methods

# A novel direct homogeneous assay for ATP citrate lyase[S]

**Zhengping Ma, Ching-Hsuen Chu, and Dong Cheng[1]**

Department of Metabolic Research, Research and Development, Bristol-Myers Squibb Company, Princeton, NJ 08543-5400

**Abstract** **ATP citrate lyase (ACL) is a cytosolic enzyme that catalyzes the synthesis of acetyl-CoA and oxaloacetate using citrate, CoA, and ATP as substrates and $Mg^{2+}$ as a necessary cofactor. The ACL-dependent synthesis of acetyl-CoA is thought to be an essential step for the de novo synthesis of fatty acids and cholesterol. For this reason, inhibition of ACL has been pursued as a strategy to treat dyslipidemia and obesity. Traditionally, ACL enzyme activity is measured indirectly by coupling to enzymes such as malate dehydrogenase or chloramphenicol acetyl transferase. In this report, however, we describe a novel procedure to directly measure ACL enzyme activity. We first identified a convenient method to specifically detect [$^{14}$C]acetyl-CoA without detecting [$^{14}$C]citrate by MicroScint-O. Using this detection system, we devised a simple, direct, and homogeneous ACL assay in 384-well plate format that is suitable for high-throughput screening. The current assay consists of *1*) incubation of ACL enzyme with [$^{14}$C]citrate and other substrates/cofactors CoA, ATP, and $Mg^{2+}$, *2*) EDTA quench, *3*) addition of MicroScint-O, the agent that specifically detects product [$^{14}$C]acetyl-CoA, and *4*) detection of signal by TopCount. This unique ACL assay may provide more efficient identification of new ACL inhibitors and allow detailed mechanistic characterization of ACL/inhibitor interactions.**—Ma, Z., C-H. Chu, and D. Cheng. **A novel direct homogeneous assay for ATP citrate lyase. *J. Lipid Res.* 2009.** 50: **2131–2135.**

**Supplementary key words** fatty acid • acetyl-CoA • obesity • dyslipidemia

ATP citrate lyase (ACL) catalyzes the production of cytosolic acetyl-CoA and oxaloacetate from citrate. In mammals, the formation of acetyl-CoA is an essential step for the de novo synthesis of fatty acid (FA) and cholesterol for converting the carbohydrate carbon energy source into lipids. Hence, it has been thought that ACL inhibition would be beneficial for the treatment of obesity and dyslipidemia through the simultaneous inhibition of endogenous synthesis of FA and cholesterol.

Several citrate analogs have been reported as ACL inhibitors in the literature. Hydroxycitrate (HCA), a potent competitive inhibitor of ACL ($K_i$ = 150 nM) (1), has been shown to inhibit the synthesis of both FA and cholesterol and increase the LDL receptor activity (2). In vivo administration of HCA reduced plasma cholesterol and triglycerides in rats (3). Of note, HCA is also thought to be the active ingredient of CitriMax, a nutritional supplement used in controlling human body weight (4). In obese rodent models, HCA reduced food intake and body weight gain (5). Weight loss was attributed to selective lowering of body fat with no change in body protein composition. In addition, SB-201076, another citrate analog with a reported $K_i$ of 1.0 μM, has been tested in both rats and dogs. SB-204990, the cell-permeable γ-lactone prodrug of SB-201076, inhibited cholesterol and fatty acid synthesis in a dose-dependent manner in HepG2 cells (91% and 82%, respectively) and rats (76% and 39%, respectively). When administered for 1 week, SB-204990 decreased plasma cholesterol by 46% and plasma triglycerides by 80% in rats. SB-204990 (dosed at 25 mg/kg for 2 weeks) also decreased plasma cholesterol up to 23% and plasma triglycerides up to 38% in dogs (6).

Recently, Li et al. (7) described a 2-hydroxy-N-arylbenzenesulfonamides class of compounds as ACL inhibitors that structurally deviate from citrate and demonstrate higher cell permeability and in vivo bioavailability. Chronic administration of Compound 9 (or BMS-303141), the leading inhibitor in this class, in high-fat–fed mice reduced weight gain and lowered plasma cholesterol, triglycerides, and glucose. All these data are consistent with the hypothesis that ACL might be an attractive target for the treatment of metabolic disorders, including obesity and dyslipidemia.

Traditionally, ACL activity is measured using coupling enzymes such as malic dehydrogenase (MDH) (8) or chloramphenicol acetyl transferase (9). This coupling approach, however, is an indirect measure of ACL enzyme activity, and detailed enzymatic features or enzyme/inhibitor interactions may be missed. To avoid the shortcomings inherent in the coupled enzymatic assays, we describe

Abbreviations: ACL, ATP citrate lyase; HCA, hydroxycitrate; MDH, malic dehydrogenase.

[1] To whom correspondence should be addressed.
e-mail: dong.cheng@bms.com

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table and one figure.

*Manuscript received 5 February 2009 and in revised form 6 March 2009 and in re-revised form 9 March 2009.*

*Published, JLR Papers in Press, March 12, 2009*
*DOI 10.1194/jlr.D900008-JLR200*

in this report a novel procedure to directly measure ACL enzyme activity in 384-well plate format suitable for high-throughput screening.

## MATERIALS AND METHODS

### Materials

We obtained [$^{14}$C]citric acid from GE Healthcare and MicroScint-O and 384-well PolyPlate from Perkin Elmer. All other reagents are the highest purity from Sigma.

### Human ACL expression and purification

Recombinant human ACL was expressed and purified according to Lord et al. (10) with minimal modifications. Full-length human ACL coding region was cloned and expressed in Baculovirus Bac-N-Blue expression system (Invitrogen). Sf9 cells were infected by the recombinant baculovirus at multiplicity of infection of 5 for 55 h. The infected cells were resuspended and sonicated in Buffer A (10 mM HEPES, pH 7.2, 280 mM sucrose, 2 mM DTT, 0.24 mM EGTA, and 1 mM EDTA) supplemented with 1 mM PMSF, 1 mM leupeptin, and 1 mM pepstatin A to break the cells. The lysate was centrifuged at 45,000 rpm in a Ti60 rotor for 1 h. The supernatant was precipitated with 40% ammonium sulfate and the resultant pellet was resuspended and dialyzed against Buffer B (20 mM Tris, pH 8.0, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM EGTA). The dialysate was then purified by ion exchange DEAE chromatography followed by gel-filtration Sephacryl S300 chromatography in Buffer C (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 1 mM $MgCl_2$, 10% glycerol). The active peaks were pooled and stored in Buffer C at −80°C for future use. The recombinant ACL purified with this method is about 90% pure as assessed by SDS-PAGE (**supplementary Fig. I**).

### Direct ACL assay

The enzymatic reaction of purified human ACL was carried out in 20 μL of Buffer D (87 mM Tris, pH 8.0, 20 μM $MgCl_2$, 10 mM KCl, 10 mM DTT) containing substrates 100 μM CoA, 400 μM ATP, 150 μM [$^{14}$C]citrate (specific activity: 2 μCi/μmol) in a 384-well PolyPlate at 37°C for 3 h. The reaction was terminated by the addition of 1 μl 0.5 M EDTA stock to reach ~24 mM final concentration. An aliquot of 60 μl MicroScint-O was then added to the reaction mixture and incubated at room temperature overnight with gentle shaking. The [$^{14}$C]acetyl CoA signal was detected in a TopCount NXT liquid scintillation counter (Perkin-Elmer). The count time is 1 min/well and the unit of signal is expressed as counts per minute (CPM).

## RESULTS

### Selective detection of [$^{14}$C]acetyl CoA by MicroScint-O

Equation 1 illustrates the biochemical reactions catalyzed by ACL that are conducted in the current study.

$$\left[^{14}\mathrm{C}\right]-\mathrm{Citrate}+\mathrm{CoA}+\mathrm{ATP}\xrightarrow{\mathrm{ACL,\,Mg^{++}}}$$
$$\left[^{14}\mathrm{C}\right]-\mathrm{Acetyl}-\mathrm{CoA}+\mathrm{Oxaloacetate}+\mathrm{ADP}+\mathrm{Pi}$$

**Figure 1** demonstrates that MicroScint-O differentiates the signal of [$^{14}$C]acetyl CoA from that of [$^{14}$C]citrate. A series of concentrations of [$^{14}$C]acetyl CoA and [$^{14}$C]citrate (specific activity of both: 0.67 μCi/μmol) in 20 μl of dilution buffer (87 mM Tris, pH 8.0, 20 μM $MgCl_2$, 10 mM KCl, and 7.5 mM DTT) was mixed with 60 μl of MicroScint-O. The signals were counted in TopCount after shaking the mixtures in a plate at room temperature overnight. As shown in Fig. 1A, [$^{14}$C]acetyl CoA was detected in a concentration-dependent manner by MicroScint-O. The linear range of [$^{14}$C]acetyl CoA signal covers 0.25 mM, which encompasses the maximal level of [$^{14}$C]acetyl CoA expected to be generated in the ACL reaction. Further testing demonstrated that the linearity of [$^{14}$C]acetyl CoA signal can be extended to at least 1 mM (data not shown), indicating that MicroScint-O-based detection for [$^{14}$C]acetyl CoA signal is a robust system with a wide dynamic range. In contrast, the same concentrations of [$^{14}$C]citrate showed very little signal in MicroScint-O (Fig. 1A) despite the fact that the radioactive signals of the same series of [$^{14}$C]acetyl CoA and [$^{14}$C]citrate in a classic Liquid Scintillant (EcoLite, MP catalog no. 882475) were comparable when measured with a liquid scintillation counter (Fig. 1B). We speculate that the reason MicroScint-O specifically detects [$^{14}$C]acetyl-CoA, but not [$^{14}$C]citrate, is because of its special design in cocktail physical chemical characteristics that allow it to solubilize acetyl-CoA better than the more hydrophilic citrate. According to the manufacturer PerkinElmer, the patented MicroScint-O cocktail is only suitable for counting nonpolar hydrophobic samples. The cocktail does not contain surfactants; therefore, MicroScint-O is not miscible with water and is unable to detect aqueous samples.

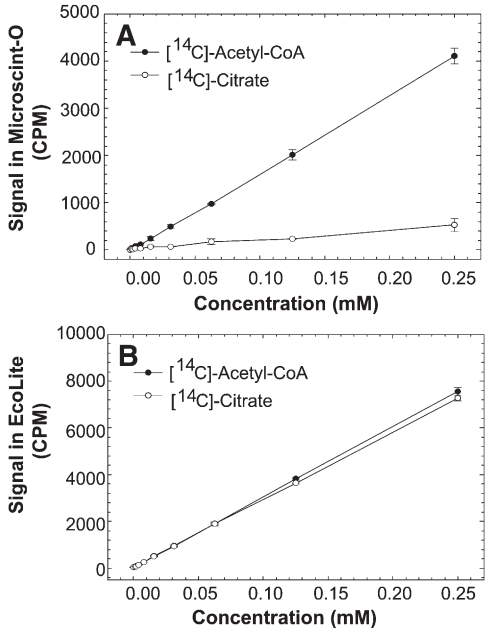


**Fig. 1.** Signal differentiation of [$^{14}$C]acetyl CoA and [$^{14}$C]citrate in MicroScint-O. Serial concentrations of [$^{14}$C]citrate or [$^{14}$C]acetyl CoA were mixed with dilution buffer (87 mM Tris, pH 8.0, 20 μM $MgCl_2$, 10 mM KCl, 7.5 mM DTT). The final specific activities of both [$^{14}$C]citrate and [$^{14}$C]acetyl CoA were 0.67μCi/μmol. A: Aliquots of 60 μl MicroScint-O were added into each well. Signals were detected with TopCount after shaking the plates overnight at room temperature. B: 4 ml of EcoLite LSC was added into each scintillation vial and counted in a liquid scintillation counter.

To mimic the signal detection conditions in a real enzymatic assay, serial concentrations of [$^{14}$C]citrate or [$^{14}$C]acetyl CoA were mixed with reaction buffer (87 mM Tris, pH 8.0, 20 μM $MgCl_2$, 10 mM KCl, 10 mM DTT, 0.1 mM CoA, 0.4 mM ATP, 0.01% β-lactoglobulin) along with 30 ng human ACL and 24 mM EDTA. MicroScint-O again demonstrated excellent selectivity regarding differentiation of [$^{14}$C]acetyl CoA signal from that of [$^{14}$C]citrate signal (data not shown). Furthermore, an overnight time course study indicated that the [$^{14}$C]acetyl CoA signal in MicroScint-O reached maximum after 3 h of incubation. The signal was shown to be stable (80% of the signal remained even after 5 days). For consistency, all remaining studies described were analyzed after an overnight incubation with shaking at room temperature.

### Effect of carrier proteins

As described above, our initial data indicated that the MicroScint-O method directly and selectively measured [$^{14}$C]acetyl CoA in a reproducible manner. However, during the enzyme titration experiment, we found that ACL started to lose linearity when the ACL concentration dropped below 100 ng/reaction (data not shown). One possibility is that the ACL enzyme may not be stable at low concentrations. Alternatively, nonspecific protein adsorption to plastic surfaces may cause loss of activity. This problem was not manifested in coupled assay systems, because high concentrations of MDH or chloramphenicol acetyl transferase coupling enzyme may provide a stabilizing protein environment that prevents the loss of ACL activity. This hypothesis is supported by data in **Fig. 2**. In the absence of carrier protein activity produced by 50 ng ACL is less than one-half of that produced by 100 ng ACL (data beyond 2 h of incubation). In addition, after 3 h, no increase of activity by 50 and 100 ng ACL was observed, indicating that the enzyme was inactivated. Notably, nonspecific carrier proteins β-lactoglobulin and BSA, at either 0.01% or 1%, significantly increased the activity produced by 50 ng ACL. Further, addition of these agents also helped sustain enzyme activity beyond 3 h at 37°C. Between these two carriers, β-lactoglobulin appeared to possess a better protective result, as 0.01% of β-lactoglobulin produced the same ACL stabilizing effects as 1% lactoglobulin and BSA. That there is no difference in activity between 0.01% and 1% lactoglobulin suggests saturation of the stabilizing effect. Glycerol at 1% did not produce any appreciable protective effect. Based on these results, 0.01% β-lactoglobulin was selected as the routine carrier protein for the direct ACL assay in all remaining studies.

### Concentration- and time-dependent ACL activity

To further determine the optimal ACL reaction conditions, ACL concentrations and reaction time were systematically titrated. As shown in **Fig. 3**, the signal increased linearly with increasing amounts of ACL and reaction time. When using 30 ng ACL, the reaction was linear up to 3 h.

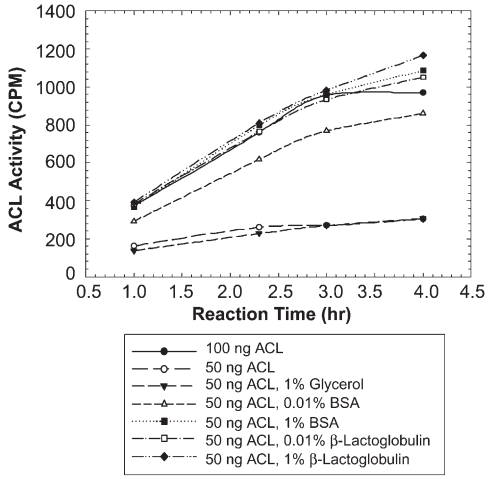


**Fig. 2.** Effect of carrier proteins on ACL activity. The ACL reactions were carried out in buffer D as indicated in Materials and Methods. Aliquots of 50 ng or 100 ng human ACL and 150 μM [$^{14}$C]citrate (0.67μCi/μmol) were incubated either with or without various indicated concentrations of carrier proteins or stabling agents at 37°C for various amounts of time. A total of 60 μl MicroScint-O was added to each well after stopping the reaction with 24 mM EDTA. Signals were detected with TopCount after shaking the plate overnight at room temperature.

### Steady-state kinetic parameters of ACL

To determine the steady-state kinetic parameters of ACL by the direct assay, substrate concentration-dependent enzyme reactions were conducted as shown in **Fig. 4**. **Table 1** summarizes the $K_m$ values: 73.8 ± 11.4 μM for citrate, 4 ± 2 μM for CoA, and 47 ± 17 μM for ATP. Although the $K_m$ value for citrate derived from the current direct assay was similar compared with that determined by the ACL/MDH coupled assay, $K_m$ values of CoA and ATP were about 3-fold less than those determined by the coupled assay.

### Assay statistics

To evaluate the consistency and reproducibility of the direct ACL assay, we adopted a statistical approach. The assays were performed in two separate plates on each day for two consecutive days. Each plate had 48 wells containing substrates, reagents, and human ACL enzyme with the

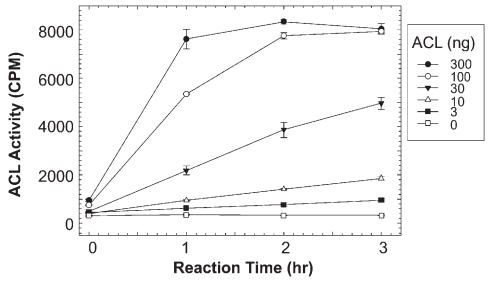


**Fig. 3.** ACL enzyme titration and reaction time course. The ACL reactions were carried out in buffer D with 150μM [$^{14}$C]citrate (2 μCi/μmol) and with various amounts of human ACL at 37°C for various amounts of time.

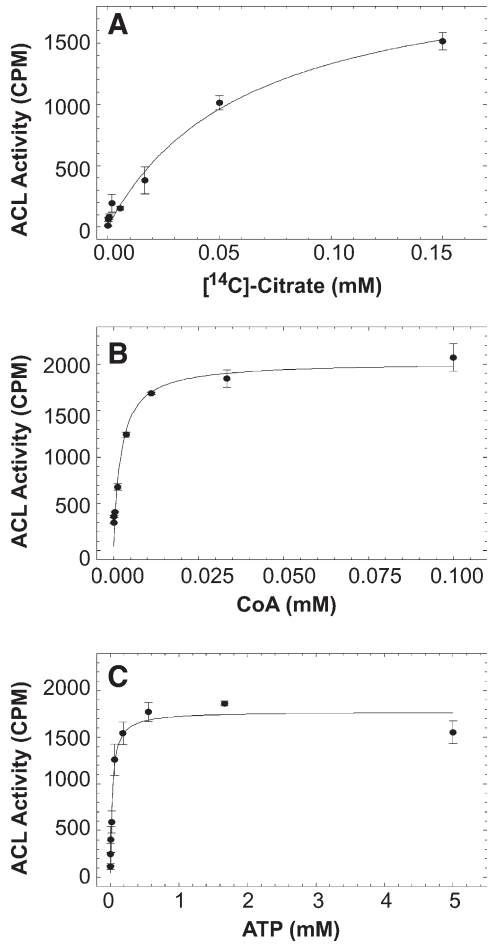


**Fig. 4.** Substrate concentration-dependent human ACL reaction. The ACL reactions were carried out in buffer D containing 30 ng human ACL and the following substrates: A: 0.1 mM CoA, 1 mM ATP, and various concentrations of [$^{14}$C]citrate (2 μCi/μmol); B: 1 mM ATP, 150 μM [$^{14}$C]citrate (2 μCi/μmol), and various concentrations of CoA; C: 0.1 mM CoA, 150 μM [$^{14}$C]citrate (2 μCi/μmol), and various concentrations of ATP. Reactions were carried at 37°C for 3 h.

quenching EDTA added before the reaction to define the background level or the minimal signal level, and 48 wells containing all of the above except that the quenching EDTA was added after the enzymatic reaction to define the maximal signal level. The statistics for each plate were calculated independently and tabulated in **Table 2** according to methods described in online supplementary data Table I. The current direct ACL assay has a Z' greater than 0.6, signal/noise and signal/background values greater than 9, indicating that the direct ACL assay is both very robust and highly reproducible.

TABLE 1. Summary of $K_m$ values for human ACL

| Substrate | $K_m$ (μM) Direct Assay (current study) | $K_m$ (μM) ACL-MDH Coupled Assay[a] |
|---|---|---|
| Citrate | 73.8 ± 11.4 | 98 |
| CoA | 4 ± 2 | 14 |
| ATP | 47 ± 17 | 120 |

[a] Values reported by Lord et al. (10).

TABLE 2. Assay statistics

| Assay performance statistics | Day 1 Plate 1 | Day1 Plate 2 | Day 2 Plate 1 | Day 2 Plate 2 |
|---|---|---|---|---|
| Signal window | 14.3 | 17.5 | 8.9 | 7.9 |
| Z' | 0.74 | 0.78 | 0.64 | 0.60 |
| Signal/noise | 13.6 | 15.9 | 9.9 | 9.3 |
| Signal/background | 9.5 | 9.7 | 11.3 | 10.1 |
| Statistics for totals | | | | |
| No. of wells | 48 | 48 | 48 | 48 |
| Mean | 2294 | 2320 | 2310 | 2325 |
| SD | 148 | 129 | 208 | 218 |
| CV | 6.5 | 5.6 | 9.0 | 9.4 |
| Statistics for NSB | | | | |
| No. of wells | 48 | 48 | 48 | 48 |
| Mean | 242 | 238 | 204 | 230 |
| SD | 27 | 24 | 46 | 60 |
| CV | 11.3 | 9.9 | 22.6 | 25.9 |

### Effect of reaction temperature, DMSO, and assessment of ACL inhibitor

We also compared temperature effects on the current direct assay. At 20°C, ACL produced one-half of the signal compared with 37°C. However, the signal-to-noise values were both approximately 15.

For inhibitor screening purpose, compounds were routinely dissolved in 100% DMSO with further dilution to 1% or lower in the final assay. DMSO up to 5% had no significant effect on ACL activity.

To assess if the current assay consistently reports inhibitory potencies of a given ACL inhibitor, we tested BMS-303141 [or compound 9 in (7)] against ACL. As shown in **Fig. 5**, when tested on separate days and in different plates, the shape of the concentration-inhibition curves and the degree of inhibition by BMS-303141 were highly reproducible. The $IC_{50}$ values were determined to be 0.80, 1.00, 1.23, and 0.74 μM using four independent curves. The

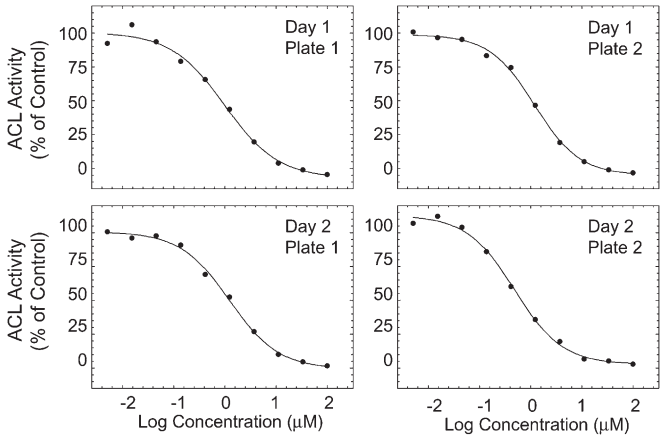


**Fig. 5.** Concentration-dependent inhibition curves of ACL by BMS-303141. The ACL reactions were carried out in buffer D containing 150 μM [$^{14}$C]citrate (2 μCi/μmol), 30 ng human ACL, and various concentrations of BMS-303141. Final DMSO concentration was 2.5%. Reactions were carried out at 37°C for 3 h.

**Table 3: Assay Comparison**

| | Direct ACL | ACL-MDH Coupled |
|---|---|---|
| **Principle** | [$^{14}$C]-Citrate + CoA + ATP $\xrightarrow{ACL}$ [$^{14}$C]-Acetyl-CoA + Oxaloacetate + ADP + Pi | (1) Citrate + CoA + ATP $\xrightarrow{ACL}$ Acetyl-CoA + Oxaloacetate + ADP + Pi<br>(2) Oxaloacetate + NADH + H+ $\xrightarrow{MDH}$ Malate + $NAD^+$ |
| **Signal** | Increase of Radioactive Signal | Decrease of Fluorescence Signal |
| **Plate Format** | 384 - well | 96 - well |
| **Assay Volume** | 20 μl | 100 μl |
| **hACL (ng/well)** | 30 | 300 |

average value of $IC_{50}$ was 0.94 μM, with a standard error of 0.19 μM, indicating that the assay reproducibly reports ACL inhibitor potencies.

## CONCLUSIONS

The work described here presents a novel assay for the direct measurement of ACL enzymatic activity. The assay design utilizes the unique ability of MicroScint-O to selectively detect [$^{14}$C]acetyl CoA signal from [$^{14}$C]citrate. **Table 3** summarizes the major differences between the current direct assay and the traditional ACL-MDH coupled assays. The direct assay offers several advantages: *1*) it is conducted in 384-well plate format with lower assay volume and 10-fold less ACL enzyme; and *2*) the assay is homogeneous where all the reagents are added and signals are detected in the same reaction vessel. In addition, BMS-303141, a BMS ACL inhibitor, exhibited a consistent and classic sigmoidal inhibition pattern using this assay. The statistics parameters of the assay performance further demonstrate that it is a robust and reliable assay amenable to high-throughput screening.

The authors thank Drs. Simeon Taylor and Mary Ann Pelleymounter for support of the project, Dr. Joelle Onorato for critical reading of the manuscript, and Ms. Luping Chen for sharing protocols for ACL-MDH coupled assay.

## REFERENCES

1. Watson, J. A., M. Fang, and J. M. Lowenstein. 1969. Tricarballylate and hydroxycitrate: substrate and inhibitor of ATP: citrate oxaloacetate lyase. *Arch. Biochem. Biophys.* **135:** 209–217.
2. Sullivan, A. C., J. G. Hamilton, O. N. Miller, and V. R. Wheatley. 1972. Inhibition of lipogenesis in rat liver by (-)-hydroxycitrate. *Arch. Biochem. Biophys.* **150:** 183–190.
3. Sullivan, A. C., J. Triscari, J. G. Hamilton, O. N. Miller, and V. R. Wheatley. 1974. Effect of (-)-hydroxycitrate upon the accumulation of lipid in the rat. I. Lipogenesis. *Lipids.* **9:** 121–128.
4. Preuss, H. G., C. V. Rao, R. Garis, J. D. Bramble, S. E. Ohia, M. Bagchi, and D. Bagchi. 2004. An overview of the safety and efficacy of a novel, natural(-)-hydroxycitric acid extract (HCA-SX) for weight management. *J. Med.* **35:** 33–48.
5. Sullivan, A. C., J. Triscari, J. G. Hamilton, and O. N. Miller. 1974. Effect of (-)-hydroxycitrate upon the accumulation of lipid in the rat. II. Appetite. *Lipids.* **9:** 129–134.
6. Pearce, N. J., J. W. Yates, T. A. Berkhout, B. Jackson, D. Tew, H. Boyd, P. Camilleri, P. Sweeney, A. D. Gribble, A. Shaw, et al. 1998. The role of ATP citrate-lyase in the metabolic regulation of plasma lipids. Hypolipidaemic effects of SB-204990, a lactone prodrug of the potent ATP citrate-lyase inhibitor SB-201076. *Biochem. J.* **334:** 113–119.
7. Li, J. J., H. Wang, J. A. Tino, J. A. Robl, T. F. Herpin, R. M. Lawrence, S. Biller, H. Jamil, R. Ponticiello, L. Chen, et al. 2007. 2-hydroxy-N-arylbenzenesulfonamides as ATP-citrate lyase inhibitors. *Bioorg. Med. Chem. Lett.* **17:** 3208–3211.
8. Linn, T. C., and P. A. Srere. 1979. Identification of ATP citrate lyase as a phosphoprotein. *J. Biol. Chem.* **254:** 1691–1698.
9. Pentyala, S. N., and W. B. Benjamin. 1995. Effect of oxaloacetate and phosphorylation on ATP-citrate lyase activity. *Biochemistry.* **34:** 10961–10969.
10. Lord, K. A., X. M. Wang, S. J. Simmons, R. C. Bruckner, J. Loscig, B. O'Connor, R. Bentley, A. Smallwood, C. C. Chadwick, P. E. Stevis, et al. 1997. Variant cDNA sequences of human ATP:citrate lyase: cloning, expression, and purification from baculovirus-infected insect cells. *Protein Expr. Purif.* **9:** 133–141.