



InnoQuant® HY Human and Male DNA Quantification & Degradation Assessment Kit – User Guide

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Kit Contents

200 reactions (Item # 21108-200)

InnoQuant® HY Primer Mix	1 yellow top tube	1.62 mL
InnoQuant® HY DNA Standard	1 clear top tube	75 µL at 100 ng/µL
InnoQuant® Dilution Buffer A	2 blue top tubes	1.8 mL each
InnoQuant® HY QPCR Master Mix	2 white top tubes	1.05 mL each
ROX Reference Dye	1 green top tube	20 µL at 1 mM

60 reactions (Item # 21108-060)

InnoQuant® HY Primer Mix	1 yellow top tube	0.5 mL
InnoQuant® HY DNA Standard	1 clear top tube	33 µL at 100 ng/µL
InnoQuant® Dilution Buffer A	1 blue top tube	1.8 mL
InnoQuant® HY QPCR Master Mix	1 white top tube	0.65 mL
ROX Reference Dye	1 green top tube	20 µL at 1 mM

NOTE: The **InnoQuant® HY Kit** has been validated and optimized for use with InnoQuant® HY QPCR Master Mix.

Storage Conditions

Upon receipt, store the kit at -20°C in a frost-free freezer. For short-term storage (i.e. ~1 wk.), store the kits components at 2-8°C. After the initial thaw, **do not** refreeze InnoQuant® HY DNA Standard. If the kit will not be used upon initial thaw in a week, refreeze can be performed on the Primer Mix, Master Mix and ROX reference dye, however minimize the number of freeze-thaw cycles. Increased Freeze-thaw cycles will result in increased background and poor efficiencies for the targets.

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

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



I. Preparation of DNA Standards

1. Equilibrate InnoQuant® HY 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.
2. Using InnoQuant® Dilution Buffer A, prepare fresh serial dilutions of the InnoQuant® HY 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 0.005 ng/μL (Std. 5).

Note: Once the standard is thawed, keep at 2-8 °C. Subjecting the InnoQuant® HY 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	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® HY Primer Mix, InnoQuant® Dilution Buffer A, InnoQuant® HY QPCR Master Mix and ROX Reference Dye to room temperature for at least 15 minutes in the dark.

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

2. Dilute the ROX Reference Dye to 4 μM by adding 2 μL of 1mM Reference Dye to 498 μL of TE⁻⁴ for a final concentration of 60 nM in the PCR reaction.

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

ROX should be diluted daily. Storage of diluted ROX for longer than one day will result in reduced intensity.

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 the 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 Components	Volume per Reaction
InnoQuant® HY QPCR Master Mix	10 µL
IQ HY: ROX Reference Dye (4 µM)	0.3 µL
InnoQuant® HY Primer Mix	7.7 µL
Total Volume	18.0 µL

6. In an optical plate, add 2 µL 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 2 µL of InnoQuant® Dilution Buffer A to the NTC reaction(s).
7. Add 18 µL 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. Instrument Setup


A. QuantStudio

Note: Prior to running, the instrument must be calibrated for FAM, HEX, TAMRA, ROX and Cy5 dyes. Calibration dyes can be purchased from InnoGenomics: InnoQuant® Spectral Calibration Kit part no. 21105.

1. Open the QuantStudio Design and Analysis Software or Quant Studio Real-Time PCR Software (QuantStudio 5/6/7)
 - a. For the QuantStudio D&A v1.5.1: Select *Open Existing Experiment*.
 - b. For the QuantStudio D&A v2.4.3: Select *Open File...*
 - c. For the QuantStudio Real-Time Software: Select *Template*
2. Locate and open the appropriate template for your software package (*InnoQuant-HY_QuantStudio_Temp*). This template contains all the set PCR run conditions, analysis settings, as well as the standards set up in Columns 1 and 2, and NTCs set up in wells F1 and F2, which was created with QuantStudio Design and Analysis Software v1.5.1.
3. Provide the necessary Name and optional information in the *Properties (D&A v1.5.1)/Actions (D&A v2.4.3)/Setup (QS RT)* tab. Ensure you are running the Standard Curve for Experiment type.
4. Verify the following PCR Cycling Parameters in *Methods/Run Method* tab:

Initial heating time:	3 min at 95 °C
Followed by 40 cycles of:	12 seconds at 95 °C
	30 seconds at 61 °C
5. For QuantStudio D&A v1.5.1:
 - a. Click the *Plate* tab
 - b. Under the Quick Setup tab select for the Passive Reference as ROX.
 - c. Under the Advanced Setup tab assign the targets to your plate for the wells in use:
 - For unknown samples select “U” for all targets
 - For Standard samples select “S” for Short, Long and Y targets and provide the known concentration for each dilution (already preset for default standard curve). Select “U” for the IPC.
 - For NTC, select “N” for Short, Long and Y targets and “U” for IPC target.

For QuantStudio D&A v2.4.3:

- a. Click the *Plate Setup* tab
- b. Set the Passive Reference as ROX
- c. The standards have been preset in the template. If changes are needed click the more options button  and adjust as needed for the *Standard Curve Setup*.
- d. Add samples as needed to the plate and assign all the targets to the each of the samples indicating unknown for the task except for IPC Target mark as IPC

For the QuantStudio Real-Time Software:

- a. Click the *Define* tab



- b. Ensure the targets, reporters and reference dye are designated correctly
 - c. Under the Samples window assign the Sample Names to your plate:
 - d. Under the *Assign* Tab, assign the samples as follows:
 - For unknown samples select “U” for all targets
 - For Standard samples select “S” for Short, Long and Y targets and provide the known concentration for each dilution (already preset for default standard curve). Select “U” for the IPC.
 - For NTC, select “N” for Short, Long and Y targets and “U” for IPC target.
6. Select File -> “Save As” to name and save the experiment.
 7. Load the plate, select the *Run* tab and select “Start Run”.

B. ABI 7500

Note: Prior to running, the instrument must be calibrated for FAM, HEX, TAMRA, ROX and Cy5 dyes. Calibration dyes can be purchased from InnoGenomics: InnoQuant® Spectral Calibration Kit part no. 21105.

1. Open the 7500 HID Real-Time PCR Analysis Software v 1.1, 1.2, or 1.3 and in the launch window, select *Custom Assays*.
2. Locate and open the appropriate template for your software package (*InnoQuant-HY_7500_Temp*). This template contains all the set PCR run conditions, analysis settings, as well as the standards set up in Columns 1 and 2, and an NTCs set up in wells F1 and F2, which was created with HID Real-Time PCR Analysis Software-Version 1.2.
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 unknown samples select “U” for all targets
 - For Standard samples select “S” for Short, Long and Y targets and provide the known concentration for each dilution (already preset for default standard curve). Select “U” for the IPC.
 - For NTC, select “N” for Short, Long and Y targets and “U” for IPC target.
5. Verify the following PCR Cycling Parameters in *Methods/Run Method* tab:

Initial heating time:	3 min at 95 °C
Followed by 40 cycles of:	12 seconds at 95 °C
	30 seconds at 61 °C
6. Select “Save As” to name and 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
Y Target	HEX:	80 bp size
Internal Positive Control (IPC)	TAMRA:	172 bp size

The InnoQuant® HY assay is 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 and the long targets than the Y target due to the higher copy number. For this reason, it is recommended to use a cut off of 30 C_T (i.e., disregard signals for all samples and negative controls higher than 30 C_T) for the short and the long InnoQuant® HY targets.

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

Run Parameters

The following parameters should be evaluated for each run:

1. The Auto analysis parameters should be used for all four targets in both the QuantStudio Design and Analysis Software and the 7500 HID Real-Time PCR Analysis Software. These parameters are already programmed in the respective run templates.

These parameters may be edited if amplification curves are non-ideal in shape, threshold is outside the exponential phase, R^2 values are below passing value or the efficiency is below or above acceptable ranges. Ensure the threshold is within the exponential phase of the amplification plot.

2. Passing reaction efficiency values are greater than 90% and less than 110% for the Short, Long, and Y 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.

Sample Analysis

To obtain the Human:Male ratio and the Degradation Index, please see the InnoQuant_Analysis_Macro.

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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 kit to 4 μ M (add 2 μ L Reference dye to 498 μ L TE ⁻⁴)
IPC C _T falls outside acceptable range	High concentration of EDTA	Verify the EDTA concentration in the unknown samples is 0.1mM EDTA.
	PCR inhibition	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).
	Analysis parameters incorrect	Ensure the baseline and threshold analysis parameters are set correctly in the software (under the Analysis Settings tab).
Low Efficiency values or slope values outside the acceptable range	Primers/probes not properly thawed	Allow primers/probes to equilibrate to room temperature for 15 minutes
	DNA standards too old	Re-dilute standards from 100 ng/ μ L stock using InnoQuant® Dilution Buffer A.
Low R ² values	Incorrect threshold setting	Verify the threshold set is in the exponential phase of the amplification curve and above baseline
	Pipette maintenance (or possible pipetting error)	Check maintenance of pipettes for calibration
S-shaped 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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