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Full Length Article

# A duplexed high-throughput mass spectrometry assay for bifunctional POLB polymerase and lyase activity

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ABSTRACT

Polymerase β (POLB), with dual functionality as a lyase and polymerase, plays a critical role in the base excision repair (BER) pathway to maintain genomic stability. POLB knockout and rescue studies in BRCA1/2-mutant cancer cell lines revealed that inhibition of lyase and polymerase activity is required for the synthetic lethal interaction observed with PARP inhibitors, highlighting POLB as a valuable therapeutic target. Traditional biochemical assays to screen for enzyme inhibitors focus on a single substrate to product relationship and limit the comprehensive analysis of enzymes such as POLB that utilize multiple substrates or catalyze a multi-step reaction. This report describes the first high-throughput mass spectrometry-based screen to measure the two distinct biochemical activities of POLB in a single assay using a duplexed self-assembled monolayer desorption ionization (SAMDI) mass spectrometry methodology. A multiplexed assay for POLB dual enzymatic activities was developed optimizing for kinetically balanced conditions and a collection of 200,000 diverse small molecules was screened in the duplexed format. Small molecule modulators identified in the screen were confirmed in a traditional fluorescence-based polymerase strand-displacement assay and an orthogonal label-free binding assay using SAMDI affinity selection mass spectrometry (ASMS). This work demonstrates the flexibility of high-throughput mass spectrometry approaches in drug discovery and highlights a novel application of SAMDI technology that opens new avenues for multiplexed high-throughput screening.

## 1. Introduction

Polymerase β (herein POLB), belonging to the X-family of polymerases [1], is a bifunctional enzyme that features lyase and polymerase activity [2–6]. A recent genetic screen revealed that POLB knockout is synthetic lethal with PARP inhibition (PARPi) [7]. The combination of POLB knockout and PARPi selectively kills BRCA-mutant cell lines while sparing healthy cells [7]. Rescue experiments using BRCA1 and BRCA2-mutant isogenic cell lines further demonstrated that the lyase and polymerase activities of POLB are required for the observed synthetic lethality with PARPi [7]. Mechanistically, POLB knockout is associated with increased single and double strand DNA breaks, accumulation of poly-ADP-ribose polymers, cell cycle arrest, and apoptosis [7]. These results suggest that POLB inhibitors in combination with PARPi have the potential to drive deep and durable responses, providing a new therapeutic avenue for BRCA1/2-mutant cancer patients.

Traditional high-throughput screening assays often lack the flexibility to simultaneously report on two distinct activities, restricting their use to initiate drug discovery efforts for POLB. Common polymerase assays rely on strand displacement with a fluorescent readout [8]. Traditional lyase assays often measure loss of radioactivity or incorporate a modified nucleotide in the substrate such that lyase activity results in release of a fluorescent reporter [9,10]. While these assays enable the identification of inhibitors against one specific functional activity, assessing the two POLB activities has not previously been possible without doubling efforts on assay development, screening, and cross validation of initial hits. A label-free and high-throughput platform that simultaneously reports on the lyase and polymerase activities would

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eliminate optical interference artifacts and deliver a data-rich output to accelerate discovery efforts tailored to both activities.

The mass spectrometry (MS) approach termed self-assembled monolayer desorption ionization (SAMDI) [11,12] has previously been reported as a label-free and high-throughput screening platform for diverse targets, including enzymes that generate multiple products such as methyltransferases and nucleases [13–18]. A recent report described the use of SAMDI MS to screen 300,000 compounds against the SARS-CoV-2 3CLpro and human rhinovirus HRV3C proteases in a multiplexed format that benefited from distinct substrate specificities of the two enzymes [19]. These data suggest that SAMDI MS could offer a valuable solution to simultaneously measure the two activities of POLB. This study describes the development and application of a bespoke multiplexed SAMDI MS assay in a high-throughput screen to identify inhibitors for the bifunctional POLB enzyme. Initial hits were validated using orthogonal approaches including traditional fluorescent strand displacement assays and a label-free binding assay using affinity selection mass spectrometry (ASMS). This is the first report of a SAMDI MS assay for measuring two distinct activities on a single substrate from a bifunctional enzyme and highlights the critical role that mass spectrometry plays in drug discovery.

## 2. Materials and methods

### 2.1. Reagents

DNA oligomers (biotinylated, fluorescently labeled, and unlabeled) were synthesized by Integrated DNA Technologies (Coralville, IA) and purified by high-performance liquid chromatograph (HPLC) to >95 % purity. Deoxythymidine triphosphate (dTTP) and other chemical reagents were purchased from Millipore Sigma (Burlington, MA). Substrate sequences included a template strand 5′-AGAAAGAGAAGGAAGGA-3′, where the first three and last three nucleotides (underlined) featured locked nucleic acid (LNA) bonds; a polymerase substrate 5′-Biotin-TCCTTCCT-3′ and a lyase substrate 5-SpC3idoexyU//CTCTTTCT-3′biotin. The inhibitor sequences included the template 5-TGAGCCGCACGGCGCATCAGC-3′, polymerase inhibitor sequence 5′-GCTGATGCGC-3′ and lyase inhibitor sequence 5′-Phos//idSp/GTGCGGCTCA-3′. The inhibitor sequence was also used for the SAMDI ASMS binding experiments. For the fluorescence strand displacement assay, the template strand included a 5′-linked BHQ2 quenching group, 5′-BHQ2-GCAAAAAAAAAAGAGTCGTACGAGGGTGA and two annealed oligos, 5′-TAMRA-CGTTTTTTTT-3′ and 5′-TCACCCTCGTACGACTCTT-3′ to form a triplex polymerase read-through substrate. The screening of compounds was conducted using the Tango High Throughput Screening library (Tango HTS library). This diversity-based library consists of over 200,000 compounds assembled from commercial sources (Enamine, Kyiv, Ukraine; Asinex, Winston-Salem, NC, USA; ChemBridge, San Diego, CA, USA) selected to maximize lead-like properties (MW 150 - 450, clogP −2 - 4, Ar ring count <4, REOS and PAINS filtered). The compounds were arrayed in 384-well plates at a concentration of 10 mM in DMSO solution. For running the screen, compounds were supplied as pre-dispensed assay-ready plates (BioAscent, Newhouse, UK) spotted with sufficient compound stock volume for a final concentration of 40 μM.

### 2.2. Protein production

Recombinant human POLB was cloned and expressed in *E. coli* as reported previously, [20] but with a modified procedure (performed at Biortus Biosciences Co., Ltd.). A full-length protein expression construct was prepared in a pET28a vector that contained an N-terminal hexahistidine tag followed by a TEV protease cleavage site. *E. coli* BL21 (DE3) cells grown in LB media were induced with 0.5 mM IPTG and POLB protein was expressed for 16 h at 15 °C. The cells were collected and resuspended in lysis buffer consisting of 50 mM HEPES (pH 7.5), 500 mM NaCl, and 5 % glycerol, followed by cell lysis using a microfluidics high pressure homogenizer (750 Bar, 3 cycles). Cell lysates were clarified by centrifugation (16,000 rpm, 60 min) and then applied to a HisTrap Fast Flow column (5 mL, Cytiva). The column was washed with buffer A (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 5 % glycerol) followed by buffer A supplemented with 20 mM imidazole. The protein was eluted with buffer A containing 300 mM imidazole. Following cleavage of the affinity tag with His-tagged TEV protease (1:40 ratio) by overnight incubation at 4 °C and dialysis into buffer A, the protein was re-applied to a HisTrap Fast Flow column and the flow-through was collected. The protein was then exchanged into a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 % glycerol and applied to a Mono S column. The protein was eluted as a single, sharp peak by running a gradient with buffer containing 1 M NaCl. As a final step, the protein was concentrated and run on a Superdex 75 column (16/600). The final buffer consisted of 20 mM Bis-tris propane (pH 7.0) and 50 mM NaCl. Pure protein at >30 mg/ml was flash-frozen as aliquots and stored at – 80 °C for future use. Following cloning to introduce the appropriate mutations, the K72A and D256A mutant POLB proteins were prepared following this same protocol.

Protein constructs containing an N-terminal Avi-tag were prepared for full-length POLB as well as for the truncation mutants POLB(1–98) and POLB(95–335), whose design was guided by prior work mapping domains of POLB [21]. In each case, an Avi-tag was introduced between the TEV cleavage site and the POLB protein sequence. Expression of these proteins in *E. coli* and the first steps of purification proceeded as described above. Following elution from the HisTrap Fast Flow column, in vitro biotinylation was performed concurrently with TEV protease treatment by incubating POLB with BirA (1:100 ratio) in a buffer that contained 50 mM bicine (pH 8.3), 10 mM ATP, 10 mM magnesium acetate, and 100 μM D-biotin at 4 °C overnight. The biotinylated protein was loaded onto a Strep-Tactin XT column to further purify the sample and it was eluted using buffer A containing 75 mM biotin. As a final step, the protein was concentrated and run on a Superdex 75 column (16/600). Purified samples were frozen as aliquots and stored at −80 °C for future use.

In addition to POLB, the full-length uracil-DNA glycosylase (UDG) from *E. coli* and UDG-specific inhibitor (UGI) from bacteriophage PBS2 were prepared for use in the biochemical assay. These proteins were cloned and overexpressed in *E. coli* as reported previously [22,23]. As above, each protein contained an N-terminal hexahistidine tag followed by a TEV protease cleavage site. Protein purification proceeded as described for POLB except that the Mono S ion exchange column was eliminated from the purification protocol and the final buffer from the Superdex 75 column consisted of 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 % (v/v) glycerol. Purified samples were frozen as aliquots and stored at −80 °C for future use.

### 2.3. Mass spectrometry biochemical assay

The biotinylated triplex substrate was prepared by heating the three oligos in an equimolar ratio to 95 °C for 5 min and allowing the reaction to cool at 1 °C / min until room temperature. To generate the 5′-dRP moiety, the triplex substrate (50 μM final) was incubated with 100 nM final UDG in 25 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.005 % Tween-20, and 1 mM TCEP for 30 min at room temperature. The reaction was quenched with 1 μM final of UGI [24]. The POLB reactions were performed with 6.5 nM final POLB, 175 nM dTTP, and 100 nM d-RP-triplex substrate in 25 mM HEPES pH 7.4, 80 mM NaCl, 2.5 mM $MgCl_2$, 0.005 % Tween-20, and 1 mM DTT. Reactions were run in 20 μL volume in 384-well V-bottom polypropylene microtiter plates (781280 Greiner Bio-One) at room temperature and quenched by the addition of a mixture of 0.25 % formic acid (final) and 50 mM EDTA (final) to quench the lyase and polymerase activities, respectively. At defined times, a 2 μL sample of the quenched reactions was transferred using a 384-channel liquid handler to SAMDI 384-spot biochip arrays

functionalized with a Neutravidin-presenting self-assembled monolayer as previously reported [16]. The arrays were incubated for 60 min in a humidified chamber to allow for the specific immobilization of the biotinylated substrates and products. The plates were then washed using deionized ultrafiltered (DIUF) water and dried with compressed air before the application of a matrix comprising 2-hydroxy-5-methoxybenzoic acid (HMB) in acetonitrile (30 mg/mL) and ascorbic acid in aqueous ammonium citrate (500 mM) at 350 nL per spot in the array. SAMDI mass spectrometry (MS) was performed using the reflector negative mode on an AB Sciex matrix assisted laser desorption ionization (MALDI) time of flight (TOF) 5800 System (Framingham, MA). Each spot was analyzed with 400 shots with a random raster sampling (20 shots/sub-spectrum and 20 subspectra with pass acceptance), 400 Hz laser frequency, bin size of 1 ns, and detector voltage multiplier of 0.55. A mass window of *m/z* 2000 to *m/z* 4000 was used and a mass threshold of *m/z* 0.8 applied for peak identification. The conversion of substrates to products was calculated using a ratio of product area under the curve (AUC) over the sum of the substrate and product AUC peaks for each distinct substrate.

### 2.4. Fluorescence strand displacement assay

The fluorescence displacement assay was performed by incubating 1 nM final POLB with 10 nM triplex substrate and 100 μM dTTP in 25 mM Tris pH 7.5, 25 mM NaCl, 2.5 mM MgCl2, 0.01 % Tween-20, 2 mM DTT, and 0.25 mM EDTA. The reactions were performed in 20 μL volume in 384-well white opaque Optiplate (Perkin-Elmer) and read on a Pherastar FSX (BMG) with a 540 nm excitation / 590 nm emission wavelength module, with focal height 10.8 and gain 525.

### 2.5. SAMDI ASMS assay

Binding assays were performed as previously reported [25]. Biotinylated POLB (400 nM final) was incubated in solution with compounds at 5 μL volume in 1x assay buffer (same as SAMDI MS assay) for thirty minutes at room temperature in 384-well low volume polypropylene plates (784201, Greiner Bio-One). In parallel, the compounds were incubated in buffer without POLB ("background") to inform on selective binding. The reactions (2 μL) were then transferred to 384-spot SAMDI biochip arrays using a 384-channel automated Thermo PlateMate, where the arrays are functionalized with Neutravidin-presenting monolayers to specifically immobilize the biotinylated protein along with compounds that are bound in complex with protein. The arrays were incubated for one hour at room temperature in a humidified chamber. Next, the SAMDI biochips were purified by a rapid (<3 s) cascade wash step with DIUF water (50 μL/spot) and dried with compressed air. The matrix solution comprised 20 mg/mL alpha cyano cinnamic acid (CHCA) [26] was prepared in 80 % acetonitrile / 20 % aqueous ammonium citrate and 0.3 % trifluoroacetic acid was applied in an automated format using a Combi Nano by dispensing 50 nL to each spot in the array. SAMDI ASMS was performed using the reflector positive mode on the AB Sciex 5800 instrument as each compound was first confirmed by MALDI to ionize in positive mode. Each spot was analyzed with 400 shots with a random raster sampling (20 shots/sub-spectrum and 20 subspectra with pass acceptance), 400 Hz laser frequency, bin size of 1 ns, and detector voltage multiplier of 0.48. A mass window of *m/z* 230 to *m/z* 900 was used and a mass threshold of *m/z* 0.5 applied for peak identification. The AUC of each peak that corresponds to the mass ID (mass tolerance of *m/z* 0.5) is measured from the raw spectra for each well on the SAMDI array and reported along with the AUC of a peak corresponding to the mass of the tri(ethylene glycol) (EG3) terminated monolayer molecule, which serves as an internal comparator having an *m/z* 335.2. To calculate the relative signal value (RSV) for each compound, the AUC of each compound is divided by the sum of that same compound's AUC and the AUC of the internal comparator.

### 2.6. Data analysis

GraphPad Prism was used to calculate enzyme kinetics and parameters such as $K_M$, $k_{cat}$, $IC_{50}$ and $EC_{50}$ values. Michaelis-Menten fits of enzyme velocities were applied. $IC_{50}$ values and Hill slopes were generated using a four-parameter fit. $EC_{50}$ values were generated using "EC anything" where $F = 50$ and the max value constrained to the saturating RSV. The quality and robustness of the assay were determined by the analysis of the Z' factor [27].

## 3. Results

### 3.1. Development of SAMDI MS POLB duplex assay

To develop a SAMDI MS assay for POLB lyase and polymerase activities, we designed a triplex DNA sequence with a longer DNA template and two shorter complementary strands that represent the sites for the catalytically distinct lyase and polymerase activities (Supplementary Figure S1). The two ssDNA substrate strands incorporate a biotin handle that enables the rapid and specific immobilization onto Neutravidin presenting self-assembled monolayer biochip arrays for SAMDI MS analysis [12]. The sequence design strategy balanced the ionization potential of the substrates and products, which often favors shorter sequences, and the melting temperature to ensure stable triplex formation. We incorporated locked nucleic acids (LNAs) to increase the melting temperatures for shorter sequences, ensuring that the triplex remains intact throughout the assay including upon immobilization, and to generate high-quality signal by MS [28,29]. Synthesizing the deoxyribose presenting substrate proved challenging and was therefore generated enzymatically using the UDG enzyme [30] (Fig. 1A). SAMDI MS analysis of the triplex substrate generated two peaks corresponding to the anticipated *m/z* values for the biotinylated polymerase substrate (*m/z* 2400.2) and the lyase substrate (*m/z* 2792.5), the latter aligning with the expected mass following a complete reaction of the UDG enzyme (Fig. 1B, top). The template strand is too large to generate a significant signal in the SAMDI spectrum (*data not shown*). Upon treatment with POLB, the SAMDI MS spectrum revealed two new peaks corresponding to the anticipated products of the lyase (*m/z* 2537.2) and the polymerase (*m/z* 2703.2) reaction (Fig. 1B, bottom), supporting its use for further assay development.

### 3.2. Assay development and kinetic parameters

To develop an assay amenable for characterizing POLB lyase and polymerase inhibitors, it is important to develop a kinetically balanced assay for both activities [31]. The assay development strategy aimed to identify optimal buffer conditions for measuring lyase and polymerase activities together, rather than optimizing for an individual enzyme activity. For example, while the lyase activity was optimal in a HEPES pH 6.8 buffer, polymerase activity was not detected (Supplementary Figure S2). Therefore, HEPES pH 7.4 was selected as each reaction exhibited similar activities. The same strategy was adopted for optimizing the buffer for monovalent and divalent salts (NaCl and $MgCl_2$), reducing agents, carrier proteins, glycerol and DMSO tolerance (Supplementary Figure S2). These data revealed an optimized buffer of 25 mM HEPES pH 7.4, 80 mM NaCl, 2.5 mM MgCl2, 0.005 % Tween-20, and 1 mM DTT.

The $K_M$ of the DNA substrate was measured by measuring POLB activity over a substrate concentration range of 78 nM to 2.5 μM and a fixed dTTP concentration of 200 nM. The initial velocities ($V_0$) were calculated using the linear portion of the reaction and fit to a Michaelis-Menten curve. The data revealed a $K_M$ value of approximately 990 nM (95 % CI 820 nM to 1.2 μM) for the lyase substrate and approximately 750 nM (95 % CI 425 nM to 1.39 μM) for the polymerase substrate (Fig. 2A). The similar $K_M$ value is consistent with previous reports characterizing the interaction between POLB and its DNA substrate [38].

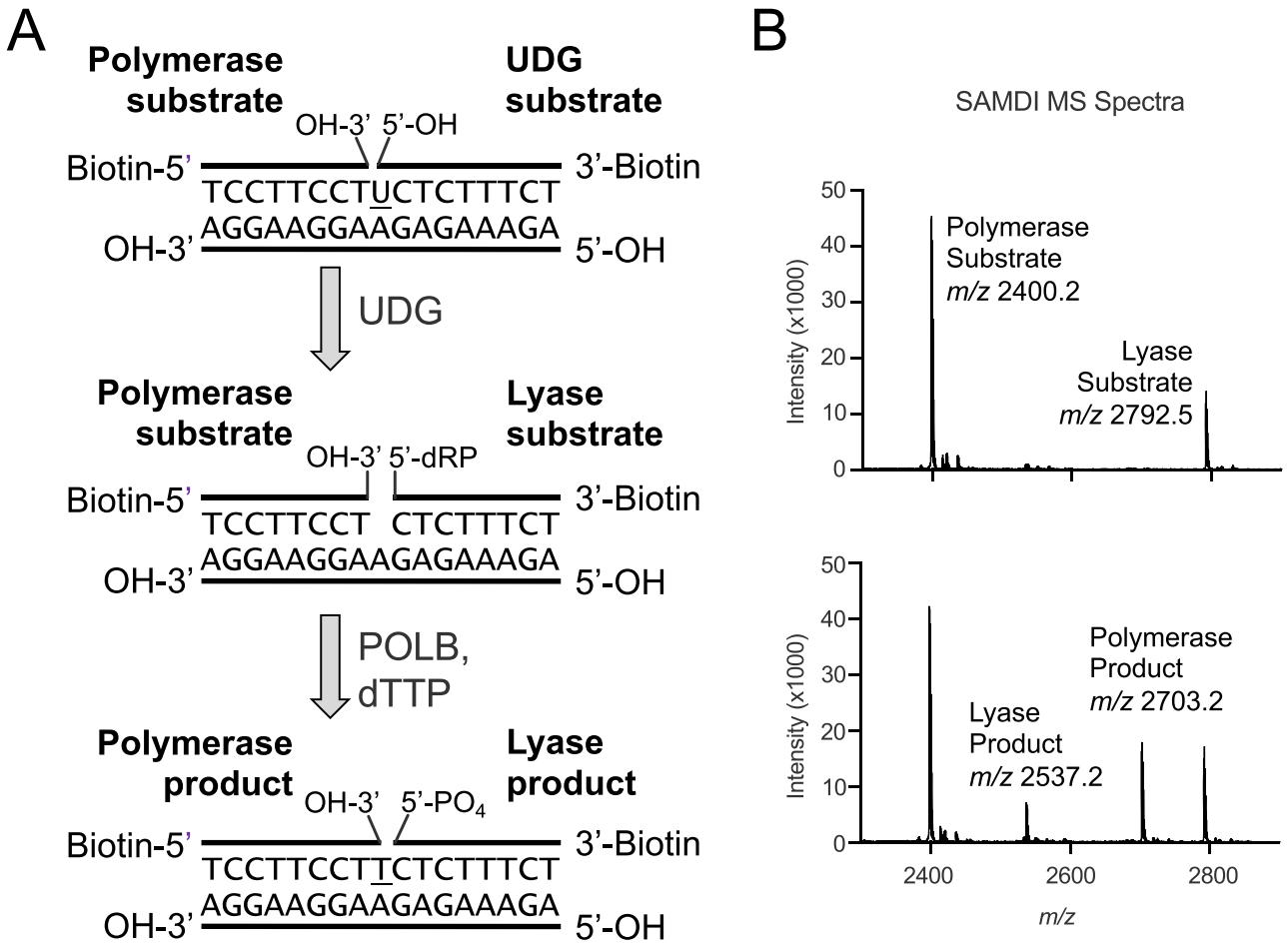



**Fig. 1.** SAMDI MS assay for POLB activity. (A) Scheme showing the enzymatic reaction. A DNA triplex is subjected to UDG treatment to remove the uracil base and leave a 5′-deoxyribosephosphate (dRP). The bifunctional POLB enzyme exhibits lyase activity to remove the 5′-dRP residue and in the presence of dTTP adds one nucleotide to the 3′ end of the abasic site (B) Representative SAMDI MS spectra before (*top*) and after (*bottom*) POLB activity.

A final substrate concentration of 100 nM (below the $K_M$) was chosen to minimize reagent consumption and allow the identification of small molecule inhibitors that act through substrate competition. Next, the $K_M$ of the dTTP cofactor was measured over a concentration range of 0.3–10 μM and fixed triplex DNA substrate concentration of 100 nM and the data revealed a linear relationship, suggesting that the $K_M$ was >10 μM (Fig. 2B). To confirm these results in an orthogonal assay, we developed a fluorescent strand displacement assay to monitor POLB polymerase activity [8]. The $K_M$ of each substrate was measured by testing the substrate over a concentration range of 2.5–80 nM and a dTTP concentration range of 15.125–1000 μM. These data revealed an oligo substrate $K_M$ of 10.8 nM (95 % CI 9.0 nM to 13.0 nM) and a dTTP $K_M$ of 275 μM (95 % CI 250 μM to 310 μM) (Fig. 2D, E). The higher affinity for the fluorescence DNA substrate is likely influenced by the longer sequence which makes more stable contacts with the enzyme compared to the shorter seven nucleotide used in the SAMDI MS assay and a different buffer that includes a lower NaCl concentration. The fluorescence assay data supports the linear relationship observed for dTTP using SAMDI MS at lower concentrations. For the SAMDI MS assay, a dTTP final concentration of 175 nM was selected because it is below the $K_M$ and generated sufficient product formation for the polymerase in a time frame that also permitted optimal lyase conversion. When using these conditions, the $IC_{50}$ of a triplex oligo that mimicked the product was calculated to be approximately 2.5 nM for the lyase and 2.3 nM for the polymerase (Fig. 2C) in the SAMDI MS assay, and an $IC_{50}$ of 5.2 nM in the fluorescence polymerase assay (Fig. 2F). The apparent higher potency for the triplex inhibitor for the enzyme compared to the measured $K_M$ of the triplex substrate is likely attributed to the additional nucleotides of the triplex inhibitor (see Materials and Methods). These data are consistent with the hypothesis that the higher affinity for the fluorescence substrate is likely influenced by the longer sequence and it makes more stable contacts.

### 3.3. *POLB wildtype and mutant substrate specificity*

To distinguish the two distinct activities of POLB, the triplex substrate was tested with wildtype POLB and two constructs featuring point mutations in the lyase and polymerase domains, respectively [32,33]. Plots of velocity as a function of enzyme concentration were generated where the wildtype enzyme exhibited similar rates for the lyase and polymerase activities (Fig. 3A, Supplemental Figure S3). These data further support that the optimized assay maintains steady-state, balanced kinetics suitable for identifying inhibitors through diverse mechanisms. The POLB K72A mutant, located in the N-terminal lyase domain, exhibits only polymerase activity (Fig. 3B) and the D256A mutant in the polymerase domain generates only the lyase product (Fig. 3C), while not significantly impacting the activity of the unaffected domain function. The mutant constructs confirm the assay specificity of the two independent POLB activities and suggest that compounds may potentially inhibit one activity without impacting the other. Taken together, these data support the rationale for developing the SAMDI MS duplexed assay for screening for POLB polymerase and lyase inhibitors.

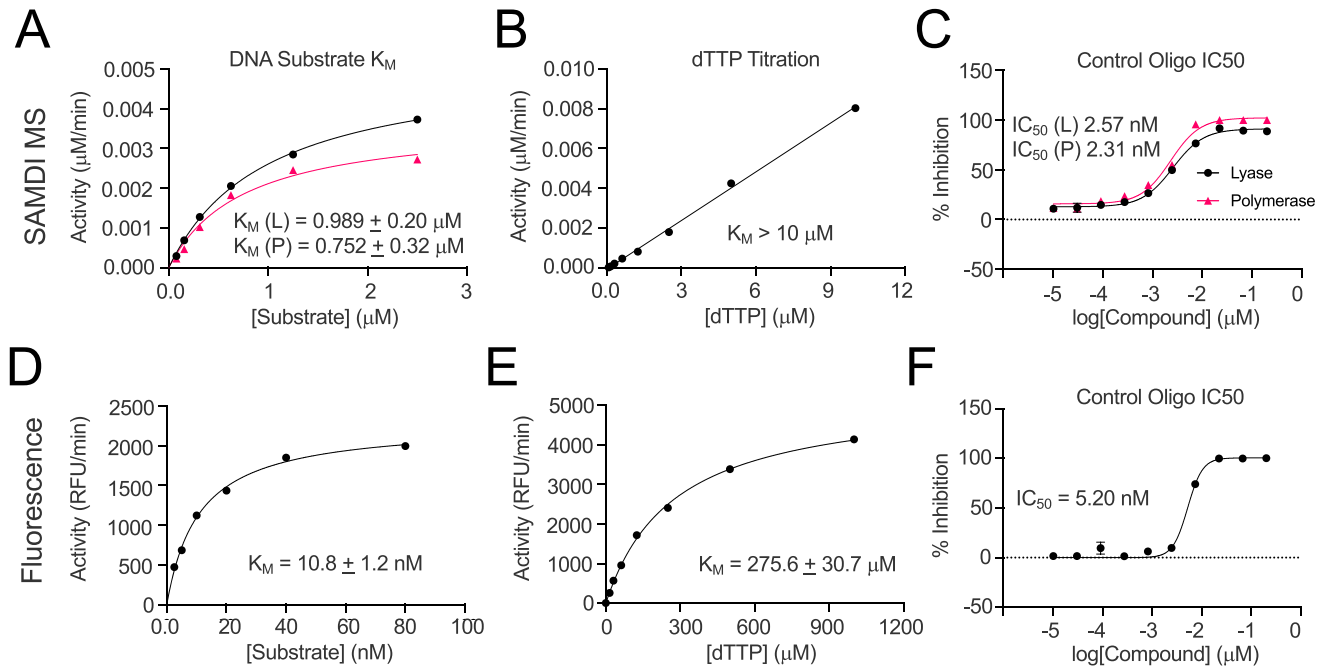



**Fig. 2.** Development of a duplexed POLB lyase and polymerase assay using (A-C) SAMDI MS and (D-F) a polymerase assay using a fluorescence strand displacement assay. (A, D) $K_M$ of the triplex substrate was determined using conditions described in the Materials and Methods. (B, E) $K_M$ of the dTTP cofactor was determined using described conditions. (C, F) $IC_{50}$ measurements of the triplex inhibitor.

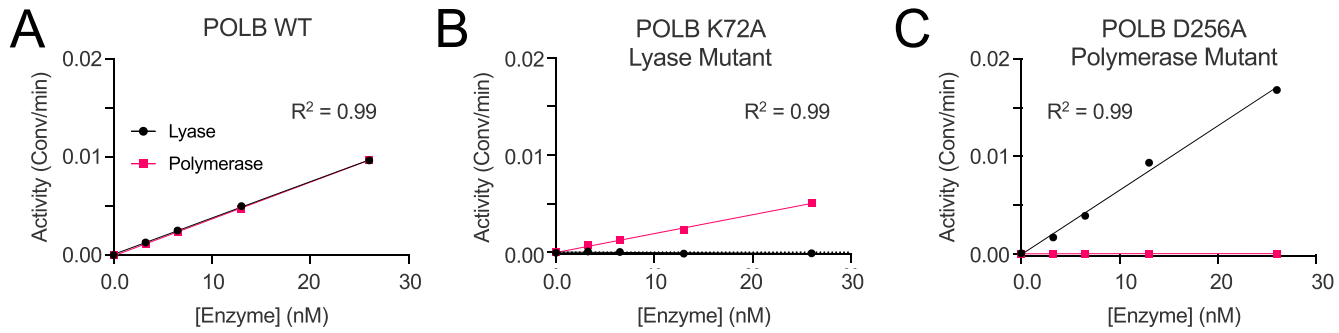
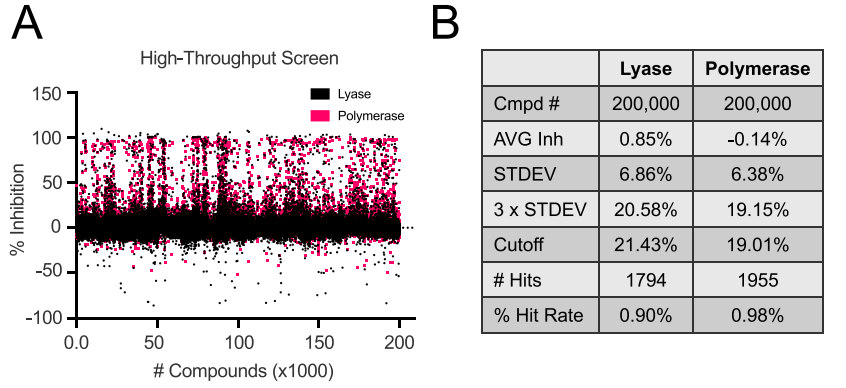



**Fig. 3.** Duplex SAMDI MS assay to measure POLB lyase and polymerase activity. Enzyme activity is measured with POLB (A) wildtype (B) K72A, and (C) D256A mutants.

### *3.4. High-throughput screen of POLB bifunctional activity*

The throughput of the SAMDI MS assay was assessed in 20 μL volumes in 384-well plate format using the optimized conditions. Each plate included 24 wells of DMSO only, 24 wells with 200 nM triplex oligo for a 100 % inhibition control, and 16 wells with 4 nM triplex oligo




B

| | Lyase | Polymerase |
|---|---|---|
| Cmpd # | 200,000 | 200,000 |
| AVG Inh | 0.85% | -0.14% |
| STDEV | 6.86% | 6.38% |
| 3 x STDEV | 20.58% | 19.15% |
| Cutoff | 21.43% | 19.01% |
| # Hits | 1794 | 1955 |
| % Hit Rate | 0.90% | 0.98% |

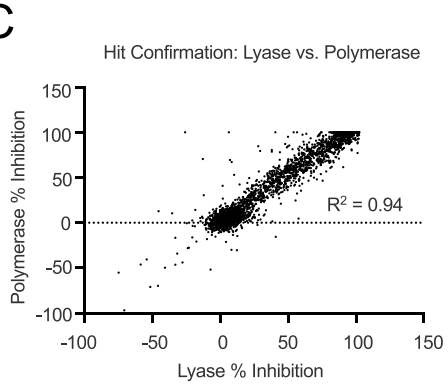



**Fig. 4.** High-throughput screen to identify inhibitors of POLB lyase and polymerase activity using SAMDI MS. (A) Inhibition data of 200,000 compounds analyzed in a duplexed SAMDI MS assay. Circles represent lyase activity, squares represent polymerase activity. (B) Table of screening statistics. (C) Hit confirmation experiment comparing the % inhibition observed for each compound against lyase and polymerase activities.

as a mid-control. The presence of positive and negative controls allows the calculation of the Z-factor (a measure of robustness) and the mid-control ensures consistent assay behavior across the screen. A library of 200,000 diverse compounds, each dissolved in DMSO, was screened at a final concentration of 40 μM and a final DMSO concentration of 1 % (Fig. 4A). The lyase and polymerase activities were consistent across each plate (Supplementary Figure S4A) with Z-factors averaging > 0.7 for the lyase and > 0.9 for the polymerase activities (Supplementary Figure S4B). Importantly, the mid-control exhibited consistent activity around 60 % inhibition for each activity across all plates (Supplementary Figure S4C). The threshold for determining a hit was calculated by summing the average inhibition of each compound to three times the standard deviation across the screen for the two distinct activities (Fig. 4B). The thresholds resulted in 1794 lyase hits and 1955 hits (0.90 % and 0.98 %, respectively), the majority of which were hits for both activities, with a selection of compounds exhibiting preferential inhibition for one enzyme activity (Fig. 4C). Each compound was then tested at 40 μM in duplicate in the primary SAMDI MS assay with excellent correlation amongst the independent replicates ($R^2$ = 0.86 for lyase, 0.94 for polymerase) (Supplementary Figure S4D, S4E). The compounds continued to exhibit similar activity on lyase and polymerase activities (Fig. 4C). These data highlight the robustness and consistency of the SAMDI MS assay to identify POLB lyase and polymerase activities and identified several initial compounds for further evaluation.

### 3.5. Potency and selectivity in orthogonal assays

To gain insight into potency, a selection of compounds was tested in a concentration response manner in the primary SAMDI MS duplex assay and in the fluorescent strand displacement assay. Consistent with the primary HTS and hit follow-up experiments, many compounds exhibited similar $IC_{50}$ values for the lyase and polymerase activities (Fig. 5A) and behaved similarly in the fluorescence format (Fig. 5B). A possible mechanism for inhibition is the intercalation of compounds into the DNA, thereby perturbing the interaction with POLB and giving rise to an inhibition signal. While intercalation assays such as thiazole orange can rule out this undesirable mechanism [17], the fluorescence readout is still susceptible to false positives due to optical interference. An assay that measures direct binding of small molecules to the POLB protein would provide more confidence that the compounds directly engage the target protein.

### 3.6. SAMDI ASMS analysis

ASMS is a powerful platform that informs on direct binding [34–37]. The SAMDI technology was first reported in an ASMS workflow in 2017 [26] and later reported to complete a screen of 100,000 compounds in less than eight hours against a viral protease [25]. In the SAMDI ASMS assay, compounds and target are incubated in solution. Next, the target-ligand complex is enriched on the SAMDI biochip arrays through

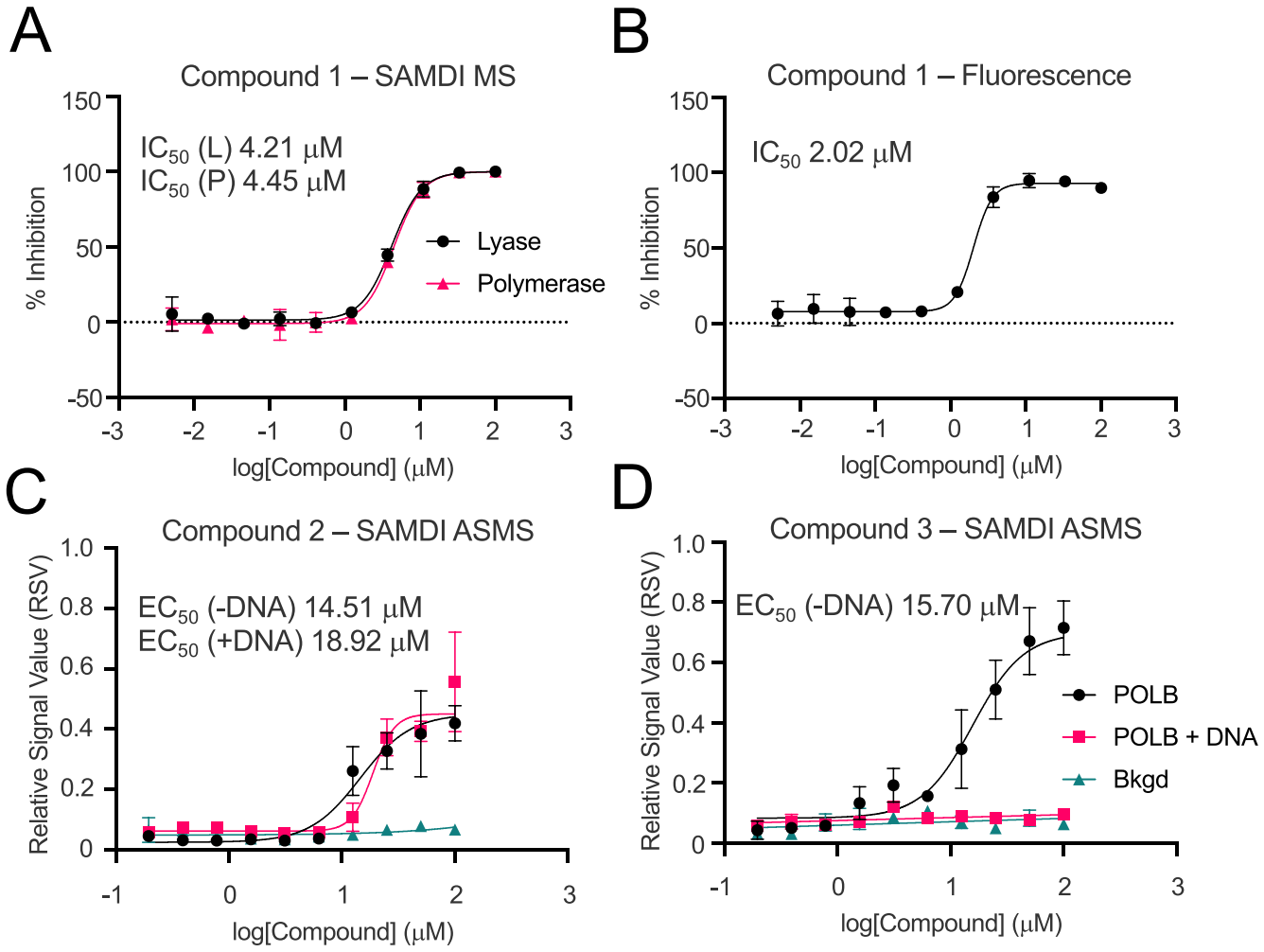



**Fig. 5.** Concentration response analysis of compounds in the (A) SAMDI MS duplex assay where circles represent lyase and triangles represent polymerase activities and (B) fluorescence displacement polymerase assay. (C, D) Concentration response analysis of compounds analyzed in the SAMDI ASMS assay in the presence of POLB alone (circles), POLB plus triplex DNA oligo (squares) and the reference surface (triangles).

specific and rapid immobilization of the target. After unbound compounds and the buffer are washed away, a matrix is applied, and upon MALDI laser activation, the target and ligand dissociate due to the non-covalent nature of the interaction, and the ligand is detected based on its mass. To demonstrate SAMDI ASMS for POLB binders, a biotinylated POLB construct was prepared and incubated with the triplex oligonucleotide used as an inhibitor control in the biochemical assays, along with the corresponding ssDNA lyase and polymerase sequences. To further shed light on where the oligos bind to POLB, each oligo was titrated over a concentration rage of 4–1000 nM in the presence of full length POLB, an N-terminal construct (AA1–98) featuring the lyase domain, a C-terminal construct (AA95–335) featuring the polymerase domain, along with the reference surface. When the oligos are presented within the triplex, the lyase and polymerase strands are detected with the full-length protein and N-terminal truncation (Supplementary Figure S5A, S5B). When the lyase and polymerase strands are incubated as individual ssDNA oligos, the data revealed the lyase strand detected with the full-length protein, with minimal signal detected for the N-terminal construct (Supplementary Figure S5C). The polymerase strand is detected with similar $EC_{50}$ curves with the full length and N-terminal construct (Supplementary Figure S5D), consistent with the characterized DNA binding domain of POLB [38]. These data support the use of SAMDI ASMS for measuring ligand binding to POLB.

A series of compounds was then tested in the SAMDI ASMS assay in two formats. The first format aimed to understand whether the compounds bound to a distinct domain. The data revealed compounds that demonstrated binding to the lyase domain and full-length protein, along with compounds that bound to the polymerase domain and full-length protein, supporting the conclusion that both domains can bind ligands (Supplementary Figure S5E, S5F). The second format aimed to address whether small molecules exhibited competition with the DNA substrate by titrating the compounds over a concentration range of 0.19–100 μM in the presence of equimolar POLB and triplex DNA (400 nM each). The data revealed compounds that likely bind independently of DNA binding (Fig. 5C) and compounds that exhibit competitive binding with DNA (Fig. 5D). Combined with the biochemical assays, the binding data aids in identifying compounds that inhibit the two functional activities while also exhibiting binding to the target, eliminating compounds that likely inhibit through an undesirable mechanism such as intercalation. The lack of signal observed in the background condition suggests that the compounds do not non-specifically bind to the Neutravidin presenting monolayer. Taken together, the use of orthogonal functional assays and binding assays provide a toolbox to identify and validate POLB inhibitors and shed light on binding sites to guide which compounds are most encouraging for further development. It also supports the flexibility of the SAMDI technology to measure biochemical activities and binding interactions for drug discovery research.

## 4. Discussion

Dual inhibitors of POLB lyase and polymerase activities remain an unmet need. A recent report developed a covalent inhibitor of the POLB polymerase activity with a small molecule that modifies a lysine residue within the POLB polymerase domain [39]. The impact of that molecule on POLB lyase activity remains an open question as traditional assay formats are restricted to a single substrate to product transition. Inhibition of lyase and polymerase activities is critical for the synthetic lethality observed with PARPi. The combination of POLB knockout with therapeutic doses of PARPi led to profound tumor regression and prevented in vivo tumor growth even after stopping treatment [7]. These findings support the development of dual POLB lyase and polymerase inhibitors. The label-free, high-throughput duplex SAMDI MS assay described in this report is a major step towards addressing this clinical need. The screen identified several hits that were validated in orthogonal biochemical and binding assays. We expect that there will be future work to test these inhibitors in relevant cancer model cell lines to evaluate their potential therapeutic value.

Mass spectrometry continues to play a critical role in drug discovery with new instruments and techniques to overcome historical hurdles and it has been extensively reviewed [40-41]. This study reports, to the best of our knowledge, the first label-free and high-throughput assay to simultaneously measure two distinct activities from a bifunctional enzyme in a single assay and showcases the value that high-throughput mass spectrometry brings to drug discovery research. The SAMDI MS technology has previously been applied to diverse enzyme activities, including post-translational modifying enzymes (lysine demethylases, protein and RNA methyltransferases, deacetylases, among others), nucleases, arginase, and proteases [13–18]. Recently, SAMDI MS was used to screen two distinct viral proteases in a multiplexed format on two peptide substrates. The ability to inform on enzyme activity and selectivity in a single assay was valuable, identifying a compound that exhibited the desired antiviral effect in cells without exhibiting cytotoxicity [19]. SAMDI MS offers several advantages over traditional MS approaches [40,41], including conventional MALDI, that benefit drug discovery. First, the ability to immobilize the analytes of interest to SAMDI biochip arrays offers rapid purification of analytes of interest out of complex, quenched reactions. In this manner, the buffer components that can lead to ion suppression [42] in mass spectrometry instruments are washed away prior to analysis. The solution allows researchers to optimize the assay conditions for the target, without being limited by instrument requirements, while benefiting from the ultra-high-throughput readout of the MALDI instrument. The data generates peaks corresponding to singly charged, intact molecules, simplifying data analysis.

The SAMDI MS platform is flexible in that it also offers a rapid workflow for measuring small molecule binders to distinct targets. The SAMDI ASMS platform has previously been reported to screen for small molecule binders to a protein target [25,26]. This paper is the first report demonstrating that SAMDI ASMS can measure the binding of short oligonucleotides to a protein target. Data analysis of oligonucleotide binding is different than SAMDI ASMS for small molecule ligands due to the MALDI and matrix conditions needed for ionization. Traditional SAMDI ASMS with small molecules generates a RSV calculated by a ratio of the AUC of a peak corresponding to the mass of the compound of interest to the sum of that AUC plus the AUC of a peak that corresponds to the mass of a monolayer molecule that acts as an internal comparator [25]. Reporting data in this manner benefits from the fact that small molecules and the internal comparator ionize utilizing similar MALDI and matrix conditions. Oligonucleotides, however, ionize using matrix and MALDI settings that are different than the internal comparator. Therefore, the data here for oligonucleotide binders is reported strictly as the AUC value. Since absolute signal on a MALDI instrument can vary, these data are utilized to inform on whether the oligo is detected. The $EC_{50}$s generated for small molecule ligands are used to rank order binders by affinity since ASMS workflows are not technically equilibrium reactions, and therefore do not report a $K_D$. The use of SAMDI ASMS in this study was critical to rule out compounds that inhibit through non-desirable mechanisms. The data for several biochemical inhibitors did not support binding in the ASMS assay, demonstrating effective compound triage using this workflow. Together, this study showcases how the evolution of SAMDI MS continues to have a positive impact for drug discovery research, with flexible solutions for diverse targets and analytes.

## CRediT authorship contribution statement

**Zachary A. Gurard-Levin:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Brian McMillan:** Writing – review & editing, Validation, Investigation, Formal analysis. **Douglas A. Whittington:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Brian Doyon:** Investigation,

Data curation. **Michael D. Scholle:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jacques Ermolieff:** Investigation, Formal analysis, Data curation. **Madhavi Bandi:** Investigation. **Mu-Sen Liu:** Investigation. **Alvaro Amor:** Investigation, Formal analysis. **William D. Mallender:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Zack Gurard-Levin reports a relationship with Society of Laboratory Automation and Screening that includes: board membership. Douglas Whittington, Brian Doyon, Madhavi Bandi, Mu-Sen Liu, Alvaro Amor, and William D. Mallender report a relationship with Tango Therapeutics that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Zack Gurard-Levin and Michael D. Scholle report a relationship with Charles River that includes employment.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slast.2024.100173.

## References

[1] Braithwaite DK, Ito J. Compilation, alignment, and phylogenetic relationships of DNA polymerases. Nucleic Acids Res 1993;21(4). https://doi.org/10.1093/nar/21.4.787.
[2] Tanabe K, Bohn EW, Wilson SH. Steady-state kinetics of mouse DNA polymerase β. Biochemistry 1979;18(15). https://doi.org/10.1021/bi00582a029.
[3] Beard WA, Wilson SH. Structure and mechanism of DNA polymerase β. Biochemistry 2014;53(17). https://doi.org/10.1021/bi500139h.
[4] Sawaya MR, Prasad R, Wilson SH, Kraut J, Pelletier H. Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: evidence for an induced fit mechanism. Biochemistry 1997;36(37). https://doi.org/10.1021/bi9703812.
[5] Kumar A, Abbotts J, Karawya EM, Wilson SH. Identification and properties of the catalytic domain of mammalian DNA polymerase β. Biochemistry 1990;29(31). https://doi.org/10.1021/bi00483a002.
[6] Kumar A, Widen SG, Williams KR, Kedar P, Karpel RL, Wilson SH. Studies of the domain structure of mammalian DNA polymerase β: identification of a discrete template binding domain. J Biol Chem 1990;265(4). https://doi.org/10.1016/s0021-9258(19)39949-1.
[7] Bandi M., Lazarides K., Engel J., et al. POLB knockout is synthetic lethal with PARP inhibition leading to complete and durable responses in BRCA-mutant tumor xenografts. Manuscript in Preparations. Published online 2024.
[8] Dorjsuren D, Wilson DM, Beard WA, et al. A real-time fluorescence method for enzymatic characterization of specialized human DNA polymerases. Nucleic Acids Res 2009;37(19). https://doi.org/10.1093/nar/gkp641.
[9] Ochkasova A, Arbuzov G, Kabilov M, Tupikin A, Karpova G, Graifer D. AP lyase activity of the human ribosomal protein uS3: the DNA cleavage sequence specificity and the location of the enzyme active center. Biochim Biophys Acta Proteins Proteom 2023;1871(2). https://doi.org/10.1016/j.bbapap.2022.140880.
[10] Maksimenko A, Ishchenko AA, Sanz G, Laval J, Elder RH, Saparbaev MK. A molecular beacon assay for measuring base excision repair activities. Biochem Biophys Res Commun 2004;319(1). https://doi.org/10.1016/j.bbrc.2004.04.179.
[11] Gurard-Levin ZA, Mrksich M. Combining self-assembled monolayers and mass spectrometry for applications in biochips. Ann Rev Anal Chem 2008;1(1). https://doi.org/10.1146/annurev.anchem.1.031207.112903.
[12] Mrksich M. Mass spectrometry of self-assembled monolayers: a new tool for molecular surface science. ACS Nano 2008;2(1). https://doi.org/10.1021/nn7004156.
[13] Gurard-Levin ZA, Scholle MD, Eisenberg AH, Mrksich M. High-throughput screening of small molecule libraries using SAMDI mass spectrometry. ACS Comb Sci 2011;13(4). https://doi.org/10.1021/co2000373.
[14] Patel K, Sherrill J, Mrksich M, Scholle MD. Discovery of SIRT3 inhibitors using SAMDI mass spectrometry. J Biomol Screen 2015;20(7). https://doi.org/10.1177/1087057115588512.
[15] Swalm BM, Knutson SK, Warholic NM, et al. Reaction coupling between wild-type and disease-associated mutant EZH2. ACS Chem Biol 2014;9(11). https://doi.org/10.1021/cb500548b.
[16] Buker SM, Gurard-Levin ZA, Wheeler BD, et al. A Mass Spectrometric Assay of METTL3/METTL14 Methyltransferase Activity. SLAS Discov 2020;25(4). https://doi.org/10.1177/2472555219878408.
[17] Scholle MD, Liu C, Deval J, Gurard-Levin ZA. Label-free screening of SARS-CoV-2 NSP14 exonuclease activity using SAMDI mass spectrometry. SLAS Discov 2021;26(6). https://doi.org/10.1177/24725552211008854.
[18] Scholle MD, Gurard-Levin ZA. Development of a novel label-free and high-throughput arginase-1 assay using self-assembled monolayer desorption ionization mass spectrometry. SLAS Discov 2021;26(6). https://doi.org/10.1177/24725552211000677.
[19] Scholle MD, O'Kane PT, Dib S, Gurard-Levin ZA. Label-free duplex SAMDI-MS screen reveals novel SARS-CoV-2 3CLpro inhibitors. Antiviral Res 2022;200. https://doi.org/10.1016/j.antiviral.2022.105279.
[20] Abbotts J, SenGupta DN, Zmudzka B, Widen SG, Notario V, Wilson SH. Expression of human Dna Polymerase β in Escherichia coli and characterization of the recombinant enzyme. Biochemistry 1988;27(3). https://doi.org/10.1021/bi00403a010.
[21] Beard WA, Wilson SH. Purification and domain-mapping of mammalian DNA polymerase β. Methods Enzymol 1995;262(C). https://doi.org/10.1016/0076-6879(95)62013-3.
[22] Stivers JT. 2-Aminopurine fluorescence studies of base stacking interactions at abasic sites in DNA: metal-ion and base sequence effects. Nucleic Acids Res 1998;26(16). https://doi.org/10.1093/nar/26.16.3837.
[23] Zhigang W, Smith DG, Mosbaugh DW. Overproduction and characterization of the uracil-DNA glycosylase inhibitor of bacteriophage PBS2. Gene 1991;99(1). https://doi.org/10.1016/0378-1119(91)90030-F.
[24] Pearl LH, Savva R. DNA repair in three dimensions. Trends Biochem Sci 1995;20(10). https://doi.org/10.1016/S0968-0004(00)89092-1.
[25] Scholle MD, McLaughlin D, Gurard-Levin ZA. High-throughput affinity selection mass spectrometry using SAMDI-MS to identify small-molecule binders of the human rhinovirus 3C protease. SLAS Discov 2021;26(8). https://doi.org/10.1177/24725552211023211.
[26] VanderPorten EC, Scholle MD, Sherrill J, Tran JC, Liu Y. Identification of small-molecule noncovalent binders utilizing SAMDI technology. SLAS Discov 2017;22(10). https://doi.org/10.1177/2472555217712761.
[27] Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999;4(2). https://doi.org/10.1177/108705719900400206.
[28] Allawi HT, Santalucia J. Thermodynamics and NMR of internal G·T mismatches in DNA. Biochemistry 1997;36(34). https://doi.org/10.1021/bi962590c.
[29] McTigue PM, Peterson RJ, Kahn JD. Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. Biochemistry 2004;43(18). https://doi.org/10.1021/bi035976d.
[30] Dai DP, Prasad R, Strauss PR, Wilson SH. The Pol β variant containing exon α is deficient in DNA polymerase but has full dRP lyase activity. Sci Rep 2019;9(1). https://doi.org/10.1038/s41598-019-45846-0.
[31] Copeland RA. Mechanistic considerations in high-throughput screening. Anal Biochem 2003;320(1). https://doi.org/10.1016/S0003-2697(03)00346-4.
[32] Prasad R, Beard WA, Chyan JY, Maciejewski MW, Mullen GP, Wilson SH. Functional analysis of the amino-terminal 8-kDa domain of DNA polymerase β as revealed by site-directed mutagenesis: DNA binding and 5'-deoxyribose phosphate lyase activities. J Biol Chem 1998;273(18). https://doi.org/10.1074/jbc.273.18.11121.
[33] Menge KL, Hostomsky Z, Nodes BR, et al. Structure-function analysis of the mammalian DNA polymerase β active site: role of Aspartic Acid 256, Arginine 254, and Arginine 258 in Nucleotidyl Transfer. Biochemistry 1995;34(49). https://doi.org/10.1021/bi00049a008.
[34] Busby SA, Carbonneau S, Concannon J, et al. Advancements in assay technologies and strategies to enable drug discovery. ACS Chem Biol 2020;15(10). https://doi.org/10.1021/acschembio.0c00495.
[35] Zehender H, Le Goff F, Lehmann N, Filipuzzi I, Mayr LM. SpeedScreen: the "missing link" between genomics and lead discovery. J Biomol Screen 2004;9(6). https://doi.org/10.1177/1087057104267605.
[36] O'Connell TN, Ramsay J, Rieth SF, Shapiro MJ, Stroh JG. Solution-based indirect affinity selection mass spectrometry-a general tool for high-throughput screening of pharmaceutical compound libraries. Anal Chem 2014;86(15). https://doi.org/10.1021/ac500938y.
[37] Annis DA, Nazef N, Chuang CC, Scott MP, Nash HM. A general technique to rank protein-ligand binding affinities and determine allosteric versus direct binding site competition in compound mixtures. J Am Chem Soc 2004;126(47). https://doi.org/10.1021/ja048365x.
[38] Howard MJ, Horton JK, Zhao ML, Wilson SH. Lysines in the lyase active site of DNA polymerase b destabilize nonspecific DNA binding, facilitating searching and DNA gap recognition. J Biol Chem 2020;295(34). https://doi.org/10.1074/jbc.ra120.013547.
[39] Yuhas SC, Laverty DJ, Lee H, Majumdar A, Greenberg MM. Selective inhibition of DNA polymerase β by a covalent inhibitor. J Am Chem Soc 2021;143(21). https://doi.org/10.1021/jacs.1c02453.

[40] Dueñas ME, Peltier-Heap RE, Leveridge M, Annan RS, Büttner FH, Trost M. Advances in high-throughput mass spectrometry in drug discovery. EMBO Mol Med 2023;15(1). https://doi.org/10.15252/emmm.202114850.

[41] McLaren DG, Shah V, Wisniewski T, et al. High-throughput mass spectrometry for hit identification: current Landscape and Future Perspectives. SLAS Discov 2021;26 (2). https://doi.org/10.1177/2472555220980696.

[42] Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49(7). https://doi.org/10.1373/49.7.1041.