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Biomek FX-ADMETox Workstation

PAMPA Evolution96
Permeability Analyzer

User's Guide

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Introduction

1.1 Introduction

The PAMPA Evolution96 is an in vitro tool for measuring drug permeability and membrane retention by using filter-immobilized artificial membranes.

It belongs to the pHTS series of high throughput analyzers from pION.

By using as little as 10 µL of purified sample presented in a 96-well microtitre plate, the measuring device performs an analysis similar to the one described by Kansy, et al. (J. Med. Chem. 1998, 41, 1007-1010), but refined over seven years of intensive research and development.

The PAMPA Evolution96 allows for unattended analysis from start to finish plus automated calculation and export of the data to Microsoft EXCEL.

1.2 PAMPA Evolution Users

Large pharmaceutical and biotech companies use parallel synthesis to make large numbers of new molecules each year. Each year a single company may have as many as one to three million new molecules to screen for biological activity.

Early testing of these molecules for their physicochemical properties is becoming more and more desirable, as a way to sooner fail deficient compounds and quickly find those with promising bioactivity and good physicochemical properties.

High-Throughput Screening (HTS) is the catch phrase. However, there is no agreement on the definition of 'high throughput'. Maybe it means more than a few assays per day, maybe thousands per day, or maybe more than we have been able to do previously.

'Screening' tends to mean 'quick and dirty,' where false positives and false negatives are expected and not easily avoided.

The PAMPA Evolution does a much better job of quantifying permeability than might be expected from a high throughput screening device.

The PAMPA Evolution, in its current version, is best suited for discovery people wanting to screen 50-100 promising, purified compounds per day for 'drugability'. In a standard configuration it is not intended for screening of thousands of compounds per day.

1.3 Operating Principle

The PAMPA Evolution technique is described as follows:

1. Dilute small volumes of at least 10 mM drug stock solutions (presented in DMSO or acetonitrile) in 1 to 2 mL System Solution.
2. Pipette the diluted samples into a donor plate (bottom half of a PAMPA Sandwich).
3. Dispense ASB-7.4 (acceptor sink buffer) into an acceptor plate (top half of the PAMPA Sandwich) of which have been 'painted' with the GIT(0) lipid solution.

4. Place the acceptor filter plate on top of the bottom plate to form the PAMPA sandwich.
5. Place the sandwich on the Gut-Box and stir for 30 minutes (longer if protocol requires).
6. Scan the UV spectra of the donor, acceptor, and reference wells.
7. Process the spectra to calculate permeability and membrane retention.
8. Print the results, or export them to a Microsoft Excel spreadsheet to send them to a corporate database.

1.4 Safety

This section discusses safety for the Beckman Coulter Biomek FX system and the PAMPA Evolution96 Permeability Analyzer.

Beckman Coulter Biomek FX System

The Biomek FX system pipetting instrument requires accurate positioning of all reagents, samples, racks, and plates on the instrument's worksurface. The operator should verify that this is the case, before executing any program.

Please refer to the instrument Operating Manual for additional hazards before using the instrument.

PAMPA Evolution96 Permeability Analyzer

The analyzer comes with a set of test compounds for training on the permeability assay. All samples and test kit components must be considered potentially hazardous agents. Additionally, the assay requires the use of laboratory chemicals. Therefore, a potential risk may arise from the liquids being handled by the pipetting instrument. Follow all state, and federal laws as well as any company policies and procedures required for the handling and disposal of the instrument waste.

Warnings and Notes

Three attention symbols appear in the text of all SNPstream documentation. Each symbol implies a particular level of observation or action as described below.

NOTE Calls attention to useful information.

CAUTION Indicates information that is necessary for proper assay function or instrument operation.

WARNING Warns the user that serious physical injury or death to the user or other persons could result if these precautions are not taken. BECKMAN COULTER, INC., DISCLAIMS ALL LIABILITIES RESULTING FROM ACTIONS TAKEN CONTRARY TO SUCH INSTRUCTIONS.

Safety Statements in this Manual

NOTE Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
Use powder-free gloves whenever possible to minimize introduction of powder particles into sample or reactions.
A lab coat, gloves and safety glasses are recommended.
Avoid microbial contamination of stock solutions which may cause erroneous results.

CAUTION Dispose of waste in accordance with federal, state, and local regulations.
SNPware reagents should be prepared and used with appropriate caution.

WARNING All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. This includes adherence to the OSHA Blood borne Pathogens Standard (29 CFR 1910.1030) for blood-derived and other samples governed by this act.
Never pipette by mouth.
Avoid specimen contact with skin and mucous membranes.

Installation

CAUTION During installation, power to all system components should be kept OFF until instructions indicate that it is time to turn the power ON.

2.1 System Requirements

Configuration

The system standard configuration consists of 7 major components:

- Robotic sample preparation system using disposable tips.
- UV microtitre plate scanning spectrophotometer.
- Computer with Windows XP® operating system and Installed Biomex Software.
- Gut-Box.™
- Installation Pack of accessories and initial consumables for Getting Started.
- PAMPA Evolution96 Command Software and a set of test compounds.
- Consumables Pack for approximately 50 assays.

Space Requirements

The space requirement is w = 6', d = 3', and h = 3' (180 x 90 x 90 cm). Additional work space on both sides is needed. Include a space of 23' (60-90 cm) below the worksurface, for a waste container of discarded volumes of system solution and small amounts of compounds and solvents.

Access to compressed air is required.

Power Requirements

Power requirement is about 1250 VA from 100-120 VAC or 220-240 VAC grounded line source.

2.2 Computer Hardware

A computer is needed to operate the robot and to run the PAMPA EVOLUTION Command software (Version 3.0 or higher). This computer is typically delivered as part of the Beckman Coulter robot and should be installed by qualified Beckman Coulter personnel. Use only with a flat screen monitor due to the magnetic field from the Gut-Box. See Gut-Box™ Installation Section 2.4 below.

If the computer is to be connected to a network and/or if company policy requires special registration of computer hardware/software, it is generally easier to have the IT department properly install the computer and hook it up to the network close to where the computer is used before the robot is installed.

If not already installed, the supplied USB to RS232-C serial port bridge must be attached to a USB port on the computer.

A Beckman Coulter service engineer should then install and calibrate the robot.

2.3 Power Switch

The supplied Computer Controlled Power Switch is used to control the power to the Gut-Box from the Command software.

Connect the Power Switch to a wall outlet close to the wall outlet used for the computer. If needed, attach the 1' (30 cm) power cord converter to output channel 1 on the Power Switch.

Attach a serial data cable to the connector onthe Power Switch at one end and the bridge port device attached to the computer at the other end.

2.4 Gut-Box™ Installation

The proper model of the Gut-Box is needed for the Biomek FX. This model can be identified by its label on the back panel. It must indicate "-FX". It can also be identified by its physical features: It has only two rubber feet at the back bottom edge of the box and two indexing screws protrude from inside the box between the front and bottom center of the box.

The remaining hardware task is to install the Gut-Box component used for the PAMPA EVOLUTION assay on the Biomek FX worksurface.

The necessary parts include:

- Gut-Box with loose lid
- External power supply unit
- Power cord with plug according to local standard
- Two sponges

1. Remove the center back panel of the Biomek FX to allow access to the worksurface from the back.
2. If the 3 by 4 ALP component support rack is installed, remove it from the worksurface to get access from the front of the robot to the opened access hole.
3. Remove and store the lid of the Gut-Box as well as the sponges provided. These items would only be needed for extended incubation periods. This use is not part of the protocol for the Biomek FX.
4. Connect the power cord to the power supply unit and its attached power supply cord to the power socket on the back panel of the Gut-Box.
5. Thread the power cord through the center opening of the robot and connect it to the power cord extender attached to output channel 1 of the Power Switch.
6. Refer to Figure 2.1 for placement of the Gut-Box on the Biomek FX worksurface. Carefully align the two indexing screws on the bottom of the Gut-Box with the indexing holes in the worksurface. The screws barely engage the holes but will secure the position

of the Gut-Box on the worksurface. When properly situated, the front of the box is lined up with the tip-feeder unit to its right. There are no screws to fasten.

Figure 2.1 Gut-Box placement on Biomek FX worksurface

7. Reinstall the 3 by 4 ALP component support rack on the worksurface. Check that the Gut-Box clears the rack by about 1/4" (6 mm) and that it is square to the rack. If this is not the case, the Gut-Box is not engaging the index holes properly. This must be corrected before operating the robot.

2.5 Software Installation

The PAMPA EVOLUTION Command Software is licensed to the user in both of two versions (one seat each for each version) one of which is able to operate the hardware needed to run the PAMPA assay (Instrument Version), the other is for data analysis and remote monitoring of the system (Desktop Version). Contact pION for extended licenses.

The PAMPA EVOLUTION Command Software installation comes on 2 CDs. The first CD is the Instrument Version, the second CD is the Desktop Version. The Instrument Version **MUST** be installed on the same computer to where the other PAMPA EVOLUTION system components are connected. The Desktop Version may be loaded on another computer.

NOTE The INSTRUMENT VERSION of the PAMPA EVOLUTION Command Software runs only under Windows 2000 or higher. The DESKTOP VERSION can run under Windows 95/98/Me and Windows NT 4.0 (Service Pack 6) or higher.

NOTE You must have Microsoft Excel loaded on the computer you run either version of the Command software.

NOTE The Windows screen setting for the computer monitor should be for a resolution of 1024×768 pixels with 256 or higher color setting.

PAMPA EVOLUTION Command Software

Turn power **ON** to all system components. When the computer has booted up, insert the PAMPA EVOLUTION Installation CD into the CD-ROM drive.

1. Click **Start | Settings | Control Panel** and then click the **Add/Remove Programs** button. Click the **Install** button and the Setup Wizard will help with the installation process. The PAMPA EVOLUTION Installation Software default is D\: drive. Accept this default if your system is set up to handle a D\: drive.
2. In the **Setup Type** dialog box, choose the appropriate setup type.

When the Instrument Version of the PAMPA EVOLUTION Command Software installation is complete, the Beckman Coulter software must be configured to work with it.

Running the Instrument Version of the PAMPA EVOLUTION Command Software

Turn power **ON** to all PAMPA EVOLUTION system components. Wait for the computer to boot.

1. Click on **Start | Programs | pION Software | PAMPA Evolution Command Software** to launch the program. You may create a short-cut on the desktop to launch this procedure. To create a Windows short-cut refer to the Microsoft Help and Support web page at http://support.microsoft.com/kb/140443/en-us.
2. Follow the instructions in Getting Started Section 4.

Running the Desktop Version of the PAMPA EVOLUTION Command Software

1. Click on **Start | Programs | pION Software | PAMPA Evolution Command Software** to launch the program. You may create a short-cut on the desktop to launch this procedure. To create a Windows short-cut refer to the Microsoft Help and Support web page at http://support.microsoft.com/kb/140443/en-us.
2. Open an existing data file to analyze the results. See the Results Section 6.

The System Solution

3.1 The System Solution (SS)

The PAMPA Evolution uses a special pION aqueous universal buffer called the System Solution (SS), especially designed for high-throughput permeability applications and other applications, including solubility determination, where concentration measurements using spectrophotometric methods are needed at a specific pH.

A buffer is needed that is based on componentsthat will not interact with the molecules being studied. For that reason, phosphate is not used due to its strong tendency to cause precipitation of salts of positively-charged drug substances [W.H. Streng, S.K. Hsi, P.E. Helms, H.G.H. Tan, J. Pharm. Sci. 1984, 73, 1679-1684]. To avoid ion-pair interactions between buffer components and the sample, lipophilic buffers must be avoided.

Making the UV detection system produce the highest possible sample signal and the smallest possible signal from the background buffers, the UV absorption of the buffer components have to be kept low. Citric acid and several other common buffers cannot be used due to their unfavorably elevated UV absorption.

The pH vs. volume-of-alkaline-titrant relationship must be as linear as possible, to allow easy adjustment of the pH to any needed value inthe range 3 to 10. Noneof the commonly known universal buffers [D.D. Perrin, B. Dempsey, Buffers for pH and Metal Ion Control, Chapman and Hall, London, 1974; A. Avdeef, J.J. Bucher, Anal. Chem. 1978, 50, 2137-2142] fit the desired profile.

The System Solution has been designed with fivedifferent ionization groups, evenly spaced in pK_a values, to produce a very constant buffer capacity in the interval pH 3-10. The ionic strength of the System Solution is about 10 mM. No NaCl or KCl have been added to boost the ionic strength to higher values.

3.2 Preparing the System Solution

What you will need:

- 2L volumetic Flask or 2 L graduated cylinder
- 1 bottle System Solution concentrate
- 2L bottle

1. Pour the 50 mL of concentrate into an empty and clean 2 L volumetric flask or 2 L graduated cylinder (The System Solution concentrate bottle should contain 50 mL of solution). Note the lot number of the concentrate.
2. Add high-quality distilled or deionized water to a total volume of 2 L, stir.
3. Add the solution to the 2 L bottle, cap, and label the bottle with the date, lot number.

CAUTION The System Solution is normally shipped in plastic bottles as a 50 mL concentrate. The concentrate contains a small amount of bacteriostatic preservative to prevent growth during storage. The concentrate should be kept refrigerated, but NOT frozen.

3.3 Preparing the System Solution for Single pH Use

When using just one pH value for all wells, the System Solution is prepared for that pH value.

What you will need:

- calibrated pH meter
- magnetic stirrer
- stir bar
- stir bar retriever
- 0.5M NaOH
- 100 mL graduated cylinder
- 1 mL disposable pipette
- pH 7 buffer

The following procedure describes adjustment of the 2L System Solution pH. A calibrated pH meter is recommended to use.

1. Place the 2 L bottle containing the System Solution on top of a magnetic stirrer. Place a clean, sufficiently large magnetic stir bar in the 2 L bottle and turn on the stirrer.
2. Insert the properly connected pH electrode into the pH 7 buffer tube.
3. Calibrate pH electrode with laboratory pH meter
4. Rinse the pH electrode with DI water and position it in the System Solution bottle. Secure it with a clamp or carefully hold it by hand.
5. Read the pH. The read value is referred to as pH_{START}.
6. Choose the pH for the assay to be run and use Figure below to find the approximate volume of 0.5 M NaOH needed to bring 2 L of System Solution from pH_{START} to pH_{TARGET}.

   Example: If the target pH is 7.4, and pH_{START} was measured at 2.8, look up in the pH_{START} = 2.8 column of Table the required volume of 0.5 M NaOH. You will see that it would take about 63 mL of 0.5 M NaOH to take the solution from pH 2.8 to pH 7.4.
7. With the graduated cylinder measure out about 60 mL of 0.5 M NaOH (about 5% less than gathered from Table , so as not to overshoot the target), and add the NaOH to the System Solution.
8. Take a new pH reading.
9. Fine tune the pH value using the 1mL disposable-tip pipette, until the desired pH_{TARGET} of 7.4 has been reached. Allow at least 2 minutes for the final pH reading to stabilize. For future use, note on the 2 L bottle the actual total volume of NaOH that was added to 2000 mL for this Lot number to reach pH_{TARGET}.
10. Proceed to Heading Detecting Microbial Growth in the System Solution on page 5.

3.4 Preparing System Solution for Double Sink ™ Multiple-pH Application

1. Prepare 1 (one) Liter of System Solution according to the Preparing the System Solution for Single pH Use Section 3.3.

2. Pour equal aliquots of System Solution in 3 clean glass bottles (about 330 mL).
3. Adjust pH to required values 7.4, 6.2 and 5.0 following the procedure described in Table 3.2.

NOTE Diluted System Solution at pH 3.0±0.5 may be safely stored at temperature 2-8 C for 7-10 days or for 3-5 days at room temperature. Diluted System Solution at pH 7.4 may be stored at temperature 2-8 °C for 2-3 days.

NOTE It is recommended to check System Solution for bacterial contamination before the assay. Bacterial contamination in the System Solution may affect the results.

The table below shows the approximate Volumes of 0.5 M NaOH to be Added to 2000 mL System Solution to reach a certain pH.

Table 3.1 Approximate Volumes of 0.5 M NaOH to be Added to 2000 mL System Solution to reach a certain pH.

pH_{START} = 2.6		pH_{START} = 2.8		pH_{START} = 3.0	
pHTARGET	VOL (mL)	pHTARGET	VOL (mL)	pHTARGET	VOL (mL)
2.8	2	2.8		2.8	
3.0	5	3.0	2	3.0	
3.2	8	3.2	5	3.2	2
3.4	11	3.4	8	3.4	5
3.6	13	3.6	11	3.6	8
3.8	16	3.8	13	3.8	11
4.0	19	4.0	16	4.0	13
4.2	22	4.2	16	4.2	16
4.4	**25**	**4.4**	**22**	**4.4**	**19**
4.6	27	4.6	25	4.6	22
4.8	30	4.8	27	4.8	25
5.0	**33**	**5.0**	**30**	**5.0**	**27**
5.2	36	5.2	33	5.2	30
5.4	38	5.4	36	5.4	33
5.6	41	5.6	38	5.6	36
5.8	44	5.8	41	5.8	38
6.0	47	6.0	44	6.0	41
6.2	50	6.2	47	6.2	44
6.4	52	6.4	50	6.4	47
6.6	**55**	**6.6**	**52**	**6.6**	**50**
6.8	58	6.8	55	6.8	52
7.0	61	7.0	58	7.0	55
7.2	63	7.2	61	7.2	58
7.4	**66**	**7.4**	**63**	**7.4**	**61**

Table 3.1 Approximate Volumes of 0.5 M NaOH to be Added to 2000 mL System Solution to reach a certain pH.

pH_{START} = 2.6		pH_{START} = 2.8		pH_{START} = 3.0	
7.6	69	7.6	66	7.6	63
7.8	72	7.8	69	7.8	66
8.0	75	8.0	72	8.0	69
8.2	77	8.2	75	8.2	72
8.4	80	8.4	77	8.4	75
8.6	83	8.6	80	8.6	77
8.8	86	8.8	83	8.8	80
9.0	88	9.0	86	9.0	83
9.2	91	9.2	88	9.2	86
9.4	94	9.4	91	9.4	88
9.6	97	9.6	94	9.6	91
9.8	100	9.8	97	9.8	94
10.0	102	10.0	100	10.0	97

Table 3.2 Approximate volumes of 0.5 NaOH to be added to 330 mL of System Solution in order to reach a certain pH.

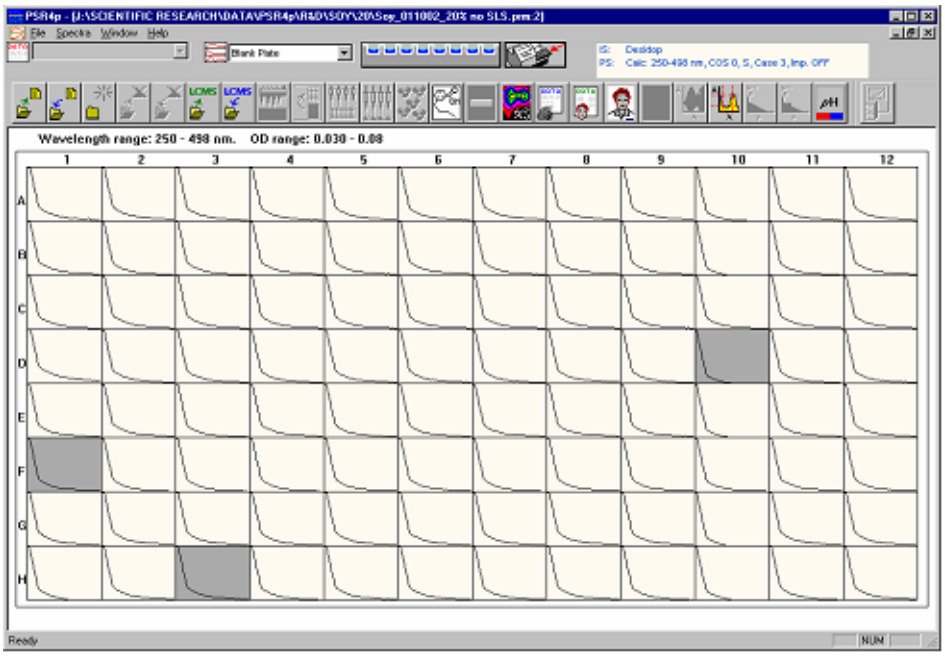
pH_{START} = 2.6		pH_{START} = 2.8		pH_{START} = 3.0	
pHTARGET	VOL (mL)	pHTARGET	VOL (mL)	pHTARGET	VOL (mL)
5.0	5.4	5.0	5.0	5.0	4.5
6.2	8.0	6.2	7.5	6.2	7.0
7.4	10.5	7.4	10.0	7.4	9.5

3.5 Detecting Microbial Growth in the System Solution

The System Solution has an anti-fungal agent in the concentrate to prevent contamination. After dilution the anti-fungal is no longer effective. It is recommended to routinely filter out the buffer using a 0.2 µM filter cellulose acetate filter. This will remove most bacterial contamination.

CAUTION If this process is not used, possible contamination is possible that can effect the sensitivity of the assay.

Figure 3.1 'View 96 Spectra' displaying blank spectra collected with uncontaminated System Solution.



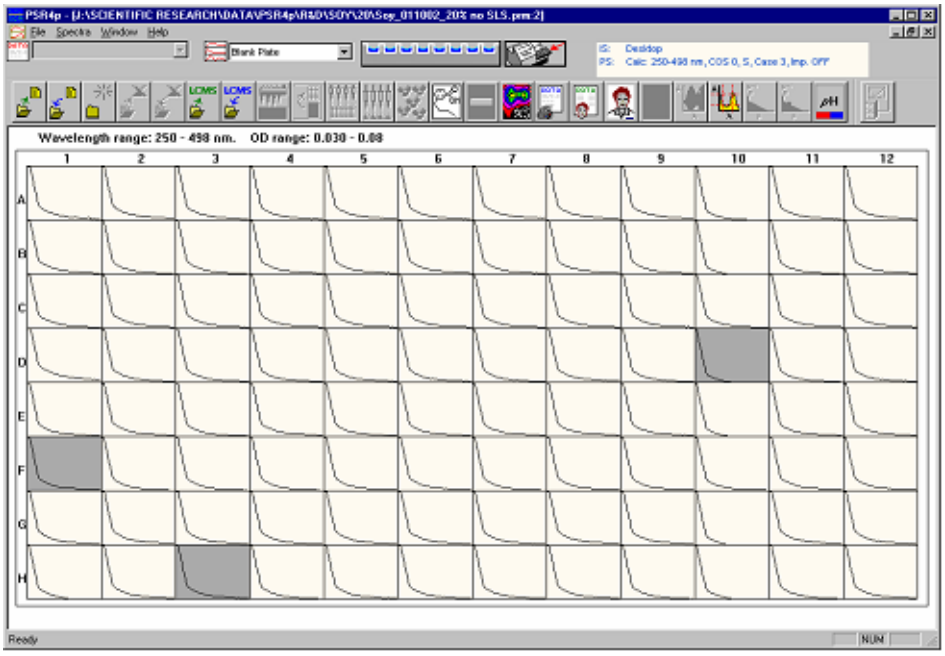
Spotting Contamination in the System Solution by UV

System Solution with no contamination

After a blank plate (containing only System Solution) is read: go to View 96 Spectra (raw UV data) and clip the spectra from 0.03 - 0.08 OD, keeping the wavelength range at 250-500nm. An UV spectrum of a blank that has no contamination should have the maximum absorbance at low wavelength (250 nm) and the minimum absorbance at the highest wavelength (500nm), as shown in the View 96 Spectra of Figure 3.1 and the maximized view in Figure 3.2.

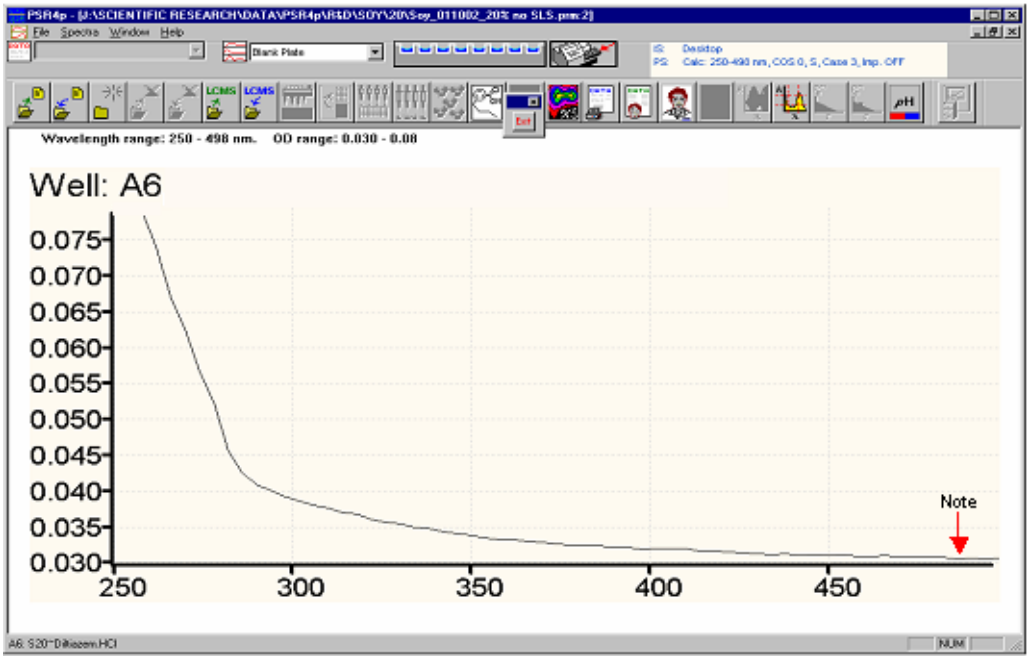
NOTE This analysis uses pION UV plates.

Figure 3.2 'View 96 Spectra' displaying blank spectra collected with uncontaminated System Solution.



To see the Expanded view as shown below, double click a single well in the 96 spectra view.

Figure 3.3 Expanded view of a well containing System Solution without contamination.



The UV absorbance of pH 7.4 System Solution and the UV plate at 300 nm should be less than 0.04 OD. UV absorbance of the System Solution and plastic plate higher than 0.04 OD can cause the processed UV spectra to be faulty.

System Solution with contamination

Contaminated System Solution can be spotted by taking a UV spectrum of the solution. It is seen that the maximum and the minimum absorbance of the spectrum is much larger than the 0.03 - 0.08 OD in the spectral range 250 - 500nm, as shown above. This is due to the higher UV absorbance of the bacteria in solution as seen in the maximized spectrum ofFigure 3.4. Then another key observation is the skewed baseline in the UV spectrum.

Figure 3.4 Spectral view of Blank Spectra with some contamination in the System Solution.

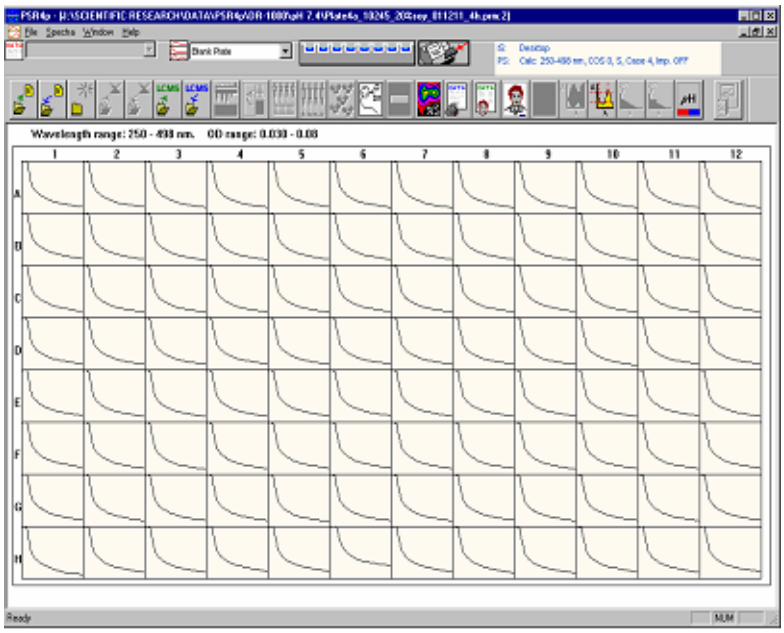
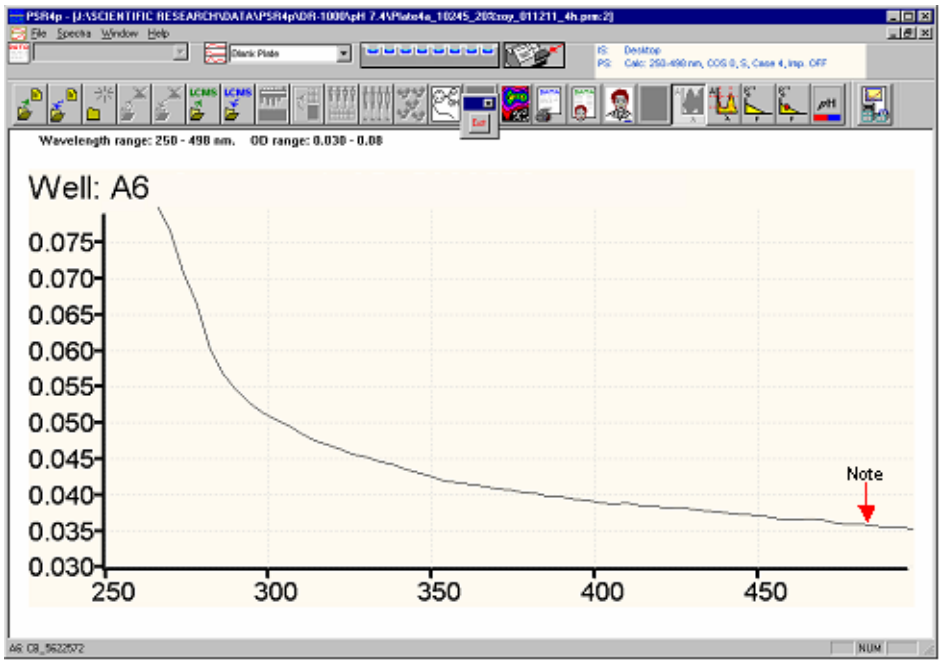
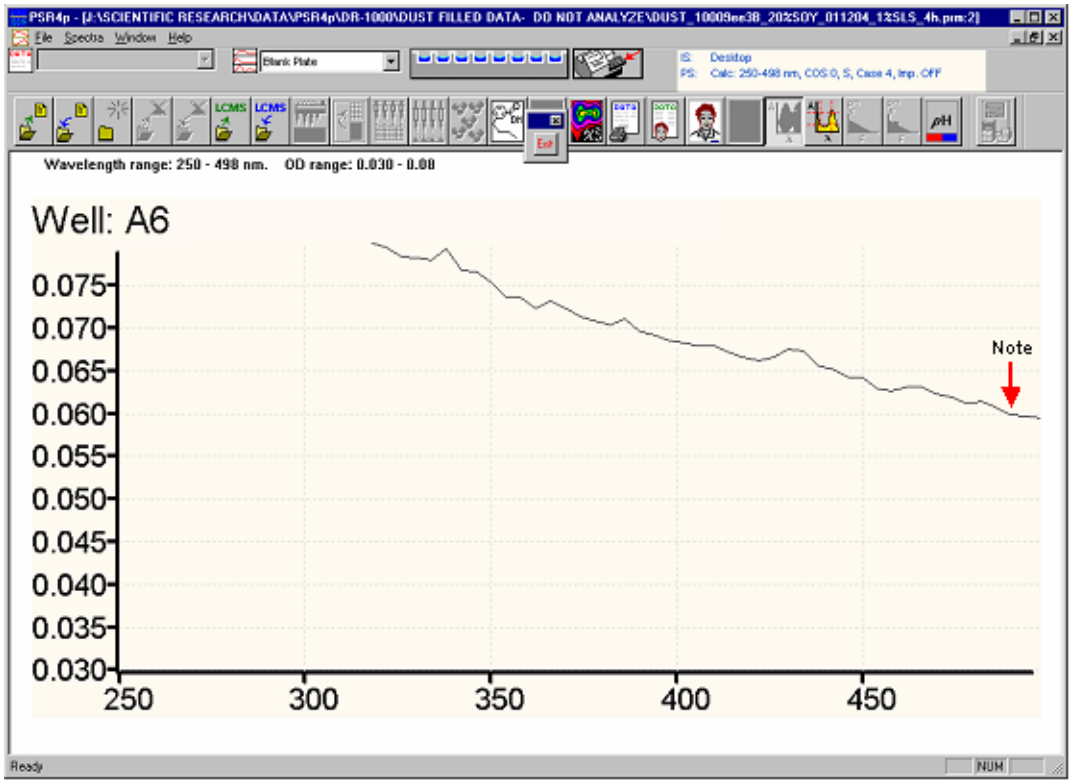


Figure 3.5 Expanded view of a well containing System Solution with contamination.



An example of pH 7.4 System Solution UV spectra containing a high amount of bacterial contamination is shown in Figure 3.5, using the same clip range as Figure 3.6. The UV spectrum is very noisy. There is a significant absorbance in the range: 250 - 500nm.

Figure 3.6 Expanded view of a well containing System Solution with high amount of contamination.



Preventing Contamination

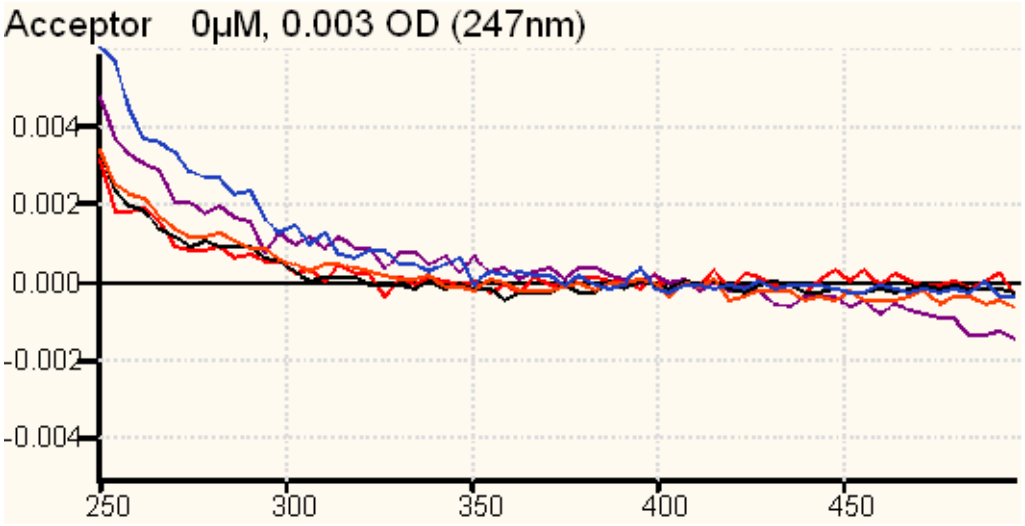
One weeks is the longest period the System Solution should be used after it has been diluted (regardless of its final pH). Should contamination occur, wash the bottle with household bleach, (in the US, one can use Clorox, which is a 6% sodium hypochlorite in water). Add 10mL of household bleach to 200mL of deionized water.

It is strongly recommend that the System Solution concentrate be stored in a refrigerator at all times to prevent contamination. **When in doubt, please filter diluted system solution through 0.2 µM filters to remove any bacteria, prior to use.**

Method for Detecting Bacteria in the System Solution

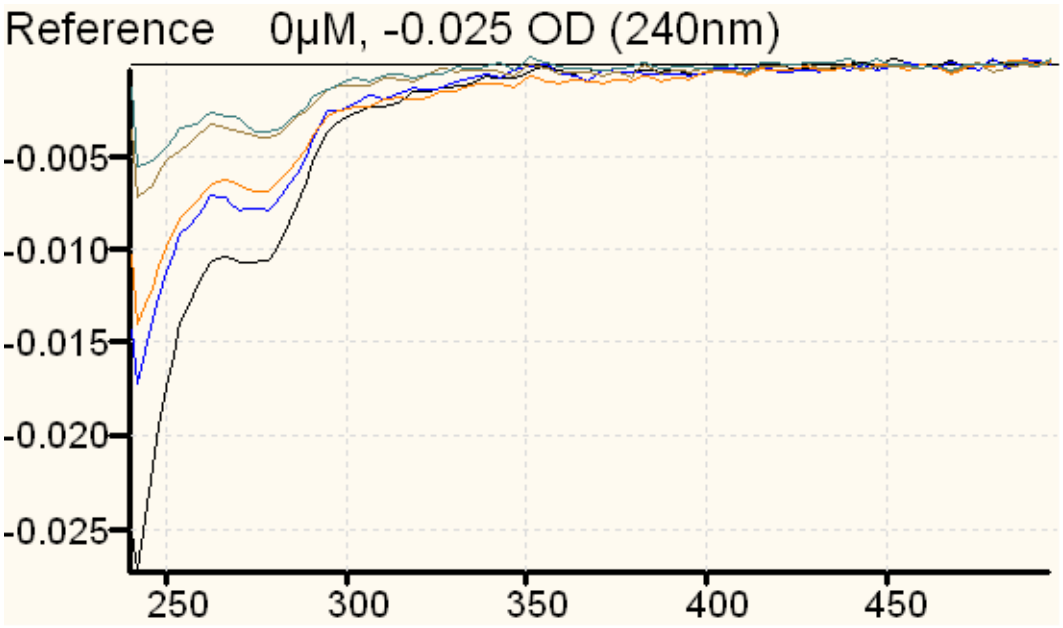
A method is described which collects four spectra, blank, reference, acceptor, donor containing only freshly prepared System Solution. Since the reference, acceptor and donor all have the blank subtracted from them, when the spectra are viewed, one should see a line very close to 0, see Figure 3.7. This file is saved as a Quality Control dataset.

Figure 3.7 Spectra with no contamination in the System Solution.



If bacterial growth is suspected in the SystemSolution, the file containing the Quality Control spectral is loaded into the program. A spectra of only the suspect System Solution is collected either as the reference, donor or acceptor. When you view the new spectra, the straight line is no longer observed, the absorption is negative. This is due to the impurity of the microbial growth, see Figure 3.7. This file is given a new name and saved to not loose the original Quality Control dataset.

Figure 3.8



The following steps describe how to detect bacteria in the System Solution.

1. Open a new file in the permeability software and import a test file of eight compounds (A1-H1) from an excel spreadsheet.
2. Save the file in the DATA directory and provide a name so that it is obvious it is a quality control dataset.
3. Dispense 150 L of a newly prepared System Solution into a washed UV plate.
4. Read the UV plate four times and save as blank, acceptor, donor and reference, 240-500nm.

This file contains information about the System Solution and subsequent System Solution can be compared to it.

To run a quality control of the System Solution:

1. Open the Quality Control file.
2. Dispense System Solution into a washed UV Plate.
3. Read the UV plate and save as the reference.
4. Save the file as a new file name.

A solution that contains no contamination will have a zero baseline, as seen in Figure 3.6. A contaminated System Solution will have some absorption at 275 nm, as seen in Figure 3.7.

Getting Started

4.1 Introduction

CAUTION Carefully follow the suggested operational steps. Damage to the robot may result from improper placement of plates, lids and other worksurface components.

The following tutorial protocol is for a 0.5 hour incubation time stirring experiment and is designed to provide a quick overview of the process. This test procedure will take approximately 2 hours to complete plus approximately 2-4 hours of laboratory preparation. As an aide, a test set of compounds is included in the installation kit. The procedure is based on processing these compounds as if they were uncharacterized samples. All steps involved in the process are included below.

For a detailed explanation of dialog boxes, definitions, and for suggestions, refer to Operation Section 5.

You will need the following items for the procedures in this chapter.

1. pION Test Compounds in a PCR microtitre plate Preparing the Stock plate Section 4.3.	❑
2. EXCEL spreadsheet, see Preparing an Input Excel File Section 4.4.	❑
3. System Solution, see Preparing the System Solution Section 3.2.	❑
4. File "Default Blank Plate.spc" containing UV spectra of BLANK Plate.	❑
5. UV Plates - 3.	❑
6. Deep Well Plate - 1.	❑
7. Pre-loaded PAMPA Sandwich - 1.	❑
8. SUPPORT Plate - 1.	❑
9. Lids - 1.	❑
10. High Profile, 12 Trough Reservoir - 1.	❑
11. Low Profile Reservoir (for ASB) - 1	❑
12. High Profile Reservoir - 1	❑
13. 250 µL Tips - 5 boxes	❑
14. PCR plate for lipid - 1	❑
15. GIT-0 Lipid - 2 vials	❑
16. 0.5 M NaOH Solution	❑
17. Acceptor Sink Buffer - 7.4	❑
18. Gut-Box TM	❑

The required laboratory equipment and reagents are:

1.	Laboratory magnetic stirrer	❑
2.	1" Stirrer bars - 3	❑
3.	Laboratory pH-meter	❑
4.	Spectroscopic Purity DMSO, CAS. 67-68-5	❑
5.	Plate Washer (optional)	❑
6.	8-channel pipettor (5-100 µL)	❑
7.	20 -100 µL one channel pipette	❑
8.	10 mL one-channel pipette or 25 mL graduated cylinder	❑
9.	Sonicator (optional)	❑

4.2 Preparing the Samples

Seven vials containing pION test compounds are included in the installation kit along with seven empty vials. To prepare the samples from the vials included you will need:

1. Spectroscopic Purity DMSO	❑
2. 1mL pipette	❑
3. Sonicator (optional)	❑
4. PCR plate 96	❑
5. Plate Washer (optional)	❑
6. Plate Lid	❑
7. 20 -100 µL Pipette	❑

Each vial contains about 50 mg of material. Prepare 10 mM solutions of the test compounds.

Use the exact amount, according to Figures 4.1 below, and weigh the compound into the empty vials.

a. Add 1 ml of DMSO.
b. Shake the vials or sonicate if necessary.

Table 4.1 Table of solution mix

Samples	MW	Weight (mg)	Volume, mL of DMSO	Expected concentration, mM
Ketoprofen	254.3	2.54	1	10
Verapamil	491.1	4.91	1	10
Antipyrine	188.2	9.40	1	50
Metoprolol	684.8	6.85	1	10
Carbamazepine	236.3	2.36	1	10
Ranitidine	350.9	3.51	1	10
Propranolol	295.8	2.96	1	10
Atenolol	266.3	2.66	1	10

The precise concentration is not needed for the permeability analysis. Any material left over after the procedure may be frozen and re-used at a later time.

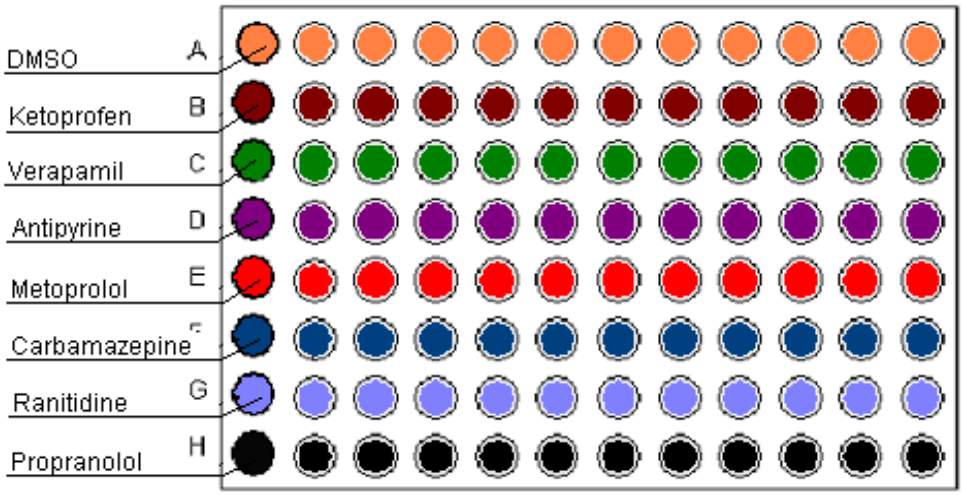
4.3 Preparing the Stock plate

1. Pipette by hand - and without cross contamination - 80 µL of each solution into the wells of a clean (use the plate washer if one is available) PCR plate.

EXAMPLE:

A correctly prepared stock plate for 7 pION test compounds, as shown in Figure 4.1 below.

Figure 4.1 A correctly prepared stock plate.



2. Cover the plate with a lid to avoid airborne particulate contamination ('dust').

4.4 Preparing an Input Excel File

The PAMPA Evolution Command program takes user-prepared data from a Microsoft® Excel spreadsheet file as its input. This same file can also be used as an output file for the assay data if so desired.

1. Start Microsoft Excel.
2. Select **File | Open**. Browse to find the default PAMPA template. The file is called "PAMPA Template.xlt" and is located in PAMPA Evolution Command Software.
3. Fill in the compound information ACCORDING TO THE PREPARED STOCK PLATE: Sample Name, Stock Conc, mM and MW, as shown Figure 4.2 below.

   Each row represents a different compound. Physicochemical constants, like pK_a, log D, are optional, as are many other entries in the Excel file. For the permeability experiment, the Sample Name, MW and concentration are the required fields.

CAUTION Fill the excel sheet by adding compounds down each column and not across rows. Do NOT leave blank spaces in the Sample Name column.

Figure 4.2 Compound Information Table.

Microsoft Excel - pION TEST COMPOUNDS_Evolution96.xls

File Edit View Insert Format Tools Data Window Help

N27

	A	B	C	D	E	F	G	H	I	J	K	L
1	Stock Plat	Sample Name	Stock Conc, mM	MW	pKa1	pKa2	logP	ABX	Theme No.	Stock Soln.	Fract.COS.	Compound No. (re
2	A1	DMSO										
3	B1	Ketoprofen	10.00	254.3	4.12		3.16					
4	C1	Verapamil	10.00	454.6	9.07		4.33					
5	D1	Antipyrine	50.00	188.2			0.56					
6	E1	Metoprolol	10.00	267.4	9.56		1.95					
7	F1	Carbamazepine	10.00	236.3			2.45					
8	G1	Ranitidine	10.00	350.9	8.31		1.28					
9	H1	Propranolol	10.00	259.3	9.53		3.48					
10	A2	DMSO										
11	B2	Ketoprofen	10.00	254.3	4.12		3.16					
12	C2	Verapamil	10.00	454.6	9.07		4.33					
13	D2	Antipyrine	50.00	188.2			0.56					
14	E2	Metoprolol	10.00	267.4	9.56		1.95					
15	F2	Carbamazepine	10.00	236.3			2.45					
16	G2	Ranitidine	10.00	350.9	8.31		1.28					
17	H2	Propranolol	10.00	259.3	9.53		3.48					
18	A3	DMSO										
19	B3	Ketoprofen	10.00	254.3	4.12		3.16					
20	C3	Verapamil	10.00	454.6	9.07		4.33					
21	D3	Antipyrine	50.00	188.2			0.56					
22	E3	Metoprolol	10.00	267.4	9.56		1.95					
23	F3	Carbamazepine	10.00	236.3			2.45					
24	G3	Ranitidine	10.00	350.9	8.31		1.28					
25	H3	Propranolol	10.00	259.3	9.53		3.48					
26	A4	DMSO										
27	B4	Ketoprofen	10.00	254.3	4.12		3.16					
28	C4	Verapamil	10.00	454.6	9.07		4.33					
29	D4	Antipyrine	50.00	188.2			0.56					
30	E4	Metoprolol	10.00	267.4	9.56		1.95					
31	F4	Carbamazepine	10.00	236.3			2.45					

conc / calc / Sheet3 /

Ready NUM

Reserve one well for DMSO or the solvent used to dissolve the compounds as "blanks". No MW or concentration information is needed for this compound.

NOTE Included with the installation software is the Test Samples Template. This file can be found on your hard drive in **D:\PAMPA Evolution Command Software\PAMPA Evolution DATA** directory after installation.

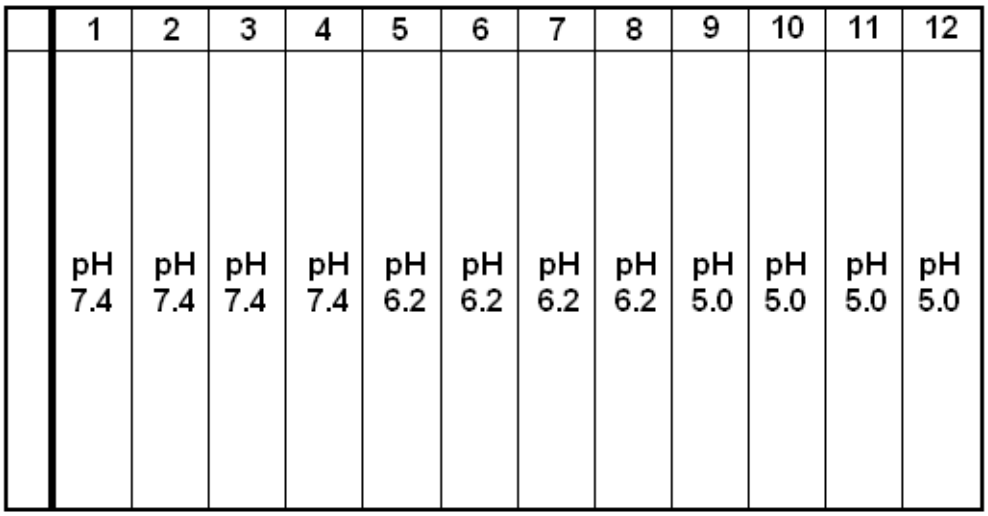
4. Name the file “YYMMDDXX Test Set” (e.g., 040525HT Test Set) where the first 6 characters represent the year (YY), month(MM), day(DD), XX (operators initials), and a text string identifying the assay.
5. Save the file in the DATA sub-folder of the PAMPA Evolution Command Software folder. **D:\PAMPA Evolution Command Software\PAMPA Evolution DATA**
6. Exit the Excel software.

4.5 Preparing Solutions

To prepare the System Solution:

1. Prepare 1 liter of the System Solution according to description in The System Solution Section 3.
2. Place 200 ml aliquots of the System Solution in the three 1L bottles supplied.
3. Take the initial pH of the System Solution using a laboratory pH-meter. It is recommended to calibrate the electrode before measurement using a one-point calibration procedure.
4. Adjust the pH values of the System Solution inthe bottles sequentially to 5.0, 6.2 and 7.4 by adding aliquots of 0.5 M NaOH. See Table 4.1 for a volume guideline.
5. Pour about 15 ml of the pH-adjusted System Solution into each partition of the High Profile, 12 Trough Reservoir as shown in Figure 4.3 below.

Figure 4.3 12 Trough Reservoir, correctly filled



6. Place 200 mL of De-ionized water into one High Profile Reservoir.
7. Place 50 mL of ASB 7.4 into one Low Profile Reservoir.

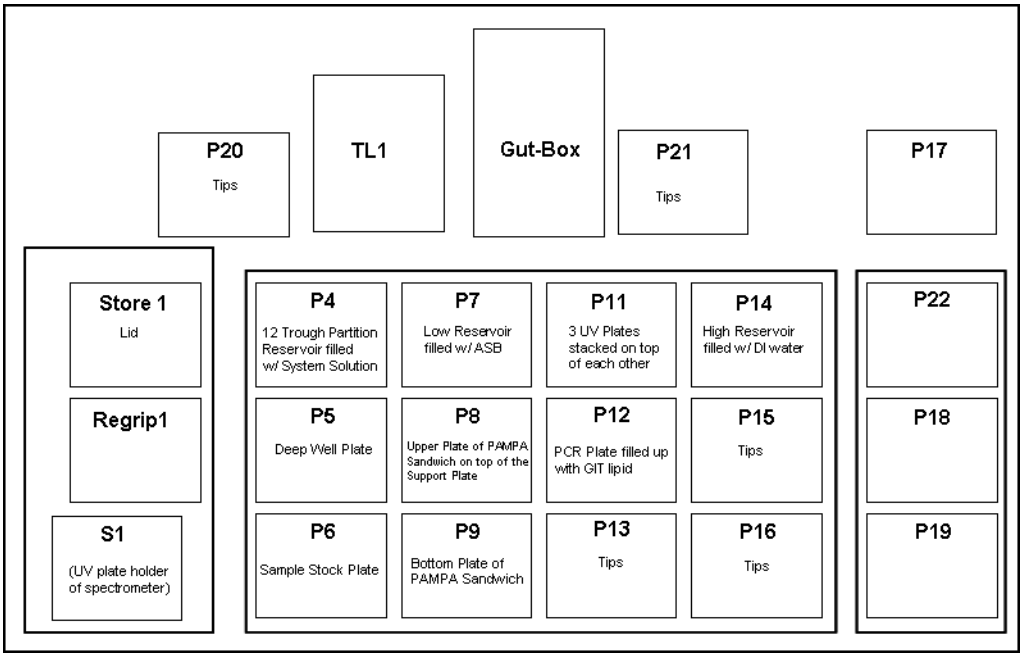
   A High Profile Reservoir can also be used. See The System Solution Section 3 for preparation of the ASB solution.

NOTE At room temperature, 25º C, the ABS solution is clear.

4.6 Setting up the worksurface

Figure 4.4 shows the arrangement of the plates and reagents for the PAMPA Experiment on the Biomek FX worksurface.

Figure 4.4 PAMPA Assay Plate Layout



NOTE Depending on the features of your Biomek-FX, the arrangement may look slightly different.

Populate the Robot worksurface with the following:

1. Place the 12 Trough High Partition Reservoir filled with pH adjusted System Solution into position P4.
2. Place the ASB solution Low Profile Reservoir into position P7.
3. Being careful not to touch the underside ofthe UV plates, place 3 new 96-well plastic UV plate into position P11 according to Figure 4.4.

   Use a plate washer to perform a thorough rinse of the UV plates prior to use to remove dust and plastic residual.
4. Place the DI Water High Profile Reservoir into position P14.
5. Place a 96-deep-well plate into position P5.

Make sure the deep well plate is dust-free. It cannot be rinsed on the plate washer but it may be rinsed with the DI water.

6. Place the SUPPORT plate in position P8. Separate the PAMPA Sandwich . Place the top part of the PAMPA Sandwich (permeation plate) on top of the SUPPORT plate.

NOTE The SUPPORT plate is used as a temporary stand for the painting of the lipid onto the top plate of the PAMPA Sandwich. DO NOT throw the Support plate away after the assay.

7. Place the bottom plate into position P9.
8. From the freezer, take out 2 glass ampules of the pION artificial membrane-forming GIT lipid solution and thaw it in your hand (approximately 2 sec.).

CAUTION Use the disposable ampule breaker provided and gloves to CAREFULLY break off the scratched-glass neck of the ampule. The ampule will have a glass edge. BE CAREFUL with the ampule! Please check your facility for proper protection, procedure, and disposal of glass.

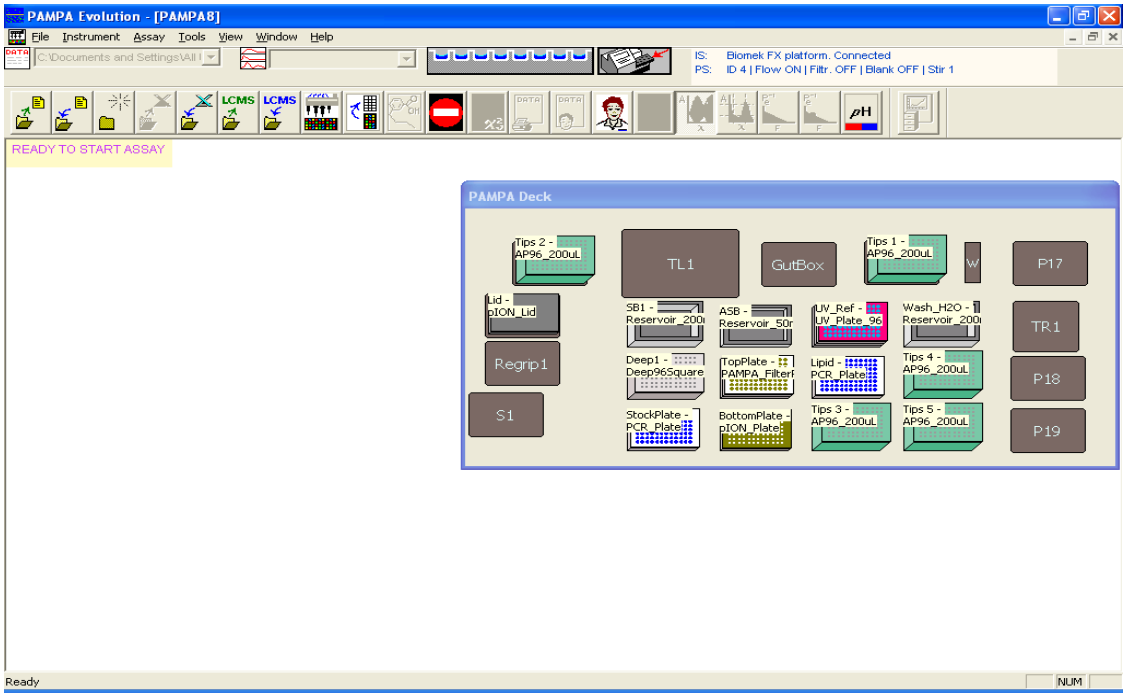
Carefully empty two vials of GIT lipid into the first column of a Low Profile, 12 Trough Reservoir or some other clean trough and transfer 18 µL per well into PCR plate using 8-channel pipettor. Place the PCR plate in position P12.

9. Place the stock plate containing samples in position P6, making sure the samples are not frozen and that the stock plate is uncovered.
10. Place five boxes of 220 µL pipette tips in positions P20, TL-1, P15, P13 and P16.
11. Place a lid in position "Store 1".

4.7 Starting the Assay Procedure

1. Launch the PAMPA Evolution96 Command Software on the computer connected to the Biomek-FX robot. The display is seen in Figure 4.5.

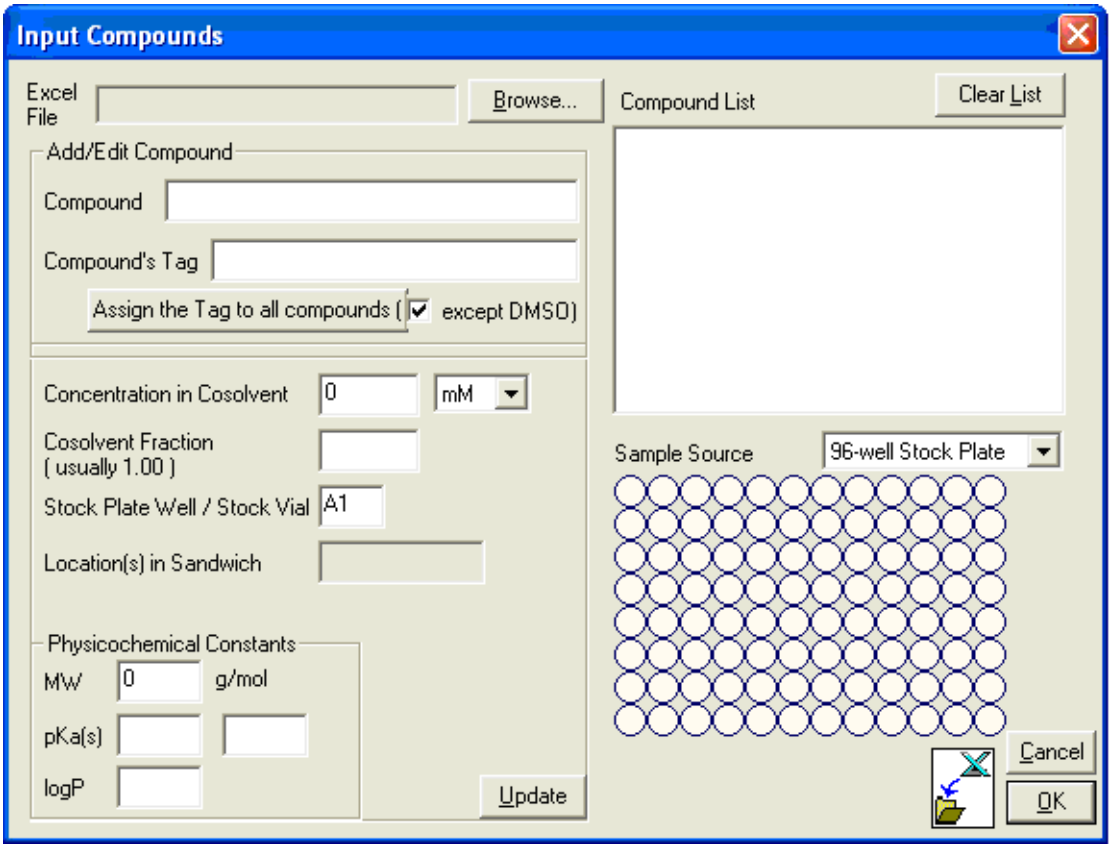
Figure 4.5 PAMPA Evolution96 Main Screen.



2. Move the cursor slowly over the toolbar buttons and examine the tool tip messages as they appear when the cursor is resting on the icons to become familiar with the menu.
3. Click the **Import from Excel** button on the software toolbar.

The Input Compounds dialog box displays as shown in Figure 4.6 below.

Figure 4.6 Input Compounds Dialog Box.

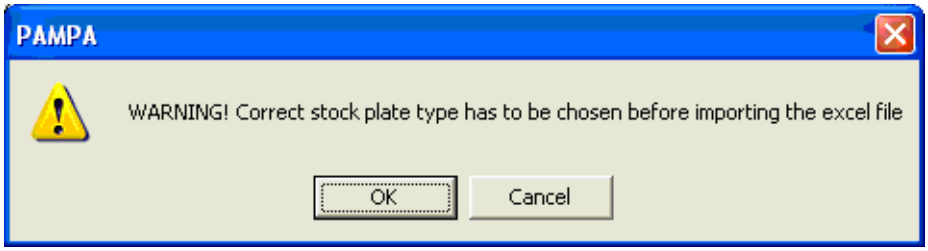


4. Click the **Browse** button to locate and import the PAMPA Input Data File prepared in Preparing an Input Excel File Section 4.4.
5. Select the 96-well Stock Plate from the Sample Source drop-down box.

NOTE If the correct stock plate has not been chosen as shown above, the following dialog box displays as shown in Figure 4.7 below.

- The stock plate should be a 96-well format.

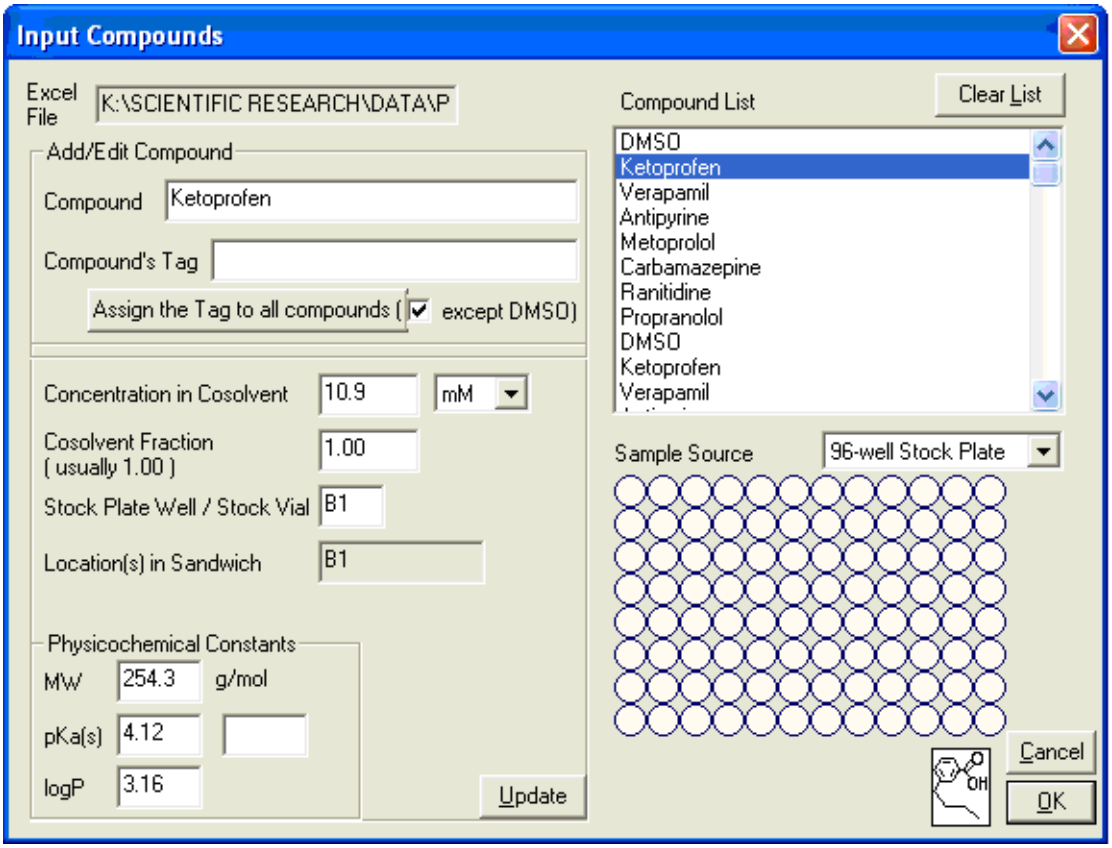
Figure 4.7 Correct Stock Plate Warning Dialog.



6. Click the **OK** button to display the proper stock plate dialog window.
7. Click the **Open** button when the file has been found.

   The Input Compounds dialog box will fill in with the information from the Excel spreadsheet. Select **Ketoprofen** from the Compound List window and verify that the information matches with the Input Compounds window as shown in Figure 4.8 below.

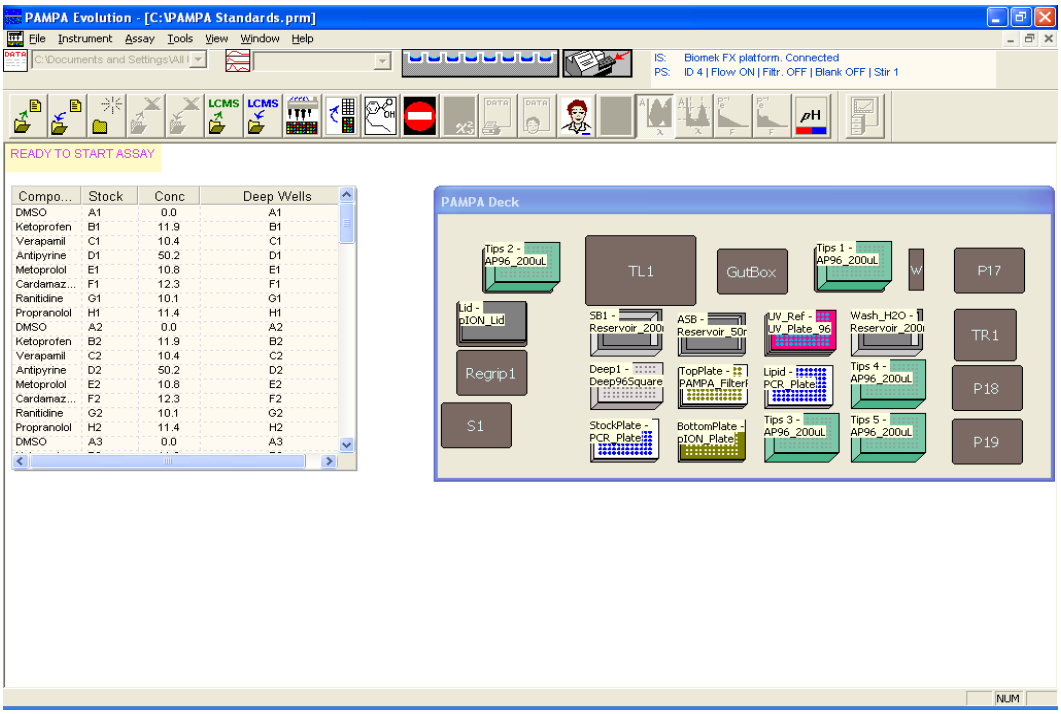
Figure 4.8 Input Compounds Dialog Box.



8. Click the **OK** button.

Figure 4.9 below shows the program`s desktop display after importing the assay template.

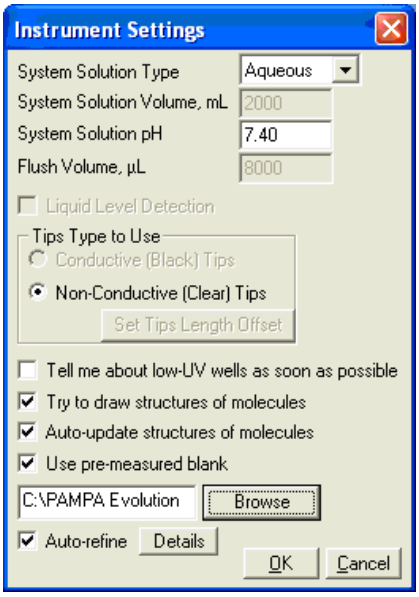
Figure 4.9 PAMPA Desktop after Excel spreadsheet import.



Next, the internal UV blank spectrum must be loaded into the file.

1. From the PAMPA software toolbar, go to **Instrument | Settings**. The following dialog box displays as shown in Figure 4.10 below.

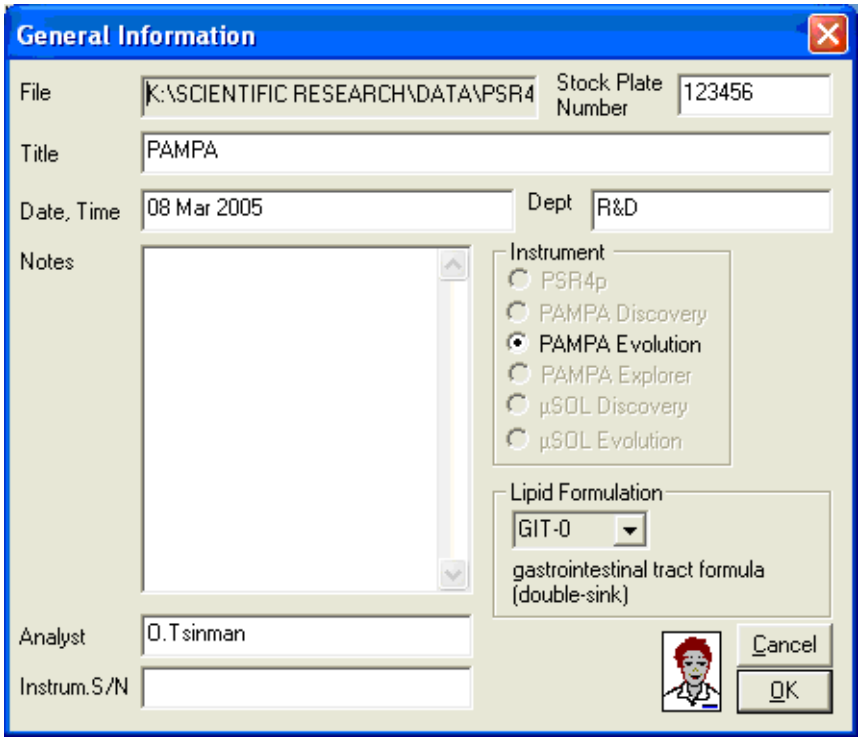
Figure 4.10 Instrument Settings Dialog Box.



2. Browse for the file "`DefaultBlankPlate.spc`" in the Evolution Software PAMPA Command folder and click the **Open** button.
3. Select the **Use pre-measured blank** option.
4. Do NOT select the **Tell me about low-UV wells...** option.
5. Select the **Auto-refine** option. The results of the experiment is automatically calculated.
6. Click the **Ok** button.

7. From the PAMPA software toolbar, check the **General Information** option in order to fill in experimental details. Figure 4.11 shows the dialog box. Fill in all the fields.

Figure 4.11 General Information Dialog.



To run this test, ddd the following text to the Notes window:

- Test r un
- 3 pH's
- Compounds in DMSO
- Stirring 30 minutes
- ABL - 40 µM

8. Click the **OK** button.

4.8 Starting the Robot

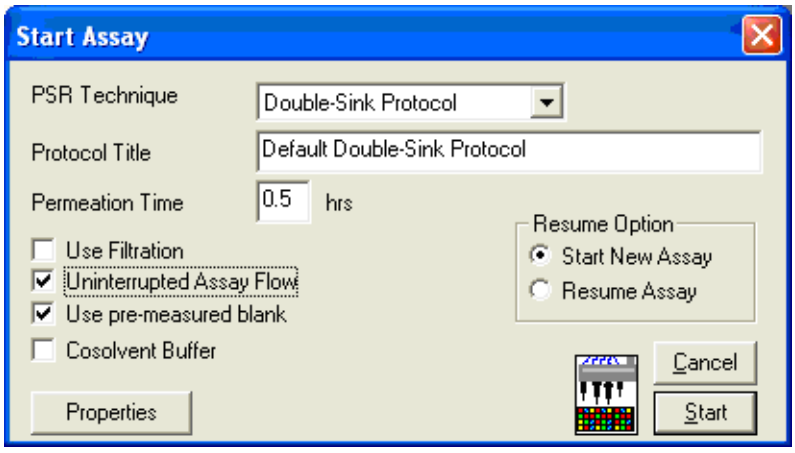
CAUTION Follow the suggested operational steps carefully. Damage to the robot may result from improper placement of plates, lids, and other worksurface components.

Start the Assay

Click the **Start** button on the PAMPA software toolbar.

The Start Assay dialog box displays, as shown in Figure 4.12 below.

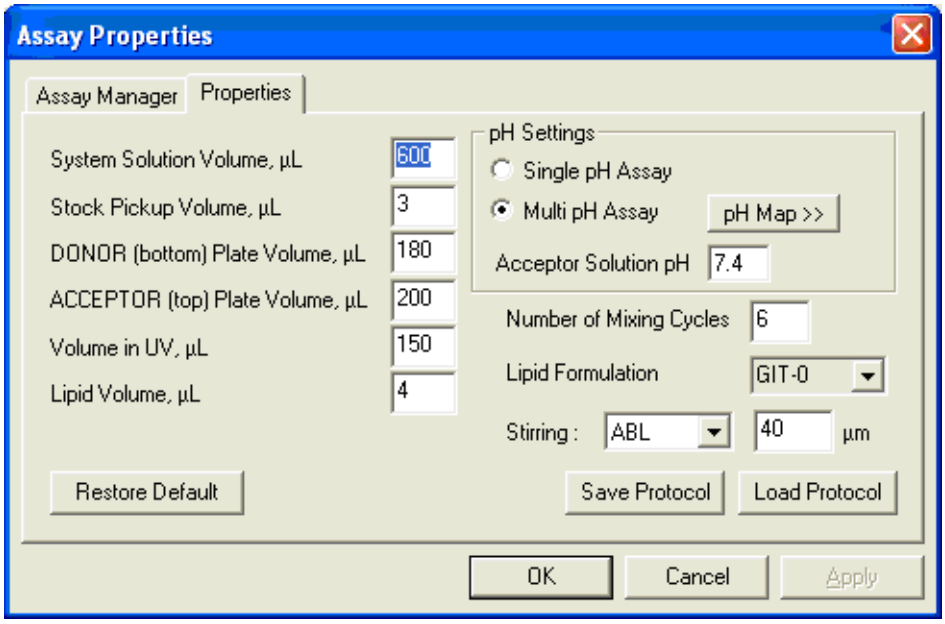
Figure 4.12 Start Assay Options.



1. Type “0.5” in the **Permeation Time** field.
2. Select the **Uninterrupted Assay Flow** and **Use pre-measured blank** options.
   Do Not select the "Use Filtration" and "Cosolvent buffer" options.
3. Select **Double-Sink Protocol** from the drop-down list.

4. Select the **Properties** tab in the Start Assay dialog box for the details of the assay. See Figure 4.13

Figure 4.13 Properties Tab.

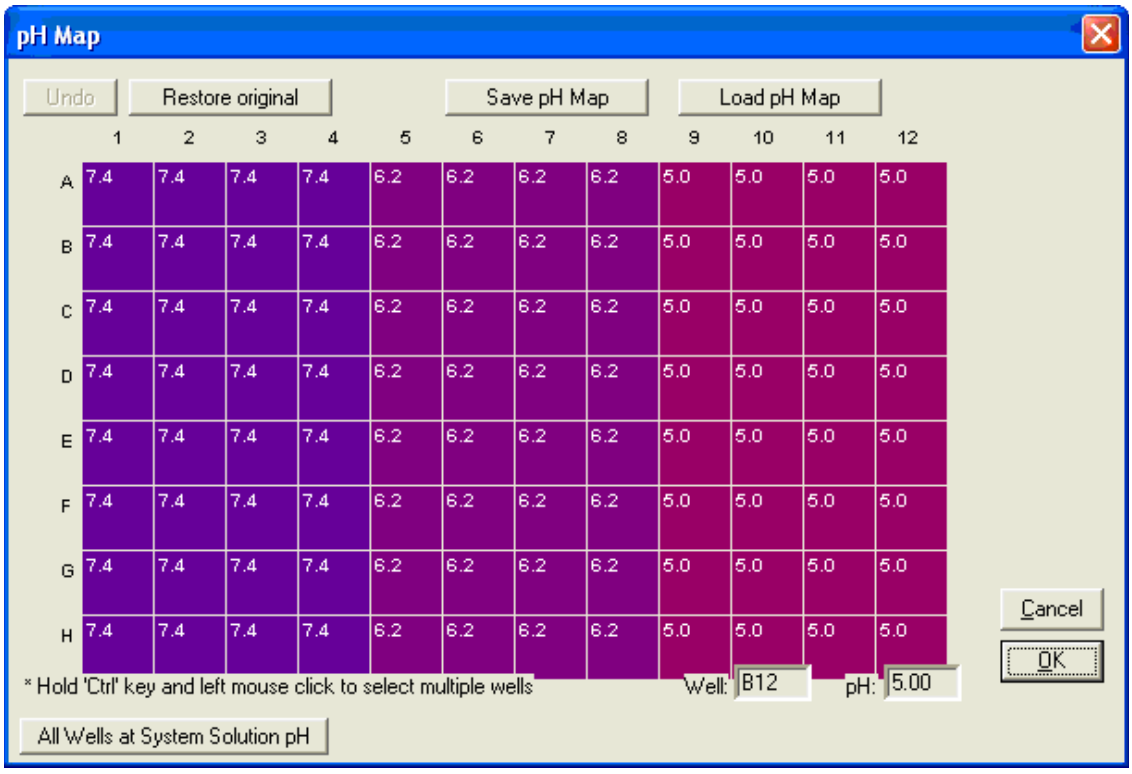


5. Set the System Solution Volume to 600 µL. This is the amount of System Solution transferred to the deep well plate.
6. Select the **Multi pH Assay** option.
7. Select **Aqueous Boundary Layer (ABL)** from the Stirring drop-down list. Set 40 µm for the **Aqueous Boundary Layer (ABL)**.

Check the pH Map

8. From **Assay Properties | Properties** tab, click the **pH Map** button to view the pH-map as shown in Figure 4.14 below.

Figure 4.14 pH Map Dialog Box.



9. Columns 1-4 are set to 7.4, columns 5-8 are set to 6.2, and columns 9-12 are set to 5.0. Click the **OK** button to return to the Assay Properties Assay Manager tab.

4.9 Automated Assay Proceedings

The stock plate (in position P6) has 8 compounds replicated in positions 1-96. From each of the stock wells, 3 µL (Stock Pickup Volume) of sample solution is transferred to the corresponding well in the deep-well plate (Pre-mix Plate Location). This stock quantity is diluted ~ 3:600 (System Solution Vol). The pre-mixing operation will typically produce 50 µM solutions containing 0.5% (v/v) DMSO. Portions of these pre-mixed solutions are distributed to the PAMPA Sandwich bottom plate and portions are used as reference solutions.

The default values in Figure 4.13 are the best choices. Click **Start** to start the Assay.

IMPORTANT A series of Instruction messages will show, indicating the actions to be taken by the robot, and the responses required of the user (if any). A blue box with an "i" displays, indicating the action is taken automatically. To stop the action, click the **Pause** button in the dialog box.

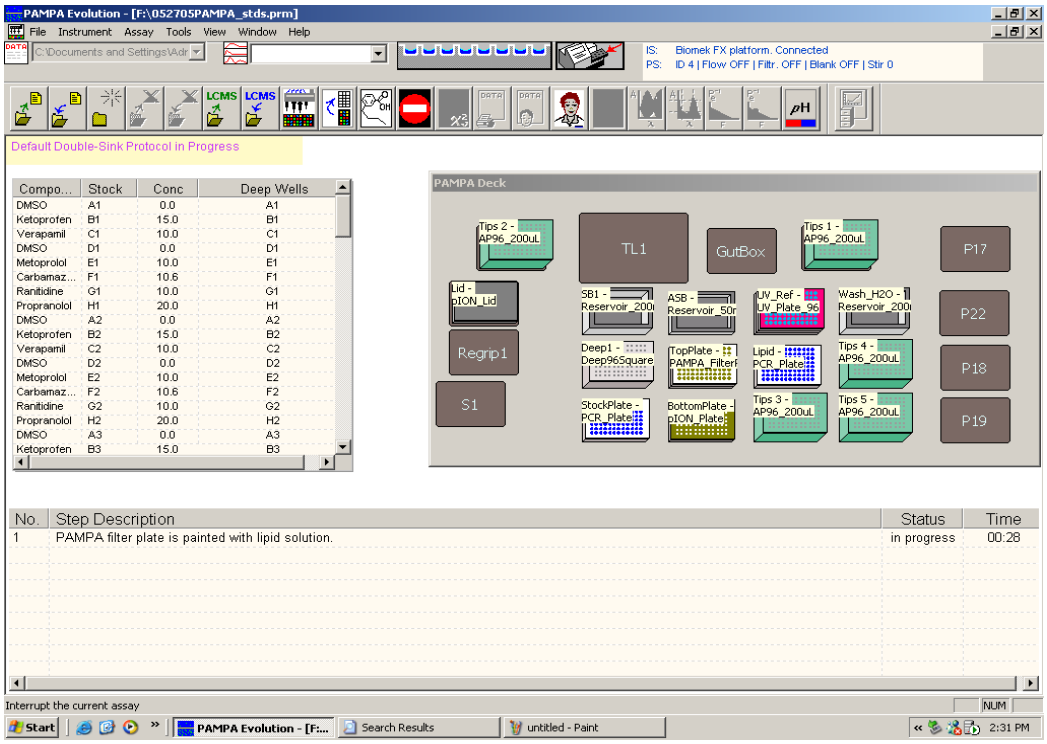
The step by step description displays on the worksurface view. After each step there is a possibility to interrupt the assay or skip the step The assay will continue if no action is taken.

Click the **Start** button after having reviewed the information in the **Start Assay** dialog box. The automated assay begins.

1. The PAMPA Filter plate is painted with lipid solution.

   While this step is occurring, the software display updates the Step Description, so the user knows what part of the assay is occurring at any time, as shown in Figure 4.15 below.

Figure 4.15 Software display with updated Step Description.



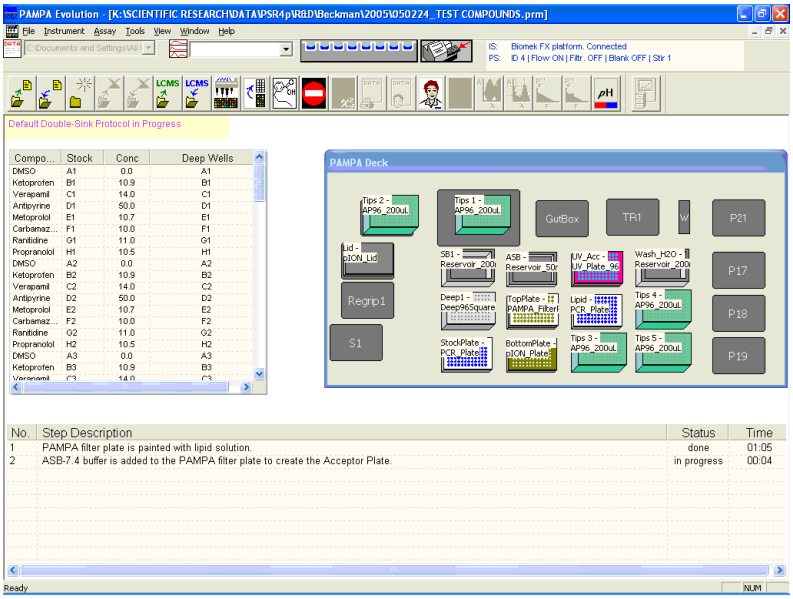
2. Add ASB to the acceptor chamber.

IMPORTANT Press the **Pause** button at this step and the ASB will not be added to the top plate of the PAMPA Sandwich until the **Continue** button is clicked. At this point, you may want to carefully pick up the top plate and look at the underside. If all of the wells are properly coated with the lipid, then they are opaque in color. When the visual inspection is completed, click the **Continue** button to resume assay.

NOTE ASB-7.4 buffer is added to the PAMPA filter plate to create the Acceptor plate.

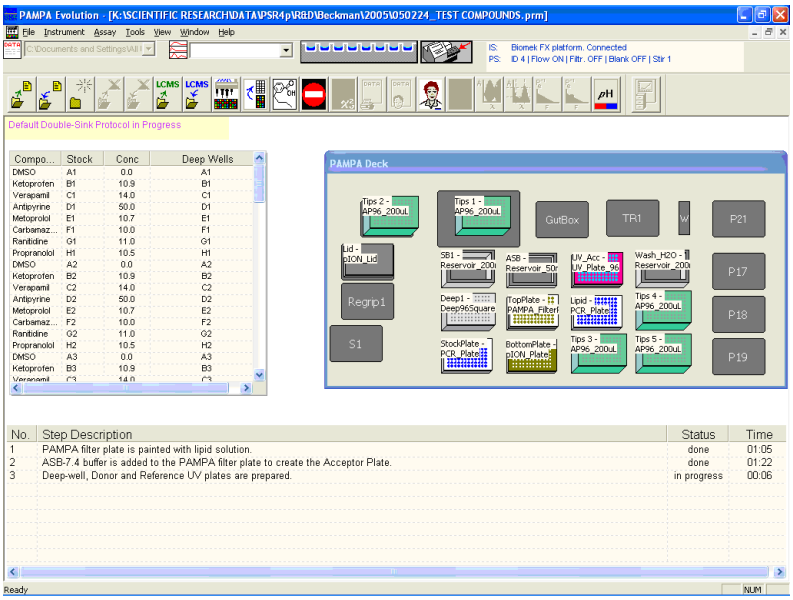
The Step Description updates.

Figure 4.16 Software display with next updated Step Description.



3. The Deep-well, Donor and Reference UV Plates are prepared.

Figure 4.17 Third updated step in Assay progression.



4. The reference UV plate is transferred into the spectrophotometer.

It takes about 15 minutes for all 96 spectra to be read. The file is automatically saved after each read operation. While the reference solution is read in the spectrophotometer, the rest of the assay will continue.

After the spectra reading is complete, the drawer slides will open. To inspect the spectrophotoemetric data, click Spectra Display button. To return to the worksurface view, click the Data Display button.

5. Prepare the PAMPA Sandwich

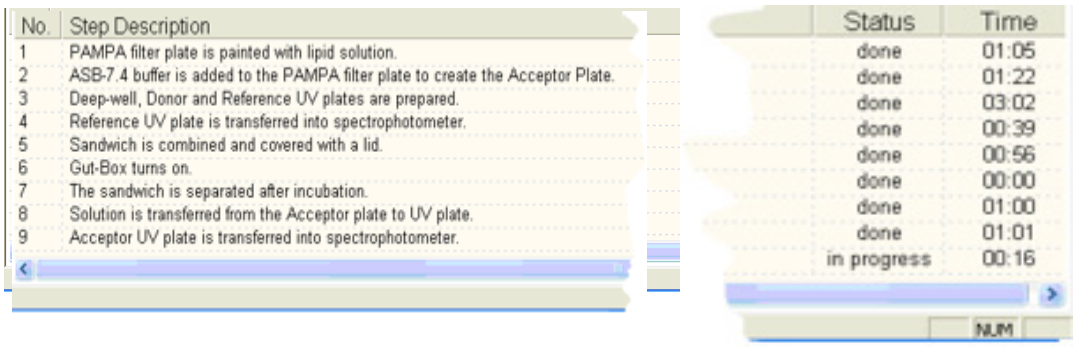
NOTE The Sandwich is combined and covered with a lid.

The Acceptor and Donor plates are combined and placed on the Gut-Box. The assay's time tracker will start at this point. The default incubation time is 30 minutes. The file is saved automatically.

6. Begin stirring and incubation time.
   - The Gut-Box will turn on for this process.
7. End the incubation time period.
   - The Sandwich is separated after incubation.
8. Prepare the Acceptor UV plate.
   - Solution is transferred from the Acceptor plate to the UV plate.
9. Quantitate the Acceptor solution.
   - The Acceptor UV plate is transferred into the spectrophotometer.

   The Spectra reading takes about 15 minutes to complete. When the reading is completed, the drawer will open. The Step Description updates for each step of the Assay, as shown in Figure 4.18 below.

Figure 4.18 Step Descriptions as noted during the entire Assay process.



10. Prepare the Donor UV plate.

NOTE Solution is transferred from the Donor plate to the UV plate.

11. Quantitate the Donor Solution.

NOTE The Donor UV Plate is transferred to the spectrophotometer. The unit will wait until the spectrophotometer has completed the reading, approximately 10 minutes.

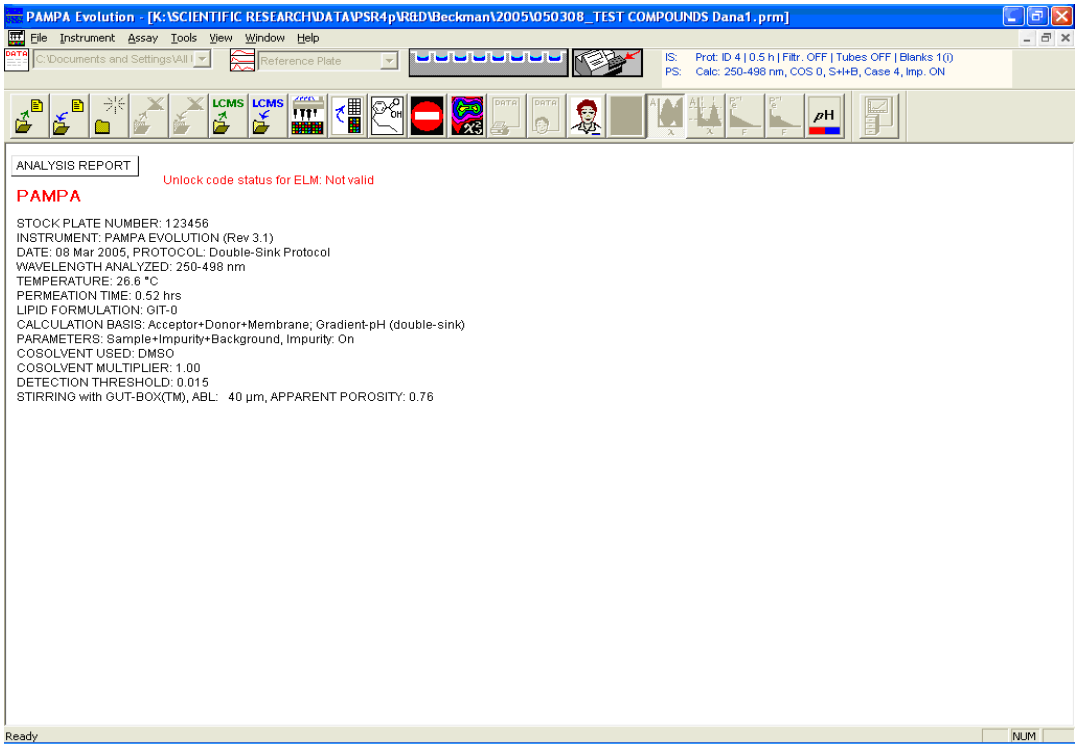
Since the position in the spectrophotometer is currently processing the Acceptor UV plate, the Donor UV plate is transferred in position “Regrip1” and the robot will pause until the Acceptor UV spectra is taken and the drawer opens.

At this time, all the spectra is collected. The data file is complete.

4.10 Processing the Data

The PAMPA software worksurface shows the completed Assay, as shown in Figure 4.19 below.

Figure 4.19 worksurface view after the completed PAMPA Assay.



Click the **Refine Permeability Constants** toolbar icon to process the data.

Operation

The Getting Started Section 4, describes the permeability experiment without the detail of individual steps and an explanation of the dialog boxes. This section describes the permeability protocol in detail.

5.1 PAMPA Evolution software

The PAMPA Evolution software has operational instructions for Permeability.

The PAMPA technique consists of several steps:

1. Dilute small volumes of 10 mM drug samples (in DMSO or another cosolvent) in 600 µL System Solution.
2. Pipette the diluted samples into a donor plate (the BOTTOM Plate of the PAMPA Sandwich).
3. Pipette Acceptor Sink Buffer - 7.4 (ASB) into the acceptor wells (the UPPER Plate of PAMPA Sandwich) which have been 'painted' by the robot with the artificial membrane-forming solution.
4. Create a "PAMPA Sandwich" by placing the acceptor plate on top of the donor plate.
5. Cover the sandwich and incubate with or without stirring, depending on the selected protocol, allowing the compounds to permeate.
6. Scan the UV spectra of the donor, acceptor, and reference wells.
7. Process the spectra to calculate permeabilities, print the results, and export them to a Microsoft Excel spreadsheet or Corporate database.

The 10 mM sample requirement is the average sample needed, based on experience, to obtain a good UV absorption signal for data processing. One may use higher or lower concentrations of samples and obtain permeability results. However, using higher concentrations (50 mM) may lead to detector saturation. Also, the exact concentration is not necessary since PAMPA is a relative measurement (donor to acceptor). Use the 10 mM as a guide for sample preparation.

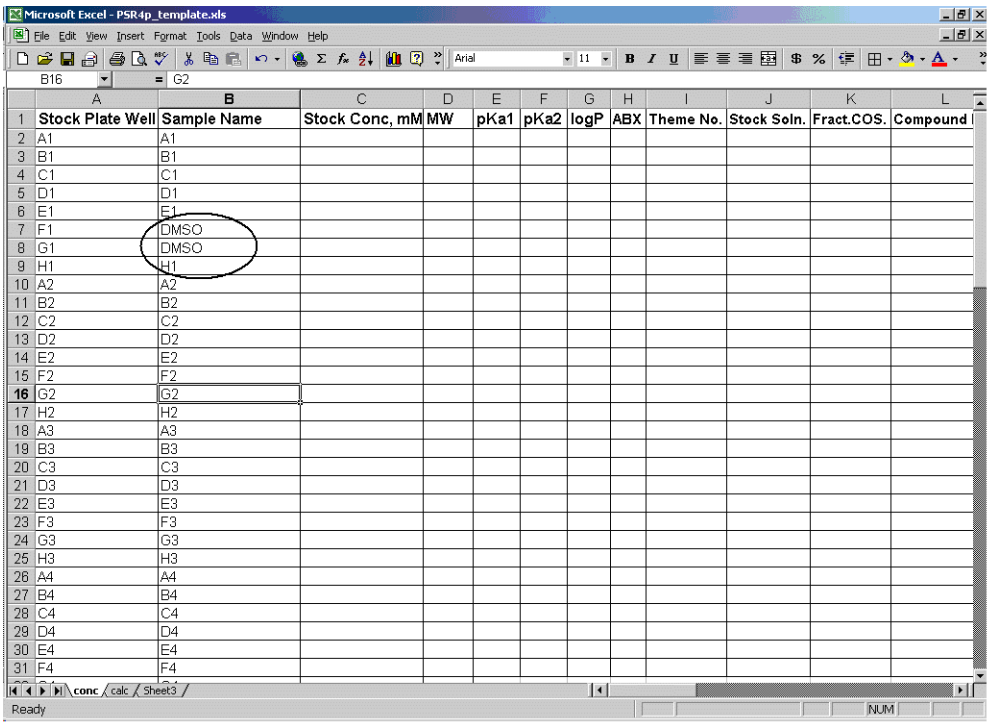
5.2 Preparing the Excel Spreadsheet

The PAMPA Evolution Command Software uses an Excel spreadsheet for its input and output Information about the compounds is contained in the spreadsheet. The information describing each sample compound is placed in a copy of a pre-formatted spreadsheet called a template. A default template, called `PAMPA_template.xlt` is included in the main PAMPA Evolution Command Software directory.

The number of samples introduced to the instrument for the assay is indicated by the number of names listed in the 'Sample Name' column. The compound name is placed in the column 'B' next to the appropriate well identifier in column 'A'. In Figure 5.1, the well identifiers

shown in column 'B' are used as default names. In consecutive rows, type in the names of the compounds to be analyzed and delete all the default names.

Figure 5.1 Template input Excel file



	A	B	C	D	E	F	G	H	I	J	K	L
1	Stock Plate Well	Sample Name	Stock Conc, mM	MW	pKa1	pKa2	logP	ABX	Theme No.	Stock Soln.	Fract.COS.	Compound
2	A1	A1										
3	B1	B1										
4	C1	C1										
5	D1	D1										
6	E1	E1										
7	F1	DMSO										
8	G1	DMSO										
9	H1	H1										
10	A2	A2										
11	B2	B2										
12	C2	C2										
13	D2	D2										
14	E2	E2										
15	F2	F2										
16	G2	G2										
17	H2	H2										
18	A3	A3										
19	B3	B3										
20	C3	C3										
21	D3	D3										
22	E3	E3										
23	F3	F3										
24	G3	G3										
25	H3	H3										
26	A4	A4										
27	B4	B4										
28	C4	C4										
29	D4	D4										
30	E4	E4										
31	F4	F4										

NOTE References to spreadsheet cells are indicated as 'A4,' 'B7,' etc. while plate wells are referenced without single quotes, A4, B7.

Template: To fill in the template, place the names of the samples in column 'B,' 'Sample name' in the row corresponding to the 'Stock Plate Well' column. If no sample is in a well, the 'Sample Name' cell for that well must be left blank. The Excel file must reflect the exact position of the compounds in the stock plate.

It is recommended to reserve at least 6 wells for DMSO or another cosolvent.

NOTE The order of the columns or rows in the spreadsheet MUST NOT be altered.

The following columns must be filled in:

Stock Plate Well	Well identifiers are supplied and protected from editing
Sample Name	The name of the sample. An Empty field means no sample. Avoid beginning the sample name with numbers. The name must contain at least one letter or underscore symbol.

The rest of the fields are optional and need not be filled in to obtain results. The table below refers to the other columns in the Excel file table.

Stock Conc, mM	A concentration of the sample in the Stock Well Plate (usually 10 mM in DMSO).
MW	Molecular Weight of the sample (g/mol).
pKa1, pKa2	pKa values of the sample (known or calculated).
log D	log D of the sample at pH 7.4 (known or calculated).
ABX	A=acid; B=base; X= ampholyte. Description of compound.
Theme No.	Can be used at the user discretion for project number, client, etc.
Stock SoIn.	The name of the solvent used (e.g., DMSO)
Fract.COS.	The Default value is 1. If a compound is not dissolved in 100% DMSO or acetonitrile, but rather in a fractional amount of DMSO in water (v/v), the decimal value of he fraction is placed here. Example: Stock samples are submitted as 45% DMSO solutions, list Cosolvent Fraction as 0.45.
Comment	Any comments entered by the operator are placed by the program in the corresponding cell in this column.
Compound No.	The User's internal alphanumeric designation for the sample.
(reserved)	This column is available for the use to rename and use. If structure are to be entered, use this column.

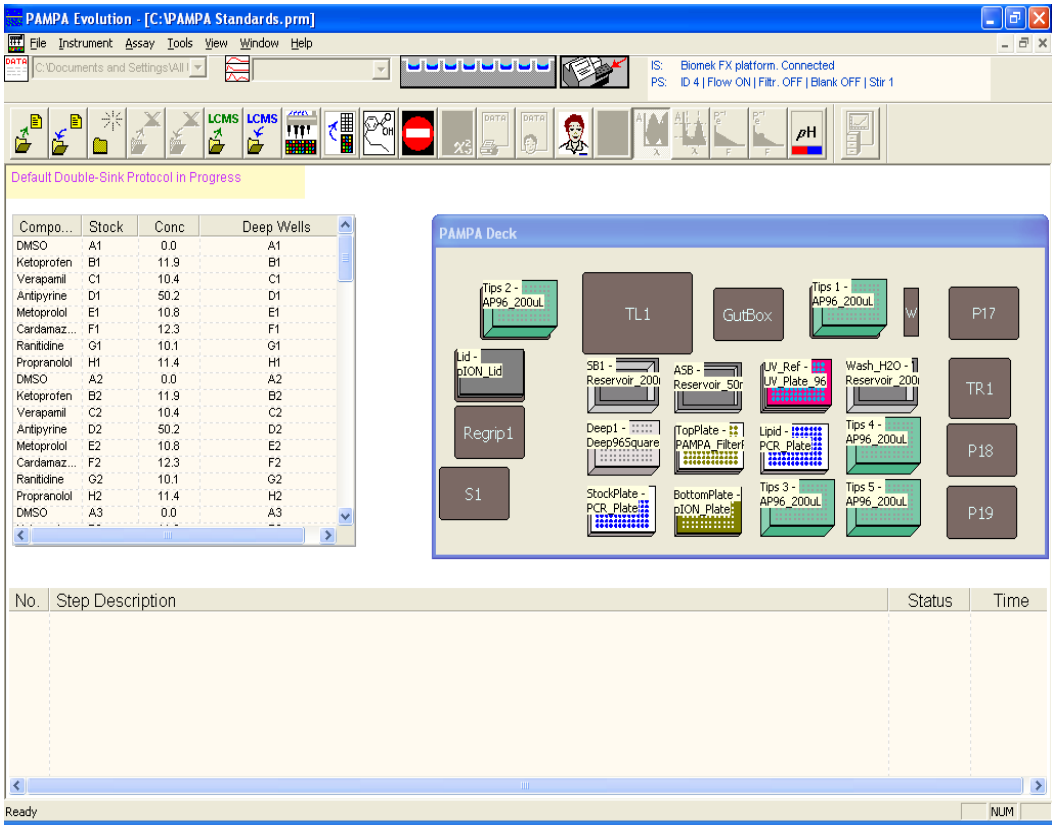
NOTE Do NOT add additional columns to the spreadsheet.

In order to add structures to the Excel file, see Inserting Structures in the Excel Import File Section i.2.

5.3 PAMPA Evolution96 Desktop

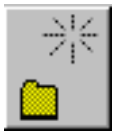
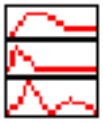
All major software functions are controlled and viewed on the PAMPA Desktop. The graphic below, Figure 5.2, shows the File Menu, the VIEW toolbar, Experimental Settings Window, the PAMPA Toolbar, Compound List Window, the PAMPA Deck layout and the Status Window.

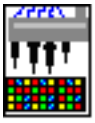
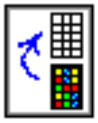
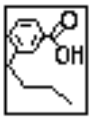
Figure 5.2 Main PAMPA Evolution96 worksurface layout.

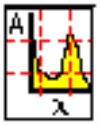
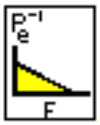


PAMPA Tool Bars

The Main Tool bar resides between the File Menu options, (File, Instrument, etc.) and the Desktop layout. It is comprised of the following software functions.

	Open a File Management software.
	Save a file in the software.
	Close a file in the software.
	View Analysis Report: Used to return to the worksurface view or analysis report.
	View 96 Spectra: Used to return to the 96 spectra matrix view from either the worksurface view or analysis view. Use the drop-down combo box to choose which spectral view to place in the active window.
	Read/Stop Spectrophotometer: Start or stop the UV plate spectrophotometer.
	Open/Close Spectrophotometer Drawer: Open or close the UV plate spectrophotometer drawer.

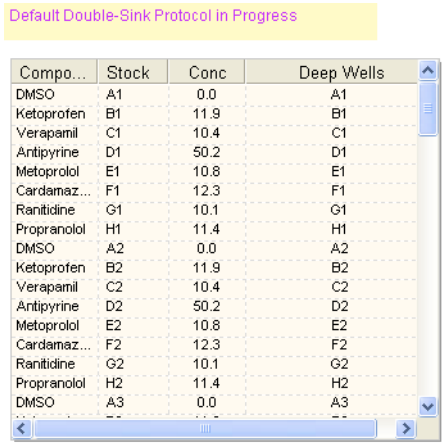
	Export to Excel: Used to export the calculated permeability values and other data to an Excel spreadsheet.
	Import from LCMS: Used to import LCMS data into the PAMPA program for processing.
	Start Assay: Starts the permeability assay.
	Finish Assay Solution Transfer: Used to interrupt incubation and manually continue the assay.
	Compound List: Review the sample names and information about the samples.
	Interrupt: Allows analyst to pause and/or abort the assay. Can be used at any time. The data is not lost.
	Refine Permeability Constants: Calculates results at the end of an experiment.
	Print Preview: Preview the data prior to printing. What is shown depends on the active view. A Zoom function is available within this view.

	Client Report: A Report generated specifically to provide all important results on one page.
	General Information: Assay and user information is entered here.
	Extended Spectra: Allows the view of all components of a spectrum at the same time (e.g., blank, cosolvent, etc.).
	Replicates Spectra: View multiple spectra of one particular compound done in several replicates and/or pH values at the same time.
	Clip Spectra: The spectra can be expanded or clipped to view a particular wavelength or absorbance region.
	Calculate Po from Multi pH: Can calculate intrinsic permeability (Po) from multiple-pH experiments; pKa needs to be provided, along with MW and other constants.
	System Solution QC: Performs a quality control assay on the System Solution. This function not activated.
	ELM: Provides access to the Evolution Library Manager add-on. (ELM is an independent add-on module for managing data. See ELM documentation for further details.)

Compound List Window

The Compound list window, in Figure 5.3 shows the compound names, the well ID in the stock plate, and the stock concentration. The ‘Deep Wells' column lists where the diluted solutions are located in the deep well plate.

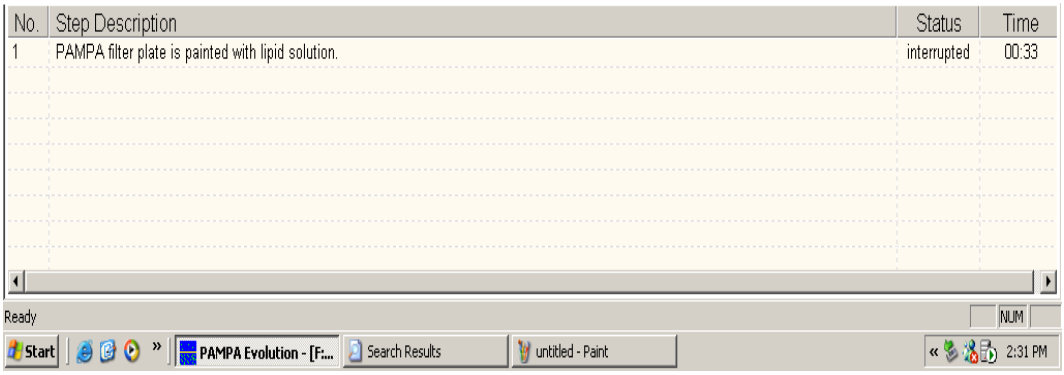
Figure 5.3 Sample of Compound List Window.



Status Window

The protocol is followed by looking at the Status window. The Status window shows each step and whether it is in progress, interrupted or completed. The last column describes how long each step took to complete.

Figure 5.4 Status Window View.



Experimental Settings Window

The instrument and protocol settings are observed in this window.

IS - Instrument Settings

Biomek FX Platform	Robotic platform of the instrument.
Connected	Status of the software. The software is ready to run the robot

PS - Protocol Status

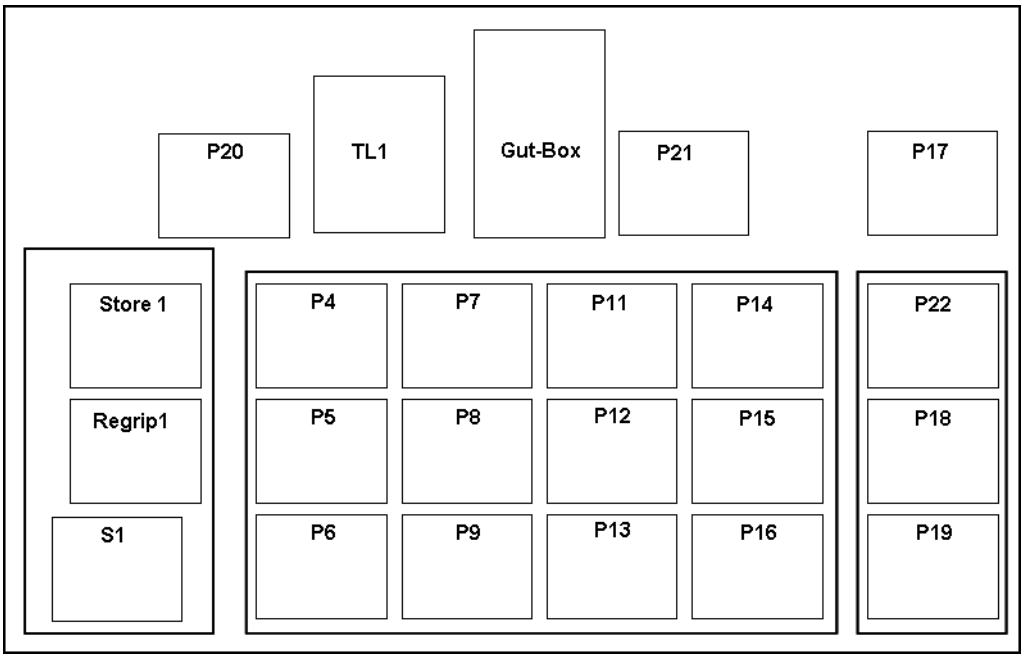
ID	ID of the Protocol chosen. 0: single pH Protocol; 1: Multi pH Protocol; 2: Cosolvent Protocol; 3: Gradient-pH Protocol and 4: Double-Sink Protocol
Flow	An option to allow the assay flow to be stopped in-between steps. On/Off setting.
Filtr	An option to allow filtration to be chosen. On/Off setting. Reserved for future use.
Blank	Whether the blank plate is to be read. The off setting, 'no', uses the pre-measured blank.
Stir	0: no stirring; 1: Stirring with Gut-Box; 2: stirring with some other device.

Figure 5.5 Experimental Settings Window View

Worksurface and PAMPA Layout

23 ALP (Automated Labware Positions) are shown in Figure 5.6 below. For a more detailed description of ALPs, refer to the Biomek FX User's Manual.

Figure 5.6 Deck view



Preparing the Worksurface

TL1	Tip loader. Position reserved for tips box only.
Store1	Position above spectrophotometer.
Regrip1	Empty position reserved for re-gripping during intermediate plate transferring operations.
S1	Position of UV plate holder of spectrophotometer.
Gut-Box	Position reserved for Gut Box platform.
P	P positions can be used to place any type of microtitre plates or reservoirs.

NOTE Minimum volume of the samples in PCR (low volume) plate is 7 µL per well.

Samples in solutions can be introduced in 96 well microtitre plates. The microtitre plate containing the samples is called the Stock Plate. The Stock Plate is placed in the position P6, see Figure 5.6.

Plate Placement for the PAMPA Assay

Different microtitre plates are used for the PAMPA assay. The plate placement on the worksurface is shown in Figure 5.7.

Figure 5.7 Positions for the plates placement.

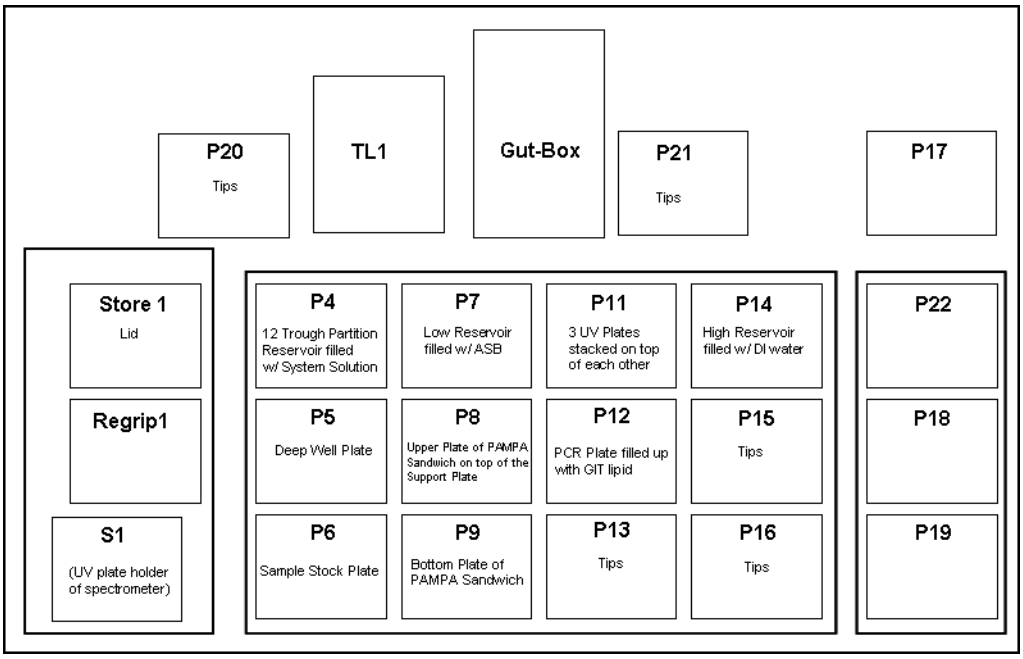


Plate positions:

P6	Holds the Stock Plate with samples in the microtitre plate.
P5	Holds the Deep Well Plate
P4	Holds the microtitre plate filled with the System Solution. 200 mL (High) 12-partitioning reservoir is recommended if PAMPA assay at several different pH values is to be setup. A 200 mL Reservoir is recommended if a Single ph PAMPA assay is to be setup.
P9	Holds the bottom half (donor) of the PAMPA Sandwich
P8	Holds UPPER plate of the PAMPA Sandwich placed on top of SUPPORT Plate.
P7	Holds reservoir filled up with ASB -7.4 solution. 50 mL reservoir is recommended to use if no more than 2-3 assay/day to be run. Otherwise, use 200 mL reservoir.
P12	Holds the plate pre-filled with lipid solution.
P11	Holds three UV plates stacked on top of each other. In case the “if Blank” plate is going to be read, four (4) UV plates have to be placed in this position.
P14	Positions reserved for the washing stations.

Lipid Placement

The lipid is transferred into the PCR plate and placed in position P12.

Two vials are transferred into an intermediate plate or trough and then 18 µL is transferred with a pipettor into the PCR plate for the PAMPA assay. Two vials of lipid holds enough lipid solution for 2 or 3 assays.

Transfer 60 µL per well is enough for daily work with about 10 PAMPA Sandwiches.

Washing Station

Positions P14 holds a 200 mL reservoir filled with Distilled water.

Tip Placement

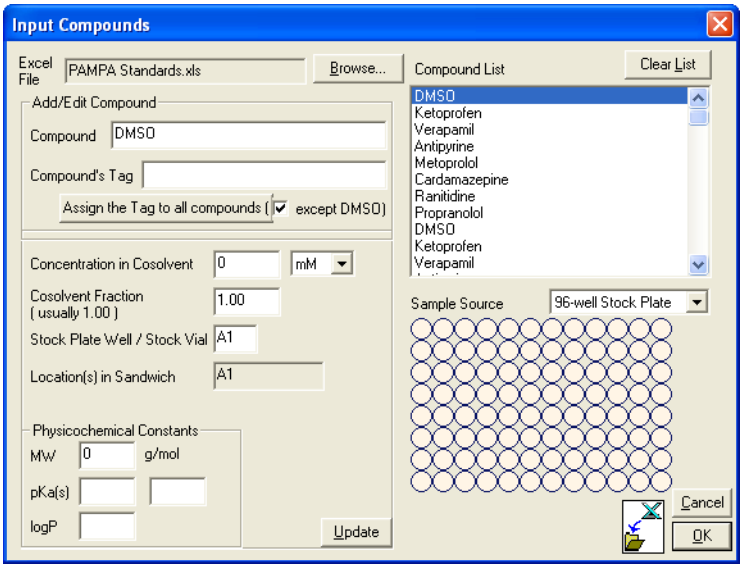
5 tip boxes are used for the PAMPA assay. Tips are placed in positions, P13, P15, P16, P20 and P21.

5.4 Import Data

Data can be read into the program by importing a Template file from Excel. Click the **Import from Excel** button  from the PAMPA Toolbar.

The Input Compounds dialog box displays as shown in Figure 5.8 below.

Figure 5.8 Input Compounds Dialog Box.



Definitions of the Input Compounds dialog box features:

Excel File	The file name of the spreadsheet containing the sample data.
Browse	Click the Browse button to choose a file to import.
Compound List	The list of the compounds imported from the Excel file.
Clear List	Allows a new Excel file to import.
Add/Edit Compound Window	Compound information can be edited or added in this area.
Sample Source	The default is 96-well Stock Plate.
Update Window	Information about a compound can be updated or filled in when a new compound is added to the list.

Add/Edit Compound Window

The following edit boxes are filled in if the information was entered into the Excel spreadsheet. If the edit boxes are blank, the information may be manually entered.

Compound	compound Name
Compound`s Tag	a string tag can be assigned to each compound for future use
Assign the Tag to all compounds	allows the same tag to be assigned to all compounds
except DMSO check box	Assign /not assign tag to DMSO

Update Window

Concentration in Cosolvent	Concentration of the sample in the stock microtitre plate well (usually around 10mM).
CoSolvent Fraction	Fraction of the cosolvent contained in the sample in the stock microtitre plate (usually 1).
Stock Plate Well / Stock Vial	Stock plate or stock vial location.
Deep-Well Plate Well	The stock sample diluted in this pre-mix plate well location.
Location(s) in Sandwich	The location of the permeability assay for this sample in the sandwich. For PAMPA Evolution96 is the same as stock plate well.
Physicochemical Constants:	
MW	Molecular weight, g/mol.
pKa(s)	Up to two pKas may be entered.
Log P	Log P value (octanol/water partition coefficient) can be entered.
Update	If any values have changed, click the **Update** button when finished.

When the **Browse** button is clicked, a Warning dialog box displays, See Figure 5.9. This dialog box makes sure the proper stock plate type is chosen, the default is 96-well.

Figure 5.9 Warning Dialog box

5.5 Defining the Protocol

Before beginning the PAMPA assay, the assay conditions and experimental settings need to be decided.

There are five protocols included in the PAMPA Evolution Command Software:

1. Double-Sink(TM) Protocol.

   The Double-Sink PAMPA assay will be performed with two kinds of sink conditions present. First a gradient-pH sink will be formed with donor pH values being pre-set to 5.0, 6.2 and 7.4 and the acceptor compartment with ASB solution at pH 7.4. The second sink condition is formed with use of special chemical scavengers in ASB solution.

   This protocol is chosen if an automated PAMPA assay is to be done. The experimental setting are set to defaults: stirring, 30 min incubation time, ASB at 7.4 in acceptor chamber and donor pH values at 5.0, 6.2, and 7.4.

2. Gradient-pH Protocol.

   The Gradient-pH Protocol may be introduced as generalized Double-Sink Protocol. Gradient-pH protocol allows the set up of double sink conditions using different pH values in the donor compartment with the acceptor compartment at one pH value. This protocol is chosen if you would like to vary the experimental conditions or change the pH Map.

3. Single-pH Protocol.

   The Single-pH Protocol assumes that user performs the PAMPA experiment at one pH value in the donor and acceptor compartments. No ASB is used and in the ASB reservoir, system solution is substituted.

4. Iso (multi)-pH Protocol.

   The Iso-pH protocol is not recommended to use and is preserved in the software for compatibility to older versions of the software

5. Cosolvent-pH Protocol.

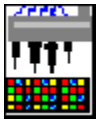
   The Cosolvent-pH protocol is not recommended to use and is preserved in the software for compatibility to older versions of the software.

5.6 Starting The Assay

CAUTION Carefully follow the suggested operational steps. Damage to the robot may result from improper placement of plates, lids, and other worksurface items.

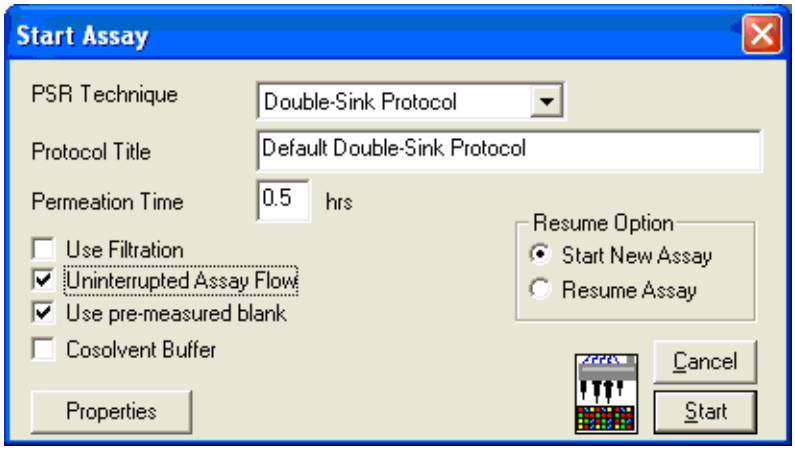
1. Before beginning the assay, complete the following:
   a. Adjust the pH values of the system Solution to the requested values.
   b. Correctly arrange all clean plates, lipids, and reagents on the worksurface.
   c. Initialize the instrument (if needed).
   d. Make sure that there are NO LIDS placed on top of the plates or reservoirs.
   e. Import an Excel file.

IMPORTANT Dialog boxes with the letter “i” or an Exclamation Mark in the top left corner describe actions which are taken, either by the robot or by the user. Please read the dialog box **Carefully**, as misplacement of plates or lids may cause damage to the robot.

2. Click the **Start Assay** button. 

The Start Assay dialog box displays as in Figure 5.10 below.

Figure 5.10 Start Assay dialog box.



PSR Technique

Use the drop-down menu to choose the Technique or Protocol. The table below defines the functions of the **Start Assay** dialog box.

PSR Technique	Use the Drop down menu to choose the Technique or Protocol.
Protocol Title	Title of the Protocol chosen
Permeation Time	The incubation time for the permeability assay
Use Filtration	Check box for filtration, not used.
Uninterrupted Assay Flow	Checkbox for automated continuation of the assay after each step.
Use pre-measured blank	Allows the option to skip the preparation and reading of the Blank UV plate by importing UV spectra of Blank from a previously saved file.
Cosolvent Buffer	Check if using Cosolvent System Solution.
Resume Option	Allows the resumption of the assay or start a new one
Properties	Describes the details of the protocol chosen.

Filtration

In the deep well plate where the samples are diluted in the PAMPA assay, precipitation may form. Prior to the preparation of the PAMPA Sandwich, the diluted samples in the deep well can be filtered.

Since this step is not yet automated, the user may choose to interrupt the PAMPA assay and take the Deep Well plate to a filtration station. Place the filtrate material back on the Biomek worksurface in the Deep Well position.

IMPORTANT Perform filtration when compounds are not expected to be very soluble.

Uninterrupted Assay Flow

To let the assay proceed automatically, select the Uninterrupted Assay Flow check box. The item is chosen at the beginning of the assay. After each step there is a possibility to interrupt the assay or skip the steps even if Uninterrupted Assay Flow is selected by choosing the **Interrupt** button on the step window.

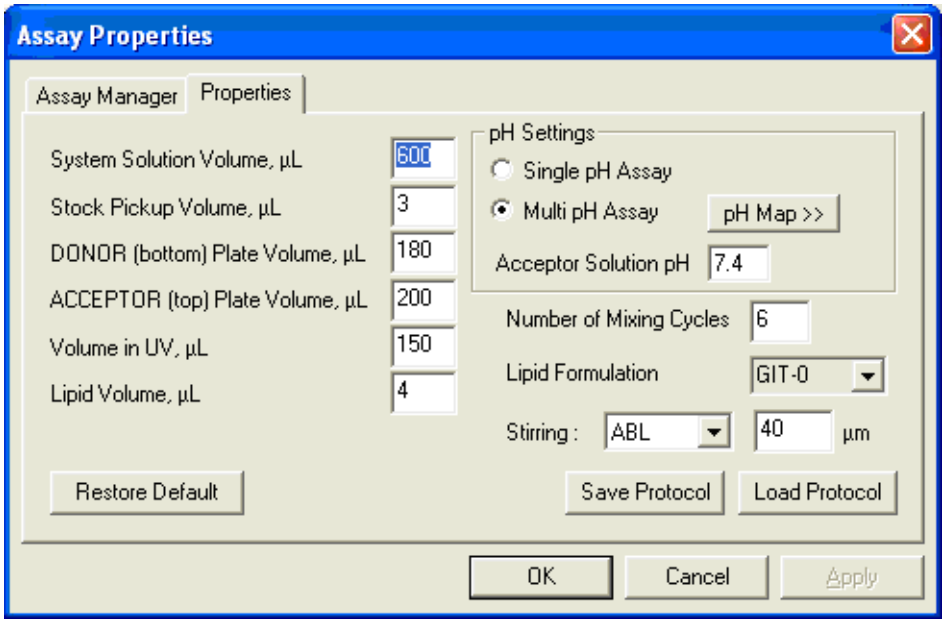
If this check box in not selected, the assay may be run by the operator. Each step of the assay waits for the user to click the **Continue** button.

Double-Sink Protocol

From the **Start Assay** dialog box, see Figure 5.10, make certain that Double-Sink Protocol is selected in the PSR Technique drop-down list.

Select the **Properties** tab for the details of the assay, as shown in Figure 5.11 below.

Figure 5.11 Assay Properties dialog box.



The functions for the Assay Properties are defined as follows:

System Solution Vol, µL	The amount of system solution used to perform the dilution in the deep-well plate. Use the default value of 600. If a different dilution of stock sample in the Deep Well is required, this amount plus Stock Pick-up volume can be changed.
Stock Pickup Volume, µL	The amount of sample picked up from the Stock Plate. The default value is 3 µL. If a different dilution of the stock sample is required, this value can be changed.
DONOR (bottom) plate Vol, µL	The volume of liquid dispensed into the donor plate. Use the default value is 180 µL. For the assay without stirring the default value is 200 µL.
ACCEPTOR (top) plate Vol, µL	The volume of reagent dispensed into the acceptor plate. The default value is 200 µL.
Number of Mixing Cycles	The number of times the diluted sample is aspirated and deaspiratied in the Deep Well plate. The default value is 6
Volume in UV, µL	The volume of liquid dispensed into the UV plate. The default value is 150 µL
Lipid Volume, µL	The volume of lipid dispensed onto the acceptor plate. The default value is settings, and 4 µL
Lipid Formulation	Choose the lipid formulation for the assay.
Stirring	Choose one of the 3 options: **None** - No stirring **ABL** - The Gut-Box™ is used for stirring. Enter the appropriate ABL thickness. **Speed** - Custom device is used for stirring. Enter the appropriate stirring speed.

pH Settings:	Choose the pH of the acceptor and donor solutions.
Single pH Assay:	pH of the System Solution
Multi pH Assay	Allows the use pH map button and adjust pH values in columns to the values used in the assay.
Acceptor Solution pH	pH of solution in the acceptor compartment. pH of Acceptor Sink Buffer is 7.4.
pH Map >>	pH Map button for setting the pH of individual columns.
Restore Default	Restores the protocol to the default software values.
Save Protocol	Saves the changed protocol.
Load Protocol	Loads a previously saved protocol.

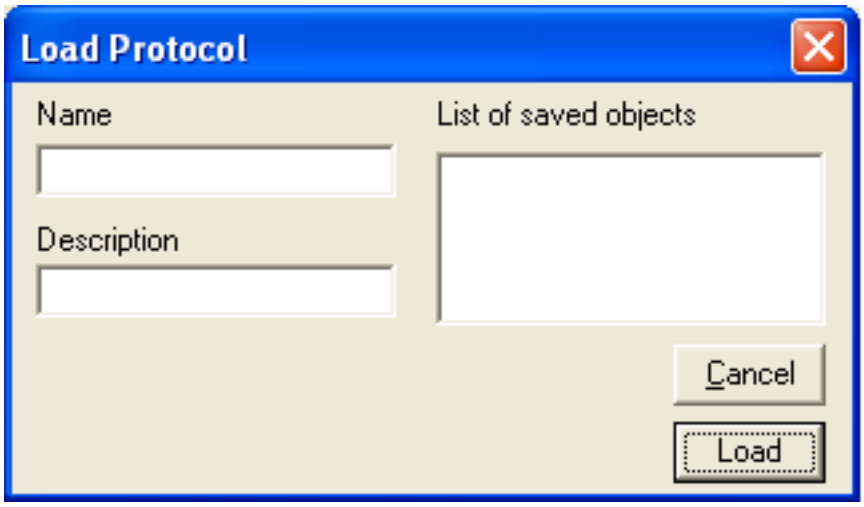
If the default setting are changed, the user can Save the Protocol and Load the Protocol for later use.

Click the **Load Protocol** button displays the Load Protocol dialog box, as shown in Figure 5.12. If more than one Protocol was saved, the name of the protocols are displayed in the 'List

of saved objects' window. If one of the Protocols is highlighted the description window is filled in with a description of the Protocol, if one was entered when the protocol was saved.

Highlight the Protocol of choice and click the **Load** button.

Figure 5.12 Save Protocol dialog box for the Double-Sink Protocol.

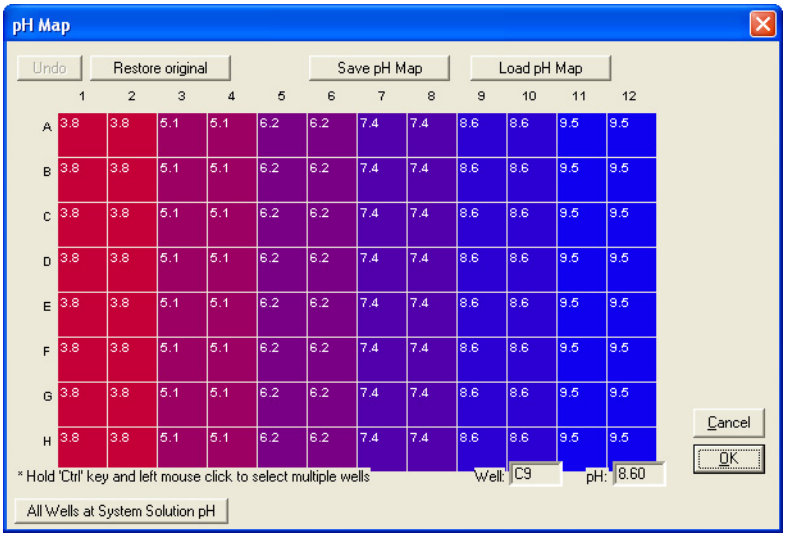


If any of the settings are changed in the **Advanced Properties** window, and you want to save the settings, Click the **Save Protocol** button. Fill in the Name and Description windows and Click the **Save** button.

pH Map

Click the **pH Map** button and the pH Map displays as in Figure 5.13.

Figure 5.13 pH-Map dialog box, showing an example for 8 compounds, 6 pH values, 2 replicates per pH.



The pH-Map has to match the System solution pHplaced in the 200 mL 12 partition reservoir in position P4.

Figure 5.14 12 partitioning reservoir filled up with System Solution according to pH map.

	1	2	3	4	5	6	7	8	9	10	11	12
A	pH 3.8	pH 3.8	pH 5.1	pH 5.1	pH 6.2	pH 6.2	pH 7.4	pH 7.4	pH 8.6	pH 8.6	pH 9.5	pH 9.5

The pH Map shows different shades of red and blue corresponding to the pH value of the well. Red corresponds to acidic pHs and blue corresponds to basic pHs. The more acidic the pH value, the more red in color for the well and vice versa.

The following functions are available on the pH Map dialog box, as show in Figure 5.13:

Restore Original button	This button restores the wells to the default pH values, if those values are changed.
Save pH Map button	This button saves the present pH Map.
Load pH Map button	This button loads a saved pH Map.
All Wells at System Solution pH button	This button places all wells at the System Solution pH.

To change pH value in column, press **CTRL+left click**, then drag the curser to select the cells. An outline of a box displays, indicating which cells are included. When you stop dragging the mouse, all the cells included are highlighted. Simply type the new pH in the field and all cells get the same pH value. Click the **OK** button when finished.

Double-Sink Protocol

In the Double-Sink Protocol the number of pH values in the donor compartment can be chosen, the default is three pH values. The Acceptor wells contain ASB solution at pH 7.4. The general method is described below:

1. Lipid is added to the top plate of the PAMPA Sandwich.
2. The top plate of the PAMPA Sandwich is filled with pION Acceptor Sink Buffer (ASB)™.
3. System Solution at pH 5.0, 6.2 and 7.4 is added to the deep well plate.
4. The stock samples are added and mixed in the Deep Well plate.
5. The diluted Stock Sample solution from the Deep Well plate is added to the donor plate.
6. The diluted Stock Sample solution is added to the UV plate and the Reference Spectra collected.
7. The Top Plate of the PAMPA Sandwich is placed on top of the donor plate to form a sandwich.
8. Lid is placed on the top plate of PAMPA Sandwich.
9. The sandwich is moved to the Gut-Box.
10. Gut-Box switched ON.The sandwich sits for 0.5 h.
11. The acceptor solution is assayed.
12. The donor solution is assayed.
13. Permeability is calculated.

Gradient-pH Protocol and Single pH Protocol

The PAMPA technique for Gradient-pH protocol and Single pH protocol is similar to the Double Sink™ protocol.

The Gradient-pH Protocol follows the same steps as the Double-Sink protocol. The user would choose the Gradient-pH Protocol if different pH values are used in Step 3 or any change in the assay settings are planned.

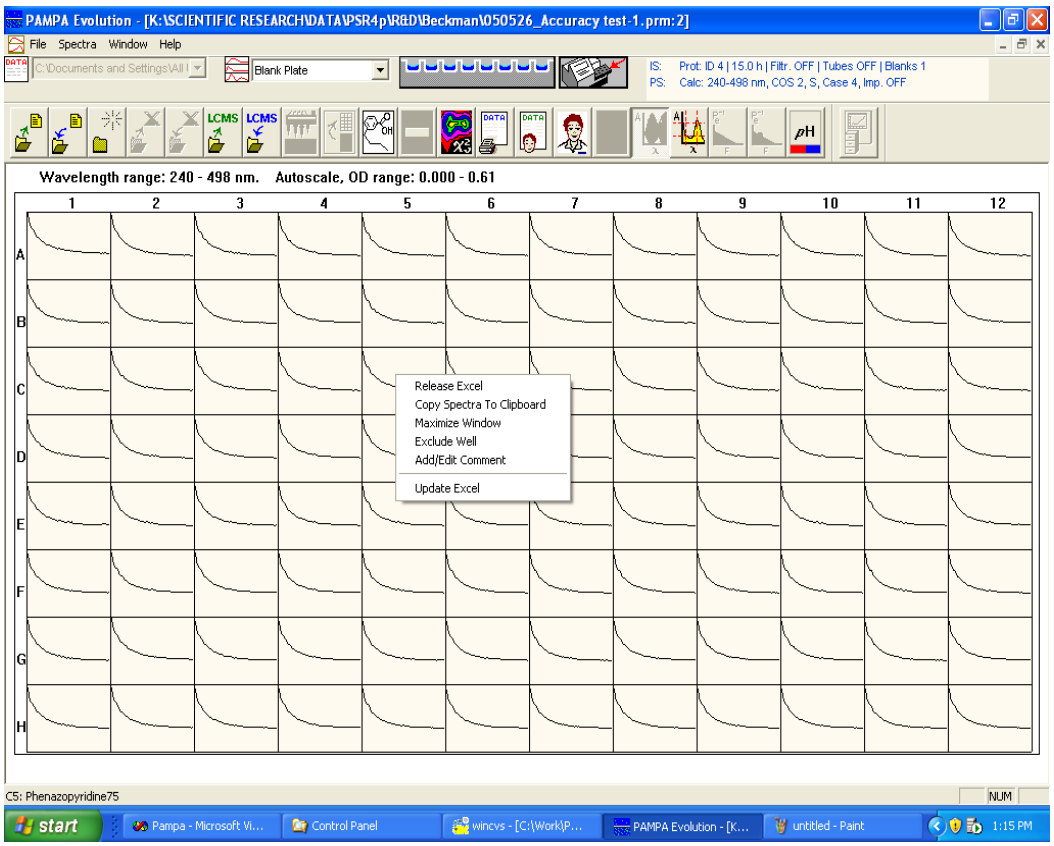
For the Single-pH Protocol, Step 2 is different. ASB is not used in the acceptor chamber, System Solution is used. Additionally, the pH of the System Solution in the donor compartment, Step 3, is the same pH as the System Solution in Step 2.

Collecting Spectra

1. The Reference spectra is collected first and is collected after the stock samples have been diluted in the Deep Well Plate. If the stock samples have precipitated, the diluted solutions can be filtered before the UV spectra is collected.
2. Acceptor Spectra is collected after the permeation time. The solution in the acceptor or TOP plate is pipetted transferred into the UV plate and the spectra collected.
3. The donor or BOTTOM plate spectra are collected last.

   If the assay is performed manually, the spectra can be collected in any order, the reference spectra may be saved to last.
4. Operator comments may be added to any set of wells (reference, blank(s), acceptor, donor) by mouse clicking any individual well on the 96-spectra view. The following dialog box displays.

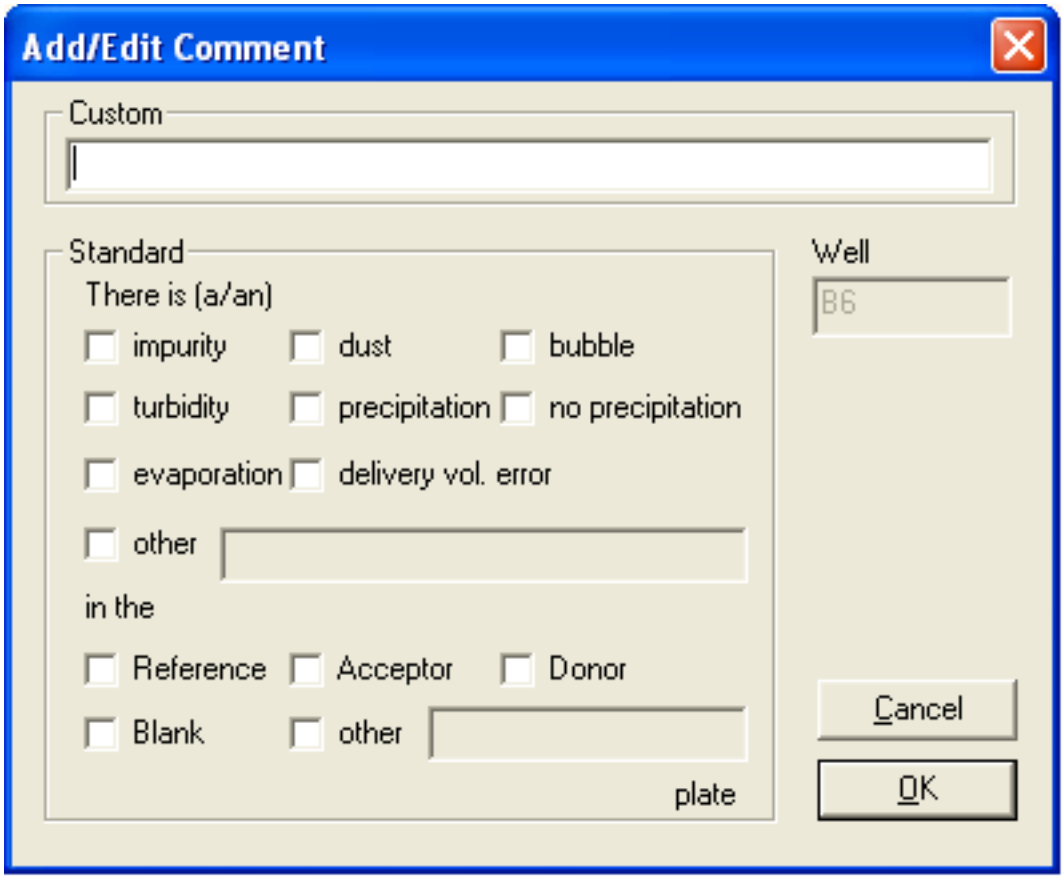
Figure 5.15 Add Commentary dialog box.



Choose the Add/EDIT Comment dialog box, see Figure 5.16 You may add a custom comment or add a standard comment using the check boxes. Wells with comments attached are

indicated with exclamation marks in 96-spectra view or exclamation marks added to the well references (e.g.B5 !).

Figure 5.16 Add/EDIT Comment dialog box.



Preparing a Blank

The blank spectra, used for quantifying the background of the UV plate, can be collected and saved for use with all files. The procedure below describes how to collect the blank spectrum.

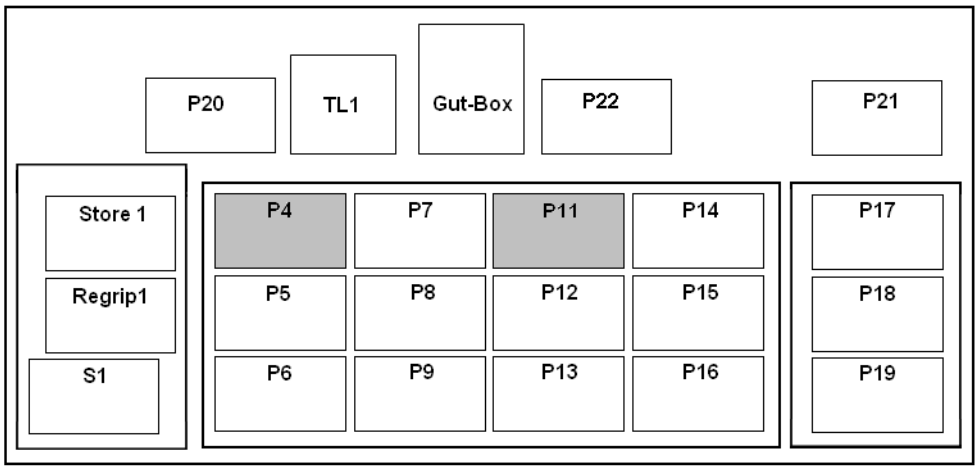
1. Launch PAMPA Evolution-96 Command Software.
2. Go to the main menu **File | Save as**, save the file as ""YYMMDD_Blank" (for example: 040701_Blank) where the first 6 characters are the year-month-day in numbers (YYMMDD).

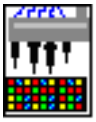
CAUTION Do not to touch the underside of the UV plates.

3. Place 4 new 96-well plastic UV plates stacked one on top of the other, into position P11 according to Figure 5.17 as shaded.

4. Place the High Profile 12 Trough Reservoir filled with System Solution into position P4 according to Figure 5.17.

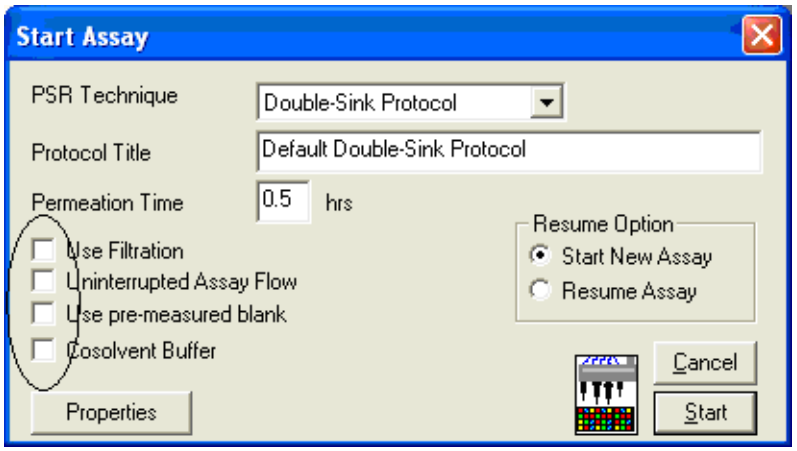
Figure 5.17 Worksurface set up for filling in the UV plate with blank solution



.5. Click the **Start Assay** button on the PAMPA toolbar. 

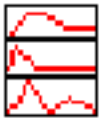
6. The **Start Assay** dialog box displays as show in Figure 5.18 below. De-select ALL the check-boxes (Use Filtration, Uninterrupted Assay Flow, Use pre-measured blank, and Cosolvent buffer).

Figure 5.18 Start Assay dialog box.



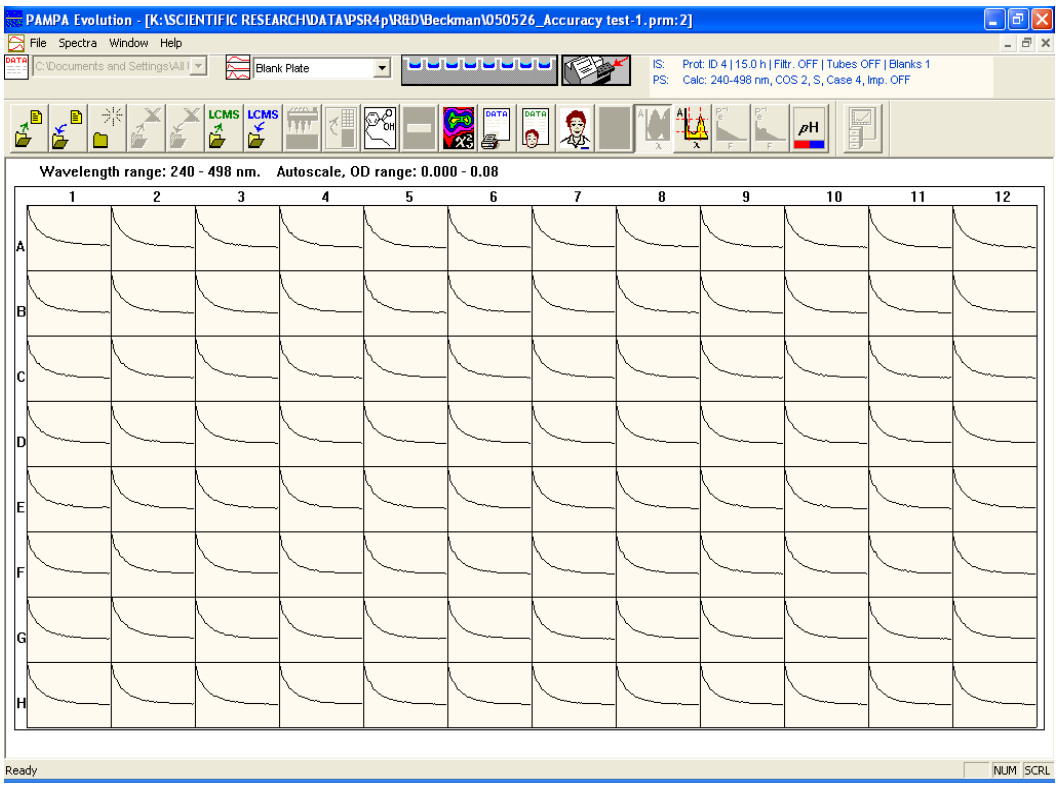
5. Click the **Start** button. System Solution from the High Profile reservoir is transferred to the top UV plate. The UV plate is then placed in the Spectrophotometer and the spectra collected.

NOTE When the instrument finishes the step, the Spectrophotometer starts reading the UV spectra of the blank plate. Abort the Assay at this point by clicking the **Interrupt** button.  Wait till the Spectrophotometer finishes reading, then remove the blank plate from the reader (this plate is longer needed).

6. To inspect the spectrophotometric data, click the **View Spectra** button. 

7. The 96 spectra view displays as shown in Figure 5.19.

Figure 5.19 All spectra view of the PAMPA Evolution software.



8. Inspect the UV spectra of the Blank as described in Load the Blank Spectra Section 5 Bacterial contamination should not be apparent.
9. To save the UV spectra, go to the main menu and select **File | Export Plate Spectra**.

10. The **Save Spectra** dialog box displays as shown in Figure 5.20 below.

Figure 5.20 Save Spectra dialog box.

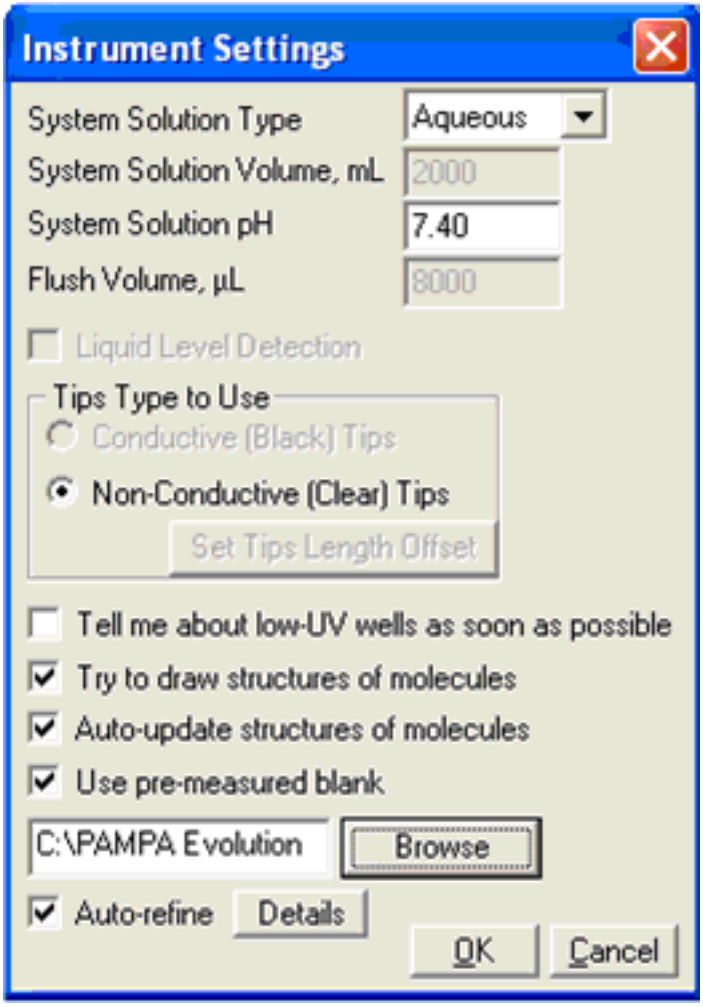
11. Choose **Blank Plate** from the **Plate to be Exported** drop-down list.
12. Click the **Save** button. A special spectra file is savedin the same folder where the data file is the name **Data file name_Blank Plate.spc** (for example: 040701_Blank_Blank Plate.spc).

NOTE The System Solution Blank may be reused for many experiments. We recommend a new Blank Spectra to be collected when new System Solution is prepared.

Load the Blank Spectra

1. Go to the main menu **Instrument-Settings**, the following dialog box will appear.

Figure 5.21 Instrument Settings dialog box



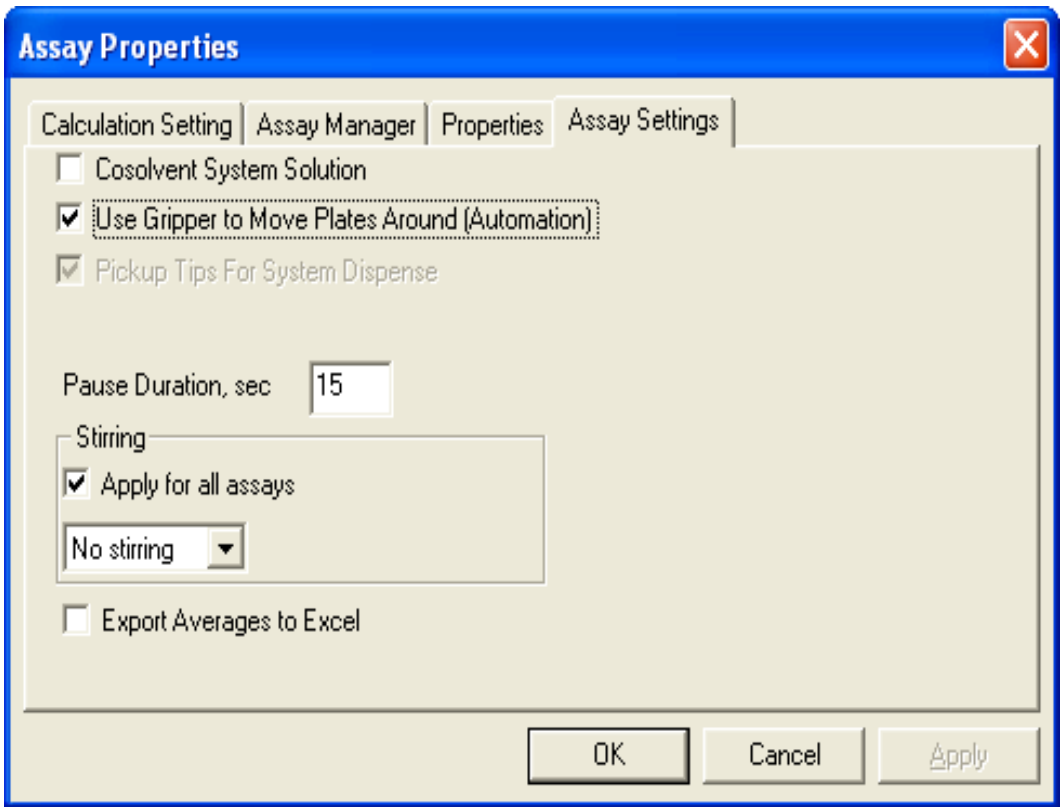
2. Browse for the file **Data file name_Blank Plate.spc**, in our example **040701_Blank_Blank Plate.spc**, Click the **Open** button.
3. Make sure "Use pre-measured blank" is selected, as shown in Figure 5.21.
4. Make sure "Tell me about low -UV wells..." is not selected, as shown in Figure 5.21.
5. Make sure 'Auto-refine' is selected. The results of the experiment are automatically calculated.
6. Click the **Ok** button.

PAMPA Assays without Stirring

The PAMPA protocols can be performed without stirring. If no stirring is chosen, changes to the assay settings and consumable are necessary. The following lists the changes required for a no stirring PAMPA protocol.

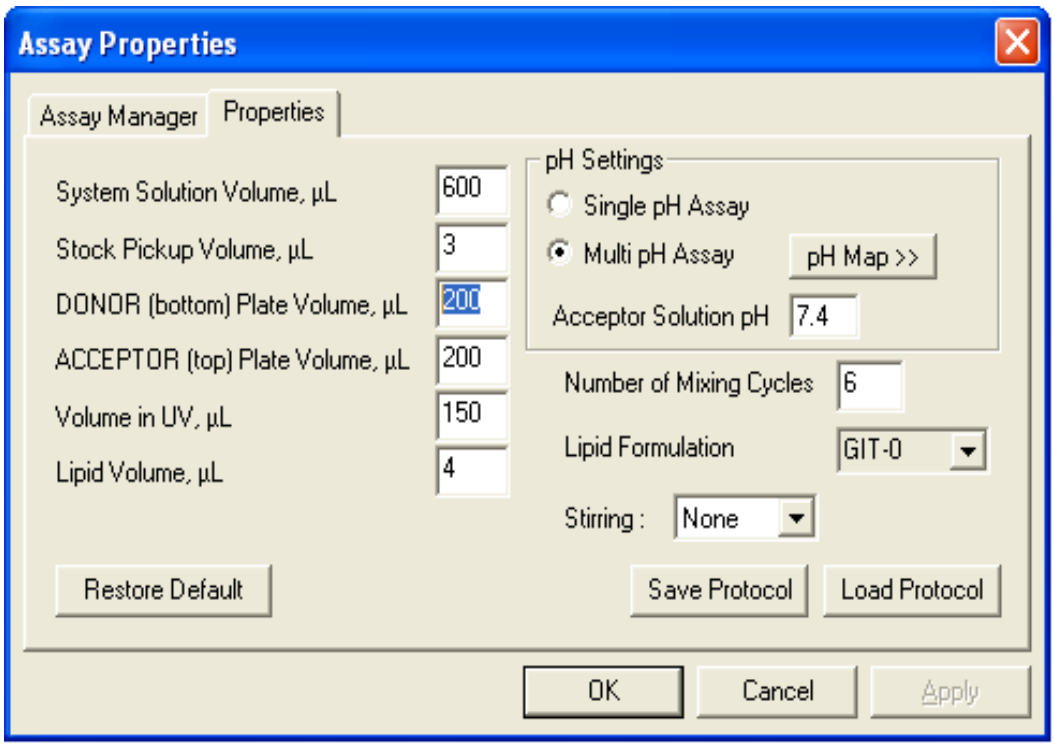
1. Use the PAMPA Sandwich instead of the Pre-loaded PAMPA Sandwich.
2. Put the bottom plate of the PAMPA Sandwich in position “Gut Box “.
3. Before starting the assay, from the main menu select **Assay -Assay Properties** and select the Assay Settings tab. Select the **No stirring** option in the drop-down menu. See Figure 5.22

Figure 5.22 Assay Settings for non stirring assay in Assay Settings dialog box.



4. After the assay is started and the Assay Properties dialog box is displayed, see Figure 5.22, click the **Properties** button. The Assay Properties dialog box displays, SeeFigure 5.23. The DONOR (bottom) plate volume must be set to 200.

Figure 5.23 Assay properties in Start Assay dialog box



5. Enter the incubation time. The Default time for the non-stirring assay is four (4) hours. For more details see Incubation Time Section 5.

IMPORTANT When the PAMPA Sandwich is combined, manually place the Sandwich into the humidity box for incubation in order to avoid evaporation problems.

Incubation Time

The incubation time or the time that the compounds in the donor compartment have to permeate through the filter barrier and get to the acceptor side is chosen by the operator. The time required for the permeability study depends on the expected permeability results and is at the discretion of the user. The guidelines are listed below.

1. Low Permeability Samples.

   Allow the sandwich to sit for 4 -15 hours without stirring. This protol provides a good separation of permeability results so that quatitation among low permeable compound can be achieved. Under this protocol, compounds with high permeability values either have a high error or are equilibrated. For compounds that have high permeability and are left to equilibrate for more than 4 hours, the permeation process may be limited by the Aqueous Boundary Layer (ABL). For protocols with sink condition in the acceptor compartment, equilibration means that all the material disappears from the donor

compartment. For non-sink protocols equilibrium is reached when the same sample concentration is found in both compartments. Low permeability compounds may end up showing unreliable low results due to an insufficient UV signal in the acceptor compartment.

The Low Permeability protocol is an optimum experiment for differentiating among low permeability compounds. To prevent evaporation, the sandwich must be placed in the Gut-Box with a wetted sponge.

2. High Permeability Samples.

   Allow the sandwich to sit for 0.5 (default) to 1 hour, applying stirring in the donor compartment by using the Gut-Box. The recommended ABL dial setting in the pION's Gut-Box is 40 µm. Using this protocol highly permeable compounds will give good results.

5.7 Aborting and Restarting the Assay

The assay can be aborted at any step. When aborted, all data is automatically saved to the file. If any spectra is collected, the spectra data is not lost and is saved with the file.

To restart the assay, click the **Start Assay** button  . The **Start Assay** dialog box displays. Un-check the selection for **Uninterrupted Assay Flow,** choose **Resume Assay** option. Click the **Start** button. The assay resumes from the beginning of the interrupted step.

In the Instructions dialog boxes, click the **Skip** button until the instructions for the needed step displays. Click **Continue**.

5.8 Menu Items

This section describes the Menu Functions in detail.

Instrument Menu

From the main menu, the Instrument drop-down list has the following selections.

Open Biomek FX software

This option allows the user access to the Biomek software. Assay cannot be started while Biomek FX software is opened.

CAUTION Keep tools, hands, and head outside the worksurface area during the following procedure and at any time the instrument is operating. Ignoring this rule may result in serious damage to the instrument and/or in interruption the robot`s movement due to light curtain violation.

Carefully read all warning dialogs. Damage to the robot may result from improper placement of lids, plates and other items.

Run Method

This option allows methods created in the Biomek FX software to run in the PAMPA Evolution software.

For additional information see the Biomek FX user's manual.

Pickup Tips

250 µL disposable tips can be picked up from the TL1 only.

Discard Tips

250 µL disposable tips can be discarded into the empty tip box in position TL1.

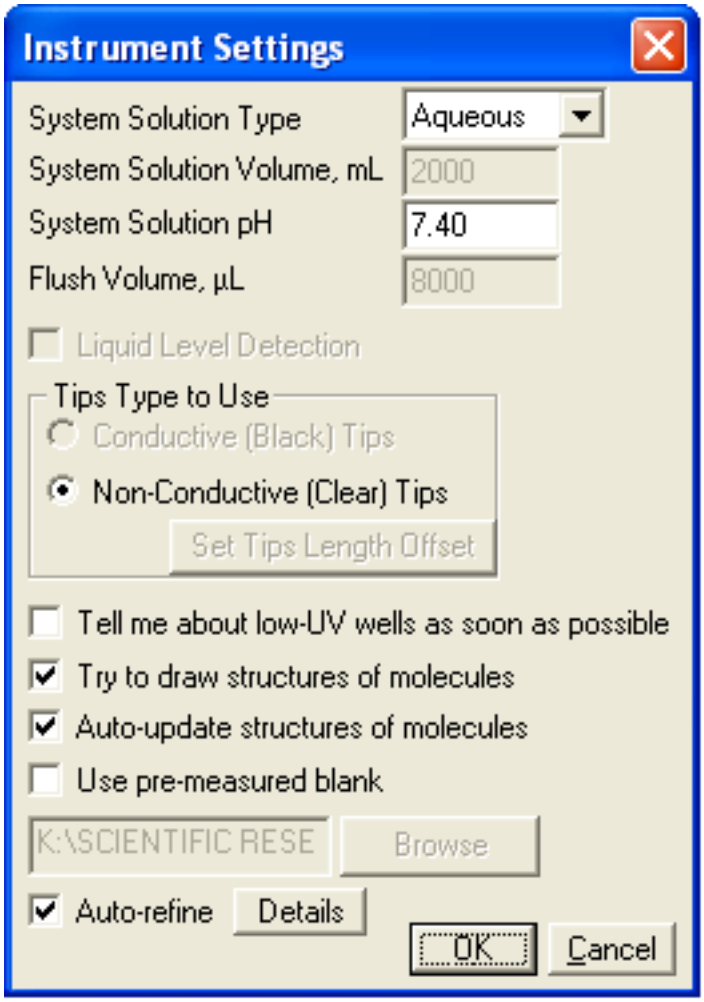
Power Controller

Not available.

Instrument Settings

The **Instrument settings** dialog box is displayed in Figure 5.24. This dialog box allows the operator to custom design a PAMPA assay or to change the defaults of the PAMPA assay

Figure 5.24 Instrument menu drop-down list.



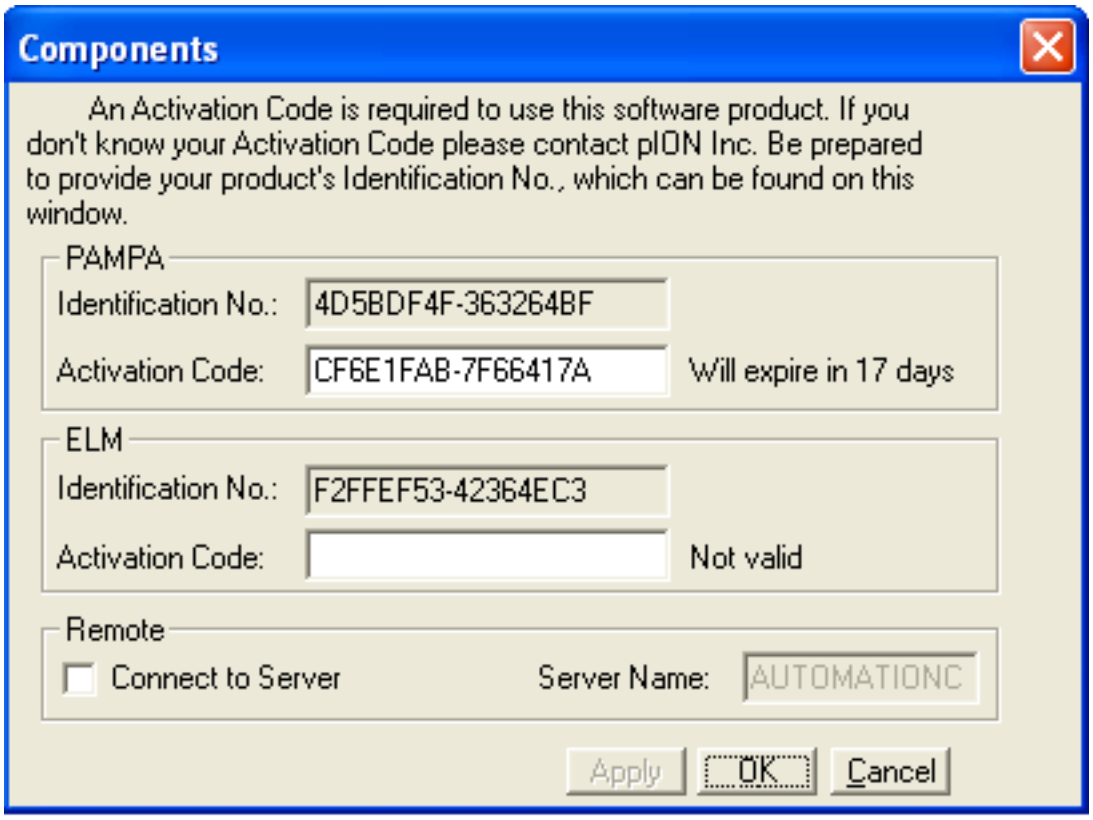
The table below describes the functions of the Instrument Settings dialog box.

System Solution Type	Choose either aqueous System Solution or Cosolvent System Solution.
System Solution Volume, mL	Not used in PAMPA Evolution96.
Liquid Level Detection	Disabled in PAMPA Evolution96.
Conductive (Black) Tips	Disabled in PAMPA Evolution96.
Non-conductive (Clear) Tips	Enabled in PAMPA Evolution96.
Tell me about low-UV wells	If selected provides the dialog aftercollecting reference UV spectra with information about low-UV wells. Allows the user to save compounds for HPLC or LCMS analysis if needed. **NOTE** This option must be deselected for Uninterrupted Assay Flow.
Try to draw structures of molecules	The software tries to draw structures of molecules.
Auto-update structures of molecules	The software tries to recover structures of molecules from "ELM"(optional software). See "ELM" documentation for details.
Use pre-measured blank	Allows the user to browse a pre-measured blank from another file. **NOTE** SHOULD BE SELECTED FOR UNINTERRUPTED ASSAY FLOW.
Browse button	Allows the user to select a file containing UV spectra collected under blank conditions. The name of the file is displayed.
Auto-refine	If selected, this option automatically processes collected data and saves it in an Excel file.

Components

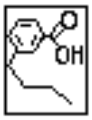
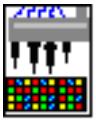
The Components dialog box, show in Figure 5.25, is reserved for present and future components of the PAMPA software. It is not necessary to make changes to this dialog box. The activation code is entered in this dialog box.

Figure 5.25 Components dialog box



Assay Menu Items

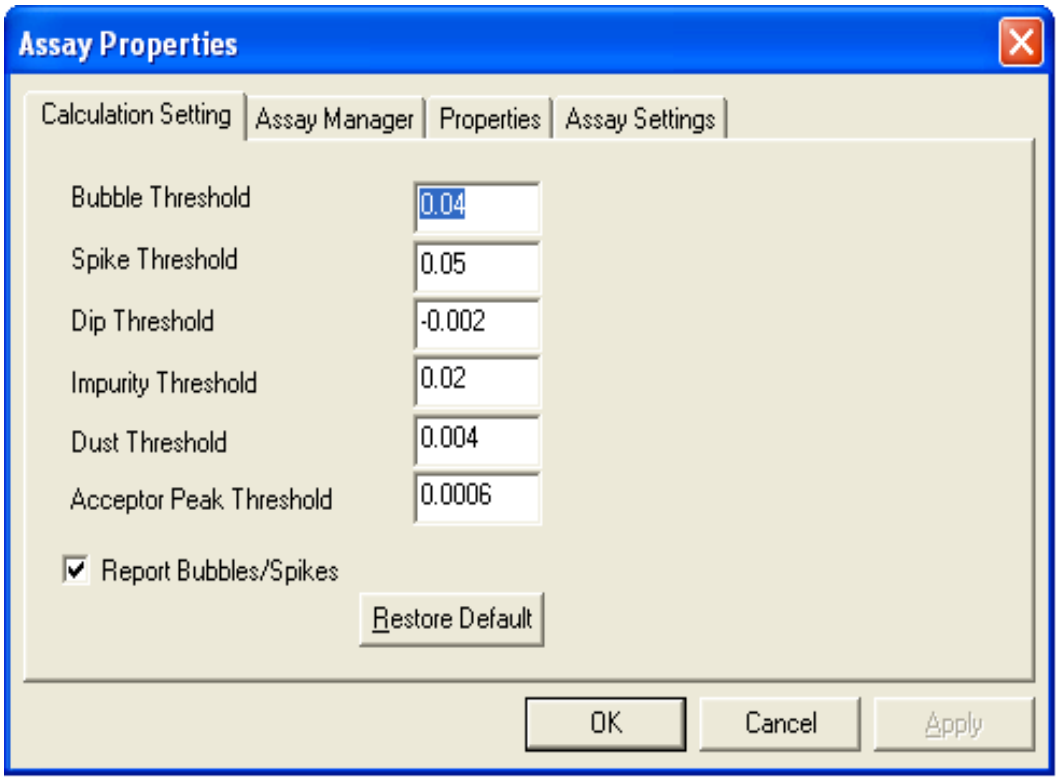
The Assay Menu items provide information about the PAMPA assay.

Compound List	The **Compound List** dialog box can also be invoked by clicking the **Compound List** button.  The Compound List dialog box is shown on Figure 5.3 and described in detail in PAMPA Evolution96 Desktop Section 5.
Start/Resume Assay	The **Start/Resume Assay** dialog box can also be invoked by clicking the **Start/Resume Assay** button.  The **Start Assay** dialog box is shown on Figure 5.10 and described in detail in Aborting and Restarting the Assay Section 5.
Abort Assay	The **Abort Assay** button is used to stop an assay and abort the step being performed. The **Abort Assay dialog** box can also be invoked by clicking the **Abort Assay** button. 
Start/Stop Timer	Allows the user to start or stop the incubation time of the assay.
Finish Assay	Skips all steps before incubation and performs all of the steps after incubation.
pH map	Another way to access the pH Map.

Properties

The **Calculation Setting** tab, as shown in Figure 5.26, contains various threshold settings used by the calculation engine of the PAMPA Evolution Command Software. The table below defines the purposes of the numbers.

Figure 5.26 Assay Properties dialog box.



Bubble Threshold	An OD (Optical Density) value. If raw spectra for any of the wells in the Blank Plate exceeds this threshold at maximum wavelength, the average "good" blank spectrum is used in calculation for those wells.
Spike Threshold	An OD value. If raw spectra for any of the wells in the Blank Plate have spikes exceeding this threshold, the average "good" blank spectrum is used in calculation for those wells.
Dip Threshold	A Threshold input designed to optimize baseline correction for the spectra in the Acceptor, Donor and Reference Plates.
Impurity Threshold	A Threshold input designed to filter out wells having impurities in the Blank Plate.
Dust Threshold	An OD value. If sum of small spikes in the well for the blank spectra exceeds this threshold, the average "good" blank spectrum is used in calculation for those wells.

Acceptor Peak Threshold	An OD value. If maximum OD for the processed spectrum in the acceptor plate well is less than this threshold, the software assumes that there is no sample in this well.
Report Bubbles/Spikes	If this option is selected, the software will report which blank wells appear to have bubbles or spikes. These wells are automatically corrected. A dialog box reports the results as discovered specifically to the results of the assay.
Restore Default	Click this button to restore the default values for all Calculation Setting thresholds.

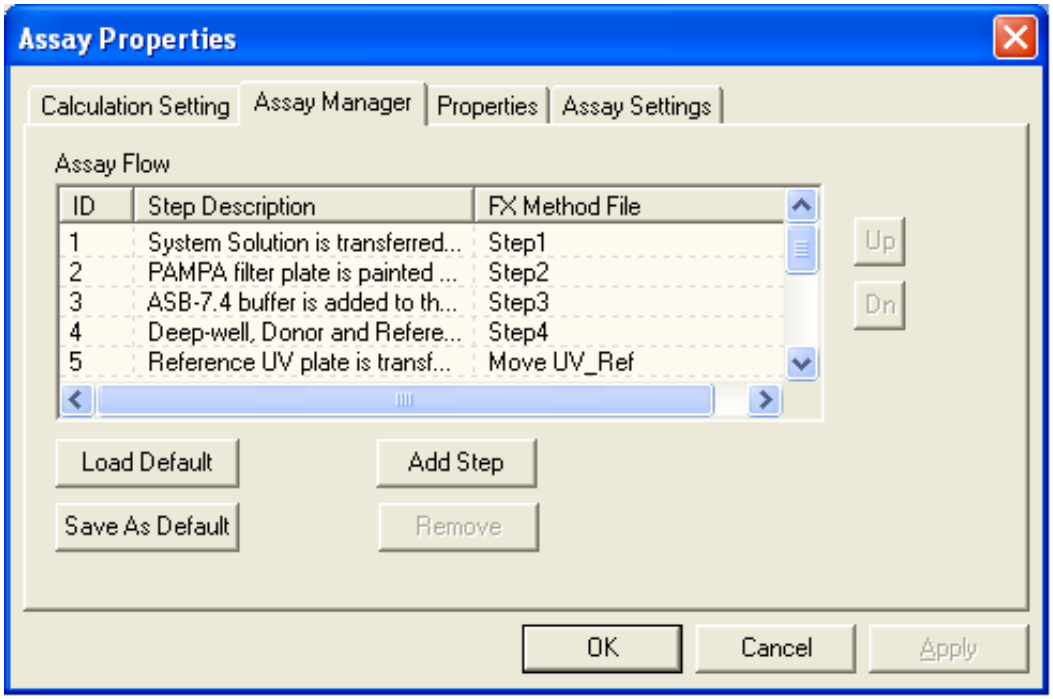
Figure 5.27 Dialog box describing dust and bubbles in the blank spectra.

Assay Manager tab

The Assay Manager tab, Figure 5.28, of the **Assay Properties** dialog box contains a list of steps performed by the robot. Each step may have a description and a Biomek FX method file associated with it. When step is finished, or skipped, it gets removed from the list. The list is

saved in a special binary file called **Default.fxp** in the same folder where the PAMPA Evolution software is installed.

Figure 5.28 Assay Manager tab of the Assay Properties dialog box.



.

Load Default button	Loads lists of steps saved in the **Default.fxp** file.
Save As Default button	Saves a list of the current display in the Assay Flow table in the **Default.fxp** file. All new files at this point are created with this list.
Add Step	Opens the "Step Configuration" dialog box (see Figure 5.3) which allows the user to write a description for a new step in order to browse to the method file and to setup some other properties of the step. For example, whether to start reading of UV spectra of a particular plate or Start/Stop the assay timer after this step. When this step is configured, press the Apply button to append the step to the end of the list.
Remove	Removes a selected step from the list.
Up/Dn	Moves a selected step up/down in the list.

Properties tab

This tab allows the user to check the assay`s properties avoiding assays interruption. For detailed description as shown in Figure 5.11 and explained in Double-Sink Protocol Section 5.

Assay Settings tab

The **Assay Settings** tab contains some additional options to perform the assay. Additional information can be found in PAMPA Assays without Stirring Section 5 and Figure 5.22

Cosolvent System Solution	This is selected when "nano-gram PAMPA Method" is in use and Cosolvent System Solution contains 20% acetonitrile. Not used in PAMPA Evolution96 software version 3.0.
Stirring section	
Apply for all assays	Applies similar conditions from the drop-down list for all following assays.
Export Averages to Excel	Enables the export of the averaged results into an additional sheet of the Excel file.

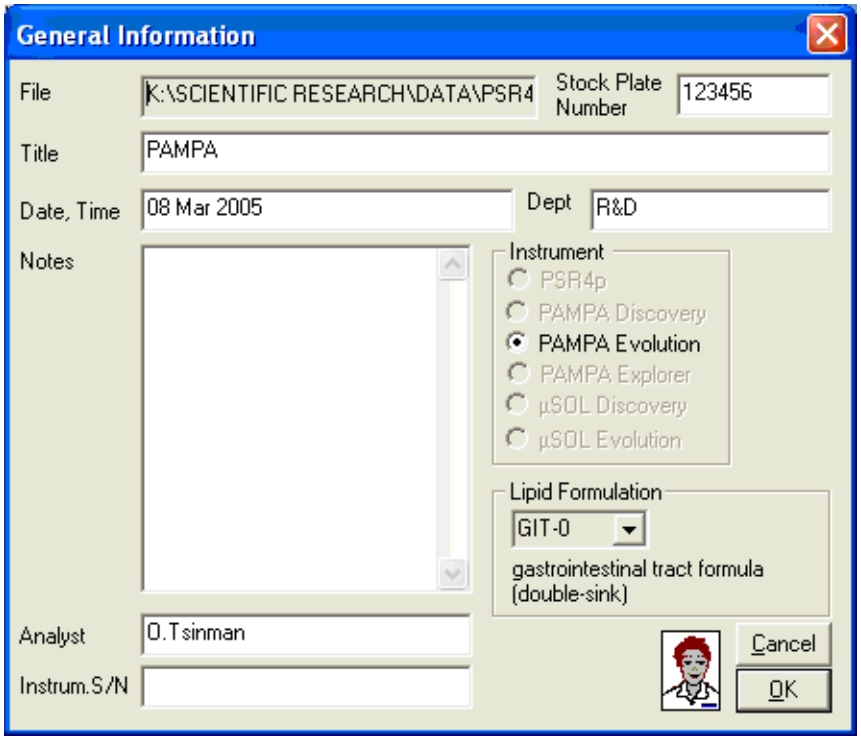
5.9 Remote Monitoring Control

The PAMPA Evolution software comes complete with a remote monitoring system. This enables a user to walk away from the instrument, but monitor the progress of tasks over a local network. This function must be installed when an instrument is purchased. It enables a user to determine the status of a current task, communication errors with the system components, and spectrophotometer status as spectral data is collected. It will not allow a user to remotely control the system.

5.10 Lipid Types

PAMPA Evolution comes with the option to select new lipid types for recording what type of experiment was performed. These values are visible from the general information dialog box.

Figure 5.29 General Information Dialog.



GIT-0	Gastrointestinal Tract formula
BLM-0	"Black Lipid Membrane" formula
Other	User defined and over-writable

All other options for the setting are reserved for future use in the software.

Results

6.1 Results

After the experiment is completed, the worksurface view switches to the Results Table View so the user can review the data. Click the **Refine Permeability Constants** button on the main toolbar.

Title	Title provided at the beginning of assay.
Temperature	Temperature inside the SpectraMax UV instrument.
Wavelength Domain for Analysis	Wavelength parameters for the refinement.
minimum	Minimum wavelength for analysis.
maximum	Maximum wavelength for analysis.
Detection Threshold	lowest OD value for analysis for all wavelengths above 250 nm. OD values< threshold are considered undetected.
COSOLVENT used	Solvent which is used to dissolve the samples in the stock plate.
Lipid Formulation	Choice of lipid formulations.
Permeation time	Incubation time of experiment.
Impurity	Internal reference correction for impurities
Set	Pull down menu to choose an acceptor well which contains the impurity only.
COSOLVENT Multiplier	Factor of cosolvent in the stock plate (usually 1).
Stirring Speed	Speed of stirring, if done. Drop down list allows to choose stirring speed for the assay in rotation per minute or as a produced thickness of ABL.
ACCEPTOR/DONOR Parameters Regression	3 Possible regression parameters.
SUBSTANCE	defines by regression, sample concentration in acceptor/donor compartments.
COSOLVENT/IMPURITY	defines by regression, cosolvent/impurity Correction concentration in acceptor/donor compartments.
BACKGROUND Correction	Correction for volume delivery errors. Adjusts blank curve to minimize residuals.
PERMEABILITY Basis	Described in detail in Definition of Permeability Units Section f.7.

Table 6.1 pH Mapping.

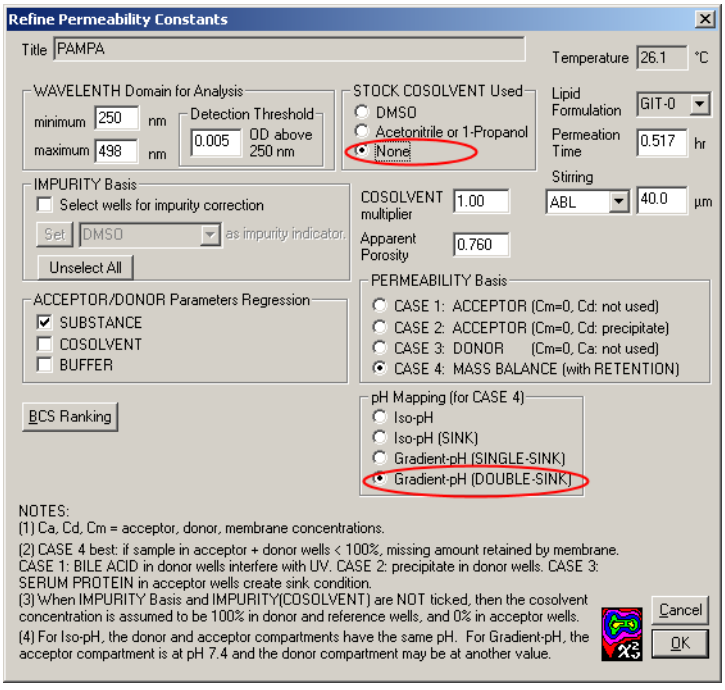
ISO-pH	Same pH in both donor/acceptor compartments
ISO-pH (SINK)	Same pH in both donor/acceptor compartments. Acceptor contains Chemical Sink.
Gradient-pH (SINGLE -SINK)	Different pH values in the donor and acceptor compartments.
Gradient-pH (DOUBLE-SINK)	Different pH values in the donor and acceptor compartments. Chemical Sink in acceptor compartment

NOTE For assays which were performed with stirring in the donor compartment, make sure that correct stirring speed in rotation per minute or produced thickness of ABL is added in "Stirring" edit box.

Setting up Impurity Correction

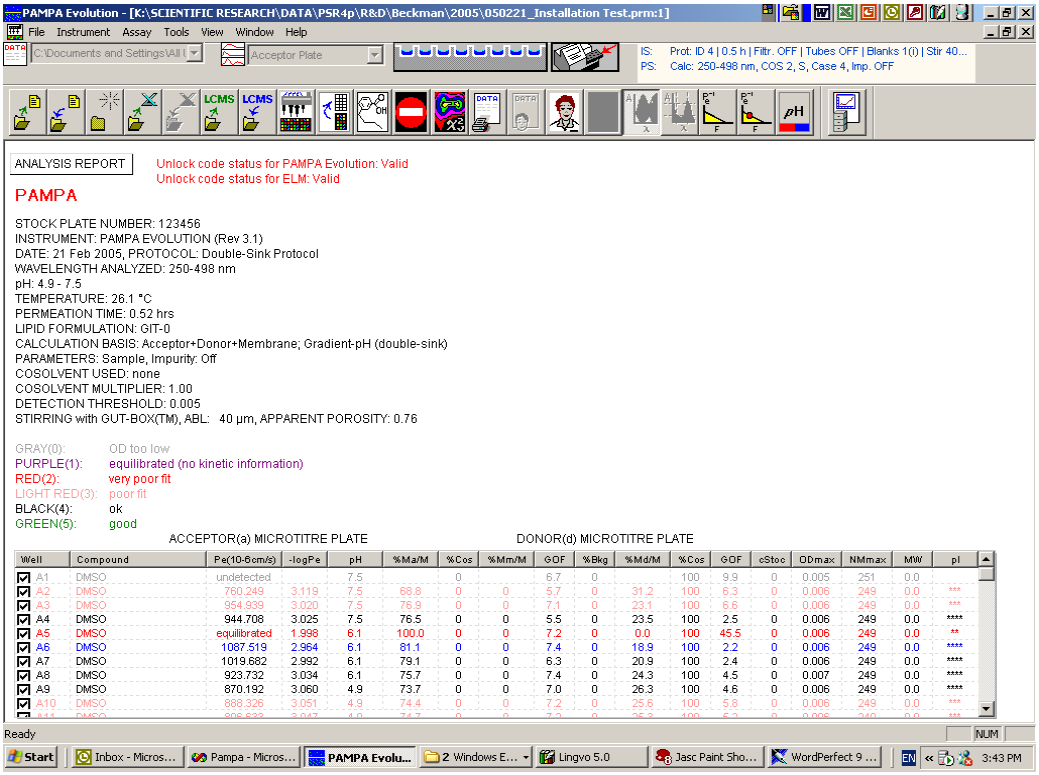
Set the Impurity Correction so that the software knows how to remove the effects of DMSO surfactant and any extraneous constituents which may affect UV absorption. Set the Impurity correction by selecting the **None** option in the "STOCK COSOLVENT Used" portion of the Refine Permeability Constants window as shown in Figure 6.1. Make sure that all other settings are the same as shown in Figure 6.1, click the **OK** button.

Figure 6.1 Impurity Correction setting.



The software generates a table of results, as shown in Table 6.2 below.

Figure 6.2 Table of results from the refinement of permeability constants.

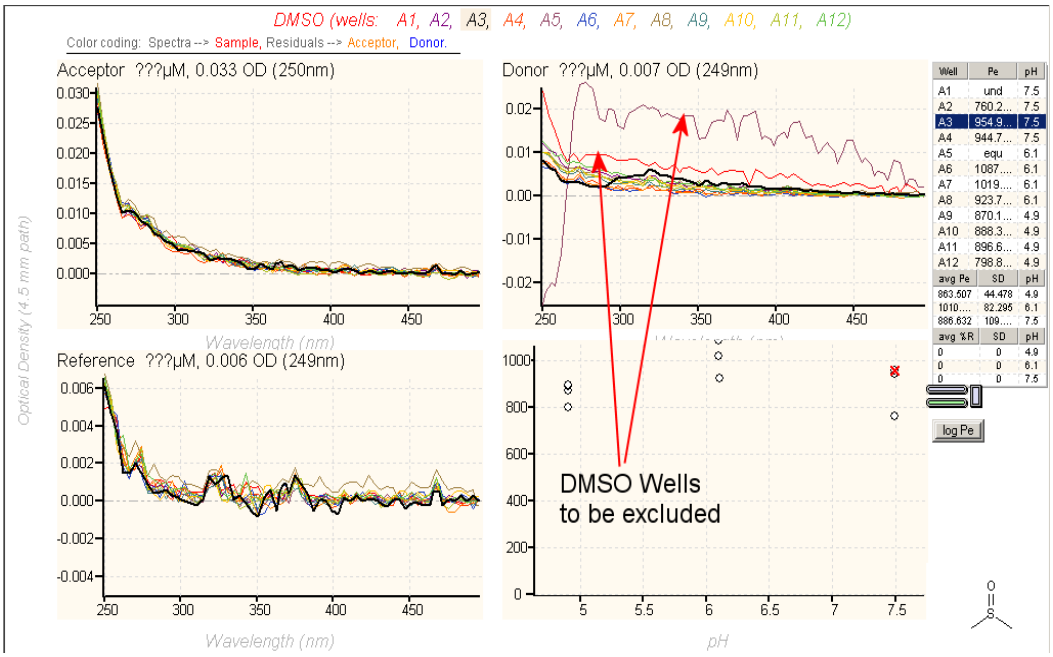


Double Click **DMSO** in the compound column of the table located in the bottom of the Analysis Report view. The UV spectra of all wells containing DMSO display in the graphics view.

The software shows Replicate Spectra for the DMSO wells, seeFigure 6.3. Use the arrows keys on the keyboard to navigate through the plate. The spectra of the current well are highlighted on the display, as shown in Figure 6.3. If the UV signal does not look correct in the reference,

acceptor or donor wells, these wells should be excluded from analysis by selecting **Exclude Well** from the drop-down menu. These objectionable spectra are shown in Figure 6.3.

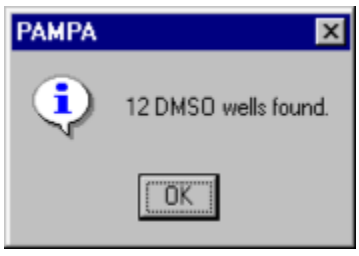
Figure 6.3 Replicate Spectra view for DMSO wells.



Now DMSO wells will be used for the correction of the sample spectra. To select the impurity correction scheme perform the following steps:

1. Click the **Refine Permeability Constants** button on the toolbar to open the dialog box.
2. Select the **DMSO** option in the **Stock Cosolvent Used** section of the **Refine Permeability Contstants** dialog box.
3. Select the **Select wells for impurity correction** option to activate impurity correction.
4. Ensure that **DMSO** is selected from the drop-down list of compounds to choose the basis for the impurity correction.
5. Click the **Set** button in the Impurity Basis section of the dialog box.

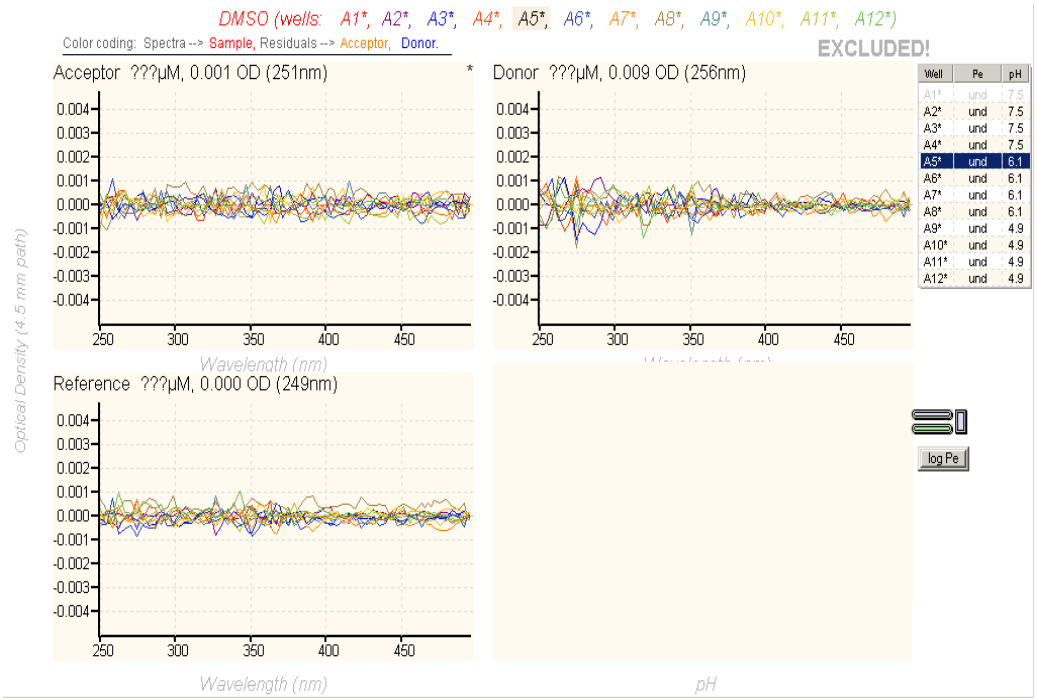
The **PAMPA** info dialog box displays confirming that the impurity correction was set.



The **Impurity** and **Buffer** check-boxes in **ACCEPTOR/DONOR Parameters Regression** Section are selected automatically.

Click the **OK** button of the **Refine Permeability Constants** dialog box to perform the calculation of permeability constants. Spectra of DMSO wells after impurity correction are displayed, see Figure 6.4.

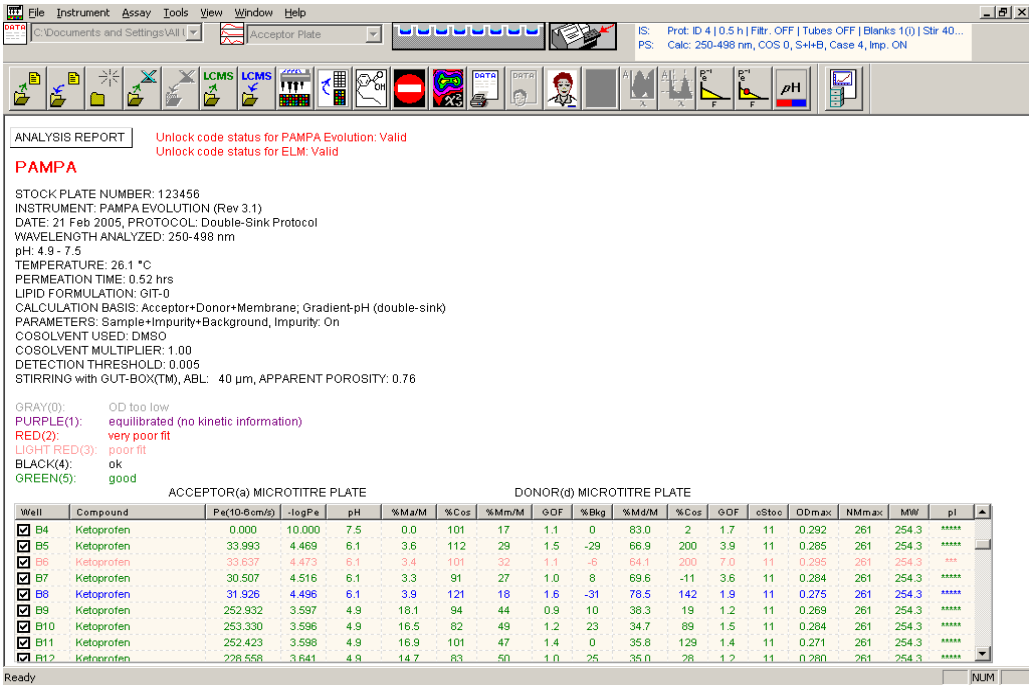
Figure 6.4 An example of UV spectra in DMSO wells after impurity correction is on and functioning.



Click the **View Analysis Report** button on the toolbar or go to the menu Window - View Analysis Report to see the table of results again.

The bottom of Figure 6.5 shows the results row B, columns 4-12. The other values are viewed by scrolling vertically. A larger view table may be displayed by selecting the menu item View|Maximize Table. The top portion of the view summarizes the assay conditions.

Figure 6.5 Table of Results after permeability constants were refined.



Color-Coded Ranking System

A color code is used to rate the results of the data refinement. A green or black result is good. Light red or red indicate problematic calculations, and suggest that the spectra should be inspected.

Possible reasons for problematic calculations are impurities present in a particular well and exhibit different permeabilities from the parent compounds to show. Another reason could be that the compound may have decomposed, or the compound has formed aggregates, which depend on concentration. If too long a permeation time is used, the most permeable compounds will have populated the acceptor wells to the maximum, and no information is available to determine the permeability constants. If the UV absorption of the compound in the original buffer solution (the "reference" solution) is below Detection Threshold (0.015 OD units by default) in the **Refine permeability Constants** dialog box, as shown in Figure 6.1, in the interval 250 - 500 nm, the compound is deemed "undetected", and color marked in gray. In case the table is printed in black-and-white, the ranking is indicated by an asterisk count in the 'Rank' column of the table, described below.

Table of Results

The columns in the results table are defined below.

Well	Check box indicating whether the compound should be included/excluded with in the results
Compound	Compound Identifier
Pe (10^{-6} cm/s)	Permeability result, in units of 10^{-6} cm/s
-log Pe	negative log of the permeability, in units of cm/s
%Ma/M	(moles of sample in acceptor compartment) / (total moles of sample) * 100%
%Md/M	(moles of sample in donor compartment) / (total moles of sample) * 100%
%Mm/M	00% - Ma/M - Md/M (compound lost to membrane portion)
%Cos(left)	FITTING PARAMETER TO BE INVOKED BY EXPERIENCED USERS ONLY. When the parameter is not selected, the calculation assumes that no DMSO (or Impurity if **Impurity Basis** is selected in the **Refine Permeability Constants** dialog box) has permeated into the acceptor compartment. Attempted refinement of the parameter may not be of any meaningful consequence in cases when Impurity is not selected, especially if the refinement wavelength range is above 250 nm, where the UV absorption of the DMSO is minimal.
%Cos(right)	FITTING PARAMETER TO BE INVOKED BY EXPERIENCED USERS ONLY. When the parameter is not selected, the calculation expects that 100% of the DMSO introduced into System Solution remains in the donor compartment (the usual situation).
GOF(left)	Refers to the standard statistical index called the "goodness-of-fit," (GOF) arising from the refinement of the permeability parameter, based on a weighted linear regression analysis incorporating a model where the shape and intensity of the acceptor spectrum are matched to that of the reference spectrum. Statistically, a value of unity would be expected if the weights are accurately assigned and the shapes of the two spectra are the same. When GOF is greater than 5, the result is marked in light red color, signifying poor fit, and prompting the user to examine the actual spectra. This may occur when compounds decompose, or form aggregates, or are impure. When GOF is greater than 10, then the results are marked in red, signifying a very poor fit.
GOF(right)	Same as the GOF(left) parameter above, but based on calculations of the donor compartment.

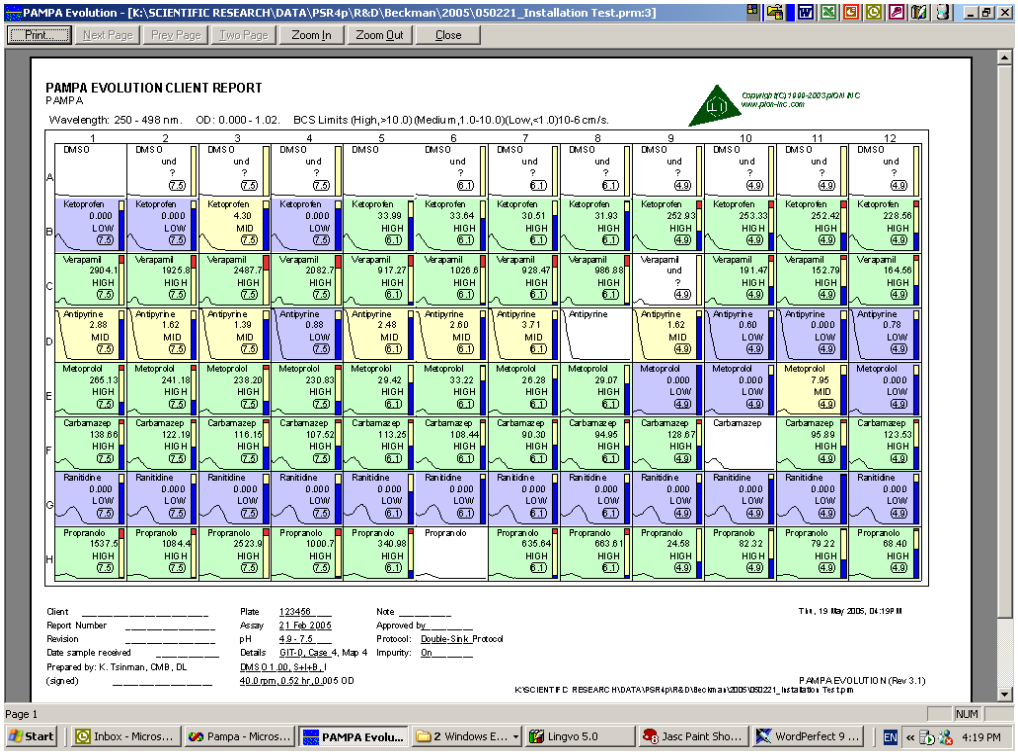
%Bkg	Background correction calculated when the box to the left of BUFFER in the **Refine Permeability Constants** dialog box is selected. This is based on a scheme where "blank" analyses (in the absence of sample) indicated a certain slight distortion of the spectral baseline, presumably due to slight "leakage" ofthe phospholipid filter coatings. The number displayed in the results table (press 'View Analysis Report') is in the range of +/- 200%, where 100% refers to the typical distortion observed in "blanks". To get a feel for this parameter, try calculating with and without such correction, and see what effects are observed in the spectral display and in the values of GOF.
CStoc	The concentration of the DMSO stock plate in millimolar units -- usually 10 mM.
ODmax	The optical density of the peak in the reference spectrum.
NMmax	The wavelength of the peak in the reference spectrum.
MW	The molecular weight (if supplied).
Rank	Symbols for color coding of results (in case B/W printer is used): ***** = good (green), **** = ok (black), *** = poor fit (light red), ** = very poor fit (red), * = equilibrated (purple), the case of highly permeable molecules reaching equal concentrations in the acceptor and donor sides. No symbol in thiscolumn signifies the 'OD too low' condition, which means that the peak maximum in the reference spectrum in the wavelength range used for refinement was below the threshold of detectibility (default 0.015 OD).

Client Report

The most convenient form for displaying all 96 results is that of the "Client Report". After the refinement results are examined, click the **View 96 Spectra** button to get the graphic view of

the 96 spectra. Then click the **Client Report** button. An example of the resulting view is given in Figure 6.6.

Figure 6.6 Example of the Client Report.



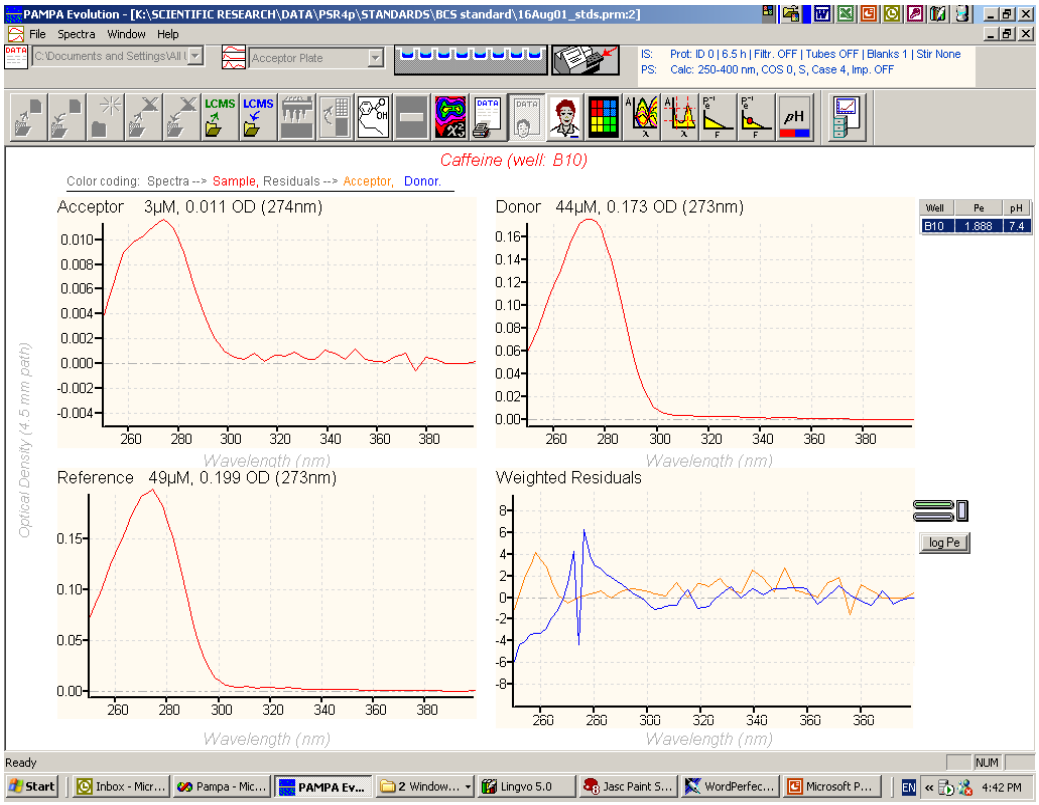
Each of the 96 frames shows

- The name of the compound.
- The spectrum of the compound in the reference well
- The effective permeability number (in units of 10^{-6} cm/s)
- The BCS ranking as HIGH or LOW, with the color indicated in green and blue, respectively.
- pH value in donor well.
- A tri-color bar, with blue, yellow and red. The colors represent the distributions of material at the end of the permeation time, with blue denoting the amount of the solute remaining in the donor well, with red denoting the amount permeated to the acceptor wells, and the yellow denoting the amount trapped in the membranes (retention).

Spectral Views

Detailed spectral information (Detailed Spectra View window) can be accessed by double-clicking on any row in the table of results or on any well in the **View 96 Spectra** window. This view shows UV spectra for a particular compound in the acceptor, donor and reference compartment. Depending on the condition of the **Replicate Data** button on the toolbar, selected or not selected, either spectra for one replicate or spectra for all replicates of this compound are displayed. For example, Figure 6.13 shows UV spectra of single replicate of the compound.

Figure 6.7 Individual Spectral View



In the bottom left corner of the image above, the spectrum of caffeine is shown in the reference solution, which had not been subjected to the permeation step. Usually the optical density (OD) of the peaks is highest in this sdution. The spectrum of the donor well after the permeation pause is shown in the upper right fame. Note that the concentration in the donor well is reduced from that of the reference well, as some of the solute transported into the membrane and some into the acceptor compartment.

The top left view is that of the solute in the acceptor well. The scale in this view is much smaller than in the other two frames. Absolute scaling displays by clicking the **Clip Spectra** button in the main menu toolbar.

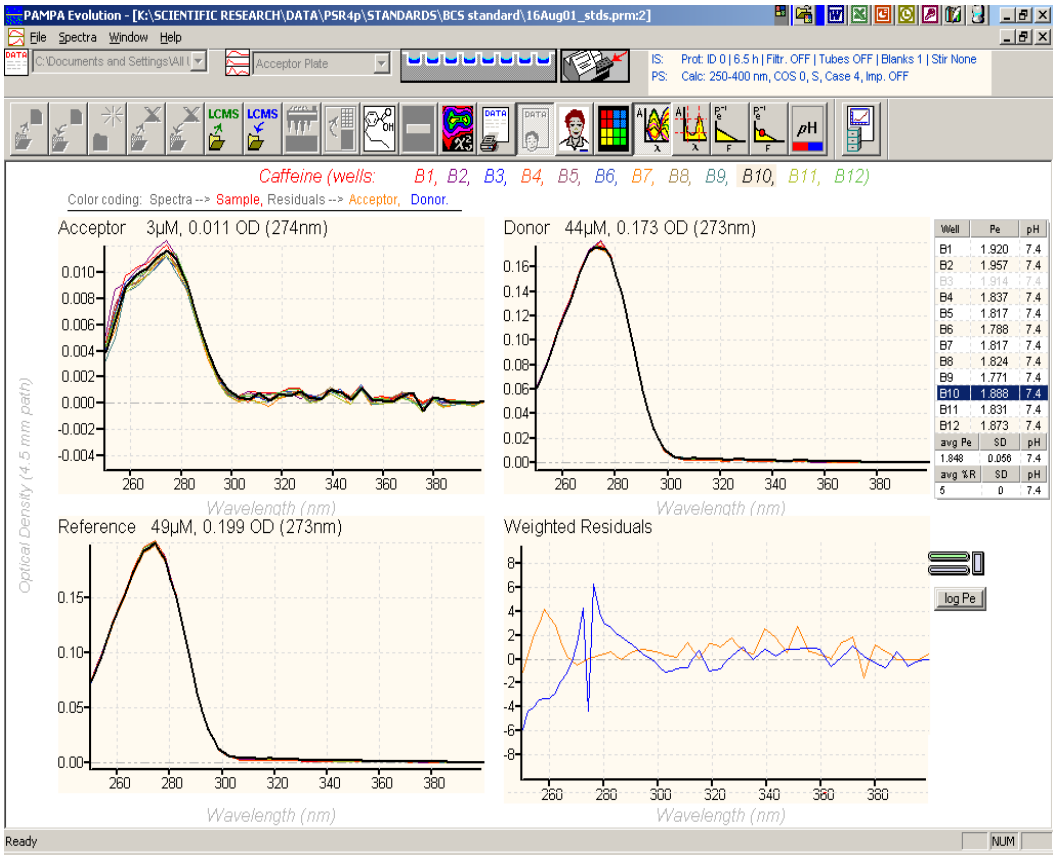
The weighted residuals plot is presented in the bottom right frame. These residuals arise from the fitting of the shape of the reference spectrum to that of the donor (blue line) and the

acceptor (orange line) spectra. PAMPA Evolution has a unique procedure to extract concentration information by weighted least squares, where the amplitudes and the shapes of the entire spectra are subjected to regression analysis. The weighted residuals plot shows how well the fitting procedure progressed. The residuals view, as shown in Figure 6.7, is considered good.

Little switch buttons located at the middle of the right edge corner of the bottom right frame is used to show either Weighted Residuals plot or Pe (logPe) vs pH plot in this frame. The**log Pe** button below these switch buttons would determine whether Pe vs pH or logPe vs pH plot is to be displayed.

Click the **Replicates Spectra** button in the main toolbar, to show all of the replicates in the same graph. As shown in Figure 6.8

Figure 6.8 Replicates drawn in the same view.

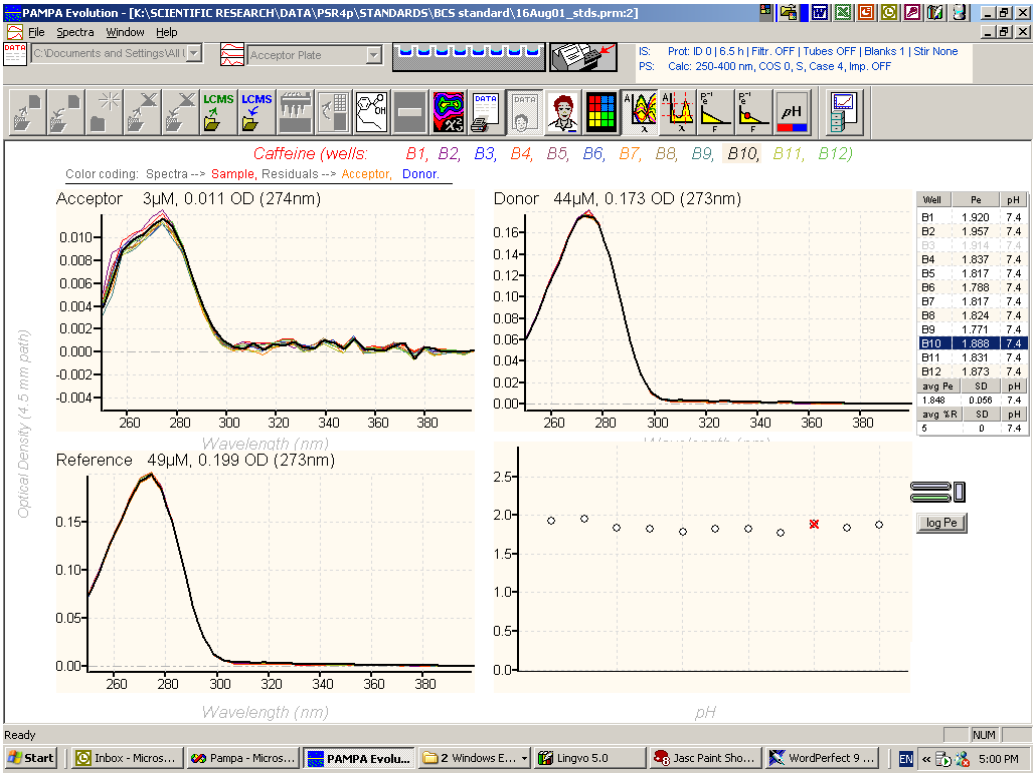


All the replicates show good agreement. The well B10 is high-lighted, and the weighted residuals plot refers to it. The arrow keys may be used to switch between the high-lighted spectra.

Click the green switch button above the **log Pe** button, the Weighted Residuals plot is replaces with Pe (or log Pe) plot in the bottom right frame. Since caffeine was performed at

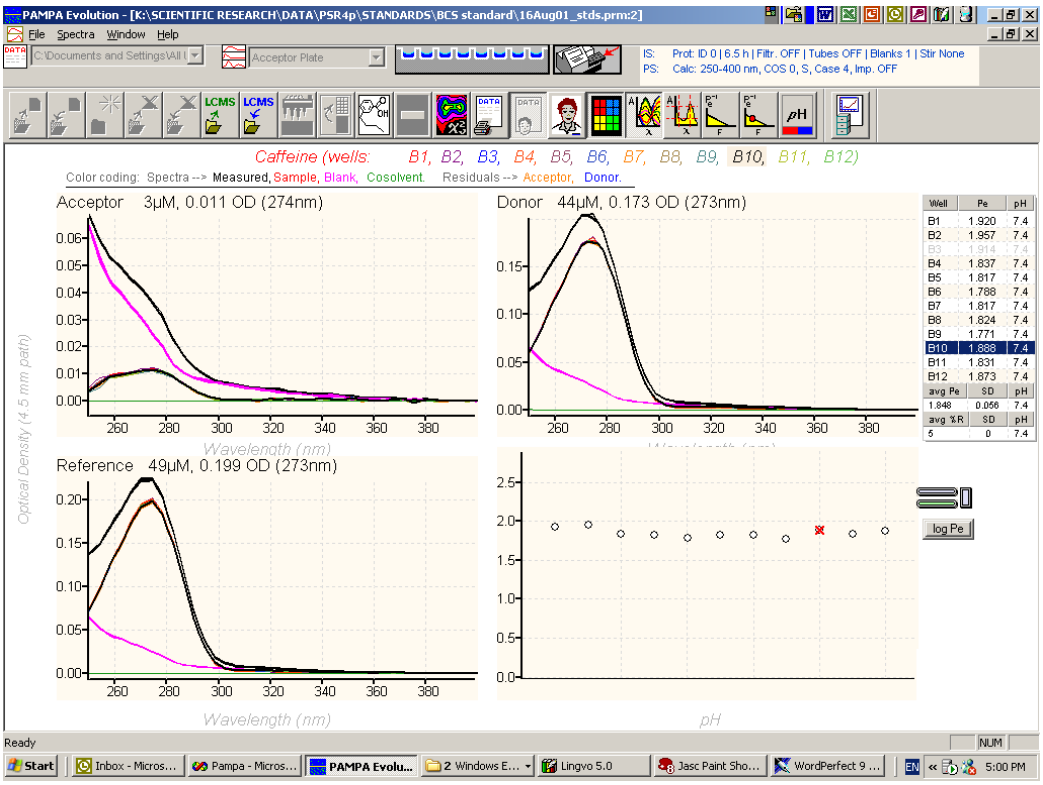
one pH, 12 times, these plots allow the viewer to see the reproducibility of the results, as shown in Figure 6.9.

Figure 6.9 Reproducibility of results for caffeine



If the **Extended Spectra** button is selected while in the **Detailed Spectra View** window, the resultant view, as shown in Figure 6.10, includes details about the various background components attributable to the absorption of the plastic plate and the buffers used.

Figure 6.10 The spectral components due to plastics and buffers.



The black lines on the UV spectra plots above represent the observed spectra; the purple lines are those arising from the "blank" plate. The green line represents the DMSO or other "impurity" contribution to the total spectra. The subtraction of purple and green lines from the black produces the net red line, due to the spectrum of the sample itself.

Advanced Impurity Correction

Due to the presence of DMSO and other components in samples an advanced Impurity correction paradigm has been developed by pION for use with PAMPA Evolution software. For the impurity correction algorithm to work correctly, blank impurity wells are used in the assay. These wells normally contain the same components as the sample wells, but without any drug. For best results plan on having at least three such wells for every pH condition. This number is not necessary proves optimum.

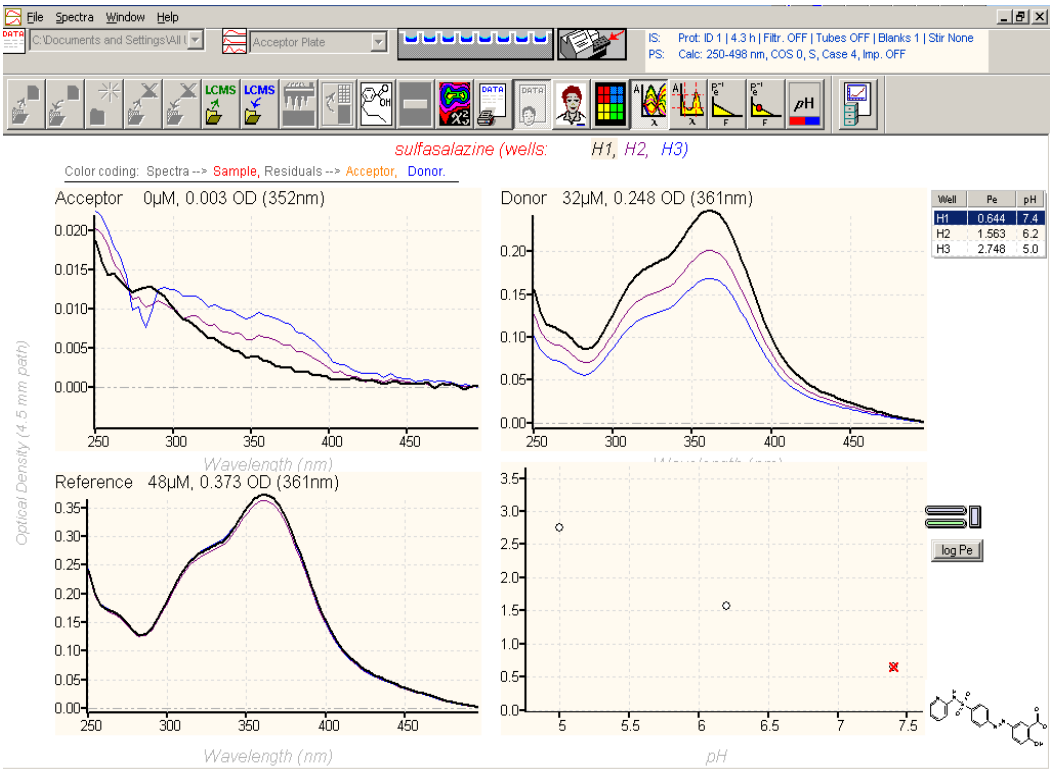
NOTE A single well of a DMSO control may prove good enough if space on the plate is a constraint.

The impurity correction technique makes use of proprietary algorithms developed at pION to allow the removal of certain components that overlap with a compounds UV absorption signal. The technique is used to help detect a drug's signal in the acceptor well and make

permeability measurements easier. It may be used under all types of protocols. An example is below showing the technique used with double-sink conditions.

The following example graphic is for sulfasalazine. This is a low permeable compound which is expected to have near zero permeability under double-sink conditions. The compound was run on a plate over three pHs (5.0, 6.2, 7.4) in the donor wells and acceptor wells of pH 7.4 using a chemical sink. The results, to mirror the observed in vivo results, should be almost no permeability. This is not the case when looking at the absorbance spectra, as shown in Figure 6.11, due to the general absorbance of the chemical sink material.

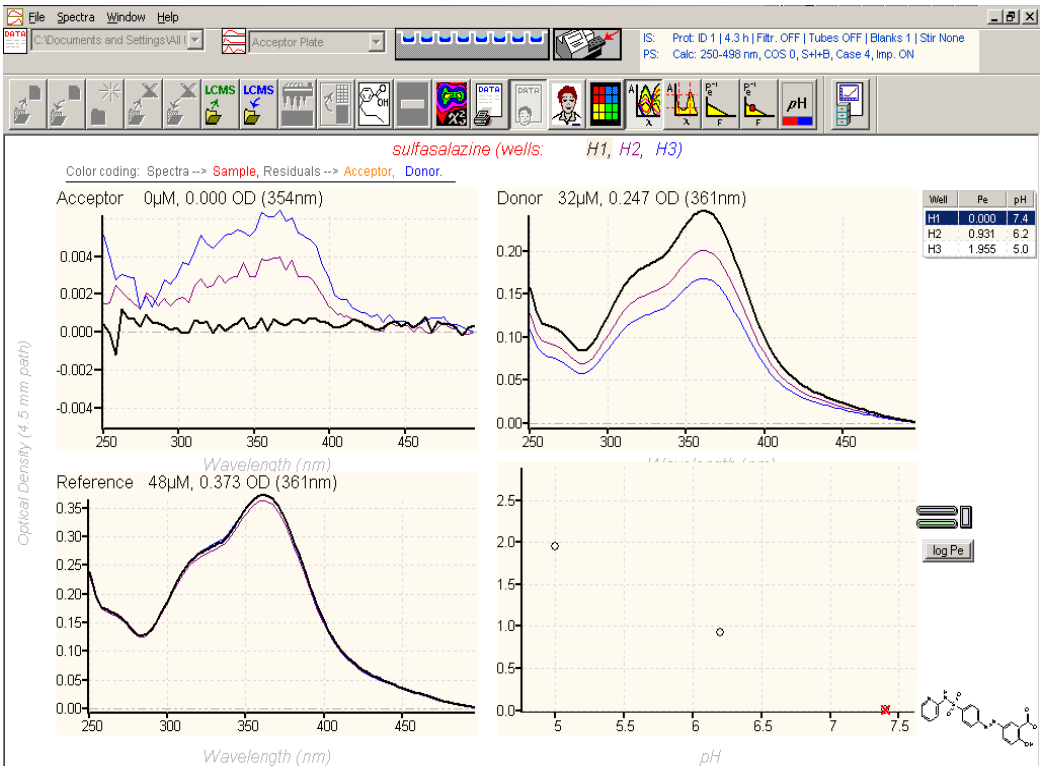
Figure 6.11 Double sink results for sulfasalazine without impurity correction.



This problem can be rectified by using a built in impurity correction to remove the contribution of the chemical sink to the absorbance in the acceptor wells. This is done by going to the **Refine Permeability Constants** dialog box and then set **DMSO** as the impurity well for the correction factor, see Setting up Impurity Correction Section 6. Performing this blank correction allows the material present in the acceptor wells from DMSO runs to be

removed from the spectral data and the true contribution from sulfasalazine to be observed, as shown in Figure 6.12.

Figure 6.12 Results for sulfusalazine assayed under double-sink conditions using impurity correction and DMSO wells as the guide for correction.



If this refinement was not performed, sulfasalazine might be incorrectly classified as being permeable. The impurity correction can also be used if contaminants are present in the buffer solution.

It is imperative that only wells called "DMSO"be used with this method. To use another well, rename it as "DMSO" using the **Compound List** dialog box for the method to work correctly.

6.2 Export to Excel

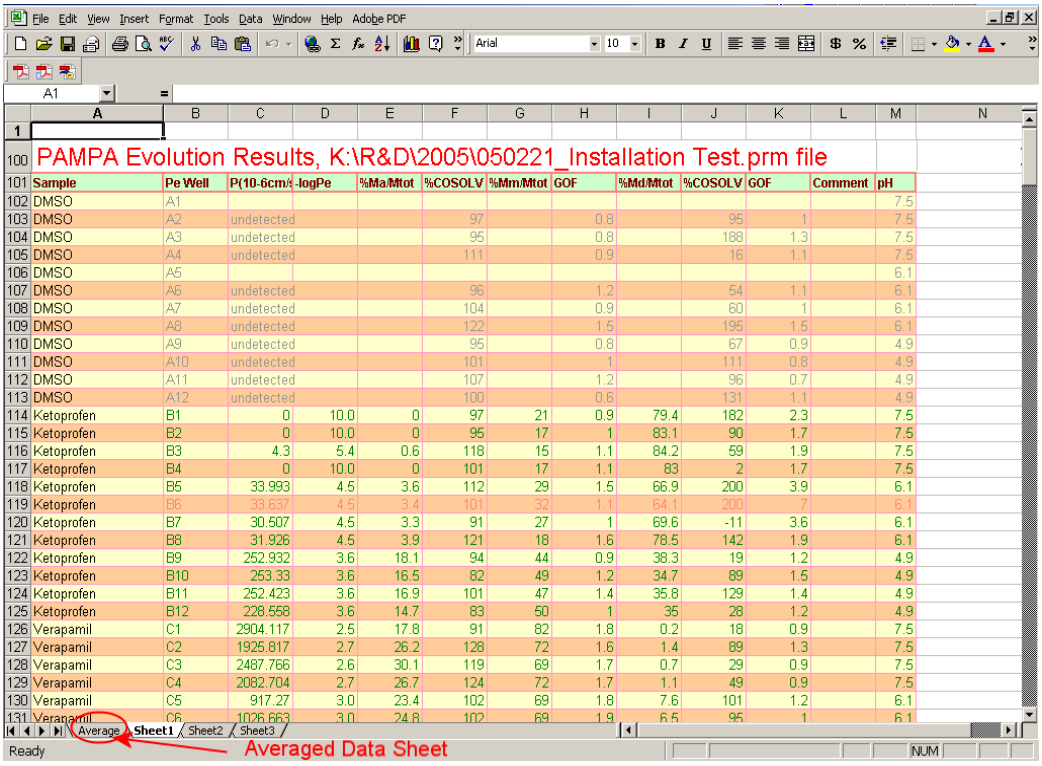
Whenever an experiment is performed the data and the results can be printed or be exported to the Excel file for presentation purposes or entered in the corporate database.

Export data to Excel

Click the **Export to Excel** icon in the main tool bar.

The **Save As** dialog box displays. Name the exported Excel file and click the **Save** button. Microsoft Excel opens with a spreadsheet filled up with the results from the PAMPA assay, see Figure 6.13.

Figure 6.13 PAMPA assay result spreadsheet in Excel.



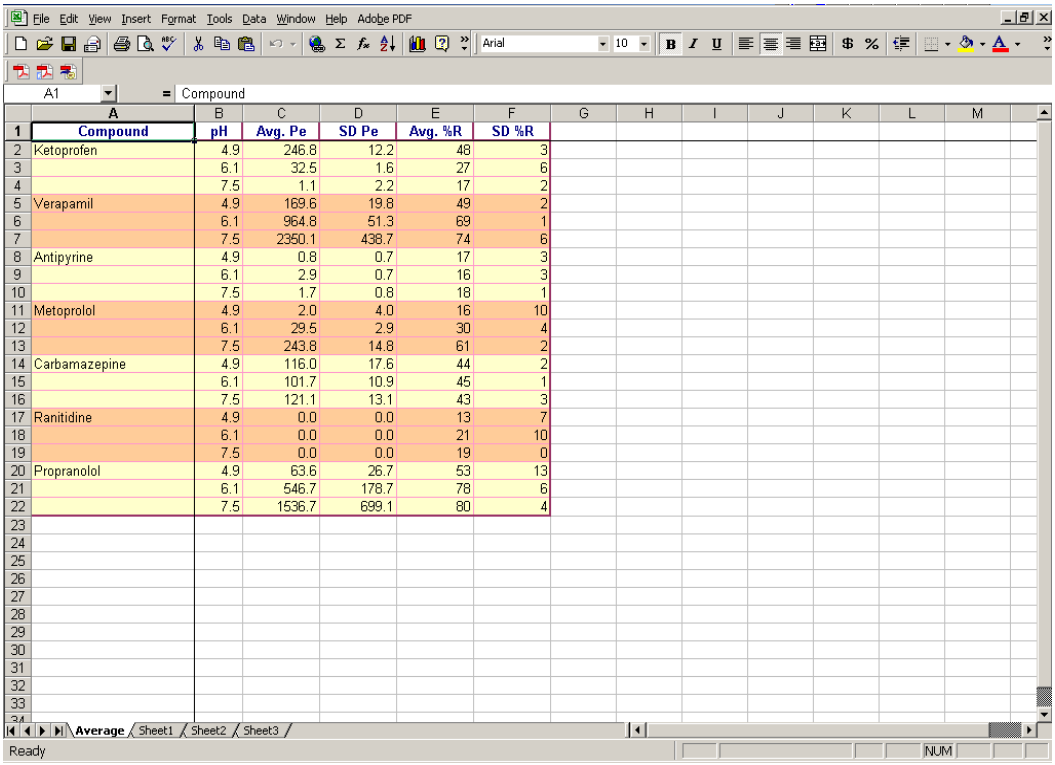
PAMPA Evolution Results, K:\R&D\2005\050221_Installation Test.prm file

Sample	Pe Well	P(10-6cm/s)	-logPe	%Ma/Mtot	%COSOLV	%Mm/Mtot	GOF	%Md/Mtot	%COSOLV	GOF	Comment	pH
DMSO	A1											7.5
DMSO	A2	undetected			97		0.8		95	1		7.5
DMSO	A3	undetected			95		0.8		188	1.3		7.5
DMSO	A4	undetected			111		0.9		16	1.1		7.5
DMSO	A5											6.1
DMSO	A6	undetected			96		1.2		54	1.1		6.1
DMSO	A7	undetected			104		0.9		60	1		6.1
DMSO	A8	undetected			122		1.5		195	1.5		6.1
DMSO	A9	undetected			95		0.8		67	0.9		4.9
DMSO	A10	undetected			101		1		111	0.8		4.9
DMSO	A11	undetected			107		1.2		96	0.7		4.9
DMSO	A12	undetected			100		0.6		131	1.1		4.9
Ketoprofen	B1	0	10.0	0	97	21	0.9	79.4	182	2.3		7.5
Ketoprofen	B2	0	10.0	0	95	17	1	83.1	90	1.7		7.5
Ketoprofen	B3	4.3	5.4	0.6	118	15	1.1	84.2	59	1.9		7.5
Ketoprofen	B4	0	10.0	0	101	17	1.1	83	2	1.7		7.5
Ketoprofen	B5	33.993	4.5	3.6	112	29	1.5	66.9	200	3.9		6.1
Ketoprofen	B6	33.637	4.5	3.4	101	32	1.1	64.1	200	7		6.1
Ketoprofen	B7	30.507	4.5	3.3	91	27	1	69.6	-11	3.6		6.1
Ketoprofen	B8	31.926	4.5	3.9	121	18	1.6	78.5	142	1.9		6.1
Ketoprofen	B9	252.932	3.6	18.1	94	44	0.9	38.3	19	1.2		4.9
Ketoprofen	B10	253.33	3.6	16.5	82	49	1.2	34.7	89	1.5		4.9
Ketoprofen	B11	252.423	3.6	16.9	101	47	1.4	35.8	129	1.4		4.9
Ketoprofen	B12	228.558	3.6	14.7	83	50	1	35	28	1.2		4.9
Verapamil	C1	2904.117	2.5	17.8	91	82	1.8	0.2	18	0.9		7.5
Verapamil	C2	1925.817	2.7	26.2	128	72	1.6	1.4	89	1.3		7.5
Verapamil	C3	2487.766	2.6	30.1	119	69	1.7	0.7	29	0.9		7.5
Verapamil	C4	2082.704	2.7	26.7	124	72	1.7	1.1	49	0.9		7.5
Verapamil	C5	917.27	3.0	23.4	102	69	1.8	7.6	101	1.2		6.1
Verapamil	C6	1026.663	3.0	24.8	102	69	1.9	6.5	95	1		6.1

To enable this option select the **Export Averages to Excel** check box in the **Assay Properties-Assay Settings** dialog box, a shown in Figure 6.14, BEFORE exporting to Excel. If

Export **Averages to Excel** has been enabled an additional sheet is added in the Excel file, as shown in Figure 6.14.

Figure 6.14 Averaged PAMPA result spreadsheet in Excel



	A	B	C	D	E	F
1	Compound	pH	Avg. Pe	SD Pe	Avg. %R	SD %R
2	Ketoprofen	4.9	246.8	12.2	48	3
3		6.1	32.5	1.6	27	6
4		7.5	1.1	2.2	17	2
5	Verapamil	4.9	169.6	19.8	49	2
6		6.1	964.8	51.3	69	1
7		7.5	2350.1	438.7	74	6
8	Antipyrine	4.9	0.8	0.7	17	3
9		6.1	2.9	0.7	16	3
10		7.5	1.7	0.8	18	1
11	Metoprolol	4.9	2.0	4.0	16	10
12		6.1	29.5	2.9	30	4
13		7.5	243.8	14.8	61	2
14	Carbamazepine	4.9	116.0	17.6	44	2
15		6.1	101.7	10.9	45	1
16		7.5	121.1	13.1	43	3
17	Ranitidine	4.9	0.0	0.0	13	7
18		6.1	0.0	0.0	21	10
19		7.5	0.0	0.0	19	0
20	Propranolol	4.9	63.6	26.7	53	13
21		6.1	546.7	178.7	78	6
22		7.5	1536.7	699.1	80	4

Update Excel

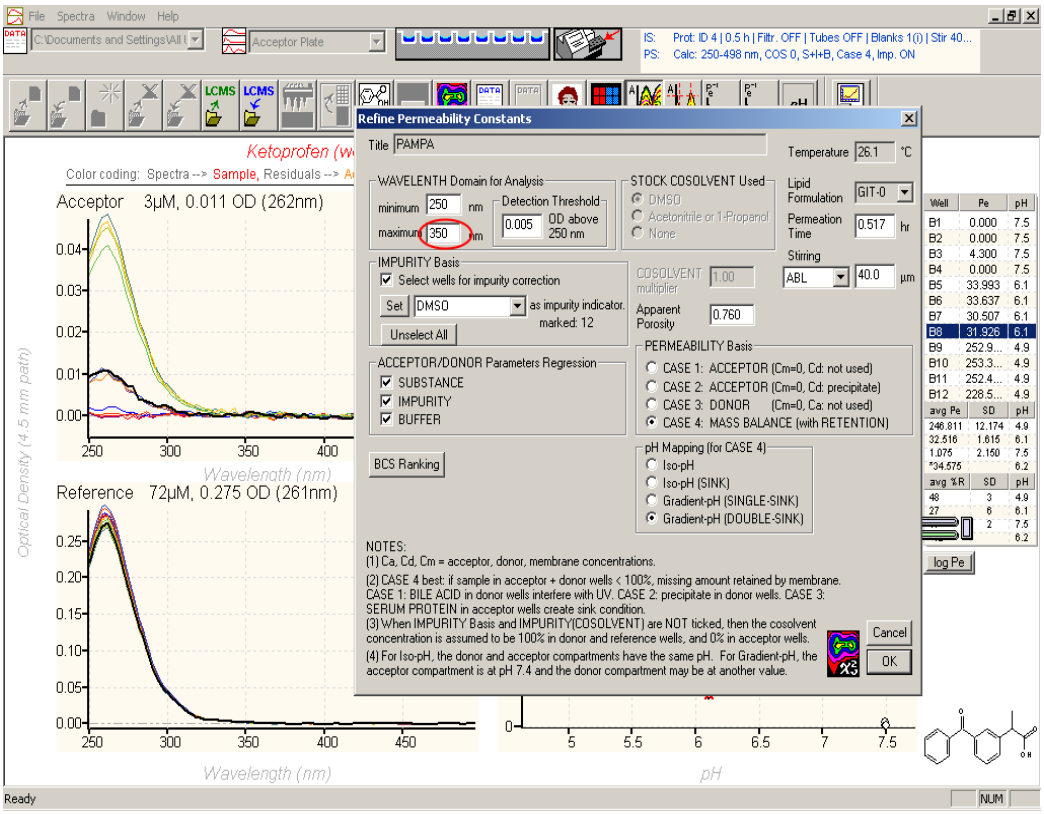
The data can be refined individually by choosing appropriate wavelength range for each compound. The software allows an update to the Excel file reflecting this individual refinement.

To set this option:

1. Open a data file and then export the data to Excel as it is described in Liquid Chromatography/Mass Spectrometry(LCMS) Section g.
2. Close the Excel file and switch back to PAMPA software.
3. Open the Spectra details view in permeability file, as shown in Spectral Views Section 6.

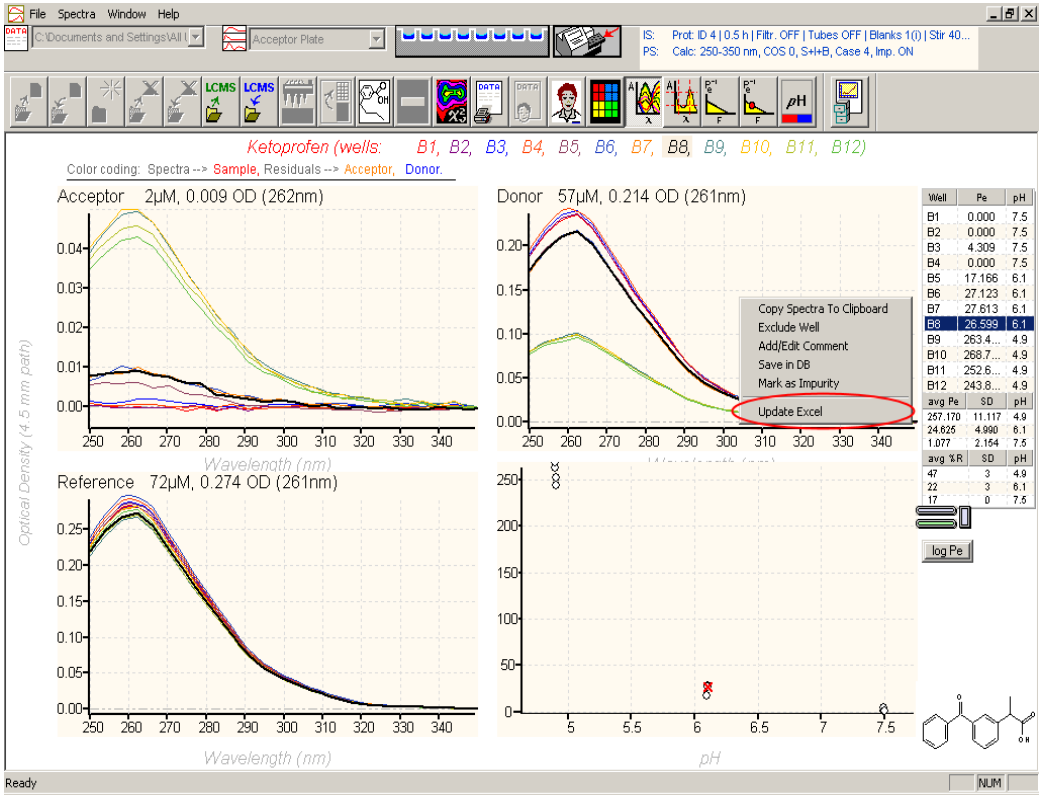
4. Click the **Refine Permeability Constants** dialog box and recalculate the results choosing appropriate wavelength domain for a particular compound, for example from 250 - 350 nm for Ketoprofen, as shown in Figure 6.15.

Figure 6.15 Recalculated results of Ketoprofen selecting wavelength range from 250 - 350 nm.



5. When the calculation is done and the results are satisfactory, RIGHT click the mouse anywhere on the screen and select **Update Excel** from the drop-down menu, as shown in Figure 6.16.

Figure 6.16 Update Excel option drop-down menu.



After selecting **Update Excel**, the software finds the file that was just exported and opens it. It also updates the results for the compound seen on the screen at the moment. Check the Excel file updated with the wavelength range which was chosen for calculation.

Figure 6.17 A fragment of updated Excel file with the saved wavelength range for Ketoprofen.

100	PAMPA Evolution Results, K:\R&D\2005\050221_Installation Test.prm file													
101	Sample	Pe Well	P(10-6cm/s	-logPe	%Ma/Mtot	%COSOLV	%Mm/Mtot	GOF	%Md/Mtot	%COSOLV	GOF	Comment	pH	
102	DMSO	A1											7.5	
103	DMSO	A2	undetected			97		0.8		95	1		7.5	
104	DMSO	A3	undetected			95		0.8		188	1.3		7.5	
105	DMSO	A4	undetected			111		0.9		16	1.1		7.5	
106	DMSO	A5											6.1	
107	DMSO	A6	undetected			96		1.2		54	1.1		6.1	
108	DMSO	A7	undetected			104		0.9		60	1		6.1	
109	DMSO	A8	undetected			122		1.5		195	1.5		6.1	
110	DMSO	A9	undetected			95		0.8		67	0.9		4.9	
111	DMSO	A10	undetected			101		1		111	0.8		4.9	
112	DMSO	A11	undetected			107		1.2		96	0.7		4.9	
113	DMSO	A12	undetected			100		0.6		131	1.1		4.9	
114	Ketoprofen	B1	0	10.0	0	92.772	17.604	0.618	82.396	168.815	3.79		7.5	250 350
115	Ketoprofen	B2	0	10.0	0	100.662	16.897	0.96	83.103	99.501	2.502		7.5	250 350
116	Ketoprofen	B3	4.309	5.4	0.548	119.772	16.724	0.738	82.728	102.99	1.63		7.5	250 350
117	Ketoprofen	B4	0	10.0	0	104.42	17.409	1.27	82.591	-14.928	3.582		7.5	250 350
118	Ketoprofen	B5	17.166	4.8	1.997	79.131	23.112	1.248	74.891	22.179	8.003		6.1	250 350
119	Ketoprofen	B6	27.123	4.6	3.068	97.896	24.677	0.818	72.255	152.831	7.885		6.1	250 350
120	Ketoprofen	B7	27.613	4.6	3.288	92.755	20.672	0.758	76.04	101.252	7.441		6.1	250 350
121	Ketoprofen	B8	26.599	4.6	3.287	112.361	17.743	1.229	78.97	151.449	2.177		6.1	250 350
122	Ketoprofen	B9	263.454	3.6	18.626	103.904	43.949	1.066	37.425	61.664	1.21		4.9	250 350
123	Ketoprofen	B10	268.724	3.6	17.798	108.944	47.293	1.459	34.909	68.727	1.059		4.9	250 350
124	Ketoprofen	B11	252.644	3.6	17.232	106.646	46.342	1.063	36.426	75.292	2.628		4.9	250 350
125	Ketoprofen	B12	243.856	3.6	15.552	101.136	50.143	0.821	34.305	28.828	1.342		4.9	250 350
126	Verapamil	C1	2904.117	2.5	17.8	91	82	1.8	0.2	18	0.9		7.5	
127	Verapamil	C2	1925.817	2.7	26.2	128	72	1.6	1.4	89	1.3		7.5	
128	Verapamil	C3	2487.766	2.6	30.1	119	69	1.7	0.7	29	0.9		7.5	

Command Software, Version 3.2.0

a.1 PAMPA EVOLUTION96, Version 3.2.0

a.2 Methods

In this portion of the manual, discover the methods for testing the BIOMEK FX system and the PAMPA Evolution96 Permeability analyzer.

Gradients	This technique allows a user to measure permeability with acceptor and donor wells of different pH and using a chemical sink in the acceptor wells.
Double Sink	This is a advanced gradient method which uses predefined multiple-pH measurements to determine permeability in the presence of a double sink.
Stirring PAMPA	This method allows a user to reduce thickness of Aqueous Boundary Layer in PAMPA assay and match the ABL found “in vivo”.

a.3 Data Refinement

Filter porosity is taken into account for calculation. Impurity correction algorithms are included.

a.4 Data Exporting

Averaged results for each compound/pH gathered with standard deviation and membrane retention.

a.5 Devices

Gut-Box™ allows stirring in the donor compartment for PAMPA assays.

a.6 Software add-on

This version is compatible with ELM (Evolution Library Manager) add-on.

a.7 Data Binding

Data may either be bound using internal standards or set values using our BCS RANKING FEATURE.

General Information

b.1 Notices

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Part Numbers

d.1 Part Numbers

Table D.1 Biomek FX-ADMETox WorkstationSales Group P/N:_____

Part Number	Quantity	Description
717001	1	Biomek FX Single Arm w/ Multi-channel pod
719368	1	Biomek 96-Channel Disposable Tip Pipetting Head - 200ul
A16170	1	Biomek Automation Controller XP and Monitor with Biomek™ System Software
719366	1	Biomek Device Controller
719356	1	Biomek Disposable Tip Loader ALP
719948	1	4x3 ALP
719357	6	Standard Single Position ALP
394593	1	SPECTRAmax 190 Reader and integration Kit

Table D.2 PAMPA Evolution96 Kit P/N: A24454

Quantity	Description
1	Gut-Box™
1	PAMPA Evolution96 Command Software
1	PAMPA Evolution96 Desktop Software
1	PAMPA Starter Kit

Table D.3 10 Plate Consumables Kit P/N: A24455

Part Number	Quantity	Description
252110	4	High Profile Reservoir, packs of 5
253110	2	Low Profile Reservoir, packs of 5
024110	4	UV Reading plates, packs of 10
186110	2	PCR plates, packs of 5

Table D.3 10 Plate Consumables Kit P/N: A24455

Part Number	Quantity	Description
669110	4	GIT(O) lipid, packs of 5
617110	20	Ampoule breakers
254110	1	High Profile 12-trough, pack of 5
255110	1	Low Profile 12-trough, pack of 5
212110	2	PAMPA Preloaded Sandwiches, packs of 5
023110	2	Deep Well Plates, packs of 5
151110	2	Bottles of System Solution
139110	2	Bottles of ASB, 7.4

Table D.4 50 Plate Consumables Kit P/N: A24456

Part Number	Quantity	Description
252110	20	High Profile Reservoir, packs of 5
253110	10	Low Profile Reservoir, packs of 5
024110	20	UV Reading plates, packs of 10
186110	10	PCR plates, packs of 5
669110	20	GIT(O) lipid, packs of 5
617110	50	Ampoule breakers
254110	5	High Profile 12-trough, pack of 5
255110	5	Low Profile 12-trough, pack of 5
212110	10	PAMPA Preloaded Sandwiches, packs of 5
023110	10	Deep Well Plates, packs of 5
151110	10	Bottles of System Solution
139110	10	Bottles of ASB, 7.4

Trouble Shooting Guide

e.1 Trouble Shooting Guide

Problems	Possible Cause	Suggestions	Comments
Bad Blank Well Message (Dust, Bubble, Impurity) If list of more than 20-30 wells reported.	Microbial contamination in System Solutions.	Follow the User's Manual Spotting Contamination in the System Solution by UV Section 3 for spotting contamination. Prepare fresh System Solution and retake blank.	Look in the User's Manual for System Solution handling and storing. More detailed information and advenced methods describes dution pION`s training courses for advense users. Contact pION for more information.
	Spectrophotomerer hardware problem.	Call Beckman Service for maintanence of SpectraMax.	
	UV plates QC issue.	Call Beckman Service.	
Abnormal Spectra View.	Microbial contamination in System Solutions.	Use fresh System Solutions.	
	Impurity in UV plates.	Need clean UV plates.	
		Do not touch the bottom of UV plates.	
	Decomposition of test compounds.	Prepare fresh test compounds.	More information how to avoid slow decomposition during PAMPA assay may be available during training course.
	Contamination in stock solvent (DMSO).	Use fresh stock solvent, spectroscopic grade.	
	Bubble in wells.	Adjust pipetting method.	
	Dust in labwares.	Use only dust-free labwares.	
	Lipid contamination.	Adjust pipetting methods to avoid lipid being aspirated from the filter surface.	

Problems	Possible Cause	Suggestions	Comments
Positive Control does not match historic data.	Wrong pH in System Buffe.	pH meter needs calibration.	If none of the listed problems observed, please, refer to "pION's Guide to Improve Double-Sink™ PAMPA" or User's Guide for data results acceptances.
	Acceptor Sink Buffer(ASB) with precipitation.	ASB needs be thawed to room temperature and appear as clear solution.	
		Do not reuse ASB for next day experiment.	
	Decomposition of compounds.	Prepare fresh stock solutions.	
	Well is not painted properly with lipid.	Ensure that there is enough lipid for painting in the lipid supply plate. Adjust pipetting method in Step 2 if needed.	
	Stirrer disk missing in the well.	Inspect donor plate prior to the experiment to ensure that stirrer disks are in all wells.	
Data Analysis.	Undetected Pe.	Adjust Detection Threshold in Refine Permeability Constants dialog box. Use only if UV spectrum can be visually confirmed.	For data analysis questions, please, refer to the User's Guide, Appendix f and "pION's Guide to Improve Double-Sink™ PAMPA" It is strongly recommended users to attend PAMPA advenced trianing course to become familiar with defferent data handling techniques.

Theory and Definitions

f.1 What is Permeability?

A drug's permeability expresses its ability to move from one medium into or through another. Specifically, it refers to its ability to move through the intestinal membrane into the bloodstream and/or from the blood stream into the receptor target.

The unit for permeability is cm/s (centimeters per second), i.e., similar to speed. In other words: Permeability is a kinetic parameter. A higher number indicates higher speed and thus higher permeability. The range of interest spans several decades (= factors of ten) of permeability, so permeability is often plotted as the logarithm of the permeability (log P_e). One log-unit represents a factor of ten.

Two substances are involved in the permeation process: the drug molecule and the membrane. The permeability number expresses a mutual property, i.e., we could assign the permeability number to either one of the two substances involved relative to the other. Since in our case, the membrane is supposedly the same for all drugs, we pin the permeability number on the drug molecule rather than the membrane, remembering that the number expresses the drug's ability to permeate the membrane in question.

Today's preferred drug is one that can be administered orally without side effects. Once ingested, there is a window of opportunity of about four hours for the dissolution and absorption of the drug in the digestive system. Of the main segments of the gastrointestinal tract, the jejunum and ileum have by far the largest surface area (each about 60 m^2) and the intestinal fluid spends a considerable time (3 to 4 hours) in these segments. It is the drug's ability to permeate the membrane of the jejunum and ileum that attracts the immediate attention. Without the ability to permeate this first membrane barrier, the drug will not make it to the organ where it is supposed to go.

f.2 Human Absorption

Human absorption is the fraction of an orally administered dose that reaches the blood, specifically the portal vein, before first passing through the liver. If the orally taken molecule does not pass across the membrane barriers in the small intestine, then the human absorption fraction will be much less than the 100% that is the maximum possible value. It should be obvious, that – for a variety of reasons – it is impossible to actually measure human absorption for the thousands of new drug molecules created every year.

What we are looking for is an easily measured descriptor of human absorption. The measured permeability of a representative membrane, or one that mimics the properties of the human body membranes, tells us how easily a drug can be expected to pass from the gastrointestinal tract into the blood stream or tissue fluid. Since membranes are made of oily substances (phospholipid bilayers), a drug's affinity to such oil – its lipophilicity, a.k.a its "oil-loving" property – may also be a descriptor for human absorption.

Lipophilicity (log P, log D at pH 7.4, or the log D/pH profile) is readily measured. It is common practice to use the octanol-water partition coefficient (log P) to predict human absorption. However, many studies have shown, that this method does not always produce a

strong correlation; after all, octanol is not at all similar to the membrane it is supposed to model. Realizing that not all human membranes are created equal, other lipophilicity models have been used as predictors for skin or the blood-brain barrier. Several good log P predictor programs are commercially available in software.

The lipophilicity approach is widely used to assess drug permeability as a predictor of human absorption. The common view is that the more lipophilic the molecule, the more likely it is to permeate the membrane. However, it has been experimentally confirmed that for highly lipophilic molecules (log $D_{7.4} > 3$), the effective permeability (log P_e) does not always increase with an increase in lipophilicity, but rather declines. The major reason for this is thought to be retention of the drug by the membrane.

pION has developed a method to actually measure retention at the same time that permeability is measured. The magnitude of the retention can be surprisingly high.

Besides poor permeability, there are several other reasons why a drug may not be absorbed even if it were permeable. Poor solubility, interference due to the drug being metabolized before it reaches the target, slow dissolution, etc. With the PAMPA Evolution we study the permeability aspect.

Measurement of membrane permeability to obtain a better predictor for human absorption has been gaining ground. There are two methods: Caco-2 and PAMPA (filter-immobilized artificial membranes).

It should be noted that permeability alone is not necessarily the best indicator to use. What is important to human absorption is the amount of drug that flows through the membrane, also known as the flux.

According to Fick's first law (applied to membranes under steady state sink conditions):

flux = $P_e \times C$(flux in units of moles/cm^2/s)

P_e is the effective permeability (in units of cm/s) and C is the dose of the drug (in units of mol/cm^3). If the dose is in excess of the solubility of the drug, C becomes the solubility. So, to get a really good handle on human absorption, we need to know the product of permeability and solubility. For that reason, the PAMPA Evolution has a companion: the µSOL Evolution for solubility determination. Flux is the name of the absorption game.

f.3 Transporting Drugs Through Membranes

Generally, drugs are transported through membranes by two main mechanisms: active transport and passive transport.

As it turns out, some drugs (10% or so) are actively transported across the membranes by several different means.

Passively transported drugs (90% of the cases, more or less) follow at least one of two major pathways:

- The transcellular pathway takes the molecule through the cell itself. It is the main pathway for the drug.

- The paracellular pathway takes the molecule around the cell through the tight junction between cells (this accounts for perhaps 0.1% of the transport). Only the smallest molecules can fit through the crevices. Most drug molecules are too big.

When measuring permeability, the value we obtain is the effective permeability P_e, which may be thought of as the total effect of the membrane permeability P_m and P_u the permeability of the so-called unstirred water layers on each side of the membrane.

f.4 Using Caco-2 Cells

Permeability can be measured by using a **filter-immobilized monolayer of human intestinal cells** (Caco-2, laboratory cultured cells) to separate a buffer solution with known drug concentration from just a buffer solution. After a certain time, the ratio of the drug concentrations in the two buffers represents the permeability of the drug.

Because the living cell monolayer exhibits most of the pathways available to the drug in the body, it should yield good permeability values. However, when correlated with available data for human absorption, it doesn't do too well because oral absorption for a diverse collection of molecules is too complicated to be predicted by just one indicator.

Studies of inter-laboratory variations in Caco-2 permeability determination show large deviations indicating difficulties of standardization of the method.

Despite its problems, the Caco-2 method has become popular and efforts to make it better standardized and suitable for fast analysis are made by several drug companies and instrument manufacturers.

A drawback of the method is the elaborate facility needed to grow the cells. The growing process takes up to 21 days under strictly controlled conditions.

Licenses make the method expensive and those who do the work, generally find it hard to do.

Despite this, Caco-2 is presently the method of choice, but it is not well suited for high workloads.

f.5 Artificial Membranes

Another way is to form a filter-immobilized artificial membrane phospholipid bi-layer (an oil more biological than octanol) and using it much the same way the Caco-2 monolayer is used. The phospholipid membrane mimics the cell membranes, but has no means for active or paracellular transport of drug molecules. It models the passive transport system only. Since about 90% of all drugs are passively transported, it is not necessarily an important limitation. The membrane is formed in seconds on a very thin filter material.

The artificial membrane is not a single bilayer, but seems to have the properties of approximately 150 to 300 bilayers.

This method lends itself well to high speed, automation, and small formats. 96-well microtitre plates are well suited for the purpose. Such plates are now becoming commercially available for the Caco-2 method too, but they are expensive.

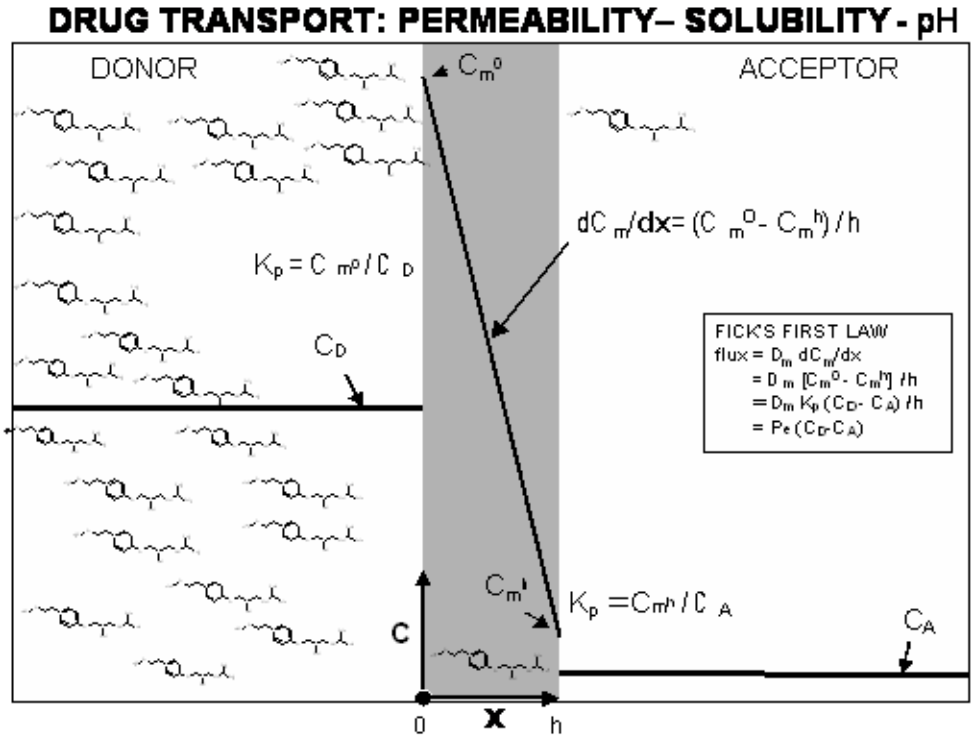
The method used in the PAMPA Evolution, is referred to as the modified-PAMPA method. Originally introduced by Kansy, et al. at Roche, PAMPA stands for parallel artificial membrane permeability analysis.

The PAMPA method should not be considered a Caco-2 replacement method. Rather, it works as a preliminary, whole-library screen before the more cumbersome and costly Caco-2 method is applied to a select group of especially promising molecules or to those that are suspected of exhibiting active transport properties.

f.6 The Theory behind the PAMPA Method

In the PAMPA assay [1-4], a "sandwich" is formed from a 96-well microtitre plate and a 96-well filter plate, so that each composite well (schematically represented in Figures f.1) is divided into two chambers: donor at the bottom and acceptor at the top. These chambers are separated by a 125 µm micro-filter disc (0.45 µm pores), coated with a 20% (wt/v) dodecane solution of a mixture of phospholipids some of which contain a net negative charge.

Figure f.1 Model for the Permeability Assay.



Fick's first law applied to homogeneous membranes at steady state [5] may be stated as

Equation f.1 Fick's First Law

$$J = D_m\, dC_m/dx = D_m\, [\, C_m(0) - C_m(h)\,]\, /\, h$$

where J is the flux, in units of mol cm^2 s^{-1}, where $C_m(0)$ and $C_m(h)$ are the concentrations, in mol cm^{-3} units, of solute within the membrane at the two water-membrane interfaces (at positions x = 0 and x = h as shown in Figure f.1, where h is the thickness of the membrane in cm units), and where D_m is the diffusivity of the solute within the membrane, in units of cm^2 s^{-1}. At a steady state, the concentration gradient, dC_m/dx, within the membrane is linear, which is why the difference, Cm(d) - Cm(h), may be used in the right side of equation, Equation f.1.

Since one can estimate (or possibly measure) the distribution coefficients between bulk water and the membrane, K_p, is possible to convert equation, Equation f.1, into a more practical form,

Equation f.2

$$J = D_m K_p (C_D - C_A) / h$$

where the substitution of K_p allows the use of bulk water concentrations in the donor and acceptor compartments, C_D and C_A, respectively, Figure f.1.

These concentrations may be readily measured by standard techniques. PAMPA Evolution uses a scanning UV plate spectrophotometer to measure the concentrations at high speeds. In a further simplification, it is a common practice to combine several constants into one composite parameter, called “effective permeability,” P_e,

Equation f.3

$$Pe = Dm\ Kp / h$$

The relevance of equation, Equation f.2, (which predicts how quickly molecules pass through artificial membranes) to solubility of compounds comes in the concentration terms. Consider “sink” conditions, where C_A is essentially zero, as would be the case on the basolateral side of the epithelial cell barrier in the small intestine. Equation f.2, reduces to the following simplified flux equation

Equation f.4

$$J = P_e C_D$$

Flux depends on the product of effective permeability of the solute (which we may presume to be most likely the uncharged molecular species) times the concentration of the species at the water-side of the donor surface of the membrane. This concentration ideally would be equal to the dose of the drug, unless the dose exceeds the solubility limit, in which case it’s equal to the solubility. If only the uncharged molecular species permeates appreciably, then equation, Equation f.4, may be restated as

Equation f.5

$$J = P_o C_o \leq P_o S_o$$

where P_o and C_o are the intrinsic permeability and concentration of the uncharged species, respectively. The intrinsic permeability does not depend on pH, but its cofactor in the flux

equation, C_o, does (for ionizable molecules). The concentration of the uncharged species is equal to or less than the intrinsic solubility of the species, S_o.

Effective permeability, P_e, can be deduced in several ways, depending on experimental design and specific assumptions made. In permeability measurements using monolayers of cultured colonic cancer cells, **Caco-2**, is customary to use the expression

Equation f.6

$$P_e = \frac{V_D}{A M_D(0)} \left(\frac{\Delta M_A}{\Delta_t} \right)$$

where V_D is the donor well volume (0.2 cm^3 in our case), A is the filter area (0.3 cm^2 in our case), $M_D(0)$ is the sample amount (mol) in the donor well at the start of the assay (time=0), and ΔM_A is the amount of sample transferred to the acceptor compartment (but removed to maintain sink conditions) after an interval of time, Δt (in sec). $\Delta M_A / \Delta t$ is determined as the slope of the plot of M_A vs. t, evaluated after steady state isreached. This is called the "one-way flux" formula, which is valid under sink conditions.

If sink conditions are not assumed, the "two-way flux" equation is more complicated[7],

Equation f.7

$$C_A(t) = \left(\frac{M}{V_D + V_A} \right) + \left(C_A(0) - \frac{M}{V_D + V_A} \right) e^{-P_e A \left(\frac{1}{V_D} + \frac{1}{V_A}\right) t}$$

where M refers to the total amount (mol) of the drug in the system, CA(t) is the concentration of the drug (mol/cm^3) in the acceptor well at time t, and V_A is the volume of the acceptor well (0.2 cm^3 in our case).

Equations, Equation f.6 and Equation f.7, do not explicitly consider the effect of mass loss to the membrane (or cell monolayer). The equation used in this instrument is a modified version of eq. Equation f.7, where the total amount is not M, but M minus the amount of sample lost to the membrane.

Acceptor and donor concentrations are measured at two time points: at t = 0 and t =.05 -15 hr (permeation time). It is assumed that the time to reach steady state is short, relative to the total permeation time. In the limit of very vigorous stirring, the time to saturate the membrane with sample and reach the steady state is estimated to be about 10 sec. [5] For highly permeable compounds, if the microtitre plate solutions are not stirred and the permeation time is short, the permeability constant calculated from eq. Equation f.7 is underestimated.

The membrane retention can be substantial with lipophilic compounds, as much as 90% in some cases.[6] If this quantity is not taken into account, then eq.Equation f.7 underestimates Pe, depending on the lipophilicity of the solute. The mass lost to the membrane is estimated by measuring both the acceptor and the donor well sample amounts, and assuming that any amount less than the total mass introduced at the start is lost to the membrane. The assumption has been validated by control measurements, where the filters were not coated by the lipid solution. The membrane retention, R, is expressed as a mol% in the instrument software.

The permeability model used by PAMPA Evolution employs a modified form of equation, Equation f.7,corrected for membrane retention. A further correction to the equation may be appropriate if the permeation time is less than 3 hours (in the presence of stirring). For lipophilic molecules, there is a lag time before any compound appears in the acceptor wells, due to the partitioning of the compound into the membrane phase.

Imagine a tub being filled with water. A certain time is needed for the tub ("membrane") to fill (called steady-state time in PAMPA Evolution, t_{SS}), before the water spills out and the neighbor in the apartment on the floor below ("acceptor" compartment) notices a leak.

In the PAMPA Evolution model, the effective permeability constant cannot be measured until the t_{SS} time has passed.

Figures f.2 shows the concentration build-up of dihydromethysticin in the acceptor compartment (in units of the initial donor well concentration) as a function of time. The points represent actual measurements of concentrations and the solid curve represents the nonlinear fit of data with the permeability equation, Figure f.7, modified for non-zero t_{SS} and retention. The analysis indicates that it takes about a half an hour to fill up the membrane, before the acceptor compartment starts to receive the permeating solute. Measurements with several other compounds indicate that the typical t_{SS} is about 20 minutes, and the values roughly correlate with lipophilicity, as indicated in Figures f.3.

Figure f.2 The appearance concentration profile of dihydromethysticin.

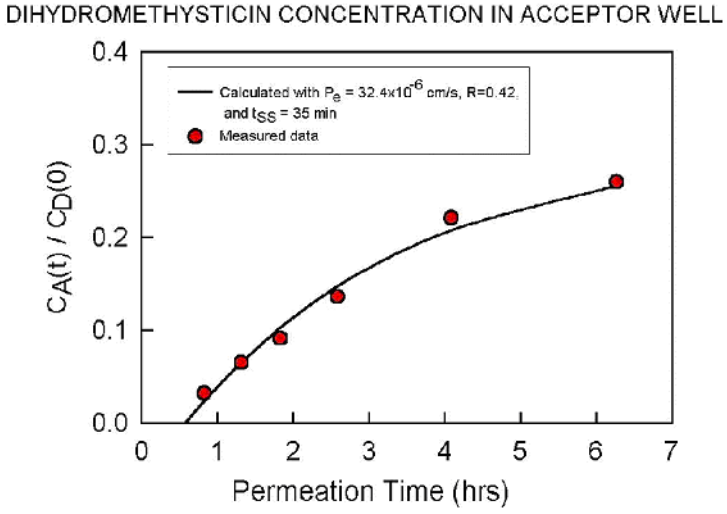
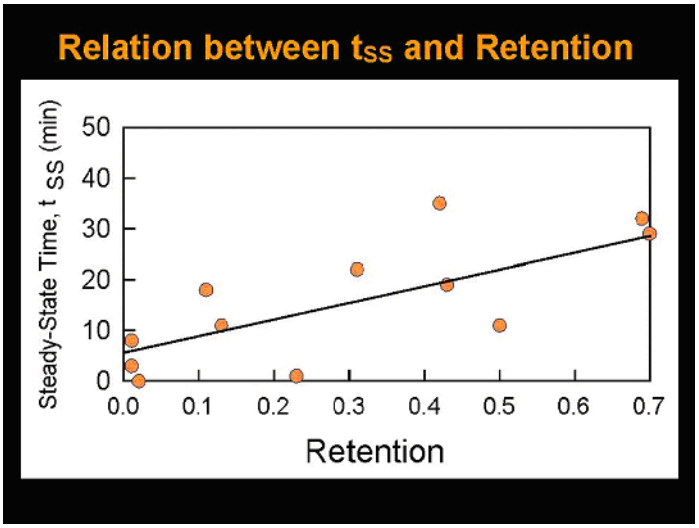


Figure f.3 Relationship between the membrane retention time, t_{ss}, and lipophilicity, as expressed by retention mass fraction.



Gradient Methods

The intestinal tract has a pH range from pH 5 – 8. The pH of the blood is constant at pH 7.4, therefore it is possible for a pH gradient to exist between the intestinal solution and the plasma that can affect the transport of ionizable molecules. A three-chamber differential equation can be derived, Equation f.8, that takes into account gradient-pH conditions and membrane retention of the drug molecule:

Equation f.8

$$P_e = -\frac{2.303 V_D}{A(t-\tau_{ss})}\left(\frac{1}{1+r_a}\right) \cdot \log_{10}\left[-r_a + \left(\frac{1+r_a}{1-R}\right) \cdot \frac{C_D(t)}{C_D(0)}\right]$$

V_D = volume of donor well

V_D = volume of acceptor well

t = permation time

R = retention

τ_{ss} = steady state time

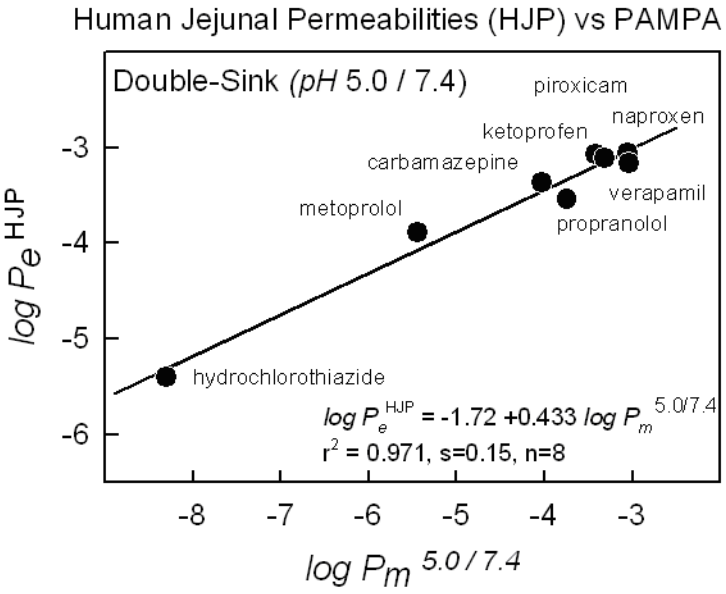
CD and CD = concentration in donor and acceptor well

Where

$$r_a = (V_D / V_A)\, P_e(A \rightarrow D) / P_e(D \rightarrow A)$$

More than 70 phospholipid compositions have been tested in the search for the "perfect" phospholipid combination to mimic human jejunal permeability values reported by Winiwarter et.al.[24]. The use of scavenger surfactant molecules in the receiver compartment along with the use of gradient-pH conditions, as well as a phospholipid mixture containing phosphotidylcholine, phosphotidylethanolamine, phosphotidyleinositol, and phosphatidic acid (net negative overall charge) produced a promising model, called Double-Sink PAMPA, see Figures f.4.

Figure f.4 Correlation between the human jejunal permeability and PAMPA under double sink conditions.

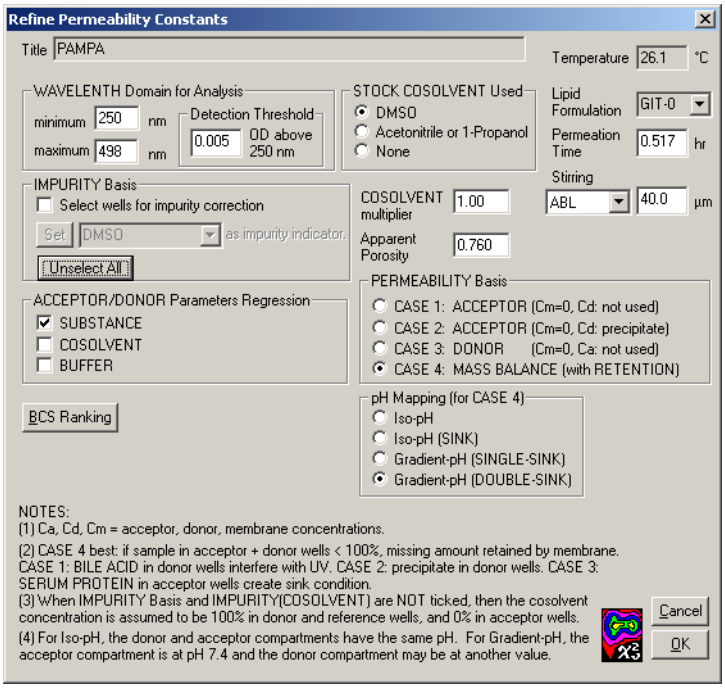


f.7 Definition of Permeability Units

We have seen that the permeability can be defined in at least four ways: sink-state equation Equation f.6, non-sink equation, Equation f.7, by the modified equation, Equation f.7, and by the pH gradient equation, Equation f.8, using the PAMPA Evolution instrument. This can be confusing, and it appears that not enough has been devoted to defining of terms in the literature. In the Refine Permeability Constants dialog box, shown in Figures f.5, there are four ways to calculate permeability constants, as indicated in the 'PERMEABILITY Basis' block: 'ACCEPTOR,' 'DONOR,' 'ACCEPTOR+DONOR,' and 'ACCEPTOR+DONOR+MEMBRANE.' The first two use an equation similar to Figure f.6. It

would only be valid to use such a definition for hydrophilic molecules, where very little membrane retention would be expected.

Figure f.5 Refine Permeability Constants Window.



We call such a constant ‘apparent' permeability, P_a. The third category represents a normalized apparent permeability basis, which is only valid for molecules showing no membrane retention. The distinction between the third case and the first two is that in the third case, mass balance is imposed. That is, if the acceptor and the donor concentrations do not add up to 100%, the individual concentrations are normalized to produce a 100% total.

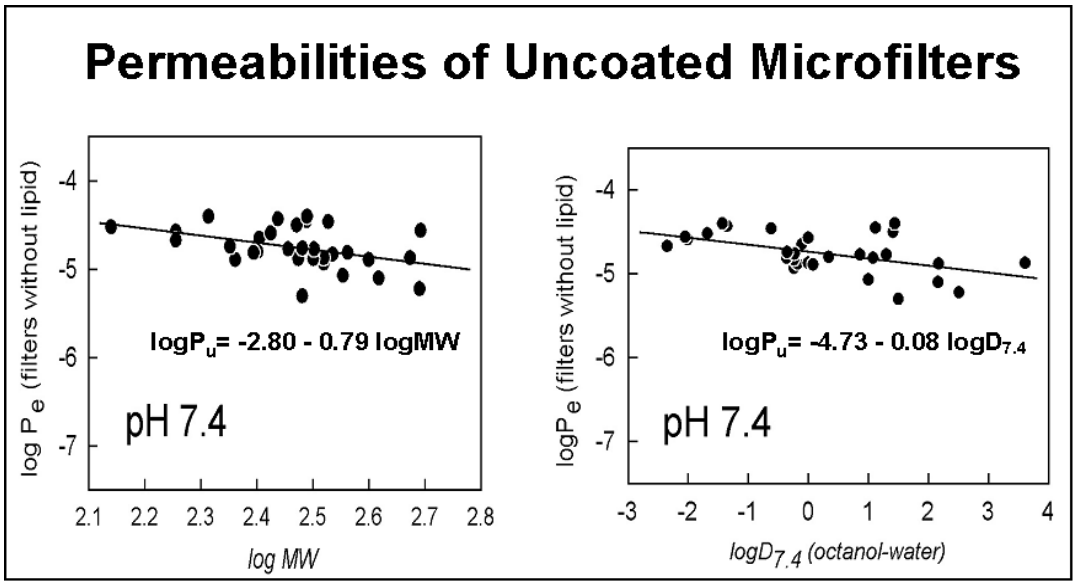
This case should not be used if the compounds may have a tendency to be retained by the membrane.

The fourth basis in the above scheme is preferred. In this basis, the membrane retention is determined as the difference between 100% and the sum of acceptor and donor concentrations. We call this the ‘effective' permeability, P_e.

There are two other fundamental definitions of permeability: ‘membrane' permeability, P_m, and ‘intrinsic' permeability, P_o. These cannot be easily measured directly, but are most often calculated. To consider ‘membrane' permeability, it is necessary to expand the simple Fick's first law model to include the unstirred water layers on the two sides of the membrane. The water layer adjacent to the membrane surface is unstirred, and molecules can only migrate across it by diffusion. Both charged and uncharged molecules migrate at about the same speed, provided they are of comparable size. Flux through the three-ply network (unstirred water - membrane - unstirred water) depends on resistance contributions from each of the lamella. In the case of highly-permeable molecules, the rate-limiting resistance to transport comes from the water layers and not the lipid membrane. It is for this reason that highly-

permeable molecules show nearly the same Pe, due principally to that of the unstirred water layer. It can be demonstrated that when the lipid coating is not applied to the filters, molecules covering a range of lipophilicities permeate the untreated filters at nearly the same high rates, as shown in Figures f.6.

Figure f.6 Permeabilities across un-coaded filters indicate the influence of the unstirred water layers.



Resistances in a series are additive. Since resistance is the inverse of permeability, inverse permeabilities are additive. So the relationship between the unstirred water layer, the membrane and the "net" effective permeabilities can be stated as

Equation f.9

$$1 / P_e = 1 / P_m + 1 / P_u$$

If the unstirred water layer permeabilities can be estimated, as in the case of Figures f.6, then one can use equation, Equation f.9, to derive the membrane component from the overall effective permeability.

Having the P_m value is not the end of the story when the molecule is ionizable. Values of P_m can depend on pH, because of the pH partition hypothesis. If the pKa of the molecule is known, then the P_m values can be corrected for ionization, to obtain the intrinsic P_o values, which represent the permeability of the uncharged species.

For simple monoprotic acids and bases:

Equation f.10

$$P_o = P_m (1 + 10 - {}^{pKa + pH}) \text{ for acids}$$

Equation f.11

$$P_o = P_m (1 + 10^{pKa - pH}) \text{ for bases}$$

The various proposed definitions of permeability are summarized in Table 6.1.

Table 6.1 Definition of permeability coefficients

Definitions	
Pa (apparent)	based on acceptor concentration (uncorrected)
Pe (effective)	P_a corrected for membrane retention, P_r $1/p_a = 1/P_e + 1/P_r$
P_m (membrane)	P_e corrected for unstirred water layer, P_u $1/P_e = 1/P_m + 1/P_u$
P_o (intrinsic)	P_m corrected for ionization
P_u (unstirred water layer)	Permeability through the unstirred water layer

Liquid Chromatography/Mass Spectrometry(LCMS)

g.1 Liquid Chromatography/Mass Spectrometry(LCMS) Data Import/Export

Introduction

PAMPA Evolution systems use UV spectrophotometry as a detection system for concentration measurements. The advantages of UV detection are speed and no required method for development. The reading of a 96-well plate takes 15 minutes as opposed to at least 6 hours by High Performance Liquid Chromatography(HPLC). However, UV has some inherent limitations: compounds with no UV chromophores, such as amino acids, cannot be detected, UV sensitivity is somewhat limited, and impurities or decomposition of compounds can affect results. In these areas LCMS and HPLC detection methods have distinctive advantages over UV methods because impurities or decompositions of compounds are separated out in LCMS and HPLC. This is important since combinatorial library compounds may contain up to 20% impurities, and, since their stability is usually unknown, the compounds could have decomposed during storage or during the assay itself. Also, with LCMS, cassette dosing can be used. This decreases assay time and thus increase the system throughput.

The PAMPA Evolution Command Software lends limited support to data collection and full support to data processing from LCMS systems as described below.

The LCMS interface allows microtitre plate preparation on the PAMPA Evolution platform and Excel file transfer of data between the LCMS system and the PAMPA Evolution Command software. This automates the inputting of compound information into the LCMS unit and allows data reduction and presentation in a manner consistent with pION's Client Report and Excel export formats.

Using data from an LCMS detection unit as input to the pION PAMPA Evolution Command software has been previously established through a combined publication effort between Waters Corporation, pION, and the University of Mississippi. The study demonstrated the use of the LCMS detection system to provide concentration data for the measurement of permeability on both individual samples and cassette-dosed multiple samples.

g.2 Data Collection

Processing of data collection from LCMS systems using the PAMPA Evolution facility can only take place after the four UV spectra have been collected and the UV data refined at least once.

It is recommended that only a few wells be repeated using LCMS because of the rather lengthy process time.

Even though only a few wells may be re-analyzed, the Excel data transfer file will contain name and location of all compounds in the plate. When imported into the PAMPA Evolution Command software, compounds without LCMS data will simply be ignored as far as the LCMS data processing is concerned.

g.3 Data Import

The PAMPA Evolution Command will automatically create a proper LCMS file in Excel format.

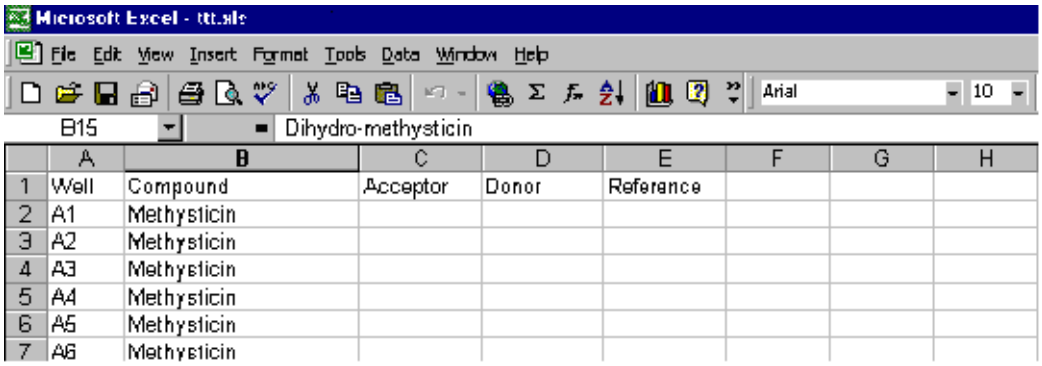
1. Click the **LCMS Button** choose a name for the file, and save it.

   An Excel file is created and has the "Well" and "Compound" columns filled in based on the data in the PAMPA Evolution file. In addition three more column headers are already named: "Acceptor," "Donor" and "Reference".

   The LCMS system may use this Excel spreadsheet as an input file regarding the names of the compounds and their locations in the sandwich plate. The LCMS operator must use it as the LCMS output file to communicate with the PAMPA Evolution Command software.

   The LCMS Excel worksheet is shown in Figure g.1.

Figure g.1 Organization of the Excel spreadsheet file used for LCMS data input and output.



	A	B	C	D	E	F	G	H
1	Well	Compound	Acceptor	Donor	Reference			
2	A1	Methysticin						
3	A2	Methysticin						
4	A3	Methysticin						
5	A4	Methysticin						
6	A5	Methysticin						
7	A6	Methysticin						

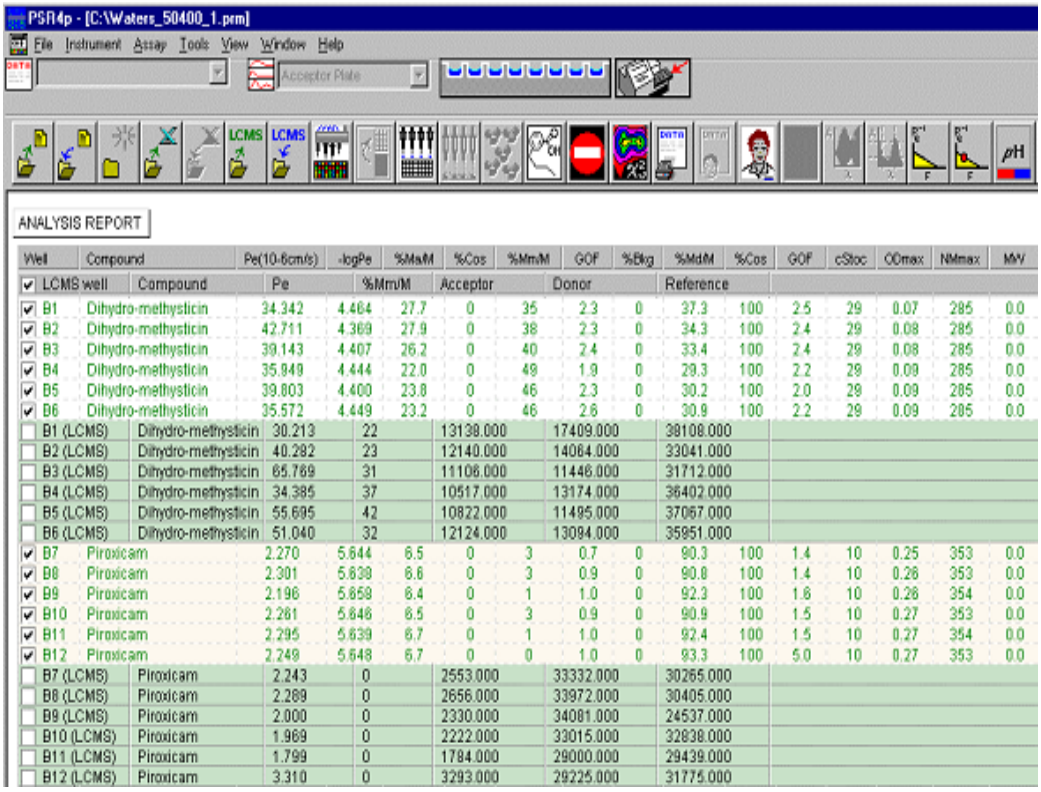
Column	Column Title?	Description
Column A	Well	Location of compound in the PAMPA sandwich
Column B	Compound	Compound Identification
Column C	Acceptor	Acceptor Concentration
Column D	Donor	Donor Concentration
Column E	Reference	Reference Concentration

To process LCMS data through the PAMPA Evolution Command software, for each compound to be processed "Acceptor," "Donor" and "Reference" column cells must be filled in with LCMS data proportional to the concentration of the compound in the corresponding plates. Integers and floating point numbers are acceptable.

2. Click the **LCMS Import** and select the proper spreadsheet file containing the collected data.

Permeability is calculated as described in the Results Section 6 using both UV and LCMS data. As shown in Figure g.2 below.

Figure g.2 Displays the results of UV data collection and imported LCMS measurements.

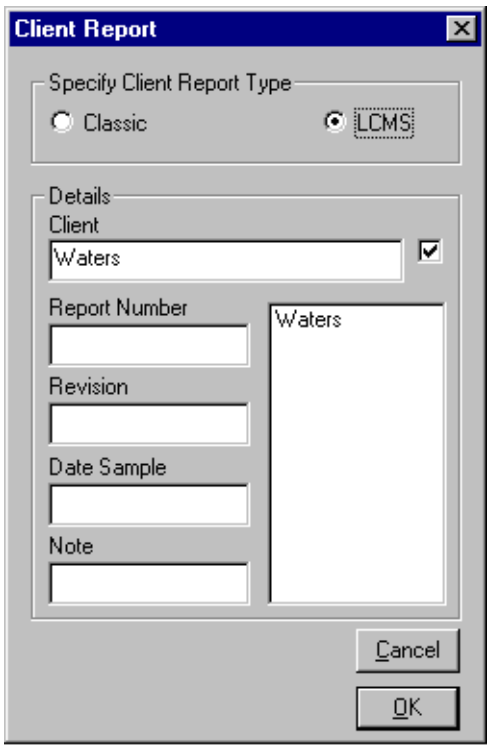


Well	Compound	Pe(10-6cm/s)	-logPe	%MaM	%Cos	%MmM	GOF	%Bkg	%MdM	%Cos	GOF	cStoc	OOmax	NMmax	MW
LCMS well	Compound	Pe		%MmM	Acceptor		Donor		Reference						
B1	Dihydro-methysticin	34.342	4.464	27.7	0	35	2.3	0	37.3	100	2.5	29	0.07	285	0.0
B2	Dihydro-methysticin	42.711	4.369	27.9	0	38	2.3	0	34.3	100	2.4	29	0.08	285	0.0
B3	Dihydro-methysticin	39.143	4.407	26.2	0	40	2.4	0	33.4	100	2.4	29	0.08	285	0.0
B4	Dihydro-methysticin	35.949	4.444	22.0	0	49	1.9	0	29.3	100	2.2	29	0.09	285	0.0
B5	Dihydro-methysticin	39.803	4.400	23.8	0	46	2.3	0	30.2	100	2.0	29	0.09	285	0.0
B6	Dihydro-methysticin	35.572	4.449	23.2	0	46	2.6	0	30.9	100	2.2	29	0.09	285	0.0
B1 (LCMS)	Dihydro-methysticin	30.213	22		13138.000		17409.000		38108.000						
B2 (LCMS)	Dihydro-methysticin	40.282	23		12140.000		14064.000		33041.000						
B3 (LCMS)	Dihydro-methysticin	65.769	31		11106.000		11446.000		31712.000						
B4 (LCMS)	Dihydro-methysticin	34.385	37		10517.000		13174.000		36402.000						
B5 (LCMS)	Dihydro-methysticin	55.695	42		10822.000		11495.000		37067.000						
B6 (LCMS)	Dihydro-methysticin	51.040	32		12124.000		13094.000		35951.000						
B7	Piroxicam	2.270	5.644	6.5	0	3	0.7	0	90.3	100	1.4	10	0.25	353	0.0
B8	Piroxicam	2.301	5.638	6.6	0	3	0.9	0	90.8	100	1.4	10	0.26	353	0.0
B9	Piroxicam	2.196	5.658	6.4	0	1	1.0	0	92.3	100	1.6	10	0.26	354	0.0
B10	Piroxicam	2.261	5.646	6.5	0	3	0.9	0	90.9	100	1.5	10	0.27	353	0.0
B11	Piroxicam	2.295	5.639	6.7	0	1	1.0	0	92.4	100	1.5	10	0.27	354	0.0
B12	Piroxicam	2.249	5.648	6.7	0	0	1.0	0	93.3	100	5.0	10	0.27	353	0.0
B7 (LCMS)	Piroxicam	2.243	0		2553.000		33332.000		30265.000						
B8 (LCMS)	Piroxicam	2.289	0		2656.000		33972.000		30405.000						
B9 (LCMS)	Piroxicam	2.000	0		2330.000		34081.000		24537.000						
B10 (LCMS)	Piroxicam	1.969	0		2222.000		33015.000		32838.000						
B11 (LCMS)	Piroxicam	1.799	0		1784.000		29000.000		29439.000						
B12 (LCMS)	Piroxicam	3.310	0		3293.000		29225.000		31775.000						

Notice the somewhat unusual table headers thatoccupy the first two rows of the table and the columns that appear not to be lined up. The grayed (green) LCMS rows belong to the second header row, while the lighter colored rows belong to the top header row.

3. Select the **LCMS** option, then click **OK** button on the Client Report dialog box, as shown Figure g.3 below, to get a printed Client Report based on the LCMS data.

Figure g.3 Selecting a Client Report based on LCMS data.

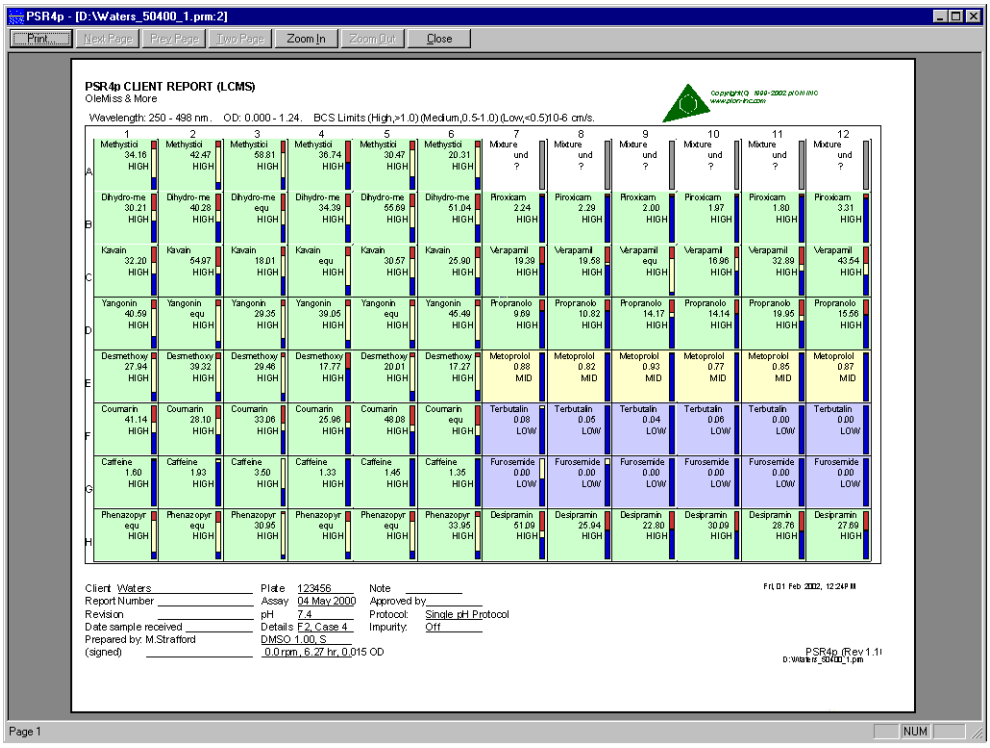


Additional information may be included in the Client Report based on entries in this dialog box.

For example, it may be advantageous to add some further information on the LCMS setup and/or a brief statement regarding the data collection in general.

Figure g.4 shows an example of a Client Report in the Print preview window based on LCMS data.

Figure g.4 Print Preview window for a Client Report based on LCMS data.



Structures Data File(SDF) Data Import Utility

h.1 Introduction

The use of the Microsoft© Excel spreadsheet program as a data communications tool for transferring compound data into the PAMPA Evolution Command software and for receiving the measured and processed data from the PAMPA Evolution Command software is described in Preparing an Input Excel File Section 4.4 and Export to Excel Section 6.2.

PAMPA Evolution Command v2.0 and higher provides support for display of molecular structures and data import support for the **SDF** and **MOL** Chemical Structure File Formats through a utility program called **SDF Wizard** included with the PAMPA Evolution Command software. This program is specifically designed to extract data and/or structures of up to 96 compounds at a time from one SDF file and identifying or assigning their specific locations in a 96-well microtitre stock plate.

The SDF Wizard parses the Structures Data File (SDF) and Molecular Design (MOL) files and enters their output data into an Excel spreadsheet to be used by the PAMPA Evolution Command software for data input/output.

NOTE These are called CTfiles, Chemical Table files.

MOL-files include just one compound name and its 2-D molecular structure data. The SDF files contains additional data pertaining to the compound. They typically include many compounds. Such files may become quite large in size. The SDF Wizard can handle large files sizes without any problems.

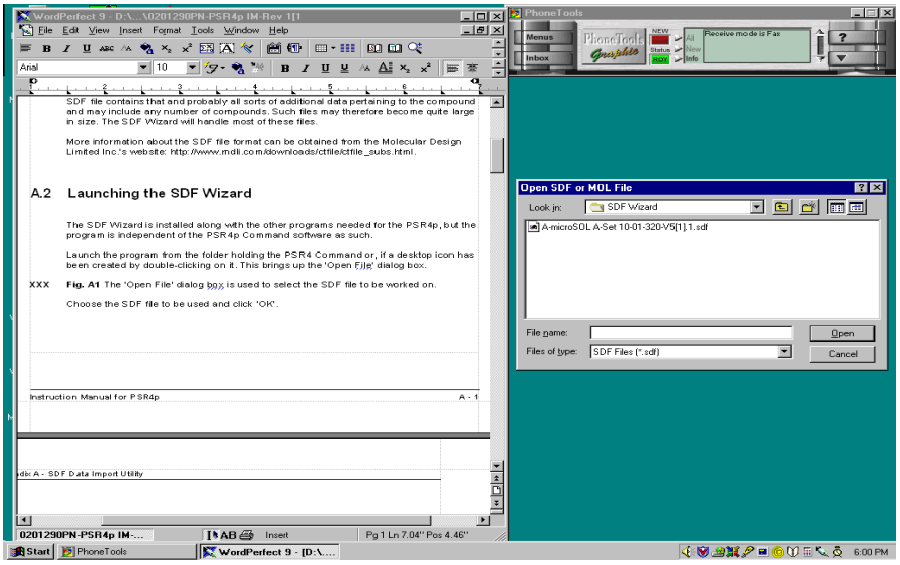
More information about the SDF file format can be obtained from the Molecular Design Limited Inc.'s website: http://www.mdl.com/solutions/white_papers/ctfile_formats.jsp.

h.2 Launching the SDF Wizard

The SDF Wizard is installed along with the other programs needed for the PAMPA Evolution. This program is independent of the PAMPA Evolution Command software.

1. Launch the program from the windows **Start | All Programs | Pion Software-SDFWizard** or, double-click the desktop icon. This displays the **Open SDF or MOL File** dialog box used to select an SDF file. As shown in Figure h.1 below.

Figure h.1 Open SDF or MOL File dialog box

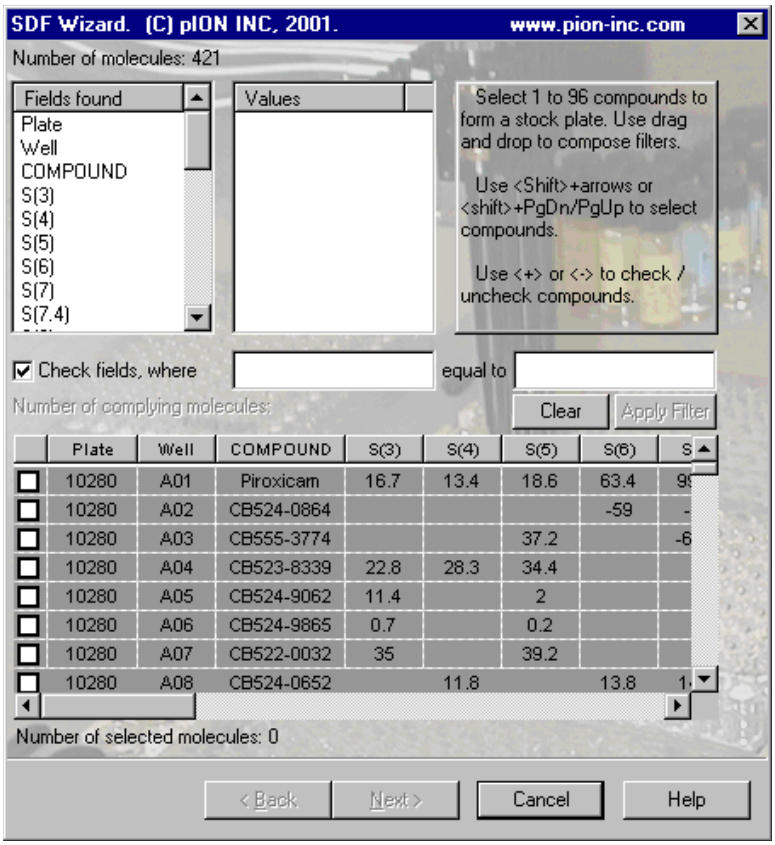


2. Choose the SDF file to use and click the **Open** button.

h.3 Extracting Data From the SDF File

The SDF Wizard selection dialog box displays. As shown in Figure h.2 below.

Figure h.2 Select Dialog Box



This dialog box selects the compounds that are included in the stock plate of compounds analyze. It is full of information regarding what is available in the SDF file.

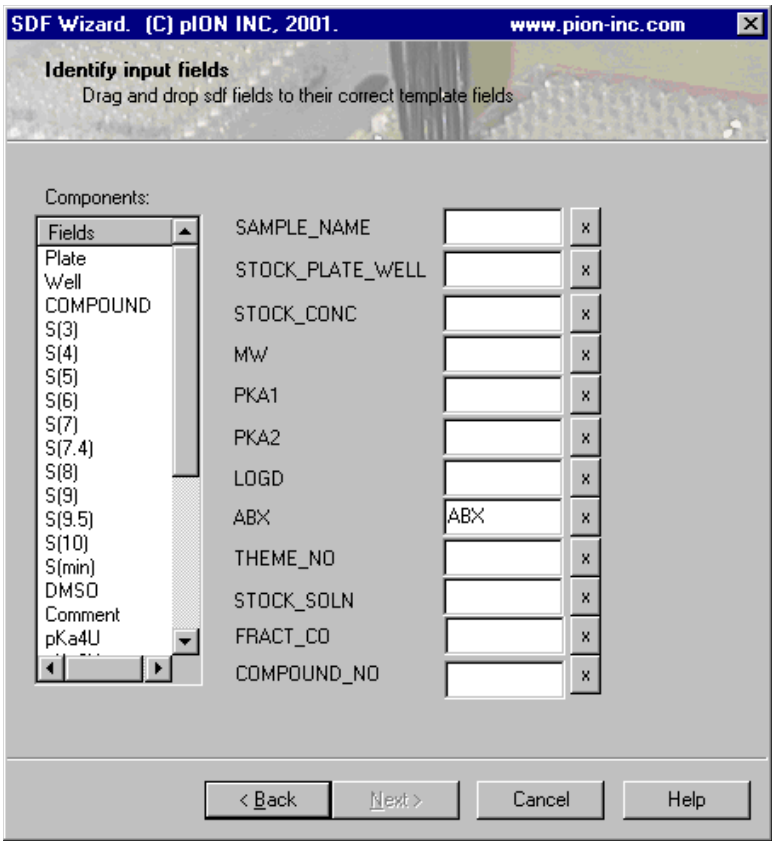
- The first line shows **Number of molecules:** with the number of compound records found in the SDF file indicated.
- The **Fields found** is a list of all the field names found in the file. Each compound may not necessarily contain all these fields or have data included in all of them.
- The **Values** listing is empty if no field has been highlighted. If the **Compound** is clicked in the **Fields found** list, all the compound names (alphanumeric) in the SDF file is shown in the **Values** list box as seen in Figures h.2.
- This table displays the data field structure of the SDF file, as well as the data recorded in each field. Each line is a record containing information for only one molecule. The width of the table columns may be adjusted by dragging.
- To select molecules for the plate, select the check box in front of the appropriate records. When a box is selected, the entire table row turns white. Do this on a molecule-by-molecule basis or to select all the records, press SHIFT+END. Press the + key to check all or the - key to uncheck all the records.

- This table cannot be sorted. It can be filtered as explained below using the two filter condition boxes above the table. All matching records are automatically found and checked.
    1) Select the check box in front of **Check fields, where** label must be checked. If it is not checked, it becomes an **Uncheck fields, where** selector for fields to uncheck.
    2) From the **Fields found** list, drag the name of the field that is used for filtering into the first box.
    3) From the **Values** list, which will now contain valid values, drag the name of the condition to be used into the second box.
    4) The amount of compounds in the SDF file that comply with the filter condition, is now indicated just above the table.
    5) Click the **Apply filter** button to check all the records according to the entered filter condition.
    6) Below the table, the **Number of selected molecules** indicator shows the number of molecules currently selected.
    7) The filter may be applied more than once. More molecules may be included by running an additional filter with the **Check fields, where** box checked. Fewer molecules may be selected by running a new filter with the box unchecked (**Uncheck fields, where**).
    8) When the number of selected molecules is in the range 1 to 96, the **Next** button becomes available. When satisfied with the selection, click it. Use the **Back** button in the next dialog box to return to this screen to make changes.

NOTE If an error is made at this step, it is not a problem. Throughout this program it is possible to return to previous steps to make corrections, by using the **Back** button. This is true even after the Excel spreadsheet has been created.

A new dialog box displays. The purpose of this dialog is to associate field names found in the SDF file with column names used in the PAMPA Evolution Excel input file. Exact names found in both, are shown when the dialog box is opened. As shown inFigure h.3 below.

Figure h.3 Association Dialog Box



In order to proceed, at least the **SAMPLE_NAME** association must be made, but other associations may be needed, too, for a meaningful data import to take place. Drag-and-drop works as well. When a **SAMPLE_NAME** association has been made, the **Next** button becomes available.

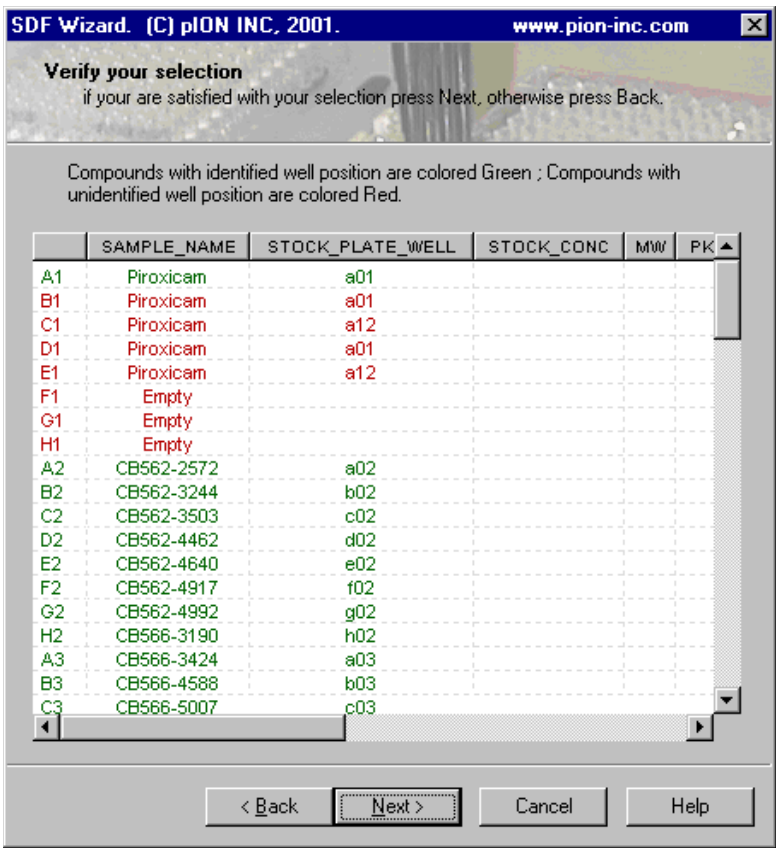
3. From the **Fields** column, drag the field name that corresponds to **SAMPLE_NAME** into the open box. If a wrong move is made, use the **x** button to clear the box and start over or simply drag a new name into the box.
4. Continue with the assignments until done.

The **STOCK_PLATE_WELL** assignment is particularly important if it is available in the SDF file along with a plate identifier, because it indicates the location of the compound in the plate. If not assigned, the SDF Wizard will automatically assign the location row-by-row, column-by-column according to the order in which the compounds are found in the SDF file.

5. When all assignments have been made, click the **Next** button to continue.
6. Use the **Back** button in the next dialog box to return to this screen to make changes.

The next dialog box, Figure h.4, is a preview of the selected data as it will look when imported into the Excel spreadsheet.

Figure h.4 Excel preview dialog box



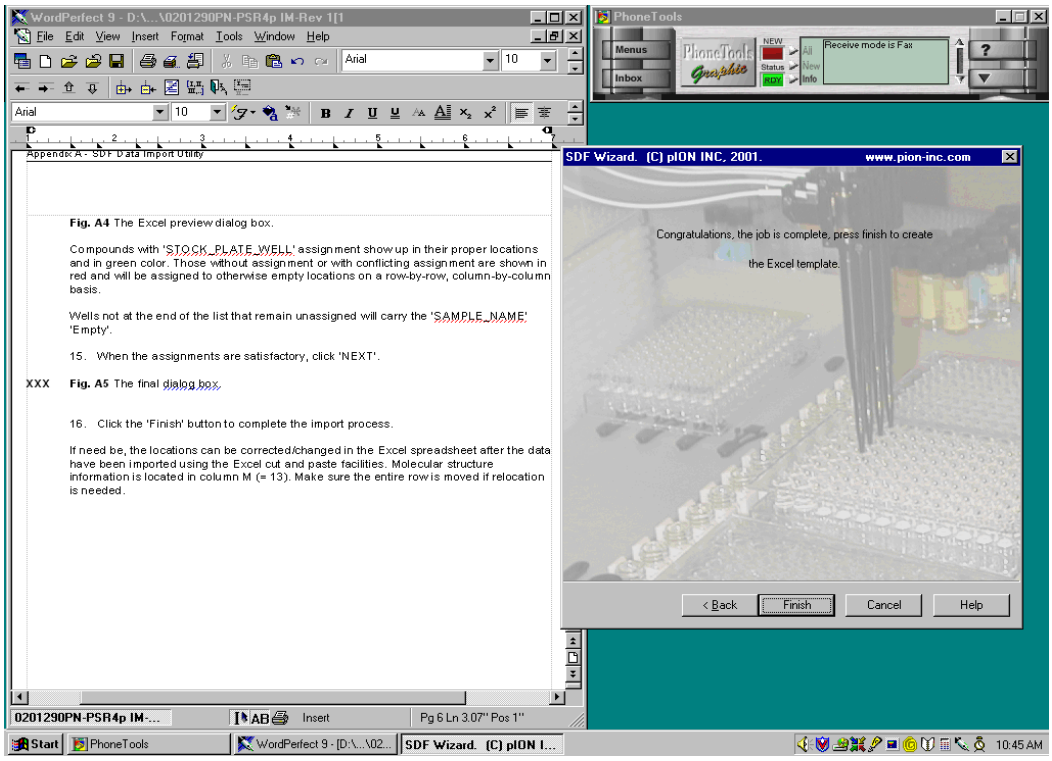
Compounds with **STOCK_PLATE_WELL** assignment show up in their proper locations and in green color. Those without assignment or with conflicting assignment are shown in red and is assigned to otherwise empty locations on a row-by-row, column-by-column basis.

Wells that are NOT at end of the list and remain unassigned, will use “Empty” for the **SAMPLE_NAME**. For PAMPA Evolution to understand the well assignments, there must be no gaps in the list of compounds in column B, but open wells in the beginning or at the end are OK. Wells not occupied by the SDF file compounds may be filled by the user with additional compounds. For proper working of the sample preparation system, no wells should be left empty, except if located at the beginning or end of the list.

This housekeeping task should be done after the Excel spreadsheet is created. Otherwise empty wells should be filled manually with 100%DMSO before the stock plate is presented to the PAMPA Evolution. The final spreadsheet must reflect the contents of the stock plate.

7. When the assignments are satisfactory, click **NEXT**. The last SDF Wizard page is displayed.
8. Click the **Finish** button to complete the import process. As shown in Figure h.5 below.

Figure h.5 Final dialog box



The Excel worksheet is created.

If needed, the assignment of the compounds can be changed after the data have been imported using Excel's cut and paste functions. If additional compounds are to be added, their names and data must be typed into the proper spreadsheet cells. The word "Empty" may be deleted from the beginning of the plate until the first compound is available, all other instances must be properly resolved by adding a compound in the empty well or by filling it with DMSO. The final spreadsheet must reflect the contents of the stock plate.

Molecular structure information is located in column M (= 13). Make sure the entire row is moved if relocation is needed.

9. Save the Excel workbook with an appropriate name in a proper folder.

The SDF Wizard stays active with the current selections preserved. If the Excel worksheet is not to your liking, simply delete it and return to the SDF Wizard to make changes before creating a new workbook.

The SDF Wizard may be closed later, click the **Cancel** button.

After processing the samples, the PAMPA Evolution output table may be saved as an SDF file. This file will also include the structure data. Please refer to Introduction Section h.1 for information on structure data.

h.4 Viewing Structures

It is possible to see molecule structures in the PAMPA/µSOL Evolution Command Software at the time of calculation and analyzing the results.

Molecule structures can be loaded into data files either from the initial excel spreadsheet, see Getting Started Section 4 and Operation Section 5,or they can be brought in.

NOTE The software component called **Accord Chemistry Control** from Accelrys Inc., located at (http://www.accelrys.com/accord/chemcont.html, must be installed on the computer to enable molecule structure visualization.

h.5 Inserting Structures in the Excel Import File

Column "M" of the Input Excel file is reserved for structures of the molecules. Structures can be entered either in the form of Simplified Molecular Input Line Entry System (SMILES) string or by inserting appropriate *.mol file in the corresponding cell of the Excel spreadsheet. See the Structures Data File(SDF) Data Import Utility Section h for the information on how to create Input Excel file from the ".sdf" file containing chemical structures of the molecules.

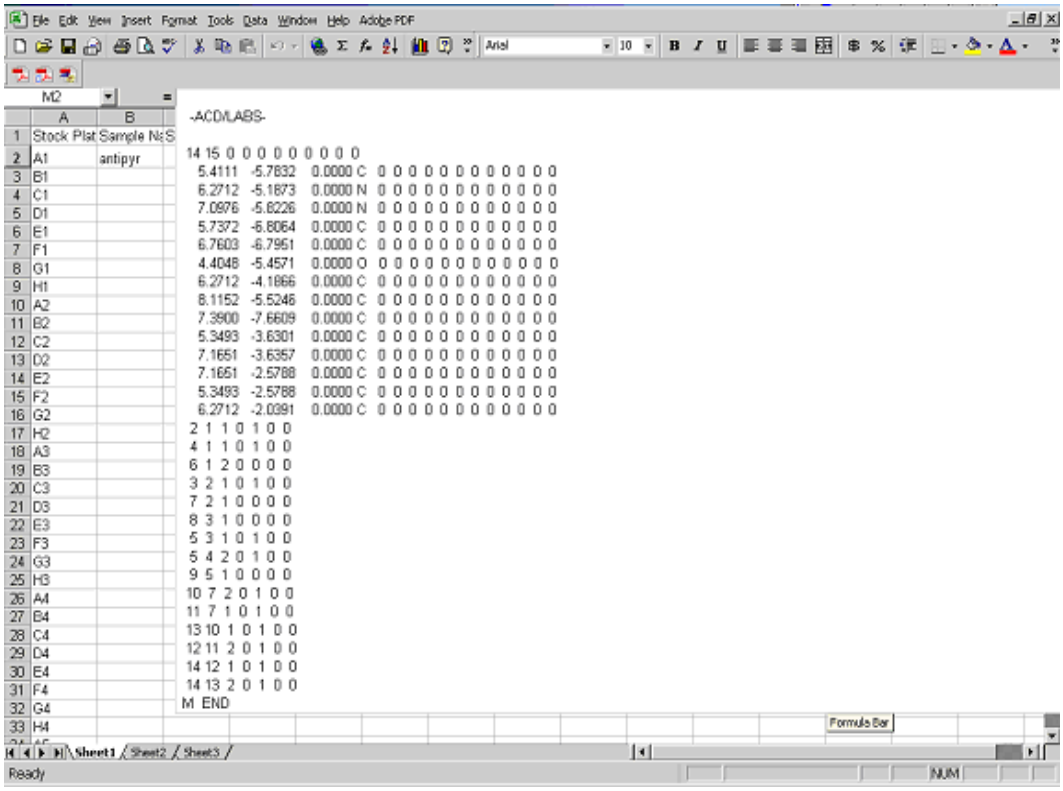
If the SMILES string is used as a source of chemical structure the string nuts be typed in.

For inserting chemical structure from MDL's "*.mol" file the SDF Wizard utility, as described in Structures Data File(SDF) Data Import Utility Section h, is usedused.

10. Launch SDF Wizard program going to **Start | Programs | pION Software | SDF Wizard**.
11. In the **Open SDF or MOL File** dialog box browse to the appropriate file containing the chemical structure (or structures if it is a "*.sdf" file).

12. Place a check mark in front of molecule, see Figure h.6, and press **Next** button several times until it changes into the **Finish** button. Press **Finish**. Microsoft Excel application will open with structure of the molecule(s) in the column M.

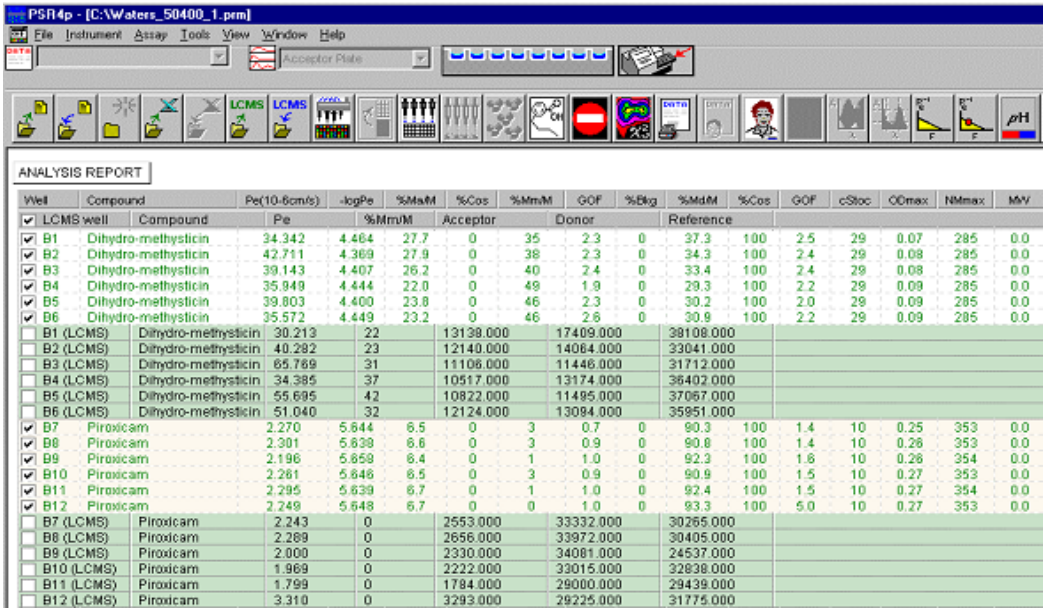
Figure h.6 SDF Wizard Utility Program



13. In the Microsoft Excel application go to menu File and choose **Hide structure** option.

14. Click on the spreadsheet cell containing the structure. For example, well M2 as inFigure h.7. Go to menu **Edit | Copy**.

Figure h.7 Example of the Excel file created by the SDF Wizard.



Well	Compound	Pe(10-6cm/s)	-logPe	%Ma/M	%Cos	%Mm/M	GOF	%Bkg	%Md/M	%Cos	GOF	cStoc	cOmax	NMmax	MV
LCMS well	Compound	Pe		%Mm/M	Acceptor		Donor		Reference						
B1	Dihydro-methysticin	34.342	4.464	27.7	0	35	2.3	0	37.3	100	2.5	29	0.07	285	0.0
B2	Dihydro-methysticin	42.711	4.369	27.9	0	38	2.3	0	34.3	100	2.4	29	0.08	285	0.0
B3	Dihydro-methysticin	39.143	4.407	26.2	0	40	2.4	0	33.4	100	2.4	29	0.08	285	0.0
B4	Dihydro-methysticin	35.949	4.444	22.0	0	49	1.9	0	29.3	100	2.2	29	0.09	285	0.0
B5	Dihydro-methysticin	39.803	4.400	23.8	0	46	2.3	0	30.2	100	2.0	29	0.09	285	0.0
B6	Dihydro-methysticin	35.572	4.449	23.2	0	46	2.6	0	30.9	100	2.2	29	0.09	285	0.0
B1 (LCMS)	Dihydro-methysticin	30.213		22	13138.000		17409.000		38108.000						
B2 (LCMS)	Dihydro-methysticin	40.282		23	12140.000		14064.000		33041.000						
B3 (LCMS)	Dihydro-methysticin	65.769		31	11106.000		11446.000		31712.000						
B4 (LCMS)	Dihydro-methysticin	34.385		37	10517.000		13174.000		36402.000						
B5 (LCMS)	Dihydro-methysticin	55.695		42	10822.000		11495.000		37067.000						
B6 (LCMS)	Dihydro-methysticin	51.040		32	12124.000		13094.000		35951.000						
B7	Piroxicam	2.270	5.644	6.5	0	3	0.7	0	90.3	100	1.4	10	0.25	353	0.0
B8	Piroxicam	2.301	5.638	6.6	0	3	0.9	0	90.8	100	1.4	10	0.26	353	0.0
B9	Piroxicam	2.196	5.658	6.4	0	1	1.0	0	92.3	100	1.6	10	0.26	354	0.0
B10	Piroxicam	2.261	5.646	6.5	0	3	0.9	0	90.9	100	1.5	10	0.27	353	0.0
B11	Piroxicam	2.295	5.639	6.7	0	1	1.0	0	92.4	100	1.5	10	0.27	354	0.0
B12	Piroxicam	2.249	5.648	6.7	0	0	1.0	0	93.3	100	5.0	10	0.27	353	0.0
B7 (LCMS)	Piroxicam	2.243		0	2553.000		33332.000		30265.000						
B8 (LCMS)	Piroxicam	2.289		0	2656.000		33972.000		30405.000						
B9 (LCMS)	Piroxicam	2.000		0	2330.000		34081.000		24537.000						
B10 (LCMS)	Piroxicam	1.969		0	2222.000		33015.000		32838.000						
B11 (LCMS)	Piroxicam	1.799		0	1784.000		29000.000		29439.000						
B12 (LCMS)	Piroxicam	3.310		0	3293.000		29225.000		31775.000						

NOTE Do not be confused by the amount of text in the cell. All this text contains information about atoms and their positions in the molecule!

15. In the Microsoft Excel application open the Input Excel file used to start the PAMPA assay where the structure is to be entered.
16. Click in the cell in column M and in the row with the name of the molecule structure which is to paste.
17. Go to menu **Edit | Paste**.
18. Save the Input Excel file.

NOTE Excel will automatically extend the cell's height to accommodate all text.

To bring it to the normal height (16) drag the bottom border of the cell or highlight the entire row by clicking on the row number right-click mouse and set the row height to 16. When the Excel file is browsed from the Evolution software, the information about chemical structures gets imported into the permeability or solubility data file.

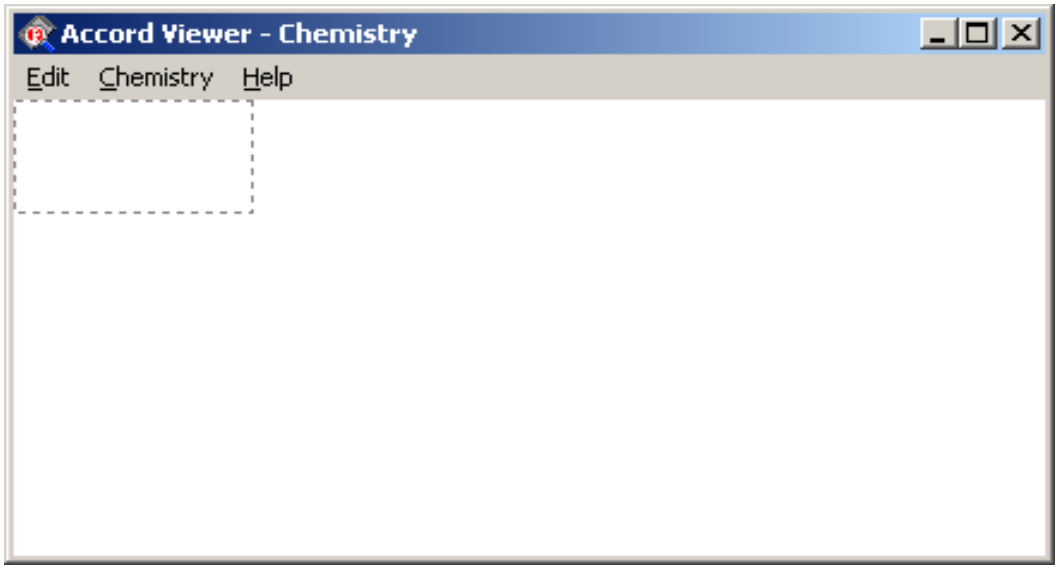
h.6 Inserting Chemical Structures into Completed Data Files

Chemical structures can be introduced into data files when the UV data collection is finished and permeability (solubility) constants have been refined at least once.

1. In the Results Table view of PAMPA (µSOL) Evolution software highlight one of the rows with the compound of interest by clicking on the name of compound.

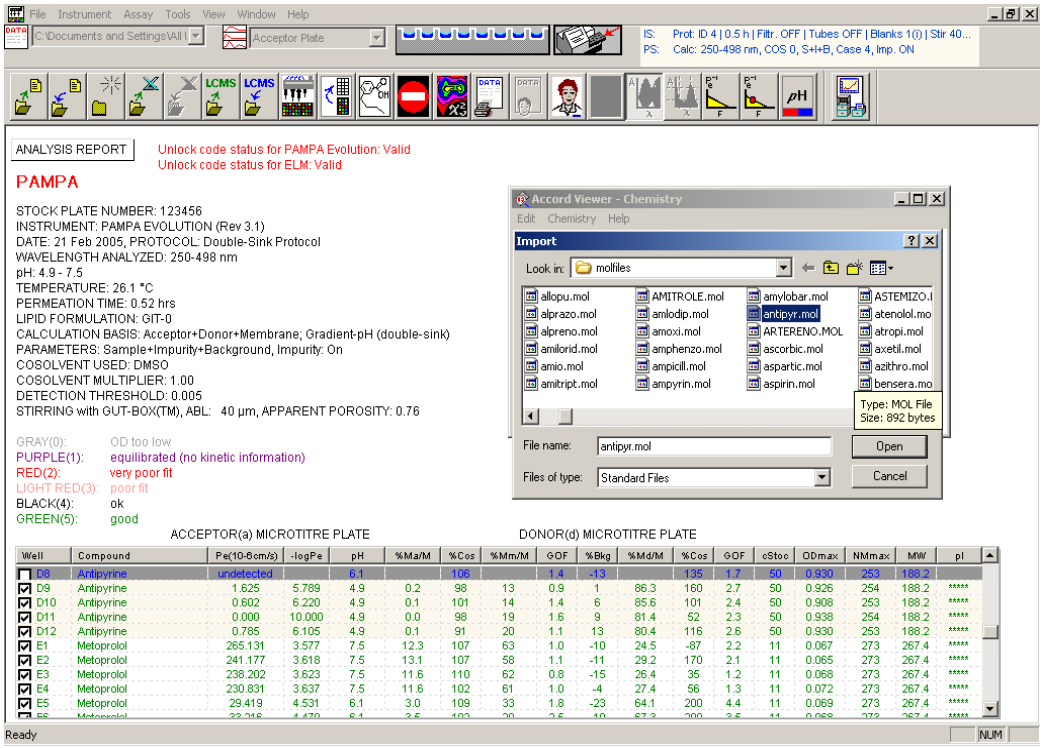
2. Right-click the mouse and select **Edit Chemical Structure** from the drop-down menu. The **Accord Viewer** window displays at the top-right corner of the screen, see Figure h.8.

Figure h.8 Accord Viewer Window.



3. Copy the chemical structure from one of the chemical editors or viewers. Go to menu Edit of the Accord Viewer and choose **Paste**. Alternatively, go to menu Chemistry and choose **Import,** as in Figure h.9.

Figure h.9 Import capability of the Accord Viewer supports a variety of different chemical formats.



4. Close the Accord Viewer to accept the structure.
5. Save the data file. On the main menu bar, select **File | Save**, or click the **Save** button on the PAMPA toolbar.

The structure of the molecule can be seen at this point either in the Result Table view while rolling the mouse over the table of results, see Figure h.10, or in the Spectra Details view, see Figure h.11.

Figure h.10 Molecule structure visualization in the Result Table view.

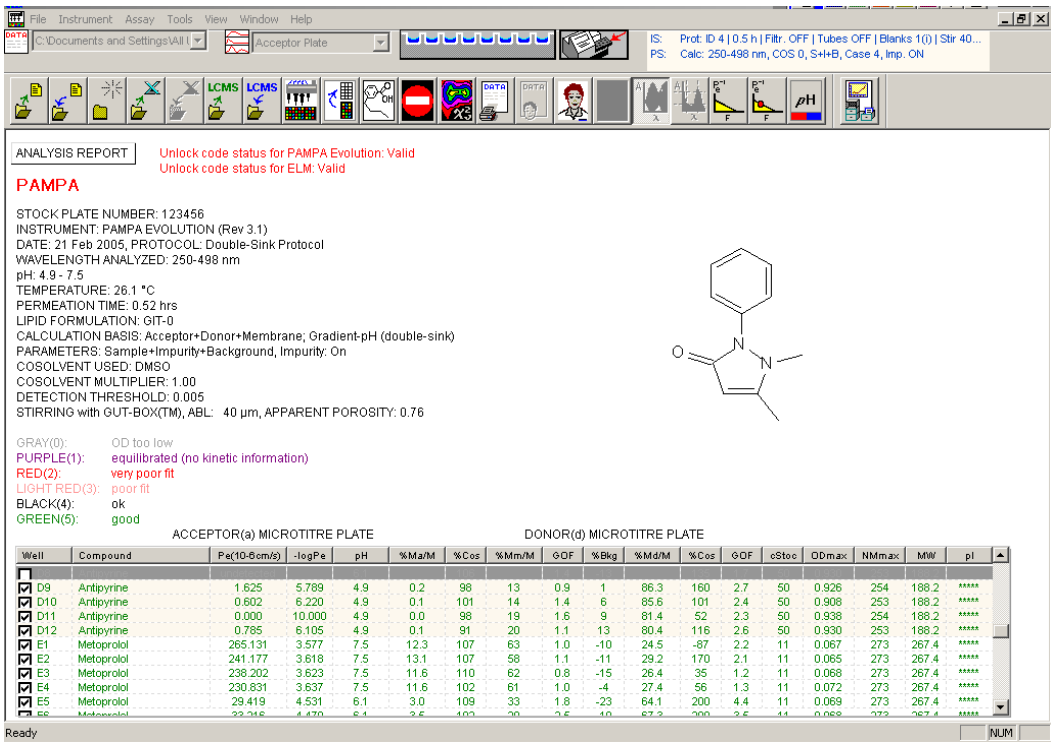


Figure h.11 Structure visualization in the Spectra Details view.