**TITLE:**

Efficient Purification and LC-MS/MS-based Assay Development for Ten-Eleven Translocation-2 5-Methylcytosine Dioxygenase

**AUTHORS AND AFFILIATION:**

Chayan Bhattacharya\*, Aninda Sundar Dey\*, Navid J. Ayon\*, William G. Gutheil, andMridul Mukherji

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO

\*These authors contributed equally

**Email Addresses of Co-Authors:**

Chayan Bhattacharya (cbg66@mail.umkc.edu)

Aninda Sundar Dey (asdmr9@mail.umkc.edu)

Navid J. Ayon (nazpd@mail.umkc.edu)

William G. Gutheil (gutheilw@umkc.edu)

**Correspondence to:**

Mridul Mukherji (mukherjim@umkc.edu)

**KEYWORDS**

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**SUMMARY**

Here, we present a protocol for an efficient single step purification of the active untagged human ten-eleven translocation-2 (TET2) 5-methylcytosine dioxygenase using ion-exchange chromatography and its assay using a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based approach.

**ABSTRACT**

The epigenetic transcription regulation mediated by 5-methylcytosine (5mC) has played a critical role in eukaryotic development. Demethylation of these epigenetic marks is accomplished by sequential oxidation by ten-eleven translocation dioxygenases (TET1-3), followed by the thymine-DNA glycosylase-dependent base excision repair. Inactivation of the TET2 gene due to genetic mutations or by other epigenetic mechanisms is associated with a poor prognosis in patients with diverse cancers, especially hematopoietic malignancies. Here, we describe an efficient single step purification of enzymatically active untagged human TET2 dioxygenase using cation exchange chromatography. We further provide a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach that can separate and quantify the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5-methyl, 5-hydroxymethyl, 5-formyl, and 5-carboxyl). This assay can be used to evaluate the activity of wild type and mutant TET2 dioxygenases.

**INTRODUCTION**

The C5 position of cytosine bases within CpG dinucleotides is the predominant methylation site (5mCpG) in mammalian genomes[1](#_ENREF_1). In addition, a number of recent studies have uncovered extensive C5 cytosine methylation (5mC) in non-CpG sites (5mCpH, where H = A, T, or C)[2](#_ENREF_2),[3](#_ENREF_3). 5mC modification serves as a transcriptional silencer at endogenous retrotransposons and gene promoters[3-5](#_ENREF_3). DNA methylation at 5mC also plays important roles in X chromosome inactivation, gene imprinting, nuclear reprogramming and tissue-specific gene expression[5-7](#_ENREF_5). Methylation of cytosine at the C5 position is carried out by DNA methyltransferases, and mutations in these enzymes cause significant developmental defects[8](#_ENREF_8). The removal of 5mC marks are initiated by TET1-3 5mC oxidases[9](#_ENREF_9), [10](#_ENREF_10). These TET-family dioxygenases convert 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by sequential oxidation steps[11-13](#_ENREF_11). Finally, thymine-DNA glycosylase replaces 5fC or 5caC to unmodified cytosine using the base excision repair pathway[11](#_ENREF_11).

The human *TET2* gene was identified as a frequently mutated gene in diverse hematopoietic malignancies including myelodysplastic syndromes (MDS)[14-16](#_ENREF_14), MDS-myeloproliferative neoplasms (MDS-MPN), and acute myeloid leukemia (AML) originating from MDS and MDS-MPN[16](#_ENREF_16). The levels of 5hmC modification in the bone marrow DNA are lower in patients with TET2 mutations compared to those with wild type (wt)-TET2[14](#_ENREF_14). A number of groups have developed TET2-knockout mouse models to elucidate its role in normal hematopoiesis and myeloid transformation[17-20](#_ENREF_17). These mice with mutations in the TET2 gene were initially normal and viable, but manifested diverse hematopoietic malignancies as they aged causing their early death. These studies showed the important roles played by the wt-TET2 in normal hematopoietic differentiation. In these mouse models, the heterozygous hematopoietic stem cells (TET2+/– HSCs) and homozygous TET2–/– HSCs had a competitive advantage over homozygous wt-TET2 HSCs in repopulating hematopoietic lineages as both TET2+/– and TET2–/– HSCs developed diverse hematopoietic malignancies[17](#_ENREF_17),[18](#_ENREF_18). These studies demonstrate that haploinsufficiency of TET2 dioxygenase alters the development of HSCs and results in hematopoietic malignancies.

Similar to mice with mutations in the TET2 gene, most leukemia patients manifest haploinsufficiency of TET2 dioxygenase activity. These mostly heterozygous somatic mutations include frame-shift and nonsense mutations dispersed throughout the TET2 gene body while missense mutations that are most clustered in the dioxygenase domain[12](#_ENREF_12). To date, little characterization of wt- and mutant-TET2 is reported in the literature mainly due to difficulties with the production of TET2 dioxygenase and its assay[21](#_ENREF_21). Here, we report a simple single-step purification of native TET2 dioxygenase using ion exchange chromatography. Further, a quantitative LC-MS/MS assay was optimized and used to measure the enzymatic activity of native TET2 dioxygenase.

**PROTOCOL**

1. **Cloning and Purification of Untagged Human TET2 Dioxygenase**
   1. Clone human TET2 dioxygenase (TET2 1129-1936, ∆1481-1843) into the pDEST14 destination vector using the site-specific recombination technique as previously described[22](#_ENREF_22).

Note: Previous studies have demonstrated that the C-terminal TET2 dioxygenase (TET2 1129-1936, ∆1481-1843) domain is the minimal catalytically active domain[21](#_ENREF_21),[23](#_ENREF_23). In order to express the untagged TET2 dioxygenase domain using the pDONR221 vector, Shine Dalgarno and Kozak sequences were incorporated in the forward primer during PCR (**Table 1**).

* 1. For bacterial transformation, add 1 µL of recombinant pDEST14 expression vector containing untagged human TET2 dioxygenase (TET2 1129-1936, ∆1481-1843) to 100 µL of chemically competent *E. coli* BL21 (DE3) cells in a 1.7 mL tube. Keep the mixture on ice for at least 15 minutes followed by heat shock at 42 °C for 30 s in a water bath.
     1. Immediately after heat shock, keep the cells back on ice for minimum of 2 min. Following this, add 250 µL of super optimal broth with catabolite repression (S.O.C media) to cells. Incubate the bacterial cells for 1 h at 37 °C in a shaker.
     2. After incubation, spin down the cells by centrifuging the tube at 9,000 x g for 1 min. Discard 70% supernatant by pipetting and dissolve the pellet in left over media.
     3. Spread the cell suspension on a Luria broth (LB) agar plate containing 100 µg/mL ampicillin. Incubate the plate for 16 h at 37 °C.
  2. Select one isolated colony and inoculate it into 100 mL of LB-ampicillin media as a primary culture. Incubate the flask at 37 °C in a shaker for overnight. The next day, use 6 mL each of primary culture to inoculate 15 flasks, each containing 600 mL of LB-ampicillin media. Incubate the flasks at 37 °C on a shaker at 180 rpm.

Note: For verifying transformed clones, perform DNA sequencing or restriction digestion with the isolated plasmid DNA.

* 1. To check the density of bacterial culture, measure its OD600 using a spectrophotometer. After the culture reaches a density of 0.8 at OD600, induce the expression of TET2 protein with 300 µL of 1 M (final concentration of 0.5 mM in 600 mL) IPTG in each flask and further grow the culture for 16 h at 17 °C.
  2. After 16 h, transfer the bacterial culture to centrifuge bottles. Centrifuge the bacterial culture expressing the TET2 enzyme at 5,250 x g for 45 min. Use the bacterial pellet for TET2 purification.

Note: Perform all remaining protein purification steps either on the ice or at 4 °C.

* 1. Resuspend the bacterial pellet in 100 mL of 50 mM MES (2-(N-morpholino) ethane sulfonic acid) buffer, pH 6 and sonicate for 5 × 30 s at power 20 with 60 s cooling intervals.
  2. After 10 min of stirring, spin the lysate at 5,250 x g for 45 min. Collect the supernatant containing the soluble TET2 enzyme and pass through 0.45-µm filters before loading on an FPLC system.

Note: In these experiments, the TET2 enzyme was very stable, but if needed a protease inhibitor cocktail containing 1 mM benzamidine-HCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.5 mM 1,10-*o*-phenanthroline can be added to the cell lysate to prevent degradation of TET2 enzyme. Avoid using EDTA or EGTA in the inhibitor cocktail as these may interfere with the following cation-exchange chromatography.

* 1. Pack 10 mL of a strong cation exchange resin into a FPLC column. Equilibrate the column with 10 bed volumes of wash buffer (50 mM MES buffer, pH 6) at the constant flow rate of 0.3 mL/min using a FPLC system.
  2. Load the clarified lysate onto the pre-equilibrated column and wash with ∼10 bed volumes of wash buffer until the flow through becomes clear.
  3. Elute TET2 using a 0-100% gradient from the wash buffer to the elution buffer (50 mM MES buffer, pH 6, 1 M NaCl) in 15 bed volumes followed by holding at 100% elution buffer for two-bed volumes.

Note: Collect samples (100 µL each) of cell lysate before and after column loading along with all the elution fractions and analyze on 10% resolving SDS-PAGE.

* 1. Pool the fractions containing TET2 protein, freeze dry, dissolve enzyme pallet in 10 mL water and store at –80 °C.

1. **5mC Oxidation by TET2 Dioxygenase**
   1. Perform all demethylation reactions in triplicate with 3 µg of substrate (25-mer double stranded DNA, **Table 2**).
      1. Add 100 µg of purified TET2 enzyme in 50 μL of total reaction buffer containing 50 mM HEPES (pH 8.0), 200 μM FeSO4, 2 mM 2OG (2-oxoglutarate/α-ketoglutarate), and 2 mM ascorbate and incubate at 37 °C for 1 h[21](#_ENREF_21).
      2. Quench TET2 catalyzed oxidation reactions with 5 μL of 500 mM EDTA.
   2. After quenching TET2 reactions, prepare samples for LC-MS/MS analysis by separating the DNA from TET2 reaction mixture using oligo purification columns.
      1. Add 100 µL of oligo binding buffer to 55 µL of quenched reaction.
      2. Following this, add 400 µL of 100% ethanol to the mixture. Pass this mixture through an oligo binding column.
      3. Wash bound DNA with 750 µL wash buffer and elute in 20 µL water.
   3. Digest the isolated DNA with 2 units of DNase I and 60 units of S1 nuclease at 37 °C for 12 h to produce individual nucleoside-monophosphates.
   4. Following digestion, add 2 units of calf intestinal alkaline phosphatase (CIAP) in the samples and incubate for addition 12 h at 37 °C to remove the terminal phosphate groups from nucleoside-monophosphates to obtain nucleosides.
   5. Quantify all nucleosides, especially modified cytosines, using the LC-MS/MS method described below.
2. **Quantitative LC-MS/MS-based Assay Development**
   1. Prepare 100 µM stock solution of all modified cytosine nucleosides [5-methyl-2′-deoxycytidine (5mdC), 5-hydroxymethyl-2′-deoxycytidine (5hmdC), 5-formyl-2′-deoxycytidine (5fdC), and 5-carboxy-2′-deoxycytidine (5cadC)] and normal DNA bases (adenine, thymine, cytosine, and guanine) in HPLC-grade water for the development of the LC-MS/MS method.
   2. Optimize nucleoside-dependent MS/MS parameters by infusing stock solutions, one at a time, in mass spectrometer at a flow rate of 10 µL/min in EMS scan mode. Optimize following parameters: Declustering Potential (DP), Entrance Potential (EP), Collision Cell Entrance Potential (CEP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) for each DNA nucleoside using automated quantitative optimization feature of the software.
   3. Optimize source-dependent MS/MS parameters by injecting 10 µL of stock solution using a gradient with 25% solvent B at a flow rate of 0.3 mL/min where solvent A is 10 mM ammonium acetate (pH 4.0) and solvent B is 20% acetonitrile with 10 mM ammonium acetate (pH 4.0). Optimize following parameters: Curtain Gas (CUR): 10-50, Temperature: 0-600 °C, Gas Flow 1 (GS1): 0-50, Gas Flow 2 (GS2): 0-50, Collisionally Activated Dissociation (CAD): Low-Medium-High, Ion spray Voltage (IS): 4000-5500 for each DNA nucleoside using manual quantitative optimization feature of the software in FIA (Flow Injection Analysis) mode.
   4. To separate all eight DNA nucleosides, perform liquid chromatography using the following gradient: 0% solvent B (0-2 min), 0-20% solvent B (2-5 min), 20-60% solvent B (5-9 min), 60-0% solvent B (9-10 min) and then equilibrate with solvent A for 5 min at a flow rate of 0.3 mL/min on a C18 column (Particle Size: 5 µm, Pore Size: 120 Å).
   5. Using the optimum MS/MS parameters (**step 3.2 and 3.3**) coupled with the above-mentioned liquid chromatography gradient (**step 3.4**), determine the response linearity, limit of detection (LOD), and lower limit of quantification (LLOQ) using a two-fold serial dilution of a 100 µM standard mixture containing all eight nucleosides. Draw standard curves for all eight DNA nucleosides.
   6. Detect and quantify all nucleosides, especially modified cytosines, produced in **step 2.4** using the LC-MS/MS method and standard curves.

**REPRESENTATIVE RESULTS**

Dynamic modification of 5mC in DNA by TET-family dioxygenases plays important roles in epigenetic transcriptional regulations. TET2 dioxygenase is frequently mutated in diverse hematopoietic malignancies[12](#_ENREF_12). To investigate the role of the TET2 enzyme in normal development and disease, we have cloned its minimal catalytically active domain without any affinity tag into the pDEST14 vector[22](#_ENREF_22). The untagged TET2 dioxygenase was produced at ∼5% of the total soluble protein by SDS-PAGE analysis in bacterial *E. coli* BL21 (DE3) cells. Since the catalytic domain of TET2 has a relatively high isoelectric point (∼7.49), compared with most indigenous *E. coli* proteins[24-26](#_ENREF_24), an efficient purification process utilizing a cation exchange chromatography was developed. This purification yielded >90% pure TET2 enzyme in a single step (**Figure 1**).

In order to separate and quantify different deoxycytidines derivatives and other four natural DNA bases following the TET2 enzymatic reaction, a sensitive LC-MS/MS-based assay was optimized. The liquid chromatography used a reversed-phase C18 columns. Standard curves were drawn using serial dilutions of a mixture containing all nucleosides (**Figure 2**). The gradient used for liquid chromatography, described in the experimental procedure, was able to resolve all eight nucleosides (**Figure 3**). The LC retention times (tr) for all eight nucleosides are described in **Table 3**. We further optimized the MS detection of each parent ion nucleoside (Q1), the most intense product ion (Q3) by determining their declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), limit of detection (LOD), and lower limit of quantification (LLOQ) (**Table 3**). Finally, an LC-MS/MS method was developed that can separate and quantify the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5-methyl, 5-hydroxymethyl, 5-formyl, and 5-carboxyl) (**Figure 3**).

The activity of untagged TET2 dioxygenase was determined using a 25-mer dsDNA containing one 5mC in a CpG island in each DNA strand (**Table 2**). After TET2 enzymatic reactions, DNA oligonucleotides were purified and converted into nucleosides. Then these nucleosides were subjected to LC-MS/MS assay. In the reactions without the TET2 enzyme (negative control), only dA, dT, dG, dC, and 5mdC peaks were observed. However, in the positive control reaction, which contained the TET2 dioxygenase, two new peaks corresponding to d5hmC and d5fC were observed. We were not able to detect the formation of d5caC nucleoside possibly due to its poor detection levels (**Figure 2**). These results demonstrate that the untagged TET2 dioxygenase purified in this procedure is catalytically active and can be used to characterize the wt-TET2 enzyme and its clinical mutants.

**Figure and table Legends:**

**Figure 1.** SDS-PAGE analysis of purified TET2 dioxygenase from *E. coli* BL21 (DE3) cells. Lane A indicates marker while lane B indicates TET2 protein purified using SP sepharose ion exchange resin. The total size of the untagged TET2 dioxygenase is ∼54 kDa as indicated by the arrow.

**Figure 2.** Standard curves was draw for four natural DNA nucleosides and different cytosine derivatives, which were then used for their quantification.

**Figure 3.** Liquid chromatography (bottom) and MS/MS (above) method used to separate and characterize four natural DNA nucleosides and different cytosine derivatives.

**Table 1:** Sequence of DNA oligonucleotide primers used for PCR amplification of the catalytic domain of untagged human TET2 dioxygenase.

**Table 2:** Sequence of the sense and anti-sense 25-mer dsDNA oligonucleotide used as a TET2 substrate for *in vitro* oxidation reactions.

**Table 3:** Optimized LC-MS/MS parameters of four natural DNA nucleosides and different cytosine derivatives under positive ion mode. For each parent ion nucleoside (Q1), the most intense product ion (Q3) was detected.

**DISCUSSION**

Mutations in TET2 gene are some of the most frequently detected genetic changes in patients with diverse hematopoietic malignancies. To date hundreds of different TET2 mutations, which include nonsense, frame-shift, and missense mutations, have been identified in patients[12](#_ENREF_12). Patients with *TET2* mutations show low levels of genomic 5hmC in the bone marrow compared to those with wt-TET2[14](#_ENREF_14). Mutant *TET2* knock-in experiments have recapitulated the effects of these mutations on 5hmC levels in transfected cells[14](#_ENREF_14). Results from TET2-knockout mouse models demonstrated that level of TET2 enzyme inversely correlated with the progression of hematopoietic malignancies[17-20](#_ENREF_17). Consistently, Zhang *et al.* recently demonstrated that down-regulation of TET2 expression levels is a potential prognostic and predictive biomarker in cytogenetically normal acute myeloid leukemia[27](#_ENREF_27).

Despite growing evidence that TET2 plays a fundamental role in normal hematopoiesis and myeloid transformation, biochemical characterization of wt- and mutant TET2 remain at rudimentary stages due to difficulties associated with the production of active TET2 and its assay. Most studies have produced recombinant TET2 either using time-consuming baculovirus system in insect cells[14](#_ENREF_14), or as a glutathione S-transferase affinity tag in bacterial cells which requires removal of affinity tag[21](#_ENREF_21).

In this experimental procedure, we described the cloning of untagged human TET2 dioxygenase catalytic domain using a site-specific recombination technique and its efficient expression using destination vector (pDEST14) in *E. coli*. Because the isoelectric point of untagged TET2 is relatively high (∼7.49) compared with most indigenous *E. coli* proteins, we developed an efficient purification process utilizing a cation exchange chromatography yielding >90% pure untagged TET2 enzyme in a single step.

Further, additional challenges exist in the quantification of wt- and mutant TET2 dioxygenase activity. For these experiments, most studies have relied on antibody-based assays such as dot-blots[14](#_ENREF_14), [28](#_ENREF_28), enzyme-linked immunosorbent assays (ELISA)[29](#_ENREF_29), *etc.* Because these assays generally use only one antibody, *e.g.,* 5hmC or 5fC or5caC, for detection of 5mC modification in substrate DNA, they do not provide a full picture of the catalytic reaction carried by TET isoforms. For these reasons, the LC-MS/MS-based assay has emerged as the only assay to quantify different cytosine modifications. In this regard, we have developed a novel liquid chromatography method that can separate the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5mC, 5hmC, 5fC, and 5caC).

To quantify the eight nucleosides from TET2 catalyzed reactions, we have coupled our improved liquid chromatography method with tandem mass spectrometry. This sensitive LC-MS/MS assay was then utilized to determine the activity of recombinant untagged human TET2 enzyme. The approach described here will greatly enhance the evaluation of wt- and mutant TET2 dioxygenase activities.

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DISCLOSURES

The authors have no financial interests to declare.

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