CHAPTER FIVE

Development of a rapid mass spectrometric method for the analysis of ten-eleven translocation enzymes

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Contents

Abstract

In DNA, methylation at the fifth position of cytosine (5mC) by DNA methyltransferases is essential for eukaryotic gene regulation. Methylation patterns are dynamically controlled by epigenetic machinery. Erasure of 5mC by Fe^{2+} and 2-ketoglutarate (2KG) dependent dioxygenases in the ten-eleven translocation family (TET1–3), plays a key role in nuclear processes. Through the event of active demethylation, TET proteins iteratively oxidize 5mC to 5-hydroxymethyl cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC), each of which has been implicated in numerous diseases when aberrantly generated. A wide range of biochemical assays have been developed to characterize TET activity, many of which require multi-step processing to detect and quantify the 5mC oxidized products. Herein, we describe the development and optimization of a sensitive MALDI mass spectrometry-based technique that directly measures TET activity and eliminates tedious processing steps. Employing optimized assay conditions, we report the steady-state activity of wild type TET2 enzymes to furnish 5hmC, 5fC and 5caC. We next determine IC_{50} values of several

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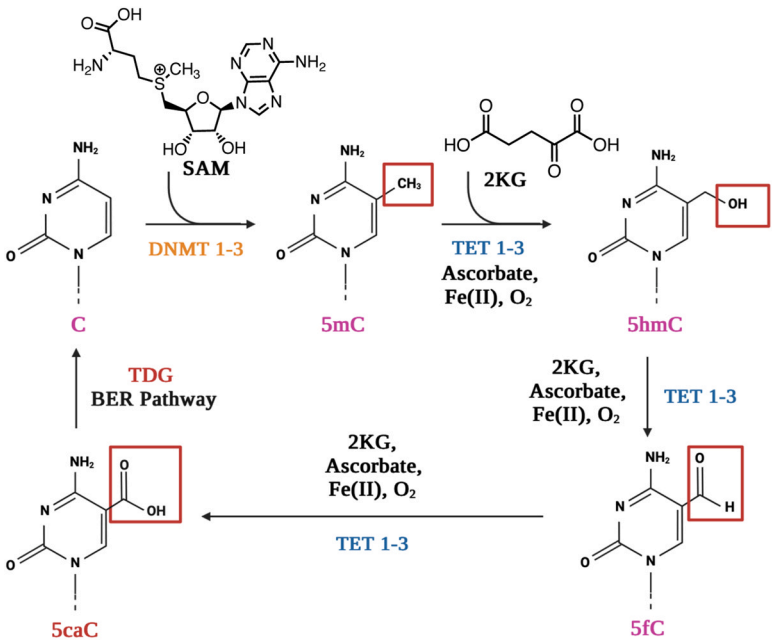

Fig. 1 Generation of 5mC by DNMTs using SAM as methyl donor and its successive oxidation by Fe^{2+}-dependent TET enzymes using 2KG as cofactor.

small-molecule inhibitors of TETs. The utility of this assay is further demonstrated by analyzing the activity of V1395A which is an activating mutant of TET2 that primarily generates 5caC. Lastly, we describe the development of a secondary assay that utilizes bisulfite chemistry to further examine the activity of wildtype TET2 and V1395A in a base-resolution manner. The combined results demonstrate that the activity of TET proteins can be gauged, and their products accurately quantified using our methods.

1. Introduction

All cells in eukaryotic organisms have the same genomic DNA but have different functions and phenotypes due to differential expression (activation or repression) of specific genes (Gibney & Nolan, 2010; Jaenisch & Bird, 2003). Methylation of cytidine at carbon 5 (5mC) by DNA methyltransferases (DNMTs) using *S*-adenosyl methionine (SAM), constitutes an important mechanism for gene expression (Fig. 1) (Jurkowska, Jurkowski, & Jeltsch, 2011). 5mC is successively oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by the Ten-Eleven Translocation (TET) enzymes, which are

members of the Fe (II) and 2- ketoglutaric acid (2KG)-dependent dioxygenases family (Loenarz & Schofield, 2008; Lu, Zhao, & He, 2015). 5caC subsequently undergoes thymine DNA glycosylase (TDG)-mediated base excision followed by repair to unmodified cytidine (Fig. 1), thus providing a biochemical basis for active DNA demethylation which is essential to early mammalian development (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009). Aside from playing a role as intermediates in the demethylation pathway, growing evidence suggests that each of the TET oxidized products (5hmC, 5fC and 5caC) can independently regulate gene expression (Bachman et al., 2015; Song & He, 2013; Spruijt et al., 2013). TET-mediated oxidation of 5mC is critical to cellular differentiation (Kohli & Zhang, 2013). Consistently, overexpression and catalytically inactive mutations of TET enzymes have also been associated with the development of human diseases such as neurodegenerative disorders and cancers (Huang & Rao, 2014; Joshi, Liu, Breslin, & Zhang, 2022; Ko et al., 2010).

Development of novel methods to detect and quantify the enzymatic activity of TET proteins as well as their oxidized products both *in-vitro* and *in-cellulo* have been driven by efforts to understand the functions of TET proteins in eukaryotic gene regulation (Bhattacharya, Dey, & Mukherji, 2023; Dey, Ayon, Bhattacharya, Gutheil, & Mukherji, 2020; Liu, DeNizio, & Kohli, 2016; Liu, Wang, Liu, Xu, & Zhang, 2022; Liu, Zhang, Hu, & Zhang, 2022; Ma et al., 2024; Marholz, Wang, Zheng, & Wang, 2016; Shen & Zhang, 2012; Sudhamalla, Dey, Breski, & Islam, 2017; Treadway et al., 2024). A majority of current approaches involve multiple enzymatic reactions and purification steps that lead to the loss of the oxidized products and subsequent errors in quantification (Sudhamalla et al., 2017). Fluorescence, quantum dots and NMR-based methods have also been applied to measure TET activity, but these newer assays are limited in scope and practicality due to their complexity often requiring amplification technologies or high DNA substrate and enzyme concentrations in order to obtain a desired sensitivity, and generation of isotope-labeled protein which is expensive and time consuming (Ma et al., 2024; Treadway et al., 2024).

Herein we report the step-by-step development and optimization of a novel assay that facilitates direct and efficient measurement of enzymatic activity of TET proteins (Sudhamalla et al., 2017). The salient features include (i) use of AG® 50W-X8 cation-exchange resin beads to desalt oxidized oligonucleotide products, thus eliminating the need for their

subsequent enzymatic digestion and often laborious purification, and (ii) direct analysis of product distribution (5hmC *vs* 5fC *vs* 5caC) by matrix-assisted laser desorption/ionization (MALDI) based mass spectrometry. Employing the optimized assay conditions, we precisely measured steady-state activity of wild type TET2 enzymes to furnish 5hmC, 5fC and 5caC. We next determine IC_{50} values of the selected small-molecule inhibitors of TETs and show that our results are comparable to those obtained by other methods. We further demonstrate the utility of the *in-vitro* assay to analyze the activity of V1395A which is an activating mutant of TET2 known to generate primarily 5caC on short and genomic oligonucleotide substrates (Sappa, Dey, Sudhamalla, & Islam, 2021).

Several chemical approaches coupled with next-generation sequencing techniques have been developed for base-resolution mapping of the 5mC oxidized products in cell- and tissue-specific manners (Booth, Raiber, & Balasubramanian, 2015; Song, Yi, & He, 2012). Herein, we describe the development of a secondary assay to further examine the activity of wildtype TET2 and V1395A in a base-resolution manner on a 76-mer oligonucleotide substrate carrying 5mC at a specific site by treating the oxidized products with bisulfite and coupling with Sanger sequencing (BS-Seq), (Sappa et al., 2021; Sudhamalla et al., 2017). The results corroborate well with the data obtained using MALDI-based *in-vitro* assay, further supporting the ability of our methods to accurately gauge the activity of the TET proteins and likely the related family of 2KG-dependent nucleic acid modifying enzymes.

2. Preparation of the materials

2.1 Expression and purification of wild type TET2

2.1.1 Materials and reagents

Item	Supplier and catalog number
Kanamycin monosulfate	Fisher Cat # K004725G
Escherichia coli strain BL21 star (DE3) competent cells	Fisher Cat # C601003
SOC medium	Corning Cat #46-003-CR
LB agar	Fisher Cat # BP1425-500
LB broth	Fisher Cat # BP9723-2

Isopropyl β-d-1-thiogalactoyranoside (IPTG)	Fisher Cat # BP162010
Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, pH 8.0)	Fisher
Sodium chloride	Fisher Cat #BP358-10
β-Mercaptoethanol	Fisher Cat #BP176-100
Glycerol	Fisher Cat #BP2291
Imidazole	Fisher Cat #AC12202-5000
Lysozyme	Sigma Cat #10837059001
DNase	Fisher Cat #4536282001
Pierce™ protease inhibitor mini tablets, EDTA-free	Thermo Cat #A32955
Ni-NTA agarose resin beads	Thermo Cat #R90101
Bovine serum albumin (BSA) standards	BioRad Cat #5000206
Bradford dye reagent (1 L)	Fisher Cat #AAJ61522K
Cell spreader	Fisher Cat # 50-751-5036
0.2 μm filter	Thermo Cat #CH2225-NH
Syringe needle (18 G × 1 ½″ TW)	BD PrecisionGlide™
10 mL syringe	BD Luer-Lok™ Tip
1.5 mL eppendorf tube	Fisher
Falcon™ round-bottom polystyrene test tubes	Fisher Cat # 14-959-11B
Falcon™ standard tissue culture dishes	Thermo
Semi-micro, 1.5 mL cuvette	Fisher Cat # 14955127
50 mL Falcon™ tube	Corning
SDS-PAGE gel	
Plastic scoopula, 210 mm, blue	SmartSpatula Cat # 17211

2.1.2 Equipment

Item	Supplier
Ultra-10k centrifugal filter device	Amicon
Large centrifuge	Thermo Sorvall Legend XTR
Weighing scale	Mettler Toledo XPE504

CO_2 incubator for bacterial cell culture	Thermo HERATherm
Innova 44® Incubator shaker	New Brunswick Scientific
Vortex mixer	Corning
Water bath	Thermo
BioSpectrometer	Eppendorf
Pulsed sonication device with microtip	Qsonica-Q700
3D shaker rocking plate	Chemglass Life Sciences
Superdex-200 column	GE Healthcare
FPLC instrument	GE Healthcare
0.2 μm filtered water	Thermo Barnstead GenPure
ChemiDoc MP imaging system	BioRad
Ultra-low temperature freezer	New Brunswick

2.1.3 Protocol

1. Bacterial transformation

The catalytic domains of TET enzymes carry a Cys-rich domain and a Double-Stranded Beta Helix (DSβH) motif separated by a large low-complexity insert (Hu et al., 2013, 2015; Tahiliani et al., 2009). The Xu group at Fudan University developed a TET2 construct (1129–1936) in which residues 1481–1843 were replaced with a 15-residue GS-linker (GGGGSGGGGSGGGGS) and found that this construct to be the smallest catalytically proficient enzyme among the larger constructs examined (Sudhamalla et al., 2017; Hu et al., 2013, 2015). A 6xHis tag was introduced at the *N*-terminus for affinity purification. The TET2 expression construct in pET-28b vector was a kind gift from the Xu group. The plasmid was transformed into *Escherichia coli* strain BL21 star (DE3) competent cells (Fisher) using the following protocol.

a. In a flame sterilized environment, 10 ng of the wild-type TET2 plasmid with pET-28b kanamycin-resistant vector was pipetted into an autoclaved 1.5 mL Eppendorf tube with 9 μL *E. coli* strain BL21 star (DE3) competent cells (Fisher).

b. This mixture was allowed to incubate on ice for 30 min before placed in a 40 °C water bath for 25 s and immediately submerged in ice for 2 min.

c. After the full 2 min had elapsed, 200 μL Super Optimum broth with Catabolite repression (SOC) medium was added to the cells and placed in a shaker at 37 °C for 1 h.

d. The full mixture was spread evenly onto a kanamycin resistant plate and left to incubate overnight in a 37 °C incubator for colony growth.

2. Starter culture inoculation

a. A single colony in close proximity to the edge of the plate away, from colony clusters, was gently picked up with a pipette tip and placed in 10 mL Luria-Bertani (LB) broth and grown for no longer than 16 h at 37 °C in presence of 50 μg/mL kanamycin.

b. It is important to observe the growth of this starter culture. The inoculates should appear significantly foggier than before, but there should be no sign of debris at this stage. Debris is a sign that the cells have begun to die, and the amount of protein obtained at the end of expression will be minimal. If this debris formation is observed, repeat the starter culture inoculation, and incubate for less time.

3. Induction and Expression of Protein

a. The starter culture was diluted 100-fold into 1 L of LB with the addition of 50 μg/mL kanamycin. 1 mL of this solution was put to the side in a cuvette, and the remaining solution was allowed to grow at 37 °C to an optical density at 600 nm (OD_{600}) of 0.8 in an Innova 44® Incubator Shaker. It is important to keep a close eye on the OD_{600} value. If the OD_{600} value increases above 0.8, the amount of protein obtained at the end of expression will be minimal.

b. Once an OD value of 0.8 was reached, protein expression was induced by adding 0.8 mM isopropyl β-d-1-thiogalactoyranoside (IPTG) and incubating for 20 h at 17 °C in an Innova 44® Incubator shaker (New Brunswick Scientific).

4. Affinity Purification

It is important to complete as many of these steps as possible in one day to ensure optimal catalytic activity at the end of this process. Completing each of these steps at 4 °C is crucial for the same reason as TET is a heat sensitive protein.

a. Each 1 L culture was centrifuged at 4 °C for 30 min at 4000 rpm and the supernatant was subsequently discarded.

b. Each liter of harvested cells was resuspended in 15 mL lysis buffer containing 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, pH 8.0), 200 mM NaCl, 5 mM β-mercaptoethanol (BME),

10% glycerol, 25 mM imidazole, Lysozyme, DNase, and Roche protease inhibitor cocktail.

c. The cells were lysed in a falcon tube by pulsed sonication (Qsonica-Q700) in a reservoir filled with fresh ice using 10 s 50 mA pulses separated by 10 s pauses.

d. The sonicated cells were then centrifuged at 13,000 rpm for 50 min at 4 °C.

e. While centrifugation was taking place, Ni-NTA agarose resin beads (Thermo) were washed and equilibrated with buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 25 mM imidazole in a 15 mL column. When centrifugation had finished, the equilibration buffer was allowed to elute from this column before pouring in the soluble extracts from the previous step. The column was capped, and the soluble extracts were allowed to incubate with the Ni-NTA resin on a rocking plate (Chemglass Life Sciences) for 40 min at 4 °C.

f. Proteins with non-specific interactions were allowed to drain out of the column leaving proteins containing the 6×-His tag attached to the beads. To displace the His-Ni interaction, beads were incubated for 10 min with 1 mL of an elution buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 400 mM imidazole. Elution fractions were subsequentially captured in a 1.5 mL Eppendorf Tube. This step was repeated two more times, collecting a total of 3 mL from each column.

5. Fast Protein Liquid Chromatography (FPLC)

a. Proteins were further purified by gel filtration chromatography (Superdex-200) using AKTA pure Fast Protein Liquid Chromatography (FPLC) system (GE healthcare) with buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 10% glycerol. Prior to injecting into the FPLC instrument, the protein was filtered by drawing up the liquid through a 10 mL syringe and pushing through a 0.2 μm filter into a 50 mL Falcon tube. The liquid was then drawn up through the syringe once again and injected into the FPLC column for purification. For a protein of this size (~69 kDa), it took approximately 3 h for the protein of interest to elute from the column. To reduce the amount of time the protein was outside of the −80 °C and increase the lifetime of TET, fractions under the UV indication curve were collected as soon as they eluted from the column in 5 mL intervals.

b. FPLC fractions were analyzed *via* sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 4%–12%) to determine which of the 5 mL fractions contained the protein of interest.

c. To concentrate the protein, fractions containing the purified protein of interest were poured into an Amicon Ultra-10k centrifugal filter device (Merck Millipore Ltd.) and centrifuged at 3000 rpm. The volume inside the device was checked every 30 min until 500 μL remained.

d. The protein concentration was initially determined using a Bradford assay kit (BioRad Laboratories). To use this kit, 1 mL of Bradford solution was pipetted into a cuvette. With proteins that were expected to have a high concentration, a 20:2 dilution of water to protein was pipetted into these cuvettes and vortexed thoroughly before sitting at room temperature for 10 min. As this solution is moderately sensitive to light, the Bradford solutions containing the protein were placed inside a covered box during incubation. The darker the blue of the solution was representative of a higher protein concentration. As such, protein concentration was determined *via* UV–VIS spectroscopy and compared to a standard curve. The standard curve was constructed by spiking the Bradford solutions with known concentrations of bovine serum albumin (BSA) and measuring their absorbance.

e. However, the Bradford method measures the total concentration of protein and not just the protein of interest. To determine a more accurate protein concentration of the protein of interest, SDS-PAGE was conducted using three or more BSA standards that had known concentrations to compare with two or more replicates of TET aliquots. TET protein concentrations could then be quantified by comparing and averaging the band signal intensities using the FlexAnalysis program after imaging with the ChemiDoc MP Imaging System.

f. Lastly, 10 μL of the concentrated protein was aliquoted into microcentrifuge tubes and stored at −80 °C for future use to prevent repetitive freeze thaw cycles that would denature the protein, effectively resulting in reduced activity.

2.2 Mutagenesis and expression of V1395A

There is limited knowledge regarding the function of TET oxidation species 5fC and 5caC as they are not as prevalent as 5hmC in the mammalian genome (Sappa et al., 2021). Efforts to modulate the production of these products have been limited. We have recently demonstrated that replacing hydrophobic and polar residues are of particular interest to

identify important gatekeeping residues that would maximize oxidation of 5mC, providing a foundation to probe for function of TET oxidation products that are not as abundant in the mammalian genome (Sappa et al., 2021). Through a systematic mutational analysis, we showed that when valine, located at position 1395 in the catalytic pocket of TET2, wass replaced with alanine (V1395A), the resulting mutant displayed a unique ability to produce 5caC as the major enzymatic product as quantitated by the *in-vitro* MALDI-based assay (Sappa et al., 2021).

2.2.1 Materials and reagents

Item	Supplier and catalog number
QuikChange lightning site-directed mutagenesis kit	Agilent Technologies
Nuclease free water	Corning
PCR mutagenesis primers (forward and reverse)	IDT
Deoxynucleotide triphosphate (dNTP) Mix	Fisher Cat # 18427013
E. coli strain XL-10 Gold Ultracompetent cells	Agilent Technologies
Luria-Bertani (LB) broth	Fisher
Kanamycin	Fisher Cat # K004725G
GeneJET plasmid miniprep kit	Thermo Fisher Cat #K0502
PCR tubes	Fisher Cat # 14-222-292
Cell spreader	Fisher Cat # 50-751-5036
1.5 mL eppendorf tube	Fisher
Falcon™ round-bottom polystyrene test tubes	Fisher Cat # 14-959-11B
Falcon™ standard tissue culture dishes	Thermo

2.2.2 Equipment

Item	Supplier
Microspin mini centrifuge	Corning
Thermal cycler	Bio Rad T100
Water bath	Thermo
CO_2 incubator for bacterial cell culture	Thermo HERATherm
Large centrifuge	Thermo Sorvall Legend XTR

2.2.3 Protocol

1. Site-directed mutagenesis
   a. This TET2 variant was generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies).
   b. Primers were designed based on the template TET2 plasmid sequence with a focus on the catalytic domain for TET2 activity modulation. General primer design principles regarding primer lengths between 25 and 45 bases were considered and although it was not crucial, designing at least one primer with either a C or G on the 3′ end increased the probability of a successful mutagenesis. These C and G bases make one extra hydrogen bond with its corresponding base on the template strand, creating a tighter bonding interaction. The forward primer had the following sequence: 5′-GGC AGC ACA TTG GCC TGC ACT CTC ACT-3′. The reverse primer was the reverse complement of the forward primer (5′-AGT GAG AGT GCA GGC CAA TGT GCT GCC-3′).
   c. The polymerase chain reaction (PCR) mixture included 40 ng of TET2 plasmid DNA template strand, 1.5 μL of 10× reaction buffer, 130 ng of each primer, 0.4 μL of deoxynucleotide triphosphate (dNTP) mix, and 0.5 μL QuikSolution reagent. Nuclease free water was added for a total reaction volume of 14.6 μL. The contents of each tube were mixed gently and briefly centrifuged before adding 0.4 μL QuikChange Lightning (QCL) Enzyme. To ensure mutagenesis was successful, three separate PCR reactions were conducted. In two of the reactions either 130 ng of the forward primer or 130 ng of the reverse primer was added to the reaction mixture. In the third reaction, 130 ng of both primers were added to the reaction mixture. This did not change the cycling parameters, nor did it change the final reaction volume.
   d. The PCR was performed using a Thermal Cycler (Bio Rad T100) utilizing conditions provided by the manufacturer. The QCL enzyme was activated at 95 °C for 2 min before denaturation occurring at 95 °C for 20 s. Following denaturation, annealing was carried out at 63.6 °C for 10 s. The annealing temperature depended on the melting temperature of the primers and was the most important part of this step. In the case of TET2 mutants, annealing temperatures that were 3 °C below the primer melting temperature (T_m = 66.6 °C) proved to be the only temperature for a successful

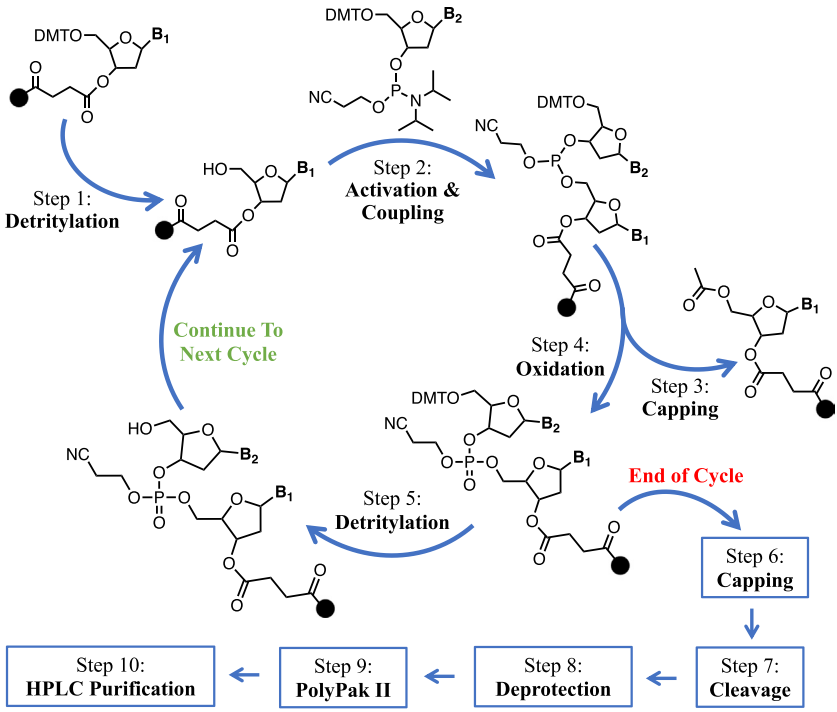

Fig. 2 Schematic showing solid-phase synthesis of oligonucleotides using dimethoxytrityl (DMT)-on protocol.

mutagenesis. Lastly, extension was performed at 68 °C for 30 s/kb of plasmid length. 25 cycles of denaturation, annealing, and extension were performed. The final extension was performed for an additional 5 min at 68 °C.

2. Bacterial Transformation and Sanger Sequencing
   - **a.** The resulting mutant plasmids were transformed into *E. coli* strain XL-10 Gold Ultracompetent cells (Agilent Technologies).
   - **b.** A single colony was picked up and grown overnight at 37 °C in 5 mL of LB broth in presence of 50 μg/mL kanamycin.
   - **c.** The cultures were centrifuged at 3000 rpm for 15 min and DNA was subsequently isolated from bacteria using GeneJET Plasmid Miniprep Kit (Thermo Fisher, Cat #K0502).
   - **d.** 800 ng of plasmid DNA was mixed with 5 μM of either T7, TET middle forward, or T7 reverse primers and sent for Sanger Sequencing through GeneWiz.

e. Resulting sequencing trace files were analyzed using Chromas software and the entire plasmid was compared to the wild-type TET2 construct to verify mutagenesis was successful using Clustal Omega.

3. TET2-V1395A Protein Expression and Purification:
   a. The mutant was transformed into BL21 star (DE3) competent cells and was expressed and purified the same way as the wild-type construct as previously described in Section 2.1.3.

2.3 Synthesis and characterization of oligonucleotides

Using the protocol described below, we synthesized several palindromic DNA oligonucleotides of various lengths to gauge TET2 enzymatic activity starting with a 58-mer DNA with a central 5mC unit: (5′-ACG ATC AGA TCC TAA GGC ATC AGC ACA C(5mC)G GTG TGC TGA TGC CTT AGG ATC TGA TCG T-3′). As the TET oxidative products were primarily confirmed by MALDI-TOF mass spectrometry, the 58-mer DNA substrate was not preferred due to poor signal-to-noise ratio in MALDI-based detection (Sudhamalla et al., 2017). Crystal structures of TET2 revealed that crucial interactions between TET2 and DNA only included a few nucleotides adjacent to the 5mC base which led to the synthesis of the shorter 22-mer, 14-mer, and 8-mer oligonucleotide substrates, using the solid-phase technique (Fig. 2), all which contained a single 5mC group (Hu et al., 2013, 2015). A schematic overview of how oligonucleotides were made using an Expedite 8909 DNA synthesizer is depicted in Fig. 2, where steps one through five were repeated until the desired sequence was obtained and steps six through ten were followed to achieve a pure synthetic oligonucleotide. As subsequent MALDI-TOF analyses utilizing these substrate oligonucleotides demonstrated successful 5mC oxidation by TET2, the 8-mer palindromic DNA was subsequently selected for future studies due to its shortest length (Sudhamalla et al., 2017).

2.3.1 Materials and reagents

Item	Supplier and catalog number
CpG resin	Glen Research
DNA phosphoramidites	Glen Research
Anhydrous acetonitrile	Glen Research
20% acetonitrile/water	Fisher

2% trifluoroacetic acid	Glen Research
2.0 M triethylamine acetate	Glen Research
Oxidant solution – Iodine solution in THF/water/pyridine	Glen Research
0.45 M tetrazole	Glen Research
Capping reagent A – acetic anhydride in pyridine/THF	Glen Research
Capping reagent B – *N*-methylimidazole in pyridine/THF	Glen Research
Wash A – acetonitrile	Glen Research
Deblock solution – trichloroacetic acid (TCA) in dichloromethane (DCM)	Glen Research
33% ammonium hydroxide/water	Fisher
Auxiliary solution	Glen Research
Activating solution – tetrazole in acetonitrile	Glen Research
Deionized water	
Frit	Glen Research
10 mL syringe	BD Luer-Lok™ Tip
Syringe needle (18 G × 1 ½″ TW)	BD PrecisionGlide™
Argon gas cylinder	

2.3.2 Equipment

Item	**Supplier**
Expedite 8909 DNA synthesizer	Biolytic
PolyPak II purification cartridge	Glen Research 60-3100-10
Column for DNA synthesis	Glen Research
Ultra-low temperature freezer	New Brunswick
SpeedVac concentrator	Thermo Savant SC210A
HPLC instrument	Agilent 1220 Infinity LC
MALDI-TOF mass spectrometer	Bruker-UltrafleXtreme
UV-Vis biospectrometer	Eppendorf

2.3.3 Protocol

1. Preparation of Expedite 8909 Nucleic Acid Synthesis System (Perspective Biosystems)
   a. The argon gas flow was set to 20 PSI with extra care not to exceed instrument pressure limits.
   b. The Expedite program was opened on the desktop connected to the DNA synthesizer. To prepare the system for synthesis the Run tab at the top of the page was opened before navigating to the 'Synthesis' option.
   c. Either position 1 or 2 was selected which corresponded to the column that would be used.
   d. 'Edit Sequence' was then selected at the bottom of the window to make a new sequence and then the following options were selected in order: 'Sequence', 'New', 'Modify', 'DNA'.
   e. The desired sequence was entered in order from 5′ to 3′. To insert an amidite that was different than the standard A, T, G, and C monomers into the sequence, a different position was selected that indicated the location of this modified phosphoramidite and appeared as a number in the sequence. Once the desired sequence was entered, the sequence was saved, and the sequence editor was closed.
   f. To choose the sequence, the drop-down menu was opened, and 'Database Sequence' was selected.
   g. The first nucleoside is bound to the support and contains a 4,4′-dimethoxytrityl (DMT) protecting group which prevents polymerization during resin functionalization. This protecting group must be removed through a process called detritylation before oligonucleotide synthesis can proceed. However, at the last step of the synthesis, this DMT group can either be left on (DMT-ON) as protection after synthesis or can be removed through a final deprotection (DMT-OFF).
   h. The DMT-ON protocol was selected so that the DMT group could be used as a purification handle due to its hydrophobic properties using a PolyPakII purification kit at the end of the synthesis.
2. Priming Reagents
   a. On the DNA synthesizer, 'Prime' was selected before selecting 'Reagents Only'
   b. The union was tightly placed on column 1 union and 'OK' was selected on the instrument. This step ensures that the reagents are

primed for the column of choice. These syntheses were run under ultra-mild conditions using tetrazole as an activator reagent.

3. Preparation of Phosphoramidites for Synthesis
   a. Synthesis of DNA oligonucleotides with one or more 5mC, 5hmC, 5fC and/or 5caC modifications required using modified phosphoramidites that carried this epigenetic mark. All canonical (A, T, G, and C) and modified phosphoramidites were synthesized using DNA phosphoramidite monomers obtained from Glen Research.
   b. When not in use, bases were stored at −80 °C in acetonitrile. Bases were allowed to reach room temperature only before they were ready for use.
   c. Each was dissolved with anhydrous acetonitrile diluent using a nitrogen balloon to offset the pressure in the diluent bottle. The volume of acetonitrile added to each base corresponded to the volume recommended by Glen Research.
4. Priming Bases
   a. Each base position was filled with acetonitrile when it was not in use.
   b. On the instrument panel, the following options were selected in order: 'Tools', 'Diagnostics', 'Fluid', 'Column 1', 'More'.
   c. Each base that would be used was selected before choosing the 'Volume' option. The prime was discontinued after 5 clicks from the instrument.
   d. The union was removed and a pre-packed column containing resin from the first base in the sequence was attached in its place.
   e. The column was washed with Wash A by selecting the following options in order: 'Tools', 'Diagnostics', 'Fluid', 'Column 1', 'Wash A', 'Volume'.
   f. The wash was terminated when a steady flow of liquid was observed in the clear tubing above the column.
5. Preparation of Column
   a. One small frit was inserted into one end of a fresh column.
   b. The CpG was chosen that corresponded to the 3′ end of the DNA sequence as the base on the solid support. This CpG was weighed out into the column according to reaction scale that was batch specific. Glen Research had previously supplied these calculations.
   c. Once the appropriate CpG was loaded into the column, a second frit was fitted to the other end of the column.

6. Synthesis
   a. To ensure a successful coupling, modified bases were set to couple for 4.5 min and standard bases were set to couple for 2 min.
   b. Once all conditions were set on the instrument, 'START' was selected on the instrument panel.
7. Post-Synthesis
   a. The crude deoxyoligonucleotide was cleaved from the column resin and deprotected by agitating the resin with ammonium hydroxide (33% v/v) at 25 °C. The ammonium solution containing the oligo is then gently stirred in a closed environment for 24 h. For 5hmC DNA, the deprotection step was heating at 75 °C for 24 h.
   b. Preliminary purification and DMT deprotection were carried out using Poly PakII purification cartridge (Glen Research, 60-3100-10) according to the standard protocol provided by Glen Research.
   c. The purified DNA was concentrated by a SpeedVac concentrator.
8. HPLC Purification
   a. Further purification was conducted utilizing High Pressure Liquid Chromatography (HPLC) by first dissolving the DNA in 950 mL nuclease free water and 50 μL triethylammonium acetate (TEAA).
   b. This mixture was passed through a 0.2 μm filter prior to injecting into the HPLC.
   c. Fractions under each peak were collected and concentrated by SpeedVac for 1–2 h before subjected to lyophilization until only a residue remained.
   d. The residue was then redissolved in nuclease free water and the concentration was measured using UV-Vis (A260/280).
   e. The quality and purity were determined by high resolution MALDI-TOF mass spectrometry (AB SCIEX Voyager DE Pro and Bruker-ultrafleXtreme™ MALDI-TOF/TOF spectrometer).

3. Biochemical assays and results

3.1 Development of a robust *in-vitro* assay

Prior to the development of our assay, exhaustive steps for the detection of TET oxidative products were required as it was necessary to pass the enzymatic products through a nucleotide cleaning kit prior to concentrating the eluent. This served to eliminate salts present in the assay components, improving signal-to-noise ratio in the mass spectrum. However, these steps led

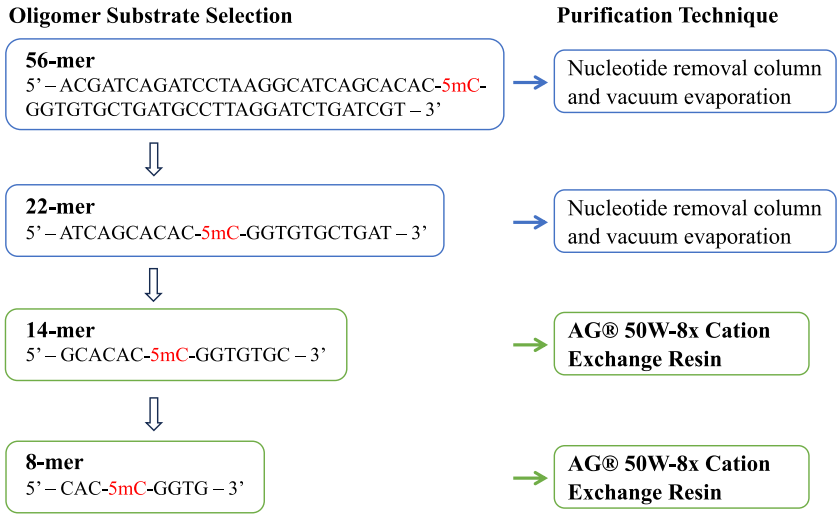

Fig. 3 Optimization of *in-vitro* assay involving substrate selection and product purification.

to the loss of TET oxidative products. With these factors in mind, we sought out a way to streamline post-assay purification procedure and eliminate the need for lengthy, expensive cleanup steps (Fig. 3). As such, we developed a simpler and more straightforward protocol for purifying TET products that greatly reduced post-assay processing (Sudhamalla et al., 2017). We reasoned that upon completion of the TET oxidation reaction, a cation exchange resin could be added directly to the assay mixture to eliminate the need for expensive desalting columns. Resin-treated samples were then spotted on the MALDI plate and subjected to mass spectrometric analysis directly utilizing 3-Hydroxypicolinic Acid (3-HPA) as the matrix. With this simplified process, TET oxidized products were detected with a significantly improved signal-to-noise ratio in the MALDI spectra without requiring multiple enzymatic digestions, liquid chromatographic optimization, and separation of digested products before analysis (Sudhamalla et al., 2017).

3.1.1 Materials and reagents

Item	Supplier and catalog number
Potassium acetate	Fisher
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5)	Fisher
Sodium chloride	Fisher Cat #BP358-10

Ammonium iron (II) hexasulfate	Fisher
Ascorbate	Thermo
Dithiothreitol (DTT)	Thermo
Adenosine triphosphate (ATP)	Thermo
2-Ketoglutarate	Fisher
Deionized water	
Agarose	Fisher
QIAquick nucleoside removal kit	QIAGEN
3-Hydroxypicolinic acid	Thermo
AG® 50W-X8 cation exchange resin	BioRad Cat #143-5441
PCR tube	Fisher Cat # 14-222-292
1.5 mL eppendorf tube	Fisher

3.1.2 Equipment

Item	Supplier
Thermocycler	Bio Rad T100
Heat block	Fisher
Medium centrifuge	Eppendorf
ChemiDoc MP imaging system	BioRad
SpeedVac concentrator	Thermo Savant SC210A
MALDI-TOF mass spectrometer	Bruker-UltrafleXtreme
UV-Vis biospectrometer	Eppendorf

3.1.3 Protocol

1. Duplexing Synthesized DNA for *in-vitro* Assays

a. In order to use synthetic DNA for TET enzymatic assays, single-stranded oligomeric DNA had to be made into double-stranded DNA through a process called duplexing. The single-stranded DNA that we used was palindromic, meaning the sequence was read the same in the forward direction as it did in the reverse direction. It was important to have one methylation mark on this single stranded DNA prior to duplexing as TET is known to oxidize 5mC-containing

double stranded DNA. DNA was duplexed using the following protocol.

b. 100 μM of pre-synthesized, single stranded DNA was added to a PCR tube with duplex buffer which included 100 mM potassium acetate, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5, Integrated DNA Technology) for a total volume of 100 μL.

c. One option for duplexing the DNA included heating the DNA mixture on a 95 °C heat block for 2 min and gradually cooling down to room temperature. However, this method did not consistently produce duplexed DNA and was discarded. This finding could have been due to the inconsistency in how fast the DNA was cooled to room temperature or that it cooled too fast.

d. Utilizing a Thermal Cycler (Bio Rad T100) for this process proved to be more reliable and consistent. With this method, the DNA was heated to 95 °C for 5 min before decreasing temperature by 1 °C each minute for a total of 40 min. The DNA was then held at 55 °C for 30 min before decreasing the temperature once again by 1 °C each minute for a total of 20 min.

e. To confirm that the DNA was duplexed, the oligonucleotides were separated on a 1% agarose gel and visualized with the ChemiDoc MP Imaging System using ethidium bromide.

2. *In-vitro* Enzymatic Activity Assay

a. 5 μM of double-stranded synthetic DNA substrates containing one methylation mark were incubated with 10 μM TET2 (1099–1936 del-insert) or its mutant (V1395A) in buffer containing 50 mM HEPES (pH 8.0), 100 mM NaCl, 100 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM ascorbate, 1 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), and 1 mM 2KG for 3 h at 37 °C.

b. After incubation, the reaction was quenched by heating the reaction at 95 °C for 10 min.

3. Product Oligonucleotide Purification

a. *Nucleotide clean up method:* The oligonucleotide products were purified using the QIAquick Nucleotide Removal Kit (QIAGEN) following the manufacturer's instructions prior to denaturation at 100 °C for 10 min. The oligonucleotides were concentrated using SpeedVac concentrator for 10 min and analyzed by MALDI-TOF mass spectrometry (AB SCIEX Voyager DE Pro) by spotting 1 μL of the

purified DNA sample with 1 μL of 3-Hydroxypicolinic Acid (3-HPA) matrix on the MALDI plate.

b. *Cation-exchange resin method:* To streamline the assay and eliminate the need for a lengthy, expensive cleanup step, we developed a simpler and more straightforward protocol for purifying TET product oligonucleotides that greatly reduced purification time. To analyze any synthetic DNA oligonucleotide *via* MALDI, reaction mixtures containing this DNA were desalted by adding 8 μL of AG® 50 W-X8 Cation Exchange Resin (BioRad, Cat. #143-5441) directly into the 20 μL reaction mixture and briefly agitating the solution. Samples were subsequentially centrifuged at 10,000 rpm for 2 min. TET oxidized products were then analyzed by MALDI-TOF MS by spotting 1 μL of sample and then mixing with 1 μL of 3-Hydroxypicolinic Acid (3-HPA) matrix on a MALDI plate. The 3-HPA matrix has a low amount of fragmentation for oligonucleotides between 1 and 30 kDa and is suitable for oligonucleotide analysis (Bruker-Daltonics, 2012; Chou & Limbach, 2000).

4. Analysis of TET2 Oxidative Products *via* MALDI

a. MALDI data was collected on a Bruker ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer using the reflectron negative TOF mode (Fig. 4).

b. The background noise was corrected for calculating signal/noise ratios using AB Sciex Data Explorer (4.0). Resulting MALDI spectra could be analyzed quantitatively by normalizing ion intensities to the highest peak and measuring apex peak intensities using GraphPad Prism software.

c. MALDI-based measurement is suitable for providing quantitative information on TET enzymatic activity due to similar ionization potentials of 5mC, 5hmC, 5fC and 5caC containing DNAs as was determined by generating a standard curve (Sappa et al., 2021). For the standard curve, the percentage of 5mC, 5hmC, 5fC and 5caC containing synthetic DNAs was varied from 0% to 100%, while the total DNA concentration remained a consistent 10 μM. Samples were mixed and analyzed by MALDI-TOF. The relative intensities of synthesized DNAs were fitted to a straight line with nonlinear regression using GraphPad Prism. Since standard curves generated from this experiment displayed similar ionization potentials of 5mC, 5hmC, 5fC and 5caC, we determined that the relative abundance of each oxidation product can be directly compared using MALDI.

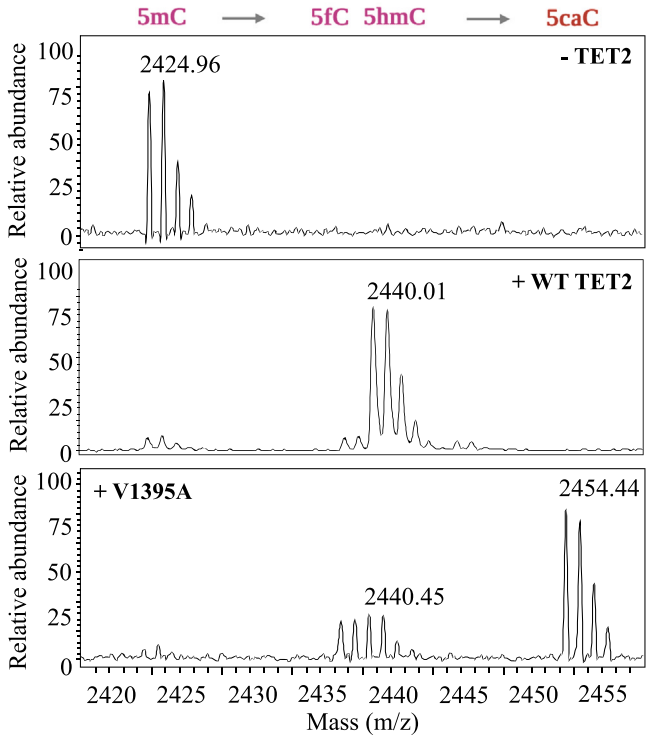

Fig. 4 MALDI-TOF-MS spectra displaying the activity of wild-type TET2 and V1395A mutant.

d. For quantitative analysis of TET enzymatic activity, peaks corresponding to 5mC, 5hmC, 5fC and 5caC were clearly labeled as displayed in Fig. 4. With respect to the wildtype enzyme, V1395A demonstrated that it was indeed acting as a superior dioxygenase, represented by its complete oxidation of 5mC to primarily 5caC. In contrast, wildtype TET2 produced a wide variety of products, but primarily produced 5hmC (Fig. 4).

3.2 Measurement of IC_{50} of TET2 inhibitors NOG and 2HG

We next applied the optimized *in-vitro* assay to measure the potency of two known TET2 inhibitors: 2-hydroxyglutarate (2HG) and *N*-oxalylglycine (NOG) (Fig. 5), (Sudhamalla et al., 2017). These two molecules have been shown to inhibit a range of 2KG-dependent dioxygenases in a cofactor-competitive manner due mainly to its structural similarity with 2KG (Hamada et al., 2009). Cancers including gliomas and acute myeloid

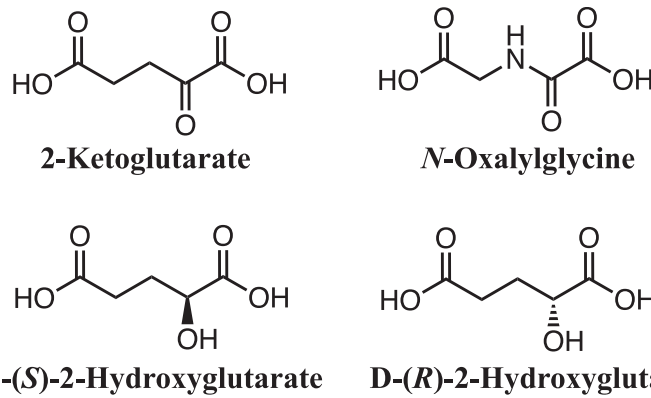

Fig. 5 Cofactor 2KG and competitive inhibitors of TET2: *N*-oxalylglycine (NOG), l-(*S*)-2-hydroxylutarate (l-2-HG) and d-(*R*)-2-hydroxyglutarate (d-2-HG).

leukemia have been associated with not only an increase in 2HG levels, but also with decreased levels of global 5hmC, suggesting that inhibition of TET activity by 2HG is crucial for hematopoietic transformation (Losman & Kaelin, 2013). Utilizing our MALDI-based assay, we show that the inhibitory effect of NOG and 2HG on the human TET2 protein can be reliably determined as it measures the formation of oxidized products. Due to the cofactor-competitive nature of the inhibition, this assay was modified by varying the concentration of 2KG from which we concluded that 80 μM 2KG (5-fold higher than $K_{M,2KG}$) was optimal for TET activity (Sudhamalla et al., 2017). Under such conditions, TET demonstrated maximum activity, but was still competitively inhibited by NOG and 2HG. NOG has been previously reported to inhibit Naegleria Tet1, with an IC_{50} of 49 ± 6 μM in a fluorescence polarization-based competitive binding assay that did not require the cofactor 2KG (Hashimoto et al., 2014; Marholz et al., 2016). However, by optimizing 2KG in our MALDI-based assay, the inhibitory activity of NOG and both enantiomers of 2HG (d-2HG and l-2HG) reflected the ability to modulate the catalytic potential of TET2 in a dose-dependent manner as our assay measures the formation of oxidized products. Under our assay conditions, we determined an IC_{50} for NOG to be 149.5 ± 7.6 μM. Using the dose-response inhibition studies, IC_{50} values were found to be 5.3 ± 0.3 mM and 12.4 ± 2.4 mM for d-2HG and l-2HG, respectively (Sudhamalla et al., 2017). The higher inhibitory effect of NOG towards TET enzymes was linked to the structure of NOG consisting of the 2-keto acid moiety as well as an internal

–NH– group that participates in strong hydrogen bonding with TET enzymes, and which is absent in 2HG (Sudhamalla et al., 2017).

3.2.1 Materials and reagents

Item	Supplier and catalog number
N-oxalylglycine	Sigma Cat #O9390
d-2-Hydroxyglutaric acid	Santa Cruz Cat #sc-227739
l-2-Hydroxyglutaric acid	Santa Cruz Cat #sc-361834
HEPES	Fisher
Sodium chloride	Fisher Cat #BP358-10
Ammonium iron (II) hexasulfate	Fisher
Ascorbate	Thermo
DTT	Thermo
ATP	Thermo
2-Ketoglutarate	Fisher
AG® 50W-X8 cation exchange resin	BioRad Cat #143-5441
Deionized water	
1.5 mL eppendorf tube	Fisher

3.2.2 Equipment

Item	Supplier
Heat block	Fisher
Ultra-low temperature freezer	New Brunswick
MALDI-TOF mass spectrometer	Bruker-UltrafleXtreme

3.2.3 Protocol

a. Assay mixture containing 10 μM TET2 was incubated with varying concentrations (0.05–5 mM) NOG or varying concentrations (0.5–30 mM) of 2HG in buffer containing 50 mM HEPES (pH 8.0), 100 mM NaCl, 100 mM $Fe(NH_4)_2(SO_4)_2$, 2 mM ascorbate, 1 mM DTT, 1 mM ATP for 5 min on ice.

b. Demethylation was initiated by adding 100 μM 2-KG and 10 μM 8-mer double stranded 5mC DNA and further incubated at 37 °C for 3 h,

(see Section 3.1.3 step 2). Variations to the demethylase assay mentioned in Section 3.1.3 were minimized to ensure that inhibition of this protein was due to NOG and not due to differences in assay conditions. This supports the decision to keep the 8-mer DNA as well as the 3-h incubation time.

c. The product DNA was denatured at 95 °C for 10 min and desalted using cation exchange resin method described above.

d. To accurately determine the inhibitory effects of NOG and 2-HG towards TET2, the concentrated oligonucleotides were analyzed by MALDI-TOF mass spectrometry, and the values were fitted to the 4-parameter non-linear regression algorithm (Y = Bottom + (Top – Bottom))/(1+10 $^{((\text{Log } IC_{50}-x)*\text{Hill Slope})}$) of the GraphPad Prism software. X: log of dose or concentration; Y: response, decreasing as X increases; Top and bottom: upper and lower values of a given curve; logIC_{50}: same log units as X; Hill Slope: Slope factor or Hill Slope, unitless. With these parameters, the effect of NOG and 2-HG was accurately determined as the oxidation of 5hmC decreased with increasing concentration of NOG or 2-HG in a dose-dependent manner.

3.3 Validating the activity of wildtype TET2 and V1395A using BS-seq

We employed a secondary assay to further examine the activity of wildtype TET2 and V1395A in a base-resolution manner by treating the oxidized products with bisulfite and coupling with Sanger Sequencing (BS-Seq) (Fig. 6) (Lu et al., 2013). Upon treatment with bisulfite under alkaline conditions, 5caC is deaminated and read as T during sequencing. In contrast, 5mC and 5hmC do not react with bisulfite and are subsequentially read as C after Sanger Sequencing (Fig. 6). Employing our optimized *in-vitro* demethylase assay, a duplexed 76-mer DNA carrying a central 5mC unit was subjected to oxidation using wildtype TET2 and the V1395A mutant followed by bisulfite treatment, PCR amplification and Sanger sequencing. While 5mC in samples exposed to either no protein or wildtype TET2 read as C, the equivalent site in the V1395A-treated sample emerged as T demonstrating the ability of the mutant to predominantly generate 5caC (Fig. 6) (Sappa et al., 2021).

3.3.1 Materials and reagents

Item	Supplier and Catalog Number
GeneJET PCR purification kit	Thermo Fisher Cat #K0701
Isopropyl alcohol	Fisher

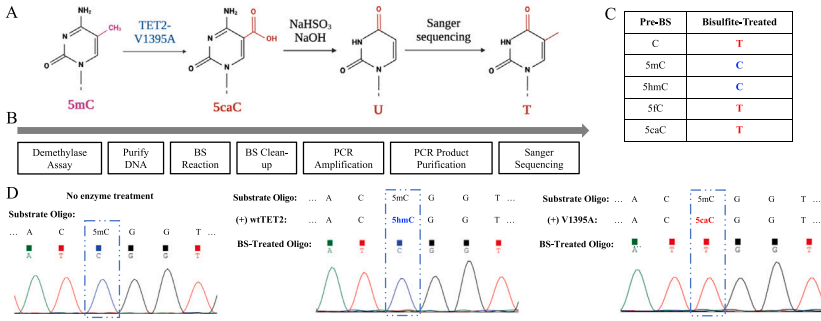

Fig. 6 Analysis of TETs oxidative products using bisulfite (BS) coupled with Sanger sequencing. (A) Scheme showing bisulfitemediated 5caC conversion to uracil which is read as thymine after Sanger sequencing. (B) Overview of steps required to perform bisulfite conversion. (C) Sanger readout of cytidine and its modified congeners upon BS treatment. (D) 76-mer DNA substrate containing a single 5mC modification subjected to bisulfite treatment before and after being oxidized by either wild-type TET2 or V1395A, confirming 5caC as the major product of V1395A utilizing the base-resolution sequencing technique.

76-mer DNA substrate	IDT
EpiTect bisulfite kit	QIAGEN Cat #59104
Hot star taq polymerase	QIAGEN Cat #203207
PCR primers (forward and reverse)	IDT
Agarose	Fisher
Nuclease free water	Corning
GeneJET plasmid miniprep kit	Thermo Fisher Cat #K0502
E. coli strain XL-10 gold ultracompetent cells	Agilent Technologies
1.5 mL eppendorf tube	Fisher
PCR tubes	Fisher Cat # 14-222-292

3.3.2 Equipment

Item	Supplier
Heat block	Fisher
Microspin mini centrifuge	Corning
Centrifuge – medium	Eppendorf
Ultra-low temperature freezer	New Brunswick

SpeedVac concentrator	Thermo Savant SC210A
MALDI-TOF mass spectrometer	Bruker-UltrafleXtreme
Thermocycler	Bio Rad T100

3.3.3 Protocol

1. *In-vitro* Demethylase assay
   a. 5000 ng of double-stranded 76-mer DNA substrate obtained from IDT (5′-CCT CAC CAT CTC AAC CAA TAT TAT ATT A TGT GTA TAC AC 5mC GGT GTT TGT GTT ATA ATA TTG AGG GAG AAG TGG TGA-3′) (Lu et al., 2013), was incubated with 20 μM of V1395A TET2 in demethylase activity buffer for 3 h at 37 °C as previously described in 3.1.3 step 2.
   b. After incubation, the reaction was quenched by heating reaction at 95 °C for 10 min.
2. Initial DNA purification
   a. Following the demethylase assay, 76-mer DNA was purified with the GeneJET PCR Purification Kit (Thermo Fisher, Cat. #K0701) following the supplier's instructions with several modifications.
   b. The reaction mixture was diluted to 100 μL with nuclease free water before adding 100 μL of 100% isopropyl alcohol and 200 μL binding buffer. After addition of the binding buffer, the solution should remain a bright orange color which indicates that the pH of the solution is optimal for DNA binding.
   c. This mixture was transferred into a purification spin column, provided in the kit, and centrifuged at 11,000 rpm for 30 s
   d. After the flowthrough was discarded, 500 μL of Wash Buffer was added to the column and the centrifugation was repeated. This step was repeated twice before the empty centrifuge tube was spun for an additional 30 s to remove any residual wash buffer.
   e. The spin column was placed in a fresh Eppendorf tube and 20 μL of nuclease free water was added. To evaporate residual ethanol left over from the wash solution, the spin column was placed on a 56 °C heat plate for 3 min before centrifuging the column once again.
   f. The 20 μL was pipetted out of the bottom of the Eppendorf tube and incubated on the center of the spin column membrane once again at room temperature. After 5 min, the column was centrifuged and the 20 μL of purified DNA was subjected to bisulfite treatment.
3. Bisulfite Treatment

a. The purified DNA was applied to EpiTect Bisulfite Kit (QIAGEN, Cat. #59104) following the supplier's instructions.

b. The bisulfite treated DNA was used for PCR amplification using Hot Start Taq polymerase (QIAGEN, Cat #203207) (forward primer: 5′-CCC TTT TAT TAT TTT AAT TAA TAT TAT ATT-3′; reverse primer: 5′-CTC CGA CAT TAT CAC TAC CAT CAA CCA CCC ATC CTA CCT GGA CTA CAT TCT TAT TCA GTA TTC ACC ACT TCT CCC TCA AT-3′). The PCR was performed in 2 steps with both primers in the PCR mixture.

c. In this PCR reaction, Hot Start Taq polymerase was activated for 15 min at 95 °C. Following activation, initial denaturation (5 min at 94 °C), denaturation (1 min at 94 °C), annealing (reverse primer, 1 min at 63 °C) and extension (1 min at 72 °C) was carried out. The final extension was carried out for 10 min at 72 °C.

d. Without purification, the mixture proceeded for another PCR round for the forward primer starting with Hot Start Taq polymerase activation (15 min at 95 °C). Following activation, initial denaturation (5 min at 94 °C), denaturation (1 min at 94 °C), annealing (forward primer, 1 min at 44 °C) and extension (1 min at 72 °C) were carried out. The final extension was carried out for 10 min at 72 °C. 30 cycles of PCR amplification were performed for each primer.

e. The resulting DNA was purified using a GeneJET PCR Purification Kit (Thermo Fisher, Cat. #K0701) as previously described in Section 3.4.3, step 2a-f.

f. To ensure the PCR reaction worked, 500 ng of each reaction was run on a 1.5% agarose gel.

4. Sanger Sequencing

a. To verify the oxidation, 800 ng of the DNA was sent for Sanger Sequencing (Genewiz) using the following sequencing primer: 5′-CCC TTT TAT TAT TTT AAT TAA TAT TAT ATT-3′.

4. Notes

We have optimized a majority of the conditions mentioned in the procedures above to offer a robust *in-vitro* assay for examining enzymatic activity of TET proteins and likely other 2KG-dependent dioxygenases. However, below are some of the issues we encountered along with suggestions on how to correct these issues should they arise in future

experimentation. We also include safety related information important to these methods. It is important that proper personal protective equipment is worn when conducting any experimentation. Disposable nitrile gloves are suitable for carrying out biochemical methods mentioned in this chapter.

1. Protein expression/purification

- **a.** In a flame sterilized environment, 10 ng of the wild-type TET2 plasmid with pET-28b kanamycin-resistant vector was pipetted into an autoclaved 1.5 mL Eppendorf tube with 9 μL *E. coli* strain BL21 star (DE3) competent cells (Fisher). To ensure that bacterial transformation is done safely, isopropyl alcohol can be used to sterilize the bench prior to turning on the flame and the flame should not be left unattended. Sterilization is important to prevent transformation of any unwanted DNA as well as to ensure the highest transformation efficiency of the plasmid of interest. The competent cells should be thawed on ice as they are sensitive to changes in temperature. If cells come into contact with the eyes or ingested, rinse impacted area thoroughly with water for 15 min. If cells come into contact with skin, wash with soap and water.
- **b.** It is important that an agar plate from bacterial transformation is not used for starting a bacterial inoculation if it is older than one week. A risk with using an older plate is that there will be little-to-no growth of the bacterial inoculate after 16 h of incubation at 37 °C.
- **c.** It is important to observe the growth of the starter culture once a single colony has been inoculated in the LB growth media with antibiotics. After 16 h, the inoculate solution should appear significantly foggier than before, but there should be no sign of debris at this stage. Debris is a sign that the cells have begun to die, and the amount of protein obtained at the end of expression will be minimal. If this debris is observed, repeat the starter culture inoculation, and incubate for less time. This may also occur if more than one colony is inoculated in the same culture tube or if a colony cluster is chosen for inoculation.
- **d.** Prior to diluting the starter culture into 1 L of LB, the flasks containing LB must be autoclaved. It is important to wear heat-resistant gloves while handling hot flasks. Do not dilute starter cultures or add antibiotics until after these autoclave flasks have cooled down to room temperature. Add 50 μg/mL antibiotic and the starter culture under a flame-sterilized environment.

e. After the starter culture is diluted 100-fold into 1 L of LB with the addition of 50 μg/mL kanamycin and allowed to grow at 37 °C, it is important to keep a close eye on the OD_{600} value. If the OD_{600} value increases above 0.8, the amount of protein obtained at the end of expression will be minimal.

f. Prior to protein purification, the 1 L cultures must be centrifuged at 4000 rpm. To ensure that this process is done safely, the rotor of the high-speed centrifuge must be balanced. This means that the mass of each culture being centrifuged must be the same. Deionized water can be added to increase the mass without decreasing the yield of final protein isolated.

g. TET is a heat sensitive protein meaning that once expressed and purified, it should not remain at room temperature for longer than absolutely necessary. As the number of freeze-thaw cycles increases, the activity of the protein will subsequentially decrease. To avoid this scenario, aliquot 10–20 μL of the purified TET protein into smaller Eppendorf tubes.

2. Site-directed mutagenesis

a. During PCR, the annealing temperature selected for the primers is the most important step and varies depending on the melting temperature of the designed primer as well as the template plasmid construct. In the case of TET2 mutants, annealing temperatures that are 3 °C below the primer melting temperature (T_m = 66.6 °C) are used. If mutagenesis is not successful, repeat the process mentioned above and increase or decrease the temperature by 1 degree. It is important to perform mutagenesis below the melting temperature of the primer to prevent primer degradation.

b. Another technique widely used to overcome difficulties in mutagenesis includes performing mutagenesis with one primer instead of both primers. Although using two primers increases the chance that mutagenesis will occur, using either the forward or reverse primers often yielded a successful mutation when using two primers did not. The overall yield of the plasmid containing the mutation will be significantly decreased with this method, but adding *Dpn*I degrades unreacted plasmid leaving only the mutated plasmid. Utilizing an ultracompetent cell line like XL10-Gold cells increases transformation efficiency and should be used in this case.

3. DNA oligonucleotide synthesis

a. Phosphoramidites are sensitive to water contamination as they are easily hydrolyzed. To prevent hydrolysis, oligonucleotide synthesis with the Expedite 8909 instrument requires a connection to argon gas for an inert environment. When using an argon gas tank, wear personal protective equipment such as gloves and goggles and follow regulations and standards set by OSHA. Use extreme caution not to exceed the instrument pressure limits during synthesis and monitor the pressure throughout use of the instrument. Precautions must also be used to ensure that reagents used are anhydrous as phosphoramidites are easily hydrolyzed.

b. Using the DMT-ON protocol leaves the final DMT on for initial purification purposes using the PolyPakII kit. Using this method serves to isolate the oligonucleotide of interest with ease. Nonetheless, the synthetic oligonucleotide can be directly purified by reverse-phase HPLC if the DMT-OFF setting is chosen by using an ion pairing agent in the mobile phase.

c. To ensure a successful coupling, modified bases are set to couple for 4.5 min and standard bases are set to couple for 2 min. When purchasing modified bases from Glen Research, there are recommended coupling times mentioned both on the website and on the information packet that is received with the phosphoramidites.

d. After synthesis, the crude deoxyoligonucleotide is cleaved from the column resin and deprotected by agitating the resin with ammonium hydroxide (33% v/v) at 25 °C. The ammonium solution containing the oligo is then gently stirred in a closed environment for 24 h. There are extra steps for deprotection that need to be followed when using modified bases. For example, oligonucleotides containing 5hmC must be deprotected by heating at 75 °C for 24 h. Recommendations for deprotections of modified bases are also found on the information packet and on the Glen Research website.

e. If the product oligonucleotide is found by MALDI to still contain the protecting group, repeat the ammonium hydroxide incubation in a closed environment and agitate by stirring.

4. Analysis of TET oxidative products *via* MALDI

a. Preparation of the MALDI plate and proper cleaning is important for visualizing a clear spectrum. To do this, gently wipe 100% isopropyl alcohol horizontally across the MALDI plate using a Kim Wipe. Wiping horizontally across the plate prevents damage while effectively removing dust, old spots, and other unwanted residues. Once

the large particles have been removed, place the MALDI plate in a beaker and fill with 100% isopropyl alcohol until it completely covers the plate and sonicate with pulsed sonication for 10 min to remove smaller particles. The clean plate should then be wiped once more with 100% acetonitrile and then air dried in a closed environment so that dust or other particles do not dry onto the plate. The final wipe with acetonitrile is crucial and improves the resolution of the MALDI peaks.

b. It is encouraged to use fresh 3-HPA matrix when spotting the product oligonucleotide on the MALDI plate to reduce the signal-to-noise ratio and achieve a clear spectrum.

c. In order to achieve a clear spectrum, it is also important to spot the product oligonucleotides without including cation-exchange resin beads and mix thoroughly with the 3-HPA matrix.

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