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大学院医療福祉学研究科博士課程

A highly sensitive and rapid enzymatic method using a biochemical automated analyzer to detect inorganic pyrophosphate generated by nucleic acid sequence-based amplification

(邦題：Nucleicacidsequence-basedamplification反応生成物ピロリン酸の高感度酵素的測定法の開発と生化学自動分析装置への応用)

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Abstract

Background and aims: Polymerase chain reaction-based techniques require expensive equipment for fluorescence detection of the products. However, the measurement of inorganic pyrophosphate (PPi) released during DNA synthesis can be used to quantify target genes without such equipment. Here, we devised a high-sensitivity enzymatic assay for detection of PPi.

Materials and methods: In our assay method, PPi was converted to hypoxanthine by hypoxanthine phosphoribosyl transferase. Xanthine dehydrogenase converted the hypoxanthine to uric acid and yielded two molecules of NADH, which in turn reduced Fe^{3+} to Fe^{2+} (mediated by 1-methoxy-5-ethylphenazinium ethylsulfate). 2-Nitroso-5-(N-propyl-N-sulfopropylamino) phenol chelated the Fe^{2+}, which resulted in an intensely colored product that could be measured using a biochemical automated analyzer.

Results: The assay was able to detect PPi within 10 min. It was linear between 0 and 10 μmol/L PPi, and intra-run and inter-run coefficients of variation were 1%–2%. Other validation tests with a biochemical automated analyzer were satisfactory. The assay could potentially be used to directly quantify samples after isothermal nucleic acid sequence-based amplification of a target gene.

Conclusion: The method developed here for detection of PPi can be used to measure

nucleic acid biomarkers in biological samples in clinical practice using a high-throughput biochemical automated analyzer.

Keywords:

Inorganic pyrophosphate, Enzymatic method, Biochemical automated analyzer, Nucleic acid sequence-based amplification, Nitroso-PSAP.

Title:

A high-sensitivity and rapid enzymatic method using a biochemical automated analyzer to detect inorganic pyrophosphate generated by nucleic acid sequence-based amplification

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Abbreviations:

Polymerase chain reaction (PCR); Nucleic acid sequence-based amplification (NASBA); Nucleoside triphosphate (NTP); Inorganic pyrophosphate (PPi); Hypoxanthine phosphoribosyl transferase (HPT); Xanthine oxidase (XOD); Xanthine dehydrogenase (XDH); Purine-nucleoside phosphorylase (PNP); Peroxidase (POD); Inorganic pyrophosphatase (PPase); 1-Methoxy-5-ethylphenazinium ethylsulfate (1-methoxy PES); 2-Nitroso-5-(N-propyl-N-sulfopropylamino) phenol (Nitroso-PSAP).

1. Introduction

Polymerase chain reaction (PCR)-based techniques, including reverse transcription-PCR and real-time PCR, are the most commonly used methods to amplify and quantify a specific target DNA. However, these techniques are not yet in widespread clinical use because the quantification of PCR products based on the coupling of fluorescently-labeled probes and amplicons requires expensive reagents, a specific detector, and well-trained staff. Nucleic acid sequence-based amplification (NASBA), which achieves amplification of target RNA, is performed isothermally (at 41°C) [1]. However, the techniques used to quantify the amplified RNA by hybridization of a fluorescent reagent with the target are similar to those for PCR [1–3]. Development of a sensitive detection method for inorganic pyrophosphate (PPi), which is formed by PCR amplification and NASBA, would, if the product were colored, contribute to simplified quantification of specific target genes in clinical laboratories.

Methods for detecting PPi, which is released from deoxynucleoside triphosphates (dNTPs) during PCR amplification, can be used as a surrogate marker to confirm whether target sequence amplification has been completed and can be employed for diagnosis (for example, of cancer, viruses, and genetic disorders) [4–8]. Various methods have been developed to detect PPi, including fluorometric [5,9–11], ion

55 chromatographic [12], enzyme-based colorimetric [4,13], and metal-complex-based
56 colorimetric methods [6–8,14]. However, the fluorometric and ion chromatographic
57 techniques are expensive, specialized, have low throughput, and require complex
58 preparation [9,10]. Although colorimetric methods are attractive as easy procedures with
59 a rapid response, conventional methods have low sensitivity and specificity and limited
60 throughput capacity.

61 Therefore, here, we devise a sensitive enzymatic assay for detecting PPi. This
62 assay uses hypoxanthine phosphoribosyl transferase (HPT) as a key enzyme. HPT is
63 widely used to catalyze pyrophosphorolysis of inosine monophosphate (IMP) [15,16].
64 There are several methods to measure IMP pyrophosphorolysis and phosphoribosyl
65 pyrophosphate using the combination of HPT and xanthine oxidase (XOD) [17,18].
66 However, few studies have reported on the detection of PPi using HPT [4]. Our assay also
67 uses 2-nitroso-5-(N-propyl-N-sulfopropylamino) phenol (Nitroso-PSAP); this reagent
68 selectively forms a chelate with Fe^{2+}, which results in a highly colored product (molar
69 absorptivity 45,000 L cm^{-1} M^{-1} at 756 nm) [19]. This chelate has been used to quantify
70 non-transferrin-bound iron in serum [20]. Moreover, we applied the assay using a
71 biochemical automated analyzer to perform the diagnostic tests with rapidity and high
72 throughput. We also developed methods combining NASBA and this enzymatic assay.

The assay developed in this study is expected to be valuable for diagnostic tests of nucleic acid biomarkers in the clinical laboratory.

2. Materials and Methods

2.1. Principle of the method

Figure 1 shows the reaction sequences in our novel assay. PPi, which is derived from DNA/RNA amplification, and IMP are converted to hypoxanthine and phosphoribosyl pyrophosphate by HPT. The hypoxanthine produced is then converted to uric acid and NADH, catalyzed by xanthine dehydrogenase (XDH). The NADH reacts with Fe^{3+} to form NAD^{+} and Fe^{2+}, mediated by 1-methoxy-5-ethylphenazinium ethylsulfate (1-methoxy PES). Nitroso-PSAP forms a green chelate complex with the Fe^{2+}.

2.2. Overproduction and purification of HPT from *Escherichia coli* K12 W3110

A synthetic gene encoding nicotinamide mononucleotide HPT from *E. coli* K12 W3110 (BAB96700) was synthesized by GenScript (Piscataway, NJ, USA). The synthetic gene (0.54 kbp) contained *Nde*I and *Hin*dIII restriction sites at the 5′- and 3′- ends, respectively. The gene was cloned into the corresponding sites in vector pET21a to yield EcHPT/pET21a. *E. coli* BL21 (DE3) cells were transformed with EcHPT/pET21a and

91 transformants were selected by growth on Luria-agar supplemented with ampicillin (50
92 μg/mL). A single colony was then picked and cultured in 1.6 L of Overnight Express™
93 Instant TB Medium (Novagen) containing 50 μg/mL ampicillin for 24 h at 30°C. Cells
94 expressing HPT were harvested by centrifugation, suspended in 10 mmol/L Tris-HCl
95 buffer (pH 8.5), and then lysed by adding 0.5% lysozyme, 1 mmol/L EDTA, and 0.05%
96 Triton X-100, followed by a 1-h incubation at 37°C. After removing the cell debris by
97 centrifugation for 30 min at 5,000 × *g*, the supernatant was loaded onto a column
98 containing 300 mL of Q Sepharose Big Beads pre-equilibrated with 10 mmol/L Tris-
99 HCl buffer (pH 8.5). After washing the column with the equilibration buffer, bound
100 protein was eluted with a linear gradient of 0 to 0.5 mol/L KCl in the same buffer.
101 Active fractions (see section 2.3 for assay details) were concentrated using a 30-kDa
102 centrifugal filter device (Millipore, Bedford, MA) and solid KCl was added to 3 mol/L.
103 The protein solution was applied to a 50-mL Phenyl Sepharose 6 Fast Flow column
104 (High Sub) pre-equilibrated with 10 mmol/L Tris-HCl buffer (pH 7.5) containing 3
105 mol/L KCl. The column was then washed with equilibration buffer and the bound
106 protein was eluted with a linear gradient of 3 to 0 mol/L KCl in the same buffer.
107 Fractions containing HPT activity were pooled, concentrated using a 30-kDa centrifugal
108 filter device, and dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.5). The protein

109 solution was applied to a 25-mL Q Sepharose high-performance column pre-
110 equilibrated with 10 mmol/L Tris-HCl buffer (pH 8.5). The column was then washed
111 with equilibration buffer and the protein was eluted with a linear gradient of 0 to 0.5
112 mol/L KCl in the same buffer. Active fractions were pooled and then concentrated using
113 a 30-kDa centrifugal filter device. The sample was desalted by gel filtration
114 chromatography using a Sephadex G-25 Superfine column (GE Healthcare, IL, USA)
115 pre-equilibrated with 10 mmol/L Tris-HCl buffer (pH 7.5). The entire purification
116 procedure was performed at room temperature (<25 °C).
117
118 2.3. Assay of HPT
119 Unless otherwise specified, HPT activity was routinely determined in a continuous
120 reaction entailing hypoxanthine formation from IMP and PPi and oxidation of
121 hypoxanthine to urate by XDH with concomitant conversion of NAD^+ to NADH.
122 Standard reaction mixtures contained 20 mmol/L Tris-HCl buffer (pH 7.5), 5 mmol/L IMP,
123 5 mmol/L potassium pyrophosphate, 5 mmol/L NAD^+, 5 mmol/L magnesium chloride,
124 and 5 kU/L XDH. The rate of NADH formation at 37°C was monitored
125 spectrophotometrically at 340 nm (molar absorptivity of NADH = 6,220 L cm^{-1} M^{-1}).
126 One unit (U) of enzyme was defined as the amount of enzyme forming 1 μmol of NADH

per min.

2.4. Reagents for the determination of optimal assay parameters and equipment

Tris-buffered saline tablets were obtained from Takara Bio, Shiga, Japan. Nitroso-PSAP and 1-methoxy PES were from Dojindo Molecular Technologies, Kumamoto, Japan. β-NAD^+ was from Oriental Yeast Co., Tokyo, Japan. Magnesium chloride, inosine-5′-monophosphte, ferric chloride, potassium sodium tartrate, and potassium pyrophosphate were obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. XDH (EC 1.17.1.4; from microorganisms) was from Asahi Kasei Pharma Corporation, Tokyo, Japan. Uridine 5′-triphosphate trisodium salt hydrate was obtained from Tokyo Chemical Industry Co., Tokyo, Japan. Guanosine 5′-triphosphate sodium salt hydrate, adenosine 5′-triphosphate disodium salt, and cytidine 5′-triphosphate disodium salt were obtained from Sigma Aldrich Japan, Tokyo, Japan. Optimal assay parameters such as reagent concentrations were determined using a Hitachi 7012 clinical spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan).

2.5. Reagents for the enzymatic assay using a biochemical automated analyzer

Tris-buffered saline tablets, Nitroso-PSAP, 1-methoxy PES, β-NAD^+, magnesium

chloride, inosine-5′-monophosphte, ferric chloride, potassium sodium tartrate, potassium pyrophosphate, and XDH were obtained from the suppliers listed in section 2.4. A two-reagent system was used in the assay in the biochemical automated analyzer. Reagent-1 for our novel method (RN1) consisted of 50 mmol/L Tris-HCl buffer (pH 8.0), 0.2 mmol/L ferric chloride, 0.4 mmol/L potassium sodium tartrate, and 0.8 mmol/L Nitroso-PSAP. Reagent-2 (RN2) contained 50 mmol/L Tris-HCl buffer (pH 8.0), 8.0 mmol/L β-NAD^+, 8.0 mmol/L inosine-5′-monophosphte, 0.5 mmol/L magnesium chloride, 0.4 kU/L HPT, 3.84 kU/L XDH, and 2.0 μmol/L 1-methoxy PES. Standard solution was prepared by dissolving 10 μmol/L PPi in distilled water.

2.6. Reagents for phosphate test kit Pi-L with PPase for use in the biochemical automated analyzer

Phosphate test kit Pi-L was obtained from Serotec Co., Hokkaido, Japan. Inorganic pyrophosphatase (PPase; EC 3.6.1.1; from *Thermococcus* sp.) was from New England Biolabs, MA, USA. A two-reagent system was used in the assay in the biochemical automated analyzer. Reagent-1 for the control method (RC1) consisted of 20 mmol/L Good's buffer (pH 7.0), 0.95 kU/L ascorbate oxidase, 1.5 kU/L XOD, 300 kU/L catalase, 12.0 mmol/L xanthosine, and 0.85 mmol/L N-(2-hydroxy-3-sulfopropyl)-3,5-

163 dimethoxyaniline, sodium salt. Then, 1.0 kU/L PPase was added to RC1. Reagent-2
164 (RC2) contained 20 mmol/L Good's buffer (pH 7.0), 5.0 kU/L purine-nucleoside
165 phosphorylase (PNP), 5.6 kU/L peroxidase (POD), and 2.88 mmol/L 4-aminoantipyrine.
166 Standard solution was prepared by dissolving 10 μmol/L PPi in distilled water.
167
168 2.7. Materials for total RNA extraction, NASBA, and gel electrophoresis
169 Human dermal fibroblasts were obtained from Kurabo Industries, Osaka, Japan. Total
170 RNA extraction reagent RNAiso Plus was obtained from Takara Bio, Shiga, Japan. An
171 NASBA kit was purchased from KAINOS Laboratories, Tokyo, Japan. FastGene 50-bp
172 DNA Ladder and Midori Green Advance DNA Stain were from NIPPON Genetic Co.,
173 Tokyo, Japan.
174
175 2.8. Biochemical automated analyzer
176 Assays were performed using a Hitachi 7180 Biochemical Automated Analyzer (Hitachi
177 High-Technologies Co.).
178
179 2.8.1 Analytical conditions for the novel method designed in this work using the
180 biochemical automated analyzer

Ten microliters of sample were incubated with 110 μL of RN1 (see section 2.5) for 5 min at 37°C. Then, 60 μL of RN2 was added. After 5 min, the absorbance at 750/600 nm (primary/secondary) was measured using a two-point end assay.

2.8.2 Analytical conditions for the conventional method for inorganic phosphate determination with PPase using the biochemical automated analyzer

Sample (3.1 μL) was incubated with 150 μL RC1 (see section 2.6) for 5 min at 37°C. Then, 50 μL RC2 were added. After 5 min, the absorbance at 600/800 nm (primary/secondary) was measured using a two-point end assay.

2.9. Correlation between the control method (using PPase, PNP, XOD and POD) and our novel method (using HPT, XDH, and Fe) for determination of PPi

The control method was performed by adding PPase to phosphate test kit Pi-L because no enzymatic assay for PPi using a biochemical automated analyzer has previously been established. Phosphate test kit Pi-L was employed as it is a commercially available product using the PNP-XOD-POD method for determination of inorganic phosphate [21,22]. PPi reference solution (0 to 10 μmol/L) was measured by the control method and our novel method using the biochemical automated analyzer.

199

200 2.10. Total RNA extraction

201 Total RNA was extracted from spheres coated with human dermal fibroblasts using total
202 RNA extraction reagent RNAiso Plus. According to the instruction manual, precipitated
203 RNA was dissolved in DNase- and RNase-free water. The amount of RNA sample was
204 quantified using a ratio beam spectrophotometer U-5100 (Hitachi High-Technologies
205 Co.) and diluted to 0.1 μg/5 μL with the DNase- and RNase-free water.

206

207 2.11. Combination of NASBA with our enzymatic method for determination of PPi

208 NASBA was performed in line with instructions for use of the NASBA kit for *β-actin* as
209 previously described [3]. First, 5 μL RNA template (0.1 μg total RNA) was mixed with
210 10 μL of the NASBA reagent and 0.4 μmol/L each of reverse and forward primers for *β-*
211 *actin*. RNase-free water was used for an amplification negative control. Then, 15 μL of
212 the reaction mixture was heated at 65°C for 5 min, followed by 41°C for 5 min for
213 annealing. Next, 5 μL of NASBA enzyme mix was added. The reaction mixture was
214 incubated at 41°C for 0, 20, 40, 60, 80, or 100 min. A ProFlex PCR system (Thermo
215 Fisher Scientific, MA, USA) was used to control the temperature for NASBA. After that,
216 5 μL of sample was analyzed by 2% agarose gel electrophoresis containing Midori Green

217 Advance DNA Stain, and 10 μL of NASBA sample was used for enzymatic PPi assay
218 using the biochemical automated analyzer.
219
220 2.12. Statistical analysis
221 Results are presented as the mean ± standard deviation (SD) and coefficients of variation
222 (CVs) were determined using Microsoft Excel® 2016. The linearity and correlation
223 between the control method and our novel method were calculated using Validation-
224 Support/Excel V4.18 guaranteed by the Japan Society of Clinical Chemistry, as
225 previously described [23].
226
227 **3. Results**
228 3.1. Determination of optimum parameters for the assay
229 HPT was expressed in and purified from *E. coli*. Approximately 5,170 U of purified
230 enzyme was obtained from 1.6 L of culture. The purified recombinant protein ran with an
231 apparent molecular mass of 20 kDa on SDS-PAGE, in good agreement with the expected
232 mass based on the amino acid sequence (20,115 Da).
233 The optimum pH for the combined assay using HPT and XDH was 8.0 (data not
234 shown). The optimum concentrations of β-NAD^+ and inosine-5′-monophosphte were

each 8.0 mmol/L, which indicates a zero-order reaction of HPT and XDH (data not shown). Graphs of the enzymatic activity of HPT and XDH toward PPi are shown in Figure 2. The absorbance at 340 nm was measured 5 min after starting the enzymatic reaction. With enzymatic activity above 0.4 kU/L for HPT and 3.84 kU/L for XDH, the absorbance at 340 nm (indicating formation of NADH) almost plateaued; thus, the enzymes reached a steady-state and the reactions were complete within 5 min. The optimum concentration of magnesium chloride was 0.5 mmol/L (data not shown). The presence of Mg^{2+} made HPT highly active. Color development from 0.2 mmol/L ferric chloride, 0.4 mmol/L potassium sodium tartrate, and 0.8 mmol/L Nitroso-PSAP was completed within 5 min using 2.0 μmol/L 1-methoxy PES (data not shown).

Thus, we determined that the optimum reagent conditions were: 50 mmol/L Tris-HCl buffer (pH 8.0), 8.0 mmol/L β-NAD^+, 8.0 mmol/L inosine-5′-monophosphte, 0.5 mmol/L magnesium chloride, 0.4 kU/L HPT, 3.84 kU/L XDH, 2.0 μmol/L 1-methoxy PES, 0.2 mmol/L ferric chloride, 0.4 mmol/L potassium sodium tartrate, and 0.8 mmol/L Nitroso-PSAP. We confirmed the linearity of the determined concentration for 0 to 100 μmol/L PPi solution under the optimized reagent conditions (data not shown).

3.2. Specificity of the assay

253 NTPs, including uridine, guanosine, adenosine, and cytidine 5′-triphosphate, are essential
254 materials for the amplification reactions of RNA; however, these materials may in
255 principle interfere with the enzymatic assay described in this work. NASBA reagent
256 contains 2 mmol/L NTPs [24]. The specificity of our enzymatic assay was examined using
257 2 mmol/L NTPs in distilled water. Each solution was measured with three replicates. The
258 concentration of each solution of NTPs was compared with that of a reference sample
259 (distilled water without NTPs). The concentrations of PPi represented by the assay were
260 the same as those in the reference sample, which indicates that the assay was not
261 significantly affected by NTPs at up to 2 mmol/L.
262
263 3.3. Linearity
264 The linearity of the assay was measured using working solutions of up to 10 μmol/L PPi.
265 Each solution was assessed with five replicates. A plot of theoretical vs. measured values
266 of PPi had a slope of 1.039 and an intercept of +0.349, which indicates satisfactory
267 linearity up to 10 μmol/L PPi.
268
269 3.4. Precision
270 Intra- and inter-assay CVs were determined using water-diluted PPi standard solution

with five and two different concentrations, respectively. Each solution for intra-assay determination was measured with 20 replicates on the same day. Each solution for inter-assay determination was measured with two replicates for 20 days, and calibration was conducted on each day. The intra-assay CVs of samples with low (2.27 μmol/L), medium (6.85 μmol/L), and high (8.70 μmol/L) PPi concentrations were 1.76%, 1.02%, and 0.57%, respectively. The inter-assay CVs during measurements over the 20-day period were 1.27% (4.74 μmol/L), and 0.69% (8.72 μmol/L).

3.5. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were estimated using water-diluted PPi standard solution with 10 replicates. LOD was based on the difference of three standard deviations between the zero-concentration sample (purified water) and the lowest concentration sample. LOQ was defined as the lowest concentration that showed a CV of ≤20%. The LOD and LOQ for detecting PPi using the biochemical automated analyzer were 0.15 μmol/L and 1.00 μmol/L, respectively.

3.6. Correlation between the control assay method (using PPase, PNP, XOD, and POD) and our novel method (using HPT, XDH, and Fe) in determination of PPi

289 The concentrations of PPi measured by the control enzymatic method were compared
290 with those measured by the novel method developed in this study. Thirty PPi solutions
291 (0–10 μmol/L) were measured by both methods. A plot of the concentrations of PPi
292 determined by the two methods had a slope of 0.938, an intercept of −0.214, Spearman's
293 rank correlation coefficient (r) of 0.999, and Sy·x of 0.32 (Figure 3). These results suggest
294 that our method is suitable for the measurement of PPi using the biochemical automated
295 analyzer.
296
297 3.7. Combination of NASBA with the novel enzymatic method
298 NASBA products were detected by agarose gel electrophoresis (Figure 4). The intensity
299 of each band was proportional to the amplification time, which indicates that the quantity
300 of product, and hence the amount of released PPi, increased with amplification time.
301 No PPi was detected in the NASBA negative control reaction by our enzymatic
302 method using the biochemical automated analyzer (Figure 4). However, PPi was
303 detectable after a 20-min amplification when a 0.1-μg total RNA sample was used, which
304 indicates that the concentration of released PPi was over the LOD and LOQ. The
305 concentration of PPi detected increased with the NASBA reaction time. These results
306 show that the combination of NASBA and our enzymatic assay method can be applied to

307 the detection of PPi released by RNA amplification, which has great potential for
308 measuring nucleic acid-based biomarkers in biological samples.
309
310 **4. Discussion**
311 PCR-based techniques are the most widely used methods for nucleic acid amplification
312 for clinical diagnostics. Several other techniques relating to signal and target
313 amplification, such as NASBA, have also been developed and used clinically. However,
314 some clinical laboratories, especially in small hospitals, have not benefited from these
315 techniques. Direct detection systems based on fluorescence-labeled hybridization probes
316 and DNA or RNA amplicon-labeling fluorescence dyes require complicated procedures,
317 skilled staff, and long detection time. Therefore, here, we developed a rapid, sensitive,
318 and specific detection method for PPi formed by nucleic acid amplification methods
319 including PCR and NASBA. The detection uses visible absorbance (i.e., color). Thus, the
320 present method can be used to measure PPi without fluorescence-based systems and
321 contribute to simple and quick quantification of target DNA and RNA in the clinical
322 laboratory.
323 Several methods involving fluorometric and colorimetric techniques have been
324 developed for the measurement of PPi produced by various biological reactions.

Fluorometric assay for the detection of PPi has high sensitivity based on fluorescence of, for example, luciferin or spiropyran [5,9–11]. However, these methods are not always applicable in clinical practice because of limited throughput capacity and the requirement for an expensive fluorescence detector. Enzyme-based colorimetric assays for PPi based on formazan, generated from tetrazolium salts, have also been reported [4,13]. However, the formazan dye is too water-insoluble to use in clinical practice; an extra procedure to dissolve it in an organic solvent or detergent is needed and repeatedly-used optical cells attached to the biochemical automated analyzer thus become dirty [25]. A new sulfonated tetrazolium salt, which generates a highly water-soluble formazan dye with molar absorptivity of 37,000 L cm^{-1} M^{-1} at 438 nm, has been developed [26]. However, the molar absorptivity of this dye is less than that of the chelate complex of Nitroso-PSAP and Fe^{2+} used in the present study (45,000 L cm^{-1} M^{-1} at 756 nm). Although an assay for the detection of PPi using HPT and XOD/XDH has been reported, it cannot be applied for clinical laboratory use because of the lack of validation with biochemical automated analyzers and the requirement for use of formazan dye with the associated problems described above [4]. The novel enzymatic method described in this report gives highly sensitive and specific detection of PPi that can be validated and used in clinical settings.

PPase catalyzes the hydrolysis of PPi to form inorganic phosphate [27].

343 Measurement methods for inorganic phosphate using PNP and XOD/XDH have been
344 established and used in the clinical laboratory [21,22,27]. A sensitive enzymatic method
345 for the determination of PPi could be established if PPase, PNP, and XOD/XDH were
346 combined; this reaction forms four molecules of NADH or H_2O_2. However, the PPase-
347 PNP-XOD/XDH method is not specific because of cross-reaction with triphosphates,
348 substrates that are required for DNA or RNA amplification. Also, use of buffers
349 containing inorganic phosphate would have an obvious effect on measurements made by
350 the PPase-PNP-XOD/XDH method. Therefore, in the present method, HPT was
351 employed as the enzyme reacting with PPi. Although the two molecules of NADH
352 produced by HPT-XDH reaction in this method are two fewer than are produced in the
353 PPase-PNP-XOD/XDH method, the present method had high sensitivity—a LOQ of 1
354 μmol/L PPi—because of the sensitive chelating reagent with high molar absorptivity that
355 was used for detection.

356 Using our novel assay, we successfully detected PPi in samples after NASBA,
357 which gives great hope for the analysis of nucleic acid-based biomarkers in biological
358 samples using a biochemical automated analyzer. According to the instructions for use of
359 the NASBA kit, two temperatures are required to complete the amplification. However,
360 the 5-min incubation at 65°C to denature the target is not required when using appropriate

primers and RNA [1]. The present method can thus be performed isothermally at 41°C, at which all the enzymes will remain active. Thus, both amplification and detection may occur in a single cell attached to a biochemical automated analyzer. Primer design that accelerates amplification of the specific target and minimizes nonspecific reactions, such as primer dimerization, would be important for high specificity of the method.

Infectious diseases and cancers are leading causes of mortality worldwide [28]. PCR-based techniques are the best strategy to detect/diagnose pathogens [29]. Gene overexpression assays have provided information on the recurrence of breast cancer [30], and the detection of circulating tumor DNA in plasma enables monitoring of early metastasis of breast cancer [31]. Thus, genome-based diagnostics play an increasingly important role in treating infectious diseases and cancers around the world. Combination of the novel method described in this work with modified NASBA with primers linked to specific target genes may be useful for the diagnosis of, for example, pathogens and cancer progression. Our method will contribute globally to clinical laboratory practice because of its simplicity and high-throughput capacity. A measurement method of target genes using PCR at a constant temperature of 37°C has been reported [32]. If this method could be combined with the present PPi assay, it would be possible to detect specific target genes rapidly and easily in a wide range of clinical laboratories.

In conclusion, we have developed an enzymatic assay for detecting PPi released during NASBA using a biochemical automated analyzer to permit wide use in daily practice.

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CRediT authorship contribution statement

Atsushi Isobe: Formal analysis, Data curation, Investigation, Methodology, Writing - original draft. Yuki Iwabuchi: Formal analysis. Miki Yajima: Formal analysis. Shin-ichi Sakasegawa: Resources, Writing - review & editing. Yoshitaka Yamaguchi: Resources, Data curation. Masanori Seimiya: Writing - review & editing. Tsukuru Umemura: Investigation, Project administration, Writing - review & editing. Susumu Osawa: Conceptualization, Investigation, Writing - review & editing, Funding acquisition, Supervision.

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Declaration of interest

Atsushi Isobe is an employee of TERUMO Corporation. However, this work was not performed in his capacity as an employee. Shin-ichi Sakasegawa is an employee of Asahi Kasei Pharma Corporation. All other authors have no conflicts of interest.

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659 **Figure legends**

660 Figure 1. Schematic representation of the reaction sequence in the novel assay method
661 developed in this study for determination of inorganic pyrophosphate (PPi).
662 HPT: hypoxanthine phosphoribosyl transferase; XDH: xanthine dehydrogenase; 1-
663 methoxy PES: 1-methoxy-5-ethylphenazinium ethylsulfate.

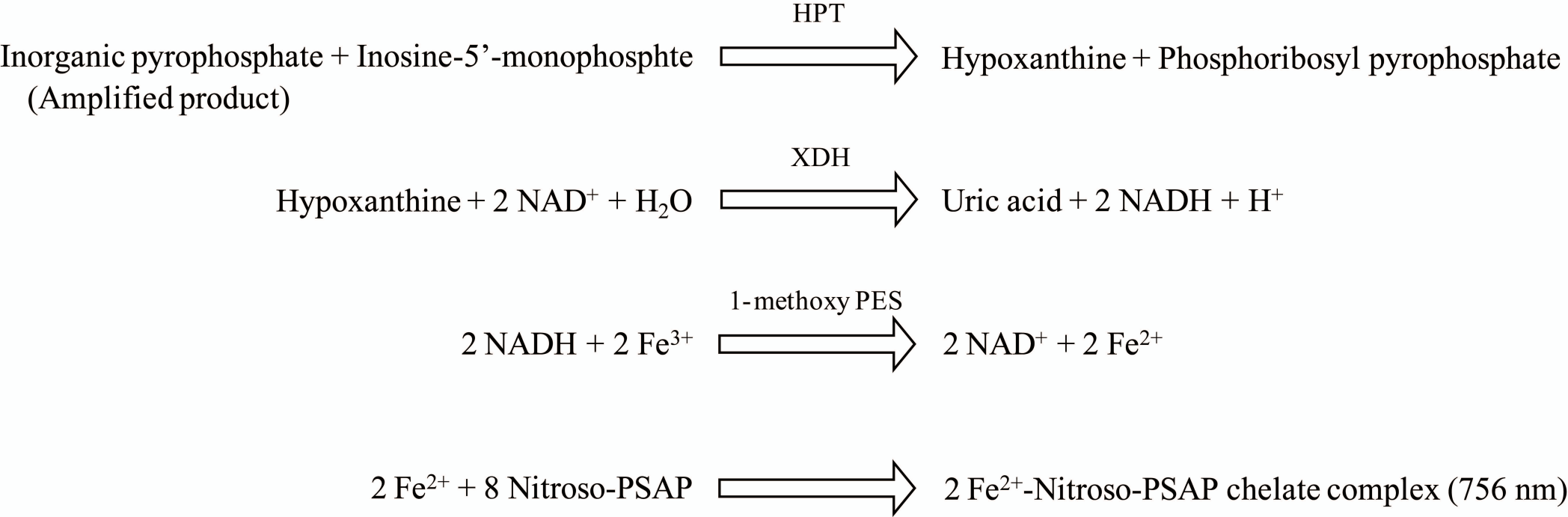
664

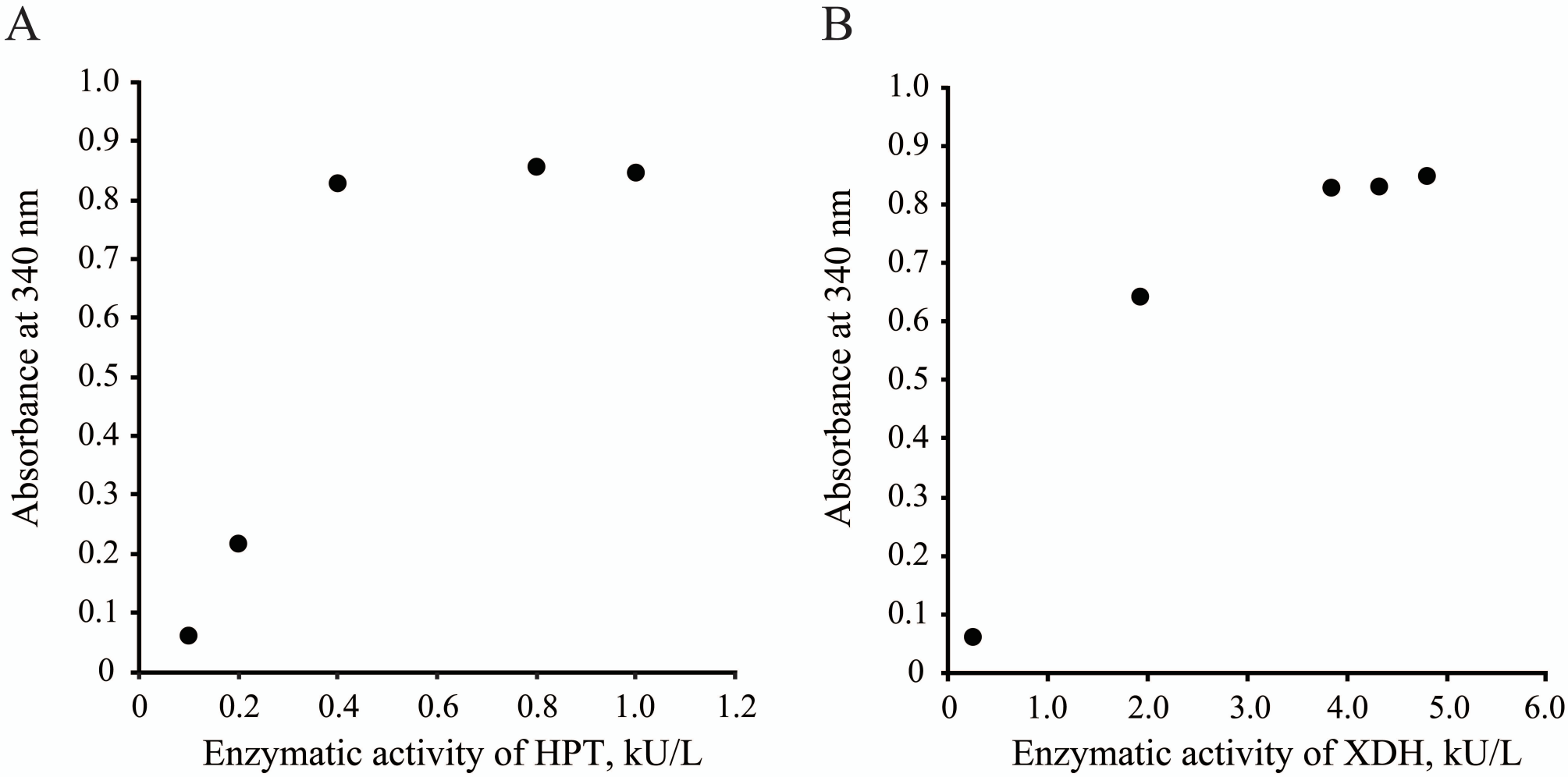
665 Figure 2. Optimization of HPT and XDH activities in the assay.
666 The determination of enzymatic activities toward PPi was performed by measuring
667 absorbance 5 min after starting the enzymatic reaction. The plot of absorbance at 340 nm,
668 the wavelength of maximum absorbance of NADH, almost plateaued despite increasing
669 the enzymatic activities above 0.4 kU/L of HPT (A) and 3.84 kU/L of XDH (B). HPT:
670 hypoxanthine phosphoribosyl transferase; XDH: xanthine dehydrogenase.

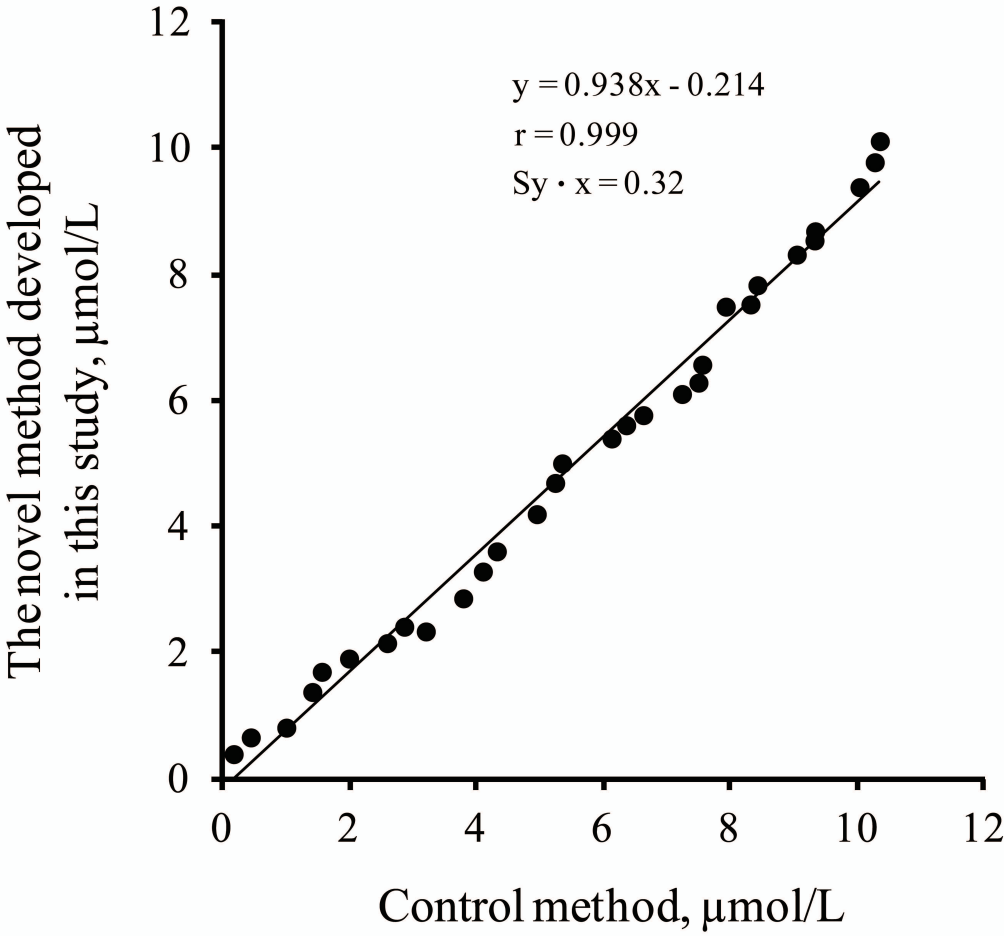
671

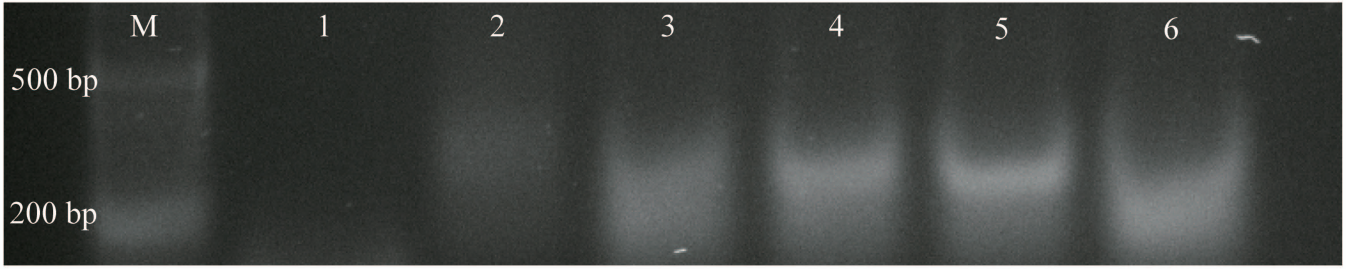
672 Figure 3. Comparison between the novel assay method developed in this study for
673 determination of PPi and a PPase-PNP-XOD-POD-based control method.
674 There was a correlation between the values determined by the two methods; the regression
675 line shows a slope of 0.938, intercept of −0.214, Spearman's rank correlation coefficient
676 (r) of 0.999, and Sy·x of 0.32. x-axis: PPase-PNP-XOD-POD method; y-axis: The novel

677 method developed in this study. PPase: inorganic pyrophosphatase; PNP: purine-
678 nucleoside phosphorylase; XOD: xanthine oxidase; POD: peroxidase.
679
680 Figure 4. Detection of nucleic acid sequence-based amplification (NASBA) product on
681 amplification of the gene *β-actin* using the novel assay method developed in this study
682 for determination of PPi.
683 Visualization of NASBA product for *β-actin* with different amplification times by 2%
684 agarose gel electrophoresis (above). Lane M: marker; Lane 1: negative control; Lane 2:
685 20-min amplification; Lane 3: 40-min amplification; Lane 4: 60-min amplification; Lane
686 5: 80-min amplification; Lane 6: 100-min amplification. Lanes 2–6 show that the quantity
687 of NASBA products consisting of DNA and RNA hybrid increased with amplification
688 time. The concentration of PPi in the NASBA product determined using the biochemical
689 automated analyzer (below). PPi was not detected in the NASBA negative control,
690 whereas it was detected after a 20-min amplification. Sample 1: negative control; Sample
691 2: 20-min amplification; Sample 3: 40-min amplification; Sample 4: 60-min
692 amplification; Sample 5: 80-min amplification; Sample 6: 100-min amplification.


HPT
Inorganic pyrophosphate + Inosine-5'-monophosphte
(Amplified product)
Hypoxanthine + Phosphoribosyl pyrophosphate
XDH
Hypoxanthine + 2 NAD+ + H2O
Uric acid + 2 NADH + H+
1-methoxy PES
2 NADH + 2 Fe3+
2 NAD+ + 2 Fe2+
2 Fe2+ + 8 Nitroso-PSAP
2 Fe2+-Nitroso-PSAP chelate complex (756 nm)


A
Absorbance at 340 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1.0
1.2
Enzymatic activity of HPT, kU/L
B
Absorbance at 340 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1.0
2.0
3.0
4.0
5.0
6.0
Enzymatic activity of XDH, kU/L


y = 0.938x - 0.214
r = 0.999
Sy · x = 0.32
The novel method developed in this study, μmol/L
Control method, μmol/L
0
2
4
6
8
10
12



Pyrophosphate (μmol/L)	0.02	6.48	8.02	9.36	9.38	9.80