LIMITING DILUTION ASSAYS FOR THE DETERMINATION OF IMMUNOCOMPETENT CELL FREQUENCIES.

II. Experimental Design for Single-Dose Assays.

EXPERIMENTAL DESIGN

FOR

LIMITING DILUTION ASSAYS

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FOOTNOTES

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[2] Abbreviations used in this paper: LDA, limiting dilution assay(s); IC, immunocompetent cell(s); CTL-P, cytolytic T lymphocyte precursor(s); SHPM, single-hit Poisson model; UAP, uninformative assay probability; CRMV, Cramer-Rao minimum variance; MUD, minimum UAP dose; MVD, minimum CRMV dose; NRP, negative response probability; ADOA, assay-design optimization analysi(e)s; all other mathematical and statistical notation as explained in the text.

[3] General statistical concepts such as the CRMV are not referenced throughout the text unless they are applied by an author specifically to limiting dilution analysis. Readers not familiar with any of the statistical concepts used in this article should consult the excellent introductory text by Freund and Walpole (1980).

[4] Standard conventions used for notation in this paper: Variable parameters and indicator variables are represented by Greek letters while random variables are represented by Latin letters. Parameter and variable names are represented by upper case letters while their values are represented by lower case letters.

SUMMARY

A statistical method was developed to optimize experimental designs for quantal response limiting dilution assays used to determine the frequencies of immunocompetent cells. Formulas for the design optimization criteria were derived by incorporating a prior distribution for the immunocompetent cell frequency into the design optimization analysis. This prior distribution was used to parameterize the advance information known about the frequency. The Cramer-Rao minimum variance and uninformative assay probability were chosen as the optimization criteria. Tables of these parameters, their corresponding optimum doses, and other experimental design parameters were computed for various frequency prior distributions. This method was developed in order to resolve the problems of previous approaches. The values computed for the optimum efficiency dose and for the minimum error of the frequency estimate are respectively higher and lower than those obtained by previous authors. Additional results predict that it is possible to perform single-dose assays successfully (with low uninformative assay probability) at doses approaching the optimum efficiency dose. (Single-dose assays should be used, however, only when the single-hit Poisson model used to estimate the frequency has already been proven valid by a multiple-dose experiment. References are provided for these validity tests.) To optimize the design of future assays in a sequence, information from past assays in the sequence should be used with the method and results presented here. The errors of frequency estimates could then theoretically be reduced to levels lower than those previously considered attainable.

INTRODUCTION

Limiting dilution assays $(LDA)^2$ can be used to determine the relative frequency of a specific type of cell defined by a biological function within a mixture constituting a general type of cell defined by a physical structure. For LDA employing immune response culture systems, the cells of the specific type are called "immunocompetent" cells (IC) and are defined by their functional activity; while the cells of the general type are called "total" cells and are defined by their structural morphology. For example, LDA are used to measure the relative frequency of cytolytic T lymphocyte precursors (CTL-P) as the IC within a mixture of leukocytes as the total cells (Taswell, MacDonald, and Cerottini, 1979). In this example, the functional activity of the IC is defined as cell differentiation and multiplication producing a clone of cells that can kill target cells (observed indirectly by the ^{51}Cr release assay), while the structural morphology of the total cells is defined as standard leukocytic morphology (observed directly by light microscopy). Quantal immune response LDA are dose-response bioassays that detect an all-or-nothing (positive-or-negative) immune response in each individual culture within groups of replicate cultures that vary in the dose of total cells tested. Analysis of dose-response data from these LDA may yield three types of results: 1) the IC frequency estimate, 2) the error of the estimate, and 3) the goodness of fit of the single-hit Poisson model (SHPM) used to estimate the frequency (Taswell, 1981).

These results can be obtained, however, only if the assay is performed at a sequence of (one or more) dilution doses such that the defined IC are limiting (neither saturating nor depleting) and all

other cell types and growth factors are saturating. If at least one of the IC doses is limiting such that some of the cultures are positive and some are negative, then a confidence interval with both lower and upper limits for the estimate (as well as the estimate itself) can be calculated. The estimate and assay can therefore be termed informative. However, if all of the IC doses are either saturating such that all of the cultures are positive or depleting such that all of the cultures are negative, then either only a lower limit or only an upper limit for the estimate (but not the estimate itself) can be calculated. The estimate and assay can therefore be termed uninformative. The nature of limiting dilution analysis thus requires the investigator to guess the frequency in advance and design the assay accordingly. With this goal in mind, the investigator should note that statistical analyses may be performed not only after an experiment (data reduction) in order to obtain the most information possible from the data available, but also before the experiment (design optimization) in order to obtain the most data possible for the information desired. The use of design optimization methods in conjunction with data reduction methods thus enables the investigator to obtain the most productive information yield for the amount of time, effort, and money invested. In the context of limiting dilution analysis, the assayist seeks to maximize the information that can be obtained from a given number of test cultures where in general the information will increase as the number of cultures increases.

In order to optimize the experimental design for LDA, it is necessary to choose criteria by which to compare alternative designs. These criteria should correspond to each of the three types of LDA

results numbered above. They could then be used to 1) minimize the probability of the cultures responding all positively or all negatively in order to obtain an informative estimate, 2) minimize the error of the estimate in order to obtain an accurate and precise estimate, and 3) maximize the trustworthiness of the SHPM goodness-of-fit (validity) test in order to obtain a valid estimate. For these three design criteria, I chose 1) the uninformative assay probability (UAP), 2) the Cramer-Rao minimum variance $(CRMV)^3$, and 3) the validity test power. In this article, I restrict my analysis to single-dose assays and present results of calculations for the UAP and CRMV design criteria. (Since any SHPM validity test requires at least two dilution doses, the restriction to single-dose assays precludes any calculation of results for the validity-test-power design criterion. Its use here is limited to the statements above and the Discussion where validity tests are reviewed briefly.) This restriction simplifies the design optimization problem to that of finding the IC dose where the UAP attains a minimum and the IC dose where the CRMV attains a minimum. I refer to these two IC doses as the minimum UAP dose (MUD) and the minimum CRMV dose (MVD). Since the probability that a culture responds negatively at each of these doses provides another measure by which to compare them, I also calculate the negative response probability (NRP) as well as several other parameters by which the assayist can judge the suitability of an assay design for his purposes.

Although most of these parameters have been calculated and published in the past (see the Discussion for a brief historical review of the literature), the assay-design optimization analyses (ADOA) of previous authors have applied to an LDA used to determine a sample

estimate rather than a sequence of LDA used to determine a population estimate. (The distinction here between samples and populations applies to biological samples and populations. It does not imply repeated assaying of the same biological sample, which is generally impractical if not impossible.) I refer to these two ADOA as the sample-estimate ADOA and the population-estimate ADOA. The unavailability of computers and algorithms and the consequent desire for simplified calculations are possible explanations for the use of the sample-estimate ADOA by previous authors. Advances in the computer sciences have made their simplifications unnecessary and the two major flaws inherent in their simplifications unjustifiable. The first flaw is a logical contradiction: In order to perform the LDA at an IC dose optimized by methods based upon the sample-estimate ADOA, the assayist needs to know the IC sample frequency in advance, but if he knows the IC sample frequency in advance, he does not need to perform the LDA. The second flaw is an unanswered question: When the assay design is optimized by two different methods, one based upon the sample-estimate ADOA and the other based upon the population-estimate ADOA, are the resulting optimum designs necessarily the same? If they are different, then the optimum design based upon the population-estimate ADOA should be used because most LDA are in fact repeated for different samples from the same population. This choice would also enable the assayist to avoid the logical contradiction stated above as well as wasting information obtained from previous LDA. Indeed, common sense leads the assayist to believe that the calculation and selection of an optimum design should depend upon the advance information known about the IC sample frequency. This advance information can be represented by a

prior distribution with a mean and coefficient of variation of the mean for the IC sample frequencies where the mean is the IC population frequency. In this article, I incorporate such a prior distribution into the ADOA and prove that the resulting assay designs optimized for the sample and population estimates are different.

MATERIALS AND METHODS

Biological assumptions. Assume that identical and independent (replicate) single-dose assays are performed for replicate biological samples at the same dilution dose of total cells added to replicate cultures . Assume furthermore that only one LDA can be performed for each biological sample under conditions for which the SHPM has been proven valid for the biological population. Then the IC sample frequency (Φ, see below) will vary due to the biological variation between different replicate samples, while the total cell dose (Λ, see below) will vary due to the error for the haemocytometer cell count used to infer its value and due to the error for the dilution procedure used after the cell count. The IC sample frequency estimate ($\hat{\phi}$, see the Appendix) will then vary as a consequence of the variation in the IC sample frequency, the error in the total cell dose, and the distribution errors for the numbers of IC and total cells added to replicate cultures in each LDA. An example of a typical population of IC is the population of C57BL/6 anti-DBA/2 murine spleen CTL-P with a mean sample frequency of 2.6×10^{-3} and a coefficient of variation of the mean of 0.5. This population estimate has been calculated from 19 sample estimates (Taswell et al, 1979).

Mathematical and statistical definitions and assumptions. Let Φ with real values[4] $0 \leq \phi \leq 1$ be the relative frequency of IC in the given sample . Φ is the conditional probability given the sample that any single cell is an IC. It is equal to the ratio of the number of IC to the number of total cells in the sample. Assume that Φ is a beta variable parameter with a prior distribution such that its probability density function is $f_\Phi(\phi) = \phi^{\alpha-1}(1-\phi)^{\beta-1}/B(\alpha,\beta)$, its mean is $\mu_\Phi = \alpha/(\alpha+\beta)$, its variance is $\sigma_\Phi^2 = \alpha\beta/[(\alpha+\beta)^2(\alpha+\beta+1)]$, and its coefficient of variation of the mean is $CV_\Phi = \sigma_\Phi/\mu_\Phi$ where

$$B(\alpha,\beta) = \int_0^1 \phi^{\alpha-1}(1-\phi)^{\beta-1}d\phi \qquad [1]$$

is the beta coefficient and $\alpha,\beta > 0$. When $CV_\Phi = 0$, then Φ is a constant parameter with value μ_Φ. Let Λ with real values $0 \leq \lambda < \infty$ be the dose of total cells added from the diluted sample to each replicate culture in the given single-dose assay. Λ is the assumed average number of total cells added to each culture. Its value is inferred from the value of a haemocytometer cell count multiplied by a dilution factor. Assume that Λ is a variable parameter with a negligible prior distribution such that it can be approximated by a constant parameter with value μ_Λ. Let Ω with real values $0 \leq \omega \leq \lambda$ be the dose of IC added from the diluted sample to each replicate culture in the given single-dose assay. Ω is the assumed average number of IC added to each culture. Its value is inferred from the value of Λ multiplied by a value assumed for Φ. Assume that Ω is a variable parameter with a prior distribution determined by those for Φ and Λ such that

$$\Omega = \Phi\Lambda \qquad [2]$$

and $\mu_\Omega = \mu_\Phi \mu_\Lambda$. Let Θ with real values $0 \leq \theta \leq 1$ be the conditional probability given $\Phi = \phi$ and $\Lambda = \lambda$ that any single culture in the given single-dose assay responds negatively according to the SHPM. Θ is the NRP. Assume that Θ is a variable parameter with a prior distribution determined by those for Φ and Λ such that

$$\Theta = e^{-\Phi\Lambda} \qquad [3] ,$$

$\mu_\Theta = E\Theta$, and $\sigma_\Theta^2 = E\Theta^2 - \mu_\Theta^2$ where E is the expectation operator and both μ_Θ and σ_Θ^2 can be expressed as functions of μ_Ω. Let n with integer values $1 \leq n < \infty$ be the number of replicate cultures tested in the given single-dose assay or sequence of replicate single-dose assays. Let Ψ_n with real values $0 \leq \psi_n \leq 1$ be the conditional probability given $\Phi = \phi$ and $\Lambda = \lambda$ that n replicate cultures in the given single-dose assay respond either all positively or all negatively according to the SHPM. Ψ_n is the UAP. Assume that Ψ_n is a variable parameter with a prior distribution determined by those for Φ and Λ such that

$$\Psi_n = e^{-n\Phi\Lambda} + (1-e^{-\Phi\Lambda})^n \qquad [4] ,$$

$\mu_{\Psi_n} = E\Psi_n$, and $\sigma_{\Psi_n}^2 = E\Psi_n^2 - \mu_{\Psi_n}^2$ where both μ_{Ψ_n} and $\sigma_{\Psi_n}^2$ can be expressed as functions of μ_Ω. Then the value of μ_Ω for which μ_{Ψ_n} attains a minimum is the MUD. Note that Φ, Λ, Ω, Θ, and Ψ_n are parameters for a given sample and single-dose assay, while μ_Φ, μ_Λ, μ_Ω, μ_Θ, and μ_{Ψ_n} are parameters for a given population and sequence of replicate single-dose assays. Furthermore, note that for the given population there is a one-to-one correspondence between the sequence of replicate samples and

the sequence of replicate assays. Let $\sigma^2_{CR_n}$ with real values $0 < \sigma^2_{CR_n}$ be the CRMV for an unbiased estimator $\hat{\mu}_\Phi$ of the IC population frequency μ_Φ based upon results observed from n replicate cultures in the given sequence of replicate single-dose assays (an estimator $\hat{\mu}_\Phi$ of μ_Φ with size n), that is,

$$\sigma^2_{CR_n} = \frac{\mu_\Theta(1-\mu_\Theta)}{n(\partial\mu_\Theta/\partial\mu_\Phi)^2} \qquad [5]$$

where $\sigma^2_{CR_n}$ can be expressed as a function of μ_Ω. Then the value of μ_Ω for which $\sigma^2_{CR_n}$ attains a minimum is the MVD. Since n is a factor independent of μ_Ω in the equation for $\sigma^2_{CR_n}$, the MVD is also independent of n. Let CV_{CR_n} with real values $0 < CV_{CR_n}$ be the Cramer-Rao minimum coefficient of variation of the mean for an unbiased estimator $\hat{\mu}_\Phi$ of μ_Φ with size n, that is, $CV_{CR_n} = \sigma_{CR_n}/\mu_\Phi = \sigma_{CR_1}/(\mu_\Phi\sqrt{n})$ where CV_{CR_n} can be expressed as a function of μ_Ω. Let N with real values $1 \leq N$ be the minimum average number of replicate cultures in the given sequence of replicate single-dose assays required to reduce CV_{CR_n} to a value such that $CV_{CR_n} \leq 0.1$, that is,

$$N = 100\,\sigma^2_{CR_1}/\mu^2_\Phi \qquad [6]$$

where N can be expressed as a function of μ_Ω. According to the asymptotic theory assumptions of the CRMV and of this analysis (see also the Appendix), the n or N replicate cultures may be apportioned to the replicate assays by any allotment sequence including, for example,

all n or N cultures in one assay. Let RE with real values $0 \leq RE \leq 1$ be the relative efficiency of the single-dose assay design. RE is the ratio of the CRMV for the MVD to the CRMV for any IC dose, that is,

$$RE(\mu_\Omega) = \frac{\sigma^2_{CR_n}(MVD)}{\sigma^2_{CR_n}(\mu_\Omega)} \qquad [7]$$

where RE is independent of n so that n = 1 can be assumed for the calculation of RE. Note that RE attains a maximum at the MVD, that is, RE(MVD) = 1. See the Appendix for derivations.

Formula for design parameters. When $CV_\phi = 0$, then

$$\mu_\Theta = e^{-\mu_\Omega} \qquad [8],$$

$$\sigma^2_\Theta = 0 \qquad [9],$$

$$\mu_{\Psi_n} = e^{-n\mu_\Omega} + (1-e^{-\mu_\Omega})^n \qquad [10],$$

$$\sigma^2_{\Psi_n} = 0 \qquad [11],$$

and

$$\sigma^2_{CR_n} = \frac{\mu_\phi^2(e^{\mu_\Omega}-1)}{n\mu_\Omega^2} \qquad [12].$$

When $CV_\phi > 0$, then

$$\mu_\Theta = \frac{1}{B(\alpha,\beta)} \int_0^1 e^{-\phi\mu_\Omega/\mu_\phi}\, \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi \qquad [13]$$

$$= \sum_{i=0}^{\infty} p_i(-\mu_\Omega)^i \qquad [14],$$

$$\sigma_\Theta^2 = \frac{1}{B(\alpha,\beta)} \int_0^1 e^{-2\phi\mu_\Omega/\mu_\Phi}\, \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi - \mu_\Theta^2 \qquad [15]$$

$$= \sum_{i=0}^{\infty} p_i(-2\mu_\Omega)^i - \mu_\Theta^2 \qquad [16],$$

$$\mu_{\Psi_n} = \frac{1}{B(\alpha,\beta)} \int_0^1 \left[e^{-n\phi\mu_\Omega/\mu_\Phi} + (1-e^{-\phi\mu_\Omega/\mu_\Phi})^n \right] \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi \qquad [17],$$

$$\sigma_{\Psi_n}^2 = \frac{1}{B(\alpha,\beta)} \int_0^1 \left[e^{-n\phi\mu_\Omega/\mu_\Phi} + (1-e^{-\phi\mu_\Omega/\mu_\Phi})^n \right]^2 \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi - \mu_{\Psi_n}^2 \qquad [18],$$

and

$$\sigma_{CR_n}^2 = \frac{\mu_\Phi^2\, \mu_\Theta(1-\mu_\Theta)}{n\left(\sum_{i=0}^{\infty} s_i p_i(-\mu_\Omega)^i\right)^2} \qquad [19]$$

where

$$\alpha = (1-t)/CV_\Phi^2 \qquad [20],$$

$$\beta = (1/\mu_\Phi - 1)\alpha \qquad [21],$$

$$p_i = \prod_{j=0}^{i-1} \frac{(1+jCV_\Phi^2 - t)}{(1+j)(1+jCV_\Phi^2\mu_\Phi - t)} \quad \text{with} \quad p_0 = 1 \qquad [22],$$

$$s_i = \sum_{j=0}^{i-1} \left[\frac{2-(3+CV_\Phi^2)\mu_\Phi}{1+jCV_\Phi^2 - t} - \frac{1-2\mu_\Phi}{1+jCV_\Phi^2\mu_\Phi - t} \right] \quad \text{with } s_0 = 0 \qquad [23],$$

$$t = (1+CV_{\Phi}^2)\mu_{\Phi} \qquad [24].$$

All other explicit formulas are identical to their definitions. See the Appendix for derivations.

Numerical computation of parameter values. Integral expressions in equations [1,13,17,15,18] were evaluated by adaptive Simpson's method according to the algorithm of McKeeman (1962,1963). The error convergence criterion, eps, in his algorithm was set to 5×10^{-7}. The limits of integration for ϕ were reset from 0 and 1 to the respective limits of a confidence interval for Φ defined by 10 standard deviations ($\mu_{\Phi} \pm 10\sigma_{\Phi}$) if the lower confidence limit was greater than 0 or the upper confidence limit was less than 1. Infinite series expressions in equations [14,16,19,22,23] were evaluated by Euler's method according to the algorithm of Naur et al (1960; see also Thacher, 1963). The convergence criteria, eps and tim, in their algorithm were set to 5×10^{-7} and 3, respectively. The MUD and MVD were found by differentiating formulas [10,17] and [12,19] respectively for the parameters μ_{Ψ_n} and $\sigma^2_{CR_n}$ with respect to μ_{Ω} and solving for the roots of these equations by Newton-Raphson's method (Dahlquist and Bjorck, 1974) with an absolute error convergence criterion of 5×10^{-6}. (The MUD and MVD can also be found simply by inspecting values of μ_{Ψ_n} and $\sigma^2_{CR_n}$ tabulated for incremented values of μ_{Ω}.) The computation methods for all other parameters are obvious from their formulas. Computations were performed on a Control Data Corporation CYBER 170 computer using programs written in PASCAL.

RESULTS

Summary of results. The CRMV σ^2_{CR} and UAP μ_{Ψ_n} design criteria were minimized as functions of the IC dose μ_Ω to find the MVD and MUD for values of the IC population frequency μ_Φ ranging from 1×10^{-4} to 1×10^{-1}, its coefficient of variation CV_Φ from 0.0 to 0.5, and the number n of replicate cultures in the given single-dose assay or sequence of replicate single-dose assays from 5 to 200. Various other design parameters such as the NRP μ_Θ and relative efficiency RE were then evaluated at the optimum IC doses μ_Ω = MVD and μ_Ω = MUD as well as other IC doses μ_Ω (see the Introduction and Materials and Methods). Identical results were obtained for parameter values calculated from both integral and infinite series expressions thus suggesting adequate convergence of the numerical methods employed. The CRMV σ^2_{CR} for the case when $CV_\Phi > 0$ is always less than for the case when $CV_\Phi = 0$. The value of the MVD is dependent upon CV_Φ but relatively independent of μ_Φ. For $0.0 \leq CV_\Phi \leq 0.5$ with $1\times10^{-4} \leq \mu_\Phi \leq 1\times10^{-1}$, the MVD range is $1.6 \leq \mu_\Omega \leq 2.6$ and corresponds to $0.20 \geq \mu_\Theta \geq 0.14$ on the NRP curve (see Figure 1). The UAP μ_{Ψ_n} for the case when $CV_\Phi > 0$ is less if n is small (eg., n = 5) and greater if n is large (eg., $n \geq 20$) than for the case when $CV_\Phi = 0$. The value of the MUD is relatively independent of CV_Φ, of μ_Φ, and of n. For $0.0 \leq CV_\Phi \leq 0.5$ with $1\times10^{-4} \leq \mu_\Phi \leq 1\times10^{-1}$ and $5 \leq n \leq 100$, the MUD range is $0.7 \leq \mu_\Omega \leq 0.8$ and corresponds to $0.53 \geq \mu_\Theta \geq 0.48$ on the NRP curve (see Figure 1) and to $0.58 \leq RE \leq 0.74$ on the relative efficiency scale. If the number of replicate cultures is sufficiently high, single-dose assays may be performed successfully at the MUD. For example, with $CV_\Phi = 0.5$,

$1\times10^{-4} \leq \mu_\Phi \leq 1\times10^{-1}$, and $20 \leq n \leq 200$, the UAP at the MUD is $\mu_{\Psi_n} < 0.004$. The relative efficiency of the single-dose assay may be increased by shifting the IC dose toward the MVD (away from the MUD) as long as the number of replicate cultures is increased concomitantly.

The minimum CRMV σ^2_{CR} dose (MVD). Figure 2 displays a plot of $\sigma^2_{CR_1}$ versus μ_Ω for $CV_\Phi = 0.0$ and $CV_\Phi = 0.5$ with $\mu_\Phi = 0.1$. The relative position of these curves is independent of n with the curve for $CV_\Phi = 0.5$ always lower than the curve for $CV_\Phi = 0.0$. $\sigma^2_{CR_1}$ attains a minimum of 1.54×10^{-2} at $\mu_\Omega = 1.6$ for $CV_\Phi = 0.0$ and a minimum of 1.07×10^{-2} at $\mu_\Omega = 2.6$ for $CV_\Phi = 0.5$. These values of μ_Ω are the values of the MVD. Table 1 summarizes the values of various design parameters evaluated as functions of μ_Ω at μ_Ω = MVD for various values of CV_Φ and μ_Φ. The MVD, μ_Θ(MVD), σ_Θ(MVD), σ_{CR_1}(MVD), and N(MVD) all vary with CV_Φ but only relatively negligibly with μ_Φ. For $CV_\Phi = 0.5$ with $1\times10^{-4} \leq \mu_\Phi \leq 1\times10^{-1}$, the MVD range is $2.56 \leq \mu_\Omega \leq 2.61$ and corresponds to $0.138 \geq \mu_\Theta \geq 0.134$ on the NRP curve (see Figure 1).

The minimum UAP μ_{Ψ_n} dose (MUD). Figure 3 displays a plot of μ_{Ψ_5} versus μ_Ω for $CV_\Phi = 0.0$ and $CV_\Phi = 0.5$ with $\mu_\Phi = 0.1$. The relative position of these curves is dependent upon n. In this example with $n = 5$, μ_{Ψ_5} attains a minimum of 14.0×10^{-2} at $\mu_\Omega = 0.69$ for $CV_\Phi = 0.0$ and a minimum of 6.3×10^{-2} at $\mu_\Omega = 0.70$ for $CV_\Phi = 0.5$. These values of μ_Ω are the values of the MUD. Table 2 summarizes the values of various design parameters evaluated as functions of μ_Ω at μ_Ω = MUD for various values of CV_Φ, μ_Φ, and n. In these examples with $20 \leq n \leq 100$, μ_{Ψ_n}(MUD) is lower for $CV_\Phi = 0.0$ than for $CV_\Phi = 0.5$ contrary to the

example with $n = 5$ in Figure 3. $\mu_{\Psi_n}(MUD)$ and $\sigma_{\Psi_n}(MUD)$ vary with CV_Φ and with n but only relatively negligibly with μ_Φ. $\sigma_\Theta(MUD)$, N(MUD), and RE(MUD) vary with CV_Φ but only negligibly with μ_Φ and with n. The MUD and $\mu_\theta(MUD)$ vary negligibly with CV_Φ, μ_Φ, and n. For $CV_\Phi = 0.5$ with $1\times10^{-4} \leq \mu_\Phi \leq 1\times10^{-1}$ and $5 \leq n \leq 100$, the MUD range is $0.70 \leq \mu_\Omega \leq 0.81$ and corresponds to $0.53 \geq \mu_\Theta \geq 0.48$ on the NRP curve (see Figure 1) and to $0.58 \leq RE \leq 0.63$ on the relative efficiency scale.

Constrained optimization of the single-dose assay design. Since the MVD and MUD are located within different IC dose ranges corresponding to different ranges of the NRP curve (see above), it is not possible to optimize the assay design simultaneously for the CRMV σ^2_{CR} and the UAP μ_{Ψ_n} criteria. It is possible, however, to constrain one criterion and optimize the other criterion subject to this constraint. Table 3 summarizes the values of various design parameters evaluated at various values of μ_Ω for $CV_\Phi = 0.5$ with $\mu_\Phi = 0.1$. For given n, it is possible to increase $RE(\mu_\Omega)$ by increasing μ_Ω until $\mu_{\Psi_n}(\mu_\Omega)$ exceeds a predetermined limit. For example, with $n = 120$, $RE(\mu_\Omega)$ can be increased to 0.95 by increasing μ_Ω to 1.8 before $\mu_{\Psi_{120}}(\mu_\Omega)$ exceeds 0.01. Note that at $\mu_\Omega = 1.8$, $n = 120$ is greater than the value of $N(\mu_\Omega)$ required to reduce $CV_{CR_{120}}$ to a value less than 0.1. Alternatively, for given μ_Ω and $RE(\mu_\Omega)$, it is possible to decrease $\mu_{\Psi_n}(\mu_\Omega)$ by increasing n. For example, with $\mu_\Omega = 1.5$ and $RE(\mu_\Omega) = 0.89$, $\mu_{\Psi_n}(\mu_\Omega)$ can be decreased to 0.005 by increasing n to 80.

DISCUSSION

Historical reviews of the theory and practice of limiting dilution analysis have been published by Halvorson and Ziegler in 1933 , Eisenhart and Wilson in 1943 , and Finney in 1978 . Although the interested reader should consult these sources for a complete bibliography, I would like to draw attention to the following investigators each of whom was responsible for a major new development. In 1908, Phelps published the earliest referenced article on LDA and introduced a method for the calculation of the "most probable number" of B. coli (analogous to an IC frequency estimate) in water and sewage samples. In 1915, McCrady applied probability theory for the first time to the analysis of LDA dose-response data, enabling estimation of the error of the most probable number as well as the most probable number itself. In 1917, Greenwood and Yule proposed a data analysis model based upon the Poisson distribution rather than the binomial distribution used by McCrady (1915). In 1919, Stein recommended an optimum dose for the first time and favored a value corresponding to a NRP of 0.35. In a series of publications beginning in 1922 and ending in 1935, Fisher progressively refined his calculations and proved that the value of the information (the original form of the CRMV and equal to its reciprocal) for a given sample and single-dose assay attains a maximum at an optimum dose of 1.59 "organisms" corresponding to a NRP of 0.20 where at least 155 replicate cultures are required to reduce the coefficient of variation of the sample estimate to a value less than 0.1. Since then, Fisher's results have also been obtained by Haldane (1939), Mather (1949), Finney (1951), Peto (1953), Thompson (1962), Porter and Berry (1963), Kerr (1971), Griffiths (1972), Fazekas de St. Groth (1982), and Swindel (1983).

Their sample-estimate ADOA suffers from the practical disadvantage that it applies only to a given sample and LDA. This situation unfortunately presents a contradiction. In order to perform the LDA at Fisher's optimum dose, the assayist needs to know in advance the value of the sample parameter that the LDA is intended to estimate; but if he knows in advance the sample parameter value, he does not need to perform the LDA. This predicament has been partially resolved in the past by designing multiple-dose assays (consult references listed in the reviews cited above) with the hope that at least one if not all of the doses would be close to Fisher's optimum dose, the truth of which could not be determined until after the assay. Multiple-dose assay designs were recommended not only for the rationale of attaining Fisher's optimum dose but also for the more important rationale of avoiding an uninformative assay, that is, one where the cultures respond all negatively or all positively. The loss in efficiency of multiple-dose assay designs relative to a single-dose assay design at the optimum dose was the price paid for the gain in assurance of successfully performing an informative assay. The price that has been paid, however, may have been unnecessarily high since the probability with which an uninformative assay would occur was never calculated. In 1935, Matuszewski, Neyman, and Supinska published an equation for the UAP based upon the sample-estimate ADOA but they used it to calculate the "range of efficacy" rather than the UAP itself. In 1963, Porter and Berry followed what appears to be the same approach (they did not provide or cite the formulas that they used for their calculation of the UAP). In 1972, Griffiths discussed the possibility of incorporating a prior distribution into the ADOA. This prior

distribution would have enabled him to improve his analysis from that of the sample-estimate ADOA to that of the population-estimate ADOA, and as a consequence, to calculate correctly the UAP itself . However, he did not derive any equations or compute any parameter values and resorted instead to recommending multiple-dose assay designs. Examination of current literature and a recent review by Fazekas de St. Groth (1982) reveals that multiple-dose assay designs continue to be recommended and used in practice.

In this article, I propose a new method for optimizing the design of LDA based upon the population-estimate ADOA rather than the sample-estimate ADOA. This method provides results not of theoretical interest after the assay but rather of practical use before the assay. The systematic quantification of all criteria relevant to the single-dose assay design distinguishes my analysis from that of previous authors. Whereas they parametrized only the error of a sample estimate for a given assay, I now parameterize the error of the population estimate for a given sequence of assays and the probability of an uninformative assay in the sequence. Formulas for these parameters, the CRMV and UAP, are based upon a prior distribution which parameterizes the advance information known about the IC frequency. The prior distribution and its parameters, the mean and the coefficient of variation of the mean, represent the advance information known whether actually obtained from past assays or merely guessed by reason, intuition, and experience. In the former case, the mean and coefficient of variation can be estimated empirically (see the example under the heading biological assumptions in Materials and Methods); while in the latter case, they must be predicted so that the mean

represents the predicted frequency and the coefficient of variation represents the assayist's confidence in his prediction. With the use of this prior distribution, I minimize the CRMV and UAP as functions of the IC dose in order to find the respective optimum doses, MVD and MUD (see the Introduction and Materials and Methods), for various values of the mean and coefficient of variation of the IC frequency prior distribution as well as various values of the number of replicate cultures.

Although the calculated design parameters depend upon the IC frequency prior distribution, as an example of results analogous to those of Fisher, the MVD occurs at an IC dose of 2.56 corresponding to a NRP of 0.14 where at least 109 replicate cultures are required to reduce the coefficient of variation of the population estimate to a value less than 0.1. This example is valid for an IC frequency prior distribution with a mean between 1×10^{-4} and 1×10^{-2} and a coefficient of variation of 0.5 which is typical of CTL-P (Taswell et al, 1979). My results are consistent with Fisher's results in the sense that his results can be obtained with my method as the limiting case where the prior distribution coefficient of variation tends to zero (see the Results). This observation reflects the fact that when the coefficient of variation is zero the population-estimate ADOA reduces to the sample-estimate ADOA. Since the coefficient of variation for an IC population is never zero, this description of Fisher's optimum dose is another way of explaining its impracticality. This statement does not imply, though, that the use of my MVD is any more practical in and of itself without consideration of my MUD. In fact, rather than approaching the design problem by starting the assay sequence at the

MVD and shifting the IC dose of each new assay in the sequence toward the MUD in order to decrease the UAP, the assayist would be more assured of performing informative assays if he approaches the problem by starting at the MUD and shifting the IC dose toward the MVD in order to increase the efficiency (see the example under the heading constrained optimization in the Results). Note that these approaches are different for two reasons. First, the tables of the MUD, MVD, and other assay design parameters are computed with the IC frequency prior distribution parameters but are consulted with estimates of these parameters (recalculated after each new assay in the sequence). Second, the tables are based upon an analysis assuming asymptotic or large-sample theory (an infinite sequence of samples and assays) rather than small-sample practice (a finite sequence of 1-10-100 samples and assays).

The tables of design parameters presented in this article are neither complete nor representative of the entire range of those that could be calculated for all possible values of the mean and coefficient of variation of the IC frequency prior distribution, the number of replicate cultures, and the IC dose. Although interpolation within the range of calculated values should not result in unreasonable approximations, extrapolation beyond the limits of this range could result in approximations far from the true values of the parameters. A further note of caution concerns the general use of single-dose assays. The appearance of single-dose assay designs in this article should be interpreted as a demonstration of a design optimization method and not as an endorsement of a design optimization solution. Single-dose assay designs are a partial but not necessarily complete solution. Perhaps a

double-dose assay design with one dose at the MVD and one dose somewhere between the MVD and the MUD would have a higher efficiency than a single-dose assay design when both are constrained to the same maximum level of the UAP. Nevertheless, according to the population-estimate ADOA, it is possible to perform single-dose assays successfully when sufficient advance information is available. However, single-dose assays should be used only when the SHPM has already been proven to apply to the culture system employed by the assay. In addition to the Pearson χ^2 goodness-of-fit test (Taswell, 1981;Finney, 1978) first used for quantitative response LDA by Fisher, Thornton, and MacKenzie in 1922 and for quantal response LDA by Barkworth and Irwin in 1938, other SHPM validity tests have been published by Stein (1922), Halvorson and Moeglein (1940), Savage and Halvorson (1941), and Moran (1954a, 1954b), Armitage and Spicer (1956), Stevens (1958), Moran (1958), Armitage (1959), Armitage and Bartsch (1960), Cox (1962), and Gart and Weiss (1967). Beginning with the SHPM validity test of Moran in 1954, those published in the past few decades are probably more powerful than the χ^2 test.

When consulting this literature on SHPM validity tests as well as historical or any other literature on LDA, the reader should bear in mind the following general principles (see also the Introduction). An LDA is an assay for a biological function of biological particles such as cells, bacteria, viruses, and plasmids. There are two types of LDA: one with (Type I) and one without (Type II) an accompanying assay for the physical structure of the biological particles. Using the notation of this article (see the Materials and Methods), both types of LDA estimate the number Ω of functional particles in a unit volume of the

sample and use the estimate of Ω to infer an estimate of either Φ or Λ according to the relationship expressed by equation [2]. However, in LDA Type I, Λ represents the known number of structural particles in a unit volume of the sample used to infer a value for the unknown relative frequency Φ of functional particles in the sample; whereas in LDA Type II, Φ represents the known dilution factor used to infer a value for the unknown absolute number Λ of functional particles in a unit volume of the sample. On the basis of this distinction, I refer to a Type I LDA as a relative frequency LDA and a Type II LDA as an absolute number LDA. Regardless of whether the experiment requires a concrete interpretation as a relative frequency LDA or an absolute number LDA, the abstract mathematical and statistical method of analysis for the optimization of the assay design remains equally applicable as long as the prior distributions for Φ and Λ are chosen appropriately. The use of these prior distributions in the population-estimate ADOA leads to equations which can be solved with available computers and numerical algorithms. The flawed simplifications of the sample-estimate ADOA are therefore no longer justifiable. According to the dogma of the scientific method, experiments must be repeated before any inferences can be made. In fact, most published experiments are performed repeatedly several times and many others are performed routinely as standard procedures. However, a cautious approach to making inferences when repeating LDA should not be pursued to an unnecessary extreme. Thus, when sequentially repeating LDA for different samples from the same population, information from past assays should be used to optimize the design of future asays in order to obtain information from data that would not otherwise have been

available. The theoretical analysis of this article predicts that the errors of sample and population estimates from LDA can be reduced when this principle of experimental design is implemented in laboratory practice.

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APPENDIX

Definitions and assumptions. In addition to those explained in the Materials and Methods, the following definitions and assumptions are required for the derivation of formulas. Let X with integer values $0 \leq x < \infty$ be the actual number of total cells added from the diluted sample to each replicate culture in the given single-dose assay. Assume that X is a random variable with a conditional Poisson distribution such that $f_{X|\Lambda}(x|\lambda) = \lambda^x e^{-\lambda}/x!$, $\mu_{X|\Lambda} = \lambda$, and $\sigma^2_{X|\Lambda} = \lambda$. Let Z with integer values $0 \leq z \leq x$ be the actual number of IC added from the diluted sample to each replicate culture in the given single-dose assay. Assume that Z is a random variable with a conditional Poisson distribution such that $f_{Z|\Omega}(z|\omega) = \omega^z e^{-\omega}/z!$, $\mu_{Z|\Omega} = \omega$, and $\mu_Z = \mu_\Omega = \mu_\Phi \mu_\Lambda$. Let Y with integer values $0 \leq y \leq x$ be the actual number of non-IC (immunoincompetent cells) added from the diluted sample to each replicate culture in the given single-dose assay. Assume that Y is a random variable such that $Y = X-Z$. Let T be the cell type indicator variable defined as $T = 1$ iff the cell is immunocompetent, and $T = 0$ iff the cell is immunoincompetent, with a conditional Bernoulli distribution such that $f_{T|\Phi}(\upsilon|\phi) = \phi^\upsilon(1-\phi)^{1-\upsilon}$, $\mu_{T|\Phi} = \phi$, and $\sigma^2_{T|\Phi} = \phi(1-\phi)$. Let Ξ be the culture response indicator variable defined as $\Xi = 1$ iff the culture responds negatively iff $Z = 0$, and $\Xi = 0$ iff the culture responds positively iff $Z \geq 1$, with a conditional Bernoulli distribution such that $f_{\Xi|\Theta}(\xi|\theta) = \theta^\xi(1-\theta)^{1-\xi}$, $\mu_{\Xi|\Theta} = \theta$, and $\sigma^2_{\Xi|\Theta} = \theta(1-\theta)$ and an unconditional Bernoulli distribution such that $f_{\Xi;\mu_\Theta}(\xi;\mu_\Theta) = \mu_\Theta^\xi(1-\mu_\Theta)^\xi$, $\mu_{\Xi;\mu_\Theta} = \mu_\Theta$, and $\sigma^2_{\Xi;\mu_\Theta} = \mu_\Theta(1-\mu_\Theta)$ (see below). Note that the definition of Ξ is the

mathematical expression of the single-hit hypothesis. Let R with integer values $0 \leq r \leq n$ be the observed number of cultures in the given single-dose assay that respond negatively according to the SHPM. Assume that R is a random variable with a conditional binomial distribution such that $f_{R|\Theta}(r|\theta) = \binom{n}{r}\theta^r(1-\theta)^{n-r}$, $\mu_{R|\Theta} = n\theta$, and $\sigma^2_{R|\Theta} = n\theta(1-\theta)$.

The NRP, UAP, and CRMV according to the sample-estimate and population-estimate ADOA. The conditional probability given the sample and single-dose assay that any one culture responds negatively is

$$P(\Xi=1|\Phi=\phi,\Lambda=\lambda) = P(Z=0|\Phi=\phi,\Lambda=\lambda) = \sum_{x=0}^{\infty} P(Y=x,X=x|\Phi=\phi,\Lambda=\lambda)$$

$$= \sum_{x=0}^{\infty} P(T_1=0,\ T_2=0,\ldots,\ T_x=0,X=x|\Phi=\phi,\Lambda=\lambda)$$

$$= \sum_{x=0}^{\infty} \left[P(T=0|\Phi=\phi)\right]^x P(X=x|\Lambda=\lambda) = \sum_{x=0}^{\infty} (1-\phi)^x\ \lambda^x\ e^{-\lambda}/x!$$

$$= e^{-\lambda} \sum_{x=0}^{\infty} (\lambda-\phi\lambda)^x/x! = e^{-\lambda}e^{\lambda-\phi\lambda} = e^{-\phi\lambda} = \theta$$

and thus

$$\theta = e^{-\Phi\Lambda} \qquad [3].$$

The probability given the population that any one culture responds negatively in any one of an infinite sequence of replicate single-dose assays is

$$P(\Xi=1) = \int_0^\infty \int_0^1 P(\Xi=1,\Phi=\phi,\Lambda=\lambda)d\phi d\lambda$$

$$= \int_0^\infty \int_0^1 P(\Xi=1|\Phi=\phi,\Lambda=\lambda)f_\Phi(\phi)f_\Lambda(\lambda)d\phi d\lambda$$

$$= \int_0^\infty \int_0^1 e^{-\phi\lambda}f_\Phi(\phi)f_\Lambda(\lambda)d\phi d\lambda$$

$$= Ee^{-\Phi\Lambda} = E\Theta = \mu_\Theta ,$$

and thus μ_Θ is the parameter for the unconditional Bernoulli distribution of Ξ (see above). The conditional probability given the sample and single-dose assay that the cultures respond all negatively or all positively is

$$P(R=n|\Phi=\phi,\ \Lambda=\lambda) + P(R=0|\Phi=\phi,\ \Lambda=\lambda) = \binom{n}{n}\theta^n(1-\theta)^{n-n} + \binom{n}{0}\theta^0(1-\theta)^{n-0}$$

$$= \theta^n + (1-\theta)^n = e^{-n\phi\lambda} + (1-e^{-\phi\lambda})^n = \psi_n$$

and thus

$$\Psi_n = e^{-n\Phi\Lambda} + (1-e^{-\Phi\Lambda})^n \qquad [4].$$

The probability given the population that the cultures respond all negatively or all positively in any one of an infinite sequence of replicate single-dose assays is $\mu_{\Psi_n} = E\Psi_n$. The CRMV for an unbiased estimator $\hat{\Phi}$ of the given IC sample frequency Φ based upon results observed from n replicate cultures in a given single-dose assay (an estimator $\hat{\Phi}$ of Φ with size n) is

$$\frac{1}{nE\left(\frac{\partial \ln f(\xi|\theta)}{\partial\phi}\right)^2}$$

where $\Theta = \theta$ is defined by equation [3] with $\Phi = \phi$ and $\Lambda = \lambda$,

$$\ln f(\xi|\theta) = \xi \ln\theta + (1-\xi)\ln(1-\theta),$$

$$\frac{\partial \ln f(\xi|\theta)}{\partial\phi} = \frac{\xi}{\theta}\frac{\partial\theta}{\partial\phi} + \frac{(1-\xi)}{(1-\theta)}\frac{\partial(1-\theta)}{\partial\phi} = \frac{(\xi-\theta)}{\theta(1-\theta)}\frac{\partial\theta}{\partial\phi},$$

and

$$E\left(\frac{\partial \ln f(\xi|\theta)}{\partial\phi}\right)^2 = \left(\frac{1}{\theta(1-\theta)}\frac{\partial\theta}{\partial\phi}\right)^2 E(\Xi-\theta)^2$$

$$= \left(\frac{1}{\theta(1-\theta)}\frac{\partial\theta}{\partial\phi}\right)^2 \sigma^2_{\Xi|\Theta} = \left(\frac{1}{\theta(1-\theta)}\frac{\partial\theta}{\partial\phi}\right)^2 \theta(1-\theta) = \frac{1}{\theta(1-\theta)}\left(\frac{\partial\theta}{\partial\phi}\right)^2$$

so that the CRMV for $\hat{\Phi}$ is

$$\frac{\theta(1-\theta)}{n\left(\frac{\partial\theta}{\partial\phi}\right)^2} = \frac{e^{-\phi\lambda}(1-e^{-\phi\lambda})}{n(-\lambda e^{-\phi\lambda})^2} = \frac{e^{\phi\lambda}-1}{n\lambda^2}.$$

The CRMV for an unbiased estimator $\hat{\mu}_\phi$ of the given IC population frequency μ_ϕ based upon results observed from n replicate cultures in an infinite sequence of replicate single-dose assays (an estimator $\hat{\mu}_\Phi$ of μ_ϕ with size n) is

$$\sigma^2_{CR_n} = \frac{1}{nE\left(\frac{\partial \ln f(\xi;\mu_\Theta)}{\partial \mu_\Phi}\right)^2} = \frac{\mu_\Theta(1-\mu_\Theta)}{n\left(\frac{\partial \mu_\Theta}{\partial \mu_\Phi}\right)^2} \qquad [5]$$

where μ_θ is defined by equations [8,13,14] with given μ_Φ, σ^2_Φ, and μ_Λ.

The NRP, UAP, and CRMV according to the population-estimate ADOA when $CV_\Phi = 0$. When $CV_\Phi = 0$, then

$$\mu_\Theta = Ee^{\Phi\Lambda} = e^{-\mu_\Phi\mu_\Lambda} = e^{-\mu_\Omega} \qquad [8],$$

$$\mu_{\Psi_n} = E\left[e^{-n\Phi\Lambda} + (1-e^{-\Phi\Lambda})^n\right] = e^{-n\mu_\Omega} + (1-e^{-\mu_\Omega})^n \qquad [10],$$

and

$$\sigma^2_{CR_n} = \frac{\mu_\Theta(1-\mu_\Theta)}{n\left(\frac{\partial \mu_\Theta}{\partial \mu_\Phi}\right)^2} = \frac{\Theta(1-\Theta)}{n\left(\frac{\partial \Theta}{\partial \Phi}\right)^2} = \frac{e^{\Phi\Lambda}-1}{n\Lambda^2} = \frac{\mu_\Phi^2(e^{\mu_\Omega}-1)}{n\mu_\Omega^2} \qquad [12].$$

Note that when $CV_\Phi = 0$, the population-estimate ADOA reduces to the sample-estimate ADOA.

The NRP, UAP, and CRMV according to the population-estimate ADOA when $CV_\Phi > 0$. When $CV_\Phi > 0$, then

$$\mu_\Theta = Ee^{-\Phi\Lambda}$$

$$= \frac{1}{B(\alpha,\beta)} \int_0^1 e^{-\phi\mu_\Lambda} \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi \qquad [13a]$$

$$= {}_1F_1(\alpha;\alpha+\beta;-\mu_\Lambda)$$

and

$$\mu_{\Psi_n} = E[e^{-n\Phi\Lambda} + (1-e^{-\Phi\Lambda})^n]$$

$$= \frac{1}{B(\alpha,\beta)} \int_0^1 [e^{-n\phi\mu_\Lambda} + (1-e^{-\phi\mu_\Lambda})^n] \phi^{\alpha-1}(1-\phi)^{\beta-1} d\phi \qquad [17a]$$

where

$$\alpha = (1-t)/CV_\Phi^2 \qquad [20],$$

$$\beta = (1/\mu_\Phi - 1)\alpha \qquad [21],$$

$$t = (1+CV_\Phi^2)\mu_\Phi \qquad [24],$$

$\mu_\Lambda = \mu_\Omega/\mu_\Phi$, $\alpha+\beta = (1-t)/(CV_\Phi^2\mu_\Phi)$, and ${}_1F_1$ is the confluent hypergeometric function (Luke, 1975). The infinite series expansion of ${}_1F_1$ is

$${}_1F_1(\alpha;\alpha+\beta;-\mu_\Lambda) = \sum_{i=0}^{\infty} \frac{[\alpha]_i}{[\alpha+\beta]_i} \frac{(-\mu_\Lambda)^i}{i!} = \sum_{i=0}^{\infty} (-\mu_\Lambda)^i \prod_{j=0}^{i-1} \frac{(\alpha+j)}{(1+j)(\alpha+\beta+j)}$$

$$= \sum_{i=0}^{\infty} \left(\frac{-\mu_\Omega}{\mu_\Phi}\right)^i \prod_{j=0}^{i-1} \frac{(1-t+jCV_\Phi^2)/CV_\Phi^2}{(1+j)(1-t+jCV_\Phi^2\mu_\Phi)/CV_\Phi^2\mu_\Phi}$$

$$= \sum_{i=0}^{\infty} (-\mu_\Omega)^i \prod_{j=0}^{i-1} \frac{(1+jCV_\Phi^2-t)}{(1+j)(1+jCV_\Phi^2\mu_\Phi-t)}$$

so that with

$$p_i = \prod_{j=0}^{i-1} \frac{(1+jCV_\Phi^2-t)}{(1+j)(1+jCV_\Phi^2\mu_\Phi-t)} \quad \text{and} \quad p_0 = 1 \qquad [22] ,$$

then

$$\mu_\Theta = \sum_{i=0}^{\infty} p_i(-\mu_\Omega)^i \qquad [14] .$$

Differentiating μ_Θ with respect to μ_Φ when σ_Φ^2 and μ_Λ are constant,

$$\frac{\partial \mu_\Theta}{\partial \mu_\Phi} = \sum_{i=0}^{\infty} \frac{(-\mu_\Lambda)^i}{i!} \frac{\partial}{\partial \mu_\Phi} \prod_{j=0}^{i-1} \frac{(\alpha+j)}{(\alpha+\beta+j)}$$

where

$$\frac{\partial}{\partial \mu_\Phi} \prod_{j=0}^{i-1} \left(\frac{\alpha+j}{\alpha+\beta+j}\right) = \sum_{j=0}^{i-1} \left[\frac{\partial}{\partial \mu_\Phi} \left(\frac{\alpha+j}{\alpha+\beta+j}\right)\right] \prod_{\substack{k=0 \\ k\neq j}}^{i-1} \left(\frac{\alpha+k}{\alpha+\beta+k}\right) ,$$

$$\frac{\partial}{\partial \mu_\Phi} \left(\frac{\alpha+j}{\alpha+\beta+j}\right) = \left[\left(\frac{2-(3+CV_\Phi^2)\mu_\Phi}{1+jCV_\Phi^2-t}\right) - \left(\frac{1-2\mu_\Phi}{1+jCV_\Phi^2\mu_\Phi-t}\right)\right] \left(\frac{1+jCV_\Phi^2-t}{1+jCV_\Phi^2\mu_\Phi-t}\right) ,$$

$$\frac{\partial}{\partial \mu_\Phi} \prod_{j=0}^{i-1} \left(\frac{\alpha+j}{\alpha+\beta+j}\right) = \sum_{j=0}^{i-1} \left[\left(\frac{2-(3+CV_\Phi^2)\mu_\Phi}{1+jCV_\Phi^2-t}\right) - \left(\frac{1-2\mu_\Phi}{1+jCV_\Phi^2\mu_\Phi-t}\right)\right] \left(\frac{1+jCV_\Phi^2-t}{1+jCV_\Phi^2\mu_\Phi-t}\right) \prod_{\substack{k=0 \\ k\neq 1}}^{i-1} \frac{(1+kCV_\Phi^2-t)/CV_\Phi^2}{(1+kCV_\Phi^2\mu_\Phi-t)/CV_\Phi^2\mu_\Phi}$$

$$= \mu_\Phi^{i-1} \sum_{j=0}^{i-1} \left[\left(\frac{2-(3+CV_\Phi^2)\mu_\Phi}{1+jCV_\Phi^2-t}\right) - \left(\frac{1-2\mu_\Phi}{1+jCV_\Phi^2\mu_\Phi-t}\right)\right] \prod_{k=0}^{i-1} \frac{(1+kCV_\Phi^2-t)}{(1+kCV_\Phi^2\mu_\Phi-t)}$$

so that with

$$s_i = \sum_{j=0}^{i-1} \left[\left(\frac{2-(3+CV_\Phi^2)\mu_\Phi}{1+jCV_\Phi^2-t}\right) - \left(\frac{1-2\mu_\Phi}{1+jCV_\Phi^2\mu_\Phi-t}\right)\right] \quad \text{and} \quad S_0 = 0 \qquad [23],$$

then

$$\frac{\partial \mu_\Theta}{\partial \mu_\Phi} = \sum_{i=0}^{\infty} \frac{(-\mu_\Lambda)^i}{i!} \mu_\Phi^{i-1} s_i \prod_{k=0}^{i-1} \frac{(1+kCV_\Phi^2-t)}{(1+kCV_\Phi^2\mu_\Phi-t)}$$

$$= \sum_{i=0}^{\infty} \left(\frac{-\mu_\Omega}{\mu_\Phi}\right)^i \mu_\Phi^{i-1} s_i \prod_{k=0}^{i-1} \frac{(1+kCV_\Phi^2-t)}{(1+k)(1+kCV_\Phi^2\mu_\Phi-t)}$$

$$= \frac{1}{\mu_\Phi} \sum_{i=0}^{\infty} s_i p_i (-\mu_\Omega)^i$$

and thus

$$\sigma^2_{CR_n} = \frac{\mu_\Phi^2 \mu_\Theta (1-\mu_\Theta)}{n\left(\sum_{i=0}^{\infty} s_i p_i (-\mu_\Omega)^i\right)^2} \qquad [19].$$

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FIGURE LEGENDS

Figure 1. The NRP as a function of the IC dose for single-dose assays. The NRP μ_Θ for $CV_\Phi = 0.0$ by equation [8] (lower curve) and $CV_\Phi = 0.5$ by equations [13,14] (upper curve) both with $\mu_\Phi = 0.1$ was computed and plotted as a function of the IC dose μ_Ω as explained in the Materials and Methods. Identical results were obtained for equations [13] and [14]. From left to right, the vertical lines indicate the MUD for $CV_\Phi = 0.0$, the MUD for $CV_\Phi = 0.5$ with n = 100, the MVD for $CV_\Phi = 0.0$, and the MVD for $CV_\Phi = 0.5$. These optimum doses are independent of the number n of replicate cultures in the given single-dose assay except for the MUD when $CV_\Phi > 0$ (see Figures 2 and 3 and the Results).

Figure 2. The CRMV as a function of the IC dose for single-dose assays. The CRMV $\sigma^2_{CR_1}$ for $CV_\Phi = 0.0$ by equation [12] (upper curve) and $CV_\Phi = 0.5$ by equation [19] (lower curve) both with $\mu_\Phi = 0.1$ was computed and plotted as a function of the IC dose μ_Ω as explained in the Materials and Methods. Values for $\sigma^2_{CR_1}$ are expressed as the number indicated on the ordinate times 10^{-2}. The MVD for $CV_\Phi = 0.0$ is $\mu_\Omega = 1.59362$ and is independent of μ_Φ, while the MVD for $CV_\Phi = 0.5$ is $\mu_\Omega = 2.60956$ and is dependent upon μ_Φ.

Figure 3. The UAP as a function of the IC dose for single-dose assays. The UAP μ_{Ψ_5} for $CV_\Phi = 0.0$ by equation [10](curve with lower minimum ordinate value) and $CV_\Phi = 0.5$ by equation [17] (curve with upper minimum ordinate value) both with $\mu_\Phi = 0.1$ was computed and plotted as a function of the IC dose μ_Ω as explained in the Materials and Methods.

The MUD for $CV_\Phi = 0.0$ is $\mu_\Omega = 0.69315$ and is independent of μ_Φ and n, while the MUD for $CV_\Phi = 0.5$ is $\mu_\Omega = 0.69703$ and is dependent upon μ_Φ and n.

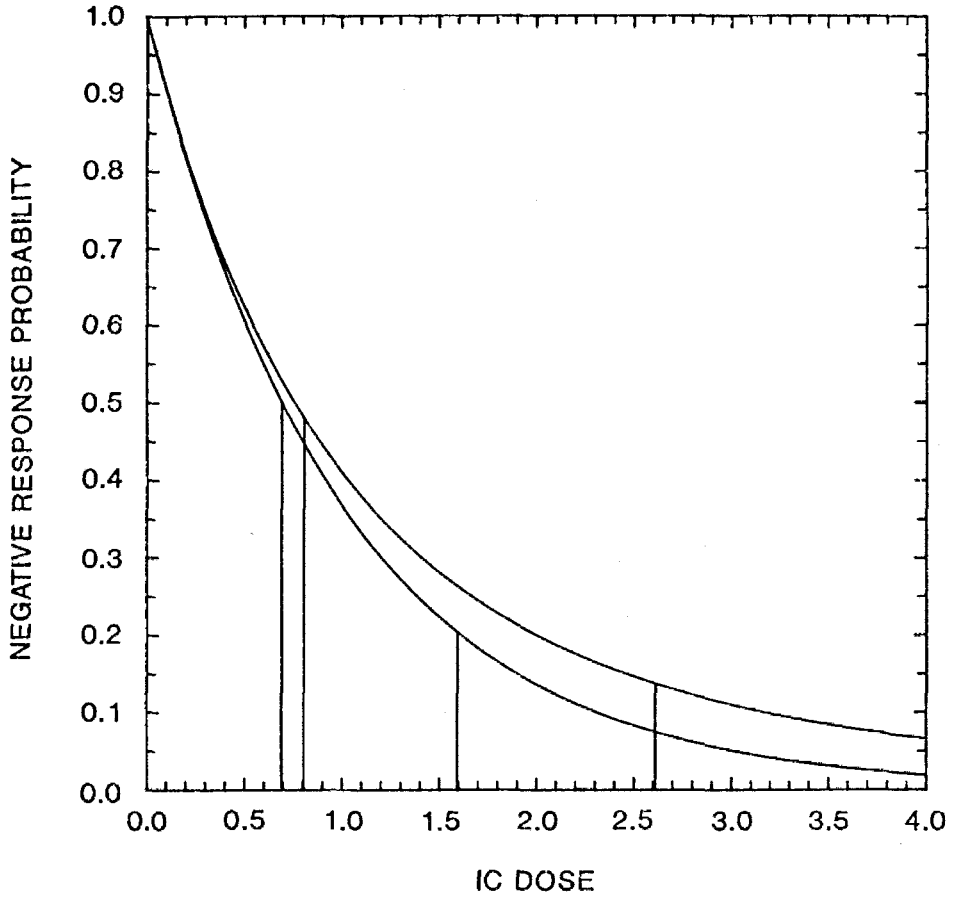
NEGATIVE RESPONSE PROBABILITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
IC DOSE

Figure 1

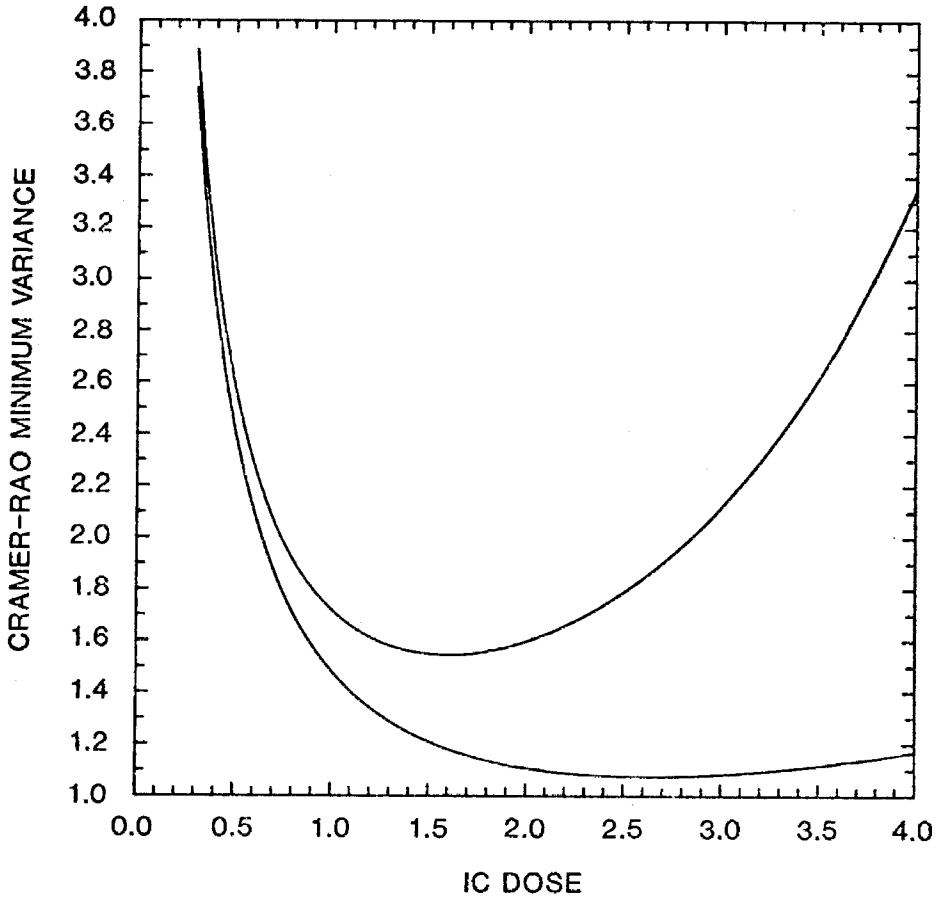
CRAMER-RAO MINIMUM VARIANCE
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
IC DOSE

Figure 2

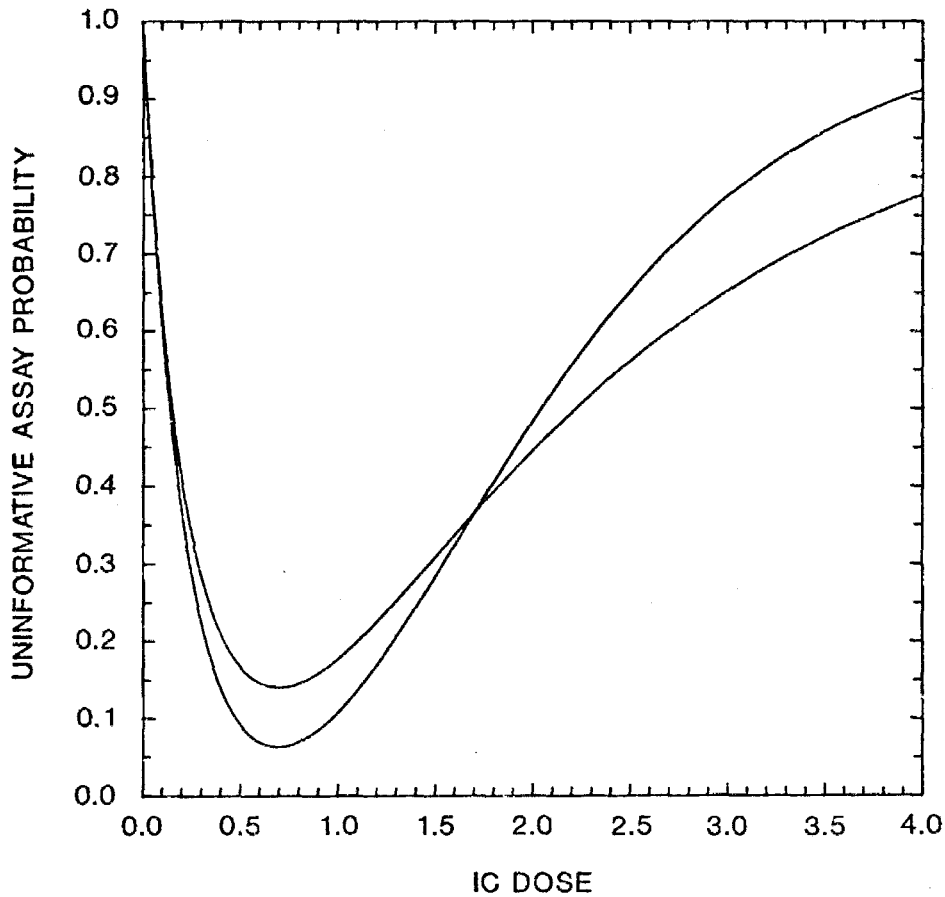
UNINFORMATIVE ASSAY PROBABILITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
IC DOSE

Figure 3

TABLE I

The MVD for various IC frequency prior distributions[a]

CV_Φ	μ_Φ	μ_θ (MVD)	σ_Θ (MVD)	MVD	σ_{CR_1} (MVD)[b]	N (MVD)
0.0	1×10^{-1}	0.20319	0.00000	1.59362	1.24263	154.41
0.1	1×10^{-1}	0.19891	0.03213	1.62803	1.23279	151.98
0.2	1×10^{-1}	0.18692	0.06278	1.73492	1.20395	144.95
0.3	1×10^{-1}	0.16978	0.09073	1.92447	1.15842	134.19
0.4	1×10^{-1}	0.15156	0.11556	2.21066	1.10054	121.12
0.5	1×10^{-4}	0.13832	0.13381	2.55900	1.04180	108.53
0.5	1×10^{-3}	0.13830	0.13385	2.55944	1.04175	108.52
0.5	1×10^{-2}	0.13811	0.13417	2.56383	1.04126	108.42
0.5	1×10^{-1}	0.13637	0.13806	2.60956	1.03643	107.42

[a]The MVD and other assay design parameters evaluated at μ_Ω = MVD were computed for various values of CV_Φ and μ_Φ. The MVD was obtained as the value of μ_Ω that minimizes $\sigma^2_{CR_1}$ which was computed by equations [12,19]. μ_Θ, σ^2_Θ, and N were computed by equations [8,13,14], [9,15,16], and [6], respectively. The standard deviation σ was computed as the square root of the variance σ^2. Identical results were obtained for equations [13] and [14] and for equations [15] and [16].

[b]Expressed as the indicated number times the same exponential power of 10 as that for μ_Φ .

TABLE II

The MUD for various IC frequency prior distributions[a]

CV_Φ	μ_Φ	n	μ_θ(MUD)	σ_θ(MUD)	MUD	μ_{Ψ_n}(MUD)	σ_{Ψ_n}(MUD)	N(MUD)	RE(MUD)
0.0	1×10^{-1}	20	0.50000	0.00000	0.69315	0.00000	0.00000	208.14	0.74189
0.1	1×10^{-1}	20	0.50085	0.03458	0.69384	0.00000	0.00001	207.02	0.73411
0.2	1×10^{-1}	20	0.50219	0.06877	0.69838	0.00003	0.00011	203.35	0.71282
0.3	1×10^{-1}	20	0.50380	0.10226	0.70727	0.00020	0.00119	197.38	0.67988
0.4	1×10^{-1}	20	0.50715	0.13458	0.71755	0.00106	0.00622	190.17	0.63688
0.5	1×10^{-4}	20	0.52157	0.16122	0.70687	0.00344	0.01727	186.61	0.58162
0.5	1×10^{-3}	20	0.52150	0.16125	0.70703	0.00345	0.01729	186.57	0.58167
0.5	1×10^{-2}	20	0.52083	0.16157	0.70862	0.00348	0.01745	186.25	0.58213
0.5	1×10^{-1}	20	0.51329	0.16520	0.72663	0.00388	0.01954	182.73	0.58787
0.5	1×10^{-1}	40	0.50051	0.16711	0.75663	0.00039	0.00582	177.60	0.60483
0.5	1×10^{-1}	60	0.49168	0.16834	0.77792	0.00009	0.00071	174.22	0.61657
0.5	1×10^{-1}	80	0.48501	0.16922	0.79431	0.00003	0.00038	171.75	0.62544
0.5	1×10^{-1}	100	0.47968	0.16989	0.80763	0.00001	0.00023	169.82	0.63254

[a]The MUD and other assay design parameters evaluated at μ_Ω = MUD were computed for various values of CV_Φ, μ_Φ, and n. The MUD was obtained as the value of μ_Ω that minimizes μ_{Ψ_n} which was computed by equations [10,17]. μ_θ, σ_θ, and N were computed as explained in Table I. $\sigma^2_{\Psi_n}$ and RE were computed by equations [11,18] and [7], respectively. The standard deviation σ was computed as the square root of the variance σ^2.

TABLE III

Constrained optimization of the single-dose assay design for a particular IC frequency prior distribution[a]

$\mu_\Theta(\mu_\Omega)$	$\sigma_\Theta(\mu_\Omega)$	μ_Ω	$\mu_{\Psi_{40}}(\mu_\Omega)$	$\mu_{\Psi_{80}}(\mu_\Omega)$	$\mu_{\Psi_{120}}(\mu_\Omega)$	$\mu_{\Psi_{160}}(\mu_\Omega)$	$\mu_{\Psi_{200}}(\mu_\Omega)$	$N(\mu_\Omega)$	$RE(\mu_\Omega)$
0.52498	0.16333	0.7	0.00042	0.00004	0.00001	0.00000	0.00000	187.67	0.57238
0.48272	0.16951	0.8	0.00041	0.00003	0.00000	0.00000	0.00000	170.92	0.62849
0.44466	0.17364	0.9	0.00065	0.00005	0.00001	0.00000	0.00000	158.15	0.67923
0.41031	0.17611	1.0	0.00130	0.00013	0.00003	0.00001	0.00000	148.17	0.72495
0.37925	0.17725	1.1	0.00255	0.00033	0.00009	0.00003	0.00001	140.24	0.76598
0.35110	0.17733	1.2	0.00463	0.00075	0.00023	0.00009	0.00004	133.83	0.80264
0.32554	0.17656	1.3	0.00775	0.00152	0.00053	0.00024	0.00012	128.61	0.83521
0.30228	0.17512	1.4	0.01209	0.00278	0.00107	0.00052	0.00029	124.33	0.86401
0.28110	0.17314	1.5	0.01779	0.00469	0.00198	0.00104	0.00061	120.79	0.88929
0.26176	0.17075	1.6	0.02492	0.00738	0.00336	0.00187	0.00116	117.87	0.91133
0.24407	0.16804	1.7	0.03353	0.01098	0.00535	0.00312	0.00202	115.46	0.93035
0.22788	0.16509	1.8	0.04358	0.01559	0.00804	0.00489	0.00328	113.48	0.94661
0.21302	0.16196	1.9	0.05503	0.02127	0.01153	0.00729	0.00505	111.86	0.96030
0.11937	0.15870	2.0	0.06779	0.02806	0.01590	0.01040	0.00739	110.55	0.97164
0.18682	0.15536	2.1	0.08174	0.03596	0.02121	0.01429	0.01040	109.52	0.98081
0.17525	0.15197	2.2	0.09677	0.04497	0.02747	0.01900	0.01413	108.72	0.98800
0.16457	0.14855	2.3	0.11274	0.05504	0.03470	0.02458	0.01863	108.14	0.99336
0.15471	0.14514	2.4	0.12952	0.06610	0.04288	0.03103	0.02393	107.74	0.99705
0.14559	0.14174	2.5	0.14697	0.07810	0.05200	0.03836	0.03004	107.50	0.99922
0.13715	0.13838	2.6	0.16497	0.09096	0.06200	0.04655	0.03697	107.42	0.99999
0.12931	0.13507	2.7	0.18339	0.10457	0.07283	0.05556	0.04470	107.47	0.99950

[a]Assay design parameters evaluated at μ_Ω for $CV_\Phi = 0.5$ with $\mu_\Phi = 0.1$ were computed as explained in Tables I and II. See the Results and Discussion for explanations and cautions about the use of this assay design optimization table.