Supporting Information for:

Botulinum Toxin as an Ultrasensitive Reporter for Bacterial and SARS-CoV-2 Nucleic Acid Diagnostics

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1. Materials and methods

1.1 Bacteria RNA extraction

All bacteria were purchased from the China Center of Industrial Culture Collection (CICC) and were cultured in trypticase soy broth until a centrifugation (6,000 r.p.m., 10 min) at 4 ℃. Lysozyme (15 mg/mL) /TE buffer and ~200 mg of zirconia beads were added to the pellets followed by a thorough vortex. The harvested bacteria pellets were broken in a tissue cell-destroyer (NewZongKe Viral Disease Control Bio-Tech LTD.) with a small tube adapter for 9 runs of 30 s, with 15 s intervals. Subsequently, BRK Lysis buffer (Bacterial RNA kit, Omega) was added to the lysis mixture according to the protocol. And the RNA was extracted by HiBind® RNA Mini Columns with the final RNA yield detected on NanoDrop Microvolume Spectrophotometers (Thermo Scientific) and agarose gel electrophoresis (DYY-6D, Beijing Liuyi Biotechnology Co. Ltd).

For clinical blood samples provided friendly from the 187th hospital of Haikou, 2 mL of sample was centrifuged to harvest pellets. ACK Lysis Buffer (Gibco) was added and then followed by centrifugations to remove the red blood cells. The RNA was extracted as described above.

1.2 Construction of plasmid pTIG-Trx-BoNT-ALC

The pTIG-Trx-RBS-BoNT-ALC plasmid was constructed based on plasmid pTIG-Trx-BoNT-ALC provided from NovoPro Bioscience Inc (Yu et al., 2011). First, Trx-RBS gene was amplified from pTIG-Trx-BoNT-ALC using primers: F-5' ATTCCATATGAGCGATAAAATTATTCACCTGA3', R- 5' GGAATTCCTCCTTACCGGATCCCGCGGCCAGGTTAGCGTCGA3'. Amplicon was cloned into pMD19-T vector to form pMD19-T-Trx-RBS and sequenced by Sangon Biotech. The pMD19-T-Trx-RBS plasmid was digested with EcoR I and Nde I to excise the Trx-RBS DNA fragment, which was then subcloned into pTIG-Trx-BoNT-ALC plasmid. The engineered plasmid was digested by the same enzymes to produce a recombinant plasmid pTIG-Trx-RBS-BoNT-ALC. The correct pTIG-Trx-RBS-BoNT-ALC plasmid was transformed into *E. coli* strain BL21 (DE3) cells (TransGen Biotech) and the cells were cultured in Luria Bertani (LB) containing 100 g/mL ampicillin to express the engineered GBP-tagged BoNT/ALC protein.

1.3 Protein expression and purification

The recombinant plasmids pET-22b(+)-Trx-SNAP25 and pTIG-Trx-BoNT-ALC were provided from NovoPro Bioscience Inc. and subsequently confirmed by DNA sequencing (Sangon Biotech). The SNAP25 protein was expressed in *E. coli* with plasmid pET-22b(+)-Trx-SNAP25 directly by using thioredoxin fusion expression system. And the GBP-tagged BoNT/A LC protein was co-expressed with thioredoxin in free form by inserting ribosome binding site (RBS) site in pTIG-Trx-BoNT-ALC between BoNT-ALC gene and thioredoxin gene (*trxA*) gene to yield a new expression vector, pTIG-Trx-RBS-BoNT-ALC. Plasmids (pET-22b(+)-Trx-SNAP25 and pTIG-Trx-RBS-BoNT-ALC) were transformed into trans BL21(DE3) pLysS chemically competent cell (TransGen Biotech, China) and were grown in Luria broth for growth at 37 °C and 180 rpm until an OD600 of 0.6. At this time, the SNAP25 and BoNT/A LC protein expressions were induced by addition of IPTG at a final concentration of 0.5 mM with cells cooled down to 25 ℃ and 16 ℃ respectively for 16 h with shaking at 180 rpm. Bacterial cells were subsequently centrifuged at 8000 rpm for 10 min at 4°C, and pellets were harvested and resuspended in lysis buffer (20 mM Na_3PO_4, 500 mM NaCl, 40 mM imidazole, pH 7.4) followed by sonication. Clear supernatants were obtained by lysate centrifugation for 30 min at 4 °C at 10,000 rpm following by filtration with 0.22 μm filters and added onto Bio-Scale Mini Nuvia IMAC Ni-Charged (Bio-Rad Laboratories, Inc., USA) for affinity chromatography purification according to the manufacturer's instruction (NGC Chromatography System, Bio-Rad Laboratories). Fractions containing SNAP25 were collected and stored at -80°C for later usage. While fractions containing BoNT/A LC were pooled and dialyzed at 4 °C against storage buffer (50 mM HEPES, 200 mM NaCl, pH 7.4). Both SNAP25 and GBP-tagged BoNT/A LC were confirmed by SDS-PAGE and western blot (Bio-Rad Laboratories, Inc., USA).

1.4 Characterization of engineered BoNT/A LC and SNAP25 proteins

The activity of engineered BoNT/A LC was detected by SNAPtide FRET assay and confirmed by

SDS-PAGE with the expressed SNAP25 protein as its cleavage substrate at the same time. BCA protein assay kit (Solarbio Life Sciences) was used to evaluate concentrations of the two proteins. To detect its activity, BoNT/A LC was diluted to different concentrations and added 5 μM SNAPtide substrate in cleavage buffer 1 (50 mM HEPES, pH 7.4, 0.05% TWEEN20) to measure the generating fluorescence in a fluorescence plate reader. Moreover, the BoNT/A LC of different concentrations were added 5 μM SNAP25 protein substrates with SDS-PAGE loading buffer mixed to terminate the cleavage reactions before being analyzed by coomassie blue stained acrylamide gel.

1.5 Binding capacities of BoNT/A LC on BNPs

For the binding capability, 200 μL of anti-TAG sequence conjugated GNP at 10 nM and 200 μL of purified GBP-tagged BoNT/A LC (0.3 μM) were incubated at 37 ℃ for 0.5 h. The resultant mixture was centrifuged for 10 min at 10000 rpm. And the forming BNP pellets were washed and resuspended by 400 μL of cleavage buffer. Then 10 μL of the SNAPtide fluorescence detection assay was carried out in cleavage buffer, consisting of 5 μM SNAPtide and 5.6 μL of the BNPs. Meanwhile, a dilution series of the purified BoNT/A LC were added to the cleavage buffer in the same way as described above to prepare the standard curve. Finally, the BC% of BoNT/A LC on BNPs was estimated according to the prepared standard curve.

Table S1. Sequences of primers and probes for bacterial and viral detection.

type	Amplicon	Forward primer	Reverse primer (5`-biotin)	Probe (3`-SH)
Escherichia	GGAGGAAGGGAGTAAAGTTAATACCTTTG CTCATTGACGTTACCCGCAGAAGAAGCAC CGGCTAACTCC	TAGCGCGACCATAGTGA AGAAATA/iSp18/GGAGGA AGGGAGTAAAGTTAAT	AAAAAAGGAGTTAG CCGGTGCTTCT	TATTTCTTCACTATGG TCGCGCTAAAAAAA
Eberthella	GGAGGAAGGTGTTGTGGTTAATAACCGCA GCAATTGACGTTACCCGCAGAAGAAGCAC CGGCTAACTC	TAGCGCGACCATAGTGA AGAAATA/iSp18/GGAGGA AGGTGTTGTGGTTA	AAAAAAGAGTTAGC CGGTGCTTCTTC	
Pseudomonas	GGAGGAAGGGCAGTAAGTTAATACCTTGC TGTTTTGACGTTACCAACAGAATAAGCAC CGGCTAACTTCGT	TAGCGCGACCATAGTGA AGAAATA/iSp18/GGAGGA AGGGCAGTAAGTT	AAAAAAACGAAGTT AGCCGGTGCTTA	
Streptococcus	GAAGAATGATGGTGGGAGTGGAAAATCC ACCAAGTGACGGTAACTAACCAGAAAGG GACGGCTAACTA	TAGCGCGACCATAGTGA AGAAATA/iSp18/GAAGAA TGATGGTGGGAGTG	AAAAAATAGTTAGCC GTCCCTTTCTG	
Staphylococcus	GGGAAGAACATATGTGTAAGTAACTGTGC ACATCTTGACGGTACCTAATCAGAAAGCC ACGGCTAACTAC	TAGCGCGACCATAGTGA AGAAATA/iSp18/GGGAAG AACATATGTGTAAGTA	AAAAAAGTAGTTAG CCGTGGCTTTCT	
Enterococcus	GAAGAACAAGGACGTTAGTAACTGAACG TCCCCTGACGGTATCTAACCAGAAAGCCA CGGCTAACTAC	TAGCGCGACCATAGTGA AGAAATA/iSp18/GAAGAA CAAGGACGTTAGTA	AAAAAAGTAGTTAG CCGTGGCTTTCT	
SARS-CoV-2 *N* gene	GGGGAACTTCTCCTGCTAGAATGGCTGGC AATGGCGGTGATGCTGCTCTTGCTTTGCTG CTGCTTGACAGATTGAACCAGCTTGAGAG CAAAATGTCTG	TAGCGCGACCATAGTGA AGAAATA/iSp18/GGGGAA CTTCTCCTGCTAGAAT	AAAAAACAGACATT TTGCTCTCAAGCTG	

2. Construction of pTIG-Trx-RBS-BoNT-ALC plasmid

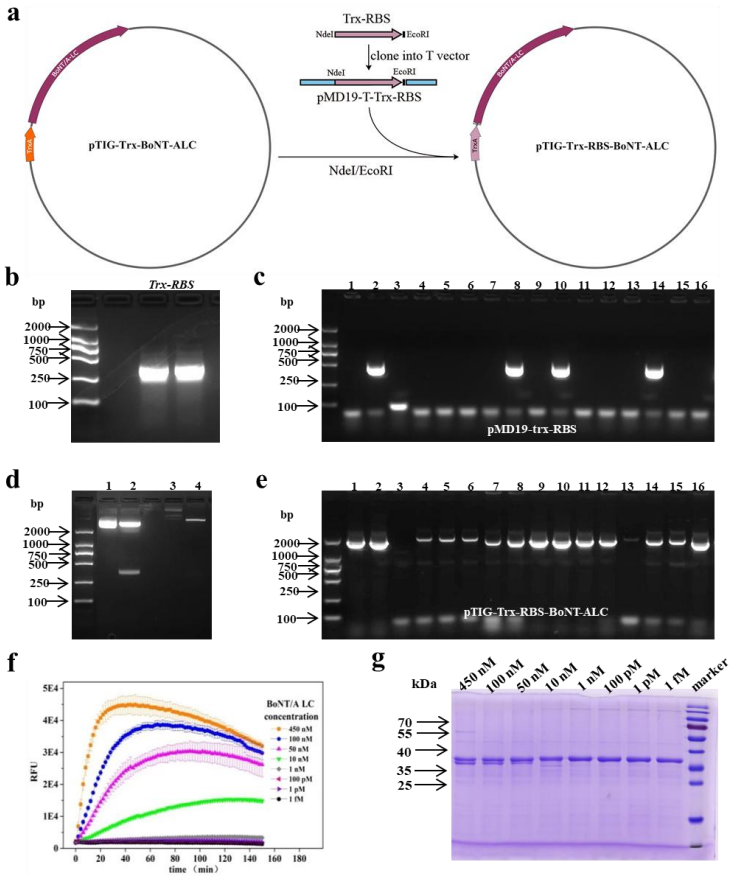

Figure S1. Construction of pTIG-Trx-RBS-BoNT-ALC plasmid. Schematic of pTIG-Trx-RBS-BoNT-ALC plasmid construction (a). Trx-RBS gene was amplified from pTIG-Trx-BoNT-ALC plasmid (b). Amplicon was cloned into pMD19-T vector, forming positive pMD19-T-Trx-RBS clones (2, 8, 10 and 14) (c). The pMD19-T-Trx-RBS plasmid (1) and pTIG-Trx-BoNT-ALC plasmid (3) were digested with EcoR I and Nde I (2 and 4) (d). Analysis (e) of *E. coli* transformant positive and correct clones (1, 2 and 4 to 16) for pTIG-Trx-RBS-BoNT-ALC. The activities of engineered BoNT/A LC were detected by SNAPtide FRET assay (f) and SDS-PAGE (g) with the expressed SNAP25 protein as cleavage substrate in cleavage buffer 1.

3. Protein expression and purification

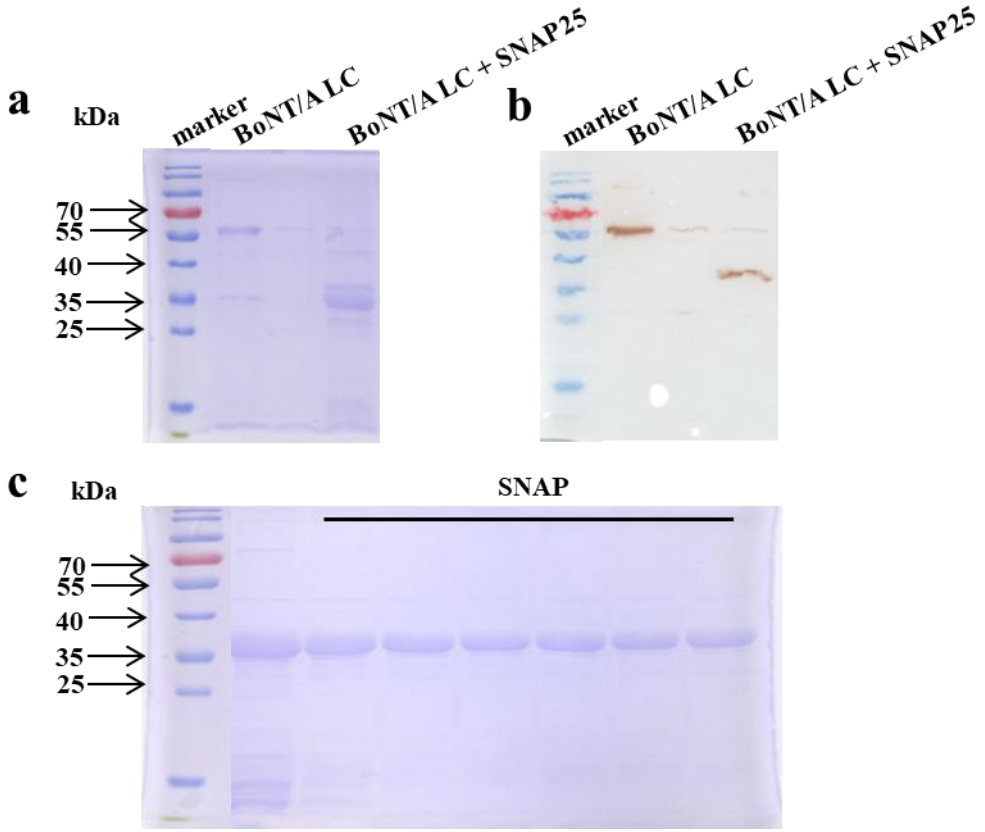

Figure S2. Protein expression and purification. Coomassie blue stained acrylamide gel (a) and western blotting detections (b) of purified BoNT/A LC and BoNT/A LC cleaved SNAP25 protein. SDS-PAGE detections of purified SNAP25 protein (c).

4. Optimization of BoNT/A LC activities

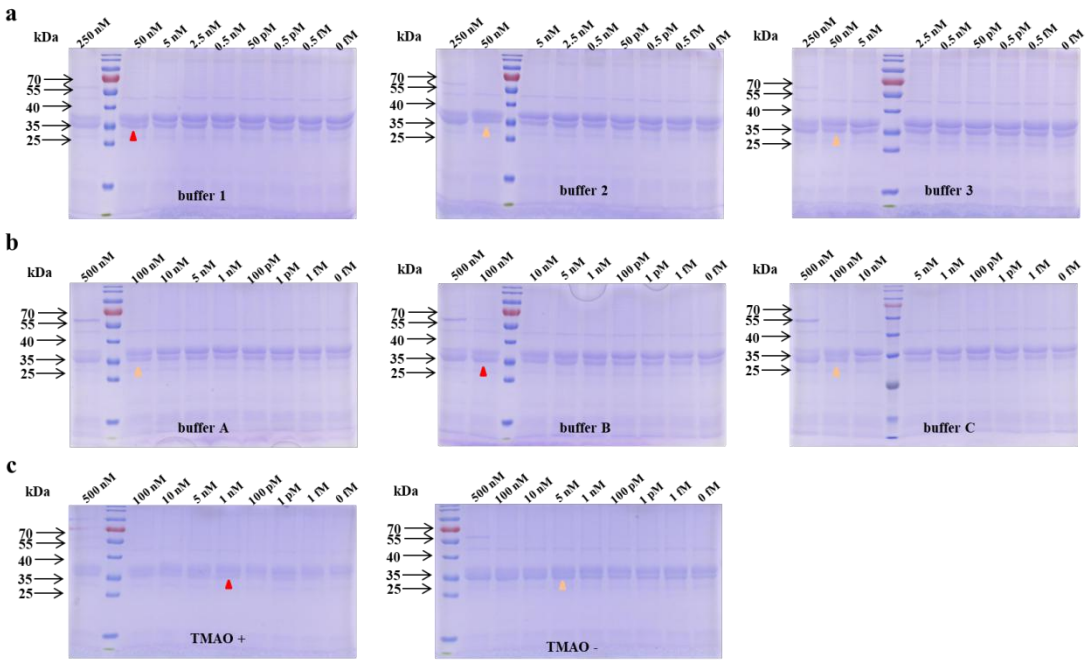

Figure S3. Optimization of BoNT/A LC activities through SDS-PAGE analyses. SNAP25 protein was used as cleavage substrate in this experiment. BoNT/A LC cleavage activity with different cleavage buffers (cleavage buffer 1: 50 mM HEPES, 0.05% TWEEN20, pH 7.4; cleavage buffer 2: 50 mM HEPES, 20 μM $ZnCl_2$, 0.05% TWEEN20, pH 7.4; cleavage buffer 3: 10 mM Tris-HCl with 20 mM NaCl, pH 7.6) (a). BoNT/A LC cleavage activity with different dilution buffers (dilution buffer A: 10 mM PB buffer at pH 7.4; dilution buffer B: 50 mM HEPES, 0.05% TWEEN20, pH 7.4; dilution buffer C: 50 mM HEPES, 200 mM NaCl, pH 7.6) (b). BoNT/A LC cleavage activity with or without TMAO in cleavage buffer (c).

5. Bacterial RNA extraction

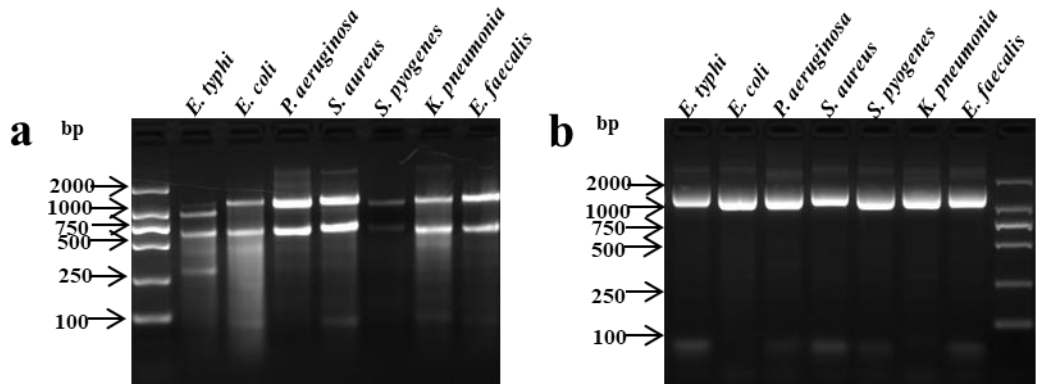

Figure S4. Bacterial RNA extraction (a) and 16S amplification of cDNA template by using 27F/1492R primers (b).

6. Preparation and fabrication of BoNT/A LC functionalized nanocomplexes

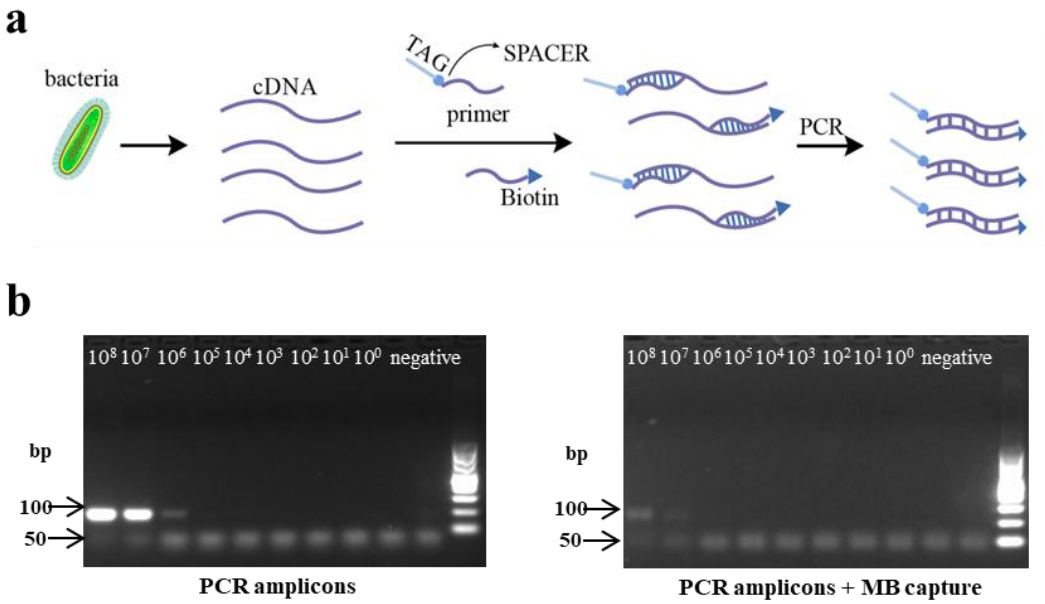

Figure S5. Amplification and capture of biotinylated TAG overhang targets. Schematic of biotinylated TAG overhang targets acquisition (a). Biotinylated TAG overhang targets amplified from a dilution series of *S. pyogenes* were captured by streptavidin magnetic beads (b).

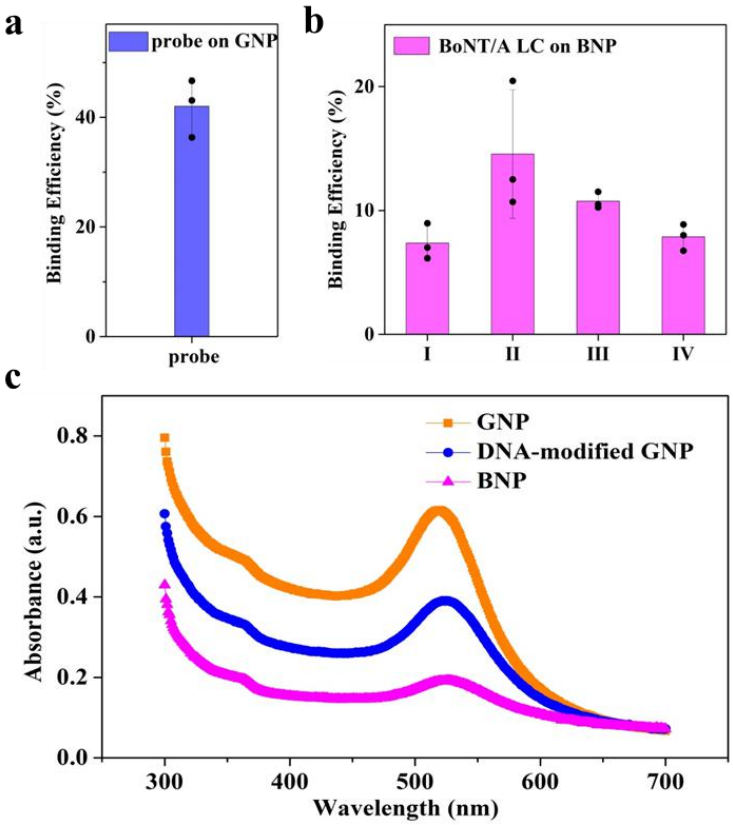

Figure S6. Construction of BNPs. Binding efficiency of anti-TAG probe conjugated onto GNP (a). Optimization of conjugation conditions (I: 0.4 μM BoNT/A LC reacted with GNPs for 0.5 h; II: 0.3 μM BoNT/A LC reacted with GNPs for 0.5 h; III: 0.2 μM BoNT/A LC reacted with GNPs for 0.5 h; IV: 0.1 μM BoNT/A LC reacted with GNPs for 0.5 h) of BoNT/A LC onto BNP (b). Ultraviolet-Visible absorption spectra (c) of GNP, DNA-modified GNP and BNP.

7. Bacteria 16S amplification using respective specific primers

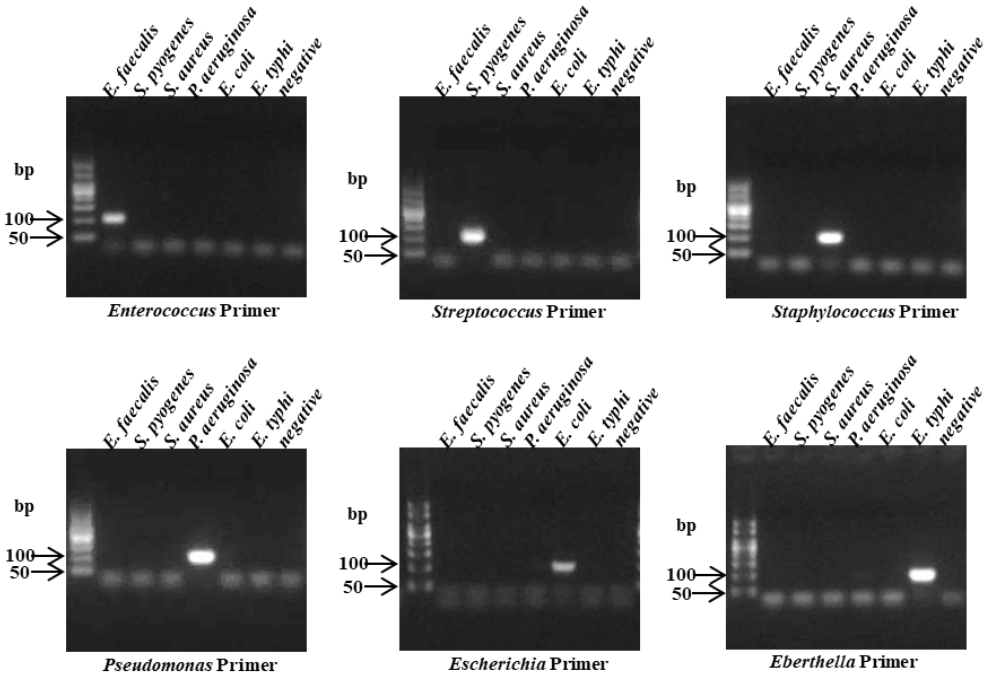

Figure S7. Bacteria 16S amplification using respective specific primers.

8. Bacterial diagnosis and DNA detection by BACA

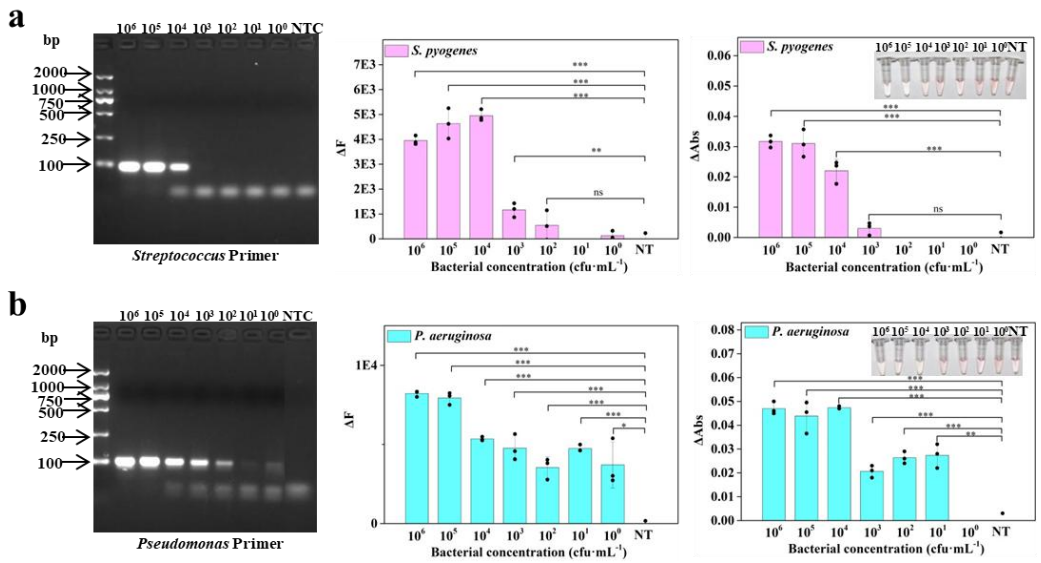

Figure S8. Bacteria diagnosis using BACA. BoNT/A LC based detections for serial dilutions of *S. pyogenes* (a) and *P. aeruginosa* (b)-derived DNA. (n=3 technical replicates, bars represent mean ±s.d.)

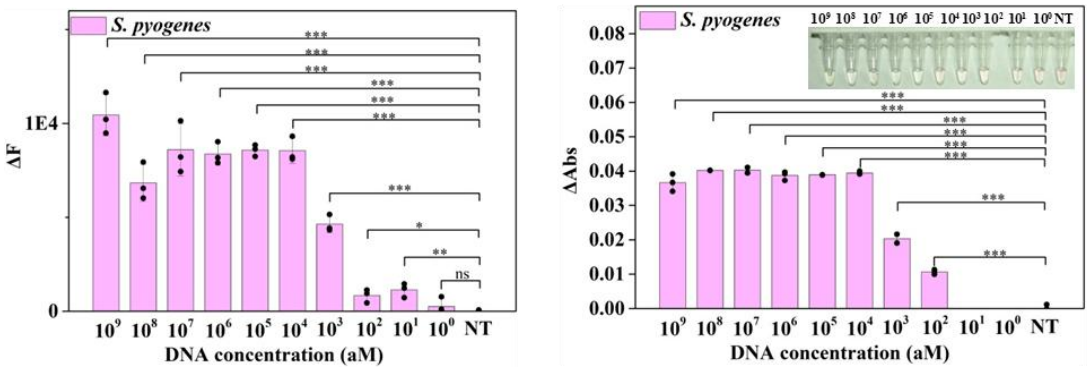

Figure S9. DNA detection using BACA method. BoNT/A LC based detection for serial dilutions of synthetic *S. pyogenes* DNA. (n=3 technical replicates, bars represent mean ±s.d. NT represents no template control)

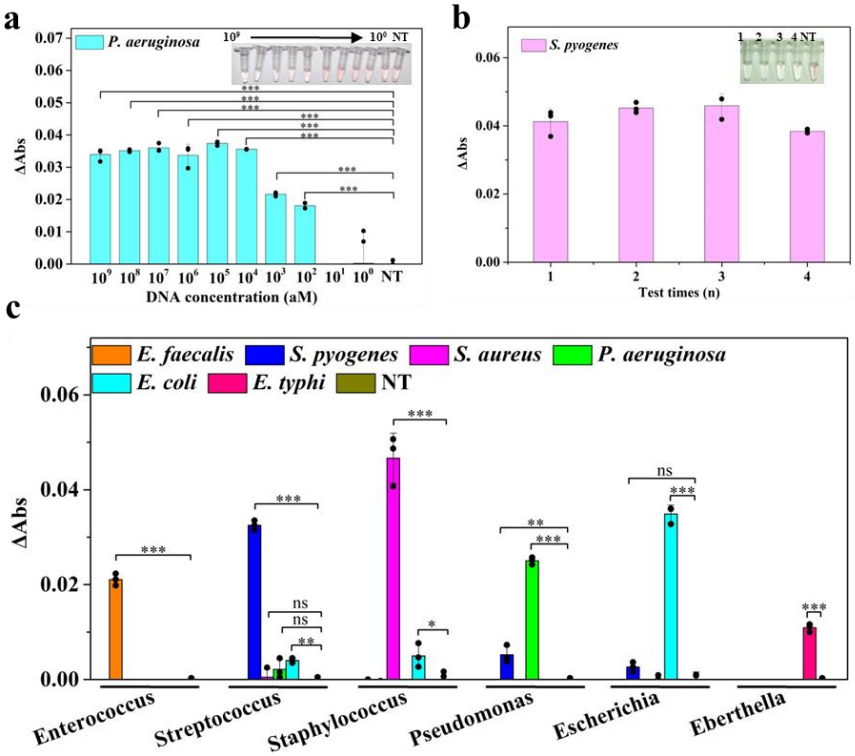

Figure S10. Bacteria detection by colorimetric BACA biosensor. BoNT/A LC based detection for serial dilutions of *P. aeruginosa* DNA (a). The reproducibility of response in relative absorbance intensity through BACA colorimetric method after exposure to the same target DNA in tests (b). Absorbance response for differential detection of 6 bacteria types (e). (n=3 technical replicates, bars represent mean ±s.d. ΔAbs represents the absolute value of background subtracted absorbance. NT represents no template control)

9. Clinical sample analysis

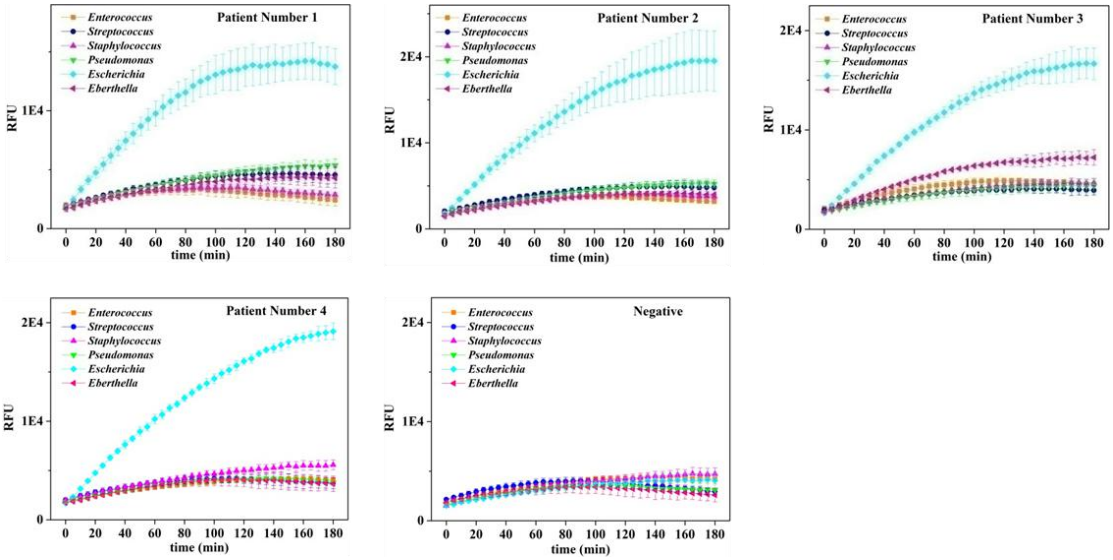

Figure S11. Clinical species-specific target detection by BACA biosensor. (n=3 technical replicates, bars represent mean ±s.d.)

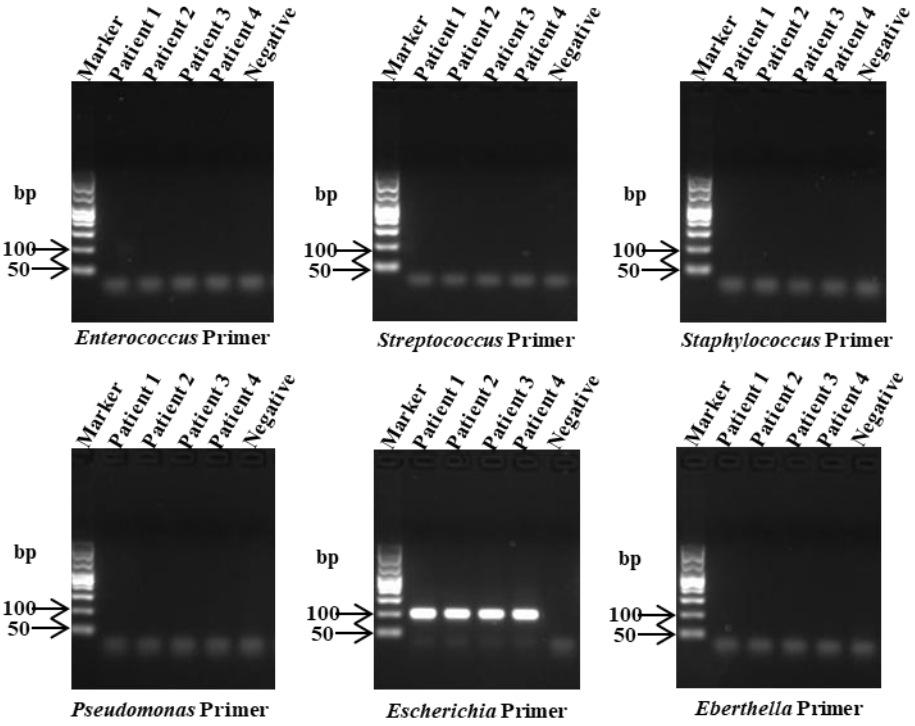

Figure S12. Clinical blood sample total RNA extraction and 16S amplification using respective specific primers.

10. Colorimetric detection sensitivity analysis for SARS-CoV-2 *N* gene

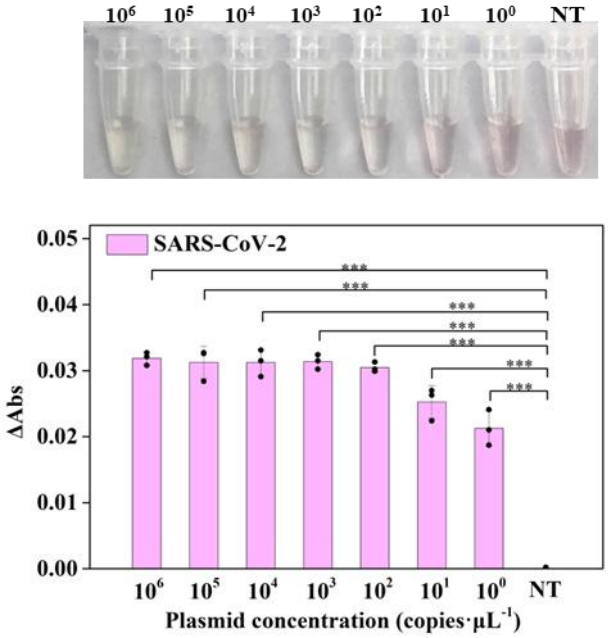

Figure S13. Sensitivity analysis of colorimetric BACA experiment for SARS-CoV-2 *N* gene plasmid. (n=3 technical replicates, bars represent mean ±s.d. NT represents no template control)

11. Pseudovrius detections using BACA biosensor

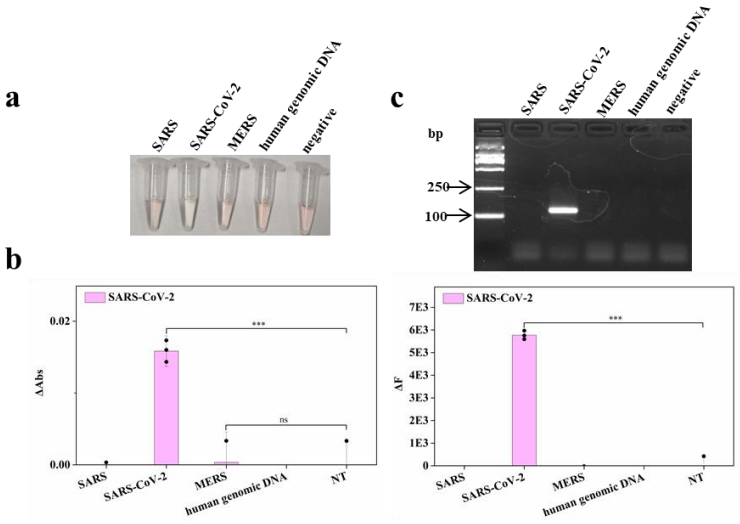

Figure S14. Pseudovrius detections using BACA biosensor. (n=3 technical replicates, bars represent mean ±s.d. NT represents no template control)

Reference:

Yu, Y.Z., Gong, Z.W., Ma, Y., Zhang, S.M., Zhu, H.Q., Wang, W.B., Du, Y., Wang, S., Yu, W.Y., Sun, Z.W., 2011. Co-expression of tetanus toxin fragment C in Escherichia coli with thioredoxin and its evaluation as an effective subunit vaccine candidate. Vaccine 29(35), 5978-5985.