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PreadyPort BCRP

24-inserts with differentiated BCRP-expressing MDCKII cells

USER'S MANUAL (Corning Plate)

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Intended Use

The contents of this product are designed to conduct monolayer transport experiments. Monolayer assays may be used for the evaluation of interactions with drug transporters and also to model pharmacological barriers.

This kit is optimized for interaction studies with the human breast cancer resistance protein (BCRP, MXR, ABCG2).

Handling and experimental procedures are provided below. The manual has been written for users with experience in cell culturing and monolayer transport experiments. For more detailed advice and training opportunities please contact:

support@solvo.com, or
helptech@readycell.com

The kit is intended for *in vitro* purposes only. Not for human or veterinary use.

Principle

In the monolayer transport experimental setup, two solute compartments are separated by a tight cell monolayer. The distribution of compounds between the two compartments is determined by qualities of the cell monolayer, most notably the presence of transporter proteins in the cell membrane.

The differentiation of the cells defines an apical and a basolateral solute compartment as well as the localization of transporter proteins. Apically localized efflux transporters will introduce a basolateral-to-apical bias in the distribution of substrate compounds.

In a standard assay design, the reaction is initiated by filling either compartment with the solution containing the test compound. The distribution is assessed over time by withdrawing and analyzing samples from both compartments. After normalization the speed of translocation is obtained in both directions. The ratio of the two values is a measure of active processes involved in the distribution of the compound.

Timelines for Delivery and Experimental Procedures

- Day 1: Starting production (Seeding of cells)
- Day 4: Pre-shipping quality control (TEER and Lucifer Yellow)
- Day 5: Package Dispatch
- Days 6-7: Package Delivery
- Day 8: Replacement of Shipping Medium
- Day 11: Quality Control Experiments, Medium Replacement
- Days 12-15: Transport Experiments

Packages are dispatched on Tuesday and are delivered within two days in EU countries. For other locations and customized schedule please contact us at

support@solvo.com, or
helptech@readycell.com
+ 34 93 403 70 77

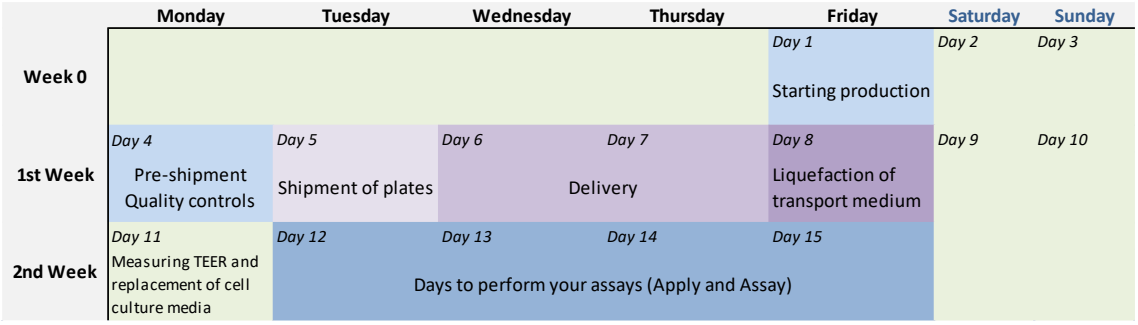


Figure 1. Timeline of manufacturing and operation for PreadyPort™ BCRP 24-well kits

The recommended timing for transport experiments is **Day 12 (Tuesday)**. If you choose to conduct the experiments later, medium must be replaced as follows:

DAY OF EXPERIMENT	MEDIUM REPLACEMENT
Tuesday (Day 12)	Monday (Day 11)
Wednesday (Day 13)	Monday (Day 11)
Thursday (Day 14)	Monday (Day 11) and Wednesday (Day 13)
Friday (Day 15)	Monday (Day 11) and Wednesday (Day 13)

Table 1. Recommended days for medium replacement

Equipment Not Included

- Cell culture laminar flow hood
- CO_2 incubator
- Aspiration system
- Multichannel pipettes
- Automatic multichannel micropipettor (volume range: 50-1200 µl)
- 24-well format vacuum manifold (Drummond, cat. No 3-000-097 recommended)
- Trans-Epithelial Electrical Resistance (TEER) meter (Ref Millicell© ERS-2 or WPI EVOM2)
- Recommended 24-well electrode (Ref WPI STX100C)
- Quantitative analytics equipment

Consumables Not Included

- 24-well receiver plates (Costar®, Ref. 3526)
- Reagent reservoirs (Costar, Ref. 4870 recommended)
- Pipette tips

Material Not Included

- **MDCKII Cell Culture Medium**. Dulbecco's Modified Eagle Medium with the following supplements (final concentration):
    - 5 mM Glucose
    - 25 mM HEPES
    - 10% V/V Fetal Calf Serum
    - 2 mM L-glutamine
    - 100 U/mL; 0.1 mg/mL Pen/Strep
    - 1x MEM non-essential amino acid solution
- **Cell Culture Medium for induction of BCRP expression**
    - MDCKII Cell Culture Medium supplemented with 1 mM sodium butyrate (Sigma-Aldrich, Ref. 303410)
- **Assay Buffer**

For the preparation, dissolve the following compounds in 800 ml purified water (dry powder weight):

- 4 .8 mM KCl (0.36 g)
- 0.96 mM KH_2PO_4 anhydrous (0.17 g)
- 1.2 mM $MgSO_4 \cdot 7H_2O$ (0.3 g)
- 12.5 mM HEPES (3 g)
- 1.53 mM $CaCl_2$ (0.17 g)
- 142 mM NaCl (8.3 g)
- 5 mM glucose (1 g)

Adjust pH to 7.4 with 1 M NaOH.

Bring total volume to 1000 ml and filter the solution using a 0.22 µm sterile filter.

Buffer can be stored at 2-8°C for a maximum of 1 month after sterilizing.

- **Recommended reporter substrate:** 1 µM Prazosin (Sigma-Aldrich, P-7791) in DMSO
- **Recommended Inhibitor:** 1 µM Ko134 (SOLVO Biotechnology, SB Ko134) in DMSO

Unpacking and Maintenance

Unpacking

Upon reception retrieve the zip-top bags containing the plates. Open the zip, and leave the bag at a dark location at room temperature until Day 8 (refer to Timelines).

Replacement of Shipping Medium

Caution: Never handle more than one plate at a time while changing the shipping medium. Re-solidification of the shipping medium may provoke mechanical damage to the cell monolayer.

These steps have to be carried out on Day 8 (refer to Timelines and see Figure 2) (Perform all manipulation under sterile conditions).

1. Retrieve the plates from the bags and remove the Parafilm wrap.
2. Incubate the plates in a 5% CO_2 humidified atmosphere at 37°C for **4 hours.**
3. Remove one PreadyPort™ plate from the incubator and place inside the laminar flow hood, along with one receiver plate.
4. Using sterile procedures (inside the laminar flow hood):
   - Fill a sterile reagent reservoir with 50 ml of pre-warmed (37°C) MDCKII culture medium.
   - Open the PreadyPort™ plate and the receiver plate, and leave the lids upwards, next to the plates.
   - Carefully lift up the apical inserts of the PreadyPort™ plate and transfer on top of the receiver plate.
   - Remove all liquefied shipping medium from the basal compartments of the PreadyPort™ plate via aspiration with the 24-well manifold.
   - Using a multichannel pipette, dispense **900 µl** of MDCKII cell culture medium from the sterile reservoir into the wells of the **basal compartments** of the PreadyPort™ plate.
   - Using the aspiration manifold connected to a vacuum (adjust aspiration flux to medium-low), aspirate the liquefied shipping medium from the apical integrated inserts of the PreadyPort™ plate placing the manifold perpendicular to the cell monolayer and close to the insert wall. Check that the shipping medium has been removed from all wells. Approximately, 70 µl of medium will be left in each well.
   - Using a multichannel pipette, dispense **300 µl** of MDCKII cell culture medium from the sterile reservoir, and fill, column by column, each of the **apical inserts** of the PreadyPort™ plate. Always add the medium against the wall of the well, not onto the center directly on the cell monolayer.
   - Carefully return the apical inserts onto the basal compartment of the PreadyPort™ plate. Replace the lid and place inside cell culture incubator, set at 37°C, with 5% CO_2.

5. Once shipping medium has been replaced by the fresh MDCKII cell medium, plates should be kept inside the incubator until next Monday (Day 11).

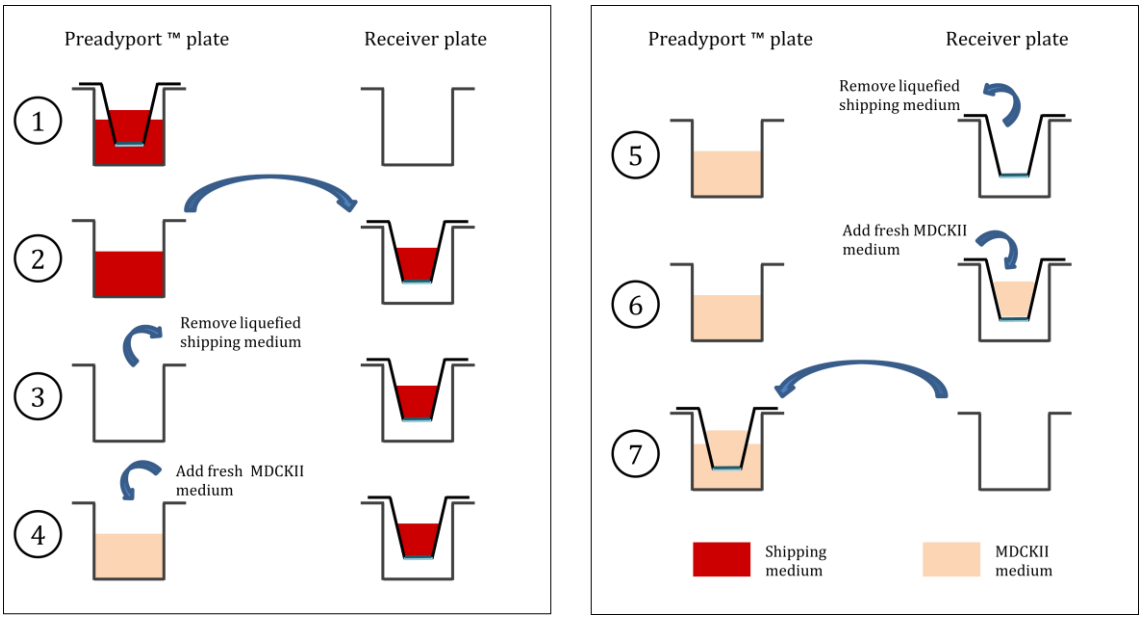


Figure 2. Scheme of the replacement of the shipping medium

Exchanging of Cell Culture Medium

Exchanging the cell culture medium has to be carried out once (at day 11) or twice (at day 11 and day 13) depending on the day of the assay, following the same procedure described in the previous section (refer to Timelines and see Figure 2). Always work under sterile conditions. If Day 12 is the set date for transport experiments, these steps have to be carried out with cell culture medium for induction of protein expression (cell culture medium supplemented with sodium butyrate), as described in the "Material Not Included" section.

Induction of Protein Expression

The final cell culture medium replacement, which takes place the day before transport experiment is conducted, must be done with **sodium butyrate** supplemented Cell Culture Medium (1 mM final concentration), as described in the "Material Not Included" section. All procedure steps are identical to those described in the section "Replacement of Cell Culture Medium".

Quality Control of the Barrier System

1. Pre-assay Quality Control - Measuring TEER

This section provides general instructions for TEER measurements. It is important to read the instructions of the TEER measurement equipment carefully in conjunction with these instructions:

On Day 11 (Monday) perform TEER measurement on the plate/plates **before any further processing.**

To proceed with the TEER measurement, follow the steps below:

1. **It is highly recommended to use the WPI STX 100C electrodes, in order to avoid any damage in the cell monolayer.**
2. Sterilize the electrodes (probe) by submerging both electrodes in ethanol (70%) for 30 minutes.
3. Equilibrate the electrodes (probe) for 30 minutes in MDCKII medium, pre-warmed at room temperature.
4. While the electrodes are equilibrating, remove the PreadyPort™ plate from the incubator and place in a laminar flow hood. Allow the plate to reach room temperature (approximately 20 minutes), as TEER measurements should be performed at room temperature.
5. Insert the probes into the insert system, so the thinner electrode is placed within the narrowest slit, which corresponds to the basal part of the inserts. The thicker electrode must be placed inside the widest slit, corresponding to the apical part of the inserts.
6. Record the TEER value for each well. A TEER value of 100 $\Omega \cdot cm^2$ (or higher) indicates that the barrier system is acceptable for a transport assay (the active membrane surface is 0.33 cm^2).

KEY DATA

Membrane area	**0.33 cm^2**
Minimum TEER value	**100 $\Omega \cdot cm^2$**

**if WT is included in the kit, TEER value has to be ≥ 83 $\Omega \cdot cm^2$*

IMPORTANT TIPS:

- TEER reading should be carried out 48 hours after medium changing in order to verify the integrity of the monolayer prior to the permeability assay. **Never perform the TEER measurement with the shipping medium.**
- Do not repeat TEER measurements on the same insert.
- For an appropriate TEER measurement, both electrodes have to be submerged well within the cell culture medium both in the apical and basal compartments.

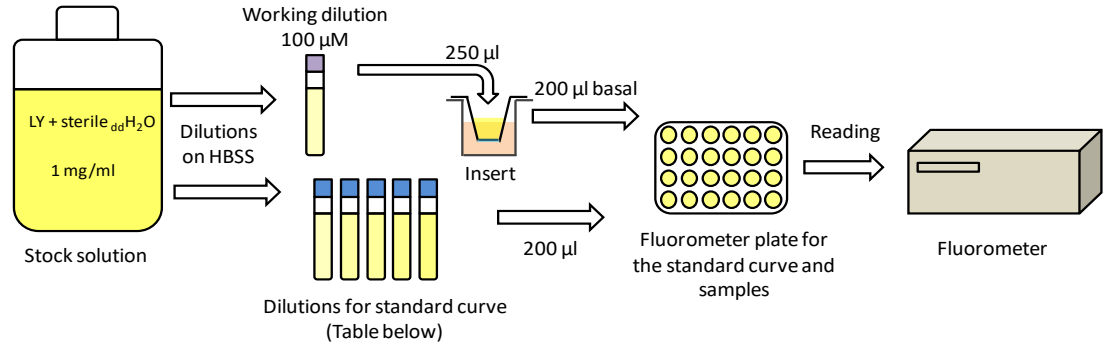
2. Post-assay Quality Control - Lucifer Yellow paracellular permeability assay

Prepare a Lucifer Yellow stock solution of 1 mg/ml in sterile ddH_2O. Aliquot (for example 500 µl) and store at –20°C.

From the stock solution, prepare a 100 µM working dilution in assay buffer to be used for the standard curve and the LY test. These dilutions of LY should be pre-warmed (37°C) before application (see *figure 3* for recommended concentrations).

To proceed with the LY permeability assay follow the steps below:

1. Rinse both the apical and the basal compartment gently with HBSS buffer working solution (250 µl apical/750 µl basal).
2. Add 250 µl of LY working dilution into the apical compartment.
3. Add 750 µl of HBSS buffer working solution to the basal compartment.
4. Incubate the PreadyPort™ plate, protected from light, in a 37°C cell culture incubator for 1 hour.
5. Recover 200 µl from each well in the basal compartment and dispense onto corresponding wells on a empty fluorometer plate (mix well and avoid bubble formation when dispensing each aliquot).
6. Read the fluorescence intensity directly in a fluorometer, using a 485 nm excitation filter and a 527 nm emission filter.



0.00 µM	0.10 µM	0.20 µM	0.30 µM	0.40 µM	0.50 µM	0.75 µM	1.00 µM	10.00 µM	25.00 µM	50.00 µM	100.00 µM

Figure 3: General procedure and recommended concentrations for the standard curve

Under standard PreadyPort™ conditions **the amount of translocated LY should not exceed 2% of initial amount on the donor side**. Results over this threshold signals damage to the barrier incurred during the transport experiment.

KEY DATA

LY Paracellular Flux	**≤ 2%**
LY Papp	**≤ 4.5 x 10^{-6} cm/s**

IMPORTANT TIPS:

- Perform the LY Assay after the test compound transport assay.
- It is highly recommended that some of the inserts are reserved and used exclusively for testing LY permeability, as a quality control test of the monolayer status.

General Protocol for Transport Assays

Recommended Controls

The following controls are recommended to be included in all plates of the PreadyPort-BCRP kit:

- Active transport (1 µM prazosin, both directions)
- Selective inhibition of prazosin active transport (1 µM Ko134, both directions)

The following controls are recommended as optional measures of passive flux:

- Passive transcellular flux (10 µM atenolol or 10 µM metoprolol, apical to basolateral)
- Paracellular flux (100 µM lucifer yellow, apical to basolateral)

1. General Protocol Apical-to-Basal studies

Test compounds are applied to the apical side of the cell monolayer (upper compartment of the insert), and the apical-to-basal (A-B) transport through the cell barrier is evaluated by sample recovery and test compound detection in the basal (lower) compartment over a defined incubation period. A-B permeability of test compounds is determined as the coefficient of apparent permeability (P_{app}) in cm/s.

The protocol provided below recommends the following conditions for performing transport assays:

- Test compound concentration: 10 µM (recommended)
- Replicates: 3
- Time points: 2 (0 and 2 hours)
- Volumes:
    - Apical compartment: 250 µl of the test compound at working solution (275 µl is added to the basal compartment and 25 µl is recovered at time 0 hr.)
    - Basal compartment: 750 µl of HBSS assay Buffer

***Note 1**: All the following manipulations must be performed under standard **sterile conditions** used for cell culture*

***Note 2**: **CAUTION**, do not add the Lucifer Yellow reagent together with the test compounds. Lucifer Yellow may interfere with certain substances, producing false results*

1- Prepare stock solutions of the test compounds in assay buffer. In case of **poorly water-soluble compounds**, DMSO may be used at a final concentration of 1% in assay buffer without significantly affecting the barrier properties.

2- Prepare working dilutions of test compounds in assay buffer.

3- Fill a sterile reagent reservoir with 50 ml of pre-warmed (37°C) assay buffer.

4- Unwrap one receiver plate in the laminar flow hood where the experiments will be performed.

5- Remove one PreadyPort™ plate from the incubator and place it in the laminar flow hood beside the receiver plate. Both plates should be oriented in the same way.

6- Open the PreadyPort™ plate and the receiver plate, and leave the lids upwards next to the plates.

7- Carefully lift the 24 apical inserts of the PreadyPort™ plate and transfer on top the receiver plate.

8- Remove all MDCKII medium from the wells of basal compartments of the PreadyPort™ plate via aspiration using the 24-well manifold.

9- Using a multichannel pipette, fill, column by column, each of the 24 wells of basal compartments of the PreadyPort™ plate with 750 µl of pre-warmed (37°C) assay buffer.

10- Remove all MDCKII medium from the apical inserts of the PreadyPort™ plate by aspiration using the 24-well manifold. Place the manifold perpendicular to the cell monolayer and close to the insert wall, and take care not to disrupt the cell layer.

11- Using a multichannel pipette, fill, column by column, each of the 24 apical compartments of the PreadyPort™ plate with 250 µl of pre-warmed (37°C) assay buffer.

12- Carefully return the 24 apical inserts onto the wells of the basal compartment of the PreadyPort™ (original position).

13- Incubate the plate for 1 minute at room temperature inside the laminar flow hood.

14- Repeat rinsing the plate (steps 8 to 12), two more times and keep the plate for 30 min at 37ºC inside a 5% CO_2 incubator.

15-Take the plate from the incubator, return it to the laminar flow hood and place it next to the receiver plate. Both plates should be oriented in the same way.

16- Carefully lift the 24 apical inserts of the PreadyPort™ plate and transfer them to the top of the receiver plate.

17- Remove the assay buffer from the basal compartments of the PreadyPort™ plate by aspiration with the 24-well manifold.

18- Using a multichannel pipette, fill, column by column, each of the 24 wells of the basal compartments of the PreadyPort™ plate with 750 µl of pre-warmed (37°C) assay buffer.

19- Remove the assay buffer from the apical inserts of the PreadyPort™ plate by aspiration with the 24-well manifold. Place the manifold perpendicular to the cell monolayer and close to the insert wall, and take care not to disrupt the cell layer.

20- Using a multichannel pipette, fill, column by column, each of the 24 apical inserts of the PreadyPort™ plate with 275 µl of the test compound working dilution.

21-Recover 25 µl from the apical compartments **(0 hr. Apical samples)** and keep them at -20°C until analysis.

22- Carefully return the 24 apical inserts onto the wells of the basal compartment of the PreadyPort™ plate (original position).

23- Cover the PreadyPort™ plate with its lid, and place it inside the incubator, at 37°C and 5% CO_2 for 2 hours.

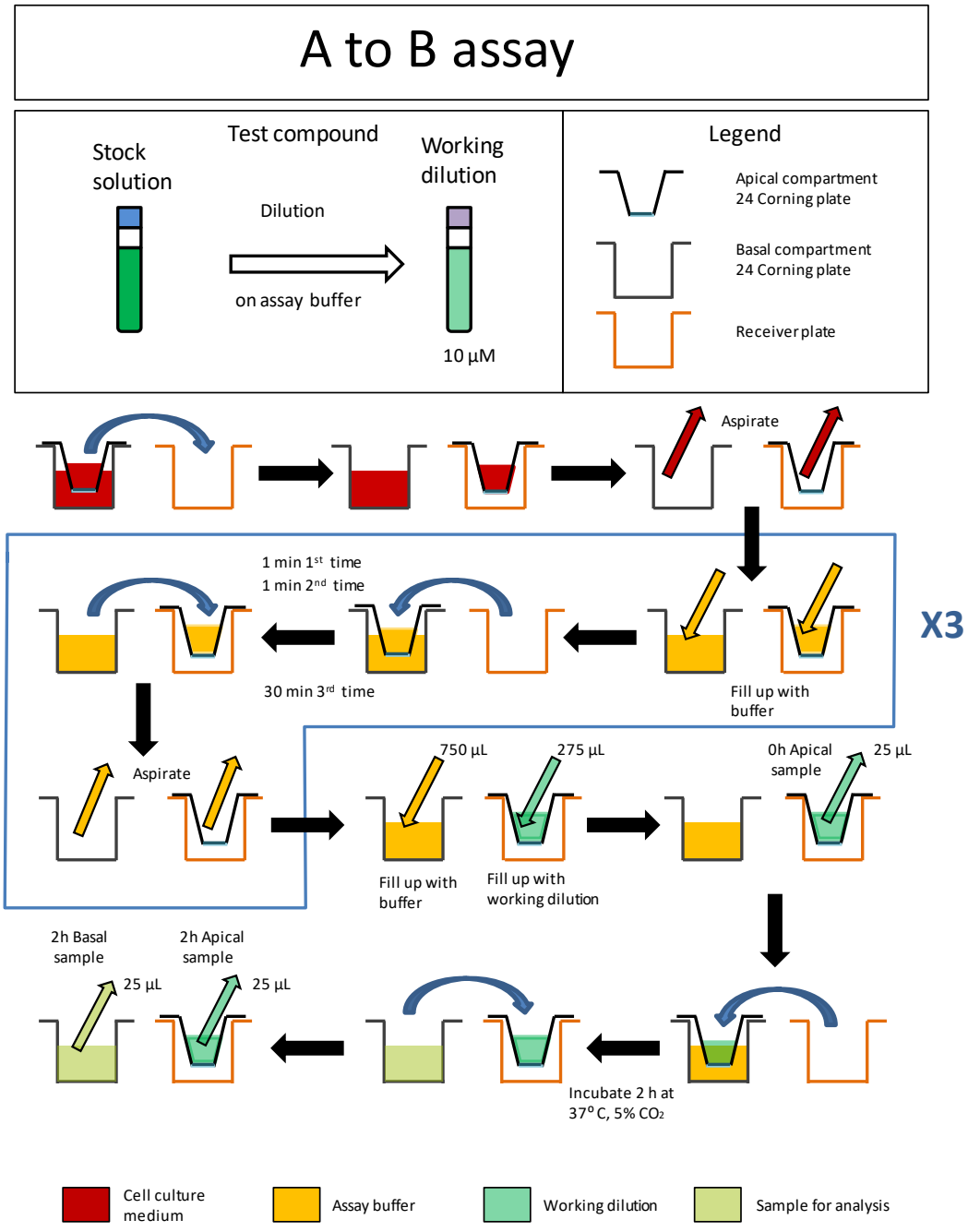
24- Take the plate from the incubator, return it to the laminar flow hood and place it next to the receiver plate. Transfer the apical inserts onto the receiver plate.

25- Recover 25 µl from the apical inserts **(2 hr. Apical samples)** and keep at -20°C until analysis.

26- Recover 25 µl from the basal compartments **(2 hr. Basal samples)** and keep at -20°C until analysis.

27- Analyze the samples (0 hr. and 2 hr. samples, above).

28- After quantification of test compound within samples, calculate the permeability coefficient (P_{app}) as indicated in section "Evaluation of the test compound transport".



Assay conditions		Quality control parameters
Concentration: 10 µM	Assay volumes	TEER (pre-assay): > 100 Ω cm²
Membrane area: 0.33 cm²	•Apical: 250 µl •Basal: 750 µl	Lucifer Yellow (post-assay) •Paracellular flux: 2% •Papp : ≤ 4.5 x 10^{-6} cm/s
Replicates: 3		
Time points: 2 (0 and 2 hours)	Sample volume: 25 µl	

Figure 4: General procedure for A to B assays

2. General Protocol Basal-to-Apical studies

Test compounds are applied to the basal side of the cell monolayer (lower compartment of the insert), and their basal-to-apical (B-A) transport through the cell barrier is evaluated by sample recovering and test compound detection at the apical side (upper compartment) over a defined incubation time. B-A permeability of test compounds is determined as the coefficient of apparent permeability (Papp) in cm/s.

- Test compound concentration: 10 µM
- Replicates: 3
- Time points: 2 (0 and 2 hours)
- Assay volumes:
    - Apical compartment: 250 µl of HBSS assay Buffer
    - Basal compartment: 750 µl of the test compound at working solution (775 µl is added to the basal compartment and 25 µl is recovered at time 0 hr.)

All other steps are identical as in the AB protocol, above.

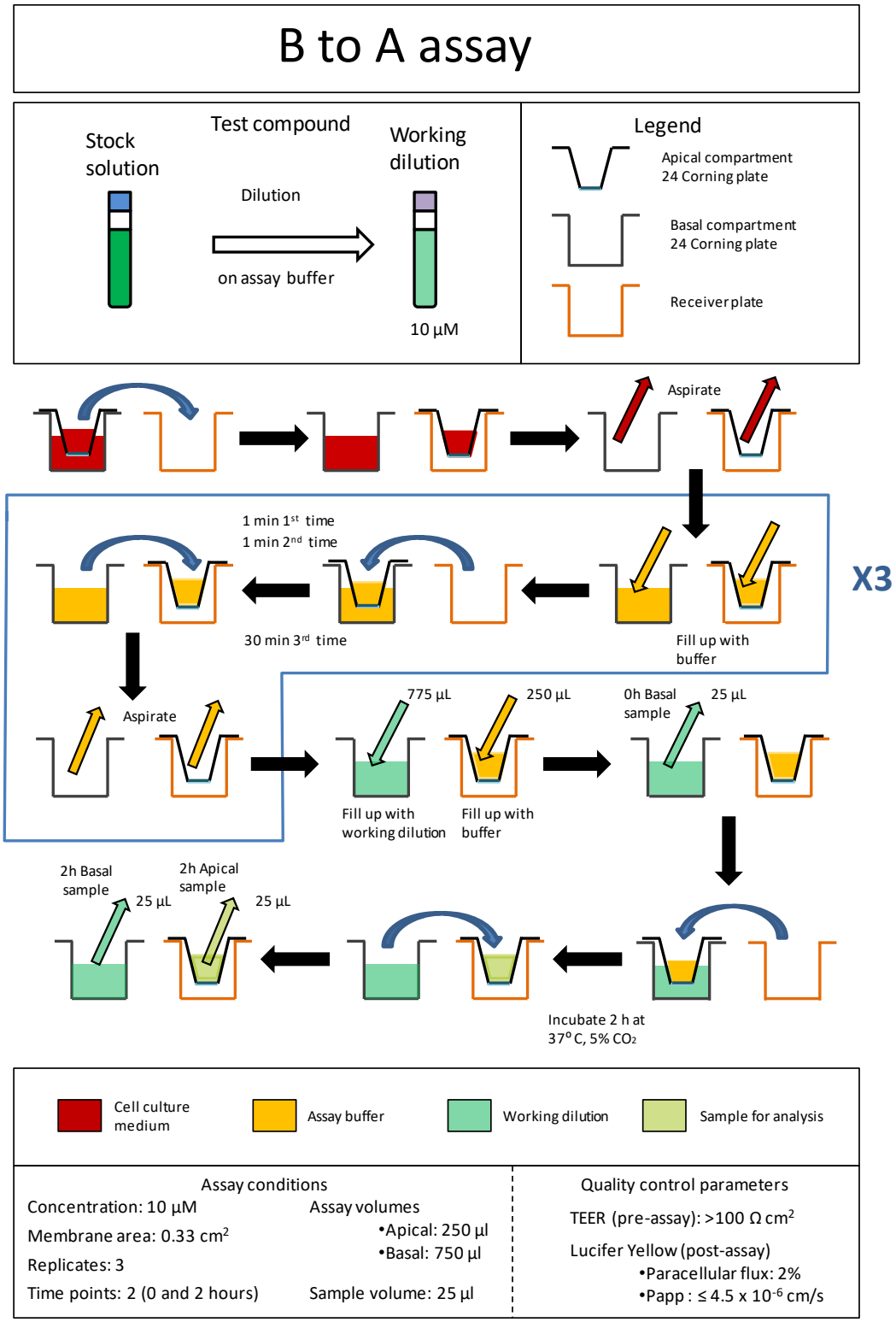


Figure 5: General procedure for B to A assays

Evaluation of the Test Compound Transport

This section contains recommendations for sample retrieval, handling and evaluation.

For test compounds with unknown behavior, the following pattern for sample retrieval is recommended:

- Donor side (apical for apical to basolateral, basolateral for basolateral to apical): t=0 and t=2 h
- Receptor side (basolateral for apical to basolateral, apical for basolateral to apical): t=0 and t=2 h

Shorter or longer periods may be required for very high or low permeability compounds.

For calculations the following formula is recommended:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

Where:

- **dQ/dt**: amount of translocated material over incubation time (nmol/s)
- **A:** area of transwell (0.33 cm^2 for PreadyPort™ BCRP-24)
- **C_0**: initial concentration of product applied in apical (A-B) or basal (B-A) compartment (nmol/ml)

The efflux Ratio ($ER=P_{app(B-A)}/P_{app(A-B)}$) is a general measure of the involvement of active processes. ER values higher than 2 indicates an active process.

Considerations for calculations:

- P_{app} is traditionally given in units of 10^{-6} cm/s. The dimensions of all parameters must be set accordingly (Q in mol, t in seconds, A in cm^2, C_0 in mol per cm^3)
- When plotting Q versus time, the amount of material lost with previous samples must be accounted for
- When applying the formula with a single time point that time point must be in the linear range otherwise the obtained P_{app} will underestimate the real value. It is not recommended to sample compounds with unknown behavior at a single time point

Normal Range of Controls (According to FDA, p. 64 Fig. A1)

- The efflux ratio of prazosin must be greater than 2 in BCRP overexpressing MDCKII cells
- In the presence of Ko134 (specific BCRP inhibitor) the efflux ratio has to significantly decrease > 50%
- Paracellular flux of Lucifer Yellow must be less than 2% per hour of incubation (internal data)