Applied Biosystems™ HIV-1 Genotyping Kit with Integrase

USER GUIDE

Catalog Number A55120

Publication Number MAN0026636

Revision B.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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

Revision history: MAN0026636 B.0 (English)

Revision	Date	Description
B.0	8 January 2024	• Sections for PR/RT and IN step for RT-PCR, nested PCR, and sequencing were combined. • Sample handling and extraction guidelines were added. • A section for analyzing sequencing traces was added ("Analyze the sequencing traces" on page 26). • Legal manufacturer was updated. • Compression pad and e-gel information was added ("Required materials not supplied" on page 9). • A recommended step was changed to required and pause points were added ("Workflow overview" on page 14). • Updates made for RT-PCR, nested PCR, and sequencing steps. • Additional observation was added and updates made to recommended actions (Appendix A, "Troubleshooting"). • Additional references added ("Related documentation" on page 34). • Updates made for general style, formatting, and branding.
A.0	14 July 2022	New document for the Applied Biosystems™ HIV-1 Genotyping Kit with Integrase.

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

Contents

1 Product information

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

IMPORTANT! Before using this product, read and understand the information in "Sample handling and extraction guidelines" on page 13.

Product description

The Applied Biosystems™ HIV-1 Genotyping Kit with Integrase is designed to detect HIV genomic mutations in the Protease (PR) codons 6-99, Reverse transcriptase (RT) codons 1-251, and Integrase (IN) codons 1-288 regions of the pol gene in RNA isolated from human immunodeficiency virus type 1 (HIV-1).

Pre-extracted HIV-1 RNA samples are run through the genotyping kit, which is based on three major processes:

- Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Nested PCR
- Cycle Sequencing
- Automated Sequence Detection to deliver an output AB1 file.

The laboratory utilizes the output file for further data interpretation analysis using public or privately available software of their choice.

This kit is for Research Use Only. Not for use in diagnostic procedures.

Contents and storage

Note: For component contents refer to Table 4.

Note: For image of kit contents refer to Figure 1.

Table 1 Applied Biosystems™ HIV-1 Genotyping Kit with Integrase (Cat. No. A55120): Contents for amplification

Contents	Part number	Amount	No. of reactions	Cap color	Storage
RNA Positive Control	100086895	2 × 100 µL	2 × 8	Purple	–25°C to –15°C
HIV RNA Negative Control	100090958	2 × 100 µL	2 × 8	White	
RT-PCR Master Mix, PR/RT	100112430	2 × 1,055 µL	2 × 24	Teal	
RT-PCR Master Mix, IN	100112431	2 × 1,055 µL	2 × 24	Teal	
Nested-PCR Master Mix, PR/RT	100098361	2 × 1,285 µL	2 × 24	Yellow	
Nested-PCR Master Mix, IN	100086894	2 × 1,285 µL	2 × 24	Light green	
SuperScript™ III One-Step RT-PCR with Platinum™ *Taq* High Fidelity Enzyme	100042782	2 × 54 µL	2 × 48	Dark green	
AmpliTaq Gold™ LD DNA Polymerase	100087086	1 × 54 µL	1 × 48	Gold	

Table 2 Applied Biosystems™ HIV-1 Genotyping Kit with Integrase (Cat. No. A55120): Contents for sequencing (IN)

Contents	Abbreviated contents	Part number	Amount	No. of reactions	Cap color	Storage
HIV Sequencing Mix R12	R12	100086899	2 × 435 µL	2 × 24	Teal	–25°C to –15°C, protected from light
HIV Sequencing Mix R11	R11	100086898	2 × 435 µL	2 × 24	Light green	
HIV Sequencing Mix F12	F12	100086897	2 × 435 µL	2 × 24	Gold	
HIV Sequencing Mix F11	F11	100086896	2 × 435 µL	2 × 24	Grey	

Table 3 Applied Biosystems™ HIV-1 Genotyping Kit with Integrase (Cat. No. A55120): Contents for sequencing (PR/RT)

Contents	Abbreviated contents	Part number	Amount	No. of reactions	Cap color	Storage
HIV Sequencing Mix R3	R3	100042791	2 × 435 µL	2 × 24	Orange	–25°C to –15°C, protected from light
HIV Sequencing Mix R2	R2	100042790	2 × 435 µL	2 × 24	Yellow	
HIV Sequencing Mix R1	R1	100042789	2 × 435 µL	2 × 24	Dark green	
HIV Sequencing Mix F3	F3	100042788	2 × 435 µL	2 × 24	White	
HIV Sequencing Mix F2	F2	100042787	2 × 435 µL	2 × 24	Purple	
HIV Sequencing Mix F1	F1	100042786	2 × 435 µL	2 × 24	Blue	

Table 4 Applied Biosystems™ HIV-1 Genotyping Kit with Integrase contents composition

Contents	Composition in aqueous solution
RNA Positive Control	Non-infectious in-vitro transcribed armored RNA construct containing PR/RT and IN sequences of HIV-1 of genome <0.1% Tris-HCl <0.1% Magnesium Chloride <0.1% Sodium Chloride <0.1% Gelatin, Bovine Skin, Type B
HIV RNA Negative Control	Tris-EDTA buffer (T10E0.1 pH 8.0)
RT-PCR Master Mix, PR/RT	<0.1% Tris base <0.1% Magnesium Sulfate <0.1% BSA and recombinant proteins <0.1% dNTP mix <0.1% Non-infectious synthetic HIV-1 specific oligonucleotide primer mix
RT-PCR Master Mix, IN	
Nested-PCR Master Mix, PR/RT	<0.1% Tris base <0.1% Non-ionic detergent <0.1% Potassium Chloride <0.1% BSA <0.01% Sodium Azide

Table 4 Applied Biosystems HIV-1 Genotyping Kit with Integrase contents composition *(continued)*

Contents	Composition in aqueous solution
Nested-PCR Master Mix, IN	<0.1% Ammonium Sulfate <0.1% dNTP mix <0.1% Non-infectious synthetic HIV-1 specific oligonucleotide primer mix
SuperScript™ III One-Step RT-PCR with Platinum™ *Taq* High Fidelity Enzyme	Recombinant Murine Leukemia Virus Reverse Transcriptase Platinum™ *Taq* High Fidelity Enzyme
AmpliTaq Gold™ LD DNA Polymerase	Recombinant hot-start enabled AmpliTaq Gold™ LD DNA Polymerase (Low DNA), 5 µ/µL
HIV Sequencing Mix R12	<0.1% Tris base <0.1% EDTA <0.1% Magnesium Chloride <0.1% Non-ionic detergent <5% Glycerol <0.1% DTT <0.1% dNTP mix <0.1%BigDye™ Terminator mix <0.1% Recombinant sequencing enzymes composition <0.1% Non-infectious synthetic oligonucleotide HIV-1 specific primer
HIV Sequencing Mix R11	
HIV Sequencing Mix F12	
HIV Sequencing Mix F11	
HIV Sequencing Mix R3	<0.1% Tris base <0.1% EDTA <0.1% Magnesium Chloride <0.1% Non-ionic detergent <5% Glycerol <0.1% DTT <0.1% dNTP mix <0.1%BigDye™ Terminator mix <0.1% Recombinant sequencing enzymes composition <0.1% Non-infectious synthetic oligonucleotide HIV-1 specific primer
HIV Sequencing Mix R2	
HIV Sequencing Mix R1	
HIV Sequencing Mix F3	
HIV Sequencing Mix F2	
HIV Sequencing Mix F1	

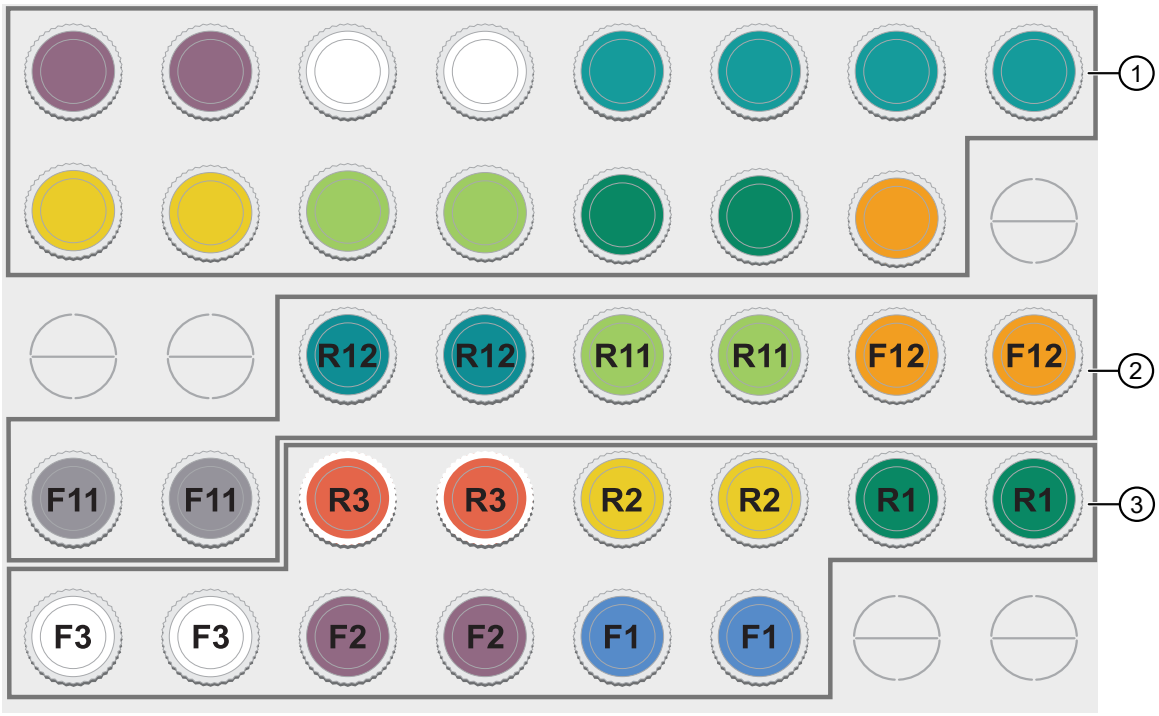


Figure 1 Applied Biosystems™ HIV-1 Genotyping Kit with Integrase (Cat. No. A55120) image of the contents

① Contents for amplification
② Contents for sequencing (IN)
③ Contents for sequencing (PR/RT)

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier. Catalog numbers that appear as links open the web pages for those products.

Table 5 Recommended materials for extraction

Item	Source
Instruments and equipment	
KingFisher™ Flex Purification System, KingFisher™ with 96 Deep-well Head	5400630
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit for HIV-1 Dried Blood Spots[1]	A53770
Reagents and other consumables	
Linear Acrylamide (5 mg/ml) (1 ml Tube)	AM9520

[1] Optimize the protocol for use with additional extraction methods as some methods require different sample input and/or elution volumes.

For additional information about RNA extraction, see “Sample handling and extraction guidelines” on page 13.

Table 6 Materials for generating nested PCR products

Item	Source
Instruments and equipment	
One of the following thermal cyclers: [1] • Veriti™ 96-Well Thermal Cycler, 0.2 mL • GeneAmp™ PCR System 9700 Thermal Cycler	Contact your local sales office.
Two PCR work stations with UV light	MLS
Bench top microcentrifuge	MLS
Plate centrifuge	MLS
Vortex mixer	MLS
E-Gel™ Power Snap Plus Electrophoresis System, or equivalent (for example, gel electrophoresis equipment and consumables, UV box, and photo documentation system)	Contact your local sales office
Adjustable micropipettes	MLS
Multi-channel pipette (1–20 µL volume)	MLS
Multi-channel pipette (20–200 µL volume)	MLS
PCR plate cold block or equivalent	MLS
Storage freezers and refrigerators	MLS
Plates and other consumables [2]	
One of the following: • MicroAmp™ Optical 96-Well Reaction Plate with Barcode • MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode	4306737 4483354
One of the following, or equivalent: • MicroAmp™ Clear Adhesive Film • Thermo Scientific™ Flat PCR Caps, strips of 8 • PolarSeal Aluminum Microplate Seals • MicroAmp™ Optical Film Compression Pad (for use only with the GeneAmp™ PCR System 9700 Thermal Cycler)	4306311 AB0783 1152A34 (Thomas Scientific) 4312639
Aerosol-resistant pipette tips	MLS

Table 6 Materials for generating nested PCR products *(continued)*

Item	Source
One of the following: • FastRuler Middle Range DNA Ladder, ready-to-use • DNA ladder that covers fragments size ranges from 100 bp to 2,000 bp	SM1113 MLS
One of the following: • E-Gel™ Sample Loading Buffer, 1X • Nuclease-free water	10482055 MLS
One of the following, or equivalent 2% agarose gel with SYBR Gold II DNA gel stain: • E-Gel™ EX Agarose Gels, 2% • E-Gel™ EX Double Comb Agarose Gels, 2%	G401002 A42346

[1] Use of an equivalent thermal cycler will require protocol optimization.

[2] Optimize the protocols for other plastics as some may not be suitable for PCR or capillary electrophoresis (CE). Use of equivalent plastics will require protocol optimization.

Table 7 Materials for cycle sequencing

Item	Source
Instruments and equipment	
One of the following thermal cyclers: [1] • Veriti™ 96-Well Thermal Cycler, 0.2 mL • GeneAmp™ PCR System 9700 Thermal Cycler	Contact your local sales office.
The following instrument: • 3500/3500xL Series Genetic Analyzers	Contact your local sales office.
Two PCR work stations with UV light	MLS
Bench top microcentrifuge	MLS
Plate centrifuge	MLS
Vortex mixer for the BigDye XTerminator™ Purification Kit; one of the following, or equivalent: • Scientific Industries ABI Digital Vortex-Genie™ 2 for microplate protocols • Fisherbrand™ Microplate Advanced Vortex Mixer (02-216-101)	MLS
Adjustable micropipettes	MLS
Reagents	
ExoSAP-IT™ PCR Product Cleanup Reagent	78200.200.UL

Table 7 Materials for cycle sequencing *(continued)*

Item	Source
BigDye XTerminator™ Purification Kit	4376486
Plates and other consumables [2]	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp™ Clear Adhesive Film, or equivalent	4306311
MicroAmp™ Optical Film Compression Pad (for use only with the GeneAmp™ PCR System 9700 Thermal Cycler)	4312639
Aerosol-resistant pipette tips	MLS
Wide-bore (>1.0 mm) pipette tips	MLS
Other plastic consumables	MLS

[1] Use of an equivalent thermal cycler will require protocol optimization.

[2] Optimize the protocols for other plastics as some may not be suitable for PCR or capillary electrophoresis (CE). Use of equivalent plastics will require protocol optimization.

Note: To analyze the sequencing traces, export the AB1 files from the 3500 Series Genetic Analyzer for use in downstream analysis and reporting software. To learn more about available commercial software, see “Analyze the sequencing traces” on page 26.

Note: The 3500/3500xL Series Genetic Analyzer instruments must be calibrated to support BigDye™ Terminator v3.1. For a complete list of required capillary electrophoresis (CE) instrument consumables, see your instrument user guide.

Kit stability

- Do not use kit components after the expiration dates indicated on the component boxes.
- Do not exceed 6 freeze-thaw cycles for the RT-PCR Master Mixes, Nested-PCR Master Mixes, RNA Positive Control, and HIV Sequencing Mixes.
- Keep the reagents on ice during reaction setup.
- Return the enzyme tubes to –25°C to –15°C immediately after use.

Warnings and precautions

The Applied Biosystems™ HIV-1 Genotyping Kit with Integrase workflow should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of samples and controls to prevent false positive results.

- No interfering substances or cross-reactants were identified to negatively impact kit performance.
- Samples and controls should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.
- Do not eat, drink, smoke, or apply cosmetic products in the work areas.
- Reagents must be stored and handled as specified in "Contents and storage" on page 6.
- Do not use the kits after the indicated expiry date.
- Dispose of waste in compliance with local, state, and federal regulations.
- Safety Data Sheets are available upon request.
- Encrypt, pseudonymize, or anonymize personal data where possible following the requirements of GDPR (General Data Protection Regulation).
- When running the instruments do not modify the kit template parameters.

Sample handling and extraction guidelines

Proceed with the following guidelines:

- Centrifuge the blood to cell-free plasma as soon as possible for best results. We recommend processsing the blood within 2 hours, if possible. Do not exceed 6 hours.
- Centrifuge the blood sample at 2,000 x g for 10 minutes at 4°C. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.
- If cell-free plasma is frozen, avoid multiple freeze-thaw cycles. Thaw plasma gently on ice and minimize time plasma is held on ice to protect the nucleic acids from degradation.
- The kit is optimized to work with an input volume of 200 µL plasma, and an elution volume of 30 µL using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit for HIV-1 Dried Blood Spots using the KingFisher™ Flex automated extraction instrument.

Note: If you use an elution volume greater than the recommended volume, align the sample input volume to avoid dilution of the viral content.

IMPORTANT! Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the target sequences.

For a complete list of required extraction consumables and the recommended extraction workflow for both plasma and DBS, see *MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit for HIV-1 Dried Blood Spots User Guide* ("Related documentation" on page 34).

For additional information on sample handling recommendations (plasma and dried blood spots), see World Health Organization (WHO) Guidelines for Using HIV Testing Technologies in Surveillance: **https://www.ncbi.nlm.nih.gov/books/NBK305270/pdf/Bookshelf_NBK3052**.

Workflow overview

1. Perform two RT-PCR reactions per sample, one for PR/RT and one for IN, to generate two RT-PCR products (RT-PCR products may be held for up to 18 hours at 4°C or <2 weeks at -15 to -25°C).
2. Perform one nested PCR reaction for PR/RT, and one nested PCR reaction for IN. This will generate two nested PCR products per sample (nested PCR products may be held for up to 18 hours at 4°C or <2 weeks at -15 to -25°C).
3. Run an aliquot of the nested PCR products on a 2% E-gel for quality control of the input material for cycle sequencing.
4. Treat all nested PCR products with ExoSAP-IT™ PCR Product Cleanup Reagent.
5. Cycle-sequence the ExoSAP-IT™-treated PR/RT nested PCR product with the R3, R2, R1, F3, F2, and F1 sequencing mixes (cycle sequencing products may be held for up to 18 hours at 4°C or <2 weeks at -15 to -25°C).
6. Cycle-sequence the ExoSAP-IT™-treated IN nested PCR product with the R12, R11, F12, and F11 sequencing mixes (cycle sequencing products may be held for up to 18 hours at 4°C or <2 weeks at -15 to -25°C).
7. Clean up the cycle sequencing reactions with the BigDye XTerminator™ Purification Kit (purified cycle sequencing products may be stored overnight at 2-8°C).
8. Perform capillary electrophoresis (CE), then use the AB1 files to determine sequence quality.

Workflow tasks by laboratory area

Preamplification Area—Reagent dispensing (no templates; PCR workstation)

- **Prepare RT-PCR mix**
- **Prepare and aliquot nested PCR mixes into the plate**
- **Aliquot cycle sequencing mixes into the plate**

Amplification Area (instrument area, low-amplicon area)

- **Aliquot extracted RNA into the plate (PCR workstation)**
- **Denature RNA before the RT-PCR step**
- **Add RT-PCR mix to denatured RNA samples (PCR workstation)**
- **Run the thermal cycler protocols**
- **Perform capillary electrophoresis (CE)**

Postamplification Area (high-amplicon area)

- **Add RT-PCR product to the nested PCR reaction plate**
- **Prepare nested PCR samples for gel analysis**
- **Perform gel analysis**
- **Add nested PCR samples to ExoSAP-IT™ Reagent**
- **Add purified nested PCR product to the sequencing mixes**
- **Perform sequencing cleanup with BigDye XTerminator™ Purification Kit**

2 Perform amplification

Guidelines

For additional information about sample handling and RNA extraction, see “Sample handling and extraction guidelines” on page 13.

- Set up separate laboratory areas to minimize the risk of contamination.

  Note: Process samples in a unidirectional manner.

  - **Preamplification**: For setting up reaction mixes
  - **Amplification**: For running thermal cycler protocols and CE
  - **Postamplification**: For gel analysis of amplified products
- Set up all reactions on a cold block or ice.
- Include the provided controls with every set of PCR reactions.
  - Positive control: RNA with mutations in the HIV-1 PR/RT and IN regions
  - Negative control: TE buffer
- Thermal cycler optimization may be required if 9600 emulation mode is not available on your thermal cycler.

Before you begin

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

  IMPORTANT! Follow your instrument guidelines to protect against exposure to UV light.

  Do not expose reagents to UV light during the sterilization process.
- Allow the RT-PCR Master Mixes to equilibrate to room temperature, gently vortex, then briefly centrifuge. Place on a cold block or ice until use.
- Clean all workspaces and pipettes with 10–15% sodium hypochlorite solution (bleach) to minimize the risk of contamination. Then remove the bleach with 70% ethanol.

Perform RT-PCR

Note: Based on user preference, the PR/RT RT-PCR and IN RT-PCR reactions can occur in parallel on the same plate with the same thermal cycler.

Set up the RT-PCR reactions

1. Briefly vortex and centrifuge RNA samples. Add 10 µL of the RNA sample or control for each PR/RT or IN reaction to a labeled reaction plate, then seal the plate with film.

   Note: Input RNA quantity and quality affect sequencing results.

2. Centrifuge briefly to collect contents at the bottom of the wells.

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

   Note: Ensure that all sides of the sample wells are completely surrounded by ice.

4. While the denatured RNA is on ice, prepare the RT-PCR reaction mix.

5. In the Preamplification laboratory area, combine the following components in a tube. Prepare sufficient RT-PCR reaction mix for the required number of reactions plus ~5% for overage (not included in calculations).

Component	Volume (number of reactions)		
	1	12	24
RT-PCR Master Mix, PR/RT or RT-PCR Master Mix, IN	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**

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

   Note: RT-PCR reaction mix should be used immediately or within 15 minutes of preparation.

7. Move the tube to the Amplification laboratory area.

8. Centrifuge the denatured RNA plate briefly to collect contents at the bottom of the wells.

9. Carefully remove the plate seal and add 40 µL of the appropriate RT-PCR reaction mix to each well of the plate, then seal the plate with film.

   IMPORTANT! Change pipette tips between wells and go to the second stop on the pipette when dispensing reaction mix.

10. Vortex all corners and the center of the plate gently, ensure complete mixing of the contents of the well, then centrifuge briefly to collect contents at the bottom of the wells.

11. Immediately proceed to “Run the RT-PCR reactions” on page 18.

Run the RT-PCR reactions

1. In the Amplification laboratory area, set the RT-PCR thermal cycling conditions according to the following table.

IMPORTANT! See the recommended thermal cyclers in Table 6.

IMPORTANT! Use 9600 emulation/simulation mode. If 9600 emulation/simulation mode is not available on your thermal cycler, contact technical support (see “Customer and technical support” on page 34) for guidance on how to adjust the ramp rates on your specific thermal cycler to mimic 9600 ramp rates when using a 50 µL reaction volume.

Note: Prior to putting the reaction plate in the thermal cycler, ensure the thermal cycler conditions are properly set and the heated cover has reached temperature.

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	

IMPORTANT! Do not the modify thermal cycler conditions.

2. Set the reaction volume to 50 µL, then start the run.

3. Load the labeled reaction plate in the thermal cycler.

4. When the run is complete, proceed to: “Perform nested PCR” on page 19.

Perform nested PCR

Note: Based on user preference, the PR/RT nested PCR and IN nested PCR reactions can occur in parallel on the same plate with the same thermal cycler.

Set up the nested PCR reactions

1. Label the nested PCR reaction plate, then place on a cold block or ice to chill.
2. In the Preamplification laboratory area, combine the following components in a chilled tube. Prepare sufficient nested PCR reaction mix for the required number of reactions plus ~5% for overage (not included in calculations).

Component	Volume (number of reactions)		
	1	12	24
Nested PCR Master Mix, PR/RT or Nested PCR Master Mix, IN	47.5 µL	570 µL	1,140 µL
AmpliTaq Gold™ LD DNA Polymerase	0.5 µL	6 µL	12 µL
Total volume of reaction mix	**48 µL**	**576 µL**	**1,152 µL**

3. Vortex the components thoroughly, then centrifuge briefly to collect contents at the bottom of the tube.
4. Add 48 µL of the nested PCR reaction mix to each well of the labeled reaction plate.
5. Seal the plate with film and move the plate to the Amplification laboratory area.

   Note: Nested PCR reaction mix should be used immediately or within 15 minutes of preparation.

6. Briefly vortex and centrifuge the RT-PCR products.
7. In a PCR hood in the Amplification laboratory area carefully remove the film from the RT-PCR products. Add 2 µL of RT-PCR products (including controls) to the plate, then seal the plate with film.

   Note: Add PR/RT RT-PCR products to PR/RT nested PCR reaction mix. Add IN RT-PCR products to IN nested PCR reaction mix.

8. Vortex all corners and the center of the plate gently, ensure complete mixing of the contents of the well, then centrifuge briefly to collect contents at the bottom of the wells.
9. Immediately proceed to “Run the nested PCR reactions” on page 20.

Run the nested PCR reactions

1. In the Amplification laboratory area, set the nested PCR thermal cycling conditions according to the following table.

IMPORTANT! See the recommended thermal cyclers in Table 6.

IMPORTANT! Use 9600 emulation/simulation mode. If 9600 emulation/simulation mode is not available on your thermal cycler, contact technical support (see "Customer and technical support" on page 34) for guidance on how to adjust the ramp rates on your specific thermal cycler to mimic 9600 ramp rates when using a 50 µL reaction volume.

Note: Prior to putting the reaction plate in the thermal cycler, ensure the thermal cycler conditions are properly set and the heated cover has reached temperature.

Step	Temperature	Time	Cycles
Initial denaturation	94°C	4 minutes	1
Denature	94°C	15 seconds	40
Anneal	53°C	20 seconds	
Extend	72°C	2 minutes	
Final extension	72°C	10 minutes	1
Hold	4°C	Maximum of 18 hours	

IMPORTANT! Do not modify the thermal cycler conditions.

2. Set the reaction volume to 50 µL, then start the run.
3. Load the labeled reaction plate in the thermal cycler.
4. When the run is complete, proceed to "Nested PCR product quality" on page 20.

Nested PCR product quality

To conserve reagents and time, we recommend confirming PCR products.

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

   (Recommended) Use an aliquot of nested PCR product (5 µL) mixed with water or E-gel sample loading buffer (5 µL) and the appropriate size marker on a 2% E-gel or traditional agarose gel to confirm PCR products.

   For additional information on running an E-gel, see **https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook.html**.

Note: Use a DNA ladder that includes bands that are near 1 kb in size. We recommend the FastRuler Middle Range DNA Ladder, which has 5 bands and loading 10 µL.

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

Sample	Criteria
Positive control	The major product is ~0.9 kb
Negative control	No amplification; no visible DNA bands
Samples	The major product is ~1.1 kb **Note:** Extra bands of shorter length and lesser intensity in the sample may reduce sequencing quality and make genotyping difficult. Extra bands of shorter length and greater intensity in the sample may give incorrect sequencing results. For more information, see Appendix A, "Troubleshooting".

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

3. Proceed according to the sample results:

If the sample	Do this
Displays no amplification	Repeat the RT-PCR and nested PCR for the sample. For more information and additional suggestions, see Appendix A, "Troubleshooting".
Displays additional bands shorter than 1 kb and/or longer than 100 bp that are of equal or brighter intensity than the target band	Repeat the RT-PCR and nested PCR for the sample.
Passes the criteria in step 2	Proceed to "Perform sequencing" on page 22.

Note: For troubleshooting due to gel issues, see Appendix A, "Troubleshooting".

3 Perform sequencing

Perform sequencing

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

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

1. Briefly vortex and centrifuge the nested PCR products.
2. Carefully remove the film and transfer 10 µL of nested PCR products to a new 96-well reaction plate.
3. Place the plate and the tube of ExoSAP-IT™ PCR Product Cleanup Reagent on a cold block or ice.
4. 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.

5. Label the plate "PR/RT + ExoSAP-IT™" or "IN + ExoSAP-IT™", then seal the plate with MicroAmp™ Clear Adhesive Film.
6. Vortex the plate for 2–3 seconds, then centrifuge at 1,000 × *g* for 5–10 seconds.
7. 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

8. For immediate use, store the plate on a cold block or ice, then proceed to "Set up cycle sequencing reactions" on page 23.

Set up cycle sequencing reactions

Note: Based on user preference, the PR/RT and IN cycle sequencing reactions can occur in parallel on the same plate with the same thermal cycler.

IMPORTANT! Protect the sequencing mixes from light.

Before you begin:

- For PR/RT, in the Preamplification laboratory area, completely thaw the following six sequencing mixes provided with the Applied Biosystems™ HIV-1 Genotyping Kit with Integrase on a cold block or ice: R3, R2, R1, F3, F2, F1. Vortex briefly, then centrifuge for 2–3 seconds to collect contents at the bottom of the tubes.
- For IN, in the Preamplification laboratory area, completely thaw the following four sequencing mixes provided with the Applied Biosystems™ HIV-1 Genotyping Kit with Integrase on a cold block or ice: R12, R11, F12, F11. Vortex briefly, then centrifuge for 2–3 seconds to collect contents at the bottom of the tubes.
- Briefly vortex and centrifuge the ExoSAP-IT™-treated nested PCR products.

1. In the Preamplification laboratory area, add 18 µL of each of the ten sequencing mixes corresponding to each ExoSAP-IT™-treated nested PCR products, to the appropriate wells of a chilled 96-well reaction plate.
2. Transfer the plate to the Postamplification laboratory area, then add the following:
   - 2 µL of ExoSAP-IT™-treated nested PCR products to each sequencing mix
3. Seal the plate, then immediately proceed to “Run the cycle sequencing reactions” on page 23.

Run the cycle sequencing reactions

1. Set the cycle sequencing conditions.

IMPORTANT! See the recommended thermal cyclers in Table 7.

IMPORTANT! Use 9600 emulation/simulation mode. If 9600 emulation/simulation mode is not available on your thermal cycler, contact technical support (see “Customer and technical support” on page 34) for guidance on how to adjust the ramp rates on your specific thermal cycler to mimic 9600 ramp rates when using a 20 µL reaction volume.

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	

IMPORTANT! Do not modify the thermal cycler conditions.

2. Set the reaction volume to 20 µL, then start the run.
3. Load the 96-well reaction plate into the instrument.
4. When the run is complete, proceed to "Purify sequencing reactions with the BigDye XTerminator™ Purification Kit" on page 24.

Purify sequencing reactions with the BigDye XTerminator™ Purification Kit

IMPORTANT! To ensure proper purification of sequencing reactions, follow the pipetting guidelines (see the *BigDye XTerminator™ Purification Kit User Guide* (Pub. No. 4374408)).

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 and wide bore tips are used.

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**

   IMPORTANT! Ensure the cycle sequencing plate has been vortexed and centrifuged prior to removing the MicroAmp™ Clear Adhesive Film.

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. Use a tissue to carefully wipe off or absorb any liquid present at the top of or between the wells, then seal the plate using a MicroAmp™ Clear Adhesive Film.
6. Vortex the plate for 30 minutes at 1,800 rpm.

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

8. Proceed immediately to "Run capillary electrophoresis (CE)" on page 25.

Run capillary electrophoresis (CE)

1. Ensure that the correct consumables are installed on the CE instrument.

CE instrument	Consumable
3500/3500xL Series Genetic Analyzers	POP-7™ Polymer for 3500/3500xL Series Genetic Analyzers
	50-cm capillary array

2. Ensure that the instrument is calibrated with BigDye™ Terminator v3.1 Dye Set Z.

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 CE instrument user guide.

Note: If the instrument does not contain the appropriate run modules, see "Customer and technical support" on page 34.

CE instrument	Run module
3500 Series Genetic Analyzer	BDxFastSeq50_Pop7
3500xL Series Genetic Analyzer	BDxFastSeq50_Pop7xl

5. Use the factory-provided analysis protocol: BDT v3.1_PA_Protocol_POP7.

6. Set up the result group for the CE run.

7. Set up the plate record for the CE run.

Note: Ensure that you follow the appropriate sample naming convention for the data analysis software used in your laboratory.

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

9. Load the plate into the instrument, then start the run.

IMPORTANT! Only run with one plate at a time.

10. When the run is complete, proceed to "Determine sequence quality" on page 26.

Determine sequence quality

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

See your CE instrument user guide for standard procedures for sequence detection and analysis.

Analyze the sequencing traces

To analyze the sequencing traces, export the AB1 files from the 3500 Series Genetic Analyzer for use in downstream analysis and reporting software.

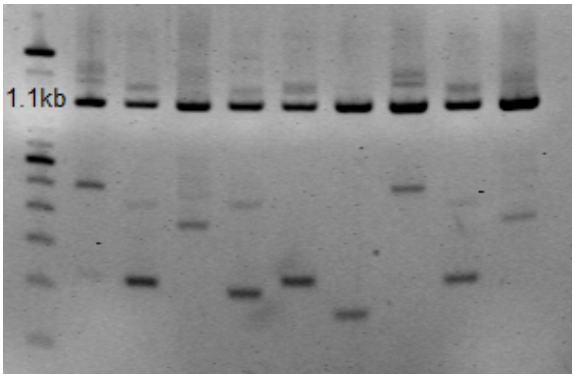
Note: For available commercial software, see **https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/genotyping-hiv-detect-drug-resistance.html**.
Navigate to the **Assay workflow**, then select **Step 4: Data analysis and results**.

To learn more about available commercial software, consult the WHO documents listed in “Related documentation” on page 34.

A

Troubleshooting

Observation	Possible cause	Recommended action
No amplification or low sample band intensity. Positive control amplifies as expected.	Too few viral HIV-1 RNA copies in the RT-PCR reaction.	Collect a fresh sample and re-process.
		Repeat RT-PCR and nested PCR and ensure thorough mixing.
		Repeat extraction and use a larger input volume of plasma, if possible. Repeat RT-PCR and nested PCR.
		Repeat extraction and use a lower elution volume, if possible. Repeat RT-PCR and nested PCR.
	Presence of an unknown PCR amplification inhibitor.	Dilute RNA and repeat RT-PCR and nested PCR.
	RT-PCR product may have been omitted when setting up the nested PCR reaction plate.	Repeat nested PCR.
	PR/RT RT-PCR product may have been used with IN nested PCR reaction mix.	Repeat nested PCR.
	IN RT-PCR product may have been used with PR/RT nested PCR reaction mix.	Repeat nested PCR.
No bands are present on the gel. Positive control fails to amplify.	The RT-PCR or nested PCR (PR/RT or IN) failed to amplify. Enzyme may not have been added to the RT-PCR Master Mix or Nested-PCR Master Mix.	Repeat with fresh reagents. Do not mix lot numbers.

Observation	Possible cause	Recommended action
Incorrect band sizes are present on the gel 	Mispriming or internal deletion occurred.	Larger bands do not affect sequencing. Proceed if major band at correct size is present.
		If smaller sized bands of equal or brighter intensity are present, repeat the entire procedure starting from RT-PCR. Smaller bands may reduce sequencing quality and make genotyping difficult.
Bands are present in the negative control well of the gel and/or 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 16.
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 **thermofisher.com**.
	There is a problem with the E-Gel™ stain or loading dye.	If you use an E-Gel™, use ethidium bromide or 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.
	Gel run at too high voltage or for too little time.	Calculate appropriate voltage and run time using recommendations from **thermofisher.com**.
Poor quality sequence	Poor PCR product cleanup.	Use fresh ExoSAP-IT™ Reagent.

Observation	Possible cause	Recommended action
Poor quality sequence *(continued)*	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.
	Contamination occurred.	Decontaminate the laboratory, then repeat the sequencing reactions with fresh reagents. For more information, see the *Troubleshooting Sanger sequencing data User Bulletin* (Pub. No. MAN0014435).
	Incorrect dye set selected.	Ensure that a BigDye™ Terminator v3.1 analysis protocol is selected.
	The wrong run module or conditions was used (for example, a standard run module instead of a BigDye XTerminator™ Purification Kit run module).	If you used the BigDye XTerminator™ Purification Kit for purification, you must use a run module with a "BDX" prefix for capillary electrophoresis.
		For more information, see the *Troubleshooting Sanger sequencing data User Bulletin* (Pub. No. MAN0014435).

B

Control sequences

Partial sequence of RNA Positive Control, PR/RT/IN

A partial sequence of the synthetic RNA Positive Control is shown below.

The sequence encompasses:

Note: The positive control will align to the reference sequence as multiple fragments.

- Partial protease (PR) region
- Partial reverse transcriptase (RT) region
- Complete integrase (IN) region

```
   1 CTTTAACTTC CCTCAAATCA CTCTTTGGCA GCGACCCCTT GTCTCAATAA AAGTAGGGGG CCAGATAAAG
  71 GAGGCTCTCT TAGACACAGG AGCAGATGAT ACAGTATTAG AAGAAATAAG TTTGCCAGGA TAATGGAAAC
 141 CAAAAATGAT AGGAGGAATT GGAGGTTTTA TGAAAGTAAG ACAGTATGAT CAAATACTTA TAGAAATTTG
 211 TGGAAAAAAG GCTATAGGTA CAGTATTAGT AGGACCTACA CCTGTCAACA TAATTGGAAG AAATATGATG
 281 ACTCAGCTTG GATGCACACT AAATTTTCCA ATTAGTCCTA TTGAAACTGT ACCAGTATGG CCATTGACAG
 351 AAGAGAAAAT AGAAGAACTG GAGAAGGAAG GAAAAATTAC AAAAATTGGG CCTGAAAATC CATATAACAC
 421 TCCAGTATTT GCCATAAAGA GGAAGGACTG AACTAAGTGG AGAAAATTAG TAGATTTCAG GGAAGTTCAA
 491 TTAGGAATAC CACACCCAGC AGGGTTAAAA AAGAATAAAT CAGTGACAGT ACTGGATGTG GGGGATGCAT
 561 ATTTTTCAGT TCCTTTAGAT GAAGACTTCA GGAAATATAC TGCATTTACC ATACCTAGTA TAAACAATGA
 631 AACACCAGGG ATTAGATATC AATATAATGT GCTTCCACAG GGATGGCCAG AAATAGTCAT CTGTCAATAA
 701 GTGGATGACT TGTATGTAGG ATCTGACCTA TTAAAGTGGG GACTTACCAC ACCAGACAAG AAACATCAGA
 771 AAGAACCCCC ATTTCTTTGG ATGGGGTATG AACTCCATCC TGACAAATGG ACAGTACAGC CTATACAGCT
 841 GCCAGAAAAG GATAGCTGGA CTGTCAATGA CATACAGAAG TTAGTGGGAA AATTAAACTG GGCAAGTCAG
 911 ATTTATGCAG GGATTAAGTC ATGGGTACCA GCACATAAAG GAATTGGAGG AAATGAACAA GTAGATGTAA
 981 GTAGTGGAAT CAGGAAAGTG CTGTTTCTAG ATGGAATAGA TAAGGCTCAA GAAGAGCATG AAAAATATCA
1051 CAGCAATTGG AGAGCAATGG CTAGTGAGTT TAATCTGCCA CCCATAGTAG CAAAAGAAAT AGTAGCTAGC
1121 TGTGATAAAT GTCAGCTAAA AGGGGAAGCC ATACATGGAC AAGTAGACTG TAGTCCAGGG ATATGACAAT
1191 TAGATTGTAC ACATTTAGAA GGAAAAATCA TCATGGTAGC AGTCCATGTA GCCAGTGGCT ACATAGAAGC
1261 AGAGGTTATC CCAGCAGAAA CAGGACAAGA AACAGCATAC TATATACTAA AATTAGCAGG AAGATGGCCA
1331 GTCAAAGTAA TACATACAGA CAATGGCAGT AATTTCACCA GTGCTGCAGT TAAGGCAGCC TGTTGGTGGG
1401 CAGGTATCCA ACAGAAATTT AGCATTCCCT ACAATCCCCA AAGTAAGGGA GTAGTAGAAT CCATGAATTA
1471 AGAATTAAAG AAAATCATAG GGCAGGTAAG AGATCAAGCT GAGCACCTTA AGACAGCAGT ACAAATGGCA
1541 GTATTCATTC ACAATTTTAA AAGAAAAGGG GGGATTGGGG GGTACAGTGC AGGGGAAAGA ATAATAGACA
1611 TAATAGCAAC AGACATACAA ACTAAAGAAT TACAAAAACA AATTATAAAA ATTCAAAATT TTCGGGTTTA
1681 TTACAGAGAC AGCAGAGACC CTATTTGGAA AGGACCAGCC AAACTACTCT GGAAAGGTGA AGGGGCAGTA
1751 GTAATACAAG ATAACAGTGA CATAAAGGTA GTACCAAGGA AGAAAGCAAA AATCATTAAG GACTATGGCA
1821 AGCAAATGGC AGGTGCTGAT TGTGTGGCAG GTAGACAGGA TGAAGATTAG AACATGGAAT AGTTTAGTAA
1891 AGCACCAGTT CAGAAGTACA CATCCCATTA G
```

Mutation profile of RNA Positive Control

The RNA Positive Control contains the following mutations (the mutations in bold cause varying levels of drug resistance).

Note: Amino acid numbering is based on the codon position in HIV-1 HXB2, not the codon position in the RNA Positive Control.

- Protease: N37S, R41*, **I54M**, and **L90M**
- Reverse transcriptase: **M41L**, **K65R**, S68*, **K103N**, K122E, **Y181C**, Y183*, **M184V**, and F214L
- Integrase: W61*, L74M, E138K, G140S, Q148K, K156*, and R263K

Note: * Protease position 41, reverse transcriptase positions 68 and 183, and integrase positions 61 and 156 encode stop codons.

C

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, visit **thermofisher.com/support**.

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number
3500/3500xL Genetic Analyzer with 3500 Series Data Collection Software 3.3 User Guide	100079380
Veriti™ Thermal Cycler User Guide	4375799
GeneAmp™ PCR System 9700 Base Module User Manual	4303481
BigDye XTerminator™ Purification Kit User Guide	4374408
Troubleshooting Sanger sequencing data	MAN0014435
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit for HIV-1 Dried Blood Spots User Guide	MAN0001765
WHO HIVResNet meeting report, Johannesburg, South Africa, 19 October 2019. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.	—
WHO HIVResNet HIV drug resistance laboratory operational framework, second edition. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.	—
WHO Guidelines for using HIV testing technologies in surveillance: selection, evaluation and implementation – 2009 update	—

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
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  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support

- 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

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.