Vitamin D Testing By Mass Spectrometry

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9/17/13

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Disclosures

- Morgan Stanley, provide consulting services
- Thermo Fisher Scientific, provide consulting services

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Vitamin D Testing By Mass Spectrometry

Learning Objectives:

- Describe CMS reimbursement criteria for Vit D
- Define ways to determine appropriate Vit D clinical decision thresholds
- Describe experimental design strategies for method validation of Vit D assays
- Describe the impact of potential interfering substances

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Vitamin D: Regulation of Ca^{2+} Homeostasis

7-dehydrocholesterol (Skin)
Diet (animal, fish) / Food Fortification

OTC supplements
Prescription Drug Form

Vitamin D3 (cholecalciferol) — **Vitamin D2 (ergocalciferol)**

Liver

25 hydroxylase

25-OH D3, 25-OH D2 (ng/mL)
t½ = 2-3 wks

Kidney

1-α-hydroxylase

1,25 $(OH)_2$ D3, 1,25 $(OH)_2$ D2 (pg/mL)
t½ = 4hr

- ***↑↑Ca^{2+} Absorption from Gut**
- ***↑↑Retention of Ca^{2+} by Kidney**
- ***↑↑Bone Mineralization**

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Vitamin D Testing: Indications

Endocrine Society Recommendations (2011):

- Total 25-OHD is the marker of choice
- Meas should only be made in individuals "at risk" for deficiency
- General population screening is *not* recommended
- 1,25$(OH)_2$D should *not* be used to dx deficiency except in narrowly defined populations

JCEM 2011; 96(7): 1911-1930

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Vitamin D Testing: CMS Reimbursement

The following ICD-9-CM codes support the medical necessity of CPT code *82306*. VIT D; 25 HYDROXY, INCLUDES FRACTION(S), IF PERFORMED

010.00 - 018.96	PRIMARY TUBERCULOUS COMPLEX UNSPECIFIED EXAMINATION - UNSPECIFIED MILIARY TUBERCULOSIS TUBERCLE BACILLI NOT FOUND BY BACTERIOLOGICAL OR HISTOLOGICAL EXAMINATION BUT TUBERCULOSIS CONFIRMED BY OTHER METHODS (INOCULATION OF ANIMALS)
135	SARCOIDOSIS
252.02	SECONDARY HYPERPARATHYROIDISM, NON-RENAL
268.0	RICKETS ACTIVE
268.2	OSTEOMALACIA UNSPECIFIED
268.9	UNSPECIFIED VITAMIN D DEFICIENCY
275.3	DISORDERS OF PHOSPHORUS METABOLISM
275.41	HYPOCALCEMIA
275.42	HYPERCALCEMIA
278.8	OTHER HYPERALIMENTATION
359.5	MYOPATHY IN ENDOCRINE DISEASES CLASSIFIED ELSEWHERE
555.0 - 555.9	REGIONAL ENTERITIS OF SMALL INTESTINE - REGIONAL ENTERITIS OF UNSPECIFIED SITE
556.0 - 556.9	ULCERATIVE (CHRONIC) ENTEROCOLITIS - ULCERATIVE COLITIS UNSPECIFIED
571.2	ALCOHOLIC CIRRHOSIS OF LIVER
571.5	CIRRHOSIS OF LIVER WITHOUT ALCOHOL
571.6	BILIARY CIRRHOSIS
576.8	OTHER SPECIFIED DISORDERS OF BILIARY TRACT
579.0 - 579.9	CELIAC DISEASE - UNSPECIFIED INTESTINAL MALABSORPTION
585.3	CHRONIC KIDNEY DISEASE, STAGE III (MODERATE)
585.4	CHRONIC KIDNEY DISEASE, STAGE IV (SEVERE)
585.5	CHRONIC KIDNEY DISEASE, STAGE V
585.6	END STAGE RENAL DISEASE
696.1	OTHER PSORIASIS AND SIMILAR DISORDERS
710.0	SYSTEMIC LUPUS ERYTHEMATOSUS
710.3	DERMATOMYOSITIS
729.1	MYALGIA AND MYOSITIS UNSPECIFIED
733.00 - 733.09	OSTEOPOROSIS UNSPECIFIED - OTHER OSTEOPOROSIS
733.90	DISORDER OF BONE AND CARTILAGE UNSPECIFIED
756.51	OSTEOGENESIS IMPERFECTA
756.52	OSTEOPETROSIS
V58.65*	LONG-TERM (CURRENT) USE OF STEROIDS
V58.69*	LONG-TERM (CURRENT) USE OF OTHER MEDICATIONS

Max reimb = 4x/yr

- **General Screening is not reimbursed**
- **Max 1x/yr**

VIT D; 1, 25 diHYDROXY

The following ICD-9-CM codes support the medical necessity of CPT code *82652*.

310.00 - 018.96	PRIMARY TUBERCULOUS COMPLEX UNSPECIFIED EXAMINATION OR HISTOLOGICAL EXAMINATION BUT TUBERCULOSIS CONFIRM
135	SARCOIDOSIS
268.0	RICKETS ACTIVE
278.8	OTHER HYPERALIMENTATION
585.3	CHRONIC KIDNEY DISEASE, STAGE III (MODERATE)
585.4	CHRONIC KIDNEY DISEASE, STAGE IV (SEVERE)
585.5	CHRONIC KIDNEY DISEASE, STAGE V
585.6	END STAGE RENAL DISEASE
756.51	OSTEOGENESIS IMPERFECTA
756.52	OSTEOPETROSIS

https://www.novitas-solutions.com/policy/mac-ab/l30273-r6.html
Accessed August, 2013

Vitamin D: Reporting Guidelines

Institute of Medicine Report, 2010

Maintain populations at 20 ng/mL to reflect RDA

< 12 ng/mL	"at risk of deficiency relative to bone health"
12 - 19 ng/mL	"potentially at risk for inadequacy"
20 - 30 ng/mL	"practically all persons are sufficient"
31 - 50 ng/mL	"not consistently associated with increased benefit"
> 50 ng/mL	"reason for concern"

http://www.iom.edu/Reports.aspx; *Accessed August, 2013*

Endocrine Society Clinical Practice Guidelines, 2011

< 20 ng/mL	Deficiency
21 - 29 ng/mL	Insufficiency
30 – 100 ng/mL	Sufficiency

JCEM 2011; 96(7): 1911-1930

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Vitamin D: Reporting Guidelines

Established in conjunction with input from
VCU Division of Endocrinology and Metabolism

VCUHS Total 25-OH D Clinical Decision Thresholds

< 10 ng/mL	Severe Hypovitaminosis D
10.0 – 19.9 ng/mL	Moderate Hypovitaminosis D
20.0 – 29.9 ng/mL	Mild Hypovitaminosis D
30.0 – 100.0 ng/mL	Optimal
> 100 ng/mL	Potential for Toxicity may exist

Osteoporos Int 2005; 16: 713–716
N Engl J Med 2007;357: 266-81

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Why Should Vit D be measured by LC-MS/MS?

Nov 09	70yr F seen for fatigue/weakness. Osteoporosis Risk. IA: 25 OH D = < 4.0 ng/mL
Jan 09	IA: 25-OH D = < 4.0 ng/mL → 50,000 U ergocalciferol, 2x/wk
May 09	Referred to endocrine specialty clinic PTH 35.0 pg/mL, Ca^{2+} 9.4 mg/dL, Urine Ca^{2+} 190 mg/24h ALP = 119 U/L IA: 25-OH D = < 4.0 ng/mL → 50,000 U ergocalciferol 2x/wk
June 09	IA: 25-OH D = 5.5 ng/mL

Patient was admonished for not taking her Vit D!! Patient cried…

July 09 IA: 25-OH D = <4.0 ng/mL

LC-MS/MS: 25-OH D3 = 6.0 ng/mL
25-OH D2 = 56.0 ng/mL
Total D = 62.0 ng/mL

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Measurement of Total 25-OH Vitamin D by LC-MS/MS Method Validation

Select Examples

Notes

- Some experimental design aspects of CLSI C60 (to be renamed C62): Liquid Chromatography-Mass Spectrometry Methods; Draft Guideline (to be renamed C62) are shown
- Draft CLSI C62 experimental design strategies are still under development and may not represent the final guidelines

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CLSI C62 Recommendations: Stability Assessment

C62: Assess analyte stability in native matrix under appropriate storage conditions

Short-term stability at RT (Bench top)
Long-term stability at 2-8˚C, -20˚C or -70˚C
Max # of Freeze/Thaw cycles, if applicable

FDA Guideline: Bioanalytical Method Validation
CLSI C55: Sample Stability in Chem/Tox (Draft Guideline)

-Stable for 3 days at 2-8˚C (data)
or RT (Clin Chem 1981, 27:773-774)

C62: Assess analyte stability during all phases of the analytical measurement process

- Determine max bench-top processing time (ex: extracts at RT)
- Determine max storage duration of extracts/preparations in the autosampler (evaporation effects, analyte degradation)

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Vit D: Stability of Extracts Stored in Autosampler

25-OH D3
Injected extracted cal, QC and native patient samples at T = 0
Extracts re-injected after 17hr and 24hr storage in autosampler (2-8˚C)

Subset of N=36 patient samples shown

Acceptability Criteria: ± 10% or ≤ 2 ng/mL

Sample ID	t=0hr	t=17hr	t=24hr	Mean (ng/mL)	%CV	Unit Diff, 17hr	% Diff, 17hr	Unit Diff, 24hr	% Diff, 24hr
Patient	**4.9**	5.3	5.5	5.2	5.4	**0.4**	**7.8**	**0.5**	**11.1**
Patient	**5.4**	6.0	4.6	5.3	12.4	**0.5**	**9.6**	**-0.8**	**-14.6**
Patient	**6.3**	6.1	6.0	6.1	2.6	**-0.1**	**-2.2**	**-0.3**	**-5.0**
Patient	**8.3**	8.3	8.7	8.4	2.5	**-0.1**	**-0.8**	**0.3**	**3.9**
Patient	**8.5**	8.5	9.2	8.7	4.7	**-0.1**	**-0.6**	**0.7**	**8.1**
Patient	**9.0**	8.4	7.5	8.3	9.2	**-0.7**	**-7.4**	**-1.5**	**-16.8**
Patient	**9.1**	8.6	8.0	8.6	6.7	**-0.6**	**-6.3**	**-1.2**	**-12.6**
Patient	**12.0**	11.5	10.4	11.3	7.1	**-0.5**	**-4.6**	**-1.6**	**-13.2**
Patient	**24.8**	25.4	24.8	25.0	1.3	**0.5**	**2.2**	**0.0**	**0.0**
Patient	**27.3**	25.2	25.6	26.1	4.2	**-2.1**	**-7.5**	**-1.6**	**-6.0**
Patient	**28.2**	28.7	28.2	28.4	0.8	**0.4**	**1.5**	**0.0**	**0.0**
Patient	**35.4**	34.4	33.0	34.3	3.4	**-1.0**	**-2.7**	**-2.3**	**-6.6**
Patient	**37.0**	40.0	40.6	39.2	4.9	**3.0**	**8.1**	**3.6**	**9.6**
Patient	**45.4**	46.6	42.5	44.8	4.7	**1.2**	**2.7**	**-2.9**	**-6.3**
Patient	**65.1**	65.1	67.8	66.0	2.4	**0.0**	**0.0**	**2.7**	**4.2**
Patient	**73.9**	76.1	78.0	76.0	2.7	**2.2**	**3.0**	**4.1**	**5.6**

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CLSI C62 Recommendations: Signal-to-Noise (S/N)

Evaluate the S/N at LLoQ

CLSI-C50A Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance; Approved Guideline

- **Minimum S/N at LLoQ of 3:1**

C62 Minimum Acceptability Criteria

- **Minimum S/N at LLoQ of 10:1**

C62 Best Practice Acceptability Criteria:

- **Minimum S/N at LLoQ of 20:1 to ensure ruggedness**

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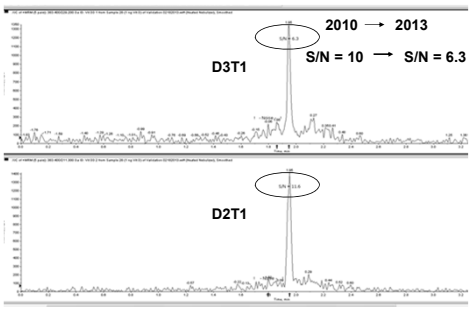
Vitamin D Method: Signal-to-Noise (S/N)

S/N degradation for 25-OH D method at LLoQ



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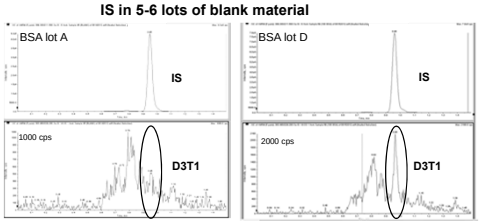
Validation of a Blank Matrix for Vitamin D Method

C62: Validate a blank matrix for use in subseq validation procedures (LLoQ, AMR, recovery, cal prep… etc.)

- Meas peak area of a **double blank matrix** (no analyte/no IS)
  - Can use BSA or stripped serum (we could not find a SS w/o 25OHD)

C62 Best Practice Acceptability Criteria:

- **No peak or peak area < 20% of LLoQ and <5% of IS in 5-6 lots of blank material**



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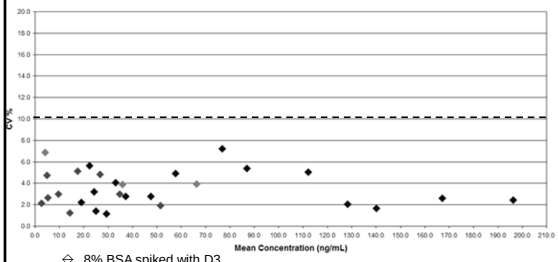
Vit D Method Imprecision Profile

Imprecision profile generated to determine %CV across AMR
Analyzed in quadruplicate/run for 2 runs



- 8% BSA spiked with D3
- Stripped Serum spiked with D3
- Native Patient Serum Pools with endogenous D3

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Vitamin D Method: Accuracy

C62: Assess accuracy using multiple approaches

C62 Best Practice:

Validate "trueness" using a "reference of higher order" (CLSI X5, ISO 17511) listed by JCTLM (approved RMPs, ref labs and RMs)
(www.bipm.org/jctlm/)

Described in CLSI EP15

1) **Method Comp vs. a JCTLM-approved RMP**
2) **Matrix-Approp CRMs (pref commutable)**
3) **Spike and Recovery – only if RMP or RMs are unavail**

C62 Alternative Approaches:

4) Accuracy-Based PT Materials
5) Method Comp vs. Previous Method using CLSI EP9-A2
(Bias vs. prev method only, not trueness)

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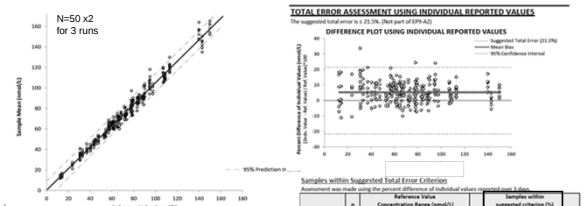
Accuracy Approach 1) Method Comp with RMP – VDSCP; EP 9



Samples within Suggested Total Error Criterion

	n	Reference Value Concentration Range (nmol/L)		Samples within suggested criterion (%)
Group 1	100	12.6	59.5	99
Group 2	100	59.5	83.0	99
Group 3	100	83.0	150	99
OVERALL	300	12.6	150	99

	n	Reference Value Conc. Range (nmol/L)		Mean Bias (%)	Mean Bias SD (%)	95% Confidence Interval (%)		Minimum Bias (%)	Maximum Bias (%)
Group 1	50	12.6	17.9	6.3	4.1	4.3	8.4	1.3	16.3
Group 2	17	59.5	79.4	4.6	3.6	2.8	6.3	0.1	10.6
Group 3	17	85.0	150	5.1	3.3	3.5	6.7	0.0	11.5
OVERALL	50	12.6	150	5.3	3.7	4.3	6.3	0.0	16.3

- ODS/CDC VDSP
(www.ods.od.nih.gov/Research/VitaminD.aspx#vdsp)
- CDC HoST
(http://www.cdc.gov/labstandards/hs.html)

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CDC Vitamin D Standardization Certification Program Procedure

Calibration/ Calibration Verification

Challenge 1 (1st Quarter)
Challenge 2 (2nd Quarter)
Challenge 3 (3rd Quarter)
Challenge 4 (4th Quarter)

Performance Assessment

40 single donor serum samples with reference values known to participant

10 single donor serum samples per challenge with reference values unknown to participant

Performance criteria*:
Mean bias: 5%
Imprecision: 10%

Participant uses serum samples to calibrate or to verify accuracy of calibration

Participant measures samples in duplicate on 2 days (n=4) and reports results to CDC

CDC issues quarterly reports to participants

CDC assesses bias and imprecision and issues certificates of performance

http://www.cdc.gov/labstandards/hs.html
Slide prepared by Hubert Vesper, CDC

*Stöckl et al. Clin Chem Acta 2009;408:8 – 13

Accuracy Approach 2) Certified Reference Material (CRM)

Assessment of traceability to NIST SRM 972a

NIST SRM 972a							
NIST D3	**NIST TARGET CONCENTRATIONS INCLUDE THE 3-EPIMER CONCENTRATIONS						
NIST Level 1	30.64	NIST Level 2	19.39	NIST Level 3	20.98	NIST Level 4	55.8
Rep 1	31.33	Rep 1	18.87	Rep 1	20.84	Replicate 1	53.13
Rep 2	32.80	Rep 2	20.67	Rep 2	21.33	Replicate 2	51.33
Rep 3	32.48	Rep 3	20.02	Rep 3	20.34	Replicate 3	52.97
Rep 4	32.31	Rep 4	19.36	Rep 4	22.64	Replicate 4	50.84
mean	32.23	mean	19.73	mean	21.29	mean	52.07
sd	0.635	sd	0.785	sd	0.987	sd	1.155
cv	2.0	cv	4.0	cv	4.6	cv	2.2
% bias from NIST	5.19	% bias from NIST	1.75	% bias from NIST	1.46	% bias from NIST	-6.69
NIST D2							
NIST Level 1	0.52	NIST Level 2	0.81	NIST Level 3	13.30	NIST Level 4	0.55
Rep 1	0.00	Rep 1	0.62	Rep 1	13.47	Rep 1	0.0
Rep 2	0.00	Rep 2	0.51	Rep 2	13.69	Rep 2	0.0
Rep 3	0.00	Rep 3	0.62	Rep 3	13.14	Rep 3	0.0
Rep 4	0.00	Rep 4	0.40	Rep 4	15.01	Rep 4	0.0
mean	0.00	mean	0.53	mean	13.83	mean	0.00
sd	0.000	sd	0.105	sd	0.819	sd	0.000
cv	N/A	cv	19.7	cv	5.9	cv	N/A
% bias from NIST	N/A	% bias from NIST	N/A	% bias from NIST	3.98	% bias from NIST	N/A

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Accuracy Approach 3) Spike and Recovery

25-OH D3 Spiked into 8% BSA (Low Conc)
25-OH D3 Spiked into Stripped Serum with back-calculation
N = 10 reps/sample over 2 runs

Acceptability Criteria:
±10% or 2 ng/mL

	Expected (ng/mL)	Mean (ng/mL)	SD	% CV	Unit Diff (ng/mL)	%Diff
8% BSA	1.0	1.0	0.1	9.0	0.0	102.3
	2.5	2.6	0.1	2.4	0.1	105.7
	5.0	5.6	0.2	2.8	0.6	111.0
Stripped Serum	5.0	4.7	0.1	2.5	-0.3	94.0
	10	10.5	0.4	4.2	0.5	104.8
	15.3	16.1	0.6	3.4	0.8	105.1
	20.3	20.8	0.5	2.4	0.5	102.6
	25.3	25.4	1.1	4.4	0.1	100.3
	30.3	31.4	1.5	4.7	1.1	103.5
	35.3	35.3	1.4	4.1	0.0	100.1
	45.3	46.2	1.1	2.5	0.9	101.9
	55.3	56.6	2.7	4.9	1.3	102.4
	65.3	66.7	2.9	4.4	1.4	102.2
	75.3	77.7	2.2	2.8	2.4	103.2
	90.3	93.0	3.0	3.3	2.7	103.0

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Accuracy Approach 4) Accuracy-Based PT Programs

CAP Accuracy Based Survey April 2012
CAP Acceptability Criteria = ± 25%

25-OH D (Total)	Result (ng/mL)	Target (ng/mL)	D3 epimer (ng/mL)	Target+3epi (ng/mL)	%Diff vs. Target	%Diff w/3 epi
ABVD-06	30.4	**29.4**	1.3	30.7	**3.4**	-1.0
ABVD-07	24.2	**22.2**	0.5	22.7	**9.0**	6.6
ABVD-08	35.4	**34.7**	2	36.7	**2.0**	-3.5
ABVD-09	23.0	**23.2**	1	24.2	**-0.9**	-5.0
ABVD-10	16.6	**14.9**	0.5	15.4	**11.4**	7.8
25-OH D2						
ABVD-06	<1.0	**0.47**			**N/A**	
ABVD-07	9.1	**9.42**			**-3.4**	
ABVD-08	1.2	**1.57**			**-23.6**	
ABVD-09	1.0	**0.94**			**6.4**	
ABVD-10	<1.0	**1.15**			**N/A**	
25-OH D3						
ABVD-06	30.4	**29.16**	1.3	30.46	**4.3**	-0.2
ABVD-07	15.1	**14.32**	0.5	14.82	**5.4**	1.9
ABVD-08	34.2	**33.37**	2	35.37	**2.5**	-3.3
ABVD-09	22.0	**22.05**	1	23.05	**-0.2**	-4.6
ABVD-10	16.6	**14.87**	0.5	15.37	**11.6**	8.0

Total 25-OHD, D2, D3 and D3-epi ref values provided
Note that 3-epi D3 is NOT included in the Target Value

Value-assigned using RMPs

- **CAP Accuracy Based Vitamin D Survey**
- **New Accuracy Based Program – DEQAS (www.deqas.org)**

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Vitamin D Proficiency Testing

CAP BGS (Peer Group)
- VCUHS does *not* participate (historical matrix problems)

CAP Vitamin D Accuracy Based
- 2x/yr
- Uses donor samples, some supplemented with vitamin D2
- Values assigned using the CDC RMP
- Total 25-OHD is graded
- 25-OHD3 and 25-OHD2 provided but "educational grade"

DEQAS (www.deqas.org)
- 4x/yr, now approved by CAP
- Becoming an accuracy-based survey

NY STATE (www.wadsworth.org)
- 25-OHD3 and 25-OHD2 peer values avail

NIST QAP (http://www.nist.gov/mml/csd/vitdqap.cfm)
- 2x/yr
- Ongoing traceability to NIST RMP

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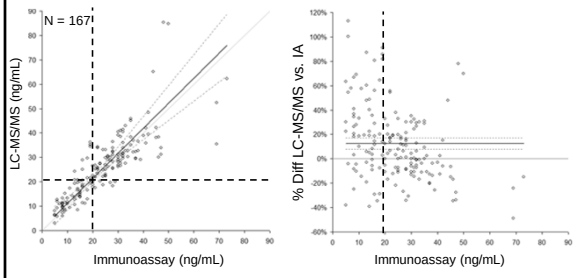
Accuracy Approach 5) Bias vs. Previous Method (IA): CLSI EP-9

Total 25-OH D: LC-MS/MS vs. IA



	Bias	95% CI	SE	p
Constant	0.87	-4.08 to 5.83	2.510	0.7286
Proportional	1.03	0.79 to 1.27	0.122	0.8098
Syx (vertical)	7.70			

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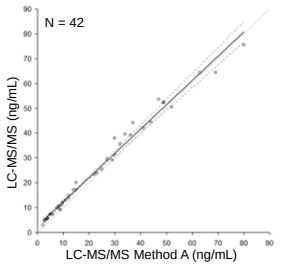
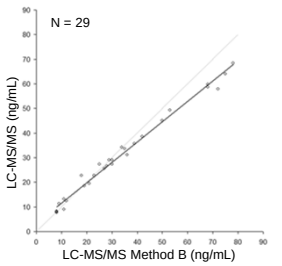
Accuracy Approach 5 continued) Bias vs. LC-MS/MS method

25-OH D3: LC-MS/MS vs. 2 independent LC-MS/MS Methods



	Bias	95% CI	SE	p
Constant	2.48	1.46 to 3.50	0.505	<0.0001
Proportional	0.98	0.92 to 1.04	0.031	0.4856
Sy|x (vertical)	2.35			



	Bias	95% CI	SE	p
Constant	3.28	1.76 to 4.80	0.742	0.0001
Proportional	0.83	0.79 to 0.87	0.020	<0.0001
Sy|x (vertical)	2.02			

- Compare to a RMP, if possible

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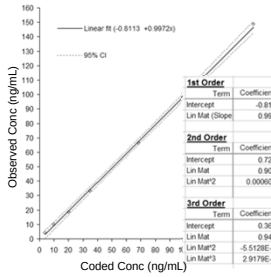
Linearity (CLSI EP6-A) – Polynomial Regression Approach



25-OH D3 analyzed used matrix-appropriate commercial linearity materials

1st Order

Term	Coefficient	95% CI	SE	t statistic	DF	p
Intercept	-0.8113	-3.0051 to 1.3824	0.85340	-0.95	5	0.3854
Lin Mat (Slope	0.9972	0.9681 to 1.0263	0.01132	88.09	5	<0.0001

2nd Order

Term	Coefficient	95% CI	SE	t statistic	DF	p
Intercept	0.7285	-0.7405 to 2.1975	0.52909	1.38	4	0.2406
Lin Mat	0.9081	0.8499 to 0.9663	0.02098	43.29	4	<0.0001
Lin Mat^2	0.0006075	0.0002229 to 0.0009920	0.00013849	4.39	4	0.0118

3rd Order

Term	Coefficient	95% CI	SE	t statistic	DF	p
Intercept	0.3686	-2.0146 to 2.7518	0.74886	0.49	3	0.6563
Lin Mat	0.9457	0.7668 to 1.1247	0.05623	16.82	3	0.0005
Lin Mat^2	-5.5128E-05	-2.9858E-03 to 2.8755E-03	9.2088E-004	-0.06	3	0.9560
Lin Mat^3	2.9179E-06	-9.8216E-06 to 1.5657E-05	4.0030E-006	0.73	3	0.5188

Nonlinearity should be assessed for clinical significance

25-OH D3 (ng/mL)	NL
3.8	0.79
9.9	0.35
20.1	-0.28
35.0	-1.00
68.5	-1.69
107.0	-0.94
148.0	1.69

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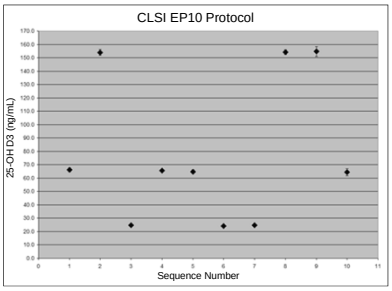
Vit D Carryover Assessment – CLSI EP10



Low: 24.3 ng/mL
Mid: 65.3 ng/mL
High: 154.3 ng/mL

Run	%Carryover
1	-0.6
2	-0.9
3	-0.8
4	0.1
5	-0.4
MEAN	**-0.52**
p	**1.000**

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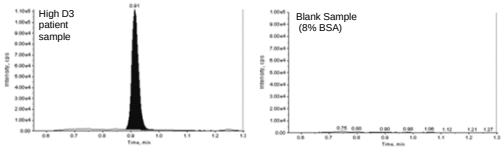
Vit D Carryover Assessment - High Sample vs. Blank

Analysis Sequence #	Sample Name	Analyte Peak Name	Analyte Peak Area (cps)	Area Ratio	IS Peak Area (cps)	Conc (ng/mL)
172	Patient 1	Vit D3 1	180000	1.15	156000	159.0
173	BLANK	Vit D3 1	0	0	169000	<1.0
175	BLANK	Vit D3 1	0	0	169000	<1.0
177	BLANK	Vit D3 1	0	0	179000	<1.0



C62 Best Practice Acceptability Criteria:

- **No peak in blank or < 20% of LLoQ**

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Matrix Effect 1) Pre vs. Post-Extract Spike and Rec (CLSI-C50)

10 ng/mL D3 spiked into Low Patient Pool (~1 ng/mL)

	Spiked Pre-Extraction			Spiked Post-Extraction			D3 in Solvent
	D3 spiked into Serum (cps)	Endogenous D3 in Serum (cps)	Diff (cps)	D3 spiked into Serum (cps)	Endogenous D3 in Serum (cps)	Diff (cps)	D3 spiked into MeOH (cps)
Rep 1	16030	2630	13400	18900	2630	16270	19100
Rep 2	15060	2960	12100	18500	2890	15610	17800
Rep 3	11220	2520	8700	19800	3170	16630	18100
Rep 4	19660	2960	16700	18100	2920	15180	18100
Rep 5	11230	2680	8550	16900	2790	14110	17700
Mean	14640	2750	**11890**	18440	2880	**15560**	**18160**
SD	3182	179		954	177		496
			Set C			**Set B**	**Set A**

% Matrix Effect (B/A*100) 85.7
% Extr Effic (C/B*100) 76.4
% Process Effic (C/A*100) 65.5

% Matrix Bias -14.3
% Matrix Bias Corr for IS -2.3

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Approach 2) Eval of Matrix Effects: Matrix Mixing (CLSI EP7)

Experiment for purpose of validating surrogate matricies
But, this approach can also be used to validate ME in patient samples

Native Patient Serum mixed in proportion with Stripped Serum or 8% BSA
N=4 reps over a single run

Native Patient Serum + Stripped Serum:

% D3 Patient Serum	Expected (ng/mL)	Rep1 (ng/mL)	Rep2 (ng/mL)	Rep 3 (ng/mL)	Rep 4 (ng/mL)	Mean (ng/mL)	SD	%CV	Unit Diff (ng/mL)	% Diff
100%	**52.7**	50.6	53.8	54.3	52.0	**52.7**	1.66	3.2	N/A	N/A
80%	**42.1**	40.4	41.5	43.8	43.5	**42.3**	1.62	3.8	0.2	0.4
50%	**26.3**	26.8	25.5	26.6	25.4	**26.1**	0.73	2.8	-0.3	-1.0
20%	**10.5**	9.8	10.1	11.5	11.5	**10.7**	0.88	8.2	0.2	1.8
0%	**0.0**	0.0	0.0	0.0	0.0	**0.0**	0.00	0.0	0.0	0.0

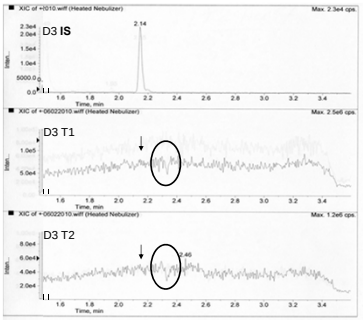
- contribution from endogenous D3 in stripped serum removed

Native Patient Serum + 8% BSA:

% D3 Patient Serum	Expected (ng/mL)	Rep1 (ng/mL)	Rep2 (ng/mL)	Rep 3 (ng/mL)	Rep 4 (ng/mL)	Mean (ng/mL)	SD	%CV	Unit Diff (ng/mL)	% Diff
100%	**52.7**	50.6	53.8	54.3	52.0	**52.7**	1.66	3.2	N/A	N/A
80%	**42.1**	39.3	40.8	42.0	42.6	**41.2**	1.49	3.6	-1.0	-2.3
50%	**26.3**	25.8	25.8	25.9	27.4	**26.2**	0.79	3.0	-0.1	-0.5
20%	**12.1**	12.1	11.4	11.9	11.7	**11.8**	0.31	2.6	-0.3	-2.5
0%	**0.0**	0.0	0.0	0.0	0.0	**0.0**	0.00	0.0	0.0	0.0

Approach 3) Evaluation of Matrix Effects: T - Infusion

D3 in MeOH infused + injection of analyte-free serum



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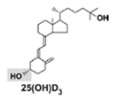
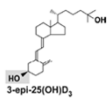
3-epimer Vit D interference





3-epi 25-OH Vitamin D

- Elevated in serum of infants and adults
- Clinical significance unknown
- **Not recognized** by most IAs
- **Not resolved** by most routine LC-MS/MS methods – source of discrepancies
- VDSP LC-MS/MS methods **do resolve** the 3-epimer

Used for NHANES measurements to establish dietary recommendations

J Clin Endocrinol Metab 2012, 97:163–168
Clinica Chimica Acta 2012, 413: 203–206

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NIST SRM 972a and 3-epimer Vit D3

Level 1 (ng/mL)		
25OH D3	**28.8 ± 1.1**	**If using to value-assign calibrators or verify traceability of method**
3-epi 25OH D3	**1.84 ± 0.08**	
25OH D2	**0.54 ± 0.06**	
Level 2		**+**
25OH D3	**18.1 ± 0.4**	
3-epi 25OH D3	**1.29 ± 0.06**	**Your method does not resolve 3-epimer**
25OH D2	**0.81 ± 0.06**	
Level 3		⇩
25OH D3	**19.8 ± 0.5**	
3-epi 25OH D3	**1.18 ± 0.13**	
25OH D2	**13.3 ± 0.3**	
Level 4		**Need to add 3-epi concentration to NIST value assignments**
25OH D3	**29.4 ± 0.9**	
3-epi 25OH D3	**26.4 ± 2.1**	
25OH D2	**0.55 ± 0.10**	***Caveat: 3-epi values are not certified**

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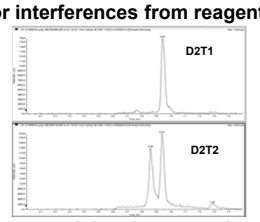
Vit D: Interference Testing

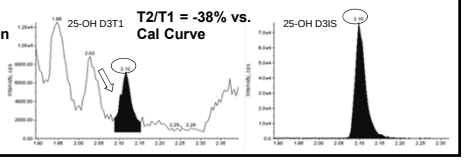
C62: Check for interferences from reagents & disposables

New lot MeOH



C62: Check for potential endogenous interferences

↑↑ Bile Salts in PBC patient



Exogenous and Other Endogenous Interferences

- Reagents and Disposables (major interference from new lot MeOH)
- Collection Tube Additives (ex: SST interference in some testo methods)
- Physiological or Disease-Associated Interferences (ex: PBC patients)
- Isobaric/Isotopomeric – Interferences resulting in shared product ions or a precursor and product ion (3-epi 25-OH D)
- Drugs/Metabolites (Vit D metabolites, use of T2/T1 ratios)

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Vit D QA Monitoring – Run Acceptability and Sample Acceptability

- Slope & Intercept PASS Range
- % Calibrator Recovery (ex: ±10%)
- r^2 or SE of Calibrator Curve
- Run-to-run IS Peak Area Recovery Criteria (ex: ±30%)
- IS Recovery Criteria for each sample (ex:±20%)
- RT, Peak Resolution, Peak Symmetry
- Presence of Interfering Peaks or Absent Peaks
- T2/T1 Ratio Criteria (CLSI-C50)
- For multiplexing, each column/stream is considered a separate method

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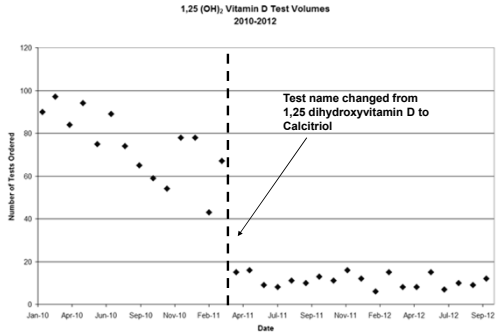
1, 25 $(OH)_2$ Vitamin D – To Test or Not To Test?



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END

Extra Information

C60: Reagent Lot Change – Patient Comps and Linearity (CAP)

Vitamin D Reagent Lot Change or Post Maintenance Patient Comparison Form
v.03.01.10. Supersedes: N/A. Implemented 03.01.10 LMB

Date:	3/23/2011	Reason for Validation:	New Column	MS Datafiles:	3022011
Old Reagent Lot#:	532600-21	Date Old Reagent Lot Implemented:		11/15/2010	
New Reagent Lot#:	545763-20	Date New Reagent Lot Implemented:		3/23/2011	
Date of Patient Comp:	3/22/2011	Pass Criteria: Difference of <= 10% or <= 2 ng/mL if % criteria fails			

25-OH D3

Sample#	Old Lot	New Lot	Unit Diff	% Diff	Criteria
1	<1.0	<1.0	N/A	N/A	Pass
2	4.5	5.2	0.7	15.6	Pass
3	8.1	8.3	0.2	2.5	Pass
4	13.7	14.5	0.8	5.8	Pass
5	16.5	15.4	-1.1	-6.7	Pass
6	20.6	19.2	-1.4	-6.8	Pass
7	27.3	27.1	-0.2	-0.7	Pass
8	37.7	36.9	-0.8	-2.1	Pass
9	42.3	40.1	-2.2	-5.2	Pass
10	59.1	61.5	2.4	4.1	Pass

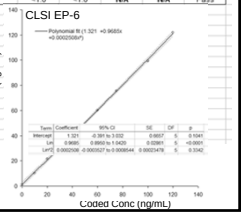
25-OH D2

Sample#	Old Lot	New Lot	Unit Diff	% Diff	Criteria
1	54.7	49.4	-5.3	-9.7	Pass
2	26.0	28.0	2.0	7.7	Pass
3	38.8	37.7	-1.1	-2.8	Pass
4	7.7	7.3	-0.4	-5.2	Pass
5	62.8	60.2	-2.6	-4.1	Pass
6	34.2	32.1	-2.1	-6.1	Pass
7	5.2	4.1	-1.1	-21.2	Pass
8	10.3	9.0	-1.3	-12.6	Pass
9	14.1	15.6	1.5	10.6	Pass
10	<1.0	<1.0	N/A	N/A	Pass

25-OH Total D

Sample#	Old Lot	New Lot	Unit Diff	% Diff	Criteria
1	54.7	49.4	-5.3	-9.7	Pass
2	30.5	33.2	2.7	8.9	Pass
3	46.9	46.0	-0.9	-1.9	Pass
4	21.4	21.8	0.4	1.9	Pass
5	79.3	75.6	-3.7	-4.7	Pass
6	54.8	51.3	-3.5	-6.4	Pass
7	32.5	31.2	-1.3	-4.0	Pass
8	48.0	45.9	-2.1	-4.4	Pass
9	56.4	55.7	-0.7	-1.2	Pass
10	59.1	61.5	2.4	4.1	Pass

Reviewed By _____ Date Reviewed _____



Plus chromatography acceptability criteria
Plus QC acceptability criteria (native pt based)

C62: Periodic Accuracy Monitoring

Using Patient Pools traceable to NIST SRM 972 (25-OHD)

1) Create Patient Pools near NIST SRM values

2) Calc Method Bias vs. NIST SRM

3) Apply a Correction Factor to Patient Pools to trace their values to NIST-assigned values

NIST SRM-972 D3	972-1	972-2	972-3	972-4
N = 9	24.6	13.3	19.6	65.3
*SRM 972-4	25.3	12.5	20.9	65.0
incl 3-epi	25.6	13.9	21.3	66.9
	25.2	12.5	20.9	68.2
	24.6	13.3	20.6	65.6
	24.4	13.5	21.1	68.3
	25.7	12.3	20.4	68.5
	25.8	12.5	19.3	67.6
	25.7	12.0	19.8	64.8
Mean (ng/mL)	**25.2**	**12.9**	**20.4**	**66.7**
SD (ng/mL)	0.5	0.6	0.7	1.5
%CV	2.1	5.0	3.5	2.3
NIST Target (ng/mL)	**25.3**	**13.1**	**19.6**	**70.7**
% Diff	**-0.3**	**-1.4**	**4.5**	**-5.7**
Correction Factor	1.003	1.014	0.955	1.057

Patient Pools D3	Pool 1	Pool 2	Pool 3	Pool 4
	25.0	11.5	19.1	72.3
	24.5	12.4	18.2	75.8
	25.2	11.2	18.4	72.9
	25.1	11.1	19.6	74.5
	25.3	12.9	18.8	70.9
	25.3	12.4	19.0	75.0
	23.9	10.9	18.6	72.4
	25.7	10.6	19.0	71.8
	23.7	11.9	17.7	72.9
Mean (ng/mL)	**24.9**	**11.7**	**18.7**	**73.2**
SD (ng/mL)	0.7	0.8	0.6	1.6
%CV	2.7	6.7	3.0	2.2
Assigned Value	**24.9**	**11.8**	**17.9**	**77.3**