Ver.260501

Non-esterified Free Fatty Acid (NEFA) Assay Kit

OBCK014(50Tests/48Samples)

FOR RESEARCH USE ONLY, DO NOT USE IT IN CLINICAL DIAGNOSIS

1. Product Principle

NEFA can combine with copper ions to form fatty acid–copper salts, which dissolve in chloroform. The concentration of fatty acid–copper salts in chloroform is directly proportional to the NEFA content. As a result, the NEFA content can be calculated by measuring the copper ion concentration using a copper reagent.

2. Reagent composition & preparation (50T/48S):

Reagent	Volume	Storage
Reagent I	**-**	**-**
Reagent II	**40mL**	**RT**
Reagent III (Copper Reagent)		
Solution A	**30mL**	**4°C**
Solution B	**30mL**	**4°C**
Solution C	**5mL**	**4°C**
Reagent III Preparation: Mix Solution A, Solution B and Solution C at ratio of 10:9:1, consider volume according to your need. Prepared reagent can be stored at 4°C for 2 weeks.		
Reagent IV (Chromogenic Agent)		
Powder	**2 vials**	**4°C**
Diluent	**10mL × 2 bottles**	**4°C**
Reagent IV Preparation: Use 1 bottle (10mL) diluent to dissolve 1 vial powder, prepared reagent can be stored at 4°C for 2 weeks.		
Reagent V (Palmitic Acid Standard)		
Powder	**2 vials**	**4°C**
Solvent	**50mL**	**4°C**
1000μmol/L Reagent V preparation: Use diluent to dissolve 1 vial powder, adjust volume to 20mL, mix sufficiently. **Note: Thoroughly rinse the small centrifuge tube containing the powder with the Solvent to ensure complete transfer.**		
Reagent VI	**40mL**	**-**

Note: Reagent I: Chloroform (Trichloromethane, analytical grade) — Prepare freshly by yourself

3. Storage

The kit is stable for up to 3 months when stored at 4°C. Reagents prepared from the kit solutions remain stable for up to 2 weeks when stored at 4°C.

4. Operation procedure:

I. **Label glass test tubes:** It is suggested to use glass grinding test tubes with stopper in order to prevent reagent volatilization and induce extraction.

II. **Operation table:**

	Blank tube	Standard tube	Sample tube
Double distilled water (mL)	**0.2**	**0.2**	**-**
1000μmol/ L palmitic acid (mL)	**-**	**0.2**	**-**
Sample to assay (mL)	**-**	**-**	**0.2**
Reagent II buffer (mL)	**0.5**	**0.5**	**0.5**
Reagent III copper reagent (mL)	**1.0**	**1.0**	**1.0**
Reagent I (mL)	**4.0**	**3.8**	**4.0**
Extract by mixing for 2 minutes, then centrifuge at 3500 rpm for 10 minutes. Remove the blue liquid from the upper layer along with the protein clot, and take 2mL of the extract solution from the lower layer for the chromogenic reaction.			
Underlayer extract (mL)	**2.0**	**2.0**	**2.0**
Chromogenic agent (mL)	**0.25**	**0.25**	**0.25**
Mix thoroughly, keep at room temperature for 2 minutes, transfer into cuvettes with a 1cm light path, and measure the OD values of all tubes at 440nm (adjust to zero using Reagent I).			

Note: Detailed Operating Procedures

1. Vortex thoroughly for 12 minutes until no phase separation is observed in the tube. If ground-glass stoppered tubes are unavailable, disposable 10mL centrifuge tubes may be used as alternatives. Prior to use, verify compatibility by adding chloroform to the centrifuge tube to check for potential chemical reactions.

2. After vortexing, centrifuge at 3500 rpm for 10 minutes. If the lower-layer liquid appears semi-coagulated, the coagulated layer is excessively thick, or the volume of the lower layer is less than 2mL, gently stir the mixture with a small glass rod or pipette tip and re-centrifuge until clear phase separation is achieved.

3. Subsequently, carefully aspirate the upper-layer liquid and the coagulated layer using a pipette, and discard them.

4. Take a syringe fitted with an epidural anesthesia needle (stylet inserted into the cannula). Carefully insert the needle into the lower-layer extract, remove the stylet, attach the syringe, and aspirate 2.3–2.5mL of the lower-layer extract into a new test tube. This method prevents contamination of the lower layer with the upper-layer liquid or coagulated substances. If contamination occurs, re-centrifuge the sample before re-collecting the lower-layer extract; otherwise, the assay results may be affected. If the extract is accidentally found to be foggy or turbid, incubate it in a 37°C water bath for 1–2 minutes to clarify.

5. Using the same syringe-equipped needle, accurately transfer 2mL of the lower-layer extract from the previously mentioned test tube into another tube. Then add the chromogenic agent to initiate the chromogenic reaction.

6. After cleaning the cuvettes with double-distilled water, rinse them thoroughly with absolute ethanol. Zero the spectrophotometer using chloroform before measurement. Failure to do so may result in water droplets mixing with the added chloroform (since chloroform and water are immiscible), leading to measurement errors.

7. Glass tubes are preferred for all operational steps. Certain types of plastic centrifuge tubes may also be used, but their compatibility must first be verified by adding chloroform to ensure that no adverse reactions occur.

Calculation

I. **Blood serum NEFA assay:**

Blood serum NEFA Content (μmol/L) = $(A_T - A_B \div A_S - A_B) \times C_S$

II. **Tissue NEFA assay:**

Tissue NEFA Content (μmol/gprot) = $(A_T - A_B \div A_S - A_B) \times C_S \div Cprot$

A_T: OD_{Sample}
A_C: $OD_{Control}$
A_B: OD_{Blank}
A_S: $OD_{Standard}$
C_S: Standard concentration 1000μmol/L
C_{prot}: Protein concentration of tissue samples gprot/L (where ‘prot’ denotes protein)

Note: For non-protein samples, the sample mass concentration (sample mass (g)/total volume of homogenate (L)) can be substituted for the protein concentration in the calculation.

5. Notes

1. This assay must be performed using glass test tubes and a standard spectrophotometer. Plastic test tubes, semi-automatic analyzers, and fully automatic biochemical analyzers must not be used, as the organic solvents used in this assay may damage these instruments.

2. While collecting the lower-layer extract solution, ensure that the pipette or needle tip does not touch the inner wall of the test tube to avoid contamination with the copper reagent. The collected lower-layer extract solution should be clear and free from turbidity. A cloudy or contaminated extract may result in falsely elevated readings.

3. Bilirubin may be extracted by Reagent I and can interfere with spectrophotometric measurements. Therefore, for icteric (yellow-coloured) serum samples, an additional control tube should be prepared using *n*-butanol instead of the chromogenic reagent to minimize bilirubin interference.

Appendix: Problems in assay

The NEFA assay often shows a high OD blank. Some researchers may obtain very high OD blank and OD standard values (even >3.000). This situation is caused by operational errors, so please take care of the following problems:

1. When performing tissue sample assays, do not prepare all homogenized samples at the same time. The components in the homogenate may degrade rapidly after preparation. Ideally, if homogenization is performed in the morning, the assay should be completed on the same day, preferably in the afternoon. If a large number of samples need to be tested, prepare and assay them in smaller batches according to the number of samples that can be completed in one day. It is recommended to include control samples in each batch to reduce variation between batches (CV).

2. It is recommended to use the reverse pipetting method when working with micropipettes for improved accuracy. Beginners should first practice pipetting with distilled water, followed by alcohol and serum samples, before performing the actual assay. This helps improve pipetting accuracy and consistency.

3. During the NEFA extraction mixing step, seal the test tube securely with a rubber stopper and hold the upper part of the tube while mixing thoroughly. Do not hold the middle or lower part of the tube, as this may reduce mixing efficiency and result in incomplete extraction.

4. All containers and glassware used in this assay must be clean and completely dry. Contamination may affect the assay results. For example, dirty flasks may cause the prepared copper reagent to become turbid, while contaminated test tubes may lead to the formation of unwanted protein layers in blank samples.

5. Prepare all reagents strictly according to the procedure described in this manual. Incorrect preparation order or improper mixing may cause reagents, such as the copper reagent, to become turbid and unsuitable for use.

6. Ensure that all reagents, especially distilled water, are free from contamination. Contaminated distilled water is a common cause of failed or inaccurate experiments.

7. After centrifugation, it is recommended to remove and discard the upper copper reagent layer using a pipette. To collect the lower extract layer, use an appropriately sized puncture or spinal needle (for example, an epidural anesthesia needle). Needles that are too large may accidentally draw the upper copper reagent layer or intermediate protein layer along with the extract. This contamination can cause abnormally high absorbance readings and inaccurate results. To avoid cross contamination, do not use the same puncture needle or glass pipette for different experiments. Dedicated pipettes or needles are recommended for each assay.

8. During spectrophotometric measurement, ensure proper handling and operation of the spectrophotometer. Always rinse and re-zero the instrument when changing cuvettes. Using a new cuvette without proper rinsing and blank adjustment may result in falsely high absorbance values due to residual colour adsorption on the cuvette surface.

9. During reagent preparation, measure all reagent volumes accurately and strictly follow the specifications provided in this manual. Do not estimate or add reagents arbitrarily, as improper measurements may affect the accuracy and reliability of the assay results.