Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

MycoScreen Real-TM

Handbook

Real Time PCR kit for detection of of fungal infectious agents from genus Candida, Malassezia, Saccharomyces and Debaryomyces: *Meyerozyma guilliermondii (C. guilliermondii), Candida albicans, Pichia kudriavzevii (C.krusei), Saccharomyces cerevisiae, Candida auris, Candida tropicalis, Clavispora lusitaniae (C.lusitaniae), Debaryomyces hansenii (C.famata), Candida dubliniensis, Candida glabrata, Candida parapsilosis, Malassezia spp., Kluyveromyces marxianus (C.kefyr), Malassezia furfur*

REF **F23-48FRT**

Σ **48**

NAME

MycoScreen Real-TM

INTRODUCTION

The MycoScreen REAL-TIME PCR Detection Kit is intended for detection and typing of fungal infectious agents from genus Candida, Malassezia, Saccharomyces and Debaryomyces: Meyerozyma guilliermondii (C. guilliermondii), Candida albicans, Pichia kudriavzevii (C.krusei), Saccharomyces cerevisiae, Candida auris, Candida tropicalis, Clavispora lusitaniae (C.lusitaniae), Debaryomyces hansenii (C.famata), Candida dubliniensis, Candida glabrata, Candida parapsilosis, Malassezia spp., Kluyveromyces marxianus (C.kefyr), Malassezia furfur. Samples are human biological material (blood, phlegm, urine, swabs/scrapes from respiratory tract, gastrointestinal and urogenital tracts, faeces, bioptates), catheter and endotracheal tube washings, and fungal cultures.

INTENDED USE

The MycoScreen REAL-TIME PCR Detection Kit is intended for research and diagnostic applications.
Indications for the use:

- a suspicion for candidiasis, candidemia, candiduria and Candida carrier state;
- monitoring of the dynamic of colonization normally non-sterile loci, lesions and catheters with fungi;
- infectious control in patients including risk groups;
- identification of fungal species in fungal cultures.

PRINCIPLE OF ASSAY

The MycoScreen REAL-TIME PCR Detection Kit is based on fluorescent modification of the PCR method. The PCR-mix contains target-specific probes, each of them bearing reporter fluorescent dyes and quencher molecules. When specific product is formed, DNA probes are disintegrated and the quencher molecule stops affecting the fluorescent dye. Thus the level of fluorescence increases and it is detected by the thermocycler data collection unit. As a result of probe activation fluorescence increases proportionally to target sequence amplification. The amount of disintegrated probes increases proportionally to target sequence amplification. The intensity of fluorescence is measured at every cycle of reaction in real time with a Real-time PCR thermo cycler.

DNA probes for target sequences of fungal pathogens contain fluorescent dyes Fam and Cy5. DNA probes for the detection of amplification products of internal control (IC) and sample intake control (SIC) contain fluorescent dye Hex. The application of four fluorescent dyes makes it possible to register the results of different amplification reactions taking place simultaneously in one tube.

The reagents in tubes 1-7 contain internal control (IC) for the estimation of the PCR efficiency.

The amplification mix in the tube N°8 contains the reagents for the detection of SIC that is required for the analysis of the efficiency of DNA extraction from human biological material and allows to determine if the amount of DNA is sufficient for the analysis.

The tube N°3 contains the oligonucleotide with fluorescent dye Rox – a marker. It is used by detecting thermocycler for the detection of strip's position in the thermoblock. After the end of the amplification program the software compares predetermined order of tubes with the real localization of the marker ROX and in case of mismatch warns an operator.

Table 1. Detection channels and color marking of the mixes

N° of tube in a strip	Detection channel				Color of the mix
	FAM	Hex	Rox	Cy5	
1	Meyerozyma guilliermondii (C.guilliermondi)	IC	-	-	Blue
2	Candida albicans	IC	-	Pichia kudriavzevii (C.krusei)	Colorless
3	Saccharomyces cerevisiae	IC	marker	Candida auris	
4	Candida tropicalis	IC	-	Clavispora lusitaniae (Candida lusitaniae)	
5	Debaryomyces hansenii (C.famata)	IC	-	Candida dubliniensis	
6	Candida glabrata	IC	-	Candida parapsilosis	
7	Malassezia spp.	IC	-	Malassezia furfur	
8	Kluyveromyces marxianus (C.kefyr)	SIC	-	-	

The automatic analysis is available on SaCycler-96 instrument.

MATERIALS PROVIDED

- **Strips-Mycoscreen**, 48 8-tube strips (20 µl in each tube), including optical strip caps;
- **Taq Polymerase**, 8 x 0,5 ml;
- **Pos cDNA C+**, 2 x 0,320 ml;
- **Negative Control**, 1,0 ml*;

Contains reagents for 48 tests.

* *must be used in the isolation procedure as Negative Control of Extraction (NCE).*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- Real Time qPCR Thermalcycler instrument
- Workstation
- Pipettes with aerosol barrier
- Tubes and tubes racks

STORAGE INSTRUCTIONS

All reagents of **Mycoscreen Real-TM** kit must be stored at 2-8°C. The kits can be transported in thermal containers with icepacks by all types of roofed transport at temperatures from 2°C to 8 °C over the transportation. Transportation is allowed in thermal containers with icepacks by all types of covered transport at a temperatures up to 25 °C inside the container, but for no longer than 5 days.

STABILITY

Mycoscreen Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification. Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Mycoscreen Real-TM can analyze DNA material obtained from human biological samples (blood, phlegm, urine, swabs/scrapes from respiratory tract, gastrointestinal and urogenital tracts, faeces, bioptates), catheter and endotracheal tube washings, and fungal cultures, depending on professional prescription.

- **Peripheral blood**

Peripheral blood sampling is carried out in vacuum plastic tube. It may be 2.0 or 4.0 mL Vacuette blood collection tubes with anticoagulant, for example (EDTA) at a final concentration of 2.0 mg/mL. After taking the material, it is necessary to mix the blood with anticoagulant inverting the tube 2 – 3 times.

- **Phlegm**

Sample taking is made in amount no less than 1.0 mL into single-use graduated sterile flasks with wide neck and screwing caps with volume no less than 50 mL. After sample collection, flask is tightly screwed and marked.

- **Urine**

The first portion of morning urine in the amount of 20–30mL is selected for the analysis. The urine is taken into a special dry sterile container with volume of up to 60 mL, equipped with a hermetical screw- cap. After the urine collection, container is tightly screwed and marked.

- **Swabs/scrapes from respiratory tract, gastro-intestinal and urogenital tracts**

Sample taking is made with special sterile single-use tools – probes, cytobrushes, swabs depending on the source of biological material according to established procedure.

After sample taking place the probe into 1.5 mL plastic tube with a transport medium for transportation and storage of biological material for PCR and then rotate for 10-15 seconds, avoiding splashing of the liquid. Then remove the probe from the solution and, by rotating it against the wall of the test tube above the level of the solution, squeeze out the excess liquid. Dispose the used probe, close the test tube and mark it.

- **Faeces**

Samples of faeces with mass (volume) 1-3 g (1-3 mL) are transferred to a sterile dry flask by a single- use filtered pipette tip or single-use shovel. After sample collection the flask is tightly closed with a lid and marked.

- **Biopsy samples**

Place a tissue biopsy sample in a 1.5 mL tube with transport medium for transportation and storage of biomaterial. Close the tube and mark it.

- **Washings from parts of intravenous catheter**

Cut with sterile scissors 5-10 mm of a catheter tip and place it into a 1.5 ml tube of Eppendorf type. Close the tube and mark it.

- **Swabs from endotracheal tubes**

Sample taking is made from a surface of endotracheal tube with special sterile single-use probes. After sample collection, place the probe into 1.5 mL plastic tube with a transport medium for transportation and storage of biological material for PCR and then rotate for 10-15 seconds, avoiding splashing of the liquid. Then remove the probe from the solution and, by rotating it against the wall of the test tube above the level of the solution, squeeze out the excess liquid. Dispose the used probe, close the test tube and mark it.

- **Washings from endotracheal tubes**

Sample taking is made in single-use 50 mL tubes. After sample collection, close the tube tightly and mark it. Invert the tube 3-5 times to mix the material.

- **Bacterial cultures**

Sample taking from liquid and solid media is made with single-use microbiological loop or spreader. Place a sole colony of cells or 100 µL of liquid media in single-use 1.5-2 mL tube with 500 µL of sterile saline. Close the tube tightly and mark it.

Specimens can be stored at +2-8°C for no longer than 48 hours, or frozen at -20°C to -80°C for longer periods. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

SAMPLE PREPARATION

Phlegm: Add mucolysin to the container with sample in the 5:1 ratio (5 parts of mucolysin to 1 part of phlegm), referring to container calibrations. Close the container, mix the container content and incubate it at room temperature for 20–30 min, shake the container every 2-3 min.

Urine: Transfer 1.0 mL of the sample to the 1.5 mL tube. Centrifuge the tube at 16000 x g for 10 min. Remove the supernatant completely. Add 1.0 mL of sterile buffered saline to the precipitate. Centrifuge the tube at 16000 x g for 10 min. Remove the supernatant, leaving the volume of precipitate+liquid fraction in the tube that is recommended in the instruction for the DNA extraction kit.

Swabs/scrapes from respiratory tract, gastro-intestinal and urogenital tracts, swabs from endotracheal tubes, bacterial cultures from liquid and solid media: Centrifuge the tube at 16000 x g for 10 min. Remove the supernatant, leaving the volume of precipitate+liquid fraction in the tube that is recommended in the instruction for the DNA extraction kit.

Faeces - preparation of the suspension: Put approximately 0,1-0,2 g (mL) of faeces into the 1.5 mL tube with 1.0 mL of sterile buffered saline. Vortex the tube for 5-10 sec. Further processing of the suspension is carried out in accordance with the instruction for the DNA extraction kit from the corresponding biomaterial.

Biopsy samples: Centrifuge the tube at 16000 x g for 10 min. Remove the supernatant, leaving the volume of precipitate+liquid fraction in the tube that is recommended in the instruction for the DNA extraction kit.

Washings from endotracheal tubes: Transfer 1,0 ml of biomaterial into 1,5 ml tube using an automatic dispenser with filtered pipette tip. Centrifuge the tube at 16000 x g for 10 min. Remove the supernatant, leaving the volume of precipitate+liquid fraction in the tube that is recommended in the instruction for the DNA extraction kit.

Fragments of intravenous catheters (only with DNA/RNA Prep Extraction Kit): Add 100 µL of distilled water or sterile saline in the tube with a fragment of catheter. Vortex the tube for 3-5 sec and spin down the drops for 1-3 sec on vortex. Add in the tube 300 µL of lysis buffer from DNA/RNA PREP-NA Extraction Kit. Vortex the tube for 3-5 sec and spin down the drops for 1-3 sec on vortex. Termostate the tube on 65 °C for 15 min. Spin down the drops for 1-3 sec on vortex and transfer the supernatant in a new 1,5 mL tube.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **DNA/RNA Prep NA** (Sacace, REF K-2-9/2);

Please carry out the DNA extraction according to the manufacturer’s instructions. Independently of DNA extraction kit used, a negative control sample should go through all stages of DNA extraction. Physiological saline solution or negative control sample from an extraction kit can be used as a negative control sample in volumes as indicated.

PCR AMPLIFICATION

Prepare required quantity of PCR strips according to the number of samples to be analyzed, 1 strip for negative control of extraction (NCE) and 1 strip for positive control of amplification (C+) (Example: to test 2 samples, mark 4 strips - 2 strips for the samples, 1 strip for "C-" and 1 strip for "C+"

1. Vortex the tube with Taq-polymerase solution for 3-5 seconds, then spin briefly for 1-3 seconds on vortex.
2. Add 10 µl of Taq-polymerase solution into each tube of the strip. Avoid paraffin layer break.
3. Vortex the tubes with DNA samples, positive control sample and negative control sample for 3-5 seconds, then spin down drops by centrifuging on vortex-microcentrifuge for 1-3 seconds.
4. Add 5 µl of DNA sample into corresponding strip tubes. Do not add DNA into the "C-", "C+" strip tubes. Avoid paraffin layer break. Close the strips tightly.
5. Add 5.0 µL of negative control (C-) which passed whole DNA extraction procedure into "C-" tube and 5.0 µL of positive control (C+) into corresponding tube. Avoid paraffin layer break. Close the strips tightly.
6. Spin the strips briefly for 1-3 seconds on vortex.
7. Set the strips into the Real-time Thermal Cycler.
8. Launch the RealTime_PCR application program

	Plate-type qPCR Instruments[1]		
Step	**Temperature, °C**	**Time**	**Cycles**
1	80	30 sec	1
2	94	1 min 30 sec	1
3	94	30 sec	5
	64	15 sec	
4	94	10 sec	45
	64	15 sec * **Fluorescence detection ****	

*[1] For example, SaCycler-96™ (Sacace), CFX-96™*** Deep Well / iQ5™ (BioRad); Mx3005P™/Mx3000P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems);*
** On ABI® 7500 Real Time PCR instrument, please set the fluorescence acquisition time to 30 seconds.*
*** Fluorescence detection on channels FAM/Green, Joe/HEX/Yellow, ROX/Orange, Cy5/Red*

NOTE: FOR CFX-96 and other plate type instruments: it is recommended to use at least two additional empty strips placing them in the last left and right columns of the thermal block to better uniform the thermolid pressure in case of not filling the complete plate.

CONTROLS

The **Mycoscreen Real-TM** contains positive control sample. Positive control is a cloned part of the virus genome. It is produced with genetic engineering techniques and characterized by automatic sequencing. The PCR-mix from the kit includes the Internal control. IC is an artificial plasmid intended to assess the quality of PCR performance. To reveal possible contamination a negative control is required.
A negative control sample should go through all stages of RNA extraction. Physiological saline solution can be used as a negative control sample in volumes indicated in supplied instructions.

The test result is considered valid when:

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not taken into account;
- the exponential growth of the fluorescence level for the specific product is absence and for internal control is present.

The test result is considered invalid when the exponential growth of the fluorescence level for the specific product and for internal control are not observed.
If positive control (C+) does **not** express growing fluorescence of the specific product or positive result, it is required to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling. If negative control (C-) expresses growing fluorescence of the specific product or positive result, all tests of the current batch are considered false. Decontamination is required.

DATA ANALYSIS

Registration and interpretation of the PCR results are held in automatic mode on *SaCycler-96™ (Sacace)* instrument. The graph will show the fluorescence dependence of the number of cycle on all detecting channels for each tube in the thermoblock. The table will show the sample ID, threshold cycles (Cp) and decimal logarithms of concentrations (lg) of target DNA copies in 1 mL of the DNA preparation through the corresponding channels and interpretation of the amplification results («+» or « - »). It is possible to create and print a report based on the analysis results.

After the end of the amplification program the SaCycler-96™ *(Sacace)* software compares predetermined order of tubes with the real localization of the Rox marker and in case of mismatch warns an operator. In this case the operator should check the localization of the strips in a thermoblock (the first tube is marked by a blue buffer) and correct identifiers of tubes in the protocol.

Analyzing the results, the values of sample intake control (SIC, tube N°8 of the strip, Hex channel) and internal control (IC, tubes N°1-7, Hex channel) must be taken into account:

1. To control the sample intake containing human cells, the parameter SIC (the sufficient amount of human DNA) is used. The SIC value less than 3,0 in case of absence of specific results in all tubes of the strip is considered as insufficient amount of biomaterial. In this case resampling is recommended.

   

   In case of analyzing biomaterial that does not contain human DNA (fragments of intravenous catheters, swabs and washings from endotracheal tubes, bacterial cultures), the SIC value is not taking into account.

2. To access the quality of DNA extraction, Internal control is used. If the IC is not present in one or more tubes in the strip and at the same time there are the absence of specific positive results in these tubes, the result in these tubes is considered invalid due to incorrect conduction of PCR. In this case repeating of PCR amplification, or reextraction of DNA, or resampling is needed (performed sequentially).

3. In the samples containing DNA of detected pathogens the software detects positive result on the corresponding detection channel (Fam or Cy5) in a corresponding tube. In the result table in the line with the name of this pathogen the result of the qualitative analysis (“+”), the value of threshold cycle (Cp) and the decimal logarithm of concentration (lg, the Lg of the number of copies of DNA target in 1 mL of sample) will be indicated. The interpretation of the result is “detected (N lg)”.

4. In the samples not containing DNA of detected pathogens the software detects negative result on the corresponding detection channel (Fam or Cy5) in a corresponding tube. In the result table in the line with the name of this pathogen the result of the qualitative analysis (“-”) will be indicated. The interpretation of the result is “not detected”.

5. In the tube N°5 of the strip the value of lg≤2,5 on Fam detection channel is not taking into account by the software. In the result table in the "Result" column the result of qualitative analysis (“-“) will be indicated. The interpretation of the result is “not detected”.

6. In the tube N°7 of the strip the value of lg≤2,5 on Fam detection channel is not taking into account by the software in case of the absence the exponential increase of fluorescence on Cy5 detection channel. In the result table in the "Result" column the result of qualitative analysis (“-“) will be indicated. The interpretation of the result is “not detected”.

7. In the tube N°8 of the strip the value of lg≤2,0 on Hex detection channel is not taking into account by the software. In the result table in the "Result" column the result of qualitative analysis (“-“) will be indicated. The interpretation of the result is “not detected”.

8. For positive and negative control samples the results must correspond to those from the Table 2. In the negative control sample the IC value must be no less than 3,5. In the positive control sample the IC value is not taking into account.

9. If results for negative control sample differ from those in table 2, the results of the whole series are considered invalid. In this case decontamination is required.

10. The positive control sample is needed to access the efficiency of PCR reaction. In case of adherence to all conditions of reaction, the amount of target DNA in the positive control sample must correspond to the Table 2. If results for positive control sample differ from those in table 2, repeat of amplification of the whole test is required.

Plate-type instruments threshold settings

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold in the log-linear phase of amplification, approximately 10% of the fluorescence level of the positive control in the last amplification cycle.

Table 2. The results of the test for positive and negative control samples

N° of the tube in a strip	Pathogen	C- (lg)	C- result	C+ (lg)*	C+ result
1	Meyerozyma guilliermondii	-	-	3,5-5,5	+
2	Candida albicans	-	-	3,5-5,5	+
	Pichia kudriavzevii	-	-	3,5-5,5	+
3	Saccharomyces cerevisiae	-	-	3,5-5,5	+
	Candida auris	-	-	3,5-5,5	+
4	Candida tropicalis	-	-	3,5-5,5	+
	Clavispora lusitaniae	-	-	3,5-5,5	+
5	Debaryomyces hansenii	-	-	3,5-5,5	+
	Candida dubliniensis	-	-	3,5-5,5	+
6	Candida glabrata	-	-	3,5-5,5	+
	Candida parapsilosis	-	-	3,5-5,5	+
7	Malassezia spp.	-	-	3,5-5,5	+
	Malassezia furfur	-	-	3,5-5,5	+
8	Kluyveromyces marxianus	-	-	3,5-5,5	+
	SIC	-	-	3,5-5,5	+

* *SaCycler-96™ (Sacace)* instrument only.

SPECIFICATIONS

The analytical specificity of the **Mycoscreen Real-TM** Kit was assessed by bioinformatics analysis using available on-line databases with up-to-date comprehensive genetic information. The specific oligonucleotides used in the test were checked against GenBank database sequences. None of the sequences showed sufficient similarity for unspecific detection. The samples of human biological material with DNA of the detected mycosis pathogens are to be registered positive for specific product through the declared detection channels. The samples of human biological material free of DNA of the detected mycosis pathogens are to be registered negative for specific product through the declared detection channels. In the samples of biological material, containing human genomic DNA, the detecting amplifier should register a positive result of SIC amplification. In the samples of biological material, not containing human genomic DNA, the detecting amplifier should register a negative result of SIC amplification. For each test in the kit, there are not cross non-specific results with all other tests from the kit and non- specific positive results of amplification in the presence of other microorganisms or human DNA in concentration up to $1,0\times10^8$ copies/mL of the sample.

Analytical sensitivity of the **Mycoscreen Real-TM** Kit is 5 copies of DNA per amplification tube ($1,0x10^3$ copies/mL DNA sample). Sensitivity is determined by the analysis of serial dilutions of the laboratory control sample (LCS).

Sensitivity depends on the type of biomaterial, the extraction kit used for DNA extraction and the final elution volume (dilution) of the extracted DNA. For example, the sensitivity of the **Mycoscreen Real-TM Kit** for yeast culture is 50 copies/sample in case of extraction with **DNA/RNA Prep NA** Extraction Kit (elution volume 50 µl).

Diagnostic characteristics
Number of samples (n) - 429;
Diagnostic sensitivity (95% CI) - 100% (98,6-100%);

Diagnostic specificity (95% CI) – 100% (97,9-100%).

TROUBLESHOOTING

Table 6. Troubleshooting

	Result	Possible cause	Solution
C+	-	Operation error PCR inhibition Violation of storage and handling requirements	Repeat whole test Dispose current batch
C-	+	Contamination	Dispose current batch Perform decontamination procedures
IC	Invalid	PCR inhibition	Repeat whole test Resample

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* ABI® is a registered trademark of Applied Biosystems

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