Validation of Vitamin D Status Point-of-Care Assessment in Healthy and Critically Ill Young Children

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ABSTRACT

Vitamin D is known to have an important role in multiple organ systems. A growing body of evidence suggests there may be clinical benefits to the rapid identification and correction of vitamin D deficiency in hospitalized populations. With current standard of care practices, patient 25-hydroxyvitamin D (25-OHD) levels are generally unavailable immediately following blood collection, thereby creating a window of time during which vitamin D deficient patients remain untreated. As such, it is important to develop and validate point-of-care tests for vitamin D status assessment. The aim of this study was to determine whether point-of-care testing for 25-OHD could accurately and precisely determine vitamin D status, based on concentration and status categories. This study was conducted using stored research serum samples from recently completed projects on healthy and ill children, as well as available DEQAS samples. Qualigen® results were compared to reference methods (LC-MS/MS, NIST, etc.). With the precision verification test, samples 1 (mean 48.8 nmol/L) and 2 (mean 66.5 nmol/L) largely passed (sample 2 failed for within-laboratory for option B), whereas sample 3 (177.7 nmol/L) failed for both repeatability and within-laboratory imprecision. To improve test accuracy, sample results were averaged per run to decrease variation. Further, the Qualigen® method is suspected of having bias for higher values which, if used in clinical or research settings, may result in missed detection of deficient patients. This would need to be confirmed prior to use in clinical practice. In conclusion, these results indicate that there may be possible benefit for the use of the Qualigen® method in assessing patient vitamin D status. Although this method failed on some aspects of the precision verification test, averaging the data may be a simple practical solution to increase the accuracy in results.

However, as with most point-of-care tests, physicians and research teams should continue to rely on standard and reliable measurement methods for true patient 25-OHD measurements.

RÉSUMÉ

La vitamine D est connue pour avoir un rôle important dans les systèmes d'organes multiples. Un nombre croissant de preuves suggère qu'il peut y avoir des avantages cliniques à l'identification rapide et la correction de la carence en vitamine D chez les populations hospitalisés. Avec norme actuelle des pratiques de soins, le patient 25-hydroxyvitamine D (25-OHD) niveaux sont généralement pas disponibles immédiatement après la collecte de sang, créant ainsi une fenêtre de temps au cours de laquelle la vitamine D patients déficients ne sont pas traités. En tant que tel, il est important de développer et de valider des tests de point de soins pour la vitamine D évaluation de la situation. Le but de cette étude était de déterminer si les tests de point de soins pour les 25-OHD pourrait exactitude et précision déterminer le statut en vitamine D, basée sur la concentration et l'état catégories. Cette étude a été réalisée en utilisant des échantillons de sérum de recherches stockées de projets récemment achevés sur les enfants en bonne santé et malades, ainsi que des échantillons DEQAS disponibles. Qualigen® résultats ont été comparés à des méthodes de référence (LC-MS/MS, NIST, etc.). Avec le test de vérification de précision, des échantillons 1 (moyenne 48,8 nmol/L) et 2 (moyenne 66,5 nmol/L) largement adoptée (échantillon 2 a échoué pour intra-laboratoire pour l'option B), tandis que l'échantillon 3 (177,7 nmol/L) a échoué pour à la fois la répétabilité et l'imprécision au sein du laboratoire. Pour améliorer la précision du test, les résultats des échantillons ont été en moyenne par cycle pour diminuer la variation. En outre, la méthode Qualigen® est soupçonné d'avoir partialité pour des valeurs supérieures qui, si elle est utilisée en milieu clinique ou de recherche, peuvent entraîner détection manquée des patients déficients. Ce devrait être confirmé avant de les

utiliser dans la pratique clinique. En conclusion, ces résultats indiquent qu'il peut y avoir avantage possible pour l'utilisation de la méthode Qualigen® dans l'évaluation des patients statut en vitamine D. Bien que cette méthode a échoué sur certains aspects du test de vérification de la précision, la moyenne des données peuvent être une solution simple et pratique pour augmenter la précision des résultats. Cependant, comme avec la plupart des tests de point de soins, les médecins et les équipes de recherche devrait continuer à compter sur des méthodes de mesure standard et fiables pour les patients de véritables mesures 25-OHD.

CONTRIBUTION OF AUTHORS

All authors contributed extensively to the work presented in this paper. P. Sekhon was the primary author included in this thesis and was a large contributor to the work included. P. Sekhon aided in obtaining ethics approval for this project. P. Sekhon prepared the experiment, performed the tests, compiled the data, and performed analyses. H. Weiler and D. McNally supervised the project, edited the paper, and aided in ethics approval, sample collection, and data analysis. M. Henderson helped with data analysis.

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ABBREVIATIONS

25-OHD	25-hydroxyvitamin D
AIN	adrenal insufficiency
ANOVA	analysis of variance
CHD	congenital heart disease
CHEO	Children's Hospital of Eastern Ontario
CLSI	Clinical and Laboratory Standards Institute
CV	coefficient of variation
DBP	vitamin-D binding protein
DEQAS	Vitamin D External Quality Assessment Scheme
DRI	Dietary Reference Intake
EAR	Estimated Average Requirement
ICU	intensive care unit
IOM	Institute of Medicine
LC-MS/MS	liquid chromatography-mass spectrometry
NIST	National Institute of Standards and Technology
PICU	pediatric intensive care unit
PTH	parathyroid hormone
RDA	Recommended Dietary Allowance
SD	standard deviation
UVL	upper verification limit
VDR	vitamin D receptor

UNITS

nmol	nanomole
L	litre
ng	nanogram
mL	millilitre
%	percent
IU	international unit
µg	microgram
mg	milligram
g	gram
dL	decilitre
PSI	pounds per square inch
°C	degree Celsius
d	day

CONVERSION FACTOR

1 ng/mL of 25-OHD= 2.5 nmo

1. INTRODUCTION/RATIONALE

Vitamin D is known to have an important role in multiple organ systems involved in the pathophysiology of adverse patient outcomes in multiple hospitalized, critically ill and post-surgical populations (1). For example, high deficiency rates and lower 25-hydroxyvitamin D (25-OHD) concentrations in adult studies have repeatedly been associated with higher illness severity scores, longer lengths of hospital stay and even death in the intensive care unit (ICU) (2). Further, recent pediatric observational studies in ICU and post-cardiac surgical settings have shown that many children are vitamin D deficient, with lower vitamin D levels associated with greater illness severity scores and worse clinical outcome (1, 2).

A growing body of evidence suggests there may be clinical benefits to the rapid identification and correction of vitamin D deficiency in pediatric patients. For example, the only large randomized placebo controlled trial of loading dose vitamin D (540 000 IU) in the critically ill adult setting suggested decreased mortality (3). Repeating this trial or applying the findings to other settings is near impossible, as the study protocol provided vitamin D levels within 4 hours of testing. With current standard of care practices, patient vitamin D levels are generally unavailable immediately following blood collection, thereby creating a window of time in which vitamin D deficient patients remain untreated. Although this time delay may be acceptable in certain healthy or stable patient populations, it may not be appropriate for acutely ill, hospitalized and/or critically ill patients. Considering the observed relationship between vitamin D and pathophysiology in these populations, this window may have: 1) a detrimental impact on patient outcomes, and 2) negatively impact the ability of clinical trials to determine the

clinical benefits of vitamin D loading dose therapy. As such, it is important to develop and validate point-of-care options for vitamin D status assessment.

Point-of-care testing refers to medical diagnostic testing performed in close proximity to the patient and providing information for immediate decision making in regards to patient health. A vitamin D point-of-care test would reduce the wait time for determination of vitamin D status, and afford medical personnel and/or researchers the ability to make immediate decisions on whether changes to dosing regimens are appropriate. This would not only be instrumental for clinical trials in determining the benefit(s) of vitamin D therapy, but also improve patient outcomes. Therefore, the main goal of this thesis is to test a point-of-care 25-OHD compared to gold standard and other reference methodology.

2. LITERATURE REVIEW

2.1. Vitamin D General Metabolism

The term vitamin D encompasses a class of fat-soluble compounds (4). The two major physiological forms encompassed in this definition are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (4). Vitamin D_2 is largely derived artificially from fungi or yeast following radiation to transform the steroid, ergosterol, into vitamin D_2 (5), and is found in certain plant beverages (6, 7). Vitamin D_3 is largely synthesized in the skin (80%) from the natural cutaneous derivative, sterol 7-dehydrocholesterol (7-DHC), but may also be ingested (20%) (6, 7, 8). Since vitamin D may be synthesized endogenously, it is not, strictly speaking, a vitamin, but a steroid (secosteroid) hormone, and more specifically a prohormone (9). The two forms of vitamin D, when activated, have been assumed to have similar biological responses, primarily through gene expression regulation (6); however, there may be differences between the two (7). For example, equipotency may be different at higher doses, where vitamin D_2 may be associated with higher toxicity (6). This is, however, subject to further exploration (6).

The process of vitamin D_3 synthesis (Figure 2.1) initiates from the sun, where ultraviolet B (UVB) light (wavelength 290 nm to 315 nm) irradiates the skin (6) and is absorbed by 7-DHC (7). This may account for up to 80% of the vitamin D source for humans (10). Solar UVB radiation varies based on solar angle, latitude, altitude, etc. (5). For hospitalized children, UVB exposure is void and thus not a source of vitamin D, making interventions using exogenous sources of vitamin D more pressing. It has been shown that within 12-24 hours of UVB exposure, circulating vitamin D_3 concentration (in the form of calcidiol (7)) reaches its maximum level (7). However, prolonged sunlight

exposure does not promote excessive vitamin D_3 production, as side products are produced in response to prolonged irradiation (9).

Vitamin D_3 synthesis may be influenced by numerous factors, such as solar angle, time of day or year, skin pigmentation, sunscreen use which prevents UVB irradiation, as well as temperature, which enables conversion of the previtamin to the vitamin (7). Numerous chromophores in the skin also influence the extent of vitamin D_3 synthesis, including melanin, DNA, ribonucleic acid, proteins and the provitamin itself, 7-DHC (7). For example, individuals with low levels of melanin have more efficient vitamin D_3 synthesis (10).

Once synthesized, vitamin D_3 is transported by vitamin-D binding proteins (DBPs), which carry the vitamin from the skin to storage tissues or for 25-hydroxylation in the liver (6). The DBPs are important for circulatory transport as well as target cell uptake of vitamin D metabolites (10). Dietary sources of vitamin D are similarly transported to storage tissues (adipose) or the liver, primarily via the lipoproteins, chylomicrons (6). Within hours, vitamin D (half-life of approximately 24 hours (8)) is promptly taken-up (6). Stored vitamin D (adipose), however, can have a have life of approximately 2 months (11).

Aside from the endogenous source, there are few dietary sources of vitamin D (4) contributing to a small portion of the total vitamin D in the body (9), such as fatty fish and egg yolks (4). Vitamin D_2 may be obtained from plant source foods, whereas vitamin D_3 may be obtained from animal source foods (9). Additionally, both forms may be obtained from supplements and fortified foods (ex. milk and orange juice) (10). These dietary sources become more important during winter months in regions beyond 40°

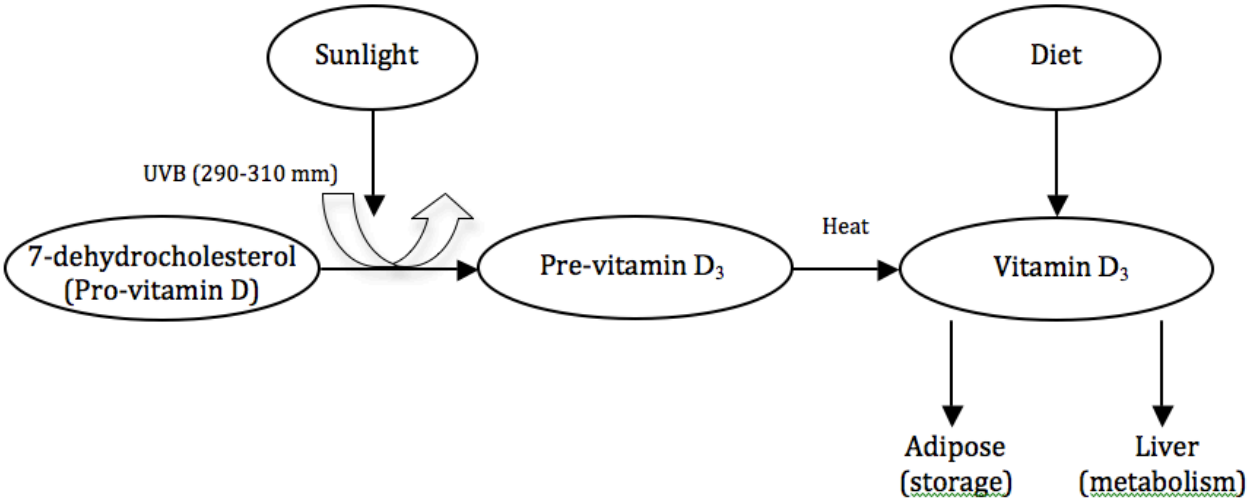

Figure 2.1 Schematic of vitamin D_3 synthesis.

latitude, for example Canada, when UVB penetration from the sun is minimal and vitamin D_3 synthesis is insignificant (5, 6).

Both forms of vitamin D are biologically inactive (7). Metabolism of dietary or cutaneous vitamin D occurs once is it absorbed by the liver (Figure 2.2) (4, 5). The liver converts vitamin D into calcidiol, or 25-hydroxyvitamin D (25-OHD), with the help of 25-hydroxylase action of hepatocytes (6). Calcidiol is then converted to biologically active, calcitriol (1α, 25-$(OH)_2$D), in the kidneys (4), catalyzed by CYP27B1 (5), an 1α-hydroxylase (6). This process is stimulated by parathyroid hormone (PTH), a calcium homeostasis regulatory hormone (5).

Calcitriol works in an endocrine manner together with PTH to maintain sufficient blood ionized calcium levels (12). When levels of ionized calcium become low, the parathyroid gland (through the calcium-sensing receptor) stimulates secretion PTH (12). PTH then increases expression of renal 1α-hydroxylase gene which leads to increased levels of 1α,25-$(OH)_2D_3$ in circulation (12). With this, 1α,25-$(OH)_2D_3$ increases intestinal absorption of calcium, whereas both 1α,25-$(OH)_2D_3$ and PTH increase bone resorption and renal reabsorption of calcium (12). Finally, 1α,25-$(OH)_2D_3$ decreases PTH expression through a negative feedback loop (12).

Calcitriol, the hormonally active product of vitamin D (9), is able to bind to vitamin D receptors (VDR) (Figure 2.2) found on numerous cells in the body (6). VDRs may be found on cells involved in functions such as gene regulation, renin production, insulin production, and proliferation of vascular muscle cells and cardiomyocytes (4). Many of these cells have the CYP enzymes for synthesis of calcitriol, where it is used in

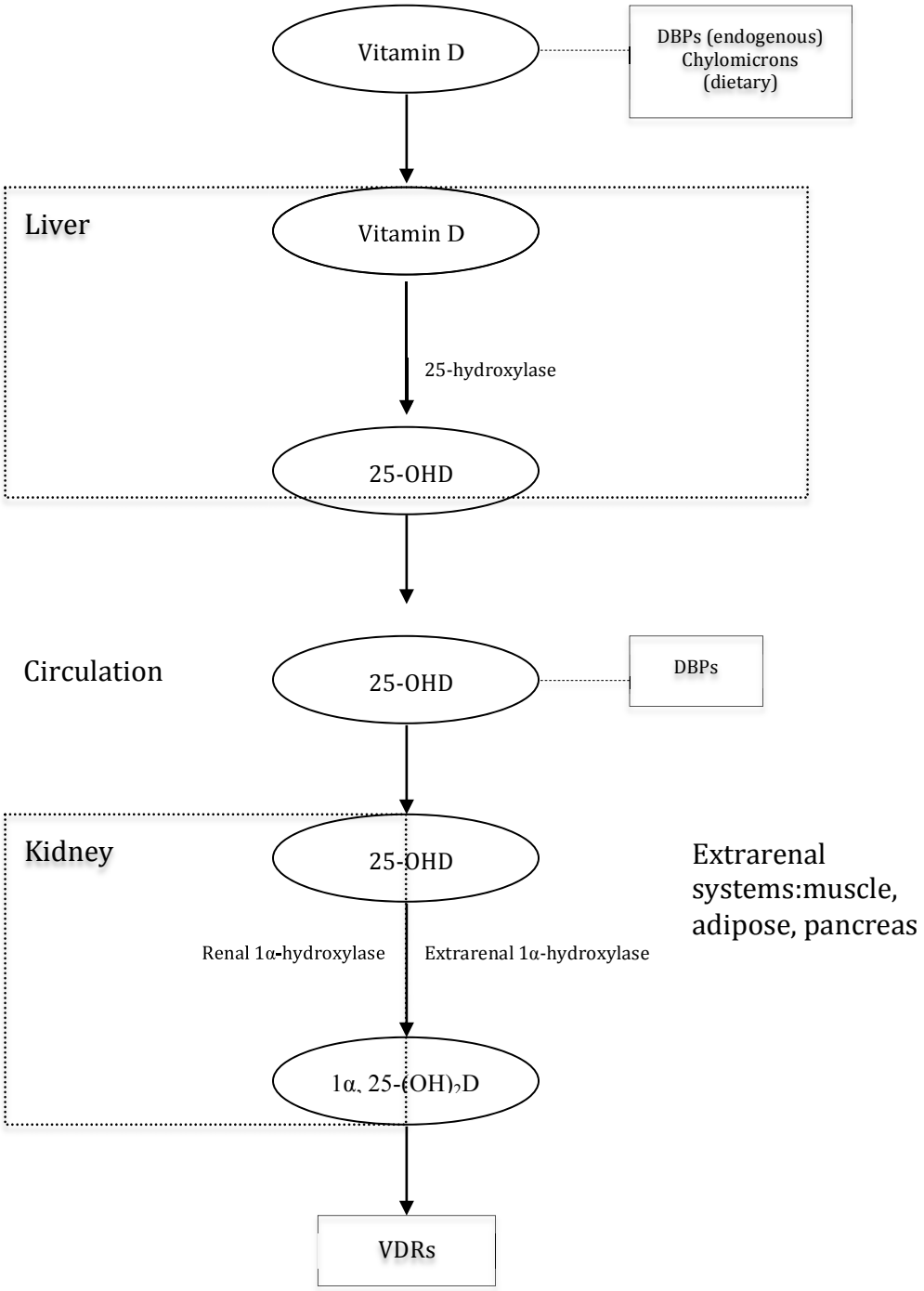

Figure 2.2 Schematic of vitamin D activation. DBP- vitamin-D binding proteins; 25-OHD- calcidiol; 1α,25-$(OH)_2D_2$ – calcitriol; VDR- vitamin-D receptor.

an autocrine and paracrine manner (6). Calcitriol may act on VDRs genomically, via binding to nuclear VDR, or non-genomically, via binding to membrane-bound VDR (13).

Genomically, calcitriol's role in different cell types may be divided into calcemic (regulating blood calcium and phosphate concentrations), as well as noncalcemic (cell differentiation and antiproliferative effects) (6). Vitamin D is best known for its actions in calcium absorption in relation to positive effects on bone formation and maintenance (9). Noncalcemic roles may be explained by the actions of extrarenal 1α-hydroxylase, which is expressed in sites outside the kidneys, thereby increasing vitamin D's roles beyond calcium and phosphate homeostasis (6).

Aside from these genomic effects, calcitriol may act nongenomically in numerous signal transduction systems (7). These include calcium influx and release from stores, activities found in many cells, including muscle cells (7). Vitamin D is also believed to act in a more immediate fashion, via ligand binding to a receptor, in pathways such as protein kinase, cAMP, and intracellular calcium and MAP kinase (9).

Calcidiol and calcitriol are catabolized through the action of the cytochrome P-450, CYP24A1 (6), a 24-hydroxylase (14). This catabolic degradation yields biologically inactive metabolites (5), largely 24,25-$(OH)_2D$ (15).

2.2. Vitamin D Measurement and Cut-Off Values

As previously mentioned 25-OHD, or calcidiol, is the major circulating form of vitamin D (4, 5). Levels of total serum 25-OHD are used for assessment of vitamin D status in reporting clinical status and encompass both forms of vitamin D (6). Serum level of 25-OHD is considered the best estimate of vitamin D status (16) for most populations

for a number of reasons. First, 25-OHD has a relatively long half-life (approximately 3 weeks or 21-30 days (8)), where levels represent vitamin D stores from both endogenous and exogenous sources (16, 17). This provides a much better estimate of vitamin D status than 1α, 25-$(OH)_2$D levels (half-life of approximately 4-15 hours (8)) (16). Additionally, 25-OHD production is not as regulated as 1α, 25-$(OH)_2$D production, and it mostly dependent on substrate concentration (16, 18). On the contrary, 1α, 25-$(OH)_2$D production is tightly regulated (16), and 1α, 25-$(OH)_2$D levels can vary in vitamin D deficient states (18). For example, there may be elevated levels of 1α, 25-$(OH)_2$D with low substrate (vitamin D) concentrations, since low vitamin D concentrations can lead to elevated levels of PTH, which increases 1α, 25-$(OH)_2$D production (18). So, in vitamin D deficient states, levels of 1α, 25-$(OH)_2$D may be low, normal, or elevated, thereby making 1α, 25-$(OH)_2$D an inappropriate indicator of vitamin D status (17). With this 1α, 25-$(OH)_2$D is rarely used in clinical settings as an indicator of vitamin D status (18). Another reason 25-OHD is considered the best estimate of vitamin D status is the ease of measurement of 25-OHD, as it is present in nanomolar concentrations in circulation, compared to picomolar concentrations of 1α, 25-$(OH)_2$D (8). Finally, 25-OHD concentrations are considered to be in equilibrium with body stores of vitamin D (19).

Vitamin D deficiency is assessed using calcidiol serum concentrations; however, there is still debate on the appropriate cut-off values of vitamin D (10). The IOM report indicates that, relative to bone health, vitamin D deficiency may be described as serum calcidiol levels below 30 nmol/L, where inadequacy is possible between 30-50 nmol/L (20). Sufficiency is defined as levels above 50 nmol/L, whereas concentrations above 125 nmol/L may begin to pose risks of adverse effects (20). It is important to note that these

cut off points have not yet been developed through broad scientific consensus beyond that of the Institute of Medicine (IOM) expert committee deliberations (20). Also, these guidelines are based on the maintenance of bone and mineral health; they do not consider non-calcemic benefits of vitamin D (21) or those of patient groups. For example, as will be discussed further, some consider 75 nmol/L as the cut-off value for adequacy, based on a plateau in PTH levels within the normal range. As such, there remains a lack of consensus on desirable vitamin D status targets.

After vitamin D deficiency was found to be the cause of rickets in the 19th century, supplementation and fortification of foods were implemented to resolve this important health crisis (20). Not only are these practices important for rickets, but they have also been shown to be effective for all other disease states associated with vitamin D deficiency (22), which will be further discussed. As there are few dietary sources of vitamin D, relying on sufficient intake through diet is problematic (22). A recent IOM review revised the Dietary Reference Intake (DRI) values for vitamin D (20). This report states vitamin D intake above the Recommended Dietary Allowance (RDA) is not associated with additional health benefits (20). The Adequate Intake (AI) for infants (0-12 months) is 400 IU, for children to adults (1-70 years) the Estimated Average Requirement (EAR) is 400 IU, and the RDA is 600 IU (20). These DRIs for vitamin D are based on maintenance of skeletal health and the assumption of minimal solar UVB exposure (20). In order to maintain these levels, measurement of 25-OHD levels and administration of supplements when necessary, is an important practice to ensure optimal health.

2.3. Vitamin D Deficiency

Specific population groups are at higher risk of becoming vitamin D deficient than others. For example, newborns/infants have a high need of vitamin D due to high rates of skeletal growth during this time (7). At birth, infants are presumed to have acquired, in utero, sufficient vitamin D reserves for the first months of life (7). However, due to low levels of vitamin D (25 IU/L to 40 IU/L (0.6-1 μg/L)) in human milk, breastfed infants are at higher risk of vitamin D deficiency if not receiving a supplemental form (7). This is further worsened by restricted sunlight exposure, particularly for infants born in warmer months (7). As a result, vitamin D_3 supplementation (400 IU/day) of breast-fed infants is recommended (7), beginning at birth and continuing until 1 year of age, to reduce the risk of rickets (20). Rickets is a disease characterized by insufficient mineralized or calcified bone matrix, with numerous clinical manifestations (ex. bowing of long bones, enlargement of epiphyses of joints, etc.) (6).

Vitamin D deficiency is associated with numerous clinical consequences, affecting disease severity, mortality, and survival time in the hospital ICU (23). Clinical consequences of vitamin D may be divided into classical (musculoskeletal) and non-classical (inflammation, cardiovascular, respiratory, etc.) consequences. For example, it is known that vitamin D deficiency leads to hypocalcemia, which when untreated, can lead to the release of PTH (23). Continued, this may develop into secondary hyperparathyroidism, thereby increasing bone resorption (23).

Vitamin D status is often unclear in hospitalized patients, even though vitamin D deficiency is common and often undiagnosed (23). A high burden of vitamin D

deficiency is importantly suggested in critically ill patients, where these patients are at a high risk of adverse outcomes as a result of vitamin D deficiency when compared to healthy populations (24). Yet assessment of 25-OHD concentrations is rarely performed (24). This is further complicated by the fact that most of the information regarding how status changes across time is obtained from healthy adult populations. For example, one study assessed vitamin D status of adult ICU patients throughout hospital stay to find that status was significantly decreased at admission and continued throughout stay (24). Therefore, there remains an important need for rapid identification and restoration of low vitamin D status, particularly in critically ill patients.

2.4. Vitamin D Illness

As previously mentioned, VDRs are found in various cell membranes in the cytoplasm and the nucleus of various cell types (6). With this, vitamin D deficiency has been linked to number of disease states such as osteoporosis, fractures, cognitive decline, type-2 diabetes mellitus, cardiovascular disease, and cancer (4). Low vitamin D levels can lead to multiple organ dysfunction and even death (4). Importantly, deficiency symptoms develop slowly and are nonspecific; therefore, they may be overlooked, leaving vitamin D deficiency untreated (4, 22). Common manifestations of vitamin D deficiency include bone discomfort (low back, pelvis, or lower extremities), muscle aches, and proximal muscle weakness (22).

2.4.1. Vitamin D and Cardiovascular Disease

As previously mentioned, vitamin D has recently been shown to play a role in cardiovascular physiology, beyond its traditional role in calcium absorption and bone health (12). Muscle cells (25), specifically cardiac myocytes and fibroblasts (26), express

both VDRs as well as the two aforementioned hydroxylases required for vitamin D metabolism (1α-hydroxylase and 24-hydroxylase) (12). It is believed that vitamin D is involved in numerous processes related to cardiac muscle (12), having direct or indirect effects on cardiac muscle (26). In addition, vitamin D has been demonstrated to have an important role in muscle health (25). For example, vitamin D deficiency has been associated with proximal muscle weakness in both children and adults (25, 27). Further, supplementation has been associated with increased muscle strength and balance, where it has been found that vitamin D supplementation (50 000 IU per week for 12 weeks) increased serum vitamin D from 51 ± 18.2 to 120.5 ± 44.8 nmol/L ($P<0.0001$) and resolved associated myalgia in 35 of 38 patients treated with statins, indicating a possible benefit to muscle function (28).

Vitamin D deficiency has been demonstrated to pose risk for cardiovascular disease and disease states (26). For example, poor vitamin D status has been shown to independently predict adverse cardiovascular events and poor clinical outcomes in adult cardiac surgery patients (29). Patients with deficient 25-OHD levels (< 30 nmol/L) had higher risk of major cardiac and cardiovascular events (2.3 (1.5-3.6)) than patients with non-deficient levels (75-100 nmol/L) (30). Further, routine angiography patients with vitamin D deficiency (25-OHD less than 25 nmol/L) were shown to be more likely to die of heart failure (2.8 (1.2-6.7)) and sudden cardiac death (5.1 (2.1-12)) than patients with optimal levels (25-OHD greater or equal to 75 nmol/L) (12, 31). Various studies have also indicated a strong link with vitamin D deficiency and increased risk of coronary artery disease (12). Studies have suggested that this deficiency is present prior to heart failure, supporting the potential cause-effect association between vitamin D deficiency

and myocardial dysfunction (26). It has been proposed that vitamin D supplementation be implemented for all vitamin D-deficient patients with or at high risk of myocardial diseases, to maintain levels above deficiency (26).

Vitamin D deficiency in children has also been associated with heart failure (6), and studies support vitamin D's association with pediatric cardiomyopathies (26). For example, one study assessed the perioperative vitamin D status and impact of congenital heart disease (CHD) surgery in 58 children (less than 18 years of age) with CHD (and undergoing surgery) in the PICU, as well as testing the association between vitamin D and clinical outcomes (1). It was found that almost all patients were vitamin D deficient (86% (CI, 77-95) with 25-OHD levels less than 50 nmol/L) or severely deficient (27% (CI, 15-38) with 25-OHD levels less than 25 nmol/L) following surgery (1). These patients had normal or low preoperative levels, and there was an observed acute intraoperative decline or 25 +/- 19.5 nmol/L (1). The study revealed that lower postoperative levels were associated with heart dysfunction and other markers of organ dysfunction, such as post-operative fluid requirements and catecholamines, which are measures of cardiovascular and immune dysfunction (1). Resultantly, it was suggested that current recommendations of vitamin D for healthy children is insufficient to maintain adequate vitamin D levels (1).

In many of these cases, there has been significant clinical improvement observed after vitamin D and calcium supplementation (26). For example, one study evaluated the effect of vitamin D_3 supplementation (1000 IU for 12 weeks) in infants with chronic congestive heart failure (32). After 12 weeks of supplementation, there was an observed increase in serum 25-OHD levels (33.4 +/- 5.5 nmol/L pre-intervention to 82.1 +/- 5.7

nmol/L; P< 0.001) versus non-significant improvement among the placebo group (32). The difference between the intervention and non-intervention groups after 12 weeks was also found to be significant (P< 0.001). With this, there was a significant improvement in heart failure score (Ross scoring system) after 12 weeks in the intervention group (9.1 +/- 2.7 to 6.4 +/- 1.2) (32).

Importantly, vitamin D deficiency is known to elevate PTH levels in an attempt to maintain normal serum calcium levels in light of the insufficient effect of vitamin D on calcium metabolism (25). Increased PTH is known to have deleterious effects on blood vessels and the myocardium, as PTH acts by increasing blood pressure, as well as adversely affecting the heart, such as through exerting pro-arrythmic actions (10). This suggests a pathophysiological link between vitamin D and myocardial diseases (25). This is supported by the finding that secondary hyperparathyroidism increases risk of cardiovascular disease (33). It is therefore believed that the ability of vitamin D supplementation to suppress PTH activity may reduce cardiovascular risk (10). This is supported by research highlighting vitamin D's protective effects with respect to cardiovascular disease, including its anti-atherosclerotic and anti-inflammatory effects (10).

Findings have indicated that serum PTH continues to decrease as serum 25-OHD increases, until a plateau is reached at 25-OHD levels of approximately 75 nmol/L (34). Many argue that optimal serum 25-OHD concentrations keep serum PTH concentrations at a minimum (35). With this, it has been suggested that maximal suppression of serum PTH occurs at approximately 75-80 nmol/L (35, 36). Table 2.1 provides a summary of major studies highlighting vitamin D's role in cardiovascular disease.

Table 2.1 Summary of studies on vitamin D and cardiovascular disease.

Authors (Year)	25-OHD measurement method	Participants	General Findings
Ahmed et al. (2009)	Two-dimensional liquid chromatography with tandem mass spectrometry	38 vitamin D deficient and myalgic adult patients treated with statins.	Vitamin D supplementation (50000 IU/week, 12 weeks) 1) Increased serum vitamin D from 51 ± 18.2 to 120.5 ± 44.8 nmol/L and 2) Resolved associated myalgia in 35/38 patients.
Zittermann et al. (2013)	Liaison assay	4418 outward adult patients hospitalized for cardiac surgery.	Patients with deficient 25-OHD levels (< 30 nmol/L) had higher risk of major cardiac and cardiovascular events than patients with non-deficient levels (75-100 nmol/L).
Pilz et al. (2008)	RIA (DiaSorin, Antony, France, and Stillwater, MN)	3299 adult patients referred to coronary angiography.	Patients with vitamin D deficiency (25-OHD < 25 nmol/L) were shown to be more likely to die of heart failure and sudden cardiac death than patients with optimal levels (≥75 nmol/L)
McNally et al. (2013)	Liquid chromatography-mass spectrometry	58 children with CHD in PICU, undergoing surgery.	86% of patient vitamin D deficient (<50 nmol/L) or severely deficient (27%) (<25 nmol/L) following surgery. Lower postoperative levels were associated with heart dysfunction and other markers of organ dysfunction.
Shedeed (2012)	RIA (DiaSorin, Stillwater, MN)	80 infants with congestive heart failure.	After 12 weeks of vitamin D and calcium supplementation, there was an observed increase in serum 25-OHD levels versus non-significant improvement among the placebo group.

2.4.2. Vitamin D and Critical Illness

Research has showed that vitamin D deficiency is common in critically ill adult and pediatric patients, and is associated with worse patient outcomes (37). These patients are at higher risk of vitamin D deficiency than their healthy counterparts, as they largely remain indoors and may have altered vitamin D metabolism (38). The prevalence of vitamin D insufficiency/deficiency in over 400 critically ill patients in a number of studies was shown to range from 38% to 100%, which is about 50% higher than general medical patients (38). Vitamin D deficiency, although associated with a wide range of chronic medical conditions, is rarely considered or adequately, or at all, corrected in patients with critical illness (38). Given vitamin D's importance in numerous organ systems, there are various clinical implications of vitamin D deficiency among critically ill patients (38). For example, vitamin D deficiency may be responsible for common conditions among critically ill patients, including sepsis and inflammation, due to immune system dysfunction, as well as hypocalcemia, due to PTH-vitamin D system imbalance (38).

One recent study assessed the prevalence of vitamin D deficiency in critically ill children, in addition to identifying influential factors on calcidiol levels upon PICU admission (37). This study assessed 511 children (under 21 years of age) admitted to the PICU with severe or critical illness over a one-year period (37). It was found that 40.1% of these children were vitamin D deficient (less than 20 nmol/L) and 71.2% were vitamin D insufficient (less than 74.9 nmol/L), which was reported as being higher than the healthy pediatric population in the US, and rather more similar to the prevalence among adult ICU patients (37). Age (older; OR 1.4 (1.2-1.7 (95% CI)) per every 5 years) and

race (darker skin; 0.51 (0.3-0.8) for non-Hispanic white versus other) were associated with the deficiency, whereas protective factors included summer season (0.3 (0.1-0.5)), supplementation (0.5 (0.4-0.8)), and formula intake (0.4 (0.2-0.6)) (39). Also, lower vitamin D levels were found among those who were most ill upon PICU admission (1.2 (1.1-1.3)) (37). This study additionally highlighted that higher vitamin D levels may decrease critical illness severity caused by infection or injury (37).

There are various clinical implications of vitamin D deficiency among critically ill patients (38). Firstly, vitamin D deficiency disrupts the PTH-vitamin D endocrine system, where hypocalcemia has been noted in critically ill patients as a result of disrupted bone formation and heightened bone resorption (38). In addition, vitamin D appears to be very important in immune system functioning, as VDRs are found in immune cells (38). Deficiency may result in dysregulation of the immune system leading to various adverse conditions, such as sepsis and inflammation, which are common to critically ill patients (38). In addition, critically ill patients have been shown to have lower innate immune system activity (38).

Additionally, it has been proposed that there is increased tissue requirement of vitamin D in critically ill patients (38). Normally, when vitamin D levels are sufficient and tissues do not require much, there is normal and healthy functioning (38). When vitamin D deficiency occurs, PTH ensures production of calcitriol through bone resorption, resulting in rapid conversion of calcidiol to calcitriol (38). As such, vitamin D deficiency in critically ill patients encompasses an imbalance between substrate supply (vitamin D) and tissue requirements (38). Organ dysfunction and mortality are results of this continued imbalance (38).

As previously mentioned, despite numerous guidelines on treating vitamin D deficiency, a universally accepted optimal level of vitamin D has yet to be established (38). The ability to apply proposed levels of sufficiency is even less clear among critically ill patients, as these patients do not have clear clinical end-points, such as fractures, which are used to define appropriate cut-off values in the general population (38). With this, current vitamin D replacement treatments are suggested as inadequate among critically ill children, where high dose regimes may be required (38).

Importantly, it has been suggested that status based on calcidiol measurements in critically ill patients should be interpreted cautiously, due to widely fluctuating levels of albumin and DBP, to which most of the vitamin D in the body is found bound (38). Critically ill patients have lower DBP, especially those with sepsis (38). The reason for these lower DBP levels is not yet clear (38). However, it remains problematic as it may lead to vitamin D wasting, since re-absorption of vitamin D metabolites in the renal tubules requires DBP binding (38). Though, it has been highlighted that appropriate timings and determination of free and total albumin, DBP and vitamin D metabolite concentrations may solve this issue (38). It has also been suggested that standardization and quality assurance is crucial in intervention studies involving critically ill patients (38). It is therefore important to establish the varying patterns of metabolites and binding proteins with different degrees of disease severity, as well as the appropriate cut-off for vitamin D deficiency among critically ill patients (38).

2.5. Vitamin D Measurement/Analysis

2.5.1. Issues with 25-OHD measurement

There are a number of issues associated with accurate measurement of 25-OHD (18). These issues can lead to significant variability between methods and even measurements (8). To start, vitamin D is a lipophilic molecule, and therefore, non-specific interference from other lipids is very likely (18). This complication may be avoided, however, in assays that utilize extraction/chromatography steps, and through the displacement of 25-OHD from DBPs in non-extraction assays (18). Further, as discussed, vitamin D exists in several molecular forms (i.e. D_2 and D_3 forms), and there are a number of vitamin D metabolites (8). For example, 25-OHD_3 makes up approximately 95% of the total amount of 25-OHD in circulation, whereas 25-OHD_2 makes up a miniscule amount, under non-supplementary conditions (40). Consequently, the D_3 form of 25-OHD has been considered the most useful marker for vitamin D status in plasma (41). That being said, measurement of a specific metabolite without interference from other metabolites may be problematic. Although currently available assays (ex. LC-MS/MS) may be able to separately quantify the (equimolar) amount of D_2 and D_3 forms in circulation, total 25-OHD levels (encompassing both forms of vitamin D) are commonly used in reporting clinical status (6, 16). Finally, 25-OHD measurements may be variable between labs and measurements, as a suitable reference standard for assays has only recently become available (8).

2.5.1.1. Vitamin D Metabolites in Measurement

Over 50 vitamin D metabolites have been identified, to date (8). Although metabolites can interfere in measuring specific molecules (8), most of these metabolites have relatively shorter half-lives and are therefore not thought to majorly affect vitamin D measurements using vitamin D assays (8).

2.5.1.1.1. 3-epi-25-OHD

One important metabolite of note is the 3-epimer (3-epi-25-OHD_3), which has the same mass as 25-OHD_3 (18), and has been shown to make up a significant proportion of total circulating 25-OHD in infants less than 1 year of age (19, 18, 41). One study found an inverse correlation between 3-epimer concentrations and age, suggesting that this epimerization may be the result of immature vitamin D metabolism (19). Consequently, it is considered a potential cofounder in 25-OHD measurements (42). It has been shown that if epimers, particularly 3-epimer, are not chromatographically separated, or accounted for in vitamin D assays, they could largely contribute to total vitamin D levels (14-55%) (43). This may have detrimental effects clinically, as vitamin D levels may be overestimated and deficiency may become hidden (8, 43). With this, when measuring serum from neonates, it has been suggested that methods should be used that do not detect or cross-react with the epimer (19), or enable 3-epimer concentrations to be excluded in total 25-OHD measurements (18). Although it has mainly been seen in young infants and not usually detectable in adults, it has recently been noted that the 3-epimer may also be found in smaller, variable concentrations in adults (8, 41). Serum 3-epimer concentrations can be very high, although its biological importance is not clear (18). Though, it has been suggested to suppress PTH with similar potency to 1α, 25-$(OH)_2$D,

but with reduced calcemic effects (19). One experimental study suggests it is equally good at maintaining bone health (44).

At present, it is practiced that the epimer is not included when assessing vitamin D status. In part this is due to many of the available vitamin D assay methods failing to separate or take into account the 3-epimer (5), and only a few, more recent, methods have the ability to separate, or not recognize, the 3-epimer (41). For example, it has been shown that presence of the epimer can lead to overestimation of total 25-OHD using some LC/MS-MS assays when compared to immunoassays, which do not cross-react with 3-epimer (8).

2.5.1.1.2. 24,25-$(OH)_2D$

As mentioned, 24,25-$(OH)_2D$ is the most abundant product of vitamin D catabolism, with a half-life of approximately 7 days (13). This metabolite is found in ng/mL concentrations (13), where its production is largely determined by 25-OHD concentrations (45). Importantly, 24,25-$(OH)_2D$ has been suggested as a potential cofounder in 25-OHD measurements. 24,25-$(OH)_2D$'s physiological effects are not very clear (46); however, it has been suggested to have a role in regulating endochondral ossification, for example (47).

It has been suggested that serum 24, 25-$(OH)_2D$ levels are not significantly different between children and adults; however, levels may be lower in neonates when compared with older children (46). One study found the median concentration of 24,25-$(OH)_2D$ as being lower in children than in adults ($P<0.01$). It was suggested that 1,25-$(OH)_2D$ production is preferred over 24,25-$(OH)_2D$ production during infancy when compared with adulthood, as infancy is a period of rapid skeletal growth (45). Finally,

this study also found that 24,25-$(OH)_2D$ concentrations were positively correlated to 25-OHD concentrations in both children ($r=0.8$, $P<0.0005$) and adults ($r=0.9$, $P<0.0005$) (45).

The presence of 24,25-$(OH)_2D$, as well as other metabolites in serum, may contribute to positive bias in measurements of 25-OHD by immunoassay methods, relative to chromatographic methods (48). This metabolite has been suggested as ranging between 2%-20% of total serum 25-OHD in adults, possibly leading to inflation of true 25-OHD concentration; however, the degree of inflation may vary based on assay type (48). The impact of 24,25-$(OH)_2D$ in measurement of serum 25-OHD using immunoassays was recently assessed where it was found that 24,25-$(OH)_2D$ contributed to a positive bias observed in some immunoassays (mean factor 2.8) relative to LC-MS/MS results (48). It was suggested that the degree of cross-reactivity is high and may be the reason for this positive bias (48). With this, adjusting for 24,25-$(OH)_2D$ led to estimates being closer to the true values (48).

Methods used to measure 24,25-$(OH)_2D$ are similar to those used to measure 25-OHD (49), as 24,25-$(OH)_2D$ and 25-OHD display similar potency in displacing 25-OHD from its binding sites in serum or kidney cell proteins (46). Since 25-OHD competitive protein-binding assays are said to be equally sensitive for multiple vitamin D metabolites, chromatographic techniques are considered important in measurement of these metabolites (46). Interestingly, serum 24,25-$(OH)_2D$ levels have been used as an index of vitamin D deficiency and catabolism in healthy individuals, and are often expressed as a molar ratio to 25-OHD (48). This ratio may also be used as an indicator of individual vitamin D supplementation responses (48).

2.5.2. Vitamin D Assays

There are a number of different vitamin D assays that can be classified into two main types: immuno/radioimmunoassay and chromatographic assays. Two important assays that will be discussed further are immunoassays and LC/MS-MS.

2.5.2.1. Immunoassays

Immunoassay methods quantify an analyte (i.e. 25-OHD) based on the reaction of the analyte with an antibody (50). Specifically, a fixed amount of labeled analyte competes with an unknown concentration of unlabeled sample analyte for limited binding to a specific, anti-analyte antibody (50). These reagents are mixed and incubated together, to yield an analyte-antibody immune complex (50). This complex is then separated from the rest of the free fraction, and either the free or bound fractions are analyzed by quantifying the labeled analyte activity (50). From this, a calibration curve, using measured signals, may be constructed to determine the concentration of the unknown analyte (50).

Immunoassays have become the most popular method for 25-OHD measurements for a number of reasons, including availability, high throughput, rapidity, etc. (8). Also, many of these assays are not able to detect the 3-epimer (18). However, immunoassays have also been criticized for problems with accuracy, specificity, fluctuating results, and poor agreement (inter-laboratory and inter-method variability) (8, 40, 42, 43).

There are few major 25-OHD immunoassay manufacturers including DiaSorin and Roche (18). In the DiaSorin assay, 25-OHD is extracted by denaturing DBPs, and chromatography is not used (18). Roche is an automated electrochemiluminescence

immunoassay that only detects 25-OHD_3 (18). This assay functions by incorporating acidic pH changes, which inactivates DBP and frees the bound 25-OHD (18).

2.5.2.2. LC-MS/MS

LC-MS/MS is considered the current gold standard in vitamin D measurement (16). It is considered as the most accurate method at present, and therefore, the method of choice of quantification of vitamin D metabolites (41). LC-MS/MS is considered to have a high degree of accuracy, due to the specificity of tandem mass spectrometry (18).

A mass spectrometer (MS) measures the mass-charge ratio of charged particles and ions are separated from each other based on these mass-charge ratios (m/Q) (40). In clinical practice, most mass spectrometers are single- or tandem (quadruple) mass filter designs, with either gas chromatography (GC) or liquid chromatography (LC) front-ends (40). LC-MS/MS is a tandem MS with LC front-ends, and is the most common instrument in clinical laboratories (40).

LC-MS/MS has become increasingly popular in use, specifically for low molecular weight analytes, due to the limitations and higher cost of immunoassays (particularly reagent costs) for these types of analytes, better analytic specificity for low molecular weight analytes than immunoassays (40), and the easier workflows and higher throughput than high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS), which were previously used for these analytes (8, 40). In addition, LC-MS/MS has shorter run times, is able to separately measure D_2 and D_3 (16), as well as total 25-OHD (summation of D_2 and D_3) (8), and does not require sample derivatization (43).

Despite these advantages, LC-MS/MS has some limitations. First, due to its manual nature and the highly complex equipment, operation and maintenance of LC-MS/MS technology requires advanced training and experience (40). Further, although LC-MS/MS is less expensive to run, given the high cost of reagents with immunoassays, the initial high cost of LC-MS/MS equipment is also a drawback (40). Next, although it has higher throughput than other chromatographic methods, LC-MS/MS is still not as efficient as immunoassays, despite the analytical and financial benefits (40). Also, vitamin D epimers (i.e. 3-epimer) may display very similar chromatography to 25-OHD (19), where they can overlap 25-OHD peaks and form the same masses (MS/MS ion pairs (19)) after ionization (43). With this, there is new LC/MS-MS technology that has been shown to be able to separate and quantify epimers, and therefore, quantify different 25-OHD forms accurately (43). However this technique has been criticized for lack of standardization among labs, as most have been criticized to not being able to separate the epimer from 25-OHD (8). Consequently, National Institute of Standards and Technology (NIST) has recently produced and released standard reference material (SRM 972) and certified reference values for the two major forms of vitamin D and the 3-epimer (8, 42). This is thought to aid in harmonization and standardization of results by all methods used to measure vitamin D levels, and improve analytical 25-OHD measurements (8). Table 2.2 highlights the major advantages and disadvantages of the use of immunoassays and LC-MS/MS for vitamin D measurement.

Table 2.2 Advantages and disadvantages of major vitamin D assays.

	Advantages	Disadvantages
Immunoassays	Availability High Throughput Rapidity Do not detect 3-epimer	Problems with accuracy Problems with specificity Fluctuating results Poor agreement (Inter-laboratory and inter-method variability)
LC-MS/MS	High degree of accuracy Specificity Ability to separately measure D_2 and D_3 Analytic specificity for low molecular weight analytes Easy workflows High throughput Shorter run times than previous methods Does not require sample derivitization	Required advanced training and experience for use High initial cost Not as efficient as immunoassays Some not able to separate and quantify epimers

2.5.2.3. Practical Issues with Vitamin D Assays

Despite their numerous advantages to afford accurate quantification of 25-OHD concentrations, current 25-OHD assays have certain practical issues that may affect their usefulness in research and clinical settings. First, 25-OHD assays may be limited in availability and use to individuals trained for laboratory equipment. This would greatly limit those who may conduct vitamin D status assessments, as physicians, nurses, and research staff may not be able to personally conduct these tests and must rely on a third party. Ultimately, this would likely increase the time of return for results. In addition, costs of these assays, whether from initial cost of equipment or continued cost of reagents, may be high. This again would limit the individuals willing to house one of these devices. Consequently, results are sent in for quantifications (ex. approximately $45 per 25-OHD test using LC-MS/MS, and $20 per 25-OHD test using immunoassay (Waters©, 2012)) but may not be available immediately. This is clearly problematic for patients whose levels may be dangerously low and require dose therapy and/or monitoring. Therefore, although the currently available methods are relied on for accurate results, there remains a need for a faster approach to provide 25-OHD concentrations in research and clinical settings.

Despite the urgent need for such methods, biochemistry tests still must be properly developed and validated. For any quantitative tests, both the precision and bias of the method need to be tested. Alternatively, for qualitative tests, sensitivity and specificity of the method are tested. In vitamin D settings, methods that provide low coefficients of variation (%CV) are sought. Defined total allowable error for vitamin D

tests were said to be 25%, based on recommendations for a coefficient of variation of 10% and bias of 5% (51).

The Clinical and Laboratory Standards Institute (CLSI) outlines guidelines for method verification of new quantitative and qualitative methods. It is stated that prior to use of a new measurement procedure for reporting patient results, its analytical performance must be evaluated (52). Performance may be assessed relative to standard regulatory requirements as well as clinically usefulness requirements (52). Importantly, CLSI (EP05-A3) states a minimum sample size of 20 samples is acceptable for evaluation of quantitative measurement procedures.

2.5.3. Vitamin D Point-of-Care Testing

Point-of-care testing refers to analytic testing performed by health care professionals that may be performed in settings outside the laboratory, at or near the site of patient care (53), such as at the patient bedside (54). This alternative to laboratory settings allows for shorter turn-around on results, the ability to monitor rapid changes (54), and results reflect patient status as close as possible to testing (53). The shorter therapeutic turn-around associated with point-of-care options are particularly beneficial in critical illness, as delay in treatment may adversely affect patient health (53). As such, point-of-care options may aid in making fast therapeutic choices (54), as well as aid in ongoing research efforts. These benefits are extended to economic advantages, as rapid results save time and money (55). Economic benefit is seen largely through reduced hospital stays, as quick results decrease time for decision-making, leading to faster treatment and discharge (55). Further, point-of-care tests provide societal benefits, such

as through prolonged years of life or work (55). Whether this could also apply to duration of hospitalization or return to school for children is not known.

Specifically, a vitamin D point-of-care test would reduce the wait time for determination of vitamin D status, and afford medical personnel and/or researchers the ability to make immediate decisions on whether changes to dosing regimens are appropriate. This would not only improve patient outcomes, but it may be instrumental for clinical trials in determining benefit of vitamin D therapy as well a numerous other research studies examining vitamin D status. Despite the advantages of point-of-care tests, it remains crucial to patient health and research progress to establish accuracy of test performance, particularly in critically ill populations.

There are a number of different, currently available vitamin D point-of-care tests. These tests may be defined in numerous ways, such as whether they measure 25-OHD levels qualitatively or quantitatively, based on the types of technology used (i.e. small bench top analyzers or single-use, hand held devices (similar to pregnancy tests) (55)), based on what metabolites they are able to measure, the different type of chemistry used to measure levels, etc. (53). One commercially available vitamin D point-of-care options is the FastPack IP Vitamin D, approved for use in Canada and developed by Qualigen®.

In comparison to commonly used 25-OHD assays, Qualigen requires minimal training (2 hour on-line training session). To house a Qualigen device, the initial cost of the analyzer is $9,995.00 (US), with each kit of 30 tests costing $500.00 (US). Based on 30 test kits (as the cost per test of the analyzer would be decreased with each subsequent kit purchased), each test would cost approximately $17.00 (US). As such, this method has an initial analyzer cost with subsequent costs per kit of 30 tests. However, it is important

to note that the cost per reportable result may be higher than cost per test, as this will take into consideration costs for ongoing quality control, validation, and other necessary costs (certification, standardizing of results, etc.). Additionally, each sample test provides results in approximately 11 minutes. It is important to note that the Qualigen® FastPack IP Vitamin D method cannot detect 25-OHD levels below 32.2 nmol/L (12.9 ng/mL), the limit of quantitation, as well as higher than 375 nmol/L (150 ng/mL), the upper end of the calibration range (56). Other pertinent performance information provided by Qualigen® include the method's interference with other substances (Table 2.3) and cross-reactivity (Table 2.4) (56). Although Qualigen® has performed an internal validation of the FastPack system, the importance of an external validation remains clear, to ensure that this system works in all types of environments.

Although this test has been evaluated in adult populations, it has yet to assessed in pediatric and/or point-of-care settings. The FastPack IP Vitamin D is Food and Drug Administration (FDA) and Canadian Standards Association (CSA) approved and has been tested by NEMKO according to electrical safety standards. It has recently received approval from Health Canada. Other point-of-care devices exist, but are not yet approved for use in Canada. Thus, the present thesis will further evaluate FastPack IP Vitamin D.

Table 2.3 Interference of substances in quantification of vitamin D by Qualigen®. Modified from Qualigen® FastPack IP Vitamin D Immunoassay Kit Complete (56).

Interfering substance	Non-spiked aliquot (nmol/L)	Spiked aliquot (nmol/L)	% Bias
Bilirubin (40 mg/dL)	147.8	132.8	-10.2
Biotin (1 μg/mL)	90	87.2	-3.1
Cholesterol (500 mg/dL)	96.2	103.2	7.3
Protein (10.7 g/dL)	70.5	77.8	10.3
Hemoglobin (500 mg/dL)	122.5	114.2	-6.7
Lipids (250 mg/dL)	245.5	221.2	-9.9

Table 2.4 Effects of potential cross-reacting compounds using Qualigen® method. Modified from Qualigen® FastPack IP Vitamin D Immunoassay Kit Complete (56).

Cross-reactant	Concentration tested (nmol/L)	% Cross-reactivity
Vitamin D_2	1250	2.0
Vitamin D_3	1250	1.9
1,25-$(OH)_2$-Vitamin D_2	250	4.0
1,25-$(OH)_2$-Vitamin D_3	250	9.8
3-epi-25-OH Vitamin D_3	1000	7.8
25-OH Vitamin D_2	250	93.0
25-OH Vitamin D_3	62.5	106.0
Paricalcitol	500	-1.2
24,25-$(OH)_2$ Vitamin D_2	100	-0.9
24,25-$(OH)_2$ Vitamin D_3	50	117.4

OBJECTIVE/HYPOTHESIS

The aim of this study is to determine whether point-of-care testing for 25 hydroxyvitamin D can accurately and precisely determine vitamin D status, based on concentration and status categories. It is hypothesized that this test will be able to immediately determine patient 25-OHD concentrations to meet manufacturer specifications and perform similar to reference methods.

3. METHODOLOGY

3.1. Summary of Study

This was a validation study evaluating a vitamin D point-of-care test in a heterogenous population. This study was conducted at the Children's Hospital of Eastern Ontario (CHEO) and McGill University using stored research serum samples collected from heterogenous pediatric and adult populations. Aside from method comparison, this validation study included a precision verification study as outlined by the Clinical and Laboratory Standards Institute (CLSI, EP-15 A3), in consultation with a clinical biochemist at the CHEO laboratory with experience in validation studies. This study also involved the participation in Vitamin D External Quality Assessment Scheme (DEQAS) quality assessment. The present study was reviewed under delegated review and was approved by CHEO Research Ethics Board (CHEOREB#14/138X) and McGill University Research Ethics Board (III) (REB File #: 315-0115).

3.2. Description of Test

The test that was evaluated in this study is the FastPack IP Vitamin D, developed by Qualigen®. This is a quantitative vitamin D test intended to determine the total 25-OHD and other hydroxylated metabolites in human serum and plasma (Qualigen®, 2014). The Qualigen® point-of-care test is comprised of sample-loaded packs that are inserted into the analyzer, which measures the amount of 25-OHD found in the sample. The FastPack IP Vitamin D is FDA and CSA approved and has been tested by NEMKO according to electrical safety standards. It has recently received approval from Health Canada.

3.3. Test Principle

The FastPack IP Vitamin D test is a paramagnetic particle chemiluminescence immunoassay, based on the competitive binding principle, where blood vitamin D competes with vitamin D-conjugate for binding sites on the anti-vitamin D antibody (Qualigen®, 2014). The sample to be tested is mixed with a pretreatment buffer before it is added into the FastPack, which is inserted into the analyzer for testing. There is a primary incubation in which a labeled (alkaline phosphatase) monoclonal (mouse) anti-vitamin D antibody reacts with the 25-OHD in the sample. During the secondary incubation, 25-OHD, that is covalently coupled to biotin and pre-bound to streptavidin-labeled paramagnetic particles, is combined with this mixture. The 25-OHD found in the sample competes with the 25-OHD-biotin-streptavidin particles for binding sites on the labeled antibody. Then, a chemiluminogenic substrate is added, resulting in chemiluminescence measured by the analyzer. With this, the amount of labeled antibody is inversely proportional to the 25-OHD concentrations in the sample.

Within the pack, the sample and reagents are automatically mixed and moved by internal pressure pads found in the analyzer to perform the quantitative test (57). These pressure pads apply pressure (50 PSI) to the compartments, through software controlling the small air compressor found in the analyzer. This moves the sample and reagents from one chamber to the next, and the various chambers found in each pack are illustrated in Figure 3.1 (57). The sample chamber receives the patient sample via the injection port. The conjugate (antibody solution) chamber contains capture and labeled antibodies, whereby this chamber attaches and captures the target molecule (25-OHD) from the sample (57). Further, the reaction chamber contains the coated paramagnetic particles,

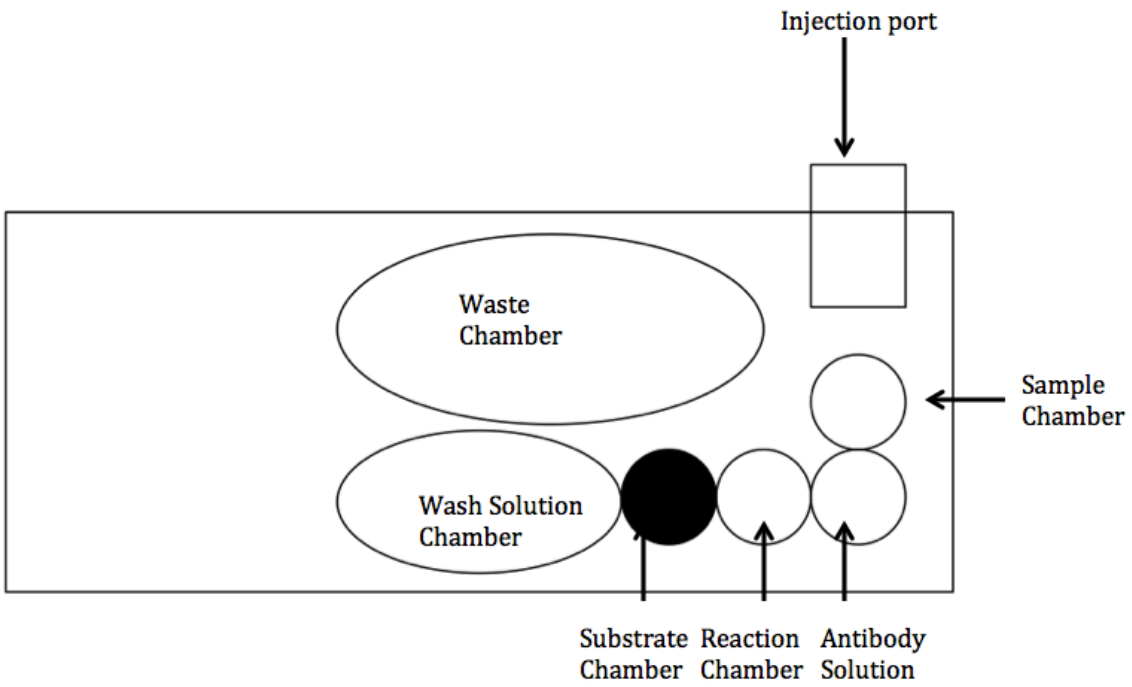

Figure 3.1 Chambers found in the Qualigen® FastPack inserts. Modified from Qualigen® FastPack IP System Assay Principle (57).

integral to the immunoassay reaction. The substrate chamber contains light sensitive substrate, which emits photons, equating to the target molecule (25-OHD) concentration. The wash solution chamber contains wash solution and is used to wash away excess, unbound materials from the reaction. Finally, the waste chamber is where all the wash solution is deposited once the test is complete (57).

The analyzer runs tests at a controlled temperature of 37°C ± 0.5°C, achieved through heated metal plates (57). With this, the analyzer performs regular self-diagnostic tests to self-monitor temperature, air pressure, background light, system power, and force profiles (seal ruptures and clamps) (57).

3.4. Description of Research Samples

Stored serum samples from recently completed projects on healthy community dwelling and hospitalized children were used for the project. Samples from the Vitamin D External Quality Assessment Scheme (DEQAS) were also used.

3.4.1. Research Sample Origin:

3.4.1.1. Title: *Impact of Anesthesia and Surgery for Congenital Heart Disease on the Vitamin D Status of Infants and Children*

The primary objective of this study was to determine the effect of congenital heart disease (CHD) surgery on vitamin D status, and to determine the relationship between postoperative levels and clinical outcome in a pediatric cardiac surgery population (1). A total of 58 children with CHD were enrolled in this prospective cohort study. Blood was collected preoperatively, intraoperatively, and postoperatively, and serum 25-OHD was measured.

The present research used 100 μL of the remaining serum from 58 children with CHD and 20 control children (minor problems undergoing surgery). As the original REB approval and consent form did not clearly permit patient samples to be used for other or related research purposes, familes were contacted to obtain permission. First, a letter was sent to families providing an update on the original study results and informing them of the desire to use the stored samples for for related studies. Second, families were contacted by phone to discuss the study and obtain telephone consent.

3.4.1.2. Title: *Effect of Different Dosages of Oral Vitamin D Supplementation on Vitamin D Status in Healthy, Breastfed Infants.*

The primary objective of this study was to investigate the efficacy of different dosages of vitamin D supplementation in supporting 25-OHD concentrations in infants (58). This was a double-blinded randomized clinical trial conducted among 132 one-month-old healthy, term, breastfed infants. Infants were randomly assigned to receive oral cholecalciferol supplements of 400 IU/d (n=39), 800 IU/d (n=39), 1200 IU/d (n=38), or 1600 IU/d (n=16). A 3 year follow-up (publication pending) on these patients has also been performed. Some of these samples were also used in the present study. Secondary analysis of data collected from this study was approved by the Institutional Review Board at McGill University.

3.4.1.3. Title: *A Prospective Multicenter Study of Adrenal Function in Critically Ill Children*

The objectives of this study were to determine the prevalence of adrenal insufficiency (AIN), risk factors, and potential developmental mechanisms, as well as its association with clinically important outcomes in critically ill children (39). This was a

prospective, cohort study conducted in seven tertiary-care pediatric intensive care units in Canada, over the course of 3 years (2005-2008). Patients were up to 17 years of age with existing vascular access. Research Ethics Board approval was obtained to measure 25-OHD and written and informed consent was obtained for all participants.

3.4.1.4. *DEQAS Samples*

Vitamin D External Quality Assessment Scheme (DEQAS) is an organization with the objective to ensure analytical reliability of 25-OHD and 1,25-$(OH)_2$D assays; in doing so DEQAS harvests serum from blood donated by adult patients (59). All samples are screened for hepatitis B, C, and HIV before being distributed to participating laboratories and researchers (59). The sample 25-OHD_3, 25-OHD_2, 3- epi-25-OHD_3 concentrations are assigned by NIST Reference Measurement Procedure (59).

3.5. Test Protocol

Each FastPack IP Vitamin D kit contains FastPacks (which are inserted into the analyzer), a calibrator, two controls, pretreatment buffers, calibration cards, and control range card (57). The FastPack analyzer, printer, 100 μL pipette and corresponding tips were separately purchased. FastPacks were stored at 4°C at the CHEO Research Institute. All appropriate laboratory safety measures were adhered during experimentation. The FastPack IP system provides unique pipettes and tips, specific to this system. With this, the sample and tip are simultaneously injected into the pouch through an injection port, whereby the tip acts as a seal for the pack (57).

Three calibrations were initially performed, followed by two controls, prior to sample testing. Calibrations were performed at any point in which the analyzer was shut down and/or moved to another location, whereas controls were performed on a weekly

basis, as indicated by Qualigen®. Research samples (serum and plasma) containing endogenous vitamin D were mixed with the provided pretreatment buffer vials. This pretreatment buffer-sample solution was then added to a FastPack, which was labeled with the sample ID and inserted into the FastPack analyzer. Testing occurred for approximately 11 minutes, after which the analyzer displayed the measured 25-OHD concentration (in ng/mL) of the sample. The FastPack was then inserted into the printer, where the results were printed onto the removable label on the FastPack. All results (removable labels) were stored in a confidential notebook and subsequently converted to molar units.

3.6. Sample Size

The samples tested were: a) 41 healthy infant samples, b) 40 ill children, c) 3 different samples (DEQAS sample, pooled DEQAS samples, Qualigen® provided control) were tested 25 times each as part of a precision verification study, and d) 29 total DEQAS samples. A minimum sample size of 20 samples has been used and is acceptable for evaluation of quantitative measurement procedures, as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (EP05-A3).

3.7. Analysis

3.7.1. Precision Verification Study

A precision verification study was conducted according to CLSI guidelines (EP-15 A3) for verifying a manufacturer's claims of precision by a measurement procedure (52). This experiment allowed for the calculation of two precision claims: repeatability (within-run imprecision) and within-laboratory imprecision. The precision verification

study requires repeatedly testing at least two different samples, using the basic 5x5 design (five days, five replicates per day) to yield 25 results per sample.

In this experiment, three different samples (25-OHD concentrations near clinical thresholds) were tested (Sample 1: DEQAS sample, and Sample 2: pooled DEQAS samples, Sample 3: Qualigen® provided control) using the 5x5 design, with all samples processed in a single run per day. Once the data was collected, it was analyzed consistent with CLSI guidelines: the results were tabulated and inspected for discordant values, repeatability and within-laboratory imprecision estimates were calculated, and these estimates were compared to their corresponding precision claims. For any estimates that exceeded their associated claims, the relevant upper verification limit (UVL) was calculated and compared with the estimate to determine whether the estimate verified the claim or not. If estimates were found to be below the UVL, the user estimates "passed" and were considered as being consistent with manufacturer claims. Conversely, if estimates were found to be above associated claims and UVLs, the test was considered to "fail". Multiple comparisons were completed for increased confidence in results. First, each sample precision estimate was compared with the manufacturer claim that was closest in mean value to the sample mean. Second, the manufacturer's data was extrapolated to obtain a claim that was identical in mean value to the sample mean. Third, Qualigen® provided its own quality assurance value, which was used as the threshold for a third comparison method.

3.7.1.1. Grubb's limit

CLSI recommends the use of Grubb's test to justify treating a result as a statistical outlier (52). In this test, a result is considered a statistical outlier if it lies more than *G*

SDs from the sample mean, where 1) the mean and SD are based on all N results for the samples (including the suspected outlier), and 2) the Grubb's factor, *G* (found in Table 5 in the EP-15 A3 document) (52). A statistical outlier may be calculated using the following equation (52):

Grubb's limits= mean±G•SD

3.7.2. DEQAS Quality Assessment

The present study involved participation in the DEQAS quality assessment of vitamin D metabolites for the April 2015 distribution cycle. Five DEQAS samples were measured using the Qualigen® measurement method and submitted to DEQAS for quality assurance of vitamin D measurements. These samples are included in 29 total DEQAS samples analyzed in this study.

3.7.3. Method Comparison

To compare the data obtained through Qualigen® to the reference values, various analyses were carried out. The resulting plots include: frequency distribution, deming regression, and Bland-Altman plots. Plots and graphs were constructed using GraphPad Prism®.

Different reference methods were used to measure 25-OHD in the samples used in this study. These were LC-MS/MS (healthy children, CHD) and NIST (DEQAS).

3.7.4. Differences Based on Pediatric Groups and Status Categories

To assess whether there were any major differences between groups (healthy and ill) when using the Qualigen device, the data was plotted, standard deviations and CVs were calculated, and Bland-Altman plots were constructed. To further assess whether there were major differences in status categories (deficient/insufficient (≤50 nmol/L) or

sufficient (>50 nmol/L) 25-OHD concentrations (20)) in children, a chi-square test for proportions was conducted. The deficient and insufficient groups were combined in view of the limited sample size.

4. RESULTS

4.1. Precision Verification Study

4.1.1. Raw Data

Table 4.1 lists the 25 results of 25-OHD concentrations (in nmol/L) for each sample vertically in the order in which they were measured, identified by the run and replicate number. The mean 25-OHD concentrations were calculated as 48.8 nmol/L for sample 1, 66.5 nmol/L for sample 2, and 177.7 nmol/L for sample 3. These concentrations represent values near important clinical thresholds: 50 nmol/L (sufficiency), 75 nmol/L (considered optimal levels by some), and 125 nmol/L (begin to pose risks of adverse effects) (20, 35, 36). Figure 4.1 displays simple plots of these datasets, which allows for surveying consistency between runs and of individual results. Visually, no points stood out as potential outliers. For sample 1 (mean 48.8 nmol/L), the measured 25-OHD concentrations ranged from 32.3 nmol/L to 64.3 nmol/L. For sample 2 (mean 66.5 nmol/L), the range of results was 41.8 nmol/L to 86.8 nmol/L, and for sample 3 (mean 177.7 nmol/L), the range of results was 151.3 nmol/L to 243 nmol/L (Table 4.2). Table 4.2 provides the basic statistics for the samples.

4.1.2. Outlier Testing

The Grubb's outlier limits for sample 1 (mean 48.8 nmol/L) were found to be [19.1, 78.5] nmol/L, for sample 2 (mean 66.5 nmol/L) were [30.4, 102.6] nmol/L, and for sample 3 (mean 117.7 nmol/L) were [105.6, 249.7] nmol/L (Table 4.2). Using the Grubb's test for outliers, no statistical outliers were found as results fell within these limits for all three samples (52). Calculations are found in Appendix A.

4.1.3. ANOVA Comparison

One-way ANOVA statistics were computed in multiple ways (including SAS software, R software) to ensure consistency (manual calculations are found in Appendix B). For sample 1 (mean 48.8 nmol/L), the user estimate for repeatability (S_r, nmol/L) is 9.4 (19.2%) and within-laboratory imprecision (S_{wl}, nmol/L) is 9.5 (19.4%). For sample 2 (mean 66.5 nmol/L), the user estimate for repeatability (S_r, nmol/L) is 11.4 (17.2%) and within-laboratory imprecision (S_{wl}, nmol/L) is 11.5 (17.4%). For sample 3 (mean 177.7 nmol/L), the user estimate for repeatability (S_r, nmol/L) and within-laboratory imprecision (S_{wl}, nmol/L) were both calculated to be 24.6 (13.9%). Table 4.3 highlights the calculated ANOVA statistics (verified with SAS and R software).

4.1.4. Comparison of User Imprecision Estimates to Manufacturer Claims.

Table 4.4 (a and b) indicates the precision claims provided by Qualigen® for various sample means. These claims assess the variation for sample 25-OHD concentrations ranging from 64.8 nmol/L to 212.2 nmol/L. Within-run variation, between-run variation, between-day variation, and total imprecision are all listed. This study tested the manufacturer claims for within-run variation (for within-laboratory imprecision) and total variation (for repeatability). Multiple comparisons of estimates to claims (options A-C) were completed. Calculations are found in Appendix C.

Option A:

Using this option, each sample precision estimate was compared with the manufacturer claim that was closest in mean value to the sample mean. The results of this comparison are presented in Tables 4.5 and 4.6.

The repeatability (% coefficient of variation (CV)) estimates for each sample were calculated to be 9.4 nmol/L (19.2%) for sample 1 (mean 48.8 nmol/L), 11.4 nmol/L (17.2%) for sample 2 (mean 66.5 nmol/L), and 24.6 nmol/L (13.9%) for sample 3 (mean 177.7 nmol/L) (Table 4.3). These samples were compared to the manufacturer claims that most resembled the calculated means for each sample. Sample 3 (mean, 177.7 nmol/L) was compared to the manufacturer claim 8 (mean, 191 nmol/L; claim, 8 nmol/L (4.1%)), and sample 1 (mean, 48.8 nmol/L) and 2 (mean, 66.5 nmol/L) were both compared to claim 5 (mean, 64.8 nmol/L; claim, 9.8 nmol/L (15.1%)) (Table 4.5). Upon initial comparison, all three sample estimates exceeded the claims; therefore, the UVLs were calculated for each claim, claim 8 (5.7%) and claim 5 (20.7%) (Table 4.5). When comparing sample imprecision estimates to the UVLs, sample 1 (19.2% < 20.7%) and 2 (17.2% < 20.7%) pass the test, and are thereby considered as consistent with manufacturer claims for repeatability, while sample 3 (13.9% > 5.7%) fails.

The within-laboratory (%CV) estimates for each sample were calculated to be 9.5 nmol/L (19.4%) for sample 1 (mean 48.8 nmol/L), 11.5 nmol/L (17.4%) for sample 2 (mean 66.5 nmol/L), and 24.6 nmol/L (13.9%) for sample 3 (mean 177.7 nmol/L) (Table 4.3). These samples were again compared to the manufacturer claims that most resembled the calculated means for each sample. Sample 3 (mean, 177.7 nmol/L) was compared to the manufacturer claim 8 (mean, 191 nmol/L; claim, 9 nmol/L (4.7%)), and sample 1 (mean, 48.8 nmol/L) and 2 (mean, 66.5 nmol/L) were both compared to claim 5 (mean, 64.8 nmol/L; claim, 9.8 nmol/L (15.1%)) (Table 4.6). Upon initial comparison, all three sample estimates again exceeded the claims; therefore, the UVLs were calculated for each claim, claim 8 (6.5%) and claim 5 (20.2%) (Table 4.6). When comparing sample

imprecision estimates to the UVLs, sample 1 (19.4% < 20.2%) and 2 (17.4% < 20.2%) pass the test, and are thereby considered as consistent with manufacturer claims for within-laboratory imprecision, while sample 3 (13.9% > 6.5%) fails.

Option B:

Alternatively, the manufacturer's data was extrapolated to obtain a claim that is identical in mean value to the sample mean. The manufacturer's data (means and CVs) were plotted to determine the lines of best fit for total (y=-7.907*ln(mean)+46.995) and within-laboratory (y=-7.355*ln(mean)+43.25) variation. These equations were then used to find the %CVs for the specific mean values of interest. With this, the subsequent steps were identical to those used in option A to test the calculated estimates against the UVLs (Appendix C). Table 4.7 provides the extrapolated repeatability and within-laboratory estimates for each sample. The extrapolated total %CV for sample 1 (48.8 nmol/L) is 16.3%, for sample 2 (mean 66.5 nmol/L) is 13.8%, and for sample 3 (mean 177.7 nmol/L) is 6.04%. Similarly, the extrapolated within-laboratory %CV for 48.8 nmol/L is 14.7%, for 66.5 nmol/L is 12.4%, and for 177.7 nmol/L is 5.2%.

The repeatability estimates (Table 4.3) were compared to the extrapolated imprecision claims for the same mean concentrations. Sample 1 (mean, 48.8 nmol/L; 19.2%) was compared to its matching extrapolated claim (mean, 48.8 nmol/L; claim, 16.3%), sample 2 (mean, 66.5 nmol/L; claim, 17.3%) was compared to its matching extrapolated claim (mean, 66.5 nmol/L; claim, 13.8%), and Sample 3 (mean, 177.7 nmol/L; claim, 13.9%) was compared to its matching extrapolated claim (mean, 177.7 nmol/L; claim, 6.03%) (Table 4.8). Upon initial comparison, all three sample estimates exceeded the claims; therefore, the UVLs were calculated for each claim (Table 4.8).

When comparing sample imprecision estimates to the UVLs sample 1 (19.2% < 22.3%) and 2 (17.2% < 18.9%) pass the test, and are thereby considered as consistent with manufacturer claims for repeatability, while sample 3 (13.9% > 8.3%) fails.

The within-laboratory estimates (Table 4.3) were compared to the extrapolated imprecision claims for the same mean concentrations. Sample 1 (mean, 48.8 nmol/L; 19.2%) was compared to its matching extrapolated claim (mean, 48.8 nmol/L; claim, 14.7%), sample 2 (mean, 66.5 nmol/L; claim, 17.2%) was compared to its matching extrapolated claim (mean, 66.5 nmol/L; claim, 12.4%), and sample 3 (mean, 177.7 nmol/L; claim, 13.9%) was compared to its matching extrapolated claim (mean, 177.7 nmol/L; claim, 5.2%) (Table 4.9). Upon initial comparison, all three sample estimates exceeded the claims; therefore, the UVLs were calculated for each claim (Table 4.9). When comparing sample imprecision estimates to the UVLs, sample 1 (19.2% < 19.6%) passes the test, and is thereby considered as consistent with manufacturer claims within-laboratory imprecision, while sample 2 (17.2% < 16.6%) and sample 3 (13.9% > 6.9%) fail.

Option C:

Finally, Qualigen® provides its own quality assurance value, which indicates that CVs below 20% are accepted as a pass (56). This test was also performed, and all three sample estimates (sample 1, CV=19.2%; sample 2, CV=17.2%; sample 3, CV=13.9%) passed (Table 4.10).

4.1.5. Averaging data

In an attempt to improve test accuracy, sample results were averaged per run. Table 4.11 provides the averaged data of each run (N, number of samples; N=5 to N=1),

where results are averages of individual, 2, 3, 4, and all 5 samples, respectively. This data allows determination of the point at which the data gives coefficients of variation below a particular threshold (i.e. 10%, 15%). This data is also expressed in graphic form with a line indicating 10% variation (Figure 4.2). From this data, it is clear that in order to get <10%CV using the Qualigen® machine, samples should be tested 2 (based on sample 3) or 3 (based on sample 1 and 2) times and averaged.

4.2. DEQAS Quality Assurance

Five DEQAS samples were submitted for quality assurance. DEQAS indicates proficiency as when at least 75% of the results fall within +/- 25% of the corresponding NIST value. The provided DEQAS samples were within a clinically relevant range of 39.6 nmol/L to 78.6 nmol/L, according to the provided NIST values. The Qualigen® measured results provided a similar range to that of NIST of 39.8 nmol/L to 87.2 nmol/L. With this, all five Qualigen® values were within 25% of the respective NIST values. Table 4.12 lists the DEQAS samples submitted with the Qualigen® and NIST results.

4.3. Method Comparison

The Qualigen® vitamin D immunoassay performance with regards to the tested samples was compared with the reference methods. Various plots were created to illustrate and test performance using the available data set. The total data set (healthy and ill children, and DEQAS samples) was used to construct the plots. It is important to note that for three of the tested samples (1 healthy, 2 DEQAS samples), the Qualigen® reported values were below the test threshold (<32.2 nmol/L). These values were excluded from the analyses. The frequency distribution graph (Figure 4.3) compares the Qualigen® method (test) with the reference methods (field). According to the plot, the

highest density of values reported with the reference methods were around 50 nmol/L, whereas the highest density of values reported with the Qualigen method were around 60 nmol/L (Figure 4.3).

The deming regression comparing Qualigen® method to LC-MS/MS is shown in Figure 4.4. The slope of the fitted regression line was 0.82 and the intercept was 19.25. The deming regression comparing Qualigen® method to NIST is shown in Figure 4.5. The slope of the fitted regression line was 0.97, and the intercept was 3.9.

Bland-Altman (difference) plots compared the Qualigen® method to LC-MS/MS (Figure 4.6), the Qualigen® method to NIST (Figure 4.7), and to all reference methods (Figure 4.8). The Qualigen® method had a mean difference of 7.2 when compared to all reference methods, 8.9 when compared with LC-MS/MS and 2.1 when compared with NIST. Paired t-tests revealed only one non-significant difference ($P>0.05$), between the Qualigen method and NIST method. The 95% confidence intervals for each comparison, the Qualigen® method to all reference methods (3.95-10.4), to NIST (-2.42-6.62), and to LC-MS/MS (4.91-12.87), reveal that the estimates of bias are roughly within +/- 4.5 nmol/L.

4.3.1. Healthy versus Ill Children

The data was further analyzed to determine if there was a difference between healthy and ill patient samples. Healthy, ill, and total (healthy and ill) 25-OHD concentrations measured using the Qualigen method were plotted against values obtained from reference methods (Figure 4.9) to visualize agreement. The slope of the trend line was 0.8 for healthy children data and 0.1 for ill children data. The slope for the combined data was 0.6. A correlation (R) of 0.11 is observed for Qualigen and healthy children, and

0.25 is observed for Qualigen® and ill children. Bland Altman plots were also created to assess any group differences (healthy and ill) (Figure 4.10-4.12). All three categories (total, healthy, and ill) seemed to have similar distributions.

Additionally, the means and standard deviations were separately calculated for the healthy and ill samples using the Qualigen® method and reference methods (Tables 4.13 and 4.14). With the Qualigen® results, the mean for healthy samples was 69.9 nmol/L and standard deviation was 26.4 (37.8%). The mean for ill children samples was 66.8 nmol/L and standard deviation was 14.4 (21.6%). With the reference method results, the mean for healthy samples was 65.3 nmol/L and standard deviation was 27.8 (42.5%). The mean for ill children samples was 53.5 nmol/L and standard deviation was 18.5 (34.6%).

4.3.2. Pediatric Status Categories

Using the reference method for actual concentrations and the Qualigen® method for reported values, the data was grouped in Chi-square categories (expected and observed) as illustrated in Table 4.15. From this, approximately 26% of the deficient/insufficient samples were reported as deficient/insufficient (95% confidence interval [0.14 to 0.43]), whereas approximately 91% of the sufficient samples were reported as sufficient (95% confidence interval [0.79 to 0.97]). The result from the Chi-square test was not statistically significant (P=0.0527). Calculations are found in Appendix D.

4.4. Tables and Figures

Table 4.1 Raw data of 25-OHD results using Qualigen® FastPack Immunoassay, in order of generation (nmol/L). Samples were tested for five days, with five replicates per day.[1]

Run	Replicate	Sample 1	Sample 2	Sample 3
1	1	56.8	73.5	203.5
1	2	47.5	74.8	163.3
1	3	39.8	59.5	176.5
1	4	56.8	65.8	166.3
1	5	44.3	74.8	163.3
2	1	43.5	51.8	202.8
2	2	32.3	86.8	176.5
2	3	57.3	69.3	179.3
2	4	32.3	53	166
2	5	44.8	72.8	161.3
3	1	64.3	63.3	243
3	2	33	57	166.5
3	3	63.8	81.8	164
3	4	53.5	47	158.3
3	5	47.3	50.3	157.5
4	1	54.5	56	192.8
4	2	32.3	75.8	180.5
4	3	48.3	66.8	169.3
4	4	53.3	73.8	159.8
4	5	49.5	41.8	161.3
5	1	58.3	73.3	218.8
5	2	52	73	175.8
5	3	56.8	76.8	218.3
5	4	53	70.8	166.5
5	5	45.5	73	151.3

[1] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.2 Summary of basic statistics and outlier limits for 25-OHD results using Qualigen® method. Samples were tested for five days, with five replicates per day. [2]

	Sample 1	Sample 2	Sample 3
N	25	25	25
Grand Mean (nmol/L)	48.8	66.5	177.7
SD, nmol/L (%CV)	9.5 (19.4%)	11.5 (17.3%)	23.0 (12.9%)
Lowest result, nmol/L	32.3	41.8	151.3
Highest result, nmol/L	64.3	86.8	243
Grubb's lower limit, nmol/L	19.1	30.4	105.6
Grubb's upper limit, nmol/L	78.5	102.6	249.7

Abbreviations: N, number of results; SD, standard deviation; %CV, coefficient of variation expressed as a percentage.

[2] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.3 ANOVA results and imprecision estimates for 25-OHD results using Qualigen® method. Samples were tested for five days, with five replicates per day.[3]

	Sample 1	Sample 2	Sample 3
N	25	25	25
MS_1 (between)	98.7	143.5	132.3
MS_2 (within)	87.7	130.5	607.3
n_0	5	5	5
V_b (between)	2.2	2.6	0
V_w (within)	87.7	130.5	607.3
Grand Mean (nmol/L)	48.8	66.5	177.7
S_r, nmol/L (%CV)	9.4 (19.2%)	11.4 (17.2%)	24.6 (13.9%)
S_{wl}, nmol/L (%CV)	9.5 (19.4%)	11.5 (17.4%)	24.6 (13.9%)

Abbreviations: ANOVA, analysis of variance; N, number of results; MS, mean squares; V_b, variance between runs; V_w, variance within run; S_r, user estimate for repeatability; S_{wl}, user estimate for within-laboratory imprecision; %CV, coefficient of variation expressed as a percentage.

[3] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.4 Manufacturer precision claims, Qualigen®. Modified from FastPack IP Vitamin D Immunoassay Kit Complete (01/14), ENG-5. 4.4a. represents values from Lot 1 (top). 4.4b. represents values from Lot 2 (bottom). Values represented in nmol/L.

Sample Number	Average	Within-run variation (%CV)	Between-run variation (%CV)	Between-day variation (%CV)	Total imprecision (%CV)
1	68.2	7 (10.2)	3.2 (4.9)	4.8 (7.1)	9.2 (13.4)
2	77.8	8.2 (10.7)	0 (0)	4.5 (5.7)	9.5 (12.1)
3	113.8	9.8 (8.5)	0 (0)	5 (4.3)	10.8 (9.5)
4	212.2	10.2 (4.8)	0 (0)	8 (3.7)	12.8 (6.1)

Sample Number	Average	Within-run variation	Between-run variation	Between-day variation	Total imprecision
5	64.8	9.8 (15.1)	0 (0)	0 (0)	9.8 (15.1)
6	81.8	9.8 (11.2)	0 (0)	5 (6.0)	10.5 (12.7)
7	115.2	8.8 (7.5)	0 (0)	3 (2.6)	9.2 (7.9)
8	191	8 (4.1)	0 (0)	4.2 (2.3)	9 (4.7)

Abbreviations: %CV, coefficient of variation expressed as a percentage.

Table 4.5 Results of precision verification study (repeatability) for 25-OHD results using Qualigen® method: user sample estimates, claims (using option A), and UVLs.[4]

	Mean (nmol/L)	Estimate, nmol/L (%CV)	Claim, nmol/L (%CV)	UVL (%)	Status
Sample 1	48.8	9.4 (19.2%)			Pass
Claim 5	64.8		9.8 (15.1%)	20.7	
Sample 2	66.5	11.4 (17.2%)			Pass
Sample 3	177.7	24.6 (13.9%)			Fail
Claim 8	191		8 (4.1%)	5.7	

Abbreviations: %CV, coefficient of variation expressed as a percentage; UVL, upper verification limit.

[4] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown..

Table 4.6 Results of precision verification study (within-laboratory) for 25-OHD results using Qualigen® method: user sample estimates, claims (using option A), and UVLs.[5]

	Mean (nmol/L)	Estimate, nmol/L (%CV)	Claim, nmol/L (%CV)	UVL (%)	Status
Sample 1	48.8	9.5 (19.4%)			Pass
Claim 5	64.8		9.8 (15.1%)	20.2	
Sample 2	66.5	11.5 (17.4%)			Pass
Sample 3	177.7	24.6 (13.9%)			Fail
Claim 8	191		9 (4.7)	6.5	

Abbreviations: %CV, coefficient of variation expressed as a percentage; UVL, upper verification limit.

[5] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.7 Extrapolated CVs for repeatability and within-laboratory claims for 25-OHD results using Qualigen® method.

Concentration (nmol/L)	Extrapolated Total %CV (r)	Extrapolated Within-laboratory %CV
48.8	16.3	14.7
66.5	13.8	12.4
177.7	6.04	5.2

Abbreviations: %CV, coefficient of variation expressed as a percentage; r, repeatability.

Table 4.8 Results of precision verification study (repeatability) for 25-OHD results using Qualigen® method: user sample estimates, extrapolated claims (using option B), and UVLs.[6]

	Mean (nmol/L)	Estimate, nmol/L (%CV)	Claim, nmol/L (%CV)	UVL (%)	Status
Sample 1	48.8	9.4 (19.2%)			Pass
PI Claim	48.8		16.3%	22.3	
Sample 2	66.5	11.4 (17.2%)			Pass
PI Claim	66.5		13.8%	18.9	
Sample 3	177.7	24.6 (13.9%)			Fail
PI Claim	177.7		6.03%	8.3	

Abbreviations: %CV, coefficient of variation expressed as a percentage; UVL, upper verification limit.

[6] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.9 Results of precision verification study (within-laboratory) for 25-OHD results using Qualigen® method: user sample estimates, extrapolated claims (using option B), and UVLs.[7]

	Mean (nmol/L)	Estimate, nmol/L (%CV)	Claim, nmol/L (%CV)	UVL (%)	Status
Sample 1	48.8	9.4 (19.2%)			Pass
PI Claim	48.8		14.7%	19.6	
Sample 2	66.5	11.4 (17.2%)			Fail
PI Claim	66.5		12.4%	16.6	
Sample 3	177.7	24.6 (13.9%)			Fail
PI Claim	177.7		5.2%	6.9	

Abbreviations: %CV, coefficient of variation expressed as a percentage; UVL, upper verification limit.

[7] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.10 Results of precision estimate comparisons using Qualigen®-provided quality assurance criteria. [8]

	Mean (nmol/L)	Estimate, nmol/L (%CV)	Criteria	Pass or Fail
Sample 1	48.8	9.4 (19.2%)	<20%	Pass
Sample 2	66.5	11.4 (17.2%)	<20%	Pass
Sample 3	177.7	24.6 (13.9%)	<20%	Pass

Abbreviations: %CV, coefficient of variation expressed as a percentage.

[8] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.11 Averaged run data for 25-OHD results using Qualigen® method (nmol/L). N=5, total average of five single replicates averaged per run per sample. N=4, total average of two replicates averaged together per run per sample. N=3, total average of three replicates averaged together per run per sample. N=2, total average of four replicates averaged together per run per sample. N=1, total average of all 5 replicates averaged from each run per sample.[9]

	N=5	N=4	N=3	N=2	N=1
Average (Sample 1)	48.8	48.7	48.6	48.6	48.5
SD	9.5	5.5	4.5	4.3	3.6
CV (%)	19.4	11.3	9.2	8.9	7.5
Average (Sample 2)	66.5	66.2	65.9	65.7	65.5
SD (%)	11.5	7.3	5.5	4.5	3.7
CV	17.3	11.0	8.3	6.9	5.7
Average (Sample 3)	177.7	177.7	178.1	177.8	177.8
SD	23	14.6	10.8	8.2	5.6
CV (%)	12.9	8.2	6.1	4.6	3.1

Abbreviations: SD, standard deviation; %CV, coefficient of variation expressed as a percentage.

[9] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table 4.12 Raw DEQAS sample data of 25-OHD results using Qualigen® method and NIST (nmol/L).

Sample	25-OHD concentration (Qualigen)	25-OHD concentration (NIST)
471	74.5	64.4
472	39.8	39.6
473	73.8	65.2
474	87.2	78.6
475	79.8	75.2

Table 4.13 Summary of basic statistics for 25-OHD results for healthy and ill samples using Qualigen® method.

	Healthy	Ill
N	40	40
Mean (nmol/L)	69.9	66.8
SD, nmol/L (%CV)	26.4 (37.8%)	14.4 (21.6%)

Abbreviations: N, number of results; SD, standard deviation; %CV, coefficient of variation expressed as a percentage.

Table 4.14 Summary of basic statistics for 25-OHD results for healthy and ill samples using reference methods.

	Healthy	Ill
N	40	40
Mean (nmol/L)	65.3	53.5
SD, nmol/L (%CV)	27.8 (42.5%)	18.5 (34.6%)

Abbreviations: N, number of results; SD, standard deviation; %CV, coefficient of variation expressed as a percentage.

Table 4.15 Summary of Chi-square data. Actual= reference methods. Reported= Qualigen® method.

	Actually Deficient/Insufficient	Actually Sufficient	Total
Reported Deficient/Insufficient	9	4	13
Reported Sufficient	25	42	67
Total	34	46	80

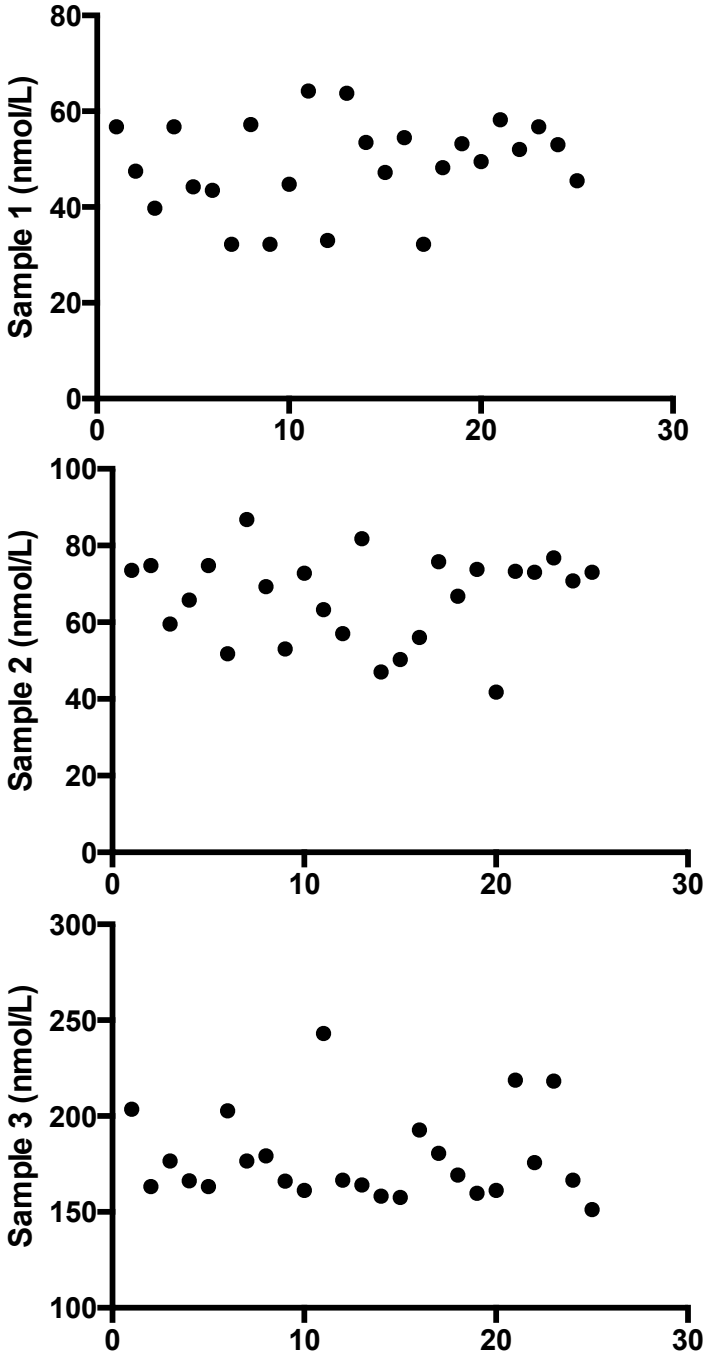

Figure 4.1 Repeated measures of 25-OHD values from precision verification test using Qualigen® FastPack Immunoassay, plotted in order of generation. Sample 1: DEQAS sample (panel a); Sample 2: pooled DEQAS samples (panel b); Sample 3: Qualigen® provided control (panel c). n=25 replicates per sample. Constructed using GraphPad Prism®.

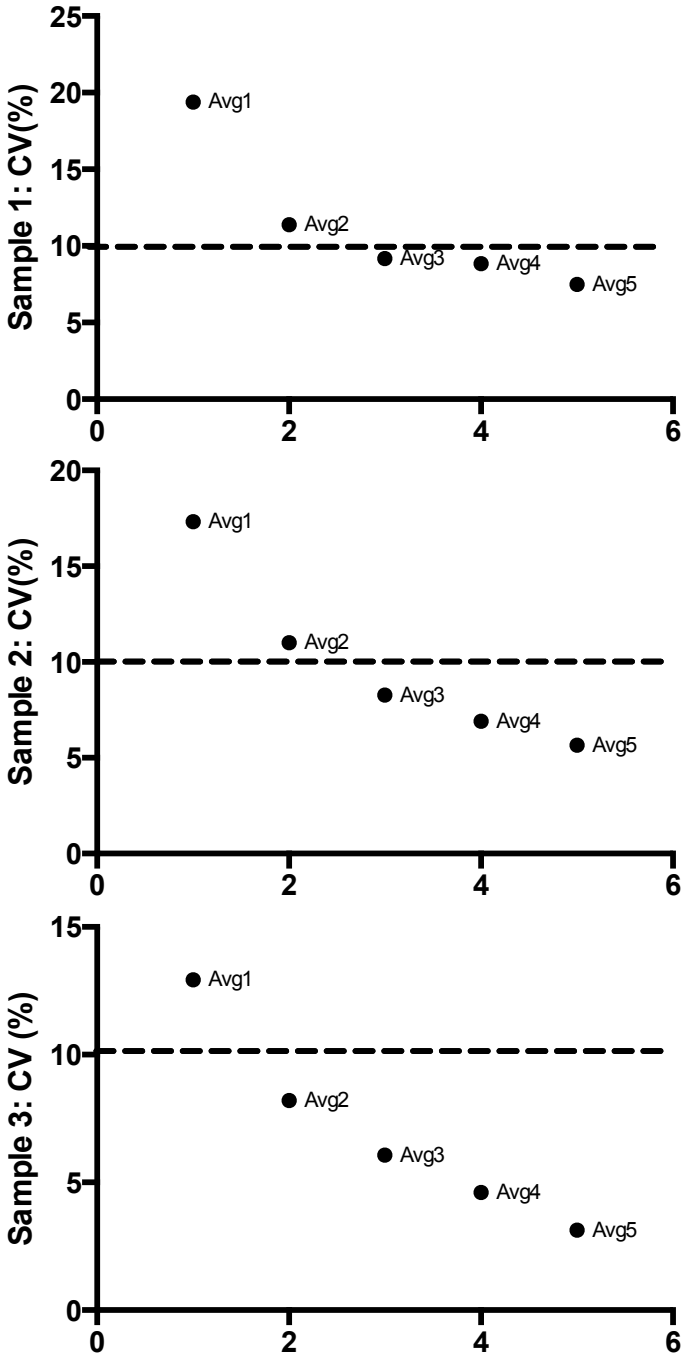

Figure 4.2 Averaged run data of 25-OHD precision verification data using Qualigen® method. Sample 1: DEQAS sample (panel a); Sample 2: pooled DEQAS samples (panel b); Sample 3: Qualigen® provided control (panel c). n=25 replicates per sample. Dotted line represents 10%CV. Abbreviations: %CV, coefficient of variation expressed as a percentage; Avg1, total average of five single replicates averaged per run per sample; Avg2, total average of two replicates averaged together per run per sample; Avg3, total average of three replicates averaged together per run per sample; Avg4, total average of four replicates averaged together per run per sample; Avg5, total average of all 5 replicates averaged from each run per sample. Constructed using GraphPad Prism®.

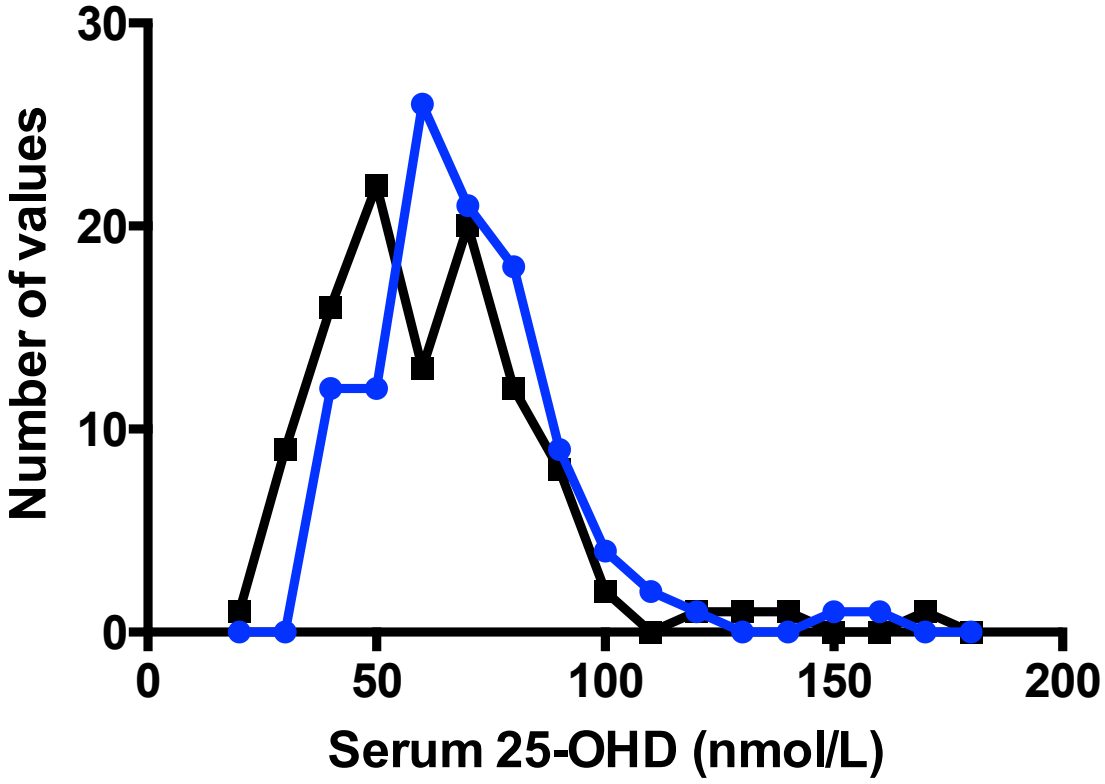

Figure 4.3 Frequency distribution for serum 25-OHD method comparison (nmol/L). Methods: field (black)=reference methods; test (blue)=Qualigen® testing. N=107. Constructed using GraphPad Prism®.

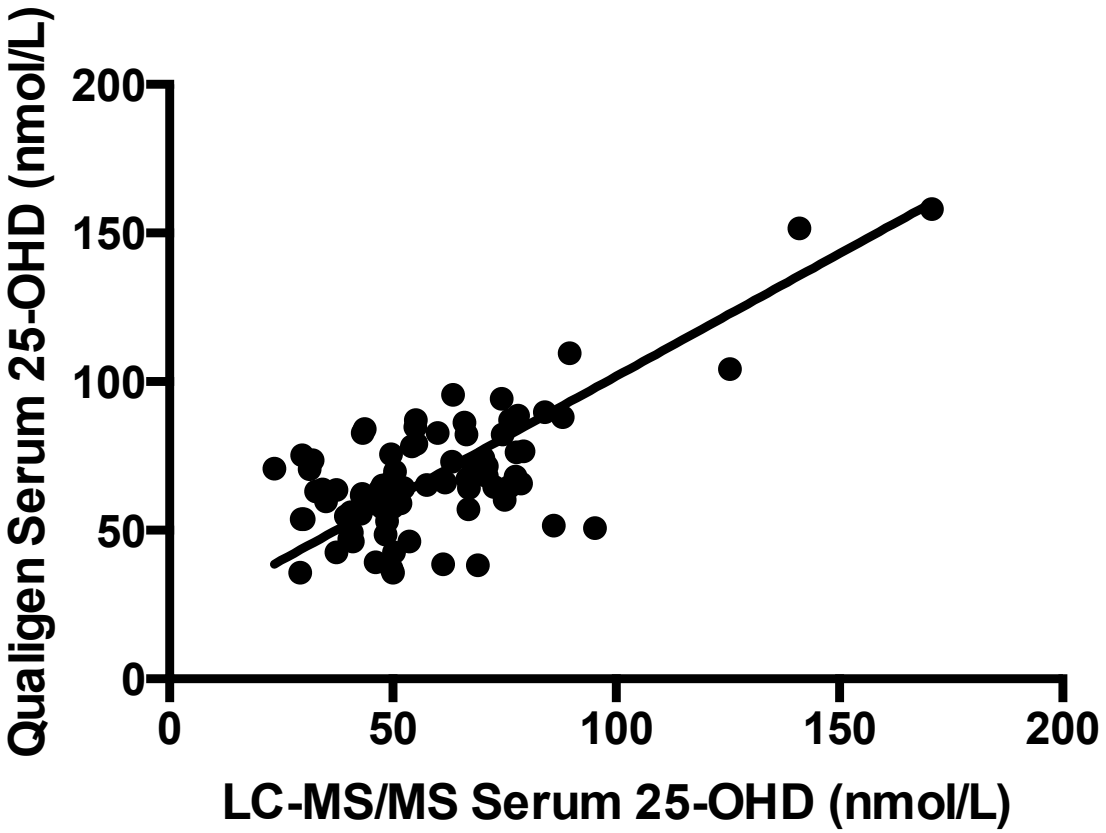

Figure 4.4 Deming regression for vitamin D method comparison (Qualigen® and LC-MS/MS). The circles represent the individual results. The line represents the fitted regression line. Equation: Y=0.82*X+19.2. R=0.687. N=80. Constructed using GraphPad Prism®.

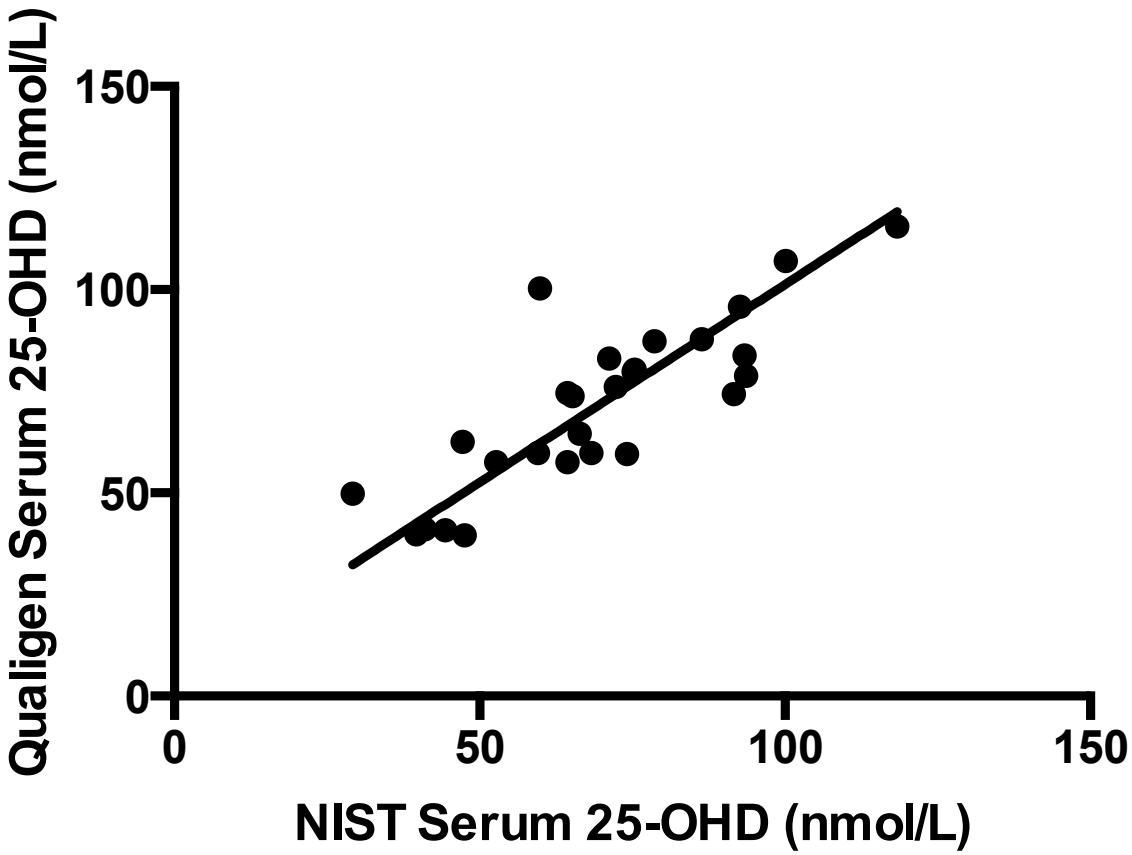

Figure 4.5 Deming regression for vitamin D method comparison (Qualigen® and NIST). The circles represent the individual results. The line represents the fitted regression line. Equation: Y=0.97*X+3.9. R=0.834. N=27. Constructed using GraphPad Prism®.

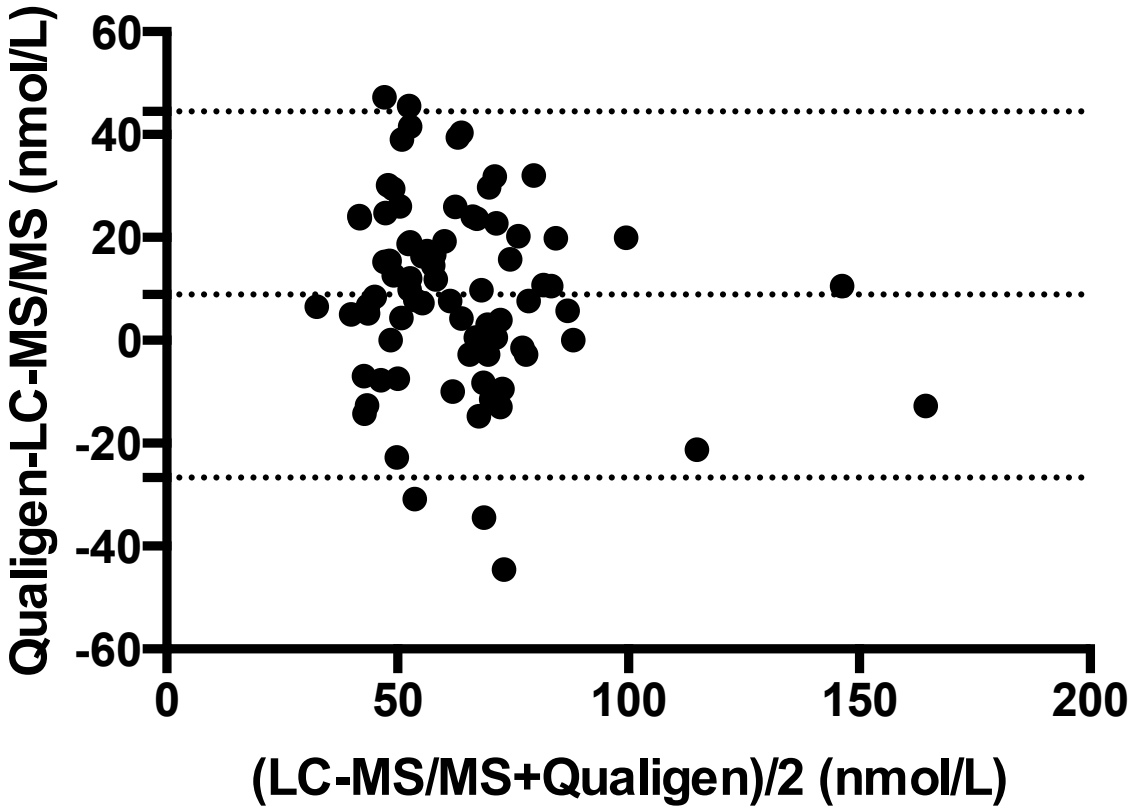

Figure 4.6 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and LC-MS/MS). The middle line represents the mean difference between the Qualigen® point-of-care test and the reference method (LC-MS/MS). The upper and lower lines represent the mean difference at ± 1.96 SD limits. N=80. Constructed using GraphPad Prism®.

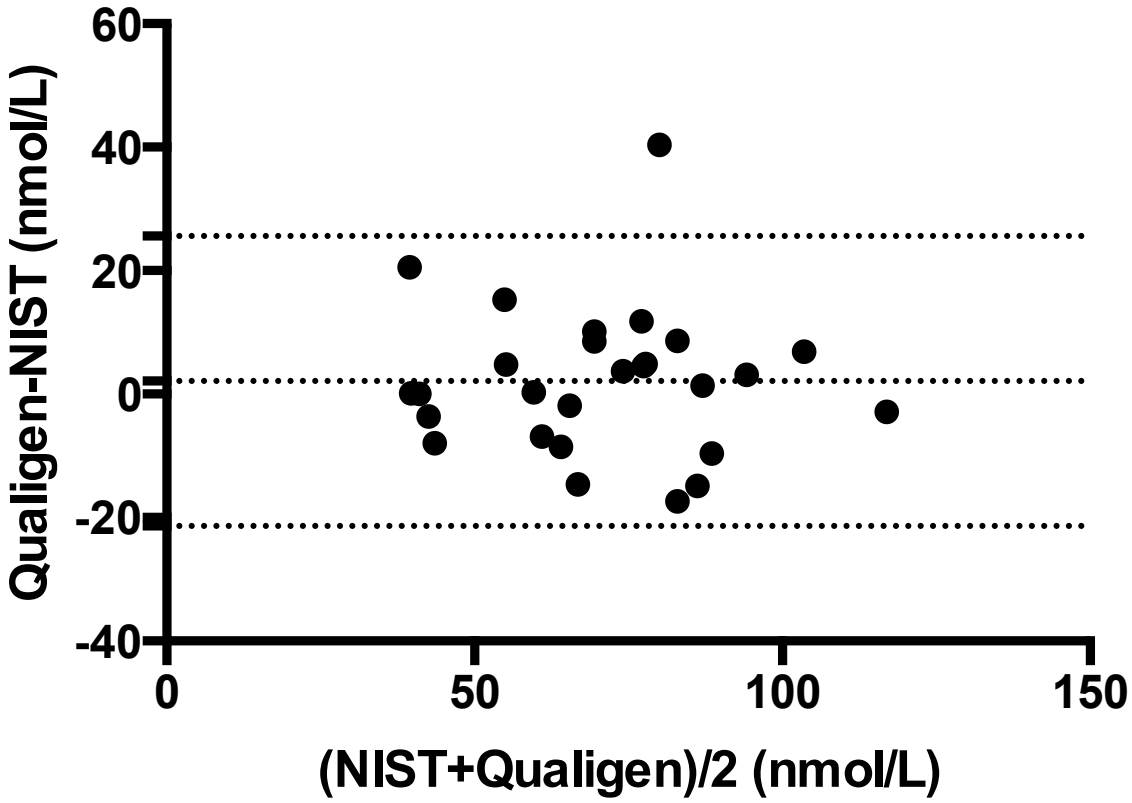

Figure 4.7 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and NIST). The middle line represents the mean difference between the Qualigen® point-of-care test and the reference method (NIST). The upper and lower lines represent the mean difference at ± 1.96 SD limits. N=29. Constructed using GraphPad Prism®.

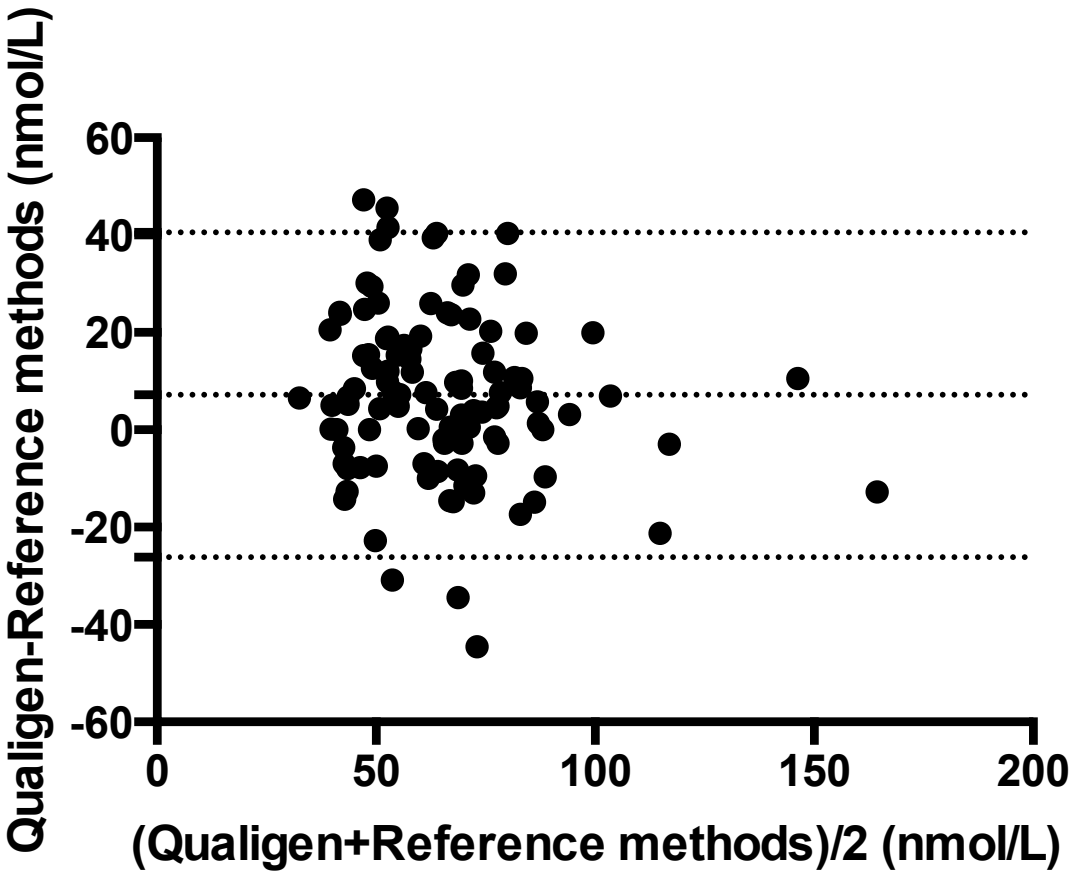

Figure 4.8 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and reference methods). The middle line represents the mean difference between the Qualigen® point-of-care test and the reference method (DEQAS). The upper and lower lines represent the mean difference at ± 1.96 SD limits. N=107. Constructed using GraphPad Prism®.

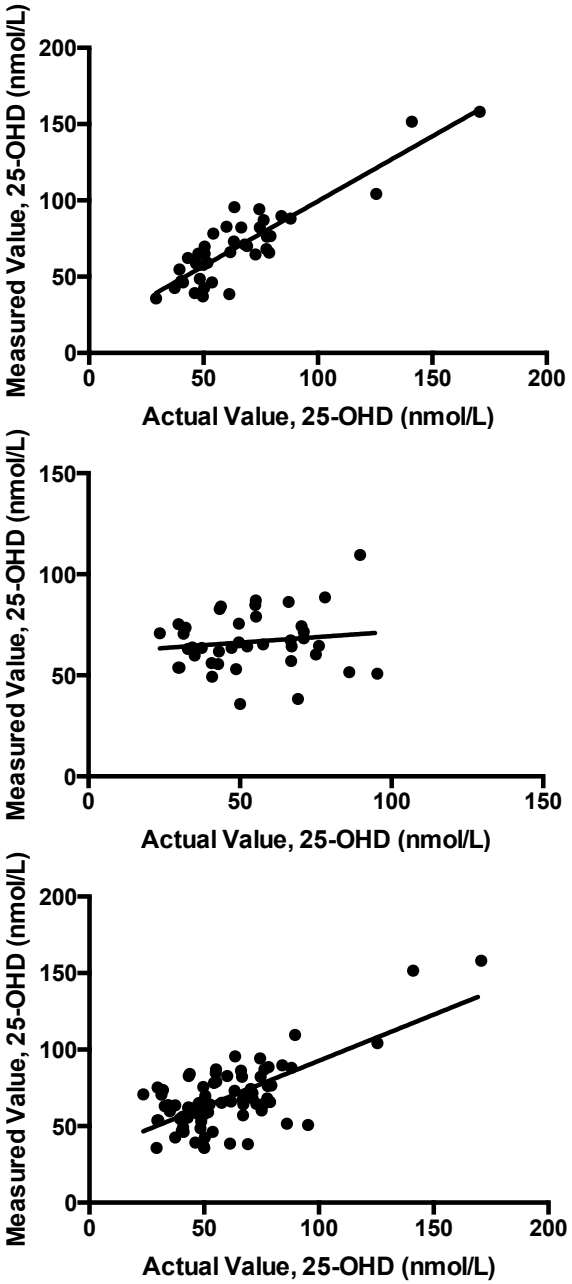

Figure 4.9 25-OHD concentrations plotted based on measurement method: measured (Qualigen®) versus actual (reference methods). Panel a: healthy children, n=40, Y=0.8508*X+14.40; Panel b: ill children, n=40, Y=0.1069*X+60.86; Panel c: total data (healthy and ill), n=80, Y=0.6021*X+32.51. Constructed using GraphPad Prism®.

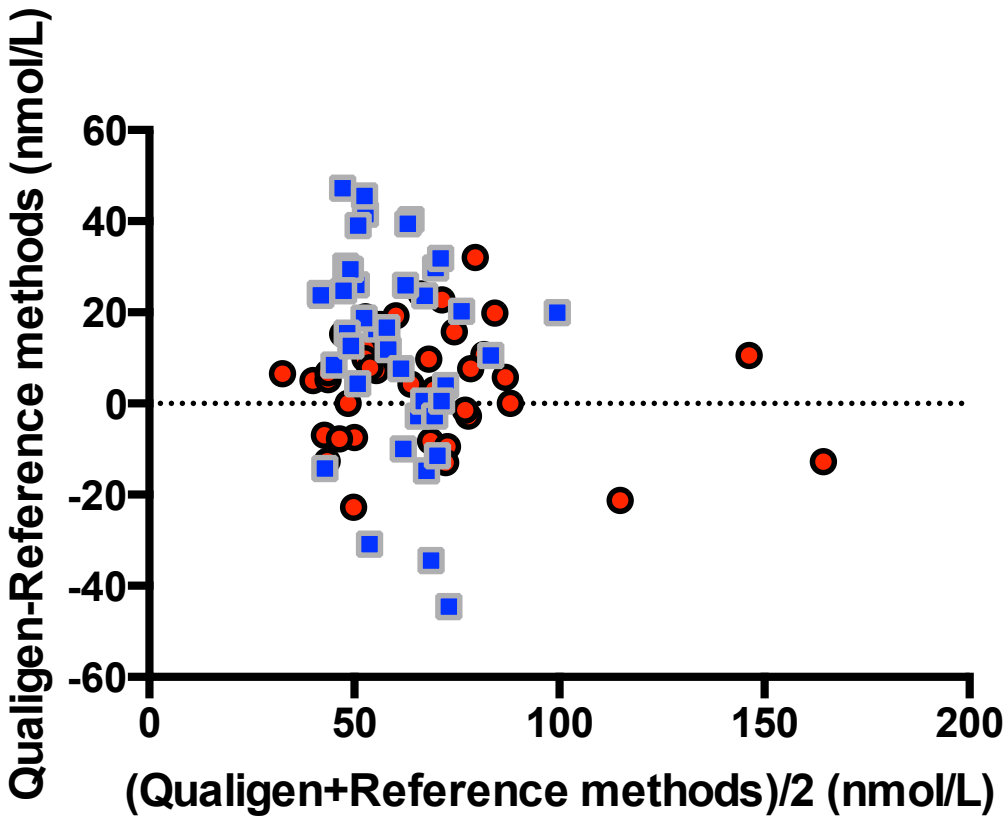

Figure 4.10 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and reference methods) of the total data (healthy and ill children) obtained using the Qualigen® immunoassay. Red dots= healthy children data. Blue squares= ill children data. N=80 samples. Constructed using GraphPad Prism®.

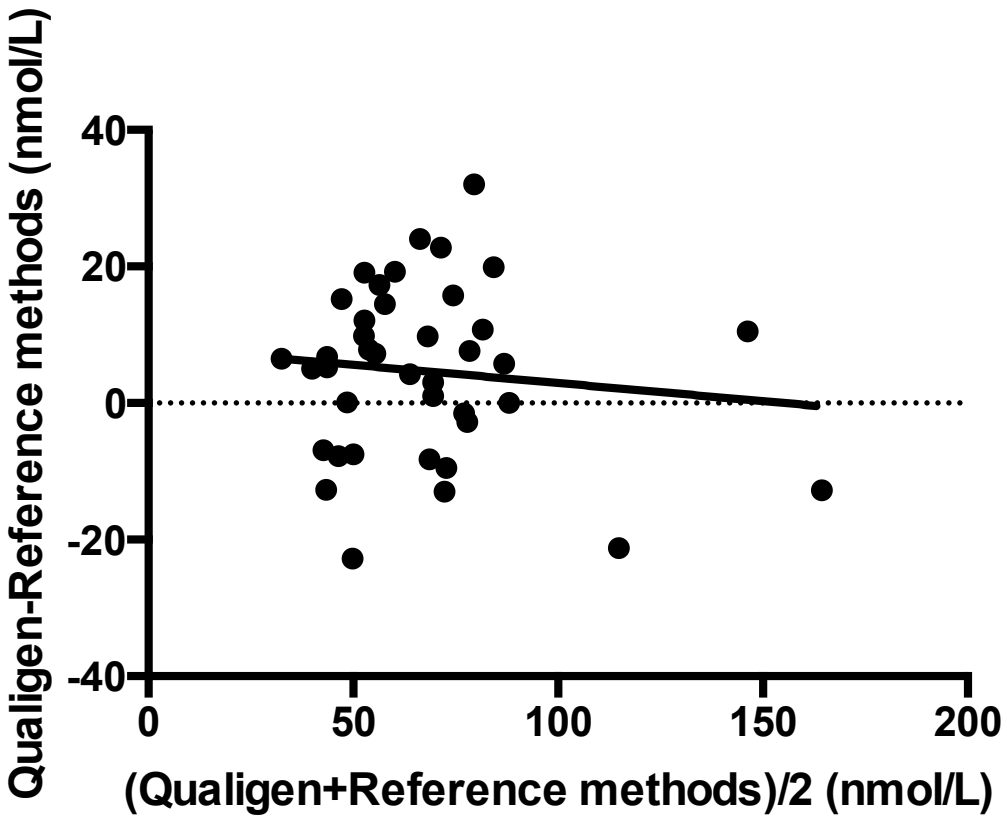

Figure 4.11 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and reference methods) of the healthy children obtained using the Qualigen® immunoassay. N=40 samples. Y=-0.05313*X+8.253. Correlation R= 0.1121. Bias=4.660. SD of bias=12.51. Constructed using GraphPad Prism®.

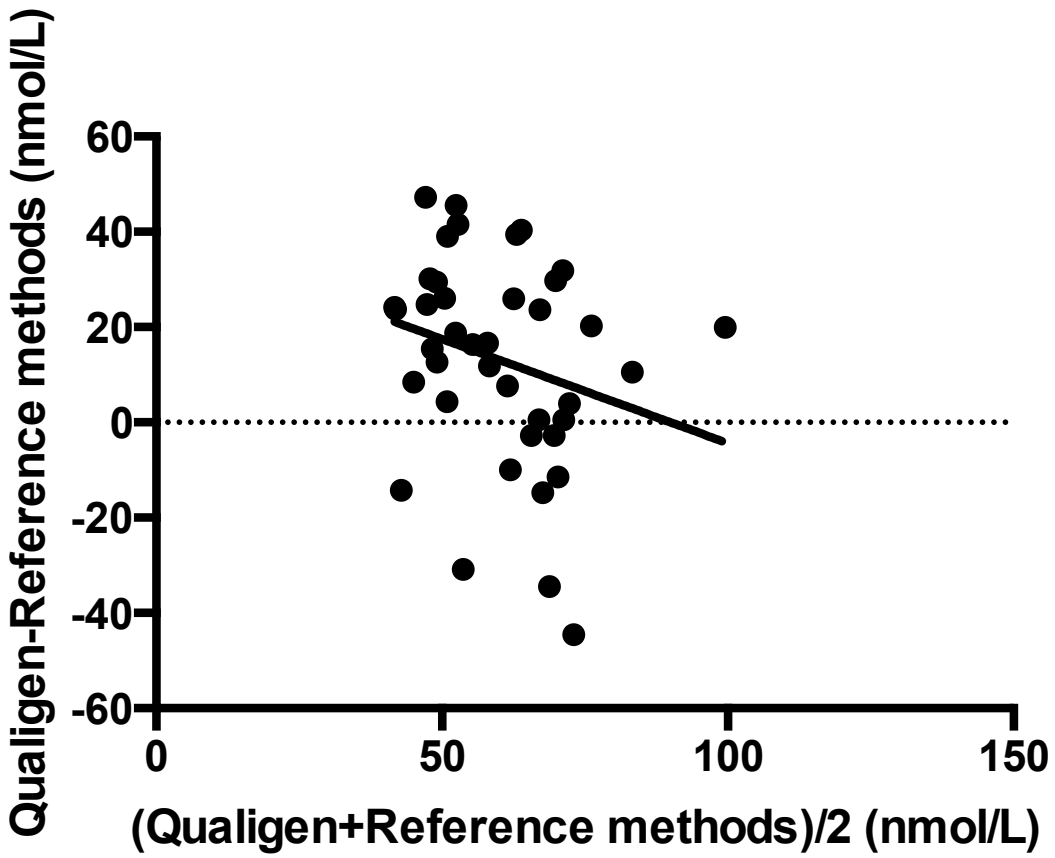

Figure 4.12 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and reference methods) of the ill children obtained using the Qualigen® immunoassay. N=40 samples. Y=-0.4372*X+39.95. Correlation R= 0.249. Bias=13.12. SD of bias=21.79. Constructed using GraphPad Prism®.

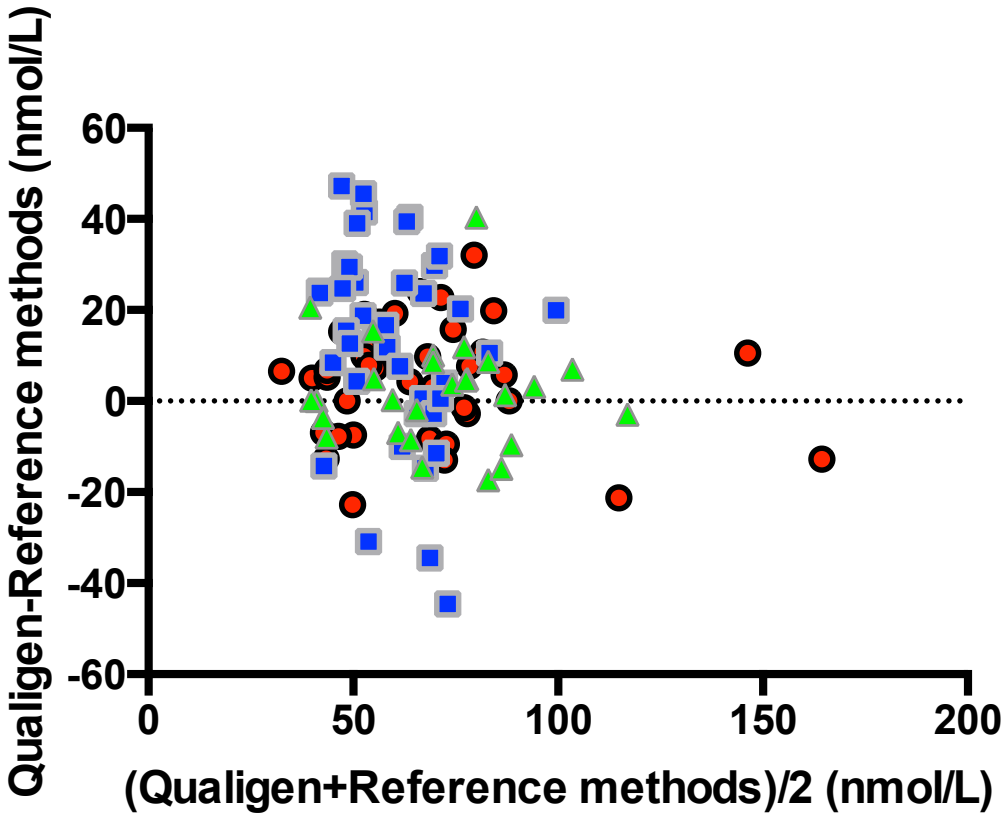

Figure 4.13 Bland-Altman (difference) plot for vitamin D method comparison (Qualigen® and reference methods) of the total data, obtained using the Qualigen® immunoassay. Lab 1 (red dots) measured the healthy samples. Lab 2 (blue squares) measured the ill children. NIST (green triangles) measured the DEQAS samples. N=107 samples. Constructed using GraphPad Prism®.

5. CONCLUSION

A growing body of evidence suggests there may be clinical benefits to the rapid identification and correction of vitamin D deficiency in ill hospitalized populations. With current standard of care practices, patient vitamin D levels are generally unavailable immediately post-blood collection, thereby creating a window of time in which vitamin D deficient patients remain untreated. As such, it is important to develop and validate point-of-care options for vitamin D status assessment.

This thesis tested the precision and bias of the vitamin D point-of-care test developed by Qualigen®. A precision verification study was conducted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (EP-15 A3) (52). Three patient samples (DEQAS sample (48.8 nmol/L), pooled DEQAS sample (66.5 nmol/L), and manufacturer provided control (177.7 nmol/L)) were tested 5 times per day for 5 days, consistent with a 5x5 precision verification design. These results were analyzed using a one-way ANOVA to calculate estimates of imprecision, which were then compared with the manufacturer claims. This comparison was used to determine whether the precision verification test passed or failed for each sample. The user's imprecision estimates (repeatability and within-laboratory) were considered to be consistent with the manufacturer's claims solely if they were less than or equal to the claim, or falling less than or equal to the UVL for that claim, at the appropriate (similar or exact) concentration (52). As such, the estimate meeting this criterion was considered to pass the test, and vice versa.

The verification analysis was performed in multiple ways: 1) comparing the calculated CVs from our experiment to the manufacturer's claim with a mean value that

was closest to the mean value of our tested sample or 2) through extrapolating the manufacturer claims and retrieving a CV claim with the same mean value as the sample being tested. Using the first option, Sample 3 was the only one to fail the precision verification test for both repeatability and within-laboratory imprecision. With the second option, Sample 3 failed for both estimates, in addition to Sample 2, which failed for within-laboratory imprecision. Additionally, Qualigen® provides a quality assurance method in which sample CVs less than 20% are considered as appropriate (56). Using this criterion, all three samples passed.

Given these results, failing the precision test simply means that the samples did not meet manufacturer claims for repeatability and/or within-laboratory imprecision. It is important to note that the internal tests performed by Qualigen® are likely conducted in ideal conditions, and may not reflect representative environments. As such, higher variability is expected in external validation settings. While some of the samples used in this study failed to meet these claims, it is not to say that this method is not clinically useful. Therefore, although some aspects of the precision study can be seen to "fail", this does not negate the potential usefulness of the Qualigen® method.

From Table 8, it can be seen that Qualigen® largely reports coefficients of variation <5%. For a vitamin D test to be useful to the user, a functional cut-off is set/implemented. It is often recommended that goals for the coefficient of variation of vitamin D assays be <10% (42, 60). With the results of the precision verification study, none of the estimates for repeatability or within-laboratory imprecision were found to be <10% (the closest being 13.9%). Consequently, results were averaged in an attempt to increase the accuracy in results.

Interestingly, Qualigen® provides a quality assurance method whereby CVs <20% are considered to be appropriate. Again, although Qualigen® may indicate this as appropriate, this variation may be too high in clinical settings, where patient vitamin D levels would be estimated from a large range of values. Therefore, although the error of this method, based on these results, may be considered high for a vitamin D assay (ranges from 13.9-19.4%), the acceptable error is at the discretion of the user.

In addition to the CLSI guidelines, options to increase the accuracy in the results provided by the Qualigen® method (decreasing the CVs) were used. As such, results of individual, 2, 3, 4, and 5 results per run were averaged to determine how many results needed to be averaged to bring the average CV down to 10%. So, the CVs obtained from averaging all five individual samples for all five runs were compared to the CVs obtained from averaging 2 samples per run and so on. It is known that the CV for a vitamin D assay varies based on concentration, where the lower the concentration, the higher the CV. As such, the number of replicates to obtain <10%CV would be determined by the concentration range in which the sample were found. With this, when using the Qualigen® method (based on sample 3, 177.7 nmol/L), patient samples would need to be tested twice and averaged in order to get a 25-OHD level with <10% variation. Alternatively, based on samples 1 (48.8 nmol/L) and 2 (66.5 nmol/L), patient samples would need to be measured three times and averaged to have similar precision in the Qualigen® method (Figure 4.2). This is useful for future applications in both research and daily clinical practice, as users can be aware of how many tests would be required to increase the confidence of patient 25-OHD levels they are obtaining using this Qualigen® method. Therefore, although the Qualigen® method initially failed on some

aspects of the precision test, it may still be useful to determine 25-OHD levels if the number of result per sample are increased and averaged. This however adds to the cost of the assay and reduces its utility.

In order to compare the Qualigen® measurement method to the reference methods, various plots were created for method verification, including frequency distribution graph, Bland-Altman (difference) plots, and deming regression plots. With this, different reference methods and laboratories were used to measure 25-OHD in the samples used in this study. These include LC-MS/MS (healthy children, CHD) and NIST (DEQAS). As previously mentioned, LC-MS/MS is considered the current gold standard in vitamin D measurement (16), and is considered to have a high degree of accuracy, due to the specificity of tandem mass spectrometry (18). Despite these advantages, LC-MS/MS has a few drawbacks including the possibility that vitamin D epimers (i.e. 3-epimer) may display very similar chromatography to 25-OHD (16), where they can overlap 25-OHD peaks and form the same masses (MS/MS ion pairs (16)) after ionization (17). Due to this, LC-MS/MS may contribute a degree of bias to the 25-OHD measurements by Qualigen® that may not be considered. For example, in the January 2015 DEQAS report for sample 470, the %CV of the LC-MS/MS method was 9.7%.

For the DEQAS samples, the provided 25-OHD concentrations were measured using NIST (using their LC-MS/MS reference measurement procedure). NIST has developed standard reference materials (SRMs) for vitamin D metabolites to help ensure reliable calibration and validation of assays for determining metabolite concentrations (61). Using these materials, isotope dilution LC-MS and LC-MS/MS methods for

measuring 25-OHD have been developed, and LC-MS/MS is the recognized reference measurement procedure (61).

Figure 4.13 illustrates a Bland-Altman plot with separate points for each laboratory/method used to measure sample 25-OHD. Based on this plot, there seems to be some variation in results between the reference methods. LC-MS/MS (laboratory 2) seems to have a higher range of differences than the other methods (y axis). Therefore, bias from reference methods still remains a potential factor when considering the accuracy of the Qualigen® method.

Based on the frequency distribution graph (Figure 4.3), which describes the frequency of values at difference 25-OHD concentrations, it is suggested that the Qualigen® method may be producing values that are slightly higher than those produced by reference methods. This is further supported by the difference plots (Figure 4.6-4.8), which suggest that the Qualigen® method may be reporting values that are on average higher than what the reference methods measure. When compared to LC-MS/MS, Qualigen® is overestimating by 8.9 nmol/L (Figure 4.6), and when compared with DEQAS measurements, Qualigen® is overestimating by 2.1 nmol/L (Figure 4.7). When compared to all reference methods, Qualigen® is overestimating by 7.2 nmol/L (Figure 4.8). This is approaching the limit of detection of most assays, and therefore is not considered as a main issue. However, this may provide some information to possible error from the LC-MS/MS methods used to measure 25-OHD concentrations, as DEQAS results are largely from NIST, and therefore an excellent alternative to increase confidence in test performance.

When comparing the deming-regression analyses (Figure 4.4 and 4.5), it can be additionally seen how the Qualigen® method more closely resembles the results from NIST (R=0.834) than the LC-MS/MS (R=0.687) methods. This further implies that perhaps there is a larger degree of error with the LC-MS/MS methods than initially realized, likely due to laboratory differences and error. It may also indicate that both the Qualigen® and NIST have similar error (P>0.05); however, NIST is the current standard reference material for 25-OHD measurements. As such, one would prefer that test results more closely resemble NIST values than others.

Finally, the frequency curve for the Qualigen® method also seems to have a higher peak (approximately at 60 nmol/L) than the reference methods, which have a slightly broader peak (Figure 4.3). This may indicate that values are reporting more similarly (around 60 nmol/L) with the Qualigen® method when compared with reference methods (around 50 nmol/L).

To test whether there was a difference in test performance between healthy and ill children, 25-OHD concentrations measured using the Qualigen method for healthy and ill children were plotted against values obtained from reference methods (Figure 4.9) to visualize agreement. From these graphs, there seems to be more agreement with the healthy children (slope=0.8) than the ill (slope=0.1) samples. This supports the previous finding where the LC-MS/MS from laboratory 2 seemed to have a higher range of differences than the other methods. This may be due to laboratory differences rather than differences in samples. For example, it is important to note that laboratory 1 separated out the 3-epimer when measuring the 25-OHD concentrations of the healthy children samples. As previously mentioned, the 3-epimer is known to be in higher concentrations in infants

and may contribute to total vitamin D levels. Lab 2 did not separate the epimer, which could be a main reason why the agreement is much lower for the ill children samples. Qualigen® reports a cross-reactivity of 7.8% by the 3-epimer (Table 2.4). So, another possibility is the potential interference of 24,25-$(OH)_2$ vitamin D metabolite, which Qualigen® reported as having a cross-reactivity of 117.4% (Table 2.4). Additionally, the higher agreement observed for healthy children samples may be partly caused by three visible outliers (Figure 4.9). It was initially thought that these points might be influencing a better agreement than there is in actuality; however, removing the three outliers yielded a similar relationship (slope=0.8). A larger sample size would better allow assessment of this possibility.

Further, from the calculated standard deviations, there seems to be degree of bias in measurements, for both healthy and ill samples and notable differences in standard deviations between the groups or laboratories/methods. Bland-Altman plots were constructed (Figure 4.10-4.12). Using these graphs, a similar pattern of results is observed whether healthy (R=0.11) or ill children (R=0.25) are assessed. This is important as it may be concluded that the test is not affected by the choice of patient samples. However, again there does not seem to be good agreement between either healthy (SD of bias=12.51) or ill samples (SD of bias= 21.79) and the Qualigen® method.

Additionally, status categories (deficient/insufficient versus sufficient) were tested using a Chi-square test for proportions. The result was not statistically significant (P=0.0527). However, from Table 17 it can be seen that the Qualigen method tends to overestimate sample concentrations, and therefore, may have bias towards higher values. As previously discussed, Figure 4.3 further supports this. This suggests that Qualigen®

may miss detection of low vitamin D levels, especially in addition to its limit of quantitation. This is problematic, as deficient patients need to be identified for proper treatment, in both clinical and research settings. As such, this observation needs to be confirmed with a larger sample size before use in clinical practice.

It may also be important to consider, when assessing the accuracy of the Qualigen® method, whether or not the observed differences in this method comparison are actual differences in Qualigen® and reference methods or whether they are the result of normal error, experimental error, etc. as these may influence not only Qualigen® measurements, but also reference methods. Additionally, it is important to test for the impact of other metabolites on Qualigen® results, for example the 3-epimer, which is known to be in high concentrations in infants. With this, since 25-OHD concentrations are often lower for children than adults and error is greater at lower values, this may account for some of the discrepancy with results from children samples and DEQAS samples. Additionally, as mentioned, the presence of 24,25-$(OH)_2$D may contribute to positive bias in measurements of 25-OHD. With this, Qualigen indicates high cross-reactivity (117.4%) of the D_3 form of this metabolite (Table 4). As such, it may be an important consideration in explaining bias.

There are a number of other possible sources of bias that may affect the consistency in results. For example, although the Qualigen® method performance is assessed relative to reference methods, the degree to which these reference methods may also be contributing to the overall bias is unknown. So, although there may be a degree of test method bias, there may also be contributions of the various methods and laboratories to the overall estimated bias. For example, older DEQAS samples may be measured

using “all methods”, rather than the currently used, and preferred, NIST method. This may contribute to a larger degree of bias, where the bias with each method would decrease confidence in the trueness of the measurement procedure relative to known concentrations of tested sample (52). As such, although the estimated mean and SD for the sample are compared to those measured through DEQAS, there may be sources of bias in these reference measurements that cannot be controlled. Although it may be difficult to decrease laboratory bias, some sources of bias that cannot be controlled in order to decrease the overall bias estimation include unifying the reference method (preferably using NIST) and testing multiple samples. In this study, NIST was the reference method for all DEQAS samples analyzed (one of the excluded samples (<32.2 nmol/L) was tested using “all methods”).

Interestingly, standard use of the Qualigen® FastPack system involves the use of a printer to obtain results that appear on the machine screen. This printer is normally connected to the Qualigen® machine, providing a physical output of the results (25-OHD value is printed onto the pack label). In an attempt to increase the applicability of the Qualigen® FastPack system, the system was alternatively connected to a computer output whereby results were sent to a file on the connected desktop computer, and ultimately transferred to a spreadsheet. A typical result would appear in the following format (txt.file): XX.X ng/mL, mm/dd/yy hh:mm. One of the major benefits of the possibility for computer output of results is in the context of clinical trials, whereby study staff would be blinded to patient results, which would be sent directly to those responsible (ex. pharmacy) for administrating either placebo or treatment. This increases the scope of use

for the Qualigen® machine, from simply point-of-care in clinical and limited laboratory use, to wider-scale research studies.

Given the possible benefit of the Qualigen® method in estimating vitamin D levels of patients, the degree of laboratory work, although fairly simple, associated with using this device may be problematic in offices and environments where laboratory work and experience is minimal. Also, this machine may prove costly for larger scale projects or daily use, particularly if results were averaged to increase accuracy in the results. Further, a few limitations in this study were noted. First, this study was a single rather than a multicenter study, where the latter may have provided more information on performance and possible sources of error. Additionally, only stored serum samples were tested in this study. As this machine is meant for serum assessment, the use of fresh samples would be more akin to the point-of-care assessment desired in certain research settings. Therefore, testing the machine with fresh serum/plasma samples would have been beneficial for these future research projects. Also, Qualigen® does provide results from their own tests for interference and cross-reactivity (Table 2.3 and 2.4), where there was a high degree of cross-reactivity 24,25-$(OH)_2$ D_3 (117.4 %). This study did not assess cross-reactivity of other metabolites, but this remains important to explore in future studies. Additionally, Qualigen® only provided claims in the optimal vitamin D level range (64.8 nmol/L and above). This prevented the accurate assessment of patient samples with insufficient or deficient 25-OHD concentrations, which are especially important in research settings.

In conclusion, the results of this study indicate that there may be possible benefit for the use of the Qualigen® method, particularly in research settings, where patient

vitamin D levels need to be approximated. Although the Qualigen® method initially failed on some aspects of the precision test, averaging the data may be a simple practical solution to correct for this and increase the accuracy in the results. Doing so may increase acceptability of results and provide values that are more representative of true values. Further, the Qualigen® method may still be useful even with the possible bias, as a correction factor may be made available to users, through future studies, in an attempt to estimate the true 25-OHD concentration in patient samples. Due to the limited number of available 25-OHD point-of-care options, Qualigen® may provide a suitable option for 25-OHD measurement for the time being, as improvements in precision and accuracy are important in order to increase confidence in results. For exact values, physicians and research teams should rely on standard and approved measurement methods for 25-OHD with lower coefficients of variation. Further use and analyses of the Qualigen® method will indeed uncover its true precision and effectiveness as a point-of-care test in measuring 25-OHD concentrations.

SIGNIFICANCE

This study will be a significant step in implementing vitamin D point-of-care options in research and even practical settings. This study will make known the benefits of point-of-care options for vitamin D assessment and the possibility of use of the Qualigen® method, one of the limited available methods. Vitamin D measurements are often seriously delayed and the importance of rapid measurements is often ignored. As such, this study will provide a voice to the importance of rapid 25-OHD assessments in research and clinical settings and the use of point-of-care devices in this respect.

SUMMARY

There may be clinical benefit to the rapid identification and correction of vitamin D deficiency in critically ill populations. With current standard of care practices, patient vitamin D levels are generally unavailable for days to weeks post-blood collection, thereby creating a window of time in which vitamin D deficient patients remain untreated. As such, it is important to develop and validate point-of-care options for vitamin D status assessment.

This was a validation study evaluating a vitamin D point-of-care test in a heterogenous population. In this study, the precision and bias of the vitamin D point-of-care test developed by Qualigen® were tested. This study was conducted at the Children's Hospital of Eastern Ontario (CHEO) and McGill University using stored research serum samples collected from heterogenous pediatric and adult populations.

The results of this study indicate that there may be possible benefit for the use of the Qualigen® method to estimate patient vitamin D levels. Although the Qualigen® method initially failed on some aspects of the precision test, averaging the data may be a simple practical solution to correct for this and increase the accuracy in the results. Doing so may increase acceptability of results and provide values that are more representative of true values. Further, although the Qualigen® method may be biased for higher values (needs to be confirmed with a larger sample size), it may still be useful if a correction factor is made available to users. Due to the limited number of available 25-OHD point-of-care options, Qualigen® may provide a suitable option for 25-OHD measurement for the time being, as improvements in precision and accuracy are important in order to increase confidence in results. For exact values, physicians and research teams should

rely on standard and approved measurement methods for 25-OHD with lower coefficients of variation. Further use and analyses of the Qualigen® method will indeed uncover its true precision and effectiveness as a point-of-care test in measuring 25-OHD concentrations.

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APPENDIX

A) Grubb's Test for Outliers

Sample 1
5 runs, 5 replicates per run
N=25
G=3.135 (CLSI EP-15 A3, page B22, Table 3) (70)
Mean of all results: 48.8 nmol/L
SDs of all results: 9.46264586 nmol/L

Grubb's limits= mean ± G × SD
= 48.8 ± (3.135 × 9.46264586)
= 48.8 ± 29.6653948
= 19.1346052 and 78.4653948 nmol/L, respectively.

Because all of the results fall within these limits, none qualifies as a statistical outlier.

Sample 2
5 runs, 5 replicates per run
N=25
G=3.135 (CLSI EP-15 A3, page B22, Table 3) (70)
Mean of all results: 66.47 nmol/L
SDs of all results: 11.5163652 nmol/L

Grubb's limits= mean ± G × SD
= 66.47 ± (3.135 × 11.5163652)
= 66.47 ± 36.1038049
= 30.3661951 and 102.573805 nmol/L, respectively.

Because all of the results fall within these limits, none qualifies as a statistical outlier.

Sample 3
5 runs, 5 replicates per run.
N=25
G=3.135 (CLSI EP-15 A3, page B22, Table 3) (70)
Mean of all results: 177.67 nmol/L
SD of all results: 22.9809387 nmol/L

Grubb's limits= mean ± G × SD
= 177.67 ± (3.135 × 22.9809387)
= 177.67 ± 72.0452428
= 105.624757 and 249.715243 nmol/L, respectively.

Because all of the results fall within these limits, none qualifies as a statistical outlier.

B) One-Way Analysis of Variance (ANOVA) Computation

All calculations were confirmed using excel, SAS, and R.

Table B1. Basic statistics and outlier limits for 25-OHD results using Qualigen® FastPack Immunoassay.[10]

	Sample 1	Sample 2	Sample 3
N	25	25	25
Grand mean (nmol/L)	48.8	66.47	177.67
SD, nmol/L (%CV)	9.46264586 (19.3906677%)	11.5163652 (17.3256584)	22.9809387 (12.9346196%)
Lowest result, nmol/L	32.25	41.75	151.25
Highest result, nmol/L	64.25	86.75	243
Grubb's lower limit, nmol/L	19.1346052	30.3661951	105.624757
Grubb's upper limit, nmol/L	78.4653948	102.573805	249.715243

Abbreviations: N, number of results; SD, standard deviation; %CV, coefficient of variation expressed as a percentage.

Table B2. Generalized one-way ANOVA summary table format.

Source of Variation	SS	DF	MS
Between-run	SS1	DF1	MS1
Within-run	SS2	DF2	MS2
Total	SS total	DF total	

Abbreviations: SS, sum of squares; DF, degrees of freedom; MS, mean squares; ANOVA, analysis of variance.

<u>Sample 1</u>
N=25
k (number of distinct runs): 5
arithmetic average of the N results, (grand mean): 48.8 nmol/L

a) Compute sums of squares (SS) total.
Manual equivalent of obtaining SS total using the following equation (Page B69 (70)):

[10] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

$$SS_{total} = \sum_{i=1}^{N} (x_i - \bar{\bar{x}})^2 = \left(\sum_{i=1}^{N} x^2_i \right) - N\bar{\bar{x}}^2$$

= Taking the squares of the results and then sum of these squares = 61685
= N*(grand mean squared)=59536
= SStotal= 61685-59536= 2149

b) Compute SS1 (between-run SS).
Table B3. Run statistics for Sample 1.

Run	n	average of the results for each run (nmol/L)	DM2 (difference between grand mean and run average, difference square) * n (number of results for that run)
1	5	49	0.2
2	5	42	231.2
3	5	52.35	63.0125
4	5	47.55	7.8125
5	5	53.1	92.45

Abbreviations: DM2, difference between grand mean and run average, difference square; n, number of results for run.

Manual equivalent of obtaining SS1 using the following equation (Page B69 (70)):

$$SS1 = \sum_{i=1}^{k} n_i (\bar{x}_i - \bar{\bar{x}})^2 = \left(\sum_{i=1}^{k} n_i \bar{x}_i^2 \right) - N\bar{\bar{x}}^2$$

SS1= sum of k entries of DM2*n= 394.675

c) Compute SS2 (within-run SS).
SS2 can be obtained by subtraction from the two already computed SS, SStotal and SS1.

SS2=SStotal-SS1=12674.965-529.015=1754.325

d) Compute degrees of freedom (DF).
N and k are used to calculate DF entries.

DF total= N-1
= 25-1
= 24
DF1=k-1
= 5-1
= 4
DF2= DFtotal-DF1
= 24-4
= 20

e) Compute the two mean squares (MS).
MS1=SS1/DF1
= 394.675/4
= 98.66875
MS2=SS2/DF2
= 1754.325/20
= 87.71625

n_0= N/k
= 25/5
= 5 (average number of results per run)

Sample 2.
N=25
k (number of distinct runs): 5
arithmetic average of the N results, (grand mean): 66.47 nmol/L

a) Compute sums of squares (SS) total.
Manual equivalent of obtaining SS total using the following equation (Page B69 (70)):

$$SS_{total} = \sum_{i=1}^{N} (x_i - \bar{\bar{x}})^2 = \left(\sum_{i=1}^{N} x^2_i \right) - N\bar{\bar{x}}^2$$

= Taking the squares of the results and then sum of these squares = 113639.563
= N*(grand mean squared)= 110456.523
= SStotal= 113639.563- 110456.523= 3183.04

b) Compute SS1 (between-run SS).
Table B4. Run statistics for Sample 2.

Run	n	average of the results for each run (nmol/L)	DM2 (difference between grand mean and run average, difference square) * n (number of results for that run)
1	5	69.65	50.562
2	5	66.7	0.2645
3	5	59.85	219.122
4	5	62.8	67.3445
5	5	73.35	236.672

Abbreviations: DM2, difference between grand mean and run average, difference square; n, number of results for run.

Manual equivalent of obtaining SS1 using the following equation (Page B69 (70)):

$$SS1 = \sum_{i=1}^{k} n_i \left(\bar{X}_i - \bar{\bar{X}}\right)^2 = \left(\sum_{i=1}^{k} n_i \bar{X}_i^2\right) - N\bar{\bar{X}}^2$$

SS1= sum of k entries of DM2*n= 573.965

c) Compute SS2 (within-run SS).
SS2 can be obtained by subtraction from the two already computed SS, SStotal and SS1.

SS2=SStotal-SS1= 3183.04-573.965= 2609.075

d) Compute degrees of freedom (DF).
N and k are used to calculate DF entries.

DF total= N-1
= 25-1
= 24
DF1=k-1
=5-1
=4
DF2= DFtotal-DF1
= 24-4
= 20

e) Compute the two mean squares (MS).
MS1=SS1/DF1
= 573.965/4
= 143.49125
MS2=SS2/DF2
= 2609.075/20
= 130.45375

n_0= N/k
= 25/5
= 5 (average number of results per run)

Sample 3
N=25
k (number of distinct runs): 5
arithmetic average of the N results, (grand mean): 177.67 nmol/L

a) Compute sums of squares (SS) total.
Manual equivalent of obtaining SS total using the following equation (Page B69 (70)):

$$SS_{total} = \sum_{i=1}^{N} (x_i - \bar{\bar{x}})^2 = \left(\sum_{i=1}^{N} x^2_i \right) - N\bar{\bar{x}}^2$$

= Taking the squares of the results and then sum of these squares = 801840.688
= N*(grand mean squared)= 789165.723
= SStotal= 801840.688- 789165.723= 12674.965

b) Compute SS1 (between-run SS).
Table B5. Run statistics for Sample 3.

Run	n	average of the results for each run (nmol/L)	DM2* n
1	5	174.55	48.672
2	5	177.15	1.352
3	5	177.85	0.162
4	5	172.7	123.5045
5	5	186.1	355.3245

Abbreviations: DM2, difference between grand mean and run average, difference square; n, number of results for run.

Manual equivalent of obtaining SS1 using the following equation (Page B69 (70)):

$$SSI = \sum_{i=1}^{k} n_i \, (\bar{X}_i - \bar{\bar{X}})^2 = \left(\sum_{i=1}^{k} n_i \, \bar{X}_i^2 \right) - N\bar{\bar{X}}^2$$

SS1= sum of k entries of DM2*n= 529.015

c) Compute SS2 (within-run SS).
SS2 can be obtained by subtraction from the two already computed SS, SStotal and SS1.

SS2=SStotal-SS1=12674.965-529.015= 12145.95

d) Compute degrees of freedom (DF).
N and k are used to calculate DF entries.

DF total= N-1
= 25-1
= 24
DF1= k-1
= 5-1
= 4
DF2= DFtotal-DF1
= 24-4
= 20

e) Compute the two mean squares (MS).
MS1=SS1/DF1
= 529.015/4
= 132.25375
MS2=SS2/DF2
= 12145.95/20
= 607.2975

n_0= N/k
= 25/5
= 5 (average number of results per run)

Summary of ANOVA results.

Table B6. Summary ANOVA results for Sample 1.

Source of Variation	SS	DF	MS
Between-run (1)	394.675	4	98.66875
Within-run (2)	1754.325	20	87.71625
Total	2149	24	

Abbreviations: SS, sum of squares; DF, degrees of freedom; MS, mean squares; ANOVA, analysis of variance.

Table B7. Summary ANOVA results for Sample 2.

Source of Variation	SS	DF	MS
Between-run (1)	573.965	4	143.49125
Within-run (2)	2609.075	20	130.45375
Total	3183.04	24	

Abbreviations: SS, sum of squares; DF, degrees of freedom; MS, mean squares; ANOVA, analysis of variance.

Table B8. Summary ANOVA results for Sample 3.

Source of Variation	SS	DF	MS
Between-run (1)	529.015	4	132.25375
Within-run (2)	12145.95	20	607.2975
Total	12674.965	24	

Abbreviations: SS, sum of squares; DF, degrees of freedom; MS, mean squares; ANOVA, analysis of variance.

f) Variance components

Sample 1
Vw= MS2= 87.71625
Since MS1 is larger than MS2, Vb= (MS1-MS2)/n_0= (98.66875-87.71625)/5= 2.1905.
Sum of the two variances corresponds to within-laboratory precision:
Vw + Vb= 87.71625+2.1905= 89.90675

Taking the square roots of these variances provides desired precision estimates expressed as SDs:
sr= sqrt(Vw)= 9.36569538262
sb= sqrt(Vb)= 1.4800337834
swl= sqrt(Vw+Vb)= 9.48191700027

In relative terms, the %CV is calculated:
%CVr= sr*100/grand mean= 9.36569538262 * 100/48.8= 19.1919987349
%CVb= sr*100/grand mean= 1.4800337834* 100/48.8= 3.03285611352
%CVwl= swl*100/grand mean= 9.48191700027* 100/48.8= 19.4301577874

Sample 2
Vw= MS2= 130.45375
Since MS1 larger than MS2, Vb= (MS1-MS2)/n_0= (143.49125-130.45375)/5= 2.6075
Sum of the two variances corresponds to within-laboratory precision:
Vw + Vb= 130.45375+2.6075= 133.06125

Taking the square roots of these variances provides desired precision estimates expressed as SDs:
sr= sqrt(Vw)= 11.42163517190074
sb= sqrt(Vb)= 1.6147755261955143
swl= sqrt(Vw+Vb)= 11.535217813288138

In relative terms, the %CV is calculated:
%CVr= sr*100/grand mean= 11.42163517190074* 100/66.47= 17.183143
%CVb= sr*100/grand mean= 1.6147755261955143* 100/66.47= 2.42932981
%CVwl= swl*100/grand mean= 11.535217813288138* 100/66.47= 17.354021

Sample 3
Vw (repeatability variance)= MS2= 607.2975
As MS1 is smaller than MS2, Vb ("pure" between-run variance)= 0.
Sum of the two variances corresponds to within-laboratory precision:
Vw + Vb= 607.2975

Taking the square roots of these variances provides desired precision estimates expressed as SDs:
sr (repeatability)= sqrt(Vw)= 24.6434068262
sb (between-run)= sqrt(Vb)=0
swl (within-lab)= sqrt(Vw+Vb)= 24.6434068262

In relative terms, the %CV is calculated:
%CVr= sr*100/grand mean= 24.6434068262 * 100/177.67= 13.870325163
%CVb= sb=0

Table B9. ANOVA results and imprecision estimates for 25-OHD results using Qualigen® FastPack Immunoassay.[11]

	Sample 1	Sample 2	Sample 3
N	25	25	25
MS1 (between)	98.66875	143.49125	132.25375
MS2 (within)	87.71625	130.45375	607.2975
n0	5	5	5
Vb (between)	2.1905	2.6075	0
Vw (within)	87.71625	130.45375	607.2975
Grand mean (nmol/L)	48.8	66.47	177.67
Sr, nmol/L (%CV)	9.37 (19.19%)	11.42 (17.18%)	24.64 (13.87%)
Swl, nmol/L (%CV)	9.48 (19.43%)	11.53 (17.35%)	24.64 (13.87%)

Abbreviations: ANOVA, analysis of variance; N, number of results; MS, mean squares; V_b, variance between runs; V_w, variance within run; S_r, user estimate for repeatability; S_{wl}, user estimate for within-laboratory imprecision; %CV, coefficient of variation expressed as a percentage.

[11] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

C) Upper Verification Limit

Calculations for option A results:

Using claim closest to mean value for sample.

Mean concentrations:
Sample 1: 48.8 nmol/L
Sample 2: 66.47 nmol/L
Sample 3: 177.67 nmol/L

Table C1. Manufacturer precision claims, Qualigen®. Modified from FastPack IP Vitamin D Immunoassay Kit Complete (01/14), ENG-5. C1a. represents values from Lot 1 and C1b. represents values from Lot 2. Values represented in nmol/L.

Sample Number	Average	Within-run variation (%CV)	Between-run variation (%CV)	Between-day variation (%CV)	Total imprecision (%CV)
1	68.2	25.5 (2.8)	12.2 (1.3)	17.8 (1.9)	33.5 (3.7)
2	77.8	26.8 (3.3)	0 (0)	14.2 (1.8)	30.2 (3.8)
3	113.8	21.2 (3.9)	0 (0)	10.8 (2.0)	23.8 (4.3)
4	212.2	12 (4.1)	0 (0)	9.2 (3.2)	15.2 (5.1)

Sample Number	Average	Within-run variation	Between-run variation	Between-day variation	Total imprecision
5	64.8	37.8 (3.9)	0 (0)	0 (0)	37.8 (3.9)
6	81.8	28 (3.7)	0 (0)	15 (2.0)	31.8 (4.2)
7	115.2	18.8 (3.5)	0 (0)	6.5 (1.2)	19.8 (3.7)
8	191	10.2 (3.2)	0 (0)	5.8 (1.7)	11.8 (3.6)

Abbreviations: %CV, coefficient of variation expressed as a percentage.

To calculate UVLs,
k (actual number of runs)= 5
n (number of replicates per run)= 5
Dfr=N-k
= 25-5
= 20

Based on the PI claims and the means for our samples (177.67, 66.47, 48.8 nmol/L), the following claims that most resemble our calculated means can be used:

- PI Claim 5 (Sample 1, Lot 2) and PI Claim 8 (Sample 4, Lot 2).

For PI Claim 5:
p(rho)= σwl/σr =%CVwl(manufacturer's within-laboratory imprecision claim)/%CVr(manufacturer's repeatability (within-run SD) claim)
= 15.1/15.1
=1
Dfwl (number of runs= 5, p= 1) from Table 6 (CLSI EP-15 A3)
Dfwl=24

F values from Table 7 (CLSI EP-15 A3)
Fr (5 samples, df=20)= 1.37
Fwl (5 samples, dwl=24)= 1.34

UVLr= Frx%CV
= 1.37x15.1%
= 20.687%
UVLwl=Fwlx%CV
= 1.34x15.1%
= 20.234%

For PI Claim 8:
p(rho)= σwl/σr =%CVwl/%CVr
= 4.7/4.1
= 1.146
Dfwl (number of runs= 5, p= 1.146)
Dfwl= 18

F values from Table 7 (CLSI EP-15 A3)
Fr (5 samples, df=20)= 1.37
Fwl (5 samples, dwl=18)=1.39

UVLr=Frx%CV
= 1.37x4.1
= 5.617%
UVLwl=Fwlx%CV
= 1.39x4.7
= 6.533%

Table C2. Repeatability UVLs for manufacturer's claims 5 and 8.

Claim	Mean (nmol/L)	σr, nmol/L (%CV)	k	n	p	dfr	F	UVLr, nmol/L (%CV)
Claim 5	64.75	9.75 (15.1)	20	4	1	60	1.37	20.687%
Claim 8	191	8 (4.1)	20	4	1.146	60	1.34	5.617%

Abbreviations: σr, manufacturer's claim for repeatability; k, actual number of results; n, number of replicates per run; dfr, degrees of freedom for repeatability; UVLr, upper verification limit (repeatability); %CV, coefficient of variation expressed as a percentage.

Table C3. Within-laboratory UVLs for manufacturer claims 5 and 8.

Claim	Mean (nmol/L)	σwl, nmol/L (%CV)	k	n	p	dfwl	F	UVLwl, nmol/L (%CV)
PI Claim 5	64.75	9.75 (15.1)	20	4	1	60	1.37	20.234%
PI Claim 8	191	9 (4.7)	20	4	1.146	60	1.39	6.533%

Abbreviations: σr, manufacturer's claim for within-laboratory imprecision; k, actual number of results; n, number of replicates per run; dfr, degrees of freedom for within-laboratory imprecision; UVLr, upper verification limit (within-laboratory); %CV, coefficient of variation expressed as a percentage.

The sample tests may be passed or failed by comparing calculated imprecision estimates and UVLs.

Table C4. Comparing imprecision estimates to manufacturer's claims: repeatability. [12]

	Mean (nmol/L)	Estimate (%CV)	Claim (%CV)	UVLr (%)	Status
Sample 1	48.8	9.37 (19.19%)			Pass
PI Claim 5	64.75		9.75 (15.1%)	20.687	
Sample 2	66.47	11.42 (17.18%)			Pass
Sample 3	177.67	24.64 (13.87%)			Fail
PI Claim 8	191		8 (4.1%)	5.617	

Abbreviations: UVLr, upper verification limit (repeatability); %CV, coefficient of variation expressed as a percentage.

[12] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table C5. Comparing imprecision estimates to manufacturer's claims: within-laboratory imprecision.[13]

	Mean (nmol/L)	Estimate (%CV)	Claim (%CV)	UVLwl (%)	Status
Sample 1	48.8	9.48 (19.43%)			Pass
PI Claim 5	64.75		9.75 (15.1%)	20.234	
Sample 2	66.47	11.53 (17.35%)			Pass
Sample 3	177.67	24.64 (13.87%)			Fail
PI Claim 8	191		9 (4.7)	6.533	

Abbreviations: UVLwl, upper verification limit (within-laboratory imprecision); %CV, coefficient of variation expressed as a percentage.

Calculations for option B results:

Table C6. Extrapolated data. [14]

Sample	Concentration (nmol/L)	Extrapolated Total CV (r)	Extrapolated Within-lab CV
1	48.8	16.25471642	14.65574355
2	66.47	13.81129207	12.38289847
3	177.67	6.037310118	5.151630317

Abbreviations: %CV, coefficient of variation expressed as a percentage.

To calculate UVLs,
k (actual number of runs)= 5
n (number of replicates per run)= 5
Dfr=N-k
= 25-5
= 20

PI Claim for 48.8 nmol/L
p(rho)= σwl/σr =%CVwl/%CVr
= 14.65574355/ 16.25471642
= 0.901630221

Dfwl (number of runs= 5, p= 0.9016) from Table 6 (CLSI EP-15 A3)
Dfwl=24

[13, 14] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

F values from Table 7 (CLSI EP-15 A3)
Fr (5 samples, df=20)= 1.37
Fwl (5 samples, dwl=24)= 1.34

UVLr= Fx%CV
=1.37x16.25471642%
=22.269%
UVLwl=Fx%CV
=1.34x14.65574355%
= 19.639%

PI Claim for 66.47 nmol/L
p(rho)= σwl/σr =%CVwl/%CVr
= 12.38289847/ 13.81129207
= 0.896577844

Dfwl (number of runs= 5, p= 0.89658) from Table 6 (CLSI EP-15 A3)
Dfwl=24

F values from Table 7 (CLSI EP-15 A3)
Fr (5 samples, df=20)= 1.37
Fwl (5 samples, dwl=24)= 1.34

UVLr= Fx%CV
=1.37x 13.81129207%
=18.92%
UVLwl=Fx%CV
=1.34x12.38289847%
= 16.59%

PI Claim for 177.67 nmol/L
p(rho)= σwl/σr =%CVwl/%CVr
= 5.151630317/ 6.037310118
= 0.853298939

Dfwl (number of runs= 5, p= 0.8533) from Table 6 (CLSI EP-15 A3)
Dfwl=24

F values from Table 7 (CLSI EP-15 A3)
Fr (5 samples, df=20)= 1.37
Fwl (5 samples, dwl=24)= 1.34

UVLr= Fx%CV
= 1.37x6.037310118%
= 8.27%
UVLwl=Fx%CV

= 1.34x5.151630317%
= 6.903%

Table C7. Comparing imprecision estimates to extrapolated claims: repeatability.[15]

	Mean (nmol/L)	Estimate (%CV)	Claim (%CV)	UVLr (%)	Status
Sample 1	48.8	9.37 (19.19%)			Pass
Claim	48.8		16.255%	22.269	
Sample 2	66.47	11.42 (17.18%)			Pass
Claim	66.47		13.811%	18.92	
Sample 3	177.67	24.64 (13.87%)			Fail
Claim	177.67		6.037%	8.27	

Abbreviations: UVLr, upper verification limit (repeatability); %CV, coefficient of variation expressed as a percentage.

Table C8. Comparing imprecision estimates to extrapolated claims: within-laboratory imprecision.[16]

	Mean (nmol/L)	Estimate (%CV)	Claim (%CV)	UVLwl (%)	Status
Sample 1	48.8	9.48 (19.43%)			Pass
Claim	48.8		14.6557%	19.639	
Sample 2	66.47	11.53 (17.35%)			Fail
Claim	66.47		12.3829%	16.59	
Sample 3	177.67	24.64 (13.87%)			Fail
Claim	177.67		5.1516%	6.903	

Abbreviations: UVLr, upper verification limit (within-laboratory imprecision); %CV, coefficient of variation expressed as a percentage.

Calculations for option C results:
Based on the Qualigen® quality assurance manual, the accepted imprecision criteria is <20%.

[15, 16] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

Table C9. Comparing imprecision estimates to extrapolated claims: within-laboratory imprecision.[17]

	Mean (nmol/L)	Estimate (%CV)	Criteria	Pass or Fail
Sample 1	48.8	9.37 (19.19%)	<20%	Pass
Sample 2	66.47	11.42 (17.18%)	<20%	Pass
Sample 3	177.67	24.64 (13.87%)	<20%	Pass

Abbreviations: %CV, coefficient of variation expressed as a percentage.

[17] Sample 1: DEQAS sample; Sample 2: pooled DEQAS samples; Sample 3: Qualigen® provided control. Sample concentrations unknown.

D) Chi-Square Analysis

Table D1. Summary of Chi-square square data. Actual= reference methods. Reported= Qualigen®.

	Actually Deficient/Insufficient	Actually Sufficient	Total
Reported Deficient/Insufficient	9	4	13
Reported Sufficient	25	42	67
Total	34 (42% of 80)	46 (58% of 80)	80

Null hypothesis: actual=reported

Alternate hypothesis: actual ≠ reported

Expected frequencies

(0.42)(13)= 5.46 (0.58)(13)=7.54

(0.42)(67)= 28.14 (0.58)(67)= 38.86

Test statistics= $(9-5.46)^2/5.46+ (25-28.14)^2/28.14 + (5-7.54)^2/7.54+ (42-38.86)^2/38.86$

=2.295+0.3504+0.8556+0.2537

=3.7547

Df=n(number of classes)-1=1

Probability=0.05

P-value=0.052659>0.05

Therefore, we fail to reject the null hypothesis. The result is not statistically significant.