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Hepatic Steatosis Assay Kit

(Catalog # K584-100; 100 assays; Store at 4°C)

I. Introduction:

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. BioVision's 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 staining reagents enough for two 96-well plates, two 6-well plates, or four 100 mm culture dishes.

II. Application:

- Evaluate hepatic steatosis, quantify triglyceride, and visualize lipid droplet accumulation
- Screen drug candidates for steatotic risk

III. Sample Type:

Liver cells such as HepG2

IV. Kit Contents:

Components	K584-100	Cap Code	Part Number
Triglyceride Assay Buffer	25 ml	WM	K584-100-1
Triglyceride Probe (in DMSO)	200 µl	Red	K584-100-2
Lipase	Lyophilized	Blue	K584-100-3
Triglyceride Enzyme Mix	Lyophilized	Green	K584-100-4
Triglyceride Standard (1 mM)	300 µl	Yellow	K584-100-5
Chloroquine	120 µl	Purple	K584-100-6
PBS	48 ml	NM	K584-100-7
Formalin (10%)	24 ml	Green/NM	K584-100-8
Oil Red O	60 mg	NM	K584-100-9
Methyl Green	24 ml	Blue/NM	K584-100-10

V. User Supplied Reagents and Equipment:

- HepG2 cells (or other liver cells), can be purchased from ATCC
- 96-well white plate (fluorometric) or clear plate (colorimetric)
- Multi-well spectrometer (absorbance/fluorescence ELISA reader)
- Syringe and 0.2 µm syringe filter
- Light microscope
- Reagent grade isopropanol

VI. Storage Conditions and Reagent Preparation:

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. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay. Upon opening, use within two months.

- **Triglyceride Assay Buffer, PBS, Formalin, and Methyl Green**: 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 Triglyceride Assay Buffer. Aliquot and store at -20°C. Use within two months.
- **Triglyceride Enzyme Mix**: Dissolve in 220 µl 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 min. or until the standard looks cloudy and vortex for 30 sec. 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 to make a stock solution, mix well, and let it sit for 20 min. The stock solution is stable for one year. To make a working solution, add 3 parts of Oil Red O Stock Solution to 2 parts of dH_2O, mix well, and allow it to sit for 10 min. Filter with 0.2 µm syringe filter. Prepare 15 min. before use. Working solution is stable for 2 hr.

VII. Triglyceride Assay Protocol:

1. **Sample Preparation:** Grow liver cells, e.g. HepG2, until confluent. Add 1 ml 5% NP-40 per ~2 x 10^6 cells and collect cells using a cell scraper. Homogenize cells and then heat the samples to 80-100°C for 2 min. until the NP-40 becomes cloudy. Vortex and cool to room temperature. Repeat the heat/vortex/cool step one more time to solubilize all triglycerides. Centrifuge for 2 min. at 10,000 X g to remove insoluble material. Collect supernatant. For positive control, add 5 µl of Chloroquine per ml of media (= 25 µM) and filter through a 0.2 µm syringe filter under sterile conditions. Treat HepG2 cells with media containing 25 µM Chloroquine for 3 days. Homogenize cells as described above. Add 2-50 µl test samples and positive control into a 96-well plate. Adjust the volume to 50 µl/well with Triglyceride Assay Buffer.

   Notes:

   a. We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

   b. Prepare parallel sample well(s) as sample background control(s) and adjust the volume to 50 µl/well with Triglyceride Assay Buffer.

   c. Endogenous compounds in the sample may interfere with the assay, for accurate determination of Triglyceride in your sample; we suggest spiking parallel samples with a known amount of Triglyceride Standard (4 nmol for color and 0.4 nmol for fluorescence).

2. **Standard Curve Preparation:** For colorimetric assay, prepare 0.2 mM Triglyceride Standard by adding 40 µl of 1 mM Triglyceride

Standard to 160 µl Triglyceride Assay Buffer. Mix well; add 0, 10, 20, 30, 40, and 50 µl of 0.2 mM Triglyceride Standard into a series of wells in 96-well plate. Adjust the volume to 50 µl/well with Triglyceride Assay Buffer to generate 0, 2, 4, 6, 8, and 10 nmol/well of Triglyceride Standard. For fluorometric assay, dilute the Triglyceride Standard further to 0.02 mM by adding 10 µl of 0.2 mM Triglyceride Standard to 90 µl Triglyceride Assay Buffer. Mix well and use 0.02 mM Triglyceride Standard to generate the fluorometric Standard Curve by following the procedure mentioned for the colorimetric assay.

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

3. **Lipase:** Add 2 µl of Lipase to each Standard, sample and positive control well and 2 µl Assay buffer to sample background control well(s). Mix and incubate for 20 min. at room temperature to hydrolyze triglyceride.
4. **Triglyceride Reaction Mix**: Mix enough reagent for the number of assays to be performed. Prepare 50 µl of Reaction Mix per well:

	Reaction Mix
Triglyceride Assay Buffer	46 µl
Triglyceride Probe*	2 µl
Triglyceride Enzyme Mix	2 µl

Add 50 µl of Reaction Mix to each well containing Standard, sample, positive control, or background control. Mix well. Protect from light and incubate at room temperature for 30-60 min.

*For the fluorometric assay, use 0.4 µl/well of the Probe to decrease background reading and adjust the volume of Triglyceride Assay Buffer accordingly.

5. **Measurement:** Measure absorbance at 570 nm for colorimetric assay or fluorescence at Ex/Em = 535/590 nm for fluorometric assay in a microtiter plate reader. The reaction is stable for at least two hrs.
6. **Calculation:** Subtract 0 Standard reading from all readings. If sample background control reading is significant then subtract the sample background control reading from sample reading. Plot the Triglyceride (TG) Standard Curve. For unspiked samples, apply the corrected OD to the Triglyceride Standard Curve to get B nmol of Triglyceride in the sample well.

Sample TG concentration (C) = B/V X D nmol/µl or mM

Where: **B** = amount of TG in sample well from Standard Curve (nmol)
V = sample volume added into the reaction well (µl)
D = sample dilution factor

Note: For spiked samples, correct for any sample interference by subtracting the sample reading from spiked sample reading.

For spiked samples, TG amount in sample well (B) = $\left(\frac{\mathrm{OD}_{\mathrm{sample(corrected)}}}{(\mathrm{OD}_{\mathrm{sample}}+\mathrm{TG\ Std}_{\mathrm{(corrected)}})-(\mathrm{OD}_{\mathrm{sample\ (corrected)}})}\right) * \mathrm{TG\ Spike\ (nmol)}$

VIII. Oil Red O Staining Protocol

Note: All components: For 96-well plate, use 100 µl per well; for 24-well plate, use 500 µl per well; for 6-well plate, use 2 ml per well; for 100 mm culture dish, use 6 ml. Prepare sufficient amount of 60% isopropanol.

1. **Cell Fixing:** Remove media from cells and gently wash 2X with PBS. Add Formalin (10%) to each well and incubate for 30 min. to 1 hr. **Note:** Do not pipet directly onto cells, pipet to the side of well or plate and mix by rotating.
2. **Cell Staining:** Prepare Oil Red O Working Solution. Remove formalin and gently wash cells 2X with dH_2O. Make 60% Isopropanol and add to each well. Incubate for 5 min. Remove isopropanol and add Oil Red O Working Solution to evenly cover the cells. Incubate while rotating gently on an orbital shaker for 20 min. Remove Oil Red O solution and wash 2-5X with dH_2O until excess stain is no longer apparent. Add Methyl Green to stain for nuclei and incubate for 1-5 min. Remove Methyl Green and wash with dH_2O 2-5X. Keep cells covered with dH_2O at all times and immediately view under microscope. Lipid droplets appear red and nuclei appear blue. **Note:** Discard used Oil Red O Solution. Methyl Green signal washes out over time, examine the cells immediately after staining for best results.

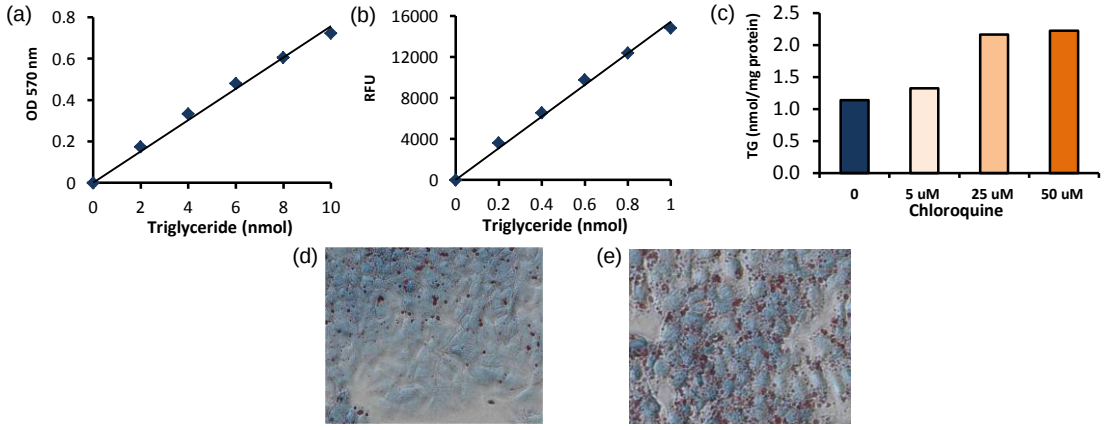

Figure: Triglyceride Standard Curve by colorimetric (a) and fluorometric (b) assay; (c) Measurement of Triglyceride in untreated HepG2 cells and cells treated with 5, 25, or 50 µM Chloroquine. (d) Untreated HepG2 cells stained with Oil Red O and methyl green, a nuclear stain. (e) HepG2 cells treated with 25 µM Chloroquine for 3 days and stained with Oil Red O and methyl green.

IX. Related Products:

Lipid (Oil Red O) Staining Kit (K580)
3T3-L1 Differentiation Kit (K579)
Triglyceride Quantification Colorimetric/Fluorometric Kit (K622)
Free Fatty Acid Quantification Colorimetric/Fluorometric Kit (K612)

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