A Universal Proximity CRISPR Cas12a Assay for Ultrasensitive Detection of Nucleic Acids and Proteins

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1. Materials. EnGen Lba Cas12a (Cpf1), 10 × NEBuffer™ 2.1 Buffer, Klenow Fragment (3'→5' exo-), 10 × NEBuffer™ 2, Deoxynucleotide (dNTP) Solution Mix, nicking endonuclease (Nb.BbvCI) were purchased from New England Biolabs Ltd. (Whitby, ON, Canada). Anti-biotin antibodies were purchased from Thermo fisher Scientific (Mississauga, ON, Canada). Human serum, magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), and 100×Tris−EDTA (TE, pH 7.4) buffer were purchased from Sigma-Aldrich (Mississauga, ON, Canada). NANOpure H_2O (> 18.0 MΩ), purified using an Ultrapure Mili-Q water system, was used for all experiments. All DNA samples and the guide RNAs were purchased from Integrated DNA Technologies (Coralville, IA) and purified using high-performance liquid chromatography. The DNA and RNA sequences are outlined in Table S1.

Table S1. DNA sequences and modifications.

Name	Sequence (5'→3')
Nucleic Acid Detection	
P2 (Template)	GCT TGT GGC CG TTTA CGT CGC CGT CCA GCT CGA CCTCAGC CGTAGA TT GAC TCT GGC TTT-InvT
P1 (Primer)	ATC TCT CTG AAG TT TCTACG
Blocking DNA	TTT TTT CGTAGA[c]
Target	AAA AGA TAA CAA GAA AGAC AAA GCC AGA GTC CTT CAG AGA GA TAC AGA AAC TCT AAT TCA
Protein Detection	
P2' (Template)	GCT TGT GGC CG TTTA CGT CGC CGT CCA GCT CGA CCTCAGC ATGCGTAGA TTT TTT TTT TTT TTT-Biotin
P1' (Primer)	Biotin-TTT TTT TTT TTT TTT TCTACG
CRISPR-Cas12a	
crRNA	UAA UUU CUA CUA AGU GUA GAU CGU CGC CGU CCA GCU CGA CC
Signal Reporter	FAM-TTATT-Quencher

2. Nucleic acid detection using proximity CRISPR Cas12a assay. For a typical test, a 50 µL reaction mixture contained 5 µL of 100 nM P1, 5 µL of 100 nM P2, 10 µL of varying concentrations of genetic target, 3.3 mmol of dNTPs, 5 units of Klenow Fragment and 0.5 unit of nicking endonuclease in 1X NEBuffer™ 2. The solution was incubated at 37 °C for 20 min. A 50 µL enzyme solution which contains 30 nM of Cas12a, 30 nM of gRNA and 60 nM of signal reporter in 1× NEBuffer™ 2.1 was added. Fluorescence was measured immediately after transferring the reaction mixture to a 96-well microplate and kept measuring every 30s for 2 hours at 37 °C using a SpectraMax i3 multi-mode microplate reader (Molecular Devices) with excitation/emission at 485/515 nm.

3. Proximity CRISPR Cas12a assay with blocking DNA. 5 µL of 100 nM P1, 5 µL of 100 nM P2, 5 µL of 200 nM blocking DNA and 10 µL of genetic target with varying concentrations were incubated at 37 °C for 30 min. This reaction mixture was then added with 3.3 mmol of dNTPs, 5 unit of Klenow Fragment and 0.5 unit of nicking endonuclease in 1X NEBuffer™ 2 to a final volume of 50 µL. The solution was incubated at 37 °C for another 20 min. A 50 µL enzyme solution containing 30 nM of Cas12a, 30 nM of gRNA and 60 nM of signal reporter in 1× NEBuffer™ 2.1 was added. Fluorescence was measured immediately after transferring the reaction mixture to a 96-well microplate and kept measuring every 30s for 2 hours at 37 °C.

4. Antibody detection using proximity CRISPR Cas12a assay. For a typical test, 5 µL of 100 nM P1', 5 µL of 100 nM P2' and 10 µL of varying concentrations of antibody were first mixed and incubated at 37 °C for 30 min. A 30 µL enzyme solution containing 3.3 mmol of dNTPs, 5 unit of DNA Polymerase (Klenow Fragment) and 0.5 unit of nicking endonuclease (Nb.BbvCI) in 1X NEBuffer™ 2 was then added. The solution was incubated at 37 °C for 20 min. Another 50 µL enzyme solution containing 30 nM of Cas12a, 30 nM of gRNA and 60 nM of signal reporter in 1× NEBuffer™ 2.1 was added. Fluorescence was measured immediately after transferring the reaction mixture to a 96-well microplate and kept measuring every 30s for 2 hours at 37 °C.

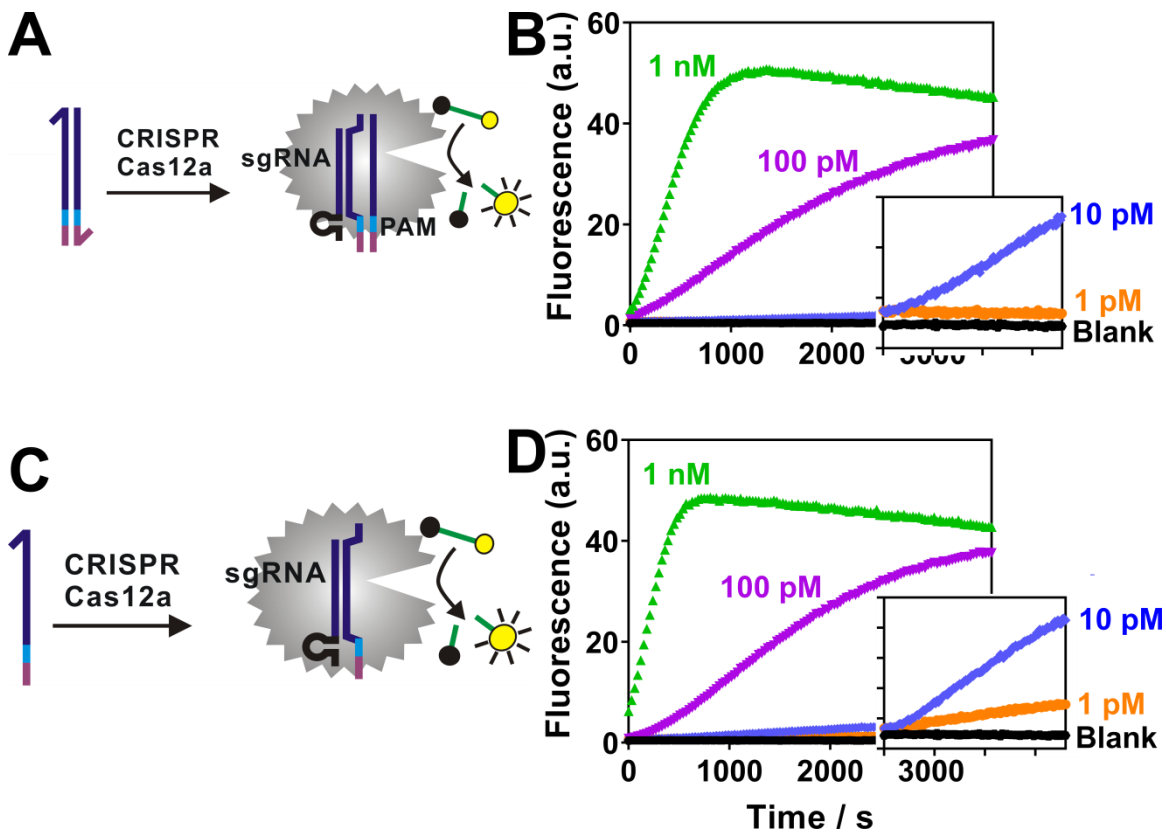

Figure S1. Direct detection of double-stranded DNA (dsDNA) (**A**, **B**) or single-stranded DNA (ssDNA) (**C**, **D**) using direct CRISPR RNA (crRNA) recognition and Cas12a cleavage. The limit of detection (LOD) was determined to be 10 pM for dsDNA (**B**) and 1 pM for ssDNA (**D**).

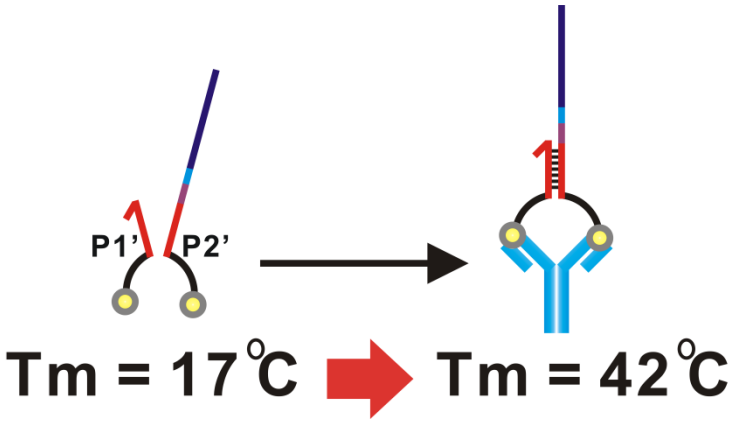

Figure S2. Estimated melting temperature (T_m) between P1' and P2' in the absence or presence of the target antibody. In absence of the target antibody, the estimated T_m = 17 °C by NuPack, suggesting that P1' and P2' do not hybridize at 37 °C. In the presence of the target, the affinity binding to the target protein brings P1' and P2' into proximity, leading to the formation of a stable duplex with an estimated T_m of 42 °C.

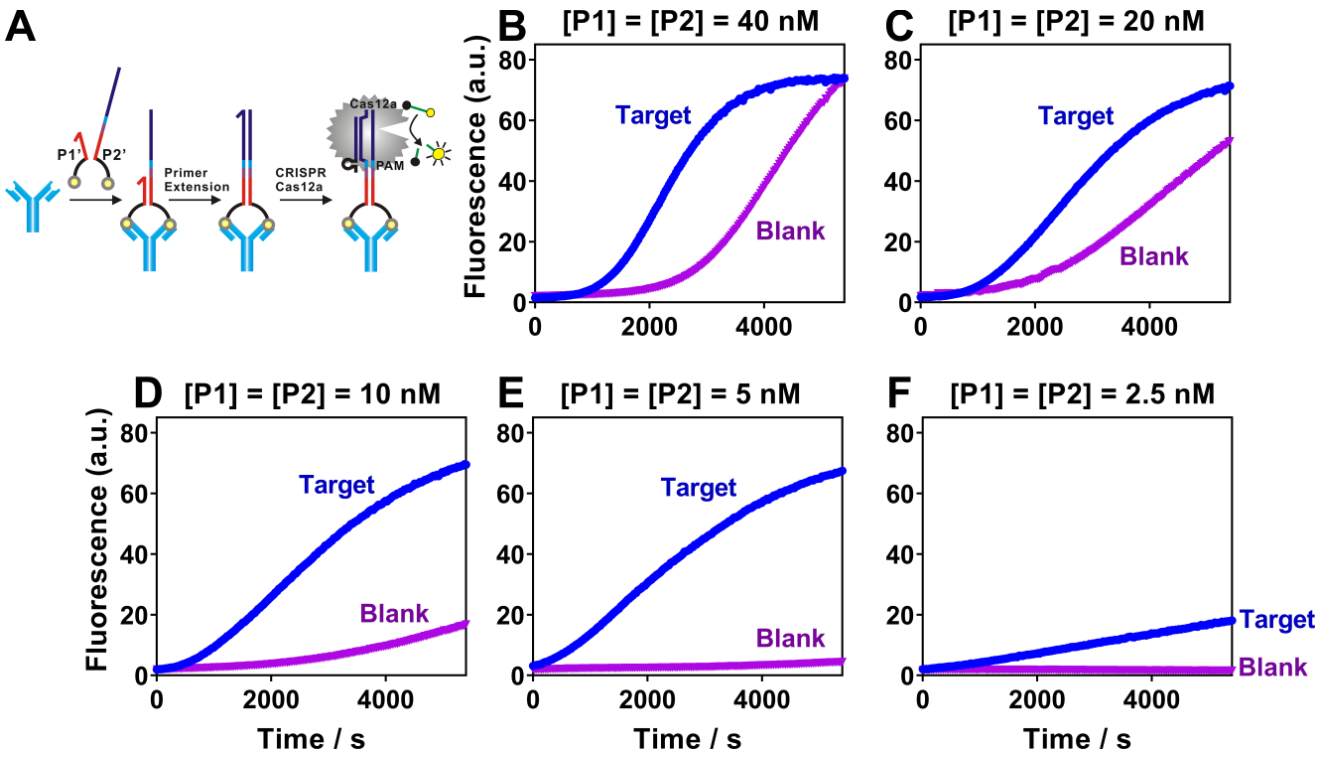

Figure S3. Optimization of the concentrations of proximity probes P1' and P2' for protein analysis. (**A**) Schematic illustration of the detection of protein using a binding-induced primer extension and then CRISPR Cas12a amplification. (**B-F**) Binding-induced primer extension using varying concentrations of P1' and P2' from 40 nM to 2.5 nM. The optimal concentration of P1' and P2' is 5 nM as it maximizes the target-dependent fluorescence signal and minimizes background signal. [Anti-biotin] = 5 nM, [Polymerase] = 1 U.

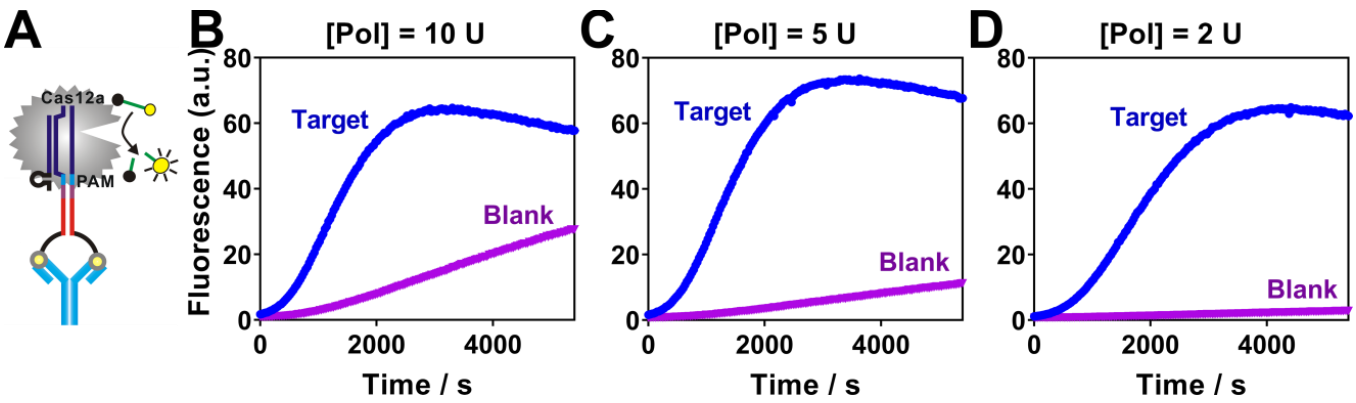

Figure S4. Optimization of the concentrations of DNA polymerase (Klenow Fragment, unit) for protein analysis. (**A**) The detection of protein was achieved using a binding-induced primer extension and then CRISPR Cas12a ampification. (**B-D**) Detection of anti-biotin antibody using varying concentrations of DNA polymerase. The optimal amount of Klenow Fragment was found to be 5 units, as it maximizes detection signals and kinetics while maintains a reasonably low background. [Anti-biotin] = 5 nM, [P1'] = [P2'] = 5 nM.

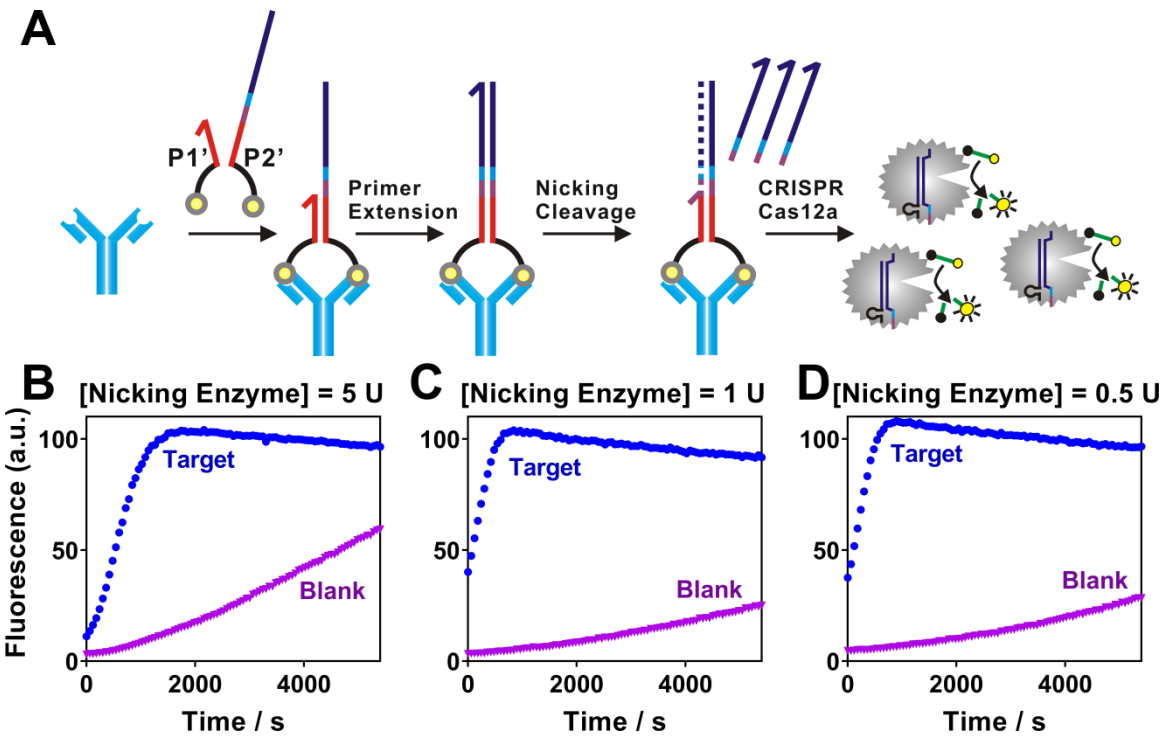

Figure S5. Optimization of nicking endonuclease for the proximity CRISPR Cas12a assay. (**A**) Schematic illustration of antibody detection using priximity CRISPR Cas12a combined with nicking cleavage. (**B-D**) Detection of anti-biotin antibody using varying concentrations of nicking endonuclease from 0.5 U to 5 U. The optimal amount of nicking endonuclease was found to be 0.5 units, as it maximizes detection signals and minimizes the background. [Anti-biotin] = 5 nM, [P1'] = [P2'] = 5 nM, [Polymerase] = 5 U.