LIGHT **DIAGNOSTICS®**

Amplifluor™ ID Human Rhinovirus Type A and B Detection Kit

Cat. No. AMP 4200

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

Application

The **Amplifluor® Human Rhinovrius Detection kit** is intended for the quantitative or qualitative evaluation of viral RNA generated by a validated *in vitro* nucleic acid amplification of conserved nucleic regions of Human Rhinovirus type A and B.

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Test Principle

Human Rhinovirus (HRV) is one of common contributors to respiratory diseases. Its frequent cause of common cold and more severe lower respiratory illness has raised the health burden to health care particularly to young children. There is increasing evident the many elderly or immunocompromised persons with underlying respiratory diseases, such as asthma, chronicle bronchitis and cystic fibrosis have higher risk of severe HRV-associated complication. Together with human enteroviruses, HRVs are classified within the *Picornaviridae* family. There are currently more than 100 distinct serotypes assigned to two major HRV groups, type A and type B. The **Amplifluor® HRV detection kit** was designed to detect conserved nucleic acid regions. Amplification reactions are performed using Polymerase Chain Reaction (PCR) utilizing the Amplifluor® hairpin forward primer and a reverse primer to produce target-specific fluorescent amplicons. The Amplifluor® hairpin primer is labeled with a reporter fluorophore *(fluorescein)* and a dark quencher *(DABSYL)*, which produce the high signal-to-noise ratio observed in the Amplifluor® assay. Upon incorporation into new amplicons, the Amplifluor® primer is fluorescent (reported in the instrument's FAM layer), while the fluorescein signal of the unincorporated Amplifluor primer is quenched (Figure 1&2). When performed on a thermal-cycler that is equipped with real-time fluorescence detection, the amplification products of the PCR reaction can be monitored directly in a quantitative fashion, allowing evaluation of template detection in a single, closed-tube system with no additional assay procedure. Where required, template standard curves may be employed to quantitatively determine viral load.

Figure.1

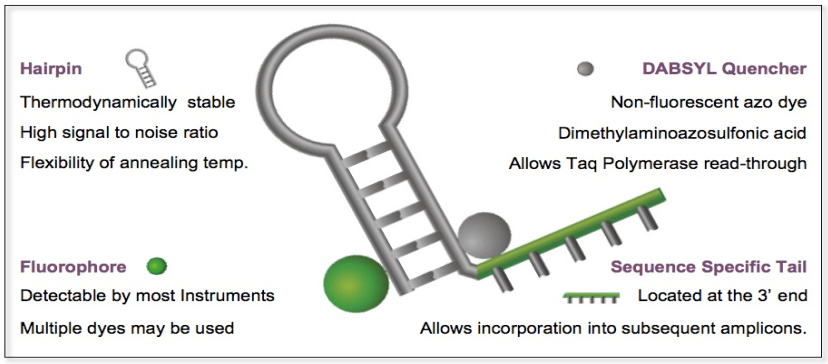
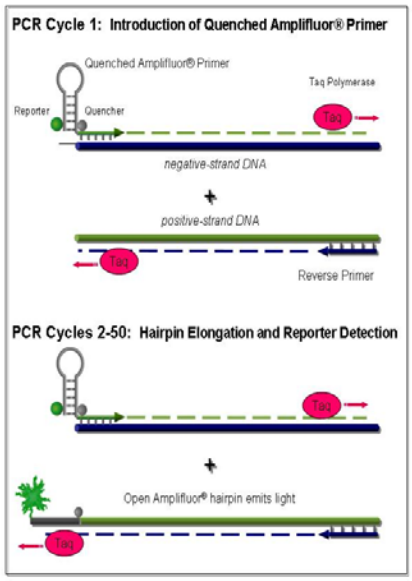


Figure 2. The Amplifluor® Primer

The Amplifluor® system consists of the fluorescent Amplifluor® hairpin primer and an unconjugated reverse primer. In combinations, these two primers produce a fluorescently labeled amplicon, which can be measured by real-time PCR (**right**). The carefully designed Amplifluor® molecule consists of four parts - a fluorophore, a hairpin structure, a DABSYL quencher, and a target-specific sequence tail (**top**). The Amplifluor® primer has little fluorescence in the native closed state (energy transfer), but upon incorporation into an amplicon during PCR, the hairpin unfolds, separating the fluorochrome and quencher creating a substantial signal which can be easily detected (**right**).



Kit Components

1. 25X Amplifluor® HRV Type A Forward Primer 100 µl (100 reactions). **Light sensitive**, **Store at or below -20°C**.
2. 25X Amplifluor® HRV Type A Reverse Primer 100 µL (100 reactions)**. Store at or below -20°C.**
3. 25X Amplifluor® HRV Type B Forward Primer 100 µL (100 reactions). **Light sensitive**, **Store at or below -20°C.**
4. 25X HRV Amplifluor® Type B Reverse Primer 100 µL (100 reactions)**. Store at or below -20°C.**
5. HRV Type A Positive Control (Part No. AMP4200-A)

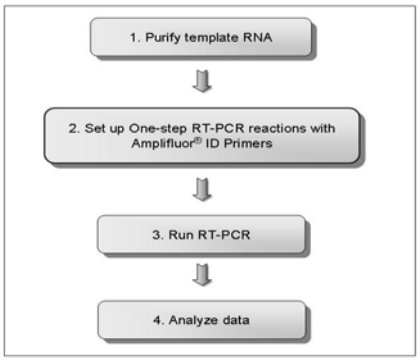
   250 µL, 10^9 copies/5 µL. **Store at or below -20°C.**
6. HRV Type B Positive Control (Part No. AMP4200-B)

   250 µL, 10^9 copies/5 µL. **Store at or below -20°C.**

Storage

Amplifluor® primers are light sensitive and must be stored in the dark at -20ºC (or at -80ºC for long term storage). Amplifluor® primers may be divided into convenient aliquots and placed in amber or foil-wrapped tubes to minimize light exposure.

Amplifluor® HRV Detection kit Protocol Flow Chart



Recommended Materials and Methods

1. Purify Template DNA:

Template RNA should be purified to remove potential PCR inhibitors. Many reagents are suitable for extraction of viral DNA from cell-culture supernatant or patient samples (cell free bodily fluids, e.g. plasma, serum, CSF, etc.). Proper aseptic technique and safety routines should be employed when handling potentially infectious material. Qiagen® (Valencia, CA) QIAamp® Viral RNA Mini Kit is recommended.

2. Setup One-step RT-PCR Reaction with Amplifluor® Primers:

Formulate a master mix for each reaction as indicated below. The **Amplifluor® HRV Detection kit** was specifically designed for use in a single tube and one step real-time PCR reaction. Although most commercially available reagents required for nucleic acid amplification can be used with the **Amplifluor® HRV Detection kit**, Bio-Rad iScript™ One step RT-PCR 2XMM was optimal for low copy detection of viral target using **Amplifluor® HRV Detection kit**. The following reaction recommendations are based on use of the Bio-Rad real time PCR reagents. For additional polymerases or reagents, please inquire with Technical Services at 1-800-645-5476.

To ensure that sufficient master mix is available, determine the number of reactions required and then add 1-2 additional sample volumes to compensate for pipetting precision and loss associated with aliquoting. The procedure provided below is based on a final PCR volume of 25 µL. Larger volumes may be analyzed in cases where sample concentration is limited.

MasterMix (Primer, Buffer, and Enzyme) for HRV Type A

Reagent	25 µL Reaction	Final Conc.
Water	4.75 µL	
2X RTi qPCR Buffer	12.50 µL	1X
25X Amplifluor® HRV type A FAM Forward Primer	1.0 µL	1X
25X Amplifluor® HRV type A	1.0 µL	1X Reverse Primer
Rox*	0.25 µL	
RT-enzyme	0.50 µL	

MasterMix (Primer, Buffer, and Enzyme) for HRV Type B

Reagent	25 µL Reaction	Final Conc.
Water	4.75 µL	
2X RTi qPCR Buffer	12.50 µL	1X
25X Amplifluor® HRV type B FAM Forward Primer	1.0 µL	1X
25X Amplifluor® HRV type B	1.0 µL	1X Reverse Primer
Rox*	0.25 µL	
RT-enzyme	0.50 µL	

***** *Rox reference dye is required for the use of ABI Real Time instrument*

Note: *Exercise extreme care in sample handling to avoid cross-sample contamination. The Amplifluor® viral detection reagents, like other PCR detection reagents, are exquisitely sensitive to PCR carry-over and other types of PCR contamination. Use of dedicated nuclease free water, aerosol resistant tips, and a template-free environment for sample preparation and setup is highly recommended.*

- Add 20 µL of master mix into each well of a 96 well plate.
- Add up to 5 µL of extracted sample to the appropriate well. If less than 5 µL is analyzed, add additional nuclease-free water to bring the final sample volume to 5 µL.
- Add 5 µL of no template control (NTC, water only) to the appropriate wells.
- Add 5 µL of Positive Control to the appropriate wells. The positive control needs to be diluted into a 10 fold serial dilution to generate a standard curve for quantitative evaluations.
- Seal the plate with optical caps or plate sealer.
- Briefly centrifuge plate to collect all components and to remove air bubbles.
- Place the plate into the real-time instrument.

3. Run Real-Time RT-PCR:

Several instruments may be used to monitor and quantitate the continuous (real-time) fluorescence emission signal generated in the Amplifluor® reaction. The ABI Prism 7700®, 7500 and the LightCycler 480® have been utilized in Amplifluor® optimization. See Appendix A and B for specific setup instructions for ABI 7500® and Roche LightCycler 480®. For real-time PCR instruments not listed, please inquire with Technical Services at 1-800- 645-5476.

The following two sets of parameters can be used with the Bio-Rad iScrip One Step RT PCR for Probes 2X Master mix:

One-step RT-PCR

1 Cycle	50ºC for	10 minutes
1 Cycle	95ºC for	5 minutes
40-45 cycles of:	95ºC for	15 seconds
	60ºC for	35 seconds

Collect Fluorescent data at 60ºC in the FAM filter.

The DABSYL quencher does not emit fluorescence. Unincorporated Amplifluor® Forward Primer also does not emit fluorescence at this temperature.

4. Data Analysis

The real-time data collected from experiments employing Amplifluor® ID reagents should be analyzed according to the instrument manufacturer's suggestions. The output of real-time PCR data is often presented as a C_T value. The C_T, or cycle threshold, is the cycle number at which the Reporter fluorescence generated by incorporating and elongating the Amplifluor® hairpin into newly amplified amplicon passing a fixed threshold above baseline. For qualitative analysis of nucleic acid target presence, replicate data from a sample should be compared to known positive and negative samples to determine sample cutoff, similar to ELISA type assays.

Alternatively, for quantitative assays, a standard curve may be generated using positive controls or calibrator samples. Copy number (or relative copy number) of experimental samples can then be extrapolated from standard curve data.

Warnings and Precautions

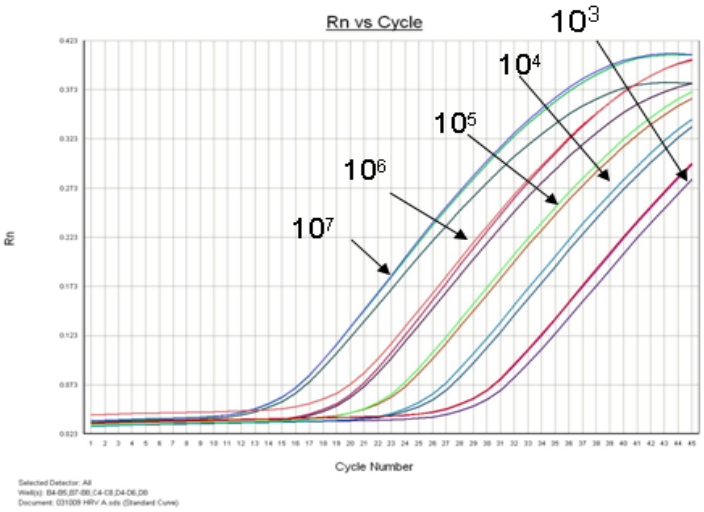
- Handle all specimens and materials coming in contact with them as potentially infectious materials. Decontaminate with 0.05% sodium hydrochloride (1:100 dilution of household bleach).
- Do not use reagents beyond expiration date.
- Nucleic acids are subject to degradation by nucleases found in the environment and on human surfaces. Clean and cover work surfaces with disposable coverings and wear powder free gloves during all procedures.
- Use pipetting techniques that will deliver correct volumes of reagents in all steps, including pre-wetting aerosol resistant pipette tips. Repeating pipettors should be primed and voided of all air bubbles.
- With nucleic acid amplification, the potential for carry-over contamination is high. To reduce this carryover contamination, NucleoClean™ Decontamination Spray (Cat. No. 3097S) or Wipes (Cat. No. 3097) are available. Further, work in a unidirectional flow from workstation to workstation and always use dedicated equipment and aerosol-barrier pipette tips.

Analytical Detection Limits

Evaluation of the Amplifluor® HRV Detection kit. A ten-fold serial dilution of HRV type A&B control plasmids were amplified using Kit primer sets and Bio-Rad iScript® One-step RT-PCR 2XMM buffer. Samples were run on the ABI 7500 Real-Time PCR instrument using the following parameters: 50ºC for 10 minutes, 95ºC for 5 minutes; followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 35 seconds. The mean C_T value of diluted control plasmid was plotted on the Y axis against the Log 10 (copy number of concentration) on the X axis. The PCR efficiency (Ex) of control plasmid was determined using the C_T slope method with 5 data points from a 5 log dilution range. The calculated efficiency for type A is 100% with an R^2 value of 0.99 and for type B is 96% with an R^2 value of 0.99. The detection of no template control (NTC) was not observed before 45 cycles (**Figure 3. A, B, C&D**)

Fig

A.



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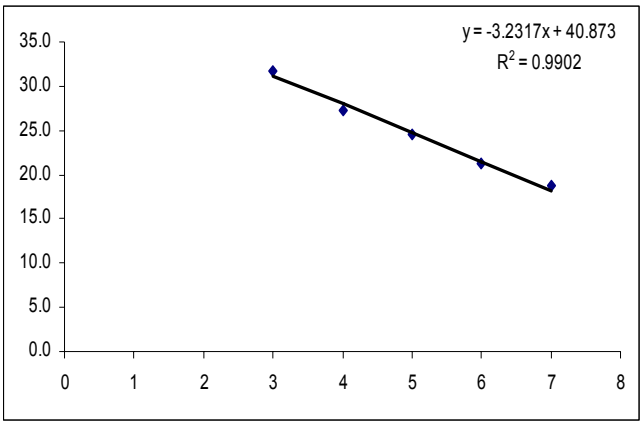
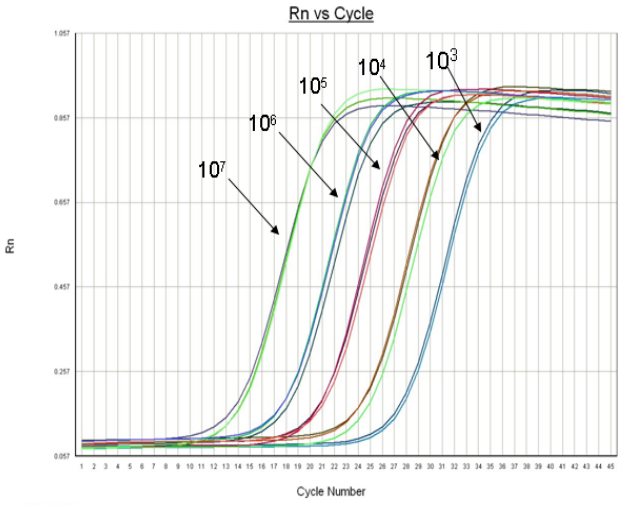
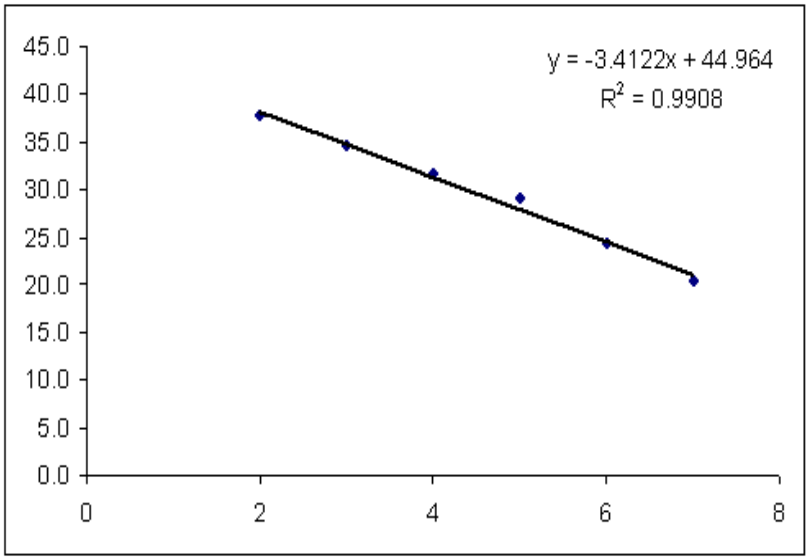


C.



D.



Trouble Shooting

- Similar to SYBR Green® real-time PCR reagents, Amplifluor® detection kit may generate signal associated with primer dimer formation, or amplification of contaminating template. The signal associated with primer dimer formation occurs in late cycles of PCR, is often associated with the NTC sample, and occurs near the lower limits of detection of each assay. If NTC signal is observed, assay cut-offs may be adjusted appropriately using known negative samples. Note that some extraction reagents may influence the formation of primer dimers in the Amplifluor® system.

Appendix A

Procedure for use with the ABI 7500

1. Click ABI 7500 System software icon to open the run program. In the Quick Startup dialog box, select “Create New Document”. In assay drop-down list, select Standard Curve (absolute Quantitative) Plate. Choose standard 7500 mode. If first time user, you could ask manufacture for a demo assay kit to verify the instrument is functioning properly.
2. Click next to move to the detector selection. This kit is designed to use FAM fluorophore dye. Select FAM from the list and add it to the dialog box. Other fluorophore dyes can be used only for custom design kit. Please contact 1-800-MILLIPORE (1-800-645-5476) or go to http:.//www.millipore.com/company/sup3/support for additional assistance.
3. Click next for the plate setup. Select the wells corresponded to sample ID (unknown, standard control, NTC etc) and sample group (triplicate). Select FAM detector for all the sample wells. You may also enter the numbers for the dilution of control template and sample dilution unit. Click finish to move to real time PCR program setup.
4. In the default thermal cycling condition, make proper changes according to the RTi qPCR method for your experiment, such as one step or two step RTi qPCR. See page 11 for the recommended PCR condition. Select reaction volume
5. Select Start and the software will ask to save the setup as Save and Continue.
6. Load the plate into the instrument. Enter the file name and click Save.

The instrument will start automatically

Appendix B

Procedure for use with Roche Lightcycler 480

1. Click LC480 software icon to open the run program. In the Startup dialog box, select “New Experiment”. In detection format drop-down list, select “Mono Hydrolysis Probe”. If first time user, you may ask manufacture for a demo assay kit to verify the instrument is functioning properly. Check the custom button to view the filter setup for correct fluorescent dye detection.
2. Click the program button to set up the cycling condition. Under Target (°C) box, enter the correct temperature for each cycle step. Enter the correct time for each temperature into the holding Time box. Enter “single” into the read function at the temperature where you want to collect your fluorophore signal. After all parameter of cycling condition is completed, enter quantitative assay under the program drop-down list.
3. Click “sample editor” to set up the plate. Select the wells corresponded to sample ID (unknown, standard control, NTC etc) and sample group (triplicate). After the plate set up, click save button to save the file under a designated macro file so that the same cycling condition can be used next time.

PCR Statement

This product is optimized for use in conjunction with the Polymerase Chain Reaction (PCR) patented process. This process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman-La Roche Ltd. No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser of this product.

List of Patents

The Amplifluor® nucleic acid amplification process is protected by US Patent Nos. 5,866,336, 6,090,552 & 6,117,635, EU Patent No. 0912597, Canadian Patent No. 2,260,973 & Japanese Patent No. 3,597,869

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