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bKIT *Lactobacillus rhamnosus*

PN bKTPR-LRHA.01

Real-Time PCR assay

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Introduction

Lactobacillus classification trace back to 1901, when, based on biochemical and morphological characteristics, Beijerinck ([1]) M.W proposed the genus. More recently, also other approaches assisted traditional classification. In fact, in 1989, using a DNA based approach, Collins ([2]) proposed the species *Lactobacillus rhamnosus*. Nowadays, many efforts focus on the correlation between taxonomic classification with traditional procedures and DNA molecular methods. Consistently with this trend, traditional culture approaches are increasingly assisted by DNA molecular methods ([3]). Among these, Real-Time PCR emerged for its sensitivity, rapidity, reliability, specificity and repeatability making it a well-established method for the detection, quantification, and typing of different microbial agents in the areas of clinical and veterinary diagnostics and food safety ([4]).

([1]) BEIJERINCK (M.W.): Sur les ferments lactiques de l'industrie. Archives Néerlandaises des Sciences Exactes et Naturelles (Section 2), 1901, 6, 212-243.
([2]) COLLINS (M.D.), PHILLIPS (B.A.) and ZANONI (P.): Deoxyribonucleic acid homology studies of Lactobacillus casei, Lactobacillus paracasei sp. nov., subsp. paracasei and subsp. tolerans, and Lactobacillus rhamnosus sp. nov., comb. nov. Int. J. Syst. Bacteriol., 1989, 39, 105-108.
([3]) Mianzhi Y, Shah NP. Contemporary nucleic acid-based molecular techniques for detection, identification, and characterization of Bifidobacterium. Crit Rev Food Sci Nutr. 2017 Mar 24;57(5):987-1016. doi: 10.1080/10408398.2015.1023761. Review.PubMed PMID: 26565761.
([4]) Kralik P, Ricchi M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. Front Microbiol. 2017 Feb 2;8:108. doi: 10.3389/fmicb.2017.00108. eCollection 2017. Review. PubMed PMID: 28210243; PubMed Central PMCID: PMC5288344

Principle

SYBR® Green Real-Time PCR (qPCR) assay for the detection of *Lactobacillus rhamnosus*. The product is intended for research purpose only.

NHPRA validation

The validation was performed on the bCUBE platform following the methods and the guidelines accepted and published on J AOAC Int. 2019 Nov 1;102(6):1774-1778.
In the validation trials performed by NHPRA (Natural Health Product Research Alliance) the following strains were tested: *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* Lr-32, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* HA-114, *Lactobacillus rhamnosus* R0011, *Lactobacillus rhamnosus* HA-111, *Lactobacillus rhamnosus* Lr-06, *Lactobacillus gasseri* Lg-36, *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* HA-196, *Lactobacillus plantarum* Lp-115, *Lactobacillus plantarum* UALp-05, *Lactobacillus bulgaricus* Lb-87, *Lactobacillus casei* Lc-11, *Lactobacillus salivarius* HA-118, *Lactobacillus brevis* Lbr-35, *Lactobacillus plantarum* HA-119, *Lactobacillus acidophilus* DDS-1, *Lactobacillus delb bulgaricus* HA-137, *Lactobacillus reuteri* HA-188, *Lactobacillus paracasei* UALpc-04, *Lactobacillus casei* UALc-03, *Lactobacillus salivarius* Ls-33, *Lactobacillus helveticus* R0052, *Bifidobacterium breve* HA-129, *Bifidobacterium bifidum* HA-132, *Bifidobacterium infantis* HA-116 and *Bifidobacterium infantis* Bi-26. Moreover, assay performances were assessed in mixtures containing the DNA of the strains listed above. All DNA solutions tested were normalized to the concentration of 1 ng/µL before use. All target and non-target DNA sample solutions were successfully classified. For more details, contact us at support@hyris.net.

bKIT *Lactobacillus rhamnosus* packaging

Part number: bKTPR-LRHA.01-50

qPCR Master Mix (1 tube, blue cap)	50 tests
Positive Control (1 tube, green cap)	14 tests
Negative Control (1 tube, red cap)	14 tests

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Part number: bKTPR-LRHA.01-100	
qPCR Master Mix (2 tubes, blue cap)	2 x 50 tests
Positive Control (1 tube, green cap)	28 tests
Negative Control (1 tube, red cap)	28 tests

Storage

-20°C. Avoid prolonged exposure to light and repeated freeze and thaw cycles.

Shelf life

If the bKIT is correctly stored, at constant-temperature freezer, its performance is guaranteed until the shelf life indicated on the tubes.

Additional material/reagents required

- DNA extraction tools and reagents.
- Nuclease-free water.
- Gloves.
- Pipettes.
- bCUBE® instrument or other Real-Time PCR instrument (*) with filters calibrated for SYBR® Green.
- bCUBE® sample loading cartridge or, if using other Real-Time PCR instrument, samples loading support according to the instrument specifications.

(*) This assay was especially developed to be used in association with the bCUBE® instrument, available from Hyris Ltd, but can be used also with any other compatible thermal cycler.

DNA extraction

The assay has been validated on DNA samples obtained through the application of one or more protocols that are available for download on the bAPP.
The procedure for the download is the following:

a. Login on the bAPP.
b. Go on bKITs section and select **bKTPR-LRHA.01**.
c. Click the button "Download DNA extraction method".

Reaction set-up

a. Thaw all the bKIT components by placing the tubes on ice.
b. Gently mix the tubes content by swirling the tubes.
c. Spin the tubes to let the content down.
d. In new tubes, one for each sample, including the Negative Control and the Positive Control of the bKIT, prepare the Reaction Mix as shown in the table below:

Components	Volume
DNA sample (normalized to the concentration of **1 ng/µL**) or Positive Control or Negative Control	1 µL
qPCR Mastermix	19 µL
Total Volume	**20 µL**

Cartridge set-up

The procedure described is for the bCUBE® cartridge, but, if using a different Real-Time PCR instrument, the same procedure can be adopted for other loading sample supports with minor modifications.

1. Samples set-up

Samples of the following types must be prepared to be loaded on the cartridge:

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Positive Control for *Lactobacillus rhamnosus.*
Negative Control for *Lactobacillus rhamnosus.*
Sample(s) to be tested.

2. Cartridge Loading

a. Load the sample prepared as described in the previous section.
b. Carefully seal the cartridge with adhesive film in order to avoid any contamination.
c. Load the cartridge onto the bCUBE®, then start the run.

Analysis set-up

Set up the run method using the proper procedure, depending on the instrument you use.

1. On the bCUBE®

a. Login on the bAPP/bPANEL.
   - If you are using the **bAPP**, set-up "New Analysis", flag bKIT, and select "**Probiotics"** → "**bKTPR-LRHA.01**"; alternatively, scan the QRCode on the front page of the present document, or on the bKIT packaging.
   - If you are using the **bPANEL**, set-up "New Analysis" and select *"***Lactobacillus rhamnosus 1.x***"* (x indicates the number of the last version available) from the "Global recipes" list.

b. Specify the "Well types" for each of the loaded sample as follows (**Fig. 1**):
"PosCtrl" for the well loaded with *Lactobacillus rhamnosus.* Positive Control.
"NegCtrl" for the well loaded with *Lactobacillus rhamnosus.* Negative Control.
"Sample" for the wells loaded with samples under analysis.

Fig 1. Cartridge set-up
An example of cartridge set-up on the bAPP for one replicate of a sample to be analyzed is shown.

2. On a compatible Real-Time PCR instrument

Please, contact us for the protocol set-up on the instrument.

Reading the results

1. On the bCUBE®

a. The presence of the target *Lactobacillus rhamnosus* in the **Positive Control** or in the **sample** under analysis will generate an amplification curve **(Fig. 2a)** and a melting curve with a specific melting peak **(Fig. 2b)**.

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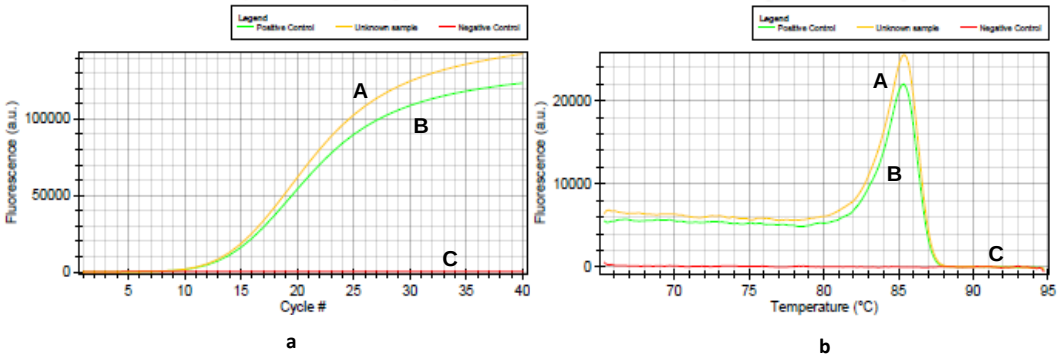

Fig.2. Amplification and melting plots
In the plots, the amplification curve (**Fig. 2a**) and the specific melting peak (**Fig. 2b**) of a *Lactobacillus rhamnosus* containing **sample** (**A**), the **Positive Control** (**B**), and the **Negative Control** (**C**) are shown.

b. At the end of analysis each well will be labelled depending on the "Well type" as described in the table below and samples classification will be shown on the pdf report of the analysis (**Fig. 3**).

Well type	Possible labels	Label meaning
Positive Control (PosCtrl)	**OK**	The positive control behavior is within the expected range.
	KO	The positive control behavior isn't within the expected range. Please see **Troubleshooting** section below.

Well type	Possible labels	Label meaning
Negative Control (NegCtrl)	**OK**	The negative control behavior is within the expected range.
	KO	The negative control behavior isn't within the expected range. Please see **Troubleshooting** section below

Well type	Possible labels	Label meaning
Sample	**Present**	The target DNA sequence, characteristic of *Lactobacillus rhamnosus*, is present in the sample**.
	Absent	The target DNA sequence, characteristic of *Lactobacillus rhamnosus*, is absent from the sample** or in amount below the limit of detection of the assay.
	Indeterminate	The test is not conclusive and should be repeated. If the "Indeterminate" classification persists, contact us at support@hyris.net.

(**) The assay has been designed to discriminate DNA polymorphisms between target and non-target sequences; nevertheless, correct label classification applies and can be ensured only with validated processing conditions, including samples and matrixes tested during the validation of the assay.

Results for target *Lactobacillus rhamnosus*	
Positive control (PosCtrl)	**OK**
Negative control (NegCtrl)	**OK**
Unknown sample (Sample)	**Present**

Fig.3. Analysis results table
An example of the results table, as reported in the pdf report of the analysis, is shown.

2. On a compatible Real-Time PCR instrument
Please, contact us for results interpretation.

Troubleshooting

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1. Results show no amplification, or anomalous amplification curves

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate/strips	Repeat the test using the appropriate materials and tools to seal correctly the plate/strips
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the supplier of the Real-Time PCR instrument
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, contact us at support@hyris.net.

2. No amplification curve is observed for the Positive Control

Possible causes	Corrective actions
The Positive Control provided with the assay was not added into the reaction well	Repeat the test adding the Positive Control. If the problem persists, contact us at support@hyris.net.
Some issues with reaction components and/or reaction conditions occurred	Repeat the experiment checking that all step required for the analysis have been performed correctly. If the problem persists, contact us at support@hyris.net.

3. An amplification curve with a specific melting peak is observed for the Negative Control

Possible causes	Corrective actions
Contamination of the Negative Control or the qPCR Master Mix with target-positive DNA	Repeat the test by applying appropriate quality procedures to prevent contamination. Correctly seal the cartridge or plate/strips. If the problem persists, contact us at support@hyris.net.

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