

DRG® 1,25-dihydroxy Vitamin D ELISA (EIA-4484)**REVISED 24 AUG. 2005****RUO IN THE USA****1 INTENDED USE**

For In Vitro Diagnostic Use. In the United States, this kit is intended for Research Use Only.

The DRG 1,25-Dihydroxy Vitamin D kit is a complete assay system intended for the purification of 1,25-dihydroxy Vitamin D (1,25D) in human serum or plasma by immunoextraction followed by quantitation by enzyme immunoassay.

Results are to be used in conjunction with other clinical and laboratory data to assist the clinician in the assessment of 1,25D deficiency associated with renal disease in adult populations.

2 SUMMARY AND EXPLANATION

Vitamin D is a commonly used collective term for a family of closely related molecules derived from naturally occurring 7-dehydrocholesterol (pro-vitamin D3). Pro-vitamin D3 undergoes photolytic conversion in the skin to 'parent' vitamin D3 (cholecalciferol) upon exposure to sunlight. This compound is biologically inactive, but enters the circulation and is hydroxylated in the liver to active 25-hydroxyvitamin D (25D). A small proportion of this becomes further hydroxylated in the kidney to the highly potent calcitropic hormone 1,25D.

1,25D is largely bound to Vitamin D Binding Protein and albumin in the circulation.

1,25D is one of the major regulators of calcium (and phosphate) metabolism, stimulating intestinal calcium absorption and increasing bone resorption. It also inhibits parathyroid hormone (PTH) production both by direct action on the parathyroid glands and indirectly by raising serum calcium levels. 1,25D production is itself stimulated by parathyroid hormone (PTH), thus providing an effective control loop.

Hypovitaminosis D is commonly associated with dietary insufficiency, most frequently with vegetarianism, and is also associated with low exposure to sunlight (e.g. the elderly and institutionalized) and skin pigmentation.

1,25D production appears to be impaired in early renal failure though this may not be a renal effect. In late-stage renal failure, 1 α -hydroxylation may be impaired, with low 1,25D levels as a result.

3 METHOD DESCRIPTION

The DRG 1,25-Dihydroxy Vitamin D kit is a complete assay system for the purification of 1,25D in patient samples by immunoextraction followed by quantitation by EIA. Patient samples are delipidated and 1,25D extracted from potential cross-reactants by incubation for 90 minutes with a highly specific solid phase monoclonal anti-1,25D. The immunoextraction gel is then washed and purified 1,25D eluted directly into glass assay tubes. Reconstituted eluates and Standards are incubated overnight with a highly specific sheep anti-1,25D. Then a portion of this is incubated for 90 minutes with shaking in microplate wells which are coated with a specific anti-sheep antibody. 1,25D linked to biotin is then added and the plate shaken for a further 60 minutes before aspiration and washing. Enzyme(horseradish peroxidase) labelled avidin is added and binds selectively to complexed biotin and, following a further wash step, colour is developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures are read in a microtiter plate reader, colour intensity developed being inversely proportional to the concentration of 1,25D.

DRG[®] 1,25-dihydroxy Vitamin D ELISA (EIA-4484)**REVISED 24 AUG. 2005****RUO IN THE USA****4 WARNINGS AND PRECAUTIONS**

The 1,25-Dihydroxy Vitamin D kit is for in vitro diagnostic use only and is not for internal use in humans or animals. In the United States, this kit is intended for Research Use Only. This product must be used strictly in accordance with the instructions set out in the Package Insert. DRG will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of non-compliance with the instructions provided.

CAUTION: this kit contains material of human and/ or animal origin. Handle kit reagents as if capable of transmitting an infectious agent.

Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. Disposal of kit reagents should be in accordance with local regulations.

Human Serum: Controls

Human material used in the preparation of this product has been tested by FDA recommended assays for the presence of antibody to Human Immunodeficiency Virus (HIV I and II), Hepatitis B surface antigen, antibody to Hepatitis C, and found negative. As no test can offer complete assurance that infectious agents are absent, the reagents should be handled in accordance at Biosafety Level 2.

Sodium Azide

Some reagents in this kit contain sodium azide as a preservative, which may react with lead, copper or brass plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent azide build up.

Elution Reagent

Elution Reagent contains ethanol.

R11 Highly Flammable (flashpoint 13°C).

S7 Keep container tightly closed.

S16 Keep away from sources of ignition - No Smoking.

0.5M hydrochloric acid

Stop Solution contains 0.5M hydrochloric acid.

R36/38 Irritating to eyes and skin.

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

S36/37 Wear suitable protective clothing and gloves.

Tetramethylbenzidine

TMB Substrate contains 3,3',5,5'-tetramethyl-benzidine.

R21/22 Harmful by contact with skin and if swallowed.

S36/37 Wear suitable protective clothing and gloves.

5 PREPARATION OF REAGENTS**Standards:**

Standards are supplied in lyophilized form. Reconstitute immediately before use.



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Add 1 mL distilled or deionized water to each bottle. Replace stopper and leave to reconstitute for 5-10 minutes, inverting several times to ensure complete reconstitution.

DO NOT RECONSTITUTE ON A ROLLING MIXER - this will result in potency loss.

Controls:

Controls are supplied in lyophilized form. Reconstitute immediately before use.

Add 1.2 mL distilled or deionized water to each bottle. Replace stopper and leave 15 - 20 minutes to reconstitute, inverting several times to ensure complete reconstitution.

If Standards or Controls are to be used more than once, they must be frozen (-20°C) within 15 minutes of reconstitution. When re-using frozen Standards or Controls, thaw at room temperature, mix well and use within 15 minutes.

Primary Antibody Solution:

Primary Antibody Concentrate 6x is supplied as a concentrate. Add the entire contents of the bottle of Primary Antibody Buffer, replace the stopper and invert several times to ensure complete mixing.

1,25D Biotin solution:

1,25D Biotin Concentrate 6x is supplied lyophilized. Add the entire contents of the bottle of the 1,25D Biotin Buffer. Replace the stopper and stand for 10-15 minutes at room temperature. Invert several times to ensure complete reconstitution.

If 1,25D Biotin solution is to be used more than once, it must be frozen (-20°C) within 2 hours of reconstitution. When using frozen 1,25D Biotin solution, thaw at room temperature, mix well and use within 2 hours.

Wash Solution:

Prepare by adding the contents of each bottle of Wash Concentrate 20x to 950 mL of distilled or de-ionized water. Store at room temperature.

All other reagents are supplied ready for use.

Allow all reagents to come to room temperature before use.

Reagents should be mixed by repeated inversion prior to use in the assay

6 SHELF LIFE AND STORAGE OF REAGENTS

This kit is stable until the stated expiry date if stored as specified. Upon receipt, store all reagents at 2-8°C. Reconstituted Standards, Controls and 1,25D Biotin solution are stable at -20°C for 4 weeks.

Antibody Solution is stable at 2-8°C for 4 weeks.

Unused Antibody Coated Plate strips must be returned to the foil pouch with the desiccant sachet and self-sealed. Store at 2-8°C for up to 4 weeks.

Wash Solution can be stored at room temperature for up to 8 weeks.

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- The presence of abnormal particulate matter in any of the reagents.
- A decrease in the maximum binding.
- A high non-specific binding.
- A shift in the slope of the curve from its normal position.

7 SPECIMEN COLLECTION AND STORAGE

The assay should be performed using serum or plasma (EDTA or heparin) specimens.

Specimens should be separated as soon as possible after collection. For long term storage, store at -20°C. Avoid repeated freeze/thaw of samples.

8 MATERIALS PROVIDED**1. Standard 0 - 6:**

Lyophilized BSA-phosphate buffer containing 1,25-dihydroxyvitamin D and 0.09% sodium azide. The exact value of each Standard is printed on the bottle label. 1 mL per bottle, 7 bottles per kit.

2. Primary Antibody Concentrate 6x

Sheep anti-1,25-dihydroxyvitamin D in BSA-phosphate buffer with 0.09% sodium azide, 2 mL per bottle.

3. Primary Antibody Buffer

Proprietary reagent containing phosphate buffer with 0.09% sodium azide. 10 mL per bottle.

4. Microtiter plate (Anti-Sheep coated plate)

Microplate with anti-sheep IgG linked to the inner surface of the polystyrene wells, 12 x 8 well strips in a foil pouch with desiccant.

5. 1,25D Biotin Concentrate 6x

Lyophilized buffer containing 1,25-dihydroxy-vitamin D labelled with biotin, and proprietary stabilizers, 2mL per bottle.

6. 1,25D Biotin Buffer Phosphate buffered saline with 0.09% sodium azide, 12 mL per bottle.**7. Enzyme Conjugate** Phosphate buffered saline containing avidin linked to horseradish peroxidase, protein, enzyme stabilizers and preservative, 24mL per bottle.**8. Controls 1 & 2** Lyophilized human serum containing 1,25-dihydroxy-vitamin D and 0.09% sodium azide, 1.2 mL per bottle, 2 bottles per kit.**9. Immunocapsules**

Capsules containing monoclonal antibody to 1,25-dihydroxyvitamin D linked to solid phase particles in suspension with vitamin D binding protein inhibitor, 80 immunocapsules per kit.

10. Delipidation Reagent A solution of dextran sulphate and magnesium chloride, 2.5 mL per bottle.**11. Elution Reagent** Ethanol, 44 mL per bottle.**12. Assay Buffer** BSA-phosphate buffer with 0.09% sodium azide, 12 mL per bottle.

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13. **TMB Substrate Solution** A proprietary aqueous formulation of tetramethylbenzidine (TMB) and hydrogen peroxide, 24 mL per bottle.
14. **Stop Solution** 0.5M Hydrochloric acid, 14 mL per bottle.
15. **Wash Buffer Concentrate 20x**
Phosphate buffered saline containing Tween, 50 mL per bottle.
16. **Adhesive Plate Sealer:** 8 per kit.
17. **Documentation** Package Insert and QC report.

9 MATERIALS REQUIRED BUT NOT PROVIDED

1. Disposable 12 x 75 mm borosilicate glass tubes.
2. Disposable 12 x 75 mm polystyrene tubes (optional).
3. Precision pipetting devices to deliver 50 µL, 100 µL, 150 µL, 200 µL, 500 µL and 1 mL.
4. Repeating pipetting devices to deliver 150µL and 500 µL, e.g. Eppendorf Multipipette 4780 or similar.
5. Precision multi-channel pipettes to deliver 100 µL and 200 µL.
6. Vortex mixer.
7. End-over-end or roller mixer.
8. Heating block or water bath at 40°C.
9. Nitrogen supply and manifold.
10. Centrifuge capable of achieving 2000g.
11. Orbital shaker.
12. Automatic microplate washer (optional).
13. Photometric microplate reader and data analysis equipment.
14. Distilled or deionized water.

10 SAMPLE PREPARATION

1. Prepare labelled glass or plastic tubes, one for each Control and unknown sample.
DO NOT DELIPIDATE Standards .
2. Add 500 µL of each Control or sample to appropriately labelled tubes.
3. Add 50 µL of Delipidation Reagent to each tube. Vortex all tubes.
4. Centrifuge all tubes at 2000 g for 15 minutes.

Note: Take care not to disturb the pellet when handling delipidated samples. If the pellet becomes suspended or if the sample is not clear, then repeat the centrifugation.

10.1 Alternative Sample Preparation:

Suitable for samples where the volume available is less than 500 µL.

1. Prepare labelled conical-bottom plastic tubes or microcentrifuge tubes, one for each sample.
2. Add sample (e.g. 250 µL) to appropriately labelled tubes.

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3. Add 0.1 x sample volume of Delipidation Reagent REAG 1 (e.g. 25 µL) to each tube. Vortex all tubes.
4. Centrifuge all tubes at 2000 g for 15 minutes, or at 10000 g for 10 minutes (microcentrifuge).

11 IMMUNOEXTRACTION PROCEDURE

1. Prepare labelled Immunocapsules, two for each Control and sample
DO NOT IMMUNOEXTRACT Standards.
Note: If a Immunocapsule shows signs of leakage or incorrect volume - do not use.
2. Vortex Immunocapsules and allow solid phase to settle. Stand Immunocapsules upright in foam rack for 3-5 minutes.
3. Remove top screw caps from Immunocapsules.
Add 100 µL of delipidated sample or control to Immunocapsules in duplicate. Replace caps securely.
4. Place Immunocapsules in foam rack and rotate end over-end at 5-20 revolutions per minute for 90 minutes at room temperature (18-25°C). Foam racks can be easily attached to a blood tube rotator by means of cut out slots. Alternatively, foam rack may be wedged inside a suitable plastic beaker and rotated on a bottle roller.
5. Stand Immunocapsules upright in foam rack for 3-5 minutes to allow gel to settle. Tap to dislodge any gel adhering to the screw caps. Allow gel to settle for a further 1-2 minutes. Remove screw cap and break off (do not twist off) bottom stopper from Immunocapsules and place each Immunocapsule in a plastic (or glass) tube. Centrifuge at low speed (500-1000g) for approximately 1 minute to remove sample.
6. Add 500 µL of deionised water to each Immunocapsule. Add carefully to avoid solid phase splashing out of the Immunocapsule. Centrifuge at low speed (500-1000g) for approximately 1 minute to wash immunoprecipitation gel.
7. Repeat the above wash step a further two times.
8. Prepare labelled borosilicate glass tubes, one for each Immunocapsule, and transfer Immunocapsules to the glass tubes.
9. Add 150 µL of Elution Reagent REAG 2 to all Immunocapsules.
Allow reagent to soak into solid phase for 1 to 2 minutes. Centrifuge at low speed (500-1000g) for approximately 1 minute to collect eluate.
10. Repeat above step a further two times. The total elution volume collected is therefore 450 µL for each sample.
11. Discard Immunocapsules and place tubes in a heating block or water bath set to 40°C.
Evaporate the eluates under a gentle flow of nitrogen. Evaporation should take 20 - 30 minutes. Ensure there is no remaining liquid in the tubes.
12. Add 100 µL of Assay Buffer to each tube and vortex to dissolve residues.

The immunopurified samples are now ready for assay.

12 ASSAY PROCEDURE

- Reconstitute Standards immediately before assay as described in Preparation of Reagents, or thaw previously reconstituted materials.

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- Allow all reagents to come to room temperature before use.
 - Mix all reagents gently before use in the assay. Prepare labelled borosilicate glass tubes, two for each Standard.
1. Add 100 µL of each Standard to the appropriately labelled tubes. Pipette directly to the bottom of the tube.
 2. Assemble sample extract tubes from step 12 above.
 3. Add 100 µL of Primary Antibody Solution to all tubes.
 4. Vortex all tubes gently without foaming. Incubate at 2-8°C overnight (16-20 hrs).
 5. Add 150 µL of solution from step 4 to the appropriate wells of the Antibody Coated plate.
Leave the first two wells empty for substrate blanks.
Cover the plate with an adhesive plate sealer and incubate the plate on an orbital shaker (500-750 rpm) at 18-25°C for 90 minutes.
 6. Add 100 µL of 1,25D Biotin solution to all wells except for the substrate blanks.
Cover the plate with an adhesive plate sealer and incubate the plate on an orbital shaker (500-750rpm) at 18-25°C for 60 minutes.
 7. Wash all wells three times with Wash Solution:
 - a. Automatic plate wash:
Set plate washer to dispense at least 300 µL of Wash Solution per well. Fill and aspirate for 3 cycles.
 - b. Manual Wash:
Decant the contents of the wells by inverting sharply. Dispense 250 µL of Wash Solution to all wells. Decant and repeat twice.
Tap the inverted plate firmly on absorbent tissue to remove excess Wash Solution before proceeding to the next step.
 8. Add 200 µL of Enzyme Conjugate to all wells except for the substrate blanks using a multichannel pipette. Cover the plate with an adhesive plate sealer and incubate the plate at 18-25°C for 30 minutes.
 9. Repeat Wash Step 7.
 10. Add 200 µL of TMB Substrate to all wells including the substrate blanks using a multichannel pipette. Cover the plate with an adhesive plate sealer and incubate the plate at 18-25°C for 30 minutes.
 11. Add 100 µL of Stop Solution to all wells using a multichannel pipette.
 12. Measure the absorbance of each well at 450nm (reference 650nm) using a microplate reader within 30 minutes of adding the Stop Solution.

13 QUALITY CONTROL

The regular use of control samples at several analyte levels is advised to ensure day-to-day validity of results. Two kit controls are provided. The controls should be tested as unknowns. Quality Control charts should be maintained to follow the assay performance.

14 CALCULATION OF RESULTS

Calculate the percent binding (B/Bo%) of each Standard, Control and unknown sample as follows:



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$$B/Bo\% = \frac{(\text{mean abs.} - \text{mean abs. substrate blank})}{(\text{mean abs. for '0' std.} - \text{mean abs. substrate blank})} \times 100$$

Prepare a calibration curve on semi-log graph paper by plotting B/Bo% on the ordinate against concentration of 1,25-dihydroxyvitamin D on the abscissa.

Calculate B/Bo% for each unknown sample and read values off the curve in pmol/L.

Alternative data reduction techniques may be employed, such as automated data reduction programs, but users should confirm that the selected curve fit is appropriate and gives acceptable results. Smoothed spline or 4PL curve fits are recommended.

DRG calculates results using MultiCalc (PerkinElmer) data reduction software with a 4PL curve fit plotting net absorbance versus log concentration.

The reportable range of the assay is 6 – 500 pmol/L.

Any value that reads below the lowest Standard, 6 pmol/L, is an extrapolated value and may be reported as “less than 6 pmol/L”.

Conversion of Units:

$$X \text{ pmol/L} \times 0.42 = Y \text{ pg/mL}$$

$$Y \text{ pg/mL} \times 2.4 = X \text{ pmol/L}$$

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14.1 Sample Assay Data

This data is for illustration only and must not be used for the calculation of any sample result.

Well	Description	Abs.	Mean Abs.	B/Bo%	Result pmol/L
A1, A2	Substrate blank	-0.006 0.006	0.000		
B1, B2	Standard 0 0 pmol/L	1.956 1.992	1.974	100.0	
C1, C2	Standard 1 5.7 pmol/L	1.746 1.776	1.761	89.2	
D1, D2	Standard 2 13.4 pmol/L	1.572 1.553	1.563	79.2	
E1, E2	Standard 3 34.0 pmol/L	1.132 1.179	1.156	58.5	
F1, F2	Standard 4 112 pmol/L	0.682 0.686	0.684	34.7	
G1, G2	Standard 5 246 pmol/L	0.419 0.457	0.438	22.2	
H1, H2	Standard 6 544 pmol/L	0.315 0.304	0.310	15.7	
A3, A4	Sample 1	1.109 1.158	1.134	57.4	37.2
B3, B4	Sample 2	0.532 0.547	0.540	27.4	169

15 LIMITATIONS OF USE

1. The assay may underestimate the amount of 1,25-dihydroxyvitamin D in circulation in patients receiving vitamin D2 therapy.
2. Samples suspected of containing analyte concentrations in excess of the highest Standard should be assayed in dilution.
3. The performance characteristics of this assay have not been established in a pediatric population.
4. As in the case of any diagnostic procedure results must be interpreted in conjunction with the patient's clinical presentation and other information available to the physician.
5. The following substances have been tested - in accordance with NCCLS EP7-A, "Interference Testing in Clinical Chemistry; Approved Guideline" - and found not to interfere in the 1,25-Dihydroxy Vitamin D assay:
 - Haemoglobin tested up to 500 mg/dL
 - Bilirubin tested up to 20 mg/dL
 - Lipid tested up to 2803 mg/dL



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16 EXPECTED VALUES

The following ranges have been determined using the 1,25-Dihydroxy Vitamin D kit and are provided for guidance only. Each laboratory should determine ranges for their local population.

The 95% reference interval for Normal Adults, collected from 120 apparently healthy adults, was calculated by a non-parametric method following the NCCLS guideline C28-A2, "How to Define and Determine Reference Intervals in the Clinical Laboratory".

Normal Adults 39-193 pmol/L (n=120)

End-stage renal disease* <6-22 pmol/L (n=24)

*Observed range of values.

17 PERFORMANCE DATA

17.1 Accuracy

The 1,25-Dihydroxy Vitamin D kit was compared against a recognized radioimmunoassay for the quantitative determination of 1,25-dihydroxyvitamin D, following NCCLS EP-9A2, "Method Comparison and Bias Estimation Using Patient Samples".

A population of 152 samples, selected to represent a wide range of 1,25-dihydroxy vitamin D [10 - 402 pmol/L], were assayed by each method. Passing & Bablok regression analysis was performed on the comparative data:

DRG = $0.94(x) + 7.2$ (95% CI of the slope and intercept were 0.89 to 1.01, and 2.1 to 12.7 respectively); correlation coefficient (r) = 0.95

17.2 Sensitivity

The sensitivity, defined as the concentration corresponding to the mean minus 2 standard deviations of 10 replicates of the zero Standard, is 6 pmol/L (2.5 pg/mL).

17.3 Precision

Precision was evaluated in accordance with NCCLS EP-5A2, "Evaluation of Precision Performance of Quantitative Measurement Methods".

Three human serum controls were assayed over 17 assay days spanning more than 49 operating days. The assays were performed by multiple operators using multiple reagents lots.

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Control	n	mean (pmol/L)	Within-run		Total	
			SD	CV%	SD	CV%
1	28	19.0	2.0	10.7	3.8	19.7
2	28	53.2	5.6	10.5	9.1	17.1
3	28	152	14.1	9.3	26.7	17.6

17.4 Recovery

Recovery was assessed by adding 1,25D3 to samples prior to extraction and assay.

Sample Conc pmol/L	125D 3 added pmol/L	Measured pmol/L	Recovery pmol/L	Recovery %
62.7	46.5	106.4	43.6	94%
62.7	93.0	140.7	78.0	84%
46.2	54.4	100.6	54.4	100%
46.2	108.8	161.3	115.1	106%
			Mean	96%

17.5 Linearity

Linearity was evaluated based on NCCLS EP-6A, "Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach".

Samples containing varying concentrations of 1,25-dihydroxyvitamin D were assayed in duplicate. The resulting mean concentrations were compared to predicted concentrations. Samples were prepared by diluting a high patient sample with a low patient sample prior to extraction and assay. The reportable range was determined to be 68-318 pmol/L.

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Predicted Concentration pmol/L	Measured Concentration pmol/L	Variation	
		pmol/L	%
65.5	68.3	2.8	4%
95.9	80.5	-15.3	-16%
126.3	130.5	4.2	3%
156.7	168.9	12.2	8%
187.1	184.4	-2.7	-1%
217.4	233.2	15.8	7%
247.8	235.7	-12.1	-5%
278.2	264.5	-13.7	-5%
308.6	317.5	8.9	3%

17.6 Specificity

The specificity of the kit was assessed with the following analytes at 50% binding of the Zero Standard.

Analyte	Cross-reactivity
1,25-Dihydroxyvitamin D ₃	100 %
1,25-Dihydroxyvitamin D ₂	39 %
24,25-Dihydroxyvitamin D ₃	0.056 %
25-Hydroxyvitamin D ₃	0.009 %

18 REFERENCES

Iqbal, SJ, Vitamin D metabolism and the clinical aspects of measuring metabolites, Ann Clin Biochem, 1994, 31, 109-124