User's Guide

RM Synthesizer

Version 1.02

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WARNINGS / INFORMATION

When the system contains alkane gas the system should be left turned on since an internal fan constantly circulates the air in the instrument case to prevent possible build-up of flammable gases.

The maximum pressure rating of this device is 1 kbar (14,500 psi). Use of the system above this pressure can result in component failure and possible injury to the user.

The system has been leak tested to the maximum pressure, however when using gases, especially expensive deuterated reagents it is prudent to not leave gases loaded in the system for extended periods of time. It is good practice to retract the gases from the RM Synthesizer back into the syringe pump, and then push it back into the gas cylinder.

The MCCS seal is single use only. Reuse of the seal or undertaking multiple displacement cycles of the piston at high pressure can cause the seal to fragment leading to the loss of pressure tolerance or causing the piston to be jammed in the cap or mixing chamber.

Do not open the HP ALKANE TO BOOSTER valve on the RM Synthesizer if the reservoir is not filled with liquid CO_2. This could displace the internal separator of the gas booster to an unknown position. Thus it is very important that the separator be reset to the position closest to the alkane inlet on the gas booster (see Figure 6) for proper function and transfer of samples to the NMR tube under pressure.

ITEMS INCLUDED WITH THE RM SYNTHESIZER

Below is the list of components that are supplied with the RM Synthesizer to facilitate setup. This list includes part numbers for possible replacement sources where appropriate.

Quantity	*Description*	*Source*
1	Elpac Power Systems 12V, 0.5A auto ranging power supply	
1	AC power cord	
30 ft.	1/16" O.D. x 0.03" I.D. stainless steel tubing (1 – 10 ft. coil, 1 – 20 ft. coil)	High Pressure Equipment Company P/N 15-9A1-030
16	15,000 psi glands – ¼"-28 threads	High Pressure Equipment Company P/N 15-2AM1
16	15,000 psi sleeves	High Pressure Equipment Company P/N 15-2A1
4	1L Pressure resistant bottles	Ace Glass P/N 5557-20
4	PTFE 3-hole GL-45 caps	VICI P/N JR-9000-0001
1	PEEK Cross w/fittings – ¼"-28 threads	VICI P/N CXMPK
3 packs	Cheminert glands and collars – ¼"-28 threads; 5/pack	VICI P/N CFL-1N
1	PEEK plug – ¼"-28 threads	VICI P/N CPPK
3	PEEK shut-off valves – ¼"-28 threads	Cole-Parmer P/N 02014-00
1	PEEK check valve – ¼"-28 threads	Cole-Parmer P/N 01355-26
12 ft	1/16" O.D. polyethylene tubing	Various sources
1	5/16" x 1/4" wrench	
1	3/4" wrench	
1	3/16" hex driver	
200	MCCS mixing chamber cap seals (primary)	Daedalus
10	WS01 mixing chamber window seals (spares)	Daedalus
2	Stir bar	
	North America only	
1	Nitrogen gas regulator; 150 psi max. out	
1	Tescom Extreme Pressure Regulator; 6000 psi max in; 2,500 psi max out	
1	0-3000 psi brass gauge	
1	0-3000 psi brass gauge	
1	CGA 320 brass nipple	
1	CGA 320 brass nut	
1	HiP 15-21AF1NMB adapter	
1	HiP 15-21AF1NMC adapter	

ITEMS TO BE SUPPLIED BY THE END-USER

Some additional tools may be required to complete the setup of the instrument.

Metal tubing cutter: A 1/16” metal tubing cutter and deburring tool will be necessary to cut sections of tubing for making connections between the syringe pump and RM Synthesizer and connections to the gas cylinders.

Teflon tape: This may be required when making attaching gas cylinder regulators.

The gas cylinder requirements are outlined below.

N_2 gas cylinder: Standard nitrogen gas cylinder. High purity is not necessary. For customers outside of North America a suitable regulator capable of outlet pressures of at least 120 psi – 150 psi (~8 bar - ~10 bar) is ideal.

CO_2 gas cylinder: Proper function of the RM Synthesizer requires that CO_2 be delivered in liquid form. A standard CO_2 gas cylinder will **NOT** work for this purpose. Many companies can apply 2,000 psi helium head pressure to a cylinder to allow the CO_2 to exit as a liquid. This requires the tank be equipped with an eductor or siphon tube so liquid CO_2 will be drawn from the bottom of the tank. High purity is not required. For customers outside North America a regulator capable of outlet pressures of at least 1,800 psi (124 bar) is preferred. Lower outlet pressures are possible, but it should not drop below the liquefaction pressure of CO_2 (~850psi @ RT).

Alkane gas cylinder: Typically the propane and ethane gas cylinders used with the RM Synthesizer do not have a regulator attached. The reason for this is the alkane is generally loaded into the cylinder at or near the liquefaction pressure. So in most cases the gas is withdrawn at maximum cylinder pressure hence no regulator is needed. However, it is likely an adapter will be required mate the 1/16” tubing from the RM Synthesizer to the cylinder outlet. Due to the variance in cylinder types Daedalus does not attempt to supply suitable adapters. Companies such as High Pressure Equipment Company (www.highpressure.com) can help identify a proper adapter once the outlet specifications for the particular alkane tank are identified.

INSTRUMENT SITE CONSIDERATIONS

The operation of this instrument involves the use of high pressure carbon dioxide and flammable gases. Quantities of these gases are vented from the system after sample preparation. For the safety of the user this instrument should be setup in an exhaust hood or other suitable ventilated environment.

The setup instructions below assume that the syringe pump to be used with the RM Synthesizer is the Daedalus Xtreme-10. The proposed arrangement of components is merely a guide. Other configurations are possible, and may be necessary depending on the space available. It is important to stress the necessity of using the equipment in a ventilated space for the safety of the user.

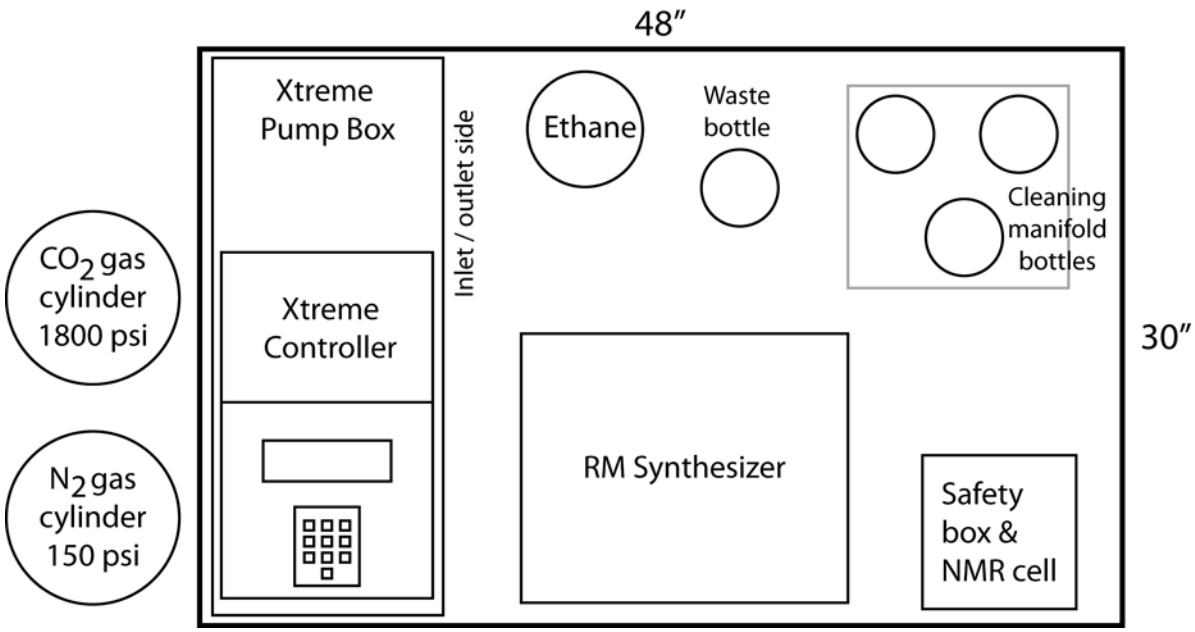

Figure 1: Possible bench-top setup

The bench-top setup in Figure 1 assumes that the instrument in installed under an appropriate exhaust hood. If that is not feasible, the instrument can be installed inside a fume hood, however many fume hoods have a smaller depth than the length of the Xtreme-10. This may require that the Xtreme-10 be placed towards the back as shown in Figure 2. The Xtreme Controller would have to be placed outside the hood, or mounted on top of the Xtreme Pump Box. Alternatively, the Xtreme-10

could be turned orthogonal to the placement in the figure with a portion protruding outside the hood. If this configuration is selected, the Xtreme-10 should be placed on the left side of the hood with the inlet / outlet side facing to the right hand side. This places the internal tubing fully in the hood for optimal safety. The Xtreme Controller could then be placed on top of the Xtreme Pump Box. Not shown are nitrogen and carbon dioxide cylinders which would be outside the fume hood.

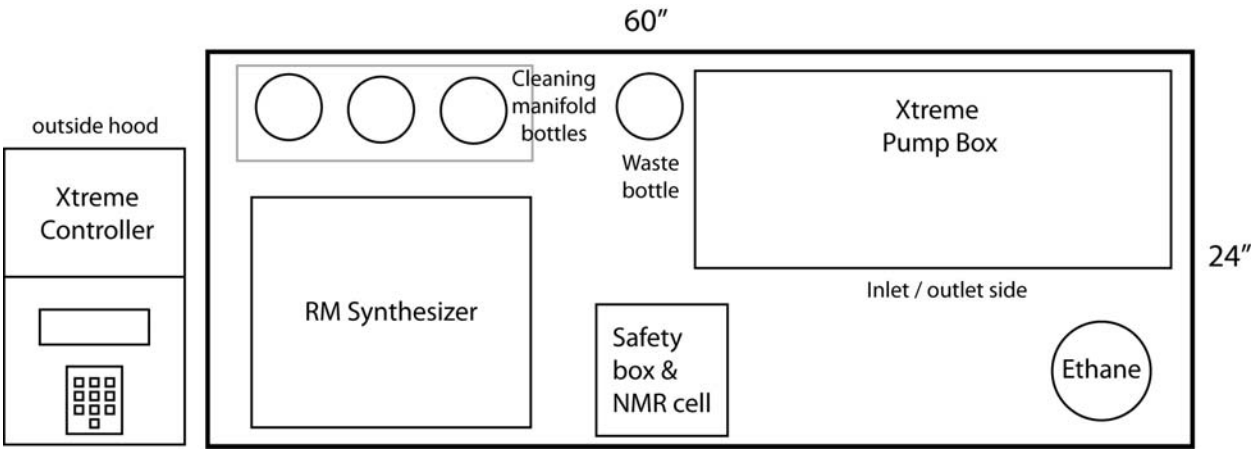

Figure 2: Possible fume hood setup

CONNECTION DETAILS

Once the instruments have been positioned the equipment should be connected using the included 1/16” stainless steel tubing, the high pressure 15-AM1 glands, and 15-2A1 sleeves. These steps should be followed when making a high pressure connection:

i) Deburr the end of the tube section.
ii) Assemble the gland then sleeve onto the tube end.
iii) Insert the end of the tube into the fitting until it bottoms.
iv) Tighten the gland to 55 in·lb. A “bottoming out” or “dead stop” should be felt when the connection is properly assembled.

Always use two wrenches when tightening fittings: one to tighten the gland and one to prevent counter rotation of the fitting receptacle. Failure to do so could break loose internal connections.

Make the following connections using high pressure tubing and fittings unless otherwise specified:

1) Connect the **OUTLET** port of the Xtreme-10 to the **HP ALKANE INLET** port on the back of the RM Synthesizer.

2) Connect the **INLET** port of the Xtreme-10 to the alkane gas cylinder.

3) Connect with high pressure the **CO_2 INLET** port on the RM Synthesizer to the CO_2 gas cylinder regulator.

4) Connect the **N_2 INLET** port on the RM Synthesizer to the N_2 gas cylinder regulator.

5) Connect a section of tubing to the **WASTE** port of the RM Synthesizer using a high pressure fitting. Assemble the Cheminert CFL-1N gland and sleeve on the free end. Feed this end through the small port of one of the three-port bottle caps until it is at the bottom of the bottle. Tighten the Cheminert gland to secure the line. The remaining ports of this bottle cap should be left open to allow gases to vent.

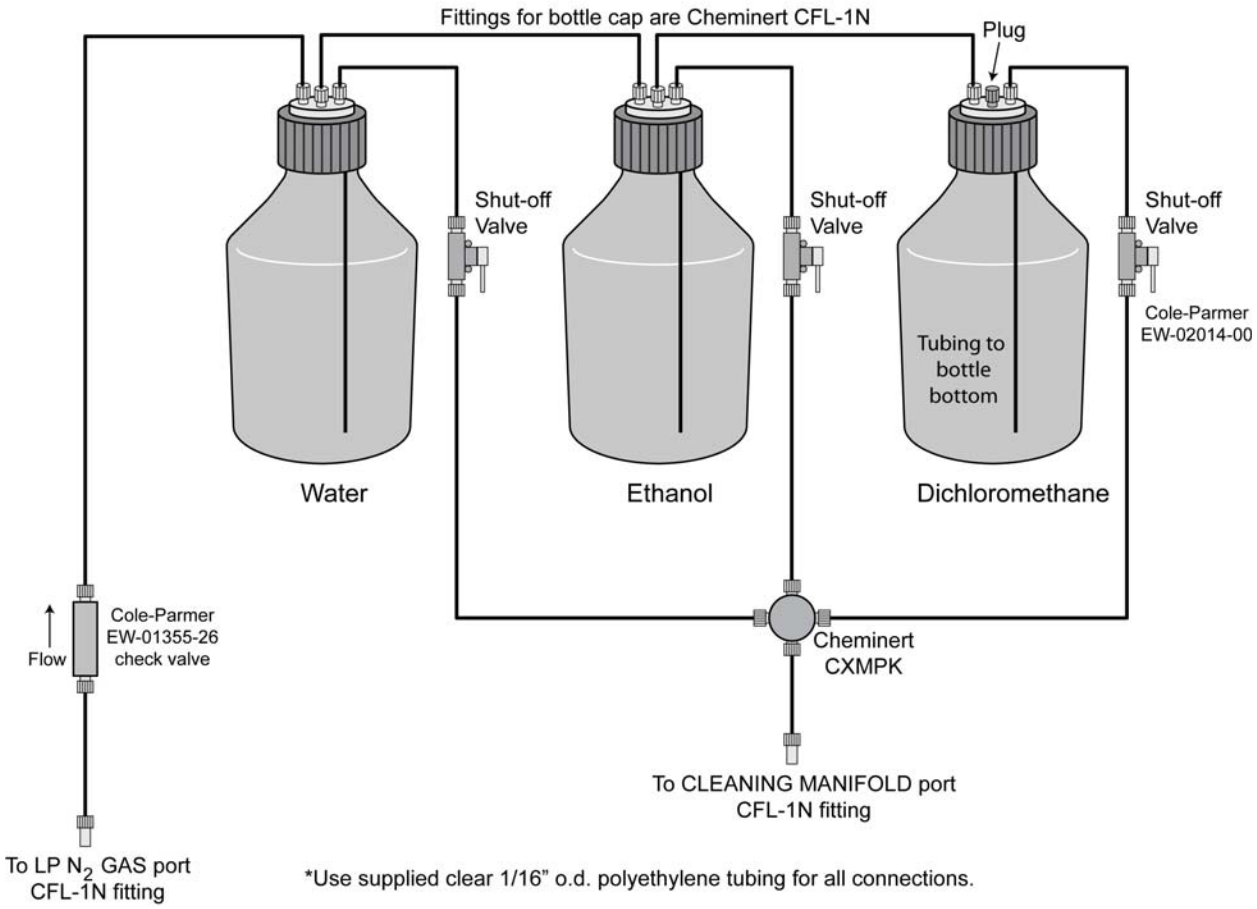

Figure 3: Cleaning manifold assembly diagram

6) Assemble the cleaning manifold according to the Figure 3 using the included parts and connect to the RM Synthesizer as indicated.

7) The NMR cell is connected to the **OUTLET/INLET** valve on the RM Synthesizer as indicated later in the instructions. This valve can also serve as the introduction point for deuterated alkane in situations where the syringe pump is not fully loaded with deuterated solvent.

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THEORY OF OPERATION

The RM Synthesizer was designed to bring together all the necessary elements for preparing reverse micelle samples in liquid alkanes at high pressures. One issue with preparing samples in a vessel where the desired analysis cannot be performed is the need to move the pressurized sample into a more appropriate container. In the case of reverse micelle samples, the efficacy of the sample is dependent on the pressure such that in moving the sample the pressure cannot dip below a certain threshold without losing integrity of the construct.

There are a variety of methods that could be used. The first is to avoid the two vessel system entirely; however, mixing in the high pressure NMR tube is not very efficient and was discarded early in the development. Another method is to use a high pressure gas to push that sample from one vessel into another. After considering a variety of gases there are not many candidates that are immiscible with liquid alkanes, readily obtainable, and do not require significant compression cycles to bring the pressure of the gas up to the level required. Nitrogen is one of the best candidates. However, for certain applications, the pressure required for the transfer step increases the density of the nitrogen gas above that of the liquid alkane such that the nitrogen is no longer pushing the sample; rather it replaces the sample in the secondary chamber. The method used by the RM Synthesizer is to displace the sample, by way of a piston, from the mixing chamber into the NMR tube. The relative piston position during preparation and transfer is shown in Figure 4.

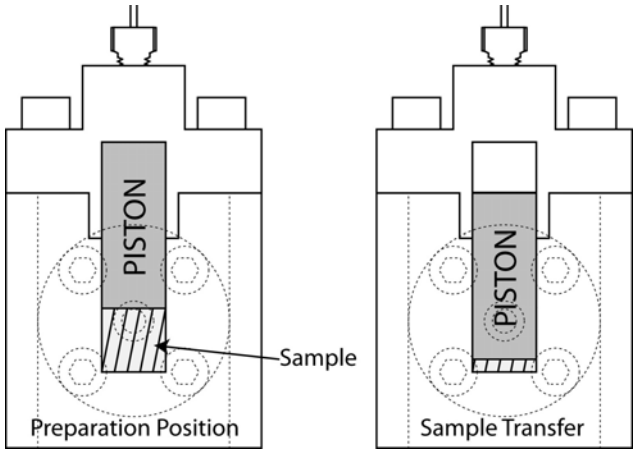

Figure 4: Piston position during operation

The force driving the piston displacement is provided by the expansion of a high pressure fluid delivered by a secondary pressure source. Again, there are a variety of alternative methods that could be used to achieve this action. Rather than explain why other methods are not used, this discussion will focus on the what RM Synthesizer provides for this step. The liquid alkanes used in the preparation of reverse micelles are highly compressible. Data from the website: http://webbook.nist.gov/chemistry/ for liquid ethane shows that ethane undergoes a decrease in volume of approximately 7% over the range of 4,000 – 7,000 psi. This

change in volume can be harnessed to rapidly move the piston when a reservoir at high pressure is allowed to relax to a lower pressure. This process is rapid, and the pressurizing fluid does not need to go through a transition from liquid to gas to deliver the needed force.

This entire action could be provided solely by the syringe pump. However, the syringe pump plays a role immediately after the actual transfer step by rapidly returning the sample to the required encapsulation pressure. This will be discussed later in the example protocols. The end result is the syringe pump has the role of maintaining the reverse micelle sample pressure over being used to drive the piston directly. In addition, and perhaps more importantly, more of the precious deuterated alkanes would be wasted using it as the fluid for this step.

Instead the secondary pressure source is provided by an internal fluid reservoir or gas booster. It turns out that carbon dioxide has nearly identical compressibility as liquid ethane. Thus the reservoir can be filled with CO_2 and this fluid pressurized to the required level using liquid ethane delivered by the syringe pump. A separator piston in the reservoir keeps the fluids apart. Once the transfer steps are performed, the CO_2 fluid can be used to push any deuterated alkane out of the reservoir keeping the losses to an absolute minimum.

The piston displacement literally rams the sample into the waiting NMR tube. Because it might be possible to deliver more pressure to the piston than the tube can withstand the dimensions of the piston and length of the connection tube to the NMR cell were selected such that the piston displaces less than the volume of the NMR cell plus tubing. For most situations this is not strictly necessary. However, for the small fraction of cases where this might be important, it is suggested this approach be maintained.

Since the volume displaced is less than the volume of the NMR cell, additional solvent molecules must be added to the mixing chamber fill this extra space. This is again done by taking advantage of the compressibility of liquid ethane. The sample in the mixing chamber is over pressurized just enough that when it is released, and the piston pushes the sample into the NMR cell, the expanded fluid volume will fully fill the volume of the NMR cell at the encapsulation pressure. The method for taking all these details into account is described later in this manual when outlining potential sample preparation protocols.

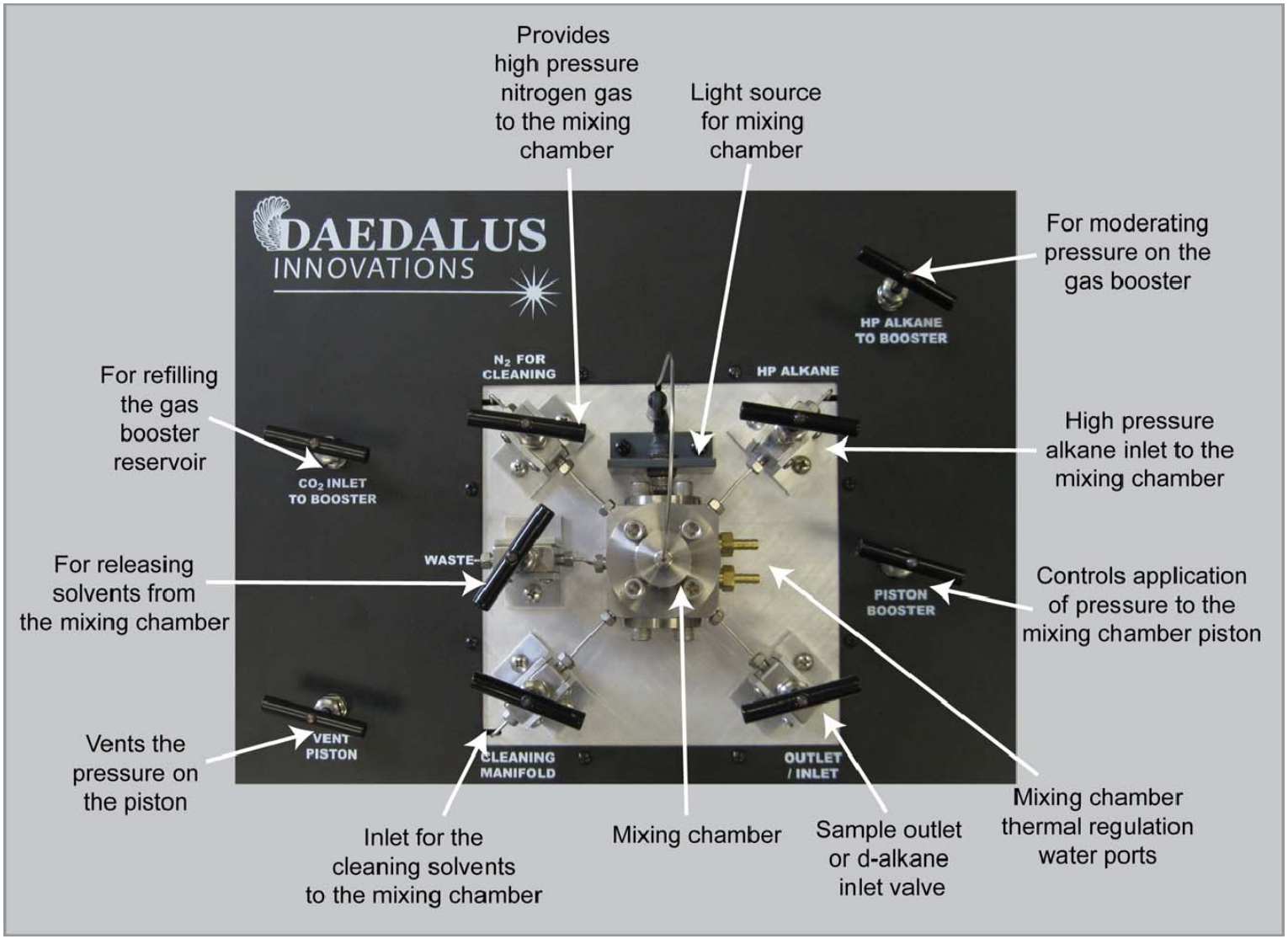

Figure 5: RM Synthesizer Operational Description

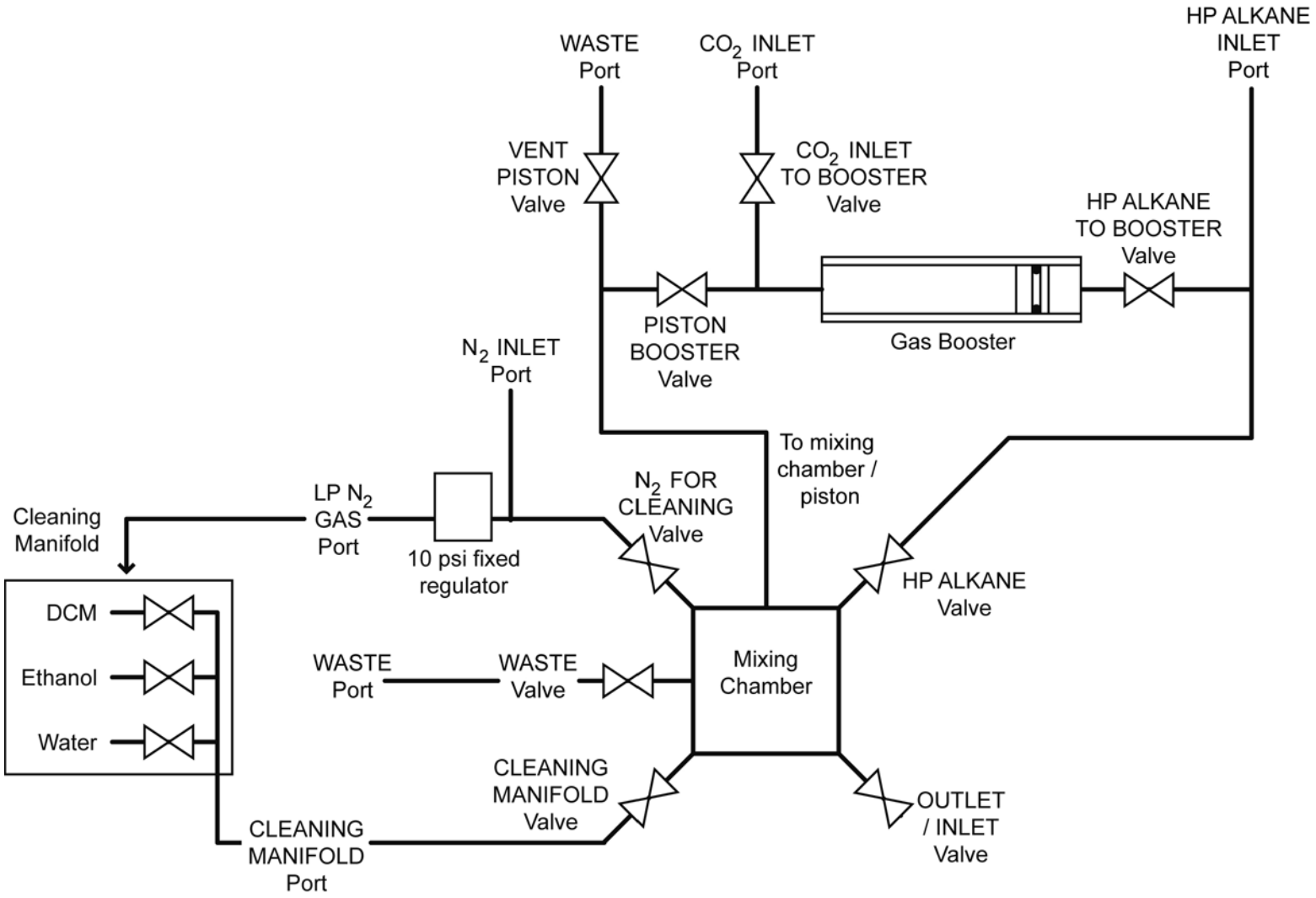

Figure 6: RM Synthesizer Flow Diagram

CONVENTIONS USED IN THIS MANUAL

When describing operations of this device the identification of the valves involved will be made by listing the valve name that is stenciled on the instrument such as **HP ALKANE** or **PISTON BOOSTER** valve. The same method will be used to identify ports on the back of the instrument. Where it is prudent, the specific sequence of valve actuation will be identified on the platform figure with indicators corresponding to the step. Shown below is a typical example.

To transfer the sample from the mixing chamber to the sample tube perform the following steps diagramed in Figure 7:

i) ***Open*** the **OUTLET / INLET** valve; wait for two seconds.
ii) ***Close*** the **PISTON BOOSTER** valve.
iii) ***Open*** the **HP ALKANE** valve to equalize the system pressure.

Figure 7: Example valve actuation progression

VALVE DESCRIPTIONS

Refer to Figure 5 and Figure 6 for pictorial representations of the valve connections and how they control fluid flow.

OUTLET / INLET: This is the connection point for the reverse micelle NMR cell. The transfer line that comes with the NMR cell should be used for this connection. In cases where the syringe pump will not be fully loaded with deuterated alkane, this valve can be used as an inlet port for the initial fill with deuterated solvents. The

syringe pump would then use protonated solvents to pressurize the sample. Due to the compressibility of ethane and propane, this will likely result is a sample that has 20-30% protonated solvent.

CO_2 INLET TO BOOSTER: This connects the CO_2 INLET port directly to the gas booster. This valve allows the gas booster to be refilled with high pressure CO_2.

VENT PISTON: This releases the pressure on the piston to the WASTE port. This valve should not be opened with the PISTON BOOSTER open as this will release all gas in the gas booster reservoir.

HP ALKANE TO BOOSTER: This connects the HP ALKANE INLET port to the opposite side of the separator in the gas booster. This valve is opened when changing the internal pressure of the gas booster or when releasing pressurized alkane back into the syringe pump.

HP ALKANE: this connects the HP ALKANE INLET to the mixing chamber. This is opened when preparing the reverse micelle samples.

PISTON BOOSTER: Opening this valve delivers the pressurized CO_2 from the gas booster to the piston contained in the mixing chamber cap. This can be used both to change the internal pressure of the mixing chamber without adding additional alkane to the sample as well as more commonly for transferring the sample from the mixing chamber into the NMR sample tube.

WASTE: Allows the contents of the mixing chamber to be flushed out to the WASTE port. This is used for venting the chamber after the sample preparation as well as for passing cleaning fluids out of the chamber.

N_2 FOR CLEANING: This connects the N_2 INLET port directly to the mixing chamber. This supplies high pressure nitrogen gas for pushing the cleaning fluids out of the mixing chamber through the WASTE port. This valve should not be opened when the chamber is at a pressure above the nitrogen gas cylinder output.

CLEANING MANIFOLD: This provides a path for the selected cleaning fluid into the chamber. This valve should only be opened when the chamber pressure is at atmospheric otherwise it will cause a backflow into the cleaning manifold bottles.

Mixing chamber water ports: There are a series of channels cut into the mixing chamber forming a continuous loop. The ends of this loop terminate at the two

brass barbed fittings on the right side of the mixing chamber. These can be connected to a water bath to provide thermal regulation of the sample.

BACK PANEL CONNECTIONS

Shown in Figure 8 are the ports used to connect the system to the external fluid sources required for operation of the RM Synthesizer.

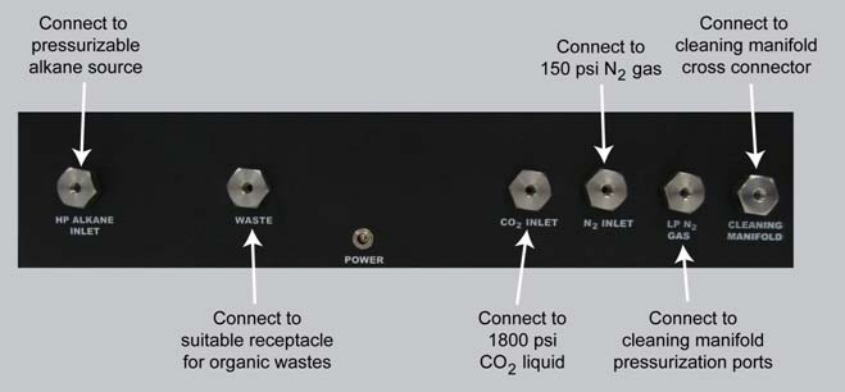

Figure 8: Back panel port connections

HP ALKANE INLET: Connects to the outlet of the Xtreme-10 Syringe Pump. The tubing should be 1 kbar rated.

WASTE: This port handles the outflow from the mixing chamber as well as the high pressure gas from the piston. The tubing should be rated for high pressure, and the end should be in a vessel that can tolerate organic solvents. It should be anchored so it does not break loose when high pressure gas is vented.

CO_2 INLET: This is the high pressure carbon dioxide inlet. Standard CO_2 cylinders are not able to deliver liquid CO_2. Instead connect to a cylinder with an eductor tube pressurized with helium to at least 1,800 psi for best performance.

N_2 INLET: This port is for connecting a standard nitrogen cylinder with an output of 125-150 psi. This inlet links to the mixing chamber as well as an internal regulator rated to a maximum of 250 psi which delivers 10 psi gas to the LP N_2 INLET.

LP N_2 INLET: The output from this port is 10 psi nitrogen gas. Connect this to the cleaning manifold pressurizing port. It is this gas pressure that promotes fluid flow out of the manifold bottles. There is no valve between the N_2 INLET and LP N_2

INLET so always have it connected or plugged to prevent discharging the nitrogen cylinder.

CLEANING MANIFOLD: The outlet of the PEEK cross from Figure 3 should be connected to this port. Solvents from the cleaning manifold flow from this port to the mixing chamber.

FRONT PANEL CONTROL

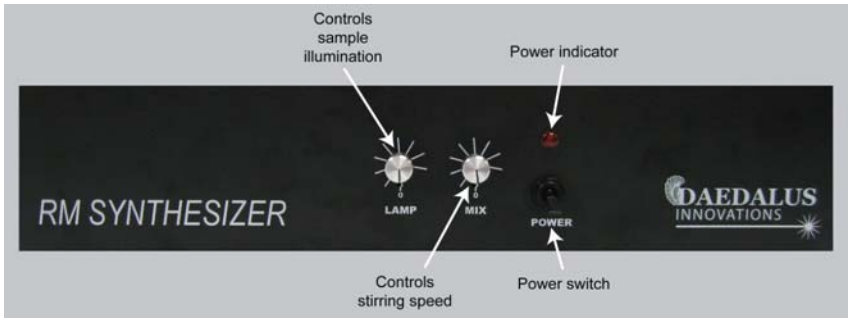

Figure 9: Front panel controls

POWER: Provides power for the lamp and mixing electronics. It also turns on a venting fan inside the instrument. Even if the lamp and mixer are not required the power should be turned on when in use to keep air circulating inside the instrument.

LAMP: This knob controls the intensity of the light source illuminating the mixing chamber

MIX: This knob controls the sample stirring speed.

PREPARING THE INSTRUMENT FOR USE

The procedure described is the basic setup required for use of the instrument. It assumes the Xtreme-10 Syringe Pump is being used as the pressure source.

(1) Fill the gas booster with 1,800 psi CO_2 by ***opening*** the **CO_2 INLET TO BOOSTER** valve.

(2) ***Open*** the **HP ALKANE TO BOOSTER** valve while filling the gas booster to allow the high pressure CO_2 to push the internal separator all the way to one end thus pushing out any ethane present in the reservoir.

(3) ***Close*** the **HP ALKANE TO BOOSTER** and **CO_2 INLET TO BOOSTER** valves. The gas booster is now filled with CO_2.

(4) ***Fill*** the syringe pump completely with liquid ethane. Follow the procedures in the Xtreme manual for refilling the pump. To extract liquid from the ethane cylinder it may be necessary to place the cylinder in warm water while filling the pump. It will take time for the liquid to transfer. Depending on the temperature ethane liquefies around 610 psi. See http://webbook.nist.gov/chemistry/ for detailed information about the properties of liquid alkanes. ***Open*** the **HP ALKANE TO BOOSTER** to fill this void as well.

(5) The nitrogen cylinder can be connected, but does not need to be open until the cleanings steps. Set the cylinder pressure to between 125-150 psi.

At this point the system is ready for reverse micelle preparation work. The remaining steps are provided as an example of how to load the syringe pump with extra grams of ethane. This is optional, and does not need to be performed for routine operation of the instrument. However, this operation might be useful if a higher starting pressure is desired, or if the time between syringe pump recharges is to be increased.

Only perform this procedure with the gas booster reservoir filled with CO_2 to act as a brake for the internal separator. If the reservoir is empty and subsequently filled entirely with alkane the piston would be moved to the fully displaced position. Later additions of liquid CO_2 may not be sufficient to push the separator back into position and as a result the transfer step may not work as expected because of insufficient distance for the piston to travel.

(6) With the syringe pump fully filled with liquid ethane and the pump **INLET** valve ***closed***, set the pump to pressurize to 7,000 psi. With the HP ALKANE TO BOOSTER valve already open this will pressurize the gas booster to 7,000 psi. Once complete proceed to the next step.

(7) ***Close*** the **HP ALKANE TO BOOSTER** and **OUTLET** valve on the pump and fully refill the pump again with liquid ethane. Be sure to ***Close*** the **INLET** valve on the pump after the refill step.

(8) ***Open*** the **HP ALKANE TO BOOSTER** and pump **OUTLET** valves to depressurize the gas booster. This will push out the ethane in the gas booster back into the syringe pump. Dependent on the pump fill factor and the initial CO_2 head pressure, the pressure in the syringe pump will be around 1,500 psi. The system is now filled with sufficient ethane to make fifteen typical ethane samples with encapsulation pressures of 4,000 psi The CO_2 in the gas booster will need to be recharged after each sample. This will be addressed later when the protocol for transferring the sample and resetting the system is outlined.

THE MIXING CHAMBER / ADDING REAGENTS

The internal volume of the mixing chamber is approximately 1.65 ml when fully assembled. There are slight variations between cells so an exact measurement should be obtained using the syringe pump (see CALIBRATING THE SYSTEM). Of this volume approximately 1.1 ml can be displaced by the piston. The dead volume is comprised of the empty volume of the connection tubing empty, the reagent plate well, and spaces created from placing windows in the mixing chamber walls. This large available sample volume is required because the NMR cell has an active volume of approximately 1.2 ml. There are two observation windows in the walls of the chamber. The lamp abuts the back facing window, while the front facing window is for observation of the sample preparation process.

In addition to the main mixing chamber body and top cap there are three additional components as shown in Figure 10. These are the piston, the reagent plate, and o-ring seal (MCCS).

Figure 10: Mixing chamber components:
Poston, reagent plate, and main seal (MCCS).

Figure 11 shows the assembly diagram of the cell and the relative position of the components. The reagent plate serves two functions: The wall of the reagent plate stops the descent of the piston and prevents the stir bar from being crushed. Second, surfactants, especially liquid surfactants, can be weighed directly on this plate prior to inserting into the mixing chamber. The reagent plate should be placed at the bottom of the mixing chamber.

Make certain the **WASTE** valve is closed prior to adding reagents to prevent the reagents from being forced out through this port in subsequent steps.

Add the reagents in the following order (not all items are used with every sample):

(1) Add the protein / aqueous phase first

(2) Add any liquid co-surfactants such as hexanol

(3) Add the liquid surfactants

(4) Add the dry surfactants

(5) Add other additives such as pentane or carbon disulfide

(6) Always add the stir bar last

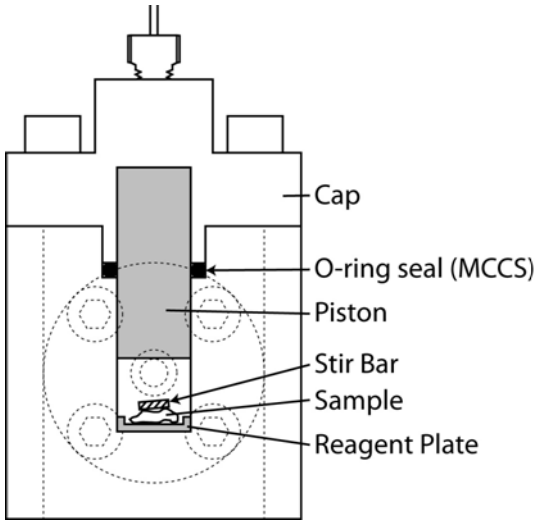

Figure 11: View showing internal placement of the mixing chamber components

SEALING THE MIXING CHAMBER

The high pressure seal is provided by the o-ring component P/N MCCS. This seal provides both a static containment seal during the sample mixing as well as a dynamic seal when the piston is driven down during the sample transfer step. This combination of forces tends to degrade the seal such that it must be replaced after every sample.

The following photos in Figure 12 show the steps for assembling the mixing chamber for the high pressure work.

(1) Insert the o-ring seal (MCCS) into the large chamber O.D. Push it down as far as it will go.

(2) ***Open*** the **OUTLET / INLET** valve, and push the piston to just slightly past the o-ring seal. Do not push it all way down as this will force the sample out the OUTLET / INLET valve. The valve is opened to relieve pressure generated by pushing the piston into place.

(3) Place the cap with the four screws into position. The small O.D. section of the cap should fit into the mixing chamber main body large O.D. section.

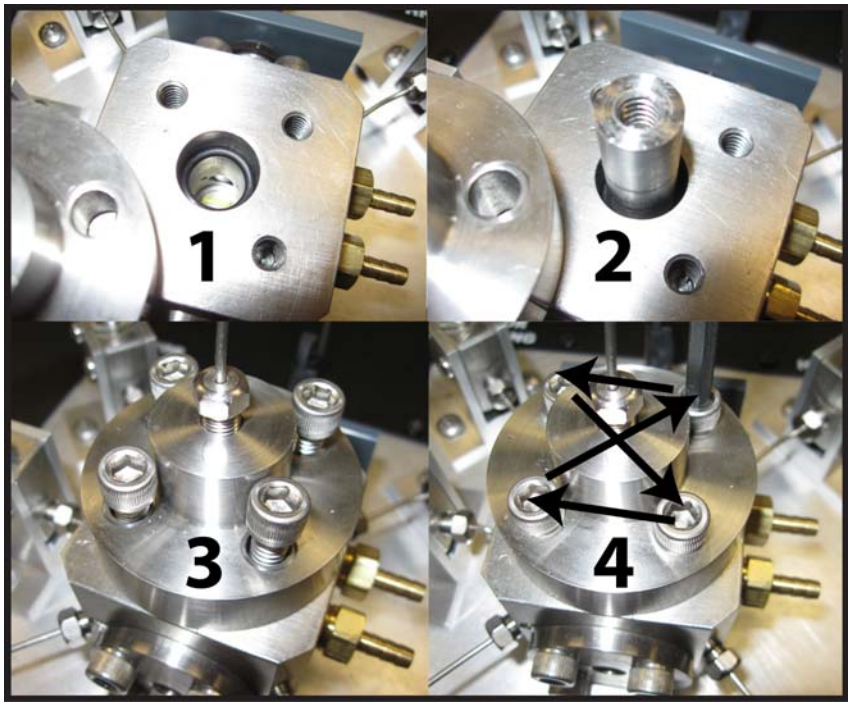

Figure 12: Progression of steps for assembling and sealing the mixing chamber prior to sample preparation

(4) Tighten the four screws in a star pattern using a 3/16” hex driver in the pattern shown in the Figure 12. Once tightened, ***close*** the **OUTLET / INLET** valve.

At this time it is recommended that all valves be checked to make sure they are closed, especially those that were actuated in the previous sample preparation process.

PROTOCOL FOR MAKING SAMPLES

The encapsulation pressure for samples in liquid ethane varies according to the surfactant, the water loading, and the co-surfactants / solvents added to the system. Typically for samples in pure ethane in CTAB with modest water loadings the encapsulation pressure is around 4,000 psi. For AOT this increases to around 8,000 psi without the addition of co-surfactants / solvents. Since there are so many variables involved the best method for learning the art of preparing samples in ethane is to practice. One way to do this is to prepare a series of ethane samples using a protein-free water stock with bromophenol blue added. The visual indicator will help the user learn the tricks and visual markers that are apparent as the pressure on the sample is increased ultimately to the encapsulation point.

For a CTAB surfactant sample with 10% hexanol, a water loading of 15, in pure ethane the steps are as follows:

(1) Add the reagents and seal the chamber.

(2) ***Open*** the **HP ALKANE** valve to introduce ethane into the chamber. Usually the target pressure for this step is around 1,500 – 2,000 psi. Increase the pressure if it is not already at this level. The **OUTLET** valve on the syringe pump should be ***open*** and remain so throughout the sample preparation process.

(3) Set the stir bar speed to around 2/3rds. At this time the water and protein solution will likely not be visually observable.

(4) Slowly step up the pressure to 3,000. Using small increments of 500 psi should make apparent when the water begins to mix by a clouding of the sample. This is the point where it is thought the CTAB solubilizes. The mixing should continue at this pressure until this phenomenon is observed to assure the sample is in fact mixing.

(5) Continue stepping up the pressure in small increments of 250 psi while continuing to observe the mixing process as noted by the cloudy nature of the sample.

(6) Around 3,500 – 4,000 psi the sample should become noticeably more clear. At this point the experience of the user becomes important. Most samples will not completely solubilize so some flocculence may be apparent in even a good sample. Stop the stirring process and allow the insoluble particles to settle. If the previous steps were followed, and a mixing transition was observed a transparent sample usually indicates it is close or at the encapsulation point. The alternative is to increase pressure some more, but doing will increase the total viscosity of the sample and defeat the purpose of encapsulating proteins in ethane. However, it may be necessary to do so to achieve stable samples so a judgment must be made. A sample that is properly encapsulated will not become clearer with the addition of more pressure.

Increasing the pressure in the chamber directly using ethane is not always the best option since this would have negative consequences on the sample viscosity as well as the pressure could not be subsequently reduced as this would draw sample out of the chamber. However, the pressure on the piston can be used to increase the chamber pressure without diluting the sample or making irreversible additions. This procedure is **optional**, and is presented only as another method available for finding the proper encapsulation pressure. To perform this piston pressurization routine refer to Figure 13 and perform the following:

i) ***Close*** the **HP ALKANE** valve to isolate the mixing chamber.
ii) ***Open*** the **HP ALKANE TO BOOSTER** valve.
iii) ***Open*** the **PISTON BOOSTER** valve to expose the piston to the pressure source.
iv) Using the syringe pump increase the pressure on the piston to a target pressure above the internal chamber pressure.
v) Watch for additional clearing of the sample.
vi) After testing is complete, reduce the syringe pump pressure to below the internal chamber pressure (if possible – see (ix))
vii) ***Close*** the **HP ALKANE TO BOOSTER** and **PISTON BOOSTER** valve.
viii) Resume preparing the sample with the newly obtained data.
ix) Optional: It may be necessary to release the piston pressure to reduce the chamber pressure back to what it was originally. If necessary, be sure

all valves are closed, and ***open*** the **VENT PISTON** valves to release the pressure. ***Close*** the valve after venting is complete.

Figure 13: Increasing the sample chamber pressure using the piston

(7) Once the sample has been encapsulated it is recommended the internal sample pressure be increased by an additional 250 psi to account for potential pressure loss from the reverse micelle NMR cell over time. This is the encapsulation pressure of the sample and will be referenced later in the protocol.

(8) As discussed in the *Theory of* Operation section, the volume displaced by the piston is less than the volume of the NMR tube. Therefore, the sample must be over pressurized to compress more molecules of ethane into the chamber such that once the transfer is initiated the sample will expand to fully fill the NMR tube. The over pressurization required is dependent on the NMR cell style as described below:

Varian: $\text{Density}_{encap} \times \mathbf{1.08} = \text{Density}_{over}$

Bruker: $\text{Density}_{encap} \times \mathbf{1.03} = \text{Density}_{over}$

Using the density data ethane obtained from http://webbook.nist.gov/chemistry/ look up the density for ethane at the encapsulation pressure, plug it into the formula, then find the corresponding density for the over pressurization target. Over pressurize the sample to the calculated target pressure then ***close*** the **HP ALKANE** valve. The sample is ready to be transferred.

TRANSFERRING SAMPLES FROM THE MIXING CHAMBER TO THE NMR CELL

With the sample now over pressurized to expand to fill the NMR cell internal volume, what remains is to apply sufficient pressure to the piston such that the sample is displaced from the mixing chamber into the NMR cell when the OUTLET / INLET valve is opened. The following steps outline the procedure.

(1) Preset the NMR cell valve. The needle in the NMR cell valve displaces volume during actuation. Thus the valve must be opened to the same position each time to assure the internal volume matches the over pressurizing ratio from the previous section. To set the position close the NMR cell valve until just snug, then open the valve (counter clockwise) two full turns, then close (clockwise) one full turn. Be sure to use the transfer tube provided as its internal volume is included in the over pressurizing ratio. Connect this tubing between the NMR cell and the **OUTLET / INLET** valve.

(2) ***Open*** the **PISTON BOOSTER** and **HP ALKANE TO BOOSTER** valves. **Be sure the HP ALKANE valve is closed before performing this operation otherwise the sample will be pushed back into the syringe pump.** This allows the syringe pump to pressurize the piston. Increase the pressure on the piston to 2000 psi above the encapsulation pressure (this is not the current mixing chamber pressure) or equal to the over pressurization pressure, whichever is higher. ***Close*** the **HP ALKANE TO BOOSTER** valve after the target pressure is reached. The piston is now set for transfer.

(3) Reset the syringe pump pressure to the encapsulation pressure of the sample. This is not the over pressurization pressure.

(4) Perform the sample transfer by actuating valves according to the following (Refer to Figure 14):

i) ***Open*** the **OUTLET / INLET** valve; wait for two seconds.
ii) ***Close*** the **PISTON BOOSTER** valve.
iii) ***Open*** the **HP ALKANE** valve to equalize the system pressure.

Figure 14: Progression of valve actuation for the sample transfer steps

(5) After the system pressure equalizes to the encapsulation pressure, the NMR cell valve can be closed. The **OUTLET / INLET** and **HP ALKANE** should remain ***open*** during this step.

(6) After the NMR cell is sealed ***close*** the **OUTLET / INLET** and **HP ALKANE** valves to prevent accidental expulsion of the pressurized fluid.

(7) Disconnect the transfer line from the NMR cell. It is now ready to be inserted into the NMR spectrometer for sample assessment.

(8) Be sure to clean the mixing chamber after each sample.

CLEANING THE MIXING CHAMBER

The RM Synthesizer is shipped with three low pressure glass reservoirs that can be filled with solvents for cleaning the mixing chamber and associated tubing after sample preparation. Typically the solvents are water, ethanol, and dichloromethane. The **LP N_2 GAS** port on the back of the instrument delivers a low pressure gas supply that plugs into the reservoirs to provide the fluid pushing force. A high pressure nitrogen line feeds directly into the mixing chamber to push the cleaning solvents out of the chamber. **Be sure the WASTE port is connected to a suitable waste container and waste gases are properly exhausted away from users.** The nitrogen gas cylinder should be opened and delivering at least 125 psi nitrogen gas.

(1) ***Open*** the **VENT PISTON** valve to release the high pressure CO_2 gas to the WASTE port. The remaining ethane in the mixing chamber will now push the piston back to the retracted position.

(2) ***Open*** the **WASTE** valve to vent the sample to the WASTE port. ***Close*** the valve after the sample is vented.

The order of cleaning solvents should be:

i) Dichloromethane
ii) Ethanol
iii) Water
iv) Ethanol
v) Dichloromethane.

Ethanol (ii) will dissolve most common surfactants and water (iii) will solubilize the protein. Both reagents influence reverse micelle formation so these need to be displaced with subsequent rinses (iv & v). Dichloromethane serves as the bridge between ethanol and alkane solvents. It evaporates quickly when the cell is opened to air however trace dichloromethane will not cause problems for reverse micelle formation.

For each cleaning solvent:

(3) ***Open*** the **CLEANING MANIFOLD** valve, and open the appropriate solvent PEEK shut-off valve in the manifold. Allow the mixing chamber to fill with the fluid. At the same time the transfer tube to the NMR cell can be flushed by opening the **OUTLET / INLET** valve for a short time. Be sure to catch the fluid in a suitable receptacle. Stir the sample in the mixing chamber to promote solubilization.

(4) ***Close*** the **CLEANING MANIFOLD** valve and the PEEK shut-off valve.

(5) ***Open*** the **N_2 FOR CLEANING** valve. This allows the high pressure nitrogen gas into the mixing chamber.

(6) ***Open*** the **WASTE** valve to expel the cleaning solvent into the waste receptacle. Also open the **OUTLET / INLET** valve to blow out the transfer tube.

(7) ***Close*** the **N_2 FOR CLEANING** first then the **WASTE** valve.

(8) Go back to step (3) and repeat for each solvent in the list.

(9) After cleaning the chamber, the cell cap can be removed. A screw from the cap can be threaded into the piston to remove it from the chamber (Figure 12). The seal (MCCS) should be removed and discarded.

(10) Wipe out any additional trace material with a cotton swab. The mixing chamber is ready for the next sample.

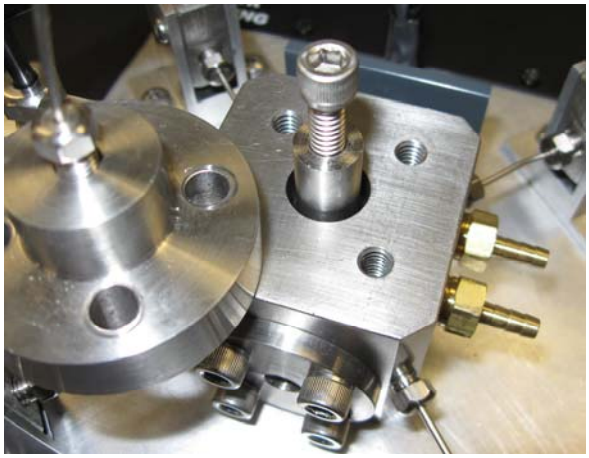

Figure 15: Removing the piston

RESETTING THE SYSTEM FOR THE NEXT SAMPLE

The following guidelines are for resetting the system; primarily resetting the internal gas booster reservoir separator piston to the proper position for the next sample. This method can be used to confirm that the separator piston has been moved to the fully reset position.

At this point the reservoir pressure and syringe pump pressure are probably well above the CO_2 gas cylinder outlet pressure (1,800 psi). Before the reservoir can be recharged the pressure in both the reservoir and syringe pump should be below this level. All the system valves should be closed at this point.

(1) ***Open*** the **HP ALKANE TO BOOSTER** valve and the **OUTLET** valve on the syringe pump. This will allow excess alkane to flow back into the syringe pump. The reservoir pressure may be higher than the syringe pump so there may be a jump in the pressure reported by the syringe pump.

(2) Change the setpoint on the syringe pump to 1,700 psi and start the pump running. The high pressure CO_2 fluid in the reservoir will now push the separator piston back towards the fully reset position. Since some fluid was used during the sample preparation process it should not be expected to complete the full displacement.

(3) With the pressure in both the reservoir and syringe pump below 1,800 psi the **CO_2 INLET TO BOOSTER** valve can be opened to allow fluid from the gas cylinder to recharge the booster. Once this valve is opened any excess alkane remaining will be forced back into the syringe pump, and a temporary pressure increase should be displayed by the syringe pump until it starts to readjust to the 1,700 psi setpoint. This process can take a few moments since the pressure differential is small.

(4) Once the syringe pump is stable at 1,700 psi it can be assumed the separator has been fully reset since the applied 1,800 psi CO_2 pressure is no longer influencing the syringe pump reading. ***Close*** the **CO_2 INLET TO BOOSTER** the **HP ALKANE TO BOOSTER** valves. The syringe pump **OUTLET** valve can be ***closed*** as well.

(5) The system is now reset and ready for the next sample preparation.

The primary goal of this protocol is to assure the gas booster reservoir is fully recharged with CO_2. This method can be adapted to the user's needs and time constraints.

TIPS

The following list contains some tips for operation of the RM Synthesizer some of which were contained in the text of this manual. It also contains tips that will hopefully help in making successful reverse micelle samples in ethane. It is by no means exhaustive.

- After the first few uses of an ethane or propane cylinder, the outlet pressure will likely be below the liquefaction pressure. Placing the tank in warm water can help deliver liquid alkane from the cylinder.

- Propane may not start to fill the mixing chamber until the pressure reaches 300 psi. The mixing chamber can be chilled to help condense propane, but it is by no means necessary to make it work.

- Propane samples can use the 1,800 psi CO_2 fluid to actuate the piston. The over pressurization method should still be followed, but it is unlikely the piston will need more pressure than 1,800 psi.

- Once encapsulation has been achieved it is good practice to go 250 psi above that to overcome leakage from the NMR cell over extended periods of time.

- Insoluble material is common even in good samples. Stop the stir bar and allow the insoluble material to settle to properly observe the sample.

- The insoluble material should be allowed to settle prior to transferring the sample to the NMR cell.

- After the sample has been transferred, and the NMR cell closed, the cell should be inverted several times to help solubilize any reagents that may have fallen out of solution during the transfer.

- The pressure in the NMR cell can be increased after it has been closed. Reconnect the NMR cell to the OUTLET/INLET valve using the transfer line. Open the OUTLET/INLET valve and pressurize the mixing chamber to the encapsulation pressure or the new pressure desired. Open the NMR cell valve and let the system equilibrate to the new pressure. Close the NMR cell valve, and the OUTLET/INLET valve. Disconnect and check the sample at the new pressure.

- Use the piston to perform test increases in pressure in the mixing chamber. This may be preferable to adding additional solvent and diluting the sample unnecessarily.

- No matter how good the seals, the system will leak over time. If the system will not be used for several weeks it is good idea to retract any deuterated solvents from the RM Synthesizer, into the syringe pump, and then push that fluid back into the alkane cylinder.

- Occasionally the stir bar becomes lodged in the surfactant slurry. It can be dislodged using a strong magnet; either on the side of the mixing chamber or balanced along the edge of the mixing chamber cap.

- Typical CTAB samples encapsulate around 4,000 psi, AOT above 8,000 psi, and a triple surfactant mix consisting of 70% $C_{12}E_4$, 25% AOT, 5% DTAB might not encapsulate around 9,000 psi. Around 8,000 psi the viscosity of ethane is equal to propane so the benefit of working in ethane is lost.

- The encapsulation pressure is dependent on the dielectric constant of the medium. The elevated pressure is required to raise the dielectric of ethane to match the dielectric of the surfactant.

- The encapsulation pressure can be lowered through the use of additives. This serves to increase the dielectric constant of the bulk solvent. Examples of additives are pentane, dichloromethane, and carbon disulfide.

- Encapsulation conditions found in pentane in general are translatable to ethane with minor changes. For example CTAB sample may require 1.5% more hexanol in ethane than pentane.

- Water loading is the ratio of molar water to molar surfactant in the reverse micelle sample. It has a large impact on the size of the particle.

- Past studies using reverse micelles have worked to keep the water loading low; typically in the range of 10-15 to keep the particle as small as possible. However, larger proteins likely require more water to maintain the proper hydration shell. Thus water loadings of 20-25 should be considered for proteins larger than 40 kDa. At this size the protein is dominating the size of the particle so the excess water has less impact.

- Ethane can support much higher water loadings than are common in pentane. This will require slightly higher encapsulation pressures.

CALIBRATING THE SYSTEM

The mixing chamber and components are designed to provide an internal volume near 1.65 ml. Of this volume only 1.1 ml can be displaced by the piston. The actual volume of the mixing chamber and the amount displaced are important numbers. The former being important for the initial reagent calculations, and the latter for determining the over pressure requirements for the transfer step to the NMR cell. These numbers can be determined by a simple protocol. Use protonated alkanes for these measurements as it will be vented from the mixing chamber. The gas booster reservoir should be charged with CO_2 to the normal operating pressure.

(1) Setup the mixing chamber as would be done for a typical preparation process. Include the reagent plate and stir bar.

(2) ***Open*** the **HP ALKANE** valve just enough to let in a little alkane gas then ***close*** the valve. The syringe pump **OUTLET** valve should be ***open*****.**

(3) ***Open*** the **WASTE** valve to vent the alkane. This will purge the mixing chamber of air.

(4) Change the setpoint on the syringe pump to 1,000 psi, and let it stabilize.

(5) Note the current volume on the syringe pump, and ***open*** the **HP ALKANE** valve just enough to fill the chamber.

(6) After the syringe pump is again stable at the setpoint note the new volume. The difference between the volume in step (5) and this new volume is the mixing chamber volume.

(7) ***Open*** the **CO_2 INLET TO BOOSTER** valve and the **PISTON BOOSTER** valve. It is assumed the CO_2 gas cylinder valve is open. This delivers 1,800 psi CO_2 to the mixing chamber piston. It will push the alkane out of the mixing chamber causing the syringe pump to compensate to maintain the 1,000 psi setpoint.

(8) After the syringe pump has again stabilized note the new volume reading. The difference between this volume, and the volume in step (6) is the volume the piston can displace for the sample transfer step.

(9) ***Close*** all valves.

(10) ***Open*** the **VENT PISTON** valve to vent the CO_2 gas behind the piston. After venting is complete ***close*** the valve.

(11) ***Open*** the **WASTE** valve to vent the alkane from the mixing chamber. After venting is complete ***close*** the valve.

(12) Repeat steps (5-11) several times to obtain an average reading for the mixing chamber and piston displacement volumes.

REPLACING THE WINDOW SEALS

The performance of the seals around the mixing chamber sapphire windows can degrade over time. Before changing the seals, be sure all other sources of leaking are eliminated. Leaking window seals are evidenced by the external pressure source noticeably not being able to maintain pressure. It can be further identified by

filling the chamber with ethanol, pressurizing with the piston, and monitoring for leaks. Do not change the seals unless necessary. To remove:

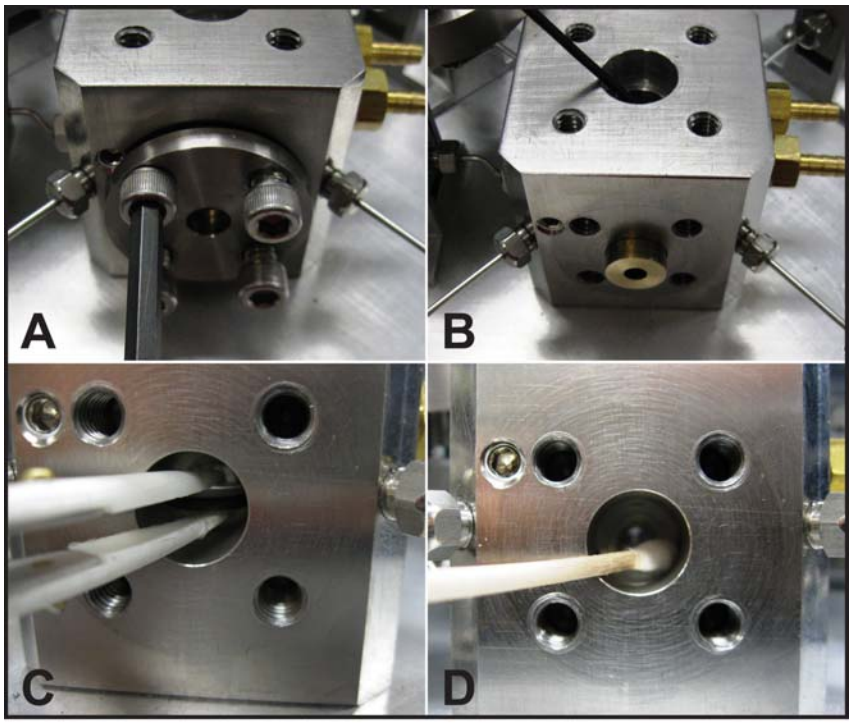

Figure 16: Replacing the mixing chamber windows

(A) remove the four face plate screws. (B) Using a small L-shape hex wrench or equivalent, push out the plug from the inside. (C) Using a forceps, remove the sapphire window and seal. Discard the seal. (D) Remove debris from the window / seal surfaces.

Use a new window seal (WS01) and reassemble the plug by placing the window in the recess of the plug. Then place the o-ring seal around the window as shown in Figure 17.

Figure 17: Reassembly of the window plug

Wet the seal with ethanol, and reinsert the assembled plug into the chamber wall (Figure 18).

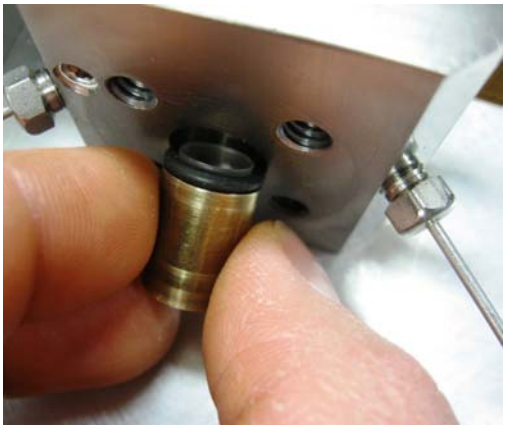

Figure 18: Reinserting the window plug

Reattach the face plate using the four socket cap screws and incrementally tighten the screws using the pattern shown in Figure 19. This will better distribute the force on the window seal.

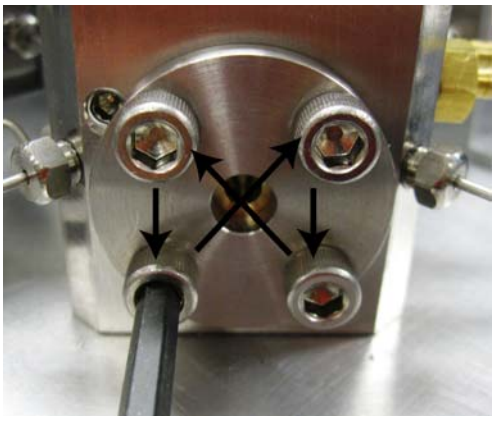

Figure 19: Face plate tightening pattern

To replace the backside window, first remove the lamp housing by unscrewing the power plug at the base and the two screws holding the housing to the mixing chamber plate (Figure 20). Proceed as previously described.

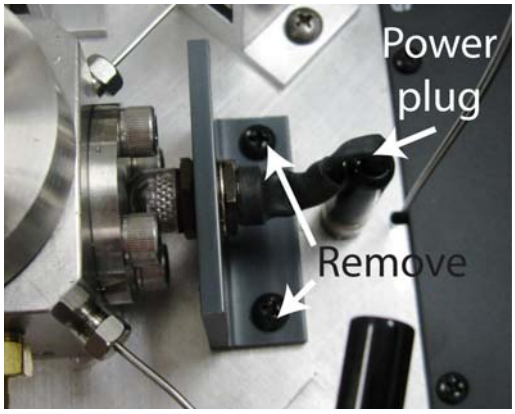

Figure 20: Removing the light housing

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SPECIFICATIONS

Power requirements	100-120 VAC / 200-240 VAC, 50/60 Hz
Input current	< 0.5A rms
Power output	12 VDC, 0.5 A maximum
Temperature range	10 °C to 70 °C
Weight	23 lbs (10.5 kg)
Dimensions	16.25” W x 13.25” D x 9” H (41.3 cm x 33.7 cm x 23 cm)
Pressure range	0-14,500 psi (1 kbar)
Wetted parts	316 stainless steel
Operating medium	Alkanes, Carbon Dioxide, Water, Oils, Alcohols, Inert Gases.
Mixing chamber volume	1.65ml nominal
Piston displacement volume	1.1ml nominal
Pressure connections	All ports are HiP AF1 (1/4”-28 UNF) for use with 1/16” tubing

This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his own expense.

This Class A digital apparatus complies with Canadian ICES-003.

This system conforms to the European Community Council Directive 2004/108/EC for electrical equipment for measurement, control and laboratory use. The standard used for emissions requirements EN 61326-1:2006; Clause 7.2, and the immunity requirements conformed to EN 61326-1:2006; Table 2.

This system conforms to the European Community Low Voltage Safety Directive 2006/95/EC. The standard used was EN 61010-1:2001 for electrical equipment for measurement, control, and laboratory use, Part 1: General requirements.

Documentation can be provided by contacting in writing:

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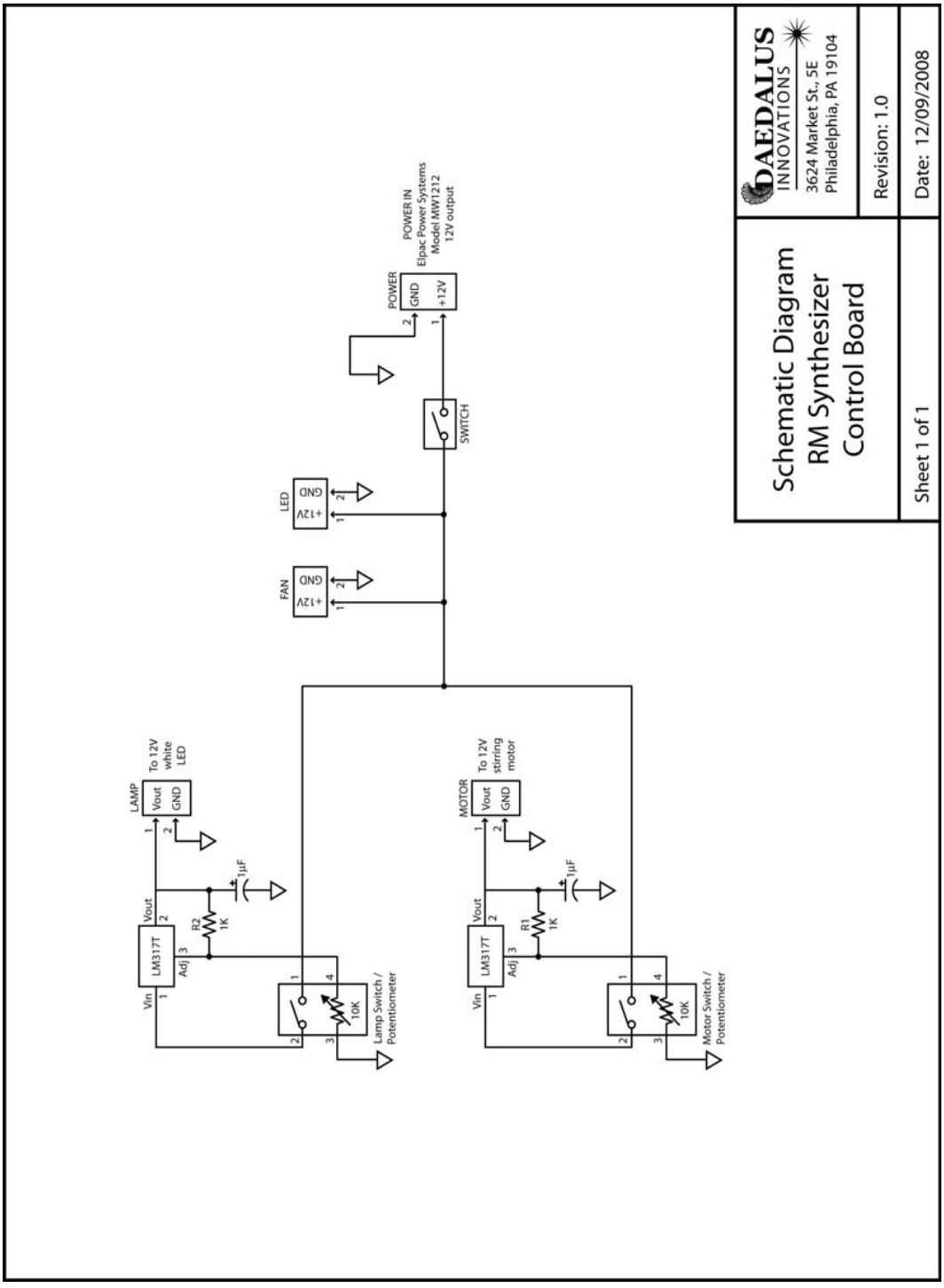

Figure 21: Internal control board schematic

FURTHER INFORMATION

This document may be updated periodically to reflect questions from users. Please check back at www.daedalusinnovations.com in the support section for more recent versions of this document.

Technical support can also be obtained by emailing questions to support@daedalusinnovations.com, or contacting Daedalus directly at 610-358-4728.

Other correspondence can be directed to:

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