Designation: E2805 – 11

Standard Practice for Measurement of the Biological Activity of Ricin[1]

This standard is issued under the fixed designation E2805; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Ricin is a member of the protein toxins that cause their physiological effect by inactivation of ribosomes. Ricin is a member of the class 2 ribosome inactivating proteins (1).[2] Other members of this class of toxins include the proteins abrin and Shiga toxin.

Ricin consists of two chains, the A-chain that is responsible for the N-glycosidase enzymatic activity and the B-chain that is needed for cell binding and intra-cellular processing. Ricin is a heterogeneous protein with molecular weights ranging from approximately 62 to 64 kilodaltons (kDa) (2). Both chains are glycosylated and of similar size (approximately 32 kDa). There are several genes encoding putative ricin and ricin-like proteins in the genome of R. communis (3) resulting in differences in the amino acid sequence of the subunits. The differences in amino acid sequence and glycosylation both contribute to the heterogeneity of ricin.

1. Scope

1.1 This guide is intended for the manufacturers and users of ricin reference material. Ricin reference materials are well-characterized materials that can be used to test detection devices and calibrate laboratory measurements. It is anticipated that ricin reference materials will be characterized by biochemical methods in addition to the measurement of biological activity.

1.2 This practice details the measurement of ricin biological activity using a cell-free translation (CFT) assay (4).

1.3 The CFT assay has been developed for use in any biotechnology laboratory where determination or confirmation of ricin biological activity is required.

1.4 The CFT assay has been validated by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) VP-016 Validation of Cell-Free Translation Assay for the Detection of Ricin Toxin Biological Activities in compliance (5) with Good Laboratory Practices (GLP) Regulations of the Food and Drug Administration (21 CFR Part 58). Strict adherence to the protocol is necessary for validity of the test results.

1.5 Appendix X1 and Appendix X2 also provide guidance for the measurement of the biological activity of ricin using cell-based assays and the use of synthetic enzyme substrates.

1.6 Ricin is a category 2 select agent and acquisition of the ricin standard must adhere to the Center for Disease Control (CDC) regulations. Ricin is listed on the select agent list (42 CFR Part 72).[3] The possession, transfer, and use of ricin are restricted under the Public Health Security Preparedness Act (CRS Report RL31263 Public Health Security and Bioterrorism Preparedness and Response Act (P.L. 107-188): Provision and Changes to Preexisting law). Access to stores of ricin is limited (USA Patriot Act, P.L. 107-56). Ricin is also a prohibited substance under the Biological Weapons Convention and the Chemical Weapons Convention (CRS Report RL31559 Proliferation Control Regimes: Background and Status).

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Ricin is an extremely dangerous toxin. See Section 9 for specific hazards information.

[1] This practice is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.01 on CBRNE Sensors and Detectors.

Current edition approved Jan. 1, 2011. Published March 2011. DOI: 10.1520/E2805-11.

[2] The boldface numbers in parenthesis refer to the list of references at the end of this standard.

[3] Available at http://www.bt.cdc.gov/Agent/agentlist.asp.

2. Referenced Documents

2.1 *ASTM Standards:*[4]

F2149 Test Method for Automated Analyses of Cells—the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions

2.2 *Code of Federal Regulations:*

21 CFR Part 58 Good laboratory practice for nonclinical laboratory studies[5]

42 CFR Part 72 Interstate shipment of etiologic agents[6]

2.3 *ANSI/ATCC Standard:*[7]

ASN-0001-2009 Standardization of In-Vitro Assays to Determine Anthrax Toxin Activities

3. Terminology

3.1 *Abbreviations:*

3.1.1 *CFT*—cell free translation.

3.1.2 *CPS*—counts per second, units of luminescence instrument.

3.1.3 *IC50*—concentration of ricin that produces inhibition of 50 % of the activity in an assay.

3.1.4 *kDa*—molecular mass in kilo Dalton units.

3.1.5 *PBS*—phosphate buffered saline.

4. Summary of Practice

4.1 The CFT assay for measuring biologically active ricin is based on its inhibitory effects on protein synthesis (6, 7). When added to a rabbit reticulocyte translation mixture containing luciferase mRNA, ricin inhibits translation of the mRNA into the enzyme luciferase. Luciferase is then detected using a buffer containing the luciferin substrate. The test is a bioluminescence assay that measures the amount of luminescence proportional to the amount of luciferase produced from protein translation (RNA → protein). When active ricin is present, the amount of luminescence decreases corresponding to a decrease in the production of the luciferase enzyme. The amount of protein (luciferase) produced is directly proportional to the amount of luminescence generated. The decrease in luminescence is directly proportional to the amount of active ricin in the sample. Confirmation that translation inhibition is caused by the presence of active ricin is determined by mixing an aliquot of the ricin samples with anti-ricin antibody before adding to the translation mixture. The neutralized ricin does not inhibit luciferase translation, and therefore, luminescence does not decrease.

4.2 Cell-based assays use mammalian cells maintained in culture to measure the effect of ricin on cell death or damage (cytotoxicity). Ricin is added to the cells and after an incubation period, the effect on cell cytotoxicity is measured. The ricin-treated cells are compared to control cells (without added ricin) maintained under the same conditions. Guidance is given in Appendix X1.

4.3 The N-glycosidase enzymatic activity of the A-chain of ricin can be measured using synthetic oligonucleotides. The enzyme activity is measured either by the released adenine or the effect on the depurinated substrate using a number of methods. Guidance is given in Appendix X2.

5. Significance and Use

5.1 The CFT assay provides a sensitive and reliable method to detect ricin biological activity and results can be generated within 3 h. The assay measures the amount of ricin biological activity when compared to a known ricin standard and provides a quantitative measurement for active ricin.

5.2 The lower limit of quantitation and the upper limit of quantitation for ricin using the CFT assay was measured at 10 ng/mL and 170 ng/mL, respectively (5).

5.3 This practice is focused on the measurement of reference materials and not environmental samples. Additional control runs may be needed for measurements of environmental samples to ensure that the presence of additional materials in the samples (also referred to as the matrix) will interfere with the measurements.

5.4 The CFT assay may be used to determine the presence of active ricin in forensic or bioterrorist samples if the appropriate controls are utilized to ensure valid results (5).

5.5 The methods described in this document measure the biological activity of ricin and do not detect the presence of inactivated ricin in a given sample.

5.6 Ricin reference materials have a number of applications, such as testing detection devices, laboratory instruments, environmental sampling methods, disinfection studies, and basic research.

6. Apparatus

6.1 *List of Equipment*—The make and model are provided as examples, however equivalent apparatus may also be used.

6.1.1 *Mixer,* vortex mixing motion

6.1.2 *Display timers.*

6.1.3 *Incubator,* capable of maintaining temperature of (37 ± 1°C).

6.1.4 *96 Well Microplate Luminometer and Luminescence Test Plate.*[8]

[4] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[5] Available from Food and Drug Administration (FDA), 5600 Fishers Ln., Rockville, MD 20857, http://www.fda.gov.

[6] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

[7] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[8] The sole source of supply of the apparatus (MicroLumi XS) known to the committee at this time is Harta Instruments, Inc., 8 Russell Ave Unit 106, Gaithersburg, MD 20877, www.hartainstruments.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

6.1.5 *Microplate Data Analysis Software,* KC4, with PowerReports,™ v3.0.[9]

6.1.6 *Plateshake.* [10]

6.1.7 *Laboratory Refrigerators (4°C), Freezer (-20°C), and Ultralow Freezer (-70°C or lower).*

6.1.8 *Water Bath,* 37 ± 1°C.

6.1.9 *Fixed Volume Pipettes, 1000 µL (200 to 1000 µL), 200 µL (20 to 200 µL), 20 µL (5 to 20 µL), 10 µL (1 to 10 µL), and 2 µL (0.5 to 2 µL), or adjustable pipettes of this range*—Pipettes should be regularly calibrated to ensure accurate dispensing of the set volumes.

6.1.10 *Multi-Channel Pipettes including 12-Channel (20 to 200 µL), 8-Channel Pipettor (2 to 20 µL), and 8-Channel pipettor (5 to 50 µL)*—Pipettes should be regularly calibrated to ensure accurate dispensing of the set volumes.

7. Reagents

7.1 *Reagents for the CFT Assay*—The validation of the assay was performed with reagents purchased from the specific vendors. The reproducibility and precision of the assay is dependent upon the quality of the reagents. The specific reagents have been tested to work in the validated assay. Substitution of reagents will require testing to ensure the same performance.

7.2 *Rabbit Reticulocyte Lysate,* nuclease treated.[11] The rabbit reticulocyte lysate is prepared from New Zealand white rabbits using a standard protocol under quality-controlled conditions (8). After the reticulocytes are lysed, the lysate is treated with micrococcal nuclease in order to destroy endogenous mRNA. The lysate is further optimized for mRNA translation by addition of an energy generating system, a mixture of tRNAs, hemin (to prevent inhibition of initiation), potassium acetate, and magnesium acetate. The rabbit reticulocyte lysate contains no endogenous mRNA and therefore translates only the mRNA added to the lysate (9). The ability to translate only one protein that can be detected permits a more accurate analysis of the biological effect of ricin's enzymatic reaction.

7.3 *Amino Acid Mixture, Complete*—The amino acid mixture, complete, has been prepared for use in the rabbit reticulocyte lysate systems and is an aqueous solution containing 1 m*M* each of the 20 essential amino acids. The mixture is sterile and RNA-free.

7.4 *Luciferase Control RNA*[11]—Luciferase control RNA used in these studies is commercially made using SP6 RNA polymerase transcription of a plasmid bearing the coding region for the luciferase gene with an additional 30 adenine residues that creates an uncapped, polyadenylated mRNA. The product of this luciferase control RNA is a monomeric protein (61 kDa) that does not require post-translational processing or modification for enzymatic activity. Only full-length luciferase is active. In most laboratories, contamination with extraneous luciferase or luciferase mRNA does not occur because luciferase is not found in laboratory environments.

7.5 *Luciferase Reporter Buffer*[11]—The luciferin reaction system is purchased as two components, lyophilized assay reagent and assay buffer. When mixed, the luciferase assay buffer provides high quantum efficiency and no background luminescence in the reticulocyte system or in the assay chemistry. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule of oxyluciferin. The luciferase reaction buffer contains coenzyme A that improves kinetics and allows for greater enzymatic turnover resulting in increased light intensity. Unlike many chemiluminescent reactions, this reaction remains constant for nearly two minutes, thereby permitting accurate measurements using a microtiter format (4). As noted, the amount of luminescence emitted with this reaction buffer is proportional to the amount of luciferase present and therefore provided a comparative measurement of the luciferase amount in samples treated with toxins.

7.6 *RNase Inhibitor*[11], a 50 kDa protein that noncovalently binds to RNases in a 1:1 ratio, is a broad-spectrum RNase inhibitor. The product is purified using a combination of ion exchange and affinity chromatography.

7.7 *Sterile Nuclease-Free De-ionized Water*[11], sterile and RNase free.

7.8 *Ricin Standard and Antibody*—Reagents should be comparable to the following products:

7.8.1 *Ricin Toxin (Ricin), Ricinus Communis Agglutinin II, 5 mg/mL*[12]—This product, purified from castor beans, is the approximately 60 kDa molecular weight protein that is highly toxic with little agglutinin activity. The toxin is purchased as a liquid (5 mg/mL) and contains 0.08 % sodium azide. At this concentration, the sodium azide does not affect the assay.

7.8.2 *Antibody to Ricinus Communis Agglutinin I and II, Affinity Purified from Goat, 2 mg/mL*[12], This product is a polyclonal anti-ricin IgG and provides excellent neutralization of ricin toxin.

[9] The sole source of supply of the apparatus known to the committee at this time is BioTek, P.O. Box 998, Highland Park, Winooski, VT 05404, http://www.biotek.com/. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[10] The sole source of supply of the apparatus (DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay), product # 1296-003) known to the committee at this time is PerkinElmer, 940 Winter St., Waltham MA 02451, http://www.perkinelmer.com/. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[11] The sole source of supply of the reagent known to the committee at this time is Promega Corporation, 2800 Woods Hollow Rd., Madison, WI 53711, www.promega.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[12] The sole source of supply of the reagent known to the committee at this time is Vector Laboratories, 30 Ingold Rd., Burlington, CA 94010, www.vectorlabs.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

7.9 *Phosphate Buffered Saline (PBS)*[13], with a pH of 7.2. Sterile PBS solutions were tested in the CFT assay and this PBS did not interfere with the assay.

7.10 *Polyoxethylenesorbitan Monolaurate*[13], molecular biology grade, ensured to be endonuclease, exonuclease, and RNAse free.

7.11 *Disinfectant*—Bleach, 5 % sodium hypochlorite. Ensure that the expiration date on the bleach has not passed and that the bleach is still active.

8. Materials

8.1 *96 V Shaped Bottom Plates,* sterile, tissue culture grade.

8.2 *96 Well Flat Bottom Plate,* black with clear bottom.

8.3 *Sterile Pipette Tip.*

8.4 *15 mL and 50-mL Conical Tubes,* sterile.

8.5 *1.5 mL Microtubes,* sterile.

8.6 *100 mL Disposable Reagent Reservoir,* sterile.

8.7 *Low-Density Polyethylene Bag,* zip-lip, 5 by 8 in.

8.8 *250 mL Glass Bottle,* sterile, for preparing buffer.

8.9 *Ice Bucket (Ice Pan).*

8.10 *Waste Container.*

8.11 *Paper Towels,* disposable.

8.12 *Nitrile Gloves,* nonsterile.

9. Hazards

9.1 Ricin is extremely toxic in very small quantities (10, 11). The user of this practice is responsible for ensuring that the procedures are carried in full compliance with the institute's safety/biosurety regulations.

9.2 Ricin should be managed as a hazardous chemical and included in the laboratory specific chemical hygiene plan. Ricin in powder form is more hazardous, therefore work with ricin powder should be avoided if possible. The institute's biosurety/biosafety office should be contacted prior to any work with powder samples containing (or thought to contain) ricin.

9.3 The use of ricin requires biosafety level 2 (BSL2) or higher and specialized procedures including training for working with toxins of biological origin (12).

9.4 Any materials containing ricin waste should be inactivated by autoclaving or treating with active solutions of bleach (10 % vol/vol) for 30 min before disposal.

10. Calibration and Standardization

10.1 Calibration of instruments should be performed in accordance with the institute's standard operating procedures or manufacturer's instructions.

10.2 The assay is standardized using the reagents listed. If different reagents are used, standardization of the materials is required.

10.3 The performance and concentration of the anti-ricin antibody used should be determined.

10.4 The purity and concentration of the ricin standard used should be verified by analytical measurements.

10.4.1 Denaturing gel electrophoresis using sodium dodecyl sulfate (SDS) and conditions that reduce the disulfide bond between the A and B subunits, results in two bands of approximately 32 kDa (13).

10.4.2 Techniques such as size exclusion chromatography can also be used to determine the heterogeneity of the ricin sample, pure samples should give a single peak with an approximate molecular weight of 60 kDa (14).

10.4.3 Other methods can be used for characterizing ricin preparations such as ion-exchange chromatography and isoelectric focusing (15).

10.5 The ricin protein concentration of pure ricin samples should be determined for reference samples. The molar absorption coefficient of ricin in phosphate-buffered saline (PBS) at 279 nm was measured as (93 900 ± 3300) L mol^{-1} cm^{-1}, amino acid analysis is used to determine the protein concentration (16).

11. Procedure

11.1 *Preparation of CFT Luciferase Reaction Buffer:*

11.1.1 Thaw the luciferase assay buffer using a 37°C water bath.

11.1.2 Add the thawed assay buffer to the luciferase assay substrate powder that is contained in a brown glass bottle. Dispense 10 mL to each tube of the reaction buffer into conical tubes. Each tube (10 mL) is enough for 2 plates.

11.1.3 Assign a lot number and label the tube with the name (luciferin assay buffer), the date made, and the expiration date (1 month if stored at -20 ± 10°C, or 1 year if stored at –70 ± 10°C after the preparation date). Wrap each tube with aluminum foil and store the buffer at a -20°C or -70°C.

11.2 *Preparation of the Assay Buffer (PBS Containing 0.02 % Tween 20):*

11.2.1 Pipette 200 mL of PBS into a sterile glass bottle and add 40 µl Tween 20. Mix gently to insure that Tween 20 is dispersed. Larger volumes can be prepared as long as sterility of the container is maintained.

11.2.1.1 Prepare assay buffer fresh each day.

11.3 *Preparation of Ricin Standard Working Solution (0.5 µg/mL):*

11.3.1 Add 5 µL of ricin (5 mg/mL) to 2495 µL of assay buffer to make ricin stock solution (10 µg/mL). Label the stock solution and store at 4°C. The stock solution is stable for 2 weeks when stored at 4°C (5), but caution should be exercised in storage of dilute concentrations of ricin to confirm the stability or if losses due to adsorption to storage containers have occurred. When in doubt prepare a fresh solution from the concentrated stock.

11.3.2 Then add 50 µL of the stock solution to 950 µL assay buffer to prepare a 0.5 µg/mL ricin working solution.

[13] The sole source of supply of the reagent known to the committee at this time is Sigma-Aldrich Corp., St. Louis, MO, www.sigmaaldrich.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

11.3.3 Place the ricin standard working solution (0.5 µg/mL) in ice pan.

11.4 *Preparation of Anti-Ricin Antibody Working (Neutralization) Solution (10 µg/mL):*

11.4.1 Add 10 µL goat anti-ricin antibody stock solution (2 mg/mL) to 1090 µL assay buffer for every set of 3 samples.

11.4.2 Keep anti-ricin antibody stock solution at 4°C.

11.5 *Equipment Check:*

11.5.1 Check to ensure that the incubator, vortex mixer, and shaker platform are working properly.

11.5.2 Turn on and test the luminometer following the manufacturer's users manual, using the luminescence filter and settings. Run the luminescence test plate to ensure that the instrument is functioning correctly.

11.6 *Prepare the Necessary Amount of Luciferase Reporter, Assay Buffer, and Reagents on Ice:*

11.6.1 Place the assay buffer, ricin working solution, anti-ricin antibody working solution, test samples, water, amino acid solution, and luciferase mRNA in the ice bath. Except for incubating ricin with antibody, all reagents shall be at a temperature between 0 to 4 in order to minimize translation before incubating the lysate.

11.7 *Serial Dilution of Ricin Standards and Unknown Test Samples:*

11.7.1 Seven concentrations of ricin have been characterized as providing a suitable sigmoid standard curve for this assay. The standards are prepared by serially diluting ricin working solution with assay buffer. The final concentrations of the standards are: S1 = 250 ng/mL, S2 = 125.0 ng/mL, S3 = 62.5 ng/mL, S4 = 31.3 ng/mL, S5 = 15.6 ng/mL, S6 = 7.8 ng/mL, and S7 = 3.9 ng/mL. The concentrations for the seven standards may need to be adjusted when using a different lot of ricin or ricin purchased from a different vendor. The ricin standards are prepared in separate tubes and then added to the plates, so that the same standards can be added to multiple plates if needed.

11.7.2 The starting dilution of test samples should be determined by the assay supervisor based on sample information or pre-test results before starting the assay. The amount used for serial dilution of unknown test samples varies, depending on quantity/amount of the sample.

11.7.3 Prepare two sets of serial dilutions for ricin standards (S), unknown test samples (T), and buffer control (BC) on a v-shaped 96-well microplate. One set will be used for CFT assay and the second set will be neutralized with ricin antibody and then analyzed by the assay to confirm ricin toxicity. The plate should be set up in accordance with Table 1.

11.7.4 Add 50 µL assay buffer to the wells for all of the BC samples, and the T samples in rows C, D, E, F, G, and H (but not row B, according to the configuration shown in Table 1).

11.7.5 Add 50 µL of the corresponding ricin standard (S1 to S9) to the wells of columns 1 and 9.

11.7.6 Add 100 µL of unknown sample starting dilution to each corresponding well of row B (columns 2, 3, 4, 10, 11, and 12).

11.7.7 Perform 2-fold serial dilution of unknown samples by transferring 50 µL/well from row B to row C, and so on continuing to row H. Remove and discard 50 µL from row H. After each transfer, mix dilutions by pipetting up and down 7 times. Clean pipette tips should be used after each serial dilution step

11.8 *Prepare Anti-Ricin Antibody Neutralization Reaction:*

11.8.1 Add 50 µL/well of the anti-ricin antibody neutralization working solution (10 µg/mL) to one set of standards, samples, and buffer control (columns 9-12). After each addition, mix solutions by pipetting up and down 5 times.

11.8.2 Add 50 µL/well of the assay buffer to the other set of standards, samples, and control (Columns 1-4). After each addition, mix solutions by pipetting up and down at least 5 times.

11.8.3 Cover and gently shake the plate on a shaker at room temperature (25 ± 3) for 20 ± 5 min.

11.9 *Transferring Solutions:*

11.9.1 Place a clean V-shaped bottom plate on ice in an ice bucket.

11.9.2 Remove the lysate plate from the shaker and place the plate on ice.

11.9.3 Transfer 5 µL/well from each set of standards, sample dilutions, and control to corresponding wells in a V-shaped bottom plate.

11.10 *Preparation of Translation Reagent:*

11.10.1 Place the reagent reservoir on ice in an ice bucket.

11.10.2 Prepare the translation reagent by adding the following reagents in the reagent reservoir.

11.10.3 All work should be done on ice (4 ± 2) in order to prevent the onset of translation.

11.10.4 Add reagents in the same order as listed in Table 2.

11.10.5 Mix reagents by pipetting the mixture gently up and down 5 times using a 1000 µL pipettor.

11.11 *Incubation of Translation/Ricin Mixture:*

TABLE 1 Configuration for 96-well Microplate for Serial Dilution of Ricin Standards and Unknown Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC[A]	BC[A]	BC[A]	BC[A]					BC[A]	BC[A]	BC[A]	BC[A]
B	S1[B]	T1–1[C]	T2–1[C]	T3–1[C]					S1[B]	T1–1[C]	T2–1[C]	T3–1[C]
C	S2[B]	T1–2[C]	T2–2[C]	T3–2[C]					S2[B]	T1–2[C]	T2–2[C]	T3–2[C]
D	S3[B]	T1–3[C]	T2–3[C]	T3–3[C]					S3[B]	T1–3[C]	T2–3[C]	T3–3[C]
E	S4[B]	T1–4[C]	T2–4[C]	T3–4[C]					S4[B]	T1–4[C]	T2–4[C]	T3–4[C]
F	S5[B]	T1–5[C]	T2–5[C]	T3–5[C]					S5[B]	T1–5	T2–5	T3–5
G	S6[B]	T1–6[C]	T2–6[C]	T3–6[C]					S6[B]	T1–6[C]	T2–6[C]	T3–6[C]
H	S7[B]	T1–7[C]	T2–7[C]	T3–7[C]					S7[B]	T1–7[C]	T2–7[C]	T3–7[C]

[A] BC = buffer control.

[B] Ricin standard samples: S1 = 250 ng/mL, S2 = 125.0 ng/mL, S3 = 62.5 ng/mL, S4 = 31.3 ng/mL, S5 = 15.6 ng/mL, S6 = 7.8 ng/mL, and S7 = 3.9 ng/mL.

[C] Sample dilutions: T1, T2, and T3 = serial dilutions of unknown samples prepared in triplicate.

TABLE 2 Translation Reagent Preparation

Reagent Name	Volume (per 3 unknown samples)
Nuclease-Free Water	1000 µL
Rabbit Reticulocyte Lysate, Nuclease Treated	1000 µL
RNasin RNase inhibitor	5 µL
Amino Acid Mixture, Complete	35 µL
Luciferase Control RNA	30 µL

11.11.1 Immediately add 25 µL of above mixed translation reagent solution into each well of the V-shaped plate containing 5 µL of the samples.

11.11.2 Gently tap the sides of the plate. Avoid creating air bubbles.

11.11.3 Place a damp paper towel around the plate and place the plate in a sealable plastic bag.

11.11.4 Incubate the plate at 37 ± 1°C for 90 ± 5 min.

11.11.5 After the 90 ± 5 min incubation, remove the plate from the plastic bag.

11.11.6 Immediately place the plate on ice in the ice pan. Leave on ice for 5 min.

11.11.7 *Transferring Reaction Mixture and Adding Luciferase Reaction Buffer:*

11.11.7.1 Transfer 5 µL per well of the reaction mixtures from the translation incubation plate to a new 96 well, clear bottom black plate.

11.11.7.2 All dilutions should be run in triplicate. Each set of samples is run on one black plate. One plate shall be used for the normal translation set and one plate for the anti-ricin antibody (neutralization) translation reaction set.

11.11.8 Set up black plates as shown in Table 3.

11.11.9 Without interruption, add 45 µL of luciferase reaction buffer to each well (in black plates) using the multichannel pipettes to the wells containing the 5 µL samples. Once the luciferase reaction buffer is added the plate shall be read by the luminometer within 2 min.

11.11.10 Gently tap the sides of the plate to mix.

11.12 *Reading the Plates:*

11.12.1 Immediately following the addition of the luciferase reporter buffer place the plate in the luminometer and read within 2 min.

11.12.2 Save raw data, counts per second (CPS) to a disk.

11.13 *Calculations:*

11.13.1 Calculate the mean CPS, standard deviation (SD), and %CV of the triplicate wells, including standards, controls, and samples, on each individual test black plate with the Bio-Tek microplate data analysis software with PowerReports™ (KC4, v3.0),[9] or a suitable statistical analysis software program.

11.13.2 The four parameters and R^2 of ricin standard curves and mean ricin concentration of sample dilutions are calculated with KC4 software, using the following four-parameter logistic (4PL) equation:

$$y = (a - d)/(1 + (x/c)b) + d \qquad (1)$$

where:

y = the expected response,
x = concentration,
a = response at zero concentration,
d = response at infinite concentration,
c = concentration resulting in a response halfway between a and d (the IC50), and
b = slope parameter

11.13.3 Specific active ricin in the test samples is confirmed by comparing CPS of each sample dilution without and with the addition of anti-ricin IgG, which neutralized the ricin toxic activity.

11.13.4 Raw data (CPS) should be stored in a computer spreadsheet program.

11.13.5 Calculate percent recovery (% recovery), percent of relative error (% RE), and all mean concentrations, standard deviation (SD), and % CV for dilution-to-dilution, plate-to-plate, and day-to-day comparison, using the following equations:

$$\%\ CPS = CPS_{sample}/CPS_{buffer\ control}\ x\ 100 \qquad (2)$$

$$\%\ recovery = observed/nominal\ x\ 100 \qquad (3)$$

$$\%\ RE = (Observed/Nominal - 1)\ x\ 100 \qquad (4)$$

$$\%\ CV = SD/mean\ x\ 100 \qquad (5)$$

Nominal concentration refers to the initial concentration of the ricin used as the standard.

11.14 The P value is calculated by paired T-test for neutralization data and un-paired T-test for stability data, using a suitable statistical analysis software program (see Fig. 1).

12. Disposal of All Ricin Solutions

12.1 All liquid waste shall be decontaminated using a final concentration of 10 % bleach solution for a 30-min contact time.

TABLE 3 Configuration for 96-well Black Micro Plates for the Luciferase Reaction

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC
B	S1	S1	S1	T1–1	T1–1	T1–1	T2–1	T2–1	T2–1	T3–1	T3–1	T3–1
C	S2	S2	S2	T1–2	T1–2	T1–2	T2–2	T2–2	T2–2	T3–2	T3–2	T3–2
D	S3	S3	S3	T1–3	T1–3	T1–3	T2–3	T2–3	T2–3	T3–3	T3–3	T3–3
E	S4	S4	S4	T1–4	T1–4	T1–4	T2–4	T2–4	T2–4	T3–4	T3–4	T3–4
F	S5	S5	S5	T1–5	T1–5	T1–5	T2–5	T2–5	T2–5	T3–5	T3–5	T3–5
G	S6	S6	S6	T1–6	T1–6	T1–6	T2–6	T2–6	T2–6	T3–6	T3–6	T3–6
H	S7	S7	S7	T1–7	T1–7	T1–7	T2–7	T2–7	T2–7	T3–7	T3–7	T3–7