Ready To Use PCR Reagents

CANINE VISCERAL LEISHMANIASIS

Cat. No. 60CVL100
INSTRUCTION MANUAL

I. Intended Use

CVL Ready to Use PCR Reagents are intended for Canine Leishmania amplifications. All reagents are ready to use for a successful amplification, from DNA extraction to obtaining PCR products suitable for loading onto Agarose gel.

II. General Information

Each pakage contains **Rapid One Step Extraction Buffer** (Tube **A**), which is intended for use with fresh or dry blood samples. The extraction step yields appropriate amount of crude DNA needed for a successful amplification of Leishmania via PCR. No purification is needed! Tubes **B**, **C**, and **D** are the components for subsequent use in PCR amplification. Tube **B** contains **CVL-PCR mix**, Tube **C** contains **CVL Activation Buffer** and Tube **D** contains the **Positive Control**. The **Extraction Buffer** (Tube **A**) also serves as **Negative Control.** Also included are **Tissue/Swab Extraction Buffer** (Tube **E**) and **Tissue/Swab Neutralization Buffer** (Tube **F**). Each PCR set up should include 3 reaction vials; each vial should be added with: **5µl CVL-PCR mix**, **10µl CVL Activation Buffer** and **5µl DNA product of the Extraction step / Positive Control / Negative Control.** Following the addition and mixing of all the above ingredients, the reaction vials are placed in thermal cycler for amplification according to the program detailed in the Step by Step chapter (**see section VIII**). At the end of the program the product should be visualized on 1.5% Agarose gel, yielding a **120bp** band.

III. Description Of The Disease

Canine Visceral Leishmaniasis (**CVL**) is an endemic zoonotic disease caused by parasites of the Leishmania donovani complex: L. d. donovani and L. d. infantum in the Old World and L. d. chagasi in the New World. Recent molecular characterization indicates that L. chagasi and L. infantum are indistinguishable Leishmania spp. Parasite is transmitted by sand-flies. Clinical signs appear progressively and cutaneous lesions may be present in combination with visceral disease. Clinical manifestations include non-pruritic skin lesions, local or generalized lymphadenopathy, weight loss, anorexia, anemia, conjunctivitis, ocular signs, diarrhea, articular involvement, onychogryphosis, weakness and decreased activity, severe muscle atrophy and renal failure, which is often the main cause of death in infected dogs. Clinical signs may be present from three months to several years post infection, although some dogs may remain asymptomatic. Any infected dog plays a role as a reservoir for further virus transmission by sand-flies.

IV. Diagnosis Of The Disease

The traditional diagnosis of **CVL** consists of serological tests (ELISA and Immunofluorescence), direct examination of smears after Giemsa staining, *in vitro* culture and histological techniques. Despite the sensitivity of the traditional diagnosis, it has been found inadequate for species discrimination, due to limitations such as cross-reactions to other phylogenetic close species, a problem which has not been solved by using mAb. A variety of molecular methods have been developed for identifying Leishmania; yet, despite their significant contribution, most of them are time consuming and expensive. A specific PCR-based method is appealing as it is rapid, sensitive, and specific, avoiding culture of parasites, thus being suitable for Leishmaniasis surveillance programs that require efficient laboratorial response for rapid and effective actions. Sampling may be done using whole blood or skin biopsy.

V. Contents (Sufficient for 48 tests)

Tube **A**	**Rapid One Step Blood Extraction Buffer**
Tube **B**	**CVL-PCR mix (Green cap)**
Tube **C**	**Specific CVL Activation Buffer (Blue cap)**
Tube **D**	**Specific CVL Positive Control (Red cap)**
Tube **E**	**Tissue/Swab Extraction Buffer**
Tube **F**	**Tissue/Swab Neutralization Buffer**
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VI. Essentials Not Included

RNAase free PCR reaction vials.
PCR Thermo-Cycler.
5-10µ, 100µl Pipettes and filter tips.
Micro-centrifuge.
Heating bath or heating block.
Agarose, DNA size marker.
Microwave for Agarose casting.
Horizontal Mini-Electrophoresis chamber, Comb and power pack.
TBE /TAE Buffer and Ethydium Bromide (EB).
UV Transilluminator (254nm for EB).
A pair of sterile scissors.
A cutter (for swab application).

VII. Storage And Handling

- Store at 4°C for 6 months or at -20°C for two years.
- Use gloves and maintain clean working conditions.
- Avoid spillage and cross contamination of solutions.
- Change tips between reagents and between reaction vials.
- Disinfect scissors before and after each cutting of blood filters.
- Do not mix reagents from different batches.
- Always treat samples with precaution, and dispose as biological material.
- Remember that Ethidium Bromide is hazardous, and use the UV transilluminator carefully.
- It is recommended to incinerate the contents after use.

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VIII. Step By Step Protocol

Blood Extraction:

(1) Into an empty clean vial, add **100µl of Rapid OneStep Blood Extraction Buffer** (Tube **A**) for **every 5µl** of fresh blood sample or approximately 3/5 mm^2 piece of Whatman/ tissue paper soaked with blood. Make sure the piece of paper is submerged underneath the extraction buffer.

(2) Incubate samples at **50°c** for **10 minutes** followed by a subsequent **95°c** for additional **10 minutes**.

(3) Centrifuge sample at **>10,000 rpm** for **1** minute to allow the paper and cell debris to pellet. The extracted DNA product is in the liquid phase, ready to be used for PCR.

Tissue/Swab Extraction:

(1) Into a clean 1.5 ml vial add **300µl** of **Tissue/Swab Extraction Buffer** (Tube **E**).

(2) When using tissue sample, cut a 3 mm^2 from the fresh or frozen tissue and add it to the 1.5 ml vial containing **300µl** of **Tissue/Swab Extraction Buffer**.

(3) Incubate the tissue within buffer **E** for **10 minutes at 95°c.**

(4) Add **300µl** of **Tissue/Swab Neutralization Buffer** (Tube **F**) and the product will be ready for PCR use.

Extracted DNA product (of any source)* may be applied immediately for PCR or stored for a few days at 4°c / several months at -20°c. Please mark the vial properly for future identification.

* Note: **The reagents have been adjusted for use with crude DNA extraction to enable better sensitivity (with less handling).**

PCR Procedure:

(1) Into a clean reaction vial add: **5µl CVL-PCR mix** (Tube **B**), **5µl** of the **Extracted DNA product** and **10µl** of the specific **CVL-Activation Buffer** (Tube **C**). Mark each reaction vial properly to avoid mistakes.

(2) Into a second clean reaction vial add **5µl CVL-PCR mix** (Tube **B**), **5µl** of the **Positive Control** (Tube **D**) and **10µl** of the specific **CVL Activation Buffer** (Tube **C**). Mark this vial as Positive Control reaction.

(3) Into a third clean reaction vial add **5µl CVL-PCR mix** (Tube **B**), **5µl** of the **Extraction Buffer** (Tube **A**) and **10µl** of the specific **CVL Activation Buffer** (Tube **C**). Mark this vial as **Negative Control** reaction.

(4) Gently mix each reaction vial (do not vortex!) and place in the thermal cycler for amplification.

PCR Program:

A. 95°C for 2 minutes

38 cycles of:

B. 94°C for 30 seconds

C. 60°C for 30 seconds

D. 72°C for 30 seconds

End cycles

E. 72°C for 2 minutes

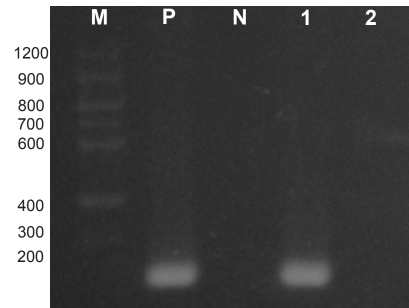
F. Stop at 8°C

(5) If not used immediately, store PCR products at 4°c until application on Agarose.

IX. Reading And Interpreting The Results

- Visualize PCR products on 1.5% Agarose gel, along with a size marker (see Fig. 1).
- Mark each well and load the whole content of each reaction vial into the relevant wells.
- The Positive Control should yield a single band at **120bp**.
- No band should be detected at the Negative Control lane.
- The expected product should be a single band at **120bp**.

Fig. 1 - Visualization Of PCR Product.



Lanes: M Size Marker, P Positive Control, N Negative Control. Lanes 1 - 2 are test samples of which 1 is positive for CVL.

X. Limitations And Troubleshooting

- For *in vitro* use only. Do not use internally or externally in humans or animals.
- A false positive result may occur, even if precaution has been taken. To eliminate inconclusive results, always use the Negative Control in each PCR set.

XI. References

- Franceschi A et al. (2007) A simple duplex-PCR protocol for routine diagnosis and follow up of canine leishmaniasis. Parassitologia. 49(1-2):43-8
- Gomes YM et al. (2008) Diagnosis of canine visceral leishmaniasis: biotechnological advances. Vet J. 175(1):45-52.
- Manna L et al. (2004) Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis. Vet Parasitol. 10:125(3-4):251-62.
- Manna L et al. (2008) Urine sampling for real-time polymerase chain reaction based diagnosis of canine leishmaniasis. J Vet Diagn Invest. 20(1):64-7.
- Manna L et al. (2008) Real-time PCR assay in Leishmania-infected dogs treated with meglumine antimoniate and allopurinol. Vet J. 177(2):279-82.

For further information and assistance please contact your local distributor or Biogal Galed Labs. Directly by e-mail: info@biogal.co.il or by tel: 972-4-9898605 / fax: 972-4-9898690.