DeepChek® Assay

PROTEASE / REVERSE TRANSCRIPTASE

Genotyping and Drug Resistance

(RUO)

24
96

User Guide

Version 1 – Revision 5

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	**101B24**	**GTIN: 05407007960866**
	101B96	**GTIN: 05407007960842**

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Document control

Date	Device version	IFU version	Description of change
2022/07/11	B	1.4	• Merging of IFUs to have only one document suitable for V2.1, V2.2 and V2.3 of kit. • Modification in assays components (color cap for version 2.3 of the kit).
2023/04/21	B	1.5	• Modification in assay components (volumes) and adding the mapping for the 96 tests format in V2.3 of kit.

Contents

Application

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The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance*** (RUO) kit is a single tube system which utilizes PCR technology for amplifying the human immunodeficiency virus protease and reverse transcriptase regions.

This nucleic acid amplification method screens the emergence of mutations in the *Pol* gene of human immunodeficiency virus, type one (HIV-1) specimens. The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance*** (RUO) can be used to process samples with viral loads between 500 and 1,000,000 copies per milliliter (cp/mL).

The ***DeepChek® Assay PR / RT 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) workflows.

Principles of the assay

The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance*** (RUO) is a reverse transcriptase-polymerase chain reaction test which includes primers, reverse and forward, designed to amplify HIV-1 extracted RNA specimens.

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 PR / RT 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 HIV-1 genome mutations according to available public reference knowledge databases.

Genotypic analysis of this region of HIV-1 facilitates the study of the relationship between mutations and viral resistance to anti-retroviral drugs, specifically the protease and RT inhibitors.

Assay components

The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance*** is provided in two models of 24 and 96 reactions (REF 101B24 / OLD REF K-16-H2-RTPR24, and REF 101B96).

DeepChek® Assay PR / RT Genotyping and Drug Resistance V2.1

Label	Volume for		Color cap	Storage
RT-PCR	**24 Rxn.**	**96 Rxn.**		
RT-PCR Buffer 5X	320 µL	2 x 450 µL	Green	-25°C to - 15 °C
dNTPs	65 µL	180 µL	Brown	-25°C to - 15 °C
RT-PCR Enzyme Mix	65 µL	180 µL	Clear	-25°C to - 15 °C
RNAsine	70 µL	180 µL	Orange	-25°C to - 15 °C
H_2O	1000 µL	1000 µL	Blue	-25°C to - 15 °C
PROT FOR RT-PCR Primers	55 µL	150 µL	Yellow	-25°C to - 15 °C
PROT REV RT-PCR Primers	55 µL	150 µL	Yellow	-25°C to - 15 °C
RT FOR RT-PCR Primers	55 µL	150 µL	Yellow	-25°C to - 15 °C
RT REV (1) RT-PCR Primers	55 µL	150 µL	Yellow	-25°C to - 15 °C
RT REV (2) RT-PCR Primers	55 µL	150 µL	Yellow	-25°C to - 15 °C
PROT SEQUENCING FOR Primers	35 µL	100 µL	Red	-25°C to - 15 °C
PROT SEQUENCING REV Primers	35 µL	100 µL	Red	-25°C to - 15 °C
RT SEQUENCING FOR RT-PCR Primers	35 µL	100 µL	Red	-25°C to - 15 °C
RT SEQUENCING REV RT-PCR Primers	35 µL	100 µL	Red	-25°C to - 15 °C
Nested PCR	**24 Rxn.**	**96 Rxn.**		
Nested Buffer 10X	160 µL	500 µL	Green	-25°C to - 15 °C
Nested dNTPs	35 µL	90 µL	Brown	-25°C to - 15 °C
Nested PCR Enzyme	10 µL	30 µL	Clear	-25°C to - 15 °C
Nested H_2O	1500 µL	1500 µL	Blue	-25°C to - 15 °C
PROT FOR Nested PCR Primers	20 µL	55 µL	Yellow	-25°C to - 15 °C
PROT REV Nested PCR Primers	20 µL	55 µL	Yellow	-25°C to - 15 °C
RT FOR Nested PCR Primers	20 µL	55 µL	Yellow	-25°C to - 15 °C
RT REV (1) Nested PCR Primers	20 µL	55 µL	Yellow	-25°C to - 15 °C
RT REV (2) Nested PCR Primers	20 µL	55 µL	Yellow	-25°C to - 15 °C
PROT SEQUENCING FOR Nested PCR Primers	40 µL	110 µL	Red	-25°C to - 15 °C
PROT SEQUENCING REV Nested PCR Primers	40 µL	110 µL	Red	-25°C to - 15 °C
RT SEQUENCING FOR Nested PCR Primers	40 µL	110 µL	Red	-25°C to - 15 °C
RT SEQUENCING REV Nested PCR Primers	40 µL	110 µL	Red	-25°C to - 15 °C

Table 1: Volumes and storage conditions of the DeepChek® Assay PR / RT Genotyping and Drug Resistance (RUO) ***V2.1***

RT-PCR (24 samples)				Nested PCR (24 samples)		
RT-PCR Buffer 5X	dNTPs	RT-PCR Enzyme mix		Nested Buffer 10X	Nested dNTPs	Nested PCR Enzyme
RNAsine	H_2O	PROT FOR RT-PCR Primers			Nested H_2O	PROT FOR Nested PCR Primers
PROT REV RT-PCR Primers	RT FOR RT-PCR Primers	$RT\ REV_1$ RT-PCR Primers		PROT REV Nested PCR Primers	RT FOR Nested PCR Primers	$RT\ REV_1$ Nested PCR Primers
$RT\ REV_2$ RT-PCR Primers		PROT SEQUENCING FOR RT-PCR Primers		$RT\ REV_2$ Nested PCR Primers		PROT SEQUENCING FOR Nested PCR Primers
PROT SEQUENCING REV RT-PCR Primers	RT SEQUENCING FOR RT-PCR Primers	RT SEQUENCING REV RT-PCR Primers		PROT SEQUENCING REV Nested PCR Primers	RT SEQUENCING FOR Nested PCR Primers	RT SEQUENCING REV Nested PCR Primers

Figure 1: mapping of the assay components for the ***101B24 V2.1 (RUO)***

DeepChek® Assay PR / RT Genotyping and Drug Resistance V2.2

Label	Volume for		Color cap	Storage
RT-PCR	**24 Rxn.**	**96 Rxn.**		
RT-PCR Buffer 5X	320 µL	2 x 385 µL	Green	-25°C to - 15 °C
dNTPs	65 µL	155 µL	Brown	-25°C to - 15 °C
RT-PCR Enzyme Mix	65 µL	155 µL	Clear	-25°C to - 15 °C
RNAsine	70 µL	155 µL	Orange	-25°C to - 15 °C
H_2O	1000 µL	1000 µL	Blue	-25°C to - 15 °C
PROT FOR RT-PCR Primers	55 µL	170 µL	Yellow	-25°C to - 15 °C
PROT REV RT-PCR Primers	55 µL	170 µL	Yellow	-25°C to - 15 °C
RT FOR RT-PCR Primers	55 µL	170 µL	Yellow	-25°C to - 15 °C
RT REV (1) RT-PCR Primers	55 µL	170 µL	Yellow	-25°C to - 15 °C
RT REV (2) RT-PCR Primers	55 µL	170 µL	Yellow	-25°C to - 15 °C
PROT SEQUENCING FOR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
PROT SEQUENCING REV Primers	40 µL	120 µL	Red	-25°C to - 15 °C
RT SEQUENCING FOR RT-PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
RT SEQUENCING REV RT-PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
Nested PCR	**24 Rxn.**	**96 Rxn.**		
Nested Buffer 10X	160 µL	370 µL	Green	-25°C to - 15 °C
Nested dNTPs	35 µL	80 µL	Brown	-25°C to - 15 °C
Nested PCR Enzyme	12 µL	25 µL	Clear	-25°C to - 15 °C
Nested H_2O	1500 µL	1500 µL	Blue	-25°C to - 15 °C
PROT FOR Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
PROT REV Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
RT FOR Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
RT REV (1) Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
RT REV (2) Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
PROT SEQUENCING FOR Nested PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
PROT SEQUENCING REV Nested PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
RT SEQUENCING FOR Nested PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C
RT SEQUENCING REV Nested PCR Primers	40 µL	120 µL	Red	-25°C to - 15 °C

*Table 2: Volumes and storage conditions of the DeepChek® Assay PR / RT Genotyping and Drug Resistance (RUO) **V2.2***

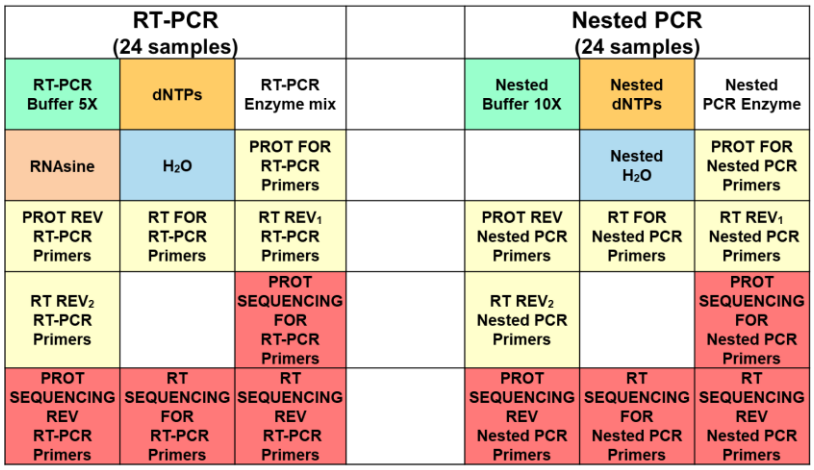

*Figure 2: mapping of the assay components for the **101B24 V2.2 (RUO)***

DeepChek® Assay PR / RT Genotyping and Drug Resistance V2.3

Label	Volume for		Color cap	Storage
RT-PCR	**24 Rxn.**	**96 Rxn.**		
RT-PCR Buffer 5X	320 µL	1200 µL	Green	-25°C to - 15 °C
dNTPs	65 µL	255 µL	Brown	-25°C to - 15 °C
RT-PCR Enzyme Mix	65 µL	255 µL	Clear	-25°C to - 15 °C
RNAsine	70 µL	255 µL	Orange	-25°C to - 15 °C
PROT FOR RT-PCR Primers	55 µL	180 µL	Yellow	-25°C to - 15 °C
PROT REV RT-PCR Primers	55 µL	180 µL	Yellow	-25°C to - 15 °C
RT FOR RT-PCR Primers	55 µL	180 µL	Pink	-25°C to - 15 °C
RT REV (1) RT-PCR Primers	55 µL	180 µL	Pink	-25°C to - 15 °C
RT REV (2) RT-PCR Primers	55 µL	180 µL	Pink	-25°C to - 15 °C
PROT SEQUENCING FOR Primers	40 µL	125 µL	Purple	-25°C to - 15 °C
PROT SEQUENCING REV Primers	40 µL	125 µL	Purple	-25°C to - 15 °C
RT SEQUENCING FOR RT-PCR Primers	40 µL	125 µL	Red	-25°C to - 15 °C
RT SEQUENCING REV RT-PCR Primers	40 µL	125 µL	Red	-25°C to - 15 °C
H_2O	1000 µL	1000 µL	Blue	-25°C to - 15 °C
Nested PCR	**24 Rxn.**	**96 Rxn.**		
Nested Buffer 10X	160 µL	600 µL	Green	-25°C to - 15 °C
Nested dNTPs	35 µL	130 µL	Brown	-25°C to - 15 °C
Nested PCR Enzyme	12 µL	40 µL	Clear	-25°C to - 15 °C
PROT FOR Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
PROT REV Nested PCR Primers	20 µL	65 µL	Yellow	-25°C to - 15 °C
RT FOR Nested PCR Primers	20 µL	65 µL	Pink	-25°C to - 15 °C
RT REV (1) Nested PCR Primers	20 µL	65 µL	Pink	-25°C to - 15 °C
RT REV (2) Nested PCR Primers	20 µL	65 µL	Pink	-25°C to - 15 °C
PROT SEQUENCING FOR Nested PCR Primers	40 µL	125 µL	Brown	-25°C to - 15 °C
PROT SEQUENCING REV Nested PCR Primers	40 µL	125 µL	Brown	-25°C to - 15 °C
RT SEQUENCING FOR Nested PCR Primers	40 µL	125 µL	Black	-25°C to - 15 °C
RT SEQUENCING REV Nested PCR Primers	40 µL	125 µL	Black	-25°C to - 15 °C
Nested H_2O	1500 µL	3 x 1500 µL	Blue	-25°C to - 15 °C

*Table 3: Volumes and storage conditions of the DeepChek® Assay PR / RT Genotyping and Drug Resistance (RUO) **V2.3***

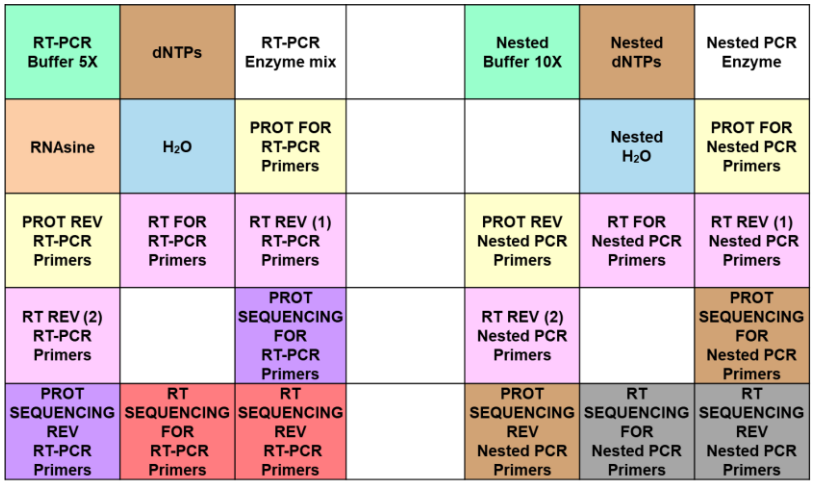

*Figure 3: mapping of the assay components for the **101B24 V2.3 (RUO)***

RT-PCR Buffer 5X	dNTPs	RT-PCR Enzyme mix		Nested Buffer 10X	Nested dNTPs	Nested PCR Enzyme
RNAsine	H_2O	PROT FOR RT-PCR Primers		Nested H_2O	Nested H_2O	Nested H_2O
PROT REV RT-PCR Primers	RT FOR RT-PCR Primers	RT REV (1) RT-PCR Primers		PROT FOR Nested PCR Primers	PROT REV Nested PCR Primers	RT FOR Nested PCR Primers
RT REV (2) RT-PCR Primers		PROT SEQUENCING FOR RT-PCR Primers		RT REV (1) Nested PCR Primers	RT REV (2) Nested PCR Primers	PROT SEQUENCING FOR Nested PCR Primers
PROT SEQUENCING REV RT-PCR Primers	RT SEQUENCING FOR RT-PCR Primers	RT SEQUENCING REV RT-PCR Primers		PROT SEQUENCING REV Nested PCR Primers	RT SEQUENCING FOR Nested PCR Primers	RT SEQUENCING REV Nested PCR Primers

*Figure 4: mapping of the assay components for the **101B96 V2.3 (RUO)***

Reagent storage and handling

The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance (RUO)*** is shipped with dry ice and should 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
- 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 pipettes 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 and refer to relevant the 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.
- 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 the 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 specimens.
- 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 HIV RNA analysis, the best representation of the viral quasispecies, it is recommended to extract **1 mL** of specimen for subsequent cDNA and amplicon generation and elute in the minimum volume required for your preferred extraction kit.

The ***DeepChek® Assay PR / RT Genotyping and Drug Resistance (RUO)*** will work with at least an extraction of 400 µL of specimen (i.e., plasma, serum, whole blood) specimens, to be eluted in 100 µL.

For specimens 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 specimen for your preferred extraction kit.
   OR
2. To extract one or 2 mL of specimen and elute in the minimum volume required for your preferred extraction kit.

PCR reaction setup Workflow

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 specimens at 10000 RPM for 10 seconds. And then pipette up and down the mix several times before the dispensing.
2. Prepare PR / RT master mix according to **Table 4**. 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.

Reagent	Protease Volume / Reaction	Reverse Transcriptase Volume / Reaction
RT-PCR Buffer 5X	5.0 µL	5.0 µL
PROT-FOR RT-PCR Primers 10 µM	1.5 µL	-
PROT-REV RT-PCR Primers 10 µM	1.5 µL	-
RT-FOR RT-PCR Primers 10 µM	-	1.5 µL
RT-REV (1) RT-PCR Primers 10 µM	-	1.5 µL
RT-REV (2) RT-PCR Primers 10 µM	-	1.5 µL
RT-PCR Enzyme Mix	1.0 µL	1.0 µL
RNAsine	1.0 µL	1.0 µL
H_2O	1.5 µL	-
dNTPs	1.0 µL	1.0 µL
Final Volume	**12.5 µL**	**12.5 µL**

Table 4: Reaction components for the PR / RT targets

3. Vortex the master mix thoroughly and dispense 12.5 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
4. Add 12.5 µL of RNA in 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 5**.

Cycle	Temperature (°C)	Time
RT step	50	30 min
Enzyme activation	95	15 min
10 cycles	94	40 sec
	54	30 sec
	72	1 min
35 cycles	94	30 sec
	54	30 sec
	72	1 min (+5 sec/cycle)
Final extension	72	10 min
1	10	∞

Table 5: PR / RT RT-PCR cycling program

6. Start the ***DeepChek® Assay PR / RT Genotyping and Drug Resistance*** (RUO) 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**.
   Δ Safe Stopping Point: After amplification, specimens can be stored overnight at 2–10°C, or at -20°C for long-term storage.
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. In that case, first use the DeepChek® Nested PCR and Sequencing PR / RT reagents.

Expected amplicons size:

- **Protease: 645 bp**
- **Reverse transcriptase: 1045 bp**

Nested PCR Step-by-Step Workflow for PR / RT (optional)

PROTEASE Nested PCR

1. Thaw the RT-PCR product, Nested PCR primer solutions, dNTP Mix and 10x Buffer and place them on ice. Load all the tubes into the centrifuge. Spin the specimens at 11000 g for 10 seconds. And then aspirate and discharge the solution several times before the dispensing.
2. Prepare PROTEASE master mix according to **Table 6**. The master mix typically contains all the components required for Nested 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.

Reagent	Protease Volume / Reaction
Nested Buffer 10X	2.50 µL
PROT FOR Nested PCR Primers	0.50 µL
PROT FOR Nested PCR Primers	0.50 µL
Nested PCR Enzyme	0.13 µL
Nested dNTPs	0.50 µL
Nested H_2O	17.87 µL
Final Volume	**22.00 µL**

Table 6: Reaction components for the PROTEASE Nested PCR target

3. Vortex the master mix thoroughly and dispense 22 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
4. Add 3 µL of the PROTEASE RT-PCR product in 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 7**.

Cycle	Temperature (°C)	Time
Enzyme activation	94	3 min
35 cycles	94	1 min
	54	30 sec
	72	1 min
Final extension	72	10 min
1	10	∞

Table 7: PROTEASE Nested PCR Cycling Program

6. Start the DeepChek® PROTEASE Nested PCR program. After amplification, specimens can be stored overnight at 2–10°C, or at –20°C for long-term storage.

7. **[Recommended]** – Nested PCR products can be checked 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:

- **Nested protease: 520 bp.**

REVERSE TRANSCRIPTASE Nested PCR

1. Thaw the RT-PCR product, Nested PCR primer solutions, dNTP Mix and 10x Buffer and place them on ice. Load all the tubes into the centrifuge. Spin the specimens at 11000 g for 10 seconds. And then aspirate and discharge the solution several times before the dispensing.
2. Prepare REVERSE TRANSCRIPTASE master mix according to **Table 8**. The master mix typically contains all the components required for Nested 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.

Reagent	Reverse Transcriptase Volume / Reaction
Nested Buffer 10X	2.50 µL
RT FOR RT-PCR Primers	0.50 µL
RT REV (1) RT-PCR Primers	0.50 µL
RT REV (2) RT-PCR Primers	0.50 µL
Nested PCR Enzyme	0.13 µL
Nested dNTPs	0.50 µL
Nested H_2O	17.37 µL
Final Volume	**22.00 µL**

Table 8: Reaction components for the REVERSE TRANSCRIPTASE Nested PCR target

3. Vortex the master mix thoroughly and dispense 22 µL into PCR tubes. Mix by pipetting the master mix up and down a few times.
4. Add 3 µL of the REVERSE TRANSCRIPTASE RT-PCR product in 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 9.**

Cycle	Temperature (°C)	Time
Enzyme activation	94	3 min
35 cycles	94	1 min
	57	30 sec
	72	1 min
Final extension	72	10 min
1	10	∞

Table 9: REVERSE TRANSCRIPTASE Nested PCR Cycling Program

6. Start the DeepChek® REVERSE TRANSCRIPTASE Nested PCR Assay program. After amplification, specimens can be stored overnight at 2–10°C, or at –20°C for long-term storage.
7. **[Recommended]** – Nested PCR products can be checked 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:

- **Nested reverse transcriptase: 937 bp.**

RT-PCR Troubleshooting Guide

1. Check the concentration, storage conditions and quality of the starting template. For optimal results use fresh/frozen specimen and proceed with a fresh RNA extraction.
2. For specimens with low viral load, we recommend performing an ultracentrifugation procedure. Pellet the specimen for 1.5 hours at 40,000 g and at 4°C. Remove enough supernatant to leave the required amount of specimen for your preferred extraction kit.
3. Before sequencing, first make sure your PCR products have been purified. In presence of very large PCR bands on the agarose gel, dilute ($1/10^1$ - $1/10^3$) of the PCR product before sequencing.

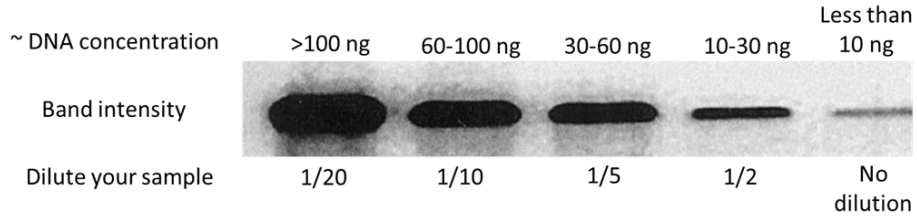

Figure 5: DNA concentration examples

PCR Products Purification

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

Sequencing

Sanger

After the amplicon verification, the specimens are ready for the Sanger sequencing kit processing using the ABL ***DeepChek® SANGER SEQUENCING REACTION V1.1*** (24 or 48 reactions) ***(REF 123A24 / 123A48)*** or ***DeepDye™ SANGER SEQUENCING REACTION V2*** (24 or 48 reactions) ***(REF 123B24 / 123B48).*** Users shall then follow the user guide.

NGS

After the amplicon verification, the specimens are ready for the NGS kit processing:

Through Illumina:

- **116A24 / 116A48 / 116A96 | ABL DeepChek® NGS LIBRARY PREPARATION V1 (24/48/96 reactions) or**
- **116B24 / 116B48 / 116B96 | ABL DeepChek® NGS LIBRARY PREPARATION V1 (24/48/96 reactions).**
- **124B24 / 124B48 / 124B96|ABL DeepChek® Adapters (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

User shall then follow the instructions for use from the manufacturer.

Data Analysis

Sanger

AB1 or FASTA files containing nucleotide sequences for Reverse Transcriptase and Protease fragments are analyzed by a downstream analysis software (i.e., the ABL **DeepChek® Software** (#S-12-023) or by the ABL **ViroScore® Software** (#S-09-14)). Users shall then follow the software user guide.

NGS

NGS files containing nucleotide sequences for Reverse Transcriptase and Protease fragments are analyzed by a downstream analysis software (i.e., the ABL **DeepChek® Software** (#S-12-023)). Users shall then follow the software user guide.

Quality controls

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.

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.

Performance Characteristics Nonclinical studies

Nonclinical studies were conducted to establish the analytical performance of the DeepChek® Assay.

Measurement procedure

The test shall amplify targeted HIV-1 coding regions known to confer resistance to approved anti-HIV drugs. The regions of interest for the DeepChek® Assay are the reverse transcriptase (RT) and the protease (PR), and for its DeepChek® Assay variant, the integrase (INT). If the Nested PCR amplicon was of quality, then it was prepared for sequencing and sequenced. Otherwise, we used the RT-PCR amplicon for downstream processing.

We used the ABL NGS Library Preparation kit (references 116A96 or 116B96 and 124A96) with a downstream NGS sequencing instrument (iSeq100, Illumina, USA; One-Channel SBS Chemistry, iSeq100 Flow Cell).

For next generation sequencing (NGS), it is required to assign a mutation as present or not in a sample. For the assays cut-off, we used the following criteria: minimal median coverage of 1000 reads for the amplicons (PR/RT and INT) and a Phred Quality Score Q30 >80% for the NGS run as reported by the Illumina Sequencing Analysis Viewer (instrument software version 2.4.5).

The number of reads per amplicon was measured by the ABL DeepChek® software (version 3.30.18; Expert System DeepChek® (v2.3); Drug Resistance Rulers algorithm for HIV (v.11.9)).

Analytical Performance Parameters

Analytical limit of detection

The analytical limit of detection (LOD) is defined as the lowest concentration at which ≥95% of tested replicates showed presumptive positive for the detection of PR, RT and IN targets of HIV-1.

Our LOD is 500 cp/mL as reported in the table below.

Concentration (cp/mL)	Number of samples tested	Number of correctly identified samples	Percentage of correctly identified samples
2000	**13**	**13**	**100%**
1000	**10**	**10**	**100%**
500	**10**	**10**	**100%**

Table 10: Limit of Detection (LoD)

We also get 100% of sequencing achieved with samples having a viral load at 10^6 cp/mL.

Note: Even if we were able to correctly amplify HIV-1 subtype B samples at a concentration of 500 cp/mL, a laboratory shall proceed to its own evaluation of the DeepChek® Assay performance for concentrations below the LOD.

Analytical cut-off

We used again the three levels of HIV-1 RNA concentrations (2000, 1000 and 500 copies/ml) together with an optimal coverage which is determined as above or equal to 1000 reads per amplicon available after NGS. We also reported the number of samples with a sub-optimal coverage per amplicon for each previous HIV-1 RNA concentration which is determined as above 50 and below 1000 reads.

We reached 100% of samples with an optimal median coverage at a concentration of 500 cp/ml (assay cut-off).

Concentration (cp/mL)	Number of samples tested	Samples with optimal median coverage (>=1000)		Samples with sub-optimal median coverage (>50x - < 1000)	
		Number	**%**	**Number**	**%**
2000	13	13	100%	0	0%
1000	10	10	100%	0	0%
500	10	10	100%	0	0%

Table 11: Assay cut-off

The median coverage per sample for the three amplicons (PR/RT and INT) was 13'237 reads.

Note: Even if we were able amplify adequately HIV-1 subtype B samples with an optimal coverage at a concentration of 500 cp/mL, a laboratory shall proceed to its own evaluation of the DeepChek® Assay performance for concentrations below the LOD.

Analytical reactivity/specificity

We used 24 clinical samples (median viral load=30600 cp/mL; 17 subtype B; 7 non-B) and 12 other samples (3 HIV negative; 3 cross-reaction (HBV positive and HCV positive); all in duplicates).

We get 100% of the samples specifically assessed:

- HIV-1 positive samples were amplified and sequenced with quality criteria achieved included 10 samples with viral load below 1000 cp/mL and/or non-B subtype.
- No amplicon products for the HIV-1 negative samples.

No interference substances were reported as no cross-reactivity occurred with the HCV and HBV spiked clinical samples.

Thus, the in-silico analytical study showed no amplification of other DNA organisms than HIV-1 (viruses, microbes or human).

Analytical reproducibility and repeatability

Analytical reproducibility and repeatability of the DeepChek® Assay was tested using a panel of 45 samples in 3 distinct NGS runs across 3 operators for 30 days, at different times of the day, using 3 different DeepChek® Assay lots, where each operator used 2 kits from 1 lot of reagents. The instruments used were the same.

High analytical reproducibility and repeatability were evidenced by Percent Agreement being 100%.

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 and date of manufacturing	**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.

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