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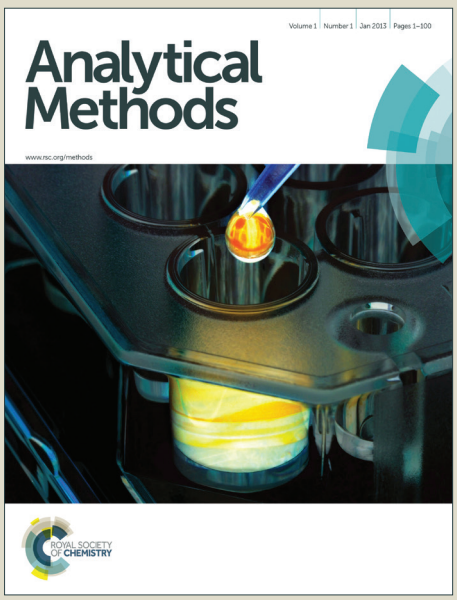

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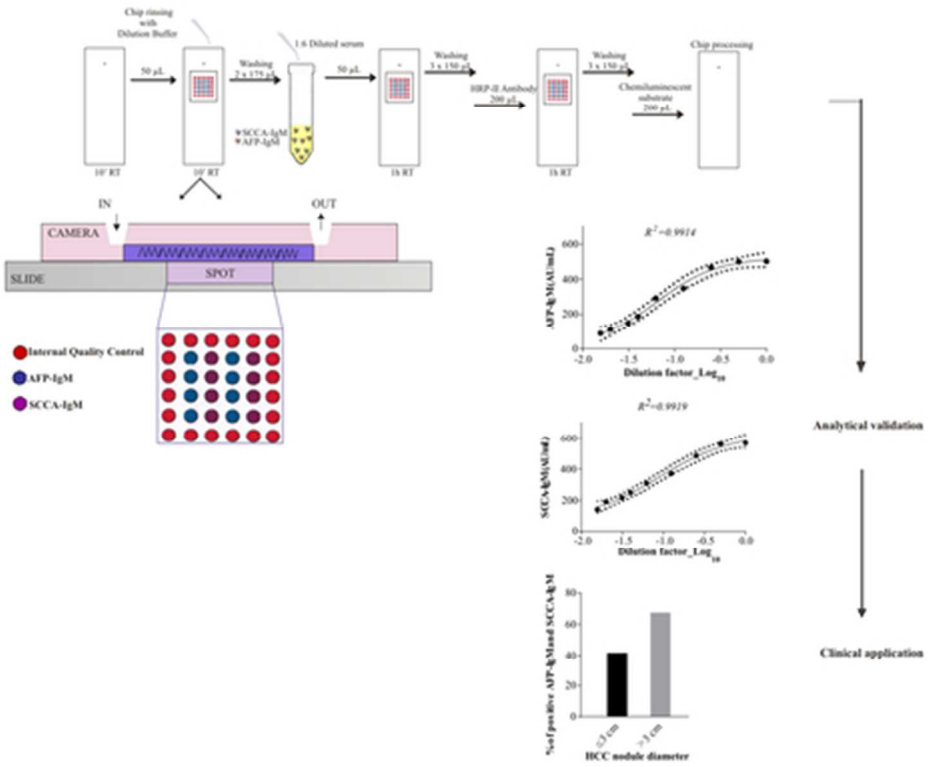

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This contribution demonstrates that a novel Biochip with ink-jet spotted antibodies for the chemiluminescent detection of AFP-IgM and SCCA-IgM is analytically reliable and has potential clinical application.

39x32mm (300 x 300 DPI)

Full Title: Analytical Validation of a Biochip prototype for integrated analysis of AFP-IgM and SCCA-IgM serum biomarkers in patients with liver cirrhosis and hepatocellular carcinoma.

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This contribution demonstrates that a novel Biochip with ink-jet spotted antibodies for the chemiluminescent detection of AFP-IgM and SCCA-IgM is analytically reliable and has potential clinical application.

ABSTRACT

Aim: This study evaluates the analytical and clinical performances of a new technology, CompleXima HCC Biochip, for the simultaneous serum measurement of alpha-fetoprotein-IgM (AFP-IgM) and squamous cell carcinoma antigen-IgM (SCCA-IgM).

Methods: AFP- and SCCA-IgM were measured by both ELISA and CompleXima HCC Biochip in 39 blood donors and in 174 patients (102 liver cirrhosis -LC- and 72 hepatocellular carcinoma -HCC-).

Results: The intra-assay coefficients of variation were lower than 12% and inter-assay were comprised between 14% and 21%. The linearity interval for CompleXima HCC Biochip was 50-300 AU/mL for AFP-IgM and SCCA-IgM. The comparison between the prototype and the ELISA test was studied by using Bland-Altman method and Passing-Bablok regression analyses. Passing-Bablok showed that the Biochip under-estimated AFP-IgM (Intercept A: -165.06; 95% CI: -313.11 to -51.32) and overestimated SCCA-IgM (Intercept A: 26.83; 95% CI: 14.47-35.86) with respect to ELISAs. Both biomarkers were higher in LC and HCC with respect to controls ($p<0.001$) with no difference between LC and HCC ($p=0.864$ for AFP-IgM and $p=0.214$ for SCCA-IgM). The thresholds for AFP-IgM and SCCA-IgM were calculated by means of ROC curves, fixing the specificity at 95%. Sensitivity for AFP-IgM and SCCA-IgM associated with CompleXima in detecting patients with liver diseases were 47% and 46%, respectively. The combined evaluation of macrocomplexes with CompleXima in diagnosing HCC with respect to LC was associated with a sensitivity of 51.4% and a specificity of 48%.

Conclusions: AFP-IgM and SCCA-IgM increase in chronic liver disease. The prototype CompleXima HCC Biochip allows their measuring with a good analytical reproducibility.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth most common cancer in the world (about 700000 new cases yearly) and the third cause of cancer-related death[1]. Although non-alcoholic steatohepatitis associated with obesity, insulin resistance, and type 2 diabetes has recently emerged as a risk factor for HCC[2], in more than 90% of the cases HCC develops within an established chronic liver disease, namely liver cirrhosis (LC). The most relevant causes of HCC, viral hepatitis B and C and alcohol abuse, in most instances first determine LC, which might evolve in established HCC through a multistep process including early stage neoplasia (early HCC)[3-7]. Since a delayed diagnosis of HCC is associated with a worse prognosis[8], the need of identifying patients with HCC as earlier as possible is advised in order to improve treatment options and ameliorate survival.

The association between LC and HCC represents the basis for preventive strategies, which contemplate only one biomarker, serum alpha-fetoprotein (AFP). AFP is a 70 kDa oncofetal antigen proposed in the mid '60 as HCC tumor marker[9]. In normal adults' sera low levels of AFP are detectable. AFP increases during pregnancy[10] but the highest levels are found in the presence of HCC, supporting its use as HCC tumor marker, as stated by several European associations for the study of the liver (EASL, ESMO, EGTM) and by the US National Academy for Clinical Biochemistry (NACB)[1,11]. However, the limitations in both sensitivity and specificity of AFP have prompted the American Association for the Study of Liver Diseases (AASLD) to rule out AFP from current guidelines for HCC[12,13].

The discovery and clinical validation of new sensitive and specific biomarkers for the early detection of HCC is strongly advised to overcome AFP limitations or improve AFP clinical utility[11-13]. Among the most promising biomarkers of HCC, squamous cell carcinoma antigen (SCCA), a 45-55 kDa member of the serpin family of ovalbumin-serine protease inhibitors, deserves major interest. Two main variants of this antigen have been correlated with HCC, SCCA1, originally identified in squamous cell carcinoma of the uterine cervix[14],

and SCCA2. These SCCA isoforms are produced by two tandemly arranged genes located on chromosome 18q21[15]; they share a 98% and 92% homology in nucleotide and amino acid sequences, their main difference being found in the reactive site loop (RSL) amino acid sequence responsible for the proteolytic activity. Overall, SCCA is expressed in stratified squamous epithelia and it is over-expressed in squamous cell carcinoma[16]. In HCC patients, the high expression levels of SCCA found in tumoral, not in peritumoral tissues, and in sera have prompted us to suggest this marker as useful for the detection of this cancer type[17,18].

Both AFP and SCCA are detectable in HCC sera as free antigens, but, as demonstrated for other tumor markers in different cancer settings, they may enter in IgM macrocomplexes[19-27]. The pathophysiological mechanism underlying the appearance in cancer patients' sera of tumor antigen-IgM macrocomplexes is not completely understood, although it has been suggested that they might be the expression of a physiological mechanism aimed at clear tumor antigens, mainly when they are abnormally glycosylated and/or at high levels[28,29]. Irrespective of the pathophysiological significance of macrocomplexes, in HCC AFP-IgM in association with AFP determination was suggested to be useful for early diagnosis[23,30] and for tumor size prediction[31], while SCCA-IgM serum levels were shown to have a prognostic role in patients with chronic HCV-related hepatitis[32] and were suggested to be HCC risk predictors in patients with LC[33]. More recently serum SCCA-IgM values were proposed to be useful for prognosis, since low values were correlated with a lower HCC risk[34] and a longer overall survival[35].

The serum determination of IgM macrocomplexes is actually performed by established ELISA assays, and recently our group demonstrated that SCCA-IgM ELISA determination is not affected by endogenous immunoglobulins, such as IgM with rheumatoid factor activity, supporting the reliability of this detection method[36]. However, to be cost-effective ELISA assays should be run when the number of samples to be analysed completely fullfill the microtiter ELISA plate. The specific clinical context for which AFP-IgM and SCCA-IgM

serum determinations appear to be appropriate might restrict the number of samples to be analysed, expecially among non-referral centres. As a consequence the time spent to collect enough samples before performing ELISA determinations might increase the overall turn around time thus unmeeting the clinical needs. A laboratory system which allows individual sample handling providing a reliable and simultaneous measurement of AFP-IgM and SCCA-IgM macrocomplexes in a short time, appears therefore advised. Nanotechnology may be a reliable tool to address this issue.

In recent years the introduction of nanotechnology in diagnostics provided several miniaturized devices which allow the simultaneous detection of hundreds to thousands of targets, including nucleic acids and proteins[37]. Micro-array systems, mainly designed to detect nucleic acids, such as the FDA approved Amplichip CYP450, allowed transferability of complex genetic-based analyses in clinical medicine because they offer the advantage to handle complexity in a reliable, easy and rapid way[37,38]. Lab-on-chip miniaturized devices to replace ELISA immunoassays have also been developed in order to simultaneously test several proteins for each patient just using small amounts of biological fluids. We developed a multiplex chip designed system to simultaneously detect AFP-IgM and SCCA-IgM, which novelty is mainly represented by a miniaturized glass surface which is deposited with antibodies by means of the precise ink-jet technology, usually employed for ink printers. Our aims were to investigate the analytical and clinical performances of this novel chip technology (CompleXima HCC Biochip) in comparison with commercially available ELISAs.

MATERIALS AND METHODS

Study design

The analytical and clinical performances of CompleXima HCC Biochip were verified using retrospective collected patients and control sera, following the experimental steps illustrated in Figure 1.

Patients

Sera from a total of 174 patients, including 102 LC and 72 HCC, stored in the Liver Bio bank of the Department of Medicine, University of Padova, were analysed. The study was approved by the Ethics Committee for Experimentation of Azienda Ospedaliera di Padova (protocol n. 1958P; approved on February 8th 2010). Participants provided their written informed consent for the study. Patients characteristics are detailed in Table 1. Thirty-nine blood donors (23 males, 16 females, mean age ± SD: 45 ± 8.5) were also included as controls. HCC diagnosis was done when focal lesions >2 cm in diameter were found by US scan and confirmed with CT or MR and it was always histologically confirmed (US-guided fine-needle biopsy). Whole blood was collected in Vacutainer tubes (BD Diagnostics, USA) from all cases and controls and centrifuged for 15' at 2000 x *g* to obtain sera, which were aliquoted and stored at -20°C until use. Samples were collected before any pharmacological or surgical treatment.

Laboratory techniques

ELISA for AFP-IgM and SCCA-IgM.

Circulating macrocomplexes AFP-IgM and SCCA-IgM were measured by the commercial ELISA kits Hepa AFP-IC and Hepa-IC respectively (Xeptagen S.p.A., Venezia, Italy) according to manufacturer's instructions. In these assays, rabbit oligoclonal anti-human AFP or anti-human SCCA antibodies were used as capture antibodies. The amount of AFP-IgM and SCCA-IgM was expressed in arbitrary units per mL (AU/mL) as described elsewhere[23,39].

AFP-IgM and SCCA-IgM determination using CompleXima Biochip.

CompleXima HCC Biochip prototype has been developed by Xeptagen S.p.A. and Olivetti I-Jet (Torino, Italy). It is based on the contemporary chemiluminescent serum detection of AFP-IgM and SCCA-IgM using a biochip technology. CompleXima HCC Biochip was prepared using a positively charged 2.1x2.1 mm silica surface which was covered with a regular distribution of 36 antibodies spots, each with a diameter of 127 μm and a surface area of 12.6 μm^2. Spotting was realized by using an ink-jet method under patent. The external ring, made

of 20 individual spots of rabbit IgG anti-human IgM, represented the internal quality control (IQC). The remaining 16 spots inside the IQC ring were replicates of synthetic anti-AFP-IgM and anti-SCCA-IgM (8 replicated spots/each analyte). The silica surface was included in a microfluidic reaction chamber, with in and out accesses. The manufacture's suggested original protocol for CompleXima HCC Biochip analysis included the following analytical steps: direct addition to the reaction chamber of 1:6 diluted sera in PBS-non fat milk, incubation at room temperature (RT) for 1 hr, two washings with PBS-Tween and addition of the secondary antibody (goat IgG anti-human IgM-HRP), incubation at RT for 1 hr, two washings, addition of the chemiluminescent HRP substrate and chip reading. The detection and transduction signal systems were based on a CCD (Charge Coupled Device) digital camera and they were part of an integrated system (called Biochip reader) which included the CCD Hamamatsu ORCA 05GX, a netbook and a chip housing system. CCD images, which were black and white with a 1344x1024 pixel resolution, were acquired and processed by a dedicated software. Instrument sensitivity was supported by a 16 bit electronics. The dedicated software, on the basis of IQC ring results, selected and distinguished valid from invalid analyses. The concentration and the intra-chip CV for AFP-IgM and SCCA-IgM were calculated by the dedicated software. The CompleXima HCC Biochip returned quantitative results above 50 AU/mL and below 600 AU/mL.

The original protocol was optimized as follow and detailed in Supplementary figure 1: any chip was kept RT for 10', then rinsed with 50 µL of dilution buffer for 10', washed twice with 175 µL of washing buffer before adding diluted sera (1:6). All incubations were performed in a humid chamber. All subsequent washings (150 µL/each) were repeated three instead of two times.

AFP-IgM and SCCA-IgM were measured in all serum samples by both ELISAs and by using the CompleXima HCC Biochip. To obtain CompleXima HCC Biochip results in individual sera, all samples were subjected to a first run to distinguish those with AFP-IgM and SCCA-

IgM results below or above the upper detection limit of the system (600 AU/mL), the latter requiring further dilution before analysis. All sera were then properly diluted and analysed in triplicate. The mean of these three replicates was used to assign CompleXima HCC Biochip AFP-IgM and SCCA-IgM values for any given patient and were used for comparison with ELISA results.

AFP determination.

AFP levels were measured using an automated method (ARCHITECT AFP, Abbott Diagnostics, Sligo, Ireland), which is a two-step assay based on CMIA (Chemiluminescent Microparticle ImmunoAssay) technology.

Statistical analysis

Bland-Altman method and Passing-Bablok regression were used to evaluate the agreement between CompleXima HCC Biochip and ELISA assays[40,41]. For these comparisons ELISAs and Biochip results of the whole studied subjects (n=213) were included. AFP-IgM and SCCA-IgM thresholds were assessed by Receiver Operating Characteristics (ROC) curve analysis. Statistical analysis was performed using the nonparametric Mann–Whitney Rank Sum Test. Logistic regression models and statistical analysis were performed with statistic software STATA (version 10). Statistical significance was considered when $p<0.05$.

RESULTS

Overall 1089 Biochips were used in this study to evaluate the performance of CompleXima HCC Biochip prototype. Among them, 34% were marked as invalid by the software since the IQC did not met the established criteria, due to heterogeneity among spots or to the presence of bubble airs.

Table 2 reports intra-assay coefficients of variation (CV_{intra}) of CompleXima HCC Biochip, which were calculated using a series of 8 different HCC serum samples with high, medium and low concentration levels of AFP-IgM and SCCA-IgM. The inter-assay coefficient of variation (CV_{inter}), which was calculated by means of 20 repeated measures of

the same sample in different days, was 14% for AFP-IgM (mean ± SD, 310 ± 42 AU/mL) and 21% for SCCA-IgM (mean ± SD, 463 ± 95 AU/mL).

Sera with high levels of AFP-IgM or SCCA-IgM were selected to define the linearity interval of CompleXima HCC Biochip. Serial dilutions of sera with the dilution buffer were performed and analysed. As shown in Figure 2, values above 300 AU/mL were outside the linearity for both AFP-IgM and SCCA-IgM.

To verify the agreement between ELISAs and CompleXima HCC Biochip results, two statistical approaches were used: the Bland-Altman method and the Passing Bablock regression analysis. Both approaches assume uncertainty of the new (CompleXima HCC Biochip) and the control (ELISA) test. Bland-Altman plot allows to detect the presence of a proportional bias[40], while Passing Bablock regression analysis is the only method that adjusts for non-normal data as frequently observed in practice[41]. Figure 3 shows the Bland-Altman plot results of AFP-IgM and SCCA-IgM values obtained with CompleXima HCC Biochip in comparison with those obtained with the respective ELISAs. Passing-Bablok regression analyses showed that the Biochip prototype underestimated AFP-IgM (Intercept A: -165.06; 95% CI: -313.11 to -51.32) and overestimated SCCA-IgM (Intercept A: 26.83; 95% CI: 14.47 to 35.86) with respect to ELISAs.

Figure 4 shows AFP-IgM (upper panel) and SCCA-IgM (lower panel) measured with CompleXima HCC Biochip in controls, LC and HCC patients. The levels of both markers were significantly higher in LC and HCC patients with respect to controls ($p<0.001$ for AFP-IgM and SCCA-IgM; Mann–Whitney Rank Sum Test). However, no difference between LC and HCC was observed ($p=0.864$ and $p=0.214$ for AFP-IgM and SCCA-IgM, respectively; Mann–Whitney Rank Sum Test).

ROC curve analysis for AFP-IgM and SCCA-IgM measured with CompleXima HCC Biochip and ELISAs, and for AFP serum levels were performed (Fig. 5). The thresholds were identified by fixing the specificity at 95%; corresponding sensitivities in distinguishing

healthy subjects from patients with advanced liver disease, including both LC and HCC, were calculated and they are reported in Table 3. The established cut-off values (60 AU/mL for AFP-IgM and 139 AU/mL for SCCA-IgM) were used to categorize as positive or negative each individual value of AFP-IgM and SCCA-IgM. We then ascertained sensitivity and specificity of their combined evaluation in diagnosing HCC with respect to LC, considering positive those patients having positive findings of at least one test: positive results were found in 37/72 HCC (sensitivity=51.4%), while negative results were found in 49/102 LC (specificity=48%). A logistic regression model was developed, by including LC and HCC patients and leaving out controls, and considering the presence or absence of HCC as outcome. The model, adjusted for age and gender, included among predictors AFP-IgM and SCCA-IgM, measured with ELISA or with CompleXima HCC Biochip, AFP and disease aetiology. Table 4 reports the results of the analysis.

None of the studied biomarkers, including macrocomplexes measured by both ELISA and Biochip and AFP, was correlated in patients with HCC with the number of nodules nor with their diameter (data not shown). However when AFP-IgM and SCCA-IgM Biochip results were evaluated together according to the above described criteria and cut-off, positive findings were more frequently encountered in HCC patients with nodule diameter above than in those with nodule diameter below 3 cm (chi-square=3.725; $p<0.05$).

DISCUSSION

In this work we evaluated a prototype device, namely CompleXima HCC Biochip, for the simultaneous measurement of serum AFP-IgM and SCCA-IgM, two emerging biomarkers suggested to be of utility for early HCC detection[23,24]. This lab-on-chip technology combines AFP-IgM and SCCA-IgM immunoassays with chemiluminescence detection and microfluidics in one platform. The micro-chip presented in this study shares with other integrated microchip-based systems, designed for the detection of the serum tumor markers AFP and PSA[42,43,44], portability, simplicity and rapid processing, which render them attractive

for future applications as low-cost point of care testing. However, before commercialization of these new products proof-of-principle studies in a laboratory environment are mandatory[45]. In this study both analytical and clinical performances of the Biochip were evaluated, in agreement with the requirements of the International Standard for Medical Laboratories Accreditation (ISO 15189: 2012)[46,47]. Analytically, intra-assay coefficients of variation were lower than 12% for low, medium and high SCCA-IgM or AFP-IgM levels, which fits with an overall good performance. The intra-assay coefficients of variation showed a decreasing trend from low to high concentration, and this probably depends on the fact that in the low and medium concentration ranges some measurements were very close or equal to the lower limit of detection (50 AU/mL), thus being more error-prone. Inter-assay was slightly higher than intra-assay variability and this is probably dependent on the fact that, differently from intra-assay experiments, Biochips for inter-assay might be of different lots. In the evaluation process of CompleXima HCC Biochip we compared the results with those obtained with the ELISAs by Bland-Altman method and Passing-Bablok regression, showing that the two methods were not perfectly aligned. On average, AFP-IgM was underestimated while SCCA-IgM was overestimated by CompleXima HCC Biochip compared to ELISAs, and this was indicated by the Bland-Altman differences mean value (-52,6 AU/mL for AFP-IgM and +35.8 AU/mL for SCCA-IgM). Moreover the AFP-IgM underestimation and SCCA-IgM overestimation by CompleXima HCC Biochip varied largely along the increasing X-axis, indicating dose-dependence and this was further confirmed by the Passing-Bablok analyses. These discrepancies might probably depend on differences in the standard curve used to calculate ELISAs and CompleXima HCC Biochip results. In the former case the standard curve is always run together with samples, while in the latter case this is not possible and the standard curve is necessarily included in the software for data analysis.

To establish whether the differences between ELISAs or CompleXima HCC Biochip AFP-IgM and/or SCCA-IgM measurements might have a different impact in the clinical

assessment of LC and HCC we studied a series of patients with chronic liver diseases including LC and HCC. In the majority of healthy subjects both macrocomplexes levels detected by CompleXima HCC Biochip were below 50 AU/mL (39/39 for AFP-IgM and 30/39 for SCCA-IgM), and this rendered the cut-off almost equal to the lower detection limit of the system, differently from what observed with ELISAs which measurable results in healthy controls allowed to calculate the threshold level at 120 AU/mL for AFP-IgM and 156 AU/mL for SCCA-IgM. While both biomarkers levels were higher in patients than in controls, no differences were found between LC and HCC (Fig. 3) and this clearly indicates that they cannot be used alone in the first clinical assessment of a patient suspected of having or not HCC. Surprisingly, as presented in Figure 4, ROC curves analyses demonstrated that the results obtained with CompleXima HCC Biochip discriminated better patients with a diseased liver from controls than the ELISAs being 60 AU/mL and 139 AU/mL the 95% specificity associated cut-offs for AFP-IgM and SCCA-IgM, respectively (Table 3). These cut-offs were, however, associated with a low sensitivity (47% and 46%, respectively). Noteworthy, sensitivity values associated with CompleXima HCC Biochip were slightly better than those obtained with the ELISAs (20% for both AFP-IgM and SCCA-IgM). Sensitivity and specificity associated with the combined evaluation of AFP-IgM and SCCA-IgM in diagnosing HCC with respect to LC were 51.4% and 48%, respectively. However, as emerged by the binary logistic regression analysis presented in Table 4, the only significant predictor able to discriminate LC from HCC was AFP ($p=0.009$).

In conclusion, with respect to ELISA, CompleXima HCC Biochip allows to perform AFP-IgM and SCCA-IgM determinations in one run and to handle each sample independently from the others. CompleXima HCC Biochip allows to obtain AFP-IgM and SCCA-IgM values characterized by a good analytical reproducibility, although they tended to be under- or overestimated with respect to ELISAs. Future chances for this new Biochip, to further implement its reproducibility and simplicity, are automation of the whole analytical process

and validation of the system for other matrices, such as whole blood, thus allowing the development of a point-of-care testing device. Moreover, due to its power-free design our device can be easily equipped with increasing number of antigens to the grid, thus being suitable for rapid implementation when new diagnostic/prognostic markers of HCC will emerge.

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FIGURE CAPTIONS

Figure 1. Study design. The flowchart illustrates the experimental steps followed to validate CompleXima HCC Biochip.

Figure 2. CompleXima HCC Biochip Linearity Interval for SCCA-IgM and AFP-IgM. Serial dilution of sera with high levels of specific biomarkers-IgM were performed and results are presented. Values above 300 AU/mL were outside the linearity for both AFP-IgM and SCCA-IgM.

Figure 3. Bland-Altman comparison of AFP-IgM and SCCA-IgM values. Comparison between CompleXima HCC Biochip and ELISA measurements. Upper panel: AFP-IgM results; lower panel: SCCA-IgM results. For any single subject the average between CompleXima HCC Biochip and ELISA results are reported on the X-axis, while the corresponding differences between the two measurements are reported in the Y-axis. Continuous lines indicate mean values, dashed lines show ±1.96 standard deviation (SD). The comparison included all studied subjects (n=213).

Figure 4. AFP-IgM and SCCA-IgM increase in patients with liver diseases. AFP-IgM (upper panel) and SCCA-IgM (lower panel) were measured with CompleXima HCC Biochip in 39 controls, in 102 patients with liver cirrhosis (LC) and in 72 patients with hepatocellular

carcinoma (HCC). Higher levels of both biomarkers were found in LC and HCC patients with respect to controls.

Figure 5. Receiver Operating Characteristic (ROC) curves analysis for determining AFP-IgM and SCCA-IgM thresholds. The figure illustrates ROC curves of AFP-IgM and SCCA-IgM measured with both CompleXima HCC Biochip (CHIP_AFP-IgM and CHIP_SCCA-IgM) and ELISA (ELISA_AFP-IgM and ELISA_SCCA-IgM). Alpha-fetoprotein (AFP) is also shown. Patients with chronic liver disease (LC and HCC together) were compared to healthy controls. The arrow indicates 95% specificity. The areas under the ROC curves with their respective standard errors (SE) are reported in the bottom table.

Table 1. Patients' characteristic included in the study.

		LC	HCC
		n=102	n=72
SEX	males	70	55
	females	32	17
AGE	mean (years)	60.29	67.86
	SD	11.96	10.24
AETIOLOGY	HBV-related	18	9
	HCV-related	44	39
	Alcohol-related	39	22
	Other*	1	2
CHILD-PUGH	A	59	37
	B	33	23
	C	10	12
TUMOR Ø	>2cm >3 cm		32
	≥3cm		24
	missing		16
N° OF NODULES	1		35
	2-3		13
	diffuse		23
	missing		1

Notes: *: aetiology autoimmune, cryptogenic, HDV or Primary Biliary Cirrhosis (PBC)

Table 2. CompleXima HCC Biochip Intra-assay coefficients of variation (CV_{intra}) of AFP-IgM and of SCCA-IgM. For both biomarkers and for any detection levels (low, medium and high) 8 samples were analysed in quadruplicate the same day using Biochips of the same batch. To calculate intra-assay CVs, first for each sample the CV from four replicated analyses was obtained. Mean intra-assay CVs were calculated from the eight individual CVs and reported in the table together with the corresponding mean values and standard deviations of AFP-IgM and SCCA-IgM results. AU= Arbitrary Units.

	AFP-IgM concentration		**AFP-IgM CV_{intra}**		**SCCA-IgM concentration**		**SCCA-IgM CV_{intra}**	
	Mean±SD (AU/mL)	Range (AU/mL)	Mean (%)	Range (%)	Mean±SD (AU/mL)	Range (AU/mL)	Mean (%)	Range (%)
Low	58 ± 14	50-110	10	0-36	96 ± 31	50-153	12	2-29
Medium	101 ± 57	50-224	12	0-21	237 ± 70	103-352	9	3-13
High	304 ± 58	211-447	9	2-22	443 ± 75	294-579	9	3-17

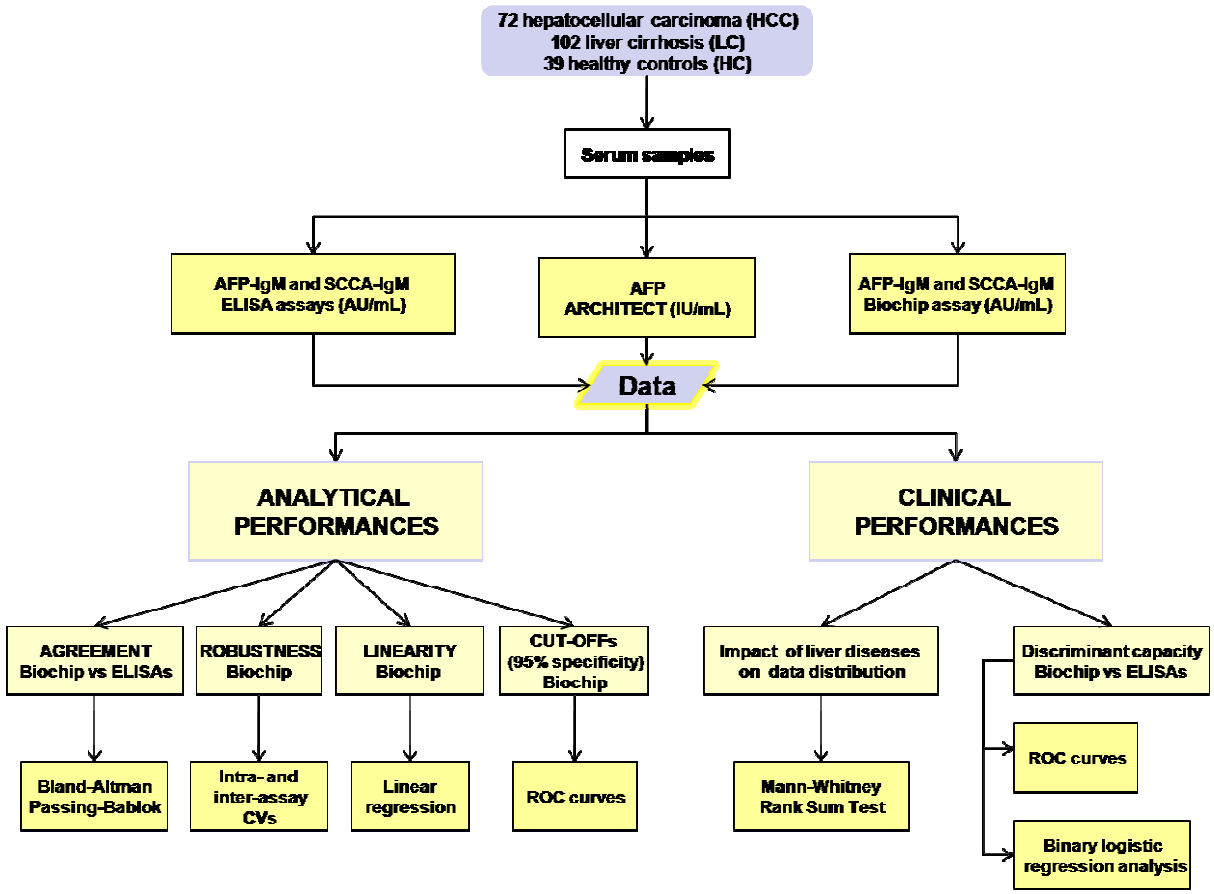
Table 3. Sensitivity%, specificity%, positive (LR+) and negative (LR-) likelihood ratios of serum biomarkers in distinguishing healthy controls from patients with LC or HCC. The cut-offs were established at a 95% fixed specificity. 95% Confidence intervals are reported in brackets.

Biomarker	Method	Cut-off	Sensitivity %	Specificity %	LR+	LR-
AFP-IgM	ELISA	319 AU/mL	20 (14-27)	95 (79-98)	2.60 (1.90-3.60)	0.87 (0.30-2.60)
	Biochip	60 AU/mL	47 (40-55)	95 (83-99)	9.19 (7.7-10.9)	0.56 (0.10-2.20)
SCCA-IgM	ELISA	231 AU/mL	20 (14-26)	95 (79-98)	2.54 (1.90-3.50)	0.87 (0.30-2.60)
	Biochip	139 AU/mL	46 (38-54)	95 (83-99)	8.97 (7.50-10.70)	0.57 (0.10-2.20)
AFP		4.2 IU/mL	59 (51-66)	95 (83-99)	11.43	0.44

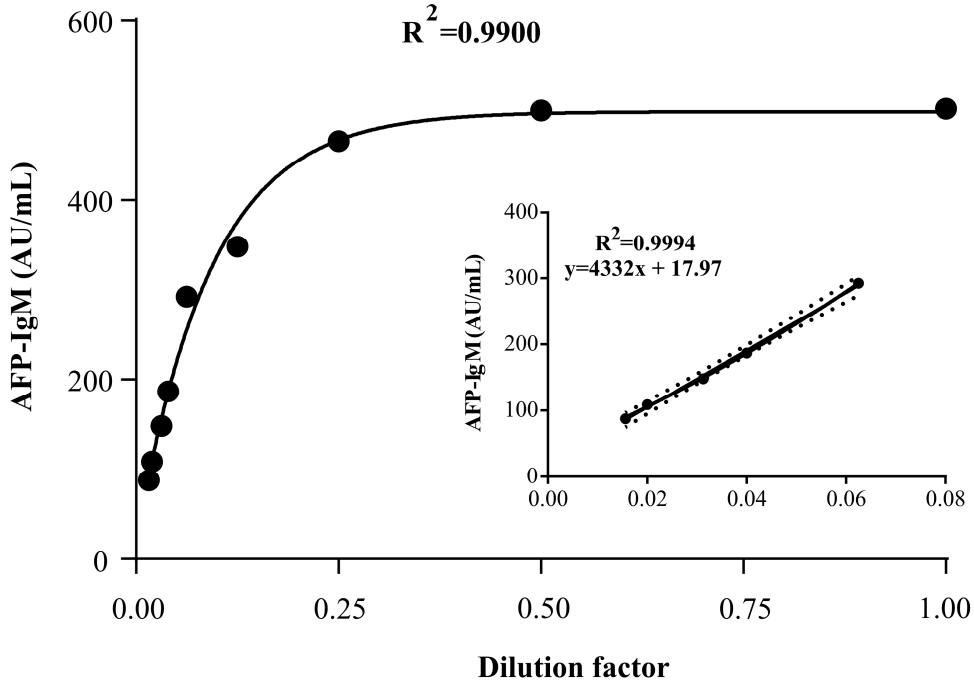
Table 4. Binary logistic regression analysis. Among patients with LC and HCC, the presence or absence of HCC was considered as outcome variable. Predictors included in the analysis were AFP, AFP-IgM and SCCA-IgM measured with both ELISA and Biochip, age, gender and disease aetiology.

	Odds Ratio	P value	95% C.I.
AFP (IU/mL)	1.02	0.009	1.00-1.03
ELISA AFP-IgM (AU/mL)	1.00	0.707	0.99-1.00
ELISA SCCA-IgM (AU/mL)	1.00	0.535	0.99-1.00
Biochip AFP-IgM (AU/mL)	0.99	0.852	0.99-1.00
Biochip SCCA-IgM (AU/mL)	0.99	0.797	0.99-1.00
Disease aetiology	1.01	0.973	0.44-2.31
Age (years)	1.08	0.0001	1.04-1.12
Gender	0.21	0.004	0.07-0.61

Figure 1



485 **Figure 2**



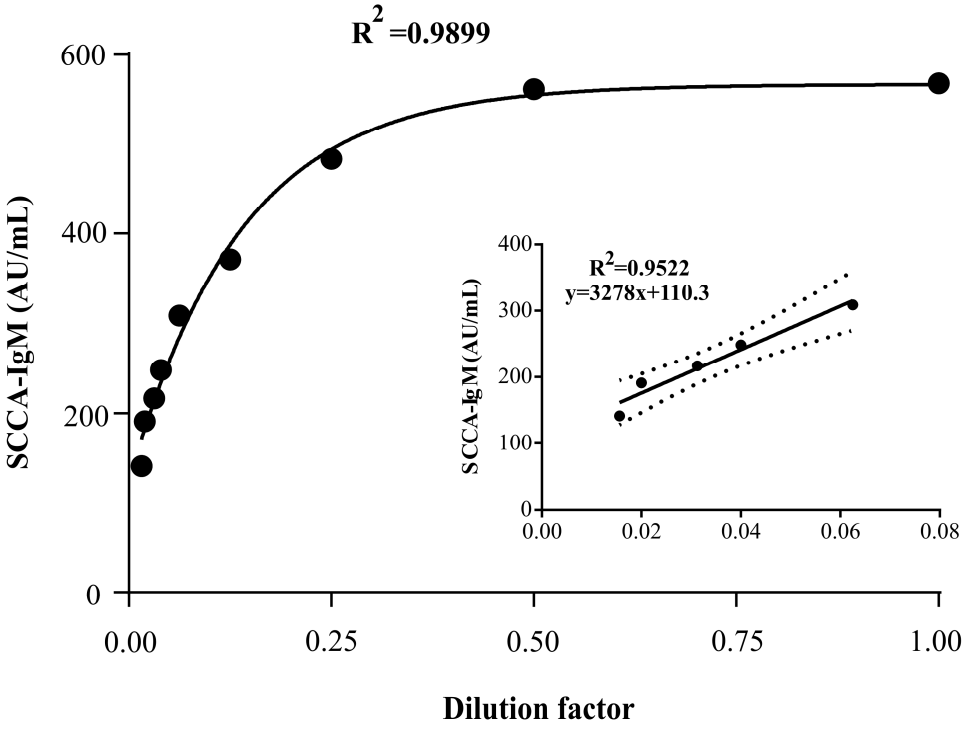


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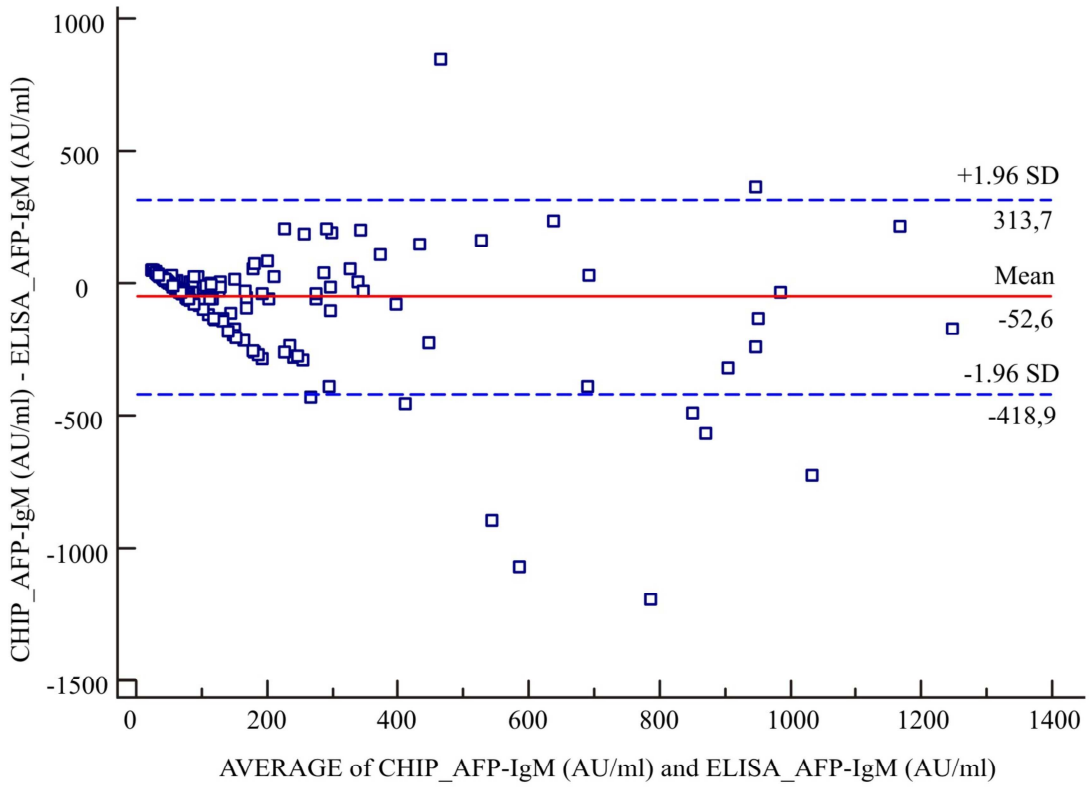
Figure 3



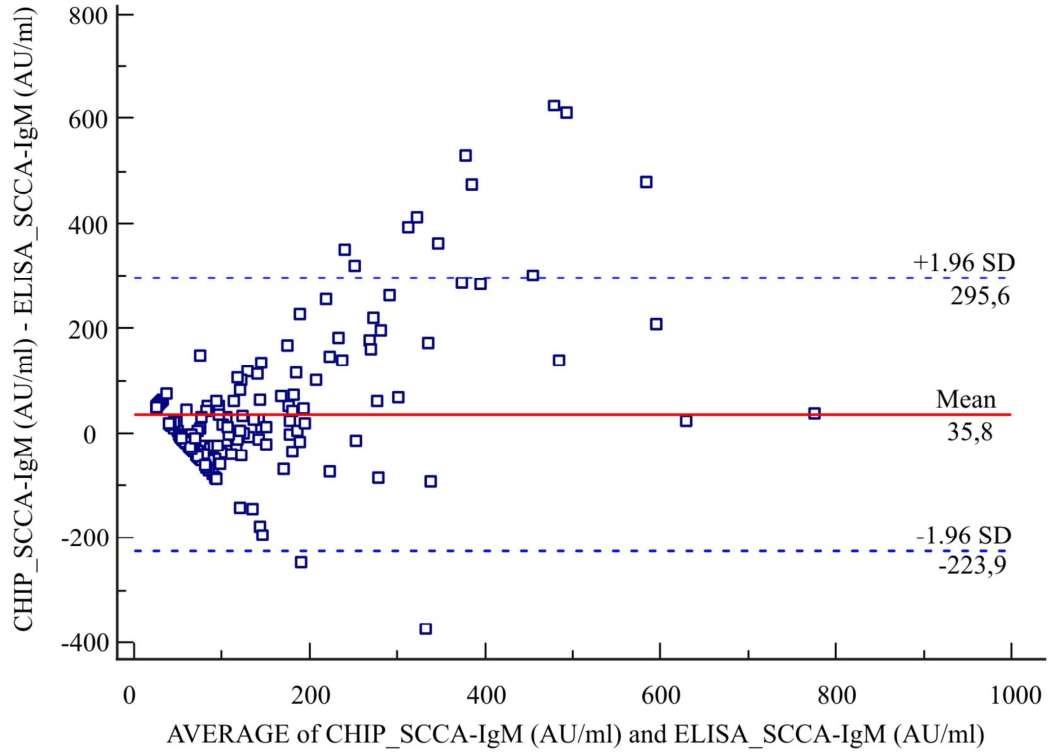
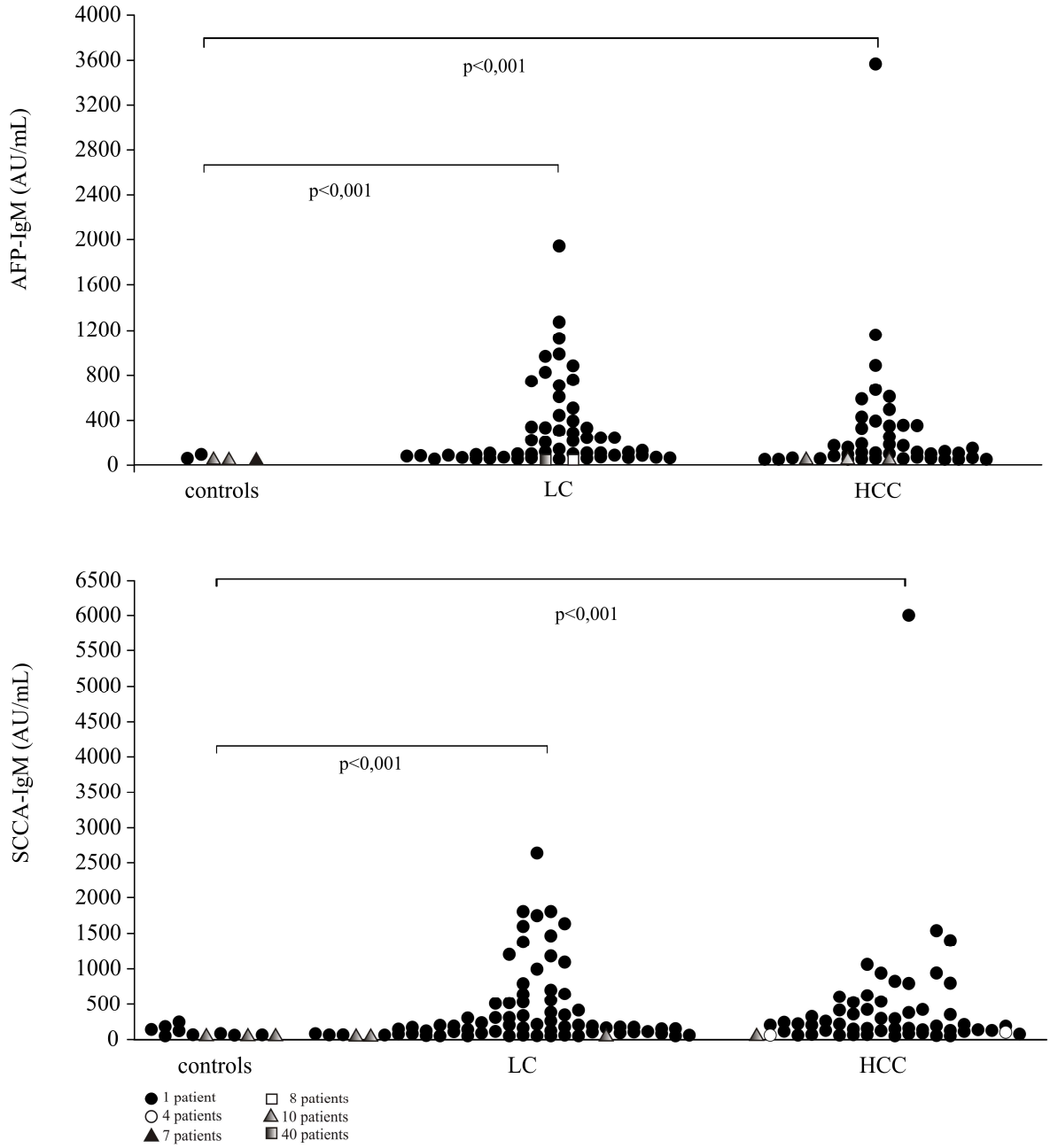
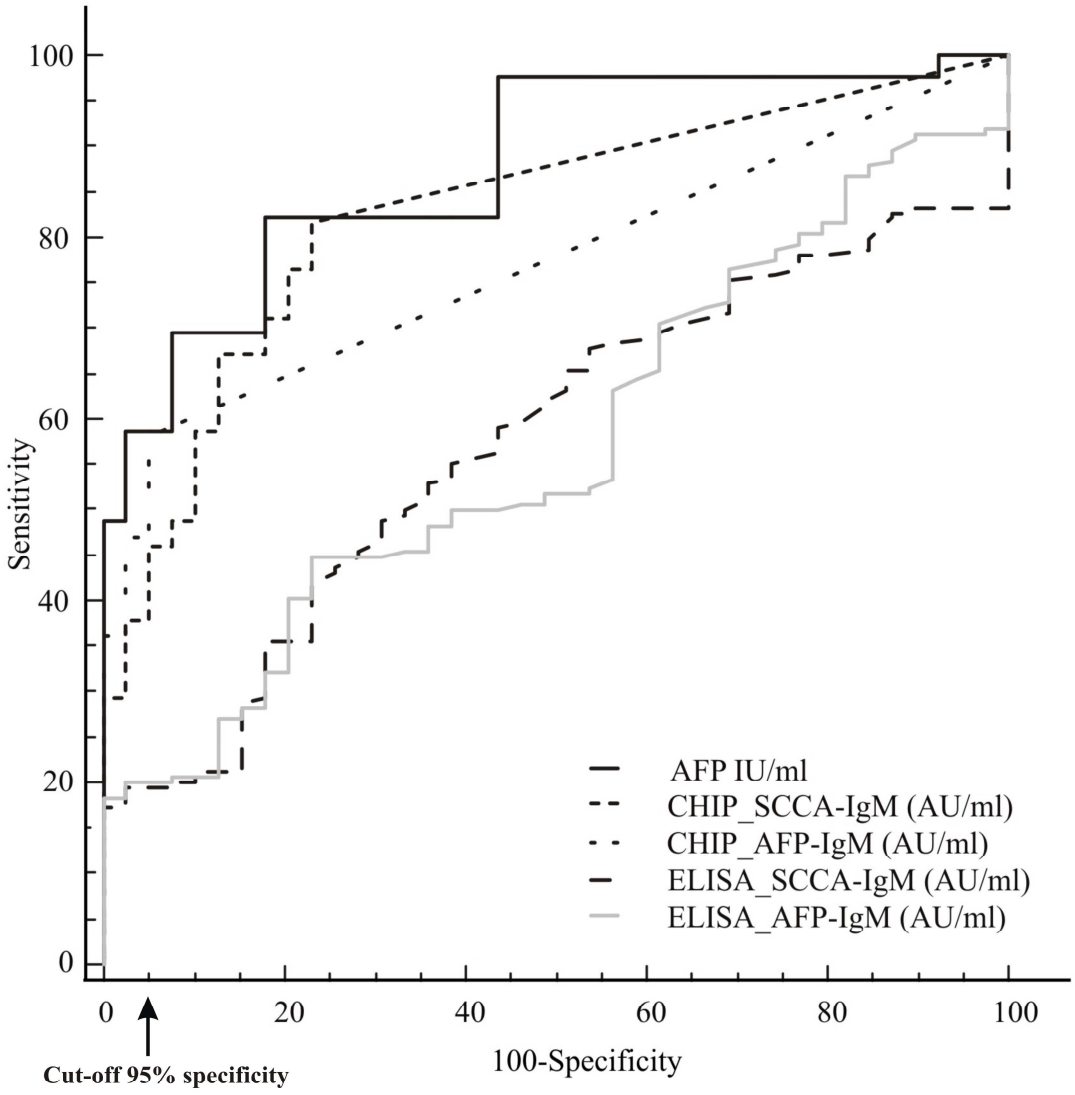


Figure 4



494 **Figure 5**



	AUC	SE	95% CI
AFP_IU/ml	0,878	0,024	0,826-0,918
CHIP_SCCA-IgM AU/ml	0,827	0,030	0,770-0,876
CHIP_AFP-IgM AU/ml	0,771	0,036	0,709-0,826
ELISA_SCCA-IgM AU/ml	0,575	0,049	0,506-0,642
ELISA_AFP-IgM AU/ml	0,572	0,049	0,502-0,639

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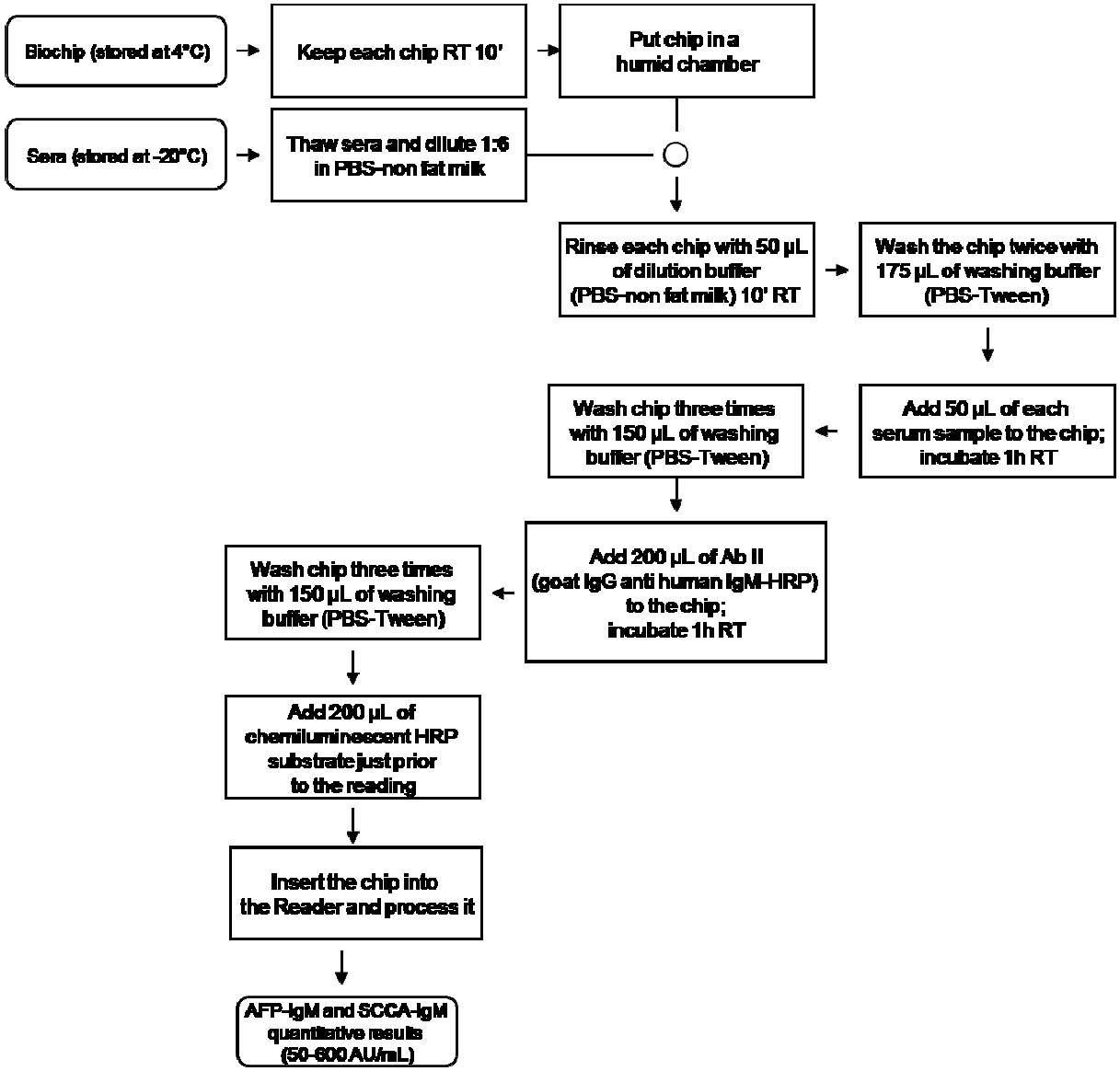
497 **Supplementary Figure 1**



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