MicroVue™ Total DPD EIA Kit

Bone Health

Reagents and Controls for assaying
Total Deoxypyridinoline Crosslinks
in Serum and Urine

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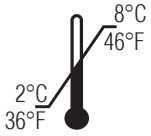
Not for Use in Diagnostic Procedures.



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REF 8032

QUICK GUIDE TO HYDROLYSIS STEPS

1. Prepare Samples
   A. FOR SERUM
      i) Combine 100 µL of each sample and Serum Hydrolysis Control with 100 µL of Total DPD Acid and mix by pipetting up and down immediately when added.
      ii) Close cap and vortex. Centrifuge all samples for 5 minutes at 10,000 x g.
      iii) Add 100 µL of supernatant to each well of the hydrolysis plate. Discard pellets.
   B. FOR URINE
      i) Dilute samples and Urine Hydrolysis Control 1:10 with deionized water.
      ii) Add 50 µL of Total Dpd Acid to Hydrolysis Plate wells using a multichannel pipette or repeat pipettor for each sample to be hydrolyzed.
      iii) Add 50 µL of diluted sample or Urine Hydrolysis Control to each well containing acid.
2. Add Hydrolysis Plate Sealer and place Hydrolysis Plate into a PCR thermocycler or Hydrolysis Unit. Seal mat in place on PCR thermocycler or seal by tightening screws on the Hydrolysis Unit.
3. Incubate at 99°C for 18-20 hours. Use an insulator in the oven for the Hydrolysis Unit.
4. Cool to 4°C (PCR thermocycler) or put on ice (Hydrolysis Unit).
5. Add 25 µL of Total DPD Assay Buffer followed by 25 µL of Total DPD Base. Do not mix buffer and base before adding to plate.

QUICK GUIDE TO ASSAY STEPS

1. Add 50 µL Total DPD Assay Buffer to all wells using a multichannel pipette or repeat pipettor.
2. Add 50 µL diluted Standards and diluted kit Controls.
3. Transfer 50 µL of each hydrolyzed and neutralized sample (and Serum and/or Urine Hydrolysis Control) to the assay stripwell after pipetting up and down in the pipette tip 5 times to mix.
4. Incubate 30 minutes at 2-8°C in the dark.
5. Add 50 µL of reconstituted Enzyme Conjugate to each well.
6. Incubate for 120 ± 5 minutes at 2-8°C in the dark.
7. Wash 3 times with 1X Wash Buffer.
8. Add 150 µL 20-28°C Working Substrate Solution.
9. Incubate 120 ± 5 minutes at 20-28°C. Check the optical density at 405 nm. If the optical density is < 1.2, incubate an additional 30 minutes.
10. Add 100 µL Stop Solution, and read the optical density at 405 nm.

SUMMARY AND EXPLANATION

Bone is constantly undergoing a metabolic process called remodeling.[1,2] This includes a degradation process, bone resorption, mediated by the action of osteoclasts, and a building process, bone formation, mediated by the action of osteoblasts.[1,2] Remodeling is required for the maintenance and overall health of bone and is tightly coupled; that is, resorption and formation are in balance.[2] In abnormal states of bone metabolism this process becomes

uncoupled and, when resorption exceeds formation, this results in a net loss of bone.[2] The measurement of specific degradation products of bone matrix provide analytical data of the rate of bone metabolism.[1,3,4]

Approximately 90% of the organic matrix of bone is type I collagen, a triple helical protein.[5] Type I collagen of bone is crosslinked by specific molecules that provide rigidity and strength. Crosslinks of mature type I collagen in bone are the pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD).[1,5] DPD is formed by the enzymatic action of lysyl oxidase on the amino acid lysine.[6] DPD is released into the circulation during the bone resorption process.[1,3,4,6] DPD is excreted unmetabolized in urine and is unaffected by diet,[7] making it suitable for assessing resorption.

In humans, the total pool of urinary DPD is approximately 45% free while the remaining fraction is bound to oligopeptides ranging from small linear peptides to very large crosslinked structures in excess of 10,000 Da.[8-11] Free and total crosslinks appear in healthy individuals and those with metabolic bone diseases,[8,10,12] thus providing the rationale for measuring the combined total forms of DPD. Improvements in immunoassay methodology have resulted in the ability to measure Total DPD levels in serum and urine, thus permitting a novel method for researching bone specific collagen degradation.[13] Since DPD is present and excreted in all mammalian species evaluated, including rodents, guinea pigs, dogs, sheep, horses, cows, and non-human primates, research applicability of a serum and urine Total DPD immunoassay extends to animal model studies.

PRINCIPLE OF THE PROCEDURE

To measure total DPD in serum or urine, samples are acid hydrolyzed. The MicroVue tDPD protocol is optimized for the detection of DPD in the hydrolysate. The MicroVue tDPD assay is a competitive enzyme immunoassay in a microassay stripwell format. DPD in the samples or standards competes with alkaline phosphatase conjugated DPD for binding to monoclonal anti-DPD antibody coated on the strip. The reaction is detected with pNPP substrate.

REAGENTS AND MATERIALS PROVIDED

96 Assays for quantifying Total Deoxypyridinoline Crosslinks in Serum and Urine.

MicroVue tDPD kit contains the following:

	Plate Tape Cover	**Part 0047**	**3 each**
	Hydrolysis Plate Sealer	**Part 0857**	**1 each**
	Hydrolysis Plate	**Part 0858**	**1 each**
S	**Total DPD Serum Hydrolysis Control** Lyophilized bovine serum	**Part 4835**	**1 each**
U	**Total DPD Urine Hydrolysis Control** Lyophilized human urine	**Part 4836**	**1 each**

A **B** **C** **D** **E** **F**	**Standards A-F** DPD purified from bovine bone in 10 mmol/L phosphoric acid containing sodium azide (0.05%) as a preservative	**Parts 4962 – 4967**	**0.3 mL, 1 each**
L **H**	**Low/High Controls** DPD purified from bovine urine in 10 mmol/L phosphoric acid containing sodium azide (0.05%) as a preservative	**Parts 4969, 4970**	**0.3 mL, 1 each**
❶	**Coated Strips** Purified murine monoclonal Anti-DPD antibody adsorbed onto stripwells	**Part 4661**	**12 each**
❷	**Stop Solution** 0.5N NaOH	**Part 4702**	**15 mL**
❸	**10X Wash Buffer** Nonionic detergent in a buffered solution containing sodium azide (0.05%) as a preservative	**Part 4703**	**55 mL**
❹	**Total DPD Assay Buffer** Phosphate buffer (pH 7.2) and solubilizing agent	**Part 4833**	**25 mL**
❺	**Substrate Buffer** A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as a preservative	**Part 4705**	**3 x 10 mL**
❻	**Substrate Tablets** p-Nitrophenyl phosphate	**Part 0012**	**3 x 20 mg**
❼	**Enzyme Conjugate** Lyophilized DPD, purified from bovine bone, conjugated to alkaline phosphatase containing buffer salts, and stabilizers	**Part 4202**	**3 each**
	Total DPD Acid 6N HCl plus solubilizing agent	**Part 4834**	**8 mL**
	Total DPD Base 10N NaOH	**Part 4837**	**6 mL**

MATERIALS REQUIRED BUT NOT PROVIDED

- Safety glasses and plastic gloves
- Oven or Polymerase Chain Reaction (PCR) thermocycler capable of maintaining 99 ± 4°C (If an oven is used, Hydrolysis Unit, REF 4838, an insulator for use in the oven, insulated gloves, glycerol, ice, and an appropriate ice container are required. See Sample and Control Hydrolysis section.)
- Micropipettes to deliver 25-100 µL
- Multichannel pipette or repeat pipettor to deliver 25-150 µL
- Labware suitable for liquid measurement of 10-500 mL
- Vortex Mixer
- Deionized or distilled water
- Glass or polypropylene tubes, or polypropylene dilution plates for dilution of standard and controls
- Microcentrifuge tubes
- Microcentrifuge capable of 10,000 x g
- Plate reader capable of reading at 405 nm
- 4-parameter calibration curve fitting software

WARNINGS AND PRECAUTIONS

1. For Research Use Only. Not for use in diagnostic procedures.
2. Treat specimen samples as potentially biohazardous material. Follow Universal Precautions and wear protective gloves when handling contents of this kit and any patient samples.
3. Dispose of containers and unused contents in accordance with Federal, State and Local regulatory requirements.
4. Use the supplied reagents as an integral unit prior to the expiration date indicated on the package label.
5. Wear suitable protective clothing, gloves, and eye/face protection when handling contents of this kit.
6. Store assay reagents as indicated.
7. Do not use Coated Strips if pouch is punctured.
8. Hydrolyze each sample in a single well of a volume sufficient to provide enough hydrolyzed sample to test in duplicate.
9. Total DPD Base (4837) and Total DPD Acid (4834) are corrosive and poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing by wearing protective eyewear and clothing. If contact is made, wash with water. If ingested, call a physician.
10. The Stop Solution (4702) and Substrate Buffer (4705) are considered corrosive and can cause irritation upon contact. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.
11. Sodium azide is used as a preservative. Incidental contact with or ingestion of buffers containing sodium azide may cause irritation to the skin, eyes, or mouth. Only use buffers for intended purposes and avoid contact with acids. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with a large volume of water to prevent azide build-up.
12. The MicroVue tDPD Kit standards and controls are in 10 mmol/L phosphoric acid. Avoid contact with skin, eyes, or clothing. Do not ingest. If contact is made, wash with water. If ingested, call a physician.
13. Use of multichannel pipettes or repeat pipettors is recommended to ensure the timely delivery of reagents.
14. For accurate measurement of samples, add samples and standards precisely. Pipette carefully using only calibrated equipment. Use the same type of pipette for standards and samples. For example, do not use an 8-channel pipette for sample addition and a single pipette for standard addition.
15. Use the same pipette to add acid and base to the samples and calibrators because ratios will be proportional even if the actual volume calibration may be slightly inaccurate. Dilution plates are useful for making calibrators when using a multichannel pipette.
17. The tDPD standards, controls, and serum samples are light sensitive. Avoid prolonged exposure to light, especially direct or indirect sunlight. Store reagents in the dark when not in use. Samples and reagents are not significantly affected by normal, artificial laboratory lighting when handled as directed in the Assay Procedure.

18. Do not allow microassay wells to dry once the assay has begun.
19. When adding or removing liquid from the microassay wells, do not scrape or touch the bottom of the wells.
20 A wash bottle or automated filling device should be used to wash the plate (*ASSAY PROCEDURE*, Step 18). For best results, do not use a multichannel pipette to wash the microassay plate.
21. Perform this assay with any validated washing method.
22. **Special Note for Customers Using a PCR thermocycler and MicroVue tDPD:**

    The Total DPD hydrolysis procedure utilizes a high-temperature acid hydrolysis to cleave low molecular weight peptides from the DPD crosslink in Type I collagen. During this heating process it is critical to ensure a complete airtight vapor seal of the hydrolysis plate. The Quidel Hydrolysis Block (Item 4838) is custom-made for this application. Quidel STRONGLY suggests the use of the Quidel Hydrolysis Block with the tDPD assay. Some customers may prefer to use a PCR thermocycler for this step of the procedure. While we have not had reports of volume loss when the hydrolysis is conducted using our Hydrolysis Block, we have had sporadic reports of PCR thermocyclers that do not hold a vapor seal across the entire plate.

    If you chose to use a PCR thermocycler for the hydrolysis step, Quidel recommends using only the interior wells of the hydrolysis plate to avoid the potential for volume loss in the samples. Avoid the outer three rows and columns of your hydrolysis plate. Prior to assaying the acid hydrolyzed sample in the tDPD ELISA, Quidel recommends confirming that your PCR device has maintained a complete seal by checking the volume recovery of the samples. You should recover by pipette 100% of the originally loaded 100 µL volume from several different positions on the hydrolysis plate. A darkening of the sample solution or particulate matter in the solution is normal and is caused during the denaturing process. Pipette up and down to re-solubilize any particulates.

    Additionally, PCR thermocyclers typically heat from both the top and bottom. Cooling should only be done on the base of the unit—not on the top as this will lead to condensation on the cover.

REAGENT PREPARATION

Total DPD Serum Hydrolysis Control

Reconstitute Total DPD Serum Hydrolysis Control with 0.5 mL deionized or distilled water and let stand for 30 minutes. Mix by inversion or vortex. Store reconstituted Control at 4°C for up to 2 weeks or at -20°C for longer periods.

Total DPD Urine Hydrolysis Control

Reconstitute Total DPD Urine Hydrolysis Control with 0.5 mL deionized or distilled water and let stand for 30 minutes. Mix by inversion or vortex. Store reconstituted Control at 4°C for up to 2 weeks or at -20°C for longer periods.

Wash Buffer

Prepare required amount of DPD 1X Wash Buffer (see table in Assay Procedure Section) by diluting 10X Wash Buffer 1:10 with deionized water. Store at 20-28°C. Use 1X Wash Buffer within 21 days of preparation.

Special Washing Instructions: **Prepare 1X Wash Buffer as above and store at 2-8°C until use.**

Enzyme Conjugate

Prepare DPD Enzyme Conjugate within 2 hours of use. Reconstitute each required vial of Enzyme Conjugate (see table) with 3.5 mL of Total DPD Assay Buffer. Store reconstituted Enzyme Conjugate at 20-28°C until use.

Working Substrate Solution

Bring the DPD Substrate Buffer to 20-28°C before beginning the assay (two hours to overnight recommended). Prepare Working Substrate Solution within 2 hours of use. Put one Substrate Tablet into each required bottle of 20-28°C Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

Total DPD Standards and Controls

Use the same pipette to add acid and base to the samples and calibrators because ratios will be proportional even if the actual volume calibration may be slightly inaccurate. Dilution plates are useful for making calibrators when using a multichannel pipette.

Add 50 µL of each standard and control to microcentrifuge tubes or a dilution plate. Then add 50 µL of Total DPD Acid followed by 25 µL of Total DPD Assay Buffer and 25 µL of Total DPD Base. Mix by vortexing (tubes) for at least 3 seconds or pipetting up and down 5 times (dilution plate). Use within 1 hour.

STORAGE

Store the kit at 2-8°C.

SPECIMEN COLLECTION AND STORAGE

Serum

Collect serum using standard venipuncture technique following an overnight fast. Process specimens to avoid hemolysis. Store the serum sample at 2-8°C for up to 4 days, or freeze the sample at ≤ 20°C for longer storage. Do not subject the samples to more than 3 freeze/thaw cycles. Avoid prolonged exposure to light, especially direct sunlight. Samples are not affected by normal, artificial laboratory lighting.

Urine

Collect urine using preservative-free First Morning Void (FMV) or Second Morning Void (SMV) collections. It is recommended collections be made prior to 10:00 a.m. to obviate any potential influence of diurnal variation. Refrigerate (2-8°C) the urine samples for storage of less than 7 days, or freeze the samples at less than -20°C for longer storage. Do not subject samples to more than 5 freeze/thaw cycles. Avoid prolonged exposure to light, especially sunlight. During routine processing, samples are not affected by normal, artificial laboratory lighting.

OVERVIEW OF THE TOTAL DPD ASSAY

This overview is intended as an introduction only. Refer to the Assay Procedure sections for step-by-step instructions. The Total DPD assay measures both free and peptide-bound DPD in serum and urine following acid hydrolysis. There are two major steps to the assay procedure: (1) hydrolyzing the samples and (2) assaying the hydrolyzed samples.

Day 1 Hydrolysis Step:

Serum and urine samples (and kit Hydrolysis Controls that come in the MicroVue tDPD kit) must be hydrolyzed overnight prior to testing. The standards do not require hydrolysis. Hydrolysis involves heating each sample with Total DPD Acid solution in a sealed plate to release the peptide-bound DPD. The acid-treated serum samples require a centrifugation step to remove precipitated proteins before hydrolysis. The heating can be accomplished by using a PCR thermocycler or by using an oven and the Hydrolysis Unit (REF 4838). Sample preparation on Day 1 takes approximately 30-60 minutes depending on the number of samples.

The next day, hydrolyzed serum and/or urine samples are quickly cooled. If an oven and the Hydrolysis Unit were used, the cooling is done in an ice bath. Once cooled, the samples are neutralized using Total DPD Base reagent and Total DPD Assay Buffer prior to testing in the assay.

Day 2 Assay Step:

Standards are prepared using the standards and controls supplied. The standards are prepared in the same mixture of acid, base, and buffer that was used for the samples.

To run the assay, the prepared samples, standards and controls are added to the microassay stripwells using a multi-channel pipettor. Total DPD Assay Buffer is also added to the stripwells first, to ensure that the samples and standards are adequately neutralized when they contact the wells.

The stripwells are incubated prior to adding the Enzyme Conjugate.

After incubation with the Enzyme Conjugate, the stripwells are washed, and substrate is added. The substrate incubation is stopped by adding Stop Solution, and the optical density in each well is read in a microassay plate reader. The total assay incubation time is 4.5 hours (not including the overnight hydrolysis).

ASSAY PROCEDURE PART 1: SAMPLE AND CONTROL HYDROLYSIS

Read entire product insert before beginning the assay.

Day 1:

1. Reconstitute Total DPD Serum and/or Urine Hydrolysis Control(s) with 0.5 mL deionized or distilled water and let stand for 30 minutes. Mix by inversion or vortex. Store reconstituted Control at 4°C for up to 2 weeks or at -20°C for longer periods.

2. Sample Preparation
   a. **SERUM**
      i) The sample hydrolysis step is for serum samples only. The assay standards do not require hydrolysis. The Serum Hydrolysis Control should be hydrolyzed at the same time and in the same manner as the rest of the serum samples. For each serum sample (and Serum Hydrolysis Control) to be hydrolyzed, place 100 µL of Total DPD Acid into a microcentrifuge tube. (A repeat pipettor speeds the process.) Add 100 µL of each sample to an acid containing tube. Immediately pipette the mixture up and down to ensure complete mixing, otherwise clumping may occur. Close cap and vortex. Centrifuge for 5 minutes at 10,000 x g.
      ii) For each treated serum sample, transfer 100 µL of supernatant from the microcentrifuge tube to a Hydrolysis Plate well. Discard pellets.
   b. **URINE**
      i) Dilute urine samples (and Urine Hydrolysis Control) 1:10 with deionized water. Samples with Total Dpd values < 3 nmol/L when diluted may be re-run undiluted.
      ii) The sample hydrolysis step is for urine samples only. The assay standards do not require hydrolysis. The Urine Hydrolysis Control should be hydrolyzed at the same time and in the same manner as the rest of the urine samples.
      iii) For each urine sample (and Urine Hydrolysis Control) to be hydrolyzed, place 50 µL of Total Dpd Acid into each Hydrolysis Plate well. (An 8-channel pipette or repeat pipettor speeds this process.) Add 50 µL of each diluted sample to an acid containing well in the Hydrolysis Plate. Do not mix.
3. Overlay Hydrolysis Plate with the Hydrolysis Plate sealer.
4. Set sealed Hydrolysis Plate into (a) a PCR thermocycler or (b) a Hydrolysis Unit.

   If oven method is used, the Hydrolysis plate is sometimes difficult to dislodge from the Hydrolysis Unit following heating. To prevent this, the operator has the option of adding ~100 µL glycerol to each of the indentations in the Unit prior to inserting the Hydrolysis Plate. The glycerol will act as a lubricant and allow for easier removal of the hydrolysis plate from the aluminum block after the assay is completed. To clamp the plate into the hydrolysis Unit, tighten the screws that are diagonally opposite in even increments until all screws are completely tightened to ensure that even pressure is applied over the entire plate. The hydrolysis plate should be completely, evenly sealed.
5. Heat at 99°C for 18-20 hours. If heating in an oven, the aluminum Hydrolysis Unit must be placed on an insulator in the oven to allow the unit to heat evenly. The bottom of the unit must not heat faster than the top of the unit or condensation will occur on the plate sealer lids, which could adversely affect the results of the assay. Good insulators include heat-resistant, plastic test tube racks, insulated fabric hotpads, etc., that will allow the unit to heat slowly and evenly.

Day 2:

6. Cool down by (a) setting PCR thermocycler to 4°C or (b) removing Hydrolysis Unit from oven and placing on ice until block is cold. Cubed ice is preferable for this step because it melts less rapidly and more efficiently cools the block. Hold the block on top of a 4-5-inch layer of ice cubes in a container so that the unit is cooled from the bottom. The unit will melt down an inch or two into the ice while being cooled, and the unit must be held to keep it from tipping as it melts the ice. The top of the unit will remain warm after the bottom is cooled, which is intended to prevent sample condensation on the sealer lid. If only crushed ice is available, place in a low, flat tray so that the ice is not deeper than the bottom half of the hydrolysis unit. The ice will melt quickly. Continue to add ice until the bottom aluminum block is cool. The top of the unit will remain warm. Do not let water get between the Hydrolysis Plate and Sealer. Remove screws and lid after the unit has cooled.

   Caution: If cooled properly, the lid may be hot though the block is cold. Do not attempt to remove Hydrolysis Plate from Hydrolysis Unit. All further assay steps may be completed while the hydrolysis plate remains in the hydrolysis unit. Removal from the unit may cause hydrolyzed samples to spill.
7. For each hydrolyzed sample and Hydrolysis Control, neutralize by adding 25 µL of Total DPD Assay Buffer followed by 25 µL of Total DPD Base, directly to the wells of the Hydrolysis Plate. (An 8-channel pipette or repeat pipettor speeds this process.) **Caution: Do not combine the Total DPD Assay Buffer and Total DPD Base together before pipetting as crystallization may occur.** Use the same pipette to neutralize the samples and control because ratios of acid and base will be proportional even if the actual volume calibration may be slightly inaccurate. Pipette sample up and down a minimum of 5 times to thoroughly mix.
8. After the assay is completed, remove the hydrolysis plate from the PCR thermocycler or the hydrolysis unit. If glycerol has been used in the hydrolysis unit, wash the unit thoroughly with soap and water to clean residual glycerol from the unit.

ASSAY PROCEDURE PART 2: TOTAL DPD

Procedural note: The Total DPD assay is sensitive to washing conditions. The entire wash step should be completed within 2 minutes. If the wash step cannot be completed in 2 minutes, follow the Special Washing Instructions located in the Reagent Preparation and Enzyme Conjugate Incubation sections.

Determine amount of each reagent required for the number of strips to be used.

# of Strips	4	6	8	12
# of Samples (tested in duplicate)	8	16	24	40
Enzyme Conjugate (vial)	1	1	2*	2*
Substrate Buffer (bottle)	1	1	2*	2*
1X Wash Buffer (mL)	100	150	200	300

*When more than one bottle or vial is to be used, combine the contents and mix prior to use.

Sample Incubation

9. Prepare Standards, Controls, samples and reagents as instructed. Use the same pipette to neutralize the samples and calibrators because ratios of acid and base will be proportional even if the actual volume calibration may be slightly inaccurate. Dilution plates are useful for making calibrators when using a multichannel pipette.

   Add 50 µL of each standard and Control to microcentrifuge tubes or a dilution plate. Then add 50 µL of Total DPD Acid followed by 25 µL of Total DPD Assay Buffer and 25 µL of Total DPD Base. Mix by vortexing (tubes) for at least 3 seconds or pipetting up and down 5 times (dilution plate). Use within 1 hour.
10. Allow pouch of Coated Strips to equilibrate to 20-28°C before opening. Remove Stripwell Frame and the required number of Coated Strips from the pouch (see table). Ensure that the pouch containing any unused strips is completely resealed and contains desiccant.
11. Add 50 µL Total DPD Assay Buffer to each well of the Coated Strips using a repeat pipettor or multichannel pipette.
12. Add 50 µL prepared Standards and Kit Controls to each well of the Coated Strips. For accurate analysis of samples, use the same pipette for standards and samples. For example, do not use an 8-channel for sample addition and a single pipette for standard addition.
13. Mix hydrolyzed samples and Hydrolysis Control(s) by pipetting up and down in the pipette 5 times before transferring the first aliquot. Add 50 µL of neutralized sample or Hydrolysis Control to each well of the Coated Strips. (An 8 channel pipette speeds the process). Then return to the same well(s) of the Hydrolysis Plate and transfer the second aliquot to a second well of the assay plate. In this way 1 column of the hydrolysis plate produces 2 columns of assay wells. For ease of transferring samples using an 8-channel pipette, the assay plate template should correspond to double the arrangement of the Hydrolysis Plate. Complete this step within 30 minutes.
14. Tap Stripwell Frame several times. Cover Strips with Tape Cover provided. Incubate for 30 minutes at 2-8°C. This incubation should be carried out in the dark.

Enzyme Conjugate Incubation

15. Prepare Enzyme Conjugate within 0.5-2 hours of use. Reconstitute each required vial of Enzyme Conjugate (see table) with 3.5 mL of Total DPD Assay Buffer. Store reconstituted Enzyme Conjugate at 20-28°C until use.
16. Add 50 µL of 20-28°C reconstituted Enzyme Conjugate to each well. Replace Tape Cover and incubate for 120 ± 5 minutes refrigerated (2-8°C) in the dark.
17. Bring the Substrate Buffer to 20-28°C before beginning the assay (two hours to overnight recommended). Prepare Working Substrate Solution within 2 hours of use. Put one Substrate Tablet into each required bottle of 20-28°C Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

18. Manually invert/empty strips (or use plate washer). Add at least 250 µL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash. While the strips are inverted, carefully wipe bottom of strips with a lint-free paper towel to ensure that the bottoms of the strips are clean.

    Special Washing Instructions: **Perform wash step as above, using cold (2-8°C) 1X Wash Buffer. After last wash, allow strips to drain for 5-10 minutes on paper towels before adding substrate.**

Substrate Incubation

19. Add 150 µL of Working Substrate Solution to each well. Incubate for 120 ± 5 minutes at 20-28°C. **NOTE:** If room temperature cannot be maintained between 20-28°C and an absorbance of > 2.0 is not compatible with your plate reader, monitor the development of signal in the zero standard wells; stop the reaction when the optical density reaches 1.2-1.5; then read the strip(s).
20. Read the optical density at 405 nm. If the optical density is < 1.2 then incubate an additional 30 ± 5 minutes.

Stop/Read

21. Add 100 µL of Stop Solution to each well to stop the reaction. Add Stop Solution in the same pattern and time intervals as the Working Substrate Solution addition.
22. Read the optical density at 405 nm. Assure that no large bubbles are present in wells and that the bottoms of the strips are clean. Strips should be read within 15 minutes of Stop Solution addition. If the optical density of the zero standard is less than 0.8, the results should be considered questionable and the assay repeated.
23. Quantitation software with a 4-parameter calibration curve fitting equation must be used to analyze Total DPD assay results:

    $(y = (A-D) / (1 + (x/C)^B) + D)$.

Sample Concentration

24. a. **SERUM**

    Determine concentration of serum samples and hydrolysis controls from the standard curve directly. No compensation is required. Report samples greater than 100 nmol/L as "> 100 nmol/L." Do not dilute serum samples.

    b. **URINE**

    Determine concentration of urine samples and Hydrolysis Controls from the standard curve. Multiply urine sample values by 10 to compensate for the initial dilution. Kit Hydrolysis Controls require no compensation because the values in the C of A are **reported as diluted**.
25. Low and High kit Controls and Hydrolysis Control(s) values should be within the range specified in the Certificate of Analysis supplied with the kit.

QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel. The optical density values are provided and are to be used as a guideline only. The results obtained by your laboratory may differ.

Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should be considered questionable and the samples should be repeated.

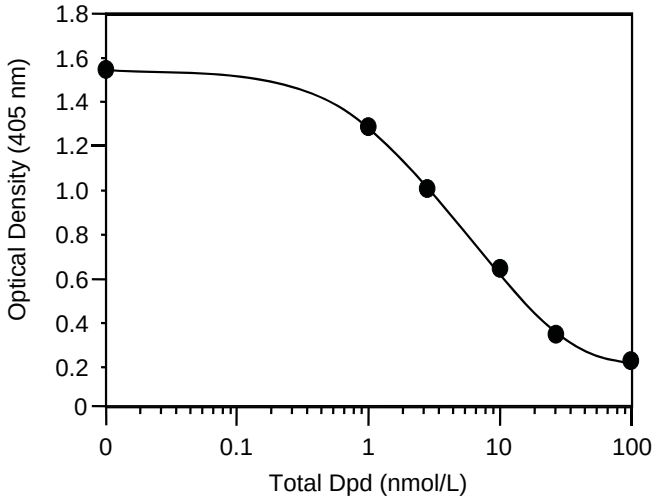
If the optical density of the zero Standard is less than 0.8, the results should be considered questionable, and the samples should be retested.

INTERPRETATION OF RESULTS

Representative Standard Curve

Total DPD Standard levels: A = 0, B = 1.0, C = 3.0, D = 10, E = 30, F = 100 nmol/L*

*The curve below is representative only. Use the standard values printed on the vial labels.



SAMPLE VALUES – SERUM TOTAL DPD

Normal Serum Total DPD reference ranges have been established for premenopausal females (n=45) and males (n=40) over 25 years of age. For the purposes of establishing reference ranges, normal subjects were defined as:

- Basically healthy, no bone, endocrine or chronic disorders
- Regular menstrual cycles (females)
- Not pregnant or breast feeding (females)
- Not currently taking any medication known to influence bone metabolism (e.g. anticonvulsants, bisphosphonates, calcitonin, corticosteroids, GnRH analogs, heparin, hormone replacement therapy, and thyroid medication)

Sex	Age	Mean (nmol/L)	SD	5th%	95th%
Females	25-44	3.43	0.64	2.56	4.79
Males	25-55	3.25	0.66	2.25	4.44

SAMPLE VALUES – URINE TOTAL DPD

A comparative study was performed to assess the correlation between measurement of urinary Total Dpd obtained using this enzyme immunoassay/hydrolysis method (EIA) and a conventional high performance liquid chromatography/hydrolysis method (HPLC).[14] The study was performed using 23 human urine samples that ranged from 19 to 325 nmol/L and yielded the following linear regression equation:

EIA = 0.998 x HPLC + 5.5 nmol/L, r = 0.99.

The 95% confidence interval of the intercept contained 0 nmol/L.

Due to the comparability of the methods, information concerning expected values can be obtained by reviewing the published literature for Total Dpd measured by properly standardized and controlled HPLC methods.

PERFORMANCE CHARACTERISTICS

Specificity

The anti-DPD antibody demonstrates selective, high affinity to hydrolyzed DPD and negligible binding to PYD, and nonhydrolyzed PYD and DPD peptides.

	% Reactivity
Free DPD	100%
Free PYD	< 1%
PYD/DPD peptides ≥ 1000 MW	< 2.5%

Reactivity to DPD in Animal Sera

The anti-DPD antibody demonstrates reactivity to hydrolyzed DPD from the following animal species: mouse, rabbit, rat, guinea pig, dog, cow, horse and primate (monkey, baboon).

Sensitivity

The minimum detection limit of the Total DPD Assay is 0.5 nmol/L, determined by the upper 3 SD limit in a zero standard study.

Precision (Serum)

Within-run and between-run precision were determined by assaying three serum samples.

DPD (nmol/L)	Within-run[1] C.V. (%)	Between-run[2] C.V. (%)
2.9	9.0	13.6
10.0	6.0	13.4
17.2	5.3	13.9

[1] n = 20 replicates [2] n = 20 runs

Precision (Urine)

Within-run and between-run precision were determined by assaying three urine samples.

DPD (nmol/L)	Within-run[1] C.V. (%)	Between-run[2] C.V. (%)
3.0	12.1	16.8
8.7	6.6	13.8
56	5.3	10.1

[1] n = 20 replicates [2] n = 20 runs

Recovery - Dilution

Dilution recovery was determined by serially diluting samples of all listed species and comparing observed values with expected values.

Serum Average recovery: 97% Absolute range: 85 - 115%
Urine Average recovery: 98% Absolute range: 83 - 118%

Linearity (Serum)

Sample linearity was determined by diluting a high serum sample with a low serum sample creating 3 intermediate levels. The observed values were compared with expected values.

Average recovery: 100% Absolute range: 87 - 111%

Linearity (Urine)

Sample linearity was determined by diluting a high urine sample with a low urine sample creating 3 intermediate levels. The observed values were compared with expected values.

Average recovery: 100% Absolute range: 85 - 113%

Recovery - Spike Recovery (Serum)

Spike recovery was determined by adding known quantities of purified DPD to serum samples of all listed species.

Average recovery: 104% Absolute range: 84 - 122%

Recovery - Spike Recovery (Urine)

Spike recovery was determined by adding known quantities of purified DPD to urine samples of all listed species.

Average recovery: 102% Absolute range: 84 - 114%

Interfering Substances (Serum)

Glucose levels above 200 mg/dL may interfere with the assay.

The following substances were tested at the specified concentrations, and were not found to interfere with the assay.

Substance	Concentration
Bilirubin, conjugated	40 mg/dL
Cholesterol	500 mg/dL
Hemoglobin	500 mg/dL
Protein, Total	12 g/dL
Protein, Albumin	6 g/dL
Protein, d-Globulin	6 g/dL
Triglycerides (lipemia)	3000 mg/dL

Interfering Substances (Urine)

The following substances were tested at the specified concentrations, and were not found to interfere with the assay.

Substance	Concentration
pH	4-9
Bilirubin	0.25 mg/dL
Creatinine	500 mg/dL
Albumin	500 mg/dL
Hemoglobin	200 mg/dL
Glucose	2 g/dL
Sodium chloride	6 g/dL
Acetone	1 g/dL
Sodium azide	0.1% wt/vol
Boric acid	0.1% wt/vol
Sodium fluoride	0.13 mg/mL

Very low samples run undiluted will be falsely elevated in diabetic subjects when Glucose levels are above 200 mg/dL.

ASSISTANCE

To place an order or for technical assistance, please contact a Quidel Representative at 800-524-6318 (in the U. S. only) or 408-616-4301, Monday through Friday, between 8:00 a.m. and 5:00 p.m., Pacific Time. Orders may also be placed by fax at 408-616-4310.

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REFERENCES

1. Delmas PD. Biochemical markers for the assessment of bone turnover. In: Riggs BL, Melton LJ,III (eds): *Osteoporosis: etiology, diagnosis, and management*. Philadelphia: Lippincott-Raven Publishers. 1995, pp. 319-333.
2. Riggs BL. Overview of osteoporosis. *West.J.Med*. 1991;154:63-77.
3. Delmas PD, Schlemmer A, Gineyts E, Riis B, Christiansen C. Urinary excretion of pyridinoline crosslinks correlates with bone turnover measured on iliac crest biopsy in patients with vertebral osteoporosis. *J.Bone Miner.Res.* 1991;6:639-644.
4. Eastell R, Colwell A, Hampton L, Reeve J. Biochemical markers of bone resorption compared with estimates of bone resorption from radiotracer kinetic studies in osteoporosis. *J.Bone Miner. Res.* 1997;12:59-65.
5. Seyedin SM, Rosen DM. Matrix proteins of the skeleton. *Curr. Opin.Cell Biol.* 1990;2:914-919.
6. Seibel MJ, Robins SP, Bilezikian JP. Urinary pyridinium crosslinks of collagen: specific markers of bone resorption in metabolic bone disease. *Trends Endocrinol.Metab.* 1992;3:263-270.
7. Colwell A, Russell RGG, Eastell R. Factors affecting the assay of urinary 3-hydroxypyridinium crosslinks of collagen as markers of bone resorption. *Eur.J.Clin.Invest.* 1993;23:341-349.
8. Robins SP. Collagen crosslinks in metabolic bone disease. *Acta Orthop.Scand.* 1995;66 (Suppl.266):171-5.
9. Seyedin SM, Kung VT, Daniloff YN, et al. Immunoassay for urinary pyridinoline: the new marker of bone resorption. *J.Bone Miner.Res.* 1993;8:635-41.
10. Robins SP, Duncan A, McLaren AM. Structural specificity of an ELISA for the collagen crosslink, pyridinoline: implications for the measurement of free pyridinium crosslinks as indices of resorption in metabolic bone diseases. [Abstract]. *J.Bone Miner. Res.* 1991;6(Suppl.1):S244.
11. Kamel S, Brazier M, Neri V, et al. Multiple molecular forms of pyridinoline crosslinks excreted in human urine evaluated by chromatographic and immunoassay methods. *J.Bone Miner.Res.* 1995;10:1385-92.
12. Robins SP, Woitge H, Hesley R, Ju J, Seyedin S, Seibel MJ. Direct enzyme-linked immunoassay for urinary deoxypyridinoline as a specific marker for measuring bone resorption. *J.Bone Miner.Res.* 1994;9:1643-1649.
13. Weitz S, Benham P, Leung S. Total deoxypyridinoline in serum and urine as measured by a novel adaptation of the DPD enzyme immunoassay [Abstract]. *J.Bone Miner.Res.* 1999;14 (Suppl. 1):S371.
14. Pratt DA, Daniloff Y, Duncan A, Robins SP. Automated analysis of the pyridinium crosslinks of collagen in tissue and urine using solid-phase extraction and reversed-phase high-performance liquid chromatography. *Anal.Biochem.* 1992;207:168-75.

Manufacturer

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0860G (2009/04)