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Effect of Microwave Irradiation On Covalent Ligand–DNA Interactions

Khondaker M. Rahman,[a] and David E. Thurston,*[a,b]

[a]*Gene Targeting Drug Design Research Group, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX and* [b]*Spirogen Ltd, The School of Pharmacy, University of London WC1N 1AX*.

Supporting Information

Single-Stranded Oligonucleotides

The single-stranded (SS) oligonucleotides (*Seq-1* and *Seq-2*) were obtained in a lyophilised form from AtdBio Ltd., Southampton. (see **Table S1**). The double-dye fluorescent hairpin oligonucleotide (*Seq-3*) used for the FRET-based DNA melting assay was obtained in a lyophilised form from Euogenetec UK.

Double-Stranded Oligonucleotide (*Seq-1*/*Seq-1*)

The single-stranded self-complementary *Seq*-1 was dissolved in 100 mM ammonium acetate (Sigma-Aldrich UK) to form a stock solution of 2 mM which was later diluted to 1 mM by addition of annealing buffer (10 mM Tris/50 mM sodium chloride/1 mM EDTA). Solutions of double-stranded DNAs were prepared by heating the complementary SS DNA sequences (1 mM) in annealing buffer (pH 8.5) to 70°C for 10 minutes in a heating/cooling block (Grant Bio UK). The solutions were then allowed to cool slowly to room temperature followed by storage at -20°C overnight to ensure completion of the annealing process. Working solutions of DS DNA duplexes at 50 μM were prepared by diluting the stored solutions with 20 mM ammonium acetate.

The hairpin oligonucleotide *Seq-2* was dissolved in 1M ammonium acetate (Sigma-Aldrich UK) to form a stock solution of 1 mM. A working solution of 50 μM was prepared by diluting the stock solution with 100 mM ammonium acetate. **Table S1** provides the masses of the oligonucleotides alone, and for their covalent adducts with the PBD dimer SJG-136 (**1**) and the PBD conjugate GW-78 (**2**).

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PBD Dimer 1 (SJG-136)

SJG-136 (**1**) was provided by Spirogen Ltd (Batch no: SG2000) and was dissolved in 50/50 v/v methanol/water to form a stock solution of 3 mM which was stored at -20^{o}C for no longer than four months. Working solutions of 200 μM were prepared by diluting the stock solution with nuclease free water. These were stored at -20^{o}C for not more than one week and then thawed to room temperature for use when required.

PBD Conjugate 2 (GWL-78)

PBD conjugate **2** (GWL-78) was provided by Spirogen Ltd (Batch No. SG2274.005) and was dissolved in methanol to form a stock solution of 10 mM which was stored at -20^{o}C for no longer than four months. Working solutions of the drug of 200 μM were prepared by diluting the above solution with 100 mM ammonium acetate. These were stored at -20^{o}C for not more than one week and thawed to room temperature for use when required.

Preparation of Ligand/DNA Complexes

Ligand/DNA complexes were prepared by incubating working solutions of **1** (in nuclease free water) or **2** (in 100 mM ammonium acetate) with single stranded (in 100 mM ammonium acetate) or duplex oligonucleotides (in 20 mM ammonium acetate/0.5 mM Tris/2.5 mM sodium chloride/50 μM EDTA) at a 4:1 molar ratio at room temperature, unless otherwise stated. Samples were withdrawn at various time intervals and subjected to ion-pair RPLC and mass spectrometry analysis as described below.

Microwave Irradiation

All microwave irradiation was performed in 0.2-5 ml sealed pyrex vials in an Emry's Optimizer Personal Chemistry Instrument (Biotage AG). The ligand/DNA solutions were subjected to microwave irradiation at the indicated temperatures for different time intervals without any pre-stirring. Depending on the temperatures required, 43-68 watts of microwaves were used to irradiate the ligand/DNA or DNA solutions. This Biotage instrument has sophisticated thermostat settings, and the reaction temperatures inside the vials were believed to be the indicated temperatures (27ºC, and 40ºC).

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Ion-Pair Reversed-Phase Liquid Chromatography (RPLC) Analysis

Chromatography was performed on a Thermo Electron HPLC system equipped with a 4.6 × 50 mm Xterra MS C18 column packed with 2.5 μM particles (Waters Ltd UK), an UV 1000 detector, an AS3000 autosampler, a SCM1000 vacuum degasser and Chromquest software (Version 4.1). A gradient system of 100 mM triethyl ammonium bicarbonate (TEAB) as buffer A and 40% acetonitrile in water (HPLC grade, Fischer Scientific UK) as buffer B was used. For buffer A, a 1 M pre-formulated buffer of TEAB was purchased from Sigma-Aldrich UK and diluted to 100 mM with HPLC grade water (Fischer Scientific UK). The gradient was ramped from 90% A at 0 minutes to 50% A at 20 minutes, and finally to 10% A at 35 minutes. UV absorbance was monitored at 254 nm and fractions containing separated components collected manually, combined when appropriate, lyophilised and analysed using ESI-QTOF and MALDI TOF mass spectrometers as described below.

Mass Spectrometry Analysis (MALDI TOF)

Samples were prepared by diluting with matrix (37 mg THAP in 1 mL ACN, 45 mg ammonium citrate in 1 mL water – mixed 1:1 for matrix) either 2:1, 1:1 or 1:5 (sample:matrix). 1 μl of sample was spotted onto the MALDI target plate and allowed to dry. Samples were analyzed on a Voyager DE-Pro with a nitrogen laser in positive linear mode using delayed extraction (500 nsec) and an accelerating voltage of 25000 V. Acquisition was between 4000 – 15000 Da with 100 shots/spectrum.

Generation of Five-Point Calibration Curves to Test the Reproducibility and Sensitivity of the HPLC Assay

The reproducibility and sensitivity of the assay were tested by constructing separate five-point calibration curves for both *Seq-2* and SJG 136 (**1**). The concentration range studied for both *Seq-2* (5, 10, 20, 40 and 80 μM) and SJG-136 (20, 40, 80, 160 and 320 μM) covered the concentration ranges of the oligonucleotides and ligands used in the HPLC assays as reported in the main text. The calibration curves were generated by plotting "Area Under the Curve" (AUC) against the respective concentrations using a scatter graph, followed by drawing a best fit line between the data points (**Figure S11 and S12**). The linear calibration curves obtained for both *Seq-2* and SJG-136 (**1**) indicated that the assay is reproducible and linear over the concentration range studied.

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Fluorescent Resonance Energy Transfer (FRET)-Based DNA Melting Assay

A fluorescence tagged *Seq-3* (Eurogentec UK) stock solution in water (20 μM) was diluted to 400 nM using FRET buffer (50 mM potassium cacodylate, pH 7.4) and annealed by heating at 85ºC for 5 minutes followed by cooling to room temperature over 5 hours. Ligand solutions were prepared at concentrations double those required for the final solutions. Dilutions from the initial 10 mM DMSO stock solutions were carried out with FRET buffer. 50 μL of annealed DNA and 50 μL of ligand solution were placed in each well of a 96-well plate (MJ Research, Waltham, MA, USA) and measured in a DNA Engine Opticon (MJ Research). Fluorescence readings were taken at intervals of 0.5°C over the range 30-100°C, with a constant temperature maintained for 30 seconds prior to each reading. The incident radiation was 450-495 nm and the detection was set at 515-545 nm. Raw data were imported into the Origin program (Version 7.0, OringinLab Corp., USA) and graphs were smoothed using a 10-point running average and subsequently normalised. Determination of melting temperatures was performed by obtaining values at the maxima of the first derivative of the smoothed melting curves using a script. The difference between the melting temperature of each sample and that of a blank (ΔTm) was used for comparative purposes. .

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Table S1: Structures and average masses of oligonucleotides used in the study, and the average mass of the adducts formed from their covalent interaction with one molecule of **1** or **2** (as measured by MS).

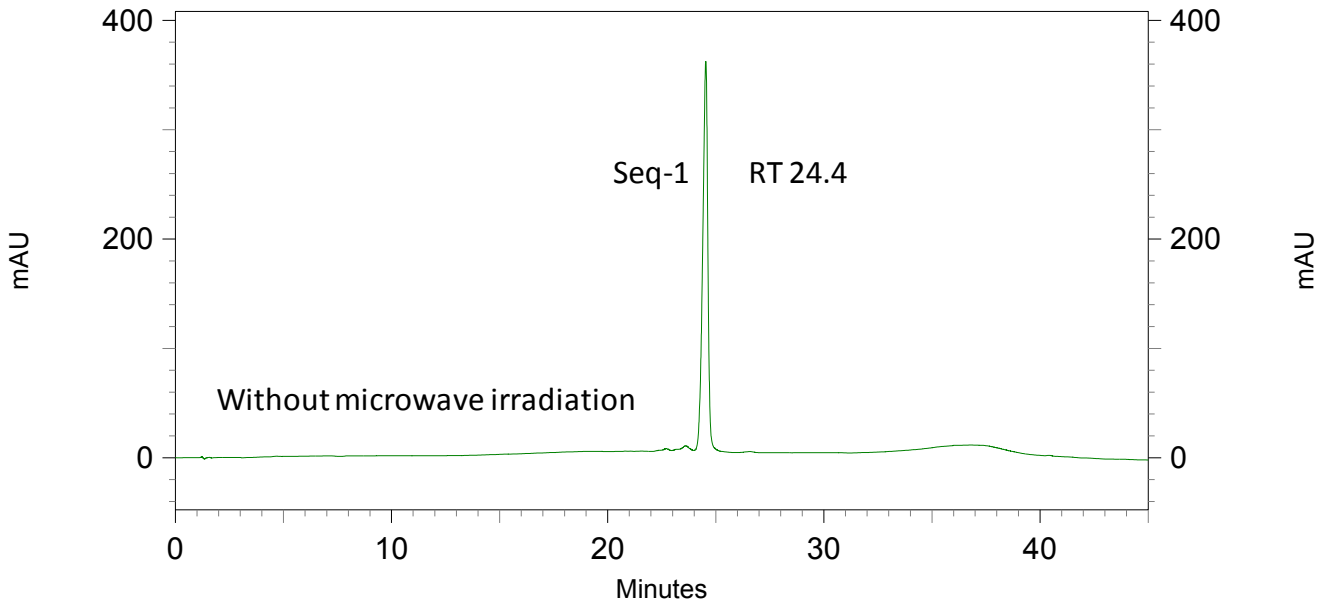
Label	DNA Sequence	Average Mass	Average Mass of Adduct	
			With **1**	With **2**
Seq-1/Seq1	5′-TATAGATCTATA-3′ 3′-ATATCTAGATAT-5′	7287.4	7844.01 (1:1)	8468 (2:1)
Seq-2	5′-TATAAGATTTTCTTATA-3′	5173.45	n/a	5764.06 (1:1)

Table S2 : Quantitative HPLC data using *Seq-1/Seq-1* duplex and *Seq-2* as external standards.

Reaction type	**% completion after immediate analysis (*i.e.*, 5 mins)**
SJG-136 (**1**) with *Seq-1/Seq-1* duplex at RT without microwave irradiation	36.7
SJG-136 (**1**) with *Seq-1/Seq-1* duplex after 30 seconds MW irradiation at 40°C	92.8
SJG-136 (**1**) with *Seq-1/Seq-1* duplex after 3 x 10 seconds MW irradiation at 27°C	92.6
SJG-136 (**1**) with *Seq-1/Seq-1* duplex after heating at 40ºC in an incubation block for 5 minutes	37.8
GW-78 (**2**) with *Seq-1/Seq-1* duplex at RT without microwave irradiation	10.8
GW-78 (2) with *Seq-1/Seq-1* duplex after 30 seconds MW irradiation at 40°C	67.1
GW-78 (**2**) with *Seq-2* hairpin at RT without microwave irradiation	27.5
GW-78 (**2**) with *Seq-2* hairpin after 30 seconds MW irradiation at 40°C	96.2
GW-78 (**2**) with *Seq-2* hairpin after 3 x 10 seconds MW irradiation at 27°C	97.1

Figure S1

A.



B.

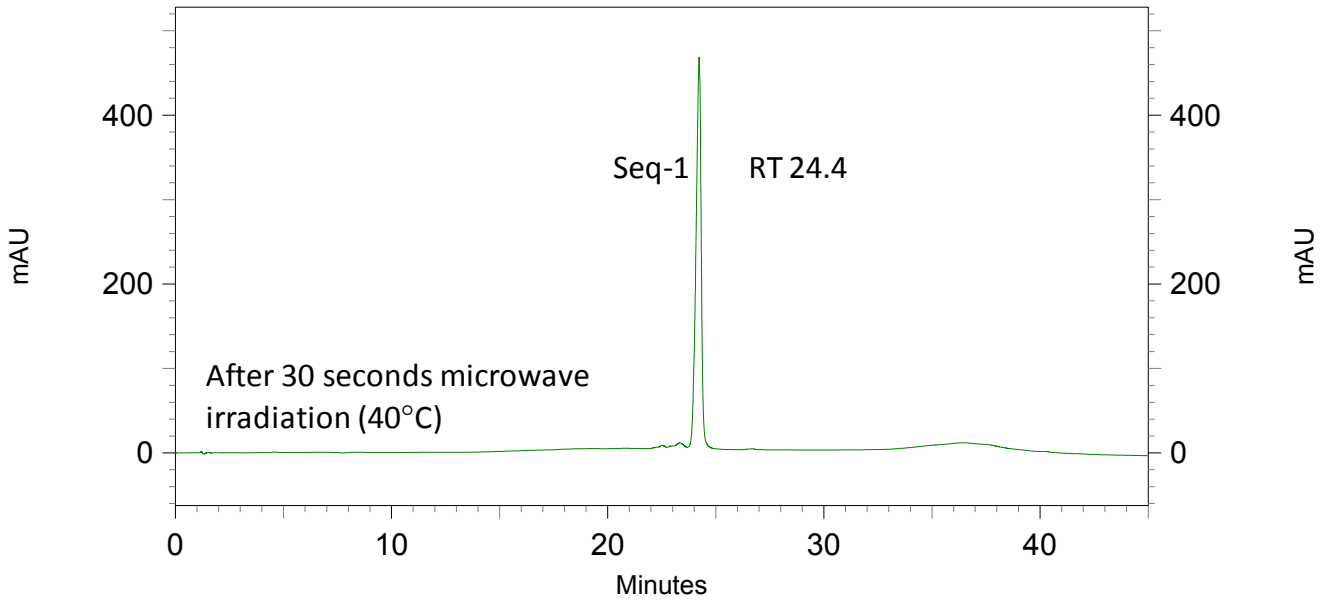
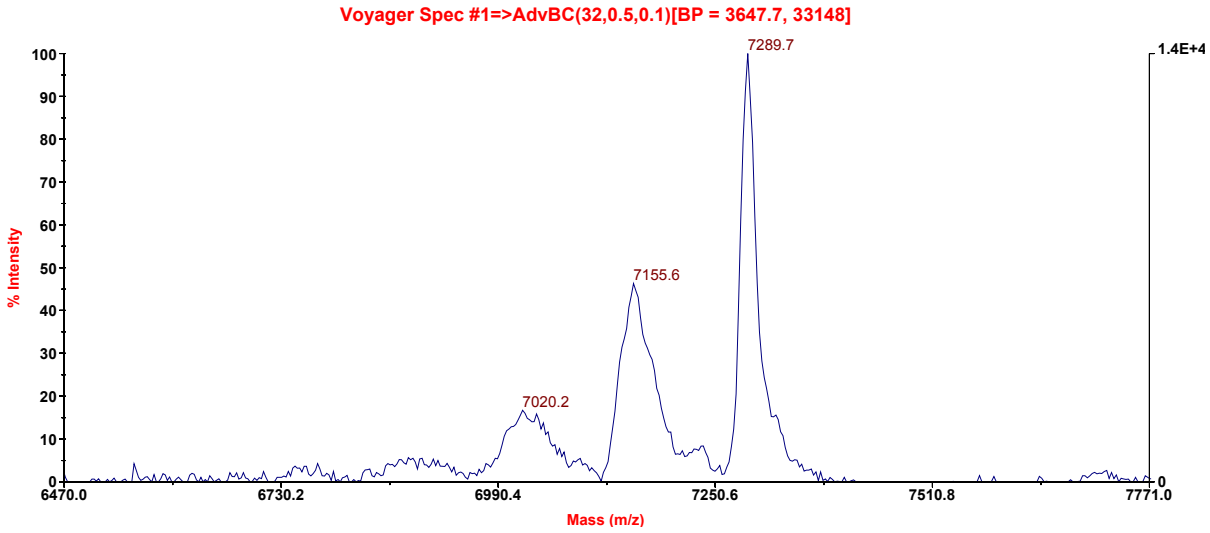


Figure S1 : HPLC chromatograms of *Seq-1/Seq1* duplex- **A**, without microwave irradiation; **B**, after 30 seconds of microwave irradiation at 40°C showing no change in retention time or peak pattern.

Figure S2

A.



B.

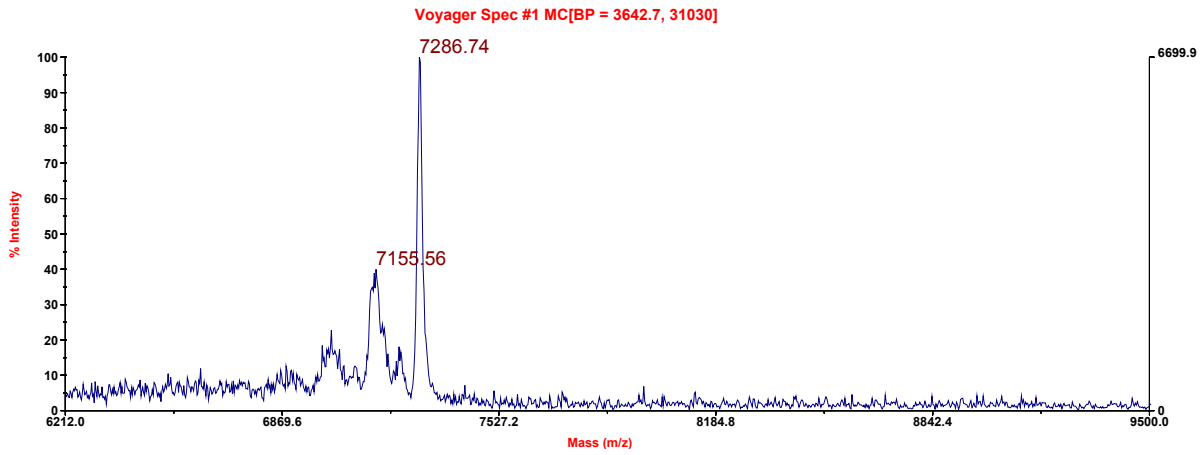
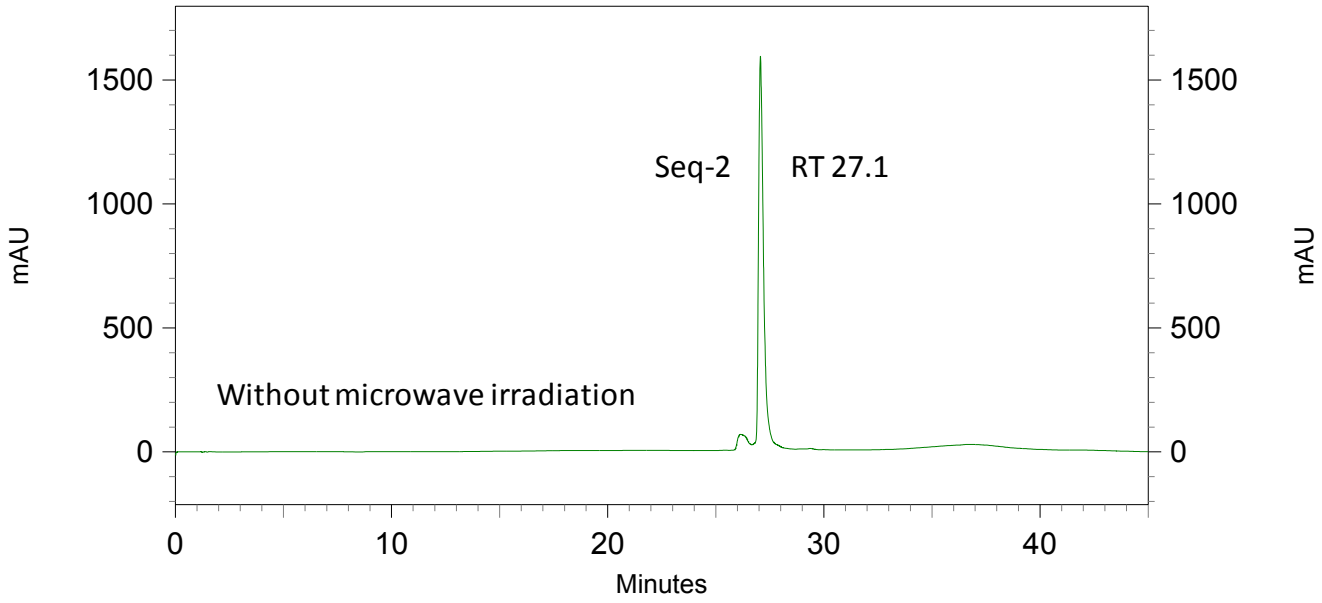


Figure S2 : MALDI-TOF MS spectrum of *Seq-1/Seq-1* duplex - **A**, without microwave irradiation, m/z 7287.4, observed m/z 7289.7; **B**, after 30 seconds of microwave irradiation at 40°C showing no change in mass, m/z 7287.4, observed m/z 7286.7.

Figure S3

A.



B.

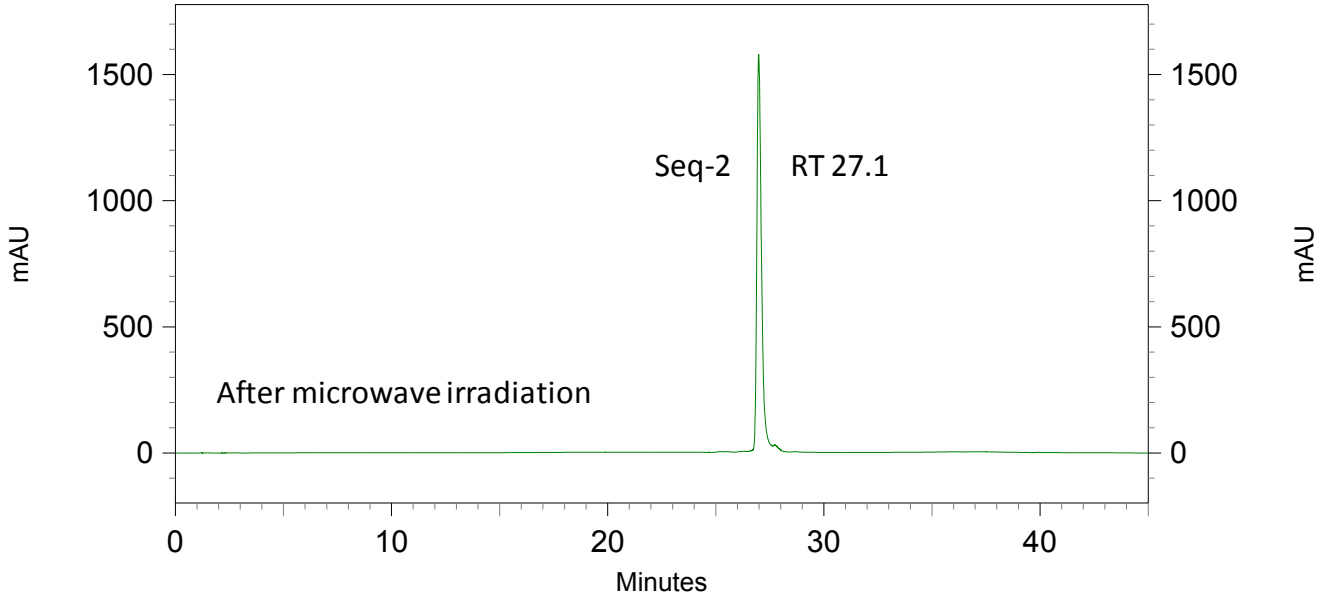
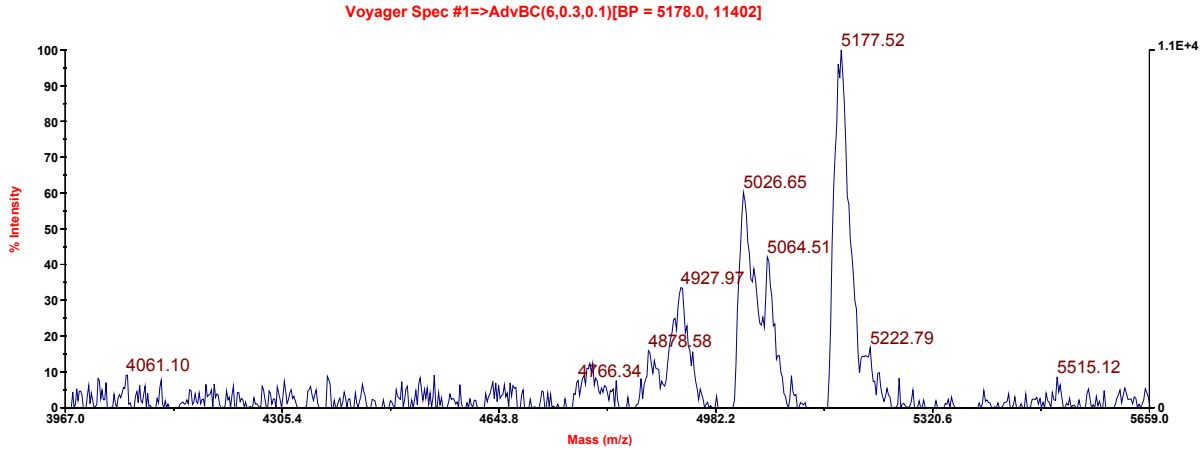


Figure S3 : HPLC chromatograms of *Seq-2* hairpin DNA- **A**, without microwave irradiation; **B**, after 30 seconds of microwave irradiation at 40°C, showing no significant change in retention time or peak pattern.

Figure S4

A.



B.

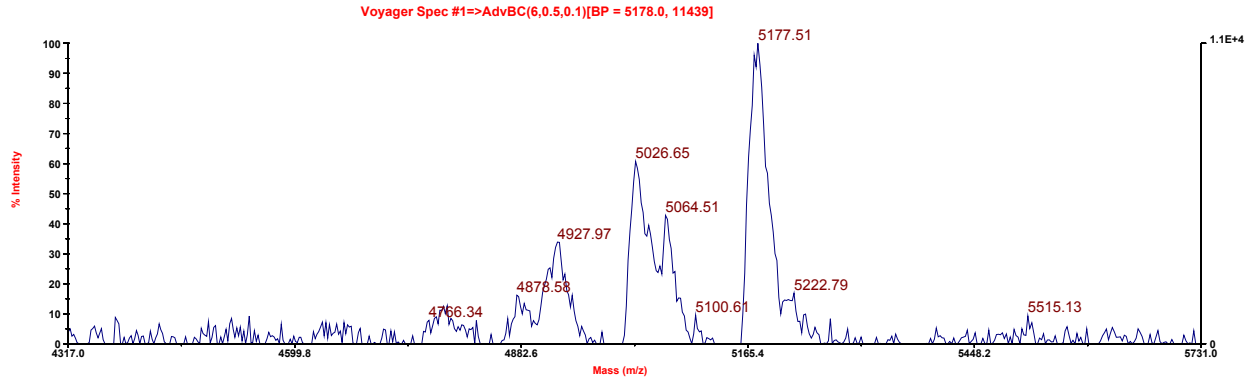


Figure S4 : MALDI-TOF MS spectrum of *Seq-2* hairpin DNA - **A**, without microwave irradiation, m/z 5173.4, observed m/z 5177.5; **B**, after 30 seconds of microwave irradiation at 40°C showing no change in mass, m/z 5173.4, observed m/z 5177.5.

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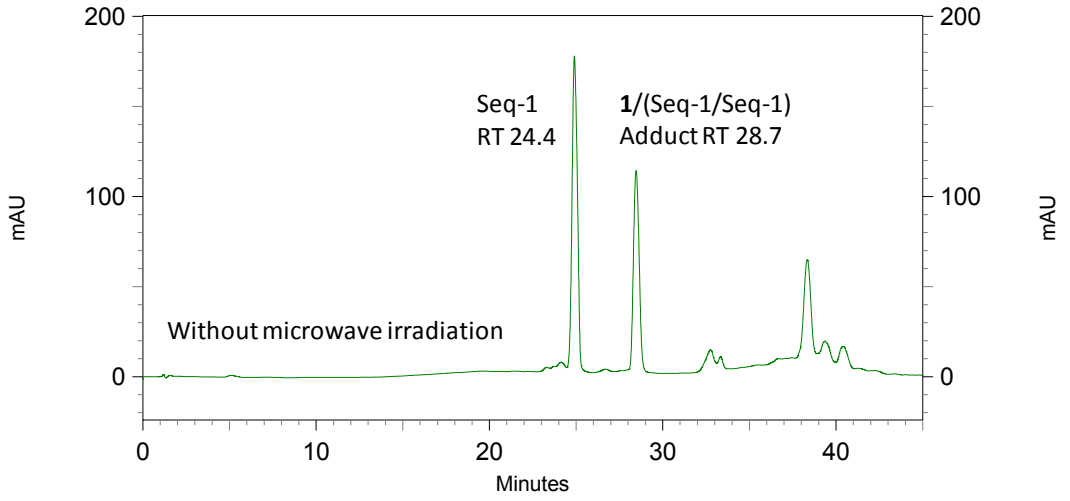
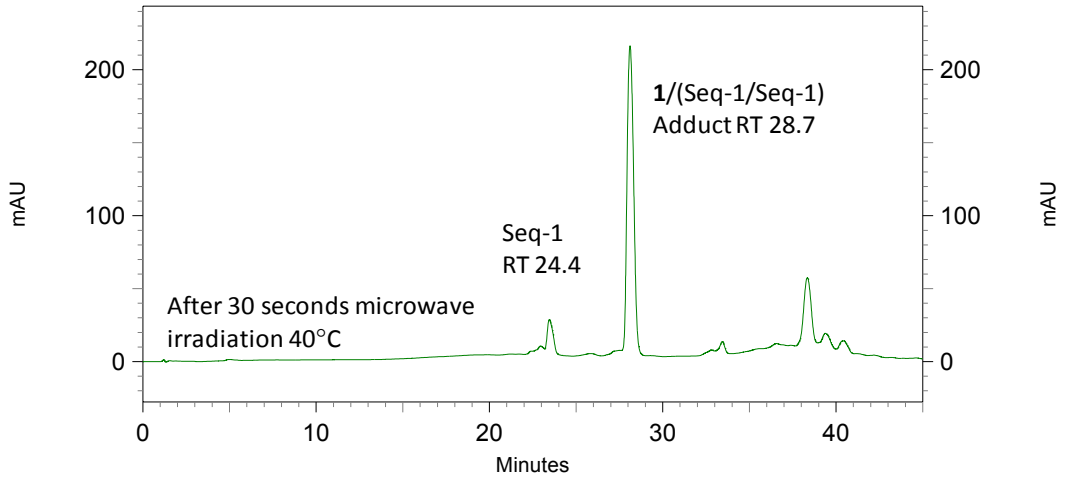
Figure S5

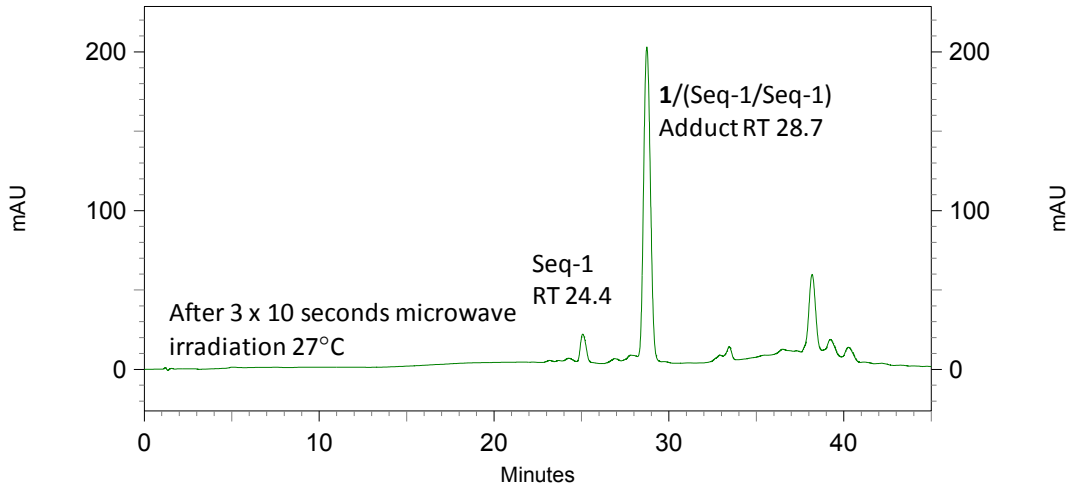
A.



B.



C.



D.

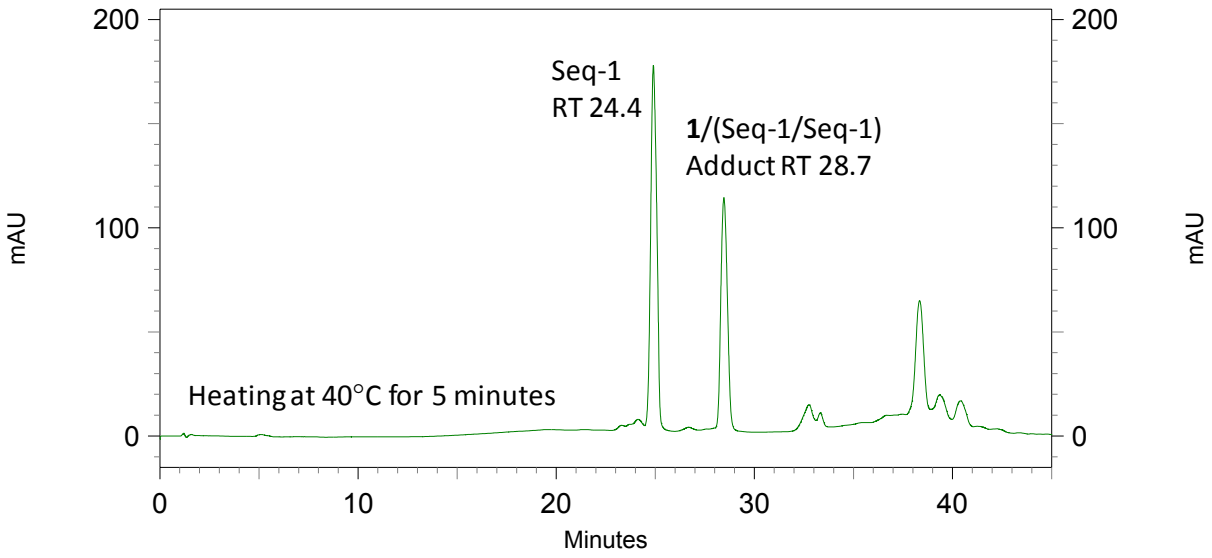


Figure S5 : HPLC chromatograms: **A, 1**/(*Seq-1/Seq-1*) duplex adduct and un-reacted/(*Seq-1/Seq-1*) duplex immediately after incubating **1** with *Seq-1/Seq-1* duplex and injecting onto the HPLC (no microwave irradiation); **B**, immediately after incubating **1** with *Seq-1/Seq-1* duplex and injecting onto the HPLC (after 30 seconds of microwave irradiation at 40°C); **C**, immediately after incubating **1** with *Seq-1/Seq-1* duplex and injecting onto the HPLC (after 3x10 seconds pulse of microwave irradiation at 27°C); **D**, immediately after heating **1** and *Seq-1/Seq-1* incubation mixture for 5 minutes at 40ºC in an incubation block.

Figure S6

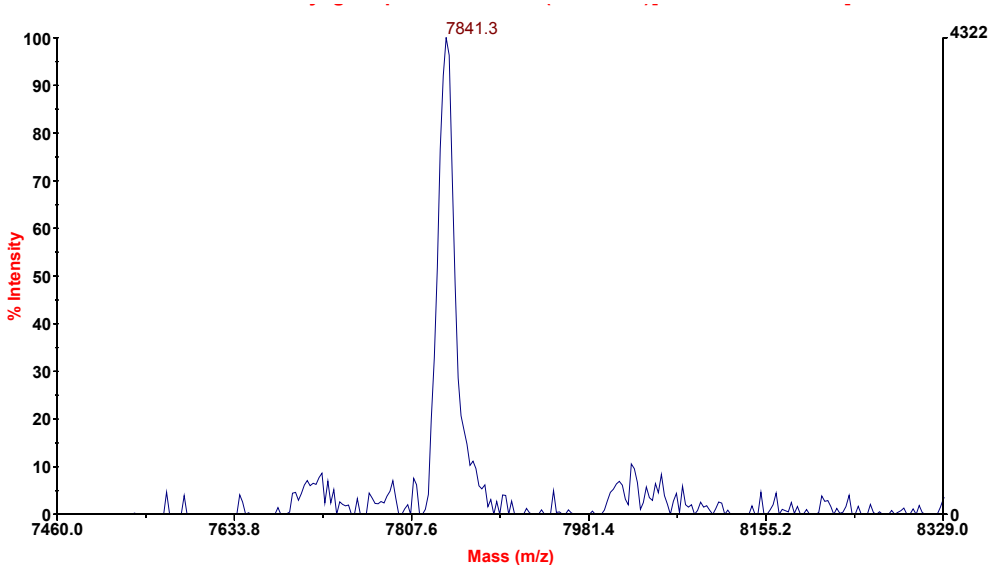
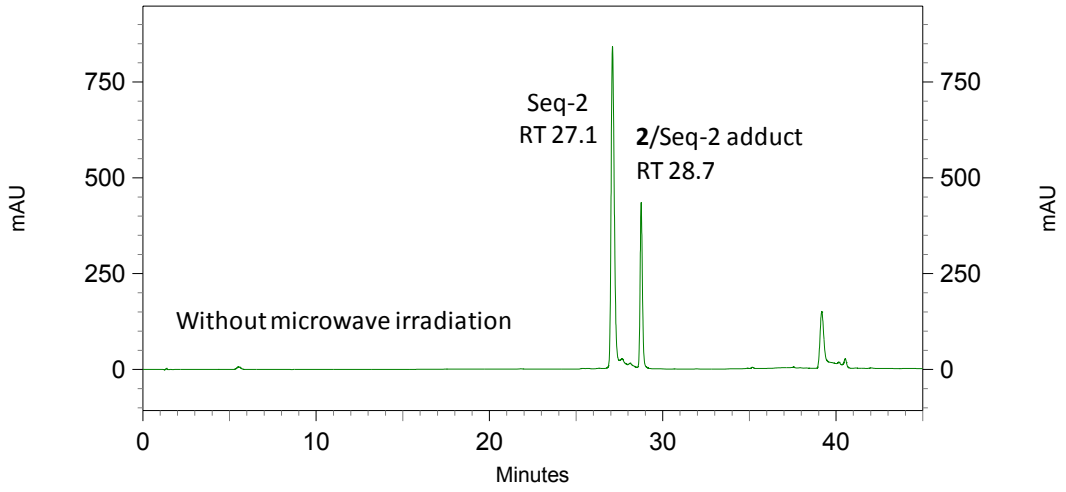


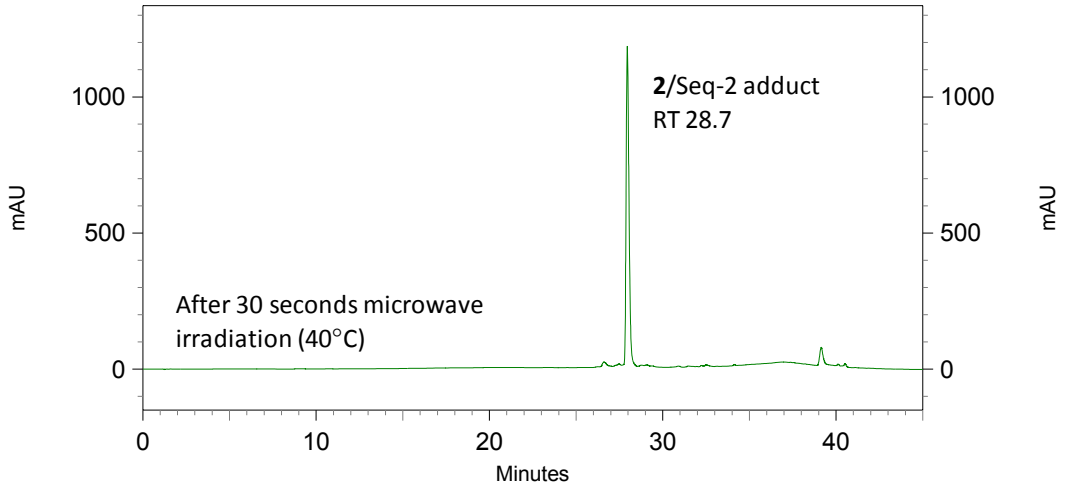
Figure S6 : MALDI-TOF MS spectrum of 1:1 **1**/(*Seq-1/Seq-1*) duplex adduct, m/z 7844, observed m/z 7841.3.

Figure S7

A.



B.



C.

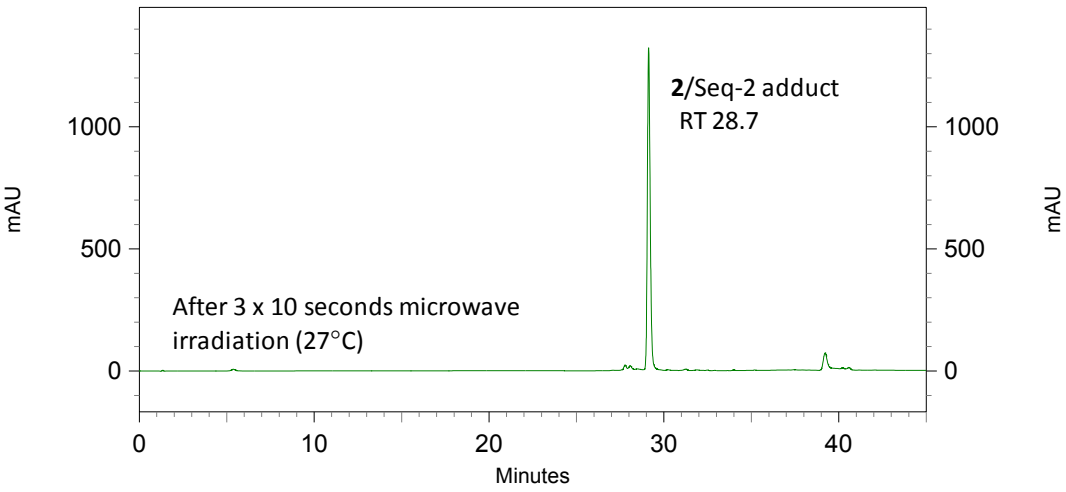


Figure S7 : HPLC chromatograms: **A, 2**/*Seq-2* hairpin adduct and un-reacted *Seq-2* hairpin , immediately after incubating **1** with *Seq-2* hairpin and injecting onto the HPLC (no microwave

irradiation) **B**, immediately after incubating **1** with *Seq-2* hairpin and injecting onto the HPLC (after 30 seconds of microwave irradiation at 40°C); **C**, immediately after incubating **1** with *Seq-2* hairpin and injecting onto the HPLC (after 3 x 10 seconds pulse of microwave irradiation at 27°C).

Figure S8

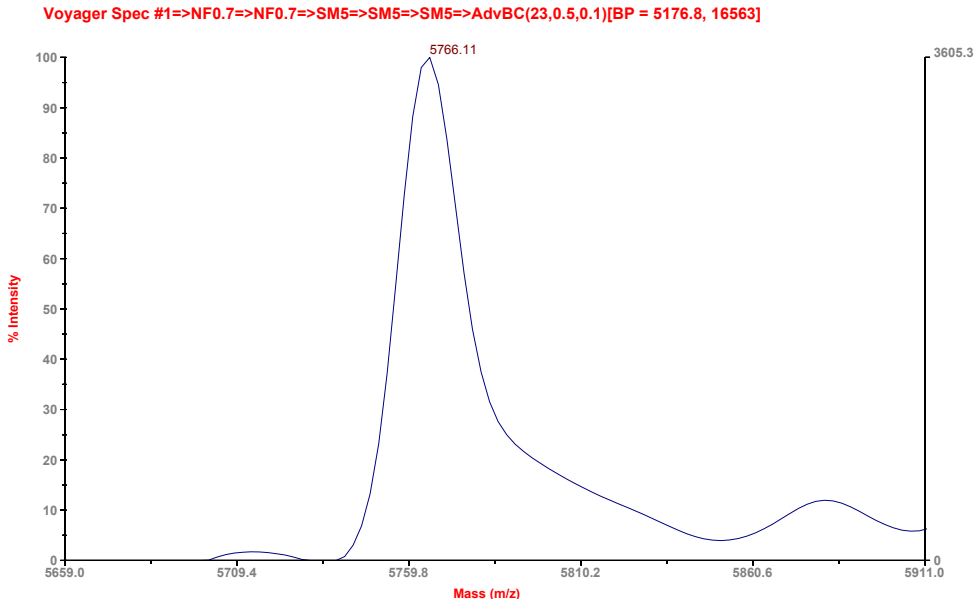
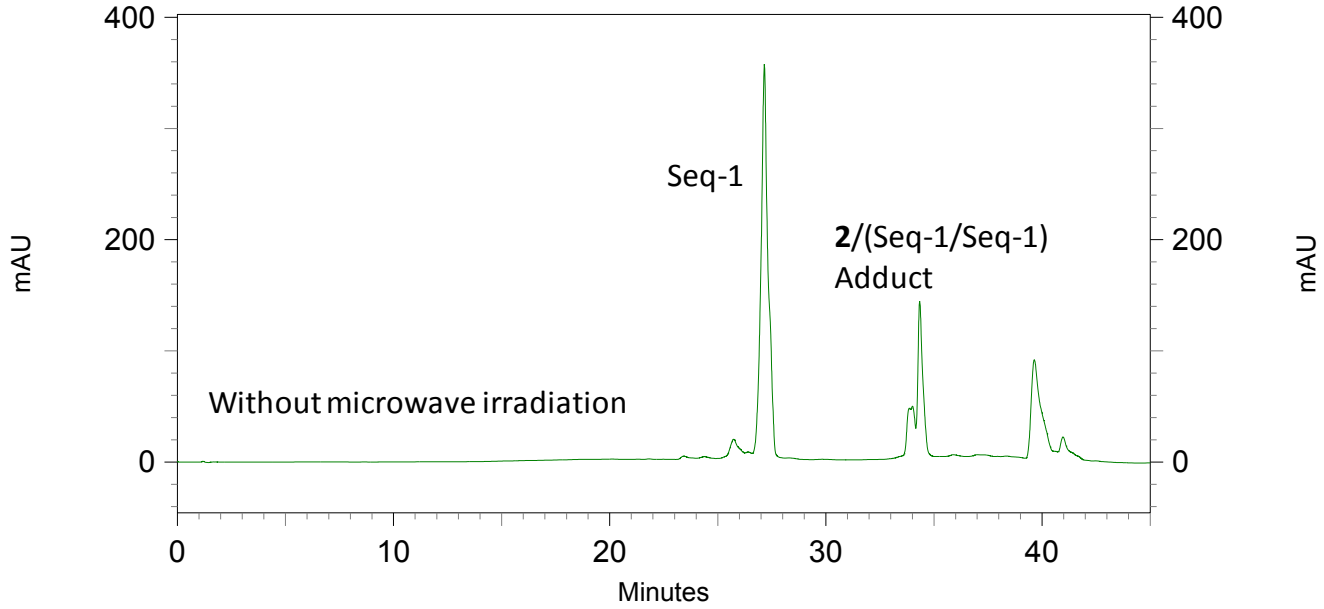


Figure S8 : MALDI-TOF MS spectrum of 1:1 **2**/*Seq-2* hairpin adduct, m/z 5764.1, observed m/z 5766.1.

Figure S9

A.



B.

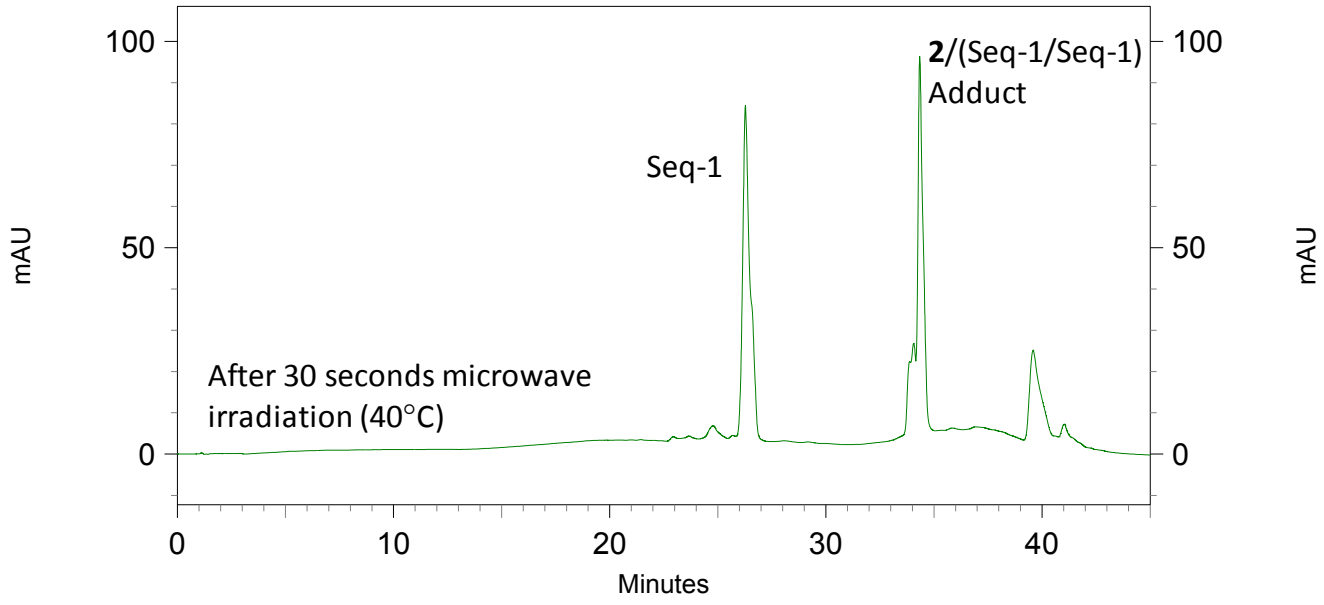


Figure S9 : HPLC chromatograms: **A,** **2**/(*Seq-1/Seq-1*) duplex adduct and un-reacted (*Seq-1/Seq-1*) duplex immediately after incubating **2** with *Seq-1/Seq-1* duplex and injecting onto the HPLC (no microwave irradiation); **B**, immediately after incubating **2** with *Seq-1/Seq-1* duplex and injecting onto the HPLC (after 30 seconds of microwave irradiation at 40°C).

Figure S10

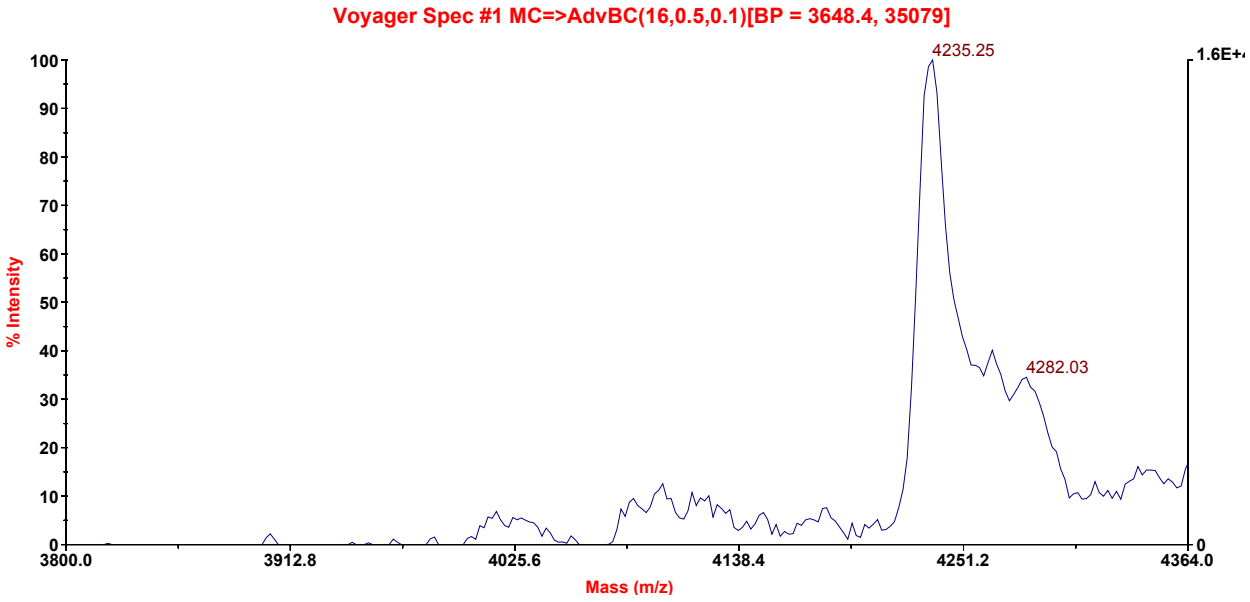


Figure S10 : MALDI-TOF MS spectrum of 2:1 **2**/(*Seq-1/Seq-1*) duplex adduct. Due to the relative instability of the **2**/(*Seq-1/Seq-1*) duplex adduct (**2** being a monoalkylator), a mass corresponding to 2:1 **2**/(*Seq-1/Seq-1*) was not observed in the MALDI spectrum. Instead, a mass for the 1:1 **2**/*Seq-1* adduct (*m/z* 4234.1, observed *m/z* 4235.25) was observed.

Figure S11

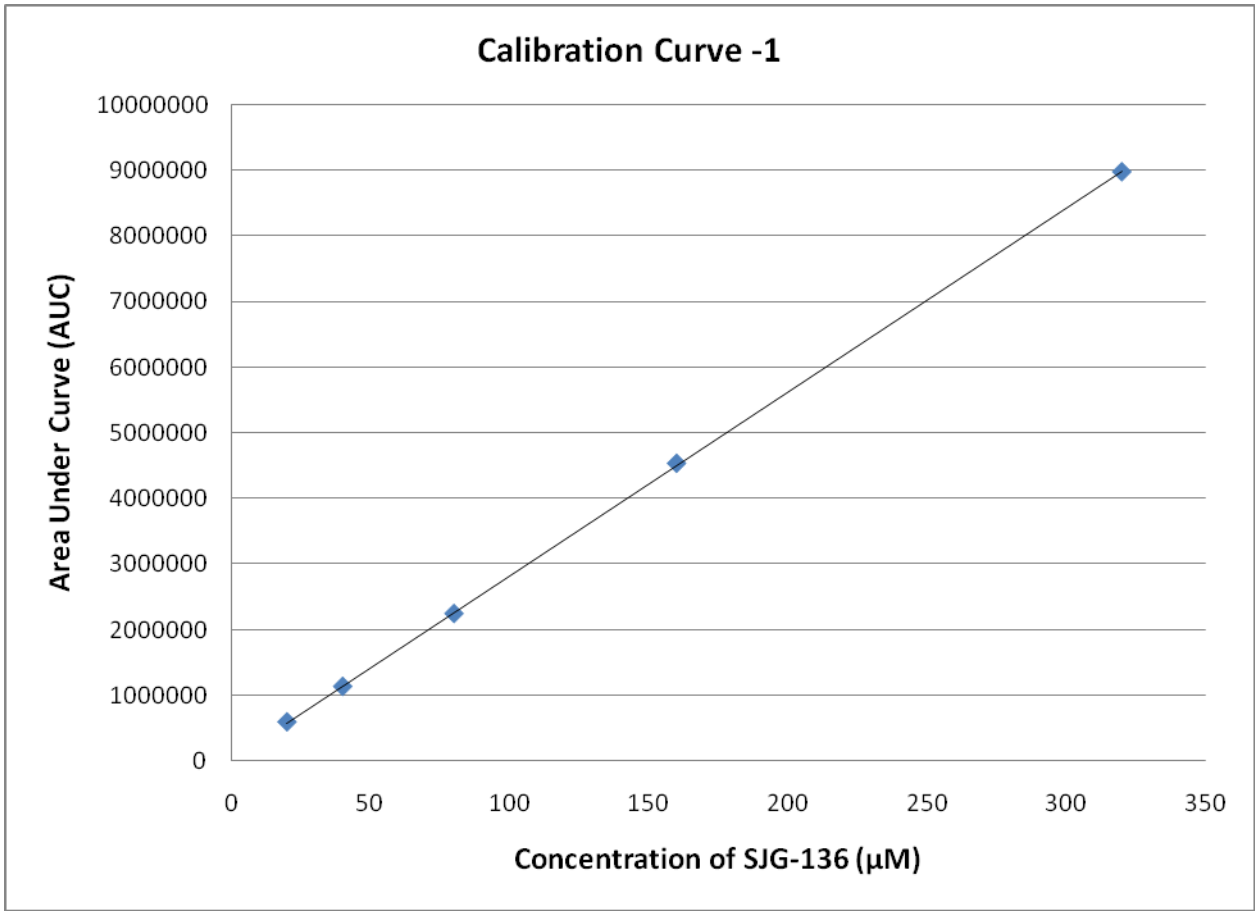


Figure S11 : HPLC calibration curve using SJG 136 (**1**) as the external standard.

Figure S12

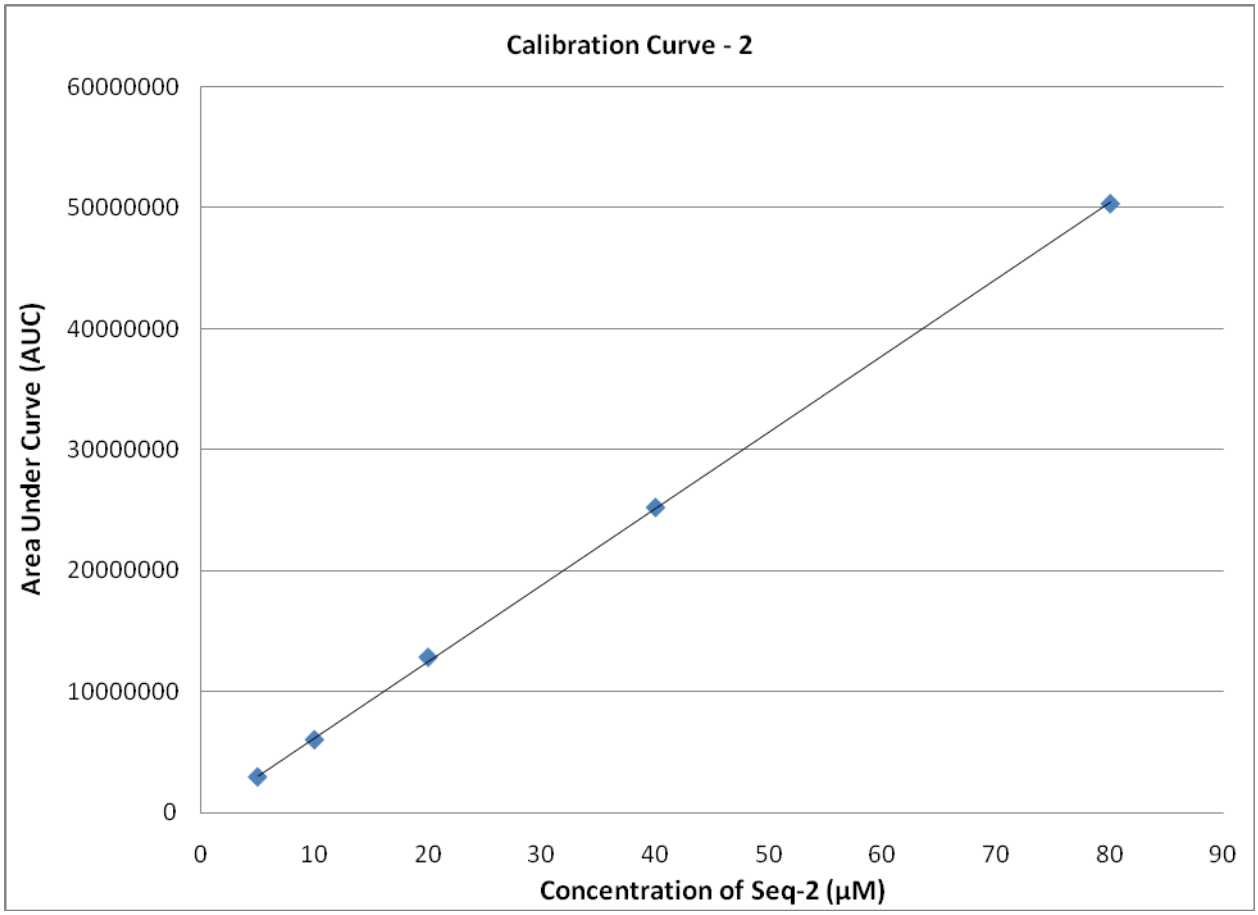


Figure S12 : HPLC calibration curve using *Seq-2* as the external standard.