1 Intended use

RealCycler ALPAGL-UX / ALPAGL-GX is an *in vitro* diagnostic kit of reagents which allows the detection of *Candida albicans*, *Candida parapsilosis* and *Candida glabrata* DNA in clinical samples. It is recommended to interpret the results with the *BioVisor RC* software that includes the validation criteria of samples and controls.

2 Principle of the test

The polymerase chain reaction (PCR) is based on the amplification of a specific region of the DNA/RNA by using complementary primers to the target sequence. Real-time PCR uses Taqman® probes labelled with fluorophores that emit fluorescence in the case of amplification. The cycle of the PCR protocol in which appears significant fluorescence is proportional to the DNA/RNA quantity present in the sample. This value is called *Cycle Threshold* (Ct).

The system includes a vial of an internal control CHIC *(Competitive Heterologous Internal Control*) out of the AmpliMix to verify the nucleic acids extraction and to prevent false negatives due to reaction inhibition.

The amplification of *Candida albicans* is detected with FAM fluorophore, CHIC with HEX, *Candida parapsilosis* with TxR and *Candida glabrata* with ATTO647N.

3 Technical specifications

Sensitivity

Candida albicans: 100 copies/µL.
Candida parapsilosis: 10 copies/µL.
Candida glabrata: 10 copies/µL.

The analytical sensitivity has been determined by limit dilution. This sensitivity has been showed in repeated assays with reproducibility over 95%.

Specificity

Candida albicans: Internal transcribed spacer ITS2.
Candida parapsilosis: Internal transcribed spacer ITS2.
Candida glabrata: rRNA 26S gene.

Specificity validation has been performed according to experimental assays and bioinformatics analysis.

4 Contents

RealCycler ALPAGL-UX / ALPAGL-GX includes the **AmpliMix ALPAGL-UX**, a **CHIC** vial and an **ALPAGL Positive Control**, which consists of a DNA artificial construction of *Candida albicans*, *Candida parapsilosis* and *Candida glabrata*. *RealCycler* Chic-Out kits do not include extraction controls (positive or negative).

All reagents are ready to use without adding or rebuilding any component.

Component	Vials		Volume
	ALPAGL-UX	ALPAGL-GX	
AmpliMix ALPAGL-UX	1	2	430 µL
ALPAGL Positive Control	1	2	120 µL
CHIC	1	2	350 µL

Number of determinations: *RealCycler* ALPAGL-UX allows to perform 30 reactions of 20 µL and 24 reactions of 25 µL. *RealCycler* ALPAGL-GX allows to perform 60 reactions of 20 µL and 48 reactions of 25 µL.

5 Stability and storage

- Stability assays have shown that this product is stable for 1 year between -18 °C and -25 ºC. See the expiration date included in the external label.
- *RealCycler* kits must be stored frozen between -18 °C and -25 ºC.
- The experimental assays performed have shown that *RealCycler* reagents are stable for at least 15 cycles of freezing / thawing.

6 Additional materials and equipment required and not supplied

- Real-time PCR instrument.
- Microcentrifuges.
- DNA purification system.
- Disposable gloves.
- Calibrated pipettes.
- Pipette tips with filter.
- Freezer (between -18 °C and -25 ºC).
- Positive and negative extraction control (positive or negative sample of the pathogen of the study).
- *BioVisor RC* (provided free of charge at the user's request).

7 Warnings and Precautions

- All components of the kit must be kept cold while you are working.
- Load tubes in the Real-time PCR instrument immediately after adding DNA.
- Do not expose tubes with AmpliMix to light for a long time.
- Repeated freezing and thawing of the reagents can decrease the sensitivity of the kit.
- Use disposable gloves.
- Use adequate and calibrated pipettes and pipette tips with filter.
- The tests must be carried out by qualified personnel and following good laboratory practices.
- It is recommended to use positive and negative controls whenever an analysis is performed.
- Do not use the kit after the expiry date.
- The presence of polymorphisms in the binding sequences of probes or primers to pathogen DNA/RNA can lead to erroneous results in a sample. If discordances appear between results and clinical observations it is recommended to check the results obtained using alternative methods.
- Negative results do not exclude an infection caused by the pathogen. The results obtained with this diagnostic kit should be used and interpreted within the context of the clinical history of the patient. Clinical decisions should not be made using solely the results of this kit.
- Signals with high intensity and low Ct could interfere in adjacent channels, generating a false positive signal. Thus, a high signal in the TxR channel could generate a residual signal in ATTO647N channel and vice versa.
- When the samples used have a pathogen concentration lower to the limit of detection, the results obtained have a reproducibility less than 95%.
- For *in vitro* diagnostic use.
- The limit of detection can be modified in Chic-Out AmpliMix depending on the extraction method used.
- It is recommended to interpret the results with the *BioVisor RC* software that includes the validation criteria of samples and controls.

8 Clinical samples

- Collect samples in sterile tubes.
- Storage and transportation frozen at -20 °C until use.
- The samples can be kept refrigerated during transport up to a maximum of 48 h.
- The kit is potentially compatible with any sample from which the pathogen's DNA can be extracted in enough quantity and quality, although the user must assess the suitability of non-validated samples through laboratory tests.

Validated clinical samples:

● *Candida albicans*: blood, bronchoalveolar lavage, culture, genital exudate, liquid cytology, plasma, sputum, upper airways aspirate and upper airways exudate.
● *Candida parapsilosis*: blood, culture, liquid cytology, sputum and upper airways exudate.
● *Candida glabrata*: blood, culture, liquid cytology, sputum and upper airways exudate.

9 Procedure

a) CHIC incorporation

- Samples: Shake the CHIC vial and add **5 µL** of CHIC to the sample volume to extract or to the lisis buffer. Continue with the usual acid nucleic purification procedure.

Note: The indicated CHIC volume allows to obtain a Ct value in channel 2 below 36 for negative non-inhibited samples (using any of the validated purification systems indicated below). If higher values are usually obtained, the user can increase the amount of CHIC to obtain a value of Ct within the range. A maximum of 10 µL of CHIC can be added for each sample. Higher amounts may cause competition with the pathogen and decrease the sensitivity of the kit.

- Positive Control: Do not add CHIC to the Positive Control of the kit.

- Negative control (not supplied): Use as negative control DNA of well characterized negative samples. Follow the same procedure used for the samples.

b) Nucleic acids purification

DNA should be purified from the clinical sample using an appropriate procedure. There are many nucleic acids purification systems available in the market. Please carry out the purification according to the manufacturer's instructions and using the recommended volume.

Validated purification systems:

● Arrow/LIAISON® IXT (reference 12.08.02 Viral NA Extraction Kit). DiaSorin.
● MagCore® Automated Nucleic Acid Extractor (references MVN400-03, MVN400-04). RBCBioscience.

RealCycler kits are compatible with most purification systems, among them:

● BioRobot EZ1. Qiagen.
● QIAamp DNA Blood Mini kit. Qiagen.
● QIAcube. Qiagen.
● Maxwell® 16 Cell LEV DNA Purification Kit. Promega Corporation.
● NucliSENS® easyMAG®. bioMérieux.

c) Thermal profile

Programme the "ALPAGL" amplification protocol according to the following specifications:

Time	Temperature	Cycles	Fluorescence
15:00	95 °C	1	OFF
0:15	95 °C		OFF
0:30	60 °C	45	ON
0:30	72 °C		OFF

d) PCR reaction set-up

- Thaw **AmpliMix ALPAGL-UX** and **ALPAGL Positive Control**.
- Prepare the necessary amplification tubes for samples and controls.

d.1) SmartCycler® (Cepheid®)

- Pipette **17,5 µL** of AmpliMix into each amplification tube.
- Add **7,5 µL** of DNA sample or control to each reaction tube.
- Spin tubes to transfer AmpliMix to optical area of tube. Check there are no bubbles in the optical area.
- Load tubes in the SmartCycler®.
- *Create Run* > *Dye Set* > FATA25.
- *Add/Remove Sites*: assign both the "ALPAGL" protocol and sites to each sample including controls.
- *Start Run*.

d.2) ABI 7500 (Thermo Fisher Scientific)

- Open ABI 7500 software (version 2.0.6).
- *Set up* > *Advanced Set up.*
- Select type of experiment *Quantitation-Standard Curve*.
- Select *TaqMan® Reagents*.
- Define a *Template* with ALPAGL thermal profile.
- Click *Add New Target* until there are 4 rows available. Enter fluorophore names according to the following table:

Target Name	*Reporter*	*Quencher*
FAM	FAM	*None*
HEX	VIC	*None*
TxR	TxR	*None*
ATTO647N	CY5	*None*

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.
- Spin tubes. Check there are no bubbles.
- Load tubes in the Real-time PCR instrument.
- *File* > *New experiment* > *From template* > Select ALPAGL protocol > Open.
- Click *Plate Setup*.
- Click to *Assign Targets* and *Samples*.
- Click *Assign* for all the *Targets* that have been defined.
- *Select the dye to use as passive reference* > *None*.
- Click *Run Method*.
- *Reaction volume per well* > 20 µL.
- *Start run*.

d.3) CFX96™ (Bio-Rad)

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.
- Spin tubes. Check there are no bubbles.
- Load tubes in the Real-time PCR instrument.
- Open *CFX™ Manager* software (version 1.6).
- Select *File* > *New Plate* > Select the whole plate or the used wells.
- Select *Sample type* > *Unknown* > OK.
- Select channels: FAM, HEX, TxR and Cy5.
- On the window *Experiment Setup* > *Protocol* > *Select Existing* > select ALPAGL protocol (indicate 20 µL of volume) > *Save* > *Start Run*.

d.4) Mic qPCR Cycler (Bio Molecular Systems)

- Open micPCR software (version 2.4.0).
- *New* > *Assay* > *Assay Setup* > *Information* > On *Chemistry Type* select *Hydrolysis Probes*.
- Click on *Target* until there are 4 *Targets* available. Enter fluorophore names according to the following table:

Target Name	*Reporter*	*Quencher*
FAM	BHQ1	*None*
HEX	BHQ1	*None*
TxR	BHQ2	*None*
Cy5	BHQ2	*None*

- *Assay Setup* > *Profile*.
- On *Temperature Control* select *Standard TAQ (v3)* and on *Volume* Indicate reaction volume 20 µL.
- Define ALPAGL thermal profile.
- *Analysis* > not indicate any analysis method > Save.
- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.

- Spin tubes. Check there are no bubbles.
- Load tubes in the Real-time PCR instrument.
- *New* > *Run Setup* > *Assays* > assign "ALPAGL" protocol and on *Samples* indicate the name of the samples.
- *Start run*.

d.5) Rotor-Gene® Q (Qiagen)

- It is indispensable to process only one *RealCycler* AmpliMix per run due to the fluorescence subtraction calculation of the instrument.
- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.
- Load tubes in the Real-time PCR instrument. It is indispensable to compensate the rotor positions not used and place the AmpliMix Positive Control on the tube 1 position.
- *File* > *New* > select ALPAGL > *New*.
- *Select rotortype* > 36 *well Rotor* > *Select Locking Ring Attached* > *Next*.
- Indicate reaction volume 20 µL > *Next*.
- On *Edit profile* confirm thermal profile and channels (*Green*, *Yellow*, *Orange* and *Red*).
- Click *Gain optimization* > on the *Auto-Gain-Optimization setup* window click *Perform Optimization Before 1st Acquisition* > Click *Optimize all* to select *Auto-Gain-Optimization* for each channel. Select *Target Sample Range: 5 Fl up to 10 Fl* > OK.
- Select *Tube position* 1 for all channels > *Close* > *Next* > *Start Run*.
- On the *New Run Wizard* window, assign the samples name (from 1 to 36) > *Finish*.

d.6) AriaDx® (Agilent Technologies)

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.
- Spin tubes. Check there are no bubbles.
- Load tubes in the instrument.
- Open *Aria Real-Time PCR* software (version 1.6).
- Select *File* > *New*.
- Select *Quantitative PCR (Fluorescence Probe)* > *Create*.
- On the *Plate setup* window select the whole plate or the used wells.
- Select *Well type* > *Unknown*.
- Select channels: FAM, HEX, ROX and Cy5.
- Select *Reference dye* > *None*.
- Define the thermal profile ALPAGL on the *Thermal profile* window.
- *Run*.

d.7) Other instruments

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of DNA sample or control to each reaction tube.
- Load tubes in the Real-time PCR instrument.
- Select the corresponding fluorophores.
- Select "ALPAGL" protocol.
- Start run.

e) Adjust the fluorescence threshold

Once the run is concluded, it is necessary to set the fluorescence threshold for each channel according to the indicated values hereafter. This adjust is indispensable for the correct results interpretation.

It is not necessary to set up these values when *BioVisor RC* software is used. These values are included on its database.

e.1) SmartCycler® (Cepheid®)

- *Analysis settings* > *Manual Thresh Fluor Units* > 30.0.

e.2) ABI 7500 (Thermo Fisher Scientific)

- On the *Analysis* window click *Analysis Settings*.
- On the emergent window click *Edit Default Settings*.
- Unselect *Automatic Threshold*.
- Enter *Threshold Target* to *Target*:

Target 1 (*C. albicans*): 20.000.
Target 2 (CHIC): 10.000.
Target 3 (*C. parapsilosis*): 60.000.
Target 4 (*C. glabrata*): 40.000.

- Click *Save Changes*.
- Click *Apply Analysis Settings*.

e.3) CFX96™ (Bio-Rad)

Adjust the fluorescence threshold for each channel:

- FAM: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.
- HEX: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.
- TxR: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.
- Cy5: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.

e.4) Mic qPCR Cycler (Bio Molecular Systems)

- *Analysis* > *Cycling*.
- For each channel on *Method* select *Dynamic* and on *Ignore Cycles Before* indicate 0.
- Indicate on *Threshold level* the following thresholds:

Green: 0,4.
Yellow: 0,1.
Orange: 0,5.
Red: 0,6.

- On *Exclusion* select *Extensive* and on *Fluorescence Cutoff Level* select 5,0%.

e.5) Rotor-Gene® Q (Qiagen)

- Select *Dynamic Tube, Slope Correct* and *Linear Scale*.

Adjust the fluorescence threshold for each channel:

Green: *CT calculation* > *Threshold* > 0,02.
Yellow: *CT calculation* > *Threshold* > 0,01.
Orange: *CT calculation* > *Threshold* > 0,05.
Red: *CT calculation* > *Threshold* > 0,06.

e.6) AriaDx® (Agilent Technologies)

- On the window *Graphic Displays* > *Amplification Plots graph*.
- Select the advanced settings of the analysis parameters.
- In *Threshold Fluorescence,* indicate the following thresholds:

FAM: 150.
HEX: 150.
ROX: 150.
Cy5: 300.

e.7) Other instruments

It is recommended to perform assays with samples of known result (positive and negative) in order to stablish the basal signal and fix the fluorescence thresholds.

f) Control results interpretation

- Valid control results

Control	Channels			
	Ch1	Ch2	Ch3	Ch4
	FAM	HEX	TxR	ATTO647N
	C. albicans	CHIC	*C. parapsilosis*	*C. glabrata*
POS	POS (Ct within the range)	NEG	POS (Ct within the range)	POS (Ct within the range)
NEG	NEG	POS (Ct within the range)	NEG	NEG

- Use as Positive Control the vial of the kit.
- Use as negative control DNA of negative samples previously extracted with CHIC.

- Invalid control results

In case of obtaining a negative result in any channel of the **Positive Control** (excepting for CHIC) the result is invalid. The results obtained in the samples included in the working series must be discarded (not assessable).

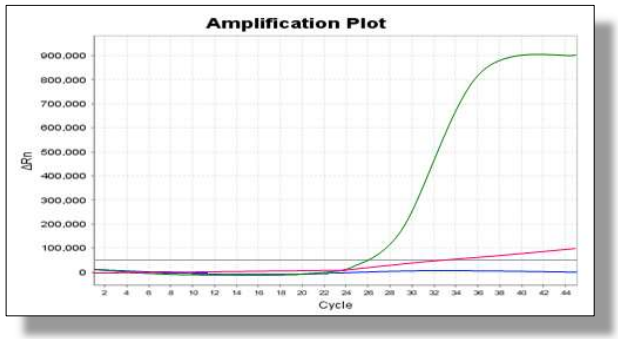
In case of obtaining a positive result (Ct > 0) in any channel of the negative control (excepting for CHIC) the result is invalid. The results obtained in the samples included in the working series must be discarded (not assessable).

g) Sample results interpretation

Interpret the result obtained in each sample by the combination of signals indicated in the following table:

Channels				Interpretation
Ch1	Ch2	Ch3	Ch4	
FAM	HEX	TxR	ATTO647N	
C. albicans	CHIC	*C. parapsilosis*	*C. glabrata*	
POS	Indifferent	NEG	NEG	POSITIVE *C. albicans*
NEG	Indifferent	POS	NEG	POSITIVE *C. parapsilosis*
NEG	Indifferent	NEG	POS	POSITIVE *C. glabrata*
POS	Indifferent	POS	NEG	POSITIVE *C. albicans* and *C. parapsilosis*
POS	Indifferent	NEG	POS	POSITIVE *C. albicans* and *C. glabrata*
NEG	Indifferent	POS	POS	POSITIVE *C. parapsilosis* and *C. glabrata*
POS	Indifferent	POS	POS	POSITIVE *C. albicans, C. parapsilosis* and *C. glabrata*
NEG	Ct within the range	NEG	NEG	NOT DETECTED
NEG	Ct out of range	NEG	NEG	NOT ASSESSABLE
NEG	0,00	NEG	NEG	NOT ASSESSABLE

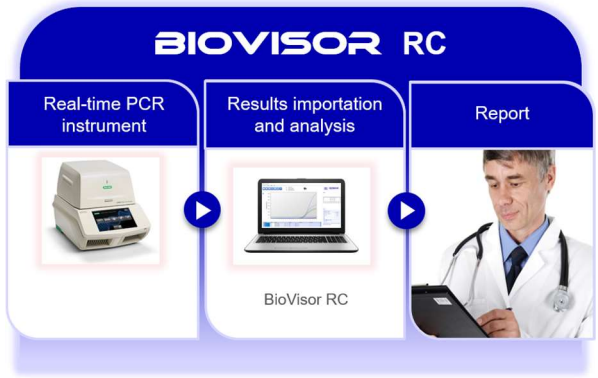
h) Example result



Result obtained when processing a positive sample (green) and a negative sample (blue). Positive sample: exponential curve. Negative sample: signal below the threshold. Other curves with different shapes must be considered abnormal and be evaluated in an individual way, such as linear signal (pink) above the threshold.

10 Results interpretation using the software *BioVisor RC*

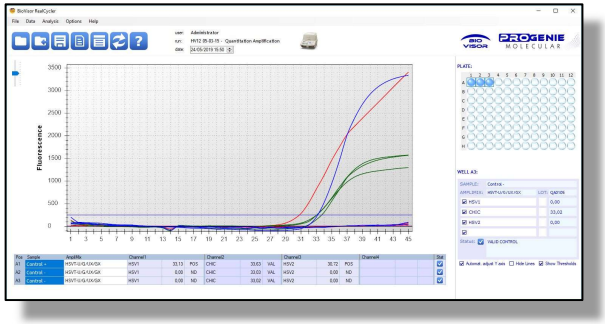
For the correct interpretation of the results obtained with the *RealCycler* products in the Real-time PCR instrument used, it is recommended to use the *BioVisor RC*.



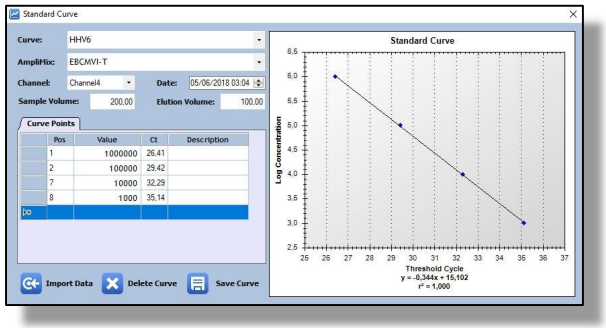
Specifications *BioVisor RC*:

- CE mark.
- Users and licenses management.
- Data importation from PCR instrument.
- Predetermined fluorescence thresholds.
- Normalization of fluorescence values.
- Visualization of all channels simultaneously.
- Interpretation of the result of each sample depending of its CHIC result.
- Series validation depending on the controls result.
- Monitoring of possible inhibitions.
- Allows the simultaneous quantification for several pathogens.
- Automatic calculation of the load of pathogen in the sample.
- Generation of a report with results.
- Conservation of calibration curves.
- Archiving of work series.
- Management of all the runs from the different instruments in a single database.
- Storage of runs obtained with the following validated instruments:
  - SmartCycler® (Cepheid®)
  - ABI 7500 (Thermo Fisher Scientific)
  - CFX96™ (Bio-Rad)
  - Mic qPCR Cycler (Bio Molecular Systems)
  - T-COR 8® (Cirrus Dx®)
- Connectivity with several T-COR 8® instruments in a local area network.

Detection example (all channels):



Quantification example:



The *BioVisor RC* is free for users of *RealCycler* products. To obtain it you can contact Progenie molecular (soporte@progenie-molecular.com) or your distributor.

11 Quality control

To validate the results, Ct values obtained for the positive control and the internal controls of each sample must be within the ranges specified in the internal label of the kit.

Every lot of *RealCycler* ALPAGL-UX / ALPAGL-GX kit has been tested according to the specifications of the real-time PCR using the SmartCycler® instrument (Cepheid®) and the validation criteria included in the *BioVisor RC*.

12 Observations

RealCycler reagents include FAM, HEX, TxR and ATTO647N fluorophores, which emit in the wavelengths indicated on the table (considered channels from 1 to 4). If an instrument does not explicitly recognise these fluorophores, it must be set up according to one of the following criteria:

1) Selection of an equivalent wavelength emission to those indicated on the table.

2) Selection of equivalent fluorophores (that emit in the same wavelength as the reagent uses).

Channel	Used fluorophores	Emission (nm)	Equivalent fluorophores
Ch1	FAM	519	——
Ch2	HEX	556	JOE, VIC, Alexa 532, CAL Fluor Orange 560
Ch3	Texas Red	610	ROX, LC Red 610, CAL Fluor Red 610
Ch4	ATTO647N	669	Cy5, Alexa 647, LC Red 670, Quasar 670, Oyster 645

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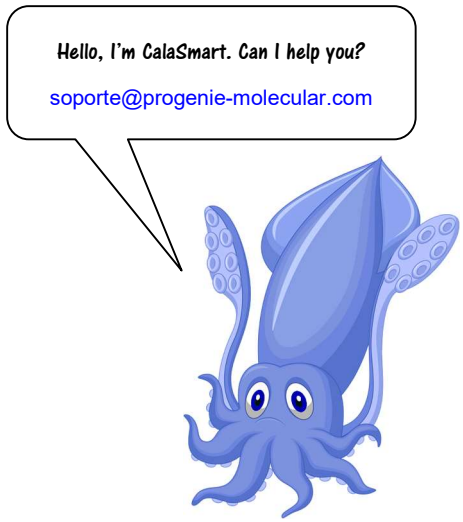

Revision history:

- Version 3 - June 2019: Change in the volume of Positive Control vial.
- Version 2 - January 2019: Change of the volume of CHIC vial.

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