PRODUCT INSTRUCTION MANUAL

QuantiDNA™ DNA Measurement Assay (Luminometer Assay)

CATALOG NUMBER

DC-08-0092R
Pack Size: 96 Reactions

Manufacturer

DiaCarta, Inc.
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INTENDED USE

Research Use Only

Document Number: MAN.0074 Instruction Version: Rev.2 Date of Revision: September 2022

CONTENTS

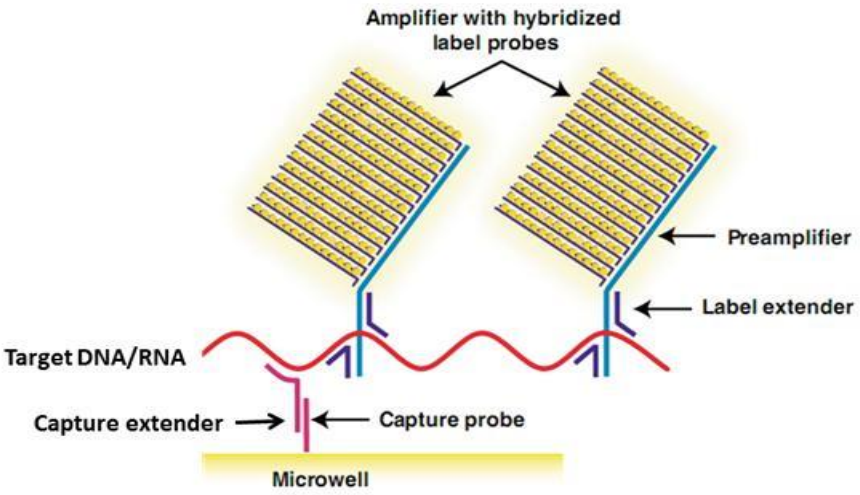
PART 1. INTENDED USE

The QuantiDNA™ DNA Measurement Assay is a cell-free DNA (cfDNA) quantification method that is based on the strong correlation between radiotherapy toxicity, such as bladder toxicity or acute GI toxicity in prostate cancer subjects and the cfDNA change in subject's plasma before and after radiation. The assay uses direct cfDNA quantification without DNA extraction or qPCR to quantify human cfDNA from plasma. The method uses DNA-hybridization-based, modified branched DNA (bDNA) technology. The target DNA is captured through DNA hybridization, and the signals associated with the quantity of the target DNA, rather than the target by itself, are amplified and quantified by a luminometer.

PART 2. PRINCIPLE OF THE QUANTIDNA™ DNA MEASUREMENT ASSAY

QuantiDNA™ is based on DiaCarta's SuperbDNA™ technology. QuantiDNA™ assay uses a modified bDNA molecule, and new probe set designs that result in increased amplification of the target gene signal. The ability to quantify DNA molecules in a sample lies in the design of a probe set. 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. The 3' tails of CEs bind to the capture probes attached to the well, and via cooperative hybridization capture the associated target DNA. Signal amplification occurs when a bDNA molecule hybridizes to the tails of the LEs. The bDNA molecules contain hybridization sites for 400 alkaline phosphatase-conjugated Label Probes, which can then be detected by the alkaline phosphatase mediated degradation of the chemiluminescent substrate, dioxetane. Luminescence is reported as relative light units (RLUs) on a microplate luminometer. The amount of luminescence is proportional to the number of DNA molecules present in the sample.

Figure 1. Schematic Diagram of SuperbDNA™ Technology



PART 3. REAGENTS AND INSTRUMENTS

3.1. Package Contents

IMPORTANT: Upon receiving, store **Component 1** at 2°C to 8°C and **Component 2** at -25°C to -15°C.

Table 1. Items stored at 2°C to 8°C (Component 1)

Item Name	Quantity	Part #	Description	Storage
1X Lysis Mixture	10 mL	1010611	Aqueous buffered solution with sodium azide (<0.1%) and other preservatives	2°C to 25°C*
Amplifier/Label Probe Diluent	36 mL	1000972	Aqueous buffered solution with a protein-containing preservative	2°C to 25°C*
Substrate Enhancer	36 µL	1000982	Aqueous solution with sodium azide (<0.1%) and other preservatives	2°C to 25°C*
Plate Seal	15 sheets	1011242	Adhesive-backed foil seal	2°C to 25°C*
10X Wash Buffer	50 mL	1001562	Aqueous buffered solution concentrate with sodium azide (<0.1%) and other preservatives	2°C to 25°C*
1X PBS	10 mL	1007703	Phosphate Buffered Saline (pH 7.4, without calcium, magnesium and phenol red)	2°C to 25°C*
Capture Plate	1 plate	1001012	Polystyrene microwells coated with synthetic oligonucleotides	2°C to 8°C
Substrate	10.6 mL	1001022	Chemiluminescent substrate	2°C to 8°C
Label Probe	110 µL	1001102	Enzyme-labeled Synthetic oligonucleotides in buffered solution	2°C to 8°C

*These reagents or materials can be stored at room temperature after receival if desired.

Table 2. Items Stored at -25°C to -15°C (Component 2)

	Component	Quantity	Part #	Description	Storage
Grey Box 1	Proteinase K	15 µL	1002011	Proteinase K solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
	Blocking Reagent	110 µL	1001042	Aqueous buffered solution containing a preservative	-25°C to-15°C
	Human Alu Probe Set	110 µL	1002033	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
	Pre-Amplifier Probe	110 µL	1001082	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
	Amplifier Probe	110 µL	1001092	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
	Positive Control-High	225 µL	1010531	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	Positive Control-Low	225 µL	1010541	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
Grey Box 2	DNA Standard 1	225 µL	1010551	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	DNA Standard 2	225 µL	1010561	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	DNA Standard 3	225 µL	1010571	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	DNA Standard 4	225 µL	1010581	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	DNA Standard 5	225 µL	1010591	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C
	DNA Standard 6	225 µL	1010601	Human genomic DNA in aqueous solution with sodium azide (<0.1%)	-25°C to-15°C

3.2. Materials Required but Not Provided with the Kit

3.2.1. Special Instruments for this Assay

- **Plate incubator** (capable of maintaining a constant temperature between 46–55 °C ±1 °C). Recommended incubator: Thermal Shaker Incubator (vendor: DiaCarta, catalog #: DC-06-0002)

- **Luminometer** (read 420nm wavelength, read time of 0.2 seconds/well, linear dynamic range ≥ 5-log (optional) and well-to-well uniformity < ± 5%). Recommended luminometer: DiaCarta Luminometer (vendor: DiaCarta, catalog #: DC-06-0001)

3.2.2. Required Materials

- Nuclease-Free or Deionized Water
- Reagent reservoirs (25mL or 100 mL capacities)
- Adjustable single-channel precision pipettes for dispensing 1–20µL, 20–200µL, and 200–1000µL (accuracy ± 5%)
- Adjustable multi-channel precision pipettes for dispensing 20–200µL (accuracy ± 5%)
- Pipette tips for dispensing 1–20µL,20–200µL, and 200–1000µL
- 15 mL sterile Nuclease-free polypropylene tubes
- 10 mL sterile-packaged serological pipettes
- 1.5 mL microcentrifuge tubes
- 500ml solution bottle
- Microcentrifuge
- Vortex mixer
- Dry block heater for 1.5 mL microcentrifuge tubes
- Receptacle (sink or large pan)
- Lint-free absorbent paper towel

3.2.3. Optional Materials

- 4-inch soft rubber roller and film-sealing paddle for plate sealing
- Microplate centrifuge that can achieve 240 x g

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. Contains: Proteinase K, Lysis Mixture.

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): Lysis Mixture, Amplifier/Label Diluent, Pre-Amplifier Probes, Amplifier Probe, Label Probe, Wash buffer, and Substrate Enhancer.

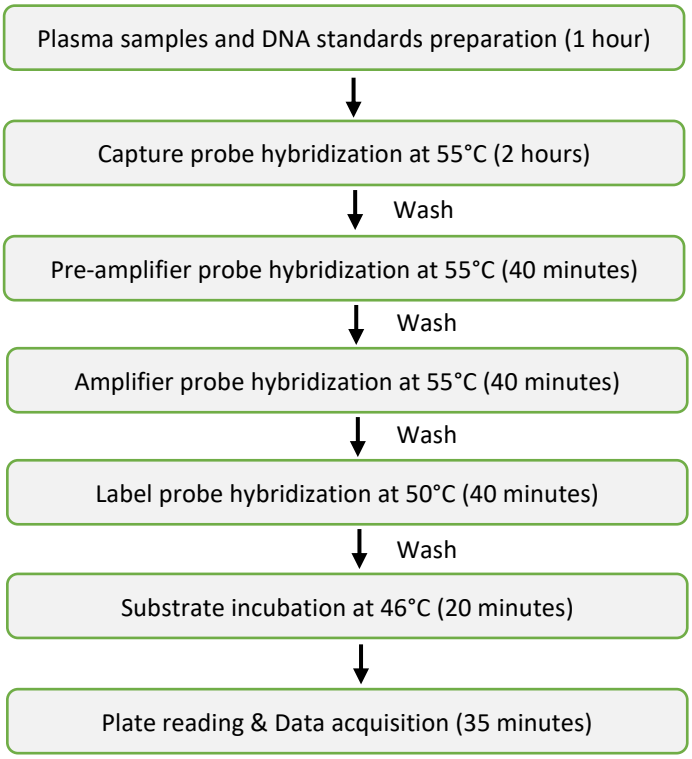
CAUTION: Sodium azide in the reagents can react with copper and lead plumbing to form explosive metal azides. On disposal flush reagents with a large volume of water to prevent the buildup of metal azides, if disposal into a drain, is in compliance with federal, state, and local requirements.

- 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 is 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 a 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.
- For stability of the working reagents, refer to the Assay Procedure section.
- Do not interchange vial or bottle caps as cross-contamination may occur.

PART 4. TEST PROCEDURE

4.1. Flow Chart of Assay Procedure

Figure 2. QuantiDNA™ DNA Measurement Assay Workflow



4.2. Assay Background Control

Assay background is the assay signal generated by the entire probe set in the absence of DNA sample input. The following is recommended:

- Run three background controls in every experiment to ensure optimal assay evaluation.
- Use 1X PBS as assay background control.
- Subtract the assay background signal from the target DNA generated signal during data analysis.

4.3. Prepare Plasma Samples

4.3.1. For blood collected in Streck Cell-free DNA BCT® blood collection tubes, centrifuge the room temperature-stored blood within 14 days (suggested stability by manufacture) at 1,600 x g for 10 min at room temperature. For blood collected in EDTA-treated polypropylene tubes, within two hours after blood collection, centrifuge blood for 10 minutes at 1900 x g at 4°C. This assay needs 1 mL or more blood.

Note: Hemolyzed samples cannot be used for testing due to DNA contamination from blood cells.

4.3.2. Then carefully transfer plasma supernatant without disturbing the buffy coat layer to a new centrifuge tube.

4.3.3. Centrifuge at 16,000 x g at room temperature (for Streck tube-stored samples) or ≥12,000 x g at 4°C (for EDTA tube-stored samples) for 10 minutes.

4.3.4. Carefully remove supernatant to a new tube without disturbing the pellet. Prepared plasma should be stored at -80°C if not used immediately.

4.3.5. Add 90 µL of 1X PBS to a 1.5 mL microcentrifuge tube, then add 10 µL of plasma prepared above. Quickly vortex and spin down.

Note: For frozen plasma samples, bring to room temperature until completely thawed. Quickly vortex and spin down. Then, dilute with 1X PBS as instructed above.

4.3.6. Put plasma samples on a dry block heater and heat at 95°C for 10 minutes.

4.3.7. Immediately chill on ice for at least 5 minutes.

4.3.8. Quickly vortex and centrifuge the plasma samples at high speed (10000xg) for 60 seconds at 4°C. Keep denatured plasma samples on ice prior to use.

4.4. Denature DNA Standards and Positive Controls

4.4.1. Bring DNA standards 1 to 6, Positive Control-High, and Positive Control-Low to room temperature until completely thawed.

4.4.2. Quickly vortex and spin down.

4.4.3. Transfer 75 µL to 1.5 ml nuclease-free microcentrifuge tubes, and then put the tubes on a dry block heater and heat at 95°C for 10 minutes.

4.4.4. Immediately chill on ice for at least 5 minutes.

4.4.5. Quickly vortex, spin down and keep on ice prior to use.

4.5. Prepare Working Probe Solution

Note: The following instructions, unless otherwise specified, are for preparation of material sufficient for processing one 96-well plate using multi-channel pipets and reagent reservoirs. Before starting the preparation, calculate the number of samples and adjust the quantities as appropriate. Prepare 15% extra working solutions to ensure there is enough for all samples.

4.5.1. Bring Blocking Reagent and Human Alu Probe Set to room temperature for at least 30 minutes. Then vortex and follow with a quick spin.

4.5.2. Bring the Lysis Mixture to room temperature for at least 30 minutes. If needed, place the Lysis Mixture at 37°C for 20 minutes to dissolve any visible crystals.

4.5.3. Set the Plate Incubator to 55°C.

4.5.4. Prepare Working Probe Solution in a 15mL polypropylene centrifuge tube following Table 3 (prepare 15% extra). One well needs 80 µL Working Probe Solution.

Table 3. Preparing Working Probe Solution

Reagent Name	Volume (µL) Required per Well
Lysis Mixture	78
Blocking Reagent	1
Human Alu Probe set	1
Proteinase K	0.1

4.6. Capture Target DNA into Capture Plate

4.6.1. Bring Capture Plate to room temperature for at least 30 minutes.

4.6.2. Create a plate layout using Table 4 as a guide. Three replicates of DNA Standards, Positive Controls, and Blank are highly recommended.

Table 4. Recommended Plate Layout for QuantiDNA™ DNA Measurement Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Blank	Blank	Blank	UN-22	UN-30	UN-38	UN-46	UN-54	UN-62
B	Std 2	Std 2	Std 2	UN-1	UN-8	UN-15	UN-23	UN-31	UN-39	UN-47	UN-55	UN-63
C	Std 3	Std 3	Std 3	UN-2	UN-9	UN-16	UN-24	UN-32	UN-40	UN-48	UN-56	UN-64
D	Std 4	Std 4	Std 4	UN-3	UN-10	UN-17	UN-25	UN-33	UN-41	UN-49	UN-57	UN-65
E	Std 5	Std 5	Std 5	UN-4	UN-11	UN-18	UN-26	UN-34	UN-42	UN-50	UN-58	UN-66
F	Std 6	Std 6	Std 6	UN-5	UN-12	UN-19	UN-27	UN-35	UN-43	UN-51	UN-59	UN-67
G	PC-Low	PC-Low	PC-Low	UN-6	UN-13	UN-20	UN-28	UN-36	UN-44	UN-52	UN-60	UN-68
H	PC-High	PC-High	PC-High	UN-7	UN-14	UN-21	UN-29	UN-37	UN-45	UN-53	UN-61	UN-69

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

4.6.3. Dispense 80 µL of Working Probe Solution to each well of the Capture Plate using a multichannel pipette.

4.6.4. Add 20 µL of denatured DNA Standards, Positive Controls, and unknown samples to the 96-well plate following the layout in Table 4. Add 20 µL of 1X PBS to blank control wells.

4.6.5. Seal the plate tightly with a Plate Seal. Use a soft rubber roller to apply pressure and aid in complete and uniform plate sealing. It is recommended to use a film-sealing paddle to ensure a tight seal across every individual well. Failure to do so causes potential well-to-well contamination and edge effects due to evaporation.

4.6.6. Place the Capture Plate in the incubator at 55°C for 2 hours to capture target DNA.

4.7. Hybridize Pre-Amplifier Probe

4.7.1. Bring Pre-Amplifier, Amplifier, Label Probe, Substrate, 10X Wash Buffer, and Amplifier/Label Probe Diluent to room temperature for at least 30 minutes before use. If needed, place the Amplifier/Label Probe Diluent and 10xWash Buffer at 37°C for 20 minutes to dissolve any visible crystals.

4.7.2. Vortex and briefly centrifuge the tube of Pre-Amplifier, Amplifier, and Label Probes after they are completely thawed.

4.7.3. Calculate the amount of Pre-Amplifier Working Solution needed based on the number of wells (make 15% extra). One well needs 100 µL Pre-Amplifier Working Solution.

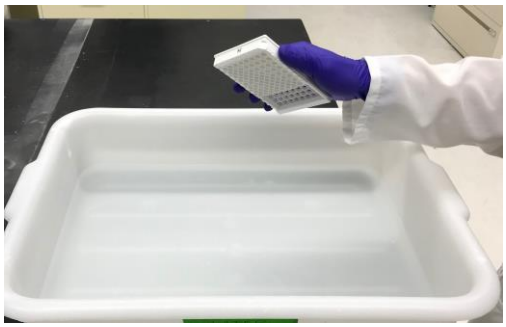
4.7.4 Prepare Pre-Amplifier Working Solution in a 15 mL polypropylene tube by 100-fold dilution of Pre-Amplifier Probe with Amplifier/Label Diluent. For example, to prepare 5 mL Pre-Amplifier Working Solution, add 50 µL Pre-Amplifier Probe to 4.95 mL Amplifier/Label Diluent.

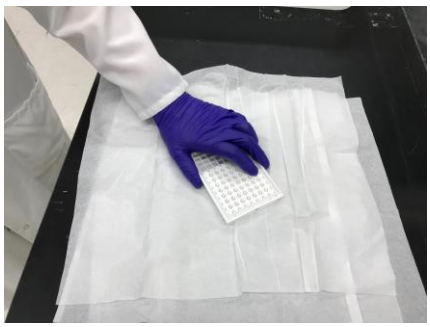
4.7.5 Gently vortex or invert 10 times to mix Pre-Amplifier Working Solution and leave it at room temperature before use.

4.7.6 Dilute 50mL of the 10X Wash Buffer with 450 mL of deionized water to obtain 1X Wash Buffer concentration.

4.7.7. Wash Capture Plate following the instructions below:

a. Remove the Capture Plate from the incubator and remove the Plate Seal.
b. Invert the Capture Plate into an appropriate receptacle and firmly expel the contents as shown below (Figure Left). Then tap the inverted plate onto lint-free absorbent paper towels (Figure Right).





c. Add 260 µL of 1X Wash Buffer to each well and let it soak for 30 seconds.
d. Repeat steps 4.7.7.b. to 4.7.7.c. two more times for a total of three times.
e. Invert the Capture Plate into an appropriate receptacle and firmly expel the contents.
f. Tap the inverted plate onto lint-free absorbent paper towels.
g. (Optional) Remove traces of Wash Buffer: Place the inverted plate onto lint-free absorbent paper inside a centrifuge bucket. Spin at 240 x g for 1 minute. Discard the lint-free absorbent paper.

4.7.8. Add 100 µL of Pre-Amplifier Probe Working Solution to each well of the Capture Plate.

4.7.9. Seal the Capture Plate tightly with an appropriate size of Plate Seal and incubate at 55°C for 40 minutes.

4.8. Hybridize Amplifier Probe

4.8.1. Prepare Amplifier Probe Working Solution into 15mL centrifuge tube by 100-fold dilution with Amplifier/Label Probe Diluent (make 15% extra). One well needs 100 µL Amplifier Probe Working Solution. For example, to prepare 5 mL Amplifier Probe Working Solution, add 50 µL Amplifier Probe to 4.95 mL Amplifier/Label Diluent.

4.8.2. Gently vortex or invert 10 times to mix Amplifier Working Solution.

4.8.3. Follow the entire step of 4.7.7. to wash the Capture Plate.

4.8.4. Add 100 µL of Amplifier Probe Working Solution to each well of the Capture Plate.

4.8.5. Seal the Capture Plate tightly with a Plate Seal and incubate at 55°C for 40 minutes.

4.9. Hybridize Label Probe

4.9.1. Prepare Label Probe Working Solution into a 15mL centrifuge tube by 100-fold dilution with Amplifier/Label Probe Diluent (make 15% extra). One well needs 100 µL Label Probe Working Solution. For example, to prepare 5 mL Label Probe Working Solution, add 50 µL Label Probe to 4.95 mL Amplifier/Label Diluent.

4.9.2. Gently vortex or invert 10 times to mix Label Probe Working Solution.

4.9.3. Follow the entire step of 4.7.7. to wash the Capture Plate.

4.9.4. Add 100 µL of Label Probe Working Solution to each well of the Capture Plate.

4.9.5. Seal the Capture Plate tightly with a Plate Seal and incubate at 50°C for 40 minutes.

4.10. Add Substrate

4.10.1. Place the Substrate Enhancer and Substrate on the bench top and bring to room temperature.

4.10.2 Calculate the amount of Substrate Working Solution to preprare based on the number of wells (make 15% extra). One well needs 100 µL Substrate Working Solution.

4.10.3 Prepare Substrate Working Solution in a 15 mL polypropylene tube by adding Substrate Enhancer to Substrate at 1:333 ratio. For example, to prepare 5 mL Substrate Working Solution, add 15 µL Substrate Enhancer to 5 mL Substrate.

4.10.4 Gently vortex or invert 10 times to mix. Store Substrate Working Reagent at room temperature in the dark before use.
Note: It is normal for the Substrate Working Reagent to become turbid after the addition of Substrate Enhancer.

4.10.5. Follow the entire step of 4.7.7. to wash the Capture Plate. After removing the Capture Plate from the incubator, set the incubator temperature down to 46°C.

4.10.6. Add 100 µL of Substrate Working Solution to each well of the Capture Plate. Immediately seal the Capture Plate tightly with a Plate Seal and incubate at 46°C for 20 minutes.

4.10.7 Remove the plate from the incubator and let it cool down for 30 minutes in room temperature without removing the Plate Seal.

4.10.8. Remove the Plate Seal from the Capture Plate and read it immediately in the luminometer.

4.11. Measure Chemiluminescence Signal in Luminometer

4.11.1. Follow the procedure below to read the Capture Plate if using DiaCarta Luminometer (vendor: DiaCarta, DiaCarta catalog #: DC-06-0001):

a. Turn on the machine and pre-warm the reader for 5 minutes before use.
b. Open the DiaCarta QuantiDNA™ Benchtop Luminometer software.
c. Load the Capture Plate into the instrument and select the desired wells.
d. Select **Run** to read the Capture Plate.

4.11.2. Follow the procedure below to read the Capture Plate if using SpectraMax L Microplate Reader (Molecular Device Inc.):

a. Open SoftMax Pro software and connect to SpectraMax L Microplate Reader.
b. Click on the Acquisition button and select appropriate parameters:

- Read Mode: Lum
- Read Type: Endpoint
- Number of Wavelengths: 1
- Plate Type: 96 wells standard opaque
- Read Area: select the desired wells
- PMT gain: automatic
- Shake: unselect “Before first read” (NO shaking required)

c. Open the drawer and load the Capture Plate.
d. Close the drawer and start the run.
e. Export the results for analysis.

4.11.3. If using other luminometers, follow the manufacturer's instructions to read the plate and export the data for analysis.

PART 5. DATA ANALYSIS

5.1. Create Standard Curve from DNA Standards and Calculate Positive Control Concentrations

5.1.1. Calculate the average relative light units (RLU) of each of DNA Standards and the Blank (background control).

5.1.2. Use point-to-point interpolation of the DNA standards to calculate the measured concentrations of unknown samples. The slopes and intercepts of each interpolating line can be calculated using simple linear regression of each pair of adjacent DNA standards/Blanks.
Note: An Excel template for data analysis can be requested through emails to information@diacarta.com.

5.1.3. Calculate the concentration of Positive Control-Low and Positive Control-High using point-to-point interpolation.
Note: The expected concentration for each Positive Control is indicated on the inside label of box-2 of the kit and may be variable for each batch.
Important: A test will be considered valid when the measured concentration of both Positive Controls falls within the expected range.

5.2. Calculate cfDNA Concentration of Samples

5.2.1. Calculate the average RLU of each unknown sample.

5.2.2. Using point-to-point interpolation of the DNA standards found in step 5.1.2. to calculate the concentration of each unknown sample.
Note: An Excel template for data analysis can be requested through emails to information@diacarta.com.

5.2.4. Since the unknown samples have been 10-fold-diluted for the QuantiDNA™ DNA Measurement Assay, the calculated concentration must be multiplied by 10 to obtain the original DNA concentration of the unknown samples.

$$\text{Concentration (ng/mL)} = [(\text{RLU-Intercept})/\text{Slope}]*10$$

5.2.5. Other samples with various dilutions. If the unknown samples have been 200-fold-diluted for the QuantiDNA™ DNA Measurement Assay, the calculated concentration must be multiplied by 200 to obtain the original DNA concentration of the unknown samples.

PART 6. ASSAY PERFORMANCE CHARACTERISTICS

6.1. Analytical Sensitivity of the Assay (LoD)

The limit of detection (LoD) of the test was determined by measuring serially diluted human genomic DNA (gDNA) which was sheared to mimic the size of cell-free DNA in human plasma until the signal was close to the blank control (background). First, the instrument limit of detection (yLoD) was calculated from the mean and standard deviation (SD) of the blank control with the following formula: $yLoD = Mean_{blank} + 3.3 \times SD_{blank}$

The yLoD was then converted to the concentration limit of detection (cLoD) using the blank-adjusted mean signal (ys) for a gDNA solution with a known concentration (Cs) by the following formula: $cLoD = Cs \times yLoD \div ys$.

The test was able to detect as low as 0.17 ng/mL DNA.

6.2. Assay Reproducibility

Three development lots of QuantiDNA™ DNA Measurement Assay reagents were used in the reproducibility experiments: DL1, DL2, and DL3. Two operators were testing the kits. Experiments were performed to evaluate the reproducibility of the assay, including intra-assay, inter-assay, lot-to-lot, and operator reproducibility. For intra-assay reproducibility, 6 replicates of each sample, including serial diluted sheared gDNA spiked with gDNA were tested in one run on one plate. For inter-assay reproducibility, 3 replicates of each sample were tested in 3 separate runs using the same lot reagents. For lot-to-lot reproducibility, reagents from 3 development lots (DL1, DL2, DL3) were used to test sheared gDNA in water at various concentrations in triplicates. For operator reproducibility, sheared gDNA at various concentrations were tested using the same lot reagents by two operators. The summarized reproducibility results are shown in the table below.

	CV
Intra-assay reproducibility	<11.9%
Inter-assay reproducibility	<21.1%
Lot - to -lot reproducibility	<11.2%

6.4. Assay Stability

The kit has a shelf-life of 12 months when stored at recommended conditions and there is no effect up to 9 freeze-thaw cycles on the performance of the kit.

PART 7. TROUBLESHOOTING

7.1. Troubleshooting Low Assay Signal or Poor Sensitivity

Probable Cause	Recommended Actions
Inactivation of alkaline phosphatase	Do not leave the Capture Plate empty for more than 5 minutes after the Label Probe Hybridization step.
	Do not exceed incubation temperature of 50°C at Label Probe and Substrate addition steps
Detection reagents diluted incorrectly	Carefully add and thoroughly mix the correct amounts of Pre-Amplifier, Amplifier, or Label Probe with Amplifier/Probe Diluent.
Plate left at room temperature for an extended time before taking a reading	Read plate immediately after removal from the incubator.
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.

7.2. 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 samples.
Plate sat at room temperature for longer than 10 minutes before washing	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.

7.3. Troubleshooting plate uniformity problems

Probable Cause	Recommended Actions
Incomplete sealing during 2-hour hybridization	Use the CTC Plate Sealer for robust plate sealing (P/N QG0400). Ensure numbers and letters are clearly visible from under the foil seal. Verify that the supplied plate seal was used.
Capture Plates exposed to moisture prior to the assay	Allow the Capture Plate to come to room temperature for 20–30 minutes before opening to minimize condensation.
Temperature gradients within the plate incubator	Verify that the plate incubator maintains a constant, even temperature. Avoid opening and closing the incubator. See instrument manual for proper maintenance procedure.

7.4. Troubleshooting High Variations Between Replicates

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.
Scratching of the capture well surface	Minimize contact with the Capture Plate well surfaces during all addition and washing steps
Inaccurate pipetting	Make sure that tips are secured onto the pipette shaft. Use a new tip for each well.
Edge effect	The outer wells of the capture plate may have different temperature and differing rates of evaporation during long-term incubation which will result in variable hybridization efficiencies. Re-test the sample and avoid using the outer wells when needed.

7.5. Troubleshooting Day to Day Variation

Probable Cause	Recommended Actions
Non-constant temperature control between incubator and plate reader	Make sure that transfer time from incubator to plate reader (luminometer) is consistent.
Variable substrate incubation times	Keep substrate incubation times consistent.

PART 8. SYMBOLS USED IN PACKAGING

Symbol	Definition	Symbol	Definition
CE	CE Marked	LOT	Batch Code
REF	Catalog Number		Expiration Date
	Temperature Limitation		
2012-11-25	Date Format (year-month-day)		
2012-11	Date Format (year-month)		

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