
Technical Note

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Title: Automation of PAMPA-Lipid using a MultiScreen®-IP PAMPA Filter Plate on a Tecan Freedom EVO® Workstation

Introduction

PAMPA-Lipid (Parallel Artificial Membrane Permeation Assay) 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 Polar Lipids, Inc. or pION Inc. The protocol can be run using either Millipore's PTFE acceptor plate or the disposable MultiScreen Transport Receiver plate.

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 (either MultiScreen PTFE or MultiScreen Transport Receiver 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 ($\text{Log } P_e$) of the drugs. At the end of the incubation time, the integrity of the artificial membrane layer can be measured using electrical resistance. Processing of the automated PAMPA-Lipid assay on the Freedom EVO workstation takes about 47 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.

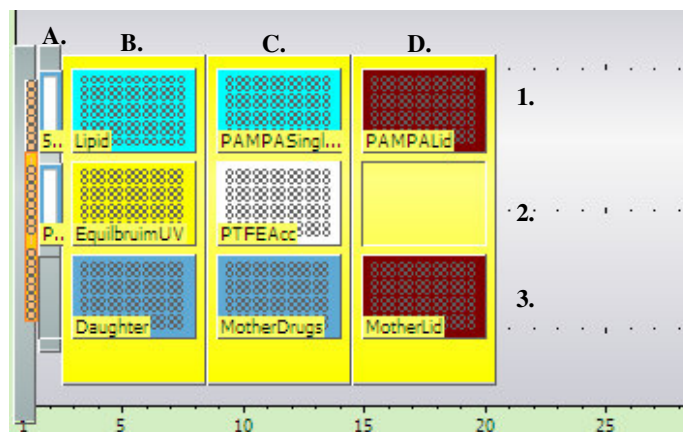
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Configuration of the Tecan Freedom EVO Deck for *MilliporePAMPALipid* or *MilliporeDispPAMPALipid* Methods

Membrane formation, drug addition, equilibrium plate creation and incubation



Important:

- Program created using Tecan Freedom EVOware Software Version 1.0, Service Pack 1, Build 1.3.29.303 on a Tecan Freedom EVO Workstation. It requires a RoMa and 8 fixed tips with 1000 μL syringes.

Prior to starting either program *MilliporePAMPALipid* (which includes the subroutine *MilliporeLipidMembrane*) or *MilliporeDispPAMPALipid* (which includes the subroutine *MilliporeDispLipidMembrane*), make sure the deck configuration is as follows:

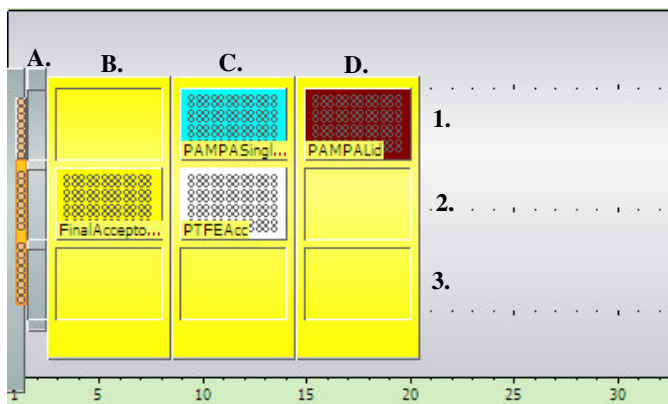
- A:** 100 mL trough carrier (2 x 100 ml reservoirs)
 - A1. 5% DMSO in Phosphate buffer
 - A2. Phosphate buffer
- B:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - B1. 12 column reservoir - lipid in first column
 - B2. UV 96-well analysis plate for the equilibrium plate
 - B3. Daughter plate (v-bottom polypropylene)
- C:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - C1. MultiScreen PAMPA filter plate (on top of a single well cell culture tray with cover on)
 - C2. MultiScreen Acceptor plate (either MultiScreen PTFE Acceptor or MultiScreen Transport Receiver)
 - C3. Mother plate (v-bottom polypropylene with cover on)
- D:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - D1. Lid for MultiScreen PAMPA filter plate (placed here during the run)
 - D3. Lid for mother plate (placed here during the run)

Procedure (*MilliporePAMPALipid* or *MilliporeDispPAMPALipid*):

1. Distribute 285 μL aliquots of PBS buffer (A2) to the daughter plate (B3).
2. Remove the lid from the mother plate (C3) and place it at D3 using the RoMa.
3. Distribute 15 μL from the wells in the mother plate (C3) to the daughter plate (B3). Mix 3 times with a volume of 275 μL after each drug addition.
4. Re-lid the mother plate using the RoMa.
5. Distribute 300 μL aliquots of 5% DMSO/buffer (A1) to the acceptor plate (C2).
6. Remove the lid from the MultiScreen PAMPA filter plate in the single well cell culture plate (C1) and place it at position D1.
7. *MilliporeLipidMembrane* or *MilliporeDispLipidMembrane* (subroutines within the methods):
 - a. The reservoir used in this program is a low profile, 12 column reservoir located at position B1. The lipid (2 – 3 mL) is placed in the first column of the reservoir.
 - b. Distribute 5 μL aliquots of lipid (B1) to each well of the filter plate (C1). There is a mix step prior to the first aspiration to wet out the tips.
8. Transfer 150 μL from the daughter plate (B3) to the filter plate (C1).
9. Move the filter plate (C1) to the acceptor plate (C2) at a slow speed using the RoMa.
10. Move the cover (D1) on top of the filter plate (C2).
11. Create equilibrium plate:
 - a. When using the MultiScreen PTFE acceptor for an acceptor plate:
 - i. Distribute 170 μL of 5% DMSO/buffer to each of the wells of the equilibrium plate (B2).
 - ii. Distribute 80 μL from each of the wells of the daughter plate (B3) to each of the wells of the UV equilibrium plate (B2). Mix 3 times with a volume of 200 μL after each drug addition.
 - b. When using the MultiScreen Transport Receiver for an acceptor plate:
 - i. Distribute 136 μL of 5% DMSO/buffer to each of the wells of the equilibrium plate (B2).
 - ii. Distribute 64 μL from each of the wells of the daughter plate (B3) to each of the wells of the UV equilibrium plate (B2). Mix 3 times with a volume of 150 μL after each drug addition.
12. Remove the UV 96-well analysis plate (equilibrium plate) at B2 and analyze with an UV/Vis microplate spectrophotometer.
13. Manually place filter plate with acceptor plate into a plastic zip lock bag and incubate for 16 hours at room temperature, if using the PTFE acceptor plate. If using MultiScreen Transport Receiver plate incubation can occur on the deck without the use of a plastic bag.

Configuration of the Tecan Freedom EVO Deck for *MilliporePAMPALipid_Analysis* or *MilliporeDispPAMPALipidAnaly* Methods

Creation of final acceptor analysis plate



Important:

- Program created using Tecan Freedom EVOware Software Version 1.0, Service Pack 1, Build 1.3.29.303 on a Tecan Freedom EVO Workstation. It requires a RoMa and 8 fixed tips with 1000 μ L syringes.

Prior to starting either program *MilliporePAMPALipid_Analysis* or *MilliporeDispPAMPALipidAnaly* make sure the deck configuration is as follows:

- A:** 100 mL trough carrier (no reservoirs) - empty
- B:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - B2. UV 96-well analysis plate for the final acceptor analysis plate
- C:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - C1. MultiScreen PAMPA filter plate (placed here during the run on top of a single well cell culture tray)
 - C2. MultiScreen Acceptor plate with the MultiScreen PAMPA filter plate on top with a lid on it. The acceptor plate is either the MultiScreen PTFE Acceptor or the MultiScreen Transport Receiver plate.
- D:** Millipore MP3 PAMPA carrier (3 position landscape, 384 MTP carrier)
 - D1. Lid for MultiScreen PAMPA filter plate (placed here during the run)

Procedure (*MilliporePAMPALipid_Analysis* or *MilliporeDispPAMPALipidAnaly*):

***Note this protocol is to be run after the 16 hour incubation.**

1. Remove lid from the filter plate (C2) and place it at position D1 using the RoMa.
2. Remove the filter plate from the acceptor plate (C2) using a slow speed and place it at position C1 (on top of the single well cell culture tray).
3. Transfer aliquots from the acceptor plate (C2) to the UV 96-well analysis (B2).
 - a. MultiScreen PTFE Acceptor plate – transfer 250 μ L.
 - b. MultiScreen Transport Receiver plate – transfer 200 μ L.
4. Remove the UV-96 well analysis plate and analyze with an UV/Vis microplate spectrophotometer.

Automation vs Manual & Assay Reproducibility – Avanti Lipid & MultiScreen PTFE Acceptor Plate

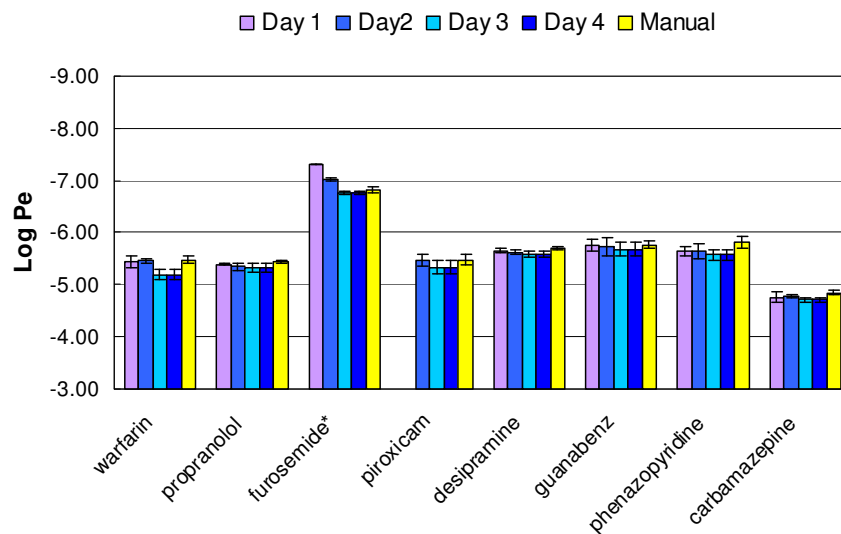


Figure 1. Each value is an average Log P_e for 12 wells per compound for each plate. One plate was run on each day (Day 1, Day 2, Day 3 and Day 4) using the Tecan Freedom EVO workstation, while only one plate was run manually on Day 2. The UV/Vis absorbance was determined for each plate using a Molecular 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 and the MultiScreen PTFE Acceptor plate was used.

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

MultiScreen PTFE Acceptor Plate vs MultiScreen Transport Receiver Plate Avanti Lipid

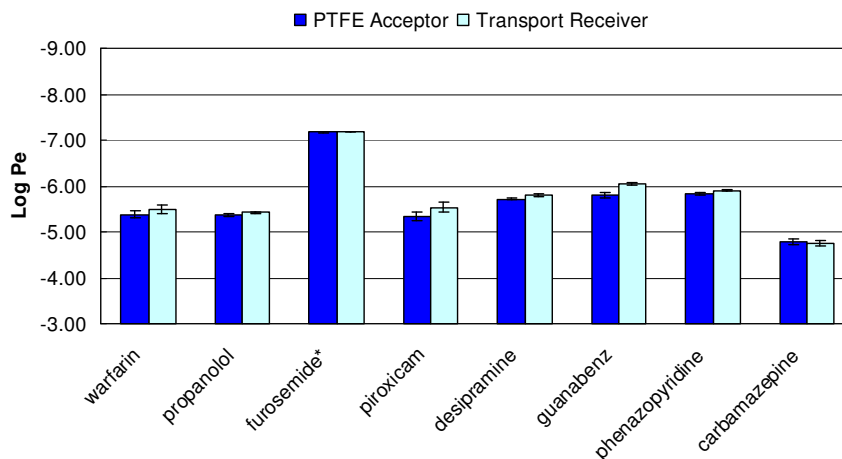


Figure 2. Each value is an average Log P_e for 12 wells per compound for each plate. Both plates were run on the same day. The lipid membrane was created using a lipid manufactured by Avanti Polar Lipids, Inc. (#790787). Log P_e -6.00 (*) or below are the result of the compounds being at the limit of detection of Uv/Vis and therefore should be analyzed using a more sensitive method, i.e. LC/MS.

Automation vs Manual – pION Lipid

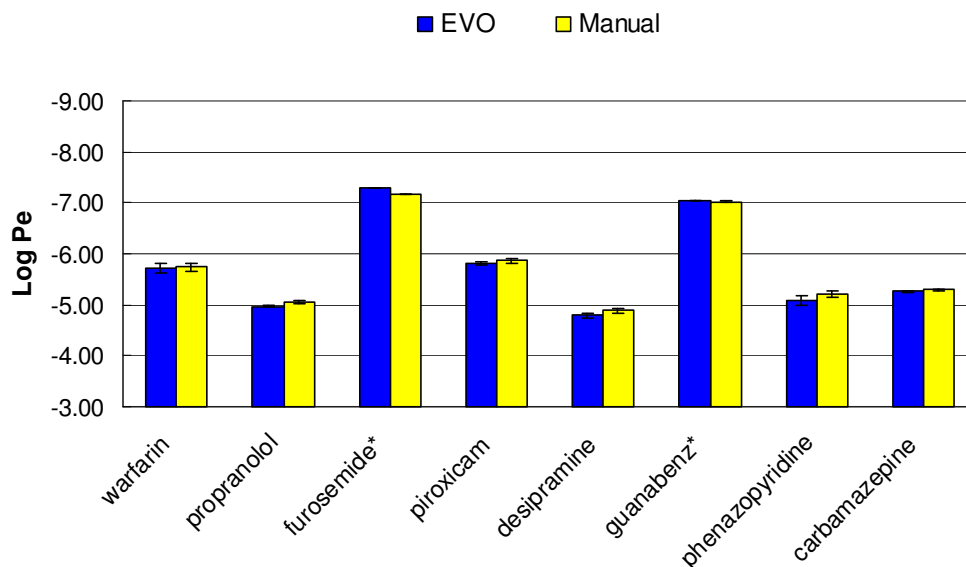


Figure 3. Each value is an average Log P_e for 12 wells per compound for each plate (automation and manual). Both plates were run on the same day. The lipid membrane was created using a lipid manufactured by pION (# 110618). Log P_e -6.00 (*) or below are the result of the compounds being at the limit of detection of Uv/Vis and therefore should be analyzed using a more sensitive method, i.e. LC/MS. The MultiScreen PTFE acceptor plate was used for the acceptor plate both manually and with automation.

Conclusion

The data above shows that the PAMPA-Lipid protocol using Millipore's MultiScreen PAMPA filter plate can be easily automated on the Tecan Freedom EVO Workstation. The results obtained from automation are comparable to the results obtained manually (Figure 1 & 3). The automation results also show day to day reproducibility of the protocol and no difference between the use of the MultiScreen PTFE Acceptor plate or the MultiScreen Transport Receiver plate for the acceptor plate (Figure 1 & 2).

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 used³. The use of either lipid with the Tecan Freedom EVO workstation allows 96 samples to be processed in 16.75 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
MultiScreen Transport Receiver Plate	MATRNPS50	50/pk
96 well MultiScreen Collection Plate – UV Analysis	MSCPNUV40	40/pk
96 well Solvinert PolyPro Collection Plate	MSCPNPP00	100/pk

Other Accessories:

Item	Vendor	Part Number
1000 µL syringes	Tecan	20725591
Standard fixed tips (8)	Tecan	10612501
3 position landscape, 384 MTP carrier (3)	Tecan	10613031
100 mL trough carrier	Tecan	10613020
100 mL troughs (2)	Tecan	10613021
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. *Physicochemical 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.

³ Avdeef, A; *Absorption and Drug Development Solubility, Permeability, and Charge State*. John Wiley & Sons, Inc. Publishers, New Jersey, 2003. p. 171 – 235.

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