Version 4a, Last updated 24 August 2023

ab211075 CYP1A2 Inhibitor Screening Kit (Fluorometric)

For the rapid, sensitive and accurate screening of potential inhibitors of cytochrome P450 1A2 (CYP1A2).

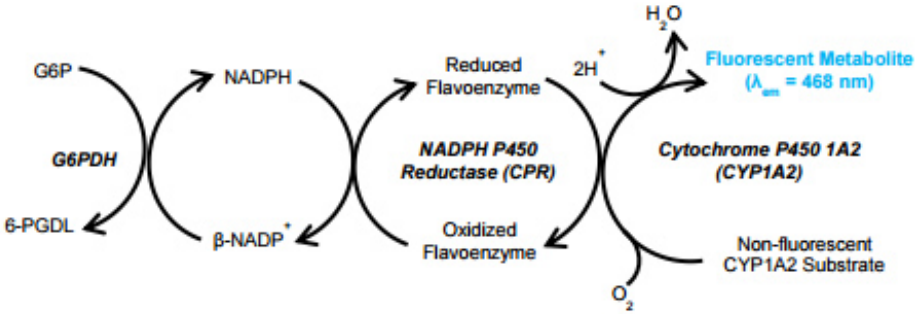
This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Table of Contents

1. Overview

CYP1A2 Inhibitor Screening Kit (Fluorometric) (ab211075) allows rapid screening of drugs and other new chemical entities (NCEs) for compound-CYP1A2 interaction in a reliable, high-throughput fluorescence-based assay. The kit provides a yeast microsomal preparation of human CYP1A2 and human cytochrome P450 reductase (CPR) enzymes. The assay utilizes a non-fluorescent CYP2C19 Substrate/CYP1A2 substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 406/468 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96 well plate format.



Cytochrome P450 1A2 (CYP1A2, EC 1.14.14.1) is a member of the cytochrome P450 monooxidase (CYP) family of microsomal xenobiotic metabolism enzymes. CYPs are membrane-bound hemeproteins responsible for Phase I biotransformation reactions, in which lipophilic drugs and other xenobiotic compounds are converted to more hydrophilic products to facilitate excretion from the body. CYP1A2 is primarily expressed in liver, intestinal and olfactory mucosal tissue and catalyzes oxidation of planar polyaromatic and heterocyclic molecules such as aromatic amines. CYP1A2 is responsible for metabolism of approximately 10% of all small molecule drugs commonly used by humans. Polymorphisms in the human CYP1A2 gene have been implicated in clinical drug/drug interactions involving widely-used drugs such as methylxanthines (caffeine and theophylline) and a number of antidepressants and antipsychotics. Isoforms of the CYP1A subfamily are also involved in metabolic activation of environmental pro-carcinogens in cigarette smoke and combustion exhaust fumes.

2. Protocol Summary

Prepare Standard curve and
measure fluorescence at Ex/Em = 406/468 nm

Set up appropriate reaction wells
(background, test compound, controls)

Add substrate mix to reaction wells

Measure fluorescence at Ex/Em = 406/468 nm
for 60 minutes in kinetic mode

** For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted CYP1A2 inhibitor should be used within 2 months.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Assay Buffer XXVI/CYP1A2 Assay Buffer	100 mL	-20°C	-20°C
3-CHC Standard	1 vial	-20°C	-20°C
100X NADPH Generating System II/NADPH Generating System 100X	1 vial	-20°C	-20°C
β-NADP Stock/β-$NADP^+$ Stock 100X	1 vial	-20°C	-20°C
CYP2C19 Substrate/CYP1A2 Substrate	1 vial	-20°C	-20°C
CYP1A2 Inhibitor (α-naphthoflavone)	1 vial	-20°C	-20°C
Recombinant Human CYP1A2	2 vials	-20°C	N/A

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 406/468 nm
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- White 96 well plate with flat bottom
- Anhydrous DMSO (reagent grade)
- Anhydrous acetonitrile (HPLC-grade)

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer XXVI/CYP1A2 Assay Buffer (100 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 3-CHC Standard:

Reconstitute the 3-CHC Standard in 110 µL of DMSO to generate a 5 mM standard stock solution. Vortex until fully dissolved. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Standard is stable for at least 3 freeze/thaw cycles.

9.3 100X NADPH Generating System II/NADPH Generating System (100X):

Reconstitute in 110 µL Assay Buffer XXVI/CYP1A2 Assay Buffer. Pipette up and down to dissolve completely. Keep on ice while in use. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid freeze/thaw cycles.

9.4 β-NADP Stock/β-NADP+ Stock (100X):

Dissolve in 110 µL Assay Buffer XXVI/CYP1A2 Assay Buffer and vortex thoroughly to prepare a 10 mM solution of β-NADP Stock/NADP+ (100X stock). Aliquot so that you have enough to perform the desired number of assays. Store at -20°C. β-NADP Stock/β-NADP+ Stock is stable for at least 3 freeze/thaw cycles.

9.5 CYP2C19 Substrate/CYP1A2 Substrate:

Reconstitute CYP2C19 Substrate/CYP1A2 substrate in 110 µL of anhydrous HPLC-grade acetonitrile to generate a 5 mM stock solution. Vortex until fully dissolved. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Allow the vial to warm to room temperature before opening and promptly retighten cap after use to avoid absorption of airborne moisture.

9.6 CYP1A2 inhibitor (a-naphthoflavone):

Reconstitute CYP1A2 inhibitor in 110 µL of acetonitrile to generate a 1 mM stock solution. Vortex until fully dissolved. Store at - 20°C. Use within 2 months.

Prepare a 30 µM working solution of inhibitor (5X final concentration) by adding 30 µL of stock solution to 970 µL of Assay Buffer XXVI/CYP1A2 Assay Buffer. The inhibitor working solution should be stored at -20°C and used within one week.

9.7 Recombinant Human CYP1A2:

Do not reconstitute until ready to use.

Each vial is sufficient for preparation of 50 reactions in a 96 well plate format.

Reconstitute recombinant CYP1A2 stock in 1 mL of Assay Buffer XXVI/CYP1A2 Assay Buffer. Mix thoroughly to ensure a homogenous solution; the solution will have a slightly opaque, milky appearance. Transfer solution to a 15 mL conical tube. Bring the volume up to 2450 µL with Assay Buffer XXVI/CYP1A2 Assay Buffer and add 50 µL of 100X NADPH Generating System II/NADPH Generating System (100X) (Step 9.3) for a final volume of 2.5 mL.

CYP1A2 stock is stable for up to 4 hours at room temperature or 1 day if kept on ice to minimize enzyme instability. Long term storage of the reconstituted enzyme system mix is not recommended.

Δ Note: Recombinant human CYP1A2 preparation may settle and should be thoroughly mixed before dispensing to ensure a homogenous solution.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 200 µM standard by diluting 20 µL of the 5 mM 3-CHC standard stock solution (Step 9.2) with 480 µL Assay Buffer XXVI/CYP1A2 Assay Buffer. Mix well.

10.2 Dilute 5 µL of the 200 µM 3-CHC standard in 995 µL Assay Buffer XXVI/CYP1A2 Assay Buffer to prepare a final 1 µM (1 pmol/µL) 3-CHC standard.

10.3 Use the 1 µM 3-CHC standard to prepare the standard curve dilution as described in the table in a plate or microcentrifuge tubes:

Standard #	3-CHC Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount 3-CHC in well (pmol/well)
1	0	300	100	0
2	6	294	100	2
3	12	288	100	4
4	18	282	100	6
5	24	276	100	8
6	36	264	100	12
7	48	252	100	16
8	60	240	100	20

Each dilution has enough amount of standard to set up duplicate readings (2 x 100 µL).

10.4 Immediately measure fluorescence in an end point mode program on a microplate reader at Ex/Em = 406/468 nm.

11. Test compound Preparation

11.1 Test compounds:

11.1.1 Dissolve each test compound in appropriate solvent.

11.1.2 Dilute to 5X working solution with Assay Buffer XXVI/CYP1A2 Assay Buffer.

Δ Note: To determine IC_{50} values of test compounds, 5X test inhibitor solution should be prepared in a range of concentration in order to generate a multi-point dose-response curve. It is also possible to perform a cursory initial screen of a large number of test compounds by observing the percent inhibition at a single fixed concentration of each test compound. We recommend a final test inhibitor concentration of 3 µM, for which 15 µM solutions (5X final concentration) should be prepared.

Δ Note: Many commonly-used organic solvents can severely impact CYP1A2 activity. Importantly, DMSO and methanol cause significant inhibition of CYP1A2 at final concentrations ≥ 0.25% (v/v). This assay is designed to use acetonitrile at ≤ 1% final concentration, which has been shown to have little impact on CYP1A2 activity.

We recommend preparing a parallel solvent control well with the same final concentration of solvent used to solubilize the test compound, particularly if using a solvent other than acetonitrile.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1 Set up Reaction wells:

Set up solvent control (SC), test inhibitor (TI), background control (BC), positive inhibition control (PIC) in a 96-well plate as described in the table below:

Component	SC	TI	BC	PIC
CYP1A2 enzyme stock (2X)	50 µL	50 µL	-	50 µL
Test inhibitor solution (5X)	-	20 µL	-	-
CYP1A2 Inhibitor (α-naphthoflavone) 30 µM solution (5X)	-	-	-	20 µL
Assay Buffer XXVI/CYP1A2 assay buffer (+5X solvent)*	20 µL	-	70 µL	-
Total	70 µL	70 µL	70 µL	70 µL

*Prepare a small volume of Assay Buffer XXVI/CYP1A2 assay buffer containing the organic solvent to be used to dissolve test inhibitors at 5X final concentration.

12.2 Assay Reaction:

12.2.1 Incubate plate for 10 – 15 minutes at 37°C to allow test compounds to permeate the microsomal membranes and interact with CYP1A2 in the absence of P450 catalytic turnover.

Δ Note During this incubation time, the plate can be read to determine if any test inhibitor is intrinsically fluorescent.

12.2.2 During the incubation, prepare a CYP2C19 Substrate/CYP1A2 substrate/ β-NADP Stock/NADP+ (3X) mixture:

Component	CYP2C19 Substrate/CYP1A2 Substrate/ β-NADP/NADP+ mixture (µL)
5 mM CYP2C19 Substrate/CYP1A2 Substrate stock solution	4
10 mM β-NADP Stock/β-NADP+ stock (100X)	50
Assay Buffer XXVI/CYP1A2 Assay Buffer	1446
TOTAL	1.5 mL

Δ Note: This reaction mix is sufficient for 100 reactions. It can be scaled down if necessary, depending on the number of reactions to be performed.

12.2.3 Start the reaction by adding 30 µL of the CYP2C19 Substrate/CYP1A2 substrate/ β-NADP/NADP+ (3X) mixture to each well using a multichannel pipette, giving a final reaction volume of 100 µL/well.

Δ Note: Suggested final concentration of CYP2C19 Substrate/CYP1A2 substrate is 4 µM, which is approximately equal to the K_m value for the recombinant CYP1A2 enzyme. This can be optimized by the user depending on the inhibitory potency of the test compound and the mechanism of inhibition.

Δ Note: To ensure maximum signal intensity, both incubations (steps 12.2 and 12.3.1) should be performed at 37°C.

12.3 Measurement:

12.3.1 Without any delay, measure output at Ex/Em = 406/468 nm on a microplate reader in kinetic mode for at least 60 minutes at 37°C.

Δ Note: Since reaction starts immediately after the addition of the CYP2C19 Substrate/CYP1A2 Substrate/ β-NADP/NADP+ mix, it is essential to preconfigure the fluorescence microplate reader settings and use a multichannel pipette with a reagent reservoir to minimize lag time among wells.

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- Use only the linear rate for calculation.

13.1 Standard curve calculation:

13.1.1 Subtract the mean fluorescence value of the blank (Standard #1) from all standard readings. This is the corrected fluorescence.

13.1.2 Average the duplicate reading for each standard.

13.1.3 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Calculate background-corrected reaction rates:

13.2.1 For all reaction wells (SC, TI, BC and PIC), choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding fluorescence values at those points (RFU1 and RFU2).

13.2.2 Calculate ΔF and ΔT as follows:

$$\Delta F = RFU2 - RFU1$$
$$\Delta T = T2 - T1$$

13.2.3 Determine the background corrected change (R) in fluorescence intensity for each well of solvent control (SC), test inhibitor (TI) and positive inhibitor control (PIC) by subtracting the ΔF value of the background control (BC), from rates of SC and TI wells.

$$R = \Delta F - \Delta F_{BC} / \Delta T$$

Δ Note: In our experience, the CYP2C19 Substrate/CYP1A2 substrate does not undergo appreciable non-enzymatic conversion to the fluorescent product. Therefore, background control (BC) well rate

calculation may yield a negative value, in which case, BC value may be ignored.

13.3 % relative inhibition due to the test inhibitor is calculated as

$$\%\ relative\ inhibition = \frac{R_{SC} - R_{TI}}{R_{SC}} \text{ x } 100\%$$

Where

R_{SC}: rate solvent control

R_{TI}: rate test inhibitor

Δ Note: If necessary, reaction rate calculations can also be expressed in terms of pmol of 3-CHC formed per unit time per unit amount of protein by interpolation from the standard curve. Each well will contain a total of 50 µg of protein when the recombinant human CYP1A2 is used at the proportions suggested in the kit protocol.

14. Typical data

Typical standard curve – data provided for **demonstration purposes** only. A new standard curve must be generated for each assay performed.

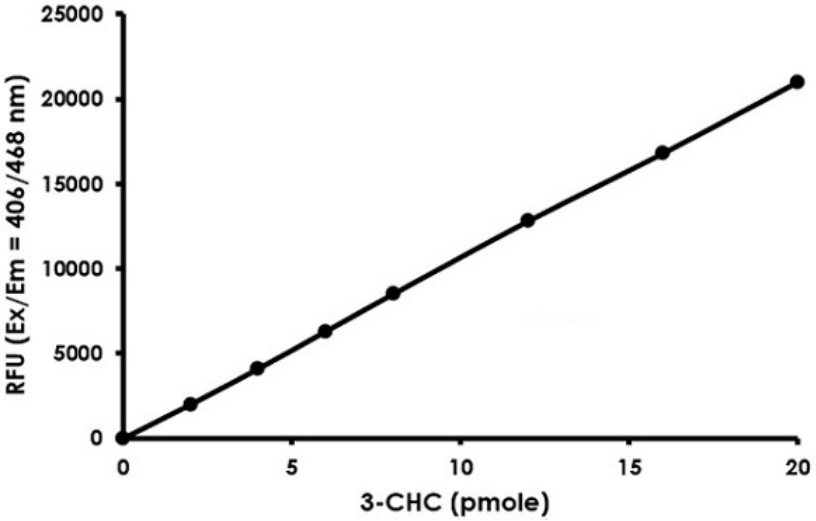


Figure 1. Typical 3-CHC standard calibration curve. One mol of 3-CHC corresponds to the metabolism of one mol of CYP2C19 Substrate/CYP1A2 substrate.

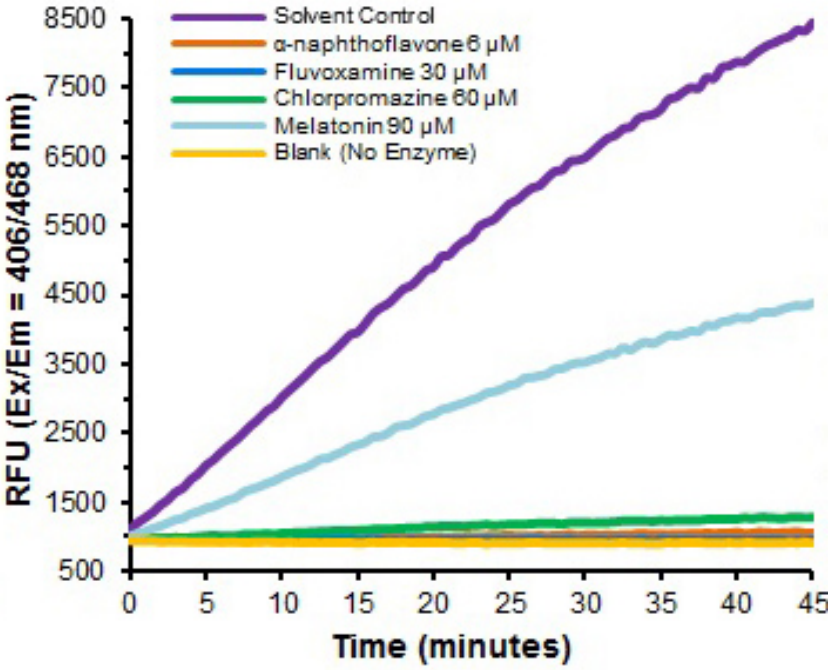


Figure 2. Reaction kinetics of recombinant human CYP1A2 enzyme at 37°C in the presence and absence of the indicated inhibitor (solvent control reaction includes final concentration of 0.6% acetonitrile).

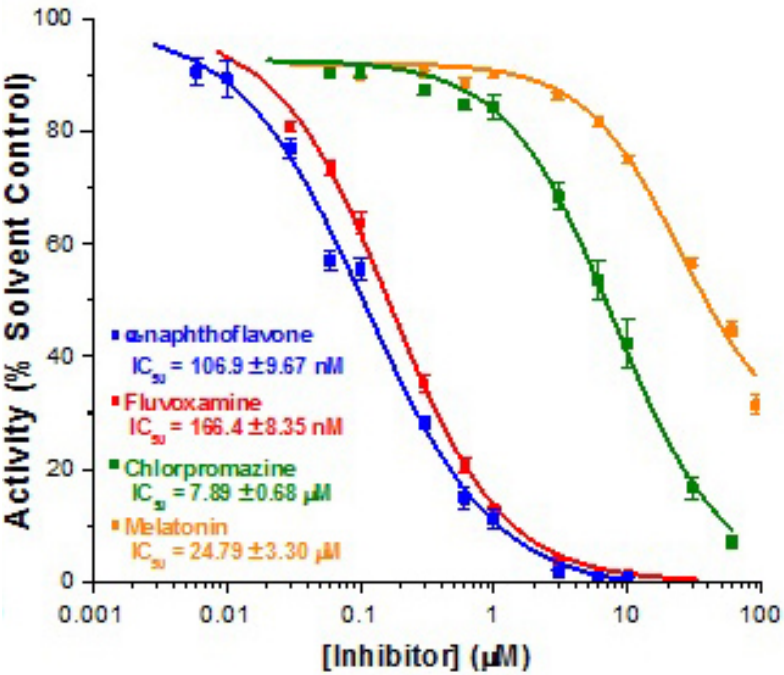


Figure 3. Dose-response curves for various CYP1A2 ligands of differing structural and mechanistic classes: the competitive CYP1A2 inhibitor a-naphthoflavone, the antidepressant fluvoxamine, the tricyclic antipsychotic chlorpromazine and the endogenous neurohormone melatonin (a CYP2C19 Substrate/CYP1A2 substrate). For dose-response curves, percent activity was calculated for each concentration of inhibitor by comparison to activity of reactions containing no inhibitor. For each CYP1A2 inhibitor, IC50 values were derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least three replicates. Assays were performed according to the kit protocol.

15. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ dilute sample so it is within the linear range

16. Interferences

– Many commonly-used organic solvents can severely impact CYP1A2 activity. Importantly, DMSO and methanol cause significant inhibition of CYP1A2 at final concentrations ≥ 0.25% (v/v). This assay is designed to use acetonitrile at ≤ 1% final concentration, which has been shown to have little impact on CYP1A2 activity.

17. Notes

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)