# Clinical and Analytical Performance of a New Molecular *C. difficile* Direct Assay

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## Revised Abstract

**Introduction:** *Clostridium difficile*, a gram-positive, anaerobic, spore-forming bacillus, is associated with significant morbidity and mortality. *C. difficile* has been linked to 50% to 75% of antibiotic-associated colitis cases, up to one-third of all antibiotic-associated diarrhea cases and over 90% of all cases of antibiotic-associated pseudomembranous colitis.

The DiaSorin Molecular Simplexa® *C. difficile* Direct Assay is FDA-cleared for sample-to-answer detection of the *Clostridium difficile* toxin B gene (tcdB) from liquid or unformed stool samples from individuals suspected of difficile infection (CDI). This test utilizes real-time PCR amplification without any nucleic acid extraction.

The analytical performance characteristics, including analytical sensitivity/limit of detection (LoD), reproducibility, analytical reactivity, analytical specificity/cross-reactivity, microbial inhibition and interfering substances, were determined. Clinical agreement studies compared Simplexa performance to direct and combination direct/enriched culture and toxin assay. Positive and negative agreement were also determined for Simplexa versus three FDA cleared NAAT assays.

**Methods:** The analytical sensitivity/limit of detection was tested for two toxigenic *C. difficile* strains, ATCC 43255 and NAP1A. A reproducibility panel with both strains was tested at 3 different laboratories. For the method comparison study, the performance of Simplexa *C. difficile* Direct was compared to direct culture and direct/enriched combined culture with toxin assay. Samples from the method comparison study were also tested with one of three FDA cleared molecular NAAT assays. Analytical reactivity was determined for an additional 32 toxigenic *C. difficile* strains. In order to evaluate analytical specificity, a panel of 127 bacteria, viruses and fungi, were tested for cross-reactivity. Microbial inhibition was evaluated by testing low levels of ATCC 43255 or NAP1A in a background of high levels of each of the same 127 microorganisms. The potential inhibitory effect of 33 endogenous and exogenous substances on detection of ATCC 43255 and NAP1A was evaluated.

**Results:** The LoD for the Simplexa® *C. difficile* Direct Assay was 0.95 CFU/mL for the ATCC 43255 strain and 0.43 CFU/mL for NAP1A. Across all 3 sites in the reproducibility study, 100% detection was observed for ATCC 43255 LP, ATCC 43255 MP and NAP1A MP. For NAP1A LP, the detection rate was 98.9%. In comparison to direct culture and toxin assay, Simplexa *C. difficile* Direct had a positive agreement of 95.7% and a negative agreement of 92.1%. When Simplexa *C. difficile* Direct was compared to direct/enriched culture and toxin assay, the sensitivity was 85.9% and the specificity was 95.1%. The performance of Simplexa *C. difficile* Direct compared to 3 FDA cleared NAAT assays, demonstrated 93.4%, 93.9% and 84.8% positive agreement and 96.6%, 94.0% and 99.2% negative agreement. Simplexa *C. difficile* Direct was able to detect all 32 toxigenic strains tested for analytical reactivity. The assay did not have any cross-reactivity to the 127 microorganisms tested; at the same time, none of the organisms tested inhibited the detection of the ATCC 43255 or NAP1A strains when tested at 2-4x LoD. The assay was able to detect ATCC 43255 and NAP1A in the presence of each potentially interfering substance tested.

## Methods

**Real-time PCR Amplification and Detection:** Simplexa *C. difficile* Direct Assay (MOL2950) contains all reagents for on-board extraction and real-time PCR. Swabs were dipped into stool specimens and excess liquid was removed. The swab was placed into Sample Prep Buffer and swirled to release the stool. For each reaction on the Direct Amplification Disc (DAD), 50 µL of sample was loaded into the sample port and 50 µL of *C. difficile* Direct Reaction Mix was loaded into the reaction port. All testing was performed using the LIAISON MDx instrument.

**Limit of Detection:** The LoD for *C. difficile* ATCC 43255 and NAP1A (Zeptometrix) strains was determined as the lowest concentration with ≥95% detection in negative stool-TE matrix.

**Reproducibility:** The panel included a positive control (PC), a negative control and 4 contrived samples: a low positive (LP) and a medium positive (MP) for the *C. difficile* ATCC 43255 and NAP1A strains. The *C. difficile* reproducibility panel was contrived in negative stool-TE matrix.

**Method Comparison:** A panel of clinical specimens from 5 geographically diverse sites was evaluated using the Simplexa Direct and Direct/Enriched Culture methods as well as 3 commercially available FDA-cleared molecular Nucleic Acid Amplification Test (NAAT) assays. Positive and negative agreements were determined.

**Analytical Reactivity:** Thirty-two toxigenic *C. difficile* strains were individually spiked into negative stool-TE matrix at 2-4X LoD and tested.

**Cross-reactivity:** The test panel consisted of $10^6$ CFU/mL for bacteria and fungi, $10^6$ cells/mL for parasites or $10^5$ $TCID_{50}$/mL for viruses and was formulated in negative stool-TE matrix.

**Microbial Inhibition:** The organisms in the cross-reactivity panel were spiked into a separate baseline sample of *C. difficile* ATCC 43255 or NAP1A at 2-4X LoD in negative stool-TE matrix.

**Potentially Interfering Substances:** Each individual substance was spiked into a baseline sample consisting of *C. difficile* ATCC 43255 or NAP1A at 2-4X LoD in negative stool-TE matrix.

## Results

**Limit of Detection was 0.95 CFU/mL or less for *C. difficile* strains tested in Table 1.**

**Table 1. Limit of Detection**

| *C. difficile* Bacterial Strain | LoD Concentration (CFU/mL) |
|---|---|
| ATCC 43255 | 0.95 |
| NAP1A | 0.43 |

## Results (continued)

**Reproducibility: All ATCC 43255 replicates were detected at all sites; for NAP1A, 100% detection for MP and 98.9% detection (89/90 replicates) for LP was observed. PC and negative control were as expected.**

**Method Comparison – all comparators: Positive Agreement was ≥ 84.8%. Negative Agreement was ≥ 92.1% (Tables 2 – 6).**

**Table 2. Simplexa vs. Direct Culture**

| | Direct Culture + Toxin Assay | | |
|---|---|---|---|
| Simplexa Results | Detected | Not Detected | Total |
| Detected | 265 | 163 | 428 |
| Not Detected | 12 | 1890 | 1902 |
| Total | 277 | 2053 | 2330 |
| Positive Agreement | 95.7% (265/277)<br>95% CI: 92.6% to 97.5% | Negative Agreement | 92.1% (1890/2053)<br>95% CI: 90.8% to 93.2% |

**Table 3. Simplexa vs. Combined Culture**

| | Combined Culture (Direct & Enriched) + Toxin Assay | | |
|---|---|---|---|
| Simplexa Results | Detected | Not Detected | Total |
| Detected | 336 | 96 | 432 |
| Not Detected | 55 | 1849 | 1904 |
| Total | 391 | 1945 | 2336 |
| Sensitivity | 85.9% (336/391)<br>95% CI: 82.1% to 89.0% | Specificity | 95.1% (1849/1945)<br>95% CI: 94.0% to 95.9% |

**Table 4. Simplexa vs. NAAT 1**

| | NAAT1 | | |
|---|---|---|---|
| Simplexa Results | Detected | Not Detected | Total |
| Detected | 114 | 24 | 138 |
| Not Detected | 8 | 683 | 691 |
| Total | 122 | 707 | 829 |
| Positive Agreement | 93.4% (114/122)<br>95% CI: 87.6% to 96.6% | Negative Agreement | 96.6% (683/707)<br>95% CI: 95.0% to 97.7% |

**Table 5. Simplexa vs. NAAT 2**

| | NAAT2 | | |
|---|---|---|---|
| Simplexa Results | Detected | Not Detected | Total |
| Detected | 138 | 38 | 176 |
| Not Detected | 9 | 591 | 600 |
| Total | 147 | 629 | 776 |
| Positive Agreement | 93.9% (138/147)<br>95% CI: 88.8% to 96.7% | Negative Agreement | 94.0% (591/629)<br>95% CI: 91.8% to 95.6% |

**Table 6. Simplexa vs. NAAT 3**

| | NAAT3 | | |
|---|---|---|---|
| Simplexa Results | Detected | Not Detected | Total |
| Detected | 112 | 5 | 117 |
| Not Detected | 20 | 584 | 604 |
| Total | 132 | 589 | 721 |
| Positive Agreement | 84.8% (112/132)<br>95% CI: 77.8% to 90.0% | Negative Agreement | 99.2% (584/589)<br>95% CI: 98.0% to 99.6% |

## Results (continued)

**Analytical Reactivity: All toxigenic *C. difficile* strains tested were detected at 512 CFU/mL or lower.**

**Table 7. *C. difficile* Strains Tested**

| Strain | Toxinotype | Toxin | Ribotype |
|---|---|---|---|
| ATCC 17857 | 0 | A+B+ | 001 |
| ATCC 17858 | 0 | A+B+ | 054 |
| ATCC 43594 | 0 | A+B+ | 005 |
| ATCC 43596 | 0 | A+B+ | 012 |
| ATCC 43598 | VIII | A-B+ | 017 |
| ATCC 43599 | 0 | A+B+ | 001 |
| ATCC 43600 | 0 | A+B+ | 014 |
| ATCC 51695 | 0 | A+B+ | 001 |
| ATCC 700792 | 0 | A+B+ | 005 |
| ATCC 9689 | 0 | A+B+ | 001 |
| BAA-1382 | 0 | A+B+ | 012 |
| BAA-1805 | IIIb | A+B+ | 027 |
| BAA-1814 | XXII | A+B+ | 251 |
| BAA-1870 | IIIb | A+B+ | 027 |
| BAA-1871 | 0 | A+B+ | 001 |
| BAA-1872 | 0 | A+B+ | 207 |
| BAA-1873 | 0 | A+B+ | 053 |
| BAA-1874 | 0 | A+B+ | 002 |
| BAA-1875 | V | A+B+ | 078 |
| CCUG 8864 (CCUG 20309) | X | A-B+ | Unknown |
| IS81 | III | A+B+ | 034 |
| R1880 | I | A+B+ | 086 |
| R7771 | VIII | A-B+ | 110 |
| R8366 | 0 | A+B+ | 001 |
| R8637 | IX | A+B+ | 019 |
| R9367 | XIII | A+B+ | 070 |
| R9385 | XV | A+B+ | 122 |
| R10456 | IV | A+B+ | 058 |
| R10725 | V | A+B+ | 078 |
| R10842 | VI | A+B+ | 045 |
| R10870 | XIV | A+B+ | 111 |
| R12425 | II | A+B+ | 103 |

**Refer to the Simplexa *C. difficile* Direct Package Insert for information on the concentration tested for each strain.**

**No interference was detected with the substances tested in Table 8.**

**Table 8. Potentially Interfering Substances Tested**

| | | | | |
|---|---|---|---|---|
| Afrin | Dramamine | Metronidazole | Palmitic Acid | Tums |
| Antacid and Anti-gas | Dulcolax | Milk of Magnesia | Pepto-Bismol | Vagisil |
| Anusol Plus | Gynol II, Nonoxynol 9 | Mineral Oil | Preparation H | Vancomycin |
| Barium Sulfate | Hydrocortisone cream | Monistat 7 | Sennosides | Vaseline |
| Benzalkonium | Imodium | Mucin | SPF 30 Sunscreen | Witch hazel |
| Blood | KY Jelly | Naproxen | SPF 50 Sunscreen | |
| Colace | Mesalazine | Nystatin | Stearic Acid | |

**Refer to the Simplexa *C. difficile* Direct Package Insert for information on the concentration tested for each strain.**

## Results (continued)

**No cross-reactivity or microbial inhibition was observed with the organisms tested in Table 9**

**Table 9. Cross Reactivity Pathogens (representative of the 127 organisms tested).**

| | | |
|---|---|---|
| Adenovirus 1 | *Clostridium sepiticum* | *Lactobacillus acidophilus* |
| *Alcaligenes faecalis* subsp. faecalis | *Clostridium sordellii* | *Listeria monocytogenes* |
| *Bacillus cereus* | *Clostridium sporogenes* | Norovirus G2 |
| *Bacteroides fragilis* | *Clostridium tetani* | *Proteus mirabilis* |
| *Campylobacter coli* | Coxsackievirus A10 | *Pseudomonas aeruginosa* |
| *Campylobacter jejuni* | Cytomegalovirus | Rotavirus |
| *Candida albicans* | Echovirus 11 | *Salmonella enterica* subsp. arizonae |
| *Chlamydia trachomatis* | *Enterobacter aerogenes* | *Salmonella enterica* subsp. enterica |
| *Citrobacter freundii* | *Enterobacter cloacae* | *Serratia liquefaciens* |
| *Citrobacter koseri* | *Enterococcus faecalis* vanB | *Shigella sonnei* |
| *Clostridium difficile* (non-toxigenic) | *Enterococcus faecium* vanA | *Staphylococcus aureus* |
| *Clostridium haemolyticum* | Enterovirus 71 | *Staphylococcus epidermidis* |
| *Clostridium innocuum* | *Escherichia coli* | *Streptococcus agalactiae* |
| *Clostridium perfringens* | *Helicobacter pylori* | *Vibrio cholerae* |

**Refer to the Simplexa *C. difficile* Direct Package Insert for the complete list of organisms tested for cross reactivity and microbial inhibition.**

## Conclusions

- The Simplexa *C. difficile* Direct kit and LIAISON® MDx from DiaSorin Molecular provide sample-to-answer detection of *Clostridium difficile* from unextracted liquid or unformed stool in approximately one hour, with a simple workflow.
- Simplexa *C. difficile* Direct does not show cross-reactivity to, nor inhibition by, other organisms found in human stool. The assay could detect all toxigenic *C. difficile* strains tested.
- This assay is FDA-cleared with a CLIA moderate complexity rating and is CE-marked.

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