CONFLUOLIP™ Continuous Fluorometric Lipase Test

Fluorometric tests for the analysis of triglyceride hydrolysis capacity in postheparin plasma (PHP) and tissue culture fluids/ cell extracts

Cat. No.: PR2003 **Total Lipase Test**
Cat. No.: PR2004 **Hepatic Lipase Test**
Size of the Kit: **24 Determinations**
Storage: **2 - 8° C**
In Vitro Test / For Research Use Only

Introduction

Two lipases are involved in the catabolism of triglyceride-rich lipoproteins: lipoprotein lipase (LPL; EC 3.1.1.34) located on glucosaminoglycan chains, anchored to the luminal surface of the capillary epithelium in adipose tissue, heart and skeletal muscle; hepatic lipase (HL; EC3.1.1.3) which is almost exclusive to the endothelial cells of the liver. Both lipases are triglyceride lipases [1].

LPL is a multifunctional protein with a central role in homeostasis and a rate-limiting enzyme for the metabolism of triglyceride-rich chylomicrons and VLDL. Triglycerides of chylomicrons and very low density lipoprotein (VLDL) are the preferred substrates of LPL. An inherited deficiency of LPL causes defective chylomicron metabolism [1]. Patients with autosomal recessive LPL defects show the symptoms of chylomicronemia and type I hyperlipidemia [2]. Heterozygote LPL deficiency has been identified as a defect of some cases of familial combined hyperlipidemia, one of the most common causes of genetic hyperlipidemia [3]. Many metabolic disorders such as diabetes, obesity, insulin resistence, hyperinsulinemia, hypothyroidism, gestational hyperlipidemia, and renal disease as well as alcohol ingestion are associated with elevated plasma triglycerides. The lipid abnormalities in these diseases can be explained as modulated or changed by LPL activity [4,5].

HL hydrolyses the triglycerides of intermediate density lipoprotein (IDL) and high density lipoprotein-2 (HDL_2) [6-9]. It was further shown that the hepatic removal of chylomicron remnants is primarily mediated by mechanisms involving HL (and apolipoprotein E). After the chylomicron remnant particles have bound to the hepatocyte surface, endocytosis ispredominantly mediated by the hepatic LDL receptor and at a slower rate by the LDL receptor-related protein (LRP) which is regulated by the receptor-associated protein (RAP) [10]. LPL and HL have been shown to bind directly to LRP [11]. HL and LPL should, therefore, be an important determinant of lipoprotein receptor pathways [12]. Retention of lipoproteins by the extracellular matrix may be another process modulated by the lipases [13].

As the affinity of lipases for heparin is higher than the heparin-sulfate-like anchor, injection of an intravenous heparin bolus displaces both enzymes into postheparin plasma (PHP), where their activity can be quantified.

The Total Lipase Test [Cat. No.: PR2003] can also be used to determine the activity of bacterial lipases (data available for Pseudomonas lipase).

Principle of the Test

PROGEN´s lipase substrate is 1-trinitrophenyl-amino-dodecanoyl-2-pyrendecanoyl-3-0-hexadecyl-sn-glycerol (12-TA-10-P-H6), a triglyceride in which the pyrene fluorescence is intramolecularly quenched by the trinitrophenyl group (Hermetter and colleagues [14-15]). Upon addition of active lipase the quencher is hydrolysed and the pyrene fluorescence can be detected. The kinetic increase in fluorescence intensity at 37°C is proportional to lipase activity. Fluorescence intensity is measured at 342 nm excitation and 400 nm emission wavelength. Distinct assay conditions (pH, NaCl and Triton X100 concentration) allow the selective determination of **Hepatic Lipase [PR2004]**. The substrate is not hydrolized by esterases and phospholipase A2.

The standard provided is the unquenched fluorescent derivate of the substrate, exhibiting endpoint fluorescence. The standard is required for calibration of the fluorometer (see step 1) and for calculation of the molar fluorescence of the pyrene group (see step 2.)

Materials Required, But not Included:

- Fluorometer (342 excitation [Ex] nm and 400 nm emission [Em] wavelength) with a thermostated cuvette holder; the appropriate excitation and emission slit width (e.g. 10 nm / 10 nm) has to be set up for the instrument used.
- Vials for sample and sample dilution (1 ml, 5 ml)
- Precision pipettes (10 µl, 1000 µl)
- Sterile pipette tips
- UV-permeable acrylic or quartz cuvettes (2 ml)

Contents of the Test Kit

3 vials of lipase Substrate (lyoph.), for 8 determinations each. Immediately before use reconstitute one vial with 16 ml Buffer.

PR2003: Substrate **A;** PR2004: Substrate **B**

4 bottles of Buffer, 30 ml each;

PR2003: Buffer **A** (pH 8.2; physiological salt concentration)

PR2004: Buffer **B** (pH 8.8; high salt concentration)

3 vials of Standard (lyoph.). Immediately before use reconstitute one vial with 4 ml Buffer to obtain a final concentration of 20 pmol/ml.

Sample Material

Postheparin plasma (PHP), heparin cell culture supernatant or cell extract, and purified lipase may be used. Appropriate dilutions should be prepared in the Buffer provided, e.g. predilute postheparin plasma **1:10**.

The Test Requires Four Working Steps:

Step 1: Adjustment of Sensitivity and Assay Range: Calibration of the Fluorometer

The test requires a linear assay range. Serial dilutions of the provided standard are prepared. The linear range is obtained by adjustment of a suitable slit width and sensitivity at the fluorometer.

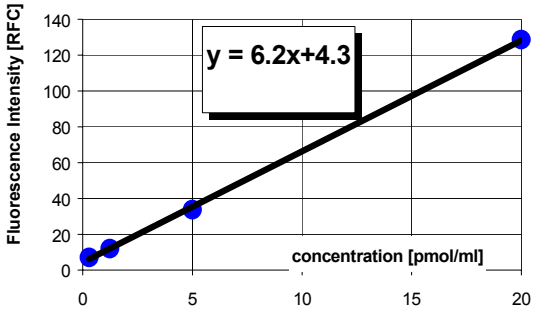
1.1 Reconstitute 1 vial of Standard with 4 ml Buffer, mix well and make standard dilution as follows:

S1:	**reconstituted standard**	**20 pmol/ml**
S2:	**1 ml S1 + 3 ml buffer**	**5 pmol/ml**
S3:	**1 ml S2 + 3 ml buffer**	**1.25 pmol/ml**
S4:	**1 ml S3 + 3 ml buffer**	**0.30 pmol/ml**

1.2 Transfer 2 ml of each standard into a cuvette and measure fluorescence at Ex 342 nm and Em 400 nm at room temperature.

1.3 Adjust the sensitivity of the instrument first with S1 (which gives the highest fluorescence of all standards). Make sure that there is no signal overflow of the photomultiplier! The other standards of the serial dilution will then show a lower fluorescence than S1. The concentration of the unquenched standards S1 - S4 and their corresponding pyrene fluorescence must result in a linear correlation over the whole range (0.3 to 20 pmol/ml).

Fig. 1: Example of a Calibration Curve with Standards



Step 2: Quantification of the Pyrene Fluorescence

The „molar fluorescence“ of the pyrene group is needed for the calculation of the concentration of the unquenched pyrene group which accumulates over time in the kinetical lipase assay (see below).

cence of the unquenched product is the slope of the straight line obtained with standards S1-S4 and their fluorescence. It can be calculated using the ratio of the differences of the standard concentrations and their corresponding fluorescence values from the calibration straight line.

Example of Calculation of Molar Fluorescence from Values in Fig. 1:

$$\frac{y_2 - y_1}{x_2 - x_1} = \frac{128.6 - 5.6}{20 - 0.3} = 6.2 \text{ RFU x pmol}^{-1} \text{ x ml}$$

Step 3: Kinetical Lipase Assay

The lipase activity in the assay corresponds to the appearance of the pyrene fluorescence of the unquenched product over time. If the calibration range is not altered, the increase of fluorescence in the lipase assay over time remains linear and no signal overflow occurs.

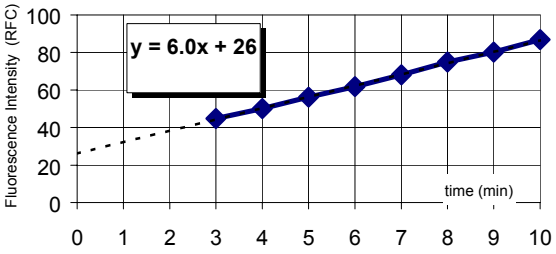
3.1 Transfer 2 ml freshly reconstituted Substrate into a cuvette and warm up to 37°C.

3.2 Add 20 µl sample and mix well.

3.3 Start kinetic measuring after 2-3 min. Measure the kinetics for 6-10 min. Very slow kinetics have to be measured over a longer period of time.

Use fluorometer set-up as for calibration (Ex 342 nm, Em 400 nm, slit width, amplification).

Fig. 2: Example



Step 4: Calculation of Lipase Activity

The „molar fluorescence“ of the pyrene group calculated from the straight line of the standards above (Step 2) is used now and the pyrene fluorescence over time released from the substrate by the action of lipase (Step 3).

First, the unquenched pyrene fluorescence resulting from cleavage of the substrate by the lipase in the assay over time represents the slope of the straight line (x-axis: time; y-axis: pyrene fluorescence). It is calculated from the ratio of the differences between time points and the corresponding fluorescence values, respectively, of the values obtained under Step 3.

Example of Calculation of Fluorescence/Time with Values of Fig. 2:

$$\frac{y_2-y_1}{x_2-x_1} = \frac{86.8 - 44.8 \text{ RFU}}{10 - 3 \text{ min}} = 6.0 \text{ RFU x min}^{-1}$$

Second, the activity of the lipase in the assay is now calculated as the ratio delta fluorescence/time in the assay and the molar fluorescence, calculated from the straight line of the standards.

Example:

$$\frac{6.0 \text{ RFU x min}^{-1}}{6.2 \text{ RFU x pmol}^{-1} \text{ x ml}} = 0.97 \text{ pmol x ml}^{-1} \text{ x min}^{-1}$$

This calculation does not yet correct for a possible dilution factor of the probe. If you work with postheparin plasma or cell culture supernatants, it may be useful to calculate the lipase activity of a test sample per ml PHP and cell culture supernatant, respectively.

Limitations:

Optimal conditions for HL activity are found at high ionic strength **and pH 8.8**, independent from activators or stimulators. LPL activity is almost completely inhibited under these conditions.

At pH 8.2 and physiological salt concentration conditions are optimal for total lipase activity measurements. However, under these conditions both lipase activities (LPL and HL) are not additive.

As a consequence, most PHP show a lower total lipase activity than the measured HL activity. **The LPL activity may not be calculated from the results of Hepatic Lipase minus Total Lipase activity**.

Additionally, the test result may be influenced by competitive inhibition of plasma lipids present in the sample. For interpretation of the results it could become necessary to analyse the lipid status of postheparin plasma and, particularly, the triglyceride values.

References

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The following monoclonal antibodies for lipid research are also available from PROGEN:

Cat. No.	Product
61080:	Human LDL, Mab
61081:	Bovine Lipoprotein Lipase, Mab
61082:	Human HDL, Mab
61085/61086:	Human Apolipoprotein E, Mab
61088:	Human Apolipoprotein E2, Mab
61087:	Human LDL Receptor, Mab
61065:	Human LRP, 515 kDa, Mab
61066/61067:	Human LRP, 85 kDa, Mab
61068:	Human RAP, 39 kDa, Mab
61098:	Pleo-Lipoprotein Receptor, rabbit
61049/61549:	Human LDL Receptor, chicken
61099/61599:	Human LDL Receptor, rabbit
62221:	RAP (Lipoprotein Receptor-associated Protein), rat recombinant

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