Version 3c, Last updated 24 October 2023

ab211078 CYP2D6 Activity Assay Kit (Fluorometric)

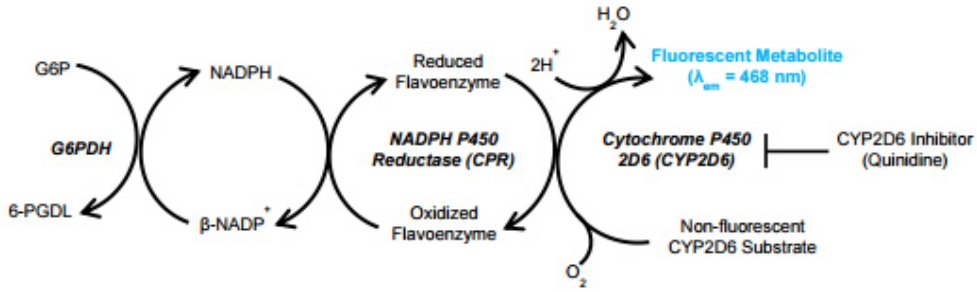
For the rapid, sensitive and accurate measurement of cytochrome P450 2D6 (CYP2D6) activity in various samples.

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

Table of Contents

1. Overview

CYP2D6 Activity Assay Kit (Fluorometric) (ab211078) allows rapid measurement of native or recombinant cytochrome P450 2D6 (CYP2D6) activity in biological samples such as liver microsomes. The assay utilizes a non-fluorescent CYP2D6-selective substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 390/468 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. CYP2D6 specific activity is calculated by running parallel reactions in the presence and absence of the potent CYP2D6-selective inhibitor quinidine and subtracting any residual activity detected with the inhibitor present. The kit contains enough reagents to perform 100 sets of paired reactions (in presence / absence of inhibitor).



Cytochrome P450 2D6 (CYP2D6, 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. CYP2D6 is responsible for metabolism of nearly 25% of all small molecule drugs used by humans, particularly psychiatric drugs such as antidepressants, antipsychotics and stimulants.

CYP2D6 catalyzes oxidation of lipophilic bases with an aromatic ring and a nitrogen atom and is highly expressed in liver and brain tissue. The CYP2D6 gene is highly polymorphic in the human population, with CYP2D6 activity ranging from complete metabolic deficiency to ultra-rapid metabolism.

2. Protocol Summary

Prepare Standard curve and
measure fluorescence at Ex/Em = 390/468 nm

Set up appropriate reaction wells
(background, sample, sample + compound, controls)

Add substrate mix to reaction wells

Measure fluorescence at Ex/Em = 390/468 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 CYP2D6 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)
CYP2D6 Assay Buffer	100 mL	-20°C	-20°C
AHMC Standard	25 µL	-20°C	-20°C
NADPH Generating System 100X (25 mg)	1 vial	-20°C	-20°C
β-$NADP^+$ Stock 100X (2 mg)	1 vial	-20°C	-20°C
CYP2D6 Substrate (200 µg)	1 vial	-20°C	-20°C
CYP2D6 Inhibitor (Quinidine) (150 µg)	1 vial	-20°C	-20°C
Recombinant Human CYP2D6 (15 mg)	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 = 390/468 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
- Opaque white 96 well plates with flat bottom
- 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 **CYP2D6 Assay Buffer (100 mL):**

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

9.2 **AHMC Standard:**

Reconstitute the AHMC Standard in 110 µL of DMSO to generate a 2 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 **NADPH Generating System (100X) (lyophilized, 25 mg):**

Reconstitute in 440 µL CYP2D6 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 (100X) (lyophilized, 2 mg):**

Dissolve in 440 µL CYP2D6 Assay Buffer and vortex thoroughly to prepare a 100X stock solution of NADP+. Aliquot so that you have enough to perform the desired number of assays. Store at -20°C. β-NADP+ Stock is stable for at least 3 freeze/thaw cycles.

9.5 **CYP2D6 Substrate (lyophilized, 200 µg):**

Reconstitute CYP2D6 substrate in 220 µL of anhydrous reagent grade acetonitrile. 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 **CYP2D6 inhibitor (Quinidine) (lyophilized, 150 µg):**

Reconstitute CYP2D6 inhibitor in 220 µL of anhydrous reagent-grade acetonitrile to generate a 2 mM stock solution. Vortex until fully dissolved. Store at -20°C. Use within 2 months.

Prepare a 15 µM working solution of inhibitor (5X final concentration) by adding 15 µL of stock solution to 1985 µL of CYP2D6 Assay Buffer. The inhibitor working solution should be stored at -20°C and used within one week.

9.7 **Recombinant Human CYP2D6 (lyophilized, 15 mg):**

Do not reconstitute until ready to use.

Reconstitute recombinant CYP2D6 protein in 460 µL of CYP2D6 Assay Buffer. Add 40 µL of NADPH Generating System (100X) (Step 9.3). Mix thoroughly to ensure a homogenous solution. Aliquot CYP2D6 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 CYP2D6 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 CYP2D6 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 10 µM (10 pmol/µL) standard by diluting 5 µL of the 2 mM AHMC standard stock solution (Step 9.2) in 995 µL CYP2D6 Assay Buffer. Mix well.

10.2 Use the 10 µM AHMC standard to prepare the standard curve dilution as described in the table in a plate or microcentrifuge tubes:

Standard #	AHMC Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount AHMC in well (pmol/well)
1	0	300	100	0
2	6	294	100	20
3	12	288	100	40
4	18	282	100	60
5	24	276	100	80
6	36	264	100	120
7	48	252	100	160
8	60	240	100	200

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

10.3 Immediately measure fluorescence in an end point mode program on a microplate reader at Ex/Em = 390/468 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 CYP2D6 Assay Buffer on ice using a Dounce homogenizer.
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 CYP2D6 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.
In human liver tissue, CYP2D6 typically accounts for only a small fraction of the total CYP450 content (2 – 4% approx). Hence, for human liver microsomes, we recommend starting with 50 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 100 -200 µg/well.

Sample	Protein amount per reaction
Human liver microsomes	50 µg/well
Liver S9 fractions	100 – 200 µg/well
Cellular lysates	100 – 200 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 CYP2D6 Assay Buffer.

Δ Note: Many commonly-used organic solvents can severely impact CYP2D6 activity. Importantly, DMSO cause significant inhibition of CYP2D6 at final concentrations ≥ 0.2% (v/v). This assay is designed to use acetonitrile at ≤ 1% final concentration, which has been shown to have little impact on CYP2D6 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.

12.1 Sample Preparation:

12.1.1 Prepare 50 µL of Sample Reaction Mix for each sample by combining 2 µL NADPH Generating System (100X) with 2 – 48 µL sample, adjusting volume to 50 µL/reaction with CYP2D6 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), in a 96-well plate as described in the table below:

Component	S	S+T	IC	BC	EC
Sample Reaction Mix	50 µL	50 µL	50 µL	-	-
Recombinant human CYP2D6	-	-	-	-	25 µL
CYP2D6 Inhibitor (15 µM)	-	-	20 µL	-	-
Assay Buffer or Test Ligand (5X)	20 µL	20 µL	-	70 µL	45 µL
TOTAL	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 CYP2D6 inhibitor and test ligands to interact with CYP2D6 in the absence of P450 catalytic turnover.

12.3.2 During plate incubation, prepare a CYP2D6 Substrate/$NADP^+$ (3X) mixture:

Component	CYP2D6 Substrate/NADP+ mixture (µL)
2 mM CYP2D6 Substrate stock solution	30
5 mM β-$NADP^+$ stock (100X)	100
CYP2D6 Assay Buffer	2870
TOTAL	3 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.

12.3.3 Start the reaction by adding 30 µL of the CYP2D6 Substrate/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 = 390/468 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 CYP2D6 Substrate/$NADP^+$ 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 CYP2D6 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 CYP2D6 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 CYP2D6 in the sample:

13.2.1 For all reaction wells (S, S+T, IC, BC and EC), 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 CYP2D6 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 CYP2D6 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 CYP2D6 metabolic activity (pmol/min/mg or µU/mg) in the sample is calculated as:

$$CYP2D6\ Activity = \frac{B}{\Delta T x\ P}$$

Where:

B = amount of substrate metabolized to AHMC by CYP2D6 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 CYP2D6 activity = amount of CYP2D6 that will generate 1.0 µmol of AHMC per minute by hydrolysis of 1 µmol of fluorogenic substrate at pH 7.7 at 37°C.

Δ Note: In our experience, the CYP2D6 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.

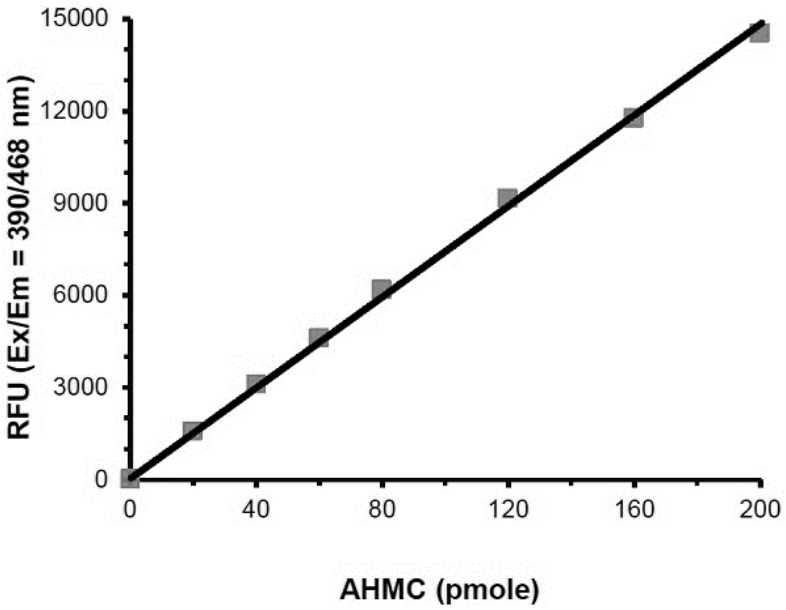


Figure 1. Typical AHMC standard calibration curve. One mol of AHMC corresponds to the metabolism of one mol of CYP2D6 substrate.

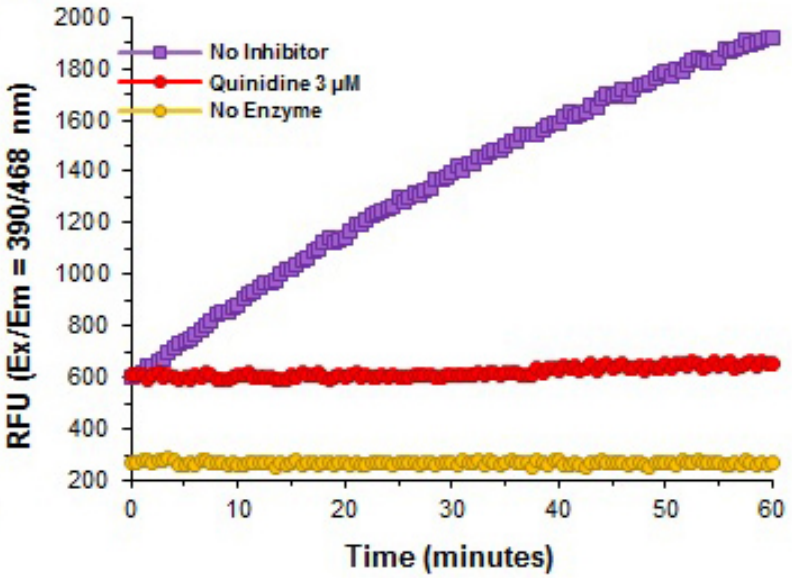


Figure 2. Reaction kinetics of fluorogenic substrate metabolism in human liver microsomes (0.5 mg/mL) at 37°C in the presence and absence of the selective CYP2D6 inhibitor quinidine (the no inhibitor reaction contained a final concentration of 0.5% acetonitrile).

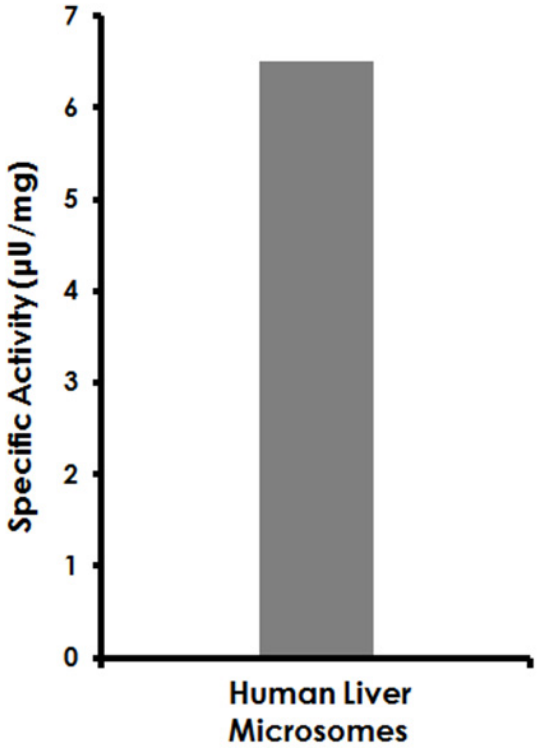


Figure 3. Specific activity of CYP2D6 in pooled human liver microsome sample (0.5 mg/mL).

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. 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)