For life science research use only. Not for use in diagnostic procedures. For *in vitro* use only.

LightMix® Modular Orthopox Virus typing

Cat.-No. 58-0551-96 Roche SAP n° 09 799 575 001

Kit with reagents for 96 PCR reactions 20 µl for detection of OPXV and typing for MPXV

1. Content, Storage and Expiry

1 Vial red cap 96 reactions OPXV/MPXV (dried)
1 Vial black cap Positive Control (dried)

Storage at Arrival:

Store cooled or at ambient temperature
Do not freeze the lyophilized reagents.

- Kits are stable for one year after production (store 4°C to 25°C in the dark). See lot-specific expiry date.
- Reconstituted reagent is stable for two weeks if stored protected from light and cooled (2°C to 8°C).
- Dissolved reagent can be stored long-term if frozen (-15°C to -25°C). Avoid multiple freeze-thaw cycles.
- Reconstituted positive controls must be stored frozen. Minimize multiple freeze-thaw cycles.

2. Additional Reagents required

LightCycler® Multiplex DNA Master Cat.-No. 07 339 585 001
or 1-step RT polymerase *90-9999-96*

We strongly recommend to use LightCycler® Multiplex DNA Master, only if this reagent is not available, 1-step RT polymerase can be used as substitute. For optimal performance with 1-step RT polymerase we recommend to adjust the annealing Tm from 60 to 64°C, as described in section 8.1.

3. Introduction

Orthopoxviruses (OPXV) are very large (200 nm like small bacteria), brick-shaped ds DNA viruses with a genome of ~ 200 kb. They cause febrile illnesses with prominent vesicular rash.

Orthopoxviruses that are known to cause disease in humans include smallpox, vaccinia virus, cowpox, and **monkeypox**.

Monkeypox (MPXV) is a rare smallpox-like disease of children in central Africa. Typical signs are fever, sore throat, headache and vesiculopustular rash, but also bronchopneumonia and diarrhoea. They are distinguished in 2 clades the Congo-Basin (**MPXV-CB**) and the West-African clade (**MPXV-WA**). MPXV-WA has been recently reported in various European countries and the USA.

4. Description

A 160 bp long fragment from the RAP94 gene specific to orthopoxviruses is amplified with primers and detected with a **R6G** labeled hydrolysis probe.

If amplification occurs, the inclusion of the MPXV WA specific **FAM** labeled probe enables differentiation between **MPXV-WA**, **MPXV-CB** and other orthopoxviruses, with the performance of additional melting analysis. To assist with interpretation, the included positive control represents MPXV-CB. Melting-peaks with a highter Tm represent MPXV-WA and melting-peaks with lower Tm other orthopoxviruses.

5. Specification

This assay detects 10 genome equivalent copies or less per reaction (plasmid DNA dilution).

6. Sample Material and Extraction

Typical samples are swabs from skin lesions.
For extraction protocols see Roche MagNA Pure or Roche manual kit instructions.

7. Material Safety Data (MSDS)

This product is not hazardous (according to regulation (EC) No 1272/2008), not toxic, not IATA-restricted. Not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and EU Directives (EC) No 1907/2006 and (EC) No 2015/830 any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

8. Instructions for Use

When run in combination with assays containing other fluorophores (channels), a Color Compensation file must be applied. To generate a Color Compensation file, see instructions in the ***Roche 06296971001 Universal Color Compensation Hexaplex*** Instructions For Use.

8.1. Programming Roche 480 Instruments

See the Instrument operator's manual for details. Start programming before preparing the solutions. The protocol consists of three program steps:

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification
- 3: Cooling: cooling the instrument

Detection Format Two (Three) Channels	**Set Quant Fact. 10, Max Int. time 1 sec, 660: 3 sec**		
LightCycler® 480 Instrument:	483-533	523-568	615-670
LightCycler® 480 II Instrument:	465-510	533-580	618-660
cobas z 480 Analyzer (open channel):	465-510	540-580	610-670

Program Step:	***Denaturation***	***Cycling***			***Melting***			***Cooling***
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			
Target [°C]	95	95	60*	72	95	40	80	40
Hold [hh:mm:ss]	00:05:00	00:00:05	00:00:15	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
							Continuous	
Acquisition Mode	None	None	**Single**	None	-	-	1**	None

**** with 1-step RT polymerase use 64°C*** Table 1

** Melting slope should be 0.19 to 0.29°C per second. If reading more channels reduce the number of acquisitions/sec.

8.2. Experimental Protocol

- **Sample material:** Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Preparation Kit').
- **Negative control:** Always run at least one no-template control (NTC) - replace the template NA with water.
- **Positive control:** Run a positive control - replace the template NA with the provided Positive Control.

For an increased sensitivity use 10 µl nucleic acid per 20 µl reaction, for sample types where inhibition may occur e.g. Fecal sample extracts, use 5 µl. For 10 µl reactions in 384 well plates use 5 µl /2.5 µl.

8.2.1. Preparation of Parameter-Specific Reagents (PSR, 96 reactions):

The reagent vial with a red cap contains the primers and probe to run 96+ LightCycler® reactions.
Add 50 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.
For robotic pipetting the volume can be extended to 55 µl (signals will decrease by 10-20%).

►**Use 0.5 µl** reagent for a 20 µl PCR reaction.

8.2.2. Preparation of the Positive Control (MPXV-CB)

Add 160 µl RNase/DNase-free 10 mM Tris buffer pH 8 - 8.5 to the vial with the black cap, if using 10 µl sample volume add **320 µl**. Mix by pipetting up and down 10 times. If vortexing spin down to collect the solution. Store dissolved controls frozen. Use of Tris increases the stability in solution.
Notes: Opening this vial may cause contamination of the workspace. Pulse spin vial prior to opening.

►**Use 5 µl** positive control for a 20 µl PCR reaction (10 µl if using 10 µl sample volume).

8.2.3. Preparation of the Reaction Mix

Multiply volumes by the number of reactions plus controls and one reserve, prepare in a cooled tube:

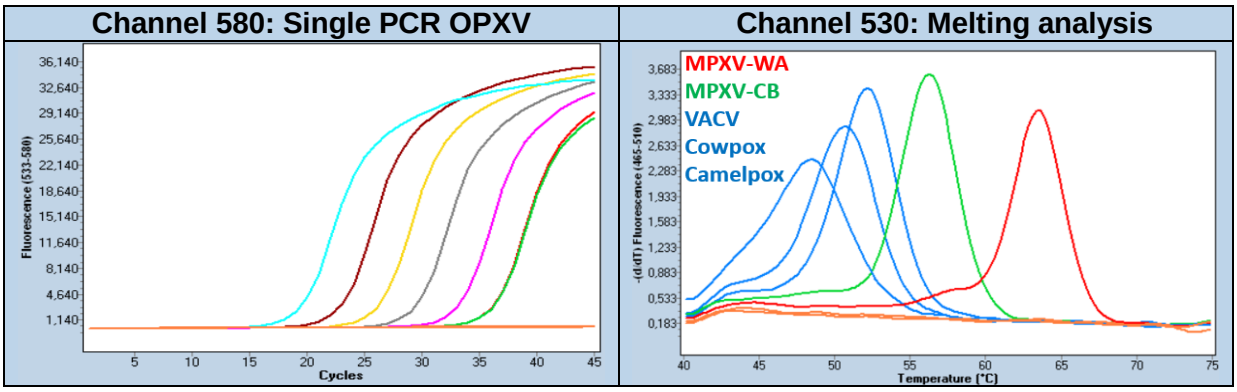
LightCycler® Multiplex DNA Virus Master		1-step RT Polymerase 90-9999-96 *	
10.5 µl	**Water**, PCR-grade	**Water**, PCR-grade	4.5 µl
0.5 µl	**Reagent** mix **(PSR)**	**Reagent** mix **(PSR)**	0.5 µl
--	Control Reaction (Multiplex PCR)	Control Reaction (Multiplex PCR)	--
4.0 µl	**Roche Master**	**1-step RT Master**	10.0 µl
15.0 µl			**15.0 µl**

Table 2

Mix gently, spin down and **transfer 15 µl** per well.
Add 5 µl of sample or control to each well for a final reaction volume of 20 µl. Seal plate and centrifuge.
Start run

* Pox is a DNA virus. No RT step required. The dried RT polymerase 90-9999 has been tested to work well.

9. Typical Results (Data from LightCycler® 480 II system)



Dilution row 1E6 to 10 copies / reaction; **red** MPXV-WA, **green** MPXV-CB, **blue** other OPXV Figure 1

10. Reading the Results

Perform data analysis as described in the operator's manual. For multiplex assays select the color compensation. We recommend using the Second Derivative Maximum method (Automated (F'' max). View results in the 530 and 580 channel. The negative control (NTC) must show no signal.

Channel 580 (sample)	Channel 660 Control Reaction	Channel 580 NTC Control	Result
No amplification	**Detectable**	**Negative**	**Not detectable**
Amplification Cp < 37 +	**Not relevant**	**Negative**	**Orthopox virus Positive**
No amplification	**Not detectable**	**Not relevant**	**PCR failure Repeat**
Amplification signal	**Not relevant**	**Positive**	**Contamination Repeat**

Note: cobas z 480 Analyzer signal levels are ~ 50% as compared to LightCycler® 480 II results.
+ Recommendation: Define the cut-off 1-2 cycles higher than observed Cp value for 10 copies.

Melt curve analysis interpretation

Channel 530	Channel 660 Control Reaction	Channel 530	Result
Melting Curve Tm ≤ 55 °C	**Not relevant**	**Not visible**	**other orthopoxviruses**
Melting Curve Tm ~ 57 °C	**Not relevant**	**Not visible**	**MPXV-CB**
Melting Curve Tm ~ 64 °C	**Not relevant**	**Positive**	**MPXV-WA**

Tm values shift depending on the instrument, speed of heating, mastermix, salt contents and detection format.
The assay detects the genetic situation and not a clade; the correlation to a reference clade describes the most likely assignment

11. References

-none-

12. Multiplex PCR Compatibility

This assay can be combined with an internal control (IC), an extraction control (EC) or a spiked extraction control (for example PhHV) as depicted below:

Multiplex PCR and Instrument Compatibility

Color Compensation 40-0320 is mandatory for Multiplex PCR

500	530	580	610	640	660
	typing	Orthopox			
	typing	Orthopox			IC/EC

480 II	z 480	LC96	LC2.0	Nano
X	X	X	X	X
X	X	X		

Table 3

recommended extraction controls are:

66-0901-96 PhHV	Roche Cat.-No. 07 093 802 001
66-0905-96 MSTN	Roche Cat.-No. 07 225 253 001
66-0907-96 RNase P	
66-0913-96 Actin	Roche Cat.-No. 07 805 993 001

13. Version History

V220530	Release version	2022-05-30
V220602	Primer moved, to improve performance with specific reagents	2022-06-02
V220621	Change the probe fluorescence label to R6G, for improved routine production	2022-06-21
V220805	use with1-step RT polymerase 90-9999-96 and melt-curve programming	2022-08-05

Note. EU / German Export Restrictions for this product (Dual Use Bioweapon Detection). End-user-certificate may be required. End user will be reported to the National Authorities.

Certificate of Analysis (CoA)

Lot n° **5250**

Expiry: **YYYY-MM-DD**

Dilution	1E6	1E5	1E4	PC	1E2	1E1	passed
Cp value							✓
Tm value							✓
Signal level							✓
Negatives	10/10						✓

***Note**:* Cp (crossing point) values collected with pDNA (single target PCR). Fluorescence (FL) levels depend on instrument settings and may vary. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (ΔCp).

DOM (manufactured): YYYY-MM-DD QC Acceptance: YYYY-MM-DD

We, the undersigned, certify that the product designated above has been obtained in accordance with the rules of production and quality control.

Name(s):

Name1 *Name2*

TIB MOLBIOL Syntheselabor GmbH | Eresburgstr. 22-23 | D-12103 Berlin | Germany
Tel. +49 30 78 79 94 55 | FAX +49 78 79 94 99 | dna@tib-molbiol.de | WWW.TIB-MOLBIOL.COM
HS code 3822 0000 | EORI DE 4806433 | Registry Court Berlin Charlottenburg HRB 93163 B
Distributed by Roche - www.lifescience.roche.com