by this method, spots of pdG-C8-IQ and pdG- N^2 -IQ, as well as a very small radioactive spot representing an unknown form of adduct, could be detected (10).

Recoveries with the modified methods are very close to those with the standard and intensification methods; in other words, very high after treatment with PNK, NP1, and PDE I. These results indicate that ^{32}P -labeled oligonucleotides have modified bases at the 5'-most position.

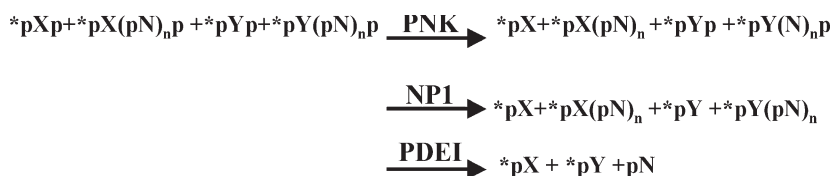


Fig.1. Principle of the modified method. *, ^{32}P -label; X and Y, modified deoxynucleosides; N, normal deoxynucleoside. 3' Phosphates of pXp and pX(pN)_np seem to show the same susceptibility to the enzymes. In method I, NP1 and PDE I treatments and in method II, PNK, NP1, and PDE I treatments are performed. NP1, nuclease P1; PDE I, phosphodiesterase I; PNK, T4 polynucleotide kinase.

A major advantage of these methods is that the number of adduct species formed in vivo and/or under in vitro conditions can be estimated by TLC.

2. Materials

2.1. DNA Digestion

1. 0.01X SSC, 0.1 mM EDTA: make as 1X SSC (0.15 M NaCl, 0.015 M Na-citrate), 10 mM EDTA. The solution can be stored at 4°C (*see Note 1*).
2. MN (Worthington, Freehold, NJ): dissolve in water to give 4 U/μL (*see Note 2*).
3. PDE II from bovine spleen (Worthington): dissolve in water to give 40 mU/μL.
4. Nuclease mixture: mix MN and PDE II solutions at a ratio of 1:1 to give a final concentration of 2 U/μL for MN and 20 mU/μL for PDE II.
5. Digestion buffer: 0.1 M sodium succinate and 0.05 M CaCl₂, pH 6.0. This can be stored at 4°C.

2.2. Postlabeling

1. [γ - ^{32}P]ATP with a specific activity of approx 260 TBq/mmol (~370 MBq/60 μL, e.g., ICN Biomedical, Irvine, CA).
2. 10 U/μL PNK (e.g., Takara Shuzo, Kyoto, Japan).
3. 10X Kination buffer: 0.3 M Tris-HCl, pH 9.5, 0.1 M dithiothreitol, 0.1 M MgCl₂, 0.01 M spermidine.
4. ATP solution: 200 μM ATP.
5. Kination solution A (10 μL used for each tube): 1.5 μL of 10X kination buffer, 1 μL of PNK, 5 μL of [γ - ^{32}P] ATP, and 3 μL of ATP solution (*see Notes 3 and 4*).
6. Kination solution B (5 μL used for each tube): 1.5 μL of 10X kination buffer, 0.5 μL of PNK, 1.5 μL of [γ - ^{32}P] ATP, and 1.5 μL of water.

2.3. Total Nucleotide Analysis

1. 20 mU/μL Potato apyrase (Sigma, St. Louis, MO).
2. Polyethyleneimine (PEI)-cellulose TLC sheets (95-mm height; Polygram CEL 300 PEI, Machery-Nagel, Duren, Germany; *see Note 5*).
3. 0.5 M LiCl.
4. Scintillation counter or BioImaging Analyzer (BIA; e.g., BAS2000 Fuji, Tokyo, Japan).

2.4. Digestion of Adducted Oligonucleotides by Method I

1. NP1: dissolve in water to give 1.6 U/ μ L (Yamasa Shoyu, Choshi, Japan).
2. PDE I: dissolve in water to give 20 mU/ μ L (Worthington).
3. Digestion buffer I: 0.3 M sodium acetate (or 0.13 M sodium citrate), pH 5.3. This can be stored at 4°C.
4. 1 mM ZnCl₂. This can be stored at 4°C.
5. 0.3 N HCl. This can be stored at room temperature.
6. 0.5 M Tris-base. This can be stored at 4°C.

2.5. Digestion of Adducted Oligonucleotides by Method II

1. NP1: same as in **Subheading 2.4., item 1.**
2. PNK: dissolve in water to give 10 U/ μ L (New England BioLabs, Beverly, MA).
3. PDE I: same as in **Subheading 2.4., item 2.**
4. Digestion buffer II: 0.2 M sodium citrate buffer, pH 5.7. This can be stored at 4°C.
5. 1 mM ZnCl₂: same as in **Subheading 2.4., item 4.**
6. 0.3 N HCl: same as in **Subheading 2.4., item 5.**
7. 0.5 M Tris-base: same as in **Subheading 2.4., item 6.**

2.6. Thin-Layer Chromatography of Labeled Adducts

1. PEI-cellulose TLC sheet (Po CEL 300 PEI, Macherey-Nagel), 20 \times 20 cm. Keep at 4°C.
2. Whatman no. 1 filter sheets.
3. D1: 2.3 M sodium phosphate buffer, pH 6.0.
4. D2: 3.4 M lithium formate, 6.4 M urea, pH 3.5 (*see Note 6*).
5. D3: 0.7 M NaH₂PO₄, 8.5 M urea, pH 8.0 (*see Note 6*).
6. D4: 1.7 M sodium phosphate buffer, pH 6.0

3. Methods

3.1. DNA Digestion

1. Dissolve DNA in 0.01X SSC, 0.1 mM EDTA at a concentration of 2 μ g/ μ L.
2. Transfer 5 μ L of the DNA solution, 1.5 μ L of water, 1.5 μ L of the nuclease mixture, and 2 μ L of digestion buffer to a 1.5-mL tube.
3. Incubate at 37°C for 3–3.5 h and centrifuge at 12,000g for 5 min at 4°C. Dilute a 2- μ L aliquot of the supernatant with 58 μ L of water (*see Note 7*).

3.2. ³²P-Postlabeling by the Standard Method or Adduct-Intensification Method

1. Standard condition: transfer an aliquot of 5 μ L of the diluted DNA digest and 10 μ L of kination solution A to a 1.5-mL tube, and incubate at 37°C for 1 h. Spin down in a microcentrifuge at 4°C (*see Note 8*). Proceed to **Subheading 3.3.**
2. Adduct intensification condition: transfer an aliquot of 5 μ L of DNA digest, 5 μ L of water, and 5 μ L of kination solution B to a 1.5-mL tube, and incubate at 37°C for 1 h.

3.3. Total Nucleotide Analysis

1. Transfer an aliquot of 2 μ L of the ³²P-labeled sample to a 0.5-mL tube, add 5.4 mU (3 μ L, 1.8 mU/ μ L) of apyrase, and incubate at 37°C for 45 min (*see Note 9*).
2. Add water to make a total of 250 μ L.
3. On a PEI-cellulose sheet, draw 1-cm² grids, 3 cm from the base.
4. Spot an aliquot of 5 μ L on a PEI-cellulose sheet and dry.

5. Develop with LiCl solution to the top edge.
6. Check separation of nucleotides (origin) from phosphate (Rf: approx 0.2) by exposure to an X-ray film for approx 3 min.
7. Carefully cut out the squares containing nucleotides. Place in scintillation vials, add 3 mL toluene cocktail, and count over the entire energy window (*see Note 10*).

3.4. Adduct Analysis by Method I

1. Adjust the pH of the remaining sample (13 μ L) of the incubate from **Subheadings 3.2., step 1 or 3.2., step 2** to approx 6.0 by adding 1.8 μ L of 0.3 *N* HCl.
2. To the tube, add 1 μ L of NP1 solution (1.6 U), 1 μ L of ZnCl₂ solution, and 1.5 μ L of digestion buffer I (pH 5.3) and incubate at 37°C for 10 min (*see Note 11*).
3. Adjust the pH to 8.0–9.0 by adding 3 μ L of 0.5 *M* Tris-base.
4. Add 1.5 μ L of PDE I solution (30 mU) to this tube and incubate at 37°C for 30 min. Proceed to **Subheading 3.6**.

3.5. Adduct Analysis by Method II

The 3'-phosphate of some adducts is resistant to NP1 phosphatase activity. In this case, prior PNK treatment is useful.

1. Adjust the pH of the remaining sample (13 μ L) of the incubate from **Subheading 3.2., step 1 or step 2** to approx 6.0 by adding 1.3 μ L of 0.3 *N* HCl.
2. Add 3 μ L of PNK solution (30 U) and 1.5 μ L of digestion buffer II pH 5.7 (the final concentration of citrate is 16 mM) to the tube and incubate at 37°C for 30 min.
3. Add another 1 μ L of PNK solution (10 U) and incubate at 37°C for 30 min.
4. Add 0.7 μ L of NP1 solution (1.1 U) and 1 μ L of ZnCl₂ solution, and incubate at 37°C for 10 min.
5. Adjust the pH to approx 8.0 by adding 3 μ L of 0.5 *M* Tris-base.
6. Add 1.5 μ L of PDE I solution (30 mU), and incubate at 37°C for 30 min.

3.6. TLC Analysis

Almost the same TLC conditions as those described in Chapter 1 are applicable, although migration distances differ from the adducted deoxynucleoside diphosphate case. Run D3 twice (*see Note 12*). Run D4 in the same direction as D3 after attaching a 35-mm Whatman filter paper to the top edge of the TLC sheet.

3.7. Imaging and Quantification

1. Visualize and quantify adduct spots as described in Chapter 1, **Subheading 3.7., step 1**.
2. Under standard conditions, calculate relative adduct labeling (RAL) according to the following equation:

$$\text{RAL} = \frac{\text{adduct radioactivity (cpm)}}{\text{radioactivity of total deoxynucleotide (cpm)} \times \text{fold dilution}}$$

3. For analysis under intensification conditions, calculate intensification factor (IF) according to the following equation:

$$\text{IF} = \frac{\text{RAL}_{\text{int}}}{\text{RAL}_{\text{std}}}$$

where RAL_{int} is RAL under intensification conditions, and RAL_{std} is RAL under standard conditions.

4. Notes

1. Ultrapure water prepared by passing through Milli-Q spUF is used.
2. All solutions should be stored at -20°C , except where otherwise stated.
3. To ensure thorough mixing, it is recommended that all tubes containing different components be vortexed and spun down in a microcentrifuge.
4. Protection from radioactivity during handling of radioactive samples is crucially important and should be performed as described in Chapter 1, **Note 8**.
5. Sheets can be stored at 4°C . Plates should be prerun with water overnight after attachment of a 12-cm filter paper wick and dried at room temperature.
6. The solvent used for IQ-DNA adduct analysis is indicated as an example. For D2, 4.5 M lithium formate, 8.5 M urea, pH 3.5 is prepared and diluted appropriately. For MeIQ-adducts 60% and for PhIP-adducts 80% solutions were used. For D3, 1.0 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 8.0, was used for MeIQ and PhIP.
7. When analysis is performed under intensification conditions (*see Subheading 3.2., step 2*), it is also necessary to perform the procedure outlined in **Subheading 3.2., step 1**, to determine the IF value of each adduct as written in **Subheading 3.7**.
8. After incubation in a tube, the contents should be spun down at 4°C .
9. Potato apyrase (20 mU/ μL) is diluted with water to give a concentration of 1.8 mU/ μL .
10. When BIA is used for quantification, a 500X dilution for the standard method (and a 50X dilution for the adduct intensification method), and exposure to X-ray film for approx 30 min are recommended. For BIA analysis, total and adduct analyses should be made on the same imaging plates.
11. This condition is appropriate for PhIP and MeIQ adducts, but the optimum pH may differ depending on specific adducts and it is necessary to check the optimum pH, which should be between 5.3 and 7.0.
12. The background usually becomes clean by running twice. Running once is enough in some cases.

References

1. Randerath, K., Reddy, M. V., and Gupta, R. C., (1981) ^{32}P -Labeling test for DNA damage. *Proc. Natl. Acad. Sci. USA* **78**, 6126–6129.
2. Gupta, R. C., Reddy M. V., and Randerath, K., (1982) ^{32}P -Postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis* **3**, 1081–1092.
3. Randerath E., Atrawal H. P., Weaver, J. A., Bordelon C. B., and Randerath K., (1985) ^{32}P -postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[a]anthracene. *Carcinogenesis* **6**, 1117–1126.
4. Wilson, V. L., Basu, A. K., Essigmann, J. M., Smith, R. A., and Harris, C. C., (1988) O^6 -alkyldeoxyguanosine detection by ^{32}P -postlabeling and nucleotide chromatographic analysis. *Cancer Res.* **48**, 2156–2161.
5. Randerath, K., Randerath, E., Danna, T. F., van Golden, K. L., and Putaman, L. L., (1989) A new sensitive ^{32}P -postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5'-monophosphates. *Carcinogenesis* **10**, 1231–1239.
6. Cameron, V. and Uhlenbeck, O. C., (1977) 3'-Phosphatase activity in T4 polynucleotide kinase. *Biochemistry* **16**, 5120–5126.

7. Frandsen, H., Grivas, S., Andersson, R., Dragsted, L., and Larsen J. C., (1992) Reaction of the N^2 -acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine with 2'-deoxyguanosine and DNA. Synthesis and identification of N^2 -(2'-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis* **13**, 629–635.
8. Fukutome, K., Ochiai, M., Wakabayashi, K., Watanabe S., Sugimura, T., and Nagao, M., (1994) Detection of guanine-C8-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine adduct as a single spot on thin-layer chromatography by modification of the ^{32}P -postlabeling method. *Jpn. J. Cancer Res.* **85**, 113–117.
9. Tada, A., Ochiai, M., Wakabayashi, K., Nukaya, H., Sugimura T., and Nagao, M., (1994) Identification of *N*-(deoxyguanosin-8-yl)-2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (dG-C8-MeIQ) as a major adduct formed by MeIQ with nucleotides in vitro and with DNA in vivo. *Carcinogenesis* **15**, 1275–1278.
10. Ochiai, M., Nakagama, H., Turesky, R. J., Sugimura, T., and Nagao, M., (1999) A new modification of the ^{32}P -post-labeling method to recover IQ-DNA adducts as mononucleotides. *Mutagenesis* **14**, 239–242.
11. Snyderwine, E. G., Yamashita, K., Adamson, R. H., et al., (1988) Use of the ^{32}P -postlabeling method to detect DNA adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in monkeys fed IQ: identification of the *N*-(deoxyguanosin-8-yl)-IQ adduct. *Carcinogenesis* **8**, 1739–1743.
12. Turesky, R. J. and Markovic, J., (1994) DNA adduct formation of the food carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline at the C-8 and N^2 atoms of guanine. *Chem. Res. Toxicol.* **7**, 752–761.



<http://www.springer.com/978-1-58829-084-7>

Molecular Toxicology Protocols

Keohavong, P.; Grant, S.G. (Eds.)

2005, XIV, 489 p., Hardcover

ISBN: 978-1-58829-084-7

A product of Humana Press