# TaqMan® Fast Advanced Master Mix

## USER GUIDE

### For two-step RT-PCR in gene expression experiments or quantitative analysis

**Catalog Numbers** 4444556, 4444557, 4444558, 4444963, 4444964, 4444965

**Publication Number** 4444605

**Revision** C

**Manufacturer's address:** Life Technologies Corporation | 2130 Woodward Street | Austin, TX 78744

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**Revision history:** Pub. No. 4444605

| Revision | Date | Description |
|---|---|---|
| C | 21 March 2017 | Update licensing, trademarks, general style and format; update instrumentation; separate miRNA assay chapters. |
| B | June 2010 | Baseline for this revision history |

# Contents

# About this guide

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

## Purpose of this guide

The *TaqMan® Fast Advanced Master Mix User Guide* describes how to perform two-step RT-PCR using TaqMan® Fast Advanced Master Mix with:

- TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, and custom TaqMan® primer and probe sets
- TaqMan® Gene Expression Array Plates (Fast and Standard plates)
- TaqMan® Gene Expression Array Cards
- TaqMan® MicroRNA Assays
- TaqMan® Advanced miRNA Assays

This User Guide provides general guidelines for analyzing data because analysis can vary between applications. See the documentation for your instrument for more information about procedures and data analysis.

# 1 Product information

## Product description

The Applied Biosystems™ TaqMan® Fast Advanced Master Mix enables PCR in any gene expression experiment or quantitative analysis, including:

- Pathogen detection
- Differential gene expression analysis
- Viral load quantitation
- MicroRNA quantitation
- Microarray verification

TaqMan® Fast Advanced Master Mix can be used with any DNA target, including complementary DNA (cDNA) or genomic DNA (gDNA). It can be used in the second step of a two-step RT-PCR protocol for RNA quantitation experiments. A cDNA template can be generated from RNA using one of our reverse transcription kits (see "Required materials not supplied" on page 8) prior to PCR with the TaqMan® Fast Advanced Master Mix.

TaqMan® Fast Advanced Master Mix is supplied at a 2X concentration and contains:

- AmpliTaq™ Fast DNA Polymerase
- Uracil-N glycosylase (UNG)
- dNTPs with dUTP
- ROX™ dye (passive reference)
- Optimized buffer components

See "Components of the TaqMan® Fast Advanced Master Mix" on page 31 for more information about each component.

TaqMan® Fast Advanced Master Mix is optimized for use with primers and TaqMan® probes designed according to our guidelines.

# Contents and storage

**Table 1** TaqMan® Fast Advanced Master Mix

| Cat. No. | Number of 20-µL reactions | Amount | Storage[1] |
|---|---|---|---|
| 4444556 | 100 | 1 × 1 mL | –20°C; 4°C after opening |
| 4444557 | 500 | 1 × 5 mL | |
| 4444963 (2 × 4444557) | 1,000 | 2 × 5 mL | |
| 4444964 (5 × 4444557) | 2,500 | 5 × 5 mL | |
| 4444965 (10 × 4444557) | 5,000 | 10 × 5 mL | |
| 4444558 | 5,000 | 1 × 50 mL | |

[1] See label for expiration date.

# Required materials not supplied

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

**Table 2** Instrument, software, equipment, plates and accessories, and consumables

| Item | Source |
|---|---|
| **Instrument, one of the following:** | |
| QuantStudio™ 3 and 5 Real-Time PCR Instruments[1] | Contact your local sales office. |
| QuantStudio™ 6 Flex Real-Time PCR System[1] | |
| QuantStudio™ 7 Flex Real-Time PCR System | |
| QuantStudio™ 12K Flex Real-Time PCR System | |
| StepOne™ Real-Time PCR System[2] | |
| StepOnePlus™ Real-Time PCR System[1] | |
| 7500 Real-Time PCR System[1] | |
| 7500 Fast Real-Time PCR System[1] | |
| ViiA™ 7 Real-Time PCR System | |
| 7900HT Real-Time PCR Instrument[1] | |
| 7900HT Fast Real-Time PCR Instrument | |
| Or use a compatible real-time PCR instrument from another supplier. Validate thermal cycling conditions on other real-time PCR instruments. | |

| Item | Source |
|---|---|
| **Software** | |
| Microsoft™ Excel™ *(Optional, to create plate layout files for import)* | microsoft.com |
| **Equipment** | |
| Centrifuge with plate adapter | MLS |
| Microcentrifuge | MLS |
| Thermal cycler, or heat block or water bath set to 95°C | MLS |
| Adjustable pipettors | MLS |
| Laboratory mixer (vortex or equivalent) | MLS |
| **Tubes, plates, and other consumables** | |
| Plastics consumables | thermofisher.com/plastics |
| Pipette tips | thermofisher.com/pipettetips |
| Disposable gloves | MLS |

[1] Not compatible with TaqMan® Array Cards.
[2] Not compatible with TaqMan® Array Plates or TaqMan® Array Cards.

**Table 3** Reagents for reverse transcription

| Item | Source |
|---|---|
| **Reagents for reverse transcription (all assays)** | |
| TE, pH 8.0 | AM9849 |
| *(Optional)* RNase Inhibitor | N8080119<br>AM2684 (Cloned; 40 U/µL) |
| Nuclease-Free Water (not DEPC-Treated) | AM9930 |
| **Reagents for reverse transcription (TaqMan® and Custom TaqMan® Gene Expression Assays, TaqMan® Array Plates, and TaqMan® Array Cards)** | |
| High-Capacity cDNA Reverse Transcription Kit | 4368814<br>4374966 (with RNase Inhibitor) |
| High-Capacity RNA-to-cDNA™ Kit | 4387406 |
| SuperScript™ VILO™ cDNA Synthesis Kit | 11754050 |
| SuperScript™ IV VILO™ Master Mix | 11756050 |
| **Reagents for reverse transcription (TaqMan® MicroRNA Assays)** | |
| TaqMan® MicroRNA Reverse Transcription Kit[1] | 4366596 |

| Item | Source |
|---|---|
| TaqMan® Advanced miRNA cDNA Synthesis Kit[2] | A28007 |

[1] TaqMan® MicroRNA Assays are optimized for use with the TaqMan® MicroRNA Reverse Transcription Kit. Assay performance cannot be guaranteed with other reverse transcription kits.

[2] TaqMan® Advanced miRNA Assays are optimized for use with the TaqMan® Advanced miRNA cDNA Synthesis Kit. Assay performance cannot be guaranteed with other reverse transcription kits.

**Table 4** Assays

| Item | Source |
|---|---|
| **TaqMan® Assays** | |
| TaqMan® Gene Expression Assays | thermofisher.com/taqmangeneexpression |
| Custom TaqMan® Gene Expression Assays | thermofisher.com/taqmancustomgeneexpression |
| Custom TaqMan® probes and primers[1] | thermofisher.com/customprimersprobes |
| **TaqMan® Array Plates** | |
| TaqMan® 96-Well Array Plates (Standard and Fast) | thermofisher.com/taqmanarrays |
| **TaqMan® Array Cards** | |
| TaqMan® Array Card | thermofisher.com/taqmanarrays |
| **TaqMan® MicroRNA Assays** | |
| TaqMan® MicroRNA Assays | thermofisher.com/taqmanmirna |
| TaqMan® Advanced miRNA Assays | thermofisher.com/advancedmirna |
| Custom TaqMan® Small RNA Assays | thermofisher.com/taqmancustommirna |

[1] Synthesized to your sequence and choice of quencher and reporter dyes.

**Table 5** Kits and reagents for RNA isolation

| Item | Source |
|---|---|
| RNA isolation products | thermofisher.com/rnaisolation |
| Supporting reagents | thermofisher.com/rnaisolationreagents |

# Workflow

**Perform reverse transcription**

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**Perform real-time PCR amplification**

Prepare the PCR reaction mix

▼

Prepare the PCR reaction plate or TaqMan® Array Card

▼

Set up a plate or card document, or experiment file

(or use the document provided with the cards or custom plates)

▼

Run the PCR reaction plate or TaqMan® Array Card

▼

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**Analyze the data**

# 2 RT-PCR for TaqMan® and Custom TaqMan® Gene Expression Assays

## Perform reverse transcription

Perform reverse transcription to obtain cDNA from RNA samples.

See Table 3 for reverse transcription kits. See *TaqMan® Gene Expression Assays Protocol* (Pub. No. 4333458) for detailed guidelines and instructions.

## Perform PCR

### Guidelines

- Store TaqMan® Assays frozen and away from light until use. Excessive exposure to light may affect the fluorescent probes.
- Multiple assays can be run on one reaction plate. Include no-template controls (NTCs) for each assay.

### Before you begin

- Determine the total number of PCR reactions, including a gene expression assay for each cDNA sample and a no-template control for each assay.

  **Note:** We recommend three replicates reactions for each assay.

- Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.
- Thaw TaqMan® Assays on ice, then vortex and briefly centrifuge to resuspend.
- Thaw samples on ice, then vortex and briefly centrifuge to resuspend.

## Prepare the PCR reaction mix

1. Combine the following components for the number of reactions required, plus 10% overage.

| Component | Volume per reaction | | Final concentration |
|---|---|---|---|
| | 384-well plate | 96- or 48-well plates[1] | |
| TaqMan® Fast Advanced Master Mix (2X) | 5.0 µL | 10.0 µL | 1X |
| TaqMan® Assay (20X) | 0.5 µL | 1.0 µL | 1X |
| Nuclease-Free Water[2] | 3.5 µL | 7.0 µL | — |
| **Total volume per reaction** | **9.0 µL** | **18.0 µL** | **—** |

[1] Standard and Fast.
[2] Adjust the volume of Nuclease-Free Water for a larger volume of cDNA.

2. Vortex briefly to mix.
3. Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate.
2. Add cDNA template (1 pg to 100 ng in Nuclease-Free Water), or Nuclease-Free Water for NTC, to each well.
   - 1.0 µL for a 384-well plate
   - 2.0 µL for 96- and 48-well plates (Standard and Fast)

   **Note:** Be sure to adjust the volume of Nuclease-Free Water in the PCR reaction mix for a larger volume of cDNA.
3. Seal the reaction plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
4. Apply a compression pad to the plate, if required by your real-time PCR system.

## Set up a plate document or experiment file

1. Set up a plate document or experiment file using the following conditions:

| Real-time PCR system | UNG incubation[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|---|
| | Hold 50°C | Hold 95°C | Denature 95°C | Anneal / extend 60°C |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System | 2 minutes | 20 seconds | 1 second | 20 seconds |
| • 7500 Fast Real-Time PCR System<br>• 7500 Real-Time PCR System | 2 minutes | 20 seconds | 3 seconds | 30 seconds |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.

2. Select the appropriate block, if this option applies to your instrument.
3. Select the appropriate experiment type, if this option applies to your instrument.
4. Select **TaqMan® Reagents** to detect the target sequence, if this option applies to your instrument.

5. Select a run mode.

| Real-time PCR system | Run mode |
|---|---|
| • 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument (384-Well and Standard 96-Well Block Modules)<br>• 7500 Real-Time PCR System | **Standard** |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System<br>• 7900HT Fast Real-Time PCR Instrument (Fast 96-Well Block Module)<br>• 7500 Fast Real-Time PCR System | **Fast** |

6. Enter the sample volume, if this option applies to your instrument.
   - 384-well plate: **10.0 µL**
   - 96- and 48-well plates (both Standard and Fast): **20.0 µL**

### Run the PCR reaction plate

1. Open the plate document or experiment file that corresponds to the reaction plate in the system software.
2. Load the reaction plate.
3. Start the run.

## Analyze data

Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.

1. View the amplification plots for the reactions.
2. Use auto baseline and auto threshold settings, or set the baseline and threshold values to determine the threshold cycles ($C_t$) for the amplification curves.
3. Use the relative standard curve method or the comparative $C_t$ method to analyze data.

# 3 RT-PCR for TaqMan® Gene Expression Array Plates

## Perform reverse transcription

Perform reverse transcription to obtain cDNA from RNA samples.

See Table 3 for reverse transcription kits. See *TaqMan® Gene Expression Assays Protocol* (Pub. No. 4333458) for detailed guidelines and instructions.

## Perform PCR

### Guidelines

Store TaqMan® Array Plates away from light until use. Excessive exposure to light may affect the fluorescent probes.

### Before you begin

- Determine the total number of PCR reactions.
  One reaction corresponds to one well in the TaqMan® Array Plate.
- Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.
- Thaw samples on ice, then vortex and briefly centrifuge to resuspend.

### Prepare the PCR reaction mix

1. Combine the following components for the number of reactions required, plus 10% overage.

| Component | Volume per reaction | |
|---|---|---|
| | 96-well plate (Fast) | 96-well plate (Standard) |
| cDNA template + Nuclease-Free Water[1] | 5 µL | 10 µL |
| TaqMan® Fast Advanced Master Mix (2X) | 5 µL | 10 µL |
| **Total volume per reaction** | **10 µL** | **20 µL** |

[1] 5–50 ng of cDNA diluted in Nuclease-Free Water.

2. Vortex briefly to mix.
3. Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of a TaqMan® Array Plate.
2. Seal the plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
3. Apply a compression pad to the plate, if required by your real-time PCR system.

## Set up a plate document or experiment file

1. Set up a plate document or experiment file using the following conditions:

| Real-time PCR system | UNG incubation[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|---|
| | Hold 50°C | Hold 95°C | Denature 95°C | Anneal / extend 60°C |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument<br>• ViiA™ 7 Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System | 2 minutes | 20 seconds | 1 second | 20 seconds |
| • 7500 Fast Real-Time PCR System<br>• 7500 Real-Time PCR System | 2 minutes | 20 seconds | 3 seconds | 30 seconds |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.

2. Select the appropriate block, if this option applies to your instrument.
3. Select the appropriate experiment type, if this option applies to your instrument.
4. Select **TaqMan® Reagents** to detect the target sequence, if this option applies to your instrument.

5. Select a run mode.

| Real-time PCR system | Run mode |
|---|---|
| • 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument (384-Well and Standard 96-Well Block Modules)<br>• 7500 Real-Time PCR System | **Standard** |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System<br>• 7900HT Fast Real-Time PCR Instrument (Fast 96-Well Block Module)<br>• 7500 Fast Real-Time PCR System | **Fast** |

6. Enter the sample volume, if this option applies to your instrument.
   - 96-well Fast plate: **10.0 µL**
   - 96-well Standard plate: **20.0 µL**

### Run the PCR reaction plate

1. Open the plate document or experiment file that corresponds to the reaction plate in the system software.
2. Load the reaction plate.
3. Start the run.

## Analyze data

Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.

1. View the amplification plots for the reactions.
2. Set the baseline and threshold values to determine the threshold cycles ($C_t$) for the amplification curves, or select relative threshold under analysis settings to obtain ($C_{rt}$) values (recommended for dried-down assays).
3. Use the relative standard curve method or the comparative $C_t$ method to analyze data.

# 4 RT-PCR for TaqMan® Gene Expression Array Cards

## Perform reverse transcription

Perform reverse transcription to obtain cDNA from RNA samples.

See "Required materials not supplied" on page 8 for reverse transcription kits. See the protocol for your kit and the *TaqMan® Array Micro Fluidic Cards User Guide* (Pub. No. 4400263) for detailed guidelines and instructions.

## Perform PCR

### Guidelines

- Store the TaqMan® Array Card in its packaging until the packaging has reached room temperature and you are ready to fill it with sample-specific PCR mix. Prolonged exposure to indoor lighting can photo-degrade the fluorescent probes contained within the card. Do not expose the card to sunlight.
- Fill each fill reservoir with sample-specific PCR mix made from a single cDNA sample.
- Use 100 µL of sample-specific PCR mix to fill each fill reservoir. Volumes smaller than 100 µL will result in insufficiently filled cards.
- Do not add the sample after centrifuging the cards. Centrifugation of the card causes the sample-specific PCR mix to resuspend the dried TaqMan® probes and primers within the wells of the card. Addition of the sample after centrifuging disrupts the resuspended assay positions.
- Schedule runs so that each card is run as soon as possible, to ensure reproducibility. After sealing, there is no measurable well-to-well contamination for up to 64 hours.

### Recommended amount of cDNA

- We recommend 30–1000 ng (0.3–10 ng/µL) of cDNA (converted from total RNA) per fill reservoir.
- The amount of cDNA to use depends on the expression level of the target genes and the number of target copies per well that need to be detected. For example:
  - Use 1000 ng (10 ng/µL) per fill reservoir to detect genes with low expression. Because the cDNA concentration is high, use high-quality cDNA without inhibitors.
  - Use 100–200 ng per fill reservoir to detect genes with moderate expression.
  - Use 30–50 ng per fill reservoir to detect genes with moderate to high expression.
- Use the same amount of cDNA sample for all reactions.

## Before you begin

- Determine the number of fill reservoirs in the TaqMan® Array Card that will be used for each cDNA sample.
- Thaw samples on ice, then vortex and briefly centrifuge to resuspend.
- Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.

## Prepare the sample-specific PCR reaction mix

1. Combine the following components for the number of reactions required, plus 10% overage.

| Component | Volume per fill reservoir |
|---|---|
| cDNA template + Nuclease-Free Water[1] | 50 µL |
| TaqMan® Fast Advanced Master Mix (2X) | 50 µL |
| **Total volume** | **100 µL** |

[1] See "Recommended amount of cDNA" on page 19.

2. Vortex briefly to mix.
3. Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

## Prepare the TaqMan® Array Card

Fill the TaqMan® Array Card with sample-specific PCR reaction mix, then centrifuge and seal (see the *TaqMan® Array Micro Fluidic Cards User Guide*; Pub. No. 4400263).

## Set up a card document or experiment file

Set up a card document or experiment file for the TaqMan® Array Card.

**Note:** Thermal cycling conditions depend on the instrument.

- For the **7900HT Fast Real-Time PCR Instrument**:
  - Thermal cycling conditions:

| UNG incubation[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|
| **Hold 50°C** | **Hold 92°C** | **Denature 97°C** | **Anneal / extend 62°C** |
| 2 minutes | 10 minutes[3] | 1 second | 20 seconds |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.
[3] To completely dissolve the primers and probes on the TaqMan® Array Card.

  - Run mode: **Standard**

  - Ramp rate: **100%**
  - Sample volume: **1.0 µL**
- For the **ViiA™ 7 Real-Time PCR System**, the **QuantStudio™ 7 Flex Real-Time PCR System**, or the **QuantStudio™ 12K Flex Real-Time PCR System**:
  - Select the appropriate template file for your real-time PCR system and experiment:

| Experiment type | Template file |
|---|---|
| **ViiA™ 7 Real-Time PCR System** | |
| Comparative $C_t$ | ViiA7_TaqMan_Array_Comparative_Ct_Fast_Template.edt |
| Standard Curve | ViiA7_TaqMan_Array_Std_Curve_Fast_Template.edt |
| **QuantStudio™ 7 Flex Real-Time PCR System and QuantStudio™ 12K Flex Real-Time PCR System** | |
| Comparative $C_t$ | TaqMan_Array_Comparative_Ct_Fast_Template.edt |
| Standard Curve | TaqMan_Array_Std_Curve_Fast_Template.edt |

  - Manually select each option:
    - Block: **Array Card Block**
    - Experiment type: **Comparative $C_t$** or **Standard Curve**
    - Reagents: **TaqMan® Reagents**
    - Properties: **Fast**
    - Thermal cycling conditions, including ramp rate: Select **Run Method**, then enter as indicated:

| UNG incubation[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|
| **Hold 50°C** | **Hold 92°C** | **Denature 95°C** | **Anneal / extend 60°C** |
| 2 minutes | 10 minutes[3] | 1 second | 20 seconds |
| 1.75°C/second | 1.75°C/second | 1.75°C/second | 1.83°C/second |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.
[3] To completely dissolve the primers and probes on the TaqMan® Array Card.

**IMPORTANT!** Do not use the default Fast settings.

## Run the TaqMan® Array Card

1. Open the card document or experiment file that corresponds to the TaqMan® Array Card in the system software.
2. Load the TaqMan® Array Card.
3. Start the run.

# Analyze data

Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.

1. View the amplification plots for the reactions.
2. Set the baseline and threshold values to determine the threshold cycles ($C_t$) for the amplification curves, or select relative threshold under analysis settings to obtain ($C_{rt}$) values (recommended for dried-down assays).
3. Use the relative standard curve method or the comparative $C_t$ method to analyze data.

# 5 RT-PCR for TaqMan® MicroRNA Assays

This chapter covers use of TaqMan® MicroRNA Assays. For use of TaqMan® Advanced miRNA Assays see Chapter 6, "RT-PCR for TaqMan® Advanced miRNA Assays".

## Perform reverse transcription

Perform reverse transcription to obtain cDNA from RNA samples.

Use the TaqMan® MicroRNA Reverse Transcription Kit, and see *TaqMan® Small RNA Assays Protocol* (Pub. No. 4364031) for detailed guidelines and instructions.

## Perform PCR

### Guidelines

- Store the TaqMan® MicroRNA Assays at –20°C and away from light until use. Excessive exposure to light may affect the fluorescent probes.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling.

### Before you begin

- Determine the total number of PCR reactions, including a microRNA assay for each cDNA sample, endogenous control assays, and a no-template control (NTC) for each assay.

  **Note:** We recommend three replicate reactions for each assay.

- Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.

## Prepare the PCR reaction mix

1. Combine the following components for the number of reactions required, plus 10% overage.

| Component | Volume per reaction | |
|---|---|---|
| | 384-well plate | 96- or 48-well plates[1] |
| TaqMan® Fast Advanced Master Mix (2X) | 5.00 µL | 10.00 µL |
| Nuclease-Free Water[2] | 3.83 µL | 7.67 µL |
| TaqMan® MicroRNA Assay (20X) | 0.50 µL | 1.00 µL |
| cDNA[3] | 0.67 µL | 1.33 µL |
| **Total volume per reaction** | **10.00 µL** | **20.00 µL** |

[1] Standard and Fast.
[2] Adjust the volume of Nuclease-Free Water for a larger volume of cDNA.
[3] Minimum final dilution of RT reaction in PCR reaction is 1:15.

2. Mix gently, then centrifuge to bring the reaction mix to the bottom of the tube.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate.
2. Seal the plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
3. Apply a compression pad to the plate, if required by your real-time PCR system.

## Set up a plate document or experiment file

1. Set up a plate document or experiment file using the following conditions:

| Real-time PCR system | UNG incubation[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|---|
| | Hold 50°C | Hold 95°C | Denature 95°C | Anneal / extend 60°C |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System | 2 minutes | 20 seconds | 1 second | 20 seconds |
| • 7500 Fast Real-Time PCR System<br>• 7500 Real-Time PCR System | 2 minutes | 20 seconds | 3 seconds | 30 seconds |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.

2. Select the appropriate block, if this option applies to your instrument.
3. Select the appropriate experiment type, if this option applies to your instrument.
4. Select **TaqMan® Reagents** to detect the target sequence, if this option applies to your instrument.

5. Select a run mode.

| Real-time PCR system | Run mode |
|---|---|
| • 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument (384-Well and Standard 96-Well Block Modules)<br>• 7500 Real-Time PCR System | **Standard** |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System<br>• 7900HT Fast Real-Time PCR Instrument (Fast 96-Well Block Module)<br>• 7500 Fast Real-Time PCR System | **Fast** |

6. Enter the sample volume, if this option applies to your instrument.
   - 384-well plate: **10.0 µL**
   - 96- and 48-well plates (both Standard and Fast): **20.0 µL**

### Run the PCR reaction plate

1. Open the plate document or experiment file that corresponds to the reaction plate in the system software.
2. Load the reaction plate.
3. Start the run.

## Analyze data

Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.

1. View the amplification plots for the reactions.
2. Use auto baseline and auto threshold settings, or set the baseline and threshold values to determine the threshold cycles ($C_t$) for the amplification curves.
3. Use the relative standard curve method or the comparative $C_t$ method to analyze data.

# 6 RT-PCR for TaqMan® Advanced miRNA Assays

This chapter covers use of TaqMan® Advanced miRNA Assays. For use of TaqMan® MicroRNA Assays see Chapter 5, "RT-PCR for TaqMan® MicroRNA Assays".

## Perform reverse transcription

Perform reverse transcription to obtain cDNA from RNA samples.

Use the TaqMan® Advanced miRNA cDNA Synthesis Kit, and see *TaqMan® Advanced miRNA Assays User Guide (Single-tube Assays)* (Pub. No. 100027897) for detailed guidelines and instructions.

## Perform PCR

### Guidelines

- Store the TaqMan® Advanced miRNA Assays at –20°C and away from light until use. Excessive exposure to light may affect the fluorescent probes.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling.

### Before you begin

- Determine the total number of PCR reactions, including a microRNA assay for each cDNA sample, endogenous control assays, and a no-template control (NTC) for each assay.

  **Note:** We recommend three replicate reactions for each assay.

- Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.

## Prepare PCR reaction mix

1. Prepare 1:10 dilutions of the cDNA template.
2. Combine the following components for the number of reactions required, plus 10% overage.

| Component | Volume per reaction | |
|---|---|---|
| | **384-well plate** | **96- or 48-well plates[1]** |
| TaqMan® Fast Advanced Master Mix (2X) | 5.00 µL | 10.00 µL |
| Nuclease-Free Water[2] | 2.00 µL | 4.00 µL |
| TaqMan® Advanced miRNA Assay (20X) | 0.50 µL | 1.00 µL |
| cDNA (1:10 dilution) | 2.50 µL | 5.00 µL |
| **Total volume per reaction** | **10.00 µL** | **20.00 µL** |

[1] Standard and Fast.
[2] Adjust the volume of Nuclease-Free Water for a larger volume of cDNA.

3. Mix gently, then centrifuge to bring the reaction mix to the bottom of the tube.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate.
2. Seal the plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
3. Apply a compression pad to the plate, if required by your real-time PCR system.

## Set up plate document or experiment file

1. Set up a plate document or experiment file using the following conditions:

| Real-time PCR system | UNG incubation (optional)[1] | Polymerase activation[2] | PCR (40 cycles) | |
|---|---|---|---|---|
| | Hold 50°C | Hold 95°C | Denature 95°C | Anneal / extend 60°C |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System | 2 minutes | 20 seconds | 1 second | 20 seconds |
| • 7500 Fast Real-Time PCR System<br>• 7500 Real-Time PCR System | 2 minutes | 20 seconds | 3 seconds | 30 seconds |

[1] For optimal UNG activity.
[2] To activate AmpliTaq™ Fast DNA Polymerase.

2. Select the appropriate block, if this option applies to your instrument.
3. Select the appropriate experiment type, if this option applies to your instrument.
4. Select **TaqMan® Reagents** to detect the target sequence, if this option applies to your instrument.

5. Select a run mode.

| Real-time PCR system | Run mode |
|---|---|
| • 7900HT Real-Time PCR Instrument<br>• 7900HT Fast Real-Time PCR Instrument (384-Well and Standard 96-Well Block Modules)<br>• 7500 Real-Time PCR System | **Standard** |
| • QuantStudio™ 3 and 5 Real-Time PCR Instruments<br>• QuantStudio™ 6 and 7 Flex Real-Time PCR System<br>• QuantStudio™ 12K Flex Real-Time PCR System<br>• ViiA™ 7 Real-Time PCR System<br>• StepOne™ Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System<br>• 7900HT Fast Real-Time PCR Instrument (Fast 96-Well Block Module)<br>• 7500 Fast Real-Time PCR System | **Fast** |

6. Enter the sample volume, if this option applies to your instrument.
   - 384-well plate: **10.0 µL**
   - 96- and 48-well plates (both Standard and Fast): **20.0 µL**

### Run the PCR reaction plate

1. Open the plate document or experiment file that corresponds to the reaction plate in the system software.
2. Load the reaction plate.
3. Start the run.

## Analyze data

Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.

1. View the amplification plots for the reactions.
2. Use auto baseline setting and threshold setting of 0.1, or set the baseline and threshold values to determine the threshold cycles ($C_t$) for the amplification curves.
3. Use the relative standard curve method or the comparative $C_t$ method to analyze data.

A

# Background information

## Components of the TaqMan® Fast Advanced Master Mix

### AmpliTaq™ Fast DNA Polymerase

The AmpliTaq™ Fast DNA Polymerase enzyme is purified through a proprietary process to reduce bacterial DNA introduced from the host organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR.

When AmpliTaq™ Fast DNA Polymerase is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Any low-stringency mispriming events that may have occurred will not be enzymatically extended and subsequently amplified. A thermal incubation step is required for activation to ensure that active enzyme is generated only at temperatures where the DNA is fully denatured.

### Uracil-N glycosylase

Uracil-N glycosylase (UNG) treatment can prevent the reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons (Longo et al., 1990). UNG prevents reamplification of carryover PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See “Use UNG to prevent false-positive amplification“ on page 35 for more information about UNG.

### dUTP

This master mix includes dUTP to enable uracil-N-glycosylase (UNG) activity and maintain optimal PCR results.

### ROX™ Passive Reference dye

The ROX™ Passive Reference dye provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

# Two-step RT-PCR

Visit **thermofisher.com/qpcreducation** for more information.

A target template is a DNA sequence, including cDNA, a gDNA, or a plasmid nucleotide sequence. An amplicon is a short segment of DNA.

Gene quantitation assays using TaqMan® Fast Advanced Master Mix and TaqMan® Assays are performed in a two-step RT-PCR.

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from RNA.
2. In the PCR step, PCR products are quantitatively synthesized from cDNA samples using the TaqMan® Fast Advanced Master Mix.

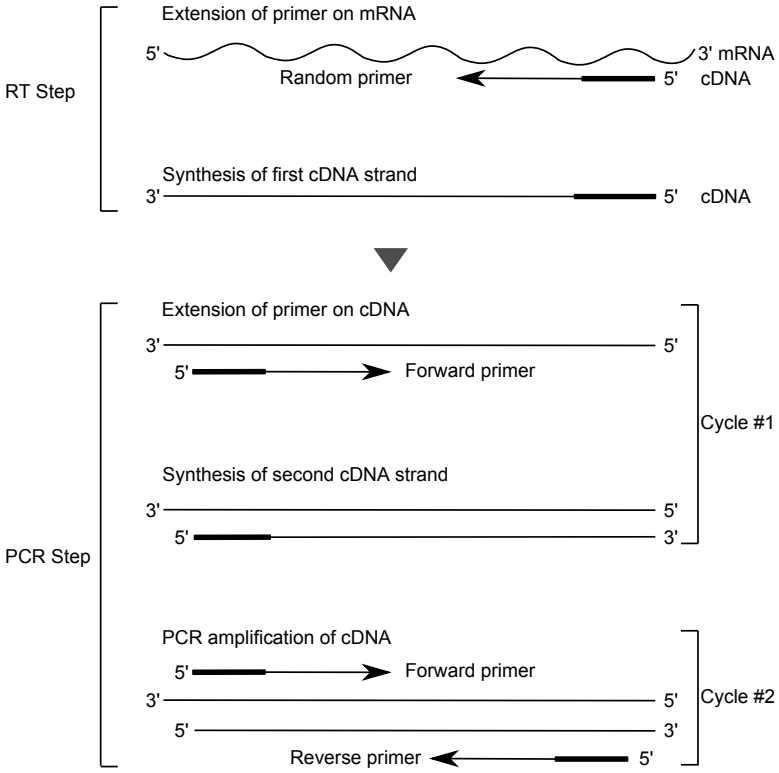


**Figure 1** Two-step RT-PCR.

This illustration does not show hybridization of the TaqMan® MGB probe. See "TaqMan® MGB probes" on page 34 for details on how the TaqMan® MGB probe is used in the PCR step.

# About the 5' nuclease assay

The 5' nuclease assay process takes place during PCR amplification. It occurs in every cycle and does not interfere with the exponential accumulation of product.

During the PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites.

When the probe is intact (Figure 3 and Figure 4), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

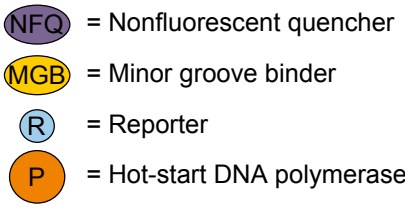


**Figure 2** Legend.

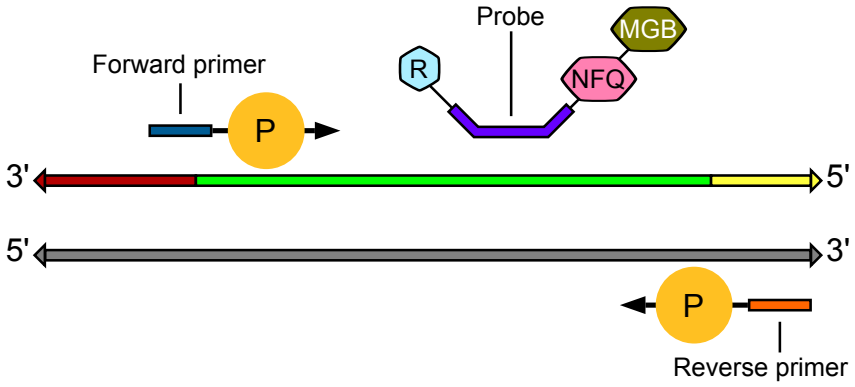


**Figure 3** Polymerization.

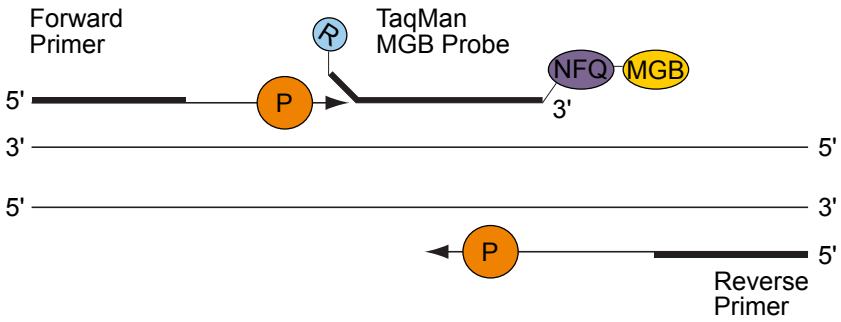


**Figure 4** Strand displacement.

The DNA polymerase cleaves only probes that hybridize to the target (Figure 5). Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to

the probe and amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

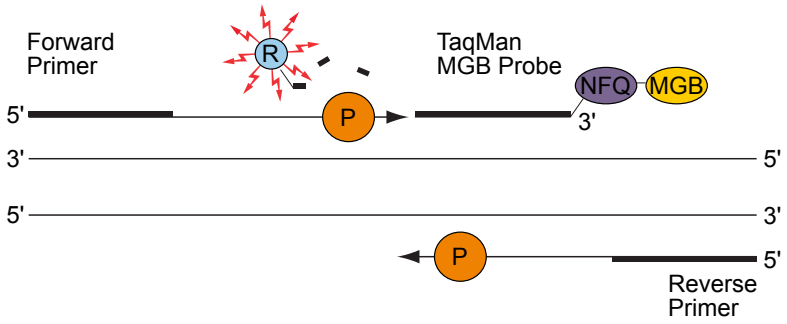


**Figure 5** Cleavage.

Polymerization of the strand continues, but because the 3' end of the probe is blocked, no extension of the probe occurs during PCR (Figure 6).

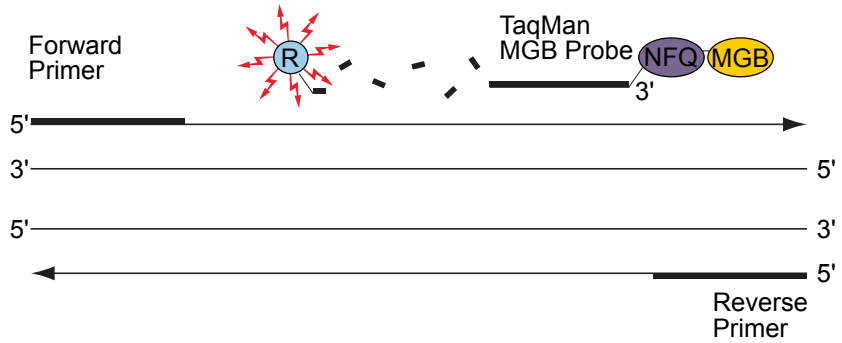


**Figure 6** Completion of polymerization.

## TaqMan® MGB probes

TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™) at the 5′ end of the probe (Afonina et al., 1997; Kutyavin et al., 1997)
- A non-fluorescent quencher (NFQ) dye at the 3′ end of the probe.

  The NFQ dye does not fluoresce, which allows the real-time PCR system to measure the reporter dye contributions more accurately.
- A minor groove binder (MGB) at the 3′ end of the probe that:
  - Increases the melting temperature ($T_m$) without increasing the probe length.
  - Allows for the design of shorter probes.

# B Best practices for PCR and RT-PCR experiments

## Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA*Zap*™ Solutions (Cat. No. AM9890).

## Use UNG to prevent false-positive amplification

Carryover amplicons can result in false-positive amplification during PCR. Use a master mix that contains uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)) to degrade many contaminating carryover amplicons.

UNG enzymatic activity occurs during an initial incubation at 50°C. UNG is partially inactivated during the 95°C incubation step for template denaturation and polymerase activation. Because UNG is not completely deactivated during the 95°C incubation, it is important to keep the annealing temperatures greater than 55°C and to refrigerate PCR products at 2°C to 8°C in order to prevent amplicon degradation.

To ensure the desired UNG activity:

- Use PCR components and thermal cycling conditions as specified.
  UNG-containing master mixes incorporate the optimal concentration of UNG to prevent cross-contamination while not affecting real-time PCR performance.
- Do not attempt to use UNG-containing master mixes in subsequent amplification of dU-containing PCR products, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR products, preventing further amplification.

Although treatment with UNG can degrade or eliminate large numbers of carryover PCR products, use good laboratory practices to minimize cross-contamination from non-dU-containing PCR products or other samples.

## Detect fluorescent contaminants

Fluorescent contaminants can generate false positive results. To help detect these contaminants, we recommend including a No-Amplification Control reaction that contains sample, but no master mix.

After PCR, if the absolute fluorescence of the No-Amplification Control is greater than the fluorescence of the no template control (NTC), fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.

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, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

# Chemical safety

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

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with 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. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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  - Safety Data Sheets (SDSs; also known as MSDSs)

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

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Sninsky and Gelfand, personal communication