HIV-1 Genotyping Workflow

Catalog Numbers A32317 and A32318

Pub. No. 100043578 **Rev.** F

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *HIV-1 Genotyping Workflow User Guide* (Pub. No. MAN0016022). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This Quick Reference is intended as a benchtop reference for the HIV-1 genotyping workflow. For detailed instructions, analysis guidelines, supplemental procedures, and troubleshooting, see the *HIV-1 Genotyping Workflow User Guide* (Pub. No. MAN0016022).

Product description

Specific types of antiretroviral (ARV) drugs are associated with mutations in the following coding regions of the Human Immunodeficiency Virus type 1 (HIV-1) *pol* gene:

- Protease (PR) region, codons 6–99
- Reverse transcriptase (RT) region, codons 1–251

The HIV-1 Genotyping Workflow supports research on HIV-1 genomic mutations in the PR and RT coding regions when used with the following kits:

- **HIV-1 Genotyping Kit: Amplification Module**—Generate nested PCR products (1.1 kb) from viral RNA isolated from plasma or dried blood spots.
- **HIV-1 Genotyping Kit: Cycle Sequencing Module**—Using six overlapping and bi-directional primers, sequence the nested PCR products and generate a consensus sequence of 1.04 kb. The consensus sequence is compared to a subtype B wild type reference sequence (HIV-1 HXB2 Genbank accession No. K03455).

Compatible instruments

Thermal cyclers	Genetic/DNA Analyzers
One of the following thermal cyclers:[1] • Veriti™ 96-Well Thermal Cycler, with 0.2-mL sample wells • GeneAmp™ PCR System 9700 96-Well • ProFlex™ 96-well PCR System with 0.2 mL sample wells	One of the following instruments:[2] • 3500/3500xL Genetic Analyzer • 3130/3130*xl* Genetic Analyzer • 3730/3730*xl* DNA Analyzer

[1] You can use an equivalent thermal cycler. Optimize the protocols for other thermal cyclers.

[2] You can use an equivalent instrument with Dye Set Z spectral calibration. Optimize the protocols for other instruments.

Contents and storage

Table 1 HIV-1 Genotyping Kit: Amplification Module (Cat. No. A32317)

Contents	Amount	No. of reactions	Storage
RT-PCR Master Mix (Normal)	2 × 1055 µL	2 × 24	–25°C to –15°C, protected from light.
Nested-PCR Master Mix	2 × 1285 µL	2 × 24	
RT-PCR Master Mix (Rescue)	1 × 550 µL	1 × 12	
SuperScript™ III One-Step RT-PCR with Platinum™ *Taq* High Fidelity Enzyme	1 × 54 µL	1 × 48	
AmpliTaq Gold™ LD DNA Polymerase	1 × 27 µL		
RNA Positive Control	2 × 40 µL	2 × 4	
RNA Negative Control	2 × 40 µL		

Table 2 HIV-1 Genotyping Kit: Cycle Sequencing Module (Cat. No. A32318)

Contents	Amount	No. of reactions	Storage
HIV Sequencing Mix F1	2 × 435 µL	2 × 24	–25°C to –15°C, protected from light.
HIV Sequencing Mix F2			
HIV Sequencing Mix F3			
HIV Sequencing Mix R1			
HIV Sequencing Mix R2			
HIV Sequencing Mix R3			
pGEM Sequencing Control	2 × 80 µL	2 × 4	

Guidelines

- Optimize viral RNA extraction protocols before use.
- Set up separate laboratory areas to minimize the risk of contamination.

  Note: Process samples in a unidirectional manner.
  - **Preamplification 1**: for setting up reaction mixes
  - **Preamplification 2**: for potentially infectious samples
  - **Amplification**: for running the PCR reactions
  - **Postamplification**: for clean up and sequencing of PCR products.
- Set up all reactions on ice.
- Include controls with every set of PCR reactions.
  - Positive control: RNA with mutations in the HIV-1 *pol* gene protease region (codons 6–99) and reverse transcriptase region (codons 1–251)
  - Negative control: TE buffer

Before you begin

- UV sterilize the PCR work stations in the Preamplification 1 and Amplification laboratory areas for at least 30 minutes before use.

  IMPORTANT! Do not expose reagents to UV light during the sterilization process.
- Thaw the provided RT-PCR Master Mix to room temperature, gently vortex, then briefly centrifuge.

Perform RT-PCR

1 Set up the RT-PCR reactions

a. In the Preamplification 1 laboratory area, prepare sufficient RT-PCR reaction mix for the required number of reactions plus 1 additional reaction for overage, in a chilled tube.

Component	Volume (number of reactions)		
	1	12	24
RT-PCR Master Mix (Normal)	39 µL	468 µL	936 µL
SuperScript™ III One-Step RT-PCR with Platinum™ *Taq* High Fidelity Enzyme	1 µL	12 µL	24 µL
Total volume	**40 µL**	**480 µL**	**960 µL**

b. Mix the components thoroughly, then centrifuge briefly to collect contents at the bottom of the tube.

c. Move the tube to the Preamplification 2 laboratory area.

d. Add 10 µL of RNA or controls to a labeled reaction plate.

Note: Input RNA quantity and quality affect sequencing results.

e. Denature the RNA and controls in a thermal cycler for 10 minutes at 65°C, then *immediately* place the plate on ice for ≥3 minutes.

f. Add 40 µL of the RT-PCR reaction mix to each well of the plate.

IMPORTANT! Change pipette tips between wells.

g. Vortex gently, then centrifuge briefly to collect contents at the bottom of the wells.

h. Move the plate to the Amplification laboratory area and immediately proceed to "Run the RT-PCR reactions".

2 Run the RT-PCR reactions

a. In the Amplification laboratory area, load the labeled reaction plate in the thermal cycler.

b. Set the RT-PCR thermal cycling conditions according to the following table.

IMPORTANT! Use 9600 emulation/simulation mode.

Step	Temperature	Time	Cycles
Reverse transcription	50°C	45 minutes	1
Enzyme inactivation	94°C	2 minutes	1
Denature	94°C	15 seconds	40
Anneal	50°C	20 seconds	
Extend	72°C	2 minutes	
Final extension	72°C	10 minutes	1
Hold	4°C	Maximum of 18 hours	

c. Set the appropriate reaction volume, then start the run.

Perform nested PCR

1 Set up the nested PCR reactions

Label the nested PCR reaction plates, then place on ice to chill.

a. In the Preamplification 1 laboratory area, prepare sufficient nested PCR reaction mix for the required number of reactions plus 1 additional reaction for overage, in a chilled tube.

Component	Volume (number of reactions)		
	1	12	24
Nested PCR Master Mix	47.5 µL	570 µL	1.14 mL
AmpliTaq Gold™ LD DNA Polymerase	0.5 µL	6 µL	12 µL
Total volume	**48 µL**	**576 µL**	**1.15 mL**

b. Mix the components thoroughly, then centrifuge briefly to collect contents at the bottom of the tube and to eliminate air bubbles.

c. Add 48 µL of the nested PCR reaction mix to each well of a chilled and labeled reaction plate.

d. Move the plate to the Amplification laboratory area.

e. In the Amplification laboratory area, add 2 µL of RT-PCR products (including controls) to the plate.

f. Vortex gently, then centrifuge briefly to collect contents at the bottom of the wells.

g. Immediately proceed to "Run the nested PCR reactions".

2 **Run the nested PCR reactions**

a. In the Amplification laboratory area, load the labeled reaction plate in the thermal cycler.
b. Set the nested PCR thermal cycling conditions according to the following table.

IMPORTANT! Use 9600 emulation/simulation mode.

Step	Temperature	Time	Cycles
Initial denaturation	94°C	4 minutes	1
Denature	94°C	15 seconds	40
Anneal	55°C	20 seconds	
Extend	72°C	2 minutes	
Final extension	72°C	10 minutes	1
Hold	4°C	Maximum of 18 hours	

c. Set the appropriate reaction volume, then start the run.

3 **Determine nested PCR product quality**

Confirmation of PCR products is recommended to conserve reagents and time.

a. In the Postamplification laboratory area, visualize the nested PCR products according to your laboratory's standard procedures.

Note: Use a DNA ladder that includes bands that are near 1 kb in size.

b. Determine whether the nested PCR products meet the following criteria:

Sample	Criteria
Positive control	The major product is 1.1 kb
Negative control	No amplification; no visible DNA bands
Test samples	The major product is 1.1 kb
	No DNA smear

IMPORTANT! If either of the controls does not meet the criteria, repeat the amplification process.

c. Proceed according to the test sample results:

If the test sample displays...	Do this...
No amplification	One of the following (in order): 1. Repeat the RT-PCR and nested PCR 2. Perform a rescue RT-PCR
Low band intensity	One of the following (in order): 1. Repeat the nested PCR using 4 µL of RT-PCR product 2. Increase the initial RNA to 20 µL 3. Perform a rescue RT-PCR
Passes the criteria in step 3b	Proceed to "Perform sequencing" on page 3.

Perform sequencing

Except as noted, perform cycle sequencing in the Postamplification laboratory area.

1 **Treat the nested PCR products with ExoSAP-IT™ PCR Product Cleanup Reagent**

a. Transfer 10 µL of nested PCR products to a new 96-well reaction plate.
b. Place the plate and the tube of ExoSAP-IT™ PCR Product Cleanup Reagent on ice.
c. Add 4 µL of ExoSAP-IT™ PCR Product Cleanup Reagent to each well containing 10 µL of nested PCR products.

IMPORTANT! Change pipette tips between wells.

d. Label the plate "+ExoSAP-IT™", then seal the plate with MicroAmp™ Clear Adhesive Film.
e. Vortex the plate for 2–3 seconds, then centrifuge at 1,000 × *g* for 5–10 seconds.
f. Place the plate into the thermal cycler, then run with the following settings.

Step	Temperature	Time
Digest	37°C	15 minutes
Heat deactivation	80°C	15 minutes
Hold	4°C	Hold

Store the plate on ice for immediate use. For longer term storage, store the plate at –15°C to –25°C.

2 **Set up cycle sequencing reactions**

IMPORTANT! Protect the sequencing mixes from light.

Before you begin:

- In the Preamplification 1 laboratory area, completely thaw the six sequencing mixes provided with the HIV-1 Genotyping Kit: Cycle Sequencing Module (F1, F2, F3, R1, R2, R3) on ice. Vortex briefly, then centrifuge for 2–3 seconds to collect contents at the bottom of the tubes.
- In the Postamplification laboratory area, completely thaw the pGEM Sequencing Control on ice. Vortex briefly, then centrifuge for 2–3 seconds to collect contents at the bottom of the tubes.

2 Set up cycle sequencing reactions *(continued)*

a. In the Preamplification 1 laboratory area, add 18 µL of each of the six sequencing mixes to the appropriate wells of a chilled 96-well reaction plate.
b. Transfer the plate to the Postamplification laboratory area, then add:
   - 2 µL of nested PCR products (treated with ExoSAP-IT™ PCR Product Cleanup Reagent) to each sequencing mix.
   - 20 µL of pGEM Sequencing Control to at least one well per run.
c. Seal the plate and immediately proceed to "Run the cycle sequencing reactions".

3 Run the cycle sequencing reactions

a. Load the 96-well reaction plate into the instrument.
b. Set the cycle sequencing conditions.

IMPORTANT! Use 9600 emulation/simulation mode.

Step	Temperature	Time	Cycles
Denature	96°C	10 seconds	25
Anneal	50°C	5 seconds	
Extend	60°C	4 minutes	
Hold	4°C	Maximum of 18 hours	

c. Set the appropriate reaction volume, then start the run.

(*If needed*) You can store sequencing products for up to 3 days at –15°C to –25°C.

4 Purify sequencing reactions with the BigDye XTerminator™ Purification Kit

Before you begin, remove the XTerminator™ Solution from 4°C storage and allow it to equilibrate to room temperature.

a. Vortex the XTerminator™ Solution for at least 10 seconds before mixing with the SAM™ Solution.

IMPORTANT! For effective BigDye XTerminator™ clean-up, ensure the materials are well mixed.

b. Prepare the bead working solution:

Component	Volume per 20-µL reaction	Volume per 96-well reaction plate
SAM™ Solution	90 µL	9.9 mL
XTerminator™ Solution	20 µL	2.2 mL
Total volume	**110 µL**	**12.1 mL**

c. Remove the MicroAmp™ Clear Adhesive Film from the 96-well reaction plate (sequencing reactions).
d. Dispense 110 µL/well of the bead working solution to each sample.

IMPORTANT! To ensure that the bead working solution is mixed thoroughly, pipette the solution up and down 3–4 times before each transfer.

e. Seal the plate using MicroAmp™ Clear Adhesive Film.
f. Vortex the plate for 30 minutes at 1,800 rpm (for the Digital Vortex-Genie™ 2).
g. Centrifuge the plate at 1,000 × *g* in a swinging bucket centrifuge for 2 minutes at room temperature.
h. Proceed immediately to capillary electrophoresis.

(*If needed*) You can store the purified sequencing reactions overnight at 2°C to 8°C.

5 Run capillary electrophoresis

a. Remove the adhesive film from the 96-well reaction plate (if present), then replace with a 96-well plate septa.
b. Load the plate into the genetic analyzer.
c. Select the 50-cm capillary length, the number of capillaries, and the POP-7™ polymer type.
d. Select or create an appropriate run module according to your specific instrument user guide.

IMPORTANT! Select a run module with a BDx prefix if sequencing reactions were purified with BigDye XTerminator™.

e. Start the run.

Determine sequence quality

1. Open the data collection software and review the AB1 files for quality control parameters.

   See your instrument user guide for standard procedures for sequence detection and analysis.
2. (*Optional*) Download the Sequence Scanner Software to review quality metrics. Go to **thermofisher.com/sangersequencing**, scroll to the **Resources** area at the bottom of the page, click **Sanger software download**, then click the link for freeware.

   For more information on determining sequence quality, see *Troubleshooting Sanger sequencing data* (Pub. No. MAN0014435).
3. If traces are of good quality, proceed to "Perform genotyping analysis" on page 4.

Perform genotyping analysis

There are multiple software platforms that can assist you with identifying HIV-1 genotypes.

For example, you can use the Exatype™ Platform by Hyrax Biosciences to facilitate analysis.

Perform surveillance and generate reports using the CPR Tool

To perform research on population-based surveillance and generate transmitted resistance reports for research use, use the Stanford University HIV Drug Resistance Database—Calibrated Population Resistance (CPR) Tool.

For more information, go to **cpr.stanford.edu/**.

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

The information in this guide is subject to change without notice.

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Revision	Date	Description
F	14 October 2019	• Add the ProFlex™ 96-well PCR System to the list of recommended thermal cyclers. • Direct users to other software platforms for performing genotyping analysis. • Minor formatting and typographical changes.
E	06 June 2017	Modify overview text to emphasize research use.
D	20 December 2016	Baseline for this revision history.