EurobioPlex

Monkeypox Screening Test

REAL-TIME RT-PCR

For **qualitative** real-time RT-PCR

EBX-060-25
EBX-060-50
EBX-060-100
EBX-060-200
EBX-060-600

25/50/100/200/600 reactions

RUO

Version 1.00 of June 13h 2022

Validated on:

- CFX96™ Real Time PCR detection system (Bio-Rad) with analysis on CFX Manager version 3.1 (Bio-Rad)

Storage conditions:

Keep all reagents between -15°C and -22°C until use and after first use

Instructions for use

Available on www.eurobio-scientific.com

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1. Introduction

Monkeypox is a viral infectious disease caused by an orthopoxvirus (MPXV). It is a DNA virus of about 200,000 base pairs. The Monkeypox virus is divided into two clades: West Africa and the Congo Basin. This zoonosis, known since the 1970s, is usually transmitted to humans in the forest areas of Central and West Africa by wild rodents or primates. Human-to-human transmission is also possible, particularly within the family home or in a care setting. The Monkeypox virus can be transmitted by direct contact with the skin lesions or mucous membranes of a sick person, as well as by droplets (saliva, sneezing, postillons...). The symptoms are comparable to those of smallpox but the disease is less severe. Several cases of indigenous Monkeypox infections (MKP) have been reported since May 2022 in several European countries, including in men who have sex with men (MSM), cases have also been reported in the United States, Canada, Australia and Israel. Several cases have been reported in France. There is no vaccine against monkeypox, however, the High Authority of Health has recommended the implementation of a reactive vaccine strategy in post-exposure with smallpox vaccines of 3rd generation.

Measles virus (also called MV for measles virus) is an RNA virus belonging to the genus Morbillivirus of the Paramyxovirus family. Measles is one of the most contagious viral infections of the respiratory tract, with complications that can be serious, and potentially eradicatable due to high vaccination coverage. In the first two months of 2022, the World Health Organization (WHO) has seen a significant increase in measles cases worldwide. Measles vaccination is mandatory for all children born on or after 1 January 2018. The first dose is given at 12 months and the second at 16 to 18 months.

Varicella-Zoster Virus (VZV) is a DNA alpha herpesvirus responsible for two diseases: chickenpox and shingles, a disabling disease in the elderly and immu-nodepressed patients. It is transmissible by inhalation followed by replication in the mucosa of the respiratory tract and oropharynx giving rise to severe varicella pneumonia in children and the elderly. Neurological complications related to VZV, in addition to early onset encephalitis after the onset of skin "rashes", include Guil-lain-Barré syndrome and Reye's syndrome. Vaccination against chickenpox is recommended from the age of 12 years for people who have not had chickenpox and are therefore not naturally immune, and who work in professions in contact with early childhood, or health professions in training in contact with subjects at risk of severe chickenpox.

The EurobioPlex EBX-060 has been designed to detect and differentiate Monkeypox, Measles and VZV viruses.

Extracted RNA is the starting material for the EurobioPlex Monkeypox Screening Test.
It is the user’s responsibility to choose extraction methods relevant to the type of samples tested.

2. Purpose of the system

EurobioPlex Monkeypox Screening Test is a test based on real-time reverse-transcription and amplification designed for *in vitro* use for qualitative determination of absence or presence of Monkeypox, Measles and VZV viruses in a RNA extract. This test is indicated to detect presumptive infection in humans.

The EurobioPlex EBX-060 must be used by qualified medical biology analysis laboratory personnel. It is for single use, it should not be recycled after use.

3. Symbols

	Reference
	Batch number
	Limits of temperature
	Expiration date
	Content sufficient for « N » reactions
	Manufacturer
RUO	Research Use Only
	Store away from light
	Do not use if packaging is damaged
	Caution

4. Principle of detection

The EBX-060 is a test using reverse-transcription and real-time amplification of viral RNA of Monkeypok, VZV and viral DNA of Measles based on amplification of 3 viruses in the same well.

The kit contains one oligomix to detect the 3 targets, as well as an encapsidated control of RNA extraction and RT-PCR inhibition. The test is performed from extracted RNA from a sample, using one RT-PCR reaction in a single distinct well/tube.

RNA of SARS-CoV-2 is detected with specific probes of each target, labeled respectively with FAM (target 1/Monkeypox), HEX (tardet 2/Measles) and Texas red (target 3/VZV). The endogenous control is detected using a CY5 labeled probe. Probes emit a specific fluorescence following their hydrolysis during the elongation of the amplification product. The measurement of the intensity of real-time fluorescence correlates with the accumulation of amplification products.

5. Content of the kit

The RT-PCR real-time EurobioPlex Monkeypox Screening Test kit is ready to use and contains all reagents and enzymes for the detection of this virus (Table 1).

Fluorescence is emitted and individually recorded through optical measurements during the PCR. The detection of the amplified fragment is performed by a fluorimeter using the channels shown in the Table 2.

Table 1: Content of the kit

ap color	Content of the kit	25 reactions	50 reactions	100 reactions	200 reactions	600 reactions	Reconstitution
Red	Enzymes	101,25 µL	195 µL	450 µL	750 µL	2 x 1237,5 µL	Ready to use
Transparent	Oligomix	81 µL	156 µL	360 µL	660 µL	2 x 990 µL	Ready to use
Yellow	Positive control CP	80 µL	80 µL	80 µL	80 µL	350 µL	Ready to use
Blue	Water = Negativ control (CN-H2O)	500 µL	500 µL	500 µL	1000 µL	3 x 1000 µL	Ready to use

Oligomix: contains primers and probes for the 3 targets and for the endogenous control

Table 2: Detection of targets by fluorophores

Targets	Fluorophore	Excitation	Emission
Target 1 / Monheypox	FAM	495 nm	515 nm
Target 2 / Measles	HEX	535 nm	555 nm
Target 3 / VZV	Texas Red	585 nm	605 nm
Endogenous internal control	Cy5	650 nm	670 nm

6. Storage

All reagents should be stored between -15°C and -22°C.
All reagents can be used until the expiration date indicated on the kit label.

The sensitivity of the assay may decrease if the kit components undergo multiple freeze/thaw cycles. The kit can be used after the initial opening for up to 5 freeze/thaw cycles.

7. Materials required not provided

- ◊ Biological cabinet
- ◊ Real time PCR instrument
- ◊ Centrifuge for microtubes
- ◊ Vortex
- ◊ Plates / tubes for real-time PCR reaction
- ◊ Micropipettes
- ◊ DNAse-free and RNAse-free filter tips for micropipettes
- ◊ Sterile microtubes
- ◊ Gloves (talc-free)

8. Real-time PCR instrument

The EurobioPlex Monkeypox Screening Test kit has been developed and validated for use with the following real-time PCR instruments:

- CFX96TM Real Time PCR detection system (Bio-Rad) with analysis on CFX Manager version 3.1 (Bio-Rad)

9. Cautions and note

Read these instructions carefully before starting the procedure.

- ◊ This experimentation should be performed by medical laboratory technicians.
- ◊ The local and national biosafety regulations in place for the detection of Monkeypox, Measles, VZV must be followed strictly at all times, especially in laboratories and with laboratory equipment in agreement.
- ◊ Ensure that instruments have been installed, calibrated and maintained in accordance with the manufacturer's recommendations.
- ◊ It is the responsibility of the user to use equipment other than that validated, and in such cases performance is not guaranteed.
- ◊ Clinical samples should be considered as potentially infectious material and should be prepared in a laminar flow cabinet.
- ◊ This experiment should be performed according to good laboratory practice.
- ◊ Do not use this kit after the stated expiration date.
- ◊ The kit is shipped under dry ice, and the kit components should arrive frozen. If one or more of the components arrive thawed, or if the tubes have been damaged in transit, contact Eurobio Scientific.
- ◊ Avoid freeze/thaw cycles of reagents as this may lead to a decrease in assay sensitivity.
- ◊ Once the reagents have been thawed, centrifuge the tubes briefly before use.
- ◊ The use of ice or an ice pack is recommended in case of long delays due to, for example, a large number of samples to be processed or high temperatures.
- ◊ It is recommended to define three separate work areas: 1) RNA isolation, 2) Preparation of the reaction mixture, and 3) Amplification/Detection of the amplified products.
- ◊ The fluorescent probes in the oligomix are light sensitive. Any prolonged exposure of the oligomix to light should be limited to the technical time required to prepare the PCR plate.
- ◊ It is recommended that the positive control be opened and handled separately from the biological samples to be tested and the other kit components to avoid cross-contamination.
- ◊ Distinct lab coat and gloves (talc-free) should be worn in each work area.
- ◊ Pipettes, reagents and other working materials should not be moved between these areas.
- ◊ Special care should be taken to maintain the purity of reagents and reaction mixtures.
- ◊ The internal endogenous control detects a detectable cell target in all samples of human origin but not in the CN-H2O negative control provided in the kit. The absence of a signal in the negative control prevents cross-contamination from occurring.
- ◊ Appropriate RNA preparation/extraction methods for quality RNA production and RT-PCR application should be used, especially to avoid contamination with RNAs.
- ◊ Use filter tips for micropipettes, RNAse-free and DNAse-free.
- ◊ Do not pipette with the mouth. Do not eat, drink or smoke in the laboratory.
- ◊ Avoid aérosols.

10. Procedure

a. Samples collection

◊ Collect samples in sterile tubes.
◊ It is the responsibility of the user to control their own sample collection, transport, storage and extraction conditions to ensure that RNA extraction using appropriate systems produces RNA of high quality.
˅ It is recommended that samples are extracted immediately, or stored according to the sample storage recommendations before extraction (Table 3).

Table 3: Storage recommendations before use

Recommendations for maximum storage of samples before extraction	
Ambient temperature	2 h
4°C	72 h
-20°C (preferably -80°C)	Long-term storage

Caution	
	◊ The user may refer to the recommendations issued by the World Health Organization or the French National Authority for Health for the proper storage of samples. ◊ Extracted RNA should be stored at -80°C. ◊ The transport of clinical samples is subject to local regulations for the transport of infectious agents.

b. RNA extraction

It is the responsibility of the user to ensure that the nucleic acid extraction system used is compatible with real-time RT-PCR technology.

c. Real-time RT-PCR

General note:

- The positive control contains high concentrations of matrix. Handling must be done carefully to avoid contamination.
- To confirm that the RT-PCR is working correctly, it is necessary to test the positive control, as well as the negative control (water supplied = CN-H2O) (see II-2/6 of the real-time RT-PCR protocol).

Diagram of the procedure:

1 - PREPARATION OF MASTERMIX

Number of reactions	N+3
Enzymes	(N+3) x 3.75 µl
Water	(N+3) x 3.25 µl
Oligomix	(N+3) x 3 µl
Total Volume Mastermix	(N+3) x 10 µl

2 - PREPARATION OF REACTIONS

Sample

10 µl Mastermix
+
5µl RNA sample

Positive Control

10 µl Mastermix
+
5µl CP

Negative Control

10 µl Mastermix
+
5µl Molecular biology water (CN-H2O)

3 – REAL TIME PCR INSTRUMENT

Program	Tempera-	Dura-	Cy-	
Reverse Tran-	45°C	5 min	1	-
Denaturation	98°C	20 sec	1	-
Amplification	98°C	3 sec	40	-
	58°C	10 sec		Acquisition of fluo-

d. Detailed procedure

1) Ensure that the reagents are completely thawed. Homogenise the enzyme tubes, and vortex the Oligomix and CP for about 15 seconds, then centrifuge briefly.
2) Prepare the Mastermix as below. N is the number of reactions (including positive and negative controls. Plan to prepare enough reagents for at least N+3 reactions.

Number of reactions	**N+3**
Enzymes	(N+3) x 3.75 µl
Water	(N+3) x 3.25 µl
Oligomix	(N+3) x 3 µl
Total Volume Mastermix	(N+3) x 10 µl

**For smaller series (≤10): preparing for N+2 is sufficient.*

1) Homogenise the Mastermix prepared in 2) and centrifuge briefly
2) Dispense 10 µL of Mastermix into separate microplate tubes/wells using a micropipette and filter tips.
3) Add 5 µL of extracted RNA sample.
4) In parallel perform the following controls:
   - Positive Control:
     - 10 µL of Mastermix + 5 µL of CP.
   - Negative Control:
     - 10 µL of Mastermix + 5 µL supplied water (CN-H2O).
5) Immediately close the tubes, or plate with an adhesive film to avoid contamination.
6) Centrifuge briefly to collect the reaction mixture at the bottom of the tubes or micro-plate wells.
9) Run the following program on the real-time PCR instrument.

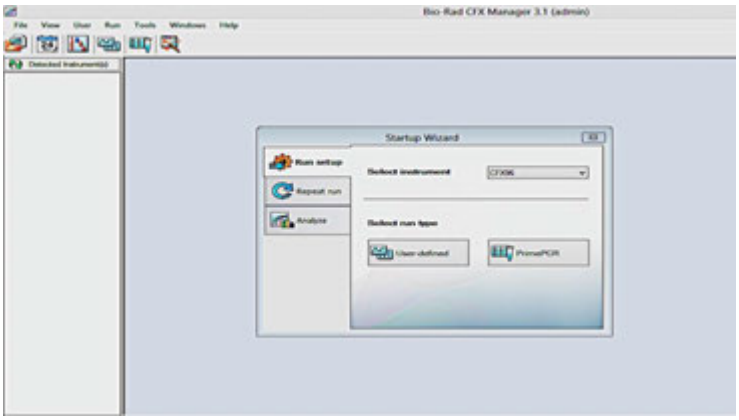
Program	**Temperat**	**Durati**	**Cycle(s**	
Reverse Transcription	45°C	5 min	1	-
Denaturation	98°C	20 sec	1	-
Amplification	98°C	3 sec	40	-
	58°C	10 sec		Acquisition of fluo-

Note: On CFX96™ (Bio-Rad), start the run using the v1.6 or later version of CFX Manager Software, and analyze with v 3.1 (see § Validation of the Experiment)

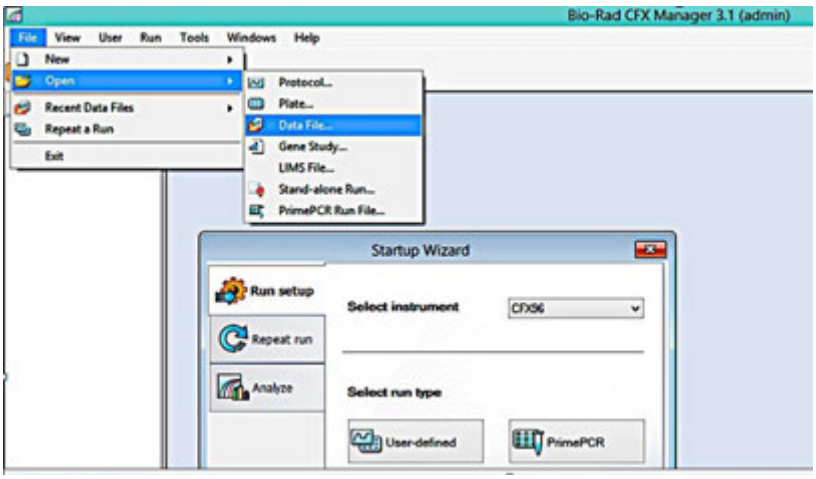
11. Validation of the experiment

Post-acquisition data analysis on a CFX96 PCR instrument (Bio-Rad) must be performed using version 3.1 of the CFX Manager software (Bio-Rad). In order to change to this version from a run started on an earlier version, please follow the procedure below: At the end of the run, the data file with the extension .pcrd must be opened and processed with CFX Manager (Bio-Rad) version 3.1.

If the run was started with CFX Manager v1.6 for example, to open a data file with CFX Manager v3.1, click on the CFX Manager v3.1 icon. The home screen appears.

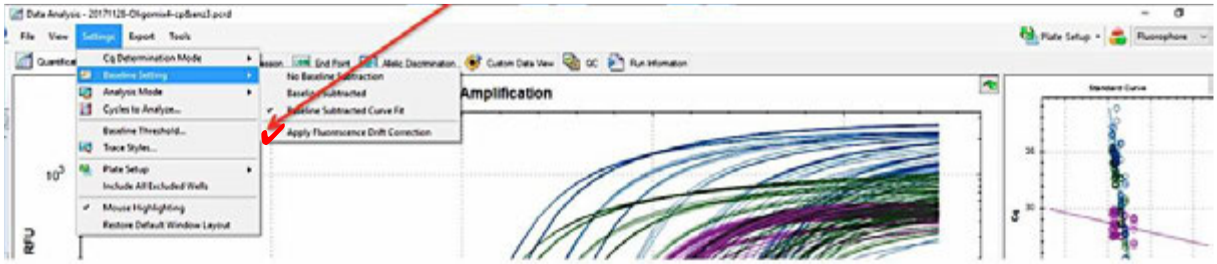


Click on "File" and select "Open" then "Data File".

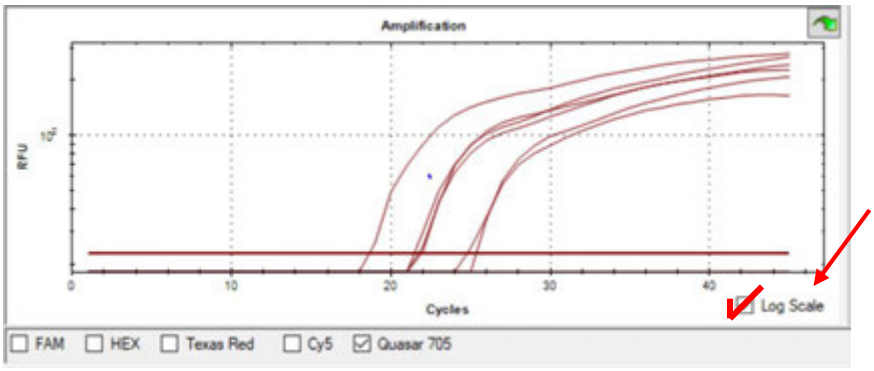


Select the file you need to analyse and click on "Open".

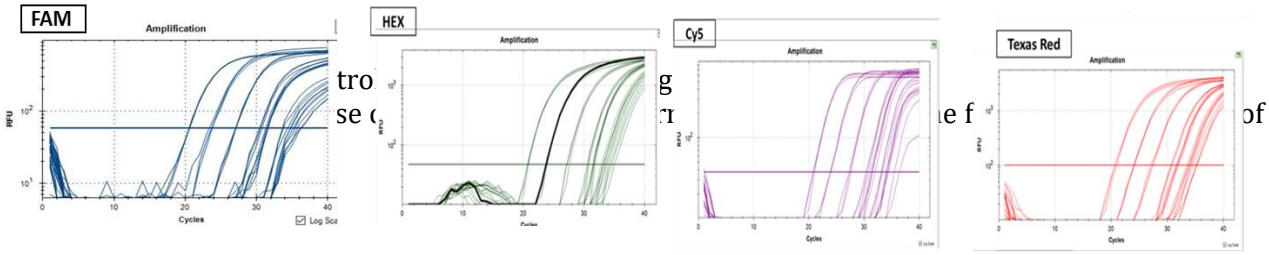
The "Drift correction" option must be applied in the "Settings" tab as shown in the image below: click on the "Settings" tab, then on "Baseline Setting" and on "Apply Fluorescence Drift Correction".



To optimise the run analysis, select the "Log Scale" checkbox for each channel analysed. Then place the threshold bar above the background noise corresponding to the middle of the exponential phase. The 5 channels of interest have high RFUs and differ according to the channel considered, so the "Log Scale" option allows for a better reading and analysis of the run.



Once this step has been completed, the analysis can proceed for the 5 channels considered **FAM**, **HEX**, **Texas Red** and **CY5**.



To validate the assay, the Ct values for the controls must be the following (Table 4). Outside of these values, the experiment cannot be validated.

Table 4: Run validation

Positive Control	
FAM	Ct ≤ 32
HEX	Ct ≤ 32
Texas Red	Ct ≤ 32
Cy5	Ct ≤ 32
Negative Control	
FAM	Undetermined Ct
HEX	Undetermined Ct
Texas Red	Undetermined Ct
Cy5	Undetermined Ct

12. Data analysis and interpretation

RNA extraction and RT-PCR inhibition control in samples:

The proper functioning of RT-PCR reaction can be evaluated on the Cy5 channel measuring the endogenous control.

In some cases, it is recommended to repeat the extraction or to dilute the sample 5 times, because the result cannot be interpreted (NI) (See column « validity of the test » on Table 5. All cases that can be encountered are described in Table 5.

For clinical samples, and determination of presence or absence of Monkeypox, Measles or VZV

Cut-off Ct values for positivity: Ct < 40

The following results are possible:

Table 5: Detection of Monkeypox, Measles or VZV

Target 1 Monkey-pox	Target 2 Measles	Target 3 VZV	Endoge-nous con-trol	Validity of the test	**Presence of one virus or no possible interpretation (NI)**
FAM	HEX	Texas Red	CY5		
+	-	-	+ / -	Yes	**YES :** Presence of Monkeypox
-	+	-	+ / -	Yes	YES : Presence of Measles
-	-	+	+ / -	Yes	YES : Presence of VZV
-	-	-	+	Yes	YES : No virus detected
-	-	-	-	*Limited*	*NI*

NI: no possible interpretation because of RT-PCR inhibition or failed extraction: no conclusion can be given. **It is then recommended to proceed to a new sampling and/or repeat the extraction and/or to dilute 5 times the sample.**

Limitations of use and interpretation:

- ❖ All samples must be treated as potentially infected, and biosafety local regulations must be thoroughly followed.
- ❖ Interpretation of results must take into account the possibility of false negatives and false positives.

False negative can be due to:

- Inappropriate collection of samples, or bad storage
- Samples outside the viremic phase
- Incorrect extraction methods or use of non-validated PCR instruments
- Manipulations that do not rigorously follow all the indications of this manual.

False positive can be due to:

- A contamination related to wrong manipulation of highly positive samples, or from the positive control, or PCR products

- Procedures that do not rigorously follow precautions to avoid contamination described in this manual

- ❖ Results must be interpreted by medical professionals in the clinical context of the patient, its history and symptoms.
- ❖ This test does not exclude the presence of other pathogens.
- ❖ A negative result for this test does not absolutely exclude a possible infection with Monkepox, Measles or VZV.

13. Performance analysis

Limit of detection/analytical sensitivity

A dilution range was performed from a plasmid mixture ranging from 10^5 to 1 copy/µL. This dilution range was used to determine the detection limit (LoD) of the kit with a Probit analysis which allowed the 95% cut-off to be determined.

Detection limitation/analytical sensitivity (95% cut-off**)**

Monkeypox (FAM) : 3,879 copies/µL Measles (HEX) : 2,289 copies/µL VZV (Texas Red) : 2,565 copies/µL Alb (Cy5) : 4,862 copies/µL

The validation of the performance was performed on:

23 positive samples	
22 VZV samples	Characterised by Biomnis Sample Library
1 vaccine sample with attenuated virus	ROR vaccine

9 negative samples	
9 negative sample	Characterised by Biomnis Sample Library

For this study, sample extraction was performed on Maelstrom 9600 (TANBead) with the OptiPure Viral Auto Plate kit (ref: W665A46), and RT-PCR on CFX96 (Bio-Rad).

<u>Positive samples</u> :
sensitivity for VZV / Measles *and specificity for virus of interest*

N=23 (+) positif / (-) négatif		**Virus**		
		Mon-key-pox	**Measles**	**VZV**
VZV samples	N=22	-	-	+ (22/22)
Sensitivity to VZV		N/A		
Specificity to virus		N/A	N/A	**100%**
Measles sample (ROR)	N=1	-	+ (1/1)	-
Sensitivity to Measles		N/A		
Specificity to virus		N/A	**100%**	N/A

Measles sensitivity : > 99% (1/1)
Measles specificity: > 99% (22/22)
Measles concordance: > 99% (23/23)

VZV sensitivity : > 99% (22/22)
VZV specificity: > 99% (1/1)
VZV concordance: > 99% (23/23)

14. Bibliography

- Vanessa Zubach, Alberto Severini, Joanne Hiebert. Development of a rapid, internally controlled, two target, real-time RT-PCR for detection of measles virus. Journal of Virological Methods 299 (2022) 11434
- Yu Li, Victoria A. Olson, Thomas Laue, Miriam T. Laker, Inger K. Damon. Detection of monkeypox virus with real-time PCR assays. Journal of Clinical Virology 36 (2006) 194–203.
- Kimberly B. Hummel , Luis Lowe, William J. Bellini, Paul A. Rotal. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. Journal of Virological Methods 132 (2006) 166–173
- Rapport annuel d'activité 2019. Centre de national de référence Laboratoire Expert Orthopoxvirus Centre de national de référence Laboratoire Expert Orthopoxvirus

15. Quality control

In accordance with Eurobio's ISO EN 13485 certified quality management system, each batch of EurobioPlex Monkeypox Screening Test is tested according to predefined specifications to ensure consistent product quality.

16. Waste disposal

Eliminate all waste in accordance with the legislation on DASRI.

17. Incident report

Serious incidents related to the system must be reported to Eurobio Scientific and to the competent authority of the Member States in which the user and/or the patient is registered.

18. Technical assistance

For assistance with our products, please contact our technical support.

Eurobio Scientific's customer service can be contacted by e-mail at adv@eurobio-scientific.com or by phone at +33 (0)1.69.79.64.80.

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