

A quantitative and highly sensitive luciferase-based assay for bacterial toxins that inhibit protein synthesis

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Inhibition of protein synthesis is a common mechanism by which bacterial and plant toxins injure human cells. Examples of toxins that inhibit protein synthesis include shiga toxins of *Escherichia coli*, diphtheria toxin, *Pseudomonas* exotoxin A and the plant toxin ricin. In order to facilitate studies on toxin pathogenesis and to enable screening for inhibitors of toxin action, a quantitative and highly sensitive assay for the action of these toxins on mammalian cells was developed. The cDNA encoding destabilized luciferase was cloned into an adenoviral expression plasmid and a high-titre viral stock was prepared. Following transduction of Vero cells, luciferase expression was found to be linear with respect to viral multiplicity of infection. Luciferase expression by as few as 10 cells was readily detected. Treatment of transduced cells with either cycloheximide or shiga toxin resulted in a decrease in luciferase activity, with a half-life ranging from 1 to 2 h. Inhibition of luciferase expression was evident at toxin concentrations as low as 1 pg ml⁻¹. The assay was adapted for use in 24-, 96- and 384-well plates, enabling rapid processing of large numbers of samples. Using this approach, susceptibility of Vero, Hep2, Chang, A549, COS-1 and HeLa cells to three different toxins was determined. These results demonstrate that the luciferase-based assay is applicable to the study of numerous cell types, is quantitative, highly sensitive and reproducible. These features will facilitate studies on pathophysiology of toxin-mediated diseases and allow high-throughput screening for inhibitors of cytotoxicity.

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INTRODUCTION

The bacterial toxins *Escherichia coli* shiga toxin (Stx), *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT), and the plant toxin ricin are widely divergent in their origins, structures and intracellular targets. However, each of these toxins causes damage to susceptible cells through their ability to inhibit protein synthesis. DT and PE inhibit protein synthesis by ADP-ribosylation of elongation factor 2 (Iglewski & Kabat, 1975; Pappenheimer & Brown, 1968; Pappenheimer, 1977). By contrast, Stx and ricin enzymically cleave an adenosine residue from 60S ribosomes, inactivating ribosome function (Endo *et al.*, 1987; Endo & Tsurugi, 1988; Obrig *et al.*, 1985, 1987; Reisbig *et al.*, 1981). These toxins are important agents of human disease and are potential agents of bioterrorism (Balint, 1974; Boulton, 2003; Bradberry *et al.*, 2003; Clarke, 2002; Dittmann *et al.*, 2000; Franz & Zajchuk, 2000; Lyczak *et al.*, 2000; Madsen, 2001; O'Brien *et al.*, 1992; Olsnes, 2004; Pickering *et al.*, 1994; Tarr, 1995). Consequently, there is considerable interest in understanding how these toxins interact with host cells, with the ultimate goal of developing inhibitors of their action (Doring & Muller, 1989;

Laithwaite *et al.*, 1999; Ohmi *et al.*, 1998; Sekino *et al.*, 2004; Valdivieso-Garcia *et al.*, 1996).

The conventional assay for measuring susceptibility to bacterial toxins that inhibit protein synthesis is a radioactive cytotoxicity assay. In this assay, cells are exposed to toxin and then incubated transiently with radioactive amino acids such as [³H]Leu, [³⁵S]Met or [³⁵S]Met-Cys (Debinski *et al.*, 1995; DeGrandis *et al.*, 1989; Elliott *et al.*, 2003; Keusch *et al.*, 1995; Obrig *et al.*, 1993; Puri *et al.*, 1996; Suhan & Hovde, 1998). After this pulse period the amount of radioactive amino acid incorporated into protein is determined, usually by lysing cells and precipitating polypeptides with 10% trichloroacetic acid (TCA). In addition to the requirement for radioactive materials, this assay is a complex multi-step procedure and suffers from a high degree of sample-to-sample variability. Moreover, the assay lacks sufficient sensitivity to detect toxin effects on small numbers of cells. Because of these limitations, we sought to develop an improved assay for cytotoxins that inhibit protein synthesis. Here we describe the development of a highly sensitive assay for these toxins that is based on luciferase content as a marker of protein synthesis.

The firefly luciferase enzyme, when in the presence of ATP, catalyses the oxidation of D-luciferin (4,5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazoline-carboxylic

Abbreviations: DT, diphtheria toxin; FS, Forssman synthetase; PE, *Pseudomonas* exotoxin A; Stx, shiga toxin.

acid) to produce light. Therefore when the substrate D-luciferin is in excess, light output corresponds to the concentration of luciferase enzyme. The *luc* gene is a commonly used reporter gene often utilized as an *in vitro* genetic reporter (de Wet *et al.*, 1987).

The assay described here aims to measure the level of protein synthesis in cells through the light output from the luciferase and D-luciferin reaction. The luciferase protein used here has been modified by Promega by the addition of a PEST sequence, resulting in a short intracellular half-life (Li *et al.*, 1998; Rechsteiner, 1990). Whereas this reporter is generally used to measure changes in luciferase content in response to transcriptional regulation, we constitutively transcribed luciferase and used enzyme activity as a measure of ongoing protein synthesis. In cells constitutively expressing the luciferase mRNA, inhibition of protein synthesis results in diminished luciferase translation and as existing protein is degraded the amount of light output decreases proportionately. Delivery of the luciferase cDNA is accomplished by transduction with an adenoviral expression system, allowing susceptibility testing on a wide variety of dividing or non-dividing mammalian cells. We demonstrate here that the assay is highly sensitive and reproducible. Using this assay, several cell lines were efficiently examined for their susceptibility to Stx, DT and PE. In 384-well plates, the light output from toxin-treated samples was highly statistically significantly decreased compared to untreated samples and those treated with a combination of toxin plus anti-toxin antibodies. The assay described here should have broad applicability to the study of bacterial toxins.

METHODS

Plasmid preparation. Plasmid pGL3(R2.1) (Promega) was digested with *KpnI* and *Sall* to release the luciferase cDNA. This was ligated into plasmid pENTR11 (Invitrogen), which had been digested with *KpnI* and *XhoI*. The resulting plasmid was digested with *NcoI* to release an approximately 100 bp region upstream of the luciferase start codon, then was religated to create plasmid pENTR-luc. The luciferase-coding region was transferred to plasmid pAD/CMV/V5-DEST (Invitrogen) using the Gateway cloning system (Invitrogen), forming plasmid pAD-Luc1.

Cell culture. Vero (African Green Monkey adult kidney cells; ATCC CCL-81), HeLa (ATCC CCL-2), Chang (ATCC CCL-13), Hep2 (ATCC CCL-23), COS-1 (ATCC CRL-1650) and A549 cells (ATCC CCL-185) from the American Type Culture Collection, 293A cells (Invitrogen) and Vero-Forsman Synthetase (Vero-FS) cells (Elliott *et al.*, 2003) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum (GibcoBRL) with 0.1 mM MEM non-essential amino acids (NEAA) and were incubated in 95% air, 5% CO₂ at 37 °C. As described previously, Vero-FS cells lose Forsman synthetase (FS) expression with successive passage without selective pressure provided by Stx. Therefore, 1 week prior to performing the susceptibility assays, Vero-FS cells were incubated overnight in the presence of 10 ng Stx ml⁻¹ to select for those highly expressing FS (Elliott *et al.*, 2003).

Toxins. Stx (100 µg ml⁻¹), DT and PE (1 mg ml⁻¹ each) were obtained from List Biological Laboratories. Toxins were aliquoted into several

tubes and stored at -80 °C. After thawing once, toxins were maintained and used at 4 °C for up to 1 month after thawing.

Adenovirus preparation and titration. 293A cells (Invitrogen) were transfected with plasmid pAD-Luc1 after digestion with *PacI*. Five micrograms of pAD-Luc1 in 800 µl OptiMem (Invitrogen) was mixed with a solution of 120 µl lipofectamine (Invitrogen) and 800 µl OptiMem. After 30 min at room temperature, the mixture was added to 6.4 ml OptiMem and placed on the monolayer of approximately 1 × 10⁶ 293A cells. The medium was removed after 24 h and replaced with complete medium. When the entire monolayer of cells was rounding and detaching, a crude viral lysate was prepared by harvesting the 293A cells and medium. Three freeze/thaw cycles were performed to release intracellular virus. The samples were then centrifuged and the pellet discarded. To amplify the crude viral lysate, 500 µl of the lysate was added to each near-confluent dish of 293A cells seeded at 9.5 × 10⁶ cells per 20-cm-diameter dish. Upon demonstration of a cytopathic effect of the lysate, the cells were harvested and viral lysate was prepared as above. A final amplification was performed by infecting 293A cells in a 20-cm-diameter dish at 95% confluence with 50 µl of the amplified lysate. Virus was harvested as above in a final volume of 45 ml. The amplified viral stock was aliquoted and stored at -80 °C. Titration of the viral stock was performed by seeding a six-well dish with 1 × 10⁶ 293A cells per well and infecting with serial dilutions of adenoviral stock. Two millilitres of each dilution was added to a well and after 2 days the number of plaques was counted. The high-titre stock was found to have a titre of 10¹¹ p.f.u. ml⁻¹.

Luciferase assays. For all luciferase-based assays, cells were seeded at 5 × 10⁵ cells per 10-cm-diameter dish 1 day prior to viral transduction. The next day, the medium was changed and virus was added at various multiplicities of infection (for most experiments an m.o.i. of 100–200 was utilized). On the day following transduction, virus-containing medium was removed and the cells were harvested by trypsinization, resuspended in fresh medium and passed through a cell strainer to remove clustered cells. The cells were aliquoted into 24-, 96- or 384-well plates. For 24-well plates, 1 × 10⁴ to 2.5 × 10⁴ cells per well were used, while 1 × 10⁴ cells were seeded per 96-well plate, and either 1000 or 5000 cells were seeded per well in 384-well plates. In experiments comparing luciferase to radioactivity assays, cells were seeded in duplicate at this point for radioactivity assays. The following day, cells were treated with toxin at various concentrations and incubated for various times. In some experiments, polyclonal antiserum raised against the shiga toxin 'B' subunit was added at 1:25 dilution to toxin samples prior to addition to cell monolayers.

Following incubation with and without toxin, SuperLight Luciferase Reporter Gene Assay (BioAssay Systems) was added at a volume equal to the volume of medium per well. The plates were incubated for 30 s to 5 min at room temperature, and light output was then measured in a luminometer. For experiments in 24-well plates, samples were transferred to disposable 12 × 75 mm glass culture tubes and counted individually in a BG-1 Optocomp 1 luminometer (Bacterial systems GEM Biomedical) with an integration time of 10 s. For experiments in 96- or 384-well plates, light output was measured in an Lmax luminometer (Molecular Devices) using an integration time of 2–5 s.

Radioactivity assay. Experiments comparing a radioactivity assay and the luciferase-based assay were performed with cells seeded and virally transduced at the same time. The cells were then aliquoted in duplicate and exposed to toxin in identical manner. Thereafter, the radioactive cytotoxicity assay was carried out as described (Elliott *et al.*, 2003). Briefly, the medium was removed from toxin-treated cells and replaced with DMEM lacking cysteine and methionine, to which was added Tran^[35S]label at 10 µCi ml⁻¹. The cells were incubated at 37 °C for 30 min then washed five times with PBS. After lysis in 50 mM Tris containing 0.2% SDS, proteins were precipitated by the addition of

TCA to 10%. After centrifugation, the supernatant was removed and the pellet was washed with 70% ethanol. This was resuspended in 100 mM Tris pH 8.0, and the amount of radioactivity was quantified in a liquid scintillation counter.

Data analysis. Half-life determinations were performed by assuming that luciferase expression underwent one-phase exponential decay. The rate constant (K) of decay was calculated using GraphPad Prism version 4.0. Comparison of replicate samples in 384-well plates was performed by paired *t*-test using GraphPad Prism. Differences were considered significant if the *P*-value was less than 0.0001.

RESULTS AND DISCUSSION

Optimization of assay sensitivity

As a first step in validating and optimizing the luminescence assay, we examined the relationship between viral multiplicity of infection, signal intensity, and response to toxin. In order to determine the viral multiplicity of infection that yielded high sensitivity while allowing optimal detection of toxin effect, Vero cells were infected with pAD-Luc1 at an m.o.i. of 0.1–1000. Stx (10 ng ml⁻¹) was added to half of the samples. After incubation for 5 h, the medium was removed from all the samples, replaced with PBS, an equal volume of SuperLight reagent was added and light output was quantified.

Whereas an m.o.i. of 1000 resulted in light output beyond the range of detection both for untreated and for toxin-treated samples, the relationship between light output and multiplicity of infection was linear in the range of m.o.i. from 1 to 100. Treatment with Stx resulted in a 60–70% decrease in light output at all m.o.i. between 0.1 and 100 (Fig. 1a). These results indicate that luciferase content was diminished by toxin treatment to a similar degree regardless of basal expression levels.

In order to determine the minimum number of cells capable of being detected with this assay, Vero cells were seeded at 5×10^5 cells per 10-cm-diameter dish. The next day, cells were infected with pAD-Luc1 at an m.o.i. of 200. Following overnight incubation, the cells were released by trypsinization and serially diluted. These dilutions were plated in 24-well plates in duplicate, ranging from 1 to 1×10^4 cells per well. The following day, SuperLight reagent was added, the samples were transferred to glass tubes and light output was determined. These experiments demonstrated a linear relationship between cell number and light output. A statistically significant signal was detected from as few as 10 cells (Fig. 1b).

Kinetics of luciferase response to protein synthesis inhibition

In order to determine the rate at which luciferase expression was decreased after inhibition of protein synthesis, a time-course experiment was performed using either Stx or cycloheximide to inhibit protein synthesis. Vero cells were infected at an m.o.i. of 50 and seeded in triplicate at 5×10^3 cells per well in a 96-well plate. After overnight incubation,

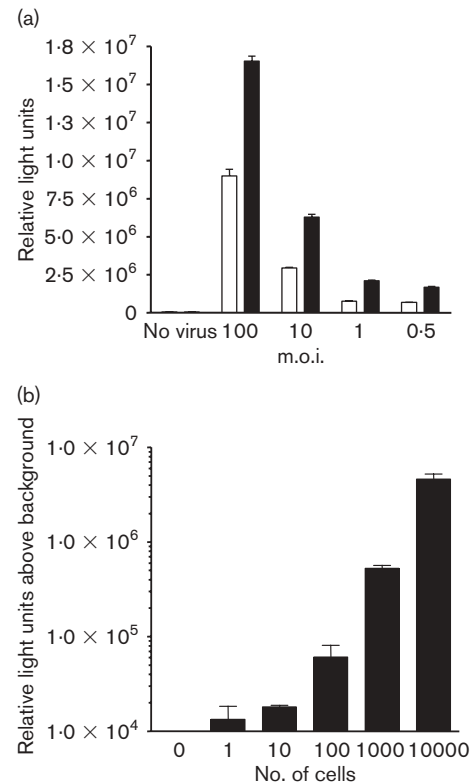


Fig. 1. (a) Determination of the effects of Stx on luciferase quantity at various pAD-Luc1 multiplicities of infection in Vero cells. Duplicate samples were tested after 6 h incubation with (white bars) or without (black bars) 10 ng Stx ml⁻¹. Light output is expressed as relative light units. (b) Quantification of light output versus cell number for duplicate samples of Vero cells infected with pAD-Luc1 at an m.o.i. of 100.

cells were left untreated, or treated with Stx (10 ng ml⁻¹) or cycloheximide (100 µg ml⁻¹). After incubation in these conditions for 0, 1, 2, 4, 6 or 12 h, luciferase content was determined by the addition of 100 µl SuperLight reagent and determining light output in a luminometer. These data demonstrate that luminescence was decreased with a half-life of 1.1 h in the cycloheximide-treated cells and plateaued at approximately 3% light output compared with untreated samples (Fig. 2a). In comparison, cells treated with Stx demonstrated diminished light output with a half-life of 2.1 h, with maximal suppression reaching approximately 85% by 12 h after toxin exposure.

Comparison of luminescence and radioactive cytotoxicity assays

Inhibition of protein synthesis has generally been demonstrated by determining the amount of radioactive amino acid incorporated into peptide during a pulse period. The luminescence-based assay described here was devised as an alternative to the radioactivity assay. We wished to compare the two assays with respect to their kinetics of detection.

Vero cells were infected at an m.o.i. of 200 and then seeded at

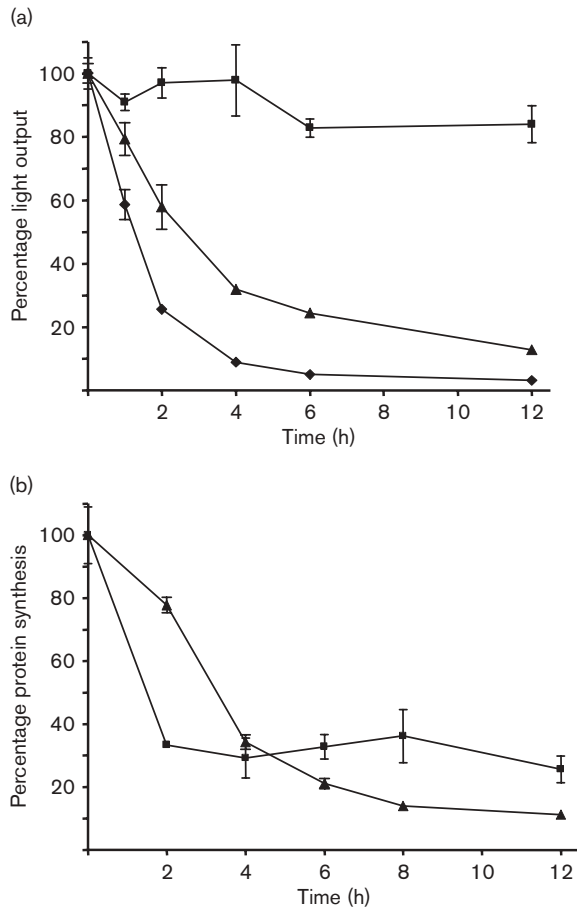


Fig. 2. (a) Kinetics of luciferase response in Vero cells infected with adenovirus pAD-Luc1 at an m.o.i. of 100. Triplicate samples were tested at various times after treatment with 10 ng Stx ml⁻¹ (▲), treatment with 100 µg cycloheximide ml⁻¹ (◆) or no treatment (■). Light output is expressed as relative light units. (b) Comparison of radioactivity and luciferase-based assay kinetics in Vero cells infected with adenovirus pAD-Luc1 at an m.o.i. of 100. Duplicate samples were tested at each time point. Symbols: ▲, luciferase-based assay; ■, radioactivity assay.

1×10^4 cells per well in a 24-well plate. After overnight incubation, Stx (10 ng ml⁻¹) was added in quadruplicate to the cells and incubated for various time periods, ranging from 0 to 12 h. Thereafter, half the samples were treated with SuperLight reagent and light output was determined as described above. The other 12 samples were analysed using a radioactive cytotoxicity assay. The medium was removed from toxin-treated cells and replaced with DMEM lacking cysteine and methionine, to which was added Tran[³⁵S]label. After a 30 min pulse, cells were washed extensively and lysed. Proteins were precipitated by the addition of TCA, protein pellets were resuspended and the amount of radioactivity was quantified in a liquid scintillation counter.

Whereas protein synthesis was calculated to have a half-life of 2.9 h in the luciferase-based assay, the radioactivity assay demonstrated a rapid half-life of 0.4 h (Fig. 2b). The

luminescence assay plateaued at approximately 10% basal protein synthesis, while the radioactivity assay plateaued at approximately 25%.

The radioactivity assay detected a decrease in protein synthesis with more rapid kinetics than the luminescence assay because the latter depends on degradation of luciferase after *de novo* protein synthesis is inhibited, whereas incorporation of radioactive amino acids is terminated as soon as toxin reaches the ribosome. Susceptibility assays are typically performed using a 4 h incubation in toxin and at this time point, the assays demonstrated almost identical decreases in protein synthesis of approximately 70%.

Some advantages of the luminescence assay are evident. This approach demonstrates lower sample-to-sample variability than the radioactivity assay. In addition, the maximal percentage inhibition was greater using the luciferase-based assay. A similarly greater maximal inhibition of protein synthesis was seen with the luciferase versus radioactivity assay when cycloheximide was used to inhibit protein synthesis (data not shown). The higher baseline in the radioactivity assay may reflect the presence of unincorporated radioactive amino acids in the TCA precipitated pellet. Since amino acids will be transported into the cell even while protein synthesis is inhibited, these amino acids may be trapped in the ensuing TCA precipitate despite extensive washing, thereby limiting the maximal level of suppression possible with the radioactivity assay.

Rapid determination of Stx susceptibility of multiple cell types

A major impetus for developing a new assay for toxin susceptibility was the inconvenience of the radioactivity assay, particularly when a number of samples were to be analysed. Having demonstrated that the luciferase assay is able to detect Stx activity on Vero cells, we wished to validate the ability of this assay to distinguish susceptibility of modified Vero cells. Our laboratory has previously shown that transfection of Vero cells with the Forssman synthetase (FS) cDNA results in depletion of Stx receptor glycolipids, and consequently renders cells Stx resistant as determined by the radioactivity assay (Elliott *et al.*, 2003). Stx susceptibility of wild-type and FS-transfected Vero cells was compared using the luciferase assay.

Vero and Vero-FS cells were infected with pAD-Luc1 at an m.o.i. of 200, and then incubated in quadruplicate for 5 h with serial dilutions of Stx, ranging from 0 ng ml⁻¹ to 100 ng ml⁻¹. We found, as expected, that wild-type Vero cells were exquisitely susceptible to Stx, with even the lowest toxin concentration resulting in a greater than 60% decrease in luciferase content. By contrast, Vero-FS cells were highly resistant to Stx, demonstrating essentially no decrease in luciferase expression even at a toxin concentration of 100 ng ml⁻¹ (Fig. 3a). These results confirm that the susceptibility of various cells to Stx is readily compared with the luminescence assay.

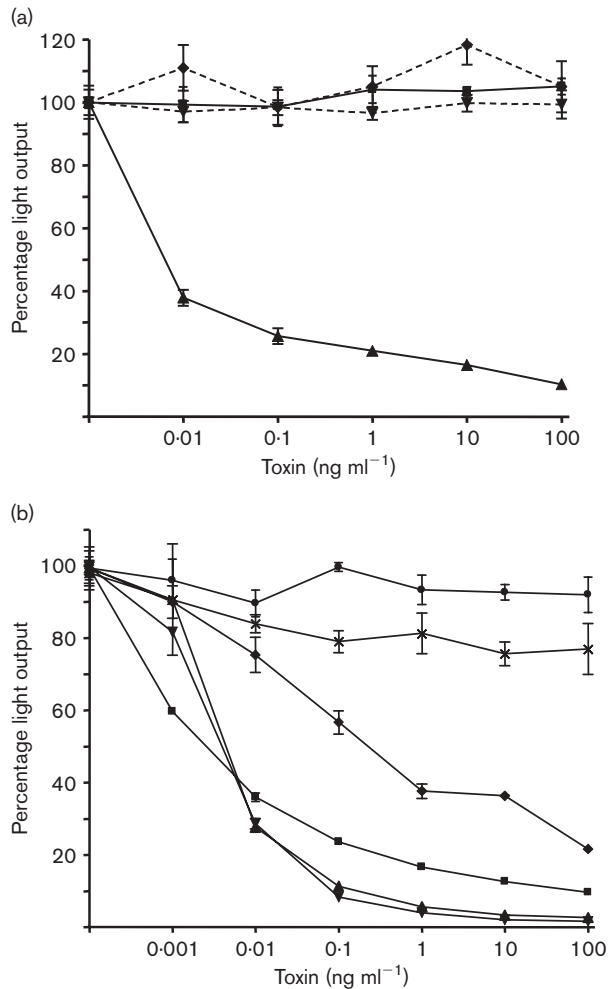


Fig. 3. (a) Effect of various Stx concentrations on light output by wild-type and FS-expressing Vero cells infected with adenovirus pAD-Luc1 at an m.o.i. of 100. Triplicate samples were tested after 5 h incubation with various concentrations of Stx. As a control, untreated samples, also in triplicate, were paired with each toxin-treated sample. Symbols: ◆, untreated FS-expressing cells; ▼, untreated wild-type cells; ■, FS-expressing cells treated with Stx; ▲, wild-type cells treated with Stx. (b) Determination of Stx susceptibility of multiple cell lines. Vero (■), HeLa (▼), Chang (▲), Hep2 (◆), COS-1 (×) and A549 (●) cells were infected at an m.o.i. of 100. Triplicate samples were tested after 5 h incubation with various concentrations of Stx.

We next examined the susceptibility of a number of mammalian cell lines to Stx using the luminescence assay. These cell lines are commonly used in laboratory research, including studies on bacterial pathogenesis and toxin-mediated diseases. Vero and HeLa cells are often used in studies on Stx biology; however, the Stx susceptibility of these two cell lines has not directly been compared previously. Stx susceptibility of the other lines used here has not been reported.

Vero, HeLa, Chang, Hep2, COS-1 and A549 cells were infected with pAD-Luc1 at an m.o.i. of 100. The cells were

then seeded into 96-well plates and treated in triplicate with serial dilutions of Stx, ranging from 0.001 ng ml⁻¹ to 100 ng ml⁻¹. Since the assay is performed with no washing, precipitation or centrifugation steps, the assay was completed in a fraction of the time that would have been required of a radioactivity assay. The resulting data allow a clear comparison of the cell lines with respect to their susceptibility to Stx (Fig. 3b). Chang and HeLa cells were at least as susceptible to Stx as Vero cells. By contrast, COS-1 cells, which like Vero cells were derived from African green monkey kidney, and A549 cells were highly resistant to Stx.

Comparison of Stx, DT and PE susceptibility of multiple cell lines

As reported previously, Stx reaches the cytoplasm after transit from the cell surface through the endoplasmic reticulum lumen and inhibits protein synthesis by enzymically removing an adenosine residue from 60S ribosomes (Yu & Haslam, 2005). PE follows a similar intracellular pathway but targets elongation factor 2 (EF2) instead of the ribosome. DT also targets EF2 but reaches the cytoplasm in a very different manner. Comparison of toxins with differences in trafficking and intracellular targets might facilitate studies on toxin biology. For example, a toxin inhibitor that had similar effects on Stx and PE without affecting DT susceptibility would suggest an effect of the inhibitor on retrograde trafficking pathways. The data presented above demonstrate that the luminescence assay is capable of determining and comparing susceptibility of multiple cell types to Stx. Since the basis of the assay is a measure of *de novo* protein synthesis, the assay should be able to detect the action of other bacterial toxins that inhibit protein synthesis.

In order to examine the ability of the luminescence assay to compare the effects of Stx, DT and PE on various cell types, Vero, HeLa, Chang, Hep2, COS-1 and A549 cells were infected at 100 m.o.i. and 5×10^3 cells were seeded per well. After incubation overnight, serial dilutions of toxins DT, PE and Stx from 0.001 ng ml⁻¹ to 1000 ng ml⁻¹ were added to triplicate samples of each cell line for a total of 558 samples (100 ng ml⁻¹ was the highest Stx concentration used). After 5 h incubation with toxin, SuperLight reagent was added and light output was determined.

The results demonstrate that the assay is able to determine the susceptibility of individual cell types to each of the three toxins (Fig. 4). Vero, HeLa and Chang cells demonstrated susceptibility to all three toxins. Among the cell lines examined, A549 cells were the least susceptible to these toxins, demonstrating inhibition in protein synthesis only at the highest toxin concentrations.

Adaptation of the luminescence assay for high-throughput applications

An anticipated use for this assay is to facilitate screening of small compound libraries for bacterial toxin inhibitors (Ward *et al.*, 2002). Luciferase is a commonly used 'read out' in small molecule screens due to its high sensitivity and

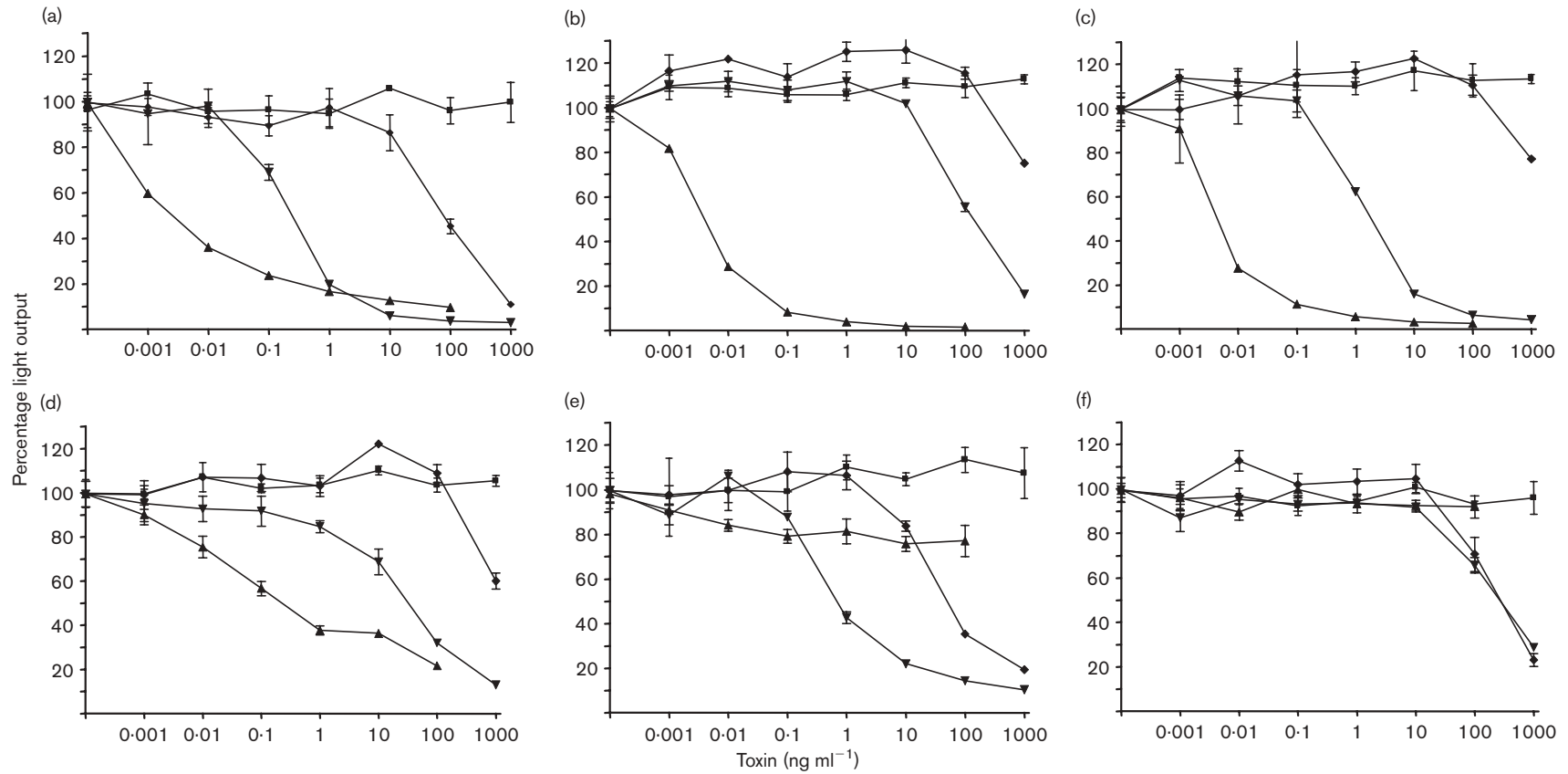


Fig. 4. Determination of Stx, DT and PE susceptibility of (a) Vero, (b) HeLa, (c) Chang, (d) Hep2, (e) COS-1 and (f) A549 cells infected at an m.o.i. of 100. Samples were tested after 5 h incubation with various concentrations of the three toxins. Symbols: ■, untreated; ▲, Stx; ▼, DT; ◆, PE.

linear signal response over several orders of magnitude. The luminescence assay was adapted to 384-well plates, and its suitability for detecting differences between samples treated with toxin, untreated or treated with toxin plus anti-toxin antibodies was examined.

HeLa and Vero cells infected at an m.o.i. of 200 were seeded at 5×10^3 cells per well in 384-well plates. The next day, Stx was added to 32 replicate samples to a concentration of 10 ng ml^{-1} . Another 32 samples were treated with Stx at 10 ng ml^{-1} combined and preincubated with antiserum against the Stx 'B' subunit, as a surrogate for a toxin inhibitor. Finally, the same number of samples was treated with medium lacking toxin or anti-toxin antiserum. After 5 h incubation, Super-Light reagent was added and light output was measured.

The results demonstrate that the assay was clearly able to distinguish toxin-treated Vero and HeLa cells from those untreated or incubated with toxin plus anti-toxin antiserum (Fig. 5). There was no overlap in light output between untreated samples and those treated with Stx. Statistical analysis demonstrated that the mean values for these samples were different, with a P -value of < 0.0001 . Interestingly, in this experiment the samples treated with toxin plus anti-toxin antiserum had an even higher mean value than

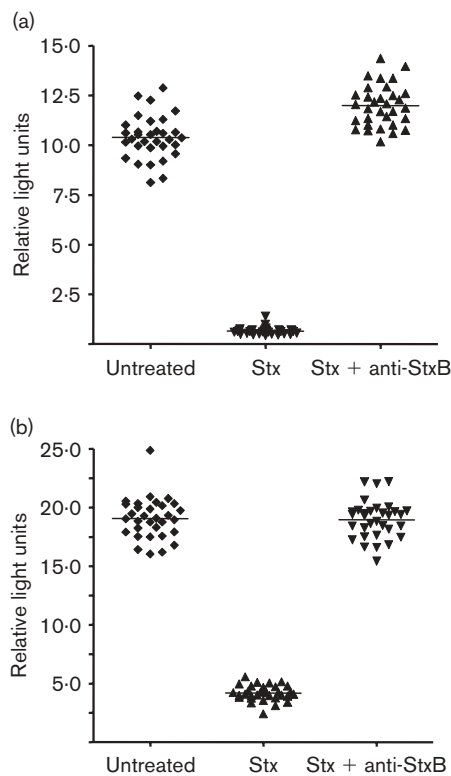


Fig. 5. High-throughput screening of (a) HeLa and (b) Vero cell samples in 384-well plates. HeLa and Vero cells were infected with adenovirus pAD-Luc1 at an m.o.i. of 200. Thirty-two replicates were tested after 5 h incubation with $10 \text{ ng Stx ml}^{-1}$ alone, $10 \text{ ng Stx ml}^{-1}$ that had been preincubated with anti-STxB antiserum treatment or no treatment.

untreated samples, indicating that the antiserum effectively inhibited toxin action, and suggesting the presence of a factor in serum that slightly stimulated protein synthesis.

In summary, we have developed a novel assay for toxins that inhibit protein synthesis. The assay described here has several advantages over existing assays for toxin susceptibility, which are typically performed either by viability assays or an assay for protein synthesis. Viability assays are performed by incubating cells with toxin and determining the percentage of viable cells at 48 h. In some commercially available assays, such as the CellTitre-Glo Assay (Promega) luciferase is used as an indicator of cellular viability. However, viability assays require a 2 day incubation period, and in general are not applicable to the testing of large numbers of samples and are poorly quantitative. Assays based on an examination of protein synthesis are more quantitative and sensitive. However, these are generally performed by examining incorporation of radiolabelled amino acids into *de novo* synthesized peptide chains. Radioactivity assays suffer from the requirement for several washing steps, significant sample-to-sample variability and insufficient sensitivity for high-throughput assays on small numbers of cells (Debinski *et al.*, 1995; Elliott *et al.*, 2003; Puri *et al.*, 1996; Suhan & Hovde, 1998). We show here that the luciferase-based assay is highly sensitive, reproducible and readily adaptable to high-throughput sample analysis. The ability to use this assay for a wide variety of mammalian cell types and its ease of use suggest that the luciferase-based approach will have broad utility in studying the pathogenesis of toxin-mediated diseases.

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