SFM-300/400

User's Manual

Version 2.7

TABLE OF CONTENTS

WARRANTY

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THIS WARRANTY SHALL NOT APPLY TO FUSES OR ANY PRODUCT OR PARTS WHICH HAVE BEEN SUBJECT TO MISUSE, NEGLECT, ACCIDENT, OR ABNORMAL CONDITIONS OF OPERATION. USING A NONCOMPATIBLE SOLVENT WITH THE SFM IS NOT COVERED BY THE WARRANTY.

IN THE EVENT OF FAILURE OF A PRODUCT COVERED BY THIS WARRENTY, THE PRODUCT MUST BE RETURNED TO AN AUTHORIZED SERVICE FACILITY FOR REPAIR AND CALIBRATION AND TO VALIDATE THE WARRANTY.

THE WARRANTOR MAY, AT THEIR DISCRETION, REPLACE THE PRODUCT OR REPAIR. WITH REGARD TO ANY INSTRUMENT RETURNED BECAUSE OF A DEFECT DURING THE WARRENTY PERIOD, ALL REPAIRS OR REPLACEMENTS WILL BE MADE WITHOUT CHARGE. IF THE FAULT HAS BEEN CAUSED BY MISUSE, NEGLECT, ACCIDENT, OR ABNORMAL CONSITIONS OF OPERATION, REPAIRS WILL BE BILLED AT NORMAL COST. IN SUCH CASES, AN ESTIMATE WILL BE SUBMITTED BEFORE WORK IS STARTED.

IN CASE ANY FAULT OCCURS:

NOTIFY BIO-LOGIC OR THE NEAREST SERVICE FACILITY, GIVING FULL DETAILS OF THE DIFFICULTY, AND INCLUDE THE MODEL NUMBER, TYPE NUMBER, AND SERIAL NUMBER. UPON RECEIPT OF THIS INFORMATION, SERVICE OR SHIPPING INSTRUCTIONS WILL BE FORWARDED TO YOU.

* EXCEPTION: ARC LAMPS SOLD BY BIO-LOGIC ARE ONLY WARRENTIED FOR A PERIOD OF 3 MONTHS FROM DATE OF PURCHASE.

1 INTRODUCTION AND SPECIFICATIONS

1.1 General Description

Each Bio-Logic stopped-flow module (SFM) consists of a mechanical subsystem and a motor power supply (MPS).

There are two SFM configurations:

SFM-300 - The mechanical sub-system consists of three machined syringes and one valve block with 3×3-way valves, with the possibility to include one or two mixers and one ageing loop.

SFM-400 - The mechanical sub-system consists of four machined syringes, one valve block with 4×3-way valves, and the possibility to include one to three mixers and one to two ageing loops.

All SFM syringes, valves, delay lines, and cuvettes are enclosed in a water jacket to allow temperature regulation of the reactants' containers. The syringe plungers of the SFM are driven by stepping motors via ball screws.

1.1.1 The mechanical designed

The mechanical part of the SFM module is carefully constructed. The parts in contact with the sample and the buffers are all machined out of materials selected for their inert characteristics: Teflon, VITON, EPDM, PEEK and quartz.

Millisecond dead time can be achieved with the SFM due to the combined effects of high-performance control of the stepping motors, and low dead volumes.

Ageing lines of various volumes can be used in the SFM. The ageing line(s) of the instrument can be replaced and secured in a few minutes.

1.1.2 Intelligent power supply

The high performance of the SFM and the high speed of the stepping motors can be achieved only because of the quality of its power supply. The MPS unit contains independent constant current power supplies for each syringe, all driven independently by their own microprocessor.

The sequences of impulses to be sent to the stepping motors are stored in the memory of each motor board. One main microprocessor board synchronizes all the power supplies and performs the communication with the microcomputer via a serial interface.

1.1.3 Microcomputer commands

The SFM module is controlled by Bio-Kine software starting from version 4.0 (for older versions, please download an older version of this manual where the use of the MPS software is fully explained). It is also advised to read the Bio-Kine user's manual to get more information about software functions. Various menus and windows permit the user to:

- know the volume of the solution contained in each syringe
- perform manual or automatic movement of the syringes
- create a sequence of reactions with complete control of time and volume delivered by the syringes
- save or recall the sequences
- program the synchronization pulse used to trigger the acquisition system

1.2 Modes of Operation

The SFM can be used in two main operating modes that are briefly described below. More details on the two modes of operation can be found in later sections of this manual.

1.2.1 Stopped-Flow (SF) mode (commercial reference SFM-X00/S)

In this configuration, the SFM is a full stopped-flow instrument with an optical observation chamber.

This configuration is described in Figure 1. In this configuration, the SFM has unique features for a stopped-flow instrument.

SFM-300/S - Two or three solutions can be mixed and injected into the cuvette, and a single delay line can be installed (Figure 1 panel 1).

SFM-400/S - Two to four solutions can be mixed and injected into the cuvette, and one to two delay lines can be installed (Figure 1 panel 2).

The speed capability of the SFM instrument (3 or 4 syringes) with all its syringes running gives a dead time below 1 ms in the observation cuvette.

1.2.2 QUENCHED-FLOW (QF) MODE (commercial reference SFM-X00/Q)

In this configuration, the SFM functions as a complete quench-flow instrument. This configuration allows for various modes of operation as described in Figure 1.

SFM-300/Q - It can be used as a three syringe quench-flow instrument with one delay line, two mixers, and a diverting valve for waste and collect (Figure 1, panel 3). Alternatively, an external flow line can be connected for direct injection of the mixture into a quenching solution. This mode may be used with or without an additional delay line.

It can also be used in a simple 3 syringe mode and direct collection of the sample in a pipette or syringe.

In another mode, the mixture can be injected onto a filter at the same time it is mixed with a flow of washing buffer.

SFM-400/Q - It can be used as a quench-flow instrument with 2 to three syringes, up to one delay line, either single or double mixing, and a diverting valve for waste and collect (panel 4). Alternatively, an external flow line can be connected for direct injection of the mixture into a quenching solution.

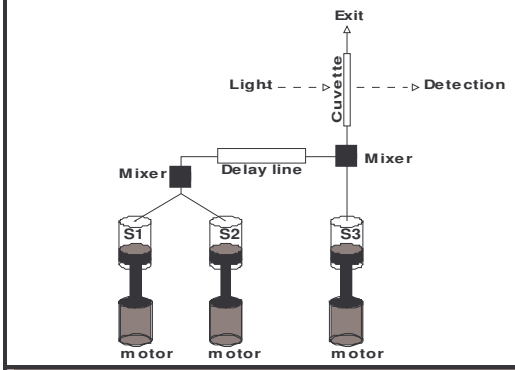
Flash quenching with a photoreactive reagent is also a mode that can be easily implemented with the SFM. Many other configurations are possible, and you are invited to inquire about their feasibility.

The commercial reference SFM-X00/QS has all the components for the two applications. An SFM-X00/S or a SFM-X00/Q can easily be updated to a SFM-X00 /QS.

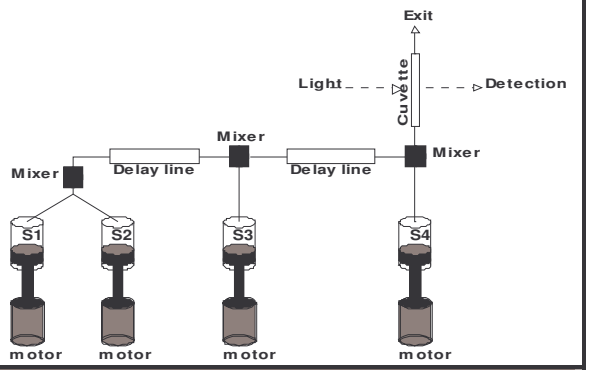
Figure 1 – SFM Modes of Operation

STOPPED-FLOW MODES

Panel 1: SFM-300

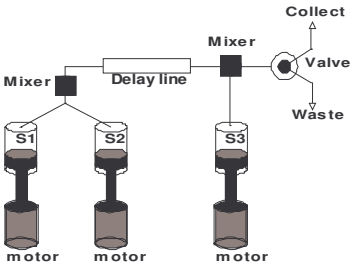


Panel 2: SFM-400

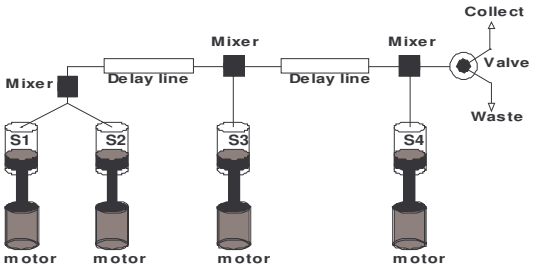


QUENCHED-FLOW MODES

Panel 3: SFM-300 with diverting valve



Panel 4: SFM-400 with diverting valve



1.3 Specifications

The general specifications of each SFM are listed in Table 1-SFM specifications below.

Table 1-SFM specifications

GENERAL SFM SPECIFICATIONS	
Number of syringes	3 (SFM-300) or 4 (SFM-400)
Driving mechanism	One stepping motor per syringe (6400 steps per motor turn)
Number of mixers	1 to 3
Ageing line between the two mixers	25 to 1000 µl
Trigger	Programmable trigger for data acquisition and synchronization of accessories
Filling range of the drive syringes	500µl to syringe limit
Minimum injection volume per syringe	10ml syringe - 28 µl 6.8ml syringe - 20 µl (standard syringe) 1.9ml syringe - 10 µl
Flow-rate range	10 ml syringe: 0.062 – 8 ml/s/syringe 6.8 ml syringe: 0.045 – 6 ml/s/syringe 1.9 ml syringe: 0.010 – 1.32 ml/s/syringe
Minimum flow rate for efficient mixing	1 ml/s (total flow rate through each mixer)
Variable ratio range	Continuously variable from 1/1 to 1/20 with single dilution, >1/100 with double dilution
Minimal dead time (SF mode)	0.8 ms with FC-08 cuvette; 0.25ms with the µcuvette accessory
Minimal ageing time (QF mode)	< 2ms with minimal volume delay line
Material	PEEK (stainless steel or Kel/F on special order)
Syringe volume	10ml standard syringes (6.8 and 1.9 mL syringes are also available)
Volume per µ-step	10 ml syringe - 0.19 µl 6.8 ml syringe - 0.14 µl 1.9 ml syringe - 0.03 µl
Duration of flow	adjustable from 1 ms to 60000 ms per phase
Power requirement	300 Watt, 110/220 Volt, 50/60 Hz
Total weight	13-14 kg

1.4 Principle of Operation

The syringes of the SFM are driven by independent stepping-motors. The stepping-motors are of hybrid technology with 200 steps per revolution and 4 phases, each phase being powered by a constant current supply (2.9 A per phase). The power supply of each motor is controlled by a microprocessor. A complex impulse sequence enables micro-positioning of the motor's rotor with an accuracy equivalent to 1/32 of the mechanical step. This gives an effective number of steps of 6400 per revolution, or a volume quantification of 0.14 µl per micro-step (µ-step), when standard (10 ml) syringes are used.

With the damping produced by the rotor inertia, this results in an almost continuous, linear movement of the syringe even at very low flow rates.

The motors can be activated manually or automatically. The manual mode is mainly used to refill or wash the syringes; the syringes can be driven independently and their speed adjusted using the microcomputer with a very simple menu. Automatic mode is used for actual experiments.

The motor impulses are counted in the positive direction (refilling) or negative direction (emptying), so that the contents of each syringe can be continuously displayed. Zero volume corresponds to the uppermost position of the syringe and referencing the zero volume position can be done using the keyboard of the microcomputer.

The movements of the syringes are completely controlled by the microprocessor, which eliminates the need for a stop syringe. Thus, the stop artifact present in most conventional stopped-flow systems is absent in the SFM. The observation system can be synchronized with the syringe "start" or "stop" by using the trigger pulses available on the front panel of the MPS unit.

The independent control of each syringe allows for high versatility of the injection sequence. It is possible to make an injection of one syringe only, unequal filling of the syringes, variable ageing times, variable concentration, variable mixing ratios, and other complicated actions with only a few keystrokes.

The reproducibility and regularity of the linear translation of the syringes and the absence of pressure artifacts allows for optical recording during the drive sequence. This feature greatly facilitates the determination of the initial phase of the reaction being monitored and makes the equipment suitable for very accurate, continuous flow experiments.

1.5 Description of the Mechanical Design

The observation chamber and the syringes of the SFM are mounted vertically. This allows for easy purging of bubbles, which are evacuated during refilling by a few up and down movements of the drive syringe.
The syringes, valves, and observation chamber are very carefully thermoregulated. This thermoregulation prevents the occurrence of temperature artifacts on a very wide temperature range and permits rapid kinetics studies even at temperatures below 0°C.

1.6 The Delay Lines

The SFM instrument can be used with delay lines, permitting various reaction delays to be obtained between the two (SFM-300) or three (SFM-400) mixers. The delay lines are machined into PEEK, Kel-F or stainless steel spacers (depending on the instrument). These spacers can be inserted between the mixers to adjust the volume and ageing time of a reaction between the mixers. See sections 3.4 and 3.5 for full description of delay line installation and calculation of volumes. Replacement of the delay lines is an easy operation which usually takes only a few minutes.

Delay lines of nominal volumes up to 1000 µl are available.

Standard equipment of an SFM-X00/S does not include ageing lines. SFM-X00/Q and /QS versions are delivered with two sets of ageing lines up to 200 µl. Ageing lines of 500 µl and 1000 µl can be obtained as additional accessories.

To evaluate the ageing time of a reaction, the entire volume between two mixers must be taken into account. This volume includes the delay line plus the dead volumes (the volumes on the both sides of the delay line and the mixers). The complete description of the volumes is described in section 3.5.

2 GENERAL INSTRUCTIONS FOR INSTALLATION

This section of the manual contains information on the installation and preliminary operation of all SFM instruments. It is recommended that the contents of this section be read and understood before any attempt is made to operate the instrument. In case of difficulties please contact Bio-Logic or its nearest representative. The SFM-300/400 can be connected to the MPS-60 or MPS-70. Please refer to the appropriate following operating features.

2.1 Operating Features with MPS-60

The general features of the MPS-60 are shown below in Figure 2 and described in Table2 – MPS-60 Panel Descriptions.

Figure 2 – MPS-60 Panels

Front **Back**

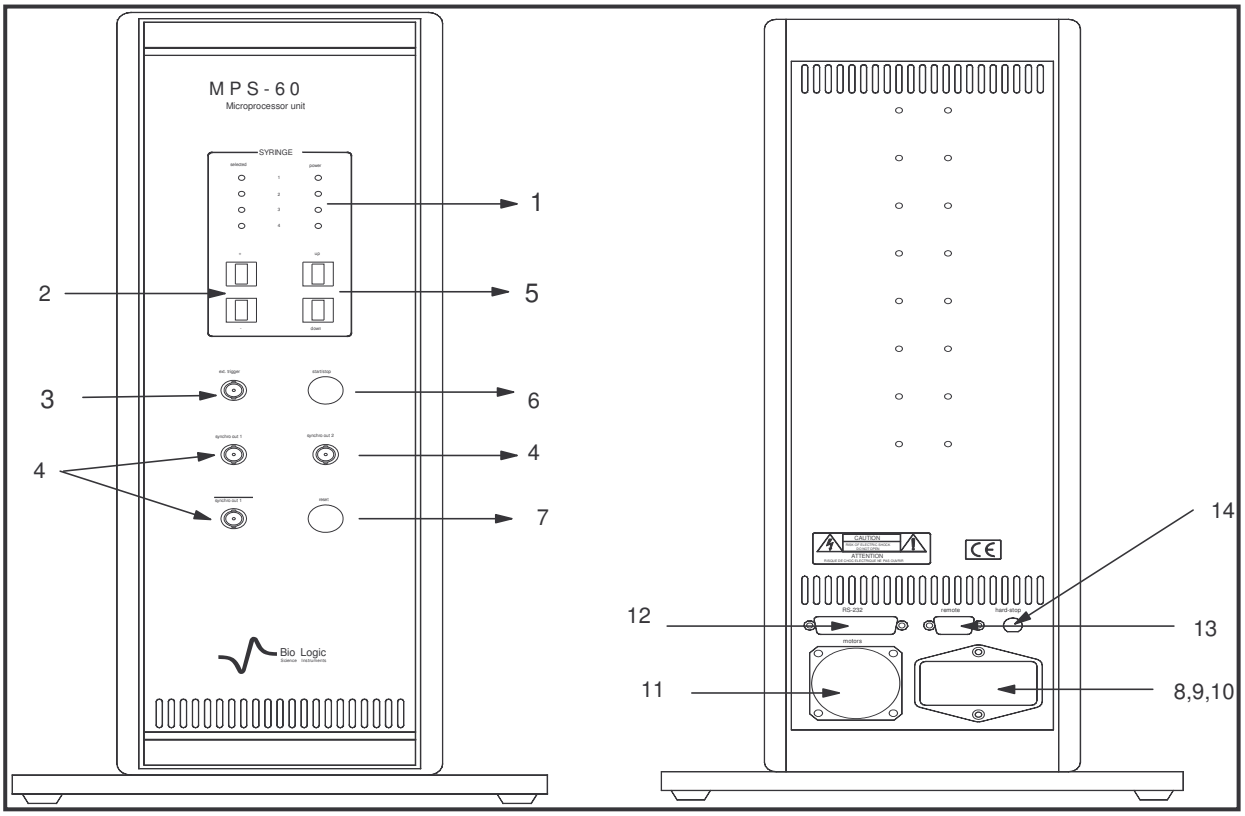


Table2 – MPS-60 Panel Descriptions

	NAME	FUNCTION
1	LCD DISPLAY	*Used to display messages (selected syringe, auto mode...)*
2	SYRINGE SELECTOR	*Selects the syringe for the manual control (5)*
3	TRIGGER INPUT	*Input for an external signal to trigger the drive sequence*
4	SYNCHRO PULSE OUTPUT	*TTL Pulse output to trigger the recording system, or any electronic device to be synchronized with the instrument*
5	MANUAL MOVEMENT	*Manual control of the syringes*
6	START/STOP	*Initiates (or stops) the programmed sequence in the automatic mode. The instrument may also be started and stopped using the keyboard of the PC.*
7	PROGRAM RESET	*Resets the MPS-60 instrument (does NOT reset syringe values)*
8	MAIN POWER FUSE	*3 A for 220 V, or 6 A for 115 V*
9	AC LINE CONNECTOR	
10	MAIN POWER SWITCH	
11	MOTOR POWER CONNECTOR	*Sends the power pulses to the stepping motors*
12	SERIAL CONNECTOR	*Connects the MPS-60 controller to the PC*
13	REMOTE CONNECTOR	*For optional remote control*
14	HARD STOP (SF) BNC CONNECTOR	*Connects to Hard Stop Valve*

2.2 AC Power and Connections of MPS-60

Before connecting the SFM to the local AC line, verify that the setting of the instrument matches the local line voltage. Prepare the SFM for operation by connecting the mechanical subsystem to the MPS-60 unit. Connect the MPS-60 to the serial port of the microcomputer. Finally, plug the MPS-60 into the appropriate AC line.

2.3 Operating Features MPS-70

The connection of the MPS-70 with the PC is done through a USB connector by plugging the USB cable on the rear panel of the MPS and installing the driver from Biokine version >4.45 (e.g. 4.49) software from the folder "driver MPS USB 441". The selection of the syringes individually or simultaneously can be done manually by pressing the buttons (2) on the front panel (select buttons).

- By pressing one button, syringe 1, 2, 3, or 4 is selected while the corresponding led is lighted on. The "select" left button allows you to switch from syringe 1 to 2 to 3 with increasing numbers. The "select" right button allows you to switch from syringe 3 to 2 to 1 with decreasing numbers.

- By pressing on the two buttons on the same time, all syringes 1, 2, 3, & 4 are selected while the entire led are lighted on. The up/down buttons allows you to move the pistons of the syringe up and down.

The general features of the MPS-70 are shown below in Figure 3-MPS-70/4 Front Panel and Figure 4-MPS-70/4 Rear Panel described in Table 3- MPS-70 Panel Descriptions.

Figure 3-MPS-70/4 Front Panel

Front Panel

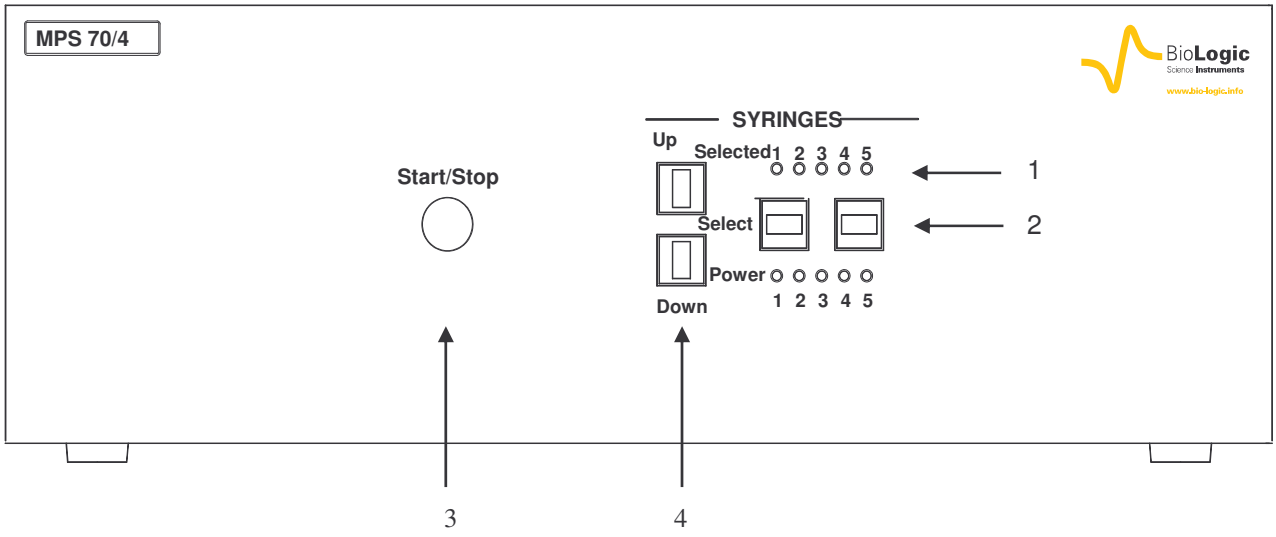


Figure 4-MPS-70/4 Rear Panel

Rear Panel

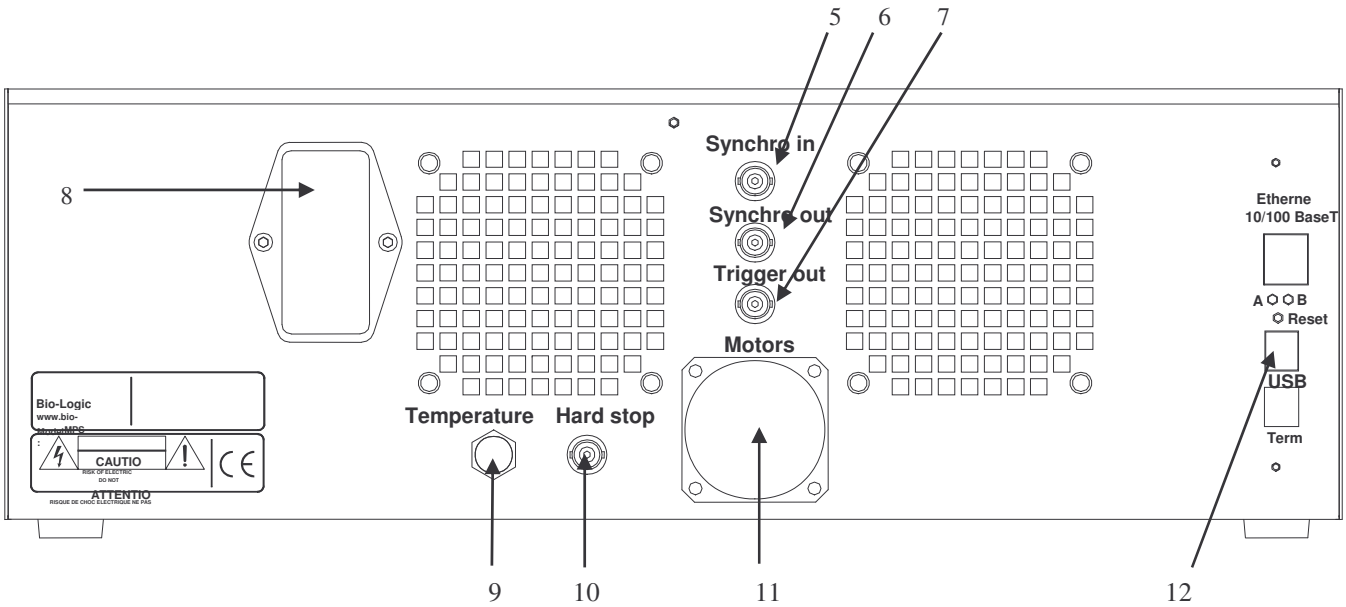


Table 3- MPS-70 Panel Descriptions

	NAME	FUNCTION
1	LCD DISPLAY	*Used to display messages (selected syringe, auto mode...)*
2	SYRINGE SELECTOR	*Selects the syringe for the manual control*
3	START/STOP	*Initiates (or stops) the programmed sequence in the automatic mode. The instrument may also be started and stopped using the keyboard of the PC.*
4	MANUAL UP/DOWN MOVEMENT	*Manual up and down movement control of the syringes*
5	SYNCHRO IN	*Input for an external signal to trigger the drive sequence*
6	SYNCHRO OUT	*TTL Pulse output for special application*
7	TRIGGER OUT	*TTL Pulse output to trigger the recording system, or any electronic device to be synchronized with the instrument*
8	MAIN POWER SWITCH	
9	TEMPERATURE PROBE CONNECTOR	*Connects to temperature probe*
10	HARD STOP (SF) BNC CONNECTOR	*Connects to Hard Stop Valve*
11	MOTOR POWER CONNECTOR	*Sends the power pulses to the stepping motors*
12	USB CONNECTOR	*Connects the MPS-70 controller to the PC*

2.4 AC Power and Connections of MPS-70

Before connecting the SFM to the local AC line, verify that the setting of the instrument matches the local line voltage. Prepare the SFM for operation by connecting the mechanical subsystem to the MPS-70 unit. Connect the MPS-70 to the USB port of the microcomputer. Finally, plug the MPS-70 into the appropriate AC line.

2.5 Temperature Regulation

The syringes, valves, and observation chamber of the SFM are designed to be temperature regulated. Organic oil (like Perfluorinated oil) may be preferred for temperature regulation to avoid corrosion, but the user should check compatibility with stopped-flow materials beforehand. Careful temperature regulation minimizes any occurrence of temperature artefacts. The SFM module may be connected to a circulating temperature bath for temperature regulation. The coolant flows through two internal circuits: around the injection syringes and through the isolation valve block and observation head. With careful temperature regulation, temperature artefacts can be avoided over a very wide temperature range (between -10 and 80℃).
For lower temperatures, the use of a cryo stopped-flow accessory is necessary. Rapid kinetics down to -90℃ can be achieved. Please contact our commercial service for any inquiries.

3 INSTALLATION OF THE STOPPED-FLOW COMPONENTS

3.1 The Observation Head

The stopped-flow observation head (Figure 5) is installed on top of the SFM. The observation head has four optical windows: one window for illumination and three for observation. This allows measurements of absorbance, transmittance, circular dichroism, single or double wavelength fluorescence emission and light scattering or fluorescence polarization without adding any reflecting or beam splitting elements. The two windows at right angles to the incoming light can be equipped with lenses to increase the efficiency of light detection.

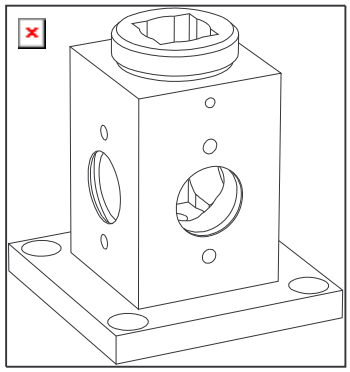


Figure 5 – Stopped-Flow Observation Head

3.2 Mixer Installation and Replacement

Each SFM comes from the factory with mixers installed. The mixers are located in the syringe block between the syringe block and the observation head (SFM-400) and at the bottom of the observation head below the cuvette as shown in Figure 8 and Figure 9. As in all stopped-flow systems, the mixer is one of the most delicate pieces of the instrument. It is recommended to check the state of the mixer regularly and also when the SFM has been unused for a prolonged period of time. Instructions for removal and replacement of the mixers are described in section 8.2.

3.3 Cuvette Installation

The observation cuvette is one of the most critical parts of all stopped-flow instruments. Indeed, it is extremely important to adapt the cuvette to the parameter being observed. For example, it would be inappropriate to use the same cuvette for measuring a small absorbance change and for measuring a fluorescence change of a compound having a high absorbance and producing strong inner filter effects. The SFM observation head can be equipped with a number of different cuvettes adapted to a variety of situations. If our standard cuvettes do not satisfy your specific experimental requirements, we invite you to contact us about custom-made cuvettes.

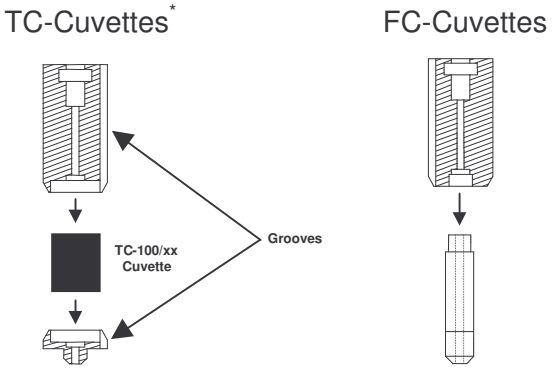
Figure 7 shows the cuvettes presently available and their specifications. There are two general styles of cuvettes:

FC (fluorescence cuvette) -FC type cuvettes have blackened edges to reduce light scattering in fluorescence configuration. The FC-15 and FC-20 cuvettes are the best choices

for CD experiments in the far UV. Their large aperture facilitates low noise recording at these wavelengths.

TC (transmittance cuvette) - TC type cuvettes have been primarily designed for absorbance and transmittance experiments. However in the TC-xx/yyF models, both sides of the light path are transparent. These models of cuvettes can also be used for fluorescence experiments using dilute samples and excitation with a laser or any other low divergence light source. Cuvettes of the TC.xx/10 type have a 1x1 mm² cross section and cuvettes of the TC.xx/15 type have a 1.5x1.5 mm² cross section.

The two styles of cuvettes have different holders that are used to install them into the SFM observation head. The assembly of the cuvette with their respective cuvette holders is shown in Figure 6.



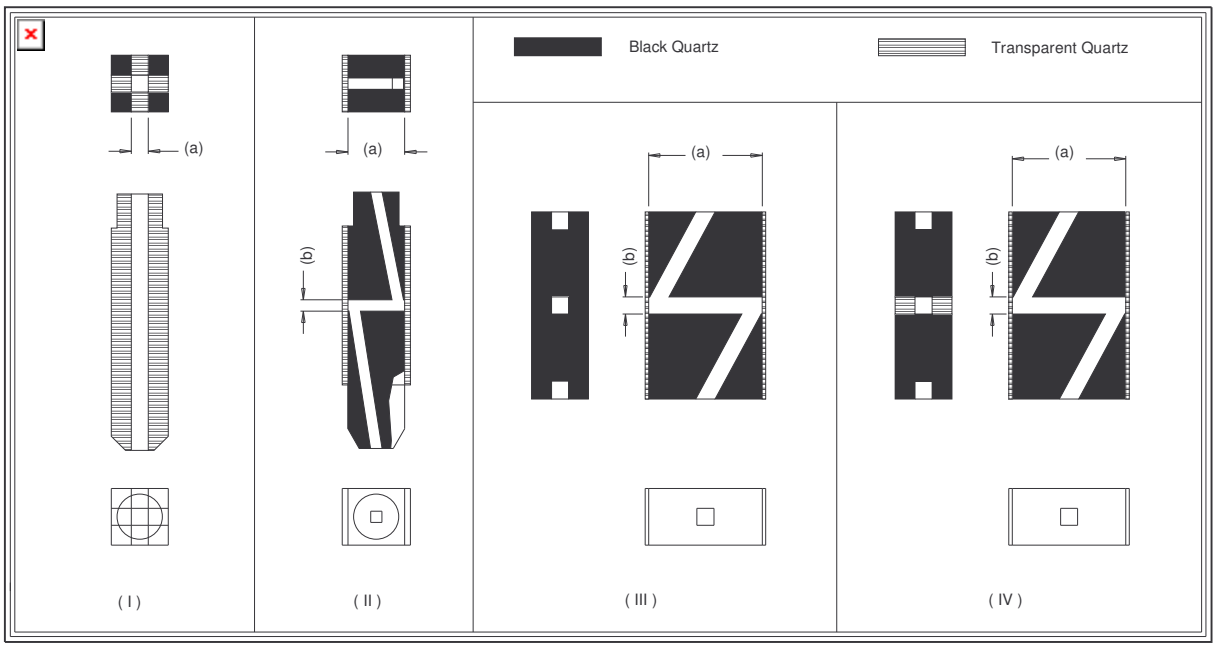
Note: *Be sure to align the grooves of the TC-Cuvette holder pieces.

Figure 6 – Cuvette Assembly

The SFM observation head has been designed so that the observation cuvette can be exchanged in a few minutes. This is often recognized by our users as one of the many advantages of the SFM systems. Removal and replacement of the cuvette is shown in Figure 12.

CUVETTE OPTICAL SPECIFICATIONS[1]				
Cuvette	Drawing	Light path (mm) (a)	Aperture (mm) (b)	Main application
FC-08	I	0.8	-	*Fluorescence, light scattering*
FC-15	I	1.5	-	*Fluorescence, light scattering, high absorbance*
FC-20	I	2.0	-	*CD, fluorescence*
TC-50/10	II	5	1.0	*Absorbance, CD, fluorescence*
TC-50/15	II	5	1.5	*Absorbance, CD, fluorescence*
TC-100/10T	III	10	1.0	*Absorbance, CD*
TC-100/10F	IV	10	1.0	*Absorbance, CD, fluorescence*
TC-100/15T	III	10	1.5	*Absorbance, CD*
TC-100/15F	IV	10	1.5	*Absorbance, CD, fluorescence*

Notes: (1) All cuvettes are made of Suprasil (transparent from 185 to 2500 nm).



	CUVETTE DEAD VOLUMES[1] AND DEAD TIMES[2]			
	With Berger Ball Mixer		With High Density (HDS) Mixer	
Cuvette	Dead Volume (µl)	Dead Time (ms)	Dead Volume (µl)	Dead Time (ms)
FC-08	15.6	1.6	30.4	3.0
FC-15	36.6	3.7	51.3	5.1
FC-20	59.3	5.9	74.1	7.6
TC-50/10	22.4	2.2	36.8	3.7
TC-50/15	40.1	4.0	54.6	5.5
TC-100/10T	30.2	3.0	45.0	4.5
TC-100/10F	30.2	3.0	45.0	4.5
TC-100/15T	46.8	4.7	61.5	6.2
TC-100/15F	46.8	4.7	61.5	6.2

Notes: (1) Dead volumes measured from mixing point to the center of the observation area.
(2) Dead times calculated at 10 ml/s flow rate. Dead time is inversely proportional to flow rate.

Figure 7 – SFM Cuvette Specifications

3.4 Installation of the Mixer Blocks and Delay Lines

In stopped-flow mode, the syringes of the SFM can be used to perform many types of mixing experiments. It is difficult to list all the possibilities here. Some are described below:

1) Load several reagents and mix them in different shots with the contents of the last syringe.

2) Use syringes loaded with reagents and a buffer to vary the concentration of one or two reagents and mix the result with the contents of the last syringe.

3) Perform sequential mixing and delays between up to 3 reagents before they are mixed with the content of the last syringe.

The observation head is installed on the SFM body differently depending on how many syringes are present and which type of experiment is being performed.

SFM-300 - The observation head and delay line are installed as shown in Figure 8. The smallest delay line comes standard and installed with the instrument
SFM-400 - The observation head and delay line(s) are installed as shown in Figure 9.
The observation head may be installed using the mixing blocks labeled **0-MIX-0**, **0-MIX-DL**, **DL-MIX-0** and **DL-MIX-DL** or no mixing block. The installation of the different mixing blocks is described in Table 4 – SFM-400 Observation Head Installation.

MIXING BLOCK	COMMENTS
0-MIX-0	Installed with no additional delay lines.
0-MIX-DL	Installed with one delay line between the mixer block and the observation head
DL-MIX-0	Installed with one delay line between the SFM body and the mixer block.
DL-MIX-DL	Installed with delay lines on both sides of the mixer block.
NONE	Only a delay line is installed between the SFM body and the observation head and the SFM-400 functions as an SFM-300 (Figure 8). Syringe 3 is blocked by the delay line and only syringes 1, 2, and 4 are useable. In this case, syringe 3 does not need to be filled.

Table 4 – SFM-400 Observation Head Installation

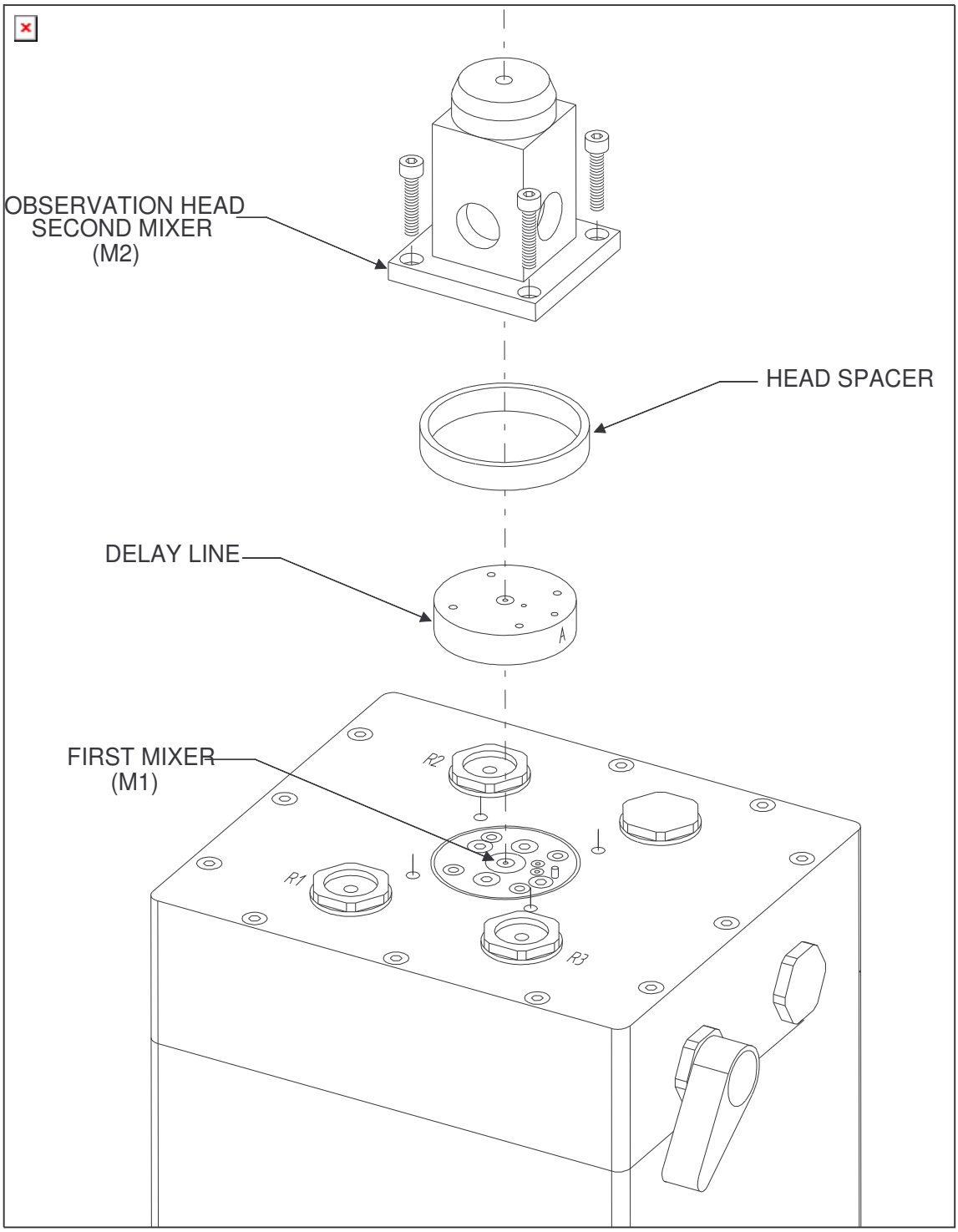


Figure 8: SFM-300: Installation of Delay Lines

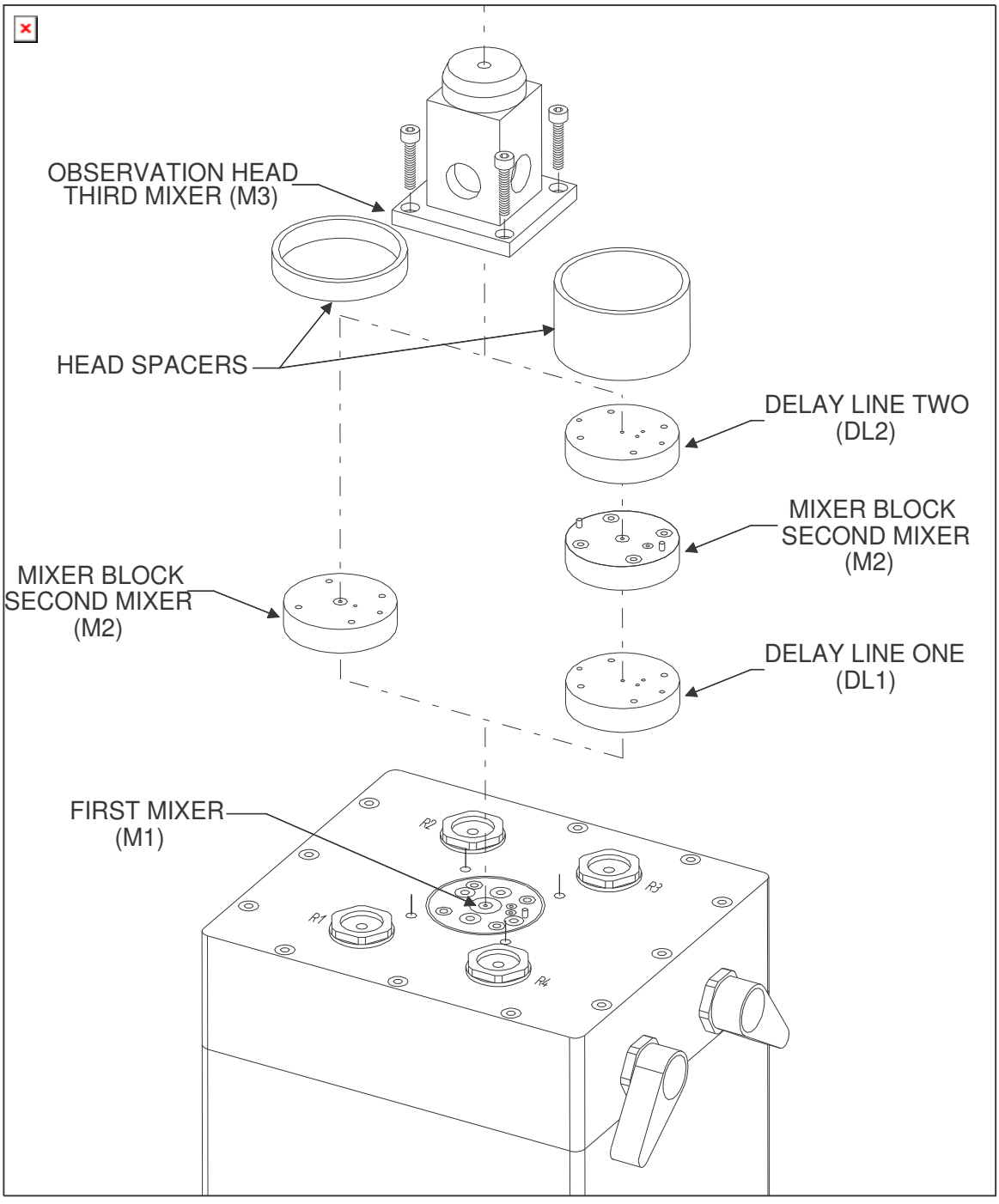


Figure 9 : SFM-400: Installation of Mixing Blocks and Delay Lines

3.5 Flow Line and Intermixer Volumes

Figure 10 (SFM-300) and Figure 11 (SFM-400) below indicate the volumes of the SFM flow lines and delay lines. The amount of time a sample ages between two mixers is given by:

Ageing time between two mixers = (Intermixer volume)/(Flow rate through intermixer volume)

It should be noted that the volumes given in the table are the mechanical volumes. The hydrodynamical volumes may vary slightly around these values. For a precise measurement of ageing times, it is recommended that the intermixer volumes be determined experimentally with known reactions. One such experimental procedure for determining the intermixer volumes is described in the Quenched-Flow section of this manual.

SFM-300/S FLOW LINE VOLUMES	
Line Number	**Flow Line Volume (µl)**
1	69
2	7
3	89
4	88
5	10
6	Delay Line
7	19
8	108
9	Cuvette (Figure 7)

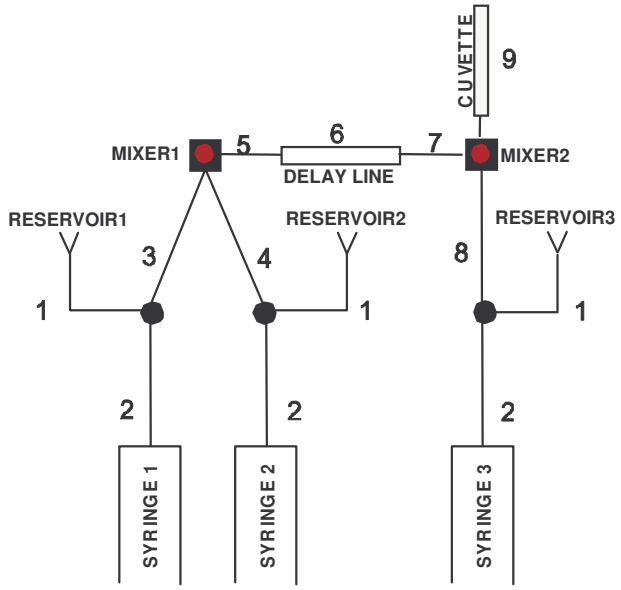
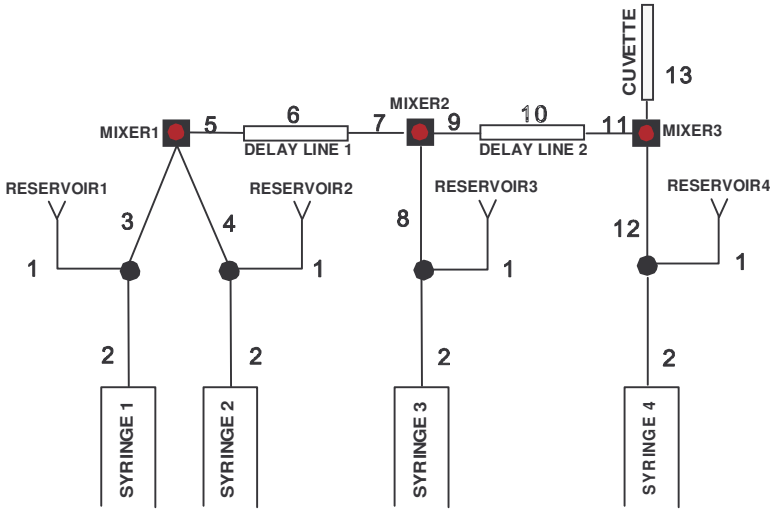


Figure 10: SFM-300/S Flow Line and Delay Line Volumes

	DELAY LINE AND INTERMIXER VOLUMES*						
	Delay Line						
	N°1(17)	N°2(40)	N°3(90)	N°4(140)	N°5(190)	N°6(500)	N°7(1000)
Volume (µl)	19	35	92	144	192	498	1003
Intermixer Volume $M1_{BB} – M2_{BB}$ (µl)	47	63	120	172	220	526	1031
Intermixer Volume $M1_{BB} – M2_{HDS}$ (µl)	43	59	116	168	216	522	1027

**Notes: Intermixer volumes are measured from the mixing point of one mixer to the mixing point of the next mixer. BB indicates a Berger Ball mixer has been installed at the position noted. HDS indicates a High Density mixer has been installed at that position.*

SFM-400/S FLOW LINE VOLUMES	
Line Number	**Flow Line Volume (µl)**
1	69
2	7
3	89
4	88
5	7
6	Delay Line 1
7	13
8	94
9	10
10	Delay Line 2
11	19
12	108
13	Cuvette (Figure 7)



	DELAY LINE AND INTERMIXER VOLUMES*							
	Delay Line							
	None	N°1(17)	N°2(40)	N°3(90)	N°4(140)	N°5(190)	N°6(500)	N°7(1000)
Volume (µl)	0	19	35	92	144	192	498	1003
Intermixer Volume $M1_{BB}$ – $M2_{BB}$ (µl)	21	39	55	112	164	212	518	1023
Intermixer Volume $M2_{BB}$ – $M3_{BB}$ (µl)	31	47	63	120	172	220	526	1031
Intermixer Volume $M2_{BB}$ – $M3_{HDS}$ (µl)	27	43	59	116	168	216	522	1027

*Notes: Intermixer volumes are measured from the mixing point of one mixer to the mixing point of the next mixer. BB indicates a Berger Ball mixer has been installed at the position noted. HDS indicates a High Density mixer has been installed at that position.

Figure 11: SFM-400/S Flow Line and Delay Line Volumes

3.6 Liquid Outlet System: Hard Stop Valve

During the injection phase, the liquid in the cuvette can reach linear velocities greater than 20 meters per second. At the flow stop, the liquid column must be immobilized in a fraction of a millisecond. Several different stop modes can be used to immobilize the liquid column. The stop mode used can result in overpressure or underpressure conditions that are potential sources of stop artifacts. The mode chosen by Bio-Logic is presented below: the hard stop system.

In this mode, the flow will be immobilized by a combination of two mechanisms: first, from the stepping motors stop and second, by a high speed electrovalve (hard-stop) which closes the output of the SFM cuvette. This hard-stop is actuated by the programmable power-supply of the SFM. No overpressure is developed in the observation cuvette because synchronization of the hard-stop with the motor halt. The result is elimination of the stop and overpressure artifact giving high quality stopped-flow traces with the lowest dead times.

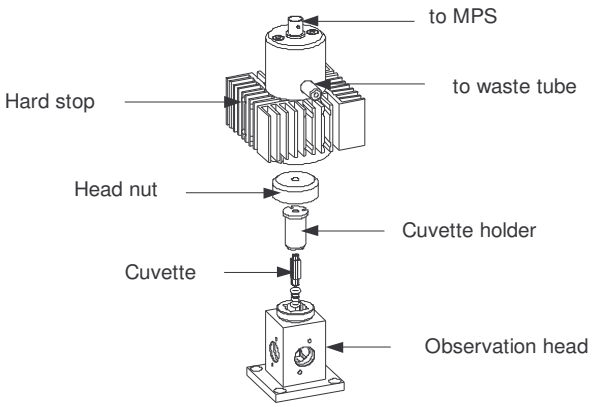


Figure 12 : Hard Stop installation

There are three operation modes of the hard-stop that can be chosen in Bio-Kine. The modes of operation are:

• **Automatic mode:** the hard-stop is controlled by the software. Using this mode, the hard-stop is closed at the end of the pushing phase and during the acquisition. However the hard-stop remains opened between shots.

• **Manual mode**: the hard-stop is programmed to open and close by the user.

• **None**: The valve is always open.

The installation of the hard-stop on the observation head is shown in Figure 12.

3.7 Special Accessories

Several accessories are available to expand the functions of the SFM. Below are the descriptions of the accessories and their functions. Custom accessories can also be designed and we invite you to contact Bio-Logic or its nearest representatives to discuss your particular needs.

3.7.1 Small drive syringe

The SFM standard syringes (10 ml) have a large driving speed range. Each syringe can be programmed for different speeds and used to make mixing ratios different from 1:1. Ratios as high as 1:20 can be obtained with the standard syringes. Ratios beyond 1:20produce poor

results due to the extremely slow movement of the syringe motor delivering the sample to be diluted.

For operation with dilution ratios higher than 1:20, we advise the use of a 1.9 ml syringe for injecting the solution to be diluted. This enables the motor pushing the 1.9 ml syringe to run at a faster and smoother rate. The specifications of the 1.9 ml syringe are given in Table 1-SFM specifications. 1.9 ml syringes can be ordered from Bio-Logic or its representatives.

Syringe disassembly and reassembly is discussed in the Technical Instructions section of this manual. We recommend that the user be familiar with this section before attempting syringe disassembly and assembly.

3.7.2 High density mixer

Mixing solutions of different densities offers a formidable challenge for stopped-flow instruments. In typical protein folding/unfolding experiments, heavy solutions of urea or guanidine chloride are mixed with pure aqueous buffers just before the cuvette. The result is an unavoidable convection reaching the observation cuvette 10 to 30 seconds after mixing. This convection creates a massive artifact that is guaranteed to ruin the kinetics being recorded. The SFM module can be equipped with a specially designed mixer (model HDS; Figure 13) that includes an internal siphon-like frame and allows blockage of convection created by density or temperature differences. Using this mixer, stopped-flow traces produced by mixing high density solutions with water can now be recorded from the first millisecond to several 100 seconds.

Installation of the HDS mixer is identical to that of a standard (Berger Ball) mixer. Instructions are provided in the Technical Section of this manual.

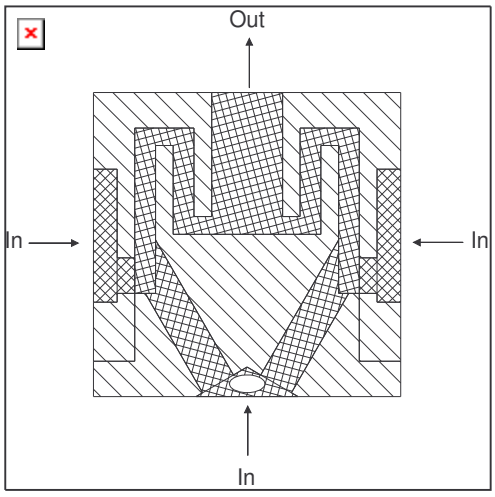


Figure 13 – HDS Mixer

3.7.3 Observation head with separate cooling

The standard observation head may be replaced with an observation head that has separate cooling (see Figure 14).

The separate cooling feature regulates temperature in the observation head as well as the main body of the instrument. This may be used in cases where mixing the solution produces a temperature change of the solution flowing into the cuvette, requiring extra cooling.

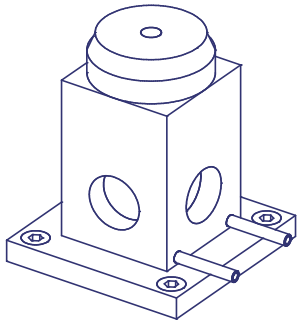


Figure 14 – Observation Head with Separate Cooling

3.7.4 MICROCUVETTE ACCESSORY

The standard observation head may be replaced with the microcuvette accessory (see Figure 15). The microcuvette accessory consists of one micromixer combined with a special μFC-08 cuvette.
The μFC-08 is a modified FC-08 cuvette with a dead volume varying from 1 to 3 μl. The dead volume of the cuvette is adjusted by the user by setting the special adapter provided with the accessory (see Figure 15). Minimum dead time obtained with this accessory is 0.25 ms.

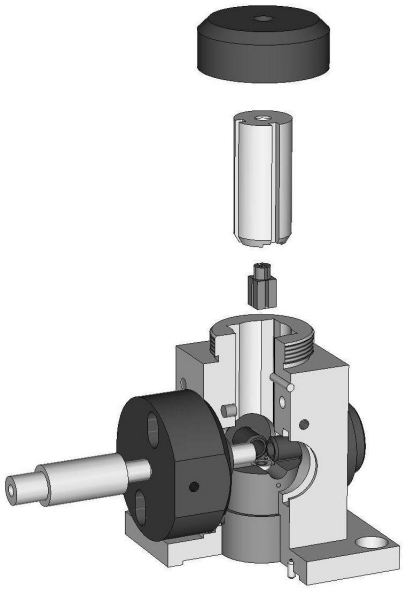


Figure 15 : microcuvette accessory

3.7.5 LOW TEMPERATURE ACCESSORY

Because standard stopped-flow cannot be operated below 0°C, a special accessory was designed to perform kinetics at temperatures as low as -90°C. A description of the low temperature accessory is given in Figure 16.
The low temperature accessory consists of an umbilical connector and a mixing compartment.

• The **umbilical connector** allows for a fluidic connection between SFM-300/400 and the observation cell. This allows for a physical separation of these two parts of the stopped-flow. It is the case when working with far subzero temperatures: the mixing unit and the solution immerged in PEEK tubes can be cooled by the cryothermostat while the rest of instrument can be kept at a more normal emperature. The fluid used for regulating the stopped-flow temperature also circulates into the umbilical connector, keeping the active solutions at a constant temperature until the end of the connector.

• **Mixer and observation cell assembly**: this part of the setup is immerged in the final cryosolvent. It contains a flow lines block that serves as a reactant reservoir. These flow lines are made of inert material (PEEK) and are of sufficient volume so as to act as a heat exchanger and allow equilibration of the reactants at the temperature of the cryosolvent before injection in the observation cuvette. In the standard configuration, the volume of these lines is 200 µl but can be easily adapted.
The mixer is fitted to the observation cell which can be any the standard stopped-flow cell (Figure 7).

• Detection is made through optical fibers which are installed on the cuvette. These optical fibers are protected from the cryosolvent by umbilical tubes. It is also possible to flush nitrogen or inert gas through these tubes for anaerobic work or to avoid condensation phenomena.

• A temperature probe is in contact with the cuvette to give the precise temperature of the reaction.

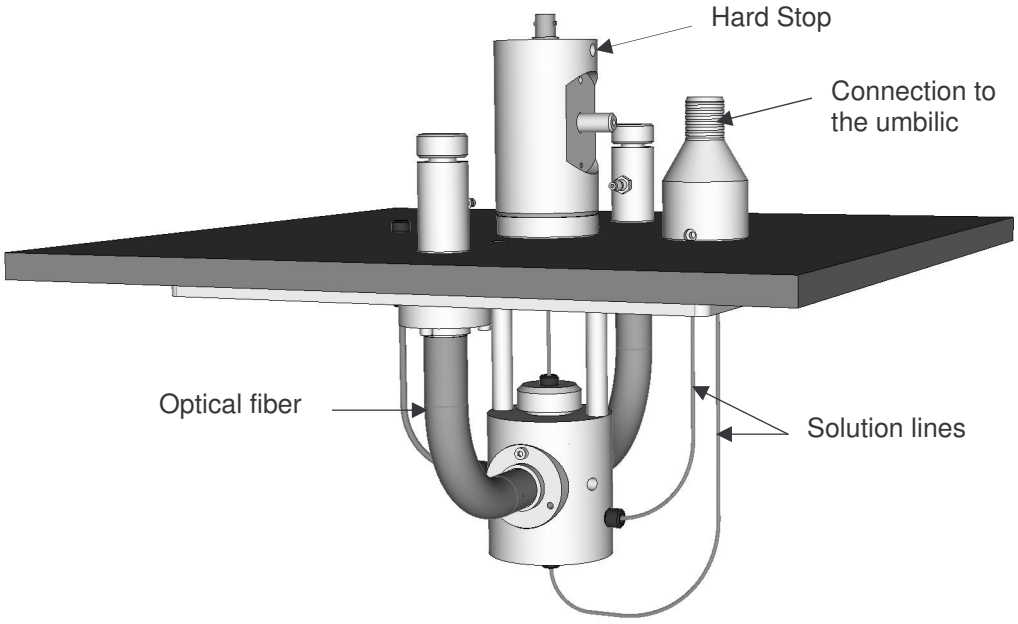


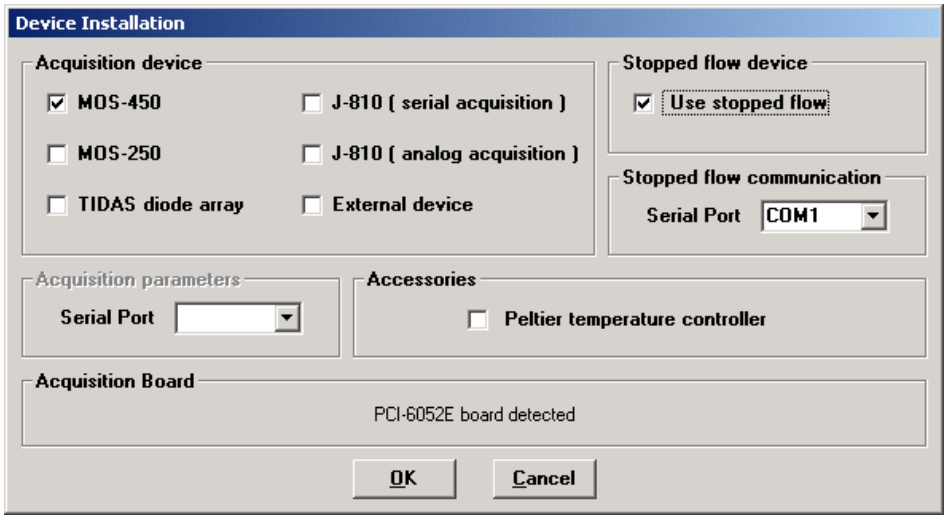
Figure 16: low temperature accessory

4 SOFTWARE CONFIGURATION IN STOPPED-FLOW MODE

The SFM is controlled by Bio-Kine software which is also used to control acquisition parameters. This section precisely describes the configuration of the software. Please note that the procedures and examples have been generalized, and configuration choices should be made based upon the equipment purchased and intended experiments.
This section assumes that the user has already installed Bio-Kine software on the host microcomputer.

4.1 Installation-SFM-300/400 with MPS-60 using Bio-KIne version up to 4.45.

Once Bio-Kine loaded, choose **Install, device installation** in the main menu. The stopped-flow communication is established from this window by checking the **stopped-flow device** box and choosing the corresponding **serial port.** Accept the parameters using the **OK** button.



4.2 Installation-SFM-300/400 with MPS-60 or MPS-70/3(4) using Bio-Kine version 4.47 and higher.

Once Bio-Kine is loaded, choose **Install, device installation** in the main menu (Figure 17-device installation.). The stopped-flow communication is established from this window by checking the **stopped-flow device** box and choosing the corresponding **Serial port** for **MPS-60** or **USB port** for **MPS-70**. Accept the parameters using the **OK** button.

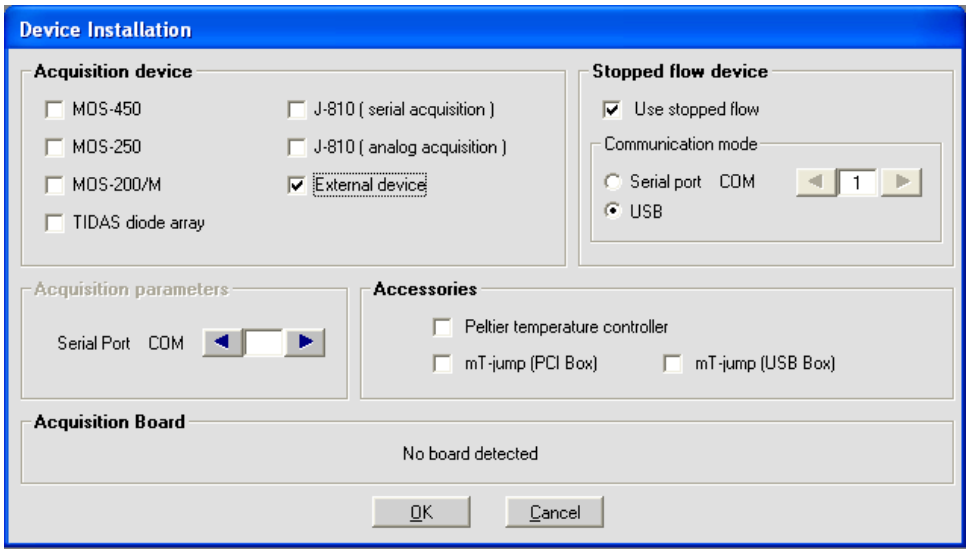


Figure 17-device installation.

4.3 Stopped-flow Configuration

Once the stopped-flow device and its serial port are selected in the **device configuration** menu (refer to section 0), choose **the Install, stopped-flow configuration** menu (see Figure 18).

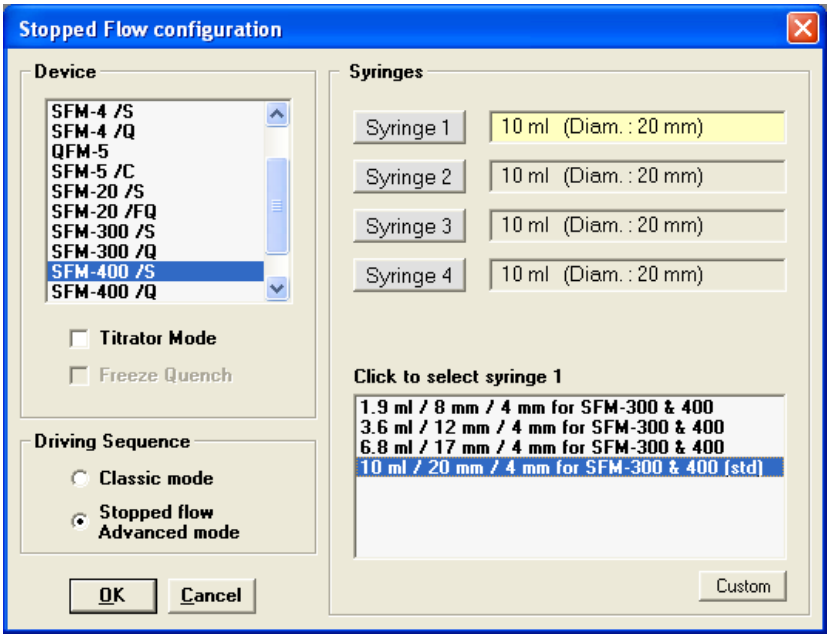


Figure 18 : stopped-flow configuration.

The device to be installed should be configured according to the instrument purchased, and the mode chosen for use must be selected in the driving sequence. The **titrator** and **freeze quench** modes are only available in a specific configuration; please refer to their respective manual for details.

Syringe configuration is made in the same window. The active syringe is displayed in yellow; select the nature of the syringes that have been installed in each syringe position of the SFM by clicking on the right one.

The SFM comes equipped with standard 10 ml syringes and these are the default syringes installed in the software. Changes only need to be made in the software when syringes of different volumes (other than standard) have been installed in the SFM.

Use the Custom button to enter syringe specifications if you have a custom syringe. In this condition the window shown in Figure 19 is displayed. It is then necessary to enter volume, piston diameter and screw pitch of the custom syringe to add it to the standard ones.

Figure 19 : custom syringe

WARNING: Incorrect syringe configuration will cause volume and flow rate calculations to be incorrect!

4.4 Stopped-flow status area

A vertical menu bar on the left of the screen is dedicated to the stopped-flow device (see Figure 20). This menu bar can be hidden or displayed using the SFM in the main menu. This menu bar gives access to the syringe control window using the button (refer to section 0), to the classic mode and to the advanced mode using the Mixing Sequence button (refer to section 5.6)
At any time, information about the configuration of the stopped-flow can be found in this bar such as device, delay lines installed and cuvette type.

Once the sequence is ready in the classic or advanced mode, the shot control window is displayed in the area as shown in Figure 20 (also refer to section 5.6).

Figure 20: stopped-flow menu bar.

5 INSTRUMENT OPERATION IN STOPPED-FLOW MODE

5.1 Manual Syringe Control

The syringes of the SFM can be controlled either manually or automatically. Automatic control of the syringes is strictly used only for experiments. The manual control of the syringes is used for initialization, filling, and emptying the syringes. Manual movement of the syringes can be made either directly from the MPS or though Bio-Kine. Both methods are described in the following sections.

5.1.1 MPS-60

Syringe control directly from the MPS is made through the use of the buttons on front panel of the MPS (Figure 2).

The (+) and (-) buttons are used to select the syringe to be moved. The (up) and (down) buttons are used to empty and fill the syringes respectively. The corresponding light on the front panel will indicate which syringe has been selected.

5.1.2 MPS-70

Syringe control directly from the MPS is made through the use of the buttons on the front panel of the MPS (Figure 2).

The "selected" buttons are used to select the syringe to be moved. The left button selects syringes from number 1 to 3(SFM-300) or 4(SFM-400) with increasing numbers. The right button selects syringes from number 3 or 4 to 1 with decreasing numbers. The (up) and (down) buttons are used to empty and fill the syringes respectively. The corresponding light at the front panel will indicate which syringe has been selected. By pushing the left and right "select" buttons at the same time, all the syringes are selected.

5.1.3 Software

Syringe control from Bio-Kine software is made through the  button in the stopped-flow status area (see Figure 21). The MPS is then initialized and communication established between Bio-Kine software and the MPS unit. The message 'MPS on line' is displayed in a green window in the stopped-flow status area

The syringe to be moved is selected by clicking on the corresponding frame or pressing the <**Left**> or <**Right**> arrows keys on keyboard. The new selected syringe will be surrounded by a red rectangle (Figure 21).

Syringes are emptied or filled using the  ,  ,  and  buttons or with the <**Up**> and <**Down**> arrows. The  button moves a syringe upwards by one elementary movement, and the  button moves a syringe downwards by one elementary movement. The  button moves the piston upwards by 10x elementary movements and the  button moves the piston downwards by 10x elementary movements.

The size of the elementary steps and syringe movement speed is controlled in the Manual Speed section of the window (Figure 21). Press the  and  buttons to change the manual speed. The display shows the speed in flow rate based on the syringe installed and moved.

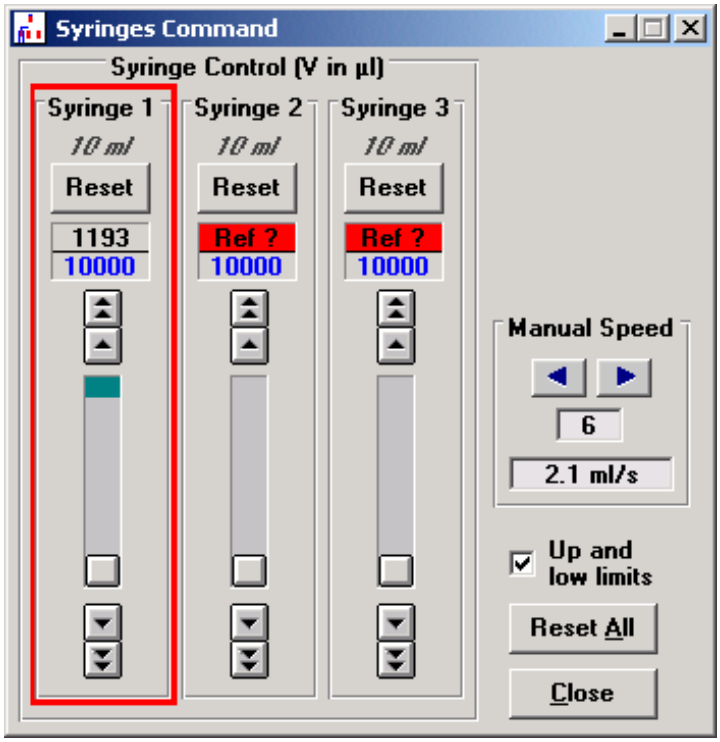


Figure 21: syringe control window

5.2 Syringe Initialization

The MPS that controls the SFM follows the movements of the syringes so that the actual residual volumes are displayed at all times (see Figure 21). When the MPS is turned on and the software started, the syringe volume counters show Ref ? and must be initialized (Figure 21).

The syringes are initialized by setting the syringes to their uppermost (empty) position and resetting the syringes in Bio-Kine. The syringes can be selected and moved to their uppermost positions either directly with the MPS (section 0) or through Bio-Kine (section 0). Once a syringe has reached its uppermost position, the syringe motor will oscillate and vibrate as it becomes out of phase with the driving pulses. There is no danger to the SFM or syringe motors when this occurs, but there is no reason to unnecessarily prolong this treatment either.

The syringes can be reset individually by pushing the Reset button for each syringe or all at once by pushing the Reset All button in the syringe control window.

IMPORTANT: Measurement of residual syringe volume is made by counting the logic pulses from the controller for each syringe. If, for any reason, a syringe is blocked during a run, the pulses will not correspond to the true volume delivered and the value displayed may become erroneous (e.g. in the case of incorrect positioning of a valve). In this case, it is advisable to reinitialize the syringes.

If, by accident, a syringe is returned to its uppermost position the syringe volume counter will again show Ref ? and the syringe must be reinitialized. To avoid such accidents, the **Up and Low Limits** checkbox may be checked. When this box is checked, Bio-Kine will not allow the syringes to be driven beyond their upper and lower limits. This also avoids

accidentally pulling the syringe plunger completely from the syringe and spilling solution onto the SFM.

WARNING: The Up and Low Limits only applies to control of the syringe from within Bio-Kine. These limits can be bypassed by manual control of the SFM directly from the MPS.

5.3 Filling the Syringes

WARNING: Utmost care should be exercised during this operation. Normal operation of the system requires that no bubbles are present in the injection syringes. If this occurs, the buffer flow through the observation chamber will not be correctly controlled by the plunger movement and artifacts may be observed. For best results it is recommended that all solutions be degassed and filtered before filling the SFM.

The syringes of the SFM can be emptied and filled manually (section 5.1). The filling of the syringes follows the steps below which are shown in Figure 22.

1) Attach a syringe (disposable plastic syringes may be used) containing sample or buffer to a syringe reservoir port on top of the SFM (Figure 22, Panel 1).

2) Set the syringe valve handle to (**R**) and fill the syringe manually (section 5.1) while exerting slight pressure on the reservoir syringe (Figure 22, Panel 2 and 3). The pressure exerted on the reservoir syringe prevents any vacuum from occurring in the reservoir syringe, which could result in bubble formation. It is suggested that 10ml syringes be filled using manual speed 4 in Bio-Kine and 1.9ml syringes be filled using manual speed 2.

3) Eliminate any bubbles in the SFM syringe by driving the SFM syringe up and down several times while it is connected to the reservoir syringe (Figure 22, Panel 4).

4) Turn the syringe valve handle to (**C**) (Figure 22, Panel 5)

5) Empty one or two elementary movements of the syringe (section 5.1) to definitively eliminate any bubbles remaining in SFM and cuvette.

6) Repeat the above process for the other syringes.

It is recommended that the syringes be filled in reverse numerical order to best remove bubbles from the SFM and cuvette.

IMPORTANT: ALL SYRINGES MUST BE FILLED EVEN IF THEY WILL NOT BE USED FOR AN EXPERIMENT! The valve handles of the unused syringes should be turned to (R) after the filling process is complete.

The Stopped-Flow-Module is now ready for operation.

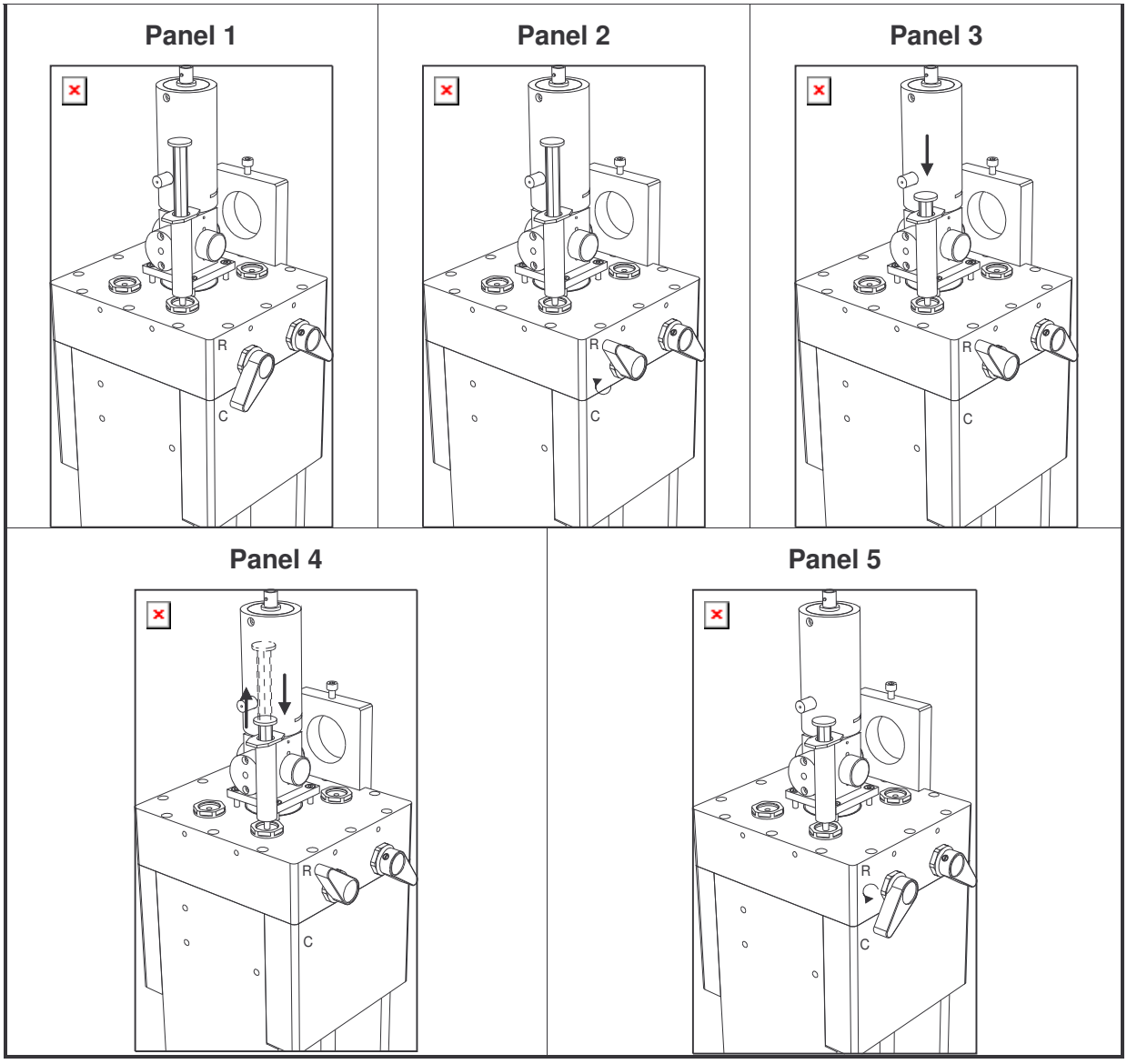


Figure 22 – SFM Syringe filling procedure

5.4 SFM Cleaning and Storage

After each day's experiments the SFM should be cleaned. A thorough cleaning of the SFM will ensure that it has a long functional life and diminish any chance of sample contamination for the next user of the instrument. The procedure below is the recommended daily cleaning procedure to be done before shutting off the instrument.

1) Remove and remaining samples or buffer from the syringes.

2) Wash the syringes and flow lines 2 – 3 times with water. This is done by filling each syringe with water to a volume at least equal to the sample volume used for experiments. With the syringe valve handles set to (**C**), empty the syringes completely. Since the liquid will exit via the cuvette, it will wash the flow lines and cuvette as well as the syringes.

3) Wash the syringes and flow lines one time with 70 – 100% ethanol. Use the same procedure as in step 2.

4) Dry the syringes, flow lines and cuvette with a single wash of air. Use the same procedure as in step 2. The syringes should be emptied in reverse numerical order so that all liquid is pushed out of the syringes, flow lines and cuvette.

Set all syringe valve handles to (**R**) and move all syringes to their lowermost positions. The syringe plungers should exit the SFM so that the plunger tips are completely visible. If this is done using Bio-Kine it will be necessary to uncheck the **Up and Low Limits** checkbox in the software **syringe control** window (Figure 21).

Note: You may observe a few drops of liquid that fall from the syringes when the syringe plungers are completely out of the SFM. This is normal as a small amount of liquid is always trapped between the plunger tip and the syringe barrel to make a tight seal.

5) Turn all syringe valve handles to (**C**).

6) Turn off the MPS.

5.5 Long-term Storage of the SFM

If the SFM will not be used for a long period of time (more than several weeks), it should be cleaned as explained in section 5.4. If the SFM is connected to a circulation temperature bath, the temperature bath should be disconnected from the SFM and the SFM drained completely of all cooling liquid. Afterwards, is recommended that the SFM cooling circuits be flushed with ethanol followed with air. The SFM is now ready for storage.

5.6 Creating a sequence using the advanced mode

5.6.1 SFM options

An advanced menu was created to improve the friendliness of the design of the stopped-flow sequence and to optimise experimental settings in order to get the best quality results. This mode can only be used for a single mixing experiment. It is still necessary to use the classic mode for a double mixing experiment or to perform pre-washing phase (refer to section 5.7 in this case). The advanced mode must be selected in the stopped-flow configuration (see 4.4) then click on 

button in the stopped-flow status area. The window shown in Figure 23 appears.

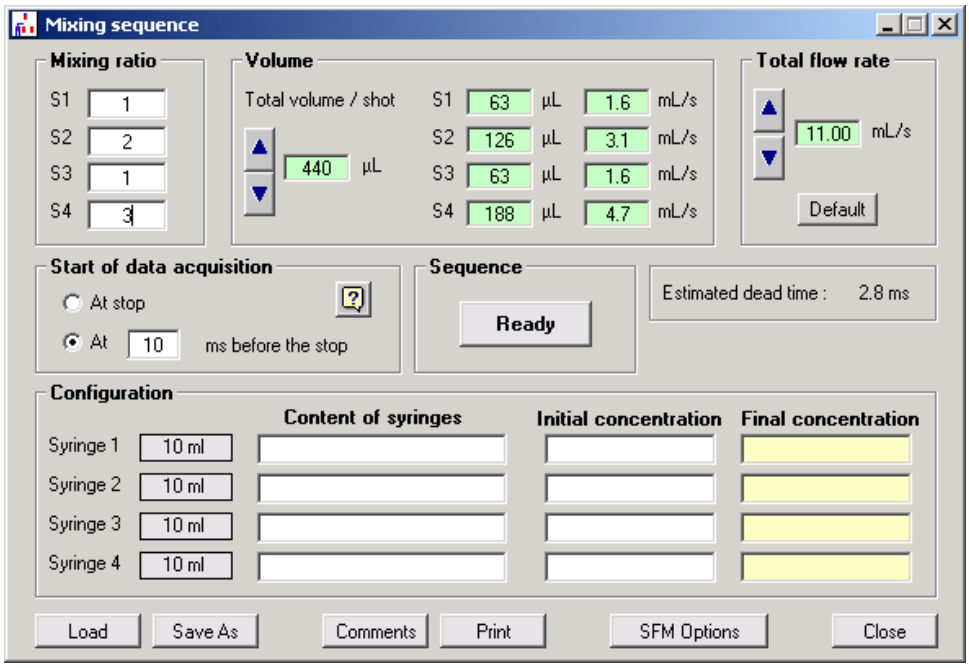


Figure 23 : driving sequence in advanced mode

First operation should be to check the configuration of the stopped-flow by clicking on the SFM Options button.

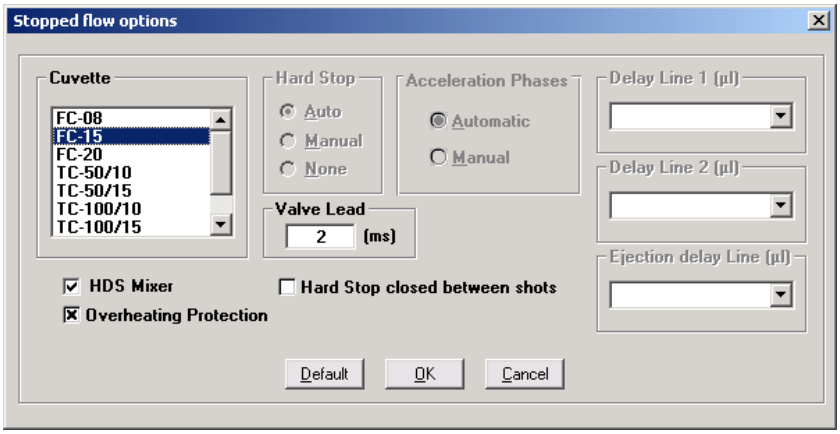


Figure 24 : SFM options in advanced mode

• Select the **cuvette** and **mixer** according to the cuvette and mixer installed in the SFM (refer to the SFM user's manual for more details).

WARNING: Incorrect cuvette and mixer configuration will cause dead time calculations to be incorrect!

• **Valve Lead:** This section of the window allows one to enter the number of milliseconds before the flow stops that the hard stop starts closing. The default value is zero. The lead time may be adjusted (from 0 – 5 ms) to fine-tune the quality of the stop. The precision of the setting is 0.1 ms.

• **Overheating Protection:** Not applicable for the recent MPS-60. The default mode is checked. It is a protection against electronic overheating after a long working day.

• **Hard-stop closed between shots**: in advanced mode, the configuration of the hard-stop is automatically set: the hard-stop closes at the end of the pushing phase (or a few milliseconds

before if a **lead time** is selected) and opens at the end of the acquisition. If the user needs to keep the hard-stop closed at the end of the acquisition (to run a spectrum for example), then it is necessary to check the corresponding box.

• **Delay lines**: In the advanced mode, delay lines cannot be changed; boxes are not active because only a single mixing experiment can be performed.

5.6.2 Design of stopped-flow sequence

The window shown in Figure 23 **: driving sequence in advanced mode** is separated into six areas: mixing ratio, volume, total flow rate, start of data acquisition, shots, and configuration. These different areas are respectively described below.

• **Mixing ratio**: it is the first parameter set. The ratio of the unused syringes must be set to 0. It is possible to enter a decimal value for the ratio.
• **Volume**: it is necessary to set the total volume of the reactants pushed into the cuvette using the 'up' or 'down' arrows. The volume is proportional to a micro-step volume in order to improve the reproducibility of results. Once the total volume is selected, the volume to be pushed for each syringe is calculated. The total volume selected should be big enough to wash the cuvette efficiently between two shots (refer to the color code for the limits).
• **Total flow rate**: total flow rate must be selected using the 'up' and 'down' arrows. Once the total flow rate is selected, the flow rate for each syringe is automatically calculated. 1 ml/s is considered the minimum value to get efficient mixing. There are also limits for a single syringe according to their respective volume (refer to colour code).
• **Start of data acquisition**: Using the 'stop' option, only the kinetics will be recorded. The acquisition is started when the hard-stop closes. To make sure the cuvette is well washed and the stationary state is reached, it is advised to start the acquisition a few ms before the stop.
• **Configuration**: In this area, it is possible to find the volume of the syringe installed and the type of cuvette. The content of the syringes can be entered with initial concentration; the final concentrations are calculated using the mixing ratio selected

• **Sequence**: Once the sequence ready, click on the Ready button.Tthe shot window is now activated on the left panel of the screen.

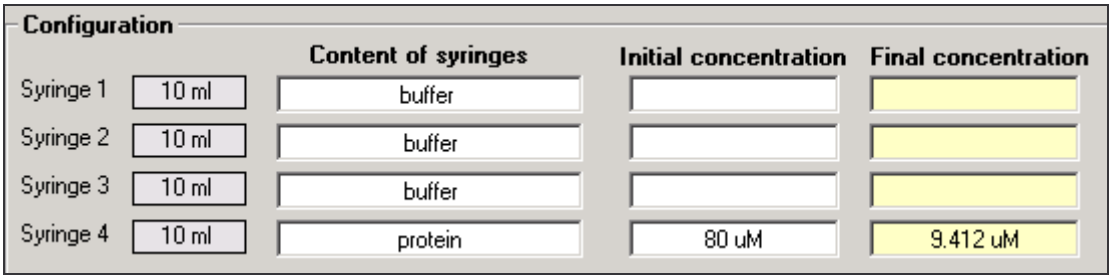
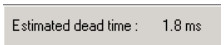


Figure 25: example of driving sequence

• The **estimated dead time** of the reaction is given in ms. The dead time is calculated using the cuvette and mixer selected (dead volume) and the total flow rate



$$\text{Dead Time} = \frac{\text{Cuvette Dead Volume}}{\text{Total Flow Rate}}$$

Figure 26 –Estimated dead time

A color code is used to warn the user about the choice of the parameters selected:

	Colour code	message
Total volume	green	OK (depending on syringe)
	yellow	total volume may be insufficient for washing the cuvette (<4 times the dead volume)
	red	Total volume too low for correct washing of cuvette
Volume per syringe	green	OK
	yellow	Syringe volume may be insufficient for washing of mixer
	red	Syringe volume too low
Total flow rate	green	OK
	yellow	Flow rate too low for correct mixing (< 1 ml/s)
	red	Flow rate may be difficult to achieve for this cuvette (>15ml/s)
Flow rate per syringe	green	OK
	yellow	Flow rate may be too high (will be dependant on cuvette and flow by other syringes)
	red	Flow rate out of range (too low or too high)

Standard operations can be made from the same window:

- Load a sequence using the Load button.
- Save a sequence using the Save As button.
- Print a sequence using the Print button.
- Close a sequence using the Close button.

• **Comments**: a text window is opened by clicking on the Comments button. Comments will be saved with the sequence.

5.6.3 *Programmable synchronization trigger using the MPS-60*

Contrary to the classic mode, only **Synchro out 1** can be used in the advanced mode. In this mode the hard-stop cannot be controlled manually from **Synchro out 2.** The use of **Synchro out 1** will result in an incorrect trigger signal and lose of signal.

Synchro out 1 is a falling trigger (5→0 V). The acquisition will start at the end of the pushing phase or few milliseconds before according to the configuration chosen by the user in the driving sequence.

5.6.4 *Programmable synchronization trigger using the MPS-70*

Only Trigger can be used in the advanced mode. In this mode the hard-stop cannot be controlled manually from **Synchro out.** The use of **Synchro out will** result in an incorrect trigger signal and loss of signal.

Trigger is a falling trigger (5→0 V). The acquisition will start at the end of the pushing phase or few milliseconds before according to the configuration chosen by the user in the driving sequence.

5.7 Creating a Driving Sequence using the classic mode

5.7.1 SFM options

Experiments are performed with the SFM through the use of a driving sequence. A driving sequence tells the SFM to automatically perform several functions such as moving the syringes, activating the hard stop, and triggering the data acquisition. Driving sequences are created in the window shown in Figure 27. The classic mode must be selected in stopped-flow configuration (see 4.4). Then click on 

button in the stopped-flow status area.

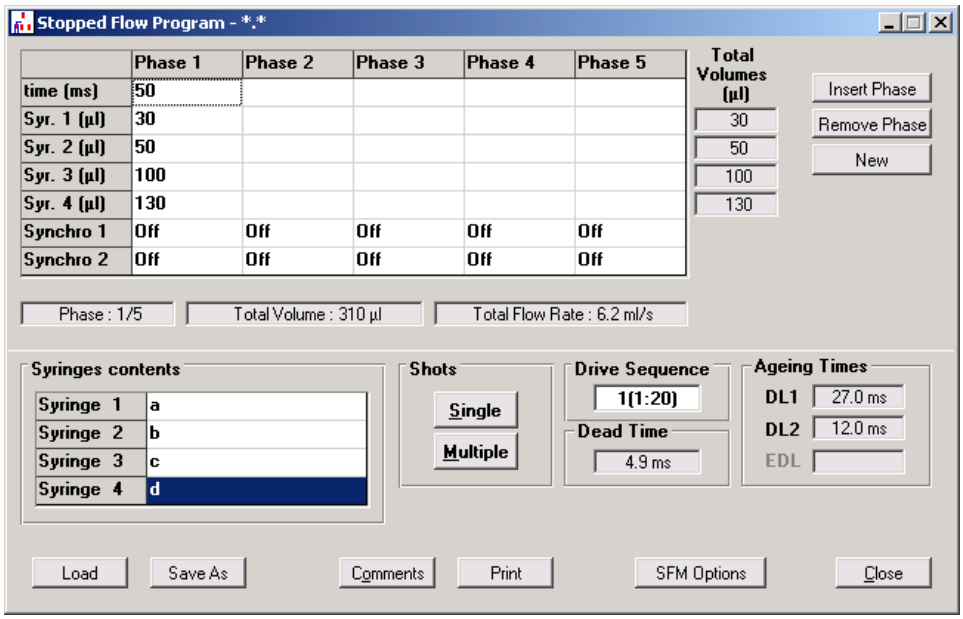
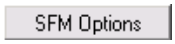


Figure 27 : driving sequence in classic mode

First operation should be to check the configuration of the stopped-flow, which is done by clicking on the 

button (refer to Figure 27).

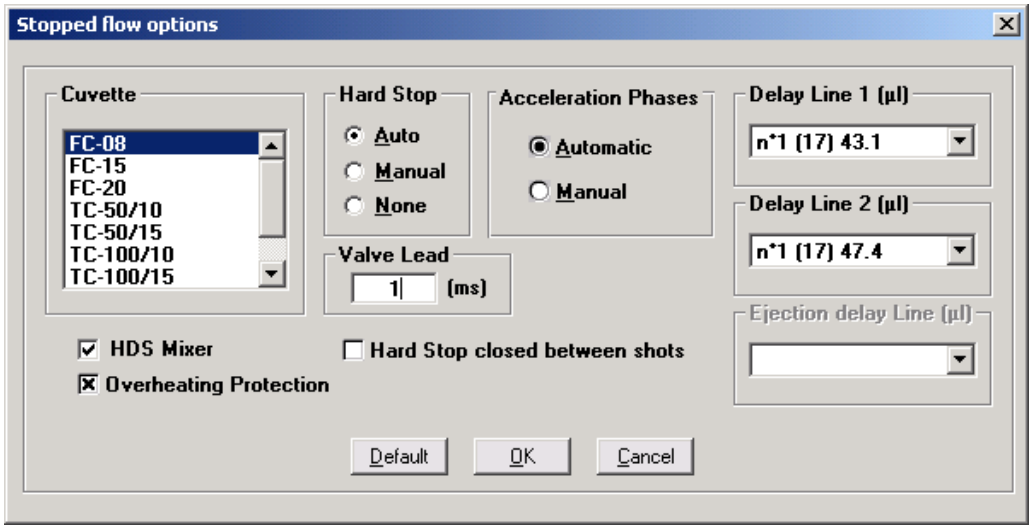


Figure 28: SFM options.

• Select the **cuvette** and **mixer** according to the cuvette and mixer installed in the SFM (refer to the SFM user's manual for more details)

WARNING: Incorrect cuvette and mixer configurations will cause dead time calculations to be incorrect!

• **Valve Lead:** This section of the windows allows one to enter the number of milliseconds before the flow stops that the hard stop starts closing. The default value is zero. The lead time may be adjusted (from 0 – 5 ms) to fine-tune the quality of the stop. The precision of the setting is 0.1 ms.

• **Overheating Protection:** Not applicable for the recent MPS-60. The default mode is checked. It is a protection against electronic overheating after a long working day.

• **Hard-stop:** '**auto**' is the default mode. In this position, the hard stop is closed at the end of the pushing phase (or few milliseconds before depending on the lead time chosen) and remains closed until the end of the acquisition. As soon as the acquisition is finished, the hard-stop opens. In case the user wants to leave the hard-stop closed after the acquisition (for example to perform a spectrum), it is necessary to choose the **Hard-stop closed between shots** option (see Figure 28). When using the '**manual**' mode the hard-stop is programmed to open and close by the user (through the synchro out 2). If '**none**' is selected, the hard-stop is always open.

• **Delay lines**: Select the delay line(s) according to the delay line(s) you have installed in the SFM. One or two delay lines must be configured depending on the type of device installed under section 3.4. Each delay line is chosen from a pull-down menu.

WARNING: An incorrect delay line configuration will cause ageing time calculations to be incorrect!

5.7.2 Design of the sequence

A driving sequence is entered in the program grid shown in Figure 29. Each column of the grid represents a driving sequence phase. Each phase contains actions for the SFM to perform. A complete driving sequence may contain from 1 to 20 phases. Although only 5 phases are shown initially, additional phases may be inserted using Insert Phase button or removed using the Remove Phase button.

Figure 29 shows an expanded view of the program grid. The duration of a phase is entered in ms (1 – 60000 ms/phase) on the first line of the program grid. The volume in µl delivered by each of the syringes during a phase is entered on the line next to the appropriate syringe. The status of the synchronization trigger is noted on the last line of the program grid.

To enter the phase duration and syringe volumes delivered, click on the corresponding cell or use the keyboard arrows keys to navigate between cells. The BACKSPACE key can be used for correction and the DEL key to clear a value. The synchronization trigger is toggled **on** or **off** by pressing "O" on the keyboard.

The selected values entered in the program grid can be cut, copied, and pasted using the **Cut, Copy**, and **Paste** functions available under the **Edit** menu. To perform a cut, copy, or paste operation, select the area of the grid desired by dragging the mouse with the left mouse button pressed and then choose the **Cut, Copy**, or **Paste** functions desired in the **Edit** menu. The values will be stored in the Windows clipboard for the **Cut** and **Copy** functions. Values will be pasted from the Windows clipboard for the **Paste** function. If the copy area is bigger than the paste area, the operation is done only for values that can fit inside the paste area.

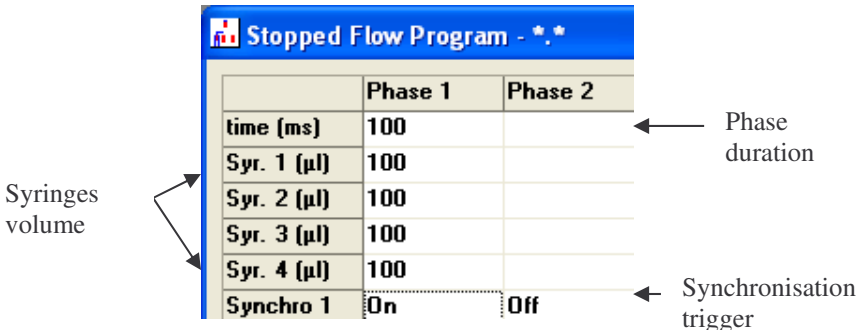


Figure 29– Expanded program grid

WARNING: Blank and non-numeric values entered in the program grid are considered as zero values. Phase duration of 0ms will cause the phase to be skipped in the execution of the drive sequence.

The contents of the syringes can be entered in the **Syringe Contents** frame of the driving sequence window (Figure 30). The text is entered from the keyboard and the BACKSPACE and DEL keys can be used for corrections.

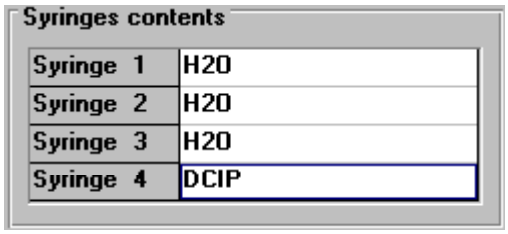


Figure 30 –Syringes Contents

Each time a program grid cell value is changed, information about the current syringe, current phase, and driving sequence, which is displayed below and to the right of the grid is updated (Figure 31). This information indicates:

1) Current phase number and total number phases in the driving sequence.

2) Volume delivered by the current syringe during the current phase or current phase total volume (if an entire phase is selected).

3) Flow rate of the current syringe during the current phase or current phase total flow rate (if an entire phase is selected).

4) Total volume delivered by each syringe during the driving sequence.

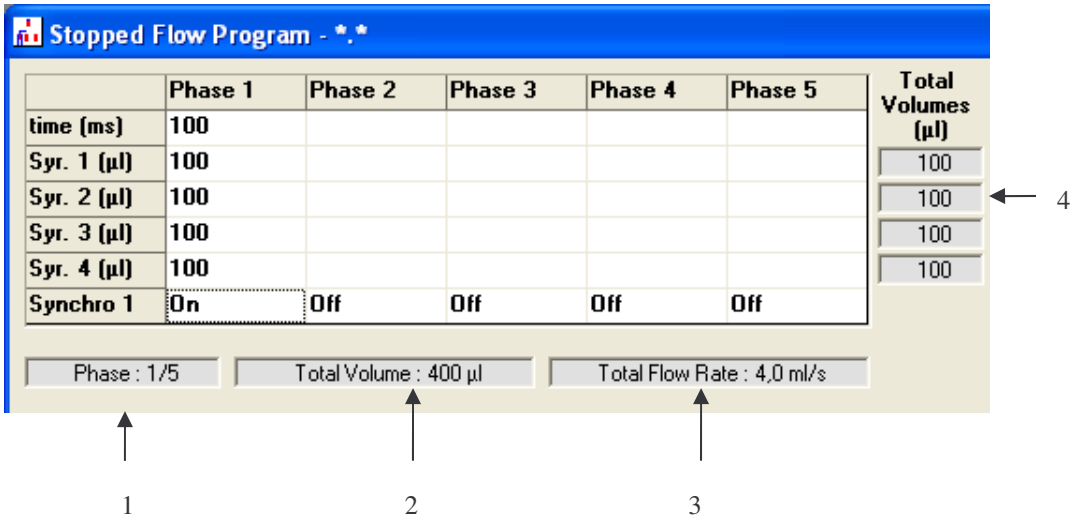


Figure 31 –Driving Sequence Information

An indication of the **Dead Time** and **Ageing Times** is shown in the driving sequence window (Figure 32). The dead time is calculated for the last valid phase, according to its flow rate and of the cuvette dead volume (Figure 7). The dead time is calculated according to the equation show in Figure 32.

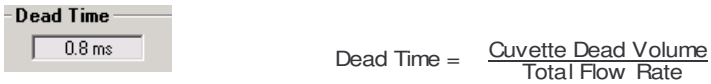


Figure 32 –Estimated dead time

The ageing times are calculated for the current phase selected based upon the syringes flow rates, delay lines installed and intermixer volumes. The ageing times are calculated according to the equations shown in Figure 33.

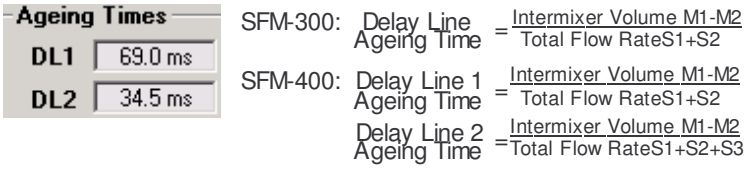


Figure 33 –Ageing Times calculation

Bio-Kine offers the ability to repeat phases a number of times in virtually any order. This is accomplished though a macro sequence entered in the Driving Sequence window (Figure 34). The macro sequence can be edited to run a single phase or many phases in a different order than present in the program grid.

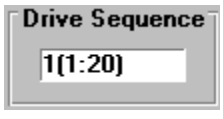


Figure 34 – MPS Software: Drive Sequence Macro

Once the sequence ready, click on the Single or Multiple button to activate the shot control window in the stopped-flow status area.

Standard operations can also be made from the same window:
- Create a new sequence using the New button
- Load a sequence using the Load button.
- Save a sequence using the Save As button.
- Print a sequence using the Print button.
- Close a sequence using the Close button.

• **Comments**: a text window is opened by clicking on the Comments button. Comments are saved with the sequence.

5.7.3 Programmable synchronization trigger using the MPS-60

The MPS can be programmed to deliver synchronization pulses (triggers). These pulses are TTL pulses (0 or 5 Volt) are delivered from BNC connectors **Synchro out 1**, $\overline{\text{Synchro out 1}}$, and **Synchro out 2** on the front panel of the MPS (see Figure 2). Both **Synchro out 1** and **Synchro out 2** are rising triggers (0→5 V). $\overline{\text{Synchro out 1}}$ is simply the inverse of **Synchro out 1** and is a falling trigger (5→0 V). $\overline{\text{Synchro out 1}}$ is used for most of the acquisition devices sold by Bio-Logic.

The triggers can be used to synchronize the SFM and data acquisition system or other instruments. If Bio-Kine software is being used for data collection, acquisition will start on the falling edge of the synchronizing pulse.

- If **Synchro out 1** is used then the acquisition will start at the end of the first active phase with a synchro set to 'On'.

- if $\overline{\text{Synchro out 1}}$ is used then the acquisition will start at the beginning of the first phase with a synchro set to 'On'

The triggers can also be used for synchronizing the SFM with other devices

The timing of the triggers with respect to the drive sequence is programmed in the last line(s) of the program grid in the driving sequence window (refer to Figure 29). The duration of the pulse will be equal to the duration of the phase. **Synchro out 2** is used to control the hard stop when the hard-stop is programmed by the user (configuration set to 'manual'). If the hard stop is not used (configuration set to 'none'), both **Synchro out 1 and 2** are available.

5.7.4 Programmable synchronization trigger using the MPS-70

The MPS can be programmed to deliver synchronization pulses (triggers). These pulses are TTL pulses (0 or 5 Volt) and delivered from BNC connector Trigger out, Synchro in and **Synchro out** on the rear panel of the MPS (see Figure 2-b). Trigger and **Synchro in/out** are falling edge triggers (5→0 V). Trigger out is used for most of the acquisition devices sold by Bio-Logic.

The triggers can be used to synchronize the SFM and data acquisition system or other instruments. If Bio-Kine software is being used for data collection, acquisition will start on the falling edge of the synchronizing pulse.

When the Trigger is used then the acquisition will start at the beginning of the first phase with a synchro set to 'On'

The triggers can also be used for synchronizing the SFM with other devices

The timing of the triggers with respect to the drive sequence is programmed in the last line(s) of the program grid in the driving sequence window (refer to Figure 29). The duration of the pulse will be equal to the duration of the phase. **Synchro out** is used to control the hard stop when the hard-stop is programmed by the user (configuration set to 'manual'). If the hard stop is not used (configuration set to 'none'), both **Synchro in**/**out** are available.

5.8 Creating a double mixing experiment

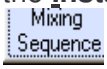
The double mixing experiment program is used in order to mix three reagents together. This means that a SFM 300 or SFM 400 has to be considered. In the SFM 300/S, all syringes will be used, for SFM 400, the three last syringes will be used (S2, S3 and S4) and S1 is not used, however this syringe has to be loaded with a solvent (water or buffer for example).

A double mixing experiment is divided in three phases:

1/ A and B are mixed in a first mixer

2/ The solution (A+B) goes through a delay line. The solution is aged in the delay line (waiting phase)

3/ The solution from the delay line is mixed with a C solution (third reageant) in the second mixer; and the final solution goes through the cuvette for analysis.

Once the mixing sequence "Double mixing mode" is selected from the **Install**, **Stopped Flow Configuration** menu, clicking on the button mixing sequence 

opens a window (Figure 35).

The double mixing experiment can be shared in two parts:

- Double mixing experiments: all parameters such as content syringe, concentration, phase conditions are defined
- Global sequence: gives information on the volume consumption for each syringe, ageing time assessed etc…

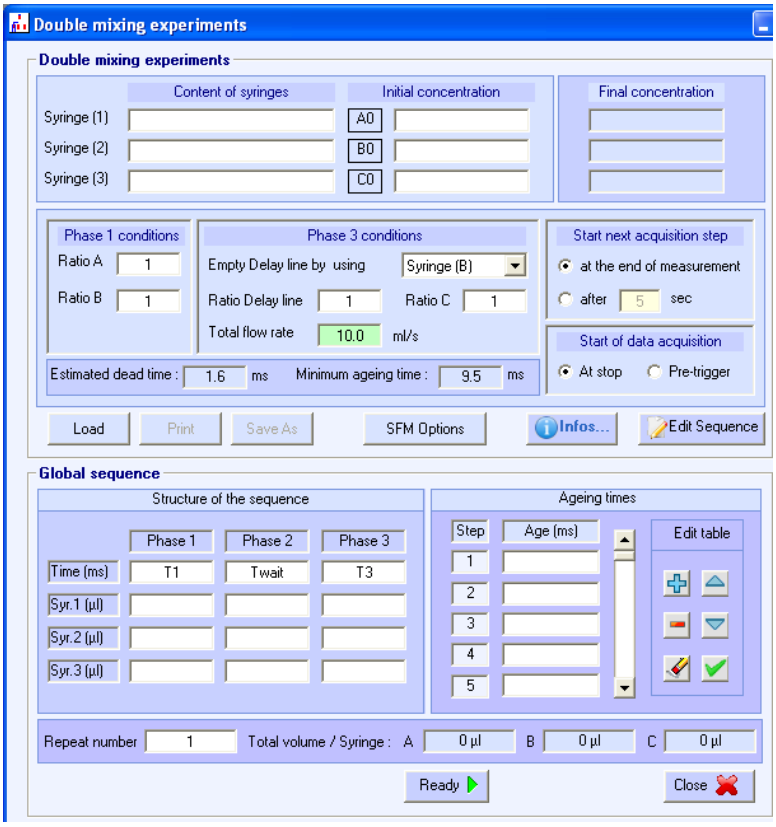


Figure 35 : Double mixing experiment, main window using SFM 300/S

- **Double mixing experiments part** (**Figure 36**):

The content of syringes and the initial concentration has to be entered. Thus the final concentration will be calculated (the final concentrations change in function of the ratio assessed: see below).

In the **Phase 1** conditions window, enter the Ratio A and ratio B,

In the **Phase 3** conditions window, select the syringe(s) used in order to empty the delay line. Product from the reaction of A+B is stored in the delay line and then mixed with the C solution. To empty the delay line of the aged solution, A or B or A+B can be used. This procedure is particularly useful to save a precious solution during this flushing phase.

Assess the ratio between the solution coming from the delay line (A+B) and C, and finally fix a total flow rate. Please notice that the flow rate must be assessed to satisfy the turburlent conditions: a color coded window orientates the user. The green font colour indicates adequate parameter conditions. The orange font color indicates a too low total flow rate.

Start next acquisition step: the next acquisition step starts at the end of the measurement or after a delay defined by the user.

Start of data acquisition: starts the acquisition at the stop of the motors or few milliseconds before the stop (this time is fixed at 2 ms and cannot be changed)

Estimated dead time: An estimated dead time is calculated in function of the flow rate assessed and the volume of the cuvette (chosen from the SFM option).

Minimum ageing time: this corresponds to the time needed to fill the delay line. This minimum ageing time is equal to the delay line volume divided by the flow rate coming from syringe 1 and 2 for an SFM 300 (A+B).

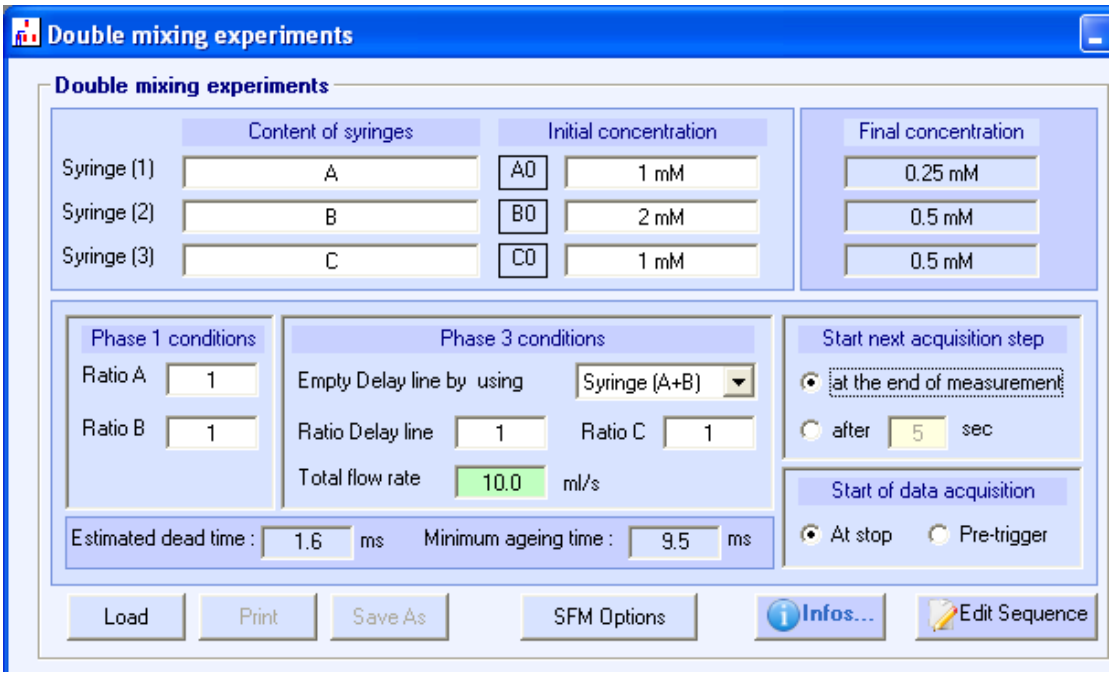


Figure 36 : editing a double mixing experiment

Clicking on SFM option 

opens a new window (Figure 37):

Select the right **cuvette** to allow a correct estimation of the dead time (Figure 36).

Select the correct delay line. For practical reasons we advise the user to choose the delay line number 3 (90 µL) or number 5 (190µL). The volume of the delay line has to be high enough to allow a correct mixing during the third phase (A+B) mixed with C.

Valve lead: this time is driving the closure of the valve of the hard stop to fine tune the quality of the stop of the kinetic.

HDS Mixer: In case a HDS mixer is used, tick this box to allow a correct calculation of the dead time

Overheating Protection: this prevents overheating of the electronic in case of intensive daily work.

Hardstop closed between shots: the hardstop closes at the end of the pushing phase and open once the acquisition is completed. Ticking this box will left the valve closed. This could be particularly useful in order to run a spectrum at the end of a shot for example.

Use Synchrout 1 trigger: used in case an external device is connected to the MPS for synchronization.

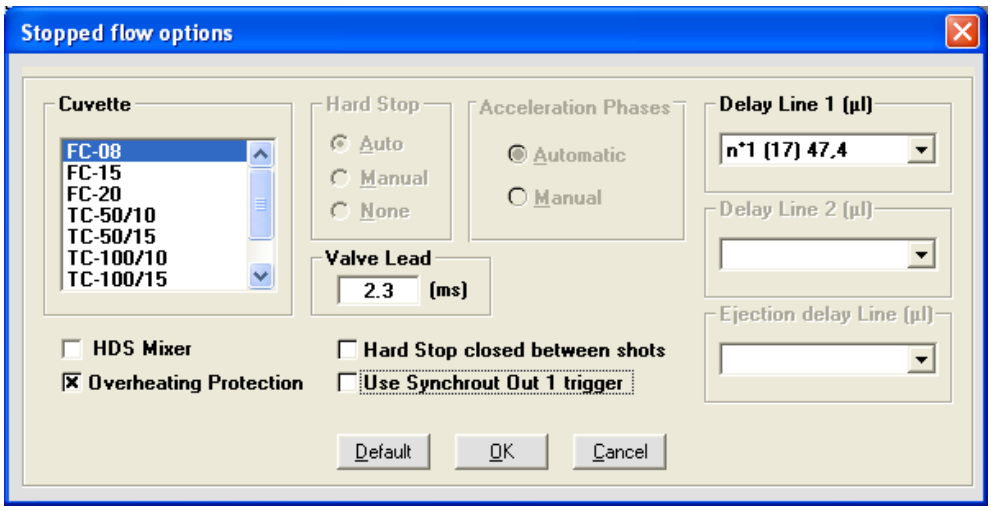


Figure 37 : stopped flow options

Once all parameters are fixed from the stopped flow option window, click on the **OK** button.

Clicking on 

, opens a new window (Figure 38), giving informations on the double mixing experiments. The theory, the description of the methods is explained.

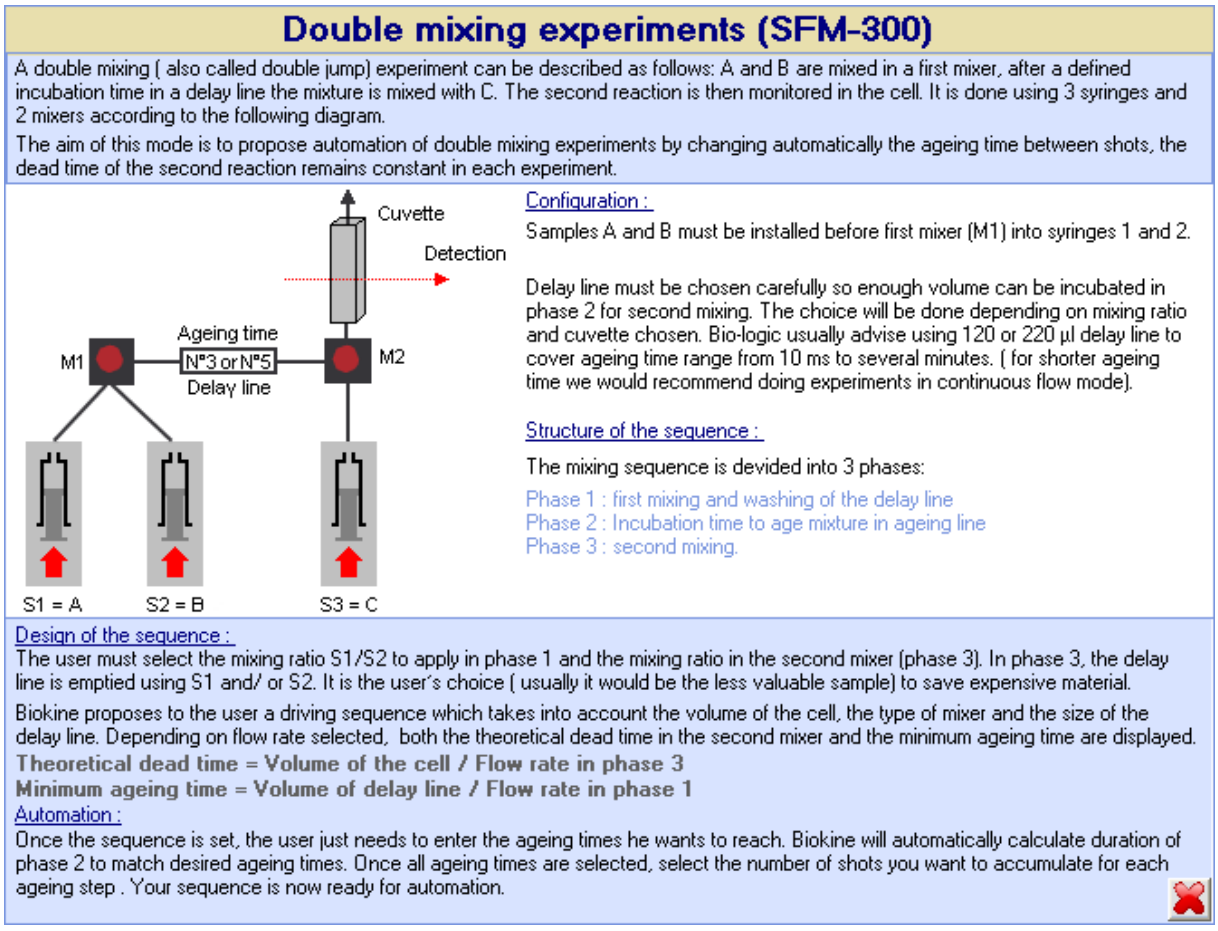


Figure 38 : Help section

Once all parameters of the double mixing sequence window are defined, click on the button Edit Sequence.

This will automatically create a sequence in the global sequence window.

- **Global sequence window part (**Figure 39)

Once the user has clicked on Edit Sequence, a structure of the sequence is automatically defined taking in account the ratios and total flow rate.

The **structure of the sequence** is made with four rows and three columns (phases).

The rows display times and volumes injection for Syringe 1, syringe 2 and syringe 3.

The columns display the three phases:

Phase 1: first mixing (S1 + S2)

Phase 2: waiting phase. Solution is stored in the delay line to be aged.

Phase 3: solution from the delay lined is emptied (using S2) and mixed with solution coming from S3. In our example in Figure 39, 47.5 µL of aged solution present in the delay line will be mixed with 47.4 µL of solution coming from S3.

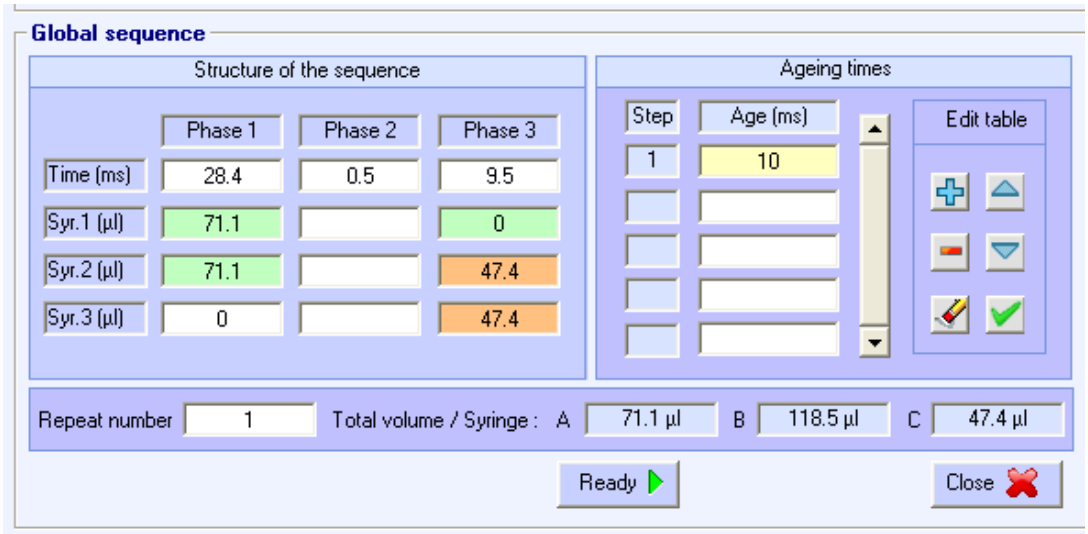


Figure 39 : Global sequence window

The volumes in the structure of the sequence can be changed manually, click on the desired box, and enter a new injection volume.

A color code is used to warn the user about the volumes selected:

orange colour: the volumes used are too small to insure an efficient washing of the cuvette or the delay line.

Green colour: the volumes used are adequate to run the kinetic.

The ageing times window is used in order to define an ageing time (Figure 39). The ageing time is equal to the waiting phase from phase 2 + minimum ageing time (time needed for the solution A+B to fill the delay line under a continuous flow).

When changing the ageing time, the waiting phase is automatically calculated.

It is possible to run several shots, using different ageing times for each shot. To do so, click on the button from the Edit table. This will add a second step in the ageing time window (Figure 40).

To remove a step, click on the button .

To erase all the ageing time sequence, click on .

When several steps are created, and in case the user wants to invert two lines, the arrow or from the edit table can be used.

At the bottom of the Global sequence window, a function allows to repeat each step. For example in case three steps are created as in Figure 40, and a "repeat number" of 2 is introduced, the sequence will be run sothat step number one is done 2 times, then, step number two is done in two times etc…

This procedure allows to record each traces and do an average

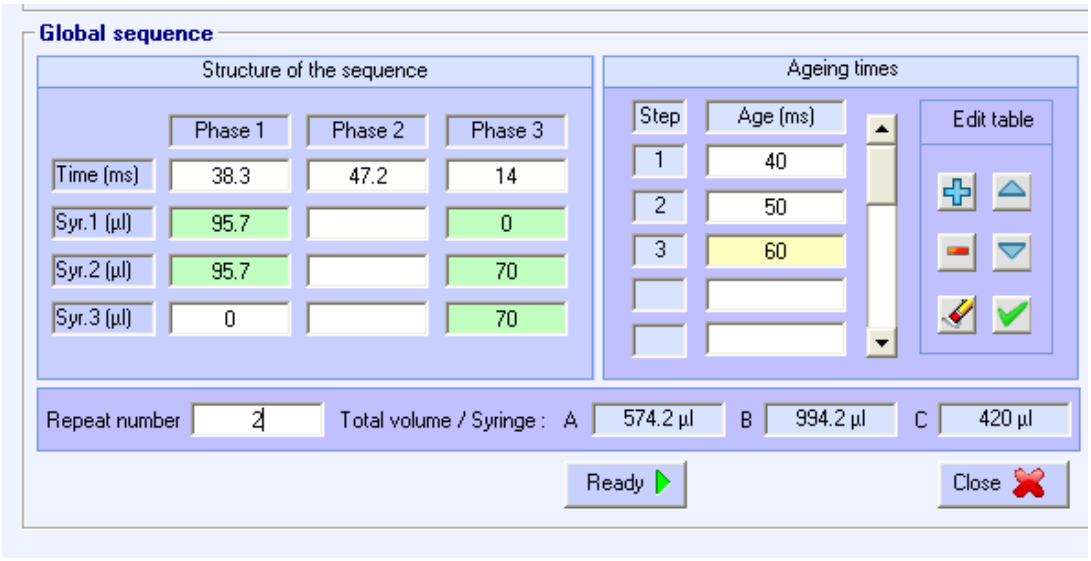


Figure 40 : step creation

SAVING/LOADING A DOUBLE MIXING EXPERIMENT

It is possible to save a sequence, by clicking on Save As , the file is saved under a .DMX file.

To load an existing sequence, click on the **Load** button.

5.9 Creating a Driving Sequence using concentration dependence studies

5.9.1 Creating driving sequences using SFM-300

The aim of this sequence is to observe different kinetic curves by increasing automatically step by step the concentration of one reactant A against B in A + B reaction. Thus the concentration of B is maintaining constant while the concentration of A is increasing in constant steps (automatic mode) or in certain steps values (manual mode).

The first mixer M1 is used to change the concentration of a reagent A by mixing it with a diluant while the concentration of reactant B is constant. The second mixer is used to mix solution A and solution B to start the reaction (Figure 41). The following window is obtained by clicking on the button 

of the main mixing sequence window.

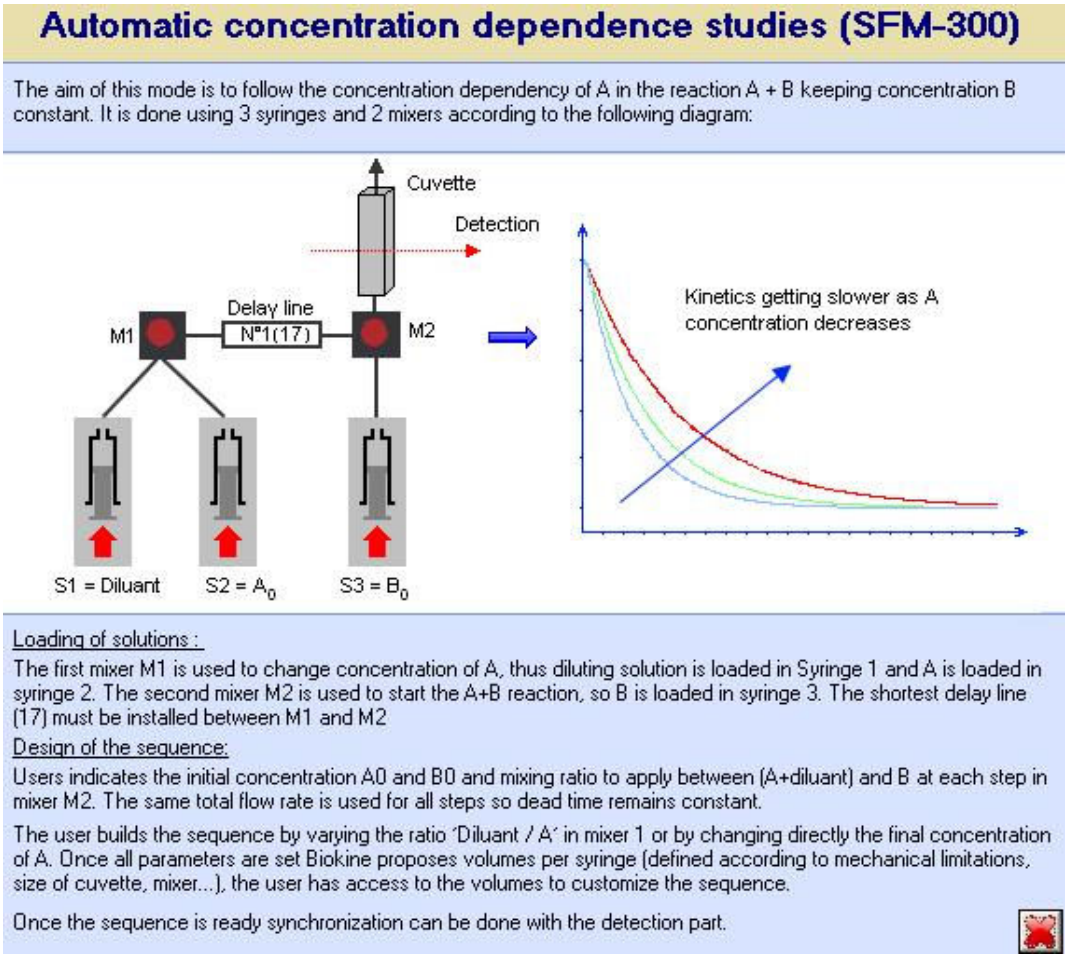


Figure 41: Concentration dependence setup

Loading of solutions:

Syringe 1 (S_1) is used to stock the diluant solution while Syringe 2 (S_2) and Syringe 3 (S_3) are filled with reagents respectively A and B

The increase of the concentration of A can be achieved in two different ways: by varying the ratios between syringe 1 and syringe 2 (i.e. ratios steps) or by increasing the concentration of the reagent A (i.e. concentration steps). In both sequences automatic mode and manual

mode are available. The delay line installed between mixer M1 and Mixer M2 should be the shortest one corresponding to a dead volume of 19 µl.

Design of the sequence:

The driving sequences is created in the window shown in Figure 42, this window can be reached from the Mixing Sequence button, in the stopped flow status area.

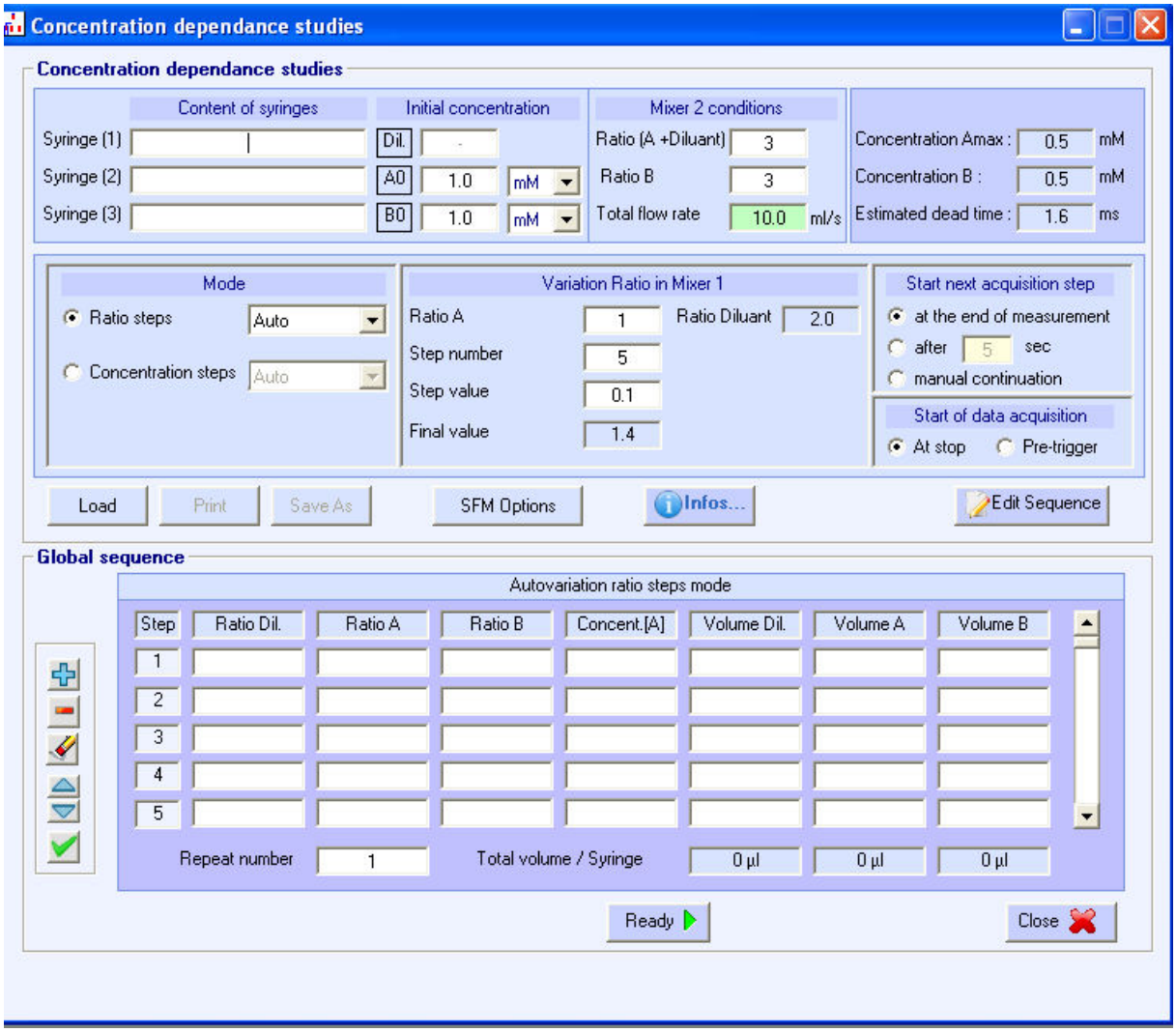


Figure 42: driving sequence for concentration dependence studies

In the sequence the user has to indicate the content of the syringes and the initial concentrations of reactant A as A_0 and reactant B thus B_0.

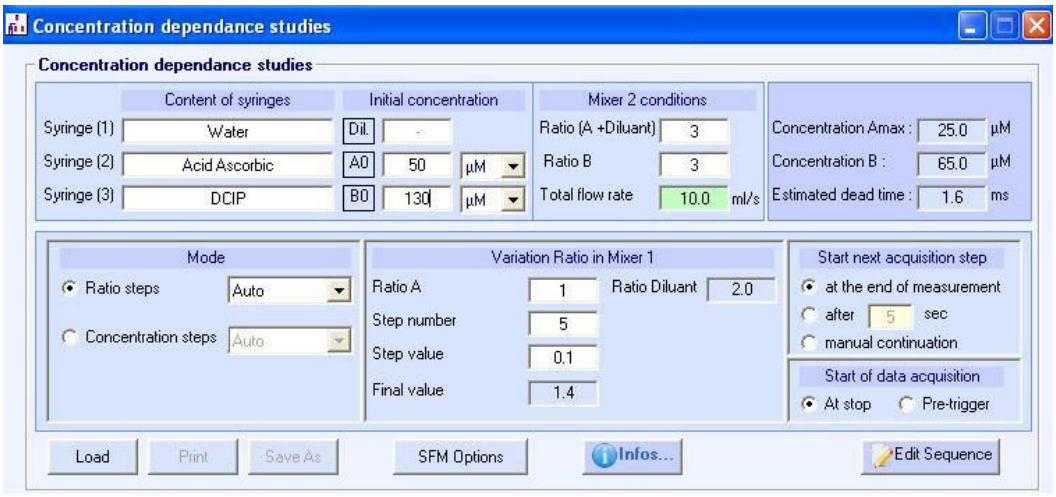


Figure 43:Mixing sequence

Then the conditions in Mixer 2 have to be entering in the following window:

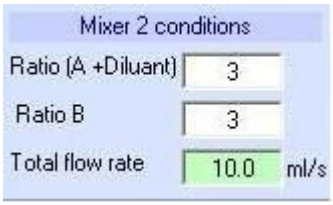


Figure 44: Ratio in mixer 2

These conditions correspond to a 1 to 1 mixing sequence in Mixer 2 with a flow rate of 10 ml/s. The concentration maximum of A " A_{max} " is automatically calculated while the concentration of B reactant is maintained as a constant value.

There are two ways to increase the concentration of A: the first is done by the increase of the ratio in mixer 1 step by step. The second is done by increasing the concentration of A step by step.

Ratio step Auto mode:

The variations of the ratios between Diluant and reactant A in mixer M1 have to be fixed by typing a value in Ratio A (i.e.1 in the example), choosing the steps numbers and the value of the step.

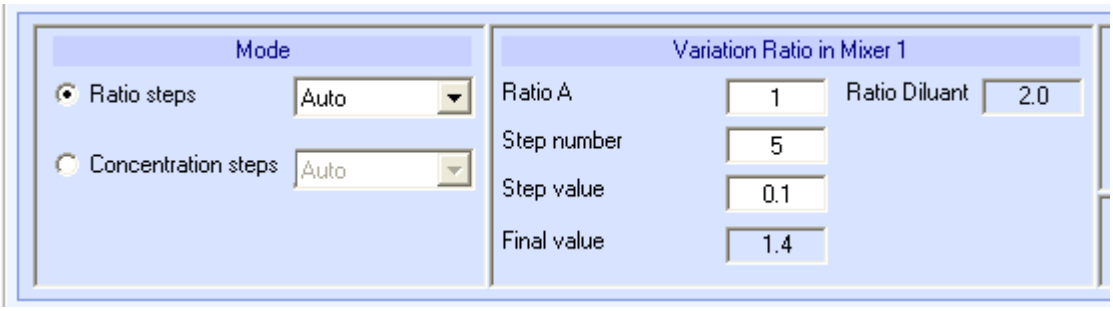


Figure 45: Ratio steps Mode

The sequence is edited by clicking on the button  as following:

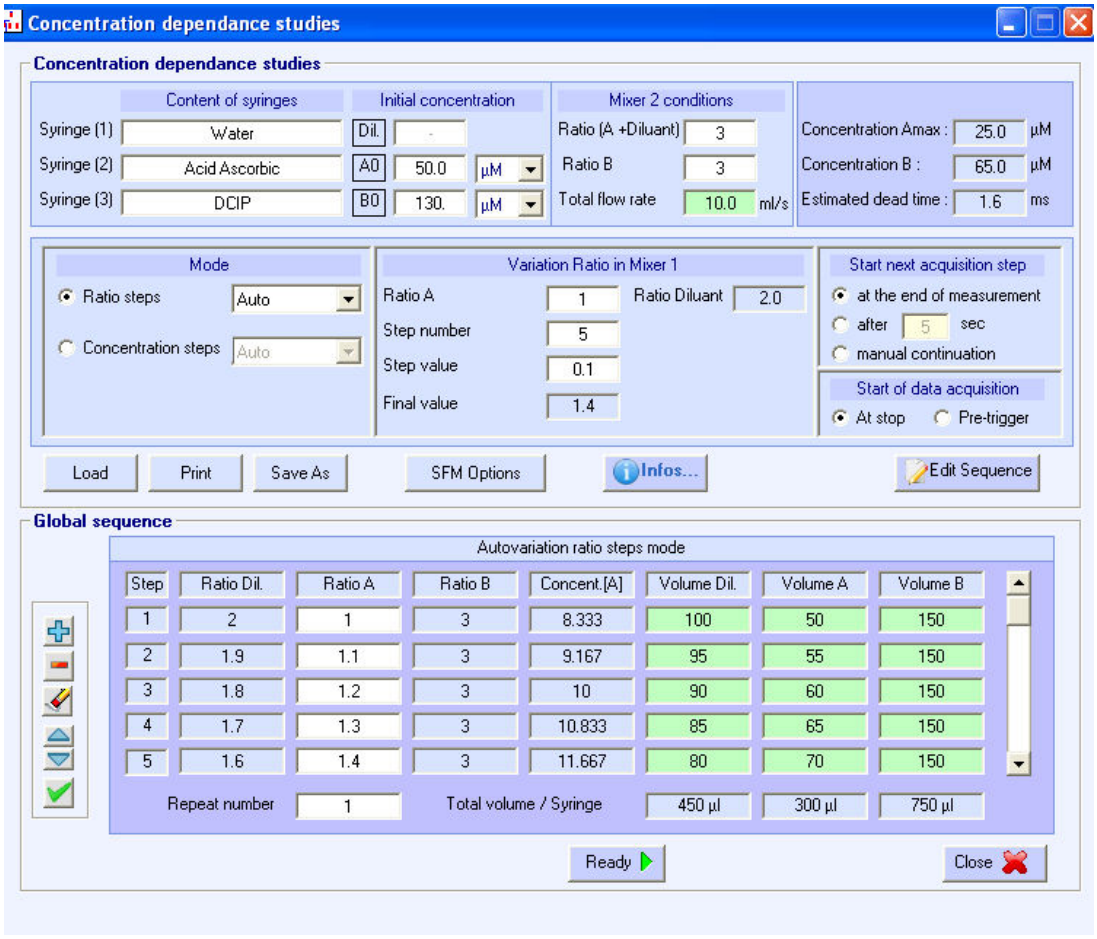
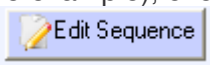


Figure 46: Global sequence in ratio steps mode

Concentration step Auto mode:

The variations of the concentration of reactant A in mixer M1 have to be fixed by typing a value in concentration A (i.e.1 in the example), choosing the steps numbers and the value of the step. By clicking on the button  , the following sequence is edited:

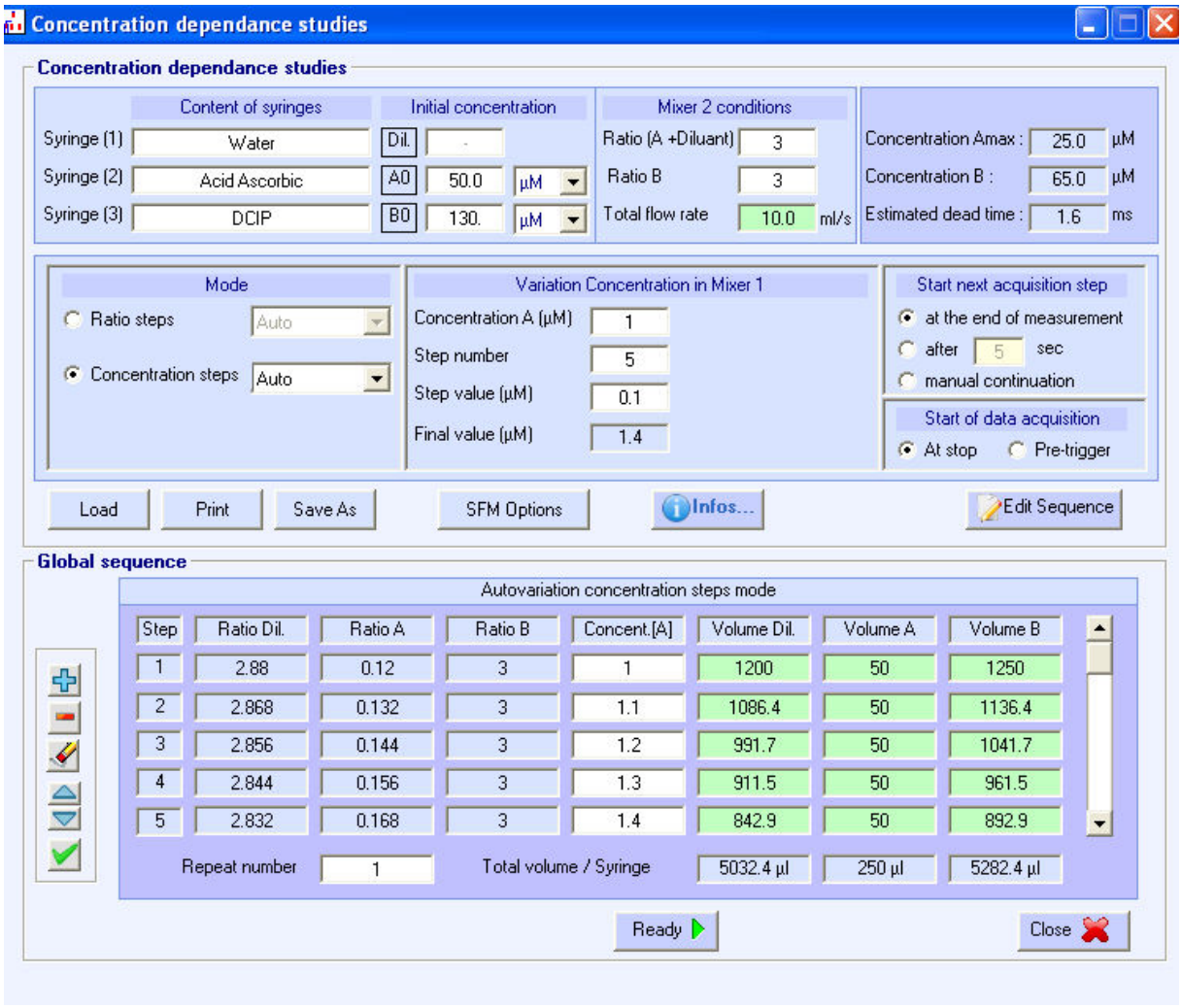


Figure 47: Global sequence in concentration steps mode

Ratio step manual mode:

By selecting Ratios steps Manual in the Mode window you have an access to the window menu then

by clicking on the button 

, the following sequence is edited or the latest sequence is automatically loaded as a default one:

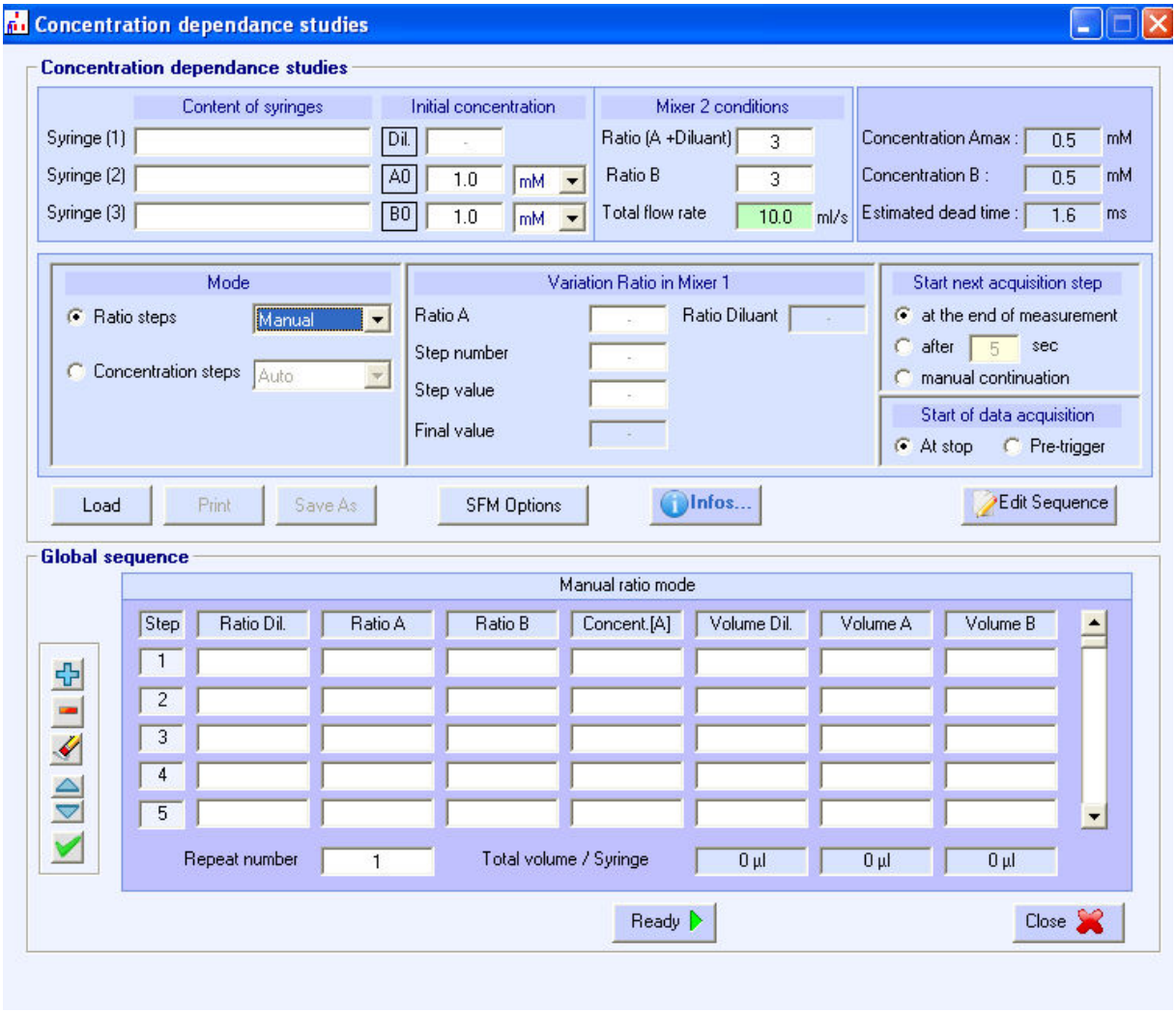


Figure 48: Global sequence in ratio steps manual mode

The Global sequence is created through the following table:

Click on  button to create a step then type the ratio in "Ratio A" that will be used during the step and indicate the volumes. All the volumes, volume Dil., volume A and volume B have to be more than 40µl.

1) Click on  button to create a second step
2) Click on  to clear all the sequence or  to remove a step.

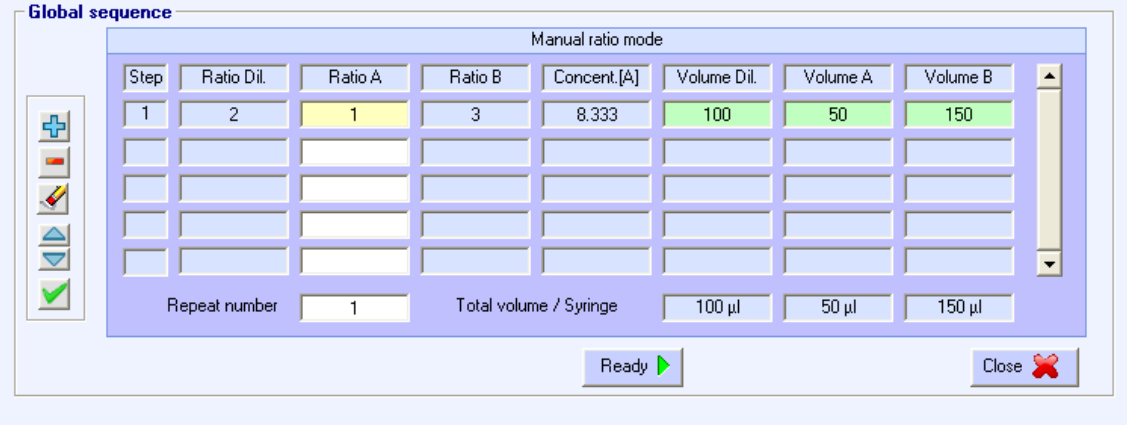


Figure 49: Global sequence window

The functions of the different buttons are the following:

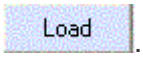
 is used to create or add a step

 is used to remove a step

 is used to remove all the sequence

 is used to move up or down a step

 is used to validate the sequence

Notice that a stored sequence can be automatically load by clicking on "load" button  .

5.9.2 *Creating driving sequence using SFM-400*

The aim of this sequence is to observe different kinetic curves by increasing automatically step by step the concentration of one reactant A against B in A + B reaction. Thus the concentration of B is maintaining constant while the concentration of A is increasing in constant steps (automatic mode) or in certain steps values (manual mode).

The second mixer M2 is used to change the concentration of reactant A by mixing reactant A with diluant while the concentration of reactant B is constant. The third mixer M3 is used to start the reaction (Figure 50). The following window is obtained by clicking on the button  of the main mixing sequence window.

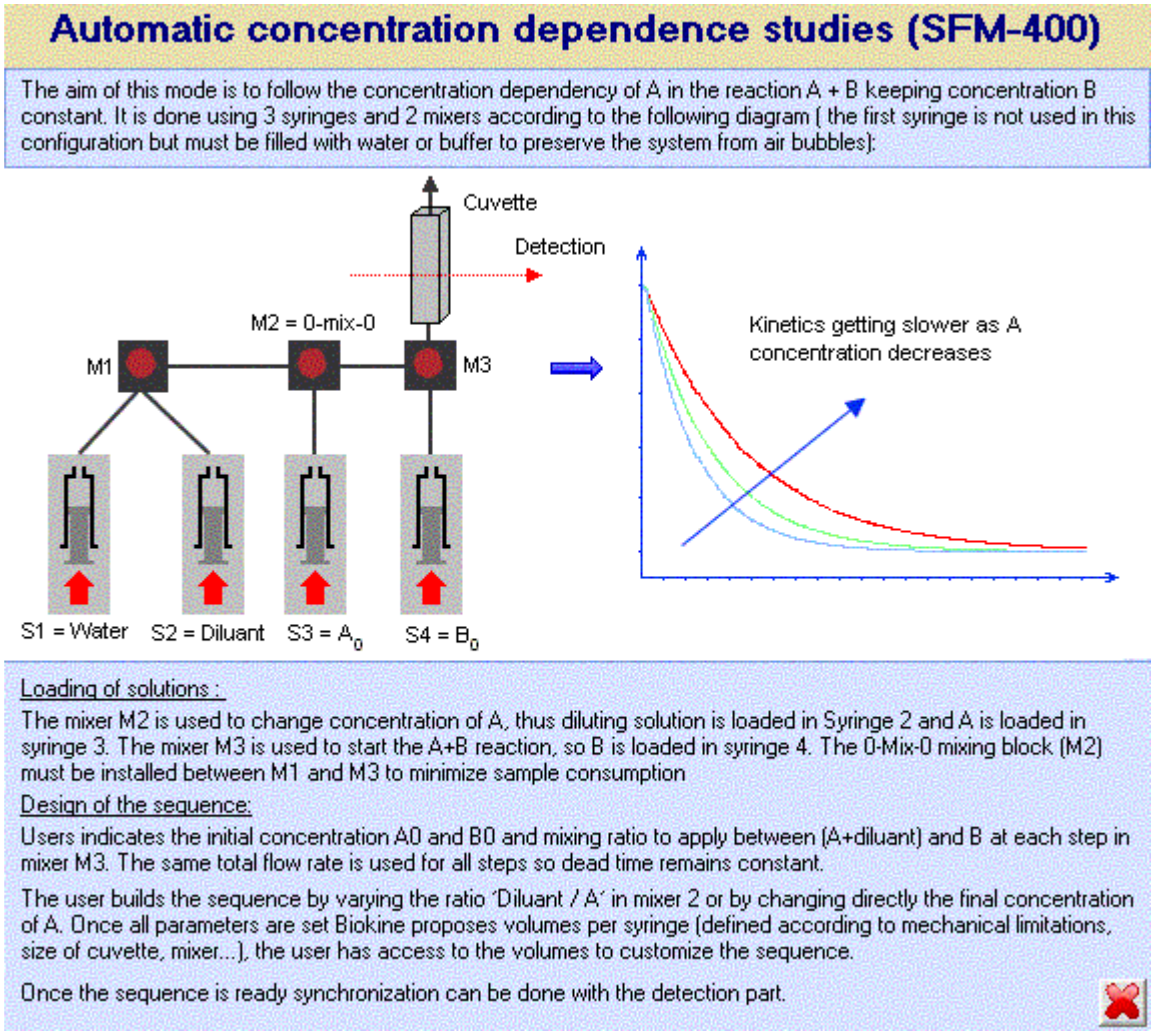


Figure 50: Automatic concentration dependence studies

Loading of solutions:

Syringe 1 (S_1) is used to stock water, Syringe 2 (S_2) to stock the diluant solution while Syringe 3 (S_3) and Syringe 4 (S_4) are filled with reactants respectively A and B. The reaction between reactants A and B starts in the third mixer M3.

The increase in A concentration can be achieved in two different ways: by varying the ratios in Mixer 2 between syringe 2 and syringe 3 (i.e. ratios steps) or by increasing the concentration of the reactant A (i.e. concentration steps). In both sequences automatic mode and manual mode are available. The delay line installed between mixer M2 and Mixer M3 should be the shortest one corresponding to a dead volume of 19 µl.

Design of the sequence:

The driving sequence is created in the window shown in Figure 51. This window can be reached from the 

button in the stopped-flow status area.

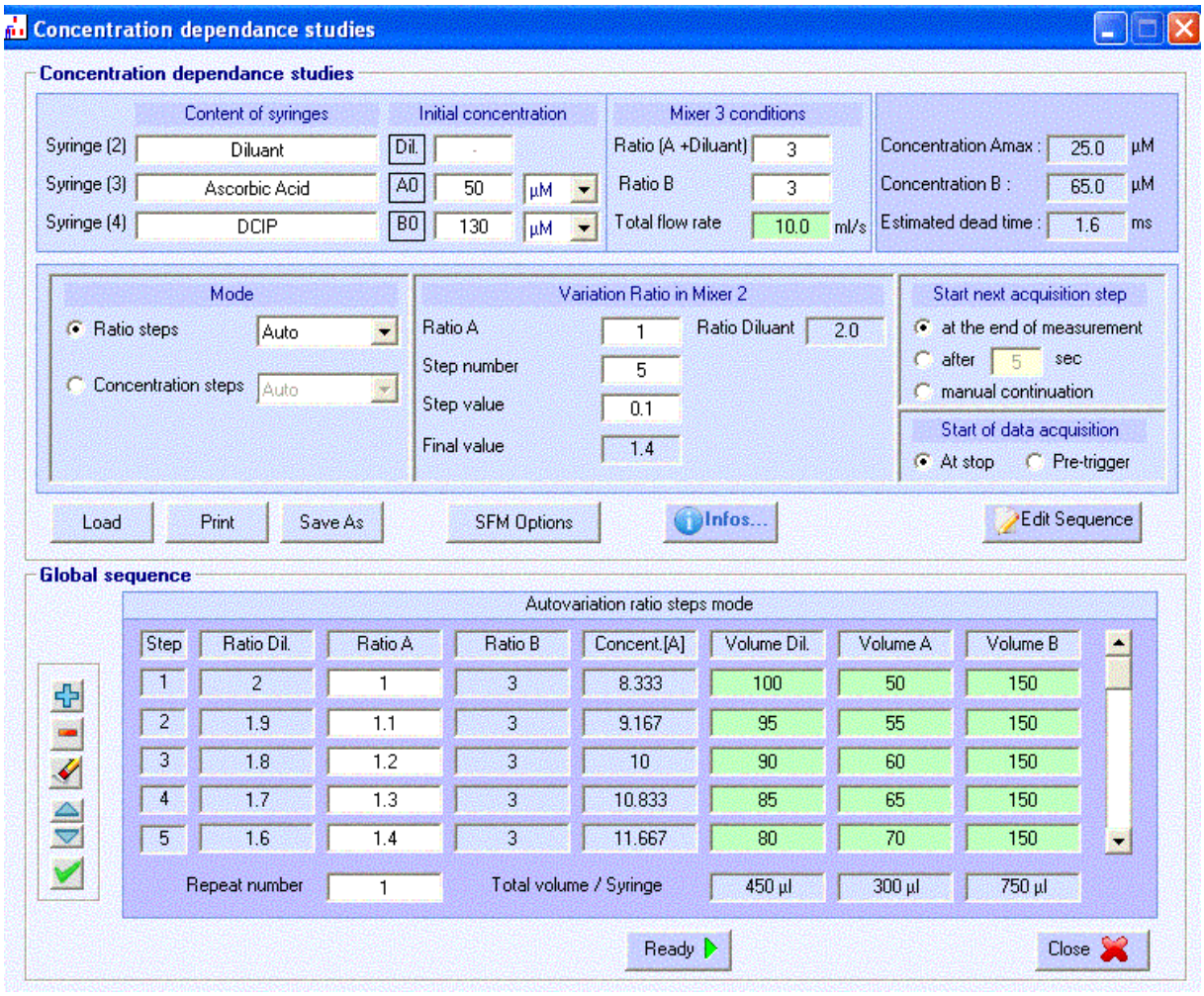


Figure 51: driving sequence

In the sequence the user has to indicate the content of the syringes and the initial concentrations of reactant A as A_0 and reactant B thus B_0 (Figure 52).

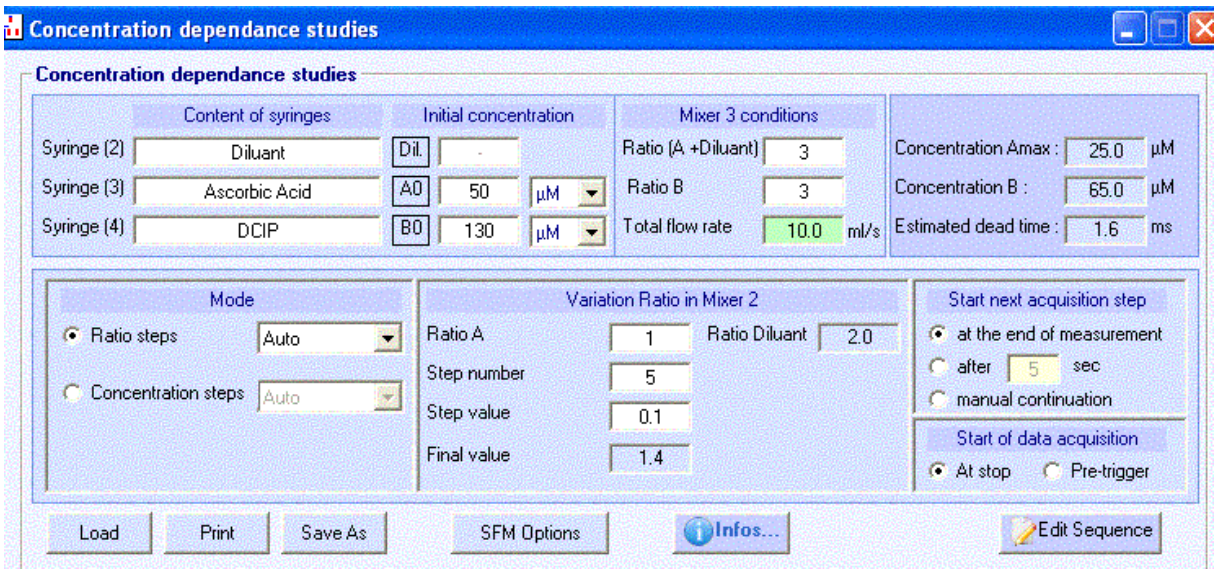


Figure 52: mixing sequence

Then the conditions in Mixer 3 have to be entering in the following window:

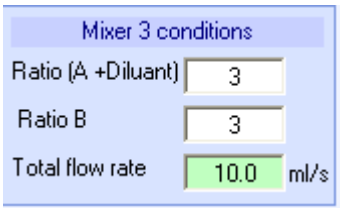


Figure 53: Ratio in mixer 3

These conditions correspond to a 1 to 1 mixing sequence in Mixer 3 with a flow rate of 10 ml/s. The concentration maximum of A " A_{max} " is automatically calculated while the concentration of B reactant is maintained as a constant value.

There are two ways to increase the concentration of A: the first is done by the increase of the ratio in mixer 2 step by step. The second is done by increasing the concentration of A step by step.

Acquisition parameters:

The parameters of the acquisition have to be entered in the following window (Figure 54: acquisition **parameters**:

Start next acquisition step: In case of a repetition of the sequence, starting the next acquisition step can be done at the end of the measurement or after a defined time.

Start of data acquisition: as in the stopped flow advanced mode, starting the acquisition can be done at the stop of the motors or few milliseconds before the stop (this time is fixed at 20 ms and cannot be changed)

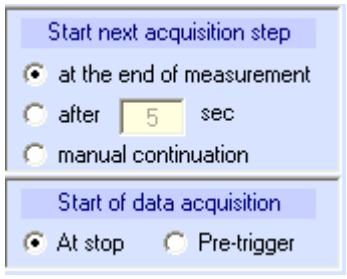


Figure 54: acquisition parameters

Ratio step Auto mode:

The variations of the ratios between Diluant and reactant A in mixer M2 have to be fixed by typing a value in Ratio A (i.e.1 in the example), choosing the steps numbers and the value of the step.

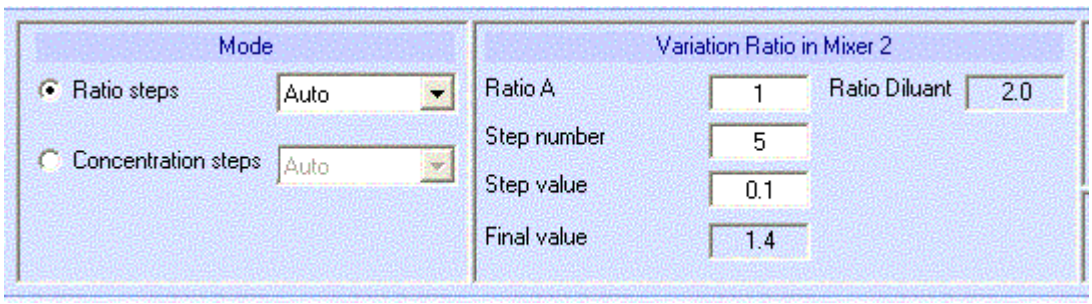


Figure 55: Ratio steps Mode

The sequence is edited by clicking on the button  as following:

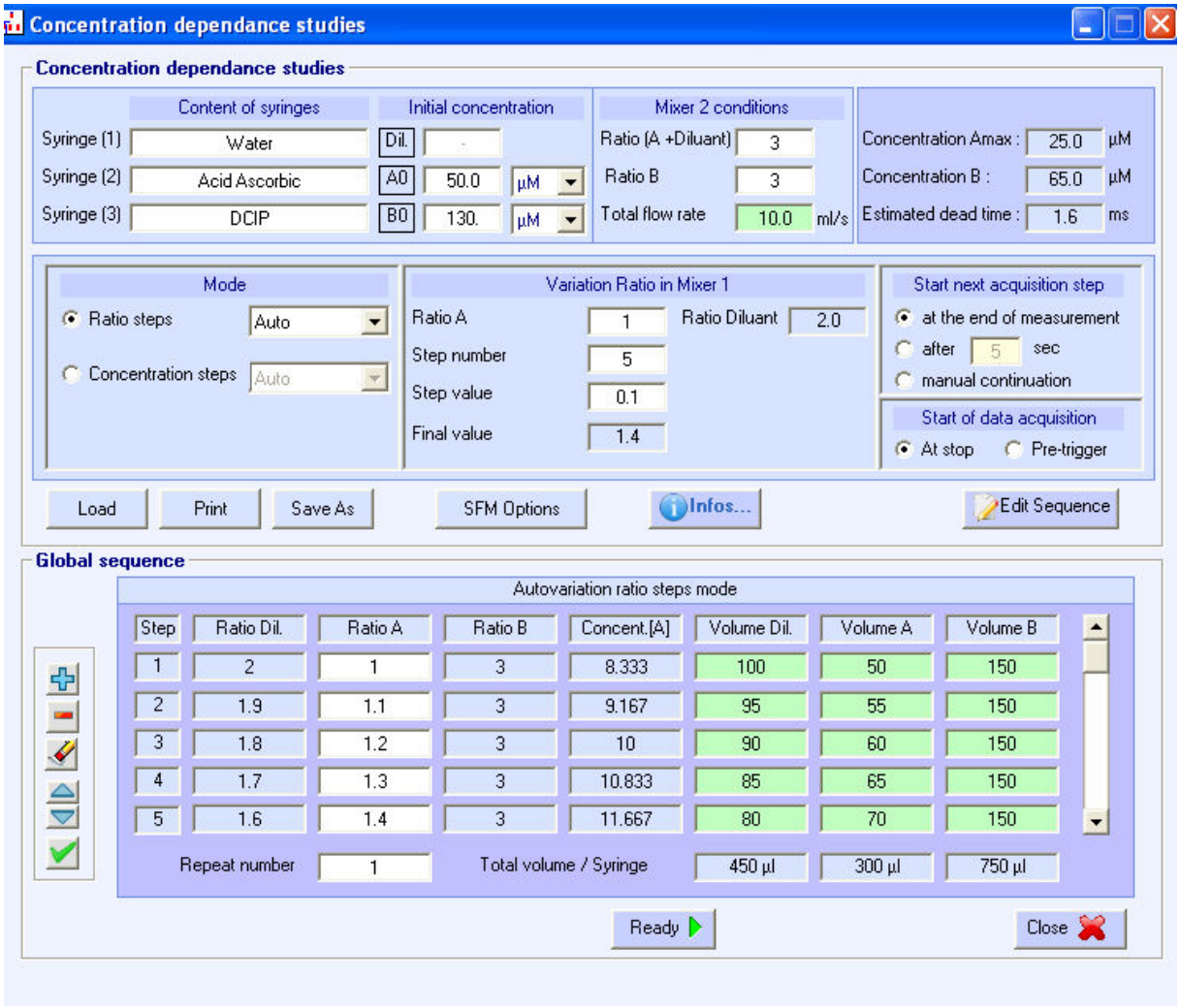


Figure 56: Global sequence

Concentration step Auto mode:

The variations of the concentration of reactant A in mixer M1 have to be fixed by typing a value in concentration A (i.e.1 in the example), choosing the steps numbers and the value of the step. By clicking on the button  , the following sequence is edited:

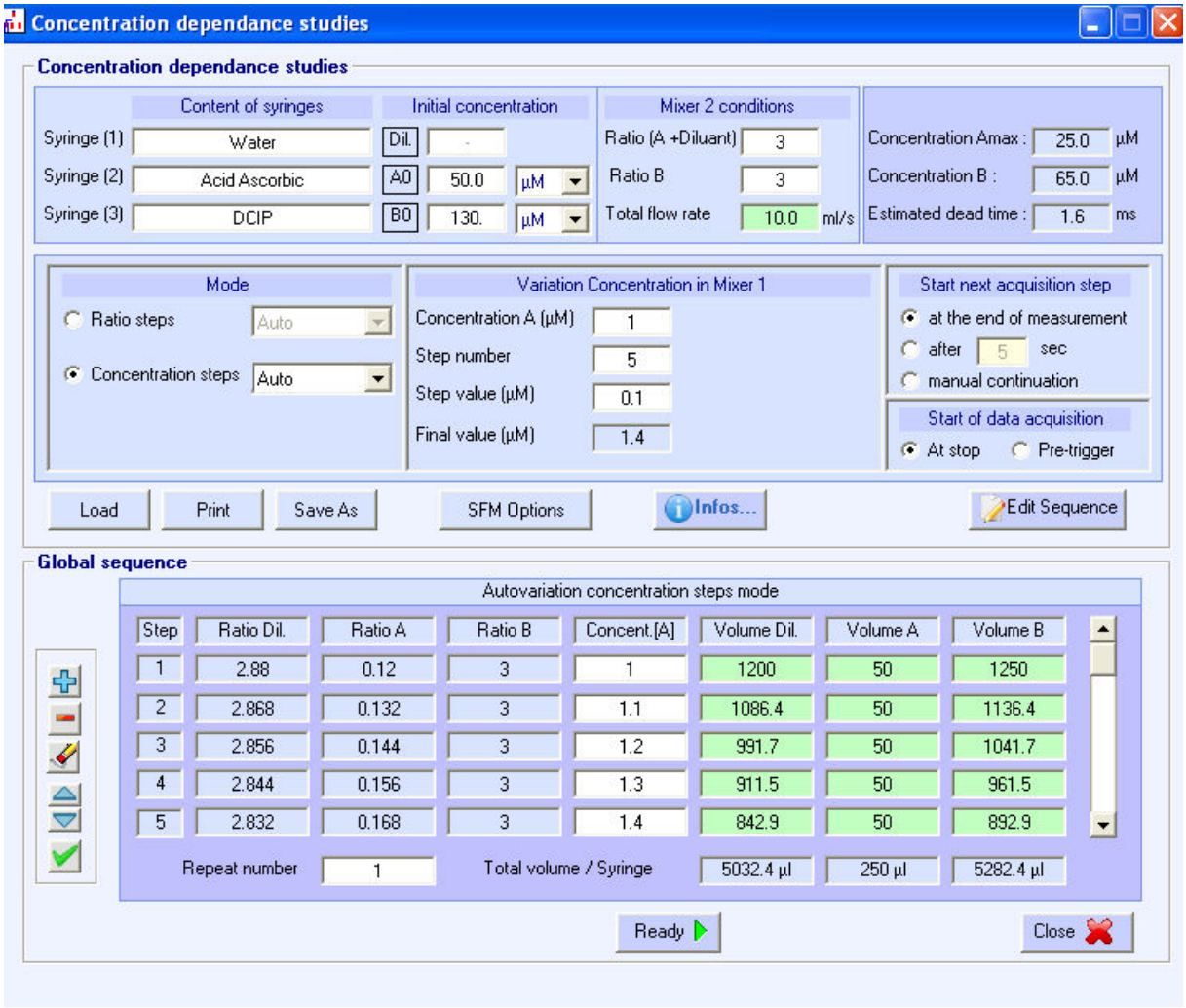


Figure 57: Global sequence in concentration steps mode

Ratio step manual mode:

By selecting Ratios steps Manual in the Mode window you have an access to the window menu then

by clicking on the button 

, the following sequence is edited or the latest sequence is automatically loaded as a default one:

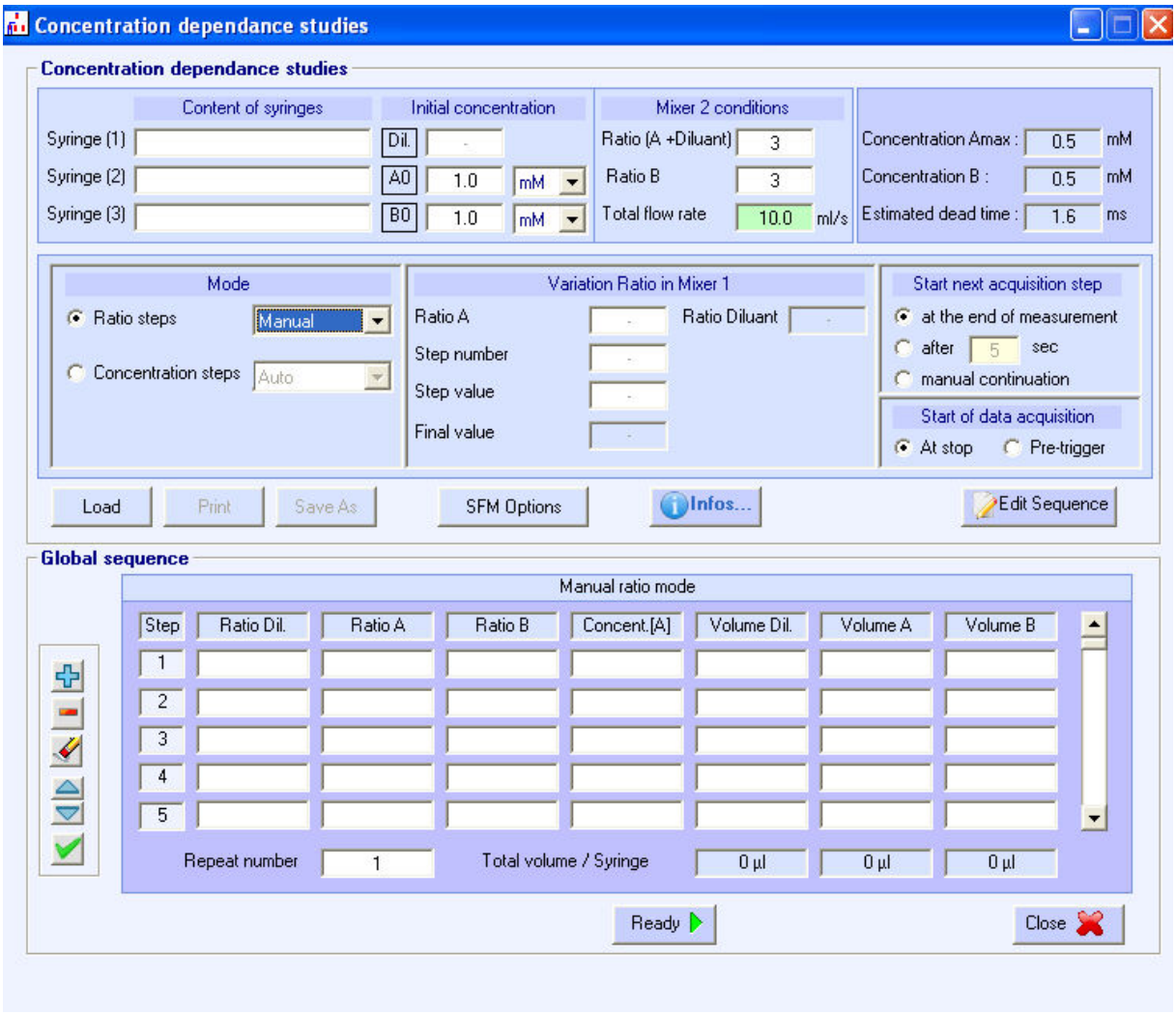


Figure 58: Global sequence in ratio steps manual mode

The Global sequence is created through the following table:

Click on  button to create a step then type the ratio in "Ratio A" that will be used during the step and indicate the volumes. All the volumes, volume Dil., volume A and volume B have to be more than 40µl.

3) Click on  button to create a second step
4) Click on  to clear all the sequence or  to remove a step
5) Repeat number: is used to repeat each step.

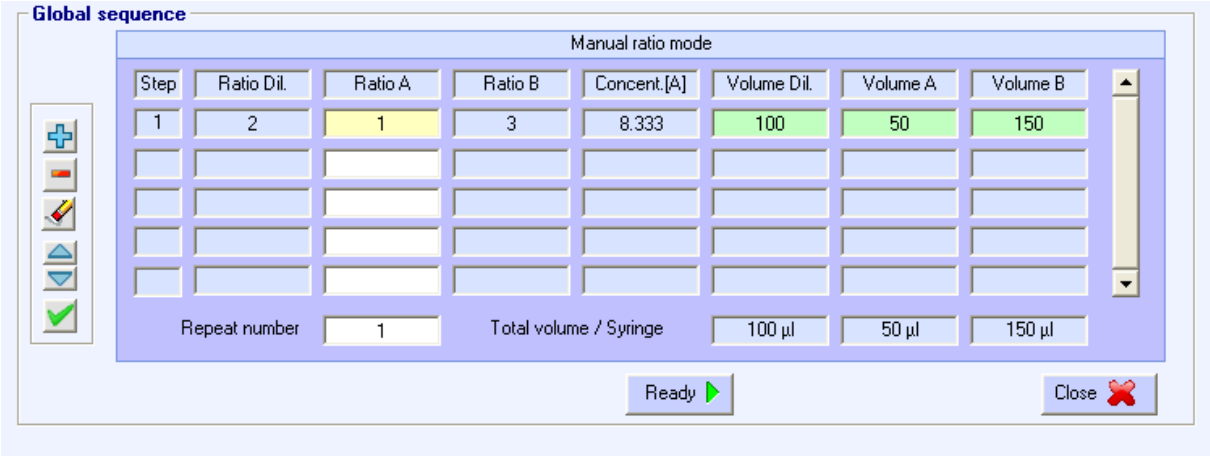


Figure 59: Global sequence

The functions of the different buttons are the following:

 is used to create or add a step

 is used to remove a step

 is used to remove all the sequence

 is used to move up or down a step

 is used to validate the sequence

Notice that the latest sequence is automatically load, in this case the manual mode permits the user to change the parameters or by clicking on  to remove all the sequence.

5.10 Running a shot

Once a driving sequence has been entered or loaded, it is transferred to the MPS by pushing the **Single** or **Multiple** buttons (in classic mode) or **Ready** (in advanced mode).

The MPS is now in automatic mode and the **shot control** window appears in the stopped-flow status (as shown in Figure 60). The **Shot control** window shows the number of shots possible based the current volumes in the SFM syringes. It also indicates whether the SFM is running a driving sequence or ready for the next shot. A driving sequence is executed by pushing the button or the **start-stop** button on the front panel of the MPS. The button can be used to stop an experiment prematurely if necessary.

If the **Single** button was used to transfer the driving sequence to the MPS, only a single shot can be made. The End button must then be pushed to return to the driving sequence and the **Single** button must be pushed again to re-transfer the driving sequence to the MPS for a subsequent shot.

If the **Multiple** buttons was used to transfer the driving sequence to the MPS, the button can be used to execute shots until the **shot** window shows that 0 shots remain. The End button is then pushed to return to the driving sequence.

Figure 60: shot control window

6 A SHORT STOPPED-FLOW PRIMER

6.1 General Principle of Experiments with the SFM

There are many variations on the stopped-flow experiment, such as multiple mixing, continuous-flows and accelerated flow. However, the simplest stopped-flow experiment occurs in two stages.

In the first stage, the flow is initiated by two plungers. The plungers force liquid through a mixer and along a flow path into an observation cuvette. The resulting mixture ages as it travels along the flow path and into the cuvette. The amount of ageing depends on the flow-rate of the mixture and the volumes of the flow path and cuvette. In this first stage, the mixer, flow path, and cuvette are initially washed by the constantly refreshed mixture. This continues until a steady-state condition arises in which the age of the mixture is completely linear with respect to the distance along the flow path. Once the steady-state condition is reached, any particular point in the flow path represents the mixture at a particular age. Furthermore, the age of the mixture in the cuvette at the point of observation during the shot is the theoretical dead-time (the time before which observation of the mixture is impossible).

The second stage of the experiment begins when the flow is stopped. At this point, the mixture in the cuvette (and elsewhere) becomes stationary but continues to age. Observation of the mixture in the cuvette after the stop, therefore, represents a time course of the reaction from the dead-time onward.

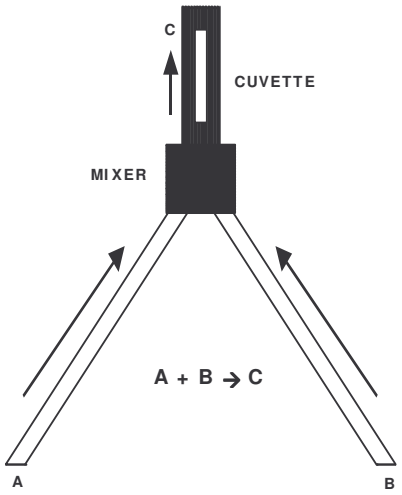


Figure 61 – A Simple Stopped-Flow Experiment

Figure 61 shows a schematic of a simple stopped-flow experiment. In the experiment, reagents A and B are pushed into a mixer where they react to form product C. Reagent A has a strong absorbance, while reagent B and product C do not. Therefore, as the reaction proceeds, the absorbance of a mixture of A and B should decrease as A is diminished. Figure 62 shows a cartoon of the experiment over time. Note the two stages of the experiment as described above.

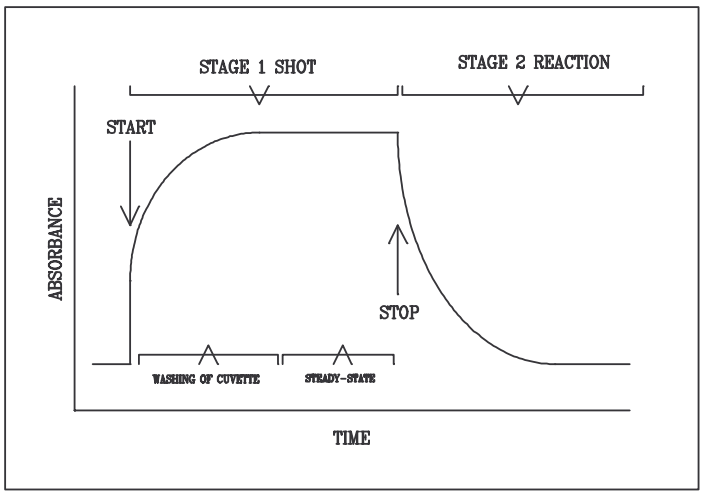


Figure 62 – Stopped-Flow Experiment Time course

IMPORTANT: In every stopped-flow experiment enough liquid must be pushed to wash the flow path and cuvette and achieve a steady-state condition. If this is not done, all samples are contaminated and the resulting signal trace does not represent the true time course of the reaction!

6.2 General Advice for Stopped-Flow Experiments

6.2.1 Achievement of fastest dead times

The dead time of a stopped-flow experiment is defined as the time before which observation of the mixture is impossible. The dead time depends on a number of factors, only some of which the researcher can control. Ideally, the dead-time depends only on the flow rate of the mixture exiting the last mixer and the volume between the last mixer and the cuvette. Thus, as the flow rate is increased, the dead-time will decrease. In addition, as the volume between the last mixer and the cuvette volume decreases, so does the dead-time.

Nevertheless, an effective stopped-flow experiment depends on a number of other inter-related factors, such as an adequate signal, complete washing of the cuvette, and prevention of cavitations and prudent use of valuable reagents. The relationships between these factors require careful consideration and experimentation. Compromises are often necessary to achieve successful stopped-flow experiments. Some of the most common actions that can be take to achieve the fastest dead times, and their consequences, are shown in Table5.

Table5 – Common Actions to Achieve Fastest Dead Times

IN ORDER TO...	ONE SHOULD...	BUT THE RISK IS...
Lower Dead Times	Increase Flow Rate	stalled motors
		cavitations
		overuse of reagent
		inadequate washing
	Decrease Cuvette volume	loss of signal

6.2.2 Washing

As mentioned in section 6.1, it is necessary to completely wash the flow path between the last mixer and cuvette and the cuvette itself during the shot. This ensures that the signal observed after the shot is only of the recently mixed samples. To accomplish this, a sufficient volume of the mixed samples needs to pass through the cuvette during the shot. This volume varies with flow rate, viscosity, and composition of the sample. It is strongly recommended that tests be performed and adequate washing conditions found before starting any series of experiments.

Cavitation occurs when turbulence creates regions of low enough pressure in a liquid that a "cavity" is formed. This cavity fills with the liquid's vapor. These cavities collapse incompletely, leaving behind small bubbles of vapor which interfere with optical observation methods. As the flow rate increases through a mixer, so does the likelihood of cavitation. The probability of cavitation also increases with increasing viscosity for a given flow rate. De-gassing of solutions decreases the probability of cavitation by eliminating gas and lowering the total vapor pressure available to fill the cavities.

6.2.3 Signal amplitude

Signal amplitude is generally proportional to the path length of the cuvette and the concentration of the signal-generating reagent. An increase in signal can then be accomplished by an increase in cuvette path length or an increase in the concentration of the reagent. However, the researcher may be limited by practical concerns such as value of sample, viscosity of sample, dead-times, inherent limitation of the signal (such as inner-filter effect) and sample precipitation. As with the achievement of the fastest dead times, compromises may be necessary to achieve a successful stopped-flow experiment. Table6 shows some of the most common actions that can be taken to improve signal amplitude and their consequences.

Table6 – Common Actions to Improve Signal Amplitude

IN ORDER TO...	ONE SHOULD...	BUT THE RISK IS...
Increase Signal	Increase Cuvette Path Length	overuse of reagent
		increased dead time
		Inadequate washing
		Inner-filter effect (fluorescence)
	Increase Reagent Concentration	overuse of reagent
		Increased viscosity causing cavitation
		Increased viscosity causing stalled motors
		Increase viscosity causing inadequate washing

6.2.4 Flow rate

The flow rate of the SFM is limited by the speed with which the stepping motors can push. At the nominal flow rate limit of 8ml/s (10 ml syringes), with all syringes in use and using the smallest cuvette, sub-millisecond dead-times may be accomplished. However, solutions of increased viscosity will lower the obtainable syringe speed. Also, lower than room temperatures often lower the obtainable syringe speed.

7 TEST REACTIONS IN STOPPED-FLOW MODE

7.1 Reduction of 2,6-Dichlorophenolindophenol by Ascorbic Acid

A complete description of the reduction of 2,6-dichlorophenolindophenol (DCIP) by ascorbic acid (AA) and its use can be found in *Tonomura et al, Analytical Biochemistry (1978), 84, 370-383*. DCIP has a strong absorbance at 524 nm, and reduction by ascorbic acid results in a nearly complete discoloration. The second order reduction rate constant is highly dependent on pH and varies from about $10^{4.6}$ $M^{-1}s^{-1}$ at pH 2.0 to $10^{2.5}$ $M^{-1}s^{-1}$ at pH 8.0. If the concentration of DCIP is sufficiently smaller than AA, the reaction can be treated as a pseudo first-order reaction whose rate constant will be directly proportional to the AA concentration.

All these properties make this reaction a very useful tool for stopped-flow calibration. The fast reaction at acid pH can be used to measure the dead time of the SFM instrument. The slow reaction at basic pH is used to check the quality of the stop, evaluate the washing of the observation cell, and test the variable ratio mixing capabilities. The following sections describe the use of this reaction for testing and exploring its capabilities.

7.2 Evaluation of the Dead Time

The dead time of the SFM can be measured using both the fast and slow reduction reactions of DCIP. An example dead time evaluation is shown in this section. As discussed in section 6.2.1, the dead time of a stopped-flow experiment depends on many factors besides simply the flow rate and cuvette volume. The technique presented here may be adapted to evaluate the dead time under many experimental conditions.

Experimental Conditions:	Syringe 1, 2 or 3 (10 ml):	20 mM Ascorbic Acid (pH 2 or 9)
	Syringe 4 (10 ml):	150 uM DCIP
	Wavelength:	524 nm
	Cuvette:	TC-100/10
	Detection method:	Absorbance
	Total Flow Rate (ml/s):	8 – pH 9 reaction, 8 – pH 2 reaction

The dead time of the experiment is the age of the solution at the observation point. In other words, it is the time for the mixed solution to go from the centre of the last mixer to the observation point. The dead time depends on many factors besides simply the total flow rate and the cuvette volume. But because the hydrodynamics phenomenon is difficult to be taken into account for software calculations, a slight difference between the estimated dead time given by Bio-Kine and the real dead time may be observed.

In basic pH conditions, the reaction is considered as a slow reaction. Therefore, the amplitude of the signal at the stop can be assimilated to the total amplitude of the reaction. In other words the change in absorbance between the mixing point and the observation point is negligible.

In acidic conditions the reaction is much faster, and the change in absorbance between the mixing point and the observation point cannot be neglected. The amplitude of the signal at the stop corresponds to the age of the solution. So knowing the amplitude of the signal and its rate constant in addition to the total amplitude measured with the slow reaction results, it is possible to determine the real dead time of the experiment.

A simplified drawing of the method used for the dead time calculation is given in Figure 63.

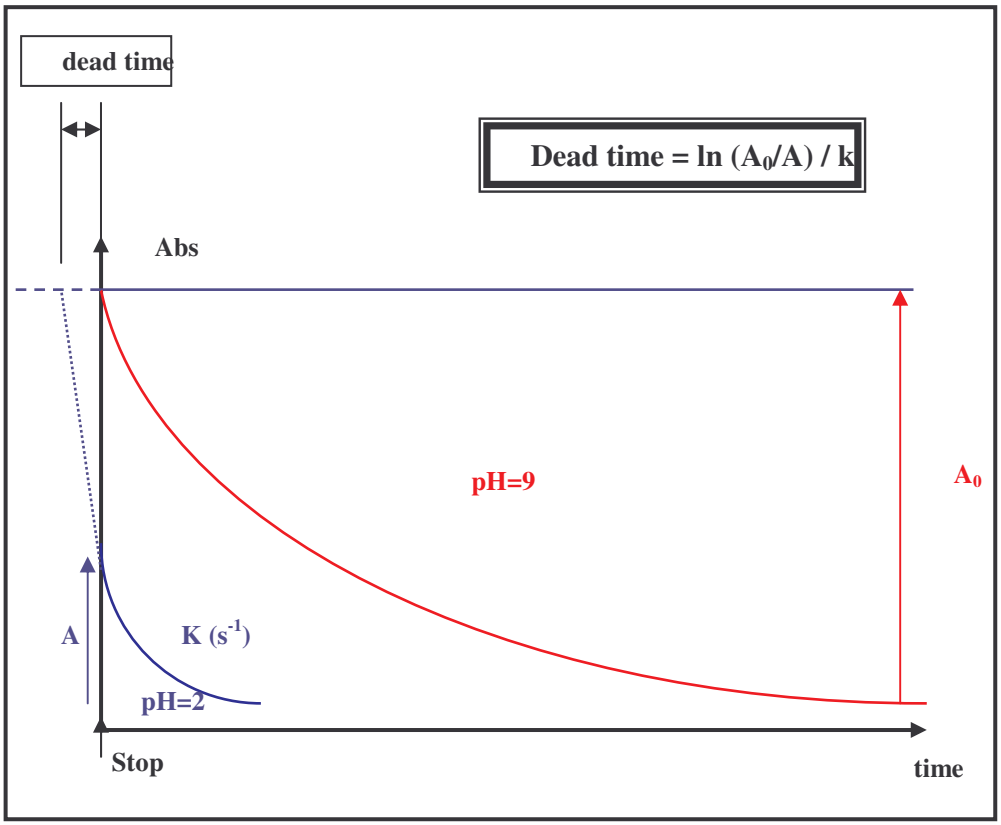


Figure 63 : dead time evaluation

Configuration of Bio-Kine:

- Load Bio-Kine.
- Enter the **Install, device** menu and select the stopped-flow device and its serial port.
- Configure the stopped-flow device and the syringe sizes.
- Push some water into the cuvette and do the absorbance reference.
- Initialise the syringes, and then fill the syringes.

Slow reaction at pH=9

- Click on the Advanced button, then on the SFM Options button: select the TC-100/10 cuvette and a 2 ms lead time. Validate by clicking the OK button.
- Edit the driving sequence shown in Figure 64 .
- In your acquisition software choose to perform one measurement every 1 ms during 3 seconds.
- Run the sequence.
- The data obtained is shown in Figure 65.

Fast reaction at pH=2

- Click on the Advanced button, then on the SFM Options button: select the TC-100/10 cuvette and a 2 ms lead time. Validate by clicking the OK button.
- Edit the driving sequence shown in Figure 66.
- In your acquisition software choose to perform one measurement every 50 µs during 0.15s.
- Run the sequence.
- The data obtained is shown in Figure 67.

Dead time calculation

From the kinetics at pH =9 we get A_0 = 0.787 A.U.

From the kinetics at pH=2 we get A = 0.151 A. U and k = 445 s^{-1}.

Therefore, **the real dead time is 3.7 ms.** The estimated dead time given by the MPS software was 3.8 ms, so very close to the real one

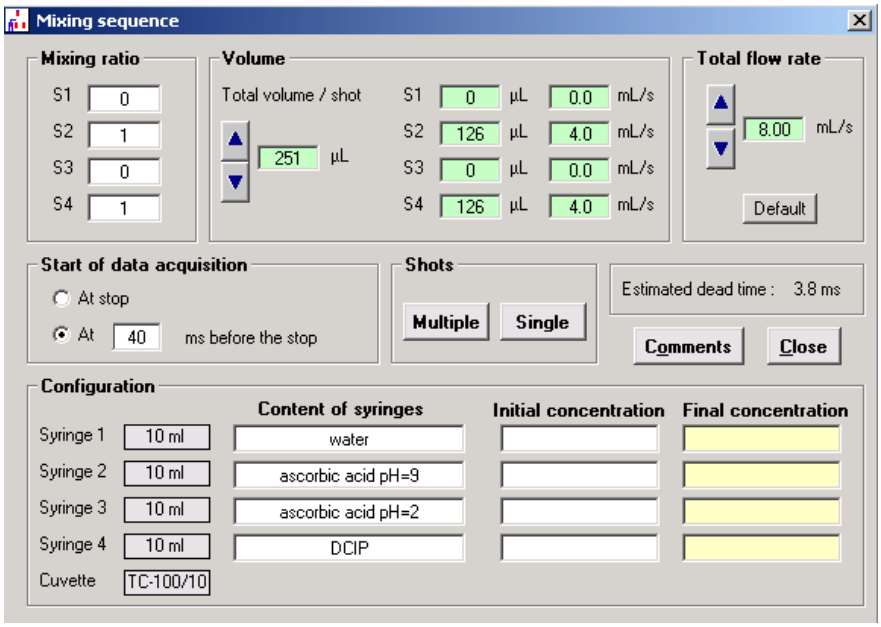


Figure 64: driving sequence of the reduction of DCIP at pH=9

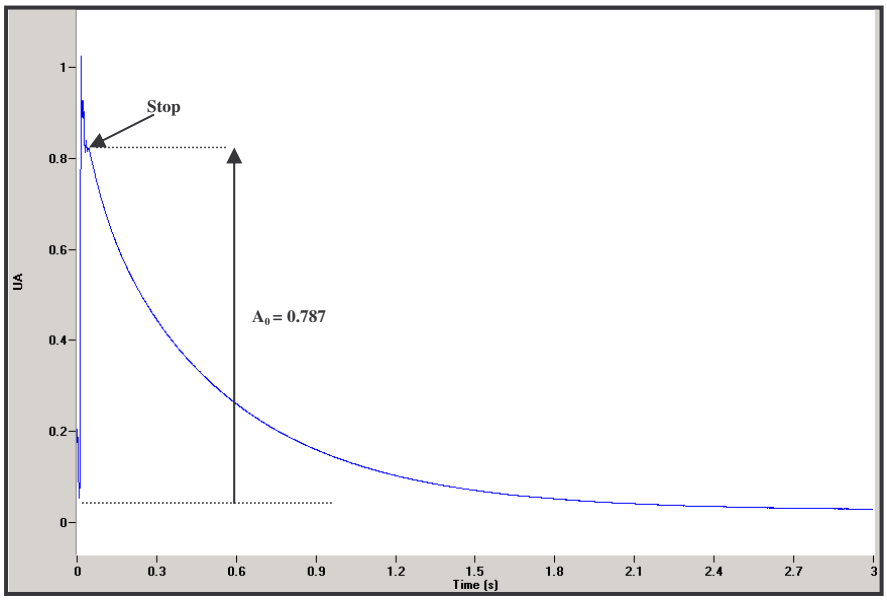


Figure 65: reduction at pH=9.

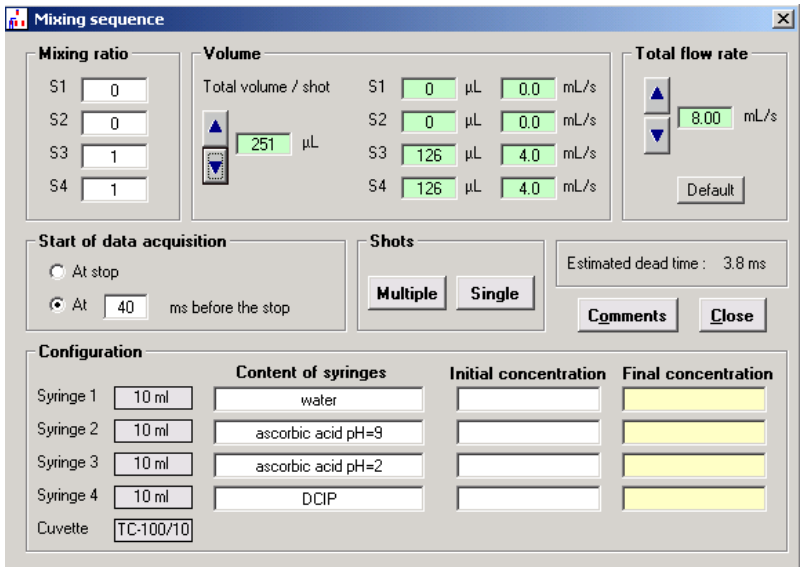


Figure 66: driving sequence of the reduction of DCIP at pH=2.

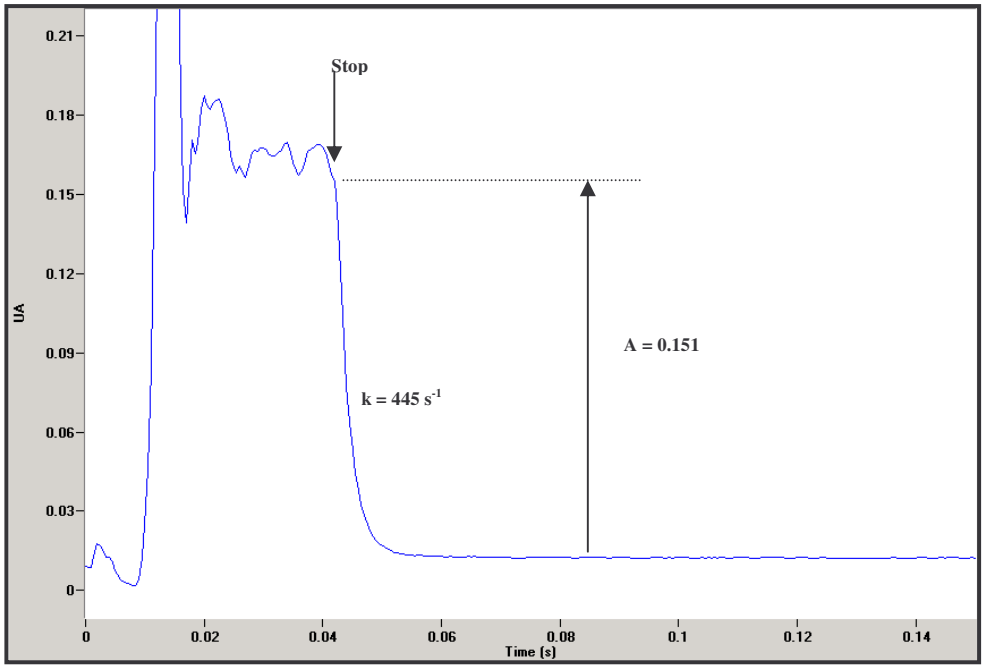


Figure 67: reduction at pH=2.

7.3 Evaluation of Washing and the Quality of the Stop

As mentioned in section 6.1, it is necessary to completely wash the flow path from the last mixer to the point of observation in the cuvette. One method of evaluating the volume needed for washing the flow path is presented in Figure 69. The reaction was the fast reduction of DCIP with ascorbic acid at pH 2.

Experimental condition:

Stopped-flow: SFM-400 equipped with 1.9 ml syringes.

Syringe 1 and 2: Water

Syringe 3: Dichloroindophenol 1 mM

Syringe 4: 10 mM ascorbic acid.

Mixer: Standard Berger ball.

Cuvette: µFC-08 (dead volume # 3 µl).

Detection: MOS-200 in absorbance mode (detection at 524 nm)

Equal volumes of each reactant were mixed. The data acquisition was started 40 ms before the shot for clear observation of the start of the shot.

The fast reaction also allows the examination of the data around the stop for any artefacts.

The results indicate that there are no stop artefacts present and that a minimum of 40 µl is needed to completely wash the flow path for this reaction.

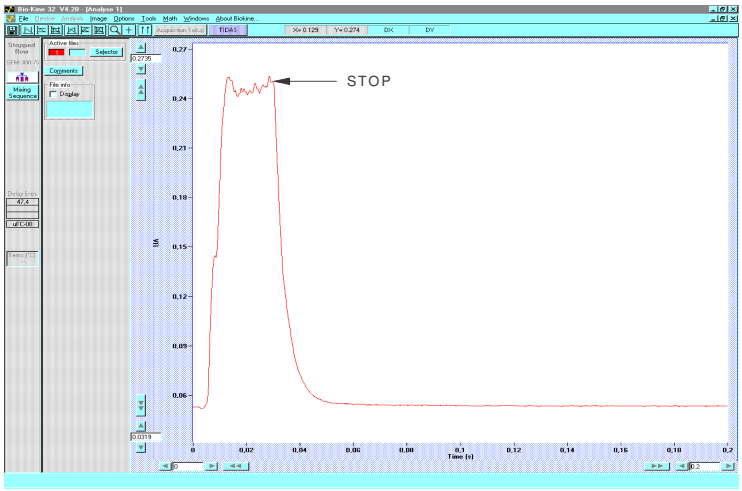


Figure 68 : kinetics obtained with total volume = 80 µl.

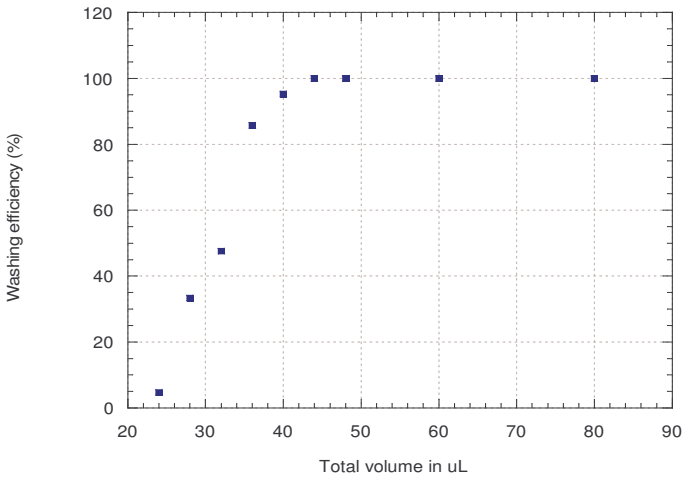


Figure 69: determination of the minimum volume to push.

7.4 Variable Ratio Mixing

The ability to obtain variable mixing ratios by a simple programming of the instrument (i.e. without changing the syringes) is one of the major advantages of the SFM instruments. The microprocessor control of the stepping motors gives 6400 steps per revolution of the motor and results in a smooth and quasi-continuous movement of the syringe over a very large range of flow rates. A few example experiments using the SFM to carry out variable ratio mixing are described below.

7.4.1 Reduction of DCIP by ascorbic acid

Experimental Conditions:

Syringe 1 (10 ml):	20mM Ascorbic Acid, pH 9
Syringe 2 (10 ml):	Buffer
Syringe 3 (10 ml):	100 µM DCIP
Wavelength:	524 nm
Cuvette:	TC-50/10
Detection method:	Transmittance

Acquisition was started at the end of the stop. A series of experiments were performed in which the concentration of ascorbic acid was varied from 0.8 mM to 10 mM. This was accomplished by programming the SFM to deliver a constant volume of DCIP (S3) and varying volumes of ascorbic acid (S1) and buffer (S2). The total volume of each shot was kept constant as was the volume of S1 + S2. The total flow rate was also kept constant in all experiments. Figure 70 shows the results of the experiments with the dilution factor of ascorbic acid noted next to each curve.

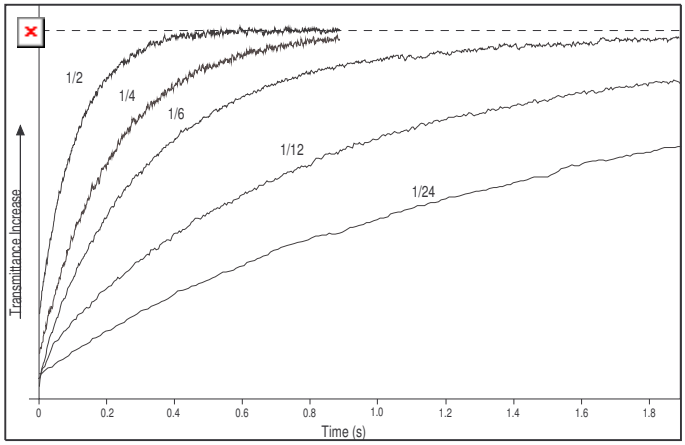


Figure 70 – DCIP Variable Ratio Mixing Experiments

Using the variable ratio mixing method, the concentration of one reactant (ascorbic acid in this case) can easily be varied while another reactant (DCIP) is kept constant.

The curves in Figure 70 were analyzed using the Bio-Kine software to determine the rate constants. The rate constants measured show a satisfactory linear relationship as a function of ascorbic acid concentration (Figure 71).

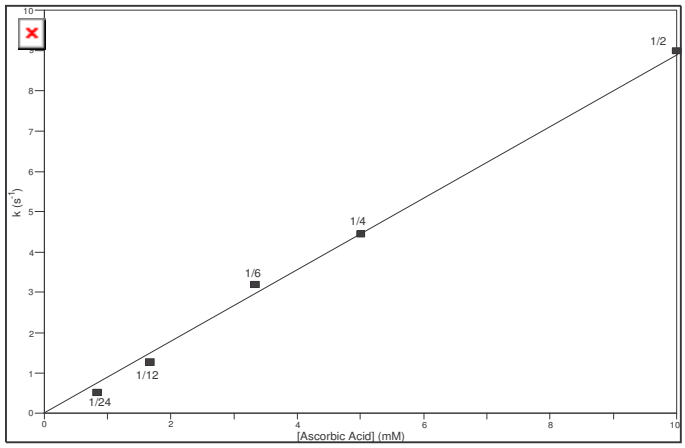


Figure 71 – DCIP Variable Ratio Mixing: k vs. [AA]

Dilution factors of 1/50 or higher can be obtained with the SFM. Figure 72 shows the results of experiments where 1 mM DCIP in S3 was mixed with various volumes of buffer from S1. In this case, there is no reaction but only dilution of the DCIP. The results indicate a satisfactory linear relationship between the absorbance measured and the final concentration of DCIP calculated according to the diluted effect.

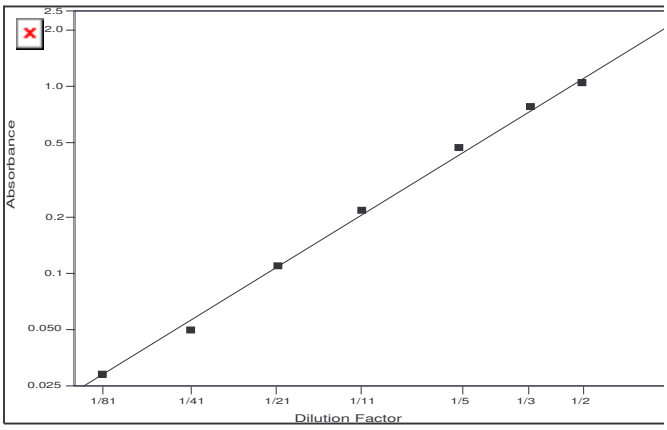


Figure 72 – DCIP Dilution Experiments

7.4.2 Alcohol dehydrogenase activity

Experimental Conditions:

Buffer: 100 mM Tris-Cl, 1 mM EDTA,5 g/l semicarbazyde-Cl and 25 mM ethanol

Syringe 1 (10 ml):	Buffer
Syringe 2 (10 ml):	Buffer + 1 mg/ml alcohol dehydrogenase (ADH)
Syringe 3 (10 ml):	Buffer + 1 mM NAD
Wavelength:	340 nm
Cuvette:	TC-50/10
Detection method:	Absorbance

Experiments were performed in a manner similar to the variable mixing ratio mixing experiments of DCIP in the previous section. The volume and concentration of NAD (S3) were kept constant. The concentration of ADH varied by varying the volumes of buffer (S1) and ADH (S2) in each experiment. The total volume and flow rate of each shot was kept constant. The dilution of ADH varied from 1/2 to 1/120 (0.5 to 0.083 mg/ml final ADH concentration). The results of the experiments are shown in Figure 73.

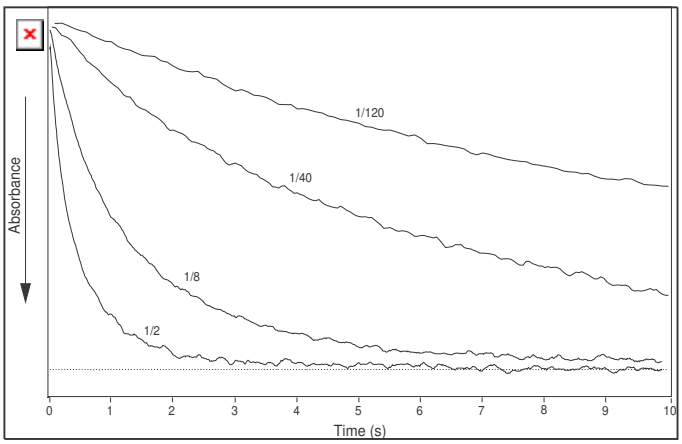


Figure 73 – ADH Variable Ratio Mixing Experiments

The initial rate of each reaction in Figure 73 was measured and plotted as a function of the dilution factor in Figure 74. The rates and dilution factors are plotted on a log-log scale. Figure 74 shows that there is reasonable alignment of the data to a line with a slope of 1. This indicates a linear relationship between the initial rate and the dilution factor.

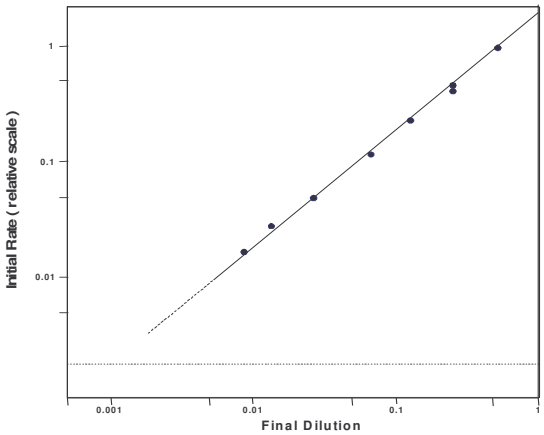


Figure 74 – ADH Variable Ratio Mixing: Rate vs. Dilution

The horizontal dashed line in Figure 74 corresponds to the remaining ADH activity after washing the cuvette. To obtain this line, the cuvette was washed with two shots containing no ADH (only S1 and S3 were used) follow by a 1/2 dilution of ADH with NAD (only S2 + S3). The contaminating activity corresponds to a 1/1000 of the initial ADH concentration. Further washing could reduce the contaminating activity, but this sets a reasonable limit for the dilutions that can be obtained with the SFM.

7.5 Mixing Solutions of Unequal Density and Viscosity

The SFM can be used to mix solutions of unequal density and viscosity in addition to simple aqueous solutions. This situation is commonly found when the kinetic of protein refolding (renaturation) is to be measured.
Figure 75 shows the result of an experiment performed with cytochrome-c.

Experimental Conditions:

Buffer:	100 mM NaCl, 20 mM MOPS, pH 7.5
Syringe 1 (10 ml):	Buffer
Syringe 2 (10 ml):	Buffer
Syringe 3 (10 ml):	50 µM cytochrome-c in 5.5 M guanidine-HCl, 20 mM MOPS, pH 7.5
Wavelength:	290 nm
Cuvette:	FC-15
Detection method:	Fluorescence (320 nm cutoff filter)
Temperature:	25°C

Cytochrome-c denatured in 5.5 M guanidine-HCl was mixed with buffer in a 1:10 ratio and the intrinsic fluoresce of cytochrome-c was observed. The final concentrations of cytochrome-c and gaunidine-HCl in the cuvette were 5 µM and 550 mM respectively. At this final concentration of guanidine-HCl, cytochrome-c shows rapid renaturation as seen in Figure 75.

The curve in Figure 75 was fitted with two exponentials and rate constants of 83 s^{-1} and 9 s^{-1}. The amplitudes of the exponentials were 38% and 62% of the total transition respectively. The fit is shown as a dotted line under the experimental curve.

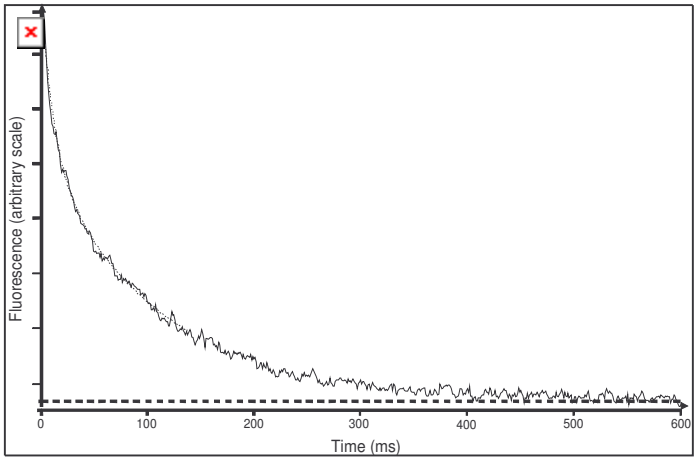


Figure 75 – Cytochrome-c Renaturation

WARNING: The mixing of solutions of unequal density and viscosity can result in convection artifacts!

Convection artifacts are due to the slow rise of light buffer from the last mixer and subsequent entry into the observation chamber after mixing. The entry of the light buffer is detected by a sudden and reproducible change in absorbance or fluorescence 10 to 100 seconds (or more) after the mixing. The existence of this artifact and the time at which it is observed are dependent on the relative densities and viscosities of the mixture and of the light buffer.
In the above example with cytochrome-c, a large dilution ratio was used so that the final mixture has a density not too different from that of the NaCl buffer. As a consequence no convection artifact was visible when data acquisition was prolonged for more than 100 seconds.
On the other hand, if a 1/1 mixing was used, the high concentration of guanidine in the cuvette (2.75 M) would have resulted in the formation of a large gradient of density at the last mixer. Under these conditions, if no precautions are taken, a rapid rise of the NaCl buffer in the observation cuvette can be observed about 20 s after mixing.
Hence, the best solution is to use the high density (HDS) mixer developed by Bio-Logic. This mixer is described in detail in section 3.7.2.

8 Technical section

8.1 Solvent compatibility

Any solution used in the SFM system will be in contact with the following materials:

- **PEEK** (the core of syringes, blocks and piston caps)
- **Teflon** (head cap or valves for special version)
- **Vitton** (o'rings)

PEEK has excellent chemical resistance to organic and inorganic liquids.

Only concentrated acids like sulfuric and nitric can attack it.

Methylene chloride, DMSO, and THF has some swelling effect, should be used under control.

Maximum operating temperature: 100°C.

Teflon is chemically inert

Viton parts – these parts are most vulnerable chemically

Other materials are available upon request (EPDM, Nitrile, Isolast…). Please contact our commercial service for enquiries.

We highly recommend Isolast o'ring with an organic solvent

Please refer to: Isolast http://www.superseal.hu/al/catalogs/busak+shamban/isocatal.pdf **for chemical compatibility guide.**

Using a solvent with a non appropriate o'ring material will be not considered under warranty by Bio-Logic.

8.2 Mixer Removal, Examination and Replacement

Removal and replacement of the mixer in the observation head is described below using Figure 76.

Removal

1) Remove any observation head caps or collimators.

2) Unscrew the nut on top of the observation head and remove it.

3) Remove the cuvette holder and attached cuvette.

4) Remove the observation head from the SFM body via the four screws at the corners of the observation head.

5) Insert a flat-end pin (diam. 1 mm) or paper clip through the bottom side of the observation head and **gently** push out the mixer and the o-ring (2.2 x 1.6).

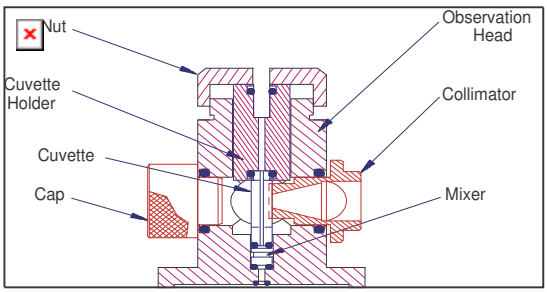


Figure 76 – Mixer Removal

Examination

1) Examine the mixer for any material blocking the holes. If found, the mixer should be cleaned before returning it to the SFM (we recommend soaking the mixer in 2% Hellmanex II cleaning solution for 15 min. or sonication).

2) Measure the height of the mixer. The mixer (Berger Ball or HDS) should measure 5.1 mm in height (Figure 77). If the mixer is smaller than this, it has been damaged and should be replaced.

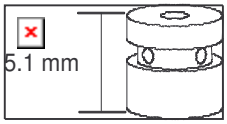


Figure 77 – Mixer Height

Replacement

1) Insert the mixer into the observation head in the orientation shown in Figure 78.
2) Insert the o-ring on top of the mixer.
3) Reattach the observation head to the SFM body.
4) Reinsert the cuvette and cuvette holder.
5) Screw on the observation head nut.

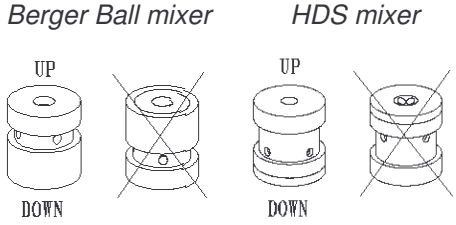


Figure 78 – Mixer Orientation

8.3 Lubrication

The drive screws should be lubricated periodically (once per year) with mineral oil. Access to the drives screws is obtained by removing the cover in front of stepping motors.

8.4 Thermostated Bath

To avoid any corrosion we recommend using a circulating oil bath

9 INSTALLATION OF THE QUENCHED-FLOW COMPONENTS

9.1 Installation of the Mixer Blocks and Delay Lines

In quenched-flow mode, the syringes of the SFM can be used to perform many types of mixing experiments. It is difficult to list all the possibilities here. A few common types are described below:

1) Load several reagents, mix them and quench the reaction with the contents of the last syringe.

2) Use syringes loaded with reagents and buffer to vary the concentration of one or two reagents, mix and then quench the resulting mix with the contents of the last syringe.

3) Perform sequential mixing and delays between up to 3 reagents before they are mixed with the content of the last syringe.

In all experiments, the final sample is recovered for analysis. All SFM/Q instruments are shipped standard with a quench exit valve (fig54) to simplify sample collection.

The exit valve and delay line(s) are installed in the SFM body differently depending on how many syringes are present and the type of experiment performed.

SFM-300 - The exit valve and delay line are installed as shown in Figure 80.

SFM-400 - The exit valve and delay line(s) are installed as shown in Figure 81. The exit may be installed using the mixing blocks labeled **0-MIX-0**, **0-MIX-DL**, **DL-MIX-0**, **DL-MIX-DL** or no mixing block. The installation of the different mixing blocks is described in Table7.

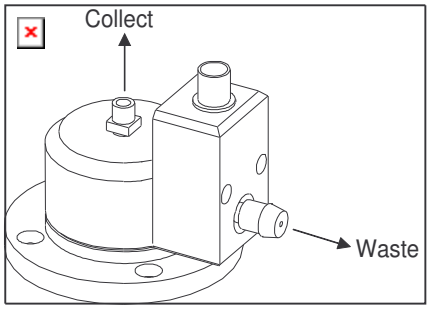


Figure 79 – Exit Valve

Table7 – SFM-400 Exit Valve Installation

MIXING BLOCK	COMMENTS
0-MIX-0	Installed with no additional delay lines.
0-MIX-DL	Installed with one delay line between the mixer block and the observation head
DL-MIX-0	Installed with one delay line between the SFM body and the mixer block.
DL-MIX-DL	Installed with delay lines on both sides of the mixer block.
NONE	Only a delay line is installed between the SFM body and the exit valve and the SFM-400 functions as an SFM-300 (Figure 80). Syringe 3 is blocked by the delay line and only syringes 1, 2, and 4 are useable. In this case, syringe 3 does not need to be filled.

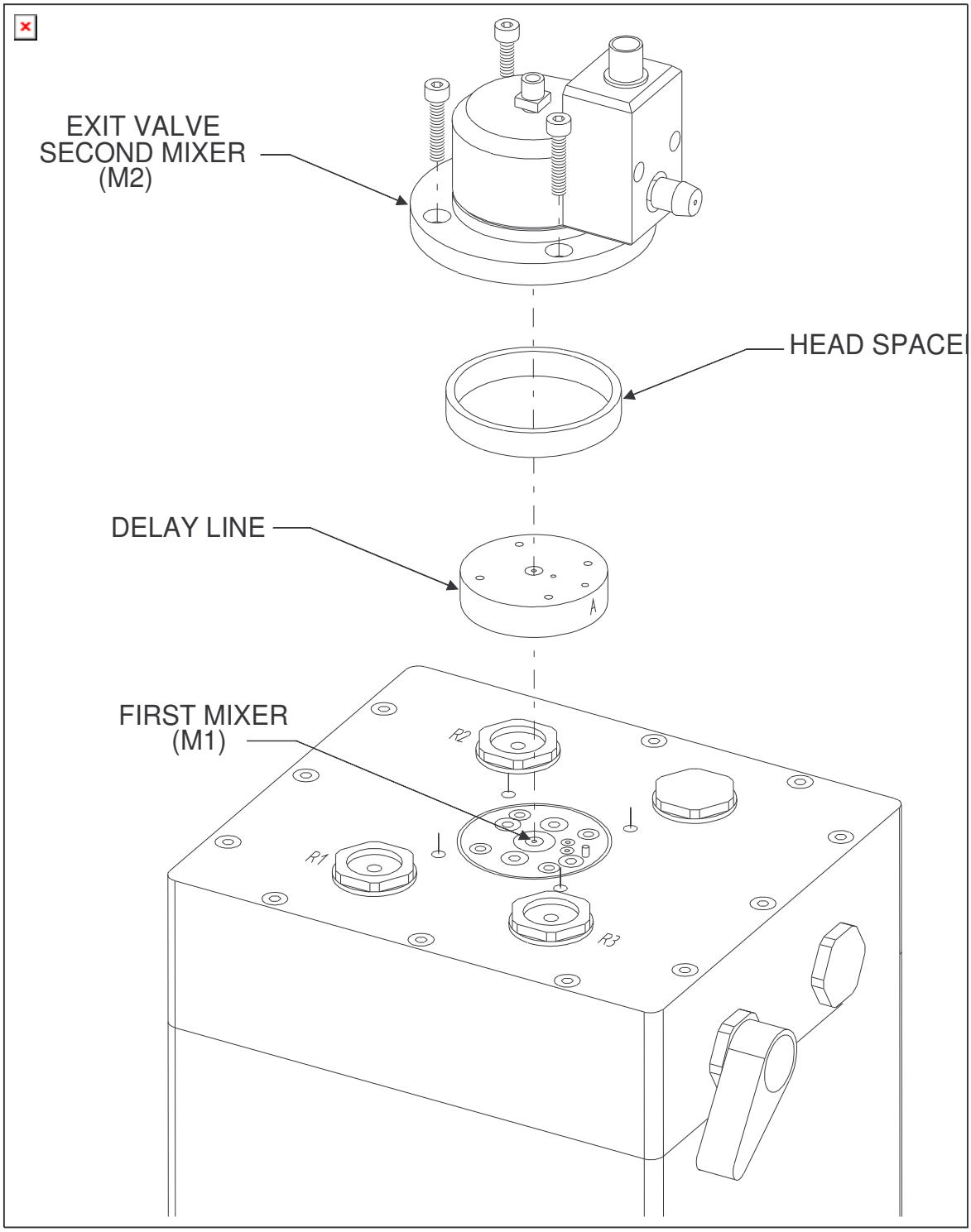


Figure 80 – SFM-300: Installation of Exit Valve and Delay Lines

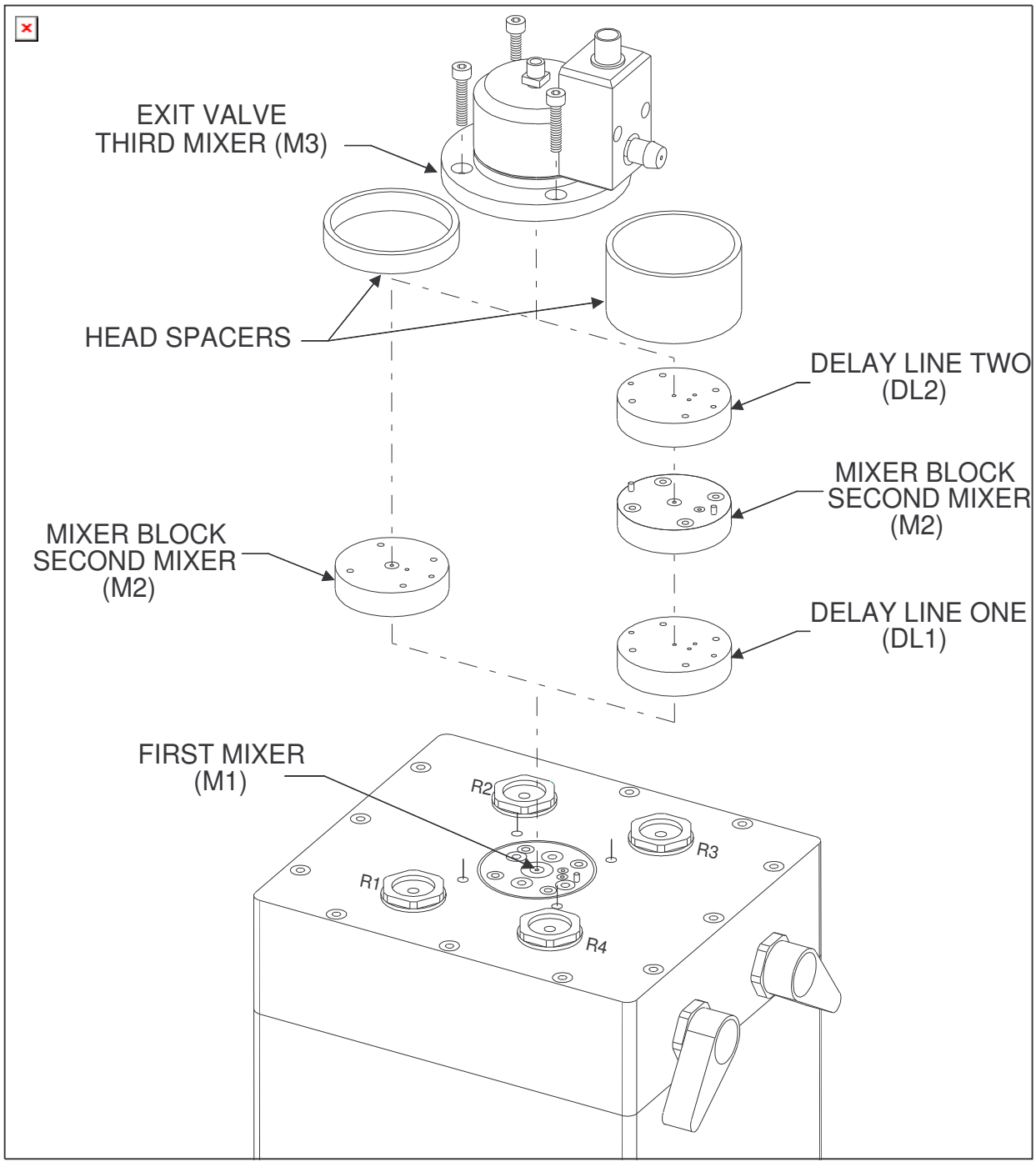


Figure 81 – SFM-400: Installation of Exit Valve, Mixer Blocks and Delay Lines

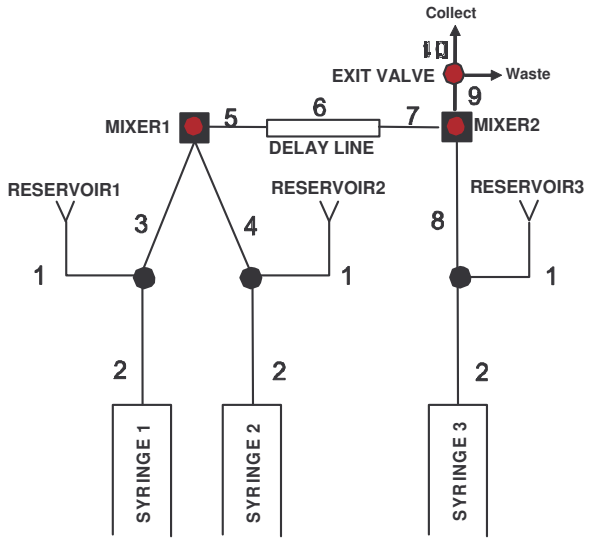
9.2 Flow Line and Intermixer Volumes

Figure 82 (SFM-300) and Figure 83 (SFM-400) below indicate the volumes of the SFM flow lines and delay lines. The amount of time a sample ages between two mixers is given by:

Ageing time between two mixers = (Intermixer volume)/(Flow rate through intermixer volume)

It should be noted that the volumes give in the table are the *mechanical* volumes. The hydrodynamic volumes may vary slightly around these values. For precise measurement of ageing times, it is recommended that the intermixer volumes be determined experimentally with known reactions. One such experimental procedure for determining intermixer volumes is described in the section 14.2 of this manual.

SFM-300/Q FLOW LINE VOLUMES	
Line Number	**Flow Line Volume (µl)**
1	69
2	7
3	89
4	88
5	10
6	Delay Line
7	14
8	108
9	36
10	55

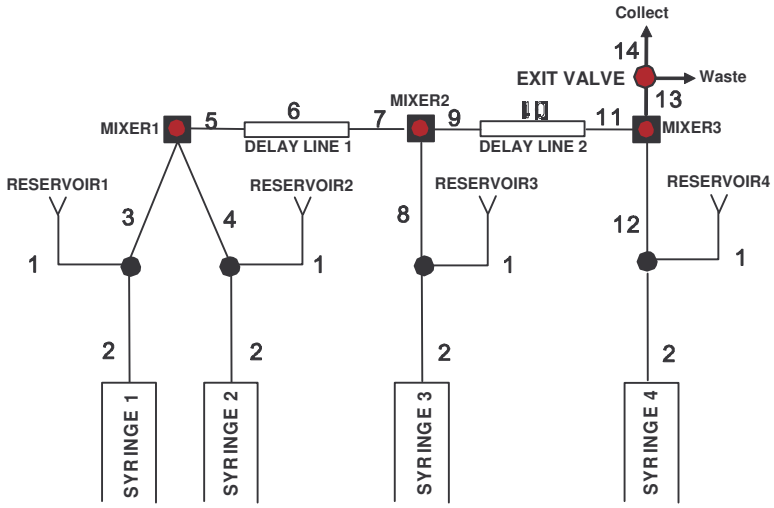


	DELAY LINE AND INTERMIXER VOLUMES*						
	Delay Line						
	N°1(17)	N°2(40)	N°3(90)	N°4(140)	N°5(190)	N°6(500)	N°7(1000)
Volume (µl)	19	35	92	144	192	498	1003
Intermixer Volume $M1_{BB} – M2_{BB}$ (µl)	43	60	116	168	216	523	1027

*Notes: Intermixer volumes are measured from the mixing point of one mixer to the mixing point of the next mixer. BB indicates a Berger Ball mixer has been installed at the position noted.

Figure 82 – SFM-300/Q Flow Line and Delay Line Volumes

SFM-400/Q FLOW LINE VOLUMES	
Line Number	**Flow Line Volume (µl)**
1	69
2	7
3	89
4	88
5	7
6	Delay Line 1
7	13
8	94
9	10
10	Delay Line 2
11	14
12	108
13	36
14	55



	DELAY LINE AND INTERMIXER VOLUMES*							
	Delay Line							
	None	N°1(17)	N°2(40)	N°3(90)	N°4(140)	N°5(190)	N°6(500)	N°7(1000)
Volume (µl)	0	19	35	92	144	192	498	1003
Intermixer Volume $M1_{BB}$ – $M2_{BB}$ (µl)	22	39	55	112	164	212	518	1023
Intermixer Volume $M2_{BB}$ – $M3_{BB}$ (µl)	27	43	59	116	168	216	522	1027

*Notes: Intermixer volumes are measured from the mixing point of one mixer to the mixing point of the next mixer. BB indicates a Berger Ball mixer has been installed at the position noted.

Figure 83 – SFM-400/Q Flow Line and Delay Line Volumes

9.3 Sample Collection Methods

The result of a quenched-flow experiment can be recovered by two different methods: total liquid collection and partial liquid collection. The method of choice will depend on the experiment. The two methods are described below.

9.3.1 Total liquid collection

In this method all the liquid that exits the SFM during a quenched-flow experiment is recovered. This includes the result of the quenched-flow experiment and any old reaction mixture that remained in the SFM before the start of the experiment. Two manners exist to recover the total liquid from a quenched-flow experiment. These are described in the next two sections.

9.3.1.1 Free flow method

A tube is connected to the **waste outlet** of the exit valve to recover all the liquid exiting the SFM (Figure 84). The liquid may be ejected into a test tube or beaker for simple collection or for quenching with an external solution. If the latter method is used, the tube acts as an additional delay line whose volume can be adjusted by the user.

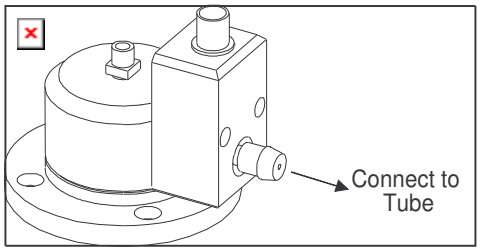


Figure 84 – External Tube Collection

CAUTION!: If the volume collected is not substantially larger than the flow line and tube volume, contamination of samples by old reacted solution may occur. It is recommended to collect sample volumes a minimum of 3-5× flow line + tube volumes (section 9.2). In addition, it is recommended to wash the old solution out of the SFM and tube with a buffer between sample collections and perform test experiments to verify the level of sample contamination is minimal.

WARNING: The inner diameter of the tube connected to the waste outlet should always be larger than that of the hole in the waste outlet. If this is not respected, back pressure can build up inside the SFM during a shot and cause the motors to stall.

9.3.1.2 Pipette/syringe collection

In this method, a pipette or syringe is connected to the **collect outlet** of the exit valve, and all the liquid is collected (Figure 85). This method allows for the complete collection of the sample and isolates the collected sample from the environment. It is recommended that a pipette be used for collection rather than a syringe. Undue back pressure from a collection syringe plunger can force liquid to exit through the waste outlet instead of being collected.

!CAUTION!: If the volume collected is not substantially larger than the SFM flow line volume, contamination of samples by old reacted solution may occur. It is recommended to collect sample volumes a minimum of 3-5× flow line volumes (section 9.2). In addition, it is recommended to wash the old solution out of the SFM and tube with a buffer between sample collections and perform test experiments to verify the level of sample contamination is minimal.

9.3.2 Partial liquid collection

This method is the most preferred method used for quenched-flow experiments. It is similar to the previous method in section 9.3.1.2 in that the sample is collected in a pipette or syringe (Figure 85). It differs from the total liquid collection method because only the portion of the liquid exiting the SFM that corresponds to completely new, uncontaminated sample is collected. The exit valve is programmed to divert the contaminated sample to waste so that only uncontaminated sample is recovered. Because of this, even very small volumes (10's of µl) of a sample can be collected, and the sample economy is high. The programming of the exit valve is described in detail in section 12.6.

As discussed in the previous section, it is recommended that a pipette be used for collection rather than a syringe. Undue back pressure from a collection syringe plunger can force liquid to exit through the waste outlet instead of being collected.

IMPORTANT: The volume diverted to waste should be a minimum of 3-5× the flow line volumes (section 9.2) to ensure only uncontaminated sample is collected. Larger volumes may be necessary, and it is recommended that test experiments be performed to optimize the volume needed to minimize sample contamination.

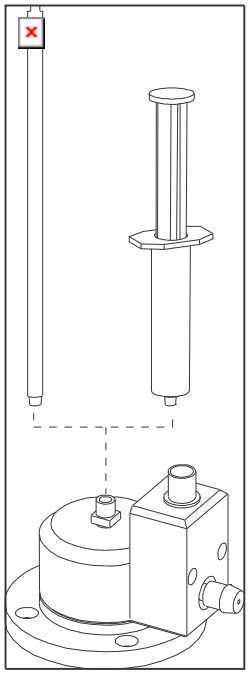


Figure 85 – Pipette/Syringe Collection

11 SOFTWARE CONFIGURATION IN QUENCHED-FLOW MODE

The SFM is controlled by Bio-Kine software which is also used to control acquisition parameters. This section precisely describes the configuration of the software. Please note that the procedures and examples have been generalised and configuration choices should be made based upon the equipment purchased and intended experiments.

This section assumes that the user has already installed Bio-Kine software on the host microcomputer.

11.1 Device installation using PMS-60 and Bio-kine version up to 4.45

Once Bio-Kine loaded, choose **Install, device installation** in the main menu. The stopped-flow communication is established from this window by checking the **stopped-flow device** box and choosing the corresponding **Serial port.** Accept the parameters using the **OK** button.

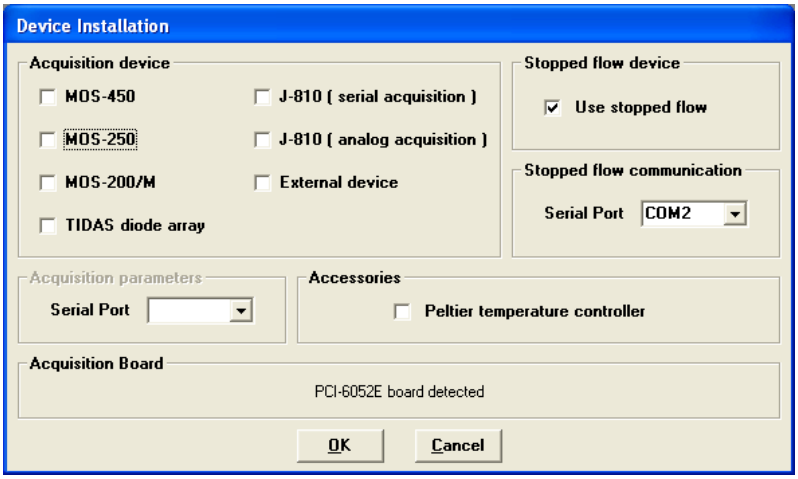


Figure 86 : device installation.

11.2 Device installation using MPS-60 or MPS-70 with Bio-kine version 4.47 and higher (4.49)

Once Bio-Kine is loaded, choose **Install, device installation** in the main menu (Figure 17-device installation.). The stopped-flow communication is established from this window by checking the **stopped-flow device** box and choosing the corresponding **Serial port** for the **MPS-60** or **USB port** for the **MPS-70.** Accept the parameters using the **OK** button.

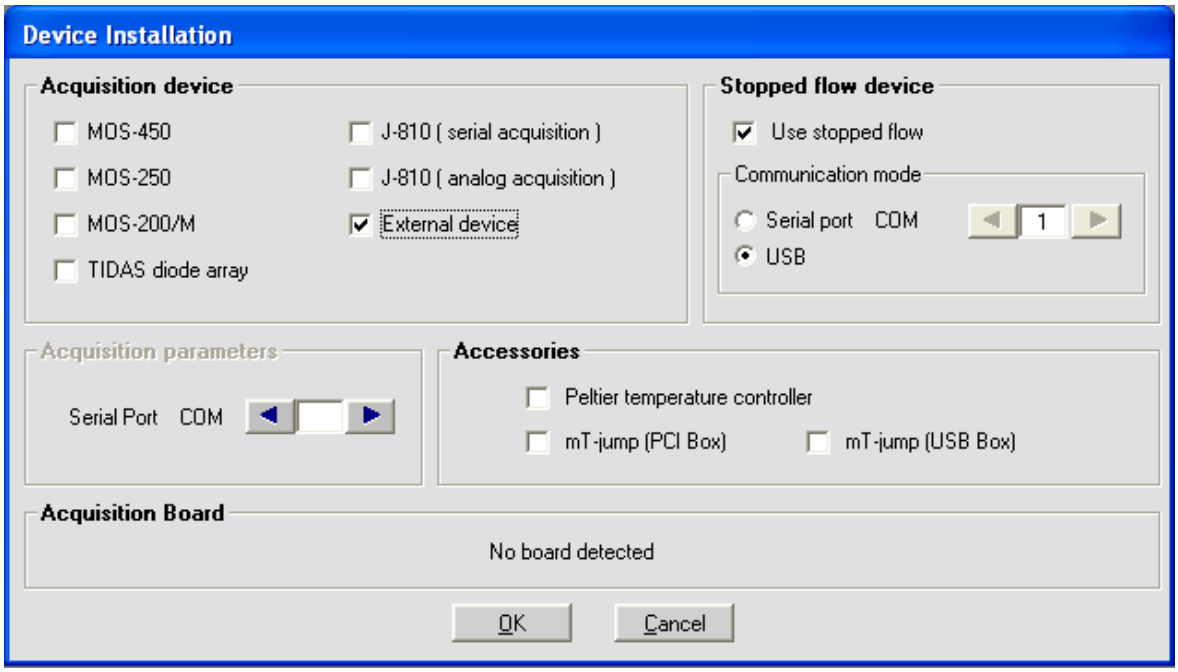


Figure 87 - device installation.

11.3 Stopped-flow Configuration

Once the stopped-flow device and its serial port are selected in the **device configuration** menu (refer to section 11.1), choose **the Install, stopped-flow configuration** menu (see Figure 88).

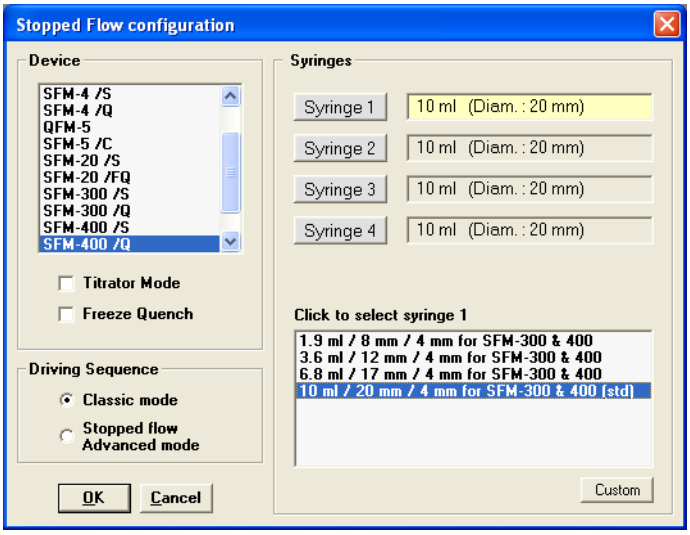


Figure 88 : stopped-flow configuration.

The device to be installed should be configured according to the instrument purchased and mode chosen for use. Check Classic mode, the advanced mode is not available in quenched-flow configuration.

Syringe configuration is made in the same window. The active syringe is displayed in yellow; select the nature of the syringes that have been installed in each syringe position of the SFM by clicking on the correct one.

The SFM comes equipped with standard 10 ml syringes, which are the default syringes installed in the software. Changes only need to be made in the software when syringes of different volumes (other than standard) have been installed in the SFM.

Use the Custom button to enter syringe specifications if you have a custom syringe. In this condition the window shown in Figure 89 is displayed, it is then necessary to enter volume, piston diameter and screw pitch of the custom syringe to add it to the standard ones.

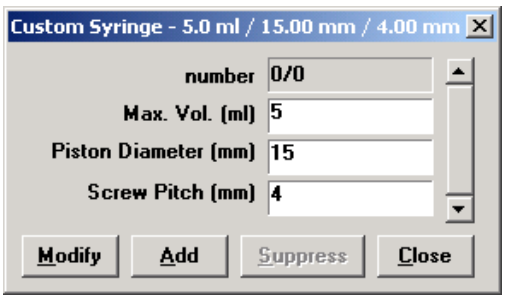


Figure 89 : custom syringe

WARNING: Incorrect syringe configuration will cause volume and flow rate calculations to be incorrect!

11.4 Stopped-flow status area

A vertical menu bar on the left of the screen is dedicated to the quenched-flow device (see Figure 90). This menu bar can be hidden or displayed using the SFM button in the main menu. This menu bar gives access to the syringe control window using the button (refer to section 12.1) and to the classic mode using the Mixing Sequence button (refer to section 12.6).

The advanced mode is not available in the quenched-flow configuration. The volumes of the delay lines installed are indicated in SFM option.
Once the sequence is ready in the driving sequence window, the shot control window is displayed in the area as shown in Figure 90.

Figure 90: stopped-flow menu bar.

12 INSTRUMENT OPERATION IN QUENCHED-FLOW MODE

12.1 Manual Syringe Control

The syringes of the SFM can be controlled either manual or automatically. Automatic control of the syringes is strictly used only for experiments. The manual control of the syringes is used for initialization, filling, and emptying the syringes. The manual movement of the syringes can either be made directly from the MPS or though Bio-Kine. Both methods are described in the following sections.

12.1.1 MPS

Syringe control directly from the MPS is made through the use of the buttons on front panel of the MPS (Figure 2).

The (+) and (-) buttons are used to select the syringe to be moved. The (up) and (down) buttons are used to empty and fill the syringes respectively. The LCD panel at the top of the controls will display which syringe has been selected and whether it is being filled or emptied.

12.1.2 Software

Syringe control from Bio-Kine software is made through the  button in the stopped-flow status area. The MPS is then initialized and communication established between Bio-Kine software and the MPS unit. The message 'MPS on line' is displayed in a green window in the stopped-flow status area.

The syringe to be moved is selected by clicking on the corresponding frame, or pressing the <**Left>** and <**Right>** arrows keys on keyboard. The newly selected syringe will be surrounded with a red rectangle.

Syringes are emptied or filled using the  ,  ,  , and  buttons or with the <**Up**> arrow, <**PageUp**>, <**Down**> arrow, and <**PageDown**> keys on the keyboard. The  button and <**Up**> arrow moves a syringe upwards by one elementary movement, and the  button and <**Down**> arrow moves a syringe downwards by one elementary movement. The  button and <**PageUp**> arrow moves the piston upwards by 10x elementary movements, and the  button and <**PageDown**> moves the piston downwards by 10x elementary movements.

The size of the elementary steps and syringe movement speed is controlled in the Manual Speed section of the window (Figure 91). The  and  buttons change the manual speed. The display shows the speed in flow rate based on the syringe installed and moved.

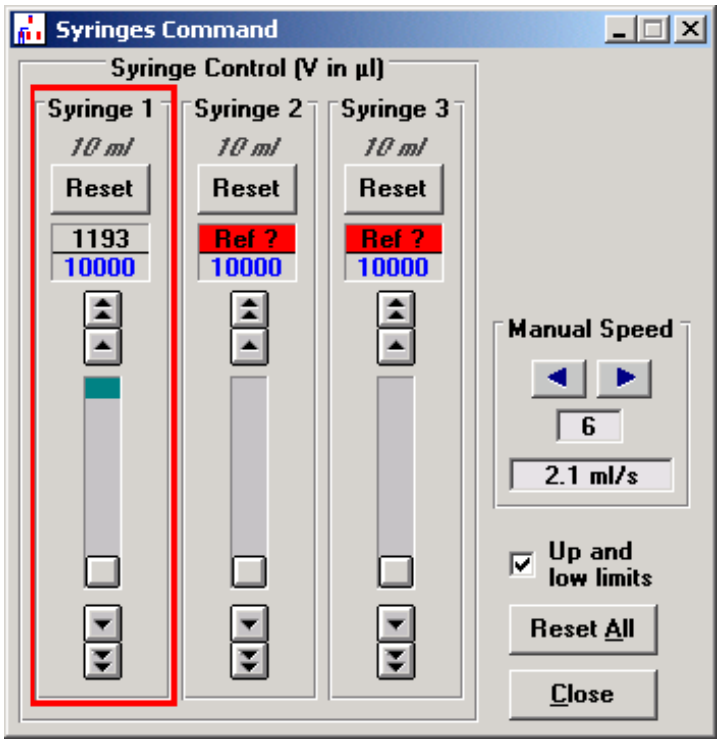


Figure 91 : syringe control window

12.2 Syringe Initialization

The MPS that controls the SFM follows the movements of the syringes so that the actual residual volumes are displayed at all times (see Figure 91). When the MPS is turned on and the software started, turned on, the syringe volume counters show Ref ? and must be initialized (Figure 91).

The syringes are initialized by setting the syringes to their uppermost (empty) position and resetting the syringes in Bio-Kine. The syringes can be selected and moved to their uppermost positions either directly with the MPS (section 12.1.1) or through Bio-Kine (section 12.1.2). Once a syringe has reached its uppermost position, the syringe motor will oscillate and vibrate as it becomes out of phase with the driving pulses. There is no danger to the SFM or syringe motors when this occurs, but there is no reason to unnecessarily prolong this treatment either.

The syringes can be reset individually by pushing the Reset button for each syringe or all at once by pushing the Reset All button in the syringe control window.

IMPORTANT: Measurement of residual syringe volume is made by counting the logic pulses from the controller for each syringe. If, for any reason, a syringe is blocked during a run, the pulses will not correspond to the true volume delivered, and the value displayed may become erroneous (e.g. in the case of incorrect positioning of a valve). In this case, it is advisable to reinitialize the syringes.

If, by accident, a syringe is returned to its uppermost position, the syringe volume counter will again show Ref ?, and the syringe must be reinitialized. To avoid such accidents, the **Up and Low Limits** checkbox may be checked. When this box is checked, Bio-Kine will not allow the syringes to be driven beyond their upper and lower limits. This also avoids accidentally pulling the syringe plunger completely from the syringe and spilling solution onto the SFM.

WARNING: The Up and Low Limits only applies to control of the syringe from within Bio-Kine. These limits can be bypassed by manual control of the SFM directly from the MPS.

12.3 Filling the Syringes

WARNING: Utmost care should be exercised during this operation. Normal operation of the system requires that no bubbles are present in the injection syringes. If this occurs, the buffer flow through the observation chamber will not be correctly controlled by the plunger movement and artifacts may be observed. For best results, it is recommended that all solutions be degassed and filtered before filling the SFM.

The syringes of the SFM can be emptied and filled manually (section 12.1). The filling of the syringes follows the steps below and shown in Figure 92.

1/ Attach a syringe (disposable plastic syringes may be used) containing a sample or buffer to a syringe reservoir port on top of the SFM (Figure 92 Panel 1).

2/ Set the syringe valve handle to (**R**) and fill the syringe manually (section 12.1) while exerting slight pressure on the reservoir syringe (Figure 92 Panel 2 and Panel 3). The pressure exerted on the reservoir syringe prevents any vacuum from occurring in the reservoir syringe which could result in bubble formation. It is suggested that 10ml syringes be filled using manual speed 4 in Bio-Kine and 1.9ml syringes be filled using manual speed 2.

3/ Eliminate any bubbles in the SFM syringe by driving the SFM syringe up and down several times while it is connected to the reservoir syringe (Figure 92 Panel 4).

4/ Turn the syringe valve handle to (**C**) (Figure 92 Panel 5)

5/ Empty by one or two elementary movements of the syringe (section 12.1) to definitively eliminate any bubbles remaining in SFM and cuvette.

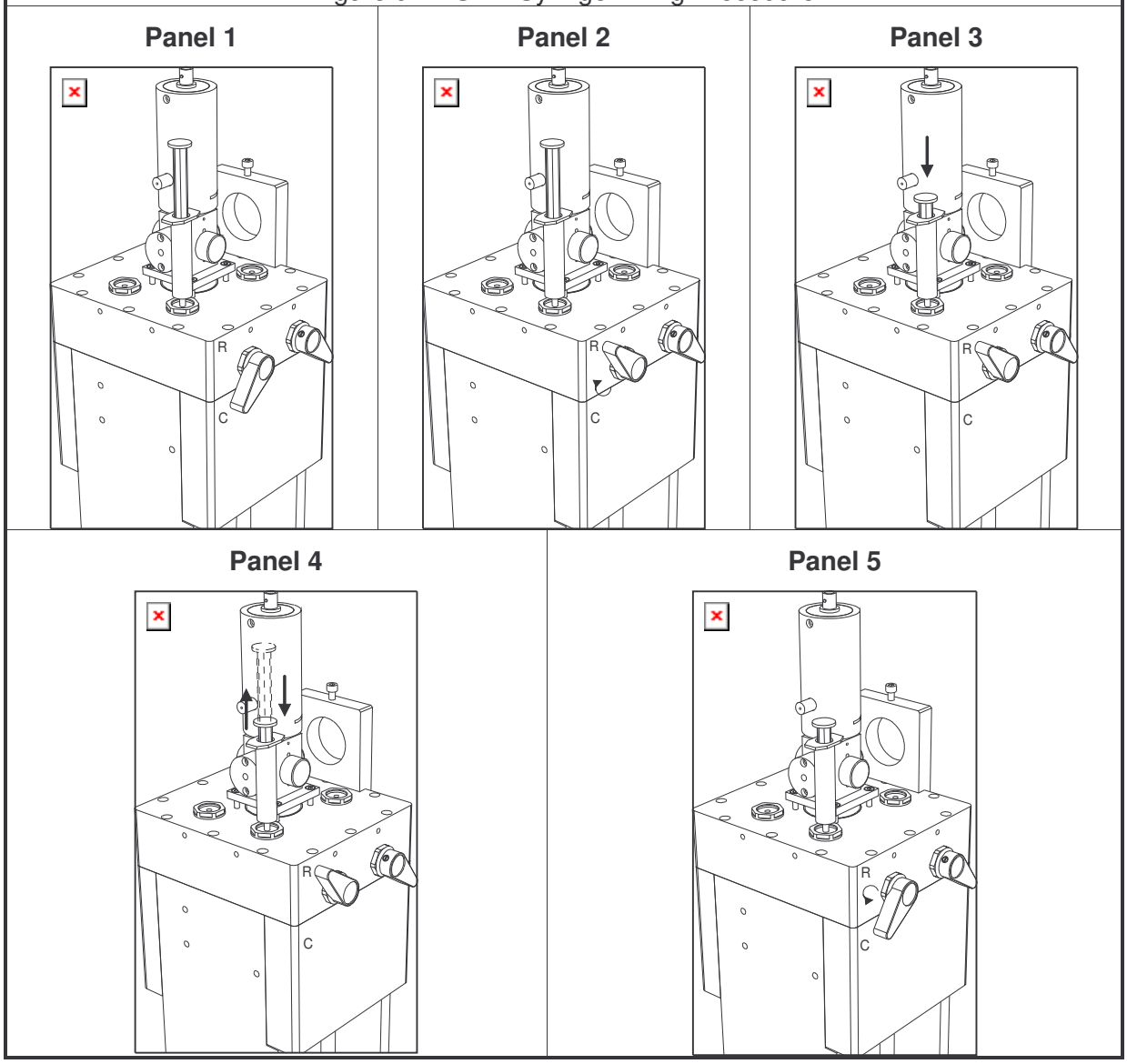
6/ Repeat the above process for the other syringes.

7/ It is recommend that the syringes be filled in reverse numerical order to best remove bubbles from the SFM and cuvette.

IMPORTANT: ALL SYRINGES MUST BE FILLED EVEN IF THEY WILL NOT BE USED FOR AN EXPERIMENT! The valve handles of the unused syringes should be turned to (R) after the filling process is complete.

The Stopped-Flow-Module is now ready for operation.

Figure 92 – SFM Syringe Filling Procedure



12.4 SFM Cleaning and Storage

After each day's experiments the SFM should be cleaned. A thorough cleaning of the SFM will ensure that it has a long functional life and diminish any chance of sample contamination for the next user of the instrument. The procedure below is the recommended daily cleaning procedure to be done before shutting off the instrument.

1/ Remove the remaining samples or buffer from the syringes.

2/ Wash the syringes and flow lines 2 – 3 times with water. This is done by filling each syringe with water to a volume at least equal to the sample volume used for experiments. With the syringe valve handles set to (**C**), empty the syringes completely. Since the liquid will exit via the waste tube, it will wash the flow lines and waste part of the electrovalve as well as the syringes. To clean the collection port, the best is to run a washing sequence where water is collected into the collection device

3/ Wash the syringes and flow lines one time with 70 – 100% ethanol. Use the same procedure as in step 2.

4/ Dry the syringes, flow lines, and electrovalve with a single wash of air. Use the same procedure as in step 2. The syringes should be emptied in reverse numerical order so that all the liquid is pushed out of the syringes, flow lines, and electrovalve.

5/ Set all syringe valves handles to (**R**) and move all syringes to their lowermost positions. The syringe plungers should exit the SFM so that the plunger tips are completely visible. If this is done using Bio-Kine it will be necessary to uncheck the **Up and Low Limits** checkbox in the software **syringe control** window (Figure 91).

Note: You may observe a few drops of liquid that fall from the syringes when the syringe plungers are completely out of the SFM. This is normal as a small amount of liquid is always trapped between the plunger tip and the syringe barrel to make a tight seal.

6/ Turn all syringe valves handles to (**C**).

7/ Turn off the MPS.

12.5 Long-term Storage of the SFM

If the SFM is not be used for a long period of time (more than several weeks), it should be cleaned as above in section 12.4. If the SFM is connected to a circulation temperature bath, the temperature bath should be disconnected from the SFM and the SFM drained completely of all cooling liquid. Afterwards, it is recommended that the SFM cooling circuits be flushed with ethanol followed with air. The SFM is now ready to be stored.

12.6 Creating a quenched-flow sequence

12.6.1 SFM options

Experiments are performed with the SFM through the use of a driving sequence. A driving sequence tells the SFM to automatically perform several functions such as moving the syringes, activating the hard stop, and triggering data acquisition. Driving sequences are created in the window shown in Figure 93. This window can be reached from the

button in the stopped-flow status area.

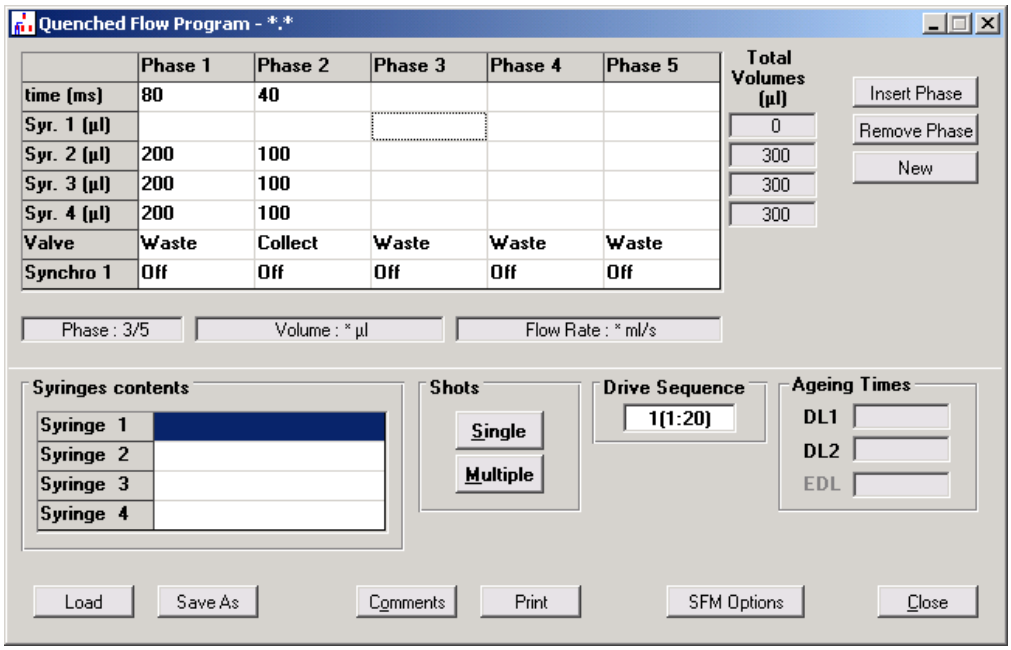


Figure 93 : example of quenched-flow sequence

The first operation should be to check the configuration of the stopped-flow. This is done by clicking on the SFM Options button (refer to Figure 94).

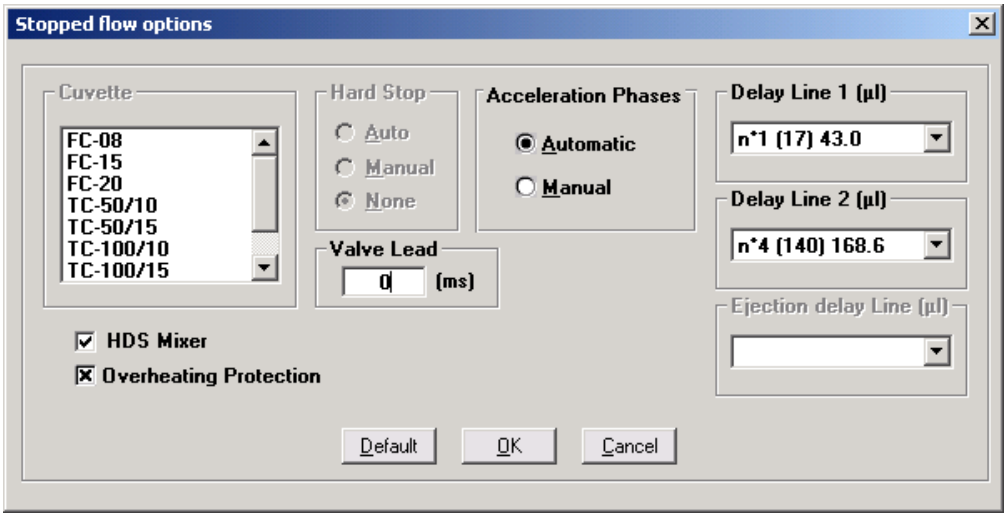


Figure 94: SFM options.

• **cuvette**: not available in quenched-flow configuration.
• **HDS mixer**: not available in standard (please contact our commercial service for special application)

• **Valve Lead:** This section of the window allows one to enter the number of milliseconds before the flow stops that the valve starts closing. The default value is zero. The lead time may be adjusted (from 0 – 5 ms) to fine-tune the quality of the stop. The precision of the setting is 0.1 ms.

• **Overheating Protection:** Not applicable for the MPS-60. The default mode is checked. It is a protection against electronic overheating after a long working day.

• **Hard-stop:** not available is this mode.

• **Delay lines**: Select the delay line(s) according to the delay line(s) you have installed in the SFM. One or two delay lines must be configured depending on the type of device installed under section 3.4. Each delay line is chosen from a pull-down menu.

WARNING: An incorrect delay line configuration will cause ageing time calculations to be incorrect!

12.6.2 Design of the sequence

A driving sequence is entered in the program grid shown in Figure 95. Each column of the grid represents a driving sequence phase. Each phase contains actions for the SFM to perform. A complete driving sequence may contain from 1 to 20 phases. Although only 5 phases are shown initially, additional phases may be inserted using Insert Phase button or removed using the Remove Phase button.

Figure 95 shows an expanded view of the program grid. The duration of a phase is entered in ms (1 – 60000 ms/phase) on the first line of the program grid. The volume in µl delivered by each of the syringes during a phase is entered on the line next to the appropriate syringe. The position of the exit valve is set near the bottom of the program grid.

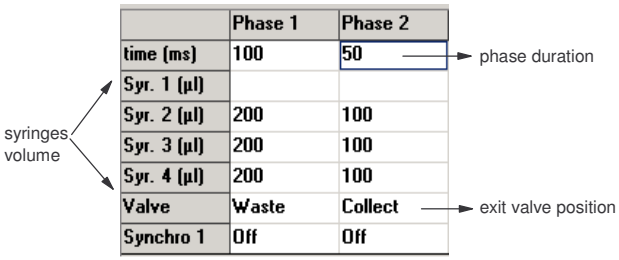


Figure 95 –Program Grid

To enter the phase duration and syringe volumes delivered, click on the corresponding cell or use the arrow keys to navigate between cells. The BACKSPACE key can be used for correction and the DEL key to clear a value. The position of the exit valve is set by pressing "W" for **Waste** and "C" for **Collect** on the keyboard.

Selected values entered in the program grid can be cut, copied and pasted using the **Cut, Copy** and **Paste** functions available under the **Edit** menu. To perform a cut, copy, or paste operation, select the area of the grid desired by dragging the mouse with the left mouse button pushed in and then choose the **Cut, Copy** or **Paste** functions desired under the **Edit** menu. The values will be stored in the Windows clipboard for the **Cut** and **Copy** functions. Values will be pasted from the Windows clipboard for the **Paste** function. If the copy area is bigger than paste area, the operation is done only for values that can fit inside paste area.

IMPORTANT: Blank and non-numeric values entered in the program grid are considered as zero values. Phase duration of 0ms will cause the phase to be skipped in the execution of the drive sequence.

Each time a program grid cell's value is changed, information about the current syringe, current phase and driving sequence is updated displayed below and to the right of the grid (Figure 96). This information indicates:

• Current phase number and the total number phases used in the driving sequence.

• Volume delivered by the current syringe during the current phase or current phase total volume (if an entire phase is selected).

• Flow rate of the current syringe during the current phase or current phase total flow rate (if an entire phase is selected).

• Total volume delivered by each syringe during the driving sequence.

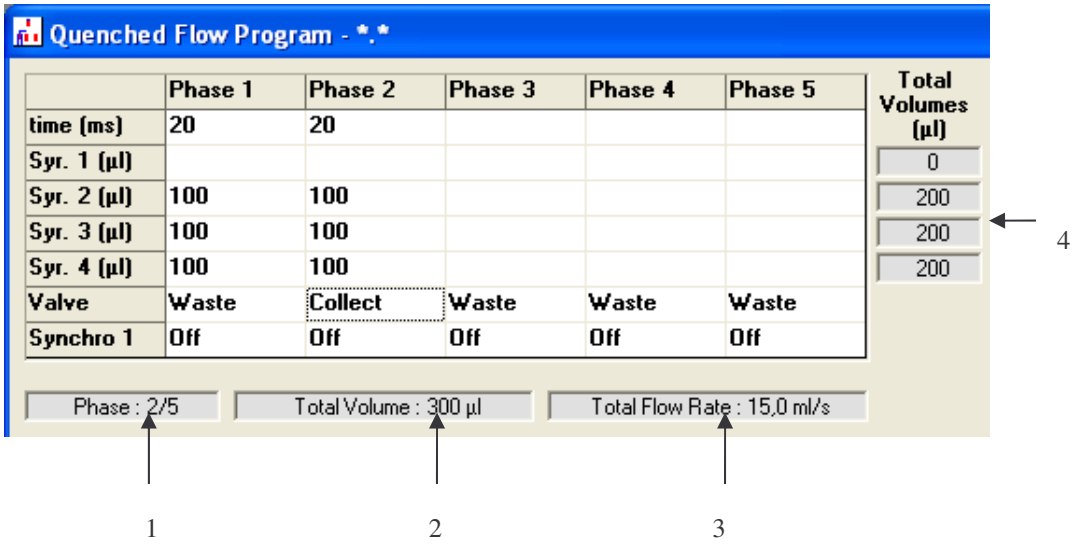
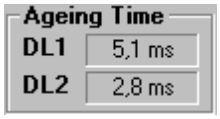


Figure 96 –Driving Sequence Information

An indication of the **Ageing Times** for a driving sequence is also displayed in the driving sequence window and the calculation is made as shown in Figure 97. The ageing times are calculated for the current phase selected based upon the syringes flow rates, delay lines installed and intermixer volumes (Figure 82 and Figure 83)



$$\text{SFM-300}: \; Ageing\ time = \frac{Intermixer\ volume\ M1-M2}{Total\ Flow\ Rates\ S1+S2}$$

$$\text{SFM400}: \; Ageing\ time\ DL1 = \frac{Intermixer\ volume\ M1-M2}{Total\ Flow\ Rates\ S1+S2}$$

$$Ageing\ time\ DL2 = \frac{Intermixer\ volume\ M2-M3}{Total\ Flow\ Rates\ S1+S2+S3}$$

Figure 97 –Ageing Times

Bio-Kine provides the ability to repeat phases a number of times in virtually any order. This is accomplished though a macro sequence entered in the Driving Sequence window shown in Figure 98. The macro sequence can be edited to run a single phase or many phases in a different order than present in the program grid.

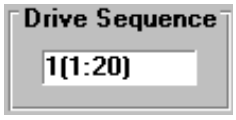


Figure 98 –Drive Sequence Macro

Standard operations can be made from the same window:
• Load a sequence using the Load button.
•Save a sequence using the Save As button.
• Print a sequence using the Print button.
• Close a sequence using the Close button.

• **Comments**: a text window is opened by clicking on the Comments button. Comments are saved with the sequence.

12.7 Running a shot

Once a driving sequence has been entered or loaded, it is transferred to the MPS by pushing the **Single** or **Multiple** buttons.

The MPS is now in automatic mode and the **shot control** window will be displayed in the stopped-flow status (as shown in Figure 99).

The **Shot control** window shows the number of shots possible based the current volumes in the SFM syringes. It also indicates whether the SFM is running a driving sequence or ready for the next shot. A driving sequence is executed by pushing the ▶ button or the **start-stop** button on the front panel of the MPS. The ■ button can be used to stop an experiment prematurely if necessary.

If the **Single** button was used to transfer the driving sequence to the MPS, only a single shot can be made. The End button must then be pushed to return to the driving sequence and the **Single** button must be pushed again to re-transfer the driving sequence to the MPS for a subsequent shot.

If the **Multiple** button was used to transfer the driving sequence to the MPS, the ▶ button can be used to execute shots until the **shot** window shows that 0 shots remain. The End button is then pushed to return to the driving sequence.

Figure 99 : shot control window

13 A SHORT QUENCHED-FLOW PRIMER

This section describes the basics of the quenched-flow technique and provides some general advice about how to design and perform quenched-flow experiments using the SFM. It is not meant to be an exhaustive reference as there are many variations on the quenched-flow experiment too numerous to describe here. We invite the user to explore the references listed below to learn more about rapid mixing and the quenched-flow technique:

Barman, T.E. and Gutfreund, H. (1964), in Rapid Mixing and Sampling Techniques in Biochemistry. (Ed. B. Chance, R.H. Eisenhardt, Q.H. Gibson and K.K. Lonberg-Holm, Eds.). Academic Press, London, pp. 339-344.

Gutfreund, H. (1969), *Methods in Enzymology*, 16, 229-249.

Barman, T.E. and Travers, F., *Methods of Biochemical Analysis* (1985), Vol. 31, 1-59.

13.1 General Principle of Quenched-Flow Experiments

The simplest quenched-flow experiment consists of three stages: mix, age and quench. Complex experiments may involve more stages, but for example purposes only a three-stage experiment is discussed here.

Figure 100 shows a schematic of a quenched-flow experiment. The reaction considered is

$$A + B \rightarrow C \uparrow Q$$

Where the reaction can be stopped at any time by the addition of quencher: **Q**.

Mix: In the first stage, flow is initiated by two plungers. The plungers force the reactants **A** and **B** through a mixer where they are mixed and the reaction initiated and starts to produce **C**.

Age: In the second stage, the plungers push the sample (reaction mixture) through a delay line to the second mixer. The sample ages (reacts) as it travels through the delay line until it reaches the second mixer where it is quenched.

Quench: As the sample passes through the second mixer it is mixed with the quencher **Q** which stops the reaction. The resulting solution is then collected for analysis of the quantity of **C** produced during the experiment.

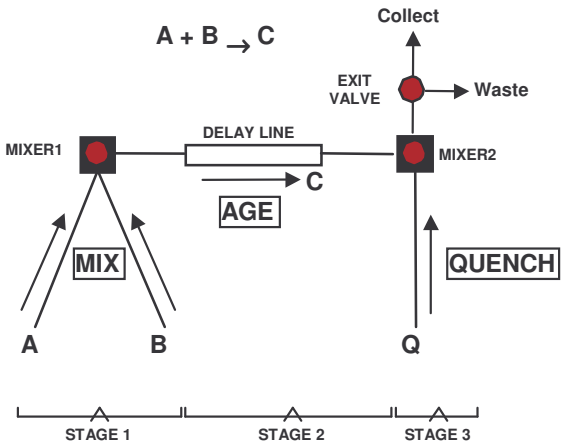


Figure 100 – Quenched-Flow Experiment Scheme

The age of the sample (t_{age}) is the total time between the start of the reaction in and the moment it is quenched. The age will depend on the total flow rate through the delay line and the intermixer volume as described in section 9.2. It can also depend on the duration of a pause in the flow that allows the sample to age for long times (see section 13.2.3). A quenched-flow study will consist of numerous experiments where t_{age} is varied for each experiment. At the end of the study, a kinetic trace can be constructed by plotting t_{age} vs. the results of each sample analysis.

13.2 AGEING METHODS

Samples can be aged with the SFM using two different methods: the continuous flow method or the interrupted flow method.

13.2.1 Continuous flow method

In the continuous flow method, the sample flow is continuous from the start of the reaction through sample collection. The sample age is dependent only on the intermixer volume and the total flow rate through the intermixer volume. In this case:

$$t_{age} = \frac{\text{intermixer volume}}{\text{flow rate through intermixer volume}}$$

The sample age can then be adjusted by changing the intermixer volume or the flow rate through the intermixer volume. The intermixer volume is modified by introducing delay lines of different volumes (section 9.1). The flow rate through the intermixer volume is modified by changing the flow rate of the syringes in the driving sequence. The use of stepping motors in the SFM allows a large range of syringe flow rates to be programmed and many t_{age} values achieved with minimal changes of delay lines. In addition, unlike pneumatic based systems, the flow rates are independent of viscosity and temperature.

An example driving sequence using the continuous flow method is shown in Figure 101. It should be noted that the experiment is performed only in Phase 2 of the driving sequence. This phase encompasses all stages (mixing, ageing, and quenching) of the experiment. DL2 is Delay line n°3; the intermixer volume for mixers 2 and 3 is then 116.7 µl and the total flow through the intermixer volume is 4 ml/s (syr2 +syr3) which indicates:

$$t_{age} = \frac{116.7\mu l}{4ml/s} = 29.2\ ms$$

The continuous flow method is generally used to study reactions from 1 to ~200ms. It is generally the most economic ageing method with respect to sample consumption within this time range. To get correct mixing, a minimum flow rate of 1 ml/s through each mixer is required.

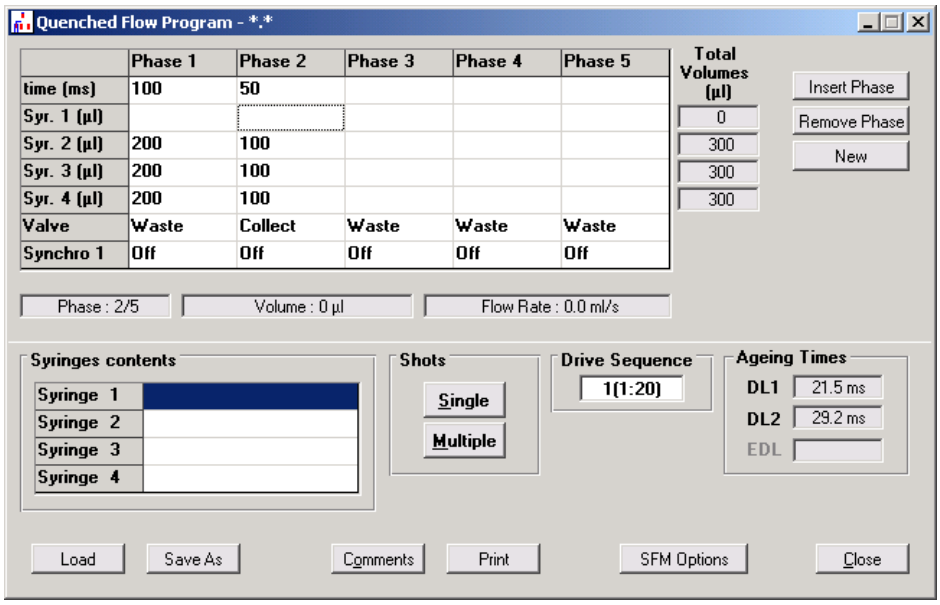


Figure 101: example of continuous flow experiment

13.2.2 *Pulsed flow method*

The pulsed flow method consists of making a few pulses to fill the delay line in order to get a turbulence flow in the delay line, followed by an incubation time, and then mixing with the quencher. Under these conditions, t_{age} depends on the number of pulses to fill the delay line, flow rate in the delay line, and incubation time.

$$t_{age} = \frac{n \times (\text{volume of the pulse})}{\text{Flow DL}} + t_{inc} \times (n-1)$$

With**:**

n: number of pulse

Flow DL: flow rate in the delay line

t_{inc} : incubation time

With this method, we only need to enter a different incubation time to reach different ageing time.

The others parameters remain unchanged.

An example of a driving sequence using the pulsed method is shown in Figure 102.

For this example, the 17/Mix/190 delay lines are installed, 8 pulses of 27 µl are needed to fill the delay line num 190(216.6µl), and 1ml/s is the flow rate of the pulse in the delay line. To reach the point 286ms, the incubation time of 10ms is entered.

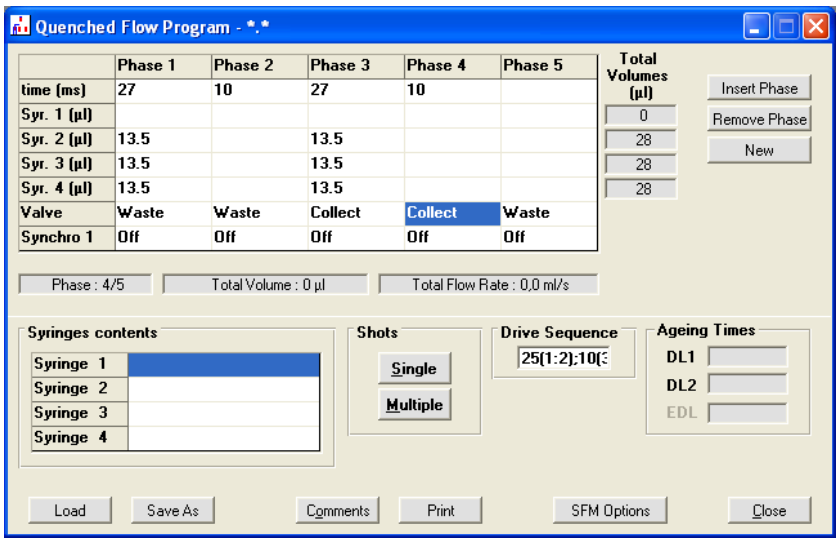


Figure 102 : example of pulsed flow experiment

Phase 1 and 2 correspond to the washing phases and are repeated 25 times. Phases 3 and 4 are the collecting phases, and they are repeated 10 times.

13.2.3 *Interrupted flow method*

In the interrupted flow method, the sample is transiently stored in the intermixer volume for a programmed incubation period before being mixed with the quencher. Under these conditions t_{age} depends on the intermixer volume, the total flow rate as the sample enters and exits the intermixer volume and the incubation period of the sample in the intermixer volume.

$$\mathbf{t_{age} = t_{flow} + t_{pause}}$$

$$t_{flow} = \frac{\text{Intermixer volume}}{\text{flow rate through intermixer volume}}$$

t_{pause} = Time sample is transiently stored in the intermixer volume

As with the continuous flow method, the intermixer volume and flow rates can be modified by introducing different delay lines and modifying syringe flow rates in Bio-Kine.

IMPORTANT: To obtain uniform ageing of the sample, the flow rate of the sample entering the delay line must always equal the flow rate of the sample exiting the delay line!

An example driving sequence using the interrupted flow method is shown in Figure 103. The experiment is performed in Phases 1 – 4. In phase 1 the reactants are mixed, and the intermixer volume filled with sample. In Phase 2, the sample is allowed to age for 400 ms. In phase 3, the leading edge of the line is pushed to the waste to get rid of contamination from syringe 4 during phase 2(see section 14.4 for details). In phase 4, the sample is pushed out of the intermixer volume, quenched, and collected. Delay line n°5 is installed for the ageing. The intermixer volume for mixers 2 and 3 is then 216.9 µl, and the total flow through the intermixer volume upon sample entry and exit is 2 ml/s which indicates:

	(t_{flow})		(t_{pause})		
t_{age} =	**108.4 (=216.9/2)**	**+**	**400 ms**	**=**	**508.4 ms**

It is important to note that not all the sample can be recovered from the intermixer volume without contamination. This is because unwanted mixing occurs at each end of the intermixer volume by diffusion during the incubation period. The fraction of the sample that remains uncontaminated must be determined experimentally, and an example procedure is provided in section 14.4.

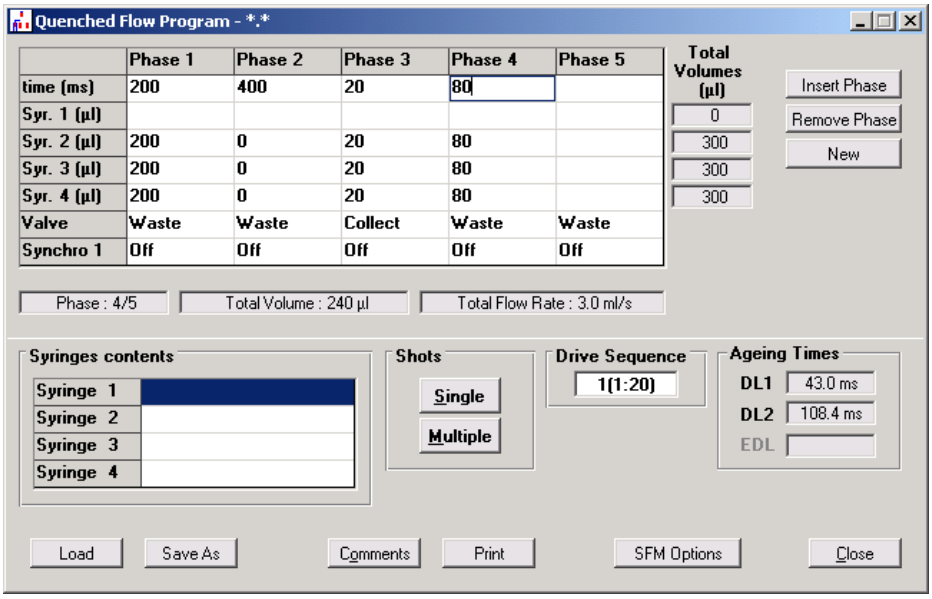


Figure 103 : example of interrupted flow experiment

The interrupted flow method allows samples to be aged for several 100 ms to several seconds or longer. It generally uses more sample than the continuous flow method. This is due to the fact that only a portion of uncontaminated sample can be recovered and sometimes necessitates multiple repetitions of the same experiment to achieve sufficient sample volume for analysis. Because the ending and leading edges need to be eliminated, delay lines 5 and 4 are generally used for this ageing method.

13.3 Double mixing experiment

Double mixing experiment can only be achieved with the SFM-400/Q. It is generally the combination of interrupted and continuous flow techniques. An example of such a sequence is given in Figure 104. In this example volumes of DL1 and DL2 are respectively 216.2 µl and 60.1 µl.

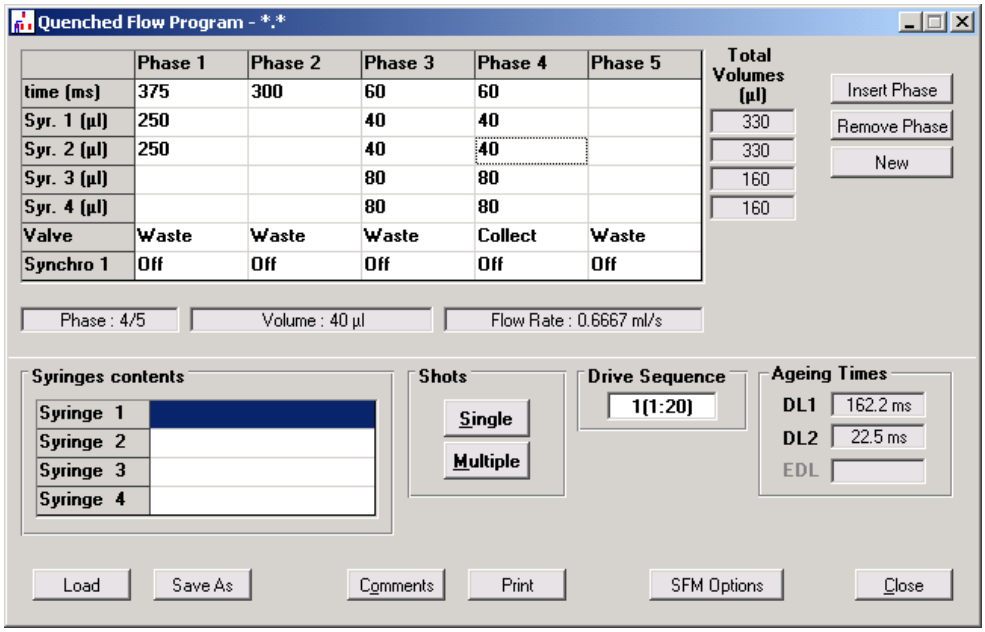


Figure 104 : double mixing sequence.

In the first phase, samples 1 and 2 are mixed, after which the solution is allowed to age in the first delay line (phase 2). After this 300 ms incubation, the solution is mixed with sample 3 in the second mixer and allowed to age 22.5 ms in the second delay line (using continuous mode). Finally, the solution is quenched by sample 4. The ageing times in DL1 and DL2 for the sequence proposed are 462.2 ms (162.2+300) and 22.5 ms respectively. It is just necessary to change the incubation time in phase two to vary the first ageing time and to keep the second one constant.

13.4 COLLECTION METHODS

Section 9.3 described how sample can be recovered from a quenched-flow experiment with the SFM. The sections below describe how sample collection is incorporated into a driving sequence.

13.4.1 Total liquid collection

If the total liquid collection method is chosen for sample recovery the exit valve position is set constant throughout the experiment. The exit valve should be set to **Waste** if the free-flow method is used and **Collect** if the pipette/syringe collection is used.

In general, total liquid collection will be used only with the continuous flow ageing method (section 13.2.1) and when large volumes (> 1 ml) of a sample need to be collected and contamination from previously sample can be neglected. In such situations, all stages (mix, age, and quench) of the experiments will occur in a single phase as shown in Figure 105.

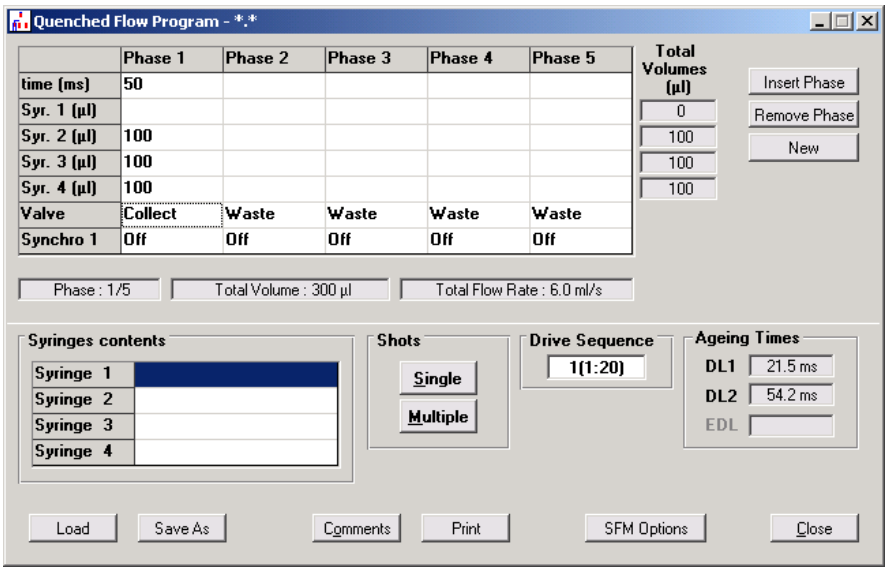


Figure 105: driving sequence for a total liquid collection

13.4.2 Partial liquid collection

Sample collection in experiments using the partial liquid collection method is divided into two parts: purge and collect.

Purge: Reactants are mixed, aged, and the reaction quenched, but the exit valve is set to **Waste** and the exiting liquid is not recovered. The purge serves to evacuate all old reaction mixtures from the SFM, wash the flow lines free of any contamination, and fill the flow lines with a new, uncontaminated sample.

Collect: The exit valve is set to **Collect**, and a new, uncontaminated sample is pushed from the SFM into a pipette or syringe.

The partial liquid collection method can be used with either the continuous or interrupted flow ageing method (section 13.2). Example driving sequences using the partial liquid collection method with continuous flow ageing and interrupted flow ageing are shown in Figure 106 and Figure 107 respectively.

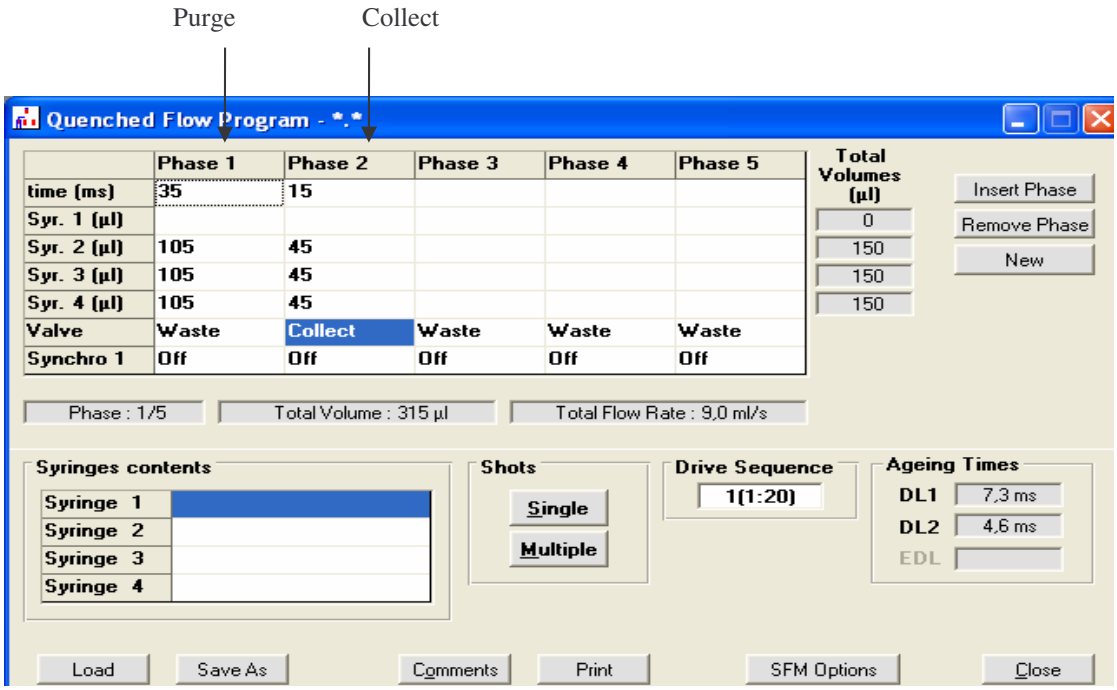


Figure 81: Partial liquid collection with continuous flow ageing

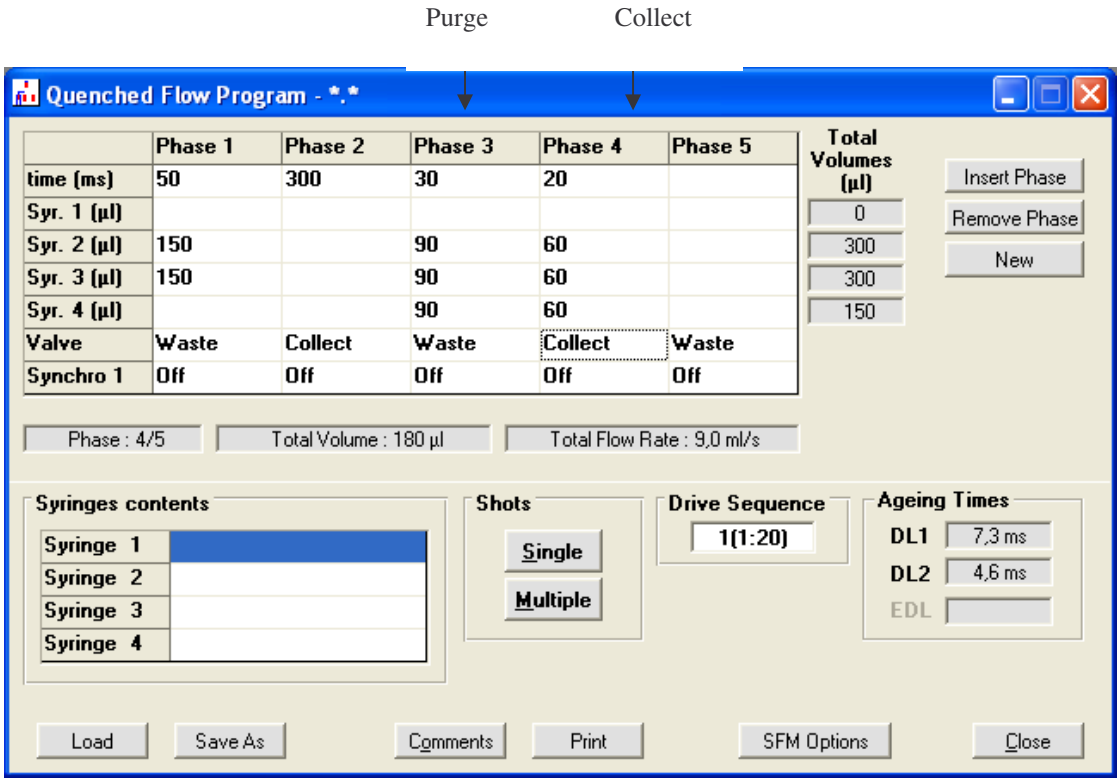


Figure 107 – Partial Liquid Collection with Interrupted Flow Ageing

IMPORTANT: The flow rate of the sample during the purge and collect steps must be Equal. If the flow rate is different during the two steps, the sample collected will not have the same age.

IMPORTANT: As noted in section 0, the purge volume should be a minimum of 3-5× the flow line volumes (section 9.2) to ensure only uncontaminated sample is collected. Larger volumes may be necessary, it is recommended that test experiments be performed to optimize the volume needed to minimize sample contamination. An example procedure to determine the purge volume needed is provided in section 14.3.

13.5 General Advice for Quenched-Flow Experiments

To achieve successful results from quenched-flow experiments and optimal performance of the SFM, it is imperative that the specifications of the SFM and its components be respected at all times. The specifications are provided in Table 1-SFM specifications. It is recommended that each experiment's driving sequence be carefully examined for compliance with the SFM specifications before execution.

It is strongly recommend that all driving sequences be tested with non-precious samples. Although such tests may be time-consuming, they maximize the experiment's success by ensuring that a majority of miscalculations and mistakes will be found and avoided.

The accuracy and precision of quenched-flow experiments depend on the quality of the sample collected from the SFM. Sample contamination can be minimized by optimizing the volume needed to wash all contamination from the SFM flow lines during a given experiment. This is best achieved by performing test experiments similar to that described in section 14 and adapting them as close as possible to true experimental conditions (temperature, viscosity, etc. . .).

As mentioned in sections 13.2.1 and 13.2.3, the continuous flow and interrupted flow ageing methods work best for ageing times of 1- ~200ms and ~200ms to several seconds respectively. These ranges are meant to be guidelines and not strict requirements. It is worthwhile to explore the application of both ageing methods to design an experiment which best economizes the use of reactants.

14 TEST REACTIONS IN QUENCHED-FLOW MODE

14.1 Alkaline Hydrolysis of 2,4-Dinitrophenyl Acetate (DNPA)

A complete description of the alkaline hydrolysis of 2,4-dinitrophenyl acetate (DNPA) can be found in: Gutfreund, H. (1969), *Methods in Enzymology*, 16, 229-249.

DNPA can be hydrolyzed by OH^- to 2,4-dinitrophenol (DNP). At 20℃ the reaction has a second order rate constant in water of 56 $M^{-1}s^{-1}$. Conditions can easily be set to make the concentration of OH^- sufficiently larger than that of DNPA so that the reaction occurs under pseudo first-order conditions with an apparent rate constant, k_{app}, of 56 $s^{-1} \times [OH^-]$ (NOTE: The $[OH^-]$ is the concentration of OH^- after mixing with DNPA). The reaction can be quenched at any time by the addition of excess acid and the amount of DNP produced determined by absorbance at 325nm. Figure 108 shows the absorbance spectrum of DNPA and DNP under various conditions of pH. It can be see that the absorbance spectrum of DNP changes with pH, but there is a clear isobestic point at 325nm.

These properties make the alkaline hydrolysis of DNPA a useful tool for the testing of a quenched-flow instrument. The reaction can also be followed by the stopped-flow technique, omitting the acid quench.

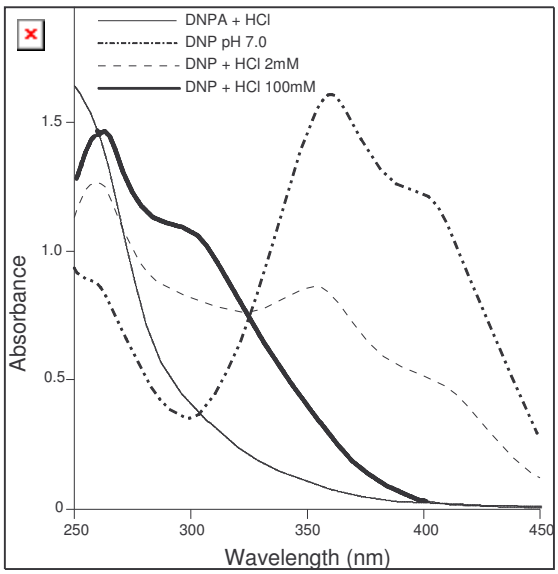


Figure 108 – DNPA/DNP Absorbance Spectra

Experimental Conditions: Syringe 1: Water

Syringe 2: 1 mM DNPA, 1%(v/v) DMSO, 2 mM HCl

Syringe 3: 1 M NaOH

Syringe 4: 2 M HCl

Delay Line 1: 17µl

Delay Line 2 190µl

Sample Preparation: Make 1 ml of 100 mM DNPA in fresh DMSO (22.6 mg DNPA/ml DMSO. The solution may turn slightly yellow as the DNPA dissolves. As the solution ages, the yellow color will intensify. For best results, it is recommended to use the freshest possible DMSO and prepare new samples each day).

1) Prepare a 2 M HCl solution by mixing 8.3 ml concentrated HCl with 50 ml of water.

2) Prepare a 1 M NaOH solution by dissolving 2g of NaOH in 50 ml of water.

3) Prepare the working DNPA solution by mixing

   49.45 ml water

   50 µl 2 M HCl

   500 µl 100 mM DNPA in DMSO

Driving Sequence: Various ageing times for the reaction are achieved by varying the intermixer volume (M2-M3) and the flow rate through the intermixer volume. The general format of the driving sequence is shown below and the delay lines and flow rates used are given in Table8.

PHASE	1	2
Time (ms)	T	T
S1: Water	-	-
S2: DNPA	300	300
S3: NaOH	300	300
S4: HCl	300	300
Exit Valve:	Waste	Collect

Table8 – DNPA Experiment Parameters

T (ms)	INTERMIXER VOLUME (M2-M3) FLOW RATE (ml/s)	t_{age} (ms)
50	12	18
100	6	36
150	4	54
200	3	72
300	2	108
450	1.333	162
600	1	216
1000	0.6	360

IMPORTANT: This reaction is very sensitive to contamination! The experiments must be performed from smallest t_{age} to the largest (least to most DNP produced) so that contamination of subsequent shots is kept to a minimum.

Three shots were performed for each t_{age}. The first shot was discarded, and the second and third shots kept for analysis. A t_{age} = 0 ms the sample was prepared by hand by mixing 300µl of the DNPA solution with 300 µl of water and 300 µl of 2 M HCl. A t_{age} = ∞ the sample was prepared by hand by mixing 300µl of the DNPA solution with 300 µl of 1 M NaOH and 300 µl of 2 M HCl

The absorbance of DNP at 325 nm was measured for each ageing time. The absorbance was measured for 500µl of each sample mixed with 500µl of water in a 1cm path length cuvette. The results were plotted against the ageing times as shown in Figure 109. The apparent first-order rate constant determined from Figure 109 is 28 s^{-1} which yields a second order rate constant of 56 $M^{-1}s^{-1}$ for a final [OH^-] of 0.5 M.

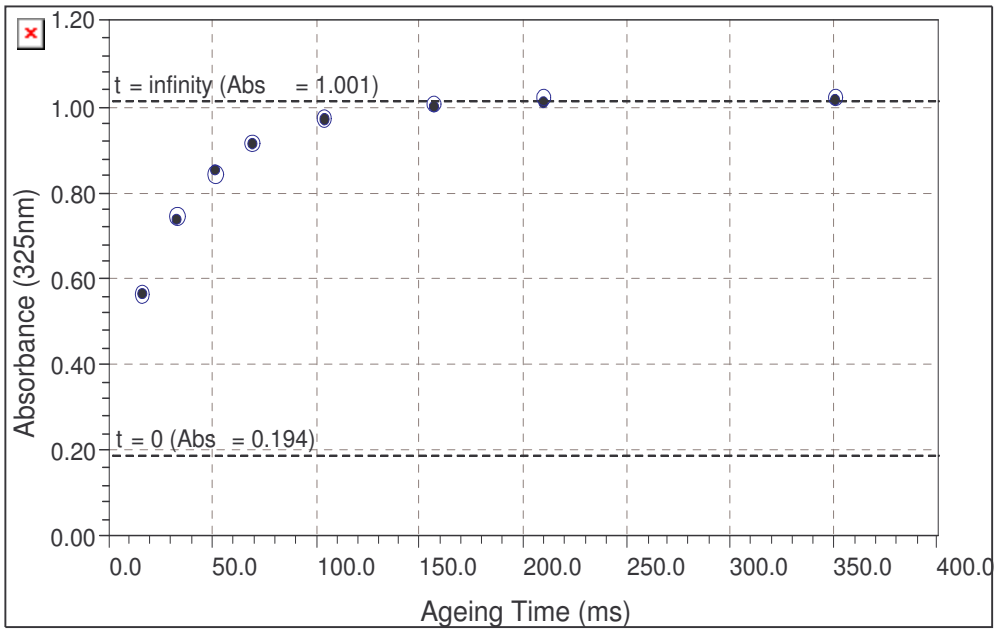


Figure 109 – DNPA Experiment Results

This experiment was done entirely using the continuous flow ageing method. The interrupted flow ageing method could also have been used for the longer time points with the same results.

14.2 Calculation of Hydrodynamic Volumes from Kinetic Data

As indicated in section 9.2, the volumes supplied in this manual are the mechanical volumes. The *hydrodynamical* volumes may vary slightly around these values and in some instances it may be necessary to determine the hydrodynamic intermixer volumes. The results of the DNPA experiment in the previous section can be used to determine the hydrodynamical intermixer volumes. A procedure for determining the hydrodynamical volume of Delay Line 2 is provided below.

1) Using the data from the DNPA experiment in the previous section, calculate the fraction of reaction complete (**Y**) for each ageing time according to the equation:

$$Y = \frac{A(t) - A(0)}{A(\infty) - A(0)}$$

Where A(t) is the absorbance at 325nm at ageing time t, A(0) is the absorbance at t_{age} = 0 and A(∞) is the absorbance at t_{age} = ∞.

2) Using **Y** from step 1 above and the pseudo first-order rate constant measured, calculate the actual ageing time **Ta** for each point from:

$$Ta = 1000 \times \frac{Ln(C(0)) - Ln(C(t))}{k}$$

$$Ta = 1000 \times \frac{Ln(0.5) - Ln(0.5 \times (1 - y))}{k}$$

Where C(0) is the DNPA concentration at t_{age} = 0 (0.5 mM for the experiment in the previous section), and C(t) is the DNPA concentration at time t and k is in s^{-1}. The units of **Ta** are ms.

3) The hydrodynamical intermixer volume (M2-M3) can then be calculated from

$$volume = \frac{F}{Ta}$$

Where **F** is the total flow through the intermixer volume in µl/s. Since the hydrodynamical intermixer volume can be calculated for each **Ta**, the mean and standard deviation of the volume can easily be determined.

14.3 Washing Efficiency

To obtain the best results from quenched-flow experiments, it is necessary to minimize sample contamination. The most common source of sample contamination is due to an inefficient washing phase of previous reacted sample coming from the flow lines and intermixer volumes before sample collection. The simplest method of determining the volume needed to create an efficient wash (or purge) phase between the flow lines and intermixer volumes is to carry out multiple experiments with increasing purge volumes until there are no differences in experimental results. In many cases this is impractical due to the cost or availability of one or more experimental components.

The procedure below uses the DNPA experiment (14.1) to provide an example of how to determine the volume needed to efficiently wash (or purge) the flow lines and intermixer volumes using inexpensive and readily available materials. In this example, the needed volume for intermixer volume M2-M3 is determined. The procedure can be adapted as needed to various experimental conditions and systems.

Experimental Conditions:		
	Syringe 1:	Water
	Syringe 2:	1 mM DNPA, 1%(v/v) DMSO, 2 mM HCl
	Syringe 3:	1 M NaOH
	Syringe 4:	2 M HCl
	Delay Line 1:	None
	Delay Line 2:	N°1 (17µl)

Driving Sequence:

PHASE	1	2	3
Time (ms)	T	0	30
S1: Water	-		-
S2: DNPA	V		60
S3: NaOH	V		60
S4: HCl	V		60
Exit Valve:	Waste	Waste	Collect

V is varying by adding small increments from 0 to V (wash) where the results indicate that a complete washing phase is achieved into the intermixer volume (volume between Mixer 2 and mixer 3). T is adapted to maintain a total flow rate in phase 1 equal to the one in phase 3.

The results of the experiments performed with no delay lines installed are shown in Figure 110. It should be remembered that the purge volume is the volume flowing into intermixer volume M2-M3 and equal to S2 + S3 = (2 × V). It can be clearly seen that the reaction products collected after using a purge volume of zero are completely contaminated by the previous reaction mixture that remained in the instrument before sample collection. The results in Figure 110 indicate that a minimum purge volume of 25-30µl is necessary to wash most of the contamination that come from previous reaction (i.e. volume in the intermixer) in a way to avoid any cross contamination.

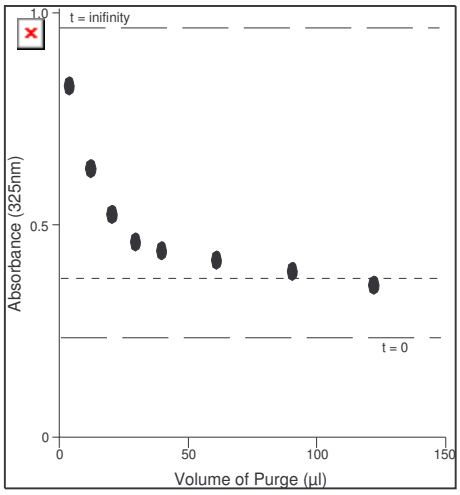


Figure 110 – Washing Efficiency

14.4 Recovery of Uncontaminated Material in Intermixer Volume

In the interrupted mode, the reaction mixture is transiently stored in the intermixer volume. During this incubation period, unwanted mixing occurs at both ends of the intermixer volume so that only a fraction of the mixture contained therein can be recovered. The experiment described below is intended to give an estimate of the uncontaminated fraction that can be recovered. The procedure provided in the experiment can easily be adapted to various incubation times and experimental conditions.

Experimental Conditions:

Syringe 1:	Water
Syringe 2:	1 mM DNPA, 1%(v/v) DMSO, 2 mM HCl
Syringe 3:	1 M NaOH
Syringe 4:	2 M HCl
Delay Line 1:	17 µl
Delay Line 2:	190 µl

Driving Sequence:

PHASE	1	2	3	4	5	6
Time (ms)	50	50	3000	0	T	20
S1: Water	-	-	-	-	-	-
S2: DNPA	150	-	-	-	V	30
S3: NaOH	150	-	-	-	V	30
S4: HCl	-	150	-	-	V	30
Exit Valve:	Waste	Waste	Waste	Waste	Waste	Collect

V is varied from 0 in small increments until ~2× the intermixer volume. T is varied so that the total flow rate in Phase 5 is equal to that in Phase 6.

This experiment is designed to test the intermixer volume M2-M3. In Phase 1, DNPA and NaOH are pushed through the delay line and then to waste. The second phase is used to wash the last mixer with HCl. The reaction mixture is then allowed to age for several seconds in the delay line (Phase 3). Phase 5 corresponds to the purge of the delay line, the solution being pushed and evacuated to waste. The purge volume is again equal to S2+S3 (2 x V). After the purge, 60µl of the reaction mixture is collected and measured.

The results of this test are shown as a function of purge volume in **MERGEFORMAT 111**. Due to the long ageing time in Phase 3, the solution collected in the last phase should correspond to the full reaction (t = ∞). Contamination on the leading edge of the liquid column contained in the delay line is observed when the volume of the purge is zero. Contamination on the trailing edge is observed for overly large purge volumes, when the fresh reactants pushing the liquid column are collected.

These results in **MERGEFORMAT 111** show that, for a delay line of 190µl (216.9µl nominal volume), the first 20 to 30µl and the last 30 to 40µl are contaminated and should be discarded.

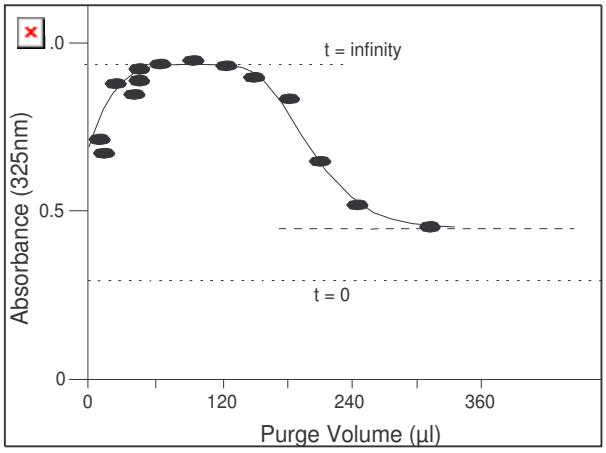


Figure 111 MERGEFORMAT 111 – Recovery of Uncontaminated Material