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Graphical Representation of a Generalized Linear Model-Based Statistical Test Estimating the Fit of the Single-Hit Poisson Model to Limiting Dilution Assays

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Standardized statistical and graphical methods for analysis of limiting dilution assays are highly desirable to enable investigators to compare and interpret results and conclusions with greater accuracy and precision. According to these requirements, we present in this work a powerful statistical slope test that estimates the fit of the single-hit Poisson model to limiting dilution experiments. This method is readily amenable to a graphical representation. This slope test is obtained by modeling limiting dilution data according to a linear log-log regression model, which is a generalized linear model specially designed for modeling binary data. The result of the statistical slope test can then be graphed to visualize whether the data are compatible or not with the single-hit Poisson model. We demonstrate this statistical test and its graphical representation by using two examples: a real limiting dilution experiment evaluating the growth frequency of IL-2-responsive tumor-infiltrating T cells in a malignant lymph node involved by a B cell non-Hodgkin's lymphoma, and a simulation of a limiting dilution assay corresponding to a theoretical non-single-hit Poisson model, suppressor two-target Poisson model. ***The Journal of Immunology*****, 2001, 167: 5725–5730.**

Limiting dilution analysis has gained widespread acceptance as a tool for quantifying the frequency of cells in the immune system that possess various functional activities (1). To analyze the data, it is usually assumed that only one limiting cell of only one cell subset is necessary and sufficient for generating a positive response (the single-hit Poisson model (SHPM)[2]. However, the immune system is a complex network of cellular and humoral (cytokines) interactions. It is thus not unreasonable to consider that experimental data do not adhere to all-or-none functionality and will yield nonlinear titration curves (1, 2). In the literature, deviation from single-hit kinetics is usually estimated by a standard χ^2 test. Previously, we demonstrated that the standard χ^2 test was insufficient for estimating the goodness-of-fit to the SHPM due to its intrinsic lack of power (3). Subsequently, we presented evidence that modeling limiting dilution assays by a log-log generalized linear model results in a statistical slope test that is better able to account for deviation from linearity than the standard χ^2 test (4). In the present paper we show that, in addition to its ability to accurately detect departure of the experimental data from the SHPM, this slope test is readily amenable to a graphical representation.

In recent years, the continuing development of more complex models giving mathematical representation of biological data, in parallel with the tremendous improvements in computing power, have led to the emergence of even more complex statistical methodologies. In this context, generalized linear theory-based statistical tests represent a major advance in evaluating the fit of the SHPM to limiting dilution analysis. However, new methods are rarely used in practice, and a major challenge for research statisticians is to explain to immunologists the benefit that they can draw from these new methods. In line with this problem, it must be emphasized that graphing can make the result of a statistical investigation clearer to the reader and more attractive than if the result has been presented merely in a table. In this way, we present an understandable graphical illustration of a generalized linear model-based statistical slope test with the aim of encouraging investigators to use a new statistical method for their limiting dilution data.

The generalized linear model-based statistical procedure, accompanied by its graphical representation, is illustrated in this paper with two examples. The first set of data is derived from a real experiment assessing the growth frequency of IL-2-responsive tumor-infiltrating lymphocyte T (TIL-T) in a malignant lymph node involved by a low-grade B cell non-Hodgkin's lymphoma. The second set of data is artificially generated and corresponds to a theoretical non-SHPM, suppressor two-target Poisson model (STTPM). Specifically, in this second example, the total cell population contains a suppressor cell subset exerting a suppressor activity on the growth of a second cell subset (3). A possible physiologic illustration of this situation is given by the functional relationship which has been described between $CD4^+$ virgin and memory T cells, where memory cells produce IL-10, which in return inhibits the growth of virgin cells (2, 5).

Materials and Methods

Abbreviations used in the text, f, k, i, n, x, r, m, and μ, are defined as follows: f is the estimate of the cell frequency according to the SHPM hypothesis; k is the number of groups of replicate wells, each group labeled i, with i = 1, 2, 3, . . . , k. At each i, n_i is the number of replicate wells, x_i is the number of cells plated in each replicate well, r_i is the number of observed negative wells, m_i is the observed fraction of negative wells, $m_i = r_i/n_i$, μ_i is the theoretical fraction of negative wells.

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[2] Abbreviations used in this paper: SHPM, single-hit Poisson model; TIL-T, tumor-infiltrating lymphocyte T; STTPM, suppressor two-target Poisson model; IRLS, iteratively reweighted least-squares.

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Generalized linear model fitting the SHPM

The first step consists of modeling limiting dilution data according to a generalized linear log-log model fitting the SHPM and checking this model by an appropriate slope test (4). According to the zero term of the Poisson distribution, the theoretical fraction μ_i of negative wells is given by $\mu_i = \exp(-fx_i)$.

This equation can be written according to a log-log linear model as $\log(-\log(\mu_i)) = \log(f) + \log(x_i)$. This equation is now in the form $Y_i = \alpha + \beta X_i$ with $Y_i = \log(-\log(\mu_i))$, $\alpha = \log(f)$, $\beta = 1$, and $X_i = \log(x_i)$.

A test of deviation from the SHPM (model checking) is a test of whether the estimate of the slope β is significantly different from 1. The appropriate statistical test z uses the normal deviates under the null hypothesis that $\beta = 1$.

$$z = \frac{\beta - 1}{\sqrt{\text{var}(\beta)}} \tag{1}$$

The hypothesis that the slope β is compatible with 1 at the 95% confidence level can also be expressed as

$$-1.96 < \frac{\beta - 1}{SE(\beta)} < +1.96$$

This statement can be rewritten as $\beta - 1.96\ SE\ (\beta) < 1 < \beta + 1.96\ SE\ (\beta)$ showing that the value, 1, is included in the 95% confidence interval of the slope β if the SHPM hypothesis holds.

Maximum likelihood estimates of the parameters α and β were obtained with the Fisher's method of scoring according to an iteratively reweighted least-squares (IRLS) procedure written for binary data (4, 6, 7). Maximum likelihood estimates satisfy the equation $\mathbf{X^T W X \beta = X^T W Z}$ with $\mathbf{X}$, the matrix with elements $X_i = \log(x_i)$; $\mathbf{W}$, the diagonal matrix of weights with elements

$$W_i = 1 \Big/ \left(\frac{dY_i}{d\mu_i}\right)^2 V_i$$

where V_i is the variance of μ_i

$$V_i = \frac{\mu_i(1 - \mu_i)}{n_i}$$

and thus

$$W_i = n_i \frac{\mu_i(\log(\mu_i))^2}{1 - \mu_i} \tag{2}$$

$\mathbf{Z}$ is the matrix of dependent variables with elements

$$Z_i = Y_i + (\mu_i - m_i)\left(\frac{dY_i}{d\mu_i}\right)$$

and thus

$$Z_i = \alpha + \beta X_i + \frac{m_i - \mu_i}{\mu_i \log(\mu_i)} \tag{3}$$

$\boldsymbol{\beta}$ is the matrix of the coefficients α and β. The revised estimate $\boldsymbol{\beta^{(m)}}$ solved iteratively by IRLS is $\boldsymbol{\beta^{(m)} = (X^T W X)^{-1} X^T W Z}$ where m is the m^{th} iteration and $\boldsymbol{\beta^{(m)}}$ is the matrix of the coefficients $\alpha^{(m)}$and $\beta^{(m)}$. The elements α and β of the matrix $\boldsymbol{\beta^{(m)}}$ are given by

$$\alpha = \frac{\left(\sum_{i=1}^{k} n_i W_i Z_i\right)\left(\sum_{i=1}^{k} n_i W_i X_i^2\right) - \left(\sum_{i=1}^{k} n_i W_i X_i\right)\left(\sum_{i=1}^{k} n_i W_i Z_i X_i\right)}{\left(\sum_{i=1}^{k} n_i W_i\right)^2\left(\sum_{i=1}^{k} n_i W_i X_i^2\right) - \left(\sum_{i=1}^{k} n_i W_i X_i\right)^2} \tag{4}$$

$$\beta = \frac{\left(\sum_{i=1}^{k} n_i W_i Z_i\right)\left(\sum_{i=1}^{k} n_i W_i X_i\right) - \left(\sum_{i=1}^{k} n_i W_i\right)\left(\sum_{i=1}^{k} n_i W_i Z_i X_i\right)}{\left(\sum_{i=1}^{k} n_i W_i X_i\right)^2 - \left(\sum_{i=1}^{k} n_i W_i X_i^2\right)\left(\sum_{i=1}^{k} n_i W_i\right)} \tag{5}$$

The estimates of the variances for α and β, var(α) and var(β), are given by the diagonal elements of the variance-covariance matrix $\mathbf{(X^T W X)^{-1}}$ evaluated with $\alpha^{(m)}$ $\beta^{(m)}$. The variance of β is given by

$$\text{var}(\beta) = \frac{\left(\sum_{i=1}^{k} n_i W_i\right)}{\left(\sum_{i=1}^{k} n_i W_i\right)\left(\sum_{i=1}^{k} n_i W_i X_i^2\right) - \left(\sum_{i=1}^{k} n_i W_i X_i\right)^2} \tag{6}$$

The 95% confidence interval for β was calculated as

$$95\%CI(\beta) = \beta \pm 1.96 SE(\beta). \tag{7}$$

The main steps of the IRLS algorithm used for calculations of α, β, and var(β) are given in the *Appendix*.

Computation of the cell subset frequency according to the SHPM

The second step consists in computation of the cell subset frequency according to the SHPM by maximum likelihood estimation. Let f be the estimate of the cell frequency; the maximum likelihood of f is the value of f that maximizes

$$\log(L) = \sum_{i=1}^{k}\left[\log\left(\frac{n_i!}{r_i!(n_i! - r_i!)}\right) + r_i \log(P_i) + (n_i - r_i)\log(1 - P_i)\right]$$

where $\log(L)$ is the natural logarithm of the likelihood function L (8), and P_i is given by $P_i = \exp(-fx_i)$ according to the SHPM.

The variance of f was calculated as the negative reciprocal of the second derivative of $\log(L)$ (8).

$$\text{var}(f) = \frac{-1}{d^2 \log(L)/df^2}$$

The 95% confidence interval for f was calculated as 95% $CI(f) = f \pm 1.96$ SE (f).

Derivation of the STPPM

For simulation experiments, it was assumed that the total $CD4^+$ T cell population contains suppressor cells with frequency ϕ, demonstrating a growth suppressor activity, for instance, by the production of IL-10, on another population of $CD4^+$ T cells capable to growth with frequency γ (3). According to the Poisson law, the fraction of wells not containing

Table I. *Data of limiting dilution assays*[a]

Cell Population or Model	Number of Replicate Wells	Number of T Cells per Well	Number of Negative Wells
TIL-T lymphocytes in B-NHL	96	5	78
	96	10	71
	96	20	44
	96	50	17
	96	100	5
Suppressor two-target Poisson model	100	0.1	96
	100	0.5	83
	100	1.5	60
	100	2	53
	100	3	45
	100	3.5	43
	100	4	42

[a] The first set of data represents a limiting dilution culture aimed at estimating the frequency of IL-2-responsive TIL-T in one case of low-grade, splenic marginal zone lymphoma. The method has been previously described (9). Briefly, total spleen cells were separated into purified TIL-T and purified malignant B cells by using an immunomagnetic procedure. Malignant B cells were used as feeder cells with 50×10^4 cells per well. Cultures were performed in 96-well round bottom plates in culture medium containing 10 ng/ml rIL-2, and fed weekly with culture medium, IL-2 and malignant B cells. After 4 wk of culture, each well was scored microscopically for growth. The second set of data is artificially generated from a theoretical suppressor two-target Poisson example modeling the ability of suppressor $CD4^+$ T cells to inhibit the growth of another subpopulation of $CD4^+$ T cells, for instance by the production of IL-10.

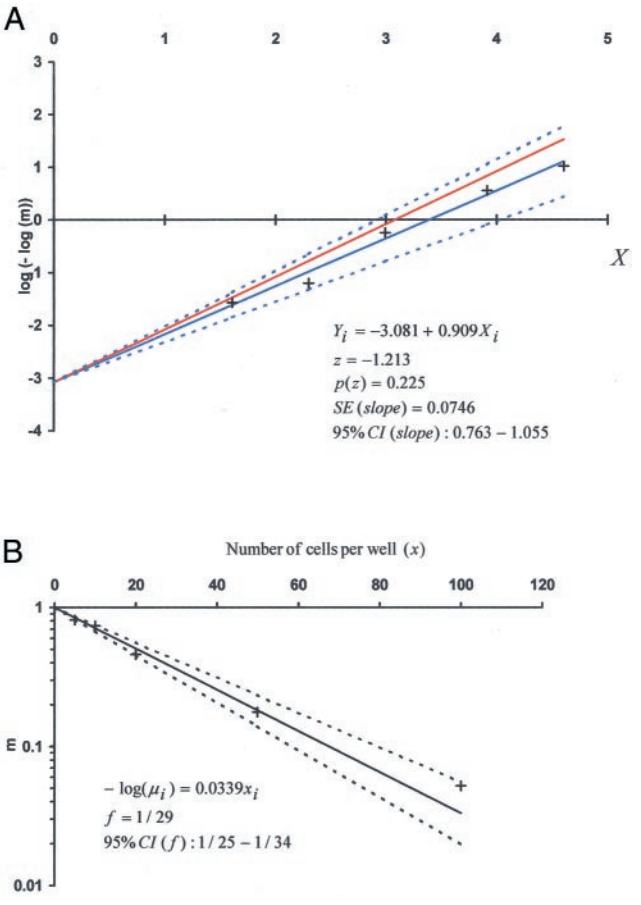

FIGURE 1. *A* and *B*, Graphical displays of limiting dilution data in a real experiment estimating the growth frequency of IL-2-responsive TIL-T in one case of B cell lymphoma. *A*, Graphical representation of the slope test *z* evaluating the adequacy of the SHPM to limiting dilution data. The log-log transform of the fraction of negative wells is plotted against the logarithm of the number of cells per well. The blue plain line represents the log-log regression line fitted to the experimental data according to the generalized linear log-log model ($Y_i = -3.081 + 0.909X_i$). The blue dotted lines represent regression lines with the same intercept α as the fitted log-log regression line, but with slope β corresponding to the lower and upper values of the 95% confidence interval for β ($Y_i = -3.081 + 0.763X_i$; $Y_i = -3.081 + 1.055X_i$). The red line represents the theoretical SHPM regression line with the same intercept α, but with slope β equal to 1 ($Y_i = -3.081 + X_i$). *B*, Plot of the fraction of negative wells as a function of the number of cells per well. The prediction equation of the regression line (plain line) is $-\log(\mu_i) = 0.0339x_i$, where 0.0339 is the cell frequency *f* estimated by the maximum likelihood method according to the SHPM hypothesis. *Upper* and *lower dotted lines* are plotted by using upper and lower values of the 95% confidence interval of *f*. The boundary equations are $-\log(\mu_i) = 0.0289x_i$ (lower) and $-\log(\mu_i) = 0.0391x_i$ (upper).

suppressor cells, but containing at least one proliferating T-lymphocyte precursor cell, is $\exp(-\phi x_i)(1 - \exp(-\gamma x_i))$. Thus, the theoretical fraction μ_i of negative wells is $\mu_i = 1 - (\exp(-\phi x_i)(1 - \exp(-\gamma x_i)))$. Calculations were made with $\phi = 0.08$ and $\gamma = 0.4$. The theoretical number of negative wells was equal to 100 μ_i (100 replicate wells for each cell dose; $n_i = 100$), and the experimental number of negative wells, r_i, was taken at the nearest integer.

Results and Discussion

Table I presents the experimental and simulated limiting dilution data. Table II indicates the results of statistical analysis of the data and precursor frequency. In the real experiment conducted with TIL-T in one case of B cell lymphoma (9), the slope test *z* derived from the generalized linear log-log model did not reject the SHPM, and it was concluded that the SHPM adequately fits the observed data. In contrast, in the simulated experiment, conducted according to the STTPM, the statistics *z* clearly rejected the hypothesis that the data conformed to single-hit kinetics. This reflects the fact that the slope β was not compatible with 1 (95% confidence interval for the slope β ranged from 0.598 to 0.936).

Fig. 1*A* and Fig. 2 are graphical representations of the slope test *z* for both examples. This graphical representation is designed to allow visualization of whether the slope β of the log-log regression line fitted to the experimental data is compatible or not with 1 (SHPM adequacy or inadequacy). The graph is constructed in the following way. The log-log transform of the proportion of negative wells is plotted against the logarithm of the number of T cells per well. First, the observed data points (X_i, $\log(-\log(m_i))$ are plotted. Then the log-log regression line fitted to the experimental data (regression line with equation $Y_i = \alpha + \beta X_i$; Fig. 1*A* and Fig. 2, blue plain line) is plotted. Next are plotted two regression lines with the same intercept α as the log-log regression line, but with slope β equal to either the upper or the lower value of the 95% confidence interval for β (boundary regression lines, $Y_i = \alpha + \beta_{upper}X_i$, $Y_i = \alpha + \beta_{lower}X_i$; Fig. 1*A* and Fig. 2, blue dotted lines). Finally, a theoretical, so-called SHPM regression line, with the same intercept α, but with slope β equal to 1, is superimposed on the graph ($Y_i = \alpha + X_i$; Fig. 1*A* and Fig. 2, red line). If the theoretical SHPM regression line with slope β equal to 1 does not lie between the two boundary regression lines for β_{upper} and β_{lower}, then the slope β of the log-log regression line fitted to the experimental data is not compatible with 1, and thus the SHPM does not fit the data. Reciprocally, if the SHPM regression line with slope β equal to 1 lies inside the limits of the two boundary regression lines for β_{upper} and β_{lower}, then the slope β of the log-log regression line fitted to the experimental data is compatible with 1, and thus the SHPM fits the data. To construct the graphs presented in Fig. 1*A* and Fig. 2, Table III gives the numerical values of the pairs (X_i, $\log(-\log(m_i))$ for plotting observed data points, and Tables IV and V show determination of the pairs (X_i, Y_i) for drawing the four regression lines.

Fig. 1*A* represents the graphical evaluation of the slope test *z* in the experiment evaluating the growth frequency of IL-2-responsive TIL-T in lymphoma. The equation of the log-log regression line fitted to the experimental data is $Y_i = -3.081 + 0.909X_i$, and the boundary equations of the two regression lines corresponding to the lower and upper limits of the slope β are $Y_i = -3.081 +$

Table II. *Statistical analysis of limiting dilution data*

Cell Population or Model	β	SE (β)	*z*	*p* Value (*z*)	95% Cl (β)	SHPM Hypothesis	Precursor Frequency (*f*)	95% CI (*f*)
TIL-T lymphocytes in B-NHL	0.909	0.0746	−1.213	0.225	0.763–1.055	accepted	0.0339	0.0289–0.0391
Suppressor two-target Poisson model	0.767	0.0864	−2.689	0.00716	0.598–0.936	rejected		

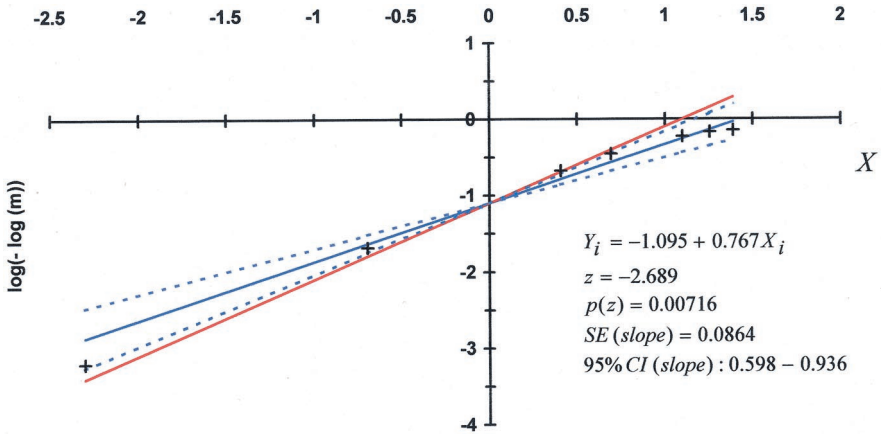

FIGURE 2. Graphical representation of the slope test *z* evaluating the adequacy of the SHPM to limiting dilution data. Data were artificially generated according to a STTPM. The log-log transform of the fraction of negative wells is plotted against the logarithm of the number of cells per well. The blue plain line represents the log-log regression line fitted to the experimental data according to the generalized linear log-log model ($Y_i = -1.095 + 0.767X_i$). The blue dotted lines represent the regression lines with the same intercept α as the fitted log-log regression line, but with slope β corresponding to the lower and upper values of the 95% confidence interval for β ($Y_i = -1.095 + 0.598X_i$; $Y_i = -1.095 + 0.936X_i$). The red line represents the theoretical SHPM regression line with the same intercept α, but with slope β equal to 1 ($Y_i = -1.095 + X_i$).

$0.763X_i$ (lower value) and $Y_i = -3.081 + 1.055X_i$ (upper value). Obviously, the regression line with β equal to 1, $Y_i = -3.081 + X_i$, lies inside the boundary regression lines for $\beta = 0.763$ and $\beta = 1.055$, thereby indicating that the experimental data are compatible with the SHPM. The graphical display of the slope test *z* in the STTPM is shown in Fig. 2. The equation of the log-log regression line fitted to the experimental data is $Y_i = -1.095 + 0.767X_i$, and the boundary equations of the two regression lines for the lower and upper limits of the slope β are $Y_i = -1.095 + 0.598X_i$ (lower value) and $Y_i = -1.095 + 0.936X_i$ (upper value). The regression line with β equal to 1, $Y_i = -1.095 + X_i$, lies outside the boundary regression lines for $\beta = 0.598$ and $\beta = 0.936$, indicating that the data do not conform to the SHPM, thus precluding the computation of the cell frequency *f* according to single-hit kinetics.

Because the limiting dilution assay with TIL-T is compatible with the SHPM, results can be presented by the usual graphical display of limiting dilution data (semilog plot, Fig. 1*B*). The fraction of negative wells on a logarithmic scale is plotted against the number of T cells per well. The prediction equation of the regression line is $-\log(\mu_i) = 0.0339x_i$, where 0.0339 is the cell frequency *f* estimated by the maximum likelihood method according to the SHPM hypothesis. *Lower* and *upper dotted lines* in Fig. 1*B* are plotted by using lower and upper values of the 95% confidence interval of *f*. The boundary equations are $-\log(\mu_i) = 0.0289x_i$ (lower), and $-\log(\mu_i) = 0.0391x_i$ (upper).

Based on these findings, we propose that immunologists adopt a standard two-graph method for presenting the results of limiting dilution data. The first graph should present the log-log transform of the results with the graphical representation of the slope test *z* designed to visualize whether the data are consistent or not with the SHPM. Four regression lines should be presented: the log-log regression line fitted to the experimental data according to the generalized linear log-log model, the two boundary regression lines for β_{upper} and β_{lower}, and the theoretical regression line with β equal to 1 (SHPM regression line). The graph should be accompanied by the equation of the log-log regression line fitted to the experimental data, the SE of β, the 95% confidence interval of β, and the numerical values of *z* and p(*z*) (Fig. 1*A* and Fig. 2).

If the SHPM hypothesis holds, the second graph should be the usual graphical representation of limiting dilution data (semilog plot), where the fraction of negative wells is plotted on a logarithmic scale against the number of cells per well. The prediction equation of the regression line is $-\log(\mu_i) = fx_i$, with the frequency *f* estimated by the maximum likelihood method according to the SHPM hypothesis. On this graph should also be plotted the two regression lines based on the extreme values of the confidence

Table III. *Tabulated values of the experimental data points [X_i, log(−log(m_i)] plotted on Fig. 1A (TIL-T in B-NHL) and Fig. 2 (suppressor two-target Poisson model)*

Cell Population or Model	Number of T Cells per Well x_i	$X_i = \log(x_i)$	Observed Fraction of Negative Wells $m_i = r_i/n_i$	$\log[-\log(m_i)]$
TIL-T in B-NHL	5	1.609	0.812	−1.871
	10	2.302	0.739	−1.198
	20	2.995	0.458	−0.248
	50	3.912	0.177	0.548
	100	4.605	0.052	1.019
Suppressor two-target Poisson model	0.1	−2.302	0.96	−3.198
	0.5	−0.693	0.83	−1.68
	1.5	0.405	0.6	−0.671
	2	0.693	0.53	−0.454
	3	1.098	0.45	−0.225
	3.5	1.252	0.43	−0.169
	4	1.386	0.42	−0.142

Table IV. *Tabulated values of the pairs of data (X_i and Y_i) used for the construction of the regression lines corresponding to the limiting dilution experiment performed with TIL-T in B-NHL (Fig. 1A)*[a]

Cell Population	α	β	Regression Line $Y_i = \alpha + \beta X_i$	Number of T Cells per Well x_i	$X_i = \log(x_i)$	Y_i
TIL-T in B-NHL	−3.081	0.909	$Y_i = -3.081 + 0.909\ X_i$			
				5	1.609	−1.618
				10	2.302	−0.988
				20	2.995	−0.358
				50	3.912	0.475
				100	4.605	1.104
	−3.081	1.055 (β_{upper})	$Y_i = -3.081 + 1.055\ X_i$			
				5	1.609	−1.383
				10	2.302	−0.652
				20	2.995	0.0787
				50	3.912	1.046
				100	4.605	1.777
	−3.081	0.763 (β_{lower})	$Y_i = -3.081 + 0.763\ X_i$			
				5	1.609	−1.853
				10	2.302	−1.324
				20	2.995	−0.795
				50	3.912	0.0961
				100	4.605	0.432
	−3.081	1	$Y_i = -3.081 + X_i$			
				5	1.609	−1.472
				10	2.302	−0.779
				20	2.995	−0.0860
				50	3.912	0.831
				100	4.605	1.524

[a] Y_i values are calculated from the prediction equations, $Y_I = \alpha + \beta X_i$.

interval for *f*. The graph should be accompanied by the numerical value of *f* (or its inverse), its 95% confidence interval, and the equation of the regression line (Fig. 1*B*).

In conclusion, this proposition broadly resembles a two-graph standard presentation of data adopted a number of years ago by immunologists dealing with radioimmunoassays. The first graph

Table V. *Tabulated values of the pairs of data (X_i and Y_i) used for the construction of the regression lines corresponding to limiting dilution data artificially generated from the theoretical suppressor two-target Poisson model (Fig. 2)*[a]

Model	α	β	Regression Line $Y_i = \alpha + \beta X_i$	Number of T Cells per Well x_i	$X_i = \log(x_i)$	Y_i
Suppressor two-target Poisson model	−1.095	0.767	$Y_i = -1.095 + 0.767X_i$			
				0.1	−2.302	−2.860
				0.5	−0.693	−1.626
				1.5	0.405	−0.784
				2	0.693	−0.563
				3	1.098	−0.252
				3.5	1.252	−0.134
				4	1.386	−0.0319
	−1.095	0.936 (β_{upper})	$Y_i = -1.095 + 0.936X_i$			
				0.1	−2.302	−3.249
				0.5	−0.693	−1.743
				1.5	0.405	−0.715
				2	0.693	−0.446
				3	1.098	−0.0672
				3.5	1.252	0.0768
				4	1.386	0.202
	−1.095	0.598 (β_{lower})	$Y_i = -1.095 + 0.598X_i$			
				0.1	−2.302	−2.475
				0.5	−0.693	−1.512
				1.5	0.405	−0.852
				2	0.693	−0.680
				3	1.098	−0.438
				3.5	1.252	−0.346
				4	1.386	−0.266
	−1.095	1	$Y_i = -1.095 + X_i$			
				0.1	−2.302	−3.397
				0.5	−0.693	−1.788
				1.5	0.405	−0.690
				2	0.693	−0.402
				3	1.098	0.003
				3.5	1.252	0.157
				4	1.386	0.291

[a] Y_i values are calculated from the prediction equations, $Y_i = \alpha + \beta X_i$.

eliminates nonspecific binding by demonstrating that the concentration of bound ligand (*y*-axis) increases nearly linearly with increasing free ligand (*x*-axis), then asymptotically approaches a plateau; this graph serves as a validation for transforming the data according to a second graph (the so-called Scatchard plot; bound/free vs bound) used to determine the number of binding sites and the ligand binding affinity. In the same manner, data from limiting dilution assays could adequately be summarized and validated according to the SHPM by routine representation using the two graphs presented in this report.

Appendix

Calculations of α, β, and var(β) according to the IRLS algorithm

A convenient feature of this algorithm is that it suggests a simple starting procedure to get the iteration under way. This consists of using the data themselves as the initial values of the dependent variable, Z_0, and the weight, W_0, and from these values are then calculated the first values of the parameter estimates, α_0 and β_0. These first values for α and β are in turn used to calculate the first value of the theoretical fraction of negative wells, μ_1, which in turn is used to obtain the revised values of weight and dependent variable, Z_1 and W_1, which in turn are used to calculate the revised values of the parameter estimates, α_1 and β_1, and so on. The iteration is continued until the absolute change in the values of α_m and β_m in two successive cycles of the iterative process is <0.0001. m is the mth cycle of the iteration. The estimates of α and β, thus obtained, are denoted by $\hat{\alpha}$ and $\hat{\beta}$, respectively. The corresponding fitted values of weights, W, are denoted by $\hat{W}$.

First step: initiation of the algorithm and calculation of the starting values, W_0, Z_0, α_0, *and* β_0

For each group of replicate wells, i,

1) compute the initial value of the weight, W_{i0}

$$W_{i0} = n_i \frac{[m_i \log(m_i)]^2}{1 - m_i}$$

2) Compute the initial value of the dependent variable, Z_{i0}

$$Z_{i0} = \log[-\log(m_i)]$$

3) Compute the first values of the parameter estimates, α_0 and β_0, according to equations 4 and 5, using W_{i0} and Z_{i0}.

Second step, the iterative process for calculating the revised values of the parameter estimates, α_m *and* β_m

1) Compute the estimates of the theoretical fraction of negative wells, μ_{im}: $\mu_{im} = \exp(-\exp(\alpha_{m-1} + \beta_{m-1} X_i))$.

2) Compute W_{im} according to equation 2 using μ_{im}.

3) Compute Z_{im} according to equation 3 using μ_{im} and α_{m-1}, β_{m-1}.

4) Compute the revised values of the parameter estimates, α_m and β_m, according to equations 4 and 5, using W_{im} and Z_{im}.

Repeat steps one to four until the absolute change in the values of α_m and β_m in two successive cycles of the iterative process is sufficiently small (<0.0001). The estimates of α and β are denoted by $\hat{\alpha}$ and $\hat{\beta}$. The corresponding fitted values of weights are denoted by $\hat{W}_i$.

Final step

1) Compute the variance of $\hat{\beta}$ and var($\hat{\beta}$), according to equation (6), using the values $\hat{W}_i$.

2) Compute the SE of $\hat{\beta}$

$$SE(\hat{\beta}) = \sqrt{\text{var}(\hat{\beta})}$$

3) Compute the value, z, of the slope test according to equation 1 using β and $\sqrt{\text{var}(\hat{\beta})}$

4) Compute the 95% confidence interval for $\hat{\beta}$ according to equation (7) using $\hat{\beta}$ and $SE(\hat{\beta})$.

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