Halochromism of Rosolic Acid: a pH-Sensitive Colorimetric Dye Combined with the Smartphone Technique for Quantification of DNA in Molecular Diagnostics

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Author Contributions

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Materials and Reagents. Rosolic acid ($C_{19}H_{14}O_3$, ≥ 99%) was purchased from Alfa Aesar. The 2× PHD LAMP premix which contains a mixture of deoxynucleoside triphosphate (dNTPs), *Bst* DNA polymerase, and Mg^{2+} salts was purchased from NanoHelix Co., Ltd (Daejeon, South Korea). 100 bp DNA size marker was obtained from Takara (Shiga, Japan). Agarose powder for gel electrophoresis was acquired from BioShop (Burlington, ON, Canada). The Luria-Bertani broth and agar bacteriology were obtained from MBcell (Seoul, Korea). Ethidium bromide dye was purchased from Dynebio (Seongnam, South Korea). A Wizard Genomic DNA Purification kit was obtained from Promega (Madison, WI, USA). An Orion Star™ A211 pH benchtop meter was purchased from ThermoFisher Scientific (Seoul, South Korea). The *esp* gene of *E. faecium* (ATCC: BAA-2127) and the *hlyA* gene of *L. monocytogenes* plasmids were purchased from ATCC and Cosmogenetech (Seoul, South Korea), respectively. The genuine *A. baumannii* sample was taken from hospitalized patients in Thailand and kept on the FTA card. All the LAMP primers were designed using the PrimerExplorer V5 program and purchased from Cosmogenetech (Seoul, South Korea).

Culture of bacteria and DNA purification

The *E. faecium* spp. was incubated in Luria-Bertani broth (5 mL) at 37°C for 17 h in the incubator with constant agitation at 200 rpm. Following the growth of *E. faecium*, pure genomic DNA was extracted from 1 mL of the bacterial culture medium by employing a Wizard Genomic DNA Extraction kit. A NanoDropTM spectrophotometer was used to measure the ultraviolet absorbance ratio at 260/280 nm in order to determine the purity of the DNA.

Table S1. Primer sequences were used for the detection of *Listeria monocytogenes Enterococcus faecium,* and *Acinetobacter baumannii*

Target gene	Primer name	Primer sequences (5'-3')
hlyA (*L. monocytogenes*)	LB	CGTCCATCTATTTGCCAGGTAACGC
	F3	TGATCACTCTGGAGGCTAC
	B3	CCATTCCCAAGCTAAACCA
	FIP	TGAACAATTTCGTTACCTTCAGGATGTTGCTCAATTCAACATCTCTT
	BIP	AGCAAGCTAGCTCATTTCACATGTGCATTCTTTGGCGTAA
esp gene (*E. faecium*)	LB	TGATGTTGACACAACAGTTAAGGG
	F3	CCAGAACACTTATGGAACAG
	B3	GTTGGGCTTTGTGACCTG
	FIP	CGTGTCTCCGCTCTCTTCTTTTTATTTGCAAGATATTGATGGTG
	BIP	ATCGGGAAACCTGAATTAGAAGAAGAACTCGTGGATGAATACTTTC
bla OXA-23-like carbapenemase (*A. baumannii*)	LF	TTTTGCATGAGATCAAGACCGA
	LB	CTGGTTGGTAGGACCATTAAAGGTT
	F3	GAAGCCATGAAGCTTTCTG
	B3	GTATGTGCTAATTGGGAAACA
	FIP	ACCGAAACCAATACGTTTTACTTCTCAGTCCCAGTCTATCAGGA
	BIP	CTGAAATTGGACAGCAGGTTGACTCTACCTCTTGAATAGGCG

Table S2. Weakly-buffered LAMP assay for the detection of *E. faecium and A. baumannii*

***E. faecium* (*esp* gene)**		***A. baumannii (*blaOXA-23-like carbapenemase gene)**	
Components	Volume (μL)	Components	Volume (μL)
2× PHD LAMP premix	12.5	2× PHD LAMP premix	12.5
80 μM FIP	0.5	80 μM FIP	0.5
80 μM BIP	0.5	80 μM BIP	0.5
10 μM F3	0.5	10 μM F3	0.5
10 μM B3	0.5	10 μM B3	0.5
40 μM LB	0.5	40 μM LB	0.5
DNA template	1	Water	10
Water	9		
Total volume	25	Total volume	25

Table S3. Weakly-buffered real-time LAMP assay for the detection of *L. monocytogenes*

***L. monocytogenes* (*hlyA* gene)**	
Components	Volume (μL)
2× PHD LAMP premix	12.5
80 μM FIP	0.5
80 μM BIP	0.5
10 μM F3	0.5
10 μM B3	0.5
40 μM LB	0.5
DNA template	1
7.5 mM RA	1
Water	8
Total volume	25

Table S4. Comparison of the introduced method with other relevant strategies for the detection of *L. monocytogenes*

Molecular methods	Sensing strategies	Sensitivity	Assay time (min)	Types of real sample	Ref
LAMP	colorimetry	62.5 fg/reaction	60	milk	1
LAMP	LFD	2.82 CFU/mL	90	chicken	2
LAMP	electrochemical	1 CFU/25g	60	milk	3
SRCA	colorimetry	4.4 CFU/mL	90	milk	4
HAMP	colorimetry	1500 CFU/g	90	chicken	5
RPA	LFD	1360 CFU/mL	45	milk	6
PCR	fluorescence	-	120	pork	7
PCR	fluorescence	10 CFU/mL	120	egg	8
LAMP	colorimetry	10 fg/μL	45	-	This work

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Table S5. Quantification of the *L. monocytogenes* (*hlyA* gene) and *E. faecium* (*esp* gene) based on the three ratios (R/RGB, G/RGB, and B/RGB) using a "color picker" APP.

		1 ng/µL	0.1 ng/µL	10 pg/µL	1 pg/µL	0.1 pg/µL	10 fg/µL		R/RGB	G/RGB	B/RGB
	RED	232	231	242	239	237	236	1.00E-09	0.517088	0.427192	0.055721
		224	235	237	244	231	233	1.00E-10	0.525185	0.432593	0.042222
		240	243	240	242	231	235	1.00E-11	0.538174	0.431886	0.02994
hylA		**232**	**236.3333**	**239.6667**	**241.6667**	**233**	**234.6667**	1.00E-12	0.559414	0.430556	0.010031
	GREEN	189	195	194	185	166	152	1.00E-13	0.581531	0.408486	0.009983
		185	192	188	186	164	149	1.00E-14	0.591597	0.381513	0.026891
		201	197	195	187	161	153				
		191.6667	**194.6667**	**192.3333**	**186**	**163.6667**	**151.3333**				
	BLUE	20	24	13	8	7	11				
		3	15	6	0	4	10				
		52	18	21	5	1	11				
		25	**19**	**13.33333**	**4.333333**	**4**	**10.66667**				
	RGB	**448.6667**	**450**	**445.3333**	**432**	**400.6667**	**396.6667**				

		1 ng/µL	0.1 ng/µL	10 pg/µL	1 pg/µL	0.1 pg/µL	10 fg/µL	1 fg/µL		R/RGB	G/RGB	B/RGB
	RED	236	241	237	243	242	246	235	1.00E-09	0.470511	0.392529	0.136959
		240	239	242	247	245	248	243	1.00E-10	0.488644	0.410186	0.10117
		242	230	239	242	249	246	240	1.00E-11	0.497574	0.404019	0.098406
		239.3333	**236.6667**	**239.3333**	**244**	**245.3333**	**246.6667**	**239.3333**	1.00E-12	0.51768	0.408769	0.07355
esp	GREEN	195	201	189	191	176	180	157	1.00E-13	0.528949	0.412928	0.027376
		203	204	197	197	181	183	166	1.00E-14	0.559696	0.388134	0.082916
		201	191	197	190	186	180	157	1.00E-15	0.585644	0.391517	0.022838
		199.6667	**198.6667**	**194.3333**	**192.6667**	**181**	**181**	**160**				
	BLUE	59	55	27	25	2	47	2				
		81	72	74	49	9	37	16				
		69	20	41	30	25	32	10				
		69.66667	**49**	**47.33333**	**34.66667**	**12**	**38.66667**	**9.333333**				
	RGB	**508.6667**	**484.3333**	**481**	**471.3333**	**438.3333**	**466.3333**	**408.6667**				

Table S6. Reproducibility test for the RA-based colorimetry method (n=3)

DNA Concentration	R/RGB	SD	RSD (%)
1 ng/μL	0.517088	0.0209	4.04
0.1 ng/μL	0.525185	0.0099	1.90
10 pg/μL	0.538174	0.0150	2.78
1 pg/μL	0.559414	0.0184	3.20
0.1 pg/μL	0.581531	0.0185	3.08
10 fg/μL	0.591597	0.0264	4.25

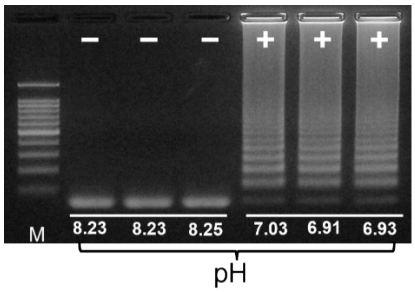

Fig. S1 Confirmation of weakly-buffered LAMP process by agarose gel electrophoresis.

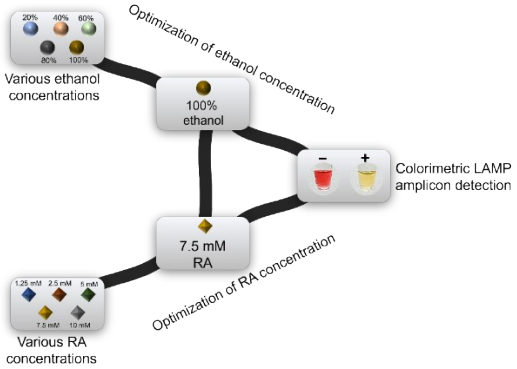

Fig S2. The flow chart illustrates the optimization of ethanol and RA concentration for the colorimetric detection of LAMP amplicons.

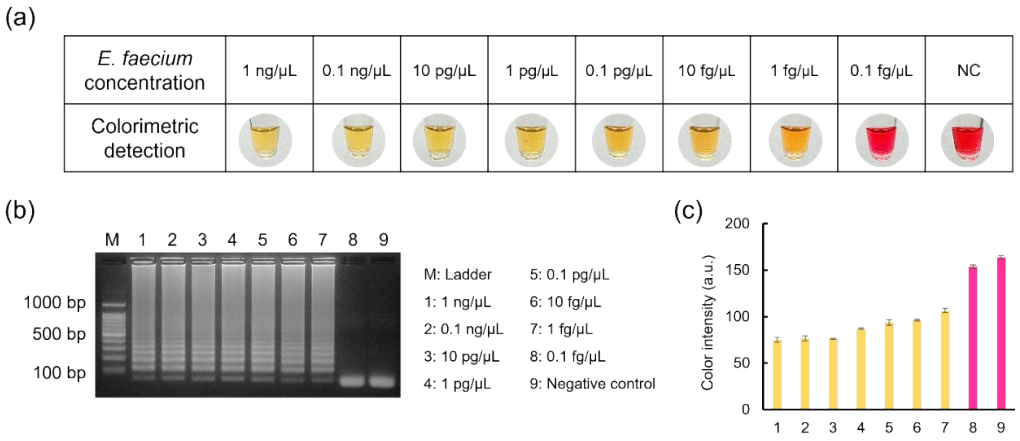

Fig. S3 Sensitivity of the RA-based LAMP assay for *E. faecium* detection (a) colorimetry, (b) agarose gel electrophoresis, and (c) grayscale analysis employed for color intensity quantification.

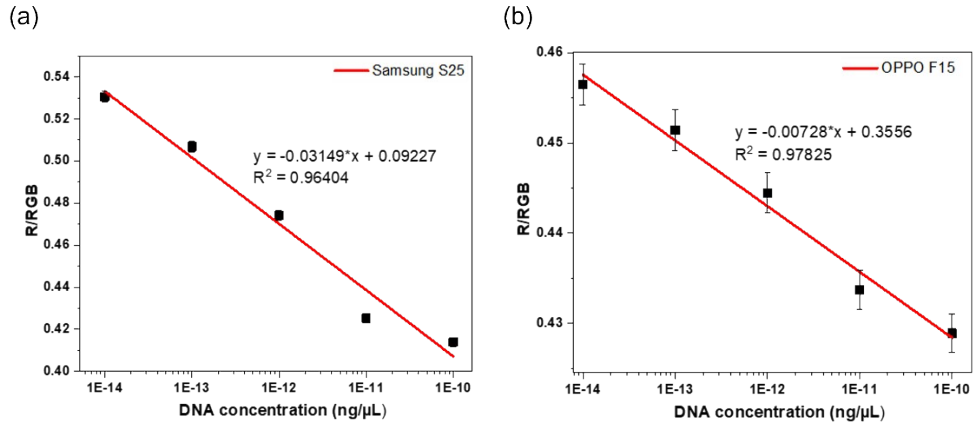

Fig S4. Results showing the impact of various smartphone models: (a) Samsung S25 and (b) OPPO F15 on image analysis for the quantification of *L. monocytogenes* based on R/RGB ratio using the "color picker" APP.

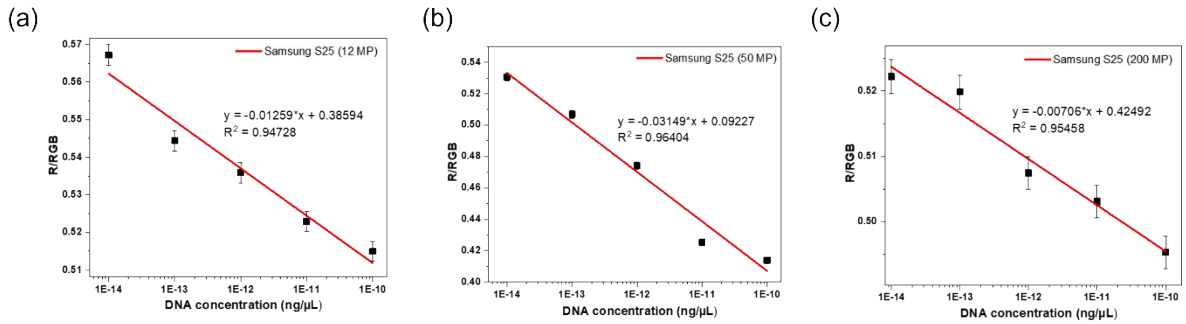

Fig S5. Results showing the impact of various megapixel cameras; (a) 12 MP, (b) 50 MP, and (c) 200 MP on image analysis for the quantification of *L. monocytogenes* based on R/RGB ratio using the "color picker" APP.