VetMAX™-Plus Multiplex One-Step RT-PCR Kit

TaqMan® probe-based multiplex one-step real-time RT-PCR amplification of RNA targets

Protocol

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

Information in this document is subject to change without notice. Life Technologies Corporation assumes no responsibility for any errors that may appear in this document.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Warranty and Liability: Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about warranties or would like information about post-warranty support.

NOTICE TO PUCHASER: LIMITED LICENSE

Use of this product is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for veterinary diagnostic services including reporting the results of purchaser's activities for a fee and for the purchaser's own internal research. No right under any other patent claim (such as apparatus or system claims) is conveyed expressly, by implication, or by estoppel. Human in vitro diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

The BHQ technology is the subject of US Patents No. 7,019,129. The CAL Fluor technology is the subject of pending patents. Both technologies are licensed and sold under agreement with Biosearch Technologies, Inc. Ambion products incorporating the BHQ and CAL Fluor dyes are sold exclusively for research and development applications and their use for human diagnostic testing is prohibited.

These products are sold for use by the end-user only and may not be re-sold, distributed or re-packaged.

TRADEMARKS:

Trademarks of Life Technologies Corporation and its affiliated companies include: AB Logo™, Ambion®, Applied Biosystems® MagMax™, ROX™, StepOne™, StepOnePlus™, VetMAX™, and Xeno™.

Black Hole Quencher is a registered trademark of Biosearch Technologies. Eclipse is a registered trademark of Epoch. NanoDrop is a trademark of Thermo Fisher Scientific.

TaqMan and AmpliTaq Gold are registered trademarks of Roche Molecular Systems, Inc.

All other trademarks are the sole property of their respective owners.

Part Number 4426015 Rev. C
09/2010

Contents

Safety information

Note: For general safety information, see this Preface and Appendix A, "Safety" on page 11. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "MSDSs" on page 13.

IMPORTANT! For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

How to use this guide

Text conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

User attention words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

How to obtain support

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

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

VetMAX™-Plus Multiplex One-Step RT-PCR Kit

Product information

Purpose of the product

The VetMAX™-Plus Multiplex One-Step RT-PCR Kit is designed for multiplex, quantitative, reverse-transcription PCR (qRT-PCR). The kit is optimized for the simultaneous amplification of up to four animal pathogen nucleic acid targets using your RNA samples and TaqMan® primer/probe sets. The kit includes Xeno™ RNA Control, which serves as an internal positive control for RNA isolation and RT-PCR amplification. You perform a single-tube, one-step procedure to reverse-transcribe the RNA and amplify your targets using the 10× Multiplex RT-PCR Enzyme Mix containing AmpliTaq Gold® DNA Polymerase. Run the reactions on a real-time PCR system.

Figure 1 below shows amplification plots from reactions that included four targets using the VetMAX-Plus Multiplex One-Step RT-PCR Kit. Three of the targets in the experiment were held constant, but the fourth was serially diluted to show the dynamic range of multiplex target amplification with the kit.

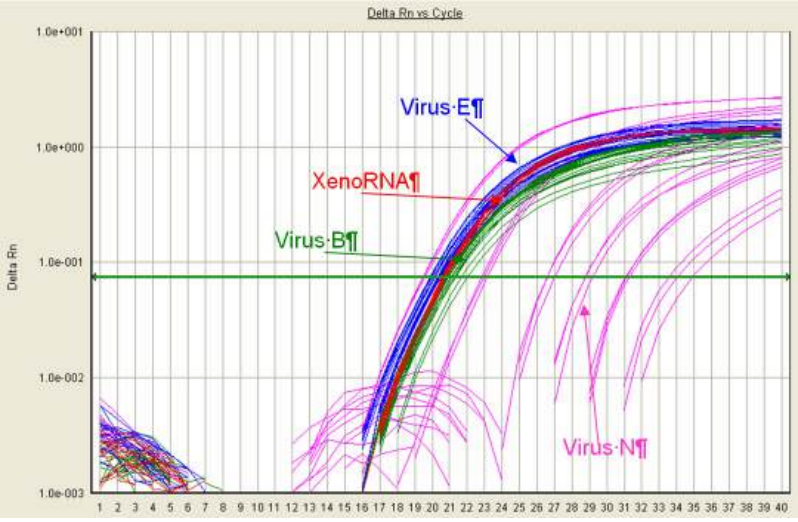


Figure 1 Four-plex amplification of control RNAs using the VetMAX™-Plus Multiplex One-Step RT-PCR Kit

Xeno RNA Control and control RNAs for virus B, virus E, and virus N were amplified in a multiplex RT-PCR using the VetMAX™-Plus Multiplex One-Step RT-PCR Kit on an Applied Biosystems 7500 Real-Time PCR System. This experiment included a sample set with fixed amounts of three of the targets and a serial dilution series of the virus N control RNA. Virus N was amplified even in four-plex reactions containing only 400 copies (~33 C_T).

Kit contents

Component	PN 4415330 100 reactions
2× Multiplex RT-PCR Buffer	1.4 mL
10× Multiplex RT-PCR Enzyme Mix	280 µL
Xeno™ RNA Control (10,000 copies/µL)‡	250 µL
Nuclease-free Water	1.75 mL

‡ Applied Biosystems recommends that you add Xeno™ RNA Control to the MagMAX™ lysis/binding solution concentrate that is used for the nucleic acid isolation. When added to the sample lysis solution, the Xeno RNA Control can serve as a positive control for the recovery of RNA and for the RT-PCR. See the workflow on page 4.

Storage

- Store the kit at –20 °C in a non-frost-free freezer.
- Store the Nuclease-free Water at –20 °C, 4 °C, or at room temperature.

Materials and equipment required

RNA sample(s)

Use pure RNA that is free of RT-PCR inhibitors in the procedure. Applied Biosystems recommends a MagMAX™ RNA isolation kit that is appropriate for your sample type. Go to **www.appliedbiosystems.com**, then search for **MagMAX**.

When isolating viral RNA from cell-free sample sources such as serum, use MagMAX™ viral RNA isolation kits, which include carrier RNA to maximize viral RNA recovery.

PCR primer/TaqMan® probe mixture

Use any licensed PCR primer/TaqMan probe mixture that is compatible with your real-time PCR system and that is designed for one-step RT-PCR. Optimization of PCR primer and probe concentrations is critical for multiplex reactions. In reactions with targets of different abundance, it is necessary to limit the PCR primer concentrations of highly abundant targets so that less abundant targets can effectively compete for the amplification reagents. The concentration of primers and probes may require optimization, but the concentrations shown in the table below typically work well.

Component	Final concentration in the reaction	25× primer/probe mix‡
Forward PCR primer	400 nM	10 µM
Reverse PCR primer	400 nM	10 µM
TaqMan® probe	120 nM	3 µM

‡ Use 1 µL per 25-µL RT-PCR of a PCR primer/TaqMan probe mixture prepared at these concentrations.

Real-time PCR systems supported

The VetMAX-Plus Multiplex One-Step RT-PCR Kit is compatible with the following Applied Biosystems systems:

- 7500 Real-Time PCR System
- 7500 Fast Real-Time PCR System
- 7900HT Real-Time PCR System (96-well and 384-well sample block)
- 7900HT Fast Real-Time PCR System (96-well and 384-well sample block)
- StepOne™ Real-Time PCR System
- StepOnePlus™ Real-Time PCR System

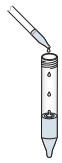
Accessories required

- Reaction plates and covers appropriate for your real-time PCR system. See the Plastic Consumables Compatibility Chart: go to **www.appliedbiosystems.com**, then select **Products ▸ Real-Time PCR ▸ Reaction Plates & Adhesive Films**.
- Nuclease-free pipettes and tips.
- Reagent reservoirs or tubes for preparing the RT-PCR mix.

Workflow

Before you begin: isolate the RNA

Add Xeno™ RNA Control to the MagMAX™ lysis/binding solution concentrate that is used for the RNA isolation



Prepare the reactions

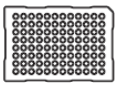
Prepare the RT-PCR mix

↓

Add the RT-PCR mix to a reaction plate or tubes

↓

Add the RNA sample or NTC to each reaction



Perform the run (RT-PCR)

Program your real-time PCR instrument using the recommended thermal-cycling conditions

↓

Start the run

Analyze the data

Analyze the data according to the instructions for your real-time PCR instrument

Procedure

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 14.

CAUTION! CHEMICAL HAZARD. 2× Multiplex RT-PCR Buffer.

CAUTION! CHEMICAL HAZARD. Multiplex RT-PCR Enzyme Mix.

Before you begin: isolate the RNA

Use a MagMAX RNA isolation kit to isolate the RNA. For optimal results, Applied Biosystems recommends the following:

- Use a MagMAX RNA isolation kit that is appropriate for your sample type. Go to **www.appliedbiosystems.com**, then search for **MagMAX**.
- Use pure RNA that is free of RT-PCR inhibitors.
- Add Xeno RNA Control to the MagMAX lysis/binding solution concentrate that is used for the RNA isolation, to serve as a positive control for the recovery of RNA and for the RT-PCR. Add 2 µL of undiluted Xeno RNA Control (20,000 copies) per isolation. For example, for ten isolations, you would:
  a. Start with enough MagMAX lysis/binding solution concentrate for ten isolations.
  b. Add 20 µL of Xeno RNA Control to the MagMAX lysis/binding solution concentrate, vortex briefly, add isopropanol, then vortex to mix.
- When isolating viral RNA from cell-free sample sources such as serum, add carrier RNA to the MagMAX lysis/binding solution concentrate to maximize RNA recovery. Include carrier RNA even if you added Xeno RNA Control to the lysis/binding solution concentrate.

Prepare the reactions

1. Prepare the RT-PCR mix on ice (see the required volumes in the table below):
   - Prepare 10% extra RT-PCR mix.
   - Include duplicate no-template controls (NTCs or negative controls) using Nuclease-free Water in place of sample.
2. Add the RT-PCR mix to a reaction plate or tubes.
3. Add sample to each reaction.

	Component	Volume (µL)
RT-PCR mix	2× Multiplex RT-PCR Buffer	12.5
	PCR primer/TaqMan® probe mixture	—
	10× Multiplex RT-PCR Enzyme Mix	2.5
	RNA sample (Nuclease-free Water for NTCs)	—
	Total volume per reaction	25.0

Perform the run (RT-PCR)

1. Program your real-time PCR instrument using the thermal-cycling conditions shown in the tables below.
   - ROX™ passive reference dye is included in the 2✕ Multiplex RT-PCR Buffer.
   - For real-time PCR instruments capable of Fast thermal cycling, set the mode to *Standard*.
   - The suggested reaction volume is 25 µL.

Non-MGB probe thermal cycling conditions‡

	Stage	Reps	Temp	Time
Reverse transcription	1	1	48 °C	10 min
RT inactivation/ initial denaturation	2	1	95 °C	10 min
Amplification	3	40	95 °C 60 °C	15 sec 45 sec§

‡ Applied Biosystems recommends the non-MGB thermal cycling conditions for non-MGB probes, such as Eclipse® Q and Black Hole Quencher® dyes.
§ For long targets, the extension time may need to be >45 seconds.

MGB probe thermal cycling conditions

	Stage	Reps	Temp	Time
Reverse transcription	1	1	48 °C	10 min
RT inactivation/ initial denaturation	2	1	95 °C	10 min
Amplification	3	40	95 °C 55 °C	15 sec 45 sec‡

‡ For long targets, the extension time may need to be >45 seconds.

2. Start the run according to the instructions for your real-time PCR instrument.

Analyze the data

Analyze the data according to the instructions for your real-time PCR instrument. Applied Biosystems recommends the following:

Recommendation	Details
Use the **Auto C_T** setting for data analysis.	This setting minimizes subjectivity when setting the threshold cycle (C_T) for the Xeno RNA Control and sample amplifications.
If the Auto C_T setting does not produce satisfactory results, manually set the thresholds that are used to determine the C_T values.	To manually set the thresholds: 1. Select **Manual C_T** and **Manual Baseline**. 2. View each amplification plot (ΔR_n vs. cycle) with the Y-axis (ΔR_n) in log scale. 3. Select **Analyze** to adjust the amplification curves. 4. Set the threshold for the RNA target at 10% of the average maximum fluorescence value of the control RNA target in duplicate positive control reactions containing ~8000 copies/reaction. 5. Set the threshold for the Xeno RNA Control target at 10% of the maximum fluorescence value of the Xeno RNA Control in duplicate positive control reactions containing ~8000 copies/reaction). Example: If the average maximum fluorescence value for the sample RNA is 3.0, set its threshold at 0.3. Likewise, if the average maximum fluorescence value for the Xeno RNA Control is 2.0, set its threshold at 0.20.
Check the raw fluorescence data.	Verify that fluorescence increases seen in the normalized data are also evident without mathematical data processing.

Troubleshooting

Observation	Possible Cause	Solution
No signal from samples expected to be positive	RNA sample contains RT-PCR inhibitors	• Use less starting sample as input for your RNA isolation procedure. -Or- • Use less RNA sample in the RT-PCR. Follow the guidelines under "Low signal from samples expected to be positive" or "No signal from the Xeno RNA Control" below.
	Poor RNA recovery	Follow the guidelines under "No signal from the Xeno RNA Control" below.
Low signal from samples expected to be positive	RNA sample contains a low level of RT-PCR inhibitors	Samples containing minimal amounts of inhibitors may yield successful RT-PCRs if less RNA sample (and therefore less inhibitor) is added to the reaction. For example: • Reduce the sample volume to 1 to 2 µL, then add Nuclease-free Water to bring the reaction to the proper volume. -Or- • Dilute the RNA sample 1:10 using the solution used to elute the nucleic acid at the end of the nucleic acid isolation procedure, then use the diluted RNA in the RT-PCR. -Or- • Dilute the RNA sample 1:10 using 10 mM Tris-HCl pH 8, 0.1 mM EDTA, then use the diluted RNA in the RT-PCR.
No signal from the Xeno RNA Control	The Xeno RNA Control was omitted	Check that the Xeno RNA Control was added to the lysis solution as described in "Before you begin: isolate the RNA" on page 5.
	Poor RNA recovery	With a NanoDrop™ spectrophotometer, check the recovery of the carrier RNA that was used in the RNA isolation.
	Poor RNA recovery -Or- RNA samples contain RT-PCR inhibitors	Use master mix with and without additional Xeno RNA Control at 250 copies/reaction, then compare the amplification results for the sample RNA. (For this experiment, the Xeno RNA Control must have been added to the lysis solution). • If the reaction run using master mix *with* additional Xeno RNA Control results in signal, but the reaction run using master mix *without* additional Xeno RNA Control does not result in signal, the Xeno RNA Control was not recovered. • If the Xeno RNA Control signal is not seen from either sample, the RNA contains RT-PCR inhibitors.

Observation	Possible Cause	Solution
No signal from the Xeno RNA Control, but high signal from the positive control	The Xeno RNA Control primers and probe are present at limiting concentrations in the RT-PCR. High levels of the positive control in a sample can compromise amplification of the Xeno RNA Control.	No signal from the Xeno RNA Control is expected in a reaction that gave a strong signal for the positive control.
No signal from the positive control	Improper handling of the positive control, resulting in RNA degradation	Use appropriate precautions against RNase contamination when handling the positive control. For example, wear clean gloves and use nuclease-free barrier pipet tips.
	The 10× Multiplex RT-PCR Enzyme Mix was stored or handled improperly, and it lost activity	Repeat the RT-PCR with fresh reagents.
	The RT-PCR mix setup was incorrect.	See “Prepare the reactions” on page 5.
	The thermal cycler was not properly programmed.	See “Perform the run (RT-PCR)” on page 6.
Signal detected in no-template control (NTC)	RT-PCR contamination	• Repeat the RT-PCR with fresh reagents and decontaminated pipettes. • Set up and run the RT-PCR in an area that is isolated from areas used for nucleic acid isolation and RT-PCR product analysis.

Appendix A

Safety

This appendix covers:

General chemical safety

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page 13.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

MSDSs

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.
- For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

Chemical alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page v.

Specific chemical alerts

CAUTION! CHEMICAL HAZARD. 2× Multiplex RT-PCR Buffer causes eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Wear appropriate protective eyewear, clothing, and gloves. **FIRST AID:** If inhaled, remove to fresh air. In case of contact, flush thoroughly with water. If symptoms develop, get medical attention. Read the MSDS, and follow the handling instructions.

CAUTION! CHEMICAL HAZARD. Multiplex RT-PCR Enzyme Mix may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Wear appropriate protective eyewear, clothing, and gloves. **FIRST AID:** If inhaled, remove to fresh air. In case of contact, flush thoroughly with water. If symptoms develop, get medical attention. Read the MSDS, and follow the handling instructions.

Part Number 4426015 Rev. C 09/2010

Applied Biosystems
850 Lincoln Centre Drive | Foster City, CA 94404 USA
Phone 650.638.5800 | Toll Free 800.345.5224
www.appliedbiosystems.com

Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at
www.appliedbiosystems.com/support