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Stopped-Flow Circular Dichroism Kinetics

To monitor the change in circular dichroism over time at a single wavelength, set the KSHU up with a circular dichroism PMT detector in the transmission port, and perform the following steps.

1. Select Circular Dichroism mode
2. Select Kinetics mode, set timebase and number of points
3. Select monochromator wavelength and bandwidth
4. Zero the detector
5. Load the reactants
6. Acquire data

These steps are described in detail below.

1) On the **Signal** panel of the Pro-Data main window select **Circular Dichroism** from the drop down list.

If you wish to record the absorbance simultaneously with the circular dichroism, tick the **Absorbance** box. In this mode the absorbance will not be measured directly, but will be derived from the known change in gain in the Chirascan electronics. The fluorescence can also be recorded by ticking the **Fluorescence** box, but this requires a second (fluorescence) detector.

2) Select **Kinetic**s mode on the **Sequencer** panel.

Select **External** from the drop down list on the **Trigger** panel to activate stopped-flow operation.

On the **Timebase** panel choose an acquisition time based on the expected timescale of the reaction. **Points** should normally be set to a value between 400 and 1000 data points.

3) On the **Monochromator** panel enter the selected wavelength and bandwidth. A bandwidth of 1.0 nm is suitable for most circular dichroism work.
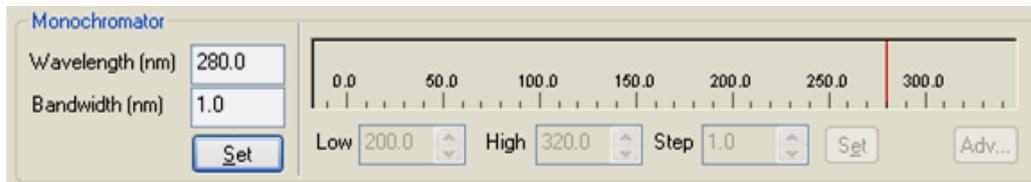
4) Fill the optical cell with a suitable reference material, usually water. Click the **Baseline** button on the **Signal** panel to zero the detector.

5) Load the reactants into the drive syringes.

6) Ensure that the drive valves are in the **Drive** (front) position and click the **Acquire** button to perform a drive and collect the data. Repeat until consistent results are obtained, normally by about the fourth or fifth drive.

Stopped-Flow Absorbance Kinetics

To monitor the change in absorbance over time at a single wavelength, set the KSHU up with a circular dichroism detector in the transmission port, and perform the following steps.

1. Select Absorbance mode
2. Select Kinetics mode, set timebase and number of points
3. Select monochromator wavelength and bandwidth
4. Zero the detector
5. Load the reactants
6. Acquire data

1) On the **Signal** panel of the Pro-Data main window select **Absorbance** from the drop down list. Ensure that **AutoHV** is ticked.
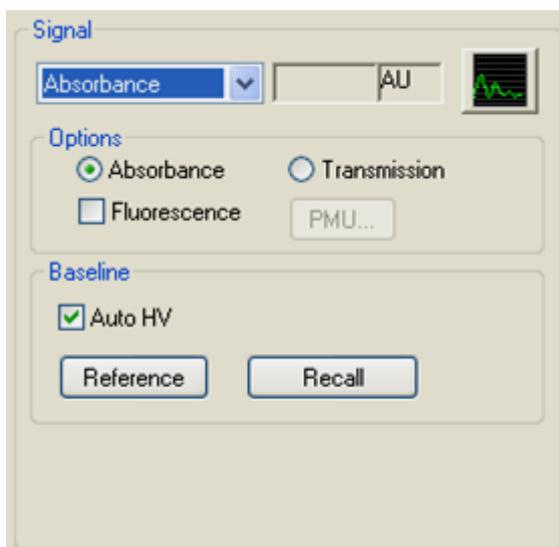
The fluorescence can be recorded simultaneously, but this requires a second (fluorescence) detector.

2) Select **Kinetics** mode on the **Sequencer** panel.

Select **External** from the drop down list on the **Trigger** panel to activate stopped-flow operation.

On the **Timebase** panel choose an acquisition time based on the expected timescale of the reaction. **Points** should normally be set to a value between 400 and 1000 data points.

3) On the **Monochromator** panel enter the selected wavelength and bandwidth. A bandwidth of 1.0 nm is suitable for most absorbance work.
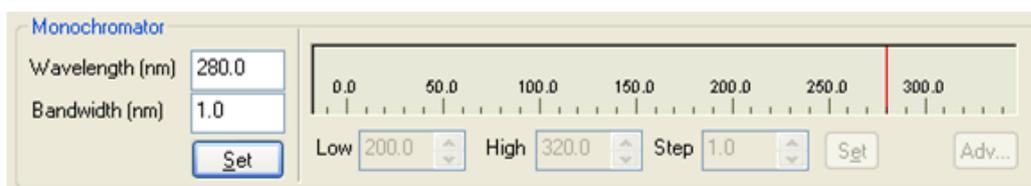
4) Fill the optical cell with a suitable reference material. This is usually water or other solvent, although particularly when looking for small changes, it may be convenient to use one of the reactants (normally colourless), or the chemical endpoint of the reaction. Click the **Baseline** button on the **Signal** panel to zero the detector.

5) Load the reactants into the drive syringes.

6) Ensure that the drive valves are in the **Drive** (front) position and click the **Acquire** button to perform a drive and collect the data. Repeat until consistent results are obtained, normally by about the fourth or fifth drive.

If water was used as the reference, the absorbance measurements will be in units of absolute absorbance. If something other than water was used, the units will be relative absorbance.

Stopped-Flow Fluorescence Kinetics

To monitor the change in total fluorescence over time, set the KSHU up with a fluorescence detector in the primary fluorescence port, and perform the following steps. If the change in fluorescence at a single wavelength is required, a second monochromator is needed.

1. Select an appropriate path length.
2. Install an appropriate cut-off filter.
3. Select Fluorescence mode
4. Select Kinetics mode, set timebase and points
5. Select excitation wavelength and bandwidth
6. Load sample
7. Set detector voltage
8. Acquire data

1) For fluorescence measurements, the choice of excitation pathlength will depend on the absorption and concentration of the sample under investigation, taking into account the risk of inner filtering effects. Inner filtering is the partial blockage of the excitation light by absorption. If there is a significant change in the absorption of the sample during the course of the experiment, the proportion of 'blocked' excitation light will also change. There are therefore two sources for any observed change in the fluorescence emission signal: the direct change produced by the changing concentrations of the reactants, and the effect of blocking. The usual procedures for obtaining kinetic information from the fluorescence signal will then be invalid. A 2 mm pathlength is therefore normally selected for fluorescence measurements.

2) Select a long pass cut-off filter appropriate for the fluorophore being used. The filter should block scattered light of the excitation wavelength but pass fluorescent emission. It should be installed between the fluorescence detector and the end window of the cell. The filter holder is accessed by removing the fluorescence detector from the KSHU.

3) On the signal panel of the Pro-Data main window select **Fluorescence** from the drop down list.
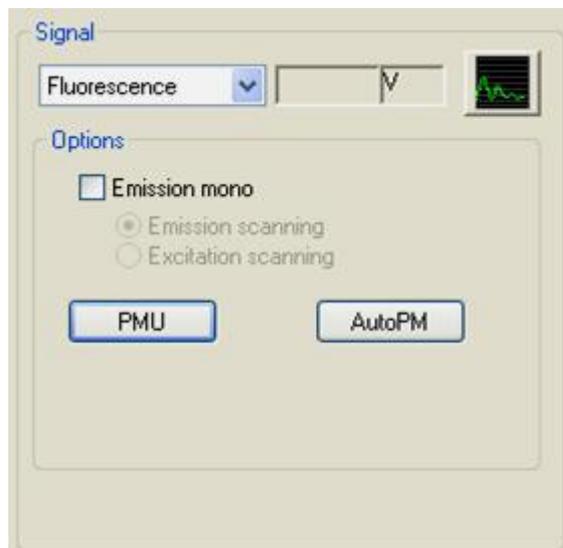
4) Select **Kinetics** mode on the **Sequencer** panel.

Select **External** from the drop down list in the **Trigger** panel to activate stopped-flow operation.

On the **Timebase** panel choose an acquisition time based on the expected timescale of the reaction. **Points** should normally be set to a value between 400 and 1000 data points.

5) On the **Monochromator** panel enter the selected excitation wavelength and bandwidth. The bandwidth used for fluorescence work is usually wider than that used for absorbance: 4.0 nm is typical.
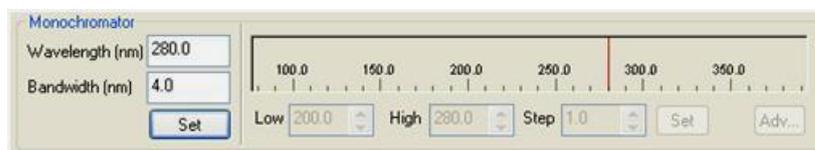
6) Load the reactants into the drive syringes. Ensure that the drive valves are in the **Drive** (front) position and press the **Drive** button on the **KSHU** panel to push sample through the flow circuit without collecting data. Repeat three or four times to ensure that the observation chamber contains the reactant mixture.

7) Click the **AutoPM** button on the **Signal** panel to set the detector supply voltage. This will by default target an 8V fluorescence signal from the endpoint of the reaction mixture currently in the optical cell. For most applications this will ensure that the signal does not exceed the detector operating range. Note that some fluorescence quenching reactions may require a lower detector supply voltage in order to measure a signal within the detector operating range. In such cases a lower supply voltage should be entered manually by the user.

8) Press **Acquire** to run a drive with data acquisition.

Stopped-Flow Circular Dichroism Spectra-Kinetics (Multi-Wavelength CD Kinetics)

In it often more informative to monitor the change in circular dichroism over a range of wavelengths, rather than at a single wavelength, although it is also more time consuming. Set the KSHU up with a circular dichroism detector in the transmission port, and perform the following steps.

1. Select Circular Dichroism mode
2. Select Spectra-Kinetics mode, set timebase and points
3. Select wavelength range, bandwidth and step size
4. Acquire baseline measurement
5. Load sample
6. Prime flow circuit
7. Acquire data

1)   On the **Signal** panel of the Pro-Data main window select **Circular Dichroism** from the drop down list.

If you wish to record the absorbance spectrum simultaneously with the circular dichroism spectrum, tick the **Absorbance** box. In this mode the absorbance will not be measured directly, but will be derived from the known change in gain in the Chirascan electronics. The fluorescence spectrum can also be recorded by ticking the **Fluorescence** box, but this requires a second (fluorescence) detector.

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2) Select **Spectra-Kinetics** mode on the **Sequencer** panel.

Select **External** from the drop down list in the **Trigger** panel to activate stopped-flow operation.

On the **Timebase** panel choose an acquisition time based on the expected timescale of the reaction. **Points** should normally be set to a value between 400 and 1000 data points.

3) On the **Monochromator** panel, select the wavelength range, bandwidth and step size. A bandwidth of 1.0 nm is suitable for most CD work. Remember that the smaller the step size, the more drives will be performed, the longer the experiment will take and the more sample will be used. Note that the monochromator always moves from a high to a low wavelength when scanning.

4) Fill the optical cell with a suitable reference material, usually water, and click the **Baseline** button to collect the wavelength dependent reference data. The monochromator will move to each required wavelength and apply an appropriate HV to the detector. This will be re-applied when running your samples and a spectrum derived.

5) Load the reactants into the drive syringes.

6) Ensure that the drive valves are in the **Drive** (front) position and click the **Drive** button on the **KSHU** panel four or five times in succession to perform a series of drives without data acquisition. This will ensure that the flow circuit is primed with fresh reagents.

Refill the drive syringes from the reservoir syringes.

7) Click the **Acquire** button to run the experiment with data acquisition. Note that spectra-kinetics data are acquired from an automated series of stopped-flow drives, and the reactant volume remaining in the drive syringes should be monitored during the course of the reaction. The acquisition can be paused and resumed from the acquisition panel if an interruption to re-fill the syringes is needed.

Stopped-Flow Absorbance Spectra-Kinetics (Multi-Wavelength Absorbance Kinetics)

In it often more informative to monitor the change in absorbance over a range of wavelengths, rather than at a single wavelength, although it is also more time consuming. Set the KSHU up with a circular dichroism detector in the transmission port, and perform the following steps.

1. Select Absorbance mode
2. Select Spectra-Kinetics mode, set timebase and points
3. Select wavelength range, bandwidth and step size
4. Acquire baseline measurement
5. Load sample
6. Prime flow circuit
7. Acquire data

1) On the **Signal** panel of the Pro-Data main window select **Absorbance** from the drop down list. Ensure that **AutoHV** is ticked.
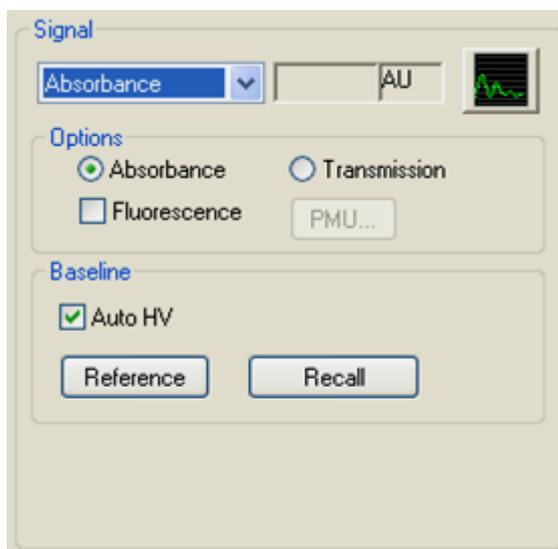
The fluorescence spectrum can be recorded simultaneously, but this requires a second (fluorescence) detector.

2) Select **Spectra-Kinetics** mode on the **Sequencer** panel.
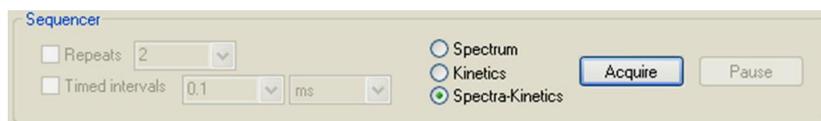
Select **External** from the drop down list in the **Trigger** panel to activate stopped-flow operation.

On the **Timebase** panel choose an acquisition time based on the expected timescale of the reaction. **Points** should normally be set to a value between 400 and 1000 data points.

3) On the **Monochromator** panel, select the wavelength range, bandwidth and step size. A bandwidth of 1.0 nm is suitable for most absorbance work. Remember that the smaller the step size, the more drives will be performed, the longer the experiment will take and the more sample will be used. Note that the monochromator always moves from a high to a low wavelength when scanning.

4) Fill the optical cell with a suitable reference material, usually water, and click the **Baseline** button to collect the wavelength dependent reference data. The monochromator will move to each required wavelength and apply an appropriate HV to the detector. This will be re-applied when running your samples and a spectrum derived.

5) Load the reactants into the drive syringes.

6) Ensure that the drive valves are in the **Drive** (front) position and click the **Drive** button on the **KSHU** panel four or five times in succession to perform a series of drives without data acquisition. This will ensure that the flow circuit is primed with fresh reactants.
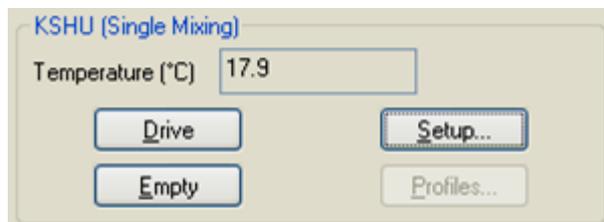
Refill the drive syringes from the reservoir syringes.

7) Click on the **Acquire** button to start the data acquisition. Note that spectra-kinetic data are acquired from an automated series of stopped-flow drives, and the reactant volume remaining in the drive syringes should be monitored during the course of the reaction. The acquisition can be paused and resumed from the acquisition panel if an interruption to re-fill the syringes is needed.

Sequential-Mixing Stopped-Flow

The sequential-mixing experiment employs a double pneumatic drive. In the first (Pre-Mix) drive, the right hand (sequential-mix) ram fires, and the contents of the two right hand drive syringes (A and B) are mixed in an ageing loop. After a user defined ageing period (from 0.010s to 1000s) the second (Flush) drive, in which the left hand (single-mix) ram fires, the contents of the ageing loop are driven from the loop by the buffer in drive syringe F, and mixed with the contents of syringe C in the optical cell. The reaction in the cell is then monitored spectroscopically in the usual way. To set up the SF.3 for sequential-mixing operation and run an experiment, set up the KSHU detectors and software as required, and then perform the following steps.

1. Fit the half-cylinder spacer under the single-mix cylinder
2. Fit the brake assembly and stop syringe
3. Drain the thermostat bath
4. Fit reservoir and drive syringes to the A and B positions
5. Configure the flow circuit for sequential mixing
6. Adjust the pre-mix ram drive volume
7. Adjust the total drive volume
8. Flush the flow circuit
9. Check the drive profiles
10. Load reactants
11. Acquire data

1) Fit the half-cylinder spacer under the left hand (single-mix) ram to reduce the travel in the C and F syringe plungers.
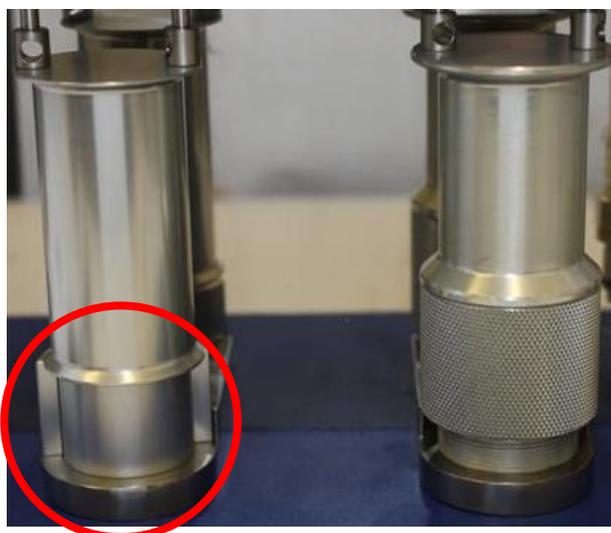
2) Fit the brake assembly and stop syringe to the AutoStop Assembly. A 2.5 ml stop syringe is recommended for sequential-mixing operation.

3) If the waterbath is full, remove the bleed screw at the top of the cell block to allow the fluid to drain out. Remove the six screws on the front of the thermostat bath to allow the cover to be opened.

4) Install drive syringes in the A and B positions. 1 ml drive syringes are recommended for sequential mixing.
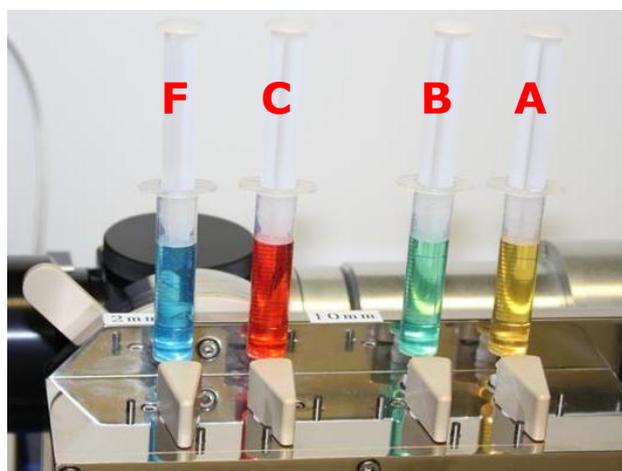
5) Set the flow circuit up as shown in the diagram below, which is colour coded for clarity.
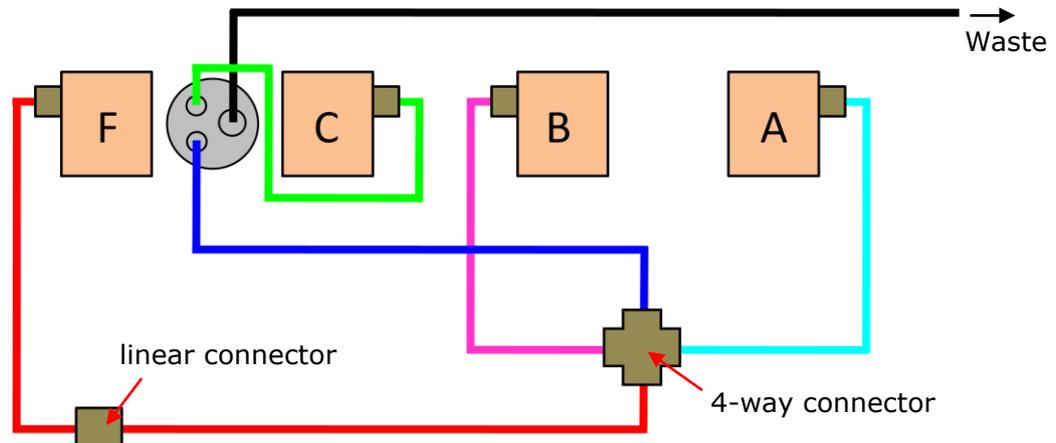
Drive valve A (cyan) connects to an arm of the 4-way connector, and drive valve B (magenta) to the opposite arm. The ageing loop (blue) connects from a third arm to the pressure plate (in single-mix set up this tubing connects to drive valve F via the straight connector). Drive valve C (green) connects directly to the pressure plate (this connection is unchanged from the single-mix set up). Drive valve F (red) connects to the fourth arm of the 4-way connector via the straight connector). The connection from the pressure plate to waste is shown in black, and is unchanged form the single-mix set-up.

When the connections have been made, leak test the flow circuit and replace the front of the waterbath and the bleed screw.

6) With the stop valve control knob set to the **Waste** (front) position, set the adjuster on the right hand (sequential-mix) drive ram to the zero position by rotating fully clockwise, i.e. the front moving from left to right, until there is no further travel. Set the adjuster three and a quarter rotations anticlockwise (counterclockwise). This produces a travel of 3.25 mm in the A and B drive syringes, equivalent to a total first drive volume of about 220 µl if 1 ml drive syringes are used.

7) Rotate the volume adjuster on the Auto-Stop assembly fully anticlockwise (counterclockwise), i.e. front edge moving from right to left, until there is no further travel, then rotate it four and three quarter rotations clockwise. This produces a total drive volume of approximately 400 µl for a 2.5 ml stop syringe, and therefore a second drive volume of about 180 µl.

8) Set the drive rams to their lowest positions. Fit reservoir syringes filled with distilled water or other suitable solvent to the A, B, C and F Luer fittings (5 ml or 10 ml plastic disposable syringes are recommended), and fill the drive syringes.

Set the A, B and C drive valves to the **Load** (side) position, and the F drive valve to the drive (front) position, then force the water from the drive syringe, through the flow circuit and into the stop syringe by manually raising the F drive syringe plunger. Empty the stop syringe by clicking the **Empty** button on the **KSHU** panel, and continue to flush until the drive syringe is empty. Flush from the other three drive syringes in the same manner.

Fill all four drive syringes with water and set the drive valves to the **Drive** positions.

Enable the sequential mixing mode in the Pro-Data control software by clicking the **Setup** button on the **KSHU** panel, going to the **Sequential** tab, and ticking the **Enable** box. Set the required delay time (i.e. the time between the two drives) and click **Apply**.
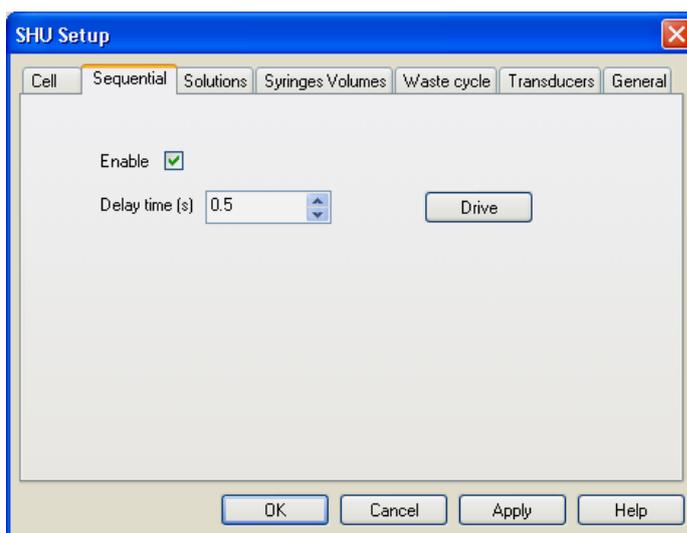
The **KSHU** panel on the main window will indicate that the sequential mixing mode is activated and the delay time currently set.

9) Run a drive by clicking the **Drive** button on the **KSHU** panel. Check the drive volumes by clicking the **Profiles** button on the **KSHU** panel. Drive 1 Volume should be 220 ± 10 µL, Drive 2 volume should be 180 ± 10 µL. Make any necessary volume adjustments.
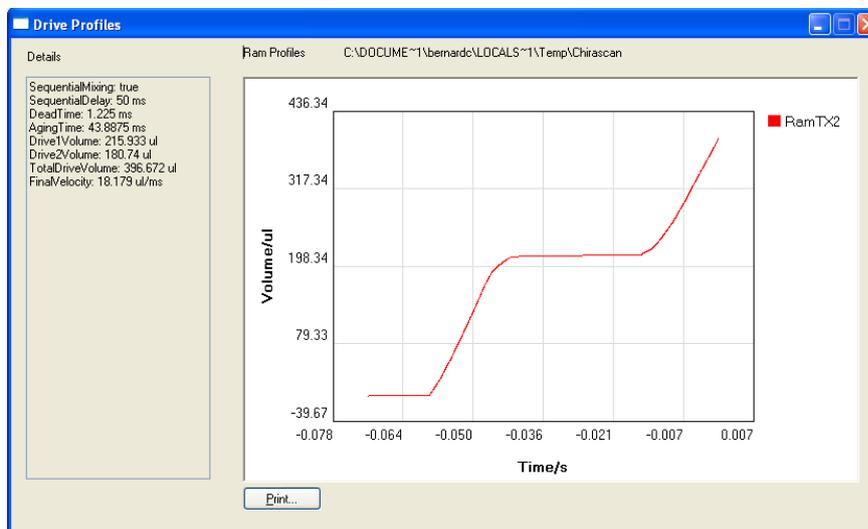
Repeat until the correct volumes are obtained reproducibly.

10) Refill the reservoir and drive syringes with the appropriate fluids. Reactants A, B and C are loaded into drive syringes A, B and C respectively, a neutral buffer is loaded into drive syringe F. Ensure that the rams are in their lowest positions, the drive syringes plungers are in contact with the rams, and the drive valves are all set to the **Drive** (front) positions.

11) Run a drive with data acquisition by clicking the **Acquire** button. If a repeat drive is required, reload the drive syringes and ensure that that the rams are in their lowest positions, the drive syringes plungers are in contact with the rams, and the drive valves are all set to the **Drive** (front) positions before running the drive.