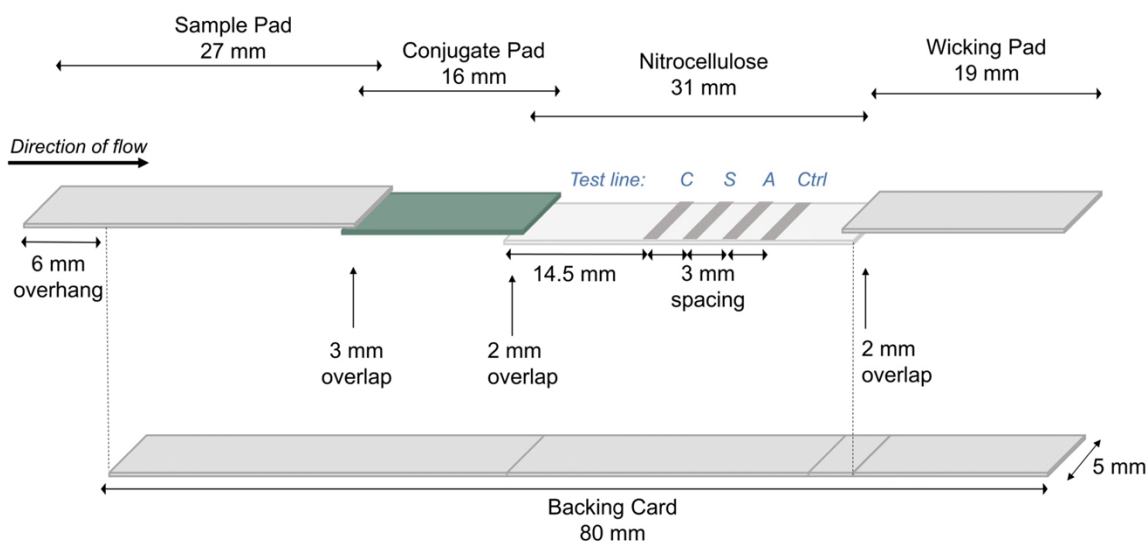1 **Electronic Supplementary Information**

2 The supplementary information includes details on primer sequences, lateral flow detection,

3 multiplex reaction optimization, and images of lateral flow strips from clinical sample testing.

4

5

6 Figure S1. Lateral Flow Strip Schematic. Lateral flow strips were adapted from [25] to include
7 capture antibodies

8

Primer Name	Primer Sequence (5' → 3')
fP-S	/5BiosG/AGGGCAGTAACGGCAGACTTCTGC+A
fP-S-1	/5BiosG/AGGGCAGTAACGGCAGACTTCTGCA
fP-S-2	/5BiosG/AGGGCAGTAACGGCAGACTTCT+GC+A
fP-S-3	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+A
fP-S-4	/5BiosG/AGGGCAGTAACGGCAGACTTCTC+CA
fP-S-5	/5BiosG/AGGGCAGTAACGGCAGACTTCTC+C+AC
fP-S-6	/5BiosG/AGGGCAGTAACGGCAGACTTCT+C+C+A

9 Table S1. β^S allele-specific forward primer candidates. All primer candidates used during the β^S
10 primer screen in Fig. 1, where "+" precedes LNA nucleotides. 5BiosG: 5' biotin.

Primer Name	Primer Sequence (5' → 3')
fP-A	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+TG
fP-A-v1	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+TC
fP-A-v2	/5BiosG/AGGGCAGTAACGGCAGACTTCTCAT+C
fP-A-v3	/5BiosG/AGGGCAGTAACGGCAGACTTCTCA+T+C
fP-A-v4	/5BiosG/AGGGCAGTAACGGCAGACTTCTCAC+C
fP-A-v5	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+CC
fP-A-v6	/5BiosG/AGGGCAGTAACGGCAGACTTCTCA+TC
fP-A-v7	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+T+C
fP-A-v8	/5BiosG/AGGGCAGTAACGGCAGACTTCTCA+CC

Table S2. β^A allele-specific forward primer candidates All primer candidates used during the β^A primer screen in Fig. 2, where "+" precedes LNA nucleotides. 5BiosG: 5' biotin.

| Primer Name	Primer Sequence (5' → 3')
fP-C	/5BiosG/AGGGCAGTAACGGCAGACTTCTCAT+T
fP-C-v1	/5BiosG/AGGGCAGTAACGGCAGACTTCTCC+TT

Table S3. β^C allele-specific forward primer candidates. All primer candidates used during the β^C primer screen in Fig. S3.

Figure S2. Multiplexed Assay Genotype Prediction Algorithm. The combination of test lines that cross the corresponding positivity threshold can be used to predict a sample's genotype using the table above.
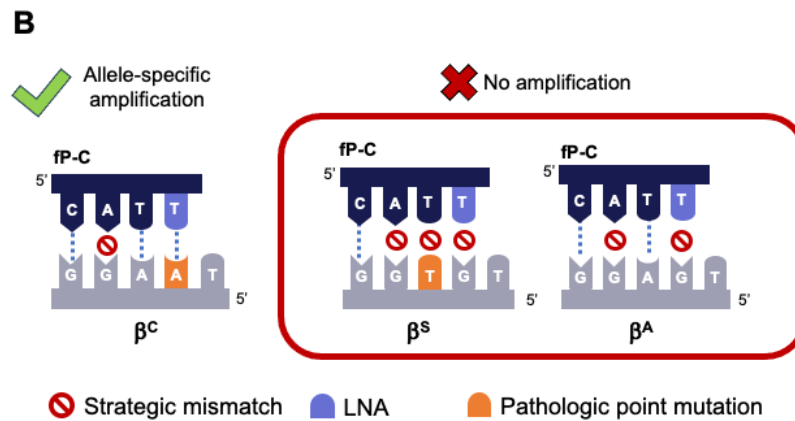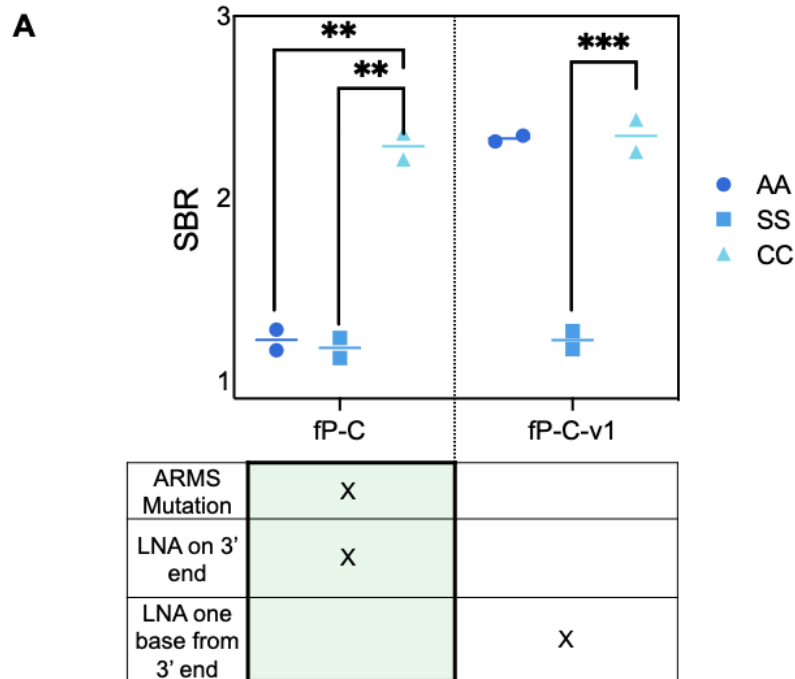
Figure S3. β^C allele-specific primer screen. (A) Allele-specific forward primers for the β^C allele were designed and screened using 10,000 input copies of AA, SS, or CC target. Signal-to-background ratios of the resultant lateral flow assays are graphed. Individual replicates are represented by a circle, and horizontal lines represent the mean (n=2). ***p<0.01; ***p<0.001; ****p<0.0001; significance determined for each primer pair by a one-way ANOVA with post-hoc Tukey's test.* (B) Schematic of specificity enhancing modifications incorporated into fP-C that achieve allele-specific amplification of DNA encoding for the β^C globin allele.

29 **Optimization of multiplexed allele-specific RPA**

30 *Methods*

31   5 M betaine (ThermoFisher J77507), Tte UvrD helicase (20 µg/mL, New England

32 BioLabs, Ipswich, MA), and T4 gene 32 single-stranded binding protein (10 mg/mL, New England

33 BioLabs) were explored as specificity enhancing additives. For multiplex optimization

34 experiments, each lyophilized enzyme pellet was rehydrated with 37.5 µL master mix containing

35 29.5 µL of rehydration buffer, 0.35 µL–1.13 µL of each allele-specific primer, 1.05 µL unmodified

36 reverse primer, 0.5 µL biotinylated probe, specificity enhancing additives when noted, and

37 supplemented with nuclease-free water to reach a final volume of 37.5 µL. 10 µL multiplex RPA

38 reactions contained 7.5 µL of the rehydrated enzyme pellet mixture, 2 µL of target at a

39 concentration of 5000 copies per µL, and 0.5 µL of 280 mM magnesium acetate. For the 10 mM

40 Mg condition, 0.5 µL of magnesium acetate at 200 mM was used; for the 17.5 mM Mg condition,

41 reactions contained 7.375 µL of rehydration mixture, and an increased volume of 0.625 µL of 280

42 mM magnesium – the additional volume of magnesium required was subtracted from the water

43 volume. For multiplex optimization reactions, the singleplex positivity thresholds were averaged

44 to form a composite positivity threshold to inform optimization of the multiplexed reaction – the

45 multiplexed assay formulations that achieved sensitive amplification of all three alleles and

46 resulted in clear differentiation of genotypes of interest were considered optimal.

47 *Forward primer optimization in multiplex reaction*

48   Using DNA containing only one allele of interest (AA, SS or CC), we first optimized the

49 total forward primer concentration (Fig. S4). We found that decreasing the total forward primer

50 concentration while maintaining a 1:1:1 ratio of fP-C, fP-S and fP-A resulted in stronger signal

51 formation at the S test line and more consistent signal formation at the A test line. This perhaps

52 indicates that the forward primers are inhibiting each other and preventing efficient amplification

53 of DNA containing the allele of interest. Given the strong S line signal formation and the more

54 consistent A line signal formation, 700 nM total forward primer was chosen for additional

55 optimization.

A

B

% fP-S	30	25	20
% fP-C	35	37.5	40
% fP-A	35	37.5	40
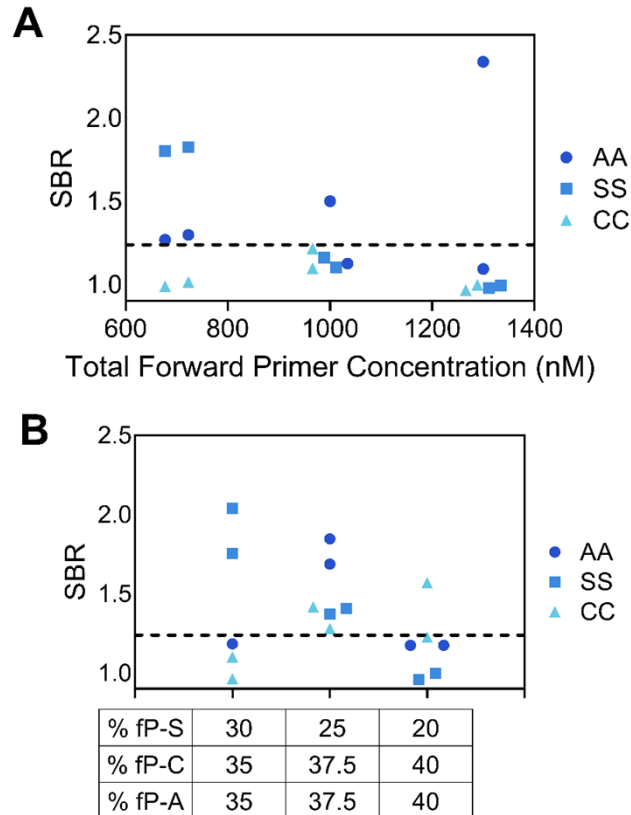
56

57 Figure S4. **Optimization of forward primer concentrations and ratios in multiplexed reaction**
58 **with homozygous target DNA.** (A) Purchased DNA of genotypes AA, SS, and CC (n=2) were
59 amplified in a multiplexed reaction containing 700, 1000, or 1300 nM total of forward primer,
60 divided equally between fP-S, fP-A, and fP-C. Signal-to-background ratio (SBR) at the test line of
61 interest for each genotype is plotted against increasing total forward primer concentration. As the
62 total amount of forward primer increases, the corresponding test line signals decrease. A composite
63 positivity threshold, calculated by averaging the three test line positivity thresholds from Fig. 3, is
64 indicated by the dashed line. (B) SBR for AA, SS, and CC target (n=2) at the test line of interest
65 is plotted against decreasing percentages of S primer while holding the total forward primer
66 concentration at 700 nM based on results from (A). The ratio between fP-A and fP-C ratios was
67 maintained at 1:1. A composite positivity threshold is indicated by the dashed line. One AA
68 replicate was excluded from the 30% fP-S condition due to pipetting errors.
69

70   Next, we sought to optimize the relative forward primer ratios to avoid primer inhibition

71 and achieve sensitive detection at all three test lines. Since fP-S resulted in the strongest

72 amplification and signal generation when all primers had equal concentrations, we hypothesized

73 that it was the most efficient forward primer and that lowering its percentage while maintaining

74 fP-C and fP-A at a 1:1 ratio would allow strong signal formation at all three test lines. Therefore,

75 we tested decreasing the percentage of fP-S from 33% down to 30%, 25% and 20% while holding

76 the total forward primer concentration constant at 700 nM using purchased DNA with genotypes

77 AA, SS, and CC (Fig. S4B). We found that as the percentage of fP-S decreased, the S test line

78 signal also decreased. However, the signals at the A and C test lines did not have a corresponding

79 increase as their concentrations increased, indicating that perhaps fP-A and fP-C are mutually

80 inhibitory. The best performing primer ratio combination, 25% fP-S, 37.5% fP-A, and 37.5% fP-

81 C, resulted in signal generation above the positivity thresholds for all the homozygous targets

82 tested.

83   We then wanted to validate that the optimal primer ratios for homozygous targets would

84 also allow sensitive detection and differentiation of heterozygous targets of interest, AS and SC.

85 We used the previously best performing ratio of 25% fP-S, 37.5% fP-A and 37.5% fP-C to amplify

86 purchased DNA with genotypes AA, AS, SS and SC (Fig. S5A). Homozygous targets AA and SS

87 had signal above the positivity threshold. However, AS target only had one replicate generate a

88 signal above the positivity threshold at the A line, and SC target had no signal above the positivity

89 threshold at the test lines of interest. Given our hypothesis that fP-A and fP-C are mutually

90 inhibitory, we decided to lower the concentration of fP-A to 35% and slightly increase the

91 concentration of fP-S to 27.5% to increase its amplification efficiency with heterozygous targets -

92   this altered forward primer ratio was able to restore positive signal formation for heterozygous

93 targets AS and SC (Fig. S5B).

94
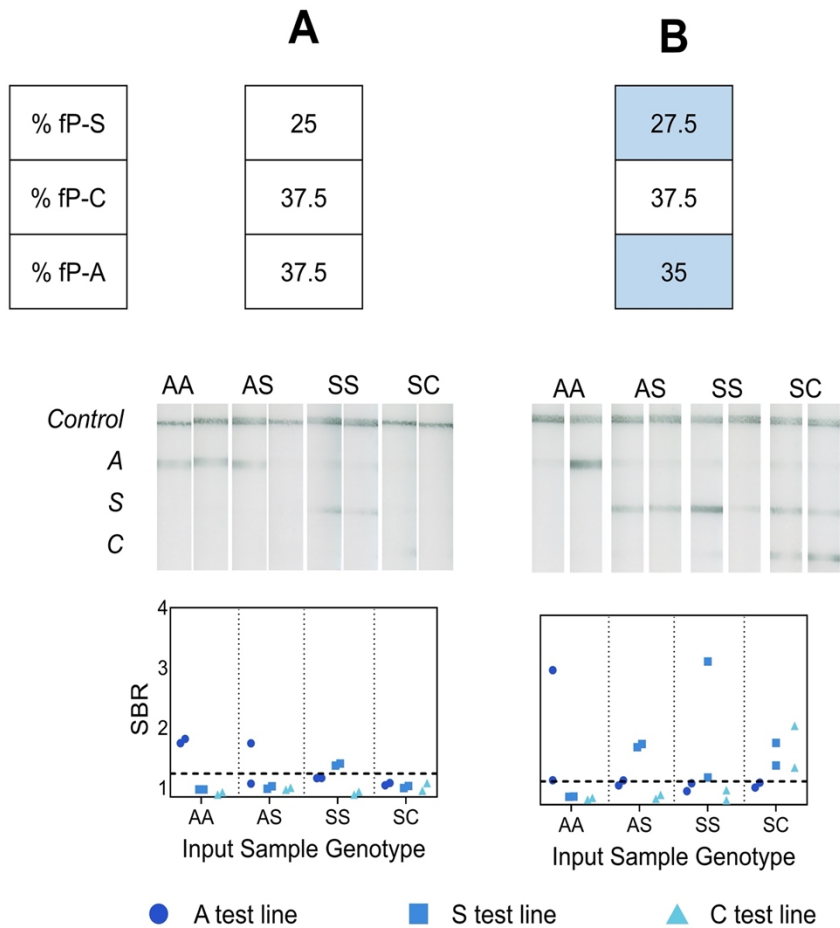
Figure S5. **Primer ratio validations with heterozygous targets.** (A) The best performing primer ratios from Fig. S4 was evaluated with purchased DNA with both homozygous genotypes (AA, SS) and heterozygous genotypes (AS, SC) of interest. The resultant lateral flow strips are shown in the middle, with analyzed signal-to-background ratio (SBR) below. (B) Slight changes to the primer ratios (highlighted in blue) result in stronger signal formation at the test lines of interest for heterozygous targets AS and SC.

Of note, faint non-specific signal formation is observed at the A test line even when the β^A allele is not present, likely due to concentration dependent specificity (i.e. fP-A is unable to maintain allele specificity as millions of copies of off-target amplicons are generated). These results indicated that the reaction needed further optimization to better differentiate between genotypes AS, which does not require treatment, and SS, which does require treatment, either by completely suppressing non-specific signal formation at the A line from targets containing off-target alleles or improving the amplification efficiency of fP-A.

Magnesium, additives, and temperature optimization

110   To further improve the performance of the multiplexed reaction, we explored three
111 different strategies: 1) magnesium catalyst concentration optimization; 2) specificity enhancing
112 additives; and 3) reaction temperature optimization. Previous publications offer conflicting reports
113 of whether decreased [18]or increased [19] magnesium concentrations results in greater RPA
114 specificity for differentiating point mutations. Therefore, we evaluated both lowering the
115 magnesium concentration to 10 mM and increasing the magnesium concentration to 17.5 mM
116 compared to the kit's recommended concentration of 14 mM (Fig. S6).
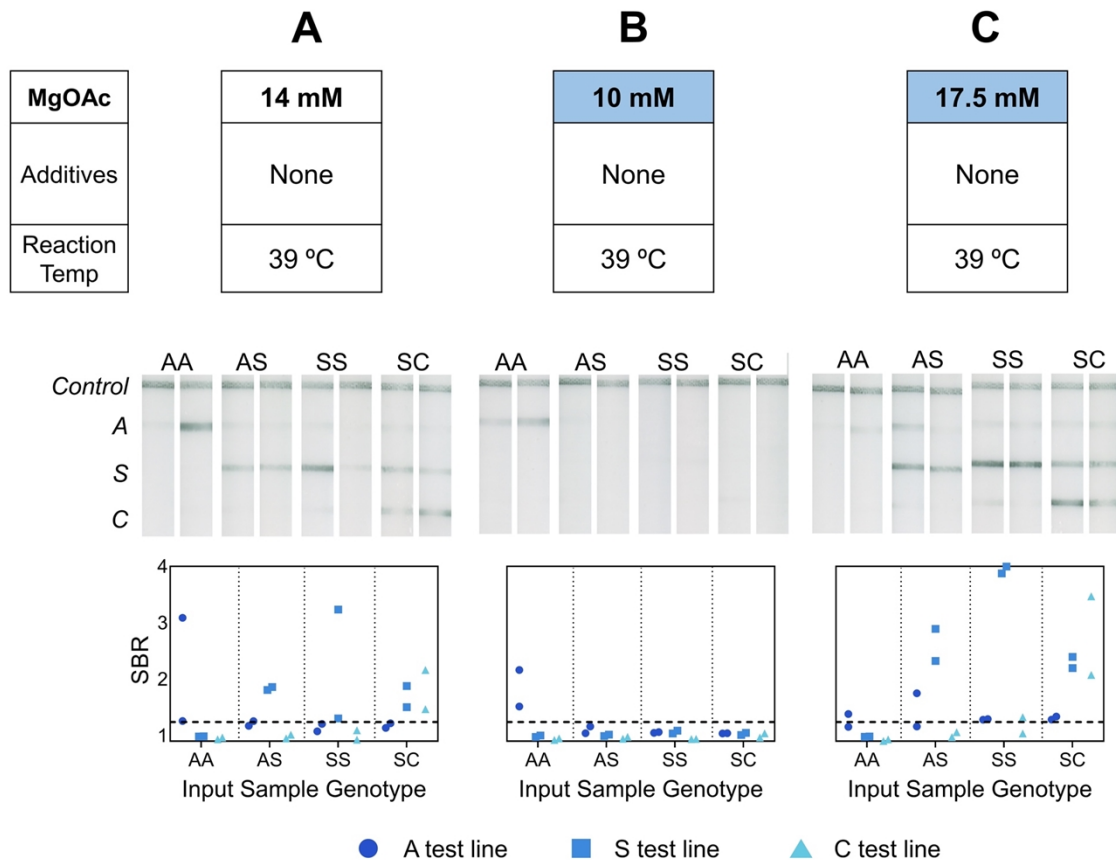

117

118 Figure S6. Magnesium concentration effects on multiplex reaction. Magnesium acetate catalyst
119 was included in the multiplexed reaction at (A) 14 mM, the manufacturer's recommended
120 concentration, (B) 10 mM, and (C) 17.5 mM. The reaction was evaluated with purchased DNA
121 with genotypes AA, AS, SS, and SC. The lateral flow strips are shown in the center row and the
122 analyzed signal-to-background ratio (SBR) is in the bottom row. Magnesium was found to inhibit
123 amplification efficiency at 10 mM, and increase amplification efficiency of fP-S and fP-C at 17.5
124 mM, but no impacts on allele specificity for fP-A were observed. Data in panel (A) was reproduced
125 from Fig. S5 to better visualize effects of altered magnesium concentrations.
126

127   Results indicated that amplification efficiency was greatly reduced at lower magnesium
128 concentrations, as only reactions containing DNA with genotype AA produced valid lateral flow
129 strips (Fig. S6A). On the other hand, increasing the magnesium concentration seemed to greatly
130 improve the amplification efficiency of fP-S and fP-C, but faint non-specific signal at the A test
131 line could be still seen for off-target genotypes SS and SC (Fig. S6C), thus making it difficult to
132 distinguish target DNA with genotype AS from target DNA with genotype SS. Interestingly, the
133 efficiency of fP-A seemed to be inhibited at this higher concentration of magnesium, which is most
134 clearly seen in the faint test lines in target DNA with genotype AA.

135   We next evaluated whether the inclusion of RPA additives could improve the sensitivity
136 and specificity of fP-A in the multiplexed reaction. We first evaluated the effects of incorporating
137 Tte UvrD helicase at 0.4 ng and 0.6 ng per reaction, as Tte UvrD helicase has previously been
138 shown to reduce non-specific amplification in isothermal amplification [55]. Including 0.4 ng of
139 Tte UvrD helicase in the multiplexed reaction resulted in good genotype differentiation between
140 purchased DNA of genotypes AA, AS, SS, and SC using the composite positivity threshold (Fig.
141 S7A), but increasing the amount of helicase to 0.6 ng per reaction did not provide additional
142 specificity in comparison to 0.4 ng per reaction, and resulted in reduced amplification efficiency
143 of fP-A (Fig. S7B).
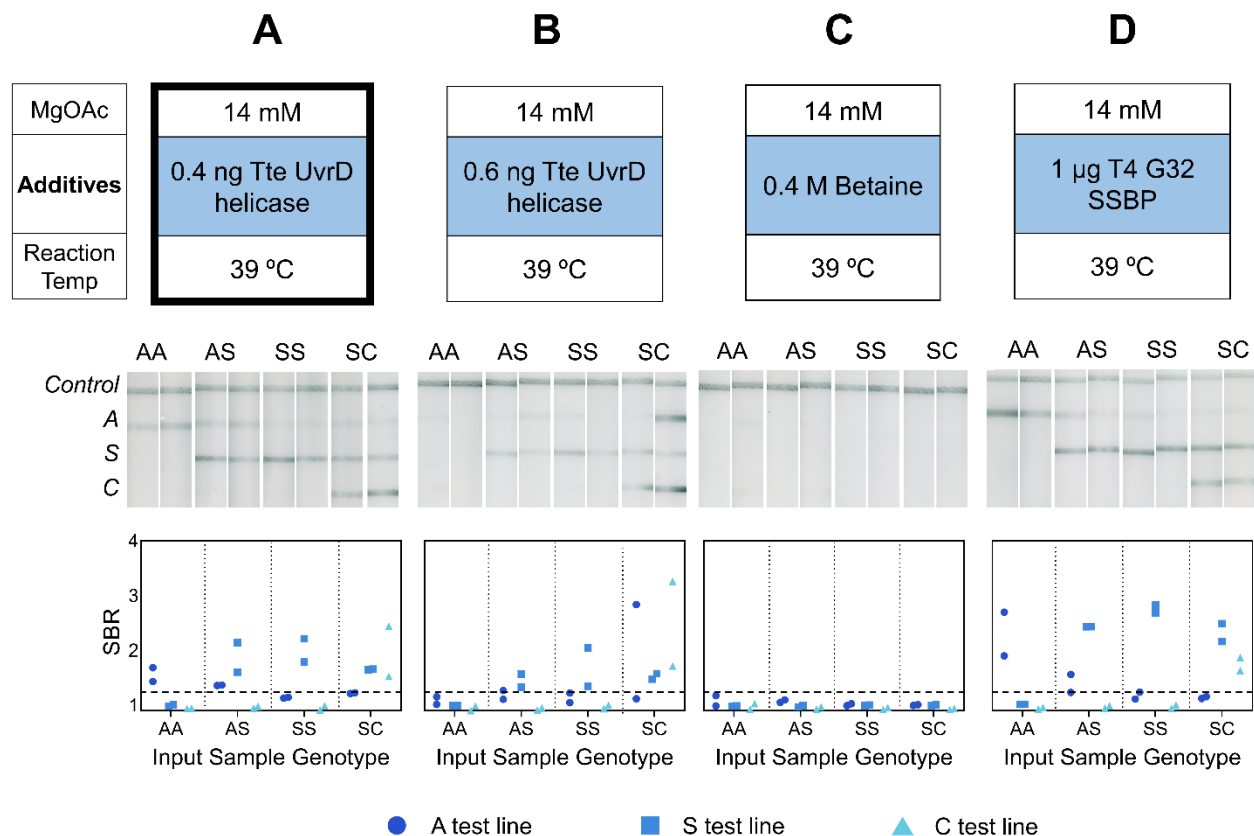
Figure S7. **Effects of specificity enhancing additives on multiplex reaction.** Multiplexed reactions containing (A) 0.4 M betaine, (B) 0.4 ng of Tte UvrD helicase per reaction, (C) 0.6 ng Tte UvrD helicase per reaction, or (D) T4 G32 single-stranded binding protein were evaluated with DNA of genotypes AA, AS, SS, and SC. Lateral flow strips are shown on the middle row, and the analyzed signal-to-background ratio of those strips is plotted on the boom. Condition (A) was chosen as the best performing multiplex assay formulation.

We also tested if adding 0.4 M betaine (Fig. S7C) could improve the multiplexed reaction performance, as previous reports have indicated betaine helps reduce non-specific product formation in RPA [56] and improves recombinase-based amplification [57]. However, at a concentration of 0.4 M in the multiplexed reaction, betaine completely inhibited amplification. T4 gene 32 single stranded binding protein (Fig. S7D) was also evaluated as an additive- we hypothesized that additional single stranded binding protein could stabilize primers and reduce off-target interactions. The addition of single-stranded binding protein was also promising at reducing non-specific A test line formation for samples containing genotypes SS and SC. However, fP-A amplification was again negatively impacted, especially for target DNA with

genotype AS. Of the strategies tested, 0.4 ng of Tte UvrD helicase was chosen as the most promising condition and used for additional optimization.

Next we hypothesized that increasing the reaction incubation temperature could improve reaction performance. We evaluated the multiplexed reaction incubated at 42 °C and 45 °C (Fig. S8) and found that increased temperatures resulted in decreased amplification, likely due to polymerase and enzyme deactivation at these higher temperatures.

Of all the multiplexed assay formulations tested in these optimization experiments, the multiplexed reaction with a standard concentration of 14 mM magnesium, the addition of 0.4 ng per reaction of Tte UvrD helicase, and an incubation temperature of 39 °C (Fig. S7A) was chosen as the optimal reaction conditions.
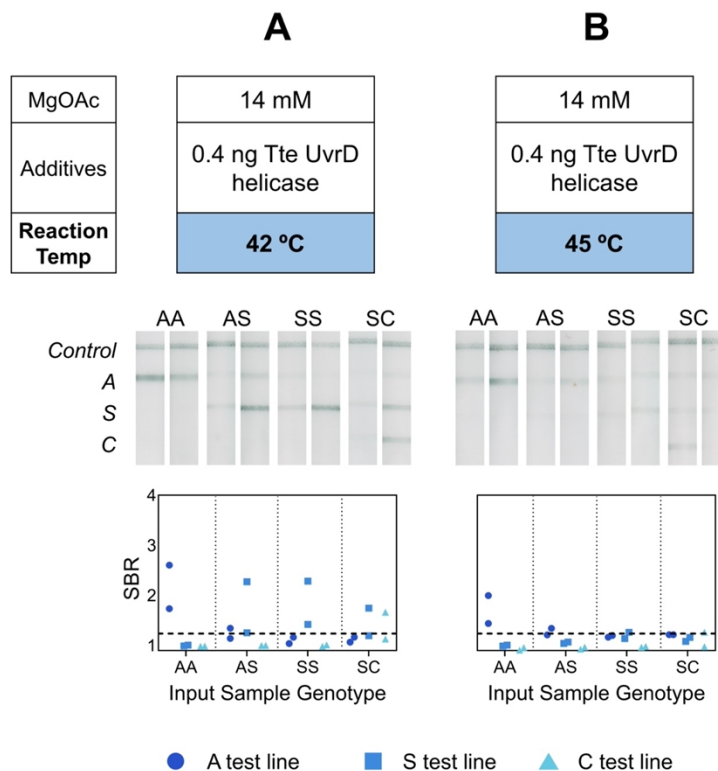
Figure S8. **Multiplex reaction temperature optimization.** The multiplexed RPA reaction was incubated at an increased temperature of (A) 42 °C or (B) 45 °C. The resultant lateral flow strips with target DNA of genotypes AA, AS, SS and SC are shown in the middle, and the analyzed signal-to-background ratio at the A, S, and C test lines are plotted below them for each reaction temperature tested. Increased reaction temperature negatively affected the performance of the multiplexed reaction.

179
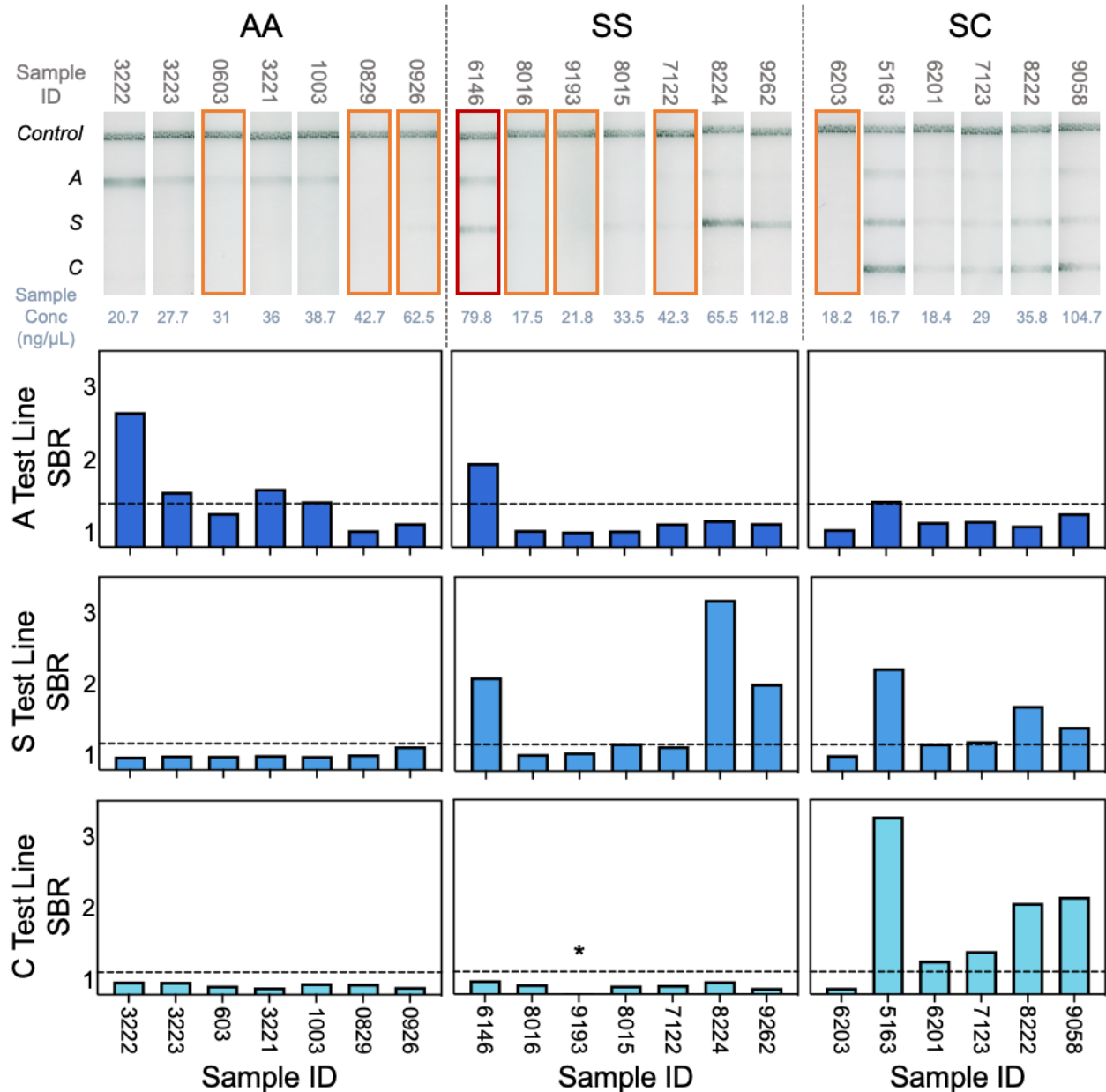
Figure S9. Analyzed lateral flow strips from clinical sample testing. Scanned lateral flow strips (top) resulting from testing of extracted genomic DNA from clinical samples of genotypes AA, SS, or SC. Lateral flow strips for which the multiplexed assay assigned an incorrect genotype are outlined in red, and lateral flow strips for which the multiplexed assay classified as invalid are highlighted orange. Sample DNA concentrations, as measured by NanoDrop, are listed below each corresponding lateral flow strip. The analyzed signal-to-background at the A, S, and C test lines are plotted below the lateral flow strips, with dashed lines indicating test line-specific positivity thresholds. Asterisks indicate SBRs less than the y-axis lower bound.

Test Component	Material	Cost per Test (USD)
Amplification	RPA nfo reagents	0.85
	Primers and Probe	0.01
Lateral Flow Detection	Capture Antibodies	0.91
	Paper/Plastic Components	0.08
	Elution Buffer	0.01
	Gold Nanoshells	5.17
	Total	7.03

Table S4. Estimated reagent and materials costs for the multiplexed 10 µL sickle cell disease detection assay with small batch purchasing. Pre-conjugated streptavidin coated gold nanoshells are the most expensive reagent; however, that cost is expected to significantly decrease with manufacturing at-scale.