

# A semi-automated, microplate version of the SOS Chromotest for the analysis of complex environmental extracts<sup>1</sup>

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## Abstract

Environmental monitoring for genotoxicity requires that a large number of measurements be made across space and time. This requirement demands a rapid and efficient bioassay system. The SOS Chromotest is a rapid, efficient bacterial system for the detection of DNA damaging agents. Over 100 publications have described its use on a variety of samples. Relatively few studies have used the test to examine complex mixtures. Effective testing of complex samples poses a variety of problems. Although solutions have been proposed, few have validated the resulting protocol. In this work we present a semi-automated microplate version of the SOS Chromotest for the examination of complex mixtures. Experiments were conducted to determine the optimal cell concentration, exposure time, substrate conversion time and S9 enzyme concentration. The performance of the method was evaluated using 6 reference genotoxins and 3 complex mixtures. The complex mixtures examined are extracts of diesel particulate matter, urban dust and coal tar. The results obtained indicate that optimal responses often require fewer cells ( $\approx 5\text{--}10 \times 10^6$  CFU/ml) and a longer exposure (3 h) than that recommended in the original protocol. Interfering effects of colored and turbid samples are removed using centrifugation and initial optical density readings taken 60 min after cell resuspension and lysis. The performance of the established protocol was evaluated using mitomycin C and benzo[a]pyrene results for 207 microplates and solvent control results for 293 microplates. The results indicate that the established method is accurate, sensitive and precise. Coefficient of variation on mean SOSIP values for mitomycin C and benzo[a]pyrene are  $< 5\%$ . Solvent control data indicate that the standard threshold for determination of a positive response (induction factor  $> 1.5$ ) is excessively conservative. All liquid transfers were automated using the Biomek™ automated laboratory workstation. Automation permits a throughput of up to 72 samples per day and maintains excellent precision and accuracy.

**Keywords:** SOS Chromotest; Genotoxicity; Complex mixture; Automation

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## 1. Introduction

Genotoxic and mutagenic substances have been detected in a wide range of environmental samples

including urban air particulates (reviewed by Chrisp and Fischer, 1980), gasoline and diesel engine emissions (e.g., Nishioka et al., 1983; Saleem et al., 1984; Wang et al., 1978) river sediments (e.g., Langevin et al., 1992; Metcalfe et al., 1990; Durant et al., 1992), contaminated soils (e.g., Donnelly et al., 1983; Brown and Donnelly, 1988), surface waters (reviewed by Stahl, 1991), snow (White et al., 1995) and industrial wastes (reviewed by Houk, 1992). A thorough understanding of the sources, pathways, sinks and potential hazard of these substances for both humans and other biota requires extensive environmental monitoring of the relevant environmental media. To be effective, environmental monitoring research requires that a large number of measurements be made over time and across locations (Lewtas, 1991). In addition, bioassay directed fractionation for the identification of genotoxins in complex samples requires that large numbers of samples be examined (e.g., Marvin et al., 1993; Meier et al., 1987; Savard et al., 1992; Schuetzle and Lewtas, 1986). Therefore, research investigating the genotoxicity of complex mixtures requires inexpensive, rapid and sensitive (micro)bioassays (Brusick, 1987; Blaise et al., 1988). The bioassay system that is most frequently used for the detection of genotoxic substances in complex environmental samples is the Salmonella/mammalian microsome assay (Ames et al., 1973, 1975; Maron and Ames, 1983). The Salmonella test detects mutagenic substances via their ability to revert histidine auxotrophs of *Salmonella typhimurium* to wild-type. The test system was recently used in an extensive inter-laboratory, collaborative study on the mutagenicity of complex mixtures (Claxton et al., 1992).

Since the advent of the Salmonella test many other short-term bacterial assays have been developed. Several assays (e.g., the Microscreen phage induction assay of Rossman et al., 1984; the SOS/*umu* test of Oda et al., 1985; the SOS Chromotest of Quillardet et al., 1982) use the error-prone DNA repair pathway of *Escherichia coli* for the detection of DNA damaging agents. The error-prone repair pathway, also known as the SOS response, is a complex regulatory network that is induced by DNA damage (Walker, 1984). Activation of the SOS system results in the coordinated regulation of a series (> 17) of unlinked, *damage-inducible* genes that

have a variety of known and unknown functions (Kenyon and Walker, 1980). Using the specialized transducing phage Mu *dI*(Ap, *lac*) developed by Casadaban and Cohen (1979), Huisman and D'Ari (1981) constructed a strain of *E. coli* in which the expression of  $\beta$ -galactosidase is under the express regulatory control of the SOS response pathway. This strain, known as PQ37, became the cornerstone of the SOS Chromotest for the detection of DNA damaging agents. The test involves incubation of the bacteria with the substance under investigation and subsequent spectrophotometric determination of  $\beta$ -galactosidase activity – i.e., the level of SOS induction. Activity values are usually normalized to account for the background level of SOS induction in cells that are exposed to a solvent control. Constitutive synthesis of alkaline phosphatase, an enzyme not under SOS control, permits an indirect measure of bacteriostatic effects.

The SOS Chromotest has many advantages that make it an attractive choice for environmental monitoring: (1) survival of the tester strain is not required; (2) the results can be obtained in a single working day; (3) sample sterility is not usually required; (4) the organism responds to a wide range of DNA damage scenarios (Elespuru, 1987; Houk and DeMarini, 1988); (5) bacteriostatic effects can be simultaneously monitored; (6) the microplate version of the assay is readily amenable to automation; and (7) the test can easily accept biological samples such as tissue extracts and biological fluids (histidine contamination of biological samples can effect Salmonella test performance (Sparks et al., 1981; Parry, 1985; Fish et al., 1987; Kohn et al., 1988; De M  o et al., 1988; Bosworth and Venitt, 1986)). Since its inception in 1982, the test has been validated (Von der Hude et al., 1988; Quillardet et al., 1985; Ohta et al., 1984; Quillardet and Hofnung, 1993) and used to measure the genotoxicity of over 750 chemicals. However, despite well over 100 publications, relatively few studies have used the assay to detect genotoxicity in complex environmental extracts. The test has recently undergone a number of modifications that optimize performance and efficiency. Microsuspension methods permit the entire assay to be carried out in microtiter plates. This facilitates sample processing and spectrophotometric measurements. Fish et al. (1987) described a kit version of

the microtiter SOS Chromotest. Although convenient and efficient, the cost of the kit system (Environmental Biodetection Products, Brampton, Ontario) often prohibits extensive environmental monitoring.

In this work we present a semi-automated, micro-suspension SOS Chromotest for the detection of genotoxicity in complex mixtures. The method decreases the time and expense of processing large numbers of samples. Three standard reference materials and six reference genotoxins were used to assess assay performance. The Biomek™ 1000 automated laboratory workstation (Beckman Instruments, Palo Alto, CA) performed all liquid transfers and serial dilutions required in the assay.

## 2. Materials and methods

### 2.1. Culture media, reagents, reference materials and laboratory equipment

**LB (Luria-Bertani) medium:** 10 g Bacto tryptone (Difco Laboratories, Detroit, MI), 5 g Bacto yeast extract (Difco), 10 g NaCl (CAS No. 7647-14-5, Fischer Scientific, Fair Lawn, NJ) and 20 mg ampicillin (CAS No. 69-52-3, Sigma Chemicals, St. Louis, MO) per liter of Super Q™ (Millipore Corp., Bedford, MA) water.

**Enzyme assay/cell lysis reagent or SOS Chromogen:** 40% (v/v) methanol (CAS No. 67-56-1, spectrophotometric grade: Fischer), 0.5% (v/v) toluene (CAS No. 108-88-3, spectrophotometric grade: Fischer), 10% (v/v) *N,N*-dimethyl formamide (CAS No. 68-12-2, spectrophotometric grade: Fischer), 4 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (CAS No. 7240-90-6, Vector Biosystems, Toronto, Ontario), 1 mg/ml *p*-nitrophenyl phosphate (CAS No. 4262-83-9, Sigma). The reagent is prepared in Super Q™ water adjusted to a pH of 9 with 0.1 N NaOH. Immediately prior to use the reagent is warmed to 37°C and filtered (0.2  $\mu$ m cellulose-nitrate filter, Nalgene Labware, Rochester, NY). The final pH of the reagent is 7.8 to 7.9.

**S9 activation mixture:** 33 mM KCl (CAS No. 7447-40-7, CAS No. 7786-30-3, Sigma) 8 mM MgCl<sub>2</sub> (J.T. Baker Chemical Co., Phillipsburg, NJ),

100 mM Tris(hydroxymethyl)aminomethane, pH 7.4 (CAS No. 77-86-1, Sigma), 5 mM glucose 6-phosphate (sodium salt, CAS No. 54010-71-8, Boehringer-Mannheim Canada, Laval, Québec), 2 mM NADP (nicotinamide adenine dinucleotide phosphate, disodium salt, CAS No. 1184-16-3, Boehringer-Mannheim) and 20–160  $\mu$ l Aroclor 1254-induced rat liver S9 (Molecular Toxicology, Annapolis, MD) per ml of activation mixture. Activation mixture was kept on ice until the start of the assay. S9 concentration values provided in the text reflect the concentration of S9 (in %, v/v) in the final assay mixture (i.e., not in the S9 activation mixture).

**Resuspension buffer:** 200 mM Tris(hydroxymethyl)aminomethane, pH 7.5 (Sigma).

**Reference genotoxins:** Mitomycin C (CAS No. 50-07-7, Sigma), 4-Nitroquinoline-1-oxide (CAS No. 56-57-5, Aldrich Chemical Co., Milwaukee, WI), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (CAS No. 70-25-7, Sigma), Captan (3*a*,4,7,7*a*-tetrahydro-2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione, CAS No. 133-06-2, Chevron Chemical Co., San Francisco, CA), *N*-nitrosodimethylamine (CAS No. 62-75-9, Sigma), 2-naphthylamine (CAS No. 91-59-8, Sigma), Benzo[*a*]pyrene (CAS No. 50-32-8, Sigma).

**Standard reference materials:** All standard reference materials (SRMs) were obtained from the U.S. National Institute of Standards and Technology (Gaithersburg, MD). The three SRMs used in this study are: SRM 1597, complex mixture of polycyclic aromatic hydrocarbons from coal tar; SRM 1649, urban dust/organics; and SRM 1650, diesel particulate matter. SRM 1597 is a toluene extract of medium-crude coke oven tar. SRM 1649 is intended to typify atmospheric particulate matter obtained from an urban area. The material is a time-integrated sample collected over a 12-month period in the Washington, DC area. The sample was screened through a fine sieve to remove extraneous matter. SRM 1650 is intended to be a representative of heavy-duty diesel engine particulate emissions. The material was collected from several four-cycle diesel engines, operating under a variety of conditions.

**Spectrophotometers:** Titertek Multiskan MCC/340 microplate spectrophotometer (Flow Laboratories, Lugano, Switzerland) for microtiter plates,

Beckman DU-70 UV-VIS spectrophotometer (Beckman Instruments) with sipper flowcell for monitoring bacterial cultures.

*Microplate centrifuge:* Heraeus Varifuge RF

(Heraeus Sepatech GmbH, Am Kalkberg, Germany) with microplate rotor (model No. 4690).

Automated laboratory workstation- Biomek™ 1000 (Beckman Instruments), controlled by the Gen-

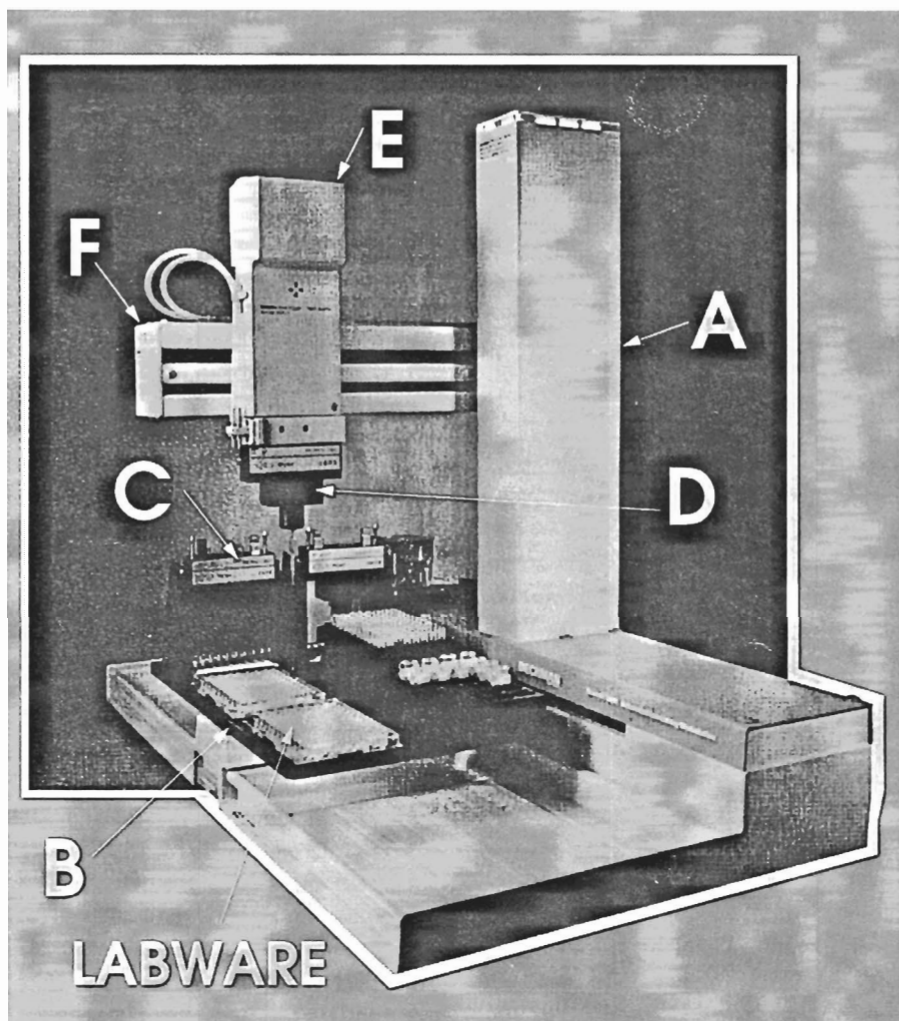


Fig. 1. The Biomek™ 1000 Automated Laboratory Workstation. The instrument performed all liquid transfers and serial dilutions required for the SOS Chromotest. The instrument is made up of a series of moving parts that collectively permit three-dimensional movement. The elevator (A) provides vertical movement for the bridge (F) and pod (E). The bridge and the attached pod move up and down over the tablet (B). The pod moves back to front along the bridge and across the width of the tablet. The tablet moves side to side beneath the pod and bridge. The tablet contains four locations that hold currently used labware and four locations for storing pipetting tools currently not in use (C). The tool currently in use (D) is attached to the pod and carries out automated liquid handling and measurement functions. Compatible labware include a variety of microtiter plates and tube racks, a variety of pipette tips for volumes from 2–1000  $\mu$ l and a wide range of reagent reservoirs. The instrument can be equipped with an optional sideloader that automatically removes used labware from the tablet and replaces them with a fresh supply as required. Labware shown are not necessarily used on the SOS Chromotest procedure.

esis™ (version 2.11) software running on an IBM PS/2™ model 50 computer (IBM Corp., Armonk, NY). The instrument (without computer) is illustrated in Fig. 1. Both multichannel and single-channel pipette tools were used to manipulate volumes from 5 to 200  $\mu$ l. When properly adjusted, the instrument can routinely deliver volumes from 2 and 1000  $\mu$ l with less than 1% error (usually in the 0.2% to 0.6% range). The Genesis™ software contains a user-friendly interface that permits custom programming of the instrument. Custom subroutines permit liquid transfers, liquid mixing and serial dilutions. To provide optimal performance, liquid transfer parameters such as dispense rate, tip height, mixing speed and height, tip prewetting, liquid blowout or *to contain* volume, liquid level sensing and tip touching can be adjusted to suit the physical properties of the reagents and the desired degree of accuracy and precision. A custom program for the SOS Chromotest was written in our laboratory and is available on diskette from the corresponding author. Sterile polypropylene pipette tips (TiterPak™) were obtained from Robbins Scientific Corp. (Sunnyvale, CA).

## 2.2. Tester strain maintenance and culture methods

*E. coli* strain PQ37 (*lac* $\Delta$ U169 *uvrA* *rfa* *sulA*::Mud(Ap, *lac*) PhoC) was kindly provided by Philippe Quillardet (Unité de Programmation Moléculaire et Toxicologie Génétique, Institut Pasteur, Paris). The complete genotype, as well as strain construction details, can be found in Quillardet and Hofnung (1985). Frozen permanent copies of the tester strain were prepared and stored according to Maron and Ames (1983). The integrity of several genetic markers was verified semi-annually. The *rfa* mutation and alkaline phosphatase constitutivity (PhoC) were verified according to Quillardet and Hofnung (1985). The *uvrA* mutation was verified according to Ames et al. (1975). The integrity of the *sulA*::*lacZ* fusion was verified using a modified version of the standard SOS spot test (Mamber et al., 1986).

To prepare a PQ37 culture, a 1 ml frozen permanent copy was thawed and diluted in 50 ml of fresh LB broth (20  $\mu$ g/ml ampicillin) in a 125-ml Erlenmeyer flask fitted with a cotton/cheesecloth plug.

Flasks were incubated overnight (15 h maximum) with agitation (200 rpm) at 37°C.

## 2.3. Extraction of SRMs (Standard Reference Materials)

SRM 1597 was supplied as a liquid in 5 ml sealed ampoules (1.3 ml toluene/ampoule). The 'Certificate of Analysis' indicates that each  $\mu$ l of sample is equivalent to approx. 8  $\mu$ g of coal tar. SRM 1650 and SRM 1649 were both supplied as dry powders. Dichloromethane (CAS No. 75-09-2, Anachemia Science, Montréal) extracts of SRM 1650 and SRM 1649 were prepared using the combined blender/sonication method described by White et al. (1996a). The method is a variation on those developed by Marble and Delfino (1988), Maggard et al. (1987) and Williams (1989). Maggard et al. (1987) determined that, for mutagenicity studies, the performance of blender methods is similar to, or better than that of Soxhlet extraction. Nielsen (1992) determined that dichloromethane is the best choice as a general solvent for extraction of complex environmental samples. Briefly, each sample (2.0 g of SRM 1649 and 0.25 g of SRM 1650) was blended with 250 ml of dichloromethane in stainless steel cups using a high-speed (3500 rpm) industrial blender (Eberbach Corp., Ann Arbor, MI). Each sample was blended for 3  $\times$  4 min and subsequently sonicated (ultrasonic cell disrupter: Branson Sonic Power Co., Danbury, CT) on ice for 2  $\times$  3 min. Blending and sonication periods were alternated with 3–4 min cooling periods. Extracts were dried with anhydrous sodium sulfate (CAS No. 7757-82-6, Mallinckrodt Specialty Chemicals, Mississauga, Ontario), filtered through coarse sintered glass and reduced to approx. 5 ml by rotary evaporation at 30°C. Where necessary, fine particulate matter was removed via filtration through 0.45  $\mu$ m Teflon™ filters (Gelman Science, Ann Arbor, MI). Extracts were taken to dryness under vacuum at low temperature (Speedvac AS290 Concentrator, Savant, Farmingdale, NY). Both extracts were resuspended in dimethyl sulfoxide (DMSO) (CAS No. 2206-27-1, Sigma). Samples of SRM 1597 were taken to dryness and resuspended in DMSO. Extracts were resuspended at the following concentrations: 250 mg of equivalent start-

ing material per ml of DMSO for SRM 1650, 2.0 g of equivalent starting material per ml for SRM 1649 and approx. 8 mg of coal tar equivalent per ml for SRM 1597. Maximum tested concentrations were 100 mg per assay ml for SRM 1649, 12.5 mg per assay ml for SRM 1650 and approx. 0.4 mg of coal tar equivalent (50  $\mu$ l of SRM) per assay ml for SRM 1597.

#### 2.4. Initiation of SOS chromotest procedure

Overnight (log phase) PQ37 culture was diluted in fresh LB medium (warmed to 37°C). Where S9 was required, the culture was diluted in 3 parts LB broth and 1 part S9 activation mixture. The sample (or solvent blank), dissolved in 10  $\mu$ l DMSO, was then added to 190  $\mu$ l of diluted culture (plus S9 if desired) present in the first well of each dilution series. The automated multichannel pipette then mixed the well contents and performed a 2-fold serial dilution with an adjacent well that already contained 95  $\mu$ l of diluted culture and 5  $\mu$ l of DMSO. The 2-fold serial dilution was continued in four additional wells for a total of six, 2-fold serial dilutions. This dilution system permits full use of the automated laboratory workstation, while not permitting any change in the concentration of carrier solvent or cells. A minimum of 12 control wells received PQ37 culture and a solvent blank. All extracts examined for genotoxicity were dissolved in pesticide grade DMSO. The final background concentration of DMSO was always 5% (v/v).

#### 2.5. Exposure of bacteria and assay of enzyme activities

Exposure times of 2, 3 and 4 h at 37°C were examined. Plates were subsequently centrifuged at 1200  $\times$  g for 20 min at 37°C. The supernatant was removed and the bacterial pellet resuspended in 100  $\mu$ l of resuspension buffer and 100  $\mu$ l of *SOS Chromogen*. Plates were mixed for 60 s (Titertek microplate vortex, Flow Laboratories) and returned to the 37°C incubator. A variety of experiments were conducted to determine the optimal time for conversion of the substrates to their colored products. Incubation times of 30, 60, 90, 120 and 150 min were examined. Optical density measurements were taken

at 620 nm to measure  $\beta$ -galactosidase activity and 405 nm to measure alkaline phosphatase activity.

#### 2.6. Analysis of results

Simultaneous measurements at both 405 and 620 nm requires that the measurements of alkaline phosphatase activity are corrected for the *spillover* of the blue  $\beta$ -galactosidase product into the 405 nm range (Organics Ltd., 1990). Experiments conducted in our laboratory indicated that the ratio of the optical density of 5-bromo-4-chloroindole at 405 nm to its optical density at 620 nm is  $0.80 \pm 0.0050$  units. We verified that this ratio is constant for optical density values between 0.02 and 1.3. Therefore, corrected 405 nm measurements (i.e., corrected alkaline phosphatase activity) were calculated as:

$$\text{Corrected OD}_{405 \text{ nm}} = \text{Raw OD}_{405 \text{ nm}} - (0.80 * \text{OD}_{620 \text{ nm}}) \quad (1)$$

Induction of the SOS response pathway at a sample concentration  $C$ , denoted  $R(C)$ , is the ratio of  $\beta$ -galactosidase activity to alkaline phosphatase activity. To account for the background induction of the *sulA* gene, a normalized SOS response induction factor was calculated as  $IF = R(C)/R(0)$ , where  $R(0)$  is the ratio of  $\beta$ -galactosidase to alkaline phosphatase activity averaged over the 12 controls that received blank solvent. A normalized induction factor that exceeded the upper 95% confidence limit of the control was regarded as a significant positive. Since normalized induction factor values are ratios of two means, variance values were calculated according to Welsch et al. (1988)<sup>2</sup>. Welsch et al. (1988) described methods for calculating the variance associated with various functions of mean values (reciprocals, sums, products, ratios etc.). Concentration response curves of normalized induction factor against sample concentration (mg sample equivalent per assay ml for SRM extracts and  $\mu$ g per assay ml for pure compounds) were inspected and the results qualitatively categorized as positive if the induction factor exceeded the upper confidence limit of the control at a minimum of three doses.

<sup>2</sup> We used the older, more complicated method of EC Fieller (described in Kendall and Stuart, 1979) to empirically validate this calculation method.

The genotoxic potency of each sample was assessed via its SOS Response Inducing Potency or SOSIP (Quillardet and Hofnung, 1985). The SOSIP is the initial slope of the concentration-response curve, expressed as SOS Induction factor units per  $\mu\text{g}$  of pure compound or equivalent mg of original SRM. SOSIP values were calculated for each significant, positive response. Values were determined using ordinary, least-squares linear regression (SAS Institute, 1988). In addition, the maximum induction factor obtained was recorded and denoted MaxIF.

### 2.7. Protocol development

Adaptation of the SOS Chromotest protocol proceeded in 7 separate stages.

1. Development of the enzyme assay/cell lysis reagent (*SOS Chromogen*).
2. Investigation of the colorimetric interference of complex SRM extracts.
3. Automation of the protocol.
4. Adjustment of cell density in the assay mixture.
5. Determination of optimal exposure time.
6. Determination of optimal substrate conversion time.
7. Determination of optimal S9 concentration.

Steps 4 through 7 were carried out using both pure substances and extracts of SRMs.

The following abbreviations are used throughout the text: 4-NQO, 4-nitroquinoline-1-oxide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MitC, mitomycin C; NDMA, *N*-nitrosodimethylamine; 2-NA, 2-naphthylamine; BaP, benzo[*a*]pyrene; Tris, Tris(hydroxymethyl)aminomethane; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; SRM, standard reference material; SOSIP, SOS response inducing potency; IF, SOS induction factor; MaxIF, maximum SOS induction factor observed; DMSO, dimethyl sulfoxide; DCM, dichloromethane; CFU, colony forming unit; PNPP, *p*-nitrophenyl phosphate.

## 3. Results

### 3.1. The SOS Chromogen

Preliminary experiments were conducted to design an effective alternative to the standard enzyme

assay and cell lysis reagent. We avoided phosphate buffer since  $\text{PO}_4^{2-}$  can impair alkaline phosphatase activity (Marzin et al., 1986; Courtois et al., 1988). We replaced the buffer with Super Q™ water adjusted to pH 9 with 0.1 N NaOH. The net result is a hypotonic solution that invokes an osmotic shock and causes a sudden expansion of the cell membrane (Neu and Heppel, 1965) against the already fragile (deep rough) cell wall. We replaced SDS with 0.5% (v/v) toluene. Toluene has been used to disrupt the cell membrane of *E. coli* and permit quantitative

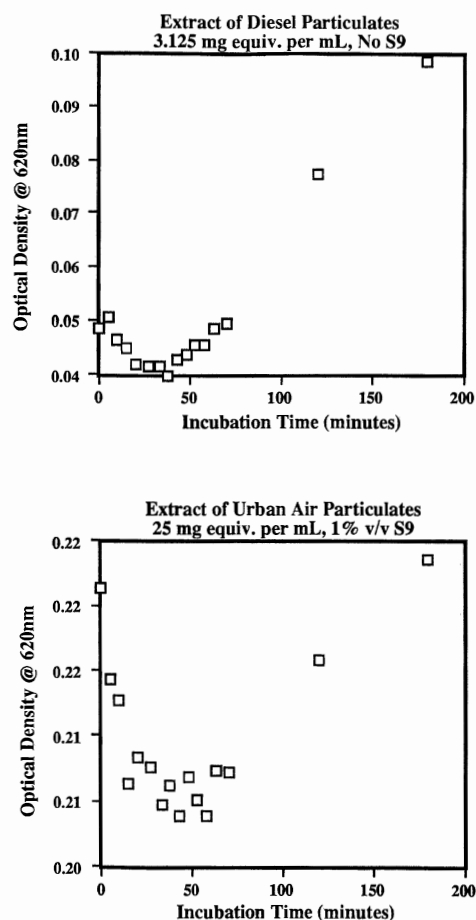


Fig. 2. Time course of SOS Chromogen development at 37°C. Following a 2-h exposure cells were harvested via centrifugation at 1200–g for 20 min. Measurements at 620 nm were taken each 5 min after cell resuspension and SOS Chromogen addition and followed for a total of 180 min. Optical density readings at 620 nm follow the conversion of the  $\beta$ -galactosidase substrate (X-Gal) to its blue product.



determination of  $\beta$ -galactosidase activity (Miller, 1972; Goulding, 1986). The efficacy of toluene is increased by the *rfa* mutation. Deep rough mutants (*rfa*) are highly sensitive to organic solvents by virtue of the fact that they are missing a substantial portion of their outer membrane (Nikaido and Vaara, 1987). The solvent mixture (40% (v/v) methanol and 10% (v/v) *N,N*-dimethylformamide) effectively dissolves both the enzyme substrates and their products. The final pH of the reagent is between 7.8 and 7.9. This value is very close to the optimal pH values for measurement of both  $\beta$ -galactosidase (7.75) and alkaline phosphatase (8.05) activity described by Mersch-Sundermann et al. (1991).

### 3.2. Color interference

We explored the use of centrifugation for post-exposure removal of colored samples. However, we found that in many instances it was not completely effective. Insoluble, colored components remained adhered to the sides and bottom of the microplate wells. Moreover, initial optical density readings did not effectively compensate for this interference. Optical density readings were often observed to fall for the first 40–60 min after cell resuspension and addition of the SOS Chromogen. This phenomenon is illustrated in Fig. 2. The reduction in optical density is presumably caused by a decrease in turbidity resulting from cell lysis and dissolution of organic substances that were insoluble in the original assay medium. In all subsequent experiments initial optical density readings were taken 60 min after cell resuspension and SOS Chromogen addition. Final readings were taken 30–120 min after the initial reading.

### 3.3. Cell density, substrate conversion time, exposure time and S9 concentration

Preliminary experiments were conducted to determine the optimal concentration of cells in the assay mixture. The results are illustrated in Fig. 3. The results indicate that both the MaxIF and the SOSIP of 4-NQO are highest at  $5 \times 10^6$  CFU/ml. For the diesel particulate extract, maximum SOSIP and MaxIF were obtained at  $1 \times 10^7$  CFU/ml. All subsequent experiments were carried out using a cell density of  $6\text{--}8 \times 10^6$  CFU/ml (30–40% of standard protocol).

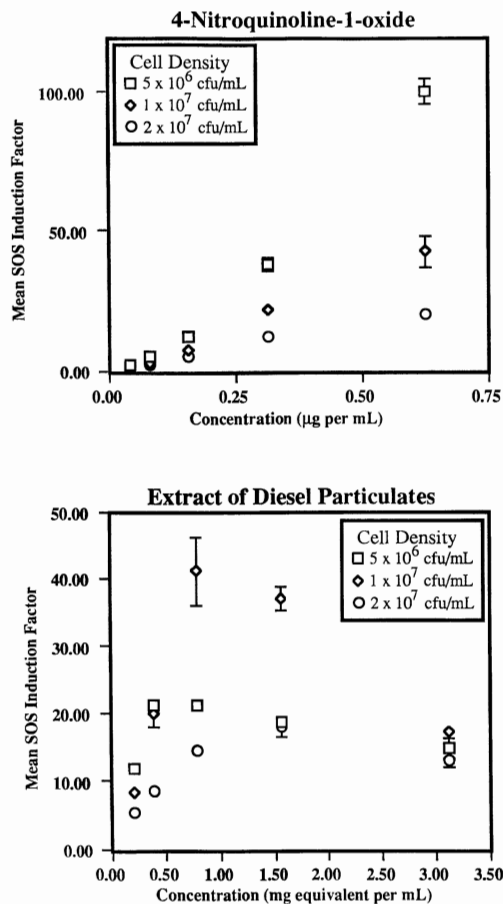


Fig. 3. The effect of cell concentration on SOS Chromotest results. The upper panel illustrates the effect of cell density on the SOS genotoxicity of 4-NQO. The corresponding SOSIP values are (lowest to highest cell density): 170.1 IF  $\mu\text{g}^{-1}$ , 72.5 IF per  $\mu\text{g}$  and 32.6 IF  $\mu\text{g}^{-1}$ . The lower panel illustrates the effect of cell density on the SOS genotoxicity of the diesel particulate extract. The corresponding SOSIP values are: 49.0 IF  $\text{mg}^{-1}$ , 55.7 IF  $\text{mg}^{-1}$  and 15.5 IF  $\text{mg}^{-1}$ . Induction factor values are means of five replicates. Error bars are 1 SE (standard error of the mean). Where error bars are not shown, they were smaller than the plotting symbol. Exposure time = 2 h. Substrate conversion time = 120 min total. Cell concentrations were determined by serial dilution and ordinary plate counts on LB agar (20  $\mu\text{g}/\text{ml}$  ampicillin).

Further experiments were carried out to determine the optimal exposure time, substrate conversion time and, for progenotoxic samples, the optimal S9 concentration. Results were interpreted using SOSIP and MaxIF values. Fig. 4 illustrates some of the results of experiments to determine an effective substrate



conversion time (i.e., enzyme assay incubation time). The results indicate that in some cases substrate conversion time has little effect on the SOS Chromotest results (e.g., MNNG and coal tar extract). Similar results were obtained for 2-NA with S9, Captan with S9 and urban dust extract with S9. For MitC the highest SOSIP and MaxIF were obtained for substrate conversion times of 30–60 min after an initial reading at 60 min. For several other samples net substrate conversion times of 60 min resulted in small (< 3-fold) increases in SOSIP and MaxIF. These samples include 4-NQO, NDMA, and coal tar extract with S9. Substrate conversion time had a

particularly dramatic effect on the diesel particulate extract results. The SOSIP associated with a 60 min net incubation time is at least an order of magnitude higher than other values. Overall, the results indicate that for 8 out of the 9 substances tested with or without S9 activation (extract of urban dust elicited an erratic, marginal response in the absence of S9), the SOSIP calculated for a 60 min net substrate conversion time was either higher than, or not significantly different from, that calculated for other incubation times. Fig. 5a provides a summary of the effect of substrate conversion time on the mean SOSIP of the complex mixtures examined. Mean

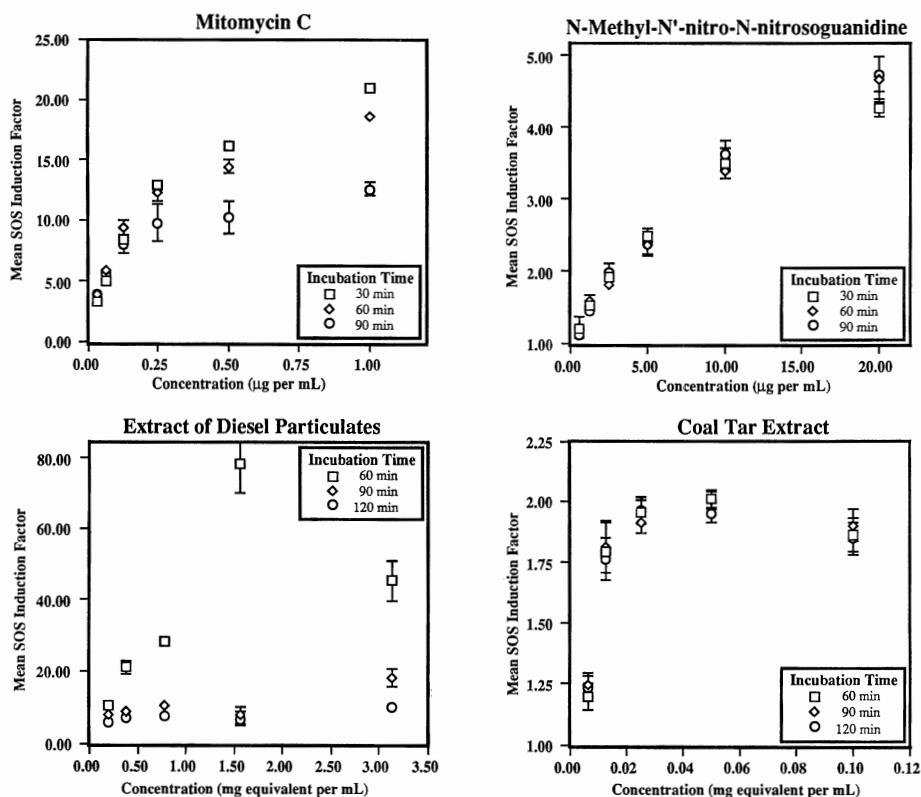


Fig. 4. The effect of substrate conversion time on SOS Chromotest results. Exposure time was held constant at 3 h. In all cases conversion time values indicate minutes *after* an initial measurement taken 60 min after cell resuspension and SOS Chromogen addition. All samples were tested without S9 activation. The two upper panels illustrate the effect of substrate conversion time on the SOS genotoxicity of MitC and MNNG. The corresponding SOSIP values are (shorter to longer incubation time): (1) MitC, 43.5 IF  $\mu\text{g}^{-1}$ , 58.2 IF  $\mu\text{g}^{-1}$  and 41.9 IF  $\mu\text{g}^{-1}$ ; (2) MNNG, 0.29 IF  $\mu\text{g}^{-1}$ , 0.22 IF  $\mu\text{g}^{-1}$  and 0.25 IF  $\mu\text{g}^{-1}$ . The lower panel illustrates the effect of substrate conversion times on the SOS genotoxicity of the diesel particulate matter extract and the coal tar extract. The corresponding SOSIP values are: (1) diesel particulate extract, 49.1 IF  $\text{mg}^{-1}$ , 3.6 IF  $\text{mg}^{-1}$  and 1.2 IF  $\text{mg}^{-1}$ ; (2) coal tar extract, too few points to calculate SOSIP, values are similar and in 35 IF  $\text{mg}^{-1}$  range. Induction factor values are means of triplicate samples. Error bars are 1 SE. Where error bars are not shown, they were smaller than the plotting symbol. Initial cell concentration was  $6\text{--}8 \times 10^6$  CFU per mL.

values were calculated using all experimental data. The figure confirms that the highest SOSIPs often occur when the final optical density readings are taken 60 min after the initial reading.

Fig. 6 illustrates the effect of exposure time (i.e., contact time between cells and sample) on the SOS genotoxicity of MitC, 4-NQO, diesel particulate extract and coal tar extract. The results indicate that in several instances exposure time has a large effect on genotoxic potency. For diesel particulates without S9 the SOSIP calculated for a 3-h exposure time is at

least 3-fold higher than that calculated for other exposure times. The highest MaxIF for the coal tar extract without S9 was obtained following a 3 h exposure. Results for diesel particulates with S9 and urban dust with S9 revealed a similar pattern. In addition, the potency of MitC (Fig. 6), Captan with S9 and 2-NA with S9 are higher when the exposure time is increased to 3 h. Exposure time had a relatively minor (< 2-fold) effect on the potency of 4-NQO (Fig. 6), MNNG and NDMA with S9. Fig. 5b provides a summary of the effect of exposure time on the genotoxic potency of the complex mixtures examined. The figure indicates that in most cases a 3-h exposure provides the highest mean SOSIP values. The exception is coal tar extract with S9, which elicited a strong response following a 4-h incubation.

Fig. 7 summarizes the effect of S9 enzyme concentration on the SOS genotoxicity of captan, NDMA, 2-NA, diesel particulate extract, urban dust extract and coal tar extract. The results indicate that there is no single S9 concentration that produces the highest genotoxic potency in the majority of samples. Pure substances such as 2-NA elicit a high SOSIP at high S9 concentration (3%, v/v). Captan demonstrates the opposite trend (max. at 0.75%, v/v). For complex mixtures, the coal tar extract elicits a high SOSIP at high S9 concentrations (4%, v/v), while the diesel particulate extract demonstrates the opposite trend (max. at 0.5%, v/v). Maximal SOSIP values for the urban dust extract were obtained for S9 concentrations in the 1–2% range.

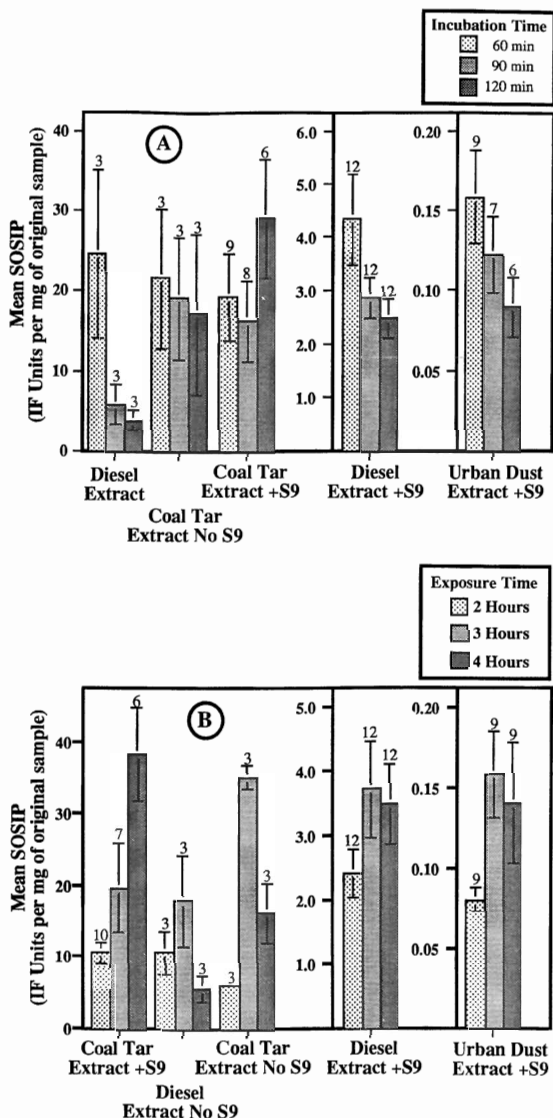


Fig. 5. Summary of the effect of exposure time and substrate conversion time on the SOS Chromotest results for 3 complex mixtures. Potency is expressed as mean SOSIP in IF per equivalent mg of original sample. Mean values were calculated from all available experimental data. (A) The effect of substrate conversion time on the genotoxic potency of coal tar extract, diesel particulate extract and urban dust extract. In all cases incubation times are minutes after an initial optical density reading taken 60 min after cell resuspension and SOS Chromogen addition. Error bars are 1 SE. Numbers above error bars indicate sample size. Each individual SOSIP value is based on three concentration-response replicates. (B) The effect of exposure time on the genotoxic potency of coal tar extract, diesel particulate extract and urban dust extract. Error bars are 1 SE. Numbers above error bars indicate sample size. Each individual SOSIP value is based on three concentration-response replicates.

Based on the results shown in Figs. 2–7, the following standard protocol was adopted for investigations of complex environmental extracts:

1. Cell density =  $6-8 \times 10^6$  CFU/ml.
2. Exposure time = 3 h @ 37°C.
3. Substrate conversion time (SOS Chromogen incubation time) = 120 min total with initial reading at 60 min.
4. Final S9 concentration in assay mixture = 1% (v/v). If results are negative, questionable or erratic repeat with 2–4% (v/v).

### 3.4. Automation

Automation of the modified SOS Chromotest protocol dramatically increased sample throughput. The

Biomek™ workstation permitted processing of up to 12 microplates per day. Up to six samples can be tested on each plate, resulting in a maximum daily throughput of 72 samples. This maximum assumes that each sample is tested in duplicate with six, 2-fold serial dilutions and each plate contains 12 solvent control wells and 6 positive control wells. To streamline the assay procedure, 12 microplates were processed in three batches of 4 plates. The automated system is capable of adding the culture media and appropriate culture controls to a batch of 4 plates in 25–30 min. Sample addition and serial dilution requires an additional 15 min. Therefore, assay of up to 24 samples can be initiated in under 45 min. The completed plates are placed in the 37°C incubator

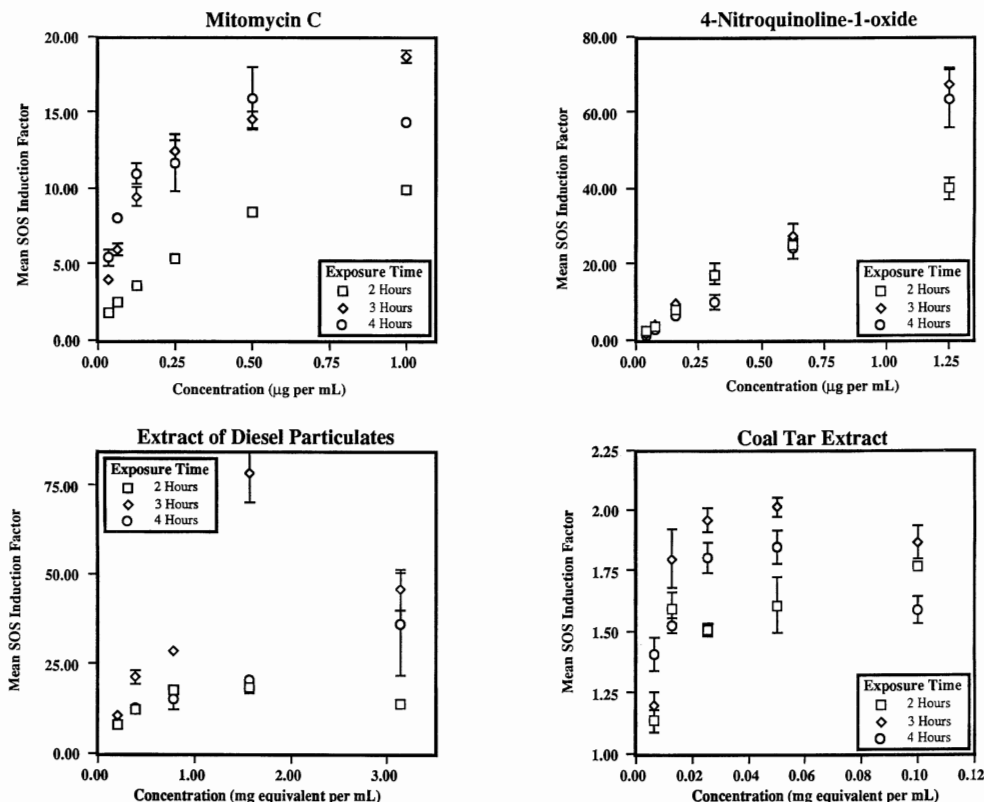


Fig. 6. The effect of exposure time on SOS Chromotest results. *Net* substrate conversion time was held constant at 60 min. All samples were tested without S9 activation. The two upper panels illustrate the effect of exposure time on the SOS genotoxicity of MitC and 4-NQO. The corresponding SOSIP values are (shorter to longer exposure time): (1) MitC, 13.9 IF  $\mu\text{g}^{-1}$ , 58.2 IF  $\mu\text{g}^{-1}$  and 57.7 IF  $\mu\text{g}^{-1}$ ; (2) for 4-NQO, 54.3 IF  $\mu\text{g}^{-1}$ , 52.5 IF  $\mu\text{g}^{-1}$  and 50.8 IF  $\mu\text{g}^{-1}$ . The lower panel illustrates the effect of exposure time on the SOS genotoxicity of the diesel particulate matter extract and the coal tar extract. The corresponding SOSIP values are: (1) diesel particulate extract, 15.7 IF  $\text{mg}^{-1}$ , 49.1 IF  $\text{mg}^{-1}$  and 9.0 IF  $\text{mg}^{-1}$ ; (2) coal tar extract, too few points to calculate SOSIP, highest values appear to correspond to an exposure time of 3 h (SOSIP in the 35 IF  $\text{mg}^{-1}$  range). Induction factor values are means of triplicate samples. Error bars are 1 SE. Where error bars are not shown, they were smaller than the plotting symbol. Initial cell concentration was  $6-8 \times 10^6$  CFU  $\text{ml}^{-1}$ .

prior to beginning culture addition for the next batch of 4 plates. Separate overnight cultures (staggered by 1 h) were prepared for each batch of microplates. Culture dilution and S9 mix preparation (where necessary) were carried out immediately prior to assay initiation. Once exposure and centrifugation is completed, cell resuspension, addition of SOS Chromogen and mixing of each microplate requires 3 min. Optical density measurements at both 405 and 620 nm requires less than 10 s per microplate.

Including initial and final optical density measurements at both 405 and 620 nm, 12 microplates generate over 4600 optical density values. To handle this quantity of data, we have also partially automated the data analyses. All raw data were processed as a batch using the SAS system version 6.08 (SAS Institute, 1993). We have written several programs that perform all necessary calculations and produce concentration response plots for each tested sample.

### 3.5. Test performance

Using the described protocol we have conducted extensive tests on organic extracts of industrial wastes (White et al., 1996a White et al., 1996b), river sediments (White et al., 1996c) and a wide range of aquatic biota (White et al., 1996d, e). The large number of assays performed in our laboratory permits an analysis of the long-term variability in the response to both positive controls and blank solvent. Fig. 8 summarizes the results of 150 microplate assays conducted without S9 and 57 assays conducted with S9. The positive controls were MitC and BaP, respectively. The results indicate that the variability in response across microplates is very small. The coefficient of variation on the SOSIP values for BaP and MitC are 2.8 and 3.2%, respectively. The lower portion of the figure is a comparison of the SOSIP values obtained using our protocol and values presented in the recent literature. The results indicate that the SOSIP values obtained using the semi-automated, microplate method presented here are very similar to previously published values.

The variability in the response to the carrier solvent can determine the sensitivity of the assay system. Well behaved control wells, result in tight confidence intervals and an increased ability to detect weak, positive responses. We calculated the

upper 95% confidence interval (Welsch et al., 1988; Zar, 1984) on the mean control values from 293 microplate assays conducted in our laboratory. The results obtained are summarized in Fig. 9. The results indicate that the upper 95% confidence limit of

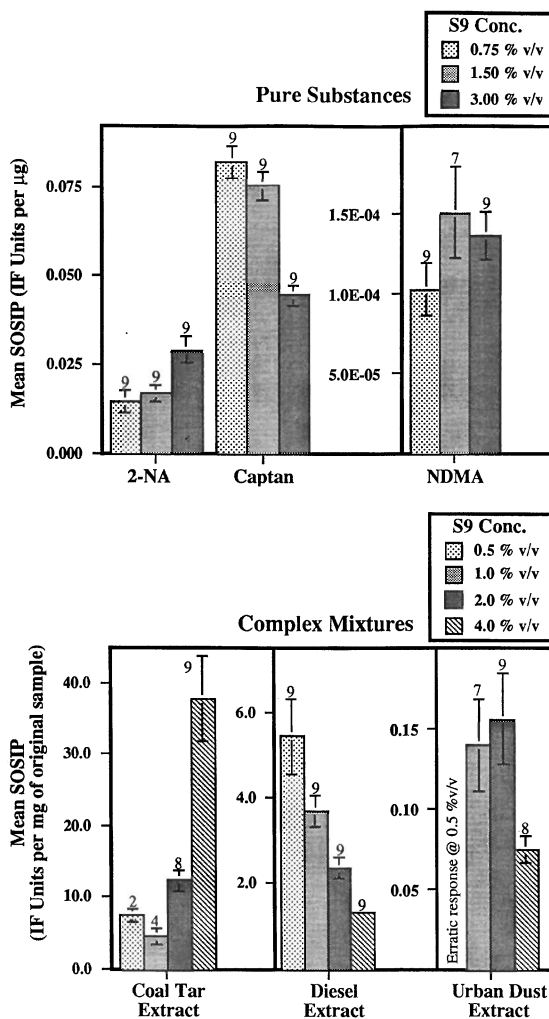


Fig. 7. Summary of the effect of S9 concentration on SOS Chromotest results for 3 complex mixtures and 3 reference genotoxins. Potency is expressed as mean SOSIP in IF per equivalent mg of original SRM or IF per  $\mu\text{g}$  of pure substance. Mean values were calculated from all available experimental data. The upper panel illustrates the effect of final S9 concentration on the mean SOSIP of 2-NA, Captan and NDMA. The lower panel illustrates the effect of S9 concentration on extracts of coal tar, diesel particulates and urban dust. Error bars are 1 SE. Numbers above error bars indicate sample size. Each individual SOSIP value is based on three concentration-response replicates.

the solvent control never exceeded 1.302 IF Units and rarely ( $\approx 2\%$  of microplates) exceeded 1.20 IF Units. In over 75% of the assays conducted in our laboratory the upper (95%) confidence limit was less than 1.10. Upper confidence limits can be used to calculate minimum genotoxic concentration values (e.g., Langevin et al. (1992) and White et al. (1995, 1996a).

## 4. Discussion

### 4.1. Published SOS Chromotest modifications for the examination of complex samples

Approx.  $\frac{1}{4}$  of the SOS Chromotest publications have examined complex mixtures. Tested samples include bodily fluids and excreta, foodstuffs, indus-

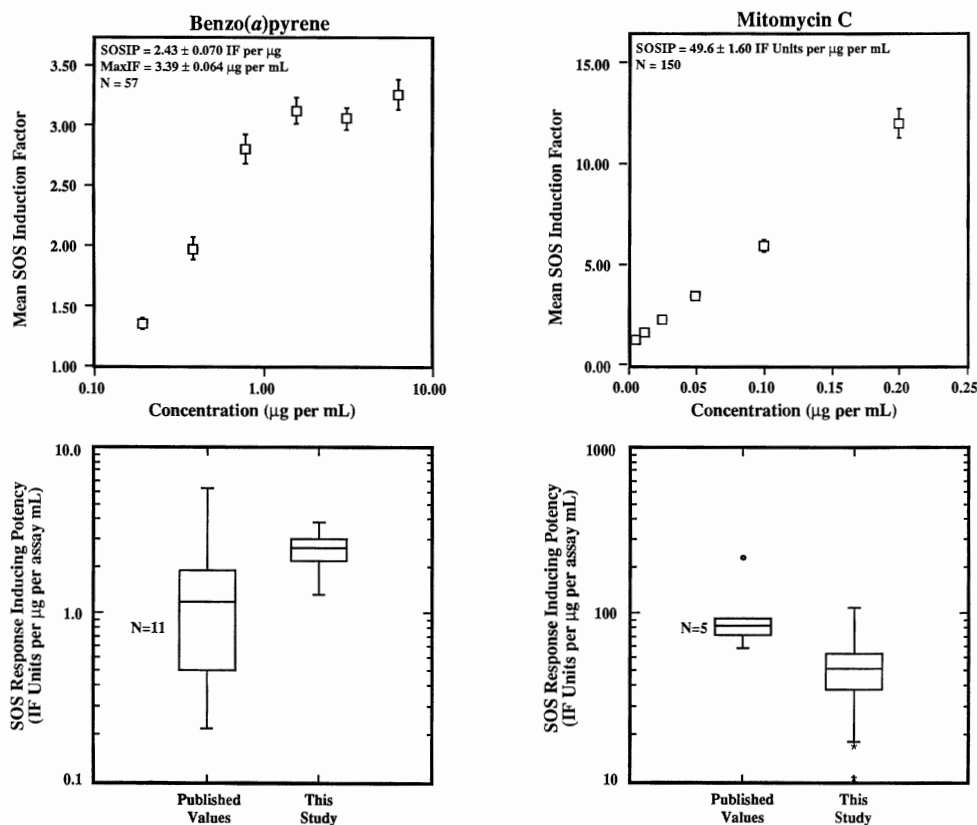


Fig. 8. Historical summary of SOS Chromotest results for two reference genotoxins used as positive controls. The SOS Chromotest protocol discussed in the text was used in 150 assays conducted in the absence of S9 and 57 assays conducted in the presence of S9 activation. The upper panels show the concentration-response relationships based on average IF values. Error bars are 2 SE. Where error bars are not shown, they were smaller than the plotting symbol. The lower panels compare the SOSIP values obtained using the protocol described in this study with those presented in the recent literature. Values are summarized using box and whisker plots (Wilkinson, 1987). The horizontal lines dividing the boxes are the median values. The edges of the boxes represent the interquartile range (from the 25th percentile to the 75th percentile). Vertical lines or whiskers extend 1.5 interquartile ranges beyond the box. Values beyond the whiskers are plotted as circles or asterisks. Asterisks represent values 1.5 to 3 interquartile ranges from the box edge. Circles are values more than 3 interquartile ranges from box edge. Literature values for BaP were obtained from: Mersch-Sundermann et al. (1992), Von der Hude et al. (1988), P. Quillardet (pers. comm.), Venier et al. (1989), Mersch-Sundermann et al. (1993), Schleibinger et al. (1989), Tudek et al. (1988), Marzin et al. (1986), Rempola et al. (1986). Literature values for MitC were obtained from: Auffray and Boutibonnes (1987), P. Quillardet (pers. comm.), Venier et al. (1989), Ohta et al. (1984), Rempola et al. (1986).

trial wastes, surface waters, sediments and airborne particulate matter. Table 1 provides a summary of the published studies that used the SOS Chromotest to investigate the genotoxicity of complex samples. These complex samples present several technical challenges for the SOS Chromotest. In overcoming these problems many researchers have modified the original SOS Chromotest protocol. For example, the dilute nature of liquid samples and the incompatibility of solid samples requires the preparation of organic extracts and/or sample concentrates (see Table 1 for examples). Organic extracts, as well as oily samples, are not easily mixed with the aqueous assay medium (Raabe et al., 1993; White et al., 1996a). Some researchers have attempted to use non-ionic detergents to overcome this problem (Janz et al., 1990; Raabe et al., 1993; Braun et al., 1993). Complex environmental extracts and concentrates also cause color interference problems. These are compounded by the poor solubility of many organic substances and extracts in the aqueous assay system. Precipitation of sample components can contribute to sample turbidity. Turbidity, like color, can interfere

with the measurement of enzyme activity (Langevin et al., 1992; White et al., 1996a; Venier et al., 1989; Hoflack et al., 1993; Lan et al., 1991). These problems are often overcome by post-exposure centrifugation and supernatant removal (Bosworth and Venitt, 1986; White et al., 1996a; Legault, pers. comm.; Von der Hude et al., 1988) and/or initial optical density readings taken immediately after addition of the enzyme assay reagent(s) (Wong et al., 1994; McDaniels et al., 1993; Lan et al., 1991; Nair et al., 1990; Venier et al., 1989; Poirier et al., 1989; Von der Hude et al., 1988).

In addition to protocol modifications that permit the testing of complex mixtures, additional modifications have been introduced. The majority of these concern culture methods, the concentration of cells in the assay mixture, incubation times, the concentration of S9 and cofactors in the activation mixture and the composition of the cell lysis/enzyme assay reagent. The net result is a wide range of SOS protocols for the analysis of complex samples, few of which have been validated in any way. Several researchers have mentioned that although the SOS

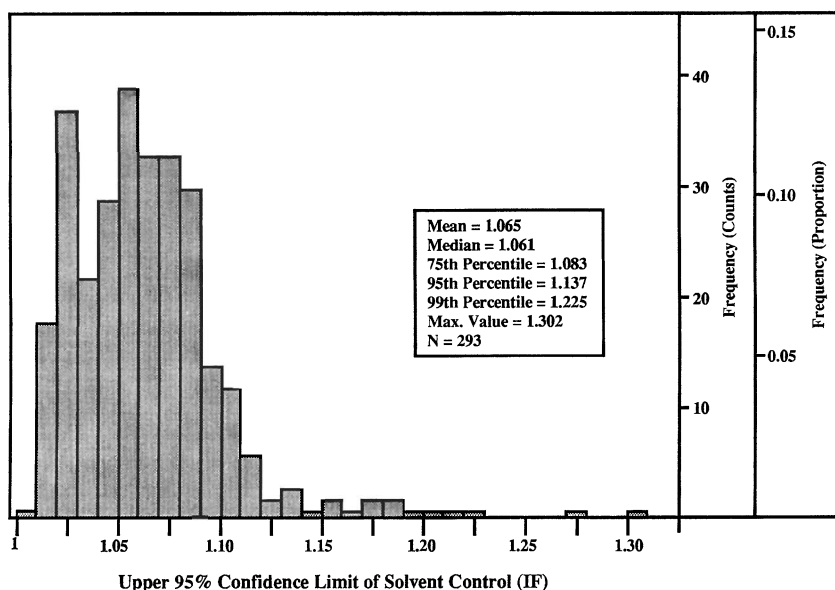


Fig. 9. Histogram of upper 95% confidence limits on solvent controls. Confidence limits on mean control values were calculated for 293 microplates. Each confidence limit value is based on the results for 12 wells on a given microplate. All solvent control wells contained 5% (v/v) DMSO. All assays were conducted using the Biomek™ automated laboratory workstation and the SOS Chromotest method described in the text. Variance of mean IF values for solvent controls were calculated according to Welsch et al. (1988). Confidence limits were calculated according to Zar (1984).

Table 1

Summary of published studies which used the SOS Chromotest to investigate the genotoxicity of complex mixtures

Sample studied	Results obtained	Source <sup>a</sup>
(1) URINE AND URINE CONCENTRATES		
Unconcentrated urine.	Positive response for subjects exposed to toxic mineral oils.	1
XAD-2 <sup>b</sup> extract.	No positive response.	2
XAD-2 and C <sub>18</sub> <sup>c</sup> SEP-PAK extracts.	Positive response for patients receiving antineoplastic drugs (+ S9 where required).	3
XAD-2 extract.	Positive response (+ S9 and – S9) for patients receiving coal tar treatment.	4
(2) AIRBORNE PARTICULATES		
DCM <sup>d</sup> and acetone extracts of samples from Paris area.	Several positive responses. No response in presence of S9.	5
DCM extracts of samples from Berlin area.	Several positive responses. Higher activity with S9.	6
DCM extracts of samples from Gangzhou, China.	Several positive responses without S9.	7
(3) INDUSTRIAL WASTES AND EFFLUENTS		
Unconcentrated effluents from 7 industries.	Several positive responses. Algal activation documented.	8
Several waste samples.	Investigated the effect of detergents on the response of complex mixtures.	9
Unidentified industrial waste.	Effect of complex waste on 4-NQO response.	10
Solid waste from semiconductor manufacture.	Several positive responses (PQ37 and PQ243) both with and without S9.	11
Aqueous soil and waste leachates.	Positive responses without S9.	12
DCM extract of aluminum plasma etching waste.	Several positive responses without S9. S9 addition reduced response.	13
XAD-8, XAD-4 extracts of bleached Kraft mill effluent.	Positive response on several fractions (both with and without S9).	14
Kraft pulp spent liquors (pulp and paper waste).	Several positive responses without S9. No response for hardwood pulps.	15
Petroleum refinery effluents (10-fold concentrate).	Positive response for one sample.	16
DCM extracts of final effluents from 42 industries.	Samples of varying potency. S9 frequently caused reduction in potency.	17
DCM extracts of effluent suspended particulates.	High potency positive responses (relative to aqueous filtrate).	18
(4) SURFACE WATERS, SEDIMENT AND SOILS		
Surface water and aqueous sediment extracts.	Several positive responses on surface waters and sediments (with S9).	19
DMSO extracts of marine sediments.	Several positive responses both with and without S9.	20
DMSO extracts of river sediment.	Several positive responses with S9.	21
DCM extracts of surface water and sediments.	Positive responses on water, suspended and bottom sediments (both + S9 and – S9).	22
Unaltered river sediments.	Direct sediment test procedure (DSTP).	23
DCM and cyclohexane extracts of contaminated soils.	Several positive responses both with and without S9.	24
C <sub>18</sub> extracts of groundwater.	Weak, but detectable responses at several sites (without S9).	25



Table 1 (continued)

Sample studied	Results obtained	Source <sup>a</sup>
Raw surface waters and sediment extracts (aqueous and DMSO).	Several positive responses on water and bottom sediment extracts without S9.	26
Aqueous extract of river sediment.	No evidence of genotoxicity.	27
DCM extracts of suspended particulates and sediments.	Suspended particulates – positive without S9. Sediments – frequently require S9.	28
(5) MISCELLANEOUS SAMPLES		
Orange juice.	Detected Aflatoxin B <sub>1</sub> genotoxicity in juice.	29
Cow's milk.	Aflatoxin M <sub>1</sub> not detected in milk extract	30
Various complex mixtures.	Discussed effects of extraction solvents on test results.	31
Aqueous extract of faeces.	No clear positive response. Exogenous catalytic activity documented.	32
Aqueous extract of faeces.	No positive response.	33
XAD-2 extract of red wine.	Weak positive responses both with and without S9.	34
Refined hard-wood smoke flavor.	No positive response with or without S9.	35
Aqueous and organic extracts of preserved foods.	Several weak, positive responses without S9.	36
Ethyl acetate extract of faeces.	Positive responses without S9.	37
Aqueous extract of medicinal plant.	Positive response on SOS spot test.	38
DCM extracts of SRM 1650 and SRM 1649.	Strong, positive response to SRM 1650. Questionable response to SRM 1649.	39
Nitrosated red wine.	Toxic effect. Genotoxicity results inconclusive.	40
CHL <sup>c</sup> extracts of molds in uranium mine samples.	Positive responses, particularly with S9.	41
DCM extracts of melted snow.	Several positive samples. Most required S9 activation.	42
DCM extracts of bivalve molluscs ( <i>Mya arenaria</i> ).	Several positive responses without S9. Evidence of genotoxin bioaccumulation.	43
DCM extracts of freshwater macroinvertebrates and fish.	Several positive responses without S9. Evidence for bio-diminution.	44

<sup>a</sup> Literature cited: (1) Fish et al., 1987; (2) De Méo et al., 1988; (3) Kohn et al., 1988; (4) Venier et al., 1989; (5) Courtois et al., 1988; (6) Schleibinger et al., 1989; (7) Qian et al., 1994; (8) Harwood et al., 1989; (9) Raabe et al., 1989; (10) Janz et al., 1990; (11) Braun et al., 1993; (12) Hoflack et al., 1993; (13) Raabe et al., 1993; (14) Rao et al., 1994; (15) Nylund et al., 1994; (16) Sherry et al., 1994; (17) White et al., 1996a; (18) White et al., 1996b; (19) Dutka et al., 1987; (20) Xu et al., 1987; (21) Lan et al., 1991; (22) Langevin et al., 1992; (23) Kwan and Dutka, 1992; (24) McDaniels et al., 1993; (25) Pfeil et al., 1994; (26) Wong et al., 1994; (27) Naikai and Ahlf, 1994; (28) White et al., 1996c; (29) Riesenfeld et al., 1985; (30) Fremy and Quillardet, 1985; (31) Carver and Machado, 1986; (32) Bosworth and Venitt, 1986; (33) Venitt and Bosworth, 1986; (34) Rueff et al., 1986; (35) Jenek et al., 1988; (36) Poirier et al., 1989; (37) Nair et al., 1990; (38) Vargas et al., 1990; (39) Nylund et al., 1992; (40) Laires et al., 1993; (41) Srám et al., 1993; (42) White et al., 1995; (43) White et al., 1996d; (44) White et al., 1996e.

<sup>b</sup> Non-polar polystyrene resin (Amberlite<sup>®</sup>). <sup>c</sup> Non-polar, bonded-phase extraction cartridge. <sup>d</sup> Dichloromethane. <sup>e</sup> Chloroform.

Chromotest is convenient and practical, its use on complex mixtures requires protocol validation (Courtois et al., 1988; Nylund et al., 1992) such as that performed by Whong et al. (1986) for the SOS/*umu* assay. The goal of this work was to produce a rapid, cost-effective microplate version of the SOS Chromotest and validate its performance on several complex samples.

#### 4.2. SOS Chromotest protocol examined in this study

The protocol described here includes several modifications of the standard SOS Chromotest protocols (Quillardet and Hofnung, 1985; Organics Ltd., 1990; Fish et al., 1987; Mersch-Sundermann et al., 1991). The original test tube version of the test (Quillardet and Hofnung, 1985) recommends that  $\beta$ -galactosi-

dase and alkaline phosphatase activities be measured in a Tris (Tris(hydroxymethyl)aminomethane) or phosphate buffer containing sodium dodecyl sulfate (SDS) to promote cell lysis. The standard microtiter kit version of the test contains a ready-to-use *chromogen* for the determination of both  $\beta$ -galactosidase and alkaline phosphatase activity. However, its composition is not provided (Orgenics Ltd., 1990). Several researchers have recommended modification of the *SOS Chromogen* or enzyme assay/cell lysis reagent (Mersch-Sundermann et al., 1991; Marzin et al., 1986; Nair et al., 1990). However, for the most part the enzyme assay reagent has remained unchanged. The *SOS Chromogen* advocated here does not contain SDS and contains both the  $\beta$ -galactosidase (X-Gal) and the alkaline phosphatase (PNPP) substrates in a solvent mixture that promotes cell lysis. The removal of SDS avoids bubbles and possible detrimental effects on  $\beta$ -galactosidase. De M  o et al. (1988) and Whong et al. (1986) demonstrated that SDS concentrations as low as 0.1% can reduce  $\beta$ -galactosidase activity by 1–2% per min. Addition of both X-Gal and PNPP to the *SOS Chromogen* is advantageous since it permits the simultaneous measurement of both  $\beta$ -galactosidase and alkaline phosphatase activity (Fish et al., 1987).

To avoid problems introduced by colored samples we advocate centrifugation and sample removal prior to enzyme activity measurements. While initial optical density measurements taken immediately after *SOS Chromogen* addition can correct for the color or turbidity of the tested substance or extract, it cannot correct for interference with enzyme activity. Several researchers have documented specific inhibition of  $\beta$ -galactosidase and/or alkaline phosphatase. Courtois et al. (1988, 1992) and Marzin et al. (1986) have demonstrated that components of the S9 activation mixture can passively inhibit  $\beta$ -galactosidase. Olivier and Marzin (1987), Hoflack et al. (1993) and Venitt and Bosworth (1986) demonstrated that some substances (e.g.,  $\text{AlCl}_3$ , landfill leachates, faecal extracts) can alter the activity of  $\beta$ -galactosidase and/or alkaline phosphatase. These alterations can likely be attributed to the presence of metal ions (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ni}^{2+}$ ) that are known to inhibit  $\beta$ -galactosidase (Cohn and Monod, 1951; Biswas, 1987) and alkaline phosphatase activity (Asthana et al., 1992; Rueter, 1983). Differential,

post-exposure inhibition of either  $\beta$ -galactosidase or alkaline phosphatase by the tested substance will result in ambiguous results. The results presented in Fig. 2 indicate that the optical density values reach a minimum after approx. 40–60 min after Chromogen addition. Thus, in addition to centrifugation and supernatant removal we advocate initial optical density readings taken 60 min after cell resuspension and Chromogen addition.

Recent publications indicate that recommended cell concentrations in the *SOS Chromotest* assay mixture vary about 5-fold from  $\approx 4 \times 10^6$  cells per ml (e.g., Janz et al., 1989) to  $\approx 2 \times 10^7$  cells/ml (e.g., standard procedure of Quillardet and Hofnung, 1985). Both Mersch-Sundermann et al. (1991) and Janz et al. (1989) observed that although reductions in cell number cause reductions in enzyme activity, induction of the *SOS* response (measured by IF and *SOSIP* values) is increased. In addition, Janz et al. (1988) found that the variability in *SOS* induction factor values was minimized at cell densities in the  $4 \times 10^6$  cells/ml range. The results obtained here also indicate that lower cell concentrations in the assay mixture can increase *SOSIP* values. For routine testing of complex mixtures, we advocate from  $6 \times 10^6$  to  $8 \times 10^6$  cells/ml of assay mixture. In addition, like Mersch-Sundermann et al. (1991), we recommend culturing of bacteria in glassware that provides a large surface area for gas exchange and dilution of overnight cultures (15 h max.) in pre-warmed broth.

*SOS Chromotest* publications recommend a variety of exposure times. The standard procedure of Quillardet and Hofnung (1985) as well as the Orgenics Ltd. microplate kit procedure and the Bioscreen Analyzer<sup>®</sup> microplate procedure (Janz et al., 1988) recommend an exposure time of 2 h at 37°C. The majority of published complex mixture studies (e.g., Xu et al., 1987; Lan et al., 1991; Wong et al., 1994; Venitt and Bosworth, 1986) employed a 2-h exposure period. Mersch-Sundermann et al. (1991) and Quillardet and Hofnung (1985) determined that, with respect to several pure substances, there is no reason to expose PQ37 for longer than 90 min. However, they both acknowledge that in some instances exposure times greater than 2 h may be required for maximum *SOS* response induction, particularly when cell density is reduced. Nair et al. (1990) and Pfeil et

al. (1994) employed a 3-h exposure period in their study of faecal (Nair et al., 1990) and groundwater (Pfeil et al.) extracts. The results obtained here indicate that optimal results are frequently obtained following a 3-h exposure (see Fig. 5b and Fig. 6). It is possible that microplate methods require longer incubations due to the reduced aeration of microplate wells compared with that of agitated 15 ml glass tubes (standard method of Quillardet and Hofnung, 1985).

Recent publications indicate that recommended substrate conversion times vary from 10 min to 180 min. Much of this variability can likely be attributed to variations in the composition of the SOS Chromogen and its concomitant efficacy for cell lysis and substrate conversion. The standard tube method of Quillardet and Hofnung (1985) uses ONPG (O-nitrophenyl- $\beta$ -D-galactopyranoside) as a  $\beta$ -galactosidase substrate and recommends 10–90 min incubation with the SOS Chromogen. Lan et al. (1991) indicated that optimal results (highest IF values) were obtained after 60 min incubation with SOS Chromogen. Xu et al. (1987) observed that optimal results are frequently obtained when a 90 min incubation time is employed. Poirier et al. (1989) employed a 120 min incubation time for measurement of  $\beta$ -galactosidase activity. Mersch-Sundermann et al. (1991) examined incubation times up to 60 min and concluded that SOS Chromogen incubation time has a negligible effect on test performance. They recommend a 25 min incubation.

It appears that SOS Chromogen mixtures containing X-Gal require longer incubations. However, recommended times still vary considerably. The standard kit method recommends an incubation of 60–90 min. McDaniels et al. (1990) recommended an incubation time of 120 min. Legault et al. (1994) recommended 60 min without S9 and 90 min in the presence of S9. Nair et al. (1990) described a modified SOS Chromogen and recommended incubation for 3 h. In this study we determined that optimal results (high SOSIP and high MaxIF) are frequently obtained when the final optical density reading is taken 60 min after an initial reading taken 60 min after Chromogen addition. For some samples (e.g., diesel particulate extract), the net Chromogen incubation time had a large effect on the result (see Figs. 4 and 5a). For other samples the effect was small or

negligible. Recent genotoxicity analyses of 150 industrial effluent extracts (White et al., unpublished) revealed that net Chromogen incubation times of more than 60 min are rarely required for optimal results.

SOS Chromotest publications advocate a wide range of S9 concentrations. The standard method of Quillardet and Hofnung (1985) recommends a final S9 concentration in the assay mixture of 9% (v/v). However, Courtois et al. (1988), Marzin et al. (1986) and Mersch-Sundermann et al. (1993) have determined that when used at high concentration, S9 enzymes can inhibit the activity of  $\beta$ -galactosidase. For optimal results, Mersch-Sundermann et al. (1993) recommends final S9 concentrations in the 1.8 to 4.5% (v/v) range (20–50% of the standard method). Other researchers studying both complex mixtures and pure substances often recommend S9 concentrations far lower than that originally recommended by Quillardet and Hofnung (1985). For example, Poirier et al. (1989) used several concentrations of S9 between 0.9 and 18% (v/v) in their examination of preserved food extracts. Nylund et al. (1992), McDaniels et al. (1993), De M  o et al. (1988), Langevin et al. (1992), Nylund et al. (1994) and White et al. (1995, 1996a) used < 2% (v/v) to examine complex extracts of standard reference materials, contaminated soil, human urine, surface water and sediment, melted snow and industrial effluents. This concentration is close to the concentration recommended by Maron and Ames (1983) for the plate incorporation version of the Salmonella/mammalian microsome assay (20–50  $\mu$ l S9 per plate or  $\approx$  0.74–1.85%, v/v). The results obtained here indicate that there is no single concentration of S9 that is appropriate for all tested substances. For complex mixtures the optimal S9 concentration ranged from 0.5% to 4% (v/v) (see Fig. 7). Similar results have been obtained for the Salmonella test (Courtois et al., 1992). As a result, several researchers (e.g., Venitt et al., 1985; Ashby, 1986; Marzin et al., 1986) recommend that tests be carried out at several S9 concentrations.

#### 4.3. SRM results: comparisons with published results

The results obtained indicate that the potency of the diesel particulate extract is inversely related to the final S9 concentration (see Fig. 7). Nylund et al.

(1992) and Savard et al. (1992) also observed that the addition of S9 can cause large decreases in the mutagenic potency of diesel particulate extracts (SRM 1650). Many other researchers (e.g., Møller et al., 1982 and Saleem et al., 1984) have documented the direct-acting genotoxicity of diesel exhaust particulates. Our results further indicated that although the coal tar extract (SRM 1597) is genotoxic in the absence of S9 enzymes, the maximal response was obtained in the presence of 4% (v/v) S9. The IPCS collaborative study on complex mixtures did not obtain a positive Salmonella test result for SRM 1597 in the absence of S9 activation (Claxton et al., 1992). However, Goto et al. (1992) obtained a weak positive response on Salmonella TA98 without S9 and Whong et al. (1986) obtained a positive response without S9 for a coal dust extract examined using the SOS/*umu* test. For SRM 1649, our results indicated that in the absence of S9 activation, the urban dust extract was highly toxic and an extremely weak inducer of the SOS response (MaxIF < 1.4). The optimal response for this sample was obtained at an S9 concentration of 1–2% (v/v). Although organic extracts of airborne particulates frequently demonstrate enhanced potency in the presence of S9 enzymes (Schleibinger et al., 1989; Møller et al., 1982; Pitts et al., 1982; Matsumoto and Inoue, 1987), Courtois et al. (1988) indicated that extracts of particulates collected in Paris invoked the SOS response only in the absence of S9. The IPCS collaborative study determined that the mutagenic potency of SRM 1649 extracts on Salmonella TA98 and TA100 was not substantially altered when S9 was added.

#### 4.4. Protocol performance

At the present time we have used the described protocol for almost 300 microplate assays. The results obtained for 150 assays with Mitomycin C and 57 assays with benzo[a]pyrene indicate that SOSIP variability is very low (see Fig. 8). The low standard error associated with mean SOSIP values can be attributed to the precision and accuracy of the automated Biomek™ system. Biomek™ precision and accuracy also reduced the variability of the solvent controls. Our results (see Fig. 9) indicate that the upper 95% confidence limit of the solvent control is rarely above 1.20 SOS IF Units. The original SOS

Chromotest protocol of Quillardet and Hofnung (1985) recommends that only samples which invoke induction factors above 1.5 be denoted positive. Although some researchers also require evidence of a dose-related response (Nylund et al., 1992; Legault et al., 1994; Hoflack et al., 1993; Rao et al., 1994; Quillardet and Hofnung, 1993; Poirier et al., 1989), most only categorize induction factors above 1.5 as 'significant' positives (e.g., Legault et al., 1994; Von der Hude et al., 1988; Braun et al., 1993; Venier et al., 1989; Nylund et al., 1992; Poirier et al., 1989). Mersch-Sundermann et al. (1992) only considered induction factors greater than 2.0 as 'significant' positives. Other researchers have reduced this threshold to 1.3 (Wong et al., 1994; Dutka et al., 1987; Kwan and Dutka, 1992). An alternative to an arbitrary threshold value is a statistical comparison of sample IF values to the mean IF of the solvent control (Xu et al., 1989; Lan et al., 1991; Langevin et al., 1992; White et al., 1995, 1996a). While this approach is less subjective, it requires that the standard error of the control be calculated correctly. We have routinely used the method of Welsch et al. (1988) to calculate this standard error and determined that the somewhat arbitrary value of 1.5 is unnecessarily high. At the present time we routinely categorize SOS Chromotest results as follows:

1. Negative: induction factor never exceeds the upper confidence limit of the control.
2. Marginal: induction factor exceeds the upper confidence limit of the control at one or two concentrations only.
3. Positive: induction factor exceeds the upper confidence limit of the control at a minimum of three doses.
4. Erratic: induction factor may exceed the control at three or more concentrations, but the concentration-response relationship is highly erratic and can not be interpreted.

#### 4.5. Conclusion

We have described a semi-automated, microplate version of the SOS Chromotest for the analysis of complex environmental extracts. We have exploited the rapid growth rate of *E. coli* and the aforementioned advantages of the SOS Chromotest to produce a protocol that can process up to 72 samples in a

single working day. We have already used this method to analyse over 750 complex environmental extracts. Although we have not performed simultaneous comparisons between the SOS Chromotest and the Salmonella test, some researchers claim that the SOS Chromotest is more sensitive for the detection of genotoxicity in complex mixtures (McDaniels et al., 1993; Lan et al., 1991; McDaniels et al., 1990). The use of the Biomek™ automated laboratory workstation permitted increased accuracy, precision and throughput. While the Biomek™ is a specialized piece of laboratory equipment, it is important to realize that it is not dedicated to performing the SOS Chromotest. The instrument is readily available and can carry out the reagent transfers and dilutions required for a wide range of bioassays and other laboratory procedures. In our laboratory it has also been used to perform a sub-lethal bioassay with the green alga *Selenastrum capricornutum*. While the instrument is expensive ( $\approx$  \$25 000 US), it is no more costly than many other laboratory instruments (e.g., HPLC, ultracentrifuge, scintillation counter, spectrophotometer etc.).

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