Electronic Supplementary Information

Applying Biosensor Development Concepts to Improve Preamplification-Free CRISPR/Cas12a-Dx

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Electronic Supplementary Information for this work includes Supplementary Table (Sequences of CRISPR guide RNA (gRNA), DNA substrate, and DNA target) and Supplementary Figures (Cas12a-Dx reacting with either HEX-labeled substrate or DNaseAlert substrate yield comparable fluorescence signals from same DNA target concentrations, Adjustments in concentrations LbCas12a and gRNA fail to enhance Cas12a-Dx, Lengthening the target region of gRNA from 20-nt to 24-nt fails to enhance Cas12a-Dx, Technical replicates for quantitatively measuring the LOD of our optimal Cas12a-Dx, Piecewise linear fitting outperforms linear fitting in fitting the fluorescence signals from detecting 100 fM dsDNA target via our Cas12a-Dx).

Table S1. Sequences of CRISPR guide RNA (gRNA), DNA substrate, and DNA target. The 20-nt target region in gRNA and DNA target is shown in bold print. The additional 4-nt target region in the gRNA with 24-nt target region and in DNA target is shown in bold italic print. The protospacer adjacent motif (PAM) sequence in DNA target is underlined. (Abbreviations: LbCas12a, New England Biolabs' EnGen Lba Cas12a from Lachnospiraceae bacterium ND2006; AsCas12a, Integrated DNA Technologies' recombinant Acidaminococcus sp. BV3L6 Cas12a; AltR1 and AltR2, Integrated DNA Technologies' proprietary Alt-R modifications; IAbRQSp, Iowa Black RQ; IABkFQ, Iowa Black FQ; TS, target strand; NTS, non-target strand)

Type	Name	Sequence (5' to 3')
CRISPR Guide RNA (gRNA)	LbCas12a gRNA-20	/AltR1/UAAUUUCUACUAAGUGUAGAU**CUACAUUACAGGCUAACAAA**/AltR2/
	LbCas12a gRNA-24	/AltR1/UAAUUUCUACUAAGUGUAGAU**CUACAUUACAGGCUAACAAA*AGUG***/AltR2/
	AsCas12a gRNA	/AltR1/UAAUUUCUACUCUUGUAGAU**CUACAUUACAGGCUAACAAA**/AltR2/
DNA Substrate	FAM	/6-FAM/TTATT/IABkFQ/
	HEX	/HEX/TTATT/IABkFQ/
	Alexa647	/Alex647N/TTATT/IAbRQSp/
	DNaseAlert	Proprietary
DNA Target	TS	GAACTT***CACT*****TTTGTTAGCCTGTAATGTAG**TAAAGTCCAT
	NTS	ATGGACTTTA**CTACATTACAGGCTAACAAA*AGTG***AAGTTC

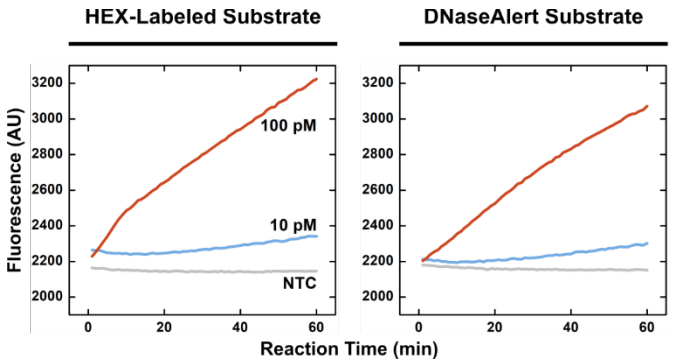

Figure S1. Cas12a-Dx reacting with either HEX-labeled substrate or DNaseAlert substrate yield comparable fluorescence signals from same DNA target concentrations. Both Cas12a-Dx here employ NEB2.1 buffer, 200 nM LbCas12a, 100 nM gRNA, 2 mg/mL bovine serum albumin (BSA), 0.01% Tween 20, and 100 nM of their respective substrate to detect 100 pM (red curves) and 10 pM (blue curves) DNA target (TS only) and no-target control (NTC; gray curves) at 37 °C. DNaseAlert substrate, which has been employed in previous Cas12a-Dx, is manufactured by Integrated DNA Technologies, Inc. (IDT) and is also labeled with HEX, but its sequence is proprietary.

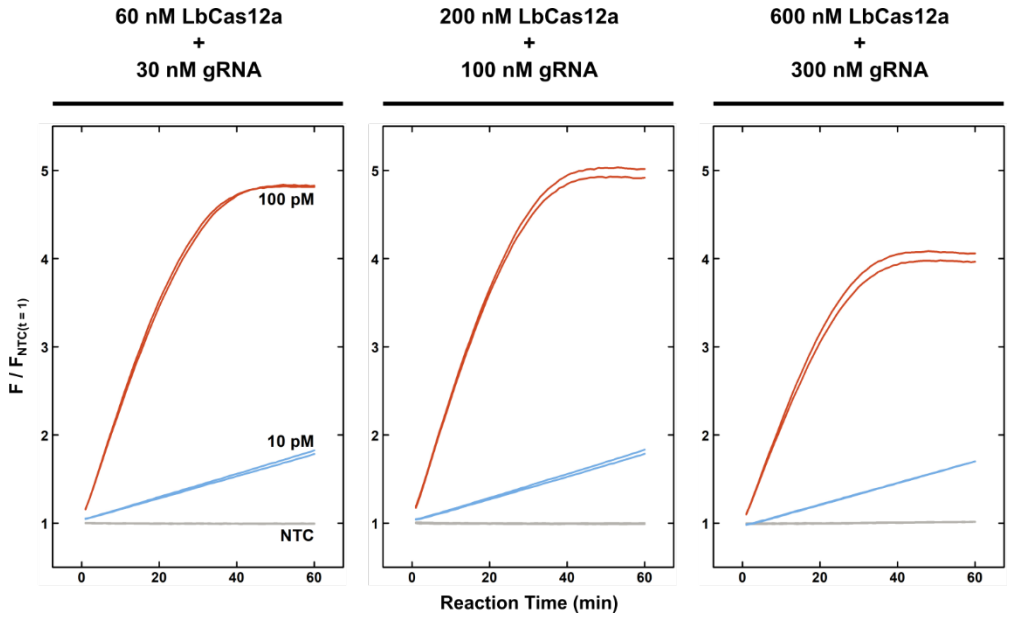

Figure S2. Adjustments in concentrations LbCas12a and gRNA fail to enhance Cas12a-Dx. At a fixed 2:1 LbCas12a:gRNA ratio, 3 versions of Cas12a-Dx employing 60 nM:30 nM, 200 nM:100 nM, and 600 nM:300 nM are compared in detecting 100 pM (red curves) and 10 pM (blue curves) dsDNA target, as well as NTC (gray curves), all in technical duplicates (n = 2). Here, all 3 Cas12a-Dx use NEB2.1 buffer and 300 nM Alexa647-labeled substrate while reacting at 45 °C. Interestingly, the Cas12a-Dx with 600 nM LbCas12a and 300 nM gRNA yields lower fluorescence relative to the NTC than its counterparts. This is resulted from both slightly increased fluorescence from the NTC and notably decreased fluorescence from the targets, which may be explained by binding between substate molecules and inactive LbCas12a-gRNA complex molecules that are free of DNA target molecules. Such binding may have kept the fluorophore and the quencher of the substrate further from each other, thereby slightly increasing the fluorescence of the NTC. Moreover, inactive LbCas12a-gRNA complex likely cannot cleave the substrate. Consequently, when these inactive LbCas12a-gRNA complex molecules (600 nM:300 nM) are in a comparable concentration as the substate (300 nM), they may take up substrate molecules from active LbCas12a-gRNA complex molecules, thus leading to decreased fluorescence in the presence of DNA targets. This is admittedly a presumption. The precise mechanistic underpinning of our observation will be investigated in future studies.

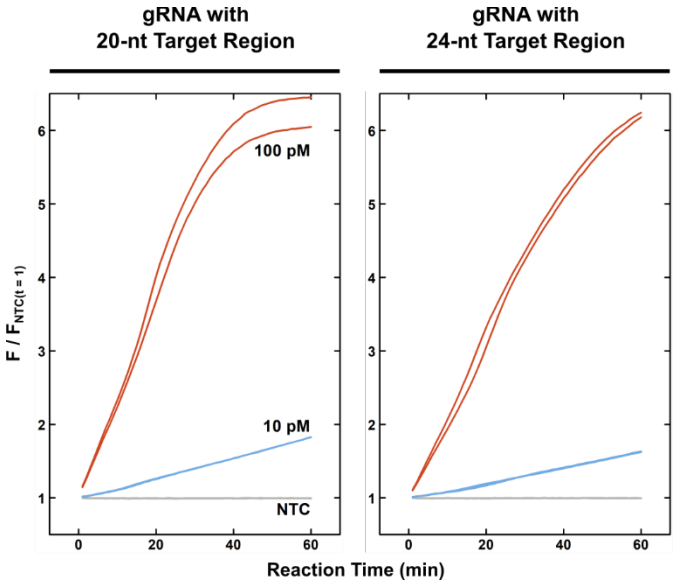

Figure S3. Lengthening the target region of gRNA from 20-nt to 24-nt fails to enhance Cas12a-Dx. 2 versions of Cas12a-Dx employing either gRNA with 20-nt target region (the default length used in this work and in previous Cas12a-Dx) or 24-nt target region compared in detecting 100 pM (red curves) and 10 pM (blue curves) dsDNA target, as well as NTC (gray curves), all in technical duplicates (n = 2). Here, both Cas12a-Dx use NEB2.1 buffer, 100 nM LbCas12a, 100 nM of either gRNA, and 300 nM Alexa647-labeled substrate while reacting at 45 °C. The Cas12a-Dx that employs gRNA with 24-nt target region performs comparatively worse than its 20-nt target region counterpart, as it leads to decreased rate of fluorescence generation from 100 pM dsDNA targets and decreased signal-to-background ratio from 10 pM dsDNA targets.

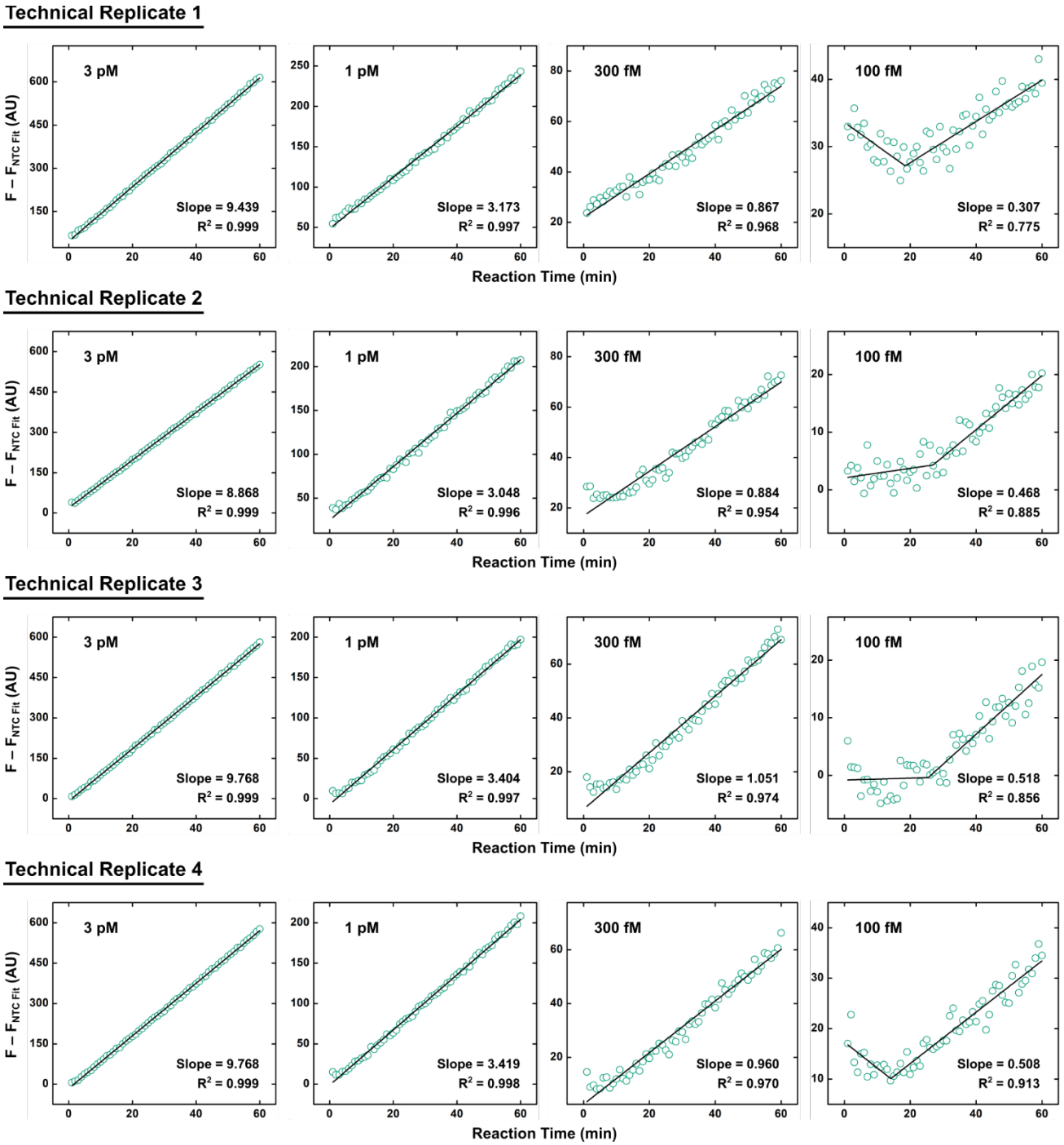

Figure S4. Technical replicates for quantitatively measuring the LOD of our optimal Cas12a-Dx. Here, our Cas12a-Dx employs NEB2.1 buffer, 200 nM LbCas12a, 100 nM gRNA, 300 nM Alexa647-labeled substrate and reacts at 45 °C to detect 3 pM, 1 pM, 300 fM, and 100 fM dsDNA target. Each target titration and NTC are replicated 4 times (n = 4), and the slopes from the real-time fluorescence measurements are determined. To do so, the fluorescence of each NTC (not shown) is first fitted (*i.e.*, $F_{NTC\ Fit}$) and then subtracted from the fluorescence signals of all target titration (*i.e.*, $F - F_{NTC\ Fit}$). For 3 pM, 1 pM, and 300 fM dsDNA, each $F - F_{NTC\ Fit}$ is highly linear, allowing its slope to be computed through simple linear fitting. For 100 fM dsDNA, the $F - F_{NTC\ Fit}$ is segmented, thus necessitating piecewise linear fitting for computing only the slope of the increasing $F - F_{NTC\ Fit}$ that more

accurately reflects the fluorescence increase due to 100 fM dsDNA target. These results demonstrate that our Cas12a-Dx can detect as little as 100 fM dsDNA target.

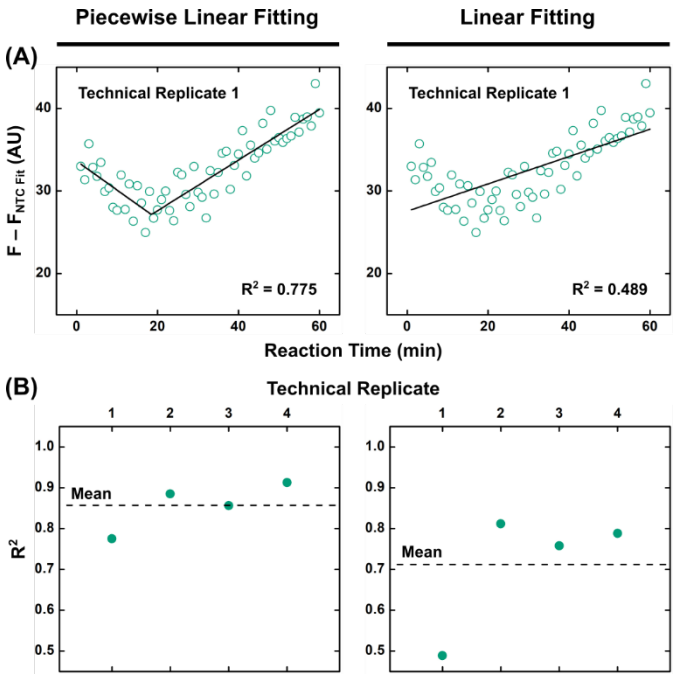

Figure S5. Piecewise linear fitting outperforms linear fitting in fitting the fluorescence signals from detecting 100 fM dsDNA target via our Cas12a-Dx. (A) For example, for our technical replicate 1, R^2 values from piecewise linear fitting and linear fitting are 0.775 and 0.489, respectively. (B) Piecewise linear fitting also results in higher R^2 values across 4 technical replicates.