USER GUIDE

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Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR

Compatible with Tempus® and PAXgene® Blood RNA Tubes

Catalog Numbers 4449079, 4449080, 4449082

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TRADEMARKS

Part Number 4449675 Rev. C
June 2011

Contents

Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR

Product information

Compatible sample source

The Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR is compatible with:

- Tempus® Blood RNA Tubes
- PAXgene® Blood RNA Tubes

Purpose of the product

The Stabilized Blood-to-C_T kit allows you to prepare stabilized blood samples for quantitative RT-PCR (qRT-PCR) without conventional RNA purification.

Traditionally, the first step in gene expression experiments that use stabilized blood has been to recover pure RNA. However, even the quickest and simplest techniques for RNA isolation are time consuming (often requiring 2 hours or more of hands-on sample manipulation). The Stabilized Blood-to-C_T kit technology allows you to reverse-transcribe digested pellets from 500-µL aliquots of stabilized blood without isolating or purifying RNA. You can perform real-time PCR analysis immediately afterwards. Eliminating the RNA isolation step substantially expedites and simplifies gene expression analysis.

The digested stabilized blood samples exhibit sensitivity and specificity similar to that from purified RNA in real-time RT-PCR. The digestion procedure simultaneously prepares samples for RT-PCR and removes genomic DNA in under 10 minutes. The digestion step is simple to automate with robotic platforms for high-throughput processing of MagMAX™ Express-96 Deep Well Plates, because it takes place entirely at room temperature.

Applications

You can use the Stabilized Blood-to-C_T kit for:

- **Low-throughput applications** – Perform the RNA isolations in 1.5-mL microfuge tubes (for example, Ambion® Non-Stick RNase-free Microfuge Tubes).
- **Medium-throughput applications** – Perform the RNA isolations in MagMAX™ Express-96 Deep Well Plates.

Kit contents and storage conditions

Upon receipt store each component as described in the following table.

Kit part number	Kit name	Kit contents			
		Box	Component	Quantity	Storage conditions
4449080	Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR (Compatible with Tempus® Blood RNA Tubes), 200 reactions	1	Digestion Solution	20 mL	4°C
			Tempus® Pellet Enhancer	4 mL	
			Tempus® Wash #1	150 mL	
			Tempus® Wash #2	150 mL	
			2X PBS for Tempus®	50 mL	
		2	Stop Solution	2 mL	–20°C
			DNase 1	200 µL	
4449082	Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR (Compatible with PAXgene® Blood RNA Tubes), 200 reactions	1	Digestion Solution	20 mL	4°C
			Wash Solution (Compatible with PAXgene® Blood RNA Tubes)	300 mL	
		2	Stop Solution	2 mL	–20°C
			DNase 1	200 µL	
4449079†	Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR (50 reactions)	1	Digestion Solution	5 mL	4°C
			Tempus® Pellet Enhancer	1 mL	
			Tempus® Wash #1	37.5 mL	
			Tempus® Wash #2	37.5 mL	
			2X PBS for Tempus®	12.5 mL	
			Wash Solution (Compatible with PAXgene® Blood RNA Tubes)	75 mL	
		2	Stop Solution	0.5 mL	–20°C
			DNase 1	50 µL	

† Kit part number 4449079 is a starter kit that contains reagents for both the Tempus® and PAXgene® Blood RNA Tubes.

User-supplied materials

Unless otherwise noted, reagents and materials are available from **www.lifetechnologies.com** (Applied Biosystems).

IMPORTANT! For the Safety Data Sheet (SDS) of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Sample collection and stabilization

Item	Source (Part no.)
Tempus® Blood RNA Tubes (50 tubes)	4342792
PAXgene® Blood RNA Tubes (100 tubes)	PreAnalytiX GmbH (762165)
Blood samples collected in the Tempus® or PAXgene® Blood RNA Tubes	–

Reagents

Control kits

Life Technologies recommends the TaqMan® Cells-to-C_T Control Kit for general use. You can use the Xeno™ RNA Control included in the kit as a positive control for inhibition testing. For a reference on inhibition test setup, see Appendix B, "Inhibition Experiment" on page 29.

Kit	Source (Part no.)
TaqMan® Cells-to-C_T Control Kit	4386995

RT-PCR

Life Technologies recommends the reverse-transcription and PCR reagents listed below for samples prepared with the Stabilized Blood-to-C_T kits.

Kit	Source (Part no.)
High Capacity RNA-to-cDNA™ Kit	4387406
SuperScript® VILO™ cDNA Synthesis Kit	Life Technologies (Invitrogen) (11754-050)
TaqMan® MicroRNA Reverse Transcription Kit	4366596
TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4309169
TaqMan® Gene Expression Master Mix	4369016
TaqMan® Universal Master Mix II, no UNG	4440040
TaqMan® Universal Master Mix II, with UNG	4440038

Additional reagents

The table below lists the additional reagents that are not included in any of the kits.

Reagent	Source (Part no.)
Phosphate-buffered saline (PBS)	MLS†
TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays	Life Technologies (Applied Biosystems)
DNA*Zap*™ Solution	AM9890
RNase*Zap*® Solution	AM9780
Nuclease-free water	MLS

† Major laboratory supplier

General laboratory equipment

Item	Source (Part no.)
Disposable gloves	MLS†
Pipette tips (aerosol-resistant, nuclease-free)	MLS
Pipettes (positive/air-displacement or multichannel)	MLS
Vortexer	MLS
Centrifuge, with plate adapter (capable of 5000 × *g*)	MLS
Microcentrifuge	MLS
Non-Stick RNase-free Microfuge Tubes (1.5-mL)	AM12450
RNase-free Microfuge Tubes (1.5-mL)	AM12400
MagMAX™ Express-96 Deep Well Plates (for medium-throughput applications	4388476

† Major laboratory supplier

Thermal cycler and PCR system tubes, plates, and accessories

The table below lists the reaction tubes, plates, and accessories available for Applied Biosystems thermal cyclers and real-time PCR systems.

Note: The Stabilized Blood-to-C_T kits were developed using Applied Biosystems thermal cyclers for the RT reaction and real-time PCR instruments for the PCR. However, the technology is expected to be compatible with any thermal cycler for the RT reaction and any real-time PCR system for the RT reaction and PCR.

Instrument	Reaction tubes, plates, and accessories (Part no.)
GeneAmp® PCR System 9700	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (4326659) – 20 plates (4306737) • ABI PRISM® 384-Well Clear Optical Reaction Plate with Barcode: – 1000 plates (4343814) – 500 plates (4326270) – 50 plates (4309849) • MicroAmp® Optical Adhesive Film (4311971) • MicroAmp® Clear Adhesive Films, 100 films (4306311) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (4323032)
Veriti® 96-Well Thermal Cycler	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (4326659) – 20 plates (4306737) • MicroAmp® Optical Adhesive Film (4311971) • MicroAmp® Optical Film Compression Pad (4312639)
7500 Fast Real-Time PCR System	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (4366932) – 20 plates (4346906) • MicroAmp® Optical Adhesive Film (4311971)

Instrument	Reaction tubes, plates, and accessories (Part no.)
7900HT Fast Real-Time PCR System, 384-Well Block Module or Standard 96-Well Block Module	• MicroAmp® Optical 384-Well Reaction Plate with Barcode (4326270) • MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (4326659) – 20 plates (4306737) • MicroAmp® Optical Adhesive Film (4311971) • MicroAmp® Optical Film Compression Pad (4312639) for use with one plate • MicroAmp® Snap-On Optical Film Compression Pad (4333292) for use with automation accessory
StepOne™ or StepOnePlus™ system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (4366932) – 20 plates (4346906) • MicroAmp® Optical Adhesive Film (4311971)

Sample preparation procedure for Tempus® Blood RNA Tubes

Required kit

Use one of the following kits for this procedure:

Kit name	Part no.
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR (Compatible with Tempus® Blood RNA Tubes)	4449080
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR	4449079†

† Kit part number 4449079 is a starter kit that contains reagents for both the Tempus® and PAXgene® Blood RNA Tubes.

Workflow

(Optional) Collect blood samples (page 10)

Pellet and wash the sample (page 11)

Pellet the sample

Wash the sample

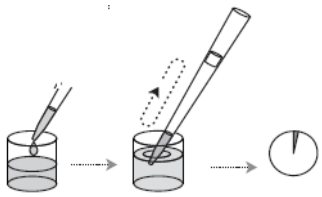
Perform digestion (page 13)

(Optional) Mix the Digestion Solution with DNase I

Add the Digestion Solution and incubate

(Optional) Mix the Stop Solution with Xeno™ RNA Control

Add the Stop Solution and incubate



(Optional) Collect blood samples

This section provides brief procedures for collecting blood samples with the Tempus® Blood RNA Tubes. For additional information, refer to the product documentation for your blood collection set.

Note: If you are using the Greiner Vacuette® Safety Blood Collection Set, go to the Vacuette Web site for additional information (**www.vacuette.com**).

Collect blood samples

WARNING! Biological samples have the potential to transmit infectious diseases. For safety and biohazard guidelines, see "Biological hazard safety" on page 34.

1. Draw 3 mL of blood directly into Tempus® Blood RNA Tubes according to your laboratory's standard procedures.

   Note: The black mark on each tube label indicates approximately 3 mL.

2. Immediately after filling each Tempus tube, vigorously shake or vortex the tube for 10 seconds to ensure that the Stabilizing Reagent makes uniform contact with the sample.

   IMPORTANT! Failure to mix the stabilizing reagent with the blood leads to inadequate stabilization of the gene expression profile and the formation of microclots that can potentially compromise the RNA purification procedure.

Storage conditions

We recommend that you store Tempus tubes containing stabilized samples in the following order of preference:

Storage option	Temperature
Store refrigerated for up to 7 days.	4°C
Freeze for long-term storage. **IMPORTANT!** Do not let the samples come into contact with the dry ice.	–20 to –80°C
Store at room temperature for up to 5 days.	18 to 25°C

Pellet and wash the sample

Pellet the sample

1. If the Tempus tubes have been frozen, allow them to thaw at room temperature (~15 minutes).
2. Transfer 500 µL of Tempus stabilized blood to:
   - A 1.5-mL microfuge tube

     OR
   - MagMAX™ Express-96 Deep Well Plate (also referred to in this guide as *Express-96 plate*)

     IMPORTANT! You must use a MagMAX™ Express-96 Deep Well Plate. This protocol has not been optimized for use with any other plate.

3. Add 250 µL of 2✕ PBS for Tempus® to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp® adhesive film.

   IMPORTANT! Make sure that the Express-96 plate is sealed securely; if the wells are not sealed firmly, cross-contamination of samples may occur.

4. Vortex the tube or plate for 10 seconds, then centrifuge briefly to remove droplets from inside of the cap or adhesive film.
5. Add 20 µL of Tempus® Pellet Enhancer to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp adhesive film.
6. Vortex the tube or plate for 10 seconds, then centrifuge at 5000 × *g* for 10 minutes to pellet the sample.
7. With a pipette, remove and discard the supernatant, being careful not to disturb the pellet. Some residual supernatant may be left behind (up to 50 µL).

Wash the sample

1. Add 750 µL of Tempus® Wash #1 to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp adhesive film.
2. Vortex the tube or plate until the pellet is dissolved.

   Note: The pellet may resuspend in Tempus Wash #1 immediately before vortexing. If the pellet is large and does not resuspend easily, vortex for 2 minutes, then proceed to step 3.
3. To pellet the sample, centrifuge the:
   - Tube at 5000 × *g* for 2 minutes
   - Plate at 5000 × *g* for 5 minutes
4. With a pipette, remove and discard the Tempus Wash #1, being careful not to disturb the pellet. Some residual supernatant will be left behind (~20 µL).
5. Add 750 µL of Tempus® Wash #2 to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp adhesive film.
6. Vortex the tube or plate until the pellet is dissolved.

   Note: The pellet may resuspend in Tempus Wash #2 before vortexing. If the pellet is large and does not resuspend easily, vortex for 2 minutes, then proceed to step 7.
7. To pellet the sample, centrifuge the:
   - Tube at 5000 × *g* for 2 minutes
   - Plate at 5000 × *g* for 5 minutes
8. With a pipette, completely remove and discard Tempus Wash #2, being careful not to disturb the pellet.
9. Place the tube or plate on ice.

Perform digestion

Thaw the Stop Solution

1. Thaw the Stop Solution at room temperature.
2. Mix the thawed Stop Solution by flicking or inverting the tube several times, then place the tube on ice.

(Optional) Mix the Digestion Solution with DNase I

To remove genomic DNA during digestion, dilute the DNase I into the Digestion Solution at 1:100.

1. Per the table below, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 reaction
Digestion Solution	99 µL
DNase 1	1 µL
Total volume required for 1 reaction	**100 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.

Add the Digestion Solution and incubate

1. Add 100 µL of room-temperature Digestion Solution (with or without DNase 1) to the microfuge tube or Express-96 plate (containing the pellet).
2. Mix by pipetting up and down 5 times to break up the pellet. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.
3. Incubate at room temperature (19 to 25°C) for 8 minutes.

   Note: The Digestion Solution and samples may appear cloudy at room temperature; this is expected.

(Optional) Mix the Stop Solution with Xeno™ RNA Control

To include an endogenous control using the TaqMan® Cells-to-C_T Control Kit, add Xeno™ RNA Control to the Stop Solution.

1. Per the table below, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 reaction
Stop Solution	10 µL
Xeno™ RNA Control	2 µL
Total volume required for 1 reaction	**12 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.
3. Place the mixture on ice.

Add the Stop Solution and incubate

1. Add 10 µL of the Stop Solution, or 12 µL of the Stop Solution/Xeno RNA Control mixture, to the microfuge tube or Express-96 plate (containing the sample).

   Touch the surface of the liquid sample with the opening of the pipette tip to ensure that all of the Stop Solution is added to the sample.
2. Mix by pipetting up and down 5 times. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.

   IMPORTANT! Be sure to thoroughly mix the Stop Solution into the sample.
3. Incubate at room temperature (19 to 25°C) for 2 minutes.

   Note: After adding the Stop Solution, do not allow the samples to remain at room temperature for longer than 20 minutes.

   STOPPING POINT You can store the samples at 4°C for up to 1 hour, or at –20 to –80°C for up to 5 months.

Sample preparation procedure for PAXgene® Blood RNA Tubes

Required kit

Use one of the following kits for this procedure:

Kit name	Part number
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR (Compatible with PAXgene® Blood RNA Tubes)	4449082
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR	4449079†

† Kit part number 4449079 is a starter kit that contains reagents for both the Tempus® and PAXgene® Blood RNA Tubes.

Workflow

(Optional) Collect blood samples (page 15)

Pellet and wash the sample (page 16)

Pellet the sample

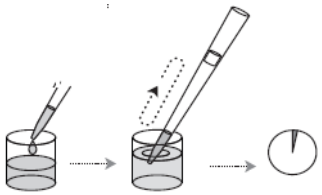
Wash the sample

Perform digestion (page 17)

Thaw the Stop Solution

(Optional) Mix the Digestion Solution with DNase I

Add the Digestion Solution and incubate



(Optional) Mix the Stop Solution with Xeno™ RNA Control

Add the Stop Solution and incubate

(Optional) Collect blood samples

This section provides brief procedures for collecting blood samples with the PAXgene® Blood RNA Tubes. For additional information, refer to the product documentation for your blood collection set.

Note: If you are using the BD Vacutainer® Safety-Lok™ Blood Collection Set, go to the Becton, Dickinson and Company Web site for additional information (**www.bd.com**).

Collect blood samples

WARNING! Biological samples have the potential to transmit infectious diseases. For safety and biohazard guidelines, see "Biological hazard safety" on page 34.

1. Draw 2.5 mL of blood directly into PAXgene® Blood RNA Tubes according to your laboratory's standard procedures.

   Note: Collecting 2.5 mL of blood into a PAXgene tube requires at least 10 seconds.

2. Immediately after filling each PAXgene tube, gently invert the tube 8 to 10 times to ensure that the RNA stabilizing additive makes uniform contact with the sample.

   IMPORTANT! Failure to mix the stabilizing additive with the blood leads to inadequate stabilization of the gene expression profile and can potentially compromise the RNA purification procedure.

3. Incubate the samples at room temperature (18 to 25°C) for 2 to 72 hours before processing.

Storage conditions

Refer to the current *PreAnalytiX® RNA Tube Product Circular* for recommendations on storing PAXgene tubes that contain stabilized blood samples.

Pellet and wash the sample

Pellet the sample

1. If the PAXgene tubes have been frozen, allow them to thaw at room temperature for at least 2 hours.
2. Transfer 500 µL of PAXgene stabilized blood to:
   - A 1.5-mL microfuge tube

     OR
   - MagMAX™ Express-96 Deep Well Plate (also referred to as *Express-96 plate*)

     IMPORTANT! You must use a MagMAX™ Express-96 Deep Well Plate. This protocol has not been optimized for use with any other plate.
3. Centrifuge the tube or plate at 5000 × *g* for 10 minutes to pellet the sample.
4. With a pipette, remove and discard the supernatant, being careful not to disturb the pellet. Some residual supernatant may be left behind (up to 50 µL).

Wash the sample

1. Add 750 µL of Wash Solution (Compatible with PAXgene® Blood RNA Tubes) to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp® adhesive film.

   IMPORTANT! Make sure that the Express-96 plate is sealed securely; if the wells are not sealed firmly, cross-contamination of samples may occur.

2. Vortex the tube or plate until the pellet is dissolved.

   Note: The pellet may resuspend in Wash Solution before vortexing. If the pellet is large and does not resuspend easily, vortex for 2 minutes, then proceed to step 3.

   STOPPING POINT You can store the mixture overnight at –20°C.

3. To pellet the sample, centrifuge the:
   - Tube at 5000 × *g* for 2 minutes
   - Plate at 5000 × *g* for 5 minutes
4. With a pipette, remove and discard the Wash Solution, being careful not to disturb the pellet. Some residual wash may be left behind (up to 50 µL).
5. Add 750 µL of Wash Solution to the tube or plate, then cap the tube or seal the plate firmly with a MicroAmp adhesive film.
6. Vortex until the pellet is dissolved.

   Note: The pellet may resuspend in Wash Solution before vortexing. If the pellet is large and does not resuspend easily, vortex for 2 minutes, then proceed to step 7.
7. To pellet the sample, centrifuge the:
   - Tube at 5000 × *g* for 2 minutes
   - Plate at 5000 × *g* for 5 minutes
8. With a pipette, completely remove and discard Wash Solution, being careful not to disturb the pellet.
9. Place the tube or plate on ice or hold at 4°C.

Perform digestion

Thaw the Stop Solution

1. Thaw the Stop Solution at room temperature.
2. After thawing, mix by flicking or inverting the tube several times, then place the tube on ice.

(Optional) Mix the Digestion Solution with DNase I

To remove genomic DNA during digestion, dilute the DNase I into the Digestion Solution at 1:100.

1. Per the following table, calculate the total volume required for each component: *volume per reaction × the total number of reactions*

Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume per reaction
Digestion Solution	99 µL
DNase 1	1 µL
Total volume required for 1 reaction	**100 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.

Add the Digestion Solution and incubate

1. Add 100 µL of room-temperature Digestion Solution (with or without DNase 1) to the microfuge tube or Express-96 plate (containing the pellet).
2. Mix by pipetting up and down 5 times to break up the pellet. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.
3. Incubate at room temperature (19 to 25°C) for 8 minutes.

   Note: The Digestion Solution and samples may appear cloudy at room temperature; this is expected.

(Optional) Mix the Stop Solution with Xeno™ RNA Control

To include an endogenous control using the TaqMan® Cells-to-C_T Control Kit, add Xeno™ RNA Control to the Stop Solution.

1. Per the table below, calculate the total volume required for each component:
   volume per reaction × the total number of reactions

   Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume per reaction
Stop Solution	10 µL
Xeno™ RNA Control	2 µL
Total volume required for 1 reaction	**12 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.
3. Place the mixture on ice.

Add the Stop Solution and incubate

1. Add 10 µL of the Stop Solution, or 12 µL of the Stop Solution/Xeno RNA Control mixture, to the microfuge tube or Express-96 plate (containing the sample).

   Touch the surface of the liquid sample with the opening of the pipette tip to ensure that all of the Stop Solution is added to the sample.

2. Mix by pipetting up and down 5 times. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.

   IMPORTANT! Be sure to thoroughly mix the Stop Solution into the sample.

3. Incubate at room temperature (19 to 25°C) for 2 minutes.

   Note: After adding the Stop Solution, do not allow the samples to remain at room temperature for >20 minutes.

STOPPING POINT You can store the samples at 4°C for up to 1 hour, or at –20 to –80°C for up to 5 months.

Perform qRT-PCR

This section provides guidelines for using the prepared samples in reverse transcription and real-time PCR with the following recommended RT-PCR reagent combinations:

- High Capacity RNA-to-cDNA™ Kit and TaqMan® Gene Expression Master Mix (this page)
- SuperScript® VILO™ cDNA Synthesis Kit and TaqMan® Gene Expression Master Mix (page 20)
- TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal Master Mix II (page 21)
- TaqMan® One-Step RT-PCR Master Mix Reagents Kit (page 21)

Appendix A, "Example qRT-PCR Procedures" includes detailed qRT-PCR procedures for the High Capacity RNA-to-cDNA™ Kit and TaqMan® Gene Expression Master Mix. You can use Appendix A as an example when performing the qRT-PCR procedures with other reagents.

High Capacity RNA-to-cDNA™ Kit and TaqMan® Gene Expression Master Mix

RT master mix

Recommended volumes for the RT master mix, using the High Capacity RNA-to-cDNA Kit:

Component	Volume per reaction
2× RT Buffer	10 µL
20× RT Enzyme Mix	1 µL
Nuclease-free water	5 µL
Sample	4 µL
Total volume of RT master mix for 1 reaction	**20 µL**

PCR mix

Recommended volumes for the PCR mix, using TaqMan® Gene Expression Master Mix:

Component	Volume per reaction
TaqMan® Gene Expression Master Mix (2X)	10 µL
TaqMan® Gene Expression Assay (20X)	1 µL
Nuclease-free water	5 µL
cDNA	4 µL
Total volume of PCR mix for 1 reaction	**20 µL**

SuperScript® VILO™ cDNA Synthesis Kit and TaqMan® Gene Expression Master Mix

RT master mix

Recommended volumes for the RT master mix, using the SuperScript® VILO™ cDNA Synthesis Kit:

Component	Volume per reaction
5X VILO™ Reaction Mix	4 µL
10X SuperScript® Enzyme Mix	2 µL
Nuclease-free water	10 µL
Sample	4 µL
Total volume of RT master mix for 1 reaction	**20 µL**

PCR mix

Recommended volumes for the PCR mix, using TaqMan® Gene Expression Master Mix:

Component	Volume per reaction
TaqMan® Gene Expression Master Mix (2X)	10 µL
TaqMan® Gene Expression Assay (20X)	1 µL
Nuclease-free water	7 µL
cDNA	2 µL
Total volume of PCR mix for 1 reaction	**20 µL**

TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal Master Mix II

RT master mix

Recommended volumes for the RT master mix, using the TaqMan® MicroRNA Reverse Transcription Kit:

Component	Volume per reaction
10× RT Buffer	0.8 µL
dNTPs with dTTP (100 mM)	0.2 µL
$MgCl_2$ (25 mM)	0.9 µL
RNase Inhibitor (20 U/µL)	0.1 µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	1.5 µL
RT Primers (10×)	0.8 µL
Nuclease-free water	0.2 µL
Sample	3.0 µL
Total volume of RT master mix for 1 reaction	**7.5 µL**

PCR mix

Recommended volumes for the PCR mix, using TaqMan® Universal Master Mix II:

Component	Volume per reaction
TaqMan® Universal Master Mix II, with or without UNG (2×)	10 µL
TaqMan® Assay (20×)	1 µL
cDNA + nuclease-free water†	9 µL
Total volume of PCR mix for 1 reaction	**20 µL**

† To each reaction, add 1 to 100 ng of cDNA diluted to the correct volume using nuclease-free water.

TaqMan® One-Step RT-PCR Master Mix Reagents Kit

Recommended volumes for the RT-PCR master mix, using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit:

Component	Volume per reaction
TaqMan® RT Enzyme Mix (40×)	0.5 µL
TaqMan® RT-PCR Mix (2×)	10.0 µL
TaqMan® Gene Expression Assay (20×)	1.0 µL
Nuclease-free water	7.5 µL
Sample	1.0 µL
Total volume of RT-PCR master mix for 1 reaction	**20.0 µL**

Troubleshooting

Observation	Possible cause	Recommended action
No PCR product	There were problems with adding or mixing the Stop Solution	Components in the Digestion Solution may inhibit RT-PCR if they are not fully inactivated by the Stop Solution: • Be sure to add the Stop Solution directly to the liquid: Touch the liquid sample with the opening of the pipette tip when adding the Stop Solution to make sure that the entire 10 or 12 µL of Stop Solution is added to each sample. • Mix by pipetting up and down 5 times.
	RNA was degraded before starting the procedure	To avoid RNA degradation, collect the blood sample and freeze and thaw the Tempus or PAXgene tubes according to the manufacturer's instructions.
	Inhibitors are present in the sample	Blood components or debris could inhibit reverse transcription or PCR: • Generally, this protocol removes inhibitors from 500 µL of stabilized blood samples. If the RT or PCR fails, try diluting the sample 1:5 or 1:10 in RNase-free water to reduce inhibitor concentration in the digested sample. • Consider performing an inhibition experiment to determine whether or not inhibitors are present in the digested blood samples. See "Inhibition Experiment" on page 29.
	Digested samples sat too long before going into the RT reaction	After adding the Stop Solution, do not let the digested samples sit longer than 20 minutes at room temperature. Either freeze the digested samples at –20 to –80°C, or immediately start the RT reactions. Alternatively, you can safely store the samples on ice for up to 1 hour after digestion.
	The pellet was removed with the supernatant during the wash steps	To avoid removing the pellet with the supernatant in: • Tubes – Slowly draw the supernatant into the pipette tip, with the tip point on the side of the tube that is opposite from the pellet. • MagMAX™ Express-96 Deep Well Plates – Slowly draw the supernatant from the top of the well; carefully lower the pipette tip while drawing up the supernatant.
	The 96-well plate used was not a MagMAX™ Express-96 Deep Well Plate	Suboptimal performance is observed in other 96-well plates. The Stabilized Blood-to-C_T kit protocols consistently perform best with the MagMAX™ Express-96 Deep Well Plates.

Observation	Possible cause	Recommended action
No PCR product *(Continued)*	Sample does not contain the target RNA	Negative results are often difficult to confirm as valid. Consider running the following experiments before concluding that the sample does not contain the RNA of interest: • Verify that the procedure is working by including Xeno™ RNA Control in the sample (see "(Optional) Mix the Stop Solution with Xeno™ RNA Control" on page 14 or page 18). Then use the Xeno RNA Control assay to amplify a Xeno RNA Control target when you perform the PCR. If product is generated in the Xeno RNA Control amplification but no product is seen in the PCR for the gene of interest, then it is possible that the RNA of interest is not expressed in the blood sample and/or is undetectable with this protocol. • Prior to digestion, the pellet can be difficult to see and could become dislodged by the pipette. It may be desirable to verify that the pellet was not lost during the procedure. Check that samples contained pellets with intact RNA by real-time RT-PCR with a PCR assay for a highly expressed endogenous control, such as β-actin. The ACTB assay included in the TaqMan® Cells-to-C_T Control Kit (sold separately) are designed for this purpose. • Check that the PCR for your target works with your PCR assay, reagents, and equipment by using cDNA generated from purified RNA from the same source (or a similar one) in the PCR. If the amplification does not give good results using cDNA from purified RNA, it will not work with Stabilized Blood-to-C_T samples.
RT-PCR products in the negative control reactions	PCR products are in the no-template control (NTC)	PCR products in the NTC most commonly indicate that this sample is contaminated with DNA, typically from completed PCRs. Contamination of PCR reagents, pipettes, and benchtops with DNA should be considered: • Careful laboratory practices are essential to avoid contaminating reactions with PCR products. Keep concentrated DNA solutions (for example, PCR products, plasmid preps) away from the area where PCRs are assembled. Routinely clean the benchtops and pipettes with *DNA*Zap™ Solution or another DNA decontamination product. Use barrier tips to pipette PCR reagents, and store completed PCRs in a different location from the PCR reagents. If the reagents are contaminated, they must be replaced. • It is always a good idea to routinely include a negative NTC reaction with experimental PCRs. If NTCs routinely yield PCR products, more stringent steps may be taken to control contamination.

A Example qRT-PCR Procedures

This appendix provides a detailed qRT-PCR procedure for the High Capacity RNA-to-cDNA™ Kit and TaqMan® Gene Expression Master Mix. You can use this procedure as an example when performing the qRT-PCR procedures with other reagents.

Required materials

Kit	Kit part number	No. of reactions
High Capacity RNA-to-cDNA™ Kit	4387406	50
TaqMan® Gene Expression Master Mix	4369016	200

About the volumes in this procedure

- **RT reaction volumes** – We recommend that you use 4 µL of sample in a 20-µL RT reaction. If a 20-µL RT reaction will not provide enough material for all of the planned PCRs, you can scale up the RT master mix components proportionally.
- **PCR volumes** – We recommend that 20% of the PCR volume be cDNA.

Workflow

Perform reverse-transcription (RT) (page 26)

Prepare the RT master mix

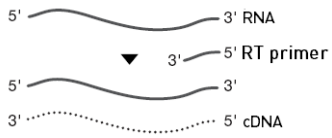
↓

Prepare the RT reaction tubes or plate

↓

Run the RT reaction

↓



Perform real-time PCR (page 27)

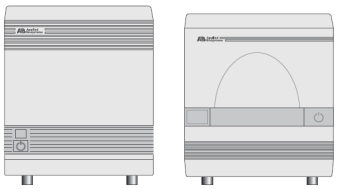
Prepare the PCR mix

↓

Prepare the reaction tubes or plate

↓

Perform the real-time run



Perform reverse-transcription (RT)

Prepare the RT master mix

1. Per the table below, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include ~10% excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 20-µL reaction	
	Plus-RT reaction	Minus-RT reaction
2× RT Buffer	10 µL	10 µL
20× RT Enzyme Mix†	1 µL	NA
Nuclease-free water	5 µL	6 µL
Total volume of RT master mix for 1 reaction	**16 µL**	**16 µL**

† For the minus-RT control, use nuclease-free water in place of the 20× RT Enzyme Mix.

2. Working on ice, add the required components to nuclease-free microcentrifuge tubes: one tube for the plus-RT reaction; one tube for the minus-RT reaction.
3. Mix gently (but thoroughly), then centrifuge briefly.
4. Place the tubes back on ice.

Prepare the RT reaction tubes or plate

1. Transfer 16 µL of the RT master mix to a nuclease-free PCR tube or to one well of a multi-well plate.
2. Add 4 µL of sample to each tube or well, for a final volume of 20-µL for each RT reaction.
3. Mix gently, then centrifuge briefly to collect the contents at the bottom of the tubes or wells.

STOPPING POINT You can store the assembled RT reactions at 4°C for up to 4 hours.

Run the RT reaction

1. Program a thermal cycler (or real-time PCR system) as described below:

Step	Stage	No. of cycles	Temperature	Time
Reverse-transcription (HOLD)	1	1	37 °C	60 min
RT Enzyme inactivation (HOLD)	2	1	95 °C	5 min
HOLD	3	1	4 °C	Indefinite

2. Start the run.

STOPPING POINT After the run is complete, you can store the RT reactions at –20°C.

Perform real-time PCR

Prepare the PCR mix

1. Mix the TaqMan® Gene Expression Master Mix by swirling the bottle.
2. Mix the PCR assay by vortexing briefly or flicking the tube, then centrifuge the tube.
3. Per the table below, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include ~10% excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 20-µL reaction	
	Xeno™ RNA Control reaction	β-Actin reaction
TaqMan® Gene Expression Master Mix	10 µL	10 µL
TaqMan® Xeno™ RNA Control Assay	1 µL	–
TaqMan® B-Actin Assay	NA	1 µL
Nuclease-free water	5 µL	5 µL
Total volume of PCR mix for 1 reaction	**16 µL**	**16 µL**

4. Working at room temperature, add the required components to a nuclease-free microcentrifuge tube.
5. Mix by gently vortexing.

Prepare the reaction tubes or plate

IMPORTANT! Be sure to use the reaction tubes or plate and the accessories that are compatible with your real-time PCR system. See "Thermal cycler and PCR system tubes, plates, and accessories" on page 8.

1. Working at room temperature, transfer the following components to individual reaction tubes or to wells of a reaction plate:

Component	Volume for 1 reaction
PCR mix	16 µL
RT master mix (containing cDNA)	4 µL
Total volume for 1 reaction	**20 µL**

2. Cover the plate or cap the tubes.
3. Mix by gently vortexing, then centrifuge briefly to remove bubbles and collect the contents at the bottom of the tubes or wells.

Perform the real-time run

1. Program a real-time PCR system with the following conditions:

Step	Stage	No. of cycles	Temperature	Time
Enzyme activation (HOLD)	1	1	95 °C	10 min
PCR (CYCLE)	2	40	95 °C	15 sec
			60 °C	1 min

2. If you are using an Applied Biosystems real-time PCR system that is capable of Fast mode thermal cycling, select **Standard mode**.
3. Load the tubes or plate into the instrument, then start the run.
4. After the run is complete, analyze the data using the real-time PCR system software. Refer to your real-time PCR system documentation for information on analyzing the data.

B Inhibition Experiment

About the inhibition experiment

Purpose

The purpose of the inhibition experiment is to determine whether or not the digested stabilized blood sample contains inhibitors to RT or qPCR. Although inhibitor concentrations are reduced through the pellet and wash steps of the Stabilized Blood-to-C_T™ kit procedure, some inhibitors may remain and affect the RT reactions or qPCRs. If inhibitors are present, dilute the digested sample 1:5 and 1:10 in RNase-free water and repeat the RT-PCR.

Experiment overview

To perform the inhibition experiment:

1. Aliquot 500 µL of stabilized blood in duplicate to 1.5-mL microfuge tubes or to a MagMAX™ Express-96 Deep Well Plate.
2. Pellet and wash the samples according to the Tempus® or PAXgene® Blood RNA Tubes procedure in this user guide.
3. Perform digestion, preparing enough Digestion Solution and Stop Solution with Xeno™ RNA Control for stabilized blood samples and no-blood control reactions in duplicate. We recommend that you add Xeno RNA Control to the Stop Solution used to prepare the digested samples.
4. Using the digested samples, perform real-time RT-PCR for an endogenous control gene, such as β-actin (a PCR assay for β-actin is included in the TaqMan® Cells-to-C_T Control Kit).
5. Amplify the cDNA in parallel using the Xeno RNA Control assay from the TaqMan® Cells-to-C_T Control Kit. In contrast to the series of PCRs for an endogenous control gene, stabilized blood in the digestion reaction should have no effect on the C_T values in the Xeno RNA Control amplification reactions, because each reaction contains the same amount of Xeno RNA Control target.

Procedure

Prepare stabilized blood aliquots

In 1.5-mL microfuge tubes or in an Express-96 plate, prepare replicate stabilized blood aliquots at 500 µL.

Pellet and wash the sample

Pellet and wash the samples according to the appropriate procedure:

- For Tempus® Blood RNA Tubes, see page 10.
- For PAXgene® Blood RNA Tubes, see page 15.

Perform digestion

Thaw the Stop Solution

1. Thaw the Stop Solution at room temperature.
2. Mix the thawed Stop Solution by flicking or inverting the tube several times, then place the tube on ice.

(Optional) Mix the Digestion Solution with DNase I

To remove genomic DNA during digestion, dilute the DNase I into the Digestion Solution at 1:100.

1. Per the table below, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 reaction
Digestion Solution	99 µL
DNase 1	1 µL
Total volume required for 1 reaction	**100 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.

Add the Digestion Solution and incubate

1. Add 100 µL of the Digestion Solution to each 1.5-mL microfuge tube or to wells of the Express-96 plate (containing pellets).
2. Add 100 µL of the Digestion Solution to new duplicate 1.5-mL microfuge tubes or to wells of the Express-96 plate (without pellets).
3. Mix by pipetting up and down 5 times to break up the pellet. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.
4. Incubate at room temperature (19 to 25°C) for 5 minutes.

   Note: The Digestion Solution and samples may appear cloudy at room temperature; this is expected.

Mix the Stop Solution with Xeno™ RNA Control

To include an endogenous control using the TaqMan® Cells-to-C_T Control Kit, add Xeno™ RNA Control to the Stop Solution.

1. Per the following table, calculate the total volume required for each component:
   volume for 1 reaction × the total number of reactions

   Include excess volume in your calculations to compensate for the loss that occurs during pipetting.

Component	Volume for 1 reaction
Stop Solution	10 µL
Xeno™ RNA Control	2 µL
Total volume required for 1 reaction	**12 µL**

2. Add the components to a microcentrifuge tube, then mix gently by pipetting up and down several times.
3. Place the mixture on ice.

Add the Stop Solution and incubate

1. Add 12 µL of the Stop Solution/Xeno RNA Control mixture to each tube.

   Touch the surface of the digestion reaction with the opening of the pipette tip to ensure that all of the Stop Solution is added to the digestion reaction.
2. Mix by pipetting up and down 5 times. To avoid bubbles, mix with the pipette set at 70 µL and do not completely empty the pipette tip.

   IMPORTANT! Be sure to thoroughly mix the Stop Solution into the digestion reaction.
3. Incubate at room temperature (19 to 25°C) for 2 minutes.

   Note: After adding the Stop Solution, do not allow the samples to remain at room temperature for longer than 20 minutes.

STOPPING POINT (Optional) You can store the samples at 4°C for up to 1 hour, or at 20 to –80°C for up to 5 months.

Perform qRT-PCR

Perform reverse-transcription and PCR according to "Perform qRT-PCR" on page 19. Use all samples and use primer-probe mixture for the following key targets:

- Amplify any endogenous control target. For example, use the β-actin PCR assay included in the TaqMan Cells-to-C_T Control Kit.
- If Xeno RNA Control was added to the samples, set up a separate PCR using the Xeno RNA Control assay included in the TaqMan Cells-to-C_T Control Kit.

Analyze the data

Endogenous control

Graph the C_T values for the stabilized blood and no-blood control in the digestion reaction. If the data show:

- Low C_T values for the stabilized blood samples and high C_T values for the no-blood control reactions, then the stabilized blood is compatible with the protocol
- High C_T values for both the stabilized blood samples and the no-blood control reactions, then the stabilized blood samples exceed the capacity of the system, which results in incomplete digestion or RT-PCR inhibition

  For future experiments, dilute the digested stabilized blood sample 1:5 and 1:10 in RNase-free water and repeat the RT-PCRs.

Xeno™ RNA Control

The C_T values from the Xeno RNA Control should be consistent (±1 C_T value), regardless of the initial volume of stabilized blood in the digestion reaction. Consistent C_T values indicate that no RT-PCR inhibitors are present in the sample. Greater C_T values for the stabilized blood samples than for the no-blood control reactions indicate that inhibitors were introduced into the RT-PCR.

For future experiments, dilute the digested stabilized blood pellets 1:5 and 1:10 in RNase-free water and repeat RT-PCR analysis.

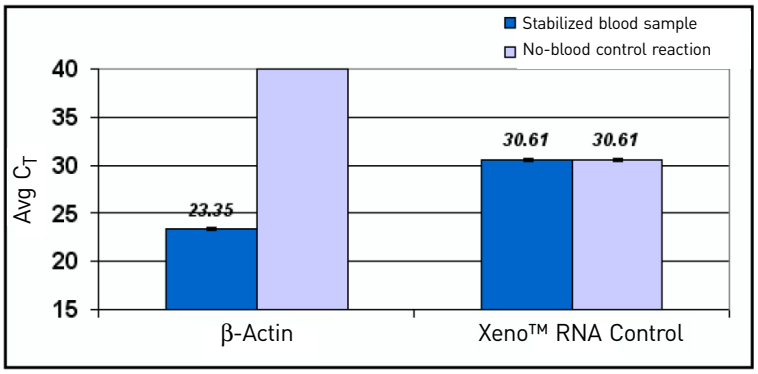
Example results

The figure below displays representative inhibition experiment results. In this example:

- Replicate 500-µL aliquots of stabilized blood were pelleted and washed, then digested along with replicate no-blood control reactions following the steps in the inhibition experiment.
- Samples were reverse-transcribed and amplified using the indicated assays in duplicate reactions.
- The resulting C_T values were graphed for stabilized blood and no-blood controls in the digestion reaction.

This experiment shows that even at 500 µL of stabilized blood per digestion reaction, the RT-PCR is not compromised by reaction inhibitors carried over from the stabilized blood samples.

The figure below shows the results for stabilized blood samples and no-blood control reactions. The samples were prepared from 500-µL replicates of stabilized blood and were pelleted and washed. The no-blood control reactions (consisting of Digestion Solution and Stop Solution plus Xeno RNA Control) were prepared and reverse-transcribed. β-actin and Xeno RNA Control qPCR assays were performed.



C

Safety

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, and 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 adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's 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 necessary) 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. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Documentation

Kit documentation

The following documents are available for the Stabilized Blood-to-C_T™ kits:

Document	Part number	Description
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR User Guide	4449675	Provides detailed instructions for preparing RT-PCR-ready lysates from blood samples collected with the Tempus® and PAXgene® Blood RNA Tubes, including troubleshooting and supplemental procedures.
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR Quick Reference (Tempus® Tubes)	4449676	Provides step-by-step instructions for preparing RT-PCR-ready lysates from blood samples collected with Tempus® Blood RNA Tubes.
Stabilized Blood-to-C_T™ Nucleic Acid Preparation Kit for qPCR Quick Reference (PAXgene® Tubes)	4456313	Provides brief procedures for preparing RT-PCR-ready lysates from blood samples collected with PAXgene® Blood RNA Tubes

Related documentation

When using this protocol, you may find the documents listed below useful. To obtain this and additional documentation, see "Obtaining support" on page 36.

Document	Part number
Tempus® Blood RNA Tube and Large-Volume Consumables User Guide	4345218
High Capacity RNA-to-cDNA™ Kit User Guide	4387951
SuperScript® VILO™ cDNA Synthesis Kit manual	NA†
TaqMan® MicroRNA Reverse Transcription Kit User Guide	4367038
TaqMan® Gene Expression Master Mix User Guide	4371135
TaqMan® Universal Master Mix II User Guide	4428173
TaqMan® One-Step RT-PCR Master Mix Reagents Kit User Guide	4310299

† The *SuperScript® VILO™ cDNA Synthesis Kit* manual does not have a part number. To download the manual, go to **www.invitrogen.com**, then search for the kit part number: **11754-050**.

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.appliedbiosystems.com/sds**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

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