emulate

Protocol for Emulate Organ-Chips:

Compound Distribution Kit

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Understanding Distribution of Compounds in System Components

In both *in vitro* and *in vivo* experiments, researchers must consider compound distribution within the biological model and experimental setup prior to quantitative drug studies, as distribution determines exposure — the concentration of a compound that cells truly experience.

In *in vivo* systems, this is addressed by volume of distribution studies, which relate compound dosage to its effective concentration. However, in both *in vivo* and *in vitro* studies, the distribution effects of system components, such as infusion tubing, syringes, tissue-culture plates, and pipette tips, are often missed.

With Organ-Chip experiments, we proactively address compound distribution in a number of ways. Several of these are embedded in our protocol designs, where we have selected experimental conditions to optimize compound exposure. Additionally, we have developed the Compound Distribution Kit to directly evaluate distribution and compound exposure.

The Compound Distribution Kit is intended to be used as a specialized control experiment — the distribution control experiment — prior to the intended Organ-Chip study. As such, the contents of the Compound Distribution Kit mirror the contents of an Organ-Chip Bio-Kit, and the protocol used for the distribution control experiment mirrors a simplified version of the intended study (e.g., without cells or ECM coating). The distribution control experiment's output indicates whether any compound may be distributed into the system and away from cells. Moreover, in some cases, the distribution control can be used to quantitatively correct the experimental results of the intended study and assign it appropriate error bars.

General Guidelines for Use

The Compound Distribution Kit should be run using the same protocol as the intended Organ-Chip experiment, except with certain simplifications (most notably, do not include cells). In particular, the distribution control experiment should use the same dosing, perfusion, and sampling design as the intended experiment. This allows the Compound Distribution Kit to assess compound exposure at the same timepoints and under the same experimental conditions that are relevant for the intended experiment.

The kit, protocols, and associated calculator are generally applicable to all drug studies, allowing for the determination of cellular exposure concentration ranges. The kit also includes an additional calculator intended for pharmacodynamic studies (including toxicity studies) that plots dose-response curves based on the measured distribution data.

When to Use the Compound Distribution Kit

We strongly suggest using the Compound Distribution Kit ahead of experiments that involve compounds smaller than 1,000 Da. However, if you have indication that the compound in question suffers from loss or "stickiness" (e.g., absorption or adsorption) in plate-based systems, please consider using the kit even for small-molecules larger than 1,000 Da and biologics.

Equipment and Supplies

You will receive the following items in each Compound Distribution Kit:

- 3 Test-Chips
- 3 Pod™ Portable Modules
- 2 Steriflip® Filter Units

The Test-Chips provided in the Compound Distribution Kit are similar to the Chip-S1™ Stretchable Chip, with the exception that the membranes of the Test-Chips are not porous. This prevents direct fluid flow between the chip's top and bottom channels that would otherwise be blocked by cell layers.

Output samples from the distribution control experiment typically need to be analyzed using liquid chromatography-mass spectrometry (LCMS).

To run the kit, you will need the following equipment and supplies:

- At least 1 Zoë™ Culture Module
- At least 1 Orb™ Hub Module
- Incubator
- Biosafety Cabinet (BSC)
- Liquid Chromatography — Mass Spectrometer (LCMS)
- 70% Ethanol
- At least one 150 mm Petri dish
- At least two 50 mL conical tubes
- At least 10 Eppendorf Tubes® (or equivalent)
- Pipette tips
- 1 pipette aid or gun
- Aspirator and aspirator tips
- Media (same media with supplements that will be used for running the Organ-Chip study)
- DMSO (or equivalent)
- Kimwipes™

Protocol

The Compound Distribution Kit is to be run as a distribution control experiment that mirrors the protocol of the intended Organ-Chip study. Accordingly, to develop the specific steps to use the Compound Distribution Kit, first define the protocol for the intended Organ-Chip study — we refer to this as the "intended-study protocol."

To run the distribution control experiment, follow the intended-study protocol with the following changes:

Study Setup

- There is no need to perform ECM coating — you may skip this part of the intended-study protocol.
- There is no need to seed cells — you may skip this part of the intended-study protocol.
- Please be sure to use the same media as the intended-study protocol, including the same additives and supplements. This is because medium composition and additives can interact with test compounds, for example through protein binding.
- Please be sure to adhere to any media equilibration, Regulate cycles, and bubble clearing steps that the intended-study protocol specifies, as these are important with the Compound Distribution Kit as well.

Dosing

- If the intended-study protocol compares several concentrations of a test compound, the distribution control experiment only needs to be run for one of these concentrations. We recommend using the highest compound concentration planned for the intended study in order to maximize LCMS sensitivity. However, you may want to select a lower concentration of test compound if there are concerns about the compound's solubility limits or it crashing out of solution at the higher concentration.
- Dose the compound for the same duration, at the same flow-rate, and in the same channel(s) as for the intended-study protocol. That is, if the intended-study protocol specifies dosing only in the bottom channel (with no compound in the top channel), do so in the distribution control experiment as well.
- If the intended-study protocol calls for dosing in both channels, dose both channels but with an equal concentration. It is suggested to dose using the higher of the two possible concentrations.

Sample Collection

- Collect effluent samples from the distribution control experiment at the very same time points as in the intended-study protocol. Compound distribution can be a highly dynamic process, so matching timepoints ensures that the results of the distribution control experiment correspond closely to the intended study. Crash the effluent samples as recommended by the LCMS provider.
- In addition to the effluent samples, also collect samples from each of the inlet reservoirs during each input media exchange, at the same time as effluent samples are collected, right before

exchanging the media. This helps ensure no compound is disappearing from the system through an unexpected means, such as compound crashing out of solution or photodegradation.

Sample Analysis

- Generate standard curves for LCMS analysis. We recommend using a 5-point standard curve in triplicate using a volume of at least 50 µL per sample. Serial dilutions of media used in the top and bottom channels should be prepared. We suggest using the following dilution ratios to generate the 5-point standard curve:
    - Undiluted dosing medium with drug
    - 1:10 dilution
    - 1:100 dilution
    - 1:1,000 dilution
    - Blank (media without drug)
- Analyze the inlet, effluent, blank media, and standard curve samples using LCMS, and plug the results into the provided calculator (please see following section).

Using the Data Analysis Calculator

The Compound Distribution Kit Data Analysis Calculator (EC001) is an Excel spreadsheet that can be obtained at emulatebio.com/protocol-archive/EP129-v1-0.

Inputting Data into the Calculator

1. In the “User Inputs” tab, enter the top and bottom channel flow rates, select the dosing channel from the drop-down menu, and type in the units of concentration. Enter the timepoints in column H and take note of the total number of LCMS samples needed to be analyzed.
2. After receiving LCMS results, enter the concentration data in the same tab under the “Concentration Results” section of the calculator.
    a. Note: If inlet values have not been measured for certain timepoints, leave these cells blank.
3. View the results in the “Results” tab.

Calculator Outputs (“Results” tab)

1. Recovered (effluent) concentrations are plotted against time for both top and bottom channels. A representative example is shown in Figure 1. This is a visualization of the “User Inputs” data, reflecting a ratio of the recovered concentration divided by the inlet concentration. A recovered concentration close to 1 at any given time point means that little compound distributes into the system, and that the effective exposure concentration is similar to the input concentration. The curves typically rise with time as the gradients that drive compound distribution diminish.

2. The range of potential cellular exposure concentrations is plotted for each collection time period for both channels. A representative example is shown in Figure 2. This plot illustrates how compound distribution in the system creates an uncertainty in the drug concentration that cells experience. For example, if all compound distribution occurs upstream of the cells, the cells would experience a lower compound concentration than if the compound

distribution occurs entirely downstream of the cells (in which case the cells would experience the full dosing concentration). Specifically, in the 6–24-hour time period of the experiment in Figure 2, cells experience between 64% and 100% of the dosing concentration. If a compound is minimally distributed into the system, you will observe a tighter range near the top of the graph, which means that the cells are expected to be exposed to most of the dosed compound.

3. Table 1 represents the information in Figure 2, indicating the range (max and min) of cellular exposure concentrations in both channels. The same exposure concentration information is presented both as “fraction of dosing”, and in terms of exposure concentration.

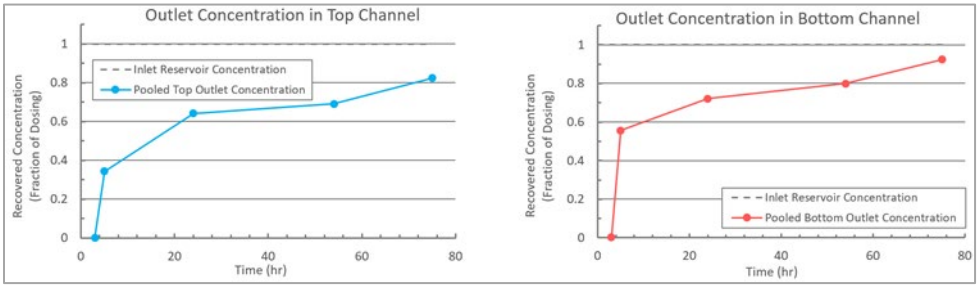


Figure 1

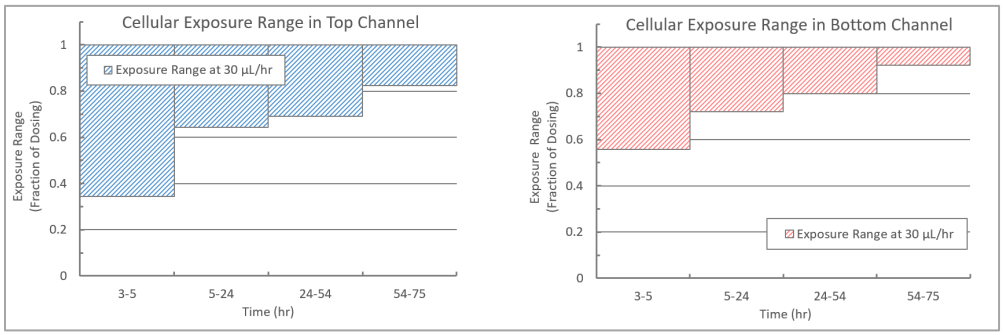


Figure 2

Potential Exposure Concentration Range (Fraction of Dosing)						
Exposure	Top Channel		Bottom Channel		Range	Range
Period (hr)	Minumum	Maximum	Minumum	Maximum		
Dosing-3	0.002	1.000	0.002	1.000	0.998	0.998
3-6	0.343	1.000	0.556	1.000	0.657	0.444
6-24	0.642	1.000	0.721	1.000	0.358	0.279
24-54	0.691	1.000	0.799	1.000	0.309	0.201
54-75	0.824	1.000	0.923	1.000	0.176	0.077
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-

Potential Exposure Concentration Range (μM)				
Exposure	Top Channel		Bottom Channel	
Period (hr)	Minimum	Maximum	Minimum	Maximum
Dosing-3	0.171	101.333	0.150	93.900
3-6	34.733	101.333	52.247	93.900
6-24	65.100	101.333	67.733	93.900
24-54	70.033	101.333	75.067	93.900
54-75	83.467	101.333	86.700	93.900
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-

Table 1

Making Use of the Calculator's Outputs

1. Ahead of performing the intended Organ-Chip study: for each timepoint, examine the potential range of cellular exposure (either in the form of Figure 2 or Table 1) and determine whether the expected uncertainty is acceptable for the intended study. For example, the aforementioned uncertainty range of 64% to 100% in the 6–24-hour time period of the example in Figure 2 may be acceptable for some studies, but not others. If the uncertainty is unacceptable, consider eliminating that timepoint from the intended Organ-Chip study or replacing it with a later one.
2. After performing the intended Organ-Chip study, use the potential cellular exposure range (most conveniently in the form of Table 1) to draw horizontal error bars around each measurement — these indicate the uncertainty in the concentration that the cells are actually exposed to. This action can also be performed within the "Dose-Response" tab in the calculator by entering the dosing concentrations and selecting the corresponding time period for which to adjust these concentrations. A "Best Estimation" of average exposure concentrations as well as the minimum and maximum possible exposure concentrations will be returned. Additionally, if data has been generated for a toxicity or other dose-response study, the response data can be entered, and a dose-response graph will be generated for each channel. An example of this is shown in Figure 3. The figure illustrates a representative dose-response curve with the estimated average cellular exposure concentration plotted versus cellular response. The data is plotted with error bars or confidence intervals for the exposure concentration at each dosed concentration, based on the results of the distribution control study. This process can be repeated for each time period being analyzed.

   A similar process can be applied to calculated quantities such as IC_{50} values. To illustrate this, we turn again to the 6–24 hour time period of the example in Table 1: Suppose that measurements from this time period of the intended study show an IC_{50} value of 1μM as measured by the dosing concentration; we would then translate this value using Table 1 to indicate that the IC_{50} value (as relating to the compound concentrations the cells experience) is actually a concentration range between 0.642μM – 1μM.

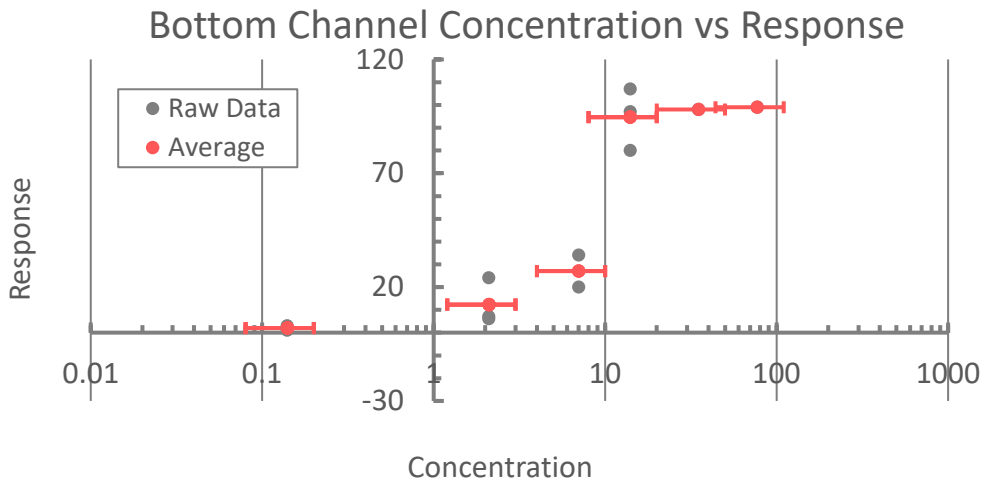


Figure 3

Appendix A – Sample Protocol

The following example illustrates the use of the Compound Distribution Kit in the case where the intended Organ-Chip experiment is a Liver-Chip toxicity study.

Media Gas Equilibration

1. Warm top channel medium and bottom channel medium in 50 mL conical tubes at 37°C in a water or bead bath for at least 1 hour.
    a. Prepare at least 4 mL of each medium type per chip — it is recommended to test at least 3 chips per compound.
    b. Media should be prepared in the same way as the media used when dosing cells with compound, matching all media components / supplements, with the exception of the compound to be tested at this stage.
2. Transfer conical tubes to the biosafety Cabinet (BSC) and immediately Steriflip medium:
    a. Connect the 0.45 μm Steriflip unit to the conical tube and apply vacuum to assembled unit for 10 seconds prior to inverting.
    b. Invert the assembled Steriflip and ensure that medium passes through the filter in a continuous stream.
    c. It should take approximately 2 seconds for each 10mL of medium to pass through the filter — if it takes longer, STOP and see the troubleshooting section at the end of each Organ-Chip culture protocol, as medium will not be equilibrated properly.
3. Leave the filtered medium under vacuum for 5 minutes.

4. Remove conical tube with medium from Steriflip unit while still under vacuum and then turn off pump. Replace the lid inside the BSC, and immediately place conical tube in an incubator or bath to maintain temperature.
5. Store this media with cap slightly loose in the incubator prior to use.

Chip Washing

1. Unpackage gamma irradiated Test-Chips in the BSC and place in a 150 mm Petri dish.
2. Wash each channel with 200 µL of equilibrated media.
    a. Place the pipette tip perpendicular to channel inlet.
    b. Ensure tip is snug in port and introduce media into top and bottom channel.
    c. Aspirate outflow liquid from the outlet of the chip.
3. Aspirate and discard any excess media from the surface of the chip, but keep channels filled with media.
4. If bubbles are observed anywhere in the chip channels or ports, aspirate each test chip port to remove media from channels, then reintroduce media.
5. Place small equilibrated medium droplets on each inlet and outlet.
6. Cover the culture dish and place in the incubator until Pods are primed.

Pod Priming

1. Sanitize the exterior of Pod packaging with 70% ethanol and transfer Pods into the BSC.
2. Retrieve trays from the Zoë and sanitize with 70% ethanol before transferring into the BSC.
    a. Orient the tray with the handle to the user's left inside the BSC.
3. Open Pod package in the BSC, and place the Pods into trays.
4. Add 3 mL of equilibrated medium to the appropriate inlet reservoir.
5. Add 300 µL of equilibrated medium to the appropriate outlet reservoir, directly over each outlet via.
6. Prime Pods in Zoë.
    a. Use the rotary dial to highlight the “Prime” cycle.
    b. Press the dial to select the “Prime” cycle.
    c. Rotate the dial to select “Start” and press the dial again to begin the cycle.
    d. Close the incubator door and wait 1 minute for the cycle to complete.
    e. The status bar will read “Ready,” confirming the cycle is finished.
7. Transfer Trays to the BSC.

8. Inspect the underside of each Pod — observe droplets have formed on all four ports.
    a. If any Pod does not show droplets, re-run Prime cycle on those Pods.
9. Set Pods aside and retrieve chips from the incubator.

Chip-to-Pod and Regulate

1. Hold Pod with non-dominant hand.
2. With chip in dominant hand, slide the arms of chip carrier into the tracks on the underside of the Pod until the chip carrier is fully seated in the Pod.
3. Place thumb on the carrier tab and gently depress tab in and up to engage the tab with the Pod.
4. Aspirate any excess medium from Pod window.
5. Place the Pod with chip into the tray, with the reservoirs along the back wall.
6. Repeat for each Pod and chip carrier and transfer loaded tray to Zoë.
7. Select flow rate settings on Zoë.
    a. Flow Rate: 30 µL / hr for the top and bottom channel.
8. Run Regulate Cycle.
    a. The cycle will take 2 hours to complete, after which the Zoë will switch to the set flow rate.

Second Regulate Cycle

1. The morning after running the Regulate cycle, pause Zoë by pressing the silver Activation button located over the bays.
2. Slide tray out and transfer to the BSC.
3. Remove Pod lids and using a 200 µL pipette, perform a via wash on each Pod inlet and outlet reservoir:
    a. Using media within the Pod reservoir, pipette 200 µL of media directly over the top of the via to dislodge any bubbles that may be present.
    b. Repeat this wash step for each of the four Pod reservoirs.
4. Replace Pod lids and return the trays to Zoë.
5. Run the Regulate cycle again.
6. Dosing is ready to commence following completion of the second Regulate cycle.

Preparation of Dosing Solution

Note: Use the same media with supplements that will be used for running the Organ-Chip study for the compound distribution control study.

1. Prepare the stock solution of compound by dissolving in vehicle of choice.
2. Dilute stock solution in the appropriate gas-equilibrated media. An additional, simple calculator can be used to estimate the total volume of dosing media required for the experiment: see Media Volume Preparation Calculator (EC002).
    a. Add details of the planned dosing experiment in the designated spaces (planned flow rates, study duration, and re-dosing period).
    b. Take note of the total volume of both media types to be prepared and the volumes to be added to the Pod inlets.

Dosing and Sample Collection

1. Pause Zoë by pushing the silver Activation button
2. Take trays to the BSC
3. Aspirate the media out of the inlets and outlets making sure to avoid bringing the aspirator tip too close to the vias (there will be a small amount of media remaining near the via and this is acceptable).
4. Refer to the Media Volume Preparation Calculator and add the total media volume needed to run the study to completion (or to the next media replenishment timepoint) into the top and bottom inlet reservoirs of the Pod.
5. Sample 50 uL from the top and bottom inlet reservoir of each Pod to capture the t=0 dosing media concentration.
6. Reserve 200 µL dosing media from the conical tube, as well as “blank” media with no compound, for standard curve preparation. Store these samples according to user standard practices.
7. Return the tray into Zoë and flush the system with dosing media by setting the flow rate to 600 µL / hr and run for 5 minutes. This replaces the media in the chip with dosing solution.
8. Pause Zoë and transport trays to the BSC
9. Completely aspirate the effluent collected in the outlet reservoir so as not to dilute the compound effluent collected in the later timepoints.
10. Return the tray to Zoë and set the flow rate as directed in the study. Begin timing for sample collection once flow is initiated on Zoë.
11. Use the remaining dosing solution to prepare serial dilutions as samples for a standard calibration curve. We recommend generating samples for a 5-point standard curve in a triplicate of volume 50 µL using serial dilutions of the top and bottom media using the following ratios (Dosing solution : media)
    a. Undiluted media with drug
    b. 1:10 dilution
    c. 1:100 dilution

d. 1:1000 dilution
e. Blank (media without drug)

12. Store these samples according to User standard practices
13. Sample 50 µL from inlet and outlet reservoirs at the remaining timepoints until the conclusion of the study
    a. In addition to the effluent samples, it is recommended to collect samples from each of the inlet reservoirs during each input media exchange, at the same time as effluent samples are collected, right before exchanging the media. This helps ensure no compound is disappearing from the system through an unexpected means.
    b. Handle and process samples per user standard practices
    c. Aspirate outlets completely before returning Pods to Zoë

    NOTE: In case the volume collected is less than 50 µL, record the volume collected.
14. Send samples to LCMS upon completion of the dosing experiment.

Data Analysis

1. After receiving LCMS results, enter the concentration data into the “User Inputs” tab under the “Concentration Results” of the calculator, as well as the corresponding times.

   Note: In this same tab, enter the parameters used in the distribution control study, including top and bottom channel flow rates, duration of experiment, dosed channel, and units of concentration.

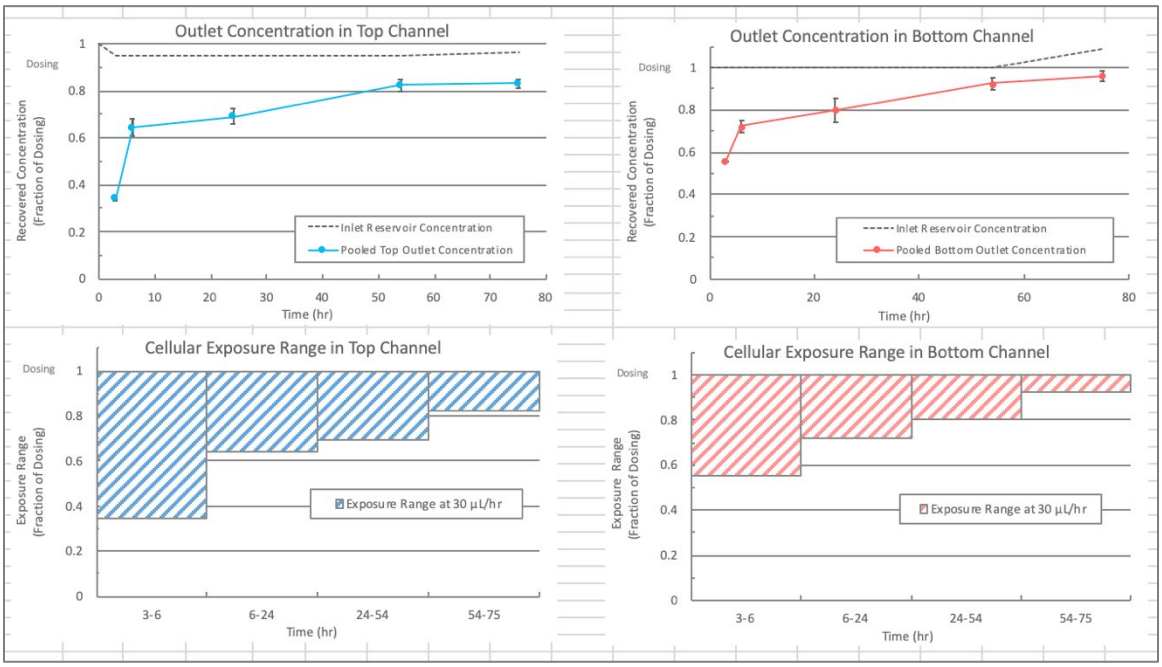
2. View the results in the “Results” tab.
   a. Recovered concentrations are plotted with time for both top and bottom channels.
   b. The range of potential cellular exposure concentrations are plotted for each collection time period and both channels.
   c. Tables indicate the range (max and min) cellular exposure concentrations in both channels with time, as well as the exposure expressed as a fraction of the dosing concentration.
3. After performing an Organ-Chip toxicity or other pharmacodynamics study, enter the dosing concentrations for both the top and bottom channels and the corresponding response observed in the “Dose-Response” tab. Additionally, select the time period over which the data was collected from the drop-down menu and the concentration adjustment factor corresponding to that period will be applied to the inputted concentration information. Graphs plotting the dose-response curve will be automatically populated with the adjusted concentration data and exposure ranges. Adjusted data can be copied from the table in this Excel tab and pasted into other software packages for further analysis, if desired.

Duration of Dosing (hours)	Recommended Time Points (hours)
3	1, 2, 3
6	1, 2, 4, 6
12	1, 2, 5, 8, 12
24	1, 2, 8, 16, 24
48	1, 2, 17, 32, 48
72+	1, 2, 24, 48, 72

Table 2. Recommended Dosing Timepoints from the Calculator for Possible Experimental Durations based on a flowrate of 60 µL/hr

Examples

Compound distribution into system is low — Between the 3 hr to 6 hr timepoint, a very low concentration of the compound is recovered in the effluent. Over time, the concentration of the compound in the effluent stream increases to reach 80% of the dosing concentration at the 72 hr timepoint. Consequently, the confidence interval significantly decreases over the same time period. This type of curve is also representative of a highly adsorbing compound, where the initial effluent concentration is low, but very quickly the recovered concentration increases to near dosing concentration.



Compound distribution into system is high — this compound shows low recovery and large confidence intervals maintained over time. This is indicative of non-saturating compound distribution, which may be prohibitive for many applications, even at longer time points.