Seattle Sensor Systems
CORPORATION

February 2013

The Measurement of Free-Cortisol from Saliva Samples

A fast and sensitive quantitative assay for use in the monitoring of animal stress

Free-Cortisol Measurement Assay - CT 1317

Associated Consumable Kit – KCT 1317

CONTENTS

1. Limitations and Cautionary Notices 3
2. Materials Provided and Storage Instructions 4
   1. Hardware and Core Equipment (specialized items) 4
   2. Consumables (standard lab items) 4
   3. Specific Assay Components (Can be ordered separately as a pre-configured kit – KCT 1317) 4
3. Preparing the SPIRIT Instrument for Use 6
   1. Setup 6
   2. Initialization 6
   3. Shut down and storage 7
4. Assay Procedures 8
   1. Instrument Familiarization - Cortisol Measurement in Buffer 8
   2. Free-Cortisol Assay and Analysis Protocol 9
Appendix 1 – Loading Parameters for the Cortisol Assay 10
Appendix 2 - Data Analysis via the Spirit Analysis Software 14
   1. Introduction: 14
   2. Installation: 14
   3. Analysis Operation: 16
Appendix 3 - Instrument Familiarization - Cortisol Measurement in Buffer 18
   Detailed Protocol 18
Appendix 4 – Saliva Depletion and Spiking for Saliva Reference Curve Generation 24
   Detailed Protocol 24
Appendix 5 – Assay Procedure for Reference Curve Generation and Endogenous Free-Cortisol Analysis 26
   Detailed Protocol 26

1. Limitations and Cautionary Notices

NOT FOR USE IN DIAGNOSTIC PROCEDURES - FOR RESEARCH USE ONLY

- The kit should not be used beyond six months of the date of manufacture.
- Do not substitute reagents or mix with those from other kits or sources.
- Before using this kit, read the user manual for the SPIRIT instrument and familiarize yourself with the use and operation of the device. Any variation in techniques used, kit age or reagents may cause variation in molecular binding rates and impact results achieved from this kit.
- Differences in results may be introduced by variations in sample preparation and treatment, including storage.
- While this assay has been designed to eliminate interference through non-specific binding by other contaminants and factors that may be present in samples used in this assay, until all such potential contaminants and factors have been tested, the possibility of interference cannot be excluded.

2. Materials Provided and Storage Instructions

1. Hardware and Core Equipment (specialized items)

Item	Quantity	Description	Storage and Handling	Usage
#1	1	1ml pipettor (p1000)	Store at room temperature	Sample preparation
#2	1	200ul pipettor (p200)	Store at room temperature	Bead and antibody
#3	1	20ul pipettor (p20)	Store at room temperature	Bead and antibody
#4	25mg	Anti-cortisol antibody coated immunomagnetic beads in PBST with 0.05% azide 2.5ml	Keep refrigerated and sealed until use. Handle with care, reusable.	Extraction of analyte from saliva or plasma
#5	1	Magnetic separation rack (MSR) for analyte separation	Store at room temperature	Separation of beads from matrix

2. Consumables (standard lab items)

Item	Quantity	Description	Storage and Handling	Usage
#1	1 box	1ml pipettor tips	Store at room temperature	Sample and reference
#2	1 box	200ul pipettor tips	Store at room temperature	Antibody and bead
#3	1 box	20ul pipettor tips	Store at room temperature	Antibody and bead
#4	1	Small petri dish	Keep with kit	Rinsing sensors
#5	1 bag	1.5ml microfuge tubes	Store at or below room temperature	Preparing samples and controls
#6	1 box	15 ml conical tubes	Store at room temperature	Preparing controls in bulk

3. Specific Assay Components (Can be ordered separately as a pre-configured kit – KCT 1317)

Item	Quantity	Description	Storage and Handling	Usage
#1	3	Buffer Solution with 0.05% azide 50ml	Store at or below room temperature	General buffer
#2	1	15 mM NaOH Regeneration	Store at or below	Regeneration of

		Solution 15ml	room temperature	sensor surface
#3	2	Waste Repository Tube	Dispose following regulations	Waste collection
#4	10	Sample Injection Syringe (1ml)	Dispose following regulations	Sample injection
#5	10	Medium syringes for washing (3ml)	Dispose following regulations	Fluidics rinse following initialization
#6	2	Cortisol Biosensor	Keep refrigerated and sealed until use.	Used in instrument
#7	1	BSA Biosensor	Keep refrigerated and sealed until use.	Used in instrument
#8	1	Cortisol stock (1 mg/ml)diluted in methanol -2ml tube	Keep refrigerated and sealed until use.	Used to create positive control samples
#9	2	Antibody buffer for no cortisol controls with 0.05% azide 15ml	Keep refrigerated and sealed until use.	Used to create negative control samples
#10	250ug	Anti-cortisol antibody 2 mg/ml (needs diluting before use) with 0.05% azide	Keep refrigerated until use.	Used in sample assay and in controls
#11	1	Glycine pH 2.2 with 0.05% azide 10ml	Keep refrigerated until use	Elution of cortisol from beads
#12	1	Initialization Solution with 0.05% azide 15ml	Keep refrigerated until use.	Preparing cortisol sensor following installation in instrument
#13	5	Cortisol Dilution Vials – 0.5, 1.0, 1.5, 2.0, 3.0 ng/ml in antibody buffer with 0.05% azide 10ml	Keep refrigerated until use.	Instrument familiarization, performance monitoring
#14	1	Shutdown Solution with 0.05% azide 50ml	Store at or below room temperature	Used prior to long term storage
#15	2	Syringe Needles (sample & initialization)	Store at room temperature	Sample and initialization solution injection
#16		<Reserved for future use>		

3. Preparing the SPIRIT Instrument for Use

1. Setup

Unpack all items in the provided assay kit; be sure to identify each item correctly using the table above and labels on items.

Immediately prior to use, the sensors will need to be prepared by rinsing with Buffer Solution (#1) in the provided Petri Dish (#11). Holding the sensors with the gold surface down and the connector pointed up, immerse the gold surface in the solution and agitate manually for at least one minute. Take care not to touch or scratch the gold surface or the electrical connectors on the sensors. This step will remove the protective shipping coating on the cortisol and prepare the sensors for first use.

Follow instructions in "SPIRIT Operations & Appendix" document for inserting sensors into their respective compartments in the instrument. Two cortisol biosensors are provided in the kit. They are to be used in parallel, and should be reliable for a minimum of 40 detection cycles. Place the first cortisol sensor (cortisol #1) into sensor position 4, the right-most slot as you face the instrument and the second cortisol sensor (cortisol #2) in position 3. Place the BSA background sensor in slot #3. You can use one of the sensors shipped with the instrument as a "dummy" sensor in position 1. Data read from this slot is not used in the detection algorithm.

Insert the, buffer solution (#1), regeneration solution (#2) and waste container (#3) into the instrument (see p16 of the operators manual).

Follow the instructions in the SPIRIT Operations and Appendix document for connecting the instrument to the laptop and starting the software. Note that you will select a location for storing the instrument data file. Make note of this because you will need it when you run the SPIRIT Analysis Software.

If the unit is "dry" after a period of storage or shipment, you will need to prime the fluidics circuits to remove all the air before using the instrument. Inject 1.0ml of Buffer Solution (#1) into the injection port. Using the software interface, select "Flow" and run buffer solution through the instrument for at least 5 minutes or until no more bubbles are seen in the lines and liquid is flowing into the waste container.

2. Initialization

Since this is a new assay for your instrument, you must program in the protocol sequence and store it before use. Follow the instructions at the back of this assay document in the Appendix 1 for loading the parameters for the cortisol detection assay into the instrument and operating software

Follow the instructions on pages 21 and 22 of the "SPIRIT Operations & Appendix" document for initializing the instrument and calibrating the sensors for first use. Use the provided syringe (#5) and the initialization solution (#12). Please attach the metal syringe needle to the plastic body before use, and use dedicated syringes and needles (labeled "I" [initialization] or "S" [sample injection] for the appropriate applications. Note that these are dull tipped needles.

Also take the time to install the Analysis Systems software that will be used to analyze the datasets created by the Spirit Instrument. Please follow the instructions in Appendix 2.

Initialization only has to be performed when <u>any</u> new sensor is mounted into one of the sensor slots in the instrument. So when you change sensors for any reason please repeat this initialization. Note that when changing sensors you must always de-pressurize the fluidics so that buffer solution does not leak.

3. Shut down and storage

If no more experiments are to be done within a 72 hour period, make sure the injection port, injection loop, and flow cell are flushed of any proteins. To do this, inject 5-10 ml of shutdown solution (#14) and flow for at least 5 minutes. It is recommended that the unit be depressurized – by selecting "Stop" on the user interface, and then manually releasing any residual pressure from the buffer tube by remove the tube momentarily and then reattaching. For storage beyond 72 hours, it is recommended that the unit be cleaned with DI water (see section viii Maintenance in the operator's manual). After storage, be sure to follow the instructions for Setup, section 3.1 above. Any active sensors should be replaced or returned to Seattle Sensors after this step and new functionalized sensors installed next time the unit is used.

4. Assay Procedures

Running the assay to measure cortisol levels in the 0.5 to 3.0 ng/ml range occurs in four steps, 1) extraction of cortisol from saliva samples, 2) running the assay to create the dataset, 3) calculation of binding rates (slope in μRIU/sec) using the provided data analysis software, and 4) computation of cortisol concentration, typically in Excel or with a calculator.

Note:

- In order to familiarize yourselves with the instrument it is recommended that you first run the steps in Section 1 below to familiarize yourself with the measurement of cortisol in buffer. The final assay will extract cortisol from saliva and then re-elute the cortisol into buffer for measurement. In order to get the best reference curve for converting binding rate to cortisol concentration, the creation of the actual Standard Curve is mapped out in Section 2 below.
- The protocol steps provided in the appendices may appear complex. We have provided as much detail as possible to ensure the successful running of the assay. As you become familiar with the assay many of these steps will become automatic.

1. Instrument Familiarization - Cortisol Measurement in Buffer

There are two purposes to this protocol: 1) for a new user to become familiar with the operation of the instrument and data analysis, and, 2) to create a standard curve that relates spiked concentration levels to measure changes in slope between control and spiked samples. It should be noted that the standard curve generated from this protocol will not be used for concentration calculations from saliva samples, as magnetic beads are not utilized during the procedure and the curve is buffer-based (not saliva-based). Note that this procedure can be helpful in monitoring assay performance.

The procedure will be to: 1) aliquot a set of 5 standard samples of precise free-cortisol concentration, 2) assay cortisol in the samples by adding anti-cortisol antibody , 3) inject the antibody+cortisol samples into the Spirit instrument while running a specific processing program, 4) transfer the collected dataset to the Analysis Software System to compute the binding-rate slopes for all the injected samples, and 5) use Excel, or a calculator, to compute and plot the measured changes in slopes vs. the spiked concentration.

See Appendix 3 for the detailed protocol.

2. Free-Cortisol Assay and Analysis Protocol

The purpose of this protocol is to start with pig or sheep saliva samples and end up with a digital dataset that can be converted to sample cortisol concentration.

First, a reference curve using cortisol-depleted saliva from the species to be assayed (pig, sheep, etc.) spiked with cortisol must be constructed. This reference curve will serve as the basis for all of the cortisol concentration calculations for this species.

See Appendix 4 for the detailed protocol.

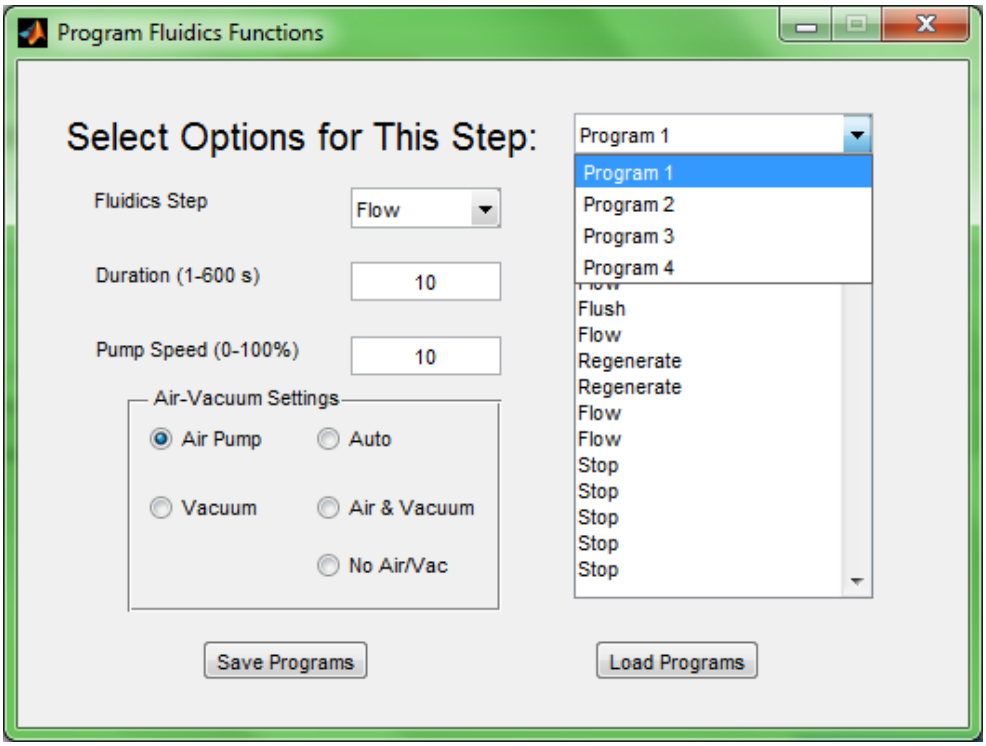
Antibody-coupled beads are used to extract the cortisol from saliva, and then the Spirit's SPR measurement capability is utilized to measure binding rates of anti-cortisol antibodies to cortisol on the biosensor surface. The binding data is captured as a time-sequence of μRIUs (refractive index units). μRIUs are the basic measurement value provided by SPR based instruments.

The measured μRIU vs. Time dataset is then read into the provided Analysis System Software which converts the data into slope measurements. These slopes, when compared to standards curves, provide a quantitative measure of the cortisol in the original saliva sample.

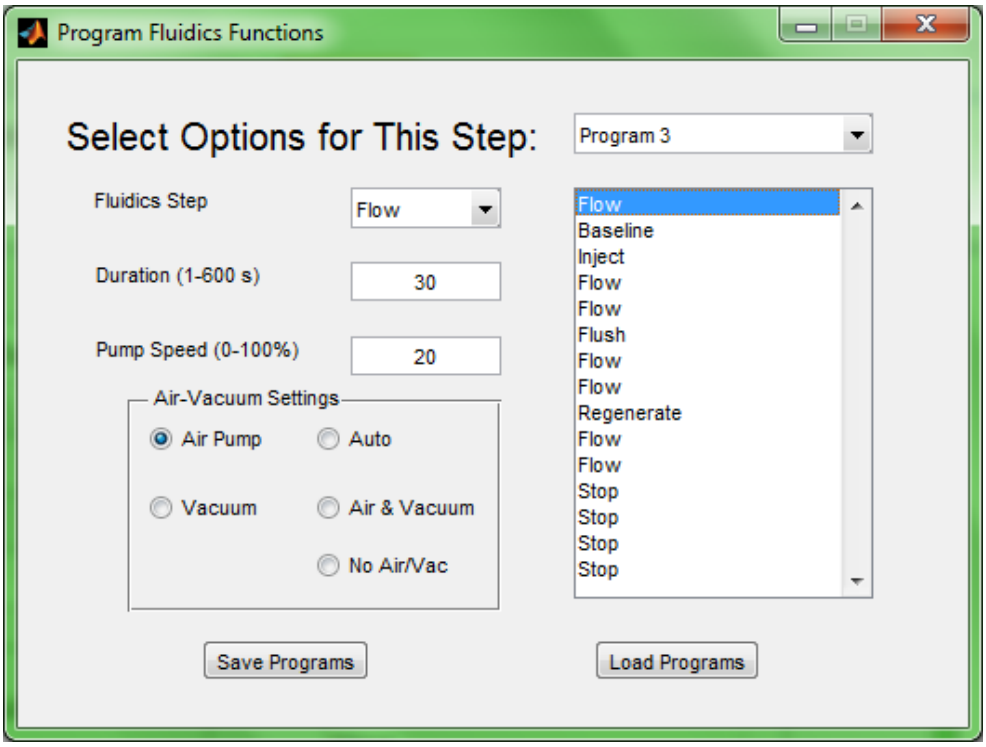
See Appendix 5 for the detailed protocol.

Appendix 1 – Loading Parameters for the Cortisol Assay

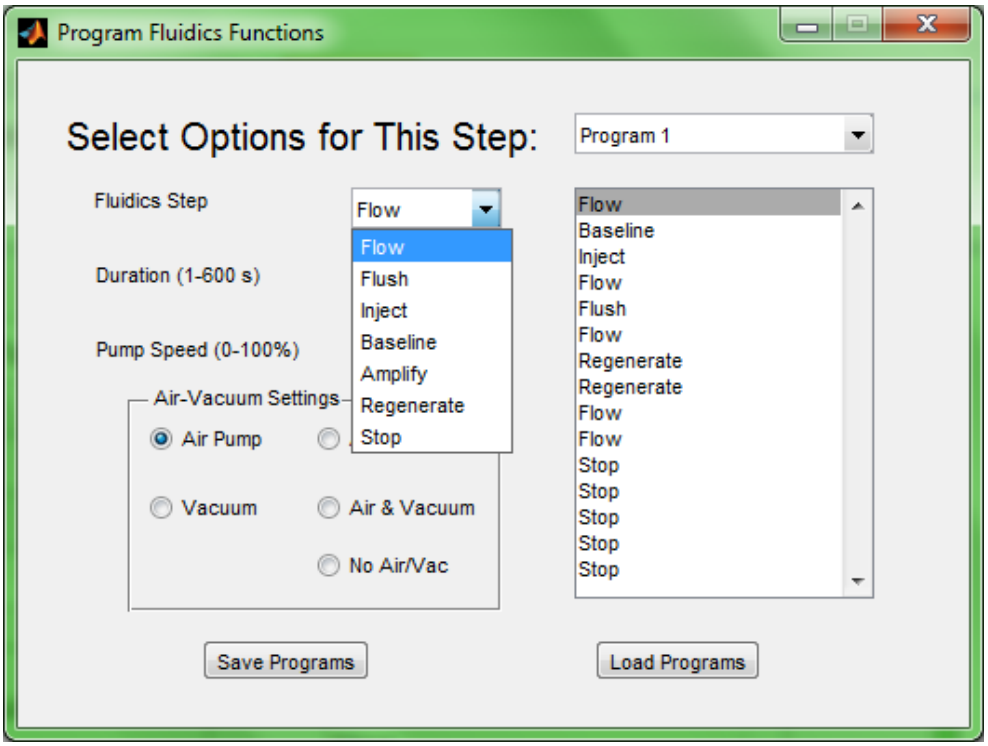
To set up the programming for the cortisol measurement, go to the “View/Set” menu in the SPR Operating Software and select “Fluidics Program”



Use the “Program” menu to select which program you will use for cortisol detection. This is stored on the instrument and the instrument is limited to four different program sequences. For the cortisol assay Programs 1 and 2 will be set up.

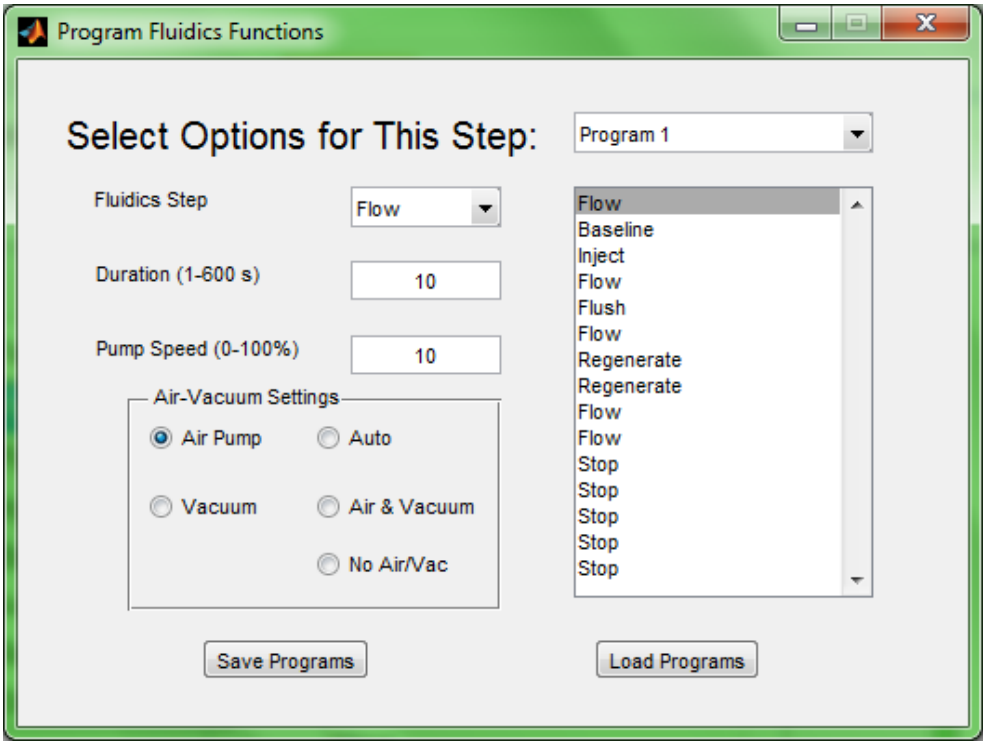


Use the list on the right to select the item in the sequence to program. This will run through each step in the list until it reaches a “Stop” step without any user input.



Select the step type in the dropdown menu. These steps will equilibrate the sensors prior to injection, zero the sensor signal, set the valves for injection, flow the sample over the sensor surface, flush the

injection loop with buffer and then “regenerate” or elute bound analyte from the sensor surface, preparing the sensor for the next detection.



Use the “Duration” and “Pump Speed” boxes to select the appropriate values for these. Use the “Air-Vacuum” Settings to select whether the air pump is turned on or off. This pressurizes the buffer chamber to prevent air bubbles from forming. This will be used for “System Preparation and Cleaning”.

Step Number	Fluidics Step	Duration	Pump Speed	Air Pump
1	Flow	3	0	ON
2	Baseline	2	0	OFF
3	Inject	20	0	OFF
4	Flow	100	20	OFF
5	Flush	20	0	ON
6	Regenerate	300	100	OFF
7	Flow	300	20	OFF
8	Stop	-	-	-

After selecting the correct values, hit the “Save Programs” button. As soon as you make a change, it gets loaded onto the instrument provided you aren’t currently running an experiment. However, if you power the system off, that currently loaded program won’t get saved unless you’ve used “Save Programs.”

Now create a second program, Program 2, with the following sequence steps. This will be used as the "Experimental Analysis" program for measuring injected samples.

Step Number	Fluidics Step	Duration	Pump Speed	Air Pump
1	Flow	3	0	ON
2	Inject	20	0	OFF
3	Flow	100	20	OFF
4	Stop	-	-	-

Appendix 2 - Data Analysis via the Spirit Analysis Software

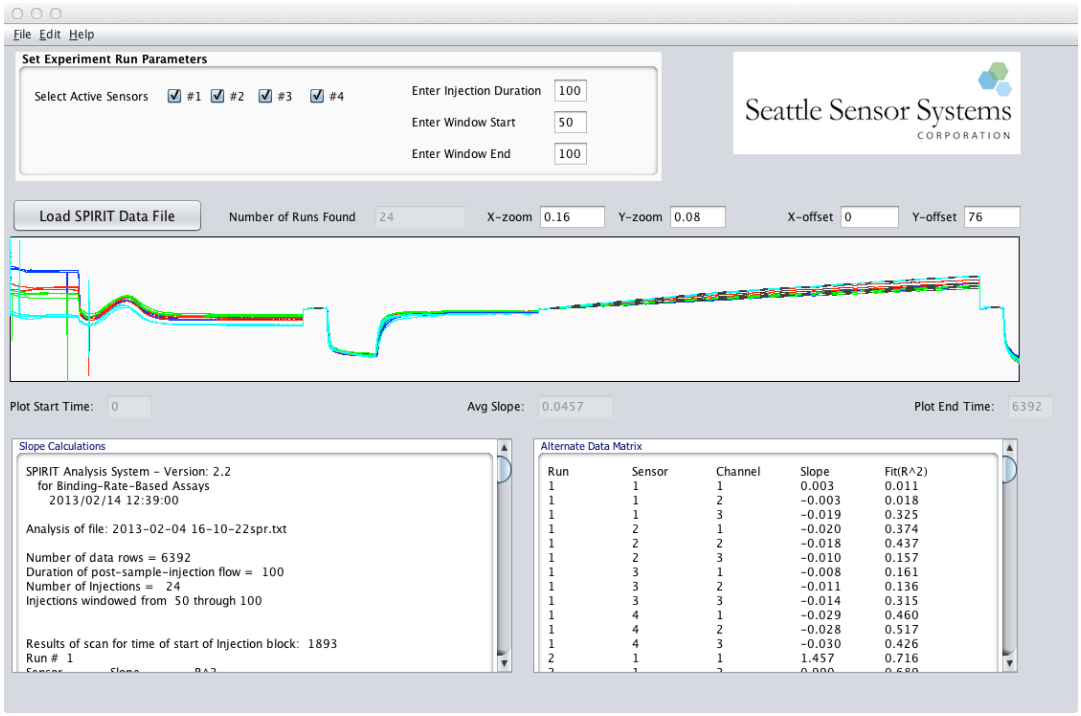
1. Introduction:

The main purpose of this software is to compute the rate of binding (slope) of the injected sample to the biosensor surface. The output of the software is the slope (in units of μRIU/sec) during the post-injection bind flow, for every injection in the selected dataset.

This software package was developed as a working tool during the cortisol assay development activity, so it reflects our experience in what to monitor for both the assay performance and in making the final call on target detected or not. Please feel free to provide us feedback on changes to the Analysis Software that you would find valuable in supporting your project goals. Currently this analysis package runs "offline" from the live data collection.

2. Installation:

The analysis system is designed to run as a companion piece of software on the same computer that is running the Spirit instrument operation systems (IOS) software. Assuming you have successfully installed the IOS onto your computer, you now need to install the analysis software. This code is written in Java so that it will run on both Windows based and Mac OS-X based computers. Your computer must have the Java Runtime Environment already loaded. Since most modern web browsers use Java technology you most likely already have this installed on your machine. The Spirit Analysis Software files were delivered to you either by a thumb-drive included in the free-cortisol kit, or by email to ensure you received the most up to date copy. The file will be a “zip” file that must first be uncompressed. Typically if you right-click on the zip file you will see an option for uncompressing. Once uncompressed, you will see a “dist” (for distribution) directory. Move into the dist directory and then double-click on SpiritAnalysisSystem.jar file. In most cases this will immediately open a new window on your screen and the software will begin to run.



On some Windows system this procedure doesn't work. If you have trouble, an alternate approach is to double click on the file SpiritAnalysisSystem.bat. This will initiate the software application in a slightly different way that we have found works on most computers. Please contact Seattle Sensors if you have any trouble installing or running your system.

Note: On some Windows based computers the graphic as shown in Figure 1 does not appear. For reasons that are unclear, the work-around is to "grab" a corner of the display window and resize it slightly, as this corrects the display problem.

3. Analysis Operation:

The procedure for analyzing a data set from the Spirit instrument is as follows:

1. Set Experiment Run Parameters: These values should be set to be compatible with the cortisol assay parameters. The default assay collects target binding data for 100 seconds after the sample is injected into the instrument. The window parameters define a subset of the 100 seconds of data. The default is to use the data between 50 seconds and 100 seconds. The goal here is to select the segment of the curve that demonstrates a linear binding rate of anti-cortisol antibody to the biosensor surface. The greater the slope, the lower the concentration of cortisol in the injected sample.

2: Load SPIRIT Data file: After setting the Experiment Run Parameters, click this button and a file system navigation and file selection window will open. Navigate to the directory that you selected as the data storage location in the Instrument Operating Software. In that directory you will see one or more text files that are automatically created and named by concatenating "date" "time" "spr.txt" (*"yyyy-mm-dd hh-mm-ss*spr.txt") Select and "Open" the file you want to analyze. (Note that the data file is a txt file and can also be easily loaded into Excel and then converted into data columns with the "Convert text to columns" command, enabling additional custom data analysis.)

3: The analysis system will calculate the slope of the line and the "goodness of fit" (R^2) of that line, and display these numerical values, along with text indicating no-detect, potential detect, or definite detection of cortisol. Note that the R^2 value is sensitive to the amount of "noise" in the data, so it is possible to get a very good slope estimate even if the R^2 value is small. For any slope that has a low R^2 value, it is good to zoom in and view the dataset in the user interface plot screen to see if the R^2 value is small because of noise, or if in fact the binding rate was non-linear.

To analyze another dataset, exit the software and restart, selecting the new dataset as described above in step 1.

When running a series of injections with the Spirit instrument, the data file is continuously appended with new data, so it is possible to copy and "Load" the current version of the dataset into the Analysis Software and do analysis of binding rates, while additional injections are still underway. This is one way of getting faster results during a field test.

The "Plot Window" of the user interface has a few interactive commands that can be used for quick inspection of the loaded dataset.

- Left click – redraw plot, potentially with new sensor activation settings changed
- Left button pushed, dragged, and released – Zoom in on highlighted, windowed section of plot
- Right button click – zoom back to full size and redraw the plot

Note that whenever the plot window is redrawn, the start and end times are updated, and for all the sensors currently active the average slope is computed and displayed under the plot as well. This gives you a way to quickly check the slope of the binding curve from any sensor, for any time sub-window of the SPR sensor data.

Appendix 3 – Instrument Familiarization – Cortisol Measurement in Buffer

Detailed Protocol

1. Remove 5 cortisol standards from kit (0.5, 1, 1.5, 2, 3 ng/ml cortisol).
2. Aliquot 1 ml of each cortisol concentration to a 1.5 ml microfuge tube (5 tubes total). Label each tube first to identify the correct tube for each aliquot.
3. Obtain anti-cortisol antibody from kit and prepare diluted antibody stock (1:75) in PBST buffer. First, label a 1.5 ml microfuge tube as the antibody dilution, then add 148 ul of PBST to the labeled microfuge tube. Add 2 ul (using the p20 pipettor) of the antibody stock to the 148 ul of PBST and mix.
4. To each of the 5 one ml cortisol standard aliquots, add 10 ul of the diluted anti-cortisol antibody, for a final antibody dilution of 1:7500. Mix each tube for 5 minutes. Do not throw away the remaining antibody dilution, as it will be used in the next step.
5. During the antibody-cortisol incubation time, prepare the no cortisol antibody controls. First, aliquot 6 ml of the antibody buffer reagent to a 15 ml conical tube. Then, add 60 ul of the diluted antibody stock prepared in step#3 to the 6 ml of antibody buffer (10 ul per ml of reagent) and mix well. Following mixing, aliquot 1 ml of the antibody buffer + antibody solution to six labeled 1.5 ml microfuge tubes. These no cortisol controls will be run before each cortisol-containing sample, and one will serve as a pre-sample "block" before the first control.
6. At the end of the 5 minute incubation, arrange the cortisol samples and no cortisol controls in order for injection into the instrument. A no cortisol control must always precede a cortisol containing sample. Therefore, the first sample will be the block followed by one of the no cortisol controls, followed by the 0.5 ng/ml cortisol sample, then another no cortisol control followed by the 1 ng/ml cortisol sample, etc. The order should be block, 0 (no cortisol), 0.5, 0, 1, 0, 1.5, 0, 2, 0, 3 ng/ml cortisol, with 5 cortisol and 6 no cortisol samples for a total of 11 samples for analysis.

<u>Instrument Preparation and Sample Analysis</u>

1. Refer to the "Spirit Operations Manual" for a general description of instrument use and programming procedures.
2. Following "initialization" of the new sensors as described in the manual, enter and save the following program as program#1:

Command	Time	Air Pump	Flow Speed
Flow	3 seconds	On	0
Baseline	2 seconds	Off	0

Inject	20 seconds	Off	0
Flow	100 seconds	Off	20
Flush	20 seconds	On	0
Regeneration	300 seconds	Off	100
Flow	300 seconds	Off	20
Stop	-	-	-

This will be the "System Preparation and Cleaning" program which should be used before each sample run if the system has been shut down for a period of time (4 hours or more) or needs to be cleaned following a sample run (described below).

3. Enter and save the following program as program#2:

Command	Time	Air Pump	Flow Speed
Flow	3 seconds	On	0
Inject	20 seconds	Off	0
Flow	100 seconds	Off	20
Stop	-	-	-

This will be the "Experimental Analysis" program for sample analysis.

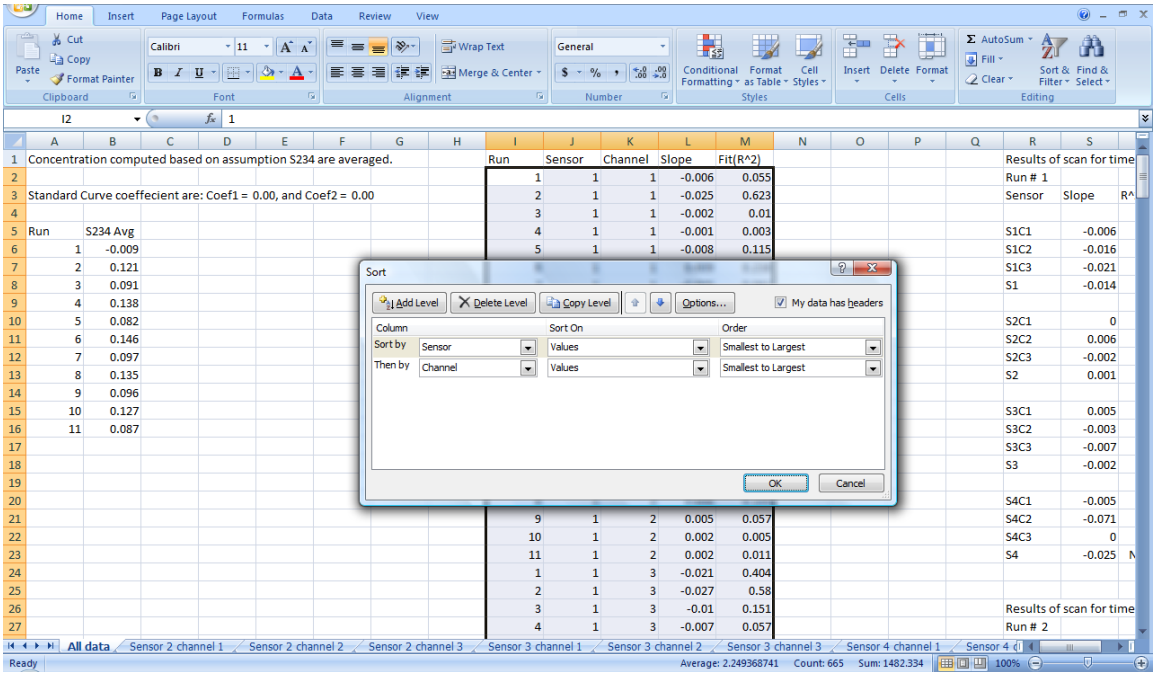
4. Aspirate 1 ml of PBST buffer into a syringe in preparation for injection into the instrument. Large air bubbles in the syringe should be removed by holding the syringe vertically (needle side up), flicking the syringe to promote movement of large air bubbles to the top of the syringe, and then expelling the air bubbles by slowly compressing the plunger while holding the needle into a clean Kimwipe or paper towel. When only fluid is expelled the sample is ready for injection and the needle can be inserted into the injection port, but do not try to inject the sample at this time.
5. Confirm or set program#1 as the selected program on the Main Screen, then click on the green "Run Experiment" button above the program selection box. You will hear the air pump as the program initiates the first command.
6. When you hear the "Inject" function valves click and the "inject" step appears in the "Messages" box, press the syringe plunger to inject the entire sample. There are 20 seconds allotted for the injection step, and the entire sample must be injected during this time frame. The syringe can be left in the injection port following injection.

7. There will be no significant slope present during the flow step as this procedure is just performed before each run to make sure the sensors and flow path are cleaned of any non-specifically bound material and the instrument is operating correctly. All of the program steps presented in step#2 should occur and be listed in the "Messages" box as the program is run.
8. At the end of the program, change the "speed" setting on the main screen to "20" and manually activate the "flow" command using the button on the main screen. This will keep buffer flowing through the system while preparing for sample injections.
9. Obtain the 11 "Standard Curve" samples prepared in the first section.
10. Set program#2 as the selected program on the Main Screen.
11. Aliquot ~30 ml of PBST into a 50 ml conical tube. This will be used to clean the syringe between injections as described below.
12. Have a waste container available for expelling buffer used to wash the syringe.
13. Remove the syringe needle from the injection port, and then move the syringe plunger back-and-forth 5-10 times while aiming the needle into a waste container to empty the syringe.
14. Wipe the syringe needle on a clean paper towel or Kimwipe to remove any excess fluid.
15. Aspirate the first sample ("block," see step#6, Instrument Familiarization section above) into the syringe and expel large air bubbles as described above. Insert the needle into the injection port but do not attempt to inject the sample at this time.
16. Click the "Zero Sensors" button in the upper right corner of the screen to bring all of the sensor traces to zero.
17. Click the green "Run Experiment" button above the program selection box.
18. When the "Inject" function valves click as described above, inject the entire sample during the 20 second time frame for injection.
19. The "flow" step will now start and sensor traces possessing particular slopes will begin to appear, depending on the amount of free-cortisol antibody present in the sample.
20. Once the flow step begins, it is important to prepare for the next sample injection as described below.
21. Remove the syringe needle from the injection port and expel residual fluid from the needle as described above.
22. Wipe the needle tip on a clean paper towel or Kimwipe as described above, and then aspirate 1 ml of PBST from the aliquot prepared in step#11.
23. Expel the PBST into the waste container then move the plunger back-and-forth as described above to expel any remaining fluid.
24. Repeat steps #21 to #23.
25. Wipe needle tip on a clean paper towel or Kimwipe, then aspirate the next sample ("0" cortisol) into the syringe, expel large air bubbles, and insert into the injection port in preparation for the next injection.
26. When the previous sample is complete and the program stops, wait 5-10 seconds then click the green "Run experiment" button on the Main Screen and inject the sample as described in step#18.
27. Follow steps 17-26 for each sample until all of the samples have been run.

28. Following the end of the last sample run, aspirate 1 ml of PBST into the cleaned syringe, insert the syringe into the injection port, set program #1 as the selected program, and click the green "Run Experiment" button. Inject the buffer sample at the appropriate time and let the program run until complete. This will clean the instrument, which can now either be manually set to "flow" until required for further experiments or disconnected from the software then shut off if no longer needed.

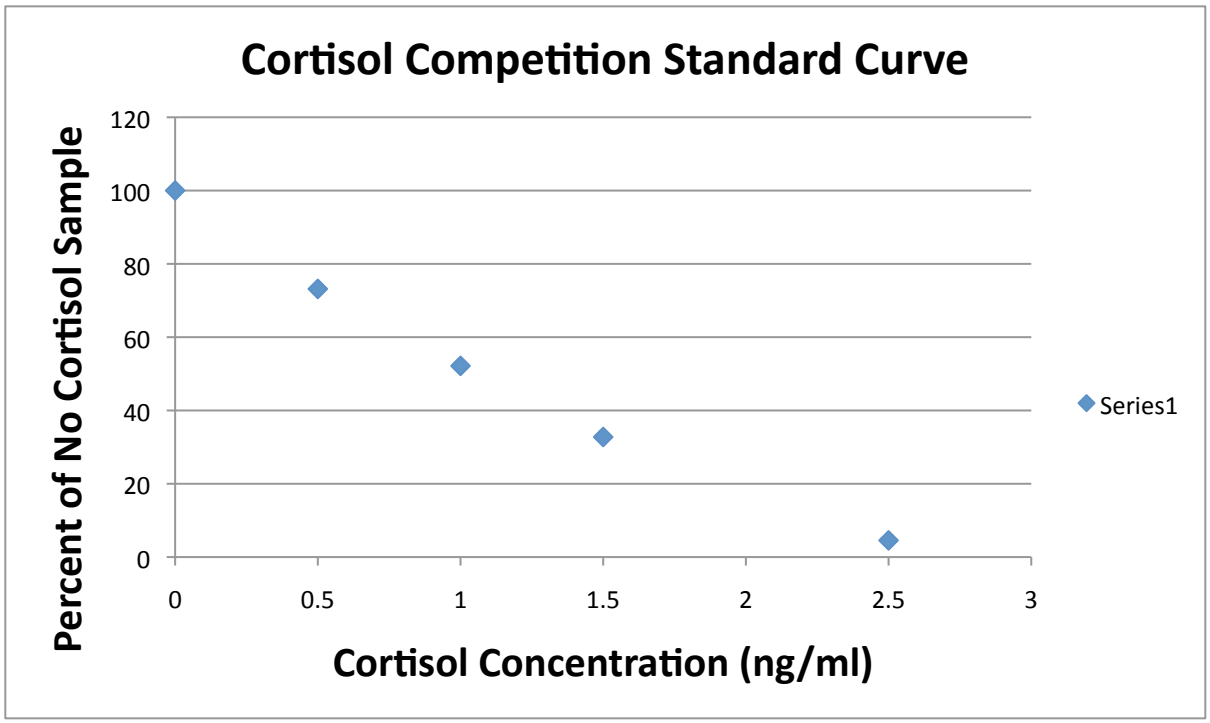
Data Analysis

1. Set the Experimental Run Parameters to 50-100 seconds out of a 100 second run. The latter part of the 100-second flow step is where the most accurate slope values are typically obtained.
2. Load the file as described in "Appendix 2" above.
3. The software output will include slope calculations for each channel in each sensor (there are 3 channels per sensor). The highest quality analysis is obtained by examining individual channels. We will QC the sensors before shipment to identify the most accurate channels for cortisol analysis.
4. We have found that copying the output to an Excel file is the easiest method for data analysis. First, select the data in each of the 2 windows (separately) and past into an Excel worksheet. Use "control C" to copy the selected data. The 2 data types would be: (1) the labeled, individual channel and sensor data. The beginning of this data type will indicate the date, SPR file ID, number of data rows, etc. (2) the data from individual channels grouped together as a table without extra labels and designed for Excel analysis. This is the easiest file to manipulate in Excel. By copying all of the data into the Excel file, it is centralized for easy access and future analysis if required.
5. Select the tabled data (data type #2 mentioned in step#4) and use the "Custom Sort" command under the "Sort & Filter" button to group the data as needed. You can also analyze the data by a different approach depending on your preference.
6. In the" Custom Sort" box, select "My data has headers" then first sort by sensor number (smallest to largest), then add a level and sort by channel (smallest to largest). The screen shot below shows an Excel worksheet containing the 2 types of data output mentioned in step#2 as well as the Sort box under "Custom Sort."



7. Following the data sorting, the runs will be in increasing order (see column "I" above), as well as the sensors, channels, and slopes (columns "J,"K," "L," respectively). This arrangement facilitates the selection of all of the slopes from one sensor's channel for all of the runs, which is the method we utilize for quantifying the cortisol concentration.
8. We usually sort the individual channel slopes to separate worksheets where they can be charted and analyzed separately (see tab labels at bottom of screen shot).
9. Once all of a particular channel's slopes have been grouped into a new worksheet (for example, sensor 2, channel 1), the percent decrease in slope (which equals the amount of free-cortisol competing for antibody binding to the sensor surface) as a function of the preceding no cortisol sample is calculated for each cortisol-containing sample. The correlation of this value with a particular cortisol concentration will define a point in the reference curve. For example, if the slope of a no cortisol sample was 0.2 and the following sample contained 1 ng/ml cortisol with a slope of 0.1, the percent decrease in slope mediated by 1 ng/ml cortisol would be 50% (0.1/0.2 X 100). Thus, saliva or other samples that similarly mediated a 50% decrease in slope compared to a no cortisol control would be quantified to contain cortisol at a concentration of 1 ng/ml based on this example.
10. Using this approach, the percent decrease in slope for each cortisol standard (0.5, 1, 1.5, 2, 3 ng/ml cortisol) is charted using the "Scatter Chart with only Markers" charting function in Excel. Percent competition values from unknown cortisol samples (obtained by comparison to no cortisol controls) can then be used in an equation derived from the curve assigned to the cortisol standard points to determine cortisol concentrations of the unknown samples. We have found that the most accurate cortisol concentrations can be obtained by plotting the 0.5, 1, 1.5,

and 2 ng/ml cortisol samples then using an equation derived from a linear fit trendline. An example of a cortisol competition standard curve is shown below.



Appendix 4 – Saliva Depletion and Spiking for Saliva Reference Curve Generation

Detailed Protocol

1. Obtain 6 mls of saliva from the species of interest (pooled or from a single animal).
2. Retrieve 15ml tube containing antibody-coated beads, the magnetic separation rack (MSR), and the p1000 pipettor.
3. Remove the plastic tube rack surrounding the magnet of the MSR by pulling it up and off the magnet.
4. With the magnet on a flat surface, hold the tube containing the antibody-coated beads directly against the magnet so the beads are lining the tube surface adjacent to the magnet.
5. Carefully remove the bead buffer using the p1000 pipettor set to 1 ml. Try to avoid removing any beads. Repeat until the entire buffer volume has been removed. The buffer can be saved for re-addition to the beads at the end of the procedure (note that it contains sodium azide).
6. Using the p1000 pipettor, pipette the 6 mls of saliva into the tube containing the magnetic beads. The bead tube does not need to be adjacent to the magnet at this point and can be put into any tube rack.
7. Once all of the saliva had been added to the beads, use the p1000 pipette to resuspend the beads in the saliva by pipetting up-and-down multiple times.
8. Mix the saliva with the beads for 5 minutes using a rotator or other mixing device.
9. Take the 15 ml tube containing the saliva and beads and put it next to the magnet as in step#4.
10. Using the p1000 pipettor (and a new tip), remove all of the saliva and pipette into a new 15 ml tube, being very careful to not transport any beads to the new tube. This saliva has now been depleted of cortisol, which is now bound to the antibody-conjugated beads.
11. Following removal of saliva from the beads, use the p1000 pipettor to pipette 3 ml of PBST buffer to the beads and resuspend by pipetting up-and-down. Use the magnet to remove the buffer wash and repeat one more time.
12. Obtain glycine solution from the kit. Following removal of the 2nd PBST wash, add 1 ml of glycine to the beads, resuspend the beads in glycine with the p1000 pipettor, and let incubate for 5 minutes. This will remove the bound cortisol from the beads.
13. Following completion of the bead+glycine incubation, use the magnet to collect the beads and remove the glycine, which can be discarded (note it contains sodium azide).
14. Wash the beads 2X with PBST as in step#11, and then add 2.5 ml of PBST. The beads have now been stripped of bound cortisol and are ready to be used in the assay of spiked (or endogenous) cortisol-containing saliva samples. **Important Note**: The bead stripping procedure (steps #11-#14) must be performed following any bead-based cortisol extraction to ensure consistent results in the next assay (the beads can be stripped at any time, as long as it precedes the next assay).
15. Obtain cortisol stock solution, 7 1.5 ml microfuge tubes, p1000 and p20 pipettors.

16. Label 5 of the 1.5 ml tubes as 0.5, 1, 1.5, 2, 3, and add 1 ml of depleted saliva from step #10 to each of the 5 tubes.
17. Label the 2 remaining 1.5 ml tubes as 10 ug or 100 ng, and add 990 ul of PBST to each tube. Then, using the p20 pipettor, add 10 ul of the cortisol stock to the 10 ug tube. The cortisol is in methanol and quickly runs out of pipette tips, so carefully observe the tip as you transfer the 10 ul to the 990 ul of buffer as rapidly as possible.
18. Once transferred to the 10 ug tube (which now contains 10 ug of cortisol), mix the tube's contents well, and then transfer 10 ul from the 10 ug tube to the 100 ng labeled tube and mix well. The 100 ng/ml cortisol dilution tube will be used for spiking the depleted saliva samples as described below.
19. Using the p20 pipettor, pipette 5 ul of the 100 ng/ml cortisol dilution to the saliva-containing microfuge tube labeled 0.5 and mix. This tube now contains 0.5 ng/ml cortisol.
20. Add the appropriate volume from the 100 ng/ml tube to the 4 remaining labeled saliva tubes, being sure to change tips following each addition of cortisol to the saliva. The volumes are 10 ul to the "1" tube, 15 ul to the "1.5" tube, 20 ul to the "2" tube, and 30 ul to the "3" tube. These 5 cortisol-spiked saliva samples can now be used for generation of a saliva reference curve.

Appendix 5 – Assay Procedure for Reference Curve Generation and Endogenous Free-Cortisol Analysis

Detailed Protocol

1. Obtain saliva samples. 5 samples is the maximum number of samples which can be assayed at one time.
2. Label 1.5 ml microfuge tubes to match the number of samples to be assayed and leave in rack without closing caps. Obtain tube containing cortisol antibody-conjugated beads.
3. Obtain p1000 pipette, set to 500 ul volume, then attach tip in preparation for use. Mix beads very well, making sure they are all in suspension. Rapidly open tube containing the mixed beads and pipet out 500 ul of bead suspension (= 5 mg of beads) with the pipette. Aliquot entire volume to first labeled 1.5 ml tube. Close tube with cap.
4. Re-close tube containing beads, mix well and repeat bead allocation procedure described in step#3 for all labeled tubes. The same pipette tip can be used for all samples unless it has been contaminated by contact with improper surfaces, hands, etc. The tip should be replaced with a new one if any unwanted contact is suspected.
5. Obtain magnetic separation rack (MSR). Insert tubes containing allocated beads into MSR with tops oriented to open away from the rack.
6. Set p1000 pipette to 600 ul volume, carefully open all of the tubes on the MSR, and starting with the left-most tube and going left to right, pipette out the entire bead buffer from each tube into a waste container. Be careful to not pipette out any beads, which are lining the tube adjacent to the MSR, by observing the aspirated buffer in the pipet tip. This and additional steps which result in the beads being exposed to air should be performed as rapidly but accurately as possible, as extensive drying of the beads can reduce the conjugated antibody's ability to bind cortisol.
7. Once bead buffer has been removed from the last tube, set the p1000 pipette to 1 ml and begin to add 1 ml of saliva to each tube, starting with the left-most tube which had buffer removed first and moving from left to right. Be sure to add the correct saliva sample to the correctly labeled tube, and switch pipet tips between different samples.
8. Following addition of saliva to the last tube, close all of the beads + saliva-containing tubes and remove all tubes from the MSR. Briefly mix each tube to re-suspend the beads, then place onto rotating apparatus and rotate for 5 minutes. Free-cortisol will be bound by the beads during this time.
9. During the bead-saliva incubation, label equivalent sets of tubes as either "supernatant," "elution 1," or "elution 2."
10. Following completion of the 5 minute incubation, place the tubes into the MSR as in step #6. Obtain PBST buffer for next steps.
11. Noting each saliva tube's label, set the p1000 pipette to 1 ml and begin to transfer the supernatant from each tube to the corresponding "supernatant" tube, being careful to avoid removing any beads. Change pipette tips after removing each supernatant. Supernatant

removal should occur in a left-to-right order as described above. The saved supernatants can be re-tested if there are any issues with the subsequent bead analysis. Close and freeze the supernatant tubes (can be stored on ice temporarily).

12. Moving from left to right, begin adding 1 ml of PBST to each tube on the MSR (the same pipet tip can be used for each tube unless contaminated) to wash the beads.
13. Close tube caps, and then remove each tube and mix vigorously until the beads are re-suspended in the buffer.
14. Return tubes to MSR, then remove and discard 1st wash, using a separate tip for each tube. Add PBST buffer again and repeat steps #13 to #14 for 2nd bead wash. Obtain elution buffer and p200 pipette set to 50 ul.
15. Return tubes to MSR, remove and discard 2nd wash solution from each tube. Make sure that there are no large pools of buffer at the bottom of the tubes which can interfere with the subsequent elution step, then add 50 ul of glycine (elution solution) to the bottom of each tube, being careful to avoid the beads. Use a different tip for each addition to eliminate any possibility of cross-contamination.
16. Remove all tubes from MSR, then (starting with the tube which was left-most on the MSR) use the p200 pipette set to 50 ul (and a new tip for each sample) to aspirate the glycine from the bottom of the tube and pipet it down the side of the tube where most of the beads are located. Repeat washing the beads down the side of the tube with glycine in this manner until all of the beads have collected at the bottom of the tube. This is important for assuring exposure of all of the beads to the glycine for optimal cortisol elution.
17. Repeat washing all of the bead samples with glycine as described in step #17. Following the last sample, wait 3 minutes to permit completion of the cortisol elution.
18. Return the tubes to the MSR then use the p20 pipette set to 19 ul to carefully remove the glycine from the bottom of the tubes, avoiding the beads along the side of the tube. A few aspiration steps should be performed to confirm removal of all of the glycine. Note the tube label, and pipet the glycine into the appropriately labeled “elution 1” tube. Repeat for each tube, using a different pipet tip for each sample.
19. After removing glycine from the last sample, set the p1000 pipette to 1 ml and pipet 1 ml of PBST into each bead-containing tube on the MSR. Remove tubes from the MSR and place on ice for later washing and regeneration.
20. Take the “elution 1” tubes containing glycine and return to the MSR, then again remove the glycine from the bottom of the tubes and transfer to the appropriately labeled “elution 2” tube. This step will ensure that there are no beads in the final glycine eluate.
21. Prepare an appropriate volume of diluted anti-cortisol antibody according to the number of samples being run. An initial 1:75 antibody dilution will be subsequently diluted 1:100 (10 ul per 1 ml) in a larger volume of PBST for addition to the samples (1:7500 final dilution). For example, if 5 samples are being processed, then 50 ul of 1:75 diluted antibody will be required for the samples alone. In addition, “0” cortisol antibody controls will be run before each sample+1 pre-sample “block” run, so an equal volume of diluted antibody + one extra control will be required for the controls. To compensate for any loss during pipet steps, a small excess of antibody dilution should always be prepared. Thus, adding 2 ul of the antibody stock to 148 ul of PBST

would be sufficient for 5 samples + controls. For the final dilution, aliquot 12 mls of PBST to a 15 ml conical tube using the p1000 pipette. The 1:75 diluted antibody should then be mixed by carefully flicking the tube with your finger. Using the p200 pipette, transfer 120 ul of the 1:75 diluted antibody to the 12 mls of PBST and mix well. Using the p200 pipette, transfer 50 ul of glycine to 6 1.5 ml tubes for no cortisol controls.

22. Using the p1000 pipette, transfer 950 ul of the 1:7500 diluted antibody to each of the 5 tubes containing the 50 ul of glycine eluate from step # 20 (+ the 6 controls prepared in step #21 [assuming 11 samples are being run, so this number will vary depending on the number of samples]). Mix then rotate for 5 minutes. The samples are now ready for injection into the instrument.
23. During the 5 min sample rotation, the instrument should be prepared for sample injection according to the "Instrument Preparation and Sample Analysis" section. First, inject buffer and run the "System Preparation and Cleaning Program" (Program #1) as described in this section.
24. Switching to Program #2, inject and run 2 consecutive "0" cortisol samples (prepared in step #23), followed by the first +cortisol sample, then another "0" cortisol sample, etc. The first of the 2 consecutive "0" cortisol samples is a blocking sample which will not be used to determine cortisol levels in subsequent samples, while the 2nd control sample will be used for this purpose.
25. Once the sample slopes have been obtained as described in the "Data Analysis" section, a standard curve can be generated from the spiked saliva samples (see step#10 in the "Data Analysis" section).
26. Saliva samples containing unknown levels of cortisol can now be assayed by starting at step #1 in this Appendix (#5). The standard curve produced in step #25 is then used to calculate cortisol levels in the samples. Thus, the equation of a standard curve for a particular sensor or sensor channel, which is solved for "y"(slope) in Excel, must be solved for "x" (concentration), then the obtained slope for a cortisol- containing sample is used in the equation (substituted for "y" in the equation) to obtain the sample's cortisol concentration.