CLOSTRIDIUM DIFFICILE TOXINS REAL TIME PCR KIT

REF RTPCR020-LP Σ 48

CE 0123 For *in vitro* diagnostic use

INTENDED PURPOSE

Real Time RT-PCR kit to detect nucleic acid from *Clostridioides difficile* (formerly *Clostridium difficile*), toxins A+B (codetection) and binary toxin in human stool samples.
The device is intended to be used with general population with suspected infection by the microorganism.
The test is a qualitative and automated assay, intended to be used as an aid to diagnosis.

INTRODUCTION

Clostridium difficile (CDI), a spore-forming, gram-positive anaerobic bacillary bacterium, was first described in 1935 by Hall and O'Toole as part of the intestinal microbiota of healthy neonates. In the late 1970s, CDI was identified as the causative agent of antibiotic-associated diarrhea and pseudomembranous colitis, associated with high morbidity and mortality in advanced ages. Currently, CDI is one of the most common causes of hospital diarrhea.
It is estimated that at least 15-25% of cases of antibiotic-associated diarrhea are caused by CDI, as are most cases of pseudomembranous colitis.
Symptoms range from mild diarrhea to intestinal infections of varying severity, including pseudomembranous colitis, toxic megacolon, and occasionally sepsis and death. Clinically symptomatic cases are caused by toxigenic strains of CDI that produce toxin A and/or toxin B. In recent years, the binary toxin has been correlated with more severe hypervirulent strains (such as ribotype 027) with an increased in morbidity and mortality, complications and recurrence.

TEST PRINCIPLE

It is based on the amplification in the same reaction well of specific fragments of *Clostridium difficile* (CDI), and specific detection of encoding genes for toxin A, B (A+B) and binary toxin (BT) by real time PCR.
One lyophilized master mix (RT-PCR MIX) is provided for screening and confirmation using one independent target.
PCR mix targets a specific fragment of the *tpi* gene for CDI, *tdcA* and *tdcB* genes for toxins A and B, respectively; and *cdtA* and *cdtB* sequences for the binary toxin.
An amplification control is included to check the absence of carry-over of amplification inhibitors and the correct amplification set-up. This control consists of exogenous nucleic acids and specific oligo pair/probe for its amplification.
The technique is divided into 2 main steps: DNA extraction and amplification/detection with specific oligo pairs and probes. CDI DNA is detected in FAM channel, A+B DNA is detected in HEX/VIC channel, BT DNA is detected in Texas/ROX channel. The internal control (IC) is detected in Cy5 channel.

KIT FEATURES

VIRCELL RT-PCR MIX and VIRCELL POSITIVE CONTROL are lyophilized. It is necessary to reconstitute them before use (see "Preparatory treatment of the device" section). The rest of the reagents are ready to use.
This kit is based on amplification and detection using real time PCR.

MATERIALS PROVIDED

[1] VIRCELL CDI RT-PCR MIX LP: 6 strips with 8 tubes containing Taq polymerase, buffer and specific primers/probe for CDI (*tpi* gene), toxins A+B (*tdcA* and *tdcB* genes) and BT (*cdtA* and *cdtB* genes). Also, primers/probe for internal control. 1 reaction per tube. Lyophilized.
[3] VIRCELL CDI POSITIVE CONTROL: 1 vial containing a mixture of lyophilized non-infectious nucleic acids to be used as positive control. Red cap.
[4] VIRCELL NEGATIVE CONTROL: 200 µl of deionized water to be used as negative control. Green cap.
[5] VIRCELL PCR MIX RECONSTITUTION SOLUTION: 1 ml of aqueous solution to reconstitute the PCR mix. Yellow cap.
[6] VIRCELL POSITIVE CONTROL RECONSTITUTION SOLUTION: 500 µl of aqueous solution to reconstitute the positive control. Brown cap.
[7] VIRCELL RT-PCR MIX CAPS 6: 6 strips of 8 caps RT-PCR compatible.

Special materials required but not provided:
-Microbiological safety cabinet.
-Reagents for bead beating and removal of stool inhibitors.
-DNA/RNA extraction kit (see recommendations in "Assay procedure").
-Real Time PCR thermocycler (compatible with low profile white tubes with FAM, HEX/VIC, Texas/ROX and Cy5 detection).
-Precision micropipettes.
-Sterile tips with aerosol barrier.
-Microcentrifuge.
-PCR cabinet (recommended).
-Vortexer.

STORAGE AND HANDLING CONDITIONS

Store at 2-8°C. Do not use the kit reagents beyond the expiration date. This will be valid only if reagents are stored closed and at 2-8°C.

IN-USE STABILITY

VIRCELL POSITIVE CONTROL reconstituted: store it between -25°C and -15°C and use until expiration date. Avoid more than 10 freeze-thaw cycles during this time period.
VIRCELL RT-PCR MIX reconstituted: store it between 2ºC and 8ºC and use before 60 minutes.
Rest of reagents: Refer to package label for expiration date (at 2-8°C).
VIRCELL, S.L. does not accept responsibility for the mishandling of the reagents included in the kit.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use only. For professional use only.
2. The product should be limited to personnel who have been trained in the technique.
3. The user of this kit is advised to carefully read and understand the package insert. Strict adherence to the protocol is necessary to obtain reliable test results.
4. Use only protocols described in this insert. Conditions other than specified may give erroneous results.
5. Wear personal protective equipment when handling samples and reagents. Wash hands properly after handling the samples and reagents. All procedures must be carried out in accordance with the approved safety standards.
6. Clean pipette tips must be used for every assay step. Use only clean, preferably disposable material.
7. Never pipette by mouth.
8. Do not use in the event of damage to the package.
9. Do not use the kit after expiration date.
10. Do not leave the reagents at temperature different to the recommended longer than absolutely necessary.
11. Keep containers for samples and reagents closed while they are not being handled.
12. Avoid using samples subjected to repeated freeze-thaw cycles.
13. Handle in aseptic conditions to avoid microbial contaminations.
14. Reagents in this kit could include nucleic acids. Observe the local regulations for waste disposal.
15. Dispose of unused reagents and waste in accordance with all applicable regulations.
16. Use kit components only. Do not mix components from different kits or manufacturers. Only VIRCELL NEGATIVE CONTROL, VIRCELL PCR MIX RECONSTITUTION SOLUTION and VIRCELL POSITIVE CONTROL RECONSTITUTION SOLUTION are compatible with the equivalents in other RTPCR VIRCELL references and lots.
17. Specimens should be handled as in the case of infectious samples using safety laboratory procedures. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.
18. Testing of all the samples at the earliest interval following collection will help ensure the most accurate test results. Variation in storage times during specimen shipment has not been assessed.
19. It is recommended to have two different areas to perform the test: Pre-Amplification and Amplification areas.
20. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures. All reagents should be closed monitored to purity.
21. It is recommended to use conventional DNA/RNA purification kits.

22. Any serious incident that occurs in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

CONDITIONS FOR COLLECTION, HANDLING AND PREPARATION OF THE SPECIMEN

The kit has been validated for human stool samples.
It is recommended to avoid delay on transport and laboratory investigations. If immediate delivery to the laboratory is not possible, store specimens in a refrigerator (2 to 8ºC). Store specimens for which testing will be delayed beyond 48h after collection at -70ºC or lower; avoid freezing at higher temperatures and freeze-thaw cycles.
Recommended guidance: Stool. p.6.4.14. MM13-A_Collection, Transport, Preparation and Storage of Specimens for Molecular Methods, 1st ed. CLSI.
Centers for Disease Control and Prevention (CDC) website. Guidelines for Specimen Collection. https://www.cdc.gov/foodsafety/outbreaks/investigating-outbreaks/specimen-collection.html#print. Accessed April 2024.

PREPARATORY TREATMENT OF THE DEVICE

All reagents supplied are ready to use, except for the VIRCELL RT-PCR MIX [1] and the VIRCELL POSITIVE CONTROL [3].
[1] VIRCELL RT-PCR MIX. For reconstitution add 15 µl of VIRCELL PCR MIX RECONSTITUTION SOLUTION [5] per tube.

⚠The reconstituted VIRCELL RT-PCR MIX must be used within 60 minutes of adding the reconstitution solution stored at 2-8ºC if the start of the test is delayed. In this case, a freeze rack is recommended.
[3] VIRCELL POSITIVE CONTROL. Follow the next steps to reconstitute it:
- Centrifuge the corresponding tube for 5 seconds at 5000 g.
- Add 100 µl of VIRCELL POSITIVE CONTROL RECONSTITUTION SOLUTION [6]
- Mix with vortex for 1-2 seconds.
- Centrifuge the tube for 5 seconds at 5000 g.

After reconstitution, the VIRCELL POSITIVE CONTROL [3] can be frozen at temperature between -25°C and -15°C to be used in subsequent reactions.

ASSAY PROCEDURE

1. DNA/RNA extraction (performed in the Pre-Amplification area):
   1.1. It is recommended to use a commercial extraction kit for DNA/RNA extraction. In order to use commercial extraction kits, follow the manufacturer instructions. Consult with Customer Service.
   1.2. Stool samples could contain inhibitory substances. In order to remove PCR inhibitors and improve the DNA extraction yield, it is recommended to use a pre-treatment (mechanical and chemical) before DNA extraction, using a bead beating and a reagent to remove inhibitors.
2. Amplification using RT-PCR (performed in the Amplification area):
   2.1. Preparation of the VIRCELL RT-PCR MIX tubes: Add 15 µl of VIRCELL PCR MIX RECONSTITUTION SOLUTION [5] per tube. Maintain cold after reconstitution/thawing.
   2.2. Addition of the sample: Add 5 µl of each extracted DNA/RNA sample to each tube. Add 5 µl of VIRCELL POSITIVE CONTROL [3] and VIRCELL NEGATIVE CONTROL [4] to the corresponding tubes. The negative control is water.
   2.3. Secure caps VIRCELL RT-PCR MIX CAPS [7] on the tubes.
   2.4. It is recommended to briefly centrifuge the plate/strips of tubes with the purpose of ensuring vial content is at the bottom of the tube.
   2.5. RT-PCR program: Insert the PCR tubes in the real time thermocycler and run the following program*:

1 cycle	95 ºC	3 minutes
45 cycles	95 ºC	15 seconds
	58 ºC	45 seconds*

* Fluorescence data (FAM, HEX/VIC, Texas/ROX and Cy5) should be collected.

INTERNAL QUALITY CONTROL

Each batch is subjected to internal quality control testing before releasing, complying with strict specifications.

VALIDATION PROTOCOL FOR USERS

It is recommended to include one negative control in each run performed. The negative control will monitor reagent or environmental contamination.
The positive control is recommended to be included on each run. The positive control monitors for reagent failures and for correct operation of essential procedure.
The thermocycler software is likely to automatically calculate the baseline fluorescence value (threshold) based on the amplification curve for each target (fluorescence detection). Nevertheless, it is recommended to set the thresholds for the different detection channels individually. In order to set a threshold for each target, it is recommended to use as a reference the amplification curves of the positive and negative controls. The threshold should be fixed at the beginning of the exponential reading of fluorescence and above the background signal.
The controls result interpretation is as follows:

CONTROL	CDI (FAM)	A+B[1] (HEX/VIC)	BT (Texas/ROX)	IC (Cy5)[2]	Interpretation
VIRCELL CDI POSITIVE CONTROL	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Correct
	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	Invalid
VIRCELL NEGATIVE CONTROL	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	Amplification (Ct < 40)	Correct
	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	No amplification or Ct >40	Invalid

INTERPRETATION OF RESULTS

The result interpretation is described in the tables below:

RESULT	CDI (FAM)	A+B[1] (HEX/VIC)	BT (Texas/ROX)	IC (Cy5)[2]	Interpretation
1	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	Invalid (sample/kit/setup related)
2	No amplification or Ct >40	No amplification or Ct >40	No amplification or Ct >40	Amplification (Ct < 40)	Negative
3	Amplification (Ct < 40)	No amplification or Ct >40	No amplification or Ct >40	Amplification (Ct < 40) or no amplification	CDI
4	Amplification (Ct < 40)	Amplification (Ct < 40)	No amplification or Ct >40	Amplification (Ct < 40) or no amplification	CDI + A+B
5	Amplification (Ct < 40)	No amplification or Ct >40	Amplification (Ct < 40)	Amplification (Ct < 40) or no amplification	CDI + BT
6	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40) or no amplification	CDI + A+B + BT

[1] Codetection of genes encoding toxins A and B in the same channel (HEX/VIC). There is amplification if there is presence of at least one of these two toxins.
[2] In case of a high copy number of the target nucleic acid, the amplification of the internal control (IC) in results 3 to 6 may be affected. The late amplification or absence of IC amplification does not change the interpretation of the result.

In case of amplification of the toxins A+B (HEX/VIC) and/or BT (Texas/ROX) and no amplification of CDI (FAM), the result should be considered positive for the amplified target.
In case of invalid or inconclusive result, it is recommended to re-extract DNA/RNA from original specimen and re-test it. In the case of failure of amplification of internal control, improper extraction of nucleic acids or inhibition of amplification could be assumed. Testing a new sample is recommended.

LIMITATIONS OF USE

1. The performance with other types of specimens different to preservative-free soft to diarrheal stool samples from symptomatic patients has not been evaluated.
2. The results of samples should be used in conjunction with clinical evaluation and other diagnostic procedures.
3. Detection of the pathogens nucleic acids depends on the number of organism load present in the specimen and may be affected by specimen collection methods, patient factors, stage of infection and/or strain. False negative results may also occur if amplification inhibitors are present in the specimen. The kit was validated with a specific nucleic acid extraction method. Alternative extraction procedures might be also appropriate but require user validation. A 260/280 purity ratio among 1.8-2.0 is acceptable.
4. The test provides qualitative results. No correlation can be drawn between the magnitude of a positive result and the number of microorganisms in the sample.
5. The test only works within the limits of the genomic regions from which the primers and probes have been chosen. The test targets highly conserved regions, however due to the high variability of DNA genomes it is possible that certain subtypes might not be detected. At design time, mutations of the target regions were not detected.
6. A negative test result does not exclude the presence of the target organism at levels below the detection limit of the assay.
7. A positive test does not rule out the possibility that other pathogens may be present.
8. The values obtained in the sensitivity and specificity performance study correspond to the total number of samples tested and may vary depending on the type of sample.
9. The performance results showed were generated using the thermocycler CFX96 (Bio-Rad).
10. The performance results showed correspond to comparative studies with commercial predicate devices in a defined population sample. Small differences can be found with different populations or different predicate devices.

PERFORMANCE CHARACTERISTICS

SENSITIVITY AND SPECIFICITY

Positive human stool samples (n=94) and previously confirmed negative human stool samples (n=50) were analysed. Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and run in CFX96 (Bio-Rad).
The results were as follows:

Samples No.		144
Sensitivity (%)		96
	95% CI	89-98
Specificity (%)		100
	95% CI	93-100
PPV (%)		100
NPV (%)		93
LR+/LR-		-0.97/-0.95
True Positive		90
True Negative		50
False Positive		0
False Negative		4
Borderline		0

CI: Confidence intervals
PPV: Positive predictive value
NPV: Negative predictive value
LR+: Positive likelihood ratio
LR-: Negative likelihood ratio

PRECISION

4 samples (2 positive and the positive and negative controls) were amplified twice in 2 runs per day in 2 different qRT-PCR thermocyclers on 20 consecutive days. Samples were run in CFX96 (Bio-Rad). Within-run precision, between-run precision, between-day precision and within-laboratory precision were determined.
The results were as follows:

Clostridium difficile (CDI)

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.7	0.4	0.5	0.9
Positive sample 1	0.5	0.8	1.0	1.4
Positive sample 2	0.7	0.6	1.3	1.6
Negative control	No amplification	No amplification	No amplification	No amplification

CV: Coefficient of variation

Toxins A/B (A+B)

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.9	2.0	0.1	2.2
Positive sample 1	0.8	1.1	0.7	1.5
Positive sample 2	0.8	1.1	1.1	1.7
Negative control	No amplification	No amplification	No amplification	No amplification

CV: Coefficient of variation

Binary Toxin (BT)

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.8	0.1	0.3	0.9
Positive sample 1	0.6	0.9	1.1	1.5
Positive sample 2	0.7	0.4	1.2	1.4
Negative control	No amplification	No amplification	No amplification	No amplification

CV: Coefficient of variation

INTERFERENCES

A study has been performed to evaluate the effect of potentially interfering substances.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and run in CFX96 (Bio-Rad).
The results were as follows:

Interfering substances	Samples No.	Maximum added concentration without interference
Human whole blood	1	4% v/v
Mucin	1	5 mg/mL
Triglycerides	1	200 mg/dL
Cholesterol	1	250 mg/dL
Laxative	1	50% v/v
Naproxen	1	10% v/v
Amoxicillin	1	1% v/v
Tetracycline	1	1% v/v
Hemorrhoideal cream	1	1% v/v
Loperamide Hydrochloride	1	1% v/v
Nystatin	1	50% v/v
Vagisil	1	50% v/v
Hydrocortisone cream	1	50% v/v
Diaper Rash Cream	1	50% v/v

CROSS REACTIVITY

A study has been performed to evaluate the effect of potentially cross-reactive microorganisms. Samples were run in CFX96 (Bio-Rad).
The results were as follows:

Microorganism	Samples No.	Positives No.
Adenovirus 1	1	0
Adenovirus 40	1	0
Adenovirus 41	1	0
Aeromonas hydrophila	1	0
Aeromonas veronii	1	0
Astrovirus genotype 1	1	0
Blastocystis hominis	1	0
Campylobacter coli	1	0
Campylobacter jejuni	1	0
Campylobacter lari	1	0
Candida albicans	1	0
Chlamydia trachomatis	1	0
Clostridium botulinum	1	0
Clostridium chauvoei	1	0
Clostridium haemolyticum	1	0
Clostridium histolyticum	1	0
Clostridium perfringens	1	0
Clostridium sporogenes	1	0
Clostridium tetani	1	0
Clostridium tyrobutyricum	1	0
Cryptosporidium parvum	1	0
Entamoeba histolytica	1	0
Enterococcus faecalis	1	0
Escherichia coli (EAEC)	1	0
Escherichia coli (EIEC)	1	0
Escherichia coli (EPEC)	1	0
Escherichia coli (ETEC)	1	0
Escherichia coli (VTEC)	1	0
Giardia intestinalis	1	0
Helicobacter pylori	1	0
Lactobacillus crispatus	1	0
Listeria monocytogenes	1	0
Neisseria gonorrhoeae	1	0
Norovirus genotype II	1	0
Plesiomonas shigelloides	1	0
Pseudomonas aeruginosa	1	0
Rotavirus A	1	0
Salmonella enterica subsp. enterica	1	0
Salmonella enteritidis	1	0
Salmonella typhi	1	0
Shigella boydii	1	0
Shigella dysenteriae	1	0
Shigella flexneri	1	0
Shigella sonnei	1	0
Staphylococcus aureus	1	0
Staphylococcus epidermidis	1	0
Streptococcus agalactiae	1	0
Streptococcus pyogenes	1	0
Vibrio alginolyticus	1	0
Vibrio cholerae	1	0
Vibrio diazotrophicus	1	0
Vibrio fluvialis	1	0
Vibrio furnissii	1	0
Vibrio mediterraneii	1	0
Vibrio mimicus	1	0
Vibrio parahaemolyticus	1	0
Vibrio proteolyticus	1	0
Vibrio vulnificus	1	0
Yersinia enterocolitica	1	0
TOTAL	59	0

In addition, an in-silico analysis of the primers/probes sequences comparing to other microorganisms that could be found in clinical samples was performed.
The results were as follows:

	Homology >80%			
Microorganism	CDI	Toxin A	Toxin B	BT
Acinetobacter spp.	No	No	No	Yes
Aeromonas caviae	No	No	No	Yes
Arcobacter butzleri	No	No	No	No
Bacillus subtilis	No	No	No	No
Bacteroides fragilis	No	No	No	No
Citrobacter freundii	No	No	No	No
Clostridium innocuum	No	No	No	No
Clostridium novyi	No	No	No	No
Clostridium septicum	Yes	No	No	No
Clostridium sordellii	Yes	Yes	Yes	No
Clostridium sphenoides	No	No	No	No
Clostridium tertium	Yes	No	No	No
Corynebacterium diphtheriae	No	No	No	No
Cryptosporidium hominis	No	No	No	No
Dientamoeba fragilis	No	No	No	No
Entamoeba dispar	No	No	No	No
Enterobacter cloacae	No	No	No	No
Helicobacter cinaedi	No	No	No	No
Helicobacter heilmannii	No	No	No	No
Helicobacter hepaticus	No	No	No	No
Klebsiella oxytoca	No	No	No	No
Morganella morganii	No	No	No	No
Norovirus genotype I	No	No	No	No
Proteus vulgaris	No	No	No	No
Salmonella bongori	No	No	No	No
Salmonella gallinarum	No	No	No	No
Salmonella paratyphi A	No	No	No	No
Salmonella paratyphi B	No	No	No	No
Salmonella pullorum	No	No	No	No
Salmonella typhimurium	No	No	No	No
Sapovirus	No	No	No	No

"YES" indicates microorganisms that showed > 80% homology with respect to one of the primers but not with any other primers included in the assay. Cross-reaction and/or interference with the assay due to the presence of these organisms could not be tested, but it is unlikely to occur.

ANALYTICAL SENSITIVITY

A preliminary LoD (limit of detection) was determined by testing serial dilutions of quantified *C. difficile* ribotype 027 samples. Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and run in CFX96 (Bio-Rad).
Once an approximated LoD is determined, the final concentration was confirmed by testing 3 serial dilutions. A minimum of 20 replicates is tested for each dilution. The LoD is determined as the lowest concentration where ≥ 95% of the replicates are positive.

	CDI
LoD (copies/µl)	3
LoD (CFU/ml)	700

INCLUSIVITY

An in-silico analysis for the primered genes included in the assay was performed to determine the inclusivity for the different CDI, toxins A, B and binary toxin of *Clostridium difficile* sequences available.
The criteria selected for including the different sequences in the analysis was geographic and the date when the sequence was deposited. Different lineages, types or subtypes were included in the analysis of each microorganism.
GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) was used for accessing sequences.
The results of the in-silico analysis show that the kit is predicted to detect all genome variants included in the analysis.

EXTERNAL CONTROL

Controls that are required but not provided with the kit will be the following:
- as positive extraction control, AMPLIRUN® TOTAL CLOSTRIDIUM DIFFICILE RT027 CONTROL (STOOL) Cat. MBTC026-R (Vircell) and AMPLIRUN®

TOTAL GASTROINTESTINAL BACTERIAL PANEL CONTROL (STOOL) Cat. MBTC021 (Vircell).
External controls help monitoring any cross-contamination that occurs during the extraction process, additionally serve as validation tools for extraction reagents.

SYMBOLS USED IN LABELS

IVD	*In vitro* diagnostic medical device
	Use-by (expiry date)
	Store at x-y °C
	Contains sufficient for <n> test
LOT	Batch code
REF	Catalogue number
	Consult instructions for use
RCNS Xµl	Reconstitute in <X> µl
	Manufacturer

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