EBV R-gene™ - ref.: 69-002

ARGENE

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EXTRACTION

- IN EXTRACTION ROOM -

- Homogenize **IC2** and samples to be analysed:

WHOLE BLOOD, CSF, PLASMA, BAL, BIOPSIES.

- Add 10 µL **IC2** to the appropriate volume of each sample to be extracted in 1 extraction tube.
- Add 10 µL **IC2** to the appropriate volume of **W0** in 1 extraction tube.

- Perform extraction as below:

INSTRUMENT	KIT	Sample + IC2 Volumes	SAMPLE TYPE	PROTOCOL	ELUTION VOLUME
	QIAamp® DNA Blood Mini Kit	200 µL of sample + 10 µL IC2	Whole blood		100 µL
			Plasma, CSF		50 µL
QIAcube			Whole blood	Blood and body fluid spin protocol V3	100 µL
			Plasma, CSF		50 µL
MagNAPure Compact® Instrument	MagNA Pure Compact Nucleic Acid Isolation Kit I		Whole blood	DNA_Blood_100_400	100 µL
			Plasma, CSF	Total_NA_Plasma_100_400	50 µL
MagNA Pure LC System® Instrument	MagNA Pure DNA Isolation Kit I		Whole blood	DNA I Blood_Cell High Performance	100 µL
			Plasma, CSF	Total_NA_Serum_plasma_blood	50 µL
NucliSENS® easyMAG™	NucliSens® EasyMag reagents		Whole blood	Manufacturer's Specific B protocol with 140 µL silica and 2 mL lysis buffer	50 µL
			Plasma, CSF	Generic / Specific B	50 µL
BioRobot EZ1	EZ1® DNA Blood	350 µL of sample + 10 µL IC2	Whole blood	EZ1 DNA Blood Card	200 µL
*m*2000sp™	Sample Preparation	800 µL of sample + 10 µL IC2** (extract 300 µL)	Whole blood, plasma, CSF, BAL, biopsies	DNA-Blood-LL-300-150 v081507	250 µL (eluate 150 µL)
Versant® kPCR Molecular System SP	Sample Preparation 1.0	400 µL of sample + 10 µL IC2** (extract 250 µL)	Plasma	Sample Preparation Protocol 5	65 µL (eluate 50 µL)

Store extracted samples at -18°C/-22°C

ANCILLARY REAGENTS:

- **CELL Control r-gene™ - ref.: 71-106**
- **Colour Compensation r-gene™ - ref.: 71-103**

for LightCycler® 2.0 Instruments use only.

GLOSSARY:

IC2	Internal Control 2
W0	Water for extraction
R4	EBV Amplification premix
QS	Quantification Standard
SC	Sensitivity Control
CT	Crossing Threshold
IC2sample	Sample Extraction + Inhibition Control
IC2W0	Reference Extraction + Inhibition Control

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AMPLIFICATION

- IN AMPLIFICATION ROOM -

Validated on:

LightCycler® 530 & 560	SmartCycler® FCTC / FAM & Cy3	Applied Biosystems FAM & VIC	Rotor-Gene™ GREEN & YELLOW	Stratagene® Versant® kPCR AD FAM & HEX

EBV R-gene™ AMPLIFICATION PROGRAM

- Enter the following program:

STEPS		TIME	TEMPERATURE	CYCLES	FLUORESCENCE ACQUISITION
Taq Polymerase Activation		15 min.	95°C	1	-
Amplification	Denaturation	10 sec.	95°C	45	-
		20 sec. for Stratagene			
	Annealing Elongation	40 sec.	60°C		530 + 560 nm end of the elongation

Note 1: for LightCycler® instruments, set **SEEK TEMPERATURE** to the temperature corresponding to the fluorescence acquisition.
Note 2: for LightCycler® instruments, add a cooling step: 30 sec. / 40°C / 1 cycle at the end of the PCR.
Note 3: for Applied Biosystems instruments, select **NONE** in **PASSIVE REFERENCE**.
Note 4: for Rotor-Gene™, calibrate the signal by clicking on **GAIN OPTIMISATION**.
Note 5: for Stratagene® and Versant® kPCR Molecular System AD instruments, select **NONE** in **REFERENCE DYE**.

EBV R-gene™ AMPLIFICATION PREPARATION

- Plan number of amplification tubes taking into account:
  - ⇨ The necessity to create standard curve or not;
  - ⇨ The negative/inhibition control(s), sensitivity control (**SC**).
- Distribute 15 µL of **R4** in all amplification tubes.
- Add 10 µL sample, **QS**, **SC**, extracted **W0**.
- Centrifuge for 15 sec / gently move the microplate if applicable.

LAUNCHING EBV R-gene™ PROGRAM

- Enter the concentrations of the standards according to the extraction method used.

	QUANTIFICATION (Whole blood, plasma, CSF, BAL, biopsies)				
	Extraction 200 µL Elution in 50 µL (copies/mL)	Extraction 200 µL Elution in 100 µL (copies/mL)	Extraction 350 µL Elution in 200 µL (copies/mL)	Extraction 300 µL Elution in 250 µL (copies/mL)	Extraction 250 µL Elution in 65 µL (copies/mL)
QS1	2 500 000	5 000 000	6 000 000	8 000 000	2 500 000
QS2	250 000	500 000	600 000	800 000	250 000
QS3	25 000	50 000	60 000	80 000	25 000
QS4	2 500	5 000	6 000	8 000	2 500

- Select the type of each amplification tube as: **SAMPLE** or **UNKNOWN** or **STANDARD** or **NEGATIVE CONTROL** or **POSITIVE CONTROL**.

RESULTS

1. DATA ANALYSIS

- **Import the external standard curve** (if applicable).
- **Identify and quantify the positive samples** by:
  - using "fit point" method with LightCycler®1.0;
  - using "second derivative maximum" method for LC2.0 and LC480;
  - setting **30** as default parameter in "**MANUAL THRESH FLUOR UNITS**" for SmartCycler®.
  - adjusting threshold line for Applied Biosystems, Rotor-Gene™, Stratagene® and Versant® kPCR Molecular System AD.

2. INTERPRETATION

- **Conditions for test validation:**
  - Negative Control: NO SIGNAL.
  - IC2W0 (**560 nm**): CT ≤ 32 CYCLES.
  - QS3: CT between 28 and 32 CYCLES.
  - Slope / Efficiency:

Real-Time PCR Platform	VALUABLE SLOPE / EFFICIENCY	
	If all QS are tested.	If all QS are tested to be stored for subsequent importation.
LightCycler® 1.0	-3.917 < Slope < -3.103	-3.587 < Slope < -3.208
LightCycler® 2.0 / LightCycler® 480	1.8 < Efficiency < 2.1	1.9 < Efficiency < 2.05
SmartCycler® 2.0	-0.322 < Slope < -0.255	-0.311 < Slope < -0.278
Rotor-Gene™	0.8 < Efficiency < 1.1	0.9 < Efficiency < 1.05
Applied Biosystems	-3.917 < Slope < -3.103	*NOT APPLICABLE*
Stratagene®, Versant® kPCR AD	0.8 < Efficiency < 1.1	

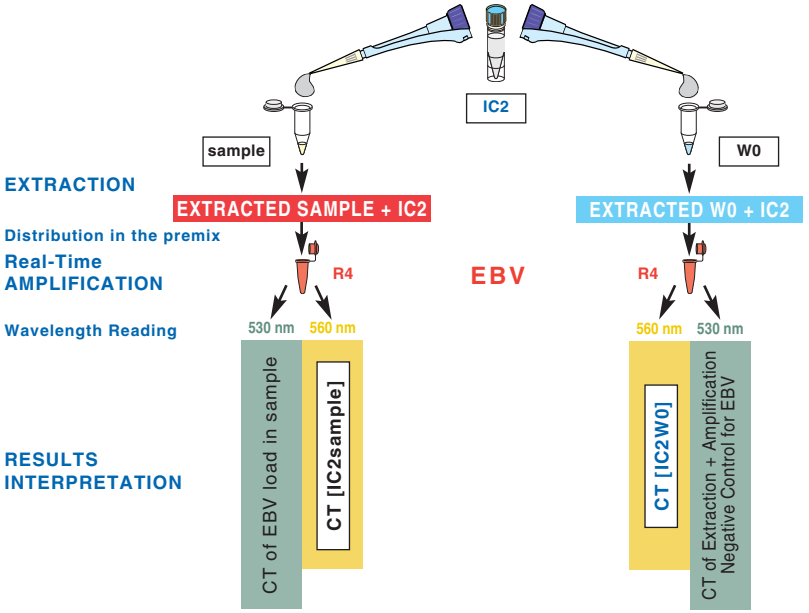
- **Quantification**

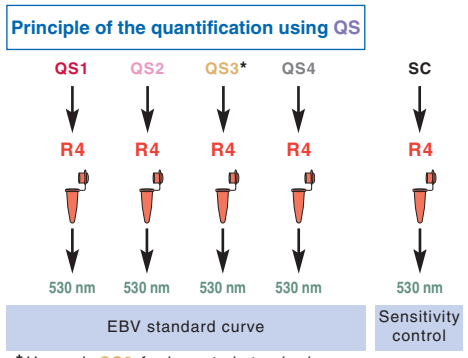
EXTRACTION and INHIBITION CONTROL	CT [IC2sample] ≤ CT [IC2W0] + 3 cycles		CT [IC2sample] > CT [IC2W0] + 3 cycles	
	NON INHIBITED and correctly extracted sample		INHIBITED or badly extracted sample	
SAMPLE	Calculated CT	Non calculated CT	Calculated CT	Non calculated CT
EBV quantification	Validated quantification	Sample validated as negative	Perform quantification again. Sample validated as positive.	NOT VALID

This outline procedure does not replace the original protocol.

O.P.69-002_vE_230710

PRINCIPLE for EBV QUANTIFICATION





Use only QS3 for imported standard curve.

Sample interpretation

CT [IC2sample] ≤ CT [IC2W0] + 3 cycles

The sample is correctly extracted and doesn't contain inhibitory agents of amplification.