Introduction and Background

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 $FADH_2$. The primary sources of reduced FAD in eukaryotic metabolism are the citric acid cycle and the beta oxidation reaction pathways. In the 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 (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.

Material and Method

A. List of component

1. FAD Assay Buffer: 25 ml.
2. OxiRed Probe (in DMSO): 0.2 ml.
3. FAD Enzyme Mix (lyophilized): 1 vial.
4. FAD Standard (1 nmol, lyophilized): 1 vial.
5. Stop Solution: 1.2 ml.

B. Storage and Handling

Store the kit at -20℃, 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.

C. Reagent preparation

- **Probe:** Ready to use as supplied. Warm to room temperature to thaw the DMSO solution before use. Store at -20℃, protect from light. Use within two months.
- **Enzyme Mix:** Dissolve in 220 µl Distilled Water. Store at -20℃. Use within two months.
- **FAD Standard:** Dissolve in 100 µl DMSO to generate 10 pmol /µl (10 µM) FAD Standard.

D. FAD Assay Protocol

1. **Standard Curve Preparation:**
   - For the colorimetric assay, dilute the 10 pmol/µl FAD Standard solution to 0.2 pmol/µl by adding 10 µl of FAD Standard to 490 µl of Assay Buffer, mix well. Add 0, 2, 4, 6, 8, 10 µl of Assay Buffer, mix well. Add 0, 2, 4, 6, 8, 10 µl into a series of wells. Adjust volume to 50 µl /well with Assay Buffer to generate 0, 0.4, 0.8, 1.2, 1.6, 2 pmol/well of the FAD Standard. Use a freshly diluted Standard each time.
   - For the fluorometric assay, dilute the FAD Standard solution to 0.02 pmol/µl by adding 10 µl of the 10 pmol/µl FAD Standard to 490 µl of Assay Buffer, mix well. Then take 10 µl to 90 µl of Assay

Buffer. Add 0, 2, 4, 6, 8, 10 µl into each well individually. Adjust volume to 50 µl/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:**

Homogenize tissue (5-20 mg) or cells (1×10^6) in 400 µl 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 to release FAD from proteins. After deproteinization, add samples directly into 96 well plate, bring the volume to 50 µl/well with Assay Buffer. Approximate sample amount per assay: ~0.1-0.5 mg tissue; ~10,000-100,000 cultured cells, ~0.1-20 µl 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 µl Reaction Mix containing:

46 µl Assay Buffer

2 µl OxiRed Probe*

2 µl Enzyme Mix

Add 50 µl 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 µl/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 µl 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.

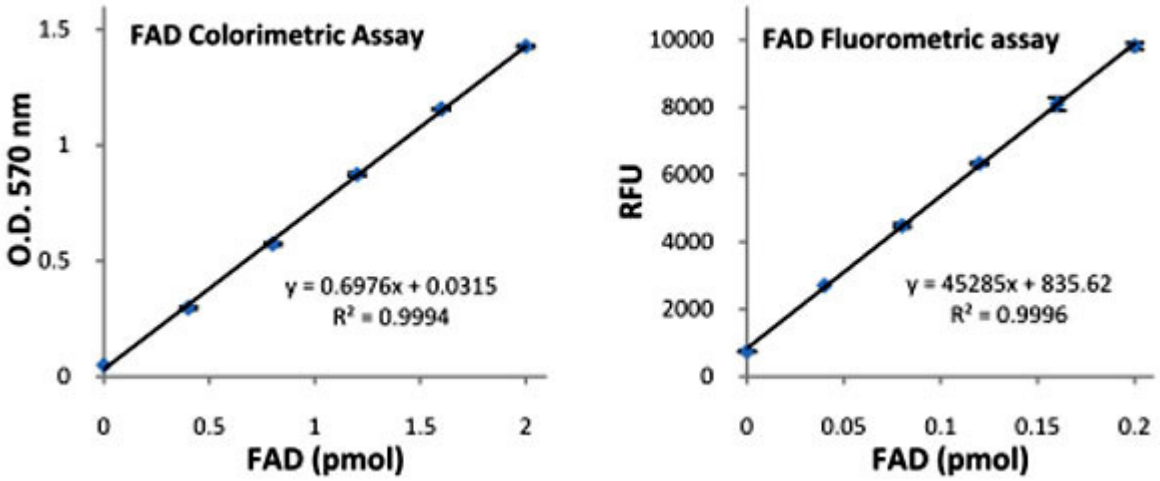
6. **Calculation:** Correct background by subtracting the value derived from the 0 FAD control from all sample readings (The background reading can be significant and must be subtracted from sample readings). Plot FAD standard Curve, FAD concentrations of the test samples can then be calculated:

C = Sa/Sv pmol/µl or µM

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

Sv is sample volume (in µl) added into the wells.

FAD Molecular Weight is 785.55 g/mol.



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