XSTEP

Software for Protein Crystallization

Software Manual

Revision 6.20, July 2006
Compiled by Patrick Shaw Stewart, Peter Baldock and James Smith

Douglas Instruments Limited
Douglas House ♦ East Garston ♦ Hungerford ♦ Berkshire, RG17 7HD ♦ UK

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INTRODUCTION

Intentions and Features of XSTEP

XSTEP is designed to allow the systematic investigation of crystallization conditions by grid searching. For this only a few solutions are required, and each of these is dispensed using a separate liquid handling channel. The use of separate channels for each solution increases the speed and accuracy of dispensing.

To allow the rapid definition of grids to be searched a spreadsheet is provided. Using this you can input values into two cells, which define the corners of a rectangular grid or matrix. The values of cells between these two extremes can be filled in automatically by interpolation. This makes it extremely simple to home in on the optimum conditions for crystallization using a series of successively finer grids.

Other Software Available for Crystallization

XSTEP allows the rapid generation and automatic dispensing of a matrix of crystallization trials containing up to 96 wells (8 x 12).

WASPRUN and the interpreter WASP (or Windows Automatic Sample Preparation) allow the ORYX system to be used to suck up and accurately dispense small volumes to and from wells. This is usually used to screen a protein with a set of standard solutions which are mixed up in advance, and may be used many times. These solutions are transferred from microtitre plate wells to microbatch trials, where they are mixed with protein. WASP can also be used to dispense protein and mix well solutions for sitting drop trials.

Dispensing Mechanisms

Three dispensing mechanisms are available for XSTEP. These are

- microbatch trials dispensed under oil
- microbatch trials dispensed dry then covered with oil
- droplet dispensing for sitting drop vapour diffusion - no reservoir dispensing

Microbatch Crystallization

In microbatch crystallization, small volumes (0.1 to 10 µl) of protein and precipitant are dispensed into the wells of a Vapor Batch plate, under oil, which prevents evaporation of the droplet. No diffusion is necessary, and no material passes into or out of the sample to bring about crystallization. The components are mixed in their final concentrations, typically in volumes of 2 µl.

The accurate dispensing of protein solutions, precipitants and additives on this scale is made possible using Douglas Instruments' unique Microtip and high resolution Syringe Drivers.

Microbatch Dispensed Dry, then Covered with Oil (Oryx Crystallization System)

Here no oil is initially put into the plate, and the droplets are dispensed straight into clean plates. A few seconds after each droplet is dispensed, oil is automatically dispensed onto the droplet to prevent evaporation.

This method gives the most accurate and reliable dispensing, but it is only available for the Oryx Crystallization System. (It is not available for IMPAX.)

At the end of dispensing with any of the three microbatch dispensing options, the plate is generally "topped up" by hand with 6 ml of oil. Generally pure paraffin oil is used for optimization experiments, although occasionally it may be helpful to mix silicone oil with the paraffin. This encourages evaporation, which may be helpful for proteins that are relatively insoluble and so difficult to crystallize. (The 50:50 mixture of paraffin and silicone oil known as "Al's Oils" is recommended for routine screening but not for optimization.)

Microbatch Dispensed Under Oil (IMPAX Crystallization System)

Figure 1 shows the microbatch dispensing method where droplets are dispensed under oil. This method is often used with the IMPAX Crystallization System.

First, medium viscosity paraffin oil is dispensed by hand into a Nunc HLA tissue culture plate so that all of the wells are covered by a layer of oil. When automatic dispensing starts, the Microtip, which is formed from two or more fluoropolymer tubes drawn to a fine tip, is moved to a position just above the bottom of the well. The protein, precipitant, and any additives are dispensed simultaneously from the Microtip, and may be actively mixed by stirring with the Microtip, or mixed by diffusion. The Microtip is then withdrawn above the level of the paraffin oil, which causes the droplet to become detached from the Microtip. The Microtip then moves to the next well until all specified wells have been dispensed.

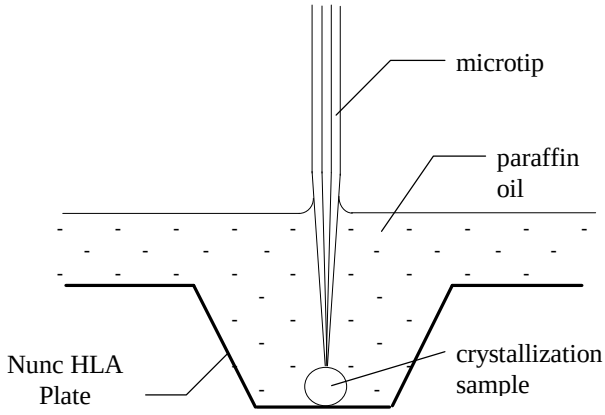

Figure 1: Microbatch Dispensing Under Oil.

When all microbatch trials have been dispensed into the plate, the plate is covered and stored at the appropriate temperature for crystallization.

Sitting Drop Vapor Diffusion

Figure 2 shows sitting drop dispensing using CrystalClear strips. The reservoir volumes must first be filled by hand with a pipette. The droplet is dispensed in air above the well. The Microtip is then lowered until the droplet comes into contact with bottom of the well depression, where it adheres centrally to the plastic.

As each strip is dispensed, it must be removed and sealed with tape, before being stored in another frame.

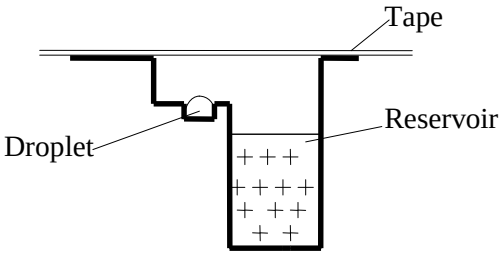

Figure 2: CrystalClear plate for Sitting Drop Vapor Diffusion.

Features of Microbatch

Microbatch has many advantages, including the following:

1. SAVING TIME. It takes approximately 15 minutes to design an experiment and load solutions into the Microtip, and to prime the gas-tight syringes. After this five 4 x 6 matrices of crystallization trials can be set up in under 4 minutes each. The most time-consuming operation is generally designing experiments, although this is greatly facilitated by XSTEP's powerful editing features.

2. LOWER COST. The system uses fewer consumable items and less stock solution than other automatic protein crystallization systems.

3. LOW PROTEIN CONSUMPTION. Typically around 0.7 µl is used per trial, which is much less than is used by other automatic crystallization systems. This is made possible by the high accuracy and repeatability of dispensing, typically $\pm$ 1.6%.

4. MORE STABLE CRYSTALS. When hanging drop crystallization trials are subjected to changing temperatures or heat flows, water often condenses on the coverslips. This can result in dilution of samples causing crystals to redissolve. This may occur, for example, when plates are taken out of a cupboard, or placed under a microscope. Microbatch trials do not suffer from these problems since they do not rely on diffusion, and it is very unusual for crystals to redissolve in microbatch.

5. EASE OF EXPERIMENT DESIGN. Using the XSTEP software package, droplet conditions are generally set up at the opposite corners of a (6 x 4) matrix. All intermediate values are determined by linear interpolation between these values. Thus a two-dimensional scan of conditions is generated in minutes.

6. KNOWLEDGE OF CRYSTALLIZATION CONDITIONS. In the microbatch technique the composition of a droplet is known exactly, which is useful for theoretical studies of crystal nucleation and growth. This contrasts with the situation with vapour diffusion. See Chayen et al., Journal of Applied Crystallography (1990), 23, page 301 for a discussion of the uncertainty of the conditions of crystallization in vapour diffusion. For example, the volume of the sample is not exactly fixed, and the sample contains unknown concentration gradients during the diffusion process, which may lead to the formation of skins on the surfaces of droplets. Also the diffusion of volatile acidic or basic components such as carbon dioxide and ammonia, which are present in very small quantities, may result in unpredictable changes in pH.

Other advantages include the ability to vary protein concentration, and the lack of skins on the surfaces of droplets. However, approximately 50% of proteins crystallize better in vapor diffusion, while 50% crystallize better in microbatch. We therefore recommend that both methods be used routinely for optimization.

XSTEP ORGANIZATION

Menu and Dialogue Options

Once a project file has been loaded into XSTEP, the menu and dialogue options are as follows:

MAIN MENU

- File
  - New Project
  - Open Project
  - Save Project
  - Save Project As
  - Close Project
  - Exit
  - View this Project's Log
  - Export Experiment as XML
  - Export Project as Multiple XML
  - Set XML Output Folder
  - AutoName XML Export Files
  - Print
  - Recent files
- Edit
  - Copy Selected Wells
  - Paste Selected Wells
  - Cut Selected Wells
  - Clear Selected Wells
  - Insert New Experiment
  - Change Name and Description
  - Delete Current Experiment
  - Copy Current Experiment
  - Insert Copied Experiment
- View
  - Solution Volumes
  - Plate Layout
  - Status Bar
  - Show Ingredient Units
  - Change Error Marker
  - Concentration Mode
  - Volume Mode
  - Volume% Mode
  - Steps Mode
- Mark
  - Mark Selected Wells for Execution
  - Unmark Selected Wells for Execution
  - Mark All Wells for Execution
  - Unmark All Wells for Execution
  - ReEnable Executed Wells
- Experiment
  - General
  - Plate
  - Stock Solutions
  - Dispensing Parameters
  - Execute Experiment
  - Execute Selected Wells
- Tools
  - Interpolate
  - Autodesign
  - Clear Errors
- Configuration
  - Use Hardware
  - File Locations
  - Colors
  - Buffer List
  - Advanced Menu Items
  - Execute in Background
- Window
  - Cascade
  - Tile Horizontal
  - Tile Vertical
  - Freeze Ingredients Legend
- Help
  - Quick Reference Card
  - XStep Manual…
  - About Xstep

SPREADSHEET

Layout of Spreadsheet

The spreadsheet displays a matrix of cells. Each cell represents a well and shows the composition of a droplet or crystallization *trial*. An XStep file represents a crystallization *project*, which may contain several spreadsheets - each spreadsheet corresponds to a plate or *experiment*.

On the left of the spreadsheet the first 6 (4 for Oryx6) ingredients of droplets are shown. These are typically protein, precipitant, additives, and buffers. The last channel always contains water, and it is not shown.

The spreadsheet is normally viewed in **Concentration Mode,** which shows concentrations and pHs. However, you can, if you choose, show the volume of each ingredient in a well, the percentage volume of each ingredient, or the number of steps that the dispensing syringes move. These alternative viewing modes can also be found on the **View** menu.

Editing Ingredients

Double clicking on the ingredients at the left of the spreadsheet allows them to be edited.

1. Click the specific ingredient to be edited.
2. Specify if this ingredient is or contains a buffer.
3. Enter the unit and name of the ingredient. You must specify well solution concentrations using the same unit as you used for the ingredient.
4. If there is a buffer present, enter the pKa of the buffer solution, followed by its pH.
5. If you are using viscous solutions such as PEG or MPD, enter the viscosity in the **Viscosity** box. Typical PEG solutions are around 40.
6. Enter or scan the solution bar code if available.
7. Click on **OK**.

Editing Values in Cells

Double clicking on a cell allows its values to be edited.

The white channel on the ORYX chassis is conventionally used for water to obtain the concentrations specified in the other channels. The amount of water added from the white channel is not shown on the spreadsheet due to lack of space. However, the amount can be seen when you are editing a well.

Errors or corrections are indicated by a cell with a symbol adjacent to the affected channel. While editing a cell the nearest possible solution can be achieved by clicking on the adjust button. In all cases, however, you will get what you see – the affected cells have already been corrected and can be used safely.

Interpolate

If you enter values in opposite corners of a block of wells, you can then automatically fill in all the intervening wells by interpolation. The principle of Interpolate is to provide 1-D or 2-D gradients of ingredient concentration. Typically, vary one ingredient in the Y direction (maybe Protein or Precipitant) and another in the X direction (e.g. Precipitant or Buffer/Additive) - this gives a 2-D interpolation.

It is also possible to do a big 1-D interpolation - using the **X then Y** variation (or the **Y then X**) in the **Interpolate** dialog. This means that the ingredient changes continuously in each well going from a minimum value in one corner to a maximum value in the other. (i.e. the sequence is like reading a book.)

1. Enter values into the wells at opposite corners of the block to be interpolated. (Note that this block need only be a part of the spreadsheet. Also, if you interpolate from the bottom left corner to the top right, then the resulting experiment reads like a phase diagram which may be helpful.)
2. Select the block to be interpolated by dragging the mouse over it.
3. Right-click on the numbers of one of the selected wells, and click on **Interpolate Selected Block**.
4. Click on the down arrow in each **Varying** box and choose whether you would like to vary the corresponding ingredient in the X direction (**X Only**) or the Y direction (**Y Only**). (Advanced users may like to interpolate in the **X Then Y** directions – which gives a sequence like reading a book – or in the **Y Then X** directions.)
5. Select **Bottom Left to Top Right**, or **Top Right to Bottom Left**.
6. Select **Interpolate**.

Autodesign

This is generally the most effective method of optimization, since it uses "multivariate" designs, where all of the important variables are changed in each experimental run. This allows you to place points evenly around a central point in a multi-dimensional experimental space. The central point is your best guess for optimal crystallization.

In XSTEP you can use the well-known Box-Behnken and Central Composite designs, but you can also invent your own designs based on the same principles, depending on the number of wells that you want to dispense.

For more information about XSTEP, multivariate designs and their advantages, see http://www.douglas.co.uk/rat_des.htm (this article is also published in *the Journal of Crystal Growth 196 (1999) 665 – 673.*)

1. Move the cursor to the well around which you would like to base your experimental design.
2. Right-click on this well and select **Autodesign**.
3. For each variable, edit the **Variation (%)** value. This value might be anything from 100% (e.g. when you want to know whether an ingredient is necessary or not), to 5% (e.g. near the end of crystal optimization) or 0% (Constant).
4. Select the number of **Center Points** that you would like. (Textbooks often recommend several center points so that you have an idea of the experimental error of the system.)
5. Adjust the boxes for **Wells with 1, 2, … 7 Parameters Varying** until you have the number of wells that you want. In all cases the resulting wells will be positioned reasonably evenly in all directions around the center point. Alternatively, select the well-known **Central Composite** or **Box-Behnken** designs.
6. Click on **OK** to generate the experiment automatically.

Executing a Spreadsheet One Well at a Time

A spreadsheet can obviously be executed by selecting *Execute Experiment.* It may sometimes be very useful to execute part of an experiment, or to execute an experiment one well at a time. This can most easily be achieved by right-clicking on a well or wells and selecting *Execute Selected Wells*. This can, for example, be used to find the precipitation point of a protein using a particular precipitant. For example, a linear gradient can be set up across the whole plate varying only precipitant. A well near the center of the gradient is first dispensed, and precipitation looked for. The precipitation point is now searched for by dispensing one well at a time using a "binary chop" procedure. Eventually the precipitation point is found, using the minimum of protein. This is explained in more detail in Acta Crystallographica D. 50 (1994), pp 441-442.

EXPERIMENT PARAMETERS

General

Oil in Wells (for Microbatch)

None – no oil is should be applied
Pre Oiled – Dispensing under oil
Oil Dropping – Oryx will automatically cover the drops with oil

Air Gap Volume

The volume of air, which is loaded before loading protein, is entered.

Sound Enabled

A bleep sound can be obtained after each well is dispensed. This may be important for manual dispensing etc.

Plate

Crystallization Plate

You may select plates other than the default Vapor Batch plate, for performing droplet dispensing of sitting drop vapour diffusion experiments, for example. We recommend use of the CrystalClear strips for miniature vapor diffusion experiments.

Dispensing Parameters

Default Volume

The default droplet volume (in microlitres) of each microbatch trial is entered. Individual volumes may be set when editing a specific well.

Maximum Flow

Largest flow rate for non viscous solutions

Pause

The delay in seconds between dispensing successive droplets is entered. This delay should usually be between 1 and 5 seconds. Use longer delays for viscous solutions.

Stirring Number and Distance

After dispensing each droplet, the droplet is stirred by moving the Microtip around the four quadrants of the droplet. This gives better stirring than moving in a circular or square pattern. The number of turns and the number of millimeters spanned by the tip's movements can be set. Stirring is not active with the Laying On option.

INGREDIENTS

Additive Model

You may design and execute experiments using ingredients such as protein which may contain a buffer, precipitant and additives which may or may not be or contain buffers, buffers and a diluent (generally water).

Note that each channel may contain an ‘additional buffer’. This is only sensible for ingredients which are not, themselves, a buffer.

This model may use any number of different buffers, which may have different pKa's. These pKa's may be input by picking from a table, or any other legitimate values may be typed in.

The ratio of buffers is determined by a generalization of the Henderson-Hasselbach equation, which is an nth degree polynomial in hydrogen ion concentration, where n is the number of negative ion species. For a two buffer system, n is two, and the equation is a quadratic in hydrogen ion concentration:

$$([Salt1]+[Salt2])\cdot[H]^2 + (Ka1\cdot([Salt2]-[Acid1]) + Ka2\cdot([Salt1]-[Acid2]))\cdot[H] - Ka1\cdot Ka2\cdot([Acid1]+[Acid2]) = 0$$

The generalized equation simplifies to the Henderson-Hasselbach equation when pKa1=pKa2. A volumetric ratio is obtained for the composition of the buffer.

After the pH and pKa values are input, the table for setting ingredients is displayed.

THE PRINCIPLES BEHIND XSTEP

The Scope of XSTEP

The XSTEP software implements three volumetric dispensing models, namely an additive model, a single buffer pH model and a double buffer pH model. The use of syringes varies according to the model chosen as follows :

Additive	1.	Protein.
	2.	Precipitant.
	3.	Additive A1 plus precipitant.
	4.	Additive A2 plus precipitant.
	5.	Water (for five channel mode only)
Single buffer pH	1.	Protein.
	2.	Precipitant.
	3.	Buffer B (made up to pH1), of arbitrary concentration, together with arbitrary concentration of precipitant.
	4.	Buffer B (made up to pH2), of arbitrary concentration, together with arbitrary concentration of precipitant.
	5.	Water (five channel mode only).
Double buffer pH	1.	Protein.
	2.	Precipitant.
	3.	Buffer B1 (made up to pH1), of arbitrary concentration, together with arbitrary concentration of precipitant.
	4.	Buffer B2 (made up to pH2), of arbitrary concentration, together with arbitrary concentration of precipitant.
	5.	Water (five channel mode only).

The ingredients may be mixed in variable proportions, to generate a wide range of solution conditions varying independently as follows :

Four channel systems (variation in three parameters)

(a) protein, precipitant, and additive 1 (second additive implicit)
or (b) protein, precipitant and pH (buffer concentration implicit)

Five channel systems (variation in four parameters)

(a) protein, precipitant, additive 1 and additive 2.
or (b) protein, precipitant, pH and buffer concentration.

Mathematical Basis of XSTEP

According to the model chosen, XSTEP calculates intermediate values of cell parameters by linear interpolation when the INTERPOLATE function is invoked while editing the spreadsheet. When

using the Additive model, amounts of all solutions dispensed are calculated by linear interpolation of concentration between two extreme values (when interpolating in concentration mode). However, with either of the pH models, where the first two channels are filled with buffer solutions of different pH's, the INTERPOLATE function calculates the amounts dispensed by linear interpolation of pH for the two buffer solutions using a development of the Henderson-Hasselbach equation.

Additive model

Given the arrangements of the syringes above, the concentrations (C1-C6) of their contents are specified according to the table below.

SYRINGE	CONTENTS	CONCENTRATION		VOLUME
		stock	required	
1	Protein	C1	Prot	V1
2	Precipitant	C2	Prec	V2
3	Additive 1	C3	A1	V3
4	Additive 2	C4	A2	V4
3	Precipitant (in 3)	C5	Prec	V3
4	Precipitant (in 4)	C6	Prec	V4
5	Water	--	--	V5

In four channel mode, the volume V4 and hence the required concentration A2 of the second additive is implicit - i.e. it is calculated by the program. In this case the water volume V5=0.

However, in five channel mode, A2 is specified by the user and it is the water volume V5 which is implicit.

Relationships between the variables :

Microbatch

$Vtot = V1 + V2 + V3 + V4 + V5$	Total volume
$Prot \cdot Vtot = V1 \cdot C1$	
$Prec \cdot Vtot = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$	Syringes 2, 3 and 4 contribute to precipitant Prec.
$A1 \cdot Vtot = V3 \cdot C3$	
$A2 \cdot Vtot = V4 \cdot C4$	

Well Solution Dispensing, Sitting Drop

$Vres = V2 + V3 + V4 + V5$	Total reservoir volume
$Prot \cdot Vdrop = V1 \cdot C1$	Protein volume for sitting drop
$Prec \cdot Vres = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$	Syringes 2, 3 and 4 contribute to precipitant Prec.
$A1 \cdot Vres = V3 \cdot C3$	
$A2 \cdot Vres = V4 \cdot C4$	

pH Model - single buffered model

SYRINGE	CONTENTS	CONCENTRATION		VOLUME
		stock	required	
1	Protein	C1	Prot	V1
2	Precipitant	C2	Prec	V2
3	Buffer 1, pKa1, pH1	C3	B1	V3
4	Buffer 2, pKa1, pH2	C4	B2	V4
3	Precipitant	C5	Prec	V3
4	Precipitant	C6	Prec	V4
(5)	Water (5 channel only)	--	--	V5

In four channel mode, the volume V4 and hence the required buffer concentration (B1+B2) is implicit - i.e. it is calculated by the program. In this case the water volume V5=0.

However, in five channel mode, (B1+B2) is specified by the user and it is the water volume V5 which is implicit.

Microbatch

$Vtot = V1 + V2 + V3 + V4 + V5$ Total Volume

$Prot \cdot Vtot = V1 \cdot C1$

$Prec \cdot Vtot = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$ Syringes 2, 3 and 4 contribute to precipitant Prec.

$B1 \cdot Vtot = V3 \cdot C3$

$B2 \cdot Vtot = V4 \cdot C4$

$Vbuff = V3 + V4$

Well Solution Dispensing, Sitting Drop

$Vres = V2 + V3 + V4 + V5$ Total Volume

$Prot \cdot Vdrop = V1 \cdot C1$

$Prec \cdot Vres = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$ Syringes 2, 3 and 4 contribute to precipitant Prec.

$B1 \cdot Vres = V3 \cdot C3$

$B2 \cdot Vres = V4 \cdot C4$

$Vbuff = V3 + V4$

The ratio of B1 to B2 is determined by the Henderson-Hasselbach equation from the required hydrogen ion concentration, producing a volumetric ratio for the composition of the buffer volume, Vbuff.

pH Model - double buffered model

The approach is broadly similar to the single buffer model, but the algebra is substantially more complicated.

SYRINGE	CONTENTS	CONCENTRATION		VOLUME
		stock	required	
1	Protein	C1	Prot	V1
2	Precipitant	C2	Prec	V2
3	Buffer 1, pKa1, pH1	C3	B1	V3
4	Buffer 2, pKa2, pH2	C4	B2	V4
3	Precipitant	C5	Prec	V3
4	Precipitant	C6	Prec	V4
(5)	Water (5 channel only)	--	--	V5

Note that two pKa's are accommodated, one for each buffer.

Microbatch

$Vtot = V1 + V2 + V3 + V4 + V5$ Total Volume

$Prot \cdot Vtot = V1 \cdot C1$

$Prec \cdot Vtot = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$ Syringes 2, 3 and 4 contribute to precipitant Prec.

$B1 \cdot Vtot = V3 \cdot C3$

$B2 \cdot Vtot = V4 \cdot C4$

$Vbuff = V3 + V4$

Well Solution Dispensing, Sitting Drop

$Vres = V2 + V3 + V4 + V5$ Total Volume

$Prot \cdot Vdrop = V1 \cdot C1$

$Prec \cdot Vres = V2 \cdot C2 + V3 \cdot C5 + V4 \cdot C6$ Syringes 2, 3 and 4 contribute to precipitant Prec.

$B1 \cdot Vres = V3 \cdot C3$

$B2 \cdot Vres = V4 \cdot C4$

$Vbuff = V3 + V4$

The ratio of B1 to B2 is determined by a generalization of the Henderson-Hasselbach equation, which is an nth degree polynomial in hydrogen ion concentration, where n is the number of negative ion species. For a two buffer system, n is two, and the equation is a quadratic in hydrogen ion concentration:

$$([Salt1+Salt2])\cdot[H]^2 + (Ka1\cdot([Salt2]-[Acid1]) + Ka2\cdot([Salt1]-[Acid2])\cdot[H] - Ka1\cdot Ka2\cdot([Acid1]+[Acid2]) = 0$$

The generalized equation simplifies to the Henderson-Hasselbach equation when pKa1=pKa2.

Dealing with Errors and Impossible Specifications

When the equations cannot be satisfied, because, for instance, impossible concentrations have been specified, then a best fit is found by adjusting all the independent variables (eg the first three required concentrations, volumes or %volumes in the case of four channel mode) by a scaling factor to obtain a realizable set of requirements. This will usually result in zero volume of the last component (A2 or buffer volume in 4 channel mode, water in 5 channel mode).

It may also be necessary to subtly correct for the actual volumes achievable with the resolution of the hardware. If the correction is significant (typically greater than 0.1%) it will cause a resolution rounding error to be indicated.

Under all circumstances, errors or corrections are indicated by a symbol adjacent to the affected channel in the appropriate cell on the XSTEP spreadsheet. The error indicator usually shows that a value required has not been achieved, and that a new (possible) value has been substituted. Consequently, re-entering the recalculated values (as though they were specified values) will clear the error indicators.