Protocol Data Sheet for BCRP-HEK293 Vesicular Transport Inhibition Assay

Membrane: **SB-BCRP-HEK293**
Control membrane (optional): **SB-HEK293-Mock-CTRL**
Probe substrate: **E3S**

Version No.: 1.6
Version No. replaced: 1.5
Effective date: 14-Aug-2020

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
Estrone 3-sulfate sodium salt	Sigma E0251	<-15°C
Ko143	Sigma K2144	RT
Magnesium chloride	Sigma M8266	RT
Sodium chloride	Sigma S5886	RT
Sucrose	Sigma S0389	RT
Trizma® base	Sigma T1503	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 0.381 g $MgCl_2$ in 10 ml of 0.1 M Tris-HCl 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		
Ko143, 0.02 mM, in DMSO		<-15°C, < 1 year
Unlabeled substrate		
Estrone 3-sulfate sodium salt, 750 µM, in DMSO		<-15°C, < 1 year
Radiolabeled substrate	**Manufacturer & Cat #**	
^{3}H-E3S: Estrone Sulfate, [^{3}H]-, 1 mCi/mL, in EtOH:water 1:1	BRC Radiolab BL-104	as indicated by the supplier

3. Assay buffers

3.1 Transport buffer with sucrose

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 with sucrose

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 E3S concentration in 75 µl reaction volume	1 µM
Specific activity per well	0.2 µCi
Protein content of membrane suspension per well	12.5 µg
Final concentration of Ko143 (optional)	0.2 µM
Incubation time	1 min
Incubation temperature	32°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-E3S, 1 mCi/mL, 39700 mCi/mmol, 25.2 µM chem.cc.	20.4 µL
Estrone 3-sulfate sodium salt, 750 µM	9.5 µL
Membrane suspension	255 µL
Transport buffer	4815.1 µL
Total volume	5100 µL

5. Special instructions

Wet each well of the filter plate with 100 µl of 100 µM E3S. Incubate for 10 min. Remove liquid from wells by applying vacuum.

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General Protocol for Vesicular Transport Inhibition Assays

Effective from: February 22, 2022

Version: 1.7

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Attn.: Solvo Contract Research Laboratory

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Date: 21-Feb-2022

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
- Calibrated timer

2.2 Consumables

- Pipette tips
- 96-well clear flat bottom cell culture plate for reaction mixture preparation
- 96-well filter plate *(Millipore: MultiScreen®$_{HTS}$-FB, MSFBN6B50* OR *MultiScreen®$_{HTS}$-DV, MSDVN6B50; Corning: FiltrEx^{TM} 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 reagent on ice (except for DMSO stocks).
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 µl of this suspension to all wells of a standard 96-well F-bottom 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 100× 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.

   NOTE: In case of water-based test drug stock solution, a 50x more concentrated stock can be used. In that case, use 1.5 µl of the test drug dilution series in the next step.
4. Add test drugs (0.75 µl) 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.

   NOTE: Test drug dilution series with the appropriate controls could be added to the plate before dispensing the membrane suspension.

NOTE2: If the solvent of the test drug and reference inhibitor is the same and the test drug is applied with 100x dilution factor, using only one solvent control is acceptable (preferably the control lane of the positive control).

5. Mix 90 µl of Mg-ATP with 1410 µl of transport buffer on ice. Be sure that the ATP stock is mixed well before using.
6. Mix 90 µl of AMP with 1410 µl of transport buffer on ice.
7. Preincubate plate, ATP and AMP at the appropriate temperature (see **Protocol Data Sheet**) for 15 min with shaking at 250 rpm.
8. Wet the filter plate as recommended by the supplier for at least 10 minutes and set up the filtering apparatus.

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

9. Stop the shaker and add 25 µl of ATP and AMP (prepared in *steps 5* and *6*) to the wells as indicated on the plate setup below. Preferably start with the AMP wells. Start timer after adding ATP to the wells. 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 (starting with ATP containing wells) by adding 200 µl ice-cold washing buffer. Transfer samples to the filter plate and filter.
11. Wash wells with 5×200 µl of ice-cold washing buffer. Do not keep the filter plate under vacuum for too long without liquid in the wells.
12. Dry filter plate (you can use a hair drier or drying cabinet to speed up the process).

   NOTE: For Memmert Universal drying cabinet use 40 °C for 3-24 hours (Fan: 80 %, Flap: 100 %). Higher temperature could cause the deformation of the plate.

13. Pipette 10 µl of the membrane suspension (prepared in *step 2*) 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*).
14. Add 100 µl 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 13* by 5. Calculate the rate of transport in pmol/mg membrane protein/min using the following formula.

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

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:

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

Calculate standard deviation of Activity% values using the formula:

$$SD_{Activity\,\%_i} = 100 \times \sqrt{\left(\frac{VarB}{A}\right)^2 + \frac{VarA \times B^2}{A^4}}$$

where A is the ATP dependent transport in drug free control
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 µl of the appropriate concentration of the reference inhibitor (see **Protocol Data Sheet**).

5. Recommendations for Laboratory Practice

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.

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General Protocol for Vesicular Transport Substrate Assessment Assay

Effective from: October 19, 2021

Version: 1.5

Previous version: 1.4

Attn.: Solvo Contract Research Laboratory

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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. Considerations for buffers

The components of transport and washing buffers described in the **Protocol Data Sheets** may vary among the different transporter assays, and some of these specifications are substrate dependent. Therefore, the following alterations from the **Protocol Data Sheet** of a certain transporter are recommended:

- *Washing buffer for BSEP* contains unlabeled taurocholate to decrease the binding of the labeled substrate to the filter plate. It is not needed for substrate assessment; however, it has no other effect on the assay than decreasing the potential non-specific binding of the substrate to the filter plate. One exception when the measured substrate is also unlabeled taurocholate, in which case it should be changed to another bile salt (e.g. glycocholate).
- MRPs might co-transport their substrates with glutathione (GSH), therefore the addition of GSH to the transport buffer should be considered.
- *Washing buffer with sucrose and BSA* contains BSA to decrease the binding of ^{3}H-DHEAS to the filter plate in the MRP4 inhibition assay. *Washing buffer with sucrose* could be used instead for substrate assessment.

If the test article (TA) has high non-specific binding to the filter plate:

- when measuring labeled TA, the addition of the unlabeled compound to the washing buffer or wetting the filter plate with substrate solution might be considered.
- the addition of 1 % BSA to the washing buffer might be considered.

3. 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**).
Alternatively, the positive control can be measured on separate plate.

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.** To decrease the organic solvent content, it is recommended to use 200-1000x more concentrated stock instead of the 100x concentrated from the **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, except DMSO stocks. 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. Calculate with total samples and add at least 10 % excess of the final volume of every mixture for easier pipetting; assemble reaction mixture(s). It is recommended to use 1000x concentrated substrate stock and mix at least 1000 ml to reduce solvent content and avoid pipetting small volumes. Even out vehicle content in all mixtures but keep it under 2 v/v%. 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 at 250 rpm.
4. Wet the filter plate with destilled water for at least 10 minutes and set up the filtering apparatus.
5. Start reactions by adding 25 µl of ATP or AMP. Preferably start with the AMP wells. Start timer after adding ATP to the wells. Longer incubations should be conducted first, and shorter incubations as second.
6. Stop reactions reaction (starting with ATP containing wells) 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. Do not keep the filter plate under vacuum for too long without liquid in the wells.
7. In case of unlabeled TA, prepare TA samples for cold analysis according to **PR-ASY-Sample-General Protocol for Sample Preparation**.

8. In case of radiolabeled TA, dry filter plate (you can use a hair drier or drying cabinet to speed up the process) and add 100 µl cocktail to all sample wells.

   NOTE: For Memmert Universal drying cabinet use 40 °C for 3-24 hours (Fan: 80 %, Flap: 100 %). Higher temperature could cause the deformation of the plate.

9. 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, except DMSO stocks. 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. Calculate with total samples and add at least 10 % excess of the final volume of every mixture for easier pipetting; assemble reaction mixture(s). It is recommended to use 1000x concentrated substrate stock. Even out vehicle content in all mixtures but keep it under 2 v/v%. 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 at 250 rpm.
4. Wet the filter plate with destilled water for at least 10 minutes and set up the filtering apparatus.
5. Start reactions by adding 25 µl of ATP or AMP. Preferably start with the AMP wells. Start timer after adding ATP to the wells. Longer incubations should be conducted first, and shorter incubations as second.
6. Stop reactions reaction (starting with ATP containing wells) 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. Do not keep the filter plate under vacuum for too long without liquid in the wells.
7. In case of unlabeled TA, prepare TA samples for cold analysis according to **PR-ASY-Sample-General Protocol for Sample Preparation**.
8. In case of labeled TA, dry filter plate (you can use a hair drier or drying cabinet to speed up the process) and add 100 µl cocktail to all sample wells.

NOTE: For Memmert Universal drying cabinet use 40 °C for 3-24 hours (Fan: 80 %, Flap: 100 %). Higher temperature could cause the deformation of the plate.

9. 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 1-8.

- 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 1-8.

4. 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:

$$Activity\%_i = 100 \times \frac{A_i - B_i}{C - D}$$

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:

$$SD_{Activity\%_i} = 100 \times \sqrt{\frac{Var_{A_i} + Var_{B_i}}{(C - D)^2} + \frac{(Var_C + Var_D) \times (A_i - B_i)^2}{(C - D)^4}}$$

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.

5. Recommendations for Laboratory Practice

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.