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Author manuscript

J Immunol Methods. Author manuscript; available in PMC 2018 December 01.

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Published in final edited form as:

J Immunol Methods. 2017 December ; 451: 54–60. doi:10.1016/j.jim.2017.08.008.

Sample Processing Approach for Detection of Ricin in Surface Samples

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Abstract

With several ricin contamination incidents reported over the past decade, rapid and accurate methods are needed for environmental sample analysis, especially after decontamination. A sample processing method was developed for common surface sampling devices to improve the limit of detection and avoid false negative/positive results for ricin analysis. Potential assay interferences from the sample matrix (bleach residue, sample material, wetting buffer), including reference dust, were tested using a Time-Resolved Fluorescence (TRF) immunoassay. Test results suggested that the sample matrix did not cause the elevated background fluorescence sometimes observed when analyzing post-bleach decontamination samples from ricin incidents. Furthermore, sample particulates (80 mg/mL Arizona Test Dust) did not enhance background fluorescence or interfere with ricin detection by TRF. These results suggested that high background fluorescence in this immunoassay could be due to labeled antibody quality and/or quantity issues. Centrifugal ultrafiltration devices were evaluated for ricin concentration as a part of sample processing. Up to 30-fold concentration of ricin was observed by the devices, which serve to remove soluble interferences and could function as the front-end sample processing step to other ricin analytical methods. The procedure has the potential to be used with a broader range of environmental sample types and with other potential interferences and to be followed by other ricin analytical methods, although additional verification studies would be required.

Keywords

Ricin contamination; Sample processing; Ultrafiltration; Concentration; Bleach decontamination; Time Resolved Fluorescence immunoassay

Introduction

Several ricin contamination incidents [1] since the 2001 anthrax bioterrorism attacks in the US suggest a need for rapid and accurate methods for ricin analysis from environmental samples to complement the methods available for clinical samples. Ricin toxin for

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bioweapon production can be present in waste material generated during oil production from castor beans (*Ricinus communis*) or from purification and refinement of the castor bean pulp. Ricin toxicity may occur from inhalation (median lethal dose, LD₅₀ ~ 21–42 µg/kg), ingestion (LD₅₀ ~ 1–20 µg/kg), dermal penetration, or injection (LD₅₀ ~ 1–1.75 µg/kg) [2]. The ricin holotoxin is a heterodimeric, Type 2 ribosome-inactivating protein, consisting of an A- and B-chain, linked by a disulfide bond. The 34 kilodalton (kDa) A-chain with N-glycoside hydrolase activity de-purinates a key adenine residue in the 28S rRNA (ribosome), stops protein synthesis, and ultimately causes cell death. The 32 kDa Bchain is a lectin that is catalytically inactive. However, the lectin mediates specific binding (to carbohydrates) and transport of the holotoxin into host cells, after which the disulfide bond is cleaved, and the A-chain is fully functional. Therefore, the A-chain has extremely low toxicity outside the cell without the B-chain.

Several analytical methods are used to detect ricin, including immunoassays [3,4] and handheld devices [5], *in vitro* cytotoxicity assays [6], cell-based activity assays [7], mass spectrometric proteomic analysis [8], and real-time PCR for *R. communis* deoxyribonucleic acid (DNA) present in the ricin preparation [9,10]. As part of a public health investigation of a white powder incident, many of these approaches were used [11], as well as a novel mass spectrometry-based activity assay that detected enzymatically active ricin [12].

The time-resolved fluorescence (TRF) immunoassay is a potential screening method using ricin-specific antibodies to determine the presence of ricin in environmental samples. TRF has also been used for detection of other toxins and bacterial pathogens [13]. The TRF assay is a solid-phase, immunosorbent assay that uses a biotin-labeled capture antibody attached to a streptavidin-coated support and a europium-labeled detector antibody that acts as a reporter for bound ricin, forming an antibody-ricin-antibody complex (i.e., “sandwich”). Upon excitation, the europium ions are extremely stable and emit fluorescence with a longer lifetime than non-specific background fluorescence, usually > 100s of microseconds to > one millisecond [14], thereby producing a more selective and sensitive assay. The large difference between europium excitation (340 nm) and emission wavelengths (615 nm) and a sharp emission fluorescence peak (full width at half maximum of ~10 nm) further contribute to assay sensitivity. Finally, the Dissociation-Enhanced Lanthanide Fluorescence Immunoassay (DELFI[®]; PerkinElmer [PE]) system used for TRF analysis includes an enhancement solution to release europium and stabilize it within surfactant micelles, which further acts to enhance the fluorescence [15].

Although the TRF assay is an accepted method for ricin detection, it has not been evaluated with the pre- and post-decontamination surface samples expected to result from environmental response to a ricin incident. Recently, high background fluorescence levels have been noted for surface samples after bleach decontamination for the Tupelo, Mississippi (MS) ricin incident [16,17], leading to unsatisfactory and unreliable results. Specifically, elevated backgrounds were observed and were speculatively attributed to bleach residue or other substances extracted from the surface or from the sampling device with associated wetting buffer. In addition, high backgrounds (~8,600 to 10,400 fluorescence counts) were reported from test samples (lacking ricin) prepared by sampling bleach-dried surfaces; these values exceeded typical background fluorescence values of <2,000 counts. It

was not clear whether bleach residue, sampling material, sample wetting buffer, or a combination of these components led to high background fluorescence. In addition, environmental samples contain particulates that could lead to assay interferences, including elevated background fluorescence, resulting in inaccurate results.

To mitigate this issue, a sample processing approach was developed and evaluated with both sponge-stick and swab sample types typically used for ricin incidents including postdecontamination surface sampling. These materials were pre-wetted with neutralizing buffer (NB) and tested with and without bleach residue from mock surface sampling. The sample processing procedure also enabled concentration of ricin and an improved detection limit with 10 kDa centrifugal ultrafiltration (UF) devices, which (depending on the device type [0.5 or 2.0 mL]) provided ~12- to 30-fold ricin concentration (based on fluorescence counts). These devices have not reportedly been used for biotoxin cleanup and concentration for bioterrorism incident samples, although UF has been used to concentrate ricin for toxicity studies [18]. Based on these findings, the sample cleanup and concentration approach may reduce false negative results in complex environmental samples that could lead to human exposure if contaminated facilities were reopened prematurely, or to reduce false positive results that could trigger additional unwarranted decontamination activities.

Materials and Methods

Immunoassay Reagent Preparation

For capture antibody, affinity-purified polyclonal goat anti-ricin antibody (CRP, Cat. No. ABAG_RIC) from the U.S. Department of Defense (DOD) Critical Reagents Program (CRP), now known as the Defense Biological Product Assurance Office (DBPAO), was used. The capture antibody was biotin-labeled using an EZ-Link™ NHS-PEG4 Biotinylation kit (Life Technologies, Cat. No. 21455) following manufacturer's instructions. Absorbance measurements (500 nm) were used with the 4-hydroxyazobenzene-2-carboxylic acid displacement assay to estimate biotin incorporation following the manufacturer's procedure. Using this method, labeled capture antibody had approximately two or seven biotins per antibody for two different lots. The capture antibody was diluted in DELFIA® Assay Buffer from a 200–400-fold concentrated stock solution just prior to use (final antibody concentration ~200 ng per well).

For detector antibody, Monoclonal Anti-Ricin Toxin A-Chain, Clone RAC18 (BEI Resources, Inc., Manassas, VA; Cat. No. NR-9571 [IgG2aK antibody class]) was used. Detector antibody was europium [Eu]-labeled and quantified by PE Custom Labeling Service (Waltham, MA) with Eu:protein ratios of 3.5:1, 3.9:1, and 1.64:1 for different lots. The labeled detector antibody was diluted in assay buffer 100-fold to 1000-fold (used at ~37 to 370 ng/well, depending on the lot) to determine optimal fluorescence signal in the TRF assay. The optimal dilution was experimentally determined by finding the lowest dilution for which the counts for buffer without ricin were <2,000, and for buffer with ricin (100 pg/well) were $10^4 - 10^5$. The detector antibody was prepared as appropriate aliquots and stored at -80 °C. For individual experiments, detector antibody aliquots were thawed, diluted appropriately in assay buffer, and filtered through a 0.22-µm Millex-GV filter unit (Millipore, Cat. No. SLGV033RS), just prior to use.

Sample Preparation

Toxin samples were generated from unconjugated *Ricinus communis* Agglutinin II (RCA 60, Ricin; Vector Laboratories, Cat. No. L-1090; Burlingame, CA). Dilutions of ricin holotoxin were made in endotoxin-free 1X-phosphate buffered saline (PBS) (Cat. No. P0300, Teknova, Hollister, CA). Other buffers were also tested to assess TRF performance including PBS with 0.05% Tween-80 (PBST), PBS with 3% Bovine Serum Albumin (BSA; Fraction V, VWR, Radnor, PA; Cat. No. EM2930), and PBST with 3% BSA. These buffers were compared with PBS by making 10-fold serial dilutions in the appropriate buffer from the original ricin stock solution (in PBS) at 100 µg/mL. Dilutions were performed to generate 1 µg/mL to 1 ng/mL concentrations in 1-mL volume (e.g., 100 µL ricin solution added to 900 µL buffer). Since 10 µL of ricin solution were added per well, the final concentrations ranged from ~10 ng to 10 pg ricin per well. Triplicate TRF analyses were performed for each sample replicate (dilution).

Ricin A-chain (Cat. No. L9514, Sigma-Aldrich, St. Louis, MO) was used as a positive control. The A-chain was suspended in 40% glycerol containing 10 mM phosphate, pH 6.0, 0.15 M NaCl, 10 mM galactose, and 0.5 mM dithioerythritol. For initial experiments, the A-chain was diluted in PBS to make a ~1 µg/mL stock; however, poor stability was noted because counts decreased significantly upon storage for even 1 day (data not shown). A-chain dilutions were subsequently made in DELFIA® Assay Buffer (Cat. No. 1244–111) just prior to use, and the dilutions were used within one hr of preparation. Two serial dilutions (~11-fold; 10 µL added to 100 µL) were performed in assay buffer on the plate; fluorescence counts for the three dilutions were used to ensure consistent assay performance between experiments. Solutions containing ricin holotoxin or A-chain were handled and processed in a ducted, Class II biosafety cabinet.

Preparation of Surface Coupons with Bleach Residual

Fresh 10% bleach was prepared using Ultra Clorox® Germicidal Bleach (one part) and autoclaved double distilled water (nine parts). Prior to use, 10 × 10 inch (25.4 × 25.4 cm) stainless steel coupons (20 Gauge 304 – 2B; Alro Steel, Cat. No. 14812194) were disinfected with 10% bleach, rinsed with water and then 70% isopropyl alcohol, and dried in the biosafety cabinet. For swab sampling, 4 × 4 inch (10.2 × 10.2 cm) sections were taped off on the 10 × 10 inch squares. To mimic bleach decontamination conditions for the Tupelo, MS incident, coupons (placed horizontally) received 10% bleach applied by hand sprayer and were allowed to remain wet for 10 min. Additional bleach was added during the 10 min when areas on the coupons appeared to dry. All coupons were dried overnight prior to sampling. Triplicate samples from bleach-disinfected surfaces, along with one sample from a water-treated (control) surface, were processed and analyzed by TRF.

Swab Sample Preparation and Processing

Foam-tipped swab samples (Puritan Medical Products, Cat. No. 25–1607 1PF SC; Guilford, ME) were pre-wetted with NB (10-mL, Cat. No. K105; Hardy Diagnostics, Santa Maria, CA). The NB was composed of the following per L: 5 g aryl sulfonate complex, 160 mg sodium thiosulfate, 42.5 mg potassium phosphate, and 8 mg sodium hydroxide. The swab was pre-wetted by immersing into the solution, and excess fluid was expressed prior to

wiping the coupon surface. Foam-tipped swabs were used to sample three 4" × 4" surfaces (swabs composited) using S-strokes to sample the entire surface [19]. Each swab was then placed in a sterile 15-mL conical tube, and the stick was cut with sterile scissors. A 1-mL aliquot of 1X PBS (Teknova Cat. No. P0300) with 3% BSA (Fraction V) was added to each swab head. The tube was vortex-mixed at ~3,200 rpm in 15 sec bursts for 2 min. Using a sterile 1-mL serological pipette, the liquid sample was transferred to a new 15-mL tube. The original sample tube containing the swab was re-vortexed in 15 sec bursts for 1 min. The remaining liquid was removed with a sterile transfer pipette, expressing as much liquid as possible, and added to the appropriate 15-mL tube. Then, the 15-mL tube with swab was briefly centrifuged for up to 1 min at ~3,000 × g, and any fluid was transferred to the same pre-labeled 15-mL tube.

Sponge-Stick Sample Preparation and Processing

Cellulose SS samples were pre-wetted with 10-mL NB (Solar Biologicals Cat. No. SH10NB; Ogdensburg, NY). In this case, the NB was composed of the following as a weight percent: 0.7 lecithin, 0.12 sodium bisulfite, 0.1 sodium thioglycolate, 0.6 sodium thiosulfate, 1.25 potassium phosphate dibasic, 0.39 potassium phosphate monobasic, and 0.5 Tween 80 (polysorbate). The SS samples were used to sample 10" × 10" coupon surfaces [19] using S-strokes as described. The SS type and sampling method were used to mimic protocols used for the Tupelo, MS, ricin incident [16, 17]. The head of the sponge was placed directly into a sterile specimen cup (Mountainside Medical Equipment, Cat. No. P250400) using the release mechanism, and 1–2 mL of sterile 1X PBS with 3% BSA was added (sufficient volume to obtain up to 2-mL expressed liquid). The cups were vortexed at ~3,200 rpm in 15 sec bursts for 3 min. A 2-mL serological pipette was used to push against the sponge to express sufficient liquid. The recovered liquid sample was transferred to a 15-mL conical tube.

Preparation of Arizona Test Dust

Arizona Test Dust (ATD; Powder Technology Inc., 2006; ISO 12103–1, A3 Medium Test Dust) was used to as a source of debris to evaluate performance of 10 kDa UF devices for cleanup of samples containing particulates. Based on the manufacturer's analysis, the material consisted of: SiO₂ (68–76%), Al₂O₃ (10–15%), Fe₂O₃ (2–5%), Na₂O (2–4%), CaO (2–5%), MgO (1–2%), TiO₂ (0.5– 1.0%), and K₂O (2–5%). The dust usually contained background microbes, including fungi and bacterial spores [20]. Dust was not sterilized, and a slurry was prepared in NB that was expressed from an SS sample. The slurry was prepared at 1.0 g/mL, and 250 µL was added to yield 250 mg ATD per sponge.

Sample Processing Using UF Devices

Different Amicon® Ultra centrifugal UF devices were used for sample processing to purify and concentrate ricin from the matrix prior to analysis, including 0.5 mL and 2-mL 10 kDa devices (Amicon® Ultra-0.5 10K device, Millipore®, Cat. No. UFC501024 and Amicon® Ultra-2 10K device, Millipore®, UFC201024, respectively), with 10 kilodaltons (kDa) nominal molecular weight limit (NMWL) cutoff. The 2-mL 10 kDa UF devices were used to enable processing larger volume samples (up to 2-mL) and provide up to 20-fold volume reduction (for 100 µL retentate).

For ricin solutions (in PBS) or swab and sponge sample extracts (in PBS with 3% BSA) with ricin added after extraction, a 400–500 μL aliquot was first filtered through a 0.22- μm Ultrafree[®] MC GV 0.5 mL filter (Millipore[®] Cat. No. UFC30GV0S), and the resulting filtrate(s) were processed using the 0.5-mL or 2-mL 10 kDa UF devices following manufacturer's instructions. In this case, up to 0.45-mL or 2-mL sample volume was loaded onto the 0.5-mL or 2-mL 10 kDa UF device, respectively. For the 0.5-mL UF devices, centrifugation was conducted using an Eppendorf 5417R instrument with fixed angle rotor at $12,900 \times g$ for 11 min at 25 °C for collection and wash steps. The same 0.5-mL UF device was used for multiple rounds of centrifugation to concentrate a 1-mL starting sample volume. For the 2-mL UF devices, an Eppendorf 5810R instrument with swinging bucket rotor was used at $3,180 \times g$ for 60 min at 25 °C. After two wash steps, the 2-mL UF device was inverted within the collection tube for retentate recovery, whereas the 0.5-mL UF device was not inverted (after washes) due to concerns about potential cross-contamination when handling with forceps (the 2-mL UF device does not require use of a forceps to invert the device). For both UF device types, the sample retentate after wash steps was measured and adjusted to 100 μL with PBS.

Time-Resolved Fluorescence (TRF) Assay

The TRF immunoassay adapted from that reported by Schieltz et al. [11] was used with the exception that serial 10-fold ricin dilutions (normally used when the ricin concentration is unknown) were not performed in the assay plate. DELFIA[®] reagents and equipment were used for TRF analysis (PE, Waltham, MA). Streptavidin-coated microtitration strips (Cat. No. 4009–0010) were prewashed with 750 μL Wash Buffer (prepared from Wash Concentrate [Cat. No. 1244–114] by diluting 1:25 with sterile endotoxin-free water) using the DELFIA[®] PlateWash (Cat. No. 1296026) followed by addition of 100 μL biotinylated anti-ricin capture antibody solution (containing ~200 ng). The plate was covered loosely with a plastic lid (from the strip plates) and aluminum foil and incubated for 2 hr at room temperature with the PlateShake shaker (PE, Cat. No. 1296–004) set to “high”. Microtitration strips were then washed two times with 750 μL Wash Buffer to remove unbound capture antibody, and excess liquid was removed. Sample wells then received 100 μL of Eu-labeled detector antibody (~37 or 100 ng antibody, depending on the lot) in assay buffer.

Triplicate negative controls each contained 10 μL PBS buffer. The positive control wells contained 10 μL ricin A-chain (5.9 μL of 850 $\mu\text{g}/\text{mL}$ into 5-mL assay buffer; final 1 $\mu\text{g}/\text{mL}$) and two serial dilutions using 10 μL into 100 μL assay buffer for each. The fluorescence counts for all three A-chain concentrations were expected to be greater than 1.5 times the negative control average; controls met these requirements for all experiments. Matrix control wells for samples and positive and negative controls received 100 μL of assay buffer instead of detector antibody solution to test for potential Eu-contamination. To each sample well, 10 μL of the appropriate sample or ricin standard was added. The plate was then covered and incubated for 1 hr at room temperature with the shaker set to “high”. Wells were subsequently washed eight times with 750 μL Wash Buffer and excess liquid was removed. Finally, 200 μL Enhancement Solution (PE, Cat. No. 1244–105) were added to each well, the plate was covered, and incubated for 10 min at room temperature with shaking at the

“low” setting. Fluorescence counts were measured on a Victor X4 plate reader (PE, Cat. No. 2030–0040) with the following settings: 400 μ s delay, 400 μ s window, and 1,000 μ s cycle time.

Data Analysis

In most cases, the average and standard deviation for fluorescence counts are reported for triplicate TRF analyses per sample. Cases where duplicates were used for controls are noted. Statistical analysis included two-tailed, paired or unpaired t-tests using a 95% confidence level. Comparison was made between non-UF treated and UF-treated individual replicates. In some cases where noted, the untreated replicates used ricin concentrations equivalent to those expected after UF-treatment and concentration (i.e., 10-fold concentration when starting with 1 mL and recovering 100 μ L retentate).

Results and Discussion

Evaluation of TRF Assay and Potential Interferences from Environmental Sample Matrices

The TRF assay was evaluated initially in buffer without added confounders to establish the assay detection limit. Background fluorescence counts for the negative control (PBS) were reduced from >5,000 counts to <2,000 counts by using endotoxin-free PBS buffer with filtration through a 0.22-micron filter (compared to a 0.45-micron filter), as well as 0.22-micron filtration of the 1X detector antibody solution. Typical fluorescence counts vs. ricin concentration (ng/mL) are shown (Figure 1), with the assay sensitivity at ~10 pg (10 μ L from 1 ng ricin/mL in the assay). Some variation due to antibody production lots was observed, as discussed below. The combined effects of optimizing the capture and detector antibody concentrations (200 ng and 37 – 100 ng per well, respectively) and filtering the PBS and the capture antibody solution (0.22-micron filter) resulted in lower, more consistent fluorescence counts for the negative control (PBS), typically between ~900 and 1,700.

Experimental conditions were then set up to reproduce the elevated fluorescence values for negative controls (PBS) in the absence of ricin that were observed in previous ricin incident sampling and TRF analysis efforts [16, 17], in which 10% Ultra Clorox® Germicidal Bleach was prepared and applied to surfaces as described above. Typically, 0.5-mL to 1 mL sample extract volumes were obtained from swabs and sponges using the protocols described above. The sample extracts with bleach residue appeared yellow with a pH of ~10 (measured by pH paper). Ricin solution spiked into these extracts (10 μ L of 1 ng ricin/ μ L added to 90 μ L extract) was degraded and not detected by TRF analysis, as expected (data not shown). Two separate experiments were conducted without added ricin for both sample types, showing no elevated background fluorescence with bleach residue; fluorescence counts were statistically similar to the samples from water treated coupon surfaces and from negative controls (Table 1). The ricin A-chain positive controls gave results within the expected ranges (data not shown). Although the sample extracts appeared to have high levels of bleach residue (based on the color), when added to assay buffer (10 μ L added to 100 μ L assay buffer with detector antibody), the pH was maintained at 8 (measured by pH paper in replicate samples). In some cases, particulates from the sponges could also be observed in the samples, perhaps due to

the vortex-mixing step. Regardless, these particulates did not appear to interfere with the TRF assay.

The data obtained from testing new Eu-labeled detector antibody lots in this study pointed to other possible causes for the elevated fluorescence levels observed in samples from the MS incident [16, 17], namely, either improper dilution or insufficient quality of Eu-labeled antibody stock solutions. The amount of labeled detector antibodies in the assay needs to be optimized to minimize background fluorescence counts. As an example, for one detector antibody lot, background fluorescence (in the absence of ricin) ranged from ~12,700 to 1,950 counts (~sixfold difference) for use at 370 ng to 37 ng detector antibody, respectively. However, the signal from 100 pg ricin (10 μ L of a 10 ng/mL solution) ranged from 35,800 to 18,400 counts (~twofold difference) for these detector antibody concentrations. If too much detector antibody is used or if poor quality detector antibody is used (i.e., sub-optimal ratio of Eu:protein, improper antibody purification, etc.), elevated fluorescence for negative controls could also result. Finally, high background fluorescence could occur from use of unfiltered or insufficiently filtered reagents.

Evaluation of 0.5-mL 10 kDa Ultrafiltration Devices for Ricin Recovery and Concentration

Although elevated background fluorescence could not be attributed to sample matrix effects in this study, a sample processing protocol was still needed to remove any potential interferences from complex environmental samples, as well as to concentrate ricin for improved detection. The presence of potential interferences is especially the case for decontamination scenarios if ricin is not quantitatively inactivated and remains at lower but still toxic levels. The 0.5-mL 10 kDa UF device was tested for concentration of ricin by processing 1-mL samples (10-ng ricin/mL) and recovering 100- μ L retentate (as described previously). These UF-treated samples were analyzed along with untreated samples (“No UF”) at 100 ng/mL, to directly compare theoretical (based on volumes) and observed (based on fluorescence counts) concentration factors. Two separate experiments were conducted to evaluate this.

The results showed that UF-treated samples had an average of 12.9 ± 1.2 fold-concentration based on fluorescence counts for UF-treated and untreated replicates and initial ricin concentrations (Table 2), where the UF treatment represented a tenfold volume reduction. These data suggested that the ricin loss from ultrafiltration was not statistically significant. In addition, the UF-treated replicate fluorescence counts for ricin were statistically greater than the untreated replicate fluorescence counts (i.e., statistically more than tenfold concentration) with a 95% confidence level (p-values were <0.004 using unpaired t-tests between individual UF-treated samples and the untreated sample). A similar test using the 10 kDa UF device for cleanup but not concentration also did not show ricin loss, even with four wash steps (data not shown). In this case, the average fold-difference from the “No UF” treatment was 1.3 ± 0.2 , which were statistically significant at a 95% confidence level ($p = 0.012$).

Evaluation of 2-mL 10 kDa UF Devices for Ricin Recovery and Concentration from Samples Containing ATD

Because surface samples could contain other interferents such as particulate substances (referred to as “debris”) that could lead to elevated background fluorescence and/or inaccurate results, Arizona Test Dust (ATD) was used as a source of debris to challenge the sample processing procedure. ATD represented a uniform challenge material, although it should be noted that ATD is largely inorganic, containing metal oxides, with some fungal and bacterial spores or cells present [20]. ATD was used to gain some information about robustness of the UF-treatment and the TRF assay although other types of environmental backgrounds including soluble organic materials could also affect sample processing and analysis. Two separate experiments were conducted to evaluate this.

The data from the first experiment showed greater than 20-fold concentration of ricin (based on fluorescence counts) with the use of the 2-mL 10 kDa UF devices, with an average ~32-fold concentration factor for clean extracts and an average ~24-fold concentration factor for ATD-containing extracts (Table 3). Concentration of ricin in the sample would improve the detection limit afforded by the overall method because the initial fluorescence counts prior to concentration were only approximately 1.5 – 2-fold above the fluorescence counts of the negative control. Similar trends with lower fold-difference values were observed for the second experiment (Table 3), using the same ricin concentrations and debris level. In this case, there was an average ~22-fold concentration factor for clean extracts and an average ~18-fold concentration factor for dirty extracts (Table 3).

In both experiments, the average fold-difference (based on counts) for dirty extracts was lower than the difference for clean extracts. The difference between average fluorescence counts for UFTreated clean and ATD-containing samples was significantly different with 95% confidence level ($p = 0.016$, two-tailed, unpaired T-test of TRF averages) for the first experiment, whereas the second experiment at this confidence level did not show a significant difference ($p = 0.052$). For both experiments, the difference between untreated clean and untreated ATD-containing samples was not significant ($p = 0.44$ and 0.52 , respectively, for two-tailed, unpaired t-tests of TRF averages). The addition of 250 mg ATD per SS sample did not significantly impact the TRF results; however, these results cannot necessarily be generalized to all environmental surface samples. In other types of samples, different types of background debris and interferences could affect the assay results making additional sample processing prior to TRF analysis necessary. However, in principle, the sample processing procedure that includes 0.22-micron pre-filtration and subsequent washes in the UF device may alleviate TRF assay interferences for other environmental samples.

The fold-differences in fluorescence counts for UF treatment were typically greater than those expected from volume reduction alone. According to the vendor, the 10 kDa UF devices include glycerol and possibly other compounds with the NMWL cutoff membrane that cannot be completely removed by wash steps. Furthermore, ricin dilutions prepared in assay buffer typically showed approximately 3- to 4-fold higher fluorescence counts than those prepared in PBS (data not shown), possibly due to improved ricin stability or Ab-ricin binding efficiency. The assay buffer composition is proprietary (vendor-supplied); however, it is known to contain a surfactant and BSA, often used to stabilize proteins and prevent

adherence to surfaces. Therefore, the effect of buffer composition on the TRF assay was tested to better understand how these types of materials could influence immunoassay results. Results are shown in Figure 2 for ricin dilutions made in PBS, PBST, PBS/BSA, and PBST/BSA.

The data showed the highest fluorescence counts using PBS with BSA, followed by PBST/BSA and PBST with similar counts ($p = 0.09 - 0.82$ for different ricin concentrations), and followed by PBS. The difference between the PBS/BSA buffer and PBS was statistically significant for all ricin concentrations (p -values ranged from 0.006 to 0.0006 for the range of ricin concentrations). The lower ricin concentrations showed greater improvements, with up to ~2.0-fold higher counts for PBS/BSA compared to PBS for 10 pg and 100 pg ricin, respectively. These data may help explain improved counts from UF treatment above those expected from sample volume reduction alone. Based on these findings, it may be advisable to use PBS with 3% BSA to increase the stability of ricin and/or the binding efficiency of ricin to antibodies, and thereby, enhance TRF assay performance. Dilutions of ricin solutions are prepared only for testing purposes, and actual environmental samples would either be analyzed only undiluted or could also be diluted in assay buffer in the plate. However, use of PBS with 3% BSA for ricin extraction from sample material including white powders is supported by these results.

Conclusions

In this effort, we investigated the potential causes of previously reported elevated background fluorescence in TRF analysis of post-decontamination samples. The TRF immunoassay reported herein did not show interference with high concentrations of bleach residue, wetting buffer, and materials from sampling devices (sponge-sticks and macrofoam swabs) because no elevated background fluorescence (i.e., > 7,000–8,000 fluorescence counts) was observed. Furthermore, samples containing particulates (from a reference test dust) up to 250 mg/SS did not contribute to high background fluorescence. However, high fluorescence counts were observed for samples lacking ricin (PBS controls) when detector antibody preparations with high background fluorescence were used at more concentrated levels; for example, >10,000 counts were evident for 200-fold dilutions of these antibody lots with PBS controls, whereas other antibody lots did not show elevated background fluorescence when used at similar concentrations. Therefore, it is possible that reported high background fluorescence leading to unsatisfactory results reported for the MS ricin incident [16,17], may not have been due to the post-bleach decontamination samples but were rather due to a reagent issue.

Using the 10 kDa UF devices, up to ~30-fold concentration factors were achieved based on fluorescence counts. The UF devices did not show significant losses of ricin, even when 2–4 wash steps were performed. Incorporation of the sample processing procedure prior to TRF analysis may enable improved assay sensitivity for real world complex environmental samples and provide greater usability of the TRF data by elevating the fluorescence response/signal above the background. Therefore, when the reported processing procedure is used, more consistent results could be expected for both pre- and post-decontamination samples, providing higher quality data for high consequence decisions concerning public

health. A processing procedure for surface samples (swabs, sponge-sticks) for both sample cleanup and ricin concentration could be useful for any assay including fluorescence-based and electrochemiluminescence immunoassays to minimize false positive and false negative results. Because the sample processing procedure developed in this effort is intended for use following the sample extraction steps, it could be used with any ricin analytical method, although further verification and validation would be required (i.e., for different surface types and potential interferences).

Acknowledgments

The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here under an Interagency Agreement (EPA IA DW-89-92328201-0). The DOE contractor role did not include establishing Agency policy. It has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names, products, or services does not convey official EPA approval, endorsement, or recommendation.

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We thank scientists Brad Bowzard, Todd Parker, and Laura Rose from the Centers for Disease Control and Prevention, and Dee Pettit from the North Carolina State Laboratory of Public Health and Christina Browne from the California Department of Public Health for technical advice concerning the ricin time-resolved fluorescence immunoassay. We acknowledge Mike Nalipinski and Terry Smith of the EPA Office of Emergency Management, Benjamin Franco, EPA Region 4-On Scene Coordinator, and Worth Calfee of EPA NHRSC for technical advice on ricin decontamination and sampling. We acknowledge Bruce Goodwin and his team (Kim Williams, Eric Thompson, Melody Zacharko, and Bryan Necciai) at the Defense Biological Product Assurance Office of the Department of Defense Joint Program Executive Office for providing affinity-purified anti-ricin antibody. We acknowledge the following technical reviewers: Matthew Magnuson, EPA National Homeland Security Research Center, and Francisco Cruz, EPA Office of Emergency Management.

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Figure 1. TRF Assay Sensitivity of Detection for Ricin.

Europium fluorescence counts vs. ricin holotoxin concentration after background fluorescence (from negative controls, PBS buffer) subtraction are presented. Data are from two separate experiments with triplicate TRF analyses per data point. Average fluorescence counts for negative controls for the two experiments were 1520 ± 110 and 1600 ± 200 . Error bars represent $\pm$ one standard deviation. For each TRF analysis, 10 μ L of solution was added to 100 μ L of assay buffer. The data were generated using 100 ng detector antibody per well.

Figure 2. Evaluation of Dilution Buffers for Ricin Detection with TRF Assay.

Europium fluorescence counts vs. ricin holotoxin concentration (ng/mL added as 10 μ L per well) for dilutions made in different phosphate-buffered saline (PBS) solutions are shown. T = Tween 80 at 0.05% final concentration; BSA = Bovine Serum Albumin, at 3% final concentration. Bars represent the average of triplicate TRF analyses, and error bars are $\pm$ one standard deviation. For each TRF analysis, 10 μ L of solution were added to 100 μ L of assay buffer. Average counts for 100 and 10 ng ricin per mL (added as 10 μ L) are from two separate experiments with triplicate TRF analyses per data point; whereas, those for 1000 and 1 ng ricin per mL are from a single experiment with triplicate TRF analyses per data point. The data were generated using 100 ng detector antibody per well.

Table 1.

Testing of the Sample Matrix (Sampling Device, Bleach Residue, and Neutralizing Buffer) for Background Fluorescence Interference in the TRF Assay

Sample Matrix	Experiment Number – Sample Replicate	Fluorescence Counts		
		Avg (SD) *	Expt Avg (SD)	Overall Avg (SD)
Swabs with Bleach Residue	1–1	1400 (220)	1330 (150)	1420 (160)
	1–2	1300 (150)		
	1–3	1300 (70)		
	2–1	1700 (200)	1500 (230)	
	2–2	1500 (200)		
	2–3	1300 (70)		
Swabs with Water	1–1	1300 (120)	NA	1350 (130)
	2–1	1400 (200)	NA	
Sponge-Sticks with Bleach Residue	1–1	1500 (250)	1430 (390)	1300 (230)
	1–2	1600 (630)		
	1–3	1200 (80)		
	2–1	1200 (160)	1170 (110)	
	2–2	1100 (40)		
	2–3	1200 (90)		
Sponge-Sticks with Water	1–1	1400 (90)	NA	1450 (70)
	2–1	1500 (40)	NA	
PBS (Control)	1–1	1300 (140)	NA	1340 (115)
	2–1	1300 (130)	NA	

* Average (Avg) and standard deviation (SD) from triplicate TRF analyses per sample (Bleach Residue) or PBS control. For Sponge-Sticks with Water controls, duplicate TRF analyses were conducted per sample. NA = Not Applicable. The data were generated using 100 ng detector antibody per well.

Table 2

Evaluation of the 0.5-mL 10 kDa UF Device for Ricin Recovery and Concentration

Treatment*	Experiment Number – Sample Replicate	Fluorescence Counts Avg (SD)	Avg FoldDifference From No UF Treatment	Concentration Factor**
10 ng/mL Ricin UF	1 – 1	$3.9 (0.1) \times 10^4$	$1.19^{\dagger}$	11.9
	1 – 2	$3.5 (0.04) \times 10^4$	$1.09^{\dagger}$	10.9
	1 – 3	$3.9 (0.08) \times 10^4$	$1.21^{\dagger}$	12.1
	Expt Avg (SD)	$3.8 (0.2) \times 10^4$	1.16 (0.07)	11.6 (0.7)
	2 – 1	$4.9 (0.03) \times 10^4$	$1.41^{\dagger}$	14.1
	2 – 2	$4.9 (0.01) \times 10^4$	$1.43^{\dagger}$	14.3
	2 – 3	$4.9 (0.1) \times 10^4$	$1.42^{\dagger}$	14.2
	Expt Avg (SD)	$4.9 (0.08) \times 10^4$	1.42 (0.007)	14.2 (0.07)
	Overall Avg (SD)	$4.35 (0.49) \times 10^4$	1.29 (0.12)	12.9 (1.2)
100 ng/mL Ricin No UF	1 – 1	$3.3 (0.1) \times 10^4$	NA	NA
	2 – 1	$3.4 (0.07) \times 10^4$	NA	NA
	Overall Avg (SD)	$3.35 (0.07) \times 10^4$	NA	NA
PBS (Control) No UF	1 – 1	$9.4 (1.0) \times 10^2$	NA	NA
	2 – 1	$1.7 (0.1) \times 10^3$	NA	NA
	Overall Avg (SD)	$1.3 (0.3) \times 10^3$	NA	NA

* Average (Avg) and standard deviation (SD) are from triplicate TRF analyses per sample. UF = ultrafiltration with 0.5 mL 10 kDa UF device. NA = not applicable.

** Since 10-fold lower ricin concentrations were used for the 1-mL aliquot concentrated to 100 μ L, than for the ricin solution not processed by UF, the average fold difference (concentration factor) between UF-treated and untreated was actually ~12-fold and ~14-fold for Experiments 1 and 2, respectively.

[†] Denotes a statistically significant difference compared to the “No UF” control (95% confidence level for Ttests on individual UF-treated samples compared to the “No UF” sample). The data were generated using 100 ng detector antibody per well.

Table 3

Evaluation of 2-mL 10 kDa UF Devices for Ricin Recovery and Concentration from Samples With or Without ATD

Treatment*	Sample Replicate	Fluorescence Counts Avg (SD)*		Avg (SD) FoldDifference From No UF Treatment
		No UF	UF	
Experiment #1				
SS Expressed Solution	1	$3.9\ (0.2) \times 10^3$	$1.2\ (0.01) \times 10^5$	31.5 (3.0)
	2	$3.6\ (0.07) \times 10^3$	$1.1\ (0.03) \times 10^5$	
	3	$3.3\ (0.1) \times 10^3$	$1.1\ (0.04) \times 10^5$	
	Avg (SD)	$3.6\ (0.1) \times 10^3$	$1.1\ (0.03) \times 10^5$	
SS Expressed Solution With 250 mg ATD	1	$3.8\ (0.1) \times 10^3$	$9.8\ (0.4) \times 10^4$	24.2 (2.4)
	2	$3.7\ (0.4) \times 10^3$	$8.2\ (0.06) \times 10^4$	
	3	$3.7\ (0.2) \times 10^3$	$9.3\ (0.06) \times 10^4$	
	Avg (SD)	$3.8\ (0.2) \times 10^3$	$9.1\ (0.2) \times 10^4$	
Experiment #2				
SS Expressed Solution	1	$5.4\ (0.1) \times 10^3$	$1.2\ (0.05) \times 10^5$	21.7 (2.8)
	2	$4.8\ (0.07) \times 10^3$	$1.1\ (0.02) \times 10^5$	
	3	$5.0\ (0.7) \times 10^3$	$1.0\ (0.005) \times 10^5$	
	Avg (SD)	$5.1\ (0.4) \times 10^3$	$1.1\ (0.03) \times 10^5$	
SS Expressed Solution With 250 mg ATD	1	$5.1\ (0.6) \times 10^3$	$9.2\ (0.4) \times 10^4$	18.3 (1.8)
	2	$4.9\ (0.2) \times 10^3$	$9.4\ (0.06) \times 10^4$	
	3	$4.7\ (0.01) \times 10^3$	$8.3\ (0.1) \times 10^4$	
	Avg (SD)	$4.9\ (0.4) \times 10^3$	$9.0\ (0.3) \times 10^4$	

* Average (Avg) and standard deviation (SD) are from triplicate TRF analyses per sample. SS = sponge-stick; ATD = Arizona Test Dust; UF = Ultrafiltration with 2.0 mL 10 kDa UF device. Neutralizing Buffer was expressed from clean, pre-wet SS samples and used as a sample matrix for addition of ATD and ricin (to initial concentration of 10 ng/mL). The data were generated using 37 ng detector antibody per well.