（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-K013-S

Specification：50 Assays (17 samples)/ 100 Assays (42 samples)

Measuring instrument：Spectrophotometer（715 nm）

Detection range：0.05-2.0 mmol/L

Elabscience® Non-esterified Free Fatty Acids (NEFA/FFA) Colorimetric Assay Kit

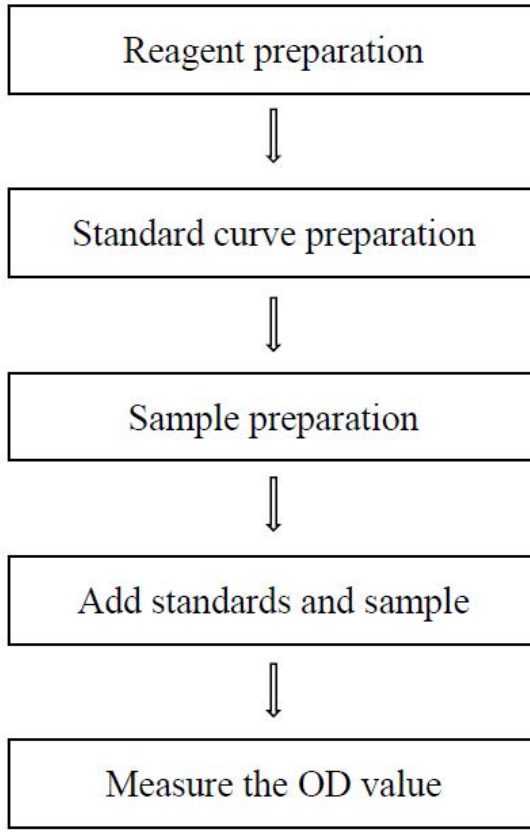
This manual must be read attentively and completely before using this product.
If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623
Tel: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

Table of contents

Assay summary



Intended use

This kit can be used to measure non-esterified free fatty acids (NEFA) content in animal tissue samples.

Detection principle

Under the condition of weak acidity, NEFA react with nantokite to form copper soap, which has a specific absorption peak at 715nm. The content of NEFA can be calculated by measuring the OD value at 715 nm.

Kit components & storage

Item	Component	Size 1 (50 assays)	Size 2 (100 assays)	Storage
Reagent 1	Extracting Solution	60 mL ×2 vials	60 mL × 3 vials	2-8℃, 12 months
Reagent 2	10 mmol/L Palmitic Acid Standard	1.8 mL ×1 vial	1.8 mL × 2 vials	2-8℃, 12 months
Reagent 3	Control Solution	14 mL ×1 vial	28 mL × 1 vial	2-8℃, 12 months
Reagent 4	Reaction Solution	22.5 mL ×1 vial	45 mL × 1 vial	2-8℃, 12 months

Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

Materials prepared by users

Instruments:

Spectrophotometer (715 nm), Vortex mixer, Micropipettor

Reagent preparation

① Equilibrate other reagents to room temperature before use.

② The preparation of standard curve：

Always prepare a fresh set of standards. Discard working standard dilutions after use.

Dilute 10 mmol/L standard solution with extracting solution to a serial concentration. The recommended dilution gradient is as follows: 0.00, 0.10, 0.25, 0.50, 1.00, 1.50, 2.00 mmol/L. Reference is as follows:

Item	①	②	③	④	⑤	⑥	⑦
Concentration (mmol/L)	**0**	**0.1**	**0.25**	**0.5**	**1.0**	**1.5**	**2.0**
10 mmol/L standard (μL)	0	30	75	150	300	450	600
Extracting solution (μL)	3000	2970	2925	2850	2700	2550	2400

Sample preparation

① Sample preparation

Tissue sample:

① Harvest the amount of tissue needed for each assay (initial recommendation 200 mg).

② Wash tissue in cold PBS (0.01 M, pH 7.4).

③ Homogenize 0.2 g tissue in 2.4 mL extracting solution with a dounce homogenizer at 4℃.

④ Oscillate at 4℃ for 2 hours to extract the NEFA.

⑤ Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection.

② Dilution of sample

The recommended dilution factor for different samples is as follows (for reference only):

Sample type	Dilution factor
Rat liver tissue homogenate	1-3
Rat heart tissue homogenate	1
Rat kidney tissue homogenate	1
Mouse liver tissue homogenate	1-3

Note: The diluent is extracting solution. For the dilution of other sample types, please do pretest to confirm the dilution factor.

The key points of the assay

① The samples should be fresh collected and detect within 24 hours.

② The supernatant after centrifugation must be clarified for the pretreatment of tissue samples. Otherwise take the turbid supernatant to another centrifuge tube and centrifuge again.

③ The reagent has a pungent smell. Please operate in the draught cupboard.

Operating steps

① Standard tube: add 1 mL of standards with different concentrations into the standard tubes, and add 0.5 mL reaction solution.

Control tube: add 1 mL of supernatant of sample and 0.5 mL of control solution into the control tubes.

Sample tube: add 1 mL of supernatant of sample and 0.5 mL of reaction solution into the sample tubes.

② Oscillate for 5 min and stand at room temperature for 5 min.

③ Set spectrophotometry to zero with extracting solution and take 0.8 mL of the upper layer liquid into 1 mL cuvette and measure the OD value at 715 nm.

Calculation

The standard curve:

1. Average the duplicate reading for each standard.

2. Subtract the mean OD value of the blank (Standard #①) from all standard readings. This is the absoluted OD value.

3. Plot the standard curve by using absoluted OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (**y = ax + b**) with graph software (or EXCEL).

The sample:

Tissue sample:

$$NEFA(\mu mol/g) = (\Delta A_{715} - b) \div a \times \frac{V}{m} \times f$$

[Note]

ΔA_{715}: Absoluted OD value, OD_{Sample} - $OD_{Control}$.

V: The volume of extracting solution added during the pretreatment of tissue sample, 2.4 mL.

m: Fresh weight of tissue, 0.2 g.

f: Dilution factor of sample before test.

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

Three tissue samples were assayed in replicates of 20 to determine precision within an assay (CV = Coefficient of Variation).

Parameters	Sample 1	Sample 2	Sample 3
Mean (mmol/L)	0.25	1.20	1.70
%CV	2.4	2.2	2.0

Inter-assay Precision

Three tissue samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (mmol/L)	0.25	1.20	1.70
%CV	5.2	6.7	6.4

Recovery

Take three samples of high concentration, middle concentration and low concentration to test the samples of each concentration for 6 times parallelly to get the average recovery rate of 100%.

	Standard 1	Standard 2	Standard 3
Expected Conc. (mmol/L)	0.08	0.45	1.3
Observed Conc. (mmol/L)	0.1	0.4	1.3
Recovery rate (%)	102	98	100

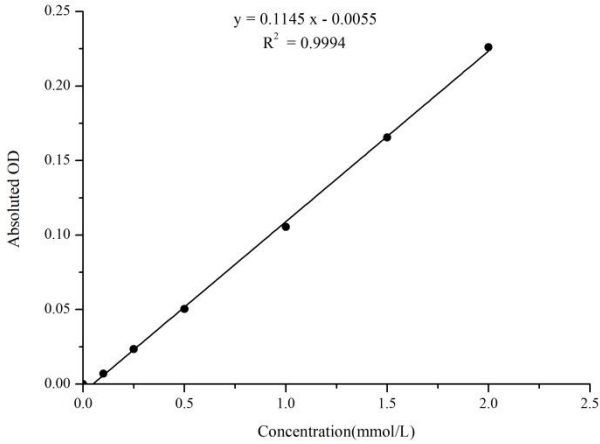
Sensitivity

The analytical sensitivity of the assay is 0.05 mmol/L NEFA. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

2. Standard curve:

As the OD value of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique or temperature effects), so the standard curve and data are provided as below for reference only:

Concentration (mmol/L)	**0**	**0.1**	**0.25**	**0.5**	**1.0**	**1.5**	**2.0**
Average OD	0	0.007	0.024	0.051	0.106	0.166	0.226
Absoluted OD	0	0.007	0.024	0.051	0.106	0.166	0.226



Appendix II Example Analysis

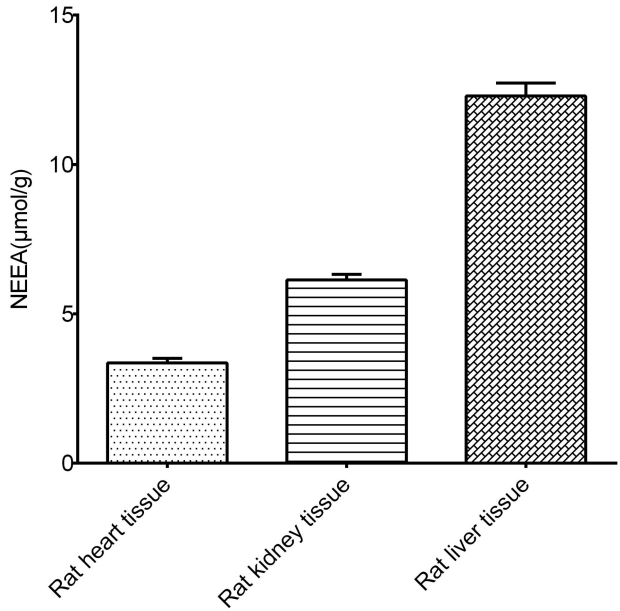
Example analysis：

For rat liver tissue, take 0.2 g of fresh rat liver sample, add 2.4 mL of extracting solution, then homogenize the sample and oscillate at 4℃ for 3 hours to extract the NEFA. Centrifuge the sample at 2000 g for 10 min at 4℃, take the supernatant, and carry the assay according to the operation steps. The results are as follows:

Standard curve: y = 0.10882 x - 0.001, the average OD value of the sample tube is 0.109, the average OD value of the control tube is 0.000, and the calculation result is:

$$\text{NEFA content (μmol/g)} = (0.109+0.001) \div 0.10882 \times 12 = 12.13 \text{ μmol/g}$$

Detect rat heart tissue (m=0.2 g, V_3=2.4 mL), rat kidney tissue (m=0.2 g, V_3=2.4 mL), rat liver tissue (m=0.2 g, V_3=2.4 mL) according to the protocol, the result is as follows：



Appendix III Publications

1. Meng J J, Shen J W, Li G, et al. Light modulates glucose metabolism by a retina-hypothalamus-brown adipose tissue axis[J]. Cell, 2023, 186(2): 398-412. e17.
2. Richa Verma,Ming Fu,Guangdong Yang,Lingyun Wu,Rui Wang.Hydrogen Sulfide Promotes Adipocyte Differentiation, Hyperplasia, and Hypertrophy[J]. 2023(1):13.DOI:10.1016/j.eng.2022.09.010.
3. Schreyer E , Obringer C , Messaddeq N ,et al.PATAS, a First-in-Class Therapeutic Peptide Biologic, Improves Whole-Body Insulin Resistance and Associated Comorbidities In Vivo[J].Diabetes, 2022, 71(9):2034-2047.DOI:10.2337/db22-0058.
4. Mu Y, Luo L B, Huang R, et al. Cardiac-derived CTRP9 mediates the protection of empagliflozin against diabetes-induced male subfertility in mice[J]. Clinical Science, 2024, 138(21): 1421-1440.
5. Kamel M A .The Anti-Obesity Potential of Superparamagnetic Iron Oxide Nanoparticles against High-Fat Diet-Induced Obesity in Rats: Possible Involvement of Mitochondrial Biogenesis in the Adipose Tissues[J].Pharmaceutics, 2022, 14.DOI:10.3390/ pharmaceutics14102134.

Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.