

Abstract

Background: Many sample types may potentially be encountered in a bioterrorism event. Idaho Technology's fast and sensitive JBAIDS platform detects nucleic acids from biowarfare agents and other pathogens with real-time PCR. While many protocols and kits exist for purification of DNA or RNA, most are optimized for a single sample type or organism type. Our goal was to develop a minimal set of kits and protocols that can process a variety of sample and organism types, including bacterial spores from biothreat agents such as *Bacillus anthracis*. Here, we present four new IT 1-2-3™ Sample Purification Kits (FLOW, SWIPE, SCOOP and VIBE) that can isolate nucleic acids from bacteria, and DNA and RNA viruses from a variety of environmental, food, and biological samples. **Methods:** IT 1-2-3 Kits and protocols were developed for 17 sample types. The protocols use mechanical lysis to release nucleic acids from the pathogens, and a spin-filter column for subsequent purification. All protocols use similar buffer configurations for binding, washing and eluting, and incorporate custom steps for difficult sample types. Purified samples were evaluated using freeze-dried PCR assays on the JBAIDS platform. Two types of testing will be presented: (1) testing for PCR inhibitors, and (2) sensitivity validations with 7 bacterial organisms, 5 RNA viruses, and 1 DNA virus (all inactivated by gamma-irradiation). **Results:** Sensitivity with JBAIDS freeze-dried assays has been determined to be at least 1000 cfu or pfu per swab or ml of sample. Representative data from bacteria and DNA and RNA viruses in all 17 sample types will be presented. **Conclusion:** This sample purification system can process a wide array of organisms and matrices for analyses on the JBAIDS platform, thus facilitating greater bioterrorism preparedness.

	Whole Blood	Culture	Nasal Swab	Sputum	Stool	Plus Swab	Gastric Washing	Lymph node aspirate	Surface Swab	PBS	Soil	Water	Milk	Vegetable Wash	Ground Beef	Tuna Salad
<i>B. anthracis</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>B. melitensis</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>Burkholderia</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>Y. pestis</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>C. burnetii</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>F. tularensis</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>R. prowazekii</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Orthopox	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Varicella	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
EEE	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
VEE	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
WEE	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Ebola	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Marburg	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

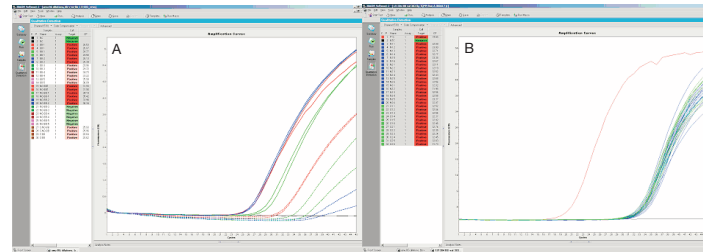


Figure 2. Lysis of Bg spores by bead beating. A) A dilution of Bg spores subjected to bead-beating (solid lines) or no lysis (dashed lines). B) Spores lysed in 2 mL tubes (green) versus 15 mL tubes (blue).

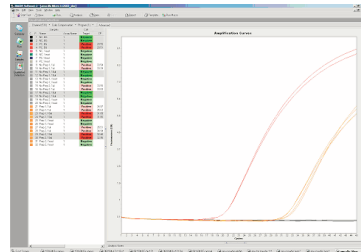


Figure 3. IT 1-2-3 FLOW kit effectively removes inhibitors found in air samples collected on a DFU filter and washed. Bg spore DFU samples were purified with the IT 1-2-3 FLOW kit (orange) or not purified (grey) prior to testing.

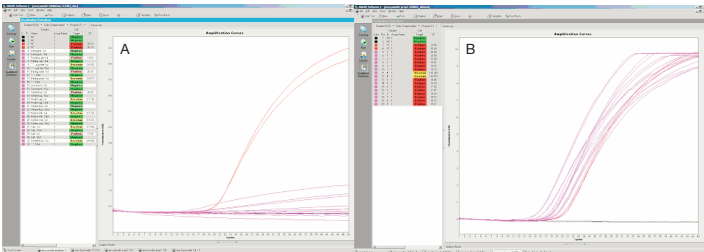


Figure 4. IT 1-2-3 SWIPE purifies common components of powder. Different types of powder were added to Bg powder (containing spores) and tested directly or purified. A) No purification prior to testing. B) Purification with the IT 1-2-3 SWIPE kit prior to testing.

Table 1. Validation completed for listed combinations. Note: Sensitivity validated at 1000 cfu/mL or 10,000 pfu/mL (or per swab or gram where appropriate). Protocols are color-coded by kit type: IT 1-2-3 FLOW, IT 1-2-3 SWIPE, IT 1-2-3 VIBE, and IT 1-2-3 SCOOP.

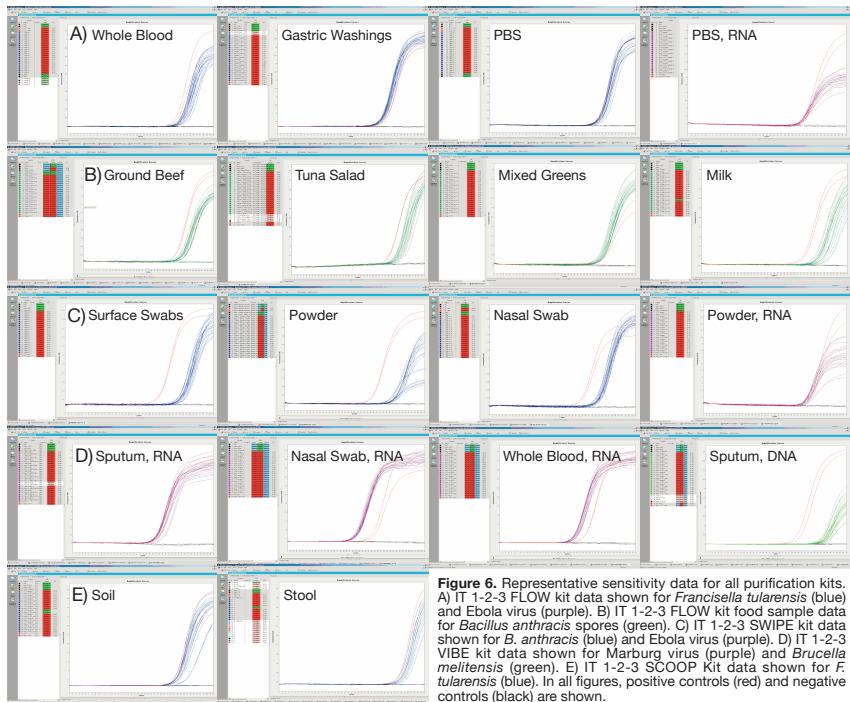


Figure 6. Representative sensitivity data for all purification kits. A) IT 1-2-3 FLOW kit data shown for *Francisella tularensis* (blue) and Ebola virus (purple). B) IT 1-2-3 FLOW kit food sample data for *Bacillus anthracis* spores (green). C) IT 1-2-3 SWIPE kit data shown for *B. anthracis* (blue) and Ebola virus (purple). D) IT 1-2-3 VIBE kit data shown for Marburg virus (purple) and *Brucella melitensis* (green). E) IT 1-2-3 SCOOP Kit data shown for *F. tularensis* (blue). In all figures, positive controls (red) and negative controls (black) are shown.

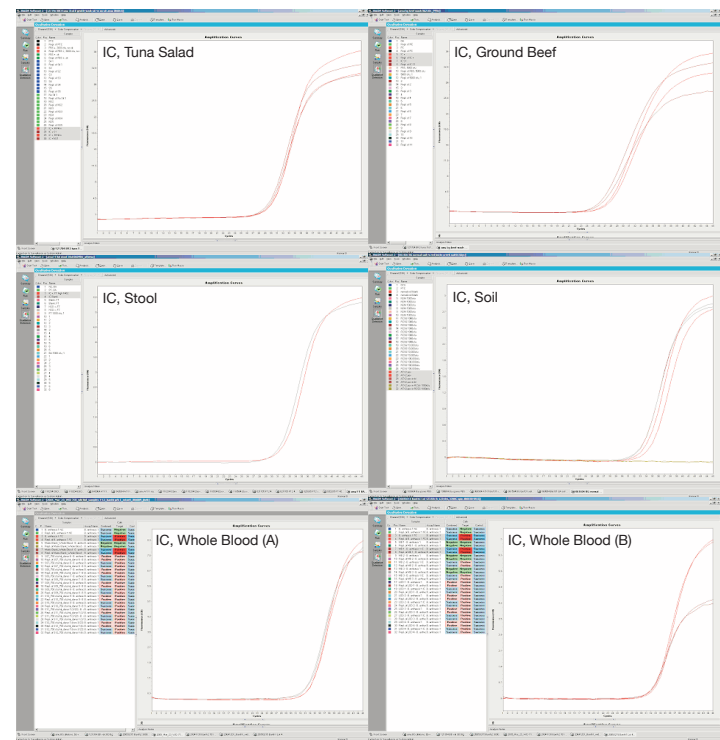


Figure 5. Inhibition controls show that inhibitors of PCR are removed. In every figure, red curves are positive controls and brown curves are positive controls spiked with purified sample that does not contain PCR target.

Background

A new simple set of sample purification kits was designed to meet the needs of biowarfare detection from the many sample types encountered. The kits were designed for Idaho Technology's fast and sensitive JBAIDS platform that detects nucleic acids from biowarfare agents and other pathogens with real-time PCR. Many existing protocols and kits for purification of DNA or RNA are available, but they are usually optimized for a single sample type or organism type. Our goal was to develop a minimal set of kits and protocols that can process a variety of sample and organism types, including bacterial spores such as *Bacillus anthracis*. Here, we present four new IT 1-2-3 Sample Purification Kits (FLOW, SWIPE, SCOOP and VIBE) that can isolate nucleic acids from bacteria, and DNA and RNA viruses in a variety of environmental, food, and biological samples and are highly compatible with the JBAIDS and R.A.P.I.D.® detection

Materials and Methods

Optimization of Protocols: Sample purification protocols were adapted from the simple procedures in the IT 1-2-3 RAPID DNA Purification Kit previously designed for use with environmental swab samples. The same basic techniques (lysis and spin filter binding) and buffers were used in the new generation kits. More complex sample types require proteases or new buffers, but the basic procedures are generally followed. The kits were primarily designed to purify bacterial and viral nucleic acids from samples, remove inhibitors of PCR, and provide enough purified material for multiple tests using JBAIDS freeze-dried reagents.

Lysis by Bead Beating: Mechanical lysis, accomplished with bead beating, is the primary method the IT 1-2-3 kits use to break open bacterial and viral organisms. Bead beating parameters were optimized for various volumes (200 uL and 5 ml samples) using Bg (*Bacillus atrophaeus* (globigii)) spores. For large volumes, antifoam is added to the bead tube to ensure free movement of the beads through the sample.

Protocols and Kits: Protocols from each kit are briefly outlined in Figure 1. Pictures of each kit with components and sample size information are contained in Figure 1.

Organisms and Reagents: Most development occurred with live *Bacillus atrophaeus* (formerly globigii, referred to as Bg) (spores and vegetative cells) or Inactivated Organism (IO, inactivated by gamma irradiation) provided by the Department of Defense Critical Reagents Program. PCR assays used for development and validation are proprietary or assays developed for the JBAIDS program. Validation testing was performed with freeze-dried reagents on JBAIDS or RAPID instruments.

Inhibition Controls: For inhibition controls, positive control freeze-dried reagents (containing target nucleic acid) are spiked with purified samples. A non-spiked positive control is run for comparison. If the spiked and non-spiked positive controls amplify similarly, the sample is considered free of PCR inhibitors. **Sensitivity Testing:** To determine the sensitivity of the JBAIDS system, which includes the IT 1-2-3 Kits, inactivated organism was spiked into multiple sample matrices then purified using the appropriate IT 1-2-3 Kit. All protocols were optimized to detect biowarfare pathogens at or below 1000 cfu/mL or 10,000 pfu/mL (or per swab or gram) using the JBAIDS freeze-dried reagents and detection system. Sensitivity was determined for 165 matrix/organism combinations (see Table 1).

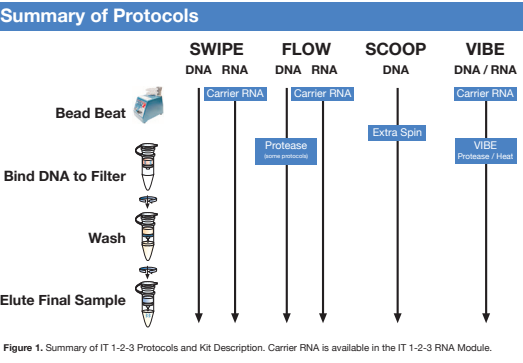


Figure 1. Summary of IT 1-2-3 Protocols and Kit Description. Carrier RNA is available in the IT 1-2-3 RNA Module.



IT 1-2-3 FLOW Kit
Start Volume: 3-5 mL
End Volume: 800 uL
Time to completion: 1 hour

Large volume liquid samples
DNA/RNA

IT 1-2-3 VIBE Kit
Start Volume: 400 uL or swab
End Volume: 100-200 uL
Time to completion: 1 hour

DNA/RNA from Sputum
and viral RNA from blood
or nasal swabs



IT 1-2-3 SWIPE Kit
Start Volume: Swab
End Volume: 200 uL
Time to completion: 30 min

Swab samples
DNA/RNA



IT 1-2-3 SCOOP Kit
Start Volume: 0.5 g or 0.5 mL
End Volume: 200 uL
Time to completion: 1 hour

Soil and stool
DNA

Results and Discussion

Bead beating is an effective way to lyse spores as well as other bacteria and viruses. Live Bg spores (treated with DNase to remove exogenous DNA) are effectively lysed with IT 1-2-3 bead tubes using Scientific Industries Disruptor Genie with 2.0 mL tube adaptor, as well as with IT's Large Bead tube adaptor (PREP-ASY-0001) for 5 mL volumes of liquid in 15 mL conical tubes. Spores lysed by bead-beating show a 100-fold (6-8 cycle difference) increase in detection over non bead-beaten spores (see figure 2). Bead beating results are comparable in both 2.0 mL tubes (200 microliter sample size) and 15 mL tubes (5 mL sample size) (see figure 2). All IT 1-2-3 kits utilize bead beating, in small or large volumes, for lysis. This allows for kits with minimal protocol variations to increase the number of organisms, including spore forming bacteria and RNA viruses, tested for from a single purified sample. Bead beating is effective for all bacteria and viruses tested.

IT 1-2-3 Kits effectively remove inhibitors found in samples. Many inhibitors of downstream analysis are present in the sample types tested. Most of these PCR inhibitors are removed by the IT 1-2-3 Kits. Inhibitors found in various types of powder and air samples, common environmental samples, and air samples tested with no purification versus purification with the appropriate IT 1-2-3 protocol. Inhibition control tests for five difficult sample types are shown in Figure 5, which shows that the IT 1-2-3 Kits remove most inhibitors of PCR. One exception is that inhibitors are not entirely purified from some types of soil. However, after purification, an additional 1/2 or 1/10 dilution removes all inhibitors found in soil.

IT 1-2-3 Kits met the sensitivity goals for all samples. The IT 1-2-3 kits met or exceeded the sensitivity goals of 1000 cfu/mL or 10,000 pfu/mL (or per swab or gram) for all 165 matrix/organism combinations when using JBAIDS freeze-dried reagents and detection system. Representative data for sensitivity testing is shown in figure 6.

Results Summary:

- Bead beating is utilized for mechanical lysis of spores, gram-negative and gram-positive bacteria, and viruses
- PCR Inhibitors from dirty samples are effectively removed with IT 1-2-3 kits for downstream analysis with the JBAIDS or RAPID systems.
- Sensitivity: Protocols are validated to detect at least 1000 cfu/mL or 10,000 pfu/mL (or per swab or gram) of listed pathogens

Conclusions

We developed four new sample purification kits for use in biowarfare agent detection. These sample purification kits, when used with the JBAIDS assays and detection platform, provide clean samples well suited for the detection of fourteen bacteria and viruses in a variety of sample types. The kits also provide samples suited for any assay on the JBAIDS or RAPID platforms. It is a challenge to create a simple kit system to purify many different samples and organisms (bacteria, including spore-formers, DNA and RNA viruses). We have attempted to limit protocol variations and the result is a simple system containing four kits that can purify DNA and RNA from 17 sample types.

Advantages of the IT 1-2-3 system include:

- Kits include beads and reagents for mechanical lysis of spores
- The system includes everything needed for sample pre-purification steps
- Protocols can purify nucleic acids from bacteria and viruses in the same sample
- Protocols provide enough end material to test for 10 organisms
- Protocols do not include time-consuming enrichment steps
- Kits contain all consumables needed for extractions (buffers, spin filters, tubes, and transfer pipets) except pipette tips

Acknowledgments

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