Technical Note

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Title: **Automation of PAMPA-Lipid using a MultiScreen-IP® PAMPA Filter Plate on a Sciclone™ ALH 3000 Advanced Liquid Handler Workstation**

Introduction

PAMPA-Lipid (**P**arallel **A**rtificial **M**embrane **P**ermeation **A**ssay) is a non-cell based assay that utilizes a 96-well filter plate.[1, 2] The PAMPA assay is designed to predict passive transcellular permeability of drugs in early drug discovery. The assay is carried out in a 96-well MultiScreen-IP PAMPA filter plate and measures the ability of compounds to diffuse from a donor to an acceptor compartment separated by a PVDF membrane pretreated with a lipid containing solvent. The protocol is robust and easy to automate when using lipids from Avanti or pION Inc.

A lipid artificial membrane is applied to the PVDF membrane in a 96-well filter plate (Donor plate). The Donor plate is filled with buffer solutions containing the compounds to be tested. The Donor plate is placed in a 96-well Acceptor plate filled with sufficient buffer to ensure liquid contact between the liquid in the Acceptor plate and the PVDF membrane. The Donor and Acceptor plates are incubated together for 16 hours after which time the Donor plate is removed from the Acceptor plate. Samples from the Acceptor plate are analyzed by LC/MS or transferred to a UV compatible 96 well plate and analyzed immediately in a UV/Vis spectrophotometer. An equilibrium plate (compounds at the theoretical equilibrium, i.e. the resulting concentration if the donor and the acceptor solutions are combined) is also created and analyzed. This equilibrium plate is used to calculate the permeability rate (Log P_e) of the drugs. At the end of the incubation time, the integrity of the artificial membrane layer can be measured using electrical resistance. Automation of PAMPA-Lipid on the Sciclone ALH 3000 workstation takes about 15 minutes (this does not include the 16 hours incubation). This time includes the formation of the artificial membrane, dilution of compounds from a mother plate, addition of compounds to the Donor plate, creating the equilibrium plate for analysis and removal of samples for UV/Vis analysis from the Acceptor plate.

Note: Refer to Tech Note #'s AN1728EN00, AN1729EN00 and PC040EN00 for more detailed information on running the PAMPA-Lipid.

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In the US and Canada call toll free 1-800-Millipore (645-5476)
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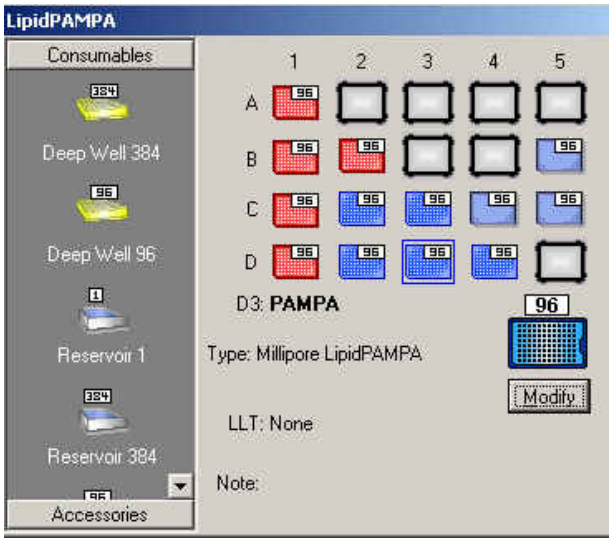
Configuration of the Sciclone ALH 3000 Deck for LipidPAMPA Method

Membrane formation, drug addition to plate and incubation



Important:

- Program created using Sciclone Software V 3.4.24 on a Sciclone ALH 3000 Workstation with a right side gripper.

Prior to starting the program *LipidPAMPA* (which includes the subroutine methods *CreateMembrane* and *PlatesTogether*), make sure the deck configuration is as follows:

Column 1:

A1. 200 µL pipette tips (Adding DMSO/buffer to acceptor plate)
B1. 200 µL pipette tips (Adding buffer to daughter plate)
C1. 200 µL pipette tips (Transfering samples from mother plate to daughter plate)
D1. 200 µL pipette tips (Creating membrane: uses 8 tips from last column of rack)

Column 2:

B2. Upside down pipette rack cover to collect ejected tips used in membrane creation
C2. Mother plate (v-bottom polypropylene with cover on)
D2. Lid for MultiScreen PAMPA filter plate

Column 3:

C3. Daughter plate (v-bottom polypropylene with cover on)
D3. MultiScreen PAMPA filter plate (in a single well cell culture tray)

Column 4:

C4. Reservoir – 12 column (lipid in first column)
D4. MultiScreen Acceptor plate

Column 5:

B5. Reservoir – standard (DMSO/buffer)
C5. Reservoir – standard (PBS buffer)

Procedure (*LipidPAMPA*):

1. *Create Membrane* (subroutine within the method):

    a. The reservoir used in this program is a low profile, 12 column reservoir located at position C4. The lipid (2 – 3 mL) is placed in the first column of the reservoir.
    b. Distribute 5 µL aliquots (2 aspirations with multi-dispense) of lipid (C4) to each well of the filter plate (D3) with the Membrane Tips (D1) using the 96 head. The head only picks up 8 tips (column 12 of rack) with column 1 of the 96 head.
    c. Eject 8 tips (B2).

2. Distribute 300 µL aliquots of DMSO/buffer (B5) to the acceptor plate (D4) using the DMSO Buffer Tips (A1).

3. Distribute 285 µL aliquots of PBS buffer (C5) to the daughter plate (C3) using the Buffer Tips (B1). The gripper will remove and hold the cover of the daughter plate (C3) while dispensing into the plate, then replace lid.

4. Transfer 15 µL of drug compound from the mother plate (C2) to the daughter plate (C3) using the Sample Tips (C1). Mix in the daughter plate 5 times with a volume of 195 µL. The gripper will remove and hold the covers of both the mother plate (C2) and the daughter plate (C3) while aspirating and dispensing into the plates, then replace lid.

5. Transfer 150 µL from the daughter plate (C3) to the filter plate (D3) using the Sample Tips (C1). The gripper will hold the cover of the daughter plate (C3) while aspirating out of the plate, then replace lid.

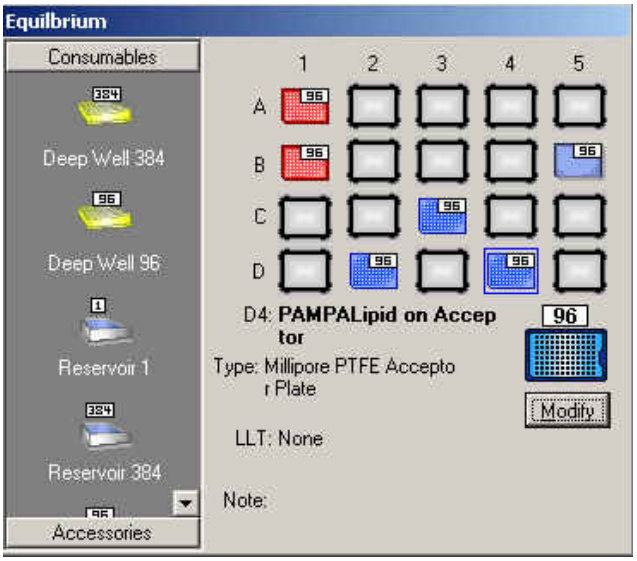
6. *PlatesTogether* (subroutine within the method):

    a. Move the filter plate (D3) to the acceptor plate (D4) at a slow speed.
    b. Move the cover (D2) on top of the filter plate (D3)

7. Manually place filter plate with acceptor plate into a plastic zip lock bag and incubate for 16 hours at room temperature.

Configuration of the Sciclone ALH 3000 Deck for LipidPAMPA-Equilb Method

Creation of Equilibrium Plate



Important:

- Program created using Sciclone Software V 3.4.24 on a Sciclone ALH 3000 Workstation with a right side gripper.

Prior to starting the program *LipidPAMPA_Equilb*, make sure the deck configuration is as follows:

Column 1:

A1. 200 µL pipette tips (Adding sample to UV 96-well analysis plate)
B1. 200 µL pipette tips (Adding buffer to UV 96-well analysis plate)

Column 2:

D2. UV 96-well analysis plate for the equilibrium plate

Column 3:

C3. Daughter plate (v-bottom polypropylene with cover on)

Column 4:

D4. MultiScreen PAMPA filter plate assembled with the MultiScreen Acceptor plate (from previous program)

Column 5:

B5. Reservoir – standard (DMSO/ buffer)

Procedure (*LipidPAMPA_Equilb*):
***Note this protocol <u>can be</u> run while the 16 hour incubation is occurring.**

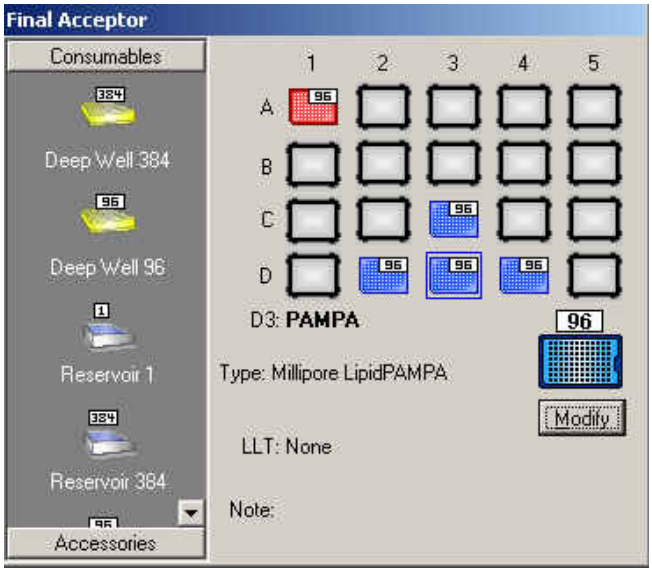
1. Transfer 80 µL from the daughter plate (C3) to the UV 96-well analysis plate (equilibrium plate) at position D2 using the Equilibrium Tips (A1). The gripper will remove and hold the cover of the daughter plate (C3) while aspirating out of the plate.

2. Distribute 170 µL aliquots of DMSO/buffer (B5) to the UV 96-well analysis plate (D2) using the Buffer Tips (B1). Mix 5 times with a volume of 190 µL.

3. Remove the UV 96-well analysis plate (D2) and analyze with an UV/Vis microplate spectrophotometer.

Configuration of the Sciclone ALH 3000 Deck for Final Acceptor Method

Creation of Final Acceptor Plate



Important:

- Program created using Sciclone Software V 3.4.24 on a Sciclone ALH 3000 Workstation with a right side gripper.

Prior to starting the program *Final Acceptor* (which includes the subroutine *Plates Apart*), make sure the deck configuration is as follows:

Column 1:

A1. 200 µL pipette tips (Adding sample from acceptor plate to UV 96-well analysis plate)

Column 2:

D2. Lid for MultiScreen PAMPA filter plate (will be placed here during the run)

Column 3:

C3. UV 96-well analysis plate for the final acceptor plate
D3. Single well cell culture tray for MultiScreen PAMPA filter plate

Column 4:

D4. MultiScreen PAMPA filter plate assembled with the MultiScreen Acceptor plate (from 16 hour incubation)

Procedure (*Final Acceptor*):
***Note this protocol is to be run <u>after</u> the 16 hour incubation.**

1. *PlatesApart* (subroutine in the method):

    a. Remove lid from the filter plate (D4) and place it at position D2.
    b. Remove the filter plate from the acceptor plate (D4) using a slow speed and place at position D3 (on top of the single well tray).

2. Transfer 250 µL aliquots from the acceptor plate (D4) to the UV 96-well analysis (C3) using the Acceptor Tips (A1).

3. Remove the UV-96 well analysis plate and analyze with an UV/Vis microplate spectrophotometer.

Assay Reproducibility – Avanti Lipid

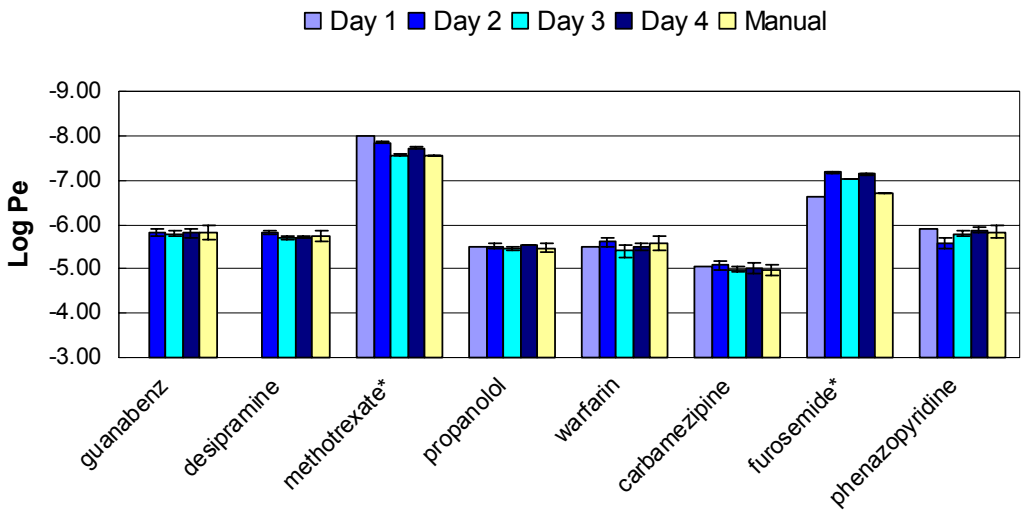


Figure 1. Each value is an average Log P_e for 12 wells per compound for each plate. One plate was run on each day (Day 1, Day 2, Day 3 and Day 4) using the Sciclone, while only one plate was run manually on Day 2. The UV/Vis absorbance was determined for each plate using a Molecular Devices SpectraMax® Plus plate reader. Donor drug concentration was 500µM. A DOPC lipid (Synthetic Phospholipid Blend I, #790787 Avanti Polar Lipids Inc.) was used for creating the membrane.

*These compounds are at the limit of detection with UV/Vis and should be analyzed using a more sensitive method, i.e. LC/MS.

Automation vs Manual – pION Lipid

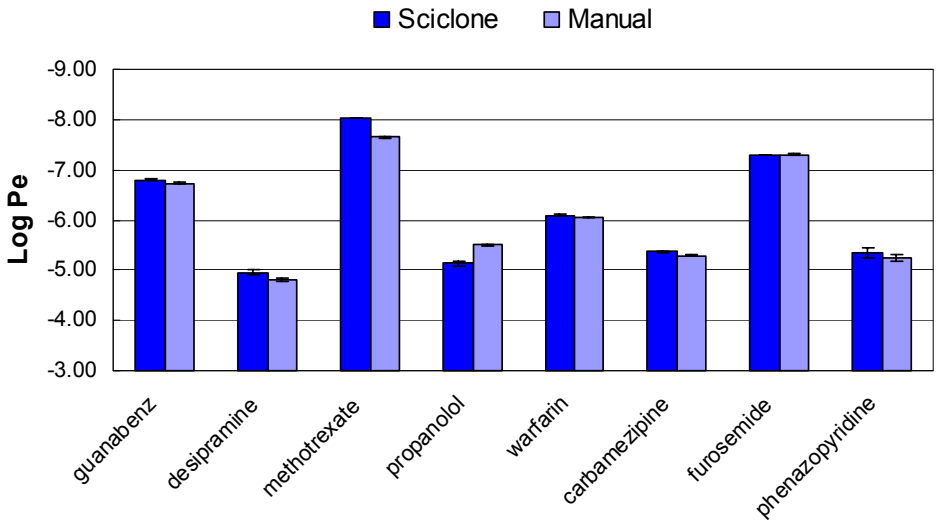


Figure 2. Each value is an average Log P_e for 12 wells per compound for each plate (automation and manual). Both plates were run on the same day. The lipid membrane was created using a lipid manufactured by pION (# 110618). Log P_e -6.00 or below are the result of the compounds being at the limit of detection of Uv/Vis and therefore should be analyzed using a more sensitive method, i.e. LC/MS.

Conclusion

The data above shows that the PAMPA-Lipid protocol using Millipore's MultiScreen PAMPA filter plate can be easily automated on the Sciclone ALH 3000 Advanced Liquid Handler Workstation. The results obtained from automation are comparable to the results obtained manually (Figure 1 & 2). The automation results also show day to day reproducibility of the protocol.

This automated method can be used with either lipid (Avanti or pION) without any changes to the programs. The permeability rates may vary between lipids due to differences in the lipid composition. It has been documented that permeability rates can vary with changes in concentrations of lipids and types of lipids used[3]. The use of either lipid with the Sciclone ALH 3000 allows 96 samples to be processed in just over 16 hours (this includes the incubation period).

Millipore Ordering Information:

	Part Number	Package Size
MultiScreen-IP Filter Plate	MAIPN4510	10/pk
MultiScreen Cell Culture Tray	MAMCS0110	10/pk
MultiScreen Acceptor Plate	MSSACCEPT0R	each

Other Accessories:

Item	Vendor	Part Number
200 µL Tempo™ disposable pipette tips	Caliper	56362
Gripper – Contact Caliper for determination based on hardware configuration	Caliper	105408 or 105409
Reservoir (2) – standard, low profile, 86 mL	Innovative Microplate	S30018
Reservoir (1) – 12 columns, low profile, partitioned	Innovative Microplate	S30028
Synthetic Phospholipid Blend I	Avanti Polar Lipids, Inc	790787
pION Lipid	pION Inc	110618

Note: The part numbers for the other accessories are U.S. part numbers and are subject to change. Please check with each company prior to any purchase.

References:

[1] Kansey, M.; Senner, F.; Gubernator, K. *Physiochemical High Throughput Screening: Parallel Artificial Membrane Permeation Assay in the Description of Passive Absorption Processes*, J. Med. Chem., 1998; 41, p. 1007 – 1010.

[2] Kansey, M; Fischer, H,; Kratzat, K.; Senner, F; Wagner, B; Parrilla, I. *High-Throughput Artificial Membrane Permeability Studies in Early Lead Discovery and Development*, Pharmacokinetic Optimization in Drug Research, 2001; XII, p. 448-464.

[2] Avdeef, A; *Absorption and Drug Development Solubility, Permeability, and Charge State.* John Wiley & Sons, Inc. Publishers, New Jersery, 2003. p. 171 – 235.