EVALUATION OF THE PERFORMANCE OF NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 (CLOSTRIDIUM DIFFICILE TOXINS REAL TIME PCR KIT) AND COMPARISON RESULTS WITH Xpert® *C. difficile* BT (Cepheid)

Clostridioides difficile CDI, a Gram-positive, spore-forming anaerobe, is the causative agent of CDI infection, a gastrointestinal infection typically characterized by high levels of inflammation and diarrhoea. Therefore, accurate clinical and laboratory diagnosis is essential for prompt patient management and effective infection control measures. Detection of *C. difficile* is normally performed on symptomatic patients by collecting a watery, soft, or semi-formed stool sample.

Molecular diagnostic methods have been prioritized for this diagnosis.

VIRCELL developed a CLOSTRIDIUM DIFFICILE TOXINS REALTIME PCR KIT (RTPCR020) for qualitatively detection and identification of toxigenic strains; toxin A, toxin B and Binary toxins (BT) or non-toxigenic strains (absence of these toxins), both in stool samples from adult and paediatric patients with suspected CDI-associated infection.

Stool samples typically contain PCR inhibitors and can cause false-negative PCR results. The quality and quantity of PCR inhibitors vary between samples, depending on clinical, dietary, gut microbiota, or other factors in terms of environment and lifestyle. Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhibitors generally act at one or more of three essential points in the reaction in the following ways: they interfere with the cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation or capture, and they inhibit polymerase activity for amplification of target DNA. Inhibition may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity of detection. In some cases, inhibition may be the cause of false-negative reactions. RTPCR020 includes an internal control that helps monitoring the carry-over of inhibitors to PCR.

DNA extraction methods are critical to extract a sufficient quantity and quality of genetic material from stool samples for disease diagnosis. There are DNA extraction kits specific for stool samples that are commercially available, using different lysis methods. The bead-beating method is a common mechanical procedure for improving DNA extraction efficiency and increasing DNA recovery, both in specific organism studies and in microbiome studies when using stool samples.

During the development of CLOSTRIDIUM DIFFICILE TOXINS REALTIME PCR KIT Vircell tested a battery of possible extractions protocols in order to determine the best performance solution, considering as a reference method result obtained with Xpert® *C. difficile* BT (Cepheid).

Those tests lead Vircell to develop and produce NA LYSIS KIT (Ref. NALK001), a laboratory reagent for pre-treating stool samples with both, mechanical and chemical processes before extraction.

NA LYSIS KIT reagent contains:

- VIRCELL LYSIS TUBES: 48 x 1 ml of inhibitor removal buffer and a mix of optimized size beads for the specimen disruption by mechanical lysis (*).
- SAMPLE COLLECTION SWABS: 1 x 48 swabs.

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The use of NALK001 reagent is very simple with a minimum specimen manipulation.

1. Remove the cap of the one or more VIRCELL LYSIS TUBES (depending the number of samples to be analysed: one tube/sample).

   Depending of the specimen consistency:

   i. **Semi-solid sample**: use one of the SAMPLE COLLECTION SWABS provided and transfer approximately 0.1 g of sample into the VIRCELL LYSIS TUBES.
   Ensure that the sample has been released into the VIRCELL LYSIS TUBES. In order to facilitate the release of the sample, the swab may be rubbed against the walls of the tube or vortexed.

   ii. **Liquid sample**: homogenize the sample and soak the SAMPLE COLLECTION SWABS in the sample tube, then transfer it to VIRCELL LYSIS TUBES. Homogenise the mixture by vortexing.

2. Shake VIRCELL LYSIS TUBES during 10 minutes at 3000 rpm to disrupt the sample and lyse the cells mechanically (*).

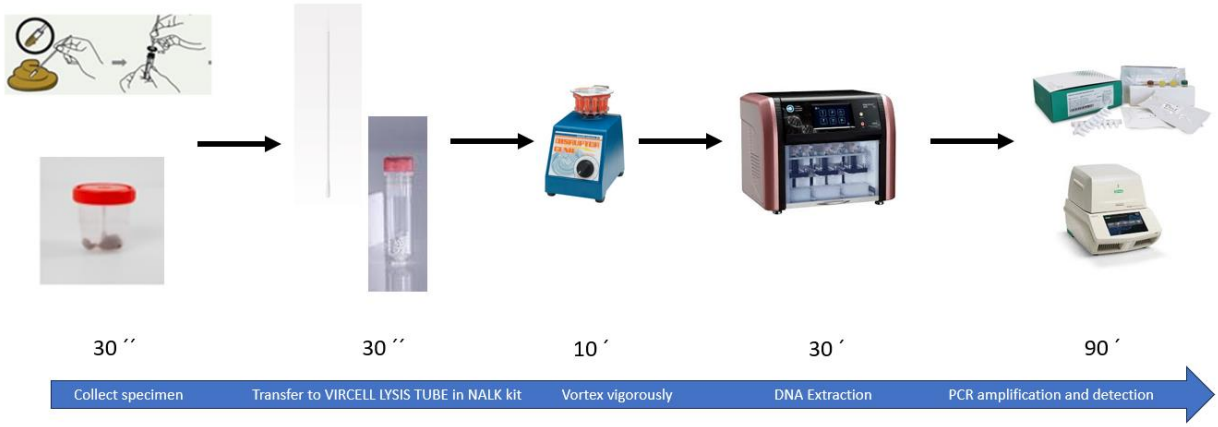
3. Wait 2 minutes to avoid foaming. These samples should be processed in the following 60 minutes. They could be at room temperature.

4. Isolation of nucleic acids can be performed using a manual or automated system compatible with real-time PCR assays following the manufacturer's instructions for use. It is recommended to use a commercial extraction kit for DNA/RNA extraction designed or adapted for stool specimens. In order to use commercial extraction kits, follow the manufacturer’s instructions.

(*) There are some compatible disruptor systems to be used with NALK001 such us:

A. Scientific Industries SI™ Disruptor Genie™ Digital Cell Disruptor by Fisher Scientific
B. MagNA Lyser Instrument (Roche).

Vircell carried out studies using the CLOSTRIDIUM DIFFICILE TOXINS REALTIME PCR KIT with different extraction kits to compare to NA LYSIS KIT pretreatment reagent + VIRAL 665 TANBead (modified protocol) + RTPCR020.



VIRAL 665 TANBead (modified protocol) in M48 series:

Step	Well	Temp (°C)	Mixing Time (m)	Mixing speed (RPM)	Collect (m)	Vapor (m)	Pause
1	1	70	0	500	0	10	Off
2	5	-	0,1	3000	0,1	0	Off
3	1	Off	2	3000	0,1	0	Off
4	2	-	2,5	3000	0,1	0	Off
5	3	-	1,5	3000	0,1	0	Off
6	4	-	1,5	3000	0,1	0	Off
7	6	56	3	3000	0,1	0	Off
8	3	-	0,1	2500	0	0	Off

m: minutes

The results obtained are shown in the following table:

	DNA extraction without NA LYSIS KIT		DNA Extraction with NA LYSIS KIT
Sample	**Extraction alternative 1 + RTPCR020**	**Extraction alternative 2 + VIRAL 665 TANBead (modified protocol) + RTPCR020**	**NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020**
1	35	38	33
2	31	30	25
3	35	35	31
4	28	27	25
5	36	30	24
6	38	33	29
7	24	28	26
8	28	21	22
9	29	29	25
10	27	27	26
11	33	28	25
12	24	27	26
13	36	37	34
14	21	21	21
15	34	31	30
16	22	20	20

The data shown are Ct values obtained for Toxin B with RTPCR020.

Extraction alternative 1: Stool DNA extraction kit (Magnetic bead based) (TANBead).
Extraction alternative 2: INHIBITEX solution (Qiagen) + VIRAL 665 TANBead (modified protocol).

NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 showed the best results compared to extraction alternative 1 and 2. In 87,5 % of the cases the lowest Ct value obtained for the detection of CDI was observed with this method, what might indicate that in cases where the bacterial load of *Clostridium difficile* could be limited or inhibitors are carried over, the solution NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 could potentially detect the presence of DNA while other systems (extraction alternative 1 and 2) might miss those samples**.**

Additionally, results using NA LYSIS KIT + Optipure VIRAL 665 kit (TANBead) + RTPCR020 were compared to a set of positive and negative samples previously analysed with Xpert® *C. difficile* BT, by Cepheid, as reference method.

Results are shown in the following table:

Sample	NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020	Reference method (Xpert® *C. difficile* BT kit)
1	CDI+ABTox (Ct 28)	Tox B (Ct 25,3)
2	CDI+ABTox (Ct 23)	Tox B (Ct 19,7)
3	CDI+ABTox (Ct 25)	Tox B (Ct 22,1)
4	CDI+ABTox (Ct 21)	Tox B (Ct 29,5)
5	CDI+ABTox (Ct 30)	Tox B (Ct 29,5)
6	CDI+ABTox (Ct 28)	Tox B (Ct 26,5)

Sample	NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020	Reference method (Xpert® *C. difficile* BT kit)
7	CDI+ABTox+BT (Ct 24)	Tox B (Ct 24,5), BT (Ct 23,5)
8	CDI+ABTox (Ct 31)	Tox B Ct 30,5)
9	CDI (Ct 32)	Tox B (Ct N/A), BT (Ct N/A)
10	CDI+ABTox (Ct 23)	Tox B (Ct 19,5)
11	CDI+ABTox (Ct 26)	Tox B (Ct 23,1)
12	CDI+ABTox(Ct 25)	Tox B (Ct 26,3)
13	CDI+ABTox(Ct 31)	Tox B (Ct 30,3)
14	CDI+ABTox (Ct 26)	Tox B (Ct 21)
15	CDI+ABTox (Ct 25)	Tox B (Ct 23,9)
16	CDI+ABTox (Ct 30)	Tox B (Ct 26,9)
17	CDI+ABTox (Ct 25)	Tox B (Ct 25,6)
18	CDI+ABTox (Ct 24)	Tox B (Ct 23,3)
19	CDI+ABTox (Ct 31)	Tox B (Ct 31,1), BT (Ct 30,2)
20	CDI+ABTox (Ct 30)	Tox B (Ct 30,1)
21	CDI+ABTox (Ct 30)	Tox B (Ct 25,8)
22	CDI+ABTox (Ct 23)	Tox B (Ct 20,6)
23	CDI+ABTox (Ct 31)	Tox B (Ct 31,5)
24	CDI+ABTox (Ct 27)	Tox B (Ct 27,8)
25	CDI+ABTox (Ct 31)	Tox B (Ct 30,2)
26	CDI+ABTox (Ct 26)	Tox B (Ct 30)
27	CDI+ABTox+BT (Ct 23)	Tox B (Ct 27,6), BT (Ct 26,8)
28	CDI+ABTox (Ct 27)	Tox B (Ct 24,3)
29	CDI+ABTox (Ct 21)	Tox B (Ct 18,9)
30	CDI+ABTox (Ct 24)	Tox B (Ct 26,7)
31	CDI+ABTox (Ct 25)	Tox B (Ct 21,2)
32	CDI+ABTox (Ct 25)	Tox B (Ct 21,9)
33	CDI+ABTox (Ct 27)	Tox B (Ct 26)
34	CDI+ABTox+BT (Ct 23)	Tox B (Ct 23,1), BT (Ct 22,6)
35	CDI+ABTox (Ct 29)	Tox B (Ct 31,1)
36	CDI+ABTox (Ct 23)	Tox B (Ct 23,3)
37	CDI+ABTox (Ct 34)	Tox B (Ct 33,8)
38	CDI+ABTox (Ct 30)	Tox B (Ct 28,2)
39	CDI+ABTox (Ct 28)	Tox B (Ct 28,8)
40	CDI+ABTox (Ct 32)	Tox B (Ct 29)
41	CDI+ABTox (Ct 24)	Tox B (Ct 22,5)
42	CDI+ABTox (Ct 28)	Tox B (Ct 27,8)
43	CDI+ABTox (Ct 25)	Tox B (Ct 28,1)
44	CDI+ABTox+BT (Ct 28)	Tox B (Ct 27,4), BT (Ct 28,2)
45	CDI+ABTox (Ct 26)	Tox B (Ct 27,3)
46	CDI+ABTox+BT (Ct 31)	Tox B (Ct 30,4), BT (Ct 29,7)
47	CDI+ABTox (Ct 25)	Tox B (Ct 25,7)
48	CDI+ABTox+BT (Ct 23)	Tox B (Ct 20,8), BT (Ct 20,2)
49	CDI+ABTox (Ct 21)	Tox B (Ct 22,3)
50	CDI+ABTox+BT (Ct 24)	Tox B (Ct 23,1), BT (Ct 22,6)

Sample	NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020	Reference method (Xpert® *C. difficile* BT kit)
51	CDI+ABTox (Ct 28)	Tox B (Ct 28,2)
52	CDI+ABTox (Ct 29)	Tox B (Ct 28,8)
53	CDI+ABTox+BT (Ct 27)	Tox B (Ct 27,4), BT (Ct 28,2)
54	CDI+ABTox (Ct 26)	Tox B (Ct 28,1)
55	CDI+ABTox+BT (Ct 27)	Tox B (Ct 26,3), BT (Ct 25,9)
56	CDI+ABTox+BT (Ct 30)	Tox B (Ct 30,4), BT (Ct 29,7)
57	CDI+ABTox (Ct 27)	Tox B (Ct 27,5)
58	CDI+ABTox (Ct 30)	Tox B (Ct 29,2)
59	CDI+ABTox (Ct 27)	Tox B (Ct 24,2)
60	CDI+ABTox (Ct 30)	Tox B (Ct 27,1)
61	CDI+ABTox (Ct 27)	Tox B (Ct 28)
62	CDI+ABTox+BT (Ct 27)	Tox B (Ct 24,9), BT (Ct 24,2)
63	CDI+ABTox (Ct 28)	Tox B (Ct 25,8)
64	CDI+ABTox (Ct 30)	Tox B (Ct 27,1)
65	CDI+ABTox (Ct 27)	Tox B (Ct 24,2)
66	CDI+ABTox (Ct 25)	Tox B (Ct 23,5)
67	CDI+ABTox (Ct 29)	Tox B (Ct 26,1)
68	CDI+ABTox (Ct 29)	Tox B (Ct 27,2)
69	CDI+ABTox (Ct 26)	Tox B (Ct 23,7)
70	CDI+ABTox (Ct 25)	Tox B (Ct 24,4)
71	CDI+ABTox (Ct 23)	Tox B (Ct 20,9)
72	CDI+ABTox (Ct 25)	Tox B (Ct 23,1)
73	CDI+ABTox (Ct 21)	Tox B (Ct 22,8)
74	CDI+ABTox (Ct 28)	Tox B (Ct 25,2)
75	CDI+ABTox (Ct 25)	Tox B (Ct 24,6)
76	CDI+ABTox (Ct 27)	Tox B (Ct 27,1)
77	CDI+ABTox (Ct 25)	Tox B (Ct 24,3)
78	CDI+ABTox (Ct 25)	Tox B (Ct 24,3)
79	CDI+ABTox (Ct 24)	Tox B (Ct 22,5)
80	CDI+ABTox (Ct 31)	Tox B (Ct 29)
81	CDI+ABTox+BT (Ct 23)	Tox B (Ct 20,8), BT (Ct 20,2)
82	CDI+ABTox (Ct 23)	Tox B (Ct 22,3)
83	CDI+ABTox (Ct 25)	Tox B (Ct 25,2)
84	CDI+ABTox+BT (Ct 30)	Tox B (Ct 28,2), BT (Ct 27,6)
85	CDI+ABTox+BT (Ct 26)	Tox B (Ct 24,5), BT (Ct 24,1)
86	CDI+ABTox (Ct 22)	Tox B (Ct 21,2)
87	CDI+ABTox (Ct 21)	Tox B (Ct 19,5)
88	CDI+ABTox (Ct 26)	Tox B (Ct 26)
89	CDI+ABTox (Ct 24)	Tox B (Ct 21,5)
90	CDI+ABTox (Ct 20)	Tox B (Ct 20,5)
91	CDI+ABTox (Ct 22)	Tox B (Ct 19,6)
92	CDI+ABTox+BT (Ct 22)	Tox B (Ct 20,7), BT (Ct 20,1)
93	CDI+ABTox (Ct 23)	Tox B (Ct 22,3)
94	CDI+ABTox (Ct 26)	Tox B (Ct 24,7)
95	CDI+ABTox (Ct 22)	Tox B (Ct 27,3)

Sample	NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020	Reference method (Xpert® *C. difficile* BT kit)
96	CDI+ABTox (Ct 31)	Tox B (Ct 25,6)
97	CDI+ABTox (Ct 30)	Tox B (Ct 21,6)
98	CDI+ABTox (Ct 32)	Tox B (Ct 33,2)
99	CDI+ABTox (Ct 23)	Tox B (Ct 21,3)
100	CDI+ABTox (Ct 25)	Tox B (Ct 20,9)
101	Negative	Tox B (Ct 26)
102	CDI+ABTox (Ct 33)	Tox B (Ct 27)
103	CDI+ABTox (Ct 32)	Tox B (Ct 24,7)
104	Negative	Tox B (Ct 28,3)
105	CDI (Ct 33)	Tox B (Ct 28,2)
106	CDI+ABTox (Ct 28)	Tox B (Ct 20,6)
107	CDI+ABTox (Ct 34)	Tox B (Ct 30,7)
108	CDI+ABTox (Ct 23)	Tox B (Ct 18,8)
109	CDI+ABTox (Ct 27)	Tox B (Ct 21,8)
110	CDI+ABTox (Ct 35)	Tox B (Ct 29,6)
111	CDI+ABTox (Ct 30)	Tox B (Ct 25,4)
112	CDI+ABTox (Ct 34)	Tox B (Ct 28,1)
113	CDI+ABTox (Ct 33)	Tox B (Ct 32,7)
114	CDI+ABTox (Ct 35)	Tox B (Ct 32,7)
115	CDI+ABTox (Ct 36)	Tox B (Ct 27,2)
116	Negative	Tox B (Ct 32,8)
117	CDI+ABTox (Ct 28)	Tox B (Ct 23)
118	CDI+ABTox (Ct 28)	Tox B (Ct 23,1)
119	CDI+ABTox (Ct 31)	Tox B (Ct 27,3)
120	CDI+ABTox (Ct 31)	Tox B (Ct 27,6)
121	CDI+ABTox (Ct 32)	Tox B (Ct 29,6)
122	CDI (Ct 33)	Tox B (Ct N/A), BT (Ct N/A)
123	CDI (Ct 26)	Tox B (Ct N/A), BT (Ct N/A)
124	CDI (Ct 39)	Tox B (Ct N/A), BT (Ct N/A)

The concordance between both methods was 95,83% in detecting positive samples for *Clostridium difficile* producing either Toxins A/B or binary toxin (BT) as well as both toxins.

NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 was able to detect 115 out of 120 positive samples.

Regarding positive samples only detecting Toxin A/B, the concordance between both methods was 96,15%.

NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 was able to detect 100 out of 104 positive samples for *Clostridium difficile* producing Toxins A/B.

Regarding positive samples detecting Toxin A/B + BT the concordance between both methods was 93,75%.

NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020 was able to detect 15 out of 16 positive samples for *Clostridium difficile* producing binary toxin (BT).

Xpert® C. difficile BT kit reported 4 negative samples which were positive for *Clostridium difficile* non-toxigenic strains by NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020, previously confirmed by a GDH test.

A summary of these results is shown in the following table:

	NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020	**Reference method (Xpert® *C. difficile* BT kit)**	**CONCORDANCE**
Positive samples	115	120	95,83%
Positive samples for Toxin A/B	100	104	96,15%
Positive samples for Toxin A/B + BT	15	16	93,75%

Conclusions:

Data shown in this technical note describes **NA LYSIS KIT + VIRAL 665 TANBead (modified protocol) + RTPCR020** solution is a suitable tool for helping laboratories in the detection of toxigenic *Clostridium difficile* and could be considered an alternative to Xpert® *C. difficile* BT kit.

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