Copy Number Analysis for Pharmacogenomics Experiments

Set up and run CYP2D6 TaqMan™ Copy Number Assays

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

These instructions are intended as a benchtop reference for copy number analysis of the drug metabolizing enzyme gene CYP2D6. For detailed instructions for TaqMan™ Copy Number Assay experiments, see *TaqMan™ Copy Number Assays User Guide* (Pub. No. 4397425).

Download the `OpenArray Genotyping Calculation Sheet` from **thermofisher.com/oaqrc**. The `OpenArray Genotyping Calculation Sheet` includes formulas for reagent mix setup, calculation of DNA concentration, and normalization of DNA concentration.

Procedural guidelines

- Use 10 ng of high-quality DNA per reaction.
- Run quadruplicate reactions for each DNA sample. The `OpenArray Genotyping Calculation Sheet` assumes quadruplicate reactions in 384-well plates.
- For quality metrics calculations, each plate should contain enough samples such that there will be at least 7 samples with the same copy number.
- Include a no template control (NTC) reaction in each plate. In place of the DNA sample, use the same diluent used to dilute the DNA.
- Include one or more samples of known copy number as controls (reference or calibrator sample).

Before you begin

- Normalize the gDNA samples: dilute each DNA sample to 5 ng/µL in nuclease-free water or 1X TE buffer.

  Use the `OpenArray Genotyping Calculation Sheet` to calculate the dilutions based on the concentration determined from the RNase P assay.

- Generate the CNV Sample TXT file from the `OpenArray Genotyping Calculation Sheet`.

Set up the PCR reactions

See “Recommended assays, master mix, and instruments” on page 3.

1. Prepare the reagents:
   a. Completely thaw the TaqMan™ Assays, gently vortex, then briefly centrifuge to bring the contents to the bottom of the tube.
   b. For large-scale copy number assays (60X) only, dilute the assay 1:3 (final concentration 20X) with 1X TE, pH 8.0.

      For example, combine 10 µL of 60X assay with 20 µL of TE.
   c. Swirl the master mix to mix contents thoroughly.
2. Prepare the reaction mix: combine the following components, invert or flick the tube to mix, then centrifuge briefly to bring the contents to the bottom of tube.

Component[1]	Volume per 10-µL reaction[2]
TaqPath™ ProAmp™ Master Mix	5.0 µL
TaqMan™ CYP2D6 Copy Number Assay (20X); Hs00010001_cn[3]	0.5 µL
TaqMan™ Copy Number Reference Assay, human, RNase P (20X)	0.5 µL
Nuclease-free water	2.0 µL
Total volume	**8.0 µL**

[1] For other reagent options, see “Recommended assays, master mix, and instruments” on page 3.
[2] Use the `OpenArray Genotyping Calculation Sheet` to calculate volumes for multiple reactions.
[3] Other TaqMan™ Copy Number Assays are available and can be used.

3. Transfer 8 µL of the reaction mix to the sample and control wells of a 384-well reaction plate.
4. Vortex the normalized gDNA samples (5 ng/µL), then add 2 µL of each sample or control to the appropriate wells of the plate, and mix by pipetting up and down several times.
5. Briefly centrifuge the plate to ensure the reaction mixes are at the bottom of each well and to minimize air bubbles.
6. Seal the plate with MicroAmp™ Optical Adhesive Film, ensuring that all 4 edges have a tight seal.

Set up and run the PCR

1. Create an experiment in the instrument software with the following properties.

Property	Setting
Experiment type	**Standard Curve**
Reagents	**TaqMan™ Reagents**
Run speed	**Standard**

2. In the **Define** screen, define targets as indicated:

Target name	Reporter	Quencher
CYPD2D6	FAM™	NFQ-MGB
RNase P	VIC™	TAMRA™

3. In the **Assign** screen, perform the following steps.
   - Import the CNV Sample TXT file that is generated from the `OpenArray Genotyping Calculation Sheet`, or assign sample names to the appropriate wells.
   - Assign both assays to each well.

4. In the **Run Method** screen, perform the following steps.
   - Set the reaction volume at 10 µL.
   - Confirm that the thermal cycling conditions are set up.

Step	Temperature	Time	Cycles
Enzyme activation	95°C	10 minutes	1
Denaturation	95°C	15 seconds	40
Annealing / extension	60°C	60 seconds	

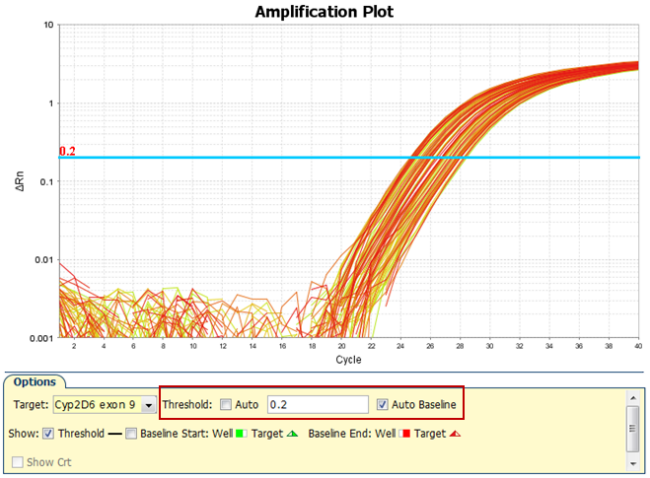
5. Save the experiment, load the sealed plate into the instrument, then start the run.

Analyze the run in the instrument software and export the results

1. In the **Analysis** tab of the experiment, click **Analyze**.

2. In the **Amplification Plot** for each target, deselect **Auto Threshold**, then set the C_t threshold to ***0.2***. Alternatively, use 0.1 as needed to ensure that the threshold is in the middle of the log phase.

   Keep **Auto Baseline** selected.



3. Click **Analyze**, then save the experiment.

4. In the **Export** screen, ensure that the only **Results** is selected.

5. Select a location and file name, select ***.txt** in the **File Type** dropdown list, then click **Start Export**.

Analyze the results in CopyCaller™ Software

The recommended settings in CopyCaller™ Software are described. For other analysis options, see “CopyCaller™ Software analysis options” on page 4.

1. In the software, select **File** ▸ **Import**, select the file that was exported from the instrument software, then click **Open**.

2. In the **Assay Selection Table**, click row(s) to select one or more assays, then click **(Analysis Settings)** .

3. In the **Analysis Settings** window, perform the following tasks.
   - Select **Without calibrator sample**
   - In the **Most Frequent Sample Copy Number** field, enter the expected value.
     The expected value is usually ***2***.

4. Click **Apply**.

   The data are analyzed using the selected analysis settings.

Review the copy number analysis data

1. In the **Assay Selection Table**, select the checkbox to the left of the analyzed assay to display the copy number analysis data.

2. Review the copy number analysis data to confirm that it meets the following criteria:

Criteria	Viewing tool
Samples have comparable VIC™ C_t values	Well and Results tables
Standard deviation is low for replicates (<0.15)	Results table
Calculated copy number values are close to integer values	Results table and Copy Number Plot
Confidence and |Z-score| values are acceptable for the predicted copy number calls	Results table
Copy number of control samples is as expected	Results table and Copy Number Plot
Copy number variation frequency is within the expected range	Results table and Copy Number Plot
Samples cluster into well-defined, well-separated copy number groups	ΔC_t plot

3. Review the quality metrics.

|Z-score|	Status
< 1.75	Pass
2.65 > *z* ≥ 1.75	Pass with caution
≥ 2.65	Fail

For sample copy number calls having confidence values ≥95%, consider passing or failing the call based on the |Z-score| value as shown in the table. |Z-score| is only evaluated for high confidence samples.

Passing the default 95% confidence threshold and |Z-score| of <1.75 is very achievable for good quality samples having 1–3 copies and is more difficult to achieve for lower quality samples carrying duplications or for higher copy samples.

Consider passing samples with calls of ≥3 copies that fall below the 95% confidence level if:

- The calculated copy numbers are close to integer values.
- The samples cluster with passing samples of the same copy number group in the ΔC_t plot.

Supplemental information

Recommended assays, master mix, and instruments

Item	Description
CYP2D6 assay	
Required: CYP2D6 exon 9 (Hs00010001_cn)	Cat. Nos. 4400291, 4400292, 4400293
Optional: CYP2D6 intron 2 (Hs04083572_cn) CYP2D6 intron 6 (Hs04502391_cn)	Use these assays in addition to the CYP2D6 exon 9 assay when detection of both full length CYP2D6 and hybrid alleles is desired. For more information, see *Pharmacogenomics Experiments Application Guide* (Pub. No. MAN0009612). **IMPORTANT!** Always use the CYP2D6 exon 9 copy number assay to detect true gene duplication events. CYP2D6 intron 2 or intron 6 copy number assays should not be used alone to detect CYP2D6 duplications as they will also detect nonfunctional hybrid alleles that do not represent duplications and that do not contribute to CYP2D6 metabolizer status.
Reference assay	
Recommended: TaqMan™ Copy Number Reference Assay, human, RNase P	Cat. Nos. 4403326 and 4403328
Alternative: TaqMan™ Copy Number Reference Assay, human, TERT	Cat. Nos. 4403316 and 4403315 Recommended if the RNase P assay functions poorly due to sample chromosomal aberrations or other issues.

Item	Description
Master mix	
Recommended: TaqPath™ ProAmp™ Master Mix	Cat. No. A30865 Recommended for optimal performance.
Alternative	If samples contain PCR inhibitors that result in poor quality or incorrect copy number calls, inhibitor-tolerant master mixes may perform better. Contact your local Field Application Scientist or call Technical Support to discuss alternative master mix options.
Compatible instruments	
QuantStudio™ 12K Flex Real–Time PCR System QuantStudio™ 7 Flex Real-Time PCR System QuantStudio™ 7 Pro Real-Time PCR System QuantStudio™ 5 Real-Time PCR System ViiA™ 7 Real-Time PCR System	Copy number variation experiments can be performed on any of these Applied Biosystems™ instruments.

CopyCaller™ Software analysis options

Analysis method	Description	How to select
Recommended: Median C_t	Copy number results are based on the copy number of the sample with the median C_t or median C_t value. This method works well when most samples have the same copy number.	1. Select **With Calibrator Sample**. 2. In the **Calibrator Sample Name** drop-down list, select **Median C_t**.
Most frequent copy number	Copy number results are based on the assumption that most samples have the same copy number.	1. Select **Without Calibrator Sample**. 2. In the **Most Frequent Sample Copy Number** field, enter the expected value (Usually 2. Enter 1 for GSTM1 & GSTT1).
Calibrator	Copy number results are based on the copy number designated for one calibrator sample. Include a calibrator sample on each plate. **IMPORTANT!** The calibrator should be of the same sample type as the test samples.	1. Select **With Calibrator Sample**. 2. In the **Calibrator Sample Name** drop-down list, select or enter a sample to use as the calibrator. 3. (*Optional*) Change Calibrator Sample Copy Number (default = 2).

Related documentation

Document	Pub. No.
Pharmacogenomics Experiments Application Guide	MAN0009612
TaqMan™ Copy Number Assays User Guide	4397425
CopyCaller™ Software v2.0 User Guide	4400042

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision history: Pub. No. MAN0014350

Revision	Date	Description
B.0	20 April 2022	• The total volume for the diluted gDNA was removed. The gDNA can be diluted into any volume ("Before you begin" on page 1). • The master mix was changed to TaqPath™ ProAmp™ Master Mix. • Information was added to specify that TaqMan™ Copy Number Assays other than Hs00010001_cn are available and can be used ("Set up the PCR reactions" on page 1). • When analyzing the run in the instrument software, the threshold can be set to 0.1 in order to ensure that the threshold is in the middle of the log phase. • The recommended analysis settings in the CopyCaller™ Software were updated to not include a calibrator sample ("Analyze the results in CopyCaller™ Software" on page 2). • The list of compatible real-time PCR instruments was updated.
A.0	23 October 2015	New document.