Technical Bulletin

Hepatic Steatosis Kit

Catalog Number MAK258

Product Description

Hepatic steatosis, also known as fatty liver, is a disease characterized by an excessive accumulation of lipid droplets within the liver. This can be caused by multiple factors, including excessive alcohol intake, obesity, and other diseases with abnormal fat metabolism. Hepatic steatosis is commonly used as a marker to determine the hepatotoxicity of drug candidates.

The Hepatic Steatosis Kit provides reagents for measurement of intracellular triglyceride accumulation and staining reagents to visualize the lipid droplet formation in hepatocytes. The kit can be used to screen and evaluate steatosis risk of drug candidates and to study the mechanism of steatosis development. Chloroquine is included as a positive control for inducing steatosis in liver cells, such as HepG2.

The kit provides enough reagents for 100 assays for triglyceride quantitation and enough staining reagents for two 96-well plates, two 6-well plates, or four 100 mm culture dishes.

Components

The kit is sufficient for 100 assays in 96-well plates.

- Triglyceride Assay Buffer 25 mL
  Catalog Number MAK258A
- Triglyceride Probe (in DMSO) 200 µL
  Catalog Number MAK258B
- Lipase 1 vial
  Catalog Number MAK258C
- Triglyceride Enzyme Mix 1 vial
  Catalog Number MAK258D
- Triglyceride Standard (1 mM) 300 µL
  Catalog Number MAK258E
- Chloroquine 120 µL
  Catalog Number MAK258F
- PBS 48 mL
  Catalog Number MAK258G
- Formalin (10%) 24 mL
  Catalog Number MAK258H
- Oil Red O 60 mg
  Catalog Number MAK258I
- Methyl Green 24 mL
  Catalog Number MAK258J

Reagents and Equipment Required but Not Provided

- 96-well flat-bottom plate. Use black plates for fluorescence assays and use clear plates for colorimetric assays. Cell culture or tissue culture treated plates are **not** recommended.
- Fluorescence or spectrophotometric multiwell plate reader
- Syringe and 0.2 µm syringe filter
- Light microscope
- Reagent grade isopropanol (Catalog Number 190764 or equivalent)
- Nonidet™ P 40 (NP-40) Substitute (Catalog Number 492016 or equivalent)
- Water bath capable of >80 °C

- Microcentrifuge capable of RCF ≥10,000 × g
- Microcentrifuge tubes
- Vortex
- Dounce tissue grinder set
- (Catalog Number D9063 or equivalent)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Upon receiving the kit, store Assay Buffer, Probe, Lipase, Enzyme Mix, Standard, and Chloroquine at –20 °C. Store components for lipid staining (PBS, Formalin, Oil Red O, and Methyl Green) at room temperature.

Upon opening, use within two months.

Preparation Instructions

Briefly centrifuge small vials prior to opening. Use ultrapure water for the preparation of reagents.

Triglyceride Assay Buffer, PBS, Formalin, and Methyl Green: Ready to use as supplied. Warm to room temperature before use.

Triglyceride Probe: Ready to use as supplied. Protect from light. Warm to room temperature before use. Store at –20 °C.

Lipase: Dissolve in 220 µL of Triglyceride Assay Buffer. Aliquot and store at –20 °C. Use within two months.

Triglyceride Enzyme Mix: Dissolve in 220 µL of Triglyceride Assay Buffer. Aliquot and store at –20 °C. Use within two months.

Triglyceride Standard: The Triglyceride Standard may separate during storage. To redissolve, keep the cap tightly closed, place in a hot water bath (>80 °C) for 1 minute or until the standard looks cloudy and vortex for 30 seconds. The Standard should become clear. Repeat heat and vortex one more time. The Triglyceride Standard is now in solution and ready to use.

Chloroquine: Warm to room temperature before use. Store at –20 °C.

Oil Red O: Dissolve Oil Red O in 20 mL of 100% isopropanol (not included) to make a stock solution, mix well, and let it sit for 20 minutes. The stock solution is stable for one year.

Oil Red O Working Solution: Add 3 volumes of Oil Red O Stock Solution to 2 volumes of water, mix well, and allow it to sit for 10 minutes. Filter with 0.2 µm syringe filter. Prepare 15 minutes before use. Oil Red O Working Solution is stable for 2 hours.

Procedure

All samples and standards should be run in duplicate. Read the entire protocol before performing the assay.

Sample Preparation

1. Grow liver cells, e.g., HepG2, until confluent.
2. Add 1 mL of 5% NP-40 solution per ~2 × 10^6 cells and collect cells using a cell scraper.
3. Homogenize cells and then heat the samples to 80–100 °C for 2 minutes until the NP-40 becomes cloudy.
4. Vortex and cool to room temperature.
5. Repeat the heat/vortex/cool step one more time to solubilize all triglycerides.
6. Centrifuge for 2 minutes at 10,000 × *g* to remove insoluble material. Collect supernatant and retain for assay.

Positive Control

1. Add 5 µL of Chloroquine per mL of medium (25 µM final concentration) and filter through a 0.2 µm syringe filter under sterile conditions.
2. Treat HepG2 cells with medium containing 25 µM Chloroquine for 3 days.
3. Homogenize cells as previously described under Sample Preparation.

Add 2–50 µL of test Samples and Positive Control into separate wells of a 96-well plate. Adjust the total volume of each well to 50 µL with Triglyceride Assay Buffer.

Notes:

(a) It is suggested to use different volumes of Sample to ensure readings are within the Standard Curve range.
(b) Prepare parallel Sample well(s) for use as Sample Background Control(s) and adjust the total volume of each well to 50 µL with Triglyceride Assay Buffer.
(c) Endogenous compounds in the sample may interfere with the assay. For accurate determination of triglycerides in the sample, we recommend spiking parallel samples (Spiked Samples) with a known amount of Triglyceride Standard (e.g., 4 nmol Triglyceride for colorimetric assays and 0.4 nmol Triglyceride for fluorimetric assays).

Colorimetric Standard Curve Preparation

1. Prepare 0.2 mM Triglyceride Standard by adding 40 µL of 1 mM Triglyceride Standard to 160 µL of Triglyceride Assay Buffer. Mix well.
2. Prepare Triglyceride Standards in separate wells of the 96-well plate according to Table 1.

Table 1.
Preparation of Triglyceride Standards for Colorimetric Assay

Well	0.2 mM Triglyceride Standard	Assay Buffer	Triglyceride (nmol)
1	-	50 µL	0
2	10 µL	40 µL	2
3	20 µL	30 µL	4
4	30 µL	20 µL	6
5	40 µL	10 µL	8
6	50 µL	-	10

Fluorometric Standard Curve Preparation

1. Prepare 0.2 mM Triglyceride Standard by adding 40 µL of 1 mM Triglyceride Standard to 160 µL of Triglyceride Assay Buffer. Mix well.
2. Dilute the 0.2 mM Triglyceride Standard further to 0.02 mM by adding 20 µL of 0.2 mM Triglyceride Standard to 180 µL of Triglyceride Assay Buffer. Mix well.
3. Prepare Triglyceride Standards in separate wells of the 96-well plate according to Table 2.

Table 2.
Preparation of Triglyceride Standards for Fluorometric Assay

Well	0.02 mM Triglyceride Standard	Assay Buffer	Triglyceride (nmol)
1	-	50 µL	0
2	10 µL	40 µL	0.2
3	20 µL	30 µL	0.4
4	30 µL	20 µL	0.6
5	40 µL	10 µL	0.8
6	50 µL	-	1.0

Note: Detection sensitivity is 10 to 100-fold higher for the fluorometric assay than the colorimetric assay.

Lipase

Add 2 µL of Lipase to each Standard, Sample, and Positive Control well. Add 2 µL of Triglyceride Assay Buffer to each Sample Background Control well. Mix and incubate for 20 minutes at room temperature to hydrolyze triglyceride.

Triglyceride Reaction Mix

1. Mix enough Reaction Mix for the number of assays to be performed. Prepare 50 µl of Reaction Mix per well according to Table 3 (Colorimetric assay) or Table 4 (Fluorometric assay).

Table 3.
Preparation of Reaction Mix for Colorimetric Assay

Reagent	Volume
Triglyceride Assay Buffer	46 µL
Triglyceride Probe	2 µL
Triglyceride Enzyme Mix	2 µL

Table 4.
Preparation of Reaction Mix for Fluorometric Assay

Reagent	Volume
Triglyceride Assay Buffer	47.6 µL
Triglyceride Probe	0.4 µL
Triglyceride Enzyme Mix	2 µL

2. Add 50 µL of Reaction Mix to each well containing Standard, Sample, Positive Control, or Sample Background Control. Mix well.
3. Protect from light and incubate at room temperature for 30–60 minutes.

Measurement

Measure absorbance at 570 nm (A_{570}) for colorimetric assay or fluorescence at λ_{Ex} = 535 nm/λ_{Em} = 590 nm (RFU) for fluorometric assay in a multiwell plate reader. The reaction is stable for at least two hours.

Oil Red O Staining Protocol

Note:

- For 96-well plate, use 100 µL of each component per well
- For 24-well plate, use 500 µL of each component per well
- For 6-well plate, use 2 mL of each component per well
- For 100 mm culture dish, use 6 mL of each component.
- Prepare sufficient amount of 60% isopropanol solution.

Cell Fixing

1. Remove medium from cells and gently wash twice with PBS.
2. Add Formalin (10%) to each well and incubate for 30 minutes to 1 hour.

   Note: Do not pipette PBS or Formalin (10%) directly onto cells. Pipette to the side of well or plate and mix by rotating.

Cell Staining

1. Prepare Oil Red O Working Solution (see Preparation Instructions).
2. Remove Formalin and gently wash cells twice with water.
3. Make a 60% isopropanol solution and add to each well. Incubate for 5 minutes.
4. Remove isopropanol solution and add Oil Red O Working Solution to evenly cover the cells.
5. Incubate while rotating gently on an orbital shaker for 20 minutes.
6. Remove Oil Red O Working Solution and wash 2–5 times with water until excess stain is no longer apparent.
7. Add Methyl Green to stain for nuclei and incubate for 1–5 minutes.
8. Remove Methyl Green and wash with water 2–5 times. Keep cells covered with water at all times and immediately view under microscope. Lipid droplets appear red and nuclei appear blue.

Note: Discard used Oil Red O Working Solution. Methyl Green signal washes out over time; examine the cells immediately after staining for best results

Results

Triglyceride Concentration

1. Subtract 0 Standard reading (A_{570} or RFU) from all readings.
2. Plot the corrected Triglyceride Standard reading (A_{570} or RFU) against the concentration and calculate the standard curve.
3. If Sample Background Control reading is significant, subtract the Sample Background Control reading from the Sample reading.
4. For unspiked Samples, apply the corrected reading (A_{570} or RFU) to the Triglyceride Standard Curve to get Triglyceride (B, in nmole) in the sample well.

   Triglyceride concentration (nmol/µL or mM) =

$$\frac{B}{V} \times DF$$

where

B = Amount of Triglyceride in Sample well from standard curve (nmol)

V = Sample volume added into the reaction well (µL)

DF = Sample dilution factor (DF = 1 for undiluted samples)

5. For spiked samples, correct for any sample interference by subtracting the Sample reading (A_{570} or RFU) from the corresponding Spiked Sample reading (A_{570} or RFU). For spiked samples, calculate the amount of triglyceride in the Sample well:

Triglyceride (TG) amount in Sample well (B) =

$$\frac{A_{570_{Sample\ (Corrected)}}}{[A_{570_{Spiked\ Sample\ (Corrected)}} - A_{570_{Sample\ (Corrected)}}]} \times TG\ Spike\ (nmol)$$

or

$$\frac{RFU_{Sample\ (Corrected)}}{[RFU_{Spiked\ Sample\ (Corrected)} - RFU_{Sample\ (Corrected)}]} \times TG\ Spike\ (nmol)$$

where

TG = Triglyceride Standard spike added to Spiked Sample (recommended 4 nmol for colorimetric assay method and 0.4 nm for fluorescence assay method)

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold Triglyceride Assay Buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96-well plate used	For fluorescence assays, use black plates. For colorimetric assays, use clear plates.
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of improperly stored reagents	Check the storage conditions and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a pooled Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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