

# Development of a simple and easy-to-adapt nucleic acid test for *Clostridium difficile*-associated diarrhea

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## BACKGROUND

*Clostridium difficile* causes antibiotic-associated diarrhea and colitis in animals and humans and is one of the most common nosocomial infections seen in hospitalized patients. An estimated 10-12 million adults are infected with this organism each year in the USA. Enzyme immuno-assays (EIA) are commonly used for diagnosis of *C. difficile*-associated diarrhea (CDAD). However, these EIA assays for toxin A and toxin B only have approximately 70% sensitivity (1). Accurate identification of pathogenic *C. difficile* in a timely fashion has great therapeutic, prognostic and economic significance. We developed and validated the IsoAmp<sup>®</sup> Rapid CDAD Assay which uses Helicase-Dependent Amplification (HDA) coupled with a disposable detection device, the BEST<sup>™</sup> (BioHelix Express Strip) Cassette (BioHelix Corp), for rapid identification of toxigenic *C. difficile* in feces. HDA is a unique isothermal DNA amplification technique that relies on the use of a DNA helicase enzyme to unwind double stranded DNA and DNA-RNA hybrid (2, 3, 4). The BEST<sup>™</sup> Cassette is a disposable device designed for instrument-free, cross-contamination-proof detection of amplicons derived from HDA, PCR, and other nucleic acid amplification reactions.

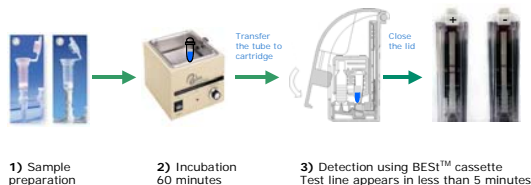


Figure 1. Workflow of IsoAmp<sup>®</sup> Rapid CDAD Assay.

## MATERIALS AND METHODS

The workflow of the IsoAmp<sup>®</sup> Rapid CDAD Assay is described in Figure 1. DNA samples can be extracted from stool specimens of CDAD suspected patients by either the NucliSENS easyMAG automated sample preparation system from bioMérieux or the manual QIAamp Stool DNA Mini Kit from Qiagen. HDA was set up by using an IsoAmp<sup>®</sup> Rapid CDAD Test Kit (BioHelix Corp). In brief, 25 µl of Mix A containing the extracted stool DNA samples and an internal control (IC) plasmid was mixed with 21.5 µl IsoAmp<sup>®</sup> CDAD Reaction Mix and 3.5 µl IsoAmp<sup>®</sup> Enzyme Mix provided by the kit in a 200-µl PCR reaction tube. The IC plasmid can also be spiked directly into the stool specimens to monitor the performance of the whole assay process from sample preparation to detection. After a 60-minute incubation at 65°C in a water bath or heat block, the reaction tube is directly placed into the BEST<sup>™</sup> Cassette for amplicon detection.

## REFERENCES

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## RESULTS

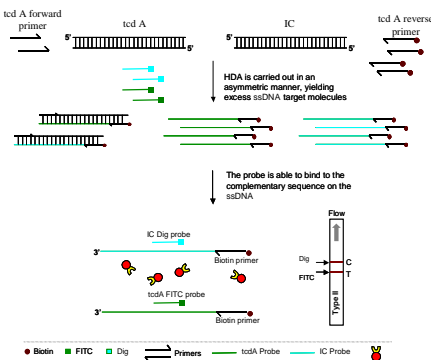


Figure 2. Schematic diagram showing the mechanisms of the IsoAmp<sup>®</sup> Rapid CDAD Assay. By using a biotin labeled primer, a 3' FITC labeled *tcdA* gene probe, and a 3' digoxigenin (Dig) labeled IC probe that can bind to the biotin labeled DNA strand generated by the HDA reaction in the amplification of *C. difficile* toxin gene *tcdA* and a competitive IC, it is possible to detect amplicon-probe complexes via a sandwich immunosay format. Amplification is asymmetric because the biotin labeled primer is present in excess such that more biotin labeled strands are produced than unlabeled complementary strands, and thus no denaturation is required to make the biotin strand available for binding by the FITC or the Dig labeled probe.

Figure 2 illustrates the mechanisms underlying the IsoAmp<sup>®</sup> Rapid CDAD Assay. The procedure of using a BEST<sup>™</sup> Cassette for amplicon detection is demonstrated in Figure 3A. The IsoAmp<sup>®</sup> Rapid CDAD Assay displayed high sensitivity in the analytical sensitivity study with a limit of detection at 5 copies of *C. difficile* genomic DNA per assay (Figure 3B). The performance of the IsoAmp<sup>®</sup> Rapid CDAD Assay was evaluated by testing 85 DNA samples prepared from stool specimens of CDAD suspected patients by easyMAG (bioMérieux), which have been previously validated by an EIA method and a PCR method in the CDAD test. The IsoAmp<sup>®</sup> Rapid CDAD Assay correctly identified all the EIA positive samples (60 out of 60, see Table 1A). Among the 25 EIA negative samples, 5 were tested positive by the IsoAmp<sup>®</sup> Rapid CDAD Assay of which 3 were also tested positive in the PCR assay and 2 were inconclusive in the PCR test (Table 1A). The performance of the IsoAmp<sup>®</sup> Rapid CDAD Assay was also evaluated by testing 27 DNA samples prepared manually using the QIAamp DNA Stool Mini Kit (QIAGEN Inc). IsoAmp<sup>®</sup> CDAD Assay detected all 14 EIA-positive samples (Table 1B). In addition, IsoAmp<sup>®</sup> Assay also identified two possible *C. difficile* positives from 13 EIA-negative samples (Table 1B), one of which was isolated from a patient with strong clinical symptoms for CDAD. Using the same HDA-BEST<sup>™</sup> Cassette platform, we have also developed a simple, rapid, and user-friendly procedure, the IsoAmp<sup>®</sup> Rapid Staph Assay, to identify *Staphylococcus aureus*, and determine its methicillin resistance directly from Gram positive cocci in clusters-containing blood culture medium. The analytical sensitivity of the assay was 50 cells per reaction, and the clinical sensitivity and specificity were both 100% for *S. aureus* detection, and 100% for methicillin-resistance (Table 2).

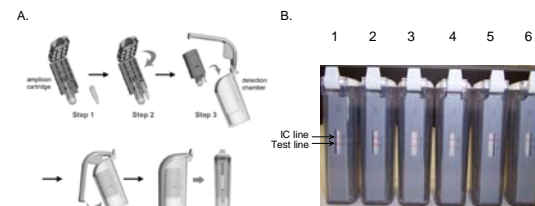


Figure 3. Amplicon detection using the BEST<sup>™</sup> Cassette. A. The procedure of using a BEST<sup>™</sup> Cassette for amplicon detection. The amplification reaction vessel is placed in an amplicon cartridge (step 1), the cartridge is closed to immobilize the reaction vessel (step 2), the amplicon cartridge is inserted into the detection chamber (step 3), the handle of the detection chamber is closed to seal the vessel into the chamber, and the reaction vessel (step 4), after 10 minutes the detection window of the chamber is read by eye to score the assay result (step 5). B. An example from the results of the analytical sensitivity study of the IsoAmp<sup>®</sup> Rapid CDAD Assay. 2–200 copies of *C. difficile* genomic DNA mixed with 5x10<sup>7</sup> copies of internal control plasmid and 2 µl of purified DNA from CDAD negative stool sample were used as the template in the IsoAmp<sup>®</sup> Rapid CDAD Assay. The amounts of *C. difficile* genomic DNA input used in the IsoAmp<sup>®</sup> Rapid CDAD Assay are: 1, 200 copies; 2, 20 copies; 3, 10 copies; 4, 5 copies; 5, 2 copies; 6, 0 copy.

Table 1. Performance of the IsoAmp<sup>®</sup> Rapid CDAD Assay in the CDAD test. A. Comparison of the performance of the IsoAmp<sup>®</sup> Rapid CDAD Assay of the DNA samples prepared by the easyMAG method with an EIA and a PCR assay in the CDAD test. B. Comparison of the performance of the IsoAmp<sup>®</sup> Rapid CDAD Assay of the DNA samples prepared by the QIAamp method with an EIA assay in the CDAD test.

A.		IsoAmp+		IsoAmp-	
EIA+/PCR+	60	0			
EIA-/PCR-	0	20			
EIA+/PCR-	0	0			
EIA-/PCR+	3	0			
EIA-/PCR±*	2	0			

B.		IsoAmp+		IsoAmp-	
EIA+	14	0			
EIA-	2	11			

\*PCR: PCR inconclusive.

Table 2. Comparison of the 119 blood culture reference results (R) with the test (T) results obtained in the same sample set using the IsoAmp<sup>®</sup> Rapid Staph Assay.

	R+/T+	R-/T+	R+/T-	R-/T-	Sens. %	Spec. %
<i>S. aureus</i>	39	0	0	80	100	100
mecA	21	0	0	18	100	100

## CONCLUSIONS

The IsoAmp<sup>®</sup> Rapid CDAD Assay is a simple nucleic acid amplification test for CDAD. It combines an isothermal amplification technology with a self-contained disposable amplicon detection device that can be easily adapted to different sample preparation methods. The IsoAmp<sup>®</sup> CDAD Assay has potentially higher sensitivity than the immunoassay on DNA samples prepared by both an automatic sample preparation method and a manual method. It requires no expensive equipments and can be performed on a random access basis.

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