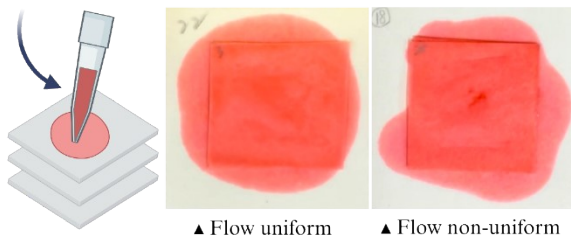


### Supplementary Information

Experimental ID	Lot No.	Thickness ( $\mu\text{m}$ )	Pore size ( $\mu\text{m}$ )	Wet burst strength (psi)
①	1004-240	205	20-25	0.22
②	1441-185	220	20-25	0.22
③	1454-185	185	22	7
④	1002-150	190	8	0.29
⑤	1440-150/185	210	8	0.29
⑥	1003-185	390	6	0.4
⑦	1006-185	180	3	0.25
⑧	1542-185	150	2.7	6
⑨	1005-185	200	2.5	0.4
⑩	1442-185	200	2.5	0.4
⑪	5A	220	7	1.45
⑫	5B	210	4	1.74
⑬	5C	220	1	1.74

1 mL of commercial red ink diluted 100-fold



In this study, commercially available water-soluble red ink was diluted 100-fold, and 1 mL was applied dropwise onto layered paper-based materials. The effect of different paper stacking combinations on ink flow was evaluated by observing flow behavior (flow time) and stability (flow uniformity).

**Supplementary Table 1.** Paper-based materials from Whatman and ADVANTEC, and their physical properties. The paper-based materials used in this study were primarily sourced from two major brands. Materials labeled ① to ⑩ correspond to products manufactured by Whatman, while materials labeled ⑪ to ⑬ were produced by ADVANTEC. The specific product models and their physical properties were extracted from official sources, including the Whatman website (<https://www.cytivalifesciences.com/en/us>) and the ADVANTEC website (<https://www.advantec.co.jp/en/>).

Experimental group	Paper layer stacking order (from top to bottom, arranged right to left)
1	①④⑦
2	①④⑧
3	①④⑨
4	①⑤⑦
5	①⑤⑧
6	①⑤⑨
7	①⑥⑦
8	①⑥⑧
9	①⑥⑨
10	②④⑦
11	②④⑧
12	②④⑨
13	②⑤⑦
14	②⑤⑧
15	②⑤⑨
16	②⑥⑦
17	②⑥⑧
18	②⑥⑨
19	③④⑦
20	③④⑧
21	③④⑨
22	③⑤⑦
23	③⑤⑧
24	③⑤⑨
25	③⑥⑦
26	③⑥⑧
27	③⑥⑨

**Supplementary Table 2.** Detailed layer configurations of Whatman paper materials for red ink flow testing. Various Whatman paper materials were stacked vertically with a blotting paper at the bottom to absorb 1 mL of water-soluble red ink. The effect of the top layer on ink flow was visually assessed, while flowability through the second and third layers was evaluated by recording the time required for the ink to reach the absorbent layer.

Experimental group	Paper layer stacking order (from top to bottom, arranged right to left)
1	③①①②
2	③①①⑦
3	③①①⑧
4	③①①⑩
5	③①①⑬
6	③⑥②
7	③⑥⑦
8	③⑥⑧
9	③⑥⑩
10	③⑥⑬
11	①①②③

**Supplementary Table 3.** Optimized parameters for Whatman paper materials and detailed stacking combinations with ADVANTEC materials for red ink flow testing. ADVANTEC paper materials were combined with previously tested Whatman papers into 11 new stacking groups based on pore size. Each combination was tested for ink flowability, uniformity, and timing to identify optimal configurations for vertical flow assay development.




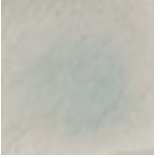

Experimental group	Paper layer stacking order (from top to bottom, arranged right to left)	Flow time (s)	Flowability and uniformity
1	①④⑦	27 s	Uniform spreading
2	①④⑧	36 s	Uneven spreading
3	①④⑨	100 s	Uneven spreading
4	①⑤⑦	45 s	Uneven spreading
5	①⑤⑧	51 s	Uneven spreading
6	①⑤⑨	73 s	Uneven spreading
7	①⑥⑦	31 s	Uniform spreading
8	①⑥⑧	45 s	Uniform spreading
9	①⑥⑨	70 s	Uneven spreading
10	②④⑦	36 s	Uneven spreading
11	②④⑧	51 s	Uniform spreading
12	②④⑨	58 s	Uneven spreading
13	②⑤⑦	37 s	Uniform spreading
14	②⑤⑧	50 s	Uneven spreading
15	②⑤⑨	73 s	Uneven spreading
16	②⑥⑦	29 s	Uniform spreading
17	②⑥⑧	40 s	Uniform spreading
18	②⑥⑨	39 s	Uniform spreading
19	③④⑦	30 s	Uniform spreading
20	③④⑧	25 s	Uneven spreading
21	③④⑨	33 s	Uneven spreading
22	③⑤⑦	33 s	Uniform spreading
23	③⑤⑧	35 s	Uniform spreading

24	③⑤⑨	32 s	Uneven spreading
25	③⑥⑦	27 s	Uniform spreading
26	③⑥⑧	26 s	Uniform spreading
27	③⑥⑨	45 s	Uneven spreading

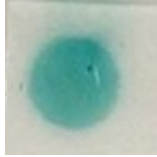
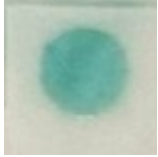
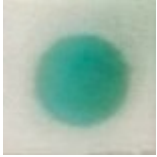
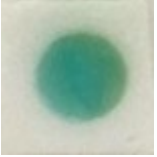
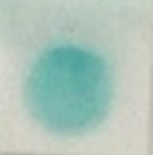
**Supplementary Table 4.** Red ink flow test results for stacked combinations of Whatman paper materials. Red ink flow tests were performed on stacked combinations of Whatman paper materials, with stacking sequences. Flow performance was evaluated based on visual observation and timing methods to assess uniformity and suitability for vertical flow assay applications.

Experimental group	Paper layer stacking order (from top to bottom, arranged right to left)	Flow time (s)	Flowability and uniformity
1	③①①①②	40 s	Uneven spreading
2	③①①⑦	25 s	Uneven spreading
3	③①①⑧	26 s	Uniform spreading
4	③①①⑩	50 s	Uneven spreading
5	③①①⑬	33 s	Uniform spreading
6	③⑥①②	20 s	Uniform spreading
7	③⑥①⑦	24 s	Uniform spreading
8	③⑥①⑧	23 s	Uniform spreading
9	③⑥①⑩	40 s	Uneven spreading
10	③⑥①⑬	23 s	Uniform spreading
11	①①①②①③	141 s	Uneven spreading

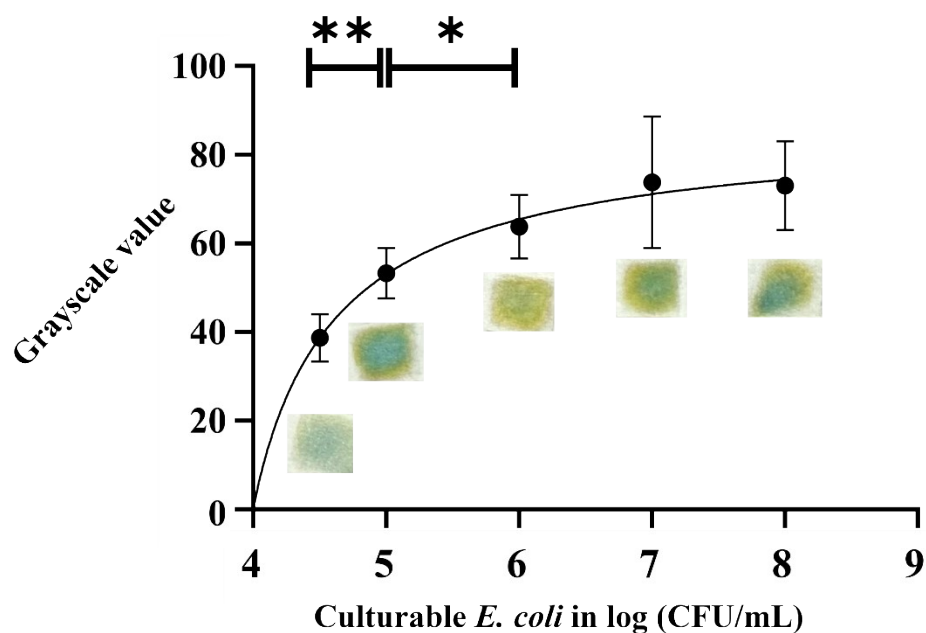
**Supplementary Table 5.** Red ink flow test results for stacked combinations of optimized Whatman and ADVANTEC paper materials. Based on the analysis in Supplementary Table 4, the most suitable paper materials for the first and second layers were identified. To further optimize the stacking design, 11 new combinations integrating untested ADVANTEC materials with the selected Whatman papers were evaluated using the same 1 mL red ink test.

<i>S. aureus</i> (CFU/mL)	$5 \times 10^4$	$10^5$	$10^6$	$10^7$	$10^8$
					
Grayscale value, n=5 (mean $\pm$ SD)	$16.94 \pm 8.90$	$15.28 \pm 2.83$	$18.05 \pm 2.06$	$20.39 \pm 5.58$	$20.88 \pm 4.2$

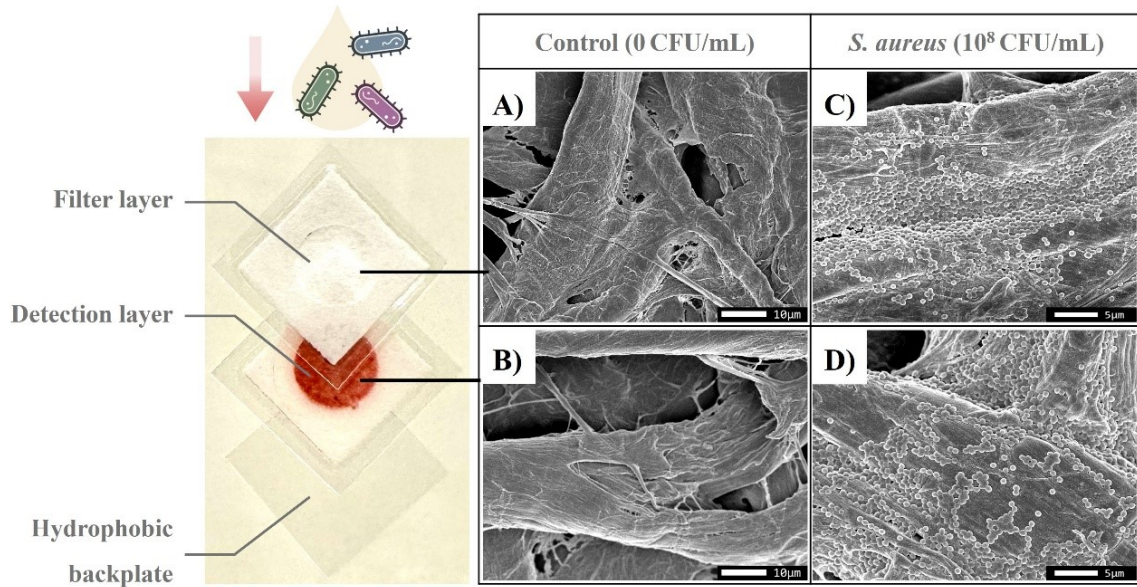
**Supplementary Table 6.** Specificity test against *Staphylococcus aureus* without plastic container integration. *Staphylococcus aureus* samples ranging from  $5 \times 10^4$  to  $10^8$  CFU/mL were applied to evaluate potential cross-reactivity. Colorimetric signals remained faint and close to the blank control across all concentrations, supporting the initial specificity of the anti-*E. coli* antibody and minimal cross-reactivity against the tested Gram-positive non-target bacterium. These results were obtained prior to integration into the plastic container system.

<i>E. coli</i> (CFU/mL)	$5 \times 10^4$	$10^5$	$10^6$	$10^7$	$10^8$
					
Grayscale value, n=5 (mean ± SD)	$89.79 \pm 10.64$	$76.53 \pm 7.51$	$91.70 \pm 8.36$	$99.47 \pm 10.26$	$63.53 \pm 4.2$
CV	11.85%	9.82%	9.12%	10.31%	6.63%

**Supplementary Table 7.** Colorimetric detection of *Escherichia coli* using Experimental ID ③ Whatman Lot No. 1454-185 paper-based material. Colorimetric signals appeared uniformly intense across the entire concentration range of  $5 \times 10^4$  to  $10^8$  CFU mL<sup>-1</sup>, resulting in high background and poor discrimination among concentrations. The grayscale values showed no clear concentration-dependent trend, rendering quantitative analysis unreliable.



**Supplementary Figure 1.** Colorimetric detection of *E. coli* using PT-R7 as the detection layer. The original Whatman paper material (experimental ID ③; Lot No. 1454-185) was replaced with PT-R7 conjugate release matrix (R-1343 / 1554; Lot No. 343438F) from Membrane Technologies Inc. to enhance species-specific detection. A significant increase in signal intensity was observed with rising bacteria concentrations, with differences between  $5 \times 10^4$  and  $10^5$  CFU/mL (\*\*,  $p < 0.01$ ) and between  $10^5$  and  $10^6$  CFU/mL (\*,  $p < 0.05$ ),  $n=5$ . Grayscale intensity was quantified by averaging pixel values over a fixed circular region of interest (ROI, 0.6 cm diameter) corresponding to the designed reaction zone, rather than point sampling. This area-averaging approach accounts for spatially non-uniform color development inherent to porous paper substrates and yields a representative bulk signal. Hill equation fitting yielded  $\text{Grayscale} = (87.04 \times [E. coli]^{0.9601}) / (0.6424 + [E. coli]^{0.9601})$  ( $R^2 = 0.7051$ ) and linear regression yielded  $\text{Grayscale} = -0.0603 \times [E. coli] + 17.596$  ( $R^2 = 0.6136$ ).



**Supplementary Figure 2.** Scanning electron microscopy verification of bacterial transport through the two-layer WST-8–mPMS module. **Left:** schematic of the two-layer configuration consisting of a filter layer, detection layer, and hydrophobic backplate, illustrating sample infiltration and flow direction. **Right:** scanning electron microscopy images of the control (0 CFU/mL) filter layer (A) and detection layer (B), showing only fibrous paper microstructures, and the corresponding *S. aureus*-treated ( $10^8$  CFU/mL) filter layer (C) and detection layer (D), where spherical bacteria were observed on the fiber surfaces. These observations support that bacteria would traverse the upper filtration layer and reach the underlying detection layer under capillary-driven flow, consistent with the design of the WST-8–mPMS module for total bacteria-load assessment.