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Method Article

Simple and rapid real-time monitoring of LPL activity *in vitro*

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A B S T R A C T

Since elevated plasma triglycerides are an independent risk factor for cardiovascular diseases, lipoprotein lipase (LPL) is an interesting target for drug development. However, investigation of LPL remains challenging, as most of the commercially available assays are limited to the determination of LPL activity. Thus, we focused on the evaluation of a simple *in vitro* real-time fluorescence assay for the measurement of LPL activity that can be combined with additional cell or molecular biological assays in the same cell sample. Our procedure allows for a more comprehensive characterization of potential regulatory compounds targeting the LPL system.

The presented assay procedure provides several advantages over currently available commercial *in vitro* LPL activity assays:

1. 12-well cell culture plate design for the simultaneous investigation of up to three different compounds of interest (including all assay controls).
2. 24 h real-time acquisition of LPL activity for the identification of the optimal time point for further measurements.
3. Measurement of LPL activity can be supplemented by additional cell or molecular biological assays in the same cell sample.

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A R T I C L E I N F O

Method name: Cell culture based real-time fluorescence assay for the measurement of LPL activity

Keywords: Lipoprotein lipase (LPL), LPL activity assay, Fluorescence, Real-time assay

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Abbreviations: ANGPTL, angiopoietin-like; FBS, fetal bovine serum; FFA, free fatty acid; FI, fluorescence intensity; LPL, lipoprotein lipase; MTT, methylthiazolyl-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PPAR, proliferator-activated receptor; PSG, L-glutamine-penicillin-streptomycin; RFU, relative fluorescence units; VLDL, very low-density lipoprotein.

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Specification Table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Investigation of lipoprotein lipase activity
Method name:	Cell culture based real-time fluorescence assay for the measurement of LPL activity
Name and reference of original method:	Name: Abcam Lipoprotein Lipase Assay Kit (Fluorometric) (ab204721) Source: [1]

Method details

Background

Lipoprotein lipase (LPL) mediates the release of free fatty acids (FFAs) from triglyceride-rich lipoproteins, like chylomicrons and very low-density lipoproteins (VLDL). Therefore, LPL represents a key enzyme for the regulation of cellular lipid homeostasis by providing FFAs for cellular energy supply and intracellular energy storage as well as a control mechanism for plasma triglyceride levels [2]. The activity of LPL is primarily regulated by post-translational modifications. The family of angiopoietin-like (ANGPTL) proteins, whose expression is controlled by peroxisome proliferator-activated receptors (PPARs), appeared as potent physiological inhibitors of LPL activity [3–5]. LPL dysfunction or dysregulation can result in elevated plasma triglycerides [6,7]. There is growing evidence that elevated plasma triglycerides are an independent risk factor for cardiovascular diseases [8,9], making the LPL system an interesting target for drug development [10,11]. Hence, determination of LPL activity is a useful tool for the identification of potential lead compounds from natural or synthetic origins. Commercially available kits for the measurement of LPL activity are based on radiolabeled (^3H or ^{14}C), fluorogenic or chromogenic substrates [12–14]. These substrates are degraded by LPL and their reaction products can be detected at defined times [15]. Unfortunately, most commercial kits are optimized for post heparin plasma samples and are therefore not suitable for initial characterization of potential drug compounds in *in vitro* systems. Next, the few commercial LPL assays that are optimized for *in vitro* application require cell harvesting and homogenization. Thus, cells cannot be used for further cell or molecular biological investigations, which would allow a more comprehensive characterization of the respective test compound [1,16]. For example, the measurement of LPL activity combined with subsequent RT-qPCR or Western blot analyses can serve as a useful tool for the identification of transcriptional regulators of LPL activity.

For this reason, we decided to develop a simple cell culture based real-time fluorescence assay for the measurement of LPL activity that can be combined with cell and molecular biological analyses of the same cell sample. In our method, LPL activity is measured using a fluorescently labeled and quenched LPL substrate in combination with isolated VLDL for stimulation of LPL activity.

Required reagents and equipment

- (1) *VLDL isolation*
 - 50 ml fasted blood sample
 - EDTA monovettes
 - Centrifuge applicable for at least $1870 \times g$
 - Ultracentrifuge thick wall tubes
 - Ultracentrifuge
 - Ultracentrifuge rotor applicable for at least $200,000 \times g$
- (2) *Cell culture*
 - 12-well cell culture plates
 - Adherent cells
 - Fetal bovine serum (FBS)
 - L-glutamine-penicillin-streptomycin (PSG)
 - Cell type specific cell culture medium
 - Cell type specific phenol red free cell culture medium (for THP-1 macrophages: RPMI-1640, R7509, Sigma Aldrich)

- VLDL isolated from blood samples of healthy volunteers (alternatively: order commercially available VLDL)
 - Fluorescently labeled, quenched LPL substrate (ab214552, Abcam, Cambridge UK)
 - Orlistat (O4139, Sigma Aldrich; negative control)
- (3) *Fluorescence-based real-time measurement of LPL activity*
- Microplate reader coupled to an atmospheric control unit applicable for fluorescence measurements (Ex/Em = 485/520 nm)
 - Microplate reader temperature: 37 °C
 - CO₂ concentration: 5% (v/v)

Procedure

The description of the following experimental procedure will be illustrated by the human THP-1 macrophage cell model. However, this method may be suitable for various adherent cell lines that express active LPL.

(1) *Isolation of VLDL (alternatively: order commercially available VLDL)*

Note: VLDL is an essential part of the assay procedure. It is used as positive control, because stimulation of LPL activity by VLDL has been already established as positive control for plasma measurements of LPL activity [12].

- Use 50 ml blood obtained from fasted donors with plasma triglyceride concentrations of > 0.90 mmol/l

Note: Plasma triglyceride concentrations of > 0.90 mmol/l was previously described as a suitable range for VLDL-based LPL assays [12].

- Collect the blood in 9 ml EDTA-monovettes (02.1066.001, Sarstedt)
- Centrifuge blood samples at 1870 × g for 10 min at 15 °C for plasma separation
- Transfer plasma into 4 ml thick-wall polycarbonate tubes (355,645, Beckmann Coulter) for ultracentrifugation
- Ultracentrifugation is performed for 4 h at 15 °C and 269 200 × g (50,000 rpm, used rotor: Type 50.4 Ti, Beckman Coulter)
- Collect separated VLDL in 2 ml tubes

Note: The separated VLDL phase is very narrow and can easily be mixed with the plasma fraction below. Be careful not to shake the thick-wall tubes when taking them out of the ultracentrifuge. Use a 1 ml pipette for collecting the VLDL phase by placing the pipette tip on the tube wall and moving it carefully around the tube.

- Determine the protein concentration of the VLDL samples (e.g., Lowry or Bradford assay)
- Isolated VLDL can be stored under nitrogen atmosphere at 4 °C

Note: It is necessary to utilize the isolated VLDL within one week for the respective experimental procedures to ensure high VLDL quality and to avoid lipid oxidation.

(2) *Cell culture*

- The assay procedure described below is based on the use of 12-well cell culture plates (92,012, TPP Techno Plastic Products, Trasadingen, Switzerland)
- Use 1 × 10⁶ THP-1 monocytes per well for macrophage differentiation (add 100 ng/ml phorbol-12-myristate-13-acetate (P1585, Sigma Aldrich) and 50 μmol/l β-mercaptoethanol (4227.3, Carl Roth) together with RPMI-1640 (R8758, Sigma Aldrich) cell culture medium supplemented with 10% (v/v) FBS Superior (S0615, Sigma Aldrich) and 0.1% (v/v) PSG solution (G1146, Sigma Aldrich))

Note: The number of wells used for the assay depends on the experimental design. However, there are four fixed controls included in each assay run to ensure reliability of the procedure: (i) an untreated control, (ii) the orlistat negative control without VLDL, (iii) the VLDL positive control, and (iv) the orlistat negative control in combination with VLDL. Keep in mind to include these four controls in your experimental design. The current 12-well assay design including all assay controls allows the simultaneous analysis of up to three different test compounds.

- THP-1 monocytes are differentiated for 96 h in 2 ml cell culture medium per well
- Remove cell culture supernatant from the fully matured THP-1 macrophages
- Wash cells twice with phosphate-buffered saline (PBS)
- Pre-incubate cells according to the respective experimental procedure (here for 24 h) in 1 ml phenol red-free RPMI-1640 medium (R7509, Sigma Aldrich) under serum-free conditions
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: It is necessary to replace standard RPMI-1640 cell culture medium by phenol red-free RPMI-1640 for incubation to avoid fluorescence interferences. For the basic assay procedure without any test compounds, cells are only pre-incubated with 50 μ M orlistat in the respective wells for 24 h.

- Add VLDL in a concentration equivalent to 50 μ g/ml protein together with 0.5 μ l quenched, fluorescently labeled LPL substrate to the pre-incubated cells
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: The LPL substrate used for the experimental procedure is a standardized, commercially available product from Abcam (Cambridge, UK ab214552). It is a component of the commercial LPL assay kit offered by the company and therefore validated for reliable functionality. Nevertheless, we recommend to pooling LPL substrates of different batches to reduce variability between measurements. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured fluorescence intensity (FI) values are proportional to the amount of hydrolyzed substrate and thus LPL activity. The use of commercially available substrates for determination of LPL activity has also been described in other methodical approaches [12,13].

(3) Fluorescence based real-time measurement of LPL activity

Note: In our experimental setup, the FLUOstar Omega microplate reader coupled to an atmospheric control unit (BMG Labtech, Ortenberg, Germany) was used for FI determination. As an initial step, a suitable measurement procedure should be prepared for the respective experimental design of every assay procedure. In our approach, we determined FI values of the used wells hourly over 24 h at Ex/Em = 485/520 nm (recommended wavelength for the LPL substrate). As an additional preparation step, a temperature of 37 °C and a CO₂ concentration of 5% (v/v) should be set at least one hour before each measurement.

- Place the cell culture plate in the plate reader and start the prepared measurement procedure.
- The determined FI values can be used for the assessment of LPL activity for any test compound.
- Adherent cells can be used for subsequent investigations.

Note: The described assay conditions do not require harvesting of the cells. Thus, LPL activity measurement can be supplemented by additional cell or molecular biological analyses for a more comprehensive characterization of the test compounds. We have currently performed Nile red staining and cell viability assays after the determination of LPL activity. However, the combination with other molecular biological methods, such as Western blot, RT-qPCR or any other *in vitro* application is possible in principle.

Method validation

The aim of this study was to establish a simple fluorescence-based *in vitro* assay to allow the initial characterization of potential regulatory compounds targeting the LPL system. We decided to develop this procedure because commercially available LPL assays were not suitable for this demand.

Preliminary experiments revealed that the standard RPMI-1640 cell culture medium had to be replaced by phenol red-free RPMI-1640 for cell incubation to avoid fluorescence interferences. To enable determination of LPL activity, we used a quenched, fluorescently labeled LPL substrate (similar approach as described in [12,13]) in our incubation procedure. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured FI values are proportional to the amount of hydrolyzed substrate and thus LPL activity. Further, we decided to use VLDL as positive control, because stimulation of LPL activity by VLDL has already been shown in plasma measurements [12]. In addition, orlistat, a well-established and clinically used LPL inhibitor [17–19] was added as a negative control.

For the initial establishment of the assay procedure, human THP-1 macrophages were treated as described in the section “Supplemental Material/and or additional information”. After 24 h real-time measurement, we noticed that incubation with VLDL (in a concentration equivalent to 50 µg/ml protein) enhanced the measured FI values, indicating increased LPL activity, in a time dependent manner. Fluorescence intensity values of the untreated control and the VLDL-treated sample (positive control) evolved in a significantly different range ($p < 0.01$) (Fig. 1(A)). Maximum FI values have been determined after 24 h as an increase to 268.75 ± 24 relative fluorescence units (RFU) in the VLDL-incubated sample compared to 187 ± 14.5 RFU in the untreated control. As expected, orlistat (negative control) significantly blocked LPL activity compared to VLDL-treatment and the untreated control. Fluorescence intensity values of the VLDL-incubated sample and the combination of VLDL and orlistat evolved over time in a significantly different range ($p < 0.001$). After 24 h, FI value for the cells incubated with a combination of VLDL and orlistat was determined at 150 ± 1.3 RFU and as significantly lower ($p < 0.01$) compared to the VLDL-incubated cells (268.75 ± 24 RFU) (Fig. 1(A)). These results indicate that our methodical approach and the used assay controls are suitable for the *in vitro* measurement of LPL activity.

As a further validation of the established assay procedure, we decided to use GW0742 as a reference test compound. GW0742 is a well-studied PPAR- δ agonist [20,21], that enhances the expression of ANGPTL4 mRNA [22], a potent physiological and endogenously produced inhibitor of LPL. Consequently, stimulation of THP-1 macrophages with the PPAR- δ agonist GW0742 should enhance ANGPTL4 expression, in turn causing a reduction of cellular LPL activity [5]. To confirm the reported effects of GW0742 under our conditions, human THP-1 macrophages were treated with 100 nM GW0742 and harvested at different time points for RT-qPCR analysis. As expected, GW0742 enhanced ANGPTL4 mRNA expression already after 1 h by approximately 20-fold ($p < 0.001$) and after 24 h by approximately 200-fold ($p < 0.001$) (Fig. 1(B)). To examine the effects of GW0742 on cellular LPL activity, we expanded our initial working procedure by an additional 24 h pre-incubation with 100 nM GW0742. In line with our initial experiment, we were able to generate similar FI values for untreated control cells, VLDL-incubated cells as well as cells cultured with orlistat alone or in combination with VLDL. GW0742 treatment slightly, but not significantly increased basal LPL activity (206 ± 20 RFU vs. 230 ± 16 RFU) compared to the untreated control. However, combination of GW0742 and VLDL did significantly reduce the induction of LPL activity by VLDL ($p < 0.05$). The strongest reduction was achieved after 24 h, where GW0742 reduced FI values of VLDL incubated cells from 291.3 ± 29 RFU to the control level of 196.6 ± 20 RFU (Fig. 1(C)). In summary, the observed effect of GW0742 on cellular LPL activity is consistent to the literature.

For the combination of the LPL assay procedure outlined here with additional cell or molecular biological methods, we studied cell viability at the end of the LPL activity measurement. For this, methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed after completing the 24 h real-time measurement. None of the applied compounds did significantly reduce cell viability compared to the untreated control. Treatment with orlistat led to a slightly but not significantly reduced cell viability of 85% of the control, representing the lowest viability in our experimental setup (Fig. 1(D)). According to ISO 10993-5:2009, a reduction of cell viability by 15% is not regarded as a cytotoxic effect [23]. For final validation of the assay procedure, we combined our LPL assay

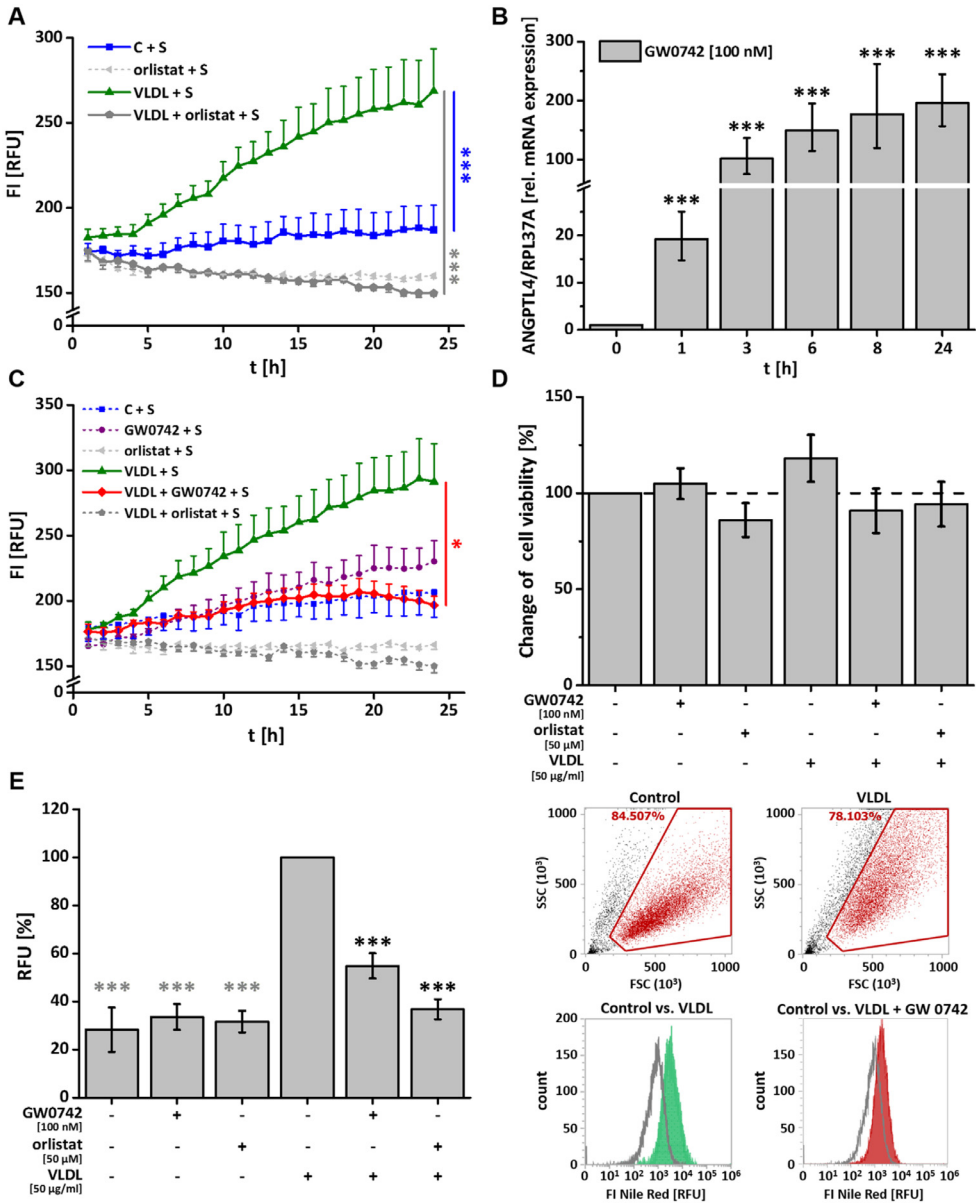


Fig. 1. Establishment and validation of an *in vitro* real-time fluorescence assay for the measurement of LPL activity. (A) Initial establishment of the LPL assay procedure. Human THP-1 macrophages were pre-incubated with 50 µM orlistat (negative control). After 24 h, VLDL (positive control, protein concentration of 50 µg/ml) and the fluorescently labelled LPL substrate were added to the corresponding wells. Fluorescence intensity (FI) of each well was determined hourly over 24 h at Ex/Em = 485/520 nm ($n = 4$; $*** p < 0.001$ vs. VLDL incubation). All further experiments were performed for assay validation, using GW0742 as reference test compound. (B) RT-qPCR of human THP-1 macrophages to investigate ANGPTL4 (LPL inhibitor) mRNA expression after GW0742 treatment ($n = 4$, $*** p < 0.001$, vs. untreated control). (C) LPL activity assay with GW0742 as test compound ($n = 3$, $* p < 0.05$ vs. VLDL incubation). (D) MTT assay for the assessment of cell viability after measurement of LPL activity ($n = 3$). (E) Measurement of the accumulation of neutral lipids accumulation by flow cytometry using Nile red staining after completion of the real-time LPL activity assay ($n = 3$, $*** p < 0.001$, vs. VLDL treatment).

with a subsequent measurement of neutral lipid accumulation by flow cytometry using Nile red staining. This experimental setting was chosen from reports on VLDL-induced cellular accumulation of neutral lipids via LPL [24,25]. Further, ANGPTL4 has been shown to reduce the uptake of triglyceride-derived fatty acids from VLDL by human THP-1 macrophages [26]. Consequently, VLDL treatment should enhance neutral lipid accumulation, while GW0742 treatment should prevent this. As expected, incubation of THP-1 macrophages with VLDL induced accumulation of neutral lipids by almost threefold compared to the untreated control. Co-incubation with GW0742 significantly reduced VLDL-induced accumulation of neutral lipids to 54% ($p < 0.001$). Co-incubation with orlistat resulted also in a significant reduction of VLDL-induced accumulation of neutral lipids to 36% ($p < 0.001$). As expected, the relative amount of neutral lipids in cells treated with GW0742 or orlistat but without VLDL did not differ from the control (Fig. 1(E)). The obtained results confirm that our LPL assay can be easily combined with other cell and molecular biological methods to produce more comprehensive information on the interaction of a compound of interest with the cellular LPL system.

Conclusion

We here provide a simple and rapid fluorescence-based *in vitro* assay for the assessment of the interactions of test compounds with the LPL system. The assay procedure provides several advantages over currently available *in vitro* LPL assays: (i) 12-well cell culture plate design for the simultaneous investigation of up to three different test compounds (including all assay controls); (ii) 24 h real-time acquisition of LPL activity data for the identification of the optimal time point for further measurements; and (iii) LPL activity measurement can be complemented by additional cell and molecular biological analyses using the same cell samples. Nevertheless, we are aware that the current assay design has limitations and needs further improvements:

- (i) In our experiments, VLDL was isolated from only a single normolipidemic male donor with plasma triglyceride concentrations of 0.90 mmol/l. However, the approach could be improved by using a mixture of VLDL obtained from multiple donors as described in [12]. Here, samples from ten normolipidemic donors (plasma triglycerides < 1.75 mmol/l) were pooled to create a more representative mean VLDL substrate for their assay procedure. Further, the use of a VLDL pool from different donors may also reduce the variation of the lipid and apolipoprotein composition between individuals.
- (ii) For further optimization of VLDL composition, the triglyceride content of the VLDL should be determined before. Di Filippo and coworkers used seven VLDL pools with varying triglyceride concentrations ranging from 0.45 to 3.45 mmol/l for the determination of LPL activity in post-heparin plasma [12]. The authors report that LPL activity reached a steady level in the range between 1.5 and 2.2 mmol/l, while lower concentrations (< 0.90 mmol/l) decreased LPL activity and high concentrations (> 2.7 mmol/l) slightly increased LPL activity in post-heparin plasma. To avoid distortion of the measured LPL activity by suboptimal triglyceride concentrations of the applied VLDL, the authors determined 1.8 mmol/l as the optimal triglyceride concentration for their assay. Hence, optimization of the triglyceride concentrations in the VLDL could improve our assay.
- (iii) The assessment of LPL activity in the current assay design is based on the comparison of FI values for the different compounds and controls at various times. This might be sufficient for a first impression of the effect of compounds on LPL activity and is therefore suitable for the intention of our assay procedure. Nevertheless, exact quantification of LPL activity based on the calculation of the released amount of substrate over time, as it has already been described for plasma measurements [1,12,13], should be an aim for future improvements. Unfortunately, no detailed information about their fluorescently labeled LPL substrate (concentration, composition, exact chemical name/structure etc.) is available from the supplier (Abcam, Cambridge UK). It was therefore not possible to adapt the procedure for the use of standard calibration curves as described in the assay manual to our conditions [1].
- (iv) For the here presented LPL activity assay, a mean coefficient of variation (CV) for inter-assay variability was 10.6% for low (untreated control) and 12% for high values (VLDL treatment).

Although both CVs are below 15%, which is in general acceptable for biological assays [27], inter-assay variability is an issue, in particular for high FI values. In general, the higher the measured FI values the higher the variation between each measurement. We were able to partially reduce this problem using a pool of different batches of the LPL substrate. Given the fact that the current assay procedure is only designed for initial compound screening and not as a diagnostic tool, the variation between each measurement seems acceptable. Nevertheless, reproducibility and the accuracy of the assay procedure should further be improved.

- (v) All experiments and optimizations for the current assay procedure were performed with human THP-1 macrophages. However, to fully understand the global impact of a given drug on lipolysis the use of further LPL-expressing cells, like adipocytes, may be necessary.

Despite of its limitations, our assay design can serve as a reliable tool for *in vitro* measurements of the effects of test compounds modulating the activity of the LPL system.

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Declaration of Competing Interest

The authors declare to have no competing interests.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.100865.

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