Tissue TG & TC Protocol

Version: 1
Replaced by version
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Summary:

The lipid extraction method is based on work by Folch 1957. Tissue is homogenized and put into a 2:1 chloroform and methanol mix. The mix is separated into two phases by adding a salt solution. The upper phase contains any non-lipid substances and the lower phase contains the chloroform with the lipids. The upper phase is removed and a sample is taken from the bottom phase and evaporated. The sample is reconstituted with 2-propanol with a volume to bring it to the desired concentration to assay.

Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate. Glycerol-3-phosphate is oxidized by dihydroxyacetone phosphate (DAP) by glycerolphosphate oxidase producing hydrogen peroxide (H2O2). In a Trinder5 type color reaction catalyzed by peroxidase, the H2O2 reacts with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) to produce a red colored dye. The absorbance of this dye is proportional to the concentration of triglycerides present in the sample.

Cholesterol esters are enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with HBA and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which may be quantitated at 500-550nm.

Reagents and Materials:

Reagent/Material	Vendor	Stock Number
Sodium Sulfate	Fisher	S415-212
Methanol	Fisher	A412-4
Chloroform	Fisher	C298-4
2-Propanol	Fisher	A417-4
Sodium Chloride	Fisher	S271-3
Calibrator	Fisher Diagnostics	TR43002
Reagents	Fisher Diagnostics	TR22203
PBS		

Microplate		
Platereader		

Protocol:

1. Weigh out tissue (minimum of 50 mg) accurately and record, then transfer to a mortar and pestle along with 2 g of sodium sulfate (Fisher S415-212). Grind the tissue with the mortar and pestle until completely homogenous with salt.

2. Transfer homogenized mix to a 16×125mm screw-cap glass tube (Fisher 14-959-35A) using a funnel (Fisher 03-865).

3. Add 4ml of ACS grade methanol (Fisher A412-4) with a volumetric pipet (Fisher 13-650-2E) then 8ml of ACS grade chloroform (Fisher C298-4) with a volumetric pipet (Fisher 13-650-2J). Screw the cap on (Fisher 14-959-36A), vortex, and store overnight at 4°C. Repeat steps 2-5 for all samples.

4. Add 2.4ml of a 0.7% sodium chloride (Fisher S271-3) solution to each tube and DO NOT VORTEX! The liquid will separate into 2 phases with the salt solution and methanol on top and the chloroform on the bottom. Store for at least 24 hours at 4°C.

5. Aspirate off the supernatant and any particulate matter floating on top of the chloroform layer. Be careful not to aspirate too much of the chloroform.

6. Take a 5ml sample of the chloroform using a volumetric pipet (Fisher 13-650-2F) and then transfer into a 16×125mm open top glass test tube (Fisher 14-961-30).

7. Put all the test tubes containing the chloroform sample into the Evap-O-Rac (Cole-Parmer EW-01610-25) and evaporate with 100% nitrogen gas until completely dry. Takes about 4 hours.

8. Add ACS grade 2-propanol (Fisher A417-4) to each tube (volume will depend on amount of sample and concentration of TG or TC in tissue, 0.25 ml for liver and 0.15 ml for muscle is a good estimate), vortex at high speed for 1 minute with parafilm over the top of the tube, then use a pipet to transfer the 2-propanol to a microfuge tube.

9. There will be bits of tissue floating in the 2-propanol so centrifuge the microfuge tube for 1 min at 10000×g, then transfer the clean supernatant to another threaded microfuge tube (Fisher 02-681-339) and cap (Fisher 02-681-358).

10. Reconstitute powdered TG or TC reagent with only 25 ml of distilled water to make a 2X solution.

11. Add 150 µl of PBS to each well.

IMPORTANT: Make sure not to add any bubbles to the wells when dispensing reagents, this will interfere with reading in the platereader.

12. Add 3 µl of calibrator and sample to each well. Read at 540 nm.

IMPORTANT: Add PBS to wells first and then add samples rather than adding samples to blank wells and adding PBS after. The 2-propanol will evaporate quickly if the 3µl is added to a blank well.

13. Add 150 µl of 2X reagent to each well. Incubate at 37°C for 5 minutes. Read at 540 nm.

14. Subtract blank readings from final readings. The assay will be linear so the unknown samples can be calculated as (Sample Absorbance ÷ Calibrator Absorbance) × Calibrator Concentration.

15. Convert the assay TG or TC value in mg/dl to mg/ml then multiply by the total ml of 2-propanol used to get the total mg in the 5 ml chloroform sample that was evaporated. Multiply by the ratio of the total chloroform used and the chloroform sample taken (8/5) to get the total mg extracted from the chloroform. Divide by the tissue weight to get mg of TG or TC per g of tissue.

Reagent Preparation:

Sodium Sulfate – ready to use
Methanol – ready to use
Chloroform – ready to use
2-Propanol – ready to use
Sodium Chloride – make a 0.7% NaCl solution with distilled water
PBS – ready to use
Reagent – reconstitute with distilled water to make a 2X solution