Version 3a, Last updated 24 August 2023

ab211074 CYP1A2 Activity Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of cytochrome P450 1A2 (CYP1A2) activity in various samples.

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.

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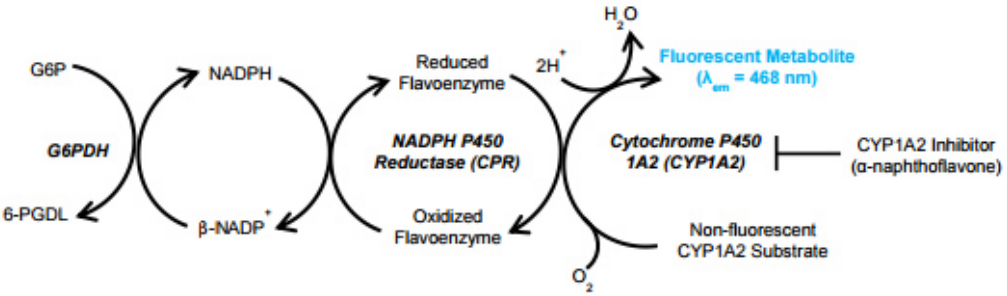
1. Overview

CYP1A2 Activity Assay Kit (Fluorometric) (ab211074) allows rapid measurement of native or recombinant cytochrome P450 1A2 (CYP1A2) activity in biological samples such as liver microsomes. 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. A selective CYP1A2 inhibitor is provided for determination of CYP1A2 activity in heterogeneous biological samples, where other CYP isozymes may contribute to substrate metabolism. The inhibitor displays greater than 20-fold selectivity for CYP1A2 over other CYPs, ensuring targeted inhibition. CYP1A2 specific activity is calculated by running parallel reactions in the presence and absence of the selective inhibitor and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for performing 50 sets of paired reactions (in the presence and absence of inhibitor).



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 xenobiotic compounds are converted to more hydrophilic products to facilitate excretion from the body.

CYP1A2 is responsible for metabolism of approximately 10% of all small molecule drugs commonly used by humans. CYP1A2 is primarily expressed in liver, intestinal and olfactory mucosal tissue and catalyzes oxidation of polyaromatic and heterocyclic molecules such as aromatic amines.

2. Protocol Summary

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

Set up appropriate reaction wells
(background, sample, sample + 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 recombinant human CYP1A2 protein and inhibitor should be used within 1 month.

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 (a-naphthoflavone)	1 vial	-20°C	-20°C
Recombinant Human CYP1A2	1 vial	-20°C	-80°C

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
- 96 well plate with clear flat bottom, preferably black
- Anhydrous DMSO
- Anhydrous acetonitrile (reagent grade)
- Dounce homogenizer (if using tissue)
- (Optional) Protein quantification assay to quantify CYC2C19 specific activity in terms of sample protein content. We recommend Protein Quantitation Kit (Bradford Assay) (ab102535)

  For microsome preparation:

- Microsome Isolation Kit (ab206995)

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 (lyophilized):**

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) (lyophilized):**

Reconstitute in 220 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) (lyophilized):**

Dissolve in 110 µL Assay Buffer XXVI/CYP1A2 Assay Buffer and vortex thoroughly to prepare a 10 mM solution of 100X β-NADP Stock/NADP+ (100X stock). Aliquot 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.

9.5 **CYP2C19 Substrate/CYP1A2 Substrate (lyophilized):**

Reconstitute CYP2C19 Substrate/CYP1A2 substrate in 110 µL of anhydrous reagent-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) (lyophilized):

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

Prepare a 2.5 µM working solution of inhibitor (5X final concentration) by adding 5 µL of stock solution to 1995 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 (lyophilized):

Do not reconstitute until ready to use.

Reconstitute recombinant CYP1A2 protein in 230 µL of Assay Buffer XXVI/CYP1A2 Assay Buffer. Add 20 µL of 100X NADPH Generating System II/NADPH Generating System (100X) (Step 9.3). Mix thoroughly to ensure a homogenous solution – the solution will have a slightly opaque, milky appearance. Aliquot CYP1A2 protein so that you have enough volume to perform the desired number of assays. Store at -80°C. Avoid freeze/thaw cycles.

Use aliquots within one month – recombinant human CYP1A2 will lose approximately 10% activity/week in storage.

Thaw aliquots rapidly at 37°C and keep on ice until use – use within 4 hours of thawing.

Δ Note: Recombinant human CYP1A2 preparation may settle and should be thoroughly mixed before dispensing.

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 10 µL of the 5 mM 3-CHC standard stock solution (Step 9.2) with 240 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. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- Commercially available microsomal preparations (eg. donor-pooled human liver microsomes) can be used for this assay.

11.1 Microsome:

Microsomes from liver tissue or cultured cells can be prepared using Microsome Isolation Kit (ab206995).

11.2 Tissue or cell lysate:

Alternatively, you can prepare a crude enriched lysate with following the protocol:

11.2.1 Harvest ~50 mg tissue or 5 x 10^6 pelleted cells.
11.2.2 Wash in cold PBS.
11.2.3 Homogenize in 500 µL ice-cold Assay Buffer XXVI/CYP1A2 Assay Buffer on ice (use a Dounce homogenizer for the tissue).
11.2.4 Incubate the homogenate on ice for 5 minutes.
11.2.5 Centrifuge homogenate at 15,000 x *g* for 15 minutes in a cold centrifuge at 4°C.
11.2.6 Collect supernatant and transfer to a pre-chilled new tube.
11.2.7 Keep on ice.

Δ Note: To quantify CYP1A2 specific activity in terms of sample protein content, measure total protein content.

Amount of sample per reaction and dilution factor required will vary based upon the nature of the sample.
For human liver microsomes, we recommend starting with 25 µg of microsomal protein per well.
For liver S9 fractions or other cellular lysates, the amount of protein required will be significantly higher. In this case, we recommend starting at 50 -100 µg/well.

Sample	Protein amount per reaction
Human liver microsomes	25 µg/well
Liver S9 fractions	50 – 100 µg/well
Cellular lysates	50 – 100 µg/well

Table 1. Initial suggested sample ranges per well.

11.3 Test ligand compound:

11.3.1 Dissolve test ligand compound in appropriate solvent.

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

Δ Note: Many commonly-used organic solvents can severely impact CYP1A2 activity. Importantly, DMSO causes 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.

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.
- CYP2C19 Substrate/CYP1A2 substrate is also metabolized by CYP2C19; therefore, the use of selective inhibitors is necessary in order to determine the contributions of each CYP450 isozyme in heterogeneous biological samples. The a-naphthoflavone concentration used in this assay is ≥10-fold greater than the *Ki* for recombinant CYP1A2 and has been shown not to affect activity of other CYPs. In human liver microsomes, the concentration results in 60 – 70% inhibition of 3-CHC formation, which represents the CYP1A2-mediated metabolic activity.
- In samples with significant CYP2C19 expression, contribution of CYP2C19 to substrate metabolism may need to be tested using the selective inhibitor (+)-N-3-benzylnirvanol at a final concentration of 30 µM.

12.1 Sample Preparation:

12.1.1 Prepare 50 µL of Sample Reaction Mix for each sample by combining 2 µL 100X NADPH Generating System II/NADPH Generating System (100X) with 2 – 48 µL sample, adjusting volume to 50 µL/reaction with Assay Buffer XXVI/CYP1A2 Assay Buffer. Amount of sample may vary depending on nature of sample (see Table 1).

12.2 **Set up Reaction wells:**

Set up Sample wells (S), Sample + Test compound (S+T), Inhibitor control (IC), Background control (BC), Enzyme control (EC), Enzyme + Inhibitor control (EIC) in a 96-well plate as described in the table below:

Component	S	S+T	IC	BC	EC	EIC
Sample Reaction Mix	50 µL	50 µL	50 µL	-	-	-
Recombinant human CYP1A2	-	-	-	-	25 µL	25 µL
CYP1A2 Inhibitor (2.5 µM)	-	-	20 µL	-	-	40 µL
Test Ligand (5X)	-	20 µL	-	20 µL	-	-
Assay Buffer	20 µL	-	-	50 µL	45 µL	5 µL
TOTAL	70 µL	70 µL	70 µL	70 µL	70 µL	70 µL

12.3 **Assay Reaction:**

12.3.1 Incubate plate for 10 – 15 minutes at 37°C to allow CYP1A2 inhibitor and test ligands to interact with CYP1A2 in the absence of P450 catalytic turnover.

12.3.2 During plate incubation, prepare a CYP2C19 Substrate/CYP1A2 Substrate/β-NADP/$NADP^+$ (3X) mixture:

Component	CYP2C19 Substrate/CYP1A2 Substrate/β-NADP/NADP+ mixture (µL)
5 mM CYP2C19 Substrate/CYP1A2 Substrate stock solution	6
10 mM 100X β-NADP Stock/β-$NADP^+$ stock (100X)	50
Assay Buffer XXVI/CYP1A2 Assay Buffer	1444
TOTAL	1.5 mL

Δ Note: This reaction mix is sufficient for 50 reactions. It can be scaled down if necessary, depending on the number of reactions to be performed.

12.3.3 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.

12.4 Measurement:

12.4.1 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.

Δ Note: Incubation time depends on the CYP1A2 activity in the samples. We recommend measuring RFU in a kinetic mode, and choosing two time points (T1 and T2) within the linear range to calculate the CYP1A2 activity of the samples.

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 and sample 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 Measurement of CYP1A2 in the sample:

13.2.1 For all reaction wells (S, S+T, IC, BC, EC and EIC), 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 as follows:

$$\Delta F = RFU2 - RFU1$$

13.2.3 Determine the background corrected change in fluorescence intensity for each well of sample (S), sample with test compound (S+T) and Inhibitor Control (IC) by subtracting the ΔF value of the background control (BC).

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 Calculate the specific fluorescence generated by CYP1A2 activity (*C*) in the samples (and/or sample with test compounds) by subtracting the inhibition control (IC) from each sample:

$Cs = (\Delta F_S - \Delta F_{BC}) - (\Delta F_{IC} - \Delta F_{BC}) = \Delta F_S - \Delta F_{IC}$

13.4 CYP1A2 metabolic activity (pmol/min/mg or µU/mg) in the sample is calculated as:

$$CYP1A2\ Activity = \frac{B}{\Delta Tx\ P}$$

Where:

B = amount of substrate metabolized to 3-CHC by CYP1A2 in sample well calculated from standard curve (Step 10.4) (pmol).

ΔT = linear phase reaction time T2 – T1 (minutes).

P = amount of protein in the well (mg).

Unit definition:

1 Unit CYP1A2 activity = amount of CYP1A2 that will generate 1.0 µmol of 3-CHC per minute by hydrolysis of 1 µmol of fluorogenic substrate at pH 7.7 at 37°C.

Δ Note: CYP2C19 Substrate/CYP1A2 substrate is also metabolized by CYP1A2; therefore, the use of selective inhibitors is necessary in order to determine the contributions of each CYP450 isozyme in heterogeneous biological samples.
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.

14. Typical data

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

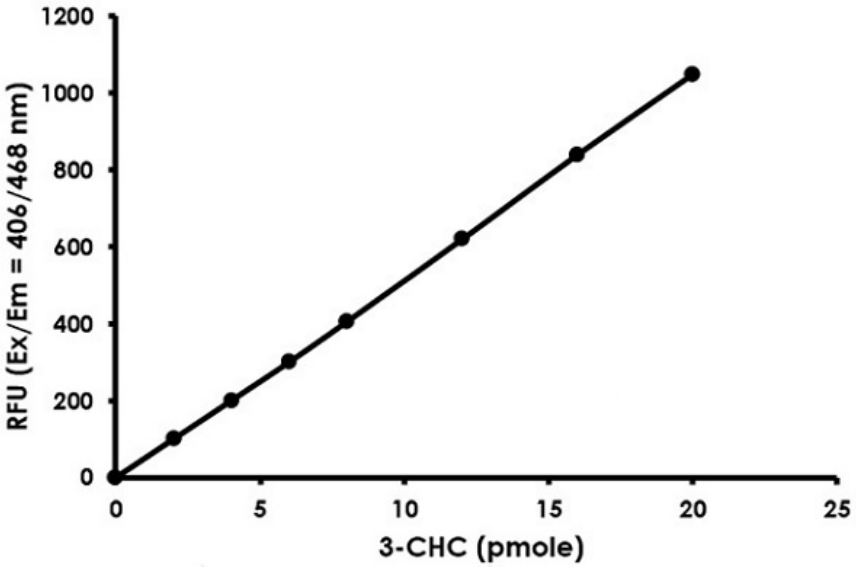


Figure 1. Typical 3-CHC standard calibration curve. One mole of 3-CHC corresponds to the metabolism of one mole of CYP2C19 Substrate/CYP1A2 substrate.

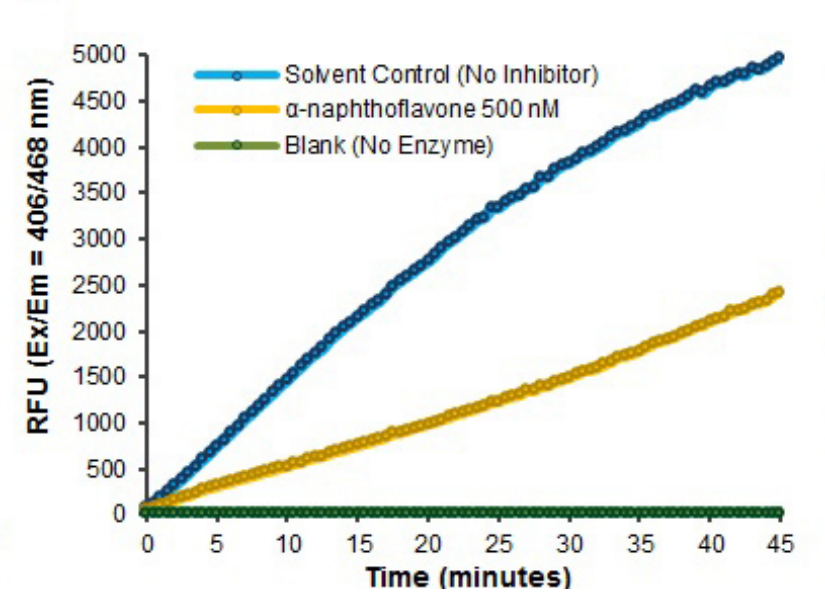


Figure 2. Reaction kinetics of fluorogenic substrate metabolism in donor-pooled human liver microsomes (0.25 mg/mL) at 37°C in the presence and absence of the CYP1A2 inhibitor α-naphthoflavone (the solvent control contained assay buffer with 0.4% acetonitrile).

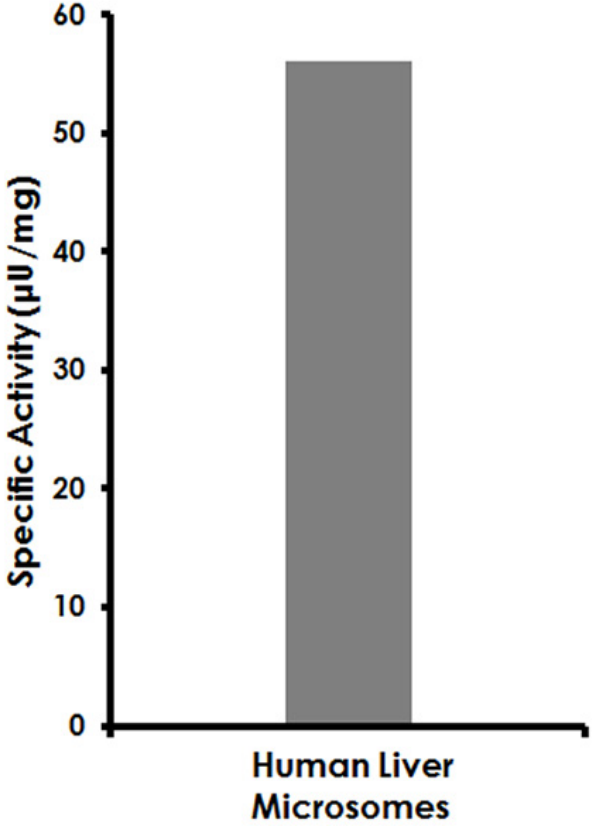


Figure 3. Specific activity of CYP1A2 in pooled human liver microsome sample.

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
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering enzyme in the sample	Check protocol for interfering substances
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

Problem	Reason	Solution
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

- CYP2C19 Substrate/CYP1A2 substrate is also metabolized by CYP2C19; therefore, the use of selective inhibitors is necessary in order to determine the contributions of each CYP450 isozyme in heterogeneous biological samples. The a-naphthoflavone concentration (0.5 µM) used in this assay is ≥10-fold greater than the K_i for recombinant CYP1A2 and has been shown not to affect activity of other CYPs. In human liver microsomes, the concentration results in 60 – 70% inhibition of 3-CHC formation, which represents the CYP1A2-mediated metabolic activity.
- In samples with significant CYP2C19 expression, contribution of CYP2C19 to substrate metabolism may need to be tested using a selective CYP2C19 inhibitor (such as (+)-N-3-benzylnirvanol).

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)