CYSTIC FIBROSIS PHENYLALANINE 508 MUTATION

SNP qPCR TEST HANDBOOK

For Research Use Only. Not intended for diagnostic use.

CAT NO.: YS-SNP-CFTR-100-DEV

100 reactions

VERSION 6.2

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INTENDED USE

This SNP qPCR test offers an efficient and user-friendly solution for determining the genotype of the polymorphism of interest in extracted nucleic acid samples. It is intended for use by trained professionals in a suitable molecular biology laboratory.

The test has been designed using available reference genome sequences. The Wild Type sequence is detected in the FAM channel and the Mutant is detected in the VIC/HEX Channel.

CONTENTS

Component	Cap Colour	Volume
Cystic Fibrosis phenylalanine 508 mutation specific primer/probe mix Wild Type (WT) probe (FAM) Mutant (MUT) probe (VIC/HEX)		100 µL
Cystic Fibrosis phenylalanine 508 mutation WT Positive Control (PTC)		500 µL*
Cystic Fibrosis phenylalanine 508 mutation MUT Positive Control (PTC)		500 µL*
Tetra™ 2X qPCR MasterMix		1 mL
Template Resuspension Buffer (TRB)		1.5 mL
DNase/RNase Free Water		1.5 mL

* Supplied dried – requires resuspension. See instructions in resuspension section.

RECOMMENDED ADDITIONAL REAGENTS & MATERIALS

Nucleic Acid extraction kit

General laboratory equipment (pipettes, pipette tips, (micro)centrifuge tubes, compatible strip tubes/plates, plate seals, etc.).

qPCR instrument with channels to detect FAM and VIC/HEX, that is capable of allelic discrimination.

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BEST PRACTICE

Sample preparation:
The recommended extracted DNA input is 10 ng to 100 ng per reaction in a total volume of 5 µL.

Decontamination:
Before beginning laboratory work, thoroughly decontaminate any work surfaces and pipettes being used, to eliminate potential contamination.

General use and set-up:
All components should be fully defrosted with contents at the bottom of the tube before opening. To ensure contents are at the bottom, centrifuge or gently tap the tube. After use, reagents should be returned to the freezer.

Once any reagents are resuspended, mark the tick box on the tube for future reference. After this, or after combining reagents, the tube should be pulse vortexed to ensure it is mixed well.

It is advised to set up the tubes/plate and reaction mix on ice to minimise artefact formation, which may reduce sensitivity.

When preparing the qPCR reaction mix, it is recommended to incorporate an overage when calculating the total number of reactions to compensate for potential volume losses incurred during pipetting.

Set-up environments:
It is best practice to set up qPCR tubes/plates in two different environments - a clean (no template) lab and PCR (template) lab.

No Template Control(s) (NTC) and Positive Control(s) (PTC) should be included in every run. To reduce contamination, NTCs and samples can be set up and sealed in a clean lab before moving to the PCR lab.

BENCH SIDE PROTOCOL

RESUSPENSION

Before first use, resuspend the designated component with the correct reagent and specified volume, as per the table below:

1. Add the resuspension reagent and pulse vortex the tube to ensure each is mixed well.

Component	Reagent	Volume	Location
Cystic Fibrosis phenylalanine 508 mutation WT Positive Control (PTC)	TRB	500 µL	PCR lab
Cystic Fibrosis phenylalanine 508 mutation MUT Positive Control (PTC)	TRB	500 µL	PCR lab

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SNP qPCR REACTION SET-UP

1. Retrieve the required components and appropriate plasticware for qPCR reaction set-up.

2. In an appropriately sized (micro)centrifuge tube, combine the following reagents to create a reaction mix that will cover all required tube/wells (e.g. samples, NTCs and PTCs).

Please note: When calculating required reactions, include an overage to allow for volume loss during pipetting

Component	Volume (per reaction)
Tetra™ 2X qPCR MasterMix	10 µL
Cystic Fibrosis phenylalanine 508 mutation specific primer/probe mix	1 µL
DNase/RNase Free Water	4 µL
Reaction mix volume	15 µL

3. Mix the combined reagents by briefly vortexing or inverting.

Please note: For best results reaction mixes should be prepared and kept on ice until the amplification protocol is ready to start.

4. Dispense 15 µL of the reaction mix into all required tubes/wells.

5. For the NTC(s), add 5 µL of DNase/RNase Free Water into required tube/well(s).

6. For each sample, add 5 µL of the extracted DNA into required tube/well(s).

Please note: A recommended amount of 10 ng to 100 ng of extracted DNA is required per reaction in a total volume of 5 µL.
Please note: It is best practice to seal the NTC(s) & sample tubes/wells before proceeding to the positive control steps.

7. For a homozygous Wild Type (WT) or homozygous Mutant (MUT) positive control reaction, each PTC should be run seperately. Add 5 µL of the appropriate PTC into the designated positive control tube/well.

Please note: The PTC is a contamination risk and should be handled in a designated PCR (template) laboratory.

8. Seal the tube(s)/plate.

9. Briefly vortex the sealed tube(s)/plate, then spin in a centrifuge to ensure all reagents are fully homogenous and at the bottom of the tubes/wells before proceeding.

qPCR AMPLIFICATION PROTOCOL

This YouSeq kit will work with any qPCR instrument capable of allelic discrimination through the FAM and VIC/HEX channels. Ensure that the genotyping experiment type is selected to enable cluster-based analysis.

1. Load the tubes/plate onto the qPCR instrument and set up the qPCR protocol following the table below.

2. Set the total reaction volume to 20 µL.

Temperature	Time	Number of Cycles
95°C	3 minutes	-
95°C	15 seconds	x 45
60°C	60 seconds	

Collect fluorogenic data through FAM and VIC/HEX channels during this step.

3. Start the run.

INSTRUMENT SPECIFIC INSTRUCTIONS

This YouSeq kit will work with any qPCR instrument capable of cluster-based allelic discrimination through the FAM and VIC/HEX channels. Below are specific instructions to assist in instrument set-up.

For QuantStudio Instruments:

Open the completed run in the compatible software (YouSeq recommends QuantStudio™ Design and Analysis software v2.X or later). Ensure the Genotyping Analysis Module is enabled and confirm the targets, controls, samples and well assignment before analysing the data. YouSeq recommends using “Real Time Data” for run analysis. Go to Actions > Genotyping Analysis Setting > Select "Real Time Data”.

Allele to fluorophore assignment:

Set the instrument software's allele-to-fluorophore assignment as follows:

- . QuantStudio™: Allele 1 = VIC/HEX; Allele 2 = FAM
- All other instruments: Retain the software's default assignment.

Please note: The QuantStudio automated genotype-calling workflow relies on this reporter assignment to call samples correctly. Do not reverse this allocation.

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INTERPRETATION OF RESULTS

Ensure that all controls, wells and fluorophores are correctly assigned and that the instrument default baseline correction and fluorescence normalisation settings have been applied. To set the Cq threshold for each target assay, YouSeq recommends:

- WT (FAM) threshold – set at 10% of the End Point Fluorescence (EPF) of the WT PTC amplification.
- MUT (VIC/HEX) threshold – set at 10% of the EPF of the MUT PTC amplification.

Ensure any failed wells or obvious technical artefacts are excluded before interpreting results.

Genotype determination will be performed using cluster-based allelic discrimination analysis based on EPF.

Positive Control

Each PTC should amplify in a Cq range of 18.5 ± 2 for the target in the representative channel. If this Cq range is not achieved, the sample test result is invalid and should be repeated.

Low-level fluorescence may occasionally be observed in the non-target allele channel of a PTC well due to the competitive probe chemistry. This may be treated as background provided that the control amplifies in the expected range in the target allele channel and occupies the expected region of the allelic discrimination plot.

Please note: The positive control is a sequence representative of the target region and does not contain the organism's entire genome.

No Template Control

The NTC tube/well(s) should be negative, with no amplification.

Please note: Background laboratory contamination can result in a very late signal in NTC tube/well(s). If the NTC has amplification, comparison to the sample test result is necessary:

- If the NTC is ≥5 Cq later than the sample signal, it can be considered negative, and the sample test result is valid.
- If the NTC is <5 Cq later, the sample test result is invalid – the test should be repeated after potential sources of contamination have been removed.

NTC wells should remain close to the origin or within the software-defined no-amplification region.

Sample(s)

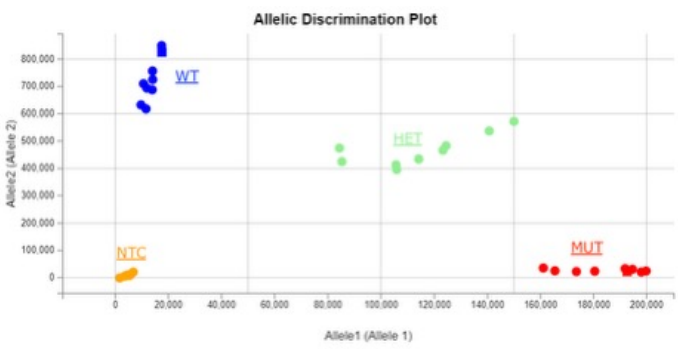
Within the required input range of 10 ng to 100 ng, valid amplification is expected between Cq 20 – Cq 30.

Use cluster-based allelic discrimination analysis for genotype determination. The allelic discrimination plot compares the EPF of the WT and MUT signals. Samples with the same genotype should group into distinct clusters:

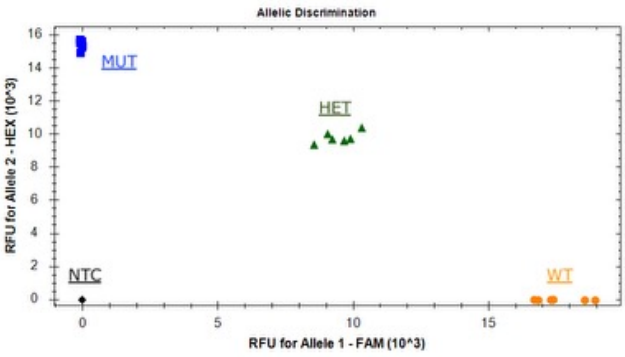
- WT homozygous samples will show higher endpoint fluorescence in the FAM channel and low or background level fluorescence in the VIC/HEX channel, forming the WT cluster.
- MUT homozygous samples will show higher endpoint fluorescence in the VIC/HEX channel and low or background-level fluorescence in the FAM channel, forming the MUT cluster.
- Heterozygous samples will show fluorescence in both the FAM and VIC/HEX channels, forming a distinct heterozygous cluster between the two homozygous clusters.
- If a sample does not fall clearly within a defined genotype cluster, review the well and reporter assignments and the analysis settings. Correct any identified errors and re-analyse the data. If the result remains unclear, report the sample as inconclusive and repeat testing.

Cluster examples are shown below, with the X axis representing the Allele 1 fluorescence signal and the Y axis representing the Allele 2 fluorescence signal.

QuantStudio



Bio-Rad



PRODUCT SPECIFICATIONS

Specificity

Rare alternative variants located within the probe binding regions may occasionally be encountered. The presence of such variants may affect probe binding efficiency, potentially impacting genotype determination.

Storage

Store at -20°C from arrival. The product's shelf life is outlined as an expiry date on the pouch label.

Suitable input material

This SNP qPCR test will work well with any source of good quality nucleic acid commonly used for genotyping. Good quality is defined as nucleic acid with high integrity (not degraded). Poor quality input nucleic acid is a leading cause of test failure.

Regulatory status

This product has been developed for Research Use Only (RUO) and is not intended for diagnostic use. It should not be used for diagnostic purposes unless specifically approved by the regulatory authorities in the country of use.

Quality Control

In accordance with the YouSeq Ltd ISO EN 13485-certified Quality Management System, each lot of YouSeq Cystic Fibrosis phenylalanine 508 mutation SNP qPCR Test is tested against predetermined specifications to ensure consistent product quality. Design of the kit met our robust bioinformatic analysis requirements resulting in a clinically relevant detection profile based on available sequence information.

Technical Assistance

For customer support, please contact:

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