1,25-Dihydroxy Vitamin D  RIA

Radioimmunoassay for the quantitative determination of 1,25-dihydroxyvitamin D in human serum or plasma

Radioimmuno-Assay (RIA) zur quantitativen Bestimmung von 1,25-dihydroxyvitamin D in menschlichem Serum oder Plasma
**Intended Use**

*For In Vitro Diagnostic Use*

The IDS 1,25-Dihydroxy Vitamin D kit is a complete assay system for the purification of 1,25-dihydroxyvitamin D (1,25D) in human serum or plasma by immunoextraction followed by quantitation by $^{125}$I radioimmunoassay. 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.

**Note:** The immunoextraction reagents are not supplied with this version of the kit. It is recommended that the extraction of 1,25-dihydroxyvitamin D is carried out using the IDS immunoextraction method. If an alternative extraction method is employed, it is the responsibility of the customer to validate its use with this $^{125}$I radioimmunoassay.

**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 D$_3$). Pro-vitamin D$_3$, primarily of dietary origin, undergoes photolytic conversion in the skin to 'parent' vitamin D$_3$ (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. Biosynthetic vitamin D (vitamin D$_2$ or ergocalciferol) is widely used as a food supplement (e.g. margarine) and follows an identical hydroxylation pathway to form bioactive 1,25D$_2$.

As a result, vitamin D status in many subjects will be determined by the presence of both 1,25D$_3$ and 1,25D$_2$. It is therefore essential to measure both forms of active vitamin D for maximum diagnostic utility. 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 institutionalised) 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, 1α-hydroxylation may be impaired, with low 1,25D levels as a result.

**Method Description**

The IDS 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 $^{125}$I RIA. Patient samples are delipidated and 1,25D extracted from potential cross-reactants by incubation for 3 hours 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 calibrators are incubated overnight with a highly specific sheep anti-1,25D. 125I-1,25D is added and incubation continued for 1 hr. Separation of bound from free is achieved by a short incubation with Sac-Cel® followed by centrifugation, decantation and counting. Bound radioactivity is inversely proportional to the concentration of 1,25D.

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. This product must be used strictly in accordance with the instructions set out in the Package Insert. IDS Limited 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.

Radioactive Materials
1. This kit contains radioactive material (Iodine-125; T½=60,14d; γ 35,5 KeV; X 27-32 KeV). Appropriate precautions and good laboratory practices must be used in storage, handling and disposal of material. Radioactive material must be received, acquired, possessed and used only by physicians, clinical laboratories or hospitals, and only for in vitro laboratory tests. Its receipt, acquisition, possession, use and transfer are subject to local regulations.
2. Store radioactive materials in the original container, in a specifically designated, properly labelled area. Access to radioactive materials must be limited to authorised personnel only.
3. Do not eat, drink, smoke or apply cosmetics in areas where radioactive materials are stored or handled. Do not pipette radioactive material by mouth.
4. Always wear a protective laboratory coat and disposable gloves when handling radioactive materials. Wash hands thoroughly afterwards.
5. Areas where spills occur should be wiped immediately with suitable absorbent material, which should then be disposed of as radioactive waste. The contaminated area should then be washed using an alkaline detergent or radiological decontamination solution.
6. Disposal of radioactive material should be in accordance with local regulations.
7. Persons under 18 should not be permitted to handle radioactive material or enter radioactive areas.
8. Radioactive areas must be kept clean. Use disposable or easily decontaminated laboratory ware and absorbent covers on laboratory bench surfaces to minimise contamination.

Elution Reagent REAG 2
R11 Highly Flammable (flashpoint 13°C).
S7 Keep container tightly closed.
S16 Keep away from sources of ignition - No Smoking.

**Sodium Azide**

Xn. Harmful:
Calibrators **CAL** and Controls **CTRL** contain sodium azide (NaN₃) >0.1% (w/w) (<1%).

R 22 Harmful if swallowed.
R52/53 Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

S46 If swallowed, seek medical advice immediately and show this container or label.
S36/37 Wear suitable protective clothing and gloves.
S60 This material and/or its container must be disposed of as hazardous waste.

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.

**Human Serum:**

Controls **CTRL**

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.

**Preparation of Reagents**

**Calibrators** **CAL**

Calibrators **CAL** are supplied in lyophilised form. Reconstitute immediately before use. Add 1 mL distilled or deionised 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** **CTRL**

Controls **CTRL** are supplied in lyophilised form. Reconstitute immediately before use. Add 1.2 mL distilled or deionised water to each bottle. Replace stopper and leave 15 - 20 minutes to reconstitute, inverting several times to ensure complete reconstitution.

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

**Wash Solution** **WASHBUF SOLN**

Prepare by adding the contents of each bottle of Wash Concentrate **WASHBUF 20x** to 475 mL of distilled or de-ionised water. Store at 2-8°C. 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.

**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.
Calibrators and Controls are stable at -20°C for 8 weeks.

**Indications of possible deterioration of kit reagents**
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.

**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.

**Procedure**

**Materials Provided**

1. **CAL** 0 – **CAL** 5 - Calibrators
   
   Lyophilised BSA-MOPS buffer containing 1,25-dihydroxyvitamin D and <0.4% sodium azide (0.01% reconstituted). The exact value of each calibrator is printed on the bottle label. 1 mL per bottle, 6 bottles per kit.

2. **Ab** - Primary Antibody
   
   Sheep anti-1,25-dihydroxyvitamin D in BSA-phosphate buffer with 0.09% sodium azide, 15 mL per bottle.

3. **Ag** $^{125}$I - $^{125}$I-1,25-Dihydroxy Vitamin D
   
   1,25-dihydroxyvitamin D in BSA-phosphate buffer with 0.09% sodium azide and proprietary stabilisers. Radioactive content <111 kBq (3µCi) per bottle, 15 mL per bottle.

4. **Sac-Cel**
   
   Anti-sheep IgG coupled to cellulose suspended in buffer with 0.09% sodium azide, 10 mL per bottle.

5. **CTRL** 1 – **CTRL** 2 - Controls
   
   Lyophilised human serum containing 1,25-dihydroxyvitamin D and <1% sodium azide (0.09% reconstituted), 1.2 mL per bottle, 2 bottles per kit.

6. **BUF** - Assay Buffer
   
   BSA-MOPS buffer with 0.01% sodium azide, 10 mL per bottle.

7. **WASHBUF** 20x - Wash Concentrate
   
   Phosphate buffered saline containing Tween, 25 mL per bottle.

**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, 300 µL, 500 µL, 1 mL and 4 mL.
4. Vortex mixer.
5. End-over-end or roller mixer.
6. Heating block or water bath at 30°C.
7. Nitrogen supply and manifold.
8. Refrigerated centrifuge capable of achieving 2000g.

9. Gamma counter capable of counting $^{125}$I.

10. SORB - Immunocapsules (REF AA-5406).

11. REAG 1 - Delipidation Reagent (REF AA-5407).

12. REAG 2 - Elution Reagent (REF AA-5408):
Sample Preparation
1. Prepare labelled glass or plastic tubes, one for each Control \textit{CTRL} and unknown sample \textit{SPE}. DO NOT DELIPIDATE CALIBRATORS \textit{CAL}.

2. Add 500 \( \mu \text{L} \) of each Control \textit{CTRL} or sample \textit{SPE} to appropriately labelled tubes.

3. Add 50 \( \mu \text{L} \) of Delipidation Reagent \textit{REAG} \textit{1} to each tube. Vortex all tubes.

4. Centrifuge all tubes at 2000 g for 30 minutes.

\textbf{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.

\textbf{Alternative Sample Preparation:}
Suitable for samples where the volume available is less than 500 \( \mu \text{L} \).

1. Prepare labelled conical-bottom plastic tubes or microcentrifuge tubes, one for each sample \textit{SPE}.

2. Add sample (e.g. 250 \( \mu \text{L} \)) to appropriately labelled tubes.

3. Add 0.1 x sample volume of Delipidation Reagent \textit{REAG} \textit{1} (e.g. 25 \( \mu \text{L} \)) to each tube. Vortex all tubes.

4. Centrifuge all tubes at 2000 g for 30 minutes, or at 10000 g for 10 minutes (microcentrifuge).

Immunoextraction Procedure
1. Prepare labelled Immunocapsules \textit{SORB}, two for each Control \textit{CTRL} and sample \textit{SPE}. DO NOT IMMUNOEXTRACT CALIBRATORS \textit{CAL}.

\textbf{Note}: If a Immunocapsule \textit{SORB} shows signs of leakage or incorrect volume - do not use.

2. Vortex Immunocapsules \textit{SORB} and allow solid phase to settle. Stand Immunocapsules \textit{SORB} upright in foam rack for 3-5 minutes.

3. Remove top screw caps from Immunocapsules \textit{SORB}. Add 100 \( \mu \text{L} \) of delipidated sample or control to Immunocapsules \textit{SORB} in duplicate. Replace caps securely.

4. Place Immunocapsules \textit{SORB} in foam rack and rotate end-over-end at 5-20 revolutions per minute for 3 hours at room temperature (18-25\(^{\circ}\)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 \textit{SORB} 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 \textit{SORB} and place each Immunocapsule \textit{SORB} in a plastic (or glass) tube. Centrifuge at low speed (500-1000g) for approximately 1 minute to remove sample.

6. Add 500 \( \mu \text{L} \) of diluted wash solution \textit{WASHBUF SOLN} to each Immunocapsule \textit{SORB}. Add carefully to avoid solid phase splashing out of the Immunocapsule \textit{SORB}. Centrifuge at low speed (500-1000g) for approximately 1
minute to wash immunoextraction gel.

7. Repeat the above wash step.

8. Prepare labelled borosilicate glass tubes, one for each Immunocapsule SORB, and transfer Immunocapsules SORB to the glass tubes.

9. Add 150 µL of Elution Reagent REAG 2 to all Immunocapsules SORB. 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 SORB and place tubes in a heating block or water bath set to 30°C. Evaporate the eluates under a gentle flow of nitrogen. Evaporation should take 20 - 30 minutes and leave a white residue of buffer salts inside the tubes.

12. Add 100 µL of Assay Buffer BUF to each tube and vortex to dissolve residues.

The immunopurified samples are now ready for assay.
**Assay Procedure**

**Reconstitute**

Calibrators \text{CAL} immediately before assay as described in Preparation of Reagents, or thaw previously reconstituted materials. 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 Calibrator \text{CAL} and non-specific binding (NSB) tubes.

1. Add 100 µL of each Calibrator \text{CAL} to the appropriately labelled tubes. Pipette directly to the bottom of the tube. Add 300 µL of Assay Buffer \text{BUF} to NSB tubes.

2. Assemble sample extract tubes from step 12 above.

3. Add 200 µL of Primary Antibody \text{Ab} to all tubes except NSB tubes.


5. Add 200 µL of \text{^{125}I-1,25-Dihydroxy Vitamin D} \text{Ag} to all tubes including two additional tubes to be set aside as Total Counts (TC). Vortex all tubes gently without foaming. Incubate at 18-25°C for 1 hour ±5 minutes.

6. Add 100 µL of Sac-Cel® to all tubes (except TC tubes). Note: Mix well to re-suspend immediately before use. Vortex all tubes gently without foaming. Incubate at 18-25°C for 30 minutes.

7. Add 4 mL of dilute Wash Solution \text{WASHBUF} to all tubes (except TC tubes). Centrifuge at 2000g for 20 minutes.

8. Decant supernatants (except TC tubes). Allow inverted tubes to drain on a pad of absorbent paper for 1 minute. Blot rims to remove remaining drops of liquid.

9. Count all tubes in a suitable gamma counter for at least 1 minute.

**Additional Guidance Notes**

1. Kit protocol, "Assay Procedure" section, step 1:
   
   **Pipette the calibrators directly to the base of the assay tube.**
   
   Do not pipette down the side of the tube. Analyte may be lost due to absorption to the tube wall.

2. "Assay Procedure", step 5:
   
   **The tracer \text{^{125}I-1,25-Dihydroxy vitamin D} must be at room temperature before addition.**
   
   The tracer incubation time must be timed accurately, 1 hour (+/- 5 minutes).
   
   The assay is sensitive to time and temperature of the tracer incubation.

As the assay antibody incubation is at 4°C and the tracer incubation is at room temperature, the assay tubes warm during the tracer incubation. If “solid” or “heavy duty” test tube racks are used, the time taken for the centre of the rack to warm to room temperature may cause a certain amount of “edge effect”, where the tubes on the outer edges of the rack warm to room temperature more rapidly than the remainder of the rack. This effect has been observed to cause elevated controls and variable sample values.

It is suggested that an “open style” or “Unwire” rack (part # 5976-0013)
fitted with a decanting retainer (# DS5979-0013) are particularly suitable. Alternatively, racks can be incubated in a room temperature water bath.

3. “Assay Procedure” step 8:

After centrifugation, smoothly decant the supernatant and place the tubes (still inverted) on absorbent paper. Allow the inverted tubes to drain undisturbed for 1 - 2 minutes. While holding the rack of tubes (still inverted) gently tap the rack of tubes onto the absorbent pad to remove any remaining liquid from the rims of the tubes.

It is important to allow the supernatant to drain thoroughly. NSB counts greater than 2% (NSB/Total cpm) indicate that the supernatant may not have been drained sufficiently. NSB of 0.7% to 1.4% indicates good draining of the pellet.

Note: if tubes are left to drain for too long, then loss of Sac Cel may occur, causing poor precision or variable sample values.

Pellets that slip or break up immediately on decanting and draining, may indicate “rough” handling of tubes after centrifugation. A poorly balanced centrifuge, or a worn (or faulty) breaking system on the centrifuge may also cause pellets to slip or break up during decanting and draining.

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. To ensure good precision it is important that the detectors in multi-well counters are well matched and not contaminated.

Calculation of Results

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

\[
B/Bo\% = \frac{(\text{mean counts} - \text{NSB counts})}{(\text{mean counts for '0' - NSB counts})} \times 100
\]

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 but users should confirm that the selected curve fit is appropriate and gives acceptable results. Smoothed spline or 4PL curve fits are recommended.

The reportable range of the assay is 8 - 500 pmol/L. Any value that reads below the lowest calibrator, 8 pmol/L, is an extrapolated value and may be reported as “less than 8 pmol/L”.

Conversion of Units:

\[ X \text{ pmol/L} \times 0.42 \Rightarrow Y \text{ pg/mL} \]
\[ X \text{ pmol/L} \leq \times 2.4 \]
Sample Assay Data
This data is for illustration only and must not be used for the calculation of any sample result.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>cpm</th>
<th>Mean cpm</th>
<th>B/Bo%</th>
<th>Result pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>Total Counts</td>
<td>38445</td>
<td>39054</td>
<td>38750</td>
<td></td>
</tr>
<tr>
<td>3, 4</td>
<td>NSB</td>
<td>400</td>
<td>418</td>
<td>409</td>
<td></td>
</tr>
<tr>
<td>5, 6</td>
<td>Calibrator 0</td>
<td>7703</td>
<td>7383</td>
<td>7543</td>
<td>100%</td>
</tr>
<tr>
<td>7, 8</td>
<td>Calibrator 1</td>
<td>7277</td>
<td>7157</td>
<td>7217</td>
<td>95.4%</td>
</tr>
<tr>
<td>9, 10</td>
<td>Calibrator 2</td>
<td>6348</td>
<td>6235</td>
<td>6292</td>
<td>82.5%</td>
</tr>
<tr>
<td>11, 12</td>
<td>Calibrator 3</td>
<td>5045</td>
<td>4964</td>
<td>5005</td>
<td>64.4%</td>
</tr>
<tr>
<td>13, 14</td>
<td>Calibrator 4</td>
<td>3182</td>
<td>3330</td>
<td>3256</td>
<td>39.9%</td>
</tr>
<tr>
<td>15, 16</td>
<td>Calibrator 5</td>
<td>1933</td>
<td>1989</td>
<td>1961</td>
<td>21.8%</td>
</tr>
<tr>
<td>17, 18</td>
<td>Sample 1</td>
<td>4850</td>
<td>4900</td>
<td>4875</td>
<td>62.6%</td>
</tr>
<tr>
<td>19, 20</td>
<td>Sample 2</td>
<td>2860</td>
<td>2725</td>
<td>2793</td>
<td>33.4%</td>
</tr>
</tbody>
</table>

Typical Calibration Curve
This sample calibration curve is for illustration only.
**Limitations of Use**

1. The assay may overestimate the amount of 1,25-dihydroxyvitamin D in circulation in patients receiving vitamin D₂ therapy.
2. The performance characteristics of this assay have not been established in a pediatric population.
3. Samples suspected of containing analyte concentrations in excess of the highest calibrator should be assayed in dilution.
4. Samples that contain appreciable background radioactivity should not be used. Any suspect samples should be screened for radioactivity before performing the assay. The sample should be stored until the radioactivity has decayed, or a further sample obtained.
5. 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.
6. The following substances have been tested - in accordance with NCCLS EP7-P, "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 3000 mg/dL
   - Urea tested up to 500 mg/dL
7. The immunoextraction reagents are not supplied with this version of the kit. It is recommended that the extraction of 1,25-dihydroxyvitamin D is carried out using the IDS immunoextraction method. If an alternative extraction method is employed, it is the responsibility of the customer to validate its use with this ¹²⁵I radioimmunoassay.
8. The performance claims made in this package insert were determined using the IDS immunoextraction method and are not valid for any other extraction method.

**Expected Values**
The following ranges have been determined using the IDS 1,25-Dihydroxy Vitamin D RIA 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 140 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 43-168 pmol/L (n=140)
- End-stage renal disease <5-43 pmol/L (n=43)

**Performance Data**

**Sensitivity**
The sensitivity, defined as the concentration corresponding to the mean minus 2 standard deviations of 20 replicates of the zero calibrator, is 5 pmol/L (2.1 pg/mL).

**Precision**
Precision was evaluated in accordance with NCCLS EP-5A, "Evaluation of Precision Performance of Clinical Chemistry Devices". Three human serum controls were assayed in quadruplicate over 20 assay days. The assays were performed
by multiple operators using multiple reagents lots.

<table>
<thead>
<tr>
<th>Control</th>
<th>n</th>
<th>Mean (pmol/L)</th>
<th>Within-run SD</th>
<th>Total SD</th>
<th>CV%</th>
<th>Total CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>19</td>
<td>3.2</td>
<td>3.8</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>46</td>
<td>4.0</td>
<td>8.8</td>
<td>6.0</td>
<td>13.0</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>162</td>
<td>14.0</td>
<td>8.6</td>
<td>19.4</td>
<td>11.9</td>
</tr>
</tbody>
</table>

**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 patient samples with analyte free serum prior to extraction and assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Predicted Conc. pmol/L</th>
<th>Measured Conc. pmol/L</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>-</td>
</tr>
</tbody>
</table>
Recovery
Recovery was assessed by adding 1,25\textsubscript{D}\textsubscript{3} to samples prior to extraction and assay.

<table>
<thead>
<tr>
<th>Sample Conc pmol/L</th>
<th>125D\textsubscript{3} added pmol/L</th>
<th>Measured pmol/L</th>
<th>Recovery pmol/L</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>162 38.8</td>
<td>195</td>
<td>32.2</td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>162 117</td>
<td>264</td>
<td>101.8</td>
<td>87%</td>
<td></td>
</tr>
<tr>
<td>88 27.0</td>
<td>114</td>
<td>25.3</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>88 73.0</td>
<td>171</td>
<td>82.7</td>
<td>113%</td>
<td></td>
</tr>
</tbody>
</table>

**Mean 94%**

Specificity
The specificity of the kit was assessed with the following analytes at 50% binding of the zero calibrator.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-Dihydroxyvitamin D\textsubscript{3}</td>
<td>100.0 %</td>
</tr>
<tr>
<td>1,25-Dihydroxyvitamin D\textsubscript{2}</td>
<td>91.0 %</td>
</tr>
<tr>
<td>24.25-Dihydroxyvitamin D\textsubscript{3}</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D\textsubscript{3}</td>
<td>&lt;0.01 %</td>
</tr>
</tbody>
</table>