

Nonesterified Fatty Acid (NEFA) Assay Kit

(Colorimetric Method)

Serial No: A042

Pack: 50T/48S

Note: You must use glass tubes in this assay, semi-automatic and automatic spectrophotometer can not be used in this assay.

1. Assay principle:

NEFA can combine with copper ion to form fatty acid copper salt which dissolves in chloroform. Fatty acid copper salt content in chloroform appears direct proportion with NEFA content. As result, it is able to calculate NEFA content by measuring copper ion content by copper reagent.

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

Reagent 1: Liquid 60ml×4 bottles, can be stored at room temperature. This reagent is easy to volatilize, so please complete assay in 1 month.

Reagent 2: Buffer 40ml×1 bottle, can be stored at room temperature for 6 months.

Reagent 3: Copper reagent, Liquid A 30ml×1 bottle, Liquid B 30ml×1 bottle, Liquid C 5ml×1 bottle. Can be stored at 4°C for 6 months.

Reagent 3 copper reagent preparation: Mix Liquid A, Liquid B and Liquid C at ratio of 10:9:1, consider volume according to you need. Prepared reagent can be stored at 4°C for 2 weeks.

Reagent 4: Chromogenic agent, Powder×2 vials, Diluent 10ml×2 bottles, can be stored at 4°C for 3 months.

Reagent 4 chromogenic agent preparation: Use 1 bottle (10ml) diluent to dissolve 1 vial powder, prepared reagent can be stored at 4°C for 2 weeks.

Reagent 5: Palmitic acid standard powder×2 vials, Solvent 50ml×1 bottle, can be stored at 4°C for 1 month.

1000μmol/L palmitic acid standard preparation: Use diluent to dissolve 1 vial powder, adjust volume to 20ml, mix sufficiently (wash small vial of powder completely by solvent.)

Reagent 6: Double distilled water 20ml×1 bottle (for blank tube).

3. Operation procedure:

(1) **Label glass test tubes** (it is suggested to use glass grinding test tubes with stopper in order to prevent reagent volatilization and induce extraction.)

(2) **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 2 buffer (ml)	0.5	0.5	0.5
Reagent 3 copper reagent (ml)	1.0	1.0	1.0
Reagent 1 (ml)	4.0	3.8	4.0

Extract by mixing sufficiently for 2 minutes, centrifugate at 3500rpm for 10 minutes, remove blue liquid of upper layer and protein clot, take 2ml extract solution of underlayer for chromogenic reaction.*

Underlayer extract (ml)	2.0	2.0	2.0
Chromogenic agent (ml)	0.25	0.25	0.25
Mix sufficiently, place at room temperature for 2 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 440nm (adjust zero by Reagent 1).			

Note:

- ① Extract by mixing sufficiently for 2 minutes accurately (use seconds-counter). If you haven't grinding test tubes, then you can use normal test tubes instead but please seal opening by handi-wrap in order to avoid liquid splashes out of tube.
- ② After extraction, centrifugate at 3500rpm for 10 minutes. If underlayer liquid appears semi-coagulated and coagulated layer is quite thick or underlayer liquid is less than 2ml, then you can stir underlayer by small glass bar or micropipet tip softly and centrifugate again to demix clearly.
- ③ Use injector with anesthesia spinal needle to draw upper layer liquid and discard.
- ④ Use another injectoe with anesthesia spinal needle to transfer 2.3~2.5ml underlayer extract solution in another test tube. If you draw some upper layer liquid or coagulated layer matter, then centrifuge again or it will affect result. If extract solution appears fog-like turbid, then please place it in 37℃ for 1~2 minutes.
- ⑤ Transfer 2ml underlayer extract solution in another test tube, add chromogenic agent for chromogenic reaction.

⑥ After washing by double distilled water, cuvettes should be rinsed by dehydrated alcohol, then add Reagent 1 to adjust zero. If you skip this step, water droplets will mixed in added Reagent 1 (Reagent 1 and water is insoluble to each other).

⑦ You should use glass test tubes in all steps above (can not be instead by plastic test tubes).

These 7 steps above are very important to achieve this assay.

4. Calculation:

(1) Blood serum NEFA assay:

① Formula:

$$\text{Blood serum NEFA content } (\mu\text{mol/L}) = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Standard}} - \text{OD}_{\text{Blank}}} \times \frac{\text{Standard concentration}}{(1000\mu\text{mol/L})}$$

② Example:

Take 0.2ml human blood serum to measure NEFA content, in results, OD_{Blank} is 0.045, $\text{OD}_{\text{Standard}}$ is 0.311, $\text{OD}_{\text{Sample}}$ is 0.159, calculate as follows:

$$\begin{aligned} \text{Blood serum NEFA content } (\mu\text{mol/L}) &= \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Standard}} - \text{OD}_{\text{Blank}}} \times \frac{\text{Standard concentration}}{(1000\mu\text{mol/L})} \\ &= \frac{0.159 - 0.045}{0.311 - 0.045} \times 1000 = 428.6 (\mu\text{mol/L}) \end{aligned}$$

(2) Tissue NEFA assay:

① Formula:

$$\text{Tissue NEFA content } (\mu\text{mol/gprot}) = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Standard}} - \text{OD}_{\text{Blank}}} \times \frac{\text{Standard concentration}}{(1000\mu\text{mol/L})} \div \frac{\text{Sample protein concentration}}{(\text{gprot/L})}$$

② Example:

Take 0.2ml 10% rat liver tissue homogenate supernatant to measure NEFA content, in results, OD_{Blank} is 0.045, $\text{OD}_{\text{Standard}}$ is 0.311, $\text{OD}_{\text{Sample}}$ is 0.293, protein concentration in 10% liver homogenate 12.24gprot/L, calculate as follows:

$$\begin{aligned} \text{Tissue NEFA content } (\mu\text{mol/gprot}) &= \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Standard}} - \text{OD}_{\text{Blank}}} \times \frac{\text{Standard concentration}}{(1000\mu\text{mol/L})} \div \frac{\text{Sample protein concentration}}{(\text{gprot/L})} \\ &= \frac{0.293 - 0.045}{0.311 - 0.045} \times 1000 \div 12.24 = 76.17 (\mu\text{mol/gprot}) \end{aligned}$$

5. Announments:

- (1) You should use glass test tubes and normal spectrophotometer in this assay, plastic test tubes and semi-automatic/automatic biochemical analyser can not be used (organic solvent is harmful for semi-automatic/automatic biochemical analyser)
- (2) When you take underlayer extract solution, do not let tip contacts with tube surface in order to avoid take copper reagent. Underlayer extract solution must be

limpid or result will be higher than true value.

- (3) Bilirubin can be extracted by Reagent 1 and disturb spectrophotometry, so auriginous blood serum need 1 contrast tube which use n-butanol to instead of chromogenic agent.

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Appendix: Problems in assay

NEFA assay always has high OD_{Blank} , some researchers may get very high OD_{Blank} and $OD_{Standard}$ (even >3.000), this situation is caused by operations, please take care of problems as follows:

1. When you do tissue sample assay, do not prepare all samples to homogenate in one time, because various parameters in homogenate will decrease fastly after preparing homogenate. In general, if you prepare homogenate in morning, then you should complete assay in afternoon. If you have too many samples which can not be assayed in one time, then please control “homogenate number” according to “how much samples you can assay today”. It is suggested to take samples in batches, each batch needs samples to participate in order to decrease CV between batches.
2. It is suggested to use “retrogression adding method” when you use micropipets. Fresh-hand operator should do adding practise with distilled water at first, then do practise with alcohol and blood serum, it is able to enhance adding sample accuracy.
3. During NEFA mixing procedure, seal test tube by rubber stopper, hold superior part of test tube to mix sufficiently, do not hold middle part or inferior part of test tube or it will cause mixing insufficiently and lead to extraction insufficiently.
4. All containers used in this assay must be cleansed and dried, pollution may cause unnecessary loss. For example, when you prepare copper reagent, if flask is dirty, then prepared reagent may appear turbid. If test tubes are dirty, then some hybridproteins may cause protein middle layer exists in blank tube.
5. Reagent preparation should follow the right order according to this mannual. For example, if you prepare copper reagent in mess, then prepared copper reagent may become turbid and can not be used.
6. Required reagents shouldn't be polluted. Pay attention to your distilled water, various fault experiments are caused by distilled water pollution (polluted by ions or