Supplementary Information

Beacon-mediated Exponential Amplification Reaction (BEAR) Using a Single Enzyme and Primer

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1. Experimental Procedures

Preparation of DNA Oligonucleotides
Sequences of the hairpin probe (**HP**) and fluorophore-conjugated stand (**FS**) are listed in Table S1. All DNA oligonucleotides (oligos) were synthesized and purified by Integrated DNA Technologies (IDT, Coralville, IA). Oligos **HP** and **FS** were resuspended to 30 µM using 20 mM TrisHCl pH 7.4. Target oligos (MERRF target, negative control, and mismatches) and primers were resuspended to 100 µM using 20 mM TrisHCl (pH 7.4), and Milli-Q ultrapure water. The stock oligonucleotides were stored at -18°C.

Preparation of the amplifiable beacon
Solutions of **HP** and **FS** mixtures to anneal the amplifiable beacon were prepared at room temperature. The solutions contained 5 µM **HP**, varying concentrations of **FS** depending on the desired **HP**:**FS** ratio, 250 mM $MgCl_2$, 20 mM TrisHCl (pH 7.4), and Milli-Q ultrapure water. Solutions were slowly cooled in a benchtop thermocycler (Biorad, USA) from 90 °C to 20 °C in 2 hr. Annealed amplifiable beacon solutions were stored at 4 °C for up to two weeks.

Procedures of Beacon-mediated Exponential Amplification Reaction (BEAR) and fluorescence detection
Reaction solutions were prepared at room temperature in a master mix and typically contained 100 nM of annealed amplifiable beacon solution, 0.1 U/µL Bst 2.0 DNA Polymerase (New England Biolabs, Whitby, ON, Canada), 500 nM primer, 200 µM Deoxynucleotide Solution Mix (dNTP) (New England Biolabs, Whitby, ON, Canada), 1X ROX Reference Dye (Thermofisher Scientific, Canada), 1X Isothermal Buffer that contained 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, and 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20 (pH 8.8) (New England Biolabs, Whitby, ON, Canada), and Milli-Q ultrapure water. Reaction solutions were added to 2 µL of either MERRF target sequence, negative control, mismatch target or Milli-Q ultrapure water for a total volume of 20 µL per reaction and incubated at 40 °C in StepOnePlus Real-Time PCR System (Thermofisher Scientific, Canada) for isothermal fluorescence detection every 2 min for a total reaction run time of 80 min. Fluorescence intensity was normalized against a reference dye (Normalized Fluorescence), ROX. Normalized fluorescence was calculated by dividing the fluorescence emission of the reporter probe, **FS**, by that of the reference dye, ROX Reference Dye. Thresholds were set by the determination of a baseline by measuring the initial fluorescence in all samples prior to detection of an increase in fluorescence and multiplying the average value by 10 standard deviations.

MCF-7 Cell Culture and Preparation of Cell Lysate

MCF-7 adherent epithelial cells were cultured in 50 mL T-25 vented flasks treated for cell cultures. The DMEM medium was supplemented with 10% FBS and 1% of penicillin/streptomycin. Cells were grown in 5% CO_2 at 37 °C with 90% humidity. Cells were sub-cultured passaging every 3-4 days at 85-90% confluency using 0.25% (w/v) trypsin-EDTA to detach the cells. Detached cells were centrifuged, resuspended and added into new flasks with fresh, pre-warmed media.

Cells were detached from flasks, counted, and diluted according to the protocols by Osborn et al.[1] where 80 µL of Tris-HCl (pH 7.4) was added for every 10^7 cells. The cell suspension was sonicated to lyse cells and checked for complete lysis visually under microscope. The suspension was centrifuged for 14 min at 13000 rpm (16200xg rcf) to remove cellular debris, and the supernatant was saved. The supernatant was heated to 65°C for 10 min to inactivate DNAses. The resulting solution was stored in 4 °C and used as cell lysate in subsequent experiments (Section 10, ESI).

2. Specific design parameters

Table S1. Sequences of oligonucleotides.

Name of Oligonucleotide	Sequence of Oligonucleotide (5'-3')
MERRF Target	GTT AAA GAT TAA GAG AGC CAA CAC CAA A[2]
Negative Control	GTT AAA GAT TAA GAG AAC CAA CAC CAA A[2]
HP	Dark Quencher -TGT GTG TCG TGC GCG TTA AAG GGT GT TGG CTC TCG GAC GCG TTA AAG GGT GTT GGC TCT CTT AAT CTT TAA CGC GTC CGT GAC TTT T
FS	AGA GCC AAC ACC CTT TAA CGC GCA CGA CAC ACA - FAM

Careful design of our oligonucleotides is important for the correct formation of the amplifiable beacon and the efficiency of the technique. This section will outline important considerations for designing **FS** and **HP**. Scheme S1 shows the sequences of **HP** and **FS** forming the amplifiable beacon separated into domains.

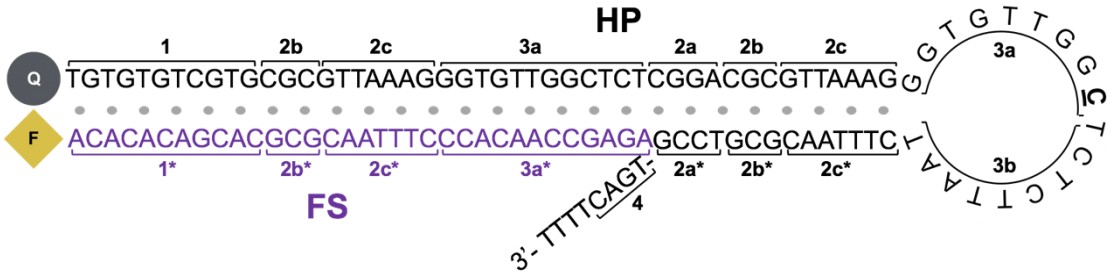
FS is responsible for enabling exponential amplification of the detection signal. In **BEAR**, one amplifiable beacon releases two reaction initiators, the displaced target and the displaced **FS**. Therefore, **FS** must be designed to bind to both the overhang of **HP** to form the amplifiable beacon and the loop and stem domains to initiate **BEAR**. As a result, **FS** contains complementary regions to both the overhang of **HP** and the loop and stem region of **HP**.

To drive **FS** to preferentially bind to the overhang instead of the loop and stem for the correct formation of the amplifiable beacon, we added domain **1** on the overhang (and **1*** on **FS**) to increase the melting temperature of the duplex forming the stable amplifiable beacon. As domain **1** is not dependent on target sequence, the sequence and length can be adjusted to tune the desired melting temperature of the duplex formed between **FS** and the overhang of **HP**. We designed the complementary domains of **1**/**1*** to increase the melting temperature of the **FS**:**HP** duplex by about 25 °C versus the loop toehold, **3a**/**3a***.

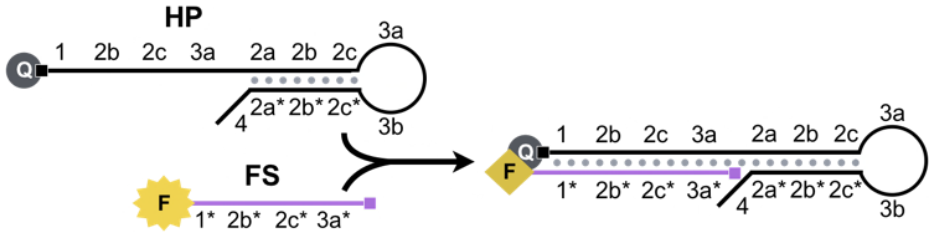
The stem of **HP** must also be designed to balance the speed of the reaction while ensuring amplifiable beacons do not open in the absence of the target. Increasing the length of the stem and increasing GC content will increase the likelihood that the stem does not open in the absence of any reaction initiators. However, too long of a stem and too high of GC content may inhibit or slow the reaction. We designed the stem to be 14-bp with 57% GC content.

HP has a 3' overhang of four T residues. The function of this overhang is to prevent polymerase extension of the stem using the 5' overhang as a template. The 3' overhang also provides additional nucleotides upstream of the primer for polymerase binding.

Another important design consideration is the location of the 25-nt MERRF target when bound to the loop and stem of **HP**. We aimed to minimize the ability of the negative control sequence, the clinically healthy sequence, to open the stem. The complementary sequence to capture the MERRF target was oriented such that when bound to the amplifiable beacon, the location of the point mutation was placed in the centre of the loop with 8-nt and 9-nt flanking either side of the point mutation location. Further discussion is provided in Section 6 of the Supporting Information, Target Mismatch Placement. Additionally, we designed the target to have a 3-nt long 3' unhybridized region when bound to its complementary domains on the loop and stem of the amplifiable beacon. The function of this unhybridized region on the target is to inhibit the polymerase from extending the target as if it were a primer.



Scheme S1. The sequence and domains of the amplifiable beacon. The amplifiable beacon consists of HP in a hairpin conformation with FS bound to its overhang. The 5' end of HP is conjugated to a quencher and the 3' end of FS is conjugated to a fluorophore. The close proximity of the fluorophore to the quencher quenches its fluorescence. The complementary base of the MERRF target point mutation in the loop is bolded and underlined.



Scheme S2. The amplifiable beacon is formed by annealing **HP** and **FS** together. **HP** folds into a hairpin with a long single-stranded overhang and is conjugated on its 5' end to a quencher. **FS** is complementary to the **HP** overhang and is conjugated to a fluorophore on its 3' end. The proximity of the quencher to the fluorophore in the amplifiable beacon results in quenched fluorescence. The small square at the end of the DNA sequences indicates the 5'-end.

3. Amplifiable Beacon: Ratios of HP to FS

The amplifiable beacon was prepared by mixing 5 µM of **HP** and various amounts of **FS** in a single tube with annealing solution and heat denatured at 95°C for 5 min, followed by slow cooling to room temperature in 2 hr. Denaturation of **HP** and **FS** converts any dsDNA secondary structures into ssDNA. Slow cooling the solution to room temperature results in **HP** folding into a hairpin first, followed by **FS** annealing to the 5' overhang of **HP**. **FS** preferentially binds to the overhang of **HP** instead of the loop and stem of **HP** due to the greater duplex stability attributed to the additional **1/1*** domain interaction. Although it is possible that **HP** can dimerize because of the nature of the hairpin stem, the domains that make up the stem are too short (14-bp) to withstand the initial denaturation and the formation of the more favourable intrastrand hairpin conformation will precede over **HP** interstrand dimerization.

The ratio of **HP** to **FS** is important when annealing the amplifiable beacon. In ratios with an excess of **HP** to **FS**, the resulting solution contains amplifiable beacon and **HP** in a hairpin conformation (Fig S1). In ratios with an excess of **FS** to **HP**, the resulting solution contains amplifiable beacons bound to **FS**. High amounts of **FS** in annealing mixes may lead to the formation of amplifiable beacons with an additional **FS** bound to the loop and/or stem. This is undesirable because the additional **FS** bound to amplifiable beacons can initiate **BEAR** in the absence of the target, thereby increasing the background signal. Our strategy to prevent background due to **FS** binding to amplifiable beacons in the annealing process is to use annealing solutions where **HP** is always in excess of **FS**. Adjusting the ratios of [**HP**] to [**FS**] can control the correct formation of the amplifiable beacon and can be further analyzed by reaction with the target and blank in **BEAR**.

Figure S1 shows the effect on **BEAR** when using amplifiable beacon annealed from various ratios of [**HP**] to [**FS**]. Nominal ratios 1:0.85 and 1:0.83 are presumed to have *de facto* excess **FS** relative to **HP**, as both of these ratios show an increase in both the reaction and blank signal, where the target-initiated reaction and blank signal become indistinguishable. On the other hand, if **HP** is in excess, there would be some free **HP** with its overhang not hybridized to **FS**. Because the complementarity of **FS** to the 5' overhang is more favourable than that of the loop and stem of **HP**, it is likely that the displaced **FS** from the target-initiated reaction will preferentially bind to the free 5' overhang on **HP** instead of the loop, which halts the propagation of the reaction. We suspect that the lag time prior to the exponential amplification for each of the curves may partially be due to displaced **FS** binding to the overhang of free **HP** in solution, rather than the loops and stems of **HP** within amplifiable beacons, resulting in linear amplification. This prediction is supported by the results obtained with excess of **HP** over **FS**. With an **HP**:**FS** ratio of 1:0.75, the high **HP** concentration relative to **FS** does not produce detectable exponential amplification for the duration of the 80 min. The annealing solution corresponding to a nominal ratio of 1:0.80 of **HP** to **FS** maximized the amount of the correctly formed amplifiable beacon without increasing the blank signal substantially and was used for subsequent reactions.

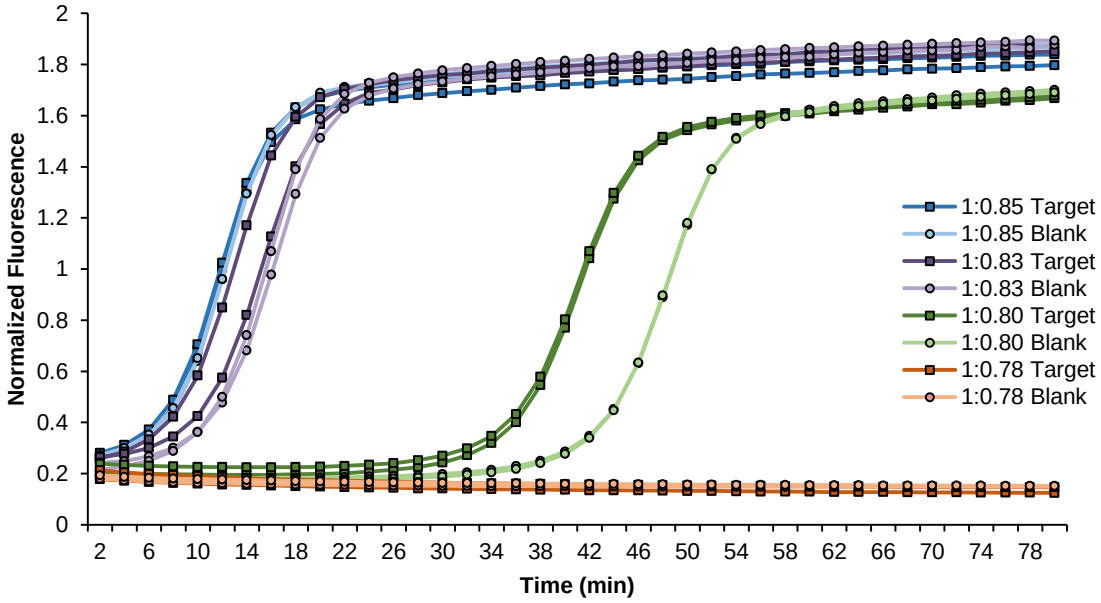


Fig. S1 Amplification curves showing the effect of varying the nominal amount of **FS** (0.85, 0.83, 0.80, 0.78) relative to **HP** in amplifiable beacon annealing solutions. Target refers to 100 pM MERRF target sequence, and blank refers to all reagents in the **BEAR** mixture without the MERRF target.

4. Reaction Progression

The typical amplification curve of **BEAR**, shown in Figure S2, can be split into three phases. The phases include lag (0 - 26 min), exponential (26 min - 54 min) and plateau (>54 min). The lag phase consists of an abundance of available amplifiable beacons. The signal output generated in the lag phase is below the detection limits. The exponential phase consists of many displaced target and **FS** that are continually reacting with other amplifiable beacons to produce detectable fluorescence signals and more displaced **FS**. The plateau phase is a result of the exhaustion of amplifiable beacons. In the plateau, all amplifiable beacons are converted to waste. Because there are no available amplifiable beacons for **FS** to react with, **FS** is free in solution.

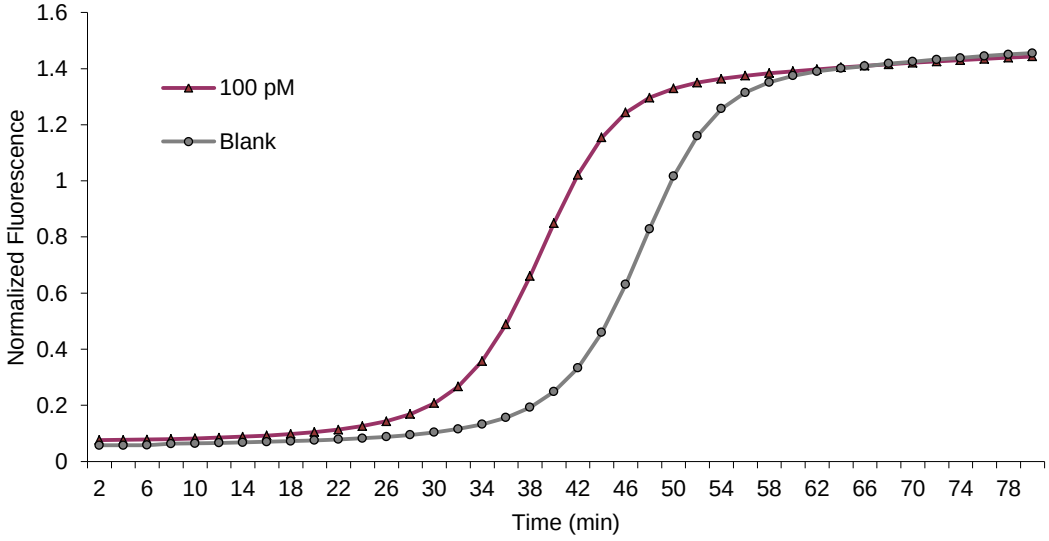


Fig. S2 Typical signal amplification curves of BEAR, showing the progression of fluorescence from reactions with either 100 pM of MERRF target or water as blank. The sigmoidal shape of the curve is characteristic of exponential amplification. Fluorescence was normalized against a reporter dye and monitored over time in minutes.

Figure S3 shows **BEAR** amplification products at various time points separated by PAGE. At 0 min, the only apparent band corresponds to the annealed amplifiable beacon structure (iii). This band disappears as the reaction proceeds and yields no fluorescence. *Waste* (ii), a higher molecular weight than the amplifiable beacon, increases throughout the reaction and does not fluoresce. Both displaced **FS** and **FS** bound to the loop/stem of **HP** within amplifiable beacons retain fluorescence. Thus, the fluorescing product at 46-48 min corresponds to the high molecular weight complex of amplifiable beacons bound to **FS**. Amplifiable beacons bound to **FS** appears in the exponential phase but disappears as the reaction proceeds. The appearance and

disappearance of this transient structure is consistent with our scheme. Low molecular weight, free **FS** (iv), can be seen fluorescing from 36-48 min when there the amount of *waste* is greater than that of amplifiable beacons.

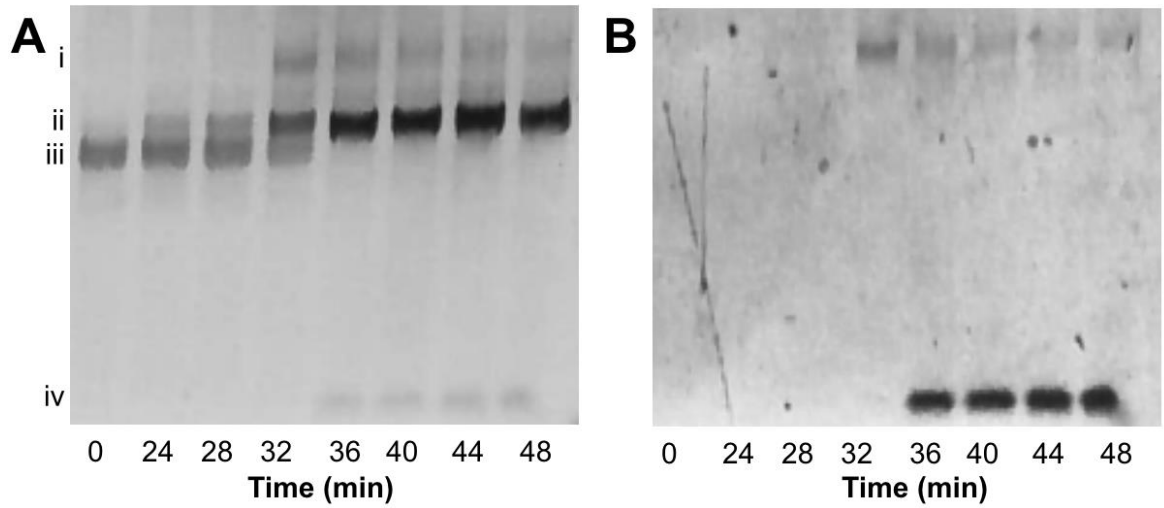


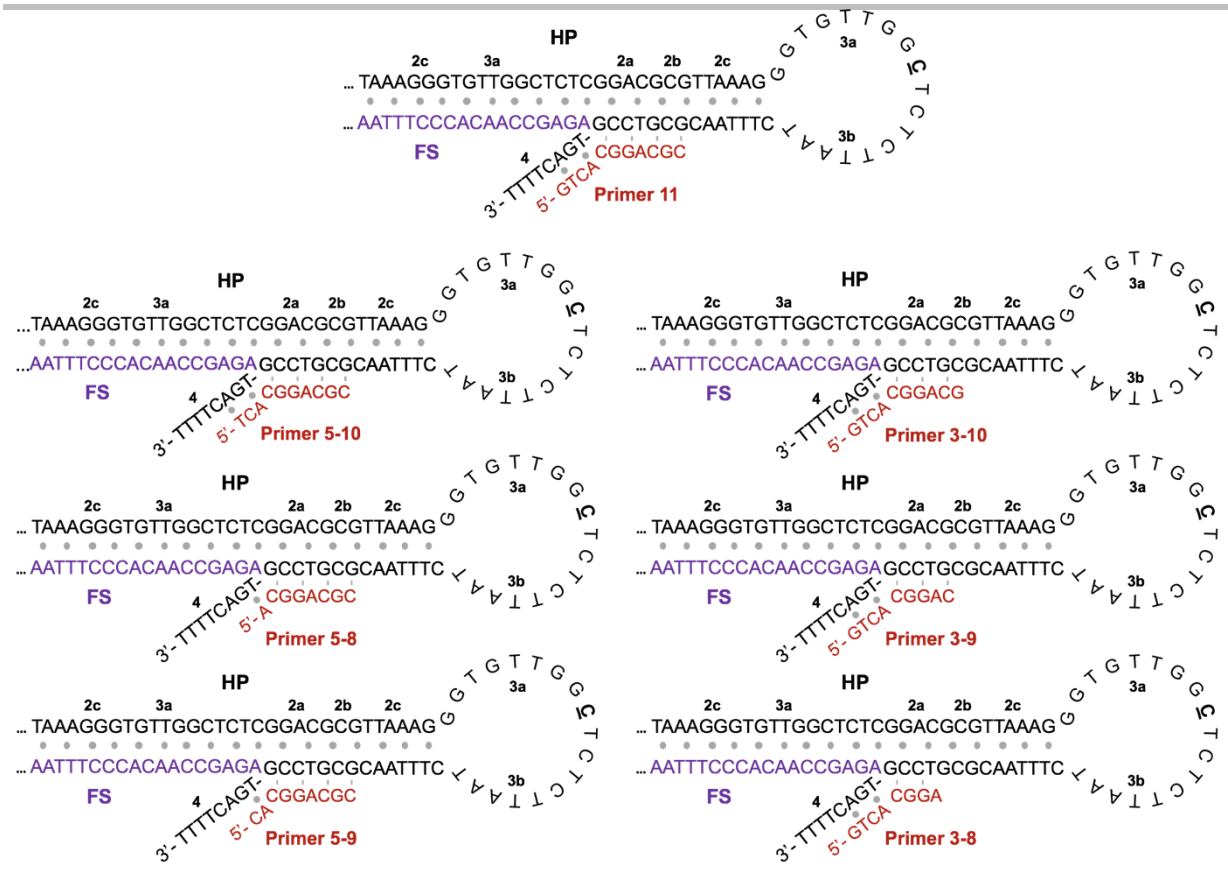
Fig. S3 BEAR was stopped at various time points with chilled EDTA. The reaction components at each time point are separated on a 10% Native PAGE and imaged after (A) SYBR gold staining and using the detection of (B) FAM fluorescein.

5. Primers

Various primer lengths and primer binding positions were tested. Two sets of primers were designed. In one set, the sequence of the primer on the 5' end was fixed and changed the length of the domain complementary to the stem of **HP**. This set is referred to as "Primer 5-n", where n is the number of nucleotides making up the primer. In the second set of primers, the sequence of the primer on the 3' end was fixed and the length of the domain complementary to **4** on **HP** was changed. This set is referred to as Primer "3-n". The names and sequences of each primer used are shown in Table S2, and the binding positions of each primer are shown in Scheme S3. Primer 11 refers to the full length primer. The effect of each primer on **BEAR** is shown in Figures S4A and S4B and are discussed in the main text.

Table S2. Primer sequences with the original, 9 nt primer, and variations in length and position.

Name of Oligonucleotide	Sequence of Oligonucleotide (5'-3')
Primer 11	GTCACGGACGC
Primer 5-10	TCACGGACGC
Primer 5-9	CACGGACGC
Primer 5-8	ACGGACGC
Primer 5-7	CGGACGC
Primer 3-10	GTCACGGACG
Primer 3-9	GTCACGGAC
Primer 3-8	GTCACGGA



Scheme S3. The binding locations of the 11-nt primer (Primer 11) and each truncated primer (Primer 5-n and Primer 3-n) to **HP** within amplifiable beacons. Stem opening via either the target or **FS** is not shown. The location of the base in the loop of **HP** that is complementary to the point mutation in the MERRF target is bolded and underlined.

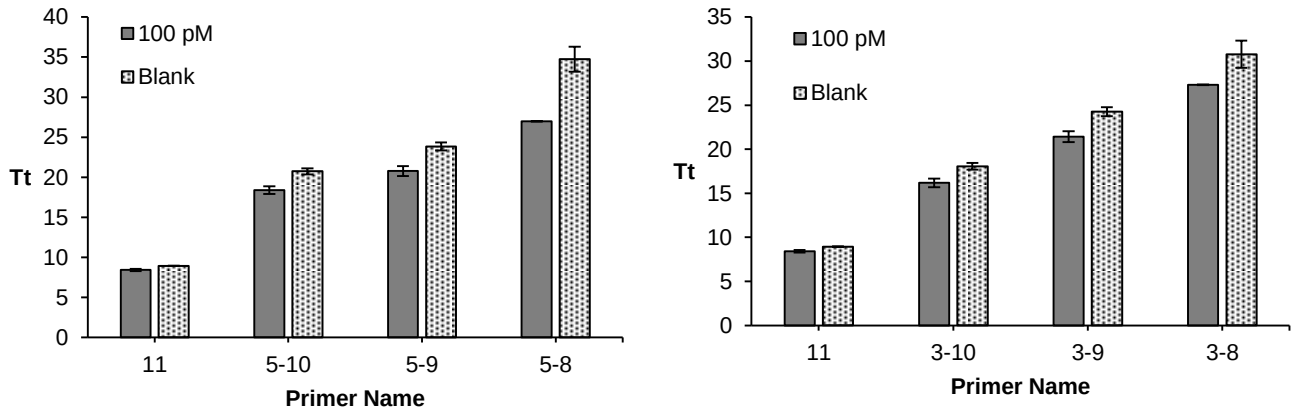


Fig. S4 The effect on **BEAR** when using 100 pM of MERRF target (100 pM) or water (Blank) when truncating the primer from 11 nt to 10 nt, 9 nt, 8 nt from the (**A**) 5' end (Primer 5-n) or (**B**) 3' end (Primer 3-n). Primer 5-7 yielded no detectable signal in the experimental timeframe.

6. Effect of varying conditions

The rate of the **BEAR** can be increased or decreased by varying the temperature and concentration of polymerase used. Figures S5 and S6 show that although the reaction is increased at higher temperatures or increased concentrations of polymerase, respectively, the background is also increased. Reaction conditions of a temperature of 40 °C with 0.1 U/L of Bst 2.0 polymerase for resulted in the most optimal target to blank signal ratio in <1 hr.

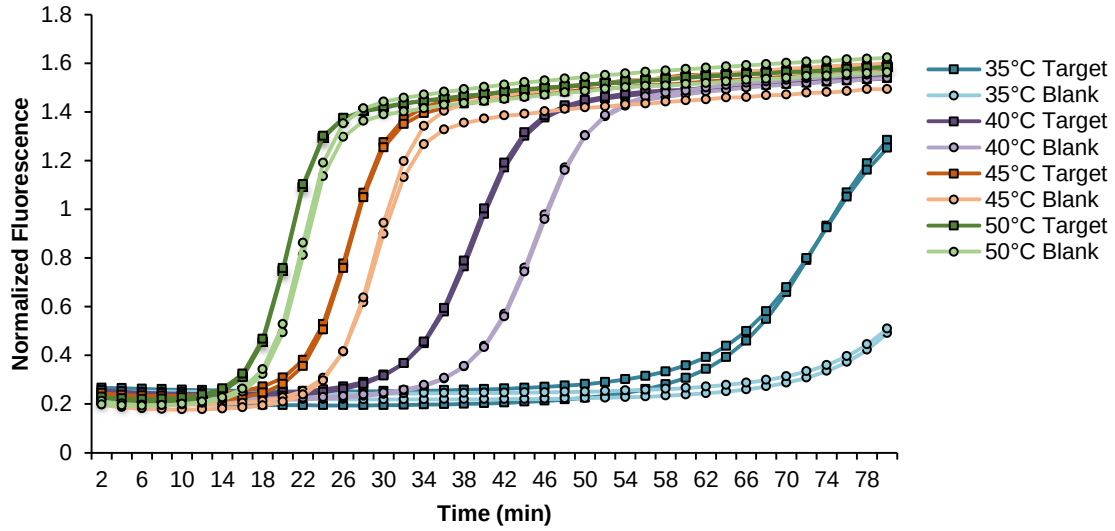


Fig. S5 Amplification curves showing the effect of temperature (35, 40, 45, 50°C) on **BEAR** with 100 pM of MERRF target and blank.

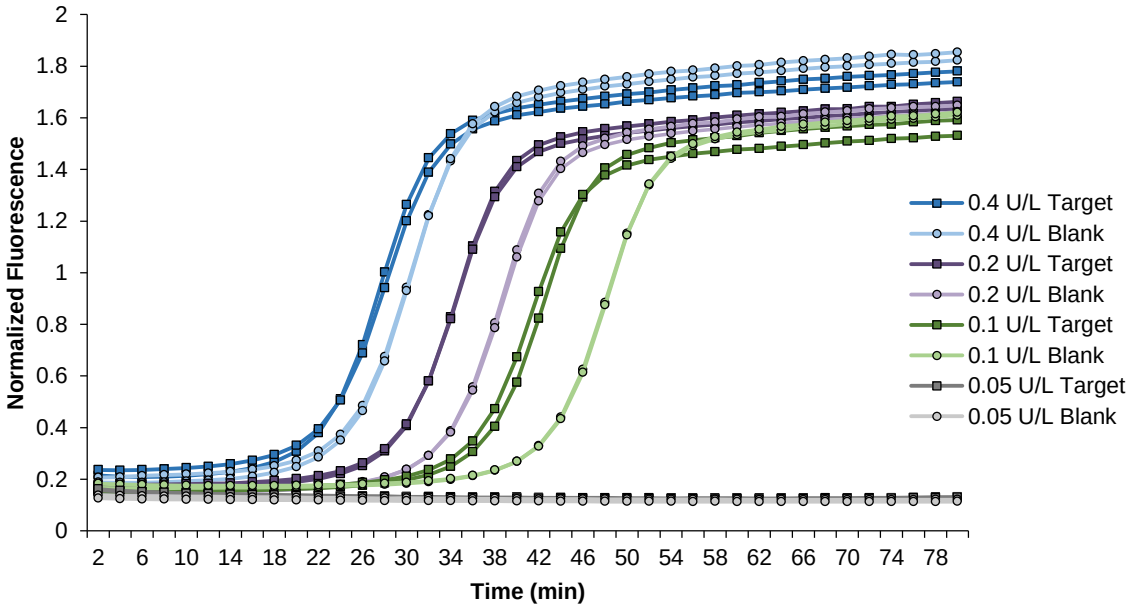


Fig. S6 Amplification curves showing the effect of varying Bst 2.0 polymerase concentrations (0.4, 0.2, 0.1, 0.05 U/L) on **BEAR** with 100 pM of MERRF target and blank.

7. Dynamic Curve: Low Concentrations

The dynamic range of **BEAR** was tested using various concentrations of the MERRF target (Figure 2). The curves of the low concentrations (10 pM to 1 fM) are shown in Figure S7 from 32 to 56 min.

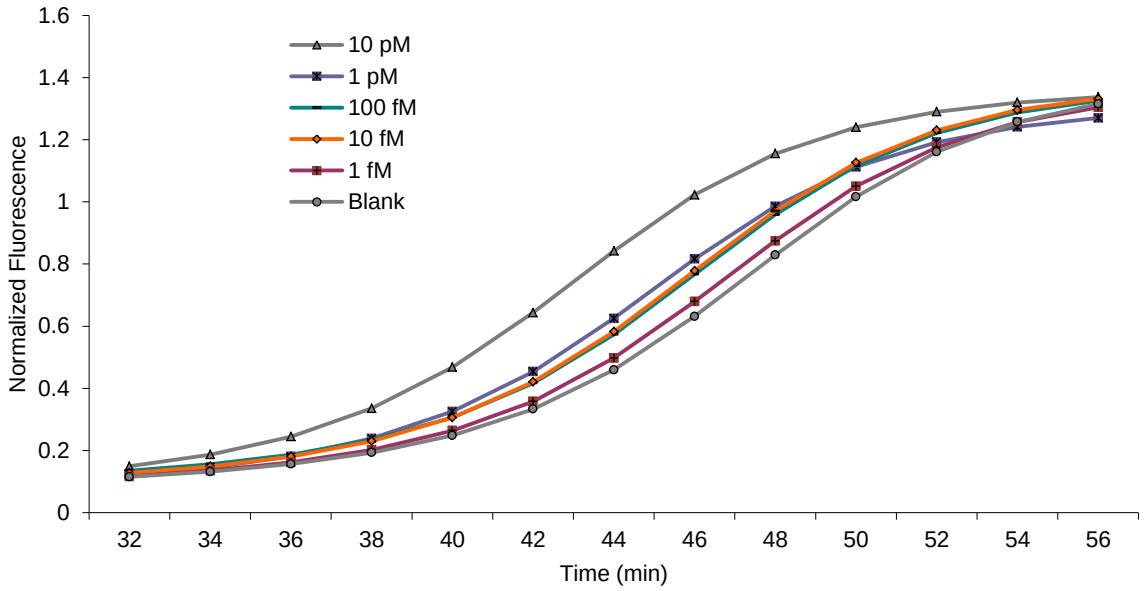


Fig. S7 Amplification curves of concentrations from reactions containing low concentrations MERRF target, ranging from 10 pM to 1 fM. Reactions times from 32 min to 56 min were shown. The 8-nt primer was used.

8. Sources of Background

Similar to other strategies with exponential amplification, any small background can be exponentially amplified as well. We considered the various sources of background and attempted to minimize them in our optimization processes. The background may come from two main sources: (1) malformation of the **HP** stem and (2) **FS** bound to the loop of amplifiable beacons. Malformation of the **HP** stem may arise in events such as base mismatch or DNA synthesis errors. We have attempted to reduce the contribution of background from stem malformation through careful design of the stem sequence and through denaturation followed by the 2 hr slow cooling process to create the amplifiable beacon. We also chose the primer length and position combination that produced the best signal to background ratio.

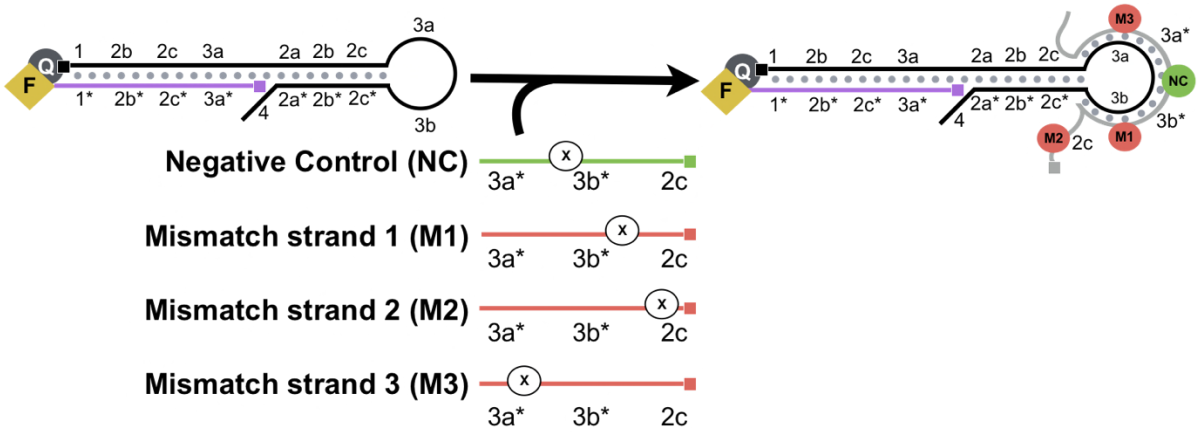
Secondly, **FS** bound to the loop of amplifiable beacons can increase background. In the design of **HP** (Section 2, ESI), inclusion of Domain 1 helps to drive the binding of **FS** to the overhang of **HP** to form the amplifiable beacon. When forming the amplifiable beacon, **FS** is mixed with an excess amount of **HP**. However, it is possible that even if **HP** is in excess, some **FS** may bind the loop and stem of **HP** in amplifiable beacons, which initiates the reaction and increases the background. We have minimized the contribution to background from this source by optimizing the ratio of **HP** to **FS** when annealing the amplifiable beacon prior to target analysis.

9. Target Mismatch Placement

Our **BEAR** technique for the detection of the 25-nt nucleic acid sequence corresponding to the 8344A>G point mutation resulting in MERRF situates the location of the point mutation in the centre of the loop of **HP** when the target is bound and therefore, the clinical negative control (Negative Control) contains a mismatch at this location. The location of the mismatch can affect the specificity of the reaction by its ability to open the stem after binding to the loop. To test the optimal location of the point mutation, we tested our MERRF target, Negative Control and three mismatches located on either side of the loop, and on the stem nearest to the 3' end of **HP**. To do this, we designed three arbitrary mismatches (Mismatch 1, 2, and 3) in the MERRF target sequence. The sequences for the MERRF target, negative control, and arbitrary mismatches are listed in Table S3 and the locations when bound to the amplifiable beacon are shown in Scheme S4.

Table S3. Sequences of the MERRF target A8344G mutation in comparison to the clinical negative control (wildtype) and other mismatches tested to determine the optimal complementary region on **HP** for the position of the point mutation. Location of MERRF point mutation is underlined. Arbitrary mismatches created are bolded.

Name of Oligonucleotide	Mismatch Position Relative to HP	Sequence of Oligonucleotide (5'-3')
MERRF Target	Centre of loop	GTTAAAGATTAAGAGAGCCAACACCAAA
Negative Control	Centre of loop	GTTAAAGATTAAGAGAACCAACACCAAA
Mismatch 1	On loop near **HP** 3' end	GTTAAAGAT**G**AAGAGAGCCAACACCAAA
Mismatch 2	On stem near **HP** 3' end	GTT**G**AAGATTAAGAGAGCCAACACCAAA
Mismatch 3	On loop near **HP** 5' end	GTTAAAGATTAAGAGAGCCAA**G**ACCAAA



Scheme S4. Locations of each mismatch when the clinical negative control (NC) and arbitrary mismatch strands to simulate the effect of mismatches at each location, Mismatch 1 (M1), Mismatch 2 (M2), and Mismatch 3 (M3), are bound to **HP**. The (x) positioned on each sequence indicates the general location of the mismatch within the strand.

The ΔTt resulting from 100 pM of MERRF target, Negative Control, and the three mismatches (Mismatch 1-3) are shown in Figure S8. A large ΔTt for the MERRF target, and low ΔTt for the Negative Control is desirable for specificity. Aside from the Negative Control, Mismatch 1 produced the largest ΔTt. This mismatch was placed on the loop adjacent to the stem binding domain, **2c**. Mismatch 2, which places the mismatch within **2c**, yielded a similar ΔTt to the MERRF target. Because this mismatch has the complete loop toehold complementary sequence, it is able to bind to the loop as does the MERRF target, yielding poor specificity. Mismatch 3 also produced ΔTt similar to that of the MERRF target. When comparing Mismatch 1 and 3, Mismatch 1 was more effective in producing better specificity than that of Mismatch 3, indicating that the position of the mismatch on the loop relative to the stem is an important factor in opening the stem. Mismatch 1 is located adjacent to the stem complementary domain **2c**, which may have inhibited stem opening. Thus, as Mismatch 3 was placed further away from **2c**, it allowed complete binding to the loop adjacent to the stem domain and as a result, stem opening for the initiation of the reaction. The Negative Control yielded the most optimal specificity as the point mutation was placed in the centre of the loop. This location reduced the ability for the Negative Control to effectively bind the loop toehold to initiate the reaction.

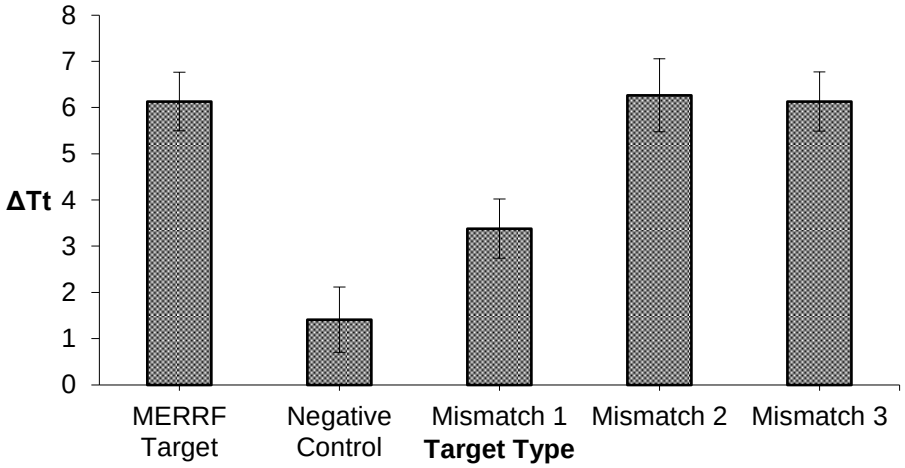


Fig. S8 The ΔTt (difference between target and blank time at threshold) of Mismatch locations, the MERRF clinical negative control, and the MERRF target. Reactions initiated with 100 pM of each respective oligonucleotide and performed in triplicates.

10. Detection of the MERRF Target in Cell Lysate

Because mitochondrial DNA mutations can occur in epithelial cells, we tested detection of the MERRF target DNA spiked in the lysate of MCF-7 human epithelial cells. A measured amount of MERRF target DNA was added to the lysate of 10^7 MCF-7 human epithelial cells so that the concentration of the MERRF target DNA was 10 pM. Representative amplification curves from the detection of 10 pM MERRF target DNA in the lysate of MCF-7 human epithelial cells and from the reagent blank are shown in Fig S9. Triplicate analyses of the cell lysate, using a calibration of the MERRF target DNA in buffer solutions, determined that the concentration of the MERRF DNA was 9.1 ± 0.8 pM. These results, representing an average recovery of 91%, are consistent with the expected concentration of 10 pM. No MERRF DNA was detectable in the cell lysate without the addition of the MERRF DNA.

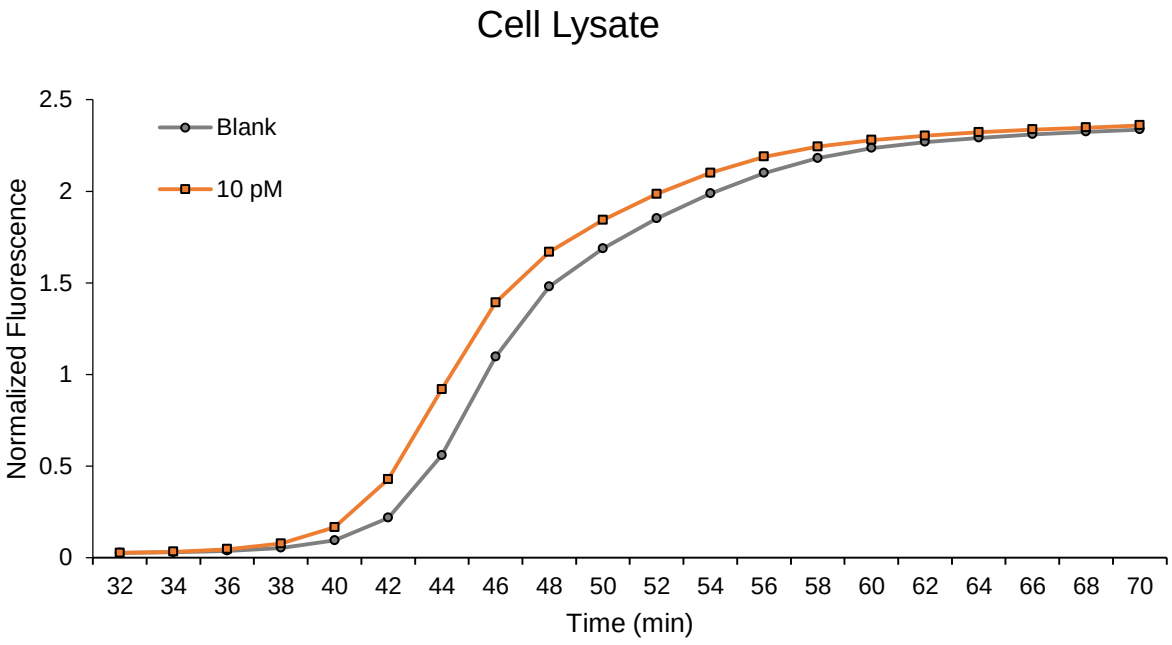


Fig. S9 Representative amplification curves of 10 pM of MERRF target in MCF-7 cell lysate and reagent blank from 32 min to 70 min.

11. References

[1] L. Osborn, S. Kunkel, G. J. Nabel, *Proc. Natl. Acad. Sci*. **2006**, *86*, 2336-2340.
[2] GenBank [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1982] - [cited 2019 May 01]. Available from: https://www.ncbi.nlm.nih.gov/nuccore/251831106