Instructions for use

AmpliX HCV Quantitative
Lyo-Format

REAL TIME PCR DETECTION AND QUANTITATION KIT OF HEPATITIS C VIRUS DNA

Research Use Only (RUO)

<table>
<thead>
<tr>
<th>AmpliX HCV Quantitative (Lyo-format)</th>
<th>VBD0794</th>
<th>96 rcs</th>
</tr>
</thead>
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<td>June 2012</td>
<td></td>
</tr>
<tr>
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**Table of content:**

1. STORAGE AND TRANSPORTATION .......... 4
2. KIT CONTENTS .......... 4
3. INTRODUCTION .......... 4
4. PRINCIPLES OF THE PROCEDURE .......... 5
5. PRECAUTIONS .......... 5
6. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED .......... 6
7. REAGENT AND SAMPLE PREPARATION .......... 6
8. PROCEDURE PROTOCOL .......... 7
9. DATA ANALYSIS .......... 7
10. ATTACHMENT 1: CALCULATION OF VIRAL DNA CONCENTRATION. .......... 9
11. ATTACHMENT 2: ANALYTICAL SYSTEM VALIDATION. .......... 9
HEPATITIS C VIRUS DNA REAL TIME PCR DETECTION AND QUANTITATION KIT

Research Use Only

1. STORAGE AND TRANSPORTATION
   - Store assay kit at (2-8) °C in the manufacturer’s packing. Transportation at 25°C for 10 days is allowed.
   - Do not freeze reagents.
   - Shelf life of the kit – 12 months after production.
   - Do not pool reagents from different lots or from different vials of the same lot.
   - Strictly follow the Instruction manual for reliable results.

2. KIT CONTENTS

<table>
<thead>
<tr>
<th>Item</th>
<th>VBD0794 96 tests</th>
<th>SVBD0794 12 tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control sample (PC), lyophilized</td>
<td>2 vial</td>
<td>1 vial</td>
</tr>
<tr>
<td>Weak Positive Control Sample (WPC HBV/HCV/HIV), lyophilized</td>
<td>1 vial</td>
<td>1 vial</td>
</tr>
<tr>
<td>Ready Master Mix (RMM) for reverse transcription and PCR, lyophilized.</td>
<td>96 test tubes (12 strips x 8 tubes)</td>
<td>12 test tubes</td>
</tr>
<tr>
<td>Solution for Restoration of Control samples (SRC)</td>
<td>2 vials, 4ml each;</td>
<td>1 vial, 4ml each;</td>
</tr>
<tr>
<td>Samples for calibration, lyophilized (CS1 and CS2) - are used when the adequacy of analytical system has to be checked, see the Attachment 2</td>
<td>1 vial each;</td>
<td>1 vial, each;</td>
</tr>
<tr>
<td>Adhesive Foils for PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Passport” for the concentration of Control PC, WPC, CS1 and CS2</td>
<td></td>
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</tbody>
</table>

3. INTRODUCTION
The assay kit “AmpliX HCV quantitative” is intended for the quantitative detection of hepatitis C virus (HCV) RNA in patient plasma and serum. The method is based on the reverse transcription of viral RNA to generate complementary DNA (cDNA), with subsequent amplification of target cDNA by Polymerase Chain Reaction (PCR) with fluorescent detection of amplified DNA in the real-time mode.

The kit “AmpliX HCV quantitative” is intended for use in conjunction with clinical practice for diagnosis of hepatitis C disease and for clinical management of HCV infected patients disease progress.

Assay kit is adapted for real-time PCR detection systems like AmpliX NG 48 Thermocycler, AmpliX Exicyclex 96, IQ iCycler, iQ5 iCycler, CFX96 (Bio- Rad, USA), or their analogues.

The kit (VBD0794) contains reagents sufficient for 96 test runs. It is strongly recommended to use three replicas of Positive Control sample, one Weak Positive Control sample and one Negative
Control sample in each test run. The kit “AmpliX HCV quantitative” is designed to detect HCV RNA isolated from serum (plasma) using the RNA extraction kit: AmpliX Viral DNA/RNA Extraction kit.

Specificity
The assay kit is designed for in vitro determination of HCV genotypes 1a, 1b, 2a, 2b, 2c, 2i, 3, 4, 5a, 6 regardless of subtype. The samples containing HCV RNA with concentration above the detection limit will be determined as positive. If specimen does not contain HCV RNA, analysis will give negative result (in 100% of cases).

Dynamic range of estimated concentration (linearity area): from 15 IU/ml to \(10^8\) IE/ml HCV RNA for the RNA isolation from 1 ml of serum (plasma).

1 IU = 2,5 copies of HCV RNA (National Institute of Biological Standards and Control for WHO international standard for Hepatitis C virus NIBSC Code: 96/798).

Sensitivity
Assay kit securely determines HCV RNA in concentration not less than 15 IU/ml for the RNA isolation from 1 ml of serum (plasma).
4. **PRINCIPLES OF THE PROCEDURE**

Principle of analysis is based on the reverse transcription of viral RNA with subsequent PCR amplification of target cDNA by PCR with fluorescent detection of amplified DNA in the real-time mode.

Reliability of analysis is provided by application of Weak Positive control sample.

Quantification of Hepatitis C virus RNA is provided by application of the Positive Control (PC) in each test run. Positive Control is characterized in international units (IU/ml) by World Health Organization *(WHO international standard for Hepatitis C virus NIBSC Code: 96/798)*, and serves as a Quantitation Standard for calculation of viral quantity. Threshold cycle value – *Ct* – is the cycle number at which fluorescence generated within a reaction crosses fluorescence threshold, a fluorescent signal significantly above the background fluorescence. Quantity of viral RNA in initial sample is calculated by comparison of the threshold cycle value of analyzed sample and Positive Control. Also efficiency of sample preparation and reverse transcription should be considered. For convenience of the user it is recommended to use magnetic rack through workout. Specimen preparation for 1ml or 100 µl of serum (*plasma*) is allowed

5. **PRECAUTIONS**

Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents.

Avoid microbial and ribonuclease contamination of reagents when removing aliquots from reagent vials. The use of sterile disposable pipettes and RNase-free pipette tips is recommended.

Do not pool reagents from different lots or from different vials of the same lot.

Dispose unused reagents and waste in accordance with country, federal, state and local regulations.

No warranty for using kit after the expiry date.
6. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED
- Real time PCR device AmpliX NG Thermocycler, AmpliX Exicycler 96, iQ/iQ5 iCycler, CFX96 (Bio-Rad, USA), or equivalent;
- Disposable gloves, powder-free;
- Pipettes (capacity 10-100 µl) with filters (aerosol barriers);
- Disposable DNase/RNase-free tips with filters
- 0.2 ml microtube racks.

7. REAGENT AND SAMPLE PREPARATION
Prepare specimens for the assay with Extraction kit AmpliX Viral DNA/RNA extraction according to Extraction kit manual. It is strongly recommended to use three replicas of Positive Control sample, one Weak Positive Control sample and one Negative Control sample in each test run.

Attention! In case of necessity to check the adequacy of analytical system, three replicas of both Calibration sample 1 and 2 (CS1 and CS2) should be used (see the Attachment 2).

7.2. Reagent preparation
- Preparation of Control samples.
  Add 1 ml of Solution for Restoration of Control samples (SRC) into each vial with Positive Control (PC) sample and Weak Positive Control sample (WPC), mix gently, keep for 15 minutes, then carefully mix once again.
  PC and WPC should be stored at (2-8) °C and used within 1 month after preparation.

- Preparation of Ready Master Mix.
  Prior to use, warm reagents (do not open!) at room temperature (18-25)°C. Open the package, separate an appropriate number of reaction tubes with Ready Master Mix (RMM) using razor or scalpel.
  Keep the tubes which were not used for the test in the original bag. Try to squeeze excess of the air out of the bag before closing the clip.
8. **PROCEDURE PROTOCOL**

8.1. Place the tubes with processed specimens and controls to Magnetic Rack.

8.2. Prepare an appropriate number of reaction tubes with Ready Master Mix (RMM). Label each reaction tube for each patient specimen and control sample. **Attention! Put marks on the lateral part of a reaction tube.**

8.3. Add 50 µl of each processed specimen and control to the appropriately labeled reaction tube using a new RNase-free tip with aerosol barrier for each sample. **Do not grasp a sorbent particles!**

8.4. Place reaction tubes into the thermal block of real time PCR device.

8.5. Program real time PCR device as follows

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45°C</td>
<td>30 min</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>3</td>
<td>94°C, 60°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>4</td>
<td>10°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

50 cycles

**Fluorescence measurements should be done at 60°C.**

8.6. Collect real-time PCR data through the FAM channel for detection of amplification of IC DNA.

8.7. Collect real-time PCR data through the ROX channel for detection of amplification of HCV DNA.

9. **DATA ANALYSIS**

9.1. In **Positive Control** sample and **Weak Positive Control** sample (also for CS1 and CS2) the program should detect:

“ROX” fluorescent signal increase and Ct value (**HCV cDNA amplification**);

9.2. “FAM” fluorescent signal increase and Ct value (**IC cDNA amplification**).

9.3. In **Negative Control** sample the program should detect:

“FAM” fluorescent signal increase and Ct value, and no significant “ROX” fluorescent increase should appear.

If Ct value for NC along “ROX” channel is less than 40, this indicates the presence of contamination.
9.4. The program should detect an amplification signal increase for IC cDNA *(channel “FAM”)* in each sample and define **Ct** for IC. Probe analysis is valid if **Ct** of IC for this sample is equal to or less than 40.

9.5. In the case of contamination *(when NC is determined as positive)* all positive results in this test should be repeated from the RNA extraction stage. Negative samples of such test run are considered reliable.

9.6. Calculate *(IC Ct)_m* as the average **Ct** value of IC for all samples *(including PC and NC)*. Samples with **Ct** of IC, that differ from *(IC Ct)_m* by more than 2 fold, should be ignored. After screening, recalculate *(IC Ct)_m* for remaining samples.

9.7. The sample is considered **negative** if **Ct** value along the “**ROX**” channel **exceeds 40 or is not determined**.

9.8. If **Ct** of IC for this sample differs from *(IC Ct)_m* by more than 2 fold, then result for this sample should be considered as equivocal. The test should be repeated from the sample RNA extraction stage.

9.9. The sample is considered **positive** if **Ct** value along the “**ROX**” channel **does not exceed 40**. If **Ct** of IC for this sample differs from *(IC Ct)_m* more then 2 fold, the sample is considered as positive without quantitative analysis. For quantitative analysis, repeat the test beginning from the RNA isolation stage.

9.10. The test results are considered reliable only when Positive and Negative controls perform as expected.

**HCV quantitation analysis.**

9.11. For quantitative analysis calculate the HCV RNA concentration in analyzed samples in accordance with Attachment 1.

**Attention!** For RNA isolation from 100 µl the result have to be multiplied by 10.

9.12. If calculated HCV RNA concentration is in dynamic range from 100 IU/ml to 10^8 IU/ml result should be reported as positive with indication of calculated HCV RNA concentration in the sample (in IU/ml).

9.13. Test results greater than 10^8 IU/ml are above the upper limit of quantitation of the Standard test and should be reported as “Greater than 10^8 IU/ml”.

9.14. Test results less than 100 IU/ml are below the lower limit of quantitation of the Standard test and should be reported as “HCV RNA detected, less than 100 IU/ml”.

9.15. The analyzed sample is considered as negative if it obtains no **Ct** value along the **“ROX”** channel or **Ct** value exceed 40.
10. ATTACHMENT 1: CALCULATION OF VIRAL DNA CONCENTRATION.
Calculate the viral NA concentration using the following equation:

\[ C_{samp^k} = \frac{C_{PC} \times 2^{(Ct_{PC} - Ct_{SAMP^k})} \times 2^{(Ct_{IC} - Ct_{ICPC})}}{\text{sample number}} \]

Where:
- \( k \) – sample number;
- \( C_{PC} \) – PC concentration, specified in the passport of the assay kit;
- \( Ct_{PC} \) and \( Ct_{ICPC} \) – \( Ct \) value of the PC sample “ROX” and “FAM” channels, respectively;
- \( Ct_{SAMP^k} \) and \( Ct_{IC} \) – \( Ct \) value of the sample numbered \( k \) along “ROX” and “FAM” channels, respectively, if amplification efficiency is \( (Ea) = 100\% \).

11. ATTACHMENT 2: ANALYTICAL SYSTEM VALIDATION.
Calculation of viral NA concentration for PC using calibration graph.
- For precise calculation of viral NA concentration in analyzed samples, the validation of the analytical system should be done by comparison of Positive Control PC concentration, specified in the passport of the assay kit with PC concentration, calculated using calibration graph.
- Prior to use, warm reagents at room temperature (18-25)°C for 30 minutes. Prepare NC, PC and IC as recommended in the instruction.
- Add 1 ml of Solution for Restoration of Control samples (SRC) into a vial with Calibration sample 1 (CS1) and Calibration sample 2 (CS2). Mix gently, keep for 15 minutes, then carefully mix once again.

Add 30 µl of IC to each tube.

For NC, add to the tube, marked NC, 1 ml (100 µl) of Negative Control.
For PC, add to each of three tubes, marked PC, 970 (70) µl of Negative Control, and 30 µl of Positive Control.
For CS1 add to each of three tubes marked CS1 970 (70) µl of Negative Control, and 30 µl of Calibration sample 1.
For CS2 add to each of three tubes marked CS2 970 (70) µl of Negative Control, and 30 µl of Calibration sample 2.

Run the isolation of NA as recommended in the instruction.
Run the PCR (or reversed PCR). Insert concentration of CS1 and CS2, specified in the passport of Assay kit, as it recommended in the instruction of PCR instrument.
For each sample calculate correct Ct value, using the following equation:

\[ Z = \text{Ct SAMP} - (\text{Ct IC}_m - \text{Ct IC}). \]

Calculate average Z value for PC, CS1 and CS2 (Z_{PCm}, Z_{CS1m}, Z_{CS2m}).

Samples with Z, that differs from Z_m more then 2 fold, should be ignored. After screening, recalculate Z_m for samples remained.

Calculate B – coefficient for calculating of specific viral NA, using following equation:

\[ B = [\text{Lg(C_{CS1})} - \text{Lg(C_{CS2})}] / (Z_{CS2m} - Z_{CS1m}), \]

Where:

- \( C_{CS1} \) and \( C_{CS2} \) – specific viral NA concentration in Calibration samples (specified in the passport of Assay kit);
- For amplification efficiency 100% \( B = \text{Lg2} = 0.3 \).

Using received results, calculate concentration of specific viral NA for Positive Control sample, using the following analytical equation:

\[ C_{PC} = 10^{x_{PC}} \text{ (IU/ml)}, \]

Where:

- \( X_{PC} = \text{Lg} (C_{CS1}) + B \times (Z_{CS1m} - Z_{PCm}); \)
- \( Z_{CS1m} \) – average Z value for Calibration Sample 1 repeats;
- \( Z_{PCm} \) – average Z value for Positive Control sample repeats;

If the calculated value of specific viral NA concentration in PC differs no more than in 2 times from the value specified in the passport of a set, PC can be used as the sample of comparison for the further calculations of specific viral NA concentration in investigated samples.

At performance of all specified conditions it is possible to spend calculation of concentration specific virus NA (in IU/ml) in investigated samples under the formula (Attachment 1) using factor B value, calculated on calibration graph.