eSensor® HCVg *Direct* Test
Assay Manual

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Intended Use
The eSensor® HCVg *Direct* Test is intended for use on the eSensor XT-8™ system for Research Use Only. This test is not for use in diagnostic procedures.

Summary and Explanation of Test
The eSensor HCVg *Direct* Test is designed to genotype a panel of nine (9) prevalent HCV type/subtypes (1a, 1b, 2, 2a/c, 2b, 3, 4, 5 and 6), using multiplex RT (reverse transcription) - PCR amplification of extracted nucleic acid followed by a direct analysis on the electrochemical eSensor XT-8 detection system.

The eSensor HCVg *Direct* Test Application Software has been designed to detect mixed infections of 1a and 1b, 1a and 2b, 1b and 2b, 1a and 2a/c, 1b and 2a/c, 1a and 3, 1b and 3 and 1 and 4; however, the performance characteristics of the test to detect mixed infections have not been established.

Principle of eSensor Technology
The eSensor technology uses a solid-phase electrochemical method for determining the presence of one or more of a defined panel of virus target sequences. Purified DNA/RNA from an HCV positive sample is amplified using specific primers with RT-PCR enzyme mix. The amplified DNA is converted to single-stranded DNA via exonuclease digestion and is then combined with a signal buffer containing ferrocene-labeled signal probes that are specific to the different types / subtypes. The mixture of amplified sample and signal buffer is loaded onto the eSensor cartridge, which contains single-stranded oligonucleotide capture probes bound to gold-plated electrodes. The cartridge is inserted into the XT-8 instrument where the single-stranded targets first hybridize to the matched signal probes then hybridize to the complementary sequences of the capture probes. The final target/signal probe and capture probe structure is shown in Figure 1. The presence of each target is determined by voltammetry, which generates specific electrical signals from the ferrocene-labeled signal probe.

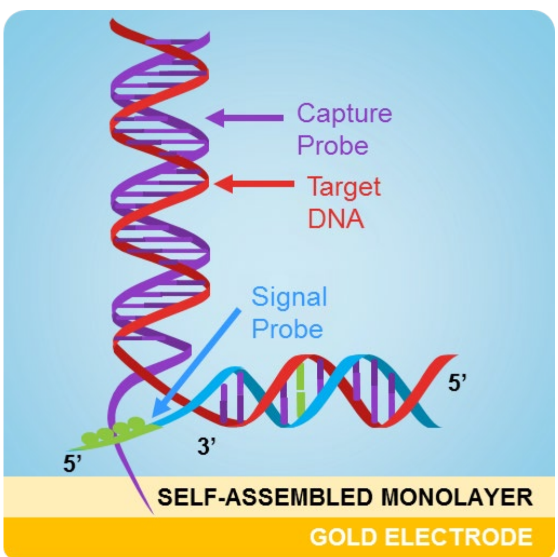

Figure 1: Hybridization complex formed at the surface of each electrode. Different, target-specific capture probes are covalently attached to the gold electrodes in the eSensor microarray. The amplified viral target DNA hybridizes to the capture probe and to a complementary ferrocene-labeled signal probe. The ferrocene label is detected at the electrode surface using voltammetry.

Limitations
The eSensor HCVg *Direct* Test is for research use only (RUO). This test is not for use in diagnostic procedures.

Warnings and Precautions

Safety
CAUTION! REAGENTS CAUSE IRRITATION TO SKIN, EYES, AND RESPIRATORY TRACT.
HARMFUL IF SWALLOWED OR INHALED!
OXIDIZING LIQUIDS!

Follow routine laboratory safety procedures for handling of reagents, e.g. do not pipette by mouth and wear appropriate protective clothing and eye protection.

Wash hands thoroughly with soap and water after handling reagents. Launder contaminated clothing prior to re-use.

Reagents are not considered a fire hazard. Liquid dries to leave a residue that may support a fire when combined with combustible materials and reducing agents. Reagents will emit hazardous fumes under fire conditions.

EXTINGUISHING MEDIA: Water, CO2, Dry Chemical, Foam
Not considered an explosion hazard.

Handle all specimens in accordance with Universal Precautions. Waste must be classified and disposed of in accordance with all Federal, State, and Local environmental regulations.

General
- Do not substitute eSensor HCVg *Direct* Test reagents with alternate reagents.
- Do not reuse cartridges.
- Do not insert a cartridge that is wet on the outside into the XT-8 instrument.
- Do not combine different lots of reagents.
- Follow the procedure as described in this Assay Manual.
- After test completion, remove cartridges from the XT-8 instrument. Do not leave cartridges in the instrument for an extended period of time.

Table 1: Components and Reagents Provided for 24 Tests

Box	Component	Packaging & Quantity	Storage
eSensor HCVg *Direct* Test Cartridges	eSensor HCVg *Direct* Test Cartridges	3 foil bags with 8 cartridges in each cartridge pack	10-25 ºC
eSensor HCVg *Direct* Test Amplification Reagents	RT-PCR Enzyme	2 vials with 40 µL	-20 ºC (in a designated pre-PCR location)
	HCVg *Direct* Test RT-PCR Mix	1 vial with 600 µL	
eSensor HCVg *Direct* Test Detection Reagents	Exonuclease	1 vial with 145 µL	-20 ºC (in a designated post-PCR location)
	HCVg *Direct* Test Signal Buffer	1 vial with 2200 µL	
	Buffer-1	1 vial with 350 µL	
	Buffer-2	1 vial with 700 µL	

Required / Recommended Materials and Equipment Not Provided

Equipment

- Vortex Mixer (with platform head for 96-well PCR plate mixing)
- Dry Heat Block
- Cold Block or Ice
- Adjustable Pipettes
- PCR Thermal Cycler compatible with 0.2 mL reaction tubes and 96-well reaction plates
- Microcentrifuge (with adaptor if using PCR tubes or strips)
- Plate Centrifuge (with adaptor if using 96-well PCR plates)
- Dead Air Hood or Dedicated PCR workstation

Consumables

- RNase/DNase-free PCR tubes and caps (0.2 mL, thin-walled), strips of 8 tubes with **individual** caps, or 96-well PCR plates and seals
- Water, Molecular Biology Grade, RNAase/DNase-free
- Disposable Gloves
- Microfuge Tubes, RNase/DNase-free
- Microfuge Tube Racks
- Pipette Tips, Aerosol Resistant, RNase/DNase-free
- Nucleic acid decontaminating solutions or 10% bleach for appropriate surfaces

Storage, Stability, and Handling Requirements

- eSensor HCVg *Direct* Test components should be stored with a unidirectional workflow in mind (i.e., maintaining physical separation of the Amplification Box from the Detection Box and Cartridge Pack).
- The unopened product should be stored as recommended in the table above. Storage of product components at conditions outside the recommended temperatures may result in assay failure. Store frozen reagents in a non-frost-free freezer. Avoid storing reagents in the door of the freezer.
- Place thawed reagents back into non-frost-free freezer immediately after use.

Assay Controls

Good laboratory practice recommends the use of controls to assure functionality of reagents and proper performance of the assay procedure. Controls are intended to monitor for substantial reagent failure. Controls used with the eSensor HCVg *Direct* Test are described in detail in this section.

Negative Control

A negative assay control should be included with each eSensor HCV *Direct* Test run. This negative control is run through the assay process from viral nucleic acid isolation to RT-PCR and XT-8 detection. The sample is identified as the negative control by the user on the XT-8, and the software will determine whether the negative control is valid.

Viral Nucleic Acid Isolation

Test samples, negative controls, and external viral controls should be prepared for testing by extraction of purified nucleic acid by a validated method. The Total Nucleic Acid® extraction protocol starting with 500 µL of plasma or serum performed on the Roche MagNA Pure® or the bioMérieux NucliSENS® easyMAG® have been evaluated for use with the HCVg *Direct* Test. Other extraction methods and input volumes have not been evaluated.

Procedural Notes

General

- Throughout the procedure section of this Assay Manual, there are references to specific actions. The details of these actions are defined below:

    Thaw: Frozen reagents, except enzymes, and samples are thawed at 37°C for up to 15 minutes. It is acceptable to thaw at room temperature, although this may take longer.

    Vortex: Thoroughly mix volumes together using a vortex set at maximum speed for 3-5 seconds.

    Centrifuge: Consolidate liquids by centrifuging briefly (5-10 seconds) in a centrifuge or mini-centrifuge.

- Do not pool reagents or mix and match kit components from different lots.
- Immediately after use, close all vials to prevent spillage or contamination.
- Store all vials in an upright position
- Do not use a product after its labeled expiration date.
- This test is for use with human serum or EDTA plasma samples containing Hepatitis C virus.
- All instruments and pipettes should be calibrated.
- Avoid use of a repeating pipette to reduce risk of contamination.
- Routinely wipe down pipettes with 10% bleach solution followed by 70% isopropanol before and after each run.
- Thoroughly decontaminate the lab, using 10% bleach solution followed by 70% isopropanol, between tests. Wipe down all surfaces, equipment, and pipettes. Remove and replace any trash containing potentially contaminated material.
- Specific instructions provided below must be carefully followed; a schematic diagram of the assay workflow is shown in Figure 2.

PCR Amplification

- All steps prior to PCR thermal cycling should be performed in a pre-amplification area.
- Dedicated pipettes, tips, and equipment are recommended for all pre-amplification area activities.
- Precautions must be taken to avoid genotype amplicon contamination throughout the procedure; samples, consumables, and lab areas should be protected from aerosol or direct contamination with amplicon. Decontaminate laboratory areas and affected equipment before and after each assay run.
- The Enzyme Mix should be handled with care to minimize the risk of inactivation; it should be kept on ice or in a cold block during use with any unused portion promptly returned to -20˚C storage conditions.
- Once the procedure has been initiated all steps should be followed continuously without interruption until the thermal cycler has been started.

Exonuclease Digestion

- This post-PCR step should be performed only in laboratory areas designated for PCR products.
- Dedicated pipettes, tips, and equipment are recommended for all activities involving PCR products.
- Precautions must be taken to avoid amplicon contamination throughout this procedure; samples, consumables, and lab areas should be protected from aerosol or direct contamination with amplicon. Decontaminate laboratory areas and affected equipment before and after each assay run.
- The Exonuclease should be handled with care to minimize the risk of inactivation; it should be kept in a cold block during use and then promptly returned to proper storage conditions.

- Visually verifying the addition of exonuclease directly into the amplicon solution is highly recommended.
- To ensure complete mixing of the enzyme solution with the amplicon solution, it is IMPORTANT that each tube is vortexed for at least 5 seconds prior to centrifuging.

Cartridge Assay

- This post-PCR step should be performed only in laboratory areas designated for PCR products.
- Dedicated pipettes, tips, and equipment are recommended for all activities involving PCR products.
- Precautions must be taken to avoid amplicon contamination throughout this procedure; samples, consumables, and lab areas should be protected from aerosol or direct contamination with amplicon. Decontaminate laboratory areas and affected equipment before and after each assay run.
- Avoid contact of Signal Buffer with the skin, eyes, or mucous membranes. If contact does occur, refer to MSDS. If left untreated, burns may result.
- Add Buffer-1 to the Signal Buffer BEFORE adding Buffer-2.
- A milky white precipitate may form following addition of Buffer-2. If vortexing for 10-20 seconds does not dissolve the precipitate, heat solution to 37ºC for 5 minutes and vortex again.
- Once hybridization solution is added to the amplicon, it should be loaded into the cartridge and the scanning protocol started within 8 hours.
- **IMPORTANT!!!** Do not freeze the hybridization solution.
- Unused cartridges from an open pouch should be kept in their original foil pouch in a dry place at room temperature with the zip-lock closure sealed.

eSensor® HCVg *Direct* Test Workflow

PCR SET-UP
RT-PCR Mix 22.2 µL
Enzyme Mix 2.8 µL
RT-PCR Master Mix 25 µL
RT-PCR Reaction Mix 35 µL
Extracted RNA 10 µL
PCR
EXONUCLEASE SET-UP
dsDNA Amplicon 40 µL
5 µL
Exonuclease 5 µL
EXONUCLEASE DIGESTION
Signal Buffer 70 µL
Buffer-1 10 µL
Buffer-2 20 µL
GENOTYPING SETUP
125 µL
100 µL
ssDNA Amplicon
Hybridization Solution 100 µL
eSensor® XT-8 Cartridge
HYBRIDIZATION / DETECTION

Figure 2: Overview of the eSensor HCVg *Direct* Test workflow procedure. The test consists of 3 steps for each sample: 1) multiplex RT-PCR to generate genotype amplicon, 2) exonuclease digestion to yield single-stranded target, and 3) hybridization and detection. The volumes indicated are per sample.

Detailed Procedure

PCR Set-Up

1. Decontaminate the work area used for setting up the RT-PCR.
2. Remove the HCVg *Direct* Test Enzyme Mix and HCVg *Direct* Test RT-PCR Mix from the Amplification Box. Place the Enzyme Mix on ice (or in a pre-cooled cold-block).
3. Thaw and vortex the frozen HCVg *Direct* Test RT-PCR Mix.
4. Centrifuge the HCVg *Direct* Test Enzyme Mix and the HCVg *Direct* Test RT-PCR Mix and place both reagents on ice or a cold block.

5. Prepare the RT-PCR Master Mix by using "RT-PCR Master Mix Set-Up Table" from the eSensor HCVg *Direct* Test Worksheet to calculate the volumes of reagents required to formulate the RT-PCR Master Mix. The calculations are designed to make 10% excess to ensure that there will be adequate solution for aliquot dispensing. Ensure that adequate reactions are prepared to accommodate all controls in the run.
6. Combine all reagents in a tube of sufficient volume.
7. **IMPORTANT!!!** Vortex and centrifuge the RT-PCR Master Mix tube
8. Place RT-PCR Master Mix tube on ice or in a cold-block.
9. Label PCR tubes/plate with sample numbers.
10. Place labeled tubes/plate on ice or in a cold-block.
11. Dispense 25 µL of RT-PCR master mix into each labeled PCR tube/plate well.
12. Return the HCVg *Direct* Enzyme Mix and the HCVg *Direct* RT-PCR Mix to the -20°C freezer.
13. If necessary, thaw, vortex, and centrifuge all tubes containing extracted samples.
14. Change gloves before adding samples to PCR tubes/plate.
15. Add 10 µL of extracted sample to each PCR tube/plate well containing master mix.
16. Cap/seal the PCR tubes/plate. Mix the solution by vortexing.
17. **IMPORTANT!!!** RT-PCR reactions **MUST** be vortexed and centrifuged after the addition of the HCVg *Direct* Enzyme Mix.
18. Program the thermal cycler according to the protocol outlined in Table 2. If the PCR instrument has adjustable ramp rates, set the ramp transition to average 2.0 to 2.5°C per second.

Table 2: Thermal Cycling Protocol – RT-PCR

Step	Temperature	Duration
RT Step		
Reverse Transcription	50°C	30 minutes
Initial PCR Activation Step	95°C	15 minutes
3-Step Cycling (45 Cycles)		
Denaturation	95°C	30 seconds
Annealing	58°C	30 seconds
Extension	72°C	30 seconds
Cool Down		
Hold	4°C	Until use in next step

19. Place the reaction tube(s)/microplate in the thermal cycler and start the RT-PCR protocol.
20. After completion of RT-PCR, remove the reaction tube(s)/microplate from the thermal cycler for exonuclease digestion and XT-8 detection steps. Alternatively, samples can be stored frozen at -15 to -30°C for up to one week before processing.

Single-Stranded DNA Generation/Exonuclease Digestion

1. If the PCR products are frozen, thaw, vortex, and centrifuge.
2. Remove the exonuclease from freezer, centrifuge, and place in a cold block. Do NOT vortex the exonuclease enzyme.
3. Add 5 µL of exonuclease directly into the liquid in each PCR reaction tube/well and close the cap or seal the well depending on the PCR set up. After dispensing, visually confirm liquid has been expelled from the pipette tip.

4. Vortex and centrifuge each tube/microplate.
5. Place PCR tube(s)/microplate in a thermal cycler and perform exo-digestion according to the protocol outlined in Table 3 below.

Table 3: Exonuclease Digestion Protocol

Thermal Cycling Protocol – Exonuclease		
Step	**Temp.**	**Duration**
Digestion	37°C	20 minutes
Inactivation	95°C	2 minutes
Hold	4°C	Until use in next step

6. After completion of incubation, remove the tubes/microplate from the thermal cycler. Proceed immediately with the next step.

Genotyping/Detection Set-Up

1. Thaw HCVg *Direct* Test Signal Buffer, Buffer-1, and Buffer-2. Mix by vortexing 3-5 seconds and then lightly tap on bench to minimize liquid left on the sides and inside the cap.
2. Use the “Hybridization Solution Set-Up” table from the eSensor HCVg *Direct* Test Worksheet to calculate the required reagent volumes. The calculations are designed to make excess to ensure that there will be adequate solution for aliquot dispensing.
3. Label a tube of sufficient volume as “Hyb.”
4. Combine the appropriate volumes of each reagent into the Hyb tube. It is important that Buffer-1 is added to the HCVg *Direct* Test Signal Buffer prior to Buffer-2. A cloudy white precipitate may form after addition of Buffer-2, but the solution will turn clear with vortexing in the next step.
5. Vortex the tube at maximum speed for 3-5 seconds, or until precipitate clears. If vortexing does not dissolve the precipitate, heat the solution to 37ºC for 5 minutes before vortexing again. Centrifuge or lightly tap the tube on a bench to minimize the amount of liquid left on the sides and inside the cap.
6. Transfer 100 µL of “Hyb” solution to each sample tube or microplate well, changing pipette tips between samples to avoid cross contamination.
7. Label cartridge(s) with the accession number(s) and place in cartridge tray(s).
8. Pipette 125 µL of Hyb-sample mix into the appropriately labeled cartridge.
9. Close each cartridge by firmly pressing the attached cap until the top of the cap is level with the top of the loading reservoir to ensure proper sealing.
10. Insert cartridges logo side up into the appropriate slots of the XT-8 instrument.
11. Slide the lever to the left to engage the cartridge. The appropriate scanning protocol will automatically show up and the LED for this slot will turn from blue to orange.
12. Enter the Accession Number or Sample ID for each cartridge into the XT-8 Software. Accession numbers can be entered by barcode, work list or manually (see eSensor XT-8 User Manual for additional instructions). The LED for this slot will turn from orange to yellow, meaning that this test is ready to start.
13. Scan in the Reagent Barcode located on the detection box and optional batch number in the appropriate box prior to touching the Start button.
14. Start the hybridization by clicking the Start button.

Results Analysis

Test Results Reports

Results may be printed, viewed electronically, or exported for additional analysis. See the eSensor XT-8 User Manual for additional details. The eSensor HCVg *Direct* Test assay generates two different report types:

1. eSensor HCVg *Direct* Test Report: Summary of the results obtained from all genotypes/subtypes (Detected or Not Detected) and the Viral Target Detection (Detected or Not Detected). Reporting options are flexible to view results in the following manner:
    - Full Panel (1a, 1b, 2, 2a/c, 2b, 3, 4, 5, and 6)
    - 1-4, 6 (1a, 1b, 2, 2a/c, 2b, 3, 4, and 6)
    - 1-4 (1a, 1b, 2, 2a/c, 2b, 3, 4)
2. eSensor HCVg *Direct* Test PCR Blank: Indicates Pass or Fail for the PCR Blank sample.

Assay Calling Parameters

In order to minimize the likelihood of an incorrect result while also minimizing the frequency of "no-call" results that require repeat testing, the eSensor HCVg *Direct* Test uses the principle of redundant electrodes. Signal generated from each electrode is evaluated against a pre-established signal threshold by the application software. This threshold ensures sufficient signal has been generated to discriminate from background levels.

Results Section

Valid results: If the assay controls pass and a valid genotyping call can be made from the signals generated from the assay, the appropriate HCV type/subtype will be displayed in the Summary Section of the report.

When an HCV virus is not detected, the Result column of Summary Section shall display "HCV Not Detected.". A repeat test is recommended when such a result is obtained. If the repeat test produces a similar result, it is recommended that GenMark Diagnostics Technical Support be contacted.

A 'No Genotype Detected' result will be displayed when HCV is detected, but no HCV genotype/subtype signal is present.

An 'Unable to Genotype' result will be displayed when HCV is detected and either a novel or partial genotype/subtype pattern is detected. Refer to the Troubleshooting Table for additional information on 'Unable to Genotype' result.

PCR Blank

It is routine laboratory practice to perform a no template control (PCR blank) as part of a PCR run to determine if amplicon contamination from prior reactions has been introduced during reaction set-up.

The PCR Blank report will provide a summary result of 'Passed PCR Blank test' or 'Failed PCR Blank test'. The description indicates what action, if any, is required based on the results of the PCR blank test.

- A Passed PCR Blank result indicates no DNA contamination was present during PCR set-up, and no further action is recommended.
- A Failed PCR Blank result indicates a risk of incorrect results due to DNA contamination. Decontamination of the PCR set-up laboratory is strongly recommended.
- An Invalid PCR Blank result indicates the sample cannot be evaluated. Please contact Technical Support.

In the event of a failure of the PCR blank test, it may be necessary to perform a retest of all samples in the batch due to potential contamination risk. Contact Technical Support to review the data.

Controls

Hardware and Software Controls: The system contains controls in the hardware and software to enable proper performance. See the eSensor XT-8 User Manual for details.

Instrument Maintenance and Calibration

No routine calibration or user maintenance is required. Please refer to the eSensor XT-8 User Manual for system

diagnostic testing recommendations.

Troubleshooting Table

The following table may be helpful in resolving issues that may arise when using this product. For more troubleshooting information, please refer to the eSensor XT-8 User Manual. If there are any additional questions regarding the eSensor HCVg *Direct* Test, please call Technical Support at 1-800-eSensor (373-6767), Option 2.

Observation	Cause / Remedy
1. Insufficient volume of RT-PCR or hybridization solutions after preparation	Some of the possible causes for this are: ▪ Calculation errors Double-check all calculations. ▪ Pipetting error Check that pipettes are properly calibrated and set at the appropriate volume settings when in use. ▪ Accidental omission of a required reagent Discard suspicious formulation and repeat formulation. ▪ PCR tubes or plates are not properly sealed Verify secure closure prior to loading into the thermal cycler. ▪ Spill Repeat RT-PCR or hybridization solution set up process.
2. Hybridization buffer precipitates upon formulation	Some of the possible causes for this are: ▪ Insufficient mixing Vortex all reagents as directed. ▪ Expired components were used Check that all components are still within their expiration dates. ▪ Improper storage and handling of reagents Check that all reagents have been stored as specified in the protocol. ▪ Pipetting error Check that pipettes are properly calibrated and set at the appropriate volume settings when in use.
3. Message in summary section of report says “Unable to Genotype”	Some of the possible causes for this are: ▪ Degraded sample ▪ Poor recovery from extracted sample ▪ Poor amplification of sample ▪ Contamination
4. Message in summary section of report says “Invalid”	Some of the possible causes for this are: ▪ System or cartridge test error Re-test is recommended

GenMark Technical Support

GenMark Diagnostics, Technical Support
5964 La Place Court, Carlsbad, CA 92008
Phone: 1-800-eSensor (1-800-373-6767), Option 2
Email: technicalsupport@genmarkdx.com
7 am to 6 pm (Pacific Time), Monday - Friday

Glossary of Symbols

Symbol	Description	Symbol	Description
LOT	Batch Code		Use by YYYY-MM-DD
	Caution, Consult Accompanying documents	SN	Serial number
Σ	Contains sufficient for <n> tests	REF	Catalog number
i	Consult instructions for use		Upper limit of temperature
	Manufacturer		Temperature limitation
	Oxidizers		Irritant, dermal sensitizer, acute toxicity (harmful), narcotic effects, respiratory tract irritation

Trademarks
eSensor®, GenMark™, GenMark Dx® and XT-8™ are trademarks of Clinical Micro Sensors d/b/a GenMark Diagnostics.
MagNA Pure® is a registered trademark of Roche.
NucliSENS® and easyMAG are registered trademarks of BioMerieux.

Patent Information
The GenMark Diagnostics eSensor® HCVg *Direct* Test and/or use thereof features technology claimed in one or more of the following patents owned or licensed by GenMark, with multiple additional foreign and domestic patents pending: U.S. Patent Nos. 5,620,850, 6,013,170, 6,013,459, 6,063,573, 6,090,933, 6,096,273, 6,197,515, 6,221,583, 6,232,062, 6,248,229, 6,264,825, 6,265,155, 6,291,188, 6,306,584, 6,322,979, 6,361,958, 6,432,723, 6,472,148, 6,479,240, 6,495,323, 6,518,024, 6,541,617, 6,600,026, 6,602,400, 6,642,046, 6,740,518, 6,753,143, 6,761,816, 6,809,196, 6,824,669, 6,833,267, 6,875,619, 6,942,771, 6,960,467, 6,977,151, 7,014,992, 7,018,523, 7,033,760, 7,045,285, 7,056,669, 7,087,148, 7,090,804, 7,125,668, 7,160,678, 7,172,897, 7,267,939, 7,312,087, 7,381,525, 7,381,533, 7,384,749, 7,393,645, 7,514,228, 7,534,331, 7,560,237, 7,566,534, 7,579,145, 7,582,419, 7,595,153, 7,601,507, 7,655,129, 7,713,711, 7,759,073, 7,863,035, 7,935,481, 8,012,743, 8,114,661, 8,383,356, 8,486,247, and 8,501,921; European Patent Nos. 0871642, 0923595, 0988534, 1075549, 1183102, 1218541, 1246699, and 1350568.

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Effective Date: July 2019
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eSensor® HCVg *Direct* Test Worksheet

Operator:____________________________ **Run:**___________________ **Date:**_____________

RT-PCR Master Mix Set-Up Table			
Component	**Volume/Reaction**	**Calculation†**	**Volume (µL)**
HCVg *Direct* Test RT-PCR Mix	22.2 µL	22.2 * N * 1.1=	
HCVg *Direct* Test Enzyme Mix	2.8 µL	2.8 * N * 1.1=	
Total Volume	25 µL	25 * N * 1.1=	
†N = Total number of reactions in run, including PCR Blank			

Thermal Cycling Protocol – RT-PCR		
Step	**Temperature**	**Duration**
RT Step		
Reverse Transcription	50°C	30 minutes
Initial PCR Activation Step	95°C	15 minutes
3-Step Cycling (45 Cycles)		
Denaturation	95°C	30 seconds
Annealing	58°C	30 seconds
Extension	72°C	30 seconds
Cool Down		
Hold	4°C	Until use in next step

Thermal Cycling Protocol – Exonuclease		
Step	**Temp.**	**Duration**
Digestion	37°C	20 minutes
Inactivation	95°C	2 minutes
Hold	4°C	Until use in next step

Hybridization Solution Set-Up			
Component	**Volume/ Reaction**	**Calculation†**	**Volume (µL)**
HCVg *Direct* Test Signal Buffer	70 µL	70 * N * 1.2 =	
Buffer-1	10 µL	10 * N * 1.2 =	
Buffer-2	20 µL	20 * N * 1.2 =	
Total Hybridization Solution Volume =			
†N = Total Number of Reactions in Run, including PCR Blank			