IMMY

CE IVD

*Cand*ID

REF #: OLM2007

qPCR kit for the detection of clinically relevant *Candida* species – 50 tests

INTRODUCTION

Each year, fungal infections are responsible for around 1.4 million deaths worldwide (1). Despite this, fungal infections are often misunderstood and receive less public awareness compared to diseases caused by bacteria, viruses and parasites. Individuals with impaired immune systems, such as cancer and HIV patients, or those who have undergone bone marrow or organ transplants, are most at risk of suffering from an invasive fungal infection (2). The fungal pathogens that pose the most risk are *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus spp.*(3). Candida is ranked 4th among the most common bloodstream infections (4). Patients who develop septicaemia caused by *Candida* have only a 60% chance of surviving, despite the use of antifungal medications (5-11). *Candida* is becoming increasingly resistant to first-line and second-line antifungal medications (12). The few available treatment options are expensive and can be toxic to already sick patients.

*Cand*ID is a kit containing 2 multiplex qPCR tests, each detecting 3 commonly isolated *Candida* species; *Cand*ID detects *C. albicans, C. glabrata* and *C. parapsilosis,* whilst *Cand*ID PLUS detects *C. tropicalis, C. krusei* and *C. dubliniensis.* The *Cand*ID kit is being targeted for use as an aid in the assessment and evaluation of patients with suspected Candida infection.

KIT CONTENTS

- *CandID* Primer/Probe Mix (50 REACTIONS). (RED CAP)
  FAM, HEX, Cy5 and ROX labelled (see table below)

Target	Fluorophore	Absorption (nm)	Emission (nm)
C. albicans	FAM	494	518
C. glabrata	HEX	535	556
C. parapsilosis	Cy5	646	669
Internal extraction control	ROX	575	602

Table 1. Primer/probe mix contents and characteristics of fluorescent dyes

- *Cand*ID Positive Ctrl (*C. albicans/glabrata/parapsilosis*). (**RED CAP**)
- Internal Extraction Ctrl DNA (IEC). (**WHITE CAP**)
- qPCR Master Mix. (**WHITE CAP**)
- RNase/DNase-free water. (**WHITE CAP**)
- *Cand*ID PLUS Primer/Probe Mix (50 reactions). (**BLUE CAP***)*
  FAM, HEX, Cy5 and ROX labelled (see table below)

Target	Fluorophore	Absorption (nm)	Emission (nm)
C. tropicalis	FAM	494	518
C. krusei	HEX	535	556
C. dubliniensis	Cy5	646	669
Internal extraction control	ROX	575	602

Table 2. *CandID* PLUS Primer/probe mix contents and characteristics of fluorescent dyes

- *CandID* PLUS Positive Control (*C. tropicalis*/*C. krusei*/*C. dubliniensis*). (**BLUE CAP**)

MATERIALS REQUIRED BUT NOT PROVIDED

- qPCR instrument
- Pipettes and tips
- Vortex and centrifuge
- 1.5 mL microcentrifuge tubes
- qPCR plate and plate seals
- DNA extraction kit

KIT STORAGE

IMMY's *Cand*ID kit should be stored at -20°C, where it is stable for 18 months from date of manufacture. Components should be kept at 0°C, not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. Please note that exposure to light leads to photo bleaching of the fluorescent reporters and so reduces the sensitivity of the assay. IMMY does not recommend using the kit after the expiry date stated on the pack.

SUITABLE SAMPLE MATERIAL

DNA should be extracted from culture or suitable clinical specimen. Good laboratory practice recommends including at least one positive and negative extraction control per analysis. Extracted samples should be stored between -80°C and -20°C for long term storage. Please be aware that low concentrations of DNA can be unstable if stored for long periods. Repeated freeze/thaw cycles should be avoided. IMMY's *Cand*ID kit can be used with any sample prepared by a nucleic acid extraction method compatible with PCR amplification. Please ensure that the samples are appropriate in terms of purity, concentration, and DNA integrity. Always run at least one negative (no template) control with the samples. To prepare a negative-control, replace the template DNA sample with RNase/DNase-free water.

DYNAMIC RANGE OF TEST

Under optimal PCR conditions the primers in IMMY's *Cand*ID kits result in amplification efficiencies of >95%. The test has a broad dynamic range of at least six orders of magnitude and is sensitive to <1 *Candida* genome.

RISK ANALYSIS

There are no hazardous substances included in the manufacture of the *Cand*ID qPCR kit. The kit reagents present no known specific risk to the user. Additional chemicals and materials may be required for the procedures described in this handbook. IMMY advises the careful reading of all warnings, instructions and material safety data sheets provided by the supplier. General safety regulations must be followed when handling chemicals, biohazards and other materials.

GENERAL PRECAUTIONS

- The *Cand*ID qPCR kit is for in vitro diagnostic use.
- The test procedure should be performed as outlined in the "Instructions for Use" provided. Any deviation from the outlined protocols may result in assay failure or the generation of erroneous results.
- Standard precautions and local laboratory/institution guidelines should be followed when handling samples.
- Low concentrations of DNA can be unstable if stored for long periods. IMMY advises that sample storage time should be minimised before testing.
- Do not mix reagents from different kits.
- A separate PCR premix must be prepared for each of the 3-plex assays (*Cand*ID and *Cand*ID PLUS).
- Do not mix the 2 Primer/Probe reagents provided in this kit.
- Ensure the appropriate Positive Control is used with its corresponding assay (Tubes are colour-coded)
- Do not load the 2 different premixes into the same reaction well of your PCR plate
- As both assays (*CandID* and *CandID* PLUS) operate in the same fluorescence channels, please ensure the wells of your reaction plate are labelled appropriately, to differentiate between respective assay targets.
- Do not substitute reagents from different manufacturers.
- Avoid reagent contamination by following good laboratory practice and segregating workflow as appropriate.
- Ensure all additional required consumables are RNase/DNase-free.
- Wear protective clothing and disposable gloves when performing the test.
- Use disposable filter tips for all pipetting.
- Thaw DNA samples on ice and keep on ice.
- Always ensure *Cand*ID reagents are defrosted thoroughly, mixed, spun down and kept on ice.
- Do not use reagents after their expiry date.

ASSAY LIMITATIONS

- Positive results do not rule out co-infection with other organisms.
- Extraction efficiency can have a negative impact on *Cand*ID test results. IMMY advises that the internal extraction control provided is used in all sample extractions to monitor for this.

PRINCIPLES OF THE TEST

REAL TIME PCR

Individual primer and probe designs for detection of six clinically relevant *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. dubliniensis*), have been combined into 2 distinct 4-plex assays and their DNA can be detected through the different fluorescent channels as described in the kit contents.

IMMY's *Cand*ID makes use of the most widely used qPCR chemistry, which is based on the detection of light emitted by hydrolysis probes (Figure 1).

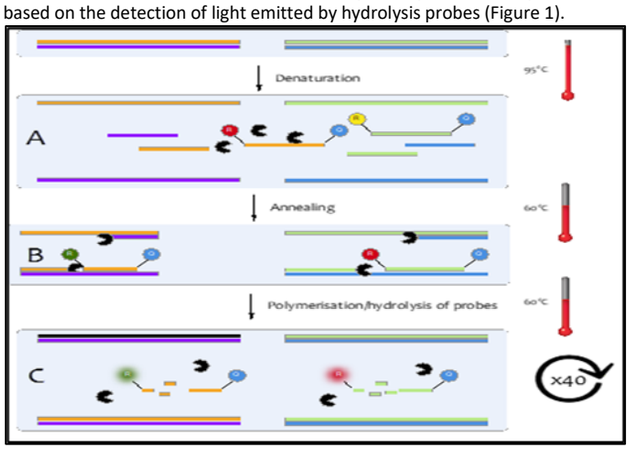


Figure 1. Principle of hydrolysis probe-based test. A. Double stranded genomic DNA, represented by the orange and purple strands, is denatured as the sample is briefly heated to 95°C. If added prior to the extraction of DNA, the assay also contains an internal extraction control, represented by blue and green strands.

B. During the annealing phase of the PCR the temperature is reduced and forward and reverse primers (short coloured lines) hybridise to any complementary target DNA present. The same reaction mixture also contains fluorogenic probes, which consist of target-specific DNA oligonucleotides labelled with a 5`-fluorescent reporter dye (R) and a quencher (Q). In the case of IMMY's *Cand*ID kit, these probes are specific for (i) *C. albicans* (FAM), *C. glabrata* (HEX), *C. parapsilosis* (Cy5) and the internal extraction control (ROX), using the *Cand*ID premix (RED cap), and (ii) *C. tropicalis* (FAM), *C. krusei* (HEX), *C. dubliniensis* (Cy5) and the internal extraction control (ROX), using the *Cand*ID PLUS premix (BLUE cap). **C.** During the polymerisation phase of the PCR, hybridised bound probes are displaced and cleaved by the 5' nuclease activity of the *Taq* polymerase (black circles), resulting in the physical separation of the reporters and quenchers. This results in the emission of light at a fluorophore-specific wavelength (see Tables 1 and 2), which can be detected in the appropriate channels of a qPCR instrument.

POSITIVE CONTROL

The kit contains 2 vials of positive control, each containing a template specifying three *Candida* species targets, corresponding to the *Cand*ID and *Cand*ID PLUS assay targets. The positive control is handled like a normal nucleic acid extract and indicates that the primers and probes for detecting *Candida* species are working properly in the run. Inclusion of the positive control depends on the preference of the end user and is not mandatory in every run. However, IMMY recommends its use in every run as it provides more confidence in results if all samples are negative. The positive control does not need to be subjected to a nucleic acid extraction procedure. Care should be taken to avoid cross-contamination of other samples when adding the positive control to the run. Any risk can be minimised by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

NO TEMPLATE CONTROL (NTC)

To confirm the absence of contamination, at least one no template control (NTC) reaction must be included in every PCR run. For this reaction, RNase/DNase-free water should be used instead of template.

INTERNAL EXTRACTION CONTROL (IEC)

The internal extraction control (IEC) is added to distinguish true negative samples from false negative samples, which can result from nucleic acid degradation, failure of nucleic acid extraction step, PCR inhibition or qPCR instrument malfunction. The primers and probe necessary to detect the IEC are included in the multiplex primer and probe mix. The IEC template is added either to the DNA lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer. The IEC will give a quantification cycle (Cq) value of >25, with the variation depending on the efficiency of sample extraction and level of sample dilution.

TESTING PROTOCOL

Remove the *Cand*ID kit from the freezer and allow the reagents to thaw. Thawed reagents must be kept on ice. Briefly vortex and centrifuge all *Cand*ID reagent tubes prior to use.

DNA EXTRACTION

The IEC DNA can be added either to the DNA lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer.
DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss of signal.

1. Add 5 µL of the IEC DNA to each sample in DNA lysis/extraction buffer for elution into 50 µL. For eluting into different volumes adjust volume of the IEC DNA accordingly.
2. Complete the DNA extraction according to the manufacturer's recommended protocols.

PCR DETECTION PROTOCOL

1. Prepare a PCR premix according to Table 3. Ensure that *Cand*ID and *Cand*ID PLUS premixes are prepared separately.

Volumes are given per reaction well and should be multiplied by the number of reaction wells required, include sufficient reactions for positive and negative controls. The prepared PCR premix should be thoroughly mixed and briefly spun down.

Component	Volume/Reaction
qPCR Master Mix	10 µL
*Cand*ID / *Cand*ID PLUS Primer/Probe Mix	2 µL
RNase/DNase-free Water	2 µL
Final Volume	14 µL

Table 3. *Cand*ID / *Cand*ID PLUS assay premix (volumes per reaction).
Due to small variations in pipetting accuracy, we recommend that you allow an additional 10% for the final volume, i.e. if you are assaying ten samples, make sufficient reaction mix for 11 tests.

2. Pipette 14 µL of prepared PCR premix into each reaction well according to your qPCR experimental plate set up.
3. Prepare sample DNA templates for each of your samples (see DNA extraction step).
4. Pipette 6 µL of DNA template into each well according to your experimental plate set up.
5. For negative control wells use 6 µL of RNase/DNase-free water. For positive control use 6 µL of *Cand*ID/*Cand*ID PLUS Positive Control, as appropriate. The final volume in each well is 20 µL.

Ensure that your PCR reaction plate is sealed and briefly centrifuged before transferring to a validated thermocycler for amplification.

AMPLIFICATION PROTOCOL

1. If using an instrument that uses ROX as a passive reference then the passive reference must be turned off or set to "none" for no passive reference, as the IEC uses the ROX channel.
2. Please refer to your instrument manual for instructions on setting up an amplification run. Amplification should be carried out according to the conditions detailed in Table 4.

	Step	Time	Temp
	Enzyme Activation	2 mins	95 °C
Cycling x40	Denaturation	5 secs	95 °C
	Data Collection*	20 secs	60 °C

** Fluorogenic data should be collected during this step through the FAM, HEX, ROX and Cy5 channels*
Table 4. PCR protocol

INTERPRETATION OF RESULTS

Please refer to your thermocycler instruction manual for information on how to operate the qPCR instrument and perform data analysis. Both *Cand*ID and *Cand*ID PLUS assays operate in the FAM, HEX, ROX and Cy5 channels. Therefore, appropriate labelling of the reaction plate wells is required. Remember to apply colour compensation if appropriate to your qPCR instrument.

It is important to visually inspect the amplification plots for each sample to ensure that the results recorded are due to true amplification and cannot be attributed to background noise recorded above the defined thresholds.

Note: Cq estimations stated below are calculated with threshold positioned at the centre of the log-linear range of the PCR amplification curve.

POSITIVE CONTROL

The supplied positive controls should each record characteristic amplification plots through the FAM, HEX and Cy5 channels, with Cq of 28 ± 2. There is no internal control template within the positive control so the ROX channel should record no signal (flat amplification plots). Each positive control generates signal to indicate that the assay is working correctly for detection of three target *Candida* species.

NO TEMPLATE CONTROL (NTC)

The NTC should give a flat line (flat amplification plots) through all channels. Signals in the NTC may indicate cross contamination during plate set up.

SAMPLE DATA (SUMMARISED IN TABLES 5 AND 6)

1. Successful sample extraction is indicated by a positive signal in the ROX channel (Cq>25), with lower Cqs indicative of more efficient extraction.
2. Presence of a signal in the FAM/HEX/Cy5 channel indicates that the sample contains *C. albicans/glabrata/parapsilosis* (for *Cand*ID), and *C. tropicalis/krusei/dubliniensis* (for *Cand*ID PLUS), respectively. Presence of signal in more than one of these channels indicates a mixed infection (mixed *Candida* species).
3. It is possible to obtain a signal in the FAM/HEX/Cy5 channel without also recording a signal in the ROX channel. Typically, this occurs when a high target load results in early amplification (i.e. low Cqs) and the accumulation of target DNA inhibits the amplification of the IEC DNA.
4. If there is no signal in any of the channels, the assay has failed and no conclusions are possible.

				***Cand*ID: detection channels**
FAM	**HEX**	**Cy5**	**ROX**	**Result**
+	-	-	+	***C. albicans* POSITIVE sample. IEC PASS. Valid result.**
-	+	-	+	***C. glabrata* POSITIVE sample. IEC PASS. Valid result.**
-	-	+	+	***C. parapsilosis* POSITIVE sample. IEC PASS. Valid result.**
-	-	-	-	**Invalid result.**

Table 5. Summary of data interpretation: *Cand*ID

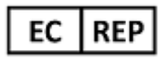
				***Cand*ID PLUS: detection channels**
FAM	**HEX**	**Cy5**	**ROX**	**Result**
+	-	-	+	***C. tropicalis* POSITIVE sample. IEC PASS. Valid result.**
-	+	-	+	***C. krusei* POSITIVE sample. IEC PASS. Valid result.**
-	-	+	+	***C. dubliniensis* POSITIVE sample. IEC PASS. Valid result.**
-	-	-	-	**Invalid result.**

Table 6. Summary of data interpretation: *Cand*ID PLUS

REFERENCES

1. Brown GD *et al*. Sci Transl Med 2012;4(165): 165rv13.
2. Rüping MJ *et al*. Drugs 2008;68(14): 1941-62.
3. Schmiedel Y *et al*. Swiss Med Wkly 2016;146: w14281.
4. Wisplinghoff *et al*. Clin Infect Dis 2004;39: 309-17.
5. Kollef M *et al*. Clin Infect Dis 2012;54: 1739-1746.
6. Hadley S *et al*. Crit Care Med 2002;30(8): 1808-14.
7. Leroy O *et al*. Crit Care Med 2009;37(5): 1612-8.
8. Guzman JA *et al*. Clin Med Res 2011;3: 65-71.
9. Charles PE *et al*. Intensive Care Med 2003;29(12): 2162-9.
10. Nolla-Salas J *et al*. Intensive Care Med 1997;23(1): 23-30.
11. Bougnoux ME *et al*. Intensive Care Med 2008;34(2): 292-9.
12. Alexander BD *et al*. Clin Infect Dis 2014;58(5): 754.

INTERNATIONAL SYMBOL USAGE

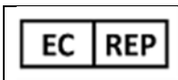
	Manufacturer
	Expiration Date
	Consult instructions for use
	Lot Number
	Product Reference Number
	In Vitro Diagnostic
	Irritant
	EU Authorized Representative

MANUFACTURER INFORMATION

For further information please contact

IMMY, Inc.

2701 Corporate Centre Dr
Norman, OK 73069 USA
+1 (405) 360-4669 / (800) 654-3639
Fax: +1 (405) 364-1058
Email: info@immy.com
www.immy.com

	Advena Ltd. Tower Business Centre, 2nd Flr., Tower Street, Swatar, BKR 4013 Malta