DeepChek® RT-PCR and Sequencing

NS3 Drug Resistance Assay (K-16-NS3DR)

24x NS3 tests

Technical Laboratory Protocol

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A. Reagents, Materials and Equipment

	Reagent / Material / Equipment	Quantity	Vendor	Cat. Number
	DeepChek® RT-PCR and Sequencing NS3DR		**ABL SA (included in the Assay)**	**K-16-NS3DR**
RT & PCR reaction	NS3 FOR1 PCR Primers	1 tube (Clear Cap / 20µM) – 18µL	ABL SA (included in the Assay)	---
RT & PCR reaction	NS3 FOR2 PCR Primers	1 tube (Clear Cap / 20µM) – 18µL	ABL SA (included in the Assay)	---
RT & PCR reaction	NS3 REV PCR Primers	1 tube (Clear Cap / 20µM) – 35µL	ABL SA (included in the Assay)	---
RT & PCR reaction	RT & PCR Buffer 2X	1 tube (Clear Cap / 2x) – 435µL	ABL SA (included in the Assay)	---
RT & PCR reaction	RT Enzyme	1 tube (Clear Cap / 5U/L) – 9µL	ABL SA (included in the Assay)	---
RT & PCR reaction	PCR Enzyme	1 tube (Clear Cap / 5U/L) – 9µL	ABL SA (included in the Assay)	---
RT & PCR reaction	Water	1 tube (Clear Cap) – 200µL	ABL SA (included in the Assay)	---
RT & PCR reaction	BP Solution	1 tube (Clear Cap / 10mg/mL) – 4.5µL	ABL SA (included in the Assay)	---
RT & PCR reaction	RP Solution	1 tube (Clear Cap / 50µM) – 9µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	NS3 FOR1-NESTED-PCR Primers	1 tube (Clear Cap / 20µM) – 15µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	NS3 FOR2-NESTED-PCR Primers	1 tube (Clear Cap / 20µM) – 15µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	NS3 REV-NESTED-PCR Primers	1 tube (Clear Cap / 20µM) – 29µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	NESTED Buffer 2X	1 tube (Clear Cap / 2x) – 365µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	Water	1 tube (Clear Cap) – 250µL	ABL SA (included in the Assay)	---
NESTED PCR reaction	NESTED PCR Enzyme	1 tube (Clear Cap / 5U/µL) – 9µL	ABL SA (included in the Assay)	---
	NS3 SEQUENCING FOR Primers	1 tube (Red Cap / 3.2µM) - 29µL	ABL SA (included in the Assay)	---
	NS3 SEQUENCING REV Primers	1 tube (Red Cap / 3.2µM) - 29µL	ABL SA (included in the Assay)	---
	Thermocycler	---	ABI9700	---
	96 well plate cooler	---	Eppendorf	22510509
	96-well PCR plates	---	Eppendorf	951020303
	Plate thermo seals	---	Thermo Scientific	AB-0558
	Plate centrifuge	---	Many	---
	0.2 mL thin-walled 8 tube & domed cap	---	Thermo Scientific	AB-0266
	1.5 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 NS3DR Assay is shipped with dry ice and should be maintained and stored immediately upon receipt at –20°C C in order to avoid compromising cold chain integrity.
Expiration date: please refer to the label on the kit box.

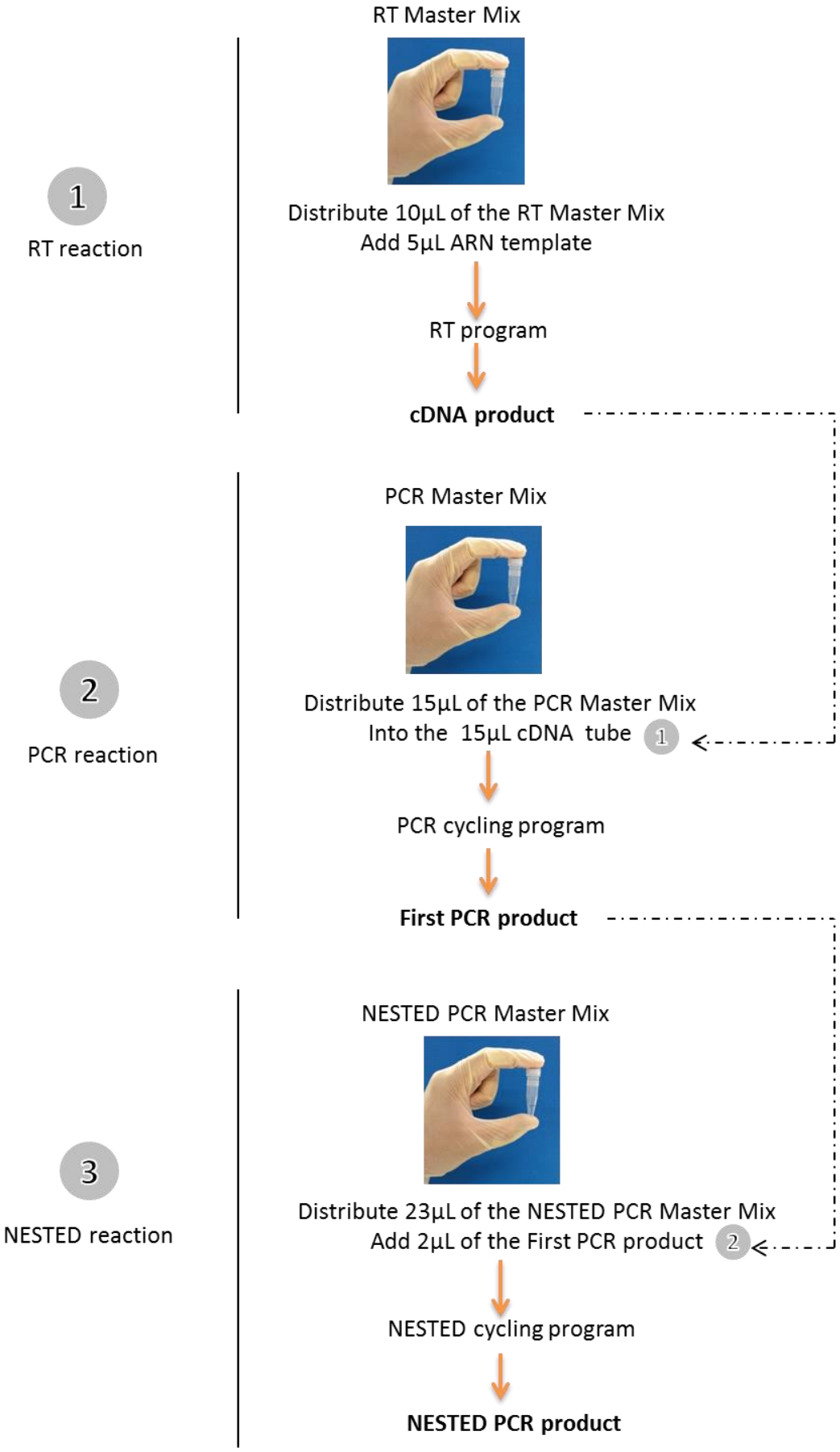
C. RNA Extraction

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 NS3DR Assay will work with at least an extraction of 400µL 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:
-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
-To extract one or two mL of plasma/serum and elute in the minimum volume required for your preferred extraction kit.

D. RT-PCR Workflow Overview



E. RT and PCR Step-by-Step Workflow for NS3 Target

STEP 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 during 10 seconds. And then aspirate and discharge the solution several times before the dispensing.

STEP 2 - Prepare a RT master mix 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-PCR NS3 target

Reagent	Volume
RT & PCR Buffer 2X	7.5 µl
RP Solution	0.3 µL
BP Solution	0.15 µL
RT Enzyme	0.3 µL
Water	1.75 µl

STEP 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.

STEP 4 - Add 5µL of RNA to the 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 2**.

Table 2. RT NS3 Program

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

STEP 6 - Start the RT NS3 program.

STEP 7 – During RT NS3 reaction, Prepare a PCR master mix 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 RT-PCR NS3 target

Reagent	Volume
RT & PCR Buffer 2X	7.5 µl
NS3 FOR1 PCR Primers	0.6 µL
NS3 FOR2 PCR Primers	0.6 µL
NS3 REV PCR Primers	1.2 µL
PCR Enzyme	0.3 µL
Water	4.8 µl

STEP 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.

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

Table 4. PCR NS3 Program

	Time	Temperature
	3 min	94°C
x 45 cycles	15 sec	94°C
	1 min	53°C
	1 min	72°C
	∞	4°C

STEP 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:

- 776 bp

F. NESTED PCR Step-by-Step Workflow for NS3 Target

STEP 1 - Prepare a NESTED PCR master mix according to **Table 5**. Prepare a volume of master mix greater (n+1) than that required for the total number of reactions to be performed.

Table 5. Reaction components for NESTED PCR NS3 target

Reagent	Volume
NESTED PCR Buffer 2X	12.5 µl
NS3 FOR1-NESTED-PCR Primers	0.5 µL
NS3 FOR2-NESTED-PCR Primers	0.5 µL
NS3 REV-NESTED-PCR Primers	1 µl
NESTED PCR Enzyme	0.3 µl
Water	8.3 µL

STEP 2 - Vortex the NESTED PCR master mix thoroughly and dispense 23 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.

STEP 3 - Add 2µL of PCR product (first PCR) to the PCR tubes. Mix by pipetting the master mix up and down a few times.

STEP 4 - Program the thermal cycler according to the program in **Table 6**.

Table 6. NESTED PCR NS3 Program

	Time	Temperature
	5 min	94°C
x 5 cycles	30 sec	94°C
	1 min	53°C
	1 min	72°C
X 40 cycles	15 sec	94°C
	30 sec	67°C
	30 sec	72°C
	∞	4°C

STEP 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:

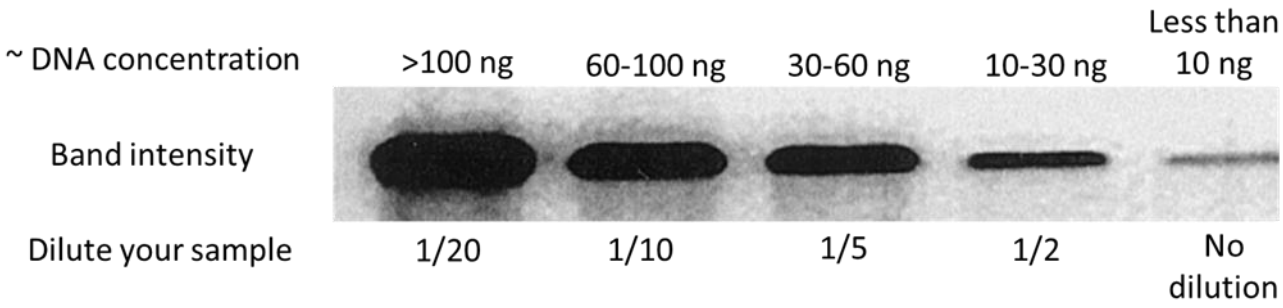
- 650 bp

G. 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 PCR bands on the agarose gel, dilute ($1/10^1$ - $1/10^3$) of the product before sequencing.



H. PCR Products Purification

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

I. 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 NS3 sequencing, use the 2 red cap tubes containing 29µL of each NS3 Sanger sequencing primer (forward and reverse - 3.2 µM). **One µL** of each primer will be used for each sample.

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	2µL
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	2µL
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	
5 min	96°C	
10 sec 5 sec 4 min	96°C 50°C 60°C	X 25 cycles
∞	4°C	

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

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

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 - PCR Cleanup reagent

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 4000rpm;
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 185rpm;
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 4000rpm;
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 4000rpm;
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® Library preparation kit (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

AB1 or FASTA files containing nucleotide sequences for NS3 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 NS3 are analyzed by the **DeepChek®** software. User shall then follow the DeepChek software procedure to complete the data analysis and reporting processes.