

**ab204710**

**Flavin Adenine  
Dinucleotide (FAD) Assay  
Kit**

Instructions for Use

For rapid, sensitive and accurate measuring of Flavin Adenine Dinucleotide (FAD) in a variety of samples.

[View kit datasheet: www.abcam.com/ab204710](http://www.abcam.com/ab204710)

(use [www.abcam.cn/ab204710](http://www.abcam.cn/ab204710) for China, or [www.abcam.co.jp/ab204710](http://www.abcam.co.jp/ab204710) for Japan)

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

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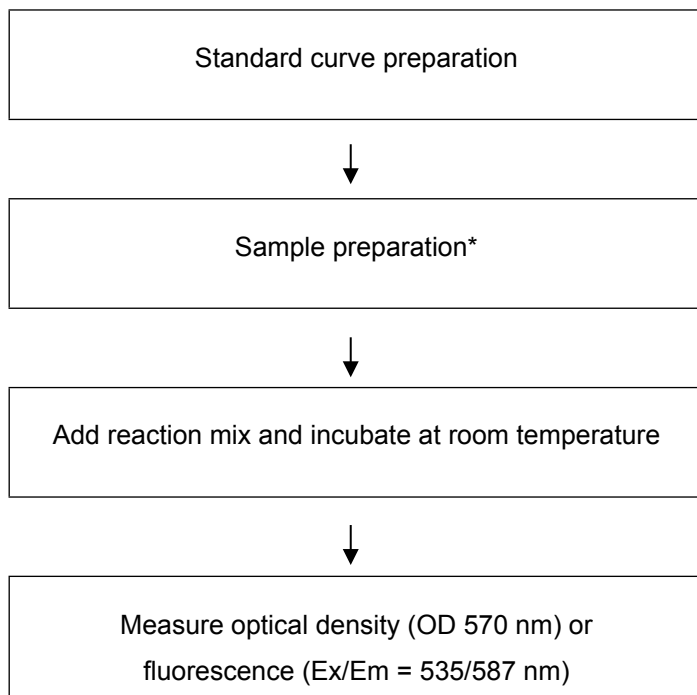
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## **1. BACKGROUND**

Flavin Adenine Dinucleotide (FAD) Assay Kit (ab204710) is an assay where FAD functions as the cofactor of an oxidase which catalyze the formation of a product that reacts with OxiRed probe generating color and fluorescence. FAD can be detected by either colorimetric (OD=570 nm) or fluorometric (Ex/Em=535/587 nm) methods. The kit provides a rapid, simple, ultra-sensitive, and reliable test suitable for high throughput assay of FAD. The lower limit of detection is less than 1 nM FAD.

Flavin Adenine Dinucleotide (FAD) is a redox cofactor which plays an important role in metabolism. FAD exists in different redox states and cycles between FAD, FADH and FADH<sub>2</sub>. The primary sources of reduced FAD in eukaryotic metabolism are the citric acid cycle and the beta oxidation reaction pathways.

## 2. ASSAY SUMMARY



\*Samples might require deproteinization.

### **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 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. **Reconstituted components are stable for 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	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
FAD Assay Buffer	25 mL	-20°C	-20°C
OxiRed Probe (in DMSO)	200 µL	-20°C	-20°C
FAD Enzyme Mix (lyophilized)	1 vial	-20°C	-20°C
FAD Standard (1 nmol, lyophilized)	1 vial	-20°C	-20°C
Stop Solution	1.2 mL	-20°C	-20°C

## 7. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH<sub>2</sub>O)
- DMSO
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD 570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (for fluorometric assay)
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)
- Deproteinizing Sample Preparation Kit – TCA (ab204708): for deproteinization step

### **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.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- 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.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

## 9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

### 9.1 FAD Assay Buffer:

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

### 9.2 FAD Standard (1 nmol, lyophilized) :

Reconstitute the FAD Standard (1 nmol) in 100 µL of DMSO to generate a 10 pmol/µL (10µM) standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

### 9.3 OxiRed Probe – in DMSO:

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use.

**NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the probe is thawed, use within two months. Keep on ice while in use.

### 9.4 FAD Enzyme Mix:

Reconstitute in 220 µL ddH<sub>2</sub>O. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months.

### 9.5 Stop Solution:

Ready to use as supplied. Store at -20°C.



## 10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and should not be stored for future use.

### 10.1 For the colorimetric assay:

10.1.1 Prepare a 0.2 pmol/ $\mu$ L FAD standard by diluting 10  $\mu$ L of the reconstituted FAD standard with 490  $\mu$ L of FAD Assay Buffer.

10.1.2 Using the 0.2 pmol/ $\mu$ L FAD standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard ( $\mu$ L)	Assay Buffer ( $\mu$ L)	Final volume standard in well ( $\mu$ L)	End Conc. FAD in well (pmol/well)
1	0	150	50	0.0
2	6	144	50	0.4
3	12	138	50	0.8
4	18	132	50	1.2
5	24	126	50	1.6
6	30	120	50	2.0

Each dilution has enough amount of standard to set up duplicate readings (2 x 50  $\mu$ L).

## 10.2 For the fluorometric assay:

- 10.2.1 Prepare a 0.2 pmol/μL FAD standard by diluting 5 μL of the reconstituted FAD standard with 245 μL of FAD Assay Buffer.
- 10.2.2 Prepare 200 μL of 0.02 pmol/μL FAD Standard by diluting 20 μL of 0.2 pmol/μL FAD standard with 180 μL of FAD assay buffer.
- 10.2.3 Using 0.02 pmol/μL FAD standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (μL)	Assay Buffer (μL)	Final volume standard in well (μL)	End Conc. FAD in well (pmol/well)
1	0	150	50	0
2	6	144	50	0.04
3	12	138	50	0.08
4	18	132	50	0.12
5	24	126	50	0.16
6	30	120	50	0.20

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 μL).

**NOTE:** *If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.*

## 11. SAMPLE PREPARATION

### **General Sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze your sample in liquid nitrogen upon extraction and store the samples immediately at  $-80^{\circ}\text{C}$ . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

### 11.1 **Cell (adherent or suspension) samples:**

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation =  $1 \times 10^6$  cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 400  $\mu\text{L}$  of ice cold FAD Assay Buffer.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times.
- 11.1.5 Centrifuge sample for 2 – 5 minutes at  $4^{\circ}\text{C}$  at  $10,000 \times g$  using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.
- 11.1.7 Keep on ice.
- 11.1.8 Deproteinize sample using trichloroacetic acid (TCA) precipitation with our Deproteinizing Sample Preparation Kit – TCA (ab204708) to release FAD from proteins. Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.4.

### 11.2 **Tissue samples:**

- 11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 5 – 20 mg).
  - 11.2.2 Wash tissue in cold PBS.
  - 11.2.3 Resuspend tissue in 400  $\mu$ L of ice cold FAD Assay Buffer.
  - 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
  - 11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at 10,000 x g using a cold microcentrifuge to remove any insoluble material.
  - 11.2.6 Collect supernatant and transfer to a clean tube.
  - 11.2.7 Keep on ice.
  - 11.2.8 Deproteinize sample using trichloroacetic acid (TCA) precipitation with our Deproteinizing Sample Preparation Kit – TCA (ab204708) to release FAD from proteins. Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.4.
- 11.3 **Plasma and Serum:**
- Deproteinize sample using trichloroacetic acid (TCA) precipitation with our Deproteinizing Sample Preparation Kit – TCA (ab204708) to release FAD from proteins. Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.4.
- 11.4 **Deproteinization step:**
- Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.
- Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.
- For this step you will need additional reagents:
- Perchloric acid (PCA) 4M, ice cold
  - Potassium hydroxide (KOH), 2M

- 11.4.1 Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. **NOTE:** *high protein concentration samples might need more PCA.*
- 11.4.2 Incubate on ice for 5 minutes.
- 11.4.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.
- 11.4.4 Precipitate excess PCA by adding ice-cold 2M KOH that equals 34% of the supernatant to your sample (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.
- 11.4.5 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). If necessary, adjust pH with 0.1 M KOH or PCA.
- 11.4.6 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

#### 11.4.7 **Sample recovery:**

The deproteinized samples will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

$$\begin{aligned} & \% \text{ original concentration} \\ &= \frac{\text{initial sample volume}}{(\text{initial sample volume} + \text{vol PCA} + \text{vol KOH})} \times 100 \end{aligned}$$

**NOTE:** *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

## 12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

### 12.1 Set up Reaction wells:

- Standard wells = 50  $\mu$ L standard dilutions.
- Sample wells = 1 - 50  $\mu$ L samples (adjust volume to 50  $\mu$ L/well with Assay Buffer).

**Approximate sample amount per assay: 0.1 – 0.5 mg tissue;  $1 \times 10^4$  –  $1 \times 10^5$  cultured cells, 0.1 - 20  $\mu$ L serum.**

### 12.2 Reaction Mix:

Prepare 50  $\mu$ L of Reaction Mix for each reaction

Component	Colorimetric Reaction Mix ( $\mu$ L)	Fluorometric Reaction Mix ( $\mu$ L)
FAD Assay Buffer	46	47.8
OxiRed Probe	2	0.2
Enzyme Mix	2	2

*\*For fluorometric readings, using 0.4  $\mu$ L/well of the probe decreases the background readings, therefore increasing detection sensitivity.*

Mix enough reagents for the number of assays (samples and standards) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X  $\mu$ L component x (Number reactions + 1).

- 12.3 Add 50  $\mu$ L of appropriate Reaction Mix into each standard and sample wells.
- 12.4 Mix and incubate at room temperature protected from light.
- 12.5 Measure output on a microplate reader.
  - Colorimetric assay: measure OD570 nm.
  - Fluorometric assay: measure Ex/Em = 535/587 nm.

- 12.6 Read the sample and standard wells every 5 minutes. Stop the reactions by adding 10  $\mu$ L of Stop Solution; gently shake the plate to mix.

The reaction is stable for 24 hours after adding stop solution. Use the data on the time that shows maximum linear readings. The reaction is linear with time until OD 570 nm reaches 1.8 in the colorimetric assays.

## 13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
  - 13.1 Average the duplicate reading for each standard and sample.
  - 13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
  - 13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of FAD.
  - 13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
  - 13.5 Concentration of FAD (pmol/μl or μM) in the test samples is calculated as:

$$FAD = \left(\frac{A}{B}\right) * D$$

Where:

A = Amount of FAD in the sample from the standard curve (pmol).

B = Sample volume added into the reaction well (μL).

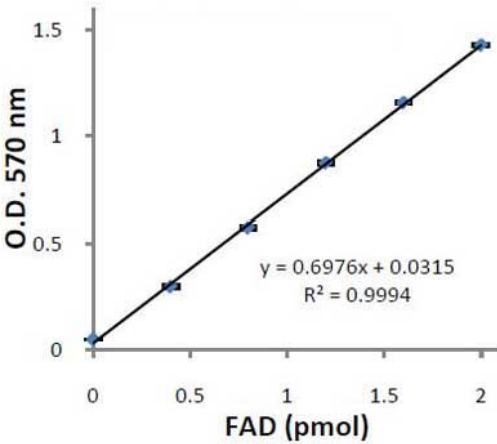
D = Sample dilution factor.

FAD molecular weight: 785.55 g/mol.

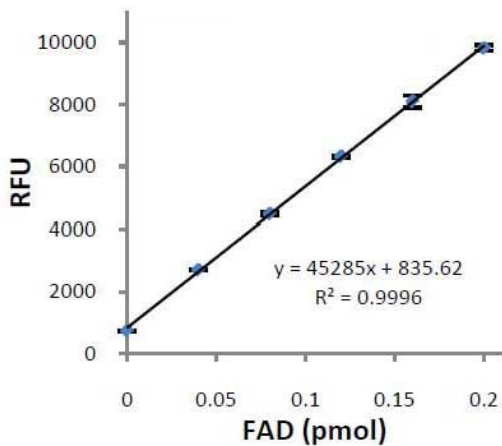


## 14. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



**Figure 1.** Typical FAD Standard calibration curve using colorimetric reading after 15 minutes incubation.



**Figure 2.** Typical FAD Standard calibration curve using fluorometric reading after 15 minutes incubation.

## 15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, FAD probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings), including deproteinization step.
- Set up plate for standard (50  $\mu$ L) and sample (50  $\mu$ L) wells.
- Prepare FAD Reaction Mix (Number samples + standards + 1).

Component	Colorimetric Reaction Mix ( $\mu$ L)	Fluorometric Reaction Mix ( $\mu$ L)
FAD Assay Buffer	46	46
OxiRed Probe	2	2
Enzyme Mix	2	2

- Add 50  $\mu$ L of FAD Reaction Mix to the standard and sample wells.
- Incubate plate at room temperature protected from light.
- Measure plate at OD 570 nm (colorimetric) or Ex/Em= 535/587 nm (fluorometric) assay. Read sample and standard wells every 5 minutes.
- Stop reaction by adding 10  $\mu$ L of Stop Solution.

## 16. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black plates
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	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 substance in the sample	Check protocol for interfering substances; deproteinize samples
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

## RESOURCES

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 $\mu\text{L}$ ) 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 on 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

### **17. FAQ**

**Your protocol mentions that samples and standards should be read every 5 minutes. For how long and how many times should these readings be performed?**

Take readings every 5 minutes until the signal reaches the highest point on the standard curve.

**What needs to be done after addition of the Stop Solution? It is necessary to wait 24 hours? Should the samples be read within those 24 hours?**

The sample reading can be taken right after the stop solution is added. Sample readings can be taken any time after the stop solution is added, for up to the 24 hour – time point.

**What is the exact volume of sample required for this assay?**

There is no specific volume we can recommend for the amount any sample to be used since it is completely sample concentration and quality based. You have to do a pilot expt with multiple sample volumes to determine the optimal volume which gives a reading within the linear range of the standard curve. Please refer to the citations for this product to see what other clients have used with similar sample types.

**How do I normalize my samples against protein concentration?**

You can use a protein quantitation assay on the supernatants you get from cell/tissue lysates or with any other liquid sample in the assay buffer.

## 18. NOTES

**UK, EU and ROW**

Email: [technical@abcam.com](mailto:technical@abcam.com) | Tel: +44-(0)1223-696000

**Austria**

Email: [wissenschaftlicherdienst@abcam.com](mailto:wissenschaftlicherdienst@abcam.com) | Tel: 019-288-259

**France**

Email: [supportscientifique@abcam.com](mailto:supportscientifique@abcam.com) | Tel: 01-46-94-62-96

**Germany**

Email: [wissenschaftlicherdienst@abcam.com](mailto:wissenschaftlicherdienst@abcam.com) | Tel: 030-896-779-154

**Spain**

Email: [soportecientifico@abcam.com](mailto:soportecientifico@abcam.com) | Tel: 911-146-554

**Switzerland**

Email: [technical@abcam.com](mailto:technical@abcam.com)

Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

**US and Latin America**

Email: [us.technical@abcam.com](mailto:us.technical@abcam.com) | Tel: 888-77-ABCAM (22226)

**Canada**

Email: [ca.technical@abcam.com](mailto:ca.technical@abcam.com) | Tel: 877-749-8807

**China and Asia Pacific**

Email: [hk.technical@abcam.com](mailto:hk.technical@abcam.com) | Tel: 400 921 0189 / +86 21 2070 0500

**Japan**

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

[www.abcam.com](http://www.abcam.com) | [www.abcam.cn](http://www.abcam.cn) | [www.abcam.co.jp](http://www.abcam.co.jp)