

ORIGINAL ARTICLE

Multiplexed immunobead-based assay for detection of oral cancer protein biomarkers in saliva

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OBJECTIVE: For clinical applications of biomarkers, there is a need for multiplex assays using high throughput platforms. The objective of this study was to determine the efficacy of Luminex Multianalyte Profiling (xMAP) technology for measurement of salivary proteins and to evaluate whether multiplex assays are as effective as single-plex assays and enzyme-linked immunosorbent assay (ELISA).

RESULTS: The average levels of interleukin-8 (IL-8) from the single-plex assay were 3313.2 ± 3759.8 pg ml⁻¹ [oral squamous cell carcinoma (OSCC), $n = 20$] and 1061.7 ± 1978.8 pg ml⁻¹ (control, $n = 20$). The IL-1 β average levels from the single-plex assay were 945.2 ± 1134.8 pg ml⁻¹ (OSCC, $n = 20$) and 314.2 ± 444.8 pg ml⁻¹ (control, $n = 20$). The average levels of IL-8 from the multiplex assay were 2834.9 ± 3385.6 pg ml⁻¹ (OSCC, $n = 20$) and 947.3 ± 2036.8 pg ml⁻¹ (control, $n = 20$). The IL-1 β average levels from the multiplex assay were 1013.5 ± 1221.1 pg ml⁻¹ (OSCC, $n = 20$) and 376.3 ± 576.3 pg ml⁻¹ (control, $n = 20$). The correlation coefficient between Luminex and ELISA assay for IL-8 ($n = 19$) and IL-1 β ($n = 19$) was 0.91 and 0.84, respectively.

CONCLUSION: Luminex xMAP single-plex and multiplex assays are as effective as ELISA assays for quantification of proteins in saliva. Both IL-8 and IL-1 β were expressed at significantly higher levels in OSCC subjects than in the matched healthy control subjects.

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Keywords: Luminex; saliva diagnostics; immunobead-based assay; oral cancer; OSCC; ELISA

Introduction

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is a high impact disease in the oral cavity, affecting more than 34 000 people in the United States each year (American Cancer Society, 2007). OSCC tumors arise through a series of molecular mutations that lead to uncontrolled cellular growth from hyperplasia to dysplasia to carcinoma in situ followed by invasive carcinoma. Major risk factors for OSCC include tobacco and alcohol consumption along with environmental and genetic factors (Figuerido *et al*, 2004; Brinkman and Wong, 2006; Turhani *et al*, 2006; Hu *et al*, 2007). OSCC is usually detected at late stages when the cancer has advanced and therefore results in poor prognosis and survival. Presently, surgery and radiotherapy are the primary treatments, but because of the location of OSCC in the head and neck; this usually results in postoperative defects and functional impairments in patients (Thomson and Wylie, 2002). Therefore, early disease detection is imperative because it can result in more effective treatment with superior results.

Saliva has gained notable attention as a diagnostic fluid because of its simple collection and processing, minimal invasiveness and low costs. Many researchers have studied salivary proteins as potential diagnostic markers for various diseases such as breast cancer, ovarian cancer, Sjögrens syndrome, hepatocellular carcinoma, leukoplakia and oral cancer (Yio *et al*, 1992; Streckfus *et al*, 2000; Gorelik *et al*, 2005; Rhodus *et al*, 2005; Brailo *et al*, 2006; Ryu *et al*, 2006; Hu *et al*, 2007). These potential disease markers, if successfully developed, can lead to simple clinical tools for early detection and the monitoring of disease prognosis and treatment in saliva, a non-invasive body fluid (Kingsmore, 2006; Hu *et al*, 2005).

Luminex Multianalyte Profiling (xMAP) technology, previously known as FlowMetrix and LabMAP (Elshal and McCoy, 2006), is a multiplex bead-based flow cytometric assay that is gaining recognition as a method for analyte quantitation. This technology utilizes

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5.6-micron polystyrene beads that are internally dyed with different intensities of red and infrared fluorophores. Currently, there are 100 beads, each with a unique spectral make up, which allows the mixing of several bead sets and, in theory, enabling the detection of up to 100 different analytes per assay (Vignali, 2000). The beads can be bound by various capture reagents such as antibodies, oligonucleotides, and peptides, therefore facilitating the quantification of various proteins, ligands, DNA and RNA (Fulton *et al*, 1997; Kingsmore, 2006; Nolan and Mandy, 2006). The assays are run on a 96-well plate format, followed by detection on a Luminex 100 instrument. As the beads run through the instrument, the internal dyes are excited by a laser, which results in the classification of each bead. Another laser excites the reporter dye which is directly proportional to the amount of analyte bound to each bead (Vignali, 2000; Ray *et al*, 2005). The resulting fluorescence is recorded by the instrument which then provides the median fluorescence unit obtained from measuring 100 beads.

Luminex xMAP technology has many applications including protein expression profiling, gene expression profiling, genotyping, immunodiagnostics, and genetic disease diagnostics. Although single-plex bead-based assays have been available for a long time; technological developments have enhanced the development of multiplex bead-based assays enabling the utilization of this method for quantitation of a panel of protein markers simultaneously (Prabhakar *et al*, 2002; Linkov *et al*, 2007). The advantage of Luminex xMAP technology lies in its high sensitivity, throughput and efficiency (Vignali, 2000; duPont *et al*, 2005). Significant reduction in time and costs results from multiplexing when compared with enzyme-linked immunosorbent assay (ELISA). ELISA is more expensive and time-consuming to perform when many proteins are to be measured using many single-plex protein specific assays (de Jager and Rijkers, 2006). On the contrary, many protein analytes can be measured by the multiplexed bead-based assay with a single plate. This is extremely important for clinical studies where sample volumes are limited (Liu *et al*, 2005). Bead-based assay is more accurate because the median fluorescence is obtained from the readout of at least 50–100 beads. Thus, each bead functions as a duplicate making this assay more reliable (Kettman *et al*, 1998; Vignali, 2000).

The disadvantage of xMAP technology is the possible cross-reactivity between antibodies. Sensitivity may also be compromised because of the increasing number of beads per well. In addition, the performance in the multiplex assays can be variable as a result of the multipurpose diluent, which may not optimize each analyte to the same extent as in the case with single analyte measurements by ELISA (Carson and Vignali, 1999). In the laboratory, interleukin-8 (IL-8) and IL-1 β protein biomarkers are measured by ELISAs. Previous studies have shown significantly higher levels of these proteins among OSCC patients than control subjects (Hoffmann *et al*, 2007). In preparation for clinical applications, there is a need to perform multiplex biomarker assays using high throughput platforms.

Although Luminex multiplex bead-based assay kits have been optimized to measure protein levels in serum and cell lines, the purpose of this study was to examine if this technique can be used to measure IL-8 and IL-1 β levels in the saliva of OSCC and matched healthy control subjects.

Materials and methods

Patients

All participants in this study signed the University of California-Los Angeles Institutional Review Board-approved consent form agreeing to donate saliva for experiments. Twenty Caucasian OSCC patients with ages ranging from 32 to 79 years were recruited from the Division of Head and Neck Surgery/Otolaryngology at the University of California Los Angeles (UCLA) Medical Center. A majority of the OSCC patients were diagnosed with early stage oral cancer, as shown in the patient demographics in Table 1. No patient had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis or HIV infection as well as prior treatment in the form of chemotherapy, radiotherapy, surgery or alternative medicine. All the 20 healthy control subjects were Caucasian ranged in age from 24 to 75 years (Table 1). To examine if IL-8 is significantly elevated in periodontitis patients, we also included 10 periodontitis patients and 10 healthy control subjects recruited at the UCLA School of Dentistry for this study.

Saliva collection

Unstimulated whole saliva samples were collected between 9 AM and 10 AM. Patients were asked to refrain from eating, drinking, smoking, and oral hygiene procedures for at least 1 h before saliva collection. Samples were collected by asking subjects to first rinse their mouth with water and then expectorate saliva into a 50-ml centrifuge tube kept on ice for 10 min. The oral cancer and healthy control subjects were not formally screened for periodontal disease but were asked not to donate saliva if they suffered from bleeding gums. Samples which contained visible traces of blood were discarded from this study. Protease inhibitor cocktail (1 μ l ml⁻¹ of aprotinin, 10 mg ml⁻¹ of phenylmethylsulfonyl fluoride, 400 mM sodium orthovanadate; Sigma-Aldrich, St. Louis, Mo, USA) was added immediately after sample collection to minimize protein degradation. Briefly, 2 ml of clear whole saliva was obtained from patients after centrifugation at 2600 g for 15 min to remove cell pellets and debris. The samples were then divided into 20 μ l aliquots to reduce freeze thaw cycles; consequently, the samples used for this study was freeze thawed twice.

Bead-based assays

Human IL-8 and IL-1 β Fluorokine MultiAnalyte Profiling systems (Fluorokine MAP) were performed according to R&D systems protocol (R&D systems, Minneapolis, MN, USA). Saliva samples were diluted five times with calibrator diluent for the IL-1 β assay and eight times for the IL-8 assay. Initially, the 96-well filter bottom plate

Table 1 Demographics of patients (a) and healthy control subjects (b)

Patient	Diagnosis	Staging	TNM staging	Location	Age (years)	Ethnicity	Sex	Tobacco
(a)								
1	OSCC	I	T1N0M0	Tongue	32	Caucasian	M	Yes
2	OSCC	I	T1N0M0	Tongue	55	Caucasian	M	Yes
3	OSCC	I	T1N0M0	Tongue	63	Caucasian	F	No
4	OSCC	I	T1N0M0	Right alveolar ridge	79	Caucasian	F	Yes
5	OSCC	I	T1N0M0	Right tonsil	66	Caucasian	F	Yes
6	OSCC	II	T1N1M0	Tongue	53	Caucasian	M	No
7	OSCC	I	T2N0M0	Tongue	49	Caucasian	F	No
8	OSCC	II	T2N0M0	Tongue	66	Caucasian	F	No
9	OSCC	II	T2N0M0	Floor of mouth	69	Caucasian	F	No
10	OSCC	II	T2N0M0	Left Tonsil	55	Caucasian	M	Yes
11	OSCC	II	T2N0M0	Base of tongue	58	Caucasian	M	No
12	OSCC	II	T2N0M0	Left tonsil	59	Caucasian	M	No
13	OSCC	II	T2N0M0	Right tongue	77	Caucasian	M	Yes
14	OSCC	II	T2N1M0	Base of tongue	40	Caucasian	M	No
15	OSCC	III	T2N1M0	Left tonsil	65	Caucasian	M	Yes
16	OSCC	IV	T2N2aM0	Right tonsil	43	Caucasian	F	Yes
17	OSCC	IV	T2N2aM0	Base of tongue	53	Caucasian	M	Yes
18	OSCC	III	T2N2bM0	Base of tongue	65	Caucasian	M	Yes
19	OSCC	III	T2N2M0	Pharynx	62	Caucasian	M	Yes
20	OSCC	IV	T4N2M0	Tongue and floor of mouth	73	Caucasian	F	No
Subject	Diagnosis		Age (years)		Ethnicity		Sex	Tobacco
(b)								
1	Normal		31		Caucasian		F	No
2	Normal		34		Caucasian		F	Yes
3	Normal		59		Caucasian		F	Yes
4	Normal		59		Caucasian		F	No
5	Normal		75		Caucasian		F	No
6	Normal		24		Caucasian		M	No
7	Normal		24		Caucasian		M	No
8	Normal		25		Caucasian		M	No
9	Normal		26		Caucasian		M	No
10	Normal		30		Caucasian		M	Yes
11	Normal		39		Caucasian		M	Yes
12	Normal		55		Caucasian		M	Yes
13	Normal		24		Caucasian		M	No
14	Normal		24		Caucasian		M	No
15	Normal		52		Caucasian		M	Yes
16	Normal		41		Caucasian		M	Yes
17	Normal		40		Caucasian		M	Yes
18	Normal		38		Caucasian		M	Yes
19	Normal		26		Caucasian		M	Yes
20	Normal		49		Caucasian		F	Yes

OSCC, oral squamous cell carcinoma; TNM, TNM classification of malignant tumors, T: size of primary tumor, N: degree of spread to regional lymph nodes, M: presence of metastasis.

were prewet. Fifty microlitre of diluted microparticle solution and 50 μ l of sample were added to each well in duplicate. Next the plate was incubated for 3 h and washed three times with wash buffer. Afterwards, 50 μ l of diluted biotin antibody was added to each well and incubated for 1 h. The plate was then washed as described above and 50 μ l of diluted Streptavidin-PE was added to each well and incubated for 30 min. All incubations were performed at room temperature on an orbital shaker set at 15 g. Finally, the plate was washed again with 100 μ l of wash buffer. The median relative fluorescence units were measured using the Luminex 100 analyzer (Luminex, Austin, TX, USA). For the multiplexed assays, the same procedure was followed except that the IL-8 and IL-1 β microparticles were pooled and then subsequently added to each well.

ELISA

Enzyme-linked immunosorbent assay (Pierce, Rockford, IL, USA) was performed to determine the IL-8 levels in the saliva samples of OSCC ($n = 40$) and control patients ($n = 42$). Samples were diluted 1:8 in sample diluent and 50 μ l was loaded, in duplicate, onto a 96-microwell Plate coated with anti-human IL-8 antibodies. Similarly, the IL-1 β ELISA assay (Pierce, Rockford, IL, USA) was performed in the saliva samples of OSCC ($n = 36$) and control patients ($n = 42$) with a dilution factor of 1:5. After incubation for 1 h on a rotator (2.5 g), the microwell strips were washed three times with approximately 300 μ l of washing buffer, followed by the addition of 50 μ l of biotinylated antibody reagent to each well. The plate was incubated again for 1 h followed by a wash (3 \times) with the washing buffer.

Subsequently, 100 μl of streptavidin-HRP solution was added to all wells and incubated for 30 min. After another wash (3 \times), 100 μl of premixed 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well and incubated, in the dark, for 30 min. Finally, stop solution (100 μl) was added to each well and the absorbance was measured. The IL-8 ELISA assay was also performed on the saliva samples of periodontitis ($n = 10$) and control subjects ($n = 10$) using the same method described above.

Statistical analysis

Single-plex and multiplex raw data were transformed by natural logarithm to obtain normal distribution. Student *t*-test was employed for the comparison of single-plex and multiplex data and the critical alpha level of 0.05 was defined for statistical significance. The Pearson correlation coefficient of log transformed single-plex and multiplex data was calculated and represented by R^2 values. Using this set of data, we conducted receiver operating characteristic (ROC) curve analyzes to evaluate overall performance of the predictive power of each of the biomarkers. The optimal cut-point was determined for each biomarker by searching for those that yielded the maximum corresponding sensitivity and specificity. ROC curves were then plotted on the basis of the set of optimal sensitivity and specificity values. Area under the curve was computed via numerical integration of the ROC curves. All statistical data analysis was performed by the statistical software packages R 2.5.0 and Bioconductor.

Results

We demonstrated that both single-plex and multiplex assays could be used to determine salivary IL-8 and IL-1 β levels using xMAP. IL-1 β level in saliva was found to be statistically higher in OSCC patients than in control subjects. The boxplot in Figure 1b shows the distribution of IL-1 β levels in OSCC and control subjects. The average level of IL-1 β from the single-plex assay was

determined as $945.2 \pm 1134.8 \text{ pg ml}^{-1}$ for OSCC subjects ($n = 20$) and $314.2 \pm 444.8 \text{ pg ml}^{-1}$ for matched control subjects ($n = 20$). ROC analysis resulted in an ROC value of 0.77 with a sensitivity of 75% and specificity of 80%, respectively. The IL-8 concentration in saliva was also statistically higher in OSCC than in control (Figure 2b). The average level of IL-8 from the single-plex assay was $3313.2 \pm 3759.8 \text{ pg ml}^{-1}$ for OSCC ($n = 20$) and $1061.7 \pm 1978.8 \text{ pg ml}^{-1}$ for controls ($n = 20$). The ROC analysis showed an ROC value of 0.80 with a sensitivity of 75% and specificity of 80%, respectively. These results confirmed the use of IL-8 and IL-1 β as biomarkers for OSCC detection (St John *et al*, 2004).

Similar results were obtained for both IL-1 β and IL-8 from the multiplexed assays (Figures 1a and 2a). The average levels of IL-1 β were $1013.5 \pm 1221.1 \text{ pg ml}^{-1}$ in OSCC subjects ($n = 20$) and $376.3 \pm 576.3 \text{ pg ml}^{-1}$ in control subjects ($n = 20$). The ROC analysis resulted in an ROC value of 0.74 with a sensitivity of 80% and a specificity of 65%, respectively. The average levels of IL-8 were $2834.9 \pm 3385.6 \text{ pg ml}^{-1}$ in OSCC subjects ($n = 20$) and $947.3 \pm 2036.8 \text{ pg ml}^{-1}$ in control subjects ($n = 20$). The ROC analysis revealed an ROC value of 0.81 with a sensitivity of 75% and specificity of 80%, respectively. These results are summarized in Table 2. The multiplex and single-plex Luminex assays show a high correlation coefficient of $R^2 = 0.9025$ (Figure 3).

We also measured salivary IL-8 and IL-1 β levels in the same patients with OSCC and control subjects using ELISA. The average levels of IL-8 using the ELISA assay were 3347.7 ± 2929 (OSCC, $n = 40$) and $759.4 \pm 563 \text{ pg ml}^{-1}$ (control, $n = 42$). The ROC analysis indicated an ROC value of 0.82 and a sensitivity and specificity of 87.5% and 64.3%, respectively. The average levels of IL-1 β were $591.5 \pm 618.7 \text{ pg ml}^{-1}$ (OSCC, $n = 36$) and $79.6 \pm 57.8 \text{ pg ml}^{-1}$ (control, $n = 42$). The ROC analysis indicated an ROC value of 0.84 and a sensitivity and specificity of 63.9% and 100%, respectively. Luminex assay and ELISA gave

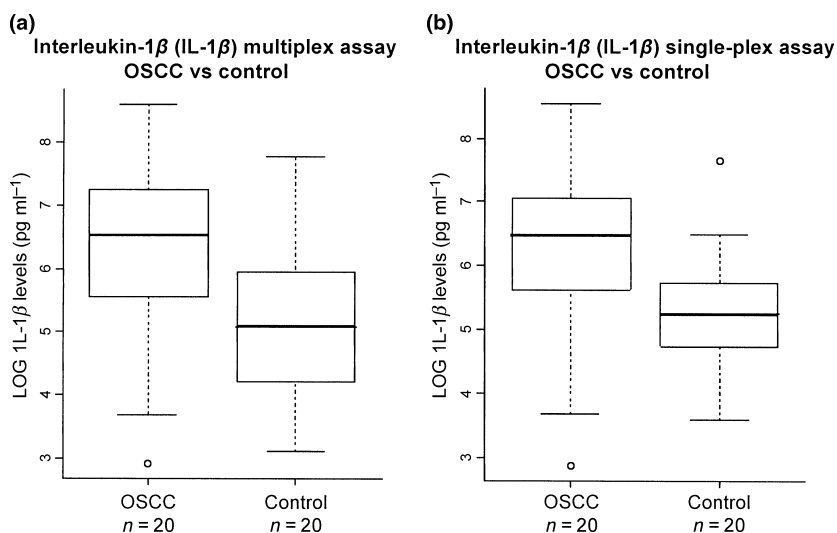


Figure 1 Comparison of multiplex (a) and single-plex (b) assays for the measurement of IL-1 β in the saliva of oral squamous cell carcinoma (OSCC) versus control subjects. For the multiplex assay (a), the average levels of IL-1 β are $1013.5 \text{ pg ml}^{-1}$ in OSCCs ($n = 20$) and 376.3 pg ml^{-1} in controls ($n = 20$). For the single-plex assay (b), the average levels of IL-1 β are 945.2 pg ml^{-1} in OSCCs ($n = 20$) and 314.2 pg ml^{-1} in controls ($n = 20$)

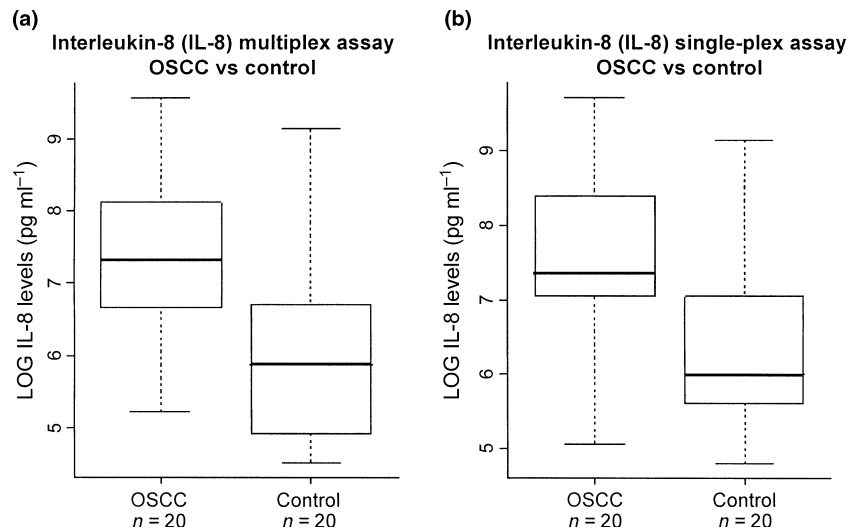


Figure 2 Comparison of multiplex (a) and single-plex (b) assays for the measurement of IL-8 in the saliva of OSCC versus control subjects. For the multiplex assay (a), the average levels for OSCCs ($n = 20$) and controls ($n = 20$) are 2834.9 and 947.3 pg ml^{-1} , respectively. For the single-plex assay (b), the average levels for OSCCs ($n = 20$) and controls ($n = 20$) are 3313.2 and 1061.7 pg ml^{-1} , respectively

highly correlated results. The correlation coefficient (R^2) was 0.91 for IL-8 ($n = 19$) and 0.84 for IL-1 β ($n = 19$). Figure 4 shows the correlation coefficient between the single-plex Luminex assay and ELISA for IL-8 and IL-1 β combined is $R^2 = 0.8794$.

Salivary IL-8 levels in periodontitis patients and matched controls were also measured using ELISA. The average level of IL-8 for the periodontitis patients ($n = 10$) was $818.82 \pm 228.41 \text{ pg ml}^{-1}$ and $589.24 \pm 370.29 \text{ pg ml}^{-1}$ for the control subjects ($n = 10$). The P -value between the periodontitis and control groups were determined as 0.098.

Discussion

The results of this study indicate that the Luminex xMAP technology is a useful platform for validation of salivary protein biomarkers with both single-plex and multiplex assays. Previous studies have reported achievable results using the multiplex bead-based assay for serum, plasma and cell culture supernatant samples (Oliver *et al*, 1998; Kellar *et al*, 2001; de Jager *et al*, 2003; Brailo *et al*, 2006; Allen *et al*, 2007; Linkov *et al*, 2007). Carlson and Vignali reported that the multiplex assay performed as well as the single-plex assay for cytokines but provided better sensitivity than ELISA (Carlson and Vignali, 1999). A recent study also found a

high correlation between bead-based assays and ELISA (duPont *et al*, 2005). Therefore, multiplex bead-based assays may substitute the ELISA method when a large number of protein analytes need to be validated. The measured levels of IL-8 in the saliva of OSCC subjects from this study are also comparable with those measured by ELISA from another group of researchers (Rhodus *et al*, 2005). We also demonstrated that there was a high correlation between the Luminex assay data and the ELISA data, and the multiplex assays were found as effective as the single-plex assays for measuring salivary proteins.

The average level of IL-1 β in OSCC patients obtained from the single-plex assay differed from the multiplexed assay by 7.0%, whereas the measurements in control subjects differed by 18.0% between the two assays. As for the measurements of IL-8, the average level of IL-8 in OSCC patients differed by 15.6% between the single-plex and multiplex assays whereas the difference between these two assays for IL-8 in control subjects was 11.4%. In our study, the IL-8 levels and the IL-1 β levels obtained with the single-plex and multiplex assay are comparable to the levels obtained using ELISA. The high correlation between the Luminex assay and ELISA for IL-8 and IL-1 β shows the effectiveness of the Luminex assays for detecting protein levels in saliva. Furthermore, the similar results obtained with the

Table 2 Comparison of single-plex and multiplex assays for the measurement of interleukin (IL)-8 and IL-1 β proteins in saliva of oral squamous cell carcinoma (OSCC) and matched control subjects

Protein	Mean level (pg ml^{-1})		P-value	ROC	Sensitivity (%)	Specificity (%)
	OSCC ($n = 20$)	Control ($n = 20$)				
IL-8 (single-plex)	3313.2 ± 3759.8	1061.7 ± 1978.8	0.02	0.80	75	80
IL-8 (multiplex)	2834.9 ± 3385.6	947.3 ± 2036.8	0.04	0.81	75	80
IL-1 β (single-plex)	945.2 ± 1134.8	314.2 ± 444.8	0.03	0.77	75	80
IL-1 β (multiplex)	1013.5 ± 1221.1	376.3 ± 576.3	0.04	0.74	80	65

ROC, receiver operating characteristic.

IL-8 and IL-1 β multiplex vs. single-plex assay, $R^2 = 0.78$

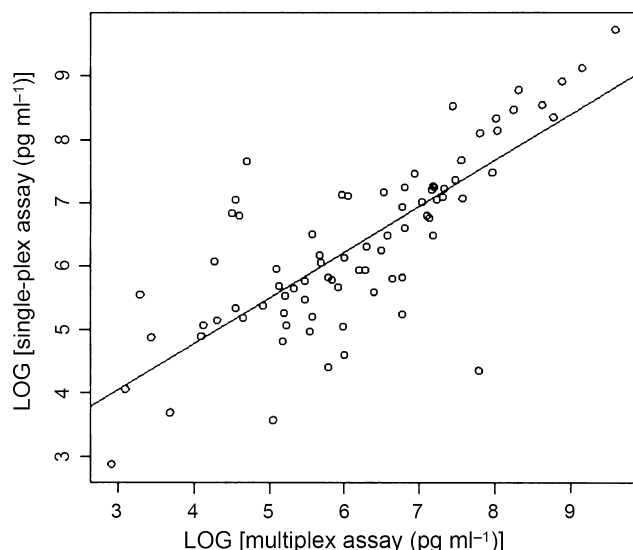


Figure 3 Correlation between the multiplex and single-plex assays of interleukin (IL)-8 and IL-1 β . The R^2 value is 0.78

single-plex and multiplex assays confirm the benefits of multiplexing by Luminex xMAP technology without compromising the accuracy.

There has been concern for using inflammatory cytokine proteins such as IL-8 and IL-1 β as biomarkers although previous studies have firmly shown significantly higher levels of these proteins among OSCC patients than control subjects (Hoffmann *et al*, 2007). Cytokines are intercellular signaling proteins which play a role in regulating growth, cellular proliferation, angiogenesis and tissue repair. They also

function in immune responses to infection, injury and inflammation (Ray *et al*, 2005; de Jager and Rijkers, 2006). Several conditions can also give rise to increased levels of inflammatory proteins such as rheumatoid arthritis, periodontal disease, osteoporosis, Sjögren's syndrome, chronic parotitis, severe exercise and diabetes. Therefore, an immunological disease control group may be included for further validation of IL-8 and IL-1 β as truly discriminatory markers for OSCC. We assayed salivary IL-8 levels in patients with severe periodontal disease ($n = 10$) and found that while they have elevated IL-8 in saliva, patients with oral cancer have significantly higher saliva levels of IL-8. This confirms that although an immunological disease may increase cytokines levels, the levels are not as high as those found in OSCC patients. It should be noted that cytokines are one of the most low-abundant proteins in human saliva. Therefore, this validation platform should be applicable to most of the salivary proteins.

There are limitations in this study that need to be acknowledged. First, the sample size is relatively small. A larger sample cohort would allow for a greater statistical comparison between the cancer and healthy control groups. Another limitation of this study is that the multiplex assay had slightly lower values for measurement of IL-8 and IL-1 β than the single-plex assay. The lower values can be attributed to the beads not being specific to one protein. A previous study comparing bovine plasma IL-1 β levels using single-plex and multiplex assays also reported a difference of 6.4% between the assays (Dernfalk *et al*, 2007).

In summary, we have demonstrated that Luminex xMAP technology can be used to validate and quantitate protein levels in saliva. The high correlation between the Luminex assays and ELISA confirms that Luminex xMAP technology is a reliable method for quantification of salivary proteins. We have also showed that the multiplex assay provided comparable results to the single-plex assays therefore demonstrating the efficacy of this technology. Our results suggest that saliva can be a promising and valuable diagnostic fluid because it contains measurable proteins, such as IL-8 and IL-1 β , at differential levels that can discriminate disease process. Future validation studies may need to be performed using a larger patient cohort, including periodontal disease group that should be included to validate firmly IL-8 and IL-1 β as protein biomarkers for OSCC. The ability to engage a high throughput platform such as the Luminex xMAP for multiplex protein biomarkers detection in saliva is a significant technological advancement towards the eventual utilization of saliva as a clinical diagnostic fluid.

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IL-8 and IL-1 β ELISA vs. single-plex assay, $R^2 = 0.81$

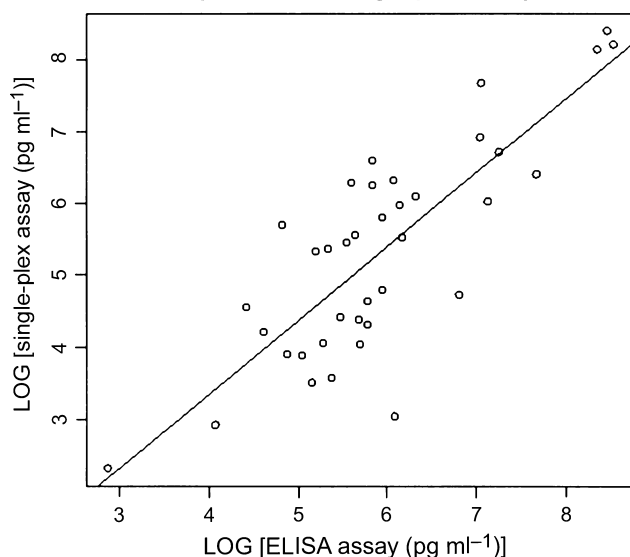


Figure 4 Correlation between the single-plex and ELISA assays of interleukin (IL)-8 and IL-1 β . The R^2 value is 0.81

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Author contributions

Martha E. Arellano-Garcia contributed to the designing of the experiment, acquisition, analysis and interpretation of the data. She also drafted the paper. She Hu provided his expertise and designed the experiment. He had a major contribution in the analysis and interpretation of data. He also helped draft the paper and critically revised it. Hui Zhou is the statistician who analysed the data and made statistical calculation. Jianghua Wang is the clinician who collected the patient samples and obtained written consent. Bradely Henson is the clinician who helped collect the patient samples. David Chia provided his expertise and knowledge about multiplexing technology and provided the instrument for data acquisition. David T. Wong provided expertise in designing the study and critically revised the manuscript.

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