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Versatile antibody-sensing Boolean logic for the simultaneous detection of multiple bacterial toxins†

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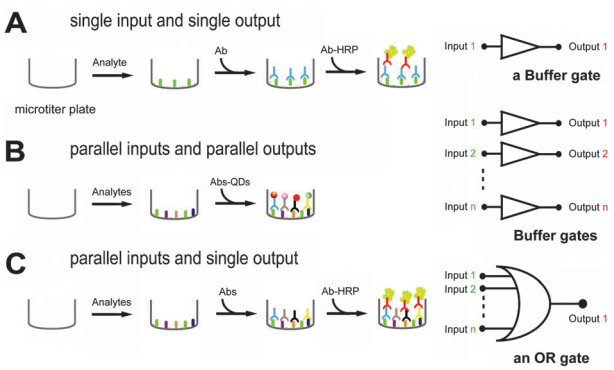
We present an OR gate based on monoclonal antibodies for the simultaneous detection of multiple toxins in a single tube. To further simplify the operating procedure, the Boolean rule of *simplification* was used to guide the selection of a marker toxin among the natural toxin profiles.

Bacterial pathogens can cause various infectious diseases in humans and animals and may have a negative impact on environmental health as well.[1] To improve food safety, there is a strong demand to shift end-of-line product inspection to inline assessment during food production.[2] Therefore, a broad spectrum of methods has been developed to replace conventional culture methods, which usually require several days to provide results. The most promising alternative methods include genetic probes and immunochemical assays as well as instrumental analyses.[3] Particularly single-component immunoassays (Scheme 1A) are indispensable tools to detect the presence of bacterial toxins in food samples. A common way to achieve multi-component detection is the fabrication of microarray-based assays, in which the targets of interest are bound by antibodies, which are physically separated on the microarray.[4] Also, parallel quantification of four bacterial toxins has been achieved by measuring the fluorescence signals of antibodies labeled with different quantum dots (Scheme 1B).[5] However, relying on sophisticated biochemical reagents and/or equipment is still a major limitation of these methods. On the other hand, for in-field controls simple assays covering a broad range of analytes and providing a simple 'YES/NO' answer would be sufficient. As a promising solution to this analytical problem, the application of a mixture of antibodies for the detection of multiple chemicals in a single well of a microtiter plate has been described.[6] These assays can be considered as a first step towards a simple and generic assay design, which can be easily adopted to serve specific analytical purposes.



Scheme 1 Main types of assays for the detection of bacterial toxins in microtiter plates. (A) ELISA for a single analyte; (B) parallel detection of multiple analytes with multiple outputs; (C) a single output for the detection of multiple analytes.

During the past few years, another successful approach for simple qualitative analyses has been achieved by the development of Boolean logic-based biosensors, which hold very promising perspectives for bioanalytical applications and enable the generation of a 'YES/NO' response.[7] The logic-based biosensors generally fall into two groups, the cellular logic systems and the abiotic logic systems.[8] The cellular logic systems are based on bacterial or mammalian cells and can perform sophisticated tasks, *e.g.*, mammalian cell-based logic gates have been used to assay the interaction between bacterial toxins and cells.[9] On the other hand, many versatile abiotic logic biosensors have been developed based on various chemicals, biomolecules and nanoparticles for implementing challenging tasks.[10]

Herein, we integrate seven monoclonal antibodies (mAbs) to demonstrate simultaneous multiplex assays for seven target proteins present in the same sample in a single well or tube with a binary 'YES/NO' answer (Scheme 1C). These proteins from *Bacillus cereus* (*B. cereus*) include a protein marker for the monomeric emetic toxin (cereulide, Cer) as well as the individual components of two tripartite enterotoxin complexes (the nonhemolytic enterotoxin, NheA, B and C, and hemolysin BL, Hbl L1, L2 and B).[11] To our best knowledge, this is the first example of a simple immunoassay for the simultaneous detection of multiple

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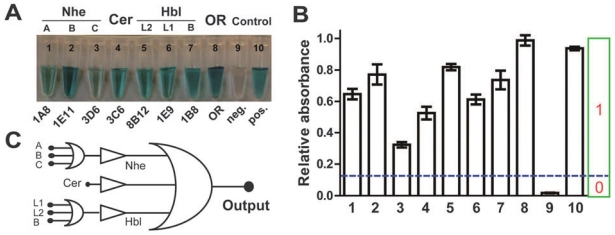

Fig. 1 Typical photograph (A) and corresponding relative absorbance (B) of the heterogeneous mAbs based OR gate for the detection of seven target proteins from *B. cereus* strain MHI 3016 (expressing all seven proteins). The mAbs used are listed in (A) and the colorless tetramethylbenzidine (TMB) is oxidized by the horseradish peroxidase–antibody conjugate to blue colored TMB diimine; (C) the equivalent circuit of an OR gate based logic circuit.

bacterial toxins in a single tube with a colorimetric readout based on Boolean logic operations. In addition, the Boolean rule of *simplification* is used to guide the selection of a marker toxin among the natural toxin profiles and is confirmed by experimental results.

The OR gate based assay showed good response to the toxins in the single tube assay as well as in the single well assay, with significant color changes compared to the negative control (Fig. 1A and Fig. S1, ESI†). The corresponding absorbance values of each tube were quantified in Fig. 1B. The presence or absence of bacterial toxins is defined as an input, 'True/False' or '1/0'; the relative absorbance value is defined as an output, and the threshold value used was '0.1', as indicated by the blue dashed line in Fig. 1B. Due to the high specificity of the individual antibodies, there was no cross-reaction between the different target proteins and their corresponding mAbs. The assay is therefore suitable for a first screening of the targets of interest in biological samples providing a rapid 'YES/NO' answer. In principle, this Boolean logic-based method could also be used for the detection of other multiple contaminants such as the residues of antibiotics in food samples or bacterial resistance genes. For example, the combination of YES and OR gates allowed the reduction of molecular beacon probes required for both the detection of *Mycobacterium tuberculosis* and the presence of rifampin resistance genes from five to two.[12]

Although qualitative and quantitative data about the toxin profile of a bacterial strain are essential to enable an unbiased assessment of the organism's virulence, there is also an urgent need to select only one or a few markers for virulence in order to improve speed and efficiency of detection. Toward this end, we introduced the Boolean rule of *simplification* to reduce the selection of target toxins among the different possible combinations of Nhe, Hbl and Cer in food related strains. All naturally occurring combinations of the three toxins are shown as a logic circuit in Fig. 2A, namely Nhe alone (MHI 1491), Nhe plus Hbl (MHI 1505), Nhe plus Cer (MHI 165), and Nhe plus Hbl plus Cer (MHI 3016). To introduce Boolean algebra for the demonstration of *simplification*, we use three letters 'a', 'b' and 'c' to represent Nhe, Hbl and Cer, respectively. According to the logic circuit, we get the Boolean expression of all the combinations 'a + ab + ac + abc', and simplify it to 'a' (Fig. 2B).

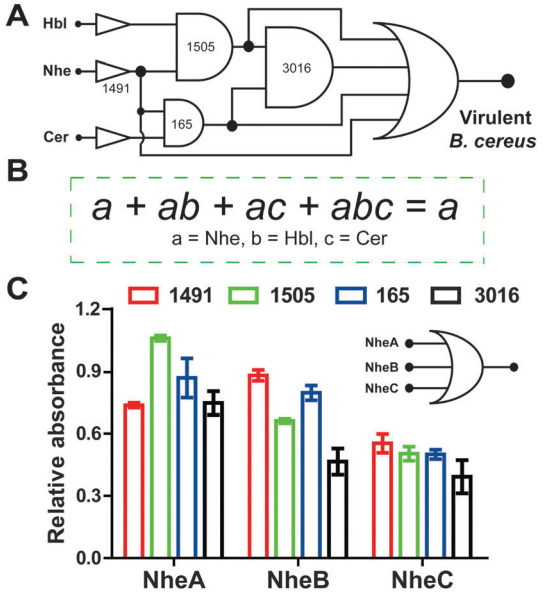

Fig. 2 Selection of the marker toxin from different *B. cereus* strains based on the Boolean rule of *simplification*. (A) All naturally occurring combinations of toxins presented as a logic circuit; (B) algebraic expression of the *simplification*; (C) the OR gate of the Nhe complex.

The corresponding proof of *simplification* is in Fig. S2 (ESI†). The resulting term 'a' is much simpler than the original expression 'a + ab + ac + abc', yet possesses the same meaning, *i.e.* Nhe is the main toxin in all combinations of three toxins and it could serve as the marker toxin for *B. cereus* strains.

This conclusion is supported by a previous report showing that the overall *B. cereus*-associated toxic activity is correlated with the Nhe expression level.[13] The former finding was based on the extensive characterization of 100 *B. cereus* strains originating from food poisoning cases as well as from randomly collected food samples. Selecting Nhe as the major virulence factor, based on Boolean logic as well as experimental data, will certainly simplify the current analytical procedures and accelerate the identification of virulent *B. cereus* isolates. Altogether, this is a convincing proof of principle of an experimental analogue circuit by using combinations of extracellular toxins of naturally occurring strains of *B. cereus*.

The fact that most *B. cereus* strains harbor a complete set of Nhe components provides a natural three-input OR gate for analytical purposes. In other words, measuring the expression levels of the individual Nhe components (NheA, B and C, Fig. 2C) indicates that the analytical procedure could be even further simplified by reducing measurement to single component detection. For this purpose we chose NheB, which can be detected by different assay types. Using antibodies 1E11 and 2B11, which recognize different epitopes of NheB, in a single antibody assay, represents an OR gate (Fig. 3A). An AND gate using the same mAbs can be established by applying a double antibody (sandwich) assay (Fig. 3B). Considering that *B. cereus* strains may produce NheB owing to mutations within the epitopes of the antibodies, but retaining the toxic activity, we analyzed several hundred strains by both assay types. Our results, which are exemplarily shown in Fig. 3C (left) and D, indicate that at least two

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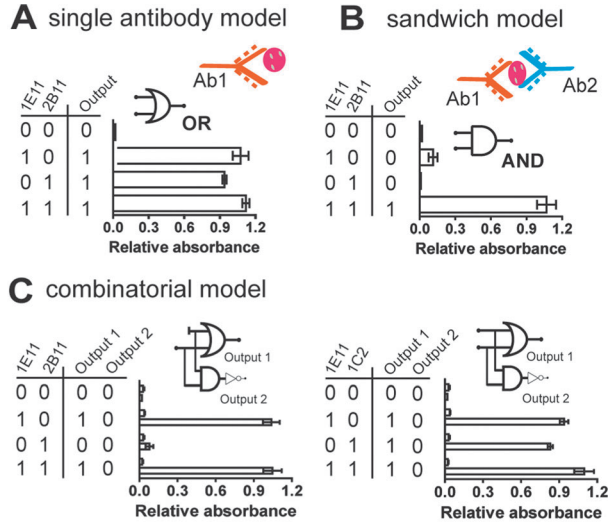

	MHI (No.)	2B11	1E11	2B11 1E11
1	1489	0	1	0
2	2970	0	1	0
3	3038	0	1	0
4	3086	0	1	0
5	control	1	1	1

Fig. 3 Three antibody-based strategies for the detection of NheB in the Nhe complex with a single antibody model (A), a sandwich model (B) and a combinatorial model (C). Table containing typical results for mutant strains (D).

different antibodies in the single antibody assay are necessary for reliable detection. The examples shown here are mutant strains harboring amino acid exchanges in the range of amino acid residues 122–150 representing the epitope of mAb 2B11 and, therefore, drastically decrease the affinity of the antibody.[14] The output is 'TRUE' only for 1E11 in the single antibody model and 'FALSE', when combining mAbs 1E11 and 2B11 in the sandwich model. In a second example, a change in conformation upon adsorption of NheB onto a surface (polystyrol or cell membrane) is demonstrated by using mAb 1C2. Although Buffer and OR gates could be set up when replacing mAb 2B11 by mAb 1C2 (Fig. S3, ESI†) in the single antibody model, no AND gate could be constructed in the sandwich model, Fig. 3C (right). This finding indicates that the epitope of NheB recognized by 1C2 is not accessible in solution, but only after adsorption of the protein onto a surface, which has been shown to be a prerequisite for binding of NheA.[14] Altogether, these examples demonstrate that the antibody-based logic gates enable sophisticated tasks not only for accurate determination of bacterial toxins but also for the elucidation of structural properties of the individual toxin components.

In conclusion, this new antibody-based single tube assay generating a bioanalytical result with a qualitative 'YES/NO' answer demonstrates a robust approach for the detection of multiple bacterial toxins and could serve as a general model for simultaneous analysis of multiple analytes. In addition, Boolean logic can not only improve the detection of multiple targets, but also holds the sophisticated power to simplify the selection of markers in complex biological systems. Therefore, the antibody-based logic system represents a versatile platform for the determination of multiple targets of interest and to perform sophisticated logic operations.

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