DeepChek® Assay

NS5A Genotyping and Drug Resistance (RUO)

V1.x

User Guide

Version 1 – Revision 2

For Research Use Only (RUO). Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.

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Contents

Application 3
Principles of the assay 3
Assay components 4
Reagent storage and handling 5
Materials required but not provided 5
Warnings and precautions 6
Starting 6
RNA Extraction 6
RT-PCR Workflow Overview 7
RT and PCR Step-by-Step Workflow for NS5A Target 8
Nested Step-by-Step Workflow for NS5A Target 9
RT-PCR Troubleshooting Guide 10
PCR Products Purification 10
Sequencing 10
1. Purification 10
2. Sanger sequencing 11
3. NGS 12
Data Analysis 13
1. Sanger 13
2. NGS 13
Product quality control 13
Symbols 13
Contact Information 13
Manufacturer 14

Application

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The ***DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)*** kit is a single tube system which utilizes PCR technology for amplifying relevant portions of the human C virus (HCV) NS5A gene from input RNA extracted from plasma/serum.

This nucleic acid amplification method might aid in the typing of HCV viruses and DR. This test is NOT intended to be used as a screening or confirmation test for the detection, confirmation, and quantification of HCV infection.

The ***DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)*** is intended for use by trained laboratory personnel specifically instructed and trained in the techniques of PCR, SANGER and next generation sequencing (NGS) workflow.

Principles of the assay

The ***DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)*** is a reverse transcriptase-polymerase chain reaction test which includes primers, reverse and forward, designed to amplify HCV RNA from plasma/serum specimens.

First, reverse transcription is performed and the HCV reverse primers anneal to their respective targets and are extended during a prolonged incubation period. After a denaturation step, in which the temperature of the reaction is raised above the melting point of the double-stranded DNA, a second primer anneals to the DNA strand and is extended by the DNA polymerase activity of the enzyme to create a double-stranded DNA product.

During each round of thermal cycling, amplification products dissociate to single strands at high temperature allowing primer annealing and extension as the temperature is lowered. Exponential amplification of the product is achieved through repeated cycling between high and low temperatures, resulting in a billion-fold or greater amplification of target sequences.

The ***DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)*** is performed on a PCR instrument.

Subsequently, the amplicons can be used for SANGER or next generation sequencing and analyzed with a downstream analysis software to list in a report HCV genotypes according to available public reference knowledge databases.

Assay components

The ***DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)*** is provided in one model of 24 reactions (REF 105A24 / OLD REF K-16-NS5ADR).

Label	Volume for 24 Rxn	Color cap	Storage
RT-PCR			
RT-PCR Buffer 2X	1 x 480 µL	Green	-25°C to - 15 °C
RP Solution	1 x 15 µL	Purple	-25°C to - 15 °C
RT Enzyme	1 x 15 µL	White	-25°C to - 15 °C
BP Solution	1 x 12 µL	Pink	-25°C to - 15 °C
PCR Enzyme	1 x 15 µL	White	-25°C to - 15 °C
NS5A REV (1) PCR Primers	1 x 25 µL (20 µM)	Yellow	-25°C to - 15 °C
NS5A REV (2) PCR Primers	1 x 25 µL (20 µM)	Yellow	-25°C to - 15 °C
NS5A FOR PCR Primers	1 x 40 µL (20 µM)	Yellow	-25°C to - 15 °C
H_2O	1 x 500 µL	Blue	-25°C to - 15 °C
Nested PCR			
Nested Buffer 10X	1 x 65 µL	Green	-25°C to - 15 °C
Nested PCR $MgCl_2$	1 x 35 µL	Black	-25°C to - 15 °C
Nested PCR dNTPs	1 x 35 µL (10 mM)	Brown	-25°C to - 15 °C
Nested PCR Enzyme	1 x 20 µL	White	-25°C to - 15 °C
NS5A REV (1) Nested PCR Primers	1 x 35 µL (10 µM)	Yellow	-25°C to - 15 °C
NS5A REV (2) Nested PCR Primers	1 x 35 µL (10 µM)	Yellow	-25°C to - 15 °C
NS5A FOR Nested PCR Primers	1 x 65 µL (10 µM)	Yellow	-25°C to - 15 °C
NS5A Sequencing FOR Primers	1 x 35 µL (3.2 µM)	Red	-25°C to - 15 °C
NS5A Sequencing REV Primers	1 x 35 µL (3.2 µM)	Red	-25°C to - 15 °C
Nested PCR H_2O	1 x 1000 µL	Blue	-25°C to - 15 °C

*Table 1: Volumes and storage conditions of the **DeepChek® Assay NS5A Genotyping and Drug Resistance (RUO)***

RT-PCR Buffer 2X	RP Solution	RT Enzyme		Nested PCR Buffer 10X	Nested PCR $MgCl_2$	Nested PCR dNTPs
BP Solution	H_2O	PCR Enzyme		Nested PCR H_2O	Nested PCR Enzyme	
NS5A REV (1) PCR Primers	NS5A REV (2) PCR Primers	NS5A FOR PCR Primers		NS5A REV (1) Nested PCR Primers	NS5A REV (2) Nested PCR Primers	NS5A FOR Nested PCR Primers
			NS5A SEQUENCING FOR Primers			
			NS5A SEQUENCING REV Primers			

Picture 1: Mapping of the assay components by color cap

Reagent storage and handling

The ***DeepChek® Assay NS5AD Genotyping and Drug Resistance (RUO)*** is shipped with dry ice and should be maintained and stored immediately upon receipt at –20°C in order to avoid compromising cold chain integrity.

Expiration date: please refer to the label on the kit box.

Materials required but not provided

- Thermocycler ABI9700 or equivalent
- 96-well plate cooler (Eppendorf / 22510509)
- 96-well PCR plates (Eppendorf / 951020303)
- Plates thermo seals (Thermo Scientific / AB-0558)
- Plate centrifuge
- 0.2 mL thin-wall 8 tubes & domed caps (Thermo Scientific / AB-0266)
- 1.5 mL centrifuge tubes (Dot Scientific Inc. / RN1700-GST)
- Centrifuges tubes (see your specific centrifuge manual)
- Mini centrifuge (see your specific centrifuge manual)
- Microliter pipets dedicated to PCR (0.1-2.5 µL; 1-10 or 1-20 µL; 20-200 µL)
- Ice

Note:

- Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.
- Please refer to relevant manufacturer's Instructions for Use (IFU) to proceed with the instrument.

Warnings and precautions

- **For Research Use Only (RUO). Not for use in diagnostic procedures.** No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.
- Laboratories maybe required to report all test results to the appropriate public health authorities.
- Store assay reagents as indicated on their individual labels.
- Do not mix reagents from different kit lots.
- Reagents must be stored and handled as specified in these instructions for use. Do not use reagents past expiration date.
- Work surfaces and pipettes should be cleaned and decontaminated with cleaning products such as 10% bleach, "DNAZap™" or "RNase AWAY®" to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.
- Dispose of waste in compliance with the local, state, and federal regulations.
- Frequent cleaning of the wells of the PCR instrument plate is recommended to prevent contamination.
- To avoid contamination, use separated and segregated working areas.
- Check whether the PCR reaction tubes are tightly closed before loading on the PCR instrument to prevent contamination of the instrument from leaking tubes.

Starting

- Identify the product.
- Verify the expiration date.
- Verify the latest instruction for use available for the product lot number.
- Verify if the product was used already. If yes, check the remaining tests available.

RNA Extraction

To achieve optimal and sensitive HCV RNA analysis, the best representation of the viral quasispecies, it is recommended to extract **1 mL** of plasma for subsequent cDNA and amplicon generation and elute in the minimum volume required for your preferred extraction kit - MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics) is recommended.

The ***DeepChek® Assay NS5ADR V1 (RUO)*** will work with at least an extraction of 400 µL of plasma or serum, ideally from fresh samples, to be eluted in 100µL (related sensitivity evaluated to 1250 UI/mL).

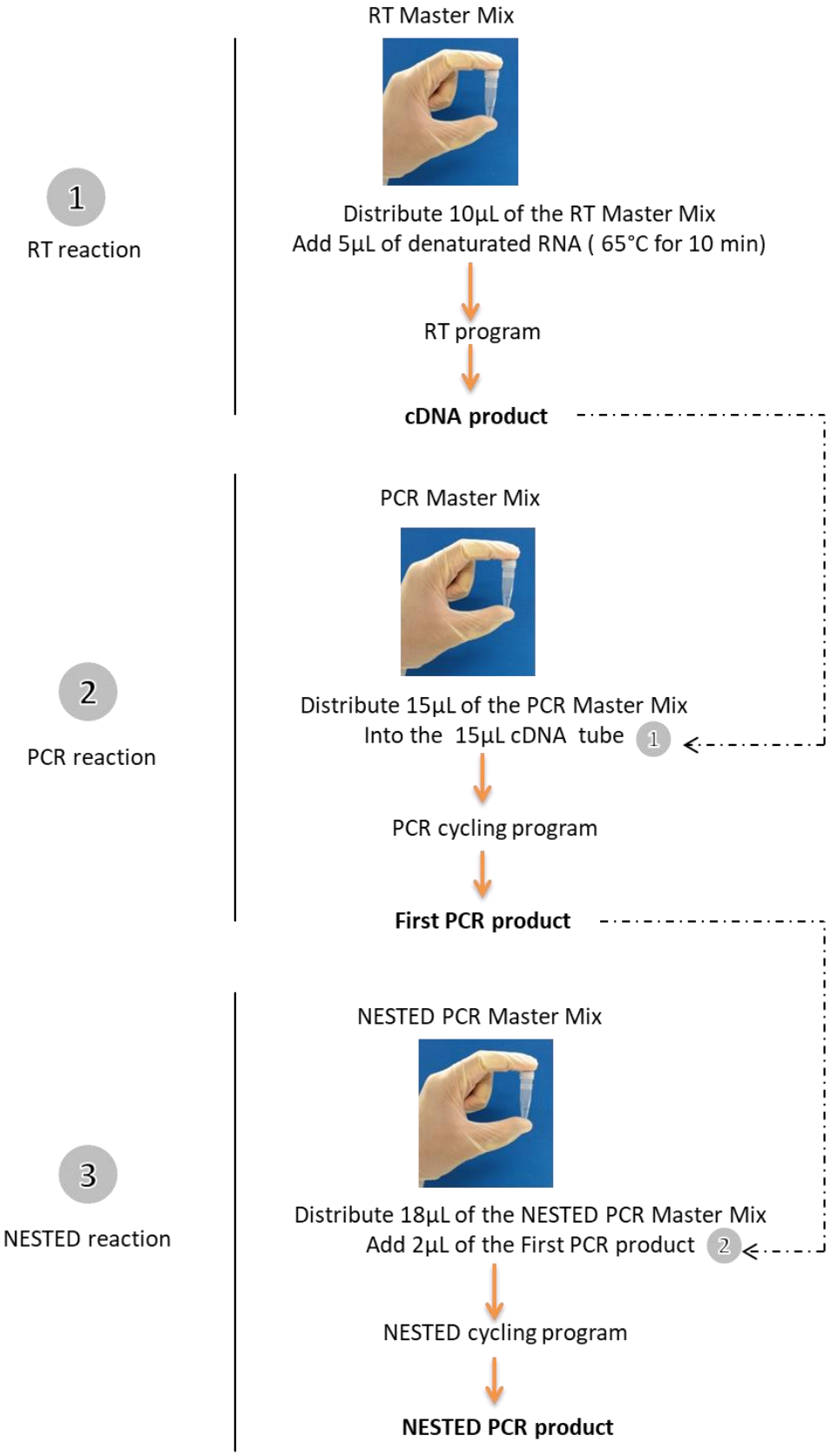
For samples with low viral load, we recommend:

1. To perform an ultracentrifugation procedure. Pellet the virus for 1.5 hours at 40,000 g (or alternatively for 2 hours at 24,000g), and at 4°C. Remove enough supernatant to leave the required amount of sample for your preferred extraction kit.

OR

2. To extract one or 2 mL of plasma/serum and elute in the minimum volume required for your preferred extraction kit.

RT-PCR Workflow Overview



RT and PCR Step-by-Step Workflow for NS5A Target

1. Thaw extracted template RNA, primer solutions, dNTP Mix, 5x Buffer and RNase-free water and place them on ice. Load all the tubes into the centrifuge. Spin the samples at 11000 g for 10 seconds. Then aspirate and discharge the solution several times before the dispensing.
2. Prepare a RT master mix according to **Table 2**. Prepare a volume of RT master mix greater (n+1) than that required for the total number of reactions to be performed.

Reagent	Volume
RT-PCR Buffer 2X	7.5 µL
RP Solution	0.3 µL
BP Solution	0.15 µL
RT Enzyme	0.3 µL
H_2O	1.75 µL

Table 2: Reaction components for RT-PCR NS5A target

3. Vortex the master mix thoroughly and dispense 10 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
4. **Incubate the RNA sample at 65°C for 10 min**. And then add 5µL of RNA to the PCR tubes. Mix by pipetting the master mix up and down a few times.
5. Program the thermal cycler according to the program in **Table 3**.

Temperature (°C)	Time
30	5 min
42	5 min
95	15 sec
4	∞

Table 3: RT NS5A Program

6. Start the RT NS5A Program.
7. During RT NS5A reaction, prepare a PCR master mix according to **Table 4**. Prepare a volume of PCR master mix greater (n+1) than that required for the total number of reactions to be performed.

Reagent	Volume
RT-PCR Buffer 2X	7.5 µL
NS5A REV (1) PCR Primers	0.6 µL
NS5A REV (2) PCR Primers	0.6 µL
NS5A FOR PCR Primers	1.2 µL
PCR Enzyme	0.3 µL
H_2O	4.8 µL

Table 4: Reaction components for RT-PCR NS5A target

8. Vortex the PCR master mix thoroughly and dispense 15 µL into RT product tubes. Mix by pipetting the master mix up and down a few times.
9. Program the thermal cycler according to the program in **Table 5**.

Cycle	Temperature (°C)	Time
1	94	3 min
45 cycles	94 48 (+ 0.3°C / cycle) 72	15 sec 30 sec 1 min
1	4	∞

Table 5: PCR NS5A Program

10. **[Recommended]** - PCR products can be controlled through electrophoresis on an agarose gel. After amplification, samples can be stored overnight at 2–10°C, or at –20°C for long-term storage.

Expected amplicons size: 817 bp

Nested Step-by-Step Workflow for NS5A Target

1. Prepare a NESTED PCR master mix according to **Table 6**. Prepare a volume of master mix greater (n+1) than that required for the total number of reactions to be performed.

Reagent	Volume
NESTED PCR Buffer 10X	2 µl
Nested PCR dNTPs	0.8 µL
Nested PCR $MgCl_2$	1 µL
NS5A REV (1) NESTED PCR Primers	1 µL
NS5A REV (2) NESTED PCR Primers	1 µL
NS5A FOR NESTED PCR Primers	2 µL
NESTED PCR Enzyme	0.5 µL
Nested PCR H_2O	9.7 µL

Table 6: Reaction components for Nested PCR NS5A target

2. Vortex the NESTED PCR master mix thoroughly and dispense 18 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
3. Add 2µL of PCR product (first PCR) to the PCR tubes. Mix by pipetting the master mix up and down a few times.
4. Program the thermal cycler according to the program in **Table 7**.

Cycle	Temperature (°C)	Time
1	95	15 min
35 cycles	94 55 72	30 sec 30 sec 1 min
1	**72**	**10 min**
1	4	∞

Table 7: Nested PCR NS5A Program

5. **[Recommended]** – NESTED PCR products can be controlled through electrophoresis on an agarose gel. After amplification, samples can be stored overnight at 2–10°C, or at –20°C for long-term storage.

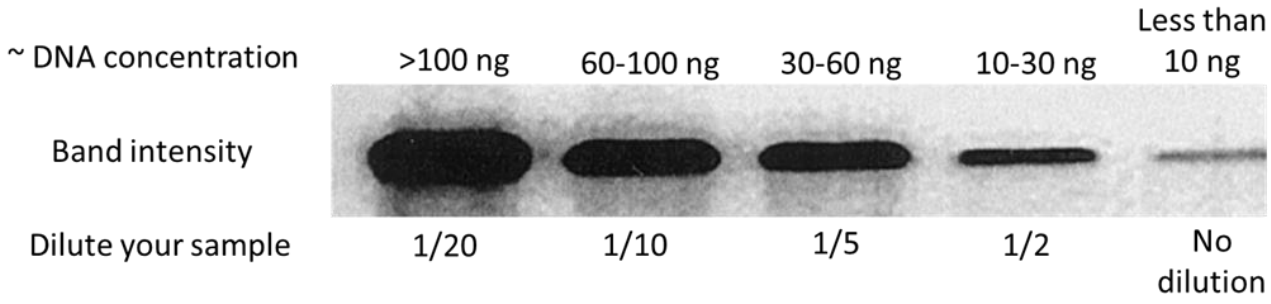
Expected amplicons size: 707 bp

RT-PCR Troubleshooting Guide

1. Check the concentration, storage conditions and quality of the starting template. For optimal results use fresh/frozen plasma/serum and proceed with fresh RNA extraction.

2. For samples with low viral load, we recommend to perform an ultracentrifugation procedure. Pellet the virus for 1.5 hours at 40,000 g and at 4°C. Remove enough supernatant to leave the required amount of sample for your preferred extraction kit.

3. In presence of very large PCR bands on the agarose gel, dilute (1/101 - 1/103) of the product before sequencing.



PCR Products Purification

Before sequencing, first make sure your Nested PCR products have been purified.

Sequencing

1. Purification

For amplicons greater than 500bp, use a 0.6x AMPure XP cleanup (30 µL volume of beads). For amplicons less than 500bp, use a 1.8x AMPure XP cleanup (90 µL volume of beads) to maximize yield.

2. Sanger sequencing

In presence of very large PCR bands on the agarose gel, make dilution ($1/10^1$ - $1/10^3$) of the RT-PCR product before sequencing.

For NS5A sequencing, use the 2 red cap tubes containing 35 µL of each NS5A Sanger sequencing primer (forward and reverse - 3.2 µM). **1 µL** of each primer will be used for each sample.

1. Prepare the sequencing reaction according to the **Table 8a** (Big Dyes Terminator kit v1.1) or **8b** (Big Dyes Terminator kit v3.1).

Reagent for *Forward* Sequencing	**Volume**
Big Dye Terminator v1.1	1 µL
Sequencing Buffer	1 µL
Forward Primer (3,2µM)	**1 µL**
Purified RT-PCR	0.7 – 2 µL
Water	q.s. to 10 µL

Reagent for *Reverse* Sequencing	**Volume**
Big Dye Terminator v1.1	1 µL
Sequencing Buffer	1 µL
Reverse Primer (3,2µM)	**1 µL**
Purified RT-PCR	0.7 – 2 µL
Water	q.s. to 10 µL

Table 8a: Sequencing reaction for Big Dyes Terminator kit v1.1

Reagent for *Forward* Sequencing	**Volume**
Big Dye Terminator v3.1	2 µL
Sequencing Buffer	2 µL
Forward Primer (3,2µM)	**1 µL**
Purified RT-PCR	0.7 – 2 µL
Water	q.s. to 15 µL

Reagent for *Reverse* Sequencing	**Volume**
Big Dye Terminator v3.1	2 µL
Sequencing Buffer	2 µL
Reverse Primer (3,2µM)	**1 µL**
Purified RT-PCR	0.7 – 2 µL
Water	q.s. to 15 µL

Table 8b: Sequencing reaction for Big Dyes Terminator kit v3.1

2. Program the thermal cycler according to the program in **Table 9a** (Big Dyes Terminator kit v1.1) or **9b** (Big Dyes Terminator kit v3.1).

Cycle	**Temperature (°C)**	**Time**
1	96	5 min
25 cycles	96	10 sec
	50	5 sec
	60	4 min
1	4	∞

Table 9a: Thermal cycler for Big Dyes Terminator kit v1.1

Cycle	Temperature (°C)	Time
1	96	1:30 min
25 cycles	96	20 sec
	50	15 sec
	60	1:30 min
1	4	∞

Table 9b: Thermal cycler for Big Dyes Terminator kit v3.1

3. **Sephadex purification** - Complete all the sequencing reaction with water (q.s. to 20µL). Purify all sequencing reaction (20µL) with Sephadex gel before the final Sanger sequencing.

OR

3. **PCR Cleanup reagent**

OR

3. **Ethanol purification**
   a) Add 4ul of EDTA 125mM and sodium acetate 3M (1:1) solution;
   b) Add 50ul Ethanol 100 %;
   c) Seal the plate well and gently vortex;
   d) Incubate at room temperature (protected from light) for 15 minutes; While the plate is incubate, change the temperature of the centrifuge to 4ºC;
   e) Centrifuge the plate at 4ºC for 35 minutes, at 4000 rpm;
   f) Remove the plate immediately, remove the sealing, and place the plate face down on a paper towel.
   g) Briefly centrifuge the place face down until 185 rpm;
   h) Add 55ul of Ethanol 70 %;
   i) Seal the plate well and vortex for 15 seconds;
   j) Centrifuge the plate at 4ºC for 15 minutes, at 4000 rpm;
   k) Remove the plate immediately, remove the sealing, and place the plate face down on a paper towel;
   l) Centrifuge the place face down for 1 minute at 4000 rpm;
   m) Place the plate at 94ºC for maximum 1 minute.

4. **Denaturation:** Add 10 µL of formamide and incubate at the thermocycle at 94°C for 5 minutes then immediately incubate the plate at 4°C for thermal shock for at least 5 minutes.

3. NGS

After the amplicon verification, the samples are ready for the NGS kit processing:

Through Illumina MiSeq

- **116AX or 116BX | DeepChek® NGS LIBRARY PREPARATION (24 / 48 or 96 reactions).**
- MS-103-1003 | MiSeq Reagent Nano kit, v2 (500 cycles).

Through Ion Torrent

- **4471269** | Ion Xpress™ Plus Fragment Library Kit
- **4471250** | Ion Xpress™ Barcode Adapters 1-16 Kit
- **4484355** | Ion 318™ Chip Kit v2

Data Analysis

1. Sanger

AB1 or FASTA files containing nucleotide sequences for NS5A are analyzed by the **DeepChek®** software. User shall then follow the DeepChek software procedure to complete the data analysis and reporting processes.

2. NGS

NGS files containing nucleotide sequences for NS5A are analyzed by the **DeepChek®** software. User shall then follow the DeepChek software procedure to complete the data analysis and reporting processes.

Product quality control

In accordance with ABL's Quality Management System, each lot of the assay is tested against predetermined specifications to ensure consistent product quality. Certificates of Analysis are available upon request.

Symbols

 <N>	Contains reagents enough for <N> reactions		Consult instructions for use
	Caution		Temperature limitation
	Catalog number		Serial Number
	Use by	**R*n***	R is for revision of the Instructions for Use (IFU) and n is the revision number
	Manufacturer		Country of manufacturing
	Distributor		

Contact Information

For technical assistance and more information, please see our Technical Support Center at Online: https://ablsa.odoo.com/fr_FR/issue; Email: support-diag@ablsa.com; Call +339 7017 0300 Or contact your ABL Field-Application Specialist or your local distributor. For up-to-date licensing information or product-specific disclaimers, see the respective ABL Assay User Guide. ABL User Guides are available at **www.ablsa.com/ifu** or can be requested from ABL Technical Services or your local distributor.

Manufacturer

Advanced Biological Laboratories (ABL) S.A.

52-54 Avenue du X Septembre, L-2550 Luxembourg, Luxembourg

The customer is responsible for compliance with regulatory requirements that pertain to their procedures and uses of the instrument. The information in this guide is subject to change without notice. DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, ABL (S.A) AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Version 1.2

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