Intended Use
For **In Vitro Diagnostic** use in the manual or automated, quantitative, determination of Nonesterified Fatty Acids (NEFA) in serum or plasma.

Clinical Significance (1)
Fatty acids are absorbed through the intestine as insoluble oils. Solubilization (emulsification) is accomplished by the presence of bile salts synthesized in the liver. In the blood, this important lipid form is carried by albumin. Problems with fatty acid metabolism are associated with the process of fatty acid ß-oxidation, carnitine deficiency which is observed in newborns, and patients undergoing dialysis. Deficiency of the enzyme Carnitine Palmitoyltransferase results in recurrent muscle pain, fatigue and myoglobinuria. Another enzyme deficiency, acyl–CoA Dehydrognenase, will cause symptoms of vomiting, lethargy and frequently coma after a prolonged fasting period. In Refsum's disease accumulation of Phytanic Acid due to blockage of ß-oxidation results in cerebellar ataxia, retinitis pigmentosa, nerve deafness and peripheral neuropathy.

Method Principle (2-4)
Most previously used procedures for Nonesterified Fatty Acids were based on organic solvent extractions, titration and gas liquid chromatography. These procedures are complicated, time consuming and restricted to manual assays. Catachem's NEFA procedure is based on the enzymatic synthesis of thiols esters of CoA, known as Acyl-CoA, by the activity of Acyl-CoA Synthetase (ACS) in the presence of ATP and CoA. The Acyl-CoA thus formed is then oxidized in a second reaction by Acyl-CoA Oxidase (ACOX) to produce 2, 3-trans-trans-enoyl-CoA and Hydrogen Peroxide. The Hydrogen Peroxide is then quantitated by the oxidative condensation of N-Ethyl-(3-sulfopropyl) Aniline (ALPS) with 4-Aminoantipyrine to produce a Quinoneindamine dye with maximum absorption at 550nm. The increase in absorbance is directly proportional to the concentration of NEFA in the original serum sample. The following reaction scheme illustrates the reactions that take place in this NEFA procedure.

$$\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + \text{PPi}$$

$$\text{Acyl-CoA} + O_2 + \xrightarrow{\text{ACOX}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$H_2O_2 + 4\text{AA} + \text{ALPS} \xrightarrow{\text{Peroxidase}} \text{Quinoneindamine Dye} + 4H_2O$$

SAMPLE DILUENT (R1)
Each liter contains:

Buffer	
ATP	4.0 mmol
4-Aminoantipyrine	1.0 mmol
Coenzyme-A (CoA)	350 mg
Ascorbate Oxidase	≥ 3000U
Acyl-CoA Synthetase	≥ 150 U

ENZYME COLOR REAGENT (R2)
Each liter contains:

Buffer	
ALPS	1.62 mmol
Acyl-CoA Oxidase	≥ 15000 U
Peroxidase	≥ 15000 U
Stabilizer and nonreactive ingredients	

REAGENT DILUENT
Each liter contains:

Magnesium Chloride	3.0 mmol
Surfactant	
Stabilizer and nonreactive ingredients	

Precautions
Handle this reagent using good laboratory practice. **DO NOT PIPETTE REAGENT BY MOUTH.** Avoid contact with skin and eyes. If contact occurs, wash affected area with plenty of cold water. Clean spills immediately.

Reagent Storage and Stability
Store the Catachem NEFA reagents at 2-8°C. When stored as directed, these reagents are stable until the expiration date stated on the label.

Working Reagent Preparation
Reconstitute the required number of vials of Catachem NEFA Sample Diluent (R1) and Enzyme Color Reagent (R2) with the volume of Catachem Reagent Diluent indicated on the label of each reagent vial. Mix reconstituted reagents by inversion, gently to prevent foaming. Store the Working Reagents at 2-8°C. When stored and prepared as directed the Catachem NEFA Working Reagents are stable for 30 days.

Reagent Indications of Deterioration
- Turbidity
- Absorbance > 0.5 OD, 1 cm light path - 550 nm
- Quality control values out of assigned ranges

If these reagent characteristics are observed, call Catachem technical service.

Specimen Collection and Stability
Test sera should be collected after fasting and should be fresh, clear and unhemolyzed. When blood is drawn, it should be processed as soon as possible and the serum should be isolated from the clot without delay. If immediate assays are not possible samples should be frozen at -20°C. If plasma specimens are collected, do not use heparin as is known to interfere with the NEFA reaction. Anticoagulants suitable for this assay are sodium citrate, EDTA, and sodium fluoride.

Procedure
These instructions are outlined for performing the Catachem NEFA assay using a manual procedure. Contact Catachem Technical Service if instrument applications are required.

Materials Provided
Catachem NEFA Reagents

Materials required but not provided
- Spectrophotometric analyzer equipped with 550nm wavelength
- NEFA Calibrator material with assigned value
- NEFA Quality Control material with assigned values

Calibration
Catachem NEFA Calibrator (C514-10) which contains a known NEFA value is recommended.

Assay Procedure

1. Label cuvettes or appropriate test tubes as: a) Calibrator Blank (CAL-BLK), b) Calibrator (CAL), c) Control 1 Blank (C-1BLK), d) Control 1 (C1), e) Control 2 Blank (C-2BLK), f) Control 2 (C2), g) Sample Blank (SAMP BLK), h) Sample (SAMP).
2. Pipette the reagent and sample volumes into the cuvettes or test tubes as shown in table below. Pipette Catachem NEFA Enzyme Reagent I (R-1) first, followed by the sample.
3. Incubate for 5 minutes.
4. Immediately, after the incubation period is over, add Catachem NEFA Enzyme Reagent II (R-2) to the assay samples and water to the blank samples. Mix all cuvettes.
5. Set a timer for exactly 4 minutes.
6. At the end of the 4 minutes read all cuvettes at 550nm. Record all absorbencies.

	CAL BLK	CAL	C-1 BLK	C-1	C-2 BLK	C-2	SAMP BLK	SAMP
	ml	ml	ml	ml	ml	ml	ml	ml
RGT 1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
SAMP	0.050	0.050	0.050	0.05	0.050	0.05	0.050	0.050
INCUBATE FOR 5 MINUTES								
RG2 2	0.00	0.10	0.00	0.10	0.00	0.10	0.00	0.10
H2O	0.10	0.0	0.10	0.00	0.10	0.00	0.10	0.00
MIX AND INCUBATE FOR 4 MINUTES								
MIX AND READ ALL CUVETTES								

Calculations and Results

$$\text{NEFA mmol/L} = \frac{\Delta\text{- Abs. Samp.}}{\Delta\text{- Abs. Cal.}} \times \text{Cal. Value (mmol/L)}$$

Example:

	Samp. Abs.	Blk. Abs.	Δ-Abs.
Sample	0.400	0.300	0.100
Calibrator	0.150	0.030	0.120

Calibrator assigned value = 0.5 mmol/L

$$\text{Sample NEFA mmol/L} = \frac{0.100}{0.120} \times 0.5 \text{ mmol/L}$$

$$= 0.42 \text{ mmol/L}$$

Quality Control

To monitor the quality performance of the procedure used, Catachem NEFA Control Level I and Control Level II should be included in the assay procedure each time the assay is run.

Procedure Limitations

Samples with NEFA values greater than 2.5mmol/L should be diluted 1:2 with physiological saline and reassayed. Multiply results obtained by 2 to adjust for the sample dilution.

Interfering Substances

The following substances have no significant effect on the accuracy of this NEFA procedure at the concentrations stated.

- Hemoglobin ≤ 200 mg/dL
- Bilirubin 10.0 mg/dL
- Ascorbic Acid ≤ 20 mg/dL

Other substances and certain drugs are also known to influence the NEFA values (6).

Method Performance Characteristics

Sensitivity: Using a path length of 1 cm, a Δ-absorbance of 0.1-0.20 per mmol/L should be obtained.

Linearity: This procedure is linear over the range of 0-2.5 mmol/L.

Precision: Precision data was obtained using five levels of protein based controls and following the NCCLS EP5-T2 procedure (5). The following results were observed:

Precision

NEFA	Within-Run Precision		Total Precision	
Mean	SD	CV	SD	CV
mmol/L	mmol/L	%	mmol/L	%
0.169	0.0060	3.550	0.0074	4.379
0.443	0.0087	1.953	0.0100	2.257
1.02	0.0149	1.461	0.0162	1.588
1.516	0.0163	1.078	0.0172	1.134

ACCURACY

Using an automated analyzer, correlation studies were carried out between this Catachem NEFA procedure (Y) and a commercially available NEFA test kit as reference (X). Serum samples were assayed and the results compared by the least squares regression. The following statistics were observed:

N	=	44
Range	=	0.1-2.3 mmol/L
Mean Y	=	0.5295 mmol/L
Mean X	=	0.5841 mmol/L
Y	=	0.9137x – 0.0042
r	=	0.9949
Sy.x	=	0.0541

Bibliography

1. Fundamentals of Clinical Chemistry. Edited by Norbert Tietz 2nd. Ed. Philadelphia: W.B Saunders; 1976.
2. Itaya, K and Ui, M. Colorimetric determination of free fatty acids in biological fluids. J. Lipids Research; 6, 16 (1965).
3. Novak, M. Colorimetric ultramicro method for the determination of fatty acids. J. Lipid Research; 6, 431 (1965).
4. Trout, D.L., Estes, E.H. and Friedberg, S.J. J. Lipid Research; 1,199 (1960).
5. Evaluation of Precision Performance of Clinical Chemistry Devices. Second Edition. NCCLS Document EP5-T2. Vol. 12, No. 4.
6. Young D.S., Pestamer L.C., Gibberman V. Effects of drugs on clinical laboratory tests. Clin. Chem. 21, No. 5(1975).

REV: 051215DT

353 Christian Street, Suite 2 ● Oxford, Connecticut ● 06478 USA ● Phone (203) 262-0330 ● Fax (203) 262-9836 ● email: catachem@catacheminc.com