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

Instructions For Use

LightMix® Modular EAV RNA Extraction Control 610

Cat.-No. 61-0909-96 Roche SAP n° 07 654 235 001

Kit with reagents for 96 PCR reactions 20 µl [lyophilized]

2016-4: Assay changed

1. Content, Storage and Expiry

Storage at Arrival:

1 Vial purple cap 96 reactions EAV (lyophilized)
1 Vial black cap containing the Extraction Control Target RNA

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

- Lyophilized kits are stable for at least 6 months (4°C to 25°C in the dark). See lot-specific expiry date.
- Dissolved reagents are stable for at least 2 weeks if stored protected from light and cooled (4°C).
- Dissolved reagents can be stored long-term at -20°C (within expiry). Avoid multiple freeze-thaw cycles.
- Dissolved Extraction Control RNA must be stored at -20°C. Avoid multiple freeze-thaw cycles.

2. Additional Reagents required

LightCycler® Multiplex RNA Virus Master Cat.-No. 06 754 155 001

3. Introduction

PCR analysis of biological samples may occasionally result difficult due to problems related to the extraction process. In particular, false negative results in pathogen testing can be due to the presence of inhibitors, degraded target material or an unsuccessful extraction of nucleic acids. The presence of amplifiable nucleic acids can be verified by running an extraction control PCR.

4. Description

A 70 bp long fragment from the Equine Arteritis Virus genome is amplified with specific primers and detected with a LC610 labeled hydrolysis probe.

This product is intended to be used with Equine Arteritis Virus RNA or the provided Extraction Control Target (ECT) as target. The ECT is *in-vitro* transcribed RNA and not viral RNA or a virus. The ECT is not encapsulated nor protected and might be degraded rapidly after added to biological samples; reduce the time until extraction to a minimum OR add ECT to the lysis buffer instead to the sample.

The amount of target spiked to the samples has to be adjusted to yield a Cp value in the range of 30. Note: With a MagNA Pure Compact the recovery rate for the EC target can be very low or even get lost.

5. Specification

These reagents detect 10 copies of the target. Typical amount used as extraction control is 500 - 5,000 copies.

6. Sample Material and Extraction

Typical sample type are vaginal swabs or urine. See ModularDx Document ***Extraction Protocols.***

7. Material Safety Data (MSDS)

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

8. Instructions for Use

• Instrument programming see document ***ModularDx Programming***
• Color Compensation see instructions in ***40-0320 Universal Color Compensation Hexaplex***
• Pipetting instructions multiplex PCR see ***ModularDx Multiplex***

8.1. Programming Roche 480 Instruments

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

• 1: Reverse Transcription of the viral RNA
• 2: Denaturation: sample denaturation and enzyme activation
• 3: Cycling: PCR-amplification
• 4: Cooling: cooling the instrument

Detection Format 610 Channel **Set Quant Factor 10, Max Integration time 2 sec**
LightCycler® 480 Instrument: 558-610
LightCycler® 480 II Instrument: 533-610
cobas z 480 Analyzer (open channel): 540-610

Program Step:	***RT Step***	***Denaturation***	***Cycling***			***Cooling***
Parameter						
Analysis Mode	***None***	None	Quantification mode			None
Cycles	*1*	1	45			1
Target [°C]	55	95	95	60	72	40
Hold [hh:mm:ss]	00:05:00	00:05:00	00:00:05	00:00:15	00:00:15	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	4.4	2.2	4.4	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	4.6	2.4	4.6	2.0
Acquisition Mode	None	None	None	**Single**	None	None

Table 1

8.2. Experimental Protocol

• **Sample material:** Use aqueous nucleic acid preparations (e.g. 'High Pure Viral Nucleic Acid Kit').

This is a control assay to verify that extraction and reverse transcription worked. There is no need to include a 'negative control' (NTC) for the extraction control. Pathogen-assay negative controls (NTC) must be positive for the extraction control; negative control PCR results in pathogen-negative samples indicate an inhibition, extraction or any other PCR or pipetting failure.

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

One reagent vial with a purple cap contains all 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 per 20 µl PCR reaction.

8.2.2. Preparation of the Target Nucleic Acids (NA)

Add 1,200 µl RNase/DNase-free Tris buffer, PBS, or water to the **black** cap vial. Mix by pipetting up and down 10 times. If vortexing spin down to collect drops. The NA is not protected / encapsulated. Use of Tris buffer pH 8.0-8.5 increases the stability in solution. Store dissolved target NA frozen.

►**Add 10 µl** target NA to 200 µl of the sample to be extracted and extract immediately OR add during the lysis step. The amount of target NA is adjusted for a standard procedure with an extraction volume to 100 µl and a sample to PCR volume of 5-10 µl. The Cp value of the control reaction should be higher than 25. The amount of target NA may be varied to achieve a Cp value in the range of 27 to 33.

8.2.3. Preparation of the Reaction Mix

Multiply volumes by the number of reactions plus controls and one reserve and prepare in a cooled tube. The table contains the amounts for a duplex reaction for one analytical parameter (red) and the control:

Duplex PCR - Instruction for use with the Roche LightCycler® Multiplex RNA Virus Master		
for 5 µl extract	**Component**	**10 µl extract**
9.9 µl	**Water**, PCR-grade (colorless cap, provided with the Roche Master kit)	4.9 µl
0.5 µl	**EAV Control Reaction** and	0.5 µl
0.5 µl	**PSR Reagent** mix additional assay(s) (Multiplex PCR))	0.5 µl
-	For each additional assay reduce the amount of water by 0.5 µl	-
4.0 µl	**Roche Master** (see Roche manual)	4.0 µl
0.1 µl	**RT Enzyme**	0.1 µl
15.0 µl	**Volume of Reaction Mix**	**10.0 µl**

Table 2

Mix gently, spin down and **transfer 15 µl (10 µl)** per well.

Add 5 µl (10 µl) of sample or control to each well for a final reaction volume of 20 µl. Seal plate and centrifuge.

Start run

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

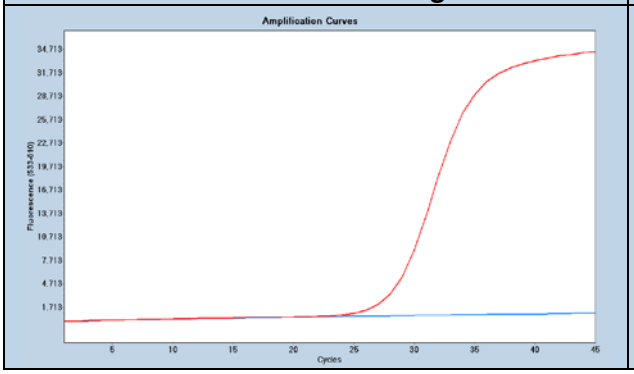
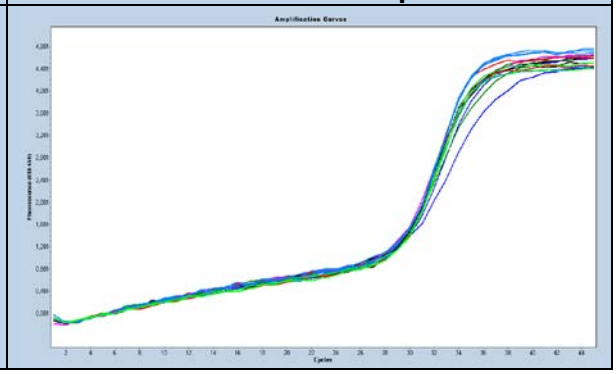
Channel 610 : EAV – Single PCR	Channel 610 : Multiplex PCR
	

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 610 channel. The negative control (NTC) and negative samples must show a signal.

Other channel than 610 Analytical PCR	Channel 610 Control Reaction	Other channel than 610 NTC Control	Result
No amplification	**Amplification +**	**Negative**	**Parameter - Negative**
Amplification signal	**Not relevant**	**Negative**	**Parameter - Positive**
No amplification	**Not detectable**	**Not relevant**	**PCR failure Repeat**
Amplification signal	**Not relevant**	**Positive**	**Contamination Repeat**

Notes: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.
+ Cp depends on the respective dilution during extraction (might have to be adjusted).

11. References

Comparison of two real-time reverse transcription polymerase chain reaction assays for the detection of Equine arteritis virus nucleic acid in equine semen and tissue culture fluid. Lu et al., 2008

12. Multiplex PCR Compatibility

The EAV extraction control / reference assay can be combined with up to one (LightCycler® 2.0), three (LightCycler® 96), four (cobas z 480) or five (LightCycler® 480 system) other assays :

Multiplex PCR and Instrument Compatibility

Color Compensation 40-0320 is mandatory for Multiplex PCR

500	530	580	610	640	660	480 II	z 480	LC96	LC2.0	Nano
	Assay 1		EAV			X	X	X	X	X
	Assay 1	Assay 2	EAV		control	X	X	X		
	Assay 1	Assay 2	EAV	Assay 3	control	X	X			
Assay 4	Assay 1	Assay 2	EAV	Assay 3	control	X				

Table 3

13. Version History

V140909	Release version	2014-09-18
V150101	Editorial changes	2015-02-28
V150404	Roche SAP number	2015-04-10
V151001	2015 protocol Multiplex Master, 5/10 µl extract and 60°C acquisition	2015-10-01
V160216	Correction in 'Content'	2016-02-26
V160313	Amplicon pos. moved starting lot 38451602 1. Storage. 8.2. buffer	2016-05-21

Certificate of Analysis (CoA)

Lot n°
Expiry :

TIB MOLBIOL

Dilution	1E6	1E5	1E4	ECT	1E2	1E1	passed
Cp range				27-30			
Measured							
Signal level				25-45			
Measured							
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).

QC Acceptance Date:

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

Name(s) :

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