

ABSTRACT

The JBAIDS requires nucleic acid purification and identification of five RNA viral threat agents from seven sample matrices. Assay development and validation primarily involved the use of inactivated organism (IO). Because viral RNA genomes present unique challenges due to RNA instability, two inactivation methods were compared: gamma-irradiation (γ -IO) and Trizol-treatment (Trizol-IO). Trizol-IO and γ -IO lots of Eastern Equine Encephalitis, Western Equine Encephalitis, Venezuelan Equine Encephalitis, Marburg, and Ebola virus were prepared from the same stock of live virus. The lots of inactivated organism were purified using the IT 1-2-3 SWIPE kit following procedures for RNA viruses. Every purified sample was tested with two reverse transcription-polymerase chain reaction (RT-PCR) assays specific for the spiked virus. The real-time PCR amplification data was used to compare the relative performance of template extracted from Trizol-IO to that of γ -IO template. Sample purification recovery of γ -IO relative to Trizol-IO was also evaluated for six sample matrices. For all RNA viruses and assays tested, Trizol-IO stocks have 10-10,000 fold higher concentration of amplifiable target than γ -IO. Of interest, a large variation was observed in the relative target concentration between two assays for the same virus. This variation can be correlated to amplicon size. Recovery of amplifiable target from all tested matrices was similar for γ -IO and Trizol-IO, except in human whole blood where Trizol-treated organism recovery was roughly 500-fold lower. Trizol-treatment is significantly better than gamma-irradiation for preserving the integrity of viral RNA genomes. However, results from whole blood samples show that Trizol-treated IO can interfere with sample extraction and/or downstream RT-PCR.

BACKGROUND

Viruses with RNA genomes are fragile, especially when viruses are structurally compromised, due to the prevalence and robustness of RNases. For safety reasons, testing of RT-PCR assays for RNA viruses preferentially occurs with inactivated organism. However, methods that inactivate RNA viruses (such as gamma-irradiation, UV light, or β -propiolactone) can lead to degradation of the RNA genome. An alternate method for RNA virus inactivation, treatment in Trizol reagent, maintains the integrity of the RNA, while disrupting cells and dissolving cell components. In this poster, we report the results of studies using gamma-irradiation or Trizol-treatment to inactivate Eastern Equine Encephalitis, Western Equine Encephalitis, Venezuelan Equine Encephalitis, Marburg, and Ebola virus, and the effects of both inactivation methods on recovery of RNA from samples in complex matrices.

METHODS

Live organism was inactivated by trizol-treatment (1:4 or 1:5 dilution of stock virus in Trizol) or gamma-irradiation (β -propiolactone inactivation followed by cobalt (γ) irradiation at 3.0×10^6 rads, total dose). Before RNA purification, gamma-irradiated organism (γ -IO) was diluted in Trizol or water at a ratio of 1 part organism to 3 or 4 parts Trizol or water. For RNA purification, 10 μ l of organism (Trizol-IO, γ -IO diluted in Trizol, and γ -IO diluted in water) was added to a small bead tube containing 16 μ g of carrier RNA. Samples were processed using the IT 1-2-3 SWIPE kit following the culture protocol for RNA purification. Three or four consecutive 10-fold dilutions of each of the purified RNAs were subjected to RT-PCR amplification using two assays specific for the organism. Relative concentration of γ -IO to the Trizol-IO was calculated by using the Trizol-IO as the standard curve and using the calculated concentrations to determine the relative concentration factor. Relative concentrations obtained with the SWIPE kit were compared to results from two additional purification methods, IT 1-2-3 FLOW kit and standard Trizol extraction.

After adjustment to the Trizol-IO, both stocks (Trizol-IO and γ -IO) of Eastern Equine Encephalitis, Western Equine Encephalitis, and Venezuelan Equine Encephalitis organism were purified from six matrices using sample purification kits developed at Idaho Technology. Eleven to thirty samples were processed for each matrix, on each type of organism at the levels listed below.

Table 1. Sample Purification Kit Information

Matrix	Sample Purification Kit	Concentration	Starting Amount
Whole Blood	IT 1-2-3 VIBE Kit	1000 pfu/ml	400 μ l
Nasal Swab	IT 1-2-3 VIBE Kit	1000 pfu/swab	1 swab
Air Sample into PBS/0.1% Triton-X	IT 1-2-3 FLOW Kit	1000 pfu/ml	5 ml
Powder	IT 1-2-3 SWIPE Kit	100 pfu/1 μ g	10 μ g
Surface Swab	IT 1-2-3 SWIPE Kit	1000 pfu/swab	1 swab
Culture Media	IT 1-2-3 SWIPE Kit	1000 pfu/20 μ l	40 μ l

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RESULTS

Relative concentrations for γ -IO compared to Trizol-IO are shown in Table 2. For all organisms, Trizol-IO stocks have a higher concentration (10-10⁵ times more) of amplifiable template than γ -IO stocks. Of interest, the effect of gamma-irradiation (as calculated in the relative concentrations) varies greatly between organisms and even between assays for the same organism. Representative RT-PCR quantification curves are shown in Figure 1. All normalization factors were confirmed through additional testing (data not shown).

Relative concentrations determined with other extraction methods (IT 1-2-3 FLOW kit and Trizol extraction) provided similar results. Average crossing points (Ave Cp) and relative concentration data for Ebov3 are shown in Table 3. Quantification curves, for all Ebov3 extractions, are shown in Figure 2.

For all assays tested, Trizol-IO consistently performed worse in blood than γ -IO. Selected RT-PCR amplification curves are shown in Figure 3. Results in other matrices varied depending on organism.

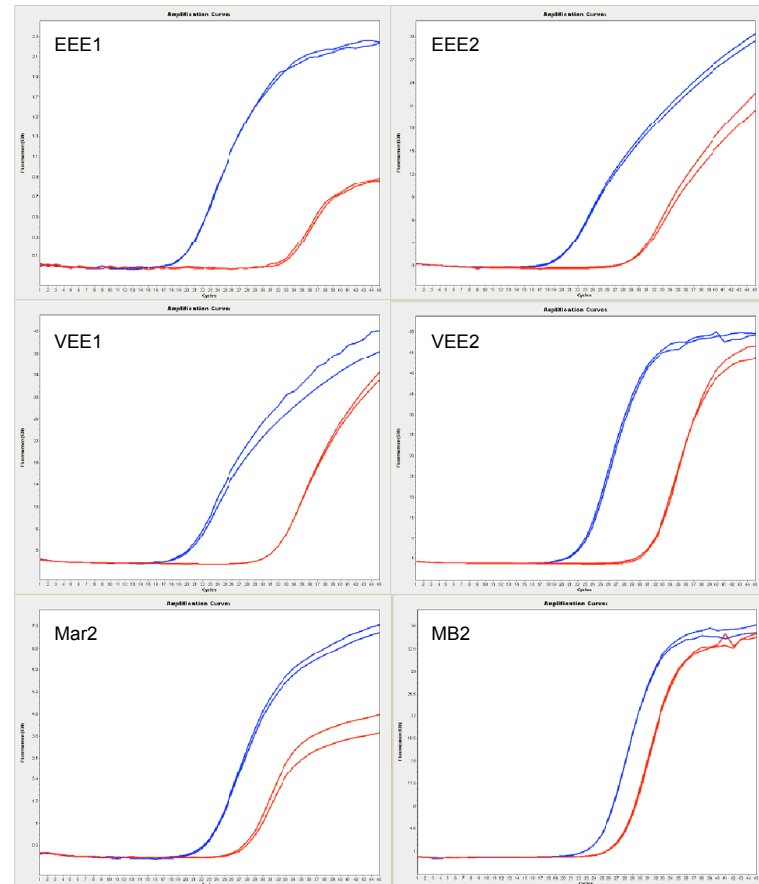


Figure 1. Selected RT-PCR Results Comparing Trizol-IO and Gamma-IO after Purification with the IT 1-2-3 SWIPE Kit. Trizol-treated organism (blue) and gamma-irradiated organism (red) of the same concentration are shown.

DISCUSSION

Trizol-Treatment and Gamma-Irradiation for RT-PCR Assay Testing Data from all organisms and assays tested show that γ -IO performs worse than Trizol-IO. The Trizol method of RNA virus inactivation is gentler than that of gamma-irradiation. Moreover, Trizol reagent maintains the integrity of RNA by inactivating nucleases, thus providing a stable, protective environment for the RNA template to be stored. With Trizol, more of the target template remains intact and suitable for PCR, as is expected with live virus in real-life samples. These findings are substantiated by the published studies of Hu *et al.* and Studer *et al.* which report that RNA treated by gamma-irradiation is damaged and less amplifiable (1, 2). A drawback to Trizol treatment is inhibition in many downstream analyses (including RT-PCR) and toxicity of the reagent. In addition, Trizol-IO should not be used to assess sensitivity in whole blood using the IT 1-2-3 VIBE kit due to unknown interactions between the matrix, Trizol, and/or reagents used for sample purification.

Relative Concentration Variations between Organisms The level of target degradation in γ -IO was variable. The difference between γ -IO and Trizol-IO is larger for the Equine Encephalitis (EE) viruses than it is for Ebola and Marburg. Virus structure may play a role in viral susceptibility to gamma-irradiation (3). Factors such as viral concentration and components in the stock organism (growth factors, host cells, etc) may also influence virus sensitivity to gamma-irradiation.

Relative Concentration Variations between Assays for a Given Organism Damage due to gamma-irradiation has different effects on different assays for a given organism when tested with RT-PCR. Gamma-irradiation directly damages RNA, causing sections of the genome to be unamplifiable (1). A simple explanation for assay differences is that assays with larger targets are more likely to encounter a section of RNA with gamma-induced damage and therefore yield a lower relative functional concentration. Another explanation may be that virus structure is damaged during gamma-irradiation causing RNA to be exposed to ribonucleases present in the stock. Damage by this mechanism is minimized by Trizol because it inhibits ribonuclease activity. Ribonucleases activity can reduce amplification of longer targets. The assay information summarized in table 4 suggests amplicon size affects assay performance with gamma-irradiated organism. However, inconsistencies within the data set suggest the mechanism for target damage due to gamma-irradiation is more likely a complex interaction of several factors.

Table 2. Crossing Point and Relative Concentration Results for All Viruses.

Virus/Strain	Inactivation Method	Ave. Crossing Point (Cp)	Relative Conc: Gamma to Trizol
EEE PE6	Trizol	EEE1 19.6	
		EEE2 19.0	
EEE PE6	Gamma	EEE1 32.7	EEE1 0.00007 X
		EEE2 28.7	EEE2 0.002 X
VEE 1A/B	Trizol	VEE1 19.6	
		VEE2 21.7	
VEE 1A/B	Gamma	VEE1 31.3	VEE1 0.0009 X
		VEE2 30.6	VEE2 0.006 X
WEE CBA 87/4	Trizol	WEE1 20.4	
		WEE2 19.4	
WEE CBA 87/4	Gamma	WEE1 33.0	WEE1 0.0001 X
		WEE2 30.3	WEE2 0.0003 X
Ebola Zaire	Trizol	Ebov2 27.1	
		Ebov3 23.8	
Ebola Zaire	Gamma	Ebov2 31.2	Ebov2 0.09 X
		Ebov3 27.5	Ebov3 0.2 X
Marburg Musoke	Trizol	Mar2 22.0	
		MB2 23.9	
Marburg Musoke	Gamma	Mar2 33.0	Mar2 0.05 X
		MB2 31.6	MB2 0.1 X

Table 3. Results for Ebov3 on Three Extraction Methods

Extraction Method	Trizol-IO Ave Cp	Gamma-IO Ave Cp	Relative Conc: Gamma to Trizol
IT 1-2-3 SWIPE	27.3	29.2	0.2 X
IT 1-2-3 FLOW	27.3	29.4	0.2 X
Trizol Extraction	28.5	30.3	0.1 X

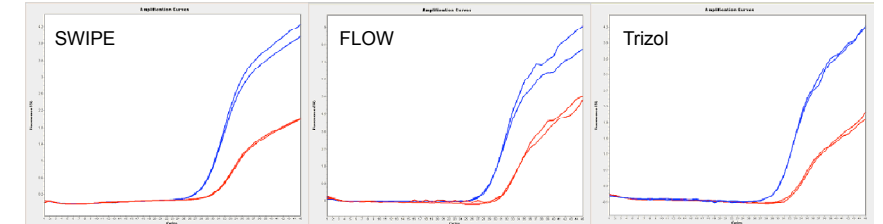


Figure 2. Ebov3 Quantification Curves of Trizol-IO and Gamma-IO after Purification with Three RNA Extraction Methods. Trizol-treated organism (blue) and gamma-irradiated organism (red) of the same concentration are shown.

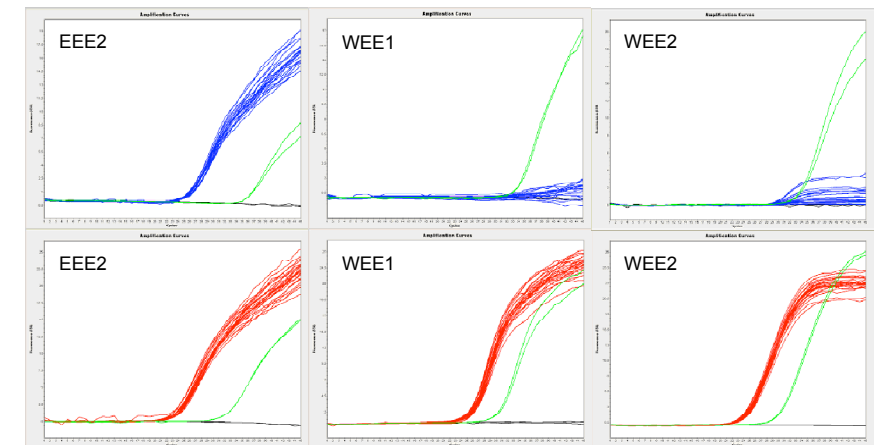


Figure 3. Selected RT-PCR Results of Trizol-IO and Gamma-IO after Purification from Whole Blood using the IT 1-2-3 VIBE Kit. Trizol-treated organisms (blue) and gamma-irradiated organism (red) were run separately. Positive controls (green) and negative controls (black) were included in every run.

CONCLUSIONS

Neither method of inactivation, gamma-irradiation or Trizol-treatment, provided an ideal alternative to live virus. RNA from γ -IO was damaged, reducing the amount of target amplifiable by RT-PCR. Trizol-IO reacted unfavorably when RNA was purified with the IT 1-2-3 VIBE kit in the presence of whole blood. Since relative concentrations of γ -IO to Trizol-IO were reproducible in multiple purification systems, all verification and validation testing performed with RNA virus for JBAIDS was conducted with γ -IO functionally normalized to Trizol-IO. This solution allowed us to test at levels as similar as possible to live intact virus to more accurately assess the sensitivity of the JBAIDS in real life situations.

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Table 4. Relation of Relative Concentration and Amplicon Size

Assay	Relative Conc.	Amplicon Size (bp)
EEE1	0.00007 X	216
EEE2	0.002 X	69
VEE1	0.0009 X	158
VEE2	0.006 X	73
WEE1	0.0001 X	193
WEE2	0.0003 X	163
Ebov2	0.09 X	121
Ebov3	0.2 X	105
Mar2	0.05 X	108
MB2	0.1 X	80