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TaqMan® Mutation Detection Assays

Competitive Allele-Specific TaqMan® PCR

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Note: For safety and biohazard guidelines, refer to the "Safety" section in the *TaqMan® Mutation Detection Assays Protocol* (Part no. 4467011). For every chemical, read the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Prepare the samples

1 Extract and purify gDNA

Extract and purify gDNA samples according to your laboratory practices. We recommend the gDNA extraction and purification kits listed below. For gDNA extraction from:

- **FFPE tissue samples** – RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE or QIAamp® DNA FFPE Tissue Kit
- **Fresh-frozen tissue samples** – MELT™ Total Nucleic Acid Isolation System or Gentra Puregene Tissue Kit
- **Cell lines** – PureLink® Genomic DNA Mini Kit or kit from other major supplier

2 Quantitate the gDNA

Quantitate the gDNA by measuring the UV absorbance (A_{260}/A_{280}). Be sure that the human gDNA that you use has an A_{260}/A_{280} ratio >1.7.

Note: Given that gDNA quantitation by UV absorbance methods may not accurately reflect the concentration of amplifiable gDNA template in some sample types (e.g. FFPE samples), also consider using gene reference assays to assess the amount of functional template in a sample that can be amplified by PCR.

Perform the PCR

Recommended reaction types

When setting up the reaction plate, we recommend that you include the following reaction types:

Reaction type	Experiment type	
	Detection ΔC_T cutoff determination	Mutation detection
Controls	No template controls (NTC) are optional, negative control samples are required (inherent to the experiment), and positive controls are not necessary.	NTCs are optional, negative sample controls are highly recommended, and positive control samples are recommended, if available.

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Reaction type	Experiment type	
	Detection ΔC_T cutoff determination	**Mutation detection**
Assays and replicates	To generate accurate detection ΔC_T cutoff values for a mutant allele assay(s), run the assay and corresponding gene reference assay on at least three wild type gDNA samples and use three technical replicates per sample. The sample type must be the same as the test sample. The same amount of amplifiable DNA should be used for each sample tested.	Run gDNA test samples of unknown mutation status with a mutant allele assay(s) and corresponding gene reference assay. Technical replicates are not required. The same amount of amplifiable DNA should be used for the test sample as was used to establish the detection ΔC_T cutoff value.
	In order for the Mutation Detector™ Software to compute ΔC_T cutoff values or sample mutation status, you must run each mutant allele assay, the corresponding gene reference assay, and (if applicable) technical replicates on the same reaction plate with the same sample. For ΔC_T cutoff determination, the software can combine wild type sample ΔC_T values from multiple plates to generate an assay ΔC_T cutoff value.	
(Optional) TaqMan® Mutation Detection IPC Reagents Kit	You can duplex the IPC reagents with any TaqMan® Mutation Detection Assay to distinguish true target negatives from PCR failure or inhibition.	

Prepare the PCR mix and the PCR plate

Detection ΔC_T cutoff determination experiments

In a detection ΔC_T cutoff determination experiment, run three or more wild type gDNA samples, and three technical replicates of each sample, with a mutant allele assay(s) and paired gene reference assay. The same amount of gDNA must be used for each sample. Prepare the PCR mix and the PCR plate as follows:

1. For each sample, calculate the total number of reactions required.
2. Calculate the total volume required for each reaction component:
   volume for 1 reaction × total no. of reactions + 10%
   Note: Include 10% extra volume to compensate for pipetting errors.

Component	Volume for 1 reaction	
	20 µL reaction (96-well plate)	**10 µL reaction (384-well plate)**
TaqMan® Genotyping Master Mix, 2X	10.0 µL	5.0 µL
Prepared gDNA sample†	2.0 – 4.0 µL	1.0 – 2.0 µL‡
(Optional) 50X Exogenous IPC Template DNA	0.4 µL	0.2 µL
(Optional) 10X Exogenous IPC Mix	2.0 µL	1.0 µL
Nuclease-free water	Add water to 18.0 µL	Add water to 9.0 µL
Total volume of super mix	**18.0 µL**	**9.0 µL**
Example: Total volume for 3 technical replicates (includes 10% extra volume for pipetting errors)	**59.4 µL**	**29.7 µL**

† We recommend that you input 20 ng of gDNA; the volume of gDNA sample should not be greater than 20% of the total reaction volume.
‡ For 10-µL reactions, we recommend that you use the same amount of gDNA as for 20-µL reactions to obtain the same sensitivity of mutation detection.

3. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, cap the tube, then vortex the tube briefly to mix the components.
4. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

5. For each set of technical replicates, transfer aliquots of super mix to a microcentrifuge tube, then add the TaqMan® Mutation Detection Assay (mutant allele or gene reference assay) to each tube.

If the total reaction volume for one reaction is...	Add the following volumes to the microcentrifuge tube...†	
	Super mix	TaqMan® Mutation Detection Assay (10X)
20 µL	59.4 µL	6.6 µL
10 µL	29.7 µL	3.3 µL

† The super mix and assay volumes listed are for three technical replicates.

6. Add the appropriate volume of PCR mix to each reaction well of a PCR plate:

PCR plate	Volume of PCR mix per reaction
96-well	20 µL
384-well	10 µL

7. Cover the plate with an optical adhesive film.

8. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

Mutation detection experiments

In a mutation detection experiment, run the test sample with a mutant allele assay(s) and corresponding gene reference assay. Technical replicates are not required. The amount of test sample gDNA used should be similar to the amount of wild type gDNA sample that was used for your detection ΔC_T cutoff determination experiments. Prepare the PCR mix and the PCR plate as follows:

1. For each sample, calculate the total number of reactions required.

2. Calculate the total volume required for each reaction component:
   volume for 1 reaction × total no. of reactions + 10%
   Note: Include 10% extra volume to compensate for pipetting errors.

Component	Volume for 1 reaction	
	20- µL reaction (96-well plate)	10- µL reaction (384-well plate)
TaqMan® Genotyping Master Mix, 2X	10.0 µL	5.0 µL
Prepared gDNA sample†	2.0 – 4.0 µL	1.0 – 2.0 µL‡
(Optional) 50X Exogenous IPC Template DNA	0.4 µL	0.2 µL
(Optional) 10X Exogenous IPC Mix	2.0 µL	1.0 µL
Nuclease-free water	Add water to 18.0 µL	Add water to 9.0 µL
Total volume of super mix	**18.0 µL**	**9.0 µL**

† We recommend that you input 20 ng of gDNA; the volume of gDNA sample should not be greater than 20% of the total reaction volume.
‡ For 10-µL reactions, we recommend that you use the same amount of gDNA as for 20-µL reactions to obtain the same sensitivity of mutation detection.

3. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, cap the tube, then vortex the tube briefly to mix the components.

4. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

5. Add the appropriate volume of super mix to each reaction well of a PCR plate:

PCR plate	Volume of super mix per reaction
96-well	18 µL
384-well	9 µL

6. Add the appropriate volume of TaqMan® Mutation Detection Assay (mutant allele or gene reference assay) to each reaction well:

PCR plate	Volume of TaqMan® Mutation Detection Assay (10X) per reaction
96-well	2 µL
384-well	1 µL

7. Cover the plate with an optical adhesive film.

8. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

Set up the plate document or experiment and start the run

In the real-time PCR system software:

1. Select the experiment type: **Absolute Quantitation** or **Quantitation - Standard Curve**.

2. For each well that contains a reaction, apply a sample name, assay name, and target or detector name. For downstream analysis with the Mutation Detector™ Software, note the following:

Parameter	Comments
Sample Name	If you enter the sample name as **NTC**, the Mutation Detector™ Software treats the sample as a No Template Control.
	Apply the same sample name to all wells containing the sample and mutation detection assays that will be analyzed together. If the sample name is not identical for each well across the reaction plate, the Mutation Detector Software treats these as different samples.
	If you are using technical replicates, apply the same sample name to the wells of each technical replicate group. The Mutation Detector™ Software combines data from replicate wells only if the wells share the same sample name. If the replicate wells are named differently, the software analyzes the wells as different samples.
Target Name or Detector Name	In order for the Mutation Detector™ Software to correctly analyze the data, you must use Applied Biosystems assay names. Using Applied Biosystems assays names ensures that a mutant allele assay will be paired with the appropriate gene reference assay in the analysis.
	If the IPC reagents are duplexed in the reaction, enter **IPC** as the detector name.
Reporter and Quencher Names	For wells that contain: • TaqMan® Mutation Detection Assays, FAM™ dye is the reporter and Non Fluorescent or NFQ-MGB is the quencher • IPC reagents (from the TaqMan® Mutation Detection IPC Reagent Kit), VIC® dye is the reporter and TAMRA™ dye is the quencher **Note:** Instrument results files exported from the 7500 SDS v1.X software do not contain a Reporter column with dye names. In this case, the Mutation Detector™ Software uses the assay name (from the Detector/Target column) to determine the dye name. If the assay name is *IPC*, the software assumes that the reporter is VIC® dye; for all other assay names, the software assumes that the reporter is FAM™ dye.

3. Enter sample quantity values in the real-time PCR system software:

   a. Select **Standard** as the task for each well of interest.

   b. Enter a numeric value. We recommend that the numeric values you enter are relevant to the ng amount of gDNA or copies of DNA input.

For downstream analysis with the Mutation Detector™ Software, note the following:

Parameter	Comments
Amount of sample DNA	For all mutation detection assay results that will be used to calculate a detection ΔC_T cutoff value or mutation status, load the same amount of sample DNA into the wells. If the sample quantity is not specified in the real-time PCR system software, then the Mutation Detector™ Software assumes that the sample amounts in each well are equivalent.
Sample quantity value	The sample quantity value must be equal and must be provided in the real-time PCR system software for all samples that will be analyzed together if you are calculating calibration ΔC_T values in real-time (comparing positive control sample C_T values between a mutant allele assay and a corresponding gene reference assay).

4. Set the following thermal-cycling conditions:
   - Run mode – **Standard**
   - Sample volume – **10 µL** (384-well plates) or **20 µL** (96-well plates)
   - Thermal-cycling profile – See the table below

Stage	Temp.	Time (mm:ss)	Cycles	Data collection
1	95°C	10:00	1	No
2	92°C	00:15	5	No
	58°C	01:00		No
3	92°C	00:15	40	No
	60°C	01:00		FAM™ or VIC® dye†

† FAM dye is the reporter for TaqMan Mutation Detection Assays; VIC dye is the reporter for the IPC reagents (from the TaqMan Mutation Detection IPC Reagent Kit).

5. Load the reaction plate into the real-time PCR instrument, then start the run.

Analyze the data

For detailed analysis procedures, refer to the *Mutation Detector™ Software User Guide* (Part no. 4467102). Briefly, the analysis steps are:

1. Analyze the data in the real-time PCR system software, using the following analysis settings:
   - Manual C_T (threshold cycle): **0.2**
   - Automatic Baseline: **On**

   The real-time PCR system software determines the C_T values for the mutation detection assays and (optional) IPC reagent reactions.

2. View the amplification plots and/or C_T values for all reaction wells as follows:

Reaction type	What to look for
Samples tested with gene reference assays (FAM™ dye signal)	Verify that the amplification curves have a distinct, linear amplification phase and that the C_T values are within a range of ~18 to 28 for a 20-µL reaction and ~17 to 27 for a 10-µL reaction.
Samples tested with mutant allele assays (FAM™ dye signal)	Review the amplification curves and C_T values. The presence or absence of a distinct, linear amplification phase and C_T values depend on the amount of mutant allele present in the sample.
Positive control samples	Verify that the amplification curves have a distinct, linear amplification phase and that the C_T values are within the expected range for the quantity of target present in the sample. **Note:** Positive control samples that are used to calculate calibration ΔC_T values must contain 100% target sequence for the mutation detection assay that is run with the positive control.
Negative control samples	Verify that the negative control samples *either* do not amplify *or* have very high C_T values.

Reaction type	What to look for
No template control (NTC) samples	Verify that the NTC samples do not amplify.
Technical replicates	Verify that the C_T values are similar between replicates. **Note:** Some variance is expected between replicates for samples that contain low amounts of target allele and have high C_T values.
(If using) IPC reagents, from the TaqMan® Mutation Detection IPC Reagent Kit	Verify that the amplification curves (VIC® dye signal) in all samples have a distinct, linear amplification phase and that the C_T values are similar for all wells that contain the same sample

Note: Some mutant allele assays are expected to give low level non-specific amplification of wild type gDNA samples. The TaqMan® Mutation Detection Assay Index file (download from: **www.lifetechnologies.com/castpcr**) provides off-target amplification C_T values determined for each mutant allele assay that can be used to evaluate an assay's performance. Note that the off-target C_T value may differ for different sample types.

3. Export the Results or Results Table from the real-time PCR system software as a *.csv or *.txt file.

4. Import the *.csv or *.txt file(s) into the Mutation Detector™ Software. The Mutation Detector Software can:
   - Calculate detection ΔC_T cutoff values
   - Determine the presence or absence of a mutation in a sample *and*
   - Quantitate the percent mutation in a sample (when assay calibration values are available)

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