

## ZYGOSITY AND EXPRESSION TESTING

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Charles River Laboratories, the industry leader in DNA-based services for the genetic monitoring of transgenic and knockout rodents, offers quantitative molecular-based services to aid in the characterization of genetic mutant models: zygosity, expression testing, and transgene copy number determination. Zygosity testing allows us to go beyond carrier status determination to discover the true transgenic nature of your test animal (i.e., wild-type, hetero/hemi, or homozygote). Zygosity information is imperative for effective breeding and colony maintenance and zygosity has been shown to correlate with gene expression levels. Determination of transgene copy number in several founder lines is also important in characterizing the model. Expression testing measures the *in vivo* transcriptional activity of your transgene, knockout, or knockdown. Quantitative assessments of transgene expression ultimately determine the relative worth of each founder in a transgenic line. Each test utilizes quantitative Polymerase Chain Reaction (PCR) combined with fluorogenic probe technology. The addition of a fluorogenic probe allows for real-time monitoring within the exponential phase of the PCR, improving assay precision and reproducibility.

### PCR – An Overview

In a typical PCR, two primers are designed to bind in opposite directions to complementary strands of target DNA. The sequence between the two primer-binding sites is amplified exponentially with each PCR cycle, which consists of three steps. In the first step, nucleic acid isolated from the clinical specimen is denatured at a high temperature (e.g., 95°C). In the second step, primers anneal to their complementary amplification target sequences at a lower temperature (e.g., 55°C). In the final step, the reaction tube is heated to temperatures (e.g., 72°C) optimal for DNA polymerases, which synthesize copies of the target sequences by extending the primers. A PCR assay consists of 30-50 cycles, performed automatically by a programmable heating block called a thermocycler.

### Fluorescence-Based PCR Detection

Charles River offers a state-of-the-art diagnostic PCR assay utilizing fluorescent probe technology. This system combines the sensitivity of PCR with the specificity of probe hybridization. An oligonucleotide probe, labeled with both a reporter and quencher dye, anneals specifically to template DNA between the forward and reverse PCR primers. During the extension phase of the PCR cycle, the annealed probe is separated from the quencher dye generating a sequence-specific signal. Post-PCR processing is performed without opening the reaction tube, eliminating the release of contaminating PCR amplicons into the test environment. The probe adds a second level of specificity to the assay, reducing the likelihood of false readings based on non-specific PCR products.

### Quantitative PCR

The key to applying this technology to zygosity testing (discrimination between two-fold differences in gene copy number) and expression testing (measurement of mRNA production) is to make it quantitative. By allowing for continuous product analysis rather than a static measurement at the end of a program (real time vs. end point reading), we compare relative differences between amounts of starting material, either DNA or mRNA. By looking at cycle thresholds ( $C_t$ ), the cycle number at which probe fluorescence is measured above background, we differentiate gene copy number and accurately quantify transgene expression, relative to a known standard. This technology allows us to determine both the zygosity and transgenic-expression levels of your genetic mutant models.

### The $\Delta\Delta C_t$ Method for Determining Relative Nucleic Acid Quantities

We use the ABI 7900HT to perform all of our quantitative PCR assays and employ the  $\Delta\Delta C_t$  method to determine relative quantities of genes (copy number, zygosity) or mRNA (transgene expression). The following example utilizes the  $\Delta\Delta C_t$  method to determine the



zygosity of transgenic animals. The QPCR assay is based upon comparing the  $C_t$  values of control DNA from animals of known zygosity to the gene in question. In addition, a single copy endogenous gene is used to normalize template DNA concentration differences between samples. By comparing the  $C_t$  values of the unknowns to those of the control and endogenous sample wells, we determine if the animal carries no gene copies (wild-type), one gene copy (hemizygote) or two gene copies (homozygote). In brief, the more copies of the gene, the more quickly (fewer PCR cycles) the  $C_t$  will be reached.

Actual data from the analysis of customer samples is shown in Table 1. Real-time reactions are run comparing  $C_t$  values of an animal known to be hemizygous for the transgene (X) to those of an endogenous dual copy gene (endo). These numbers are used to calibrate the system. The average  $C_t$  value for the endogenous gene is 25.09 while the average  $C_t$  value for our hemizygous animal is 23.52. The difference between these two numbers, referred to as the  $\Delta C_t$  is -1.57 and will be used later in the calculations (Table 1).

Our real-time PCRs are run in triplicate to generate an average  $C_t$  value for each sample. These are run alongside the endogenous gene reactions for each sample, also performed in triplicate. The difference between the two averages is calculated ( $\Delta C_t$  in Table 1), and the calibrator  $\Delta C_t$  is subtracted from each experimental  $\Delta C_t$ . This value, called  $\Delta\Delta C_t$ , is then used to calculate a value indicative of transgene zygosity ( $2^{-\Delta\Delta C_t}$ ).

Sample ID	Average $C_t$ X	Average $C_t$ endo	$\Delta C_t$	$\Delta\Delta C_t$
<b>Calibrator</b>	<b>23.52</b>	<b>25.09</b>	<b>-1.57</b>	<b>0</b>
1	38.27	24.21	14.06	15.63
2	23.29	24.29	-1	0.57
3	22.15	24.37	-2.22	-0.65

Table 1. Calculation of the  $\Delta\Delta C_t$

Sample ID	$2^{-\Delta\Delta C_t}$	Genotype
1	0	WT
2	0.67	HEMI
3	1.57	HOMO

Table 2. Zygosity Determination

In this mutant model set, the zygosity ranges were as follows: wild-type value of 0, hemizygotes value of X, and homozygotes roughly 2X. The absolute values are not important. In fact, actual values will vary between different transgene assays, but the relative difference (2X) between hemizygotes and homozygotes will be the same (Table 2). A similar method is used to calculate expression level and the transgene copy number.

Although parameters differ for every genetic mutant model, basic trends hold true allowing us to custom design zygosity, expression, and transgene copy number assays for your unique model.

### Test Results and Sample Submission

Results are typically reported about two weeks after assay development. Tail snips for zygosity testing should be collected and immersed in 70% ethanol and refrigerated (4°C) prior to shipment. Please ship overnight on ice packs. Expression Testing may require other types of tissue samples as determined by the investigator. Please contact Charles River regarding sample type and collection conditions prior to shipping samples.

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