



LabChip Assay: Off-Chip Incubation, Mobility Shift

MMP9: Matrix Metalloproteinase 9 Assay

I. Introduction

The off-chip incubation, mobility shift assay uses a microfluidic chip to measure the conversion of a fluorescent peptide substrate to a cleaved product. The reaction mixture, from a microtiter plate well, is introduced through a capillary sipper onto the chip, where the peptide substrate and the cleaved product are separated by electrophoresis and detected via laser-induced fluorescence. The signature of the fluorescence over time reveals the extent of the reaction. This application note describes the assay conditions for the matrix metalloproteinase MMP9. Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis.

II. Methods

Substrate

FITC-AHA-CPLGLKAR-CONH₂

This peptide substrate has a molecular weight of 1215 and a net charge of 0.15 at pH 7.5. Upon cleavage the fluorescent-tagged product *FITC-AHA-CPL-COOH* has a molecular weight of 688 and a net charge of -2.85.

MMP9 Assay Conditions (Final in reaction)

5 nM MMP9 enzyme
 1 μ L Compound in 100% DMSO
 1.5 μ M Substrate (*FL-AHA-CPLGLKAR-CONH₂*)

31 μ L Reaction

1 μ L Compound, 15 μ L Enzyme
 60 minute pre-incubation with compound at ambient temperature
 15 μ L Substrate
 60 minute incubation at ambient temperature
 30 μ L Stop Solution

Reaction Buffer (Final concentration in reaction)

50 mM HEPES, pH 7.5
 10 mM CaCl₂
 0.05% Brij-35
 3.2% DMSO

Chip/Trough Buffer

100 mM HEPES, pH 7.5
 10 mM CaCl₂
 0.05% Brij-35
 20 mM EDTA
 0.1% Coating Reagent 3

Stop Solution (Termination Buffer)

100 mM HEPES, pH 7.5
 10 mM MgCl₂
 0.05% Brij-35
 0.1% Coating Reagent 3
 40 mM EDTA
 500 nM MMP9 Inhibitor 1

Separation Conditions

	4-Sipper
Pressure (psi)	-2.8
Upstream Voltage (V)	-500
Downstream Voltage (V)	-1700
Sample Sip Time (sec)	0.2
Post-Sample Buffer Sip Time (sec)	20

Table 1: MMP9 Assay Parameters for Screening

III. Results

Substrate/Product Peak Separation

Figure 1 shows the separation of product and substrate on a 4-sipper chip using the parameters shown in Table 1 and a 60 second post-sample buffer sip. Marker Dye is sipped between rows to enable well assignments by the data analysis software (HTSWA). Peptide substrate and the cleaved product are separated on the chip and appear as separate peaks. The HTSWA software determines peak heights from which ratios of the product to the peak sums $P/(P+S)$ are calculated. The $P/(P+S) \times 100 = \% \text{ product formed}$.

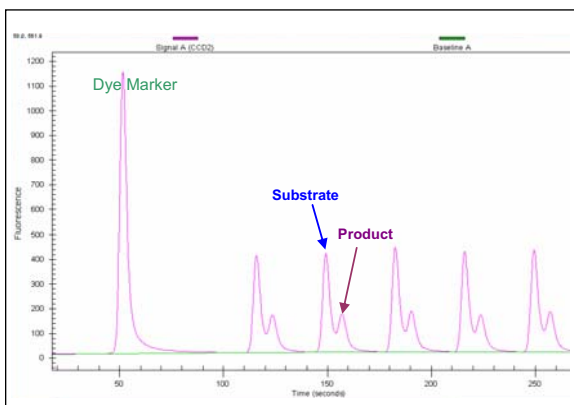


Figure 1. Caliper LabChip 3000 Data Signature; Representative peaks from 5 sips: Electropherogram illustrating the fluorescent signal of a single channel from a 4-sipper chip detected by the LabChip 3000 system.

Enzyme Titration

The initial enzyme titration is shown in Figure 2A. 60 μL reactions containing 1.5 μM peptide substrate and 4 different MMP9 enzyme concentrations were assembled in wells A1, A5, E1, and E5 on a 384-well microtiter plate. The plate was placed immediately onto the LabChip 3000 system and samples introduced onto a 4-sipper chip every 5 minutes for 80 minutes. Temperature and humidity in the reaction chamber were maintained at 20 $^{\circ}\text{C}$ and 50% respectively. Peptide and cleaved product were separated and detected using the LabChip 3000 system. The enzyme concentration resulting in 30% product formed after 60 minutes incubation was extrapolated (5 nM) and chosen for further assay development studies.

Reaction Linearity

Reaction linearity using real-time kinetic reads at a final concentration of 5 nM MMP9 was found to be approximately 80 minutes and is shown in Figure 2B. 60 μL reactions containing 1.5 μM peptide substrate and 5 nM MMP9 enzyme were assembled in wells A1, A5, E1, and E5.

The plate was immediately placed on the LabChip 3000 system and samples introduced onto a 4-sipper chip every 5 minutes for 2 hours. Temperature and humidity in the reaction chamber were maintained at 20 $^{\circ}\text{C}$ and 50% respectively. Peptide and cleaved product were separated and detected using the LabChip 3000 system. The data represents the linear regression of the average \pm std error of 4 replicate wells over 83 minutes. Linearity was lost after 83 minutes.

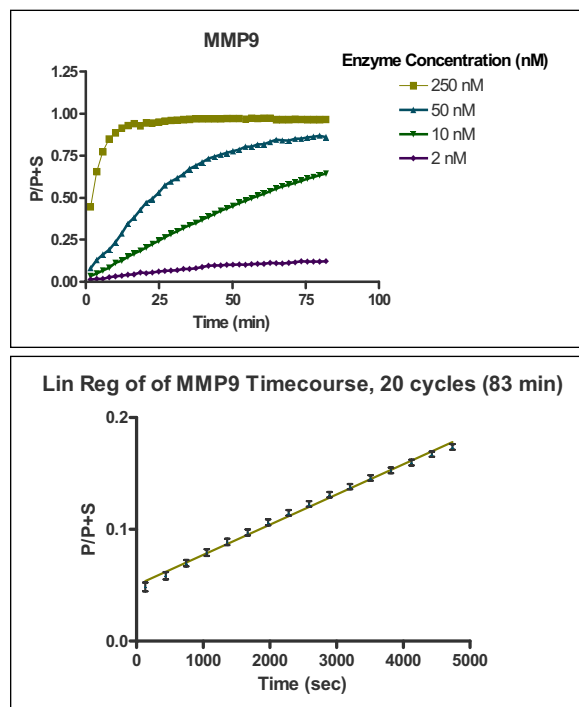
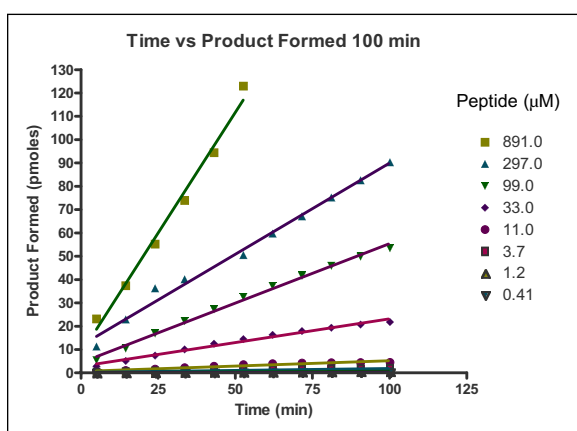


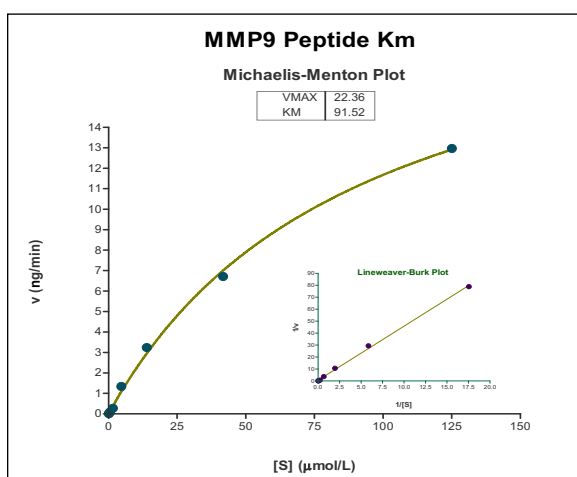
Figure 2A and 2B. MMP9 Time Course: Real Time Kinetics Figure 2A: MMP9 enzyme titration. Figure 2B: Linear regression of the product formed during reactions at 5 nM MMP9.

K_m Determinations

Peptide K_m was determined using real-time kinetic initial rates by assembling 60 μL reactions containing increasing amounts of peptide substrate and 5 nM MMP9 enzyme. The plate was immediately placed on the LabChip 3000 system and samples introduced into the chip every 5 minutes for 2 hours. Temperature and humidity in the reaction chamber were maintained at 20 $^{\circ}\text{C}$ and 50% respectively. Peptide and cleaved product were separated and detected using the LabChip 3000 system. Initial rates V (ng/min) were determined by calculating the slopes at each peptide concentration of cleaved product formed in the first 100 min of the reaction over time (Figure 3 Panel A). K_m was determined by plotting V (ng/min) vs. $[S_{\text{peptide}}]$ and non-linear regression analysis using the Michaelis-Menton equation (Figure 3 Panel B). The peptide K_m was found to be approximately 91.5 μM . V_{max} was extrapolated from the data. The inset shows the Lineweaver-Burke plot of the kinetic data.



Panel A



Panel B

Figure 3. Substrate K_m : Real Time Kinetics Panel A - initial rate linear regression for the increasing concentrations of peptide. Panel B- Michaelis-Menton non-linear regression analysis. Inset shows the Lineweaver-Burke plot of the kinetic data.

DMSO Tolerance

From the assay development parameters determined above, the final assay conditions were selected using the concentration that provides approximately 30% product formed $[P/(P+S) = 0.3]$ in one hour (see MMP9 assay conditions in Methods). The effect of DMSO on MMP9 activity was then determined by incubating increasing amounts of 100% DMSO with 15 μL each of 3.0 μM peptide substrate and 10 nM MMP9 enzyme. The reaction was incubated for 1 hour at room temperature followed by the addition of 30 μL Stop Buffer containing 20 mM EDTA and 0.5 μM MMP9 Inhibitor 1. Peptide and cleaved product were separated and detected using the LabChip 3000 system. Figure 4 shows the effect of increasing DMSO concentrations on the enzyme activity. MMP9 enzyme activity is sensitive to the presence of DMSO in the reaction. Typically the DMSO concentration is 3.2% in a 31 μL reaction containing 1 μL DMSO. At this concentration the enzyme activity is reduced approximately 25%.

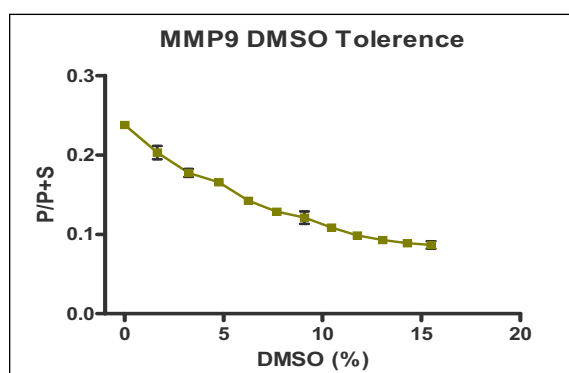


Figure 4. DMSO Tolerance: Increasing amounts of DMSO in the reaction inhibits MMP9 activity

Inhibitor IC_{50}

Known matrix metalloproteinase inhibitors, four small molecule and one peptide, were selected for analysis. 31 μL reactions containing 1.5 μM peptide substrate and 5 nM MMP9 enzyme were incubated for 1 hour in the presence of increasing concentrations of MMP inhibitors. The reactions were stopped by the addition of 20 mM EDTA and 0.5 μM MMP9 Inhibitor 1. Peptide and cleaved product were separated and detected using the LabChip 3000 system. Inhibition curves are shown in Figure 5. IC_{50} values were calculated using non-linear regression analysis and are shown in Table 2. As expected from the known literature values, the 4 small molecule inhibitors exhibit IC_{50} in the nM range. The peptide requires mM concentrations to achieve inhibition under the experimental conditions used.

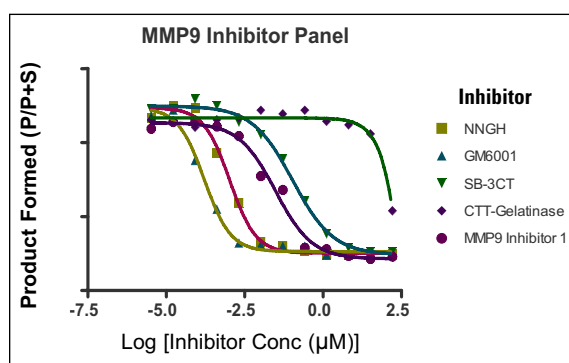


Figure 5. Inhibitor Panel IC_{50} Determinations

Compound	IC_{50} (nM)
NNGH	1.058
GM6001	0.164
SB-3CT	107.0
CTT-Gelatinase	1.02×10^7
MMP9 Inhibitor 1	30.17

Table 2: IC_{50} Values Determined for Inhibitors

V. Materials

ITEM	ITEM NAME	MANUFACTURER	CATALOG #
Microfluidic System Components	LabChip 3000 Drug Discovery System	Caliper Life Sciences	
	Caliper Chip Module TC	Caliper Life Sciences	
	Caliper Chip Module FS	Caliper Life Sciences	
	Off-chip Mobility Shift Chip, 4-Sipper, with Coating-3 Reagent	Caliper Life Sciences	761043-0266R
	Off-chip Mobility Shift Chip, 12-Sipper, with Coating-3 Reagent	Caliper Life Sciences	760137-0372R
Assay Components	MMP9 Catalytic Domain (human, recombinant) Specific Activity: 92.31U/μg Lot: T4744	BIOMOL International	SE-244
	Substrate: FITC-AHA-CPLGLKAR-NH ₂	Analytical Core Facility Tufts University	Custom
	HEPES, Free Acid ULTROL	Calbiochem	391338
	HEPES, Sodium Salt ULTROL	Calbiochem	391333
	Calcium Chloride, Anhydrous	J T Baker	1-1309
	Brij-35 Solution	Sigma	B 4184
	Coating Reagent 3	Caliper Life Sciences	760050
	EDTA, disodium salt, 0.5 M solution	Sigma	E7889
	DMSO	Burdick and Jackson	BJ081-1
	18 MΩ Water		
Inhibitors	MMP-9 Inhibitor I	Calbiochem	444278
	NNGH	BioMol	PI 115-0005
	GM6001	BioMol	EI 300-0001
	SB-3CT	BioMol	EI 325-0001
	CTT-Gelatinase	BioMol	PI 136-0001

Worldwide Offices

Benelux

Caliper Life Sciences N.V.
Klapstraat 13
B-1790 Terafene, Belgium
Telephone: +32-53-66-26-70
Fax: +32-53-66-27-32

France

Caliper Life Sciences S.A.
ZAC PARIS-NORD II
13 rue de la Perdrix
BP 48016 Tremblay en France
95911 Roissy CDG Cedex, France
Telephone: +33-1-48-63-71-35
Fax: +33-1-48-63-71-53

Germany

Caliper Life Sciences GmbH
Eisenstrasse 9c
DE-65428 Rüsselsheim, Germany
Telephone: +49-6142-834-93-0
Fax: +49-6142-162-821

Japan

Caliper Life Sciences Japan
Saito-Bldg. 2F
Yushima 2-17-15, Bunkyo-ku
Tokyo 113-0034, Japan
Telephone: +81-3-5840-6551
Fax: +81-3-5840-6554

Switzerland

Caliper Life Sciences AG
Nordstrasse 17
CH-4665 Oftringen, Switzerland
Telephone: +41-62-788-7000
Fax: +41-62-788-7017

United Kingdom

Caliper Life Sciences Ltd.
1 Wellfield
Preston Brook
Runcorn, Cheshire WA7 3AZ
United Kingdom
Telephone: +44-1928-711448
Fax: +44-1928-791228

Caliper Life Sciences has representative offices worldwide. Please visit www.caliperLS.com for locations and contact information.



Caliper Life Sciences Corporate Headquarters

68 Elm Street
Hopkinton, MA 01748-1668
1-508-435-9500
www.caliperLS.com
Fax: 1-508-435-3439
Email: cust.support@caliperLS.com

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