Nonesterified Fatty Acid (NEFA) Assay Kit

(Cat/No.:BC151 Size:50T/48S)

1. Assay principle (Colorimetric Method)

Nonesterified fatty acids (NEFAs) can chelate with copper ions to form copper fatty acid salts, which are soluble in chloroform. The concentration of these copper fatty acid salts is directly proportional to the NEFA content in the sample. By quantifying the copper ion concentration using a copper reagent, the NEFA content can be indirectly calculated.

2. Reagent composition & preparation(Kit validity period: 3 months)

Reagent 1: Chloroform (trichloromethane, analytical grade), user-prepared

Reagent 2: Buffer solution, 40 mL × 1 bottle, stored at room temperature

Reagent 3: Copper Reagent: Solution A (30 mL × 1 bottle), Solution B (30 mL × 1 bottle), Solution C (5 mL × 1 bottle); stored at 4°C.Preparation of Copper Reagent (Reagent 3): Mix Solution A, Solution B, and Solution C at a volume ratio of 10:9:1. Prepare only the required volume. The prepared reagent is stable for 2 weeks when stored at 4°C.

Reagent 4 Chromogenic Agent: Powder × 2 vials, Diluent (10 mL × 2 bottles); stored at 4°C.Preparation of Chromogenic Agent (Reagent 4): Dissolve 1 vial of powder in 1 bottle (10 mL) of Diluent immediately before use. The prepared reagent is stable for 2 weeks when stored at 4°C.

Reagent 5:

Palmitic Acid Standard: Powder × 2 vials, Solvent (50 mL × 1 bottle); stored at 4°C.Preparation of 1000 μmol/L Palmitic Acid Standard: Dissolve 1 vial of powder in the Solvent and adjust the final volume to 20 mL (Note: Thoroughly rinse the small centrifuge tube containing the powder with the Solvent to ensure complete transfer.

Reagent6: Double distilled water 40ml×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
Chloroform （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 Chloroform).			

Note: Detailed Operating Procedures

①Vortex thoroughly for12 minutes until no phase separation is observed in the tube. If ground-glass stoppered tubes are unavailable, disposable 10 mL centrifuge tubes may be used as alterna tives (prior to use, verify compatibility by adding chloroform to the centrifuge tube to check for potential chemical reactions).

②After vortexing, centrifuge at 3500 rpm for 10 minutes. If the lower-layer liquid is semi-coag ulated, the coagulated layer is excessively thick, or the volume of the lower layer is less than 2 ml, gently stir the mixture with a small glass rod or pipette tip and re-centrifuge until clear p hase separation is achieved.③Subsequently, carefully aspirate the upper-layer liquid and coag ulated layer using a pipette and discard them.

④ Take a syringe and an epidural anesthesia needle (stylet inserted into the cannula). Carefull y insert the needle into the lower-layer extract, remove the stylet, attach the syringe, and aspir ate 2.3–2.5 mL of the lower-layer extract into a new test tube. This method prevents contamin ation of the lower layer with the upper-layer liquid or coagulated substances. If contaminatio

n occurs, re-centrifuge the sample before re-collecting the lower-layer extract; otherwise, the a ssay results will be affected. If the extract is accidentally found to be foggy or turbid, incubate i t in a 37°C water bath for 1–2 minutes to clarify.

⑤Using the same syringe-equipped needle, accurately transfer 2 mL of the lower- layer extrac t from the aforementioned test tube into another tube, then add the chromogenic agent to init iate the chromogenic reaction.

⑥ After cleaning the cuvettes with double-distilled water, rinse them thoroughly with absolut e ethanol. Zero the spectrophotometer using chloroform before measurement. Failure to do s o will result in water droplets mixing with the added chloroform (chloroform and water are im miscible), leading to measurement errors.

⑦Glass tubes are preferred for all operational steps. Certain types of plastic centrifuge tube s may also be used, but their compatibility must be verified by adding chloroform to ensure n o adverse reactions occur.

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

4. Calculation and Example.

Blood serum NEFA assay:

① Formula:

$$\text{Blood serum NEFA content (μmol/L)} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Standard concentration (1000μmol/L)}$$

$C_{Standard}$: Standard concentration, 1000 μmol/L

② Example:

Take 0.2ml human blood serum to measure NEFA content, in results, ODBlank is 0.045, ODStan dard is 0.311, ODSample is 0.159, calculate as follows:

$$\text{Blood serum NEFA content (μmol/L)} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Standard concentration (1000μmol/L)}$$

$$= \frac{0.159 - 0.045}{0.311 - 0.045} \times 1000 = 428.6\ (\mu mol/L)$$

Tissue NEFA assay:

① Formula:

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

C_{Standard}: Standard concentration, 1000 μmol/L;

C_{pr}: 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.

② Example:

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

$$\text{Tissue NEFA content (μmol/gprot)} = \frac{OD_{Sample}-OD_{Blank}}{OD_{Standard}-OD_{Blank}} \times \frac{\text{Standard concentration (1000μmol/L)}}{\text{Sample protein concentration (gprot/L)}}$$
$$= \frac{0.293-0.045}{0.311-0.045} \times 1000 \div 12.24 = 76.17 \ (\mu mol/gprot)$$

5. Announments

1,You should use glass test tubes and normal spectrophotometer in this assay, plastic test tube s and semi-automatic/automatic biochemical analysator can not be used (organic solvent is har mful for semi-automatic/automatic biochemical analysator)

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 highe r than true value.

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

Appendix: Problems in assay

NEFA assay always has high ODBlank , some researchers may get very high ODBlank and ODSta ndard (even >3.000), this situation is caused by operations, please take care of problems as foll ows:

1, When you do tissue sample assay, do not prepare all samples to homogenate in one time, be cause various parameters in homogenate will decrease fastly after preparing homogenate. In g eneral, 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 “homog enate number” according to “how much samples you can assay today”. It is suggested to take s amples in batches, each batch needs samples to participate in order to decrease CV between b atches.

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 an d blood serum, it is able to enhance adding sample accurancy.

3, During NEFA mixing procedure, seal test tube by rubber stopper, hold superior part of test tu be 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 dryed, pollution may cause unnecessa ry 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 hybrid proteins may cause protein middle layer exists in blank tube.

5, Reagent preparation should follow the right order according to this manual. For example, if y ou 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 e xperiments are caused by distilled water pollution.

7, After centrifugation, it is recommended that the upper-layer copper reagent be aspirated an d discarded using a pipette. For the collection of the lower-layer extract, an appropriately size d puncture needle (i.e., an epidural anesthesia needle) should be employed. If the needle is exc essively large (e.g., a needle intended for bovine applications), it may entrain excessive amount s of the upper-layer copper reagent or the intermediate protein layer. Aspiration of the upper-l ayer copper reagent will lead to abnormally high absorbance values during spectrophotometri c measurement, resulting in significant deviations in experimental results. Furthermore, punctu re needles should not be cross-used between different experiments; ideally, dedicated glass pip ettes or puncture needles should be reserved for each specific experiment to avoid cross-conta mination.

8, During the spectrophotometric measurement, proper operation of the spectrophotomete

r must be ensured. For instance, some researchers only zero the spectrophotometer with one c uvette and directly use another cuvette without rinsing and re-zeroing. This practice will caus e an abnormally high initial absorbance value due to color adsorption by the cuvette.

9, During reagent preparation, it is crucial to accurately measure the required volumes strictly i n accordance with the specifications provided in this manual to enhance the accuracy of the ex periment. Arbitrary dispensing of reagents is not permitted.