VITASSAY

Clostridium difficile Toxin A+B

Rapid test for the simultaneous qualitative detection of Toxin A and Toxin B of Clostridium difficile in human stool samples.

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CE IVD EN

Vitassay

EN

For professional *in vitro* diagnostic use only.

INTENDED USE

Vitassay Clostridium difficile Toxin A+B is a rapid, immunochromatographic, one step assay for the simultaneous qualitative detection of Toxin A and Toxin B of Clostridium difficile in human stool samples.

Simple, non-invasive and highly sensitivity immunoassay to make a presumptive diagnosis of *Clostridium difficile* infection.

INTRODUCTION

Clostridium (Clostridioides) difficile, is a gram-positive anaerobic enterotoxigenic bacillus identified as one of the leading causes of infectious diarrhoea related to antibiotic treatment (10-15%). It is a major public health concern, as it responsible of broad outbreaks in hospitals and nursing homes. *C. difficile* is also associated with high mortality rates in the elderly (Marra et al., 2020). The main risk factors for *C. difficile* infections are age (>65 years-old), hospitalisation, being immunocompromised and/or previous infection (Abt et al., 2016).

C. difficile can be divided into two major strains: nontoxigenic and toxigenic, but only the later one causes disease in humans. These toxigenic strains produce toxin B (TcdB) alone, or in combination with toxin A (TcdA) and the pathogenicity depends on the expression of these two toxins (Burke et al., 2014). The toxins are produced and secreted in the large intestine under limit nutrient availability and undergo endocytosis by the intestinal epithelial cells. The activation of these toxins in the cytosol, produces cell necrosis and the subsequent loss of intestinal membrane integrity. This process leads to host exposure to intestinal microorganisms and the subsequent activation of the inflammatory response (CDC).

Different toxigenic strains have been reported to cause important outbreaks around the globe. In 2003, the new strain NAP1/ribotype 027 was a source of C. difficile epidemics in the US and Canada. It has also been described across Europe and Chile, and t is associated with higher morbidity and recurrent rates. It is of a particular interest since it has a mutation in an inhibitory gene, leading to an increase toxin A and B production (McDonald et al., 2005). In other parts of the world, as Asia, it is rare to find 027 ribotype, however 017 or 002 accounts for a large number of cases (Burke et al., 2014).

Treatment is based on antibiotic administration, including and vancomycin and fidaxomicin and metronidazole/vancomycin. However, antibiotics are not always affective and there is a 25% risk of having recurrent episodes. Innovative treatments, like FMT (faecal microbiota transplant), antibody-based therapy and microbiota-based drugs have risen. There are two vaccines under evaluation (Guery et al., 2019).

Signs of infection might appear after taking antibiotics or being hospitalised/during hospitalisation. Common symptoms are severe diarrhoea, fever, stomach tenderness, loss of appetite and nausea (CDC). Measurements to avoid spreading must be taking, such as good hygiene and avoiding sharing towels/linen.

Systematic testing for *C. difficile* is recommended in case a diarrhoea happens in a healthcare environment, or where the common causatives of intestinal infections have been discarded.

PRINCIPLE

Vitassay Clostridium difficile Toxin A+B is a qualitative immunochromatographic assay for the detection of Toxin A and B of *Clostridium difficile* in human stool samples.

Strip A: The test line zone of the nitrocellulose membrane is pre-coated with monoclonal antibodies against Toxin A.

Strip B: The test line zone of the nitrocellulose membrane is pre-coated with monoclonal antibodies against Toxin B.

During the process, the sample reacts with the antibodies against Toxin A (strip A) and Toxin B (strip B), forming conjugates. The mixture moves upward on the membrane by capillary action. If the sample is Toxin A positive, antibodies present on the membrane (test line) capture the conjugate complex and a red line will be visible in the strip A, and if the sample is Toxin B positive, antibodies present on the membrane (test line) capture the conjugate complex and a red line will be visible in strip B. Although the sample is positive or negative, the mixture continues to move across the membranes and the green control line always appears (for both strips).

The presence of these green lines (in the control zone (C)) indicates that sufficient volume is added; proper flow is obtained and serves as an internal control for the reagents.

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PRECAUTIONS

- For professional *in vitro* use only.
- Do not use after expiry date.
- Read the instructions for use carefully before using the test.
- Do not use the kit if the label sealing the outer carton is torn or if the bags are open or damaged on arrival.
- Do not use the tests if the desiccant material is missing or broken inside the aluminium pouch.
- Specimens should be considered potentially hazardous and should be handled in the same manner as an infectious agent, following local/national regulations. A new test should be used for each sample to avoid contamination errors.
- Material exposed to the samples should also be considered potentially hazardous and should be handled in the same manner as an infectious agent, following local/national regulations.
- Do not reuse. This is a single-use device.
- Used material should be disposed of in an appropriate biohazard container after testing.
- The reagents contain a preservative called sodium azide (<0.1%). Avoid any contact with skin or mucous membranes.
- In accordance with current regulations **Vitassay Clostridium difficile Toxin A+B** does not contain substances and/or mixtures that are hazardous or present in a concentration above the limits for their declaration. The safety data sheet is available on request (not included).
- All reagents included in the kit are approved for use with **Vitassay Clostridium difficile Toxin A+B** only. Do not mix or use the components with other batches of Vitassay. Do not use with reagents from other kits or commercial assays.
- Follow Good Laboratory Practices. These practices should include, but are not limited to, personal protective equipment (PPE), such as lab coat, surgical or appropriate mask or face shield, disposable gloves and eye protection. Take the necessary precautions during sample collection, transport, storage, handling and disposal. Each sample must be correctly and unequivocally identified to ensure proper traceability of samples.
- In case of spillage, clean thoroughly with a suitable disinfectant.
- Do not eat, drink or smoke in the workplace.
- The presence of yellow lines in the result window (control line area and test line area), before using the test, is completely normal and does not imply a failure in the functionality of the test.
- The visual interpretation of the results is done by coloured lines, the interpretation of the results should be done by a professional user without problems of visualisation and colour interpretation.
- A certificate of analysis can be provided on request (not included).

STORAGE AND STABILITY

The storage temperature of the kits should be 2-30ºC.

Do not freeze.

Under these conditions, they can be used until the expiry date indicated on the kit label.

All kit components are for single use only and must remain in their primary packaging until use. The test must remain in the sealed pouch until use.

MATERIALS

MATERIAL PROVIDED	MATERIAL REQUIRED BUT NOT PROVIDED
▪25X **Vitassay Clostridium difficile Toxin A+B** ▪Instructions for use. ▪25X **Collection Vial** with diluent	▪PPE, such as disposable gloves ▪Specimen collection container ▪Timer ▪Micropipette (in case of liquid stool)

SPECIMEN COLLECTION

Collect sufficient quantity of faeces: 1-2g or mL for liquid samples. Stool specimens should be collected in clean and dry containers.

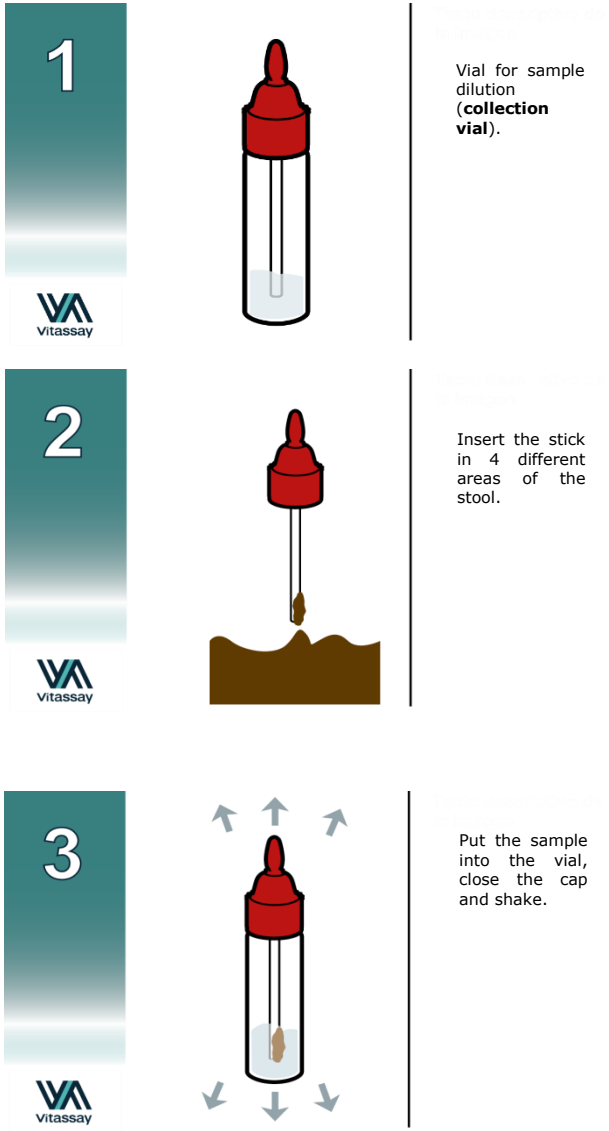
If processed the day after collection, samples can be stored at room temperature. However, we recommend testing immediately after sample collection.

Samples can be stored for up to 7 days at 2-8ºC, and also frozen at -20ºC for up to one year. Samples shall be brought to room temperature before testing.

Ensure that only the necessary quantity is thawed, as freezing freeze-thaw cycles are not recommended. Homogenise stool samples as thoroughly as possible prior to preparation.

SPECIMEN PREPARATION

1. Remove the cap of the **collection vial** with diluent for the sample dilution (figure 1).
2. Use the stick to collect sufficient sample quantity (50 mg). For solid stool, insert the stick in 4 different areas of the stool sample (figure 2), and add it into the vial with diluent for sample dilution. For liquid stool, take 125µL of the sample using a micropipette and transfer it into the vial with diluent for the sample dilution.
3. Close the tube with the diluent and stool sample. Shake vigorously the vial in order to assure good sample dispersion (figure 3).



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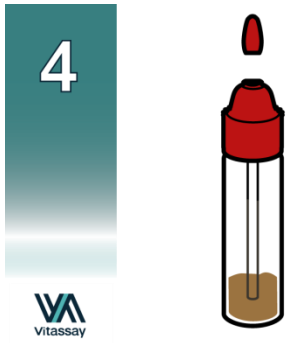
PROCEDURE

Allow the test, stool sample, controls, and diluent to reach room temperature (15-30ºC) prior to testing. Do not open pouches until the performance of the assay.

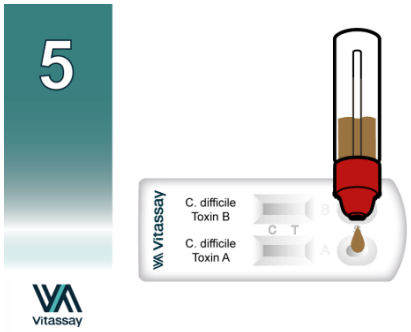
Make sure that each collection vial and each cassette are properly identified to allow a correct traceability.

1. Shake the vial with the sample vigorously to obtain a good sample dilution.
2. Remove the **Vitassay Clostridium difficile Toxin A+B** from its sealed bag just before using it.
3. Take the vial containing the diluted sample, cut the end of the cap (figure 4) and dispense 3 drops in the circular window marked with the letter A – Toxin A (figure 5) and 3 drops, using the same vial, in the circular window marked with the letter B – Toxin B (figure 6).
4. Read the results at **10 minutes**. Do not read the results later than 10 minutes.

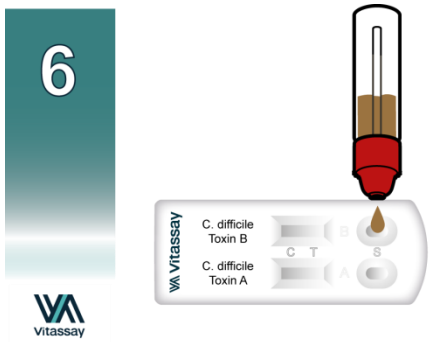
If the test does not run due to solid particles, stir the sample added in the sample window with the stick. If it does not work, dispense a drop of diluent until seeing the liquid running through the reaction zone.



Cut the end of the cap.

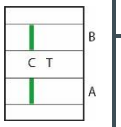
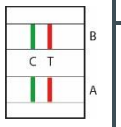
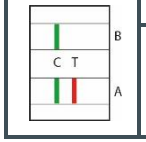
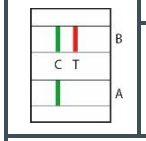


Dispense 3 drops in the circular window marked with the letter S to the strip A – Toxin A.



Dispense 3 drops in the circular window marked with the letter S to the strip B – Toxin B.

INTERPRETATION OF THE RESULTS

RESULTS	Strip A Toxin A	Strip B Toxin B	INTERPRETATION
	**Negative**	**Negative**	There is no Toxin A or Toxin B of Clostridium difficile presence. No infection caused by Clostridium difficile.
	GREEN	GREEN	
	**Positive**	**Positive**	There is Toxin A and Toxin B of *Clostridium difficile* presence. Possible infection caused by *Clostridium difficile.*
	GREEN-RED	GREEN-RED	
	**Positive**	**Negative**	There is Toxin A of *Clostridium difficile* presence. Possible infection caused by *Clostridium difficile*.
	GREEN-RED	GREEN	
	**Negative**	**Positive**	There is Toxin B presence. Infection caused by Clostridium difficile.
	GREEN	GREEN-RED	
ANY OTHER RESULTS			Invalid result, we recommend repeating the assay using the sample with another test. **Note:** Wrong procedural techniques or deterioration of the reagents are the main reasons of control line failure. If the symptoms or situation persist, discontinue using the test kit and contact your local distributor.

Notes: The intensity of the red-coloured test line in the result line zone (T) will vary depending on the concentration of antigens in the specimen.

QUALITY CONTROL

Internal procedural controls are included in **Vitassay Clostridium difficile Toxin A+B**. Green lines appearing in the in the results window are internal controls, which confirm sufficient specimen volume and correct procedural technique.

LIMITATIONS

- **Vitassay Clostridium difficile Toxin A+B** test must be carried out within 2 hours of opening the sealed bag.
- An excess of stool sample could cause incorrect results (brown bands appear). Dilute the sample with the diluent and repeat the test.
- The intensity of test line may vary depending on the concentration of antigens.
- The use of other samples different from human samples has not been established.
- The quality of **Vitassay Clostridium difficile Toxin A+B** depends on the quality of the sample; proper faecal specimens must be obtained.
- Positive results determine the presence of *Clostridium difficile* antigen GDH in stool samples; nevertheless, it can be due to toxigenic or not toxigenic strains of *Clostridium difficile*. A positive result should be followed up with additional laboratory techniques (toxigenic culture) to determine the strain. A confirmed infection should only be made by a physician after all

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clinical and laboratory findings have been evaluated and must be based in the correlation of the results with further clinical observations.

- Negative results should not be considered as conclusive; it is possible that the antigen concentration is lower than the detection limit value. If symptoms or situation persists, a *Clostridium difficile* determination should be carried out, on a sample from an enrichment culture.
- **Bloody stool samples** and/or **mucinous** stool samples can cause non-specific reactions in the test. Such positive samples should be followed up with other diagnostic techniques to confirm the result.
- This test is a screening tool. The final diagnosis must be confirmed by a physician after a thorough evaluation of the clinical history and additional confirmatory tests.

EXPECTED VALUES

Clostridium difficile is associated with 95-100% of cases of pseudomembranous colitis, 60-75% of cases of antibiotic-associated colitis and 35% of cases of antibiotic-associated diarrhoea cases. The other causes of antibiotic-associated diarrhoea are largely unknown.

According to ECDC, in 2016–2017, 72.0% of the CDI (*C. difficile* infection) cases with case-based data were above 64 years old and the majority (56.4%) were female. More than half (n=3 446/5 863; 58.8%) of the CDI cases had had contact with healthcare in the three months before the current healthcare admission, of which the vast majority (n=2 804/3 446; 81.4%) had had contact with a hospital. Fewer were reported to have had contact with a long-term care facility (LTCF) (n=431; 12.5%). There were an estimated 189 526 healthcare-associated (HA) CDI cases (cumulative 95% confidence interval (95%CI): 105 154–340 978) in EU/EEA countries/administrations annually in 2016–2017. In 2016–2017, the crude incidence density of CDI was 3.48 cases per 10 000 patient-days. This was higher in tertiary care hospitals (3.87 cases per 10 000 patient-days) than in secondary or primary care hospitals (3.46 and 2.28 cases per 10 000 patient-days, respectively).

PERFORMANCE CHARACTERISTICS

Clinical sensitivity and specificity

Two retrospective evaluations were carried out with stool samples, comparing the results obtained by **Vitassay Clostridium difficile Toxin A+B** with commercial immunochromatographic tests.

In an initial study, a total of 126 samples from patients with suspicion of *C. difficile* infection were compared. The results are shown below:

Toxin A

		Reference method (Toxin A+B)		
		Positive	Negative	Total
Vitassay Clostridium difficile Toxin A+B	Positive	28	0	28
	Negative	1	97	98
Toxin A	Total	29	97	126

Table 1. Results of Vitassay Clostridium difficile Toxin A + B (toxin A) compared to a commercial CE-IVD kit.

Vitassay Clostridium difficile Toxin A+B (Toxin A) vs Reference method (Toxin A+B)		
	Mean Value	95% confidence interval
Sensitivity	96.6%	92.2-99.9%
Specificity	100%	96.2-100%
PPV	100%	87.7-100%
NPV	99.0%	94.4-100%

Table 2: Sensitivity, specificity, positive predictive values, and negative predictive values of the Vitassay Clostridium difficile Toxin A + B (toxin A) kit compared to a commercial CE-IVD kit.

Toxin B

		Reference method (Toxin A+B)		
		Positive	Negative	Total
Vitassay Clostridium difficile Toxin A+B	Positive	36	1	37
	Negative	0	89	89
Toxin B	Total	36	90	126

Table 3. Results of Vitassay Clostridium difficile Toxin A + B (toxin B) compared to a commercial CE-IVD kit.

Vitassay Clostridium difficile Toxin A+B (Toxin B) vs Reference method (Toxin A+B)		
	Mean Value	95% confidence interval
Sensitivity	100%	90.3-100%
Specificity	98.9%	94.0-100%
PPV	97.3%	85.8-99.9%
NPV	100%	95.9-100%

Table 4. Sensitivity, specificity, positive predictive values and negative predictive values of the Vitassay Clostridium difficile Toxin A + B (toxin B) kit compared to a commercial CE-IVD kit.

Subsequently, **Vitassay Clostridium difficile Toxin A+B** was evaluated with 250 leftover samples from patients with suspected *C. difficile* infection. Again, a commercial immunochromatographic kit was used as reference kit. Inconsistent results were analysed by a CE-IVD nucleic acid amplification (qPCR) assay.

Toxin A

		Reference method (Toxin A+B)		
		Positive	Positive	Positive
Vitassay Clostridium difficile Toxin A+B	Positive	51	0	51
	Negative	1	198	199
Toxin A	Total	52	198	250

Table 5. Results of Vitassay Clostridium difficile Toxin A + B (toxin A) compared to a commercial CE-IVD kit.

Vitassay Clostridium difficile Toxin A + B (Toxin A) vs Reference method (Toxin A+B)		
	Mean Value	95% confidence interval
Sensitivity	98.1%	89.7-100%
Specificity	100%	98.2-100%
PPV	100%	93.0-100%
NPV	99.5%	97.2-100%

Table 6. Sensitivity, specificity, positive predictive values and negative predictive values of the Vitassay Clostridium difficile Toxin A + B (toxin A) kit compared to a commercial CE-IVD kit.

Toxin B

		Reference method (Toxin A+B)		
		Positive	Negative	Total
Vitassay Clostridium difficile Toxin A+B	Positive	40	0	40
	Negative	4	206	210
Toxin B	Total	44	206	250

Table 7. Results of Vitassay Clostridium difficile Toxin A + B (toxin B) compared to a commercial CE-IVD kit.

Vitassay Clostridium difficile Toxin A + B (Toxin B) vs Método de referencia (Toxin A+B)		
	Mean Value	95% confidence interval
Sensitivity	90.9%	78.3-97.5%
Specificity	100%	98.2-100%
PPV	100%	91.2-100%
NPV	98.1%	95.2-99.9%

Table 8. Sensitivity, specificity, positive predictive values, and negative predictive values of the Vitassay Clostridium difficile Toxin A + B (toxin B) kit compared to a commercial CE-IVD kit.

In summary, **Vitassay Clostridium difficile Toxin A + B** has been tested on a total of 376 clinical faecal samples. The pooled analysis of both studies shows an overall clinical **sensitivity** for toxin A of 0.97 (0.91-0-99) and for toxin B of 0.95 (0.87-0.98). On the other hand, the overall clinical **specificity** calculated for toxin A is 1 (0.98-1) and for toxin B 0.99 (0.98-1).

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Overall, the results show that Vitassay Clostridium difficile Toxin A + B has a high sensitivity and specificity to detect *Clostridium difficile* toxins A and B.

Analytical sensitivity

The limit of detection (LoD) of the **Vitassay Clostridium difficile Toxin A +B** is 2 ng/mL *Clostridium difficile* Toxin A and 3.12 ng/mL Toxin B. Note that a general reference standard has not been approved; the LoD was determined using purified *C. difficile* toxins A and B.

Cross reactivity

No cross reactivity was detected against other gastrointestinal pathogens, other organism, substances and/or faecal markers that are occasionally present in faeces:

Adenovirus	*Escherichia coli O111*	*Peptostreptococcus anaerobius*
Astrovirus	*Escherichia coli O26*	Rotavirus
Bovine Haemoglobin	*Escherichia coli O157*	RSV
Campylobacter coli	*Clostridium difficile Toxin A*	*Salmonella paratyphi A*
Campylobacter jejuni	*Giardia*	*Salmonella typhi*
Clostridium bifermentas	*Helicobacter pylori*	*Salmonella typhimurium*
Clostridium butyricum	Human Calprotectin	*Shigella boydii*
Clostridium haemolyticum	Human Haemoglobin	*Shigella dysenteriae*
Clostridium novyi	Human Lactoferrin	*Shigella flexneri*
Clostridium tetani	Human Transferrin	*Shigella sonnei*
Clostridium difficile Toxin B	*Influenza A virus*	*Staphylococcus aureus*
Clostridium perfringens	*Influenza B virus*	*Streptococcus pneumococcal*
Clostridium septicum	*Salmonella enteritidis*	*Streptococcus pyogenes*
Cryptosporidium parvum	Norovirus GII	*Yersinia Enterocolitica O:3*
Entamoeba histolytica	Porcine haemoglobin	*Yersinia Enterocolitica O:9*

Interferences

Possible interferences were assessed using the following **exogenous compounds and drugs** at the given concentrations (no interferences were found):

Aceltylcysteine (Fluimucil)	3.0 mg/mL	No interference
Acetyl Salicylic (*Adiro*)	0.3 mg/mL	No interference
Afrin *(Oxymetazoline)*	5.0·10-2 mg/mL	No interference
Almagato (Almax)	3.0 mg/mL	No interference
Amantadine	0.3 mg/mL	No interference
Ambroxol hydrochloride (Mucosan)	0.3 mg/mL	No interference
Amoxicillin	3.0 mg/mL	No interference
Ampicillin	3.0 mg/mL	No interference
Benzocaine (*Angileptol*)	0.3 mg/mL	No interference
Biotine	100.0 µg/mL	No interference
Carbocisteine (*Iniston mucolítico*)	3.0 mg/mL	No interference
Ciprofloxacin	0.3 mg/mL	No interference
Cloperastine (*Flutox*)	0.3 mg/mL	No interference
Codeine (*Toseina*)	0.2 mg/mL	No interference
CVS Nasal Drops *(Phenylephrine)*	10.0 mg/mL	No interference
CVS Nasal Spray *(Cromolyn)*	4 mg/mL	No interference
Dexchloropheniramine (*Polaramine*)	0.3 mg/mL	No interference
Dexketoprofen trometamol (Enantyum)	0.3 mg/mL	No interference
Ebastine (*Ebastel*)	3.0 mg/mL	No interference
Fluticasone Propionate	5.0·10-2 mg/mL	No interference
Fosfamycin (Monurol)	3.0·10-3 mg/mL	No interference
Heparin (Hibor)	350.0 IU/mL	No interference
Hydroxyzine dihydrochloride	0.3 mg/mL	No interference
Homeopathy	1/10 dilution	No interference
Ibuprofen (*Espidifen*)	3.0 mg/mL	No interference
Levofloxacin	3.0 mg/mL	No interference
Loperamide hydrochloride (Fortasec)	0.15 mg/mL	No interference
Loratadine	0.3 mg/mL	No interference
Lorazepam	3.0·10-3 mg/mL	No interference
Lysine Carbocysteinate (Pectox)	3.0·10-2 mg/mL	No interference
Macrogol 3350 (Movicol)	3.0 mg/mL	No interference
Mercaptopurine	0.3 mg/mL	No interference
Metamizole (*Nolotil*)	5.0 mg/mL	No interference
Metronidazole	3.0 mg/mL	No interference
Mupirocin	2.5·10-2 mg/mL	No interference
Naso GEL	0.9 mg/mL	No interference
Omeprazole	2.0·10-3 mg/mL	No interference
Oseltamivir	0.3 mg/mL	No interference
Paracetamol (*Dolocatil*)	5.0 mg/mL	No interference
Phenoxymethylpenicillin potassium	3.0 mg/mL	No interference
Phenulpropanolamine	0.3 mg/mL	No interference
Prednisone	0.3 mg/mL	No interference
Ribavirin	3.0 mg/mL	No interference
Rifampicin (Rifaldin)	0.3 mg/mL	No interference
Sore Throat Phenol spray	0.5 mg/mL	No interference
Tobramycin	0.3 mg/mL	No interference
ZICAM	1.0 mg/mL	No interference

In addition, the following human **endogenous substances** were also analysed. As before, no interferences were found:

Human blood	5.0 %	No interference
Human calprotectin	5.0 µg/mL	No interference
Human haemoglobin	5.0 µg/mL	No interference
Human lactoferrin	5.0 µg/mL	No interference
Human transferrin	0.5 µg/mL	No interference
Mucine	0.5 %	No interference

Repeatability and Reproducibility

Repeatability and reproducibility studies performed with different positive and negative samples yielded no differences between the evaluations.

Hook effect

No inhibitory hook effect was demonstrated at concentrations higher than 10^2 times the limit of detection (10^2xLoD) for toxin A, nor at concentrations 10^4 times the limit of detection (10^4xLoD) for toxin B in the **Vitassay Clostridium difficile Toxin A + B** kit.

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SYMBOLS FOR IVD COMPONENTS AND REAGENTS

IVD	*in vitro* diagnostic device		Keep dry
	Consult instructions for use		Temperature limitation
	Use by		Manufacturer
LOT	Batch code	Σ n	Contains sufficient for <n> test
DIL	Sample diluent	REF	Catalogue number
CE	CE Marking		

Vitassay