High-Throughput Direct Detection of *Clostridium difficile* From Stool Specimens Using the Simplexa™ *C. difficile* Universal Direct Assay

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Abstract

Introduction/Background: The increasing prevalence of *C. difficile* associated disease (CDAD) has resulted in the need for accurate and rapid methods for diagnosis. Detecting toxin producing organisms in stool samples is now routinely done using polymerase chain reaction methodologies. However, high throughput testing options are currently unavailable. We have developed the Simplexa™ *C. difficile* Universal Direct Assay which enables direct detection of organisms in stool without nucleic acid isolation procedures, and which enables testing of up to 96 samples under 2 hours.

Methods: Fresh stool samples were transferred into dilution buffer and incubated at 97ºC for 10 minutes on a heating block. Samples were then applied directly to a 96 well Universal Disc, along with the reaction mix, and real-time PCR was carried out using the 3M™ Integrated Cycler. Assay performance was determined by performing analytical limit of detection (LoD) and reproducibility studies. Results were compared with those of 3 other methods: 1) a method involving a complete nucleic acid extraction step; 2) cytotoxicity assay; and 3) two FDA cleared PCR assays. The effect of potential interfering substances was assessed by adding relevant concentrations of these substances to samples prior to testing.

Results: The Simplex *C. difficile* Universal Direct assay was at least as sensitive as the comparator methods, while providing greater throughput and more rapid test results. The LoD of the assay was 0.04 CFU/reaction. There was no interference from any of the 20 potential interfering substances tested, including blood, antibiotics, and diarrhea remedies. Inter- and intra-assay reproducibility assays yielded CVs of <2%. Results from a single specimen could be obtained in approximately 70 minutes, and up to 96 samples could be processed in <2 hours.

Conclusion: The performance of the Simplexa *C. difficile* Universal Direct assay was comparable to the other methods. With no requirement for nucleic acid extraction, the assay provides significantly reduced turnaround time without compromising detection sensitivity.

Methods

Sample Preparation: De-identified unformed stool samples were collected from patients suspected of having CDAD. For direct detection, a flocked swab was used to transfer a portion of each sample to a vial containing 1 mL TE buffer. The samples were then incubated at 97°C for 10 minutes on a heating block. Alternately, samples were subjected to a nucleic acid isolation procedure using the MagNA Pure LC instrument and the corresponding Total Nucleic Acid Isolation Kit (Roche, Indianapolis, IN) with the variable elution protocol (to enable elution in 50 µL).

***C. difficile* real-time PCR:** The Simplexa Direct *C. difficile* Real-Time PCR included a primer pair that targeted a well-conserved region of toxin B gene, and was labeled with 6-carboxyfluorescein (FAM). The assay also included an internal control primer pair targeting a plant gene and labeled with Quasar 670 (Q670). For the Simplexa *C. difficile* Universal Direct assay, 2 µL of heat-treated stool samples were mixed with the master mix to a final volume of 10 µL. For the real-time PCR method using extracted specimens, 5 µL of extracted DNA was added to the master mix to make a final volume of 10 µL. Samples were then added to the 96 well Universal Disc and nucleic acid amplification and detection was performed using the 3M Integrated Cycler. Cycling included an initial denaturation step at 97°C for 2 minutes, followed by 40 cycles of 97°C for 10 seconds and 60°C for 30 seconds.

Methods (Cont.)

Results

Comparison of Simplexa *C. difficile* Universal Direct Assay with a cytotoxicity assay (CTA): A panel of 58 stool samples were examined with both the Simplexa *C. difficile* Universal Direct Assay and CTA. As shown in Table 1, the Simplexa *C. difficile* Universal Direct Assay resulted in 100% sensitivity and 91.5% specificity in comparison to CTA.

Table 1. Method comparison between the Simplexa *C. difficile* Universal Direct Assay and Cytotoxicity Assay

		Cytotoxicity Assay			
		Positive	Negative	Total	Note
Simplexa Direct	Positive	11	4	15	Sensitivity 100%
	Negative	0	43	43	Specificity 91.5%
	Total	11	47	58	

Comparison of Simplexa *C. difficile* Universal Direct Assay to FDA cleared real-time PCR kits: A panel of 89 stool samples was examined with the Simplexa *C. difficile* Universal Direct Assay in parallel with a FDA cleared real-time PCR method. As shown in Table 2, the performance of the Simplexa *C. difficile* Universal Direct Assay achieved 100% sensitivity and 100% specificity in comparison to that of the commercial test kit. A second panel of 189 stool samples was examined with the Simplexa *C. difficile* Universal Direct Assay in parallel with another FDA cleared real-time PCR. As shown in Table 3, the Simplexa *C. difficile* Universal Direct Assay resulted in 99.1% sensitivity and 91.1% specificity in comparison to the commercial kit.

Table 2. Method comparison between the Simplexa *C. difficile* Universal Direct Assay and a FDA cleared *C. difficile* real-time PCR.

		FDA Cleared PCR (1)			
		Positive	Negative	Total	Note
Simplexa Direct	Positive	14	0	14	Sensitivity 100%
	Negative	0	75	75	Specificity 100%
	Total	14	75	89	

Results (Cont.)

Table 3. Method comparison between the Simplexa *C. difficile* Universal Direct Assay and another FDA cleared real-time PCR

		FDA Cleared PCR (2)			
		Positive	Negative	Total	Note
Simplexa Direct	Positive	109	7	116	Sensitivity 99.1%
	Negative	1	72	73	Specificity 91.1%
	Total	110	79	189	

Limit of detection of the Simplexa *C. difficile* Universal Direct Assay: A *C. difficile* bacteria stock (Cat# 0801620) was obtained from ZeptoMetrix. Serial dilutions of the stock were made and multiple aliquots were examined with the Simplexa *C. difficile* Universal Direct Assay to determine the assay LoD. As shown in Table 4, the LoD was determined to be 0.04 CFU/rxn based on Probit analysis.

Table 4. LoD of the Simplexa *C. difficile* Universal Direct Assay

Obs	Concentration Level (cfu/rxn)	# of Replicates Detected	Total # of Replicates	Probability
1	0.00000	0	23	0.00
2	0.00625	11	24	0.46
3	0.01250	18	24	0.75
4	0.02500	22	24	0.92
5	0.05000	24	24	1.00
6	0.07500	23	24	0.96
7	0.10000	24	24	1.00
8	0.20000	24	24	1.00
9	0.40000	24	24	1.00
LoD (cfu/rxn) with 95% CI 0.04 (0.025 – 0.072)				

Reproducibility of the Simplexa *C. difficile* Universal Direct Assay: Panels of *C. difficile* low positive (2-4X LoD) and medium positive samples were made using the ZeptoMetrix *C. difficile* stock in negative stool sample matrix and used to examine the reproducibility of the Simplexa *C. difficile* Universal Direct Assay. The CV of combined variability including inter-assay, intra-assay, inter-day, and inter-instrument for the low positive, medium positive, and positive control (based on Ct Values) were 1.52%, 1.48%, and 1.81%, respectively (Table 5).

Table 5. Reproducibility of the Simplexa *C. difficile* Universal Direct Assay

Quantitative Summary of Reproducibility													
			Inter-Instrument		Inter-Day		Inter-Run		Intra-Run		Total	
Sample Category	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Low Positive	79*	35.43	0.00	0.00	0.00	0.00	0.22	0.61	0.49	1.40	0.54	1.52
Medium Positive	79*	34.13	0.00	0.00	0.00	0.00	0.16	0.48	0.48	1.39	0.50	1.48
Positive Control	80	32.80	0.00	0.00	0.00	0.00	0.07	0.20	0.59	1.80	0.59	1.81
Negative	80	0.00	Not Applicable									
NTC**	20	0.00	Not Applicable									

*One Replicate was "Invalid".
**One Replicate of NTC included in each run

Results (Cont.)

Interference study: A panel of *C. difficile* low positive (2-4X LoD) samples was prepared using the ZeptoMetrix *C. difficile* stock in negative stool sample matrix. Twenty various potential interfering substances including blood, mucin, and antibiotics (Table 6) were spiked into the panel and examined for possible inhibition to the Simplexa *C. difficile* Universal Direct Assay. No inhibition was observed when the substances were present in the relevant concentration in the sample as shown in Table 6.

Table 6. Potential interfering substances tested with the Simplexa *C. difficile* Universal Direct Assay

Substance	Active Ingredient	Final Concentration
Mucin	Immunoglobulins, Lysozyme, Polymers	3mg/mL
Metronidazole	Metronidazole	14mg/mL
Vancomycin	Vancomycin	1.4mg/mL
Stearic acid	Stearic acid	4mg/mL
Palmitic acid	Palmitic acid	2mg/mL
Barium sulfate	Barium sulfate	5mg/mL
Nystatin	Nystatin	10,000 USP units/mL
whole blood	Glucose, Hormones, Enzymes, Ions, Iron	5% (v/v)
Antacid and Anti-gas generic	Magnesium Hydroxide, Magnesium Hydroxide	0.1mg/mL
Milk of Magnesia	Magnesium Hydroxide	0.2mg/mL
Imodium AD	Loperamide	0.005mg/mL
Pepto-Bismol	Bismuth Subsalicylate	0.175mg/mL
Moist towelettes generic	Benzalkonium Chloride	10% (v/v)
antacid generic	Calcium Carbonate	0.1mg/mL
Preparation H	Phenylephrine	2% (w/v)
Trojan with nonoxynol-9	Nonoxynol-9	1.4mg/mL
1% Hydrocortisone Cream	Hydrocortisone	2% (w/v)
Fleet	Mineral Oil	2% (w/v)
Laxative generic	Sennosides	0.1mg/mL
Aleve	naproxen sodium	14mg/mL
KY Jelly	water	2% (w/v)

Conclusions

- Sensitivity and specificity of the Simplexa *C. difficile* Universal Direct Assay is comparable to sensitivity and specificity of a cytotoxicity assay, and to that of two FDA cleared real-time PCR test kits.
- Simplexa *C. difficile* Universal Direct Assay requires no nucleic acid extraction, resulting in significantly reduced turnaround time without compromising detection sensitivity.
- The limit of detection of the Simplexa *C. difficile* Universal Direct Assay is 0.04 cfu/rxn.
- Reproducibility of the Simplexa *C. difficile* Universal Direct Assay is less than 2% (CV) based on Ct values.

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The Simplexa Direct assays are not commercially available yet, but we expect to submit assay in this format for FDA clearance in the near future.

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