Protocol Data Sheet for
MDR1-K
Vesicular Transport Inhibition Assay

Membrane: **SB-MDR1-K**
Control membrane (optional): **SB-K-CTRL**
Probe substrate: **NMQ**

Version No.: 1.2
Version No. replaced: 1.1
Effective date: 08-Jan-2019

Related document: PR-ASY-VT-General Protocol for Vesicular Transport Inhibition Assays

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1. Chemicals

Name	Manufacturer & Cat #	Storage
Purified water		
Dimethyl sulfoxide (DMSO)	Sigma D2650	RT
Adenosine 5'-monophosphate sodium salt (AMP)	Sigma A1752	<-15°C
Adenosine 5'-triphosphate sodium salt hydrate (ATP)	Sigma A2383	< 0°C
Magnesium chloride	Sigma M8266	RT
N-methyl-quinidine	SOLVO	RT
Sodium chloride	Sigma S5886	RT
Sucrose	Sigma S0389	RT
Trizma® base	Sigma T1503	RT
Verapamil	Sigma V4629	RT

2. Stock solutions

Stock solution	Storage & stability
Tris, 1.7 M Dissolve 20.587 g of Trizma® base in 100 ml purified water.	2-8°C, < 1 year
Tris-HCl, 0.1 M Dissolve 1.21 g of Trizma® base in 90 ml purified water, adjust pH to 7.4 with 1 M HCl. Bring solution to 100 ml with purified water.	2-8°C, < 1 year
Sucrose, 1 M, in purified water Sterile filter.	2-8°C, < 1 year
NaCl, 1 M, in purified water	2-8°C, < 1 year
$MgCl_2$, 0.1 M, in purified water	2-8°C, < 1 year
Mg-ATP, 0.2 M Dissolve 2.2 g ATP and 0.813 g $MgCl_2$ in 10 ml of purified water and adjust pH to 7.4 with 1.7 M Tris. Bring solution to 20 ml with purified water. Aliquot and store in freezer.	<-15°C, < 1 year
AMP, 0.2 M Dissolve 1.39 g AMP in 10 ml of purified water and adjust pH to 7.4 with NaOH. Bring solution to 20 ml with purified water. Aliquot and store in freezer.	<-15°C, < 1 year
Reference inhibitor	
Verapamil, 10 mM, in DMSO	<-15°C, < 1 year
Unlabeled substrate	
N-methyl-quinidine, 500 µM, in DMSO	<-15°C, < 1 year
Radiolabeled substrate	**Manufacturer & Cat #**
^{3}H-NMQ: N-methyl-quinidinium chloride, [^{3}H(N)-], 0.5 mCi/mL, in DMSO	BRC Radiolab BL-110	as indicated by the supplier

3. Assay buffers

3.1 Transport buffer

Ingredient	Amount to add	Final cc.
Tris-HCl, 0.1 M	1 ml	10 mM
Sucrose, 1 M	2.5 ml	250 mM
$MgCl_2$, 0.1 M	1 ml	10 mM
Purified water	5.5 ml	
Total volume	10 ml	

Sterile filter. The solution can be stored at 2-8°C.

3.2 Washing buffer

Ingredient	Amount to add	Final cc.
Tris-HCl, 0.1 M	50 ml	10 mM
Sucrose, 1 M	125 ml	250 mM
NaCl, 1M	50 ml	100 mM
Purified water	275 ml	
Total volume	500 ml	

Sterile filter. The solution can be stored at 2-8°C.

4. Assay parameters

Total NMQ concentration in 75 µl reaction volume	2 µM
Specific activity per well	0.02 µCi
Protein content of membrane suspension per well	50 µg
Final concentration of Verapamil (optional)	100 µM
Incubation time	3 min
Incubation temperature	37°C
Reaction mix (sample calculation)*, for one 96-well plate	

*The volumes shown apply to a particular isotope batch with the given specifications. If these specifications differ, please re-calculate.

^{3}H-NMQ, 0.5 mCi/mL, 58000 mCi/mmol, 8.62 µM chem.cc.	4.1 µL
N-methyl-quinidine, 500 µM	30.5 µL
Membrane suspension	1020 µL
Transport buffer	4045.4 µL
Total volume	5100 µL

5. Special instructions

None.

General Protocol for

Vesicular Transport Inhibition Assays

Effective from: November 20, 2018

Version: 1.3

Previous version: 1.2

Attn.: Solvo Contract Research Laboratory

Approved by:

11/20/2018

X 

Head of R&D
Signed by: Zsuzsanna Gáborik

Checked by:

19/11/2018

X 

QA/QC manager
Signed by: Zsuzsanna Rajnai

1. Introduction

Most ABC transporters transport substrates across the cell membrane using ATP as an energy source. One of the simplest methods invented for measuring this transport is the vesicular transport assay. This assay protocol describes the determination of the interaction of test drugs with the efflux transporter of interest, using the vesicular transport assay. The interaction is detected as the modulation of the initial rate of the probe substrate transport into inside-out membrane vesicles purified from the transporter-expressing cells.

2. Equipment, Consumables, Chemicals

2.1. Equipment

Plate incubator/shaker

Single and multichannel pipettes with corresponding tips

Rapid filtration apparatus (Millipore 96-well plate filtration system or equivalent) Liquid scintillation counter with 96-well plate capability

Timer

2.2. Consumables

Pipette tips

96-well clear flat bottom cell culture plate for reaction mixture preparation

96-well filter plate (Millipore: MultiScreen HTS MSFBN6B50; Corning: FiltrEx™ CLS3511)

Scintillation cocktail (Ultima Gold XR, PerkinElmer:6013119)

Scintillation plate foil

2.3. Items supplied

Frozen membrane vesicles in vials containing 5 mg/ml membrane protein, labeled with volume, catalog number and date of production.

Membrane Data Sheet indicating protein content, volume, date of expiry of frozen membrane stocks.

Protocol Data Sheet describing materials and stock solution preparation along with general protocol.

Note: The mentioned suppliers and products are recommended to use as those can influence the result of the assay.

3. Assay steps

1. **Thaw every reagents on ice.**
2. **Based on the described parameters in the related Protocol Data Sheet, mix the membrane suspension on ice with ice-cold transport buffer, then add the probe substrate solutions. Add 50 l of this suspension to all wells of a standard 96-well plate (not the filterplate) placed on ice.**
3. **Prepare a serial dilution of test article (TA) in its solvent in a final volume of 20 µl per well on a helper plate. 0.75 µl of the dilution series stock per well will be needed for the reaction. A 10× more concentrated stock is recommended to provide the final maximum concentration in the assay. E.g. a 30 mM TA stock concentration is recommended to provide a final maximum concentration of 300 µM. Final maximum concentration depends on solubility limit of TA.**

   Example for a 7-steps, 3-fold dilution: add 30 µl of the highest TA stock into well A1 and 20 µl solvent into wells B1 to H1, respectively. Transfer 10 µl of TA solution from well A1 to well B1. Mix thoroughly with gentle pipetting. Transfer 10 µl of TA solution from well B1 to the next (C1) well. Repeat this process until well G1 with thorough mixing after every dilution step. Well H1 will serve as positive control containing only the solvent.

4. **Add test drugs (0.75 l) and solvent; and reference inhibitor and its solvent (0.75 µl) as indicated on the plate setup below. This will result a 100-fold dilution of the TA, reference inhibitor and solvent(s) in the assay.**
5. **Mix 90 l of Mg-ATP with 1410 l of transport buffer on ice. Be sure that the ATP stock is mixed well before using.**
6. **Mix 90 l of AMP with 1410 l of transport buffer on ice.**
7. **Pre-incubate plate, ATP and AMP at the appropriate temperature (see Protocol Data Sheet) for 15 min with shaking.**
8. **Wet the filter plate as recommended by the supplier and set up the filtering apparatus.**

   NOTE: check Protocol Data Sheet if special modification of this step is applied

9. **Add 25 l of ATP and AMP (prepared in steps 4 and 5) to the wells as indicated on the plate setup below. Shake plate with the shaker. Incubate at the appropriate temperature for the indicated time (see Protocol Data Sheet).**

   NOTE: Depending on your equipment you can run the assay with one row at a time, or in blocks. The general consideration is that filtration should take place in 2 minutes after stopping the assay with cold washing-buffer.

10. **Stop the reaction by adding 200 l ice-cold washing buffer. Transfer samples to the filter plate and filter.**

11. **Wash wells with 5×200 l of ice-cold washing buffer.**

12. **Pipette 10 l of the membrane suspension (prepared in step 1.) into three empty/clean wells of a filter plate. The radioactivity (cpm) measured on these filters will be used to calculate total activity in one well (see Calculations).**

13. **Dry filter plate (you can use a hair drier to speed up the process).**

14. **Add 100 l of scintillation cocktail to each well.**

15. **Seal scintillation plate with foil and store in the dark for at least 60 minutes to allow samples mix with the cocktail.**

16. **Count samples in a scintillation counter.**

Suggested assay layout

	1	2	3	4	5	6	7	8	9	10	11	12
	Compound						Positive control					
	+ ATP			-ATP (AMP)			ATP			-ATP (AMP)		
A	C1 TA			C1 TA			+ reference inh.			+ reference inh.		
B	C2 TA			C2 TA			solvent			solvent		
C	C3 TA			C3 TA								
D	C4 TA			C4 TA								
E	C5 TA			C5 TA								
F	C6 TA			C6 TA								
G	C7 TA			C7 TA								
H	solvent			solvent						Total		

Helper plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1 TA											
B	C2 TA											
C	C3 TA											
D	C4 TA											
E	C5 TA											
F	C6 TA											
G	C7 TA											
H	solvent											

Suggested membrane negative control: see Protocol Data Sheet.

4. Calculations

ATP dependent transport (cpm): Subtract the mean cpm values measured in the absence of ATP from the mean cpm values measured in the presence of ATP for control and samples.

ATP dependent transport (pmol/mg/min): Calculate Total activity (cpm) by multiplying the average of the cpms measured in the designated wells prepared in step 11 by 5. Calculate the rate of transport in pmol/mg membrane protein/min using the following formula.

$$\frac{\text{ATP dependent transport (cpm)}}{\text{Total activity (cpm)}} * \frac{\text{Substrate concentration (nM) *Volume (ml)}}{\text{membrane protein(mg)* time(min)}}$$

ATP dependent transport (%): Calculate the percent activation or inhibition of the test drug. In this representation, the ATP dependent transport determined in the drug free control is taken as 100% and all other values are represented on this relative scale. Use the following formula:

$$\frac{\text{ATP dependent transport in the presence of test drug (cpm)}}{\text{ATP dependent transport in drug free control (cpm)}} * 100$$

Calculate standard deviation of Activity% values using the formula:

$$SD_{\text{Activity \%}_i} = 100 \sqrt{\frac{VarB}{A^2} \quad \frac{VarA \; B}{A^4}^2}$$

Where A is the ATP dependent transport in drug free control, and B is the ATP dependent transport in the presence of drug

Positive control: The probe transport of the transporter is fully (or under 20% of the drug free control) inhibited by the given reference inhibitor. You can assay this inhibition by replacing test drug with 0.75 l of the appropriate concentration of the reference inhibitor (see Protocol Data Sheet).

5. Handling of TA dilution series

Preparation of dilution series:

Every experimental day a fresh dilution series is prepared.

Dilution series are prepared in U-bottom plate.

Multiple dilution series can be prepared on the same U-bottom plate.

Already prepared dilution series are kept at room temperature.

Dilution series with purified water or DMSO can be used in a time frame of maximum 4 hours.

Dilution series of solvents with high evaporation rate must be prepared freshly for every usage.

General Protocol for Vesicular Transport Substrate Assessment Assay

Effective from: November 20, 2018

Version: 1.3

Previous version: 1.2

Attn.: Solvo Contract Research Laboratory

Approved by:

11/20/2018

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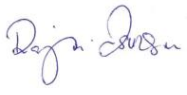
Head of R&D
Signed by: Zsuzsanna Gáborik

Checked by:

19/11/2018

X 

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1. Introduction

Efflux transporters play key role in the penetration of pharmacological barriers. Apical efflux transporters typically confer active clearance from the body through the kidney or liver, or from protected fluid compartments such as the brain. Basolateral efflux transporters block entry into the bloodstream or another fluid compartment.

Assessment of interactions with these transporters during drug development can provide early data on prospective absorption, distribution and elimination properties of developed compounds.

This protocol is applicable for both labelled and unlabeled test articles. The protocol makes use of vesicles prepared from transporter expressing and control cell lines as used for inhibition studies.

This protocol is a general guideline for all efflux transporters with no transporter specific parameters, those may be found in the respective vesicular transport (VT) protocol data sheet and general protocol together with the used equipment, consumables and materials.

As with inhibition studies, the experiment relies on differential accumulation of substrates in the transporter expressing vesicles as opposed to control cells.

The protocol divides direct transport assessment into four different stages to separate early feasibility and spare resources in case of negative results.

Level 1 is a proof of concept feasibility assessment using 2 time points and 2 concentrations or 1 time point and 4 concentrations. Optional follow-up is a one-point inhibition at the best condition.

Level 2 is a time curve experiment at an arbitrary concentration, if Level 1 is successful.

Level 3 is the descriptive determination of kinetic constants of the interaction, if Level 2 is successful.

Level 4 is inhibition of uptake with literature inhibitors. This step refers to existing inhibition protocols.

Read the protocol carefully before conducting experiments.

2. Experimental Procedures

Level 1. Proof of Concept

This level requires a partial 96-well-plate with respective efflux transporter expressing and control vesicles. Assay is conducted at 37°C using 50 µg membrane/well (except for positive control if indicated on the corresponding Protocol Data Sheet).

Experimentally the plate is laid out as below or equivalent:

2 time points – 2 concentrations setup:

			ATP			AMP			ATP			AMP		
			1	2	3	4	5	6	7	8	9	10	11	12
A	TR	T1	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	PC	PC	PC	PC	PC	PC
B			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	RI	RI	RI	RI	RI	RI
C		T2	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}						
D			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}						
E	C	T1	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	PC	PC	PC	PC	PC	PC
F			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	RI	RI	RI	RI	RI	RI
G		T2	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}						
H			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}						

T1, T2: time points; TA_{C1}, TA_{C2}: concentrations of the test article; PC: positive control; RI: reference inhibitor of positive control; TR: transporter expressing vesicles, C: parental/mock/not active control vesicles

1 time point – 4 concentrations setup:

			ATP			AMP			ATP			AMP		
			1	2	3	4	5	6	7	8	9	10	11	12
A	TR	T1	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	PC	PC	PC	PC	PC	PC
B			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	RI	RI	RI	RI	RI	RI
C			TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}						
D			TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}						
E	C		TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	PC	PC	PC	PC	PC	PC
F			TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	RI	RI	RI	RI	RI	RI
G			TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}						
H			TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}						

T1: time point; TA_{C1}-TA_{C4}: concentrations of the test article; PC: positive control; RI: reference inhibitor of positive control; TR: transporter expressing vesicles, C: parental/mock/not active control vesicles

Follow-up: for reference inhibitor see the corresponding uptake Protocol Data Sheet

		ATP			AMP			ATP			AMP		
		1	2	3	4	5	6	7	8	9	10	11	12
A	TR	TA	TA	TA	TA	TA	TA	PC	PC	PC	PC	PC	PC
B		RI	RI	RI	RI	RI	RI	RI	RI	RI	RI	RI	RI
C	C	TA	TA	TA	TA	TA	TA	PC	PC	PC	PC	PC	PC
D		RI	RI	RI	RI	RI	RI	RI	RI	RI	RI	RI	RI
E													
F													
G													
H													

T1: time point; TA: test article; RI: reference inhibitor; TR: transporter expressing vesicles, C: parental/mock/not active control vesicles;

Note: Positive control on the control membrane is optional.

Assay steps:

1. **Conduct the positive control experiment on the transporter expressing vesicles according to the General Protocol for Vesicular Transport Inhibition Assay and the corresponding Protocol Data Sheet. Proceed if the result meets the acceptance criteria for the corresponding membrane.**

2. **Thaw every reagent on ice. Assess reaction mix with test article (TA) and add 50 µl per well according to the plate setup. Note that final volumes will be diluted by 1.5 after the addition of ATP/AMP. Prepare sufficient amount of 12 mM ATP/AMP solution in the corresponding transport buffer.**

   Note: In case of radiolabeled TA, 0.1 µCi ^{3}H or 0.01 µCi ^{14}C isotope is recommended unless the sponsor requires otherwise. Different concentrations of the TA can be adjusted by using only the labeled compound or complement with unlabeled compound.

3. **Incubate plate, ATP and AMP at 37°C for 15 minutes with shaking.**

4. Wet the filter plate as recommended by the supplier and set up the filtering apparatus.

5. Start reactions by adding 25 µl of ATP or AMP. Shake plate with the shaker. Preferably longer incubations should be conducted first, and shorter incubations as second.

6. Stop reactions with 200 µl of ice cold buffer and transfer samples to a 96-well filter plate. Wash wells 5 times with 200 µl washing buffer.

7. Prepare TA samples for cold analysis according to LC-MS Sample Preparation Protocol.

8. For evaluation, plot total accumulated amount of TA in the transporter expressing and control vesicles in all concentrations and time points (grouped graph format is recommended for graphical representation). The result should be considered positive if the signal-to-noise ratio is over 2 and standard deviation is under 20%.

Level 2. Time curve

This level requires a full 96-well-plate with respective efflux transporter expressing and control vesicles, and a partial plate for positive control. Assay is conducted at 37°C using 50 µg membrane/well (except for positive control if indicated on the corresponding Protocol Data Sheet). Finetuning of these two parameters might be needed based on the POC experiments.

Experimentally the plate is laid out as below or equivalent:

	TR						C					
	ATP			AMP			ATP			AMP		
	1	2	3	4	5	6	7	8	9	10	11	12
A	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1
B	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2
C	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3
D	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4
E	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5
F	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6
G	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7
H	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8

T1 through T8: time points of the curve; TR: transporter expressing vesicles, C: parental/mock/not active control vesicles

Assay steps:

1. **Conduct the positive control experiment on 96-well plate according to the General Protocol for Vesicular Transport Inhibition Assay and the corresponding Protocol Data Sheet as in Level 1. Proceed if the result meets the acceptance criteria for the corresponding membrane.**

2. **Thaw every reagent on ice. Assess reaction mix with test article (TA) and add 50 µl per well according to the plate setup. Note that final volumes will be diluted by 1.5 after the addition of ATP/AMP. Prepare sufficient amount of 12 mM ATP/AMP solution in the corresponding transport buffer.**

   Note: In case of radiolabeled TA, 0.1 µCi ^{3}H or 0.01 µCi ^{14}C isotope is recommended unless the sponsor requires otherwise. Different concentrations of the TA can be adjusted by using only the labeled compound or complement with unlabeled compound.

3. **Incubate plate, ATP and AMP at 37°C for 15 minutes with shaking.**

4. **Wet the filter plate as recommended by the supplier and set up the filtering apparatus.**

5. **Start reactions by adding 25 µl of ATP or AMP. Shake plate with the shaker. Preferably longer incubations should be conducted first, and shorter incubations as second.**

6. **Stop reactions with 200 µl of ice cold buffer and transfer samples to a 96-well filter plate. Wash wells 5 times with 200 µl washing buffer.**

7. **Prepare TA samples for cold analysis according to LC-MS Sample Preparation Protocol.**

8. **For evaluation, plot total accumulated amount of TA over time. The result should be considered positive if the signal-to-noise ratio is over 2 and deviation is under 20%.**

Level 3. Determination of Kinetic Constants

At this level kinetic parameters (K_m, V_{max}) are extracted from the underlying interaction.

Consider these guidelines to design substrate saturation and time curve experiments:

The aim is to obtain a dataset that can be fitted with a Michaelis-Menten saturation equation without constraints, where the error of the estimated K_m and V_{max} values are less than 30%. To achieve this at least 8 well placed concentration points should be considered with three replicates. For this at least one 96-well plate and a partial plate for positive control experiment are needed as in Level 2.

	TR						C					
	ATP			AMP			ATP			AMP		
	1	2	3	4	5	6	7	8	9	10	11	12
A	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}	TA_{C1}
B	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}	TA_{C2}
C	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}	TA_{C3}
D	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}	TA_{C4}
E	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}	TA_{C5}
F	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}	TA_{C6}
G	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}	TA_{C7}
H	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}	TA_{C8}

TA_{C1} through TA_{C8}: concentration points of the test article; TR: transporter expressing vesicles, C: parental/mock/not active control vesicles

Positive control experiment should be conducted as in Level 1 and 2.

Assay steps follow Level 1 steps 1-7.

From a given number of wells more concentration points with less replicates produce better fits than less points with more replicates.

Incubation time should be selected from Level 2 experiments to be in the initial linear range. Protein content and incubation temperature must fit Level 2 parameters.

The obtained K_m and V_{max} values is recommended to be confirmed with at least one independent experiment.

A linear set of concentration points produce better fits than a logarithmic set (dilution series).

Reaction speed should be expressed in the unit pmol substrate/minute incubation/mg total protein.

Level 4. Inhibition with Literature Inhibitors

At this level substrate interaction with the transporter is corroborated by inhibition with known inhibitors. For this study, the standard vesicular transport protocol data sheet should be considered with these amendments:

The aim is to obtain a dataset that can be fitted with a non-linear regression – variable slope equation without constraints, where the error of the estimated IC_{50} values (if any) are less than 30%. To achieve this at least 7 well placed concentration points should be considered with three replicates and one vehicle control. For this at least one 96-well plate and a partial plate for positive control experiment are needed as in Level 2.

	TR						C					
	ATP			AMP			ATP			AMP		
	1	2	3	4	5	6	7	8	9	10	11	12
A	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}	IH_{C1}
B	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}	IH_{C2}
C	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}	IH_{C3}
D	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}	IH_{C4}
E	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}	IH_{C5}
F	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}	IH_{C6}
G	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}	IH_{C7}
H	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC

IH_{C1} through IH_{C7}: concentration points of the inhibitor; VC: vehicle control; TR: transporter expressing cells, C: parental/mock/not active control cells

Positive control experiment should be conducted as in Level 1 and 2.

Probe compound is replaced by the TA. TA concentration should be set to meet these criteria:

- **Preferably below K_m/3. This ensures detected IC_{50} is close to K_i, that is a system independent value is obtained.**
- **Ensure reliable detection. Vehicle control should provide a dynamic range of at least 3-fold.**

Incubation time should be set to be in the linear range as obtained in Level 2, and provide for above criteria. Protein content and incubation temperature must fit Level 2 parameters.

Assay steps follow Level 1 steps 1-7.

3. Calculations

Calculate averages and variances from parallels.

Calculate pmol substrate/mg protein with standard deviations in Level 2 and plot result versus time.

Calculate pmol substrate/mg protein/min and standard deviations in Level 3 and fit Michaelis-Menten curve to the data points versus TA concentration.

Calculate percent transport activity values in Level 4 using the following formula:

$$\text{Activity}\%_i = \frac{100 \cdot \frac{A_i}{B_i}}{\frac{C}{D}}$$

A_i: average of TA at inhibitor concentration i on transporter-expressing vesicles

B_i: average of TA at inhibitor concentration i on control wells

C: average of TA in the Vehicle Control wells on transporter-expressing vesicles

D: average of TA in the Vehicle Control wells on control wells

Calculate standard deviation of Activity% values using the formula:

$$SD_{\text{Activity}\%_i} = 100 \sqrt{\frac{Var_{A_i} + Var_{B_i}}{C \cdot D^2} + \frac{Var_C \cdot Var_D \cdot A_i^2 \cdot B_i^2}{C^2 \cdot D^4}}$$

A_i, B_i, C, D: as in the equation above.

Var_{Ai}, Var_{Bi}, Var_C, Var_D: variance of A_i, B_i, C, and D, respectively.

Plot percent activity values with standard deviations versus inhibitor concentration in a scatter graph with a logarithmic x axis. Apply non-linear regression (variable slope dose-response curve) to determine the best-fit IC_{50} value.

Calculate percent activity and deviation for the positive control using the above equations and plot as a column graph.

4. Handling of TA dilution series

Preparation of dilution series:

Every experimental day a fresh dilution series is prepared.

Dilution series are prepared in U-bottom plate.

Multiple dilution series can be prepared on the same U-bottom plate.

Already prepared dilution series are kept at room temperature.

Dilution series with purified water or DMSO can be used in a time frame of maximum 4 hours.

Dilution series of solvents with high evaporation rate must be prepared freshly for every usage.