Development and Characterization of Protein Induced by Vitamin K Absence or Antagonist II Immunoassay

January, 2018

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Life Sciences and Bioengineering)

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Abbreviations

HCC: Hepatocellular carcinoma

HBV: hepatitis B virus

HCV: hepatitis C virus

AFP: alpha-fetoprotein

PIVKA-II: Protein induced by vitamin K absence or antagonist-II

DCP: des-gamma-carboxy prothrombin

AFP-L3: AFP fraction with affinity to the Lens culinaris agglutinin

Glu: glutamic acid residues

Gla: γ-carboxy glutamic acid

GANP: germinal center-associated nuclear protein

RST: rapid serum tube including thrombin

GPC: gel permeation chromatography

RLUs: relative light units

SST: serum separator tube

IC50: half maximal Inhibitory Concentrations

%CV: percentage coefficient of variation

LoB: limit of blank

LoD: limit of detection

LoQ: limit of quantitation

CLSI: Clinical and Laboratory Standards Institute

SD: standard deviation

BD: Becton, Dickinson and Company

aa: amino acid

Contents:

Chapter I: Preface

Chapter II: Characterization of an anti-PIVKA-II antibody and evaluation of a fully automated chemiluminescent immunoassay for PIVKA-II

Summary

Introduction

Materials and methods

Results

Discussion

Chapter III: Development and evaluation of analytical performance of a fully automated chemiluminescent immunoassay for protein induced by vitamin K absence or antagonist II

Summary

Introduction

Materials and methods

Results

Discussion

Chapter IV:

Conclusion remarks

Acknowledgements

References

Chapter I:

Preface

Introduction

Liver cancer is the second most common cause of death from cancer worldwide and is given very poor prognosis [1]. Hepatocellular carcinoma (HCC) is the major histologic type among primary liver cancers occurring worldwide, accounting for 70% to 85% of total burden [2]. Chronic infection by either hepatitis B virus (HBV) or hepatitis C virus (HCV) is a major risk factor for HCC. Approximately 80% of HCC is estimated to be attributable to HBV or HCV worldwide [3]. Carcinogenic risk of HBV carriers is 223-fold that of non-carriers [4]. Infection by HCV is the most common predisposing factor in some developed countries, including Japan [5].

One of the reasons for the high fatality rate of HCC is the difficulty of detection for HCC in early stage since it is an asymptomatic disease; therefore many patients are not treated in the early stages. Early detection of HCC by surveillance in high risk groups is an appropriate way to improve the survival rate in patients with HCC [6-11]. HCC can be treated by resection, liver transplantation, or local ablation with radiofrequency for patients diagnosed at an early stage [12]. However, although alpha-fetoprotein (AFP) is the most commonly used tumor marker for HCC, its clinical diagnostic accuracy is unsatisfactory due to low sensitivity and specificity [13]. Therefore, there is an urgent need for

developing better HCC-specific biomarkers. Several serological biomarkers have been suggested as other potential biomarkers for early detection of HCC.

Protein induced by vitamin K absence or antagonist-II (PIVKA-II) as a tumor marker for HCC

PIVKA-II, also known as des-gamma-carboxy prothrombin (DCP), is a useful marker for the diagnosis of HCC [13, 14]. PIVKA-II level is not correlated with the levels of AFP or AFP fraction with affinity to the Lens culinaris agglutinin (AFP-L3), which are other representative biomarkers for HCC. Therefore, PIVKA-II can be used along with AFP or AFP-L3 as a complementary biomarker for HCC. Thus, the combination of two markers enhances the sensitivity of diagnosis of HCC, but minimizes the decrease in specificity [15-18]. The Japan Society of Hepatology recommends measurement of two or more tumor markers for the diagnosis of HCC [19]. Also, the measurement of AFP, AFP-L3 and PIVKA-II is covered by the National Health Insurance in Japan. PIVKA-II has been used as an aid for diagnosis of HCC and monitoring of high risk patients (HCV infections, HBV infections, and hepatitis/cirrhosis) for development of HCC for more than 20 years in Japan [19].

Many studies showed serum PIVKA-II levels are related to tumor size, vascular invasion, intrahepatic metastasis and frequency of recurrence after treatment [20-24]. PIVKA-II secreted from HCC cells strongly promotes HCC growth and metastasis through the mechanisms of autocrine

stimulation and paracrine interaction factor between HCC and human vascular endotherial cells [25, 26]. The autocrine secretion actives c-Met phosphorylation and affects self-proliferation of HCC through the c-Met-JAK-STAT3 pathway [27]. Also, PIVKA-II stimulates HCC invasion through activation of the ERK1/2 MAPK pathway [27]. The paracrine secretion stimulates proliferation and migration of vascular endothelial cells via release of VEGF, TGF-α, and bFGF and activation of the KDR-PLC-γ-MAPK pathway [27]. Recently, the clinical report showed enhanced antitumor action of sorafenib, which is a molecular-targeting anti-cancer agent that inhibits the serine-threonine kinase Raf and the tyrosine kinase activity if VEGF receptors, by vitamin K. It is suggest that vitamin K may have the synergistic effect by suppressing production of PIVKA-II [28].

Prothrombin, a blood coagulation protein synthesized in the liver, is converted to an active form after the 10 glutamic acid residues (Glu) in the γ-carboxy glutamic acid (Gla) domain at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 32 are γ-carboxylated to Gla by vitamin-K dependent γ-glutamyl carboxylase. In patients with HCC, γ-carboxylation of prothrombin is impaired so that PIVKA-II is formed instead of prothrombin [29]. The production of normal prothrombin and PIVKA-II in hepatic cells is shown in Figure 1. Liebman et al. [16] first reported that the serum PIVKA-II concentration was significantly higher in patients with HCC by radioimmunoassay using a polyclonal antibody. They purified PIVKA-II from ascites fluid of patients with HCC and analyzed its

Gla content [30]. Then, Motohara et al. [31, 32] made the MU-3 monoclonal antibody against PIVKA-II and established an assay system with higher sensitivity. Clinical studies have demonstrated that the assay system using MU-3 antibody shows high sensitivity and specificity to PIVKA-II in patients with HCC [33]. Sugo et al. [34] reported that the reactivity of MU-3 antibody increased when the number of Gla was less than five per molecule. Furthermore, Naraki et al. [35] described the epitope of PIVKA-II reacting to MU-3 antibody. The epitope of MU-3 antibody was located within the amino acid residues 17–27 in the Gla domain. They reported that MU-3 antibody reacted to PIVKA-II with less than four Gla and PIVKA-II variants preferentially synthesized in HCC patients have less than four Gla whereas PIVKA-II variants in benign liver diseases have more than five Gla [35]. The MU-3 antibody is used for the almost diagnostic reagents to achieve the high sensitivity and specificity. It was necessary that a new monoclonal antibody against PIVKA-II is established instead of MU-3 antibody to distribute the PIVKA-II diagnostic reagent in worldwide. Therefore, the establishment of the new monoclonal antibody against PIVKA-II was attempted. It is important that the monoclonal antibody can detect the difference between normal prothrombin and PIVKA-II molecule to develop the high sensitivity and specificity diagnostic reagent. The germinal center-associated nuclear protein (GANP) transgenic mouse has an advantage in preparation of monoclonal antibodies against various epitopes, for which conventional mice hardly generate high-affinity monoclonal antibodies by the standard

procedures [36]. Yoshimura et al. successfully developed the high-affinity monoclonal antibody against PIVKA-II, which is named 3C10, using the GANP transgenic mice [37]. Then, a fully automated quantitative immunoassay for PIVKA-II was established.

In the chapter II, the following studies are presented to characterize the epitope of 3C10 and evaluate the reactivity to PIVKA-II of the developed assay: (1) to identify the 3C10 epitope, (2) to evaluate the dilution linearity and correlation with existing assay using MU-3 antibody, (3) to evaluate and compare the tube type equivalency with existing assay using MU-3 antibody [38].

In the chapter III, the development of the immunoassay for quantitative determination of PIVKA-II with ARCHITECT® analyzer is presented [39]. Currently, some existing PIVKA-II assays need the manual pretreatment step and cannot measure plasma samples. The developed assay is an automated immunoassay and has a high sensitivity against PIVKA-II in serum and plasma. I demonstrated its precision, sensitivity, linearity, high dose hook effect, tube type equivalency including serum and plasma tubes, assay interferences of potential interfering materials, and correlation with the existing assay using MU-3 antibody.

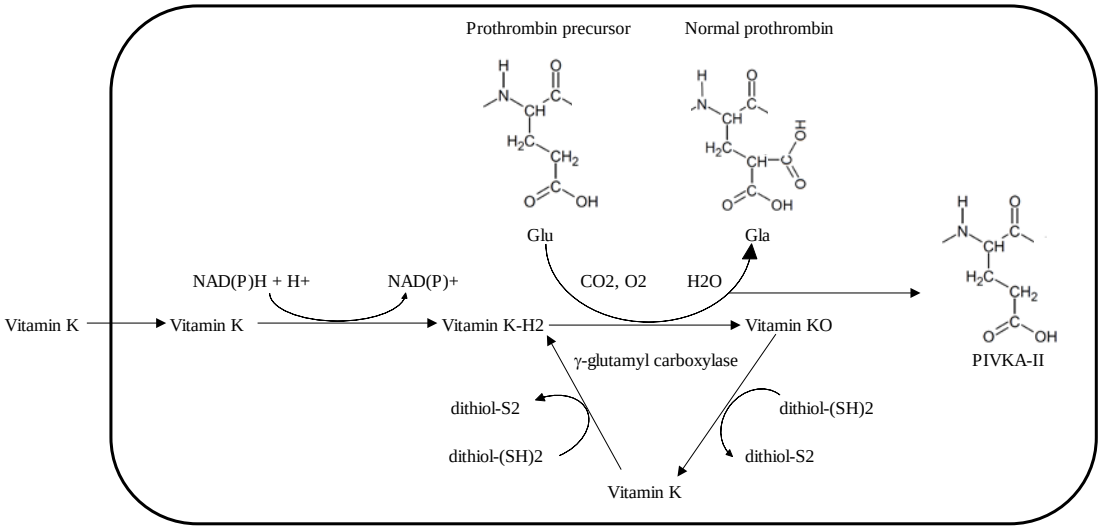


Figure 1. The production of normal prothrombin and PIVKA-II in hepatic cells

Chapter II:

Characterization of an anti-PIVKA-II antibody and evaluation of a fully automated chemiluminescent immunoassay for PIVKA-II

Summary

PIVKA-II has been used as a tumor marker to aid in the diagnosis of HCC. Yoshimura et al. [37] developed an anti-PIVKA-II monoclonal antibody, 3C10. I established a fully automated quantitative immunoassay for PIVKA-II on the ARCHITECT® i-systems using 3C10 monoclonal antibody. The aim of this study was to characterize the epitope of 3C10 and to evaluate the reactivity to PIVKA-II of this assay.

The epitope characterization was examined by using prothrombin Gla domain polypeptides which are amino acid residues 17–27 that include four Gla residues at positions 19, 20, 25 and 26. The correlation with Picolumi PIVKA-II MONO (Eidia, Tokyo, Japan) and tube type equivalency was evaluated by using the developed fully automated quantitative immunoassay.

Peptides having Glu at Gla domains strongly reacted to 3C10 but lost reactivity when the Glu at positions 19 or 20 was changed to Gla. The results were equivalent with an existing in vitro diagnostics product for PIVKA-II using the MU-3 antibody. A correlation study with the Picolumi PIVKA-II MONO gave a correlation coefficient of 0.99 and a

regression slope of 0.92. No difference between a plain serum tube and a rapid serum tube including thrombin (RST) was observed on ARCHITECT PIVKA-II.

The results demonstrate that this anti-PIVKA-II antibody detects equivalent epitopes with MU-3 and has equivalent reactivity to PIVKA-II as MU-3. Moreover, the ARCHITECT PIVKA-II assay has good correlation with the existing PIVKA-II product, and is applicable for use with RST.

Introduction

Liver cancer is the seventh most common cancer and the second cause of death from cancer worldwide. The incidence rate and mortality rate were 10.1 and 9.5 per 100,000 persons in 2012, respectively [1].

HCC is the major histologic type among primary liver cancers occurring worldwide, accounting for 70% to 85% of the total burden [2]. HCC can be treated by resection, liver transplantation, or local ablation with radiofrequency for patients diagnosed at an early stage [12]. However, AFP is the most commonly used tumor marker for HCC, its clinical diagnostic accuracy is unsatisfactory due to low sensitivity and specificity [13]. Therefore, there is an urgent need for developing better HCC-specific biomarkers. Several serological biomarkers have been suggested as other potential biomarkers for early detection of HCC. PIVKA-II, also known as DCP, is a useful marker for the diagnosis of HCC, and the combined measurement of PIVKA-II and AFP is superior to AFP alone [13, 14].

Prothrombin, a blood coagulation protein synthesized in the liver, is converted to an active form after the 10 Glu in the Gla domain at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 32 are γ-carboxylated to Gla by vitamin-K dependent γ-glutamyl carboxylase. In patients with HCC, γ-carboxylation of prothrombin is impaired so that PIVKA-II is formed instead of prothrombin [29]. Liebman et al. [16] first reported that the serum PIVKA-II concentration was significantly higher in patients with

HCC by radioimmunoassay using a polyclonal antibody. They purified PIVKA-II from ascites fluid of patients with HCC and analyzed its Gla content [30]. Then, Motohara et al. [31, 32] made the MU-3 monoclonal antibody against PIVKA-II and established an assay system with higher sensitivity. Clinical studies have demonstrated that the assay system using MU-3 antibody shows high sensitivity and specificity to PIVKA-II in patients with HCC [33]. PIVKA-II has been used as an aid for diagnosis of HCC in Japan.

Sugo et al. [34] reported that the reactivity of MU-3 antibody increased when the number of Gla was less than five per molecule. Furthermore, Naraki et al. [35] described the epitope of PIVKA-II reacting to MU-3 antibody. The epitope of MU-3 antibody was located within the amino acid residues 17–27 in the Gla domain. They reported that MU-3 antibody reacted to PIVKA-II with less than four Gla and PIVKA-II variants preferentially synthesized in HCC patients have less than four Gla whereas PIVKA-II variants in benign liver diseases have more than five Gla [35]. Yoshimura et al. developed the monoclonal antibody against PIVKA-II, 3C10 [37], and I established a fully automated quantitative immunoassay for PIVKA-II. The aim of this study was to characterize the epitope of 3C10 and to evaluate the reactivity to PIVKA-II of this assay.

Materials and methods

Preparation of 3C10 antibody

The 3C10 hybridoma cell line was prepared by immunization of mice with the synthesized PIVKA-II peptide 13–27 described by Yoshimura et al. [37]. Purification of 3C10 antibody was performed by using Protein G Sepharose 4 Fast Flow (GE Healthcare Japan, Tokyo, Japan). Analyses of the purified 3C10 antibody were carried out by SDS-PAGE, gel permeation chromatography (GPC), and Western blotting.

Preparation of PIVKA-II antigen

Human prothrombin (Enzyme Research Laboratories, South Bend, IN, USA) was dialyzed against 0.1M ammonium acetate solution and lyophilized. After the lyophilization, the prothrombin was heated for 8 hours at 105 °C for decarboxylation [40]. The decarboxylated prothrombin was purified by a 3C10 coated affinity column and a Superdex 200 column (GE Healthcare Japan, Tokyo, Japan). Analysis of the PIVKA-II antigen was carried out by GPC.

Assay principal of the automated chemiluminescent immunoassay for PIVKA-II on ARCHITECT

The ARCHITECT PIVKA-II assay is a two-step sandwich immunoassay, using chemiluminescent paramagnetic microparticle technology for quantitative determination of PIVKA-II. The ARCHITECT PIVKA-II

assay utilizes microparticles coated with 3C10 antibody which recognizes an epitope in PIVKA-II within the Gla domain at the N-terminus as the solid phase to capture PIVKA-II. The analyte-microparticle complex is detected with an acridinium labeled murine anti-prothrombin monoclonal antibody MCA 1–8 (Abbott Laboratories, IL, USA) conjugate, which recognizes an epitope in prothrombin part of the Gla domain (amino acid 33–46) shown in Figure 2.

Following a wash step, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). The RLUs detected by the ARCHITECT optical system are related to the amount of PIVKA-II Ag in the sample.

Gla domain polypeptide

Peptides used for epitope characterization were purchased from the Toray Research Center (Tokyo, Japan). The purity of all peptides was confirmed to be more than 95% by high performance liquid chromatography.

Specimens

The AFP positive sera for the dilution linearity study of the ARCHITECT PIVKA-II were obtained from ProMedDx, LLC (Norton, MA, USA). Serum specimens for the correlation of the ARCHITECT PIVKA-II with Picolumi PIVKA-II MONO were obtained from ProMedDx, LLC and Bioreclamation IVT (Hicksville, NY, USA). Normal matched

specimens from blood collected in a plain serum tube, a serum separator tube (SST) and a RST were obtained from ProMeDx, LLC. All human specimens used for this study were collected under institutional review board approved protocols.

Evaluation methods

The epitope characterization studies of 3C10 and MU-3 antibodies were examined with competing peptides against PIVKA-II antigens by the ARCHITECT PIVKA-II assay and Picolumi PIVKA-II MONO assay, respectively. The ten kinds of peptides prepared at various concentrations were mixed with 480 mAU/mL PIVKA-II, and inhibition by each peptide was measured by the ARCHITECT PIVKA-II and Picolumi PIVKA-II MONO assays.

Reactivity of 3C10 to the decarboxylated prothrombin and to native PIVKA-II from five individual serum specimens was tested in the dilution linearity study of ARCHITECT PIVKA-II assay. The decarboxylated prothrombin and native PIVKA-II adjusted to 30,000 mAU/mL were diluted to 30.03%, 9.02%, 2.71%, 0.81%, 0.24%, 0.07%, and 0.02% with the ARCHITECT PIVKA-II calibrator diluent and measured by ARCHITECT PIVKA-II assay. The values of coefficient of determination (R^2) for a linear regression were calculated.

Correlation of the ARCHITECT PIVKA-II with the Picolumi PIVKA-II MONO across the measuring range was evaluated using 119 serum specimens from various hepatic diseases including AFP positive patients,

liver cancer, non-alcoholic fatty liver disease, liver cirrhosis, hepatitis C, etc. Correlation was analyzed by using the Passing–Bablok regression method and the Spearman's correlation coefficient.

Sample type equivalency was evaluated with specimens drawn from 5 normal individuals using 3 types of blood collection tubes (plain serum tube, SST and RST). Both neat samples and samples spiked with 100 mAU/mL of PIVKA-II antigen were assayed by the ARCHITECT PIVKA-II and Picolumi PIVKA-II MONO assays. The PIVKA-II value of each sample was compared to the value of the corresponding plain serum tube sample. Statistical differences versus plain serum tube were evaluated using the paired t-test.

Data were analyzed using Analyse-It version 2.22 (Analyse-It Software Ltd., Leeds, UK).

Results

Characterization of the 3C10 antibody epitope

To identify the epitope of the 3C10 and MU-3 antibodies, the inhibition between PIVKA-II and the competing peptides against PIVKA-II antigens was tested by the ARCHITECT PIVKA-II and Picolumi PIVKA-II MONO assays. Ten peptides were used to analyze the epitope of 3C10 and MU-3 antibody. The amino acid sequences of the 10 peptides are shown in Table 1. There are four Gla residues (positions 19, 20, 25 and 26) between amino acid 17 to 27 in the Gla domain. I prepared 8 possible combinations (peptide-1 to 8) to convert each Gla to Glu in positions 19, 20 and 25. Furthermore, I prepared 4 possible combinations (peptide-1, 4, 9 and 10) to convert each Gla to Glu in positions 25 and 26 with Glu in positions 19 and 20.

The inhibition rates by peptide-1 to 8 on ARCHITECT PIVKA-II assay and Picolumi PIVKA-II MONO assay are shown in Figures 3 A and B. Peptide-1, where all 4 positions are Glu residues, most strongly inhibited the binding of 3C10 and MU-3 to PIVKA-II antigen. The half maximal Inhibitory Concentrations (IC50) to 3C10 and MU-3 by peptide-1 were 0.84 μM and 0.98 μM, respectively. Peptide-4, with Gla at position 25, showed slightly lower inhibition to 3C10 and MU-3 than peptide-1. The IC50 to 3C10 and MU-3 by peptide-4 were 3.10 μM and 5.75 μM, respectively. Peptide-2 and 3, with Gla at positions 19 and 20, respectively, weakly inhibited the reaction to 3C10 and MU-3. Peptides 5,

6, 7, and 8 contain 2 or more Gla residues and did not inhibit the reaction to 3C10 and MU-3. The inhibition rates by peptide 1–8 to 3C10 were similar with the inhibition to MU-3. These results showed that at least one Glu at positions 19 or 20 is essential for 3C10 antibody binding and the further addition Glu at position 25 enhances the 3C10 antibody binding. Moreover, the 3C10 binding affinities to the peptides were equivalent to the MU-3 binding affinities.

The inhibition rates by peptide-1, 4, 5, 8, 9 and 10 on ARCHITECT PIVKA-II assay and Picolumi PIVKA-II MONO assay are shown in Figures 3 C and D. Peptide-9 strongly inhibited the reaction to 3C10 and MU-3. The inhibition rates were equivalent with peptide-1. The IC50 to 3C10 and MU-3 by peptide-9 were 1.10 μM and 1.49 μM, respectively. Peptide-10 showed slightly lower inhibition to 3C10 and MU-3 than peptide-9. The inhibition rates were equivalent with peptide-4. The IC50 to 3C10 and MU-3 by peptide-10 were 4.95 μM and 9.23 μM, respectively. These results showed that the further addition Glu at position 26 has no effect on the 3C10 antibody binding as well as the MU-3 antibody binding.

Reactivity of 3C10 antibody to decarboxylated prothrombin and to native PIVKA-II in serum specimens

The dilution linearity study of the ARCHITECT PIVKA-II was examined by using decarboxylated prothrombin and native PIVKA-II from five individual patients. The linearity of this assay is shown in Figure 4.

The R^2 value for a linear regression through the measurement range of ARCHITECT PIVKA-II assay of the decarboxylated prothrombin was 0.985. The R^2 values of native PIVKA-II were from 0.976 to 0.982.

Correlation between the Picolumi PIVKA-II MONO and the ARCHITECT PIVKA-II assay

I evaluated the correlation between the ARCHITECT PIVKA-II assay and the Picolumi PIVKA-IIMONO assay (existing PIVKA-II product in Japan). The result is shown in Figure 5. The regression slope calculated using the Passing–Bablok regression method was 0.92. The Spearman's correlation coefficient was 0.99.

Evaluation of tube type equivalency between plain serum tube, SST and RST

I evaluated the value differences by using three types of serum tubes. The value differences of the neat samples and samples spiked with 100 mAU/mL of PIVKA-II antigen between plain serum tube and the two other serum tubes on ARCHITECT PIVKA-II and Picolumi PIVKA-II MONO assays are shown in Figure 6. The mean differences of SST and RST versus plain serum on ARCHITECT PIVKA-II were 1% and 4%, respectively. The mean differences of SST and RST versus plain serum on Picolumi PIVKA-IIMONO were −4% and −16%, respectively. The differences of SST and RST versus plain serum on ARCHITECT PIVKA-II and the difference of SST versus plain serum on Picolumi

PIVKA-II MONO were non-significant. However the PIVKA-II level using RST on Picolumi PIVKA-II MONO was significantly lower than PIVKA-II level using the plain serum tube.

Discussion

In this study, I characterized the epitope of 3C10 antibody that was developed by immunization with the synthesized peptide 13–27 of PIVKA-II. To identify the epitope in Gla domain of PIVKA-II that is detected by 3C10, ten combinations of changes of the four Gla to Glu amino acid residues in the Gla domain were evaluated. The results showed that binding to 3C10 was significantly decreased when one or both of the Glu at positions 19 and 20 was changed to Gla. Therefore, the Glu at positions 19 or 20 are essential for 3C10 antibody binding. The addition of Glu at position 25 enhanced the 3C10 antibody binding. The further addition of Glu at position 26 is no effect on the 3C10 antibody binding. The inhibition results to 3C10 using ten peptides were equivalent with those for MU-3. The inhibition results to MU-3 were similar to the results reported by Naraki et al. [35]. The results indicate that the epitope of 3C10 antibody is equivalent to that of the MU-3 antibody.

The 3C10 antibody showed equivalent reactivity to decarboxylated prothrombin and to native PIVKA-II from the suspected patient with HCC through the measurement range of ARCHITECT PIVKA-II assay. These results indicate that the 3C10 antibody binds to native PIVKA-II in the patient serum equivalent to a decarboxylated prothrombin. I evaluated the correlation between ARCHITECT PIVKA-II using the 3C10 antibody and Picolumi PIVKA-II MONO using the MU-3 antibody by using liver

diseases specimens including patients with liver cancer. The slope and correlation coefficient were good. From these results, the ARCHITECT PIVKA-II assay using 3C10 antibody gave similar detection of PIVKA-II in clinical specimens to the existing PIVKA-II assays using theMU-3 antibody. However, the numbers of tested clinical specimens were limited in this study. A more extensive clinical study of the ARCHITECT PIVKA-II assay should be performed in the future.

In Japan, the PIVKA-II assay has been commonly used as an aid of diagnosis and monitoring for the patients with HCC. However, some commercial available PIVKA-II assays have limitations to the usage of blood collection tubes. I performed the tube type study by using three kinds of serum tubes on ARCHITECT PIVKA-II and Picolumi PIVKA-IIMONO assays. In these results, no differences between serum tube types were observed on the ARCHITECT PIVKA-II assay, whereas the PIVKA-II levels collected by RST were significantly decreased on Picolumi PIVKA-II MONO assay. Thrombin cleavages in human prothrombin were identified at Arg^{51}-Thr^{52}/Arg^{54}-Asp^{55}, Arg^{155}-Ser^{156}, and Arg^{284}-Thr^{285} [41–43]. Petrovan et al. [44] reported that the rate of cleavage at Arg^{51}-Thr^{52}/Arg^{54}-Asp^{55}, Arg^{155}-Ser^{156}, and Arg^{284}-Thr^{285} is greatly affected by the presence of calcium ions. In the absence of calcium ions, thrombin relatively efficiently proteolyzes peptide bonds at Arg^{51}-Thr^{52}/Arg^{54}-Asp^{55} and Arg^{155}-Ser^{156}. Church et al. [45] reported the cleavage of prothrombin by thrombin at Arg^{155}-Ser^{156} is inhibited in the presence of calcium ions, and the protective effect has been attributed to

conformational changes mediated by calcium ions binding to the Gla domain. It was reported that the Gla domain in PIVKA-II does not bind to calcium ions [46]. Thrombin may cleavages the peptide bonds at Arg^{51}-Thr^{52}/Arg^{54}-Asp^{55} and Arg^{155}-Ser^{156} on PIVKA-II. The ARCHITECT PIVKA-II uses two different antibodies each which can recognize a peptide in the Gla domain. Therefore, the assay is not significantly affected by the PIVKA-II cleavages by proteases. The differences of PIVKA-II levels collected by RST versus serum plain tube between ARCHITECT PIVKA-II and Picolumi PIVKA-II MONO might be caused by the PIVKA-II cleavage by thrombin.

In conclusion, the results demonstrate that the anti-PIVKA-II antibody 3C10 has equivalent epitopes and reactivity to PIVKA-II with MU-3. Moreover, the fully automated ARCHITECT PIVKA-II assay using 3C10 has good correlation with existing PIVKA-II product, and is applicable to a rapid serum tube.

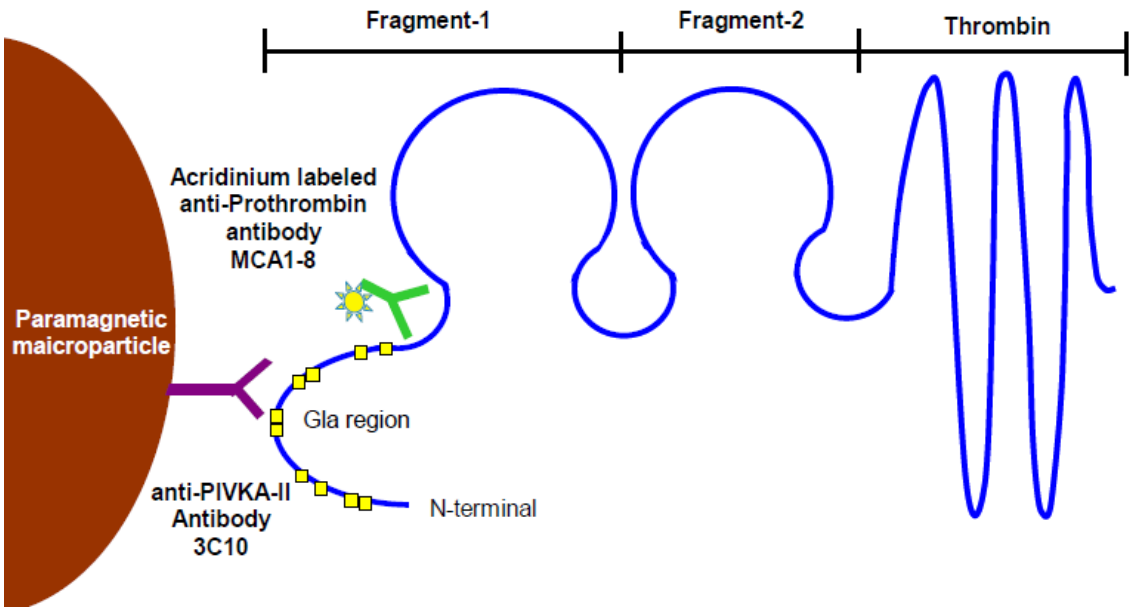
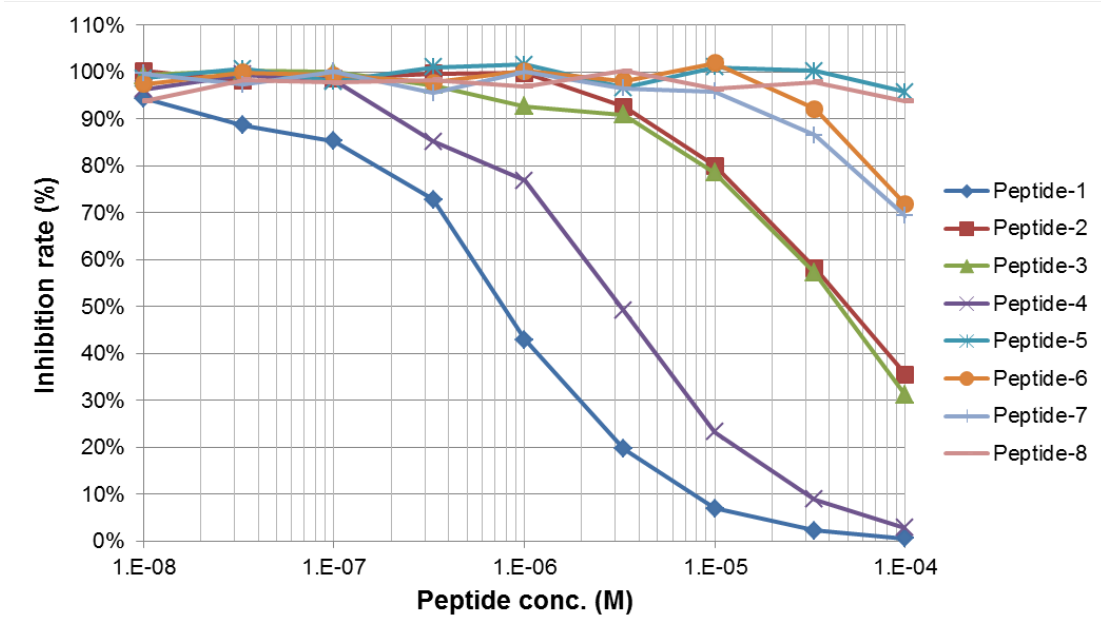


Figure 2 Assay principal of the ARCHITECT PIVKA-II.

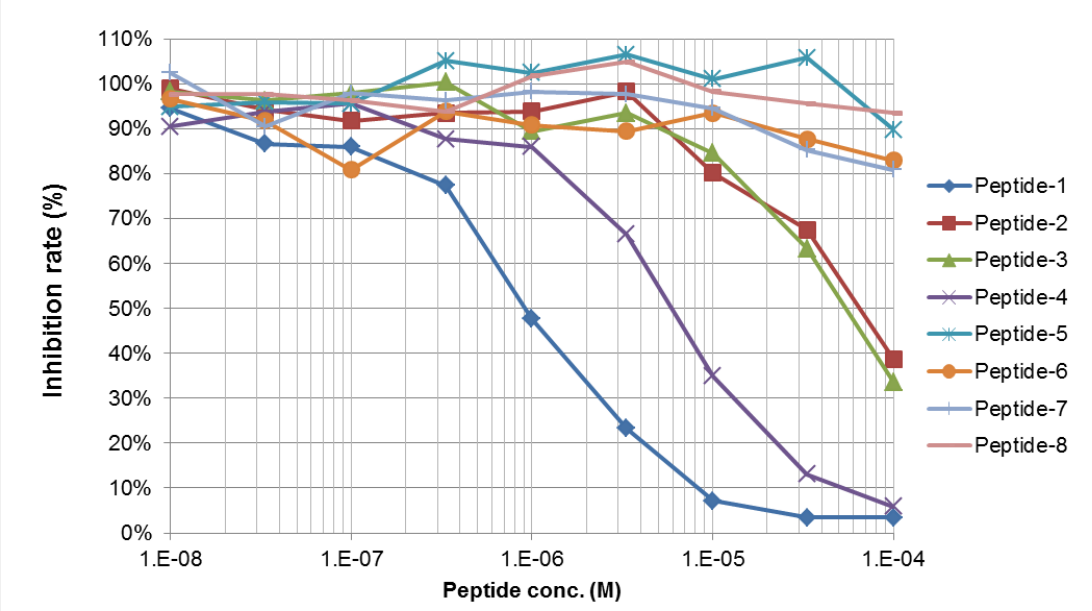
Table 1　Amino acid sequences of the Gla domain and peptides used for epitope determination.　Gla residues are denoted by γ.

Peptide	17	18	19	20	21	22	23	24	25	26	27
	┌──── SS ────┐										
Peptide-1	C	V	E	E	T	C	S	Y	E	E	A
	┌──── SS ────┐										
Peptide-2	C	V	γ	E	T	C	S	Y	E	E	A
	┌──── SS ────┐										
Peptide-3	C	V	E	γ	T	C	S	Y	E	E	A
	┌──── SS ────┐										
Peptide-4	C	V	E	E	T	C	S	Y	γ	E	A
	┌──── SS ────┐										
Peptide-5	C	V	γ	γ	T	C	S	Y	E	E	A
	┌──── SS ────┐										
Peptide-6	C	V	E	γ	T	C	S	Y	γ	E	A
	┌──── SS ────┐										
Peptide-7	C	V	γ	E	T	C	S	Y	γ	E	A
	┌──── SS ────┐										
Peptide-8	C	V	γ	γ	T	C	S	Y	γ	γ	A
	┌──── SS ────┐										
Peptide-9	C	V	E	E	T	C	S	Y	E	γ	A
	┌──── SS ────┐										
Peptide-10	C	V	E	E	T	C	S	Y	γ	γ	A

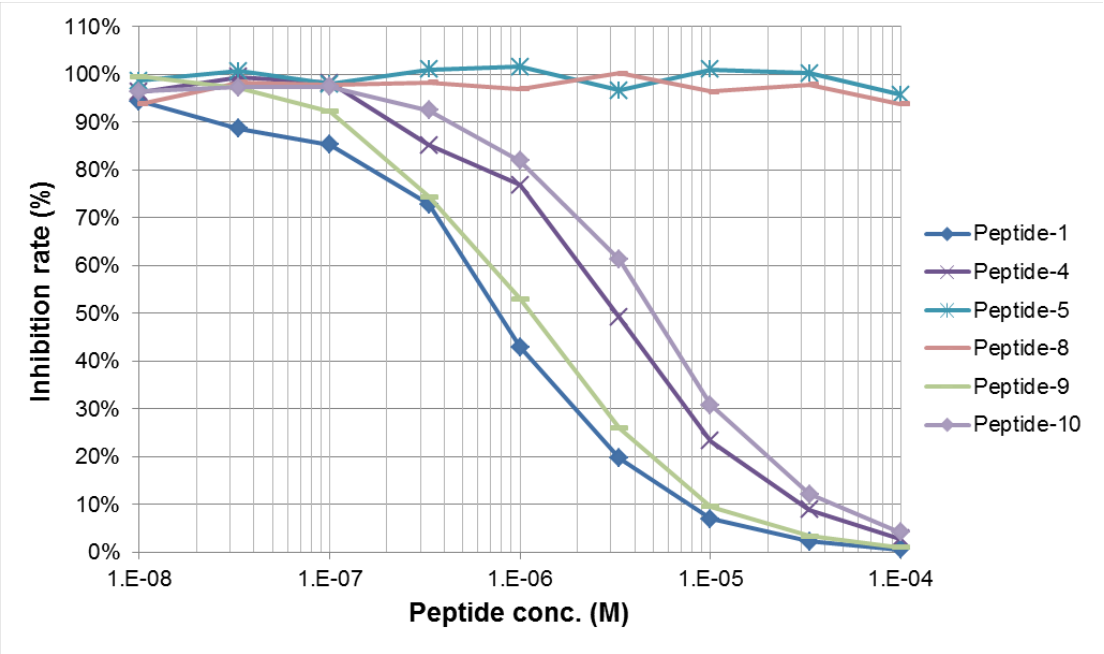
A.


110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Inhibition rate (%)
1.E-08
1.E-07
1.E-06
1.E-05
1.E-04
Peptide conc. (M)
Peptide-1
Peptide-2
Peptide-3
Peptide-4
Peptide-5
Peptide-6
Peptide-7
Peptide-8

B.


110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Inhibition rate (%)
1.E-08
1.E-07
1.E-06
1.E-05
1.E-04
Peptide conc. (M)
Peptide-1
Peptide-2
Peptide-3
Peptide-4
Peptide-5
Peptide-6
Peptide-7
Peptide-8

C.



D.

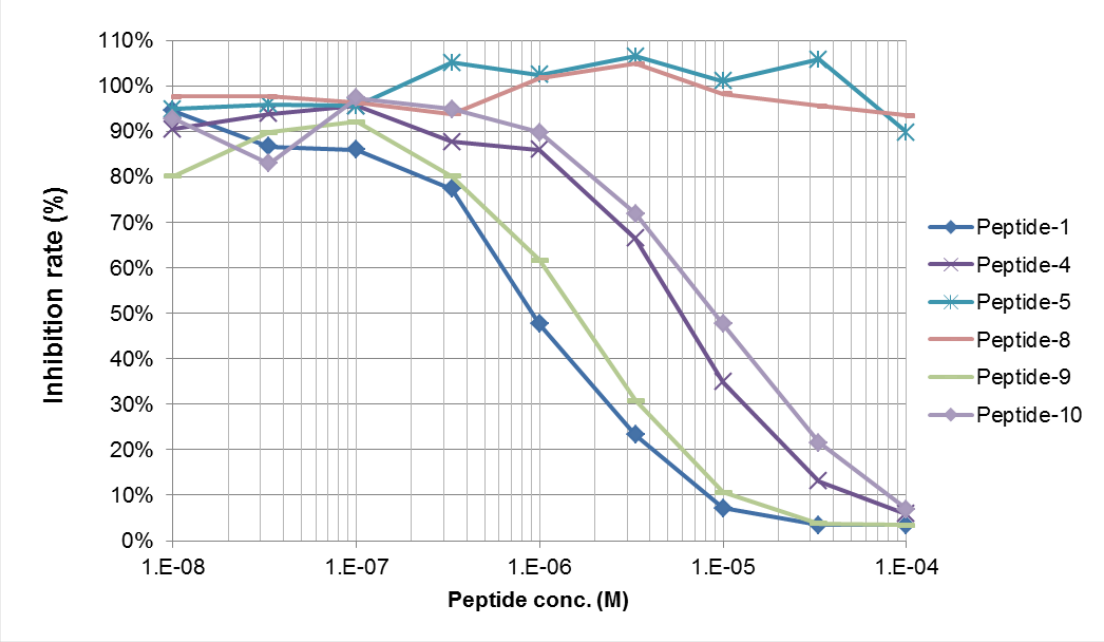


Figure 3　Inhibition of 3C10 andMU-3 antibody reactivity to PIVKA-II by various peptides with residues at positions 19, 20, 25 and 26 having different Gla and Glu.　PIVKA-II was mixed with the peptide 1–8, and the

inhibition rate to anti-PIVKA-II antibody by each peptide was measured by ARCHITECT PIVKA-II assay (A) and Picolumi PIVKA-IIMONO (B). PIVKA-II was mixed with the peptide 1, 4, 5, 8, 9 and 10, and the inhibition rate to anti-PIVKA-II antibody by each peptide was measured by ARCHITECT PIVKA-II assay (C) and Picolumi PIVKA-IIMONO (D).

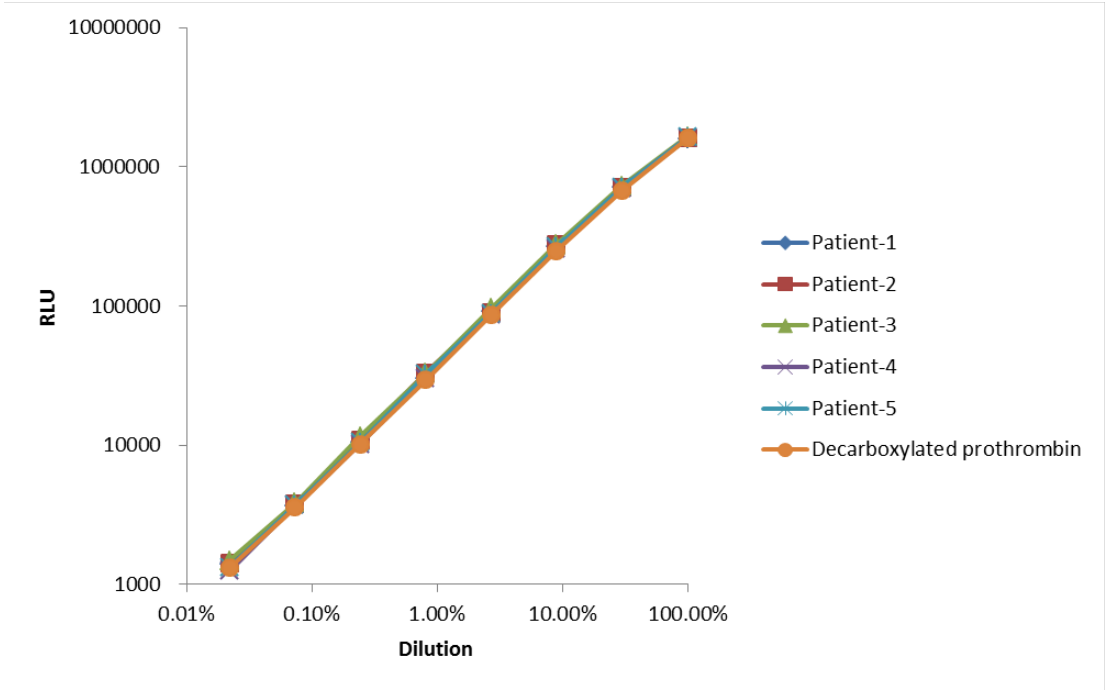


Figure 4 Reactivity of 3C10 to the decarboxylated prothrombin and to native PIVKA-II from five serum specimens by the dilution linearity study of ARCHITECT PIVKA-II assay. The R^2 values of patient-1, 2, 3, 4, 5, and decarboxylated prothrombin were 0.976, 0.979, 0.979, 0.982, 0.982, and 0.985, respectively.

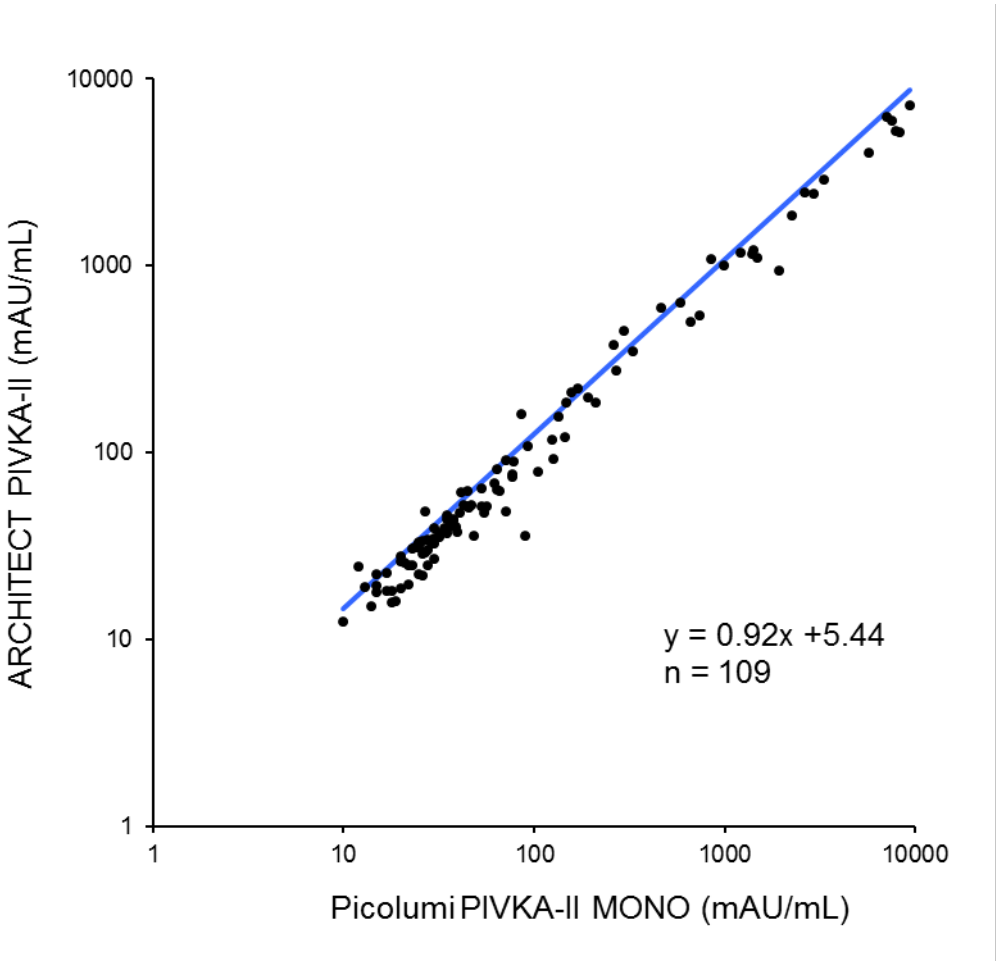


Figure 5 Correlation between the Picolumi PIVKA-II MONO and the ARCHITECT PIVKA-II assay.

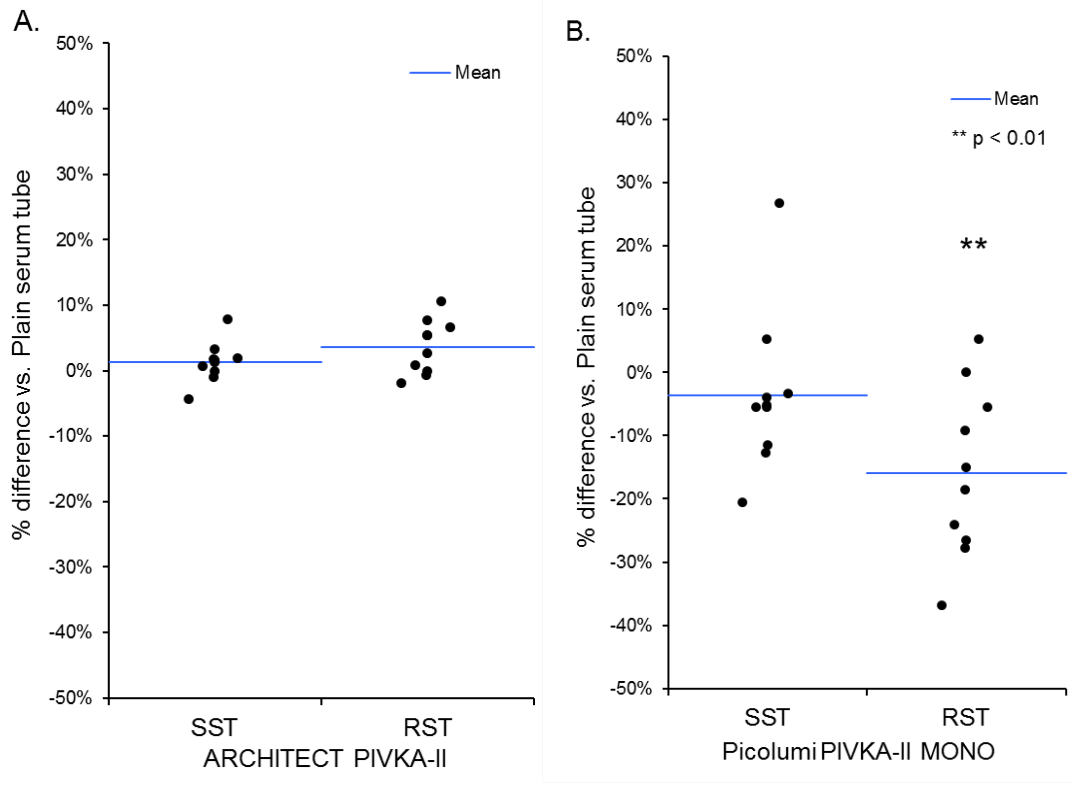


Figure 6　Evaluation of tube type equivalency between plain serum tube, SST and RST.　The neat samples and spiked samples which were added 100 mAU/mL of PIVKA-II antigen into each specimen were assayed by the ARCHITECT PIVKA-II (A) and Picolumi PIVKA-II MONO (B).　The PIVKA-II value of each sample was compared to the value of the corresponding plain serum tube sample.　The p-value of the statistical differences versus plain serum was calculated by the paired t-test.

Chapter III:

Development and evaluation of analytical performance of a fully automated chemiluminescent immunoassay for protein induced by vitamin K absence or antagonist II

Summary

PIVKA-II, an abnormal form of prothrombin, has been used as an aid in the diagnosis of HCC as a tumor marker. I developed a fully automated quantitative immunoassay for PIVKA-II on the ARCHITECT® i systems. The aim of this study was to evaluate the analytical performance of this assay.

Assay imprecision, sensitivity, dilution linearity, high dose hook effect, sample type equivalency, assay interferences of potential interfering materials and correlation with Picolumi PIVKA-II (Eidia, Tokyo, Japan) were evaluated.

The percentage coefficient of variation (%CV) of total imprecision ranged from 2.8% to 5.4% with 10 levels of samples. The limit of blank (LoB), limit of detection (LoD), and limit of quantitation (LoQ) were less than 0.63mAU/mL, 1.62mAU/mL, and 8.25mAU/mL, respectively. Linearity up to 30,000mAU/mL, no high dose hook effect, no difference among sample types and no interference of common drugs and endogenous substances were observed. Correlation study with the Picolumi PIVKA-II

gave a correlation coefficient of 0.93 and a regression slope of 1.07.

The results demonstrate that the fully automated prototype ARCHITECT PIVKA-II assay is an accurate, highly sensitive and precise assay for the measurement of PIVKA-II levels in human sera and plasmas.

Introduction

Liver cancer is the fifth most common cancer in men and the seventh in women but is the third leading cause of cancer death, because of its high fatality rate (mortality is 93% of incidence) [1]. About 85% of the burden is in developing counties with more than 50% in China alone. The regions with high incidence of liver cancer are West and Central Africa, and East and Southeast Asia. In contrast, incidence rates are generally low in developed countries except Japan and Southern Europe [1]. HCC is the major histologic type among primary liver cancers occurring worldwide, accounting for 70% to 85% of total burden [2].

Chronic infection by either HBV or HCV is a major risk factor for HCC. Approximately 80% of HCC is estimated to be attributable to HBV or HCV worldwide [3]. Carcinogenic risk of HBV carriers is 223-fold that of non-carriers [4]. Infection by HCV is the most common predisposing factor in some developed countries, including Japan [5].

Recently in some historical high risk regions, liver cancer rates decreased, possibly due to a reduction in HBV infection by improvement of hygiene and sanitation conditions. In contrast, incident rates are increasing in some developed areas, including the United State and Central Europe, possibility due to the obesity epidemic and the rise in HCV infection through continued transmission by injection drug users [47]. During the past three decades from 1980 to 2010, the overall age adjusted incidence rate of liver cancer in the United State has tripled from 2.63 to

8.07 per 100,000 persons [48].

One of the reasons for the high fatality rate of HCC is the difficulty of detection for HCC in early stage since it is an asymptomatic disease, therefore many patients are not treated in the early stages. Early detection of HCC by surveillance in high risk groups is an appropriate way to improve the survival rate in patients with HCC [6–11].

PIVKA-II, also known as DCP, is an abnormal form of the coagulation protein, prothrombin. PIVKA-II is a functionally defective prothrombin resulting from decline of the carboxylation of 10 glutamic acid residues at the N-terminus [15].

The level of PIVKA-II is elevated in patients with HCC. PIVKA-II level is not correlated with the levels of AFP or AFP-L3, which are other representative biomarkers for HCC. Therefore PIVKA-II can be used along with AFP or AFP-L3 as a complementary biomarker for HCC. Thus, the combination of two markers enhances the sensitivity of diagnosis of HCC, but minimizes the decrease in specificity [15–18]. The Japan Society of Hepatology recommends measurement of two or more tumor markers for the diagnosis of small HCC [19].

Many studies showed serum PIVKA-II levels are related to tumor size, vascular invasion, intrahepatic metastasis and frequency of recurrence after treatment, so PIVKA-II also can be used as a prognostic predictor in patients with HCC [20–24]. PIVKA-II may assume a crucial role to decide the most effective therapy according to tumor characterization.

PIVKA-II has been used as an aid for diagnosis of HCC and monitoring

of high risk patients (HCV infections, hepatitis/cirrhosis, and HBV infections) for development of HCC for more than 20 years in Japan [19].

I report here the development and evaluation of the analytical performance of a fully automated immunoassay for PIVKA-II on the ARCHITECT i systems (Abbott Japan, Tokyo, Japan).

Materials and methods

Assay principle

The ARCHITECT PIVKA-II assay is a two-step immunoassay, using chemiluminescent paramagnetic microparticle technology for quantitative determination of PIVKA-II. The analyte, PIVKA-II, is captured by paramagnetic microparticles coated with a recombinant murine monoclonal anti-PIVKA-II antibody 3C10 (Abbott Laboratories, IL, USA), which recognizes an epitope in PIVKA-II within the Gla domain (amino acid 13–27) at the N-terminus. The analyte microparticle complex is detected with an acridinium labeled murine anti-prothrombin monoclonal antibody MCA 1–8 (Abbott Laboratories) conjugate, which recognizes an epitope at the N-terminus in prothrombin (Figure 7).

In the first step, 30 μl of sample, 50 μl of assay buffer and 50 μl of anti-PIVKA-II antibody coated microparticles are combined and incubated for 18 min. After washing, 50 μl of acridinium labeled anti-prothrombin antibody conjugate is added and incubated for 4 min. Following an additional wash step, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as RLUs. The RLUs detected by the ARCHITCT optical system is related to the amount of PIVKA-II Ag in the sample. The assay is fully automated and an assay result can be obtained within 30 min after initial sample aspiration. The measuring range of this assay was designed from 10 to 30,000 mAU/mL. Calibrators were prepared from PIVKA-II antigen

diluted in buffer solution containing bovine serum albumin. The calibrator levels are 0, 40, 100, 300, 5000 and 30,000 mAU/mL.

PIVKA-II antigen

PIVKA-II antigen was prepared from human prothrombin (Enzyme Research, IN, USA) by a thermal decarboxylation method described by Bajaj et al. [40]. PIVKA-II value of the antigen was determined by comparison to the Picolumi PIVKA-II Assay (Eidia, Tokyo, Japan).

Specimens

AFP positive specimens were obtained from ProMedDx, LLC (MA, USA). Normal specimens from apparently healthy individuals were obtained from ProMeDx, LLC, C-C Biotech (CA, USA) and Denka Seiken Co., Ltd. (Tokyo, Japan). All human specimens used for this study were collected under institutional review board approved protocols.

Evaluation methods

Assay imprecision was evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guideline EP5-A3 [49]. Three levels of buffer based PIVKA-II quality controls, four levels of serum based panels and three levels of plasma based panels were assayed in replicates of three at two separate times per day for over 20 days using three lots of reagents and four instruments including three different versions (two i1000SR, one i2000SR and one i2000). A high plasma

panel spiked with PIVKA-II antigen at 75,000 mAU/mL level was tested using the 1:10 auto-dilution protocol.

The LoB, LoD and LoQ were determined according to the CLSI guideline EP17-A2 [50]. Four zero-concentration samples and eight low concentration PIVKA-II samples (0.625, 1.25, 2.5, 5, 7.5, 9, 10, 15 mAU/mL) were assayed in replicates of two during five different days using three reagent lots and five instruments (two i1000SR and three i2000). LoB was set as the 95th percentile of the zero-concentration samples' values. For LoD calculation, normality of the distribution of the lowest concentration PIVKA-II sample which 95% replicate showed over LoB was confirmed using the Shapiro–Wilk test. After the confirmation, LoD was calculated using the equation: $LoD = LoB + (C\beta * SDS)$, where $C\beta = 1.645 / [1 - 1 / (4 * f)]$, f = degrees of freedom of SDS, SDS = standard deviation (SD) of the low concentration samples. LoQ was set as the lowest concentration that showed a total error of 30% (10% bias + 2 × 10% CV). LoB, LoD and LoQ were calculated from merged data of all reagent lots on each instrument.

Reagent on-board stability studies were performed with three lots of reagents using nine instruments. Three levels of buffer based PIVKA-II quality controls, four levels of serum based panels and three levels of plasma based panels were assayed in replicates of five at 20 test points using reagents stored on-board the instrument during 30 days. A calibration curve was established on the initial day and concentration of each sample was determined using the stored calibration curve.

Concentrations of the samples were evaluated for trending over time. Dilution linearity was assessed according to the CLSI guideline EP06-A [51] with two serum and one plasma specimens spiked with PIVKA-II antigen. The specimens were diluted from 100% to 0.01% with the ARCHITECT PIVKA-II calibrator diluent across the measuring range. Regression analyses of the observed diluted concentrations were compared to expected values based on the corresponding concentration of the undiluted specimen.

High dose hook effect was assessed by testing of high level PIVKA-II samples beyond the measuring range of the assay. The high level PIVKA-II samples were prepared from pooled serum spiked with PIVKA-II antigen to 60,000, 100,000, 200,000, 300,000, 400,000, and 600,000mAU/mL. All samples were assayed in replicates of five using four instruments (three i2000 and one i1000SR). Mean RLU of each sample was compared with mean RLU of Calibrator-F, the highest level calibrator.

Sample type equivalency was evaluated with specimens drawn from 16 normal individuals using seven types of blood collection tubes (plain serum tube (Terumo, Tokyo, Japan), SST tube (Becton, Dickinson and Company (BD), NJ, USA), EDTA K2 tube (Terumo), EDTA Na2 tube (Terumo), lithium heparin tube (Terumo), lithium heparin PST tube (BD) and sodium heparin tube (Terumo)). The 20 mAU/mL or 200 mAU/mL of PIVKA-II antigen was spiked into each specimen and assayed in replicates of three. The mean of the PIVKA-II value of each sample was compared to mean

value of the corresponding plain serum tube sample.

Assay interferences of potential interfering materials including endogenous substances, nutritional supplements and therapeutic agents were evaluated based on the CLSI guideline EP7-A2 [52]. Each material was diluted with appropriate diluents and spiked into the five low level serum samples and five high level serum samples prepared by spiking with a high titer specimen. The diluent used for the preparation of the spiked materials was spiked into the low level and high level samples in the same manner for use as controls. All samples were assayed in replicates of twelve. The mean of the PIVKA-II value of each test sample was compared to mean value of the corresponding control sample.

Correlation of the prototype ARCHITECT PIVKA-II with Picolumi PIVKA-II across the measuring range was evaluated using 81 AFP positive sera and 151 normal sera. Statistical analyses for the correlation were performed using Analyse-it version 2.30 (Analyse-it Software, Ltd., Leeds, UK).

Results

Performance evaluations of the ARCHITECT PIVKA-II assay

The total imprecision determined with each sample type and instrument type is shown in Table 2. The %CV was between 2.8 and 5.4 over the investigated concentration range from 20.28 to 78,800.74 mAU/mL.

The LoB, LoD and LoQ ranged from0.00 to 0.63, from0.73 to 1.61 and from 2.38 to 8.25 mAU/mL, respectively (Table 3).

The reagent on-board stability results showed no significant change of sample concentrations. The 95% confidence interval of concentration shift% from initial day to 30 days was 2.0% to 3.2%.

The linearity of this assay over the range of 3.85 to 39,991.70 mAU/mL using three samples is shown in Figure 8-A. The Spearman's correlation coefficient between expected values and observed values of each sample was 1.00. The recovery of diluted samples ranged from 89% to 105%.

No high dose hook effect was observed in the testing conditions, up to 600,000 mAU/mL (Figure 8-B). Higher concentration samples gave higher RLU and no samples showed lower RLU than Calibrator-F RLU (30,000 mAU/mL, upper limit of the measuring range).

The value differences between plain serum and various types of specimens are shown in Table 4-a. The mean percentage differences of each specimen type ranged from −0.4% to 3.7%.

The effects of potential interfering materials are shown in Table 4-b. The mean percentage differences between test samples and control samples

ranged from -6.4% to 7.2%.

Correlation between the Picolumi PIVKA-II and the ARCHITECT PIVKA-II assay

The correlation between the Picolumi PIVKA-II and the prototype ARCHITECT PIVKA-II is shown in Figure 9. The regression slope calculated using the Passing-Bablok regression method was 1.07. The Spearman's correlation coefficient was 0.93.

Discussion

In the present study, the analytical performance of ARCHITECT PIVKAII assay was evaluated. The assay demonstrated good precision and high sensitivity. There is variability in LoQ (2.38–8.25 mAU/mL), but the highest LoQ was less than 10 mAU/mL against the reported cut-off of 40mAU/mL [53]. The good linearity over the measuring range, no hook effect up to 600,000 mAU/mL and no interference of common therapeutic agents, nutritional supplements and endogenous substances were confirmed. I demonstrated that the ARCHITECT PIVKA-II Assay can be used with various types of serum and plasma samples. The correlation data showed the prototype ARCHITECT PIVKA-II gives equivalent PIVKA-II values to those of the Picolumi PIVKA-II, the regulatory approved PIVKA-II assay in Japan.

In Japan, PIVKA-II has been commonly employed as a HCC specific tumor marker. The Japan Society of Hepatology recommended the measurement of tumor markers combined with ultrasonography at intervals of 2–6 months for regular screening in high risk patients with type C chronic liver disease, type B chronic liver disease and cirrhosis [19]. Since PIVKA-II, AFP and AFP-L3 are complementary biomarkers, measurement of two or more tumor markers is useful for a precise diagnosis of HCC [15-18]. The National Health Insurance in Japan covers measurements of AFP, PIVKA-II and AFP-L3. In contrast, outside Japan only ultrasonography and AFP are commonly used for

monitoring of the high risk patients and there are no guidelines regarding PIVKA-II usage for diagnosis of HCC so far.

Although prompt determination of the PIVKA-II level is important for an appropriate treatment decision, it has not been achieved so far even in Japan. Because the instrument used for the most predominant assay kit for PIVKA-II in Japan is not prevalent in clinics and hospitals. Therefore most samples are shipped to and assayed in commercial clinical laboratories which take several days to report the assay results after the initial sample collection. Because the second major assay kit, Lumipulse PIVKA-II, in Japan can't use plasma samples, collected blood samples must be held for more than 30 minutes to allow the serum to clot, it is difficult to obtain the assay result before medical treatment for ambulatory in a routine medical care process within a day.

Although human prothrombin is digested to several sub-fragments by serine proteases activated in the coagulation cascade [41], the assay can detect PIVKA-II in both serum and plasma without the influence of degradation of analyte and the interference by the sub-fragments because both antibodies used for the ARCHITECT PIVKA-II assay recognize epitopes at the N-terminus in PIVKA-II.

Current assay kits for PIVKA-II on market have some weaknesses. Picolumi kit needs manual dilution steps for sample preparation and Lumipulse and μTAS Wako DCP can't be used for plasma samples. The ARCHITECT PIVKA-II assay is a convenient fully automated assay which has a good analytical performance with high throughput (200 tests / hour)

without the pretreatment of specimens. The ARCHITECT PIVKA-II assay was designed to be used with various types of plasma as well as serum samples. Since the incubation time for coagulation is not required for plasma samples, the assay results can be obtained earlier than serum samples after the sample collection. These features allow to test and obtain the test result before medical treatment in a day when ambulatory patients visit.

This assay provides a convenient automated method for measurement of PIVKA-II in both serum and plasma in clinics, hospitals and clinical laboratories. It can support the global use of PIVKA-II for management of HCC and the early detection of HCC in high risk groups.

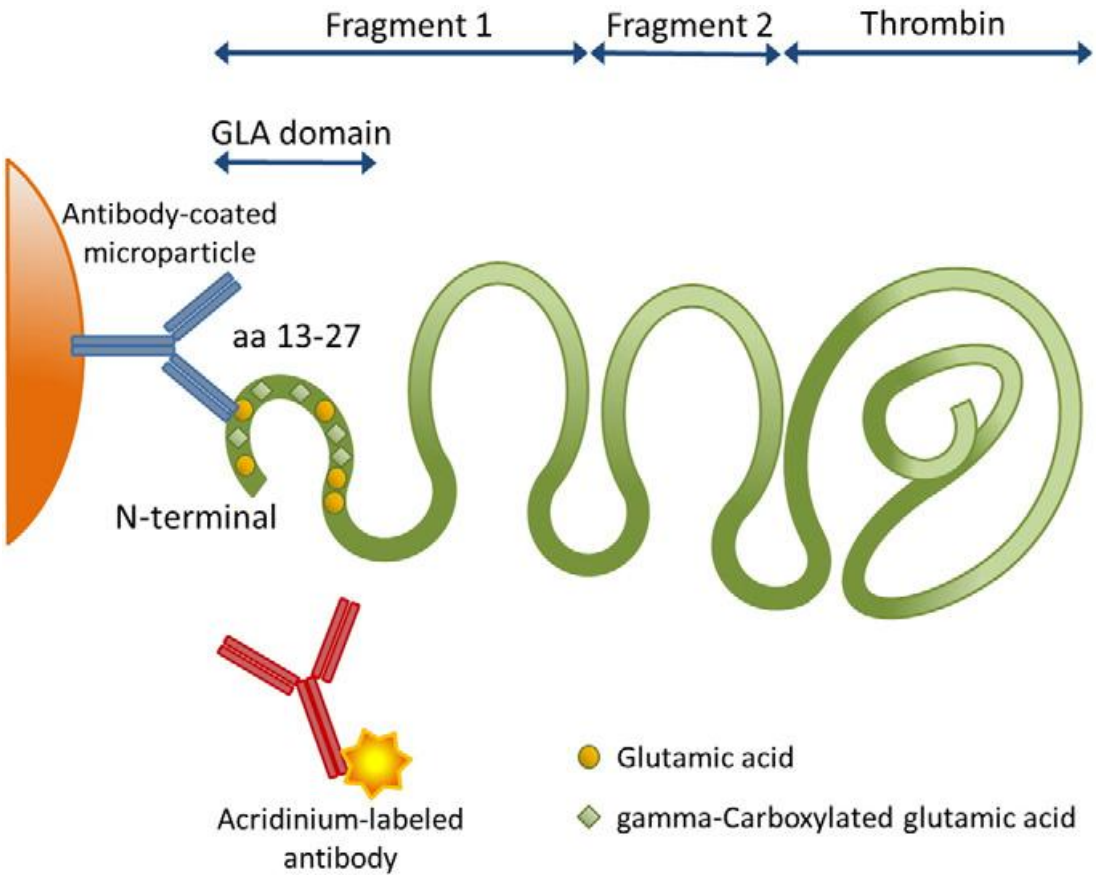


Figure 7. Assay principal of the prototype Architect PIVKA-II assay. Capture antibody recognize an epitope in the Gla domain (amino acid (aa) 13–27), tracer antibody recognize an epitope in the prothrombin part.

Table 2 Total assay imprecision (%CV).

n = 120

Sample	Instrument type	*i*1000$_{SR}$			*i*1000$_{SR}$			*i*2000			*i*2000$_{SR}$		
	Reagent lot number	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
Quality control-1	Mean (mAU/mL)	49.39	51.22	48.52	48.58	47.48	49.04	52.20	47.91	48.67	50.92	49.12	50.01
	SD (mAU/mL)	2.07	1.62	1.83	2.08	1.68	2.06	1.73	1.70	1.83	1.63	1.50	1.47
	CV	4.2%	3.2%	3.8%	4.3%	3.5%	4.2%	3.3%	3.5%	3.8%	3.2%	3.1%	2.9%
Quality control-2	Mean (mAU/mL)	484.59	512.08	470.81	478.84	484.71	475.68	490.87	471.31	477.95	488.49	487.93	496.88
	SD (mAU/mL)	18.56	16.56	14.70	16.51	17.88	19.14	14.94	15.00	13.50	13.90	13.90	14.75
	CV	3.8%	3.2%	3.1%	3.4%	3.7%	4.0%	3.0%	3.2%	2.8%	2.8%	2.8%	3.0%
Quality control-3	Mean (mAU/mL)	9779.61	9810.52	9392.19	9807.30	10,020.86	9505.93	9988.98	9284.40	9441.06	9877.19	9591.38	10,273.99
	SD (mAU/mL)	388.25	358.94	312.28	368.43	413.75	385.90	486.79	374.25	271.04	530.93	441.68	398.87
	CV	4.0%	3.7%	3.3%	3.8%	4.1%	4.1%	4.9%	4.0%	2.9%	5.4%	4.6%	3.9%
Serum panel-1	Mean (mAU/mL)	20.43	22.10	21.38	20.63	20.70	22.29	21.67	20.28	21.18	22.15	21.49	22.51
	SD (mAU/mL)	0.92	0.97	0.90	1.00	1.06	1.03	1.04	0.88	0.85	1.01	0.91	0.98
	CV	4.5%	4.4%	4.2%	4.8%	5.1%	4.6%	4.8%	4.3%	4.0%	4.6%	4.2%	4.4%
Serum panel-2	Mean (mAU/mL)	43.76	46.85	45.52	44.27	44.49	46.75	45.97	43.59	45.08	46.89	45.46	47.17
	SD (mAU/mL)	1.66	1.53	1.38	1.79	1.85	1.68	1.33	1.75	1.61	1.85	1.87	1.66
	CV	3.8%	3.3%	3.0%	4.0%	4.1%	3.6%	2.9%	4.0%	3.6%	3.9%	4.1%	3.5%
Serum panel-3	Mean (mAU/mL)	243.29	253.92	247.70	247.88	253.26	256.67	245.00	236.37	244.29	252.81	250.30	257.94
	SD (mAU/mL)	7.98	7.75	8.91	9.45	8.04	8.26	6.95	7.67	7.30	7.56	8.13	8.38
	CV	3.3%	3.1%	3.6%	3.8%	3.2%	3.2%	2.8%	3.2%	3.0%	3.0%	3.2%	3.2%
Serum panel-4	Mean (mAU/mL)	1934.50	1980.87	1909.98	1984.26	2048.24	1982.26	1922.53	1875.93	1911.39	1940.36	1974.14	2041.62
	SD (mAU/mL)	57.55	64.10	55.73	71.37	69.50	59.05	61.05	59.63	61.92	66.57	69.08	68.79
	CV	3.0%	3.2%	2.9%	3.6%	3.4%	3.0%	3.2%	3.2%	3.2%	3.4%	3.5%	3.4%
Plasma panel-1	Mean (mAU/mL)	26.33	28.51	28.49	26.52	26.60	29.63	27.42	25.84	27.92	27.95	26.92	29.08
	SD (mAU/mL)	1.03	1.36	1.04	1.11	1.32	1.14	1.22	1.06	1.20	1.16	1.09	1.17
	CV	3.9%	4.8%	3.6%	4.2%	5.0%	3.9%	4.4%	4.1%	4.3%	4.2%	4.0%	4.0%
Plasma panel-2	Mean (mAU/mL)	251.77	264.19	256.71	256.86	260.46	265.01	254.59	246.51	257.36	261.02	259.16	268.43
	SD (mAU/mL)	7.17	8.16	10.30	7.62	9.27	8.22	8.27	7.25	7.93	8.07	9.43	8.02
	CV	2.8%	3.1%	4.0%	3.0%	3.6%	3.1%	3.2%	2.9%	3.1%	3.1%	3.6%	3.0%
Plasma panel-3	Mean (mAU/mL)	77,267.12	78,785.77	74,362.72	78,800.74	81,700.78	76,808.19	75,779.80	71,850.25	72,301.50	75,424.25	76,125.15	78,788.15
	SD (mAU/mL)	2302.29	2864.63	2426.01	2505.25	2351.07	2837.68	3225.02	2580.41	2507.28	3713.16	3626.03	2888.44
	CV	3.0%	3.6%	3.3%	3.2%	2.9%	3.7%	4.3%	3.6%	3.5%	4.9%	4.8%	3.7%

CV: coefficient of variation, SD: standard deviation.

Table 3 LoB, LoD and LoQ using 5 instruments.

Instrument type/number	LoB (mAU/mL)	LoD (mAU/mL)	LoQ (mAU/mL)
*i*2000-1	0.58	1.61	6.92
*i*2000-2	0.34	1.17	4.91
*i*2000-3	0.37	0.85	2.38
$i1000_{SR}$-1	0.00	0.73	8.25
$i1000_{SR}$-2	0.63	1.37	4.81

LoB: limit of blank, LoD: limit of detection, LoQ: limit of quantitation.

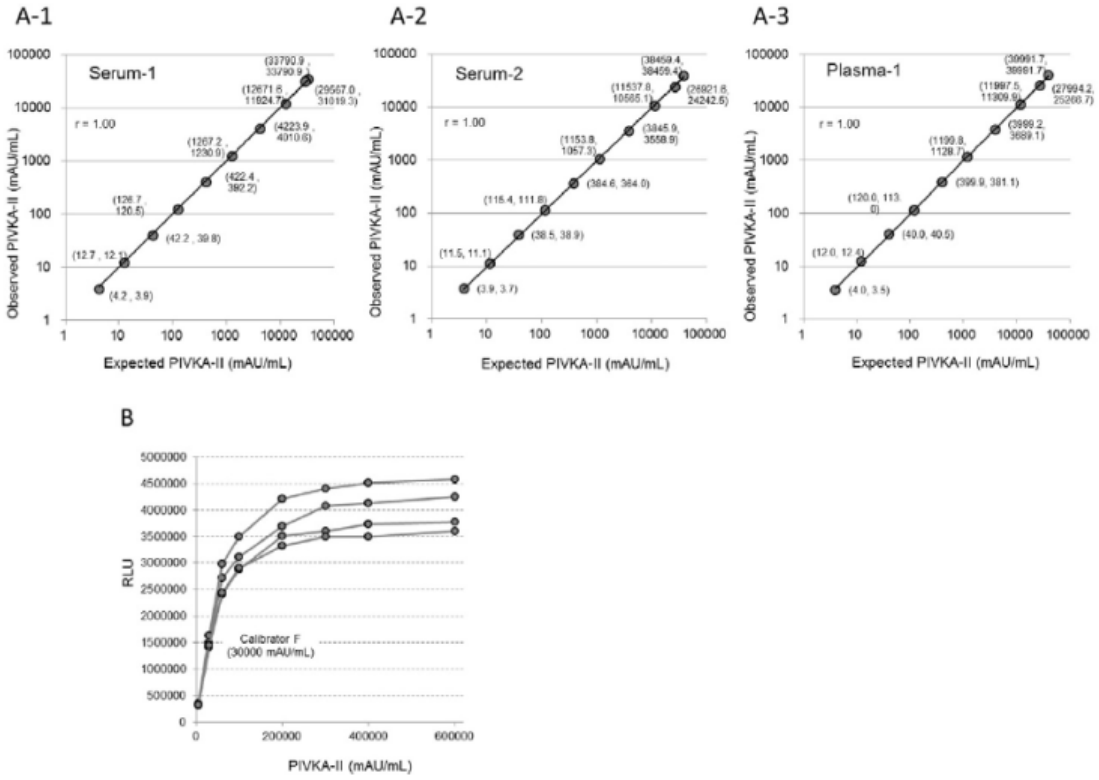


Figure 8. Evaluation of linearity and high dose hook effect.

A) Relationship between expected PIVKA-II concentrations calculated from dilution ratio and neat samples' concentration and observed concentrations in three samples (A-1 and A-2: serum samples, A-3: plasma sample).

B) Relationship between PIVKA-II concentrations and relative light units (RLUs) on 4 instruments. Higher concentration gave higher RLU up to 600,000 mAU/mL.

Table 4 Evaluation of sample type equivalence and potential interfering materials including endogenous substances, nutritional supplements and therapeutic agents.

a) Sample type equivalence

n = 48 (16 donors × 3 replicates) per condition

Specimen type	Spiked PIVKA-II concentration	% difference from plain tube serum	
		Mean	95% confidence interval
SST serum	20 mAU/mL	2.1%	0.1% to 4.1%
	200 mAU/mL	3.7%	1.6% to 5.8%
EDTA K2 plasma	20 mAU/mL	1.5%	−0.6% to 3.6%
	200 mAU/mL	1.1%	−1.0% to 3.2%
EDTA Na2 plasma	20 mAU/mL	0.4%	−1.3% to 2.1%
	200 mAU/mL	0.9%	−0.8% to 2.6%
Li Heparin plasma	20 mAU/mL	−0.4%	−2.1% to 1.3%
	200 mAU/mL	1.8%	0.0% to 3.6%
Li Heparin (PST) plasma	20 mAU/mL	0.8%	−1.0% to 2.6%
	200 mAU/mL	2.6%	1.0% to 4.2%
Na Heparin plasma	20 mAU/mL	1.0%	−0.7% to 2.7%
	200 mAU/mL	3.1%	1.4% to 4.8%

b) Potential interfering materials

n = 60 (5 donors × 12 replicates) per condition

Potentially interferents	Concentration	PIVKA-II low level (20 mAU/mL)		PIVKA-II high level (200 mAU/mL)	
		% Difference from control		% Difference from control	
		Mean	95% confidence interval	Mean	95% confidence interval
Bilirubin (conjugated)	20 mg/dL	−2.0%	−3.5% to −0.5%	−1.6%	−2.3% to −0.9%
Bilirubin (unconjugated)	20 mg/dL	1.2%	−1.9% to 4.3%	0.8%	−1.4% to 3.0%
Hemoglobin	500 mg/dL	7.2%	5.8% to 8.6%	5.2%	3.8% to 6.6%
Prothrombin	15 mg/dL	2.2%	−1.2% to 5.6%	0.0%	−1.5% to 1.5%
Total protein	12 g/dL	−4.0%	−6.9% to −1.1%	−6.4%	−7.5% to −5.3%
Triglycerides	3000 mg/dL	2.4%	−0.2% to 5.0%	2.6%	−0.5% to 5.7%
Acetaminophen	600 μg/mL	1.6%	−0.1% to 3.3%	−1.2%	−2.2% to −0.2%
Acetylsalicylic acid	500 μg/mL	1.0%	−0.5% to 2.5%	−1.8%	−6.5% to 2.9%
Ascorbate	500 μg/mL	−0.2%	−3.8% to 3.4%	0.2%	−2.0% to 2.4%
Cisplatin	7.24 μg/mL	−1.0%	−4.9% to 2.9%	−0.2%	−1.8% to 1.4%
Fluorouracil	100 μg/mL	−2.2%	−6.3% to 1.9%	−0.4%	−3.4% to 2.6%
Galactose	2 mg/mL	−2.0%	−4.9% to 0.9%	0.6%	−0.8% to 2.0%
Glucose	10 mg/mL	−2.2%	−4.6% to 0.2%	−0.1%	−1.6% to 1.4%
Ibuprofen	400 μg/mL	−2.0%	−6.9% to 2.9%	−0.4%	−3.0% to 2.2%
Interferon α	3000 IU/mL	1.8%	−0.2% to 3.8%	0.6%	−0.5% to 1.7%
Interferon β	3000 IU/mL	−1.8%	−4.6% to 1.0%	0.4%	−1.3% to 2.1%
Interferon γ	3000 IU/mL	0.8%	−1.2% to 2.8%	0.0%	−0.9% to 0.9%
Vitamin B1	140 μg/mL	−2.3%	−5.1% to 0.5%	−0.6%	−2.7% to 1.5%
Vitamin B6	500 μg/mL	−2.2%	−4.9% to 0.5%	−0.2%	−1.8% to 1.4%
Vitamin B12	250 μg/mL	−0.2%	−3.9% to 3.5%	−0.6%	−2.0% to 0.8%

a) Evaluation of sample type.
b) Evaluation of potential interfering materials.

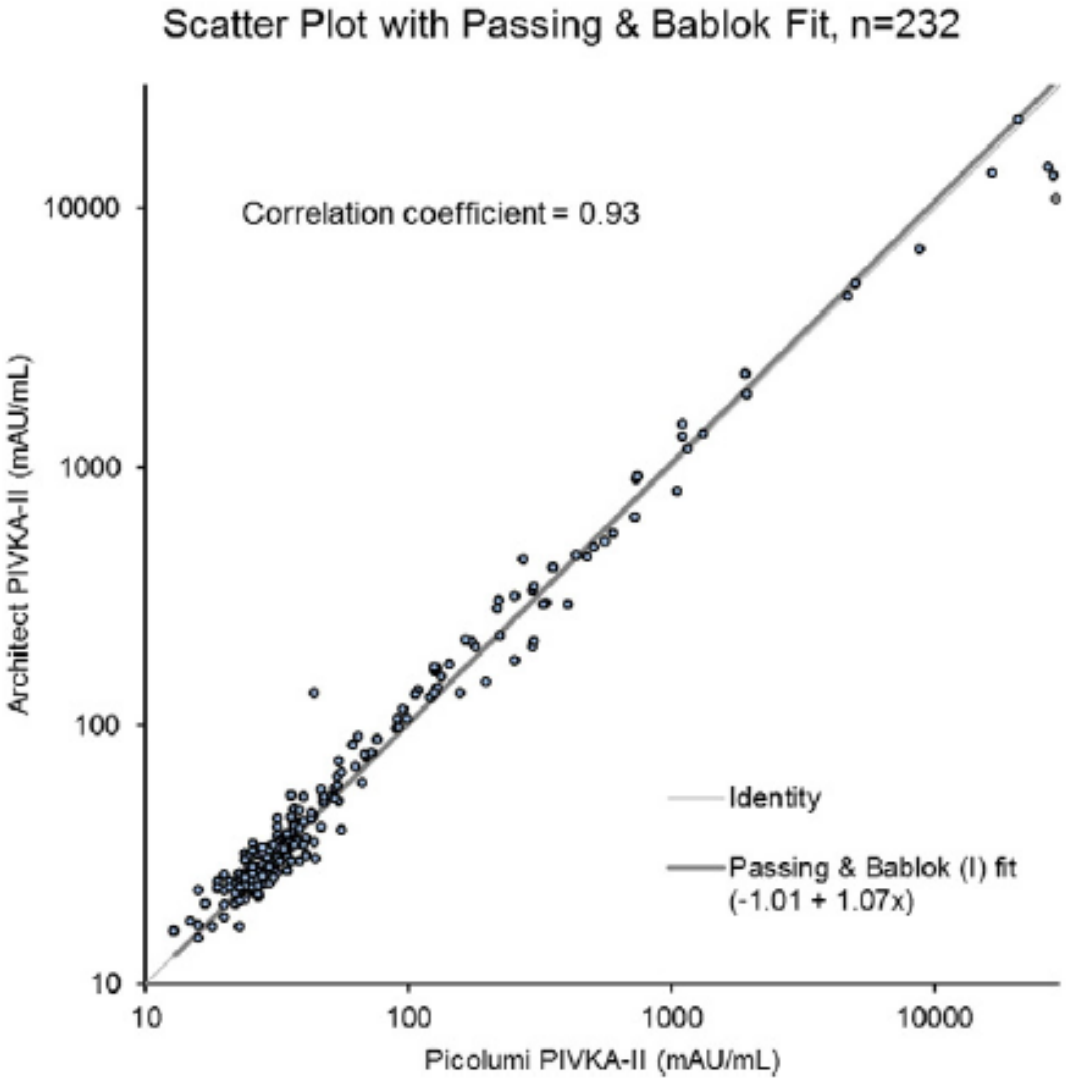


Figure 9. Correlation between the Japan-approved PIVKA-II assay kit, Picolumi PIVKA-II, and the Architect PIVKA-II assay.

Chapter IV:

Conclusion Remarks

I characterized the epitope of 3C10 antibody that was developed by immunization with the synthesized peptide 13–27 of PIVKA-II. The results showed that the Glu at positions 19 or 20 are essential for 3C10 antibody binding. The addition of Glu at position 25 enhanced the 3C10 antibody binding. The results indicate that the epitope of 3C10 antibody is equivalent to that of the MU-3 antibody. Also, the results of the linearity and correlation with the existing assay using MU-3 antibody indicate that the 3C10 antibody binds to native PIVKA-II in the patient serum equivalent with MU-3 antibody.

Some commercial available PIVKA-II assays have limitations to the usage of blood collection tubes. Although human prothrombin is digested to several sub-fragments by serine proteases activated in the coagulation cascade [39], the ARCHITECT PIVKA-II assay can detect PIVKA-II without the influence of degradation of analyte and the interference by the sub-fragments because the assay is designed using two different antibodies each which can recognize a peptide in the Gla domain. Therefore, the limitations for tube types were improved.

The fully automated ARCHITECT PIVKA-II assay demonstrated good precision and high sensitivity. The good linearity over the measuring range, no hook effect up to 600,000 mAU/mL and no interference of

common therapeutic agents, nutritional supplements and endogenous substances were confirmed. I demonstrated that the ARCHITECT PIVKA-II Assay can be used with various types of serum and plasma samples. The correlation data showed the prototype ARCHITECT PIVKA-II gives equivalent PIVKA-II values to those of the existing PIVKA-II assay in Japan.

In Japan, PIVKA-II has been commonly employed as a HCC specific tumor marker. The Japan Society of Hepatology recommended the measurement of tumor markers including PIVKA-II combined with ultrasonography at intervals of 2–6 months for regular screening in high risk patients with type C chronic liver disease, type B chronic liver disease and cirrhosis [19]. The National Health Insurance in Japan covers measurements of AFP, PIVKA-II and AFP-L3. In contrast, outside Japan only ultrasonography and AFP are commonly used for monitoring of the high risk patients and there are no guidelines regarding PIVKA-II usage for diagnosis of HCC so far.

Current assay kits for PIVKA-II on market have some weaknesses. The some existing assays need manual dilution steps for sample preparation and some assays have a limitation of measurement for plasma samples. The ARCHITECT PIVKA-II assay is a convenient fully automated assay which has a good analytical performance with high throughput (200 tests / hour) without the pretreatment of specimens. The ARCHITECT PIVKA-II assay was designed to be used with various types of plasma as well as serum samples. These features allow to test and

obtain the test result before medical treatment in a day when ambulatory patients visit.

This assay provides a convenient automated method for measurement of PIVKA-II in both serum and plasma in clinics, hospitals and clinical laboratories. The ARCHITECT PIVKA-II has been launched worldwide including Japan in 2015. The assay has been used as an aid in the diagnosis of HCC and in monitoring of high risk patients for development of HCC. It can support the global use of PIVKA-II for management of HCC and the early detection of HCC in high risk groups.

Acknowledgments

The authors thank the following for their contributions. For technical support in the 3C10 antibody, ARCHITECT PIVKA-II assay development and support for the evaluation: the assay development teams at Abbott Japan, Denka Seiken and Abbott Laboratories.

For critical discussions: Akiyoshi Fukamizu, PhD, Life Science Center, Tsukuba Advanced Research Alliance, University of Tsukuba.

The following two articles which were published on the Clinical Biochemistry, ELSEVIER are quoted by this thesis.

[38] Epitope characterization of an anti-PIVKA-II antibody and evaluation of a fully automated chemiluminescent immunoassay for PIVKA-II http://www.sciencedirect.com/science/article/pii/S0009912015003975

[39] Development and evaluation of analytical performance of a fully automated chemiluminescent immunoassay for protein induced by vitamin K absence or antagonist II http://www.sciencedirect.com/science/article/pii/S0009912015003033

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