Technical Brief

Human Anti-Mouse Antibody Protected ELISA for the Quantification of Squamous Cell Carcinoma Antigen

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Running Title: Squamous Cell Carcinoma ELISA

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Abbreviations: SCC, squamous cell carcinoma antigen; BSA, bovine serum albumin; PBS, phosphate buffered saline; HAMA, human anti-mouse antibodies; IIR, Immunoglobulin Inhibiting Reagent; AMR, analytical measurement range.

Squamous cell carcinoma antigen (SCC) was first isolated from squamous cell carcinoma tissue of the uterine cervix and initially reported as TA-4 (1). The antigen has been characterized as a glycoprotein with a molecular weight between 45,000 and 55,000 daltons (2,3). Studies suggest that SCC may function as a protease inhibitor (4-7).

SCC is expressed in normal epithelium and epithelial tissues (1,8-11). The antigen primarily consists of over ten protein fractions with isoelectric points ranging from 5.9 to 6.6 which are generally divided into two groups: The acidic group with isoelectric points < 6.25, and the neutral group 6.25 or greater (12). The neutral form of SCC normally remains inside the cell, whereas acidic SCC is easily released from the cell and is often elevated in the serum of patients having squamous cell carcinomas or other nonmalignant squamous cell lesions (3,12). It has been proposed that SCC may be involved in the malignant behavior of squamous cell cancers having a role in invasion and/or metastasis (9). Consequently, SCC levels in the serum are used for monitoring carcinomas of various tissues including uterine cervix, lung, skin, head and neck, esophagus, urothelium, anal canal and vulva (1,8,13-19).

Despite the demonstrated clinical utility of SCC testing (20), reagents (investigational use only) were no longer available in the US by the late 1990s. Yet, SCC testing continues to be commercially available in Europe and Asia. We therefore, have developed and validated a 96-well microtiter plate formatted ELISA as an option for SCC testing. The ELISA includes protection against human anti-mouse antibodies (HAMA) to a theoretical limit of at least 3.2 mg/mL.

Anti-SCC monoclonal antibodies and stock SCC antigen were acquired from Abbott Japan, LTD. (Tokyo, Japan). Microtiter strips (F8 MaxiSorp) and holders were purchased from Nunc™. Biotin protein labeling kits were obtained from Roche. Immunoglobuling Inhibiting Reagent®

(IIR) was purchased from Bioreclamation Inc. The SPECTRAmax® PLUS plate reader was manufactured by Molecular Devices Corp. and controlled using their ProMax software. Data analysis was performed using Microsoft Excel.

High concentrations of SCC antigen are present in normal saliva and sweat (8,21). Therefore, protective equipment, especially face masks, were worn throughout all procedures to prevent accidental SCC contamination of the reagents and samples.

Microtiter wells were coated overnight at 4 °C with capture anti-SCC monoclonal antibody (clone F2H7C1131) at a volume of 100 µL/well (2 µg/mL antibody in 0.1 mol/L carbonate buffer, pH 9.6). The wells were then washed once with wash solution (0.098 mol/L Na_2HPO_4, 0.009 mol/L KH_2PO_4, 0.137 mol/L NaCl, 0.05 % v/v Tween 20, pH 7.2). A blocking buffer (0.008 mol/L Na_2HPO_4, 0.003 mol/L KH_2PO_4, 0.150 mol/L NaCl, 50 g/L sucrose, 20 g/L bovine serum albumin, 0.05% v/v Tween 20, pH 7.2, 250 µL/well) was then added and the microtiter strips incubated with shaking at room temperature for 7-8 hours. The strips were then lyophilized overnight at 4 ºC and stored with desiccant in sealed plastic bags at 4-8 ºC until use.

The anti-SCC detection antibody (clone F1H3C1151) was biotinylated according to the Roche labeling kit protocol. The procedure incorporates the separation of labeled antibody from non-bound biotin by column chromatography. Eluent fractions were chosen and combined to generate a final antibody concentration of approximately 0.5 mg/mL. Aliquots were stored at -70 °C.

Calibrators and controls were prepared by diluting SCC stock antigen with bovine serum albumin phosphate-buffered saline (BSA in PBS; 0.008 mol/L Na_2HPO_4, 0.003 mol/L KH_2PO_4, 0.150 mol/L NaCl, 10 g/L BSA, pH 7.2) to the desired target values (1, 5, 20, 40 and 70 ng/mL

for calibrators; 2, 20 and 50 ng/mL for controls). Aliquots (100 µL) were then lyophilized and stored at -70 °C.

Serum samples were stored at 4-8 °C short term (two weeks or less). For longer periods, samples were frozen at -70 °C.

The SCC ELISA was performed by first reconstituting the calibrators and controls with 100 µL of distilled water. Calibrators, controls and patient serum unknowns were then diluted twenty-fold with BSA in PBS containing 0.1 mg/mL of IIR. This diluent was also used as the zero calibrator. In duplicate, 100 µL of the diluted samples were placed into the appropriate wells. The plate was sealed with an acetate sealer and incubated 2 hours at room temperature while shaking. After incubation, the wells were washed three times with 250 µL wash solution. SCC biotinylated detection antibody was diluted 1:3000 with BSA in PBS and 100 µL added to each well. The plate was resealed and the incubation repeated for 1 hour. After a second washing, 100 µL of streptavidin/horseradish peroxidase conjugate (DAKO; diluted 1:10,000 with BSA in PBS) was added to each well. The plate was resealed and incubated for 30 minutes. After a final washing, 100 µL of 3,3',5,5'-tetramethyl benzidine/H_2O_2 substrate (Enhanced K-Blue TMB Substrate, Neogen Corp.) was placed in each well and a final 10 minute incubation followed. The reaction was then stopped by the addition of 0.5 mol/L H_2SO_4 (100 µL/well). The absorbance of each well was measured at 450 nm. A calibration curve (absorbance vs. ng/mL SCC) was then constructed using a 4-Parameter fit of the calibrators. Comparison of control and unknown absorbances to the calibration curve generates results in ng/mL SCC.

The assay was linear to 70 ng/mL SCC with a slope and y-intercept of 0.989 and 0.202 respectively ($R^2 = 0.997$, n = 8). Ten replicates of the BSA in PBS diluent (zero calibrator) generated a detection limit of 0.3 ng/mL (mean = 0.07, SD = 0.12 ng/mL). Therefore, the

analytical measurement range is 0.3 to 70 ng/mL SCC. Assay imprecision was as follows: for intra-assay imprecision (n = 10), mean (SD) results of 1.7 (0.20), 19.7 (0.77) and 48.4 (1.37) ng/mL with CVs of 11.9, 3.9 and 2.8% respectively; for inter-assay imprecision (n = 10), 2.2 (0.40), 19.9 (0.82) and 48.0 (1.40) ng/mL with CVs of 18.5, 4.1 and 2.9% respectively.

A reference interval study was accomplished by measuring sera from 136 healthy individuals spanning 19 to 75 years of age. At a 95% confidence limit [mean (SD), 1.0 (0.62) ng/mL] an upper reference limit of 2.3 ng/mL SCC was calculated. A minimal difference between males (n = 66) and females (n = 70) was evident with calculated upper limits of 2.5 and 2.0 ng/mL respectively [mean (SD): male, 1.0 (0.62); female, 0.8 (0.57) ng/mL]. Non-parametric analysis of the data at a 95% limit generated a similar upper normal of 2.1 ng/mL SCC, with male and female based values of 2.2 and 1.8 ng/mL respectively. The bias between genders is displayed as cumulative frequency plots shown in Figure 1A. As a function of age, no obvious tendencies in SCC concentration is observed as demonstrated in Figure 1B. Overall, we deem the variances between gender and/or the calculation method used to be minimal clinically. Therefore, a straightforward upper reference level of 2.2 ng/mL was resolved from the mean.

The 2.2 ng/mL SCC reference interval agrees favorably with the value of 2.0 ng/mL employed at our facility until the reagent unavailability mentioned previously. The value also compares acceptably with a study of healthy individuals using the IMx® SCC assay (n = 885), which resulted in 99% of the subjects having SCC levels below 2.5 ng/mL (22).

Because of the increasing attention in recent years regarding the effects of HAMA in immunoassays (23-25), we integrated HAMA protection into our SCC ELISA. We chose the IIR because of its suggested advantages over other HAMA blocking reagents (26-28).

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Using sera with confirmed HAMA levels (Biorecamation, Inc. using the Roche HAMA ELISA kit; 11,300, 33,500 and 161,000 ng/mL HAMA), the IIR was tested at various concentrations. At an IIR concentration of 5 µg/mL, all HAMA samples were successfully blocked including the 161,000 ng/mL HAMA sera, (Table 1). Therefore, at a working IIR concentration of 100 µg/mL, the calculated hypothetical limit of the assay to block HAMA interference is at least 3.2 mg/mL HAMA.

In order to investigate any potential interference of IIR in the SCC ELISA, three samples were assayed using sample diluent minus IIR and then repeated using diluent containing four times (400 µg/mL) the working IIR concentration. Comparison of the measured values produced mean (SD) results of 2.0 (0.04), 19.8 (0.74) and 51.8 (1.03) ng/mL with CVs of 2.2, 3.8 and 2.0% respectively. Since these results are well within the established precision of the assay, no systematic interference caused by the IIR is suggested.

In conclusion, the SCC ELISA described herein demonstrates acceptable accuracy, precision, sensitivity and reliability for quantifying SCC. The established reference interval of 2.2 ng/mL agrees satisfactorily with previous and current intervals employed in SCC testing. In addition, the ELISA has clearly demonstrated a high effectiveness in blocking potential interference from HAMA, thus minimizing the potential of falsely elevated or depressed results.

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Figure Caption

Fig. 1. Reference interval study for SCC antigen ELISA.

Sera from136 healthy volunteers were assayed as described. (A), cumulative frequency plots the data segregated by gender, 66 males ($\circ$) and 70 females (Δ). (B), the data plotted as SCC antigen concentration versus age. The dashed lines denote the interval's upper limit of 2.2 ng/mL SCC.

Figure 1.
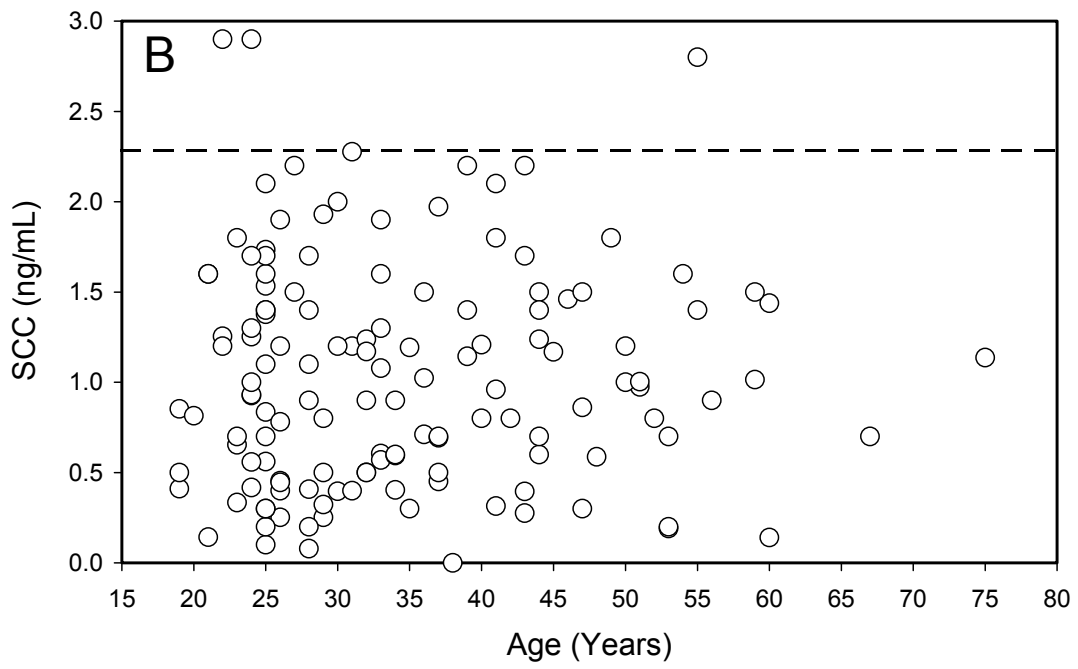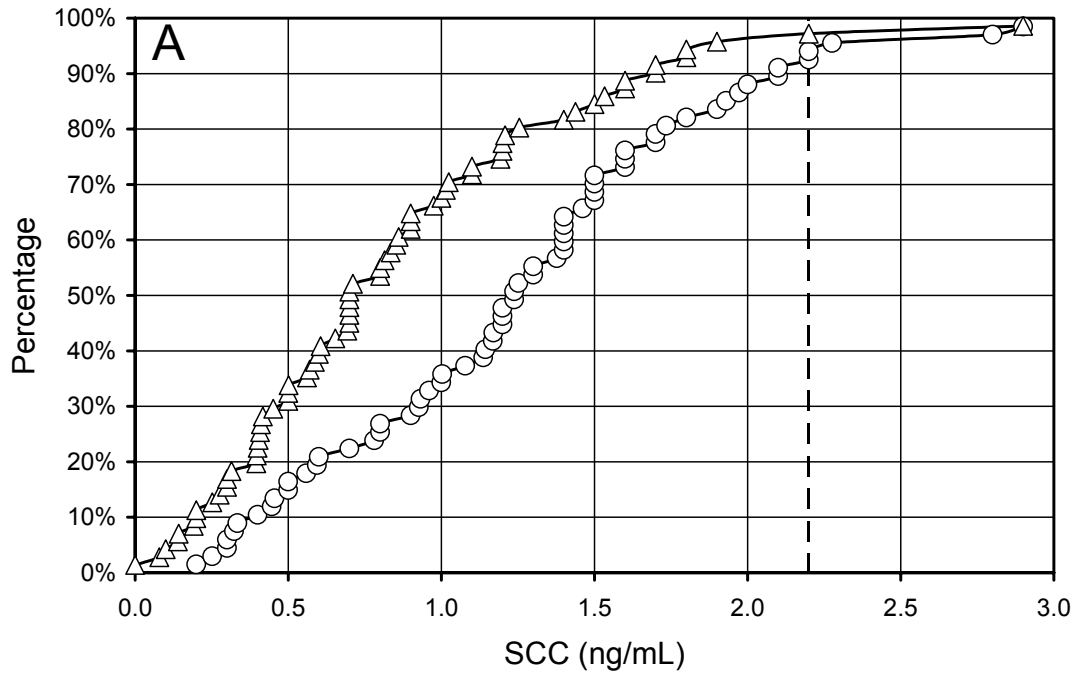
Table 1. IIR Performance Blocking HAMA in SCC ELISA

| | | SCC Measured (ng/mL) | | |
| | | IIR in Sample Diluent (µg/mL) | | |
HAMA (ng/mL)	No IIR	200	25	5
11,300	87.7	<0.3	0.4	0.5
33,500	> AMR[a]	<0.3	0.5	0.3
161,000	> AMR[a]	1.2	1.6	1.6

[a] Analytical Measurement Range

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