Instrumentation and Data Reduction for Radioassay

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This is the final article in a continuing education series on radioimmunoassay. After reading and studying this article, the reader should be able to: 1) discuss instrumentation available for RIA; 2) compare and contrast various methods of data reduction for RIA; and 3) describe quality assurance parameters of curve fitting.

During the past 10 years, the instrumentation available for RIA has evolved rapidly. In 1975, the state-of-the-art was a single NaI crystal with associated photon energy selection electronics coupled to a snaking arrangement of sample holders that sequentially counted a large series of tubes. Data analysis typically used a standards curve drawn through an eyeball french-curve technique or a logit-log transformation that, hopefully, created a straight line standards curve. Presently, a modern RIA system can be simply a series of reagent bottles connected to a microcomputer that performs the biochemistry, counts the tubes, and analyzes the results. The trick is to acquire a properly designed automated system with the reasoning power of an experienced technologist. Although the automated systems have many advantages (notably speed and low recurring costs), there are also disadvantages such as initial system cost, lack of adaptability to new assays, or lack of knowledge about the accuracy of the standards curve which results from the black-box nature of the computer system. The user contemplating purchase of an RIA system would be advised to consider the biochemistry equipment, the counter, and the analysis computer as an integral package. This article will review these components of an RIA system separately.

AUTOMATED AND MANUAL BIOCHEMISTRY: A COMPARISON

If funding is not a problem, then the average RIA laboratory could make good use of one of the automated RIA systems (*1*). These automated systems perform the entire assay—from serum sample to patient results. These systems typically consist of the automated reagent system for the RIA, coupled to NaI detector, all controlled by a microcomputer. There are two basic types of automated RIA systems. One is the batch system which is an automated process for handling a coated tube assay. The system automatically pipets the sample and combines it with reagents, incubates the reaction tube and, after separating free from bound, counts the tube and then calculates the results. The second type of system is a cycle-type process. In this case, the sample is combined with reagent in a continuous flow line which allows ligand and antibody to react. The sample is then passed through a sold phase reuseable antibody chamber for the purpose of separating bound and free activity. The separated fractions each pass through the counting coil where a microcomputer stores the information and later assembles the calculations of the assay. The entire system is then recycled in preparation for the next consecutive sample. The throughput of the two systems will vary because of the incubation time in the batch system as compared to the cycle time of the cycle system. Depending on the length of the assay runs involved, this factor may be a significant consideration.

The principal advantages of automated RIA are as follows:

1. The assay is automated so that less technologist skill is required than that of a manual assay.
2. The automation frees the technologist to perform other functions.
3. The cost of an assay is less than that of the manual method because of reagent savings and labor costs.
4. The precision and accuracy is comparable to and, in some cases, better than that of the manual method.

The principle disadvantages include:

1. The initial equipment purchase may be cost-prohibitive.
2. A limited number of assays are offered by the various manufacturers.
3. New assays cannot be added by the user.
4. Other manufacturers' reagents or methodologies cannot be used.

It would appear, therefore, that an automated analysis system is most suitable as one of the methods, but not the only method, for RIA in a hospital where skilled technologists are available. A hospital with technologists less experienced in RIA could use an automated system to begin an RIA program for the commonly available thyroid and digoxin assays.

NUCLEAR COUNTING SYSTEM

A single or dual NaI well counter is typically used in automated RIA analysis. For those hospitals performing the RIA biochemistry by manual methods, a multi-detector system greatly increases throughput. One manufacturer* offers a 20-well system so that 200 tubes can be counted for 1 min each in only 10 min. This system requires technologist attention to sequentially load racks of 20 tubes. Alternatively, the

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user can purchase a system with fewer detectors, typically four, and an auto sample changer. The advantage of an auto sample changer is that it frees the technologist to perform other tasks. For assays with fewer than 100 tubes, the speed advantages of a 20-well counter probably outweigh the advantages of an auto sample changer. Of course, any auto sample changer invites mechanical failure, and the potential user of such equipment is advised to contact other current users to assess reliability.

Regardless of the type of detector selected, it is necessary to evaluate the ease of pulse height analysis (window) setting. A multi-channel analyzer spectrum display with highlighted window channels would be most desirable. This type of display provides rapid and easy visual assessment of window setting and is more convenient than the old single-channel analyzer with its confusing array of controls for amplifier gain and number of channels per keV. Some of the newer counting systems are computer controlled, and the user only has to specify the radionuclide, typically ^{125}I or ^{57}Co. The computer then selects an appropriate window but may not display spectrum information. This black-box window setting, while very convenient, can make it difficult to insure adequate detector quality assurance. Any multi-well counter must have the provision to calibrate the energy scale for each detector and balance each detector to have equal counting efficiency. A method of monitoring this calibration and detector balancing should be provided.

Lastly, the interface between the detector and the analysis system should be considered. A counter which simply prints raw counts and leaves the user to enter the data by hand into the analysis system is most tedious. Many counters provide output compatible with a teletype or other printer and paper tape which can then be read by the analysis system.

CURVE FITTING

The task of curve fitting for RIA standards is universal, regardless of which type of biochemistry or counter system is chosen. The data consist of approximately eight standard data points that represent an antigen concentration (x-axis) and percent bound (y-axis). Percent bound (%B) is generally taken as the standard counts compared to the total counts added to each tube although percent counts compared to B_0 may also be used. The task is to join the standard points with a smooth curve and to read patient concentrations from this curve based on the patient %B. Mathematically, the task is to find the function (F) of concentration (C) that adequately describes the standards data, %B = F(C), both at and between the standard data points. The literature (*2–4*) contains discussions that attempt to create a mathematical formula based on the biochemical processes involved in RIA. In a radioimmunoassay, the patient sample antigen (Ag) competes in the test tube with added radioactively labeled antigen (Ag*) for a limited amount of added antibody (Ab):

$$Ag + Ab + Ag^* \rightleftharpoons AgAb + Ag^*Ab + Ag + Ag^*$$

The bound antigen-antibody complexes are counted in a scintillation counter following chemical separation from the remaining free antigen. As concentration of patient antigen Ag (the x-axis) increases, the radioactive bound fraction Ag*Ab (the y-axis) will decrease because of competitive binding between Ag and Ag* for the antibody. An alternative assay procedure is an immunoradiometric assay (IRMA), which involves the addition of excess radiolabeled antibodies:

$$Ag + Ab^* \rightleftharpoons AgAb^* + Ab^*$$

Here the radioactive bound fraction is proportional to the patient antigen level so that the %B increases as concentration increases. Theoretical models for these processes have been proposed based on either mass action theory (the Scatchard plot), dilution principles, or probability analysis (*2*). These models lead to equations that explain, at least for some assays, the shape of the standards curve in a theoretically satisfying manner. These theories do not guarantee a more accurate or precise standards curve than the following empirical approach: If the fitting function appears to adequately describe the standards data, then use it. Certainly, it would be preferable if one theory could completely explain all of RIA, but the wealth of assumptions in the theories (e.g., first order kinetics, univalent antibodies, lack of allosteric effects on binding, and independence of binding on antigen concentration) and the vast array of different formats for RIA kits suggests that no one theory is likely to explain all of RIA. We have followed the empirical approach by treating the standards curve as a smooth and slowly varying function of concentration.

Given a set of %B and C data values, the goal is to find the best fit curve using the following formula:

$$f(\%B) = F\,[g(C)]$$

The curve is defined by the functions f, F, and g, which are determined empirically by inspection of the shape of the standards plot. The least squares, or regression method, is then used to solve for parameters in these functions (*5,6*). Historically, several fitting functions have been found to provide good results (*2*). Three classes of fitting functions (logit-log, polynomial, and spline) will be discussed.

Logit-Log

The first method used for analysis of RIA data was logit (%B) as opposed to log (C) as a linear least squares straight line fit. The logit (%B) is defined as log [%B/(100 − %B] or log (%B/% Free), where log is the natural log function. The logit was popularized for various statistical purposes earlier in this century (*7*). In this method, the function f is the logit function, g is the natural log function, and F is a straight line with Y-intercept a_0 and slope a_1:

$$\text{logit}\,(\%B) = a_0 + a_1 \log\,(C)$$

Or let Y = logit (%B) and X = log (C):

$$Y = a_0 + a_1 X$$

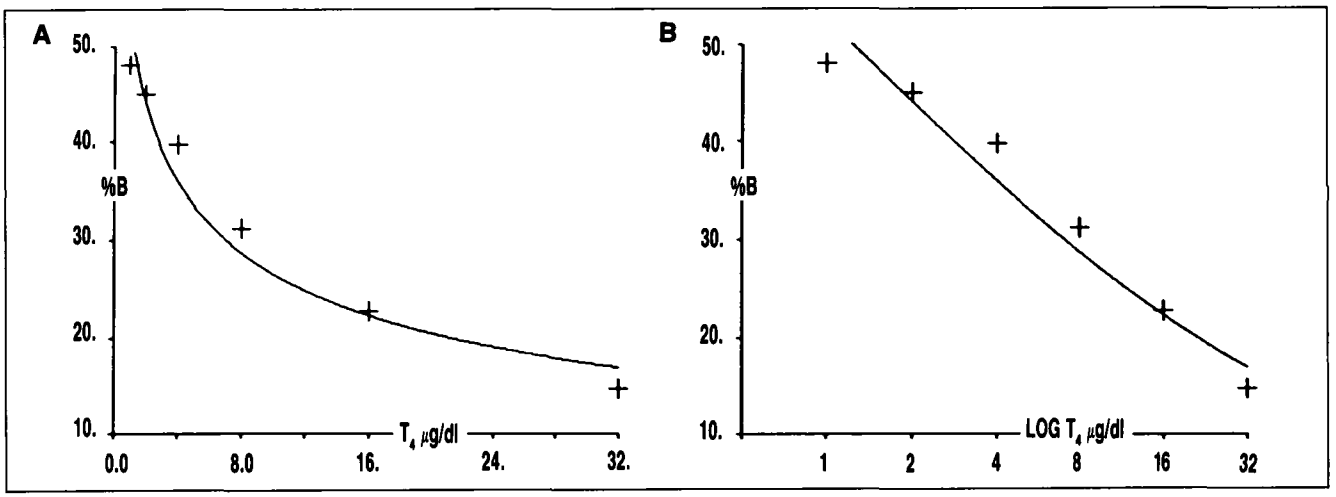

FIG. 1. A logit-log fit to the T_4 standard data as listed in Appendix A, B, and Table 1. (A) A linear x-axis is convenient to read, but it results in uneven x-axis spacing between the data points. (B) A log x-axis, which is not as convenient to read, equalizes the x-axis spacing for standard concentrations that are a common multiple (e.g., 2×) of each other.

The best fit values of a_0 and a_1 are found by the least squares technique of minimizing the sum of the squares of the differences in %B between the standard data points and the straight line fit. The a_0 and a_1 (which define the straight line fit) are the values which minimize the quantity:

$$X^2 = \sum_{i=1}^{N} [(i\text{th } \%B \text{ data value}) - (\%B \text{ on the fitted line at } i\text{th conc})]^2$$

or

$$X^2 = \sum_{i=1}^{N} [Y_i - (a_0 + a_1 X_i)]^2$$

In this instance, there are N standard data points. The equations for a_0 and a_1 are straightforward and easily amenable to calculation on a preprogrammed pocket calculator or on a microcomputer in BASIC. Figure 1 shows a logit-log least squares fit to T_4 data. The fitted curve is shown on both a linear x-axis (Fig. 1A) and with a log x-axis (Fig. 1B). The log x-axis is preferred by some people since it equalizes the x-axis distance between the standard data points. Appendix A shows the mathematical details, and Table 1 shows the predicted concentrations for the standards. A similar analysis, though less precise, can be obtained using logit-log graph paper and an eyeball best fit straight line.

Once the standards curve is determined, the patient concentration for some %B is obtained by inverting the fitting equation:

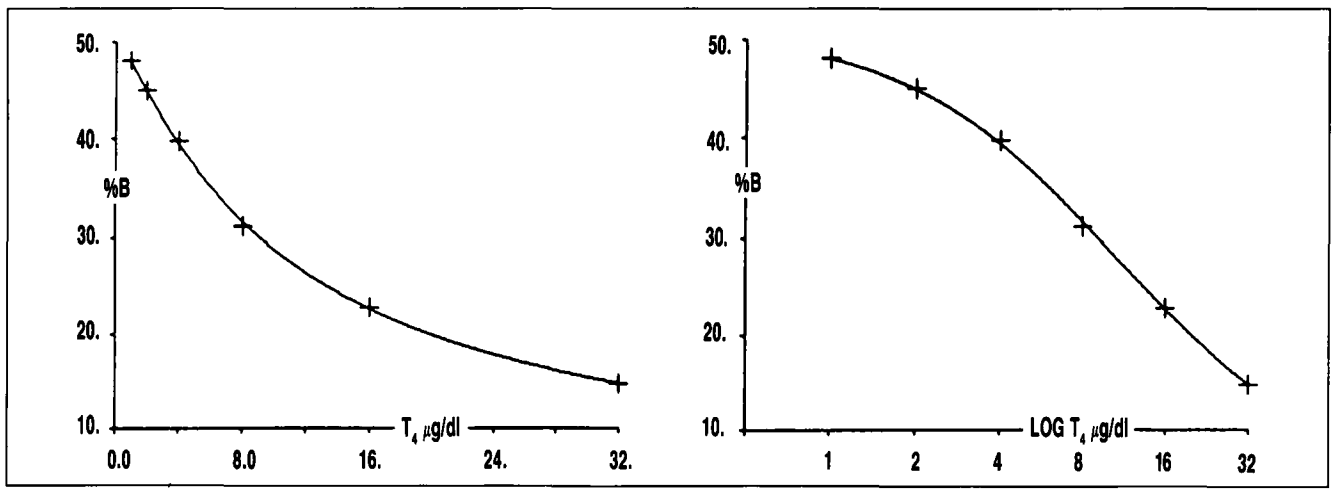

FIG. 2. A four-parameter or end-point adjustment logit-log defines the standards curve (see Appendix C).

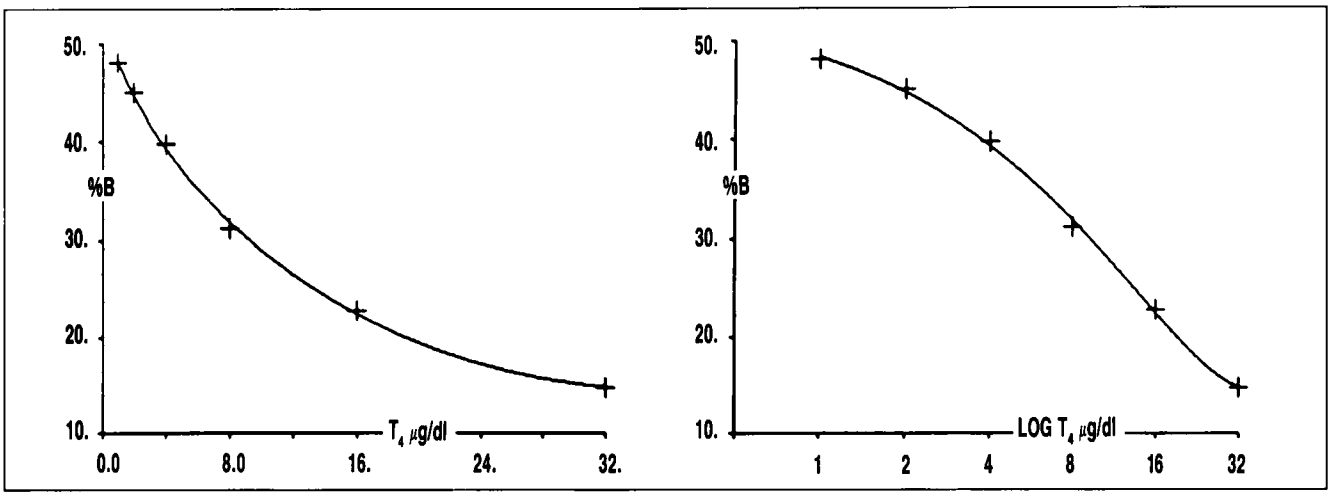

FIG. 3. A cubic polynomial on the square root of concentration defines the standards curve (see Appendix D).

$$C = \exp \{[\text{logit } (\%B) - a_0]/a_1\}$$

The pocket calculator or microcomputer can be used to calculate the concentration predicted by the straight line for some %B (see Appendix B). Unfortunately, the simple logit-log transformation often does not fit the data well as seen in figure 1. A point may appear close to the logit-log fit, but because of logarithmic compression of the x-axis, this small distance of the data point from the line may result in a large (10–20%) inaccuracy in concentration. This method will always produce a "best fit" line, but if the data are not truly linear then the logit-log fit is unacceptable. The data points in figure 1B clearly appear to have structure not described by the logit-log fit. We have completely abandoned the use of the simple logit-log transform in favor of more powerful methods.

An improvement over the simple logit-log, referred to as four-parameter logit or end-point adjustment, is to modify the method to account for its extreme sensitivity to end points of the data by using:

$$\text{logit } [(\%B - \text{BMIN})/(\text{BMAX} - \%B)] = a_0 + a_1 \log (C)$$

In this instance, the fitted curve is described by four parameters to be found by a least squares analysis (BMIN, BMAX, a_0, a_1). The parameter BMIN is related to the nonspecific binding in the RIA, and BMAX is related to the %B at zero concentration (*8,9*). The assay need not include a measure of BMIN or BMAX. The initial values of BMIN and BMAX are estimated from the minimum and maximum %B in the standards data set, and these parameters are then adjusted iteratively (adjust and retry the fit until the answers converge to a best fit). The computer defines the four parameters of a best fit curve. Figure 2 shows the T_4 results with a standards curve that is much improved over the simple logit-log in figure 1. This fitting equation results in a nonlinear curve which appears to accurately describe the data points. Table 1 and Appendix A contain mathematical details. This method is used by several of the automated analysis systems,† and has proven to be satisfactory for T_3 RIA, T_4, and digoxin assays. This method requires use of a microcomputer or programmable calculator since it involves more complex calculations than the simple logit-log.

An additional important point is to use a weighted least squares fit, which means that data points which lie far from the fitted line (outliers) are given less importance in calculating the best straight line fit. This factor prevents outliers from unduly influencing the standards curve (*10*). Another useful weighting technique is to allow standards in the mid-range of concentration to have more effect on the curve than that of standards at the low and high end.

Regardless of the logit-log curve fitting adopted, it is necessary to carefully inspect the entire curve fit and to perform quality assurance checks on the goodness-of-fit by examining the correlation coefficient and standard error of the estimate (SEE) as detailed below. It is far too easy to accept an inappropriate curve (such as Fig. 1) just because it was produced by the computer.

Polynomial Curve Fitting

The agreement between the standard data points and the curve is governed by the possible curve shapes allowed by the fitting function. If the data are not on a straight line in logit-log space, then a logit-log fit will never be as accurate as a curvilinear approach in which the curve is not restricted to a straight line shape. Polynomials have often been used to describe the shape of the standards curve, which often resembles (all or only a segment of) a half bell-shaped, sigmoid, or dose response curve (*11*). A polynomial of order n is defined as:

$$Y = a_0 + a_1X^1 + a_2X^2 + \ldots + a_nX^n$$

or

$$Y = \sum_{i=0}^{n} a_iX^i$$

The coefficients a_i define the best fit curve. A straight line is a first order polynomial, a quadratic is a second order polynomial, and a cubic is a third order polynomial. Polynomials are popular as fitting functions because the shape of the curve is governed by the order of the polynomial in a regular fashion, and because the least squares regression equations for a polynomial are solvable for the parameters a_i. It is known that an (n-1)th order polynomial exactly fits n data points (e.g., a straight line or first order polynomial passes exactly through any two data points). It is therefore important to avoid choosing too high an order polynomial which may lead to overfitting or unrealistic wiggles in the curve. If the standards data are flatter at low and high concentrations, with a steeper response at middle concentrations, then a cubic polynomial is suggested to follow this curve shape. A straight line could not follow this shape, nor could a quadratic which has a constant curvature. A polynomial of order higher than a cubic is not necessary. Experience over the past 10 yr (*2,11*) has suggested that a cubic polynomial on the square root of the concentration will provide a satisfactory curve fit to all assays we have tried, which include a variety of RIA and IRMA assays (B-12, CEA, digoxin, ferritin, folate, gastrin, gentamicin, prolactin, T_3, T_4, and TSH). The task is to find the coefficients a_0–a_3 for the best fit cubic in the following equation:

$$\%B = \sum_{i=0}^{3} a_i \, (C^{0.5})^i$$

The least squares technique yields four equations in the four unknown coefficients. Obtaining a solution requires a computer program, and such software is readily available in the literature (*5*) or as a commercial package from microcomputer software vendors. Figure 3 shows a cubic fit for the same T_4 data as in figures 1 and 2. Table 1 shows the concentration values predicted for the standards.

Upon determining the best fit, it is necessary to carefully inspect the fitted curve and to examine the goodness-of-fit parameters including correlation and SEE. If the curve fit is acceptable (as described below), then the equation is inverted to find the patient concentration based on the measured %B (*12*). Appendix D contains additional details. This factor is analogous to solving a quadratic equation with two roots with the exception in this instance of a cubic with three roots, or three possible answers for the concentration for any given %B. Deciding which of the three roots to use for the patient concentration divides into two possible categories, depending on the coefficients a_0–a_3. If the solution for the three concentrations yields one real root and two imaginary roots, then the real root is chosen as the patient concentration. If the solution for the three concentrations yields three real roots (two of which may be identical), the cubic curve has a maximum (it humps up) or a minimum (it dips down) value so that further consideration is necessary to properly choose the correct concentration. These considerations will be handled by the computer program involved, but the user is ultimately responsible for insuring that the standards curve has no minimum or maximum within the range of concentration of the standards. We have termed a minimum or maximum of the cubic as pathological curve behavior. It generally occurs at very low or very high concentrations, in which the standards curve shows little slope. The problem is best avoided by using linear interpolation between the two lowest and the two highest standards (*2*).

In this instance, a weighting procedure may be helpful in removing the undue effects of outliers, but we have not found this to be necessary. Nor is it trivial to decide on a proper weighting method for a cubic fit. One of the commercially available auto counter and microcomputer systems* uses polynomial regression for the standards curve. We have found that it produces satisfactory standard curves for all the assays listed above under polynomial curve fitting. Another system‡ offers both polynomial regression and a four-parameter logit-log regression.

Spline Fitting

A spline is defined mathematically as a function that joins the standard data points in a connect-the-dots fashion with a smooth curve between each pair of data points. By definition, the spline curve, unlike a regression fit which attempts to fit a specified function of restricted shape to the data points, passes precisely through each standard data point. The spline is usually a cubic between the data points, which gives it sufficient freedom to wriggle and change slope between data points. The spline function, for instance, will pass through any data point and includes replicates with the same concentration but different %B. Splines are commonly used to form highly irregular contours such as the borders of a geographical map or the outline of an automobile shape in three dimensions. It is this pliable nature that dooms the spline as far as RIA is concerned. We believe that the assay biochemistry is represented by a curve between data points that is free of up-down wiggles. We also realize that each standard data point is contaminated by systemic error (e.g., pipette error) and by statistical noise so that requiring the standard curve to actually pass through each data point is unrealistic.

Quality Assurance for Curve Fitting

It is mandatory to perform a visual examination of the standard curve plot with the data points and curve clearly shown as in figures 1–3. A simple printout of the predicted concentration for each standard (as in Table 1) is not sufficient because the goodness of the curve fit between the data points must be evaluated by the experienced eye. This printout can be of critical importance if the method chosen is polynomial regression in which the curve can hump up or dip down bizarrely between data points. For fits based on simple logit-log, it is important to insure that an outlier has not unduly shifted the curve. Furthermore, the data should actually appear to lie on the fitted curve rather than as illustrated in figure 1. Remember that the curve fitting methods will always produce a best fit line, but this is no guarantee that the line truly describes the data points.

If the visual examination is satisfactoy, the user should proceed to examine the correlation coefficient (r) and the SEE

TABLE 1. T_4 Concentration μg/dl*

Standard Data Value	Model Prediction: Logit-Log	Four-Parameter Logit-Log	Cubic Polynomial
1	1.44	0.99	1.07
2	1.85	1.95	1.89
4	2.90	3.90	3.85
8	6.25	8.26	8.43
16	15.3	15.8	15.6
32	44.8	32.1	32.4
	Goodness-of-Fit		
r	0.975	0.999	0.999
SEE	3.4	0.4	0.7

*In the T_4 concentration data with the results predicted by three different curve fitting methods, the logit-log fit is inferior to the four-parameter logit and cubic polynomial as judged by the SEE. The curves are illustrated in figures 1–3.

or S_{yx}, both of which are useful goodness-of-fit parameters (*13,14*). The magnitude of the correlation coefficient (i.e., ignoring its sign) describes the strength of the relationship between concentration and %B; 1 is a perfect fit and 0 implies no relationship. The closer the correlation is to 1, the better the fit. The correlation coefficients from figures 1–3 are 0.975, 0.999, and 0.999, respectively. Figure 1 is not as good a fit as figures 2 and 3, but the correlation appears to be less sensitive than a visual appraisal of the differences in these fits. The correlation should be monitored from one day to the next with lowered correlation (beyond −2 s.d. of previous results) indicating a cause for concern. Checking the probability value of the correlation is of little help since a significant probability will always result from any curve that your eye would accept.

The SEE is a similar but slightly more descriptive goodness-of-fit parameter which describes how close the data points are to the fitted curve. Crudely, the SEE can be thought of as the average vertical distance on the standards plot between the data points and the fitted curve. For example, figure 1 has a SEE of 3.4% which indicates that the average data point disagrees with the fitted curve by 3.4 %B units. A SEE of zero is a perfect fit (the curve passes through each point), and any points far from the curve will quickly cause a large increase in the SEE. The SEE is a more sensitive indicator of goodness-of-fit than correlation. The SEE for figures 2 and 3 is 0.4 and 0.7, respectively. The SEE should be monitored just like any RIA quality assurance parameter, perhaps by Levey-Jennings plots, which compares today's results to previous results.

The coefficients in the fitting equation can also be monitored as quality assurance (QA) parameters for logit-log in which the intercept and slope have simple geometric interpretations.

Many software vendors may generate their own particular QA parameters that are probably related to the correlation or SEE. It is advisable to monitor these vendor parameters after deciding which parameters correspond most closely to correlation and SEE.

CONCLUSION

Choose an analysis system with both polynomial regression and end-point adjustment logit-log when available. The polynomial may be more versatile, but the end-point adjustment logit is not subject to unwanted slope changes between data points as is the polynomial (*15*). Experiment with the software on various assays and adopt the fitting method that produces the most satisfactory curve fit (best correlation and smallest SEE). Be sure to include a visual judgment of the entire curve fit. Beware of straight line fits to nonlinear data and strange kinks at the low and high end of polynomial fits. A simple printout of results for the standard data points is insufficient. The system should also allow calculations for a variety of assays that do not require curve fitting such as T_3 uptake and a variety of hepatitis assays. Software can be purchased from commercial RIA vendors,*†‡ or users with FORTRAN imaging computers are welcome to adopt our RIA software.

The software should include an easy method of modifying existing assay protocols and of adding new assays that were not previously considered by the software vendor. Do not take the word of the vendor in this matter without an actual demonstration of adding a new assay. Black-box software, which cannot be modified by the user, is unacceptable.

If the analysis software should be interfaced directly to the nuclear counter, the user must consider how to transport data from the counter to the analysis computer. This is usually accomplished by cumbersome paper tape or keyboard entry of data. An auto analyzer system should handle all data from beginning biochemistry to final concentration printout. The more advanced department should consider interfacing the RIA computer to the hospital's main computer for immediate transmission of RIA results to the terminals on the patient wards.

FOOTNOTES

*NML, Irving, TX.
†Becton-Dickinson, Orangeburg, NY.
‡Micromedic Inc., Horshaw, PA.

APPENDIX A
T_4 Data

The %B for the standards is calculated after background subtraction as percent of total. Other assays may use percent of B_0. Standards are not replicated to keep the details simple.

T_4 Concentration (μg/dl)	(%B)
1	47.9
2	44.9
4	39.6
8	31.3
16	22.7
32	14.8

APPENDIX B
Logit-Log Fit

The least squares straight line fit is made to Y = logit (%B) versus X = log (C). Example calculations for the C = 4 standard (where %B = 39.6) are as follows:

$$X = \log (4) = 1.39 \text{ (where log is natural logarithm)}$$

$$Y = \text{logit } (39.6) = \log [39.6/(100 - 39.6)] = -0.422$$

X = log(C)	Y = logit(%B)
0.0	−0.0841
0.693	−0.205
1.39	−0.422
2.08	−0.795
2.77	−1.23
3.47	−1.75

$Y = a_0 + a_1X$ by least squares (*9*)

intercept	a_0	= 0.0937
slope	a_1	= −0.485
correlation	r	= 0.975
std. err. est.	SEE	= 3.4

Figure 1 shows the curve generated by these parameters. The SEE is unacceptably large. An example calculation of straight line prediction of concentration for %B = 39.6 (C = 4 standard) is as follows:

$$\text{logit}\%B = a_0 + a_1 \log C, \text{ find C given } \%B = 39.6$$

or

$$C = \exp [(\text{logit}\%B - a_0)/a_1]; \text{ substitute } a_0 \text{ and } a_1 \text{ from above}$$

$$C = \exp [(-0.422 - 0.0937)/-0.485]$$

$$C = \exp [1.06]$$

$$C = 2.9 \ \mu g/dl$$

All predicted standard concentrations are tabulated in Table 1. The concentration for patients would be calculated in the same way.

APPENDIX C
Four-Parameter Logit-Log

This formula finds a curve based on four parameters: BMIN, BMAX, a_0, a_1. Note that BMIN and BMAX need not be measured in the assay.

$$\log [(\%B - \text{BMIN})/(\text{BMAX} - \%B)] = a_0 + a_1 \log (C)$$

The computer generates the best fit parameters (*6*) as follows:

BMIN	=	3.50
BMAX	=	51.0
a_0	=	2.65
a_1	=	− 1.10
r	=	0.999
SEE	=	0.4

Figure 2 shows the curve generated by these parameters. Sample calculation of concentration predicted for the C = 4 standard, where %B = 39.6 (i.e., find C given %B = 39.6) is as follows:

$$C = \exp \{[\log((\%B - \text{BMIN})/(\text{BMAX} - \%B)) - a_0]/a_1\}$$

$$C = \exp \{[\log((39.6 - 3.5)/(51.0 - 39.6)) - 2.65]/-1.1\}$$

$$C = \exp \{[\log(3.17) - 2.65]/-1.1\}$$

$$C = \exp \{[1.15 - 2.65]/1.1\}$$

$$C = \exp \{1.36\}$$

$$C = 3.9 \ \mu g/dl$$

Concentrations for other standards (Table 1) and patient data are calculated similarly.

APPENDIX D
Cubic Polynomial

The formula for a curve based on four parameters a_0–a_3 is as follows:

$$\%B = \sum_{i=0}^{3} a_i (C^{0.5})^i$$

The computer generates the following best fit parameters:

a_0	=	55.89
a_1	=	− 6.708
a_2	=	− 1.191
a_3	=	0.1935
r	=	0.999
SEE	=	0.7

Figure 3 shows the curve generated by these parameters. A sample calculation of the predicted concentration for the C = 4 standard, where %B = 39.6, is not detailed here. The calculation is detailed elsewhere (*10*), and is not generally attempted by hand calculation because of the complex algebra. Basically, one must solve for X, the square root of C, in the equation:

$$39.6 = 55.89 - 6.708 \times -1.191X^2 + 0.1935X^3$$

The solution X = 1.962 satisfies this cubic equation so that:

$$X = 1.962 = \text{square root (C)}$$

or

$$C = (1.962)^2$$

$$C = 3.9\ \mu g/dl$$

In general, there are three values of X that satisfy the cubic equation. The software must eliminate imaginary values and choose a real value in the range of concentrations used in the assay. Table 1 shows the results for all the standards.

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