Technical Note

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Title: **Automation of PAMPA-HDM using a MultiScreen® Filter Plate on a Beckman Biomek® FX Workstation**

Introduction

PAMPA-HDM is a non-cell based assay designed to predict passive, transcellular permeability of drugs in early drug discovery[1]. The assay is carried out in a 96-well MultiScreen filter plate and measures the ability of compounds to diffuse from a donor to an acceptor compartment separated by a hexadecane/hexane artificial membrane on a polycarbonate membrane support. PAMPA-HDM can be used to determine the effect of pH on compound permeability by adjusting the pH of the solutions used in the analysis. The pH permeability profiles in the permeability assay are valuable in predicting gastrointestinal absorption of ionizable drugs.

An artificial membrane is applied to the polycarbonate 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 the MultiScreen PTFE plate or the MultiScreen Transport Receiver plate) filled with sufficient buffer to ensure liquid contact between the liquid in the Acceptor plate and the polycarbonate membrane. The Donor and Acceptor plates are incubated together for 5-7 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 acceptor solutions were 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. Processing of the automated PAMPA-HDM assay on the Beckman Biomek FX workstation takes about 1 hour and 12 minutes (this does not included the 5 – 7 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, creation of the equilibrium plate and removal of samples for UV/Vis analysis from the Acceptor plate.

Note: Refer to Tech Note #'s AN1729EN00 and AN1725EN00 for more detailed information on running the PAMPA-HDM.

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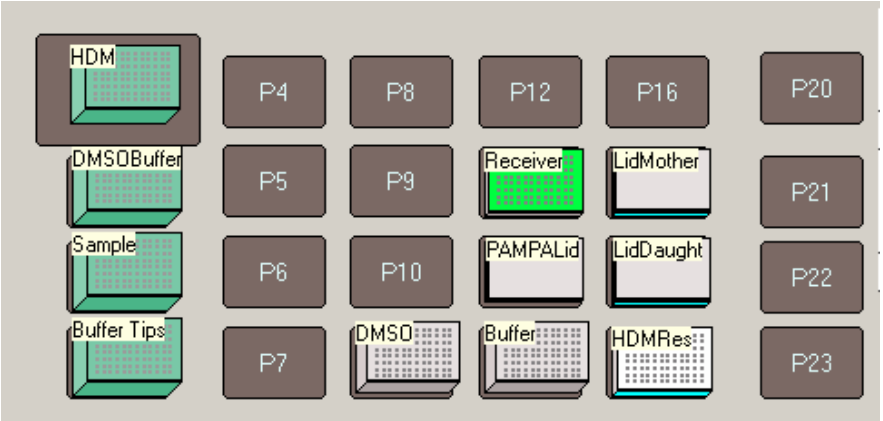
Configuration of the Biomek FX Deck for *Milipore_PAMPAHDM_V2.5_Pod1* or *Millipore_PAMPAHDM_PTFE_V2.5_Pod1* Methods

Membrane formation, drug addition to plate and incubation



Important:

- Program created using Biomek FX Software Version 2.5, Build 11 on a Biomek FX Workstation with a 4x4 deck.

Prior to starting either *Millipore_PAMPAHDM_V2.5_Pod1.bmt* (method used with the MultiScreen Transport Receiver), or *Millipore_PAMPAHDM_PTFE_V2.5_Pod1.bmt* (method used with the MultiScreen PTFE Acceptor) make sure the deck configuration is as follows:

TL1: P200 tips (HDM membrane tips)
P1: P200 tips (DMSO buffer tips)
P2: P200 tips (Sample tips)
P3: P200 tips (Buffer tips)
P4 – P10: Empty
P11: Large Reservoir (5% DMSO in Phosphate buffer)
P12: Empty
P13: MultiScreen Acceptor plate (either MultiScreen PTFE Acceptor or MultiScreen Transport Receiver)
P14: MultiScreen filter plate (on top of a single well cell culture tray with cover on)
P15: Large Reservoir (Phosphate buffer)
P16: Empty
P17: Mother plate (v-bottom polypropylene with cover on)
P18: Daughter plate (v-bottom polypropylene with cover on)
P19: Small reservoir (5% hexadecane/95% hexane for membrane layer)
P20 – P23: Empty

Procedure (*Millipore_PAMPAHDM_V2.5_Pod1* or *Millipore_PAMPAHDM_PTFE_V2.5_Pod1)*:

1. Remove the lid from the MultiScreen filter plate in the single well cell culture tray (P14) and place at position P12.

2. Distribute 15 µL aliquots of hexadecane/hexane (P19) to each well of the filter plate (P14) using the HDM pipette tips. There is a mix step prior to the aspiration to wet out the tips. There will be a post dispense back to the reservoir (P19) after dispensing into the filter plate.

3. Incubate membrane for 60 minutes (the whole system will pause).

4. Distribute 150 µL aliquots (2X for total of 300 µL) of 5% DMSO/buffer (P11) to the acceptor plate (P13) using the DMSO buffer tips.

5. Remove the lid from the daughter plate (P18) and place the lid at P16.

6. Distribute 285 µL aliquots (185 µL first, then 100 µL) of PBS buffer (P15) to the daughter plate (P18) using the Buffer tips.

7. Remove the lid from the mother plate (P17) and place the lid at P8.

8. Distribute 15 µL from the wells in the mother plate (P17) to the daughter plate (P18) using the Sample tips. Mix 5 times with a volume of 190 µL after each drug addition.

9. Transfer 150 µL from the daughter plate (P18) to the filter plate (P14).

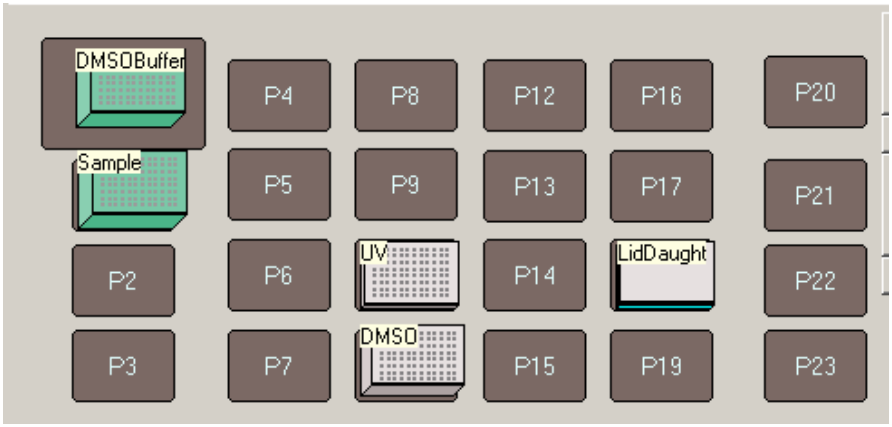
10. Re-lid the mother plate and daughter plate.

11. Move the filter plate (P14) to the acceptor plate (P13) at a slow speed.

12. Move the cover (P12) on top of the filter plate (P13). Incubate for 5 hours at room temperature.

Configuration of the Biomek FX Deck for *Milipore_HDMEquilb_V2.5_Pod1* or *Millipore_HDMEquilb_PTFE_V2.5_Pod1* Methods

Equilibrium plate formation



Important:

- Program created using Biomek FX Software Version 2.5, Build 11 on a Biomek FX Workstation with a 4x4 deck.

Prior to starting either *Millipore_HDMEquilb_V2.5_Pod1.bmt* (method used with the MultiScreen Transport Receiver), or *Millipore_HDMEquilb_PTFE_V2.5_Pod1.bmt* (method used with the MultiScreen PTFE Acceptor) make sure the deck configuration is as follows:

TL1: P200 tips (DMSO membrane tips)
P1: P200 tips (Sample buffer tips)
P2 – P9: Empty
P10: UV 96-well analysis plate for equilibrium plate
P11: Large Reservoir (5% DMSO in Phosphate buffer)
P12 – P17: Empty
P18: Daughter plate (v-bottom polypropylene with cover on)
P19 – P23: Empty

Procedure (*Millipore_HDMEquilb_V2.5_Pod1* or *Millipore_HDMEquilib_PTFE_V2.5_Pod1)*:

1. Remove the lid from the daughter plate (P18) and place it at P16.

2. Distribute 5% DMSO/buffer (P11) to each of the wells of the equilibrium plate (P10):
    a. When using the MultiScreen Transport Receiver plate – distribute 136 µL
    b. When using the MultiScreen PTFE acceptor plate - distribute 170 µL

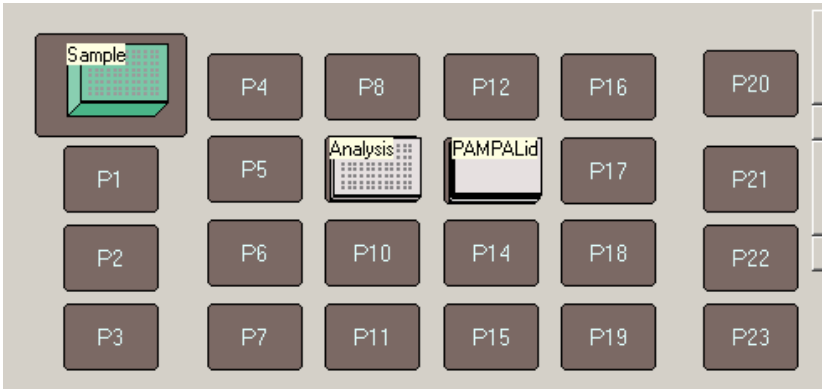
3. Distribute sample from the daughter plate (P18) to each of the wells of the equilibrium plate (P10):
    a. When using the MultiScreen Transport Receiver plate – transfer 64 µL and mix 5 times with a volume of 150 µL.
    b. When using the MultiScreen PTFE acceptor plate – transfer 80 µL and mix 5 times with a volume of 190 µL.

4. Recover daughter plate.

5. Remove the UV 96-well analysis plate (equilibrium plate) at P10 and analyze with an UV/Vis microplate spectrophotometer.

Configuration of the Biomek FX Deck for *Milipore_HDMAnaly_V2.5_Pod1* or *Millipore_HDMAnaly_PTFE_V2.5_Pod1* Methods

Analysis plate formation



Important:

- Program created using Biomek FX Software Version 2.5, Build 11 on a Biomek FX Workstation with a 4x4 deck.

Prior to starting either *Millipore_HDMAnaly_V2.5_Pod1.bmt* (method used with the MultiScreen Transport Receiver), or *Millipore_HDMAnaly_PTFE_V2.5_Pod1.bmt* (method used with the MultiScreen PTFE Acceptor) make sure the deck configuration is as follows:

TL1: P200 tips (Sample tips)
P1 – P8: Empty
P9: UV 96-well analysis plate for final acceptor analysis plate
P10 – P12: Empty
P13: MultiScreen Acceptor plate (either PTFE or Transport Receiver) with the MultiScreen filter plate on top with lid on it.
P14: Single well cell culture tray (MultiScreen filter plate will be placed here)
P15 – P23: Empty

Procedure (*Millipore_HDMAnaly_V2.5_Pod1* or *Millipore_HDMAnaly_PTFE_V2.5_Pod1)*:
***Note these protocols are to be run <u>after</u> the 5 hour incubation.**

1. Remove lid from the filter plate (P13) and place it at position P12 using the gripper.

2. Remove the filter plate from the acceptor plate (P13) using a slow speed and place it at position P14 (on top of the single well cell culture tray).

3. Transfer aliquots from the acceptor plate (P13) to the UV 96-well analysis (P9).
    a. MultiScreen Transport Receiver plate – transfer 200 µL.
    b. MultiScreen PTFE Acceptor plate – transfer 250 µL.

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

Automation *vs* Manual – MultiScreen Transport Receiver Plate

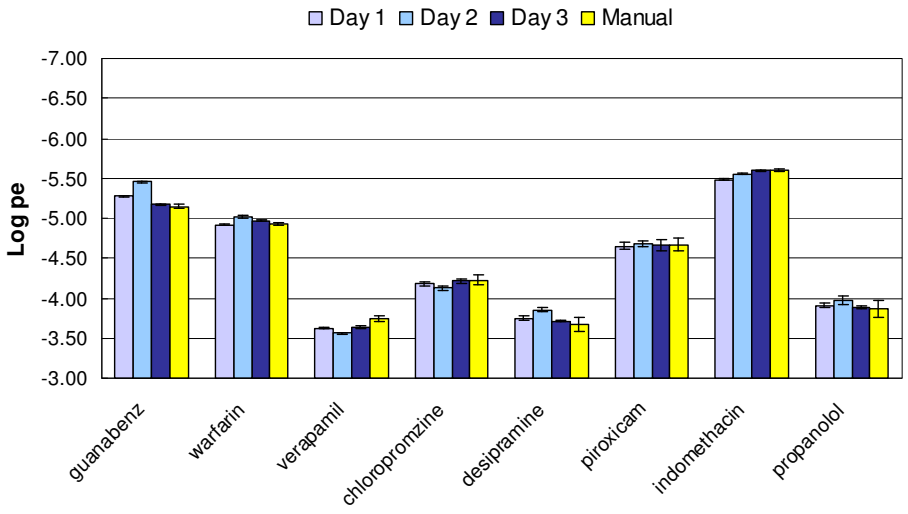


Figure 1. Each value is an average Log P_e for 12 wells per compound for each plate. One plate was run on each day (Day 1, Day 2, and Day 3) using the Biomek FX workstation, while only one plate was run manually on Day 3. The UV/Vis absorbance was determined for each plate using a Molecular Devices SpectraMax® Plus plate reader. Donor drug concentration was 500µM.

MultiScreen Transport Receiver Plate
vs
MultiScreen PTFE Acceptor Plate

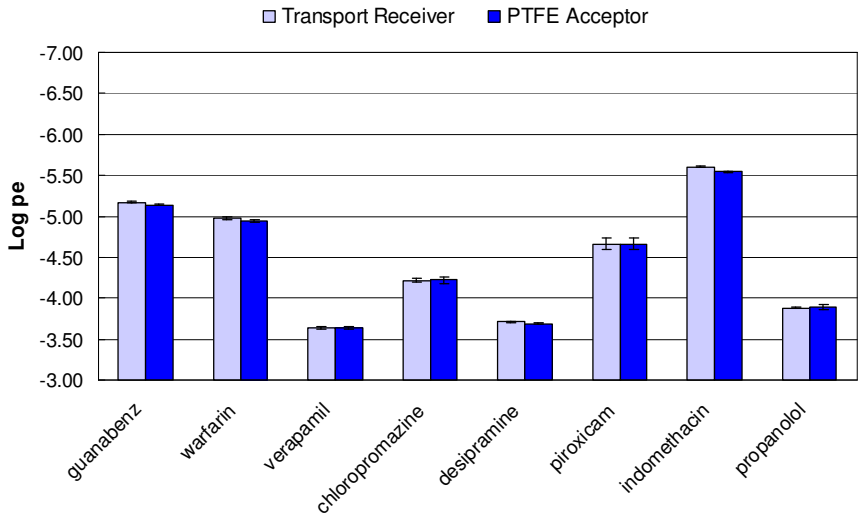


Figure 2. Each value is an average Log P_e for 12 wells per compound for each plate. Both plates were run on the same day using the Biomek FX workstation. The UV/Vis absorbance was determined for each plate using a Molecular Devices SpectraMax® Plus plate reader. Donor drug concentration was 500µM.

Conclusion:
The data above shows that the PAMPA-HDM protocol using Millipore's filter plate with either the MultiScreen Transport Receiver plate or the Multiscreen PTFE Acceptor plate can be easily automated on the Beckman Biomek FX Workstation. The results obtained from automation are comparable to results obtained manually. The automation results also show day to day reproducibility of the protocol. The use of either acceptor plate will not alter the results. The Biomek FX workstation allows 96 samples to be processed in just over 6 hours (this includes the 5 hour incubation period).

Millipore Ordering Information:

	Part Number	Package Size
MultiScreen 0.4 µM PCTE Filter Plate	MPC4NTR10	10/pk
MultiScreen PTFE Acceptor Plate	MSSACCEPT0R	each
MultiScreen Transport Receiver Plate	MATRNPS50	50/pk
96 well MultiScreen Collection Plate – UV Analysis	MSCPNUV40	40/pk
96 well Solvinert Collection Plate	MSCPNPP00	100/pk

Other Accessories:

Item	Vendor	Part Number
200 µL pipette tips	Beckman	717251
Reservoir (2) – standard, 300 mL	Innovative Microplate	S30014
Reservoir (1) – Low profile, 86 mL	Innovative Microplate	S30018

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] Wohnsland, F.; Faller, B. High-throughput Permeability pH Profile and High-throughput Alkane/Water Log P With Artificial Membranes, J. Med. Chem., 2001; 44, p. 923–930.