

ab113461 – KDM4/JMJD2 Activity Quantification Kit (Colorimetric)

Instructions for Use

For the measurement of activity/inhibition of KDM4/JMJD2 using nuclear extracts or purified enzymes from a broad range of species

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

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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 H3-K9 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.

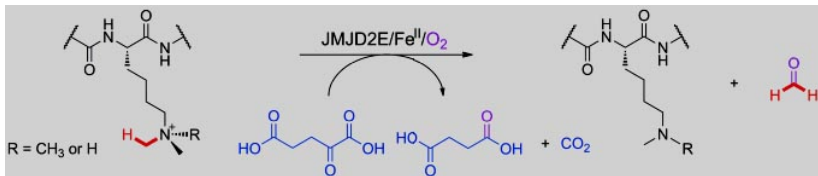


Figure 1. Histone H3-K9 demethylation reaction catalyzed by JMJD2 demethylase. (Hopkinson et al: *Chembiochem*, 4: 506-510, 2010)

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. ab113461 is designed to address these issues.

Compared to a formaldehyde release-based method, this kit has the following advantages:

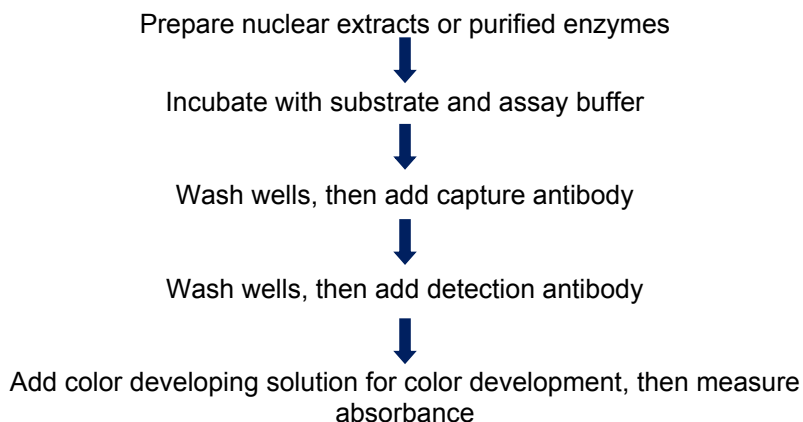
- 3 hour colorimetric procedure in a 96 stripwell microplate format allows for either manual or high throughput analysis.
- Directly measures JMJD2 activity via a straightforward detection of JMJD2-converted demethylated products, rather than by-products, thus eliminating assay interference caused by 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 1,000 times higher than formaldehyde release-based JMJD2 assays, allowing activity to be colorimetrically detected from as low as 10 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.

In Abcam's KDM4/JMJD2 Activity Quantification Assay Kit (Colorimetric) a histone H3K9me³ 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 colorimetrically measured by reading the absorbance in a microplate spectrophotometer at a wavelength of

450 nm. The activity of the JMJD2 enzyme is in turn proportional to the optical density intensity measured.

ab113461 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.

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 upon receipt away from light.

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

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

Check if 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.

Check if a blue color is present in Developer Solution which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of Developer Solution required into a secondary container (tube or vial) before adding Developer Solution into the assay wells.

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 |
| Developer Solution* | 5 mL | 10 mL | 4°C |
| Stop Solution* | 5 mL | 10 mL | RT |
| 8-Well Assay Strip (with Frame) | 6 | 12 | 4°C |
| 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 |
| Adhesive Covering Film | 1 | 1 | RT |

*Spin the solution down to the bottom prior to use.

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
- Microplate reader capable of reading absorbance at 450 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

Prepare fresh reagents immediately prior to use.

9.1 **1X Wash Buffer**

Prepare Diluted 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 **Complete Assay Buffer**

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

9.3 **Diluted Capture Antibody Solution**

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

9.4 **Diluted Detection Antibody Solution**

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

9.5 Diluted Standard Solution

First, dilute JMJD2 Assay Standard with Assay Buffer to 5 ng/μL by adding 1 μL of JMJD2 Assay Standard to 9 μL of Assay Buffer. Then, further prepare five concentrations by combining the 5 ng/μL diluted JMJD2 Assay Standard with Assay Buffer into final concentrations of 0.2, 0.5, 1.0, 2.0, and 5.0 ng/μL according to the following dilution chart:

| Tube | JMJD2 Assay Standard (μL) | Assay Buffer (μL) | Assay Standard conc (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.*

9.6 Suggested Working Buffer and Solution Setup

The table below shows the 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 (3 strips) |
|----------------------------|--------|----------------------|------------------------|------------------------|
| 1X Wash Buffer | 2.5 mL | 20 mL | 40 mL | 120 mL |
| Complete Assay Buffer | 50 µL | 400 µL | 800 µL | 2400 µL |
| Substrate | 1 µL | 8 µL | 16 µL | 50 µL |
| 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 |
| Developer Solution | 0.1 mL | 0.8 mL | 1.6 mL | 4.8 mL |
| Stop solution | 0.1 mL | 0.8 mL | 1.6 mL | 4.8 mL |

10. SAMPLE PREPARATION

Input Amount

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

Nuclear Extracts

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.

Nuclear Extract or Purified JMJD2 Storage

Nuclear extract or purified JMJD2 enzyme should be stored at –80°C until used.

11. ASSAY PROCEDURE

11.1 Enzymatic Reaction

11.1.1 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).

11.1.2 Blank Wells: Add 49 μL of Complete Assay Buffer and 1 μL of Substrate to each blank well.

Standard Wells: For a standard curve, add 49 μL of Complete 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 $\text{ng}/\mu\text{L}$ (based on the dilution chart in Section 9.5).

Sample Wells Without Inhibitor: Add 45 μL - 48 μL Complete Assay Buffer, 1 μL of Substrate, and 1 - 4 μL of your nuclear extracts or 1 - 4 μL of your purified JMJD2 enzyme to each sample well without inhibitor. Total volume should be 50 μL per well.

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

Note: Follow the suggested well setup diagram below. It is recommended to use 2 μg – 10 μg of nuclear extract per well or 10 ng – 100 ng of purified enzyme per well. The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 μM – 1000 μM). However, the final concentration of the inhibitors before adding to the wells should be prepared with Assay Buffer at a 1:10 ratio (e.g., add 0.5 μL of inhibitor to 4.5 μL of 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.

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

Note: *The incubation time may depend on intrinsic JMJD2 activity. In general, 60-90 min incubation is suitable for active purified JMJD2 enzymes and 90-120 min incubation is required for nuclear extracts. The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.*

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

11.2 Antibody Binding & Signal Enhancing

- 11.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

- 11.2.2 Remove the Diluted Capture Antibody solution from each well.

- 11.2.3 Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

- 11.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

- 11.2.5 Remove the Diluted Detection Antibody solution from each well.

- 11.2.6 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.*

11.3 Signal Detection

- 11.3.1 Add 100 µL of Developer Solution to each well and incubate at room temperature for 1 - 10 min away from light. Begin monitoring color change in the sample wells and control wells. The Developer Solution will turn blue in the presence of sufficient methylated DNA.

ASSAY PROCEDURE

11.3.2 Add 100 μL of Stop Solution to each well to stop enzyme reaction when color in the positive control wells turns medium blue. The color will change to yellow after adding Stop Solution and the absorbance should be read on a microplate reader within 2 - 10 min at 450 nm with an optional reference wavelength of 655 nm.

Note: Most microplate readers have the capability to carry out dual wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice, once at 450 nm and once at 655 nm. Then, manually subtract the 655 nm ODs from 450 nm ODs. If the strip-well microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

Suggested Strip Well Setup: The suggested strip-well plate setup for the JMJD2 activity assay in a 48-assay format is shown in the table below. 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 | Assay Standard 0.2 ng | Assay Standard 0.2 ng | Sample | Sample | Sample | Sample |
| C | Assay Standard 0.5 ng | Assay Standard 0.5 ng | Sample | Sample | Sample | Sample |
| D | Assay Standard 1.0 ng | Assay Standard 1.0 ng | Sample | Sample | Sample | Sample |
| E | Assay Standard 2.0 ng | Assay Standard 2.0 ng | Sample | Sample | Sample | Sample |
| F | Assay Standard 5.0 ng | Assay Standard 5.0 ng | Sample | Sample | Sample | Sample |

ASSAY PROCEDURE

| | | | | | | |
|----------|---------------|---------------|---------------|---------------|---------------|---------------|
| G | Sample | Sample | Sample | Sample | Sample | Sample |
| H | Sample | Sample | Sample | Sample | Sample | Sample |

12. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Simple Calculation

Calculate JMJD2 activity or inhibition using the following formulae:

$$\text{JMJD2 activity (OD/min/mg)} = \frac{\text{Sample OD} - \text{Blank OD}}{(\text{Protein Amount } (\mu\text{g})^* \times \text{min}^{**})} \times 1000$$

*Protein amount (μg) added into the reaction at step 11.1.2.

**Incubation time (minutes) at step 11.1.3.

Example calculation:

Average OD450 of sample is 0.65

Average OD450 of blank is 0.05

Protein amount is 5 μg

Incubation time is 2 hours (120 minutes)

$$\begin{aligned} \text{JMJD2 activity} &= [(0.65 - 0.05) / (5 \times 120)] \times 1000 \\ &= 1 \text{ OD/min/mg} \end{aligned}$$

For accurate or specific activity calculation

Generate a standard curve and plot OD value versus amount of Assay Standard at each concentration point.

Determine the slope as OD/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of JMJD2-converted demethylated product using the following formulae:

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

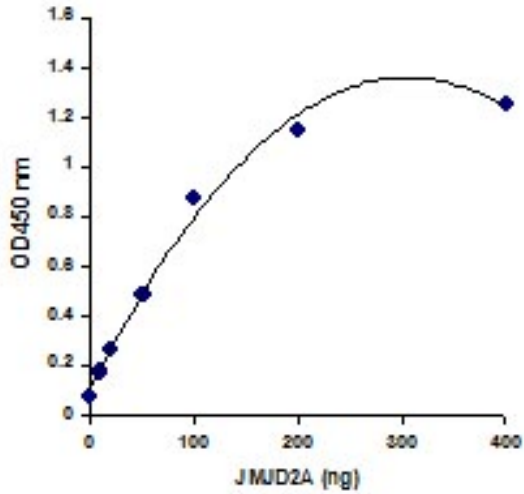
$$\text{JMJD2 activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{(\text{Protein Amount } (\mu\text{g}) \times \text{min}^*)} \times 1000$$

*Incubation time (minutes) at step 11.1.3.

For inhibition calculation:

$$\text{Inhibition \%} = 1 - \left(\frac{\text{Inhibitor Sample OD} - \text{Blank OD}}{\text{No Inhibitor Sample OD} - \text{Blank OD}} \right) \times 1000$$

Typical Data:



Demonstration of high sensitivity of the KDM4/JMJD2 activity assay achieved by using recombinant KDM4/JMJD2 with ab113461.

13. 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 absorbance reading | Check if appropriate absorbance wavelength (450 nm) 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 11.1.3 | Ensure a sufficient amount of standard is added |
| | The standard is degraded due to improper storage conditions | Follow the Storage guidance on Section 9 of this User Guide for storage instructions of JMJD2 Assay Standard. |

RESOURCES

| Problem | Cause | Solution |
|---|--|---|
| 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 |
| High Background Present for the Blank | Incubation time with detection antibody is too long. | The incubation time at section D, step 4 should not exceed 2 hours |
| | Over development of color. | Decrease the development time in section 11.3.1 before adding Stop Solution in section 11.3.2 |
| 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 |

RESOURCES

| | | |
|--|--|---|
| <p>No signal or weak signal only in sample wells</p> | <p>Sample amount added into the wells is insufficient.</p> | <p>Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in section 9. The sample can be titrated to determine the optimal amount to use in the assay</p> |
| | <p>Sample was not stored properly or has been stored for too long</p> | <p>Ensure sample is stored in aliquots at -80°C, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing</p> |
| | <p>Little or no activity of JMJD2 contained in the sample</p> | <p>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</p> |
| <p>Uneven color development</p> | <p>Insufficient washing of the wells</p> | <p>Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible</p> |
| | <p>Delayed color development or delayed stopping of color development in the wells</p> | <p>Ensure color 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)</p> |

14. NOTES

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