

(Catalog #K357-100; 100 assays; Store kit at -20°C)

**I. Introduction:**

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. In BioVision's FAD Assay Kit, 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 ( $\lambda=570$  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.

**II. Kit Contents:**

Components	100 assays	Cap Color	Part Number
FAD Assay Buffer	25 ml	WM	K357-100-1
OxiRed Probe	1 vial	Red	K357-100-2
Dimethylsulfoxide (DMSO; Dried)	0.4 ml	Brown	K357-100-3
FAD Enzyme Mix (lyophilized)	1 vial	Green	K357-100-4
FAD Standard (1 nmol)	1 vial	Yellow	K357-100-5
Stop Solution	1.2 ml	Blue	K357-100-6

**III. Storage and Handling:**

Store the kit at -20°C, protect from light. Allow Assay Buffer to warm to room temperature before use. Briefly centrifuge vials before opening. Read the entire protocol before the assay.

**IV. Reagent preparation:**

**Probe:** Dissolve in 220  $\mu$ l DMSO (provided) before use. Store at -20°C.

**Enzyme Mix:** Dissolve in 220  $\mu$ l Distilled Water. Store at -20°C. Use within two months.

**FAD Standard:** Dissolve in 100  $\mu$ l DMSO to generate 10 pmol/ $\mu$ l (10  $\mu$ M) FAD Standard.

**V. FAD Assay Protocol:****1. Standard Curve Preparation:**

**For the colorimetric assay,** dilute the 10 pmol/ $\mu$ l FAD Standard solution to 0.2 pmol/ $\mu$ l by adding 10  $\mu$ l of FAD Standard to 490  $\mu$ l of Assay Buffer, mix well. Add 0, 2, 4, 6, 8, 10  $\mu$ l into each well individually. Adjust volume to 50  $\mu$ l/well with Assay Buffer to generate 0, 0.4, 0.8, 1.2, 1.6, 2 pmol/well of the FAD Standard. Use fresh dilute each time.

**For the fluorometric assay,** dilute the FAD Standard solution to 0.02 pmol/ $\mu$ l by adding 10  $\mu$ l of the 10 pmol/ $\mu$ l FAD Standard to 490  $\mu$ l of Assay Buffer, mix well. Then take 10  $\mu$ l to 90  $\mu$ l of Assay Buffer. Add 0, 2, 4, 6, 8, 10  $\mu$ l into each well individually. Adjust volume to 50  $\mu$ l/well with Assay Buffer to generate 0, 0.04, 0.08, 0.12, 0.16, 0.2 pmol/well of the FAD Standard.

**2. Sample Preparations:**

Tissue (5-20 mg) or cells ( $1 \times 10^6$ ) can be homogenized in 400  $\mu$ l of Assay Buffer, centrifuge to remove insoluble material at 13,000 g for 3 minutes. Serum sample should be collected using EDTA at final concentration 5 mM to inhibit enzymes that may degrade FAD.

Deproteinize sample using perchloric acid precipitation method (BioVision, Cat.# K808-200, Deproteinizing Sample Prep Kit) to release FAD from proteins. After deproteinization, add samples directly into 96 well plate, bring the volume to 50  $\mu$ l/well with Assay Buffer. Approximate sample amount per assay: ~0.1-0.5 mg tissue; ~10,000-100,000 cultured cells, ~0.1-20  $\mu$ l serum. For unknown samples, we suggest testing several doses of your sample to ensure the readings are within the standard curve range.

3. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50  $\mu$ l Reaction Mix containing:

46  $\mu$ l Assay Buffer  
2  $\mu$ l OxiRed Probe\*  
2  $\mu$ l Enzyme Mix

Add 50  $\mu$ l of the **Reaction Mix** to each well containing the FAD Standard and test samples. Mix well.

**\*Note:** Fluorometric Assay is 10 fold more sensitive than Colorimetric Assay. Using 0.4  $\mu$ l/well of the probe will significantly decrease the background reading in fluorometric assay.

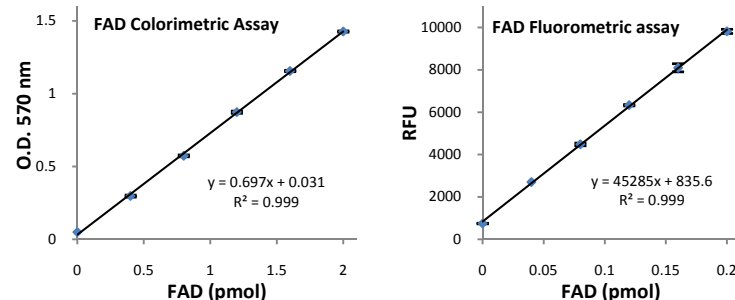
4. Incubate the reaction at room temperature.
5. Measure O.D. 570 nm for the colorimetric assay or Ex/Em = 535/587 nm for the fluorometric assay in a micro plate reader. Reading the samples and standards every 5 minutes. You can stop the reactions by adding 10  $\mu$ l of Stop Solution, gently shake 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 OD570nm reaches 1.8 in the colorimetric assays.
6. **Calculation:** Correct background by subtracting the value derived from the 0 FAD control from all sample readings (The background reading can be significant and must be subtracted from sample readings). Plot FAD standard Curve, FAD concentrations of the test samples can then be calculated:

$$C = S_a/S_v \text{ pmol}/\mu\text{l or } \mu\text{M}$$

Where:  $S_a$  is the sample amount of unknown (in pmol) from standard curve,

$S_v$  is sample volume (in  $\mu$ l) added into the wells.

FAD Molecular Weight is 785.55 g/mol.



**Figure: FAD Standard Curve.** Assays were performed following the kit protocol. The reading is at 15 min incubation.

**VI. Related Products:**

NAD/NADH Quantification Kit

ADP/ATP Ratio Assay Kit

Glucose Assay Kit

Ethanol Assay Kit

Pyruvate Assay Kit

Creatinine Assay Kit

Hemin Assay Kit

Total Antioxidant Capacity (TAC) Assay Kit

NADP/NADPH Quantitation Kit

Ascorbic Acid Quantification Kit

Fatty Acid Assay Kit

Uric Acid Assay Kit

Lactate Assay Kit/ II

Triglyceride Assay Kit

Glutathione Detection Kit

Nitric Oxide Assay Kit