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Article

Simple and Practical DNA Quantification Method for DNA-Encoded Library Synthesis

Hangke Ma, Zhaomei Sun, Lijun Xue, Xue Zhao, Jie Zhang, Huanqing Zhang, Kexin Yang, and Yun Jin Hu*

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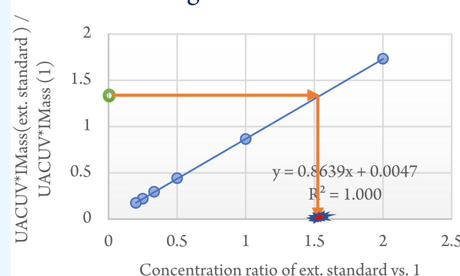
ABSTRACT: Over the past three decades, DNA-encoded library (DEL) technologies have become one of the most relevant strategies for hit-finding. Recent advances in synthetic methodologies for DNA-encoded libraries rendered the increased chemical space available, but it is unknown how every variety of chemistry affects DNA's integrity. Available assays to quantify DNA damage are restricted to electrophoresis, ligation efficiency, and mostly qPCR quantification and sequencing, which may contain predisposition and inconsistency. We developed an external standard method through LC-MS analysis to accurately quantify DNA damage throughout the chemical transformations. An assessment was conducted on on-DNA chemical reactions that are frequently employed in DEL synthesis, and these results were compared to traditional qPCR measurements. Our study provides a simple, practicable, and accurate measurement for DNA degradation during DEL synthesis. Our finding reveals substantial disagreement among the usual DNA-damaging assessment methods, which have been largely neglected so far.



1. INTRODUCTION

Brenner and Lerner's mythical 1992 PNAS article demonstrated that a novel type of combinatorial chemistry could be conveyed by DNA, paving the way for the building of DNA-encoded libraries (DELs).[1] Since then, DNA-encoded compound libraries for discovering small-molecule protein ligands have evolved into a highly desirable technology for facilitating the identification of protein "binders" in affinity-based selection assays.[2,3] DEL technologies combine synthetic organic chemistry and DNA barcoding to generate libraries of compounds covalently affixed to a unique, amplifiable tag that encapsulates the synthetic reactions from which each compound derives.[4] DELs are most commonly employed to identify molecules that interact with an immobilized protein target.[5] This strategy has yielded binders of therapeutically relevant proteins, such as kinases, phosphatases, G-protein-coupled receptors, and RNAs, to name only a few.[6,7]

It is conceivable that the potential of DEL-mediated target-based screening has not yet been fully exploited yet. Indeed, despite recent advances in DNA-compatible chemistry, the structural characteristics of DELs remain limited.[8] To resolve the structural limitations of DEL compounds, there is a pressing need to investigate a larger range of chemical reactions that are, or could be, compatible with DNA.[9] Nevertheless, DNA is vulnerable and susceptible to damage under a variety of chemical reaction conditions.[10] Thus, there is an imperative necessity to develop a method that is simple, rapid, practicable, and accurate for monitoring the DNA's viability during chemical conversion.[10−12] Consequently, this should not only facilitate the application of known DNA-compatible reactions to a wide variety of substrates but would also guide the exploration of new on-DNA chemistry.[13−20] As part of our efforts to develop novel DNA-compatible reactions and their subsequent implementation in routine DEL synthesis, we recognized DNA damage as a critical and imperative matter to address. Our efforts have resulted in the development of an LC-MS-based method meeting these requirements.

During the synthesis of DELs, DNA must be exposed to a number of toxic environments, such as an aqueous cosolvent mixture that breaks down the DNA double helix, electrophilic or nucleophilic reagents, toxic transition metal catalysts, and high temperatures. Oligomer hydrolysis, for instance, is the result of protracted reaction times at an elevated pH and temperature. In the presence of an alkaline pH and metal ions, hydrolytic deamination and mutations may occur.[21] As protonation of the N-7 of purines may result in depurination, i.e., the loss of purine bases from the oligomer, acidic conditions (such as below pH 4) are likely the most deleterious for DEL synthesis.[22] Strong nucleophiles, such as

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hydrazines and hydroxylamines, are known carcinogens that can harm DNA by hydrolyzing the phosphate backbone, adding to the 6-position of pyrimidines, and substituting nucleobase amines.[23] Radiation, which can result in the dimerization of thymine, and radicals, which can contribute to the formation of 8-oxopurine, cause additional DNA-damaging reactions and, as a result, hamper or even eliminate their encodability.[24,25]

To overcome the difficulty, Paegel and Malone developed DNA-encoded reaction rehearsal, an integrated analysis of reaction yield through HPLC-based analysis and the impact on DNA damage on the magnetic DNA-functionalized sensor beads via qPCR quantification.[10] However, this may not correlate exactly with the actual % of DNA remaining after solution-phase library synthesis.

Recently, Ratnayake et al. proposed measurement of ligation efficiency, assessment of chemical fidelity through analysis of reaction yield and purity, and readability (DNA compatibility) as an alternative method for evaluating DNA damage, which correlates with encoding issues.[11] As the overhang is merely two nucleotides long and any substantial damage there could compromise the encoding of diversity elements, ligation efficacy is a crucial metric in headpiece libraries. Nevertheless, it might not capture the damage that occurred in the center of the DNA tags.

Parallelly, Gillingham's group revealed that the encoding strategy can influence mutation rates, suggesting that the DNA compatibility of specific reactions should not only be measured with qPCR but also with deep sequencing.[12] This method would identify any bioinformatics issues during codon design, and the knowledge gathered from the current experience could guide the design of future codon systems to reduce the likelihood of point mutations during chemical conversion. Based on their findings, codons exposed to the conditions of CuAAC, for instance, should have a lower proportion of G/C pairs due to the risk of oxidation and the resulting G-T transversion.

Collectively, these prior studies demonstrated the capacity to detect and analyze DNA damage. Various techniques, such as ligation efficiency, qPCR, and Sanger sequencing, have been disclosed for quantifying a portion of DNA damage caused by chemical reactions. Although Sanger sequencing can detect significant point mutations, it cannot quantify less prevalent ones. In contrast, qPCR permits the rapid and accurate measurement of the DNA concentrations. Consequently, optimization and validation performed to ascertain the specificity and sensitivity of an assay are rarely reported in the scientific literature.[26] However, the extent to which qPCR can efficiently detect DNA damage not usually considered to be polymerase-blocking, such as 8-hydroxydeoxyguanosine (8-OHdG), has not been rigorously investigated.[27] Additionally, when DNA damage is detected, the type of DNA damage cannot be determined due to the nondiscriminatory nature of qPCR. In addition, the amplification of nonspecific products is common and independent of DNA template concentration or qPCR efficiency values.[28] Unnoticed or undetectable amplification caused by a partially damaged DNA sequence, primer dimerization, or a nonspecific product may result in a false positive readout and thus distort the quantification.[29] A further limitation of qPCR is that it provides a relative rather than absolute quantification of DNA damage since control samples are defined as "undamaged" for the purposes of DNA damage calculations and template concentrations were predetermined by other methods, such as by a NanoDrop. In addition, multiple sample processing or preparation steps are required prior to qPCR analysis, which may contribute to variations in quantification. A further concern is that the qPCR assay cannot detect regiospecific DNA damage outside the amplification region of the primer set (the headpiece cannot be amplified). Thus, the results may be biased if the DNA-damaging agent targets the amplified region or a nonamplified region specifically.

2. EXPERIMENTAL SECTION

We observed that contaminants in the reaction medium can affect the outcome of qPCR and result in significant measurement errors. LC-MS analysis of on-DNA chemical conversions during DEL synthesis is ubiquitous. We considered employing the LC-MS-based external standard method for DNA measurement. An external standard is akin to an internal standard, excluding the fact that it does not contribute to the unknown. Instead, it is analyzed separately, as a sample, and typically at varying concentrations, so that a standard curve can be generated.[30] External standards do not take into consideration losses that can occur during sample preparation, such as DNA precipitation, transferring, etc. Adding internal standards to the unknown prior to sample preparation would rectify the situation. Since the standard sample must satisfy UV absorption sensitivity and ionization requirements, DNA is the optimal choice as an external standard. External standards are preferred to prevent interference or damage to the standard samples. In our study, the standard sample is a DNA headpiece (HP), as depicted in Figure 1.

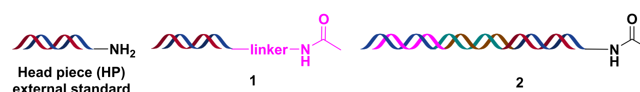

Figure 1. Descriptive structures of the headpiece and probes.

As external reference compounds, *N*-acetyl-capped substrates **1** (with a DNA headpiece) and **2** (with an amplifiable sequence) were chosen as probes to study DNA damage in reactions (Figure 2).

As per the study protocol, aqueous stock solutions of HP (20.00 μM) and **1** (10.00 μM) were prepared. Then, study samples of HP and **1** were prepared via sequential dilution (each with 50, 100, 200, 300, 400, and 500 pmol of **1**). Each sample was analyzed by LC-MS following mixing and dilution with water to a total volume of 100 μL (see SI Sections S8 and S9 for the standard curve calculation and LC-MS spectrum data).

For the standard curve analysis, six sets of experiments were conducted on six calibration standard solutions (ranging from 50 to 500 pmol), and each experiment was replicated three times. Therefore, the calibration curve provides a means of relating the concentration of an external standard to the concentration of the analyte (Figure 2). The probe compounds **1** and **2** were sequentially exposed to nonreactive substrates in well-established on-DNA chemical reactions. **1** contains a short DNA oligo, which is frequently used as an instrument for validation studies during DEL synthesis. Alternatively, due to the extended sequence exposure to chemical transformation conditions, **2** with 70 base pairs was more pertinent to DEL production. First, **1** and **2** were applied to a well-established

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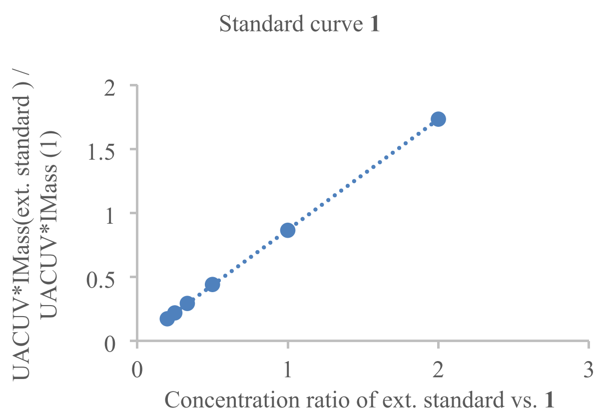

Figure 2. DNA quantification to assess DNA damage. Calibration curve derived from a succession of experimental DNA samples with known concentration (R = 1.000). The ratio of the area under the curve readouts of UV absorption (UAC_{UV}) multiplied by the ionization intensity (I_{Mass}) from mass spectra corresponds to the concentration ratio of **1** and external standard HP.

DMT-MM-mediated acylation to assess DNA damage during the process (Table 1). Each experiment was run in triplicates.

Table 1. DNA Damage during Acylation with DMT-MM

entry	probe	reaction replicates	DNA remaining (%)	mean value (%)	standard deviation (%)
1	**1**	1-1	89.01	88.55	2.02
2	**1**	1-2	90.31		
3	**1**	1-3	86.34		
4	**2**	2-1	94.24	94.52	0.49
5	**2**	2-2	95.09		
6	**2**	2-3	94.24		

Through LC-MS analysis, the UV absorption and mass ionization of **1** and an external standard, HP, were determined (Figure 3). Given the potential limitations of UV detection and mass ionization methods alone, the integration of UAC_{UV} and I_{Mass} provides a more accurate measurement. Deploying the calculation curve deployed, the measured ratio of HP to **1** in each reaction was converted to the remaining DNA percentage. The remaining DNA fraction is the ratio of the measured DNA quantity to the expected DNA quantity. It was

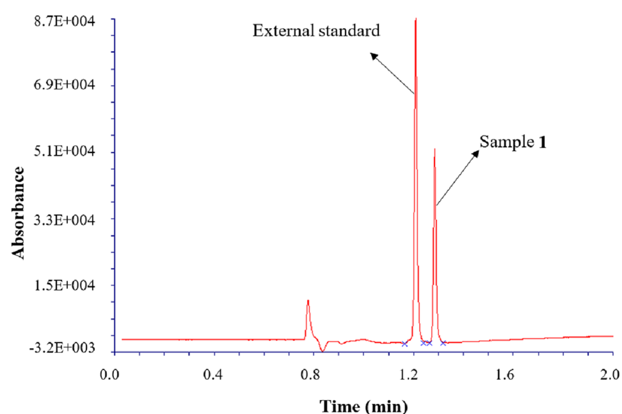

Figure 3. Typical example of the LC-MS spectrum in which the sample and external standard can be distinctly differentiated and quantified.

determined that the remaining DNA of **1** after DMT-MM acylation was, on average, 88.55%, with a standard deviation of 2.02%, whereas the remaining DNA of **2** was, on average, 94.52%, with a standard deviation of 0.49%.

3. RESULTS AND DISCUSSION

There was a less than 10% difference between the DNA damage rates of the two samples. DNA damage under the DMT acylation conditions is negligible when considering the loss during precipitation and instrument measurement errors. Next, we applied the established procedure to other reactions (Table 2).

For the above-described DMT-MM acylation, the qPCR study revealed no DNA damage, in contrast to the LC-MS results, which indicate that short DNA **1** had approximately 5% damage but longer DNA did not (entry 1). A second HATU acylation is commonly employed in DEL synthesis. **1** had 5% damage, while **2** had 11% damage, which was closer to the qPCR measurement, although the latter had a 7% data deviation (entry 2). Next, we investigated reductive amination, usually regarded as an efficient and nontoxic method for DEL synthesis (entry 3). According to the LC-MS method and qPCR quantification, there was essentially no DNA damage. The deprotection of Fmoc groups is ideally suited for DEL synthesis (entry 4). Our findings revealed that short DNA sequences had negligible DNA damage, whereas extended DNA sequences had appreciable DNA damage with outstanding data consistency. However, the qPCR study revealed a significant variance (21%) in the degree of damage. The hydrolysis reactions can be well-tolerated on-DNA (entry 5) with minimal DNA damage, which is consistent with the results of the qPCR study but with a much larger data deviation. Similar results were observed with Boc group deprotection (using $MgCl_2$/NaOAc) (entry 6).

Intriguingly, Boc deprotection (with $MgCl_2/CF_3COONa$) was harmless for shorter DNA, but it was damaging for longer DNA oligos, as confirmed by LC-MS owing to depurination. However, the qPCR analysis demonstrated that no DNA damage occurred (entry 7). Under on-DNA chemistry-compatible conditions, sulfonylation, copper-catalyzed oxidation and amination of terminal alkynes are well-tolerated (entries 8 and 9). Suzuki coupling is a significant chemistry for constructing biaryl compounds, and it has been successfully adapted to DNA. This versatile C−C bond forging reaction causes minimal DNA damage to both short and long DNA oligos. However, the qPCR quantification method showed significant variations and is deemed inaccurate (entry 10) likely due to the inhibition of enzymatic activity by reaction contaminates. The on-DNA Kinugasa reaction, alkyne iodination, and acetylenic coupling (entries 11 to 13) caused minimal DNA damage. The click reaction has had a significant impact on organic chemistry. For DEL synthesis, it has been utilized as a chemical ligation method.[31] It was also reported as one of the most DNA-damaging reactions.[12] Our study reveals that this triazole synthesis from alkyne (entry 14) causes only modest DNA damage. The on-DNA coupling reaction promoted by EDCI is an excellent approach to amide synthesis (entry 15). Both azide reduction and urea formation are effective on-DNA (entries 16 and 17), but the qPCR quantification method cannot be relied upon to measure DNA damage. Thiol aerial oxidation is a versatile reaction that can be carried out under benign conditions and is nearly innocuous to the integrity of DNA (entry 18).

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Table 2. DNA Damage Quantification of Commonly Rehearsed Reactions

entry	reaction types	reaction conditions	yield of 1[a]	yield of 2[b]	amplifiable of 2[c] (qPCR method)
0	NTC		94 ± 2%	96 ± 2%	94 ± 16%
1	condensation (DMT-MM)	borate buffer (pH = 9.5, 250 equiv, 250 mM in H_2O), acid (200 equiv, 200 mM), DMT-MM (80 equiv, 200 mM), in DMSO, 25 °C, 2 h	89 ± 2%	95 ± 0%	109 ± 5%
2	acylation (HATU)	borate buffer (pH = 9.5, 250 equiv, 250 mM in H_2O), acid (150 equiv, 200 mM), HATU (100 equiv, 200 mM), DIEA (200 equiv, 200 mM), in DMSO, 25 °C, 2 h	95 ± 1%	89 ± 4%	85 ± 7%
3	reductive amination	sodium phosphate buffer (pH = 5.5, 250 equiv, 250 mM in H_2O), benzaldehyde (200 equiv, 200 mM), $NaCNBH_3$ (200 equiv, 100 mM), in MeCN, 25 °C, 24 h	96 ± 3%	96 ± 1%	103 ± 8%
4	de-Fmoc	10% piperidine, 10 μL, 25 °C, 2 h	94 ± 2%	88 ± 1%	106 ± 21%
5	hydrolysis	LiOH (300 equiv, 200 mM), in H_2O, 25 °C, 1 h	92 ± 2%	91 ± 2%	89 ± 17%
6	de-Boc (NaOAc)	$MgCl_2$ (200 equiv, 200 mM), NaOAc (24 equiv, 7.0 mM), in H_2O, 80 °C, 24 h	88 ± 2%	91 ± 1%	88 ± 18%
7[a]	de-Boc (CF_3COONa)	$MgCl_2$ (200 equiv, 200 mM), CF_3COONa (24 equiv, 50.0 mM), in H_2O, 95 °C, 24 h	95 ± 1%	65 ± 2%	110 ± 4%
8	amine sulfonylation	borate buffer (pH = 9.5, 250 equiv, 250 mM in H_2O), benzenesulfonyl chloride (200 equiv, 200 mM), in MeCN, 25 °C, 4 h	96 ± 2%	93 ± 2%	109 ± 9%
9	alkyne oxidization and amidation	borate buffer (pH = 9.5, 250 equiv, 250 mM in H_2O), CuI (20 equiv, 20 mM), nitrone (100 equiv, 200 mM), amine (200 equiv, 200 mM), in DMSO, 50 °C, 4 h, DDTC (600 equiv, 200 mM in H_2O), 50 °C, 0.5 h	90 ± 1%	97 ± 1%	99 ± 3%
10[b]	Suzuki reaction	K_2CO_3 (120 equiv, 200 mM in H_2O), boric acid (200 equiv, 200 mM in IPA), $PdCl_2$ (5 equiv, 3 mM in H_2O), TPPTS (10 equiv, 25 mM in H_2O), 80 °C, 4 h, DDTC (600 equiv, 200 mM in H_2O), 80 °C, 0.5 h	93 ± 2%	89 ± 1%	39 ± 26%
11	Kinugasa reaction	nitrone (50 equiv, 100 mM), CuI (10 equiv, 20 mM), Cy_2NMe (100 equiv, 200 mM), in MeCN, N_2, 25 °C, 24 h, DDTC (600 equiv, 200 mM in H_2O), 25 °C, 0.5 h	91 ± 2%	90 ± 2%	93 ± 9%
12	on-DNA alkyne iodination	piperidine (50 equiv, 100 mM), NIS (20 equiv, 40 mM), CuI (5 equiv, 10 mM), in MeCN, 40 °C, 12 h, DDTC (600 equiv, 200 mM in H_2O), 40 °C, 0.5 h	87 ± 2%	93 ± 3%	73 ± 8%
13	acetylenic coupling	ethynylbenzene (400 equiv, 200 mM), CuI (10 equiv, 20 mM), pyrrolidine (400 equiv, 200 mM), in MeCN, 25 °C, 12 h, DDTC (600 equiv, 200 mM in H_2O), 25 °C, 0.5 h	95 ± 1%	97 ± 1%	100 ± 9%
14	click reaction	4-(prop-2-yn-1-yl) morpholine (50 equiv, 50 mM), TBTA (20 equiv, 20 mM), $CuSO_4$ (5 equiv, 5 mM), erythorbic acid (5 equiv, 5 mM), in MeCN, 25 °C, 4 h, DDTC (600 equiv, 200 mM in H_2O), 25 °C, 0.5 h	85 ± 1%	85 ± 3%	99 ± 6%
15	condensation reaction (EDCI)	4-formyl-3-hydroxybenzoic acid (120 equiv, 200 mM), EDCI (1200 equiv, 400 mM), DIEA (1200 equiv, 200 mM), HOAT (240 equiv, 100 mM), in DMSO, 25 °C, 1 h	97 ± 1%	89 ± 1%	115 ± 6%
16	azide reduction	triphenylphosphine (50 equiv, 50 mM), in MeCN, 50 °C, 2 h	97 ± 4%	92 ± 2%	111 ± 24%
17[c]	urea formation	phosphate buffer (pH = 3.5, 250 equiv, 250 mM in H_2O), 1-isocyanato-4-methoxybenzene (500 equiv, 500 mM in MeCN), 25 °C, 24 h	95 ± 2%	90 ± 3%	193 ± 22%
18	disulfide synthesis	2-fluorobenzenethiol (50 equiv, 20 mM), TMG (10 equiv, 4 mM), in THF, 25 °C, 10 min	97 ± 2%	93 ± 1%	93 ± 13%
19	SNAr	borate buffer (pH = 9.5, 250 equiv, 250 mM in H_2O), 2-bromoacetyl chloride (200 equiv, 200 mM in MeCN), 25 °C, 2 h	90 ± 3%	94 ± 3%	94 ± 14%

[a]More than 20% depurination detected by the LC-MS method but not by the qPCR study. [b]The low recovery from the qPCR study is probably because the chemical reagent inhibits the enzymatic activity during ligation. [c]Impurities or byproducts affected fluorescence acquisition of qPCR.

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Table 3. DNA LC-MS Quantification vs qPCR Methods: Pros and Cons

comparison aspects	DNA LC-MS method	qPCR quantification method
preconditions for study	None	Substrates must contain amplifiable primers, impurities must be minimized, and amplifiable-influence artifacts must be eliminated.
sample processing capability	Hundreds or thousands of samples per day can be processed by one LC-MS instrument.	Leading time includes ligation and workup; study condition optimization takes many days. Once conditions are optimized, dozens of samples per instrument can be processed per day.
factors influencing the results	Minor problems due to the potential resolution of liquid chromatography separation in rare cases	Multiple factors impact the results: ligation efficiency, impurities, artifacts, substrate concentration, etc.
accuracy of results	Minimal variation among replicated study outcomes, superb data repeatability, and high precision	Large deviations; poor reproducibility
insights into DNA damage	Providing proof for depurination and other nucleotide or phosphate group's loss	Only providing DNA quantification readout

The nucleophilic aromatic substitution reaction (SNAr) has been widely employed in DEL synthesis. Our research revealed minimal DNA damage (entry 19). In total, we evaluated 19 different on-DNA reactions that have been frequently implemented in DEL synthesis. Except for the Boc group deprotection with $MgCl_2/CF_3COONa$, in which 35% DNA was compromised, most of these reactions resulted in minor or negligible DNA damage. However, the qPCR method is, in certain cases, incapable of detecting DNA damage. As previously said, certain DNA alterations will not alter the action of polymerase, or modifications can occur on non-amplified regions. Also, a variety of metal-catalyzed reactions, such as the previously mentioned Suzuki coupling here, produce metallic byproducts, which greatly hamper the action of polymerases. The use of LC-MS-mediated workflows circumvents these biases by providing an actual analysis of the oligonucleotide structure.

Table 3 outlines the benefits and disadvantages of the developed LC-MS analytical method compared to the commonly used qPCR quantification technique for DNA damage assessments. In comparison to the qPCR method, the LC-MS DNA quantification approach has the advantages of an easy sample preparation process, a high throughput capacity, and the ability to generate accurate results.

4. CONCLUSIONS

Using LC-MS analysis, we developed a method to quantify DNA damage during chemical transformations using an external standard with moderate precision. In this study, common on-DNA chemical reactions were analyzed and compared to qPCR measurements, the most widely employed method to assess DNA integrity in DEL synthesis. As it is based on LC-MS, which enables a careful structural analysis of the oligonucleotide structure, our research provides a straightforward, practically applicable, and reasonably accurate method for measuring DNA damage caused by chemical reactions. This technique for quantifying DNA has been implemented in our practice during DEL synthesis, in addition to serving as a validation study. This study's findings will aid DELT practitioners in the development of new on-DNA reactions, thereby facilitating the construction of a novel chemical space for molecular recognition during the earliest phases of drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06768.

Experimental details and procedures, optimization studies, and spectral data for all on-DNA compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Yun Jin Hu − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China;* orcid.org/0000-0001-6943-2459; Email: yunjin.hu@pharmaron.com

Authors

Hangke Ma − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Zhaomei Sun − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Lijun Xue − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Xue Zhao − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Jie Zhang − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Huanqing Zhang − *Pharmaron (Ningbo) Technology Development Co., Ltd., Hangzhou Bay New Zone, Ningbo 315336, China*

Kexin Yang − *Pharmaron Beijing Co., Ltd., Beijing 100176, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06768

Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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