HANDBOOK

Bio-T kit®
Lumpy Skin Disease – DIVA
Wild Strains

Cat. N° BioTK055 - 25 reactions

Detection of LSDV wild strains
by real time qPCR
with endogenous internal positive control (IPC)

CATTLE

Sample type

- Whole blood (EDTA tubes), oro-nasal swabs, scrapings of skin lesions of cattle
- Individual analysis
- Storage at 4°C or frozen at <-20°C before analysis

Recommended DNA extraction

- Magnetics beads (ex: BioSellal – BioExtract® SuperBall® Cat. N° BES384)
- Silica column (ex: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)

Other extraction kits are available, please contact our Technical Service for further information.

Veterinary use only

PRESENTATION

Description of the Bio-T kit® LSDV-DIVA

Real time PCR (qPCR) highlights the presence of targeted nucleic acid (NA) in an accurate and quick way. The Bio-T kit® Lumpy Skin Disease is a duplex qPCR that can detect simultaneously and in the same assay, the presence of:

- **LSDV – wild strains and Neethling vaccine strain (6-FAM labelled)**
- **LSDV non-vaccine wild strains (VIC labelled)**
- and **an endogenous internal positive control (IPC) targeting an endogenous DNA (Cy5 labelled) of ruminant's cells** to assess the sample integrity, the nucleic acids extraction quality and amplification.

This kit is used for individual analysis of whole blood, swabs and skin lesions from cattle.

Outline of the steps to perform from sample extraction to qPCR result

Step 1:

Extraction and the purification of total nucleic acids on silica columns or magnetic beads. Two protocols are available and described below:

- BioExtract® SuperBall®
- BioExtract® Column

Step 2:

Addition of the Master Mix in the plate wells or in strips of qPCR.

Step 3:

Addition of the extracted DNA to the Master Mix.

Step 4:

Real-time qPCR: simultaneous amplification of the viral DNA and endogenous Internal Positive Control (IPC) DNA.

Kit contents and storage conditions

Table 1. Kit contents				
Description	**Reference**	**Volume /tube**	**Presentation**	**Conservation**
Ready to use Master Mix (MM)	MMLSDDIVA-A	410 µl	1 tube white cap Bag A	-20°C in a dark place, « MIX » Zone
External Positive Control (EPC) = LSDV and LSDV-DIVA Amplification control	EPCLSDDIVA-A	110 µl	1 tube orange cap Bag B	-20°C « Nucleic Acid addition » Zone
Water RNase/DNase free	Aqua-A	1 ml	1 tube blue cap Bag B	4°C or -20°C « NA addition » Zone

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of reagents and consumables not provided with the kit

Table 2. Reagents and consumables not provided with the kit			
Consumables / Reagent	**Description**	**Supplier**	**Cat. N°**
BioExtract® Column	Extraction Column kit (DNA/RNA) (50)	BioSellal	BEC050
BioExtract® Column	Extraction Column kit (DNA/RNA) (250)	BioSellal	BEC250
BioExtract® SuperBall®	Magnetic beads Extraction kit (4 x 96)	BioSellal	BES384
ATL Buffer	Tissue Lysis buffer	Qiagen	ATL19076

For consumables related to the thermocycler, refer to the instrument manual.

General precautions

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "MIX" (ready to use MM storage, qPCR plates preparation), "NA Addition" (NA storage and addition of extracted NA and controls in the qPCR plate), "PCR" (final area containing the thermocycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- Open and close the tubes individually step by step and limit opening times to avoid contact with RNases potentially present in the environment (skin, dust, working surfaces…).
- Avoid the repetition of freezing and thawing cycles for samples, lysates and extracted DNA.

DNA EXTRACTION

BioSellal offers two extraction kits:

- **BioExtract® Column (Cat. N° BEC050 or BEC250)** based on silica membrane column, recommended for 1 to 12-20 samples extraction in parallel.

- **BioExtract® SuperBall® (Cat. N° BES384)** based on magnetics beads and automated extraction system such as KingFisher™ Duo, mL, 96 or Flex, recommended for 12 or more samples extraction in parallel.

A simplified protocol for each method is proposed below for the whole blood matrix.

For swab and dermal matrices, pre-treatment is required prior to nucleic acid extraction (NA) extraction.

For more information, please contact our technical support.

Pre-treatment of scraping of skin lesions:

Pre-treatment of scraping of skin lesions
before nucleic acids extraction and purification

Take **20-25 mg of scrapings from skin lesions** and place it in a sterile Petri dish.

Finely dissect the tissues using a sterile scalpel.

Place the tissues in a 1.5 ml micro-tube and **add 200 µl of sterile PBS** grade molecular biology.

Add 20 µl of Proteinase K and 180 µl of ATL buffer (Qiagen).

Incubate for **15 minutes at 56 °C.**

Centrifuge quickly to drop the drops of condensation and pellet the remaining tissue.

Use all **400µl of supernatant** to perform extraction using kits either **BioExtract® Column, BioExtract® SuperBall®** or **Kit Viral Total Nucleic Acid Purification.**

Pre-treatment of nasal, oral and conjunctival swabs:

Pre-treatment of swabs before nucleic acids extraction and purification

Place the swab head in a 1.5 ml micro-tube and cut the stem.

Add **300µl of sterile PBS** grade molecular biology.

Close the micro-tube pendant and vortex for 30 seconds.

Remove the swab head by pressing on the wall of the tube to recover the maximum amount of liquid.

Use **200µl for extraction** using kits either **BioExtract® Column, BioExtract® SuperBall®** or **Kit Viral Total Nucleic Acid Purification**.

Column Extraction:

BioExtract® Column Kit

Cat. N° BEC050 or BEC250

Please refer to the extraction kit protocol for solutions preparation.

1. Lysis and Adjustment of adsorption conditions

Into a 1.5 ml micro-centrifuge tube: add 20 **µl** Proteinase K.

Add in this tube **100 µl of vortexed sample** (whole blood with EDTA).

Add in this tube: **100 µl of LA-carrier Lysis Solution**. See Table 3 below for the LA-carrier Lysis Solution:

Table 3. LA-carrier Lysis Solution

Reagent	Number of samples				
	1	**6***	**12***	**24***	**30***
Buffer LA	100 µl	660 µl	1.32 ml	2.64 ml	3.3 ml
Carrier RNA (1 µg/µl)	1 µl	6.6 µl	13.2 µl	26.4 µl	33 µl

* To ensure the pipetting volume, the prepared volume contains an additional volume of 10%.

Vortex and incubate 15 min at 15-25 ° C (room temperature).

Centrifuge briefly and add **350 µl of Buffer LB**.

Vortex and centrifuge briefly.

2. Adsorption on the silica membrane

Carefully transfer the entire volume **(570µl)** on the **BioExtract® Mini Spin Column** (placed into a clean 2 ml collection tube).

Centrifuge at **6 000 x g for 1 min**. Change the collection tube (Place the BioExtract® Mini Spin Column into a clean collection tube and discard the collection tube containing the filtrate).

3. Washes and Drying of the silica membrane

Add **600 µl Buffer W1**.

Centrifuge at **6 000 x g for 1 min**. Change the collection tube.

Add **600 µl Buffer W2**.

Centrifuge at **6 000 x g for 1 min**. Change the collection tube.

Centrifuge at **20 000 x g for 2 minutes** to dry the membrane.

4. Elution of Nucleic Acids

Place the **BioExtract® Mini Spin Column** into a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate.

Add gently **60 µl Buffer EL** (at room temperature) onto the center of the membrane.

Incubate at room temperature **(15-25 °C) for 1 min**.

Centrifuge at **20 000 x g for 1 min**.

Transfer the eluate (60 µl) into a labelled tube or conserve the eluate into the 1.5 ml tube and discard the column.

The extracted DNA can be stored at 4°C if the qPCR is done within the 8 hours following the extraction, otherwise it is recommended to store it at <-20°C for 6 months or at <-70°C for a better conservation.

Figure 1. Extraction with the BioExtract® Column Kit (Cat. N° BEC050 or BEC250)

1 Lysis and Adjustment of adsorption conditions		20 µl Proteinase K 100 µl Sample 100 µl of LA-carrier lysis solution (For 1 : 100 µl Buffer LA / 1 µl carrier RNA) Room temperature (RT) 15 min 350 µl of Buffer LB
2 Adsorption on the silica membrane		Load the BioExtract® Mini Spin Column Carefully (570 µl) 6 000 x g 1 min
3 Wash Drying the silica membrane		1st Wash 600 µl W1 6 000 x g 1 min 2nd Wash 600 µl W2 6 000 x g 1 min - - 20 000 x g 2 min
4 Elution of nucleic acids		60 µl of Buffer EL (RT) RT 1 min 20 000 x g 1 min

Magnetic Beads Extraction:

BioExtract® SuperBall® Kit

Using the KingFisher™ Flex,96, Duo or mL or equivalent workstation

Cat. N° BES384

Please refer to the extraction kit protocol for solutions preparation.

1. Preparation of plates or strips

Prepare the consumables for the extraction:

Flex: 4 plates Deep-well and 2 microplates. Annotate it depending on the element to add (see Table 5).

Duo: 1 plate Deep-well and 1 elution strip.

mL: 1 strip per sample. Get out the sliding worktable from the workstation and place the strips on it.

Add in the « Deep-well lysate » plate for KingFisher™ (Flex, 96), in the Row A for KingFisher™ (Duo) or in Position A for KingFisher™ (mL):

20 µl Proteinase K.

100 µl vortexed sample (whole blood with EDTA).

500 µl thoroughly vortexed LAB-SMB-carrier solution (See Table 4 below for composition)

Table 4. LAB-SMB-carrier Lysis Solution

Reagent	Number of samples*						
	1	5	10	12	15	48	96
Buffer LA	100 µl	550 µl	1.1 ml	1.32 ml	1.65 ml	5.28 ml	10.56 ml
Buffer LB	400 µl	2.2 ml	4.4 ml	5.28 ml	6.6 ml	21.12 ml	42.24 ml
SMB (SuperBall Magnetic Beads) ‡	25 µl	137.5 µl	275 µl	330 µl	412.5 µl	1.32 ml	2.64 ml
Carrier RNA (1 µg/µl)	1 µl	5.5 µl	11 µl	13.2 µl	16.5 µl	52.8 µl	105.6 µl

* To ensure the pipetting volume, the prepared volume contains an additional volume of 10%. The exceeding volume of lysis solution can be stored for maximum 8 days, beyond this duration, the solution should be discarded.
‡ Thoroughly vortex for 3 minutes before first use and for 1 minutes for the following uses.

Prefill deep-well plates and microplates according to Table 5 below:

Table 5. KingFisher™ Flex, Duo and mL Configuration and Reagent volume

Position on the strip or on the plate			Element to add	Volume per well (µl)
Flex or 96	Duo*	mL		
Deep-well Lysate	Row A	Position A	Lysate†	620†
Deep-well Wash 1	Row E	Position B	Buffer W1	700
Deep-well Wash 2	Row F	Position C	Buffer W2	700
Deep-well Wash 3	Row G	Position D	Ethanol (96–100%)	750
Elution Microplate	Elution strip	Position E	Buffer EL	60
Rod Cover Microplate (Large 96-Rod Cover)	Row B	*Placed manually*	Rod cover	—

* Row C, D and H are empty.
† Includes 20 µl Proteinase K, 100 µl Sample and 500 µl LAB-SMB-carrier Lysis Solution

2. Launch of the KingFisher™

Select the program **« BioExtract_KF_Flex», « BioExtract_KF_Duo »** or **« BioExtract_KF_mL »** and load the plates into the workstation.
At the end of the extraction program, keep the elution plate containing the extracted nucleic acids and discard the other plates.
The extracted DNA can be stored at 4°C if the qPCR is done within the 8 hours following the extraction, otherwise it is recommended to store it at <-20°C for 6 months or at <-70°C for a better conservation.

Figure 2. Extraction with the BioExtract® SuperBall® Kit (Cat. N° BES384)

	KingFisher™ Flex	KingFisher™ Duo	KingFisher™ mL	
1 Plate or Strip Preparation	Deep-well Lysate	Row A	Position A	20µl of Proteinase K 100 µl Sample 500 µl of LAB-SMB-carrier Lysis Solution (For 1: 100µl Buffer LA / 400µl Buffer LB / 25µl SMB / 1µl carrier RNA)
	Deep-well Wash 1	Row E	Position B	700µl Buffer W1
	Deep-well Wash 2	Row F	Position C	700µl Buffer W2
	Deep-well Wash 3	Row G	Position D	750µl Ethanol (96-100%)
	Elution microplate	Elution strip	Position E	60µl Buffer EL
	Rod Cover Microplate	Row B (Rows C, D and H are empty)	*Rod cover placed manually*	Rod Cover
2 KingFisher™	• Switch on the KingFisher™ Flex, Duo or mL. • Slide open the front door of the protective cover. • Select the "BioExtract_KF_Flex", "BioExtract_KF_Duo" or "BioExtract_KF_mL" program. • Press START and follow the messages to load the different slots of the worktable.			

To get the KingFisher™ program corresponding to the KingFisher™ system you are using (Flex, Duo or mL), please contact our technical support (tech@biosellal.com).

DETECTION OF LSDV WILD STRAINS by qPCR with the Bio-T kit® LSDV-DIVA

Global procedure

Establish a plate setup defining each sample position and including the controls described below:

- **Negative Control Sample (NCS)**: water replaces the sample from the initial extraction step to the end of the process.
  This control is **mandatory** for each run of extraction.

- **Negative PCR Control (NC):** water replaces the extracted DNA in the qPCR plate.
  This control is **recommended** when using the kit for the first time or to verify the absence of master mix contamination.

- **LSDV and LSDV-DIVA External Positive Control (EPC – orange cap):** synthetic DNA provided at 10^4 copies/qPCR.
  This control is **mandatory.**

Extemporaneous Plate Preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMLSDDIVA-A (white cap)** in each well of interest (samples and controls).

In the "NA addition" dedicated area

2. **Add 5 µl extracted DNA (or NCS, or water or EPC)** in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

3. **Seal the plate with an optically clear sealer or close the strip caps.**

In the amplification dedicated area

4. **Define the thermocycler parameters** (see Tables 6 and 7).

5. It is recommended to **spin the plate down prior to place it in the thermocycler**, to prevent drops in the well pit walls.

6. **Start the program**. Approximate run time: 65 min.

Thermocyclers settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied BioSystems) and AriaMx™ (Agilent).

	ABI PRISM® 7500 Fast	ABI PRISM® 7500	AriaMx™
Mode	Quantitation – Standard curve	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Ramping Standard or Ramping Fast	Ramping Standard by default	Ramping Fast by default
Passive reference	**ROX**	**ROX**	**ROX**

For other thermocyclers, please contact our technical support.

Table 6. Thermocycler Settings

Target	Detectors		Volume final/ well
	Reporter	Quencher	
LSDV	FAM	NFQ-MGB or None*	**20µl** = 15µl MM + 5µl extracted DNA or controls†
LSDV-DIVA	VIC	NFQ-MGB or None*	
IPC	Cy5	NFQ-MGB or None*	
to assign to samples and controls†			

* Depends on the thermocycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com).

† The controls are NC (water), NCS (water extracted) and EPC (LSDV).

Table 7. Amplification Program

Standard or Fast Ramping		
Cycles	**Time**	**Temperature**
1 cycle	5 min	95°C
40 cycles	15 s	95°C
	30 s + data acquisition	60°C

This program is compatible with all the Bio-T kits®, except the ones from the PIG and AVIAN lines. A previous reverse transcription step before the PCR has no incidence on the quality of the results.

RESULT INTERPRETATION

To analyse and interpret the signals obtained by qPCR, the threshold line must be assessed carefully to obtain most reproducible results between different manipulations. A coherent set of characteristic positive curves is used to position the threshold above the fluorescence background and in the middle of the exponential phase.
The threshold Cycle (named Ct or Cq depending on thermocyclers) corresponds to the intersection between the amplification curves and the threshold line.
The qPCR run is validated if controls (EPC, NCS or NC) present valid results, then each result sample can be interpreted.

Main Scenarios

Controls Interpretation

Table 8. PCR Controls Interpretation

	Targets			Interpretation
	LSDV (FAM)	LSDV-DIVA (FAM)	Endogenous IPC (Cy5)	
NCS = Negative Process Control **MANDATORY**	Neg	Neg	Neg	Validated
	Neg / Pos	Neg / Pos	Pos	▪ Contamination with a negative or positive sample during the extraction or the plate preparation.
NC = Negative Amplification Control **OPTIONAL**	Neg	Neg	Neg	Validated
	Neg / Pos	Neg / Pos	Pos	▪ Contamination with a negative or positive sample during plate preparation or contamination of the Master Mix.
EPC = LSDV and DIVA Detection Positive Control **MANDATORY**	Pos Ct conform to CA	Pos Ct conform to CA	Neg	Validated
	Neg	Neg	Neg	▪ Problem during qPCR preparation: Master Mix Error? EPC omission?
	Pos	Pos	Pos	▪ Contamination during plate preparation.

Samples Interpretation

The type of LSDV strain detected can be deduced according to the comparative Ct value obtained for targets FAM and/or VIC.

Table 9. Samples Interpretation

Targets			Interpretation
LSDV (FAM)	LSDV-DIVA (VIC°)	Endogenous IPC (Cy5)	
Neg	Neg	Pos	**Negative or Not detected** Non-detection of LSDV wild strains nor Neethling vaccine strain
Pos Ct x	Pos Ct x	Pos	**Positive or Detected –** Presence of only LSDV wild strains
Pos Ct y	Pos Ct z>y	Pos	**Positive or Detected –** Presence of both LSDV wild strains and Neethling vaccine strain
Pos	Neg	Pos	**Positive or Detected –** Presence of only Neethling vaccine strain
Pos	Pos	Neg or Ct>35	**Positive or Detected – see above for interpretation** ▪ Presence of PCR inhibitors? ▪ Competition with the target?
Neg	Neg	Neg or Ct>35	**Uninterpretable** ▪ Omission of extracted DNA during plate setup or addition not in contact with the MM during plate preparation? ▪ Presence of inhibiters in the sample? ▪ Degradation of the DNA in the sample?

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