Omunis

2016
Version 1.0

We make it accessible

Instruction Manual

PUMA LEPTO Kit

PCR. Universal. Molecular. Access.

Cap Alpha – 9 Avenue de l'Europe – 34830 Clapiers, France
Tel : +33 (0)4.34.35.91.18 – www.omunis.com – Email : contact@omunis.com

TEST PRINCIPLE

1. Purpose of use

The PUMA Lepto kit is a real-time PCR test that allows the detection and quantification of leptospira DNA in human plasma/serum. Combined with the clinical presentation and other biological markers of the status of the disease, the measurement of the Lepto DNA is to be used for the detection of the disease and for the follow-up of patients infected with Leptospirosis. The test is based on the principle of real-time amplification of the bacterial DNA (*lipL32* gene) in the samples, allowing the precise quantification of Polymerase Chain Reaction (PCR) products during the exponential phase of the amplification process.

This product is developed, designed and sold for research use only (RUO). It is not intended for human diagnostic purposes.

2. Advantages

With real-time detection of the fluorescent signal during and/or after each PCR cycle, the PCR quantitative data can be obtained in a very brief period of time. Thus, no post-PCR treatment is required, which not only leads to a considerable reduction in the risk of contamination of the PCR product but also permits the avoidance of ulterior contamination of samples by amplicons produced in previous reactions.

.

3. Molecular mechanisms

The PUMA Lepto test exploits the principle of PCR by hydrolysis of a nucleotide probe marked with both a 5' fluorescent reporter (e.g. FAM™) and a 3' non-fluorescent quencher (e.g. TAMRA). During PCR, the forward and reverse primers hybridize to a specific sequence at the amplicon level. The probe, contained in the same reaction mixture, hybridizes to a target sequence of the amplicon. When the probe is intact, the spatial proximity between the reporter and the quencher inhibits the fluorescence of the reporter, mainly by a transfer of Förster type energy. During PCR, the probe sets itself up specifically between the two sites where the forward and reverse primers are hybridized, and inhibits all activity of the Taq polymerase. Simultaneously, its 5'-3' exonuclease function, that cleaves the probe between the reporter and the quencher, is activated. The reporter, freed from the quencher, emits a fluorescent signal, which is registered in real-time by the sensors. Thus rid from probe fragments, the target sequence can be read and amplified by the Taq polymerase.

The augmentation of the fluorescent signal is only detected if the target sequence is complementary to the probe and if it is amplified during PCR. Thus, a non-specific amplification cannot be detected. With this reaction principle, the fluorescent signal is directly proportional to the amplification of the target during PCR.

4. Reaction phases

The evolution of the amplification is represented by a sigmoid progression curve which can be divided into two phases:

a) An exponential amplification phase, over the course of which the amount of PCR product obtained at every moment is a direct function of the number of initial copies. At the beginning of the

exponential amplification phase, the moment where the signal exits peak noise, corresponds to a number of cycles called Ct (cycle threshold).

b) The exponential amplification phase is followed by a plateau phase, which corresponds to a slow in amplification, due to the depletion of the reagents.

5. Operational steps

The PUMA Lepto kit has two main operational steps:

a) Preparation of the sample

The bacterial DNA is extracted, purified and concentrated by means of a chosen extraction technique (reagents not provided in this kit).

E.g. : QIAmp DNA minikit (Qiagen) ; sample volume: 250 µL ; elution volume : 70 µL.

b) Amplification / Analysis: Quantification and measurement of Lepto DNA

The bacterial load of the tested sample is obtained by extrapolating the calibration curve (range of standards provided in the kit), to the Ct value of the sample.

The PUMA Lepto kit allows for the testing of up to 100 samples including patient samples, the range of standards and the controls.

COMPOSITION OF THE KIT

Reagents	Type	Volume or quantity / tube	Number of tubes	State	Storage
Lepto Enzyme Mix	DNA polymerase and buffer	1100 µl	1	Liquid	-20°C
Lepto DNA Panel	Lepto Plasmid DNA* and buffer	50 µl	1	Liquid	-20°C
Set Primers and Probes	Primers and probes for Lepto and internal control	231 µl	1	Liquid	-20°C
Water	Water for molecular biology	1,5 ml	1	Liquid	-20°C
DNA Internal Control	Extraction and amplification Internal Control	550 µl	1	Liquid	-20°C
Lepto Positive Control	Lepto Plasmid DNA* and buffer	50 µl	1	Liquid	-20°C
Lepto Negative Control	Buffer	50 µl	1	Liquid	-20°C

* The concentration of the plasmid is given in the Quality Control certificate

REAGENT STORAGE

- The PUMA Lepto kit is transported at -20°C.
- Upon receipt, the box must be stored at -20°C. Stored under these conditions, the reagents will remain stable until the expiry date indicated on the label.
- Once prepared from the “Lepto DNA” tube, the 5 points of the range can be frozen at -20°C. It is up to the user to ascertain the impact of the freezing/unfreezing cycles before any handling.

In all cases, it is advised to prepare non pyrogenic and “nuclease free” aliquots in sterile microvials, to close them with the appropriate lids and to label them carefully, indicating the name of the reagent, the batch and the expiry date.

MATERIAL REQUIRED BUT NOT PROVIDED

- 1-10 µL, 20-200 µL and 100-1000 µL pipettes.
- Pipette tips with filters.
- Benchtop centrifuges.
- Benchtop vortex.
- Open real-time PCR thermocycler.
- Single use gloves.
- Sterile microvials.
- Microplates with adhesive film.
- Manual or automated nucleic acid (DNA) extraction kit.
- Refrigerator.
- Tube racks.

The real-time PCR instrument used for the test must be an « open » system with at least the following main characteristics:

- quantitative real-time PCR trials.
- a programmable thermocycling block.
- an excitation source: LEDs, light or laser.
- detection sets (length of excitation/emission wave) suitable for the detection of reporter fluorophores of FAM and Cy5 probes.
- connection to a computer using specific analysis software allowing for the collection of fluorescence data, the conducting of trials of absolute quantification and the interpretation of results.

REMARKS AND PRECAUTIONS

The material used contains less than 0.1 % hazardous or carcinogenic substances, thus MSDS sheets are not required. However it is recommended to take appropriate precautions, as with any biochemical product, and to wear appropriate clothing.

USE PRECAUTIONS

The PUMA LEPTO kit is a test that is to be exclusively used by qualified laboratory personnel subject to biology laboratory good practice, conscious of the biological risks and trained in molecular biology analysis. The interpretation of the results obtained from this test is the responsibility of the laboratory's chief biologist or of a duly authorized laboratory technician.

Differences in the treatment of samples and in the technical procedure can lead to non-interpretable results.

- For all handling, gloves and a lab coat must be worn. The wearing of protective goggles is strongly recommended for the handling of blood/plasma samples and other components of the kit presenting a potential risk of infection.
- Do not eat, drink or smoke in the different work zones of the laboratory.
- Never pipette the reagents by mouth. Avoid contact with skin and mucous membranes.
- In case of contact of skin or eyes with samples or reagents, wash thoroughly with a large quantity of water and contact a doctor if irritation develops.
- Use new cones and filters to avoid cross-contamination between the samples and the reagents.
- Use nonpyrogenic and nuclease free consumables (pipettes, tubes, cones etc).
- The samples must be handled and eliminated as if they present an infective risk. They require use precautions such as those described in "*Biosafety in Microbiological and Biomedical Laboratories* (BMBL)" (*www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf*).
- Carefully clean and disinfect all work surfaces with an extemporaneously prepared solution of 0.5% sodium hypochlorite in demineralised or distilled water.
- After each trial, the consumables (and waste) must be considered contaminated and treated with a 0.5% sodium hypochlorite solution or with any other inactivating agent.
- Carefully wash hands after all handling.
- Dispose of unused reagents, waste and tested samples in accordance with the rules in place in your country at the local, regional and national levels.

The reagents were specially formulated to be used in this real-time PCR quantitative test. For optimal performance, no substitutions or modifications should be made.

The components of this kit are tested as a single group: do not mix the reagents from different batches.

The quality of the amplification also depends on the quality of the DNA extraction and on its conservation. Preference should be given to the recommended extraction techniques. Omunis is not responsible for the quality of the results obtained with non-recommended elements, material and protocol.

Do not use extraction reagents after the indicated expiry date has passed.

Do not use the kit after its expiry date has passed.

TERMS OF USE

The workflow in the molecular biology laboratory must be unidirectional, starting in the pre-amplification zone and ending in the amplification zone.

The reaction mixture must be prepared in a separate room or within a biological safety cabinet.

To assure the separation of the phases of preparation of the sample, mixture and amplification, each work zone and/or room must have its own equipment (cf. required materials that are not provided) including pipettes, cones, reagents, etc… This equipment must not leave the zone it has been assigned to.

The use of the method for detection and quantification of the bacterial load of Lepto DNA must be limited to personnel that are well trained in molecular biology techniques and more specifically, in the process in its entirety (preparation of the sample, extraction of nucleic acids and real-time PCR trial).

BIOLOGICAL SAMPLE COLLECTION AND PREPARATION

1. Sample collection

Serum or plasma samples can be tested indifferently with the PUMA Lepto kit. In the case of an analysis of plasma, the blood must be collected in sterile tubes with citrate or an EDTA type anticoagulant. Heparin inhibits the real-time PCR reaction and must not be used with the PUMA Lepto kit. The addition of anticoagulants is not necessary in the case of serum (dry tube) analysis.

2. Sample preparation

A serum/plasma sample can be frozen and unfrozen twice without any significant negative impact on the extracted DNA yielded, and thus on the signal obtained after amplification.

The extraction and purification of Lepto DNA from a serum/plasma sample will be done with a non-provided extraction kit. The PUMA Lepto kit is currently validated with the QIAmp DNA Mini Kit (Qiagen) manual extraction kit for tissues extraction. A centrifugation step of the sample can be added at the beginning of the extraction (10 min at 7500 rpm). The pellet will be resuspended in 180 µl of ATL buffer supplied by the provider.

EXTRACTION OF THE INTERNAL CONTROL

The PUMA Lepto kit includes a DNA extraction and amplification internal control. This must be added directly to the sample before extraction, **at 5µL per sample after the first step of centrifugation, once bacteria are in contact with lysis buffer.** The DNA extraction can then be done in accordance with the manufacturer's instructions.

When performing DNA extraction, it is often advantageous to have an internal control of extraction that is spiked into the lysis buffer. This internal control is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the extraction control also indicates that PCR inhibitors are not present at a high concentration. A primer and probe mix are supplied (tube with red cap) with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations, which allows multiplexing with the target sequence primers. Amplification of the extraction control does not interfere with detection of the Leptospira DNA even when present at low copy number. The Internal control is detected through the CY5 channel and gives a Ct value of 31+/-3.

PREPARATION OF THE RANGE OF STANDARDS AND CONTROLS

The PUMA Lepto kit includes a standard (for the bacterial load refer to the Quality Control certificate) ("Lepto DNA tube"). In order to prepare the range of standards that will be used in the real-time PCR reaction for the quantification of the Leptospira bacterial load of the samples, a serial dilution from this tube is necessary. After dilution the range will include 5 points:

1) Pipet 90µl of water into 4 tubes and label them 2 to 5.
2) Pipet 10µl of **Lepto** DNA tube (white) into tube 2.
3) Vortex thoroughly.
4) Change pipette tip and pipet 10µl from tube 2 into tube 3.
5) Vortex thoroughly.

Repeat steps 4 and 5 to complete the dilution series in tube 4 and 5.

The concentration of the "Lepto DNA panel" tube is indicated in the Quality Control certificate.

PREPARATION OF THE PCR REACTION

1. Preparation of the reaction mixture

Calculate the number of samples to test, including the 5 Lepto standards with decreasing bacterial charges, the positive control, the negative control and an additional negative control (e.g. water for molecular biology).

In a sterile, nuclease free, 1.5 or 2 ml microvial, prepare the following reactive mixture for each sample. For N samples, multiply each of the indicated volumes by N+10%:

Reagents	Volume (µl)
Set Primers and probes	2,1 µl
Lepto Enzyme Mix	10 µl
Water	2,9 µl
Total volume of reaction	15 µl

2. Preparation of the PCR plate

- Homogenize the solution yielded by vortexing. Briefly centrifuge the tube to collect any droplets present on the edges or in the lid of the tube.
- Allocate 15 µL of the previous reaction volume to a PCR microplate suitable for the amplification system.

⚠ *Be sure not to make any bubbles when pipetting in the microplate.*

- Homogenize each tube containing the standard range, the Controls (positive and negative) and the samples (patient or water) by vortexing for a few seconds. Then briefly centrifuge the tubes

before opening to make the drops in the lid fall. Put 5 µL of each sample to be tested in the wells of the plate already containing 15 µl of reaction mixture. Each well to be tested therefore contains a total volume of 20 µl.

Note: for detailed instructions concerning a quantification trial, refer to the real-time PCR instrument and software user manuals.

a) After having carefully sealed the PCR plate with the suitable adhesive film, place the plate inside the real-time PCR instrument.

Note: from this step and up to the elimination of the plate containing the amplified targets after analysis, do not move or remove the adhesive film sealing the plate.

b) Conduct the real-time PCR trial based on the programming of the following parameters:

Steps	Repetition	Temperature	Duration
Incubating UNG	1	50°C	2 minutes
Enzyme activation	1	95°C	10 minutes
Denaturation	50	95°C	15 seconds
Amplification	50	60°C	1 minute

Note: the fluorescence reading is taken at the end of the elongation phase at 60°C

The PUMA Lepto kit can be used with different open amplification/analysis kits. To date, the tests have been optimized with the **LightCycler 480 II** Real-Time PCR (Roche).

⚠ *It is necessary to verify that the systems being used have been installed, calibrated and verified per the instructions and recommendations of the manufacturer.*

DATA ANALYSIS AND INTERPRETATION OF RESULTS

1. Software Configuration

When specific software is used for the real-time PCR system, consult the instruction manual describing data collection and the operating method for the quantification of the bacterial load.

The software must at least allow one to:

- Choose the type of fluorophore;
- Adjust the threshold or establish the threshold automatically for each well test.
- Carry forward the spread of range standards, controls and samples to test in the plate plan.
- Map the calibration curve used for the quantitative dose.
- Establish a result sheet describing the data of each well test, including: the position of the well, the fluorophore measured, the identification of the sample, the Ct value and the initial target quantity in the sample (e.g. value in l/µl or GE/µl).
- Report all of the analytical data.

After the real-time PCR trial and data collection:

1. Verify and determine the threshold setting;
2. Note de Ct values of each point in the Lepto range (intersection between the threshold and the amplification curve);
3. Verify the validation requirements of the trial before interpreting the results of each sample.

2. Trial validation requirements

✓ **Range of Standards**:

- The value of the slope is between -3,6 and 2,9 (included).
- The correlation coefficient R^2 of the curve, is between 0,95 and 1,00 (included), ideally $R^2 \geq 0,98$.

✓ **Negative Control**:

Ideally, the fluorescence emitted by a negative control must not intersect the threshold. It is a non-specific amplification indicator. If the fluorescence passes the threshold, check for an atypical curve (see below). In the case of an amplification curve, a contamination or distribution error in the microplate is to be assumed.

✓ **Positive Control**:

The value of the positive control must preferably be measured before 32 cycles. The absence of amplification of a positive control is indicative of the presence of a PCR inhibitor or an error in the distribution of the reagents. In this case, it is necessary to repeat the analysis series.

✓ **Atypical Curve**:

Some samples present atypical curves that are not characteristic of amplification curves. In this case, the result is considered as uninterpretable and the analysis of the sample must be repeated.

✓ **Extraction and amplification internal control** (« Lepto Internal Control » tube)

For internal control use as mentioned in the protocol, assuming the extraction was 100% effective and using 1:10 of the DNA extracted in the reaction, a Ct value of 31 is to be expected. However, this value can vary significantly depending on the effectiveness of the extraction and the parameters of the analysis instrument used. Ct values in a range of 31±3, are to be considered normal.

When amplifying a Leptospira sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result. This phenomenon is the result of a competition between the internal control and the bacterial target during amplification of the target, in the case of a high copy number.

3. Interpretation of the results

If the properties of the range of standards are not reached, or if the validation requirements of the positive and negative controls are not fulfilled, the entire series of analyses must be repeated.
A result is considered to be positive when the Lepto bacterial load measured is above the threshold of 0,1 l/µL (detection limit of the test)

A result is negative or “not detected” when no value is obtained or if the value is below the detection threshold (<0,1 l/µl).

The result of quantification is in leptospira/µl of eluate (l/µl).

To obtain results in leptospira/ml :

- Extraction rate = Volume of sample for extraction / Volume of eluate
- Bacterial load in leptospira/ml = (N x 1000) / extraction rate l/ml
  (N = raw data from the thermocycler analysis in l/µl of eluate)
- Bacterial load in GE/mL = bacterial in leptospira / mL x 5
  One leptospira contains 5 genomes

The test is validated according to criteria outlined in the table below. The results cannot be reliably interpreted if any of the criteria are not met.

Control	*Expected results*	*Acceptability criteria*
Internal control	*Detected in Cy5 in each sample*	*+/- 3 Ct of control reported to quality control certificate*
Negative control	*No detection in FAM*	*Complete absence of characteristic curve*
Positive control	*Detected in FAM*	*+/- 3 Ct of control reported to quality control certificate*
Standard panel	*Minimum 4 dilutions in the range detected in FAM*	*Efficiency value between 85 and 115 %* *R^2 >0,980*

Analysis	*Target signal (FAM)*	*Internal control signal (Cy5)*	*Interpretation*
Relative qualitative	*Detected*	*Detected or not detected*	*Positive for Q fever*
	Not detected	*Not detected or Ct > Ct_{QCref} +/- 3*	*Inhibition*
	Not detected	*Not detected*	*Not detected for Q fever*
Quantitative	*Detection of x bacteria / mL of sample*	*Detected or not detected*	*Positive at x bacteria / mL of sample*

If inhibition is observed perform a new amplification run, following the procedure below
1/ dilute the extracted DNA 10-fold in nuclease free water.
2/ repeat the amplification phase in this dilution.
3/ Use the table above to interpret the results.

NOTE

The manufacturer and the competent authority in the member state where the user is established should be notified in the case of any serious incident linked to the device.

SYMBOLS MEANING

Reference

Number of reactions


Storage temperature


Manufacturer


Expiration date

Lot number


Warning