DeepChek® SingleRound RT-PCR and Sequencing

CORE Assay (K-16-CORE)

24x CORE tests

Technical Laboratory Protocol

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A. Reagents, Materials and Equipment

Reagent / Material / Equipment	Quantity	Vendor	Cat. Number
DeepChek® SingleRound RT-PCR and Sequencing CORE Assay		ABL SA (included in the Assay)	**K-16-CORE**
CORE FOR Primers	1 tube (Clear Cap / 10µM) – 58µL	ABL SA (included in the Assay)	---
CORE REV Primers	1 tube (Clear Cap / 10µM) – 58µL	ABL SA (included in the Assay)	---
CORE SEQUENCING FOR Primers	1 tube (Red Cap / 3.2µM) - 29µL	ABL SA (included in the Assay)	---
CORE SEQUENCING REV Primers	1 tube (Red Cap / 3.2µM) - 29µL	ABL SA (included in the Assay)	---
RNAsin	1 tube (Clear Cap / 40U/µL) – 4.5µL	ABL SA (included in the Assay)	---
RT-PCR Enzyme Mix	1 tube (Clear Cap) – 58µL	ABL SA (included in the Assay)	---
RT-PCR Buffer 5X	1 tube (Clear Cap) – 290µL	ABL SA (included in the Assay)	---
RNAse-Free water	1 tube (Clear Cap) - 650µL	ABL SA (included in the Assay)	---
dNTP	1 tube (Clear Cap / 10mM) - 58µL	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® SingleRound RT-PCR and Sequencing CORE 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.

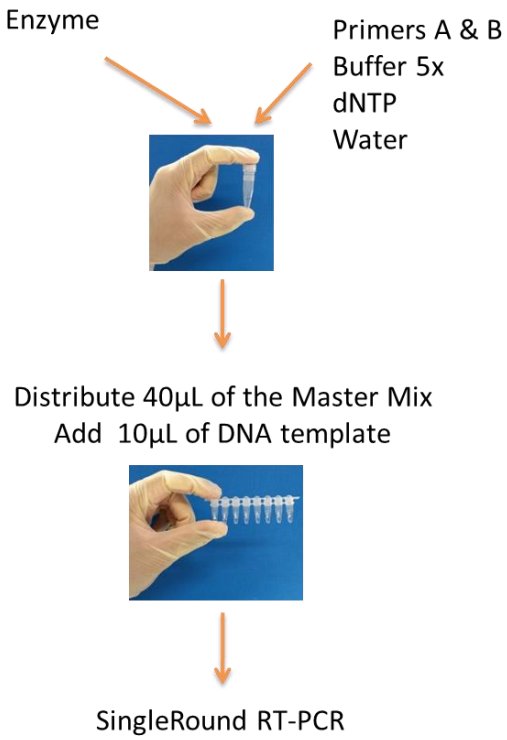
The DeepChek® SingleRound RT-PCR and Sequencing CORE Assay will work with at least an extraction of 400µL of plasma or serum, ideally from fresh samples, to be eluted in 100µL (related sensitivity evaluated to 1250 UI/mL).

For samples with low viral load, we recommend:
- To perform an ultracentrifugation procedure. Pellet the virus for 1.5 hours at 40,000 x g (or alternatively for 2 hours at 24,000 x g), and 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. SingleRound RT-PCR Workflow Overview



E. SingleRound RT-PCR Step-by-Step Workflow for CORE 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 x g during 10 seconds. And then aspirate and discharge the solution several times before the dispensing.

STEP 2 - Prepare a master mix on ice according to **Table 1**. The master mix typically contains all the components required for RT-PCR except the template RNA. Prepare a volume of master mix greater (n+1) than that required for the total number of reactions to be performed.

Table 1. Reaction components for SingleRound RT-PCR CORE target

Reagent	Volume
Buffer 5X	10 µL
dNTP 10 mM	2 µL
CORE Forward Primer 10 µM	2 µL
CORE Reverse Primer 10 µM	2 µL
Enzyme Mix	2 µL
RNAsin 40U/µL	0.15 µL
RNAse Free water	21.85 µL
Final Volume	**40 µL**

STEP 3 - Vortex the master mix thoroughly, spin down and dispense 40 µL into PCR tubes. Keep the tubes on ice.

STEP 4 - Add 10µL 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. SingleRound RT-PCR CORE Cycling Program

Time	Temperature	Cycle
40 min	50°C	1
15 min	95°C	1
30 sec 30 sec 1 min 10sec	94°C 64°C 72°C	50
10 min	72°C	1
∞	10°C	1

STEP 6 - Start the DeepChek® SingleRound RT-PCR and Sequencing CORE cycling program while PCR tubes are still on ice. **Wait until the thermal cycler has reached 50°C. Then place the PCR tubes in the thermal cycler.** After amplification, samples can be stored overnight at 2–10°C or at - 20°C for long-term storage.

STEP 7 [Recommended] - RT-PCR products can be controlled through electrophoresis on an agarose gel. Check the intensity of the signal. Even if low-intensity bands usually lead to a successful sequencing, it is recommended to avoid the process if no band can be observed.

Expected amplicons size:

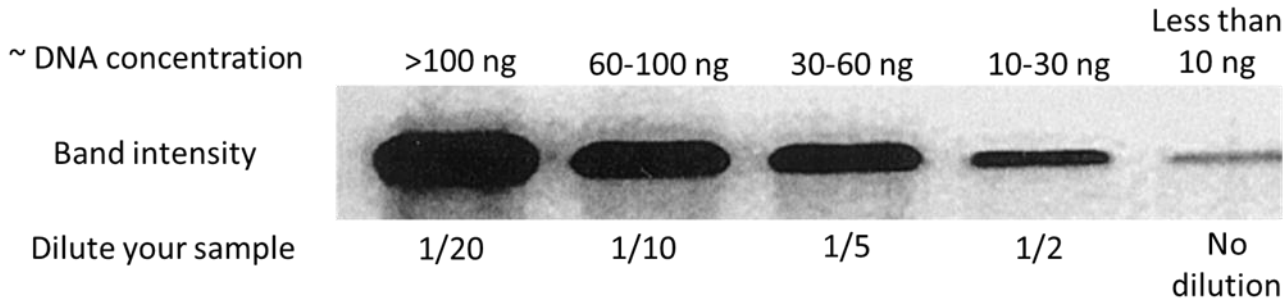
- CORE: 463 bp

F. SingleRound 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 x 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, make dilution (1/101 - 1/103) of product before sequencing.



G. PCR Products Purification

Before sequencing, first make sure your RT-PCR products have been purified. In presence of very large PCR bands on the agarose gel, dilute (1/101 - 1/103) of the SingleRound RT-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 SingleRound RT-PCR product before sequencing.

For CORE sequencing, use the 2 red cap tubes containing 29µL of each CORE Sanger sequencing primer (forward and reverse - 3.2 µM). **1µL** 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	2µL
Sequencing Buffer (5x)	3µL
***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)	3µL
***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 6a. 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 incubating, 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;

Denaturation:

1) Add 10ul of formamide and incubate in the thermocycler 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 on the 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 Kits
- **4484355** | Ion 318™ Chip Kit

J. Data analysis

1. Sanger

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