



LabChip Assay: Off-Chip Incubation, Mobility Shift

Sphingosine Kinase 1 and Sphingosine Kinase 2 Assays

I. Introduction

The off-chip incubation, mobility shift sphingosine kinase assay uses a microfluidic chip to measure the conversion of fluorescent non-phosphorylated lipid substrate (sphingosine, SPH) to a phosphorylated product (sphingosine-1-phosphate, S1P). The reaction mixture, from a microtiter plate well, is introduced through a capillary sipper onto the chip, where the phosphorylated product and non-phosphorylated lipid substrate are separated by electrophoresis and detected via laser-induced fluorescence. This application note describes assay conditions for the sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2). S1P has been implicated as a modulator of membrane signal transduction systems and as a regulatory element in important biological events, such as proliferation, differentiation, apoptosis and stress responses. Sphingosine kinase 1 and 2, responsible for the generation of S1P, have emerged as important candidates for drug targets due to their role in controlling cellular life and death events. The sensitive and rapid off-chip mobility shift assay provides a novel means of screening compounds which interfere with the activities of SPHK1 and SPHK2.

II. Methods

Substrate

Sphingosine-fluorescein has molecular weight of 672.81 and its chemical structure is shown in Figure 1. S1P is generated by both enzymes, SPHK1 and SPHK2, which share a high degree of homology in the catalytic domain.

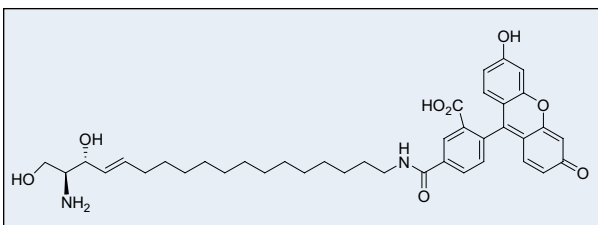


Figure 1. Chemical structure of sphingosine-fluorescein.

Separation Conditions	4-Sipper
Pressure (psi)	-2.2
Upstream Voltage (V)	-500
Downstream Voltage (V)	-1800
Sample Sip Time (sec)	0.2
Post-sample Buffer Sip Time (sec)	45

Table 1. Separation conditions for SPHK1 and SPHK2 assays.

III. Results

Substrate/Product

Figure 2 shows the separation of product and substrate on a 4-sipper chip using the separation parameters shown in Table 1. Phosphorylated product and non-phosphorylated substrate are separated on a chip and appear as a distinctive peaks. The data analysis software (HTSWA) determines peak heights from which the ratio of product to the peak sum P/(P+S) is calculated. The P/(P+S) value x 100 = % product formed.

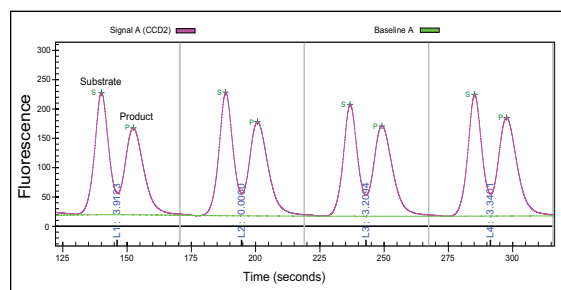


Figure 2. Caliper LabChip 3000 System Data Signature. Representative product and substrate peaks for SPHK1 and SPHK2 assays. The electropherogram illustrates the fluorescent signal detected from a single channel of a 4-sipper chip during 4 consecutive sips from different microtiter plate wells containing stopped SPHK reactions.

SPHK1 and SPHK2 Assay conditions (Final in reaction).	Reaction Buffer (Final Concentration in reaction)	51 μ l reaction	Chip/Trough Buffer	Stop Solution (Termination Buffer)
SPHK1 0.3 ng/ μ l enzyme	100 mM HEPES pH 7.5	1 μ l Compound	100 mM HEPES pH 7.5	100 mM HEPES, pH7.5
SPHK2 0.75 ng/ μ l enzyme	0.05% Triton X-100	25 μ l Enzyme	15.5 mM EDTA	2.5 mM MgCl ₂
1 μ l compound in 100% DMSO	10% Glycerol	25 μ l Substrate	0.05% Triton X-100	0.05% Brij-35
1 μ M substrate (SPH-fluorescein)	4 mM DTT	60 min reaction at 20°C	2.5 mM Glycerol	0.1% Coating reagent 3
	20 mM MgCl ₂	40 μ l Stop Solution	2.5 mM MgCl ₂	40 mM EDTA
	50 μ M ATP (for SPHK1)		0.1% CR-3	
	150 μ M ATP (for SPHK2)		0.6% DMSO	

Table 2. Reaction conditions and buffer composition

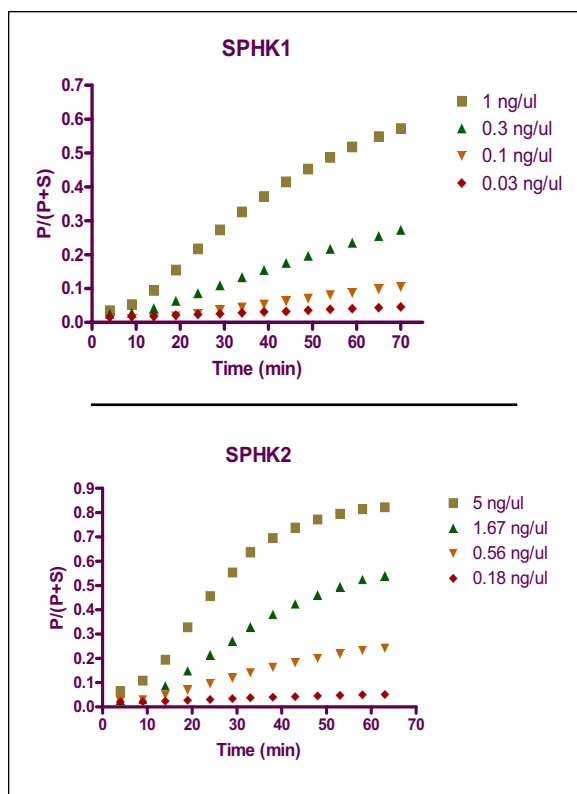


Figure 3. Enzyme titrations. Real time kinetics of SPHK1 and SPHK2 phosphorylation reactions containing varying enzyme concentrations. Data represents averages from duplicate reactions.

Enzyme Titrations

The initial titrations for SPHK1 and SPHK2 are shown in Figure 3. Reactions containing 50 μ l total volume with 1 μ M substrate and 4 different enzyme concentrations were assembled in duplicate on a 384-well microtiter plate. The plate was placed immediately onto the LabChip 3000 system and samples were introduced onto a 4-sipper chip every 5 minutes for 70 minutes. Temperature and humidity in the reaction chamber were maintained at 20° C and 50%, respectively. Substrate and phosphorylated product were separated and detected on a chip. The enzyme concentration resulting in 30% product formed after 60 min incubation was extrapolated (SPHK1: 0.3 ng/ μ l and SPHK2: 0.75 ng/ μ l) and chosen for further assay development studies.

Km Determinations

ATP Km values were determined for each enzyme using real-time kinetics (Figure 4). Initial reaction rates were determined by assembling 50 μ l reactions containing increasing concentrations of ATP (0.21, 0.62, 1.9, 5.6, 16.7, 50 μ mol/L) with either 0.3 ng/ μ l SPHK1 or 0.75 ng/ μ l SPHK2. The plate was immediately placed on the LabChip3000 system and samples were introduced onto 4-sipper chip every 5 minutes for 60 min. Temperature and humidity in the reaction chamber were maintained at 20° C and 50%, respectively.

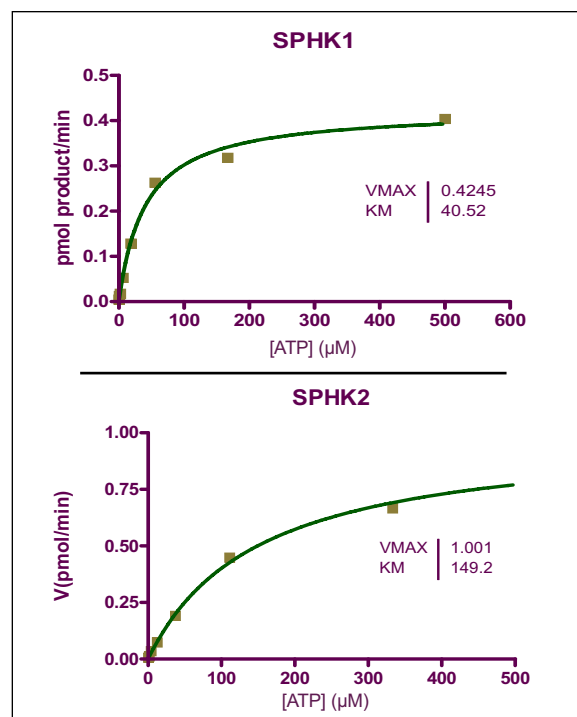


Figure 4. ATP Km determinations for SPHK1 and SPHK2. The plots show Michaelis-Menten non-linear regression analysis of initial reaction rates vs substrate concentration.

SPH and phosphorylated product were separated and detected on the chip. Initial rates V(pmol/min) were calculated for each ATP concentration by finding the slopes of product formed vs time during the first 30 minutes of the reaction. Km values were determined by plotting V(pmol/min) vs ATP concentration [S] (μ mol/L) and applying non-linear regression analysis using the Michaelis-Menten equation. The substrate Km values for SPHK1 and SPHK2 were found to be 40.5 μ M and 149.2 μ M, respectively.

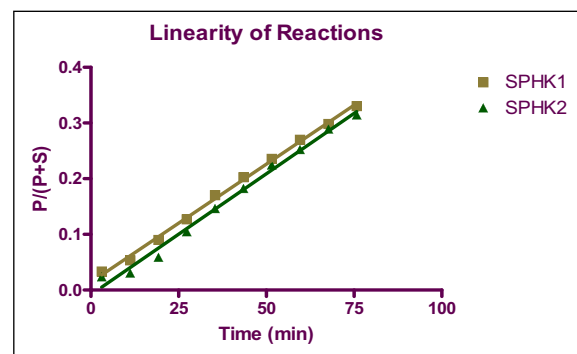


Figure 5. Linearity of the SPHK1 and SPHK2 reaction rates.

Reaction linearity

Real-time kinetics were used to show that kinase reactions remained linear for approximately 70 min. Reactions containing 50 μ l total volume with 1 μ M substrate- fluorescent SPH and enzyme (SPHK1, 0.3 ng/ μ l or SPHK2, 0.75 ng/ μ l) were assembled in duplicate on a 384-well microtiter plate.

The plate was placed immediately onto the LabChip 3000 system and samples were introduced onto a 4-sipper chip every 7 minutes for 70 minutes. Temperature and humidity in the reaction chamber were maintained at 20° C and 50%, respectively. Substrate and phosphorylated product were separated and detected on the chip. The lines represent the linear regression from data points, collected during the 70 minutes of reaction time. As it shown, the linearity is maintained over the course of the assay.

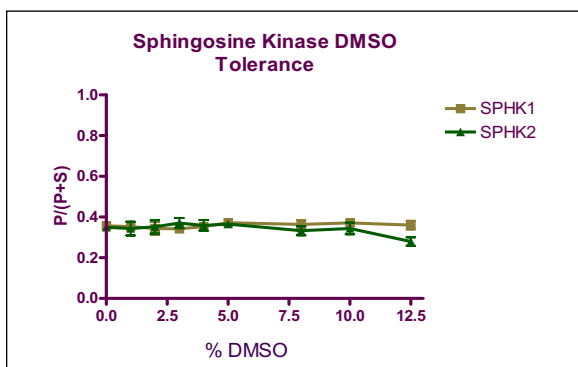


Figure 6. DMSO tolerance. Increasing DMSO concentration has no significant effect on the activity of either SPHK1 or SPHK2.

DMSO Tolerance

The effect of DMSO on SPHK1 and SPHK2 activity was determined by running 50 μ l kinase reactions with increasing amounts of added DMSO (Figure 6). 100% DMSO (0.5 μ l to 6.5 μ l) was added to wells of a microtiter plate, mimicking the addition of compounds dissolved in DMSO. Reactions were then assembled in the wells incubated as described in Methods section.

Substrate and phosphorylated product were separated and detected on a 4-sipper chip. As shown in Figure 6, increasing DMSO concentration has no significant effect on the activity of either SPHK1 or SPHK2. Both enzymes should tolerate the addition of up to 12.5% DMSO in a reaction mixture.

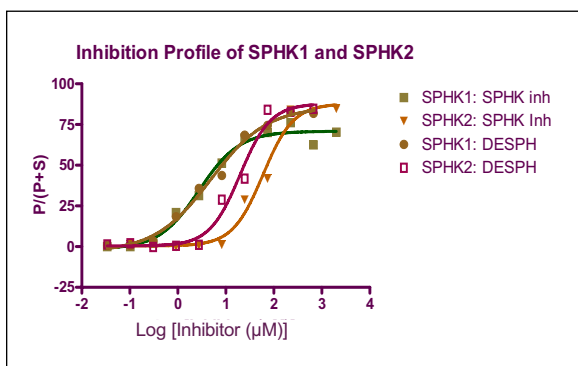


Figure 7. Inhibition curves showing the effects of the 2 known SPHK inhibitors on the activities of SPHK1 and SPHK2.

Inhibitor IC₅₀ Determinations

Known SPHK inhibitors, one N,N'-dimethyl-D-erythro-Sphingosine (DESPH) and 2-(p-Hydrozanyilino)-4-(p-chlorophenyl) tiazole (SPHK Inh), were selected for analysis. Reactions were incubated for 1 hr in the presence of increasing concentrations of SPHK Inh or DESPH. The reactions were stopped by addition of 40 μ l Stop Solution containing 35 mM EDTA. Substrate and phosphorylated product were separated and detected on a 4-sipper chip. IC₅₀ values (Table 3) were calculated using non-linear regression analysis of the fraction of product formed (P/P+S) vs Log of inhibitor concentration. The inhibition curves for both enzymes are shown in Figure 7. As expected from the literature, SPHK1 IC₅₀ values for inhibitors were below 10 micromolar, while SPHK2 demonstrated reduced sensitivity to both inhibitors.

Compound	SPHK1 IC ₅₀ (μ M)	SPHK2 IC ₅₀ (μ M)
SPHK Inhibitor	2.8	59.5
DESPH	4.9	19.8

Table 3. Compound IC₅₀ values determined for SPHK1 and SPHK2 inhibitors.

Data Stability

For high throughput screening applications, it is necessary to assemble, incubate and terminate enzymatic reaction in multiple microtiter plates during a single run of the assay. The LabChip 3000 Screening System may be programmed to read results from up to 60 plates in a single run. However, the quality of the results will depend on the stability of substrate in the terminated reaction. As shown in Figure 8, SPH and S1P show a high degree of stability over 7 hours. The terminated reaction plate remained in the LabChip 3000 system chamber with temperature and humidity maintained at 20° C and 50%, and wells were sampled repeatedly over the course of 7 hours. The data from the first and last analysis are nearly identical in terms of substrate and product peak integrity, separation and relative intensity.

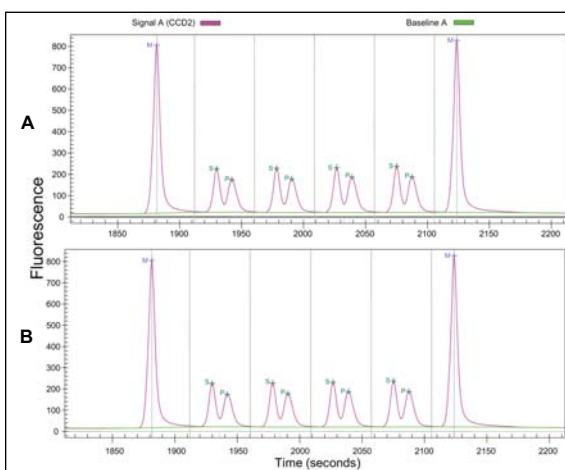


Figure 8. Stability of the substrate and phosphorylated product in the terminated reaction Mixture. Data collected immediately after reaction termination (A) and collected from the same reaction wells after 7 hours (B) in the LabChip 3000 chamber.

IV. Materials

ITEM	ITEM NAME	MANUFACTURER	CATALOG #
Microfluidic System Components	LabChip 3000 Drug Discovery System	Caliper Life Sciences	
	Caliper Off-chip Mobility Shift Chip, 4 – Sipper with Coating reagent	Caliper Life Sciences	761043-0266R
	Caliper Off-Chip Mobility Shift Chip, 4 – Sipper with Coating reagent	Caliper Life Sciences	761037-0372R
Assay components	Sphingosine Kinase 1	BPS Bioscience, Inc	40610
	Sphingosine Kinase 2	BPS Bioscience, Inc	40611
	Sphingosine-fluorescein	Echelon Biosciences, Inc	S-100F
	HEPES, Free Acid ULTROL	Calbiochem	391338
	HEPES, Sodium salt, ULTROL	Calbiochem	391333
	Magnesium Chloride	Sigma	M2670
	Coating Reagent-3	Caliper Life Sciences	760050
	EDTA, disodium salt, 0.5 M	Sigma	E7889
	DTT	Calbiochem	233153
	Glycerol	ICN Biomedical, Inc	800688
	Triton X-100	Sigma	T8787
	18 MΩ Water		
	Sphingosine Kinase Inhibitor (2-(p-Hydroxyanilino)-4(p-chlorophenyl) tiazole	EMD Biosciences, Inc	567731
	N'N'-dimethyl-D-erthyro-Sphingosine	EMD Biosciences, Inc	310500

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