InnoQuant® Human DNA Quantification & Degradation Assessment Kit Using 7500 Real-Time PCR System

For use with:

InnoQuant®	(product numbers: 21107-250, 21107-60)
InnoQuant® H-dye	(product numbers: 21106-250, 21106-60)

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InnoQuant® Kit Contents for 250 reactions (Item # 21107-250)

InnoQuant® Primer Mix	2 purple 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

InnoQuant® Kit Contents for 60 reactions (Item # 21107-60)

InnoQuant® Primer Mix	1 purple 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

InnoQuant® H-dye 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

InnoQuant® H-dye 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® Kits** have 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 initial use, store the kit at +4°C.

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

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

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® Primer Mix contains probes that are labeled with light-sensitive dyes. Thaw completely at room temperature protected from light.

1. 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 µLDilution 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 µLDilution 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® Primer Mix, InnoQuant® Dilution Buffer A and Agilent Brilliant Multiplex QPCR Master Mix and Reference Dye to room temperature for at least 15 minutes in the dark.

2. Dilute the Agilent Reference Dye to 2 µM by adding 1 µL of 1mM Reference Dye to 499 µL nuclease-free water for a final concentration of 30 nM in the PCR reaction.

Note: Protect all tubes containing the reference dye and Primer Mix from light as much as possible.

3. 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.

4. 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.

5. Prepare the reaction mix by combining the Master Mix, Primer Mix, and Reference Dye as indicated below and vortex briefly.

PCR Component	Volume per Reaction
Agilent Brilliant Multiplex QPCR Master Mix	10 µl
Agilent Reference Dye (2 µM)	0.3 µl
InnoQuant® Primer Mix	7.7 µl
Total Volume	**18.0µl**

6. 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).

7. 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.

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

9. Centrifuge the plate for 1 minute at 1500 rpm to collect the contents of the wells at the bottom. If bubbles are still present after centrifuging, repeat this step one time.

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. 7500 Instrument Setup

Note: Prior to running, the instrument must be calibrated for the following dyes: FAM, Cy5, and Cy3 (or HEX instead of Cy3 for the InnoQuant H-dye kit). Calibration kits can be purchased from InnoGenomics: InnoQuant® Spectral Calibration Kit part no. 21105. Calibration plates can also be purchased from Life Technologies: Spectral Calibration Kit I, part number 4360788 and Spectral Calibration Kit II, part number 4351151.

1. Open the 7500 HID Real-Time PCR Analysis Software v 1.1 or 1.2 and in the launch window select *Custom Assays.*

2. Import *2013 InnoQuant HID Run Template_v1.2* or the *InnoQuant H-dye HID Run Template_v1.0.* These templates contain all the set PCR run conditions, dye configurations, as well as the standard curve replicates set up in Columns 1 and 2, and an NTC.

3. Under *Define Targets and Samples*, select the *Define Samples* tab and name all unknown samples.

4. Under *Assign Targets and Samples*, assign the unknowns in the appropriate wells. For NTC, select "N" for Short and Long targets and "U" for IPC target. For unknown samples, select "U" for all three targets. Standards have been previously selected in the template.

5. 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

6. Select "Save As" to save the experiment.

7. Load plate and select "Start Run"

IV. Result Interpretation

Short Target	FAM:	80 bp size
Long Target	Cy5:	207 bp size
Internal Positive Control (**IPC**)	Cy3 or HEX*:	172 bp size

***** InnoQuant® kit contains the IPC labeled in Cy3 dye, and InnoQuant® H-dye kit contains the IPC labeled in HEX dye.

Run Parameters

The following parameters should be evaluated for each run:

1. When using the HID software, the following analysis parameters should be used:

Target	C_T threshold	Baseline
Short	Manual set at 0.3	Auto
Long	Manual set at 0.3	Auto
IPC	Auto	Auto

These parameters may be edited if the slope or R^2 values are outside the acceptable ranges. Ensure the threshold is within the logarithmic part of the amplification plot.

2. When using the SDS software, the following analysis parameters should be used:

Target	C_T threshold	Baseline
Short	Manual set at 0.2	Manual set at 3-12
Long	Manual set at 0.2	Manual set at 3-15
IPC	Manual set at 0.02	Manual set at 3-15

These parameters may be edited if the slope or R^2 values are outside the acceptable ranges. Ensure the threshold is within the logarithmic part of the amplification plot.

3. 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.

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

5. 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.

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.

Sample Analysis

To obtain the Degradation Index, run the InnoQuant Degradation Index Macro as follows:

1. Export the Well Table excel output file from the HID 1.1/1.2 software, with all samples selected.

2. Open Macro file – (Degradation_Index_Macro_HIDv1.2_v.1.02) and Enable Content when prompted.

3. From this window, open the exported HIDv1.2 excel output file.

4. From the View tab select Macros -> View Macros.

5. Run the Degradation_Index_Macro. This will create a new tab containing a table with sample name, quantity values for both targets and the Degradation Index.

V. Troubleshooting

Observation	Possible Causes	Recommended Actions
High baseline observed in the amplification plot	Reference dye problems	Re-dilute the 1mM reference dye stock in the Agilent kit to 2 µM (add 1 µl Reference dye to 499 µl nuclease-free water)
IPC C_T falls outside acceptable range	High concentration of EDTA PCR 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^2 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 that the baseline settings are adequate, 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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