MOLOGIC
BIG IN TINY SCIENCE

B.I.T.S.

Desmosine ELISA kit

Catalogue Number: BDESV1

For the quantitative determination of
desmosine concentration in urine samples.

For research use only.
Not for use in clinical diagnostic procedures.

Instruction for use must be read in its entirety
before using this product.

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INTRODUCTION

Desmosine is a 526 dalton, tetrafunctional, pyridinium ring-containing amino acid that covalently links mature elastin fibres. During leukocyte-mediated inflammation, elastin is degraded by matrix metalloproteinases and neutrophil elastase. When the elastin is degraded, desmosine is excreted into the urine, either as free form, or as variously sized peptide fragments.

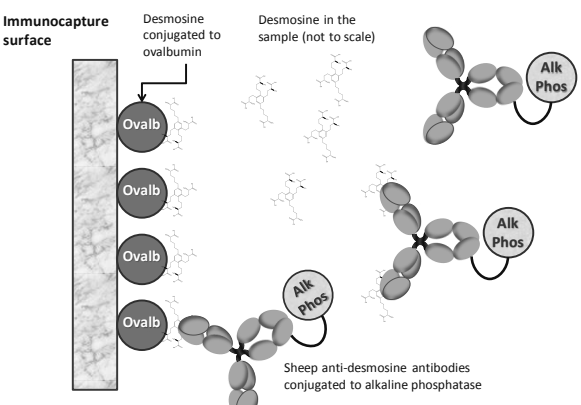
The amount of desmosine that collects in the urine will give an indication of how much leukocyte-driven tissue damage is occurring[1]. Desmosine is therefore an indicator of elevated lung elastin fibre turnover and a marker of the effectiveness of agents with the potential to reduce elastin breakdown. Increased excretion in the urine has been associated with a number of chronic diseases including chronic obstructive pulmonary disease (COPD), cystic fibrosis, aortic aneurysms and atherosclerosis[2, 3].

Level of free desmosine in the urine of healthy subjects is 4.6±0.8 ng/mg creatinine, and 6.3±1.0 in COPD patients[4].

PRINCIPLES OF THE ASSAY

This assay is a quantitative competitive enzyme immunoassay. An ovalbumin-desmosine conjugate has been pre-coated onto a 96 well microplate. Standards and samples are pipetted into the well, followed immediately by the addition of an enzyme-linked polyclonal antibody specific for desmosine. Following a wash to remove unbound desmosine-antibody reagent, a substrate solution is added to the wells and colour intensity reduces in proportion to the amount of desmosine-antibody reagent dislodged. The intensity of the colour is measured with a plate reader.

Figure 1. Schematic of assay



LIMITATIONS OF THE ASSAY AND TECHNICAL HINTS

- For research use only. Not for use in clinical diagnostic procedures.
- Use suitable protective clothing and gloves when handling patient samples and test components, and while performing the assay.
- Never pipette by mouth or allow reagents or patient sample to come into contact with skin.
- This test kit contains components of animal origin.
- The test is designed for single use only.
- Optimal results will be obtained by strict adherence to this protocol. Reagents must be added carefully to maintain precision and accuracy.
- Performing the assay outside the prescribed time and temperature ranges may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
- The components in this kit have been quality control tested as a master batch. Do not mix or substitute components from different lot numbers. Do not mix with components from other manufacturers.
- Care should be exercised to protect the reagents in this kit from contamination. Do not use if there is evidence of microbial contamination. Biological contamination of dispensing equipment, containers or reagents can lead to false results.
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing pipette tips between sample, standard and reagent additions.
- Keep storage boxes dry.
- Do not use plate if foil pouch is punctured or damaged.
- If sample results fall outside the dynamic range of the assay, dilute the samples and repeat assay.
- The kit should not be used beyond the expiration date on the kit label.
- Testing materials must be disposed of in accordance with local regulation.
- Recycle outer packaging if possible.
- Reagents must be added carefully to maintain precision and accuracy.

The B.I.T.S. desmosine ELISA has been rigorously validated with human urine samples, by reference to desmosine concentrations independently measured by LC MS/MS. Desmosine in different animal species is identical to desmosine in humans so there is no basis for designating desmosine antibodies or assay kits as specific for any particular species. However, this B.I.T.S. ELISA has been optimised (in terms of assay range, specificity and interference) for the assay of desmosine in human urine. For use with other sample types, the user should either confirm that the sample characteristics are equivalent to human urine, or validate the kit performance with the sample type they intend to analyse.

MATERIALS PROVIDED

DESCRIPTION	PART #	NO
Desmosine Microplate–96 well polystyrene microplate (12 strips of 8 wells) coated with an ovalbumin - desmosine conjugate.	DV1001	1 plate
Alk-Phos conjugate–80µL/vial of polyclonal antibody against desmosine conjugated to alkaline phosphatase with preservatives.	DV1003	1 vial
Desmosine standard - 5µg/vial of desmosine in a buffered protein base with preservatives; lyophilized.	DV1002	1 vial
Sample diluent concentrate 10X–7mL/vial	GC002	1 vial
Wash buffer concentrate 20X- 25mL/vial	GC001	2 vials
pNPP substrate -12mL/vial	GC003	1 vials
Plate sealers	GC004	2 strips

STORAGE

Store entire kit at 4°C immediately upon receipt, apart from the Alk-phos conjugate, which should be stored at -20°C.

UNOPENED KIT	Alk-Phos conjugate	-20°C
	Rest of the components	1 vial. Store at 4°C. Do not use past expiration date.
OPENED RECONSTITUTED REAGENTS	Microplate	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 4°C. *
	Alk-Phos conjugate	Discard dilutions after use, stock can be stored at -20°C for up to 1 month.*
	Desmosine standard	Discard dilutions after use, stock can be stored at -20°C for up to 1 month.*
	Sample diluent	Store at 4°C for up to 1 month.*
	Wash buffer	Store at 4°C for up to 1 month.*
	pNPP substrate	Store at 4°C for up to 1 month.*

* Provided this is within the expiration date of the kit

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 405nm.
- Pipettes and pipette tips to deliver 1µL to 1mL volumes.
- Deionized or distilled water.
- Wash bottle, or automated microplate washer.
- Horizontal orbital shaker capable of maintaining a speed of 500 ± 50rpm.
- 100mL and 1000mL graduated cylinder.
- Polypropylene tubes for dilution.
- Computer and computer software for data analysis.

SAMPLE COLLECTION AND STORAGE

Aseptically collect urine mid-stream (not the first sample of the day) directly into a sterile container. Assay immediately or aliquot and store at ≤-20°C, Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Urine samples require a 5-fold dilution into sample diluent.
A suggested dilution is 30µL +120µL sample diluent.

REAGENT PREPARATION

Bring all reagents to room temperature (18-25°C) before use. Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

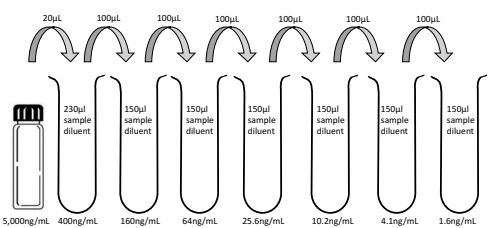
Wash buffer – Dilute 25mL of wash buffer concentrate (20X) into 475mL deionized or distilled water to prepare 500mL of wash buffer. Mix gently and thoroughly.

Sample diluent – Dilute 5mL of sample diluent concentrate (10X) into 45mL deionized or distilled water to prepare 50mL of sample diluent. Mix gently and thoroughly.

Alk-phos conjugate – The stock antibody must be diluted with 1X sample diluent. Calculate the required amount of 1X Alk-phos conjugate solution to use in the assay procedure according to how many wells you wish to use and the following calculation:

NUMBER OF WELLS STRIPS	NUMBER OF WELLS	TOTAL VOLUME OF 1X ALK-PHOS CONJUGATE (µL)	CONJUGATE (µL)	DILUENT(µL)
4	32	2333	20	2313
6	48	3500	30	3470
8	64	4667	40	4627
10	80	5833	50	5783
12	96	7000	60	6940

Desmosine standard – Reconstitute the desmosine standard with 1mL of sample diluent provided. This reconstitution produces a stock solution of 5µg/mL. Allow the standard to stand for a minimum of 10 minutes with gentle agitation prior to making dilutions.



Use polypropylene tubes throughout. Pipette 20µL of stock standard to 230µL sample diluent to produce top standard of 400ng/mL. Pipette 150µL of sample diluent into each tube and use the top standard to produce a dilution series (below). Mix each tube thoroughly before each transfer. The sample diluent serves as the zero standard (0ng/mL).

STANDARD #	VOLUME TO DILUTE (µL)	VOLUME DILUENT (µL)	TOTAL VOLUME (µL)	STARTING CONC. (ng/mL)	FINAL CONC. (ng/mL)
1	20	230	250	400	200
2	100	150	250	160	80
3	100	150	250	64	32
4	100	150	250	25.6	12.8
5	100	150	250	10.24	5.12
6	100	150	250	4.1	2.05
7	100	150	250	1.64	0.82
8	-	250	250	0	0

ASSAY PROCEDURE

Equilibrate all materials and prepared reagents to room temperature (18-25°C) prior to use.

It is recommended to assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame and return them immediately to the foil pouch containing the desiccant and reseal.
3. Wash plate four times with 300µL per well of 1X Wash buffer manually or three times with 300µL of 1X Wash buffer if using a plate washer. Tap the microplate 4-5 times on absorbent paper towel to completely remove the liquid.
4. Add 50µL of desmosine standard or diluted sample per well followed by 50µL Alk-Phos conjugate per well. Cover the well with a plate sealer and incubate for one hour at room temperature on a shaker. Start the timer after the last sample addition.
5. Wash microplate as previously described.
6. Add 100µL pNPP substrate solution to each well and incubate for 20-30 minutes or until the optimal yellow colour density develops. Protect from light.
7. Read the absorbance on a microplate reader at a wavelength of 405nm immediately.

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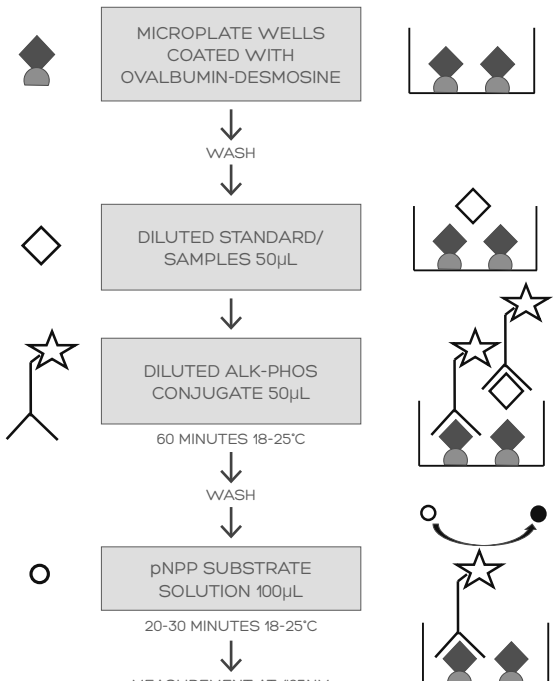
ASSAY PROCEDURE SUMMARY



CALCULATIONS

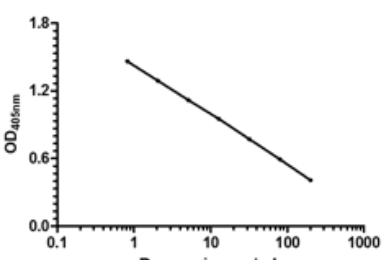
Calculate the mean value of the readings for each standard and sample. To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 405nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using four-parameter logistic curve fit.

Determine the unknown sample concentration from the standard curve and multiply the value by the dilution factor.

TYPICAL STANDARD CURVE

Data provided for demonstration purposes only.

A typical standard curve is shown below with the standards starting at 200ng/ml. The standard curve can be used to determine unknown levels of desmosine in urine samples using a 4-parameter fit.



ng/mL	AVERAGE
0	1.778
0.8192	1.427
2.048	1.219
5.12	1.055
12.8	0.923
32	0.739
80	0.532
200	0.393

SENSITIVITY

The lowest level of detection (LLOD) of desmosine is typically 0.819ng/mL. The LLOD was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

PRECISION

Intra-assay precision Four samples of known concentration were tested twenty-four times on one plate using a nested layout. This was repeated four times.

SAMPLE	HIGH	MED	LOW	NEG
n	96	96	96	96
Mean	0.51	1.15	1.68	2.31
SD	0.03	0.05	0.04	0.06
CV	5.37	3.94	2.22	2.60

Inter-assay precision Eight samples of known concentration were tested thirty-six times on three different batches of plates by three different operators.

SAMPLE	BATCH 1	BATCH 2	BATCH 3
CV	<8 %	<6%	<8%

RECOVERY

The recovery of desmosine spiked into 6 urine samples was evaluated.

Standard added value: 250ng/mL

Recovery %: 90-116.

Average recovery %: 104

LINEARITY

To assess the linearity of the assay, 7 samples spiked with high concentrations of desmosine (500ng/mL) were diluted to produce samples with values within the dynamic range of the assay.

URINE DILUTION	AVERAGE % EXPECTED VALUE
1:5	100
1:10	104.9
1:20	108.4
1:40	114.9

SPECIFICITY

The specificity of the assay was evaluated by measuring the degree of cross-reactivity of various compounds that were used in place of desmosine. The amount of antibody that was bound at each concentration was calculated and the approximate percentage of cross reactivity was calculated from the amount of compound that produced a signal equivalent to 50%.

CROSS REACTANT	% CROSS REACTIVITY
Isodesmosine	3.2
Pyridinoline (PYD)	0.04
Deoxypyridinoline (DPD)	0.08

SAMPLE RANGE

20 urine samples from healthy volunteers were evaluated for the presence of desmosine using this assay. No medical histories were available for the donors used in this study.

Median: 28ng/mL
Interquartile range: 23.1-34.63ng/mL

REFERENCES

1. CA Lindberg et al. (2012) Total desmosines in plasma and urine correlate with lung function. **Eur Respir J**, 39: 839-845
2. S Viglio et al. (2000) MEKC of desmosine and Isodesmosine in urine of chronic obstructive lung disease patients. **Eur Respir J**, 15(6): 1039-1045
3. T Osakabe et al. (1999) Characteristic change of urinary elastin peptides and desmosine in the aortic aneurysm. **Biol Pharm Bull**, 22(8): 854-857
4. S Ongay et al. (2014) Quantification of free and total desmosine and isodesmosine in human urine by liquid chromatography tandem mass spectrometry: A comparison of the surrogate-analyte and the surrogate-matrix approach for quantitation. **J. Chrom. A**, 1326: 13-19

NOTES

NOTES

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

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