DeepChek® Assay

NS5A (GT2) Drug Resistance

V1

24

User Guide

Version 1 – Revision 0

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 **106A24 (old reference: K-17-NS5ADR-GT2)**

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Contents

Application

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The ***DeepChek® Assay NS5A (GT2) Drug Resistance (RUO)*** kit is a single tube system which utilizes PCR technology for amplifying relevant portions of the human hepatitis C virus (HCV) for genotype 2 (GT2) NS5A gene from input RNA extracted from plasma/serum.

This nucleic acid amplification method might aid in the drug resistance. 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 (GT2) 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 (GT2) 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 (GT2) 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 drug resistance according to available public reference knowledge databases.

Assay components

The ***DeepChek® Assay NS5A (GT2) Drug Resistance V1 (RUO)*** is provided in one model of 24 reactions (REF 106A24 / OLD REF K-17-NS5ADR-GT2).

Label	Volume for 24 Rxn. (nb tube x volume)	Color cap	Storage
RT & PCR Buffer 2X	1 x 435 µL	Green	-25°C to - 15 °C
RP Solution	1 x 22 µL	Purple	-25°C to - 15 °C
RT Enzyme	1 x 9 µL	Clear	-25°C to - 15 °C
BP Solution	1 x 4.5 µL	Pink	-25°C to - 15 °C
H_2O	1 x 500 µL	Blue	-25°C to - 15 °C
PCR Enzyme	1 x 9 µL	Clear	-25°C to - 15 °C
NS5A GT2 FOR PCR Primers	1 x 17.5 µL	Yellow	-25°C to - 15 °C
NS5A-GT2 REV PCR Primers	1 x 17.5 µL	Yellow	-25°C to - 15 °C
NS5A GT2 SEQUENCING FOR Primers	1 x 29 µL	Red	-25°C to - 15 °C
NS5A GT2 SEQUENCING REV Primers	1 x 29 µL	Red	-25°C to - 15 °C

Table 1: Volumes and storage conditions of the **DeepChek® Assay NS5A (GT2) Drug Resistance V1 (RUO)**

Reagent storage and handling

The ***DeepChek® Assay NS5AD (GT2) 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

Instruments

The following thermal cycler, ThermoFisher Scientific ProFlex™ PCR System (model 3 x 32-well (Catalog #4484073) or 96-well (Catalog #4484075) and associated specific material), is validated for use with this test.

Note: Any laboratory validated thermal cycler with enough ramp rate of ≥ 1°C/s shall be sufficient.

Master mix preparation

- Benchtop centrifuge with rotor for 0.5 mL/1.5 mL reaction tubes (capable of attaining 10,000 rpm).
- Benchtop vortex mixer.
- Microliter pipets dedicated to PCR (0.1-2.5 µL; 1-10 or 1-20 µL; 20-200 µL; 1000 µL).
- Pipetting Robot (optional).
- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters.
- Adjustable pipettes & fitting filtered pipette tips.
- Appropriate PPE & workspaces for working with potentially infectious samples.
- Surface decontaminants such as DNAZap (Life Technologies), DNA Away (Thermo Fisher Scientific), RNAse Away (Thermo Fisher Scientific), 10% bleach.
- Nuclease-free dH2O.
- 0.5 ml or 1.5 ml RNase- and DNase-free PCR tubes.
- Ice/Icebox or even cooling blocks.
- 96 well plate cooler (optional).
- 96 well PCR plates.
- Plate thermo seals.
- Plate centrifuge.
- 0.2 mL thin walled 8 tube & domed cap.

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

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 NS5A (GT2) Drug Resistance (RUO)*** will work with at least an extraction of 400 µL of plasma or serum, ideally from fresh samples, to be eluted in 100 µL (related sensitivity evaluated to 1250 UI/mL).

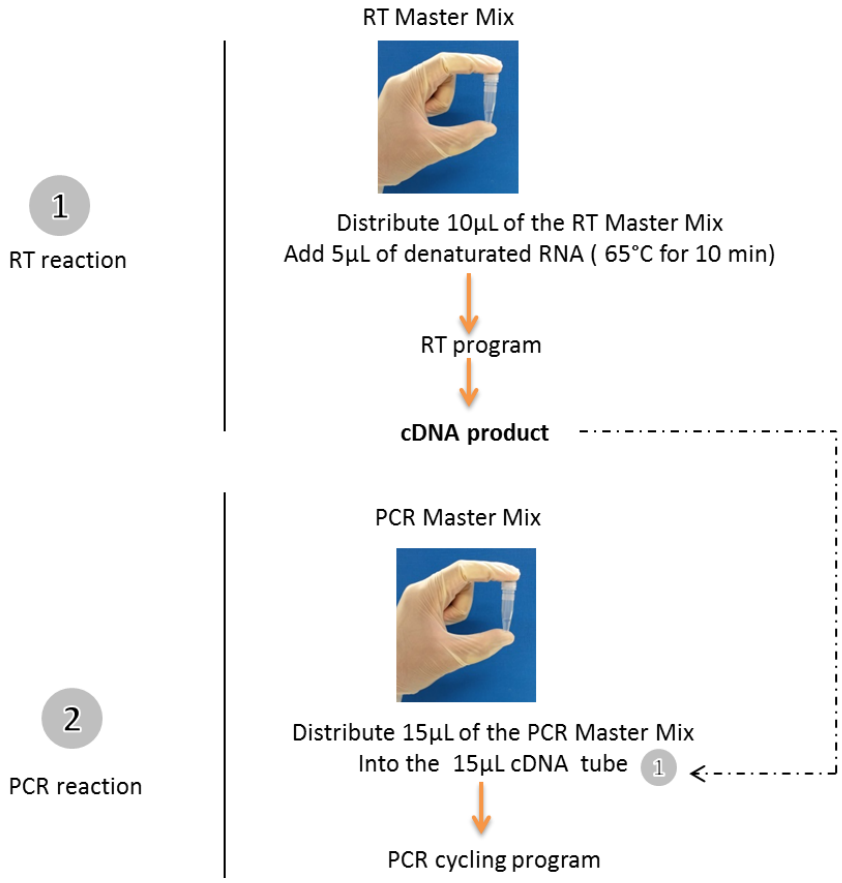
For samples with low viral load, we recommend:

1. To perform an ultracentrifugation procedure. Pellet the virus for 1.5 hours at 40,000 *g* (or alternatively for 2 hours at 24,000 *g*), 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 mix the solution by aspirating and discharging 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 / Reaction
RT & PCR Buffer 2X	7.5 µL
RP Solution	0.75 µL
BP Solution	0.15 µL
RT Enzyme	0.3 µL
H_2O	1.3 µL
Final volume	**10 µL**

Table 2: Reaction components for NS5A (GT2) targets RT

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 5 µL 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.

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

Table 3: Reverse transcription NS5A (GT2) cycling program

6. Start the RT NS5A Program.
7. During RT NS5A reaction, prepare a PCR master mix according to Table 44. Prepare a volume of PCR master mix greater (n+1) than that required for the total number of reactions to be performed.

Reagent	Volume / Reaction
RT & PCR Buffer 2X	7.5 µL
NS5A-GT2 FOR PCR Primers	0.6 µL
NS5A-GT2 REV PCR Primers	0.6 µL
PCR Enzyme	0.3 µL
H_2O	6 µL
Final volume	15 µL

Table 4: Reaction components for NS5A (GT2) targets PCR

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
Enzyme activation	94	3 min
45 cycles	94	15 sec
	61	30 sec
	72	1 min
	4	∞

Table 5: PCR NS5A (GT2) cycling program

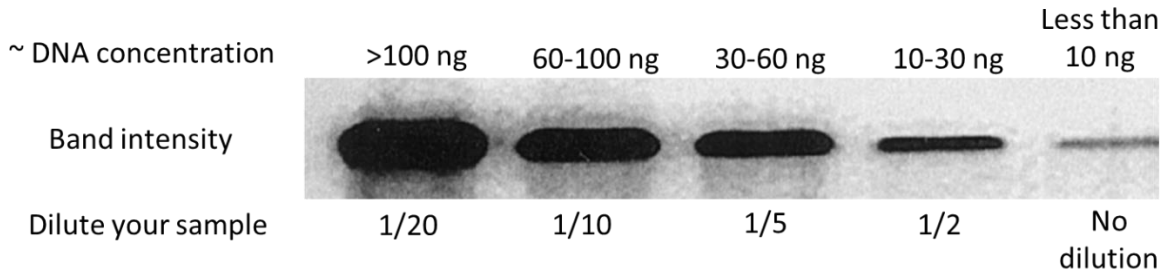
10. **[Recommended]** - PCR products can be controlled through electrophoresis on an agarose gel. After amplification, samples can be stored overnight at 2–10°C, or at –20°C for long-term storage.

Expected amplicons size: 815 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 ($1/10^1$ - $1/10^3$) of the product before sequencing.



PCR Products Purification

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

Sequencing

1. Purification

For amplicons greater than 500 bp, use a 0.6x AMPure XP cleanup (30 µL volume of beads). For amplicons less than 500 bp, 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 6 (Big Dyes Terminator kit v1.1) or Table 7 (Big Dyes Terminator kit v3.1).

Reagent for *Forward* Sequencing	**Volume**		**Reagent for *Reverse* Sequencing**	**Volume**
Big Dye Terminator v1.1	1 µL		Big Dye Terminator v1.1	1 µL
Sequencing Buffer	1 µL		Sequencing Buffer	1 µL
Forward Primer (3,2µM)	1 µL		Forward Primer (3,2µM)	1 µL
Purified RT-PCR	0.7 – 2 µL		Purified RT-PCR	0.7 – 2 µL
H_2O	q.s. to 10 µL		H_2O	q.s. to 10 µL

Table 6: Sequencing reaction for Big Dyes Terminator kit v1.1

Reagent for *Forward* Sequencing	**Volume**		**Reagent for *Reverse* Sequencing**	**Volume**
Big Dye Terminator v3.1	2 µL		Big Dye Terminator v3.1	2 µL
Sequencing Buffer	2 µL		Sequencing Buffer	2 µL
Forward Primer (3,2µM)	1 µL		Forward Primer (3,2µM)	1 µL
Purified RT-PCR	0.7 – 2 µL		Purified RT-PCR	0.7 – 2 µL
H_2O	q.s. to 15 µL		H_2O	q.s. to 15 µL

Table 7: Sequencing reaction for Big Dyes Terminator kit v3.1

2. Program the thermal cycler according to the program in Table 8 (Big Dyes Terminator kit v1.1) or Table 9 (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 8: 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 9: 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 (20 µL) with Sephadex gel before the final Sanger sequencing.

OR

PCR Cleanup reagent

OR

Ethanol purification

a) Add 4 µL of EDTA 125 mM and sodium acetate 3M (1:1) solution
b) Add 50 µL 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 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 55 µL of Ethanol 70 %
i) Seal the plate well and vortex for 15 second
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

- 116B24 / 116B48 / 116B96 | ABL DeepChek® NGS LIBRARY PREPARATION V2 (24/48/96 reactions).
- 124B24 / 124B48 / 124B96|ABL DeepChek® Adapters V2 (24 / 48 / 96).
- MS-103-1003 |MiSeq Reagent Nano Kit, v2 (500 cycles) or
- FC-420-1003 | Mid Output kit Reagents (2x150) or
- 20021533 | iSeq 100 i1 Reagent (2x150) or
- 20024908 | NextSeq 500/550 High Output Kit v2.5 (300 Cycles).User shall then follow the Denature and Dilute Libraries Guide and instructions for use from the manufacturer

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 analysed 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 analysed 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: support-diag.ablsa.com; 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 via support-diag.ablsa.com or can be requested from ABL Technical Services or your local distributor.

Manufacturer and distributors

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AdvancedDx Biological Laboratories USA Inc.
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Version 1.0

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