Supplementary Information

Multiple gene detection by selective fluorophore probe-RNA hybridization/graphene-oxide quenching system

Tasnima Alam Asa,[a] and Young Jun Seo[a,*]

[a]Department of Chemistry, Jeonbuk National University, Jeonju 54896, South Korea

*Tel.: +82-63-270-3417; Fax: +82-63-270-3408; E-mail: yseo@jbnu.ac.kr

Contents

1. **Table S1:** Oligonucleotides used in this study. Mismatched bases are marked in red, T7 promoter marked in purple, T7 promoter complementary marked in blue. Each probe color corresponds to its emission region. The underlined part of each gene for the corresponding probe region and "PHO-" indicates a 5′-phosphorylated terminus.

Name	Sequence (5`-3`)
T7 Promoter (20mers)	**TAA TAC GAC TCA CTA TAG GG**
O LigT-1 (51 mers)	PHO- AGG CCA GCA GCA ACG AGC AAA AGG TGT GAG T**CC CTA TAG TGA GTC GTA TTA**
O LigT-1 1mm	PHO- A**C**G CCA GCA GCA ACG AGC AAA AGG TGT GAG TCC CTA TAG TGA GTC GTA TTA
O LigT-2 (54 mers)	PHO- TT TAT ACT CTG CAA GAA GTA GAC TAA AGC ATA AAG ATA GAG AAA AGG GGC TTC A
O LigT-2 2mm	PHO- TT TAT ACT CTG CAA GAA GTA GAC TAA AGC ATA AAG ATA GAG AAA AGG GGC TT**G T**
O LigT-3 (54 mers)	**TAA TAC GAC TCA CTA TAG GG**G CAA AGC CAA AGC CTC ATT ATT ATT CTT ACA AAG
O gene (120 mers)	ACTCACACCT TTTGCTCGTT GCTGCTGGCC TTGAAGCCCC TTTTCTCTAT CTTTATGCTT TAGTCTACTT CTTGCAGAGT ATAAACTTTG TAAGAATAAT AATGAGGCTT TGGCTTTGC
FAM probe (for 'O' gene)	TTTGCTCGTTGCTGCTGGCCTTGAAGCCCCTTTTCTCTAT-**FAM**
E LigT-1 (51 mers)	PHO- CAA GAA TAC CAC GAA AGC AAG AAA AAG AAG T**CC CTA TAG TGA GTC GTA TTA**
E LigT-1 1mm	PHO- **G**AA GAA TAC CAC GAA AGC AAG AAA AAG AAG T**CC CTA TAG TGA GTC GTA TTA**
E LigT-2 (83 mers)	PHO- GA AGG TTT TAC AAG ACT CAC GTT AAC AAT ATT GCA GCA GTA CGC ACA CAA TCG AAG CGC AGT AAG GAT GGC TAG TGT AAC TAG
E LigT-2 2mm	PHO- GA AGG TTT TAC AAG ACT CAC GTT AAC AAT ATT GCA GCA GTA CGC ACA CAA TCG AAG CGC AGT AAG GAT GGC TAG TGT AA**G** TA**C**
E LigT-3 (84 mers)	**TAA TAC GAC TCA CTA TAG GG**T TAG ACC AGA AGA TCA GGA ACT CTA GAA GAA TTC AGA TTT TTA ACA CGA GAG TAA ACG TAA AAA
E gene (178 mers)	ACTTCTTTT TCTTGCTTTC GTGGTATTCT TGCTAGTTAC ACTAGCCATC CTTACTGCGC TTCGATTGTG TGCGTACTGC TGCAATATTG TTAACGTGAG TCTTGTAAAA CCTTCTTTTT ACGTTTACTC TCGTGTTAAA AATCTGAATT CTTCTAGAGT TCCTGATCTT CTGGTCTAA
Cy3 probe (for 'E' gene)	TCTTGCTTTCGTGGTATTCTTGCTAGTTACACTAGCCATC-**CY3**
N LigT-1 (51 mers) **N LigT-1 1mm**	PHO- TTG TGC AAT TTG CGG CCA ATG TTT GTA ATC A**CC CTA TAG TGA GTC GTA TTA** PHO- TT**C** TGC AAT TTG CGG CCA ATG TTT GTA ATC A**CC CTA TAG TGA GTC GTA TTA**
N LigT-2 (70 mers)	PHO- A CGT TCC CGA AGG TGT GAC TTC CAT GCC AAT GCG CGA CAT TCC GAA GAA CGC TGA AGC GCT GGG GGC AAA
N LigT-2 2mm	PHO- A CGT TCC CGA AGG TGT GAC TTC CAT GCC AAT GCG CGA CAT TCC GAA GAA CGC TGA AGC GCT GGG GG**G** AA**T**
N LigT-3 (70 mers)	**TAA TAC GAC TCA CTA TAG GG**G AAA TTT GGA TCT TTG TCA TCC AAT TTG ATG GCA CCT GTG TAG GTC AAC C
N LigT-3* (without T7p-50 mers)-single amplification purpose	G AAA TTT GGA TCT TTG TCA TCC AAT TTG ATG GCA CCT GTG TAG GTC AAC C
N gene (151 mers)	TGATTACAAA CATTGGCCGC AAATTGCACA ATTTGCCCCC AGCGCTTCAG CGTTCTTCGG AATGTCGCGC ATTGGCATGG AAGTCACACC TTCGGGAACG TGGTTGACCT ACACAGGTGC CATCAAATTG GATGACAAAG ATCCAAATTT C
Cy5 probe (for 'N' gene)	CATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAG-**CY5**
Cy5 probe* (for 'N' gene)-single amplification purpose	CTGAAGCGCTGGGGGCAAATTGTGCAATTTGCGGCCAATG-**CY5**

2. **Table S2:** Detection time and limit of detection of RT-qPCR and other isothermal assay.

Assay Name	Detection Time	Sensitivity (LOD)	References
RT-qPCR (Gold standard)	~1 hour	~2.9 copies/rxn or ~580 copies/ml	40
		250-1000 copies/ml	41
RPA	1 hour	6.8 copies/rxn or 1360 copies/ml	42
RCA	<2 hours	1 copy/rxn or 1000 copies/ml	43
dLig-LAMP	1 hour	69.2 copies/rxn or 13840 copies/ml	14
NASBA	1-2 hours	<50 copies/rxn or ~33,000 copies/ml	44
Lig-RPA	30 minutes	1160 copies/ml	11
LDT	45 mintues	~ 370-408 copies/ml	This study

3. Supportive data:

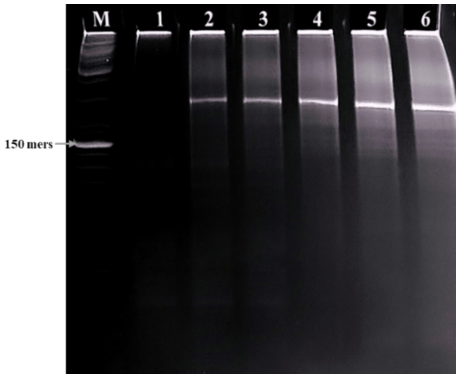

Figure S1: Time dependent study of transcription reaction by LDT-mediated fluorometric detection system with fixed 10 min ligation reaction time. Lane M: 25/100 bp DNA marker, Lane 1: 0 min, Lane 2: 15 min, Lane 3: 30 min, Lane 4: 45 min, Lane 5: 1 hour, Lane 6: 2 hours. In PAGE, the RNA band is always located at a higher position compared to DNA of the same size.

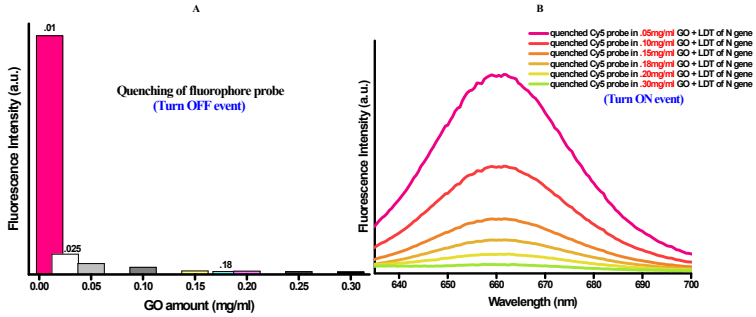

Figure S2: Optimization of LDT-mediated fluorometric detection system. **(A)** Quenching property of FAM/Cy3/Cy5 fluorophore-labeled probe oligos with different concentrations of graphene oxide (from .01 mg/mL to .30 mg/mL). **(B)** Fluorescence spectra of fluorophore probes from quenched state to turn ON state, because of hybridization between the fluorophore probes and complementary amplified RNAs. All fluorescence (normalized) emission peak intensities were measured at the wavelength of 660 nm (Cy5).

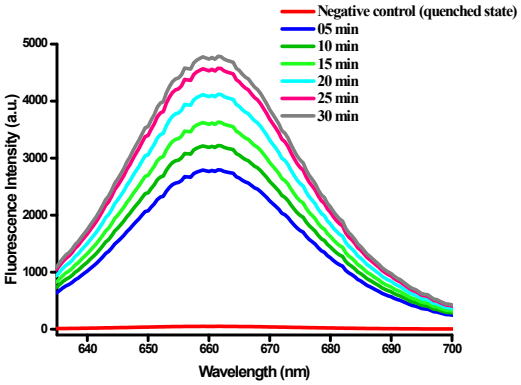

Figure S3: Analysis of dependence of the turn ON fluorescence measurement on the incubation of the "N" gene targeted LDT (ligation-double transcription) reaction with quenched solution [0.1 µM Cy5 probe/GO (0.05mg/mL) in 1X trizma buffer] after various durations (from 5 min to 30 min) against negative control/quenched state. All fluorescence intensities were measured at 660 nm (Cy5).

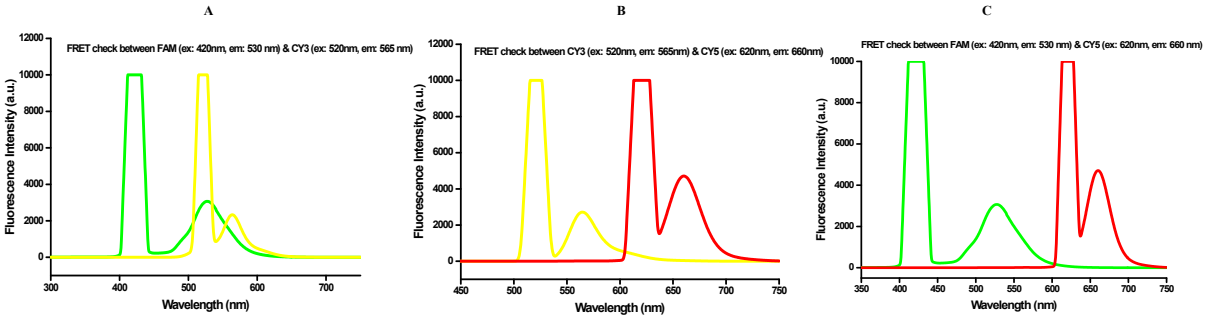

Figure S4: Analysis of FRET between 3 different fluorophore probes [FAM (emission in green region), Cy3 (emission in yellow region) and Cy5 (emission in red region)] in1x trizma buffer. The fluorescence emission peak intensities were measured at the wavelengths of 530 nm (FAM), 565 nm(Cy3) and 660 nm (Cy5).

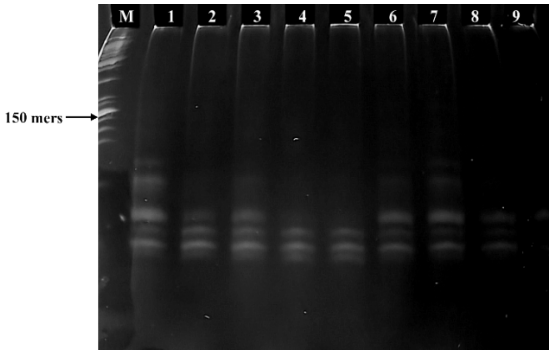

Figure S5: Analysis of 20% denaturing PAGE of bacterial selectivity study of multiple gene detection by LDT-mediated fluorometric assay. Here, Lane M: 25/100 bp DNA marker, Lane 1: *Enterococcus feacium*, Lane 2: *Staphylococcus epidermidis*, Lane 3: *Klebsiella pneumoniae*, Lane 4: *Enterococcus faecalis*, Lane 5: *Enterobacter cloacae*, Lane 6: *Escherichia coli*, Lane 7: *Acinetobacter baumannii*, Lane 8: *Pseudomonas aeruginosa*, Lane 9: *Staphylococcus aureus.* In PAGE, the RNA band is always located above the band of DNA of the same size.

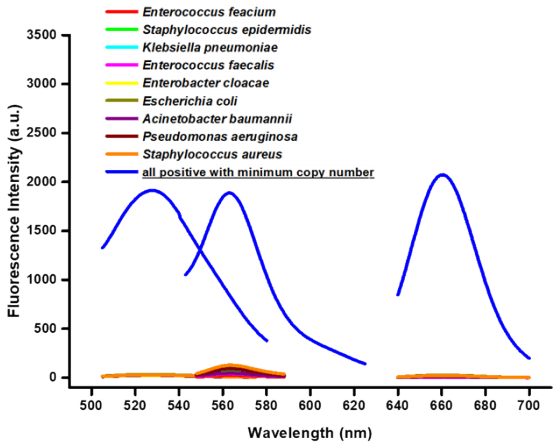

Figure S6: Fluorescence spectra of bacterial selectivity analysis of multiple gene detection by LDT-mediated fluorometric assay. All fluorescence emission peak intensities were measured at wavelengths of 530 nm (FAM), 565 nm(Cy3) and 660 nm (Cy5), respectively.

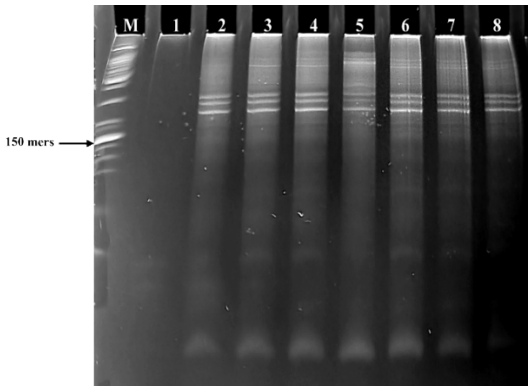

Figure S7: Analysis of sensitivity experiment of multiple gene detection by LDT-mediated fluorometric assay by 20% denaturing PAGE. Here, Ladder M : 25/100 bp marker, Lane 1: 0 copy, Lane 2: 0.5 copy, Lane 3: 1 copy, Lane 4: 2 copies, Lane 5: 5 copies, Lane 6: 10 copies, Lane 7: 20 copies, Lane 8: 50 copies. In PAGE, the RNA band is always located above the band of DNA of the same size.

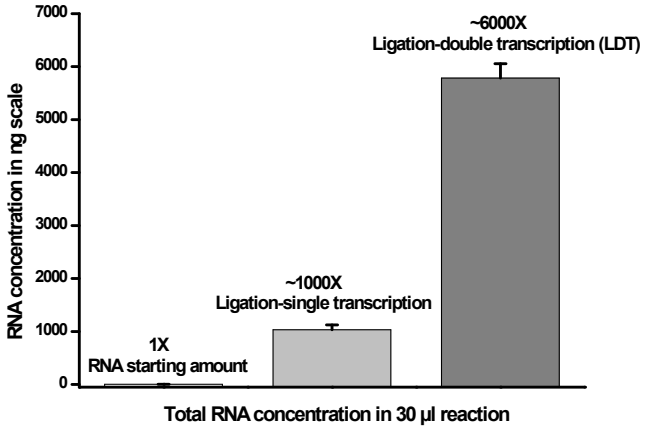

Figure S8: The bar diagram illustrates the concentration of RNA at different stages of the experiment. The first white bar represents the initial concentration of target RNA before the reaction, assumed to be 5 copies (1X). The second bar represents the concentration of RNA after ligation and single transcription, resulting in an amplification of approximately 1000-fold compared to the starting RNA (1X). The third bar represents the concentration of RNA after LDT (ligation-double transcription), where the total amplified product is approximately 6000-fold compared to the starting RNA (1X). In the case of LDT, the starting RNA (target for the first transcription) and the first amplified RNA (target for the second transcription) together contribute to the total RNA throughout the LDT reaction. As a result, there is an approximately 5000-fold increase in the second amplified RNA, specifically for detection purposes. This substantial amplification through double amplification from a small amount of target RNA significantly enhances the detection sensitivity. [For this experiment, readings are taken using the RNA 40 method of Microvolume Spectrophotometer just after RNA purified by kit-column and eluted in 30 µl (here, measurements are performed in 1 µl and later multiplied by total volume 30), and all readings are the mean of three repetitions].