TECHNICAL MANUAL

Non-esterified Free Fatty Acids (NEFA) Colorimetric Assay Kit

- **SKU CODE:** MAES0026
- **SIZE:** 48 Tests
- **DETECTION PRINCIPLE:** Assay Kit
- **RUO:** Research-Use-Only

Revised: 01/2026

1. Assay summary

- Reagent preparation
- Standard curve preparation
- Sample preparation
- Add standards and samples to microplate
- Measure OD value at 715 nm

2. Intended use

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

3. Detection principle

Under weak acidic conditions, NEFA react with cupric chloride to form copper soap, which has a specific absorption peak at 715 nm. The content of NEFA can be calculated indirectly by measuring the OD value at 715 nm.

4. Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Extracting Solution	60 mL x 1 vial	60 mL x 2 vials	2-8 °C, 12 months
Reagent 2	10 mmol/L Palmitic Acid Standard	1 mL x 1 vial	1 mL x 2 vials	2-8 °C, 12 months
Reagent 3	Control Solution	6 mL x 1 vial	12 mL x 1 vial	2-8 °C, 12 months
Reagent 4	Reaction Solution	10 mL x 1 vial	20 mL x 1 vial	2-8 °C, 12 months
	Microplate	48 wells	96 wells	No requirement
	Plate Sealer	2 pieces	2 pieces	

5. Materials prepared by users

Instruments:

Microplate reader (690-730 nm, optimum wavelength: 715 nm), micropipettor, vortex mixer

6. Reagent preparation

1. Equilibrate all reagents to room temperature before use.
2. **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 serial concentrations. The recommended dilution gradient is as follows: 0, 0.3, 0.4, 0.6, 0.9, 1.0, 1.2, 1.5 mmol/L.

7. Standard dilution table

Item	1	2	3	4	5	6	7	8
Concentration (mmol/L)	0	0.3	0.4	0.6	0.9	1.0	1.2	1.5
10 mmol/L standard (µL)	0	45	60	90	135	150	180	225
Extracting solution (µL)	1500	1455	1440	1410	1365	1350	1320	1275

8. Sample preparation

1. **Tissue sample preparation:** 1) Harvest the amount of tissue needed for each assay (initial recommendation 100 mg). 2) Wash tissue in cold PBS (0.01 M, pH 7.4). 3) Homogenize 100 mg tissue in 1200 µL extracting solution with a Dounce homogenizer at 4 °C. 4) Oscillate at 4 °C for 2 hours to extract the NEFA. 5) Centrifuge at 10,000 xg for 10 min at 4 °C to remove insoluble material. Collect supernatant and keep it on ice for detection.
2. **Sample dilution:** The recommended dilution factor for different samples is as follows (for reference only): Rat liver tissue homogenate: 1; Rat heart tissue

homogenate: 1; Rat kidney tissue homogenate: 1; Mouse liver tissue homogenate: 1. Note: The diluent is extracting solution. For the dilution of other sample types, please perform a pretest to confirm the dilution factor.

9. The key points of the assay

1. The samples should be freshly collected and detected within 24 hours.
2. The supernatant after centrifugation must be clarified for the pretreatment of tissue samples. Otherwise, transfer the turbid supernatant to another centrifuge tube and centrifuge again.
3. The reagent has a pungent smell. Please operate in a fume hood.

10. Operating steps

1. Standard tube: add 0.5 mL of standards with different concentrations into the standard tubes, and add 0.25 mL reaction solution. Control tube: add 0.5 mL of supernatant of sample and 0.25 mL of control solution into the control tubes. Sample tube: add 0.5 mL of supernatant of sample and 0.25 mL of reaction solution into the sample tubes.
2. Vortex for 3 min and stand at room temperature for 3 min.
3. Take 0.3 mL of the upper layer liquid to microplate and measure the OD value at 715 nm with microplate reader.

11. 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 absolute OD value.

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

The sample:

Tissue sample:

$NEFA(\mu mol/g) = (deltaA_{715} + b) \div a \times V_1 / (m \times f)$

[Note]

$deltaA_{715}$: Absolute OD value, $OD_{Sample} - OD_{Control}$.

m: The fresh weight of tissue sample, 0.1 g.

V_1: The volume of Reagent 1 added during the pretreatment of tissue sample, 1.2 mL.

f: Dilution factor of sample before test.

12. Appendix I Performance Characteristics

Parameter:

Intra-assay Precision

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

Intra-assay Precision Results

Parameters	Sample 1	Sample 2	Sample 3
Mean (mmol/L)	0.35	0.70	1.20
%CV	3.5	3.1	3.3

Inter-assay Precision

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

Inter-assay Precision Results

Parameters	Sample 1	Sample 2	Sample 3
Mean(mmol/L)	0.35	0.70	1.20
%CV	5.2	5.4	4.7

Recovery

Three samples of high concentration, middle concentration and low concentration were tested with 6 replicates each to obtain an average recovery rate of 101%.

Recovery Results

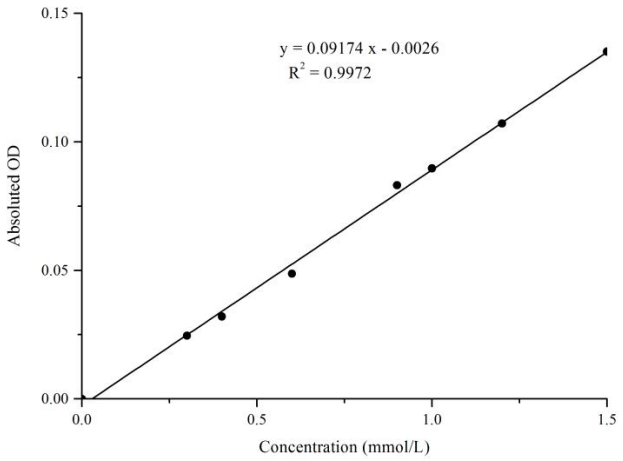
	Standard 1	Standard 2	Standard 3
Expected Conc. (mmol/L)	0.35	0.8	1.1
Observed Conc. (mmol/L)	0.4	0.8	1.1
Recovery rate (%)	103	99	101

Sensitivity

The analytical sensitivity of the assay is 0.15 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.

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), the standard curve and data are provided below for reference only:



Standard curve data

Concentration (mmol/L)	0	0.3	0.4	0.6	0.9	1	1.2	1.5
Average OD	0.043	0.067	0.075	0.091	0.126	0.132	0.15	0.178
Absolute OD	0	0.024	0.032	0.048	0.083	0.089	0.107	0.135

13. Appendix II Example Analysis

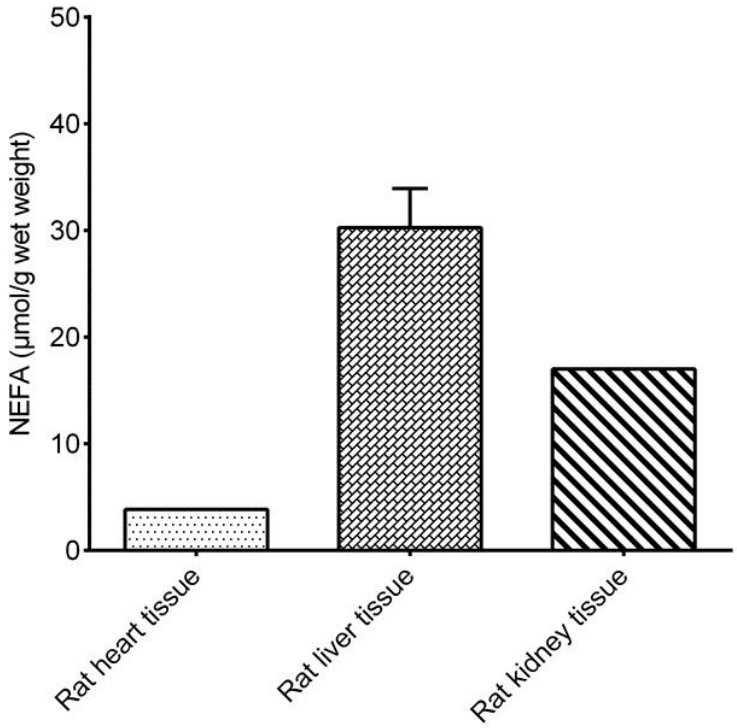
Example analysis:

For rat liver tissue, take 0.1 g of rat liver tissue, add 1.2 mL extracting solution, oscillate at 4 °C for 2 hours to extract the NEFA, centrifuge at 10,000 xg for 10 min, dilute the supernatant with extracting solution 3-fold, and carry out the assay according to the operation steps. The results are as follows:

Standard curve: y = 0.09174x - 0.0026, the average OD value of the sample tube is 0.108, the average OD value of the control tube is 0.049, and the calculation result is:

NEFA content (μmol/g) = (0.108 - 0.049 + 0.0026) ÷ 0.09174 × 1.2 / (0.1 × 3) = 24.17 μmol/g

Detect rat heart tissue (m=0.1 g, V_1=1.2 mL), rat liver tissue (m=0.1 g, V_1=1.2 mL), rat kidney tissue (m=0.1 g, V_1=1.2 mL) according to the protocol, the result is as follows:



14. Statement

1. This assay kit is for Research Use Only. Assay Genie assumes no responsibility for any problems or legal liabilities arising from the use of this kit for clinical diagnosis or any other purpose.

2. Please read the instructions carefully and calibrate the instruments before performing the experiments. Follow the instructions strictly throughout the procedure.

3. Appropriate protective measures must be taken, including wearing a lab coat and latex gloves.

4. If the concentration of the substance falls outside the detection range, perform an additional dilution or concentration step on the sample.

5. It is recommended to perform a pre-test if your sample type is not listed in the instruction manual.

6. Experimental results are closely related to reagent quality, operator technique, environmental conditions, and other factors. Assay Genie guarantees the quality of the kits only and is NOT responsible for sample consumption resulting from use of the assay kits. It is advisable to estimate the expected sample usage and reserve sufficient samples before starting the experiment.

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Manufacturers Statement: This final kit system is assembled and quality-released by Assay Genie Limited.