APPLICATION NOTE

CE IVD

Diagnosis of Enteropathogenic Viruses from Clinical Stool Samples Using EchoLUTION™ Nucleic Acid Extraction Technology

Maika Schiwy[1], *Christoph Schönfels*[1], *Maximilian Weiter*[2], and *Laura Torres Benito*[2]

[1] Department of Molecular Biological Diagnostic – PCR, Medizinisches Labor Ostsachsen MVZ GbR, Cottbuser Str. 11, 02826 Görlitz, Germany.
[2] BioEcho Life Sciences GmbH, Biocampus Cologne, Main Building, Nattermannallee 1, 50829 Cologne, Germany.

Correspondence
Dr. Laura Torres Benito
BioEcho Life Sciences GmbH
Email: laura.torres.benito@bioecho.de

Viral gastroenteritis is one of the most common infectious diseases in humans and is a significant cause of morbidity and mortality worldwide. State-of-the-art clinical diagnostics of stool viruses is performed using the real-time polymerase chain reaction (qPCR). The detection sensitivity of this method partly depends on the reliability and efficiency of nucleic acid extraction. The aim of this work was to evaluate the EchoLUTION technology for nucleic acid extraction of enteropathogenic viruses from patient stool samples. The EchoLUTION Viral RNA/DNA Swab Kit Plus from BioEcho Life Sciences, which we used for nucleic acid extraction in this study, operates on the principle of single-step purification. We assessed the purification efficiency, reliability, and practicality of the method, and we compared it with an established and automated standard method (MagNA Pure® 96 System from Roche®). For this purpose, a total of 173 clinical samples were collected and processed in parallel with both methods. The results confirmed that the EchoLUTION Viral RNA/DNA Swab Kit Plus is suitable for the extraction of nucleic acids from enteropathogenic viruses and ensures reliable diagnosis faster than with the other method.

Introduction

Enteropathogenic viruses, such as adenoviruses, rotaviruses, astroviruses, noroviruses, and sapoviruses, are responsible for one of the most prevalent infectious disorders affecting people: gastroenteritis. Children under the age of five are particularly affected, and more than 700 million cases of acute diarrhea are estimated to occur in this age group each year. Mortality associated with gastroenteritis is calculated at three to five million cases per year, and most of these cases occur in developing countries[1]. Infectious gastroenteritis is thus one of the five leading causes of death worldwide[2]. Whereas mortality is less common in developed countries, gastroenteritis frequently leads to doctor visits and hospitalizations. Due to the increasing number of infectious diseases caused by viruses, reliable and rapid clinical diagnostic tests are essential to better assess the course of diseases and, thus, design an optimal treatment for the patient.

In recent years, PCR has become the gold standard for detecting nucleic acids from viral pathogens. This detection method assures great sensitivity and allows high-throughput implementation. Importantly, the quality of the sample extract is decisive for a reliable diagnosis and detection and mainly depends on the efficacy of the nucleic acid extraction procedure. The quality of extraction is of particular importance when using native stool samples, as successful removal of accompanying substances and microorganisms is necessary. In particular, PCR inhibitors must be eliminated to prevent amplification failure and the corresponding false-negative results.

In this application note, we evaluate an alternative method for extracting nucleic acids from enteropathogenic viruses within clinical patient samples and compare it with an established standard method. For this purpose, a total of 173 patient samples were collected and processed with two nucleic acid extraction kits. We analyzed performance and handling of the kits as well as reproducibility of the methods. The two kits used were the EchoLUTION Viral RNA/DNA Swab Kit Plus from BioEcho Life Sciences, which provides a single-step purification, and the MagNA Pure 96 System from Roche, which is a standard automated extraction platform based on magnetic beads. The Ct values at which targets were detected by qPCR was slightly lower with MagNA Pure than with EchoLUTION. The PCR efficiency for both methods was comparable for all parameters tested. However, EchoLUTION required significantly less time, and handling was easier compared to the automated standard method, even though the manual EchoLUTION workflow was used (automated processing is also possible with EchoLUTION). Our results suggest that the EchoLUTION Viral RNA/DNA Swab Kit Plus is a robust method that is suitable for the extraction of nucleic acids from enteropathogenic viruses to ensure rapid and reliable diagnosis.

Materials and Methods

Clinical sample preparation

For this study, fresh human patient stool samples were collected over a two-month period. Samples were identified beforehand as positive or negative for gastroenteritis with the routinely used MagNA Pure 96 (Roche) diagnostic system in the Medizinisches Labor Ostsachsen MVZ. From each fresh stool sample, we took approximately 45–50 mg using a 10 µL sterile inoculating loop and resuspended the sample in 2 mL TE buffer (1 x, pH 8.0 low EDTA, PanReac- AppliChem). The samples were then vortexed at 300 rpm for 5 seconds and subsequently centrifuged at 10,000 x g for 15 seconds. The supernatant was aliquoted and frozen at –20 °C. In total, 168 positive and 5 randomized negative control samples were collected. Just before nucleic acid extraction, the aliquots were thawed at room temperature and processed immediately.

Internal control

An internal control (IC) containing viral RNA was used in individual experiments to confirm successful nucleic acid extraction, to check the integrity of the reagents, to assess contamination, and to determine PCR inhibition (amplification control). We used the IC provided in the PCR assay RIDA® GENE Viral Stool Panel I Assay/Sapovirus Assay (R-Biopharm®).

Nucleic acid extraction

Nucleic acids were extracted in parallel with two different methods: the standard extraction method of the Medizinisches Labor Ostsachsen MVZ (MagNA Pure 96 kit from Roche using an automated magnetic bead-based extraction) and the EchoLUTION Viral RNA/DNA Swab Kit Plus (BioEcho).

For the automated extraction with the MagNA Pure 96 platform, we used the ready-to-use reagent kit (DNA and Viral NA Small Volume Kit 2.0, Roche) according to the manufacturer's instructions. To the 180 µL patient sample, we added 20 µL of the IC described above. After initialization of the program, the samples were processed fully automated. The elution volume of the purified nucleic acids was 100 µL.

For the EchoLUTION Viral RNA/DNA Swab Kit Plus, we processed 96 samples at a time manually using the single-step process. The manufacturer's instructions were followed with the following deviations: Instead of 50 µL LyseNtact buffer, we transferred 70 µL LyseNtact and 20 µL IC (RIDA®GENE VSP I/Sapovirus, R-Biopharm)

into each well. The reason for that is that we considered the IC as part of the sample. To reach a 1:1 dilution of lysis buffer with sample, we increased the volume of lysis buffer to 70 µL. Subsequently, 50 µL of well-mixed patient sample was pipetted into each well in the prepared lysis plate. After centrifuging for one minute, the flowthrough was collected and used for diagnostic testing.

Qualitative nucleic acid detection with real-time PCR

The RIDA®GENE Viral Stool Panel I Assay (R-Biopharm) was used for direct qualitative detection of adenovirus DNA, norovirus RNA, rotavirus RNA, and astrovirus RNA by multiplex qPCR. The RIDA®GENE Sapovirus Assay (R-Biopharm) was used for the detection of sapovirus RNA. Master mixes for both assays were prepared according to the user manual. However, to reduce costs, the recommended volume of the master mix used was reduced by half, resulting in the following composition for each reaction: 9.65 µL reaction mix, 0.35 µL enzyme mix, and 2.5 µL sample, positive control, or negative control. The initial concentration of the positive control for each pathogen (10^5 copies/µL) was diluted to add a final concentration of 2.5×10^5 copies/µL to each PCR run. The processed samples were transferred to the master mix in the PCR plate, which was then sealed with an optical film. PCR amplification was performed by the LightCycler® 480 System (Roche) using the RIDA®GENE Universal Protocol and the RIDA®GENE Color Compensation Kit IV (CC4) and Kit II (CC1).

Results

Determination of the clinical decision limit

Clinical decision limits (CDLs) are essential for the interpretation of numerical clinical pathology results. For the qualitative detection of the individual enteropathogenic viruses, the detected Ct value is evaluated. The lower limit of the detectable Ct range for positive samples is defined in relation to the ICs and the CDL. To determine the CDL (also referred to as the cut-off value for a test parameter), we first calculated the mean Ct values of all measurements for each positive control. Subsequently we added 6.6 cycles (corresponding to two log levels; see Table 1). If the Ct value of the sample

Table 1. Determination of decision limits for the individual assay parameters based on positive control (PC) Ct values. *AdV, adenovirus DNA; NV, norovirus RNA; RV, rotavirus RNA, AsV, astrovirus RNA; SV, sapovirus RNA; calculation of arithmetic mean ($\bar{X}$) and standard deviation (s) of the sample.*

Measurement	Ct values (PC)				
	AdV	NV	RV	AsV	SV
1	21.21	22.60	20.61	24.68	25.75
2	21.12	21.66	18.89*	23.81	24.33
3	21.33	21.99	23.03	24.05	24.25
4	21.46	22.21	26.92	26.95	23.43
5	21.73	22.71	32.72*	24.67	23.18
6	21.44	22.01	27.53	24.02	25.88
7	21.53	22.03	23.04	24.07	25.92
8	21.43	23.89	28.92	25.82	23.79
9	22.19	23.81	25.86	23.92	23.76
$\bar{X}$	21.5	22.55	25.13	24.67	24.57
s	0.32	0.81	2.98	1.06	1.07
CDL or cut-off value (Ct)	**28.1**	**29.15**	**31.73**	**31.27**	**31.17**

**Outliers were discarded for the statistical calculations.*

is below the defined CDL, the sample is considered "positive (+)", meaning that the virus was detectable in the sample. If, however, the Ct value is above the CDL or no signal can be measured, the sample is evaluated as "negative (-)" and the target sequence was not detected. It must be mentioned that the CDL in this case was determined using the established method (MagNA Pure). For further experiments, we recommend determining the cut-off value for each method used individually, in this case also for the EchoLUTION technology.

Table 2. Samples of the individual test parameters (adenovirus, norovirus, rotavirus, astrovirus, and sapovirus). *Except for the negative controls, all the samples were previously determined to be positive.*

Parameter	Sample size	Positive	Negative
AdV	34	33	1
NV	39	38	1
RV	61	60	1
AsV	19	18	1
SV	20	19	1
Total	173	168	5

Qualitative detection by qPCR: evaluating EchoLUTION

After determining the CDL, the patient samples were analyzed for the presence of adenovirus, norovirus, rotavirus, astrovirus, and/or sapovirus. We analyzed a total of 173 samples (168 positives, 5 negatives) to compare the performance of two different methods: the EchoLUTION Viral RNA/DNA Swab Kit Plus and the automated MagNA Pure 96 System (Table 2). An IC was included in each sample to verify successful extraction. For simplicity, the test parameters are listed individually, even when adenovirus DNA, norovirus RNA, rotavirus RNA, and astrovirus RNA were detected within one reaction set using multiplex PCR.

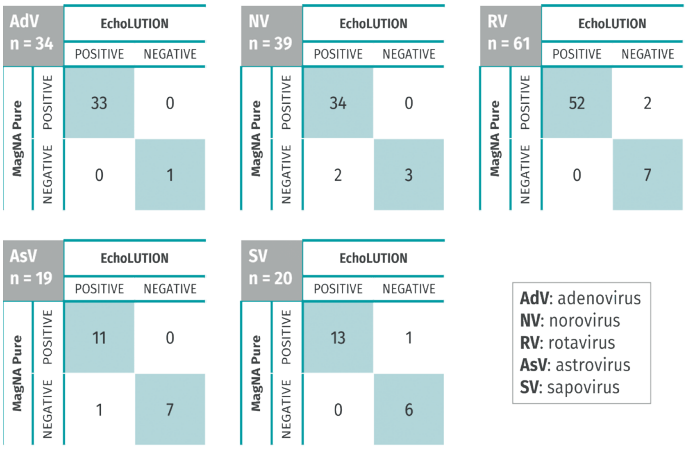
The assay result was the same for both processing methods for 167 of 173 samples (96.5 %), indicating that the performance of both methods is almost equivalent (Figure 1). In total, 24 stool samples were assessed as "negative" or "not detectable" for both processing methods. With the inclusion of the five internal negative controls, we determined that there was no cross-contamination during the processing. The six samples for which the assay result was different were as follows: RV2 and SV1 were not detected and RV49 was negative with the EchoLUTION extraction technology; AsV17, NV8 and NV36, were not detected with the MagNA Pure 96 System.

To further assess the performance of both methods, we wanted to compare the overall results of the qPCR (Figure 2). Ct values from adenovirus, norovirus, astrovirus, and sapovirus were slightly higher with EchoLUTION (adenovirus ΔCt = 4.17 ± 1.08, norovirus ΔCt = 2.6 ± 0.77, astrovirus ΔCt = 2.62 ± 2.54, sapovirus ΔCt = 2.74 ± 1.7) compared to the values from MagNA Pure.

This difference is due to the different dilution of the samples during the extraction process (see Material and Methods section): the samples prepared with MagNA Pure were more concentrated.

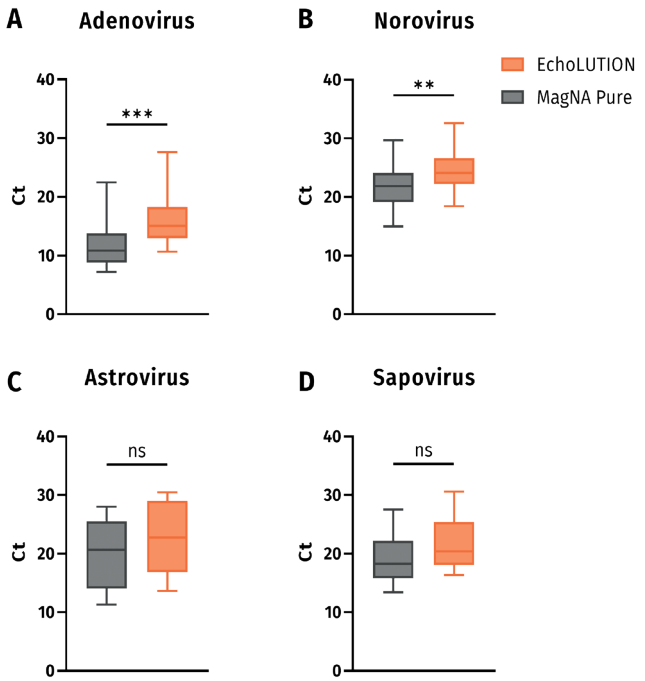
The rotavirus RNA samples extracted with the EchoLUTION Viral RNA/DNA Swab Kit Plus exhibited a ΔCt of 10.60 ± 0.81 that was higher than the samples extracted with MagNA Pure 96 System (data not shown). The reason for that is that rotaviruses are challenging pathogens to lyse because of their capsid structure. Based on these results, we developed an optimized protocol for stool samples that includes an additional heating step during lysis (see discussion).

Figure 1. *Clinical results based on the qPCR data for each test parameter. Data are represented as a paired matrix to compare the performance of EchoLUTION and MagNA Pure for all the samples. Samples that gave the same results with both methods are indicated with a light blue background and the samples that gave different results with white.*



To test the sensitivity achieved with both methods, we estimated a detection limit by means of a dilution series. Since we did not use standardized samples with a defined copy titer, it was not possible to determine a detection limit based on copy number; nonetheless, a comparison between both methods as well as determination of PCR efficiency could be achieved.

Figure 2. Mean Ct comparison between samples extracted with EchoLUTION Viral RNA/DNA Swab Kit Plus and with MagNA Pure 96 System. *Adenovirus, n = 33; norovirus, n = 45; astrovirus, n = 11; sapovirus n = 13. ** p < 0.005; *** p < 0.0005; ns, not significant.*



For this assay, on the day of the experiment, we thawed one clinical stool sample per parameter (with a Ct < 20), and then mixed and briefly centrifuged the sample. We prepared a dilution series of five logs (10^1 to 10^5) from the stock solution of each test parameter, extracted the nucleic acids with both methods (EchoLUTION and MagNA Pure) and performed a qPCR (Figure 3).

These data show that amplification of the test parameter could be detected in the 10^4 dilutions for all samples from both extraction methods. Because of the low initial viral concentration, we did not observe amplification with samples extracted with EchoLUTION for the 10^5 dilutions of norovirus and rotavirus (data not shown). We can conclude that both methods are appropriate for highly sensitive detection, and just two samples were not detectable with EchoLUTION due to low viral concentrations. Further, it was possible to estimate the PCR efficiency by calculating the slope of the curves for the dilution series (Table 3). Samples isolated with the EchoLUTION kit and with MagNA Pure 96 showed comparable qPCR efficiency.

Reproducibility and precision

The reproducibility of results can provide information about the consistency of the extraction methods as well as about the robustness of the downstream assay. To calculate the intra-run precision of the test parameters (adenovirus, norovirus, rotavirus, and astrovirus), we ran three replicates of each sample in a multiplex qPCR and calculated the coefficient of variation (CV). The CV ranged from 0.14 to 1.30 %, indicating the robustness and comparable performance of the EchoLUTION extraction technology (Table 4).

Table 3. qPCR efficiency for each test parameter with both nucleic acid extraction methods. *The calculated qPCR efficiency (%) is based on the slope of the standard curve.*

Sample	EchoLUTION		MagNA Pure	
	Slope	Efficiency %	Slope	Efficiency %
AdV	-3.129	108.7	-3.539	91.7
NV	-2.903	121.0	-2.819	126.3
RV	-3.308	100.6	-3.420	96.1
AsV	-3.043	113.1	-3.429	95.7
SV	-2.919	120.1	-2.864	123.4

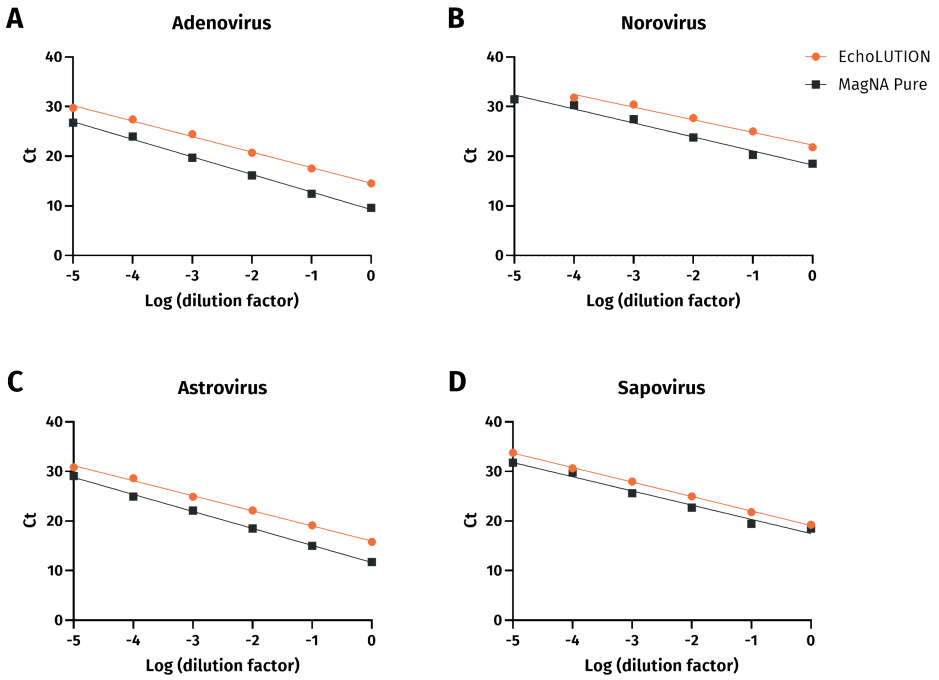


Figure 3. qPCR of a stool sample dilution series extracted either with the EchoLUTION Viral RNA/DNA Swab Kit Plus or the MagNA Pure 96 System. *One positive patient sample per test parameter was diluted in a 10-fold dilution series (10^1 to 10^5), and the corresponding Ct values were represented in a logarithmic scale with linear regression.*

To calculate the inter-run precision, three measurements per sample were performed on different days. Based on the CV values (they are all below 5 %), we concluded that there were only minimal differences in the Ct values between measurements, indicating a high reproducibility (Table 5).

Table 4. Intra-run precision. *The table summarizes the data from three replicates per sample in a single run. SD: standard deviation; CV: coefficient of variation.*

Sample	Single run		
	Mean (Ct)	SD (Ct)	CV (%)
AdV1	15.54	0.202	1.30
AdV2	24.66	0.195	0.79
NV1	22.22	0.032	0.14
NV2	20.54	0.040	0.19
RV1	23.13	0.281	1.21
RV2	27.12	0.123	0.45
AsV1	22.42	0.113	0.50
AsV2	14.52	0.070	0.48

Table 5. Inter-run precision. *The table summarizes the data from three independent runs. SD: standard deviation; CV: coefficient of variation.*

Sample	Mean (Ct)	SD (Ct)	CV (%)
AdV	14.76	0.703	4.76
NV	20.94	0.661	3.15
RV	21.54	0.624	2.89
AsV	22.09	0.624	3.05
SV	18.55	0.619	3.33

Discussion

The qualitative detection of enteropathogenic pathogens is of primary importance for clinical diagnostics. The main objective of this work was to evaluate the EchoLUTION Viral RNA/DNA Swab Kit Plus for the extraction of enteropathogenic virus nucleic acids from stool samples. The results indicate that the EchoLUTION technology gave competitive results compared to the standard MagNA Pure 96 System. Overall, positive samples were confirmed for 146 of 168 positive patient samples (~87 %) with both methods. Six patient samples that were previously evaluated as positive during the initial determination were evaluated as negative in our experiment for both MagNA Pure and EchoLUTION. There are several possible reasons for these false-negative samples: 1) The viral load in the fresh sample was low and was further reduced during sample preparation (during resuspension in TE and centrifugation to collect the supernatant). 2) Inhomogeneity of the initial sample could cause differing results: it is possible that, during the initial diagnostic assessment, a portion of sample containing higher viral load was used. 3) In addition, pre-analytical conditions should be considered. To ensure enough sample was available for a thorough comparison, fresh stool samples were collected, aliquoted, and frozen at –20 °C for storage. During thawing, shear forces and crystal structures forming during the freezing process might have damaged the virus particles. 4) It is possible that enzymatic degradation of nucleic acids occurred when nucleases were released by the lysis of the accompanying microorganisms[3]. In general, the primary structure of DNA and RNA is prone to instability and decay triggered by hydrolysis, non-enzymatic methylation, oxidative damage, and enzymatic degradation[4]. All these factors presumably contribute to reducing the amount of detectable virus in the samples used in this study. But the relevant point is, that both extraction methods detected the same number of samples as positive.

During the qualitative analysis of the samples, we observed slight differences in the performance of the two tested methods. The comparison of the Ct values from EchoLUTION and MagNA Pure samples showed that the values were between 2 to 4 Cts higher with the EchoLUTION extraction procedure for all viruses except rotavirus (see below). The difference between the Ct values can be easily explained by the initial input volume of the patient sample, which was lower for EchoLUTION compared to MagNA Pure method. Additionally, the EchoLUTION technology does not include a concentration step that the MagNA Pure method includes. And as mentioned before (see results Determination of the clinical decision limit section) we recommend determining the cut-off value for the EchoLUTION technology itself, since it will most probably lead to a different CDL than determined for the MagNA Pure 96 method.

Concerning rotavirus results, the mean ΔCt value from the EchoLUTION method was significantly above values obtained with the standard method (ΔCt = 10.60 ± 0.81). The lysis efficiency for this virus could account for this observation. Rotaviruses have a triple capsid structure composed of an inner core structure and an inner and outer protein layer, which is considered a morphological peculiarity[5]. This structural design could make extraction more challenging and could subsequently result in fewer nucleic acids being released during lysis. Based on these results, we optimized the lysis step for enteropathogenic viruses with an additional heating step (see the application note ***Evaluation of the EchoLUTION Viral RNA/DNA Kit for nucleic acid extraction of respiratory and enteropathogenic viruses***[6]). The optimization solved the issue with the rotaviruses completely. The ΔCt values of the other viruses could also decrease due to the additional heating step. Please contact BioEcho for further details.

Our evaluation of the EchoLUTION Viral RNA/DNA Swab Kit Plus versus MagNA Pure 96 considered additional factors that are relevant to laboratories where extraction is performed, including processing time, workload, and total cost balance. Both methods require the same basic laboratory equipment during sample preparation. However, compared to the manual EchoLUTION method, the automated pipetting platform MagNA Pure 96 System requires a considerable financial investment as well as additional reagents, consumables, and regular maintenance. The reagents, cartridges, and pipette tips required for the extraction must be manually placed in the processing instrument. These

manual efforts contribute to additional workload in terms of preparation and post-processing. The simpler EchoLUTION workflow eliminates elaborate washing steps and extended incubation times as in enzymatic digestions. As a result, the total processing time with EchoLUTION is significantly shorter compared to MagNA Pure (30 minutes and 80 minutes, respectively, for 96 samples), and the training effort for employees is much smaller. Over time, personnel costs can be saved by using EchoLUTION, since the process is significantly faster. In addition, the EchoLUTION technology has a valuable advantage in terms of sustainability and waste reduction. Up to 70 % of plastic consumption can be avoided, which reduces the costs of consumables as well as the overall disposal costs.

NOTE. BioEcho has introduced a new kit, the EchoLUTION Viral RNA/DNA Kit, which contains a modified lysis buffer (LyseNtact Buffer New Formula) as well as the improved protocol for enteropathogenic viruses mentioned earlier, including the heating step.

In the application note ***Evaluation of the new EchoLUTION Viral RNA/DNA Kit for nucleic acid extraction of respiratory and enteropathogenic viruses***[6], we demonstrate that the performance of the new kit is the same as that of the EchoLUTION Viral RNA/DNA Swab Kit Plus. Accordingly, all data shown in this application note are still applicable.

References

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Ordering Information

Product	Reactions	Product No.
EchoLUTION Viral RNA/DNA Kit	2 × 48 8 × 48 16 × 48	012-051-002-Dx 012-051-008-Dx 012-051-016-Dx
	2 × 96 8 × 96 16 × 96	012-102-002-Dx 012-102-008-Dx 012-102-016-Dx

*The EchoLUTION Viral RNA/DNA Swab Kit Plus has been replaced with the new EchoLUTION Viral RNA/DNA Kit. The difference between the kits is the modified lysis buffer (LyseNtact Buffer New Formula), all other components of the new kit stayed the same.

contact@bioecho.de

orders@bioecho.de

BioEcho Life Sciences GmbH
Biocampus Cologne, Main Building
Nattermannallee 1
50829 Köln/Cologne, Germany

www.bioecho.de

orders_US@bioecho.com

BioEcho Life Sciences, Inc.
400 Tradecenter Suite 6900
Woburn, MA 01801, USA

www.bioecho.com