

Development of a Sample Processing Approach for Post Bleach-Decontamination Ricin Sample Analysis

FINAL REPORT

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by

Sanjiv R. Shah, Ph.D.
Threat and Consequence Assessment Division
National Homeland Security Research Center
Cincinnati, Ohio 45268

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Lawrence Livermore National Laboratory
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Livermore, California 94551

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Sanjiv R. Shah, Ph.D.

National Homeland Security Research Center
U.S. Environmental Protection Agency
1300 Pennsylvania Avenue, NW
USEPA-8801RR
Washington, DC 20460
(202) 564-9522
shah.sanjiv@epa.gov

If you have difficulty accessing these PDF documents, please contact Nickel.Kathy@epa.gov or McCall.Amelia@epa.gov for assistance.

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List of Acronyms and Abbreviations:

Ab:	antibody
ATD:	Arizona Test Dust
Avg:	average
BR:	bleach residue
BSA:	bovine serum albumin
BSC:	biosafety cabinet
CDC:	Centers for Disease Control and Prevention
CRP:	Critical Reagents Program
DBPAO:	Defense Biological Product Assurance Office
DELFA:	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
DOD:	U.S. Department of Defense
DOE:	U.S. Department of Energy
DOH:	Department of Health
DQO:	data quality objective
EDTA:	Ethylenediaminetetraacetic acid
EPA:	U.S. Environmental Protection Agency
ERLN:	Environmental Response Laboratory Network
ETF:	endotoxin-free
Eu:	europium
HABA:	4'-hydroxyazobenzene-2-carboxylic acid
HEPA:	high-efficiency particulate air
hr:	hour(s)
kDa:	kilodalton
kg:	kilogram
L:	liter
LD ₅₀ :	median lethal dose
LLNL:	Lawrence Livermore National Laboratory
LRN:	Laboratory Response Network
mL:	milliliter
MS:	Mississippi
NB:	Neutralizing Buffer
ng:	nanogram
NHSRC:	National Homeland Security Research Center
NIOSH:	National Institute for Occupational Safety and Health
NIST:	National Institute of Standards and Technology
nm:	nanometer
NMWL:	Nominal Molecular Weight Limit

PBS: phosphate-buffered saline
PBST:phosphate-buffered saline with Tween-80
PCR:polymerase chain reaction
PE: PerkinElmer
pg: picogram
QA:quality assurance
QC: quality control
R. communis: *Ricinus communis*
RCF: relative centrifugal force
RIP: ribosome-inactivating protein
rpm: revolutions per minute
SS: sponge-stick
TRF: time-resolved fluorescence
UF: ultrafiltration
 μg : microgram
 μL : microliter

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Millipore [®]	EMD Millipore Corp.	Billerica, MA
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Solar-Cult [®]	Solar Biologicals, Inc.	Ogdensburg, NY
Teknova [®]	Teknova, Inc.	Hollister, CA
Tween [®] -80	Sigma-Aldrich Corp.	St. Louis, MO
Ultracel [®]	EMD Millipore Corp.	Billerica, MA
Ultrafree [®]	EMD Millipore Corp.	Billerica, MA
VICTOR [®]	PerkinElmer, Inc.	Waltham, MA

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Research Team

Lawrence Livermore National Laboratory

Staci Kane, Anne Marie Erler, Teneile Alfaro, and Tuijauna Mitchell-Hall (Quality Assurance)

EPA Technical Lead

Sanjiv Shah, EPA National Homeland Security Research Center

Technical Reviewers

Matthew Magnuson, EPA National Homeland Security Research Center

Francisco Cruz, EPA Office of Emergency Management

Jafrul Hasan, EPA Office of Chemical Safety Pollution Prevention – Office of Pesticide Programs Microbiology Laboratory

Quality Assurance Reviewers

Eletha Brady-Roberts and Ramona Sherman, EPA National Homeland Security Research Center

Edit Reviewer

Marti Sinclair, Alion Science and Technology, NHSRC Contract GS35F4594G

Executive Summary

There have been several ricin contamination incidents since the 2001 anthrax bioterrorism attacks. The time-resolved fluorescence (TRF) immunoassay is a primary screening method to determine the presence of ricin, the *Ricinus communis* (castor bean) toxin, in environmental samples. However, during the EPA response to the Tupelo, Mississippi, ricin incident in June 2013, unsatisfactory results were obtained due to high fluorescence backgrounds such that the TRF method could not be used for samples for ricin analysis collected from surfaces to which chlorine bleach had been applied for decontamination. The assay interferences were attributed to various potential factors including bleach residue, sampling material, and wetting buffer. To mitigate the TRF immunoassay interference issue for post-decontamination phase ricin sample analysis, the EPA's National Homeland Security Research Center (NHSRC), in partnership with the Lawrence Livermore National Laboratory (LLNL), executed a research project and developed a sample processing approach.

In the absence of appropriate sample processing prior to analysis, bleach residue and/or other factors associated with environmental samples could cause assay interferences that could ultimately lead to false negative or false positive results. False negative results could occur if high background fluorescence masked actual ricin presence, possibly leading to human exposure if facilities were re-opened prematurely. False positives could occur if controls were within range, but samples showed elevated fluorescence, thereby triggering additional, unwarranted decontamination activities. Furthermore, without sample concentration, ricin could be present below the assay's ability to detect it, while still being present at potentially hazardous levels. The current effort was conducted to resolve these issues and provide more confidence in using the TRF assay for analysis of environmental samples and for post-decontamination clearance decisions.

The TRF assay is essentially a sandwich immunoassay that uses two different ricin-specific antibodies that bind ricin, forming an antibody-ricin-antibody complex (i.e., "sandwich"). The biotin-labeled *capture antibody* attaches to a streptavidin-coated support, and the europium-labeled *detector antibody* acts as a reporter for bound ricin. Upon excitation, the europium ions emit fluorescence with a longer lifetime (ideally) than non-specific background fluorescence, thereby producing a more selective and sensitive assay.

Prior to developing a sample processing approach, the TRF immunoassay dynamic range for ricin detection was evaluated. In the current effort, the dynamic range varied with the quality of the labeled detector antibodies. The lower limit of the dynamic range was 10–100 pg ricin. With regard to the upper limit of the dynamic range, up to 10 ng ricin was tested and consistently detected. The lower limit was dependent on the production lot of

europium-labeled detector antibody produced by the vendor. Therefore, the amount of antibody used in the assay needed to be optimized for each production lot, such that background fluorescence counts (in the absence of ricin) were low while counts for ricin-containing samples were high. For detector antibody with high background fluorescence, a lower antibody concentration was required, thus negatively impacting the assay sensitivity. In addition, use of affinity-purified capture antibody (antibody bound to, and later eluted from a ricin-containing purification column) was shown to be necessary for proper performance of the TRF immunoassay in this application.

In this study, post-bleach decontamination samples (swabs and sponge-sticks) were prepared following typical operating procedures; however, even with a worst-case bleach residue present, no elevated background fluorescence was observed. Both swab and sponge-stick samples contained levels of bleach residue sufficient to cause a pH of ~10 in the expressed liquid, yellow-color, and visible dried crystals from surfaces after sampling the surface. Regardless, the TRF assay buffer essentially maintained a pH ~8 upon addition of the sample (pH ~10). Furthermore, no elevated fluorescence levels were observed for any of the post-decontamination samples suggesting that bleach residues were not responsible for elevated fluorescence. These results along with high fluorescence counts seen for poor quality Eu-labeled detector antibody in this study suggest that reagents, rather than the sample matrix, could have caused the reported high fluorescence backgrounds in the MS ricin incident. In this effort, high counts (> 10,000 counts) were observed in the absence of ricin when certain detector antibody lots were used at higher concentrations (e.g., used as a 200-fold dilution rather than a 1,000-fold dilution). The neutralizing buffer usually used to pre-wet the swab or sponge for sample collection also did not show any assay interference.

To mitigate the TRF immunoassay interference by other potential interferents, a sample processing procedure, which included sample clean up and concentration, was developed using 10-kilodalton (kDa) ultrafiltration (UF) devices, which are readily commercially available. The procedure yielded 10- to 20-fold-plus ricin concentration (for 0.5-mL and 2-mL devices, respectively) based on increased fluorescence counts, thus enhancing detection of ricin in the samples. Sample processing procedures were effective for sponge-stick samples containing as much as 250 mg of a reference test dust that is thought to be typical of dust found on potentially sampled surfaces.

In total, this study demonstrated that for the environmental surface type tested, inclusion of the sample processing procedure can improve performance of the TRF assay by elevating the fluorescence response above the background (negative control) by sample concentration (10 to 20-fold), which essentially improves the assay's signal-to-noise ratio. The combined outcome of sample cleanup and toxin concentration may enable detection of ricin at lower concentrations in environmental TRF assays, thus helping to prevent false

negative results. A broader range of environmental sample types and potential interferences should be tested to confirm that the cleanup procedure adequately maintains TRF assay performance. Since the sample processing procedure developed in the current effort is intended for use after sample extraction steps, it has the potential to be used with other ricin analytical methods, although performance should be verified due to unanticipated interfering processes potentially inherent in other methods.

1 Introduction

The U. S. Environmental Protection Agency (EPA) has a need for rapid methods for accurate analysis of ricin from environmental samples because EPA is the lead federal agency to respond, decontaminate, and restore facilities/sites in case of a contamination incident that falls within its jurisdiction. Ricin analytical methods must be reproducible, sensitive, and specific, even in complex environmental backgrounds. Furthermore, the EPA through its Environmental Response Laboratory Network (ERLN), EPA's national network of laboratories that can be accessed as needed to support large scale environmental responses, may be called on to help conduct sample analysis to determine the extent of ricin contamination and whether facilities and areas have been restored to safe conditions after decontamination. Decision-makers at local, state, federal, and tribal levels require rapid, high-confidence results that are not unduly impacted by false positives or false negatives for safely reopening facilities or for clearing areas. Therefore, appropriate analytical protocols are needed.

Ricin is a toxin found in castor beans from the plant, *Ricinus communis* (*R. communis*). It is present in the waste material when castor oil is made for legitimate purposes. The toxin can also be developed into a bio-weapon by partial purification or refinement of the castor bean pulp. Ricin toxicity may occur from inhalation, ingestion, dermal penetration, or injection. The median lethal dose (LD₅₀) of ricin is approximately 21–42 µg/kg for inhalation, 1–20 µg/kg (8 castor beans) for ingestion, and 1–1.75 µg/kg for injection (Grundmann and Tebbett, 2008). For inhalation, symptoms including respiratory distress, fever, cough, nausea, and chest tightness may appear as early as 4–8 hr, and symptoms from ingestion (nausea, vomiting, and diarrhea) typically develop in less than 10 hr. Death usually occurs after 36–72 hr depending on the exposure route and the dose received.

The ricin holotoxin is a Type 2 ribosome-inactivating protein (RIP) consisting of two different protein chains, A- and B- chains, linked by a disulfide bond (heterodimer). The 34 kDa A-chain has N-glycoside hydrolase activity, which de-purinates a key adenine residue in the 28S rRNA (ribosome), disrupts binding of elongation factors, and, thus, stops protein synthesis, causing cell death. The 32 kDa B-chain is catalytically inactive, but it is a lectin that mediates specific binding (to carbohydrates) and transport of the holotoxin into host cells. Outside the cell, the A-chain has extremely low toxicity without the B-chain. After transport of the holotoxin to the endoplasmic reticulum of the cell, the disulfide bond is cleaved, and the A-chain is fully functional.

In order to detect ricin, several analytical methods are used, including immunoassays, *in vitro* cytotoxicity assays, cell-based activity assays (e.g., Rastogi et al., 2010), mass-spectrometric proteomic analysis, and real-time PCR for *R. communis* deoxyribonucleic acid (DNA) present in the ricin preparation (U.S. Department of Health and Human Services, 2006). As part of a public health investigation of a white powder incident, many of these approaches were used, as well as

a novel mass spectrometric based activity assay that detected enzymatically active ricin (Schlietz et al., 2011).

This effort focused on a time-resolved fluorescence (TRF) immunoassay for ricin, which is similar to solid-state “sandwich” immunoassay approaches. The TRF assay uses a capture antibody specific to ricin bound to a solid support. The capture antibody is allowed to react and bind with ricin from a sample. A detector antibody that is also specific to ricin, binds to the ricin, thus making an antibody-ricin-antibody complex, i.e., “sandwich”. Instead of linking the detector antibody with an enzyme, it is labeled with the lanthanide europium, Eu (See Figure 1). The use of europium rather than an enzyme enables more sensitive fluorescence-based detection and a wider dynamic range (as opposed to colorimetric detection).

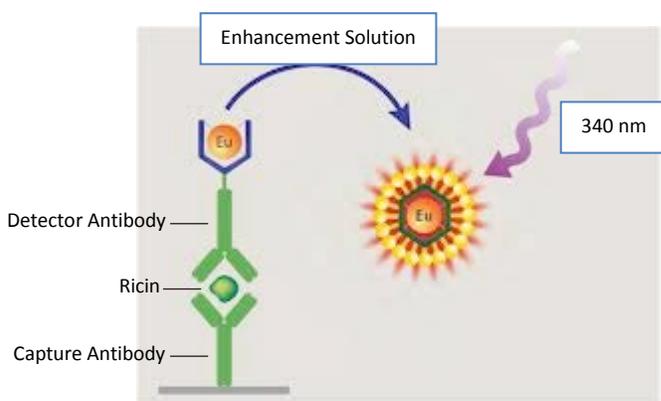


Figure 1 Schematic of the DELFIA® (Dissociation-Enhanced Lanthanide Fluorescence Immunoassay; PerkinElmer [PE], Inc.), a time-resolved fluorescence (TRF) intensity technology.

The DELFIA® is essentially a sandwich-based TRF immunoassay. It uses a stable europium (Eu) label that exhibits a long-lived emission such that it can be potentially distinguished from short-lived auto-fluorescence background. The schematic was adapted from PerkinElmer (2015).

Lanthanide chelates such as europium are extremely stable and have long fluorescence lifetimes (usually over several hundred microseconds to greater than one millisecond for europium) (Yuan and Wang, 2005). The specific fluorescence from the long-lived label can be potentially distinguished from short-lived background fluorescence. The other favorable fluorescence properties of europium include a large difference between excitation and emission wavelengths, 340 nm and 615 nm, respectively, along with a sharp emission fluorescence peak (full width at half maximum of approximately 10 nm). In the current effort, the Dissociation-Enhanced Lanthanide Fluorescence Immunoassay (DELFIA®) system was used for ricin TRF analysis. DELFIA® was developed by PerkinElmer (PE) and has been used by several groups to standardize the reagents and procedural steps. DELFIA® reagents include an Assay Buffer used

to dilute antibody solutions, Wash Concentrate used to make wash buffer for removal of unbound reagents, and an Enhancement Solution, which is an acidic chelating detergent solution.

While the TRF immunoassay has many desirable characteristics, the performance of the assay for ricin detection could potentially be negatively impacted by the presence of substances found in environmental decontamination samples, such as bleach, as well as by the presence of particulates. For example, the Mississippi (MS) Department of Health (DOH) Laboratory, which used the TRF assay as part of a Tupelo, MS ricin response in 2013 (U.S. EPA, 2013a), reported the assay to be “unsatisfactory for ricin toxin detection by fluoroimmunoassay due to high background fluorescence” with post bleach-decontamination samples. One possible cause of background fluorescence was thought to be disruption of the antibody coating on the plate by bleach residues, which could lead to non-specific binding of the Eu-labeled detector antibody, and thus an elevated fluorescence signal in the absence of ricin.

To determine whether bleach residue caused unsatisfactory TRF results, EPA scientists shipped samples mimicking field samples to the MS DOH Laboratory, which included swabs and sponges (used to sample bleach-disinfected non-porous surfaces) without ricin present (essentially negative controls). The MS DOH Laboratory reported elevated TRF values of approximately 8,600 to 10,400 counts (i.e., false positive results) for these samples, when negative controls are typically < 2,000 counts. It is possible that the analytical protocol used by the MS DOH Laboratory was not validated for bleach-treated environmental samples.

In order to meet the need for improved methodology for ricin detection from post-decontamination clearance samples, EPA National Homeland Security Research Center (NHSRC) partnered with Lawrence Livermore National Laboratory (LLNL) to develop protocols to mitigate interferences and improve the robustness on the TRF immunoassay. In addition, since the cause(s) of the assay interferences reported by the DOH Laboratory was not clear, one task of this effort was to recreate the problem and investigate possible causes of the reported background fluorescence and false positive test results by separately examining the individual components (i.e., sample matrix, wetting buffer, bleach residue). Other tasks were focused on development of a sample processing procedure that was compatible with TRF immunoassay analysis, as well as subsequent testing with relevant environmental samples to confirm the procedure’s utility. The TRF assay reported by Schieltz et al. (2011) was used although ricin dilutions and samples processed by ultrafiltration (UF) were analyzed in triplicate without serial 10-fold dilutions performed in the plate (except where noted). The sample processing procedure developed in this effort could (in principle) be usable as a front-end to other analytical methods because, in addition to cleaning-up the sample, it enables a better assay sensitivity of detection in complex environmental samples.

2 Project Objectives and Experimental Plan

For environmental ricin samples, especially after decontamination, sample cleanup is needed prior to TRF analysis; therefore, sample-processing protocols are needed for a range of sample types such as sponge-sticks and swabs. The premise of the current effort is that sample cleanup and concentration will help alleviate false positive results caused from interferences in the sample matrix and/or associated liquid or arising from the presence of bleach used in (and other activities performed during) decontamination operations. A further goal of the current effort is to reduce the possibility that false negative results occur if the sample extract is not concentrated prior to analysis resulting in ricin levels below limits of detection.

This project included investigation of approaches and development of protocols for processing post bleach-decontamination samples for ricin analysis by TRF in light of the current challenges. The proposed sample processing approach was targeted towards not only physically separating the interference from the ricin but also toward concentrating the ricin to improve the assay sensitivity. It should be noted that the same sample processing procedures might be useful for analysis of DNA from *R. communis* by real-time PCR or analysis by mass spectrometry, although additional verification studies would be required.

At the project onset, it was not known which of the potential sources of sample interferences—bleach residue (BR), sponge-stick (SS), Neutralizing Buffer (NB) individually or in combination—were contributing to background fluorescence; therefore, the plan was to evaluate potential sources of interferences, alone and in combination. In order to separately test the sample material and the wetting buffer, the plan was to use gauze wipes in place of SS and to use phosphate-buffered saline with Tween[®]-80 (PBST) in place of NB as wetting buffer. Prior to investigating the cause(s), one objective was to recreate the interference problem following the sample preparation procedure and sample processing and analysis procedures that presumably led to the interferences, via the use of protocols from the EPA scientists (not the authors of this study) who prepared the aforementioned test samples for the MS DOH Laboratory. The EPA scientists used both macrofoam swabs (Cat. No. 25-1607 1PF SC, Puritan Medical Products, Guilford, ME) and Solar-Cult[®] sponges (Cat. No. SH10NB, Solar Biologicals, Inc., Nepean, ON, Canada) to sample surfaces containing BR. Swabs and sponges were extracted following the same procedure as the protocol reported in Appendix A for the current study. This protocol uses a minimum volume of PBS with 3% Bovine Serum Albumin (BSA), namely 1–2 mL, to remove ricin (and microorganisms for other analysis methods) by vortex mixing.

For sample processing, centrifugal UF devices with different nominal molecular weight limits (NMWL) were used. These devices are designed for protein and other macromolecule concentration and purification such as DNA (i.e., Amicon[®] Ultra centrifugal filter devices, Millipore, Inc., Billerica, MA). Basically, the devices allow washing out or removal of soluble materials that could interfere with analysis while also enabling analyte concentration.

Specifically, a 1-mL sample can be concentrated to 100 μ L (10-fold concentration) with a 0.5-mL device (by using multiple loadings on the same device), and a 2-mL sample can be concentrated to 100 μ L (20-fold concentration) with a 2-mL device. The goal was to minimize losses of the analyte (ricin) to the devices resulting from toxin precipitation and/or adherence. Different devices including 10 kDa (10K) and 30 kDa (30K) NMWL membranes were tested. Because the ricin holotoxin is about 66 kDa (made up of A-chain at \sim 32 kDa and B-chain at \sim 34 kDa), any device could in principle be effective. It was considered possible that the 10K device could have better performance characteristics for the A-chain or both chains from partially denatured holotoxin. However, there were concerns that the 10K device could be more susceptible to clogging and/or excessively long filtration times. In order to remove potential assay interferences by particulate matter that could be present in the sample and lead to background fluorescence, a pre-filtration (0.22 micron) step was used in the sample processing procedure. Such pre-filtration and UF treatment leading to ricin sample cleanup and concentration could lead to better performance for other assays.

For different starting ricin concentrations, recovery efficiencies or fold-differences for UF-treated solutions were determined by comparison with ricin solutions not treated by UF. In some cases (where noted), the untreated ricin solutions were at a higher initial concentration that was equivalent to the expected concentration after UF treatment. For these cases, statistical analysis included T-tests (paired, two-tailed) comparing individual UF-treated (concentrated) samples with untreated ricin samples, with a confidence level of 95%. For other cases (where noted), the same ricin solution used for UF treatment was analyzed directly by TRF without UF treatment to determine the fold-increase from UF treatment.

Experiments were conducted using ricin at different concentrations without sample material, with clean sample materials, and with samples containing potential interferents (bleach residue, SS, NB wetting buffer, particulates) sampled from surfaces or added to sample extracts (i.e., liquid expressed from pre-wet swabs or sponges). The holotoxin (A/B complex) was used for the testing TRF assay performance, because it is most relevant to actual scenarios. Ricin A-chain was also included as a positive control. Negative controls (lacking ricin) were used in triplicate in each experiment. In addition, matrix controls containing all components (including 10 μ L sample) except the detector antibody were included for each sample.

A systematic approach was taken to address protocol development, with the following tasks:

- Establishment of the TRF assay dynamic range and the appropriate ricin concentration for use in the study (Task 1)
- Investigation and characterization of TRF assay interference (Task 2)
- Determination of ricin recovery/loss for samples processed by ultrafiltration devices (Task 3)

- Evaluation of a sample processing procedure using UF for complex environmental samples (Task 4)

A flow-chart outlining project tasks, subtasks, and experiment descriptions is shown in Figure 2.

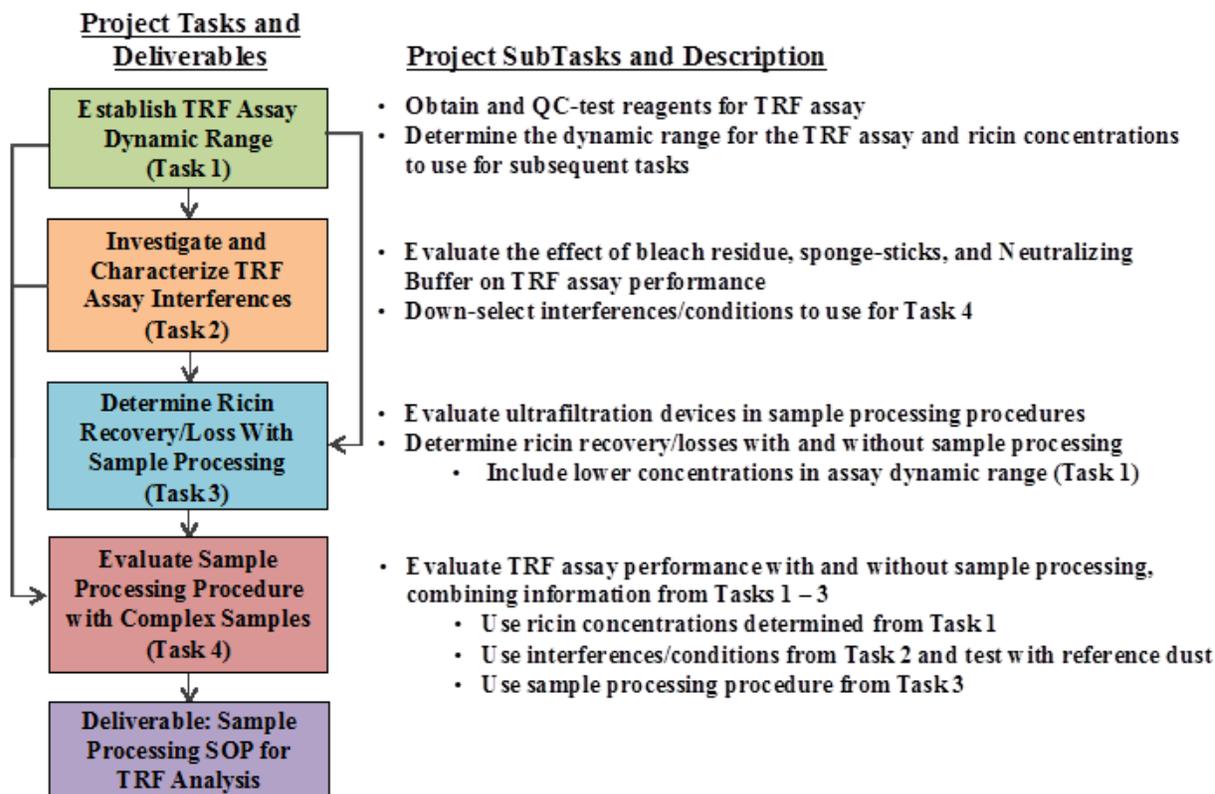


Figure 2 Tasks coordination for the effort including project tasks, subtasks, descriptions, and deliverables.

3 Materials and Methods

3.1 Antibodies and Labeling

Anti-ricin antibodies were obtained from BEI Resources, Inc. (Manassas, VA) and from the U.S. Department of Defense (DOD) Critical Reagents Program (CRP) with proper permits and non-disclosure agreements. The CRP has recently been disestablished and replaced with the Defense Biological Product Assurance Office (DBPAO). Initially, polyclonal anti-ricin toxin (immune globulin G from rabbit; BEI Cat. No. NR-862; BEI Resources, Manassas, VA) was used as capture antibody, although based on poor performance, this was replaced with affinity-purified (antibody bound to, and later eluted from a ricin-containing purification column) polyclonal goat anti-ricin

(CRP, Cat. No. AB-AG_RIC; CRP, Fort Detrick, MD). Monoclonal anti-ricin toxin (BEI Resources Inc., Cat. No. NR-9571 [IgG2aK antibody class]) was used for the detector antibody.

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 (HABA) displacement assay to estimate biotin incorporation following the manufacturer's procedure. Using this method (microtiter plate version), the first lot of labeled capture antibody had approximately seven biotins per antibody while the second lot had approximately two biotins per antibody.

PE Custom Labeling Service performed Eu-labeling of the detector antibody because the vendor was experienced with potential issues such as over-labeling, which can lead to aggregation and an elevated background. PE noted in product literature that polyclonal antibodies tend to have lower labeling efficiencies, and a labeling ratio of 3:1–6:1 Eu:protein was considered optimal. The ratios of labeled antibodies prepared by PE for this effort were 3.5:1, 3.9:1, and 1.64:1 for Lot #1, Lot #2 and Lot #3, respectively. A discussion of different reagent lots is included in Section 4.6.

3.2 Preparation of Ricin Holotoxin and Ricin A-Chain

All toxin samples were processed in a Class 2 biosafety cabinet (BSC) that was externally ducted and equipped with high-efficiency particulate air (HEPA) filters. Unconjugated *Ricinus communis* Agglutinin II (RCA 60, Ricin) obtained from Vector Laboratories (Cat. No. L-1090; Burlingame, CA) was used. Another ricin lot obtained from Sigma (Lectin from *Ricinus communis*, Toxin RCA₆₀, Cat. No. L8508; Sigma-Aldrich, St. Louis, MO) was also used for some experiments, as noted in the report. Dilutions of ricin holotoxin were made in endotoxin-free 1X-phosphate buffered saline (PBS) (Teknova, Cat. No. P0300; Hollister, CA).

Ricin A-chain (Sigma-Aldrich, Cat. No. L9514-1MG, 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. The A-chain concentration was estimated at ~850 µg/mL based on the measured volume, and assuming the vial contained 1 mg A-chain as stated. For initial experiments, A-chain was diluted in PBS; however, poor stability was noted because counts significantly decreased upon storage even for 1 day. After consultation with state public health laboratory scientists (D. Pettit and C. Browne, personal communication), A-chain dilutions were made in DELFIA® Assay Buffer (Cat. No. 1244-111) just prior to use and used within one hr of preparation. Specifically, 5.9 µL (at ~850 µg/mL) were added to 5 mL assay buffer to make a ~1 µg/mL stock. Each plate had at least three wells containing ricin A-chain including 10 µL of the stock diluted in assay buffer (1 µg/mL) added directly to the plate (10 µL added to 100 µL detector antibody in assay buffer), and two serial ten-fold dilutions were performed directly in the plate. This was a ~10-fold dilution since 10 µL were added to 100 µL in the plate (i.e., actually the dilution was ~11-fold).

3.3 Preparation of Anti-Ricin Capture and Detector Antibodies

Polyclonal biotin-labeled capture antibody (Section 3.1) stock was diluted appropriately (typically, 200-fold [denoted as 200X] to 400-fold [denoted as 400X]) in DELFIA[®] Assay Buffer just prior to use as described in Section 4.6). Antibody concentrations that produced less than 2,000 fluorescence counts for the negative control but still allowed sufficient counts from the positive control (>100,000 for 10 ng concentration per well) were selected. For the reported data, the capture antibody concentration was ~200 ng per well.

Monoclonal Anti-Ricin Toxin A-Chain, Clone RAC18 antibody (BEI Resources, Cat. No. NR-9571) was used as detector antibody. Detector antibody was europium-labeled and quantified by PE Custom Labeling Service (Waltham, MA). Once received from the vendor (on dry ice), the labeled detector antibody was thawed and QC-tested at different concentrations (diluted in assay buffer 100-fold [denoted as 100X] to 1000-fold [denoted as 1000X]) to determine optimal fluorescent signal in the TRF assay. The optimal dilution was determined as described in Section 4.6. The detector antibody was then transferred into screw-cap tubes as ~25–40 µL aliquots which were sufficient for use in one experiment (for up to 5–6 strips of 12 wells each), in order to avoid excessive freezing and thawing. Aliquots were 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 (Millipore, Cat. No. SLGV033RS) filter unit just prior to use. For the reported data, the detector antibody concentrations ranged from 37 to 100 ng per well, depending on the reagent lot.

3.4 Preparation of Surface Coupons

Fresh 10% bleach was prepared using Ultra Clorox[®] Germicidal Bleach (one part) and autoclaved double distilled water (nine parts). Each stainless steel coupon (excised sample of a material) was wiped down with 10% bleach, rinsed with double distilled water, and then rinsed with 70% isopropyl alcohol and wiped dry prior to use in an experiment. Each 10 × 10 inch (25.4 × 25.4 cm) stainless steel coupon (20 Gauge 304 - 2B; Alro Steel, Cat. No. 14812194) was labeled and set on a clean surface. For composite swab samples, three 4 × 4 inch (10.2 × 10.2 cm) sections were taped off on the 10 × 10 inch squares, and then labeled and placed on a clean surface. All coupons received 10% bleach applied by hand sprayer and allowed to remain wet for 10 min. Additional bleach was added during the 10 min when areas on coupons appeared to dry. All coupons were dried overnight prior to sampling. Bleach application was similar to that conducted for the Tupelo MS incident (EPA, 2013a; 2013b). Ricin was not applied to the coupons in any of the testing.

3.5 Swab Sample Preparation and Processing

Foam-tipped swab samples (Puritan Medical Products, Cat. No. 25-1607 1PF SC; Guilford, ME) were pre-wet with NB from Hardy Diagnostics (10-mL, Cat. No. K105; 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-wet by immersing into the solution and expressing excess fluid prior to wiping the coupon surface. Foam-tipped swab samples were used to sample coupon surfaces according to the Centers for Disease Control and Prevention (CDC) National Institute for Occupational Safety and Health (NIOSH) Sampling Procedure (CDC-NIOSH, 2010). S-strokes were used to sample the entire surface. Once sampling was complete, each swab was 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, VWR, Radnor, PA, Cat. No. EM2930) was added to each swab head. The 1X PBS was composed of the following: 137 mM sodium chloride, 1.4 mM potassium phosphate monobasic, 4.3 mM sodium phosphate dibasic, and 2.7 mM potassium chloride. 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 pre-labeled 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 and added to the appropriate 15-mL tube. The swab was pressed against the tube wall with the transfer pipette to express as much liquid as possible. The remaining liquid was transferred to the same pre-labeled 15-mL tube, and care was taken to not transfer any debris. The 15-mL tube containing the swab was briefly centrifuged for up to 1 min at $\sim 3,000 \times g$ to collect the buffer to the bottom of the tube; any fluid was then transferred to the same pre-labeled 15-mL tube.

3.6 Sponge-Stick Sample Preparation and Processing

Cellulose Sponge-Sticks (SS) samples were obtained pre-wet with 10-mL neutralization buffer (Solar Biologicals Cat. No. SH10NB; Ogdensburg, NY) with a different recipe than that used for the swabs. In this case, the neutralization buffer (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 coupon surfaces according to the CDC-NIOSH Sampling Procedure (CDC-NIOSH, 2010). As for the swab samples, S-strokes were used. After sampling, 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 were 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 retrieve the liquid and used to push against the sponge to express sufficient liquid. The recovered liquid sample was transferred to a 15-mL conical tube.

3.7 Sample Processing Using Centrifugal UF Devices

Different Amicon[®] Ultra centrifugal filter devices were used for sample processing to purify and concentrate ricin from the matrix prior to analysis. The devices were Amicon[®] Ultra-0.5 Centrifugal Filter Concentrator with Ultracel[®] 10 Membrane for 0.5 mL sample volume

(Millipore[®], Cat. No. UFC501024) and Amicon[®] Ultra-2 Centrifugal Filter Concentrator with Ultracel[®] 10 Membrane for 2.0 mL sample volume (Millipore Cat. No. UFC201024), both with a 10 kilodalton (kDa) cut off nominal molecular weight limit (NMWL). These devices contain UF membranes and thus, allow for rapid UF, high concentration factors, and easy recovery of retentates from dilute and complex sample matrices. For convenience, in this report, the Amicon[®] Ultra-0.5 10K and the Amicon[®] Ultra-2 10K centrifugal filter devices are referred to as 0.5-mL 10K UF devices and 2 mL 10K UF devices, respectively. The 2 mL 10K UF devices were used for some experiments (Task 4) to enable processing larger volume samples (up to 2-mL) and providing up to 20-fold concentration (with 100 μ L retentate). The procedure for using the Amicon[®] UF devices is included in Appendices B and C for 0.5-mL and 2-mL UF devices, respectively. Initial testing was done with 0.5-mL 30K UF devices (Millipore Cat. No. UFC503024).

For ricin solutions (in PBS) and for swab and sponge extracts (in PBS with 3% BSA) from Sections 3.5 and 3.6, respectively, and with ricin added after extraction, a 400–500 μ L aliquot was first filtered through a 0.22- μ m Ultrafree[®] MC GV 0.5 mL Filter Unit (Millipore[®] Cat. No. UFC30GV0S), followed by processing using the 0.5-mL 10K UF device as described in Appendix B. For both UF device types, the sample retentate after wash steps was measured and adjusted to 100 μ L with PBS. Triplicate samples from bleach-disinfected surfaces, along with one sample from a water-treated (control) surface, were processed and analyzed by TRF.

3.8 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 10K 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 (Rose et al., 2011). Dust was not sterilized, and a slurry was prepared in NB that was expressed from a SS sample (Solar-Cult[®] Cat. No. SH10NB, pre-wet with 10 mL NB). The slurry was prepared at 1.0 g/mL and 250 μ L were added to yield 250 mg ATD per sponge (approx. 80 mg ATD/mL).

3.9 Time-Resolved Fluorescence (TRF) Assay

For the current effort, the TRF immunoassay adapted from that reported by Schieltz et al. (2011) was used with the exception that serial 10-fold ricin dilutions were not performed in the assay plate. The TRF assay is summarized below. Additional detail with step-by-step instructions is included in Appendix A. DELFIA[®] reagents and equipment were used for TRF analysis (PE, Waltham, MA). A schematic of the major steps of the TRF immunoassay is shown in Figure 3.

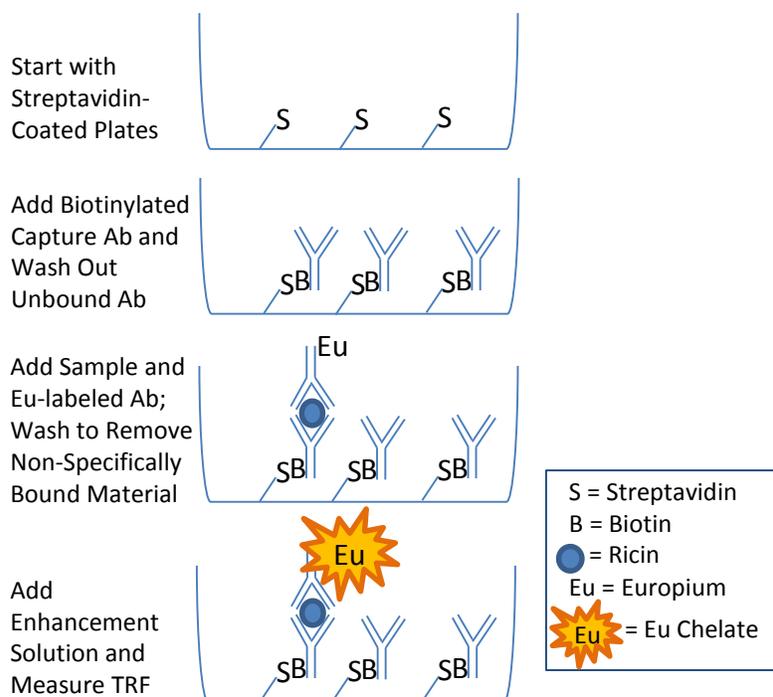


Figure 3 Schematic diagram of the TRF immunoassay steps from the perspective of a single well. Streptavidin-coated wells enable binding of biotin-labeled capture antibody. After binding the capture antibody, the sample and Eu-labeled detector antibody are added. If the sample contains ricin, it binds to both antibodies and forms a “sandwich”. The fluorescence signal is enhanced when europium is released from the detector antibody by addition of enhancement solution.

Microtitration strips (streptavidin-coated clear plate, 8 × 12 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. 1296-026). Next, 100 µL of biotinylated anti-ricin capture antibody solution (Section 3.3) were added to each well. The plate was covered loosely using a plastic lid (from the original packaging for strip plates) and then covered with aluminum foil and incubated at room temperature with the PlateShake shaker (PerkinElmer, Cat. No. 1296-004) set to “high” for 2 hr. Microtitration strips were then washed two times with 750 µL Wash Buffer on the plate washer to remove unbound capture antibody. After tamping off excess liquid, sample wells received 100 µL of Eu-labeled detector antibody solution in assay buffer (Section 3.3). Matrix control wells for samples and positive and negative controls received 100 µL of assay buffer instead of detection antibody solution because this tested for potential Eu-contamination. Triplicate negative controls each contained 10 µL PBS buffer. The positive control wells contained 10 µL ricin A-chain (5.9 µL of 850 µg/mL into 5-mL assay buffer; final 1 µg/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. To each sample well, 10 μL of the appropriate sample or ricin standard were added after vortex mixing for 3-5 seconds. The plate was then covered as described above, and incubated at room temperature with the shaker set to “high” for 1 hr. After this step, wells were washed eight times with 750 μL Wash Buffer and tamped off to remove excess liquid. Then, 200 μL Enhancement Solution (PerkinElmer, Cat. No. 1244-105) were added to each well and the plate was covered as described above, followed by shaking at “low” setting for 10 min at room temperature. Fluorescence counts were then measured on a Victor X4 plate reader (PerkinElmer, Cat. No. 2030-0040) with the following settings: 400 μs delay, 400 μs window, and 1,000 μs cycle time. The dynamic range on the Victor X4 instrument was stated to be linear to 15×10^6 counts with saturation at 25×10^6 counts (PerkinElmer, personal communication).

3.10 Data Analysis, Interpretation, and Presentation

Typically, the average and standard deviation for fluorescence counts are reported for triplicate TRF analysis per sample. Cases where duplicates were used for controls are noted. In addition, the overall average and standard deviation were calculated in order to compare treatments with one another (i.e., UF-treated vs. untreated sample extracts).

The overall standard deviation from all sample replicates was calculated using the following equation,

$$\text{Overall or joint SD} = \sqrt{\{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + (n_3 - 1)s_3^2 + (n_1 \times [X_1 - \bar{X}]^2) + (n_2 \times [X_2 - \bar{X}]^2) + (n_3 \times [X_3 - \bar{X}]^2)\} / (n_1 + n_2 + n_3 - 1)}$$

where n_1 , n_2 , and n_3 = the number of TRF analyses per sample for sample replicates 1, 2, and 3; s_1 , s_2 , and s_3 = the standard deviation of the fluorescence counts for the individual samples; X_1 , X_2 , and X_3 = the average fluorescence counts for the individual samples; \bar{X} = the overall average fluorescence counts for the samples. In some cases, data is shown in both graph and table formats. This was intended in that the graphs would show the general trend of the data, whereas exact values would be provided in the tables. Sufficient significant figures are included in the tables to allow for quality assurance review. Statistical analysis included two-tailed, paired or unpaired T-tests using a 95% confidence level; calculated p-values are reported in each case. 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 μL retentate).

4 Quality Control, Quality Assurance, and Data Quality Objectives

Prior to initiating experimental work, the data quality objectives (DQO) were reviewed by the EPA Technical Lead and the EPA quality assurance (QA) manager. In addition, the work reported here followed the quality guidelines. Monthly data reports were submitted and discussions were conducted with EPA's Technical Lead to review progress and adherence to the DQOs. The key quality control (QC)/QA provisions are described below.

4.1 Laboratory Inspections

Monthly laboratory inspections were conducted by the project principal investigator to comply with U.S. Department of Energy (DOE) and LLNL safety and security policies. In addition, the LLNL responsible official and/or biosafety officer conducted annual laboratory inspections. Inspections included the following:

- Documenting laboratory cleanliness
- certifying laboratory safety equipment, including the BSCs and autoclave
- reviewing ricin toxin inventories
- reviewing waste handling procedures
- reviewing personnel training

4.2 Calibration

The Victor X4 plate reader was calibrated by the vendor and set-up and inspected by a PE service technician prior to use. All work was conducted within the first six months of instrument procurement. Micropipettors were inspected and calibrated by the vendor annually. In addition, LLNL conducted quarterly pipettor calibration using a gravimetric method. Balances were calibrated annually using National Institute of Standards and Technology (NIST)-traceable standard weights. Records from these calibration activities were documented and reviewed by the project principal investigator.

4.3 Storage Conditions

An alarm system was used for refrigerators and freezers to ensure storage conditions were within acceptable ranges. The temperature on the monitoring device was also noted when reagents were removed or returned to storage locations to ensure the proper range was maintained. NIST-traceable thermometers were placed in storage units as well to provide temperature monitoring.

4.4 Replication

In general, for each treatment in an experiment a minimum of three replicate samples was analyzed. In some cases, where specified, duplicate controls were used. Replicate samples were prepared at the same time using the same ricin holotoxin or A-chain concentration (or same

buffer type and preparation method for negative controls) and processed at the same time following the same laboratory processes. Results are presented as average (Avg) fluorescence counts, with corresponding standard deviation (SD).

4.5 Controls

Negative controls included in the experiments used the same matrix as the test samples with no ricin holotoxin or A-chain added. These controls served as a check on cross-contamination and issues with assay components (such as the capture or detector antibodies). If the negative control showed high fluorescence counts, extra care was taken to prepare new negative control solutions (or TRF assay reagents) followed by repeating the TRF analysis. In addition, only endotoxin-free buffer was used, and buffers were passed through 0.22-micron filters prior to use. Buffers were also prepared fresh for each experiment. Matrix controls, identical to the sample except lacking detector antibody, were included for each sample. Values typically ranged from ~400–600 counts, showing an absence of europium carryover or cross contamination. The plate washer was disinfected as directed by the instrument vendor after each use to prevent salt build-up and potential cross-contamination issues.

4.6 Qualification of New Antibody Lots

Prior to using either capture or detector antibody in the TRF immunoassay, the concentrated antibody stocks were diluted in assay buffer and dilution factors for working concentrations were determined by performing the assay with set amounts of ricin holotoxin or A-chain and different dilutions of antibodies. Initially, the detector antibody concentration was held constant, and the capture antibody concentration was varied. For example, the detector antibody can be used at 200-fold dilution (denoted as 200X), while the capture antibody can be tested at both 200-fold dilution (denoted as 200X) and 400-fold dilution (denoted as 400X). Once the capture antibody working concentration was determined, this level was used to qualify a new lot of labeled detector antibody. The selection of the concentration of antibody required analysis of both the negative controls and the samples containing analyte (ricin). As fluorescence counts were enhanced with higher antibody concentration, this selection requirement ensured that the fluorescence counts for negative controls remained in the proper range (i.e., < 2,000) to prevent false positive and false negative results for the assay.

In this section, qualification data from new, labeled detector antibody lots are presented. For the majority of the experiments, a single lot of detector antibody (referred to as Lot #1) was used; however, because all experiments could not be completed with this lot, a new lot was procured and tested (referred to as Lot #2). The results from testing with negative controls and 1 ng ricin A-chain (Figure 4) showed high levels of background fluorescence counts (~12,000–13,000) when Lot #2 was used as 200X diluted (the working concentration used for Lot #1; 100 ng per well). In order to achieve < 2,000 fluorescence counts for the negative control with this antibody lot, the antibody needed to be used as 1,000X diluted; however, when diluted to this extent, the

fluorescence counts for 1 ng ricin A-chain were reduced to about 50%. Likewise, fluorescence counts for 10 ng ricin A-chain were reduced from about 150,000 to about 65,000 counts (~57% reduction) when this lot was used as 200X diluted and 1,000X diluted, respectively (Figure 5). Based on this poor performance, Lot #2 did not meet the requirements for use in the TRF immunoassay.

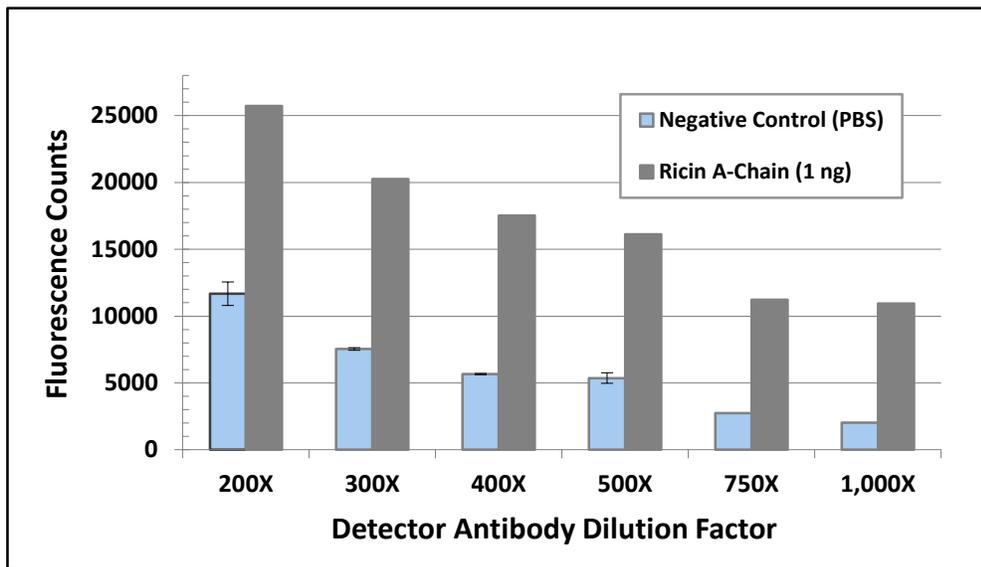


Figure 4 TRF assay results with 1 ng ricin A-chain and PBS (negative control) using different concentrations of detector antibody (Lot #2). The fluorescence counts data were from two separate experiments with the detector antibody used as 200X to 500X dilutions for the first experiment, and used as 750X and 1,000X dilutions for the second experiment. For the negative control, the bar graphs and error bars represent the average and \pm one standard deviation of three replicates, respectively. The bar graphs for ricin A-chain represent single replicates.

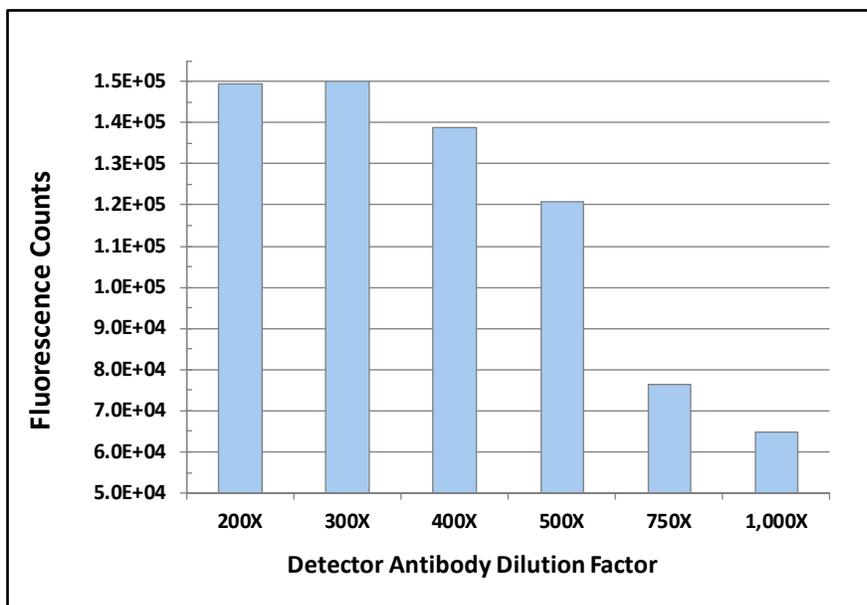


Figure 5 TRF assay results with 10 ng ricin A-chain using different concentrations of detector antibody (Lot #2). The fluorescence counts data were from two separate experiments with the detector antibody used as 200X to 500X dilutions for the first experiment, and used as 750X and 1,000X dilutions for the second experiment. Bar graphs represent single replicates.

The production process between labeled lots was the same, and similar ratios of Eu:protein were measured, with 3.5:1 and 3.9:1 for Lots #1 and #2, respectively. Additional trouble-shooting was performed to ensure that no other parameters had been varied causing the difference in background fluorescence. A new labeled antibody lot was prepared, referred to as Lot #3. In this case, the Eu:protein ratio reported was 1.6:1. A lower ratio was targeted due to the high background fluorescence count issues with the higher Eu:protein ratio. Similar testing was conducted as that described above for Lot #2, although fewer concentrations of detector antibody were tested (as 100X, 200X, 400X, and 1,000X diluted). Ricin holotoxin (100 pg) was used in addition to A-chain in order gain efficiency by directly verifying performance with holotoxin. The results for the negative control and 100 pg ricin are shown in Figure 6.

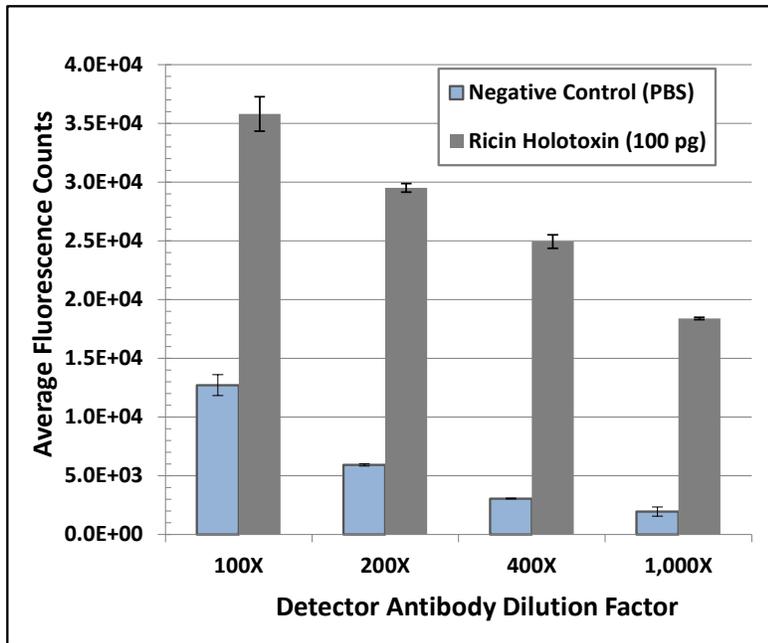


Figure 6 TRF assay results with 100 pg ricin holotoxin and PBS (negative control) using different concentrations of detector antibody (Lot #3). Bar graphs and error bars represent the averages and \pm one standard deviation, respectively.

Similar to Lot #2, Lot #3 also showed elevated background fluorescence such that it needed to be used at a 1000X dilution (~ 37 ng per well) in order for fluorescence counts to be $< 2,000$ for the negative control (Figure 6). However, in this case there was less of a decrease ($\sim 27\%$) in counts for the ricin A-chain positive control from use of the antibody as 200X to 1,000X diluted (Figure 7). There was a $\sim 37\%$ decrease in counts for 100 pg ricin when the detector antibody was more dilute (1,000X diluted compared to 200X diluted). Although the quality was not as good as Lot #1, it still met the minimum requirements for the purposes of this study and was used at 1000X dilution for the remaining experiments (Task 4). This task was focused on relative differences in TRF assay performance and not on optimizing the TRF assay detection limit.

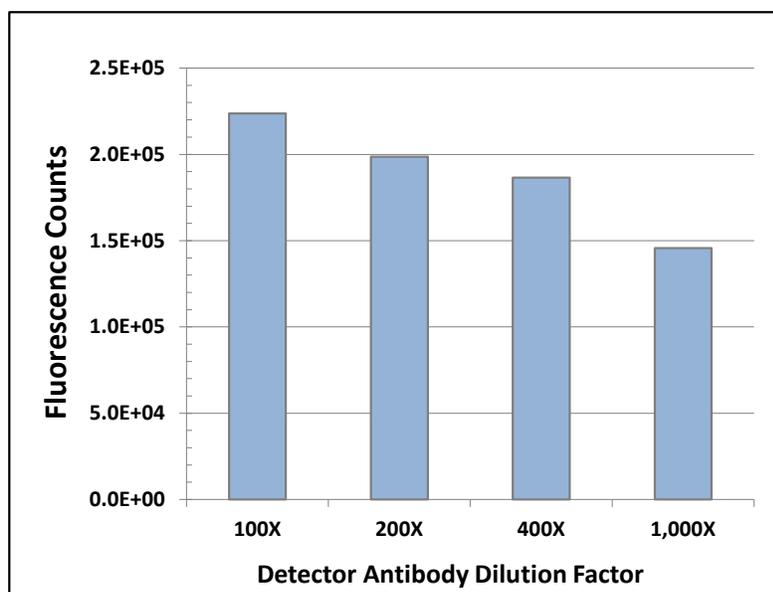


Figure 7 TRF assay results for 10 ng ricin A-chain (per well) using different concentrations of detector antibody (Lot #3). Bar graphs represent single replicates.

In addition, a new lot of biotin-labeled capture antibody was qualified for use in the remaining experiments. This lot was tested with Lot #1 of detector antibody, and tested as 100X, 200X, and 400X dilutions. Results showed optimal assay performance including low backgrounds for the negative control (average 700 ± 50 fluorescence counts) and sufficient counts with ricin holotoxin (average $1.4 \pm 0.03 \times 10^4$ fluorescence counts) when used at 200X (~200 ng per well), so this working concentration was selected for subsequent experiments (Task 4). These average fluorescence counts were similar to those for the original lot used at 400X dilution (~200 ng per well). These results were expected since only 0.25 mg protein was labeled for this lot of capture antibody compared to 0.5 mg labeled for the first lot.

4.7 Data Quality Objectives/Data Quality Indicators

The balance was calibrated annually and the pipettors were calibrated quarterly; the equipment was found to be within $< 0.01\%$ of the expected values. Calibration of the plate reader, plate washer, and plate shaker were not required and therefore were not performed. According to the vendor (PerkinElmer), the reader self-calibrates and does not need to be calibrated by the user. In addition, PerkinElmer stated that for the plate washer there were no maintenance requirements for data quality but only for preventative measures (to prevent carryover and cross-contamination), which were adhered to during the study. These included decontamination of the plate washer after each use by flushing with a solution of 50 mM potassium hydrogen phthalate and 0.01% EDTA. The washer also automatically conducted washing while in standby mode after given intervals to prevent drying of sample materials during inactivity. Likewise, the plate

shaker was configured with appropriate speed settings by the manufacturer (PerkinElmer) and did not require calibration. In addition, all equipment was used within six months of receipt. Equipment initially planned for use in reagent preparation and TRF analysis (e.g., autoclave for reagent preparation and incubators with calibrated thermometers for temperature-controlled incubation) was not required. Reagents and materials were stored under conditions specified by the vendor(s) and used within listed expiration dates. Refrigerators and freezers were on emergency (backup) power and had high and low temperature alarms; the temperature limits were not exceeded during the study. Finally, replicate experiments showed consistent trends; any deviations as well as potential explanations for any discrepancies are included in the relevant sections of the report.

A key data quality objective (DQO) of this effort was to produce data with positive, negative, and matrix control results in the range of expected values for the TRF assay, namely $\sim 1 \times 10^5$ fluorescence counts for the undiluted positive control, and $< 2,000$ fluorescence counts for negative and matrix controls (Schieltz et al., 2011; PerkinElmer, personal communication). In this study, samples either consisted of ricin holotoxin or A-chain dilutions for which the concentration was known, or they were filtrates from UF treatment for which the expected concentrations were known. The samples were analyzed in triplicate without dilution in the plate (except where this dilution procedure was being tested), and triplicate negative controls (without dilution) were included per plate. The fluorescence counts data were reported as averages with the standard deviation for both samples and negative controls.

For ricin A-chain positive controls, one replicate set was included per plate, which consisted of 10 μL of the initial 1- $\mu\text{g}/\text{mL}$ ricin A-chain solution (10 ng ricin A-chain) and two serial 10-fold dilutions prepared in the plate (in assay buffer). The ricin A-chain fluorescence counts for the three dilutions were used to compare assay performance across different experiments and used qualitatively to ensure that positive values were obtained (i.e., the assay was conducted correctly). There was no requirement for all three dilutions to exceed a minimum number of counts to be called “positive” for the purposes of this study. Likewise, for the negative control, results were qualitatively assessed and used as a general indicator of data quality. A negative cut-off value for fluorescence counts was determined by multiplying the average fluorescence counts value for the negative control by 1.5 for data comparison purposes (between experiments); however, this value was not used as a criterion for determining positive or negative results for the samples but simply to provide a point of reference.

The data typically met the DQOs when appropriate antibody sources and quality metrics were obtained. It was determined that only affinity-purified anti-ricin polyclonal antibody could be used as capture antibody for the TRF immunoassay since a non-affinity-purified version did not produce data in the expected range. This is discussed in more detail in Section 5.1. Furthermore, the quality of europium-labeled monoclonal antibody used as detector antibody clearly impacted the TRF assay performance as discussed in Section 4.6. TRF analysis with titrated amounts of

both antibodies—where a new lot of capture antibody is tested at different concentrations with already qualified detector antibody at a prescribed concentration, and vice versa—led to selection of optimal concentrations for the experiments. These variations in antibody preparations (based on specificity toward the analyte, labeling efficiency, etc.) contributed to variability in TRF assay performance. However, in this effort, variations in antibody quality from lot to lot did not negatively impact the ability to determine relative differences between conditions within an experiment. The data reported were generated from two different lots of labeled capture antibody that originated from the same lot of affinity-purified polyclonal anti-ricin antibody. Likewise, the data were from two different lots of labeled detector antibody that originated from the same lot of monoclonal antibody. Overall, the data met the requirement for less than 20% coefficient of variation (CV or the ratio of the standard deviation to the mean), with typically < 20% CV observed between replicates.

5 Results and Discussion

5.1 Task 1 Establish the TRF assay dynamic range and the appropriate ricin concentration for use in the study

5.1.1 Preliminary Experiments to Establish the TRF Assay Dynamic Range

The initial experiments were designed to establish the TRF assay dynamic range for detection using ricin dilutions from 1 ng to 100 ng per mL. Since 10 μ L were added per well, the ricin mass per TRF assay replicate was 10 pg to 1 ng. Originally, non-affinity purified anti-ricin polyclonal antibody was used as capture antibody because this was the only version available (through BEI Resources, Inc.) at the project start. However, a consistent titration was not observed with ricin concentration, and fluorescence counts for negative controls were similar to samples containing ricin (up to 1 ng), showing < 2,000 counts for all. However, when the detector antibody was analyzed in the presence of enhancement solution as a check on the europium labeling, values of $\sim 10^6$ counts were measured. In addition, europium standards showed expected values with the instrument. After data review and discussion, it was determined that the issue was likely with the capture antibody quality and that an affinity-purified version of the capture antibody should be used. Without affinity-purification, antibody specific to the analyte may be < 5% of the total antibody (Life Technologies, personal communication). The Critical Reagents Program (CRP) supplied affinity-purified anti-ricin polyclonal antibody (produced in goat) that was used for all subsequent experiments.

The TRF assay was then evaluated using 10 pg to 10 ng ricin A-Chain per well, along with PBS as a negative control. TRF assay results showed titration with ricin concentration using the affinity-purified capture antibody (Table 1), confirming the issue with low fluorescence counts was due to use of non-affinity purified antibody. Two different levels of capture antibody (400X

and 200X dilutions) were used to determine which level provided better signal-to-noise discrimination (i.e., high fluorescence counts for ricin samples and low counts for negative controls). Fluorescence counts of approximately 1.0×10^5 were obtained for both capture antibody levels for 10 ng ricin A-chain. Although the counts were slightly reduced with 400X dilution of capture antibody at 10 ng ricin A-chain ($p = 0.01$), the counts for 1 ng ricin A-chain were not significantly different for different antibody dilutions ($p = 0.14$) using a 95% confidence level. Ricin could not be detected down to 100 pg per well since the background counts were high and the 100 pg concentration was not significantly different from the negative control.

Table 1 TRF Assay Results for Dilutions of Ricin With Different Concentrations of Capture Antibody

Ricin A-Chain Level	Fluorescence Counts*			
	Capture Ab Diluted 400X		Capture Ab Diluted 200X	
	Average	SD	Average	SD
10 ng	1.028×10^5	4.981×10^3	1.290×10^5	9.034×10^3
1 ng	1.269×10^4	4.888×10^2	1.545×10^4	1.253×10^3
100 pg	3.845×10^3	1.785×10^2	4.107×10^3	1.045×10^2
10 pg	3.525×10^3	8.886×10^2	3.000×10^3	2.332×10^2
PBS	ND	ND	2.969×10^3	2.177×10^2
PBS 1:10 dilution	ND	ND	2.850×10^3	1.684×10^2

* Average and standard deviation (SD) from triplicate analyses. ND = not determined; Ab = antibody; PBS = phosphate-buffered saline.

Additional effort was then focused on reducing the background fluorescence counts for the negative control by using endotoxin-free PBS buffer with filtration through 0.22-micron filters, as well as filtration of the diluted detector antibody solution. Use of a 0.22-micron filter resulted in lower counts compared to a 0.45-micron filter.

A subsequent experiment tested both ricin A-chain and negative controls (PBS) with capture antibody dilutions used as 200X (~400 ng/well) and 400X (~200 ng/well). For both antibody levels, endotoxin-free PBS was used after 0.22-micron filtration in place of unfiltered, standard PBS buffer. For PBS controls, the fluorescence counts averaged $1,700 \pm 100$ and $2,700 \pm 500$ with capture antibody dilutions used as 400X and 200X, respectively. The counts for the positive control also decreased when using more dilute capture antibody (400X dilution), about $32 \pm 9\%$ on average (approximately 127,000 counts for 10 ng A-chain); however, this signal was still sufficient and provided data that met the criterion that negative controls should be $< 2,000$ counts. While 400X antibody dilution led to the largest improvement in assay performance,

filtering the PBS and the capture antibody solution (using 0.22-micron filters) also contributed to more consistent and lower fluorescence counts for the negative control.

5.1.2 Evaluation of the Ricin Holotoxin Dilution Method and TRF Assay Dynamic Range

An experiment was conducted to determine the optimal buffer for preparing ricin dilutions to maintain stability. Ricin A-chain was diluted in assay buffer in the plate to make ~10-fold and ~100-fold dilutions as a quality control check on assay performance. Ricin dilutions were also prepared in this manner (referred to as dilution in plates) and compared with ricin dilutions prepared in PBS (referred to as dilution in tubes) and added to the well for analysis. The Sigma ricin lot was used for this experiment. The results are shown in Figure 8 for triplicate TRF analyses for each treatment and concentration. Data points represent the average count per treatment with error bars for the standard deviation. The positive (ricin A-chain) and negative (endotoxin-free water) controls were also analyzed in triplicate.

The data showed that ricin dilutions made in assay buffer within the analysis plate produced significantly higher fluorescence counts compared to dilution in tubes (in PBS). The highest concentrations between treatments showed similar counts since these were essentially the same treatment. Dilution in the plate showed about 2.6-fold higher counts for the first dilution (~1 ng ricin) and about 6-fold higher counts for the second dilution (~100 pg ricin) compared with dilution in tubes. The counts for 10 ng ricin A-chain were about 3.8×10^4 which were about 4-fold lower than later experiments where A-chain dilutions were performed directly into assay buffer instead of in PBS first. In this case, the A-chain dilution had been prepared in advance and stored at 4°C. Based on the data, ricin A-chain and holotoxin stability in PBS was of concern. After discussion with technical experts from NC Department of Health and Human Services (D. Pettit and C. Browne, personal communication), in subsequent experiments A-chain dilutions were freshly prepared in assay buffer. Furthermore, the background fluorescence for the experiment was relatively high compared to later experiments. As mentioned above, the background counts were later reduced by filtration of the detector antibody solution through a 0.22-micron filter as opposed to a 0.45-micron filter used in this case.

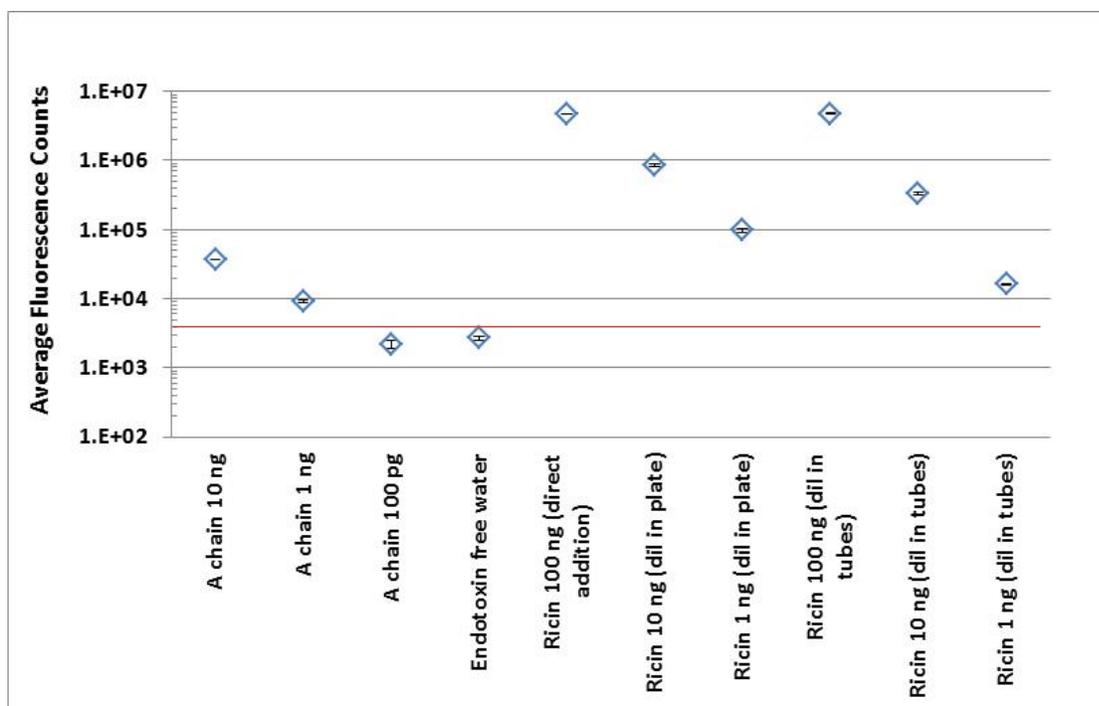


Figure 8 Evaluation of toxin dilution methods comparing dilutions made in the plate with assay buffer to dilutions made outside the plate (in tubes) with PBS. Data points represent the average fluorescence counts from three replicate TRF analyses. Error bars represent \pm one standard deviation. A red line shows the negative (endotoxin-free water) cut-off value (\sim 4,070 counts). Ricin A-chain controls showed lower counts possibly due to degradation since the 1- μ g/mL stock was not freshly prepared in assay buffer as for later experiments.

According to the vendor, preparations made in assay buffer must be analyzed within 1 hr; therefore, assay buffer *per se* could not be used for dilution to prepare solutions with different ricin concentrations for testing purposes. 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, in this effort, different buffers were tested to determine the effect on TRF assay performance, including PBS with 0.05% Tween-80 (PBST), PBS with 3% BSA, and PBST with 3% BSA. These buffers were compared with PBS by making 10-fold dilutions in the appropriate buffer from the original stock solution (in PBS) at 100 μ g/mL. Dilutions were performed to generate 1 μ g/mL to 10 ng/mL concentrations in 1-mL dilutions (i.e., 100 μ L ricin solution added to 900 μ L buffer), and because 10 μ L were added per well, the final concentrations were approximately 10 ng to 100 pg ricin. Results for the first replicate experiment are shown in Table 2.

Table 2 Evaluation of Dilution Buffer Effects on Detection of Ricin Holotoxin Using the TRF Assay

Buffer Type	TRF Replicate	Fluorescence Counts by Holotoxin Level*		
		10 ng	1 ng	100 pg
PBS	1	1.190×10^6	1.370×10^5	1.587×10^4
	2	1.134×10^6	1.382×10^5	1.472×10^4
	3	1.039×10^6	1.372×10^5	1.445×10^4
	Average (SD)	$1.121 (0.0760) \times 10^6$	$1.375 (0.00647) \times 10^5$	$1.502 (0.0754) \times 10^4$
PBST	1	1.497×10^6	2.089×10^5	2.544×10^4
	2	1.513×10^6	2.100×10^5	2.544×10^4
	3	1.455×10^6	2.208×10^5	2.511×10^4
	Average (SD)	$1.489 (0.0288) \times 10^6$	$2.132 (0.0661) \times 10^5$	$2.533 (0.0187) \times 10^4$
PBS/BSA	1	1.614×10^6	2.735×10^5	3.170×10^4
	2	1.530×10^6	2.605×10^5	3.331×10^4
	3	1.548×10^6	2.668×10^5	3.214×10^4
	Average (SD)	$1.563 (0.0442) \times 10^6$	$2.669 (0.0651) \times 10^5$	$3.238 (0.0831) \times 10^4$
PBST/BSA	1	1.365×10^6	2.038×10^5	2.308×10^4
	2	1.347×10^6	1.991×10^5	2.666×10^4
	3	1.447×10^6	2.033×10^5	2.560×10^4
	Average (SD)	$1.386 (0.0531) \times 10^6$	$2.021 (0.0257) \times 10^5$	$2.511 (0.184) \times 10^4$
Negative Control Average (SD)	$1.5 (0.1) \times 10^3$			

* Average and standard deviation (SD) from triplicate analyses. The negative cut-off value was ~2,270 counts.

The data showed the highest fluorescence counts using PBS with BSA, followed by PBST/BSA and PBST with similar counts, and PBS. The lower ricin concentrations showed greater improvements, with up to 2.1-fold higher counts for PBS/BSA compared to PBS for 100 pg ricin.

A replicate experiment was conducted using the same conditions as those described for the first replicate experiment; however, in this case, the ricin dilutions ranged from 100 ng/mL to 1 ng/mL, equivalent to 1 ng to 10 pg per well. This change was made in order to investigate the dynamic range of the assay further. The other concentrations were replicated in a subsequent experiment. The results showed the same trends with up to 2.2-fold improvement with PBS/BSA relative to PBS for 100 pg ricin (Table 3). For 10 pg ricin, fluorescence counts ranged from 2,800 for PBS to about 5,640 for PBS/BSA (~2-fold higher counts). Similar fold-increases

were noted for the other ricin concentrations (dilutions). Based on these findings, use of PBS with 3% BSA is recommended to increase the stability or solubility of ricin and, subsequently, TRF assay performance. It is understood that dilutions of ricin solutions are only prepared for testing purposes and that actual environmental samples would either be analyzed only undiluted or could also be diluted in assay buffer in the plate.

Table 3 Evaluation of Dilution Buffer Effects on Detection of Ricin Holotoxin Using the TRF Assay

Buffer Type	TRF Replicate	Fluorescence Counts by Holotoxin Level*		
		1 ng	100 pg	10 pg
PBS	1	1.317×10^5	1.340×10^4	2.799×10^3
	2	1.187×10^5	1.308×10^4	3.033×10^3
	3	1.129×10^5	1.261×10^4	2.568×10^3
	Average (SD)	$1.211 (0.0961) \times 10^5$	$1.303 (0.0395) \times 10^4$	$2.800 (0.233) \times 10^3$
PBST	1	1.795×10^5	2.024×10^4	4.012×10^3
	2	1.777×10^5	2.114×10^4	4.095×10^3
	3	1.743×10^5	1.987×10^4	4.554×10^3
	Average (SD)	$1.772 (0.0262) \times 10^5$	$2.042 (0.0653) \times 10^4$	$4.220 (0.292) \times 10^3$
PBS/BSA	1	2.446×10^5	2.836×10^4	5.679×10^3
	2	2.446×10^5	2.932×10^4	6.001×10^3
	3	2.311×10^5	2.827×10^4	5.263×10^3
	Average (SD)	$2.401 (0.0781) \times 10^5$	$2.865 (0.0584) \times 10^4$	$5.648 (0.370) \times 10^3$
PBST/BSA	1	1.862×10^5	1.965×10^4	4.261×10^3
	2	1.777×10^5	2.003×10^4	4.511×10^3
	3	1.701×10^5	2.085×10^4	4.422×10^3
	Average (SD)	$1.780 (0.0806) \times 10^5$	$2.018 (0.0616) \times 10^4$	$4.398 (0.127) \times 10^3$
Negative Control Average (SD)	$1.60 (0.202) \times 10^3$			

* Average and standard deviation (SD) from triplicate analyses. The negative cut-off value was 2,300 counts.

An additional replicate experiment was conducted to obtain data for the entire range of ricin concentrations tested in the initial replicate experiments (Table 2 and Table 3) and provide data on reproducibility. In this case, only the PBS/BSA treatment was tested with 10 ng, 100 pg, and 10 pg final concentration per well, because this provided the PBS/BSA buffer provided the best assay performance in the previous replicate experiments. The results are shown in Table 4.

Table 4 Analysis of Ricin from Dilutions Prepared in PBS/BSA Buffer – Replicate Experiment

Buffer Type	TRF Replicate	Fluorescence Counts by Holotoxin Level*		
		10 ng	100 pg	10 pg
PBS/BSA	1	ND	1.379×10^4	ND
	2		1.423×10^4	
	3		1.390×10^4	
	Average (SD)		$1.397 (0.0228) \times 10^4$	
Negative Control Average (SD)	$7.00 (0.47) \times 10^2$			
Buffer Type	TRF Replicate	Fluorescence Counts by Holotoxin Level**		
		10 ng	100 pg	10 pg
PBS/BSA	1	8.750×10^5	1.403×10^4	2.261×10^3
	2	8.746×10^5	1.332×10^4	2.144×10^3
	3	8.814×10^5	1.328×10^4	2.290×10^3
	Average (SD)	$8.770 (0.0380) \times 10^5$	$1.354 (0.0421) \times 10^4$	$2.232 (0.077) \times 10^3$
Negative Control Average (SD)	$9.16 (0.67) \times 10^2$			

* Average and standard deviation (SD) from triplicate analyses. A new lot of biotin-labeled capture antibody was used at 200X dilution (same antibody lot from vendor).

** The original lot of biotin-labeled antibody was used at 400X (same antibody lot from vendor).

ND = not determined. The negative cut-off value was 1,340 counts.

In this case, the fluorescence counts were a bit lower likely representing the normal variability due to different lots of labeled capture antibody, pipetting variation, and assay set-up differences. However, the one ricin level, 100 pg, where both the original and the new lot of labeled capture antibody were tested (with all other components the same), showed comparable results, $\sim 1.4 \times 10^4$ counts. The replicate analyses showed about a 44% decrease for 10 ng and a 60% decrease for 10-pg compared with the other replicate experiments using these ricin concentrations, Table 2 and Table 3, respectively.

5.2 Task 2 Investigation and Characterization of TRF Assay Interferences

5.2.1 *Experimental Approach for Testing TRF Assay Interferences from Environmental Sample Matrices – Bleach Residue, Sampling Materials, Wetting Buffer*

While Task 3 was focused on development of a sample processing protocol for swabs and sponges, the goal of this task was to reproduce the elevated fluorescence values observed in previous ricin sampling and TRF analysis efforts, and to evaluate mechanistically what interferents could contribute to false positive results (from high background fluorescence). During the 2013 Tupelo MS ricin incident, the TRF assay was shown to produce unsatisfactory results due to high background fluorescence (presumed associated with bleach residue or other substances extracted from the surface) such that the assay could not be used for reliable analysis (U.S. EPA, 2013a; U.S. EPA, 2013b). Furthermore, high backgrounds (~ 8,600 to 10,400 fluorescence counts) were reported from test samples (lacking ricin). These test samples were prepared by EPA scientists who applied bleach, allowed the surfaces to dry overnight, and used sponge-sticks to sample surfaces following the CDC-NIOSH protocol (CDC-NIOSH, 2012). When these samples were analyzed by the MS DOH, the reported values exceeded typical background fluorescence values of < 2,000 counts. While these samples lacked ricin, they contained bleach residue from following established ricin decontamination and sampling methods for swabs and sponges. Elevated fluorescence values (in the same range) were also observed for surfaces that were rinsed with water prior to sampling.

Following the methods described in Sections 3.4 through 3.6, pre-wet macrofoam swabs and Solar-Cult sponges were used to wipe stainless steel surfaces that were treated with 10% bleach with a hand sprayer and kept wet for 10 min, followed by overnight drying, to mimic ricin decontamination conditions used for the Tupelo MS incident. An example 10 × 10 inch surface sprayed with 10% bleach is shown in Figure 9, and a similar surface allowed to dry overnight is shown in Figure 10. The 4 × 4 inch areas sprayed with 10% bleach and allowed to dry are shown in Figure 11, and these are compared with other 4 × 4 inch areas treated with water and allowed to dry (Figure 12). Surfaces contained dried bleach solutions showed a large amount of salt crystals that were largely removed upon sampling efforts (image not shown).



Figure 9 Photo of a 10 × 10 inch stainless steel coupon with 10% chlorine bleach applied for 10 min, with re-spraying every 2 min, to mimic conditions for ricin decontamination.

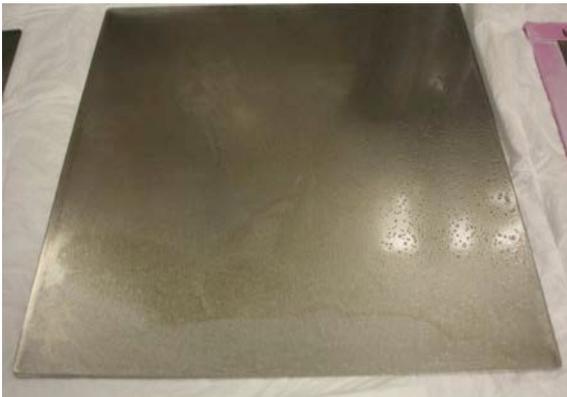


Figure 10 Photo of a 10 × 10 inch stainless steel coupon after application of 10% chlorine bleach and drying overnight.

Surfaces were sampled following the procedures used by CDC and EPA (outlined in Section 3.5 for swabs and 3.6 for sponges) for post-decontamination sampling (CDC-NIOSH, 2012). One swab was used to sample three 4 × 4 inch areas, and one sponge was used to sample one 10 × 10 inch area. Samples were then processed as described in Section 3.5 for swabs and 3.6 for sponges. Typically, 0.5-mL to 1 mL sample extract volumes were obtained from swabs and sponges for analysis by TRF. The sample extracts with bleach residue were yellow in color and had a pH of about 10 (as measured by pH paper). Based on these features of the extract, it was assumed that extracts contained high levels of bleach residual, and in fact ricin spiked into these extracts was degraded and not detected by TRF analysis (data not shown). In this experiment, replicate samples were prepared with and without bleach residual for processing using the protocol for 0.5-mL 10K UF devices (Appendix B), with pre-filtration through a 0.22- μ m

Ultrafree® MC GV filter unit. Swab and sponge extract volumes of 400 μL were applied to UF devices and after four wash steps, 100 μL were recovered. This served to streamline the effort by including treatments with and without UF processing in one experiment rather than sequential experiments.

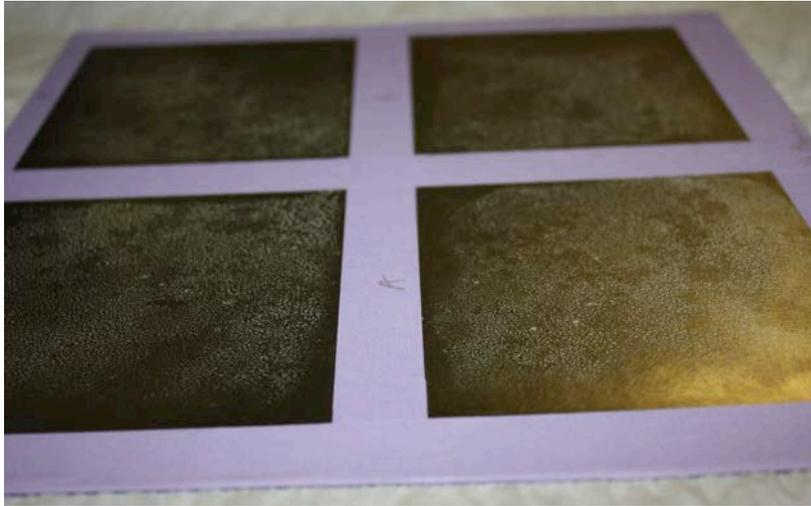


Figure 11 Photo of 4×4 inch sections on a stainless steel coupon for which 10% chlorine bleach was applied by hand sprayer for 10 min and allowed to dry overnight.

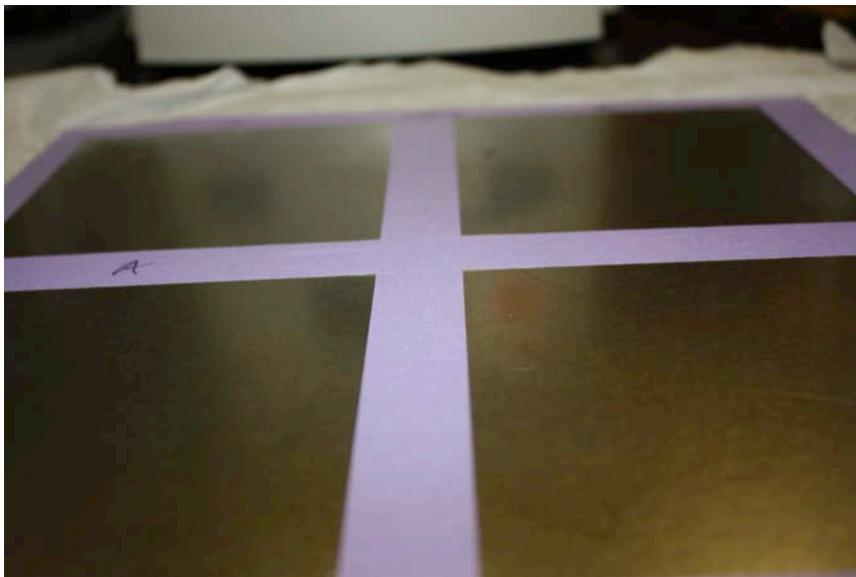


Figure 12 Photo of 4×4 inch sections on a stainless steel coupon for which water was applied by hand sprayer for 10 min (as a control) and allowed to dry overnight.

5.2.2 Evaluation of TRF Assay Interferences from Environmental Sample Matrices – Swabs and Sponge-Stick Samples

Results from TRF analysis of swab samples from bleach-applied surfaces are shown in Figure 13 and Table 5. Similarly, results from TRF analysis of sponge samples are shown in Figure 14 and Table 6. These experiments were also repeated, although the replicate experiment did not include any additional replicates processed by UF prior to TRF analysis (Table 7). In all cases, samples with bleach residue either with or without UF treatment showed fluorescence counts similar to the samples from water-treated surfaces and from negative controls, < 2,000 counts. Although the sample extracts had high levels of bleach as evidenced by a yellow color and measured pH values of ~10, once the extracts were added to assay buffer (10 µL to 100 µL assay buffer and detector antibody), the pH was maintained at 8 (measured by pH paper on replicate samples). In some cases, particulates from the sponges could also be observed in the samples. Although only smooth surfaces were sampled, the vortex-mixing step may have led to disintegration of the sponge. However, these particulates did not appear to negatively impact the TRF assay.

These data were from multiple attempts to reproduce the post-decontamination sample generation and analysis that led to the reported elevated fluorescence backgrounds. As mentioned, these efforts produced worst-case bleach residuals and used the same sampling materials, reagents, and protocols that led to the problem reported by the MS DOH Laboratory; however, no elevated backgrounds were observed in any of the testing performed for this effort, for either swabs or sponges. Data from positive and negative controls was not included along with the sample data showing elevated fluorescence values from the MS DOH Laboratory; therefore, it was difficult to trouble-shoot what other factor may have led to elevated fluorescence.

However, the data obtained from QC-testing new europium-labeled detector antibody lots in this study pointed to other possible causes for the elevated fluorescence levels, either improper dilution or improper quality control testing of antibody stock solutions. If too much detector antibody was used in the assay, this would also show elevated fluorescence counts in the negative controls, but unfortunately the data from controls for the MS DOH Laboratory was not available. Likewise, if poor quality detector antibody was used (i.e., sub-optimal ratio of Eu:protein, improper antibody purification, etc.) this could also lead to elevated fluorescence values in negative controls. 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.

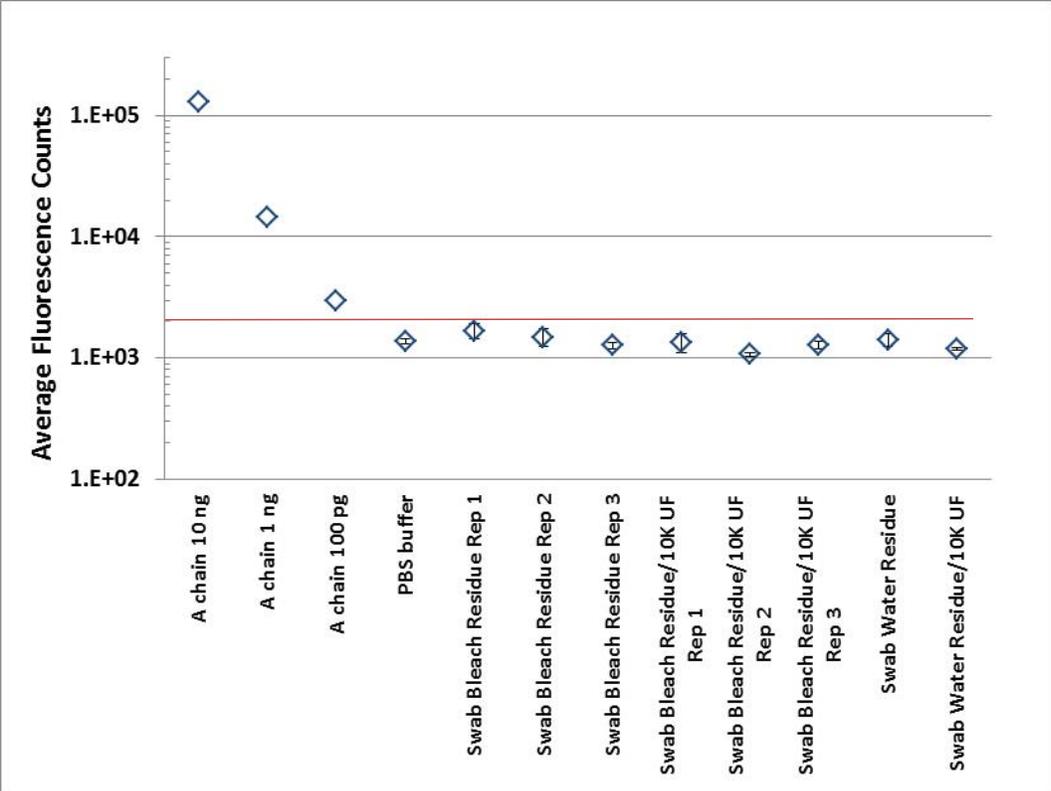


Figure 13 Effect of Surface Sample Matrix (Bleach Residue, Neutralizing Buffer) on TRF Assay Performance for Swabs. Data points represent the average fluorescence counts from three replicate TRF analyses (except for A-chain data points which are single measurements). Error bars represent \pm one standard deviation. Samples were processed by 0.5-mL 10K UF devices (Appendix B). A red line shows the negative (PBS) cut-off value (\sim 2,050 counts).

Table 5 Testing of the Sample Matrix (Sampling Device, Bleach Residue, and Neutralizing Buffer) for Background Fluorescence Interference in the TRF Assay Using Swabs

Swab Treatment	Sample Replicate – TRF Replicate	Fluorescence Counts*	
		No UF	UF
Swabs with Bleach Residue	1 – 1	1497	1389
	1 – 2	1934	1076
	1 – 3	1572	1548
	Average (SD)	1668 (234)	1338 (240)
	2 – 1	1280	1122
	2 – 2	1753	1057
	2 – 3	1425	1033
	Average (SD)	1486 (242)	1071 (46)
	3 – 1	1245	1310
	3 – 2	1205	1165
	3 – 3	1340	1366
Average (SD)	1263 (69)	1280 (104)	
Swab with Water	1 – 1	1537	1163
	1 – 2	1306	1200
	Average (SD)	1422 (163)	1182 (26)
PBS (Control)	1	1442	ND
	2	1320	ND
	3	1342	ND
	Average (SD)	1368 (65)	NA

* Average and standard deviation (SD) from triplicate analyses, except for water controls where duplicate analyses were conducted. UF = Ultrafiltration; ND = not determined; NA = not applicable.

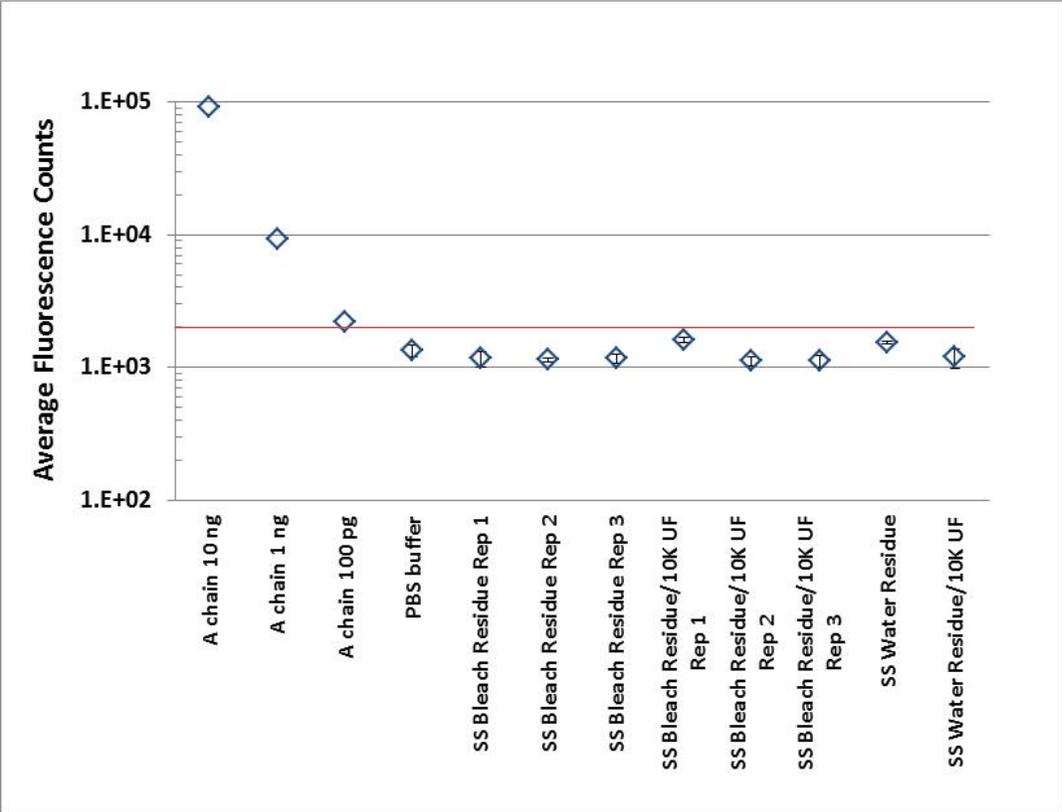


Figure 14 Effect of Surface Sample Matrix (Bleach Residue, Neutralizing Buffer) on TRF Assay Performance for Sponge-Stick Samples (SS). Data points represent the average fluorescence counts from three replicate TRF analyses (except for A-chain data points which are single measurements). Error bars represent \pm one standard deviation. “10K UF” samples were processed by 0.5-mL 10K UF devices (Appendix B). A red line shows the negative (PBS) cut-off value (~2,000 counts).

Table 6 Testing of the Sample Matrix (Sampling Device, Bleach Residue, and Neutralizing Buffer) for Background Fluorescence Interference in the TRF Assay Using Sponge-Sticks

SS Treatment*	Sample Replicate – TRF Replicate	Fluorescence Counts	
		No UF	UF
SS with Bleach Residue	1 – 1	1232	1632
	1 – 2	1282	1522
	1 – 3	990	1680
	Average (SD)	1168 (156)	1611 (81)
	2 – 1	1174	1018
	2 – 2	1104	1207
	2 – 3	1166	1120
	Average (SD)	1148 (38)	1115 (95)
	3 – 1	1143	1079
	3 – 2	1092	1044
	3 – 3	1260	1254
Average (SD)	1165 (86)	1126 (113)	
SS with Water	1 – 1	1571	1332
	1 – 2	1519	1047
	Average (SD)	1545 (37)	1190 (202)
PBS (Control)	1	1451	ND
	2	1356	ND
	3	1187	ND
	Average (SD)	1331 (134)	NA

* Average and standard deviation (SD) from triplicate analyses, except for water controls where duplicate analyses were conducted. SS = Sponge-stick; ND = not determined; NA = not applicable.

Table 7 Testing of the Sample Matrix (Sampling Device, Bleach Residue, and Neutralizing Buffer) for Background Fluorescence Interference in the TRF Assay Using Swabs and Sponge-Sticks

Treatment	Sample Replicate – TRF Replicate	Fluorescence Counts	
		Swab	Sponge-Stick
Bleach Residue	1 – 1	1254	1508
	1 – 2	1568	1764
	1 – 3	1276	1272
	Average (SD)	1366 (175)	1515 (246)
	2 – 1	1477	2337
	2 – 2	1177	1210
	2 – 3	1387	1280
	Average (SD)	1347 (154)	1609 (631)
	3 – 1	1336	1174
	3 – 2	1340	1320
	3 – 3	1213	1215
Average (SD)	1296 (72)	1236 (75)	
Water	1 – 1	1382	1450
	1 – 2	1209	1322
	Average (SD)	1296 (122)	1386 (91)
PBS (Control)	1	1478	
	2	1220	
	3	1250	
	Average (SD)	1316 (141)	

* Average and standard deviation (SD) from triplicate analyses, except for water controls where duplicate analyses were conducted.

5.3 Task 3. Determination of Ricin Recovery/Loss for Samples Processed by UF Devices

5.3.1 Evaluation of 0.5-mL 30K UF Devices for Ricin Recovery

A preliminary experiment was conducted using 0.5-mL 30K UF devices with 1 µg/mL and 100 ng/mL solutions of ricin to assess ricin recovery efficiency. As mentioned, these devices were used with multiple wash steps to remove interferents resulting from bleach decontamination protocols. In order to compare with and without UF directly for ricin recovery, the 30K device was not used to concentrate the samples. Rather, 100 µL of each ricin solution were applied to

the UF devices (in triplicate) and 300 μL PBS were added to bring the total volume to 400 μL . The UF procedure was followed (see Appendix B) except that four washes instead of two were conducted, and 100 μL retentate was recovered. These UF-treated samples were analyzed in triplicate by the TRF assay and compared with the original dilution—1 $\mu\text{g}/\text{mL}$ or 100 ng/mL —for which nine replicates were analyzed.

The results showed high background fluorescence for the negative control PBS (Table 8), with an average 5,500 fluorescence counts making the negative cut-off value at $\sim 8,200$ counts, such that only the 1 $\mu\text{g}/\text{mL}$ solution data could be used to compare ricin recovery efficiencies from 30K-UF. PBS that was filtered through a 0.22-micron filter was also analyzed, although the filtered buffer also showed high counts (avg. 4,200 counts). The detector antibody (diluted to 1X in assay buffer) was passed through a 0.45-micron filter instead of a 0.22-micron filter; it was thought that the larger pore filter could have contributed to the high counts since more detector antibody aggregates could be present. In addition, data for 1 $\mu\text{g}/\text{mL}$ replicates (corresponding to 10 ng per well since 10 μL were analyzed) which were UF-treated showed about 72% loss relative to the untreated solution. It should also be noted that the counts for 10 ng ricin were lower compared to subsequent experiments with this ricin concentration because assay conditions (i.e., antibody concentrations, reagent preparation) were not optimal in this case. Regardless, the poor recovery observed by processing through the 0.5 mL 30K UF device required testing a different device, namely the lower NMWL device, 0.5 mL 10K UF device. Therefore, no additional testing was done using the 0.5 mL 30K UF devices.

Table 8 Evaluation of the 0.5-mL 30K UF Device on Relative Percent Recovery of Ricin

Treatment*	Sample Replicate – TRF Replicate	Fluorescence Counts
10 ng Ricin 30K UF	1 – 1	1.535×10^4
	1 – 2	1.509×10^4
	1 – 3	1.493×10^4
	Average (SD)	$1.512 (0.0212) \times 10^4$
	2 – 1	1.751×10^4
	2 – 2	1.781×10^4
	2 – 3	1.775×10^4
	Average (SD)	$1.769 (0.0156) \times 10^4$
	3 – 1	1.546×10^4
	3 – 2	1.716×10^4
	3 – 3	1.558×10^4
	Average (SD)	$1.607 (0.0946) \times 10^4$
	Overall Average (SD)	$1.630 (0.124) \times 10^4$
Treatment*	TRF Replicate	Fluorescence Counts
10 ng Ricin No UF	1	5.509×10^4
	2	5.688×10^4
	3	5.592×10^4
	4	5.755×10^4
	5	5.633×10^4
	6	5.453×10^4
	7	5.828×10^4
	8	6.282×10^4
	9	5.824×10^4
	Average (SD)	$5.729 (0.245) \times 10^4$
PBS (Control) 30K UF	1	3.624×10^3
	2	4.442×10^3
	3	5.842×10^3
	Average (SD)	$4.636 (1.122) \times 10^3$
PBS (Control) No UF	1	9.491×10^3
	2	4.070×10^3
	3	3.233×10^3
	4	4.247×10^3
	5	5.418×10^3
	6	6.448×10^3
	Average (SD)	$5.485 (2.262) \times 10^3$

* Ricin samples were approximately 10 ng per sample replicate (10 μ L) for UF treatment or per TRF replicate for the untreated ricin solution (1 μ g/mL). Ricin solutions were prepared in unfiltered PBS using the Sigma ricin lot. UF = Ultrafiltration. Results are averages and standard deviations (SD) for three or more replicate analyses (as shown).

5.3.2 Evaluation of 0.5-mL 10K UF Devices for Ricin Recovery

An experiment was conducted using 0.5-mL 10K UF devices to determine ricin recovery. As for the data shown in Table 8, this experiment was not designed to test the ability to concentrate ricin but rather to determine losses by comparing solutions that should have the same ricin concentration if there were no losses to the UF device. Ricin was tested at 1 µg/mL as 100 µL added to the UF device and 300 µL PBS added to bring the volume to 400 µL. Triplicate samples were processed through 0.5-mL 10K UF devices (with four wash steps as for Section 5.3.1) with 100 µL retentate, and then analyzed by the TRF assay to allow comparison with the original ricin solution that was untreated.

In this case, the data did not show loss of ricin, but rather showed 100%+ recovery relative to the untreated solution (Table 9). The average fold-difference between UF-treated and untreated samples ranged from 1.1 to 1.5 with an average of 1.3 ± 0.2 . Using unpaired T-tests (2-tailed) on individual UF-treated replicates (sample and TRF replicates) compared with the untreated control, the UF-treated replicates had statistically higher counts than the control at a 95% confidence level but not at a 99% confidence level (p-values were 0.012). The slightly higher fluorescence values for UF-treated samples would not affect the results interpretation for the assay. The 10K UF device did not show loss of ricin, even with four wash steps, such that this device showed promise for processing complex environmental samples prior to TRF analysis. However, it was also important to evaluate performance of the UF devices for lower ricin concentrations as well (as was done in subsequent experiments), especially because, for lower ricin levels, the UF devices could enable detection where there would otherwise be no positive detection without ricin concentration and purification. In addition, impact of the UF devices on ricin recovery could be masked by high levels of ricin.

Table 9 Evaluation of the 0.5-mL 10K UF Device for Ricin Recovery

Treatment*	Sample Replicate – TRF Replicate	Fluorescence Counts	Avg. Fold-Difference From No UF Treatment**
10 ng Ricin 10K UF	1 – 1	6.370×10^5	1.3
	1 – 2	6.296×10^5	
	1 – 3	6.232×10^5	
	Average (SD)	$6.299 (0.0692) \times 10^5$	
	2 – 1	5.360×10^5	1.1
	2 – 2	5.306×10^5	
	2 – 3	5.346×10^5	
	Average (SD)	$5.337 (0.0283) \times 10^5$	
	3 – 1	7.163×10^5	1.5
	3 – 2	7.061×10^5	
	3 – 3	7.167×10^5	
	Average (SD)	$7.13 (0.0600) \times 10^5$	
	Overall Avg (SD)	$6.256 (0.779) \times 10^{5***}$	1.3 (0.2)
Treatment*	TRF Replicate	Fluorescence Counts	
10 ng Ricin No UF	1	4.844×10^5	NA
	2	4.910×10^5	
	3	4.755×10^5	
	Average (SD)	$4.836 (0.0774) \times 10^{5***}$	
PBS (Control) 10K UF	1	1.607×10^3	NA
	2	1.516×10^3	
	3	1.150×10^3	
	Average (SD)	$1.424 (0.242) \times 10^3$	
PBS (Control) No UF	1	1.468×10^3	NA
	2	1.276×10^3	
	3	1.464×10^3	
	Average (SD)	$1.403 (0.110) \times 10^3$	

* Ricin samples were approximately 10 ng per sample replicate (10 μ L) for UF treatment or per TRF replicate for the untreated ricin solution (1 μ g/mL). Ricin solutions were prepared in PBS using the Sigma ricin lot. 10K UF = ultrafiltration with 10K device. Average (Avg) and standard deviation (SD) are from triplicate TRF analyses. NA = not applicable.

** Denotes no statistically significant difference compared to the “No UF” control at the 95% confidence level ($p = 0.012$).

5.3.3 Evaluation of 0.5-mL 10K UF Devices for Ricin Recovery – Comparison of Lots of Ricin Holotoxin

An experiment was conducted using the 0.5-mL 10K UF devices to confirm that no loss of ricin occurred and furthermore, to evaluate a new Vector Labs ricin lot in comparison with the Sigma ricin lot to investigate whether similar trends were observed. A new lot was procured from Vector Labs, Inc. (Unconjugated *Ricinus communis* Agglutinin II [RCA60, ricin]; Section 3.2) since this form of ricin holotoxin was no longer available from Sigma, who originally supplied the material. The new lot was obtained to confirm there were no differences in assay performance compared with the Sigma lot (in case there was some degradation over time). As for the previous experiment, the 0.5-mL 10K UF devices were not used to concentrate the ricin but rather to mimic purification through the wash steps and subsequent recovery of retentate in the protocol; namely, 100 μ L aliquots were loaded onto UF devices, and the final retentate volume was also 100 μ L. The experiment used 100 μ L of 100 ng/mL plus 300 μ L PBS added to the UF device, and processed with 100 μ L retentate recovered. This was compared with the original solution of 100 ng/mL (1 ng final concentration since 10 μ L were loaded per well). In this case, only two wash steps on the 10K UF devices were used (Appendix B).

The results showed that there were no significant differences between average fluorescence counts between the Sigma and Vector Labs ricin lots for untreated samples (Figure 15), with p -value = 0.46 (two-tailed, unpaired T-test); however, the Sigma ricin lot had significantly higher counts than the new lot for UF-treated samples, p -value = 2×10^{-4} . It was possible that the Sigma ricin lot was more degraded such that essentially a larger number of epitopes were exposed enabling different binding. It is also possible that differences in ricin quality (other than degradation) or quantity between the two lots led to the differences. Regardless, for both ricin lots, the average counts for UF-treated samples were similar or had slightly higher counts relative to those for untreated samples showing that ricin was not lost to the UF devices. While UF-treated samples showed slightly higher average counts for some replicates relative to the unfiltered controls (on average ~30% higher counts for the Vector Labs ricin lot and ~48% higher counts for the Sigma ricin lot), these differences are not expected to affect the interpretation of results for this qualitative analysis. The positive and negative controls (A-chain and PBS, respectively) gave expected results. The majority of the experiments were conducted with the Vector Labs ricin lot and where noted the Sigma ricin lot was used.

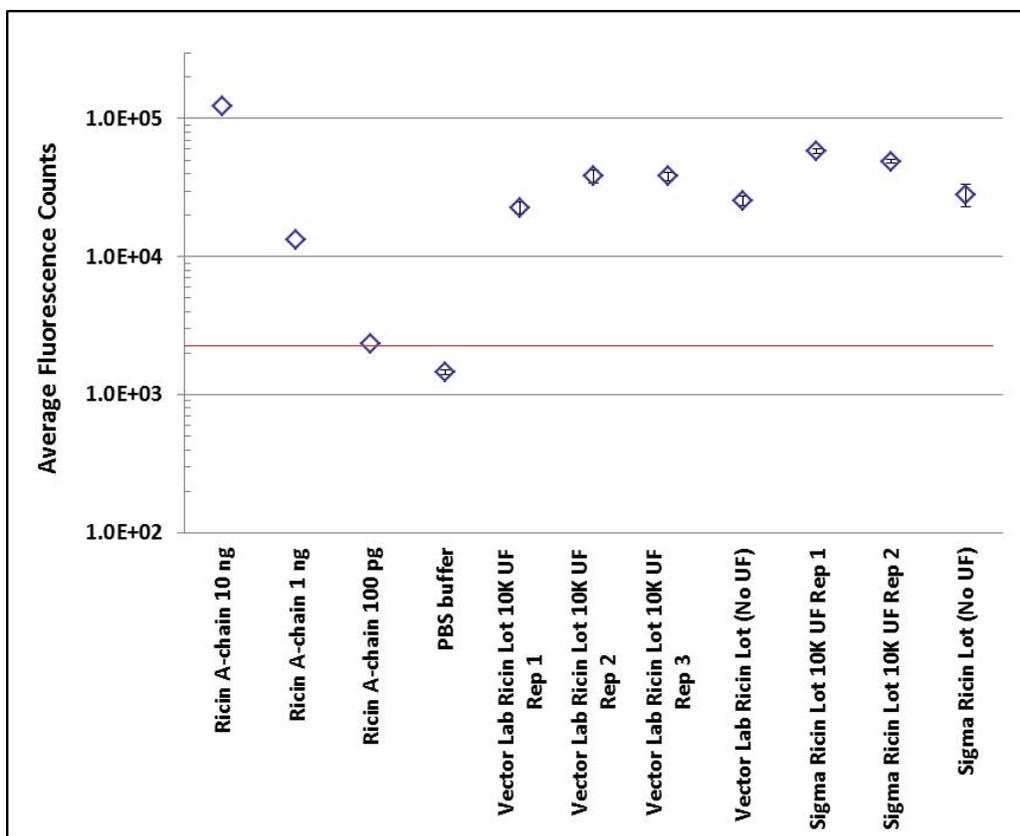


Figure 15 TRF assay results for 100 pg/mL ricin solutions processed by 10K UF devices compared to untreated 100 pg/mL solutions (ricin concentration per sample was ~1 ng) for ricin holotoxin lots from Sigma and Vector Labs. Data points represent the average fluorescence counts from three replicate TRF analyses (except for A-chain data points which are single measurements). Error bars represent \pm one standard deviation. A red line shows the negative (PBS) cut-off value (~2,190 counts).

5.3.4 Evaluation of 0.5-mL 10K UF Devices for Ricin Recovery and Concentration

An experiment was used to test whether the 0.5-mL 10K UF devices could concentrate ricin from a more dilute solution. In this effort, a protocol for processing 1-mL samples was developed and evaluated. In this case, 1-mL samples at 10-ng/mL ricin were processed and compared with untreated samples at 100-ng/mL ricin. Ten-fold different concentrations were used such that comparable fluorescence counts would be obtained after 10-fold concentration of the sample volume using the UF device (1 mL concentrated to 100 μ L).

The results are shown in Figure 16 for UF-treated (“10K UF”) and untreated extracts (“No UF”) showing that a 10-fold concentration factor (based on the fluorescence counts) was achieved with the protocol and thus, ricin losses were not observed. The UF-treated samples actually

showed more than 10-fold concentration ranging from ~10.9 to 12.1-fold concentration. Positive (A-chain) and negative (PBS buffer) controls provided expected results. The results are also shown in Table 10 where fold differences between sample treatments are included. In each case, 10 μL of the resulting solution were analyzed by TRF. Because 10-fold lower ricin concentrations were used for the 1-mL aliquot concentrated down to 100 μL (10-fold concentration by volume) than for the ricin solution not processed by UF, the average fold difference between UF-treated and untreated sample counts was actually ~11- to 12-fold (or 11- to 12-fold concentration).

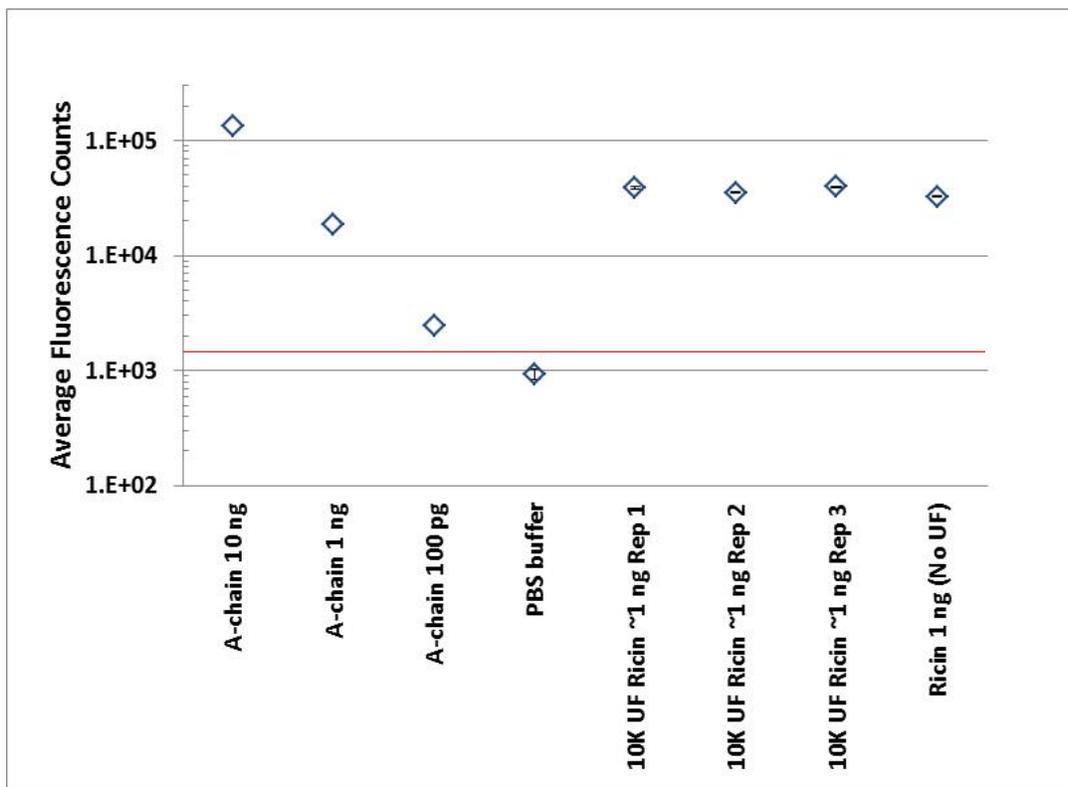


Figure 16 TRF assay results for 1-mL ricin solutions (10 ng/mL) processed by 0.5-mL 10K UF devices with 100 μL retentate compared to untreated 100 ng/mL solutions (ricin mass per well in each case was ~1 ng for 10 μL analyzed). Results are from the Vector Lab holotoxin lot. Data points represent the average fluorescence counts from three replicate TRF analyses (except for A-chain data points which are single measurements). Error bars represent \pm one standard deviation. A red line shows the negative (PBS) cut-off value (~1,400 counts).

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

Treatment*	Sample Replicate – TRF Replicate	Fluorescence Counts	Avg Fold-Difference From No UF Treatment**
10 ng/mL Ricin 10K UF	1 – 1	3.987×10^4	1.19 [†]
	1 – 2	3.856×10^4	
	1 – 3	3.767×10^4	
	Average (SD)	$3.870 (0.111) \times 10^4$	1.09 [†]
	2 – 1	3.581×10^4	
	2 – 2	3.499×10^4	
	2 – 3	3.518×10^4	1.21 [†]
	Average (SD)	$3.533 (0.0427) \times 10^4$	
	3 – 1	3.923×10^4	
	3 – 2	4.032×10^4	1.16 ± 0.07
	3 – 3	3.862×10^4	
	Average (SD)	$3.939 (0.0860) \times 10^4$	
	Overall Avg (SD)	$3.781 (0.202) \times 10^4$	
100 ng/mL Ricin No UF	1	3.295×10^4	NA
	2	3.191×10^4	
	3	3.277×10^4	
	Average (SD)	$3.254 (0.0558) \times 10^4$	
PBS (Control) No UF	1	1.024×10^3	NA
	2	9.48×10^2	
	3	8.36×10^2	
	Average (SD)	$9.36 (0.95) \times 10^2$	

* The negative cut-off value was 1,400 counts. 10K UF = ultrafiltration with 10K device. Average (Avg) and standard deviation (SD) are from triplicate TRF analyses. NA = not applicable. UF = ultrafiltration.

** Since 10-fold lower ricin concentrations were used for the 1-mL aliquot concentrated down to 100 μL, than for the ricin solution not processed by UF, the average fold difference between UF-treated and untreated was actually ~12-fold.

[†] Denotes a statistically significant difference compared to the “No UF” control (95% confidence level).

A replicate experiment was conducted using the sample parameters as that for Table 10 with results listed in Table 11 and plotted in Figure 17. Similar results were obtained showing about 14-fold average ricin concentration based on differences in fluorescence counts between UF-treated (1 mL of 10 ng/mL concentrated to 100 μL) and untreated ricin solutions (100 ng/mL). In each case 10 μL of the resulting solution were analyzed by TRF. Statistical analysis showed that UF-treated samples had significantly higher fluorescence counts based on a theoretical

concentration factor of 10-fold, with fold-differences of 14-fold. As for the previous experiment, the positive and negative controls gave expected results although the negative controls showed higher background counts.

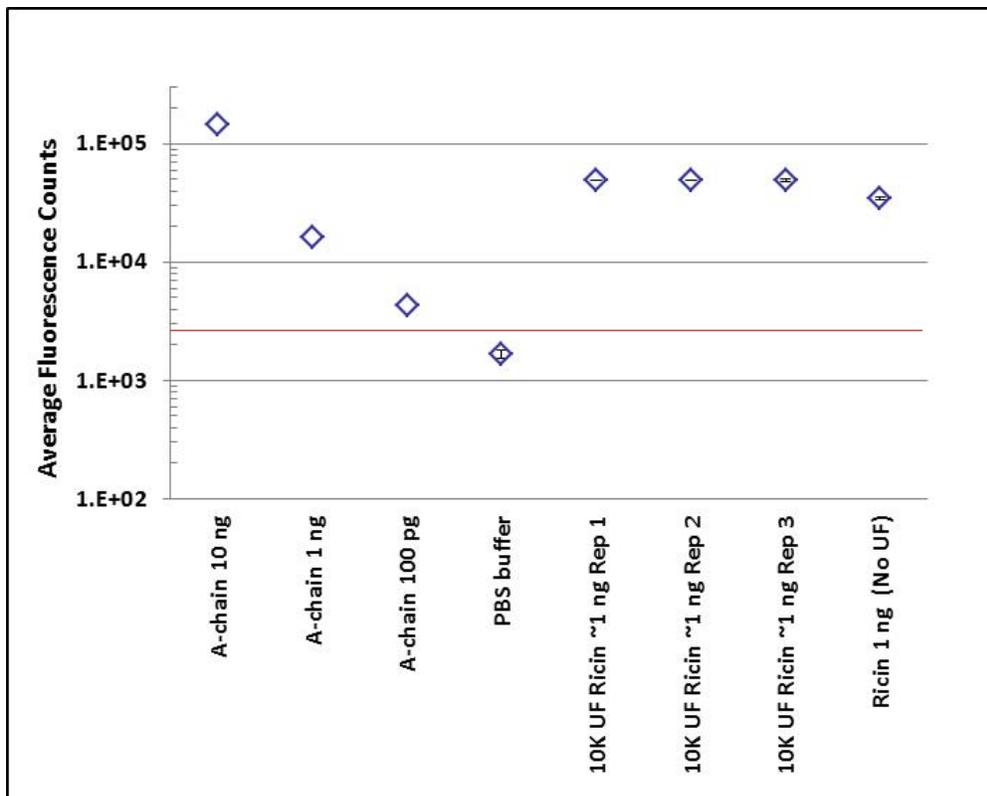


Figure 17 TRF assay results for 1-mL ricin solutions (10 ng/mL) processed by 0.5-mL 10K UF devices with 100 μ L retentate compared to untreated 100 ng/mL solutions (ricin mass per well in each case was ~1 ng for 10 μ L analyzed). Results are from the Vector Labs holotoxin lot and represent a replicate experiment of that described above. Data points represent the average fluorescence counts from three replicate TRF analyses (except for A-chain data points which are single measurements). Error bars represent \pm one standard deviation. A red line shows the negative (PBS) cut-off value (~2,500 counts).

Table 11 Evaluation of the 0.5-mL 10K UF Device on Ricin Recovery and Concentration – Replicate Experiment

Treatment*	Sample Replicate – TRF Replicate	Fluorescence Counts	Avg Fold-Difference From No UF Treatment**	
10 ng/mL Ricin 10K UF	1 – 1	4.866×10^4	1.41	
	1 – 2	4.893×10^4		
	1 – 3	4.838×10^4		
	Average (SD)	$4.865 (0.0276) \times 10^4$		
	2	2 – 1	4.927×10^4	1.43
		2 – 2	4.902×10^4	
		2 – 3	4.905×10^4	
		Average (SD)	$4.911 (0.0138) \times 10^4$	
	3	3 – 1	4.726×10^4	1.42
		3 – 2	4.971×10^4	
		3 – 3	4.991×10^4	
		Average (SD)	$4.896 (0.147) \times 10^4$	
	Overall Avg (SD)	$4.891 (0.0779) \times 10^4$	1.42 (0.007)	
100 ng/mL Ricin No UF	1	3.523×10^4	NA	
	2	3.398×10^4		
	3	3.396×10^4		
	Average (SD)	$3.439 (0.0726) \times 10^4$		
PBS (Control) No UF	1	1.776×10^3	NA	
	2	1.690×10^3		
	3	1.502×10^3		
	Average (SD)	$1.656 (0.140) \times 10^3$		

* The negative cut-off value was 2, 480 counts. 10K UF = ultrafiltration with 10K device. Average and standard deviation (SD) are from triplicate TRF analyses. Avg = Average.

** Since 10-fold lower ricin concentrations were used for the 1-mL aliquot concentrated down to 100 μ L, than for the ricin solution not processed by UF, the average fold difference between UF-treated and untreated was actually ~14-fold. UF = ultrafiltration.

5.4 Task 4 Evaluation of Sample Processing Procedure Using UF for Complex Environmental Samples

5.4.1 Evaluation of 2-mL 10K UF Devices for Ricin Purification and Concentration from Samples Containing ATD

As mentioned, bleach residue, sample materials (swab, sponge), and wetting buffer did not contribute to elevated fluorescence counts in our assessment; however, post-decontamination

samples could contain other interferents such as particulate substances (referred to as “debris”) that could lead to elevated fluorescence backgrounds and/or inaccurate results. This task focused on evaluating whether sample processing with UF devices could be used to purify ricin from complex sample matrices; therefore, Arizona Test Dust (ATD) was used as a source of debris to challenge the sample processing procedure developed in Task 3. ATD represented a challenge material that has been used by the CDC and others, although it should be noted that it is largely inorganic, containing metal oxides, with some fungal and bacterial spores or cells present (Rose et al., 2011). ATD was used in the current study in a limited effort was conducted with debris to gain some information about robustness of the UF-treatment and the TRF assay while it is understood that other types of environmental backgrounds including soluble organic materials could also affect sample processing and analysis.

An ATD slurry was prepared in the NB expressed from SS samples (as 1 g/mL) in order to use the same recipe of NB as that used for sampling. The ATD was included at 250 mg per SS sample (by addition of 250 μ L slurry per SS). The pre-wet sponge samples were then extracted with 1-mL PBS containing 3% BSA (Section 3.6). In each case, 3 mL of extract were obtained. A 2-mL aliquot of this extract was spiked with ricin holotoxin to a final concentration of 10 pg/ μ L (10 ng/mL) by adding 20 μ L of 1 μ g/mL ricin to 1980 μ L SS extract. Ricin added to SS extract without ATD was included as a control. The resulting extracts were analyzed both with and without purification and concentration with 10K UF devices.

In addition, since the TRF assay results could be affected by debris particulates in the sample, the procedure included an initial 0.22-micron pre-filtration step. Specifically, four 500- μ L aliquots of extract/toxin solution were filtered through four separate 0.22-micron filter units. The filtrates were combined, and the entire 2-mL were then transferred into a single 2-mL 10K UF device. The manufacturer’s protocol was used as guidance, with the actual procedure listed in Appendix C. Each centrifugation step to reduce 2-mL to \sim 100- μ L in the device took about 1 hr; therefore, only two wash steps were included. Interferences from bleach and environmental samples were not observed (Task 2); consequently, a systematic study of how many wash steps are required to eliminate the interferences could not be conducted. Aliquots (2 mL) of this extract were spiked with ricin to a final concentration of 10 pg/ μ L (10 ng/mL), by addition of 20 μ L of a 1- μ g/mL ricin solution to 1980 μ L SS extract (NB with or without ATD).

The data showed greater than 20-fold concentration of ricin (based on fluorescence counts) by use of the 2-mL 10K UF devices, with an average \sim 32-fold concentration factor for clean extracts and an average \sim 24-fold concentration factor for ATD extracts (Table 12). Concentration of ricin in the sample improved the detection limit afforded by the overall method (by about 24- to 30-fold) since the initial values prior to concentration were only about 1.5 – 2-fold above the negative control. The difference between average fluorescence counts for UF-treated clean and ATD-containing samples were significantly different at the 95% confidence level but not at the 99% confidence level ($p = 0.016$, two-tailed, unpaired T-test), whereas, the

difference between average values for untreated clean and ATD-containing samples was not significant at both confidence levels ($p = 0.44$, two-tailed, unpaired T-test).

Table 12 Evaluation of 2-mL 10K UF Device for Ricin Recovery and Concentration from Samples With or Without ATD

Sample Type	TRF Replicate	Fluorescence Counts*					
		Without UF -- Sample Replicates			With UF -- Sample Replicates		
		1	2	3	1	2	3
SS Solution	1	4.117×10^3	3.509×10^3	3.202×10^3	1.185×10^5	1.128×10^5	1.127×10^5
	2	3.902×10^3	3.609×10^3	3.235×10^3	1.203×10^5	1.109×10^5	1.059×10^5
	3	3.754×10^3	3.624×10^3	3.455×10^3	1.175×10^5	1.160×10^5	1.061×10^5
	Avg (SD)	3.924 $(0.183) \times 10^3$	3.581 $(0.063) \times 10^3$	3.297 $(0.138) \times 10^3$	1.188 $(0.0145) \times 10^5$	1.132 $(0.0256) \times 10^5$	1.082 $(0.0386) \times 10^5$
	Overall Avg (SD)	3.601 (0.297) $\times 10^3$			1.134 (0.0517) $\times 10^5$		
SS Solution With 250 mg ATD	1	3.860×10^3	3.791×10^3	3.623×10^3	9.848×10^4	8.141×10^4	9.381×10^4
	2	3.853×10^3	4.081×10^3	3.648×10^3	1.008×10^5	8.145×10^4	9.273×10^4
	3	3.690×10^3	3.370×10^3	3.885×10^3	9.343×10^4	8.238×10^4	9.359×10^4
	Avg (SD)	3.801 $(0.096) \times 10^3$	3.747 $(0.358) \times 10^3$	3.719 $(0.145) \times 10^3$	9.756 $(0.375) \times 10^4$	8.175 $(0.0546) \times 10^4$	9.338 $(0.0571) \times 10^4$
	Overall Avg (SD)	3.756 (0.202) $\times 10^3$			9.089 (0.735) $\times 10^4$		

* The negative cut-off value was 1,390 counts. Average (Avg) and standard deviation (SD) are from triplicate TRF analyses. The initial ricin concentration was 10 ng/mL prior to UF treatment. SS = sponge-stick; ATD = Arizona Test Dust; UF = Ultrafiltration.

5.4.2 Evaluation of 2-mL 10K UF Devices for Ricin Purification and Concentration from Samples Containing ATD – Replicate Experiment

Similar results were observed for a replicate of this experiment (Table 13), with the same ricin concentrations and 250 mg concentration of ATD used per SS sample. In this case, there was an average ~22-fold concentration factor for clean extracts and an average ~18-fold concentration

factor for dirty extracts. In both experiments, the concentration factor (based on counts) for dirty extracts was lower than the difference for clean extracts. Similar to the first experiment, the difference between average fluorescence counts for UF-treated clean and ATD-containing samples were not significantly different at the 95% confidence level ($p = 0.052$, two-tailed, unpaired T-test), as was also the case for the difference between average values for untreated clean and ATD-containing samples ($p = 0.52$, two-tailed, unpaired T-test). The addition of 250 mg ATD per SS sample did not significantly impact the TRF results; however, these results cannot be generalized to all environmental surface samples. In other types of samples, different types of background debris and interferences could affect the assay results, such that additional sample processing prior to TRF analysis is necessary. However, in principle, the sample processing procedure that includes 0.22-micron pre-filtration and subsequent washes of the analyte in the UF device may alleviate TRF assay interferences for other environmental samples.

Table 13 Evaluation of 2-mL 10K UF Devices for Ricin Recovery and Concentration from Samples With or Without ATD—Replicate Experiment

Sample Type	TRF Replicate	Fluorescence Counts*					
		Without UF -- Sample Replicates			With UF -- Sample Replicates		
		1	2	3	1	2	3
SS Solution	1	5.539×10^3	4.705×10^3	4.913×10^3	1.243×10^5	1.068×10^5	1.019×10^5
	2	5.440×10^3	4.826×10^3	5.824×10^3	1.280×10^5	1.040×10^5	1.019×10^5
	3	5.342×10^3	4.823×10^3	4.376×10^3	1.174×10^5	1.044×10^5	1.028×10^5
	Avg (SD)	5.440 (0.099) × 10^3	4.785 (0.069) × 10^3	5.038 (0.732) × 10^3	1.232 (0.0537) × 10^5	1.051 (0.0147) × 10^5	1.022 (0.00525) × 10^5
	Overall Avg (SD)	5.088 (0.469) × 10^3			1.102 (0.103) × 10^5		
SS Solution With 250 mg ATD	1	5.778×10^3	4.814×10^3	4.574×10^3	9.620×10^4	9.484×10^4	8.136×10^4
	2	4.965×10^3	5.123×10^3	4.678×10^3	9.297×10^4	9.384×10^4	8.269×10^4
	3	4.685×10^3	4.887×10^3	4.794×10^3	8.790×10^4	9.376×10^4	8.387×10^4
	Avg (SD)	5.143 (0.568) × 10^3	4.941 (0.162) × 10^3	4.682 (0.110) × 10^3	9.235 (0.418) × 10^4	9.414 (0.0601) × 10^4	8.264 (0.125) × 10^4
	Overall Avg (SD)	4.922 (0.361) × 10^3			8.971 (0.580) × 10^4		

* The negative cut-off value was 1,530 counts. Average (Avg) and standard deviation (SD) are from triplicate TRF analyses. The initial ricin concentration was 10 ng/mL prior to UF treatment. SS = sponge-stick; ATD = Arizona Test Dust; UF = Ultrafiltration.

6 Conclusions

In this effort, the potential causes of previously reported, elevated background fluorescence in TRF analysis for post-decontamination samples were investigated. It should be noted that the effort was solely to troubleshoot the TRF assay for post-decontamination applications and was not meant to directly inform the protocol used for unknown samples. Specifically, since samples contained known amounts of ricin, the multiple dilutions used for real-world, unknown samples were not prepared and analyzed. Unknown samples should be prepared in multiple serial dilutions so the fluorescence counts are in proper range for accurate detection/quantification; furthermore, there would be more confidence in the results when sequential dilutions were positive.

The TRF immunoassay reported herein appeared to tolerate high concentrations of bleach residue, wetting buffer, and materials from sampling devices (sponge-sticks and macrofoam swabs) since it did not show elevated background fluorescence responses (i.e., > 7,000–8,000 fluorescence counts) with these potential interferents. Furthermore, samples containing particulates (from a reference test dust) up to ~250 mg/Spoge-Stick 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, whereas other antibody lots did not show elevated fluorescence backgrounds when used at similar concentrations. Therefore, it is possible that reported high backgrounds leading to unsatisfactory results reported for the Tupelo, MS ricin incident, may not have been due to the post-bleach decontamination samples but rather due to a reagent issue. Because the antibodies must be appropriately diluted prior to use, elevated backgrounds could also occur if antibodies are used at too high concentration levels as a result of improper dilution.

This effort led to development of a processing procedure for surface samples (swabs, sponge-sticks) for both sample cleanup and ricin concentration that might be useful for any assay including fluorescence-based and electrochemiluminescence immunoassays to minimize false positive and false negative results. Using 0.5-mL or 2-mL UF devices, 10- to 20-fold or greater concentration factors were achieved based on fluorescence counts. The 10 kDa UF devices provided better recovery with 2–4 wash steps compared with 30 kDa UF devices. Incorporation of the sample processing procedure prior to TRF analysis may enable improved assay sensitivity of detection and provide greater usability of the TRF data by elevating the fluorescence response above the background. Therefore, more consistent results are expected for both pre- and post-decontamination samples, providing high quality data for high consequence decisions concerning public health.

The combined process of sample clean up and toxin concentration can enable detection of ricin at low concentrations. Because the sample processing procedure developed in this effort is intended for use following the sample extraction steps, it might be used with any ricin analytical method, although further verification and validation may be required, e.g., by expanding the number of surface types and potential interferences.

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Appendix A

Appendix A: Protocol Used for Ricin Detection

Materials

- *Affinity-purified polyclonal anti-ricin antibody (Critical Reagents Program, CRP, Ft. Detrick, MD, Cat. No. AB-AG_RIC)*
- *EZ-link[®] NHS-PEG4 Biotinylation Kit (Life Technologies, Waltham, MA, Cat. No. 21455)*
- *Monoclonal Anti-ricin Toxin A-Chain, Clone RAC18 antibody (BEI Resources, Manassas, VA, Cat. No. NR-9571)*
- *0.22 Micron Syringe Filter for Detector Antibody filtration (Millipore[®] Millex[®] GV, Billerica, MA, Cat. No. SLGV033RS)*
- *Polypropylene bioblock (Beckmann Coulter[®] Square well PP Plate, Cat. No. 609681 or equivalent reservoir for transferring antibody solutions to strip plates)*
- *Ricin A-chain used as positive control (Sigma-Aldrich[®], St. Louis, MO, Cat. No. L9514-1MG)*
- *PerkinElmer DELFIA[®] 25X Wash Concentrate (PerkinElmer, Inc., Waltham, MA, Cat. No. 1244-114)*
- *PerkinElmer DELFIA[®] Assay Buffer (Cat. No. 1244-111)*
- *PerkinElmer DELFIA[®] Enhancement Solution (Cat. No. 1244-105)*
- *PerkinElmer DELFIA[®] Streptavidin-coated clear plate, 8 x 12 strips, 10 plates (Cat. No. 4009-0010)*
- *Tubes, sterile 2 mL DNase- and RNase-free, gasketed, screw caps (National Scientific, Rockwood, TN, Cat. No. BC20NA-PS)*
- *Gibco[®] Water for Injection (WFI) for cell culture, VSP, EP Antimicrobial Water (Life Technologies, Cat. No. A12873-02)*
- *1X PBS (Teknova[®], Hollister, CA, Cat. No. P0300; endotoxin free filtered through 0.22-micron filter sterilization unit)*
- *Amicon[®] Ultra- 0.5 10K Centrifugal UF Devices with Ultracel[®] 10 Membrane (Millipore[®], Cat. No. UFC501024)*
- *Amicon[®] Ultra collection tubes for use with Amicon[®] Ultra Centrifugal Filters (Amicon[®]/Millipore[®], Cat. No. UFC50VL96)*
- *0.22 µm Ultrafree[®] MC GV Sterile 0.5 mL Centrifugal Filter Unit with Durapore[®] PVDF Membrane, Yellow Cap (Millipore[®], Cat. No. UFC30GV0S)*
- *Bovine Serum Albumin, Fraction V (VWR, Radnor, PA, Cat. No. VWR Cat. No. EM2930)*

- *Amicon[®] Ultra-2 10K Centrifugal UF Devices with Ultracel[®] 10 Membrane (Millipore[®], Cat. No. UFC201024)*
- *Neutralizing Buffer (Hardy Diagnostics[®], Santa Maria, CA, Cat. No. K105)*
- *Cellulose Sponge-Stick samples pre-wet with Neutralizing Buffer (Solar Biologicals, Ogdensburg, NY, Cat. No. SH10NB)*
- *Foam-tipped swab samples (Puritan[®] Medical Products, Guilford, ME, Cat. No. 25-1607 1PF SC)*
- *Victor[®] X4 Plate Reader (PerkinElmer[®], Cat. No. 2030-0040)*
- *Disposable sterile polystyrene forceps, individually wrapped (Cole Parmer[®], Vernon Hills, IL, Cat. No. YO-06443-20 or equivalent)*
- *BD[®] Falcon, Polypropylene 15 mL conical tubes, Sterile, (Becton, Dickinson and Co., Franklin Lakes, NJ, Cat. No. 352098 or equivalent)*
- *DELFI[®]A PlateWash (PerkinElmer, Cat. No. 1296-026)*
- *Pro-Advantage[®], Sterile specimen cup, 4 ounces, Polyethylene, Mountainside Medical Equipment, Marcy, NY, Cat. No. P250400*
- *DELFI[®]A PLATESHAKE (PerkinElmer, Cat. No. 1296-004)*
- *Potassium Hydrogen Phthalate 100 g (Fisher Scientific, Waltham, MA, Cat. No. P243-100 or equivalent)*
- *0.5 M EDTA, pH 8.0 (Ambion[®], Fisher Scientific, Cat. No. AM9260G)*
- *Bleach Wipes, Dispatch[®] (Clorox, Oakland, CA, Cat. No. 69150 or equivalent)*
- *50 mL serological pipettes, sterile, individually wrapped (Corning[®], Corning Inc., Corning, NY, Cat. No. 29442-440)*
- *10 mL serological pipettes, sterile, individually wrapped (Corning[®] Cat. No. 29442-430)*
- *25 mL serological pipettes, sterile, individually wrapped (Corning[®] Cat. No. 29442-436)*
- *5 mL serological pipettes, sterile, individually wrapped (Corning[®] Cat. No. 29442-422)*
- *Table top centrifuge with adapters for 15-mL tubes (Eppendorf[®], Eppendorf North America, Hauppauge, NY, 5810R or equivalent)*

Reagent Preparation for Processing Sponges and Swabs

1. *Prepare phosphate buffered saline (PBS) with 3% bovine serum albumin (BSA) (Fraction V, VWR Cat. No. EM2930).*
2. *Use sterile 1X PBS, pH 7.2–7.4 (does not need to be endotoxin-free).*
3. *Make 10 mL solution with 0.3 g BSA (or prepare 100 mL with 3 g BSA and store at 2–8°C for up to 1 month)*
4. *Filter sterilize through a 0.22 micron filter.*
5. *This solution is used to process the swab and sponge samples after surface sampling.*

Swab Surface Sampling and Sample Processing Procedure to Collect Bleach Residue and Other Matrices in the Absence of Ricin

Note: The procedure was used to generate a realistic sample matrix for testing the UF protocol and time-resolved fluorescence (TRF) assay performance. Ricin was not applied to surfaces and ricin recovery from surfaces was not evaluated.

- 1. Prepare stainless steel coupons by wiping them with isopropanol and air drying in the biosafety cabinet (BSC).*
- 2. Tape off 4 x 4 inch (10.2 × 10.2 cm) squares, 3 total squares per plate. Use 3 plates for bleach treatment and one plate for the negative control (water sprayed on—same water used to make the 10% bleach solution).*
- 3. Prepare 10% bleach just before use (9 parts sterile filtered water, 1 part Ultra Clorox[®] Germicidal Bleach). Autoclaved, double distilled water can be used.*
- 4. Apply 10% bleach by hand sprayer, completely covering surface with layer of aqueous solution. Monitor plates and reapply bleach if any drying is observed within 10 min period. Allow samples to air dry overnight.*
- 5. On the next day, sample coupons by wiping with swabs pre-wet by dipping into 10 mL NB (Hardy Diagnostics[®] Cat. No. K105) according to Macrofoam Swab Procedure (CDC-NIOSH source <http://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthraxis.html>), and pressing against the tube to express liquid from swab head. Make sure there is sufficient liquid to keep wet for all three 4 x 4 inch areas. Record weight of tube with liquid before and after dipping the swab and expressing the liquid.*
- 6. Use S-strokes to sample the entire surface.*
- 7. After sampling, break off swab stick by bending at the notch so that the swab head is inside the 15-mL conical tube.*
- 8. Add 1 mL of 1X PBS with 3% BSA to each swab head in 15-mL conical tube.*
- 9. Vortex the tube at the highest setting (~3,200 rpm) in 15 sec bursts for 2 min.*
- 10. Using a sterile transfer pipette (or serological pipette), transfer the liquid sample to a new pre-labeled 15 mL conical tube.*
- 11. Re-vortex the original sample tube containing the swab in 15 sec bursts for 1 min.*
- 12. Using a sterile transfer pipette, draw up the remaining liquid. Use the bottom of the transfer pipette to press the head of the swab against the tube wall to express as much liquid as possible.*
- 13. Transfer the remaining liquid to the same pre-labeled 15 mL conical tube that contains liquid from step 10. Avoid transferring any debris from the bottom of the tube. Briefly centrifuge the 15 mL tube with the swab for up to 1 min at approximately 3,000 RCF to collect the liquid to the bottom of the tube. Transfer any fluid to the appropriate conical tube.*

14. *Avoiding any debris at bottom of tube, transfer liquid into 2 mL screw-cap tube. Proceed to the UF Sample Processing Procedure for sample clean-up and concentration depending on the desired sample volume to treat (Appendix B or C).*

Sponge-stick Surface Sampling and Sample Processing Procedure to Collect Bleach Residue and Other Matrices in the Absence of Ricin

Note: The procedure was used to generate a realistic sample matrix for testing the UF protocol and TRF assay performance. Ricin was not applied to surfaces and ricin recovery from surfaces was not evaluated.

1. *Prepare stainless steel coupons by wiping them with isopropanol and air drying in BSC.*
2. *Use 10 x 10 inch (25.4 × 25.4 cm) stainless steel plates. Use 3 plates for bleach treatment and one plate for the negative control (water sprayed on—same water used to make the 10% bleach solution).*
3. *Prepare 10% bleach just before use (9 parts sterile filtered water, 1 part Ultra Clorox[®] germicidal bleach). Autoclaved, double distilled water can be used.*
4. *Apply 10% bleach by hand sprayer, completely covering surface with layer of aqueous liquid. Monitor plates and reapply bleach if any drying is observed. If possible, apply more bleach even if significant drying is not observed. Allow samples to air dry overnight.*
5. *Next day, sample coupons by wiping with pre-wet Sponge-sticks according to Cellulose Sponge Procedure (CDC/NIOSH source)
<http://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthraxis.html>).*
6. *Use S-strokes to sample entire surface.*
7. *After sampling, place the head of the sponge directly into the sterile specimen cup using the release mechanism.*
8. *Add 1 mL of 1X PBS with 3% BSA to each sponge head in the specimen cup. Note: Use up to 2 mL of 1X PBS/3% BSA if no fluid can be recovered from sponge. Press against the sponge with a pipet tip or serological pipet to express sufficient liquid.*
9. *Vortex the specimen cup at the highest setting in 15 sec bursts for 2 min.*
10. *Recover sample extract and process by pre-filtration through yellow-top Ultrafree-MC filter units (Millipore[®] Cat. No. UFC30GV0S).*
11. *To pre-filter 1-mL extract, carefully transfer 0.5 mL each into two yellow-top filter units with collection tube (follow manufacturer's directions for use). Note: 2 mL may be pre-filtered if four yellow-top filter units are used.*
12. *Centrifuge at 7,000 rpm for 3 minutes.*
13. *Proceed to the UF Sample Processing Procedure for sample cleanup and concentration (Appendix B for 0.5-1 mL sample volume or Appendix C for up to 2-mL sample volume).*

DELFLIA[®] Time-Resolved Fluorescence Assay:

Note: This protocol was slightly modified from Schieltz et al. (2011)¹ since the purpose in the present study was to evaluate the sample processing approach and determine the cause of high background fluorescence rather than for TRF analysis of unknown samples. Therefore, for actual ricin sample analysis, additional steps may be required for the TRF assay.

1. Waste set up: All tip waste must be rinsed with bleach prior to placement into sharps container. Set up sharps and freshly prepared 2.5% bleach reservoir in secondary container/tray inside BSC. Ensure that items such as counter tops, pipets, tubes, gloves, etc. that may be used in TRF assays are free of bleach residue and bleach reservoirs are not located in close proximity to sample processing activities.
2. Equipment preparation
 - a. Decontaminate the plate washer by flushing with 50 mM Potassium Hydrogen Phthalate, 0.01% EDTA in distilled endotoxin-free (ETF) water (Prepared with 0.2 g potassium hydrogen phthalate, 1.4 mL 5 mM EDTA, 198.6 mL ETF H₂O)
 - b. Rinse the plate washer with distilled, ETF water.
 - c. Wipe the plate reader internal plate platform with 95% ethanol to remove dust.
 - d. Start the plate reader instrument prior to starting the associated computer.
3. Reagent Preparation
 - a. Bring DELFLIA[®] reagents to room temperature; Assay Buffer (PerkinElmer[®] Cat. No. 1244-111), Wash Buffer (PerkinElmer[®] Cat. No. 1244-114) and Enhancement Solution (PerkinElmer[®] Cat. No. 1244-105).
 - b. Place an aliquot of each antibody (capture and detector), and positive control materials on ice.
 - c. Prepare wash buffer by 1:25 dilution in sterile distilled ETF water. (150 mL per strip of 12 wells)
4. Positive Control Preparation (Ricin A-chain)
 - a. Just before use (no more than 1 hr), prepare a fresh dilution of Ricin A chain in Assay Buffer (add 5.9 μ L of stock at \sim 850 μ g/mL into 5 mL Assay Buffer for a final concentration of \sim 10 ng/10 μ L). Discard any unused preparation after use.
5. Antibody Preparation

¹ Schieltz, D.M., S.C. McGrath, L.G. McWilliams, J. Rees, M.D. Bowen, J.J. Kools, L.A. Dauphin, E. Gomez-Saladin, B.N. Newton, H.L. Stang, M.J. Vick, J. Thomas, J.L. Pirkle, and J.R. Barr. 2011. Analysis of active ricin and castor bean proteins in a ricin preparation, castor bean extract, and surface swabs from a public health investigation. *Forensic Sci. Int.* 209:70-79.

- a. Thaw aliquots of each antibody at 4°C (detector and capture). Calculate the number of concentrated capture and detector antibody needed based on the sample number (~1.4 mL of antibody in assay buffers per 12 samples).
 - b. Make working dilutions of each antibody in DELFIA[®] Assay Buffer.
Note: Dilutions must be used within 1 hr.
 - Capture Antibody
 - Detector antibody

Note: when calculating detector antibody amount to use, the matrix control samples do not receive detector antibody so omit those samples from the calculation.
- 6. Sample Preparation (Ricin holotoxin)**
- a. Just before use (no more than 1 hr), prepare fresh dilution of ricin holotoxin. Discard any unused preparation after use.
- 7. Coating the assay plate with capture antibody**
- a. Determine the number of strips needed to test all samples and controls according to the plate layout. Remove excess 12-well strips (DELIFIA[®] Streptavidin Microtitration Strips; PerkinElmer[®] Cat. No. 4009-0010) from the microtiter tray, leaving enough in the plastic frame to perform the assay. The extra strips may be stored in a sealed plastic bag, or a spare frame, with the desiccant that is included with each plate. Refrigerate the extra strips until needed.
 - b. Centrifuge antibody aliquots in a microcentrifuge for 10 sec to collect the solution to the bottom of the vial.
 - c. Dilute the capture antibody with Assay Buffer in a polypropylene tube. Since the assay requires 100 µL of capture antibody in Assay Buffer per well, prepare 10% more of the antibody dilution than needed to compensate for loss during dispensing (i.e., 1.3 mL per 12-well test strip or 10.5 mL per plate).
 - d. Pre-wash all strips once (1X) with 750 µL Wash Buffer per well using the plate washer. Invert plate over an absorbent pad and tamp vigorously 1-2 times to remove any residual fluid.
 - e. Add 100 µL of the capture antibody (at working dilution in Assay Buffer) to each well using a calibrated multichannel pipette. NOTE: Avoid contact between pipette tips and the microtiter strips since touching of the tips to the plate may cause cross-contamination of adjacent wells, loss of reagent or improper dilution of controls.
 - f. Place the plate on the PlateShake and cover it with the bottom portion of the plastic container in which the plate and strips came. Cover strips/plate and plastic cover with aluminum foil before starting plate shaker.
 - g. Incubate for 2 hr at room temperature on the PlateShake set to “high”.
- 8. Addition of detector antibody**
- a. Ten minutes before the end of the above incubation, centrifuge the detector antibody in a microcentrifuge for 10 sec to collect the solution to the bottom of the vial.
 - b. Dilute the detector antibody with Assay Buffer in a polypropylene tube. Prepare extra detector antibody working solution (1.4 mL per 12-well test strip or 11 mL per plate) to cover any loss of volume during filtering and dispensing.
 - c. Filter the detector antibody-buffer solution through a 0.22-micron low-protein binding filter syringe to remove any particulates.

Note: Make additional antibody-buffer solution to account for losses during filtration (~1 mL extra volume).

- d. Wash all wells twice with the plate washer using 750 μ L Wash Buffer per well. Invert plate over absorbent pad and tamp vigorously 1-2 times to remove residual fluid.
 - e. Add 100 μ L of Assay Buffer to matrix control wells. Note: Do not add detector antibody to matrix control wells.
 - f. Add 100 μ L of the detector antibody in Assay Buffer to all wells used for negative controls, positive controls and samples.
9. Addition of sample and controls
- a. Following the plate layout, add 10 μ L of sample to each well on the assay plate, excepting the Ricin A-chain with will be direct addition of 10 μ L into first well, then two serial dilutions on the plate.
 - b. Once all samples and controls are added to the plate, cover strips/ plate and plastic cover with aluminum foil before starting the plate shaker. Incubate for 1 hr at room temperature on the PlateShake set on “high”.
10. Addition of Enhancement Solution
- a. Wash strips eight times, followed by two times with 750 μ L Wash Buffer per well. Invert plate and tamp vigorously 1-2 times to remove residual fluid.
 - b. Using a multichannel pipette dedicated solely for the addition of Enhancement Solution, add 200 μ L of Enhancement Solution per well.
 - c. Incubate for 5 min at room temperature with the PlateShake set to “low”.
 - d. Turn on the Victor[®] X4 plate reader during this incubation and load the appropriate reading procedure for the europium TRF assay.
 - e. Read the plate on the Victor[®] X4 plate reader. See manufacturer’s instructions for setting up the europium procedure.

Data Analysis and Interpretation

1. Review the counts for the negative control replicates; counts should be less than 2,000. Higher readings may indicate inadequate washing, contamination of washer with unbound europium, or improper detector antibody concentrations used (i.e., too high concentration).
 - a. Calculate the negative cut-off value by averaging the negative control well values and multiplying by 1.5
2. Review the data for the sample matrix control(s). Values should be less than those for the negative control wells, < 2,000 counts. If elevated values are consistently observed, this could indicate europium contamination. Corrective actions include additional rinses on the plate washer. In addition, phthalate buffer (50 mM potassium hydrogen phthalate, 0.01% EDTA in distilled water) could be run through the plate washer followed by additional rinses. Analysis of samples showing elevated matrix control values should be repeated.
3. The positive control (ricin A-chain) is expected to be positive in all three dilutions. If the proper concentration of ricin A-chain was used, failure to give a positive result for the three dilutions of the positive control may indicate an assay component is not functional. In this case, implement corrective actions and repeat the test.

- a. When all controls are acceptable, a sample is considered negative for this assay if the average count values are below the higher of the plate average negative control value or the matrix control value (i.e., referred to as the negative cut-off value).
 - b. When all controls are acceptable, a sample is considered “positive” (reactive) if the average counts are above the negative cut-off value.
4. Sample values must be reproducible across the sample replicates with no more than 20% variability (from mean value or $\leq 20\%$ CV) between wells. Variability greater than this may indicate inadequate washing of the plate or cross-contamination of wells during pipetting.
5. The estimated detection limit for this assay is ~10-100 pg total protein (1-10 ng ricin toxin/mL) which is dependent on the quality of Europium labeling for the anti-ricin antibody lot and resultant background fluorescence.
6. Environmental samples (e.g., soil extracts or metal containing materials) may have europium present (i.e., elevated counts for matrix controls without detector antibody), which may interfere with the TRF assay. In this case, dilution of the sample would be expected to reduce the signal caused by europium contamination.

Appendix B

Appendix B: Sample Processing Procedure for Post-Decontamination Ricin Samples using 0.5 mL 10K UF Devices

This procedure uses commercially available UF devices typically used for protein or DNA purification and concentration, namely Amicon® Ultra-0.5 10K Centrifugal UF Devices (EMD Millipore, Billerica, MA; Cat. No. UFC501024). Schematic diagrams are from the Amicon® Ultra-0.5 Centrifugal Filter Devices (for volumes up to 500 µL) User Guide².

This procedure can be used for environmental sample cleanup and concentration prior to ricin analysis.

Recommendation for Sample Collection: The post-decontamination SS samples after collection should be placed in a specimen cup containing 1–2 mL 1X PBS (Teknova Inc., Hollister, CA, Teknova® Cat. No. P0300) with 3% BSA (Fraction V, VWR, Radnor, PA, Cat. No. EM2930). Alternatively, if the sponge-stick was pre-wet with 10 mL Neutralizing Buffer (NB) (from the vendor), liquid remaining in the bag holding the sponge after excess liquid is expelled, prior to sampling, could then be used to extract ricin from the sponge after sampling and used for ricin analysis; however, addition of 3% BSA could enhance ricin stability and thus, detection.

Equipment and Supplies:

1. Millipore® 0.22 µm Ultrafree® MC GV Sterile 0.5 mL Centrifugal Filter Unit with Durapore® PVDF Membrane: EMD Millipore, Billerica, MA, Cat. No. UFC30GV00
2. Amicon® Ultra- 0.5 10K Centrifugal UF Devices: EMD Millipore, Billerica, MA, Cat. No. UFC501024
3. Amicon® Ultra- 0.5 Collection Tubes: EMD Millipore, Billerica, MA, Cat No. UFC50VL96
4. Eppendorf® centrifuge: 5424/5424R (formerly, 5415/5415R) (Eppendorf North America, Hauppauge, NY) or equivalent

² User Guide: Amicon® Ultra-0.5 Centrifugal Filter Devices;
http://www.emdmillipore.com/US/en/product/Amicon-Ultra-0.5%20mL-Centrifugal-Filters-for-DNA-and-Protein-Purification-and-Concentration,MM_NF-C82301#documentation.

- 1X PBS, endotoxin-free, pH 7.3 ± 0.2: Teknova, Hollister, CA, Cat. No. P0300 or equivalent
- 1.0 mL micropipettor and 1.0 mL pipet tips
- 2.0 mL microcentrifuge screw cap tubes

Sample Pre-filtration

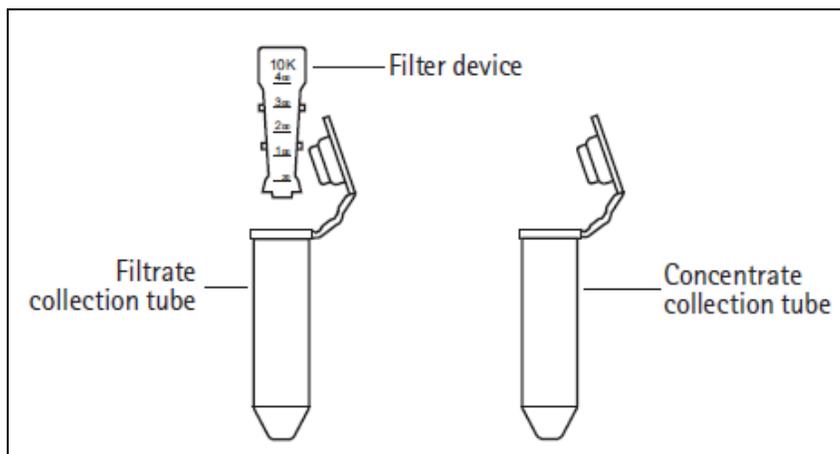
Note: For environmental samples, it is recommended to pre-filter the sample through a 0.22 micron filter prior to concentration by ultrafiltration.

- Using a micropipettor, carefully transfer 500 µL of sample into a 0.22 µm Ultrafree[®] MC GV Sterile 0.5 mL Centrifugal Filter Unit with Durapore[®] PVDF Membrane (Millipore[®] Cat. No. UFC30GV00) with collection tube (for 1 mL sample use 2 units, each with a collection tube).
- Centrifuge tubes at 5,200 RCF for 9 minutes.

Note: Ensure that the supernatant has been completely filtered. Clean samples may take less than 9 minutes to complete filtration. Centrifuge for an additional 2 minutes if there is any liquid remaining in the filter.

- Remove the filter unit using sterile disposable forceps, gripping only on the sides, and dispose to waste. Cap the tube and proceed to the next section.

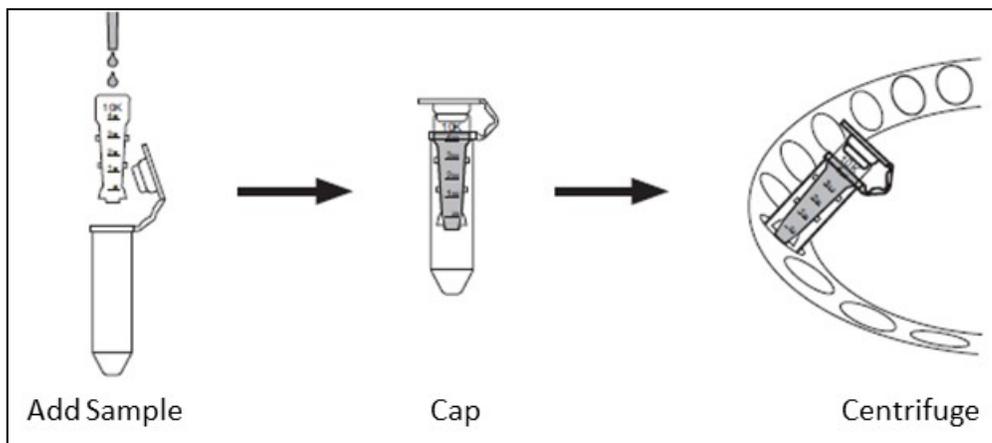
Sample Processing Using the Millipore Amicon[®] Ultra-0.5 Centrifugal UF Devices



Amicon Ultra-0.5 Centrifugal Filter Device Parts

- In a biosafety cabinet (BSC), set up for each sample one Amicon[®] Ultra-0.5 10K Centrifugal UF Device (Millipore[®], Cat. No. UFC501024) with Filtrate and Concentrate Collection microcentrifuge tubes. Label each UF device and concentrate collection tube. Labeling the filtrate collection tubes is optional as long as the UF device is clearly labeled.

2. In a BSC, briefly vortex the sample tubes (3–5 sec at ~3,200 rpm).
3. Allow the particulate matter to settle for 1 min.
4. Process each sample as described in the following steps.
5. Insert the Amicon[®] Ultra-0.5 Centrifugal filter device into the filtrate collection microcentrifuge tubes making sure that the device is fully seated in the tube.
6. Using a 1.0 mL micropipettor and respective pipet tip, carefully transfer the sample (up to 0.45 mL at a time) to the Amicon[®] Ultra-0.5 Centrifugal filter device (try to avoid particulate matter).
7. Cap the Amicon[®] Ultra-0.5 Centrifugal filter device. This step generates a filter assembly.
8. Place the filter assembly in a fixed angle microcentrifuge (Eppendorf[®] 5424/5424R or equivalent), aligning the cap strap toward the center of the centrifuge rotor. Counter balance the centrifuge rotor with a similar assembly.



9. Centrifuge at $11,200 \times g$ for 11 min at 4°C. If a refrigerated microcentrifuge is not available, this step could be performed at room/ambient temperature (no more than 25°C).
10. If the sample volume is more than 0.45 mL, remove the filter assembly from the centrifuge and transfer the Amicon[®] Ultra-0.5 Centrifugal filter device to a new filtrate collection tube. Dispose of the used filtrate collection tube with filtrate. Transfer the remainder of the sample (up to 0.45 mL) to the respective Amicon[®] Ultra-0.5 Centrifugal filter device and repeat steps 12 to 15. For a 1 mL sample, perform two centrifugation

steps with 0.45 mL and one with the remaining volume, ~0.1 mL (the last centrifugation step with less volume can be completed in ~5 min).

Note: Ensure that the sample has been filtered to ≤ 0.1 mL. If more than 0.1 mL retentate is left, spin for additional time depending upon the volume left in the device.

11. Remove the filter assembly from the centrifuge and transfer the Amicon[®] Ultra-0.5 Centrifugal filter device to a new filtrate collection tube. Dispose of used filtrate collection tube with filtrate.
12. Carefully pipet 0.45 mL PBS into the filter device for the first wash.
13. Cap the device and centrifuge at 11,200 RCF for 11 min at 4°C.

Note: The manufacturer states the device should be centrifuged at 14,000 RCF for 10 – 30 min dependent on the NMWL of the device.

14. Remove the filter assembly from the centrifuge and transfer the filter device to a new filtrate collection tube. Dispose of used filtrate collection tube with filtrate.
15. Repeat steps 12 and 13 for second wash. Ensure that ~100 μ L remains after centrifugation.
Note: While two wash steps are described, additional wash steps may be used as described, for samples containing large quantities of potential interferences.

16. Remove the filter assembly from the centrifuge.
17. Using a 1.0 mL micropipettor and respective pipet tip, carefully transfer the sample to a 2.0 mL microcentrifuge screw cap tube. (Try to avoid particulate matter at the bottom of the filter).
18. Measure the sample retentate volume and adjust the volume to 0.1 mL with PBS for replicate sample analysis.
19. Store the processed sample at 4°C until the time-resolved fluorescence (TRF) immunoassay analysis is initiated.

Appendix C

Appendix C: Sample Processing Procedure for Post-Decontamination Ricin Samples using 2 mL 10K UF Devices

This procedure uses commercially available UF devices typically used for protein or DNA purification and concentration, namely Amicon[®] Ultra-2 Centrifugal Devices (EMD Millipore, Cat. No. UFC201024). Schematic diagrams are from the Amicon[®] Ultra-2 10K Centrifugal Filter Devices (for volumes up to 2 mL) User Guide³.

This procedure can be used for environmental sample cleanup and concentration prior to ricin analysis.

Recommendation for Sample Collection: The post-decontamination sponge-stick (SS) samples after collection should be placed in a specimen cup containing 1–2 mL 1X Phosphate Buffered Saline (PBS) (Teknova[®] Cat. No. P300) with 3% BSA (Fraction V, VWR, Radnor, PA, Cat. No. EM2930). Alternatively, if the sponge-stick was pre-wet with 10 mL Neutralizing Buffer (NB) (from the vendor), liquid remaining the bag holding the SS after excess liquid is expelled, prior to sampling could then be used to extract ricin from the SS after sampling and used for ricin analysis; however, addition of 3% BSA could enhance ricin stability and thus, detection.

Equipment and Supplies:

1. Millipore[®] 0.22 µm Ultrafree[®] MC GV Sterile 0.5 mL Centrifugal Filter Unit with Durapore[®] PVDF Membrane: EMD Millipore, Billerica, MA, Cat. No. UFC30GV00
2. Amicon[®] Ultra-2 10K Centrifugal Filter UF Device: EMD Millipore, Billerica, MA, Cat. No. UFC201024
3. Eppendorf Centrifuge (Eppendorf North America, Hauppauge, NY): 5430/5430R with fixed angle rotor, or equivalent that can accommodate 15 mL conical centrifuge tubes. An Eppendorf centrifuge 5810/5810R with swinging bucket rotor may also be used.
4. 15-mL conical tubes

³User Guide: Amicon[®] Ultra-2 Centrifugal Filter Devices;

https://www.emdmillipore.com/US/en/product/Amicon-Ultra-2-mL-Centrifugal-Filters-for-DNA-and-Protein-Purification-and-Concentration,MM_NF-C86533#documentation

5. 1X PBS, endotoxin-free, pH 7.3 ± 0.2: Teknova, Hollister, CA, Cat. No. P0300 or equivalent
6. 1.0 mL micropipettor and respective 1.0 mL pipet tips
7. 2.0 mL microcentrifuge screw cap tube

Sample Pre-filtration

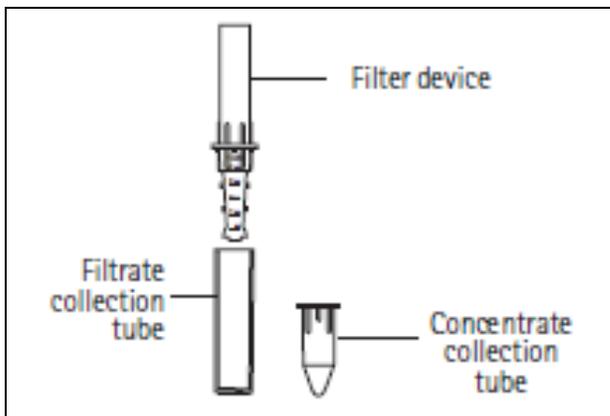
Note: For environmental samples, it is recommended to pre-filter the sample through a 0.22 micron filter prior to concentration by ultrafiltration.

1. Using a micropipettor, carefully transfer 500 µL of sample into a 0.22 µm Ultrafree[®] MC GV Sterile 0.5 mL Centrifugal Filter Unit with Durapore[®] PVDF Membrane, Yellow Color Coded (Millipore[®] Cat. No. UFC30GV00) with collection tube (for 2 mL sample use 4 units each with a collection tube).
2. Centrifuge tubes at 7,000 rpm for 9 minutes.

Note: Ensure that the supernatant has been completely filtered. Clean samples may take less than 9 minutes to complete filtration. Centrifuge for an additional 2 minutes if there is any liquid remaining in the filter.

3. Remove the filter unit using sterile disposable forceps, gripping only on the sides, and dispose to waste. Cap the tube and proceed to the next section.
4. The filtered sample aliquots will be combined (up to 2.0 mL) into the Amicon Ultra-2 10K Centrifugal device as described below.

Sample Processing using the Millipore Amicon Ultra-2 Centrifugal Devices



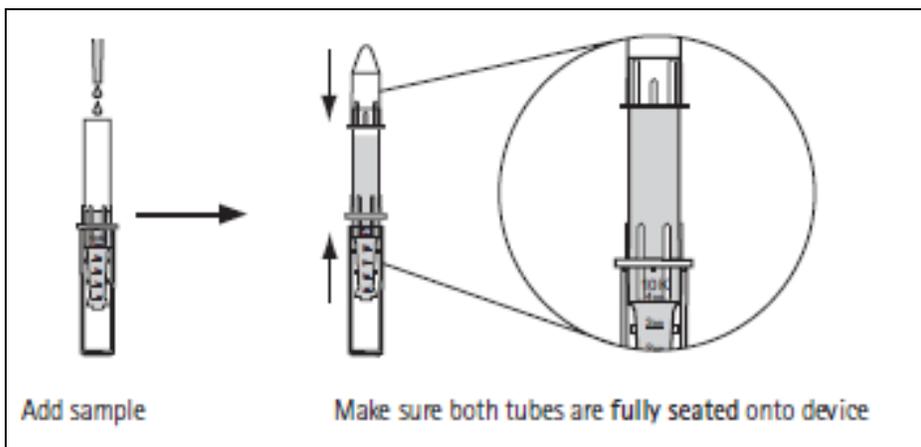
Amicon Ultra-2 Centrifugal Filter Device Parts

1. In a biosafety cabinet (BSC), set up for each sample, one Amicon[®] Ultra-2 10K Centrifugal Filter UF Device (Millipore[®], Cat. No. UFC201024) and one 1.5 mL microcentrifuge tube. Label each UF device, concentrate collection tube, and one 1.5 mL

microcentrifuge tube. Labeling the filtrate collection tubes is optional as long as the UF device is clearly labeled.

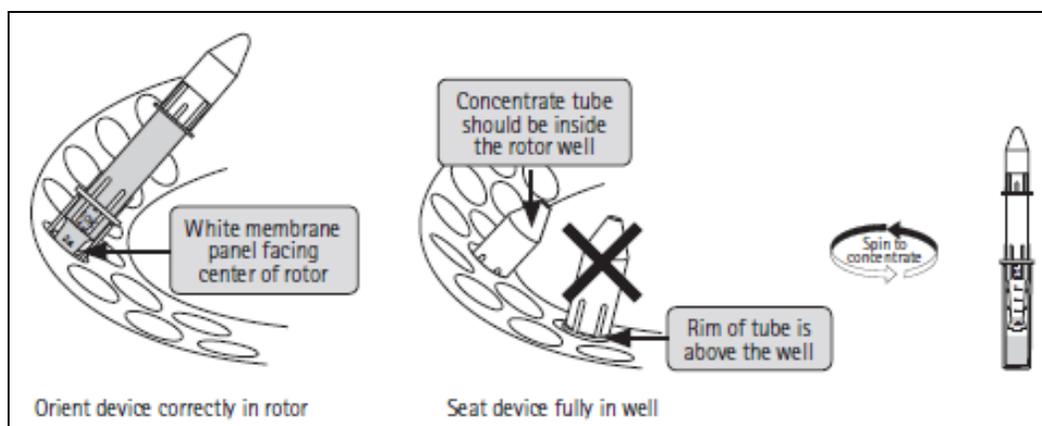
Note: It may not be necessary to label all the collection tubes as long as the Amicon® Ultra filter insert is clearly labeled.

2. In a BSC, briefly vortex-mix the samples in sample tubes (3–5 sec at ~3,200 rpm).
3. Allow the particulate matter to settle at the bottom of the tube for 1 min.
4. Process each sample as described in the following steps.
5. Insert the Amicon® Ultra-2 Centrifugal filter device into the filtrate collection tube making sure that the device is fully seated in the tube.
6. Using a 1.0 mL micropipettor and respective pipet tip(s), carefully transfer the sample (up to 2.0 mL) to the Amicon® Ultra-2 Centrifugal filter device (try to avoid particulate matter at bottom of the sample tube).
7. Using the concentrate collection tube, cap/cover the top of the Amicon® Ultra-2 Centrifugal filter device. This step generates a filter assembly.



Amicon Ultra-2 Centrifugal Filter Device Assembly

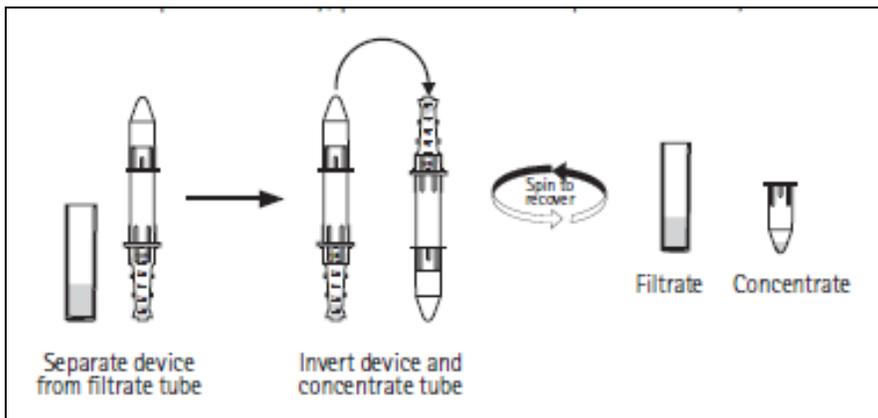
8. Place the filter assembly in a fixed angle microcentrifuge (Eppendorf® 5430/5430R or equivalent) or place into a 15-mL conical tube for a swinging bucket tabletop centrifuge (Eppendorf® 5810/5810R or equivalent) with 15-mL tube adapter. Counter balance the centrifuge rotor with a similar assembly. Make sure the device is seated on the bottom of the rotor well and that the rim of the concentrate collection tube is completely inside the well for the fixed angle rotor, or the device is completely within the 15-mL conical for the swinging bucket rotor/adapter.



9. For a fixed angle rotor, centrifuge at a maximum of 7,500 RCF for 10–60 min at 4°C, to leave 100 μ L in the device. For a swinging bucket rotor, centrifuge at a maximum of 4,000 RCF for ~60 min at 4°C, to leave 100 μ L in the device. *Note: For the 5810/5810R the maximum RCF is ~3,200, therefore 60 min was required for centrifugation.*
 10. If the sample volume is more than 2.0 mL, remove the filter assembly from the centrifuge and transfer the Amicon[®] Ultra-2 Centrifugal filter device with the concentrate collection tube on top to new filtrate collection tube. Dispose of the used filtrate collection tube with filtrate. Transfer the remainder of the sample to the respective Amicon[®] Ultra-2 Centrifugal filter device and repeat steps 6 to 9.
- Note: Ensure that the sample has been filtered, but the filter device is not dry. If there is more than 0.2 mL retentate is left, spin for additional time depending upon the volume left in the device.*
11. Remove the filter assembly from the centrifuge and transfer the Amicon[®] Ultra-2 Centrifugal filter device with the concentrate collection tube on top to new filtrate collection tube. Dispose of used filtrate collection tube with filtrate.
 12. Open the Amicon[®] Ultra-2 Centrifugal filter device and carefully pipet 1.5 mL of PBS for the first wash. Try to rinse the wall of the device while dispensing the buffer.
 13. Cap/cover the Amicon[®] Ultra-2 Centrifugal filter device again with the respective concentrate collection tube and centrifuge as described in step 9. *Note: if a lower rpm must be used, longer centrifugation times may be required.*
 14. Remove the filter assembly from the centrifuge and transfer the Amicon[®] Ultra-2 Centrifugal filter device with the concentrate collection tube on top and transfer to new filtrate collection tube. Dispose of used filtrate collection tube with filtrate.
 15. Repeat steps 12 and 13 for second wash.

Note: While two wash steps are described, additional wash steps may be used as described, for samples containing large quantities of potential interferences.

16. Remove the filter assembly from the centrifuge.
17. Remove the filtrate collection tube with filtrate and discard.
18. Invert the Amicon[®] Ultra-2 Centrifugal filter device with the concentrate collection tube and place back in the centrifuge. Counter balance the centrifuge rotor with similar assembly.



19. Centrifuge at 1000 RCF for 2 min at 4°C to collect the sample retentate in the concentrate collection tube.
20. Carefully, remove the Amicon[®] Ultra-2 Centrifugal filter device with the concentrate collection tube from the centrifuge.
21. Separate the concentrate collection tube containing the sample retentate from the filter device and discard the filter device.
22. Using a 1.0 mL micropipettor and respective pipet tip, transfer the sample retentate from the concentrate collection tube to a labeled 2.0 mL screw cap tube. Cap the tube.
23. Measure the sample retentate volume and adjust the volume to 0.1 mL with PBS for replicate sample analysis.
24. Store the processed sample at 4°C until the time-resolved fluorescence (TRF) immunoassay analysis is initiated.

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