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Hybrid Pathogen DNA Detector:Users? Manual v1.5

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Hybrid Pathogen DNA Detector

Users' Manual v1.5

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Introduction of the Hybrid Machine and Its' Uses

The Hybrid Unit uses an advanced fluidic design to move very small reagent samples through many unit operations to complete complex molecular biology experiments. The primary use of this machine is to analyze a small liquid sample for the highly specific presence of select agents known to be used in bio-warfare. The Hybrid Unit is coupled with a Luminex bead detection unit for the multiplexing of many assays in one tube. Because of this, multiple controls can be included in each run to avoid false positives. The built-in flow through PCR unit amplifies specific DNA signatures and increases sensitivity, thereby limiting exposure of handlers to highly concentrated (and potentially hazardous, spore containing) sample volumes. The reproducible precision of the Hybrid Unit also gives confidence when a signal is given that detects an agent in a given sample.

General Overview

The Hybrid unit consists of three major hardware components, custom software, and reagents for assays. While it is possible to design or adapt other protocols to this unit (part of the flexibility of the system) there are two defined baseline assays that the system is designed to run. What follows is information regarding Hardware, Software and Assay overviews. It is recommended that the user is familiar with all three sections prior to attempting to run assays or even initialize the machine.

Hardware Overview

The hardware that comprises the Hybrid can be divided into 3 major sub-groups. At the base of the unit is a Luminex^{100} IS Bead assay device. A separate manual is available for the details of this machine as well as the software to run it. On top of the Luminex^{100} is the fluidic manifold. The fluidic manifold contains the hardware for performing most of the unit operations in the assay. The manifold houses a flow-through thermocycler, heated hybridization chambers, chilled reagent reservoirs, 2 multi-position valves with manual control and position indicators, stirring bead reservoir and a Cavero pump for moving the fluids. See figure #1 for the locations of specific components. The fluidic manifold is connected to the Luminex^{100} IS with a manual 3-way valve. This allows the Luminex^{100} to be used independently for running control assays or calibrating the machine.

The final piece of hardware is the laptop computer. By use of the custom software (see next section), complete assays can be performed from the keyboard once the proper reagents are loaded. This cuts down on manually handling the reaction through many steps which could result in contamination of the reaction or the worker (or both). Protocols are included in this manual for keeping the system free of unwanted DNA and they should be followed closely.

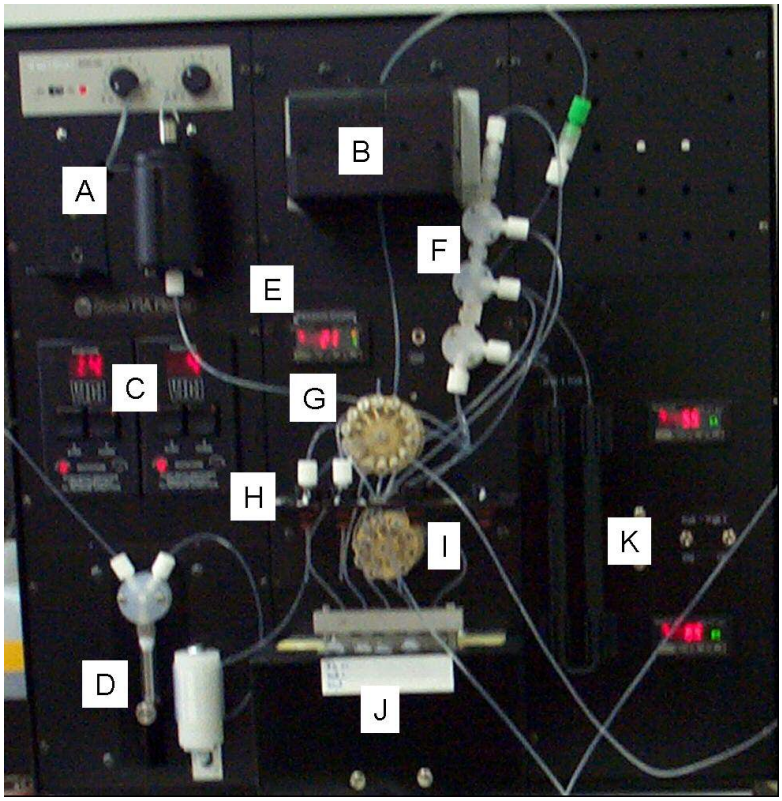


Figure 1: Fluidic Manifold layout

A) Bead stirrer and speed control
B) Flow-through Thermocycler
C) Manual valve controls and position display
D) Syringe pump
E) Reagent chiller temp. control
F) Bead sequestering cell
G) Valve #1
H) Reagent tube holder
I) Valve #2
J) Reagent chiller
K) Hybridization Chamber and temp controls

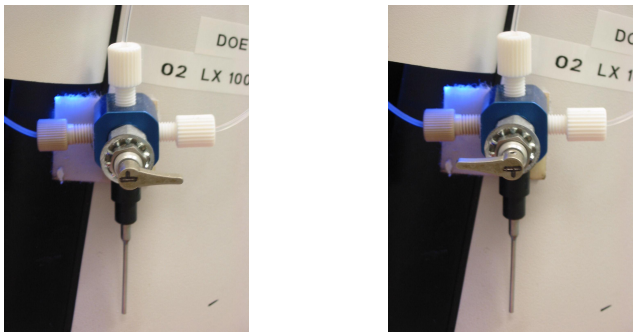


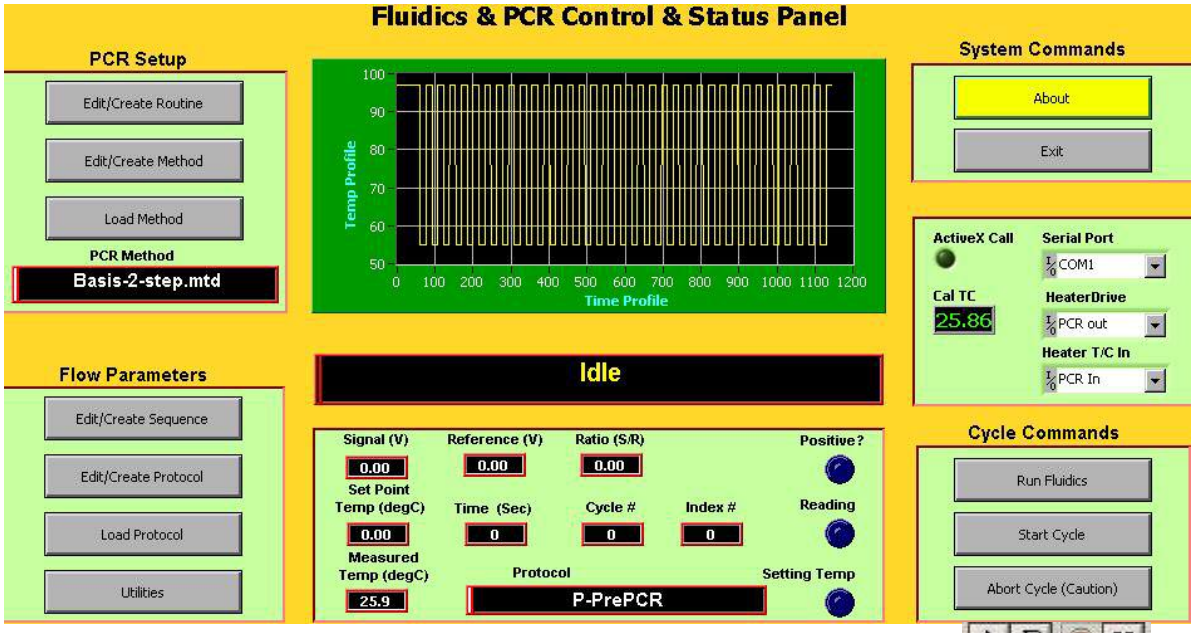
Figure 2: **A** **B**

A) Valve in UP position for connection to Fluidic manifold
B) Valve in DOWN position for use of needle probe

Software Overview

After the Fluidic Manifold and Luminex^{100} have been turned on (done by flipping the power switches on the rear panel of both instruments) the laptop computer should be turned on. You will be asked to log in. Current username and password is: raidds, and dna/rna1. The password is case- sensitive. Once the computer has completed the boot up procedure, there are two programs that must be loaded to provide control to the system. The first, the Luminex^{100} control software, is optional. If only Real-Time assays are to be run there is no need to load this software, or to even turn the Luminex^{100} itself on. If a bead assay is desired, simply double-click the Luminex icon on the screen to launch the software (included version is 1.7). The word "standby", seen in the upper, right corner of the screen will appear when the computer has connected to the Luminex and is ready to accept commands. Selecting the "Warm-up" button immediately is usually advisable since this will take 30 minutes to complete. Once this is started, minimize the program to visualize the desktop.

There is in icon labeled "- PCR -" next to the one for the Luminex. Launch this program by double-clicking it. Once completely loaded you will be presented with the main fluidic control software interface as seen below.



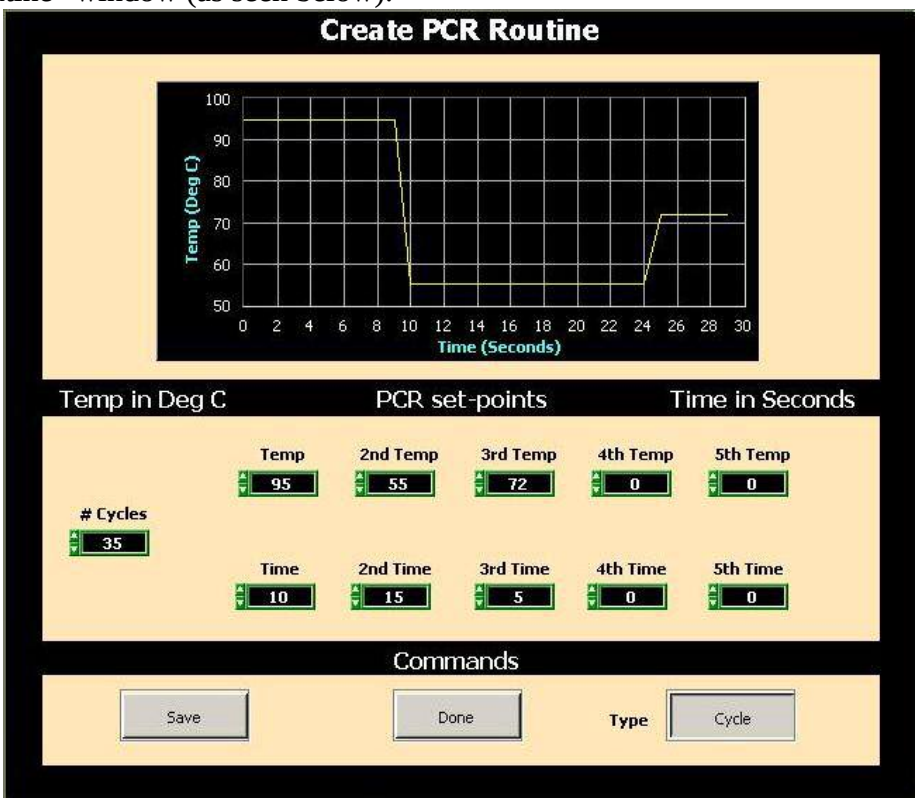
The first thing to notice is the arrow in the upper left corner. If it is white,  the system not in run mode. Click this arrow to select run mode and it will change to black,  . Notice also, that the red "stop" button is now active. You can select this button to stop the fluidics at anytime, however, if the syringe is already in motion it will complete its' current command before stopping.

There are three main control areas on the screen: PCR Setup, Flow Parameters and Cycle Commands. Each one will be explained individually.

PCR Setup

The PCR Setup section of the interface has three control buttons and a "PCR Method" readout. The "PCR Method" listed, if any, is the Method currently loaded to be executed. Prior to starting the run, a graphical representation of the pending method is displayed in the middle of the screen, bordered by green. If either of these is not present you have not correctly loaded the method as intended and you should not proceed with the assay.

The top button is "Edit/Create Routine". Clicking on this button brings up the "Create PCR Routine" window (as seen below).



From this window, thermocycling protocols can be designed and saved. A routine can be a hold of a given temperature for a set period of time (i.e. 95°C for 2 minutes), or a multi-step PCR cycling of up to 5 temperatures. Shown above is an example of a cycling parameter routine. This is a typical, 3-step PCR routine of 35 cycles. It can be combined with hold steps to create a complete method (see next paragraph) or be run by itself. If a hold is desired, clicking the "Type:" button will change the interface from "Cycle" to "Hold" mode (picture not shown). Entering the desired temperature and time is all that is required. Once a given routine is designed, it can be saved using the "Save" button. To return to the main interface, simply click the "Done" button.

The middle button, "Edit/Create Method" allows you to assemble Routines into a complete thermocycling protocol that is run by the software. Once you click this button you will be asked which Routine to open. Select the first Routine in your program (i.e. 95°C for 2 minutes). Click "Open". Continue to open Routines in the order that you would like them run. When you are finished, Save this Method using a unique name.

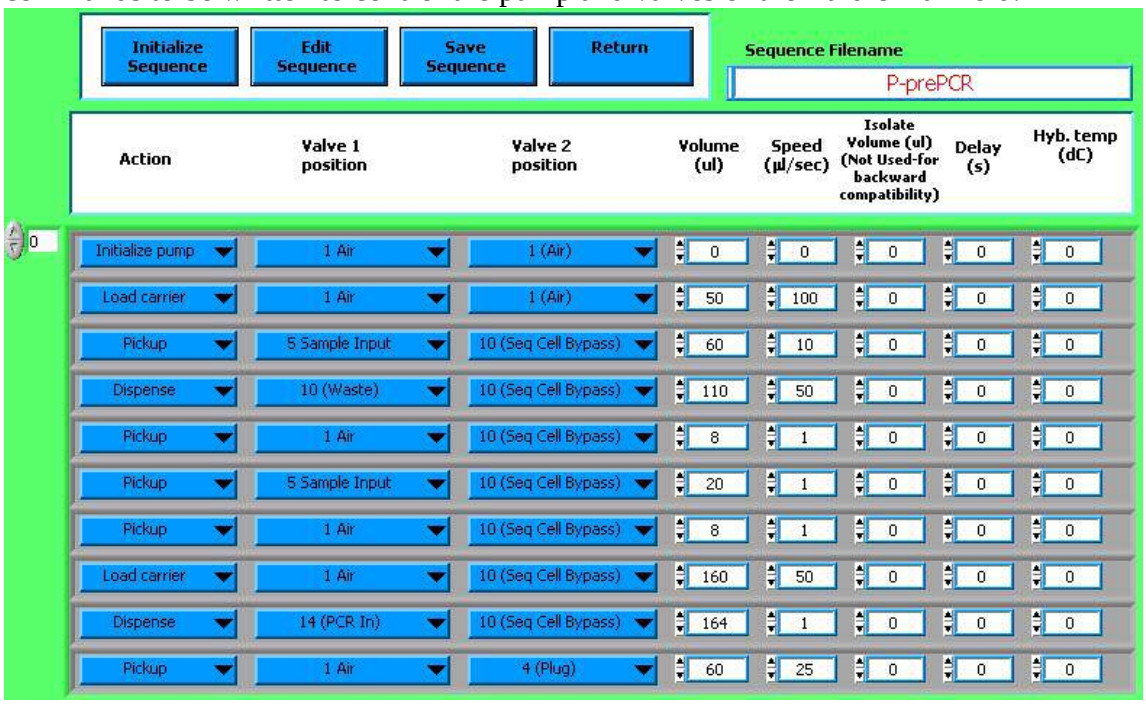
This process creates a Method file that is independent of subsequent changes to the parent Routine files. Changes to Routines DO NOT take effect in the Methods that have already been created. When a method is written it creates a file of NEW data, NOT a reference to a data file. Therefore, if changes are desired in a Method, a new Routine must be made, and a new Method using that Routine MUST be created.

The final button in this section is "Load Method". When this button is selected, you are presented with a list of created Methods that can be loaded. Select the one desired and click "Open". When this is done, the center of the main interface will display a representation of the Method on a graph of time vs. temperature. Visually inspecting this graph to verify that the Method follows the desired parameters is suggested to avoid errors. The Hybrid unit ships with a single Method in memory, (though many Routines are included, ready to build your own Methods) so as long as no new ones are created there is no chance of loading the wrong one. The standard 35-cycle, 2-step thermocycling Method included works with both the Real Time and the Multiplex Luminex Bead Assays that are described in a later section.

Flow Parameters

The Flow Parameters section of the interface contains four buttons, three of which control the fluidic manifold and a fourth that accesses misc. utilities that will not be covered in this manual (and will most likely not be needed by the common user).

The top button, "Create/Edit Sequence", will bring up an interface that allows complex commands to be written to control the pump and valves of the fluidic manifold.



There are four buttons at the top and they are as follows:

"Initialize Sequence" – clears all current commands and resets all fields.

"Edit Sequence" – allows you to select a previously saved sequence for changes.

"Save Sequence" – saves the current commands as a new sequence (will not automatically overwrite existing sequences).

"Return" – returns the user back to the main interface.

Designing a fluidic sequence, though it appears complicated at first, is actually very straightforward. All actions are performed by a single pump and controlled by the first field, "Action". Your choices are:

"Initialize Pump" – Empties the pump out the waste port and sets it to zero volume.

"Load Carrier" – Draws the requested volume from the supply bottle.

"Empty Syringe" – similar to Initialize Pump command, but the valve positions can be manually selected.

"Pick-up" – draws the selected volume into the pump with the valves at user defined positions.

"Dispense" – pushes the selected volume through the valves at user defined positions.

Note: It is typical to begin a lengthy sequence with a command to Initialize the pump. Sequences are connected to form Protocols and if the pump is sent a request to draw or dispense more volume than there is space for it will by-pass that command and proceed with the next. Therefore, resetting the pump is desirable as a safety measure.

Select the pump action required. Next, set the positions for the 2 valves with the pull-down menus. Each position is labeled to make programming more straightforward (e.g. "1 Bleach"). Add the volume to be moved by the syringe (1-1000uL), and the speed for that movement (range of 1-200uL/sec). Once a rate is set, the pump will continue to operate at this rate until a new value is supplied. For example, if you have a sequence that involves 10 steps at a rate of 50uL/sec. each, you only need to set the rate to 50 for the first step and leave the rest at 0.

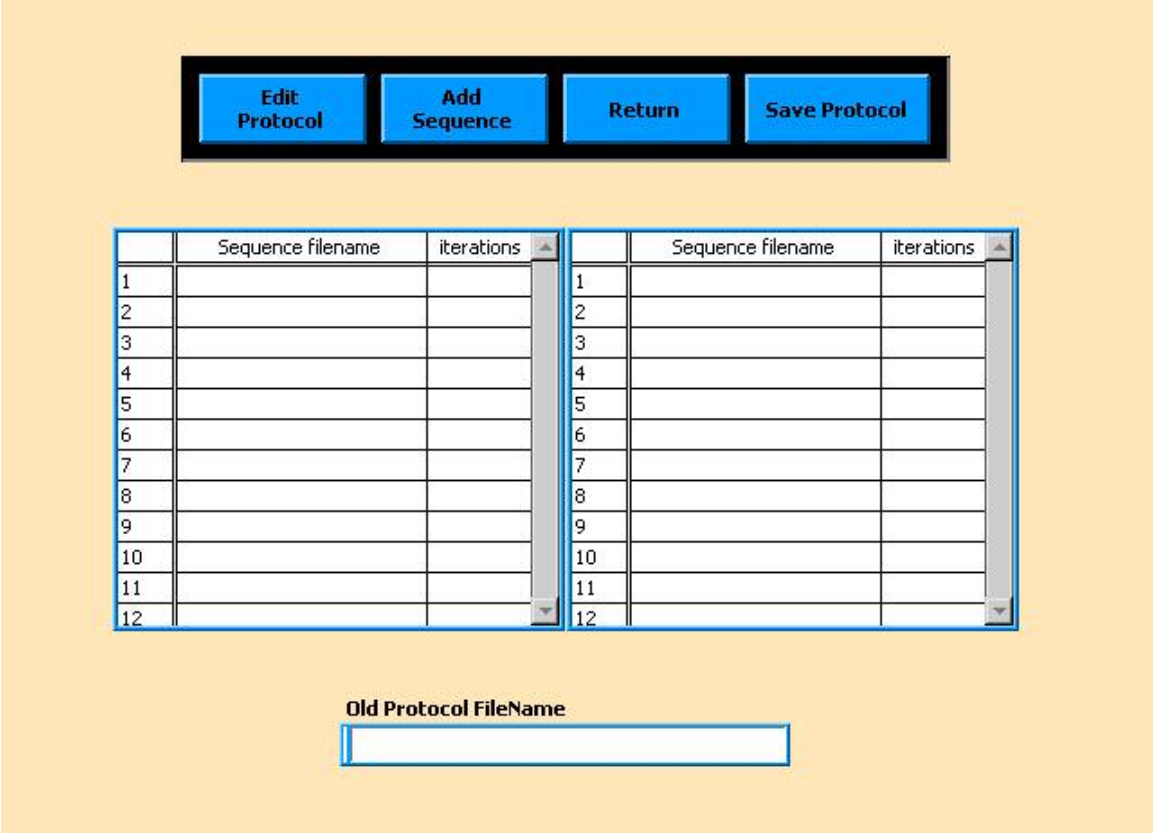
If required, a delay in seconds can be added. If the system has air in the lines, (a compressible medium) it may take a few seconds to equalize pressure after a pump action. By adding a delay prior to the next valve motion you maintain zero system pressure between steps for increased reproducibility in moving fluids. In general, however, minimizing the amount of air in the system is highly desirable.

On the far left of the screen are two arrows and a counter. If the sequence contains more steps than can be view on the screen at a time, you can move up and down the list by clicking the arrows.

Saving a sequence is as simple as clicking the "Save Sequence" button and giving the file a name. Clicking "Return" will bring you back to the main interface.

The second button in this section is to “Edit/Create Protocol”. Sequences are linked together to form executable Protocols, much in the same way the Routines are linked to form Methods. There is one distinct difference between the two that is important to remember. Changes directly to Sequences WILL affect the Protocols that run them and Protocols do NOT have to be rewritten if a change is made. The Protocol files are references to the sequence files themselves so care must be taken that a change in one Protocol that uses a Sequence does not affect a second Protocol that uses the same Sequence for a different assay. In this regard, it is advised that unique Sequences are used for each Protocol.

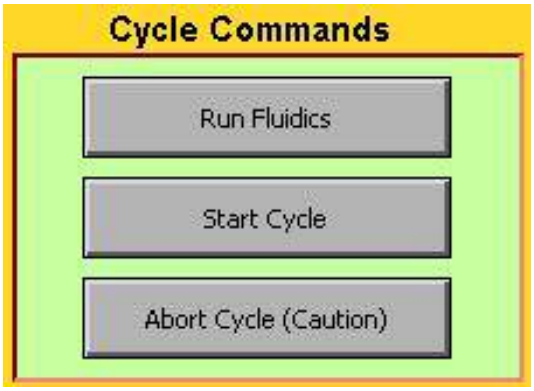
Once the sequence is saved, return to the main interface and select “Edit/Create Protocol”. This will bring up the window below.



To view an existing Protocol, click “Edit Protocol” and select the file to be viewed. A list of the Sequences in this Protocol will be shown on the Left column and the Filename will be seen in the lower box. This Protocol can not be edited in the “word processing” sense of the word. Clicking “Add Sequence” and selecting a Sequence from those listed will place this file in the first row of the Right column. It will NOT concatenate onto the end of the existing list, nor can it be simply added to the beginning or in the middle. To make changes you will need to re-enter all sequences in order and then Save the new Protocol (possibly with the old name if the update is to replace an outdated file). If a new Protocol is to be written, or if the old file does not need to be viewed first, simply select “Add Sequence” to begin adding files and “Save Protocol” when you are finished. The “Return” button will close this box and return you to the main interface.

The third button in this section, “Load Protocol” will actually load three Protocols in sequence. When selected, a box will open that allows the user to select the Pre-PCR Protocol. Once selected, the same box will ask for a Post-PCR Protocol, followed by a Post-Positive Protocol. The Pre-PCR Protocol will be the first set of sequences run by the fluidic manifold. Once these sequences are completed, the thermocycler will automatically run the loaded Method (if one has been loaded). If a Positive is detected, which will only happen with a Real Time assay (see section below), then the Post-Positive Protocol will be run once the Method is complete. If no positive is detected, the Post-PCR Protocol will run. This allows for automated decontamination or further manipulation of the PCR product, as will be seen in the specific sections.

Running the Desired Protocols

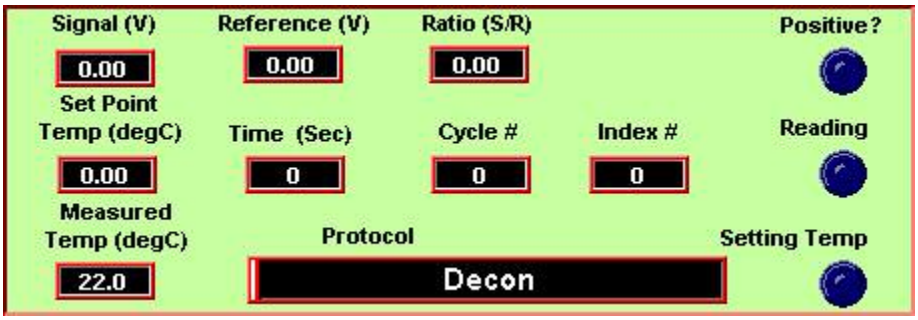


There are two buttons for starting a loaded protocol and/or method. The first, “Run Fluidics” will run the fluidics protocol that is loaded, but will NOT require, nor run any temperature method in the thermocycler. Though the software requests Post-PCR and Post-Positive PCR Protocols, they will not be run. Therefore, the user should select “Dummy” when asked for these protocols. The primary use of this button is to run decontamination or initialization protocols that are separate from thermocycling.

“Start Cycle” will run the pre-PCR fluidics protocol that is loaded, followed by the cycling Method, then the Post-PCR fluidics protocol. If a positive signal is detected (see section below) the Post-Positive PCR Protocol will be run instead of the Post-PCR Protocol.

Signal Detection and Feedback

The middle, lower section of the main software interface (see below) has several read-outs that inform the user of the state of the thermocycler.



- Signal: The reading of the florescence in the thermocycler of the FAM dye used in the Real Time assay. The values at each time-point are recorded as channel 1 (see next section for details)
- Reference: The signal of the TAM dye that can be used as a background florescence. The values are recorded as channel 2
- Ratio: The ratio of Channel 1/Channel 2 is displayed here. If this value reaches or exceeds 1.2, the software will call the reaction a "Positive".
- Set Point Temp: The temperature that the thermocycler is currently set to reach by the Method.
- Measured Temp: The current, actual temperature of the thermocycler.
- Time: A countdown in seconds. When the counter reaches 0 (zero), the software will proceed to the next step in the Method.
- Cycle #: An indication of the current cycle in the Method.
- Index: a counter not used in the current configuration of the software.
- Protocol: Lists the Pre-PCR Protocol loaded and running. The window is a good way to verify that the correct Protocol has been loaded.
- Positive: Will change to Red (from Blue) if the Ratio reaches 1.2. This will stay on for the remainder of the run as a warning that DNA has been detected.
- Reading: Will be Red while the thermocycler is taking a florescence reading.
- Setting Temp: Will be Red while the thermocycler is changing temperature to reach a Set Point. Will return to Blue when the set point has been achieved within 1°C.

Note: Signal will be detected regardless of the assay being run. Background florescence is ~0.33. If a Luminex, multiplex assay is being run that does not have a FAM floraphore included, the optics will only detect the background values.

Morning Initialization of the Unit

- Turn on the laptop, Hybrid box and Luminex. The switches are toggles are located in the back of the machines by the power cord. Log into the laptop using "raidds" as the user name, and "dna/rna1" as the password.
- Turn on the Hybridization heaters (Figure 1, K). Make sure Hyb. #1 is set to 83°C and Hyb. #2 is set to 55°C.
- Verify thatthe 3 -way valve that connects the fluidics manifold to the Luminex has the arrow pointing UP (Figure 2, A). The arrow pointing DOWN (Figure 2, B) will allow for calibration of the Luminex through the needle probe, but this must not be done while the fluidics manifold is operating or system pressure could build up and damage the system.
- Launch the Luminex control software by double-clicking the red, circle icon in the middle of the screen labeled, "Luminex". When the software has completely loaded, you will see "Standby" in the upper right-hand corner, begin the warm-up protocol as outlined in the Luminex documentation.
- On the Hybrid unit, refill the H2O supply bottle with PCR grade water and empty the waste bottle. Launch the control software by double-clicking the icon labeled "- PCR –".
- Decontaminate the system using the protocol from the next section.

Decontaminating the System

One of the most crucial steps in running the Hybrid unit is decontaminating the tubing of amplified product after each run. There are 3 steps, selecting from 2 decontaminating protocols. For each, the loading and running procedure for the software is the same. For each step, the appropriate solution should be placed in a standard 15ml conical tube and place on the regent tube holder (Figure #1, H). This holder has 4 places for tubes. 3 of these will hold 1.5ml tubes. The correct holder is identified by a **RED** finger nut.

- Click the "Load Protocol" button and Select **Decon** for the pre-PCR protocol. Select **I-ini** for the Post-PCR and Post-Positive PCR protocols.
- Place a 15mL conical tube containing 0.02% TWEEN 20 on the holding rack. Do not exceed 13mL total. Each run requires ~2mL.
- Click the "Run Fluidics" button on the main interface. Wait until the run is complete as evidenced by this button being available again.
- Click the "Load Protocol" button and Select **Decon2** for the pre-PCR protocol. Select **I-ini** for the Post-PCR and Post-Positive PCR protocols
- Place a 15mL conical tube containing 20% Bleach on the holding rack. Do not exceed 13mL total. Each run requires ~6mL.
- Click the "Run Fluidics" button on the main interface. Wait until the run is complete as evidenced by this button being available again.
- Click the "Load Protocol" button and Select **Decon** for the pre-PCR protocol. Select **I-ini** for the Post-PCR and Post-Positive PCR protocols.
- Place a 15mL conical tube containing 70% Ethanol on the holding rack. Do not exceed 13mL total. Each run requires ~2mL.
- Wipe the outside of the sample loading tube, identified by the **BLUE** finger nut and attached to position 13 on the upper valve. Wiping the outside of the tubing with 20% Bleach, followed by 70% Ethanol and finally clean water will remove any genomic DNA that is stuck from the previous sample.

Repeat this series as many times is necessary if a persistent positive is detected with negative control. Though a single run of washes is generally sufficient, if the system can not be cleaned with 3-4 washes and all reagents have been shown to be uncontaminated, the upper valve may need to be disassembled and cleaned. Extreme care must be taken with the valve face as it can be cracked if too much pressure is applied. This procedure is recommended only if all other washing attempts have failed.

- Remove all lines going into the valve face by simply unscrewing them with fingers only. Label with tape since the lengths of each tube is specific to its' location.
- Using an Allen wrench, remove the 3 screws holding the valve face to the valve body. (Loosen each one full turn before completely removing).
- Soak all parts in 20% Bleach for 1 hour and rinse with LARGE amounts of water.
- Reassemble in the reverse order taking care to balance the tightening of the Allen screws. Re-attach all tubing lines to finger tightness only.
- Run the standard Decontamination protocol above to prime tubing with fresh fluids.

Running a RT (Real Time) PCR assay

In order to run an RT assay in the Hybrid you will need to first decontaminate the system using instructions found in the previous section. Appendix A gives the required components for the PCR reaction mix. Probe concentration is not listed and must be determined for each new batch of probe received. When the system takes its' first optic reading, it is best to have a Signal reading from 0.6-1.0. Prepare the reaction mix including DNA (unless this is to be a negative). Prepare 1X Mg/Buffer to a volume of at least 120uL.

Click "**Load Method**" and select "**35-cycle-2-step**".

Click "**Load Protocol**" and select, in order, "**PrePCR**", "**decon**" and "**decon**".

Place the tube of containing >2ml of 0.02% TWEEN 20 onto the bleach loading tube. (RED finger nut as seen on Figure #1)

Place the tube of containing 80ul of 1X Mg/Buffer onto the sample loading tube. (**BLUE** finger nut as seen on Figure #1)

Click "**Start Cycle**".

The system will draw up the buffer, send an aliquot to the thermocycler, and clear the line. Once the line is clear, the system will pause for 10 seconds. During this time, remove the tube containing the 1X Mg/Buffer and replace it with one containing the 25uL PCR reaction to be amplified.

The system will draw up the full volume, but will only send a 20uL plug into the thermocycler. The remaining 5uL will be sent back into original tube. Once the plug is in position and the temperature cycling has started it is important not to manually change the valve positions. The system is designed to place the valves in a position such that the pressure that is built up by the increased temperature is not able to shift the plug away from the heater. Switching the valves to an open line would allow the pressure to shift the plug and thermocycling would then no longer act on the PCR reaction mix.

The optics will begin taking a reading at the end of the first 55°C step. The ideal reading should be between .6 and 1.0. Varying the amount of probe in the reaction will directly affect this reading and should be tested for each new batch of probe received.

When the cycling has reached its' final cycle it will stop and generate a data file containing the florescence reading at each cycle. The file is named containing the date and time of the run and found in the following path. C:\PCR\PCR_Data. An example file would be P07_10_03_1200.dat. This file would be for a run completed at noon on July 10, 2003. The .dat file can be opened with Excel. The "A" column contains the reading from channel #1 and is the relevant information for this assay.

Post run analysis is the final step and can be performed while the decontamination of the system is running. Highlight the cells of column A in the file containing the run data. Select Copy (from the Edit pull-down menu or by Right-clicking). Open the Real-Time Data Analysis spreadsheet from the shortcut on the desktop. This sheet contains space to compare up to 10 runs against a standard curve. Select the first cell under the name of the sample you wish to enter (eg. Sample A). Right-click and select Paste Special. Click the radio button for "values only" and click "OK". On the sheet, the run will show up on the graph to the far left. A normalization and background subtraction will be performed and an estimation of Mass in the sample will be generated.

Running a Luminex Bead Assay

Similar to the Real Time assay, the system must be properly decontaminated prior to running a Luminex Multiplex Bead assay. Many of the steps from the Real Time assay will be followed here as well as several additional ones. Most of the complexity of the assay comes from properly setting the Luminex software and hardware. To handle this, the user is directed to the Luminex 100 User's Manual version 1.7. Only details specific to its' integration with the fluidic manifold and the test assay will be covered here.

- While performing the washes, the blue, 3-way valve must be in the DOWN position to allow fluid to be drawn up the needle. Do not run any protocols on the fluidics manifold with the valve in this position.
- If any loading of beads is done through the needle, such as calibration or enumeration of bead stocks, do not change the valve position until a bleaching and 3 washes have been performed through the needle, followed by a wash with the valve in the UP position. Failure to do this may result in carryover into the next run.
- Though the optics are still active during the amplification, there is no florescent probe in the mix to give a signal, therefore be aware that the output file generated by the software will only contain background signal.\
- Decontamination must be performed AFTER the Luminex run has completed. If it is run before the Luminex has finished, the beads will be washed away by the cleaning solution and no signal will be obtained.

Reagents and PCR mix details can be found in Appendix A. Prepare the following tubes:

Reagent	**Volume/run**	**Loading tube**
1X PCR Lead Buffer	80uL	BLUE
PCR mix	25uL	BLUE
SAPE	100uL	YELLOW
Bead mix	40uL	GREEN

Verify that the settings of the hybridization heaters are set correctly as displayed by the digital readout (Figure #1, K). Hyb #1 should be set at 82°C and Hyb #2 at 53°C.
Click "**Load Method**" and select "**35-cycle-2-step**".
Click "**Load Protocol**" and select, in order, "**PrePCR**", "**Full_Protocol**" and "**I-ini**".
Place the tube of containing 80ul of 1X Mg/Buffer onto the sample loading tube. (**BLUE** finger nut as seen on Figure #1)
Click "**Start Cycle**".
The system will draw up the buffer, send an aliquot to the thermocycler, and clear the line. Once the line is clear, the system will pause for 10 seconds. During this time, remove the tube containing the 1X Mg/Buffer and replace it with one containing the 25uL PCR reaction to be amplified.
Once the amplification step has completed, the fluidics manifold will automatically run through the necessary steps including bead addition, hybridization, SAPE addition and incubation. When the entire protocol has completed, the greyed-out button for "Run Fluidics" will return to normal and valve # will be set to position #4. Once the system

reaches this step, change to the Luminex control software and simply click the Start button. The beads will be drawn into the Luminex and counted as though it were being run manually through the needle.
Once the count is complete, proceed with the decontamination steps. One addition to the protocol is that once the decontamination steps are completed, select “WASH” from the Luminex menu to pull water through the 3-way valve to clear residual beads.

Appendix A

Assay Components

Taqman Real-Time Components and Mix

PCR reaction mix			**1X PCR Lead Buffer**	
Water	12.0 ul		Water	78 ul
10X PCR Buffer	2.5 ul		10X PCR Buffer	10 ul
50mM Mg2+	3.0 ul		50mM Mg2+	12 ul
10uM Fwd primer	0.5 ul		**Total/rxn**	100 ul
10uM Rev primer	0.5 ul			
10uM FAM probe	0.5 ul			
10mM dNTPs	0.5 ul			
5Unit/ul plat. Taq	0.5 ul			
DNA sample	5.0 ul			
Total/rxn	25.0 ul			

Thermocycling Protocol	
Temperature	**Time**
97°C	60”
35 Cycles of:	
55°C	15”
97°C	15”

Appendix A (cont.)

Assay Components

Luminex Components and Mix

PCR reaction mix			**1X PCR Lead Buffer**	
Water	12.5 ul		Water	78 ul
10X PCR Buffer	2.5 ul		10X PCR Buffer	10 ul
50mM Mg2+	3.0 ul		50mM Mg2+	12 ul
10uM Fwd primer	0.5 ul		**Total/rxn**	100 ul
10uM Rev primer	0.5 ul			
10mM dNTPs	0.5 ul		**1X SAPE**	
5Unit/ul plat. Taq	0.5 ul		1 mg/ml SAPE	6 ul
DNA sample	5.0 ul		1X TMAC Buffer	994 ul
Total/rxn	25.0 ul		**Total**	1000 ul
			(use ~100 ul/rxn)	
1X TMAC (100ml)				
5M Tetramethyl Ammonium Chloride		45.0 ml		
20% SDS		0.375 ml		
1M Tris HCl, pH 8.0		3.75 ml		
0.5 M EDTA		0.300 ml		
Purified Water		50.575 ml		

Thermocycling Protocol			**Hybridization Protocol**	
			(set within Hybrid software)	
Temperature	**Time**			
97°C	60”		**Temperature**	**Time**
35 Cycles of:			82°C	3’
55°C	15”		53°C	5’
97°C	15”			

Appendix B

Sequence and Protocol List

Protocol	Sequence list
Decon	PCR_Prime Bleach Line
	Bleach Lines
	waterflush
	waterflush
	Prime Lines
Decon2	PCR_Prime Bleach Line
	Bleach Lines
	Bleach Lines
	Bleach Lines
	waterflush
	waterflush
	Prime Lines
I-ini	I-ini
waterflush	waterflush
P-prePCR	P-prePCR
Full_Protocol	H-MeterPCR
	B-PrimeBeads
	B-MeterBeads
	B-DispMeteredBeads
	B-EmptyBeadLine
	B-mixBeadPCR
	H-beadPCRHyb
	B-SeqBeads
	B-MeterSAPE
	B-LiberateBubble
	B-LiberateBeads
	L-ToLuminex

Appendix C

Sequence Details

Action Key:
1: initialize pump
2: load carrier
3: empty syringe
4: pick up
5: dispense

VP1= Position of Valve #1
VP2= Position of Valve #2

PCR_Prime Bleach Line

Action	VP1	VP2	Vol.	Rate			
2	1	1	100	200	0	0	0
4	1	1	130	20	0	0	0
5	12	1	230	20	0	0	0

waterflush

Action	VP1	VP2	Vol.	Rate			
1	12	2	0	0	0	0	0
2	14	2	1000	200	0	0	0
5	14	2	1000	0	0	0	0
2	10	2	1000	0	0	0	0
5	10	2	1000	0	0	0	0
2	11	2	1000	0	0	0	0
5	11	2	300	0	0	0	0
5	11	10	700	0	0	0	0
2	9	3	1000	0	0	0	0
5	9	3	1000	0	0	0	0
2	4	1	1000	0	0	0	0
5	4	1	1000	0	0	0	0
2	3	1	1000	0	0	0	0
5	3	1	1000	0	0	0	0
2	13	1	1000	0	0	0	0
5	13	1	1000	0	0	0	0

Bleach Lines

Action	VP1	VP2	Vol.	Rate			
2	2	1	50	50	0	0	0
4	2	1	20	0	0	0	0
4	1	1	600	0	0	0	0
5	4	1	200	0	0	0	0
5	3	1	200	0	0	0	0
5	13	1	200	0	0	0	0
3	12	1	0	200	0	0	0
2	12	1	1000	0	0	0	0
3	12	1	0	0	0	0	0
2	12	1	1000	0	0	0	0
3	12	1	0	0	0	0	0
4	2	1	20	50	0	0	0
4	1	1	780	0	0	0	0
4	2	1	20	0	0	0	0
3	14	10	0	0	0	0	0
2	12	1	1000	200	0	0	0
3	12	1	0	0	0	0	0
2	12	1	1000	0	0	0	0
3	12	1	0	0	0	0	0
4	2	1	50	50	0	0	0
4	1	1	650	0	0	0	0
5	11	2	200	0	0	0	0
5	10	2	200	0	0	0	0
5	9	3	200	0	0	0	0
3	12	1	0	0	0	0	0
4	2	1	200	0	0	0	0
5	1	1	200	0	0	0	0

Prime Lines

Action	VP1	VP2	Vol.	Rate			
2	1	1	50	50	0	0	0
4	2	1	350	0	0	0	0
5	4	1	100	0	0	0	0
5	3	1	100	0	0	0	0
5	13	1	100	0	0	0	0
3	12	1	0	0	0	0	0

I-ini

Action	VP1	VP2	Vol.	Rate			
1	10	5	0	0	0	0	0

P-prePCR

Action	VP1	VP2	Vol.	Rate		Delay	
1	2	1	0	0	0	0	0
2	2	1	50	100	0	0	0
4	13	10	89	10	0	0	0

5	12	10	139	50	0	0	0
4	2	10	8	10	0	0	0
4	13	10	50	10	0	0	0
4	2	10	3	10	0	0	0
5	14	10	61	10	0	0	0
4	2	1	89	50	0	0	0
5	13	1	89	50	0	10	0
2	13	1	50	50	0	0	0
4	13	1	89	10	0	0	0
3	12	10	0	0	0	0	0
4	2	1	8	10	0	0	0
4	13	1	20	10	0	0	0
2	2	10	160	50	0	0	0
5	14	10	157	1	0	0	0
4	2	4	89	25	0	0	0
5	13	4	89	10	0	0	0

H-MeterPCR

Action	VP1	VP2	Vol.	Rate			
1	13	4	0	0	0	0	0
2	13	4	284	100	0	0	0
5	14	2	284	5	0	0	0

B-PrimeBeads

Action	VP1	VP2	Vol.	Rate			
2	14	2	50	100	0	0	0
4	3	2	83	10	0	0	0
5	12	2	133	50	0	0	0

B-MeterBeads

Action	VP1	VP2	Vol.	Rate		Delay	
4	2	2	5	10	0	0	0
4	3	2	25	10	0	2	0
4	2	2	5	10	0	0	0

B-DispMeteredBeads

Action	VP1	VP2	Vol.	Rate		Delay	
2	10	10	52	50	0	0	0
5	10	10	87	10	0	2	0

B-EmptyBeadLine

Action	VP1	VP2	Vol.	Rate		Delay	
4	2	10	65	20	0	2	0
5	3	10	65	30	0	2	0

B-mixBeadPCR

Action	VP1	VP2	Vol.	Rate			
2	14	10	55	50	0	0	0
5	14	10	2	5	0	0	0
5	10	10	40	5	0	0	0

H-beadPCRHyb

Action	VP1	VP2	Vol.	Rate		Delay	
5	12	9	5	0	0	120	0
2	10	9	50	50	0	0	0
5	10	10	50	5	0	0	0
5	12	9	5	0	0	300	0

B-SeqBeads

Action	VP1	VP2	Vol.	Rate			
2	11	2	200	100	0	0	0
5	11	2	200	20	0	0	0
2	10	3	114	100	0	0	0
5	10	3	114	5	0	0	0

B-MeterSAPE

Action	VP1	VP2	Vol.	Rate		Delay	
4	4	3	84	10	0	0	0
5	12	3	84	50	0	0	0
4	2	3	5	20	0	0	0
4	4	3	80	10	0	0	0
4	2	3	5	0	0	0	0
2	11	10	90	100	0	0	0
5	11	10	120	10	0	0	0
5	11	3	60	0	0	0	0
4	2	10	84	20	0	60	0
5	4	10	84	0	0	0	0

B-LiberateBubble

Action	VP1	VP2	Vol.	Rate		Delay	
2	2	10	83	100	0	0	0
4	2	10	20	20	0	1	0
5	11	10	30	10	0	0	0
5	11	3	73	10	0	1	0

B-LiberateBeads

Action	VP1	VP2	Vol.	Rate		Delay	
2	9	3	60	100	0	0	0
5	9	2	60	167	0	5	0

L-ToLuminex

Action	VP1	VP2	Vol.	Rate			
2	11	2	355	100	0	0	0
5	11	2	355	5	0	0	0
4	1	4	5	0	0	0	0

Appendix D

Valve Positions

Valve 1 (upper)

1	Air
2	Bead reservoir
3	Small volume bead supply
4	SAPE supply
5	Sample Input
6-9	Chilled reagent
10	Waste
11	Bead liberation line
12	Sequestering Cell Input
13	Hybridization Cell Input
14	PCR Chamber Input
Center	To Cavero Pump

Valve 2 (lower)

1	Air
2	Sequestering Cell Out
3	Sequestering Waste Out
4-9	Plug
10	Sequestering Cell Bypass
Center	To Luminex Valve