# BCRP PREDIVEZ Protocol

## CAT. NO. SBPV04

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# Determination of the interaction of drugs using the BCRP-M Fluorescent PREDIVEZ Kit

**For the following membrane product:**
SB-BCRP-M-VT

**Version Number:**

**1.3**

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**Replaces:**
**1.2**

**Related Procedures:**

**SOP FFSS01**

**Signatures:**

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# 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 BCRP-M transporter using the vesicular transport assay. The interaction is detected as the modulation of the initial rate of Lucifer yellow transport of BCRP into membrane vesicles purified from mammalian cells expressing the transporter.

# 2. Principle

The human BCRP transporter can be stably expressed in mammalian cells. Membrane preparations prepared from these cells always contain some closed membrane vesicles that are in inside-out orientation. Due to the orientation of the transporter, the transported substrates accumulate inside the vesicle. In case of low permeability substrates, such as Lucifer Yellow, the molecules get trapped inside the vesicle. The rate of this transport is temperature and ATP dependent.

Rapid filtration of the membrane suspension through a filter that retains membrane vesicles allows us to separate the transported molecules trapped from the rest of the buffer.

The quantity of transported molecules can be determined by any adequate method like HPLC, LC/MS/MS separation and detection. Also, the transported molecule can be labeled by fluorescent or radioactive tags. This protocol utilizes a fluorescent drug surrogate, Lucifer yellow, for the detection of the transported substrate in a competition type assay.

BCRP mediates the transport of Lucifer yellow efficiently. Drugs that interact with the transporter modulate the initial rate of Lucifer yellow transport measured without

any other compounds added. If a test drug is a transported substrate of the transporter it might compete with Lucifer yellow thus reducing the rate of Lucifer yellow transport. If a compound is an inhibitor of the transporter, it will block the transport of Lucifer yellow into the membrane vesicles.

## 3. Deliverables

- SOLVO Biotechnology's SB-BCRP-M Fluorescent PREDIVEZ Vesicular Transport Assay Kit for BCRP transporter sufficient for the analysis of 3, 6 or 9 test compounds.
- Data sheet indicating protein content, volume, ATP dependent transport at 50 μM Lucifer yellow concentration and date of expiry of frozen membrane stocks.
- Data CD containing assay protocol, MS Excel file for calculations and data representation, and material safety data sheets.

## 4. Equipment and Materials needed

- Plate incubator/shaker.
- Automatic pipettes and multichannel pipettes with corresponding tips
- 96-well plates (Costar, Cat. No. 3585, or equivalent)
- Filterplates [Millipore multiscreen HTS 96 well filter plates with FB filters (Cat. No. MSFBN6B10) or equivalent]
- Rapid filtration apparatus [Multiscreen™ HTS Vacuum Manifold from Millipore (Cat. No MSVMHTS00) or equivalent]
- Fluorimeter suitable for the 96-well format (Ex: 430 Em: 538)
- 2 ml, 5 ml tubes
- 150 ml cylinder and Tip-Tubs (Eppendorf, Cat. No. 0030 058.607)

- MilliQ water
- Dimethyl sulfoxide (DMSO, A.C.S reagent spectrophotometric grade, ≥99,9%, Sigma 154938)

## 5. Materials

Your kit contains the following materials in amounts depending on the requested Kit size:

| Kit size (number of cpds) | | 3 | 6 | 9 | Storage | Assay conditions |
|---|---|---|---|---|---|---|
| **Vial** | **Substance** | **Amount** | | | | |
| **A** | Membrane stock (5 mg/ml) | 3x420 µl | 6x420 µl | 9x420 µl | -80 °C | on ice |
| **B** | 10x Assay Mix | 1.0 ml | 2.0 ml | 3.0 ml | ≤+4 °C | on ice |
| **C** | Lucifer yellow (10 mM) | 110 µl | 220 µl | 330 µl | ≤-20 °C | RT |
| **D** | MgATP solution (0.2 M) | 120 µl | 240 µl | 360 µl | ≤-20 °C | on ice |
| **E** | Inhibitor drug stock (30 mM Omeprazole) | 50 µl | 100 µl | 150 µl | ≤-20 °C | RT |
| **F** | Lucifer yellow for calibration (200 µM) | 50 µl | 100 µl | 150 µl | ≤-20 °C | RT |
| **G** | 10x Washing Mix | 14.5 ml | 2x14.5 ml | 3x14.5 ml | ≤+4 °C | on ice |
| **H** | 10x Detector Solution | 1.75 ml | 3.5 ml | 5.25 ml | ≤RT | RT |
| **I** | Negative control membrane stock (5 mg/ml) | 200 µl | 2x200 µl | 3x200 µl | -80 °C | on ice |
| **J** | AMP solution (0.2 M) | 120 µl | 240 µl | 360 µl | ≤-20 °C | on ice |

Keep the kit compounds during the assay procedure at the temperature specified in this table. Material safety data sheets of the compounds in your Vials are available as pdf files in the MSDS folder on the CD-ROM attached to the KIT box.

**Do not use substances from any other type of PREDIVEZ Kit.**

## 6. Suggested assay layouts

**Assay Layout 1. (Relative Transport values)**

**Assay layout for presenting results in percentages:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Compound 1 | | | | Compound 2 | | | | Compound 3 | | | |
| | + ATP | | -ATP (AMP) | | + ATP | | -ATP (AMP) | | + ATP | | -ATP (AMP) | |
| **A** | 300 µM | | 300 µM | | 300 µM | | 300 µM | | 300 µM | | 300 µM | |
| **B** | 100 µM | | 100 µM | | 100 µM | | 100 µM | | 100 µM | | 100 µM | |
| **C** | 33.3 µM | | 33.3 µM | | 33.3 µM | | 33.3 µM | | 33.3 µM | | 33.3 µM | |
| **D** | 11.1 µM | | 11.1 µM | | 11.1 µM | | 11.1 µM | | 11.1 µM | | 11.1 µM | |
| **E** | 3.7 µM | | 3.7 µM | | 3.7 µM | | 3.7 µM | | 3.7 µM | | 3.7 µM | |
| **F** | 1.23 µM | | 1.23 µM | | 1.23 µM | | 1.23 µM | | 1.23 µM | | 1.23 µM | |
| **G** | 0.41 µM | | 0.41 µM | | 0.41 µM | | 0.41 µM | | 0.41 µM | | 0.41 µM | |
| **H** | DMSO | | DMSO | | DMSO | | DMSO | | DMSO | | DMSO | |

Note: If your test drug is not dissolved in DMSO replace DMSO with that solvent.

**Assay Layout 2. (Absolute Transport values)**

**Assay layout for calculating ATP dependent transport (pmol/mg/min) transport values:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Calibration curve | | | | Compound 1 | | | | Compound 2 | | | |
| | Lucifer yellow | | | | + ATP | | -ATP (AMP) | | + ATP | | -ATP (AMP) | |
| **A** | 200 pmol | | | | 300 µM | | 300 µM | | 300 µM | | 300 µM | |
| **B** | 100 pmol | | | | 100 µM | | 100 µM | | 100 µM | | 100 µM | |
| **C** | 50 pmol | | | | 33.3 µM | | 33.3 µM | | 33.3 µM | | 33.3 µM | |
| **D** | 25 pmol | | | | 11.1 µM | | 11.1 µM | | 11.1 µM | | 11.1 µM | |
| **E** | 0 pmol | | | | 3.7 µM | | 3.7 µM | | 3.7 µM | | 3.7 µM | |
| **F** | +ATP | | -ATP | | 1.23 µM | | 1.23 µM | | 1.23 µM | | 1.23 µM | |
| **G** | +ATP | | -ATP | | 0.41 µM | | 0.41 µM | | 0.41 µM | | 0.41 µM | |
| **H** | +ATP | | -ATP | | DMSO | | DMSO | | DMSO | | DMSO | |

**Dark grey wells represent measurement with negative control membrane**

## 7. Assay steps

Prepare your solutions freshly before use. Always use MilliQ water as distilled water to prepare the solutions. The steps are for assaying **1 compound** (see Assay Layout on page 6)!

1. Prepare serial dilution of the drug to be assayed or of the Inhibitor (Vial **E**). (Use DMSO as solvent).
2. Dilute reagents as follows:

   Dilute 250 µl 10x Assay Mix (Vial **B**) to 2.5 ml with 2.25 ml distilled water. (Store 1x Assay Mix on ice)

   Dilute 4.25 ml 10x Washing Mix (Vial **G**) to 42.5 ml with 38.25 ml distilled water. (Store 1x Washing Mix on ice or in the fridge)

   Dilute 400 µl 10x Detector (Vial **H**) to 4 ml with 3.6 ml distilled water (Keep this solution at room temperature).
3. Prepare the MgATP solution

   Dilute 30 µl 0.2 M MgATP solution (Vial **D**) to 500 µl with 470 µl Assay Mix.

   Keep the MgATP solution on ice.
4. Prepare the AMP solution

   Dilute 30 µl 0.2 M AMP solution (Vial **J**) to 500 µl with 470 µl Assay Mix.

   Keep the AMP solution on ice.
5. Prepare the Membrane Suspension in Assay Mix.

   Homogenize your Membrane stock with gentle pipeting. Add 360 µl Membrane stock (Vial **A**) and 13.5 µl Lucifer yellow (Vial **C**) to 1426.5 µl Assay Mix. (Mix well, gently!)

   Keep your suspensions on ice.
6. Place a 96 well plate on ice.

7. Add 50 µl Membrane Suspension to the wells of the first 4 columns. This way one well will contain 50 µg total membrane protein.
8. Add 0.75 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells (see Assay Layout on page 6).
9. Preincubate your plate, MgATP and AMP solution at 37°C for 10 minutes.
10. Start reaction by adding 25 µl MgATP or AMP solution to the appropriate wells (see Assay Layout).
11. Incubate your plate at 37 °C for 10 minutes
12. Wet the first four columns of the Millipore filter plate with 100 µl distilled water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.
13. Stop the reaction by adding 200 µl of ice cold 1xWashing Mix to every well.
14. Transfer all the solution from the 96 well plate to the Millipore filter plate.
15. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1x Washing Mix per well.
16. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
17. Add 100 µl 1xDetector to every well and incubate for 10 minutes at room temperature.
18. Transfer the liquid under vacuum to a clear 96 well plate (a black-walled, clear-bottom plate for fluorescence can be used as well).
19. Measure fluorescence at Ex: 430, Em: 538.
20. Analyze your data.

## Optional assay steps:

### Preparation of Lucifer yellow calibration curve

With the help of the calibration curve, the interaction of the test drug and the reporter substrate can be presented in absolute transport values (pmol/mg protein/min). The

measurement is optional and can be performed on a separate plate as well. However, we suggest preparing a calibration curve before the first experiment is done.

1. Dilute 250 µl 10x Detector (Vial **H**) to 2.5 ml with 2.25 ml distilled water.
2. Prepare 2 µM Lucifer yellow solution by adding 10 µl of Vial **F** to 990 µl 1x Detector.
3. Prepare 1 µM Lucifer yellow solution by mixing 500 µl 2 µM Lucifer yellow solution with 500 µl 1x Detector solution.
4. Prepare 0.5 µM Lucifer yellow solution by mixing 500 µl 1 µM Lucifer yellow solution with 500 µl 1x Detector solution.
5. Prepare 0.25 µM Lucifer yellow solution by mixing 500 µl 0.5 µM Lucifer yellow solution with 500 µl 1x Detector solution.
6. Wet the appropriate wells of the Millipore filter plate with 100 µl distilled water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.
7. Add 100 µl of these solutions to the wells of the 96-well filter plate (see Assay Layout 2. on page 6)
8. Filter the solutions to a clear-bottom 96-well plate using the plate-to-plate filtration system.
9. Measure fluorescence at Ex: 430, Em: 538
10. Analyze data using raw data template

**Lucifer yellow transport by SB-M (negative control)**

The Kit contains a vial of SB-M membrane as well (Vial **I**), that serves as a negative control. These vesicles show minimal accumulation of Lucifer yellow. Transport in the absence (DMSO – recommended) or in the presence of a test drug (one concentration – highest is recommended) can be tested. The measurement is optional and can be performed on a separate plate as well.

1. Dilute reagents as follows:

Dilute 120 µl 10x Assay Mix (Vial **B**) to 1200 µl with 1080 µl distilled water. (Store 1x Assay Mix on ice)

Dilute 1.6 ml 10x Washing Mix (Vial **G**) to 16 ml with 14.4 ml distilled water. (Store 1x Washing Mix on ice or in the fridge)

Dilute 150 µl 10x Detector (Vial **H**) to 1.5 ml with 1.35 ml distilled water (Keep this solution at room temperature).

2. Prepare the MgATP solution

   Dilute 15 µl 0.2 M MgATP solution (Vial **D**) to 250 µl with 235 µl Assay Mix.

   Keep the MgATP solution on ice.

3. Prepare the AMP solution

   Dilute 15 µl 0.2 M AMP solution (Vial **J**) to 250 µl with 235 µl Assay Mix.

   Keep the AMP solution on ice.

4. Prepare the Membrane Suspension in Assay Mix.

   Homogenize your Membrane stock. Add 160 µl Membrane stock (Vial **I**) and 6 µl Lucifer yellow (Vial **C**) to 634 µl Assay Mix. (Mix well, gently!)

   Keep your suspensions on ice.

5. Place a 96 well plate on ice.
6. Add 50 µl Membrane Suspension to the wells indicated on the Assay Layout 2. This way one well will contain 50 µg total membrane protein.
7. Add 0.75 µl of DMSO/ test drug (in DMSO or in your solvent) to the wells
8. Preincubate your plate and MgATP solution at 37°C for 10 minutes.
9. Start reaction by adding 25 µl MgATP or AMP solution to the appropriate wells (see Assay Layout 2.).
10. Incubate your plate at 37 °C for 10 minutes

11. Wet the appropriate wells of the Millipore filter plate with 100 µl distilled water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.
12. Stop the reaction by adding 200 µl of ice cold 1xWashing Mix to the wells.
13. Transfer all the solution from the 96 well plate to the Millipore filter plate.
14. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl Washing Mix.
15. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
16. Add 100 µl 1xDetector to the wells and incubate for 10 minutes at room temperature.
17. Transfer the liquid under vacuum to a clear 96 well plate (a black-walled, clear-bottom plate for fluorescence can be used as well).
18. Measure fluorescence at Ex: 430, Em: 538.
19. Analyze your data.

## 8. Calculations

**ATP dependent transport (fluorescence):** Subtract fluorescence values measured in the absence of ATP from the fluorescence values measured in the presence of ATP for control and samples. Take the average of the duplicates.

**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:

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

**ATP dependent transport (pmol/mg/min):** For this calculation use Assay Layout 2! Set up a calibration curve with the help of the measured fluorescence values and the Lucifer yellow concentrations used. Substitute the fluorescence values into the equation of the calibration curve and calculate the amount of Lucifer yellow / well (pmol). Divide this value by the amount of protein per well (mg) and by the time (min).

**Calculation of results using the raw data template file**

Use your Excel Template file to calculate results in case of applying the suggested Assay Layouts (see page 8.). The Template file is designed to analyze one test drug at a time!

All required fields are highlighted with light green and are editable. Fields that you do not need to change are read only. Charts are editable. Copy your raw data to the RAW

DATA field of the template file. Fill header (date, membrane batch, membrane amount/well, incubation time, etc.) carefully. Check your test drug concentrations and change the value of the highest final concentration if it is necessary. Fill the DRUG NAME field.

The file can be used in both calculation modes: percentages and absolute transport values as well. Analyze your results.

## 9. Expected Results

Relative transport values (%)

This curve shows the effect of the test drug on Lucifer yellow transport by BCRP in percentages. 100% represent Lucifer yellow transport by BCRP in the absence of test drug (row H in the plate setup), while 0% is the transport in the absence of ATP (non-specific binding of Lucifer yellow). This representation is very helpful if the activities of multiple test drugs are compared.

If the test drug interacts with the Lucifer yellow transport, then a dose-dependent decrease in transport is observed. The $IC_{50}$ value for the test drug is the concentration where the Lucifer yellow transport is inhibited by 50%. In case of a non-interactor, the curve does not fall below 100%.

Absolute transport values (pmol/mg protein/min)

This curve shows the effect of the test drug on Lucifer yellow transport by BCRP in absolute transport values. This representation is important to check the performance of the transporter or for other purposes, e.g. publications.

## 10. Troubleshooting

In case of a fluorescent test drug, the analysis of the results might be difficult, especially if the excitation and emission spectra of the compounds overlap. In cases

like this, we recommend that the fluorescent test compound should be tested using a Vesicular Transport assay utilizing a radioactive reporter substrate.

The sensitivity and scaling of fluorimeters change from instrument to instrument. Even if you prefer to analyze your data using relative transport values, we recommend the preparation of a calibration curve, in order to see the fluorescence values your instrument produces. Differences among fluorimeters may account for higher background fluorescence values.

Some test compounds that are not highly soluble in aqueous solutions may precipitate in high concentrations, which might not be visible. In cases like this an increase in fluorescence in both ATP + and ATP – wells is observed, due to the incomplete filtration. We recommend the use of lower test drug concentrations, in order to get valid results.