

Optimal Clinical Relevance for LC-MS/MS Testing of Vitamin D

ZRT Laboratory is committed to giving its clientele the most accurate results possible. For vitamin D analysis, this issue is not as simple as it may seem. Results may be *analytically* accurate yet still lack *clinical* accuracy. ZRT, with the help of experts in the field of vitamin D testing, is helping to change the industry towards more clinically relevant results.

- Vitamin D status is monitored by measuring blood levels of 25-hydroxyvitamin D3.
- There are three common methods used for measuring 25-hydroxyvitamin D:
 - Liquid chromatography – tandem mass spectrometry (LC-MS/MS)
 - Radioimmunoassay (RIA) most commonly from DiaSorin
 - Liaison, an automated immunoassay by DiaSorin that has largely replaced the RIA
- LC-MS/MS is the preferred method for many labs (Mayo Clinic, Quest Labs, Esoterix, ZRT, and others), while Liaison is favored by other testing labs like LabCorp.
- Most LC-MS/MS analysis correlates strongly to DiaSorin, but LC-MS/MS values, on average, are higher across the entire range of results by 20-30% (see graph on Page 6).
- Whether LC-MS/MS or DiaSorin values are more accurate *analytically* is not fully understood. *Clinical* accuracy is ultimately what matters to physicians and patients.
- The DiaSorin RIA was used in almost every major vitamin D study (these are listed later in this document). From these studies have come the recommended optimal levels of 25-hydroxyvitamin D (many experts prefer 32-100ng/ml while some prefer a range that begins at 50ng/ml).
- For LC-MS/MS labs to have clinical relevance, our results must agree with the DiaSorin RIA results because they were used to generate currently accepted ranges.
- As of August 11, 2008, ZRT results have been calibrated against DiaSorin RIA values.
- ZRT's reference range, based on the majority of vitamin D studies, will be 32-100ng/ml.
- The graph in Figure 1 shows excellent agreement between the DiaSorin RIA and ZRT blood spot results for total 25-hydroxyvitamin D (D2 and D3).

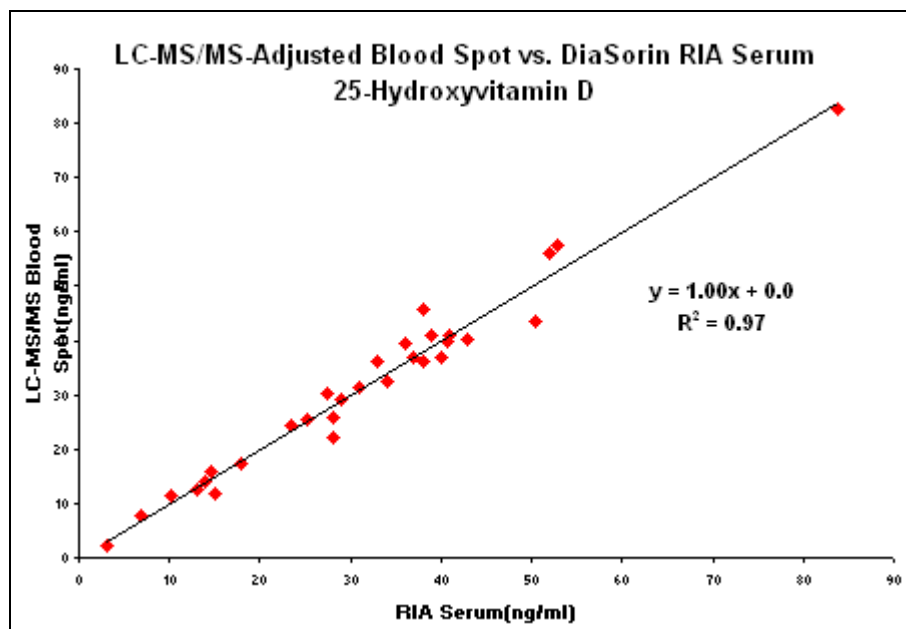


Figure 1. 25-Hydroxyvitamin D: ZRT LC-MS/MS-Adjusted vs. DiaSorin RIA

Different methods have been used over the past 30 years for measuring 25-hydroxyvitamin D. In 1985, Hollis and Napoli developed a radioimmunoassay (RIA) that accurately measured total 25-hydroxyvitamin D (Hollis and Napoli 1985; Hollis et al. 1993). This RIA assay was commercialized by DiaSorin and became a popular choice for vitamin D analysis. More recently, DiaSorin has developed an automated chemiluminescence immunoassay called Liaison that correlates reasonably well with the RIA. For purposes of this publication, we will simply refer to both the RIA and the Liaison tests as “DiaSorin.”

There are two potential drawbacks to the DiaSorin test. First, because of the lipophilic nature and low concentration of hydroxyvitamin D, this test is difficult to use for blood spot analysis. Secondly, if individuals are interested in separate measurements of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, this is not possible with the DiaSorin system (you simply get a ‘total’ 25-hydroxyvitamin D). Both of these limitations can be circumvented by the use of liquid chromatography – tandem mass spectrometry (LC-MS/MS), which has become the method of choice for serum analysis for some labs (most notably Quest Labs and the Mayo Clinic).

Because ZRT developed a blood spot test, LC-MS/MS was the obvious choice. But, how do we know that this method is *analytically* accurate?

- Serum samples measured for 25-hydroxyvitamin D by ZRT were sent to other labs using LC-MS/MS, and a very strong correlation was seen (see Figure 2).

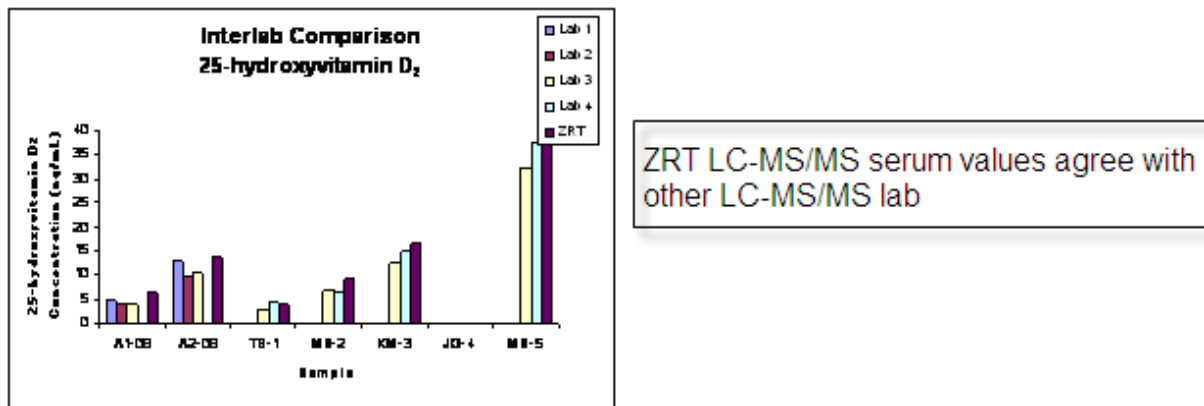
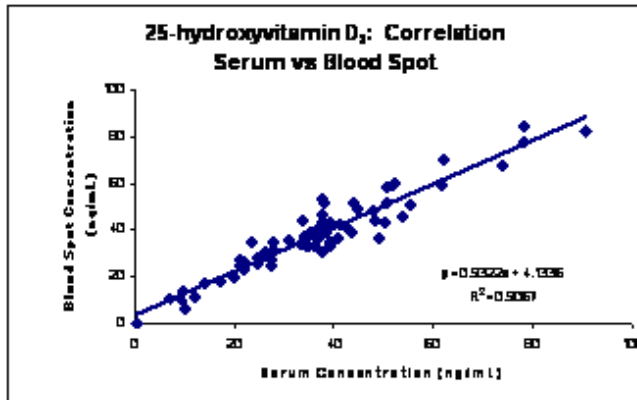


Figure 2. Interlab Comparison of 25-hydroxyvitamin D

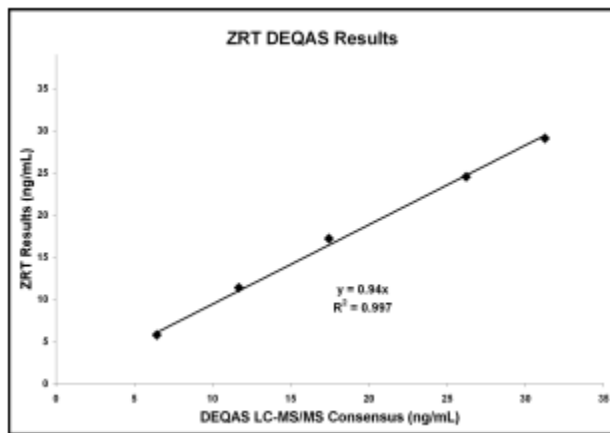
- When blood serum and blood spot samples taken simultaneously from the same individual are measured by ZRT, strong correlation is seen (see Figure 3).



ZRT LC-MS/MS blood serum and blood spot methods generate the same results, making the blood spot assay a viable option for testing

Figure 3. Serum 25-Hydroxyvitamin D3: Correlation Serum vs. Blood Spot

- ZRT participates, along with hundreds of other labs, in DEQAS (Vitamin D Quality Assessment Scheme). In this scheme, serum samples of varying concentrations of 25-hydroxyvitamin D are sent to labs and results are compared. As you can see from Figure 4, ZRT results compare very well with the consensus of all labs using LC-MS/MS.



DEQAS proficiency results show that ZRT results agree with the consensus from many other labs using LC-MS/MS, but the chart on page 5 shows that these results are higher than RIA generated results

Figure 4. ZRT DEQAS Results

Because LC-MS/MS is considered to be an accurate method, and results compare well with other LC-MS/MS labs, we can conclude with reasonable certainty that results are *analytically* accurate. Does this ensure that results are also *clinically* accurate? Not necessarily.

If, for example, ZRT developed its reference range by measuring levels of 25-hydroxyvitamin D in thousands of individuals but did so in the dead of winter in the Pacific Northwest (where the sun rarely makes an appearance), our reference range would be inappropriately low. This would make our results clinically inaccurate because we would be reporting “normal” results for vitamin D-deficient individuals.

Thanks to the prevalence of indoor jobs, high use of sunscreen, and people living at high latitudes, much of the population is vitamin D deficient. Therefore, reference ranges based solely on measuring large groups of people (particularly if they do not live in sunny areas) are inadequate. So, what have labs done to establish reference ranges? They have relied on large

studies that have measured levels of 25-hydroxyvitamin D levels along with different clinical outcomes like incidence of cancer, diabetes, etc. Some of these studies include:

- The Third National Health and Nutrition Examination Study (NHANES III)
 - Vitamin D status linked to risk of cardiovascular disease ([Conchol and Scragg 2007](#))
- The Nurses Health Study (NHS)
 - Vitamin D status linked to colon and breast cancer ([Bertone-Johnson et al. 2005](#); [Feskanich et al. 2004](#))
- The Health Professionals Follow-Up Study (HPFS)
 - Vitamin D status linked to risk of myocardial infarction in men ([Giovannucci et al. 2008](#))
- The Framingham Heart Study (FHS)
 - Vitamin D status linked to bone health ([Bischoff-Ferrari et al. 2004](#))

After years of examining the body of literature regarding vitamin D status, experts in the field have begun to narrow in on a consensus of what levels of 25-hydroxyvitamin D should be recommended. Initially, levels were recommended based on what was necessary to prevent rickets (about 20ng/ml). As the body of evidence emerged with respect to preventing other diseases, recommended levels have begun to rise. The chart in Figure 5 shows a collaboration of many of the major studies to date. From this data, along with the opinion of many vitamin D experts, ZRT has determined a reference range to be 32-100ng/ml with an optimal range of 50-80ng/ml.

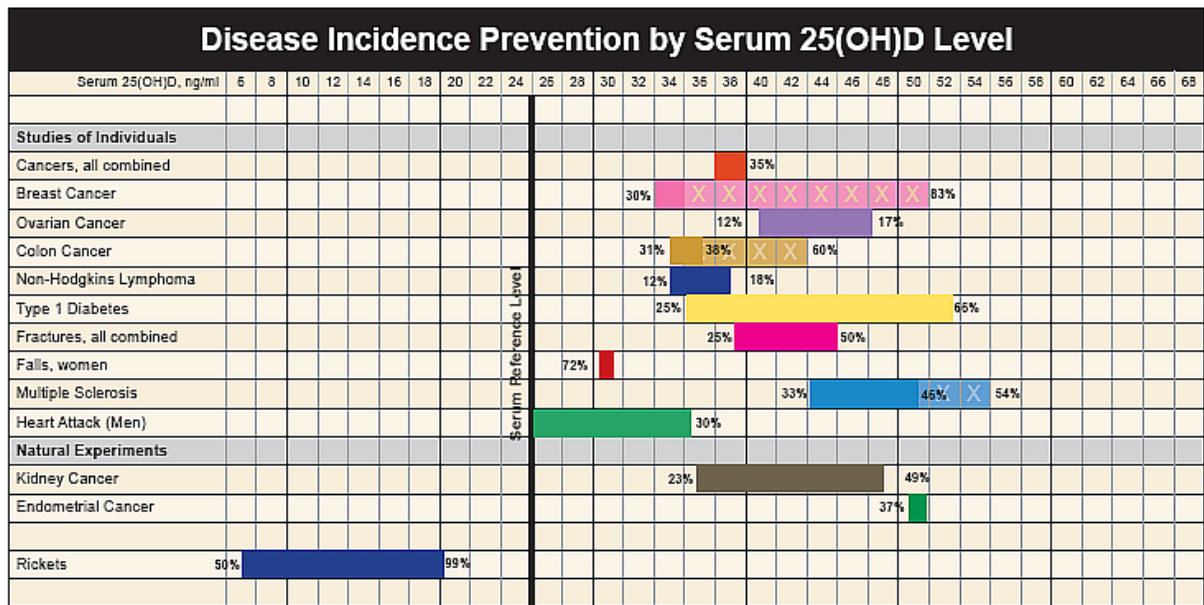


Chart prepared by: Garland CF, Baggerly CA

Figure 5. Disease Incidence Prevention by Serum 25(OH)D Level

**All percentages reference a common baseline of 25 ng/ml as shown on the chart.

References:

All Cancers: Lappe JM, et al. Am J Clin Nutr. 2007;85:1586-91. Breast: Garland CF, Gorham ED, Mohr SB, Grant WB, Garland FC. Breast cancer risk according to serum 25-Hydroxyvitamin D: Meta-analysis of Dose-Response (abstract). American Association for Cancer Research Annual Meeting, 2008. Reference serum 25(OH) D was 5 ng/ml. Garland, CF, et al. Amer Assoc Cancer Research Annual Mtg, April 2009. Colon: Gorham ED, et al. Am J Prev Med. 2007;32:210-6. Diabetes: Hyppönen E, et al. Lancet 2001;358:1500-3. Endometrium: Mohr SB, et al. Prev Med. 2007;45:323-4. Falls: Broe KE, et al. J Am Geriatr Soc. 2007;55:234-9. Fractures: Bischoff-Ferrari HA, et al. JAMA. 2005;293:2257-64. Heart Attack: Giovannucci et al. Arch Intern Med/Vol 168 (No 11) June 9, 2008. Multiple Sclerosis: Munger KL, et al. JAMA. 2006;296:2832-8. Non-Hodgkin's Lymphoma: Purdue MP, et al. Cancer Causes Control. 2007;18:989-99. Ovary: Tworoger SS, et al. Cancer Epidemiol Biomarkers Prev. 2007;16:783-8. Renal: Mohr SB, et al. Int J Cancer. 2006;119:2705-9. Rickets: Amdur SB, et al. Pediatrics. 1976 Feb;57(2):221-5.

Note that one large colon cancer study used an alternative method (neither DiaSorin nor LC-MS/MS) to show that colon cancer risk was reduced by higher levels of vitamin D (Garland et al. 1989). However, a meta-analysis by some of the same authors, which included this study, reported on four other colon cancer studies, all of which used DiaSorin testing. The meta-analysis concluded that levels >32ng/ml were associated with a 50% reduction in risk for colorectal cancer (Gorham et al. 2007).

Now that ZRT has determined what optimal reference ranges should be from available major studies, our results are both *analytically* and *clinically* accurate, right? Not necessarily. There are two very important issues to consider.

- All of the major studies listed (NHANES III, NHS, HPFS, FHS) used DiaSorin for their measurements of 25-hydroxyvitamin D and not LC-MS/MS because most of the studies predate the rise in popularity of LC-MS/MS testing.
- According to DEQAS results, the average DiaSorin RIA analysis of 25-hydroxyvitamin D is 20-30% lower than the same measurement with LC-MS/MS. The summaries of the last two reported rounds of DEQAS are shown below in Figure 4. Note that the correlation is very strong, but the RIA values are consistently lower than those generated by LC-MS/MS.

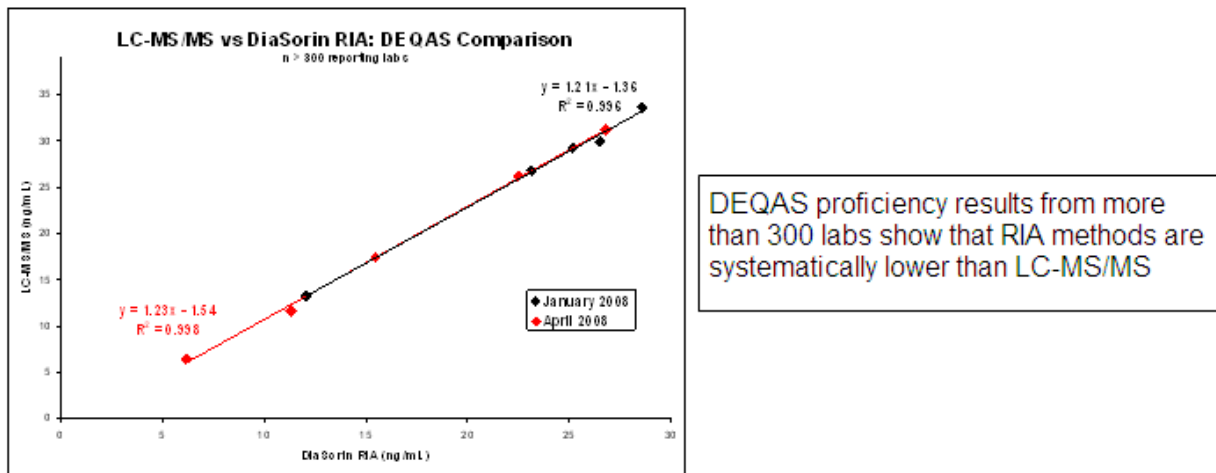
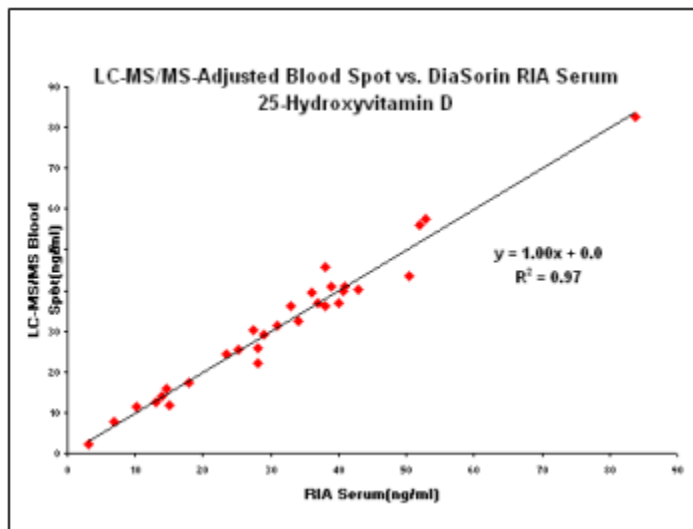


Figure 6. LC-MS/MS vs. DiaSorin RIA: DEQAS Comparison

What does this all mean? Simply put, if LC-MS/MS labs do not generate values that agree with DiaSorin, it is not appropriate to use a reference range that is generated from studies in which DiaSorin was used (even if LC-MS/MS is more accurate analytically).

By measuring many samples for 25-hydroxyvitamin D by the ZRT LC-MS/MS method and by DiaSorin RIA, ZRT results have been adjusted to be equivalent to the DiaSorin RIA test. In Figure 5, you can see a comparison of ZRT measurements compared to DiaSorin RIA.



When adjusted properly, ZRT blood spot values agree very well with DiaSorin RIA measurements

Figure 7. 25-Hydroxyvitamin D: ZRT LC-MS/MS-Adjusted vs. DiaSorin RIA

As of August 11, 2008, ZRT results have been RIA-adjusted. Results generated prior to this date were analytically accurate, but this adjustment generates optimal clinical accuracy. We urge clients using LC-MS/MS assays for 25-hydroxyvitamin D to ensure that these values correlate similarly to the gold standard DiaSorin RIA. It is important to note that the DiaSorin RIA is the gold standard not because it is more accurate than the LC-MS/MS method, but because it was the method used for the most important studies involving measurement of 25-hydroxyvitamin D. If clinically accurate, both RIA and LC-MS/MS can be successfully used to monitor vitamin D status, but for blood spot analysis LC-MS/MS is the preferred analysis. ZRT Laboratory wishes to thank Bruce Hollis, PhD for his advice and assistance.

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