ab113462 – KDM4/JMJD2 Activity Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of activity/inhibition of total KDM4/JMJD2 using nuclear extracts or KDM4/JMJD2 subtypes (JMJD2A-JMJD2F) using purified enzymes from a broad range of species

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

Version 1 Last Updated 1 September 2014

Table of Contents

1. BACKGROUND

Lysine histone methylation is one of the most robust epigenetic marks, and is essential for the regulation of multiple cellular processes. The methylation of H3K9 seems to be of particular significance, as it is associated with repression regions of the genome. H3K9 methylation was considered irreversible until the identification of a large number of histone demethylases indicating that demethylation events play an important role in histone modification dynamics. So far at least 2 classes of H3K9 specific histone demethylase, JMJD1 (JHDM2), and JMJD2 (JHDM3) have been identified. The JMJD1 family, including JMJD1A, JMJD1B, and JMJD1C can remove di- and mono-methylation from H3K9 while the JMJD2 family, including JMJD2A, JMJD2B, JMJD2C, and JMJD2D, JMJD2E, and JMJD2F can remove tri-methylation from H3K9 and H3K36. JMJD2 demethylases are Jumonji domain proteins and catalyze the removal of methylation by using a hydroxylation reaction with iron and alpha-ketoglutarate required as cofactors.

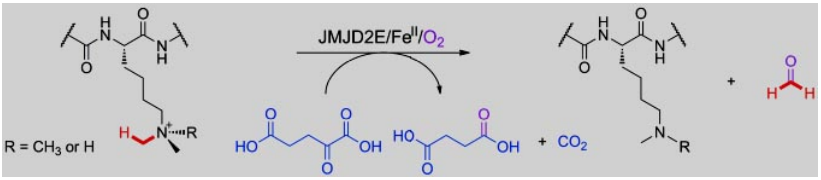


Figure 1. Histone H3K9 demethylation reaction catalyzed by JMJD2 demethylase. (Hopkinson et al: Chembiochem, 4: 506-510, 2010)

JMJD2 demethylases are found to have potential oncogenic functions. For example, JMJD2A is amplified in prostate cancer and JMJD2C overexpression is observed in oesophageal carcinoma. Detection of activity and inhibition of JMJD2 would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing, as well as benefiting cancer diagnostics and therapeutics.

There are only a couple of methods used for detecting JMJD2 activity and inhibition. These methods are based on the measurement of formaldehyde release, a by-product of JMJD2 enzymatic reaction, and have significant weaknesses: (1) Large amounts (at µg level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues cannot be used; (3) Redox-sensitive JMJD2 inhibitors are not suitable for testing with these methods; (4) High interference by SDS, DMSO, thiol-containing chemicals, and ions, which are often contained in enzyme solutions, tested compound solvents, and assay buffers; and (5) Less accuracy than a direct measurement of JMJD2-converted demethylated product.

ab113462 is designed to address these issues this kit has the following advantages:

- 3 hour fluorometric procedure in a 96 stripwell microplate format allows for either manual or high throughput analysis
- Directly measures JMJD2 activity via a straightforward detection of JMJD2-converted demethylated products, rather than by-products, thus eliminating assay interference caused by thiol-containing chemicals such as DTT, GSH and 2-mercaptoethanol, or caused by detergents/ions such as tween-20, SDS, triton X-100, Fe, and Na
- Both cell/tissue extracts and purified JMJD2 can be used, which allows for the detection of inhibitory effects of JMJD2 inhibitors in vivo and in vitro
- Sensitivity is up to 2,000 times higher than formaldehyde release-based JMJD2 assays, allowing activity to be fluorometrically detected from as low as 2 ng of purified JMJD2 enzyme
- Demethylated H3K9 standard is included, allowing specific activity of JMJD2 to be quantified
- Accurate, reliable, and consistent with extremely low background signals. High throughput analysis can be completed within 3 hours

In ab113462 a tri-methylated histone H3K9 substrate is stably coated onto microplate wells. Active JMJD2s bind to the substrate and remove methyl groups from the substrate. The JMJD2-demethylated products can be

recognized with a specific antibody. The ratio or amount of demethylated products, which is proportional to enzyme activity, can then be fluorometrically measured by reading the fluorsecence in a fluorescent microplate spectrophometer at 530 excitation and 590 emission. The activity of the JMJD2 enzyme is in turn proportional to the fluorescent intensity measured.

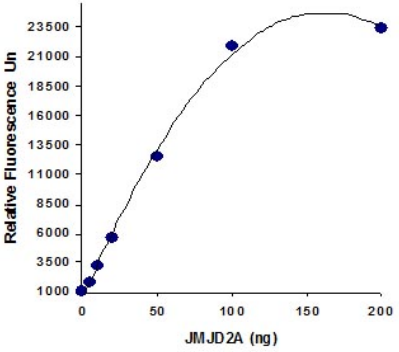


Figure 2. Demonstration of high sensitivity of the KDM4/JMJD2 activity assay achieved by using recombinant KDM4/JMJD2 with ab113462.

ab113462 is suitable for measuring activity or inhibition of total JMJD2 using nuclear extracts or JMJD2 subtypes (JMJD2A-JMJD2F) using purified enzymes from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues. Nuclear extracts can be prepared by using your own successful method. For your convenience and the best results, Abcam offers a nuclear extraction kit (ab113474) optimized for use with this kit. Nuclear extracts can be used immediately or stored at -80°C for future use. Purified enzymes can be active JMJD2s from recombinant proteins or isolated from cell/tissues.

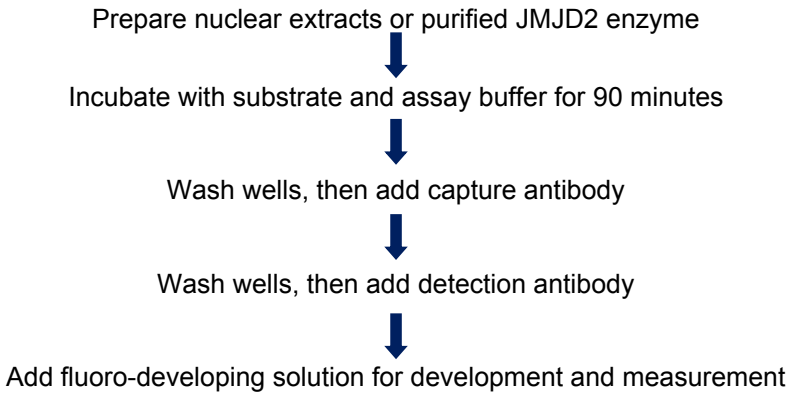
Input materials can be nuclear extracts or purified JMJD2 enzymes. The amount of nuclear extracts for each assay can be 0.5-20 µg with an optimal

range of 5-10 µg. The amount of purified enzymes can be 2-500 ng, depending on the purity and catalytic activity of the enzymes.

The JMJD2 assay standard (demethylated histone H3K9) is provided for the quantification of JMJD2 enzyme activity. Because JMJD2 activity can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

2. ASSAY SUMMARY



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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table and away from light upon receipt.

Observe the storage conditions for individual prepared components in sections 9, 10 & 11.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if the 10X Wash Buffer contains salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
JMJD2 Assay Buffer	4 mL	8 mL	RT
JMJD2 Substrate, 50 µg/mL	60 µL	120 µL	-20°C
JMJD2 Assay Standard, 50 µg/mL	10 µL	20 µL	-20°C
Capture Antibody, 1000 µg/mL	5 µL	10 µL	4°C
Detection Antibody, 400 µg/mL	6 µL	12 µL	-20°C
Fluoro Developer	10 µL	20 µL	-20°C
Fluoro Enhancer	10 µL	20 µL	4°C
Fluoro Diluter	4 mL	8 mL	RT
Co-factor 1	30 µL	60 µL	4°C
Co-factor 2	30 µL	60 µL	4°C
Co-factor 3	30 µL	60 µL	4°C
8-Well Assay Strip (with Frame)	6	12	4°C
Adhesive Covering Film	1	1	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Fluorescence microplate reader capable of reading fluorescence at 530 excitation and 590 emission nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzymes
- Parafilm M or aluminium foil

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- 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
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- 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
- Complete removal of all solutions and buffers during wash steps
- **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**

9. REAGENT PREPARATION

9.1 1X Wash Buffer

Add 13 mL of 10X Wash Buffer to 117 mL of distilled water and adjust pH to 7.2-7.5. This 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 JMJD2 Completed Assay Buffer

Add Co-factor 1, Co-factor 2, and Co-factor 3 to JMJD2 Assay Buffer at a ratio of 1:100 for each Co-factor (e.g. add 1 µL of each Co-factor to 100 µL of JMJD2 Assay Buffer for a total of 103 µL).

9.3 Diluted Capture Antibody

Dilute the Capture Antibody with 1X Wash Buffer at a ratio of 1:1000 (e.g. add 1 µL of Capture Antibody to 1000 µL of 1X Wash Buffer). 50 µl of the Diluted Capture Antibody will be required for each assay well.

9.4 Diluted Detection Antibody

Dilute the Detection Antibody with 1X Wash Buffer at a ratio of 1:2000 (e.g. add 1 µL of Detection Antibody to 2000 µL of 1X Wash Buffer). 50 µL of the Diluted Detection Antibody will be required for each assay well.

9.5 Fluorescence Development Solution

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer to every 500 µl of Fluoro Diluter.

Note: *Keep each of the diluted solutions (except 1X Wash Buffer) on ice until use. Any remaining diluted solutions, other than 1X Wash Buffer, should be discarded if not used within the same day.*

Table 1. Approximate amount of required buffers and solutions for defined assay wells, based on the protocol:

Reagents	1 Well	8 wells (1 strip)	16 wells (2 strips)	48 wells (6 strips)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL
JMJD2 Assay Buffer	50 µL	400 µL	800 µL	2400 µL
JMJD2 Substrate	1 µL	8 µL	16 µL	50 µL
JMJD2 Assay Standard	N/A	N/A	1 µL (optional)	2 µL
Diluted Capture Antibody	50 µL	400 µL	800 µL	2400 µL
Diluted Detection Antibody	50 µL	400 µL	800 µL	2400 µL
Fluorescence Development Solution	0.05 mL	0.4 mL	0.8 mL	2.4 mL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be 0.5-20 µg with optimized range of 5-10 µg. The amount of purified enzymes can be 2-500 ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extraction: You can use your own method of choice for preparing nuclear extracts. Abcam also offers a Nuclear Extraction Kit (ab113474) optimized for use with this kit. The nuclear extract or purified JMJD2 enzyme should be stored at -80°C until use.

11. STANDARD PREPARATION

Suggested Standard Curve Preparation:

11.1 First, dilute JMJD2 Assay Standard with JMJD2 Assay Buffer to 5 ng/µL by adding 1 µL of JMJD2 Assay Standard to 9 µL of JMJD2 Assay Buffer.

11.2 Then, further prepare five concentrations by combining the 5 ng/µL diluted JMJD2 Assay Standard with JMJD2 Assay Buffer into final concentrations of 0.2, 0.5, 1.0, 2.0, and 5 ng/µL according to the following dilution table:

Tube	JMJD2 Assay Standard 5 ng/µL (µL)	JMJD2 Assay Buffer (µL)	Resulting JMJD2 Assay Standard concentration (ng/ µL)
1	1.0	24.0	0.2
2	1.0	9.0	0.5
3	1.0	4.0	1.0
4	2.0	3.0	2.0
5	4.0	0.0	5.0

Note: *Keep each of the diluted solutions (except 1X Wash Buffer) on ice until use. Any remaining diluted solutions, other than 1X Wash Buffer, should be discarded if not used within the same day.*

12. PLATE PREPARATION

Table 2. The suggested strip-well plate setup for the JMJD2 activity assay in a 48-assay format. The controls and samples can be measured in duplicates.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	JMJD2 Assay Standard 0.2 ng	JMJD2 Assay Standard 0.2 ng	Sample	Sample	Sample	Sample
C	JMJD2 Assay Standard 0.5 ng	JMJD2 Assay Standard 0.5 ng	Sample	Sample	Sample	Sample
D	JMJD2 Assay Standard 1.0 ng	JMJD2 Assay Standard 1.0 ng	Sample	Sample	Sample	Sample
E	JMJD2 Assay Standard 2.0 ng	JMJD2 Assay Standard 2.0 ng	Sample	Sample	Sample	Sample
F	JMJD2 Assay Standard 5.0 ng	JMJD2 Assay Standard 5.0 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

13. ASSAY PROCEDURE

13.1 Enzymatic Reaction

13.1.1 Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive control) to ensure that the signal generated is validated. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

13.1.2 Blank Wells: Add 49 µL of Completed Assay Buffer and 1 µL of JMJD2 Substrate to each blank well.

13.1.3 Standard Wells: For a standard curve, add 49 µL of JMJD2 Completed Assay Buffer and 1 µL of Diluted Assay Standard solution to each standard well with a minimum of five wells, each at a different concentration between 0.2-5 ng/µL (based on the dilution chart in Section 11 - Standard Preparation; see the suggested plate layout in Section 12 as an example).

13.1.4 Sample Wells Without Inhibitor: Add 44-48 µL of JMJD2 Completed Assay Buffer, 1 µL of JMJD2 Substrate, and 1-4 µL of your nuclear extracts or 1-4 µL of your purified JMJD2 enzyme to each sample well without inhibitors. Total volume should be 50 µL per well.

13.1.5 Sample Wells With Inhibitor: Add 40-43 µL of JMJD2 Completed Assay Buffer, 1 µL of JMJD2 Substrate, 1-4 µL of your nuclear extracts or 1-4 µL of your purified JMJD2 enzyme, and 5 µl of inhibitor solution. Total volume should be 50 µl per well.

Note: *(1) Follow the suggested well setup diagrams in Section 12; (2) It is recommended to use 5-10 µg of nuclear extract per well or 10-100 ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g. 1-1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with JMJD2 Assay Buffer at a 1:10 ratio (e.g. add 0.5 µl of inhibitor to 4.5 µl of JMJD2 Assay Buffer), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less. The*

Jumonji demethylase general inhibitor, N-Oxalylglycine can be used as a control inhibitor.

13.1.6 Tightly cover the strip-well microplate with the Adhesive Covering Film to avoid evaporation, and incubate at 37°C for 60-120 minutes.

Note: *(1) The incubation time may depend on intrinsic JMJD2 activity. However, in general, 60-90 minutes incubation is suitable for active purified JMJD2 enzymes and 90-120 minutes incubation is required for nuclear extracts;*

(2) The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used

13.1.7 Remove the reaction solution from each well. Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

13.2 **Antibody Binding and Signal Enhancing**

13.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 60 minutes.

13.2.2 Remove the Diluted Capture Antibody solution from each well.

13.2.3 Wash each well with 150 µL of 1X Wash Buffer each time for three times.

13.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.5 Remove the Diluted Detection Antibody solution from each well

13.2.6 Wash each well with 150 µL of the 1X Wash Buffer each time for four times

Note: *Ensure any residual wash buffer in the wells is thoroughly removed as much as possible at each wash step*

13.3 **Signal Detection**

13.3.1 Add 50 µL of Fluorescence Development Solution to each well and incubate at room temperature for 2-4 minutes away from direct light. The Fluorescence Development Solution will turn pink in the presence of sufficient demethylated products.

13.3.2 Read the fluorescence on a fluorescence microplate reader within 2-10 minutes at Ex/Em = 530/590 nm.

Note: *If the strip-well microplate frame does not fit in the fluorescence microplate reader, transfer the solution to a standard 96-well microplate*

13.3.3 Calculate JMJD2 activity or inhibition using the formulae provided in Section 14 – Data Analysis.

14. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Calculate the JMJD2 activity or inhibition using the following formula:

$$\text{JMJD2 activity (RFU/min/mg)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{Protein amount (µg)}^{*} \times \text{Incubation time}^{**}} \times 1000$$

*Protein amount (µg) added into the reaction at step 13.1.4.

**Incubation time (minutes) at step 13.1.6.

Example calculation:

Average OD450 of sample is 6800.

Average OD450 of blank is 800.

Protein amount is 5 µg

Incubation time is 2 hours (120 minutes)

JMJD2 activity = [(6800– 800) / (5 x 120)] x 1000
= 10000 RFU/min/mg

For an accurate or specific activity calculation, generate a standard curve and plot RFU versus amount of JMJD2 Assay Standard at each concentration point. Determine the slope as RFU/ng then calculate the amount of JMJD2-converted demethylated product using the following formula:

$$\text{Demethylated Product (ng)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{Slope}}$$

$$\text{JMJD2 activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{\text{Protein amount (µg)} \times \text{Incubation time*}} \times 1000$$

**Incubation time (minutes) at step 13.1.6.

For inhibition calculation:

$$\text{Inhibition \%} = 1 - \left(\frac{\text{Inhibitor Sample RFU} - \text{Blank RFU}}{\text{No Inhibitor Sample RFU} - \text{Blank RFU}} \right) \times 100\%$$

15. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the positive control and sample wells	Reagents are added incorrectly	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake
	The well is incorrectly washed before enzyme reaction	Ensure the well is not washed prior to adding the positive control and sample
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol is followed correctly
	Incorrect fluorescence reading	Check if appropriate fluorescent wavelength (Ex/Em = 530/590 nm filter) is used
No signal or weak signal in both the positive control and sample wells	Kit was not stored or handled properly	Ensure all components of the kit were stored at the appropriate temperature and caps are tightly capped after each opening or use
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in step 13.1.3	Ensure a sufficient amount of standard is added
	The standard is degraded due to improper storage conditions	Follow the Storage guidance in Sections 4 and 5 for storage instructions of JMJD2 Assay Standard

High Background Present for the Blank	Insufficient washing of wells	Check if washing recommendations at each step is performed according to the protocol
	Contaminated by sample or positive control	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time with Diluted Detection Antibody is too long	The incubation time at step 13.2.4 should not exceed 45 minutes
	Over development of color	Decrease the development time in step 13.3.1 and measure absorbance as quickly as possible
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified	Ensure your protocol is suitable for JMJD2 protein extraction. For the best results, it is advised to use Abcam's Nuclear Extraction Kit (ab113474). Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity

	Sample amount added into the wells is insufficient	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in 13.1. The sample can be titrated to determine the optimal amount to use in the assay
	Sample was not stored properly or has been stored for too long	Ensure sample is stored in aliquots at -80ºC, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing
	Little or no activity of JMJD2 contained in the sample	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared nuclear extracts or purified enzymes
Uneven fluorescent development	Insufficient washing of the wells	Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible

	Delayed fluorescence development in the wells	Ensure fluorescence development solution is added sequentially and consistent with the order you added the other reagents (e.g. from well A to well G or from well 1 to well 12)

16. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp