Evaluation of commercial kits for extraction of DNA and RNA from *Clostridium difficile*

D Metcalf, JS Weese

University of Guelph, Guelph, Ontario, Canada

INTRODUCTION

The increased use of nucleic acid amplification techniques for detection and characterization of *C. difficile*, along with evaluation of gene expression, necessitates techniques for production of adequate amounts of high quality DNA and RNA.

Commercial nucleic acid extraction kits are a rapid, cost-effective, and efficient strategy for DNA and RNA isolation from bacteria. While widely used, there are no standardized or recommended methods to produce the required high purity, quality and DNA or RNA yield from *C .difficile* for genetic, sequencing and diagnostic applications.

An ideal commercial nucleic acid extraction kit for use with *C. difficile* would provide efficient cell lysis from vegetative cells and spores (if present), remove inhibitors that may interfere with downstream enzymatic reactions, and be strain independent, as well as simple, rapid and cost-effective.[1]

OBJECTIVE

To evaluate commercial nucleic acid extraction kits based on different technologies and levels of automation for use in *C. difficile*.

MATERIALS AND METHODS

Strains: NAP1/toxinotype III/ribotype 027 and NAP7/toxinotype V/ribotype 078.

Vegetative cell production: Overnight cultures of 3 biological replicates for each isolate were inoculated into pre-warmed and pre-reduced growth medium and 10 ml of culture was harvested at an OD_{600} of 1.0, then centrifuged at 13 000 x g for 60 s. The supernatant was removed and the pellet was resuspended in 5 volumes of RNAlater reagent[3] (Ambion, Austin, TX) and stored at -80˚C immediately until use.

Spore production: *C. difficile* strains were cultured anaerobically at 37 °C in duplicate, using 200 ml of the *C. difficile* growth medium described above. Spores were enriched as previously described.[4]

Cell quantification: Vegetative cell and spore suspensions were quantified by serial dilution and inoculation onto blood agar. Approximately 10^6 spores and vegetative cells were used for each of the DNA extraction kits.

RNA production: 10 ml of culture were harvested at an OD_{600} of approximately 0.4, centrifuged and the pellet was resuspended in 5 volumes of RNAlater RNA stabilization reagent (Ambion). These samples were stored at -80ºC until use.

DNA/RNA extraction: Four DNA and 3 RNA kits were evaluated. DNA extraction was performed on 3 biological replicates of ~10^6 vegetative cells and 2 biological replicates of 10^6 spores of ribotypes 027 and 078.

DNA/RNA assessment: DNA concentration, purity and quality were assessed using a NanoDrop ND-1000 (ThermoScientific, Wilmington, DE, USA), by gel electrophoresis and by qPCR amplification of *gluD* as previously described.[2] DNase treated RNA concentration, purity and quality was assessed using a NanoDrop ND-1000, an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and by qPCR amplification of the *gluD* gene fragment on cDNA as previously described.[2]

Analysis: The student's t-test or a single factor analysis of variance (ANOVA) was used to determine statistical significance.

Acronym	Commercial DNA and RNA extraction kit s
MU	MoBio Laboratories Inc. UltraClean® Microbial DNA Isolation Kit
RH	Roche High Pure PCR Template Preparation Kit
PMD	Promega Maxwell® 16 SEV Tissue DNA preparation kit
RMD	Roche MagNA Pure LC DNA Isolation Kit III
QR	Qiagen RNeasy Mini Kit
RMR	Roche MagNA Pure LC RNA Isolation Kit
PMR	Promega Maxwell® 16 LEV Cell RNA preparation kit

Table 1. Commercial kits that were evaluated.

RESULTS

Table 2 summarizes the features of each kit such as reagents needed, cost and processing time. Figure 1 and Tables 3 and 4 summarize DNA or RNA kit specific yields and purities.

Figure 2 depicts the Bioanalyzer analysis of 12 RNA samples. Note the small RNAs resulting in low RIN values in the RMR samples and the evidence of degradation in the 027 PMR sample.

Method	Processing Time (hr:min)		Max. Starting Conc.	Cost per sample[A]	Recovered vol. (µl)	Required reagents not included
	Hands On	Total				
DNA extraction kits						
MU	0:50	0:50	1.8 ml of culture	~$2.50	50	None
RH	0:35	1:00	10^9 cells	~$3.00	200	PBS Lysozyme
PMD	0:19	2:55	10^9 cells	~$5.00	300	TE buffer Lysozyme
RMD	0:45	3:00	10^6 cells	~$11.00[B]	100	Lysozyme Lysostaphin PBS
RNA extraction kits						
QR	0:50	1:00	10^7 cells	~$6.00	50	β-mercapto-ethanol
RMR	0:30	2:00	10^6 cells	~$13.00[B]	50	DTT PBS
PMR	0:10	0:55	10^6 cells	~$6.00	50	TE Buffer Lysozyme

[A]All prices are in CAD and exclude the cost of additional reagents. [B]Estimate based on 8 samples per run. Running the maximum samples (32) reduces the cost per sample to ~$9.00 for the RMR kit and ~$7.00 for the RMD kit.

Table 2. Comparison between commercial DNA and RNA extraction kits

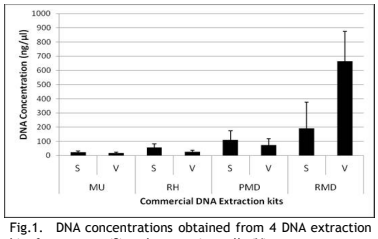

Fig.1. DNA concentrations obtained from 4 DNA extraction kits from spores (S) and vegetative cells (V).

Kit	Ribotype	Mean conc. ±SD	Avg. $A_{260/280}$ Values ± SD
MU	027	20.36 ± 2.94	2.00 ± 0.17
	078	21.39 ± 1.91	2.22 ± 0.41
	Combined	20.88 ± 1.66	2.11 ± 0.33
RH	027	46.24 ± 11.32	2.01 ± 0.06
	078	30.10 ± 8.09	2.10 ± 0.14
	Combined	38.17 ± 7.08	2.06 ± 0.12
PMD	027	74.75 ± 20.76	1.92 ± 0.04
	078	102.67 ± 28.06	1.93 ± 0.08
	Combined	88.71 ± 17.06	1.92 ± 0.06
RMD	027	441.35 ± 94.53	2.18 ± 0.03
	078	650.51 ± 399.6	2.21 ± 0.01
	Combined	545.93 ± 291.14	2.16 ± 0.05

Table 3. Summary of the average DNA concentration and purity values from spores and vegetative cells.

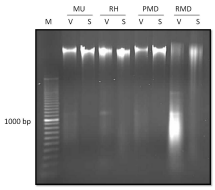

Figure 2. Spore and vegetative cell genomic DNA preparations (200 ng) from the 4 commercial extraction kits (V: vegetative cells, S: spores). M:100 bp ladder.

All kits produced samples of intact DNA with the exception of RMD, where evidence of shearing was present in both vegetative and spore DNA samples (Figure 2). Despite this shearing, PCR amplification of both a 135 bp and 3100 bp amplicon was successful.

RESULTS

qPCR of *gluD* was used to assess the influence of kit specific differences in DNA quality on Cq values. Cq values for spores ranged from 12.37 – 17.13 and from 14.84-19.69 for vegetative cells (data not shown).

Method	Strains	Mean Conc. ± SD (ng/µl)	Avg. 260/280 Values ± SD	No. of DNase Tx	RIN	Cq
QR	027	213.34 ± 149.8	2.08 ± 0.03	3	9.50	20.48
	078	163.94 ± 228.6	1.98 ± 0.12	3	8.75	20.98
	combined	188.64 ± 175.0	2.03 ± 0.09	-	9.13	20.73
RMR	027	31.83 ± 10.91	2.02 ± 0.09	3	5.25	23.75
	078	24.56 ± 14.89	2.01 ± 0.04	3	4.95	23.96
	combined	28.20 ± 12.33	2.02 ± 0.06	-	5.10	23.86
PMR	027	31.89 ± 11.65	1.78 ± 0.12	3	6.60	21.38
	078	53.11 ± 23.88	1.95 ± 0.11	3	7.10	22.19
	combined	42.50 ± 20.44	1.86 ± 0.14	-	6.85	21.79

Table 4. Summary of the RNA concentrations and purity values.

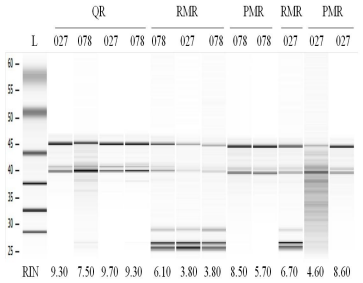

Figure 2. Agilent bioanalyzer electrophoretic analysis of RNA samples from ribotypes 027 and 078. RNA integrity numbers (RIN) are indicated for each sample.

DISCUSSION

The RMD kit produced DNA preparations of high purity and concentration, but with evidence of shearing in all samples that might affect downstream applications. Despite this, all samples amplified the *gluD* gene fragment with similar efficiency and specificity indicating some DNA degradation may not significantly impact PCR amplification of small gene fragments.

All spore samples produced earlier Cq values than vegetative cells, suggesting a difference in quality of DNA between the 2 cell forms affecting the spectro-photometric measurements, although there was no statistically significant differences between Cq values.

For RNA extractions, the QR kit yielded RNA of the highest concentration, purity and RIN value.

This study identified some degree of fallibility in methods to assess RNA integrity. The Bioanalyzer electropherogram (Fig. 2) demonstrated the presence of small RNAs (5S rRNA, tRNA) in the RMR samples. They were interpreted as degradation and resulted in a low RIN values.

The critical importance of good DNA and RNA yield are often overlooked and this study highlights the potential for variation between established commercial systems.

REFERENCES

1 Rantakokko-Jalava, K., J. Jalava. 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. 40:4211-4212.

2 Metcalf et al. 2010. Evaluation of candidate reference genes in Clostridium difficile for gene expression normalization. Anaerobe. 16:439-443.

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4 Bertolo L. et al. 2012. *Clostridium difficile* carbohydrates: glucan in spores, PSII common antigen in cells, immunogenicity of PSII in swine and synthesis of a dual *C. difficile*-ETEC conjugate vaccine. Carbohydr. Res .354:79-86.