

1 *Review*

2 **Ultrasensitive ELISA Developed for Diagnosis**

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20 **Abstract:** For the diagnosis of disease, the ability to quantitatively detect trace amounts of the causal
21 proteins from bacteria/viruses as biomarkers in patient specimens is highly desirable. Here we
22 introduce a simple, rapid, and colorimetric assay as a de novo, ultrasensitive detection method. This
23 ultrasensitive assay consists of sandwich enzyme-linked immunosorbent assay (ELISA) and
24 thionicotinamide-adenine dinucleotide (thio-NAD) cycling, forming an ultrasensitive ELISA, in
25 which the signal substrate (i.e., thio-NADH) accumulates in a triangular manner, and the
26 accumulated thio-NADH is measured at its maximum absorption wavelength of 400 nm. We have
27 successfully achieved a limit of detection of ca. 10–18 moles/assay for a target protein. As an example
28 of infectious disease detection, HIV-1 p24 could be measured at 0.0065 IU/assay (i.e., 10⁻¹⁸
29 moles/assay), and as a marker for a lifestyle-related disease, adiponectin could be detected at 2.3 ×
30 10⁻¹⁹ moles/assay. In particular, despite the long-held belief that the trace amounts of adiponectin in
31 urine can only be detected using a radioisotope, our ultrasensitive ELISA was able to detect urinary
32 adiponectin. This method is highly versatile, because simply changing the antibody enables the
33 detection of various proteins. This assay system requires only the measurement of absorbance, thus
34 it requires equipment that is easily obtained by medical facilities, which facilitates diagnosis in
35 hospitals and clinics. Moreover, we describe an expansion of our ultrasensitive ELISA to a non-
36 amplification nucleic acid detection method for nucleic acids using hybridization. These de novo
37 methods will enable simple, rapid, and accurate diagnosis.

38 **Keywords:** adiponectin; diagnosis; HIV; insulin; non-amplification nucleic acid detection;
39 ultrasensitive ELISA

40 **Abbreviations:** DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; FITC,
41 fluorescein isothiocyanate; HIV, human immunodeficiency virus; LOD, limit of detection; LOQ,
42 limit of quantification; NAD, nicotinamide adenine dinucleotide; NAT, nucleic acid test; PCR,
43 polymerase chain reaction; WHO, World Health Organization.
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46 1. Introduction

47 Examining patient specimens for the presence of proteins from pathogenic bacteria and viruses
48 or as biomarkers enables rapid and accurate diagnosis, resulting in early treatment.
49 Immunochromatography and enzyme-linked immunosorbent assay (ELISA) are commonly used
50 methods for this purpose [1,2]. The sensitivity of these assays, however, requires further
51 improvement. Although the use of radioisotopes enhances the sensitivity of these methods,
52 preparing the special reagents and facilities for radioisotopes is difficult in hospitals and clinics.
53 Another approach, mass spectrometry, is useful for identifying and measuring small quantities of
54 proteins [3], but requires large and expensive equipment that may not be available at most medical
55 facilities. More specifically, a user-friendly, simple, and rapid detection method for various marker
56 proteins is needed for diagnosis.

57 The major challenge for protein quantification is that the proteins cannot be amplified like
58 nucleic acids using polymerase chain reaction (PCR). We hypothesized that we could, however,
59 amplify the detection signal of specific proteins. For the production of a user-friendly diagnosis
60 system, we designed a method for amplifying the signals produced by the antigen-antibody reaction
61 in ELISA. How can ELISA signals be amplified? One method is enzyme cycling [4]. One of the co-
62 authors of the Kato et al. (1973) paper [4], O. H. Lowry, wrote in 1980, "Enzymatic cycling provides
63 a methodology for virtually unlimited amplification of analytical sensitivity. The most widely
64 applicable cycling systems are those for NAD and NADP, since these can be used to increase the
65 sensitivity of methods for a host of other substances" [5]. Here NAD is nicotinamide-adenine
66 dinucleotide. We thus considered that a combination of ELISA and enzyme cycling could be useful
67 for early detection and herein describe our method for ultrasensitive ELISA.

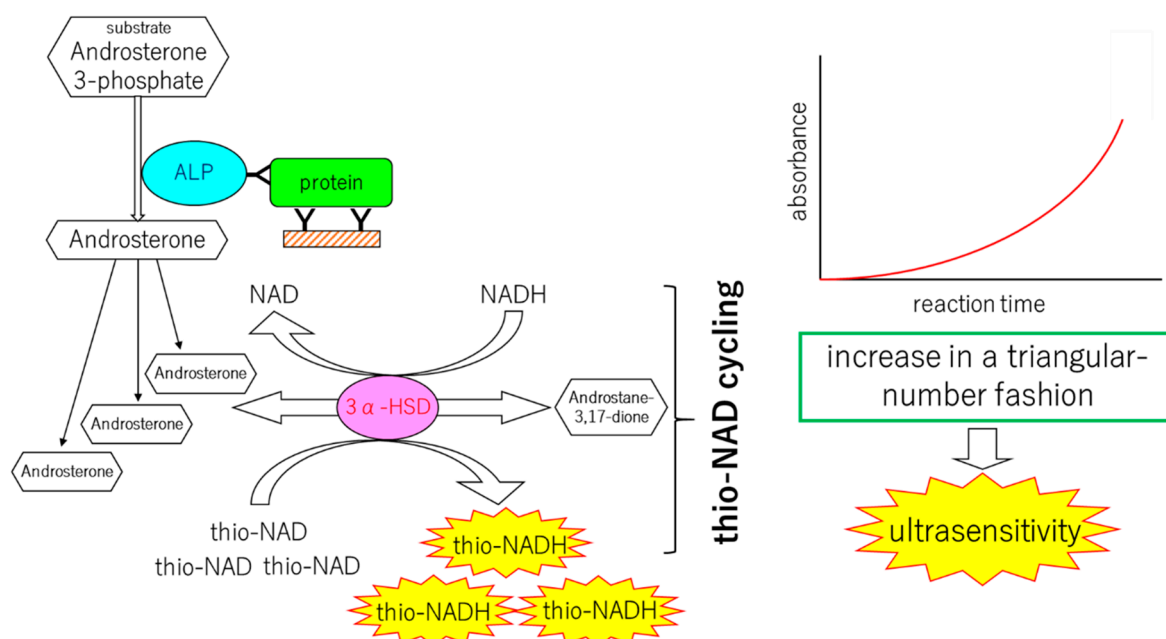
68 The present review describes the detailed mechanisms of an ultrasensitive ELISA for diagnosis.
69 This ultrasensitive ELISA consists of sandwich ELISA and thio-NAD cycling. In the thio-NAD
70 cycling, thio-NAD is reduced to thio-NADH, and the accumulated thio-NADH is measured at an
71 absorbance of 405 nm using a microplate reader [6,7]. Examples of ultrasensitive detection of trace
72 amounts of proteins for disease diagnosis have been described, including the detection of p24 for the
73 diagnosis of an infectious disease (human immunodeficiency virus type 1: HIV-1) [8], and detection
74 of adiponectin and insulin for diagnosis of a lifestyle-related diseases [9-11]. We also introduce our
75 attempt to expand the ultrasensitive ELISA to a non-amplification nucleic acid detection method, by
76 combining nucleic acid hybridization and thio-NAD cycling.

77 2. Mechanisms of the ultrasensitive ELISA

78 2.1. Principle of protocol

79 The ultrasensitive ELISA that we developed consists of two parts: a sandwich ELISA and thio-
80 NAD cycling (Figure 1) [6,7]. In the sandwich ELISA, the first antibody is used to immobilize the
81 target protein to a microplate, and the second antibody is conjugated to an enzyme that converts the
82 substrate to another form. Alkaline phosphatase (ALP, EC. 3.1.3.1) is used as this conjugated enzyme
83 in our assay. A standard sandwich ELISA produces a color change in a substrate, resulting in a
84 detectable signal. This signal increases in a linear fashion with time and thus the sensitivity is limited.
85 We therefore considered that a substrate that can be hydrolyzed by ALP should be amplified. For
86 this signal amplification, we adopted enzyme cycling. As described above, enzyme cycling is a
87 method for amplifying a substrate [4]. It is generally expected that two kinds of enzymes are needed
88 for the cycling reaction to act on the same substrate in different manners. In our version of enzyme
89 cycling, however, we used a single enzyme, 3 α -hydroxysteroid dehydrogenase (3 α -HSD, EC.
90 1.1.1.50). The cofactors were NADH and thio-NAD, and we named our enzyme cycling 'thio-NAD
91 cycling' [6,7].

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Figure 1. Schematics of ultrasensitive ELISA. A sandwich ELISA is combined with thio-NAD cycling. Two antibodies used in ELISA specifically target a pathogenic protein. The first antibody is used for immobilization, whereas the second antibody is labeled with alkaline phosphatase (ALP), which hydrolyzes a substrate containing phosphate. The hydrolyzed substrates are used in thio-NAD cycling that employs a main enzyme (dehydrogenase) and its cofactors (NADH and thio-NAD). Thio-NADH accumulates in a triangular manner, and can be measured at 405 nm. The standard ELISA shows the signals in a linear function, resulting low sensitivity, whereas our ultrasensitive ELISA has higher sensitivity than the standard ELISA. 3α-HSD is 3α-hydroxysteroid dehydrogenase.

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The first substrate applied to ALP in the sandwich ELISA is 17β-methoxy-5β-androstan-3α-ol 3-phosphate. ALP hydrolyzes this substrate to 17β-methoxy-5β-androstan-3α-ol, which is not measured in our system. The 17β-methoxy-5β-androstan-3α-ol is used as the substrate for thio-NAD cycling. 3α-HSD with the cofactor thio-NAD oxidizes 17β-methoxy-5β-androstan-3α-ol to 17β-methoxy-5β-androstan-3-one, resulting in thio-NADH. Then, 3α-HSD with the cofactor NADH reduces 17β-methoxy-5β-androstan-3-one to 17β-methoxy-5β-androstan-3α-ol by the opposite reaction, resulting in NAD. Thio-NADH accumulates in a triangular fashion expressed as follows:

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$$a \times b \times \sum_{k=1}^n k = a \times b \times \frac{n(n+1)}{2}.$$

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Here, a is the turnover ratio of ALP per min; b is the cycling ratio of 3α-HSD per min; and n = minutes of measurement time. The amount of the target protein is determined by measuring the absorbance using a microplate reader at 405 nm, which corresponds to the maximum absorption wavelength of thio-NADH (exactly 400 nm). Therefore, the signals to be used for quantifying the proteins can be obtained in a short period of time.

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2.2. Experimental protocol

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We show the typical protocol to detect a reference 'adiponectin' antigen using ultrasensitive ELISA as follows. This protocol was modified from Watabe et al. [6].

(1) Coat a primary antibody.

Dilute the first antibody with 50 mM Na₂CO₃ (pH 9.6) to a concentration of 2 μg/mL. Add 100 μL of the antibody into each well of 96-well microplates. Incubate for 1 h at room temperature.

(2) Wash microplates.

Wash the microplates 3 times with TBS including 0.05% Tween 20.

(3) Block nonspecific binding sites.

127 Dilute the TBS including 10% BSA 10 times with TBS. Block nonspecific binding sites by filling
128 wells this solution at 300 μL /well. Incubate for 1 h at room temperature.

129 (4) Wash microplates.

130 Wash the microplates 3 times with TBS including 0.05% Tween 20.

131 (5) Add an antigen.

132 Dilute the antigen with TBS including 0.1% BSA to 5 - 40 pg/mL . Add 100 μL of this antigen
133 solution to each well. Incubate overnight at 4°C.

134 (6) Wash microplates.

135 Wash the microplates 9 times with TBS including 0.05% Tween 20.

136 (7) Add an enzyme-linked secondary antibody.

137 Dilute an enzyme-linked secondary antibody with TBS including 0.1% BSA and 0.02% Tween 20
138 to 10 pmol/mL . Add 100 μL of this antibody solution to each well. Shake the microplates for 1 h at
139 room temperature.

140 (8) Wash microplates.

141 Wash the microplates 9 times with TBS including 0.05% Tween 20.

142 (9) Add a thio-NAD cycling solution.

143 Dissolve 1 mM NADH, 3 mM thio-NAD, 0.1 mM 17 β -methoxy-5 β -androstan-3 α -ol 3-phosphate
144 and 30 U/mL 3 α -HSD into 0.1 mM Tris-HCL (pH 9.0). This is referred to as a thio-NAD cycling
145 solution. Add 100 μL of this thio-NAD cycling solution to each well.

146 (10) Measure absorbance.

147 Measure the absorbance at 405 nm and 660 nm with a microplate reader every 5 min for 1 h at
148 37°C. The absorbance at 660 nm is used as reference for background correction.

149 For detection of the specimens obtained from patients (i.e., serum, urine, and saliva), we always
150 checked a spike-and-recovery test. The spike-and-recovery test is a technique for analyzing and
151 accessing the accuracy of ELISA for particular specimens types. It is used to determine whether
152 analyte detection can be affected by the difference between the diluent used for preparation and the
153 experimental specimens matrix. To perform a spike-and-recovery test, a known amount of analyte
154 (i.e., target protein) is added to a matrix (i.e., specimens). This 'addition' is called 'spike'. The
155 concentration of the added analyte in the matrix is determined from standard curves prepared. The
156 concentrations denote the spike recovered in the matrix. All the experimental procedures for this test
157 were the same as for the above ultrasensitive ELISA experiments without the antigen solution.

158 We determined the limit of detection (LOD), minimum limit of quantification (LOQ), and
159 coefficient of variation (CV) for the following sections. The experimental data were obtained by
160 subtracting the mean value of the blank signals from each of the corresponding measured data points.
161 The LOD was estimated from the mean of the blank, the standard deviation of the blank, and a
162 confidence factor of 3. The minimum LOQ was estimated by the same method as used for the limit
163 of detection, but with a confidence factor of 10. The CV calculated from 3 data points was obtained
164 for a reference antigen at a given concentration, and the value should be under 10%.

165 3. Detection of trace amount of proteins

166 3.1. Detection of proteins for an infectious disease

167 As an example of diagnosis of an infectious disease, we applied our ultrasensitive ELISA to
168 detect a specific protein for diagnosing HIV infection [8]. The fourth- and fifth-generation HIV tests
169 need to detect the HIV-1 and HIV-2 antibodies and the HIV-1 p24 antigen [12]. Thus, we focused on
170 the detection of HIV-1 p24 protein. For a CE-marked HIV antigen/antibody assay, the LOD of HIV-1
171 p24 must be less than 2 IU/mL [13,14]. A recent study revealed that the LOD of HIV-1 p24 among
172 various FDA-approved HIV antigen/antibody combination tests ranges from 0.19 to 1.77 IU/mL [15].

173 The LOD and minimum LOQ for HIV-1 p24 in our ultrasensitive ELISA was 0.0065 and 0.0242
174 IU/assay (i.e., *ca.* 10^{-18} and 10^{-17} moles/assay), respectively [8]. Because our assay contained 50 μL of
175 solution, the LOD value is expressed as 'per mL' (0.13 IU/mL). Our ELISA system included a washout
176 process, therefore we consider that the absolute value, and not the concentration, is important (i.e.,

177 0.0065 IU). The result of our ultrasensitive ELISA was thus better than those of the FDA-approved
178 tests, indicating that our ultrasensitive ELISA for HIV-1 p24 can reduce the 'window period' required
179 for the diagnosis of HIV by detecting the protein earlier in the infection process.

180 In our previous study, we compared the LOD between our ultrasensitive ELISA and a nucleic acid
181 test (NAT) [8]. The number of HIV-1 p24 proteins in the virion is thought to be larger than the number
182 of RNA copies (approximately 3000 HIV-1 p24 proteins vs. 2 RNA copies per virion) [16]. The value
183 of 10^{-18} moles is almost the same as 10^6 proteins, corresponding to *ca.* 10^3 RNA copies. The NAT (i.e.,
184 real-time PCR) can detect 10^1 - or 10^2 -order copies of nucleic acids [17]. That is, the LOD of our
185 ultrasensitive ELISA is approaching the LOD of the NAT with a margin of only one or two orders of
186 magnitude. In addition, spike-and-recovery tests using blood confirmed the reliability of our
187 ultrasensitive ELISA [8].

188 3.2. Detection of proteins for a lifestyle-related disease

189 Adiponectin is an adipocyte-derived vasoactive peptide [18]. Serum adiponectin enhances
190 insulin sensitivity, and individuals with obesity, type 2 diabetes mellitus (DM), and other metabolic
191 disorders have low serum adiponectin levels [19]. On the other hand, urinary adiponectin is a useful
192 marker of the progression of diabetic nephropathy, indicating that DM patients have high urinary
193 adiponectin levels [20]. The sensitivity of commercially available ELISA kits, however, is insufficient
194 to measure the low level of urinary adiponectin in normal subjects [21,22]. We thus attempted to
195 apply our ultrasensitive ELISA to detect urinary adiponectin levels in normal subjects and
196 distinguish between normal subjects and DM patients [10,11].

197 The LOD of adiponectin and minimum LOQ were 1.4 pg/mL (i.e., *ca.* 2.3×10^{-19} moles/assay) and
198 54.9 pg/mL (i.e., *ca.* 9.1×10^{-18} moles/assay), respectively, for our ultrasensitive ELISA, when the
199 molecular mass was assumed to be 300 kDa and the volume of the assay was 50 μ L. Our ultrasensitive
200 ELISA, therefore, succeeded in detecting urinary adiponectin at the subattomole level. We then
201 attempted to determine the urinary adiponectin concentrations in healthy subjects and DM patients.
202 The urinary adiponectin concentrations were corrected on the basis of the creatinine concentrations.
203 The mean urinary adiponectin levels of healthy subjects were 0.73 ± 0.50 (ng/mg creatinine, mean \pm
204 SEM), and those of the DM patients were 12.02 ± 3.85 (ng/mg creatinine). That is, the urinary
205 adiponectin levels were significantly higher in DM patients ($P < 0.05$) than in healthy subjects.
206 Further, a threshold of urinary adiponectin levels could be set at 4 ng/mg creatinine to distinguish
207 between healthy subjects and DM patients [11].

208 The second target protein for a lifestyle-related disease was insulin [6,9]. An ELISA for insulin
209 was established in the 1980s and 1990s, and the LOD was on the order of μ IU/mL, corresponding to
210 tens of femtomoles/mL. The molecular weight of human insulin is 5807, and for this conversion 1 IU
211 was estimated as *ca.* 43 μ g [23]. We needed for our test to exceed this sensitivity.

212 We were faced with a fundamental problem for detecting insulin. Although many hospitals and
213 diagnostic companies use them, the reference material of insulin, i.e., the quality of the WHO
214 international standard insulin reference or its equivalent products is poor. As a practical manner, the
215 use of recombinant human insulin is strongly advised from the standpoint of calibration traceability
216 [24]. When the WHO international standard insulin reference (see [22]) was applied to our
217 ultrasensitive ELISA, the LOD was 19 nIU/assay, corresponding to *ca.* 1.4×10^{-16} moles/assay [9]. These
218 values are 0.38 μ IU/mL and *ca.* 2.8×10^{-15} moles/mL for a 50- μ L assay volume, and thus they were
219 somewhat better than the previously reported values.

220 When we used a recombinant insulin reference (MP Biomedicals, MP Bio Japan, Tokyo, Japan)
221 in our ultrasensitive ELISA, the LOD was 0.0047 pg/assay (i.e., 8.0×10^{-19} moles/assay) [6]. Because
222 the assay volume was 50 μ L, we could detect insulin in the order of tens of attomoles/mL. That is, the
223 LOD of our assay was at least 3 orders of magnitude more sensitive than those of the previously
224 reported assays.

225 Ultrasensitive detection of proteins involved in lifestyle-related diseases is important. Patients
226 with these diseases, such as DM, must continuously provide blood samples for repeated testing. If
227 the LOD of a specific protein can be improved for an early diagnosis, it may obviate the need for

228 blood sampling. Urine, saliva, and tears may include the same proteins, but in very small amounts
229 as compared to blood. Thus, our ultrasensitive ELISA can be used for the noninvasive detection of
230 proteins in urine, saliva, and tears, thereby alleviating patient pain and discomfort.

231 We are now applying this ultrasensitive ELISA to detect specific proteins from active bacilli [2]
232 and pathogenic viruses as well as proteins that can be used as cancer markers.

233 4. Challenges: expansion of the ultrasensitive ELISA to a non-amplification nucleic acid 234 detection method using hybridization and thio-NAD cycling

235 The value of 10^{-18} moles in the protein experiments is equivalent to approximately 10^6 molecules.
236 We hypothesized that if we replaced the antigen-antibody reaction for proteins with hybridization of
237 nucleic acids, nucleic acid detection could be performed at the level of 10^6 copies in another new
238 assay. In the planned system, we will prepare two different nucleic acid probes, similar to the two
239 different antibodies in ELISA, one of which is used to immobilize the target nucleic acid sequence
240 and the other for ALP labeling. Here, we should consider the size of ALP compared with that of the
241 nucleic acid probes. The molecular mass of ALP is 80000 - 150000 Da. As expected, our preliminary
242 experiments indicated that locating the ALP near the probes inhibits hybridization, resulting in poor
243 sensitivity. We thus consider that a small spacer is needed between the probes and the ALP. We are
244 now thinking that fluorescein isothiocyanate (FITC, molecular mass 389 Da) could function as this
245 spacer. Thus, the secondary probe is linked to FITC, and the FITC binds to an anti-FITC antibody that
246 is labeled with ALP. Our preliminary data suggested that we could measure nucleic acids at 106
247 copies/assay. Further, by increasing the number of secondary probes, we expect to improve the
248 sensitivity.

249 Why are we working to develop a new nucleic acid detection system with a sufficiently high
250 sensitivity to replace PCR? We think that PCR has many critical drawbacks, including, e.g., (1) non-
251 specific or false positive amplifications, (2) target sample volume limits, (3) requires deactivation of
252 amplification enzymes, (4) complicated techniques, (5) difficulty in designing probe sequences, and
253 (6) expense. Non-specific or false-positive amplifications occur due to excess DNA input, long
254 targets, or contamination. The sample amount used is *ca.* 1 μ L, which means that at least 1000 copies
255 must be included in a 1-mL volume. This low concentration may contribute the production of false
256 negative results. The deactivation of enzymes further deteriorates the amplification efficiency.
257 Further, the PCR techniques are complicated. A new method that can handle a large volume and
258 avoid amplifying the nucleic acids is therefore needed. We refer to our new nucleic acid detection
259 system as a 'non-amplification nucleic acid detection method'. This assay can be used not only in
260 laboratories, but also on-site in place of PCR.

261 5. Conclusions

262 We developed an ultrasensitive ELISA comprising sandwich ELISA and thio-NAD cycling to
263 detect trace amounts of proteins for diagnosis of disease. This system is also suitable for single-cell
264 analysis [7]. Expanding this ultrasensitive ELISA to develop an assay for nucleic acids will overcome
265 the drawbacks of PCR, and this non-amplification nucleic acid detection method is potentially also
266 widely applicable for diagnosis.

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