

The program QUALITY, and the method on which it is based, is described in detail in Rodrigo et al. (2). The method is a variant of the minimum variance (MC) method described by Taswell (3) for limiting dilution assays, and for which he has demonstrated by simulation desirable properties of minimum variance (i.e., high precision) and minimum bias. Our method modifies the MC method to allow the user to specify the probabilities of a false negative and a false positive PCR.

The numerical method

The MC method, as described by Taswell (3) and as it applies to PLDAs, begins with an estimation of the probabilities of obtaining a negative or positive PCR at each dilution, but assumes that these probabilities are solely due to the absence or presence of a target molecule. In a given homogeneous volume of sample we assume that the probability distribution of the number of molecules in each replicate PCR is Poisson with parameter $\lambda_i = cd_i$ where c is the number of copies per unit volume of sample and d_i is the inverse of the dilution factor of the i^{th} dilution, or the quantity of sample used in the i^{th} dilution. The probability, then, that no target molecules are available in any one replicate at the i^{th} dilution is given by:

$$P_i(0) = e^{-\lambda_i} \quad (1)$$

The probability that at least one target molecule is present in the PCR is therefore

$$P_i(> 0) = 1 - e^{-\lambda_i} \quad (2)$$

In our modification of Taswell's method, a negative PCR can be the result of two possibilities: 1) the absence of a target molecule, after correcting for the possibility of a false positive PCR; and 2) a false negative PCR, given that a target molecule was indeed present in the PCR. The probability of a false positive PCR is the conditional probability of obtaining a positive result given that a target molecule was absent from the reaction; conversely, the probability of a false negative PCR is the conditional probability of obtaining a negative result given that a target molecule was present in the reaction. The probability of a negative PCR at the i^{th} dilution, $P_i(-)$, then is given by:

$$P_i(-) = P_i(0)(1 - P_f(+)) + P_i(> 0)P_f(-) \quad (3)$$

where $P_i(+)$ and $P_i(-)$ are the probabilities of false positive and false negative results respectively. As can be seen from (2), if $P_i(+)$ and $P_i(-)$ are zero, then the probability of a negative result is solely determined by the absence of a target molecule. The probability of a positive PCR is $P_i(+)=1-P_i(-)$.

If n_i replicate PCR amplifications are performed at the i^{th} dilution, then the distribution of the number of negative reactions is binomial with mean $n_i P_i(-)$ and variance $n_i P_i(-) P_i(+)$. Similarly, the expected number of positive reactions is $n_i P_i(+)$ and variance $n_i P_i(-) P_i(+)$. A χ^2 statistic (with $k-1$ degrees of freedom), as a measure of the agreement between observed and expected numbers of positive and negative PCR amplifications, can therefore be calculated as:

$$\chi^2_{k-1} = \sum_i \left[\frac{(r_i - n_i P_i(-))^2}{n_i P_i(-)} + \frac{((n_i - r_i) - n_i P_i(+))^2}{n_i P_i(+)} \right] \quad (4)$$

where r_i is the number of negative PCR amplifications at the i^{th} dilution, and k is the number of dilutions. Equation (4) reduces to:

$$\chi^2_{k-1} = \sum_i \left[\frac{(r_i - n_i P_i(-))^2}{n_i P_i(-) P_i(+)} \right] \quad (5)$$

The number of copies per unit volume of sample is estimated to be the value of c that minimizes the value of χ^2 . The standard error of the number of copies is estimated as:

$$SE(c) = \sqrt{\frac{2}{F''(c)}} \quad (6)$$

where $F''(c)$ is the second derivative of χ^2 at c .

The computer program

The computer program, QUALITY, has been written in C++. The program runs on Unix and MacOS platforms. A Macintosh binary file and ANSI-compatible sources are available by anonymous ftp from *ftp://ubik.microbiol.washington.edu/download/cbu/quality/* on the Internet.

In the program, Newton's method (1) for finding the root of an equation is used to find the value of c that minimizes Equation (5). The function in Equation (5) has one global minimum, so we may apply Newton's method to find the value of c at which the first derivative of χ^2 equals zero. To obtain a final estimate of c , the following equation is computed iteratively:

$$c_{n+1} = c_n - \frac{F'(c_n)}{F''(c_n)} \quad (7)$$

where c_n is the estimate of copy number at the n^{th} iteration, and $F'(c_n)$ is the first derivative of χ^2 at c_n . The values of these derivatives were estimated numerically by calculating the gradient of the function in a small neighborhood of c_n . The convergence criterion, $|c_{n+1} - c_n| < 0.0001$ is used as a stopping rule. The starting estimate of c_0 is $s_o/(d_o p_o)$ where s_o is the number of positive PCR amplifications at the last serial dilution at which a positive PCR is recorded, d_o is the dilution factor at this dilution, and p_o is the number of PCR amplifications performed at this dilution (Note: this starting estimate simply inverts the dilution at the last set of PCR amplifications for which a positive result has been observed, and multiplies by the proportion of positive reactions. Some researchers use this formula as a simple estimate of copy number). Note that for some input values, the χ^2 function may have both a minimum and a maximum, and for those values we have examined, the maximum is found at a higher copy number than the minimum. To ensure that we find the copy number when the function is at a minimum, we check the value of the second differential at the final estimate of c . If the second differential is negative, the χ^2 function is at a maximum, and the value of c obtained is divided by 10 (an arbitrarily-defined factor). This new value of c is used as a starting value for a new estimation process.

The program also estimates the standard error of the estimate of copy numbers according to the formula in Equation (6), as well as the p-value associated with obtaining a χ^2 -statistic as large or larger than the estimated value. Strictly, this p-value represents the

conditional probability of obtaining the numbers of positive and negative PCR amplifications, given that the estimated number of copies is in fact correct and the model is correctly specified. We expect this p-value to be conservative because our null estimate of copy number is based on a value that minimizes the χ^2 . It is not, therefore, equivalent to the p-values typically obtained in hypothesis tests in which null values are specified a priori. Nonetheless, a small p-value (e.g., < 0.05) may indicate that the distribution of molecules amongst replicate PCR amplifications is not appropriately described by the model specified above; this could occur if the original sample was not sufficiently homogeneous, or if the aliquots varied slightly in volume, or indeed, if the experimental conditions varied from PCR to PCR. If a p-value < 0.05 is detected, researchers are advised to examine their experimental conditions and/or redo the LDA.

Finally, the program performs a sensitivity analysis to illustrate the effect, at each dilution, of having one more or one less positive PCR than observed. We believe that this particular feature is useful as an exploratory tool: users are able to determine how sensitive their experimental design (in particular, the numbers of replicates at a particular dilution, and the dilution factors used) is to slight differences in the number of positive or negative PCR amplifications recorded before beginning a PLDA.

1. **Atkinson, K.** 1985. Elementary Numerical Analysis. John Wiley & Sons, New York.
2. **Rodrigo, A. G., P. C. Goracke, K. Rowhanian, and J. I. Mullins.** 1997. Quantitation of target molecules from PCR-based limiting dilution assays. AIDS Res. Human Retrovir. **13**:737-742.
3. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. J. Immunol. **126**:1614-1619.