Title: Ultrasensitive detection of proteins for early diagnosis of disease

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Background: We have been developing an ultrasensitive ELISA to detect proteins at 10^{-20} moles/test by use of enzyme cycling, in which a cycling reaction is conducted by a dehydrogenase (3α-hydroxysteroid dehydrogenase; 3α-HSD) with co-factors (NADH and thio-NAD) and substrates (androsterone derivatives). In the present study, we applied this ultrasensitive ELISA to a checkup for the *Mycobacterium tuberculosis* (TB) complex. Tuberculosis remains a major threat to human health around the world. For example, in 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease (WHO News, Fact sheet No. 104). To fight tuberculosis, a rapid checkup and accurate treatment for tuberculosis are crucial. Our proposed method provides same-day (4-hour) results without any TB culture.

Methods: We used MPB64, a specific protein secreted from *Mycobacterium* species, as a tuberculosis biomarker. BCG was used for the TB complex, and it was added into sputum obtained from people without TB. As a pre-treatment for the ultrasensitive ELISA, we warmed up BCG in the sputum and enhanced the secretion of MPB64. In the sandwich ELISA, two specific antibodies for MPB64 were used, one of which was conjugated with alkaline phosphate (ALP). An androsterone derivative with a phosphate was hydrolyzed by ALP, and this derivative was then employed in the enzyme cycling. Consequently, MPB64 could be determined by the accumulated amount of thio-NADH in the enzyme cycling.

Results and Discussion: The spike-and-recovery test using BCG and sputum demonstrated reasonable results. We succeeded in detecting TB (i.e., BCG) in the sputum at the level of 3×10^2 CFU/mL within only 4 hours. This rapidity can contribute to the prevention of disease spread, because potential patients can be isolated during the 4 hours that the results take. The present available tests for active TB detection are the sputum smear test and the sputum culture test. The smear test has low sensitivity (> 10,000 CFU/mL), whereas the culture test is highly sensitive (tens to hundreds CFU/mL) but requires a long culture period (at least 10 days). On the other hand, a nucleic acid amplification test (i.e., the PCR method) can be used as a rapid test. However, the PCR test is not routinely recommended. The PCR test detects dead bacteria, and it sometimes shows a false negative because of a small

amount of bacteria in the sputum. Our present method, on the other hand, is a user-friendly ELISA without any specialized apparatus: it has almost the same sensitivity as the culture method but with same-day results. We believe that our method is a de novo, same-day, highly sensitive checkup for tuberculosis that is available worldwide.