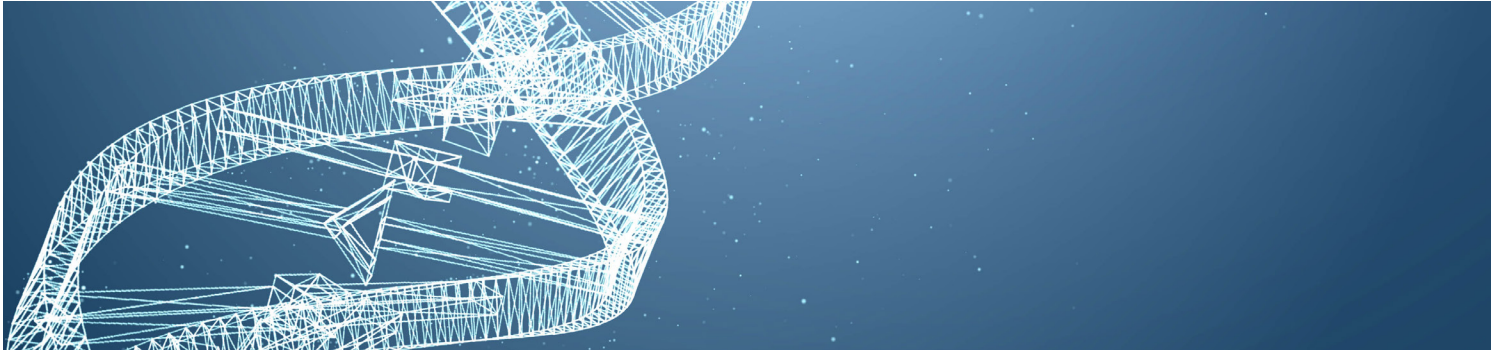


# Calculating Threshold Values

Authors: Kevin O'Brien MS, Ph.D., and Benjamin A Katchman, Ph.D.

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## Keywords:

- DNA
- Thresholding

## INTRODUCTION

The PathogenDx analysis software (“Augury”) determines whether any given probe is ON or OFF by simple thresholding. If the relative fluorescent units (RFU) value of the probe is measured above a pre-set threshold, that probe is said to be ON. If it is below the threshold, the probe is said to be OFF. Every probe has a different hybridization affinity; therefore, the chosen threshold will vary from probe to probe. As this relates to the PathogenDx assays:

- **Detect<sup>X™</sup>** assay utilize a threshold RFU cutoff to determine if an organism is present or absent down to 1 colony forming unit per gram (CFU/g). The cutoff value for the Detect<sup>X</sup> assay was determined by screening *E. coli* 0157:H7, *S. enterica*, *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* at the 1 CFU/g level to determine the median values and standard deviation of the assay (refer to Detect<sup>X</sup> Validation Report).
- **Enviro<sup>X™</sup>** assay utilizes a threshold RFU cutoff to determine if an organism is present or absent. The Enviro<sup>X</sup> assay is designated as a screening tool that contains approximately 50 different organisms. The lower limit of detection for each organism varies depending on primer and probe efficiency.
- **Quant<sup>X™</sup>** assay is designed as a quantitative assay with a narrower dynamic range in order to provide CFU/g calculations between 10<sup>2</sup> and 10<sup>6</sup> CFU/g. Similar principles are utilized in this assay as the threshold cutoff values in terms of calculating the RFU values. Further calculations are made when converting the RFU values to CFU/g values (refer to Quant<sup>X</sup> Validation Report).

## METHODS

Thresholds are calculated based on the distribution of RFU values measured when the probe is known to be OFF. The first step is to mathematically model the measured fluorescent distribution of the probes. In our case we have chosen to model the distribution as an exponential decay. Figure 1 shows the measured distribution for a *Golovinomyces* probe (blue line) and is typical of the OFF distributions for our probes. The red line represents an exponential decay model. Also shown is the normal distribution (grey line) in order to clarify why we chose to use the exponential decay model as the mathematical model.

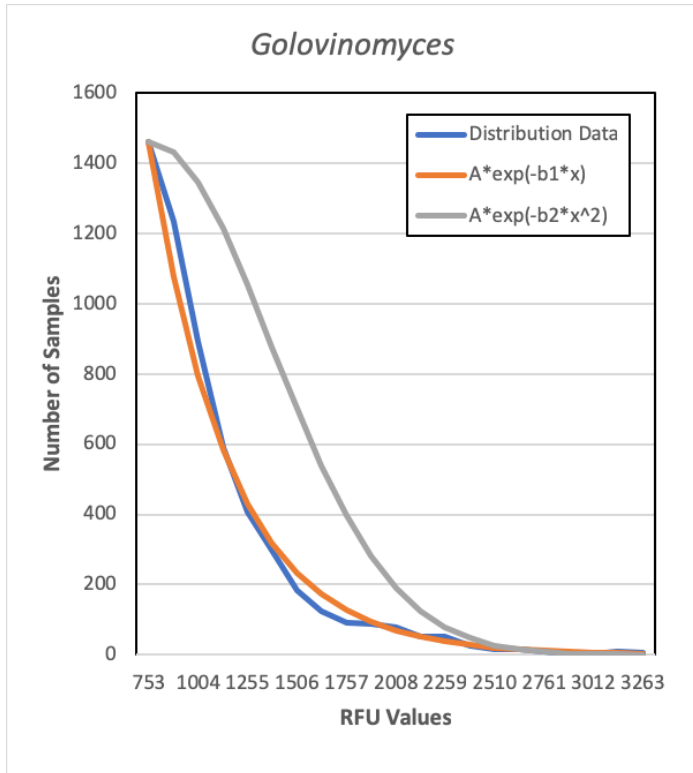


Figure 1. Sample Distribution of probe *Golovinomyces*.

The final step is to decide a reasonable choice of false positive rate and then simply solve for  $\lambda$ . As an example, if the decay rate for the probe was measured to be 0.005, the coefficient is 1000, and the false positive rate was set to 1%, we get a threshold of

$$\lambda = \frac{-\ln(0.01 \cdot 0.005 / 1000)}{0.005} \approx 3362$$

Note that a probe with a lower decay rate would have a higher threshold, while increasing the acceptable false positive rate would have the effect of driving the threshold lower.

## CONCLUSION

The method described above is utilized to determine the RFU threshold values for all PathogenDx assays. The mathematical modeling is built into the Augury software and calculations are automatically performed within the program. We have validated this software internally and externally with thousands of samples and over a dozen independent users for confirmation. Please refer to the Detect<sup>x</sup> and/or Quant<sup>x</sup> validation report when comparing a measured value to the threshold calculation.

In this particular example,  $A=1460$ ,  $b1=0.002432$ , and  $b2=1.29e-6$ . We will refer  $A$  as the coefficient and  $b1$  as the decay constant in this discussion.

Since this is a distribution plot, the probability of a probe having an RFU value larger than  $\lambda$  is the same as the area under the curve that lies above  $x = \lambda$ .

The area under the entire curve, from the origin out to infinity, is given by

$$A \int_0^{\infty} e^{-bx} dx = \frac{A}{b} \tag{Equation 1}$$

While the area from the origin to  $\lambda$  is given by

$$A \int_0^{\lambda} e^{-bx} dx = \frac{A}{b} [1 - e^{-b\lambda}] \tag{Equation 2}$$

Therefore the area under the curve where  $x \geq \lambda$  is simply

$$P(\lambda) = \frac{A}{b} e^{-b\lambda} \tag{Equation 3}$$