Free Fatty Acid Fluorometric Assay Kit

Item No. 700310

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700311	FFA Assay Buffer	1 vial/40 ml
700312	FFA Assay Cofactor Mixture 1	2 vials
700304	FFA Assay Cofactor 2	2 vials
700305	FFA Assay Cofactor 3	1 vial/15 mg
700313	FFA Assay Enzyme Mixture	2 vials
700314	FFA Assay Fluorometric Detector	2 vials
700001	DMSO Assay Reagent	1 vial/1 ml
700316	FFA Assay N-Ethylmaleimide	1 vial/20 mg
700317	FFA Assay Sample Buffer	1 vial/15 ml
700318	FFA Assay Developer Solution	1 vial/15 ml
700319	FFA Assay Standard	2 vials
400017	96-Well Solid Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm
2. Adjustable pipettors and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Fatty acids are usually ingested as triglycerides. In the intestine, triglycerides are hydrolyzed into free fatty acids (FFA) and glycerol by the enzyme pancreatic lipase. Once across the intestinal barrier, they are reformed into triglycerides and packaged into liposomes, which are released into the capillaries of the lymph system and then into the blood. Eventually, they bind to the membranes of hepatocytes, adipocytes, or muscle fibers, where they are either stored or oxidized for energy.[1] When blood sugar is low, glucagon signals the adipocytes to activate hormone-sensitive lipase, and to convert triglycerides into FFAs. Since fats are insoluble in water, they are bound to plasma protein albumin for transport around the body. The levels of FFA in the blood are limited by the number of albumin binding sites available.

While plasma FFA plays important physiological roles in skeletal muscle, heart, liver, and pancreas, chronically elevated levels appear to have pathophysiological consequences and have been linked to obesity, insulin resistance, and type 2 diabetes.[2-4] In the blood, chronically elevated FFA are believed to play a role in the pathogenesis of certain forms of type 2 diabetes by both inhibiting insulin-stimulated peripheral glucose uptake and contributing to β-cell dysfunction.[2] An increase in FFA induces oxidative stress resulting in a proinflammatory effect *via* the nuclear factor κB pathway leading to a potential link between inflammation and insulin resistance.[2] Therefore, the measurement of FFA can be useful in determining metabolic status and the reduction in elevated plasma FFA could be an important therapeutic target in obesity and type 2 diabetes.

About This Assay

Cayman's Free Fatty Acid (FFA) Fluorometric Assay provides a simple, reproducible, and sensitive tool for measuring FFAs in plasma, serum, and urine. The FFA Fluorometric Assay utilizes a coupled enzymatic reaction to determine FFA concentrations (see Figure 1). Acyl CoA synthetase (ACS) first catalyzes fatty acid acylation of coenzyme A. The acyl CoA produced is oxidized by acyl CoA oxidase (ACOD) and generates hydrogen peroxide (H_2O_2). Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with 10-aceyl-3,7-dihydroxyphenoxazine (ADHP) in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. Resorufin fluorescence is measured with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm.

$$\text{RCOOH + ATP + CoA} \xrightarrow[\text{Ascorbate Oxidase}]{\text{ACS}} \text{Acyl CoA + AMP + PPi}$$

$$\text{Acyl CoA + } O_2 \xrightarrow[\text{Oxidase}]{\text{Acyl CoA}} \text{2,3-}\textit{trans}\text{-Enoyl-CoA + } H_2O_2$$

$$2H_2O_2 + \text{ADHP} \xrightarrow{\text{Peroxidase}} \text{Resorufin } (Ex_{530}/Em_{590})$$

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **FFA Assay Buffer - (Item No. 700311)**

   The vial contains 40 ml of 50 mM Tris-HCl, pH 8.6, containing 5 mM magnesium chloride. Thaw the Assay Buffer at room temperature. This buffer should be used to reconstitute the FFA Assay Cofactor Mixture 1 (Item No. 700312). When stored at 4°C, this Assay Buffer is stable for at least six months.

2. **FFA Assay Cofactor Mixture 1 - (Item No. 700312)**

   Each vial contains a lyophilized mixture of acyl coenzyme A synthetase and ascorbate oxidase. Reconstitute one vial with 6 ml of FFA Assay Buffer (Item No. 700311). Place vial on ice. The Cofactor Mixture 1 is stable for three hours. One vial is enough to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial. *NOTE: Ascorbate oxidase eliminates ascorbic acid interference.*

3. **FFA Assay Cofactor 2 - (Item No. 700304)**

   Each vial contains a lyophilized mixture of ATP. Reconstitute one vial with 3 ml of FFA Assay Buffer (Item No. 700311). The reconstituted mixture is stable for three hours on ice. Add this solution to the reconstituted vial of FFA Assay Cofactor Mixture 1 (Item No. 700312). One vial is enough to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial.

4. **FFA Assay Cofactor 3 - (Item No. 700305)**

   The vial contains 15 mg of coenzyme A. To a separate test tube, add 5 mg of FFA Assay Cofactor 3 to evaluate 60 wells. Dissolve with 3 ml of FFA Assay Buffer (Item No. 700311). Add this solution to the reconstituted vial of FFA Assay Cofactor Mixture 1 (Item No. 700312) and FFA Assay Cofactor 2 (Item No. 700304). Gently mix FFA Assay Cofactor Mixture 1, FFA Assay Cofactor 2, and FFA Assay Cofactor 3 by inversion. The combined Cofactor Mixture is stable for three hours. If additional wells are being utilized, weigh out additional FFA Assay Cofactor 3.

5. **FFA Assay Enzyme Mixture - (Item No. 700313)**

   Each vial contains a lyophilized mixture of acyl coenzyme A oxidase and horseradish peroxidase. It will be used to prepare the 'Developer' (see **Performing the Assay** on page 16 for instructions on reconstitution). One vial is enough to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial.

6. **FFA Assay Fluorometric Detector - (Item No. 700314)**

   Each vial contains a lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). It will be used to prepare the 'Developer' (see **Performing the Assay** on page 16 for instructions on reconstitution). Once reconstituted, ADHP is stable for 60 minutes. After 60 minutes, increased background fluorescence will occur. One vial is sufficient to assay 60 wells. Use the second vial if utilizing the entire plate.

7. **FFA Assay N-Ethylmaleimide - (Item No. 700316)**

   The vial contains 20 mg of N-ethylmaleimide (NEM). Weigh 8 mg of NEM into another vial and dissolve with 1 ml of FFA Assay Developer Solution (Item No. 700318). The reconstituted NEM will be used to prepare the 'Developer' (see **Performing the Assay** on page 16 for instructions). This is enough NEM to assay 60 wells. Store unused crystalline NEM at -20°C.

8. **FFA Assay Sample Buffer - (Item No. 700317)**

   The vial contains 15 ml of 50 mM Tris-HCl, pH 7.4, containing 5 mM magnesium chloride. Thaw the Sample Buffer at room temperature. This buffer should be used to prepare the standard curve (see **Standard Preparation** on page 15) and to dilute samples. When stored at 4°C, this sample buffer is stable for at least six months.

9. **FFA Assay Developer Solution - (Item No. 700318)**

   The vial contains 15 ml of a solution containing 2-phenoxyethanol and Triton X-100. Thaw the Developer Solution at room temperature. The solution is ready to use as prepared. This solution is used to prepare the 'Developer' (see **Performing the Assay** on page 16 for further instructions). When stored at 4°C, this solution is stable for at least six months.

10. **FFA Assay Standard - (Item No. 700319)**

    Each vial contains a lyophilized powder of oleic acid that will be used to prepare the standard curve (see **Standard Preparation** on page 15).

Sample Preparation

Plasma

Typically normal human plasma has a FFA concentration in the range of 150-450 µM.[6]

1. Collect blood using an anticoagulant such as EDTA, sodium citrate, sodium fluoride, or ammonium oxalate. **Do not use heparinized plasma as heparin has been found to interfere with the assay.**
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
3. Typically, plasma samples do not need to be diluted before assaying.

Serum

Typically normal human serum has a FFA concentration in the range of 100-700 µM.[7,8]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Typically, serum samples do not need to be diluted before assaying.

Urine

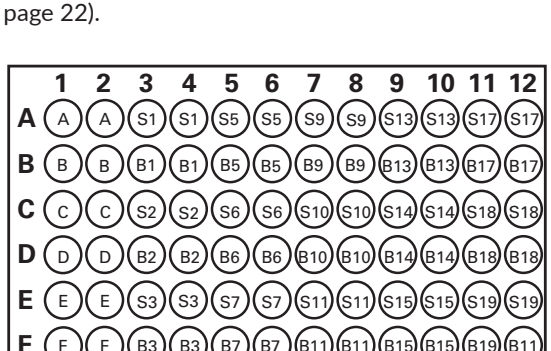
1. Collection of urine does not require any special treatment.
2. If not assaying urine on the same day, freeze at -80°C. The urine sample will be stable for one month while stored at -80°C.
3. Typically, urine samples do not need to be diluted before assaying.

It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that each sample and standard be assayed at least in duplicate (triplicate is preferred) along with duplicate or triplicate wells designated as background wells for each sample. A typical layout of samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).



A-H = Standards
S1-S20 = Sample Wells
B1-B20 = Background Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 310 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- Use the combined Cofactor Mixture 1, Cofactors 2, and 3 (see pages 8 and 9) when performing the assay.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm.

Standard Preparation

Reconstitute a vial of the FFA Assay Standard (Item No. 700319) with 500 µl of water to obtain a stock solution of 1,000 µM. Take eight clean test tubes and label them A-H. Add the amount of 1,000 µM FFA Standard and FFA Sample Buffer (Item No. 700317) to each tube as described in Table 1. We recommend that you store these diluted standards in an ice bath for no more than one hour.

Tube	FFA 1,000 µM (µl)	Sample Buffer (µl)	FFA Concentration (µM)
A	0	500	0
B	12.5	487.5	25
C	25	475	50
D	37.5	462.5	75
E	50	450	100
F	75	425	150
G	100	400	200
H	125	375	250

Table 1. Preparation of FFA standard curve

Performing the Assay

1. **Standard Wells -** Add 10 µl of standard (tube A-H) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2 on page 13).
2. **Sample Wells -** Add 10 µl of the sample to at least three wells. To obtain reproducible results, the amount of free fatty acids added to the wells should fall within the range of the assay. When necessary, samples should be diluted with FFA Assay Sample Buffer.
3. **Sample Background Wells -** Add 10 µl of the sample and 200 µl of FFA Assay Buffer to at least three wells.
4. Add 200 µl of the combined FFA Cofactor Mixture to all standard wells and sample wells. **DO NOT** add the combined FFA Cofactor Mixture to the sample background wells.
5. Cover the plate with the plate cover, and incubate for 30 minutes at 37°C.
6. Prepare the 'Developer' as follows within five to ten minutes prior to adding to the wells:
    a. To one vial of FFA Assay Enzyme Mixture (Item No. 700313), add 4.5 ml of FFA Assay Developer Solution (Item No. 700318), 1 ml of reconstituted NEM, (Item No. 700316), and vortex.
    b. Next, add 50 µl of DMSO Assay Reagent (Item No. 700001) to one vial of the FFA Assay Fluorometric Detector (Item No. 700314) and mix until dissolved. Then add 450 µl of Developer Solution (Item No. 700318) to the Fluorometric Detector in DMSO and vortex.
    c. Add all of the resuspended Fluorometric Detector solution prepared in step b to the resuspended vial of Enzyme Mixture prepared in step a and vortex. This is enough 'Developer' for 60 wells. Prepare additional 'Developer' as needed.
7. Add 100 µl of 'Developer' to all wells being used, including sample, sample background, and standards. Cover and incubate for 15 minutes at 37°C.
8. Remove the plate cover and read using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, sample, and sample background.
2. Subtract the fluorescence value of the sample background from the fluorescence of the sample wells to yield the corrected sample fluorescence (CSF).
3. Determine the average fluorescence of the standards. Subtract the fluorescence value of standard A (0 µM) from itself and all other standards. This is the corrected fluorescence.
4. Plot the corrected fluorescence values (from step 3 above) of each standard as a function of the final concentration of free fatty acid from Table 1. See Figure 3, on page 19 for a typical standard curve.
5. Calculate the free fatty acid concentration of the samples using the equation obtained from the linear regression of the standard curve, substituting the corrected sample fluorescence (CSF) for each sample.

$$\text{Free Fatty Acid Concentration (µM)} = \left[\frac{\text{CSF - (y-intercept)}}{\text{Slope}}\right] \times \text{Sample dilution}$$

Performance Characteristics

Precision:

When a series of forty-eight plasma measurements were performed on the same day, the intra-assay coefficient of variation was 5.9%. When a series of sixteen plasma measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 5.6%.

Assay Range:

Under the standardized conditions for the assay described in this booklet, the dynamic range of the kit is 0-250 µM free fatty acid.

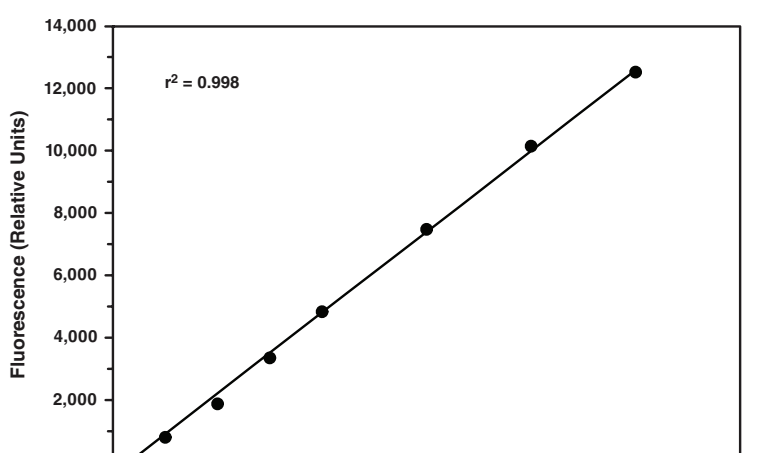


Figure 3. Free fatty acid standard curve

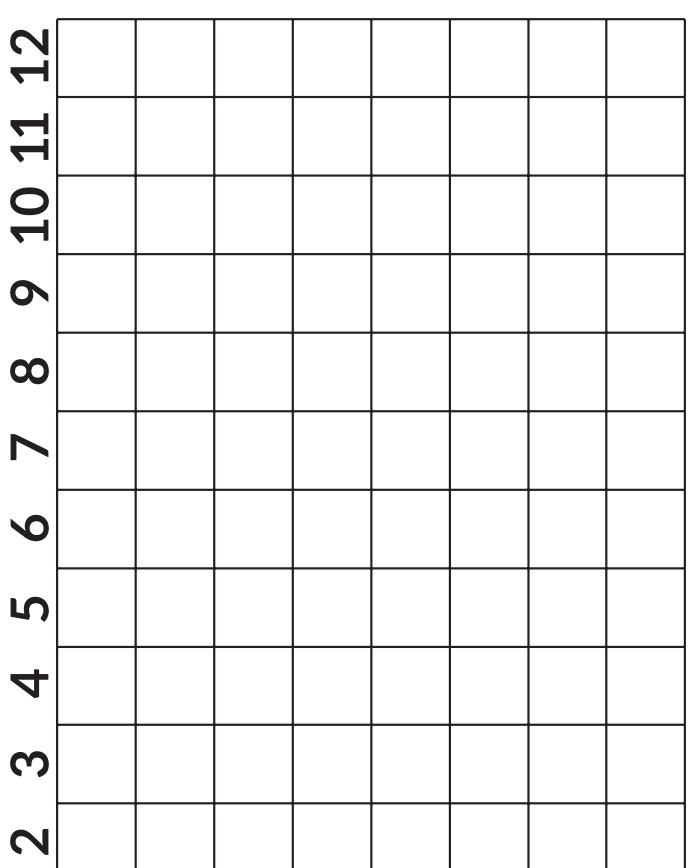
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No FFA concentration was obtained above background in the sample	The FFA concentration is too low to detect	A. Re-assay the sample using a lower dilution
FFA concentration was above the highest point in the standard curve	The FFA concentration was too high in the sample	Dilute samples with sample buffer and re-assay; *NOTE: Remember to account for the dilution factor when calculating FFA concentration*
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read

References

1. Camp, H.S., Ren, D., and Leff, T. Adipogenesis and fat-cell function in obesity and diabetes. *Trends in Molecular Medicine* **8(9)**, 442-447 (2002).
2. Dandona, P., Aljada, A., and Bandyopadhyay, A. Inflammation: The link between insulin resistance, obesity and diabetes. *TRENDS in Immunology* **25(1)**, 4-7 (2004).
3. Gade, W., Schmit, J., Collins, M., *et al.* Beyond obesity: The diagnosis and pathophysiology of metabolic syndrome. *Clin. Lab. Sci.* **23(1)**, 51-61 (2010).
4. Shi, Y. and Burn, P. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nature Reviews Drug Discovery* **3**, 695-710 (2004).
5. Adams, L.A., Angulo, P., and Lindor, K.D. Nonalcoholic fatty liver disease. *CMAJ* **172(7)**, 899-905 (2005).
6. Guerci, B., Meyer, L., Sallé, A., *et al.* Comparison of metabolic deterioration between insulin analog and regular insulin after a 5-hour interruption of a continuous subcutaneous insulin infusion in type 1 diabetic patients. *J. Clin. Endocrinol. Metab.* **84(8)**, 2673-2678 (1999).
7. Duncombe, W.G. The colorimetric micro-determination of long-chain fatty acids. *Biochem. J.* **88**, 7-10 (1963).
8. Husek, P., Simek, P., and Tvrzická, E. Simple and rapid procedure for the determination of individual free fatty acids in serum. *Anal. Chim. Acta* **465**, 433-439 (2002).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.