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Product Information

FAD Assay Kit

Catalog Number **MAK035**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Flavin Adenine Dinucleotide (FAD) is a coenzyme, synthesized from riboflavin, which plays critical roles in many metabolic pathways. FAD functions as an electron carrier in multiple redox reactions, cycling between FAD, FADH, and $FADH_2$. The primary sources of reduced FAD in eukaryotic metabolism are the tricarboxylic acid cycle and the beta oxidation reaction pathways. In eukaryotes, FAD in the endoplasmic reticulum is critical for the proper oxidative folding of proteins.

FAD concentration is determined by a coupled enzyme assay, which results in a colorimetric (570 nm)/ fluorometric (λ_{ex} = 535/λ_{em} = 587 nm) product, proportional to the FAD present.

Components
The kit is sufficient for 100 assays in 96 well plates.

FAD Assay Buffer Catalog Number MAK035A	25 mL
Fluorescent Peroxidase Substrate, in DMSO Catalog Number MAK035B	0.2 mL
FAD Enzyme Mix Catalog Number MAK035C	1 vl
FAD Standard, 1 nmole Catalog Number MAK035D	1 vl
Stop Solution Catalog Number MAK035E	1.2 mL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Fluorescence or spectrophotometric multiwell plate reader.
- Perchloric Acid (PCA) 70% (Catalog Number 244252, ACS Reagent)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

FAD Assay Buffer – Allow buffer to come to room temperature before use.

Fluorescent Peroxidase Substrate – Warm to room temperature to melt frozen solution prior to use. Mix well by pipetting, then aliquot and store, protected from light and moisture, at –20 °C.

For the fluorescence assay, dilute an aliquot of the Fluorescent Peroxidase Substrate 5 to 10-fold with FAD Assay Buffer just prior to use. This will reduce the background of the fluorescence assay.

FAD Enzyme Mix - Reconstitute in 220 µL of water. Mix well by pipetting, then aliquot and store at –20 °C. Keep cold while in use and protect from light. Use within 2 months of reconstitution.

FAD Standard- Reconstitute in 100 µL of DMSO to generate a 10 µM (10 pmole/µL) solution. Mix well by pipetting, then aliquot and store at –20 °C. Keep cold while in use and protect from light. Use within 2 months of reconstitution.

Storage/Stability
The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure

FAD Standards for Colorimetric Detection

Dilute 10 µL of the 10 µM FAD Standard with 490 µL of FAD Assay Buffer to prepare a 0.2 pmole/µL standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 0.2 pmole/µL FAD standard solution into a 96 well plate generating 0 (blank), 0.4, 0.8, 1.2, 1.6, and 2.0 pmole/well standards. Add FAD Assay Buffer to each well to bring the volume to 50 µL.

FAD Standards Fluorometric Detection

Prepare a 0.2 pmole/µL standard solution as for the colorimetric assay. Dilute 10 µL of the 0.2 pmole/µL standard solution with 90 µL of FAD Assay Buffer to make a 0.02 pmole/µL solution. Add 0, 2, 4, 6, 8, and 10 µL of the diluted 0.02 pmole/µL FAD standard solution into a 96 well plate, generating 0 (blank), 0.04, 0.08, 0.12, 0.16, and 0.2 pmole/well standards. Add FAD Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation

Both the colorimetric and fluorometric assays require 50 µL of sample for each reaction (well).

1. Tissue (5–20 mg) or cells (1×10^6) can be homogenized in 400 µL of the FAD Assay Buffer. Centrifuge the samples at 13,000 × *g* for 10 minutes to remove insoluble material. Serum samples should be collected using EDTA at a final concentration of 5 mM to inhibit enzymes that degrade FAD.

2. Deproteinize the samples with Perchloric Acid (PCA) precipitation to release FAD from proteins. Prepare an 8% (w/v) Perchloric Acid (PCA) solution by diluting 7 mL of the 70% (w/w) PCA solution to 100 mL with water. Pipette 0.4 mL of sample into a centrifuge tube containing 0.8 mL ice-cold 8% PCA. Vortex the mixture for ~30 seconds. Keep the precipitate mixture cold for an additional 5 minutes to ensure complete protein precipitation. Centrifuge 10 minutes at ~1,500 × *g* and transfer supernatant to a new tube.
   Note: Perchloric acid solutions can become explosive if allowed to dry. Always rinse glassware and working surfaces with a large volume of water.

3. Add between 1–50 µL of deproteinized samples into duplicate wells and bring to a final volume of 50 µL with FAD Assay Buffer. For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Assay Reaction

1. Set up the Master Reaction Mix according to the scheme in Table 1. 50 µL of the Master Reaction Mix is required for each reaction (well).

Table 1.
Master Reaction Mix

Reagent	Master Reaction Mix
FAD Assay Buffer	46 µL
Fluorescent Peroxidase Substrate	2 µL
FAD Enzyme Mix	2 µL

2. Add 50 µL of the Master Reaction Mix to each well. Mix well using a horizontal shaker or by pipetting, and incubate the reaction at room temperature. Protect the plate from light during the incubation.

3. For colorometric assays, measure the absorbance at 570 nm (A_{570}). For fluorometric assays, measure fluorescence intensity (λ_{ex} = 535/λ_{em} = 587 nm).

4. Read the samples at time zero and every 5 minutes. The reaction can stopped 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 from the time point that shows maximum linear readings. The reaction is linear with time until A_{570} reaches 1.8 in the colorimetric assays.

Results

Calculations

The background for either assay is the value obtained for the 0 (blank) FAD standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings.

Use the values obtained from the appropriate FAD standards to plot a standard curve. The amount of FAD present in the samples may be determined from the standard curve.

Note: A new standard curve must be set up each time the assay is run.

Concentration of FAD

$$S_a/S_v = C$$

S_a = Amount of FAD in unknown sample (nmole) from standard curve

S_v = Sample volume (μL) added to the reaction well

C = Concentration of FAD in sample

FAD molecular weight: 785.55 g/mole

Sample Calculation

Amount of FAD (S_a) = 5.84 nmole

Assay volume (S_v) = 50 μL

Concentration of FAD in sample

$$5.84 \text{ nmole}/50 \text{ μL} = 0.1169 \text{ nmole/μL}$$

$$0.1169 \text{ nmole/μL} \times 785.55 \text{ ng/nmole} = 91.83 \text{ ng/μL}$$

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For fluorescence assays, use black plates with clear bottoms. For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the FAD Assay Buffer
	Samples were not deproteinized	Use PCA precipitation to deproteinize samples
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Master Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Master Reaction Mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range