

METRA™ Total DPD

EIA kit - Serum

Reagents and Controls for Assaying Total Deoxypyridinoline Crosslinks in Serum

For use with Metra™ Dpd Assay Kit (Catalog number 8007)

For Research Use Only. Not for use in diagnostic procedures.

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Made in USA

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Catalog number 8030

QUIDEL®

Read the entire product insert thoroughly before beginning the assay. Store the Total Dpd kit at 18-28°C until use.

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 balance². In abnormal states of bone metabolism this process becomes uncoupled and, when resorption exceeds formation, this results in a net loss of bone². 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 protein⁵. Type I collagen of bone is crosslinked by specific molecules which provide rigidity and strength. Crosslinks of mature type I collagen in bone are the pyridinium crosslinks, pyridinoline (Pyl) and deoxypyridinoline (Dpd)^{1,5}. Dpd is formed by the enzymatic action of lysyl oxidase on the amino acid lysine⁶. 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 diet⁷, 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 Da⁸⁻¹¹. 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 degradation¹³. 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

The Total Dpd kit is used in conjunction with the Dpd immunoassay kit (P/N 8007). To measure total Dpd in serum, samples are acid hydrolyzed. The Dpd protocol has been modified to optimize the detection of Dpd in the hydrolysate. The modified Dpd 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

Total Dpd part number 8030

<u>Kit Contents and Descriptions</u>	<u>Part</u>	<u>Qty/Vol</u>
Hydrolysis Plate Sealer	0857	1 each
Hydrolysis Plate	0858	1 each
Reagents:		
Total Dpd Assay Buffer	4833	25 mL
Phosphate buffer (pH 7.2) and solubilizing agent.		
Total Dpd Acid	4834	6 mL
6N HCl plus solubilizing agent.		
Total Dpd Base	4837	6 mL
10N NaOH		
Total Dpd Serum Hydrolysis Control	4835	1 each
Lyophilized Bovine serum.		
Total Dpd Urine Hydrolysis Control	4836	1 each
Lyophilized Human urine.		

WARNINGS FOR DPD KIT

- Standard 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.
- 1N NaOH is corrosive and poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.
- Sodium azide is used as a preservative. It may be fatal if swallowed or adsorbed through the skin. Do not mix 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.
- Dispose of test kits and components in a manner consistent with relevant regulations.

WARNINGS FOR TOTAL DPD KIT

- For Research Use Only. Not for use in diagnostic procedures.
- Treat serum samples as potentially biohazardous material.
- Total Dpd Base and Total Dpd Acid are corrosive and poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing by wearing protective eye wear and gloves. If contact is made, wash with water. If ingested, call a physician.
- Dispose of test kits and components in a manner consistent with relevant regulations.

PRECAUTIONS

- Discard the bottle of DPD Assay Buffer that comes with the Metra™ DPD Kit. It is not used in the Total Dpd Assay Procedure. Use the bottle of Total Dpd Assay Buffer that comes with the Total Dpd Kit for the entire Total Dpd Assay Procedure. Do not confuse these two Assay Buffers.**
- The Dpd 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.
- Use reagents supplied as an integral unit prior to the expiration date indicated on the package label.
- Store assay reagents as indicated.
- Do not use Coated Strips if pouch is punctured.
- Report samples greater than 100 nmol/L as "> 100 nmol/L". Do not dilute serum samples.
- Hydrolyze each sample in a single well that will be enough hydrolyzed sample to test in duplicate.
- Perform a standard curve with each assay.
- Use a 4-parameter calibration curve fit for accurate results.
Equation: $y = (A-D)/(1+(x/C)^B)+D$
- Perform this assay with any validated washing method.
- 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 Corporation. The OD values provided 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 OD of the zero standard is less than 0.8, the results should be considered questionable and the samples should be repeated.

PRECAUTIONS (CONT.)

- 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 substrate in the zero standard wells; stop the reaction when the OD reaches 1.2-1.5; then read the strip(s).
- Use of multichannel pipettes or repipettors is recommended to ensure the timely delivery of reagents.
- For accurate quantitation of samples, the addition of samples and standards must be precise. 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.
- 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.

REAGENT PREPARATION AND STORAGE

- 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.
- 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 room temperature (20-28°C). Use 1X Wash Buffer within 24 hours 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 room temperature (20-28°C) until use. **Caution: Do not reconstitute with Dpd Assay Buffer.**
- Working Substrate Solution**
Bring the Dpd Substrate Buffer to room temperature (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 room temperature 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**
Prepare using 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.
 - Discard the 300 nmol/L F Standard.
 - Make a 1 nmol/L standard by mixing 50 µL of the 3 nmol/L B Standard and 100 µL of the 0 nmol/L A Standard. Label this new standard as "Standard X".
 - Dilute the Low and High kit Controls by mixing 50 µL of each Control with 100 µL of deionized or distilled water.
 - Add 50 µL of each standard and diluted 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) or pipetting (dilution plate). Use within 1 hour.

SPECIMEN COLLECTION AND STORAGE

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.

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 Serum assay measures both free and peptide-bound Dpd in serum following acid hydrolysis. The Total Dpd assay is performed using two kits: the Dpd immunoassay (P/N 8007) and the Total Dpd add-on kit. *For Total Dpd, The Metra Dpd assay is not performed in the manner described in its product insert and some of the reagents that come with the Metra Dpd kit are not required (Dpd Assay Buffer and Standard F).* There are two major steps to the assay procedure: (1) hydrolyzing the samples and (2) assaying the hydrolyzed samples.

Day 1 Hydrolysis Step:

Serum samples (and kit Hydrolysis Control that come in the Metra Total Dpd 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 (P/N 4838). Sample preparation on Day 1 takes approximately 30-60 minutes depending on the number of samples.

OVERVIEW OF THE TOTAL DPD ASSAY (CONT.)

The next day, hydrolyzed serum 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 supplied in the Metra Dpd kit. The standard curve range is different, so the standards must be diluted to the required range. 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. Be aware that the Enzyme Conjugate is not reconstituted in the manner described in the Metra Dpd product insert.

After incubation with the Enzyme Conjugate, the stripwells are washed and substrate is added. The substrate incubation is stopped by adding Stop Solution (the substrate incubation is longer than it is in the Metra Dpd assay) 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).

PROCEDURE PART 1: SAMPLE AND CONTROL HYDROLYSIS

Kit Contents	Qty/Vol	Part
Hydrolysis Plate Sealer	1 each	0857
Hydrolysis Plate	1 each	0858
Total Dpd Assay Buffer	25 mL	4833
Total Dpd Acid	6 mL	4834
Total Dpd Serum Hydrolysis Control	1 each	4835
Total Dpd Urine Hydrolysis Control	1 each	4836
Total Dpd Base	6 mL	4837

Materials Required or Suggested BUT NOT Provided

Safety glasses and plastic gloves
Oven or Polymerase Chain Reaction (PCR) thermocycler capable of maintaining 99 ± 4°C (If oven is used, Hydrolysis Unit, P/N 4838, an insulator for use in 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 repipettor 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

Day 1:

- 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.
- 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 repipettor 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.
- For each treated serum sample, transfer 100 µL of supernatant from the microcentrifuge tube to a Hydrolysis Plate well. Discard pellets.
- Overlay Hydrolysis Plate with the Hydrolysis Plate sealer.
- 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.

- 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.

PROCEDURE PART 1: SAMPLE AND CONTROL HYDROLYSIS (CONT.)

Day 2:

- 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 before 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.
- For each hydrolyzed sample, 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 repipettor 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 calibrators because ratios of acid and base will be proportional even if the actual volume calibration may be slightly inaccurate.
- 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.

PROCEDURE PART 2: SERUM TOTAL DPD ASSAY

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 of each vial, and mix prior to use.

Sample Incubation

- Prepare Standards, Controls, samples and reagents as instructed, using Dpd Standards and Controls. 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 by be slightly inaccurate. Dilution plates are useful for making calibrators when using a multichannel pipette.
 - Discard the 300 nmol/L F Standard.
 - Make a 1 nmol/L standard by mixing 50 µL of the 3 nmol/L B Standard and 100 µL of the 0 nmol/L A Standard. Label this new standard as "Standard X".
 - Dilute the Low and High kit Controls by mixing 50 µL of each Control with 100 µL of deionized or distilled water.
 - Add 50 µL of each standard and diluted 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) or pipetting (dilution plate). Use within 1 hour.
- Allow pouch of Coated Strips to equilibrate to room temperature (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.
- Add 50 µL Total Dpd Assay Buffer to each well of the Coated Strips using a repipettor or multichannel pipette.
- 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.

PROCEDURE PART 2: SERUM TOTAL DPD ASSAY (CONT.)

- Mix hydrolyzed samples and Serum Hydrolysis Control by pipetting up and down in the pipette 5 times before transferring the first aliquot. Add 50 µL of neutralized sample or Serum 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.
- 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

- Prepare Dpd 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 room temperature (20-28°C) until use. **Caution: Do not reconstitute with Dpd Assay Buffer.**
- Add 50 µL of room temperature reconstituted Enzyme Conjugate to each well. Replace Tape Cover and incubate for 120 ± 5 minutes refrigerated (2-8°C) in the dark.
- Bring the Dpd Substrate Buffer to room temperature (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 room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.
- 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 bottom 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

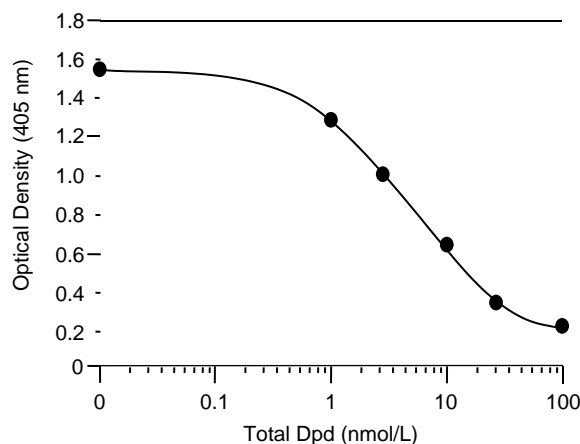
- Add 150 µL of Working Substrate Solution to each well. Incubate for 120 ± 5 minutes at room temperature (20-28°C).
- Read the Optical Density (OD) at 405 nm. If the OD is < 1.2 then incubate an additional 30 ± 5 minutes.

Stop/Read

- 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.
- Read the Optical Density (OD) at 405 nm. Assure that no large bubbles are present in wells and that the bottom of the strips are clean. Strips should be read within 15 minutes of Stop Solution addition. If the OD of the zero standard is less than 0.8 nm, the results should be considered questionable and the assay repeated.
- Quantitation software with a 4-parameter calibration curve fitting equation ($y = (A-D) / (1 + (x/C)^B) + D$) must be used to analyze Serum Total Dpd assay results.
- 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.
- Low and High kit Controls and Serum Hydrolysis Control values should be within the range specified in the Certificate of Analysis supplied with the kit.

Representative Standard Curve

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



QUICK GUIDE TO HYDROLYSIS STEPS

1. 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.
2. Close cap and vortex. Centrifuge all samples for 5 minutes at 10,000 x g.
3. Add 100 µL of supernatant to each well of the hydrolysis plate. Discard pellets.
4. Add Hydrolysis Plate Sealer and place Hydrolysis Plate into a PCR thermocycler or Hydrolysis Unit. Seal mat in place on PCR thermocycler or seal with screws on the Hydrolysis Unit.
5. Incubate at 99°C for 18-20 hours. Use an insulator in the oven for the Hydrolysis Unit.
6. Cool to 4°C (PCR thermocycler) or put on ice (Hydrolysis Unit).
7. Add 25 µL of Total Dpd Assay Buffer followed by 25 µL of Total Dpd Base. Do not mix.

QUICK GUIDE TO ASSAY STEPS

1. Add 50 µL Total Dpd Assay Buffer to all wells using a multichannel pipette or repipettor.
2. Add 50 µL diluted Standards and diluted kit Controls.
3. Transfer 50 µL of each hydrolyzed and neutralized sample (and Serum 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 minutes at 2-8°C in the dark.
7. Wash 3 times with 1X Wash Buffer.
8. Add 150 µL room temperature Working Substrate Solution.
9. Incubate 120 ± 5 minutes at room temperature. Check OD at 405 nm. If OD is < 1.2, incubate an additional 30 minutes.
10. Add 100 µL Stop Solution and read OD at 405 nm.

SERUM TOTAL DPD EXPECTED VALUES

Preliminary Serum Total Dpd reference ranges have been established for healthy 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	2.24	4.44

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

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

Dpd (nmol/L)	Within-run ¹ CV(%)	Between-run ² CV(%)
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

PERFORMANCE CHARACTERISTICS (CONT.)

Recovery - Dilution

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

Average recovery: 97%

Absolute range: 85 - 115%

Linearity

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%

Recovery - Spike Recovery

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%

Interfering Substances

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, δ -Globulin	6 g/dL
Triglycerides (lipemia)	3000 mg/dL

ASSISTANCE

If you have any questions regarding the use of this product, please call Quidel's Technical Support Number, 800-524-6318 (toll free). If outside the United States, contact your local Quidel office or distributor.

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METRA™ Total DPD

EIA kit - Urine

Reagents and Controls for Assaying Total Deoxypyridinoline Crosslinks in Urine

For use with Metra™ Dpd Assay Kit (Catalog number 8007)

For Research Use Only. Not for use in diagnostic procedures.

Quidel Corporation
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For other Quidel locations log onto our website.

Made in USA

0860A (3/01)

Catalog number 8030

QUIDEL®

Read the entire product insert thoroughly before beginning the assay. Store the Total Dpd kit at 18-28°C until use.

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 balance². In abnormal states of bone metabolism this process becomes uncoupled and, when resorption exceeds formation, this results in a net loss of bone². 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 protein⁵. Type I collagen of bone is crosslinked by specific molecules which provide rigidity and strength. Crosslinks of mature type I collagen in bone are the pyridinium crosslinks, pyridinoline (Pyl) and deoxypyridinoline (Dpd)^{1,5}. Dpd is formed by the enzymatic action of lysyl oxidase on the amino acid lysine⁶. 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 diet⁷, 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^{3,10,12}, 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 degradation¹³. 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

The Total Dpd kit is used in conjunction with the Metra™ DPD immunoassay kit (P/N 8007). To measure total Dpd in urine, samples are acid hydrolyzed. The Metra DPD protocol has been modified to optimize the detection of Dpd in the hydrolysate. The modified Metra DPD 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. Urinary total Dpd results are corrected for urinary concentration by creatinine quantitation.

REAGENTS AND MATERIALS

Total Dpd part number 8030

Kit Contents and Descriptions	Part	Qty/Vol
Hydrolysis Plate Sealer	0857	1 each
Hydrolysis Plate	0858	1 each

Reagents:

Total Dpd Assay Buffer	4833	25 mL
Phosphate buffer (pH 7.2) and solubilizing agent		
Total Dpd Acid	4834	6 mL
6N HCl plus solubilizing agent		
Total Dpd Base	4837	6 mL
10N NaOH		
Total Dpd Serum Hydrolysis Control	4835	1 each
Lyophilized Bovine serum.		
Total Dpd Urine Hydrolysis Control	4836	1 each
Lyophilized Human urine.		

WARNINGS FOR DPD KIT

- Standard 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.
- 1N NaOH is corrosive and poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.
- Sodium azide is used as a preservative. It may be fatal if swallowed or adsorbed through the skin. Do not mix 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.
- Dispose of test kits and components in a manner consistent with relevant regulations.

WARNINGS FOR TOTAL DPD KIT

- For Research Use Only. Not for use in diagnostic procedures.
- Treat urine samples as potentially biohazardous material.
- Total Dpd Base and Total Dpd Acid are corrosive and poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing by wearing protective eye wear and gloves. If contact is made, wash with water. If ingested, call a physician.
- Dispose of test kits and components in a manner consistent with relevant regulations.

PRECAUTIONS

- Discard the bottle of DPD Assay Buffer that comes with the Metra DPD Kit. It is not used in the Total Dpd Assay Procedure. Use the bottle of Total Dpd Assay Buffer that comes with the Total Dpd kit for the entire Total Dpd Assay Procedure. Do not confuse these two Assay Buffers.**
- The Dpd standards, controls, and urine 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.
- Use reagents supplied as an integral unit prior to the expiration date indicated on the package label.
- Store assay reagents as indicated.
- Do not use Coated Strips if pouch is punctured.
- Further dilute and retest urine samples greater than 1000 nmol/L in deionized or distilled water. Include the dilution factor in the final calculation. Urine samples less than 30 nmol/L may be rerun undiluted (eliminate the 1:10 dilution in the Assay Procedure).
- Hydrolyze each sample in a single well that will be enough hydrolyzed sample to test in duplicate.
- Perform a standard curve with each assay.
- Use a 4-parameter calibration curve fit for accurate results.
Equation: $y = (A-D)/(1+(x/C)^B)+D$
- Perform this assay with any validated washing method.
- 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 Corporation. The OD values provided 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.

PRECAUTIONS (CONT.)

- If the OD of the zero standard is less than 0.8, the results should be considered questionable and the samples should be repeated.
- 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 substrate in the zero standard wells; stop the reaction when the OD reaches 1.2-1.5; then read the strip(s).
- Use of multichannel pipettes or repipettors is recommended to ensure the timely delivery of reagents.
- For accurate quantitation of samples, the addition of samples and standards must be precise. 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.
- 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.

REAGENT PREPARATION AND STORAGE

- 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 room temperature (20-28°C). Use 1X Wash Buffer within 24 hours 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 room temperature (20-28°C) until use. **Caution: Do not reconstitute with Dpd Assay Buffer.**
- Working Substrate Solution**
Bring the Dpd Substrate Buffer to room temperature (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 room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.
- Total Dpd Standards**
Prepare using 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.
 - Discard the 300 nmol/L F Standard.
 - Make a 1 nmol/L standard by mixing 50 µL 3 nM B Standard and 100 µL 0 nmol/L A Standard. Label this new standard as "Standard X".
 - Dilute the Low and High kit Controls by mixing 50 µL of each Control with 100 µL of deionized or distilled water.
 - Add 50 µL of each calibrator and diluted 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) or pipetting (dilution plate). Use within 1 hour.

SPECIMEN COLLECTION AND STORAGE

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 Urine assay measures both free and peptide-bound Dpd in urine following acid hydrolysis of the specimen. The Total Dpd assay is performed using two kits: the Metra DPD immunoassay (P/N 8007) and the Total Dpd add-on kit. *For Total Dpd, the Metra DPD assay is not performed in the manner described in its product insert, and some of the reagents that come with the Metra DPD kit are not required (DPD Assay Buffer and Standard F).* There are two major steps to the assay procedure: (1) hydrolyzing the samples and (2) assaying the hydrolyzed samples.

Day 1 Hydrolysis Step:

Urine samples (and kit Hydrolysis Control that come in the Total Dpd 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 heating can be accomplished by using a PCR thermocycler or by using an oven and the Hydrolysis Unit (P/N 4838). Sample preparation on Day 1 takes approximately 30-60 minutes depending on the number of samples.

OVERVIEW OF THE TOTAL DPD ASSAY (CONT.)

The next day, hydrolyzed 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 supplied in the Metra DPD kit. The standard curve range is different, so the standards must be diluted to the required range. 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. Be aware that the Enzyme Conjugate is not reconstituted in the manner described in the Metra DPD product insert.

After incubation with the Enzyme Conjugate, the stripwells are washed and substrate is added. The substrate incubation is stopped by adding Stop Solution (the substrate incubation is longer than it is in the Metra DPD assay) 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).

PROCEDURE PART 1: SAMPLE AND CONTROL HYDROLYSIS

Kit Contents	Qty/Vol	Part
Hydrolysis Plate Sealer	1 each	0857
Hydrolysis Plate	1 each	0858
Total Dpd Assay Buffer	25 mL	4833
Total Dpd Acid	6 mL	4834
Total Dpd Serum Hydrolysis Control	1 each	4835
Total Dpd Urine Hydrolysis Control	1 each	4836
Total Dpd Base	6 mL	4837

Materials Required or Suggested BUT NOT Provided

Safety glasses and plastic gloves
Oven or Polymerase Chain Reaction (PCR) thermocycler capable of maintaining 99 ± 4°C (If oven is used, Hydrolysis Unit, P/N 4838, an insulator for use in 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 repipettor 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, controls, and urine samples
Plate reader capable of reading at 405 nm
4-parameter calibration curve fitting software
Creatinine values mmol/L for urine samples

Day 1:

- 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.
- 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.
- 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.

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 repipettor speeds this process.) Add 50 µL of each diluted sample to an acid containing well in the Hydrolysis Plate. Do not mix.

- Overlay Hydrolysis Plate with a Hydrolysis Plate Sealer.
- 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 ~100µL of 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 to be completely, evenly sealed.

- 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.

PROCEDURE PART 1: SAMPLE AND CONTROL HYDROLYSIS (CONT.)

Day 2:

- Cool down by (a) setting PCR thermocycler to 4°C or (b) removing Hydrolysis Unit from the oven and placing on ice until the 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 before 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.

- For each hydrolyzed sample, 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 repipettor speeds this process.) **Caution: Do not combine the Total Dpd Assay Buffer and Total Dpd Base before pipetting as crystallization may occur.** 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.
- 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.

PROCEDURE PART 2: URINE TOTAL DPD ASSAY

Procedural note: The Metra 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 of each vial, and mix prior to use.

Sample Incubation

- Prepare Standards, Controls, samples and reagents as instructed, using Dpd Standards and Controls. 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.
 - Discard the 300 nmol/L F Standard.
 - Make a 1 nmol/L standard by mixing 50 µL of the 3 nmol/L B Standard and 100 µL of the 0 nmol/L A Standard. Label this new standard as "Standard X."
 - Dilute the Low and High kit Controls by mixing 50 µL of each Control with 100 µL of deionized or distilled water.
 - Add 50 µL of each standard and diluted 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) or pipetting (dilution plate). Use within 1 hour.
- Allow pouch of Coated Strips to equilibrate to room temperature (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.
- Add 50 µL Total Dpd Assay Buffer to each well of the Coated Strips using a repipettor or multichannel pipette.
- 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.
- Mix hydrolyzed samples and Urine Hydrolysis Control by pipetting up and down in the pipette 5 times before transferring the first aliquot. Add 50 µL of neutralized sample or Urine 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.
- 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.

PROCEDURE PART 2: URINE TOTAL DPD ASSAY (CONT.)

Enzyme Conjugate Incubation

- Prepare Dpd 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 room temperature (20-28°C) until use. **Caution: Do not reconstitute with Metra DPD Assay Buffer.**
- Add 50 µL of room temperature reconstituted Enzyme Conjugate to each well. Replace cover and incubate for 120 ± 5 minutes refrigerated (2-8°C) in the dark.
- Bring the Dpd Substrate Buffer to room temperature (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 room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.
- 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 bottom 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

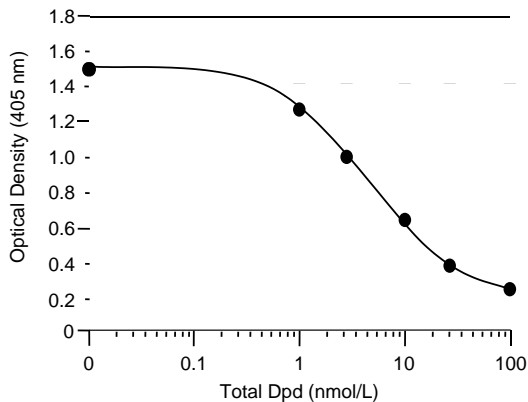
- Add 150 µL of Working Substrate Solution to each well. Incubate for 120 ± 5 minutes at room temperature (20-28°C).
- Read the Optical Density (OD) at 405 nm. If the OD is < 1.2 then incubate an additional 30 ± 5 minutes.

Stop/Read

- 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.
- Read the Optical Density (OD) at 405nm. Assure that no large bubbles are present in wells and that the bottom of the strips are clean. Strips should be read within 15 minutes of Stop Solution addition. If the O.D. of the zero standard is less than 0.8, the results should be considered questionable and the assay repeated.
- Quantitation software with a 4-parameter calibration curve fitting equation ($y = (A-D)/(1+(x/C)^B)+D$) must be used to analyze Urine Total Dpd assay results.
- 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.
- Low and High Kit Controls and Urine Hydrolysis Control values should be within the range specified in the Certificate of Analysis supplied with the kit.

Representative Standard Curve

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



QUICK GUIDE TO HYDROLYSIS STEPS

- Dilute samples and Urine Hydrolysis Control 1:10 with deionized water.
- Add 50 µL of Total Dpd Acid to Hydrolysis Plate wells using a multichannel pipette or repipettor for each sample to be hydrolyzed.
- Add 50 µL of diluted sample or Urine Hydrolysis Control to each acid containing well.
- Add Hydrolysis Plate Sealer and place Hydrolysis Plate into a PCR thermocycler or Hydrolysis Unit. Seal mat in place on PCR thermocycler or seal with screws on the Hydrolysis Unit.
- Incubate at 99°C for 18-20 hours. Use an insulator in the oven for the Hydrolysis Unit.
- Cool to 4°C (PCR thermocycler) or put on ice (Hydrolysis Unit).
- Add 25 µL of Total Dpd Assay Buffer followed by 25 µL of Total Dpd Base. Do not mix.

QUICK GUIDE TO ASSAY STEPS

1. Add 50 µL Total Dpd Assay Buffer to all wells using a multichannel pipette or repipettor.
2. Add 50 µL diluted Standards and diluted kit Controls.
3. Transfer 50 µL of each hydrolyzed and neutralized sample (and 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 minutes at 2-8°C in the dark.
7. Wash 3 times with 1X Wash Buffer.
8. Add 150 µL room temperature Working Substrate Solution.
9. Incubate 120 ± 5 minutes at room temperature. Check OD at 405 nm. If OD is < 1.2, incubate an additional 30 minutes.
10. Add 100 µL Stop Solution and read OD at 405 nm.

URINE TOTAL DPD EXPECTED VALUES

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

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

Dpd (nmol/L)	Within-run ¹	Between-run ²
	CV(%)	CV(%)
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 human urine samples and comparing observed values with expected values.

Average recovery: 98%

Absolute range: 83 - 118%

Recovery - Linearity

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

Spike recovery was determined by adding known quantities of purified Dpd to human urine.

Average recovery: 102%

Absolute range: 84 - 114

PERFORMANCE CHARACTERISTICS (CONT.)

Interfering Substances

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

If you have any questions regarding the use of this product, please call Quidel's Technical Support Number, 800-524-6318 (toll free). If outside the United States, contact your local Quidel office or distributor.

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