DeepChek® Assay

NS5B / 5'UTR Genotyping (RUO)

V3

User Guide

24

Version 1 – Revision 1

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.

REF 110C24 (old reference: K-19-NS5B5UTR V1)

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Document control

Date	Device version	IFU version	Description of change
28/03/2022	C	1.1	• Change of the analytical sensitivity to 1000 UI/mL in RNA extraction section • Update the list of next generation sequencing instruments and reagents

Contents

Application

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The DeepChek® Assay NS5B/5UTR genotyping and Drug Resistance (DR) (RUO) kit is a single tube system which utilizes PCR technology for amplifying relevant portions of the human C virus (HCV) NS5B/5UTR 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 NS5B/5UTR 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 NS5B/5UTR 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.

DeepChek® Assay NS5B/5UTR 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 NS5B/5'UTR V1 (RUO)*** is provided in one model of 24 reactions (REF 110C24 / OLD REF K-19-NS5B5UTR V1).

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 25 µL	Purple	-25°C to - 15 °C
BP Solution	1 x 5 µL	Pink	-25°C to - 15 °C
RT Enzyme	1 x 10 µL	White	-25°C to - 15 °C
PCR Enzyme	1 x 10 µL	White	-25°C to - 15 °C
NS5B-1F Primers	1 x 40 µL	Yellow	-25°C to - 15 °C
NS5B-1R Primers	1 x 20 µL	Yellow	-25°C to - 15 °C
NS5B-2R Primers	1 x 20 µL	Yellow	-25°C to - 15 °C
5'UTR FOR Primers	1 x 35 µL	Yellow	-25°C to - 15 °C
5'UTR REV Primers	1 x 35 µL	Yellow	-25°C to - 15 °C
H_2O	1 x 1000 µL	Blue	-25°C to - 15 °C
Nested PCR			
Nested PCR Buffer 10X	1 x 130 µL	Green	-25°C to - 15 °C
Nested $MgCl_2$	1 x 65 µL	Black	-25°C to - 15 °C
Nested dNTPs	1 x 55 µL	Brown	-25°C to - 15 °C
Nested H_2O	1 x 1000 µL	Blue	-25°C to - 15 °C
Nested PCR Enzyme	1 x 16 µL	White	-25°C to - 15 °C
NS5B FOR Nested PCR Primers	1 x 65 µL	Yellow	25°C to - 15 °C
NS5B REV Nested PCR Primers	1 x 65 µL	Yellow	25°C to - 15 °C
NS5B SEQUENCING FOR Primers	1 x 35 µL	Red	25°C to - 15 °C
NS5B SEQUENCING REV Primers	1 x 35 µL	Red	25°C to - 15 °C
5'UTR SEQUENCING FOR Primers	1 x 35 µL	Red	25°C to - 15 °C
5'UTR SEQUENCING REV Primers	1 x 35 µL	Red	25°C to - 15 °C
Nested H_2O	1 x 1000 µL	Red	-25°C to - 15 °C

*Table 1: Volumes and storage conditions of the **DeepChek® Assay NS5B/5'UTR V3 (RUO)***

Reagent storage and handling

The ***DeepChek® Assay NS5B / 5'UTR V3 (RUO)*** is shipped with dry ice and should be maintained and stored immediately upon receipt at –20°C to avoid compromising cold chain integrity.

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

Materials required but not provided

- Thermocycler ABI9700
- 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 NS5B / 5'UTR V3 (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 1000 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 and PCR Step-by-Step Workflow for NS5B/5'UTR GT target

1. Thaw extracted template RNA, primer solutions, 2X RT & PCR Buffer, RP Solution and RNase-free water and place them on ice. Load all the tubes into the centrifuge. Spin the samples at 11000 g during 10 seconds. Then aspirate and discharge the solution several times before the dispensing.
2. Prepare the 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 for 1 sample
RT-PCR Buffer 2X	7.5 µL
RP Solution	0.75 µL
BP Solution	0.15 µL
RT Enzyme	0.3 µL
Water	1.30 µL
Final volume	**10 µL**

Table 2: Reaction components for RT NS5B GT

3. Vortex the master mix thoroughly, spin down and dispense 10 µL into PCR tubes. Keep the master miw on ice.
4. **Incubate the RNA sample at 65°C for 10 min**. And then add 5µL of RNA to each. 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 NS5B/5UTR GT Program

6. Start the RT NS5B/5UTR GT Program.
7. During RT NS5B/5UTR GT reaction, prepare the PCR master mix on ice 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 for 1 sample
RT-PCR Buffer 2X	7.5 µL
PCR Enzyme	0.3 µL
RNase-free Water	3.8 µL
5'UTR FOR Primers	0.5 µL
5'UTR REV Primers	0.5 µL
NS5B-1F	1.2 µL
NS5B-1R	0.6 µL
NS5B-2R	0.6 µL
Final volume	**15 µL**

Table 4: Reaction components for PCR NS5B GT

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 55 72	15 sec 30 sec 1 min
1	4	∞

Table 5: PCR NS5B GT Program

10. **[Recommended]** - PCR products can be controlled through electrophoresis on a 1% agarose gel. After amplification, samples can be stored overnight at 2–10°C, or at –20°C for long-term storage.

Expected amplicons size: NS5B (1048 bp) & 5'UTR (244 bp).

Nested PCR Step-by-Step Workflow for NS5B (optional)

1. Thaw the PCR product, primer solutions, dNTP Mix, MgCl2 and 10x Buffer and place them on ice. Load all the tubes into the centrifuge. Spin the samples at 11000 g for 10 seconds. And then aspirate and discharge the solution several times before the dispensing.
2. Prepare Nested PCR NS5B GT master mix according to **Table 6**. The master mix typically contains all the components required for nested PCR except the RT-PCR product. Prepare a volume of master mix greater (n+1) than that required for the total number of reactions to be performed.

Reagent	Volume for 1 sample
Buffer 10X	4 µL
dNTP 10 mM	1.6 µL
$MgCl_2$	2 µL
NESTED FOR primers	2 µL
NESTED REV primers	2 µL
Enzyme Mix Nested PCR	0.5 µL
Water	23.9 µL
Final volume	**36 µL**

Table 6: Reaction components for the NS5B Nested PCR target

3. Vortex the master mix thoroughly and dispense 36 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
4. **Make a dilution (1/10) of the first RT-PCR** and add 4 µL of this dilution in the nested 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 7**.

Cycle	Temperature (°C)	Time
Enzyme activation	95	15 min
35 cycles	94 55 72	30 sec 30 sec 1 min
Final extension	72	10 min
-	10	∞

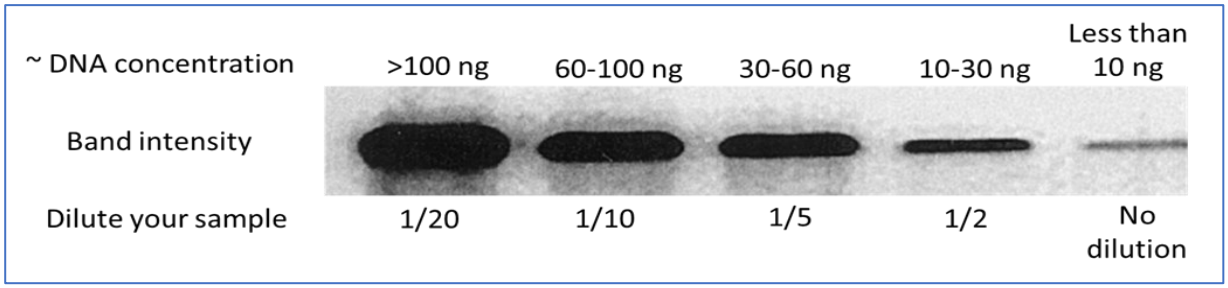
Table 7: NS5B GT Nested PCR Cycling Program

6. **[Recommended]** – PCR products can be controlled through electrophoresis on a 1% agarose gel. After amplification, samples can be stored overnight at 2–10°C, or at –20°C for long-term storage.

Expected amplicons size: NS5B GT Nested (693 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 performing 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 PCR products have been purified. In presence of very large PCR bands on the agarose gel, dilute (1/101 - 1/103) of the PCR product before sequencing.

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. Or Use PCR Clean Up.

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 29µL 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* Sequening	**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* Sequening	**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

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

Reagent for *Forward* Sequening	**Volume**
Big Dye Terminator v3.1	3 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* Sequening	**Volume**
Big Dye Terminator v3.1	3 µL
Sequencing Buffer	2 µL
Forward 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 50 60	10 sec 5 sec 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 50 60	20 sec 15 sec 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 incubated, change the temperature of the centrifuge to 4ºC;
e) Centrifuge the plate at 4ºC for 35 minutes, at 4000 x g;
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 x g;
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 x g;
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 x g;
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 ar 4°C for thermal shock for at least 5 minutes.

After these steps you can place the plate at sequencer and start run.

3. NGS

The main volume of the product output is then used for next generation sequencing (NGS). The NGS workflow can be different as the NGS technics and NGS analyzers vary. NGS sequencing instruments are general laboratory use devices. Sequencing and library preparations reagents are general laboratory use products.

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

Through Illumina (MiSeq / iSeq 100)

- **116BX** | DeepChek® NGS Library preparation V2 (24 or 48 or 96 tests)
- **124BX** | DeepChek® Assay Adapters V2 (1-24, 1-48 or 1-96)
- **MS-103-1003** | MiSeq Reagent Nano Kit, v2 (500 cycles)
- **MS-102-3003**| MiSeq Reagent Kit, v3 (600 cycles)
- **20021532** | iSeq 100 Sequencing System
- **20021533** | iSeq 100 Reagent (300 cycles)

Note: If you are using MiSeq Reagent Nano Kit, v2 (500 cycles) or MiSeq Reagent Kit, v3 (600 cycles), no need to perform the fragmentation step with the DeepChek® NGS Library preparation (**116AX**).

Through Thermo Fisher Scientific (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 NS5BGT 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 NS5BGT 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		Negative control
	Catalog number		Positive control
	Use by		Temperature limitation
	Manufacturer		Serial Number
	Country of manufacture with a date of manufacture	**R*n***	R is for revision of the Instructions for Use (IFU) and n is the revision number
	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.

17 rue des Jardiniers, L-1835 Luxembourg, Luxembourg

The customer is responsible for compliance with regulatory requirements that pertain to their procedures and uses of the device. 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 1.1

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