nature biomedical engineering

SUPPLEMENTARY INFORMATION

DOI: 10.1038/s41551-017-0126-5

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Multiplexed enrichment of rare DNA variants via sequence-selective and temperature-robust amplification

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Multiplexed Enrichment of Rare DNA Variants via Sequence-Selective and Temperature-Robust Amplification

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Section S1: BDA Mechanism and Design Principles

Fig. S1-1a shows the process of Primer displacing the Blocker off Variant templates (repeated from Fig. 1 in the main text). This reaction, however, is only one of many reactions believed to actually occur during the BDA enrichment process; Fig. S1-1b shows a more complete set of reactions that we used for modeling BDA (Section S2). When the reaction cools from the 95°C denaturing step to the 60°C anneal/extend step, the Variant template V will initially bind to either the Primer P or the Blocker B, with rate constant k_f that we believe to be similar for P and B. Consequently, the ratio of PV and BV initially formed during the anneal step will roughly correspond to that of the concentrations of P and B. At the anneal step temperature, the hybridization of both P and B to V in isolation are designed to be strong enough that the reverse reactions PV $\rightarrow$ P + V and BV $\rightarrow$ B + V are assumed to have negligible rate constants, and hence are not modeled.

After the formation of BV, P can still bind via its unique region of complementarity to the template (called the *toehold*), with a rate constant of k_{pd}, to form three-stranded complex PBV, which then can reversibly rearrange via branch migration to PBV*. Neither PBV nor PBV* are thermodynamically stable: the former will dissociate quickly into P + BV, and the latter into B + PV. PV can bind to B via the latter unique region of complementarity (the variant enrichment region, also known as the *reverse toehold*), and through a similar 3-stranded intermediate result in the formation of BV + P.

There are two mechanisms for P to be extended by the polymerase into amplicon A. The DNA polymerase can bind and extend PV with rate constant k_e; here we assume that this is a unimolecular constant because we are not changing the enzyme concentration, and believe based on literature that for short amplicons, the initial binding is the rate limiting step. Alternatively, the DNA polymerase can bind and extend PBV* using 5′ to 3′ exonuclease activity; this rate constant k_{e2} is likely different in value from k_e both because the extra bulk of B may sterically hinder the initial binding of the polymerase, and because the polymerase activity may be rate limited by exonuclease activity.

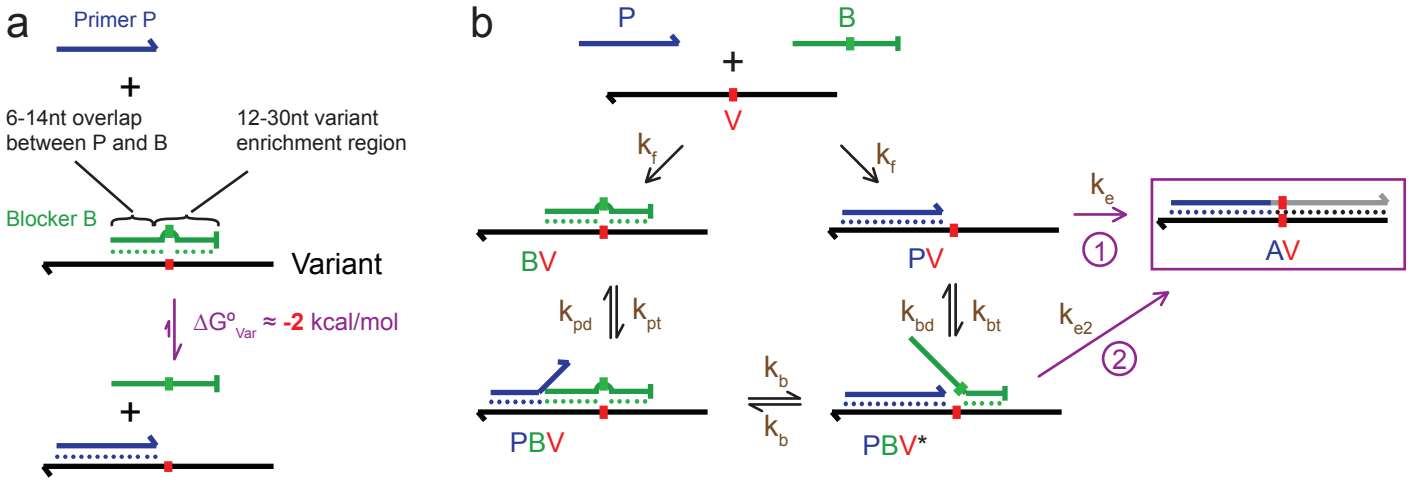


FIG. S1-1: BDA reactions. **(a)** Simplified reaction of P displacing B on a Variant template to induce high-yield amplification. **(b)** Complete reaction set modeled for each anneal/extend PCR step.

Toehold exchange. BDA achieves sequence-selective PCR enrichment of variants because the non-allele-specific Primer more favorably displaces the Blocker from Variant templates/amplicons than from Wildtype templates/amplicons. This displacement process is known as *toehold exchange* [1, 2]. In brief, toehold exchange is a competitive hybridization process in which a single-stranded primer binds to a template already hybridized to a blocker (Fig. S1-2).

The thermodynamics and kinetics of the toehold exchange reaction are primarily determined by the length and strength of the toehold and reverse toehold. The standard free energy of toehold formation $\Delta G^\circ_{\text{toe}}$ can be calculated *in silico* based on published nearest-neighbor DNA thermodynamics parameters [3], and largely determines the kinetics of the toehold exchange process (Fig. S1-2b) [1]. The standard free energy of the reaction $\Delta G^\circ_{\text{rxn}}$ can be approximated as $(\Delta G^\circ_{\text{toe}} - \Delta G^\circ_{\text{rtoe}})$, and determines the equilibrium hybridization yield of the primer (Fig. S1-2c) [2].

To achieve high sequence specificity within the timeframe of a 30 s PCR anneal/extend step, we designed P and B such that ΔG°_{toe} and ΔG°_{rtoe} are roughly -8 kcal/mol. From preliminary experiments, we noticed that

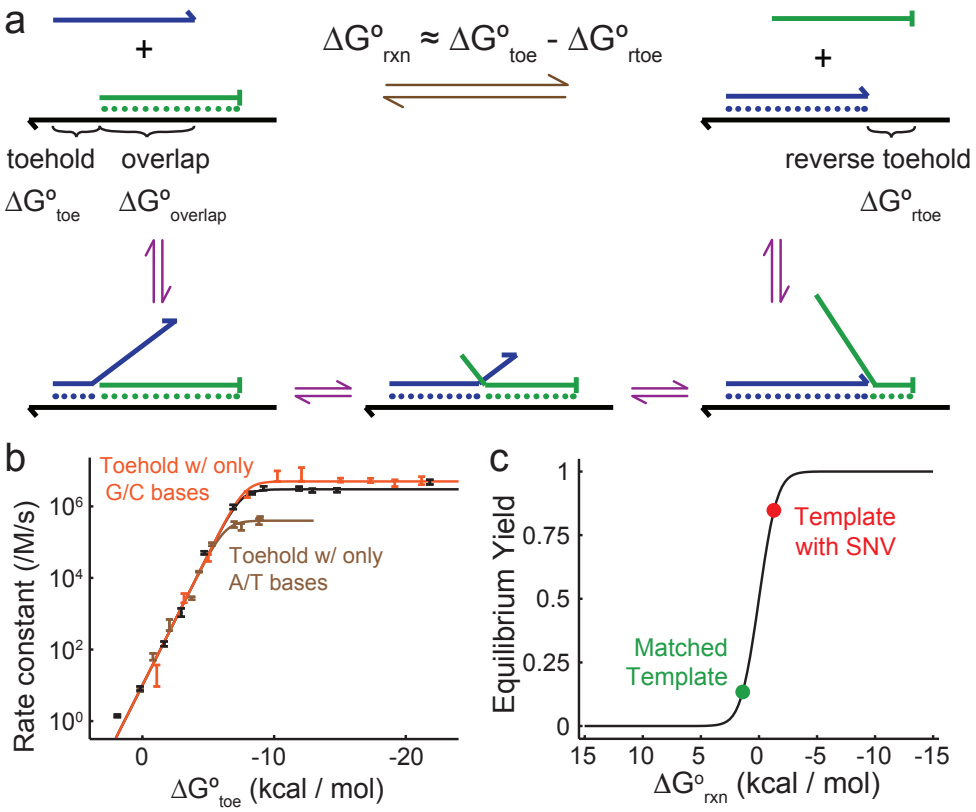


FIG. S1-2: Competitive hybridization via toehold exchange. **(a)** Mechanism by which the primer displaces the blocker. The reaction is initiated by the hybridization of the toehold, and ends with the dissociation of the reverse toehold. The reverse reaction can also proceed rapidly for properly designed reactions, with the blocker displacing the primer. **(b)** For properly designed toehold exchange exchanges, kinetics are rate-limited by the second-order process of toehold hybridization, rather than the first order rate constants of branch migration or reverse toehold dissociation. Regardless of the nucleotide composition of the toehold, the second order kinetic rate constant follows a log-linear dependence on toehold binding standard free energy $\Delta G^\circ_{\text{toe}}$ [1]. **(c)** Equilibrium hybridization yield of the primer to the template, as a function of the reaction standard free energy $\Delta G^\circ_{\text{rxn}}$. The value of $\Delta G^\circ_{\text{rxn}}$ for templates in which the blocker is imperfectly hybridized (due to the presence of a single-nucleotide variation in the reverse toehold) are more negative, resulting in a higher equilibrium hybridization yield [2].

the $\Delta G^\circ_{\text{rtoe}}$ value appear to be significantly less negative than predicted by theory, by roughly 3 kcal/mol. This systematic difference may be due to the specific buffer used in the Applied Biosystems PowerUP qPCR kit, or due to the electrostatic repulsion and thermodynamic destabilization effects of the 4 nt terminator sequence at the 3′ end of B. Consequently, the reverse toehold was designed to be longer/stronger by a corresponding number of nucleotides to compensate for this 3 kcal/mol adjustment.

Sequence selectivity. Any SNV in the reverse-toehold region of the template will result in a $\Delta G^\circ_{\text{rxn}}$ that is more negative than for the wildtype sequence ($\Delta G^\circ_{\text{WT}}$) by an amount $\Delta\Delta G^\circ_{\text{Var}}$ that is dependent on the identity and position of the SNV (Fig. S1-3a). The value of $\Delta\Delta G^\circ_{\text{Var}}$ can be approximated by $\Delta\Delta G^\circ_{\text{triplet}}$ for SNVs that are not too close to the 3′ end of the blocker. $\Delta\Delta G^\circ_{\text{triplet}}$ is the sum of the opportunity cost of two base stacks in the WT template and the mismatch penalty of a single-base mismatch (Fig. S1-3b); its value can be calculated from literature parameters [3]. The distribution of $\Delta\Delta G^\circ_{\text{triplet}}$ values for the 192 different possible WT/SNV triplets are shown in Fig. S1-3c.

BDA system design. The primer and blocker sequences should be designed so that $\Delta G^\circ_{\text{WT}}$ is marginally positive, so that $\Delta G^\circ_{\text{Var}}$ is marginally negative for a majority of possible SNVs. This ensures that the yield of the primer displacing the blocker is significantly different for WT templates as for SNV templates, allowing hypothesis-free enrichment of all variants in the reverse toehold region.

The design of a BDA system generally can be deconstructed into the following steps:

1. Selection of the forward or reverse DNA strand to act as the target template sequence, to maximize the $\Delta\Delta G^\circ_{\text{triplet}}$ value.

2. Selection of a reverse toehold region based on loci of interest, such that $\Delta G^\circ_{\text{rtoe}} \approx -9$ for the WT template.

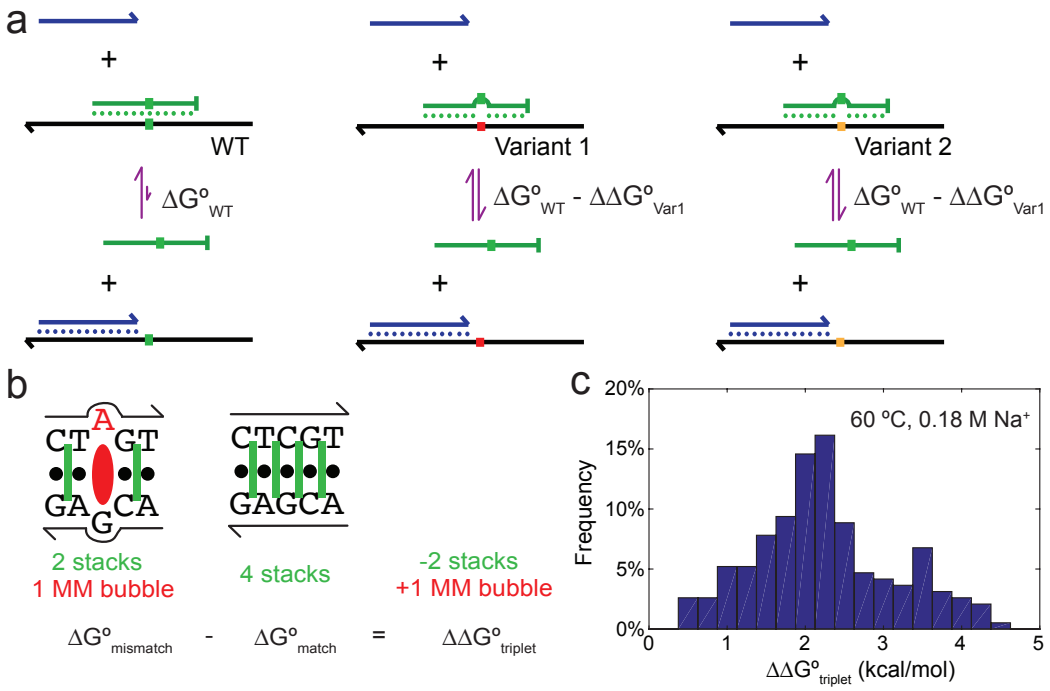


FIG. S1-3: Variation in hybridization selectivity for different SNV sequences. **(a)** The toehold exchange process efficiency recognizes sequence variations in the reverse toehold region of the template. **(b)** $\Delta\Delta G^\circ$ values for different SNVs can be approximated as the $\Delta\Delta G^\circ_{\text{triplet}}$ of the triplet, considering only local sequence information. This approximation fails in the case of templates with significant secondary structure, and in cases where the SNV lies too close to the 3′ end of the blocker. **(c)** $\Delta\Delta G^\circ_{\text{triplet}}$ values at the PCR anneal/extend step temperature and buffer conditions, based on literature-reported parameters [3].

3. Selection of a toehold region between 15 and 30 nucleotides to the 5′ of the reverse toehold, such that $\Delta G^\circ_{\text{toe}} \approx -8$ and the computed melting temperature of the primer is above 64 °C. Additionally, the toehold should not participate in any secondary structure in the template sequence.

4. Selection of a 2-4 nucleotide termination sequence at the 3′ end of the blocker to prevent blocker extension. If using a high-fidelity polymerase, use a chemical modification such as a 3-carbon spacer instead.

5. Selection of a reverse primer sequence that is not predicted to result in primer dimers with either the primer or the blocker.

The above protocol describes one process for sequence design of the primer and blocker to achieve reasonable enrichment factors for all possible variants in the reverse toehold region. In hypothesis-driven applications for enrichment and detection of a known variant, the blocker and primer sequence design may be modified to achieve $\Delta G^\circ_{\text{rxn}}$ that maximizes enrichment.

[1] Zhang, D. Y. & Winfree, E. Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc.* **131**, 17303-17314 (2009).

[2] Zhang, D. Y., Chen, S. X. & Yin, P. Thermodynamic optimization of nucleic acid hybridization specificity. *Nat. Chem.* **4**, 208-214 (2012).

[3] SantaLucia, J. Jr. & Hicks, D. The thermodynamics of DNA structural motifs. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 415-440 (2004).

Section S2: BDA Model Details

In a PCR reaction, changing temperatures and enzymatic activity means that the system never really reaches equilibrium. Consequently, the equilibrium behavior of toehold probes [1, 2] cannot provide quantitation guidance on the performance of the BDA assay. Here, we construct a kinetic model of the BDA assay, both to improve quantitative understanding of the system and also to guide the sequence design and assay formulation.

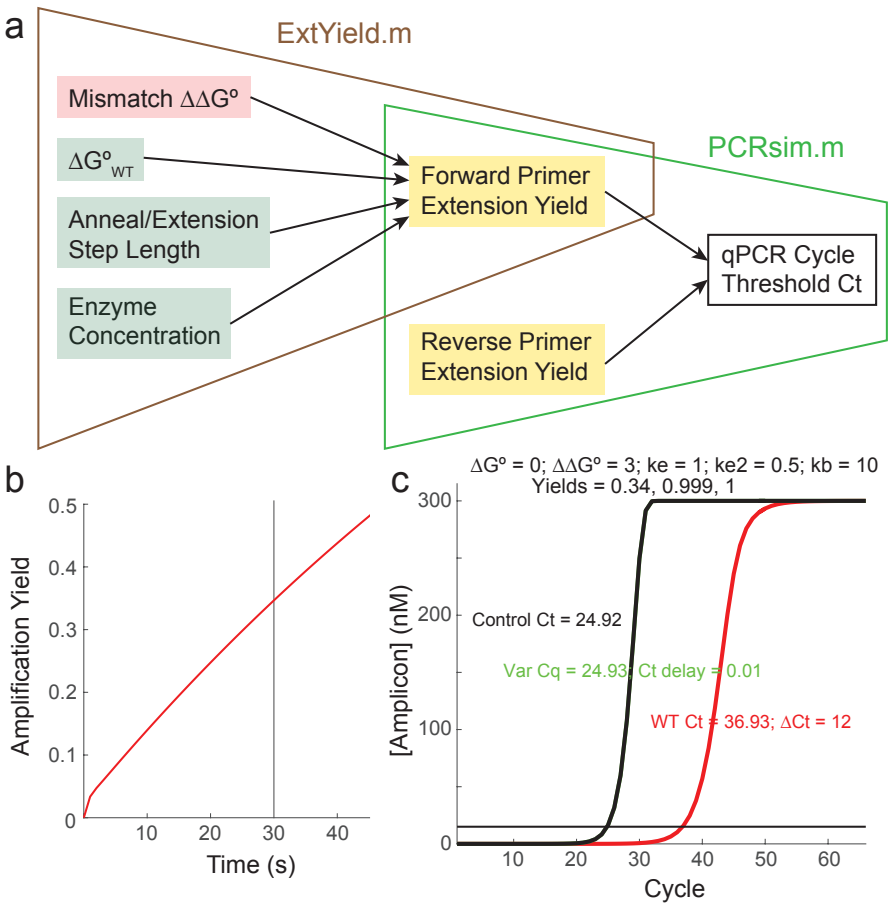


FIG. S2-1: Overview of model. **(a)** Shown in green boxes are input variables that are easily adjustable by a user. Shown in yellow boxes are hidden variables that are affected by assay design choices, but cannot be easily quantitated experimentally. **(b)** Sample simulation results for the ExtYield.m half of the simulation. For this particular set of $\Delta G^\circ_{\mathrm{WT}}$, $\Delta\Delta G^\circ$, etc., there is a roughly linear increase of amplification yield up to roughly 35% at 30 s. **(c)** Sample simulation results for PCRsim.m given the using the Amplification Yield from panel (B).

The model consists of two parts (Fig. S2-1): a kinetic simulation of the anneal/extension step that determining the per-cycle extension yield (ExtYield.m), and a discrete simulation of the PCR cycles based on the forward and reverse primer per-cycle extension yields (PCRsim.m). ExtYield.m uses the Matlab ordinary differential equation numerical method "ode23s" to kinetically simulate the binding and displacement reactions of interest; the reactions modeled are described in Fig. S1-1b. The values of the rate constant parameters used are as follows: $k_f = 10^6$ M^{-1} s^{-1}, $k_e = 1$ s^{-1}, $k_{e2} = 0.5$ s^{-1}, $k_b = 10$ s^{-1}. The larger of k_{pt} and k_{pd} is set at 10^6 M^{-1} s^{-1}, and the smaller is determined by $\frac{k_{pd}}{k_{pt}} = K_{\mathrm{eq}} = e^{-\Delta G^\circ_{\mathrm{rxn}}/R\tau}$. The larger of k_{bt} and k_{Bd} is set at 10^6 M^{-1} s^{-1}, and the smaller is determined by $\frac{k_{pd}}{k_{pt}} = K_{\mathrm{eq}} = e^{-\Delta G^\circ_{\mathrm{rxn}}/R\tau}$. PCRsim.m calculates the forward and reverse amplicon concentrations after each cycle, based on the initial template concentration and the per-cycle extension yields, using a discrete simulation.

The Matlab source code of function ExtYield.m and PCRsim.m are attached at the end of this section.

Model limitations and assumptions. In the construction of the model, we intentionally chose to limit the number of states and rate constant parameters, because complex models with too many fitted variables and reactions are more likely to be overfitted and inaccurately reflects reality. Below is a list of model assumptions that we made:

1. Polymerase binding/initiation rate constants are different depending on whether the blocker is fully dissociated from the template. When the Blocker is still attached, the extra steric constraints and negative charges (from the Blocker's phosphodiester backbone) will slow the binding of the polymerase. Additionally, extension will require enzymatic strand displacement or exonuclease activity and also be slowed.

2. Polymerase extension is instantaneous after binding to template. After an extension reaction begins, the Blocker is no longer able to displace the growing amplicon sequence from the template. The time needed to extend the amplicon (typically less than 100 nt) is expected to be less than 2 s [3].

3. Hybridization rate constants of primer and blocker to an unbound template are identical at $k_f = 10^6$ M^{-1} s^{-1}. Sequence dependence is known to affect hybridization rate constants, but a sequence-generic model will ignore these for simplicity.

4. Primer dimer interactions are not modeled and assumed not to be present. Primer dimerization can follow many complex pathways, and it is beyond the scope of the current work to model these. Experimentally, only a limited number of BDA systems tested exhibited significant primer dimerization to impact performance.

5. Temperature changes are instantaneous. The reactions at temperatures other than 95 °C and 60 °C (or other anneal/extend temperature) are not explicitly modeled.

Model vs. Experiments. The purpose of this model is to understand the effects of various design parameters on the enrichment performance of BDA; accurate quantitative match between simulation and experimental traces is secondary. Deviations between model and experimental results likely reflect both the simplicity of the model and the inaccuracy of literature reported thermodynamics parameters [4, 5]. In Fig. S2-2a, we show a comparison of experimental (solid lines) and simulated BDA qPCR traces (dashed lines) for BDA enrichment of the rs2246745 SNP.

Our model suggests that $\Delta\Delta G^\circ$ is a key parameter that sets the upper limit for the achievable enrichment by BDA. $\Delta\Delta G^\circ$ denotes the difference in the standard free energy of hybridization of the Blocker binding to the WT as compared to binding to the Variant, and is primarily dependent on the sequence identities of the WT and Variant alleles. At 60 °C and typical PCR buffer conditions, $\Delta\Delta G^\circ$ values range between 0.8 kcal/mol and 5 kcal/mol. The model suggests that larger $\Delta\Delta G^\circ$ results in larger ΔCt values, with saturation at roughly 3 kcal/mol. Thus, some Variant/WT pairs with smaller $\Delta\Delta G^\circ$ will exhibit lower enrichment by BDA due to their sequence identities. Fig. S2-2b shows model (red dots) and experimental (black dots) effects of mismatch $\Delta\Delta G^\circ$ for all SNPs tested in Fig. 2e in main text.

We also observed that the ΔCt is affected my Blocker stoichiometry, which is defined as the initial concentration of the Blocker divided by the initial concentration of the forward Primer. In Fig. S2-2c, model (dashed line) and experimental (black dots) effects of Blocker stoichiometry for BDA on the rs1884444 SNP are shown. Maximal Ct is achieved when Blocker stoichiometry ≥ 7. To be conservative against slight model errors, we chose Blocker stoichiometry of 10 for all experiments in this manuscript other than the ones shown in this panel.

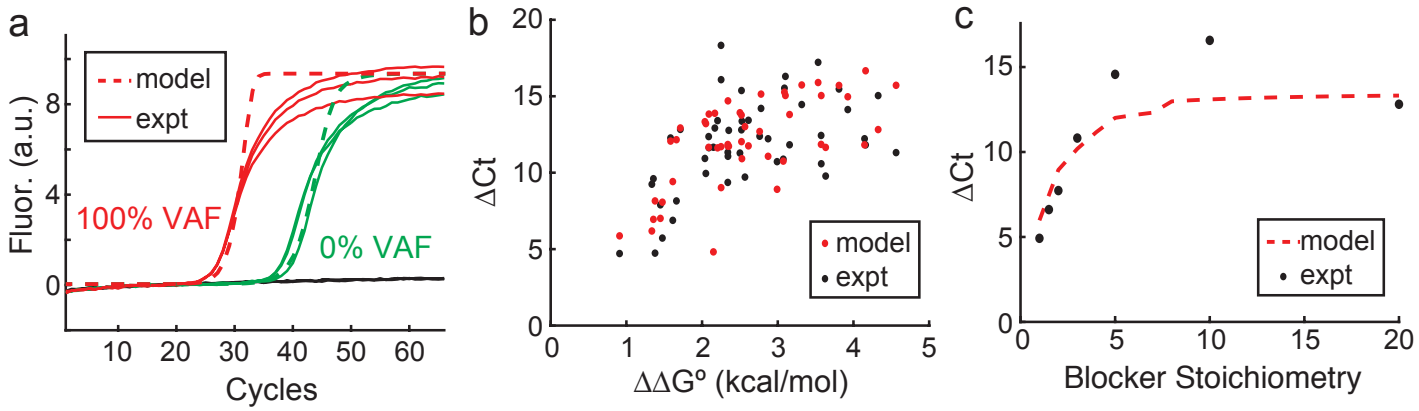


FIG. S2-2: Model vs. experimental results. **(a)** Comparison of experimental (solid lines) and simulated BDA qPCR traces (dashed lines) for BDA enrichment of the rs2246745 SNP. **(b)** Model (red dots) and experimental (black dots) effects of mismatch $\Delta\Delta G^\circ$. **(c)** Model (dashed line) and experimental (black dots) effects of Blocker stoichiometry for BDA on the rs1884444 SNP.

[1] Zhang, D. Y., Chen, S. X. & Yin, P. Thermodynamic optimization of nucleic acid hybridization specificity. *Nat. Chem.* **4**, 208-214 (2012).
[2] Wu, L. R., Wang, J. S., Fang, J. Z., Reiser, E., Pekker, I., Boykin, R., Ngouenet, C., Webster, P. J., Beechem, J., Zhang, D. Y. Continuously Tunable Nucleic Acid Hybridization Probes. *Nature Methods* **12,** 1191-1196 (2015).
[3] Kong, H., Kucera, R.B. & Jack, W.E., Characterization of a DNA polymerase from the hyperthermophile archaea Thermococcus litoralis. Vent DNA polymerase, steady state kinetics, thermal stability, processivity, strand displacement, and exonuclease activities. *Journal of Biological Chemistry* **268,** 1965-1975 (1993).
[4] SantaLucia, J. Jr. & Hicks, D. The thermodynamics of DNA structural motifs. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 415-440 (2004).
[5] Wang, C., Bae, J. H., & Zhang, D. Y. Native characterization of nucleic acid motif thermodynamics via non-covalent catalysis. *Nature communications* **7,** 10319 (2016).

Function “PCRsim.m”:

```
function [yields, Cqs] = PCRsim(dG, ddG, varargin)
% [yields, Cqs] = PCRsim(dG, ddG, varargin)
% varargin can include:
%   '-ke' (ke) [default = 1]
%   '-ke2' (ke2) [default = 0.5]
%   '-kb' (kb) [default = 10]
%   '-BMstates' (BMstates) [default = 11]
%   '-PLOT' [default = off]
%   '-fPc' (forward primer concentration) [default = 4e-7]
%   '-Bc' (blocker concentration) [default = 4e-6]
%   '-Cycles' (PCR cycles) [default = 65]
%   '-T' (temperature) [default = 60]
%   '-ATime' (anneal cycle time) [default = 30]

% yields and Cqs are 1x3 vectors in order of [WT, Var, Control]

ke = 1;
ke2 = 0.5;
kb = 10;
BMstates = 11;
plotflag = 0;
NumCycles = 65;
Primer = 4e-7;
Blocker = 4e-6;
Temp = 60;
time = 30;
counter = 1;

while (length(varargin) >= counter)
    if (strcmpi(varargin{counter}, '-KE'))
        ke = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-KE2'))
        ke2 = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-KB'))
        kb = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-BMSTATES'))
        BMstates = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-PLOT'))
        plotflag = 1;
        counter = counter + 1;
    elseif (strcmpi(varargin{counter}, '-FPC'))
        Primer = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-BC'))
        Blocker = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-CYCLES'))
        NumCycles = varargin{counter+1};
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-T'))
        Temp = varargin{counter+1};
```

```
        counter = counter + 2;
    elseif (strcmpi(varargin{counter}, '-ATIME'))
        time = varargin{counter+1};
        counter = counter + 2;
    else
        error('Unexpected input token %s found!\n', varargin{counter});
    end
end



kf = 1e6; %Arbitrary assumption
kpt = 1e6; %Arbitrary assumption
kbt_wt = kpt / max(1, dG2Keq(dG, Temp));
kbt_var = kpt / max(1, dG2Keq(dG-ddG, Temp));

kpd = 1;
kbd_wt = kpd * dG2Keq(dG, Temp) * kbt_wt / kpt;
kbd_var = kpd * dG2Keq(dG-ddG, Temp) * kbt_wt / kpt;

fPyield_wt = ExtYield(Primer, Blocker, time, kf, kpt, kbt_wt, kpd,
kbd_wt, ke, ke2, kb, BMstates);
fPyield_var = ExtYield(Primer, Blocker, time, kf, kpt, kbt_var, kpd,
kbd_var, ke, ke2, kb, BMstates);
rPyield = ExtYield(Primer, 0, time, kf, kpt, 0, 0, 0, ke, ke2, 0,
BMstates);

Amp_Fwt(1) = 1e-15; %Arbitrary template starting amount
Amp_Rwt(1) = 1e-15;
Amp_Fvar(1) = 1e-15;
Amp_Rvar(1) = 1e-15;
Amp_Fcontrol(1) = 1e-15;
Amp_Rcontrol(1) = 1e-15;

for i = 1:NumCycles
    Amp_Fwt(i+1) = Amp_Fwt(i) + rPyield*Amp_Rwt(i)*(Primer -
Amp_Fwt(i))/Primer; %#ok<*SAGROW>
    Amp_Rwt(i+1) = Amp_Rwt(i) + fPyield_wt*Amp_Fwt(i)*(Primer -
Amp_Rwt(i))/Primer;

    Amp_Fvar(i+1) = Amp_Fvar(i) + rPyield*Amp_Rvar(i)*(Primer -
Amp_Fvar(i))/Primer;
    Amp_Rvar(i+1) = Amp_Rvar(i) + fPyield_var*Amp_Fvar(i)*(Primer -
Amp_Rvar(i))/Primer;

    Amp_Fcontrol(i+1) = Amp_Fcontrol(i) +
rPyield*Amp_Rcontrol(i)*(Primer - Amp_Fcontrol(i))/Primer;
    Amp_Rcontrol(i+1) = Amp_Rcontrol(i) +
rPyield*Amp_Fcontrol(i)*(Primer - Amp_Rcontrol(i))/Primer;
end

Cq_wt = find(Amp_Fwt+Amp_Rwt > Primer*.1, 1);
Cq_var = find(Amp_Fvar+Amp_Rvar > Primer*.1, 1);
Cq_control = find(Amp_Fcontrol+Amp_Rcontrol > Primer*.1, 1);

%Interpolate fractional Cq
```

```
if (isempty(Cq_wt))
    Cq_wt = NumCycles+1;
else
    Cq_wt = Cq_wt - (Amp_Fwt(Cq_wt)+Amp_Rwt(Cq_wt) - Primer*.1) ...
        /(Amp_Fwt(Cq_wt)+Amp_Rwt(Cq_wt)-Amp_Fwt(Cq_wt-1)+Amp_Rwt(Cq_wt-
1));
end

if (isempty(Cq_var))
    Cq_var = NumCycles+1;
else
    Cq_var = Cq_var - (Amp_Fvar(Cq_var)+Amp_Rvar(Cq_var) - Primer*.1)
...
        /(Amp_Fvar(Cq_var)+Amp_Rvar(Cq_var)-Amp_Fvar(Cq_var-
1)+Amp_Rvar(Cq_var-1));
end

if (isempty(Cq_control))
    Cq_control = NumCycles+1;
else
    Cq_control = Cq_control -
(Amp_Fcontrol(Cq_control)+Amp_Rcontrol(Cq_control) - Primer*.1) ...
        /(Amp_Fcontrol(Cq_control)+Amp_Rcontrol(Cq_control)-
Amp_Fcontrol(Cq_control-1)+Amp_Rcontrol(Cq_control-1));
end

yields = [fPyield_wt, fPyield_var, rPyield];
Cqs = [Cq_wt, Cq_var, Cq_control];
%dCqs = [Cq_wt - Cq_var, Cq_var - Cq_control];

%-----
% Plotting

if (plotflag == 1)
    close all
    hold all
    set(gca, 'FontSize', 20);
    plot(1:(NumCycles+1), Amp_Fwt+Amp_Rwt, '-r', 'LineWidth', 2);
    plot(1:(NumCycles+1), Amp_Fvar+Amp_Rvar, '-g', 'LineWidth', 2);
    plot(1:(NumCycles+1), Amp_Fcontrol+Amp_Rcontrol, '-k', 'LineWidth',
2);
    plot([1, NumCycles+1], [Primer*.1, Primer*.1], '-k', 'LineWidth',
1);
    axis([1, NumCycles+1, 0, Primer*2.1]);

    curstr = ['WT Cq = ', num2str(Cq_wt,4), '; dCq = ', num2str(Cq_wt -
Cq_var, 3)];
    text(min(Cq_wt-5, 50), Primer*0.7, curstr, 'Color', 'r',
'FontSize', 18);

    curstr = ['Var Cq = ', num2str(Cq_var,4), '; Cq delay = ',
num2str(Cq_var - Cq_control, 3)];
    text(min(Cq_var, 50), Primer, curstr, 'Color', 'g', 'FontSize',
18);

    curstr = ['Control Cq = ', num2str(Cq_control,4)];
    text(min(Cq_control-10, 50), Primer*1.3, curstr, 'Color', 'k',
```

```
'FontSize', 18);

    curstr = ['dG = ', num2str(dG), '; ddG = ', num2str(ddG), '; ke =
', num2str(ke), '; ke2 = ', num2str(ke2), '; kb = ', num2str(kb)];
    text(15, Primer*2.2, curstr, 'Color', 'k', 'FontSize', 20);

    curstr = ['Yields = ', num2str(fPyield_wt, 3), ', ',
num2str(fPyield_var, 3), ', ', num2str(rPyield,3)];
    text(15, Primer*2.08, curstr, 'Color', 'k', 'FontSize', 20);

    filename = ['dG', num2str(dG), 'ddG', num2str(ddG), 'ke',
num2str(ke), 'kb', num2str(kb)];
    filename(filename == '.') = '_';
    filename = [filename, '.eps'];
    print('-depsc', filename);

end
end
```

Function “ExtYield.m”:

```
function [yield] = ExtYield(Primer, Blocker, time, kf, kpt, kbt, kpd,
kbd, ke, ke2, kb, BMstates)

%ExtYield(Primer, Blocker, time, kf, kpt, kbt, kpd, kbd, ke, kb)
%Calculates Extension yield of PCR based on input parameters
% Primer is primer concentration
% Blocker is blocker concentraiton
% time is anneal/extension time in seconds (assuming 2-step PCR)
% kf is rate constant of hybridization
% kpt is rate constant of primer toehold binding
% kbt is rate constant of blocker toehold binding
% kpd is rate constant of primer toehold dissociation
% kbd is rate constant of blocker toehold dissociation
% ke is rate constant of enzyme binding + extension without
exo/displacement (unimolecular, incorporates enzyme concentration)
% ke2 is rate constant of enzyme binding + extension with
exo/displacement
% kb is rate constant of branch migration to end-BM state
% BMstates is the number of states collapsed into PBV (number of bases
in BM region)

plotflag = 0;

Temp = 60+273.15;
Salinity = 0.18;
Template = 1e-15; %Doesn't really matter, as long as smaller than
Primer



%Simulate the Hyb reaction
options = odeset('RelTol', 1e-6, 'AbsTol', 1e-30);
[t, conc] = ode23s(@SimStep, 0:1:time, [Primer, Blocker, Template, 0,
```

```
0, 0, 0, 0, kf, kpt, kbt, kpd, kbd, ke, ke2, kb, BMstates], options);

if (plotflag == 1)
    figure
    hold all
    plot(t, conc(:,8)/Template, '-r', 'LineWidth', 1)
    set(gca, 'FontSize', 20);
end


yield = conc(end, 8) / Template;


function dy = SimStep(t, y)
%P + V -> PV
%B + V -> BV
%PV -> AV
%BV + P -> PBV
%PBV -> BV + P
%PBV -> PBV2
%PBV2 -> PBV
%PBV2 -> AV
%PBV2 -> PV + B
%PV + B -> PBV2

%y = [P, B, V, PV, BV, PBV, PBV2, AV, kf, kpt, kbt, kpd, kbd, ke, ke2,
kb, BMstates];
%     1  2  3  4   5   6    7     8   9   10   11   12   13   14, 15,
16, 17
dy = zeros(17,1);
P = y(1);
B = y(2);
V = y(3);
PV = y(4);
BV = y(5);
PBV = y(6);
PBV2 = y(7);
AV = y(8);
kf = y(9);
kpt = y(10);
kbt = y(11);
kpd = y(12);
kbd = y(13);
ke = y(14);
ke2 = y(15);
kb = y(16);
BMstates = y(17);

dy(1) = -kf * P * V - kpt * P * BV + kpd * PBV; %P
dy(2) = -kf * B * V - kbt * B * PV + kbd * PBV2; %B
dy(3) = -kf * P * V - kf * B * V; % V
dy(4) = kf * P * V - kbt * B * PV + kbd * PBV2 - ke * PV; % PV
dy(5) = kf * B * V + kpd * PBV - kpt * P * BV; % BV
dy(6) = kpt * P * BV - kb * PBV - kpd * PBV + BMstates * kb * PBV2; %
PBV
dy(7) = kb * PBV - BMstates * kb * PBV2 - ke2 * PBV2 - kbd * PBV2 + kbt
* B * PV; % PBV2
```

```
dy(8) = ke * PV + ke2 * PBV2; %AV
```

Function “dG2Keq.m”:

```
function [Keq] = dG2Keq(dG, varargin)

% [Keq] = dG2Keq(dG, <Temp>)
%Calculates Keq from input dG, and optional temperature (in Celcius)
%Temperature defaults to 37 C.

Temp = 37+273.15;

if (length(varargin) == 1)
    Temp = varargin{1} + 273.15;
end

Keq = exp(-dG * 4180 / 8.314 / Temp);

end
```

Section S3: qPCR Data for Non-pathogenic SNPs

This section contains the raw qPCR results of the 48 BDA designs of the 12 non-pathogenic SNP pairs (24 are summarized in manuscript Fig. 2e), and the temperature robustness experimental data (summarized in Fig. 3bc).

Basic qPCR Results for 12 Non-pathogenic SNPs. We selected 12 non-pathogenic SNP pairs representing two of every possible single-base substitution type in the human genome. Cell line gDNA sample NA18562 and NA18537 are homozygous of different alleles on each SNP locus (see Table S3-1 for details), based on the 1000 Genomes database. For each SNP, we designed and tested blockers complementary to each allele (marked as "Blocker for NA18562" and "Blocker for NA18537"). For each allele, we tested 2 designs: in one design, the forward primer and the blocker have the same sequences as the forward (+) genomic DNA strand in the database and is labeled "direction 1;" the other design has forward primer and blocker bearing the same sequence as the reverse (-) genomic DNA strand and is labeled "direction 2." Therefore, there are $12 \times 2 \times 2 = 48$ designs in total. We selected the better design for each of the 24 alleles, and a summary of the results are shown in manuscript Fig. 2e. Ct values of the experiments are shown in Table S3-1, a summary can be found in Fig. S3-1a, and the qPCR traces can be found in Fig. S3-2 and Fig. S3-3.

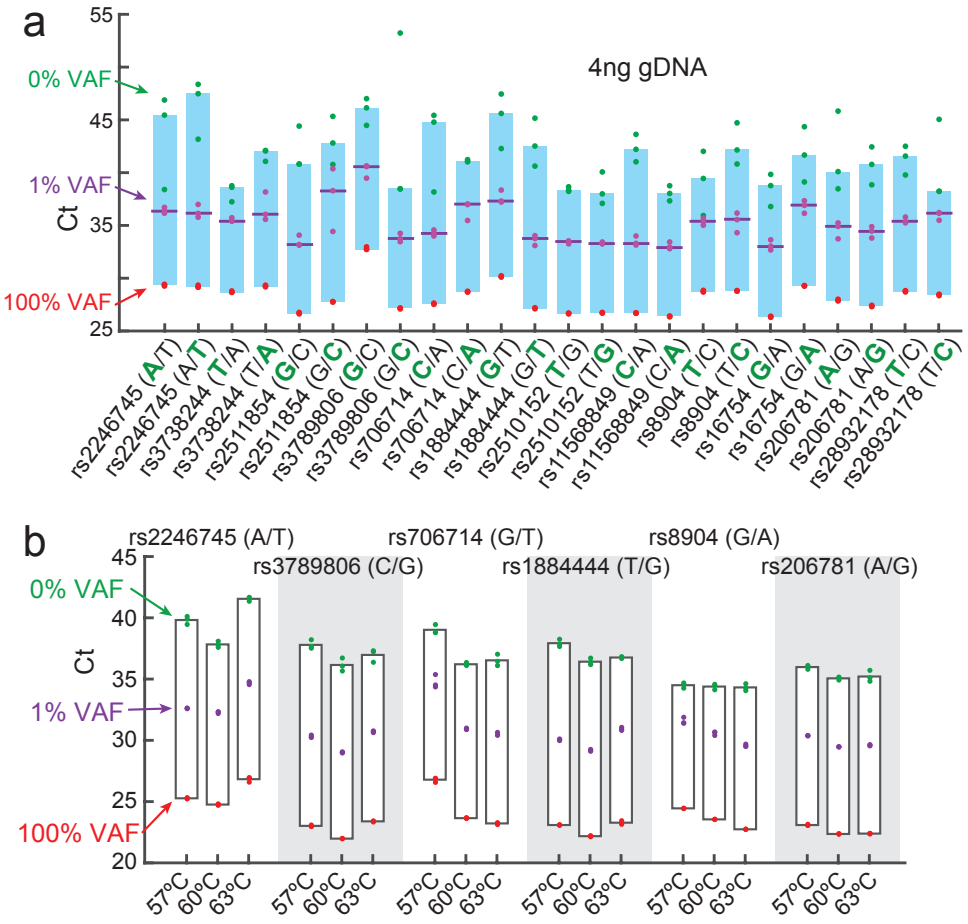


FIG. S3-1: Summary of 12 Non-pathogenic SNPs and temperature robustness. **(a)** qPCR results for 24 different BDA designs to 12 non-pathogenic SNPs with triplicate Ct values shown. **(b)** Temperature robustness validation for 5 SNPs with triplicate Ct values shown.

Temperature Robustness Tests. We performed a temperature robustness test of the BDA design for rs3789806 (NA18537 blocker, direction 1) using different anneal / extension temperature ranging from 55 °C to 65 °C (manuscript Fig. 3b); the raw qPCR traces of these experiments are shown in Fig. S3-4. We also tested the temperature robustness of 6 BDA designs at 57 °C, 60 °C, and 63 °C (manuscript Fig. 3c and Fig. S3-1b); the corresponding traces, except for the rs3789806 design in manuscript Fig. 3a, are shown in Fig. S3-5.

		Genotype						No B 100%			0% variant			1% variant			100% variant		
SNP rs-	Direction	NA18562	NA18537	Blocker	NTC			mean	std	median	mean	std	median	mean	std	median	mean	std	median
2246745	1	A/A	T/T	B18562	>66	>66	>66	26.56	0.04	26.56	40.07	2.05	40.30	33.72	0.18	33.68	27.39	0.04	27.38
2246745	1	A/A	T/T	B18537	>66	>66	>66	26.41	0.04	26.41	39.98	1.13	39.50	35.55	1.35	34.78	28.36	0.12	28.43
3738244	1	T/T	A/A	B18562	>66	>66	>66	26.51	0.02	26.51	38.21	0.84	38.62	35.52	0.18	35.44	28.71	0.07	28.67
3738244	1	T/T	A/A	B18537	54.30	>66	>66	26.43	0.04	26.43	41.74	0.58	42.06	36.60	1.38	36.05	29.25	0.08	29.23
2511854	1	G/G	C/C	B18562	51.90	60.25	59.93	27.74	0.01	27.74	44.12	1.68	43.41	37.66	0.19	37.75	30.61	0.12	30.64
2511854	1	G/G	C/C	B18537	49.63	51.36	49.32	27.63	0.04	27.63	42.97	2.28	42.81	37.70	3.01	38.29	27.77	0.02	27.76
3789806	1	G/G	C/C	B18562	>66	>66	>66	26.71	0.04	26.71	45.88	1.28	46.14	40.23	0.66	40.58	32.81	0.13	32.74
3789806	1	G/G	C/C	B18537	>66	54.04	42.02	26.46	0.07	26.48	43.39	8.53	38.48	33.82	0.40	33.74	27.14	0.05	27.17
706714	1	C/C	A/A	B18562	60.59	>66	>66	26.61	0.10	26.62	42.80	4.02	44.79	34.28	0.29	34.27	27.58	0.05	27.57
706714	1	C/C	A/A	B18537	58.62	59.37	60.09	26.46	0.02	26.45	38.59	2.08	37.64	35.80	0.61	35.63	29.47	0.08	29.48
1884444	1	G/G	T/T	B18562	54.95	>66	57.14	26.64	0.03	26.66	40.29	0.79	40.07	35.03	0.24	35.10	28.70	0.05	28.73
1884444	1	G/G	T/T	B18537	38.40	55.34	53.32	26.58	0.04	26.58	42.76	2.28	42.52	33.63	0.48	33.76	27.15	0.05	27.15
2510152	1	T/T	G/G	B18562	46.62	51.16	54.21	26.51	0.06	26.47	38.41	0.22	38.34	33.44	0.15	33.52	26.66	0.04	26.68
2510152	1	T/T	G/G	B18537	>66	>66	>66	26.60	0.05	26.63	38.38	1.53	37.99	33.32	0.09	33.28	26.72	0.03	26.71
11568849	1	C/C	A/A	B18562	>66	>66	>66	26.57	0.04	26.56	42.96	4.49	40.49	37.11	1.33	36.60	29.93	0.08	29.91
11568849	1	C/C	A/A	B18537	>66	>66	>66	26.55	0.02	26.56	43.38	1.72	43.45	42.53	1.35	42.63	36.43	0.38	36.56
8904	1	T/T	C/C	B18562	>66	>66	>66	28.02	0.06	28.00	40.17	2.26	40.49	37.24	0.33	37.13	30.84	0.13	30.89
8904	1	T/T	C/C	B18537	50.30	50.26	52.59	29.54	0.05	29.53	41.29	2.70	42.00	36.23	0.22	36.16	30.13	0.11	30.18
16754	1	G/G	A/A	B18562	>66	>66	>66	26.33	0.02	26.33	37.79	0.92	37.79	34.91	0.39	35.09	28.42	0.05	28.43
16754	1	G/G	A/A	B18537	>66	>66	>66	26.36	0.03	26.36	41.71	2.61	41.67	36.80	0.60	36.90	29.28	0.03	29.29
206781	1	A/A	G/G	B18562	>66	>66	>66	25.97	0.04	25.95	41.47	3.86	40.10	34.63	0.79	34.91	27.91	0.08	27.88
206781	1	A/A	G/G	B18537	>66	>66	>66	25.97	0.05	25.98	40.69	1.79	40.78	34.39	0.54	34.47	27.37	0.06	27.36
28932178	1	T/T	C/C	B18562	>66	>66	>66	26.20	0.05	26.20	36.65	0.14	36.60	36.13	0.47	35.90	31.84	0.08	31.87
28932178	1	T/T	C/C	B18537	>66	>66	>66	26.16	0.04	26.15	40.51	3.94	38.24	35.95	0.40	36.15	28.43	0.06	28.46
2246745	2	T/T	A/A	B18562	>66	>66	>66	27.05	0.05	27.04	43.58	4.54	45.45	36.42	0.27	36.39	29.34	0.08	29.36
2246745	2	T/T	A/A	B18537	>66	>66	54.25	26.91	0.03	26.89	46.34	2.78	47.48	36.30	0.62	36.14	29.21	0.11	29.15
3738244	2	A/A	T/T	B18562	>66	>66	>66	26.26	0.03	26.24	36.00	0.06	36.02	34.67	0.34	34.58	28.13	0.02	28.13
3738244	2	A/A	T/T	B18537	>66	>66	50.10	26.39	0.05	26.41	38.91	0.55	39.01	34.61	0.21	34.57	28.08	0.02	28.08
2511854	2	C/C	G/G	B18562	>66	>66	>66	26.49	0.02	26.49	42.01	2.08	40.82	33.47	0.53	33.19	26.69	0.07	26.69
2511854	2	C/C	G/G	B18537	>66	>66	>66	26.39	0.12	26.41	42.61	3.52	43.74	36.42	0.23	36.50	29.49	0.15	29.54
3789806	2	C/C	G/G	B18562	>66	60.54	>66	26.57	0.05	26.58	46.11	5.93	47.55	40.36	0.61	40.28	33.07	0.10	33.09
3789806	2	C/C	G/G	B18537	>66	>66	>66	26.27	0.05	26.27	43.01	0.59	43.22	38.69	0.80	38.93	31.35	0.13	31.40
706714	2	G/G	T/T	B18562	53.96	54.32	56.07	26.89	0.02	26.90	47.33	1.32	47.36	38.28	0.30	38.19	31.04	0.04	31.06
706714	2	G/G	T/T	B18537	45.02	42.44	42.13	26.79	0.04	26.78	41.11	0.10	41.07	36.49	0.89	36.98	28.72	0.03	28.71
1884444	2	C/C	A/A	B18562	>66	>66	>66	26.48	0.08	26.43	45.11	2.62	45.61	37.63	0.63	37.31	30.16	0.06	30.13
1884444	2	C/C	A/A	B18537	>66	>66	>66	26.49	0.02	26.48	36.73	0.83	36.28	37.26	0.24	37.23	31.58	0.02	31.57
2510152	2	A/A	C/C	B18562	43.82	40.89	>66	26.30	0.04	26.29	39.55	1.29	40.21	34.60	0.47	34.60	27.97	0.06	27.94
2510152	2	A/A	C/C	B18537	39.18	39.89	38.27	26.31	0.01	26.32	37.43	0.89	37.26	32.97	0.23	33.02	26.36	0.05	26.38
11568849	2	G/G	T/T	B18562	49.28	48.78	47.80	26.38	0.04	26.38	42.29	1.30	42.21	33.48	0.45	33.30	26.70	0.03	26.68
11568849	2	G/G	T/T	B18537	37.27	39.95	38.14	26.37	0.05	26.34	38.04	0.71	38.03	33.06	0.33	32.95	26.38	0.04	26.39
8904	2	A/A	G/G	B18562	60.07	51.40	47.40	27.41	0.06	27.41	39.14	3.05	39.45	35.34	0.32	35.35	28.76	0.08	28.73
8904	2	A/A	G/G	B18537	>66	48.11	56.71	27.01	0.06	27.02	42.57	1.98	42.15	35.34	0.95	35.55	28.80	0.03	28.79
16754	2	C/C	T/T	B18562	56.09	56.13	>66	26.01	0.04	26.02	38.47	1.55	38.77	33.12	0.49	33.04	26.35	0.05	26.33
16754	2	C/C	T/T	B18537	>66	>66	>66	25.87	0.05	25.84	32.94	0.26	32.93	32.16	0.02	32.17	27.20	0.03	27.21
206781	2	T/T	C/C	B18562	>66	>66	>66	26.23	0.05	26.25	36.11	0.45	36.32	33.89	0.61	34.12	27.10	0.05	27.08
206781	2	T/T	C/C	B18537	>66	>66	>66	26.08	0.01	26.08	38.36	0.88	38.55	33.03	0.58	32.81	26.34	0.04	26.33
28932178	2	A/A	G/G	B18562	>66	>66	>66	26.35	0.06	26.36	41.28	1.38	41.57	35.46	0.29	35.35	28.76	0.06	28.77
28932178	2	A/A	G/G	B18537	>66	>66	>66	26.44	0.03	26.45	38.57	1.65	37.81	34.91	0.23	35.03	28.09	0.06	28.09

TABLE S3-1: Ct values of the 48 BDA designs for non-pathogenic SNPs (4 ng gDNA input). For no template control (NTC) experiments, the 3 Ct values from the triplicates are shown separately. For other experiments, mean, standard deviation (std), and median values are shown. The "genotype" columns show the allele identity of sample NA18562 and NA18537 on the strand with the same direction as the blocker; therefore, for example, the "A/A" alleles in direction 1 become "T/T" in direction 2.

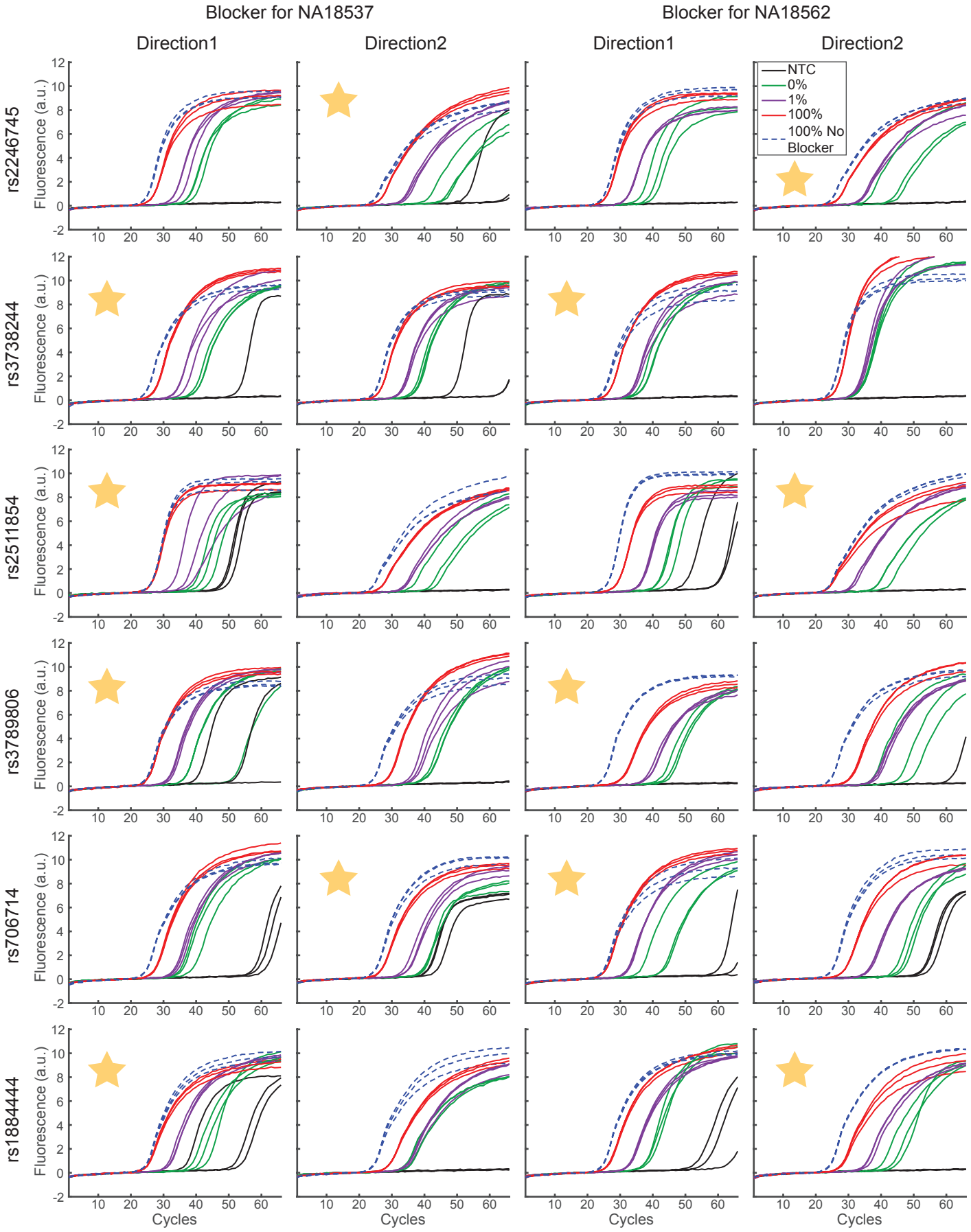


FIG. S3-2: Raw qPCR traces of the BDA designs for non-pathogenic SNPs. For each design, triplicate qPCR traces for no template control (NTC, black), 0% variant (i.e. 100% wildtype allele, green), 1% variant (purple), 100% variant (red), and 100% variant with primers only (blue, dashed) are shown. Results marked with a star were summarized in manuscript Fig. 2e. 4 ng of gDNA was added in each reaction.

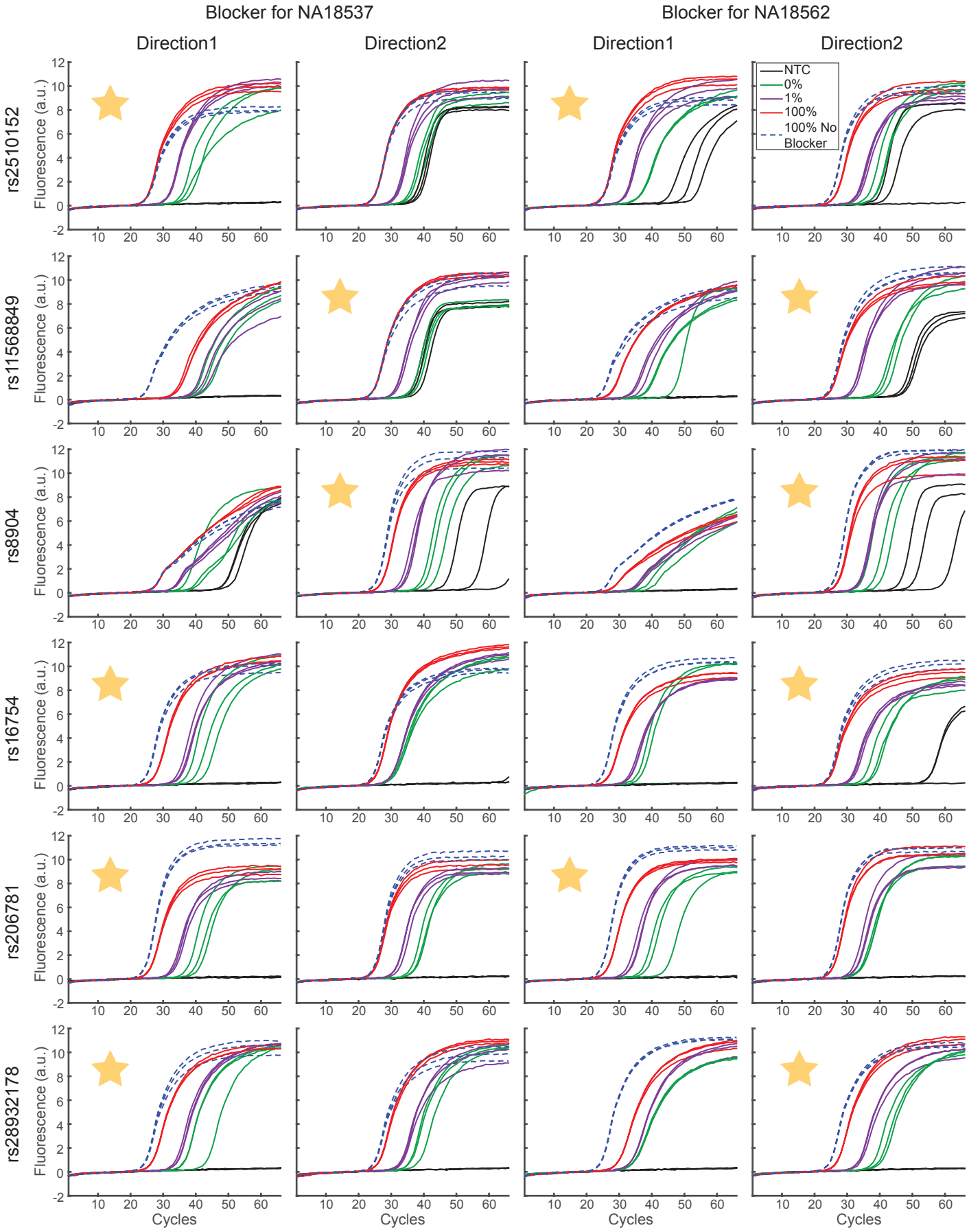


FIG. S3-3: Raw qPCR traces of the BDA designs for non-pathogenic SNPs (continued).

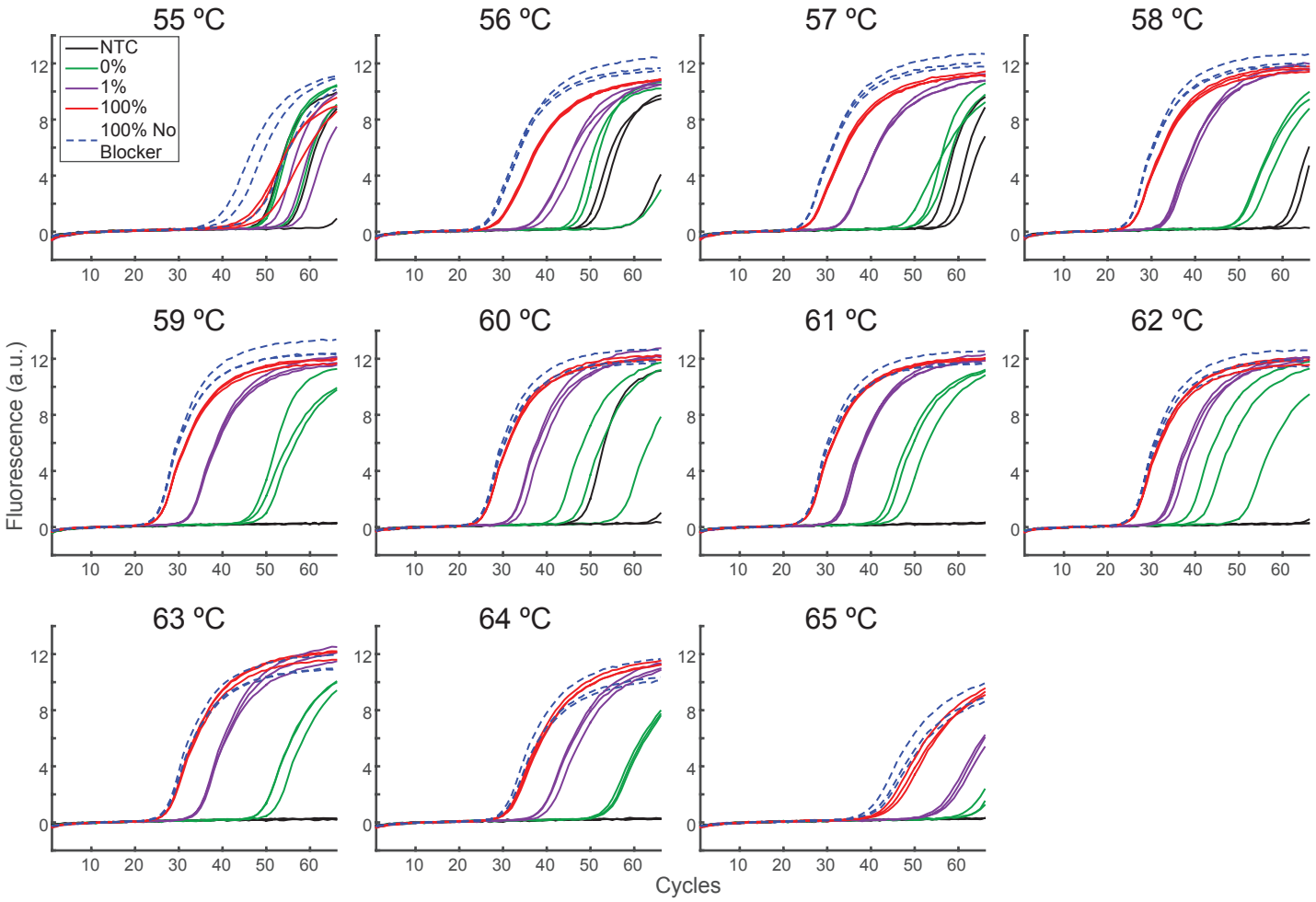


FIG. S3-4: Temperature robustness of the BDA design for rs3789806 (NA18537 blocker, direction 1). gDNA amount was 4 ng per well.

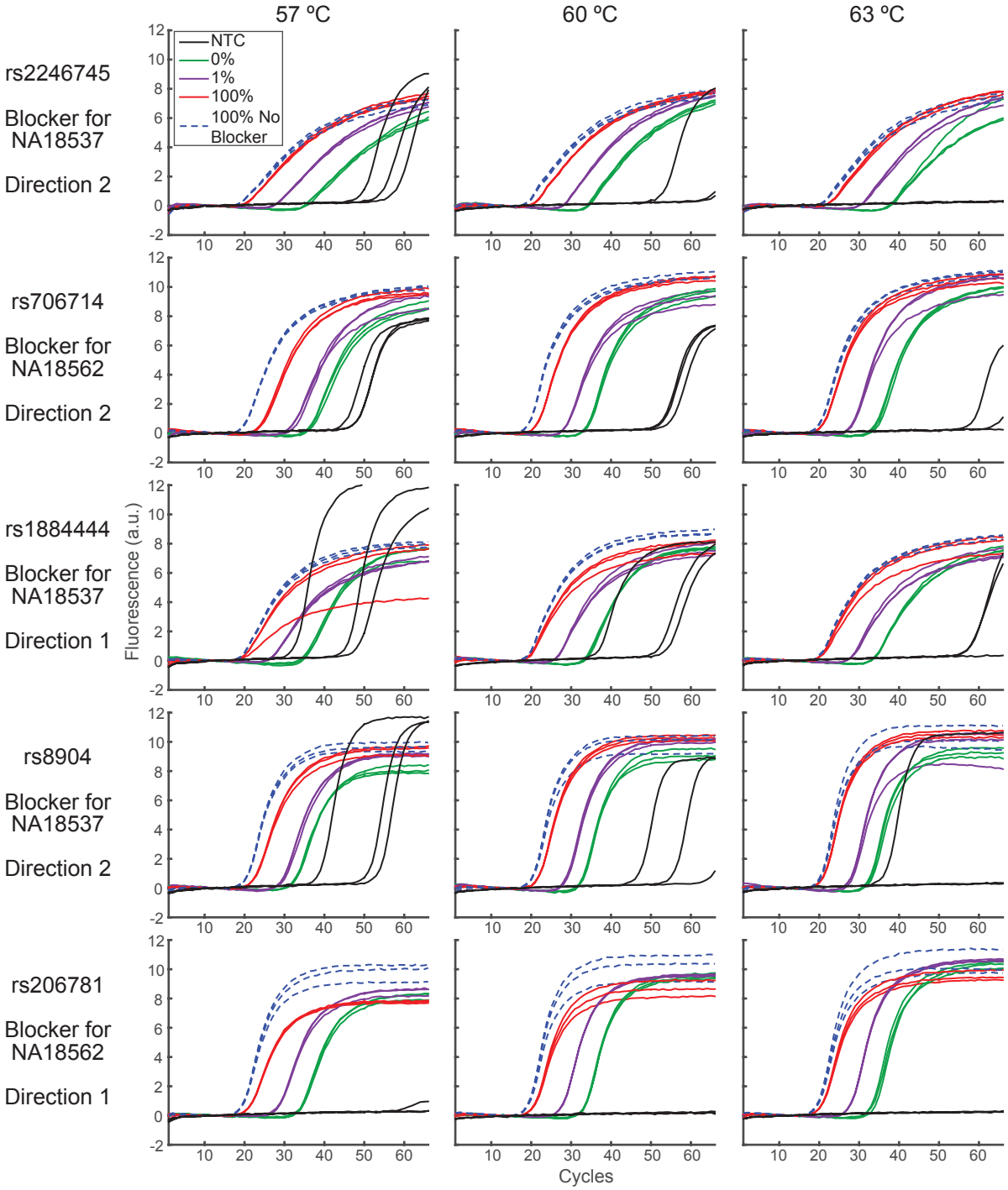


FIG. S3-5: Temperature robustness of the BDA designs for rs2246745, rs706714, rs1884444, rs8904, and rs206781. gDNA amount was 200 ng per well.

Section S4: BDA Design Flexibility

For enrichment of a specific Variant sequence, many BDA designs will yield good enrichment, as long as design principles are followed. This flexibility further facilitates the design of highly multiplexed BDA systems, because specific BDA designs for enriching different loci may be incompatible with one another (e.g. tendency to form primer dimers).

In this section, we show examples of BDA systems yielding similar ΔCt for a given variant. In Fig. SX-1, all six designs for the non-pathogenic SNP rs3789806 tested yielded ΔCt > 13 with 4 ng gDNA input.

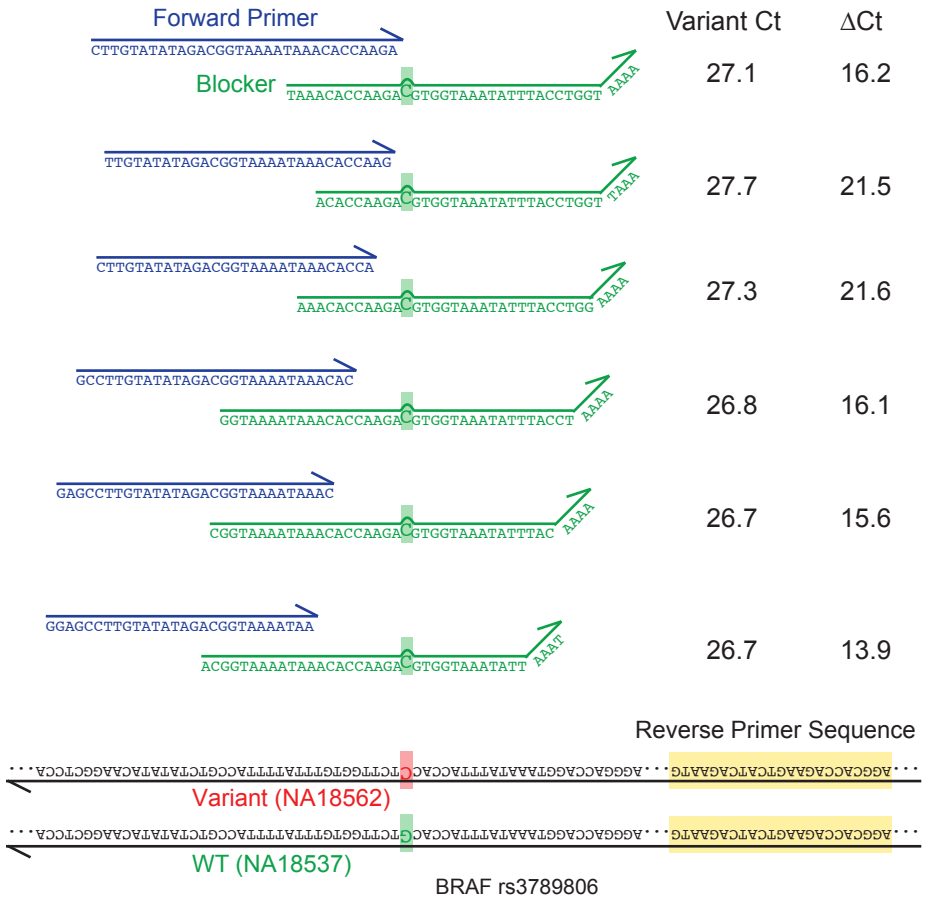


FIG. S4-1: 6 BDA systems yielding similar ΔCt for a given variant rs3789806. The Ct values shown are median of triplicate experiments.

Section S5: Variability of BDA Ct for WT Amplification (0% VAF)

For samples with 0% VAF (all Wildtype), there was significant variability observed in the Ct values when sample input was small (e.g. 4 ng in Fig. 2e). We believe that this is due to the stochastic nature of the early rounds of PCR, when the actual number of amplicon molecules generated in each cycle of PCR may differ significantly from calculated by mass-action kinetics. To obtain a good estimate for the "typical" enrichment expected from BDA, we performed 96 replicate experiments of 0% VAF at each of 4 different conditions (4 ng at 57°C, 4 ng at 60°C, 4 ng at 63°C, and 200 ng at 60°C); these results are shown in Fig. S5-1. The standard deviation of the 4 ng sample at 60°C is more than 8 times that of the 200 ng sample. The difference in Ct standard deviations based on temperature appears to be much smaller.

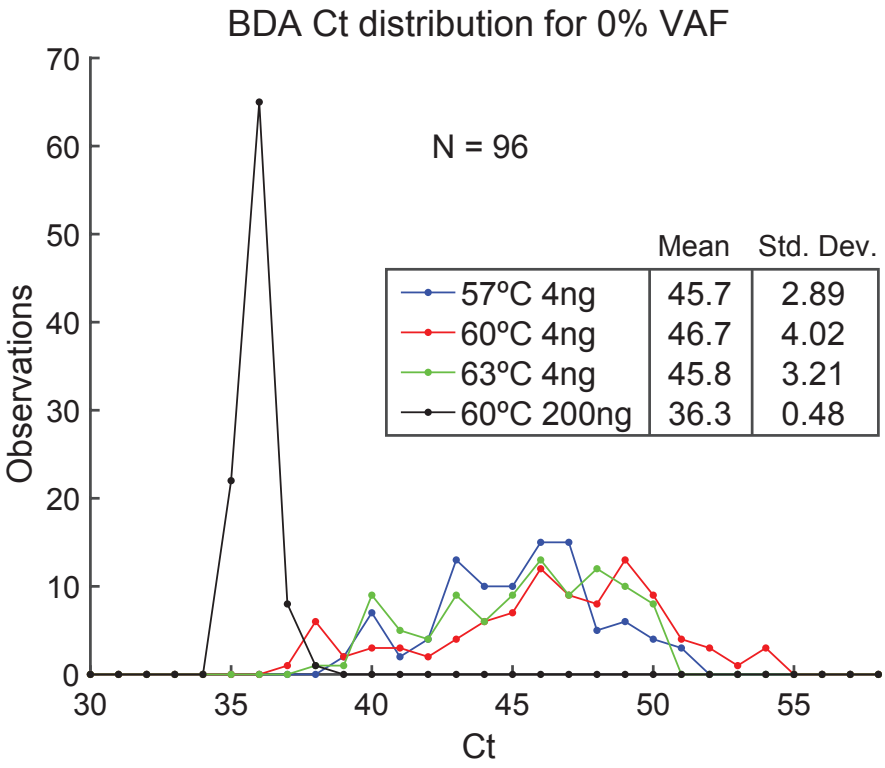


FIG. S5-1: Observed distribution of Ct values for 0% VAF from 96 independent BDA experiments for each condition. The amount of gDNA input strongly impacts the Ct variance, while anneal/extend temperature appears to have at most a minor effect.

Section S6: PCR Enrichment using an LNA Clamp Oligonucleotide

The operational principle behind locked nucleic acid (LNA) clamp enrichment PCR is that DNA polymerases are unable to displace or cleave a hybridized LNA clamp oligonucleotide bound to a template molecule (Fig. S6-1a). By designing the clamp to bind to the WT template with a melting temperature $T_{M,WT}$ that is above the PCR anneal temperature T_{anneal}, most molecules of WT templates will have an bound clamp oligo, and thus the WT template exhibits low amplification yield. Simultaneously, a Variant template should have a melting temperature $T_{M,Var}$ that is below T_{anneal}, in order to exhibit high amplification yield.

Exiqon holds the exclusive license to manufacture LNA, and provides a webtool for estimating $T_{M,WT}$ from sequence. This webtool predicted that an 8 nt LNA clamp (Fig. S6-1b) with the rs3789806 SNP in the middle would have $T_{M,WT} = 65°\text{C}$, and we expected that this would be reasonable for our PCR assays with 60°C anneal temperatures. To maximize the comparability between LNA clamp PCR and BDA, we designed the LNA clamp to bind to the same strand as the BDA Blocker, and made the mismatch position for the Variant the center of the LNA clamp to maximize $\Delta\Delta G°$. The final LNA clamp concentration was set at 4 μM, the same as the BDA Blocker concentration; the concentrations of the forward and reverse primers are 400 nM just as in the BDA experiments.

Unexpectedly, we saw no difference in the cycle threshold (Ct) values for the two SNP variants using the NA18562 and NA18537 genomic DNA samples. From these results, we hypothesized that actual melting temperatures in the qPCR solution are significantly lower than predicted by Exiqon. We next designed and tested an LNA clamp that was extended by one nucleotide, which Exiqon predicted to have $T_{M,WT} = 77°\text{C}$ (Fig. S6-1c). This LNA clamp did show significant Ct differences for 100% and 0% VAF, with maximal ΔCt at 56 and 57°C. Consequently, it is likely that LNA melting temperatures were over-estimated by 15 to 20°C for the Applied Biosystems PowerUP buffer. Even at the optimized 57°C temperature, ΔCt values observed were smaller than that for BDA.

Another limitation of LNA clamp PCR that became apparent to us through the course of these experiments is the short length of the enrichment region. Because the functional LNA clamp is only 9 nt long, and mismatches at positions 1, 2, 8, or 9 likely have weaker $\Delta\Delta G°$ values due to boundary effects, the enrichment region is thus at most 5 nt long.

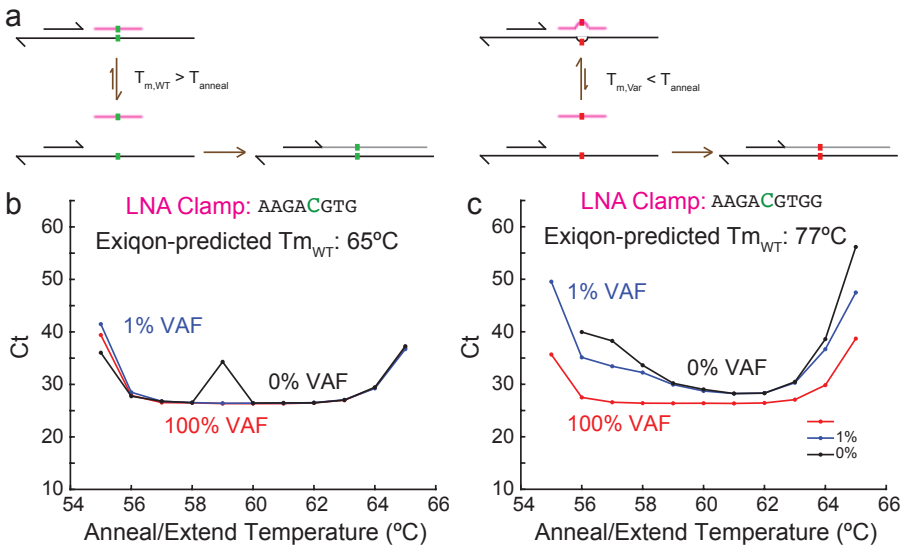


FIG. S6-1: PCR enrichment with an LNA clamp. **(a)** The LNA clamp oligonucleotide is designed to be perfectly complementary to the WT template, and harbor a mismatch against the Variant template. The melting temperature of the clamp to the WT template ($T_{M,WT}$) is designed to be higher than the PCR anneal step temperature, while the melting temperature of the clamp to the Variant template ($T_{M,Var}$) is designed to be lower than the PCR anneal step temperature. Because the DNA polymerase is unable to displace or destroy the LNA clamp, there is a differential amplification yield for the WT and Variant templates. There is a 3 nt gap between the 3′ end of the forward primer and the 5′ end of the LNA clamp. **(b)** Design and qPCR results for an LNA clamp with melting temperature predicted to be 65°C by Exiqon webtool; this design was initially expected to be optimal for our typical 60 °C anneal temperature. Experimental results indicated essentially no difference between 100% VAF and 0% VAF (except for the outlier at 59°C). **(c)** Design and qPCR results for an LNA clamp with melting temperature predicted to be 77 °C by Exiqon webtool. This is the result shown in the main paper; significant ΔCt is observed at 56°C and 57°C.

Section S7: Multiplexed qPCR BDA Results using Taqman Probe Readouts

This section contains the raw data of the 3-plex and 9-plex Taqman probe multiplexing experiments (manuscript Fig. 4bc). The triplicate Ct values of the 3-plex experiments on cfDNA samples (manuscript Fig. 4b) are shown as dots in Fig. S7-1. For the 9-plex experiments, average Ct and standard deviation values of the triplicates can be found in Fig. S7-2, and corresponding raw qPCR traces from the 3 fluorescence channels (Texas Red, HEX, and Cy5) are shown separately in Fig. S7-3 and Fig. S7-4.

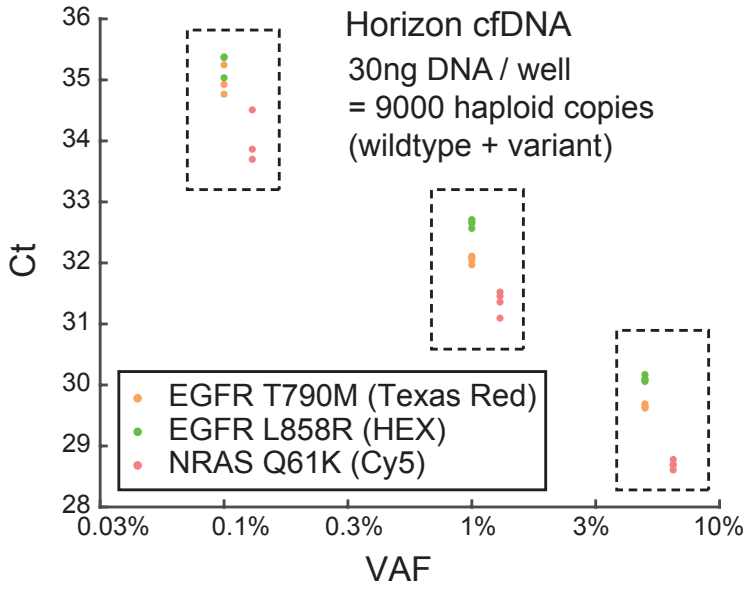
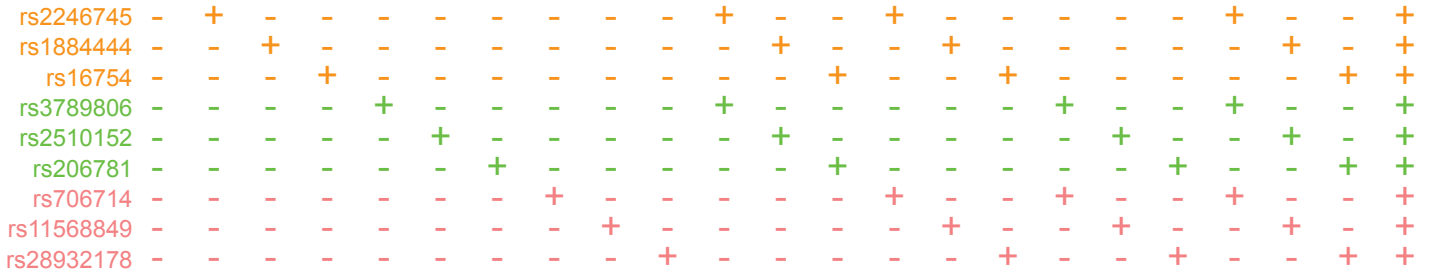


FIG. S7-1: 3-plex BDA on cfDNA reference samples with triplicate Ct values shown as dots.



Texas Red	Mean	35.23	26.69	27.32	27.60	35.39	35.21	35.08	35.36	35.75	34.93	27.03	27.01	27.73	26.78	26.99	27.76	35.43	34.90	35.48	26.87	27.45	27.89	25.47
	Std	0.50	0.11	0.08	0.25	0.24	0.05	0.27	0.07	0.87	0.47	0.08	0.36	0.09	0.28	0.30	0.20	1.16	0.23	0.48	0.19	0.19	0.33	0.08
HEX	Mean	34.16	33.96	34.00	33.78	30.13	27.04	28.29	34.04	33.97	34.01	29.87	26.84	28.02	33.87	33.99	33.79	29.74	26.91	28.29	29.85	26.74	28.31	25.79
	Std	0.11	0.12	0.17	0.12	0.05	0.05	0.06	0.18	0.34	0.14	0.01	0.07	0.05	0.21	0.16	0.37	0.08	0.02	0.17	0.14	0.02	0.16	0.04
Cy5	Mean	35.53	35.21	35.83	35.99	35.06	35.81	35.17	26.33	26.99	30.58	35.17	35.69	35.57	26.45	27.01	31.12	26.63	26.98	31.04	26.74	26.79	31.05	25.70
	Std	0.26	0.26	0.37	0.39	0.29	0.45	0.16	0.05	0.17	0.12	0.27	0.39	0.48	0.30	0.18	0.05	0.11	0.24	0.17	0.14	0.09	0.19	0.06

FIG. S7-2: Mean Ct values and standard deviation (Std) values of the triplicates for each experiment in 9-plex Taqman probe multiplexed assays.

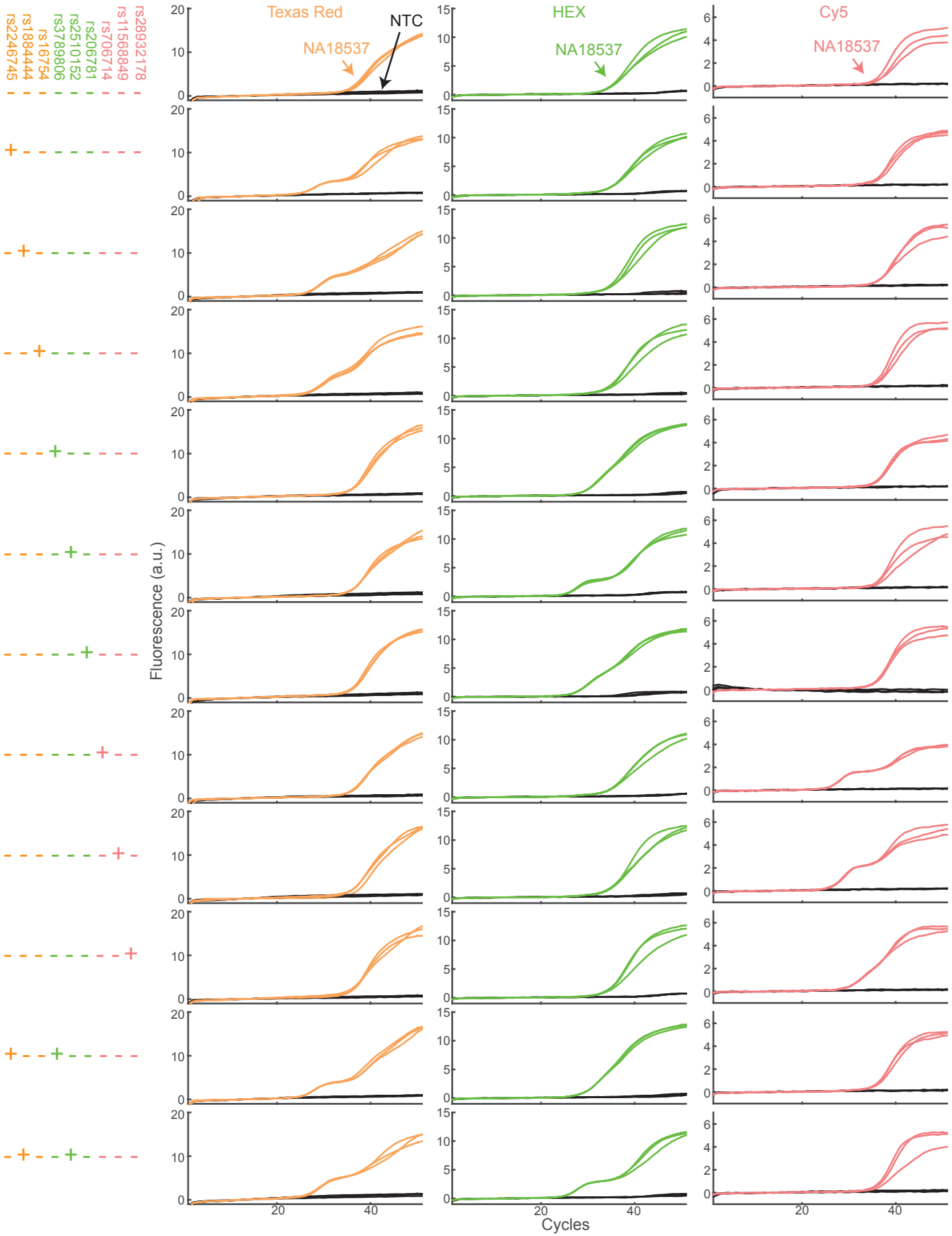


FIG. S7-3: Raw qPCR traces of the 9-plex Taqman probe multiplexed assays (experiment 1-12). No template control (NTC) experiments were performed in triplicates and shown as black lines.

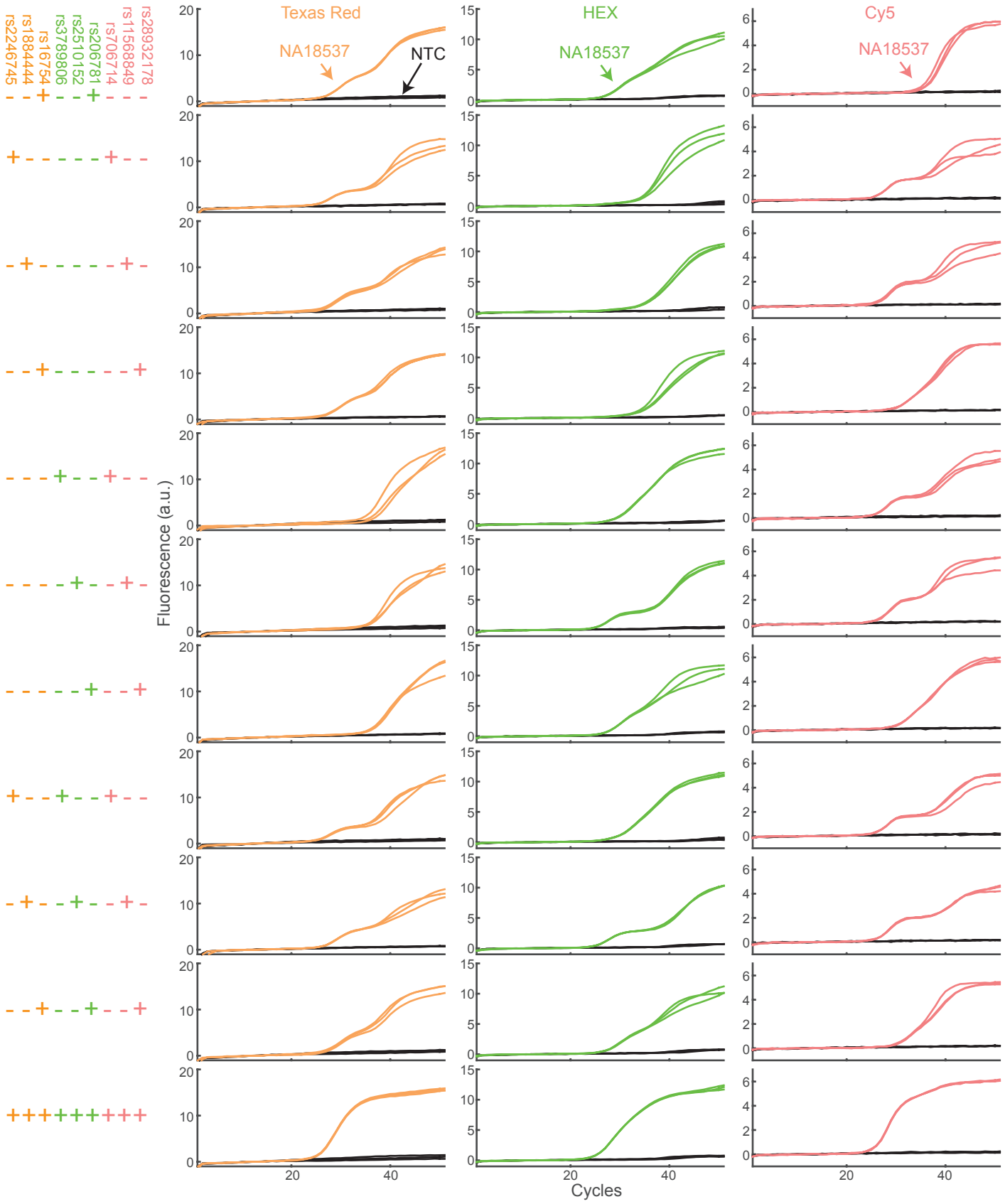


FIG. S7-4: Raw qPCR traces of the 9-plex Taqman probe multiplexed assays (experiment 13-23).

Section S8: BDA using a Low-cost Thermocycler

A variety of low-cost thermocyclers are available commercially for prices significantly below traditional high-performance PCR and qPCR machines (e.g. Eppendorf, Applied Biosystems, Roche, Qiagen). Two specific examples are the MiniPCR (Amplyus, http://www.minipcr.com/) and the OpenPCR (Chai, http://openpcr.org/); both instruments are available for less than $700 as of this writing. Furthermore, they are significantly lighter and smaller than traditional instruments, at roughly 500 g (MiniPCR) and 3.5 kg (OpenPCR). In exchange, these systems sacrifice some degree of temperature control precision and uniformity. Because BDA maintains selectivity across a wide range of anneal/extend step temperatures, it is uniquely capable for distinguishing single nucleotide variants and polymorphisms on these portable platforms. Fig. S8-1a picture of the miniPCR system, and PAGE analysis of amplicons generated by applying BDA to SNP genotyping in single-plex setting to human gDNA on the miniPCR platform.

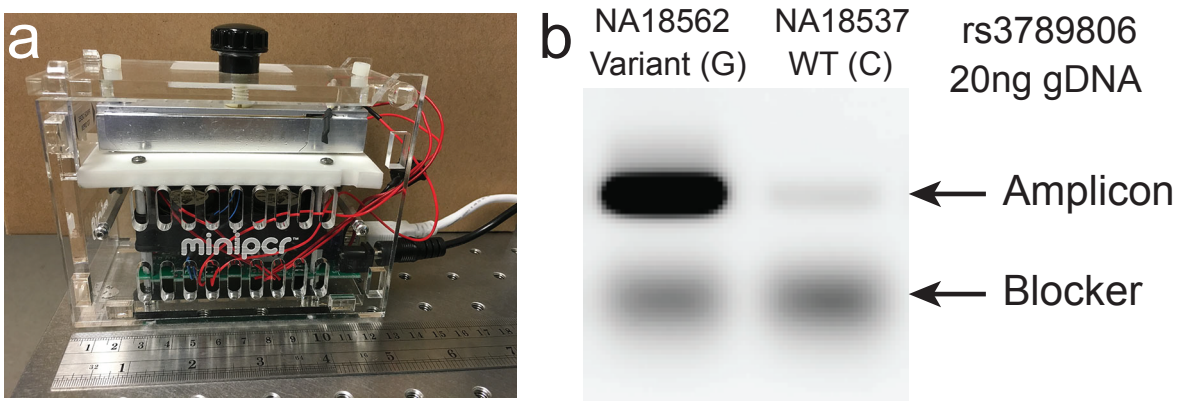


FIG. S8-1: BDA on the MiniPCR platform. **(a)** Picture of the MiniPCR instrument with a ruler as size reference. **(b)** Polyacrylamide gel electrophoresis (PAGE) analysis of BDA amplicons after 40 rounds of PCR, amplifying 20 ng of the NA18562 and NA18537 genomic DNA samples. The Blocker was designed against the NA18537 allele.

Section S9: Systematic Hotspot Assay

We tested all possible single-base replacement variants in the enrichment region of the BDA primer/blocker sets used to detect the rs706714 and rs2510152 SNPs. For the rs706714 BDA system, the full length of the blocker-unique binding region is 22 nt; based on our experience, if a variant is at the 3′-most 4 bases of the blocker binding region, BDA performance is usually suboptimal. 54 synthetic templates were constructed, each bearing a different SNV in the 18 nt enrichment region. Similarly, for the rs2510152 BDA system, 63 synthetic templates were constructed, each bearing a different SNV in a 21 nt enrichment region.

The synthetic templates (ultramers) were purchased as non-purified DNA, so a portion of the strands have truncations or deletions near the 5′ end, thus cannot be detected by qPCR. In order to ensure that the actual synthetic template concentrations are accurate and consistent with that of gDNA, we first used qPCR to quantitate each synthetic template at 20 fM (final concentration, approximate). The forward and reverse primers in the BDA set were used for these experiments. The weight of 1 copy of human genome is approximately 3.59 pg, so the equivalent gDNA concentration to 20 fM single-stranded synthetic template is 20 fM / 2 $\times N_A \times$ 3.59 pg $\approx$ 20 ng/μL. Here N_A is the Avogadro constant. We compared the obtained synthetic template Ct values ($Ct_{\text{synthetic}}$) to that of 20 ng/μL, or 200 ng per well gDNA (Ct_{gDNA}), and the equivalent gDNA concentration of the synthetic template was calculated as

$$20\text{ng}/\mu\text{L} \times 2^{(Ct_{\text{gDNA}} - Ct_{\text{synthetic}})}$$

Next, we normalized the concentrations and assayed with BDA; the Ct of normalized synthetic templates were compared to the Ct of equivalent amount of wildtype gDNA, assayed by the same primers and blocker.

The experimental ΔCt values are plotted against the calculated $\Delta\Delta G^\circ$ for each SNV in Fig. S9-1; as in Fig. 4b, ΔCt is strongly correlated with $\Delta\Delta G^\circ$ values. Raw qPCR traces of these experiments are shown in Fig. S9-2 to Fig. S9-6.

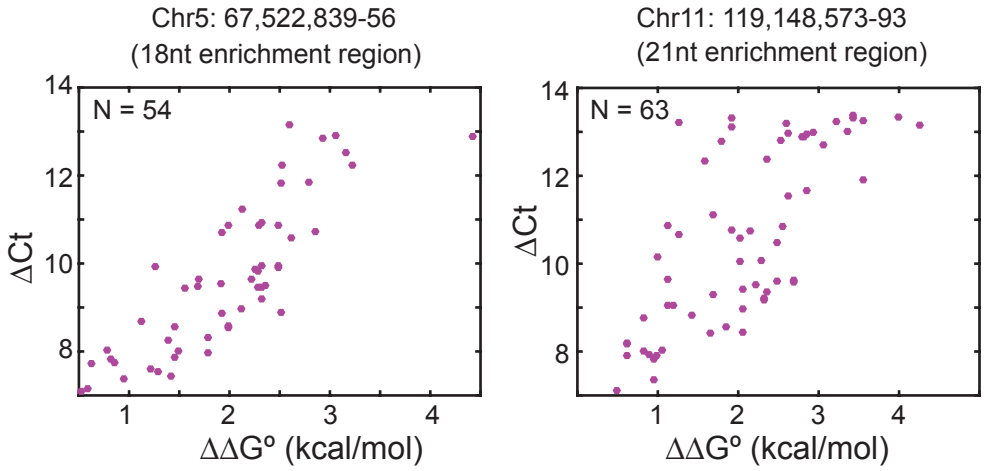


FIG. S9-1: Effect of mismatch triplet $\Delta\Delta G^\circ$ on experimental ΔCt.

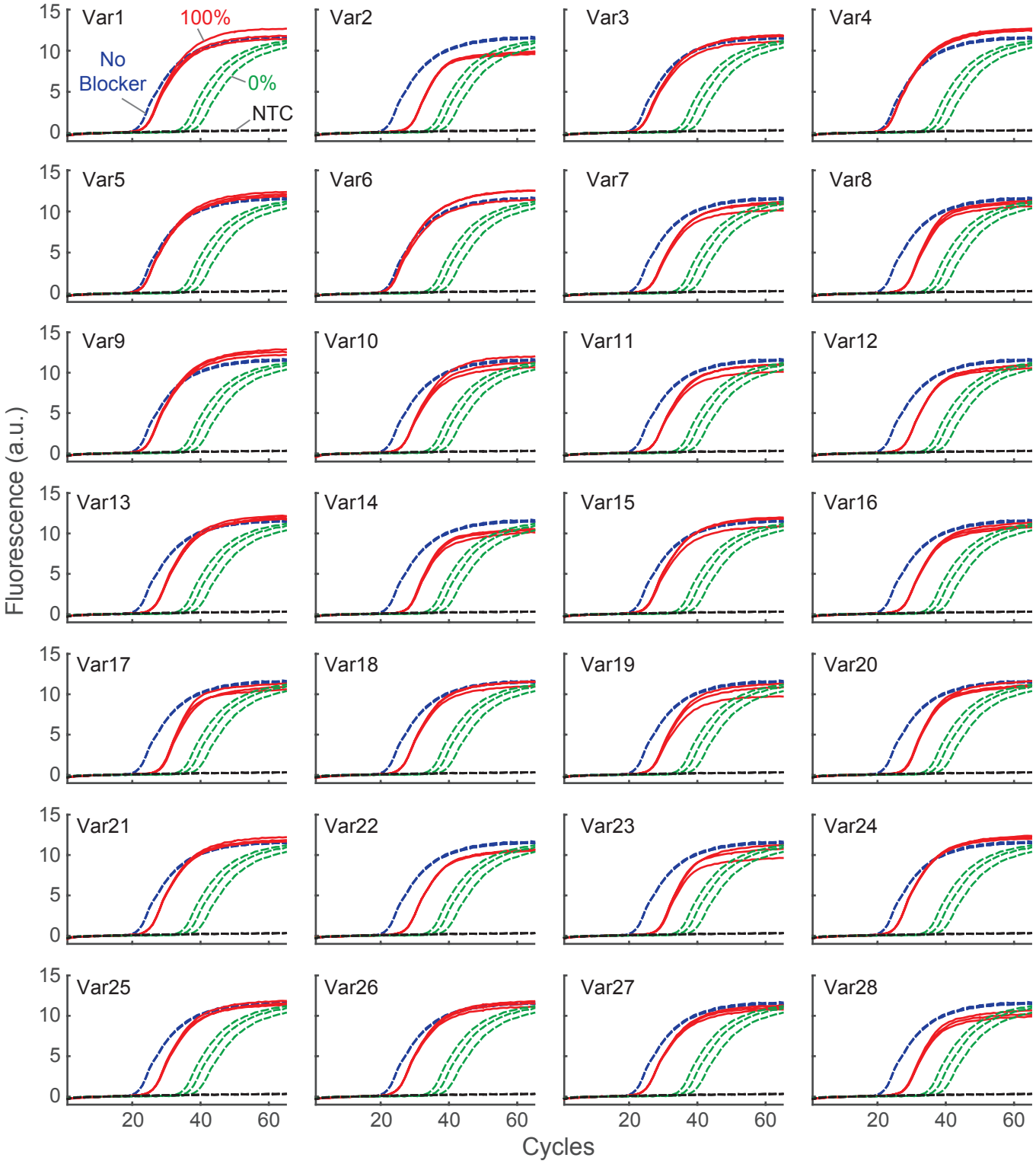


FIG. S9-2: BDA systematic hotspot assay results on synthetic templates bearing SNVs on chromosome 5, nucleotides 67,522,839 to 67,522,956 (near SNP rs706714). The BDA set used was for SNP rs706714 direction 1; 25 ng per well gDNA (NA18562) was used in the "0%" and "No Blocker" experiments. Equivalent amount of synthetic templates were used in the "100%" experiments. "0%", "No Blocker", and "NTC" traces displayed on each panel are shown as a reference, and correspond to the same set of experimental results for all plots.

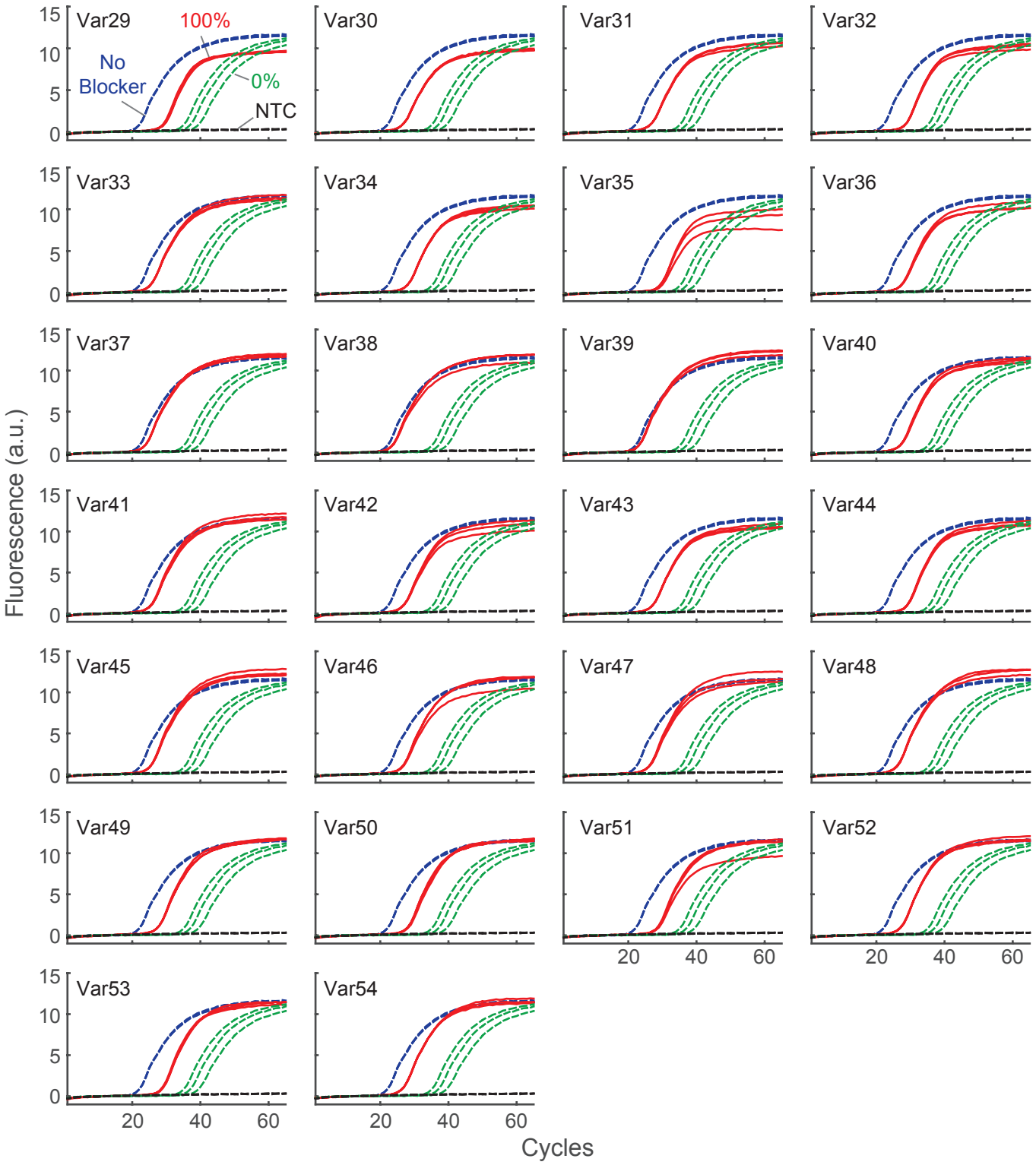


FIG. S9-3: Additional qPCR traces for BDA on synthetic templates bearing SNVs on chromosome 5, positions 67,522,839 to 67,522,956 (near SNP rs706714).

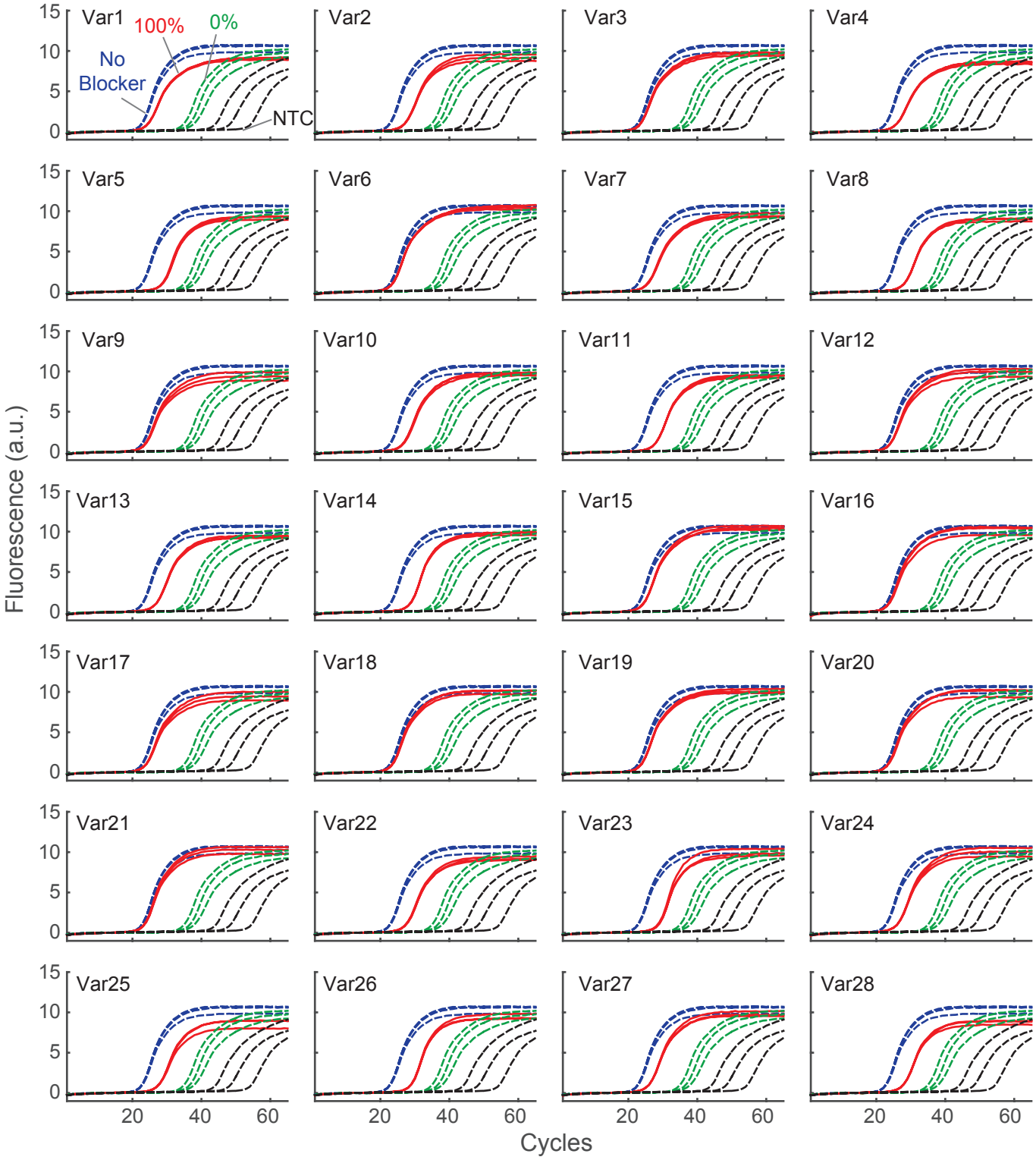


FIG. S9-4: BDA systematic hotspot assay results on synthetic templates bearing SNVS on chromosome 11, nucleotides 119,148,573 to 119,148,593 (near SNP rs2510152). The BDA set used was for SNP rs2510152 direction 1; other experimental conditions are the same as for Fig. S9-2. "0%", "No Blocker", and "NTC" traces displayed on each panel are shown as a reference, and correspond to the same set of experimental results for all plots.

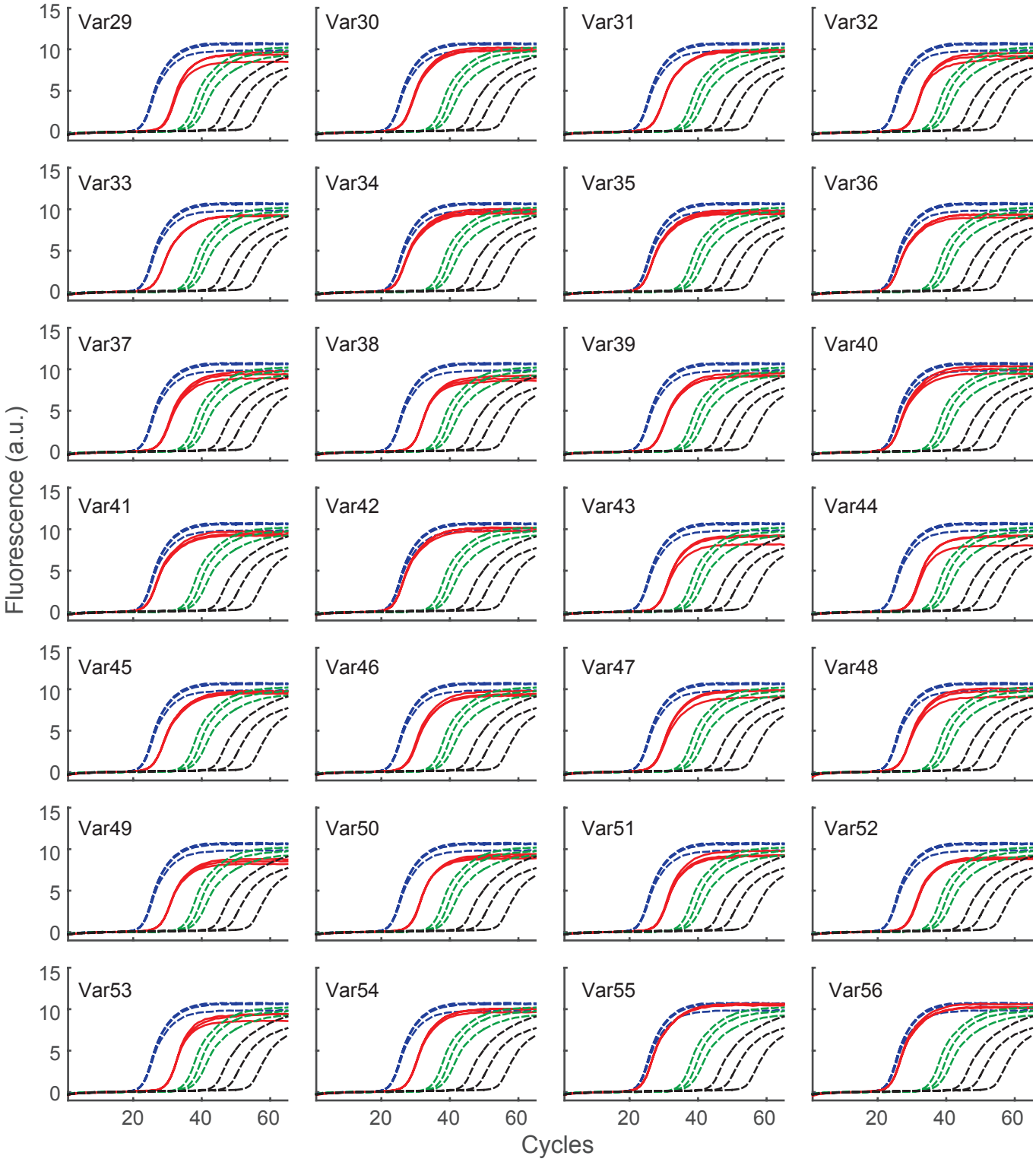


FIG. S9-5: Additional qPCR traces for BDA on synthetic templates bearing SNVs on chromosome 11, nucleotides 119,148,573 to 119,148,593 (near SNP rs2510152).

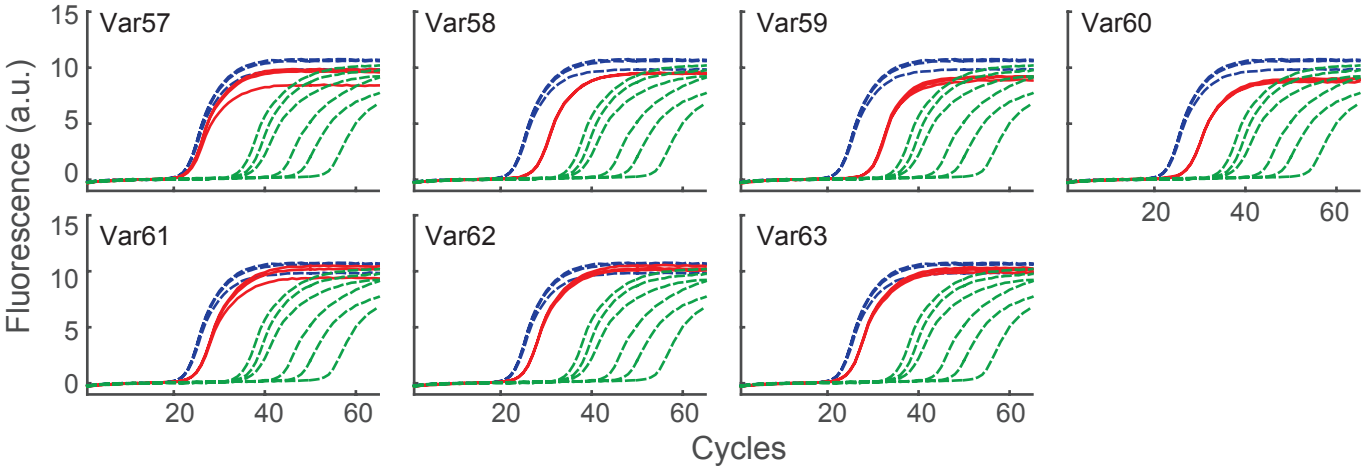


FIG. S9-6: Additional qPCR traces for BDA on synthetic templates bearing SNVs on chromosome 11, nucleotides 119,148,573 to 119,148,593 (near SNP rs2510152).

Section S10: Enhancing BDA Enrichment

The allele enrichment achieved by BDA is limited by two factors: (1) DNA polymerase nucleotide misincorporation events that create *de novo* Variants subsequently enriched by BDA [1], and (2) imperfect suppression of WT template amplification by the Blocker. Limitation (1) can be mitigated through the use of high-fidelity DNA polymerases with 3′ to 5′ proofreading activity, and (2) can be mitigated through the use of a second Blocker for the reverse primer. For different Variant/WT pairs, either (1) or (2) may be dominant; thus, addressing each of these two problems results in improved BDA performance for a subset of Variant/WT sequence pairs. Judicious application of these two approaches allows BDA enrichment to be enhanced for a large fraction of, if not all, Variant/WT pairs.

Enhancing enrichment by high-fidelity enzyme. BDA using the Taq polymerase is limited by enzyme misincorporation events. As shown in Fig. S10-1a, in the enrichment region of the BDA system amplifying a purely WT sample for rs3789806, G>A misincorporation events (boxed) resulted in a significant fraction of the amplicons bearing variant sequence. Similar BDA on pure WT sample using the KAPA high-fidelity DNA polymerase does not result in visible *de novo* variations. Because KAPA HiFi possesses 3′ to 5′ exonuclease activity, we used a 3-carbon spacer at the 3′ end of B to prevent extension.

Enhancing enrichment by dual BDA. Fig. S10-1b shows improved variant enrichment using dual BDA to block WT amplification on both the forward and reverse template strands. fB and rB denote forward Blocker and reverse Blocker that interact with the forward Primer fP and reverse Primer rP, respectively. For this (particularly challenging) Variant/WT pair, the ΔCt between 100% Variant and 100% WT is improved from 8.6 (single BDA) to 15.0 (dual BDA), using 400 ng gDNA input.

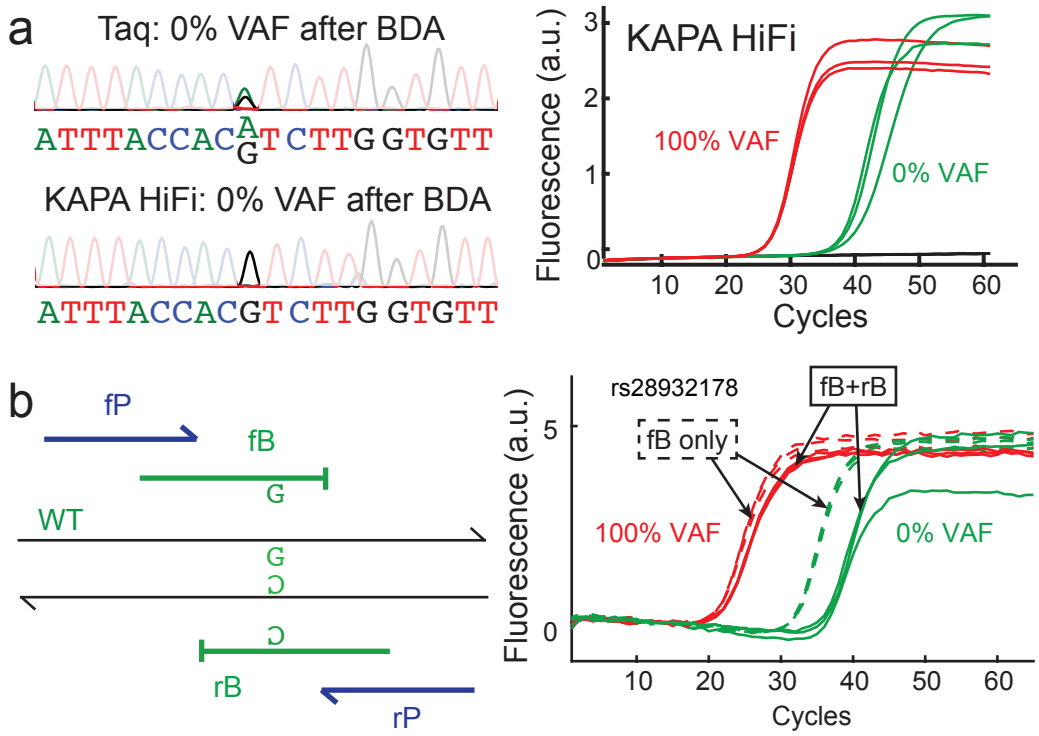


FIG. S10-1: **(a)** Sanger sequencing results and qPCR traces of BDA with KAPA high-fidelity polymerase, compared to Taq polymerase results. **(b)** Dual BDA schematic and qPCR traces compared to single BDA traces.

[1] Hiatt, J. B., Pritchard, C. C., Salipante, S. J., O'Roak, B. J., & Shendure, J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. *Genome research* **23,** 843-854 (2013).

Section S11: Quantitation with BDA

In order to validate BDA quantitation accuracy, we first prepared reference gDNA samples with different VAF and total concentrations. Droplet Digital PCR (ddPCR) quantitation was also performed for comparison. The reference samples were prepared by mixing NA18562 and NA18537 repository cell line gDNA at different ratios, and the VAF represents the fraction of NA18537 in the sample (i.e. NA18562 is WT, see Table S11-1). At the non-pathogenic SNPs of interest, NA18562 and NA18537 are homozygous for different alleles.

We also tested BDA and ddPCR quantitation using Horizon cfDNA reference samples, mixing the 5%, 1%, 0.1% variant with 100% WT samples in the Horizon Multiplex I cfDNA Reference Standard Set, which contains 8 cancer-related mutations of similar VAF (Table S11-1). In Table S11-1, we also showed the total DNA sample amount, the total molecule counts (TMC), and variant molecule counts (VMC); these values are the actual amount put in each experiment, and are the same for BDA and ddPCR experiments. The VAF and total DNA sample amount per experiment values were randomly generated using a Matlab code.

Sample	VAF (%)	ng DNA per expt.	TMC per expt.	VMC per expt.	Sample type
Ga	2.85	27.37	8211	234	gDNA
Gb	0.168	30.22	9066	15	gDNA
Gc	0.437	7.23	2169	9	gDNA
Gd	1.81	11.4	3420	62	gDNA
Ge	15.9	9.93	2979	474	gDNA
Ca	2.85	27.37	8211	234	cfDNA
Cb	0.168	30.22	9066	15	cfDNA
Cc	0.437	7.23	2169	9	cfDNA
Cd	1.81	11.4	3420	62	cfDNA

TABLE S11-1: DNA samples used in comparison of BDA and ddPCR quantitation. G samples refer to genomic DNA reference samples constructed by mixing NA18537 and NA18562. C samples refer to cell-free DNA reference samples constructed by mixing different Horizon cfDNA samples.

BDA Quantitation. In order to quantitate the number of variant molecules in the samples by BDA, we first asymmetrically split each sample: 10% of the sample was mixed with the forward and reverse primers (no Blocker), so the threshold cycle Ct value of this experiment provides information on the total number of DNA molecules bearing the region of interest; 90% of the sample was mixed with primers and Blocker, so the Ct value could be used to calculate variant molecule counts.

In a standard qPCR reaction with no Blockers, the total molecule counts (TMC) and the *Threshold* for Ct determination obey the following equation:

$$\mathrm{TMC} \times (1+\chi)^{\mathrm{Ct}} = Threshold$$

Here the *Threshold* is a fixed value for each set of primers; χ is the efficiency (or yield) of each PCR cycle, and we assumed $\chi = 1$ in a no-Blocker reaction. If we perform a calibration reaction using the a sample with known total molecule counts $\mathrm{TMC}_{\mathrm{calibration}}$ and get a threshold cycle value $\mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration})$, then the total molecule counts in the sample ($\mathrm{TMC}_{\mathrm{sample}}$) can be inferred based on the following formula:

$$\mathrm{TMC}_{\mathrm{calibration}} \times 2^{\mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration})} = 10\% \times \mathrm{TMC}_{\mathrm{sample}} \times 2^{\mathrm{Ct}_{\mathrm{NoBlocker}}(10\%\ \mathrm{sample})}$$

or

$$\mathrm{TMC}_{\mathrm{sample}} = \mathrm{TMC}_{\mathrm{calibration}} \times 2^{(\mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration}) - \mathrm{Ct}_{\mathrm{NoBlocker}}(10\%\ \mathrm{sample}))}/10\%$$

where $\mathrm{Ct}_{\mathrm{NoBlocker}}(10\%\ \mathrm{sample})$ is the Ct of the 10% sample assayed with primers only.

If we consider a sample containing both wildtype and variant molecules, the VMC, TMC, and the threshold cycle assayed by BDA ($\mathrm{Ct}_{\mathrm{BDA}}$) should obey the following formula:

$$\mathrm{Ct}_{\mathrm{BDA}} = k \times \log_2(\frac{\mathrm{VMC}}{\mathrm{TMC}}) + delay + \mathrm{Ct}_{\mathrm{NoBlocker}}$$

where $\mathrm{Ct}_{\mathrm{NoBlocker}}$ is the threshold cycle of the same sample assayed by primers only. Here $k = -1/\log_2(1+\chi)$, and χ is the amplification efficiency of the variant allele, so a more negative k value represents lower efficiency.

The *delay* value represents the Ct delay caused by addition of the Blocker. We assumed that k and *delay* values do not change with sample input amount, so that they can be inferred based on a calibration experiment using standard reference samples with known TMC and VAF (VAF = VMC / TMC). Different BDA designs may have different k and *delay* values, so we calibrated each design separately.

Because the slope k describes the amplification efficiency of the variant molecules and doesn't change with sample input amount, the 90% sample Ct with Blocker ($\mathrm{Ct}_{90\%\ \mathrm{sample}}$), the calibration Ct with Blocker ($\mathrm{Ct}_{\mathrm{calibration}}$), the VMC of the 90% sample ($\mathrm{VMC}_{90\%\ \mathrm{sample}}$), and the VMC of calibration sample ($\mathrm{VMC}_{\mathrm{calibration}}$) obey the following equation:

$$\mathrm{Ct}_{\mathrm{calibration}} - \mathrm{Ct}_{90\%\ \mathrm{sample}} = k \times \log_2 \frac{\mathrm{VMC}_{\mathrm{calibration}}}{\mathrm{VMC}_{90\%\ \mathrm{sample}}}$$

and there is also the BDA Ct equation for the calibration experiment:

$$\mathrm{Ct}_{\mathrm{calibration}} = k \times \log_2(\frac{\mathrm{VMC}_{\mathrm{calibration}}}{\mathrm{TMC}_{\mathrm{calibration}}}) + delay + \mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration})$$

based on the previous 2 equations, we can infer:

$$\mathrm{Ct}_{90\%\ \mathrm{sample}} = k \times \log_2(\frac{\mathrm{VMC}_{90\%\ \mathrm{sample}}}{\mathrm{TMC}_{\mathrm{calibration}}}) + delay + \mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration})$$

After replacing $\mathrm{VMC}_{90\%\ \mathrm{sample}}$ with ($90\% \times \mathrm{VMC}_{\mathrm{sample}}$) and rearrangement, we get

$$\mathrm{VMC}_{\mathrm{sample}} = \mathrm{TMC}_{\mathrm{calibration}} \times 2^{\frac{\mathrm{Ct}_{90\%\ \mathrm{sample}} - \mathrm{Ct}_{\mathrm{NoBlocker}}(\mathrm{calibration}) - delay}{k}} / 90\%$$

Additionally, the wildtype molecule counts (WMC) was calculated as WMC = TMC - VMC.

Calibration of BDA Designs. We performed calibration experiments on 3 BDA designs for cancer mutations, and 3 BDA designs for non-pathogenic mutations; Ct values for 2 standard samples with different VAF and no Blocker experiments are shown in Table S11-2; these Ct values are average of triplicate experiments. k and *delay* values were calculated according to the Ct of the 2 samples with different VAF.

Mutation	**Total Molecules**	**VAF1 (%)**	**VAF1 Ct**	**VAF2 (%)**	**VAF2 Ct**	$\mathbf{Ct}_{\mathrm{NoBlocker}}$	k	*delay*
EGFR L858R	6000	0.5	33.14	5	29.81	25.02	-1.004	0.45
EGFR T790M	6000	0.5	32.81	5	29.62	25.05	-0.961	0.42
NRAS Q61K	6000	0.5	32.72	5	29.34	25.17	-1.018	-0.23
rs2246745	1800	1	34.10	10	30.41	25.62	-1.112	1.10
rs706714	1800	1	33.88	10	30.38	25.81	-1.055	1.07
rs206781	1800	1	36.09	10	32.30	25.69	-1.139	2.83

TABLE S11-2: Calibration results of BDA designs for cancer mutations (first 3) and non-pathogenic mutations (last 3).

BDA Quantitation Results. Ct results for each DNA sample (10% of the sample with primers, 90% of the sample with BDA) are shown in Table S11-3. Since all experiments were done in duplicates, the 10% of the sample with primers and 90% of the sample with BDA Ct values shown are "percentage" average of duplicates, calculated as

$$\text{Percentage average} = -\log_2(2^{-(\text{Replicate1 Ct})} + 2^{-(\text{Replicate2 Ct})}) + 1$$

The inferred VMC and WMC of each sample can also be found in Table S11-3.

ddPCR Quantitation and Results. We designed ddPCR primers and Taqman probes based on online Bio-Rad ddPCR application notes and our experience with probe / primer design. There are 2 Taqman probes for each mutation: one probe bears the FAM fluorophore, and is the perfect match to the variant sequence; the other probe bears the HEX fluorophore, and is the perfect match to the WT sequence (Fig. S11-1a). The binding ΔG° of the probes are similar to those of the primers. We chose primers that are unlikely to form primer dimers, and generate short amplicons (ideally <100 bp) for cancer mutations in the Horizon cfDNA samples.

Sample	Mutation	VMC	WMC	Inferred VAF (%)	Sample VAF (%)
Ga	rs2246745	227.1	5739.9	3.81	2.85
Gb	rs2246745	17.5	7170.1	0.24	0.17
Gc	rs2246745	18.2	1976	0.91	0.44
Gd	rs2246745	59	2378.2	2.42	1.81
Ge	rs2246745	416	1800.5	18.77	15.90
Ga	rs706714	206.2	6450.9	3.1	2.85
Gb	rs706714	13.6	6775.6	0.2	0.17
Gc	rs706714	9.5	1651.4	0.57	0.44
Gd	rs706714	55.5	2626.2	2.07	1.81
Ge	rs706714	500.4	2687.5	15.7	15.90
Ga	rs206781	211.7	9234.6	2.24	2.85
Gb	rs206781	21.4	10500.3	0.2	0.17
Gc	rs206781	15	2671.5	0.56	0.44
Gd	rs206781	73.7	4049.7	1.79	1.81
Ge	rs206781	394.2	2893.2	11.99	15.90
Ca	EGFR T790M	245.6	9005.1	2.66	2.85
Cb	EGFR T790M	15.7	8987.2	0.17	0.17
Cc	EGFR T790M	8.2	2547.5	0.32	0.44
Cd	EGFR T790M	76.5	3681.6	2.03	1.81
Ca	EGFR L858R	348.4	8058.1	4.14	2.85
Cb	EGFR L858R	18.6	8301.9	0.22	0.17
Cc	EGFR L858R	5.5	2482.4	0.22	0.44
Cd	EGFR L858R	89.7	3800.3	2.31	1.81
Ca	NRAS Q61K	245.8	8011.3	2.98	3.71
Cb	NRAS Q61K	14.9	8340.9	0.18	0.22
Cc	NRAS Q61K	9.8	2181.8	0.45	0.57
Cd	NRAS Q61K	97.9	3343.3	2.84	2.35

TABLE S11-3: BDA quantitation results of gDNA and cfDNA samples.

We used customized Matlab code to process the raw droplet fluorescence data (attached at the end of this section). A HEX (WT) channel threshold and a FAM (Variant) channel threshold were selected by hand to separate positive and negative droplets; the same threshold values were applied to all the experiments using the same set of ddPCR primers and Taqman probes. Fig. S11-1b show examples of data processing: green dots are HEX (WT) positive droplets, blue dots are FAM (Variant) positive droplets, red dots are double positive droplets containing both WT and Variant templates, and black dots are negative droplets with no target molecule in them. VAF was calculated as: VAF = (VMC)/(VMC + WMC); VMC is the sum of FAM positives and double positives, and WMC is the sum of HEX positives and double positives.

Table S11-4 shows ddPCR Variant Counts and WT Counts data from all the experiments; duplicates are shown separately.

Quantitation Summary. Summary figures of BDA and ddPCR quantitation results of gDNA and Horizon cfDNA samples are shown in Fig. S11-2 and Fig. S11-3. ddPCR showed more sample loss than BDA in both gDNA and cfDNA samples.

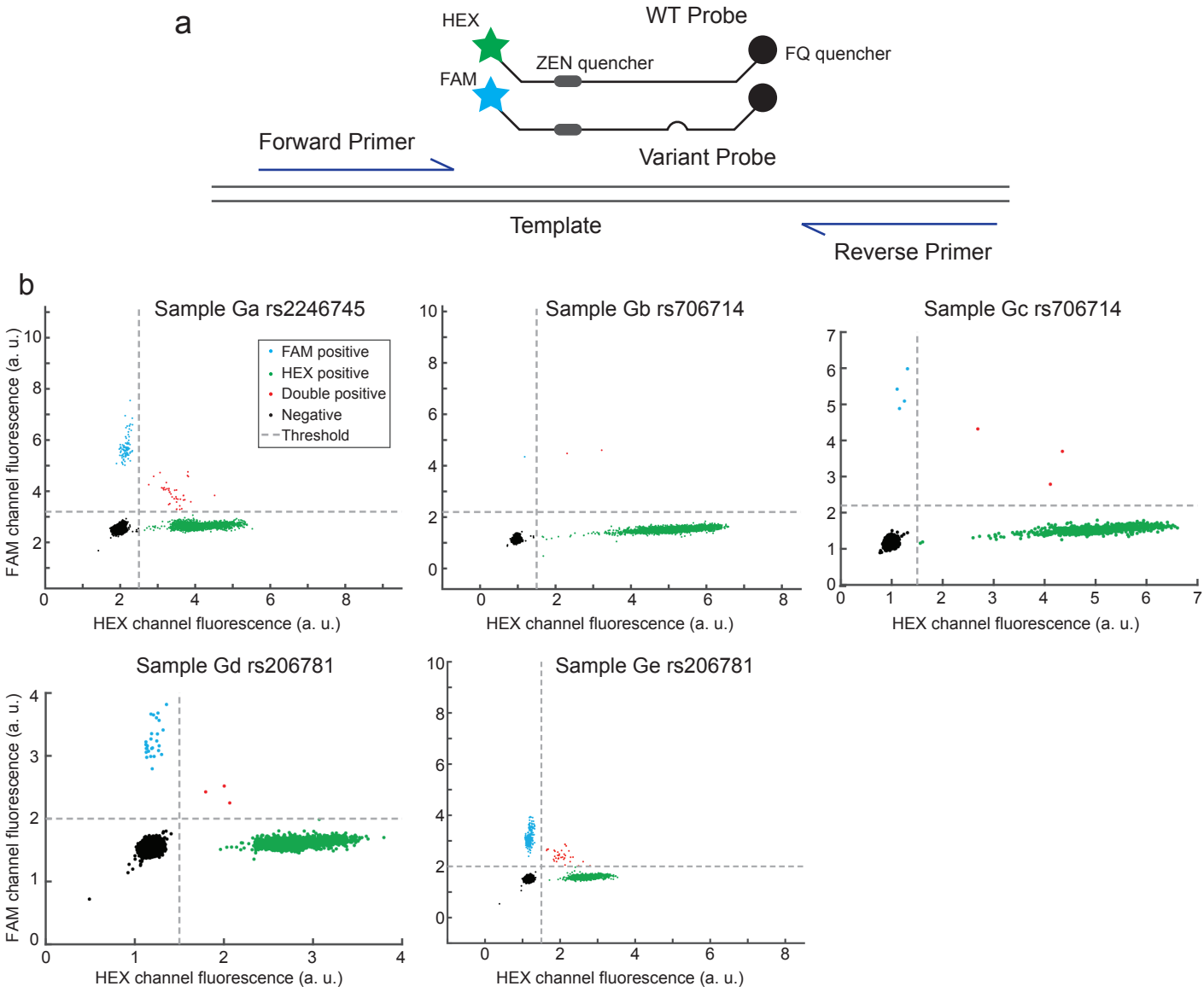


FIG. S11-1: ddPCR VAF quantitation assays. **(a)** Schematic of Primers and Taqman probes in a ddPCR design. **(b)(c)** Examples of ddPCR plot and data analysis.

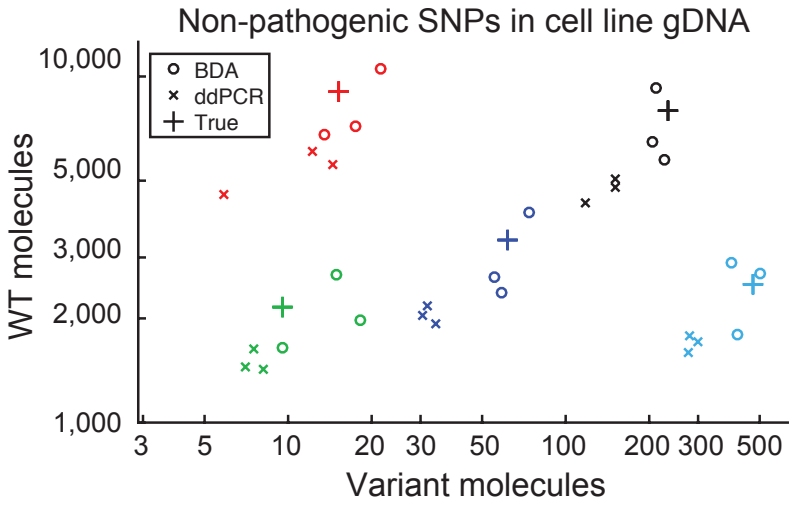


FIG. S11-2: Quantitation summary of non-pathogenic mutations. Different colors represent different gDNA samples (Ga∼Ge).

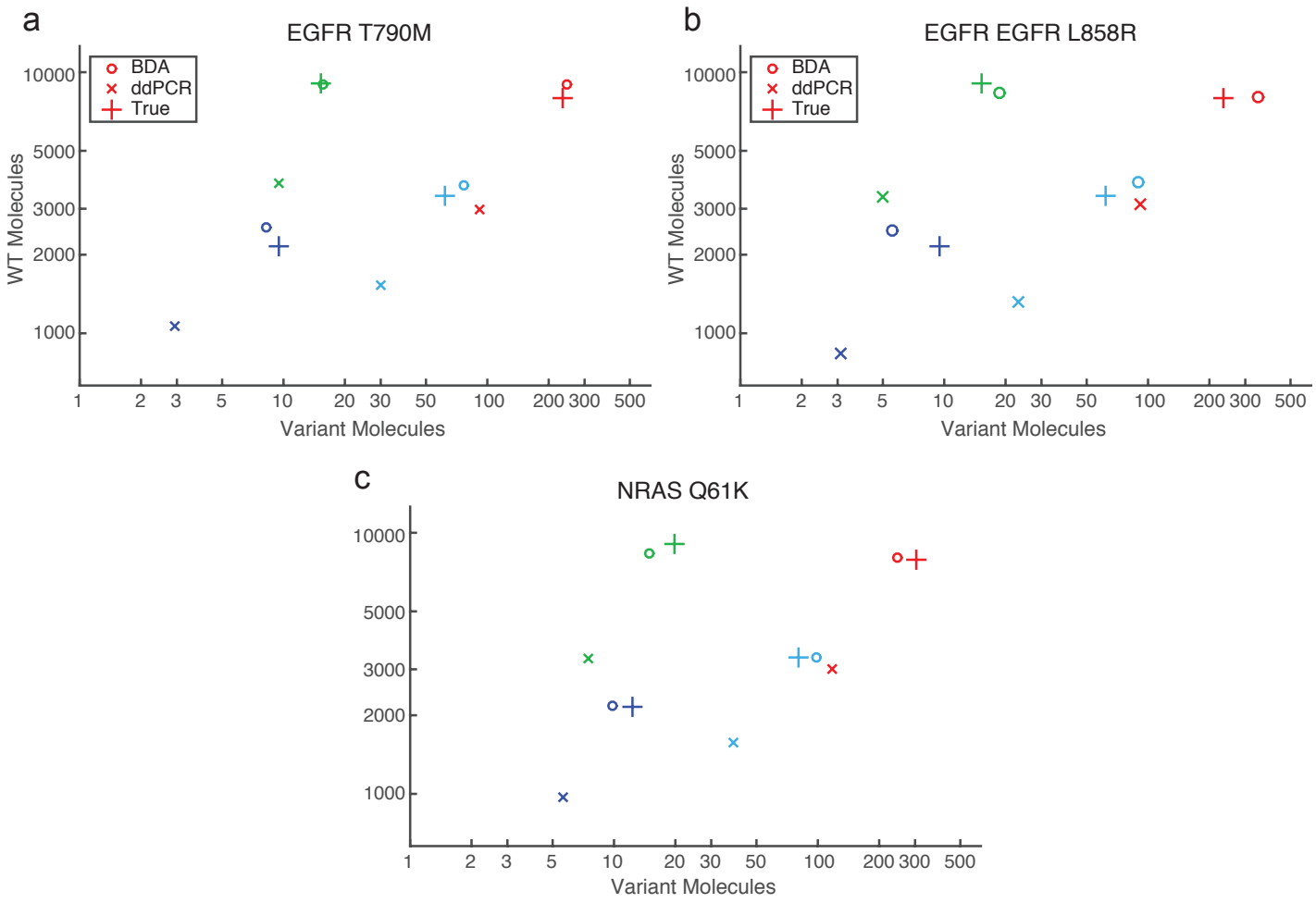


FIG. S11-3: Horizon cfDNA quantitation summary. Different colors represent different cfDNA samples (Ca∼Cd). **(a)** Summary of EGFR T790M. **(b)** Summary of EGFR L858R. **(b)** Summary of NRAS Q61K.

Sample	Mutation	VMC	WMC	Inferred VAF (%)	Sample VAF (%)
Ga	rs2246745	130	4401	2.87	2.85
Ga	rs2246745	105	4199	2.44	2.85
Gb	rs2246745	17	5760	0.29	0.168
Gb	rs2246745	12	5376	0.22	0.168
Gc	rs2246745	9	1445	0.62	0.437
Gc	rs2246745	5	1442	0.35	0.437
Gd	rs2246745	30	1955	1.51	1.81
Gd	rs2246745	38	1914	1.95	1.81
Ge	rs2246745	281	1664	14.45	15.9
Ge	rs2246745	273	1519	15.23	15.9
Ga	rs706714	162	5353	2.94	2.85
Ga	rs706714	139	4769	2.83	2.85
Gb	rs706714	8	4052	0.20	0.168
Gb	rs706714	3	5044	0.06	0.168
Gc	rs706714	9	1300	0.69	0.437
Gc	rs706714	7	1545	0.45	0.437
Gd	rs706714	27	2074	1.29	1.81
Gd	rs706714	37	2268	1.61	1.81
Ge	rs706714	262	1635	13.81	15.9
Ge	rs706714	299	1918	13.49	15.9
Ga	rs206781	125	4683	2.60	2.85
Ga	rs206781	178	4913	3.50	2.85
Gb	rs206781	14	5539	0.25	0.168
Gb	rs206781	10	6575	0.15	0.168
Gc	rs206781	8	1660	0.48	0.437
Gc	rs206781	7	1594	0.44	0.437
Gd	rs206781	31	2068	1.48	1.81
Gd	rs206781	30	2010	1.47	1.81
Ge	rs206781	297	1859	13.78	15.9
Ge	rs206781	297	1560	15.99	15.9
Ca	EGFR T790M	89	2781	3.10	2.85
Ca	EGFR T790M	94	3154	2.89	2.85
Cb	EGFR T790M	8	3457	0.23	0.17
Cb	EGFR T790M	11	4060	0.27	0.17
Cc	EGFR T790M	2	963	0.21	0.44
Cc	EGFR T790M	4	1158	0.34	0.44
Cd	EGFR T790M	30	1552	1.90	1.81
Cd	EGFR T790M	30	1507	1.95	1.81
Ca	EGFR L858R	92	3075	2.90	2.85
Ca	EGFR L858R	92	3173	2.82	2.85
Cb	EGFR L858R	3	3326	0.09	0.17
Cb	EGFR L858R	7	3352	0.21	0.17
Cc	EGFR L858R	4	764	0.52	0.44
Cc	EGFR L858R	2	918	0.22	0.44
Cd	EGFR L858R	21	1352	1.53	1.81
Cd	EGFR L858R	25	1273	1.93	1.81
Ca	NRAS Q61K	122	3019	3.88	3.71
Ca	NRAS Q61K	113	3018	3.61	3.71
Cb	NRAS Q61K	7	3305	0.21	0.22
Cb	NRAS Q61K	8	3281	0.24	0.22

Sample	Mutation	Variant Counts	WT Counts	Inferred VAF (%)	Sample VAF (%)
Cc	NRAS Q61K	7	915	0.76	0.57
Cc	NRAS Q61K	4	1032	0.39	0.57
Cd	NRAS Q61K	33	1589	2.03	2.35
Cd	NRAS Q61K	44	1556	2.75	2.35

TABLE S11-4: ddPCR quantitation results of gDNA and cfDNA samples; data of duplicates are shown separately.

Function “ddPCR2D.m” for analysis of ddPCR droplet fluorescence data

```
function report_data = ddPCR2D(filename, HEXdiv, FAMdiv)
% 2D (FAM,HEX) analysis of ddPCR data
% HEXdiv and FAMdiv: division lines on HEX/FAM channels

data1 = csvread(filename,1,0);
% column1: channel1(FAM), column2: channel2(HEX), column3: cluster
% plot FAM as y-axis, HEX as x-axis

if HEXdiv == 0 && FAMdiv == 0 % no threshold, plot only
    figname = figure;
    hold all
    plot(data1(:,2), data1(:,1), '.k');
    xlabel('HEX channel');
    ylabel('FAM channel');
    saveas(figname,filename(1:(end-4)),'epsc');
    report_data = [0];
else

    % Cluster 1 = bg, 2 = FAM pos, 3 = HEX pos, 4 = both pos
    for i = 1:size(data1,1)
        data1(i,3) = -1000; %set initial cluster
        if data1(i,1) > FAMdiv && data1(i,2) > HEXdiv
            data1(i,3) = 4;
        else if data1(i,1) > FAMdiv && data1(i,2) <= HEXdiv
                data1(i,3) = 2;
            else if data1(i,1) <= FAMdiv && data1(i,2) > HEXdiv
                    data1(i,3) = 3;
                else data1(i,3) = 1;
                end
            end
        end
    end
    cluster1 = data1((data1(:,3) == 1),:);
    cluster2 = data1((data1(:,3) == 2),:);
    cluster3 = data1((data1(:,3) == 3),:);
    cluster4 = data1((data1(:,3) == 4),:);

    figname = figure;
    hold all
    plot(cluster1(:,2), cluster1(:,1), '.k');
    plot(cluster2(:,2), cluster2(:,1), '.b');
    plot(cluster3(:,2), cluster3(:,1), '.g');
    plot(cluster4(:,2), cluster4(:,1), '.r');
    plot([HEXdiv HEXdiv], [-500 15000], '--k');
    plot([-500 15000], [FAMdiv FAMdiv], '--k');
    xlabel('HEX channel');
    ylabel('FAM channel');
    %axis([HEXdiv-2500 HEXdiv+7000 FAMdiv-3000 FAMdiv+8000]);
    saveas(figname,filename(1:(end-4)),'epsc');
    report_data = [size(cluster1,1) size(cluster2,1) size(cluster3,1)
size(cluster4,1)];
end
```

Section S12. Quantitation of Cancer Mutations in Clinical cfDNA Samples

The method of quantitation in clinical cfDNA samples using BDA is the same as described in Section S11. We first performed calibration experiments on BDA designs for different cancer mutations, and used the calibrated k, *delay*, and $\mathrm{Ct}_{\mathrm{NoBlocker}}$ values for calculation of Variant Molecule Counts (VMC) and Wildtype Molecule Counts (WMC) in cancer patient cfDNA samples. We compared the BDA quantitation results to deep sequencing results for all the samples.

Calibration of BDA Designs. The calibration data of all the BDA designs for cancer mutations is shown in Table 12-1. Note that the EGFR T790M BDA set has 2 forward primers, and we use both forward primers simultaneously. This is because 8 bases upstream of the EGFR T790M mutation, there is a non-pathogenic SNP rS1250171 (A/G) with 43% (A) minor allele frequency in the population. Therefore, we use 2 primers to tolerate the mutation at rS1250171 locus. The #5 clinical sample (containing EGFR T790M mutation) was tested with a slightly different BDA design from other EGFR T790M experiments; the calibration was done separately, and results are shown in Table S12-1 as "EGFR T790M-2".

Mutation	**Total Molecules**	$\mathbf{Ct}_{\mathrm{NoBlocker}}$	k	*delay*
EGFR L858R	6000	25.02	-1.004	0.45
PIK3CA E545K	6000	25.22	-1.116	-0.62
KRAS G12D	6000	25.17	-0.933	1.20
EGFR E746_A750del	6000	25.82	-1.031	-0.26
NRAS Q61K	6000	25.17	-1.018	-0.23
EGFR T790M	6000	25.05	-0.961	0.42
EGFR T790M-2	6000	25.45	-0.946	1.24
KRAS G13D	6000	25.33	-1.029	0.81

TABLE S12-1: Calibration results of BDA designs for cancer mutations. Ct values shown are average of triplicates.

Summary of Quantitation Results. Table S12-2 shows a summary of quantitation results of the 17 clinical cfDNA samples detected to be mutation positive by deep sequencing with molecular lineage tags. Each sample was tested only once, due to the limited amounts of cfDNA in each sample. We plotted the VAF quantitated by deep sequencing against the VAF quantitated by BDA, and also the absolute quantitation results of VMC and WMC in these samples. The results are shown in Fig. S12-1.

Sample #	**Mutation**	**BDA VMC**	**BDA WMC**	**BDA VAF (%)**	**Deep Seq. VMC**	**Deep Seq. WMC**	**Deep Seq. VAF (%)**
1	NRAS Q61K	5.1	1077.5	0.47	11	2686	0.41
2	EGFR E746_A750del	0.0	474.4	<0.1	3	925	0.32
3	EGFR E746_A750del	0.9	768.4	0.12	5	758	0.66
4	EGFR E746_A750del	4.0	897.3	0.45	3	812	0.37
5	EGFR T790M	48.9	2082.6	2.30	51	1878	2.64
6	EGFR L858R	17.3	2948.2	0.58	14	3293	0.42
7	KRAS G13D	7.0	1792.7	0.39	8	2365	0.34
8	PIK3CA E545K	0.8	493.1	0.16	4	2632	0.15
9	KRAS G12D	6.5	563.7	1.14	21	1273	1.62
10	KRAS G13D	2.2	584.5	0.38	8	1155	0.69
11	EGFR E746_A750del	1.9	1481.6	0.13	18	2172	0.82
12	EGFR E746_A750del	23.3	1599.6	1.44	27	1174	2.25
13	EGFR L858R	2.8	1798.1	0.16	11	962	1.13
14	KRAS G12D	0.8	670.8	0.12	8	1530	0.52
15	EGFR T790M	10.6	1390.9	0.75	44	2222	1.94
16	KRAS G13D	594.1	14120.6	4.04	1008	14757	6.39
17	EGFR L858R	22.5	2962.5	0.76	26	3351	0.77

TABLE S12-2: Clinical cfDNA quantitation results.

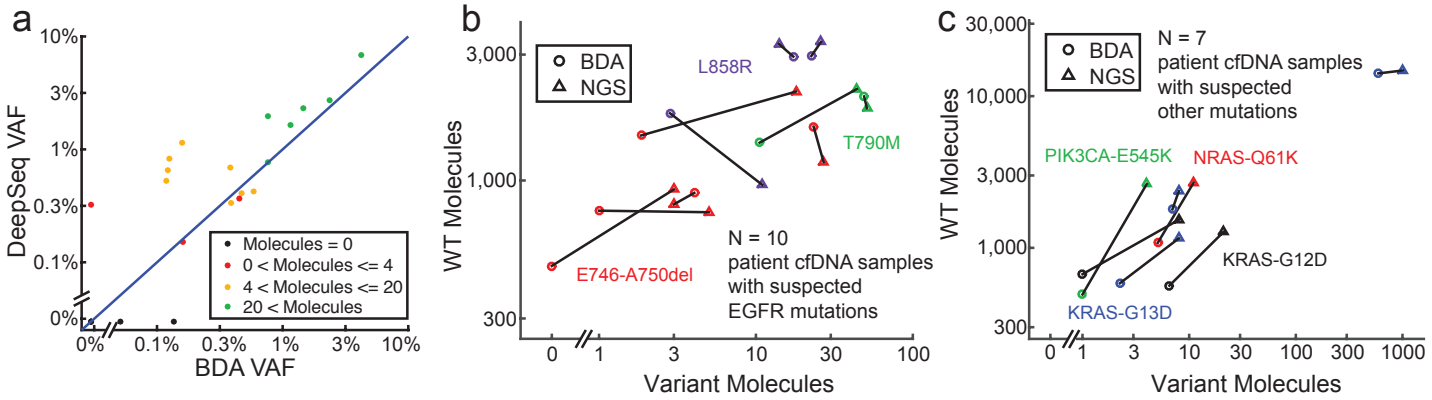


FIG. S12-1: ddPCR VAF quantitation assays. **(a)** Comparing VAF of clinical samples quantitated by deep sequencing and BDA. **(b)(c)** Quantitation of variant and wildtype molecule counts in clinical cfDNA samples.

We also used BDA to test 7 clinical samples in which deep sequencing did not detect any mutations. These results are listed in Table S12-3.

Mutation Tested	BDA VMC	BDA WMC	BDA VAF (%)
EGFR L858R	0.1	310.1	0.03
PIK3CA E545K	0.2	351.9	0.05
KRAS G12D	0.0	183.3	0.01
KRAS G13D	0.0	204.6	0.02
EGFR E746_A750del	0.1	880.7	0.01
EGFR T790M	0.6	431.6	0.14
NRAS Q61K	0.1	291.5	0.02

TABLE S12-3: BDA quantitation results of negative samples. Deep sequencing results did not detect any variant molecules in these samples.

Sanger Sequencing. After BDA enrichment, we identified the variant in the clinical samples using Sanger sequencing. The results are shown in Fig. S12-1b; in the "with Blocker" groups, the variant alleles were enriched to near 100%; in the "no Blocker" groups, there was near 100% wildtype allele.

Standard Sanger sequencing cannot provide high-quality CE traces at <60th bases (starting from the 5′ of sequencing primer), and the base call starts at roughly the 60th base, which means the variant base cannot be sequenced reliably when the amplicon length is shorter than 80 bp. Therefore, we were not able to identify the KRAS G12D, KRAS G13D, NRAS Q61K, and PIK3CA E545K mutations, because the amplicon lengths for these mutations were shorter than 80 bp (see Section S13 for sequences).

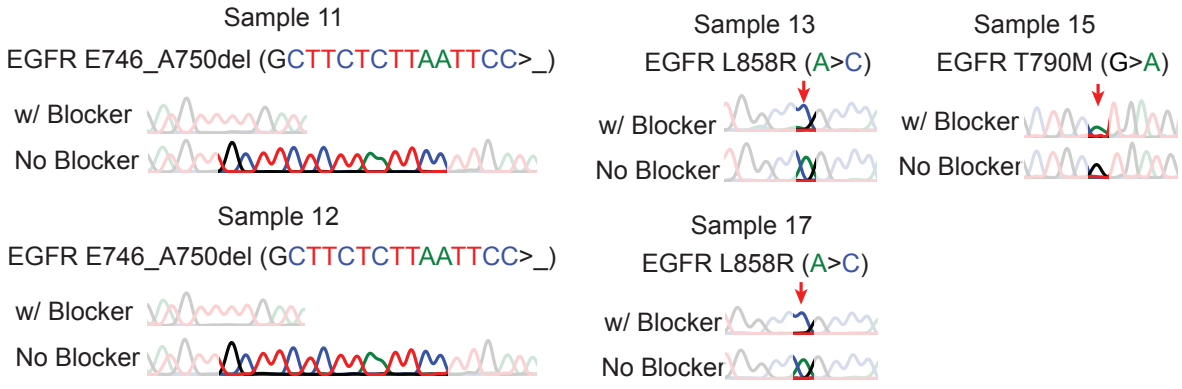


FIG. S12-2: Sanger sequencing results of sample #11, #12, #13, #15, and #17.

Receiver Operating Characteristic Curves. We next calculated the clinical *sensitivity* and *specificity* of the BDA assays against deep sequencing using different threshold values to separate the positives and the

negatives, and plotted the receiver operating characteristic (ROC) curves. The *sensitivity* was calculated as:

$$sensitivity = (\#\text{of true positives})/(\#\text{of true positives} + \#\text{of false negatives})$$

and the $specificity$ was calculated as:

$$specificity = (\#\text{of true negatives})/(\#\text{of true negatives} + \#\text{of false positives})$$

We used variant molecule counts and VAF as the classifier, and plotted their respective ROC curves (Fig. S12-3). The *accuracy* of the classification methods were calculated as the area under the ROC curve. ROC accuracy is marginally higher based on mutant molecule count than on VAF.

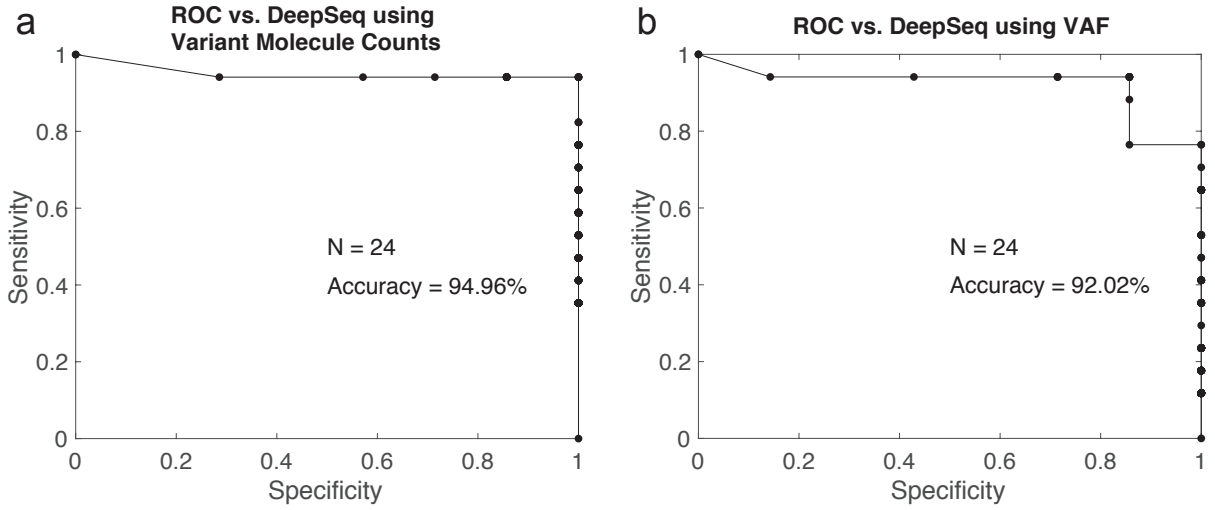


FIG. S12-3: Receiver operating characteristic (ROC) curves of BDA results on 24 clinical cfDNA samples. We used the deep sequencing results as true values. **(a)** Variant molecule counts was used as the classifier. **(b)** VAF was used as the classifier.

Section S13. List of BDA Oligonucleotide Sequences

		Genotype			Sequence		
SNP rs-	Direction	NA18562	NA18537	Blocker	fP	rP	B
2246745	1	A/A	T/T	B18562	CTCCTTGGAATCACCAACAAACAT	GGCTGCGATGAGACAGGAA	CAACAAACATGCCaTCTCCTTCTCCTGAAATA
2246745	1	A/A	T/T	B18537	CTCCTTGGAATCACCAACAAACAT	GGCTGCGATGAGACAGGAA	CAACAAACATGCCtTCTCCTTCTCCTGAAATA
3738244	1	T/T	A/A	B18562	ACTATAAATCCCTTAGGCAACAGTCC	ACTTAGGTGTAAAAACCAGATACATGAGA	ACAGTCCCATAAACATTCATGTGAtAGAGTCTTTAAT
3738244	1	T/T	A/A	B18537	ACTATAAATCCCTTAGGCAACAGTCC	ACTTAGGTGTAAAAACCAGATACATGAGA	AACAGTCCCATAAACATTCATGTGAaAGAGTCAATA
2511854	1	G/G	C/C	B18562	GAACAGACATAAACAGAAGCTTTAGTAGATT	GAAAGTTATTGTTATTCTTGATGGTTCTTTTGA	AGCTTTAGTAGATTATGCTGGTATTAgGGTGGAAAAAC
2511854	1	G/G	C/C	B18537	GAACAGACATAAACAGAAGCTTTAGTAGATT	GAAAGTTATTGTTATTCTTGATGGTTCTTTTGA	AGCTTTAGTAGATTATGCTGGTATTAcGGTGGAAAAAC
3789806	1	G/G	C/C	B18562	CTTGTATATAGACGGTAAAATAAACACCAAGA	AGGCACCAGAAGTCATCAGAATG	CACCAAGAgGTGGTAAATATTTACCTGGTCCAAAA
3789806	1	G/G	C/C	B18537	CTTGTATATAGACGGTAAAATAAACACCAAGA	AGGCACCAGAAGTCATCAGAATG	TAAACACCAAGAcGTGGTAAATATTTACCTGGTAAAA
706714	1	C/C	A/A	B18562	TGAAGCAGATGTTGAACAACAAGG	GACCAAGCTTTTATGCACCACA	CAACAAGGTCAGTATTGATAcGTGGTTGCTATTA
706714	1	C/C	A/A	B18537	TGAAGCAGATGTTGAACAACAAGG	GACCAAGCTTTTATGCACCACA	CAACAAGGTCAGTATTGATAaGTGGTTGCTATTA
1884444	1	G/G	T/T	B18562	TTCCTGCTTCCAGACATGAATCA	TGAAAGATAGCAATAGATACATAAAACACCA	CATGAATCAgGTCACTATTCAATGGGATGCTAAT
1884444	1	G/G	T/T	B18537	TTCCTGCTTCCAGACATGAATCA	TGAAAGATAGCAATAGATACATAAAACACCA	ACATGAATCAtGTCACTATTCAATGGGATGCAGATTA
2510152	1	T/T	G/G	B18562	ACCCAGGTGAGTTTTGTTTCACAT	TGAAACCACATACACACAAATTCACT	TTTTGTTTCACATtATAACCATATCACTGGACACAAGCAAAT
2510152	1	T/T	G/G	B18537	ACCCAGGTGAGTTTTGTTTCACAT	TGAAACCACATACACACAAATTCACT	TTTTGTTTCACATgATAACCATATCACTGGACACATAAA
11568849	1	C/C	A/A	B18562	TCACTCTTATCATTCTGTTGCCAGT	CCCACACAAGTAGAATTCCCATTTC	TGCCAGTAGTTTCAAAATTTcGTTTTGTTAGTCTCAATA
11568849	1	C/C	A/A	B18537	TCACTCTTATCATTCTGTTGCCAGT	CCCACACAAGTAGAATTCCCATTTC	GCCAGTAGTTTCAAAATTTaGTTTTGTTAGTCTCTCTTAT
8904	1	T/T	C/C	B18562	GGCCAGCGTCTGACGTTATG	TTTTGGGCCAGGCAGTGTG	CTGACGTTATGAGtGCAAAGGGGCTGATTAT
8904	1	T/T	C/C	B18537	GGCCAGCGTCTGACGTTATG	TTTTGGGCCAGGCAGTGTG	TCTGACGTTATGAGcGCAAAGGGGCTTTTT
16754	1	G/G	A/A	B18562	CTCTCTGCCTGCAGGATGTG	CTTCCTGCTGTGCATCTGTAAGT	GGATGTGCGgCGTGTGCCTGATTA
16754	1	G/G	A/A	B18537	CTCTCTGCCTGCAGGATGTG	CTTCCTGCTGTGCATCTGTAAGT	AGGATGTGCGaCGTGTGCCTGGTAAT
206781	1	A/A	G/G	B18562	CACTTCCTCCAGAAGGTCCAAAG	AAAAAGAAGAAACGGAAGGCAGAG	GGTCCAAAGCCaGAAGGGCCTAAAGCTAAT
206781	1	A/A	G/G	B18537	CACTTCCTCCAGAAGGTCCAAAG	AAAAAGAAGAAACGGAAGGCAGAG	GGTCCAAAGCCgGAAGGGCCTAAAAATA
28932178	1	T/T	C/C	B18562	ACTAAGAGTGCAGAGCCTGGAA	TGCTGCCCCACCCTTTATTAAC	CCTGGAACCGAGACGtCTCAGGTATTA
28932178	1	T/T	C/C	B18537	ACTAAGAGTGCAGAGCCTGGAA	TGCTGCCCCACCCTTTATTAAC	GCCTGGAACCGAGACGcCTCAGAAAT
2246745	2	T/T	A/A	B18562	TCCATGTAAAATAATCAGGAGAAGGAGA	TTTGACTGATATTGAGTGACAGCTAAAAG	GAAGGAGAtGGCATGTTTGTTGGTGAAAAA
2246745	2	T/T	A/A	B18537	TCCATGTAAAATAATCAGGAGAAGGAGA	TTTGACTGATATTGAGTGACAGCTAAAAG	AGAAGGAGAaGGCATGTTTGTTGGTGAAAAA
3738244	2	A/A	T/T	B18562	GGTAAGTACTGTATTTGCTTCATGGAA	ACCCTGCACAAAAACGGCT	TTCATGGAAGGATTGATAAGACTCTaTCACATGAATAAAA
3738244	2	A/A	T/T	B18537	GTGGTAAGTACTGTATTTGCTTCATGG	TAGGGGACAGACGGAGATTATTTCA	GCTTCATGGAAGGATTGATAAGACTCTtTCACAATTT
2511854	2	C/C	G/G	B18562	TCTTGCCTTGCCTTCCACC	TTTATTGTGAGGGATTGTCTGGAGAA	CTTCCACCcTAATACCAGCATAATCTACTAAAGCAAAA
2511854	2	C/C	G/G	B18537	TCTTGCCTTGCCTTCCACC	TTTATTGTGAGGGATTGTCTGGAGAA	CTTCCACCgTAATACCAGCATAATCTACTAAAGCAAAA
3789806	2	C/C	G/G	B18562	CCTTATTCAAACATCAACAACAGGGAC	CGTGTTGCTATTACTGCCAAAAGT	AACAGGGACCAGGTAAATATTTACCACcTCTTGAAAA
3789806	2	C/C	G/G	B18537	CCTTATTCAAACATCAACAACAGGGAC	CGTGTTGCTATTACTGCCAAAAGT	CAGGGACCAGGTAAATATTTACCACgTCTTGAAAA
706714	2	G/G	T/T	B18562	GAAAGGGAGTCATTAAGCAACCAC	GGGGGACTTTCCGGGAACT	GCAACCACgTATCAATACTGACCTTGTTGTTACTA
706714	2	G/G	T/T	B18537	GAAAGGGAGTCATTAAGCAACCAC	GGGGGACTTTCCGGGAACT	GCAACCACtTATCAATACTGACCTTGTTGTTCTTAT
1884444	2	C/C	A/A	B18562	GAGTATGTAAAGGGCTATTACTGCATC	TCCCTAATCAAAGGTTCCCATCAAA	TATTACTGCATCCCATTGAATAGTGACcTGATTCTAAA
1884444	2	C/C	A/A	B18537	GAGTATGTAAAGGGCTATTACTGCATC	TCCCTAATCAAAGGTTCCCATCAAA	CTGCATCCCATTGAATAGTGACaTGATTCATAAAA
2510152	2	A/A	C/C	B18562	AAGCTTGTGTCCAGTGATATGGTTA	CCCAGGGTTGGTTACTCTTTACA	AGTGATATGGTTATaATGTGAAACAAAACTCACCTGGAAAT
2510152	2	A/A	C/C	B18537	AAGCTTGTGTCCAGTGATATGGTTA	CCCAGGGTTGGTTACTCTTTACA	AGTGATATGGTTATcATGTGAAACAAAACTCACCTAATA
11568849	2	G/G	T/T	B18562	CGGTGCTGAGAGACTAACAAAAC	GCTCAGGCAGCCGCAT	AGACTAACAAAACgAAATTTTGAAACTACTGGCATATT
11568849	2	G/G	T/T	B18537	CGGTGCTGAGAGACTAACAAAAC	GCTCAGGCAGCCGCAT	GACTAACAAAACtAAATTTTGAAACTACTGGCAACAATTA
8904	2	A/A	G/G	B18562	GTCCATGTTCTTTCAGCCCCTT	AGTAGTGGCCTCCCCATCC	GCCCCTTTGCaCTCATAACGTCAGACAAAA
8904	2	A/A	G/G	B18537	GTCCATGTTCTTTCAGCCCCTT	AGTAGTGGCCTCCCCATCC	GCCCCTTTGCgCTCATAACGTCAGAAAAA
16754	2	C/C	T/T	B18562	AGAGTCGGGGCTACTCCAG	GGAGTGTGAATGGGAGTGGTT	CTACTCCAGGCACACGcCGCAATAA
16754	2	C/C	T/T	B18537	AGAGTCGGGGCTACTCCAG	GGAGTGTGAATGGGAGTGGTT	CTCCAGGCACACGtCGCACAAAAA
206781	2	T/T	C/C	B18562	CAGTGCTGCTTTAGGCCCTT	GGCCATTTAGAATTATCTTTGCAAAGAG	GCCCTTCtGGCTTTGGACCTTCTAATA
206781	2	T/T	C/C	B18537	CAGTGCTGCTTTAGGCCCTT	GGCCATTTAGAATTATCTTTGCAAAGAG	GGCCCTTCcGGCTTTGGACCTTAATA
28932178	2	A/A	G/G	B18562	AGATGCCTTCAGATCAGAGAGATTAAC	AGTATTCTAGGTTTGCTGCCACA	AGAGAGATTAACCTGAGaCGTCTCGGTTCATTT
28932178	2	A/A	G/G	B18537	AGATGCCTTCAGATCAGAGAGATTAAC	AGTATTCTAGGTTTGCTGCCACA	AGAGAGATTAACCTGAGgCGTCTCGGAAAA

TABLE S11-1: Sequences of BDA designs for non-pathogenic SNPs. Blocker for NA18562 (B18562) is complementary to NA18562 on the SNP locus, while blocker for NA18537 (B18537) is complementary to NA18537. The "genotype" columns show the allele identity of sample NA18562 and NA18537 on the strand with the same direction as the blocker; therefore, for example, the "A/A" alleles in direction 1 become "T/T" in direction 2. The SNP base is shown in lower case on the blocker.

SNP rs-	Direction	Taqman probe
2246745	1	/5TexRd-XN/AT GGA ATC TCA CCT GGA TAA TGA AAG ACT CCT TCC /3IAbRQSp/
3789806	1	/5HEX/TC CAG TCA T/ZEN/C AAT TCA TAC AGA ACA ATT CCA AAT GCA /3IABkFQ/
706714	1	/5Cy5/AA AGT CTT A/TAO/A GTT TGG GTT GAG TCG TTA TGT TCT GG/3IAbRQSp/
1884444	1	/5TexRd-XN/CC TTT ACA TAC TCT TCA GCT GGT GTC ATG GAG G/3IAbRQSp/
2510152	1	/5HEX/AC TGA CAG C/ZEN/A TAA TTG AAT TCA TTG GAA CCC ATT GA/3IABkFQ/
11568849	1	/5Cy5/CA GCA CTG G/TAO/A GCT GTC CTG AAG GTA CTC T/3IAbRQSp/
16754	1	/5TexRd-XN/CA TCT GAG ACC AGT GAG AAA CGC CCC TTC /3IAbRQSp/
206781	1	/5HEX/CA CTG CCA C/ZEN/T TGC TTT CTT GGA CTT CTT AGG /3IABkFQ/
28932178	1	/5Cy5/AT CTA CTC T/TAO/T GTT CAC AAA CCC CAG TCA GAT TTT AC/3IAbRQSp/

TABLE S11-2: Taqman probes used in 9-plex non-pathogenic mutation detection. Primer and blocker sequences are shown in Table S9-1. /5TexRd-XN/ represents a Texas Red fluorophore modification at the 5' end, /5HEX/ represents a HEX fluorophore, /5Cy5/ represents a Cy5 fluorophore; /3IAbRQSp/ represents a 3' Iowa Black RQ quencher, /3IABkFQ/ represents a 3' Iowa Black FQ quencher, /ZEN/ represents an internal ZEN quencher, and /TAO/ represents an internal TAO quencher.

		Genotype			Sequence		
SNP rs-	Direction	NA18562	NA18537	Blocker	fP	rP	B
3789806	1	G/G	C/C	B18537	GAGCCTTGTATATAGACGGTAAAATAAAC	AGGCACCAGAAGTCATCAGAATG	+A+A+G+A+C+G+T+G+G
3789806	1	G/G	C/C	B18537	GAGCCTTGTATATAGACGGTAAAATAAAC	AGGCACCAGAAGTCATCAGAATG	+A+A+G+A+C+G+T+G

TABLE S11-3: LNA blocker sequences and primers used with these blockers. "+N" represents an LNA base.

		Genotype			Sequence		
SNP rs-	Direction	NA18562	NA18537	Blocker	fP	rP	B
3789806	1	G/G	C/C	B18537	TTGTATATAGACGGTAAAATAAACACCAAG	AGGCACCAGAAGTCATCAGAATG	ACACCAAGACGTGGTAAATATTTACCTGGTTAAA
					CTTGTATATAGACGGTAAAATAAACACCA	AGGCACCAGAAGTCATCAGAATG	AAACACCAAGACGTGGTAAATATTTACCTGGAAAA
					GCCTTGTATATAGACGGTAAAATAAACAC	AGGCACCAGAAGTCATCAGAATG	GGTAAAATAAACACCAAGACGTGGTAAATATTTACCTAAAA
					GAGCCTTGTATATAGACGGTAAAATAAAC	AGGCACCAGAAGTCATCAGAATG	CGGTAAAATAAACACCAAGACGTGGTAAATATTTACAAAA
					GGAGCCTTGTATATAGACGGTAAAATAA	AGGCACCAGAAGTCATCAGAATG	ACGGTAAAATAAACACCAAGACGTGGTAAATATTAAAT

TABLE S11-4: Different BDA designs generating similar results predicted by our model.

	Ultramer Sequence
Var1-1	TGAAGCAGATGTTGAACAACAAGGAcagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-2	TGAAGCAGATGTTGAACAACAAGGCcagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-3	TGAAGCAGATGTTGAACAACAAGGGcagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-4	TGAAGCAGATGTTGAACAACAAGGtAagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-5	TGAAGCAGATGTTGAACAACAAGGtTagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-6	TGAAGCAGATGTTGAACAACAAGGtGagtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-7	TGAAGCAGATGTTGAACAACAAGGtcTgtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-8	TGAAGCAGATGTTGAACAACAAGGtcCgtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-9	TGAAGCAGATGTTGAACAACAAGGtcGgtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-10	TGAAGCAGATGTTGAACAACAAGGtcaAtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-11	TGAAGCAGATGTTGAACAACAAGGtcaTtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-12	TGAAGCAGATGTTGAACAACAAGGtcaCtattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-13	TGAAGCAGATGTTGAACAACAAGGtcagAattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-14	TGAAGCAGATGTTGAACAACAAGGtcagCattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-15	TGAAGCAGATGTTGAACAACAAGGtcagGattgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-16	TGAAGCAGATGTTGAACAACAAGGtcagtTttgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-17	TGAAGCAGATGTTGAACAACAAGGtcagtCttgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-18	TGAAGCAGATGTTGAACAACAAGGtcagtGttgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-19	TGAAGCAGATGTTGAACAACAAGGtcagtaAtgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-20	TGAAGCAGATGTTGAACAACAAGGtcagtaCtgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-21	TGAAGCAGATGTTGAACAACAAGGtcagtaGtgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-22	TGAAGCAGATGTTGAACAACAAGGtcagtatAgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-23	TGAAGCAGATGTTGAACAACAAGGtcagtatCgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-24	TGAAGCAGATGTTGAACAACAAGGtcagtatGgatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC

	Ultramer Sequence
Var1-25	TGAAGCAGATGTTGAACAACAAGGtcagtattAatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-26	TGAAGCAGATGTTGAACAACAAGGtcagtattTatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-27	TGAAGCAGATGTTGAACAACAAGGtcagtattCatacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-28	TGAAGCAGATGTTGAACAACAAGGtcagtattgTtacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-29	TGAAGCAGATGTTGAACAACAAGGtcagtattgCtacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-30	TGAAGCAGATGTTGAACAACAAGGtcagtattgGtacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-31	TGAAGCAGATGTTGAACAACAAGGtcagtattgaAacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-32	TGAAGCAGATGTTGAACAACAAGGtcagtattgaCacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-33	TGAAGCAGATGTTGAACAACAAGGtcagtattgaGacgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-34	TGAAGCAGATGTTGAACAACAAGGtcagtattgatTcgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-35	TGAAGCAGATGTTGAACAACAAGGtcagtattgatCcgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-36	TGAAGCAGATGTTGAACAACAAGGtcagtattgatGcgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-37	TGAAGCAGATGTTGAACAACAAGGtcagtattgataAgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-38	TGAAGCAGATGTTGAACAACAAGGtcagtattgataTgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-39	TGAAGCAGATGTTGAACAACAAGGtcagtattgataGgtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-40	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacAtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-41	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacTtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-42	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacCtggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-43	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgAggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-44	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgCggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-45	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgGggtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-46	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtAgtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-47	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtTgtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-48	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtCgtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-49	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtgAtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-50	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtgTtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-51	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtgCtTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-52	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtggATGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-53	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtggCTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var1-54	TGAAGCAGATGTTGAACAACAAGGtcagtattgatacgtggGTGCTTAATGACTCCCTTTCTTTTTCTTTTTAAGGAAAAGTCTTAAGTTTG GGTTGAGTCGTTATGTTCTGGGCAGAAGTTACCTTGGCAACTGCACCTGAATTGATGTGTGGTGCATAAAAGCTTGGTC
Var2-1	ACCCAGGTGAGTTTTGTTTCACATAataaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-2	ACCCAGGTGAGTTTTGTTTCACATCataaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA

	Ultramer Sequence
Var2-3	ACCCAGGTGAGTTTTGTTTCACATGataaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-4	ACCCAGGTGAGTTTTGTTTCACATtTtaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-5	ACCCAGGTGAGTTTTGTTTCACATtCtaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-6	ACCCAGGTGAGTTTTGTTTCACATtGtaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-7	ACCCAGGTGAGTTTTGTTTCACATtaAaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-8	ACCCAGGTGAGTTTTGTTTCACATtaCaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-9	ACCCAGGTGAGTTTTGTTTCACATtaGaaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-10	ACCCAGGTGAGTTTTGTTTCACATtatTaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-11	ACCCAGGTGAGTTTTGTTTCACATtatCaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-12	ACCCAGGTGAGTTTTGTTTCACATtatGaccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-13	ACCCAGGTGAGTTTTGTTTCACATtataTccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-14	ACCCAGGTGAGTTTTGTTTCACATtataCccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-15	ACCCAGGTGAGTTTTGTTTCACATtataGccatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-16	ACCCAGGTGAGTTTTGTTTCACATtataaAcatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-17	ACCCAGGTGAGTTTTGTTTCACATtataaTcatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-18	ACCCAGGTGAGTTTTGTTTCACATtataaGcatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-19	ACCCAGGTGAGTTTTGTTTCACATtataacAatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-20	ACCCAGGTGAGTTTTGTTTCACATtataacTatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-21	ACCCAGGTGAGTTTTGTTTCACATtataacGatatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-22	ACCCAGGTGAGTTTTGTTTCACATtataaccTtatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-23	ACCCAGGTGAGTTTTGTTTCACATtataaccCtatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-24	ACCCAGGTGAGTTTTGTTTCACATtataaccGtatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-25	ACCCAGGTGAGTTTTGTTTCACATtataaccaAatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-26	ACCCAGGTGAGTTTTGTTTCACATtataaccaCatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-27	ACCCAGGTGAGTTTTGTTTCACATtataaccaGatcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-28	ACCCAGGTGAGTTTTGTTTCACATtataaccatTtcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-29	ACCCAGGTGAGTTTTGTTTCACATtataaccatCtcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-30	ACCCAGGTGAGTTTTGTTTCACATtataaccatGtcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-31	ACCCAGGTGAGTTTTGTTTCACATtataaccataAcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-32	ACCCAGGTGAGTTTTGTTTCACATtataaccataCcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-33	ACCCAGGTGAGTTTTGTTTCACATtataaccataGcactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA

	Ultramer Sequence
Var2-34	ACCCAGGTGAGTTTTGTTTCACATtataaccatatAactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-35	ACCCAGGTGAGTTTTGTTTCACATtataaccatatTactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-36	ACCCAGGTGAGTTTTGTTTCACATtataaccatatGactggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-37	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcTctggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-38	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcCctggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-39	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcGctggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-40	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcaAtggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-41	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcaTtggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-42	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcaGtggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-43	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcacAggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-44	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcacCggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-45	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcacGggacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-46	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactAgacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-47	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactTgacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-48	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactCgacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-49	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactgAacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-50	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactgTacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-51	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactgCacacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-52	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggTcacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-53	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggCcacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-54	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggGcacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-55	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggaAacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-56	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggaTacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-57	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggaGacAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-58	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacTcAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-59	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacCcAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-60	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacGcAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-61	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacaAAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-62	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacaTAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA
Var2-63	ACCCAGGTGAGTTTTGTTTCACATtataaccatatcactggacaGAAGCTTTAGTATATTCTTTATAGAACTGACAGCATAATTGAATTCAT TGGAACCCATTGACTAGATTAGTGAATTTGTGTGTATGTGGTTTCA

TABLE S11-5: Synthetic DNA templates (ultramers) for systematic hotspot assays.

		Sequence		
SNP rs-	Direction	fP	rP	B
706714	1	TGAAGCAGATGTTGAACAACAAGG	GACCAAGCTTTTATGCACCACA	CAACAAGGTCAGTATTGATAcGTGGTTGCTATTA
2510152	1	CCCAGGTGAGTTTTGTTTCACAT	TGAAACCACATACACACAAATTCACT	TTTTGTTTCACATtATAACCATATCACTGGACACAAGCAAAT

TABLE S11-6: BDA designs used in systematic hotspot assays.

	Sequence
fP	GCTGTATCGTCAAGGCACTCTT
rP	ACCTTATGTGTGACATGTTCTAATATAGTC
WT Blocker	GCACTCTT GCCTACGCCACCAGC AAAT
G12C Blocker	GCACTCTT GCCTACGCCACaAGC AAAT
G12D Blocker	GCACTCTT GCCTACGCCAtCAGC AAAT
G12V Blocker	GCACTCTT GCCTACGCCAaCAGC AAAT
G13D Blocker	GCACTCTT GCCTACGtCACCAGC AAAT

TABLE S11-7: BDA designs for KRAS hotspot multiplexing and mutant blockers.

		Genotype			Sequence		
SNP rs-	Direction	NA18562	NA18537	Blocker	fP	rP	B
3789806	1	G/G	C/C	B18537	CTTGTATATAGACGGTAAAATAAACACCAAGA	AGGCACCAGAAGTCATCAGAATG	TAAACACCAAGAcGTGGTAAATATTTACC/3SpC3/

TABLE S11-8: BDA design used with KAPA HiFi polymerase. "/3SpC3/" represents a C3 Spacer modification at the 3' end.

		Genotype			Sequence	
SNP rs-	Direction	NA18562	NA18537	Blocker	fP	rP
28932178	1	T/T	C/C	B18537	GTACTAAGAGTGCAG AGCCTGGA	CAAGAGTAGATGCCT TCAGATCAGAG
					fB	rB
					AGCCTGGA ACCGAGACGcCTCA /3SpC3/	TCAGATCAGAG AGATTAACCTGAGgCGTC /3SpC3/

TABLE S11-9: Dual BDA sequences.

Gene	Nucleotide Mutation	Amino Acid Change	fP	B	rP	Amplicon Size	Direction
EGFR	c.2573T>G	p.L858R	GCAGCATGTCAAGATCACAGATT	GATCACAGATTTTGGGCTGGCCAAACATAA	ACCTCCTTACTTTGCCTCCTTC	92	sense
EGFR	c.2235_2249del15	p.E746_A750del	AGAAAGTTAAAATTCCCGTCGCTA	GTCGCTATCAAGGAATTAAGAGAAGCAACAAAAA	CCCCACACAGCAAAGCA	89	sense
EGFR	c.2369C>T	p.T790M	CTCCACCGTGCAGCTCA	CAGCTCATCACGCAGCTCATGCCAAAA	GGTACTGGGAGCCAATATTGTC	92	sense
EGFR	c.2369C>T	p.T790M	CCACCGTGCAGCTCATCA CCACCGTGCAACTCATCA	GCTCATCACGCAGCTCATGCCC/3SpC3/	AGGTACTGGGAGCCAATATTGTC	91	sense
KRAS	c.35G>A	p.G12D	GCTGTATCGTCAAGGCACTCTT	GCACTCTTGCCTACGCCACCAGCAAAT	TGCTGAAAATGACTGAATATAAACTTGTGG	75	anti-sense
KRAS	c.38G>A	p.G13D	GCTGTATCGTCAAGGCACTCTT	GCACTCTTGCCTACGCCACCAGCAAAT	TGCTGAAAATGACTGAATATAAACTTGTGG	75	anti-sense
NRAS	c.181C>A	p.Q61K	TGTTTGTTGGACATACTGGATACAG	TGGATACAGCTGGACAAGAAGAGTACAGTAAAT	TGTCCTCATGTATTGGTCTCTCA	73	sense
PIK3CA	c.1633G>A	p.E545K	CACTTACCTGTGACTCCATAGAAAATC	CCATAGAAAATCTTTCTCCTGCTCAGTGATTTCTTTA	AGCAATTTCTACACGAGATCCTCT	76	anti-sense

TABLE S11-10: BDA designs used for cancer mutation quantitation in clinical and Horizon cfDNA samples.

Mutation	fP	rP	Taqman WT	Taqman Variant
EGFR L858R	CGTACTGGTGAAAACACCGCA	CTCCTTCTGCATGGTATTCTTTCTCT	/5HEX/ACAGATTTT/ZEN/GGGCTGGCCAA/3IABkFQ/	/56-FAM/CAGATTTTG/ZEN/GGCGGGCCA/3IABkFQ/
EGFR T790M	CTGCCTCACCTCCACCGT	GTGTTCCCGGACATAGTCCAG	/5HEX/CTCATCACG/ZEN/CAGCTCATGC/3IABkFQ/	/56-FAM/CTCATCATG/ZEN/CAGCTCATGCC/3IABkFQ/
NRAS Q61K	TGGTTATAGATGGTGAAACCTGTTTGT	TCATGTATTGGTCTCTCATGGCAC	/5HEX/CTGGATACA/ZEN/GCTGGACAAGAAGA/3IABkFQ/	/56-FAM/ATACTGGAT/ZEN/ACAGCTGGAAAAGAAGA/3IABkFQ/
KRAS G12D	GATTCTGAATTAGCTGTATCGTCAAGG	CCTGCTGAAAATGACTGAATATAAACTTGT	/5HEX/TTGCCTACG/ZEN/CCACCAGC/3IABkFQ/	/56-FAM/TCTTGCCTA/ZEN/CGCCATCAGC/3IABkFQ/
KRAS G13D	GATTCTGAATTAGCTGTATCGTCAAGG	CCTGCTGAAAATGACTGAATATAAACTTGT	/5HEX/TTGCCTACG/ZEN/CCACCAGC/3IABkFQ/	/56-FAM/TCTTGCCTA/ZEN/CGTCACCAGC/3IABkFQ/
rs2246745	TTATGTCCAGGTCATAGCTCCTTG	TCTTTCATTATCCAGGTGAGATTCCAT	/5HEX/CAAACATGC/ZEN/CATCTCCTTCTCC/3IABkFQ/	/56-FAM/CAAACATGC/ZEN/CTTCTCCTTCTCC/3IABkFQ/
rs706714	GTTCTTCGAAAACTGAAGCAGATGT	ACTCAACCCAAACTTAAGACTTTTCC	/5HEX/TCAGTATTG/ZEN/ATACGTGGTTGCTTAATG/3IABkFQ/	/56-FAM/AGTATTGAT/ZEN/AAGTGGTTGCTTAATGACTC/3IABkFQ/
rs206781	TCACTTGGTGCCACTTCCTC	TCCAAGAAAGCAAGTGGCAGT	/5HEX/CCAAAGCCA/ZEN/GAAGGGCCTAA/3IABkFQ/	/56-FAM/CAAAGCCGG/ZEN/AAGGGCCTAA/3IABkFQ/

TABLE S11-11: ddPCR designs for cancer mutations and non-pathogenic mutations. /56-FAM/ represents a FAM fluorophore modification at the 5' end.

Mutation	fP	B	rP	Taqman Probe
EGFR L858R	GCAGCATGTCAAGATCACAGATT	GATCACAGATTTTGGGCTGGCCAAACATAA	GCTGACCTAAAGCCACCTCCTTAC	/5HEX/TTGCCTCCT/ZEN/TCTGCATGGTATTCTTTCTCTTCC/3IABkFQ/
EGFR T790M	CCACCGTGCAGCTCATCA CCACCGTGCAACTCATCA	GCTCATCACGCAGCTCATGCCC/3SpC3/	AGGTACTGGGAGCCAATATTGTC	/5TexRd-XN/TCGGCTGCCTCCTGGACTATGTCCG/3IAbRQSp/
NRAS Q61K	AAACCTGTTTGTTGGACATACTGG	ACATACTGGATACAGCTGGACAAGAAGAG/3SpC3/	ACAGAGGAAGCCTTCGCC	/5Cy5/ACAGTGCCA/TAO/TGAGAGACCAATACATGAGGACA/3IAbRQSp/

TABLE S11-12: BDA for multiplexed qPCR detection of cancer mutations in Horizon cfDNA samples.