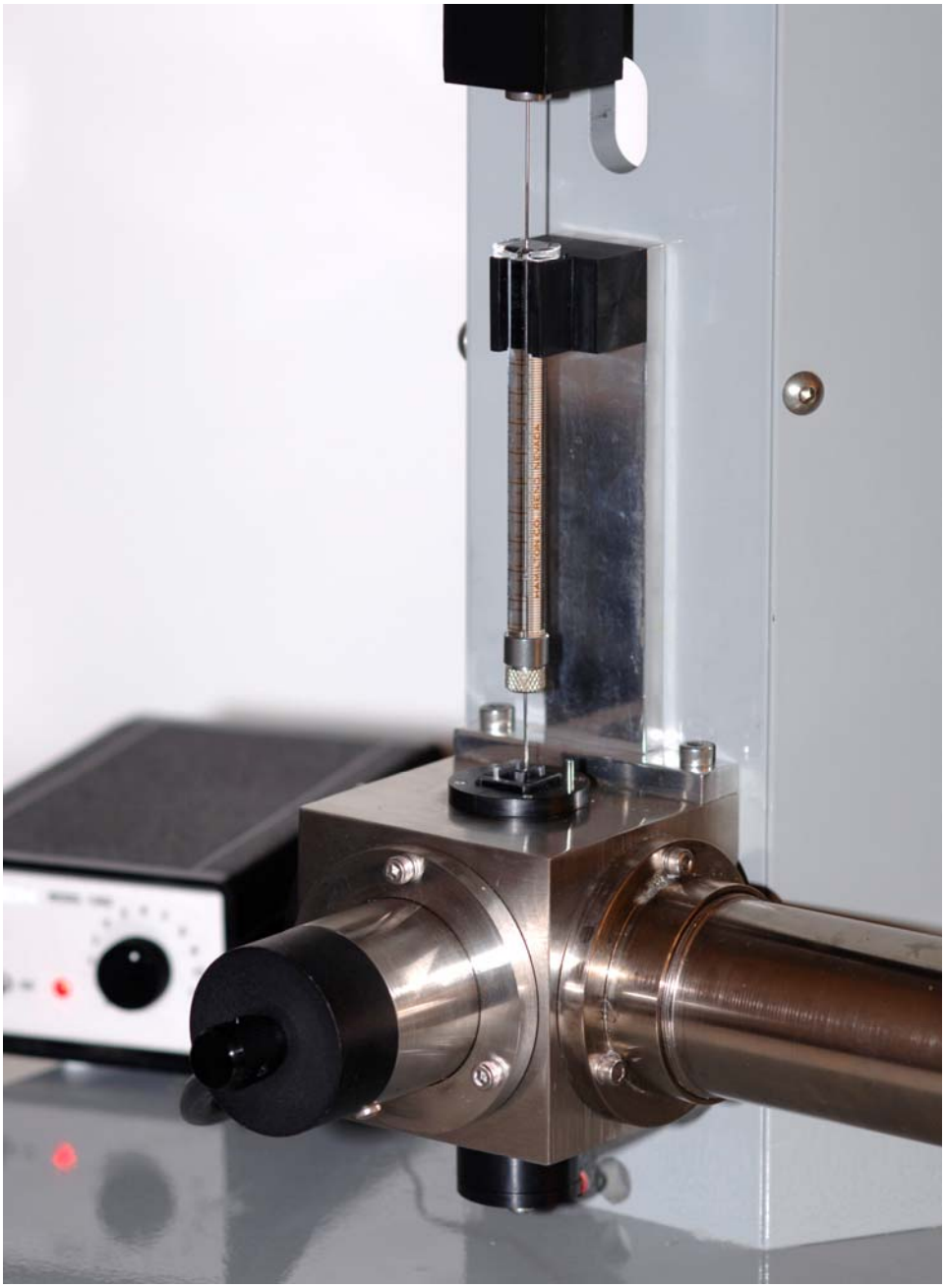
KinTek Corporation

TMX Titration Module

Instruction Manual



June 4, 2005

Introduction

Equilibrium titration data are often as important as kinetic data in working to establish a reaction mechanism and can help to decipher stopped-flow signals. The KinTek TMX Titration Module fills the need for an advanced, automated system to titrate reactions at equilibrium. For enzymology a well designed titration can define both the K_d for the binding of the ligand as well as the concentration of active sites.

Taking advantage of the smooth drive of the SF-2004 servo motor, the TMX unit allows the slow addition of titrant from a 10, 25 or 50 µL syringe into a 200 µL sample that is constantly being stirred. Simultaneously, the system records absorbance or fluorescence (or both, up to 3 channels), to provide a complete titration curve with 1000 points, in a minute. The volume of titrant added and the time of data collection are fully programmable to cover a range of concentrations and times required for the reaction to come to equilibrium.

The TMX allows the full functionality of the SF-2004 data collection including up to 3 channels of simultaneous data recording. For example, two channels of fluorescence can be recorded to compute fluorescence annisotropy as a function of titrant concentration. The TMX comes complete with thermostated cell holder, a 5 x 5 mm cuvette, a miniature stir bar and a stirring motor drive system. Simply attach it to your SF-2004 Stopped-Flow motor drive and begin collecting data.

The program includes sophisticated data fitting routines that must be applied properly to obtain valid conclusions. For more information on data fitting as well as in the design and interpretation of equilibrium and kinetic experiments, Dr. Kenneth A. Johnson teaches a 3-day intense tutorial each year. See the KinTek website for details (www.kintek-corp.com).

Instrument Setup

Refer to the photographs on the following page.

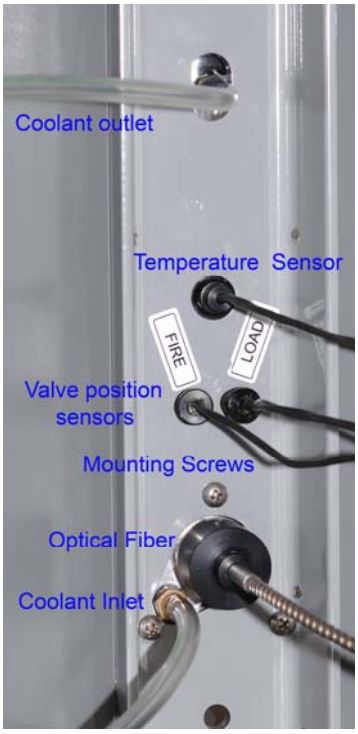
1. Remove the detectors from the stopped-flow block and set them aside. Remember to make sure the high voltage to the photomultiplier tube (PMT) is turned off before removing the PMT and to cover the PMT to protect it from ambient light. We recommend removing the PMT in a darkened room and then covering the end of the PMT with aluminum foil.

2. Disconnect the water lines, the temperature sensor, optical fiber, and then remove the three screws from the back of the stand holding the stopped-flow block in place and set it aside.

3. Attach the TMX module to the stopped-flow stand using the three screws, and then attach the circulating water lines to the inlet and outlet ports on the back and side, respectively. Attach the optical fiber cable, being careful to line up the slot with the pin on the top.

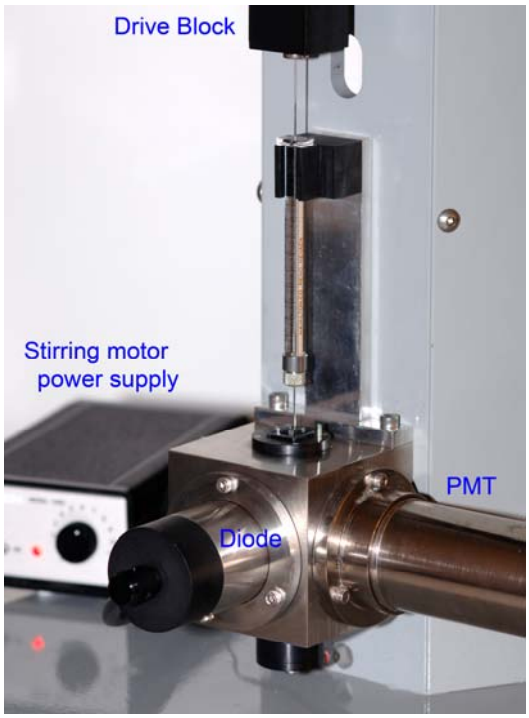
4. Attach the TMX Drive Block to the syringe drive platform. Attach the PMT and photodiode ("Diode") detectors to the TMX cell holder, placing the appropriate filter in front of the PMT for fluorescence detection. Note that two PMT detectors can be used with polarizing filters to collect fluorescence annisotropy, if desired. Plug in the stirring motor controller. Place the 5 x 5 mm cuvette into the holder, insert the 1.5 x 2 mm micro stir bar. After the sample is added to the cuvette, turn on and adjust the motor speed to a recommended setting of 8-10.

Step 1. Remove detectors from SF Cell by unscrewing the three socket head screws using the 7/64" hex driver provided.



Step 2. Remove water line, temperature sensor and optical fiber, and then remove the three mounting screws holding the SF block.

Step 3. Attach TMX block, optical fiber and cooling line.

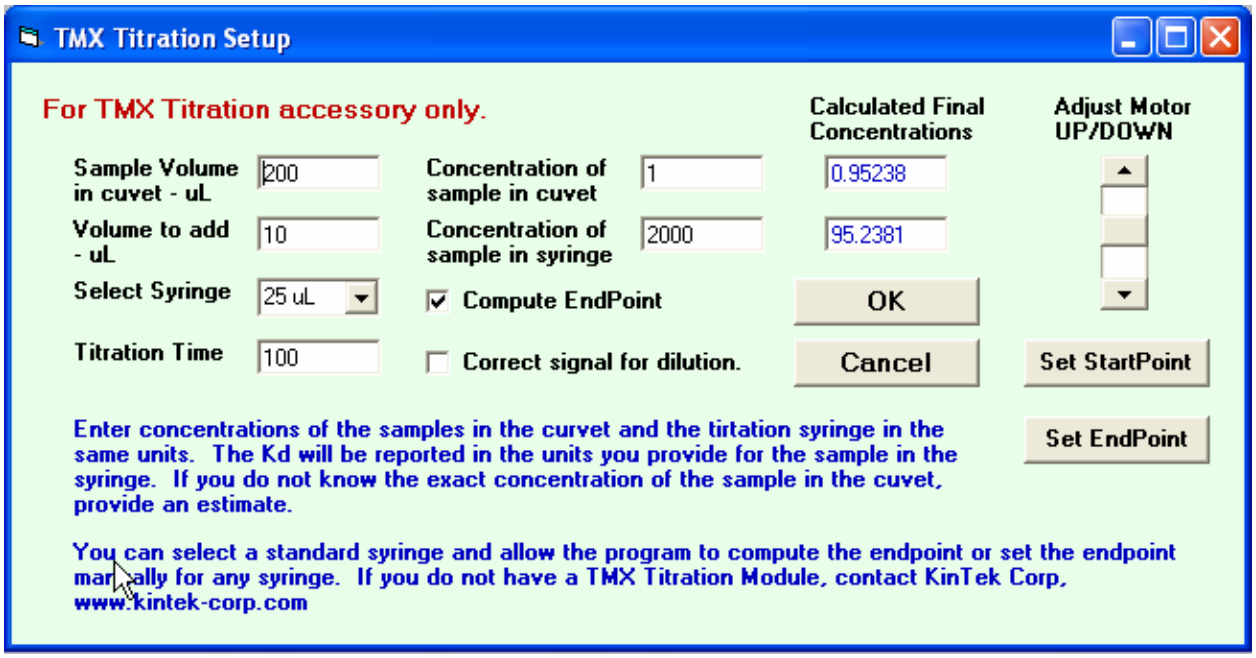


Step 4. Attach Drive Block to syringe drive, attach PMT and Diode detectors to TMX block and plug in stirring motor power supply.

Setup for Data Collection

Run the *KinTek Stopped-Flow* program. Under Setup, select *Titration Control*. If the *Titration Control* menu item does not appear, go to *Instrument Setup* and check the *Titration Module* option. Enter the volume of sample in the cuvette, the volume of titrant you wish to add, and select the total volume of the syringe you are using. Enter the initial concentrations of the sample in the cuvette and the concentration of the solution in the syringe. The computer will display the final concentrations of each reactant. Enter the total time that you would like for the titration to go to completion. A time of 300 seconds is a good starting point, but times as short as 60 seconds may be adequate.

The concentration entered can be in any units you choose, but should be consistent. The concentration entered for the sample in the syringe will be used to compute the concentration as a function of the titration.



If the syringe you are using is on the list of available syringes, then check *Compute Endpoint* so the distance traveled by the syringe drive can be calculated using the known parameters of the syringe you are using. The calibration is for Hamilton 700 series syringes. Alternatively, both *StartPoints* and *Endpoints* can be set for any syringe you wish to use.

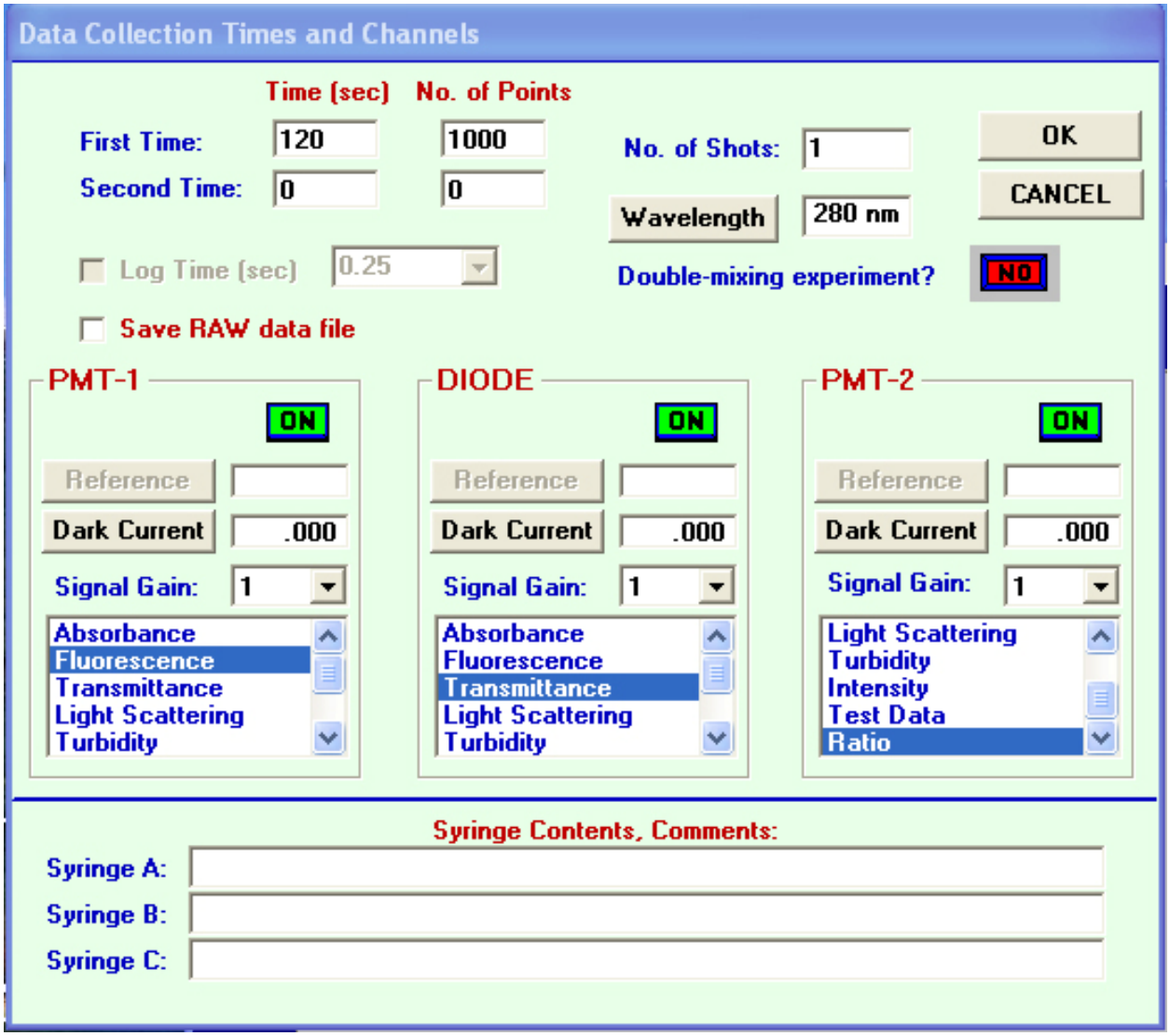
In either case, the *StartPoint* must be set. Use the UP/DOWN adjuster to move the stopped-flow platform to the desired start position with the syringe in place. This can be the 0 µL position or any other position. Then press *Set StartPoint*. Note that if you set the *StartPoint* to be greater than zero on the syringe, there is a danger that the motor will advance past the physical endpoint of the syringe and bend the plunger. The program prevents entering a volume greater than the volume of the syringe, but does not know the syringe volume of your *StartPoint*.

Check *Correct signal for dilution* only if you want the signal to be divided by the dilution factor during the titration. This will normalize fluorescence or absorbance data to correct for the dilution factor. This correction for dilution can also be performed later after the data is collected and store (see Data Analysis).

Set Time and Select Detectors

Time of data collection, the selection of the detectors, and the mode of data reduction (Fluorescence or absorbance, for example) are set using the standard Time Setup menu. Turn on or off the detectors you wish to use and select the data reduction mode from the list. Note that the Log Time and multiple shot functions are not available in titration mode.

Click OK to continue with data collection.



The menu above shows the setup for a special case involving a fluorescence titration with the addition of a titrant that absorbs some of the incident light. Although one should always choose an excitation wavelength where the titrant does not absorb, this is not always possible, and the absorption of light can lead to reductions in fluorescence intensity. The setup shows a means to correct the fluorescence intensity by simultaneously measuring the transmitted light intensity and then normalizing the fluorescence by dividing the data by the transmittance. The original data are stored in the PMT-1 (fluorescence) and Diode (transmittance) channels, while the ratio is stored in the PMT-2 channel. Thus one can easily compare the data obtained with and without normalization. If the PMT-2 channel does not appear, go to *Instrument Setup* and check the PMT-2 box.

Data Collection and Saving

Place your sample in the cuvette, including the micro stirring bar. Turn on the stirring motor and adjust to a speed setting of 8-10. As a control for the efficient mixing and the data collection time chosen, you may follow the absorption of light while adding a titrant that absorbs light. The increase in absorbance should be linear if mixing is sufficiently fast. In our control experiments, at a setting of 10, a data collection time of 60 seconds could be achieved.

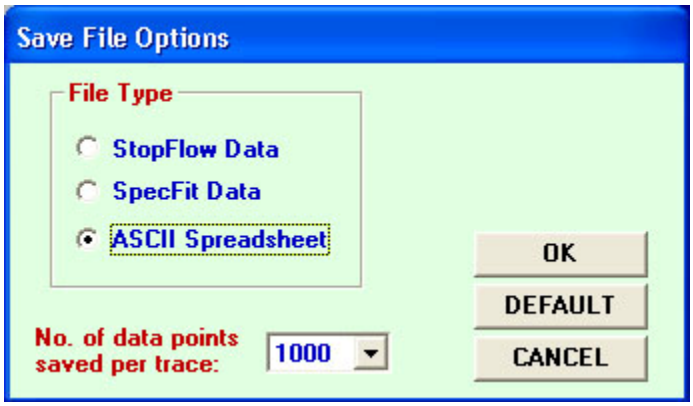
If the photomultiplier is used to collect data, set the PMT high voltage to achieve a signal of approximately 5 volts, using the Set PMT HV menu.

Attach the syringe, being careful to insert the needle through the hole in the lid and firmly fixing the syringe so that it is in proper vertical register according to your setting of the StartPoint. Also note that the syringe plunger is at the proper starting point that you defined.

To collect data, click on *Begin Titration* on the main menu. The syringe motor drive will quickly advance to the StartPoint, and then begin the slow addition of titrant, while simultaneously collecting data. After data collection, select *Save* to store the data in the directory of your choice. By default, files are saved in binary format.

The data can also be easily exported as an ASCII text file to be read into any of the many graphics packages available for the PC. Select *Save As…* from the main menu and then check the ASCII Spreadsheet option button, as shown below. The first column of the output file is the concentration (x-axis). Subsequent columns are the y-axis values, if more than one data channel, or multiple data collections are included.

You may also reduce the number of data points saved from the default of 1000. If you choose to save 100 points, for example, the program will average every 10 points to output the final 100 points. Thus, you further reduce noise in the data by averaging and retain the information content of the original data. The choice of the number of data points to export is esthetic, not scientific.



Noise in the Data

If you see spikes of noise in the data, then there may dust in the sample that is stirred into the light path. Selecting a bandpass filter for the fluorescence output that efficiently removes scattered incident light can eliminate the spikes. Alternatively, try working with a slower stirring rate, or remove the dust particles by filtration or centrifugation.

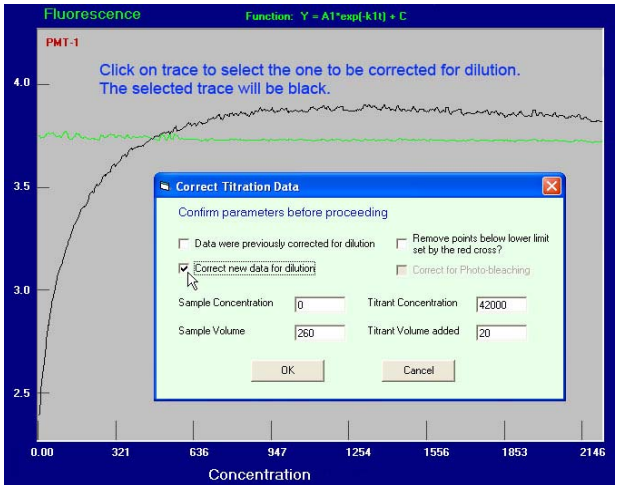
Photobleaching

You can easily perform a blank data collection to check for the stability of your signal prior the titration by omitting the titration syringe. Simply put your sample in the cuvet with stirring and collect a trace. If you see photobleaching of fluorescence that is significant relative to the magnitude of the signal you are measuring, decrease the slit width on the light source to reduce light intensity. A tenfold reduction in light intensity should produce a tenfold reduction

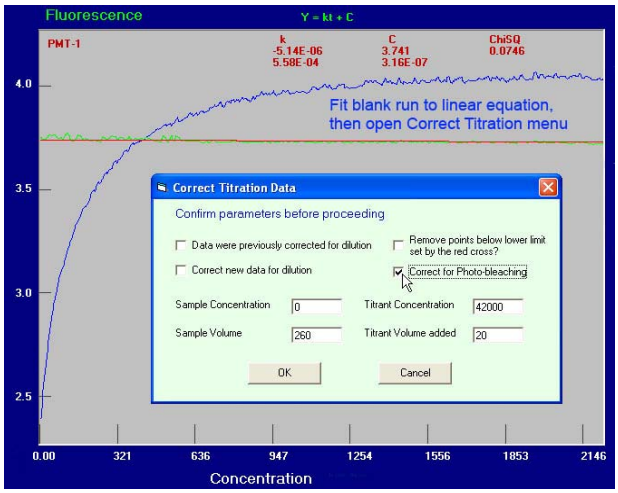
in the rate of photobleaching. You will need to increase the PMT high voltage to compensate for the lower signal, but the signal should still be adequate because of the signal averaging the takes place during data collection.

A modest amount of photobleaching in the signal can be corrected based upon the rate of change in the blank as illustrated below.

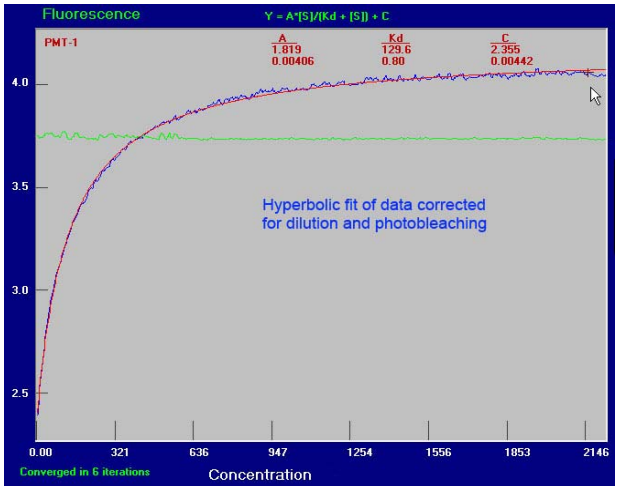
1. The figure at the right shows a blank run (green) and a titration collected without correction for dilution. Correct the data for dilution by first clicking on the trace (the selected trace will turn black. The open the *Correct Titration Data* menu and select *Correct new data for dilution.* Double check the parameters then press OK to complete the calculation.



2. This figure shows the results of the correction for dilution. The next step is to fit the blank run to a linear equation. Select *Linear* under the *Function* menu, then click on the blank trace and the select *Fit Data*. Next, open the *Correct Titration Data* menu and uncheck the box for *Data were previously corrected for dilution* to prevent undoing the dilution correction. Check the *Correct for Photo-bleaching* box and the press OK to complete correction. In this case the correction was small, approximately 0.3% of the signal.



3. This figure shows the data corrected for dilution and photobleaching and then fitted to a hyperbola.

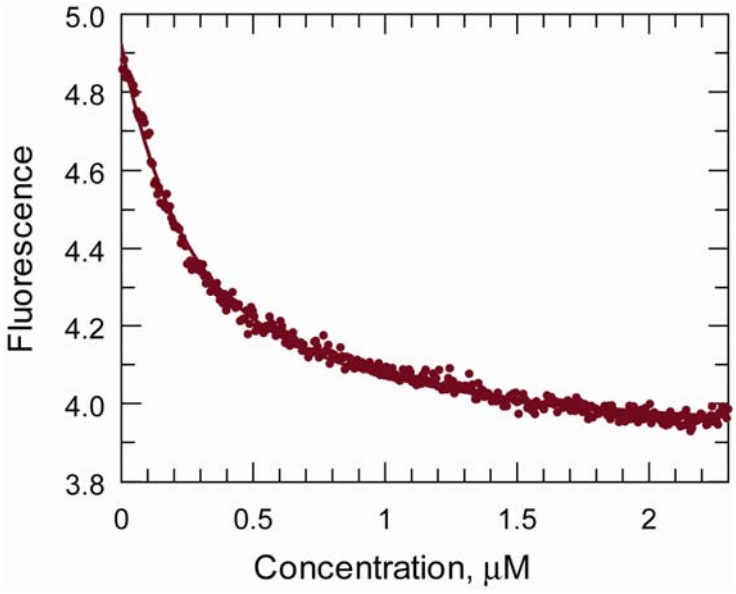


Data Fitting

Data can be fitted to either a hyperbolic or quadratic equation using routines provided with the KinTek system software. Data are fitted by nonlinear regression to the equation selected under the *Function* menu. Choice of which equation to use is critical.

The figure at the right shows data that were collected using only 100 nM enzyme and measuring a 20% change in fluorescence. The data were fitted to a quadratic equation because the K_d was only 2.5 fold larger than the enzyme concentration. The rule suggested by Dr. Kenneth A. Johnson is that the hyperbolic function is valid only when the K_d is at least 5-fold greater than enzyme concentration.

The data fitting includes both starting points (Y_0) and the change in signal (ΔY) according the function $Y = Y_0 + \Delta Y * \theta$ where θ is the faction of sites occupied.



Fluorescence Titration. This figure shows a fluorescence titration of 100 nM enzyme, showing a 20% change in signal. The smooth curve (buried under the points) is the best fit to the quadratic equation with a dissociation constant of 0.25 ± 0.01 µM. The titration required only 200 µL of solution and data collection was completed in 300 seconds.

Binding Equilibria

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S$$

Definition: $K_d = \dfrac{[E][S]}{[E \cdot S]} = \dfrac{k_{-1}}{k_1}$

Mass balance: $[E]_0 = [E] + [ES]$, $[S]_0 = [S] + [ES]$

$fraction = \theta = [ES]/[E]_0 = [ES]/([E] + [ES])$

Observed signal: $Y = Y_0 + \Delta Y * \theta$

Hyperbolic Equation

$$\theta = \frac{[S]_0}{K_d + [S]_0}$$

valid only when $[S] \approx [S]_0$ and this occurs only when $K_d >> [E]_0$

Quadratic Equation

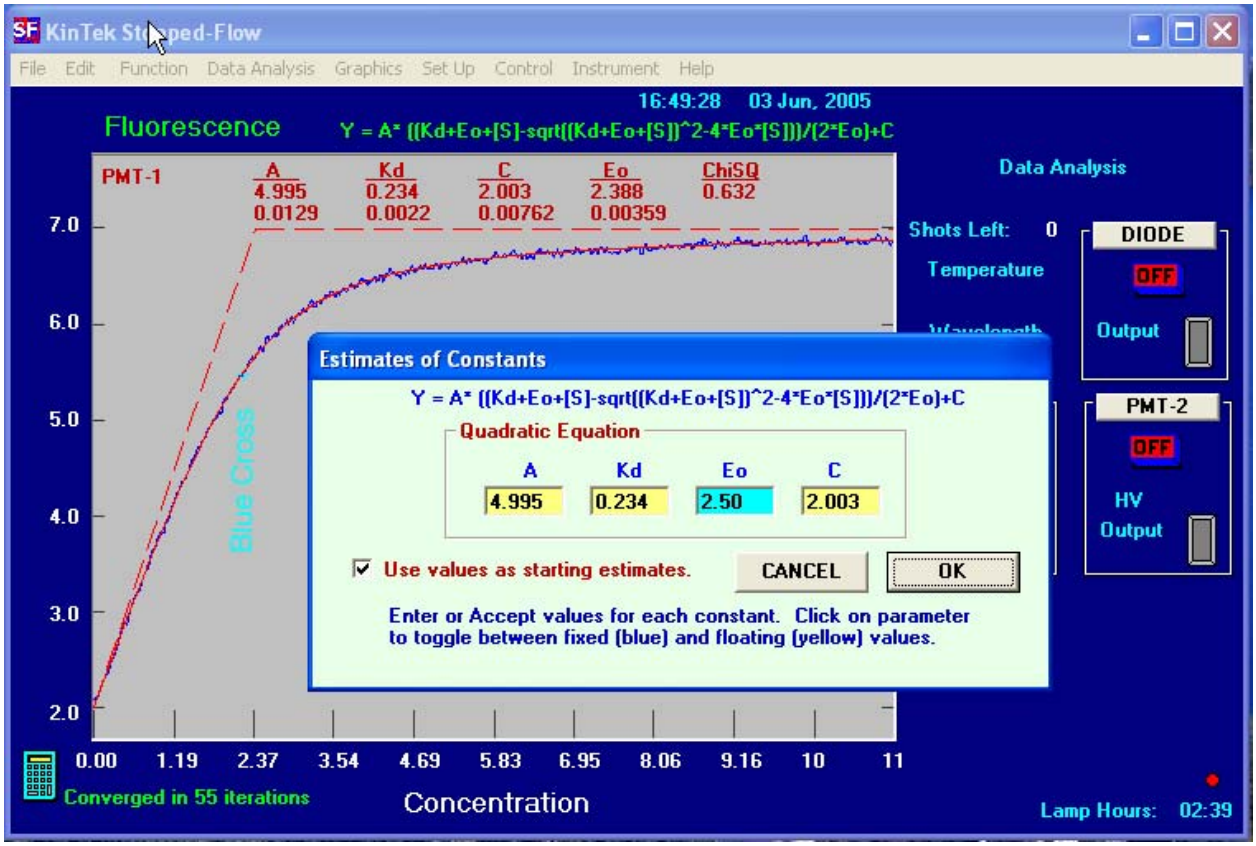
$$\theta = \frac{(E_0 + S_0 + K_d) - \sqrt{(E_0 + S_0 + K_d)^2 - 4 \cdot E_0 \cdot S_0}}{2 \cdot E_0}$$

Always valid, but you must know the E_0 concentration or have it defined by the data

Note that the equations are symmetrical with respect to [E] and [S]. Although we refer to E as the species that is held constant and S as the variable ligand, experiments can be performed with constant [S] and variable [E]. In data fitting, we have assigned S as the variable ligand.

After data are fitted, the fitted curve (solid line) and the line for infinitely tight binding (dashed line) are displayed. The figure below illustrates a fit to a quadratic equation. If the data were fitted to a hyperbola and the enzyme concentration given in the Titration Setup is greater then $K_d/5$, a warning message is displayed, stating that the fit is not valid because it violates the assumptions inherent in the derivation of the equation.

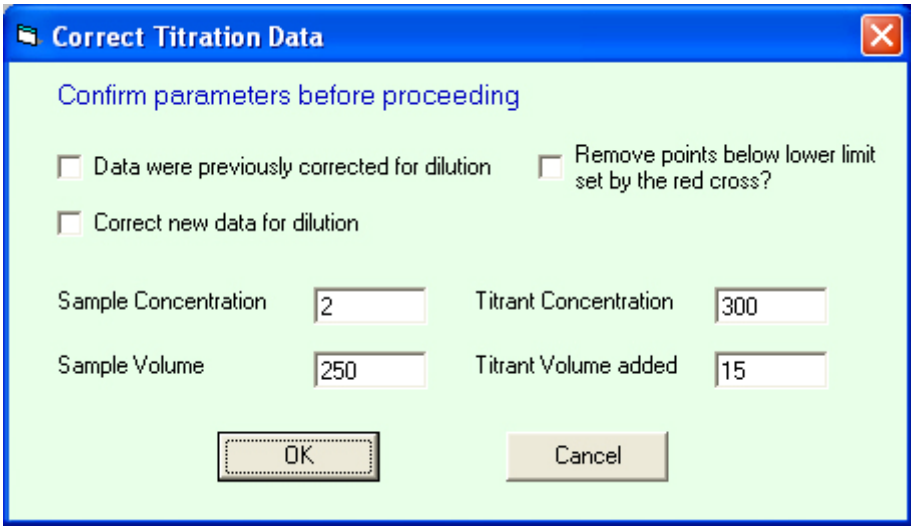
Fitting data to a quadratic equation requires knowledge of the total enzyme concentration, or a good estimate if the enzyme concentration is to be determined by data fitting. The KinTek System software provides two methods to input the enzyme concentration. If the enzyme concentration is to be obtained by data fitting, the initial guess can be easily input by placing the cursor at the appropriate concentration on the graph and then pressing simultaneously the ALT key and the left mouse button. A small light blue cross will appear at the location as shown below. If you have difficulty in fitting data, try several starting values.



This figure also illustrates the use of the *Estimates* menu item available under *Data Analysis*. Values for each of the parameters in the fitted equation can be entered into the table. If there was a previous attempt to fit data, the values from the last fitting are provided. Values for the enzyme concentration (or any parameter) can be frozen at a fixed number by clicking on the label above the number. This turns the input box to a cool blue, indicate the parameter is frozen for the subsequent fitting. The standard error will display as zero if the parameter was frozen during the fitting. After the next data fitting these settings are cleared. Check *Use values as starting estimates* if you want the program to use these values to start the fitting. If you want the program to choose starting estimates, uncheck this control.

Correcting Titration Data

Although the dilution of the sample is minimal due to the addition of 10-20 µL to a 200 µL sample, it is good practice to correct the signal for this dilution factor that varies as the titration proceeds. This can be done during data collection as controlled by the *Titration Setup* menu. Alternatively the dilution correction can be done after the data are collected and stored using the *Correct Titration Data* menu.



Check *Data were previously corrected for dilution* to restore the data to the uncorrected state. Check *Correct new data for dilution* to apply the dilution correct.

The third item, *Remove points below the lower limit set by the red cross?,* corrects the data obtained when there was an error in the syringe alignment during data collection so that a lag appears in the data. This occurs when the program starts collecting data before the motor touches the syringe. This mistake leads to errors in the computation of the concentration of the titrant as well as the dilution factor. To correct the error, place a red cross at the end of the lag by putting the cursor at the correct position and simultaneously pressing the CTRL button and the left mouse button. Then open the *Correct Titration Data* menu to apply the correction. Be sure to check *Data were previously corrected for dilution* if that is true, because the dilution correction must be removed prior to shifting the data to the correct concentration scale. Check *Correct new data for dilution* if you wish to apply the dilution correct after the data are shifted to the correct concentration scale.

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