Summary

Bicycle PCR™ is a novel patented and patent pending method which greatly improves accuracy in low-frequency cancer DNA detection. Bicycle PCR™ is the first technology that accurately detects a single fragment of cancer DNA in a sample. Bicycle PCR™ is a simple, scalable and cost-effective solution that easily augments existing qPCR, digital PCR, or NGS workflows to improve accuracy. The key innovation of Bicycle PCR™ is the use of a 'blocker' to selectively amplify target cancer DNA combined with two distinct thermocycling phases (bi-cycling). This novel combination solves the long-standing problem with blocking PCR and enables robust single molecule detection. Bicycle PCR™ is a paradigm shift in accuracy and cost, creating exciting new opportunities in cancer molecular diagnostics.

Introduction

The accurate detection of low-frequency cancer DNA has many applications in oncology, including (1) drug development, (2) cancer screening, (3) treatment response and (4) recurrence monitoring.

High-sensitivity blood tests for molecular residual disease (MRD) have been developed by several companies and the market is growing quickly. MRD can detect residual cancer up to 16-months before imaging[1]. 50% of oncologists now regularly use MRD to inform treatment decisions. Medicare now covers 19 different MRD tests, spanning 8 different cancers. Natera's test, Signatera, is a market leader and recently received FDA approval for muscle-invasive bladder cancer. The critical problem with current MRD tests is the high cost, which is a restraint on market growth and adoption.

The cost of MRD tests is high because they require (1) custom mutation panel design, (2) sequencing multiple targets, and (3) high sequencing depth. All major MRD competitors take a similar approach due to the shared problem of the technical noise of DNA sequencing which limits the accuracy of these tests and greatly increases the costs. For example, Signatera custom designs a panel of 16 mutations with an average depth of 100,000 reads[2]. A reduction in technical noise would dramatically decrease costs and increase adoption.

Blocking PCR, first described over 30-years ago, removes the effect of technical noise in NGS, digital PCR, or qPCR[3]. Despite this advantage, blocking PCR has not been commercially successful because blocking PCR cannot "distinguish a true target negative from PCR failure"[4]. This is due to the blocker very effectively blocking amplification of non-cancer ('normal') DNA, which allows for the enrich of a single copy of cancer DNA. Effective blocking results in no detectable DNA amplification for both negative samples and failed reactions.

SentryDx's breakthrough was understanding that the blocker only needs to be effective while the cancer DNA is still at very low frequency. After several rounds of exponential amplification, the blocker is no longer needed to suppress the amplification of 'normal' DNA. After that threshold is met, cycling parameters shift and allow for amplification of 'normal' and cancer DNA, resulting in successful DNA amplicon in both positive and negative samples. High enrichment of the targeted cancer DNA sequences also removes the detection step (e.g. sequencing, TaqMan probe-based detection) as a source of error because the now enriched cancer DNA sequence is the dominant sequence in positive samples (>80% of amplicon).

SentryDx new Bicycle PCR™ approach enables cost-effective and reliable detection of single fragments of cancer DNA, and additionally makes the technical noise of DNA sequencing irrelevant. Additionally, it is a cost-effective and scalable method that easily incorporates into existing workflow (e.g. digital PCR, qPCR, NGS).

Scientific Foundation of Bicycle PCR™

The premise of Bicycle PCR™ is that, at a low concentration of blocker, the percentage of normal DNA that is blocked can be modulated from high to low blocking efficiency by only changing the PCR cycling steps.

High blocking efficiency using a low blocker concentration and a blocker annealing step was demonstrated by Full COLD-PCR[5,6]. Full COLD-PCR was further improved by adding a specific blocker, instead of using the reference DNA as the blocker (Ice COLD-PCR)[7]. Bicycle PCR™ differs from COLD-PCR in that Bicycle PCR™ eliminates the PCR step where the blocker hybridizes to the

mutant DNA because it is an unnecessary step. The “temperature critical”, described in COLD-PCR, is functionally the same as a blocker annealing temperature described by Orum et al. in 1993[8]. Overall, COLD-PCR demonstrates that a blocker, at a low concentration, can hybridize before the primer, when the blocker has a higher melting temperature.

Low blocking efficiency using a low blocker concentration and skipping the blocker annealing step was demonstrated by WTB-PCR[9]. Figure 2B of the WTB-PCR paper shows that at 10 µM the blocker completely prevents normal DNA replication, but at 1 µM, the blocker allows normal DNA replication[9]. Note that the thermocycling protocol used by WTB-PCR does not contain a blocker annealing step; this shows that a blocker annealing step is not required to achieve effective blocking. However, it also shows that the blocker, at a lower concentration, allows the replication of normal DNA, when a blocker annealing step is not present in the PCR protocol. This is the premise for how to “turn-off” the blocker, by using the thermocycling protocol.

These previously well-established concepts of PCR were combined and refined into Bicycle PCR™.

Blocker Annealing Step Controls Blocking Efficiency

Bicycle PCR™ operates on the principle that the effectiveness of blocking can be modulated using distinct thermocycling parameters. The blocking effectiveness can be modulated between >95% blocking and <5% blocking by the addition of a blocker annealing step to the thermocycling program.

In Figure 1, >95% blocking occurs when thermocycling includes a blocker annealing step, and <5% blocking is observed when a blocker annealing step is not included. Samples containing KRAS G12C at a 0.02% variant allele frequency (VAF) were prepared (3 copies KRAS G12C genomic DNA was diluted into 14,500 copies of KRAS wild-type DNA). The thermocycling program was for 20 cycles, followed by Sanger sequencing. Figure 1B and 1C demonstrate the ability of Bicycle PCR™ to modulate blocker effectiveness by including or removing a blocker annealing step.

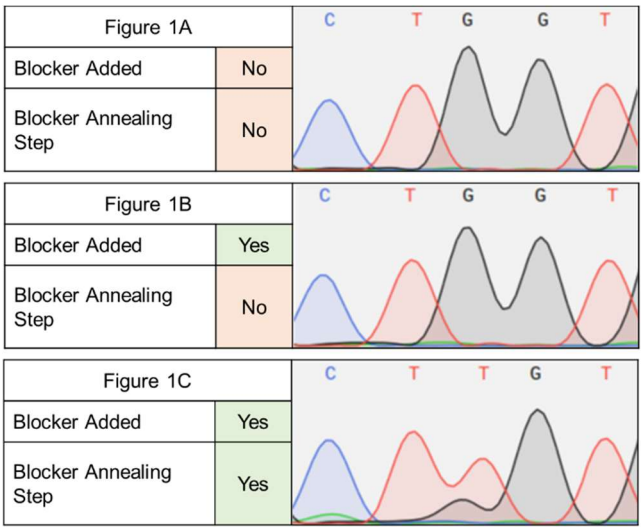

Figure 1: KRAS G12C at 0.02% VAF, a G → T mutation. 1A) normal PCR. 1B) normal PCR with the KRAS blocker. 1C) PCR with a blocker annealing step and the KRAS blocker.

The Sanger tracers were quantified to estimate blocking efficiency with or without the blocker annealing temperature (Table 1). Figure 1B and Table 1 shows that the 0.02% KRAS G12C sample is not above background noise when the blocker is present without the blocker annealing temperature: blocker alone does not prevent normal DNA from replicating. Figure 1C and Table 1 demonstrate that combining a blocker with a blocker annealing thermocycling step resulted in enrichment the 0.02% KRAS G12C sample to an average of 82.0%. Here the blocker is preventing >97% of normal DNA from replicating. These data demonstrate that Bicycle PCR™ can modulate the blocker effectiveness from between >95% to <5% by changing only the thermocycling parameters.

Table 1	Average (%) ± Standard Deviation			
	A	C	G	T
No Blocker, No Blocker Annealing	2.1 ± 1.6	0.5 ± 0.4	97.2 ± 1.6	0.1 ± 0.2
KRAS Blocker, No Blocker Annealing	1.9 ± 0.7	0.7 ± 0.8	97.4 ± 0.9	0.1 ± 0.2
KRAS Blocker, Blocker Annealing	1.5 ± 1.4	0.4 ± 0.4	16.1 ± 7.4	82.0 ± 7.8

Table 1: 6 replicates of 0.02% KRAS G12C were run for each condition for 20 PCR cycles. The average Sanger trace for each base pair at position 34, plus/minus the standard deviation. KRAS G12C is a G → T mutation.

Modulating Blocking Allows the Replication of Normal DNA

Current blocking PCR protocols are unable to distinguish between a negative result and a process failure. To compensate, both Thermo Fisher and Bio-Rad must use split workflows and an exogenous control[4].

Bicycle PCR™'s ability to modulate blocker effectiveness solves this problem by allowing the replication of normal DNA, which serves as an endogenous control and eliminates the need for a split workflow.

Figure 2 demonstrates how Bicycle PCR™ can be added to a qPCR assay to observe the delayed amplification of the wild-type (WT) DNA sequence. A dual-label fluorescence probe, containing the SUN fluorophore was added to detect the replication of KRAS WT DNA. 5 ng of KRAS WT DNA was run with or without the blocker. The thermocycling protocol contained 25 cycles with the blocker annealing temperature and 25 cycles without the blocker annealing temperature.

Figure 2

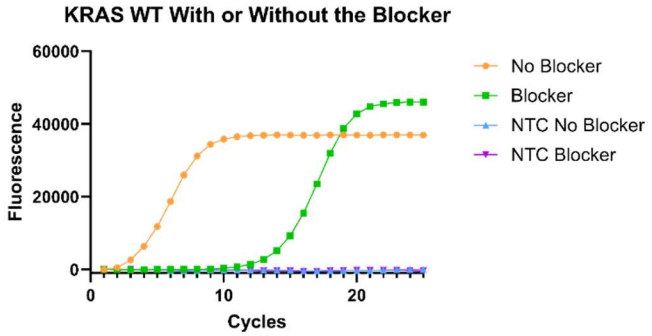

Figure 2: KRAS wild-type (WT) DNA is temporarily blocked by using Bicycle PCR™

Bicycle PCR™ Greatly Increasing the Sensitivity and Specificity of qPCR

Adding Bicycle PCR™ to qPCR increases the sensitivity of qPCR to 0.005% VAF, while maintaining 100% specificity (Figure 3). The large statistical difference between a negative and positive sample demonstrates the power that Bicycle PCR™ provides (Table 2).

A Dual-fluorescence probe for KRAS G12C containing FAM was added to the KRAS assay. KRAS G12C was tested at 0.02%, 0.01%, 0.005% VAF and 100% normal KRAS. Bicycle PCR™ has a sensitivity of 0.005% and a limit of detection of 0.01% for KRAS G12C (Figure 3). Samples containing 100% normal KRAS DNA were not positive for the KRAS G12C (FAM channel) (Figure 3A), but were positive for the endogenous control (HEX channel) (Figure 3B). Additionally, the 0.005% KRAS G12C samples were separated into called positives or called negatives. There is a clear binary distribution between the called positives and called negatives (Table 2). The called positives are statistically similar to positive KRAS G12C samples and the called negatives are statistically similar to 100% normal KRAS (Table 2). The sensitivity of KRAS G12C at 0.005% VAF was 73% (Figure 3A).

Figure 3A

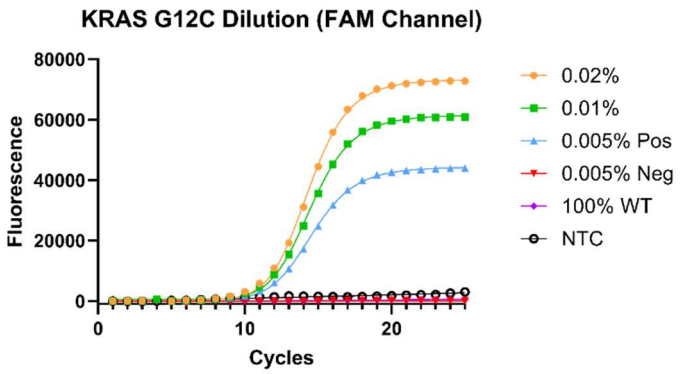

Figure 3B

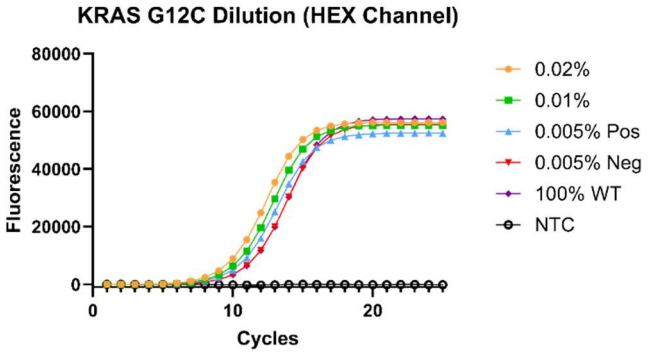

Figure 3: KRAS G12C was tested at 0.02%, 0.01%, 0.005% VAF. 100% wild-type (WT) samples and No template controls (NTC) were also run. 3A: FAM channel. 3B: HEX channel

Table 2	KRAS G12C (%)					
	0.02	0.01	0.005 (Pos)	0.005 (Neg)	0 (WT)	NTC
Average Signal	71271	59500	42718	168	437	-855
Standard Deviation	17071	17217	13850	1458	922	1196

Table 2: The average fluorescence signal and standard deviation at cycle 20 in the FAM channel

The qPCR Bicycle PCR™ KRAS 12/13 assay was further improved by combining dual-fluorescence probes for G12A, G12C, G12D, G12R, G12S, G12V and G13D. The multiplexed assay has demonstrated sensitivity of 0.02% for all 7 KRAS mutations in the 12/13 mutation hotspot region (Figure 4). Stacking all of the mutant probes into the HEX channel led to an increase in background. Further optimizations or separating into multiple fluorescence channels will reduce the background and increase the performance.

Figure 4A

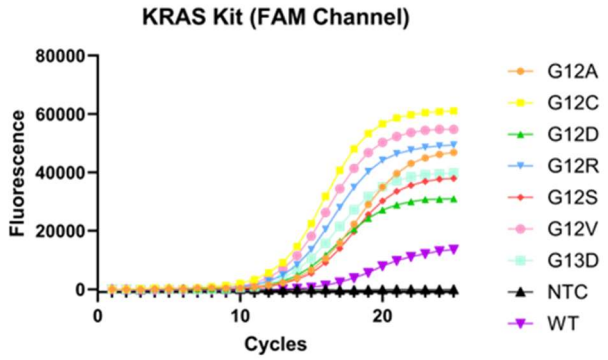

Figure 4B

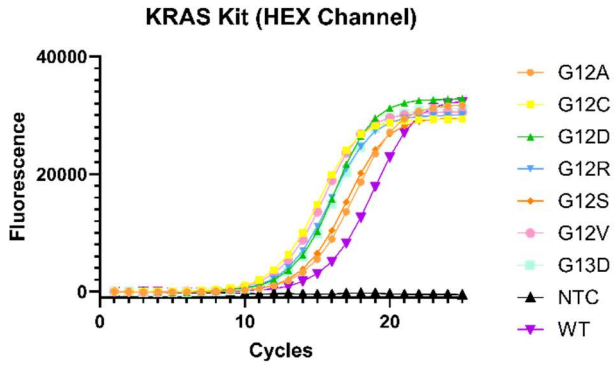

Figure 4: KRAS mutations at 0.02% VAF using the Bicycle PCR™ KRAS 12/13 Assay. 100% wild-type (WT) samples and No template controls (NTC) were also run. 3A: FAM channel. 3B: HEX channel.

A Bicycle PCR assay was developed for BRAF V600E, using the same principles as the KRAS 12/13 assay. Bicycle PCR™ BRAF V600E has a sensitivity of 0.005% and a LoD95 of 0.02% (Figure 5). Additionally, the 0.005% BRAF V600E samples were separated into called positives or called negatives. There is a clear binary distribution between the called positives and called negatives (Table 3). The sensitivity of BRAF V600E at 0.005% VAF was 78% (Figure 5A).

Table 3	BRAF V600E (%)					
	0.02	0.01	0.005 (Pos)	0.005 (Neg)	0 (WT)	NTC
Average Signal	41804	32471	28297	3761	4391	355
Standard Deviation	11121	10846	8443	1236	2014	85

Table 3: The average fluorescence signal and standard deviation at cycle 20 in the FAM channel

Figure 5A

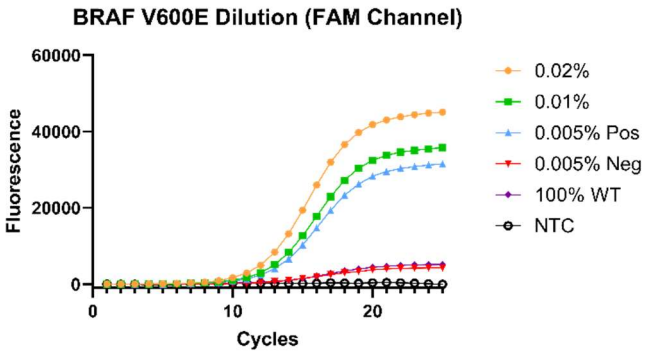

Figure 5B

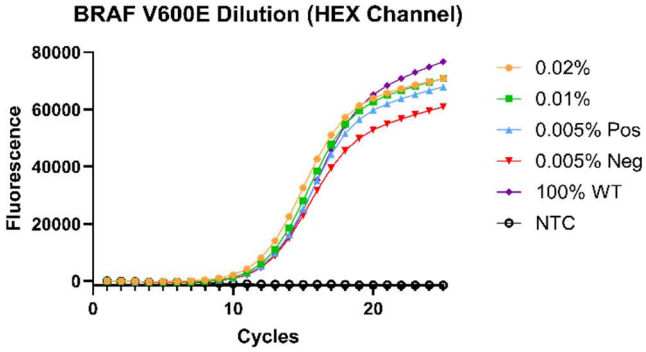

Figure 5: BRAF V600E was tested at 0.02%, 0.01%, 0.005% VAF.100% wild-type (WT) samples and No template controls (NTC) were also run. 3A: FAM channel. 3B: HEX channel

Methods

All primers, blockers and probes were purchased from Integrated DNA Technologies (IDT). KRAS DNA used from the international reference panel for KRAS cancer mutations. BRAF DNA used was from Horizon Discovery. PCR reactions were performed on a Bio-Rad PTC Tempo. qPCR reactions were performed on a Thermo Fisher QuantStudio 3.

An example qPCR protocol is below:

qPCR Protocol	Initial	Blocking			No Blocking	
Temperature	95 °C	95 °C	74 °C	60.5 °C	95 °C	60.5 °C
Time	2:00	0:15	2:00	0:30	0:15	0:30
Cycles	1	25			25	

Discussion

Bicycle PCR™ is a simple, highly sensitive and cost effective molecular diagnostic platform. Today, technical noise prevents digital PCR, qPCR, Sanger sequencing, and NGS from accurately detecting a single copy of mutant DNA without significant workarounds and cost. Quest states, “Sensitivity can also be increased by tracking more mutations-to a certain extent. A caveat is that as more mutations are tracked, more technical noise is naturally introduced in the form of sequencing errors. With increased noise, the MRD calling threshold must be set higher, decreasing sensitivity”[10]. Bicycle PCR™ opens up a new frontier in sensitivity by eliminating key sources of technical noise.

Bicycle PCR™ process removes ambiguity from the detection step; instead of sequencing an amplicon at extreme depths to find extremely rare or single copy targets, Bicycle PCR™ preferably amplifies target variants to frequencies far above the sequencing error rate. This same principle applies to TaqMan and other qPCR-based detection, enrichment makes the technical noise from probes or dyes irrelevant, enabling detection of a single DNA target.

Previous blocking PCR strategies were able to preferentially amplify the rare targets, but they lacked key process controls. The fundamental issue of a negative sample being indistinguishable from a process failure required the addition of burdensome process controls to ensure reliability. Bicycle PCR™ maintains or exceeds the sensitivity of these earlier approaches while maintaining amplification of WT (non-target) DNA to serve as a robust process control. Bicycle PCR™ unlocks the power of blocking PCR by only blocking the amplification of WT DNA until the target variant becomes the dominant amplification product.

Previously, sensitivity limitations prevented some approaches from being used for high sensitivity molecular diagnostics. The incorporation of Bicycle PCR™ to current workflows gives existing platforms democratizes access to high sensitivity applications, creating new opportunities.

Bicycle PCR™ is cost-effective compared to current high sensitivity MRD assays. Natera is a market leader in MRD, with their Signatera test. Signatera using 16 different targets, with an average coverage depth of 100,000 reads. Signatera, at 0.005% VAF has a per sample sensitivity of 61%[2]. Bicycle PCR™, at 0.005% VAF has a sensitivity of 73% for KRAS G12C and 78% for BRAF V600E. Using only qPCR for a single target, Bicycle PCR™ can deliver comparable sensitivity and specificity as Signatera at a fraction of the price. Bicycle PCR™ enables faster and more cost-effective testing for MRD.

Bicycle PCR™ is a scalable solution for cancer molecular diagnostics. It can be incorporated into NGS workflows (Figure 1) or qPCR workflows (Figure 3) by adding a blocking oligonucleotide and altering the thermocycling parameters. Additionally, Bicycle PCR™ does not use allele specific primers, meaning that multiple distinct mutations can be detected within the same amplicon using the same blocker. Bicycle PCR™ can be multiplexed different amplicons in a similar manner to regular PCR reactions and does not require any custom instruments or analysis software.

Bicycle PCR™ opens up new possibilities, such as a liquid biopsy to screen for pancreatic cancer. 90% of pancreatic cancers have a KRAS mutation[11]. Foundation Medicine estimates that 51,300 out of the 60,000 people diagnosed annually in the US with pancreatic cancer will have a KRAS mutation[12]. CancerSEEK demonstrated 100%

concordance between plasma and tumor for pancreatic cancer and detected cancer as early as Stage 1 in patients with a VAF as low as 0.01%[13].

There exists a major need for a highly sensitive pancreatic cancer screening test. The 5-year pancreatic cancer survival rate is only 9%, because most pancreatic cancer is diagnosed at a late-stage[14]. The 5-year survival rate for pancreatic cancer diagnosed at Stage 1 is 83.7%, but only 1.9% of cases are diagnosed this early[15]. Bicycle PCR™ could potentially be used as a screen for pancreatic cancer by delivering highly accurate and cost-effective test for KRAS and other driver mutations.

Conclusion

The innovation of Bicycle PCR™ enables low-cost, high accuracy detection of single copy cancer variants. Bicycle PCR™ will greatly reduce the cost of high sensitivity cancer tests. The simplicity of Bicycle PCR™, using existing workflows and instruments, is what makes it scalable. Together these reasons are why Bicycle PCR™ is a breakthrough in cancer diagnostics.

Non-Endorsement Statement

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