A new ultrasensitive assay for detection of hypermethylated tumor DNA in liquid biopsies

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BACKGROUND

The methylation pattern of cancer DNA differs from that of normal DNA, wherein some loci are hypermethylated while others are hypomethylated. The ability to detect tumor-derived DNA in body fluids such as urine and blood plasma can open the door to development of non-invasive, 'liquid biopsy' type of assays, for early detection and companion diagnostics. However, the small amounts of total cell free DNA, and the small fraction of tumor-derived DNA within the sample are both potential limiting factors for an effective liquid biopsy assay, especially for early stage detection. Additionally, sodium bisulfite treatment, which is the first step of many methylation analysis assays, degrades most of the DNA template, severely exacerbating the problem of low tumor DNA.

We developed EpiCheck – a simple, cost effective, and highly sensitive sodium bisulfite-free assay for detection of hypermethylated genomic loci associated with cancer. The assay is based on methylation-sensitive enzymatic digestion of DNA followed by real time PCR amplification of target loci and software analysis of the output file. EpiCheck is an application-agnostic platform in the sense that it can detect any type of cancer, given the right set of markers. We developed Bladder EpiCheck (15 markers) for detection of Bladder cancer in urine and Lung EpiCheck (6 markers) to detect Lung cancer in blood. We developed the markers for these tests by bioinformatics analysis of differential methylation between samples from healthy and sick patients. The biomarker panels were then assembled by a big data, machine learning based algorithm implementing an iterative process looping bioinformatics data with empirical data from testing of candidate markers' performance using EpiCheck.

METHODS

The EpiCheck assay consists of four consecutive steps (Figure 1): (1) DNA extraction from the clinical sample, (2) DNA digestion with a mix of methylation sensitive enzymes, (3) real time PCR amplification, and (4) analysis of the raw PCR data by the EpiCheck software. The output of the assay is the EpiScore – a numerical score between 0-100 which reflects the level of methylation of the sample at the panel loci. When a sample has an EpiScore that is equal to or higher than a predetermined threshold, that sample is considered positive.

The analytical sensitivity of EpiCheck was tested using mixtures of methylated DNA spiked into unmethylated DNA. The methylated DNA consisted of the HCT-15 human cell line DNA, which is completely methylated at a single predefined locus. The unmethylated DNA consisted of pGEM-T Easy (Promega) plasmids containing a cloned insert with the interrogated locus. The mixtures tested had molar ratios of methylated:unmethylated DNA of 1:8, 1:64, 1:512, 1:4,096, 1:32,768, and 1:200,000.

Overall, positive, and negative detection rates of the 1:200,000 DNA mixture were tested in 68 separate PCR reactions: 34 replicates, each containing 10 methylated and 2,000,000 unmethylated template molecules (positive detection rate), and another 34 replicates, each containing 2,000,000 unmethylated template molecules.

AIMS

The aim of this study was to determine the analytical sensitivity of EpiCheck.

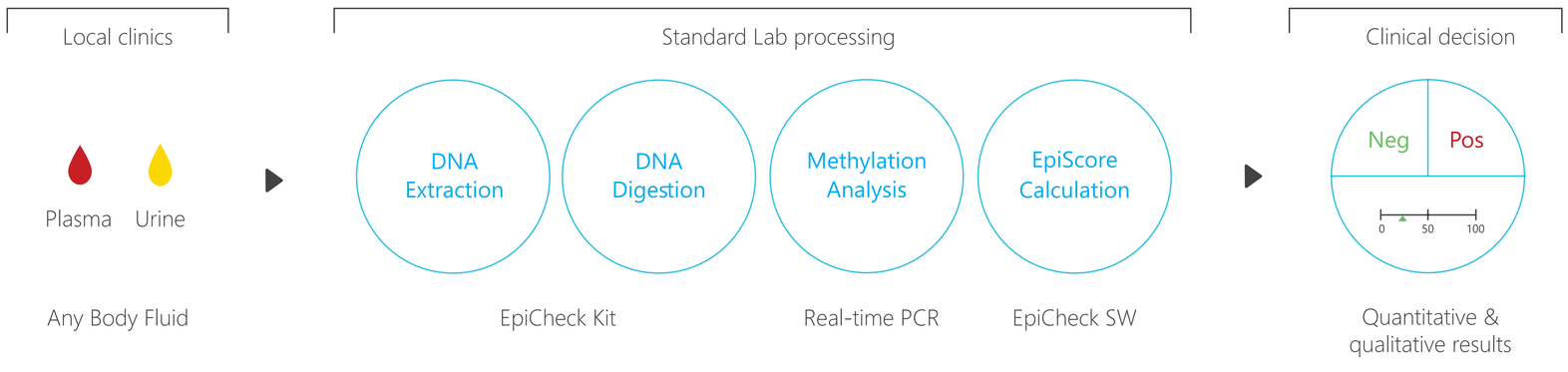


Figure 1: The EpiCheck assay process

RESULTS

Figure 2A shows the amplification plots of the mixtures of methylated and unmethylated DNA. For the mixture of DNA with 1:8 molar ratio, the ΔCQ between the test and internal reference loci is about 3 cycles. As the ratio between the methylated and unmethylated DNA samples grows, so does the ΔCQ, until it reaches 18.75 cycles for the 1:200,000 mix. When the calculated Δ CQs are plotted against the expected ΔCQs (assuming 100% PCR efficiency for both test and internal reference loci), and linear regression is performed using the least squares method, the resulting fit has an R^2 value of 0.9966 (Figure 2B), demonstrating the precision of the assay.

Figure 3 shows the amplification plots from the 1:200,000 DNA mixture ratio. The test locus was successfully amplified in all 34 replicates containing this mixture ratio, whereas in all 34 replicates containing only unmethylated DNA, no amplification of the test locus was observed.

The overall detection rate was 100.0% (68/68). The lower limit of the one-sided exact binomial 95% Confidence Interval (CI) was 95.69%, meaning that the true overall detection rate was higher than 95.69% with 95% confidence. The positive and negative detection rates were both 100.0% (34/34). The lower limit of the one-sided exact binomial 95% CI was 91.57%, meaning that the true positive and negative detection rates were higher than 91.5% with 95% confidence.

Bladder EpiCheck demonstrated 86% sensitivity and 86% specificity in high-grade tumors in a blinded, prospective, multicenter European study[1]. Bladder EpiCheck outperformed the current standard methods of cytology and cystoscopy (Figure 4) and is in commercial use in Europe and Israel. Lung EpiCheck achieved an overall sensitivity of 74% at a specificity of 91%, with an AUC of 0.887 in a blinded, prospective, multicenter European validation study[2] (Figures 5 and 6).

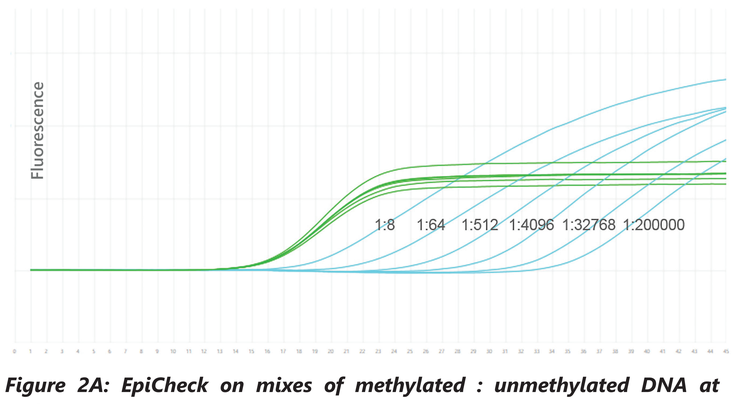


Figure 2A: EpiCheck on mixes of methylated : unmethylated DNA at different molar ratios

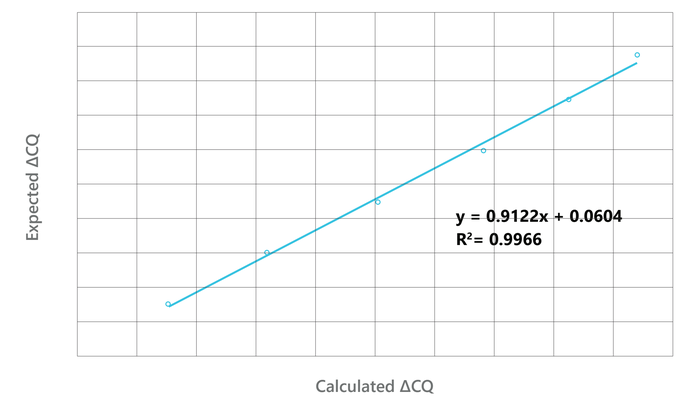


Figure 2B: Linear regression of EpiCheck results on the different mixtures

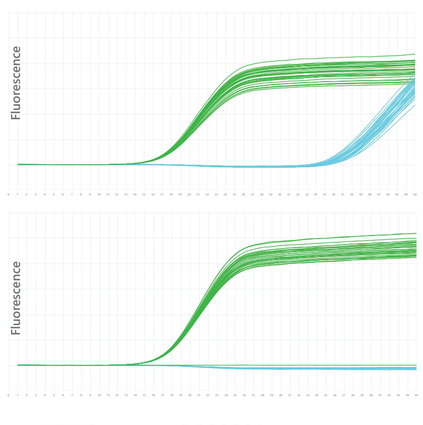


Figure 3: Replicated of EpiCheck on 1:200000 methylated:unmethylated DNA (top) and on completely unmethylated DNA (bottom)

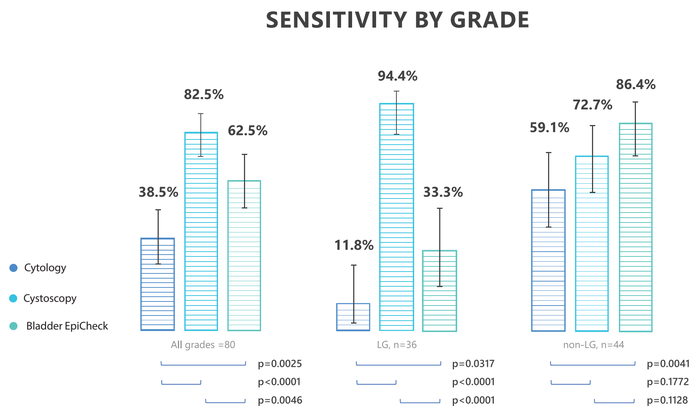
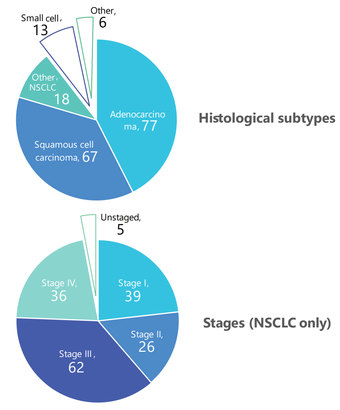


Figure 4: Results of the Bladder EpiCheck™ EU clinical trial



		Cases	Controls
n		181	141
Sex	Male Female	133 (73%) 48 (27%)	90 (63%) 51(36%)
Age	Range Average	23-90 67	51-88 65
Inclusion Criteria		• Pathologically proven primary lung cacner • Treatment naïve	• Subjects aged 50-80 years • Currently or former smokers
Exclusion Criteria		• Subjects with cancer or history of cancer from any kind	• Subjects with cancer or history of cancer from any kind

Figure 5: Lung EpiCheck™ test set samples

	Training set	Test set
AUC	0.890	0.887
Specificity	94%	91%
Sensitivity	74%	74%
Sensitivity by subtype		
Adenocarcinoma	57%	71%
Squamous Cell Carcinoma	78%	73%
Other NSCLC	88%	50%
Small cell carcinoma	90%	92%
Unknown	50%	71%
Sensitivity by stage - NSCLC		
Stage I	50%	59%
Stage II	67%	77%
Stage III	82%	76%
Stage IV	94%	83%
Unstaged	100%	40%
Sensitivity by stage - SCLC		
Limited	67%	100%
Extensive	100%	86%

Figure 6: Lung EpiCheck™ test set results

CONCLUSIONS

EpiCheck can detect 1 methylated DNA molecule among 200,000 unmethylated DNA molecules, which is the highest analytical sensitivity reported to date by any methylation analysis platform. This analytical ultrasensitivity translates to unprecedented performance of cancer detection in the EpiCheck-based products, Bladder EpiCheck and Lung EpiCheck, demonstrating the utility of EpiCheck-based assays for cancer detection and monitoring.

[1] Lozano et al, EAU19 poster 709 [2] Chorostowska-Wynimko et al. WCLC18 poster P2.12-20