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Shake-Flask Aqueous Solubility assay (Kinetic solubility)

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Protocol status: Working
We use this protocol and it's working

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Abstract

Determining compound solubility is an essential tool for the early stages of the drug discovery process, as well as for lead optimization. Low solubility can lead to unpredictable and unreliable results during in vitro testing, thereby increasing the development costs. Solubility issues at the later stages of the drug discovery may lead to poor bioavailability, underestimated toxicity, and other obstacles, lowering the chances of a given drug candidate for success.

Typically, for early-stage drug discovery, the kinetic solubility method is used, as it is fast and well-suited for the HTS format. In this case, solid compounds are first dissolved in DMSO, and then linear serial dilutions of each compound are added to an aqueous buffer and observed for precipitate formation when the compound is not completely soluble. For better precision, the solution can be subjected to high-speed centrifugation or filtration using special solubility filter plates and then the compound concentration is measured in the saturated solution directly by UV or LC-MS/MS using separately built calibration curves. Measurements were performed by the shake-flask method with UV-Vis or LC-MS/MS detection.

Materials

Equipment

- Water purification system Millipore Milli-Q Gradient A10 (Sartorius Arium™ Mini)
- Thermomixer R Block, 1.5 mL (Eppendorf, Germany)
- Matrix Multichannel Electronic Pipette 2-125 µL, 5-250 µL, 15-1250 µL (Thermo Scientific, USA)
- SpectraMax Paradigm™ Reader (Multi-Mode Detection Platform)
- MS/MS detector API 3000 PE with TurboIonSpray Electrospray module (PE Sciex, USA)
- Multi-Well Plate Vacuum Manifold (Pall Corporation, USA)
- Vacuum pump (Millipore, USA)

Material

Phosphate buffered saline, pH 7.4 (Sigma-Aldrich, USA)

Phosphate buffered saline powder, pH 7.4, for preparing 1 L solutions **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3813

Acetonitrile Chromasolv, gradient grade, for HPLC, ≥99.9% (Sigma-Aldrich, USA)

Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34851

DMSO (Sigma-Aldrich, USA)

Costar 96 Well Assay Blocks (Corning, USA)

Costar 96-Well Microplate, Deep Well, Sterile, V-Bottom, 2 mL; 25/Cs **Corning Catalog #**3960

MultiScreen HTS 96 Well Filter Plates (Millipore)

UV-Star® 96 Well Microplate (Greiner Bio-One)

Multichannel pipettors 1-30 µL, 2-125 µL, 30-850 µL (Thermo Scientific)

Flex-Tubes Microcentrifuge Tubes, 1.5mL (Eppendorf, Germany)

Eppendorf® Flex-Tubes Microcentrifuge Tubes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP022364120

Troubleshooting

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Preparation of auxiliary solutions

2w

1 1 L **PBS:** [M] 0.01 Molarity (M) **Phosphate buffer**

In a bottle for reagents with a cap of 1 L capacity, place:

- contents of 1 L sachet with phosphate buffer,
- 1 L of water and mix thoroughly and filter through a membrane filter with a pore diameter of 0.45 µm .

Note

The shelf life of the solution is 2 weeks when stored at 5 °C .

2 100 mL **A solution (AcN:PBS, 50:50, v/v):**

In a bottle for reagents with a cap of 100 mL capacity, place:

- 50 mL of AcN,
- 50 mL of PBS and mix thoroughly.

Note

The shelf life of the solution is 1 month when stored at Room temperature .

3 100 mL **B solution (AcN:PBS:DMSO, 49:49:2, v/v/v):**

In a bottle for reagents with a cap of 100 mL capacity, place:

- 49 mL of AcN,
- 49 mL of PBS,

- 2 mL of DMSO and mix thoroughly.

Note

The shelf life of the solution is 1 month when stored at Room temperature.

4 **100 mL C solution (AcN:DMSO, 98:2, v/v):**

In a bottle for reagents with a cap of 100 mL capacity, place:

- 98 mL of AcN,
- 2 mL of DMSO and mix thoroughly.

Note

The shelf life of the solution is 3 months when stored at Room temperature.

5 **Preparation of 20 mM stock compound**

Prepare [M] 20 millimolar (mM) stock solutions of test substances in DMSO. As a rule, 50 µL of solution is enough.

Sample preparation

2h

6 **Incubation of [M] 400 micromolar (µM) solution**

2h

In Matrix Storage tubes (1.4 mL), add 490 µL of buffer, followed by 10 µL of stock solution of the test and control substances. Prepare two incubation mixtures for each substance to ensure reproducibility. Place the tubes in a thermomixer. Set the thermomixer to 850 rpm and incubate for 02:00:00.

7 **Preparation of Calibration Solutions**

Using Matrix Storage tubes (1.4 mL) with 20 µL of stock solutions, prepare calibration solutions according to Table 1. Mix the solutions by pipetting 5 times. To prevent evaporation, cover the tubes with strip caps.

	A	B	C	D	E	F	G
	Calibration standard/solution (µL)	**Concentration (µM)**					
		400*	**200**	**100**	**50**	**25**	**10**
	A solution	490					
	B solution		250	250	250	250	150
	20 mM DMSO stock	10					
	400 µM		250				
	200 µM			250			
	100 µM				250		
	50 µM					250	
	25 µM						100
	10 µM						

Table 1

**Note: The 400µM standard is not used in the measurements.*

8 For the incubation mixture, add 250 µL of Solution C (well A11-12) into the tube. See Figure 1 for the arrangement of tubes in the rack.

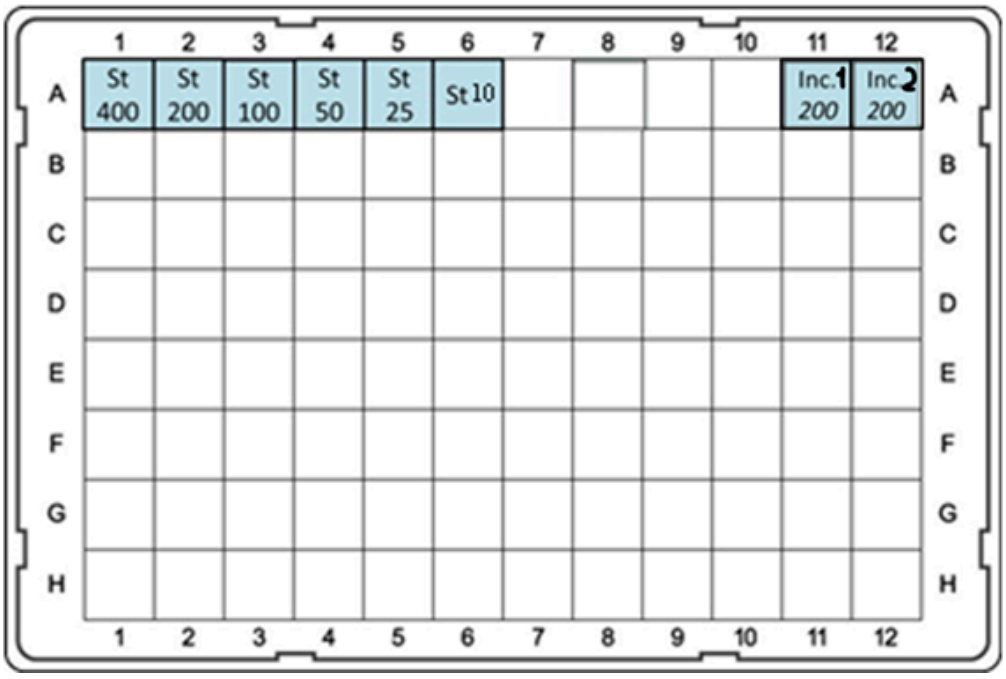

Figure 1

9 Filtration of the Incubation Mixture

9.1 Place the Deep Well Plate in the manifold and close the manifold.

9.2 Place the filtration plate on top.

9.3 Transfer 290 µL of the incubation mixture into the filtration plate, cover with a plastic lid.

9.4 Turn on the vacuum pump and gradually adjust the manifold valve to 0.2 atm.

9.5 After filtration, close the manifold valve, turn off the vacuum pump, and transfer 250 µL of the filtrate into the tube with Solution C (well A8 in Figure 1).

9.6 Mix by pipetting 5 times.

10 **Preparation for Measurement**

Transfer 200 µL of the solutions from the tubes (in duplicates) to a UV-Star® plate, following the layout in Figure 2.

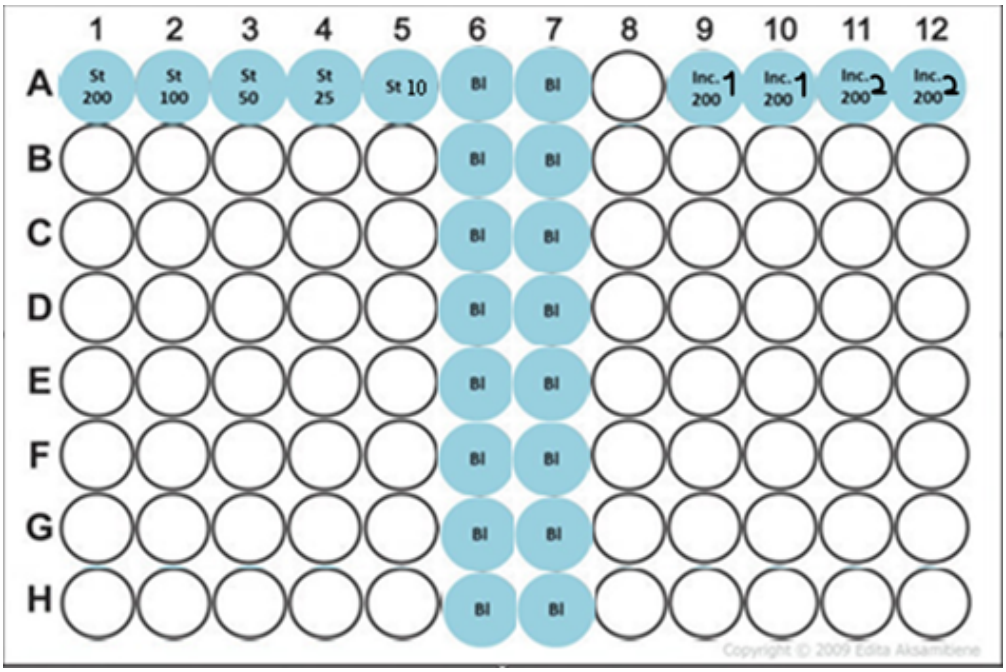

Figure 2

11 **Insert the Plate into the Reader**

11.1 Place the plate into the reader for measurement.

11.2 On the device, press the "Drawer" button, then insert the plate into the reader.

11.3 Close the reader by pressing the "Drawer" button again.

11.4 Start the measurement by clicking the "Read" button in the SoftMax Pro software.

11.5 After the measurement, remove the plates and dispose of them properly.

12 Evaluation of Results

12.1 The concentrations of compounds in PBS filtrate are calculated using a dedicated Microsoft Excel calculation script.

12.2 Proper absorbance wavelengths for calculations are selected for each compound manually based on absorbance maximums (absolute absorbance unit values for the minimum and maximum concentration points within the 0 – 3 OD range).

12.3 Each final dataset is visually evaluated by the operator, and goodness of fit (R2) is calculated for each calibration curve.

12.4 The effective range of this assay is approximately 2 µM - 400 µM and the compounds returning values close to the upper limit of the range may have higher actual solubility (e.g.5'-deoxy-5-fluorouridine).

Protocol references

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