quanty Measles

REF: RT-06

Detection and quantification of the Measles Virus with *Real Time PCR*

INTRODUCTION AND PURPOSE OF USE

The *quanty* **Measles** system is a quantitative test that allows the RNA amplification, by means of *Real Time PCR*, of N3 gene of Measles Virus.
The Procedure allows the detection and quantification of the RNA target by means a One step retro-amplification reaction.
The analysis of the results is made using a Real Time PCR analyzer (thermal cycler integrated with a system for fluorescence detection and a dedicated software).

CONTENT

The kit contains reagents enough to perform 48 tests

	Quantity	Description
R1	3 x 10 µl	**Enzyme mMix** Reverse Transcriptase e Taq Polymerase enzymes
R2	3 x 270 µl	**MeV probe Mix** Upstream primer, downstream primer, Target probe MeV (VIC), Internal Control (IC) probe (CY5), Nuclease-free water, ROX dNTPs, Tris-HCl, KCl, MgCl2
R3	3 x 35 µl	synthetic RNA corresponding to N3 region of Measles 100.000 cps/µl
R4	3 x 35 µl	synthetic RNA corresponding to N3 region of Measles 10.000 cps/µl
R5	3 x 35 µl	synthetic RNA corresponding to N3 region of Measles 1.000 cps/µl
R6	3 x 35 µl	synthetic RNA corresponding to N3 region of Measles 100.000 cps/µl
R7	3 x 35 µl	**Internal control** (murine RNA - GAPDH)
R3	1 x 50 µl	**Negative Control**

Instruction for use: **ST. RT06-ENG.0**

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge (12,000 - 14,000 rpm);
Micropipettes and Sterile tips with aerosol filter;
Vortex;
Plastic materials (microplate and optical adhesive cover);
Heat block (only for extraction)
EZ1 Advanced XL DSP Virus Card. - Ref. 9018703 - QIAGEN.

Reagents

The **quanty Measles** kit was developed and validated to be used with the following extraction method:

Manual Extraction

Ref. 52906. QIAmp Viral RNA Mini Kit
The kit allows the manual RNA extraction from tested samples . The kit contains reagents for 250 samples. (QIAGEN)

Automatic extraction

Ref. 62724. EZ1 XL DSP Virus Kit.
The kit allows the automatic viral RNA from Human samples.
The kit contains reagents for 48 samples. (QIAGEN)

Instruments

The **quanty Measles** kit was evaluated to be used with the following instruments:

Extraction System

Ref. 9001492. EZ1 Advanced XL.
Robotic Workstation for the automatic purification of the nucleic acids until 14 samples simultaneously (QIAGEN)

Real Time PCR

The **quanty Measles** kit was evaluated to be used to be used with the following real time PCR instruments:

- *7500 Fast* from Lifetechnologies
- *Rotor-Gene Q* MDx from QIAGEN

Please ensure that the instruments have been installed, calibrated, checked and maintained according to the manufacturer's instruction and raccommendations.

SAMPLES AND STORAGE

The **quanty Measles** kit must be used with extracted RNA from biological samples. Collected samples must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.
Store the sample at -20°C if it is used after 3 days.

PRECAUTIONS USE

This kit is for in *vitro* diagnostic (IVD), for professional use only and not for in vivo use.
After reconstitution, the amplification master mix must be used in one time (16 reactions). Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently. At all times follow Good Laboratory Practice (GLP) guidelines.
Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.
Avoid any contact between hands and eyes or nose during specimens collection and testing.
Handle and dispose all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.
Keep separated the extraction and the reagents preparation.
Never pipette solutions by mouth.
Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.
Wash hands carefully after handling samples and reagents.
Do not mix reagents from different lots.
It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).
Do not eat, drink or smoke in the area where specimens and kit reagents are handled.
Read carefully the instructions notice before using this test.
Do not use beyond the expiration date which appears on the package label.
Do not use a test from a damaged protective wrapper.

LIMIT OF THE METHOD

The extreme sensitivity of gene amplification may cause false positives due to cross-contamination between samples and controls. Therefore, you should:

- physically separate all the products and reagents used for amplification reactions from those used for other reactions, as well as from post-amplification products;
- use tips with filters to prevent cross-contamination between samples;
- use disposable gloves and change them frequently;
- carefully open test tubes to prevent aerosol formation;
- close every test tube before opening another one.

The proper functioning of the retro-amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.
As with any diagnostic device, the results obtained with this product must be interpreted taking in consideration all the clinical data and other laboratory tests done on the patient.
A negative result obtained with this product suggests that the RNA of Measles was not detected in RNA extracted from the sample, but it may also contain Measles-RNA at a lower titre than the detection limit for the product (detection limit for the product, see paragraph on Performance Characteristics); in this case the result would be a false negative.
As with any diagnostic device, with this product there is a residual risk of obtaining invalid, false positives or false negatives results.

STORAGE AND STABILITY

Store the product ***quanty Measles*** at **–20°C**.
The ***quanty Measles*** kit is shipped on dry ice. The kit components should be frozen.
If one or more components are not frozen upon receipt or if the tubes have been compromised during transport, contact Clonit srl for assistance.
An intact and well stored product has a stability of 6 months from the date of production. Do not use beyond the expiration date which appears on the package label.
Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.

SOFTWARE SETTING

Lifetechnologies 7500 fast

Turn the instrument and the computer on and open the control software. Click on "**Advance Setup**": by default the software will shows the page "**experiment properties**". Write in the "**experiment name**" the file name, choose the type of instrument (**7500 o 7500fast),** the type of reaction (**quantitation standard curve),** the type of used reagent (**Taqman•Reagents**) and the reaction time of analysis (**Standard ≈ 2 hours to complete a run**).
Open the page named "**page setup"** (sheet **Define Target and Samples**).
In the window **Define Targets** set:

Target	Reporter	Quencher
Measles probe:	VIC	NONE
Internal Control (IC) probe:	CY5	NONE

Set the samples' name in the window "**Define Samples**"
In the same page "**plate setup**" select the sheet "**Assign Target and Samples**". On the screen you will see the microplate draft.
Select an area of the plate where the controls will be placed: select wells of the plate and set both targets (Measles and IC). Select "**Assign target to selected wells**" in the blank, the "**task Standard (S)**" for Measles target and set the controls' concentration.
Choose an area in the plate where negative control will be placed: select "**Assign target to selected wells**" in the blank, the "**task Negative (N)**" for the Measles target.
Select an area of the plate where samples will be placed: select the wells and set both targets (Measles and IC). Link every well to a sample, through the window "**Assign samples to selected wells**".
For each sample, select in the blank "**Assign targets to selected wells**" the "**task UnKnown (U)**" for the Measles target.
Set ROX as passive reference, using it as normalizer of detecting fluorescence.
Open "**Run Method**" (sheet **Graphic View**) and set the right thermal cycling:

cycles	denaturation	annealing	extension
1	50° C 40 min		
1	95°C 2 min		
45	95° C 15 sec	58° C 45 sec	72° C 15 sec

In the window "**Reaction volume plate per well**" set a volume of 30 µl.
After preparing the plate, and correctly inserting it in the instrument, press the button "**Start Run**".

Rotor Gene Q MDx

The experiments can be **set using the Quick Start Wizard or the** Advanced Wizard, which appears when the software is started.
Select the wizard "**Advanced**". As a first step, select the model "**Two Step Reaction**" with a double click in the "**New Run**".
In the next window, select the type of rotor installed on the instrument from the list that appears. Check the "**Locking Ring Attached**", check the checkbox and then click "**Next**".
Enter the name of the operator and the reaction volume of 30 µl, and then click "**Next**".
In the next window click on "**edit profile**". Set the following thermal cycle:

cycles	denaturation	annealing	extension
1	50° C 40 min		
1	95°C 2 min		
45	95° C 15 sec	58° C 45 sec	72° C 15 sec

Select the annealing / extension from the thermal profile and click on "**Acquiring A to cycling**."
In the next window, select yellow and orange from the available channels and add it to acquiring channel along with the green channel and click "**OK**". In the next window click on "**OK**" and then click "**Next**".
Click on "Edit Gain" button and set the following values for each channel:

Reporter	Gain
Yellow	5
Red	5
Orange	5

To begin the course, click on the button "**Start Run**". You can save the model before you begin your run by clicking on "**Save Template**".
After clicking on the button "**Start Run"** window appears "**Save As**". The stroke can be saved in the desired position by the user.
Once the run started, the window "**Edit Samples**" allows you to set the name of samples and controls in the positions in which they were loaded on the instrument.
Select the locations where they were positioned the controls of known concentration and designate them as Measles Standard. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Standards**". Enter the concentrations of the controls.
Select the location where you placed the Negative Control and name it as Negative Control. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Negative Controls**"
Select the location of each sample and enter the name or code of the patient. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**UnKnown**"
At the end of the operation click "**OK**" in the "**edit samples**" and wait until the end of the race for the analysis (see "**Interpretation of Results**").

PREPARATION OF THE REACTIONS:

Defrost a tube of **Enzyme mMix**;
Defrost a tube of **MeV probes Mix;**
Mix carefully by vortex **9µl** of **Enzyme mMix** and **260µl** of **MeV probes Mix** (the mix as produced is enough to prepare **16 reactions** of amplification: **4 positive controls, 1 negative control and 11 samples**).
Distribute, in the amplification plate, **15µl of just reconstituted mix** in chosen positions as already set on the instrument software.
Distribute, in the negative control position, **15 µl** of solution taken by the negative control vial.
Distribute, in chosen position for each sample, **15 µl** of corresponding sample.
Distribute, in chosen positions for the positive controls, **15µl** of **10^2 copies/µl, 10^3 copies/µl, 10^4 copies/µl and 10^5 copies/µl**.
Seal up accurately the plate using an optical adhesive film and verify that there aren't air bubbles in the mix, to avoid the amplification interferences.
For the Rotor-Gene Q MDx, seal each tube with the appropriate cap. The air bubbles presence is not influenty; the rotor centrifugal force will allow automatic deletion.
Transfer the plate in the instrument and push the button "**Start Run**".

QUANTITATIVE ANALYSIS

Lifetechnologies 7500 Fast

At the end of the PCR run, the software automatically opens the "**Analysis**" window in the "**Amplification plot**" sheet on the menu on the left.
Select the wells corresponding to the positive control, negative control and samples for analysis.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **Measles target**. Check the correct setting of the threshold.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **IC Control target**. Check the correct setting of the threshold.
The analysis of the results is made selecting from the menu in the left the page **"Analysis"**. From the page "**Standard Curve**", maintaining open the sheet "**view well plate**" in the right side of the software select the wells containing the points of the curve and verify the parameters described in the paragraph "**Interpretation of the Results**" (coefficient of correlation, slope ecc…).
From the page "**Amplification Plot**" verify the amplification plot for every single sample.
Opening the sheet "**view well table**" in the right side of the software it is possible to verify the data obtained from experiments: Threshold Cycles, emitted fluorescence and the target quantification expressed in copies/reaction or copies/ml, depending on the settings of the calibration curve.
Clicking from the menu file and selecting the box export, the window "**export properties**" will open. Indicate the file name, select the position to save it (**Browse**) and click on button
"Start export". In this way the software will permit to save a excel file with all the data corresponding to selected experiment.

Rotor Gene Q MDx

At the end of the PCR run click "**Option**" button under the amplification plot on yellow channel. Select "**normalize to Cycling A. orange**".
Open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A.Yellow/cycling A.orange".**
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Check the correct setting of the threshold in the space provided "**CT calculation – Threshold"**.
Click "**Option**" button under the amplification plot on red channel.
Select "**normalize to Cycling A. orange**".
Open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (red)/cycling A.orange".**
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Check the correct setting of the threshold in the space provided "**CT calculation – Threshold"**.
Check the presence of a value of to CT in the table "**Quant.Results".**
Also in this case, you can print a report of the analysis by clicking on the **"Report"** window and selecting the file in the first **Quantification cycling A (yellow) /cycling A.orange"** and then the file **cycling A (red)/cycling A.orange".**

INTERPRETATION OF RESULTS

Through the Real Time PCR reaction, it is possible to obtain the RNA quantification of Measles RNA, by setting the values of the positive controls of the calibration curve. To calculate these values, all the dilutions steps that the sample has undergone during the extraction and amplification stages must be considered..
The Ct values obtained from the amplification of 4 controls of known titre are used by the software for the calculation of the calibration curve from which the unknown samples are interpolated.
A proper functioning of the amplification mix can be verified analyzing these parameters:

Parameters	Reference
RTS conc. 10^5 copie/µl (FAM)	Ct ≤ 25
Correlation Coefficients	0.990 ≤ r^2 ≤ 1
Slope	-3,6 ≤ Slope ≤-3.2
PCR Efficiency	90 ≤ Efficiency ≤ 100

If the RTS amplification reaction at a concentration of 10^5 copies produces a Ct > 25 or undetermined the session can't be considered valid and must be repeated.

Verify that the correlation coefficient value (r^2), the slope or the reaction efficiency fit to the limited indicated in the above table or do not deviate much from them, which represent the ideal range for a proper PCR reaction.

By correctly setting the standards concentration as a function of the extraction system you can get the quantization of the sample directly in copies / ml:

		Manual Extraction	Automatic Extraction EZ1 Advanced XL	Alternative Extraction
		QIAmp Viral RNA mini kit	EZ1 XL DSP Virus Kit	
		copies/ml		Copie/react
	RTS 1	35.700.000	30.000.000	1.500.000
	RTS 2	3.570.000	3.000.000	150.000
	RTS 3	357.000	300.000	15.000
	RTS 4	35.700	30.000	1.500

When alternative systems are used sample concentration expressed in copies/ml will be obtained using the formula:

where:

- **Ve:** extracted sample Volume expressed in µl
- **Ev:** eluted sample Volume during extraction stage expressed in µl
- **Ea:** extracted sample volume used for amplification expressed in µl
- **C_{reaz}:** copies provided by the instrument.

As with any diagnostic device, the results obtained with this product must be interpreted taking in consideration all the clinical data and the other laboratory tests done on the patient.
As with any diagnostic device, with this product there is a residual risk of obtaining invalid, false positives or false negatives results.
The use of positive and negative controls in each amplification session allow to verify the correct functioning of the amplification mix and the absence of any contamination.
In the amplification reaction of each sample, the Ct values for the internal control specific probe are used to validate the analysis session, from extraction process until detection step.
In the amplification reaction of each sample, the Ct values for the internal control specific probe are used to validate the analysis session, from reverse transcription process until detection step.
Be sure that emitted fluorescence from internal control amplification has not a Ct > 35 or undetermined. If a sample shows an undetermined Measles RNA and an internal control Ct >35, this means that there have been problems in the extraction stage or in the retro-amplification stage; therefore the sample could be a false negative.
Repeat the sample.
It can be considered valid the samples with a Ct > 35 for the internal control, and a high concentration of Measles RNA. In this case, the competitive nature of PCR reaction can hide or disadvantage the internal control amplification.

Detector VIC	Detector CY5	PCR Run	Sample
Ct undetermined	Ct > 35 or undetermined	Not Valid	repeat
Ct undetermined	Ct < 35	Valid	Negative
High Ct	Ct < 35	Valid	Positive
Low Ct	Ct > 35 or undetermined	Valid	HighPositive

PERFORMANCES

Analytical Specificity:

Test's specificity was guaranteed by the use of specific primers for Measles.
The alignment of the choose regions for specific primers' hybridization for Measles with available sequences of the N3 gene region present in database, demonstrated: their conservation, the absence of significative mutations and the complete specificity for the analysed target.

The analysis "in Silico " showed how the alignment of primers and probes allows the correct identification of the following subtypes :

- *Genotype B2*
- *Genotype B3*
- *Genotype D4*
- *Genotype D8*
- *Genotype D9*
- *Genotype G3*
- *Genotype H1*

INTERFERENCES:

Verify that in the RNA extracted from the sample there is no contamination from mucoproteins and haemoglobin, to exclude possible inhibition of PCR reaction. The interference due to contaminants can be detected through a spectrophotometric analysis, verifying the ratio between the absorbance readings at 260 nm (maximum absorbtion of Nucleic Acids) and 280 nm (maximum absorbtion of Proteins). A pure RNA should have a ratio of approximately 2.

QUALITY CONTROL

It is recommended to include in each analytical run, as quality control of every extraction, amplification and detection step, an already tested negative and positive sample, or a reference material with known concentration.

Each batch of quanty Measles was produced according to predetermined specifications in order to ensure compliance with the ISO EN 13485 certified quality management system

BIBLIOGRAPHY

Kimberly B. Hummel, Luis Lowe, William J. Bellini, Paul A. Rota. **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.*

World Health Organization. **Global measles and rubella laboratory network---update.** Wkly Epidemiol Rec 2005;80:384--8.

World Health Organization. **Manual for the laboratory diagnosis of measles and rubella infection,** 2nd ed. Geneva, Switzerland: World Health Organization; 2007. WHO/IVB/07.01.

TECHNICAL ASSISTANCE

For any question and support please contact our Technical support:

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IVD	*In vitro* diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
LOT	Lot (xxxx)
REF	Code
	Manufacturer
Σ	Contains sufficient for <n> tests

EDMA code: 15040540
CND: *W0105040507*

The ***quanty Measles*** kit is CE marked diagnostic kit according to the European *in vitro* diagnostic directive 98/79/CE.

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