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Simultaneous Measurement of Multiple Mouse Ear Proteins with Multiplex ELISA Assays

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Abstract

A recent advancement in enzyme-linked immunosorbent assay (ELISA) technology is the multiplex antibody array that measures multiple proteins simultaneously within a single sample. This allows reduction in sample volume, time, labor, and material costs, while increasing sensitivity over single ELISA. Current multiplex platforms include planar-based systems using microplates or slides, or bead-based suspension assay with microspheres. To determine the applicability of this technology for ear research, we used 3 different multiplex ELISA-based immunoassay arrays from 4 different companies to measure cytokine levels in mouse middle and inner ear tissue lysate extracts 24 hours following transtympanic *Haemophilus influenzae* inoculation. Middle and inner ear tissue lysates were analyzed using testing services from Quansys BioSciences, Aushon Biosystems SearchLight (both microplate-based), Milliplex MAP Sample (bead-based), and a RayBiotech, Inc. (slide-based) kit. Samples were assayed in duplicate or triplicate. Results were compared to determine their relative sensitivity and reliability for measures of cytokine related to inflammation. The cytokine pg/ml amounts varied among the multiplex assays, so a comparison also was made of the mean fold increase in cytokines from untreated controls. Several cytokines and chemokines were elevated, the extent dependent upon the assay sensitivity. Those most significantly elevated were IL-1 α , IL-1 β , IL-6, TNF α , VEGF, IL-8/MIP-2. The results of the multiplex systems were compared with single ELISA kits (IL-1 β , IL-6) to assess sensitivity over the traditional method. Overall, the Quansys Biosciences and SearchLight arrays showed the greatest sensitivity, both employing the same multiplex methodology of a spotted array within a microplate well with chemiluminescent detection. They also were more sensitive than the traditional single ELISA performed with commercial kits and matched gene expression changes determined by quantitative RT-PCR. The Quansys array showed a limit of detection for ear IL-6 down to 2–4 pg/ml, indicating it is sufficiently sensitive to detect ear proteins present in low concentrations. Thus, the multiplex ELISA procedures appear suitable and reliable for the study of hearing related proteins, providing accurate, quantitative, reproducible results with considerable improvement in sensitivity and economy.

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Keywords

Multiplex ELISA; middle ear; inner ear; protein; inflammation

1. Introduction

Hearing research often requires accurate protein measurement in the ear, brain, or serum to assess normal cellular mechanisms or progression of expression during phases of development. Also, protein characterization is becoming increasingly important in hearing disease processes and their recovery, either spontaneously or following therapeutic treatments. Since one of the first attempts at measuring specific protein levels in the ear with enzyme-linked immunosorbent assay (ELISA) (ten Cate et al. 1992), this technique has become the standard for most hearing research efforts. However, ELISA is expensive and time consuming due to the assessment of one protein at a time, even with prefabricated kits. If commercial kits are not available, ELISA may require long development times to perfect suitable capture-detection antibody pairs for assay optimization and validation. The difficulties with cochlear protein extraction and the small protein yields in mice also limit the number of proteins that can be assayed from the same sample using traditional ELISA. Thus, many animals are required to get sufficient material for studies involving multiple proteins, making experimental group comparisons more difficult because of employing different mice for the different protein ELISAs. Furthermore, the inherent folding of some cochlear proteins often prevents binding of antibodies, requiring special techniques (e.g., sodium dodecyl sulfate) to expose reactive sites (Curtis et al. 1993). This additional procedural variable, while necessary for detection, may not be optimal for quantitative comparisons with other proteins not subjected to the same protocol. Thus, the field of auditory research would gain enormous benefit from protocols that improve the accurate measurement of hearing related proteins with sufficient sensitivity, efficiency, and cost effectiveness.

A recent advancement in ELISA technology is the multiplex ELISA array that measures multiple proteins simultaneously within a single tissue sample. The basic principle entails laying down multiple capture antibodies in a small area (single well, bead). Thus, only a small volume of tissue lysate is needed to cover them, but they have sufficient intervening space to permit the photodetection of labeled detection antibodies. This allows for a significant reduction in sample volume, time, labor, and material costs, while increasing sensitivity over single ELISA (Ray et al. 2005; Elshal et al. 2006). Ultimately this means fewer animals or less patient serum is required for comprehensive assays of multiple proteins. Current multiplex platforms include planar-based systems using microplates (Backen et al. 2009) or slides (Wingren et al. 2009; Young 2009), or bead-based suspension assays (Khan et al. 2004; de Jager et al. 2006; Elshal and McCoy 2006; Leng et al. 2008). Application of this multiplex technology has now been made to a wide variety of diseases and tissues (Wingren and Borrebaeck 2009), but not the ear.

The goal of this study was to determine the applicability of this new multiplex ELISA technology for hearing research. Because of our interest in inflammatory mechanisms in the ear, we employed the technique in the measurement of inflammatory cytokine levels in mouse middle and inner ear tissue lysates following transtympanic *Haemophilus influenza* (H flu) inoculation. We tested 3 different multiplex ELISA immunoassay array formats (microplate, slide, and bead) from 4 different companies. Cytokine protein concentrations were measured to compare each array's ability to detect the small protein amounts, as well as their relative sensitivities. Traditional single ELISA was conducted for two cytokines to compare multiplex array sensitivity with current technology.

2. Material and methods

2.1 Inflammation Model

The aim of this study was to compare the ability of different multiplex ELISA immunoassays to measure inflammatory cytokine levels in mouse middle and inner ears. Female BALB/c mice (10–12 weeks of age) were bilaterally injected transtympanically with heat-killed H flu according to our standard protocol (MacArthur et al. 2006). After 24 hours, mice were killed and the middle and inner ears were harvested. For the inner ear tissue assessment, both inner ears from a mouse were pooled as one sample. For the middle ear measures, the middle and inner ear tissues were not separated in order to keep all middle ear inflammatory contents contained and not lose mucosa. However, the inner ear was dissected away from the cranial side to leave only the cochlear wall abutting the middle ear to minimize inner ear impact. Then the two middle ears from one animal were pooled as one sample. Thus, middle ear and inner ear samples for analyses were not from the same mice. Middle ear (N = 3–5) and inner ear (N = 3–5) tissues were prepared for each testing system. The only exception to this sample size was the inner ear analysis for RayBiotech in which two samples were available.

2.2 Tissue Preparation

Tissues were washed with cold 0.1 M phosphate buffer and placed in a 1.5 ml microcentrifuge tube with 100 µl of T-PER® Tissue Protein Extraction Reagent (Thermo Scientific Pierce) containing dithiothreitol (1.0 mM) and Protease Inhibitor Cocktail (1:100). T-PER is a mild tissue cell lysis solution designed for total protein extraction. Samples are stored at –80°C until homogenized and then again at –80°C until assayed.

Homogenization is carried out using the BioMasher®, composed of a disposable micro homogenizer, filter column, and collection tube. After grinding and centrifuging at 14,000 rpm at 4°C to separate the bone fragments, the supernatant was agitated at 4°C for 20 minutes in a TurboMix Vortex attachment. Then it was spun again for 10 min at 14,000 rpm at 4°C. Protein concentration was determined using the Bradford or Pierce Protein 660 nm Protein Assay. Samples are adjusted to 2–3 µg/µl and diluted further at 1:2 or 1:3 in their respective assays to achieve approximately 30 µg of protein per well to normalize the cytokine measures.

2.3 Multiplex arrays

Tissue lysates were analyzed using Sample Testing Services of Quansys Biosciences Inc. (microplate-based), Aushon Biosystems: SearchLight Assays Services (microplate-based), Milliplex MAP (bead-based), and a RayBiotech, Inc. (slide-based) kit.

Aushon SearchLight Protein Array Technology—This is a microplate-based array in which up to 16 cytokines can be detected in a single well. The final step is binding with a secondary antibody conjugated with a chemiluminescent label. (<http://www.aushon.com/Sample-Testing-Service.php>).

Quansys Biosciences Inc—This also is a microplate-based array using Q-Plex™ Array technology that can measure up to 25 analytes in a single well. This also is assessed with chemiluminescent labeling. (<http://www.quansysbio.com>).

Milliplex™ MAP—This is a bead-based suspension array using the Luminex xMAP technology in which fluorescent-coded beads, known as microspheres, have cytokine capture antibodies on the bead surface to bind the proteins. Final analysis is with flow cytometry. (http://www.millipore.com/drugdiscovery/dd3/map_portfolio)

RayBiotech, Inc. Quantibody® Technology Array—This is a glass slide-based quantitative antibody array in which a standard glass slide can be spotted with up to 60 cytokine capture antibodies in 16 wells. (http://www.raybiotech.com/Quantibody_array.asp).

Assessment was made of inflammatory cytokines available in the standard testing services of the various companies. Although the total cytokine profiles available varied among the companies, all testing services had available the routine cytokines of general interest in middle ear inflammatory disease. These included the interleukins IL-1 α , IL- β , IL-6, IL-8 (MIP-2), and IL-10, as well as tumor necrosis factor (TNF α), vascular endothelial growth factor (VEGF), and granulocyte-macrophage colony stimulating factor (GM-CSF). Previous studies have shown that these cytokines are significantly elevated in the middle ear and inner ear following middle ear inoculation with *Streptococcus pneumonia* (MacArthur et al. 2010). VEGF and IL-8 were not available from Quansys. Samples were assayed in duplicate, triplicate, or quadruplicate depending on the sample volume requirements of the commercial companies' testing services.

Results from the companies were compared to determine their relative sensitivity for measures of the inflammatory cytokines. The amount of cytokine protein was measured and statistically compared (t-tests) against untreated controls and a probability of 0.05 or less was judged as significant. Because the individual company protocols differ in their reacting antibodies, incubation times, etc., the absolute cytokine amounts can vary among the multiplex assays (Lash et al. 2006; Liew et al. 2007; Leng et al. 2008). Therefore, to compare the relative effectiveness of the techniques, a calculation was made of the mean fold change in cytokine levels (to untreated controls) for each multiplex assay.

2.4 Standard ELISA

In order to confirm that the multiplex ELISA is as sensitive as traditional ELISA techniques, the same tissue lysates were assayed with DuoSet ELISA Development System kits from R&D Systems (Minneapolis, MN) for single protein quantification. Middle and inner ear cytokines IL-1 β and IL-6 in the control and inflamed extracts were analyzed for total protein levels and fold change due to inflammation. IL-1 β or IL-6 rat anti-mouse capture antibodies were incubated overnight on medium binding Costar 96 well EIA/RIA plates and rinsed 3 $\times$ in wash buffer. 50 μ l of mouse serum (and a 7-point duplicate standard curve) in reagent diluent was incubated for 2 hours and washed 3 $\times$ with wash buffer. Wells were incubated 2 hours with IL-1 β or IL-6 specific biotinylated goat anti-mouse detection antibody, rinsed 3 $\times$ with wash buffer, reacted with streptavidin-HRP in reagent diluent for 20 minutes, washed 3 $\times$ with wash buffer, and incubated 20 minute in a 1:1 substrate solution of hydrogen peroxide and tetramethylbenzidine (R&D Systems) to generate the blue colorimetric reaction. The plates were quenched and immediately read on the BioTek EL 800 plate reader at 450 and 540nm.

2.5 Quantitative RT-PCR

Analysis of cytokine gene expression was made with quantitative RT-PCR to compare with ELISA measures of gene products. Control (N = 16) and H flu inoculated (N = 16) mice were collected as above and half of each group used for middle ear (N = 8) or inner ear (N = 8) assessment. The two middle ears from each mouse were pooled, as were the two inner ears, and stored in RNeasy lysis buffer (Qiagen, Valencia, CA) at -20°C until RNA was extracted. Tissue RNA was extracted with the Qiagen (Valencia, CA) RNeasy Mini Kit. Tissue was transferred to tubes with 600 μ l of extraction buffer and homogenized with a PowerGen 125. RNA was quantified using a NanoDrop and all samples were made up to a concentration of at least 25 ng/ μ l. Real-time RT-PCR studies were conducted with an ABI Step One Plus system (Carlsbad, CA). Total RNA (200 ng) was reverse-transcribed using RT² First Strand

Kit (SABiosciences Corp, Frederick, MD) using the manufacturer's instructions. Then samples were prepared for Real-time PCR using the RT² Real-time SYBR Green/Rox PCR master mix. Thermal cycle condition was set as: 95 °C 10 min, then 40 cycles: 95 °C 15sec, 60 °C 1 min, followed by a melt curve. Data analysis followed the suggestion of the manufacturer (SABiosciences PCR Array Data Analysis Web Portal). The parameter CT (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The statistical significance and fold change are calculated using the $\Delta\Delta C_t$ method with the aid of SABiosciences PCR Array Data Analysis Web Portal. The housekeeping gene used for this method was glyceraldehyde-3-phosphate dehydrogenase.

We used custom PCR Arrays (SABiosciences Corp, Frederick, MD) already optimized for reaction conditions, primers, and probe. Quantitative RT-PCR can be done simultaneously on primer arrays of multiple cytokine genes (RT² Profiler PCR Array System) for actual quantification of gene expression. Our custom PCR Array plates measure expression of ten key inflammation related cytokines: IL-1 α , IL-1 β , IL-6, IL-10, MIP-1 α , Mapk8, MIP-2, KC, TNF α , and VEGF. The RT-PCR results for fold change in IL-6 and IL-1 β gene expression were used in the current study for comparison with gene product quantification with the multiplex and standard ELISA methods.

3. Results

3.1 Multiplex arrays

The multiplex assays showed all of the cytokines were significantly elevated many fold at 24 hours compared to controls in most of the tests. Those most significantly increased were IL-1 α , IL-1 β , IL-6, IL-10, IL-8/MIP-2, TNF α , and VEGF. Similar patterns were seen in the middle ear (Table 1) and inner ear (Table 2). IL-6 was one of the strongest responding cytokines as it was increased several fold in both the middle ear and inner ear. The Quansys analysis showed IL-6 increased 104.3 fold in the middle ear (Table 1) and 45.6 fold in the inner ear (Table 2). Elevated levels of this protein were measured in the middle and inner ear with the other multiplex methods as well. Only the RayBiotech technique did not show it to be statistically increased in the middle ear in spite of a greater than 13 fold increase. Often the lack of a statistically significant change in a particular multiplex method, in spite of several fold increases, was the result of small sample sizes and resultant high variance. This is particularly evident in the RayBiotech results for IL-6 in the middle ear (Table 1) and inner ear (Table 2) where control and inflamed tissues showed nonoverlapping ranges, but did not reach the probability cutoff of 0.05.

IL-1 α levels in the normal middle ear showed a range of 82.8 pg/ml (RayBiotech) to 337.7 pg/ml with the Quansys method (Table 1). The fold change with H flu inflammation was similar across the four methods also, ranging from 0.8 to 3.3. In the inflamed inner ear, IL-1 α ranged from 22.2 pg/ml to 640.2 pg/ml, depending on the multiplex method (Table 2). The fold change ranged from 0.5 to 5.9. The other cytokine comparisons in the middle and inner ears showed similar patterns of a wide range of cytokine levels depending on the technique employed. This is presumably due to the specific parameters of the respective techniques.

Comparing the relative sensitivities of the multiplex methods, there appeared to be differences among the methods. Overall, the Quansys BioSciences and SearchLight arrays showed the greatest sensitivity of cytokine expression in middle ear and inner ear. These two employed the same multiplex methodology of spotted arrays within microplate wells and detection with chemiluminescent label. In every comparison with the other multiplex systems, these arrays showed the greatest fold change relative to controls. Nearly every

cytokine protein was measurably elevated (> 2 fold) compared to control tissues for the middle ear (Table 1). On the other hand, the Milliplex bead array technique and the RayBiotech slide technology often did not measure any level of cytokines due to lower sensitivity. In the bead array analysis, three of the cytokines were not measurable, reflecting a lowered sensitivity of that particular assay.

A similar pattern of assay sensitivity was seen for the inner ear (Table 2) as the Quansys and SearchLight assays were the most sensitive. Several of the cytokines were measured in higher quantities over controls. However, like the middle ear assays, the Milliplex and RayBiotech assays seldom measured cytokines at higher levels than controls. In fact, the RayBiotech assay showed most cytokines present in the inflamed tissues at levels only a fraction of controls, which seems highly unlikely given the elevated gene expression in the other methods.

3.2 Comparison against standard ELISA and RT-PCR

The single ELISA results for IL-1 β and IL-6 provided a comparison of sensitivity with the multiplex array results. IL-1 β was measured at a concentration of 90.8 pg/ml in the untreated middle ear tissues and 607.8 in the inflamed tissues (Table 1). This was considerably less than levels measured in the Quansys assay, but comparable to the other three assays. Similarly, IL-6 was measured in the single ELISA at a level of 104.8 pg/ml in the untreated middle ears and 222.4 in the inflamed. This was in the range of values determined in the multiplex arrays for controls, but considerably less than levels for the inflamed. Thus, for middle ear analytes, the standard ELISA provided protein concentration values equal to, or less than, the multiplex assays, suggesting the latter were more sensitive.

The graphical comparison of these middle ear values with quantitative RT-PCR shows the Quansys and SearchLight methods also match closely the fold change in gene expression (Fig 1A). Significant upregulation of the IL-1 β gene occurs at 6 hours, with reduced, but still significant, expression at 24 hours ($p < 0.05$). The magnitude of this gene activity is comparable to the protein level changes measured by the Quansys and SearchLight methods, both of which are microplate based. Similar patterns are seen for IL-6 where the gene expression upregulation shows fold changes comparable to the Quansys and SearchLight multiplex assays. On the other hand, the Milliplex and RayBiotech methods both show reduced levels of measured protein relative to fold changes in gene expression, but comparable to standard ELISA. Because the Quansys and SearchLight assays showed the least variability and their results paralleled gene expression analyses, these were interpreted to be the most reliable and accurate.

Inner ear standard ELISA (Table 2) showed a similar pattern to the middle ear. IL-1 β was measured at 47.7 pg/ml in the normal inner ear and 99.9 pg/ml in the inflamed inner ear. These values were considerably less than the Quansys and Searchlight assays, but comparable to the Milliplex and RayBiotech. IL-6 determinations by standard ELISA also were either comparable to, or significantly less than, values measured in the multiplex determinations. The graphical comparison of these inner ear inflammation values with quantitative RT-PCR (Fig. 1B) shows patterns similar to the middle ear. The Quansys and SearchLight methods are comparable to change in gene expression at 6 and 24 hours. This was particularly evident for the IL-1 β gene, with lower fold changes observed for the Milliplex and RayBiotech results. The same pattern is seen for IL-6 where the upregulation in gene expression is comparable to the two microplate-based multiplex assays (Quansys and SearchLight). Much lower levels of protein are measured in the Milliplex and RayBiotech methods, both of which are comparable to standard ELISA. As seen for the middle ear analyses, the Quansys and SearchLight multiplex assays were more sensitive in quantification of protein levels in the inner ear.

3.3 Limits of detection with dilution linearity

Occasionally hearing researchers need to measure inner ear proteins that occur in very small amounts, or they wish to subdivide the cochlear regions (lateral wall, organ of Corti) so that small amounts of tissue limit the amount of protein available. Application of the multiplex technique to such studies would require sensitivity comparable to, or better than, conventional ELISA. Therefore, a limit of detection comparison was made between the Quansys multiplex assay and standard ELISA method to determine if reliable protein determinations can be made when small amounts of total ear protein are present. Using the same tissue homogenates for both the standard ELISA and Quansys multiplex ELISA, dilution linearity curves were established for IL-6 with decreasing amounts of middle ear or inner ear protein in the sample (Fig. 2). The multiplex method reliably measured IL-6 down to levels of 2–3 pg/ml when the ear homogenates were diluted down to only 3 µg of normal middle ear tissue and 20–30 µg of normal inner ear tissue. Linear regression showed a middle ear correlation of 0.9847 ($p = 0.015$) and inner ear correlation of 0.9948 ($p = 0.0004$), demonstrating the significant relationship between tissue amounts and lower limit of IL-6 detection. On the other hand, standard ELISA performed on the same middle ear and inner ear homogenates did not reliably measure the same levels of IL-6 with comparable tissue amounts and failed to show linearity with sequential dilutions. It was concluded from these measures that the Quansys multiplex technique reliably measured 2–4 pg/ml of IL-6 with greater sensitivity over traditional ELISA. This suggests the multiplex technique can detect proteins that occur in very small concentrations in the ear.

1. Discussion

The results of the assays demonstrate that middle ear and inner ear proteins can be reliably analyzed by the multiplex ELISA technique. We conducted our analysis with commercially test services for inflammatory cytokines because these are the most commonly available and optimized by the various companies. The microplate based methods (Quansys BioSciences and SearchLight) provided the most sensitive results and matched the gene expression upregulation measures from RT-PCR data. The other techniques would lead one to the conclusion that there was no significant elevation in cytokines, which clearly is not the case. Interestingly, the bead array method was also reported to be less sensitive in other comparison studies (Lash et al. 2006). However, variability in techniques is often due to the selection of antibody pairs (Liew et al. 2007).

Comparison of multiplex results with standard ELISA for two of the prominent cytokines showed the multiplex assays were often much more sensitive. This is presumably due to optimization of the multiplex kits for each cytokine, as well as their use of the most sensitive chemiluminescent labeling. This matches findings of other comparison studies in which the multiplex assays were judged to be more sensitive than standard ELISA once optimized for a particular cytokine (Ray et al. 2005; Elshal and McCoy 2006). The dilutional linearity analysis also showed the limit of detection in the range of 2–4 pg/ml for the Quansys multiplex ELISA, demonstrating sufficient sensitivity to detect proteins present in small amounts in the inner ear.

While there are inherent differences between the various multiplex formats, the overall technique is rapid, cost effective, and reliable within each respective platform. This is due to commercial preparations of specific capture and detection antibody pairs being optimized for the specific protein in question, as well as eliminating cross-reactivity. When applying the multiplex ELISA for a specific research use, it is important to consider whether the method is suited for the type of tissue, required volume of analyte, matrix parameters, assay kit and data point costs, assay variability, quality of antibodies pairs, and whether it utilizes the more appropriate 5 Parameter Logistics Regression analysis (Lash et al. 2006; Liew et

al. 2007; Backen et al. 2009). A review of key criteria (Lash et al. 2006) can help investigators select a method to optimize a particular technical method for the specific tissue being analyzed. Although absolute protein pg/ml levels may not be consistent among the various multiplex formats, consistency within a particular technique can be expected. Thus, if researchers select the best format for their particular research goals, and design studies with proper controls, they can have confidence in their assay results and remain committed to that format. Generally researchers are more interested in fold change over controls rather than absolute measures of protein concentrations. However, if absolute concentrations are necessary, one would simply run protein standards in parallel with samples.

The Quansys Multiplex ELISA is now being optimized in our laboratory to measure proteins resulting from otitis media, autoimmune hearing loss, and other forms of inner ear and middle ear diseases. Quansys prepares custom arrays printed with the proteins of interest to us (ion homeostasis channels, inflammatory cytokines, tissue remodeling and growth factors). The development costs per protein may run a few thousand dollars (only the first time the company has to develop a capture/detection antibody pair), but the price includes several microplates. Furthermore, the ability to analyze multiple proteins per well and dozens of tissue samples per microplate, results in considerable cost savings compared to single ELISA. Instrumentation (camera, analytical software) also is cost effective for the Quansys method.

2. Conclusion

The multiplex ELISA procedures appear suitable and reliable for study of middle and inner ear proteins, providing accurate, quantitative, reproducible results with considerable improvement in economy. The application of this technique has enormous potential for hearing research. Any tissue that can be processed for protein separation (brain, ear, middle ear mucosa, bone, serum, plasma) can be analyzed with this technique. Thus, there is no theoretical limit to the tissues. Also, the ability to quantify so many proteins simultaneously permits investigation of factors that probably would not have been examined otherwise due to cost and time. Thus, we expect this technique will significantly enhance hearing research in the future.

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Figure 1.

A: Relationship of ELISA methods with cytokine gene expression in middle ear. Gene expression determined by RT-PCR fold change at 6 hours (PCR 6) and 24 hours (PCR 24) shows significant ($p < 0.05$) upregulation of both IL-1 β and IL-6. Comparison of RT-PCR with fold change in protein at 24 hours by the various multiplex methods reveals the Quansys and SearchLight results were comparable. The Milliplex and RayBiotech methods showed lower fold change readings, comparable to standard ELISA results.

B: Relationship of ELISA methods with cytokine gene expression in the inner ear. Patterns of cytokine gene expression for the inner ear were similar to the middle ear. The significant gene expression ($p < 0.05$) measured by RT-PCR for the inner ear was comparable to results of protein measures with the Quansys and SearchLight multiplex arrays. The standard ELISA showed lower fold change in protein present, particularly for IL-6, and these levels were similar to results in the Milliplex and RayBiotech arrays.

Figure 2.

Cytokine IL-6 measured by the Quansys multiplex array for normal middle ear and inner ear. This multiplex method measured IL-6 in the 2–4 pg/ml range reliably when minimal amounts of ear protein were sampled. The dilution linearity curves showed the regression values were statistically significant for middle ear ($r = 0.9847$; $p = 0.015$) and inner ear ($r = 0.9948$; $p = 0.0004$).

Table 1

Middle ear cytokine concentrations determined by multiplex ELISA assays

Cytokine	Group	Measured Cytokine Amount (pg/ml)					Standard ELISA
		Quansys Microplate	SearchLight Microplate	Multiplex Bead	RayBiotech Slide		
IL-1α	Control	337.7	210.0	301.1	82.8		
	Range	(118–828)	(66–477)	(249–374)	(36–144)		
	Inflamed	1124.1	972.2	582.7	70.1		
	Range	(707–1772)	(819–1132)	(512–717)	(29–104)		
	Probability	0.021	0.00004	0.007	0.76		
	Fold Change	3.3	4.6	1.9	0.8		
IL-1β	Control	632.4	26.4	60.5	75.5	90.8	
	Range	(562–686)	(19–36)	(39–82)	(10–205)	(63.2–167.8)	
	Inflamed	4402.2	614.8	225.9	98.2	607.8	
	Range	(3791–5569)	(465–696)	(165–274)	(24–199)	(423.7–803.6)	
	Probability	0.00003	0.000009	0.0001	0.80	0.0000003	
	Fold Change	7.0	23.2	3.7	1.8	6.7	
IL-6	Control	30.6	167.3	137.8	357.5	104.8	
	Range	(21–40)	(71–420)	(97–204)	(299–416)	(81.4–153.9)	
	Inflamed	3188.7	20853.4	1790.4	4716.0	222.4	
	Range	(725–4909)	(11642–29920)	(612–2635)	(3503–5929)	(132.8–283.1)	
	Probability	0.002	0.0004	0.001	0.07	0.00004	
	Fold Change	104.3	124.7	13.0	13.2	2.1	
TNFα	Control	47.3	21.9	4.6	1.0		
	Range	(32–56)	(15–30)	(4–5)	(0.7–1.2)		
	Inflamed	93.0	81.6	41.8	0.9		
	Range	(62–157)	(71–87)	(19–54)	(0.4–1.1)		
	Probability	0.03	0.000006	0.02	0.62		
	Fold Change	2.0	3.7	9.1	0.9		

Cytokine	Group	Measured Cytokine Amount (pg/ml)				
		Quansys Microplate	SearchLight Microplate	Milliplex Bead	RayBiotech Slide	Standard ELISA
	Range	22.6–49.8	12.0–22.0	29–65	32.4–42.9	
	Inflamed	74.8	27.7	41.4	38.0	
	Range	39.9–151.3	24.2–37.3	24–57	32.2–46.3	
	Probability	0.11	0.004	0.78	0.85	
	Fold Change	2.0	1.8	0.9	1.0	
VEGF	Control		4.1	30.9	206.1	
	Range		3.0–5.5	7–67	96.9–381.9	
	Inflamed		17.1	84.7	270.2	
	Range		9.7–30.2	36–143	253.5–289.5	
	Probability		0.008	0.05	0.51	
	Fold Change		4.2	2.7	1.3	
GM-CSF	Control	68.6	3.5	NM	0.4	
	Range	53.5–80.1	2.4–4.5		0.0–0.9	
	Inflamed	169.6	13.4	39.0	0.1	
	Range	91.0–317.0	7.5–22.8	34–55	0.0–0.2	
	Probability	0.06	0.005		0.33	
	Fold Change	2.5	3.8		0.3	
MIP-2/IL-8	Control		19.8	NM	246.2	
	Range		1.6–47.7		0.0–711.5	
	Inflamed		2597.2	1573.7	379.4	
	Range		2149.3–3187.9	1072–2473	324.5–487.9	
	Probability		0.000004		0.60	
	Fold Change		131.2		1.5	

Table 2

Inner ear cytokine concentrations determined by multiplex ELISA assays

Cytokine	Group	Measured Cytokine Amount (pg/ml)				
		Quansys Microplate	SearchLight Microplate	Multiplex Bead	RayBiotech ^a Slide	Standard ELISA
IL-1α	Control	46.4	144.2	260.3	43.8	
	Range	(42–55)	(137–151)	(206–318)	(41–52)	
	Inflamed	273.5	640.2	304.1	22.2	
	Range	(105–598)	(315–965)	(230–355)	(14–34)	
	Probability	0.086	0.26	0.43	0.04	
	Fold Change	5.9	4.4	1.2	0.5	
IL-1β	Control	427.4	85.2	40.7	24.2	47.7
	Range	(381–488)	(81–90)	(33–49)	(13–34)	(26.7–69.4)
	Inflamed	1302.1	467.1	85.2	28.7	196.6
	Range	(551–2454)	(416–518)	(74–96)	(26–34)	(160.6–232.5)
	Probability	0.07	0.018	0.005	0.50	0.004
	Fold Change	3.0	5.5	2.1	1.2	4.1
IL-6	Control	22.1	60.6	124.7	2328.0	58.6
	Range	(17–26)	(51–70)	(88172)	(736–3915)	(38.6–94.0)
	Inflamed	1008.2	8808.2	336.7	4798.6	44.8
	Range	(684–1398)	(4972–12645)	(255–475)	(3829–6437)	(44.1–45.4)
	Probability	0.001	0.15	0.045	0.30	0.49
	Fold Change	45.6	145.4	2.7	2.1	0.8
TNFα	Control	37.7	24.2	NM ^b	0.6	
	Range	(31–52)	(21–28)		(0.0–1.6)	
	Inflamed	41.0	38.5	11.9	0.4	
	Range	(7–55)	(38–40)	(11–14)	(0.0–1.1)	
	Probability	0.79	0.06	--	0.75	
	Fold Change	1.1	1.6	--	0.7	

Cytokine	Group	Measured Cytokine Amount (pg/ml)					Standard ELISA
		Quansys Microplate	SearchLight Microplate	Milliplex Bead	RayBiotech Slide	^a	
IL-10	Control	32.1	6.3	42.1	18.0		
	Range	4.0–40.5	5.5–7.1	26–54	0.0–54.1		
	Inflamed	58.3	14.2	33.4	14.1		
	Range	4.0–94.1	13.5–14.9	27–38	0.0–35.8		
	Probability	0.46	0.02	0.39	0.86		
	Fold Change	1.8	2.2	0.8	0.8		
VEGF	Control		2.9	14	201.5		
	Range		2.3–3.4	7–21	97.9–401.8		
	Inflamed		3.7	10	117.6		
	Range		3.3–4.0	7–13	99.9–134.5		
	Probability		0.35	0.49	0.45		
	Fold Change		1.3	0.8	0.6		
GM-CSF	Control	59.1	6.2	NM	0.1		
	Range	52.0–70.1	4.5–7.8		0.0–0.3		
	Inflamed	56.5	16.8	NM	0.0		
	Range	30.6–78.6	11.8–21.7		0.0–0.1		
	Probability	0.82	0.18		0.58		
	Fold Change	1.0	2.7		0.4		
MIP-2/IL-8	Control		28.5	NM	124.2		
	Range		25.8–31.2		0.0–345.6		
	Inflamed		188.4	209.6	58.0		
	Range		136.1–240.6	166–254	6.4–118.8		
	Probability		0.09		0.60		
	Fold Change		6.6		0.5		

^a N=2

^b Not Measureable (below assay detection)