Lipid peroxidation (TBA / TCA) Plate Reader method

Principle

Hydroperoxides originating from peroxyradicals and peroxidation of fatty acids are the cause of the disintegration of the membrane. Malonaldehyde is an example of a product from membrane deterioration. These products can be determined by TBA-measurements.

Equipment and Reagents

Machine/Product	Reference
Hotplate/Stirrer	 Jenway Model 1100
Centrifuge – cooled	Eppendorf 5415R with F45-24-11 rotor
Hot Water Bath (up to 80 °C)	Memmert Type WB14
Mixer Mill / Cryo Mill	Retsch MM 400 with 2x PTFE Adapter rack for 10 reaction vials 1.5 and 2.0 ml.
Microtube Vortex	Ika Vortex 1
Plate reader	BioTek PowerWave HT with Gen 5 software
2 ml Microtubes	Eppendorf Safe-Lock Tubes™, 2.0 ml
Crushed Ice	
Liquid Nitrogen (+ thermos jar)	
TCA (Trichloroacetic acid) *Store at 2-8 °C. Hygroscopic*	 Sigma T9159
TBA (2-Thiobarbituric acid)	 Sigma T5500

Preparations of Buffer solutions

- 0.1 % TCA: Dissolve 0.1 g TCA in 100 ml dH2O)
- 0.5 % TBA in 20 % TCA:
  1. Dissolve 20 g TCA in 80 ml dH_2O (final conc. = 20 g in 100 ml = 20%)
  2. When completely dissolved, add 0.5 g TBA (final conc. = 0.5 g in 100 ml = 0.5%)
  3. Put a glass plate on top of the beaker
  4. Heat the beaker (~80°C) while stirring in a fumehood
  5. After ~30 minutes, TBA is dissolved
  6. Let the solution cool down
  7. Transfer to a volumetric flask of 100 ml and adjust the volume with dH_2O.
- Freshly made 0.5 % TBA in 20 % TCA can be used for about one month before precipitation. Precipitation will occur more quickly if stored at 4°C

Procedure

To do before you start:

- Heat the water bath to 80°C
- Precool the centrifuge to 4°C
- Precool the tube blocks for the mixer mill in liquid nitrogen. The blocks are cool when the bubbling stops.
- Prepare a box with crushed ice and cool the TCA 0.1% solution.
- Switch on the computer and Plate reader (25°C)

Protocol

- Take the samples (~80-100 mg) out of the -80°C freezer and into liquid nitrogen.
- Add 2 Tungsten Carbide beads and put in the mixer mill for 3.5 minutes at 30 Hz. You can shred 10 samples at once (5 per block, see figure). If more tubes are placed, the risk exists that the caps of the outer tubes will snap.
- Take the tubes out and add 1 ml of 0.1 % TCA. Work on ice! Mix well on the vortex until homogenized.
- Centrifuge 10 minutes at 13 000 rpm (at 4 °C) to make a pellet.
- Puncture a small hole in the cap of a 2 ml Eppendorf tube using a syringe needle.
- Put 1 ml TBA 0.5 % in TCA 20 % in the 2 ml Eppendorf tube.
- Add 400 µl Sample (or 400 µl 0.1% TCA for blanks). This is a dilution of 3.5 times.
- Make a technical replicate for each sample.
- Close the perforated caps and incubate for *exactly* 30 minutes at 80 °C in the water bath. The tubes are held upright using a perforated float.
- After incubation, immediately put the tubes on ice until they are ice cold (~5 minutes).
- Centrifuge 5 minutes at 13 500 rpm (4 °C) to pellet any TBA precipitate.
- Transfer 200 µl supernatant to the 96-well plate. Make a technical replicate.
- Measure absorption at 532 nm and at 600 nm. [*Gen 5 protocol: Temperature: Setpoint 25°C | Shake:Medium for 0:10 | Read: (A) 532 | Read: (A) 600*]

Calculation

Absorbance is read at 532 nm subsequent to subtraction of non-specific absorption at 600 nm. The malondialdehyde (MDA) concentration is calculated using its extinction coefficient ε = 155 mM-1 cm-1. The MDA equivalents are calculated as follows:

$$nmol\ MDA/\ g\ FW = \frac{\Delta A_{corrected} * 3.5 * x * 1000}{\varepsilon * b * y}$$

Where:

- $\Delta A_{corrected}$ = A532 – A600 corrected with ΔA of the blank
- b = light path length (0.56 cm for 200 µl)
- ε = millimolar extinction coefficient (155 mM-1 cm-1)
- 3.5 (dilution factor from 400 µl extract + 1 ml TBA/TCA solution)
- x (ml) TCA 0.1 % used for extraction (1 ml)
- y (g) FW used for extraction
- 1000 = conversion factor (nmol →µmol)

Remarks / Notes:

Instead of using a water bath, it is also possible to use a heating block designed to hold Eppendorf tubes.