Supplementary Information (SI) for Analyst.

Supplementary material for:

Enzyme-free temperature resilient amplification assay with toehold stem-loop probe

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Sequence Type	Sequence Description	Sequence 5′→3′
Probe sequences for SARS-CoV-2 B.1.617.2 variant	Probe 1	CATGGATTTGTCTTCTACAATTGTTGGCATAGGCAAATTGTAGAAGACAAATCCATG
	Probe 2	AGGTTCGCGACGTGCTCGTACGTGGCTTTGGAGACTCCGTGGAGCTCTGATAAGACCTCCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAACCT
	Probe 3	ACGTGCTCGTACGTGGCTTTGGAGACTCCGTGGAGGCCTCTGATAAGACCTCCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGT
	Probe 4	TTCGCGACGTGCTCGTACGTGGCTTTGGAGACTCCGTGGAGGCCTCTGATAAGACCTCCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAA
	Probe 5	CATGTACTCAACATCAACCATATGTAGTTGATGACCCGTAGAAGTGAATAGGACACGGGTCATCAACTACATATGGTTGATGTTGAGTACATG
	Probe 6	ATCAACCATATGTAGTTGATGACCCGTAGAAGTGAATAGGACACGGGTCATCAACTACATATGGTTGAT
	Probe 7	ACACTAATTCTTTCACACGTGGTGTTTCTTTGTCAGGGTAATAAACACCACGTGTGAAAGAATTAGTGT
Same length (SL) target sequences for all the probes	Probe 1 SL	TTGCCTATGCCAACAATTGTAGAAGACAAATCCATG
	Probe 2 SL	GAGGTCTTATCAGAGCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAACCT
	Probe 3 SL	AGGTTCGCGACGTGCTCGTACGTGGCTTTGGAGACTCCGTGGAGGAGGTCT
	Probe 4 SL	AGGTCTTATCAGAGGCCTCCACGGAGTCTGGAAAGCCACGTACGAGCACGTCGCGAA
	Probe 5 SL	GTCCTATTCACTTCTACGGGTCATCAACTACATATGGTTGATGTTGAGTACATG
	Probe 6 SL	GTCCTATTCACTTCTACGGGTCATCAACTACATATGGTTGAT
	Probe 7 SL	ATTACCCTGACAAAGAAACACCACGTGTGAAAGAATTAGTGT
Truncated Target	Probe 2 Truncated target (5 nt)	GAGGTCTTATCAGAGCTCCACGGAGTCTCCAAAGC
Mutant target sequences for Probe 2	TM3	GA**C**GTCTTATCAGAGCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAACCT
	TM5	GAGG**A**CTTATCAGAGCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAACCT
	TM7	GAGGTC**A**TATCAGAGCTCCACGGAGTCTCCAAAGCCACGTACGAGCACGTCGCGAACCT

	TM12	GAGGTCTTATC**T**GAGCTCCACGGAGTCTCCAAAG CCACGTACGAGCACGTCGCGAACCT
	TM15	GAGGTCTTATCAGA**C**CTCCACGGAGTCTCCAAAG CCACGTACGAGCACGTCGCGAACCT
	TM_12_15_18	GAGGTCTTATC**T**GA**C**CT**G**CACGGAGTCTCCAAA GCCACGTACGAGCACGTCGCGAACCT
	TM25	GAGGTCTTATCAGAGCTCCACGGA**C**TCTCCAAAG CCACGTACGAGCACGTCGCGAACCT
	TM36	GAGGTCTTATCAGAGCTCCACGGAGTCTCCAAAG C**G**ACGTACGAGCACGTCGCGAACCT
	TM52	GAGGTCTTATCAGAGCTCCACGGAGTCTCCAAAG CCACGTACGAGCACGT**G**GCGAACCT
Displacer Sequences for Probe 2 Trn	Displacer V1.0	CTCCACGGAGTCTCCAAAGCCACGTACGAGCAC GTCGCGAACCT
	Displacer V2.0	CCGTGGAGAAAAAAAAAAAAAACTCCACGGAG TCTCCAAAGCCACGT
	Displacer V2.1	CCGTGGAG**GAG**AAAAAAAAA**GAG**CTCCACGGA GTCTCCAAAGCCACGT
	Displacer V2.2	CCGTGGAG**GAG**AAA**TTA**AAA**GAG**CTCCACGGA GTCTCCAAAGCCACGT
	Displacer V2.3	CGTGGAGGAGGTCTTATCAGA**C**CTCCACGGAGT CTCCAAAGCCACGT
	Displacer V2.4	CGTGGAGGAGGTCTTATCAG**TC**CTCCACGGAGTC TCCAAAGCCACGT
	Displacer V3.0	TTATCAGA**C**CTCCACGGAGTCTCCAAAGCCACGT
Probe 2 SL target sequences with truncated toeholds	T1.1	AGGTCTTATCAGAGCTCCACGGAGTCTCCAAAGC CACGTACGAGCACGTCGCGAACCT
	T1.2	GGTCTTATCAGAGCTCCACGGAGTCTCCAAAGCC ACGTACGAGCACGTCGCGAACCT
	T1.3	GTCTTATCAGAGCTCCACGGAGTCTCCAAAGCCA CGTACGAGCACGTCGCGAACCT
	T1.4	TCTTATCAGAGCTCCACGGAGTCTCCAAAGCCAC GTACGAGCACGTCGCGAACCT
	T1.5	CTTATCAGAGCTCCACGGAGTCTCCAAAGCCACG TACGAGCACGTCGCGAACCT
	T.16	TTATCAGAGCTCCACGGAGTCTCCAAAGCCACGT ACGAGCACGTCGCGAACCT

Table S1. All probe and target sequences used in the article.

Probe	Best Fit values (One Phase Decay)	
	K_{obs} (min^{-1})	Plateau (A.U.)
Probe-1	0.0108	53680
Probe-2	0.06293	269666
Probe-3	0.0141	57944
Probe-4	0.01031	117079
Probe-5	0.0167	249793
Probe-6	0.007625	169936
Probe-7	0.01628	327528

Table S2. One phase decay value for all the probes presented in Figure 1B.

Target	Best Fit values (One Phase Decay)	
	K_{obs} (min^{-1})	Plateau (A.U.)
Same length target	0.0347	200851
T1.1	0.03341	204177
T1.2	0.02384	203859
T1.3	0.02718	178859
T1.4	0.01089	137301
T1.5	0.01125	132768
T1.6	0.008442	112139

Table S3. One phase decay value for all the different targets with varying toehold length to assess the affinity of the target with the probe (Figure 2B).

Probe	Best Fit values (One Phase Decay)	
	K_{obs} (min^{-1})	Plateau (A.U.)
Probe-2 Full length (44bp)	0.02718	178859
Probe-2-1 (40bp)	0.05885	216861
Probe-2-2 (35bp)	0.06681	359953
Probe-2-3 (30bp)	0.0848	398976
Probe-2-4 (25bp)	0.04953	383964

Table S4. One phase decay value for all the probe-2 variants with different stem lengths (Figure 2C).

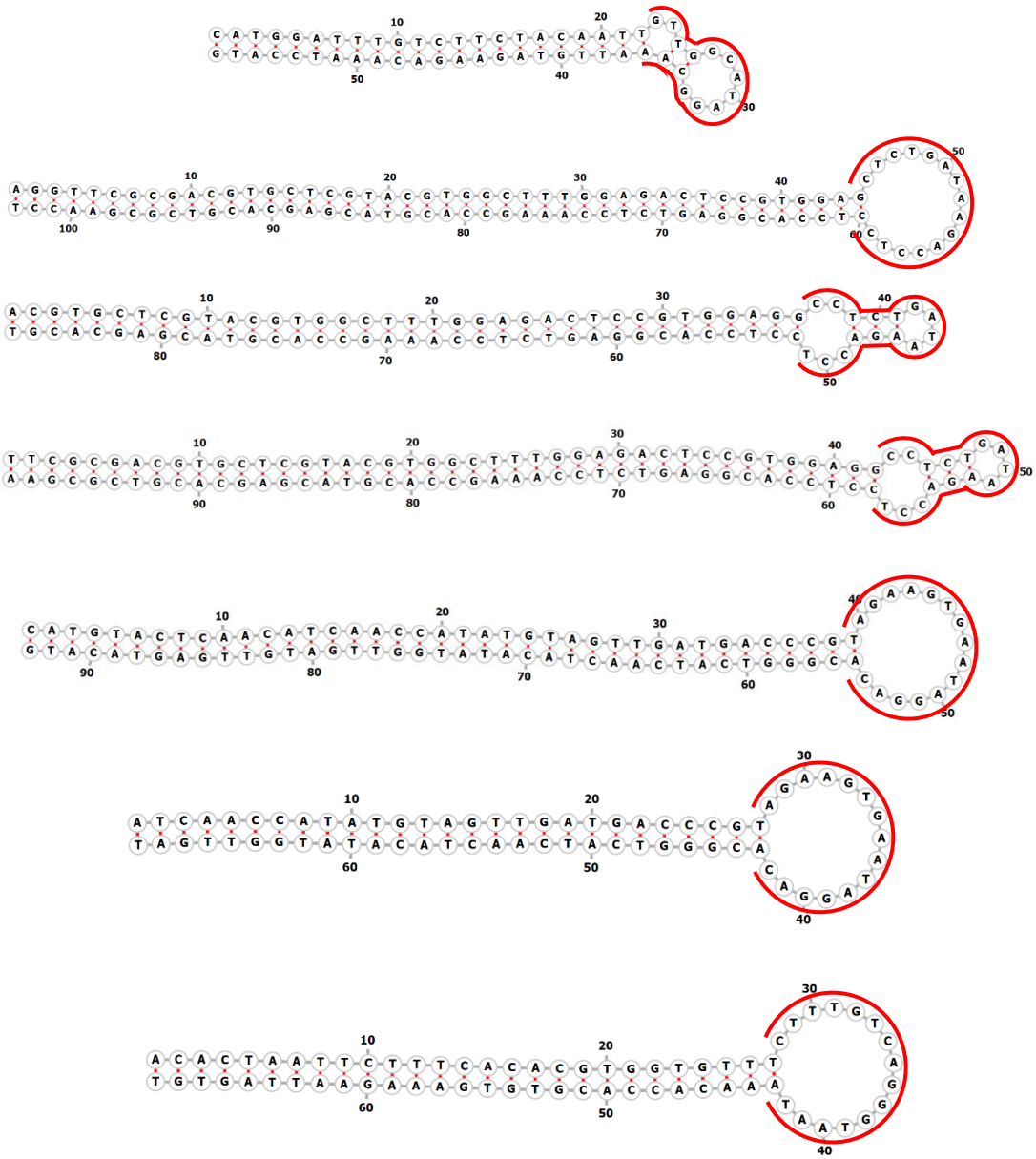

Figure S1. Probe structures predicted using Vienna RNAfold web server [1]. Probe 1 to 7 from top to bottom. The loop domain is highlighted by the red mark.

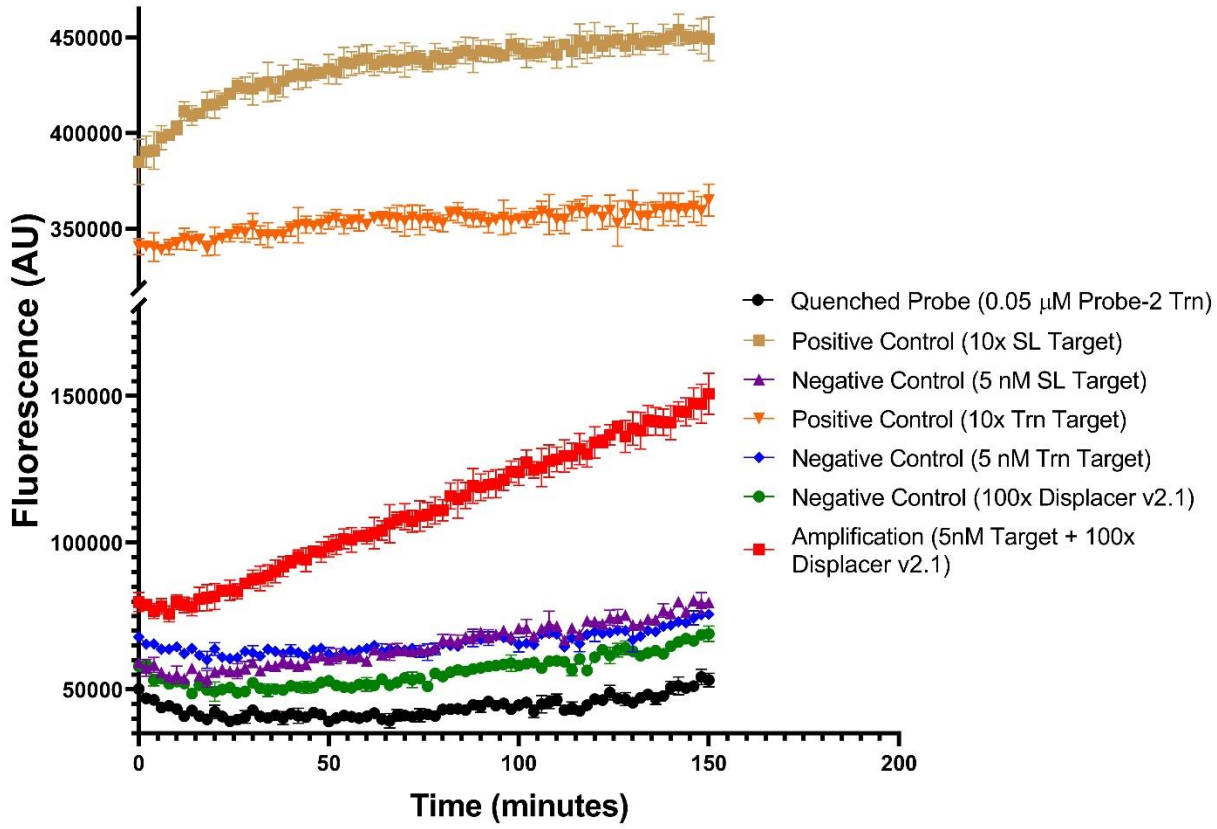

Figure S2. TMSDR assay in the presence of 1 ng/μL human genomic DNA extracted from saliva samples. Probe 2 Trn was used for the assay and Displacer v2.1 was used in 100x concentration. The assays were performed in n=3 replicates, and mean values are plotted. Standard deviations were used for error bars.

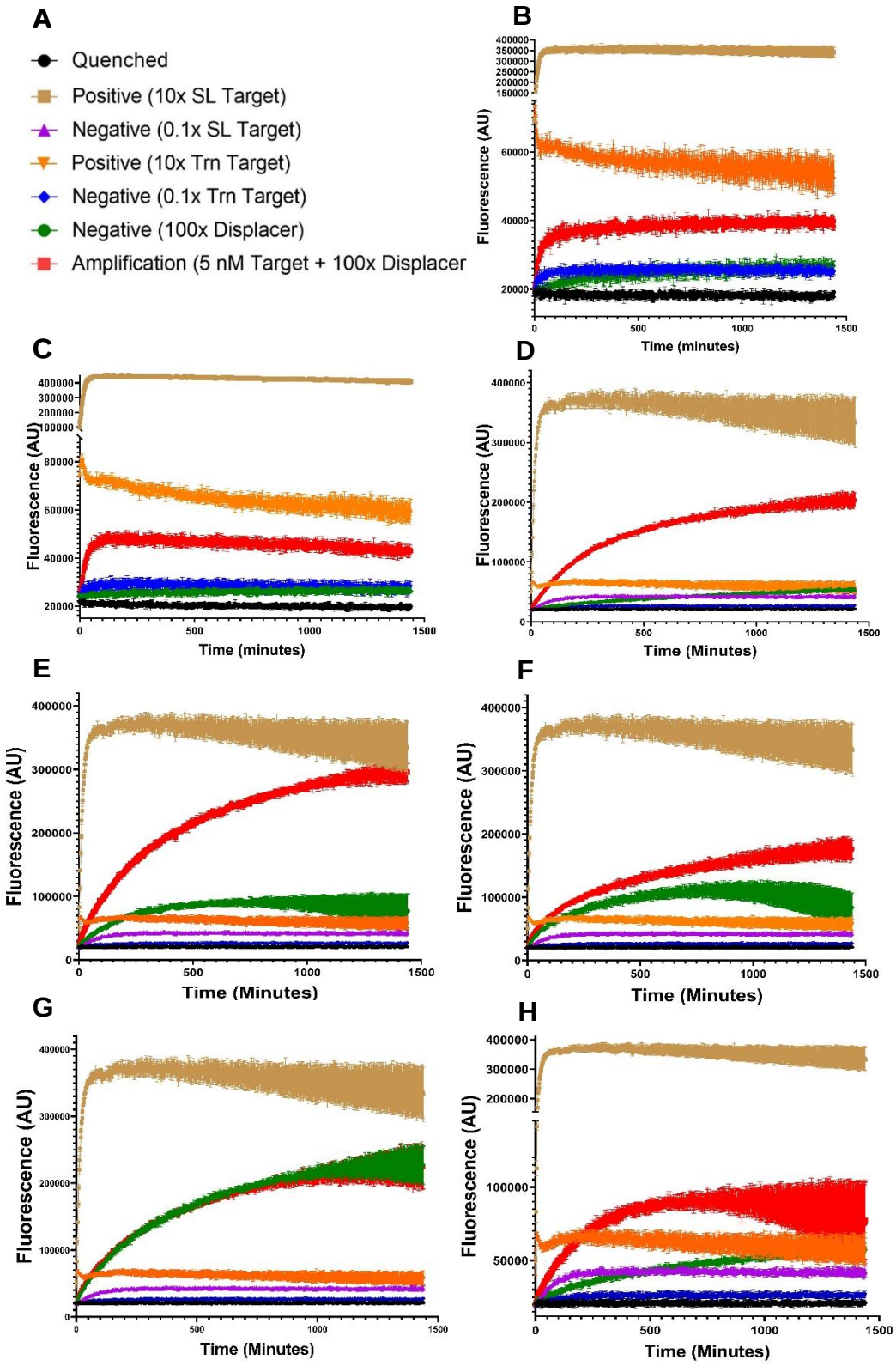

Figure S3. TMSDR amplification assay in the presence of different displacers **A)** Legends for all the graphs presented **B)** Displacer V1.0 **C)** Displacer V2.0 **D)** Displacer V2.1 **E)** Displacer V2.2

F) Displacer V2.3 **G)** Displacer V2.4 **H)** Displacer V3.0. The assays were performed in n=3 replicates and mean values are plotted. Standard deviations were used for error bars.

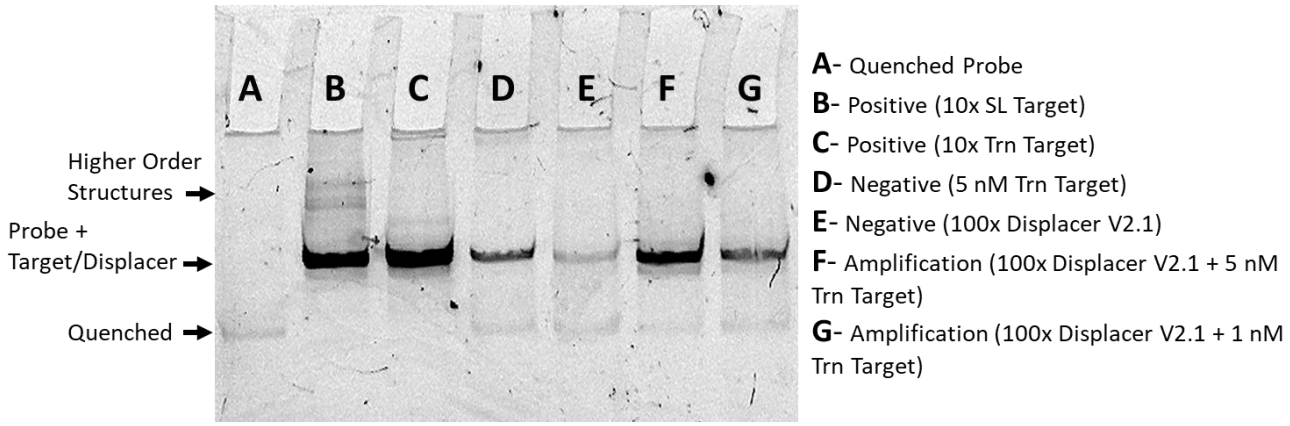

Figure S4. Fluorescence detection of TMSDR assay components using a native 12% polyacrylamide gel. The gel was scanned with a Typhoon FLA 9500 fluorescence image analyzer to detect FAM fluorescence, indicating the activation of the probes under various conditions. The assay was performed for 3h at room temperature. Probe 2-4 was used for the assay and the reaction was performed as described in materials and methods. **A)** Quenched Probe: Lane containing only the probe without any target or displacer **B)** Positive Control (10x SL target): Lane with the probe and a target of the same length that binds to the whole stem, acting as a positive control. **C)** Positive Control (10x Trn Target): Lane with the probe and a truncated target that leaves a 5-nucleotide segment at the end of the stem for the displacer. **D)** Negative Control (5 nM Trn Target) Lane with the probe and 10 times less target without the addition of Displacer V2.1 **E)** Negative Control (100x Displacer v2.1): Lane with the probe and 100 times the displacer concentration without any target **F)** Amplification (5 nM Trn Target + 100x Displacer v2.1): Lane with the probe, 5 nM target, and 100 times the displacer concentration **G)** Amplification (1 nM Trn Target + 100x Displacer v2.1): Lane with the probe, 1 nM target, and 100 times the displacer concentration. Note: After scanning for fluorescence, the gel was also stained with GelRed to detect all the DNA bands and further analyzed using Gel Doc (Bio-Rad). The faint band observed in Lane A was confirmed to be the quenched probe, exhibiting similar intensity to all other lanes. Additionally, the GelRed stain confirmed that higher-order structures were present in trace amounts, as they were not detectable with GelRed staining.

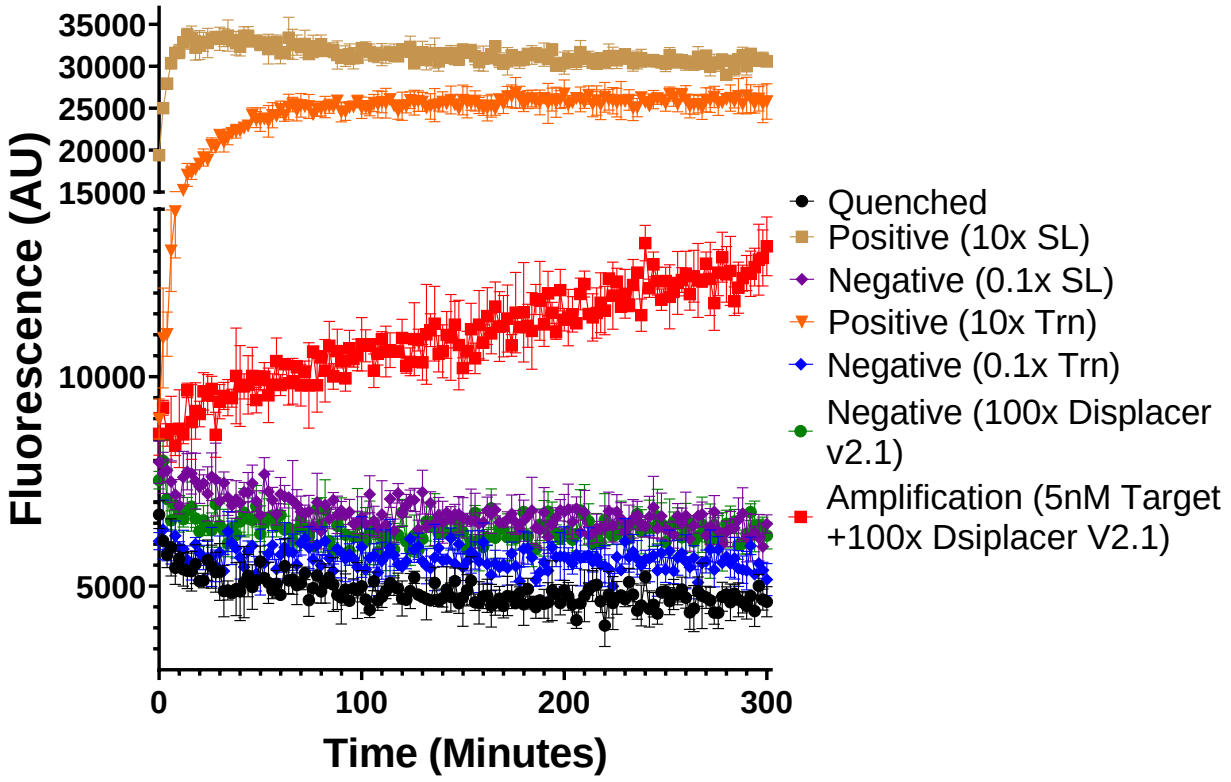

Figure S5. TMSDR assay with probe 6 and displacer v2.1 for probe 6. The graph illustrates the expected amplification with all relevant controls included. The controls validate the assay's performance and specificity, demonstrating that Probe 6 works effectively in the TMSDR setup. The assays were performed in n=3 replicates, and mean values are plotted. Standard deviations were used for error bars.

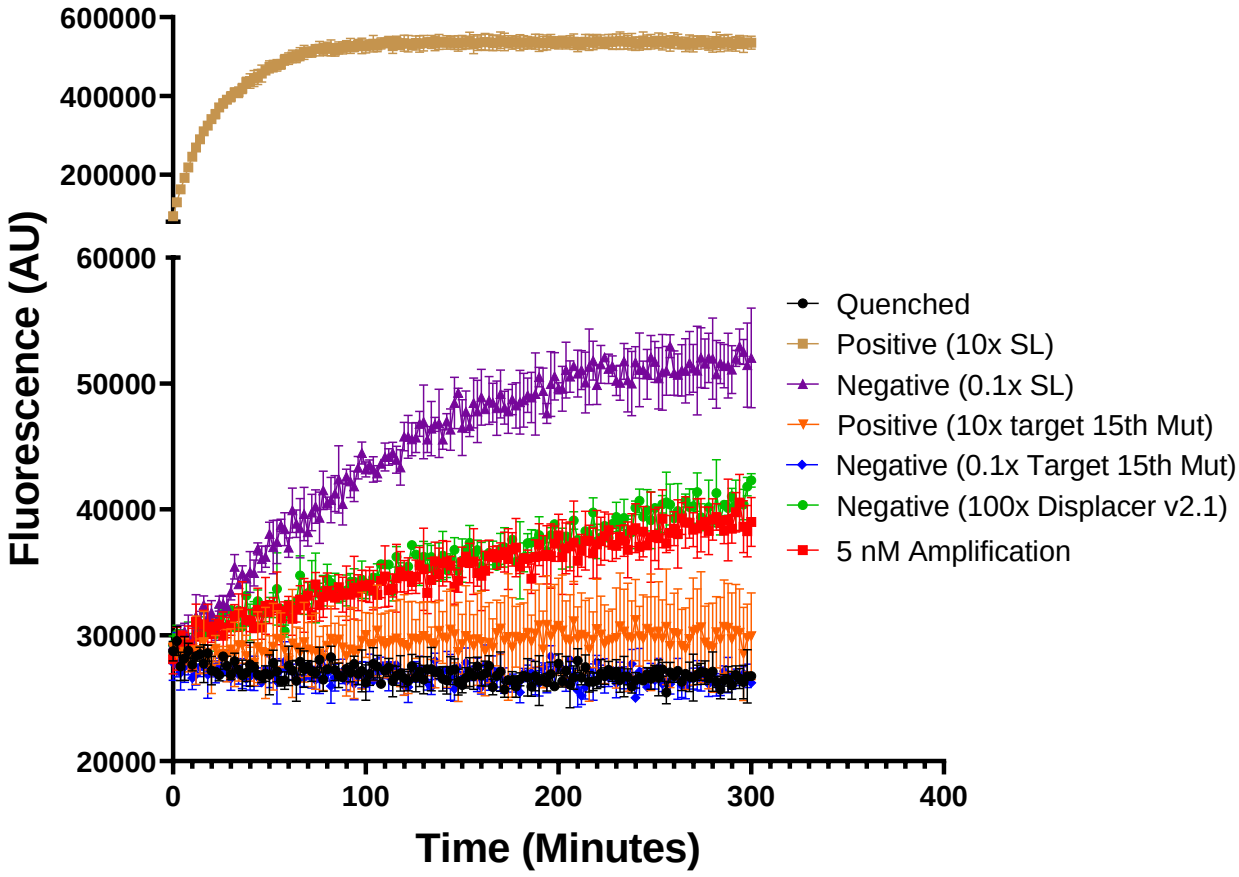

Figure S6. TMSDR amplification assay with target harbouring mutation at 15th nucleotide. Displacer V2.1 was used in 100x concentration. The assays were performed in n=3 replicates and mean values are plotted. Standard deviations were used for error bars.

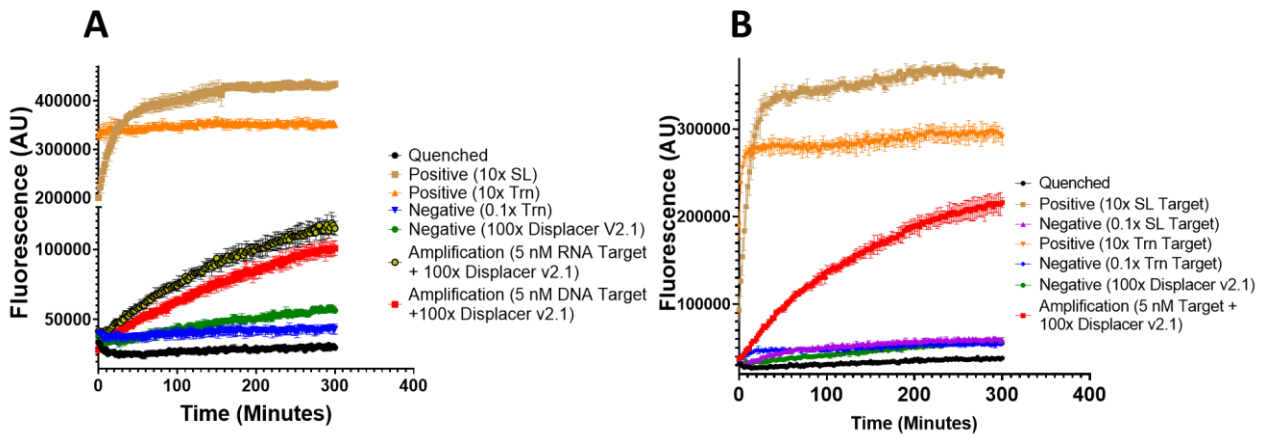

Figure S7. Comparison of TMSDR assay with DNA/RNA target and viral transport media matrix. **A)** Graph showing the amplification results of the TMSDR assay using DNA and RNA targets. The graph illustrates that the amplification efficiency for both DNA and RNA targets is nearly identical, as indicated by the error bars. **B)** Graph depicting the amplification results of the DNA target in Viral Transport Media (VTM) diluted with Viral Lysis Buffer (Galenvs Viral Lysis Kit). This figure demonstrates the TMSDR assay's capability to amplify DNA targets in the presence of VTM. The assays were performed in n=3 replicates, and mean values are plotted. Standard deviations were used for error bars. Note: For Figure S7B, the DNA target is initially dissolved in the viral transport media (VTM) and then subsequently diluted with viral lysis buffer. This viral lysis buffer, which contains chaotropic agents and detergents used for lysing the sample, was obtained from Galenvs Sciences.

References

1. Kerpedjiev, P., S. Hammer, and I.L. Hofacker, *Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams.* Bioinformatics, 2015. **31**(20): p. 3377-9.