DeepChek® RT-PCR and Sequencing

24x NS5B tests/ 24x 5UTR tests

K-19-NS5B5UTRv1

Technical Laboratory Protocol

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A. Reagents, Materials and Equipment

	Reagent / Material / Equipment	Quantity	Vendor	Cat. number
	DeepChek® RT-PCR and Sequencing NS5B/5UTR		**ABL SA (included in the Assay)**	**K-18-NS5B-GT-BackUp**
RT & PCR	2X RT & PCR Buffer	1 tube (2x)	ABL SA (included in the Assay)	---
RT & PCR	RT Enzyme	1 tube (5U/L)	ABL SA (included in the Assay)	---
RT & PCR	PCR Enzyme	1 tube (5U/L)	ABL SA (included in the Assay)	---
RT & PCR	RNase-free Water	1 tube	ABL SA (included in the Assay)	---
RT & PCR	BP Solution	1 tube (10mg/mL)	ABL SA (included in the Assay)	---
RT & PCR	RP Solution	1 tube (50µM)	ABL SA (included in the Assay)	---
	5'UTR FOR Primers	**1 tube (10µM)**	**ABL SA (included in the Assay)**	---
	5'UTR FOR Primers	**1 tube (10µM)**	**ABL SA (included in the Assay)**	---
	NS5B-1F Primers	**1 tube (20µM)**	**ABL SA (included in the Assay)**	---
	NS5B-1R Primers	**1 tube (20µM)**	**ABL SA (included in the Assay)**	---
	NS5B-2R Primers	**1 tube (20µM)**	**ABL SA (included in the Assay)**	---
Nested PCR	Nested PCR Buffer 10X	1 tube (10X)	ABL SA (included in the Assay)	---
Nested PCR	MgCl2	1 tube	ABL SA (included in the Assay)	---
Nested PCR	dNTPs	1 tube (10mM)	ABL SA (included in the Assay)	---
Nested PCR	Water	1 tube	ABL SA (included in the Assay)	---
Nested PCR	Nested PCR Enzyme	1 tube	ABL SA (included in the Assay)	---
Nested PCR	**FOR NESTED PCR Primers**	**1 tube (10µM)**	**ABL SA (included in the Assay)**	---
Nested PCR	**REV NESTED PCR Primers**	**1 tube (10µM)**	**ABL SA (included in the Assay)**	---
	5'UTR SEQUENCING FOR Primers	1 tube (3.2µM)	ABL SA (included in the Assay)	---
	5'UTR SEQUENCING REV Primers	1 tube (3.2µM)	ABL SA (included in the Assay)	---
	NS5B SEQUENCING FOR Primers	1 tube (3.2µM)	ABL SA (included in the Assay)	---
	NS5B SEQUENCING REV Primers	1 tube (3.2µM)	ABL SA (included in the Assay)	---
	Thermocycler	---	ABI9700	---
	96 well plate cooler	---	Eppendorf	22510509
	96-well PCR plates	---	Eppendorf	951020303
	Adhesive Plate seals	---	Thermo Scientific	AB-0558
	Plate centrifuge	---	Many	---
	0.2 mL thin-walled 8 tube & domed cap	---	Thermo Scientific	AB-0266
	1.7 ml centrifuge tubes	---	Dot Scientific Inc	RN1700-GST
	Centrifuge tubes	---	See your specific centrifuge manual	---
	Mini Centrifuge	---	See your specific centrifuge manual	---
	Pipette 0.1-2.5 µL	---	Many possible	---
	Pipette 1-10 or 1-20 µl	---	Many	---
	Pipette 20-200 µl	---	Many	---
	Ice	---	---	---

B. Storage Conditions

The DeepChek® RT-PCR and Sequencing Assay 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.

C. RNA Extraction

To achieve optimal and sensitive HCV RNA analysis, the best representation of the viral quasispecies, it is recommended to extract **one 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® RT-PCR and Sequencing will work with at least an extraction of 400µL of plasma or serum, ideally from fresh samples, eluted in 100µL (related sensitivity evaluated to 1250 UI/mL).

For samples with low viral load, we recommend to perform an ultracentrifugation procedure. Pellet the virus for 1.5 hours at 40,000 x g (or alternatively for 2 hours at 24,000 x g) at 4°C. Remove supernatant leaving the required amount of sample for your preferred extraction kit.
OR
- To extract one or two mL of plasma/serum and elute in the minimum volume required for your preferred extraction kit.

D. RT and PCR Step-by-Step Workflow for NS5B/5'UTR GT target

STEP 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 x g during 10 seconds. Then aspirate and discharge the solution several times before the dispensing.

STEP 2 - Prepare the RT master mix on ice according to **Table 1**. Prepare a volume of RT master mix greater (n+1) than that required for the total number of reactions to be performed.

Table 1. Reaction components for RT NS5B GT

Reagent	Volume for 1 sample
2X RT & PCR Buffer	7.50 µL
RP Solution	0.75 µL
BP Solution	0.15 µL
RT Enzyme	0.30 µL
RNase-free Water	1.30 µL
Final Volume	**10µL**

STEP 3 - Vortex the master mix thoroughly, spin down and dispense 10 µL into PCR tubes. Keep the master mix on ice.

STEP 4 – **Incubate the RNA sample at 65°C for 10 min**. And then add 5µL of RNA to each tubes. Mix by pipetting the master mix up and down a few times.

STEP 5 - Program the thermal cycler according to the program in **Table 2**.

Table 2. RT NS5B/5UTR GT Program

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

STEP 6 - Start the RT NS5B/5UTR GT program.

STEP 7 – During RT NS5B/5UTR GT reaction, prepare the PCR master mix on ice according to **Table 3.** Prepare a volume of PCR master mix greater (n+1) than that required for the total number of reactions to be performed.

Table 3. Reaction components for PCR NS5B GT

Reagent	Volume for 1 sample
2X RT & PCR Buffer	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	**15µL**

STEP 8 - Vortex the PCR master mix thoroughly, spin down and dispense 16 µL into RT product tubes. Mix by pipetting the master mix up and down a few times.

STEP 9 - Program the thermal cycler according to the program in **Table 4**.

Table 4. PCR NS5B GT Program

	Time	Temperature
	3 min	94°C
x 45 cycles	15 sec	94°C
	30 sec	55°C
	1 min	72°C
	∞	4°C

STEP 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**

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

STEP 1 - Thaw the PCR product, primer solutions, dNTP Mix, $MgCl_2$ and 10x Buffer and place them on ice. Load all the tubes into the centrifuge. Spin the samples at 11000 g during 10 seconds. And then aspirate and discharge the solution several times before the dispensing.

STEP 2 - Prepare Nested PCR NS5B GT master mix according to **Table 3**. The master mix typically contains all the components required for nested PCR except the SingleRound RT-PCR product. Prepare a volume of master mix greater (n+1) than that required for the total number of reactions to be performed.

Table 3. Reaction components for the Integrase Nested PCR target.

Nested PCR 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**

STEP 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.

STEP 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.

STEP 5 - Program the thermal cycler according to the program in **Table 4**.

Table 4. NS5B GT Nested PCR Cycling Program

	Time	**Temperature**
Enzyme activation	15 min	95°C
x 35 cycles	30 sec	94°C
	30 sec	55°C
	1 min	72°C
Final extension	10 min	72°C
	∞	10°C

STEP 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.

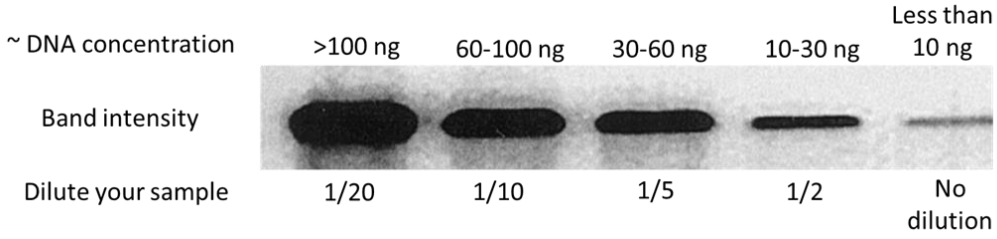
Expected amplicons size:
NS5B GT Nested :693 bp

F. RT-PCR Troubleshooting Guide

a) 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.

b) 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.

c) In presence of very large Nested PCR bands on the agarose gel, make dilution of the product before sequencing.



G. 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.

H. 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 NS5B sequencing, use the 2 red cap tubes containing 58µL of each NS5B Sanger sequencing primer (forward and reverse - 3.2 µM). **One µL** of each primer will be used for each sample.

STEP 1 - Prepare the sequencing reaction according to the **Table 5a (Big Dyes Terminator kit v1.1) or 5b (Big Dyes Terminator kit v3.1)**.

Table 5a. Sequencing reaction for Big Dyes Terminator kit v1.1

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

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

Table 5b. Sequencing reaction for Big Dyes Terminator kit v3.1

Reagent Volume for ***Forward*** Sequencing	Volume
Big Dye Terminator v3.1	3µL
Sequencing Buffer (5x)	2µL
Forward Primer **(3,2µM)**	**1µL**
Purified RT-PCR	0.7 - 2µL
Water	q.s. to 15µL

Reagent Volume for ***Reverse*** Sequencing	Volume
Big Dye Terminator v3.1	3µL
Sequencing Buffer (5x)	2µL
Reverse Primer **(3,2µM)**	**1µL**
Purified RT-PCR	0.7 - 2µL
Water	q.s. to 15µL

STEP 2 - Program the thermal cycler according to the program in **Table 6a (Big Dyes Terminator kit v1.1) or 6b (Big Dyes Terminator kit v3.1).**

Table 6a. Thermal cycler for Big Dyes Terminator kit v1.1

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

Table 6b. Thermal cycler for Big Dyes Terminator kit v3.1

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

STEP 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

STEP 3 - BigDye XTerminator® Purification Kit

OR

STEP 3 - Ethanol purification

1) Add 4ul of EDTA 125mM and sodium acetate 3M (1:1) solution;
2) Add 50ul Ethanol 100%;
3) Seal the plate well and gently vortex;
4) Incubate at room temperature (protected from light) for 15 minutes; While the plate is incubate, change the temperature of the centrifuge to 4ºC;
5) Centrifuge the plate at 4ºC for 35 minutes, at 4000 x g;
6) Remove the plate immediately, remove the sealing, and place the plate face down on a paper towel.
7) Briefly centrifuge the place face down until 185 x g;
8) Add 55ul of Ethanol 70%;
9) Seal the plate well and vortex for 15 seconds;
10) Centrifuge the plate at 4ºC for 15 minutes, at 4000 x g;
11) Remove the plate immediately, remove the sealing, and place the plate face down on a paper towel;
12) Centrifuge the place face down for 1 minute at 4000 x g;
13) Place the plate at 94ºC for maximum 1 minute;

STEP 4 - Denaturation:

1) Add 10ul of formamide and incubate at the thermocycle at 94ºC for 5 minutes;
2) Immediately incubate the plate at 4ºC for thermal shock for at least 5 minutes;

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

3. NGS

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

Through Illumina MiSeq

- **K-17-NGS-LP1** | DeepChek® NGS Library preparation (24 or 96 samples)

- **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

J. Data analysis

1. Sanger

FASTA or AB1 files 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.