INSTRUCTION FOR USE

QuantiDNA™ Direct cfDNA Test

CATALOG NUMBER

DC-08-0096R (96 Reactions)

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INTENDED USE

For Research Use Only

*Where appropriate, please disregard assay mixes that are not part of your kits.

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CONTENTS

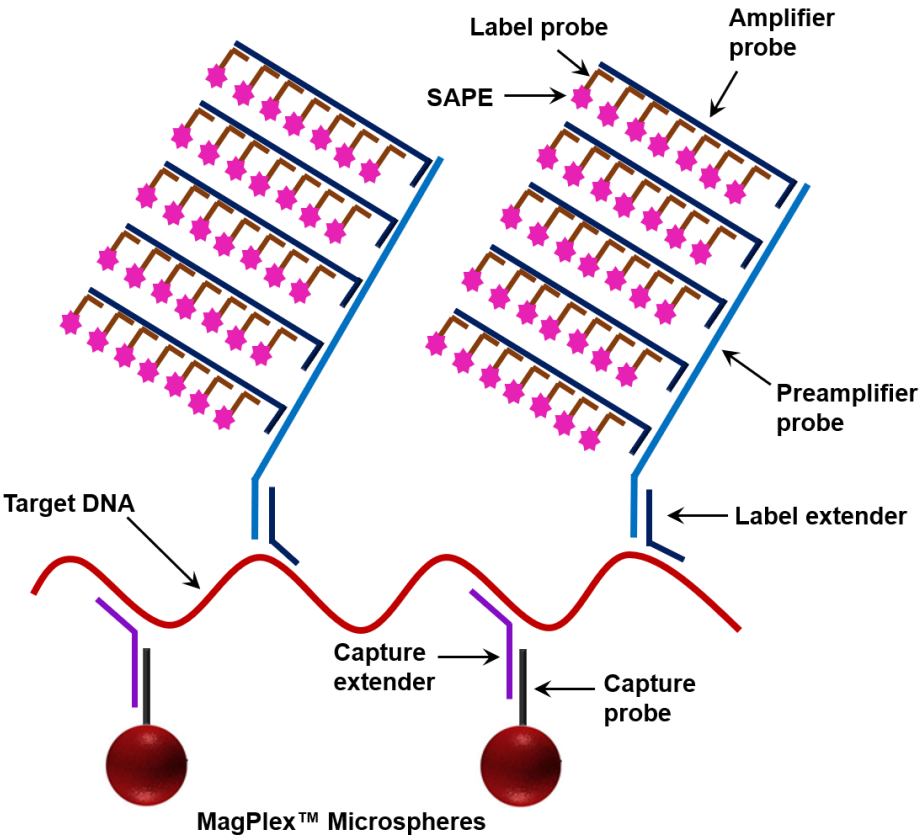
PART 1. INTENDED USE

The QuantiDNA™ Direct cfDNA Test is a testing kit that directly measures the concentration of human circulating cell-free DNA (cfDNA) in plasma. It is a nucleic acid probe hybridization assay that uses the branched DNA (bDNA) technology to amplify chemical signal generated in the presence of target cfDNA sequence without amplifying the cfDNA itself. The intensity of the chemical signal is used to calculate and quantitate cfDNA from human plasma.

PART 2. THE PRINCIPLE OF QUANTIDNA™ DIRECT CFDNA QUANTITATION ASSAY

QuantiDNA™ uses a modified bDNA molecule and new probe set designs that result in increased amplification of chemical signal that correlates with the quantity of the target DNA sequence without amplifying the target sequence itself. The ability to quantify DNA molecules in a sample lies in the design of a probe set (Fig. 1). Each oligonucleotide probe set contains two types of synthetic probes, Capture Extenders (CEs) and Label Extenders (LEs) that hybridize and span contiguous sequences of the target DNA. First, the target DNA sequence is captured by the capture probes which have been coupled to the MagPlex™ Microspheres (commonly called “beads”) through cooperative hybridization between CEs and capture probe and between CEs and target DNA. Then, the LEs bind both to the target DNA and the Preamplifier Probe. One Preamplifier Probe has 20 binding sites for Amplifier Probe which possesses 20 binding sites for Label Probe. Therefore, one signal will be eventually amplified 400 folds. Finally, Biotinylated Label Probes will bind to Streptavidin phycoerythrin (SAPE) and the fluorescence signal on the beads can be detected by Luminex instruments such as MAGPIX®.

Figure 1. Schematic Diagram of bDNA Technology



PART 3. REAGENTS AND INSTRUMENTS

3.1. Package Contents

IMPORTANT: Upon receiving, store kit **Component 1** at 2°C to 8°C and **Component 2** at -25°C to -15°C except that Plate Seal should be stored at room temperature.

Table 1. Package Contents

		Component	Quantity	Part #	Description
Component 1		1X Lysis Mixture*	10 mL	1010611	Aqueous buffered solution with sodium azide (<0.1%)
		Plate Seal	10 sheets	1010621	Adhesive-backed foil seal
		96-well plate*	1 plate	1007773	Greiner 96-well microplate, flat bottom and clear
		10X Wash Buffer*	20 mL	1001561	Aqueous buffered solution with sodium azide (<0.1%)
		1X PBS*	10 mL	1007703	Phosphate Buffered Saline (pH 7.4, without calcium, magnesium and phenol red)
		1X Tm Buffer*	20 mL	1007713	Aqueous buffered solution with sodium azide (<0.1%) and other preservatives
		Capture Beads	110 µL	1007723	MagPlex™ Microspheres conjugated with capture probe
		Pre-Amplifier Probe	13 mL	1007733	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%)
		Amplifier Probe	13 mL	1007743	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%)
		Label Probe	13 mL	1007753	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%)
		SAPE	35 µL	1007763	Streptavidin conjugated with R-Phycoerythrin (0.6 mg/mL)
Component 2	**Grey Box 1**	Blocking Reagent	110 µL	1001041	Aqueous buffered solution with sodium azide (<0.1%)
		Human Alu Probe Set	450 µL	1001541	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%)
		Proteinase K	15 µL	1002011	Proteinase K solution with sodium azide (<0.1%)
		Positive control-high	225 µL	1010531	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		Positive control-low	225 µL	1010541	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
	Grey Box 2	DNA Standard 1	225 µL	1010551	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		DNA Standard 2	225 µL	1010561	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		DNA Standard 3	225 µL	1010571	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		DNA Standard 4	225 µL	1010581	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		DNA Standard 5	225 µL	1010591	Human genomic DNA in aqueous solution with sodium azide (<0.1%)
		DNA Standard 6	225 µL	1010601	Human genomic DNA in aqueous solution with sodium azide (<0.1%)

* Those five components can also be stored at room temperature.

Shelf-Life: Approximately 12 months after production of kit. See product labels for actual expiration date.

3.2. Materials Required but Not Provided with the Kit

- Reagent reservoirs, 25 mL capacities
- Refrigerated microcentrifuge
- Dry block heater for 1.5 mL microcentrifuge tubes
- Adjustable single- and multi-channel precision pipettes for dispensing 1–20 µL, 20–200 µL and 200–1000 µL (accuracy ± 5%)
- Pipette tips
- 15 mL sterile Nuclease-free polypropylene tubes
- 10 mL sterile-packaged serological pipettes
- 1.5 mL microcentrifuge tubes
- 200 mL solution bottles
- Autoclaved deionized water or nuclease-free water (for dilution of 10X Wash Buffer)
- Vortex mixer
- Luminex MAGPIX®
- Luminex Magnetic Plate Separator (catalog number CN-0269-01)
- PlexBio™ Thermo Shaker (catalog number 80011), or Labnet VorTemp 56 shaking incubator (catalog number S2056A), or other equivalent incubators capable of maintaining a constant temperature between 37–55 °C ±1 °C and shaking at 600rpm
- 4-inch soft rubber roller for plate sealing

3.3. Warnings and Precautions

HARMFUL! IRRITANT! May cause sensitization by inhalation and skin contact. Irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing.

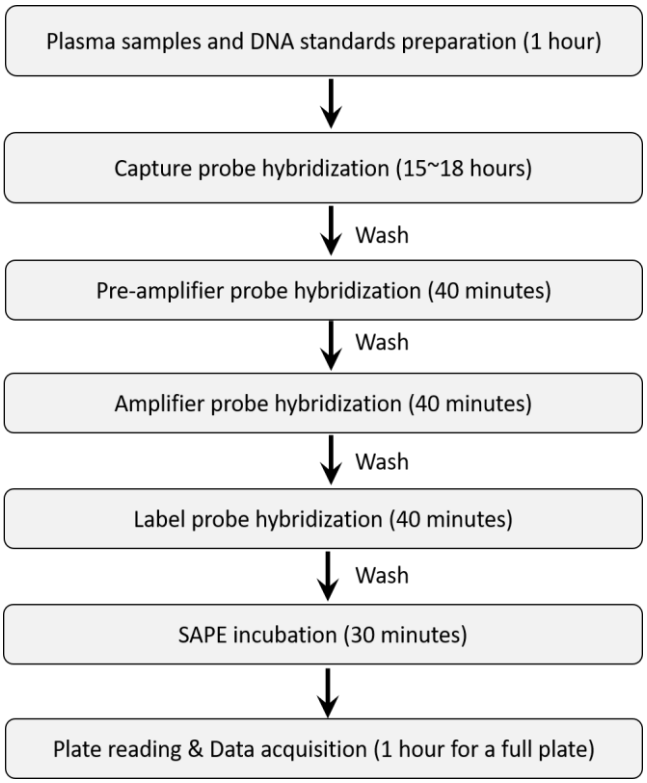
IRRITANT! Hazardous in case of skin contact (irritant), of eye contact (irritant). Hazardous in case of ingestion, of inhalation. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Wear suitable gloves and clothing. Contains Sodium Azide (CAS# 26628-22-8): Blocking Reagent, Proteinase K, Lysis Mixture, ALU probe set, Pre-Amplifier Probe, Amplifier Probe, Label Probe, 1x Tm Buffer, Wash Buffer, SAPE, Positive Control, DNA Standards.

CAUTION: Sodium azide in the reagents can react with copper and lead plumbing to form explosive metal azides. On disposal flush reagents with a large volume of water to prevent the buildup of metal azides, if disposal into a drain, is in compliance with federal, state, and local requirements.

- Perform the procedure using universal precautions.
- Disinfect spills promptly using a 0.6% sodium hypochlorite solution (1:10 v/v household bleach) or equivalent disinfectant. Handle contaminated materials as biohazardous.
- Wear personal protective apparel, including disposable gloves, throughout the assay procedure. Thoroughly wash hands after removing gloves, and dispose of gloves as biohazardous waste.
- Do not eat, drink, smoke, or apply cosmetics in areas where reagents or specimens are handled.
- Avoid the use of sharp objects wherever possible. If skin or mucous membrane exposure occurs, immediately wash the area with large amounts of water. Seek medical advice immediately. Do not pipette by mouth.
- Use all pipetting devices and instruments with care and follow the manufacturers' instructions for calibration and quality control.
- Use of aerosol-resistant pipette tips are highly recommended and use a new tip every time a volume is dispensed. Aerosol-resistant pipette tips with additives should be avoided.
- Do not use reagents if crystal or precipitate is visible after bringing to specified temperature.
- Do not use components beyond the expiration date printed on the kit boxes.
- Do not mix reagents from different lots.
- Use all kit components within 3 months after opening any component.
- Return all components to the appropriate storage condition after preparing the working reagents.
- Do not interchange vial or bottle caps as cross-contamination may occur.

PART 4. INSTRUCTIONS FOR USE

4.1. Flowchart of the Test Procedure



4.2. Plasma Sample Preparation

4.2.1. Plasma Separation

- For blood collected in EDTA-treated polypropylene tubes, within two hours after blood collection, centrifuge blood for 10 minutes at 1900x g and 4°C.
- Carefully transfer plasma supernatant without disturbing the buffy coat layer to a new 15 ml centrifuge tube.
- Centrifuge at ≥12,000 x g at 4°C for 10 minutes.
- Carefully remove supernatant to a new tube without disturbing the pellet. Prepared plasma should be stored at -80°C if not used immediately.

4.2.2. Plasma Denaturation

- Add 90 µL of 1X PBS to a 1.5 mL microcentrifuge tube, and then add 10 µL of plasma prepared above.

Note: For frozen plasma samples, bring to room temperature for 30 minutes or until completely thawed. Quickly vortex and spin down.

- Quickly vortex and spin down.
- Put plasma samples on a dry block heater and heat at 95oC for 10 minutes.
- Immediately chill on ice for at least 5 minutes.
- Quickly vortex and centrifuge the plasma samples at high speed (>8000xg) for 30 seconds at 4oC. Keep denatured plasma samples on ice prior to use.

Note: After centrifugation, a small pellet may form at the bottom of microcentrifuge tube. Do not touch the pellet when loading the plasma samples to 96-well plate.

PART 5. ASSAY PROCEDURES

5.1. Denaturation of DNA Standards and Positive Controls

5.1.1. Bring DNA standards 1 to 6, Positive Control-high and Positive Control-low to room temperature until completely thawed.
5.1.2. Quickly vortex and spin down.
5.1.3. Transfer 75 µL to 1.5ml nuclease-free microcentrifuge tubes, and then put the tubes on a dry block heater and heat at 95°C for 10 minutes.
5.1.4. Immediately chill on ice for at least 5 minutes.
5.1.5. Quickly vortex, spin down, and keep on ice prior to use.

5.2. Preparing Reagents and Setting up the Test

5.2.1. Prepare the following reagents and setting:

- Bring 1X Lysis Mixture to room temperature to make sure there is no visible crystals. If needed, place 1X Lysis Mixture at 37°C for 20 minutes to dissolve any visible crystals.
- Bring Blocking Reagent and Human Alu Probe Set to room temperature until completely thawed. Briefly vortex and spin down.
- Turn the shaker (incubator) on and set the temperature to 55°C.

5.2.2. Calculate the amount of reagents needed based on the number of wells and prepare Working Probe Solution following the table below (prepare 15% extra solutions) using a 15 ml polypropylene centrifuge tube.

Table 2. Composition of Working Probe Solution

Reagent Name	Volume (µL) Required per Well
Lysis Mixture	74
Blocking Reagent	1
Human Alu Probe Set	4
Capture Beads*	1
Proteinase K	0.1

***** Vortex the Capture Beads for 10 seconds prior to use.

5.2.3. Vortex Working Probe Solution for 10 seconds and transfer 80 µL to each well of 96-well plate.

Note: It is recommended to use a multichannel pipette to quickly dispense Working Probe Solution.

5.2.4. Add 20 µL of denatured DNA Standards, Positive Control or unknown plasma samples to 96-well plate following the layout below. Add 20 µL of 1X PBS to blank control wells.

Note: At least two replicates are recommended for blank control and unknown samples.

Table 3. Recommended Layout of the Test

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Blank	Blank	Blank	UN#8	UN#8	UN#8	UN#16	UN#16	UN#16
B	Std 2	Std 2	Std 2	UN#1	UN#1	UN#1	UN#9	UN#9	UN#9	UN#17	UN#17	UN#17
C	Std 3	Std 3	Std 3	UN#2	UN#2	UN#2	UN#10	UN#10	UN#10	UN#18	UN#18	UN#18
D	Std 4	Std 4	Std 4	UN#3	UN#3	UN#3	UN#11	UN#11	UN#11	UN#19	UN#19	UN#19
E	Std 5	Std 5	Std 5	UN#4	UN#4	UN#4	UN#12	UN#12	UN#12	UN#20	UN#20	UN#20
F	Std 6	Std 6	Std 6	UN#5	UN#5	UN#5	UN#13	UN#13	UN#13	UN#21	UN#21	UN#21
G	PC-low	PC-low	PC-low	UN#6	UN#6	UN#6	UN#14	UN#14	UN#14	UN#22	UN#22	UN#22
H	PC-high	PC-high	PC-high	UN#7	UN#7	UN#7	UN#15	UN#15	UN#15	UN#23	UN#23	UN#23

Note: Std denotes Standard; PC denotes Positive Control; UN denotes unknown plasma samples

5.2.5. Seal the plate and incubate at 55oC for 15~18 hours (overnight) with shaking at 600 rpm for PlexBio™ Thermo Shaker or at 450 rpm when using Labnet VorTemp 56 shaking incubator. Unused wells should also be sealed and can be used later if needed.

Important: Use of a rubber roller is strongly recommended for tight plate sealing to avoid any liquid evaporation.

5.3. Pre-Amplifier Probe Hybridization

IMPORTANT:

- Do not let the 96-well plate stand or dry longer than 5 minutes once the assay procedure begins.
- Do not disturb the contents of the 96-well plate or open the incubator door during the incubations.
- Prior to use, dilute the 10X Wash Buffer with autoclaved deionized water or nuclease-free water (not provided) to obtain 1X Wash buffer concentration. When stored at 2-8 °C, visible crystals may form in 10X Wash Buffer. Before making dilutions, bring 10X Wash Buffer to room temperature for 30 minutes or until visible crystals become completely dissolved.

5.3.1. Take the plate out of the incubator.
(Optional: Centrifuge Plate at 240 x g for one minute at room temperature.)

5.3.2. Remove the plate seal. Keep the unused wells sealed for later use if needed. Then put the plate on magnetic plate separator (not provided), wait 20 seconds and remove supernatant by pipetting. Use new tips for each well to avoid contamination.

5.3.3. Remove the plate from magnetic plate separator and add 150 µL of 1 x Wash Buffer.

5.3.4. Put the plate back on the magnetic plate separator, wait 20 seconds and remove the supernatant by pipetting.

5.3.5. Repeat steps 5.3.3 to 5.3.4.

5.3.6. Remove the plate from magnetic plate separator and add 100 µL of Pre-Amplifier Probe to each well.

Note: Pre-Amplifier Probe solution is viscous. Make sure the required amount is completely dispensed into each microplate well.

5.3.7. Seal the plate and incubate at 50oC for 40 minutes with shaking at 600 rpm for PlexBio™ Thermo Shaker or at 450 rpm when using Labnet VorTemp 56 shaking incubator.

5.4. Amplifier Probe Hybridization

5.4.1. Take the plate out of the incubator and wash the plate following the procedure in steps 5.3.1~5.3.5.

5.4.2. Remove the plate from magnetic plate separator and add 100 µL of Amplifier Probe to each well.

Note: Amplifier Probe solution is viscous. Make sure the required amount is completely dispensed into each microplate well.

5.4.3. Seal the plate and incubate at 50oC for 40 minutes with shaking at 600 rpm for PlexBio™ Thermo Shaker or at 450 rpm when using Labnet VorTemp 56 shaking incubator.

5.5. Label Probe Hybridization

5.5.1. Take the plate out of the incubator and wash the plate following the procedure in steps 5.3.1~5.3.5.

5.5.2. Remove the plate from magnetic plate separator and add 100 µL of Label Probe to each well.

5.5.3. Seal the plate and incubate at 50oC for 40 minutes with shaking at 600 rpm for PlexBio™ Thermo Shaker or at 450 rpm when using Labnet VorTemp 56 shaking incubator.

5.6. SAPE Incubation

5.6.1. SAPE dilution: The concentration of SAPE provided with the kit is 0.6 mg/mL (i.e. 600 µg/mL) which should be diluted to 2 µg/mL (300-fold dilution) with 1X Tm Buffer before use according to the table below. Each well needs 75 µL diluted SAPE.

Table 4. SAPE Dilution Instructions

Number of Wells	SAPE Stock Volume (µL)	1X Tm Buffer Volume (µL)
10	2.5	748
30	7.5	2243
50	12.5	3738
96	24	7176

Note:

- Volume of reagents in the above table are for the exact number of wells. End users should calculate the amount of SAPE required based on actual number of wells and prepare at least 15% extra. For example, to prepare SAPE for 50 wells, a total of 4312 µL (50 x 75 x 115%) of diluted SAPE is recommmended.
- SAPE solution is light-sensitive and should be kept in the dark prior to use.

5.6.2. Wash the plate following the procedure in steps 5.3.1~5.3.5

5.6.3. Remove the plate from magnetic plate separator and add 75 µL of 2 µg/mL SAPE to each well.

5.6.4. Seal the plate and incubate the plate at 37oC for 30 minutes with shaking at 600 rpm for PlexBio™ Thermo Shaker or at 450 rpm when using Labnet VorTemp 56 shaking incubator.

5.6.5. Put the plate on magnetic plate separator and remove the supernatant by pipetting.

5.6.6. Remove the plate from magnetic plate separator.

5.6.7. Add 75 µL of 1X Tm Buffer to each well.

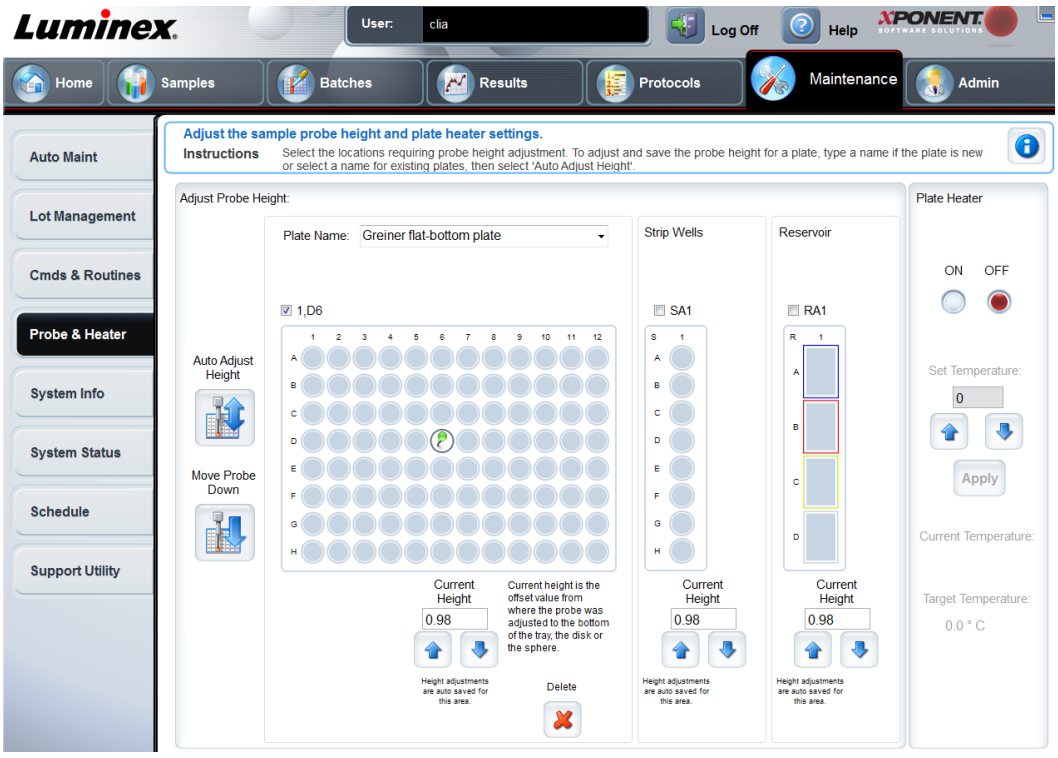
5.6.8. Load the plate to Luminex instrument and start plate reading following the procedures in 5.7.

5.7. Plate Reading and Data Acquisition

Notes:

- Described below is a general guide to data acquisition on Luminex MAGPIX® instrument. Detailed instructions may be found in the manufacturer's manual for the software version you are using.
- Perform regular calibration and verification according to the manufacturer's instructions.
- Turn on the Luminex analyzer and set the temperature at 37°C before reading the plate.
- When you run QuantiDNA™ Direct cfDNA Test on Luminex system for the first time and every time when you clean the sample probe, follow the instructions below to adjust the sample probe height to ensure that the probe drops far enough into the well to acquire sample.

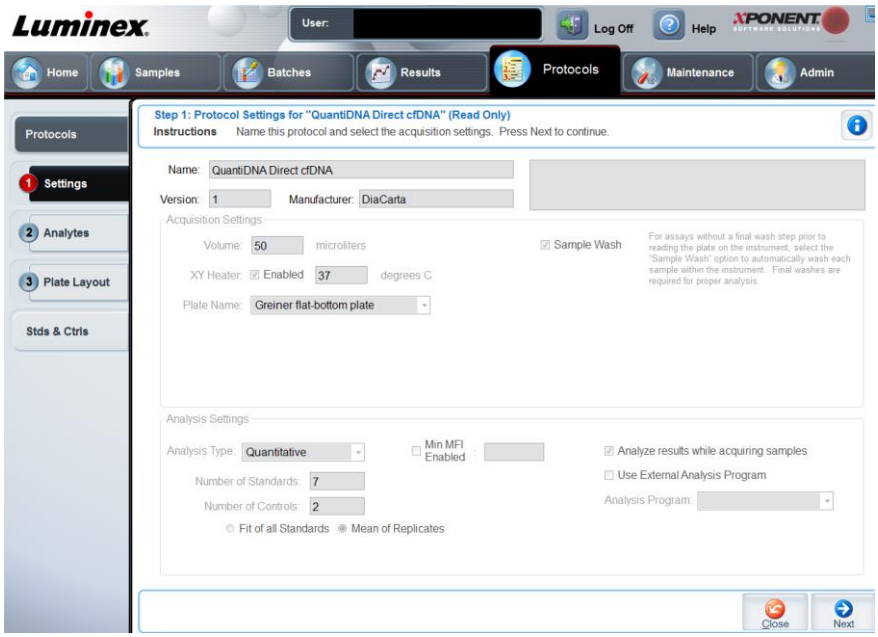
➢ Open the xPONENT software, and on the Home page, click Probe and Heater under Maintenance. The Probe & Heater tab opens. Refer to the figure below.
➢ Type a name for the plate in the Plate Name box (such as "Greiner flat-bottom plate").
➢ Click well D6 on the plate image (this is the center of a standard 96-well plate). A green pin marks the selected well.
➢ Click Eject to eject the plate holder.
➢ Place an empty Greiner flat-bottom 96-well plate on the plate holder with well A1 in the marked position, place one alignment disk in well D6, and then click Retract to retract the plate holder.
➢ Click Auto Adjust Height. The probe automatically adjusts itself to the locations you selected.
➢ Click Eject to eject the plate holder.
➢ Click Save to save the probe height settings for the plate.



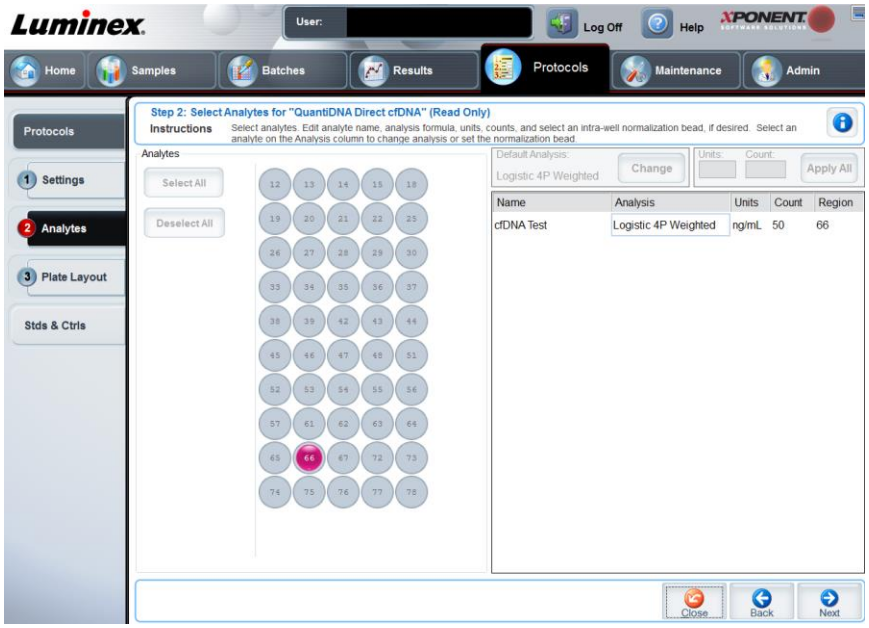
5.7.1. Creating a Test Protocol

- Open the xPONENT software, and on **Protocols** page click **Create New Protocol**.
- Type the name, version and manufacturer of the protocol following the figure below.
- In **Acquisition Settings**, type **50** for the volume, select **Enabled** and type **37** for XY Heater, enable **Sample Wash**, and select "Greiner flat-bottom plate" which has been created above.
- In **Analysis Settings**, select **Quantitative** as the analysis type, type **7** in the **Number of Standards**, type **2** in the **Number of Controls**, select **Mean of Replicates**, and select **Analyze results while acquiring samples**.

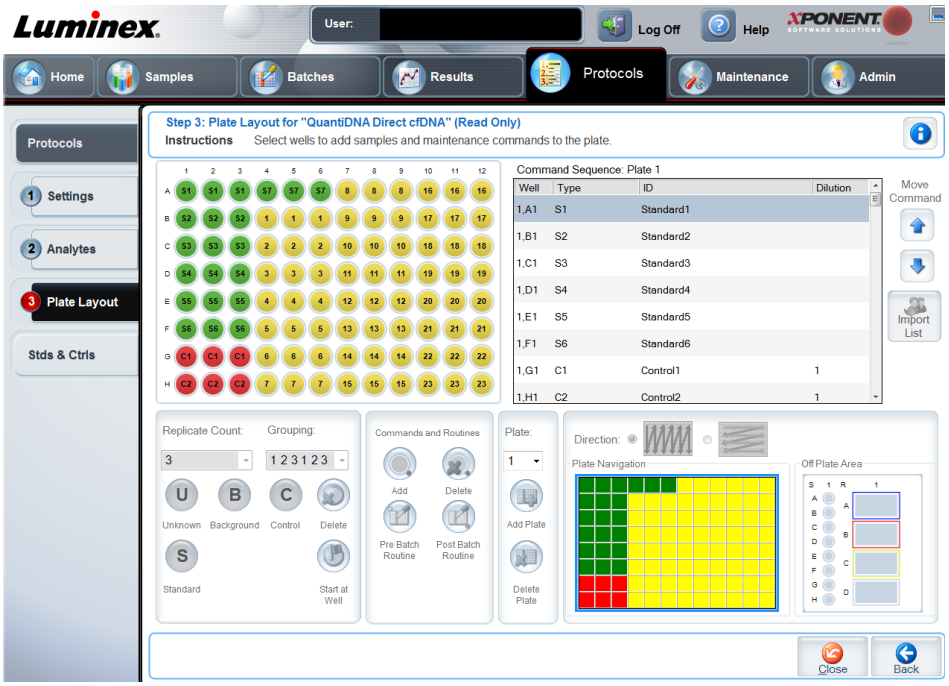
Note: *the blank wells will be used as Standard-7 where the cfDNA concentration is zero; therefore, the total number of Standards for geneating standard curve is 7.*



- Click **Next**, the **Analytes** tab opens. In the numbered analyte grid, click on **66** which is the bead ID.
- Type a name for the analyte in **Name** box (such as "cfDNA Test" shown in the figure below); Type **ng/mL** in the **Units** box, type **50** in the Count box, and click **Apply All**.
- Select **Logistic 4P Weighted** in **Analysis** box.

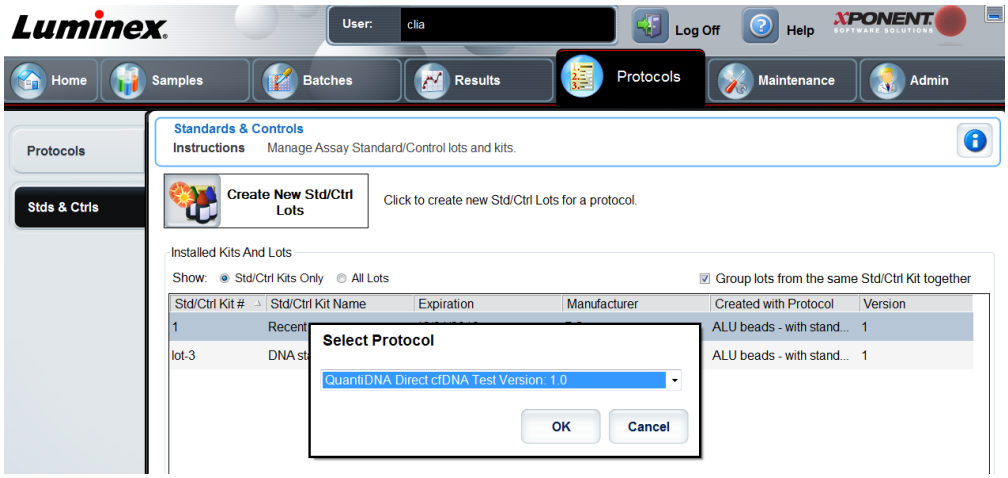


- Click Next, and the Plate Layout tab opens.
- Select the appropriate wells and mark them as standard, control, or unknown. Please note that the blank wells should be marked as S7. To run in replicate, change the Replicate Count to the appropriate number and the Grouping to your preferred grouping method.
- In “Dilution” column, enter “10” for unknown samples.
- Click Save.



5.7.2. Creating New Stds & Ctrls

- On **Stds & Ctrls** page, click **Create New Std/Ctrl Lots**.
- When **Select Protocol** box opens, select the protocol created above for “QuantiDNA™ Direct cfDNA Test Version: 1.0”, then click **OK**. See figure below.



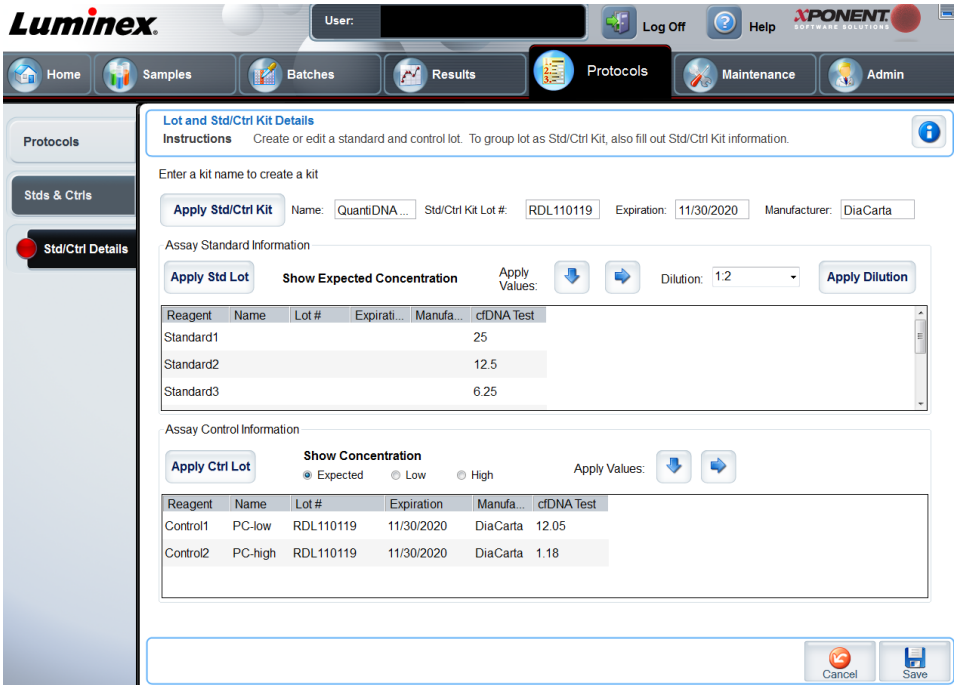
- On **Lot and Std/Ctrl Kit Details** page, type the name of the kit (such as “cfDNA Test”), the kit Lot #, Expiration date and Manufacturer which are indicated on outside label of the kit box.

- In **Std** box, type the concentration of each Standard:
  Standard 1: 25.0
  Standard 2: 12.5
  Standard 3: 6.25
  Standard 4: 3.12
  Standard 5: 1.56
  Standard 6: 0.78
  Standard 7: 0

Note: The blank wells (concentration as 0) will be used as Standard 7.

- In **Ctrl** box, type “PC-Low” as the name for **Control 1** and “PC-High” for **Control 2**. Then type the **Expected**, **Low** and **High** concentrations for each Control which are indicated on inside label of box-2 and may be variable for each batch.

- Click **Save**.



5.7.3. Create a New Batch from an Existing Protocol

- On **Batches** page, click **Create a New Batch from an existing protocol**.
- Type the batch name and select the protocol created above.
- Click **Next**, **Stds & Ctrls** page opens which shows the Standards and Controls information of the kit selected.
- Click **Next**.
- On **Plate Layout** page, assign well commands for this batch, and then click **Run Batch** to begin data acquisition.

5.8. Generating the Report and Exporting Data

- On **Results** page, click **Reports** tab and then select **Batch Reports** in **Report** drop-down list.
- Click **New Report** tab at the bottom right corner.
- A new window opens. In the **Type** drop-down list, select **Data Interpretation**.
- Select the batch of data to analyze.
- Click **Select Analytes**. Refer to the figure below.
- Click **Generate**. The report will be generated which contains detailed test information including the standard curve, recovery rate for each standard, the calculated concentration of unknown samples, etc.
- Click **Save all** to save the generated report to a desired location.
- On **Results** page, click **Saved Batches** tab, and then you can export the results by clicking **Exp Results** tab at the bottom.

Note: For instructions on other procedures such as “Creating New Samples” and “Adding a New Lot for Protocol”, please refer to xPONENT 4.2 Manual for MAGPIX Software User (IVD) which is available at https://www.luminexcorp.com/download/manual-ivd-xponent-4-2-for-magpix-software-user/.

5.9. Data Analysis

- The results will be considered valid when the measured concentration of both Positive Controls falls between the low and high concentrations.
- The measured concentration of unknown samples will be calculated automatically by the software and shown in the generated report (in .pdf format).

5.10. Reference Interval

Fifty-eight (58) blood samples from healthy donors were collected into EDTA tubes and the plasam was separated by centrifuge. The plasma samples were then tested with QuantiDNA™ Direct cfDNA kit. The mean concentration of cfDNA in normal plasma was 9.05 ng/mL (median 7.76 ng/mL, range 1.79-21.92 ng/mL).

PART 6. TROUBLESHOOTING

6.1. Troubleshooting Low Assay Signal or Poor Sensitivity

Probable Cause	Recommended Actions
Number of DNA copies below detection limit	Increase the amount of sample added to each assay well by lower dilution of the original sample
Inadequate denaturation of samples or standards	Verify the temperature of heating block for denaturation and make sure to put samples or standards on ice immediately after heating for 5 minutes
Instrument needle (probe) is partially clogged	Clean the needle or probe according to the manufacturer's recommendations and readjust the height of the probe; perform calibration and verification
Bubble introduction into Luminex fluidics	Check Luminex probe for proper height, then run instrument debubbling protocol
Expired reagents were used	Check date of manufacture (DOM) on reagents. Reagents are good for one year from DOM
Incorrect temperature in the incubator	Verify the incubator temperature with a thermometer

6.2. Troubleshooting High Background Signal

Probable Cause	Recommended Actions
Plate sat at room temperature longer than 20 minutes after the addition of samples	Avoid letting the plate sit at room temperature for longer than 20 minutes after the addition of the overnight hybridization mixture
Plate sat at room temperature for longer than 10 minutes before washing (2nd day)	Wash the plate within 10 minutes after removal from the incubator
Expired reagents were used	Check the date of manufacture (DOM) on the reagents. Reagents are good for one year from the DOM
Wash Buffer residual	Ensure that the plate wash method used completely removes all residual Wash Buffer prior to moving to the next step in the procedure
Incorrect temperature in the incubator	Verify the oven temperature with a thermometer

6.3. Troubleshooting High CVs

Probable Cause	Recommended Actions
Wash Buffer residual	Ensure that the plate wash method used completely removes all residual Wash Buffer prior to moving to the next step in the procedure.
Inaccurate pipetting	Make sure that tips are secured onto the pipette shaft. Use a new tip for each well.
Plate sealing not tight	Use a rubber roller to seal the plate tightly to avoid liquid evaporation.
Well-to-well cross contamination	Use a new tip for each well and avoid splashing reagents to other wells when pipetting.
Edge effect	The outer wells of the plate (edge wells) may generate highly variable signals due to temperature variation, especially those on the corners of the plate. Calibrate the incubator.
Instrument needle (probe) is partially clogged	Clean the needle or probe according to the manufacturer's recommendations and readjust the height of the probe; perform calibration and verification.
Bubble introduction into Luminex fluidics	Check Luminex probe for proper height, then run instrument debubbling protocol.

PART 7. PERFORMANCE CHARACTERISTICS

7.1 Limit of Detection

The limit of detection (LoD) of the assay was determined by measuring serially diluted "Seraseq ctDNA Complete" (fragment size ~170bp) until the signal was close to the blank control (background). First, the instrument limit of detection (yLoD) was calculated from the limit of blank (LoB) and standard deviation (SD) of 16 replicates of the lowest concentration control with the following formula: $yLoD = LoB + 1.645 \times SD_{(Low\ concentration\ sample)}$. The yLoD was then converted to the concentration limit of detection (cLoD) using the blank-adjusted mean signal (ys) for a diluted Seraseq ctDNA with a known concentration (Cs) by the following formula: $cLoD = Cs \times yLoD \div ys$. Limit of blank was calculated mean and 1.645 times the standard deviation (SD) of 24 replicates of the blank control with the following formula: $LoB = Mean\ blank + 1.645 \times SD_{blank}$.

Since the LoD is below the assay standard curve, the results were calculated from MFI units. The 10% (in 1xPBS) DNA-depleted human plasma was used as the blank and as the diluent for the "Seraseq ctDNA Complete". To assess the cross-contamination, we conducted an experiment that was considering both contaminations due to pipetting issues and complete wet lab process. The cross-contamination was captured by a limit of blank measurement, which is defined as the mean and 1.645 times the standard deviation of 24 replicates of the blank control.

Three lots of kits were tested. For all three-lot we run 24 replicates of the blank placed between the reference material and 16 replicates of 0.08ng/mL, 0.04 ng/mL and 0.02 ng/mL dilutions. Raw data MFI are present in Table 5, 6 and 7.

Table 5. Fluorescence signal (MFI) of three low concentration Seraseq ctDNA and blank control (Kit Lot:RDL100119)

Samples	MFI for each of replicates												Average	SD
0.08 ng/mL	127	120	110	98	106	93	90	83	121	124	105	91	103	13.53
	102	94	93.5	88	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.04 ng/mL	72	71	67	55	57	51	50	53	65	77	74	63	61	8.83
	58	57	53	50	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.02 ng/mL	47	46	47	41	44	38.5	43	39	50	65	64	54	49	7.64
	45	51	56	46	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
Blank control	19	15	13	14	13	15	12	14	15	15	14	13	15	2.96
	13	12	12	14	21	21	23	18	16	17	16	16		

n/a: not applicable

Table 6. Fluorescence signal (MFI) of two low concentration Seraseq ctDNA and blank control (Kit Lot:RDL100219)

Samples	MFI for each of replicates												Average	SD
0.08 ng/mL	373	386	369	361	345	348	325	305	359	361	352	329	344	23.41
	349	307	329	314	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.04 ng/mL	132	160	143	152	147	124	126	123	163	168	167	150	146	14.11
	153	143	152	140	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.02 ng/mL	80	75	87	68	77	73	78	64	99	79	102	108	85	12.06
	93	94	89	87	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
Blank control	15	14	17	15	14	14	13	13	15	14	13	13	14	1.93
	13	13	12	10	16	16	16	19	17	16	12	16		

n/a: not applicable

Table 7. Fluorescence signal (MFI) of two low concentration Seraseq ctDNA and blank control (Kit Lot:RDL100319)

Samples	MFI for each of replicates												Average	SD
0.08 ng/mL	72	63	68	68	77	76	95	86.5	71.5	73	76.5	77	78	9.26
	76	98.5	82	84	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.04 ng/mL	49	49	55	55	60.5	63	63	71.5	63	60	61	68	63	7.52
	73	70.5	71	70	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
0.02 ng/mL	28	28	28	31.5	32	33	34	38	38	38	38	38	36	5.66
	40	46	44	44.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
Blank control	9	9	9	8	9	9	10	13	10	9	9	9	9	0.97
	9	8	10	10.5	10	9.5	9	9	9	10	10	10		

n/a: not applicable

LoD was calculated for the three LOTs and for low concentration dilutions: 0.08ng/mL, 0.04 ng/mL and 0.02 ng/mL according to the formulas represent above, obtained results showed in table 8. The LoD ranged from 0.005 to 0.023 ng/mL determined by 0.02, 0.04 and 0.08 ng/mL ctDNA samples with three lots of kits. Therefore, the LoD of QuantiDNA™ Direct cfDNA Test is at least 0.023 ng/mL.

Table 8. Calculated cLoD from three lots of kits

Kit lot number	Concentration of diluted Seraseq ctDNA samples	Calculated cLoD (ng/mL)
RDL100119	0.08 ng/mL	0.023
	0.04 ng/mL	0.022
	0.02 ng/mL	0.015
RDL100219	0.08 ng/mL	0.005
	0.04 ng/mL	0.006
	0.02 ng/mL	0.006
RDL100319	0.08 ng/mL	0.015
	0.04 ng/mL	0.010
	0.02 ng/mL	0.009

7.2 Limit of Quantitation

The DNA depleted human plasma was used as the blank and as the diluent for the “Seraseq ctDNA Complete”. Eight different concentrations between 20-0.156 ng/mL (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/mL) were tested using three lots of kits. The limit of quantitation (LoQ) was defined as the range where the ratio of “Calculated Concentration” to “Expected Concentration” falls between 70% and 130%.

First, high concentration of “Seraseq ctDNA Complete” was prepared by dilution in ddH_2O (approximately 200ng/ml) and then diluted 1:10 in 10% plasma (first dilution -20 ng/ml), after that 2-fold serial dilution was conducted to obtain seven solutions of different concentrations. Eight solutions were tested using QuantiDNA™ Direct cfDNA test in 6-9 replicate (data presented in Table 9).

Table 9. Results of the LoQ experiments

Kit lot number	Expected Concentration	MFI		Tested Concentration (ng/mL)		Recovery
		Average	CV%	Average	CV%	(tested concentration /expected concentration) %
RDL100119	20	10357	5.38%	24.88	15.50%	124.40%
	10	7347	6.92%	11.629	12.90%	116.29%
	5	4866	5.87%	6.156	8.70%	123.12%
	2.5	2745	8.71%	3.04	10.90%	121.60%
	1.25	1487	6.28%	1.552	7.30%	124.16%
	0.625	749	7.10%	0.75	8.30%	120.00%
	0.312	413	4.30%	0.39	5.40%	125.00%
	0.156	187	6.39%	0.138	11.00%	88.46%
RDL100219	20	10806	5.69%	18.699	10.43%	93.50%
	10	7544	14.67%	10.633	22.92%	106.33%
	5	4792	11.55%	5.641	16.36%	112.82%
	2.5	2813	12.87%	2.91	16.54%	116.40%
	1.25	1544	7.02%	1.451	8.40%	116.08%
	0.625	868	11.81%	0.77	13.55%	123.20%
	0.312	462	13.70%	0.396	14.99%	126.92%
	0.156	234	8.92%	0.201	9.09%	128.85%
RDL100319	20	10330	8.70%	23.683	20.78%	118.42%
	10	7077	7.74%	11.426	13.52%	114.26%
	5	4515	8.31%	5.973	11.75%	119.46%
	2.5	2472	9.93%	2.887	12.12%	115.48%
	1.25	1286	11.26%	1.425	12.52%	114.00%
	0.625	663	9.96%	0.729	10.42%	116.64%
	0.312	335	8.58%	0.38	8.44%	121.79%
	0.156	156	4.30%	0.193	3.95%	123.72%

As shown in Table 5, the ratio of Calculated over Expected concentration was within 88.46% and 128.85%, and %CV of the concentration for the replicates was between 3.95 – 22.92% for reference fragmented DNA (“Seraseq ctDNA Complete”) in concentration 0.156- 20 ng/ml for all of 3 lots kits. Therefore, the lower limit of quantitation (LoQ) of the test was defined as 0.156 ng/mL.

7.3 Specificity

Specificity of the test was first evaluated by testing bacterial genomic DNA from ZymoBIOMICS™ Microbial Community DNA Standard which is a mixture of genomic DNA isolated from pure cultures of eight bacterial and two fungal strains (Table 10). No significant difference was observed in the signal between blank control and bacterial DNA at 500 ng/mL tested with 3 lots of kits, indicating no false positive results for bacterial DNA samples (Table 11).

Table 10. Microbial Composition of ZymoBIOMICS™ Microbial Community DNA Standard

Species	Theoretical Composition (%)				
	Genomic DNA	16S Only	16S & 18S	Genome Copy	Cell Number
Pseudomonas aeruginosa	12	4.2	3.6	6.1	6.1
Escherichia coli	12	10.1	8.9	8.5	8.5
Salmonella enterica	12	10.4	9.1	8.7	8.7
Lactobacillus fermentum	12	18.4	16.1	21.6	21.4
Enterococcus faecalis	12	9.9	8.7	14.6	14.5
Staphylococcus aureus	12	15.5	13.6	15.2	15.1
Listeria monocytogenes	12	14.1	12.4	13.9	13.8
Bacillus subtilis	12	17.4	15.3	10.3	10.2
Saccharomyces cerevisiae	2	NA	9.3	0.57	1.13
Cryptococcus neoformans	2	NA	3.3	0.37	0.73

Table 11. Fluorescence signal of blank control vs bacterial DNA

kit lot number	Sample	MFI-1	MFI-2	MFI-3	MFI-4	Average	SD
DL-1	bacterial gDNA (500 ng/mL)	7	7	7	n/a	7	0
	blank control	6	7	7	n/a	6.7	0.6
DL-2	bacterial gDNA (500 ng/mL)	8	7	7	6	7	0.8
	blank	7	8	8	10	8.3	1.3
DL-3	bacterial gDNA (500 ng/mL)	6	6	6	7	6.3	0.5
	blank	6	7	11	9	8.3	2.2

n/a: not applicable

Next, specificity of the test was further evaluated by testing chicken and bovine DNA at 1000 ng/mL with kit lot number RDL100219 and both generated fluorescence signal similar to blank control (Table 12).

Table 12. Fluorescence signal of blank control vs chicken and bovine DNA

Sample	MFI-1	MFI-2	MFI-3	Average	SD
Chicken DNA (1000 ng/mL)	7	8	8	7.7	0.577
Bovine DNA (1000 ng/mL)	8	7	7	7.3	0.577
Blank control	9	6	7	7.3	1.528

7.4 Reproducibility

For intra-assay reproducibility (repeatability), human plasma samples were tested in one run in 10 replicates and the coefficient of variation (CV) of fluorescence signal (MFI) was calculated. For inter-assay reproducibility, human plasma samples were tested in 3 separate runs and the CV of measured concentrations was calculated. As shown in Tables 13 and 14, the assay showed an average CV of 10.3% (95% CI: 8.2%~12.3%) and 13.0% (95% CI: 8.9%~17.1%) for intra-assay and inter-assay reproducibility, respectively.

Note: *95% CI was calculated with https://www.socscistatistics.com/confidenceinterval/Default3.aspx*

Table 13. Intra-assay reproducibility results for 3 lots of kits (Fluorescence signal)

Kit lot number	Sample name	MFI-1	MFI-2	MFI-3	MFI-4	MFI-5	MFI-6	MFI-7	MFI-8	MFI-9	MFI-10	Avg	SD	CV%
DL-1	Plasma sample #1	537	443	579	401	461	524	589	484	470	399	489	67	13.80%
	Plasma sample #2	2451	2423	2539	2161	2580	2907	2602	2401	2502	2130	2470	222	9.00%
	Plasma sample #3	1464	1500	1374	1413	1347	1407	1404	1333	1504	1451	1420	59	4.20%
DL-2	Plasma sample #1857	824	1047	1214	955	1058	1255	1135	1147	1238	990	1086	138	12.70%
	Plasma sample #1882	1772	1460	1821	1782	1856	2055	1396	1971	2235	1897	1825	252	13.80%
	Plasma sample #crl	992	917	997	892	911	999	996	875	811	1058	945	75	8.00%
DL-3	Plasma sample #4	8531	8475	8222	7222	9966	7288	7876	8326	8347	8239	8249	761	9.20%
	Plasma sample #5	1430	1474	1405	1523	1499	953	1337	1489	1420	1410	1394	164	11.80%
	Plasma sample #6	1160	1027	1082	1035	999	958	870	860	875	941	981	99	10.10%

Table 14. Inter-assay reproducibility results for 3 lots of kits (Measured concentration)

Kit lot number	Samples	Test- day 1	Test-day 2	Test-day 3	Average	SD	CV%
DL-1	Plasma sample #1857	4.70	5.20	4.60	4.83	0.32	6.70%
	Plasma sample #1917	10.30	12.30	12.80	11.80	1.32	11.20%
	Plasma sample #1922	8.60	5.80	6.40	6.93	1.47	21.30%
DL-2	Plasma sample #11	7.74	9.47	6.84	8.01	1.34	16.70%
	Plasma sample #22	15.13	13.90	14.70	14.58	0.63	4.30%
	Plasma sample #33	5.00	3.57	4.16	4.24	0.72	17.00%
DL-3	Plasma sample #11	7.32	7.90	8.76	7.99	0.72	9.00%
	Plasma sample #22	17.39	13.00	20.27	16.89	3.66	21.70%
	Plasma sample #33	5.57	4.59	5.03	5.06	0.49	9.60%

7.5 Interfering substance

High levels of hemoglobin and cholesterol may be present in blood with hemolysis or hyperlipidemia, respectively. To test if those two substances have any interference with the test, hemoglobin (500 mg/dL) and cholesterol (300 mg/dL) were spiked into human plasma, respsctively. The spiked plasma samples were then diluted 10-fold with 1x PBS for testing according to the manual. The anticoagulant EDTA (10 mM) which is used in blood collection tubes was also tested for interfering effect.

In addition, FDA has become aware of potential biotin interference with IVDs that use biotin/avidin interactions as part of the device technology. Biotin interference study was performed per the recommendations in the FDA draft guidance document (https://www.fda.gov/media/127915/download) on testing for biotin interferences, the amount of biotin that should not in-terfere will be used (3500 ng/mL). In this study, blood samples were collected from 11 donor blood samples into “Cell-free DNA BCT, STRECK tubes” and the donor plasma samples were tested with Biotin and without Biotin (3500 ng/mL) in 3 replicates.

As shown in Tables 15-18, no significant interference was observed for hemoglobin, cholesterol, Biotin and EDTA at tested concentrations (*t-test*, p>0.05).

Table 15. Interfering substance test_Lot DL-1 kit

Potential interfering substance tested	Group	MFI-1	MFI-2	MFI-3	Average	SD	P value with *t-test*
Hemoglobin	No hemoglobin	528	524	620	557	54	0.43
	Hemoglobin (500mg/dL)	693	444	580	572	125	
Cholesterol	No cholesterol	530	511	460	500	36	0.39
	Cholesterol (300mg/dL)	561	579	397	512	100	
EDTA	No EDTA	562	592	534	562	29	0.09
	EDTA (10mM)	527	588	475	530	57	

Table 16. Interfering substance test_Lot DL-2 kit

Potential interfering substance tested	Group	MFI-1	MFI-2	MFI-3	MFI-4	Average	SD	P value with *t-test*
EDTA	Plasma 1567: no EDTA	988	1063	894	885	957	84	0.17
	Plasma 1567: EDTA (10mM)	816	1006	989	755	892	125	
	Plasma 1692: no EDTA	1242	1403	1342	997	1246	179	0.29
	Plasma 1692: EDTA (10mM)	1084	1320	1479	1399	1321	171	
Hemoglobin	Plasma 1567: no hemoglobin	988	1063	894	885	957	84	0.34
	Plasma 1567: Hemoglobin (500mg/dL)	1117	945	931	708	925	168	
	Plasma 1692: no hemoglobin	1242	1403	1342	997	1246	179	0.19
	Plasma 1692: Hemoglobin (500mg/dL)	1571	1187	1417	1353	1382	159	
Cholesterol	Plasma 15: no cholosterol	973	1102	1056	1098	1057	60	0.35
	Plasma 15: cholesterol (300mg/dL)	1110	957	1139	882	1022	123	

Table 17. Interfering substance test_Lot DL-3 kit

Potential interfering substance tested	Group	MFI-1	MFI-2	MFI-3	MFI-4	Average	SD	P value with *t-test*
EDTA	Plasma 1567: no EDTA	844	988	1154	1508	995	286	0.11
	Plasma 1567: EDTA (10mM)	754	1079	884	1116	958	170	
	Plasma 1692: no EDTA	1049	1230	1352	1365	1249	147	0.15
	Plasma 1692: EDTA (10mM)	1295	1610	1382	1256	1386	159	
Hemoglobin	Plasma 1567: no hemoglobin	844	988	1154	1508	995	286	0.29
	Plasma 1567: Hemoglobin (500mg/dL)	1054	1058	1009	981	1025	37	
	Plasma 1692: no hemoglobin	1049	1230	1352	1365	1249	147	0.41
	Plasma 1692: Hemoglobin (500mg/dL)	1278	1360	1326	1128	1273	102	
Cholesterol	Plasma 15: no cholosterol	1194	1219	1402	1117	1233	121	0.07
	Plasma 15: cholesterol (300mg/dL)	1240	1475	1517	1198	1358	162	

Table 18. Interfering substance (Biotin) test using kit lot RDL100319

Sample name	Group	MFI-1	MFI-2	MFI-3	Average	SD	P value with *t-test*
Plasma #1	no biotin	400	563	561	508	93.38942	0.29
	Biotin (3500 ng/mL)	410	468	578	485	85.60228	
Plasma #2	no biotin	402	450	540	464	70.00774	0.13
	Biotin (3500 ng/mL)	430	477	535	481	52.83701	
Plasma #3	no biotin	814	845	936	865	63.41136	0.15
	Biotin (3500 ng/mL)	788	840	935	854	74.76352	
Plasma #8	no biotin	417	529	594	513	89.53398	0.38
	Biotin (3500 ng/mL)	461	522	522	501	35.36359	
Plasma #10	no biotin	444	653	614	570	111.1321	0.12
	Biotin (3500 ng/mL)	578	664	654	632	47.0319	
Plasma #11	no biotin	726	672	723	707	30.34798	0.22
	Biotin (3500 ng/mL)	562	676	722	653	82.37313	

7.6 Recovery rate

To perform the recovery studies, a plasma sample with high cfDNA concentration was mixed with a plasma sample with low cfDNA concentration at 1:9 ratio by volume. These 3 samples were then tested using QuantiDNA™ Direct cfDNA kit. Recovery rate was calculated following the formula below:

$$R = \frac{C_{mix} \text{ X } (V_{high} + V_{low})}{C_{high} \text{ X } V_{high} + C_{low} \text{ X } V_{low})} \text{ X } 100\%$$

R=Recovery rate
V_{high}=volume of sample with high concentration used for preparing the mixed (i.e. 1)
V_{low}=volume of sample with low concentration used for preparing the mixed (i.e. 9)
C_{mix}=measured concentration of mixed sample
C_{high}=measured concentration of sample with high concentration
C_{low}=measured concentration of sample with low concentration

For Lot DL-1 and Lot DL-2 kits, each sample was tested in 4 replictaes, and for Lot DL-3 kit, each sample was tested in 3 replicates. As show in Tables 19-21, the calculated recovery rate was 106.1%, 87.6% and 104.7%, respectively for each lot of kit used.

Note: *The samples used were different across the three Lots of kits.*

Table 19. Recovery study_Lot DL-1 kit

	Rep-1	Rep-2	Rep-3	Rep-4	Average concentration (ng/ml)
Sample-High	107.1	173.6	172.8	144.9	149.6
Sample-Low	24.5	24.5	25.0	21.5	23.9
Mixed sample	40.9	37.3	40.3	36.1	38.7
Recovery					106.1%

Table 20. Recovery study_Lot DL-2 kit

	Rep-1	Rep-2	Rep-3	Rep-4	Average concentration (ng/ml)
Sample-High	215.7	159.3	146.4	220.4	185.4
Sample-Low	15.8	18.4	14.6	19.8	17.2
Mixed sample	28.2	29.3	36.2	25.4	29.8
Recovery					87.6%

Table 21. Recovery study_Lot DL-3 kit

	Rep-1	Rep-2	Rep-3	Average concentration (ng/ml)
Sample-High	178.8	165.8	163.6	169.4
Sample-Low	14.1	15.0	13.2	14.1
Mixed sample	31.6	30.0	31.4	31.0
Recovery				104.7%

PART 8. SYMBOLS USED IN PACKAGING

Symbol	Definition	Symbol	Definition
RUO	Research Use Only		Batch Code
	Catalog Number		Expiration Date
	Temperature Limitation		
2012-11-25	Date Format (year-month-day)		
2012-11	Date Format (year-month)		

PART 9. REFERENCES

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