InnoQuant® H-dye Human DNA Quantification & Degradation Assessment Kit Using the BioRad CFX Real-Time PCR System

For use with:

InnoQuant® H-dye Kit (product numbers: 21106-250, 21106-60)

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Kit Contents for 250 reactions (Item # 21106-250)

InnoQuant® H-dye Primer Mix	2 orange top tubes	1 mL each
DNA Standard, 100 ng/µl	1 clear top tube	75 µL at 100 ng/µL
InnoQuant® Dilution Buffer A	2 blue top tubes	1.8 mL each

Agilent Technologies Brilliant Multiplex QPCR Master Mix (Agilent Cat #600553):

Master Mix	1 vial	2.5 mL
ROX reference dye	1 green top tube	100 µL at 1 mM

Kit Contents for 60 reactions (Item # 21106-60)

InnoQuant® H-dye Primer Mix	1 orange top tube	0.5 mL each
DNA Standard, 100 ng/µl	1 clear top tube	33 µL at 100 ng/µL
InnoQuant® Dilution Buffer A	1 blue top tube	1.8 mL each

Agilent Technologies Brilliant Multiplex QPCR Master Mix (Agilent Cat #5190-7274):

Master Mix	1 vial	0.5 mL
ROX Reference Dye	1 green top tube	100 µL at 1 mM

NOTE: The **InnoQuant® H-dye Kit** has been validated and optimized for use with Agilent Technologies Brilliant Multiplex QPCR Master Mix.

Storage Conditions

Upon receipt, store the kit at -20°C. After thawing, store all components at 2°C to 8°C.

IMPORTANT! The fluorescent dyes attached to the probes are light-sensitive. Protect the InnoQuant® H-dye primer mix and Agilent reference dye from light when not in use.

Please note that the InnoQuant® H-dye Kit is designed to be highly sensitive. All reasonable precautions including clean laboratory techniques should be employed.

I. Preparation of DNA Standards

1. Equilibrate InnoQuant® DNA Standard (100 ng/µL) to room temperature for at least 15 minutes. Gently vortex for 5 seconds and centrifuge for 10–15 seconds at 3000 rpm before use.

Caution: The InnoQuant® H-dye Primer Mix contains probes that are labeled with light-sensitive dyes. Thaw completely at room temperature protected from light.

2. Using InnoQuant® Dilution Buffer A, prepare fresh serial dilutions of the InnoQuant® DNA Standard using low adhesion tubes as indicated below. Before removing an aliquot for the next dilution, gently vortex for 5-10 seconds and pulse spin for 5-10 seconds to collect all liquid at the bottom of the tube. The standard dilution series ranges from 20 ng/µL (Std. 1) to 0.009 ng/µL (Std. 8). Alternatively, a 5-point 1:8 dilution standard curve may also be used ranging from 20 ng/µL (Std. 1) to 0.005 ng/µL (Std. 5).

Note: Once the standard is thawed, keep at 2-8 °C. Subjecting the InnoQuant® DNA Standard to multiple cycles of freeze-thaw is not recommended.

Note: Diluted DNA quantification standards can be stored in LOW ADHESION TUBES for up to 1 week at 2-8 °C. Longer term storage is not recommended.

NOTE: Be sure to change pipette tips between dilutions. Change gloves after handling high-concentration DNA.

Standard	Concentration (ng/µL)	Recommended Dilution Amounts	Dilution Factor
Std. 1	20	10 µL [stock 100 ng/µL] + 40 µL Dilution Buffer A	5X
Std. 2	6.67	10 µL [Std. 1] + 20 µL Dilution Buffer A	3X
Std. 3	2.22	10 µL [Std. 2] + 20 µL Dilution Buffer A	3X
Std. 4	0.741	10 µL [Std. 3] + 20 µL Dilution Buffer A	3X
Std. 5	0.247	10 µL [Std. 4] + 20 µL Dilution Buffer A	3X
Std. 6	0.082	10 µL [Std. 5] + 20 µL Dilution Buffer A	3X
Std. 7	0.027	10 µL [Std. 6] + 20 µL Dilution Buffer A	3X
Std. 8	0.009	10 µL [Std. 7] + 20 µL Dilution Buffer A	3X

Alternatively, a 5-point 1:8 dilution standard curve may also be used and prepared as follows:

Standard	Concentration (ng/µL)	Recommended Dilution Amounts	Dilution Factor
Std. 1	20	10 µL [stock 100 ng/µL] + 40 µL Dilution Buffer A	5X
Std. 2	2.5	10 µL [Std. 1] + 70 µL Dilution Buffer A	8X
Std. 3	0.3125	10 µL [Std. 2] + 70 µL Dilution Buffer A	8X
Std. 4	0.0391	10 µL [Std. 3] + 70 µL Dilution Buffer A	8X
Std. 5	0.005	10 µL [Std. 4] + 70 µL Dilution Buffer A	8X

II. Reaction and Sample Setup

1. Equilibrate InnoQuant® H-dye Primer Mix, InnoQuant® Dilution Buffer A and Agilent Brilliant Multiplex QPCR Master to room temperature for at least 15 minutes in the dark.

NOTE: Protect Primer Mix from light as much as possible.

2. Gently agitate the Master Mix for 3-5 seconds to mix and briefly vortex the Primer Mix for 3-5 seconds. Gently tap to get all liquid to the bottom of tube.

NOTE: Do not centrifuge Primer Mix after vortexing, as this may cause the concentration of primers at the bottom of the tube.

3. Determine the number of reactions to be set up, including negative control reactions and 2 additional reactions to provide excess volume for the loss that occurs during reagent transfers.

4. Prepare the reaction mix by combining the Master Mix, Primer Mix and water indicated below and vortex briefly.

PCR Components	Volume per Reaction
Agilent Brilliant Multiplex QPCR Master Mix	10 µl
diH_2O	0.3 µl
InnoQuant® H-dye Primer Mix	7.7 µl
Total Volume	**18.0µl**

5. In an optical plate, add 2µl of DNA Standard or unknown sample to the appropriate wells. Make sure to place the sample at the bottom of the well. Duplicate amplification of standards is required. Add 2µl of InnoQuant® Dilution Buffer A to the NTC reaction(s).

6. Add 18µl of the reaction mix to each appropriate well of an optical-grade PCR plate. Mix the reaction by pipetting up and down 4-5 times as the reaction mix is being added to each well.

7. Seal the plates with an optical adhesive cover using the plate cover applicator.

8. Centrifuge the plate for 1 minute at 1500 rpm to collect the contents of the wells at the bottom.

NOTE: Protect the plate from extended light exposure or elevated temperatures before cycling. Handle the plate by the edges, and avoid touching the adhesive cover and bottom of the plate.

III. Instrument Setup

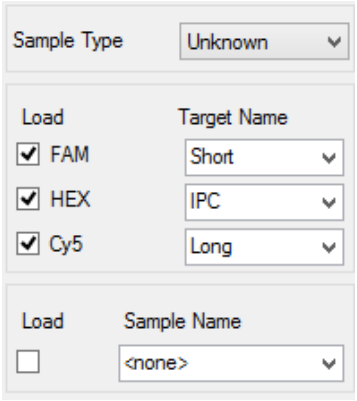
1. Import the provided protocol and plate templates titled “Innoquant.prcl” and “Innoquant.pltd”, respectively.

2. Open the BioRad CFX Manager Software v 3.1. Using the Startup Wizard tab **Run Setup**, select the instrument you are using then select run type “User-defined”.

3. Under the protocol tab, click the button **Select Existing** and navigate to the protocol “Innoquant.prcl” Verify the Cycling Parameters:

Initial heating time:	10 min at 95 °C
Followed by 32 cycles of:	15 seconds at 95 °C 2 minutes at 61 °C

4. Under the plate tab, click the button **Select Existing** and navigate to the plate file “Innoquant.pltd” Click the **Edit Selected** button, bringing up the Plate Editor. Select the wells you will be using to quantitate your samples. Select Sample Type “Unknown” and then click the boxes next to FAM, HEX and Cy5. Designate a name for each sample you are quantitating. After complete, click OK.



5. Click **Next**. Load the plate, then click **Start Run**.

IV. Result Interpretation

Short Target:	FAM – 80 bp size
Long Target:	Cy5 – 207 bp size
Internal Positive Control (**IPC**):	HEX – 172 bp size

The following parameters should be evaluated for each run:

1. When using the BioRad CFX Manager 3.1 software, the following analysis parameters should be used:

Cq Determination Mode:	Single Threshold
Baseline Setting:	Baseline Subtracted
Analysis Mode:	Fluorophore

   The BioRad CFX Manager 3.1 software automatically calculates best fit thresholds and baselines. If PCR efficiencies fall outside the acceptable range (between 90% and 110%) using the automatically calculated parameters, thresholds and baselines can be edited as follows only if needed. Ensure the threshold is within the logarithmic part of the amplification plot.

   NOTE: It is common for InnoQuant® runs to require a change of the baseline end for the first (20 ng) standard to 8

Target	C_T **threshold**	**Baseline**
Short	Manual set at 175	3-8
Long	Manual set at 175	3-11
IPC	Auto	Auto

2. Passing reaction efficiency values are greater than 90% and less than 110% for both the Short and the Long targets. Acceptable slope values are between -3.6 and -3.1.

3. Passing R^2 values are greater than 0.98.

4. The IPC is expected to have C_T values no more than 2 units above the mean IPC C_T for all quantification standards on the plate. C_T values higher than these for the IPC is indicative of a PCR inhibitor present in the reaction, including an excessively high concentration of DNA.

Degradation indices may be calculated by dividing the short quantity value (ng/µL) over the long quantity value (ng/µL) as follows:

$$DI = \frac{\text{Short target ng/µL}}{\text{Long target ng/µL}}$$

NOTE regarding NTCs and negative controls: The InnoQuant® assays are highly sensitive. Spurious signals may be obtained which are possibly the result of ambient DNA or sporadic signal from the short or long targets. Sporadic signal is more likely to be observed with the short target than the long target due to the higher copy number. For this reason, it is recommended to disregard signals in NTCs or negative controls higher than 30 C_T for the short and long InnoQuant® targets.

IMPORTANT! Before using the highly sensitive InnoQuant® Kit, assess the cleanliness of your environment. Use stringent laboratory cleanliness protocols to minimize contamination.

V. Troubleshooting

Observation	Possible Causes	Recommended Actions
IPC C_T falls outside acceptable range	High concentration of EDTA DNA inhibition	Verify the EDTA concentration in the unknown samples is 0.1mM EDTA. Purify DNA extracts using Microcon filtration units and repeat the quantitation. Alternatively, quantify a dilution of the sample DNA extract (i.e. 1/10 or 1/100 dilution).
Low Efficiency values or slope values outside the acceptable range	Primers/probes not properly thawed DNA standards too old	Allow primers/probes to equilibrate to room temperature for 15 minutes Re-dilute standards from100ng/µl stock using InnoQuant® Dilution Buffer A.
Low R-squared values	Incorrect threshold setting Pipette maintenance (or possible pipetting error)	Verify the threshold set is in the exponential phase of the amplification curve and above baseline Check maintenance of pipettes for calibration
S-shaped Long target standard curve	Incorrect threshold setting	Verify the threshold set is in the exponential phase of the amplification curve and above baseline and reanalyze

NOTE: During the development of products for forensic DNA analysis, InnoGenomics Technologies performs developmental validation studies. However, it is the responsibility of the customer laboratory to perform its own analysis and internal validation studies, and develop its own standard operating procedures and interpretation guidelines, to ensure that the products and services it obtains from InnoGenomics Technologies satisfy or will satisfy the applicable guidelines used by the forensic community and are fit for the customer laboratory's human identification applications.

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