



Studying Cytokines in EBC

Several reports of the identification of cytokines in EBC have been published in the international literature. In all cases, ELISA technology was employed for the assays

Included in the published reports are IL-4, IL-6, IL-8, IFN gamma and several others.¹⁻⁵

Levels are being reported as high as 50 pg/ml, and even higher. Assuming an EBC dilution factor of 3 logs relative to airway lining fluid (a very rough assumption that may be an underestimate), that would suggest that ALF levels are on the order of 50 ng/ml of these cytokines. This is an intriguingly high level, but perhaps not outside the realm of possibility. Little is known of actual ALF levels of any substance, as there is no unimpeachable gold standard.

Although we are certain that with sufficiently sensitive and specific tests, any substance found in the airway lining fluid will also be identifiable in EBC, we do worry that ELISA technology currently employed may sometimes be misleading. In this regard, there has been no published effort at validating the commercial ELISA assays in EBC.

Often the substances are reported at the limit of detection of the assay. It is not uncommon to have standard curves that form small valleys at the lower concentrations, leaving a given absorbance open to ambiguity as to what it represents.

An additional issue is that standards supplied with commercial kits contain a proteinaceous matrix. EBC is then added neat (straight up, as is), or sometimes after lyophilization and resuspension, to the ELISA assay, and then the absorbance results are compared to the proteinaceous standards. There may well be an "apples and oranges" effect therefore, as EBC contains only a small amount of protein. We worry that some unblocking of the plate may occur during the incubation of sample in the ELISA.

We make the following recommendations when studying cytokines in EBC:

As control experiments, consider adding the lyophilized proteinaceous matrix of the zero standard to EBC samples in order to make the matrices of the samples more alike. Alternatively, consider adding a DI water control instead of simply relying on the supplied zero standard. An additional consideration would be to dialyze aliquots of EBC sample to remove cytokines, and then assay the large-molecule-depleted sample to assure elimination or substantial reduction of signal compared to the original sample.

Perform sufficient control experiments using your collection technique and your assay. This sounds obvious, but these controls are not often reported in the published literature.

Please keep in mind that similar issues apply for ELISAs for other markers including nitrotyrosine (the ELISA for which presumably measures nitrotyrosine residues incorporated in proteins, not nitrotyrosine as a discrete peptide) and somewhat similar issues apply for *competitive* ELISAs that are often employed for a variety of smaller molecules such as leukotrienes.

References

1. Bucchioni, E., S. A. Kharitonov, L. Allegra, and P. J. Barnes. 2003. High levels of interleukin-6 in the Exhaled Breath Condensate of patients with COPD. *Respir Med* 97(12):1299-302.
2. Carpagnano, G. E., P. J. Barnes, D. M. Geddes, M. E. Hodson, and S. A. Kharitonov. 2003. Increased leukotriene b4 and interleukin-6 in Exhaled Breath Condensate in cystic fibrosis. *Am J Respir Crit Care Med* 167(8):1109-12.
3. Carpagnano, G. E., O. Resta, M. P. Foschino-Barbaro, E. Gramiccioni, and F. Carpagnano. 2002. Interleukin-6 is increased in breath condensate of patients with non-small cell lung cancer. *Int J Biol Markers* 17(2):141-5.
4. Kharitonov, S. A., and P. J. Barnes. 2001. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 163(7):1693-722.
5. Shahid, S. K., S. A. Kharitonov, N. M. Wilson, A. Bush, and P. J. Barnes. 2002. Increased interleukin-4 and decreased interferon-gamma in Exhaled Breath Condensate of children with asthma. *Am J Respir Crit Care Med* 165(9):1290-3.