Journal of Visualized Experiments

Uracil-DNA Glycosylase Assay by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry Analysis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63089R4
Full Title:	Uracil-DNA Glycosylase Assay by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry Analysis
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Taipei, Taiwan
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TITLE:

Uracil-DNA Glycosylase Assay by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry Analysis

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KEYWORDS:

Base excision repair, MALDI-TOF mass spectrometry, Uracil-DNA glycosylase, apurinic/apyrimidinic site, glycosylase inhibitor

SUMMARY:

A non-labeled, non-radio-isotopic method to assay uracil-DNA glycosylase activity was developed using MALDI-TOF mass spectrometry for direct apurinic/apyrimidinic site-containing product analysis. The assay proved to be quite simple, specific, rapid, and easy to use for DNA glycosylase measurement.

ABSTRACT:

Uracil-DNA glycosylase (UDG) is a key component in the base excision repair pathway for the correction of uracil formed from hydrolytic deamination of cytosine. Thus, it is crucial for genome integrity maintenance. A highly specific, non-labeled, non-radio-isotopic method was developed to measure UDG activity. A synthetic DNA duplex containing a site-specific uracil was cleaved by

UDG and then subjected to Matrix-assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. A protocol was established to preserve the apurinic/apyrimidinic site (AP) product in DNA without strand break. The change in the m/z value from the substrate to the product was used to evaluate uracil hydrolysis by UDG. A G:U substrate was used for UDG kinetic analysis yielding the K_m = 50 nM, V_{max} = 0.98 nM/s, and K_{cat} = 9.31 s^{-1}. Application of this method to a uracil glycosylase inhibitor (UGI) assay yielded an IC_{50} value of 7.6 pM. The UDG specificity using uracil at various positions within single-stranded and double-stranded DNA substrates demonstrated different cleavage efficiencies. Thus, this simple, rapid, and versatile MALDI-TOF MS method could be an excellent reference method for various monofunctional DNA glycosylases. It also has the potential as a tool for DNA glycosylase inhibitor screening.

INTRODUCTION:

Although uracil is a normal base in RNA, it is a common and highly mutagenic lesion in genomic DNA. Uracil can arise from spontaneous/enzymatic hydrolytic deamination of a deoxycytidine. In each living cell, this deamination occurs 100–500 times per day under physiological conditions[1,2]. If these alterations are not repaired, there can be a change in the DNA sequence composition, causing mutation. As uracil in DNA prefers to pair with dATP during replication, if cytosine deaminates to uracil, in two replication events, there will be a new G:C to A:T transition mutation in half of the progeny DNA[3].

Among the cellular strategies to maintain genetic stability, base excision repair (BER) is an essential mechanism that repairs damaged bases, such as uracil, in DNA[4]. BER is a highly evolutionarily conserved process. There are two general BER pathways: the short-patch pathway leading to a repair tract of a single nucleotide and the long-patch pathway that produces a repair tract of at least two nucleotides[5]. BER is a coordinated mechanism that occurs in several steps. The first step in BER is the enzymatic hydrolysis of the damaged nucleotide base by a damage-specific DNA glycosylase to generate an apurinic/apyrimidinic (AP) intermediate site[6]. This is followed by the cleavage of the sugar-phosphate backbone at the AP site by an endonuclease, clean-up of the DNA ends by a lyase, gap-filling by a DNA polymerase, and sealing the final nick by a ligase[5].

Uracil-DNA glycosylase (UDG) hydrolyzes the uracil from uracil-containing DNA for BER in *Escherichia coli*. Conventional UDG assays using radiolabeled DNA involving different separation techniques[6-13] are usually time-consuming, labor-intensive, with costly labeling reagents, complicated procedures, and requiring intensive training and practice to reduce risks of exposure to radioactive materials. Fluorometric oligonucleotide assays have been developed as a replacement for radioisotope labeling[14], in addition to molecular beacons and Förster resonance energy transfer technology[15-20]. However, specific labeling is required for all the aforementioned methods. Recently label-free biosensors assays[21-23] and colorimetric methods based on the formation of a G-quadruplex[24-26] have been developed. However, multiple A:U pairs or specially designed sequences in the probes complicate enzyme unit definition.

MALDI-TOF MS is a technology that could be of great use in DNA analysis. Applications developed

include single-nucleotide polymorphism genotyping[27,28], modified nucleotide analysis[29], and DNA repair intermediate identification[30-34]. MALDI-TOF MS should be readily adopted for DNA glycosylase analysis to detect AP-site-containing DNA products. However, AP-sites in DNA are prone to strand break under many experimental conditions[33]. A UDG assay is presented here using MALDI-TOF MS to directly measure AP site production without significant strand-break noise. This label-free method is easy to work with and has a high potential for the pharmaceutical application of DNA glycosylase inhibitor screening.

PROTOCOL:

1. Substrate/template preparation

1.1. Design uracil substrate/template duplex with a balanced G+C content of ~50 ± 10% and minimum melting temperature of 50 °C for the duplex region.

NOTE: One nucleotide difference between 18 nt substrates and 19 nt templates (**Table 1** and **Figure 1**) helps better MS signal interpretation and appropriate annealing. The template strand serves as complementary DNA to generate A-U or G-U mismatches (**Table 1**) but can also be used as a reference signal in MS measurements. The use of HPLC-purified synthetic oligonucleotides is satisfactory for this study.

1.2. Dissolve DNA in 1 mM EDTA and 10 mM Tris-HCl(pH 8.0 at 25 ˚C) (TE) at a concentration of 100 µmol/L as a stock and store at -20 °C. Dilute this 20 µL stock with TE to a final volume of 800 µL (25-fold dilution to 4 µmol/L). Measure the absorbance of the DNA solution in a UV-visible spectrophotometer at λ = 260 nm to ensure the manufacturer's assigned concentration. For example: A260 = 0.204 for 4 µmol/L of U+9 and A260 = 0.192 for 4 µmol/L of T1 (**Table 1**).

1.3. Perform MALDI-TOF MS analysis (sections 4–6) for oligonucleotide quality control by inspecting unique peak signals at designated m/z values as well as by checking that the signal-to-noise ratio is >100 (**Figure 1B** and **Figure 1D**).

2. DNA glycosylase assay

2.1. Using a G:U substrate of T1/U+9 duplex (**Table 1** and **Figure 1A**) for the glycosylase reaction, for example, in a 1.5 mL sterile microcentrifuge tube, add 70 µL of H_2O, 10 µL of 10x UDG reaction buffer, 5 µL of T1 stock, and 5 µL of U+9 stock (step 1.2.).

NOTE: Choose the correct type of micropipette and follow the manufacturers' instructions to handle the required volume. For example, use a 2 µL pipette to dispense 0.1–2 µL of liquid, use a 10 µL pipette to dispense 2–10 µL of liquid, and use a 100 µL pipette to dispense 20–100 µL of liquid to ensure accuracy and precision of the results. The described volume of the reagent mix is for 10 assays; adjust the volume for the desired number of reactions. The 1x UDG reaction buffer contains 1 mM EDTA, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0 at 25 ˚C). See the **Table of Materials** for the source of 10x UDG reaction buffer.

2.2. Securely close the tube; incubate in a water bath for 30 min at 65 °C, then for 30 min at 37 °C, and finally on ice for 3 min to ensure proper annealing of the substrate/template duplex.

2.3. In a 1.5 mL sterile microcentrifuge tube, add 49 µL of ice-cold 1x UDG reaction buffer and 1 µL of UDG (5,000 units/mL; see the **Table of Materials**), diluting to 0.1 units/µL. Make serial dilutions with 1x UDG buffer to the desired enzyme concentrations of 0.05, 0.02, or 0.01 units/µL. Always keep the diluted UDG on ice.

NOTE: In a 10 µL reaction, 0.1 units of UDG can cleave more than 30 pmol of uracil from a T1/U+9 duplex in 3 min.

2.4. In a 1.5 mL sterile microcentrifuge tube, add 9.0 µL of substrate mix from step 2.2 and prewarm the tube to 37 °C. Add 1.0 µL of the diluted UDG from step 2.3. Use a timer to time the reaction and flick the tube to mix contents.

NOTE: For a unit definition assay, the incubation time is 30 min. For a kinetic assay, use a time-course analysis of 0.5, 1, 2, 3, 5 min to obtain the initial rate. For a UGI inhibition assay, time the reaction for 15 min.

2.5. Centrifuge the tubes with the reaction mixture for 3–5 s at 3,200 × *g* at ambient temperature. Then, transfer the reaction immediately to 37 °C.

2.6. Reaction termination

2.6.1. Prepare solutions of 0.25 M HCl and 0.23 M Tris base. In a 15 mL test tube, add 10 mL of a solution of 1 mM EDTA and 20 mM Tris-HCl (pH 8.0 at 25 ˚C) to mimic 1x UDG reaction buffer. Acidify with 1 mL of 0.25 M HCl and check with a pH meter to ensure the pH is ~2 ± 0.5. Neutralize with 1 mL of 0.23 M Tris base and check with a pH meter for a final pH of 6.5 ± 0.5.

2.6.2. Add 1 µL of 0.25 M HCl to acidify the reaction mixture to inactivate the enzyme and place it on ice for 6 min. Add 1 µL of 0.23 M Tris base to neutralize the DNA products to avoid AP site breakage by prolonged exposure to acid. Add 13 µL TE to increase the volume of the product mixture for **matrix chip** transfer and then place it on ice.

NOTE: The AP product is chemically unstable and should be analyzed by MALDI-TOF MS within 2 days. After prolonged storage for more than a week, the accumulation of a substantial portion of strand breaks occurs due to β/δ elimination reactions of the AP products (**Figure 1D–G**).

2.7. Transfer all the 25 µL UDG reaction products from microcentrifuge tubes to a 384-well microtiter plate.

NOTE: High concentrations of cations, such as sodium or potassium in buffers, generate interference in the MALDI-TOF MS analysis and thus require desalting. As *E. coli* UDG reaction

buffer contains very low concentrations of cations, desalting is not necessary. However, modify this protocol to measure other DNA glycosylase reactions containing metal cations that require desalting as described previously[35].

3. Transfer UDG reaction products to a matrix chip

3.1. Open the door of the nanoliter dispenser (see the **Table of Materials**) and load the 384-well microtiter plate from step 2.7 onto the plate holder of the deck.

3.2. Insert the matrix chip array into the corresponding scout plate position. Place the loaded scout plate on the processing deck of the nanoliter dispenser and close the door.

3.3. Touch the **run** button on the transfer screen, and wait for the instrument to start dispensing samples from the 384-well microtiter plate to the matrix chip.

3.4. Use the **Vision tab** option to show the image of the chip and the dispense volumes for each spot during dispensing. Ensure the spotted volume on the chip is in the range of 5–10 nL.

4. Setup the assay parameters on the mass spectrometer

4.1. Use the application program (see the **Table of Materials**) to prepare a .xlsx file containing the predicted signal m/z value for importing.

NOTE: The settings in **FILE I.xlsx** (**Table 2**) are example settings for the UDG assay of G:U substrate in section 2.

4.2. Use the application program to create and define a new UDG assay by right-clicking **Import Assay Group in Designer Format** option and selecting the .xlsx file from the dropdown list (*e.g.*, **FILE I.xlsx** from step 4.1).

4.3. Right-click the **Customer:Project:Plate** button and click on the top of the dropdown **option** tree to establish a new assay plate. In the dialog box, type in a file name (*e.g.*, **CTT20210620** for the lab code and assay date), and in the **plate type** dropdown, select the **384-well plate** type and press **OK**. Look for a blank plate to appear on the right of the screen.

4.5. Click the **Assay** option; select the **assay** (*e.g.*, **FILE I.xlsx**) from the dropdown list.

4.6. To assign the selected assay (*e.g.*, **FILE I.xlsx**) to each sample spot position on the plate, move the cursor to each position of the blank plate, click to highlight the well, and right-click to select **Add Plex**.

4.7. Use a desktop or laptop computer to prepare a working list in .xlsx format with no header (*e.g.*, **0620.xlsx** of **Table 3**) for all the samples on the chip from step 2.7. Click the **Add New**

Sample Project button; select the file (*e.g.*, **0620.xlsx**) from the dropdown list to import the working list.

4.8. Look for all the test sample codes in the working list (*e.g.*, from **0620.xlsx**) on the left of the screen. Click the **sample code** in the working list, and right-click on the corresponding position of the plate to link the tests to each position.

5. Mass spectrometer operation

5.1. Use the application program to link the mass spectrometer (see the **Table of Materials**) to the sample chip (from section 3) to be analyzed.

5.2. Click on the **default setting**. In the dialog box, type in a file name from step 4.3 (*e.g.*, **CTT20210620**); in the **Experiment Name**, type in the chip ID in the **Chip Barcode**, and save the settings.

5.3. Start the mass spectrometer control program (see the **Table of Materials**).

5.4. Push the **In/Out** button of the mass spectrometer and let the deck extend. Take out the chip holder and insert the sample chip from step 3.4 into the chip holder. Place the loaded chip holder onto the extended deck and push the **In/Out** button for the sample chip to enter the instrument.

5.5. Double-click the **Acquire** icon of the application program. In the **Acquire** window, click the **auto run** tab to start the instrument and acquire mass spectra from the samples on the chip.

6. Viewing mass spectra and analyzing the data

6.1. Run the data analysis program (see the **Table of Materials**).

6.2. Browse the database tree and select the chip ID from step 5.2. Click to highlight a target well on the chip, and click the **spectrum** icon to show the mass spectrum.

6.3. Right-click to choose **Customization Dialog** to crop a specific range of spectrum in a new window. Click on the **X-Axis** to type in the upper and lower limits of m/z, and press **OK** to show the specified range spectrum, including the signals of interest.

NOTE: The amount of DNA is proportional to the peak intensity at each m/z value unit.

6.4. Measure the peak height of signals' m/z values corresponding to U substrate, AP-product, and template. Click on the peak and view the peak height in the upper left corner of the screen.

NOTE: A spectrum of 1,600 width/1,200 unit is a reasonable dimension for inspection on a computer screen as well as for recordkeeping.

6.5. To save the spectrum for recordkeeping, right-click **Export** and select **JPEG file** type in the dropdown list. Click on **Destination** and **Browse Disc** to select the **storage device** in the dropdown list (*e.g.*, flash disc E:). Type the **file name** (*e.g.*, 0620_1-2.jpg) and click **Export**.

6.6. If required, print out an exported JPEG file and measure the peak height manually using a ruler.

REPRESENTATIVE RESULTS:

Templates and substrates

Taking synthetic oligonucleotides with U in the center (U+9) paired with a G template as an example (**Figure 1A**), a blank control of equimolar amounts of template and uracil-containing substrate can be used for quality control of synthetic oligonucleotide purity (**Figure 1B**; the signals match the designated m/z and the low background noise). For the MS data analysis, the peak heights were measured (**Figure 2**). The 19 nt template DNA was expected to remain unchanged after glycosylase hydrolysis; therefore, the signal could serve as a reference for the quantitation of the AP-product (**Figure 1C**).

Reaction condition and mass spectra

This DNA glycosylase assay is simple and easy to perform using a non-labeled oligonucleotide for a standard reaction to obtain clean and reliable results **(Figure 1)**. The range of MS spectra should cover all the signals generated from substrates, templates, and reaction products (**Figure 1B**). The difference of 1 nt between the substrate and the corresponding template produced a well-separated signal profile for both the template and the substrate (**Figure 1B**). The equimolar primers U+9 and T1 showed similar peak intensities without significant differences. Extensive digestion of UDG (0.5 units for a 30 min reaction) demonstrated complete uracil cleavage. The signal of the AP-product was also well separated from that of the uracil-containing substrate (**Figure 1C** d+9 AP-product m/z = 5447.6 versus **Figure 1B** U+9 substrate m/z = 5541.6). The relatively mild MALDI ionization technique[36] used in this study minimized the signals associated with strand breaks induced by β-elimination reaction at AP sites[37], which were not significant in the mass spectra. Overall, the reaction background is very clean without noticeable noise (**Figure 1C**).

Under equimolar conditions, the relative signal intensity of the AP-product was comparable to that of the U-substrate using the T1 template as a reference (**Figure 1B,** U+9/T1 versus d+9/T1**)**. Thus, the calculation of UDG activity is based on the exact input of the U-containing substrate (50 pmol or 20 pmol) according to Eq (**1**).

$$\text{UDG activity} = (\text{signal of AP-product}) / (\text{Signal of U substrate} + \text{signal of AP-product}) \times [\text{U}] \quad (1)$$

UDG kinetic parameters determined by MALDI-TOF MS assay

As shown in **Figure 2**, throughoutf the 3 min reaction, UDG reactions from 0.01 units to 0.1 units demonstrated both dose- and time-dependence. A concentration of 3.2 pM (0.1 units per 100 µL reaction) was used for the determination of the kinetic parameters, K_m and k_{cat}, for a G:U substrate (**Table 1**). The aliquots of the UDG reaction were removed and quenched for 30 s and

60 s. Kinetic data were obtained under conditions when the uracil-containing substrate was less than 50% digested, and five substrate concentrations ranging from 10 to 200 nM were subjected to analysis. The presteady-state time course showed excellent linearity for rate analysis. An example of a reaction rate plot is shown in **Figure 3A**, where the relative MS signal intensities of the product and substrate are converted into concentration (nM). The UDG reaction rate (*v*) is presented as nM of the AP site produced per second. The *K*m and V_{max} were calculated from a Lineweaver-Burk plot (**Figure 3B**). The UDG kinetic parameters derived from the MALDI-TOF MS assay were thus determined to be *K*m = 50 nM, V_{max} = 0.98 nM s^{-1}, and K_{cat} = 9.31 s^{-1}. The values are comparable to the results of previous ^{3}H-uracil releasing assays where the *K*m = 40 nM and Kcat = 13.3 s^{-1} [38].

The G:U+9 duplex was subjected to UDG sensitivity assays. The unit measured by MALDI-TOF MS assay was plotted against the manufacturer's defined unit by a conventional ^{3}H-uracil-releasing method[38]. As shown in **Figure 3C**, the MALDI-TOF MS-measured unit was proportional to the defined unit from 0.001 units to 0.02 units with the correlation equation of y = 0.933x + 0.0003 and coefficient of determination R^2 = 0.9974. The detection limit is 0.001 units as the coefficient of variation = 9.2%, ~5 times the signal-to-noise ratio. Thus, this MALDI-TOF MS assay provides sufficient sensitivity as UDG is mostly used at the unit level[39].

Substrate specificity of UDG

One of the characteristic features of this UDG MALDI-TOF MS assay is that it is label-free, which renders high versatility for substrate design. This feature is very useful for analyzing the substrate specificity of UDG. As demonstrated in **Table 1**, uracil could be inserted at any place near the 5' or 3' end of single-stranded or double-stranded DNA for a specificity assay. For substrate specificity, it is well known that UDG is highly active in removing uracil from single-stranded DNA[38]. Indeed, as shown in **Table 1**, uracil excision from the single-stranded substrate (ssU) was decreased 3.7-fold when annealed to the complementary DNA strand, forming G:U duplex. For the commonly used A:U duplex[6-8], the reaction rate was further lowered by nearly 10-fold relative to ssU. The UDG did not act on U at the DNA terminus (**Table 1** substrates of G:U+1, G:U-1, and G:U-2), which is consistent with previous studies[40,41]. Interestingly, U at the 2nd and 3rd positions from the 5'-terminus exhibited a higher UDG catalytic rate than the U in the center by 2-fold and 1.5-fold, respectively (**Table 1**, G:U+2 and G:U+3 versus G:U). UDG excised U 3-nt from the 3'-end with 8% less activity than the U at the center (**Table 1**, G:U-3 versus G:U).

Uracil glycosylase inhibitor inhibition of the UDG reaction

Uracil glycosylase inhibitor (UGI) is a bacteriophage protein, which inhibits *E. coli* UDG by reversible protein binding with a stoichiometry of 1:1[42]. The inhibition of UDG activity by UGI is shown in **Figure 4**. In the presence of 0.05 units (100 pM) of UGI, the activity of 0.05 units of UDG (8 pM) was inhibited to an undetectable level. The IC_{50} was 7.6 pM. Thus, the UDG MALDI-TOF MS assay can be easily modified to a mass screening assay for the discovery of inhibitors of other DNA glycosylases.

FIGURE AND TABLE LEGENDS:

Figure 1: Model system for a DNA glycosylase assay. (**A**) Lesion strand containing a single uridine (U+9) was annealed to a template (T1), forming a G:U mismatch (bold). Uracil-DNA glycosylase detects and removes the uracil, forming an AP site (d). When subjected to MALDI-TOF MS, the difference in m/z values between U-containing DNA and AP products can be resolved as shown in **B**. (**B**) An 18 nt DNA containing a single uridine (U+9) annealed to a 19 nt template (T1) (**Table 1**) was tested as an enzyme blank of 50 pmol of substrate in 40 µL of reaction buffer and subjected to MS analysis. (**C**) A near-complete enzyme digestion of 50 pmol of substrate in a 10 µL reaction using 0.5 units of UDG at 37 ˚C for 30 min. (**D**) An 18 nt DNA containing a single uridine (U+3) annealed to T1 enzyme blank of 50 pmol substrate in 40 µL reaction buffer was subjected to MS analysis. (**E**) A near-complete enzyme digestion of 50 pmol of U+3 substrate in a 10 µL reaction using 0.5 units of UDG at 37 ˚C for 30 min to produce d+3 and subjected to MS analysis within 30 h. (**F**) The reaction condition was the same as in **E** except the d+3 product was in 0.1 M NaOH at 95 ˚C for 30 min to trigger a beta-elimination reaction producing B15 fragmented product and subjected to MS analysis. (**G**) The reaction condition was the same as in **E** except the d+3 product was stored at -20 ˚C for more than 7 days; a fraction of d+3 converted into B15 fragmented products. This figure is modified from [43]. Abbreviations: nt = nucleotide; MS = mass spectrometry; MALDI-TOF MS = Matrix-assisted Laser Desorption/Ionization time-of-flight MS; AP = apurinic/apyrimidinic; UDG = Uracil-DNA glycosylase; T1 = 19 nt G-containing template; U+9 = 18 nt U-containing substrate; d+9 = 18 nt AP site-containing product; U+3 = 18 nt U-containing substrate; d+3 = 18 nt AP site-containing product; B15 = 15 nt beta-elimination fragmented product.

Figure 2: Time course analysis for UDG activity at different enzyme concentrations. Substrate DNA, 50 pmol, containing a G:U lesion was incubated with 0.01, 0.02, 0.05, and 0.1 units of UDG at 37 ˚C. Aliquots (10 µL) were taken from the reaction mixture at 0, 0.5, 1, 2, and 3 min and quenched with an equal volume of phenol/chloroform. (**A**) MALDI-TOF mass spectra illustrating the concentration dependence of processing G:U substrates by UDG. (**B**) The amount of product was plotted as a function of time. UDG: 0.01 (closed triangles with solid line), 0.02 (open triangle with dashed line), 0.05 (closed circle with solid line), and 0.1 units (open circles with solid line). The data are the averages of three independent determinations, and the error bars represent 1 S.D. This figure is modified from [43]. Abbreviations: nt = nucleotide; MALDI-TOF = Matrix-assisted Laser Desorption/Ionization time-of-flight; AP = apurinic/apyrimidinic; UDG = Uracil-DNA glycosylase; T = 19 nt G-containing template; U = 18 nt U-containing substrate; AP = 18 nt AP site-containing product.

Figure 3: Determination of kinetic parameters of UDG using MALDI-TOF analysis. Michaelis-Menten curve and Lineweaver-Burk plots to determine the K_m and k_{cat} for UDG enzyme-catalyzed excision of Uracil from DNA. DNA glycosylase MALDI-TOF MS assay was performed using 0.1 units of UDG and different concentrations (10, 30, 60, 100, 200 nM) of substrates in 100 µL reaction for 30 s and 60 s. (**A**) Michaelis-Menten curve generated from three independent experiments. The error bars represent 1 SD. (**B**) Lineweaver-Burk plot for the assay; the indicated intercepts permit calculation K_m and V_{max}. (**C**) UDG assay by MALDI-TOF MS analysis compared to the manufacturer-assigned unit. Enzyme activity was measured by uracil removal forming AP site in a 10 µL reaction containing 50 pmol (0.56 µg) of G:U within an 18/19 nt DNA duplex for 30 min

at 37 °C. The UDG was diluted with buffer [50% glycerol, 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol] on ice. A unit was defined as the amount of enzyme that catalyzed the release of 60 pmol of uracil per minute. Error bars show the standard deviation of six experiments. This figure is modified from [43]. Abbreviations: nt = nucleotide; MALDI-TOF = Matrix-assisted Laser Desorption/Ionization time-of-flight; UDG = Uracil-DNA glycosylase; AP = apurinic/apyrimidinic; V, reaction velocity nM/s.

Figure 4: UDG enzyme inhibition curve by addition of UGI. The inhibition curve was determined using reactions of 20 µL with 0.05 units of UDG (8 pM) and 50 pmol of substrate for 15 min in the presence of UGI from 0.001 units (5 pM) to 0.1 units (200 pM). Data are the averages of three independent determinations, and the error bars represent 1 S.D. Data fit with an online IC_{50} calculator (see the **Table of Materials**). This figure is modified from [43]. Abbreviations: UDG = Uracil-DNA glycosylase; UGI = uracil glycosylase inhibitor.

Table 1: Substrate compositions and UDG specificities. This table shows the 18 nt U-containing DNA designated by U±N. The '+' is indicates counting the uracil position from the 5' terminus, and the '-' indicates counting the uracil position from the 3' terminus. For example, U+9 is the 9th position from the 5' terminus, U-3 is the 3rd position from the 3' terminus. The complementary 19 nt template of T1 to T7 would be paired with the U-containing DNA forming G:U or A:U mismatches as indicated. Uracil bases are in bold. Reactions were using 50 pmol of U substrates, 0.05 units of UDG, in 10 µL as described in protocol section 2. This Table is modified from [43]. Abbreviations: UDG = uracil DNA glycosylase; nt = nucleotide; ND, not detectable.

Table 2: FILE I.xlsx file. The settings were used for **Figure 1B, C**, and **Figure 2A**; 0 min entries. The MALDI-TOF MS system used in this study was designed specifically to analyze single-nucleotide polymorphism by a single nucleotide extension of the extended primer into the region of amplified gene sequence variation. For UDG assay, only six fields were used when preparing the assay group FILE I. Abbreviations: WELL = the well number assigned to the assay; TERM = termination mix; SNP_ID = name of the input sequence of the single nucleotide polymorphism; 2nd-PCRP = forward amplicon primer; 1st-PCRP = reverse amplicon primer; AMP_LEN = amplicon length; UP_CONF = uniplex amplification score; MP_CONF = multiplex amplification score; Tm = melting temperature; PcGC = percent GC content; PWARN = assay design warning codes; UEP_DIR = direction of primer extension; UEP_MASS = unextended-primer mass; UEP_SEQ = unextended-primer sequence; EXT1_CALL = name given to the analyte 1 mass peak; EXT1_MASS = mass of analyte 1.

Table 3: 0620.xlsx file. These settings were used for the UGI inhibition reactions in **Figure 4A**. Abbreviation: UGI = uracil glycosylase inhibitor.

DISCUSSION:

This paper provides a detailed procedure for using a UDG MALDI-TOF MS assay method to directly detect AP-containing DNA products. The main advantages of this method are that uracil-containing substrates are label-free, scalable, easy to work with, and afford greater flexibility in substrate design.

The UDG supplier-recommended phenol/chloroform extraction enables inactivation of the enzyme to prevent degradation of product DNA. However, the phenol extraction protocol involves tedious phase-separation of hazardous chemicals. An alternative acid termination method using HCl to lower the reaction pH to 2 ± 0.5 also effectively inactivated UDG. Subsequent neutralization with Tris base could prevent DNA damage. When the AP product was subjected to MS analysis within 30 h, there were no signs of base loss or modification in the mass spectra (**Figure 1E**). The Tris buffer in the reactions was provided with the commercial UDG. However, various amines, including Tris, can incise AP sites via beta-elimination, albeit at high reagent concentrations[44]. Some alternatives, such as HEPES and phosphate buffer, can be considered to avoid the risk of cleavage of the AP site by beta-elimination.

As a potential UDG reference method, a duplex of centered G:U substrate (**Table 1**, T1/U+9) is recommended as the standard substrate for the following reasons: 1. for physiological relevance, G:U is superior to A:U as deamination of cytosine in G:C pairs results in G:U lesions. 2. While *E. coli* UDG demonstrates higher specificity to hydrolyze U from single-stranded DNA, repair of a U lesion in single-stranded DNA is uncommon. 3. For all the G:U substrate tested, G:U+2 and G:U+3 demonstrated higher reaction rates than G:U+9. However, a DNA deamination lesion adjacent to a strand break is uncommon. For mimicking native lesions, placing a G:U at the center of the DNA duplex would be more appropriate.

Interestingly, the MALDI-TOF MS method generated UDG kinetic parameters, and the enzyme units of the G:U reaction (**Figure 3A–C**) were very similar to conventionally derived results using ^{3}H-uracil[38]. However, the single G:U substrate in this study is very different from PBS1 bacteriophage DNA previously described with multiple A:U from DNA synthesis errors[38]. Moreover, UDG showed 3 times higher activity with G:U than with the A:U substrate (**Table 1**, K_m and K_{cat} of G:U versus A:U). This seemingly contradictory result can be attributed to the DNA sequence of PBS1 substrate containing 36% of U in the form of multiple A:U lesions[45] compared to only 3% of U in the G:U substrate (**Table 1**). Meanwhile, UDG is a very 'processive' enzyme, i.e., a single protein–DNA binding event elicits multiple uracil cleavages[12]. The finding of a near-perfect one-to-one correlation between these two UDG methods makes this MS method an attractive option instead of the traditional radioisotope assay.

This approach is easily scalable. All UDG reactions in this study were performed manually using micropipettes and microcentrifuge tubes, with ~30 tests performed on a given day. Thus, less than one-tenth the capacity of the 384-well plate format chip array for the MALDI-TOF MS system (see the **Table of Materials**) described in sections 3–5 was used. In contrast, the adaptation of an automated pipetting system for a 384-well microplate could easily increase the daily output of this approach by using the acid termination method. Thus, 300 UDG reactions would take ~1 h. The MALDI-TOF MS system (see the **Table of Materials**) could output mass spectra data for up to 300 reactions in 1 h. Hence, a streamlined process would require 2 h to complete 300 UDG MALDI-TOF MS assays.

ACKNOWLEDGMENTS:

We thank the NCFPB Integrated Core Facility for Functional Genomics (Taipei, Taiwan) and the NRPB Pharmacogenomics Lab (Taipei, Taiwan) for their technical support. This work was supported by the Ministry of Science and Technology, Taiwan, R.O.C. [grant number MOST109-2314-B-002 -186 to K.-Y.S., MOST 107-2320-B-002-016-MY3 to S.-Y.C., MOST 110-2320-B-002-043 to W.-h.F.]. H.-L. C. is a recipient of a doctoral fellowship from National Taiwan University. Funding for open access charge: Ministry of Science and Technology, R.O.C.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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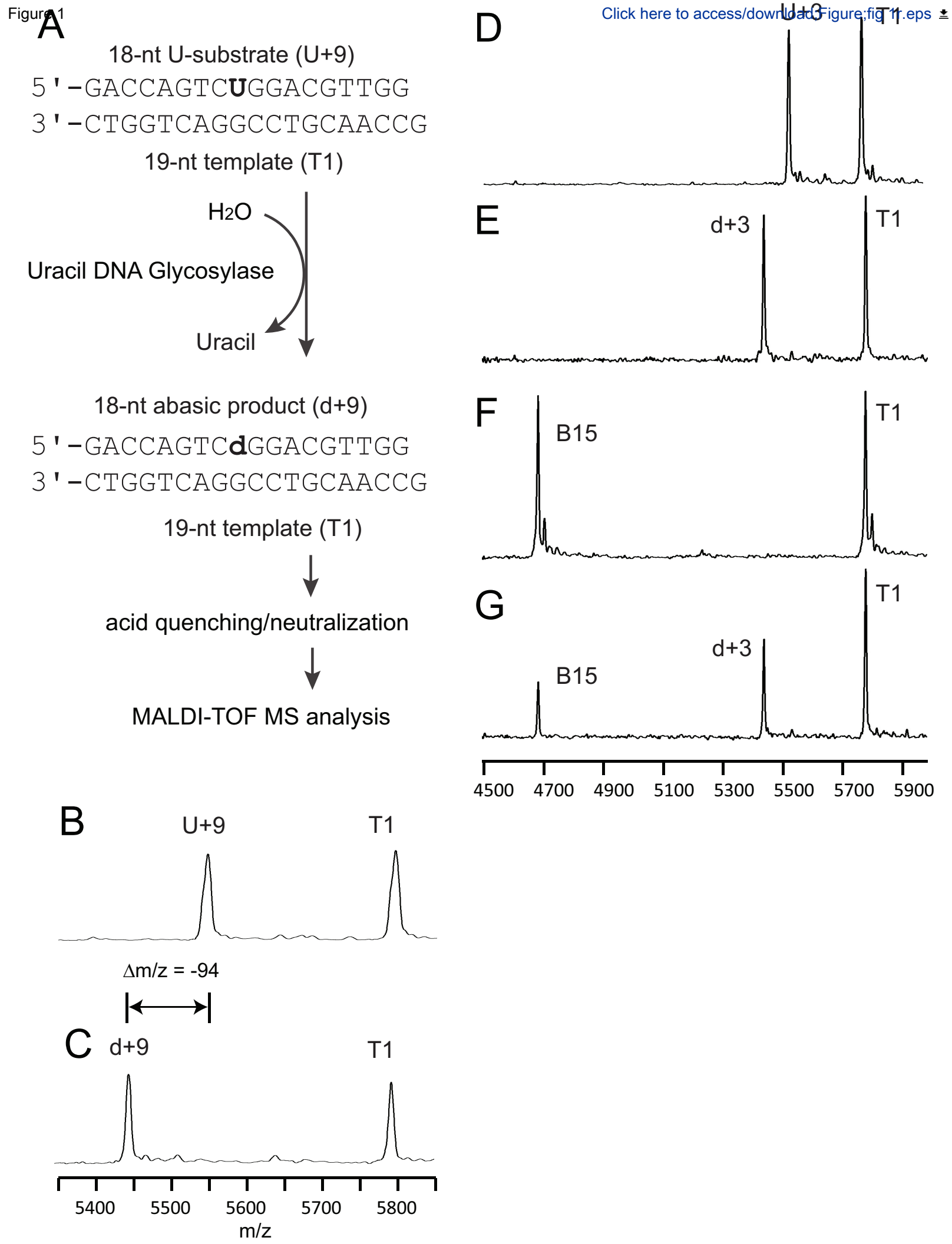
Figure 1



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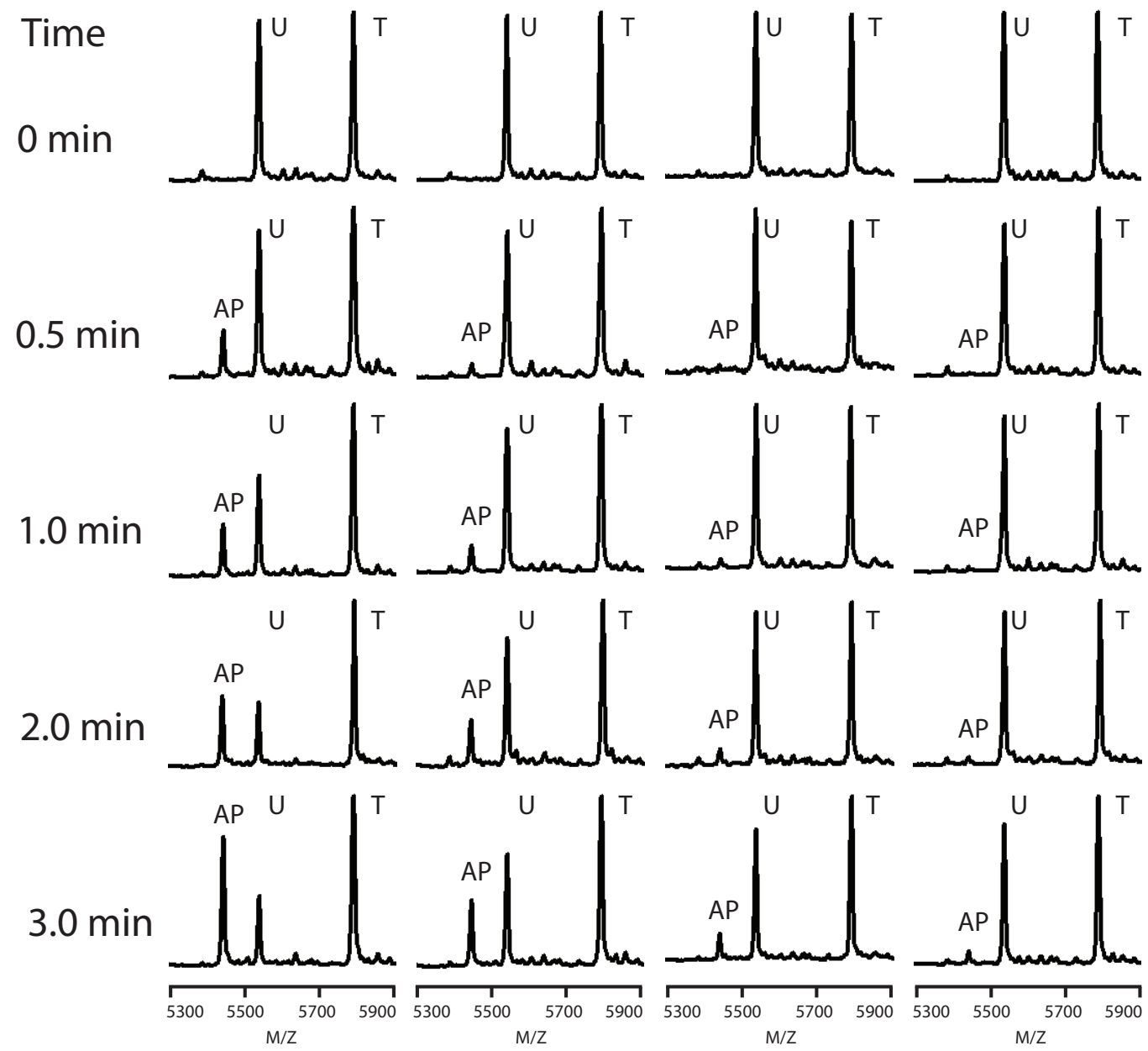

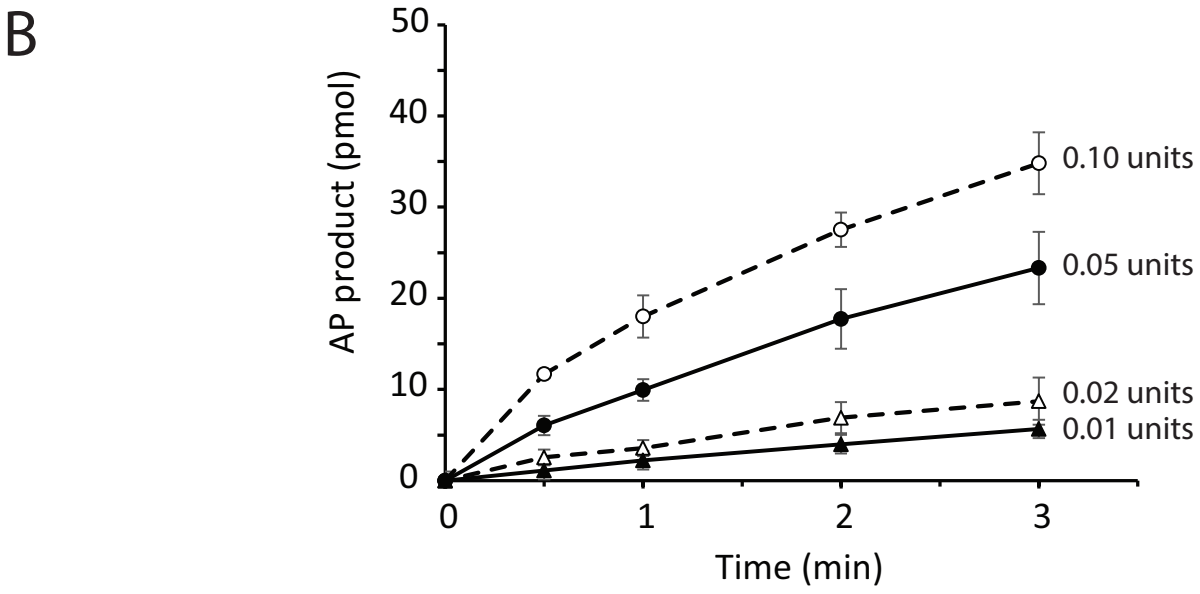

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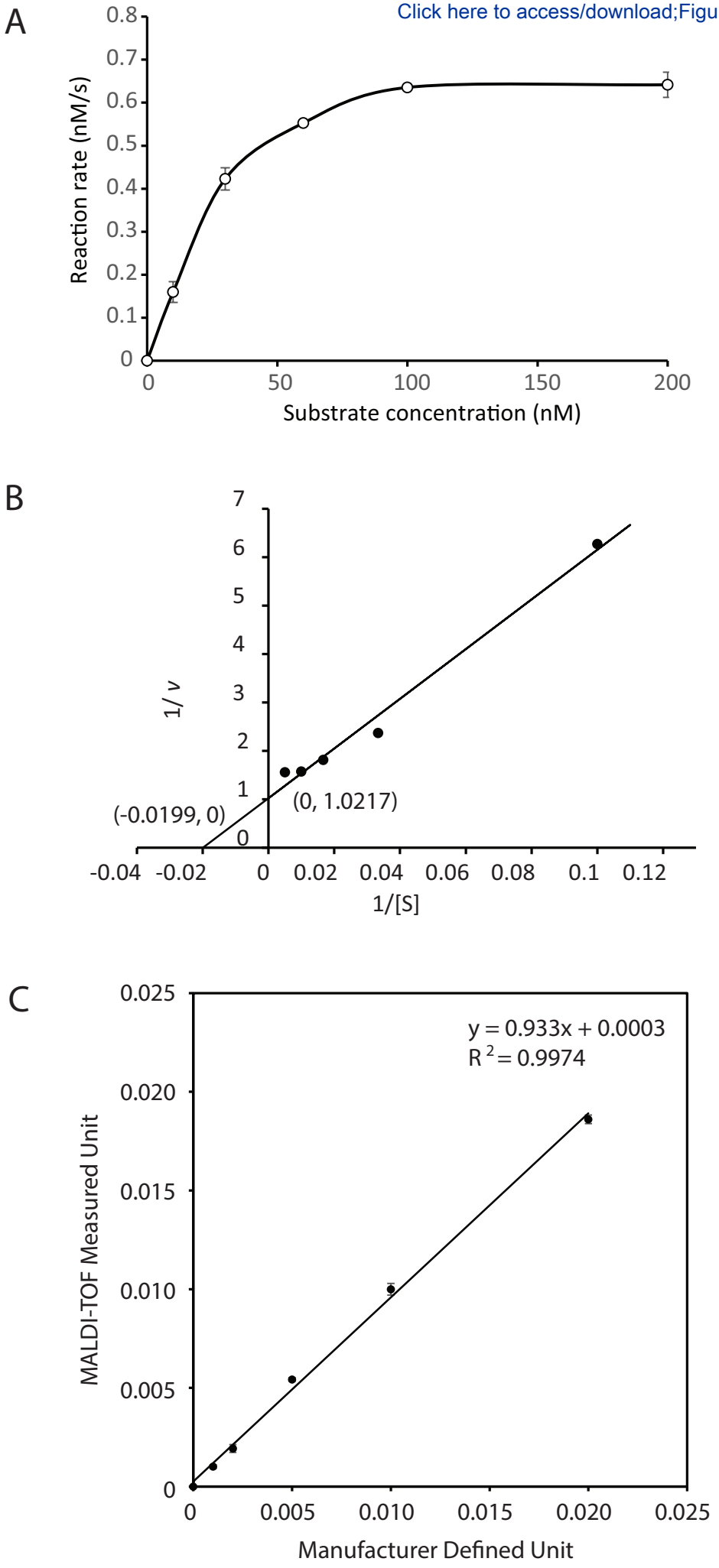

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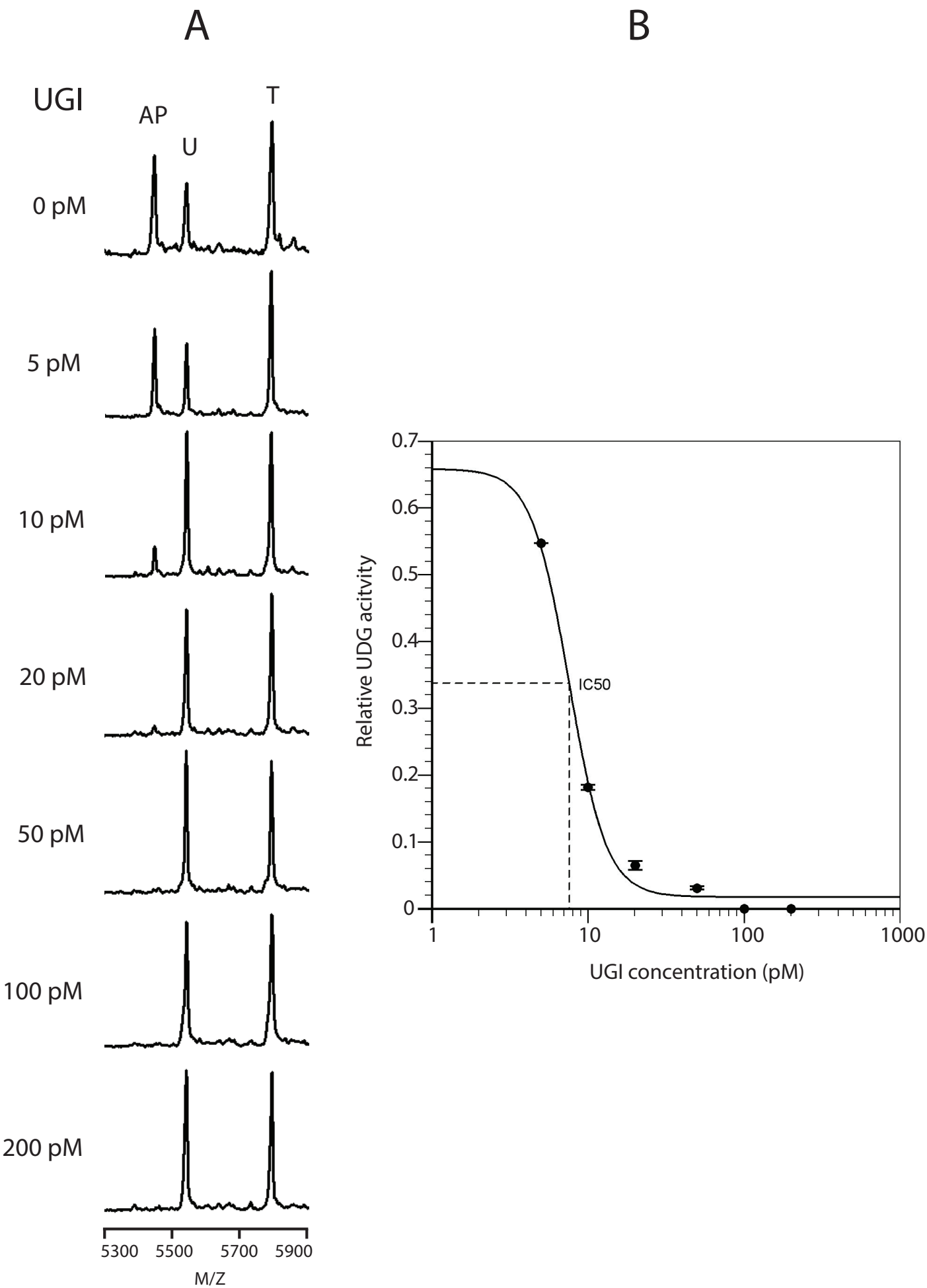

Table 1

DNA substrates	Oligonucleotides[a]	DNA Sequence[b]	Initial rate[c] (pmol/sec)	*k* cat (s^{-1})
ssU+9	U+9	5′-GACCAGTC**U**GGACGTTGG-3′	0.560 ± 0.098	35
ssU+3	U+3	5′-GA**U**CAGTCCGGACGTTGG-3′	0.756 ± 0.083	47.3
ssU-3	U-3	5′-GACCAGTCCGGACGT**U**GG-3′	0.448 ± 0.087	28
G:U	T1	3′-CTGGTCAGGCCTGCAACCG-5′	0.149 ± 0.046	9.31
	U+9	5′-GACCAGTC**U**GGACGTTGG-3′		
A:U	T2	3′-CTGGTCAGACCTGCAACCG-5′	0.053 ± 0.018	3.31
	U+9	5′-GACCAGTC**U**GGACGTTGG-3′		
G:U5′+3	T1	3′-CTGGTCAGGCCTGCAACCG-5′	0.256 ± 0.044	16
	U+3	5′-GA**U**CAGTCCGGACGTTGG-3′		
G:U5′+2	T3	3′-CGGGTCAGGCCTGCAACCG-5′	0.567 ± 0.016	35.4
	U+2	5′-G**U**CCAGTCCGGACGTTGG-3′		
G:U5′+1	T4	3′-GTGGTCAGGCCTGCAACCG-5′	ND[d]	ND
	U+1	5′-**U**ACCAGTCCGGACGTTGG-3′		
G:U3′-3	T5	3′-CTGGTCAGGCCTGCAGCCG-5′	0.138 ± 0.015	8.63
	U-3	5′-GACCAGTCCGGACGT**U**GG-3′		
G:U3′-2	T6	3′-CTGGTCAGGCCTGCAAGC-5′	ND	ND
	U-2	5′-GACCAGTCCGGACGTT**U**G-3′		
G:U3′-1	T7	3′-CTGGTCAGGCCTGCAACGG-5′	ND	ND
	U-1	5′-GACCAGTCCGGACGTTG**U**-3′		

Table 2

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WELL	TERM	SNP_ID	2nd-PCRP	1st-PCRP	AMP_LEN	UP_CONF	MP_CONF	Tm	PcGC	PWARN	UEP_DIR	UEP_MASS	UEP_SEQ	EXT1_CALL
W1	iPLEX	T28	NA	NA	NA	NA	NA	NA	NA		F	5789.8	GCCAACGTCCGGACTG	
W1	iPLEX	P21	NA	NA	NA	NA	NA	NA	NA		F	5541.6	GACCAGTCUGGACGTT	

EXT1_MASS
GTC
GG

Table 3

0620_1-2
0620_1-3
0620_1-4
0620_1-5
0620_1-6
0620_1-7
0620_1-8

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RE: Revised as in file 63089-R3_edit(1)

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This manuscript describes a mass spec based assay to examine DNA glycosylase activity

Major Concerns:
None

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None. Please correct the misspelling:" acid quenching/neutrolization .... to neutralization

RE: Revised as suggested in Figure 1A.

Reviewer #2:
Minor Concerns:
The authors have suitably addressed most of the comments. I do note that the reason they give for including Tris buffer in the reactions is that it comes with the commercial UDG that they use. But that is diluted 50-fold in the eventual reactions. On the other hand, they include Tris in practically all the other buffers, and that is not necessary: there is nothing "magic" about it with regard to Tris, and plenty of alternatives are available that don't risk cleavage of the AP site by beta-elimination. They could at least add a note to that effect for the users - perhaps for trouble-shooting problems with a high background of such cleavage.

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“The tris buffer in the reactions came with the commercial UDG. However, various amines including tris can incise AP sites via beta-elimination, but typically requires high reagent concentrations [44]. Some of alternatives can be considered such as

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