eQ-PCR™ EnV Detection Kit
User Manual

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Storage

Upon receipt of the kit, store the kit at –20°C until use. At this condition, the reagents are stable for 1 year.

After first time use, store all of reagents at 2-8°C and keep the reagents protected from direct light. At this condition, the reagents are stable for 3 months.

For research use only, not for use in diagnostic procedure

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Limited Product Warranty

It is imperative that users strictly adhere to this manual. Failure to do so will void our guarantee of this product. TrimGen Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose.

Notice to Purchaser

The purchase of eQ-PCR™ products includes a limited, nonexclusive license to use the reagents and systems. This license does not grant rights to use the reagents and systems for the reproduction of the eQ-PCR™ reagents and systems, to modify the eQ-PCR™ reagents and systems for resale, or to use the eQ-PCR™ reagents and systems to manufacture commercial products without written approval of TrimGen Corporation. No other license, expressed, implied, or by estoppels is granted.

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Product Use and Limitations

The eQ-PCR™ Kit is designed as a "For Research Use Only" (RUO) kit. For all of other applications, user should follow the instructions provided by the appropriate regulatory authorities.

Product Safety and Liabilities

Some reagents included in this product may cause harmful conditions. The user should read the instructions on the bottle or tube, and in the User Manual carefully before starting to use the reagents. Handle the chemicals with caution. When working with the reagents, always wear lab coat, gloves, and protective goggles. TrimGen Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the misuse, the results of use, or the inability to use this product.

Introduction

The eQ-PCR™ EnV Detection Kit is a real-time PCR kit used to detect enterovirus (EnV) present in samples. The EnV pathogens are detected by TrimGen's proprietary eQ-PCR (enhanced Quantitative-PCR) technology (US patent pending). This detection kit is applied to Applied Biosystems Real-Time PCR System model 7500.

Enteroviruseses are RNA viruses associated with a variety of human and mammalian diseases and can cause serious illness, especially in infants and the immunocompromised. Enteroviruses are the most common cause of aseptic meningitis and can also result in encephalitis. The diagnosis of EnV meningitis normally requires virus culture and the turnaround times are about 2-5 days. A rapid protocol for EnV identification can significantly reduce unnecessary diagnostic tests and improve patient management.

Built upon real-time PCR technology, TrimGen's eQ-PCR™ EnV Detection Kit adopts a one-step RT-PCR method to detect EnV in human tissue samples, specifically in CSF. There is no need for culturing viruses before testing.

eQ-PCR™ technology improves detection sensitivity and the assay can detect as low as 2 copies of viral RNA in the tube. The design covers 70 species of the EnV family.

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Materials Provided

The eQ-PCR™ EnV Detection Kit contains reagents for 32 reactions.

Tube Label	Cap Color	Quantity (32 Rx)
PCR Mix 2	*Purple*	400 µl
Enzyme Mix	*Red*	40 µl
EnV-TP	*White*	50 µl
EnV-CTL	*Yellow*	40 µl
NF Water	*Clear*	500 µl

Note: The identity of the reagents is printed on each tube. The PCR Oligo Mix (EnV-TP) is **light sensitive**. Keep this reagent protected from direct light.

PCR Mix 2 (*Purple* cap)
     Pre-mixed reagents for reverse transcription and PCR (RT-PCR) amplification

Enzyme Mix (*Red* cap)
     Mix of enzymes for RT-PCR

EnV-TP (*White* cap)
     Mix of PCR primers and probes for detection of EnV

EnV-CTL (*Yellow* cap)
     Positive Control DNA for EnV identification

NF Water (*Clear* cap)
     Nuclease-free water for the blank control

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Equipment Required

- Applied Biosystems Real-Time PCR System Model 7500
- Nucleic acid extraction kit for isolation of EnV viral RNA
- Pipettes
- Sterile filter tips
- Tabletop centrifuge

Sample Preparation

Reagents for nucleic acid extraction are not provided with the eQ-PCR^{TM} EnV Detection Kit.

TrimGen provides high efficiency RNA extraction kits for the following samples:

Paraffin-embedded (FFPE) tissues
Fresh or frozen tissues
Fine Needle Aspiration tissues
Cells

Kit Name	Cat No.
WaxFree RNA	WR-50 (50 extractions)

The eQ-PCR^{TM} EnV Detection Kit can detect EnV in RNA samples extracted from human tissues, CSF and whole blood. Any commercially available kits are acceptable.

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eQ-PCR^{TM} EnV Detection Kit Procedure

This kit is applied to Applied Biosystems Real-Time PCR System 7500.

A. Reaction Preparation

Thaw all reagents to 2^0C - 8^0C when ready to use. Keep all reagents at 2^0C to 8^0C during preparations. (Once thawed, do NOT refreeze remaining reagents.)

A.1. Prepare Optical 8-tube strips (Applied Biosystems, Catalog Number 4316567, not provided by the kit) or MicroAmp 96-well Reaction Plate (Applied Biosystems, Catalog Number 4306737, not provided by the kit) for the PCR reaction. Label the tubes as follows:

Blank control
Positive control
Sample #1
Sample #2
Sample #3
Sample #4
Sample #5
Sample #6
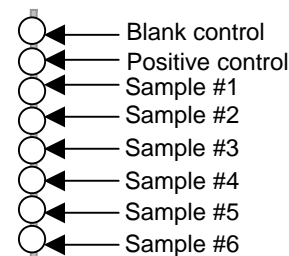
A.2. Collect **PCR Mix 2** (Purple cap)**, Enzyme Mix** (Red cap), **EnV-TP** (White cap) and **EnV-CTL** (Yellow cap) tubes from the kit box.

A.3. Using the table below, prepare the PCR Master Mix in one tube.

| Reagents	Formulation	Volume
PCR Mix 2	12.5 μl x (_____ + 2*) x 1.1** Sample number	
Enzyme Mix	1 μl x (_____ + 2*) x 1.1** Sample number	
EnV-TP	1.5 μl x (_____ + 2*) x 1.1** Sample number	
	Total Volume	

 * For the blank and positive controls.

** Recommended volume correction factor for pipetting error.

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A.4. Aliquot 15 µl of the Master Mix to each sample tube.

A.5. Add 10 µl of <u>**NF Water**</u> into the blank control tube.

A.6. Add 2 µl of EnV Control DNA from the <u>**EnV-CTL**</u> tube and 8 µl of nuclease-free water from the <u>**NF Water**</u> tube into the positive control tube.

A.7. Add 10 µl of the DNA samples into each sample tube.

A.8. Cap the tubes and mix samples well by tapping the tubes.

A.9. Centrifuge the tubes to remove air bubbles trapped in the solution.

A.10. Load the strips directly onto the Applied Biosystems Real-Time PCR System.

B. Programming ABI 7500 and PCR Set up

B.1. Enter the ABI 7500 SDS Software and select File>New. In the "New Document Wizard" window, click the "**Assay**" box, choose the **"Standard Curve (Absolute Quantification)"** in the drop-down list. Click **"Next"**.
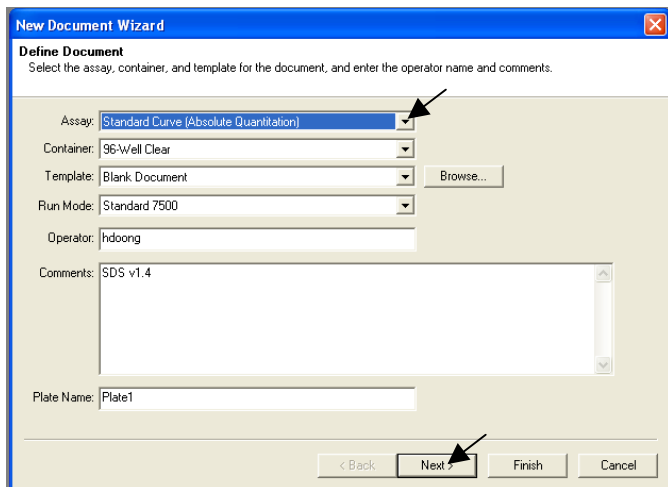

B.2. For the first time user, create the EnV detector file as follows (If the EnV detector file has been created, skip to the next step):

1) Select **"New Detector"**

2) In the "New Detector" dialogue box enter **EnV** into the "**Detector Name**". Click the **"Reporter"** and select **FAM** dye from the down list. Click "**Quencher**" and select **none**.
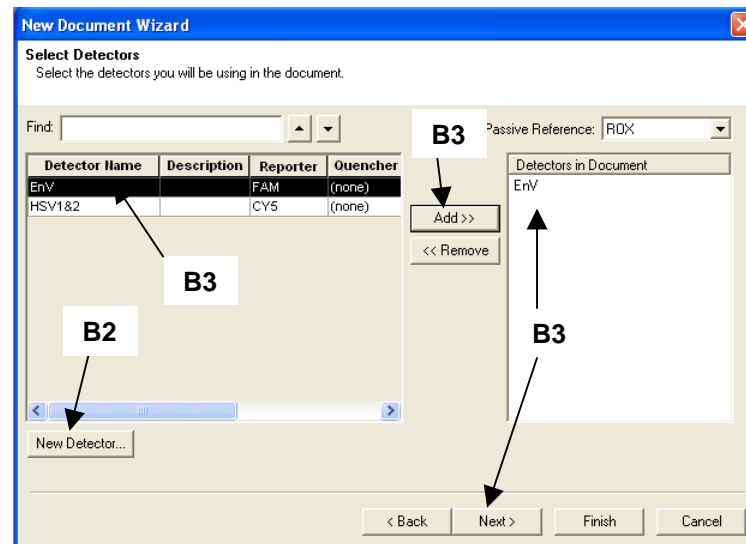
B.3. Once confirmed the target names and fluorescence options in the "**New Document Wizard**" window, select EnV. Click the "**Add>>**" button to add them to the "**Detectors in Document**" dialogue box. Select "**Next**".

B.4. In the "**Setup Sample Plate**" window, program the wells to be analyzed according to the following instructions:

1) Select the wells to be used in the plate map.

2) Select ENV detectors by clicking the check box of EnV.

3) Select "**Task**" to assign the detector task for each sample with "Unknown", "Standard" or "NTC" (blank).

4) Select "**Finish**".

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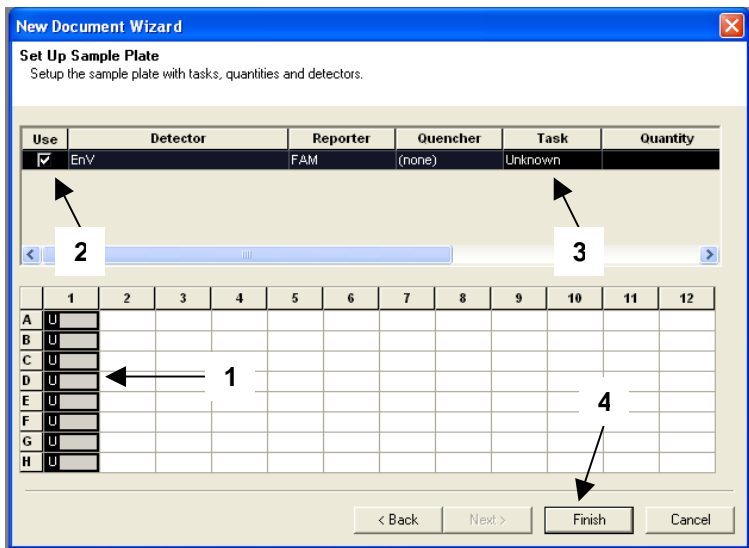
B.5. In the "**Plate Setup**" window, enter sample name by clicking a well. A "**Well Inspector**" dialogue box will then appear. Enter appropriate sample name for each well. Select the next well and enter sample name until finishing naming all the wells. Lastly, click on the **"Instrument"** tag to set up the PCR conditions.
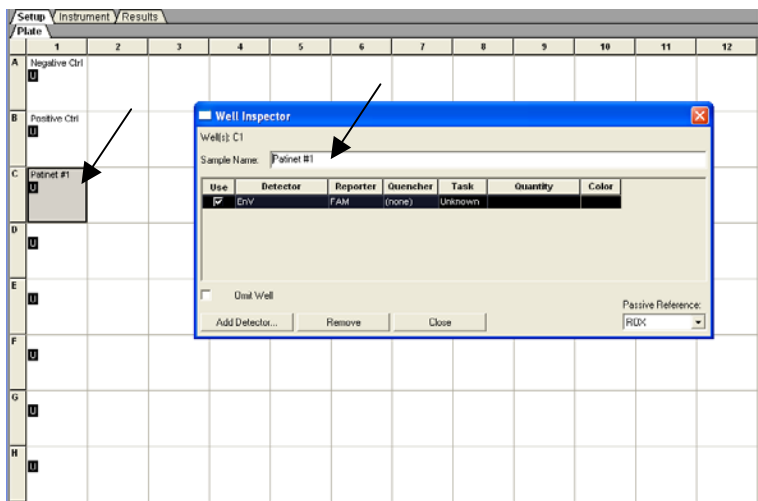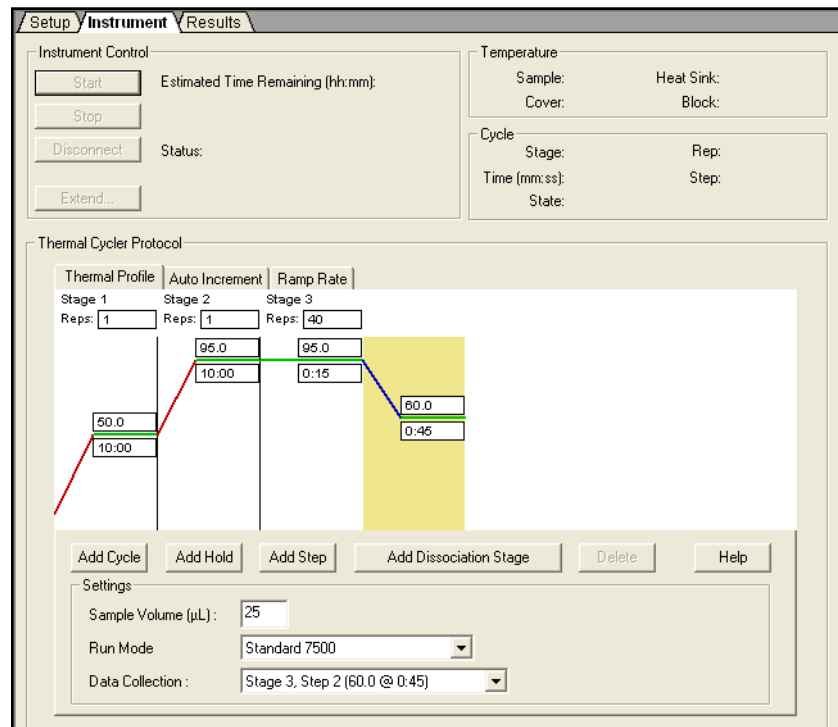

B.6. Set up the PCR conditions as follows:

	Stage 1	**Stage 2**	**Stage 3**	
Reps	1	1	40	
Temperature	50.0 ^{o}C	95.0 ^{o}C	95.0 ^{o}C	60.0 ^{o}C
Time	10:00	10:00	0:15	0:45

- In the "**Thermal Cycler Protocol** " window, enter the values for the PCR conditions (from above table) into the corresponding boxes.

- Enter "25" for the "**Sample Volume**".

- Select **Stage3. Step2 (60.0 @ 0:45)** for "**Data collection**".

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Data acquisition

(The following section is for ABI instruments; for other type of thermal cyclers, refer to the user manual for data acquisition)

A. After the PCR program has finished, locate the data file and open it.

B. Click "Results" tab. Go to the "Amplification Plot".

C.   Highlight the wells to be analyzed at the bottom of the screen. Then, select "Delta Rn vs Cycle" in the Data drop-down list.

D.   In the Detector drop-down list, select a detector.

E. Under Analysis Settings, select "Manual Ct" and "Auto Baseline".

F. Click on "Analyze" button.

G. At the next screen, open "Graph Settings" in the "Tools" drop-down list. In the "Post-Run Settings" dialog box, click on the "Linear" button to re-draw the plot.

H. Click on each sample position (from lower window) to highlight the sample that you would like to analyze. Select "EnV" in the "Detector" window to analyze samples.

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Interpretation of Results

<u>Positive sample</u>
If the fluorescent curve of a sample is greater than the fluorescent curve of negative control curve, the sample is considered positive. The Ct value of a positive sample should be less than 40.

<u>Negative sample</u>
No Ct value will be given.

Select "EnV" in the "Detector" window and use the following table to determine whether EnV is presented in the samples.

Negative control	Positive control	Test Samples	Interpretation
Negative	Positive	Positive	**EnV positive**
Negative	Positive	Negative	**EnV negative**
Positive	Positive	Positive	**Test Fail**
Negative	Negative	Positive	**Test Fail**

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