applied biosystems

HIV-1 Genotyping Workflow

USER GUIDE

for use with:
HIV-1 Genotyping Kit: Amplification Module
HIV-1 Genotyping Kit: Cycle Sequencing Module

Catalog Numbers A32317 and A32318
Publication Number MAN0016022
Revision C.0

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Revision history: Pub. No. MAN0016022

Revision	Date	Description
C.0	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.
B.0	06 June 2017	Modify overview text to emphasize research use. Remove references to NucliSENS™ products.
A.0	13 January 2017	New document

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Contents

Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

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).

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	

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 3 Materials for generating nested PCR products

Item	Source
Instruments and equipment	
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	Contact your local sales office.
Class II Biological Safety Cabinet (for specimen preparation; see Appendix C, "Supplemental procedures")	MLS
Two PCR work stations with UV light	MLS
Benchtop microcentrifuge	MLS
Refrigerated plate centrifuge	MLS
Vortex mixer	MLS
Gel electrophoresis equipment and consumables, UV box, and photo documentation system	MLS
Adjustable micropipettors	MLS
Plates and other consumables	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp™ Clear Adhesive Film, or equivalent	4306311
Aerosol-resistant pipette tips	MLS

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

Table 4 Materials for cycle sequencing and generating a consensus sequence

Item	Source
Instruments and equipment	
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	Contact your local sales office.

Item	Source
One of the following instruments:[2] • 3500/3500xL Genetic Analyzer • 3130/3130*xl* Genetic Analyzer • 3730/3730*xl* DNA Analyzer	Contact your local sales office.
Two PCR work stations with UV light	MLS
Benchtop microcentrifuge	MLS
Refrigerated plate centrifuge	MLS
Digital Vortex-Genie™ 2, or equivalent	MLS
Adjustable micropipettors	MLS
Software	
Sequence Scanner Software	resource.thermofisher.com/page/WE28396_2/
Reagents	
ExoSAP-IT™ PCR Product Cleanup Reagent (recommended), or equivalent	78200
BigDye XTerminator™ Purification Kit (recommended), or equivalent	4376486
Plates and other consumables	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp™ Clear Adhesive Film, or equivalent	4306311
Aerosol-resistant pipette tips	MLS
Other plastic consumables	thermofisher.com/plastics

[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.

Table 5 (*Optional*) Reagents for ethanol/EDTA purification

Item	Source
EDTA (0.5M), pH 8.0	AM9260G
Ethanol, absolute, for molecular biology	MLS

Methods

Workflow: procedures and laboratory areas

Laboratory area	RNA isolation	RT-PCR	Nested PCR	Cycle sequencing
Preamplification 1		Set up the RT-PCR reactions (minus RNA) ▼	Set up the nested PCR reactions (minus RT-PCR products) ▼	Set up cycle sequencing reactions (minus nested PCR products) ▼
Preamplification 2	RNA isolation ►	Add RNA ▼		
Amplification		Run the RT-PCR reactions ►	1. Add RT-PCR product 2. Run the nested PCR reactions ▼	
Postamplification			Determine nested PCR product quality ►	1. Treat the nested PCR products with ExoSAP-IT™ PCR Product Cleanup Reagent 2. Add purified PCR products 3. Run the cycle sequencing reactions 4. Purify sequencing reactions with the BigDye XTerminator™ Purification Kit 5. Run capillary electrophoresis

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.
  See "Workflow: procedures and laboratory areas" on page 9.

  – **Preamplification 1**: for setting up reaction mixes
  Eliminate DNA cross-contamination by using a hood that is equipped with ultraviolet light.
  – **Preamplification 2**: for potentially infectious samples
  Always handle potentially infectious human specimens in a Class II Biological Safety Cabinet.
  – **Amplification**: for running the PCR reactions
  Eliminate DNA cross-contamination by using a hood that is equipped with ultraviolet light.
  – **Postamplification**: for clean up and sequencing of PCR products.
  General laboratory area designated for use with 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
- Power on the thermal cycler 15–20 minutes before use.
- Run initial RT-PCR and nested PCR in batches of ≥6 samples, including the controls. This can help avoid repeated reagent thawing and reduce costs by reducing the ratio of controls to samples.

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.

Viral RNA extraction

The HIV-1 Genotyping Kit: Amplification Module accepts RNA or total nucleic acid (TNA) that has been extracted by various automated and manual methods. This workflow has been tested with the QIAamp™ Viral RNA Mini Kit (QIAGEN™).

All protocols should be optimized with your standard laboratory procedures. See the associated product information guides for more information.

Perform RT-PCR

Set up the RT-PCR reactions

IMPORTANT! Perform all steps on ice.

1. 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**

2. Mix the components thoroughly, then centrifuge briefly to collect contents at the bottom of the tube.
3. Move the tube to the Preamplification 2 laboratory area.
4. Add 10 µL of RNA or controls to a labeled reaction plate.

   Note: Input RNA quantity and quality affect sequencing results.

5. 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.
6. Add 40 µL of the RT-PCR reaction mix to each well of the plate.

   IMPORTANT! Change pipette tips between wells.

7. Vortex gently, then centrifuge briefly to collect contents at the bottom of the wells.
8. Move the plate to the Amplification laboratory area and immediately proceed to "Run the RT-PCR reactions".

Run the RT-PCR reactions

1. In the Amplification laboratory area, load the labeled reaction plate in the thermal cycler.
2. 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	

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

Perform nested PCR

Set up the nested PCR reactions

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

IMPORTANT! Perform all steps on ice.

1. 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**

2. Mix the components thoroughly, then centrifuge briefly to collect contents at the bottom of the tube and to eliminate air bubbles.
3. Add 48 µL of the nested PCR reaction mix to each well of a chilled and labeled reaction plate.
4. Move the plate to the Amplification laboratory area.
5. In the Amplification laboratory area, add 2 µL of RT-PCR products (including controls) to the plate.
6. Vortex gently, then centrifuge briefly to collect contents at the bottom of the wells.
7. Immediately proceed to "Run the nested PCR reactions".

Run the nested PCR reactions

1. In the Amplification laboratory area, load the labeled reaction plate in the thermal cycler.
2. 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	

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

(*Optional*) Store nested PCR products for up to 2 weeks at –15°C to –25°C.

Determine nested PCR product quality

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

1. 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.

2. 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.

3. 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[1]
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[1]
Passes the criteria in step 2	Proceed to "Perform sequencing" on page 15.

[1] Success varies with sample quality. See Appendix C, "Supplemental procedures" for instructions on running a rescue RT-PCR.

Perform sequencing

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

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

1. Transfer 10 µL of nested PCR products to a new 96-well reaction plate.
2. Place the plate and the tube of ExoSAP-IT™ PCR Product Cleanup Reagent on ice.
3. 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.

4. Label the plate "+ExoSAP-IT™", then seal the plate with MicroAmp™ Clear Adhesive Film.
5. Vortex the plate for 2–3 seconds, then centrifuge at 1,000 × *g* for 5–10 seconds.
6. 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.

Set up cycle sequencing reactions

See "Related documentation" on page 33 for additional information.

IMPORTANT! Protect the sequencing mixes from light.

IMPORTANT! Perform all steps on ice.

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.

1. 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.
2. 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.

     Note: The pGEM Sequencing Control contains the primers, terminator, and template. Do not use the sequencing mixes that are included in the kit.
3. Seal the plate and immediately proceed to "Run the cycle sequencing reactions".

	1	2	3	4	5	6	7	8	9	10	11	12
A	1_F1	1_F2	1_F3	1_R1	1_R2	1_R3	9_F1	9_F2	9_F3	9_R1	9_R2	9_R3
B	2_F1	2_F2	2_F3	2_R1	2_R2	2_R3	10_F1	10_F2	10_F3	10_R1	10_R2	10_R3
C	3_F1	3_F2	3_F3	3_R1	3_R2	3_R3	11_F1	11_F2	11_F3	11_R1	11_R2	11_R3
D	4_F1	4_F2	4_F3	4_R1	4_R2	4_R3	12_F1	12_F2	12_F3	12_R1	12_R2	12_R3
E	5_F1	5_F2	5_F3	5_R1	5_R2	5_R3	13_F1	13_F2	13_F3	13_R1	13_R2	13_R3
F	6_F1	6_F2	6_F3	6_R1	6_R2	6_R3	14_F1	14_F2	14_F3	14_R1	14_R2	14_R3
G	7_F1	7_F2	7_F3	7_R1	7_R2	7_R3	P_F1	P_F2	P_F3	P_R1	P_R2	P_R3
H	8_F1	8_F2	8_F3	8_R1	8_R2	8_R3	N_F1	N_F2	N_F3	N_R1	N_R2	pGEM

Figure 1 Example plate layout

The plate layout is designed for 14 test samples. Well 1_F1 refers to sample 1 with sequencing mix F1. Each sample is tested with all six sequencing mixes. P refers to the positive control, N refers to the negative control, and pGEM refers to the pGEM Sequencing Control.

Run the cycle sequencing reactions

1. Load the 96-well reaction plate into the instrument.
2. 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	

3. 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.

Note: Protect the sequencing products from light.

Purify sequencing reactions with the BigDye XTerminator™ Purification Kit

Note: This protocol is recommended for sequencing reaction purification. For an alternative ethanol/EDTA purification method, see "Purify sequencing reactions with ethanol/EDTA precipitation" on page 27.

Note: Use disposable reagent reservoirs and an 8- or 12-channel P200 pipette, if available, to help the clean-up process.

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

1. 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.

2. 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**

3. Remove the MicroAmp™ Clear Adhesive Film from the 96-well reaction plate (sequencing reactions).

4. 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.

5. Seal the plate using MicroAmp™ Clear Adhesive Film.

6. Vortex the plate for 30 minutes at 1,800 rpm (for the Digital Vortex-Genie™ 2).

   For alternative vortex mixer manufacturers and settings, see the *BigDye XTerminator™ Purification Kit Quick Reference Card* (Pub. No. 4383427).

7. Centrifuge the plate at 1,000 × *g* in a swinging bucket centrifuge for 2 minutes at room temperature.

8. Proceed immediately to capillary electrophoresis.

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

Run capillary electrophoresis

1. Remove the adhesive film from the 96-well reaction plate (if present), then replace with a 96-well plate septa.

   IMPORTANT! Plates that have been sealed with heat seal film can be placed directly into the 3730/ 3730xl instruments. All other instruments require 96-well plate septa.

2. Load the plate into the genetic analyzer.

3. Select the 50-cm capillary length, the number of capillaries, and the POP-7™ polymer type.

4. 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™.

   If your instrument does not contain BDx run modules, download them. See the *BigDye XTerminator™ Purification Kit User Bulletin* (Pub. No. 4483510).

5. Select the injection time.

   See your specific instrument user guide for information on using default settings or changing injection times.

6. Start the run.

Determine sequence quality

Controls must pass the acceptance criteria (including the pGEM Sequencing Control).

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 19.

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.

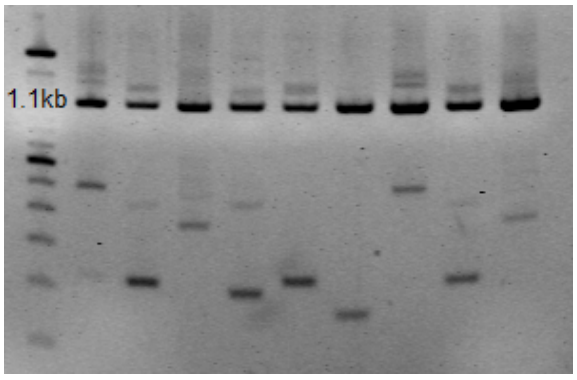
Perform surveillance and generate reports using the CPR Tool

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/**.

A

Troubleshooting

Observation	Possible cause	Recommended action
No bands are present on the gel	The RT-PCR or nested PCR failed to amplify.	Repeat with fresh reagents. Don't mix lot numbers.
	Poor quality or low concentration of RNA.	Double the input of RT-PCR products in the nested PCR reaction.
		Increase the amount of input RNA in the RT-PCR to 20 µL.
		Elute viral RNA in a lower elution volume if possible.
		Do not use Whatman FTA/FTA Elute cards (GE Healthcare Life Sciences) to prepare dried blood spots. Use Whatman 903 Sample Collection Cards (GE Healthcare Life Sciences) or grade 226 Munktell TFN (Ahlstrom Munktell) filter paper.
		Store samples as indicated in "Specimen preparation" on page 26.
		Repeat the RNA isolation step.
		Repeat the RT-PCR with RT-PCR Master Mix (Rescue). See "Perform a rescue RT-PCR" on page 28.
Incorrect band sizes are present on the gel 	Mispriming occurred.	Larger bands do not affect sequencing. Proceed if correct size band (1.1kb) is present.
		If smaller sized bands are present, or smearing, repeat the entire procedure, or extract the major band from the gel for sequencing.

Observation	Possible cause	Recommended action
Bands are present in the negative control well of the gel/ unexpected sequencing results are obtained	The RT-PCR or nested PCR reaction was contaminated.	Repeat the reactions. Always move through the laboratory areas in a unidirectional manner. Decontaminate each space after every use and follow your laboratory's standard operating procedures and PCR guidelines. For more information, see "Guidelines" on page 10.
Poor band resolution on the agarose gel	The running buffers are depleted.	Make a new gel and rerun the samples. Replace running buffers after 2 or 3 uses. For tips on agarose gel electrophoresis, go to **https://www.thermofisher.com/us/en/home/life-science/pcr/elevate-pcr-research/agarose-content-with-tips-and-tricks.html**.
	There is a problem with the E-Gel™ stain or loading dye.	If you use an E-Gel™, use ethidium bromide instead of SYBR™ Safe DNA Gel Stain to visualize the bands.
		Do not use BlueJuice™ Gel Loading Buffer.
	An incorrect percentage or type of agarose was used.	Rerun samples on a 1–2% molecular-biology-grade agarose gel. Do not use low-melting-point agarose.
Poor quality sequence	Poor nested PCR product quality (due to low- or poor-quality RNA input).	Optimize RT-PCR to obtain a single nested PCR product at the correct size. See "Determine nested PCR product quality" on page 14.
	Poor PCR product cleanup.	Use fresh ExoSAP-IT™ Reagent.
	Poor sequencing cleanup.	Use the BigDye XTerminator™ Purification Kit. Other methods can increase the incidence of dye blobs and other sequencing artifacts.
	Reagent failure.	Do not freeze/thaw sequencing reagents more than 6 times.
		Do not use expired reagents.

Observation	Possible cause	Recommended action
Poor quality sequence *(continued)*	Contamination occurred.	Decontaminate the laboratory, then repeat the sequencing reactions with fresh reagents. See *Troubleshooting Sanger sequencing data* (Pub. No. MAN0014435) for more information.
	Incorrect dye set selected.	Ensure that a BigDye™ Terminator v3.1 analysis protocol is selected.

B Control sequences

Sequence of RNA Positive Control

The complete sequence of the synthetic RNA Positive Control is shown below. The consensus sequence of the HIV-1 *pol* fragment that is generated with the HIV-1 Genotyping Kits is in bold, with the beginning and ending codons underlined.

The sequence encompasses:

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

```
   1 ACCAAATGAA AGATTGTACT GAGAGACAGG CTAATTTTTT AGGGAAGATC TGGCCTTCCT ACAAGGGAAG
  71 GCCAGGGAAT TTTCTTCAGA GCAGACCAGA GCCAACAGCC CCACCAGAAG AGAGCTTCAG GTCTGGGGTA
 141 GAGACAACAA CTCCCCCTCA GAAGCAGGAG CCGATAGACA AGGAACTGTA TCCTTTAACT TCCCTCAGGT
 211 CACTCTTTGG CAACGACCCC TCGTCACAAT AAAGATAGGG GGGCAACTAA AGGAAGCTCT ATTAGATACA
 281 GGAGCAGATG ATACAGTATT AGAAGAAATG AGTTTGCCAG GATGATGGAA ACCAAAAATG ATAGGGGGAA
 351 TTGGAGGTTT TATGAAAGTA AGACAGTATG ATCAGATACT CATAGAAATC TGTGGACATA AAGCTATAGG
 421 TACAGTATTA GTAGGACCTA CACCTGTCAA CATAATTGGA AGAAATCTGA TGACTCAGAT TGGTTGCACT
 491 TTAAATTTTC CCATTAGCCC TATTGAGACT GTACCAGTAA AATTAAAGCC AGGAATGGAT GGCCCAAAAG
 561 TTAAACAATG GCCATTGACA GAAGAAAAAA TAAAAGCATT AGTAGAAATT TGTACAGAGC TGGAAAAGGA
 631 AGGGAAAATT TCAAAAATTG GGCCTGAAAA TCCATACAAT ACTCCAGTAT TTGCCATAAA GAGAAAAGAC
 701 AGTACTAAAT GGAGAAAATT AGTAGATTTC AGAGAACTTA ATAAGAGAAC TCAAGACTTC TGGGAAGTTC
 771 AATTAGGAAT ACCACATCCC GCAGGGTTAA AAAAGAATAA ATCAGTAACA GTACTGGATG TGGGTGATGC
 841 ATATTTTTCA GTTCCCTTAG ATGAAGACTT CAGGAAGTAT ACTGCATTTA CCATACCTAG TATAAACAAT
 911 GAGACACCAG GGATTAGATA TCAGTACAAT GTGCTTCCAC AGGGATGGAA AGGATCACCA GCAATATTCC
 981 AAAGTAGCAT GACAAAAATC TTAGAGCCTT TTAGAAAACA AAATCCAGAC ATAGTTATCT GTCAATACGT
1051 GGATGATTTG TATGTAGGAT CTGACTTAGA AATAGGGCAG CATAGAACAA AAATAGAGGA GCTGAGACAA
1121 CATCTGTTGA GGTGGGGACT TACCACACCA GACAAAAAAC ATCAGAAAGA ACCTCCATTC CTTTGGATGG
1191 GTTATGAACT CCATCCTGAT AAATGGACAG TACAGCCTAT AGTGCTGCCA GAAAAAGACA GCTGGACTGT
1261 CAATGACATA CAGAAGTTAG TGGGGAAATT GAATTGGGCA AGTCAGATTT ACCCAGGGAT TAAAGTAAGG
1331
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Mutation profile of RNA Positive Control

The RNA Positive Control contains the following mutations (the mutations in bold are associated with varying levels of drug resistance):

- Protease: N37S, R41*, **I54M**, and **L90M**

  Note: * Protease position 41 encodes a stop codon.

- Reverse transcriptase: **M41L**, **K65R**, **K103N**, K122E, **Y181C**, **M184V**, and F214L

Partial sequence of pGEM Sequencing Control

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  1 GAATTGTAAT ACGACTCACT ATAGGGCGAA TTCGAGCTCG GTACCCGGGG ATCCTCTAGA GTCGACCTGC
 71 AGGCATGCAA GCTTGAGTAT TCTATAGTGT CACCTAAATA GCTTGGCGTA ATCATGGTCA TAGCTGTTTC
141 CTGTGTGAAA TTGTTATCCG CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT GTAAAGCCTG
211 GGGTGCCTAA TGAGTGAGCT AACTCACATT AATTGCGTTG CGCTCACTGC CCGCTTTCCA GTCGGGAAAC
281 CTGTCGTGCC AGCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG TTTGCGTATT GGGCGCTCTT
351 CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG CTCACTCAAA
421 GGCGGTAATA CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA
491 AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC
561 ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC
631 TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT ACCTGTCCGC CTTTCTCCCT
701 TCGGGAAGCG TGGCGCTTTC TCATAGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA
771 AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG CTGCGCCTTA TCCGGTAACT ATCGTCTTGA
841 GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG
911 TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG ACAGTATTTG
981 GTATCTGCGC TCTGCTGAAG
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C

Supplemental procedures

Specimen preparation

Optimize with your standard laboratory procedures. See the associated product information guides for further information.

IMPORTANT! Always handle potentially infectious human specimens in a Class II Biological Safety Cabinet.

Prepare plasma

1. Collect 5 mL of whole blood in sterile tubes containing EDTA anticoagulant (lavender-top), then mix according to manufacturer instructions.

   IMPORTANT! Do not use heparin as an anticoagulant because it inhibits PCR.

2. Separate plasma from the cells within 2–6 hours of collection.

   Note: Plasma can be stored for up to 6 months before testing at –65°C to –80°C. Do not freeze and thaw plasma more than 2 times.

3. Centrifuge the tubes at 1,000–2,000 × *g* for 15 minutes at room temperature (15°C to 25°C).

4. Transfer the plasma to a separate, sterile 1.5–2.0 mL polypropylene tube.

Prepare dried blood spots

1. Spot 100 µL of whole blood onto filter paper as soon as possible after collection.
   - Spot anti-coagulated EDTA venous blood within 24 hours of collection.
   - Spot blood without an anti-coagulant immediately after collection (<5 minutes).

2. Dry the blood spot at room temperature overnight on a drying rack.

3. Package the dried blood spot (DBS) according to manufacturer instructions.

Note: Store dried blood spots for up to 14 days at room temperature. For longer term storage, store at −20°C or colder.

Purify sequencing reactions with ethanol/EDTA precipitation

We recommend the BigDye XTerminator™ Purification Kit for purification of the sequencing reactions. However, you can also purify sequencing reactions with ethanol/EDTA precipitation. This method can cause subtle loss of low-molecular-weight fragments and can result in dye blobs in the sequencing traces.

IMPORTANT! Absolute ethanol absorbs water from the atmosphere, which gradually decreases its concentration and can affect sequencing results. Store absolute ethanol appropriately and replace frequently.

1. Prepare a 125 mM EDTA solution from 0.5 M EDTA, pH 8.0.
2. Prepare 70% ethanol using absolute ethanol.

   Note: Replace every 2 weeks.

   IMPORTANT! Do NOT pre-mix 125 mM EDTA solution and absolute ethanol. Pre-mixing can cause precipitation of the EDTA.
3. Centrifuge the 96-well reaction plate (sequencing reactions) in a swinging bucket centrifuge at 1,000 × *g* for 5–10 seconds.
4. Remove the MicroAmp™ Clear Adhesive Film from the plate.
5. Add the following components in the order listed:

Component	Volume
Sequencing reaction (starting volume)	20 µL
125 mM EDTA solution	5 µL
Absolute ethanol	60 µL
Total volume	**85 µL/well**

   IMPORTANT! Dispense EDTA directly into the sample in each well. If droplets are visible on the wall of the well: Before adding the ethanol, briefly centrifuge the plate to ensure that the EDTA mixes with the sequencing reactions.
6. Seal the plate with MicroAmp™ Clear Adhesive Film.
7. Vortex the plate for 2–3 seconds, then centrifuge at 1,000 × *g* for 5–10 seconds.
8. Incubate the plate at room temperature for 15 minutes.

   IMPORTANT! Timing of this step is critical.
9. Centrifuge the plate in a swinging bucket centrifuge at 1,870 × *g* for 45 minutes at 4°C.

   IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes, then proceed to the next step immediately.

10. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge, then carefully invert the plate onto the paper without dislodging the pellet. Centrifuge at 185 × *g* for 1 minute.

    Do not tip out the liquid first. Do not tap the plate to help with liquid removal.

11. Add 60 µL of 70% ethanol to each well.

12. Seal the plate with MicroAmp™ Clear Adhesive Film, then centrifuge at 1,870 × *g* for 15 minutes at 4°C.

    IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes, then proceed to the next step immediately.

13. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge, then carefully invert the plate onto the paper towel without dislodging the pellet. Centrifuge at 185 × *g* for 1 minute.

    Note: Do not tip out the liquid first. Do not tap the plate to help with liquid removal.

14. Allow the plate to air dry, face up and protected from light, for 5–10 minutes at room temperature.

    Note: Alternatively, seal the plate with MicroAmp™ Clear Adhesive Film, then store protected from light at 2°C to 8°C for CE preparation or –15°C to –25°C until use.

Perform a rescue RT-PCR

This procedure is intended for test samples that do not successfully amplify with RT-PCR Master Mix (Normal). See "No bands are present on the gel" on page 21.

1. In the Preamplification 1 laboratory area, prepare the Rescue RT-PCR reaction mix for the required number of reactions, plus 10% overage, in a chilled tube.

Component	Volume (number of reactions)		
	1	12	24
RT-PCR Master Mix (Rescue)	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**

   Note: Running the rescue RT-PCR decreases the number of normal RT-PCR reactions that can be run.

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

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

4. In the Preamplification 2 laboratory area, add 10 µL of RNA or controls to a chilled and labeled reaction plate.

5. 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.

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

   IMPORTANT! Change pipette tips between wells.

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

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Publication number
HIV-1 Genotyping Workflow Quick Reference	100043578
3130/3130xl Genetic Analyzers Getting Started Guide	4477796
3500/3500xL Genetic Analyzer with 3500 Series Data Collection Software 3.1 User Guide	100031809
User Guide: Applied Biosystems™ 3730/3730xl DNA Analyzer	4331468
BigDye XTerminator™ Purification Kit Quick Reference Card	4383427
Troubleshooting Sanger sequencing data	MAN0014435

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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