# Abstract

Microfluidic paper-based analytical devices (μPADs) are promising biosensors that may be used in a variety of bioanalytical applications. Despite the benefits of being affordable, low-volume, and portable, there are restrictions, including issues with large-scale production, multi-step operation, and particularly detection sensitivity, that may pose difficulties for users.

To address those critical issues, a new μPAD for automating competitive enzyme-linked immunosorbent assay (ELISA) for small-sized target detection was developed. Simple, precise, and rapid device fabrication was achieved by laser-cutting technology. A Sucrose valve was utilized to automate the sequential delivery of reagents, providing simple user-operation. The device was demonstrated with Aflatoxin $B_1$ ($AFB_1$) antigen, which is a hydrophobic toxin and cancer-causing agent. During an examination of various parameters, a new sample-loading method, or the so-called Direct Dropping of Sample on Antibody Location (DDoAb), was discovered to allow minimization of sample volume to 0.6 μL, while eliminating the possible loss of a target molecule by adsorption on the membrane, thus improving detection sensitivity. Under the optimization conditions, the device achieved a limit of detection of 0.1 ng/mL or 60 fg, which is 2-4 orders of magnitude lower than other reports.

To further advance the sensitivity of μPAD to an ultimate level of single molecule detection, a new method for digital counting of molecules on μPAD was proposed. Streptavidin-conjugated alkaline phosphatase (SA-ALP) was used as an analyte model. Without the need for an expensive femtoliter-sized chambers, digital counting of SA-ALP was successfully conducted using enzymatic reaction, inexpensive materials, and general laboratory equipment. This simple and low-cost digital counting platform shows potential use in other bioanalytical applications and other target molecules.



**Keywords**: μPADs, Automated ELISA, Small-sized target, Aflatoxin $B_1$, Digital counting