

Relative sensitivities of forward and reverse mutation assays in *Salmonella typhimurium*

(genetic toxicology/mutagenesis/8-azaguanine resistance)

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ABSTRACT Forward mutation to 8-azaguanine resistance and reverse mutation to histidine prototrophy were measured in *Salmonella typhimurium* after treatment with 16 mutagens of both base-substitution and frameshift classes. The two approaches were found to be equisensitive for all 16 mutagens—i.e., induction of significant mutation occurred at similar concentrations in the forward mutation assay and in the most sensitive of the five Ames tester strains.

Rapid, inexpensive bacterial assays that measure chemically induced mutation are providing "early warning signals" of possible health hazards for humans. The most widely used system at present, that developed by Ames, detects reversion to histidine prototrophy in a set of *Salmonella typhimurium* strains (1-5). In these assays, specific lesions in the histidine operon are backmutated or possibly suppressed to allow the bacterium to synthesize histidine. Several strains are used simultaneously in order to provide a variety of genetic lesions as targets because of the known specificity of certain chemical classes with regard to the kind of mutation induced and the sequence of DNA susceptible to mutation.

An alternative approach to reversion assays is a single forward mutation assay. In this type of assay the target for the mutagen can be an entire functional gene that codes for an enzyme that converts a nontoxic compound (selective agent) to a toxic metabolite. Loss of the enzyme by mutation results in resistance to the selective agent and thus provides a means of selecting for mutants in a treated population. In theory, forward assays would be sensitive to all base-pair substitutions and a large number of sequence-specific frameshift mutations. In practice, the forward assay must also be as sensitive quantitatively as a set of reversion assays—i.e., any proposed forward assay must respond to a wide variety of mutagens at concentrations equal to or less than those found to be mutagenic in the reversion assay. A number of investigators have examined bacterial forward mutation assays (6-8). Ames and coworkers have compared several possible forward assays to the histidine reversion assays (cells were treated in agar) but found the forward assays deficient in terms of sensitivity (1, 2).

Our group has developed a forward mutation assay in *S. typhimurium* based on resistance to the purine analog 8-azaguanine (8-AG) (9). Here we compare the relative sensitivity of this system to the Ames reversion assay with 16 different mutagens.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: methyl methanesulfonate and dimethylnitrosamine, Eastman Chemical Co. (Rochester, NY); ethyl methanesulfonate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG),

methylnitrosourea, and β -propiolactone from ICN/K&K Laboratories (Plainview, NY); 9-aminoacridine (9-AA), benzo[*a*]pyrene (BP), 7,12-dimethylbenzanthracene, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP, 8-AG, and 1,2,3,4-dibenzanthracene from Sigma Chemical Co. (St. Louis, MO); Aroclor 1254 from Analabs, Inc. (North Haven, CT); phenobarbital from Mallinckrodt, Inc. (St. Louis, MO); dimethyl sulfate and diethyl sulfate from Aldrich Chemical Co. (Metuchen, NJ); proflavin from Salvador E. Luria (Massachusetts Institute of Technology); aflatoxin B₁ from Gerald N. Wogan (Massachusetts Institute of Technology); ICR-191 from Hugh J. Creech (Institute for Cancer Research, Fox Chase, PA); and acetylaminofluorene (AAF) from James A. Miller (McArdle Laboratory for Cancer Research, Madison, WI). Bacto-Agar was obtained from Difco (Detroit, MI).

Bacterial Strains. *S. typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 were the generous gifts of Bruce N. Ames (University of California, Berkeley, CA). Strain TM35 is a spontaneous *his*⁺ revertant of TA1535 (9). Graham C. Walker (Department of Biology, Massachusetts Institute of Technology) provided strain TA2000 and instructed us in its use to create strain TM677, an isogenic derivative of TM35 that carries the "R-factor" plasmid pKM101, transferred from TA2000 by mating. TM677 was selected from the mated culture on a minimal agar plate containing 10 μ g of ampicillin per ml. Cultures of the various strains possessing low spontaneous *his*⁺ backgrounds (TA1535, TA1537, TA1538, TA98, and TA100) and low spontaneous 8-AG-resistant backgrounds (TM35 and TM677) were selected and stored as 1-ml frozen aliquots as described (9).

Preparation of Post-Mitochondrial Supernatant (PMS). Male Sprague-Dawley rats were pretreated either with a single intraperitoneal injection of Aroclor 1254 (400 mg/kg) 4 days prior to sacrifice or with phenobarbital administered ad lib as a 0.1% solution in drinking water during the 4 days prior to sacrifice. The PMS preparation procedure has been outlined (9). PMS was stored at -80°.

Forward Mutation Assay. For each mutation experiment, a frozen aliquot of TM35 or TM677 was quickly thawed in a 37° water bath and added to 49 ml of minimal E medium (MgSO₄·7H₂O, 0.2 mg/ml; citric acid·H₂O, 2.0 mg/ml; K₂HPO₄, 10 mg/ml; NaNH₄HPO₄·4H₂O, 3.5 mg/ml; glucose, 20.0 mg/ml; 0.05 mM biotin; pH 7.0) in a 100-ml screw-cap bottle. The culture was incubated for 30 min at 37° in a shaking water bath (200 rpm). Viable cell concentration after incubation was approximately 1 × 10⁷/ml. For compounds not requiring metabolic activation, 4.95-ml samples of the culture were placed in 25-cm² tissue culture flasks and treated for 1 or 2 hr in a 37° dry-air incubator. The compounds were delivered to each of duplicate flasks in 50 μ l of dimethyl sulfoxide. For compounds requiring activation, 4.0-ml samples of the culture

Abbreviations: 9-AA, 9-aminoacridine; AAF, acetylaminofluorene; 8-AG, 8-azaguanine; BP, benzo[*a*]pyrene; P_i/NaCl, phosphate-buffered saline; PMS, post-mitochondrial supernatant.

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Table 1. Background mutant fraction and its variation in *S. typhimurium* strains

Strain	n	Background mutant fraction*		
		Mean	SD	99% confidence interval
TA1535	23	3.1	1.7	4.3
TA1537	22	6.8	5.0	12.6
TA1538	18	2.4	2.5	6.4
TA98	20	4.0	3.1	7.8
TA100	23	22	14	35
TM35	16	1.7	1.4	3.5
TM677	15	4.7	2.4	6.2

The determination of mutant fraction on cultures derived from independent frozen aliquots was performed *n* times. Each determination involved two independent subcultures each plated for survivors and mutants or revertants in triplicate. Experiments with and without added metabolizing system or dimethyl sulfoxide were pooled because no significant effects of these variables were observed. Assays were performed on separate days and among 8–12 separate workers for each strain. The 99% confidence interval has been calculated by multiplying the SD by the appropriate *t* value of Student's *t* distribution (one-sided) given *n* independent observations and requiring 99% confidence.

* For TA1535, TA1537, TA1538, TA98, and TA100, data are $\times 10^8$ (*his*⁺ revertant fraction); for TM35 and TM677, they are $\times 10^5$ (8-AG-resistant fraction).

were placed in 25-cm² plastic tissue flasks. Each flask then received 0.5 ml of sterile PMS, 2 units of glucose-6-phosphate dehydrogenase in 50 μ l of 5 mM citrate, and 0.5 ml of minimal E medium containing 5 mg of glucose 6-phosphate, 5 mg of NADP, and 3.33 mg of MgCl₂. The flasks were incubated for 1 or 2 hr in a 37° dry-air incubator without shaking.

After treatment, each culture was transferred to plastic centrifuge tubes, centrifuged, and resuspended in phosphate-buffered saline (P_i/NaCl) (NaCl, 8.0 mg/ml; KCl, 0.2 mg/ml; Na₂HPO₄, 1.15 mg/ml; KH₂PO₄, 0.2 mg/ml; pH 7.0). Approximately 4×10^6 cells per dish were plated in triplicate on minimal E agar (minimal E medium containing 0.6% Bacto-Agar; pH 6.5) plates containing biotin and 8-AG (final concentration on plate; 50 μ g/ml) to determine the 8-AG-resistant fraction. Also, approximately 400 cells per dish were plated in triplicate on minimal E agar plates containing biotin and no 8-AG, to determine toxicity. The plating procedure has been described (9).

***his*⁺ Reversion Assay.** Strains TA1535, TA1537, TA1538, TA98, and TA100 were used in these studies. Culture inoculation, growth, and treatment were similar to those described for the forward assay, except that cell density at the beginning of treatment was approximately 1×10^8 /ml. *S. typhimurium* cultures of 10^7 or 10^8 bacteria per ml were in exponential growth phase, and no effect of cell density in this range on mutation has been observed. After treatment, the cells were centrifuged, washed in 5 ml of P_i/NaCl to ensure removal of all histidine, and resuspended in 0.5 ml of P_i/NaCl. A 0.1-ml aliquot of the suspension was added to 0.9 ml of top agar (0.6% Bacto-Agar, 0.6% NaCl), and the resulting mixture was plated on a minimal E agar (minimal E medium, 0.6% Bacto-Agar, pH 7.0) plate with biotin and no histidine to determine the *his*⁺ revertant fraction. Also, a 0.1-ml aliquot of a 10^{-6} dilution of the P_i/NaCl suspension was added to 0.9 ml of top agar, and the resulting mixture was plated on a minimal E agar plate containing biotin and 5 mM histidine to determine toxicity. Plating was performed in triplicate. The fraction of *his*⁺ revertants after treatment was calculated as follows:

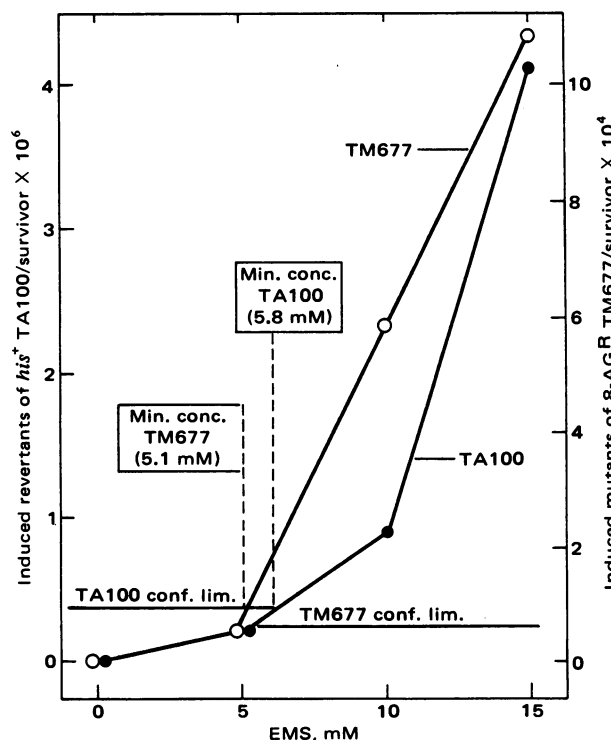


FIG. 1. Basis of comparison of sensitivity. Induced mutant fractions (mutant fraction of treated cultures minus mutant fraction of untreated culture) are plotted as a function of ethyl methanesulfonate (EMS) (for 2 hr) concentration. The concentration corresponding to the intersection of the mutation curve and the 99% confidence limit (Table 1) is used to define the minimal concentration (dashed line) to which a particular bacterial strain is sensitive.

his⁺ revertant fraction

$$= \frac{\text{no. clones on mutation plates} \times 10^{-6}}{\text{no. clones on toxicity plates}}$$

RESULTS

The rate of mutation (spontaneous and induced) is much lower in reverse mutation than in forward mutation. To compare the sensitivities of the assays directly, we have determined by interpolation the minimal concentration of each test compound at which each strain would theoretically yield a significant mutagenic response.

Table 1 records our observations of spontaneous mutant fractions for all strains. The mean spontaneous revertant mutant fractions ranged from a low of 2.4×10^{-8} for TA1538 to a high of 22×10^{-8} for TA100. The mean spontaneous forward mutant fraction was 1.7×10^{-5} for TM35 and 4.7×10^{-5} for TM677. The inclusion of PMS, cofactors, and dimethyl sulfoxide had no effect on these values. Variation about the mean ranged from a low for TA1535, for which the SD was about 55% of the mean, to a high for TA1538, for which the SD was approximately equal to the observed mean. Sufficient data are thus available to define the 99% confidence interval on the mean spontaneous mutant fraction observed for each strain. Spuriously high background mutant fractions were observed in 13 of a series of 137 independent determinations over all strains. We are unable to account for these occasional observations in terms of experimental practice. Our tentative conclusion is that they arise from an operational bias (cryptic contamination, growth as clumps prior to storing as aliquots, or some other cause) and not from random error. Thus, we have not included these results in the calculations of parameters reported in Table 1.

Table 2. Induced mutant fractions of 16 mutagens in forward and reverse mutation assays

Compound*	Assay conditions†	Conc.	TA1535	TA1537	TA1538	TA98	TA100	TM35	TM677	Surv. fraction
AAF	P; 2 hr	11 μ M	—	—	—	—	—	—	25	1.0
		22 μ M	0.5	—	45	110	15	0.2	47	0.8
		44 μ M	1.7	—	170	370	36	7.6	59	0.7
		66 μ M	0.1	—	220	370	70	6.6	82	0.6
AFB ₁	P; 1 hr	0.24 μ M	2.0	0	0	9	81	—	—	1.0
		0.32 μ M	10	0	0	18	—	—	—	1.0
	A; 1 hr	0.40 μ M	—	—	—	—	—	0	15	0.4
9AA	N; 1 hr	0.80 μ M	—	—	—	—	—	5	25	0.2
		26 μ M	0	7.3	0	1.6	13	0.2	0	1.0
		52 μ M	0	320	0.1	1.0	5	1.2	4.1	0.9
BP	A; 2 hr	78 μ M	1.3	4600	4.2	21	230	12	10	0.2
		20 μ M	4.8	82	7.8	26	74	2.8	43	0.6
		40 μ M	1.6	97	9.8	21	94	6.2	72	0.6
DBA	A; 2 hr	60 μ M	3.6	120	7.8	35	84	7.9	—	0.5
		18 μ M	2.2	30	2.8	7	82	0.2	18	0.9
		36 μ M	0.7	280	24	76	170	0.3	37	0.8
DMBA	A; 2 hr	54 μ M	3.7	370	20	73	170	1.2	85	0.8
		40 μ M	3.0	37	5.0	0.6	18	1.6	15	0.8
		60 μ M	0	73	9.0	11	19	1.4	24	0.8
DMN	A; 1 hr	80 μ M	4.0	260	15	4	38	2.9	46	0.6
		13.5 mM	12	0	0	0	10	1.2	4.0	1.0
		27 mM	30	0	0	0	105	2.3	11	1.0
DES	N; 1 hr	68 mM	760	5.3	6.0	5.3	790	49	—	0.8
		320 μ M	1.0	0.3	0	0	14	3.8	5.1	1.0
		640 μ M	1.3	0	0	1.2	55	2.4	38	1.0
DMS	N; 1 hr	960 μ M	7.5	1.9	0	0.3	110	7.6	—	1.0
		100 μ M	100	110	0.6	0	110	22	61	0.5
		200 μ M	190	220	7.8	20	340	52	90	0.2
EMS	N; 1 hr	300 μ M	420	2100	30	83	870	63	890	0.05
		5 mM	0	0	—	1.9	25	7.1	5.5	1.0
		10 mM	7.0	0	—	0.7	87	26	56	1.0
ICR-191	N; 1 hr	15 mM	480	1.5	0	0	410	56	110	0.7
		0.25 μ M	—	19	—	—	—	2.5	8.2	1.0
		2.5 μ M	—	260	—	—	—	13	14	0.9
MMS	N; 1 hr	12.5 μ M	1.9	2700	70	4.9	66	26	71	0.6
		2.5 mM	2.1	11	0.1	—	95	14	230	0.3
		5.0 mM	7.2	27	1.8	28	190	30	310	0.2
MNNG	N; 2 hr	7.5 mM	100	300	2.7	710	790	73	330	0.1
		0.34 μ M	0.6	0	0	0.9	10	2.4	5.1	1.0
		0.68 μ M	55	0.3	0	0.7	28	4.3	9.2	1.0
MNUT	N; 1 hr	2.04 μ M	230	11	0	3.4	2100	404	600	1.0
		2.5 μ M	11	—	—	—	15	3.1	1.1	0.8
		5.0 μ M	140	—	—	—	58	5.4	14	0.7
Proflavin	N; 1 hr	7.5 μ M	480	18	—	0	290	23	33	0.7
		16 μ M	1.7	0	0.6	0.6	42	1.0	7.9	0.8
		32 μ M	2.1	1.6	1.7	2.6	53	2.4	13	0.7
BPL	N; 1 hr	64 μ M	0	24	27	6.6	70	10	21	0.5
		35 μ M	62	—	—	1.8	95	38	47	1.0
		70 μ M	50	—	—	0	110	38	110	0.9
		105 μ M	150	—	3	0.3	760	68	130	0.8

* Numbers are observed mutant fraction, $\times 10^8$ for reversion assays and $\times 10^5$ for forward assays. Additional abbreviations: AFB₁, aflatoxin B₁; DBA, 1,2,3,4-dibenzanthracene; DMBA, 7,12-dimethylbenzanthracene; DMN, dimethylnitrosamine; DES, diethyl sulfate; DMS, dimethyl sulfate; EMS, ethyl methanesulfonate; MMS, methyl methanesulfonate; MNUT, methylnitrosourea; BPL, β -propiolactone.

† A, Aroclor-induced rats used for PMS; P, phenobarbital-induced rats used for PMS; N, no PMS used.

The use of the 99% confidence interval in comparing sensitivities among strains is illustrated in Fig. 1. The mutant fraction induced by ethyl methanesulfonate (mutant fraction in treated cultures minus mutant fraction in untreated culture) in strains TA100 and TM677 is plotted as a function of the initial ethyl methanesulfonate concentration in a 2-hr exposure. Also indicated in Fig. 1 is the 99% confidence interval for each strain as defined in Table 1. In order for an induced mutant fraction to be considered statistically significant, we require that it equal or exceed the 99% confidence interval on the mean background

mutant fraction. The concentration of ethyl methanesulfonate at which the induced mutant fraction for TA100 equals the 99% confidence interval for TA100 is our estimate of the minimal concentration of ethyl methanesulfonate to which strain TA100 is sensitive. In this case, interpolation indicates a minimal concentration of 5.8 mM to induce a significant mutant fraction in TA100. Similarly, interpolation of the curve for TM677 indicates that this curve equals the 99% confidence interval for TM677 background mutant fractions at a concentration of 5.1 mM.

Table 3. Minimal concentration to which each strain is sensitive

Compound*	Minimal concentration, μ M						
	TA1535	TA1537	TA1538	TA98	TA100	TM35	TM677
Base-substitution mutagens							
AFB ₁	0.26	—	—	0.21	0.10	0.68	0.17
BPL	2.4	Lost	—	—	13	3.2	4.6
DES	790	—	—	—	480	290	330
DMN	4800	—	—	—	17,000	28,000	18,000
EMS	8100	—	—	—	5,800	2,500	5,100
MNNG	0.36	—	—	—	0.68	0.54	0.43
Frameshift mutagens							
AAF	—	Lost	3.1	1.6	43	32	2.7
9AA	—	26	—	61	55	58	61
DBA	—	7.4	21	18	7.7	—	6.2
DMBA	—	13	43	54	77	—	17
ICR-191	—	0.16	1.1	—	6.6	0.46	0.19
Proflavin	—	48	38	—	13	37	13
Frameshift and base-substitution mutagens							
BP	18	3.1	16	6	9.5	24	2.9
DMS	4.3	11	180	140	32	16	10
MMS	3600	2800	—	1400	920	630	70
MNUT	1.0	5.3	Lost	—	3.7	2.9	3.5

Blanks indicate strains not sensitive at the highest concentration tested (see Table 2).

* See Table 2 for additional abbreviations.

Table 2 records the induced mutant fractions observed with each strain for all 16 compounds tested. Using this data and applying the process of Fig. 1, we calculated the minimal concentration of each compound to which each strain is sensitive (Table 3).

DISCUSSION

Table 3 separates the test compounds into three groups, based on their ability to revert strain TA1535 (base-substitution mutagens) and strains TA1537 or TA1538 (frameshift mutagens) at the concentrations tested. The known alkylating agents diethyl sulfate, dimethylnitrosamine, MNNG, β -propiolactone, and ethyl methanesulfonate exhibit specificity for reverting TA1535 and not TA1537 or TA1538. But three alkylating agents, dimethyl sulfate, methyl methanesulfonate, and methylnitrosourea exhibit ability to revert both strains TA1535 and TA1537. Aflatoxin B₁ mutates TA1535. AAF, 9-AA, 1,2,3,4-dibenzanthracene, 7,12-dimethylbenzanthracene, ICR-191, and proflavin revert TA1537 or TA1538 but not TA1535 in the range of concentrations tested.

The addition of the plasmid pKM101 to strain TA1535, creating strain TA100, increases the sensitivity of the assay for two agents, diethyl sulfate and aflatoxin B₁, but decreases sensitivity to dimethylnitrosamine, methylnitrosourea, β -propiolactone, and MNNG. However, the change in sensitivity is no greater than 6-fold in any case. Adding the plasmid to TA1535 does, however, render it sensitive to frameshift mutagens such that TA100 is found to be the most sensitive reversion strain for proflavin and shows sensitivity to all compounds of this class. However, strain TA1537 responds to compounds such as 7,12-dimethylbenzanthracene and ICR-191, and TA98 responds to AAF, at concentrations much lower than does TA100, emphasizing the need for the use of several strains in screening protocols based on reversion assays.

Among those compounds reverting TA1538, addition of the plasmid to create strain TA98 increased sensitivity to 9-AA and somewhat to AAF but decreased sensitivity to ICR-191 and proflavin. The compounds active as mutagens at similar concentrations in both TA1535 and TA1537 or TA1538 present problems of analysis beyond the scope of this discussion.

Table 3 also permits a quantitative comparison of the sensitivity of the most sensitive of the five reversion strains to the strains used in forward mutation assays. The plasmid pKM101 confers significant sensitivity to the forward assay among the frameshift mutagens. Although some actual diminution of sensitivity of the forward assay does occur with plasmid addition (e.g., β -propiolactone), the quantitative diminution is generally less than 2-fold. Thus, the sensitivity of one strain, TM677, in a forward mutation assay of each chemical may be compared to the most sensitive of the reversion assays.

In this comparison, some limits must be chosen to decide if a difference in sensitivity is (i) statistically significant and (ii) important in the bioassay of unknown chemicals. Roughly speaking, a 2-fold difference in the lowest concentration indicates a statistically significant observation, but *ii* is a matter of judgment. We suggest that differences in sensitivity within a 4-fold concentration range may not be important in actual practice.

By this arbitrary 4-fold limit, forward mutation assays with strain TM677 alone are found to be equisensitive with the set of five reversion assays for all 16 compounds tested.

Our conclusion is that the combination of Ames' physiological mutants (2) with this simple forward mutation assay in *S. typhimurium* (9) provides an improved means to perform quantitative assays of chemomutagenic potential. We should note that the studies by Ames and his colleagues (1, 2) and others (6-8) have encompassed the idea of forward mutation assay in bacteria as a bioassay. However, in distinction to 8-AG resistance, measurement of mutation to azetidine carboxylic acid resistance was reported to be relatively insensitive when compared to reversion to histidine prototrophy (1, 2). This lack of agreement may arise from complex differences between suspension-treated cells and cells treated in agar.

We have not yet discovered any chemical that is active in the forward assay but inactive in the Ames reversion assays. This fact renders questionable any hypotheses emphasizing the relative importance of the DNA sequence in limiting the general applicability of reversion assays for detecting mutagens (9). On the other hand, the virtual equisensitivity of the forward assay and reversion assays raises some interesting questions about the molecular basis of mutation. The genetic target for

forward mutation may mutate spontaneously at a number of different loci within a gene. It seems reasonable to assume that the molecular target for reversion is, in comparison, limited to a short nucleotide sequence. In the presence of a specific mutagenic stimulus that is highly active for a particular reversion site (e.g., 9-AA and TA1537; see Table 3), it would seem probable that the reversion assay would be much more sensitive than a forward mutation assay because of its apparently favorable signal-to-noise ratio. The fact that this is demonstrably not the case requires us to rethink some of our ideas about the chemotagenic process.

In terms of bioassay practice, the ability to use one strain instead of five strains of *S. typhimurium* has obvious advantages. Of particular interest to us are the characteristics of forward mutation assay that permit automation of the process. It should also be noted that forward assays can be performed with exponentially growing cultures (10^8 cells per ml) in volumes as low as 10–100 μ l. Small volumes spare the cost of expensive cofactors in large-scale bioassays and, in experimental practice, dramatically decrease the amount of compound required.

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