Adenovirus R-gene™ - ref.: 69-010

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EXTRACTION

- IN EXTRACTION ROOM -

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

Whole blood, plasma, respiratory samples, stool, CSF, 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, respiratory samples, biopsies.		100 µL
			Plasma, CSF.		50 µL
			Stool.		100 µL
	QIAamp® DNA Stool Mini Kit		Stool.		100 µL
QIAcube	QIAamp® DNA Blood Mini Kit		Whole blood, respiratory samples, biopsies.	Blood and body fluid spin protocol V3.	100 µL
			Plasma, CSF.		50 µL
			Stool.		100 µL
	QIAamp® DNA Stool Mini Kit		Stool.	Isolation of DNA from stool for pathogen detection V1.	100 µL
MagNAPure Compact® Instrument	MagNA Pure Compact Nucleic Acid Isolation Kit I		Plasma. Respiratory samples.	Total_NA_Plasma _100_400	50 µL
			Whole blood.	DNA_Blood_100_400	100 µL
MagNA Pure LC System® Instrument	MagNA Pure DNA Isolation Kit I		Plasma. Respiratory samples.	Total NA Variable_elution_volume.	50 µL
			Whole blood, Stool.	DNA I Blood_Cell High Performance	100 µL
NucliSENS® easyMAG™	NucliSens® EasyMag reagents		Respiratory samples.	Generic.	50 µL
			Whole blood.	Specific B + 140 µL silica + 2 mL lysis buffer	50 µL
			Stool.	Specific A + 100 µL silica	50 µL
BioRobot M48	Mag Attract DNA Mini M48 Kit (192)		Respiratory samples.	Bacteria DNA	100 µL
*m*2000sp™	Sample Preparation System DNA Promega	800 µL of sample + 10 µL IC2 *(extract 300 µL)*	Whole blood, respiratory samples, biopsies.	DNA_Blood_LL_300_150 V0811507	250 µL
Versant® kPCR Molecular System SP	Versant® Sample Preparation 1.0	400 µL of sample + 10 µL IC2 *(extract 250 µL)*	Plasma, CSF.	Sample Preparation Protocol 5	65 µL *(eluat 50 µL)*

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

ANCILLARY REAGENTS:

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

for LightCycler® Instruments use only.

GLOSSARY:

IC2	Internal Control 2
W0	Water for extraction
R10	ADENOVIRUS amplification premix
QS	Quantification Standard
SC	Sensitivity Control
CT	Crossing Threshold
IC2sample	Sample Extraction + Inhibition Control
IC2W0	Reference Extraction + Inhibition Control

ARGENE tel.: +33 (0)5 61 69 61 00 • infotech@argene.com

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

ADENOVIRUS 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**.

ADENOVIRUS R-gene™ AMPLIFICATION PREPARATION

- Plan number of amplification tubes taking into account:
  - ⇨ Number of samples to be amplified;
  - ⇨ The necessity to create standard curve or not;
  - ⇨ Sensitivity Control (**SC**);
  - ⇨ Negative / inhibition control (**W0**).
- Distribute 15 µL **R10** in all amplification tubes.
- Add 10 µL sample, **QS**, **SC**, extracted **W0**.
- Centrifuge for 15 sec / gently move the microplate if applicable.

LAUNCHING ADENOVIRUS R-gene™ PROGRAM

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

	QUANTIFICATION (Whole blood, plasma, respiratory samples, stool, CSF, biopsies)				
	Extraction 200 µL Elution in 50 µL (copies/mL)	Extraction 200 µL Elution in 100 µL (copies/mL)	Extraction 300 µL Elution in 250 µL (copies/mL)	Extraction 250 µL Elution in 65 µL (copies/mL)	Results in copies / PCR
QS1	1 250 000	2 500 000	2 100 000	1 250 000	50 000
QS2	125 000	250 000	210 000	125 000	5 000
QS3	12 500	25 000	21 000	12 500	500
QS4	1 250	2 500	2 100	1 250	50

- Select the type of each amplification tubes 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 ≤ 36 CYCLES.
  - QS3: CT between 29 and 33 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
ADENOVIRUS quantification	Validated quantification	Sample validated as negative	Perform quantification again. Sample validated as positive.	NOT VALID

For stool, respiratory samples and biopsies a qualitative approach is preferable.

This outline procedure does not replace the original protocol.

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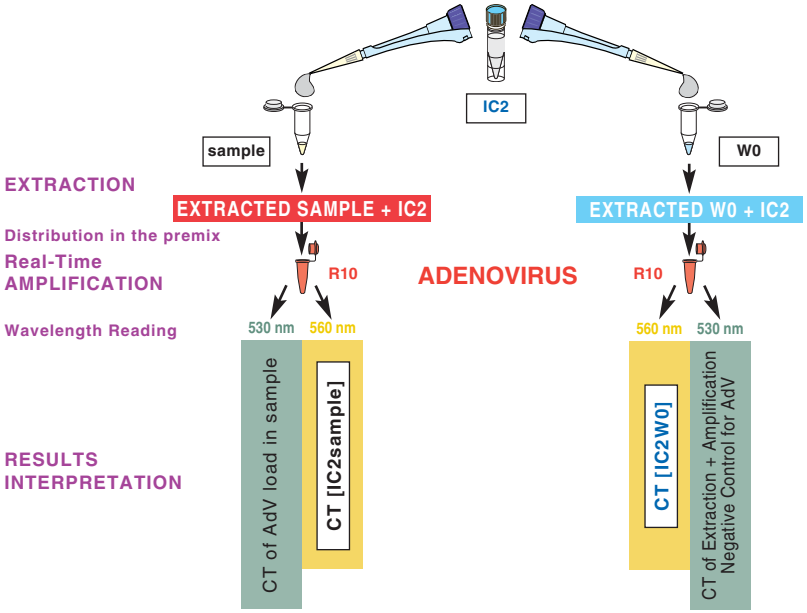
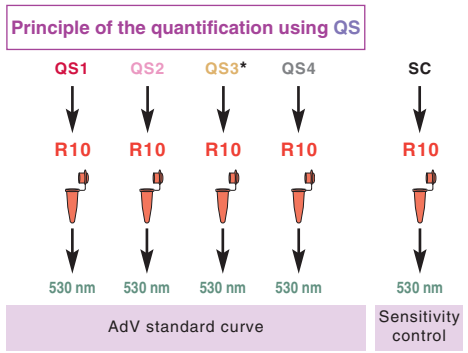
PRINCIPLE for ADENOVIRUS QUANTIFICATION





*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.