# Quantidex™ DNA Assay

## *Protocol*

| |
|---|
| *For Research Use Only.*<br>*Not for use in diagnostic procedures.* |
| REF **49539** |
| Σ **100** |

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## Background Information

Limitations in the availability of many clinical specimens drive the need for low DNA inputs into molecular assays. Highly multiplexed technologies such as Next-Generation Sequencing (NGS) often push the boundaries of input DNA material required for in-depth molecular profiling, particularly in cancer. Establishing an appropriate DNA input into these assays is needed to help assure accurate data and reliable interpretations. For example, several published studies have demonstrated the risk of false-positive and/or false-negative calls from targeted NGS data, particularly with low-input or low-quality specimens [1-4]. Although DNA quantification using spectrophotometry is suitable for many specimen types and applications, the chemical adduction, crosslinks, and fragmentation associated with FFPE DNA samples pose unique challenges, particularly for amplification-based assays. As a result, common methods for DNA quantification, such as spectrophotometry (e.g., Nanodrop®) and fluorometry using DNA-binding dyes (e.g., Qubit®) measure DNA properties that are distinct from sample-specific features that impact enzymatic function during targeted NGS library preparation. Thus, these methods fail to report "functional" DNA molecules, such as those that are amplifiable by PCR [1]. Targeted detection of FFPE DNA analytes by NGS demands a careful consideration of template library complexity and input levels to achieve accurate results [1]. Thus, metrics based on sensitive, functional, and quantitative pre-analytical sample characterization are needed to enable reliable DNA quantification and assess the fraction of template molecules that are competent for PCR amplification.

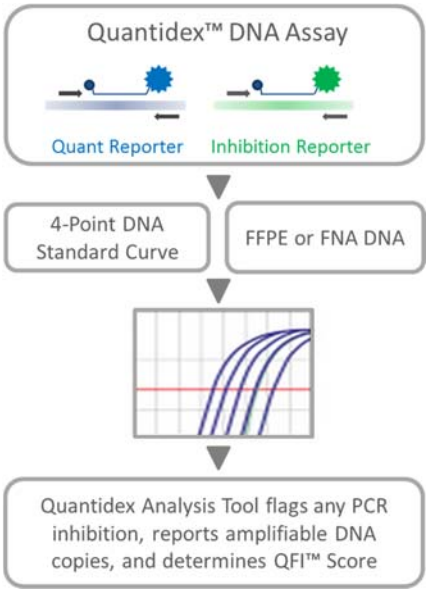
## Test Principle

The Quantidex™ DNA Assay is a multiplexed quantitative PCR assay that measures the absolute copy number of PCR-amplifiable DNA in a sample, and reports PCR inhibition. The Quant primer probe mix targets an 82 bp region in the TATA-Box Binding Protein (*TBP*) gene in the human genome, whereas the Inhibition primer probe mix assesses the amplification efficiency of an exogenous, non-human target to query the presence of PCR inhibitors.

The Quantidex Assay determines the functional quality of sample DNA using the QFI™ Score, which is the fraction of total genomic DNA copies that can be PCR amplified. The QFI™ Score and copy number of amplifiable DNA provide actionable guidance that can inform the input into NGS target enrichment and help assure analytical sensitivity and specificity. In addition, the Quantidex Assay flags PCR inhibitors in the sample and provides an opportunity to salvage such samples through a subsequent clean-up step, prior to further processing.

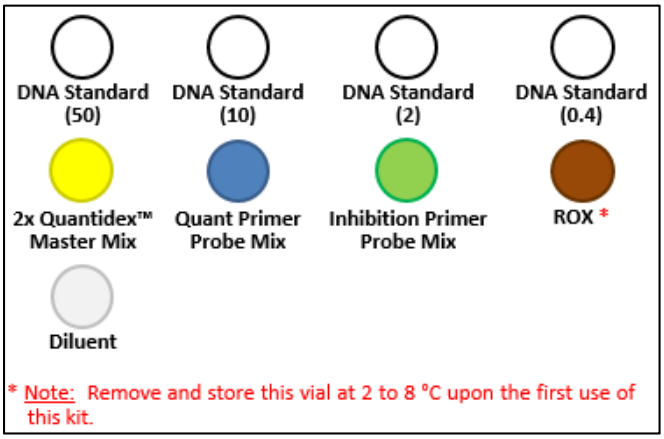
Hydrolysis probes with FAM and HEX fluorophores are used as Quant and Inhibition probes, respectively. Four pre-diluted DNA standards are provided with the kit and are required to be run in duplicate with each run.

Overview of the Quantidex DNA Assay workflow




## Kit Components

Reagents Supplied with this Kit




| Item # | Description | Volume | Storage Temp |
|---|---|---|---|
| 49539 | Quantidex™ DNA Assay | | |
| 145336 | Quant Primer Probe Mix | 50 µL | -15 to -30° C |
| 145344 | Inhibition Primer Probe Mix | 50 µL | |
| 145345 | 2x Quantidex™ Master Mix | 500 µL | |
| 145339 | Diluent | 400 µL | |
| 145340 | DNA Standard (50) | 16 µL | |
| 145341 | DNA Standard (10) | 16 µL | |

| Item # | Description | Volume | Storage Temp |
|---|---|---|---|
| 145342 | DNA Standard (2) | 16 µL | |
| 145343 | DNA Standard (0.4) | 16 µL | |
| **145346** | **ROX** | **20 µL** | **2 to 8° C** |

Handling and Storage

- Store the reagents in a non-frost-free freezer in the dark at -15 to -30°C.
- Upon first use of the ROX, store the vial at 2 to 8° C. Do not refreeze.
- Allow reagents (except 2x Quantidex™ Master Mix) to thaw at room temperature before use. Thaw 2x Quantidex™ Master Mix on ice. Vortex all reagents briefly after thawing, including 2x Quantidex™ Master Mix. Altenatively, the 2x Quantidex™ Master Mix can also flick-mixed.
- Prior to opening, briefly centrifuge each component to collect the solutions at the bottom of the vials.
- Avoid long exposure of Quant and Inhibition Primer Probe Mix (P/N 145336 and 145344) to light as these reagents are photosensitive.
- Assay setup should be performed at room temperature (approximate range of 18-25°C).

Number of Reactions

- The provided reagents are sufficient for up to 100 reactions.
- The reagents have been verified for use up to 8 freeze-thaw cycles. Additional cycles are not recommended.

Reagent Stability

- The product will maintain performance through the expiration date printed on the label when stored under the specified conditions.

Calibrators and/or Controls

- For generation of a calibration curve, pre-diluted inact DNA standards at 50, 10, 2 and 0.4 ng/µL are included in the kit.
- It is recommended that the operator include a separate intact genomic DNA as a positive control and a no-template control as a negative control within each batch run.

Reagents Required but not Provided

- Reagents for DNA isolation are not included in the kit. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high quality DNA.

Consumables & Equipment Required but not Provided

- General laboratory equipment and workspace to perform PCR
- Centrifuge capable of spinning 96-well plates
- Vortex

- Microcentrifuge
- Pipettes: Units with an accuracy range between 0.2-2 µL, 1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL
- Multi-channel pipette unit capable of pipetting 1-10 µL
- MicroAmp Optical 96-Well Reaction Plate (ABI part No. 4306737 or equivalent)
- MicroAmp® Optical Adhesive Film (ABI part No. 4311971 or equivalent)
- NanoDrop® or other spectrophotometer (needed to determine the QFI™ Score only)
- Validated real-Time PCR instrument with filters to detect FAM and HEX/VIC (e.g., Applied Biosystems® 7500 or 7900, Roche LightCycler® LC480 etc.).

## Warnings and Precautions

- Use proper personal protective equipment. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910:1030, CLSI M29, or other applicable guidance when handling human samples.
- Ensure the DNA sample being tested and DNA calibration standards are well mixed prior to use. Frozen DNA samples upon thawing can have concentration gradients that can lead to inaccurate results.
- Use nuclease-free filter pipette tips and nuclease-free tubes.
- PCR carry-over contamination can result in false-positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Do not pool components from different reagent lots. Do not use reagents after the labeled expiration date.
- Prior to use, ensure that all instruments are calibrated according to the manufacturer's instructions.

## Pre-Analytical Steps

- Genomic DNA extracted via common sample preparation methodologies is compatible with the Quantidex DNA Assay. To obtain the QFI™ Score for a sample, it is necessary to measure the absorbance (A260) of the genomic DNA using spectrophotometric methods, such as a NanoDrop instrument. In absence of this information, the assay will still report actionable output such as amplifiable copies of DNA but not the QFI™ Score.
- The recommended maximum DNA input, as measured using spectrophotometric methods (e.g., NanoDrop), into the Quantidex DNA Assay is 200 ng.

## Quantidex DNA Assay Protocol

1. In a template-free area, prepare qPCR Master Mix for the total number of samples tested, including the DNA standards and an NTC sample. For example, if 7 samples are being tested, then the master mix should be made for at least 16 reactions (7 samples + 4 standards in duplicate + 1 NTC).

   Note: Replicate testing of samples may be performed but are not required.

   Example of a 7 sample plate layout

| | 1 | 2 | 3 |
|---|---|---|---|
| A | DNA Standard (50) | Sample 1 | |
| B | DNA Standard (50) | Sample 2 | |
| C | DNA Standard (10) | Sample 3 | |
| D | DNA Standard (10) | Sample 4 | |
| E | DNA Standard (2) | Sample 5 | |
| F | DNA Standard (2) | Sample 6 | |
| G | DNA Standard (0.4) | Sample 7 | |
| H | DNA Standard (0.4) | NTC | |

2. Add the reagents in the listed order at room temperature. Volumes are shown per reaction.

| qPCR Master Mix for ABI 7500 | | |
|---|---|---|
| Part # | Description | Volume (µL) |
| 145345 | 2x Quantidex™ Master Mix | 5 |
| 145336 | Quant Primer Probe Mix | 0.5 |
| 145344 | Inhibition Primer Probe Mix | 0.5 |
| 145339 | Diluent | 2.95 |
| 145346 | ROX | 0.05 |
| | Total volume (per well) | 9 |

| qPCR Master Mix for ABI 7900 (Note 4x more ROX) | | |
|---|---|---|
| Part # | Description | Volume (µL) |
| 145345 | 2x Quantidex™ Master Mix | 5 |
| 145336 | Quant Primer Probe Mix | 0.5 |
| 145344 | Inhibition Primer Probe Mix | 0.5 |
| 145339 | Diluent | 2.8 |
| 145346 | ROX | 0.2 |
| | Total volume (per well) | 9 |

| qPCR Master Mix for Roche LC480 or similar instruments that do not need passive reference dye (Note No ROX needed) | | |
|---|---|---|
| Part # | Description | Volume (µL) |
| 145345 | 2x Quantidex™ Master Mix | 5 |
| 145336 | Quant Primer Probe Mix | 0.5 |
| 145344 | Inhibition Primer Probe Mix | 0.5 |
| 145339 | Diluent | 3 |
| | Total volume (per well) | 9 |

**Important!:** The volume or inclusion of ROX in the mastermix preparation depends on the instrument used.

3. Gently mix the master mix by flicking or by vortexing, and then pulse spin. Add 9 µL of qPCR Master Mix to each well at room temperature.
4. Add 1 µL of DNA sample or DNA calibration standard to each well.
5. Mix by gentle pipetting or briefly vortexing at low speeds. Avoid forming bubbles in the wells.
6. Cover wells with optical caps or an optical seal.
7. Spin the plate to remove any bubbles. (1 minute at 1500 rpm).
8. Place on real-time thermal cycler. Select detectors and quenchers listed below. *[The below instructions are provided using the terminology applicable to the ABI 7500 series. Terminology used for other laboratory-validated platforms may be slightly different.]*

   **Run the instrument in "Standard" mode**. The assay has <u>not</u> been tested in "FAST" mode.

   FAM for Quant; quencher=none

   VIC for Inhibition; quencher=none

   **Note:** *Select "ROX" for passive reference*

9. Run program listed below for ABI 7500 and 7900 series.

| | | |
|---|---|---|
| 95 °C | 10 minutes | |
| 95 °C | 15 seconds | 40 cycles* |
| 60 °C | 60 seconds | |

*For Roche LC480, run data collection for 45 cycles as the instrument uses the last 5 cycles for baseline calculations.

## Calibration Procedure

- To determine the target copy number in a tested sample, a standard curve must be established in every run using the provided DNA Standards at 50, 10, 2 and 0.4 ng/µL in duplicate. Standard curves are linear regression curves (y=mx+b) of the Cq versus the log of the initial template copy number generated by each Standard in the FAM channel of the instrument. No calibration is needed for the inhibition control.

## Software Procedure

- Data analysis to obtain the amplification DNA copy number, QFI™ Score, recommended volumetric input into the NGS library preparation and indication of PCR inhibition is performed using Asuragen's Quantidex™ Analysis Software (http://quantidex.asuragen.com).

## Data Interpretation

- Intact DNA samples and other non-FFPE DNA should be evaluated with the Analysis Software using the assay amplicon size of 82 bp. FFPE DNA can be analyzed by using this same amplicon length, or by using an amplicon size equivalent to the median amplicon length produced by the downstream targeted NGS enrichment step. In the latter situation, the data analysis software enlists an empirically-validated algorithm to automatically adjust the amplifiable copy number and QFI™ Score to match the user-provided amplicon size. This conversion is made possible by the highly predictable effects of PCR-inhibiting DNA modifications in FFPE samples when informed by the QFI™ Score.
- The absolute copy number is provided in copies/ul of DNA and is derived from quantification of the endogenous locus in the FAM channel. This value is then used to determine the minimum volume of sample DNA needed to achieve the *theoretical* detection of a variant at 5% abundance. This calculation is based on the requirement for at least 10 copies of mutant DNA into the NGS enrichment step to support accurate variant quantification—a determination that has been vetted across multiple target enrichment methodologies and NGS procedures evaluated at Asuragen. This said, it is important that the DNA input volume and acceptability criteria for amplifiable copy number and/or QFI™ Score be established by each laboratory in accordance with internal procedures given the diversity of possible wet and dry bench processes for targeted NGS.
- Inhibition "Pass/Fail" is reported by assessing the amplification of the spiked-in non-human target in the VIC/HEX channel. The inhibition Primer Probe mix contains templates for the exogenous target and is added to each reaction, including the NTC. In absence of an inhibitor, the PCR product in the VIC/HEX channel should always be detected. Typically, for ABI 7500 instrument using a threshold of 0.1, a Cq between 27-34 is obtained from the VIC/HEX channel for an uninhibited sample. An "undetected" Cq for a sample in the VIC/HEX channel indicates the presence of PCR inhibitors and indicates subsequent clean-up of the sample prior to further processing (e.g., OneStep™ PCR Inhibitor Removal Kit from Zymo Research).
- Refer to the User guide at http://quantidex.asuragen.com for detailed information on data analysis.

## Limitations

1. The kit is compatible with the use of only human genomic DNA
2. Samples with inhibition are identified by an increase in Cq values for the inhibition reporter. The assay is designed to respond to the presence of common PCR inhibitors but may not identify all inhibitors that may impact subsequent sample processing or other library preparation or NGS enrichment methods.
3. The QFI™ Score is dependent on an accurate measure of total bulk DNA, which is provided by the user from the spectrophotometric DNA concentration. Low absorbance readings associated with highly dilute DNA samples may be unreliable, and will warrant caution in interpreting the QFI™ Score.
4. Changes in the *TBP* copy number due to disease processes or other genomic alterations may affect the amplifiable copy number and QFI™ Score. Analyses of *TBP* copy number data in the cBioPortal database [5, 6] revealed that 99% of all reported cancer specimens across the top 20 most frequently occurring cancers were devoid of significant copy number changes in this gene.
5. Rare polymorphisms in the primer or probe binding regions of *TBP* may affect amplification. No single nucleotide polymorphisms were identified using the SNP database (NCBI build 37.1 and dbSNP Build: 138) hosted by the National Genetics Reference Lab in the UK (https://secure.ngrl.org.uk/SNPCheck/snpcheck.htm).

## Notice to Purchaser

1. ***This Product is intended for research use only. It is not intended for diagnostic use.***
2. *This Product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen.*
3. *Quantidex™ and QFI™ are trademarks of Asuragen, Inc.*
4. *The Quantidex™ DNA Assay was optimized with ABI 7500 real-time instrument. The assay has been tested for compatibility and performance with ABI 7900 and Roche LC 480 II.*
5. *Information in this document is subject to change. Asuragen assumes no responsibility for any errors that may appear in this document. In no event shall Asuragen be liable in any way (whether in contract, tort (including negligence) or otherwise) for any claim arising in connection with or from the use of this Product. Nothing in this document excludes or limits any liability which it is illegal for Asuragen to exclude or limit.*

## References


1. Sah *et al*. Functional DNA quantification guides accurate next-generation sequencing mutation detection in formalin-fixed, paraffin-embedded tumor biopsies. Genome Med 2013;5(8):77
2. Williams *et al*. A High Frequency of Sequence Alterations Is Due to Formalin Fixation of Archival Specimens. Am J Pathol. 1999; 155(5):1467
3. McCall *et al*. False positives in multiplex PCR-based next-generation sequencing have unique signatures. J Mol Diagn. 2014;16(5):541

4. Zhang *et al*. Profiling cancer gene mutations in clinical formalin-fixed, paraffin-embedded colorectal tumor specimens using targeted next-generation sequencing. Oncologist. 2014;19(4):336
5. Gao *et al.* Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Sci. Signal. 2013;6(269)
6. Cerami *et al.* The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. Cancer Discovery 2012;2(5):401

## Appendix A: Glossary of Symbols

| Symbol | Description |
|---|---|
|  | Catalog number |
|  | Batch code |
|  | Contains sufficient for 100 tests |
|  | Consult instructions before use |
|  | Temperature limitation |
|  | Use by |
|  | Date of manufacture |
|  | Manufactured by |
|  | Protect from light |
|  | Do not reuse |

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