DNA Ligase-Based Strategy for Quantifying Heterogeneous DNA Methylation without Sequencing

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BACKGROUND: DNA methylation is a potential source of disease biomarkers. Typically, methylation levels are measured at individual cytosine/guanine (CpG) sites or over a short region of interest. However, regions of interest often show heterogeneous methylation comprising multiple patterns of methylation (epialleles) on individual DNA strands. Heterogeneous methylation is largely ignored because digital methods are required to deconvolute these usually complex patterns of epialleles. Currently, only single-molecule approaches, such as next generation sequencing (NGS), can provide detailed epiallele information. Because NGS is not yet feasible for routine practice, we developed a single-molecule–like approach, named for epiallele quantification (EpiQ).

METHODS: EpiQ uses DNA ligases and the enhanced thermal instability of short (<19 bases) mismatched DNA probes for the relative quantification of epialleles. The assay was developed using fluorescent detection on a gel and then adapted for electrochemical detection on a microfabricated device. NGS was used to validate the analytical accuracy of EpiQ.

RESULTS: In this proof of principle study, EpiQ detected with 90%–95% specificity each of the 8 possible epialleles for a 3-CpG cluster at the promoter region of the CDKN2B (p15) tumor suppressor gene. EpiQ successfully profiled heterogeneous methylation patterns in clinically derived samples, and the results were cross-validated with NGS.

CONCLUSIONS: EpiQ is a potential alternative tool for characterizing heterogeneous methylation, thus facilitating its use as a biomarker. EpiQ was developed on a gel-based assay but can also easily be adapted for miniaturized chip-based platforms.

Epigenetic changes in DNA have gained recent interest as disease biomarkers (1–3). The most readily studied form of epigenetic DNA change is the methylation of the cytosine in cytosine/guanine dinucleotides (CpGs), particularly in promoter region–associated CpG islands. Whereas a region is often described as either unmethylated or methylated, the reality is more complex. In many cases, a complex mixture of unmethylated, methylated, and partially methylated DNA strands are present. This is referred to as heterogeneous methylation and the differentially methylated strands are termed epialleles (4). The role of heterogeneous methylation in cancer has been reported in numerous studies, and notable examples include tumor suppressor genes such as cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) [CDKN2B]6 and death-associated protein kinase 1 (DAPK1) (4–11).

Typically, bisulfite treatment is followed by DNA amplification using methylation-independent primers to amplify sequences regardless of their methylation status and, finally, sequencing to differentiate between methylated (M) and unmethylated (U) cytosines (12, 13). Most frequently used methodologies to measure methylation adopt an “averaging” strategy, measuring either mean methylation at single CpG sites (14–17) or the mean methylation over a region of DNA (18–23). Epialleles are, however, difficult to quantify and are usually ignored. (4, 6) An n number of CpG sites has 2n permutations, e.g., 2 CpG sites can be methylated in 4 possible permutations: MM, MU, UM, or UU (Fig. 1A). Critically, most methodologies cannot

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1

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1
distinguish between samples consisting of 50% MM and 50% UU from samples consisting of 50% MU and 50% UM, and molecularly and biologically different averaging approaches quantify both these examples as 50% methylated (4). Currently, interrogation approaches at the single-molecule level, such as next generation sequencing (NGS) and digital PCR (4, 24), are capable of providing epiallele information (4, 6, 7, 11). Although NGS is becoming more accessible, performing NGS on a routine basis is not the optimal approach for various reasons, including expensive infrastructure, the inability to do small-scale experiments, and labor-intensive protocols. Therefore, there is a need for a readily performed technique emulating the single-molecule capability of NGS to facilitate the analysis of epialleles in addition to averaging methylation data.

DNA ligases are enzymes that covalently join DNA strands by catalyzing the formation of a phosphodiester bond. Ligases have been used to amplify single-DNA base changes with the ligase chain reaction (LCR) and ligase detection reaction (LDR) assays (25, 26). Both LCR and LDR have been used to detect methylation at single CpGs and have been coupled to various readout platforms (14, 15, 26–29), but the most com-

**Fig. 1.** (A), The 4 possible methylation states of a 2-CpG region. Methylation sites (M, shaded circles) and unmethylated (U, open circles). Heterogeneously methylated DNA was indistinguishable from homogeneously methylated DNA with current averaging techniques. (B), Schematic conceptualization of the EpiQ assay. High specificity was achieved through the use of DNA ligases and probe designs. In the presence of a fully matched target, the epiallele probe is ligated to the reporter probe labelled with a fluorescent dye, resulting in higher molecular weight products. (C), Ligated products distinguished via gel electrophoresis. Example shown is for the MMM target against the 8 epiallele-specific probes. The 3 expected bands B1, B2, and B3 are as indicated. Illustrations of the expected DNA species are shown next to each band.
Common approaches still use fluorescence-based analysis (15, 26–28). Generally, DNA ligases are useful molecular tools for detecting single-base mismatches. However, DNA ligases have not been adopted for simple analysis of heterogeneous methylation. The enzyme’s specificity is dependent on various factors such as reaction temperature and complementarity of ligating DNA probes, especially at the 3’ ligation end (25, 30–32). Importantly, DNA base mismatches within 10 bases of the 3’ ligation end are not well tolerated by the DNA ligase (32) and hence function as another level of stringency. We thus envisioned that a DNA ligase-based strategy exploiting the enzyme’s sensitivity to mismatched bases should enable the discrimination and enumeration of a complex mixture of epialleles.

Here we demonstrate such a DNA ligase-based strategy, epiallele quantification without sequencing (EpiQ). Using carefully designed DNA probes, we were able to specifically detect the 8 possible epialleles of a 3-CpG cluster on the promoter of the CDKN2B (p15) tumor-suppressor gene, for which heterogeneous methylation has been implicated as a driver in a variety of cancers (4, 5, 7–9). Assay accuracy was tested on 6 patient-derived samples and validated with NGS.

Materials and Methods

PCR AND NGS

PCR was performed using the KapaHiFi PCR kit (Kapa Biosystems) as recommended by the manufacturer, in a 50-µL reaction with 10 µmol/L of each primer and 1 µL of p15 amplicons derived from clinical samples in a previous study (11). The reactions were then incubated at 95 °C for 1 min followed by 15 cycles of 95 °C for 15 s and 62 °C for 30 s. Amplicons were then gel purified with the QIAquick extraction kit (Qiagen), for which the aliquot was sequenced on the 454 NGS platform (Australian Genome Research Facility, Brisbane) and the remaining aliquot was used for interrogation with the epiallele assay. Briefly, the open source QUMA tool (33) was used on default settings to analyze raw 454 sequencing reads. Each sample had in excess of 20000 reads. The proportion of an epiallele was defined similarly in Eq. 2, where FL was substituted for the number of sequencing reads.

To generate PCR amplicons representing the 8 epialleles, a forward primer synthesized with the desired epiallele was used with a common reverse primer to “mutate” in vitro and amplify sequences of that particular epiallele. To generate a titration of MMM/UUU mixtures, the respective PCR amplicons were gel purified and quantified using spectrometry prior to mixing at the desired MMM/UUU ratios. All primers and probe (Integrated DNA Technologies) sequences used are provided in Table 1, and the characteristics of the epiallele probes provided in Table 1 in the Data Supplement that accompanies the online version of this article at http://wwwclinchem.org/content/vol61/issue1.

| Table 1. Oligonucleotide sequences used in this report. |
|-----------------|-----------------|
| **DNA oligonucleotide** | **3’–5’ Sequence** |
| P15 reporter probe | PO-cttaaatcttacatcaaaaaaac-TYE665<sup>a</sup> |
| MMM probe | caaaaaccaagacaG |
| UMM probe | caaaaaccaagacaG |
| MUM probe | caaaaaccaagacaG |
| UUM probe | caaaaaccaagacaG |
| MMU probe | caaaaaccaagacaG |
| UUU probe | caaaaaccaagacaG |
| P15 epiallele-F | gttaggcgtttttttgtagaattctaggYgYgttgg |
| P15 PCR-F | gttaggcgtttttttgtagaattctagg |
| P15 PCR-R | tacactaaactttgttaataatc |
| Capture probe | Hisgcttttttagaattctagg |
| Biotin probe | YgYgttggtagtggtagtgg |

<sup>a</sup> Interrogation sites are highlighted in bold uppercase. Modifications are as indicated. For p15 Epiallele-F and biotin probe, Y is substituted for C or T bases to generate the required epiallele sequence.

EpiQ ASSAY

The assay was performed using the Ampligase kit (Epicentre) as recommended by the manufacturer. Briefly, the 8 epialleles were interrogated individually in 10-µL reactions, each consisting of 5 ng of PCR product, 2 U of ligase, 100 mmol/L of an epiallele probe, and 50 mmol/L of the reporter probe labeled with the TYE665 fluorescent dye. The reactions were then incubated at 95 °C for 2 min followed by 15 cycles of 95 °C for 15 s and 64 °C for 3 min. Finally, ligation reactions were resolved by gel electrophoresis and visualized on the Typhoon 9100 fluorescence scanner.

DATA ANALYSIS

Densitometry was performed using ImageJ software (http://rsweb.nih.gov/ij/). A positive result typically resolved into 3 bands corresponding to the 3 expected DNA species (see Results and Fig. 1 for details), B1, B2, and B3. For each reaction, fluorescence intensities, FL, were normalized to B1 to control for sample loading as shown in Eq. 1:

\[
FL = \frac{B3 + B2}{B1}
\]

Deriving from Eq. 1, the proportion of an epiallele, P, was defined by Eq. 2 as:

\[
P = 1 - \frac{B1}{B3 + B2}
\]
where $x$ was the epiallele of interest and $\text{FL}_{\text{total}}$ was the arithmetic sum of FL for all 8 epialleles.

From Eq. 2, the methylation of a single CpG could then be inferred by the following relationship (Eq. 3):

$$\text{CpG}_{y} = \frac{\text{P}_{\text{total,} M}(\text{P}_{\text{total,} M} + \text{P}_{\text{total,} U})}{100\%},$$

where $y$ was the CpG of interest (i.e., CpG1, CpG2, or CpG3), $\text{P}_{\text{total,} M}$ was the arithmetic sum of all P, where the CpG of interest methylation status was M, and where $\text{P}_{\text{total,} U}$ was the arithmetic sum of all P where the CpG of interest methylation status was U.

From Eq. 2, the mean regional methylation was inferred by using Eq. 4:

$$\text{all epialleles}$$

$$\% \text{Regional methylation} = \sum \left[ \left( \frac{N_x}{3} \times P_x \right) \right],$$

where $N_x$ was the number of M events in epiallele pattern $x$ with P proportion.

To evaluate the stability of the EpiQ assay, $\%\text{CV}$ was calculated over 2–4 experiments, each with 2–3 replicates, using the same template input.

**ELECTROCHEMICAL DETECTION**

Microdevices were fabricated using standard photolithography techniques (fabrication details are provided in the online Data Supplement) and prepared for electrochemical detection as previously described (29). Briefly, capture sequences complementary to the reporter probe were immobilized on electrode surfaces via gold/thiol self-assembling monolayers. To generate products for electrochemical detection, the epiallele assays were performed as described above but with reporter probes that lacked the fluorescent label. Following the ligase step, 1 µL of 1 µmol/L biotin-labeled DNA oligonucleotide in 100 mmol/L EDTA solution was added and allowed to hybridize to ligase reaction products for 10 min at room temperature. Only biotin-labeled probes of complementary sequence were added to their respective epiallele reactions. Each epiallele reaction was then added to separate wells of the microdevice and allowed to incubate at room temperature with agitation for 2 h. One microliter of a 1/1000 diluted horseradish peroxidase (HRP) solution (BD Biosciences) was then added to each well and allowed to incubate for another 15 min. This was then followed by 3 washes with 100 mmol/L Tris-HCl buffer (pH 8).

Just before electrochemical measurements, 50 µL of a 100-mmol/L Tris-HCl buffer supplemented with 0.5% H$_2$O$_2$ was added to each well. All electrochemical measurements were done on a CH1040C potentiostat (CH Instruments) with a 3-electrode system consisting of DNA-modified gold microelectrodes as the working electrode, Pt counter electrode, and Ag quasi reference electrode. Electrocatalytic reduction of H$_2$O$_2$ was performed using cyclic voltammetry from 100 mV to $-600$ mV at a scan rate of 50 mV/s. The electrochemical response, EC, was defined as the current density at $-500$ mV and is given by Eq. 5:

$$\text{EC}_x = \frac{I_x}{A},$$

where $x$ was the epiallele, $I$ was current, and $A$ was the electrode area.

To determine the proportions of epialleles in the sample, EC was substituted for FL in Eq. 2 and all methylation estimations were calculated similarly.

**Results**

**THE EpiQ ASSAY**

The EpiQ assay exploited both the ability of DNA ligases to recognize DNA base mismatches at the ligation junction and the lowered melting temperatures of short mismatched DNA probes to achieve high assay specificity (Fig. 1B). In this study, we chose to interrogate the epialleles of a 3-CpG cluster in the promoter region of the CDKN2B (p15) tumor suppressor gene that had been amplified following bisulfite conversion. Because every CpG could be either methylated or unmethylated (M or U), the number of permutations for the 3 CpG cluster is $2^3 = 8$. Each of these 8 methylation patterns was recognized by a different interrogation probe, for which the CpGs of interest were at the 3’ ends of the probes. Because DNA ligases are sensitive to mismatches of up to 10 bases of the 3’ end of the ligation junction (25, 32), designing the CpGs sites at the 3’ end provided the first level of stringency. An additional level of specificity was obtained by designing short oligonucleotide probes (16–19 bases) such that one or more mismatches in the sequence could substantially destabilize hybridization at a given annealing temperature (see online Supplemental Table 1).

Depending on the target sequence, 1 of these 8 probes was ligated to a reporter sequence labeled with a fluorophore. A successful ligation event resulted in a longer oligonucleotide that had a retarded migration profile during gel electrophoresis (Fig. 1C). The fluorescence intensity of the slower-migrating DNA bands was proportional to the amount of ligation events, which in turn was proportional to the amount of the corresponding epiallele in the sample. Moreover, because the assay quantified all possible heterogeneous methylation patterns, it was also possible to calculate both regional and single CpG methylation levels if desired.
ASSAY SPECIFICITY

Assay specificity was paramount because single-base differences needed to be discriminated. To demonstrate the specificity, each of the 8 epiallele probes was individually tested against each of the 8 epiallele targets artificially generated by PCR (Fig. 2). In positive cases, 3 distinct bands corresponding to the 3 expected species of fluorescently labeled DNA were observed when visualized on the gel after electrophoresis (Fig. 1C). The low molecular weight band, B1, was the unreacted reporter probe. The B2 band was the single-stranded DNA species, which the reporter probe had ligated with an epiallele probe. The B3 band represented B2 products that had hybridized to the target DNA. As expected, ligation products were reproducibly (CV = 7.5% over 4 experiments) seen only in matching probe/target pairs (Fig. 2, A–H). In samples homogenous for a specific epiallele, the signal from the matching probe was typically between 90% and 95% of the total signal in the panel of 8 probes, indicating very high specificity. The corresponding regional and single CpG methylation levels inferred from EpiQ were consistent with the target’s expected epiallele pattern and thus also functioned as secondary validation for assay accuracy (Fig. 2, right panels). Next, we used 2-, 3-, and 4-epiallele scenarios to further demonstrate assay specificity (Fig. 3; also see online Supplemental Fig. 1). As expected, EpiQ maintained its specificity even in more complex epiallele situations, as demonstrated by estimates that were concordant with their expected ratios.

ASSAY SENSITIVITY

Assay sensitivity was defined here as the minimum detectable difference in epiallele levels. Because the proportions of the 8 epialleles could occur in a multitude of combinations for any given sample, we first chose, for simplicity, mixtures of MMM/UUU PCR-generated templates to demonstrate assay sensitivity. Targets comprising various MMM-to-UUU ratios were interrogated sequentially by the panel of 8 probes. Fig. 3 shows the linear relationship between probe-generated signal and sample input ($R^2 = 0.987$ and 0.977 for MMM and UUU probes, respectively; CV = 5.5% over 3 experiments). From the calibration plot, both the MMM and UUU probes could detect as little as 5% of their respective targets because signals saturated at 95% pure targets. Therefore, the dynamic range was between 5% and 95% and was consistent with the approximate 5% error in the system. An analysis of $P$, the signal proportions of the epiallele probes,
revealed near-equivalent signal levels in the 50% mix sample, indicating that we had an unbiased assay (Fig. 3C). However, when one target was in excess of the other, the assay tended to slightly underestimate the proportion of the epiallele in excess (Fig. 3C), thus partially accounting for the signal saturation at both ends of the titration plot.

ACCURACY COMPARISON BETWEEN EPIALLELE ASSAY AND NGS
To demonstrate assay accuracy, we then tested EpiQ directly on a panel of 6 bisulfite PCR products (amplified using methylation-independent primers across the same region of the p15 promoter) derived from the peripheral blood of leukemia patients (11). The samples were concurrently characterized on both the 454 NGS platform and by EpiQ. Comparisons were then made between both approaches (Fig. 4). Overall, the EpiQ assay performed comparably with NGS in determining heterogeneous methylation patterns for the 6 leukemia samples. However, quantitative epiallele estimations by the EpiQ assay generally were 5% to 10% higher than NGS estimates.

ADAPTING THE EPIALLELE ASSAY ON A MICRODEVICE FOR ELECTROCHEMICAL DETECTION
We have previously demonstrated electrochemical detection of DNA methylation at individual CpGs using microdevices via LCR (14, 29) and, given the various benefits of microdevice-based assays (34–36), we thus tested whether the epiallele assay could also be adapted onto such a platform. To achieve this, B2 products [representing the amount of an epiallele (Fig. 1C)] from each of the epiallele-probe reactions were immobilized onto the electrode surfaces, labeled with the redox-active HRP enzyme via a biotin/streptavidin coupling, and electrochemically stimulated (Fig. 5A). Epiallele quantification was then inferred from H2O2-mediated electrocatalytic signals, which in turn were proportional to the amount of B2 products. The 16-well device was chosen to facilitate measurements of the 8 epialleles in duplicate and was an extension of our previously reported 5-well version (14, 29). Both devices had similar electrochemical characteristics, but the 16-well version allowed for slightly higher throughput.

The feasibility of an electrochemical EpiQ assay was demonstrated using the acute myeloid leukemia (AML) sample, AML10 (Fig. 5B; also see online Supplemental Fig. 2). As expected, we could reproducibly (CV = 13.3% over 2 experiments) detect the proportions of epialleles present in AML10 to levels comparable to both fluorescence and NGS-based approaches (paired t-test, P values = 0.9 and 0.8 respectively), hence demonstrating the assay’s versatility for adaptation onto more sophisticated readout methods.

Discussion
DNA methylation is a key driver mechanism in cancer progression (1–3) and has motivated the development of new techniques to aid in both research and diagnostics (7, 14, 15, 18, 21, 22, 26–28). However, DNA strands are
frequently heterogeneously methylated with multiple differently methylated epialleles co-occurring in a sample. Because most techniques do not measure epialleles, heterogeneous methylation has been largely ignored (4, 6, 7). Currently, only single-molecule approaches like NGS can readily provide epiallele information, but the routine use of NGS is not yet feasible for various reasons, especially for diagnostic situations in which inexpensive small-scale assays are needed. Therefore, new tools emulating NGS’s single-molecule ability are needed to aid the field. We have developed the epiallele quantification assay (EpiQ), which achieved a single-molecule–like evaluation of DNA methylation. This assay is the only approach besides NGS that can provide all 3 levels of epigenetic information: epiallele, single CpG, and regional methylation.

Because the assay aims to deconvolute DNA species that differ by as little as a single nucleotide base, it is paramount that the assay be highly specific. Traditional DNA methylation methodologies detect methylation either at individual CpGs or over a region of interest. For example, restriction enzyme-based methods and all ligase-based methods to date interrogate only a single CpG site. Methylation-sensitive high-resolution melting (MS-HRM), one of few methods giving overall snapshots of methylation, often underestimates the overall methylation when methylation is heterogeneous due to the formation of heteroduplexes (4, 7, 11).

However, the combination of PCR and ligase-based approaches has now been shown to provide the specificity required for epiallele information. The PCR step first amplifies all epialleles present in a sample, and the ligase step, exploiting its sensitivity to mismatches up to 10 bases from the 3’ ligating end (14, 25, 32), is used to extract the proportion of the epiallele patterns. The results of this approach (Fig. 2–4; also see online Supplemental Fig. 1) show that each of the epiallele probes is highly specific for its respective target even in complex epiallele mixes. In addition, because gene promoter regions tend to be GC rich, the likelihood of a probe being GC rich is also high, albeit to a slightly lesser extent, after bisulfite conversion. This, in turn, leads to higher melting temperatures for probes of a given length. And since probes tend to have higher melting temperatures, shorter sequences can be designed, which in turn enhances the destabilizing effect of mismatches in the sequence for a given annealing temperature. The resulting effect is the enhanced specificity observed in the assay, which may not be possible if either ligase or probe design was used independently.

EpiQ is sensitive to approximately 5%–10% changes in epiallele proportions (Fig. 3). However, very few studies have been done on epialleles, nor is there an available alternative approach besides NGS to benchmark our assay’s analytical performance against. Nonetheless, the current preferred commercial diagnostic approach for DNA methylation is the Sequenom® MassArray. While essentially providing different methylation information, Sequenom is also sensitive to 5%–10% differences for individual CpGs (18), thus
suggesting that the detection limit of our proof-of-concept assay may still be acceptable as a general research or diagnostic tool.

The power of single-molecule analysis is undeniable. For instance, the 6 clinical samples studied had almost identical overall regional methylation (Fig. 4, right panels). However, at individual CpGs, subtle differences started becoming apparent and each patient was distinctively different epigenetically at the epiallele level (Fig. 4, left panels). Similar results have been previously reported using digital MS-HRM for the DAPK1 gene in chronic lymphocytic leukemia patients (7). This example underscores the power of single-molecule–level analysis for DNA methylation and possibly genetics in general. Notwithstanding, the epiallele profiles derived from our assay also validated well with NGS, thus accentuating the accurate analytical performance of our assay as well as highlighting its potential as a viable alternative for rapid, targeted small- to medium-scaled single-molecule–like (epi)genetic applications. NGS, on the other hand, with its longer read lengths and higher throughput, may be more suited for other applications.

The EpiQ assay was first developed and characterized using fluorescence with simple and cheap gel electrophoresis, as shown here, and may be useful as a rapid but small-scaled research tool. However, the assay is readout agnostic and is adaptable toward more sophisticated displays. The assay was successfully translated onto an electrochemical microdevice platform with good reproducibility (CV = 13%) and with similar epiallele estimates to fluorescence and NGS (Fig. 5). These data, coupled with the potential advantages (34–36) of microfabricated device platforms, suggest a plausible alternative methodology but without the limitations of NGS and digital PCR approaches (4). To the best of our knowledge, this is the first application of DNA ligases being coupled to microdevices for electrochemical detection of heterogeneous DNA methylation on individual DNA strands (epialleles). Microfabricated devices offer potential benefits, such as higher throughput, integration, portability, speed, cost, minimal solvent/reagent requirements, and high signal-to-noise ratios (34–36). Hence, adapting EpiQ onto a convenient electrochemical microdevice platform may facil-
itate routine detection of epialleles. Finally, while the assay was originally designed for epiallele detection, the assay concept may also be amendable for simultaneous interrogation of CpG sites with other genetic abnormalities (e.g., point mutations and single nucleotide polymorphisms) in close proximity.

In summary, we have developed an epiallele quantification assay (EpiQ) with single-molecule–like characteristics for quantifying epialleles as an alternative to NGS-based approaches. The assay is highly specific for all of the 8 possible epialleles in a 3-CpG region, has comparable analytical performance to NGS, is sensitive to between 5% and 10% of differences in epiallele levels, and has good reproducibility. The assay accurately profiled clinically derived samples and was validated with NGS. The assay was also shown to have the potential to be adapted for more sophisticated display methods, such electrochemical microdevice platforms, with similar performance to fluorescence and NGS.

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