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The ^{32}P-Postlabeling Protocols for Assaying Levels of Hydrophobic DNA Adducts in Fish

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NOAA Technical Memorandum NMFS-NWFSC-14

The ^{32}P-Postlabeling Protocols for Assaying Levels of Hydrophobic DNA Adducts in Fish

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PREFACE

The Environmental Conservation (EC) Division of the Northwest Fisheries Science Center is evaluating biochemical parameters for use as markers of chemical contaminant exposure and early physiological effects induced by such exposure. A very promising approach is the use of the ^{32}P-postlabeling assay for determining levels of hydrophobic aromatic compounds bound to DNA (DNA adducts) in marine organisms. Recent publications from the EC Division have shown that the ^{32}P-postlabeling method can be used to detect and measure the levels of DNA modified by environmental genotoxic compounds in feral fish. These studies have shown that 1) the levels of hepatic DNA adducts in wild fish positively correlate with the concentrations of polycyclic aromatic hydrocarbons (PAHs) present in marine sediments, and 2) that a strong positive correlation is observed between sediment concentrations of PAHs and the prevalence of neoplastic lesions in liver of marine flatfish. In addition, laboratory studies with model PAHs and sediment extracts have shown that PAH-DNA adducts formed are persistent and have chromatographic characteristics similar to adducts detected in wild fish. These findings suggest that the levels of hepatic DNA adducts found in fish tissues may function as molecular dosimeters of exposure to potentially genotoxic environmental contaminants, such as carcinogenic PAHs. The ^{32}P-postlabeling assay is currently being used as a marker of exposure to potentially genotoxic contaminants in environmental monitoring studies, such as the National Benthic Surveillance Project of NOAA's National Status and Trends (NS&T) Program and in the Bioeffects Surveys of NOAA's Coastal Ocean Program. This NOAA technical memorandum describes in detail the ^{32}P-postlabeling method and its application to marine organisms.

CONTENTS

INTRODUCTION

The ^{32}P-postlabeling (PPL) method for detection of DNA adducts was developed in Dr. Kurt Randerath's laboratory in the early 1980s (Gupta et al. 1982) and has evolved substantially since then. Currently the ^{32}P-postlabeling technique is the most sensitive method for the detection of a wide range of compounds bound to DNA. For hydrophobic, aromatic DNA adducts, such as polycyclic aromatic hydrocarbon PAH-DNA adducts, this method can detect 1 adduct in 10^9-10^{10} bases by selective removal of unmodified nucleotides by enzymatic methods (Reddy and Randerath 1986) or by partitioning the DNA adducts into *n*-butanol (Gupta 1985). The DNA adducts are then phosphorylated via enzyme catalyzed transfer of ^{32}P-phosphate from [γ–^{32}P]ATP to the deoxyribose of the adduct. Further, the nonspecific nature of the ^{32}P-postlabeling assay allows for the detection of a wide range of bulky, hydrophobic compounds bound to DNA. This attribute coupled with the high sensitivity of the assay has led to the broad use of the ^{32}P-postlabeling assay in studies with mammals and fish for assessing exposure to environmental genotoxins (Dunn et al. 1987, Varanasi et al. 1989a, Liu et al. 1991, Poginsky et al. 1990, Ray et al.1991, Stein et al. 1992) and to specific genotoxic compounds, such as benzo[a]pyrene (BaP) and 7H-dibenzo[c,g]carbazole (DBC) (Randerath et al. 1984, Schurdak et al. 1987, Varanasi et al. 1989b, Sikka et al. 1991, Stein et al. 1993).

In 1987, we initiated studies using the ^{32}P-postlabeling assay for evaluating exposure of marine fish to environmental carcinogens in order to assess relationships between carcinogen exposure and the development of hepatic neoplasia, which are observed in several benthic marine species

from contaminated coastal areas of the United States (Johnson et al. 1993, Myers et al. 1993). To date we have analyzed hepatic DNA samples from more than 20 species of marine fish sampled from numerous reference and contaminated sites on the U.S. coast as part of ongoing NOAA Programs, such as the National Benthic Surveillance Project of the National Status and Trends Program and the Bioeffects Surveys of the Coastal Ocean Program. In addition, we have conducted laboratory studies to determine the kinetics of formation and removal of DNA adducts in fish (Stein et al. 1993) and to determine the identity of the adducts observed (Varanasi et al. 1989a). Moreover, these studies led to modifications in the ^{32}P-postlabeling assay to improve the application of this technique to feral fish.

The detection of DNA adducts by ^{32}P-postlabeling is a multistep sequence (Fig. 1) involving a series of biochemical reactions. Initially, DNA is hydrolyzed enzymatically to 3'-monophosphates. The digest is then enriched in xenobiotic-modified mononucleotides by the selective removal of normal nucleotides. Following the enrichment step the adducted DNA is enzymatically labeled at the 5'-hydroxyl position with [^{32}P]phosphate to form [5'-^{32}P]deoxyribonucleoside 3',5'-bisphosphates. Separation of the ^{32}P-labeled adducts is usually accomplished by two-dimensional, thin-layer chromatography (TLC) on polyethyleneimine (PEI)-modified cellulose sheets. Autoradiography or storage phosphor imaging (Reichert et al. 1992) is then used to locate the radiolabeled adducts on the chromatogram. The radioactivity on the chromatograms can then be quantitated by liquid scintillation spectrometry or storage phosphor imaging.

The accurate quantitation of individual adducts is dependent on the specificity and efficiency of the enzymes used, which can vary substantially for each type of adduct. Optimization of each enzymatic step for specific adducts is required to increase the accuracy of measuring individual adduct levels (Watson 1987, Gorelick and Wogan 1989). Currently, the method can be considered only semiquantitative in organisms, such as feral fish, exposed to complex mixtures of environmental contaminants that have not been fully characterized. However, because mixtures of chemical contaminants are poorly characterized, the ability of the ^{32}P-postlabeling assay to detect adducts of unknown structure is a key attribute.

In this NOAA technical memorandum, the procedures and equipment we currently use in our laboratories are described. Each section discusses the function and biochemical basis for a particular step and the methodology and the procedural pitfalls. The samples we typically analyze are hepatic tissues from fish; however, the method is applicable to any tissue from which DNA can be extracted. A glossary of abbreviations is provided at the end of the text.

LABORATORY SETUP

Facilities

A dedicated laboratory is used for the ^{32}P-postlabeling analysis. This laboratory contains a fume hood, sink, -20°C freezer, -80°C freezer and a refrigerator. The fluorescent ceiling lights all have 400 nm cutoff to reduce photodegradation of samples.

Equipment

Centrifugal Vacuum Evaporator

Savant Speed-Vac SVC100H[1] (Savant Instruments, Farmingdale, NY 11735) equipped with a glass cover (unaffected by organic solvents) for removal of solvents from samples held in the microcentrifuge tubes. A liquid nitrogen trap is placed between the Speed-Vac and the vacuum pump (Duoseal Vacuum Pump) to trap solvents coming off the samples.

Refrigerated Microcentrifuge

Eppendorf Model 5402 refrigerated microcentrifuge is used in postlabeling and DNA extraction procedures.

Tabletop Centrifuge

A Sorvall T6000B (DuPont) is used for centrifuging the Plexiglas carousels holding the radioactive samples during postlabeling.

Spreader for Making Thin-Layer Chromatographic (TLC) Sheets

A DeSaga TLC spreading device (Desaga 120305 or Whatman 49961-102).

Blender

A variable speed/pulse kitchen blender to mix PEI/cellulose solutions.

Radiation Monitor

A pancake-type radiation monitor (Technical Associates PUG 1 AB, Canoga Park, CA) is used for the detection of ^{32}P contamination in the laboratory (see section on Radiation Safety).

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[1] Mention of trade names is for information only and does not constitute an endorsement by the U. S. Department of Commerce.

Autoradiography Equipment

Any 14" x 17" metal autoradiography cassette will work. The cassettes are lined on one inner side with DuPont Cronex Lightning-Plus intensifying screens.

Darkroom

The darkroom is equipped with a safelight, a sink, developer, fixer, and waterbath trays for developing the 14" x 17" negatives.

Tissue Homogenizers

A Polytron PT 1200C with a 5 mm generator (Brinkmann Instruments, Westbury, NY) or a glass Dounce homogenizer (5 to 10 mL size) is used for tissue homogenization.

Waterbaths

Any standard tabletop waterbath (20-60°C range) can be used for incubations and enzyme hydrolyses. A separate waterbath is used for radiolabeled samples.

Thermomixer

Eppendorf Thermomixer 5436 from Brinkmann Instruments (Westbury, NY).

Plexiglas Carousels

Plexiglas carousels are used for holding the microcentrifuge tubes containing radioactive samples during incubations. Please see Reddy and Blackburn (1990) for design specifications.

Thin-Layer Chromatography (TLC) Tanks

Standard glass TLC tanks (inside dimensions 275 mm x 275 mm x 75 mm) can be used; however, if large numbers of samples are processed then the Plexiglas multisheet holders (see Reddy and Blackburn 1990 for specifications) are preferable.

Spectrophotometer

Shimadzu UV/VIS Model 2100. Quartz cuvettes with a 1 cm path length and 4 mm width are used for measuring DNA absorbances.

Analytical Balance

Mettler AC100 from Mettler Instrument Co., Hightstown, NJ.

Microcentrifuge Tubes

Any high quality microcentrifuge tube (0.5, 1.5 and 1.9 mL) will work; however, all tubes should be methanol rinsed. Occasionally a residue is present on the tubes that can inhibit enzyme activity.

Freezers

A -70°C (or colder) freezer must be used for the storage of all tissues and purified DNA. A -20°C freezer may be used for overnight storage of samples but never for extended periods.

Liquid Nitrogen Dewars

Used for quick freezing of tissue samples in liquid nitrogen.

Phosphorescent Ink Pens

A phosphorescent ink pen (NEN, DuPont) is used to mark the chromatograms so that the autoradiograms can be aligned with the chromatograms. The radioactive areas on the chromatograms can then be marked for excision.

Pipettors

We use both the Eppendorf and Gilson adjustable micropipettors in sizes of 10, 20, 100, 200 and 1000 µL. One set of pipettors is used for radioactive samples and a separate set is used for nonradioactive samples.

Shielding

See section on Radiation Safety.

Liquid Scintillation Counter

We use a Packard Instrument Company Model 1900 TR liquid scintillation counter (Downers Grove, IL).

Storage Phosphor Imaging System

Storage phosphor imaging screens (14" x 17", MD23-614) are scanned on a Model 425E PhosphoImager (Molecular Dynamics, Sunnyvale, CA). This system comes with 486-33 mHz computer for data processing.

Computer

A computer is necessary if you plan to use storage phosphor imaging technology to image and quantify the chromatograms generated by ^{32}P-postlabeling. The files generated are large (up to 41 MB) and it is recommended that a 386 SX or preferably a 486 PC with at least a 120 MB hard disk and 8 MB or more of RAM be used for data processing. A high quality monitor is essential for processing the images (such as Sony 1304 monitor). Currently, we are using a 486-33 MHz with a 330 MB hard disk, 48 MB of RAM, and a video accelerator card. With this configuration we are able to process and quantitate the data in a virtual RAM drive, which is three to six times faster than working off the hard disk.

pH Meter and Electrodes

Any quality pH meter is acceptable. For preparation of Tris buffers it is recommended that a calomel rather than a silver chloride pH electrode be used.

Gloves

For all laboratory work, disposable latex gloves are used (see Radiation Safety section.).

Hot-Air Blow Dryers

Any quality hair dryer will work for drying PEI-cellulose TLC sheets between chromatography steps.

Stirring Motor

A variable speed stirring motor is used for agitating the water in the rinse tanks used to remove salts from the chromatography sheets.

Automated DNA Extractor

We use Applied Biosystems Inc. (Foster City, CA) Genepure 341 Nucleic Acid Purification System.

CHEMICALS

Carrier-free (^{32}P)phosphate (NEX-053) was obtained from New England Nuclear Research Products (E. I. DuPont, Wilmington, DE) and carrier-free [γ-^{32}P]ATP (5-6x10^3 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The following were obtained from Sigma Chemical (St. Louis, MO): Bicine (B-3876), Tris (T-1503), CHES buffer (C-2885), urea (U-1250), spermidine (S-2626), dithiothreitol (D-0632), L-glycerol-3-phosphate (G-7886), adenosine 5'-diphosphate (A-6521), adenosine 5'-triphosphate (A-6144), 2'-deoxyadenosine 3'-monophosphate (D-3014), sodium pyruvate (P-2256), lithium chloride (L-0505), proteinase K (P-0390), nuclease P1 (N-8630), micrococcal endonuclease (N-3755), spleen phosphodiesterase (P-6897), apyrase (A-6132), RNase A (R-4875), RNase T_1 (R-8251), RNase T_2 (R-3751), polyethyleneimine in 50% aqueous solution (P-3143), and α-amylase (A-6255). Cloned T4 polynucleotide kinase (70031) was purchased from United States

Biochemical (Cleveland, OH). Cellulose powder (MN-301 is manufactured by Macherey Nagel in Germany, Brinkman 6610100-8) was obtained from Brinkmann, Westbury, NY. Molecular biology grade phenol was purchased from Boehringer Mannheim (Indianapolis, IN). Chemicals used on the automated DNA extractor such as phenol/chloroform/water, lysis buffer, chloroform and 5 M sodium acetate were purchased from Applied Biosystems Inc. (Foster City, CA). All other chemicals used were reagent grade or better.

Enzymes used for the synthesis of [γ-^{32}P]ATP were as follows: glycerol-3-phosphate dehydrogenase (127124), triosephosphate isomerase (109754), glyceraldehyde-3-P-dehydrogenase (105686), lactate dehydrogenase (127230), 3-phosphoglycerate kinase (108430), and β-NAD (127302); these were purchased from Boehringer Mannheim (Indianapolis, IN).

For preparation of assay reagents, we use either double distilled, deionized water or high performance liquid chromatography (HPLC) grade water. For preparation of reagents, please see appropriate sections.

RADIATION SAFETY

The ^{32}P-postlabeling assay uses large amounts of ^{32}P, which is an energetic beta emitter (1.7 MeV). Therefore, any person using this isotope must receive detailed instruction before handling ^{32}P and must be frequently monitored for exposure to ^{32}P. Since an inexperienced person may not realize how easily radioactivity is unintentionally spread, pretraining with a fluorescent solution may be necessary. A new person should perform laboratory operations using a fluorescent solution (i.e.

fluorescein, quinine sulfate) and then turn off the overhead lights and use a black light to reveal handling errors that would have resulted in unwanted spreading of radioactivity.

Important points to help minimize and monitor ^{32}P exposure:

- All employees who handle ^{32}P must wear a whole body film badge and a thermiluminescent type finger ring dosimeter on the inside of each hand where there is the highest potential for radiation exposure. Rings and badges are monitored monthly.
- All laboratory operations are planned to minimize the time spent handling radioactivity.
- Double latex gloves are worn while handling ^{32}P and they are frequently checked for radioactivity by passing them under a pancake-type radiation monitor. If gloves are constantly needing to be changed because of ^{32}P contamination, sample handling procedures must be reviewed and revised.
- Workers wear labcoats and use disposable sleeves.
- Laboratory working surfaces are checked frequently with the radiation monitor when handling ^{32}P. The monitor probe should be covered with a thin vinyl wrap to prevent contamination of the probe.
- After completion of work with radioactivity, the workers are to check themselves and their equipment with the radiation monitor. If any radioactivity is detected then they are to wash themselves and/or the equipment until free of radioactivity.
- A senior staff member experienced in handling radioactivity routinely checks the above points to ensure that correct procedures are followed.

Equipment for Handling ^{32}P

All ^{32}P is handled behind 10 to 13-mm Plexiglas shielding. In addition, samples are kept in Plexiglas containers that are at least 13-mm thick. Most of the Plexiglas equipment we use (e.g., carousels for holding the microcentrifuge tubes, shields for the pipettors, and racks for holding the chromatograms during chromatography and drying) is similar to that described by Reddy and Blackburn (1990). This equipment increases sample handling capacity, while substantially lowering radiation exposure risk.

Radioactive Waste and Disposal

Radioactive waste is temporarily stored in a remote corner of the laboratory in a 13-mm thick Plexiglas box that has a 1.5-mm thick lead foil covering (Josefsen et al. 1993). This container is emptied regularly and the ^{32}P waste is transported to a designated storage site for radioactive materials. Radioactive waste is stored in a locked shed in a 55-gallon steel drum labeled "^{32}P only." Once a drum is filled, it is sealed and dated. After 10 half-lives (143 days) the contents of the drum are scanned with a survey meter equipped with a pancake-type detector. If there is no reading on the survey meter then the contents of the barrel are disposed of in the regular waste.

Make sure that ^{3}H-, ^{14}C-labeled compounds and carcinogens are not put into the ^{32}P waste barrel.

For additional information on the safe handling of ^{32}P see Ballance et al. (1984) and Slobodien (1980).

POLYETHYLENEIMINE-MODIFIED CELLULOSE SHEETS

Satisfactory chromatography of hydrophobic DNA adducts can be achieved with either commercially available or laboratory-prepared polyethyleneimine-modified cellulose (PEI-cellulose) TLC sheets. We have found that the laboratory prepared PEI-cellulose TLC sheets can give sharper solvent fronts and that the spot resolution is generally superior to that obtained from commercial PEI-cellulose sheets. If you plan to do ^{32}P-postlabeling on a large scale, it is cost effective to make your own PEI-cellulose sheets. However, laboratory prepared sheets are not as durable as the commercial sheets and must be handled with greater care.

Commercial PEI-Cellulose Sheets

The performance of commercially obtained PEI-cellulose sheets can be improved by shaking the sheets gently for 2-4 minutes in reagent grade methanol to remove impurities from the manufacturing process. After the methanol wash, the sheets are shaken in distilled water for 10 minutes. The washed sheets are air dried **thoroughly** and wrapped with aluminum foil or placed in a sealed container for storage; they are stable at -20°C for at least 2 months. The PEI-cellulose sheets manufactured by Macherey Nagel (available through Alltech) are satisfactory for chromatography.

Preparation of PEI-Cellulose Sheets

The procedure used for preparation of PEI-cellulose sheets is based on the method of Randerath and Randerath (1964). Currently, we use PEI in a 50% aqueous solution (P-3143) purchased from Sigma Chemical.

Note: The cellulose used is produced by Macherey Nagel and is now called MN-301. This product is more difficult to work with than its predecessor MN-300.

Preparation of 0.5% PEI Solution

For 1 liter of solution, 900 mL of deionized distilled water (ddH_2O) is added to 10 g of a 50% PEI solution in a beaker. The pH is adjusted to approximately 6 with 6N HCl while constantly stirring. Initially, PEI is a clear, viscous solution at the bottom of the beaker, but as the pH is lowered the PEI will go into solution. After the PEI has dissolved and a pH of 6 has been reached, bring the solution to a final volume of 1 liter. This solution is stable for up to 4 months when stored at 0-4°C. The retentive characteristics of the PEI-modified cellulose sheets can be increased by raising the percentage of PEI present in the solution.

Preparation of Vinyl Backing for Chromatography Sheets

1. Scrub the **dull** surface of a matte finish vinyl sheet (8.5"x 50", 0.01" thick, Universal Plastics, Seattle, WA) with a commercial cleaner and rinse thoroughly with soft tap water or distilled water.
2. Place the vinyl sheet on a clean glass plate of the same dimensions (the glass plate provides a rigid backing for the vinyl strip) with the **dull** side facing up. Be sure that one of the long edges of the glass plate barely overhangs the countertop edge. This allows the TLC spreader to slide smoothly along the vinyl sheet.
3. Dry the vinyl sheet.
4. Set the TLC spreader opening at 0.4 mm.

5. Run TLC spreader along the vinyl sheet to check for smoothness and remove any gritty material that is detected either on top of the vinyl strip or between the vinyl strip and the glass plate.

Preparation and Pouring of PEI-Cellulose Slurry

1. Place 34 g of MN-301 cellulose in a blender.
2. Add 145 mL of a 0.5% PEI solution.
3. Pulse the blender a few times and then "liquefy" on low for 15 seconds.
4. Pour the PEI-cellulose slurry into a vacuum flask and turn on the aspirator. **Swirl the flask vigorously** while aspirating to prevent the PEI-cellulose slurry from foaming. This step may take 10 to 15 minutes because the PEI-cellulose slurry is slow to degas. **Removal of the dissolved gases is important because bubbles will affect adherence of the PEI-cellulose to the vinyl sheets and leave small holes in the cellulose matrix.**
5. Place the TLC spreader at one end of the vinyl sheet.
6. Pour the PEI-cellulose slurry gently into the spreader to avoid unnecessary generation of bubbles and stir gently with glass rod to remove any bubbles.
7. Turn the spreader handle and immediately spread the slurry in one slow steady movement along the vinyl strip. At the end of the vinyl strip turn the spreader handle back to the closed position. This step will require some practice to evenly spread the slurry. The slurry layer is usually thicker at the ends of the vinyl strip. If pinhead sized holes are present in the slurry layer, press these dry spots with a fine pointed tool which will cause the slurry to fill the void. Wait for

approximately 3 hours before removing the sheets from the glass plates.

8. Dry the PEI-cellulose sheets overnight.

Cutting, Washing and Storage of PEI-Cellulose Sheets

1. Cut the sheets into 20 cm widths with a paper cutter (do not use sections that were unevenly spread or have holes in them). The sheets can be trimmed to different dimensions to meet other chromatographic needs.
2. Place the sheets in tanks and develop to the top edge with ddH_20 overnight. Remove the tank cover and continue development for 2 to 4 hours. A faint, yellow, oily band is usually present at the top of the sheet. Trim off the top edge, including the oily band, and the bottom edge of the sheets to get 14 to 16 cm x 20 cm segments.
3. Dry the sheets completely (1-2 hours). If they are dried overnight, the chromatographic characteristics can change because of PEI breakdown.

   Caution: If the sheets are placed in the freezer slightly damp, the PEI cellulose may separate from the vinyl backing later during chromatography.
4. Wrap the dried sheets in foil and label them with the preparation date, the preparer's name, the cellulose lot number, and other pertinent information. The sheets are stable for 2-4 months at -20°C.

SYNTHESIS OF [γ-^{32}P]ATP

The [γ-^{32}P]ATP used for labeling DNA adducts can either be purchased or synthesized in the laboratory starting with carrier-free inorganic [^{32}P]phosphate ($^{32}P_i$) and adenosine diphosphate (ADP). Preparation of [γ-^{32}P]ATP from $^{32}P_i$ is substantially less expensive than purchasing commercial [γ-^{32}P]ATP.

The procedure used for preparing [γ-^{32}P]ATP is based on the method of Gupta et al. (1982) and Gupta and Randerath (1988) (also see Johnson and Walseth (1979) for the original method). We prepare a synthesis premix containing all of the components for making [γ-^{32}P]ATP, except the $^{32}P_i$. The premix is stable for 2-3 months at -80°C. Preparation of [γ-^{32}P]ATP can then be easily accomplished by adding the synthesis premix to carrier-free sodium [^{32}P]phosphate.

Preparation and Storage of [γ-^{32}P]ATP Synthesis Premix

To ensure the highest possible [γ-^{32}P]ATP specific activity (curies of ^{32}P/mmol of ATP):

1. Soak all glassware, pipette tips, microcentrifuge tubes and any other objects that come in contact with the chemicals and enzymes used to make the [γ-^{32}P]ATP synthesis premix in double-distilled, deionized water for several hours to remove nonradioactive phosphates. When nonradioactive phosphate is present, it will also be used to phosphorylate the ADP along with the radioactive phosphate, thus lowering the specific activity of the synthesized [γ-^{32}P]ATP.

2. Keep all components on ice when preparing the ATP synthesis premix and immediately freeze the aliquoted ATP synthesis premix at -80°C. This precaution will yield a product with a consistently high level of specific activity.

The following stock solutions are needed for preparation of the ATP synthesis premix: enzyme premix, reagent solution, and buffer solution. **Keep all these solutions on ice!**

Enzyme[2] premix (A):

200 μL of glycerol-3-phosphate dehydrogenase (2 mg/mL)

2 μL of triosephosphate isomerase (2 mg/mL)

40 μL of glyceraldehyde-3-phosphate dehydrogenase (10 mg/mL)

4 μL of 3-phosphoglycerate kinase (10 mg/mL)

40 μL of lactate dehydrogenase (5 mg/mL)

Reagent solution (B):

62.5 μL of 2 mM ADP[3] (**add last when making solution B**)

62.5 μL of 4.4 mg/mL sodium pyruvate

150 μL of 0.1 M dithiothreitol

250 μL of 0.5 M Tris, pH 9.0

125 μL of 2.4 mM l-glycerol-3-phosphate

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[2] These enzymes are shipped individually from the manufacturer as a suspension in an ammonium sulfate solution. Before taking an aliquot, the vials containing these enzymes must be agitated to resuspend the enzymes.

[3] The ADP solution is prepared just before use and kept on ice. We use a fresh, unopened package of ADP for each premix preparation. The ADP can partially decompose with time to give adenosine monophosphate (AMP) and inorganic phosphate. The inorganic phosphate can be used by the enzyme premix to make nonradiolabeled ATP, which can substantially lower the specific activity of the synthesized [γ-^{32}P]ATP.

125 μL of 10 mM β-NAD^+

100 μL of 0.3 M $MgCl_2$

875 μL Total volume

Buffer solution (C):

42 μL of 0.1 M dithiothreitol

21 μL of 0.5 M Tris, pH 9.0

375 μL of ddH_20

438 μL Total volume

The [γ-^{32}P]ATP synthesis premix is prepared from the stock solutions as follows:

1. Place 30 μL of enzyme premix A (shake to resuspend the enzymes before taking an aliquot) in a 1.5 mL microcentrifuge tube and centrifuge at 14000 rpm for 5 minutes. Carefully remove supernatant with a pipette. The ammonium ions in the supernatant can inhibit the T4-polynucleotide kinase used to phosphorylate the xenobiotic-DNA adducts.
2. Add 400 μL of buffer solution C to dissolve the precipitated enzymes in the microcentrifuge tube. Next add a 220 μL aliquot of this mixture to 875 μL of reagent solution B and vortex. **It is critical that this solution is kept on ice.**
3. Add 70 μL of the premix to labeled 0.5 mL microcentrifuge tubes held on ice. The premix aliquots are **immediately** stored at -80°C.

[γ-^{32}P]ATP Synthesis Procedure

Caution: When ordering $^{32}P_i$ from the manufacturer request a small delivery volume (100 μL or less), otherwise the synthesized [γ-^{32}P]ATP may be too dilute.

The acid-free/carrier-free $^{32}P_i$ sometimes arrives contaminated with $^{32}P_i$-polyphosphates formed from catenation of $^{32}P_i$-monophosphates. The presence of $^{32}P_i$-polyphosphates will reduce the quality of the autoradiograms obtained. To eliminate this problem before [γ-^{32}P]ATP synthesis, the $^{32}P_i$ solution from the supplier is acid-treated with 0.1 volume of 0.1 N hydrochloric acid (HCl) for 2 hours at room temperature, followed by addition of 0.055 volume of 0.2 M Tris base. One can order $^{32}P_i$ in dilute HCl; however, the acid concentration is variable and this can substantially affect the pH of subsequent enzyme reactions.

1. To start synthesis, 50 μL of premix is added to 5 millicuries (mCi) of carrier-free $^{32}P_i$ in a 100 μL of solution. Vortex and let stand for 1 hour at room temperature. The mixture is vortexed once or twice during the synthesis period.

   Note. New England Nuclear sends their $^{32}P_i$ (NEX 053) in a lucite container surrounded with lead that provides a substantial level of radiation protection and this container can be used for the synthesis and storage of the [γ-^{32}P]ATP.

   If less than 5 mCi of $^{32}P_i$ is used, then the volume of premix added is proportionally adjusted to the amount of $^{32}P_i$ used in the [γ-^{32}P]ATP synthesis.

2. After synthesis it is necessary to determine if the reaction has gone to completion. A 0.1 to 0.3 μL aliquot of the reaction mixture is

spotted on a PEI-cellulose sheet and developed in a lithium chloride (LiCl) solution (1.3 M LiCl for commercial sheets or 1 M LiCl for laboratory prepared sheets).

3. After development in LiCl, the chromatogram is dried and exposed to autoradiographic film. A 10 second exposure at room temperature is usually sufficient. There should be one strong spot due to [γ-^{32}P]ATP with an R_f (R_f = distance of the spot from the start/distance of the solvent front from the start) of 0.4 to 0.5 and a faint spot may be present from unreacted $^{32}P_i$ at an R_f of 0.9 (see Fig. 2a). This synthesis reaction normally goes to greater than 98% completion. If the autoradiogram indicates that a substantial amount of $^{32}P_i$ is still present, then vortex the [γ-^{32}P]ATP solution and let it stand for an additional 0.5 hour and/or increase the temperature to 37°C (Talaska et al. 1992). Reanalyze for reaction completion as described above. Usually this reaction will yield [γ-^{32}P]ATP with a specific activity of 2000 to 3000 Ci per mmol ATP. The [γ-^{32}P]ATP should be used within a few days (2-3) for labeling of DNA adduct samples.

Determination of [γ-^{32}P]ATP Specific Activity

The specific activity of the [γ-^{32}P]ATP is determined by labeling a known amount of 2'-deoxyadenosine-3'-monophosphate with [γ-^{32}P]ATP and separating the products by one dimensional chromatography. The 3',(^{32}P)5'-deoxyadenosine bisphosphate spot is located by autoradiography and then quantitated by either excising the spot and liquid scintillation spectrometry or by storage phosphor imaging (see section on storage phosphor imaging).

specific activity = Ci of ^{32}P associated with the 2'-deoxyadenosine-bisphosphate spot/mmol of 2'-deoxyadenosine-3'-monophosphate labeled.

Reagents

Dissolve a small amount of 2'-deoxyadenosine-3'-monophosphate (Sigma D-3014) in double distilled, deionized water. Determine the concentration at neutral pH by measuring the absorbence at 260 nm and using a molar extinction coefficient of 15400 liter/moles for a 1 cm path length (concentration = absorbence/extinction coefficient). Based on the absorbence values obtained dilute the solution to $1x10^{-4}$ M. Aliquot this solution into 0.5 mL microcentrifuge tubes and store at -80°C. This solution is stable for several months at -80°C.

Assay Protocol

1. A 10 µL aliquot of the $1x10^{-4}$ M 2'-deoxyadenosine-3'-monophosphate solution is diluted to 10 mL with ddH_2O to give a final concentration of $1x10^{-7}$ M.
2. To four replicate 0.5 mL microcentrifuge tubes add the following:
   5 µL of $1x10^{-7}$ M 2'-deoxyadenosine-3'-monophosphate
   5 µL of a [γ-^{32}P]ATP labeling solution made by combining 3 µL of 33 µCi/µL [γ-^{32}P]ATP (each replicate requires at least 20 µCi of [γ-^{32}P]ATP), 10 µL of kinase buffer (0.1 M bicine, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0), 10 µL of ddH_2O and 1 µL of 30 units/µL T4-polynucleotide kinase. **Keep on ice.**
3. The samples are mixed and incubated for 40 minutes at 37°C. **Because small volumes (<25 µL) are difficult to mix**

adequately by vortexing or shaking, one should centrifuge the tubes to bring all liquid to the tube bottom and then mix the samples with a micropipettor.

4. After incubation, add 390 µL of H_2O to each replicate tube, mix with pipettor and spot 10 µL from each tube on a PEI-cellulose sheet.
5. Develop the chromatogram in 0.3 M ammonium sulfate, 10 mM sodium phosphate, pH 7.4. Use 80% strength for laboratory prepared sheets. The R_f values are approximately 0.15 for [γ-^{32}P]ATP, 0.4 for 3'-,(^{32}P)5'-deoxyadenosine bisphosphate and 0.9 for inorganic phosphate (Fig. 2b).
6. After chromatography, locate the 3'-,(^{32}P)5'-deoxyadenosine bisphosphate spot by autoradiography.
7. Excise the spot, place it in a liquid scintillation vial and count by liquid scintillation spectrometry. The 3'-,(^{32}P)5'-deoxyadenosine can also be quantitated on the chromatogram by storage phosphor imaging.

Sample Calculation for Determining Specific Activity

The following information is needed: amount of 2'-deoxyadenosine-3'-monophosphate labeled in moles (i.e. $5x10^{-13}$ moles), the time at which the 3',(^{32}P)5'-deoxyadenosine bisphosphate spots were counted (i.e., 8/1/93 at 800 hours), the average disintegrations per minute (dpm) for the four replicates (i.e., 70,398 dpm), time the DNA samples were postlabeled (i.e. 8/5/93 at 1600 hours), the dilution aliquot factor (i.e., 40 if a 10 µL aliquot of a total volume of 400 µL was spotted on a PEI sheet), and the conversion factor from dpm to curies (1 curie = $2.22x10^{12}$ dpm).

Specific activity at 1600 hours on 8/5/93 =(dpm on 8/1/93) x (decay correction) x (dilution factor)/(moles of 2'-deoxyadenosine-3'-monophosphate).

Decay correction = $N/N_o = e^{(-0.693T/T_{1/2})}$

T = time elapsed (decay period)

$T_{1/2}$ = half-life for isotope used (14.3 days for ^{32}P)

N = dpm at time T

N_o = dpm at T_o

Specific activity at the time of sample labeling (8/5/93)

$= (70,398) \times \{e^{[-0.693 \times 4.33d/14.3]}\} \times (40)/(5 \times 10^{-13})$

$= 4.565 \times 10^{18}$ dpm/mol

= 2,057 Ci/mmol on 8/5/93

DNA ISOLATION

DNA isolation is a critical step in the ^{32}P-postlabeling method. Care in the isolation and handling of the DNA samples improves the chromatogram quality and substantially lowers the background radioactivity on the chromatograms.

Important DNA isolation points to emphasize are

- All tissue samples are frozen immediately after collection in liquid nitrogen or on dry ice.
- All tissue and purified DNA are stored at -80°C; use of a -20°C freezer for any length of time can yield DNA with breakdown products that co-chromatograph and interfere with the quantitation of DNA adducts

derived from hydrophobic compounds, such as PAHs (Fig. 3). After several months at -20°C, the yield of DNA from tissue samples starts to decline and eventually DNA is not recoverable, whereas, ^{32}P-postlabeling grade DNA can be extracted successfully from tissues stored at -80°C for at least 2 years.

- If the DNA samples are stored for an extended period before being postlabeled, storage of whole tissue is preferable to storage of extracted DNA. DNA appears to be stable in whole tissue for at least 2 years and extracted double-stranded DNA is stable in a Tris/EDTA buffer (pH 7.4) for at least 2 months at -80°C.
- High quality chemicals must be used for the extraction of DNA. Phenol and m-cresol must be redistilled before use, saturated with nitrogen, and stored at -20°C. However, Boehringer Mannheim sells a molecular biology grade of phenol (No. 100300) that has been redistilled and stored under argon which is suitable for DNA purification. Oxidation of phenol yields quinones that crosslink nucleotides resulting in chromatograms with high backgrounds that are difficult to quantitate.

The methods used for DNA extraction from tissues are based on the procedure of Reddy and Randerath (1987) which uses a nuclei precipitation step to minimize RNA contamination. In fish hepatic tissue there is often a component capable of being postlabeled present in the cytosolic fraction that can cause intense streaking on the autoradiograms. The nuclei precipitation step minimizes this problem. Also, the concentration of EDTA used in the homogenization step was raised to 100 mM to reduce the activity of endogenous DNAses; however, for the rest of the procedure EDTA concentrations do not exceed 20 mM. High levels of EDTA (100 mM)

were found to affect the UV absorbances of DNA solutions at 230 and 260 nm.

Manual Procedure

1. Thaw tissue and keep on ice.
2. Place 125-250 mg of tissue and 1.7 mL of 10 mM Tris/100 mM EDTA, pH 7.4 in either a glass culture tube (10 mm x 75 mm) and homogenize using a Polytron 1200C at setting 2 for 5-10 seconds or in a 7 mL glass Dounce homogenizer and homogenize using 5-10 slow strokes.

   Note: If homogenization is not performed gently, the nuclear membrane may be ruptured resulting in the loss of DNA during the nuclei precipitation step.
3. Transfer the homogenate to a labeled 1.9 mL microcentrifuge tube and keep on ice.
4. Centrifuge at 4°C for 10 minutes at 6,000 rpm to pellet the crude nuclei. If large amounts of lipid are present, centrifugation at 14,000 rpm for 3 to 5 minutes may be necessary to pellet the nuclei.
5. Decant and discard supernatant. If there is a ring of lipid left on the tube wall, use a cotton swab to remove it.
6. Resuspend pellet in 850 μL of 1% SDS/20 mM EDTA (pH 7.4) solution.
7. Add 4 μL of solution containing RNAses and α-amylase and incubate for 30 minutes at 37°C. The RNAses and α-amylase solution is prepared as follows: An appropriate aliquot of α-amylase stock solution is taken (20 μg of α-amylase per sample) for the total number of samples to be processed and placed in a 1.5 mL

microcentrifuge tube. The α-amylase is included in the RNAse mixture for hydrolysis of glycogen which will interfere with the UV absorbence readings. Centrifuge at 14,000 rpm to precipitate the α-amylase and remove the supernatant with a pipet. To the α-amylase precipitate add 2 μL per sample of 50 mM Tris-HCl, pH 7.4 and 2 μL per sample of a RNAse[4] solution which contains heat-treated RNAse A (10 μg/μL) and RNAse T_1 (10 units/μL). If the ^{32}P-DNA base analysis indicates that substantial amounts of RNA remain after RNAse treatment, then add 1 unit of RNAse T_2 per sample.

8. At the end of the RNAse incubation, the proteins are digested by the addition of 0.4 mg of proteinase K in 40 μL of 1 M Tris-HCl (pH 7.4) per sample. The samples are incubated for 30 minutes at 37°C with occasional mixing.

9. Proteins are removed by sequential organic solvent extractions as follows: Add one volume of the appropriate solvent (see Reagents for DNA isolation section), mix by inverting the tube repeatedly for 1 minute, centrifuge at 14,000 rpm for the recommended time length and remove the organic layer with a pipette. Use the solvent extraction sequence outlined below.

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[4] RNAse Solution. RNAse A must be heat-treated to deactivate any DNAses present. Dissolve RNAse A powder in ddH_2O to a concentration of 10 μg/μL, heat this solution in a water bath for 10 minutes at 90°C, and then cool slowly to room temperature. Sufficient RNAse T_1 is then added to the RNAse A solution to yield a final RNAse T_1 concentration of 10 units/μL. Aliquot and freeze the solution at -80°C. The solution is stable for at least 3 months.

Extraction	Reagent	Centrifuge (min)
1	phenol	7
2	phenol:chloroform:isoamyl alcohol, 25:24:1 (CIP)	5
3	chloroform:isoamyl alcohol, 24:1 (CIA)	3

Caution: Microcentrifuge tubes sometimes fail with the chloroform solvent systems and leakage can occur during centrifugation. To avoid this problem, the DNA solution (aqueous phase) is transferred to a fresh tube after the CIP and CIA extraction steps.

When handling phenol solutions wear gloves, a longsleeve labcoat, and protective glasses. Small amounts of phenol (<50 μL) can cause severe chemical burns.

10. Add 0.1 volumes of 5 M NaCl to the aqueous phase and invert the tube repeatedly for 30 seconds.
11. Add 1 volume of cold (-20°C) 100% ethanol and gently invert the tube repeatedly for 2 minutes to mix. The DNA will precipitate out of solution as a stringy gelatinous clump unless it is sheared. If the DNA is sheared, then try precipitating the DNA by placing the tube in a -20°C freezer from 20 minutes to overnight.
12. Centrifuge for 5 minutes at 14,000 rpm to pellet the DNA.
13. Wash the DNA pellet with 1 mL of 70% ethanol by mixing, centrifuging for 5 minutes at 14,000 rpm, and decanting the supernatant. Remove residual 70% ethanol by centrifuging for a few seconds and then using a pipette.
14. Resuspend DNA in 35-50 μL of 10 mM Tris/1 mM EDTA, pH 7.4 (TE) buffer depending on the size of the pellet recovered.

Caution: The DNA precipitate often can be difficult to dissolve. Sometimes after attempting dissolution, the DNA can be a large, clear, and colorless aggregate in solution that can be either inadvertently pipetted leading to an abnormally high UV absorbence reading or the aggregate may be missed by the pipette leading to a low UV absorbence value and an erroneous amount of DNA being used for the enzyme hydrolysis step. If this is a persistent problem, and heat and vortexing do not take care of it, then pass the DNA solution through a 26-gauge needle repeatedly to obtain a homogeneous solution.

15. Take 5 μL of DNA solution and dilute to 1 mL with TE and measure the absorbances against a TE blank at 280, 260 and 230 nm.
16. Determine the absorbence ratios of 260/230 and 260/280. They should be:

$A_{260}/A_{230} > 2.3$ $\qquad$ $A_{260}/A_{280} > 1.8$

These ratios will yield information about protein contamination but not RNA contamination. The chromatograms from the ^{32}P-DNA base analysis will give an estimate of RNA contamination (Fig. 4).

17. Calculate the concentration of DNA as follows:

Concentration (mg DNA/mL) = (A_{260})(dilution factor)/ (22.9 mL/mg DNA)

Example:

dilution factor = 1000 μL final volume/5 μL original volume = 200

absorbence (A_{260}) = 0.262 OD units

Concentration = 0.262 x 200/22.9 = 2.28 mg/mL

Automated DNA Extraction Procedure

Note: The volumes used in the automated procedure are different from the manual DNA extraction method.

This procedure was designed for use on the Applied Biosystems Inc. Genepure™ 341 Nucleic Acid Purification System.

1. Weigh out 125-250 mg of tissue and place it in a 7 mL glass Dounce homogenizer.

   Note: If homogenization is not performed gently, the nuclear membrane may be ruptured resulting in the loss of DNA during the nuclei precipitation step.

2. Add 1.7 mL of 10 mM Tris/100 mM EDTA, pH 7.4 and gently homogenize. The homogenate is then transferred to a 1.9 mL microcentrifuge tube and placed on ice.
3. Centrifuge at 6,000 rpm for 10 minutes at 4°C to precipitate the nuclei. If large amounts of lipid are present, centrifuge at 14,000 rpm for 3 to 5 minutes to pellet the nuclei.
4. Decant and discard the supernatant. If there is a ring of lipid left on the tube wall, use a cotton swab to remove it. Add 1.5 mL of 1% SDS/20 mM EDTA, pH 7.4 and resuspend the pellet using a glass Pasteur pipet.
5. Add 8 µL of solution containing RNAses and α-amylase and incubate for 30 minutes at 37°C. The RNAses and α-amylase solution is prepared as follows: An appropriate aliquot of α-amylase stock solution is taken (20 µg of α-amylase per sample) for the total number of samples to be processed and placed in a 1.5 mL microcentrifuge tube. Centrifuge at 14,000 rpm to precipitate the

α-amylase and remove supernatant with a pipet. To the α-amylase precipitate add 2 μL per sample of 50 mM Tris-HCl, pH 7.4 and 6 μL per sample of a RNAse solution which contains heat-treated RNAse A (10 μg/μL) and RNAse T_1 (10 units/μL).

6. Turn on the ABI 341 Nucleic Acid Purification System, check helium pressure gauges, reagent and waste bottle levels.
7. Prompt the machine to perform a self-test of its systems, then pressurize all bottles.
8. Applied Biosystems Inc.'s DNA isolation method No. 1 (the program commands DDig, DEx1, DEx1, DEx2, DPpt are used in this method) is used with the following modifications:
   - The proteinase K digestion is done at 37°C for 45 minutes.
   - All extractions are done at room temperature with function No. 66 (slow educe) separating the phases.
   - After the DNA has been precipitated onto the filter, only one wash with 80% ethanol is performed.
9. Remove filter paper with the DNA from the precipitette cartridge with forceps, place it in a 1.5 mL microcentrifuge tube, and add 600 μL TE. **Remember! Clean forceps between samples to prevent cross-contamination.**
10. The microcentrifuge tube is gently agitated on an Eppendorf Thermomixer for 15 minutes at 37°C to dissolve the DNA and then the filter paper is removed with forceps.
11. Add 0.1 volumes of 5 M NaCl to the aqueous phase and invert the tube repeatedly for 30 seconds.
12. Add 1 volume of cold 100% ethanol (stored at -20°C) and gently invert the tube for at least 2 minutes repeatedly to mix. The DNA will

precipitate out of solution as a stringy gelatinous clump unless it is sheared. If the DNA is sheared, then try precipitating the DNA by placing the tube in a -20°C freezer from 20 minutes to overnight.

13. Centrifuge for 5 minutes at 14,000 rpm to pellet the DNA.
14. Wash the DNA pellet with 1 mL of 70% ethanol by mixing, centrifuging for 3 minutes at 14,000 rpm and decanting the supernatant. Remove residual 70% ethanol by centrifuging for a few seconds and then using a pipette.
15. Resuspend DNA in 35 to 75 µL of 10 mM Tris/1 mM EDTA, pH 7.4 (TE) buffer depending on the size of the pellet recovered. Heat and agitate DNA on Thermomixer at 37°C for 5 minutes and then mix the solution with a pipette to dissolve DNA pellet.
16. Please see Manual Procedure section on how to measure DNA concentrations.

Reagents for DNA Isolation

Some of the reagents used with the 341 Nucleic Acid Purification System were purchased from Applied Biosystems Inc. If you choose to prepare your own reagents for use with the DNA extractor, these reagents must be filtered through a 22 µm filter before use. Please see DNA extractor manual for instructions on the preparation of reagents for use on the DNA extractor:

Following is a list of reagents for DNA isolation:

Phenol Reagent

454 g	distilled phenol	25 mL	distilled m-cresol
200 mL	2 M Tris, pH 7.4	1 mL	β-mercaptoethanol

130 mL distilled water 500 mg 8-hydroxyquinoline

- Prepare all organic based reagents in a fume hood.
- Place phenol, distilled water, 100 mL of 2 M Tris buffer (pH 7.4) and 8-hydroxyquinoline in a separatory funnel and shake vigorously for 20 seconds.
- Allow the phenol layer to settle out and separate the phases.
- To the phenol phase, add 100 mL of 2 M Tris buffer, 25 mL of m-cresol and 2 mL of β-mercaptoethanol.
- Bubble inert gas through the solution for 5 minutes to remove dissolved oxygen.
- Store aliquots of aqueous and phenol phases together at -20°C.

phenol:chloroform:isoamyl alcohol, 25:24:1, v/v/v (CIP)

250 mL of phenol reagent

240 mL of chloroform

10 mL of isoamyl alcohol

- Combine components and bubble inert gas through the solution for 5 minutes to remove dissolved oxygen.
- Store at -20°C.

chloroform:isoamyl alcohol, 24:1, v/v (CIA)

To make 250 mL combine:

240 mL of chloroform

10 mL of isoamyl alcohol

- Combine components and bubble inert gas through the solution for 5 minutes to remove the dissolved oxygen.
- Store at 4°C.

70% ethanol (v/v):

- Add 300 mL of distilled water to 700 mL of 100% ethanol and store at room temperature.

5 M NaCl:

- Dissolve 292.2 g of NaCl in distilled water and bring to a final volume of 1 L. Store at room temperature.

1% SDS, 20 mM EDTA:

- Add 8 mL of 500 mM EDTA (pH 7.4) to 180 mL of distilled water.
- Bubble inert gas through the solution for 5 minutes to remove dissolved oxygen.
- Add 2 g of sodium dodecyl sulfate, and mix with a stir bar.
- Check pH and adjust to 7.4. Bring to a final volume of 200 mL and store at room temperature.

10 mM Tris/100 mM EDTA, pH 7.4 (ultra TE):

1.211 g of Tris base

38.0 g of EDTA (tetrasodium salt)

- Dissolve the Tris base and EDTA in 800 mL of distilled water.
- Adjust pH to 7.4 with 6 M HCl.
- Bring to a final volume of 1 liter with distilled water.
- Bubble inert gas through the solution for 5 minutes to remove dissolved oxygen.
- Store at 4°C.

2 M Tris, pH 7.4:

For 1 liter of buffer combine:

850 mL of distilled water

242.2 g of Tris base

75 mL of concentrated HCl

- Dissolve Tris base in water.
- Slowly add HCl to the solution.
- Add additional 6 M HCl to adjust pH to 7.4.
- Bring to a final volume of 1 liter with distilled water.
- Store at 4°C.

500 mM EDTA, pH 7.4:

- Place 190 g of EDTA (tetrasodium salt) in 800 mL of distilled water.
- Adjust pH to 7.4 with 6 M HCl.
- Bring to a final volume of 1 liter with distilled water. Store at 4°C.

50 mM Tris, pH 7.4:

- Place 25 mL of 2 M Tris-HCl, pH 7.4 into 800 mL of distilled water.
- Adjust pH to 7.4 with 1 M HCl.
- Bring to a final volume of 1 liter with distilled water. Store at 4°C.

10 mM Tris/1 mM EDTA, pH 7.4 (TE):

1.211 g of Tris base

0.380 g of EDTA (tetrasodium salt)

- Dissolve the Tris base and EDTA in 800 mL of distilled water.
- Adjust pH to 7.4 with 1 M HCl.
- Bring to a final volume of 1 liter with distilled water.
- Bubble inert gas through the solution for 5 minutes to remove dissolved oxygen.
- Store at 4°C.

ENZYMATIC HYDROLYSIS OF DNA

Enzymatic digestion of xenobiotic-modified DNA to 3'-mononucleotides is a crucial step in the ^{32}P-postlabeling process. The levels of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD) now being used are lower than what was recommended in the original procedure (Gupta et al. 1982). Excessive amounts of MN can lower the recovery of some DNA adducts (Beach and Gupta 1992). The levels of MN present in the DNA digestion solution should be in the range of 0.15-0.35 μg MN/μg DNA. However, SPD concentrations do not appear to have a negative effect on adduct recovery and a concentration of 1 μg SPD/μg DNA works well (Beach and Gupta 1992). For samples containing DNA adducts derived from PAHs, incubation of DNA with MN and SPD for 3-6 hours at 37°C is sufficient. Overnight digestion of the samples can result in the loss of some aromatic amine adducts and a lower recovery of some PAHs. Moreover, normal nucleotides can degrade during a lengthy sample hydrolysis and yield breakdown products that may substantially raise the background radioactivity levels on chromatograms or interfere with the detection of some adducts (Gupta 1989). The choice of MN and SPD concentrations and length of DNA digestion is adduct-dependent, and digestion conditions should be optimized for specific needs.

Procedure for Digestion of DNA Samples to 3'-Monophosphates

The following enzyme hydrolysis protocol is for generating a 1-10 μg DNA sample for postlabeling.

All tubes used in this procedure must be rinsed with methanol, then with distilled water and thoroughly dried to remove any residual plasticizers, fungicides or other chemicals that may interfere with enzyme activities.

1. For each sample, place 25 μg of DNA in TE buffer into a 1.5 mL microcentrifuge tube and bring to a final volume of 15 μL with distilled water. If the DNA concentration is too low, then use less DNA or concentrate the DNA solution, if the situation permits, by adding 0.1 volume of 5 M NaCl and precipitate the DNA by adding 1 volume of cold 100% ethanol. Dissolve the precipitate in the appropriate volume of TE to give a 1.6 μg/μL DNA solution.
2. Add 10 μL of the MN/SPD in buffer to each sample.

   Preparation of MN/SPD in buffer:

   Based on the number of samples, place an appropriate volume (10 μL x number of samples) of SPD solution (2 μg SPD/μL) in a microcentrifuge tube and centrifuge at 14,000 rpm for 2 minutes. Remove the ammonium sulfate supernatant with a pipettor. Then add an appropriate volume (5 μL x number of samples) of dialyzed MN (1 μg MN/μL H_2O) solution and a volume (5 μL x number of samples) of buffer (20 mM sodium succinate, 10 mM calcium chloride, pH 6.0), respectively, to the SPD residue.

Preparation of dialyzed MN:

Dissolve MN in distilled water to give a concentration of 1 μg/μL. Place MN solution in a 10 mm wide dialysis tube and dialyze overnight (4°C) against distilled water (2,000 mL volume).

3. Briefly centrifuge the samples to bring the enzyme solution to the tube bottom and then mix with a pipettor.
4. Incubate samples for 3-6 hours at 37°C and vortex occasionally.
5. Remove samples from the waterbath and briefly centrifuge to bring the tube contents to the bottom. The enzyme hydrolysate can be stored for several days at -80°C without loss of adducts.
6. A 5 μL aliquot of each hydrolysate is placed in a 1.5 mL tube along with 495 μL of ddH_2O and vortexed. This sample is used for determination of the total amount of DNA hydrolyzed.
7. A 10 μL aliquot of the enzyme hydrolysate will be taken for the DNA adduct postlabeling assay.

DNA-ADDUCT ENHANCEMENT PROCEDURES

The normal ^{32}P-postlabeling method can detect DNA adducts in the range of 1 modification per 10^6 to 10^7 nucleotides. However, sensitivity for large, hydrophobic DNA adducts can be substantially improved to detect DNA adducts in the range of 1 modification per 10^9 to 10^{10} nucleotides with the use of adduct enhancement techniques. The two enhancement methods most commonly used are 1) extraction of the DNA-adducts into water-saturated n-butanol (Gupta 1985) and 2) selective enzymatic degradation of normal mononucleotides to

nucleosides using nuclease P1 (Reddy and Randerath 1986). For PAHs, nuclease P1 generally gives better recoveries than the butanol method and is easier to perform (Gupta and Earley 1988). However, some aromatic amine adducts (e.g., dG-C8 derivatives of 2-acetylaminofluorene, 2-aminophenanthrene and 4-aminobiphenyl) are substrates for nuclease P1 and may be lost; whereas, the butanol extraction method gives better recoveries for these adducts (Gupta and Earley 1988). For DNA from fish exposed to complex mixtures, the samples should be processed by both procedures to determine adduct levels and profiles. If both methods give similar results, then the less labor-intensive nuclease P1 procedure would be the method of choice.

Butanol Adduct Enhancement

The butanol adduct extraction procedure is based on the observation that mononucleotides modified with hydrophobic structures will preferentially partition into water-saturated n-butanol. The presence of the phase transfer agent, tetrabutylammonium bromide, can enhance the extraction of some structures (Gupta 1985). This method can process up to 100 μg hydrolyzed DNA (Beach and Gupta 1992). However, some of the benefits gained by using larger quantities of DNA will be offset by increasing background radioactivity on the chromatograms.

We normally run duplicate samples of the 3'-BaPDE-dG standard through the butanol extraction process as an extraction efficiency standard to assess procedural losses. If you are using this method for a specific adduct, it would be wise to run a standard of that specific adduct through the butanol method for a recovery estimate.

Samples which have been stored at -20°C for an extended time period or tissue that is partially decomposed may contain DNA breakdown products that interfere with the detection of DNA adducts when the normal lithium choride/Tris/urea (LTU) solvent systems are used for development in the D4 direction (see chapter on Chromatography for description of solvent systems and Fig. 3). The isopropanol/4N ammonia solvent system should be used instead of LTU system for D4 as these breakdown products will migrate with the solvent front and not interfere with the autoradiographic detection of adducts. Another alternative is to use the nuclease P1 method which removes most of these breakdown products before ^{32}P-labeling.

Procedure

1. Place an aliquot of the DNA enzyme hydrolysate (up to 40 μg of hydrolyzed DNA), 20 μL of 10 mM tetrabutylammonium bromide, 20 μL of 100 mM ammonium formate, pH 3.5, in a 1.5 mL microcentrifuge tube and add sufficient distilled water to bring the volume to 200 μL. The microcentrifuge tubes should be labeled in two places (i.e., top and side) to avoid loss of sample identification from an inadvertent butanol leak.
2. Extract the enzyme hydrolysate solution twice with 180 μL water-saturated double distilled *n*-butanol[5] by vortexing for 15 seconds and centrifuging for 2 minutes at 14,000 rpm. Transfer the butanol layer

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[5] To prepare the water-saturated *n*-butanol, place equal volumes of ddH_2O and *n*-butanol in a container and vortex vigorously. The top layer will be water-saturated *n*-butanol and the lower layer will be *n*-butanol-saturated water.

containing the DNA adducts (top layer) to a clean 1.5 mL microcentrifuge tube using a pipettor.

Do not transfer any water with the butanol phase. Also, be sure to change pipette tips after each sample to prevent cross-contamination.

3. Backextract the butanol fraction with 180 µL of n-butanol-saturated distilled deionized water by vortexing for 15 seconds and then centrifuging for 2 minutes at 14,000 rpm. This step removes any normal nucleotides which may have transferred into the butanol phase. For 1-2 µg DNA do 1 backextraction, 3-10 µg DNA do 2 backextractions and for 15-40 µg DNA do 3 backextractions. Discard the water extracts (the lower phase). Remove the butanol phase after the last water backextraction and place in a methanol rinsed 0.5 mL microcentrifuge tube.
4. The samples are placed in a Savant concentrator/evaporator (heater on the unit is set at 45°C) for 30 to 60 minutes until all the butanol has evaporated. A 1.5 mL microcentrifuge tube is used as a sleeve to hold the 0.5 mL microcentrifuge tubes in the Savant rotor. Add 100 µL of ddH_2O to each tube and vortex vigorously. Use the Savant concentrator/evaporator to remove the water in the tubes. Add 15 µl ddH_2O and **flick the bottom of the tube vigorously with your finger** to dissolve adduct residue. The samples are ready for postlabeling.

Nuclease P1 Enhancement

The nuclease P1 enhancement method is easy to use and based on the observation that chemically-modified DNA is resistant to the 3'-phosphatase activity of nuclease P1; whereas, normal nucleotides are hydrolyzed to nucleosides, which are not substrates for phosphorylation by T4-polynucleotide kinase. However, the method has several potential pitfalls. The degree of resistance of DNA adducts to nuclease P1 hydrolysis is dependent on the adduct type and the physical size of the adduct (Gupta and Earley, 1988). For some DNA adducts, a variable percentage of the adducts will be hydrolyzed during nuclease P1 treatment depending on the amount of nuclease P1 used and how long the samples were hydrolyzed. If one is targeting a specific DNA-adduct, it is necessary to assess its resistance to hydrolysis by nuclease P1 and adjust the procedure accordingly to maximize recovery.

It is important that both RNAse A and RNAse T1 are used in the RNA removal steps of DNA isolation, otherwise the autoradiograms from the nuclease P1 enhancement method may have substantial smears due to RNA contamination.

Procedure

1. Place 7 μL of a nuclease P1 solution containing 2 μL of 4 μg nuclease P1/μL, 0.9 μL of 1 M sodium acetate (pH 5.0), and 4.1 μL of 1 mM zinc chloride at the bottom of a 0.5 mL microcentrifuge tube. Then add 10 μL of DNA enzyme hydrolysate directly into the nuclease P1 solution by submerging the pipet tip containing the DNA into the

nuclease P1 solution, and pumping with the pipettor to mix the solution.

This is a critical step because if any of the hydrolysate escapes nuclease P1 digestion poor quality autoradiograms will be generated with a high background.

The final concentration of the nuclease P1 in the microcentrifuge tube is 0.5 μg/μL.

2. Incubate samples for 45 minutes at 37°C. Add either 3 μL of 0.5 M CHES buffer (pH 9.6) or 0.5 M Tris base to the hydrolysate, briefly centrifuge the tube contents to the bottom, and mix with a pipettor. This step raises the pH of the hydrolysate. The samples are now ready for the addition of the [γ-^{32}P]ATP solution for postlabeling.

^{32}P-POSTLABELING OF DNA ADDUCTS AND BASES

Xenobiotic-DNA adducts and DNA bases are postlabeled using T4-polynucleotide kinase (PNK) to enzymatically transfer ^{32}P from [γ-^{32}P]ATP to 3'-mononucleotides to form [5'-^{32}P]deoxyribonucleoside-3',5'-bisphosphates. Important points for successful labeling are 1) [γ-^{32}P]ATP is in excess of the DNA, 2) sufficient PNK is present to carry out the reaction, and 3) the [γ-^{32}P]ATP used has a high specific activity. We normally use 100 μCi of [γ-^{32}P]ATP with a specific activity of 2,000 to 3,000 Ci per mmol when labeling 10 μg of DNA that has gone through either the butanol or nuclease P1 enhancement procedure. It is important to have the concentration of [γ-^{32}P]ATP in the range of 0.8 to 1.6 μM to be on a labeling plateau for PAHs (Segerbeck and Vodicka 1993); however, for

other types of DNA adducts the concentration of [γ-^{32}P]ATP used may need to be higher (Beach and Gupta 1992).

The sensitivity of adduct detection increases with the specific activity of [γ-^{32}P]ATP used. However, 100 μCi of [γ-^{32}P]ATP with a specific activity of 6000 Ci per mmol contains approximately 16 pmol of ([γ-^{32}P]ATP+ATP) versus 33 pmol when the specific activity is 3,000 Ci per mmol (the lower specific activity is due to increased presence of nonradioactive ATP). This means that 200 μCi of [γ-^{32}P]ATP with a specific activity of 6,000 Ci per mmol is needed per sample to have the same starting molar concentration of ATP that 100 μCi of 3,000 Ci per mmol [γ-^{32}P]ATP would yield. We have found that a specific activity for [γ-^{32}P]ATP in the range of 2,000 to 3,000 Ci per mmol is satisfactory.

Labeling efficiency is also dependent on the amount of PNK used and the pH of the labeling medium. In a review by Beach and Gupta (1992), they report that the amount of PNK per sample used by various laboratories ranged from 2 to 68 units and was dependent on the compounds that were labeled. Segerback and Vodicka (1993) found that a labeling plateau for individual PAHs and PAH mixtures was reached when the PNK concentration in the samples exceeds 0.3 units/μl. At pH 8.0, PNK has some residual 3'-phosphatase activity, which can affect labeling efficiency. However, at higher pH levels (>pH 8.5) this phosphatase activity is almost nonexistent. Generally, in most procedures the labeling buffer pH is 9.5 before addition to a sample. However, the actual pH in the sample during labeling is usually lower because of the buffering capacity of components present in the enzyme hydrolysate. With BaPDE-dG adducts, maximum labeling occurred when the **actual labeling pH in solution** was approximately 8.8, and there was a substantially lower labeling level

of adducts when the actual pH at the time of labeling was 9.5 or 8.0 (unpublished data).

Procedure for ^{32}P-Postlabeling of DNA Adducts

This procedure is for 1-10 μg DNA samples that have gone through an enhancement step to remove normal nucleotides (i.e., nuclease P1 or butanol extraction).

1. Make sufficient [γ–^{32}P]ATP labeling solution for the number of samples being processed and then add 10 μL of this solution to each sample.

   Each 10 μL of [γ-^{32}P]ATP labeling solution will contain:

   - 100 μCi of 2000 to 3000 Ci per mmol of [γ-^{32}P]ATP
   - 8 units of PNK in a 50% glycerol solution
   - 5 μL of labeling buffer (0.1 M bicine, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0)
   - Additional labeling buffer to bring to a 10 μl volume
   - **Keep solution on ice until added to samples.**

2. Place the samples (be sure to cut off the caps of the 0.5 mL tubes) in a preheated (37°C) Plexiglas carousel and add 10 μL of [γ-^{32}P]ATP labeling mix to each tube using a micropipettor that has a Plexiglas shield (Reddy and Blackburn 1990).

3. Centrifuge the carousel briefly to concentrate the solutions at the bottom of the microcentrifuge tubes. Mix the contents of each tube using a pipettor. **Note: Pipet slowly to avoid forming ^{32}P aerosols that will contaminate the inside of the pipettor.** Place the carousel in a 37°C waterbath for 45 minutes.

Optional Apyrase Treatment: Potato apyrase can be used to degrade any unreacted [γ-^{32}P]ATP in solution. However, this step can be omitted when labeling large bulky hydrophobic adducts, such as PAHs (the solvent system used for D1 will remove all of the unreacted [γ-^{32}P]ATP). After incubation with [γ-^{32}P]ATP labeling mix, add 4 μL of potato apyrase solution (2.5 μg apyrase/μL) to each tube. Centrifuge to concentrate solution at the bottom of the tubes and mix using a pipettor. Incubate solution at 37°C for 30 minutes.

4. After incubation, centrifuge the carousel briefly to concentrate solution at the bottom of the tubes. After mixing with a pipettor, spot 5 to 20 μL of each hydrolysate slowly on the origin of a premarked PEI cellulose sheet that has a filter paper wick stapled to it (Fig. 5). Keep the spot as small as possible by applying the sample **slowly** to the sheet. During chromatography, the spots will expand and the smaller the spot size on application to the PEI-cellulose sheet the smaller and more intense the spot will be on the autoradiogram.
5. Please go to Chromatography of Xenobiotic DNA Adducts section for specific instructions on the chromatography procedures.

Note: Spot a small aliquot (<1 μL) on a separate PEI-cellulose sheet for development in 0.3 M ammonium sulfate and 10 mM sodium phosphate, pH 7.4. This step is necessary to verify that an excess of [γ-^{32}P]ATP was present in the sample at the end of the postlabeling. The absence of [γ-^{32}P]ATP at reaction completion usually means that the enhancement step (butanol or nuclease P1) was not successful in removing the normal nucleotides which consume [γ-^{32}P]ATP and that ^{32}P-labeling of samples may

have not have gone to completion. If an apyrase step is included in the procedure, then consider taking an aliquot of the reaction solution just prior to the addition of the apyrase; otherwise the apyrase will degrade all of the [γ–^{32}P]ATP remaining in the sample.

Procedure for ^{32}P-Labeling of Bases

1. Make enough [γ–^{32}P]ATP labeling solution for the number of samples being processed and then add 10 μL of this solution to 10 μL of each diluted sample of hydrolyzed DNA (5 μL of each DNA enzyme hydrolysate is diluted to 500 μL with ddH_2O for base analysis). Each 10 μL of [γ-^{32}P]ATP labeling solution will contain:
   - 8 units of PNK in a 50% glycerol solution
   - 7.3 μL of labeling buffer (0.1 M bicine, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0)
   - 0.5 μCi of [γ–^{32}P]ATP[6]
   - 2.4 μL of nonradioactive ATP solution (0.5 mg ATP/mL)[7]
   - additional labeling buffer to volume
   - **keep solution on ice until added to samples**

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[6] About 50 μCi of [γ-^{32}P]ATP will be sufficient radioactivity for labeling approximately 30-50 samples.

[7] The nonradioactive ATP solution is prepared by dissolving 10 mg of fresh ATP in 20 mL of ddH_2O to give a concentration of 0.907 nmol ATP/μL water. It is important that the nonradioactive ATP used to make this solution has not decomposed, otherwise, the spec. act. will be low. Also, the amount of radioactive [γ-^{32}P]ATP is negligible compared to the amount of nonradioactive ATP used and will not influence the calculation of ATP concentration.

2. To determine the specific activity of the [γ-^{32}P]ATP bases labeling mix:
   - Take two 5 μL aliquots and count using a liquid scintillation counter
   - Two 0.5 μL aliquots are chromatographed on a PEI-cellulose sheet using 1 M LiCl as an eluant. If decomposition of the [γ-^{32}P]ATP to $^{32}P_i$ and ADP has occurred, then two spots will appear in the autoradiogram. The lower spot (Rf 0.4) will be [γ-^{32}P]ATP and the upper spot (Rf 0.9) will be $^{32}P_i$. Excise both spots and count them by liquid scintillation spectrometry (LSS). The percentage of [γ-^{32}P]ATP in the sample is defined as:

     % [γ-^{32}P]ATP = {[γ-^{32}P]ATP dpm}/{[γ-^{32}P]ATP dpm + $^{32}P_i$ dpm}

   Note: This step for determining the % of [γ-^{32}P]ATP will not account for the breakdown of nonradioactive ATP.

   Specific activity of bases labeling solution =

   [(% radioactivity due to [γ-^{32}P]ATP) x (average dpm in a 5 μL aliquot of the bases labeling mix)]/ [concentration of ATP in the bases labeling mix aliquot]

3. Place 10 μL of the diluted enzyme hydrolysate (from the enzyme hydrolysis section) in a 0.5 mL microcentrifuge tube for DNA base analysis and place tube in a preheated (37°C) Plexiglas carousel (be sure to trim the cap off of the tube).
4. Add 10 μL of the [γ-^{32}P]ATP labeling solution to each tube. Briefly centrifuge the carousel to bring the contents to the tube bottom and then mix the samples using a pipettor. Samples are incubated for 45 minutes at 37°C.

5. After incubation, add 4 μL of potato apyrase solution (3-5 mg potato apyrase in 2 mL of distilled water) to each sample and then centrifuge briefly to concentrate the solutions at the bottom. The potato apyrase hydrolyzes unreacted [γ-^{32}P]ATP. Mix the samples with a pipettor and incubate for 45 minutes at 37°C. At the end of this incubation, centrifuge the samples briefly to bring the liquid to the bottom of the tubes.
6. Mix with a pipettor and then spot 10 μL from each sample 2 cm above the bottom of a PEI-cellulose sheet and space them about 2 cm apart across the sheet.
7. The PEI-cellulose sheet is developed in 0.3 M ammonium sulfate and 10 mM sodium phosphate, pH 7.4 (dilute the solvent to 80% strength if you are using laboratory prepared PEI-cellulose sheets).
8. At the end of the development dry the sheets and expose the chromatograms to film for 2 hours at room temperature in an autoradiography cassette. The sequence of spots from the origin in this solvent system are dG, dA, dC, dT and the spot at the top is $^{32}P_i$ (see Fig. 4). The approximate Rf values are 0.26, 0.46, 0.52, 0.69 and 0.84, respectively (values are for laboratory prepared 0.5% PEI-cellulose sheets). If other spots are present and adjacent to these four DNA base spots, this would indicate the presence of RNA contamination (see Fig. 4). Use the developed autoradiogram to locate the [5'-^{32}P]dpGp spot by laying the film over the PEI-cellulose sheet and mark the position of the [5'-^{32}P]dpGp spot on the chromatogram with a marking pen.
9. Cut out the marked spots using a razor blade and place the piece of the PEI-cellulose sheet containing the [5'-^{32}P]dpGp spot in a 20 mL

scintillation vial, add scintillation cocktail and count on the liquid scintillation counter. The 5 μL aliquot of the [γ–^{32}P]ATP bases labeling mix for specific activity determination should be counted at the same time as the dG spots. This way, all of the samples are counted at the same time and a decay correction does not have to be made.

If an imaging system for radioactivity is available, then image the chromatograms and process directly. The 5 μL aliquot of the [γ–^{32}P]ATP bases labeling mix for specific activity determination and the excised spots from the calibration strip used for calibrating the imaging system should be counted at the same time on the liquid scintillation spectrometer.

Please see section on autoradiography and storage phosphor imaging for further information.

CHROMATOGRAPHY

The choice of solvent systems for multidimensional chromatography of postlabeled DNA adducts is dependent on the type of adducts to be chromatographed and the removal of interfering ^{32}P-labeled compounds (i.e., normal nucleotides) that can contribute to the radioactive background on the chromatograms. Urea (used to reduce the interaction of hydrophobic molecules with the PEI-cellulose sheets), pH, and salt concentrations all influence the rate at which adducts migrate. Increasing the solvent strengths of the salts or urea, or both, will generally increase migration. However, as the solubility limits of the solvent components are approached, the risk of these components

precipitating out of solution onto the PEI-cellulose sheets increases rapidly. A consequence of this precipitation is the tailing of radioactivity on the chromatograms which can hamper quantitation. Concentrations of urea greater than 8.5 M should be avoided because chromatography times increase significantly without any appreciable improvement in adduct migration. Generally, we dip the edge of the PEI-cellulose sheet in water or the buffer component of the urea/buffer solvents before placing the sheet in the solvent to reduce the problem of supersaturation and precipitation of salts in the solvent front. For large hydrophobic structures derived from PAHs a 1 M sodium phosphate, pH 6.0, solvent is generally used for D1 to migrate normal nucleotides and nonspecific radioactivity off the PEI-cellulose sheets and onto paper wicks. However, for faster migrating and less hydrophobic structures, the phosphate concentration may have to be raised to 2.3 M sodium phosphate, pH 5.5, to get the DNA adducts to salt out of solution and onto the sheets. The lower pH (5.5) is necessary to minimize the problem of sodium phosphate crystallizing on the PEI-cellulose sheets when using phosphate solutions that are 1.7 M or greater. Also, raising the laboratory temperature and sealing the tanks will help to reduce this problem. However, increasing the ionic strength of the phosphate buffer will also cause some interfering ^{32}P-labeled structures to salt out onto the sheets, and the resulting autoradiograms may not be as clean (Beach and Gupta 1992). An optional clean-up chromatography step can be done in the D2 direction (Fig. 5) using 2.5 M ammonium formate, pH 3.5, although this step is not necessary when chromatographing bulky, hydrophobic DNA adducts like those derived from PAHs (>2 rings). The solvent systems for D3 and D4 are used to separate the large, hydrophobic, xenobiotic DNA adducts. The

most commonly used D3 solvent system for large, hydrophobic adducts on commercial sheets is 3.5 to 4.5 M lithium formate, 8.5 M urea, pH 3.5 (LFU). If the DNA adducts in D3 are extremely slow migrating then consider 3.5 to 4.5 M pyridinium formate, 8 M urea, and pH 3.4 (made by neutralizing formic acid with pyridine to desired pH). The traditional solvents for D4 are 1.6 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0 (LTU) or 0.8 M sodium phosphate, 0.5 M Tris, 8.5 M urea, pH 8.2 (PTU) for commercial PEI-cellulose sheets. When using laboratory prepared sheets, dilute the solvents to 80 to 90% of the strength used for commercial sheets. The PAH-DNA adducts are generally found within a diagonally shaped region when LFU is used for D3 and LTU is used for D4. If the diagonal radioactive zone (DRZ) formed on the chromatogram (as visualized in the autoradiogram) is strong with many intense overlapping spots, then the isopropanol:4N ammonia system is an excellent alternative for D4, because it will disperse the adducts over a larger area of the chromatogram (Fig. 6).

Another promising D4 solvent system is 0.08 to 0.4 M NH_4OH that gives a zone of adducts more disperse than LTU but less than the isopropanol/ammonia system (Spencer et al. 1993). In addition, this system develops rapidly, and chromatography for D4 is complete in approximately 0.5 hours. However, if the DRZ is weak, then the LTU system would be superior because the DNA adducts are compressed into a zone of overlapping spots that can be easily quantitated. Sometimes when autoradiograms are hazy, a final development (D5) in 1 M sodium phosphate, pH 6.8, can improve autoradiogram quality by removing some of the nonspecific radioactivity from the chromatogram.

Preparation of Chromatography Solvents

Some of the solvents used for chromatography are based on formic acid or acetic acid. The concentration of these solutions is based on anion concentration, for example 4 M lithium or ammonium formate, pH 3.5 would have 4 moles per liter of formic acid and sufficient lithium hydroxide (or ammonia) would be added to bring the pH to 3.5. Another important consideration is the type of pH electrode used. With Tris solutions, a calomel electrode is essential because Tris interferes with the standard silver/silver chloride electrode in alkaline media. Also, pH must be set at the temperature of chromatography because some buffers, such as Tris, have strong temperature dependent pH profiles. Small changes in pH above 8.0 can have a substantial effect on the migration of some DNA adducts.

Chromatography of Xenobiotic DNA Adducts

Note: Some researchers report that direct exposure to fluorescent light or stray sunlight can cause photodecomposition of benzo[a]pyrene-DNA adducts on PEI-cellulose sheets (R. Roggeband and M. J. Steenwinkel, pers. commun.). These findings indicate the need to use filtered light (400 nm cutoff) in the laboratory during chromatography.

The following protocol is for large hydrophobic DNA adducts.

1. Spot 5 to 20 µl of each ^{32}P-labeled sample slowly on the origin of a premarked PEI-cellulose sheet that has an 11-cm filter paper wick

(Whatman No. 17 CHR) stapled to it (see Fig. 5) and allow the PEI-cellulose to draw the moisture from the pipette tip. If the sample is spotted too quickly, a puddle will form around the pipette tip giving a larger initial spot size. The spots will expand during chromatography. Slow, careful spotting will give better resolution of spots on the autoradiograms and this will help appreciably with quantitation. After the spot has been applied, the sheet is placed in a multisheet TLC chamber (see Reddy and Blackburn 1990 for specifications) containing 1.0 M sodium phosphate, pH 6.0, and developed in the D1 direction overnight. The wick should be saturated with the D1 solvent at the completion of this step.

2. Remove the sheet from the TLC chamber and touch the bottom edge to a piece of absorbent paper to remove any chromatography solvent adhering to the bottom edge of the PEI-cellulose sheet. Cut the sheet at Line 1 (see Fig. 5) using a paper cutter. Handle the PEI-cellulose piece with the attached wick carefully, it will have greater than 98% of the radioactivity initially spotted on the chromatogram.
3. Hold the PEI-cellulose sheet on the side opposite from the cut edge and rinse it in a slow stream of cool tap water to remove loose specks of PEI-cellulose containing radioactivity. These specks are caused by the cutting process and may give false images on the autoradiograms. They are usually easy to detect on the autoradiograms because they are intense and have a sharp border.
4. The sheet is then soaked in a tank (a 2 gallon aquarium works well with the multisheet holders) with either distilled water or soft tap water to remove the salts from the chromatography solvent. Agitation of the water in the tanks with a stirrer will enhance the

removal of salts from the PEI-cellulose sheet. Faint light refraction differences next to the sheet can be seen as the salts leach off. Usually a 10 minute soaking is sufficient.

5. The sheet is placed on a drying rack and dried under a gentle flow of warm air using hair blowers.

   Note: Do not use extremely <u>hot</u> air to dry the sheet. We have observed cracking and peeling of the PEI-cellulose. It takes approximately 10 to 20 minutes for the sheet to dry. Be sure that the sheet is completely dry, otherwise solvent migration up the sheet may not be satisfactory in the next chromatography step.

6. Dip the bottom edge (approximately 1 cm) of the sheet in 0.45 M lithium formate, pH 3.5, or water, and the excess solvent on the bottom edge is wicked away with a paper towel. Develop the sheet in the D3 direction (Fig. 5) using a lithium formate-urea solvent system (8.5 M urea, 4.5 M lithium formate, pH 3.5, for commercial sheets or 85% strength for laboratory-prepared sheets). It usually takes 2 - 3 hours for the solvent front to reach the top of the sheet.

   Caution: The level of the liquid in the chromatography chamber must be below the sample application site on the PEI-cellulose sheets.

7. Remove the sheet from the TLC chamber and touch the bottom edge to absorbent paper to remove the solvent. The PEI-cellulose sheet must be held vertical until the solvent bead has been removed from the bottom of the sheet; holding the sheet in any other position allows the solvent bead to run across the sheet, and a displaced band of radioactivity will appear on the autoradiogram. Also, the solvent

bead along the bottom of the sheet must not be allowed to wet the PEI-cellulose side of any other sheet in the chromatography tank during removal.

8. Rinse with a slow stream of tap water then place the sheet in a tank containing distilled or soft water and rinse in the same manner as D1. Remove the sheet from the water tank and cut along Line 2 (see Fig. 5). Rinse the sheet with tap water to remove any loose cellulose flecks.
9. Place the sheet on a drying rack and dry under a gentle flow of warm air.
10. Dip approximately 1 cm of the bottom edge of the sheet in a 0.05 M Tris, 0.8 M lithium chloride buffer, pH 8.0, and wick the excess solvent with absorbent paper. Develop the sheet in the D4 direction (see Fig. 5) using 0.5 M Tris, 1.6 M lithium chloride, 8.5 M urea, pH 8.0 (85% strength for laboratory prepared sheets).
11. Remove the sheet from the TLC chamber and touch the bottom edge to a tissue. Rinse in the same manner as for the D3 step.

    Optional cleanup step. Staple a 5 to 7 cm filter paper wick (Whatman No. 17 CHR) to the PEI-cellulose sheet according to Fig. 7. Dip the edge of the sheet in distilled water and wick away excess water with paper towel. Develop the sheet in the D5 direction (see Fig. 7) using a 1.0 M sodium phosphate buffer (pH 6.8) overnight. This sodium phosphate development step may improve the autoradiogram by removing some of the residual nonspecific radioactivity. After removing the wick, place sheet in a tank containing water and rinse them in the same manner as D1.

12. Trim the sheet for placement in the cassette while it is still wet (see Fig. 7). Excise the origin to remove an intense spot of radioactivity that may interfere with quantitation. Rinse the sheet gently with tap water to remove any flecks of radioactive PEI-cellulose left by the cutting process. Place the sheet on a drying rack and dry under a gentle flow of warm air. Tape the dry chromatograms face down to a piece of 14" by 17" paper and put in a cassette for autoradiography.

    Please see section on autoradiography for further information.

Alternative D4 Solvent Systems

Isopropanol/4 N ammonia is an excellent alternative D4 solvent system that will breakup the strong diagonal radioactive zones that the LTU solvent system gives and disperse the individual adduct spots over a larger area of the chromatogram (see Fig. 6).

NOTE: The isopropanol/4 N ammonia solvent system may cause some cracking to occur in Plexiglas boxes and equipment. Use glass tanks for this solvent system.

The ratio of isopropanol/4 N ammonia (v/v) can vary from 0.8:1 to 2:1; 1.2:1 works well for large hydrophobic adducts on laboratory prepared sheets and 1:1 works for commercial sheets. When using the isopropanol/4 N ammonia solvent system for D4, the sheet is placed in 10 mM Tris base after the D3 water rinse step to soak for 3 to 5 minutes (this resets the pH of the sheet). The sheet is then rinsed under the tap and dried. A 2 cm filter paper wick (Whatman No.1) is then stapled to the top edge. A longer wick stapled to the top of the PEI-cellulose sheet will

increase the distance that the adducts migrate and this can be used as an additional variable along with the isopropanol/4 N ammonia ratio to control adduct migration on the chromatogram. Place the sheet in a TLC tank containing the isopropanol/4N ammonia, seal it with plastic wrap, and develop in the D4 direction. **Do not move the tank during development.** Development time is usually 2 to 3 hr. When the solvent has reached the top of the wick, remove the sheet from the tank and let the sheet with the attached wick air dry in a fume hood. Place the dry chromatogram with the attached wick in 1 M sodium phosphate (pH 6.0) for D5. The D5 is a necessary step for the isopropanol/4 N ammonia solvent system, otherwise a strong dark band may appear in the middle of the autoradiogram. Trim sheet (see Fig. 7) for autoradiography.

Dilute ammonia (0.08 to 0.4 M) is also an excellent solvent system for D4, and the dispersion of adducts on the chromatograms falls in between the LTU and isopropanol/4 N ammonia solvent systems. The dilute ammonia solvent systems also require the Tris base pretreatment and the attachment of a 2 cm wick as described for the isopropanol/4 N ammonia system. The development time for the dilute ammonia solvent system is 20 to 40 minutes. After D4, develop in the D5 direction in the same manner as was done for the isopropanol/4N ammonia system.

IMAGING AND QUANTITATION OF RADIOACTIVITY ON CHROMATOGRAMS

At present, there are several ways to quantitate radioactivity on chromatograms. The traditional "cut and count method" aligns the autoradiogram with the corresponding chromatogram and uses a marking

pen to outline the radioactive regions on the chromatogram. Radioactive regions are then cut from the chromatogram, placed in a liquid scintillation vial containing liquid scintillation cocktail, and counted by liquid scintillation spectrometry (LSS). Cerenkov counting, which measures the light emitted when a charged particle passes through water, can also be used; however, a correction for counting efficiency is necessary. In addition, areas near radioactive spots are excised and counted to obtain background corrections. However, background corrections based on this approach can be somewhat subjective and one must exercise careful judgment when the background correction is substantial relative to the spot or region of interest. Another analytical approach is the use of computer-aided imaging systems that will locate and directly measure the radioactivity on the chromatogram.

Autoradiography

Autoradiography is used to locate the position of ^{32}P-derived radioactivity on the chromatograms; and it provides information on the relative intensity of each individual adduct. However, there are restrictions on the interpretation of autoradiograms. First, film has a limited range of response (less than 300) and the range of linear response is less than 100. Multiple exposures are usually required for a complete set of autoradiograms to show both strong and weak regions of radioactivity present on the chromatograms. Moreover, the autoradiographic images of faint spots may be only 20 to 30% of their expected densities based on the level of radioactivity present. Because of

these limitations, caution is required when making assumptions on the relative intensity of faint spots and the levels of background radioactivity present based on a visual inspection of the autoradiogram. Autoradiography of the chromatograms either can be run at room temperature or film sensitivity can be enhanced by a factor of up to 18 with the use of intensifying screens at -80°C (Swanstrom and Shank 1978).

Procedure

Handle the chromatograms carefully to avoid any flaking or loose particles being generated that are radioactive and could leave confusing images or spots on the autoradiograms.

1. Tape the chromatograms to a 14" x 17" piece of paper. The chromatograms can be taped to the paper with the vinyl side up if the samples are labeled with ^{32}P. If the chromatograms are placed on the paper with the cellulose side up, then cover with a thin plastic wrap. Be sure to make a map of the chromatogram placement on the paper sheets for future reference. The chromatograms should be marked with fluorescent ink so that they can be aligned with the autoradiograms later to be marked for cutting. If the intensifying screen surface in the film cassette is dirty, clean with Kodak screen cleaner.
2. The chromatograms are placed in a cassette and taken to the darkroom. **All darkroom operations must be done using a photographic safelight.** Add autoradiography film to each cassette, marking each film with a different scissor cut. For most samples, film exposure in the presence of intensifying screens for

2 to 72 hours at -80°C is satisfactory. For extremely radioactive samples, autoradiography at room temperature for up to several hours is often sufficient.

3. The cassette is removed from the -80°C freezer and brought to room temperature; this is done to prevent the film and PEI-cellulose sheets from cracking when the cassettes are opened.
4. In the darkroom remove the film from the cassette and place it in a tray containing Kodak GBX developer. The aqueous concentration of the developer should be about 5%. Development time varies from 2 to 4 minutes. The film is removed from the developer with tongs and rinsed in a flowing waterbath for at least 30 seconds. The film is then transferred to a tray containing Kodak GBX fixer at a concentration of 5%. Total time for fixing is 5 minutes. Do not allow fixer to accidentally get in the developer tray as it will ruin the developing solution. Remove film from the fixer and rinse it in a flowing waterbath for at least 5 minutes to remove all chemicals. To obtain high quality autoradiograms, it is important to use developer and fixer that have been made recently. As these solutions get older, the rate at which the film is developed or fixed slows down and the background "grayness" of the autoradiograms gets darker. The lights can be turned on now.
5. The autoradiograms are hung to dry and then labeled. Each autoradiogram should have the following information on it: date of autoradiography, length of exposure, temperature of film exposure, and identification of each individual chromatogram.

Photography of Autoradiograms

We use a 4 by 5 inch format Polaroid camera mounted on a stand and Polaroid type 55 positive/negative film for making prints of individual autoradiograms. Satisfactory prints can be made using a 35 mm camera and Kodak TX200 film. However, the larger format of the 4 by 5 inch Polaroid type 55 film yields a sharper print.

1. Place the autoradiogram on a lightbox that has a uniform light distribution.
2. The image is brought into focus through the Polaroid viewfinder. The viewfinder on the Polaroid camera has a centimeter scale which helps to define the print image size and is useful when more prints need to be made. The camera settings are normally f-4.5 for aperture and the shutter speed varies from 1/30 to 1/8 second. These settings can vary because of differences in lightbox intensity, film speed, the distance between the lens and the autoradiogram, and the quality of the autoradiogram.
3. After the picture has been taken, remove the film from the camera and develop for 20 to 30 seconds. Peel the paper apart to get the print and immediately wipe the surface of the print with fixer to set the image. The film pack also contains a negative which needs to be treated in a sodium thiosulfate bath immediately if you want a negative for making enlargements (see directions in the filmpack for preparing negatives).

Quantitation of Radioactivity by the "Cut and Count" Method

Place the autoradiogram on the chromatogram and align it with the fluorescent ink marks. If you do not have fluorescent ink marks to align

the chromatogram, then use the solvent front edges on 2D autoradiograms to align the autoradiogram with chromatogram. Carefully outline the regions of interest including background areas on the chromatogram with a marking pen.

A map is drawn of the radioactive regions that are to be excised and each region is numbered. This will give you a permanent record of areas quantitated and their respective positions on the autoradiograms. The regions are then excised using a sharp razor blade or a scalpel. Each of the excised chromatogram pieces is weighed (this allows a background correction to be made per unit weight of chromatogram) and placed in a 20 mL scintillation vial. Add 10 mL of scintillation cocktail to each vial and count.

Liquid Scintillation Spectrometry

Liquid scintillation counting has some restrictions. Samples containing low levels of radioactivity (i.e., faint spots and samples for estimation of background corrections) must be counted for longer time periods for adequate counting statistics. Counting statistics are based on total counts measured and not the counting rate (i.e., cpm or dpm). The following numbers give a comparison between total counts and % error (i.e., total counts / % error): 200 / ±14%, 500 / ±9%, 1000 / ±6%. Also, the liquid scintillation spectrometer has an inherent background of 20 to 25 cpm. A small variation of 3 to 10 cpm in the estimation of background per cm^2 of surface (easily achievable because of poor counting statistics for samples with low radioactivity levels) can greatly affect adduct level computations. For single, well-resolved spots, one can use a clean,

adjacent area to define background. However, for diagonal radioactive zones, which often have a faint hazy area around them (the haze may be due to adducts), the question of where to sample for defining background corrections becomes highly subjective. The subjective aspect of defining background from a remote part of the chromatogram points to the need to do the chromatography and measurement of radioactivity carefully. Moreover, proper handling and storage of tissue and purified samples is important, because DNA breakdown products will raise the overall background.

The liquid scintillation counting efficiency for ^{32}P in weakly quenched solutions is 98 to 99% which means cpm is essentially the same as dpm.

Correction for background is as follows:

corrected cpm (region of interest) = cpm (region of interest) - [wt (mg) of region of interest x (background cpm/wt of background region in mg)].

Cerenkov Counting

An alternative to counting samples by LSS is Cerenkov counting which is cheaper because it does not require the use of scintillation cocktail and eliminates disposal problems associated with some organic-based cocktails. However, the counting efficiency for radioactivity can be substantially lower. A correction factor for counting efficiency can be made by counting aliquots of ^{32}P in both liquid scintillation cocktail and water. Since the counting efficiency in the cocktail is approximately 99%, the ratio of cpm-cocktail to cpm-water will give a correction factor for converting Cerenkov counts to dpm.

Storage Phosphor Imaging

We are currently using storage phosphor imaging technology to locate and measure radioactivity associated with the DNA adducts present on the chromatograms. This technology offers high sensitivity (approximately 0.2 nmol adduct/mol nucleotides), extremely low counting background, a large linear range of response to radioactivity (10^5 to 1), and sufficient data points to allow accurate mapping and quantitation of radioactivity using computer image analysis methods. (For an in-depth discussion on storage phosphor imaging see Reichert et al. 1992). An additional benefit of this imaging system is that, unlike radioactivity measurements by liquid scintillation spectrometry, all raw data for the distribution and intensity of radioactivity on the chromatograms is available for review and reprocessing at any time in the future.

Scanning the Chromatograms

The imaging screens are first erased using an Image Eraser (Molecular Dynamics, Sunnyvale, CA). Storage phosphor imaging is performed at ambient temperature by placing storage phosphor screens (Molecular Dynamics, Sunnyvale, CA) over the radioactive chromatograms and exposing for a specified time period. A factor to convert the screen signal to dpm is generated by imaging a test strip containing a serial dilution of a ^{32}P solution concurrently with the samples. The signal response from the screen is then divided by the ^{32}P dpm in the test spots to generate a factor for converting the imaging screen signal response directly to ^{32}P dpm. The length of screen exposure to a chromatogram is dependent on the maximum levels of radioactivity present and is usually

about one-tenth the time required for autoradiography. Generally, for a 10 μg DNA sample a 6 to 24 hr exposure is sufficient. The chromatograms for DNA bases and the specific activity analyses require only a 10-minute exposure. Saturation of the screens occurs as the pixel values approach 80,000 and is evident when the radioactivity profiles of lines drawn through radioactive areas on the chromatogram images have a truncated appearance. However, this is a problem only with extremely "hot" samples where the radioactivity is concentrated in a small area. After exposure of the chromatograms to the screens, the latent radioactivity image is read by scanning the screen with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Data Storage and Computer Requirements

Image data from the PhosphorImager is stored on a 620 MB optical disk (Pioneer DEC-702 rewritable optical disk, Upper Saddle River, NJ) using a Pioneer rewritable optical disk drive (Upper Saddle River, NJ). Data are then processed on a 486-33 MHz computer equipped with a 330 MB hard drive, 32 bit local bus and 48 MB of RAM. A Diamond Stealth VRAM video accelerator card is used to increase the rate of screen repainting. Because the file sizes generated by the imaging process are up to 41 MB, it is desirable to place the files in a virtual RAM drive where processing is 3 to 6 times faster. This computer, as configured, processes large data files quickly and efficiently. The files can be processed on a 386-33 MHz; however, there is a substantial increase in data processing time. Software used to process the data is ImageQuant (version 3.15) from Molecular Dynamics.

Processing of Image Analysis Data

1. Restore the image file from the tape cartridge or other data storage device to the hard disk. If the file is on an optical disk, you can work directly from this drive, although processing will be slower.
2. If your computer has sufficient RAM, then use ImageQuant to copy the image file into a virtual RAM drive.
3. Adjust the size of the image using the "magnification"[8] command.
4. Choose the desired color scheme. If the resolution of the color image is poor, then try the gray scale.
5. The "color range" of the image can be optimized by drawing a "line object" through the strongest and weakest radioactive regions on the chromatogram and creating a profile of radioactive intensity along this line. From this graph, determine both the pixel values of the strongest spot that you are interested in and the weakest response. Set the color range using these values as a starting point for your maximum and minimum color values.
6. Draw an "object" around the region of radioactivity you wish to measure and give it a "name."
7. Determine the background value to be subtracted from your "object" by drawing "segments" through the area that surrounds the "object" that represent background. The "define background" command is used to obtain an average pixel value along each segment. From this information determine a value to be used as background.
8. "Integrate volume" for your region. (The background value will automatically be subtracted from each pixel in your "object".)

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[8] Terms in quotes represent commands in ImageQuant

9. Transfer the table of values to a spreadsheet program. See Table 1 for a typical Excel spreadsheet after the data have been transferred from ImageQuant software.

Calculations

The ^{32}P-postlabeling results are usually presented as a ratio of the number of adducts detected divided by the amount of DNA used in the assay (e.g., nmol DNA adducts/mol DNA, amol DNA adducts/μg DNA, fmol DNA adducts/μg DNA). Presenting the data in this fashion avoids the problem of not being able to extract DNA quantitatively from tissues.

It is necessary to keep track of all numbers used in the final calculations. It is also important that your laboratory protocols assure that the necessary information regarding aliquot sizes and dates when samples are counted is recorded. See Table 1 for sample calculations that include the raw data from the imaging system (in Microsoft Excel 4.0 format).

Sample calculations

Calculation for total DNA adducts measured:

fmol adduct(s) = dpm for DNA adduct spot (or zone) x labeling aliquot correction factor x (1/specific activity of (γ-^{32}P)ATP).

Where:

dpm for DNA adduct spot (or zone) = 2130

aliquot correction factor = 2 (15 μL spotted on chromatography sheet of a 30 μL ^{32}P-labeled DNA sample)

specific activity of (γ-^{32}P)ATP = 2000 Ci/mmol = 4400 dpm/fmol

fmol adduct(s) = 2130 x 2/4400 = 0.968 fmol

Calculation for total DNA analyzed for adducts:

nmol DNA = dpm for DNA base spot (dG) x factor to convert from nmol dG to nmol DNA x spotting aliquot factor x DNA enzyme hydrolysate dilution correction factor x (1/specific activity of DNA bases labeling (γ-^{32}P)ATP).

Where:

dpm for DNA base spot (dG) = 24600 dpm

factor to convert from nmol dG to nmol DNA = 100% / % of dG in DNA
= 4.76

spotting aliquot factor = 2.4 (10 µL spotted on chromatography sheet of a 24 µL ^{32}P-labeled DNA bases sample)

DNA enzyme hydrolysate dilution correction factor = (500 µL dilution volume/10 µL taken for base labeling) x 2 (to change from 5 µL of enzyme hydrolysate used for measuring DNA content to 10 µL of hydrolysate used in the DNA adduct labeling part of the assay) = 100

specific activity of DNA bases labeling (γ-^{32}P)ATP = 968055 dpm/nmol

nmol DNA = 24600 dpm x 4.76 x 2.4 x 100 x 1/(968055 dpm/nmol)

nmol DNA = 29 nmol DNA used in the DNA adduct assay

Calculation of DNA damage level:

nmol DNA adducts/mol DNA= amount of DNA adducts measured in a sample/amount of DNA used.

= 0.968 fmol DNA adducts/29 nmol DNA

= 968 amol DNA adducts/29 nmol DNA

x ($10^9/10^9$)

nmol DNA adducts/mol DNA = 33 nmol DNA adducts/mol DNA

After the calculations are completed, the results are reviewed for accuracy. An important check for computational or data errors is to compare the final numbers with the autoradiograms. The numbers should vary directly with the intensity of the autoradiograms provided that comparable amounts of DNA were used in the assay.

QUALITY ASSURANCE/QUALITY CONTROL

The PPL method is an involved procedure that requires numerous determinations to be made that are dependent on enzyme efficiencies and radioactivity measurements. The method should be considered semiquantitative when working with tissue samples from animals exposed to complex mixtures, because the identities of the adducts formed from complex mixtures are unknown, and it is not possible to simultaneously optimize the conditions for all adducts present. However, if sets of samples are run under uniform conditions, then meaningful comparisons between samples collected from different sites can be made. To have confidence in the results, appropriate controls must be used to signal the presence of significant errors. The following are quality assurance procedures that should be included in the ^{32}P-postlabeling assay:

1. Salmon testes DNA is used for measuring the efficiency of DNA hydrolysis and as a sample blank throughout the assay (chromatography and reference standard).
2. The compound 7R,8S,9S,10R-(N^2-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[a]pyrene (BaPDE-dG-3'p) is used as an external standard to monitor the efficiency of enzyme-mediated transfer of the ^{32}P-phosphate from [γ-^{32}P]ATP to polycyclic aromatic hydrocarbon derived DNA adducts. The concentration of the stock BaPDE-dG-3'p from the manufacturer should be verified by postlabeling the BaPDE-dG-3'p with ^{32}P and using the specific activity of the [γ-^{32}P]ATP to determine concentration. The concentration of the BaPDE-dG-3'p determined from the ^{32}P labeling should agree with the manufacturers stated concentration. The radioactivity measurement of the chromatographed spot will give information on the efficiency of ^{32}P labeling and can be used to verify the specific activity of the [γ-^{32}P]ATP used in the assay (specific activity of [γ-^{32}P]ATP = dpm of BaP spot/amount of BaP labeled). In addition, the postlabeled BaPDE-dG-3'p can be used as a chromatography standard.
3. In the butanol adduct enrichment method an aliquot of BaPDE-dG-3'p is used as an extraction efficiency standard for recovery of PAH derived DNA adducts. However, when a specific DNA adduct is being targeted then a standard for that compound, if available, should be used to determine extraction efficiency.
4. An aliquot of contaminant-modified DNA from fish injected with a contaminated sediment extract is also used to monitor labeling

efficiency of complex mixtures between assays and as an additional chromatographic standard.

5. A 2'-deoxyguanosine-3'-monophosphate standard is used to monitor the efficiency of enzyme-dependent labeling of the normal nucleotides by ^{32}P-phosphate from [γ–^{32}P]ATP.
6. To assess reproducibility, every 10th tissue sample is analyzed in duplicate for DNA adducts.

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GLOSSARY OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BaP	benzo[a]pyrene
CHES	2[N-cyclohexylamino]ethanesulfonic acid (a buffer)
Ci	curie (1 curie = 2.22×10^{12} dpm)
CIA	chloroform/isoamyl alcohol, 24:1, v/v
CIP	chloroform/isoamyl alcohol/phenol, 24:1:25, v/v/v
cpm	counts per minute
D1	direction for solvent development (see Fig. 5)
D2	direction for solvent development (see Fig. 5)
D3	direction for solvent development (see Fig. 5)
D4	direction for solvent development (see Fig. 5)
DBC	dibenzo[c,g]carbazole
ddH_2O	deionized distilled water
dpm	disintegrations per minute
DRZ	diagonal radioactive zone
EDTA	ethylene diaminetetraacetic acid
EtOH	ethanol
HCl	hydrochloric acid
HPLC	high performance liquid chromatography

LFU	lithium formate/urea solvent system used for developing chromatograms in the D3 direction
LiCl	lithium chloride
LSS	liquid scintillation spectrometry
LTU	lithium chloride/Tris/urea solvent system used for developing chromatograms in the D4 direction
mCi	millicuries
MN	micrococcal nuclease
PAH	polycyclic aromatic hydrocarbon
dpGp	3′, 5′-deoxyguanosine bisphosphate
PEI	polyethyleneimine
PNK	polynucleotide kinase
PPL	^{32}P-postlabeling
PTU	sodium phosphate/Tris/urea
R_f	distance of the spot from the start/distance of the solvent front from the start
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SPD	spleen phosphodiesterase
TE	10 mM Tris, 1mM EDTA, pH 7.5
TLC	thin-layer chromatography
UV	ultraviolet
v/v	volume by volume

FIGURES

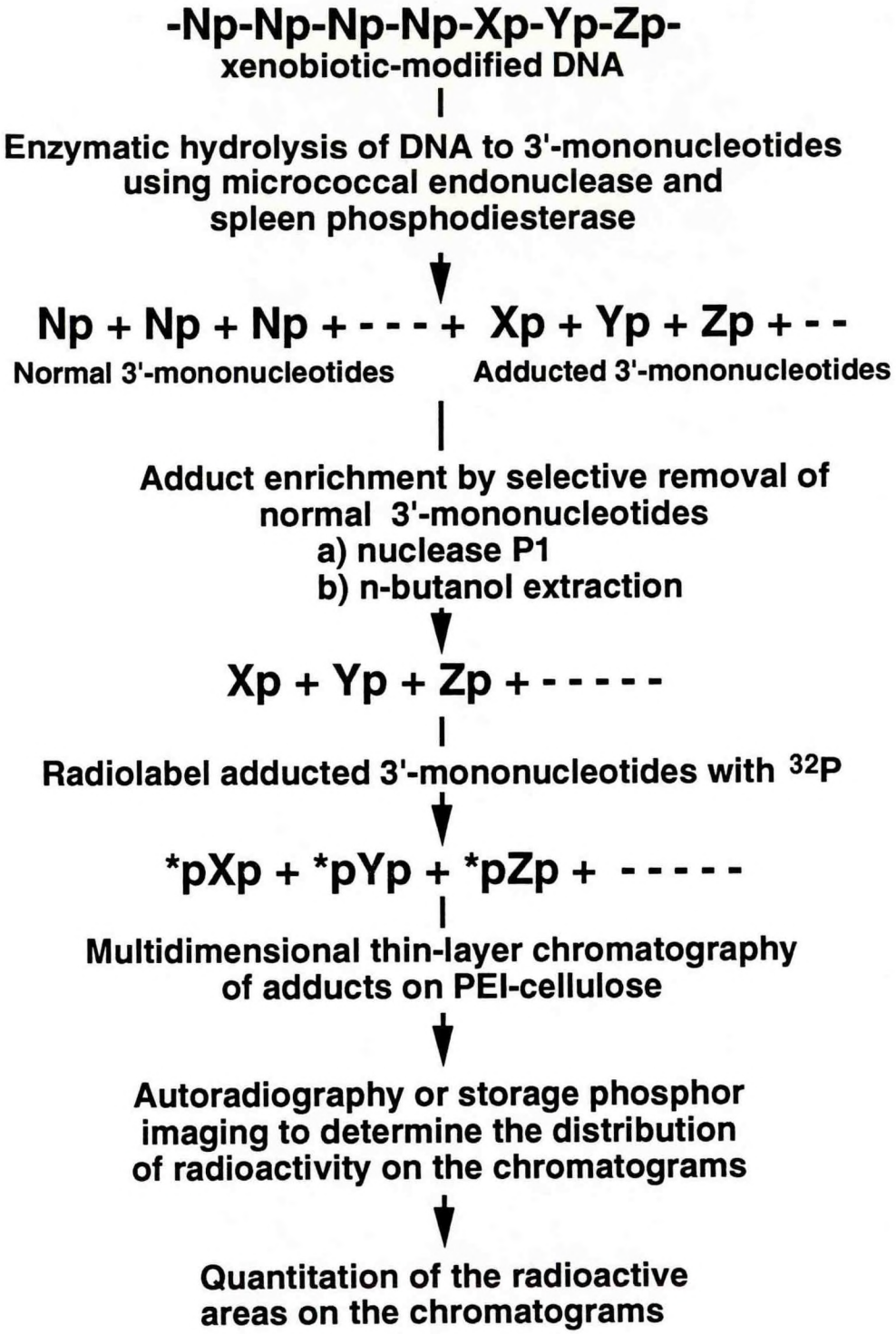

Figure 1. Schematic of the ^{32}P-postlabeling assay procedure.

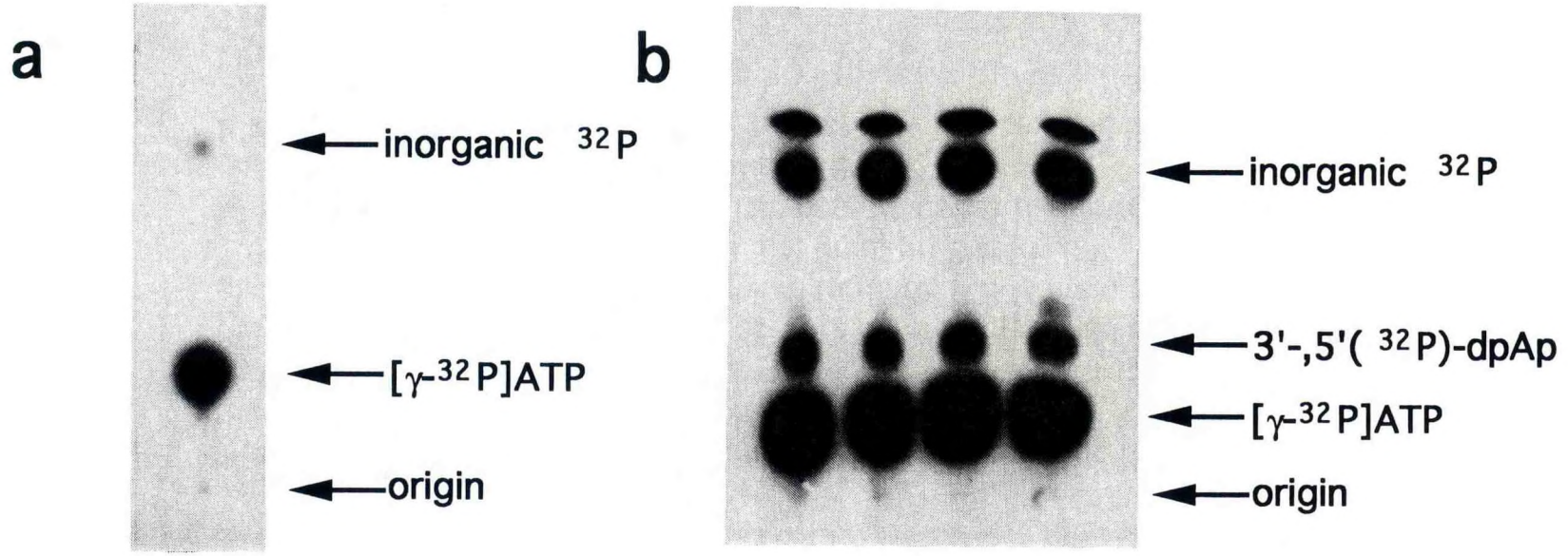

Figure 2. Autoradiograms of one-dimensional chromatograms used in the preparation of [γ-^{32}P]ATP. a) An aliquot (~ 0.1 μL) of the [γ-^{32}P]ATP synthesis mixture was eluted with 1 M lithium chloride. The autoradiogram indicates a successful synthesis of [γ-^{32}P]ATP. b) Aliquots of the specific activity determination digest were eluted in 0.24 M ammonium sulphate and 8 mM sodium phosphate, pH 7.4.

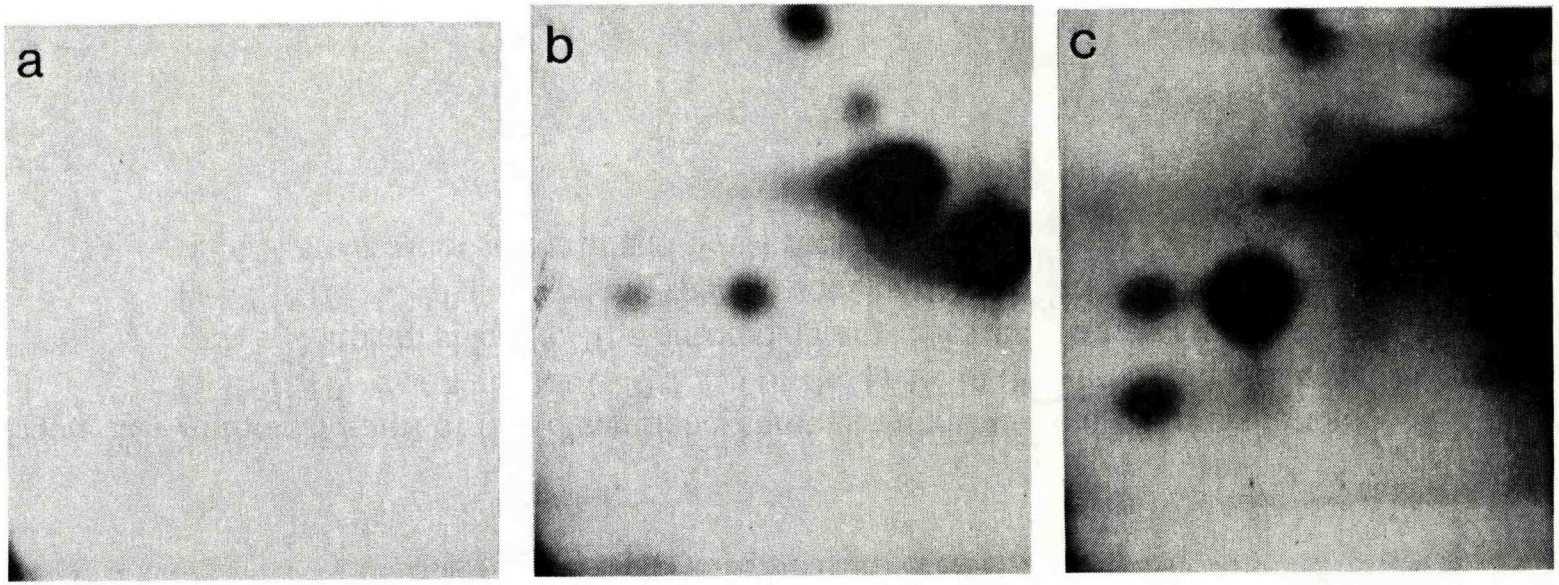

Figure 3. Profiles of ^{32}P-postlabeled 3'-mononucleotides from hepatic tissue from fish showing the chromatographic positions of DNA breakdown products. These breakdown products rarely appear in samples that were immediately frozen in liquid nitrogen and then stored at -80°C. These samples were chromatographed using 1 M sodium phosphate, pH 6.0 for D1, 4.5 M lithium formate, 8.5 M urea, pH 3.5 for D3 and 1.6 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0 for D4:

a) Profile of postlabeled DNA from fish hepatic tissue that was immediately frozen in liquid nitrogen and then stored at -80°C.

b, c) Profiles of DNA breakdown spots that may be observed in n-butanol enhanced ^{32}P-postlabeled samples.

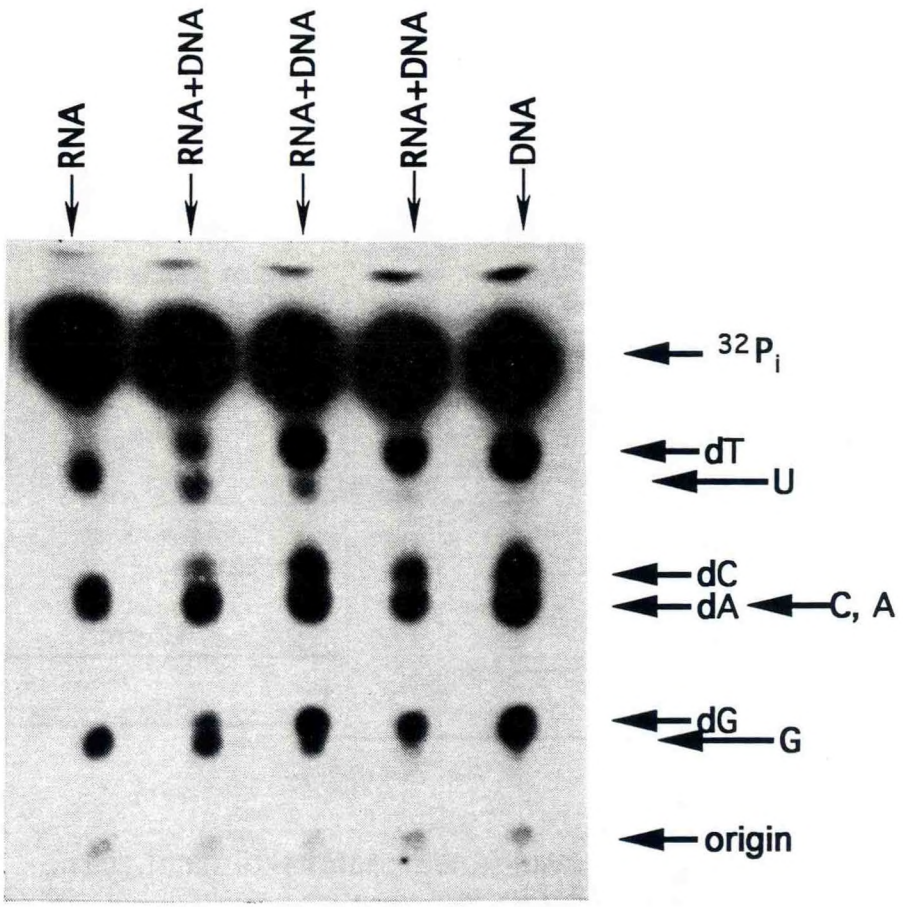

Figure 4. Representative autoradiograms showing the chromatographic profiles 3',5'- dpNp and -pNp nucleotides after micrococcal nuclease and spleen phosphodiesterase digestion of DNA and RNA samples and subsequent phosphorylation with polynucleotide kinase and [γ-^{32}P]ATP. Excess [γ-^{32}P]ATP was degraded by apyrase treatment. Samples were chromatographed on polyethyleneimine -cellulose sheets prepared in the laboratory and eluted with 0.24 M ammonium sulfate and 8 mM sodium phosphate, pH 7.4.

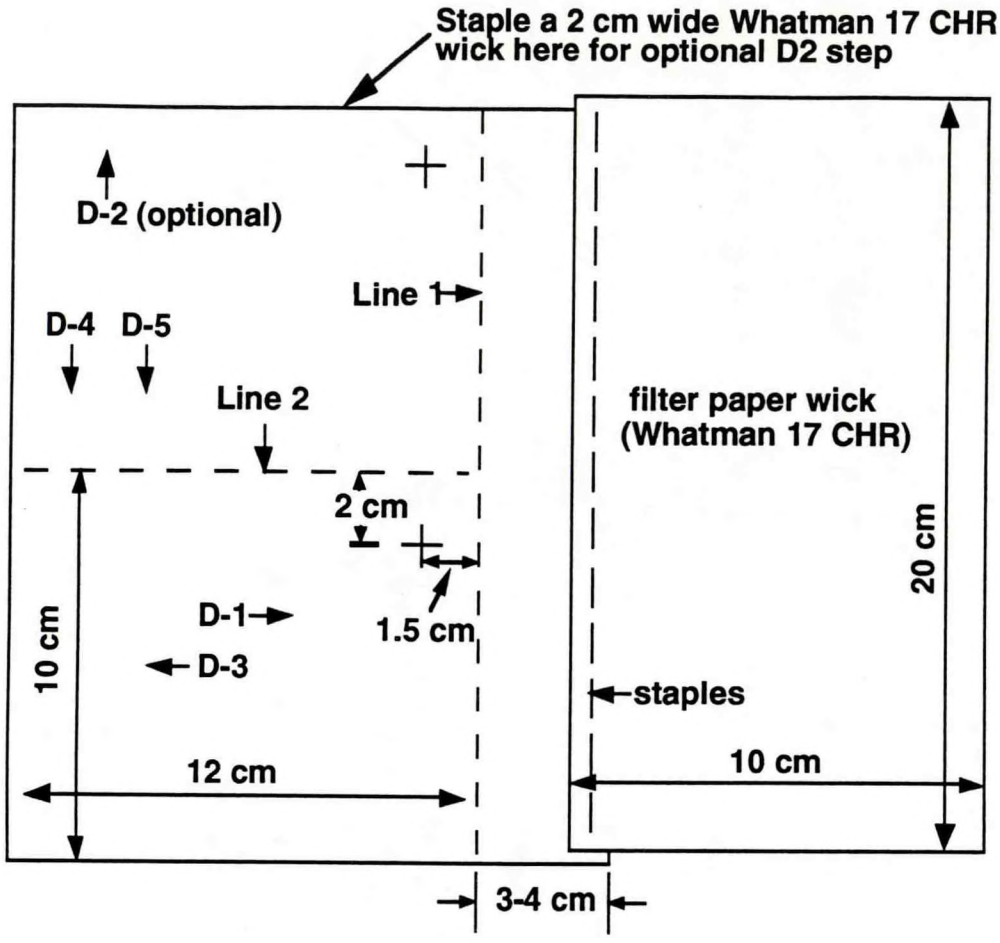

Figure 5. Diagram showing the directions of each of the chromatography steps and the cut lines (– – – –). The crosses (+) designate the samples origins.

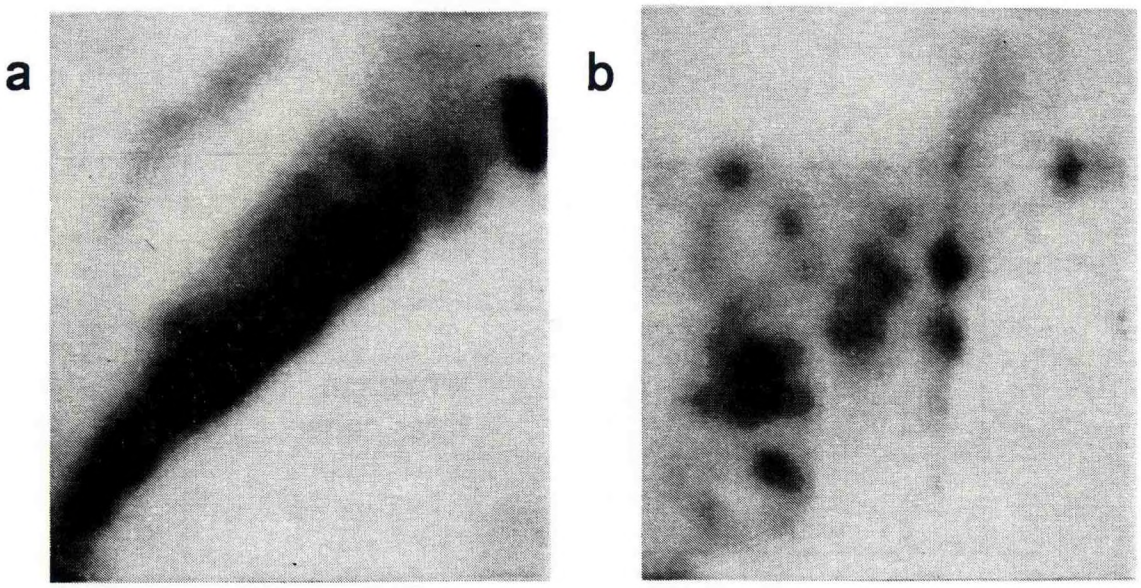

Figure 6. Influence of solvent systems on the separation of DNA adducts extracted from hepatic tissues of oyster toadfish captured at a creosote contaminated site. Commercial PEI-cellulose sheets were used :

a) D3 - 8.5 M urea, 4.5 M lithium formate, pH 3.5; D4 - 8.5 M urea, 1.6 M lithium chloride, 0.5 M Tris, pH 8.0.

b) D3 - 8.5 M urea, 4.5 M lithium formate, pH 3.5; D4 - isopropanol/4 N ammonia (1:1).

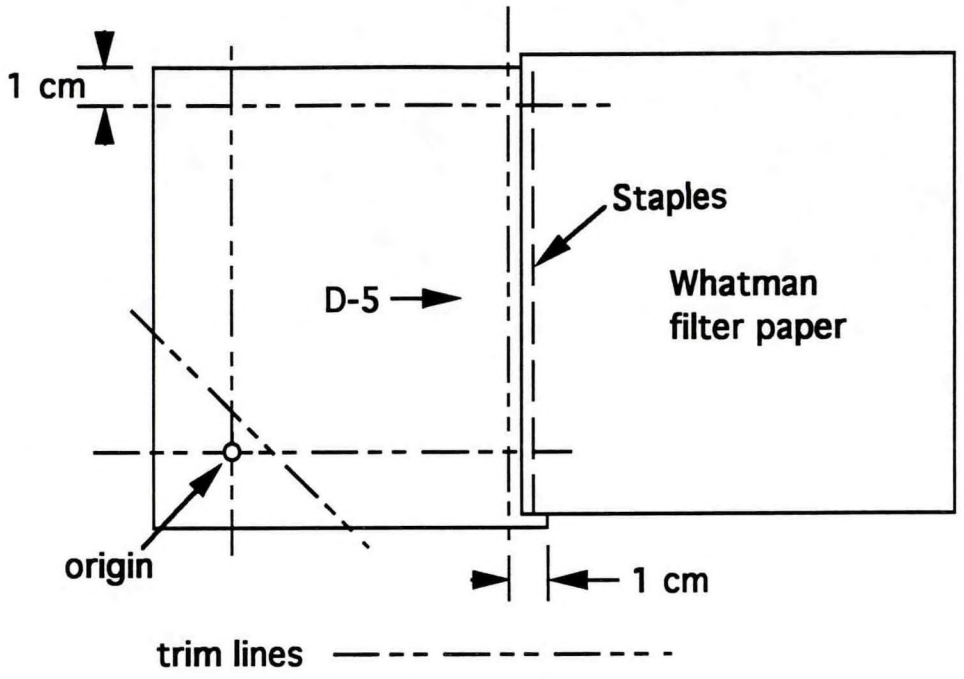

Figure 7. Diagram showing the PEI-cellulose setup for optional D5. The dashed lines show where the chromatogram is to be trimmed after D5 and before being placed in the autoradiography cassette.

TABLE

Table 1. Typical Excel worksheet showing PhosphorImager data and calculation of DNA damage.

	A	B	C	D	E	F	G	H	I	J	K	L
1	PPL#	Sample #	Study	Site	Species	PI vol-a	CF-a	cpm-a	SA-a	AQ-a	PI vol-b	CF-b
2								** F2/G2				
3	148-01	90.3180	R&F '90	Pristine Sound	P. Mudgrunt	951268	8060	118	1939	2	4024071	126.4
4	148-02	90.3181	R&F '90	Pristine Sound	P. Mudgrunt	718964	8060	89	1939	2	4761861	126.4
5	148-03	90.3182	R&F '90	Pristine Sound	P. Mudgrunt	499168	8060	62	1939	2	2406825	126.4
6	148-04	90.3183	R&F '90	Pristine Sound	P. Mudgrunt	1133085	8060	141	1939	2	2993307	126.4
7	148-05	90.3184	R&F '90	Pristine Sound	P. Mudgrunt	572750	8060	71	1939	2	2721056	126.4
8	148-06	90.3185	R&F '90	Pristine Sound	P. Mudgrunt	581683	8060	72	1939	2	1472010	126.4
9	148-07	90.3186	R&F '90	Pristine Sound	P. Mudgrunt	1003146	8060	124	1939	2	2168595	126.4
10	148-08	90.3187	R&F '90	Pristine Sound	P. Mudgrunt	1562322	8060	194	1939	2	4421854	126.4
11	148-09	91.9980	R&F '91	Urban Bay	P. Mudgrunt	10035754	8060	1245	1939	2	2093496	126.4
12	148-10	91.9981	R&F '91	Urban Bay	P. Mudgrunt	32846867	8060	4075	1939	2	3719354	126.4
13	148-11	91.9982	R&F '91	Urban Bay	P. Mudgrunt	29776508	8060	3694	1939	2	3929672	126.4
14	148-12	91.9983	R&F '91	Urban Bay	P. Mudgrunt	33118195	8060	4109	1939	2	2539131	126.4
15	148-13	91.9984	R&F '91	Urban Bay	P. Mudgrunt	45305225	8060	5621	1939	2	3358298	126.4
16	148-14	91.9985	R&F '91	Urban Bay	P. Mudgrunt	97089089	8060	12046	1939	2	4359981	126.4
17	148-15	91.9986	R&F '91	Urban Bay	Sp. Mudgrunt	4077333	8060	506	1939	2	3052623	126.4
18	148-16	91.9987	R&F '91	Urban Bay	Sp. Mudgrunt	336385	8060	42	1939	2	1089497	126.4
19	148-17	91.9988	R&F '91	Urban Bay	Sp. Mudgrunt	2638708	6852	385	1939	2	3208020	126.4
20	148-18	91.9989	R&F '91	Urban Bay	Sp. Mudgrunt	1494894	6852	218	1939	2	2414327	126.4
21	148-19	91.9990	R&F '91	Urban Bay	Sp. Mudgrunt	734904	6852	107	1939	2	2715641	126.4
22	148-20	91.9991	R&F '91	Urban Bay	Sp. Mudgrunt	1733913	6852	253	1939	2	3768276	126.4
23	148-21	91.9992	R&F '91	Urban Bay	Sp. Mudgrunt	2072451	6852	302	1939	2	2628056	126.4
24	BP					34783296	8314	4184	1939	1.333		
25	"a" refers to DNA adduct related data and "b" refers to DNA bases related data.											
26	** = arithmetic operation to obtain this cell value based on information in previous cells in this row.											
27	PI vol-a = Integrated volume units for the computer generated image from the storage phosphor imaging system.											
28	CF-a = factor used to convert PI volume units to cpm.											
29	cpm-a = cpm of the adducts measured on the chromatogram.											
30	SA-a= specific activity of [gamma-32P]ATP used for labeling adducts.											
31	AQ-a = aliquot factor (volume of DNA-adduct labeling solution/volume spotted on PEI-cellulose sheet).											
32	PI-b vol = Integrated volume of the DNA bases (dG spot) image from the storage phosphor imaging system.											

Table 1. Continued.

	M	N	O	P	Q	R	S	T
1	CPM-b	SA-b	AQ-b	dG-CF	dil-b	nmol-b	fmol-a	nmol/mol-b
2	K2/L2					(M2*O2*P2*Q2)/N2	(H2*J2)/I2	S2/(R2*0.001)
3	31836	968055	2.4	4.76	100	38	0.12	3
4	37673	968055	2.4	4.76	100	44	0.09	2
5	19041	968055	2.4	4.76	100	22	0.06	3
6	23681	968055	2.4	4.76	100	28	0.15	5
7	21527	968055	2.4	4.76	100	25	0.07	3
8	11646	968055	2.4	4.76	100	14	0.07	5
9	17157	968055	2.4	4.76	100	20	0.13	6
10	34983	968055	2.4	4.76	100	41	0.20	5
11	16562	968055	2.4	4.76	100	20	1.28	66
12	29425	968055	2.4	4.76	100	35	4.20	121
13	31089	968055	2.4	4.76	100	37	3.81	104
14	20088	968055	2.4	4.76	100	24	4.24	179
15	26569	968055	2.4	4.76	100	31	5.80	185
16	34494	968055	2.4	4.76	100	41	12.42	305
17	24150	968055	2.4	4.76	100	28	0.52	18
18	8619	968055	2.4	4.76	100	10	0.04	4
19	25380	968055	2.4	4.76	100	30	0.40	13
20	19101	968055	2.4	4.76	100	23	0.22	10
21	21485	968055	2.4	4.76	100	25	0.11	4
22	29812	968055	2.4	4.76	100	35	0.26	7
23	20792	968055	2.4	4.76	100	25	0.31	13
24							2.88	
25	CF-b = factor to convert dG image volume to cpm.							
26	AQ-b = aliquot factor (volume of solution labeled/volume spotted on PEI-cellulose sheet).							
27	dG-CF = 4.76 = total DNA%/dG% = 100%/21%							
28	dil-b = dilution factor for bases (5 uL enzyme hydrolysate diluted to 500 uL with H2O).							
29	nmol-b = nmol of bases used in the assay.							
30	fmol-a = fmol of DNA adducts labeled.							
31	nmol-a/mol-b = fmol of adducts/(nmol of bases*0.001); nmol of bases*0.001 = umol of bases							
32								

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