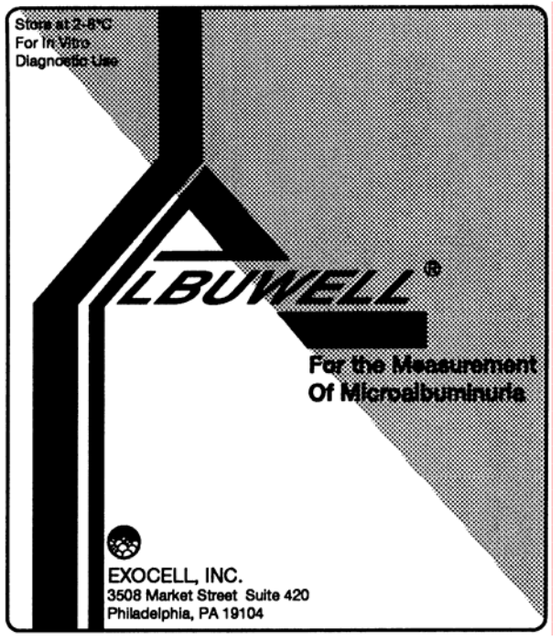

FLOW CHART

FILL PLATE WITH DILUENT

ADD STANDARDS

ADD SAMPLES

ADD CONJUGATE

INCUBATE 30 MINUTES(room temp)

WASH

ADD COLOR DEVELOPER(10 min)

ADD COLOR STOPPER

READ AT 450nm OR 490nm

96 tests in less than 1 hour total time

コスモ・バイオ株式会社

ALBUWELL

For the measurement of Microalbuminuria

INTENDED USE

The Exocell Albuwell kit is an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of albumin in human urine. Albuwell is for *in vitro* diagnostic use.

MEDICAL BACKGROUND

Diabetes mellitus is the largest single cause of kidney failure. Although the exact pathogenesis of diabetic nephropathy is incompletely defined, it is now recognized that there are detectable abnormalities in kidney structure and function a decade or more before the appearance of any overt evidence of renal disease. The most significant and well-documented of these abnormalities is a subtle increase in the urinary albumin excretion rate, known as microalbuminuria. Microalbuminuria is not measurable by conventional techniques for detecting proteinuria. It is believed that microalbuminuria represents a reversible stage of renal dysfunction, whereas overt proteinuria reflects irreversible disease. In fact, by the time kidney disease becomes clinically manifest through the appearance of persistent overt proteinuria, the renal lesions are relatively far advanced and renal function progressively and inexorably deteriorates. Proteinuria typically appears about twenty years after the onset of diabetes, whereas microalbuminuria can be detected within the first ten years. Microalbuminuria (30 - 150 (µg/min) has been established as a marker predictive of subsequent development of diabetic nephropathy. Periodic monitoring (2 - 3 times/year) of urinary albumin levels in the diabetic patient is therefore recommended, so that the initial escalation of renal damage can be detected and appropriate treatment can be instituted.

TECHNICAL BACKGROUND

ALBUWELL is an enzyme-linked immunosorbent assay (ELISA) for the detection and monitoring of microalbuminuria, that is, concentrations of albumin in the urine that are not detectable by conventional dipstick methods. It is intended strictly for *in vitro* use as an aid in monitoring the development of incipient diabetic nephropathy. ALBUWELL is simple to perform and highly specific. The assay follows a conventional ELISA format, using an antibody that recognizes antigen (albumin) in the test samples. ALBUWELL can be used to effectively measure urinary albumin levels in single void specimens or albumin excretion rates in timed-fractional collections. A comparison between ALBUWELL and Diagnostic Product Corporation's Double Antibody Albumin radioimmunoassay, performed on 45 diabetic and normal urine samples ranging between 0.75 -1,800 µg/mL, gave a correlation coefficient of 0.9995.

SPECIMEN COLLECTION

Collect a single void, timed fractional, overnight or 24-hour sample - without preservative - recording the time, duration and total volume of the collection (see section on EXPECTED VALUES). Mix well, withdraw a small portion, and clear by centrifugation or filtration. It is good practice to store a portion of the sample to permit retesting. Specimens my be stored for up to one week at 4°C or frozen at -20°C for up to two months. Prior to assay, allow the samples to come to room temperature. Do not thaw samples by applying heat. The following precautions should be observed when testing for urinary albumin:

1) Urine samples must not be contaminated with protein from non-urinary sources (blood or semen),
2) Urine samples should not be obtained following a strenuous exercise period, unless specifically indicated,
3) Individuals found to have elevated albumin levels should be retested for confirmation of microalbuminuria in another specimen collection.

KIT CONTENTS

PRECAUTIONS: Handle all kit components and patient samples as if they were capable of transmitting hepatitis and HIV (AIDS) virus.

All human body fluid sources used in the preparation of this kit were screened for the presence of hepatitis B surface antigen and for HIV antibodies and found to be negative. However, no test can guarantee that the causative agents for AIDS and hepatitis are not present.

Your ALBUWELL kit should contain the following items: PLATE MAPS (2), TEST PLATES (2), STANDARD (1), CONJUGATE (2), COLOR DEVELOPER (2), COLOR STOPPER (2), DILUENT (2), and INSTRUCTIONS with medical and technical background. STANDARD, DILUENT and CONJUGATE contain 0.02% isothiazolones as a preservative. COLOR STOPPER contains 1M sulfuric acid (12mL/vial).

ALBUWELL test plates are precoated and ready to use. The only reagent you supply is water. We suggest using distilled water for the best results. It is recommended that samples be run in duplicate, or at several dilutions, or both. The BASIC PROCEDURE will quantitate urinary albumin ranging between 1.5 - 100.0µg/mL in 80 samples when run in duplicate and requires a total of 20µL urine/sample. The ALTERNATE PROCEDURE will quantitate urinary albumin ranging between 0.15 - 2,560 µg/mL in 20 samples when run at eight dilutions and requires 100 µL urine/sample. Dilutions are performed directly in the plate. Further dilutions can be prepared if desired.

ASSAY PROCEDURE

All reagents and samples should be at room temperature before proceeding. If dilutions are performed in tubes, follow the BASIC PROCEDURE, but omit step 2 and use 100µL in step 3.

**** Standard Curve Preparation***

Standard albumin is added in twofold serial dilutions consisting of 10.0, 5.0, 2.5, 1.25, 0.625, 0.313, 0.156 and 0 µg/mL in duplicate wells. It is recommended that a standard curve be run on each plate.

1) Place 100 µL of DILUENT in columns 1 and 2 (rows A-H) of a test plate.
2) Place 100µL of STANDARD A in wells A1 and A2. There is now 200µL and 2.0µg of albumin in these wells. Mix the solution in wells A1 and A2 with a pipette.
3) Transfer 100µL of the solution in wells A1 and A2 to wells B1 and B2. Repeat the mixing and transfer steps through wells G1 and G2. Withdraw and discard 100µL from G1 and G2. Wells H1 and H2 should contain 100µL of DILUENT only.

**** Basic Procedure***

(DUPLICATE ANALYSIS at 1:10 Dilutions)

1) Prepare standard curve as described above.
2) Place 90µL of DILUENT in the remaining columns 3 - 12, rows A - H.
3) Place 10µL of urine from each sample into two adjacent wells (duplicates). Record the positions and dilution of each sample. All samples are now diluted 1:10.

**** Alternative Procedure***

(EIGHT DILUTIONS of 100µL URINE SAMPLES)

1) Prepare standard curve as described above.
2) Place 100µL of DILUENT in the remaining columns, 3 - 12, rows A - H. You will use one column/sample.
3) Place 100µL/well from each patient sample into row A of columns 3 - 12, using one column per sample. Change pipette between samples, and record the sample positions.
4) Mix the solutions in row A with a pipette or a multichannel pipette
5) Transfer 100µL of the solution in row A to row B. Repeat the mixing and transfer steps through row H. Withdraw and discard 100µL from row H. Your samples will now be in twofold serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 in rows A, B, C, D, E, F, G and H respectively.

**** Incubation***

All reagents and samples should be at room temperature before proceeding with the incubation. Once the plates are set up with standards and samples, you are ready to add CONJUGATE. Add 100µL/well of CONJUGATE to all the wells rapidly and accurately. Let the plate stand at room temperature for a minimum of 30 minutes. Longer incubation periods of up to one hour are acceptable.

**** Color Development***

After the incubation step, gently wash the plate with water or wash buffer (e.g. phosphate buffered saline containing 0.05% Tween 20) six times. Fill and empty all the wells each time. Be sure that none of the pipette tips or other materials that have been exposed to CONJUGATE come into contact with COLOR DEVELOPER. Immediately add 100µL of COLOR DEVELOPER to each well, quickly and accurately. After 2 - 10 minutes (or when the color in wells H1 and H2 has reached an absorbance of approximately 1.0) add 100µL/well of COLOR STOPPER (supplied at the appropriate working strength). Add the COLOR STOPPER to the wells in the same order that the COLOR DEVELOPER was added. Once stopped, the color is stable for up to one hour.

**** Reading the Plate***

Blank the instrument on air and read the absorbance of all the wells at 450nm. If dual mode reading is available, use a 630nm reference filter. The standard curve should have absorbances ranging between 0.1 and 1.5. If the color is overdeveloped (too dark), read the plate at 405nm. Calculate the mean value for duplicate wells. Conventional RIA and ELISA techniques of calculation are applicable.

COMPUTER or HAND CALCULATOR DATA REDUCTION

The assay has been standardized for linearity on a log-log regression of raw data if a computer or hand calculator is used. One point from either end of the standard curve may be dropped if necessary to improve linearity. Values are obtained in log form and must be converted to raw µg/mL by using the inverse log function. Multiply the raw µg/mL values times the dilution factor to get corrected µg/mL urinary albumin. When serial dilutions are used, average the corrected values from those sample dilutions that had raw absorbance values within the absorbance range of the standard curve range.

MANUAL CALCULATIONS with GRAPH PAPER

The raw standard curve data can be plotted on semi-log graph paper, placing the concentration on the x-axis (log) and the absorbance on the y-axis. Draw a curved line through the points. Raw sample concentration is taken from the x-axis by finding the point on the curve that corresponds to the sample absorbance. Multiply the raw µg/mL values times the dilution factor to get corrected µg/mL urinary albumin. When serial dilutions are used, average the corrected values from those sample dilutions that had raw absorbance values within the absorbance range of the standard curve. There should be at least two values within the standard curve range.

EXPECTED VALUES

Urine albumin concentration (µg/mL) can be converted to µg/min for timed fractional, overnight or 24-hour collections, by using the following formula:

$$\frac{(\text{µg/mL urinary albumin}) \times (\text{sample volume in mL})}{(\text{collection interval in minutes})}$$

Urinary albumin levels in single void specimens, expressed in µg/mL, have been shown to correlate closely with albumin excretion rates that are normally expressed as µg/min. In fact, patients having a urinary output of 1.4 L/day (60mL/hour) will have identical values for µg/mL and µg/min. Thus, the normal range of albumin concentration in urine is 1.0 - 20.0 µg/mL and the microalbuminuric range is 20 - 200µg/mL. Samples above 200µg/mL are proteinuric and are likely to be dipstick positive. A random screening of 31 diabetic patients

showed that 16 (50%) had normal levels of urinary albumin, 10 (33%) were microalbuminuric, and 5 (16%) were proteinuric.

REPORTING RESULTS

Results are usually reported as excretion rates, in micrograms of albumin per minute (µg/min). This is obtained by multiplying the concentration in µg/mL, as read from the curve, by the dilution factor and then by the total volume (in milliliters), and then dividing by the duration of the collection (in minutes).

QUALITY CONTROL

Record keeping: It is good laboratory practice to record for each assay the lot numbers and reconstitution dates of the components used.

Sample Handling: The instructions for handling and storing patient samples should be carefully observed. Clarify samples by centrifugation or filtration prior to assay. Dilute samples accurately, taking care to avoid foaming or splashing. Use disposable-tip micropipettes, changing the tip between samples. For the same reasons, an automatic pipettor-diluter should not be employed. Results of duplicate analysis (or of more dilutions when employed) should be inspected for agreement.

Controls: Controls or urinary pools with low, intermediate and high albumin concentrations should be routinely assayed as unknowns, and the results charted from day-to-day as described in J. O. Westgrad et al: " A multi-rule chart for quality control" in Clinical Chemistry 27: 493-501 (1987)

Data Reduction: It is good practice to construct a graph of the calibration curve as a visual check on the appropriateness of the transformation used, even where a computer handles the calculation of results.

LIMITATIONS

1) It must be remembered that there are other potential causes of microalbuminuria besides incipient diabetic nephropathy. Consideration must be given to exercise, poor diabetic control and non-diabetic renal or systemic diseases, including hypertension. Urinary tract infections and congestive heart disease are also possible causes of subclinical elevations in the albumin excretion rate.
2) Samples with a pH of less than 4 or greater than 8 may yield results which are respectively too high or too low. Acidified samples are accordingly unsuitable for use.
3) The test should not be performed if the sample exhibits significant bacterial growth or if the patient shows signs of urinary tract infection.
4) Bloody specimens are unsuitable for use, even if clarified by centrifugation, since blood is a likely sign of contamination (e.g., from menstrual flow) and albumin circulates in plasma at levels approximately 4000 times those normally encountered in urine. Semen is also a source of contamination.

TROUBLE SHOOTING

To ensure top performance of your Albuwell Kit, the causes of some foreseeable problems and methods to prevent or correct them are included herein.

1) No color appears after adding COLOR DEVELOPER. This most likely means that the kit has been exposed to temperatures above 8°C for two weeks or more or that CONJUGATE was not added. Please keep kits refrigerated until used.
2) Color in wells is too light. Longer incubation with COLOR DEVELOPER may be required. A 490 nm test filter will increase values (see 1).
3) Color in wells is too dark - absorbance of 0 µg/mL is "over". Color development was not stopped in time. Try removing 100 µL from every well and reading the plate again.
4) Poor agreement between duplicates. Be sure pipetting operations are accurate. See that the pipette tips are securely attached and that each well receives equal amounts of reagents. Isolate the plate in a drawer or cabinet during the incubation step to avoid drafts. Be sure that wash steps are thorough.

5) Dark wells appear out of sequence. Probably due to a microdroplet of conjugate contaminating a well when COLOR DEVELOPER was added. It is advisable to use separate pipettors for CONJUGATE and COLOR DEVELOPER. Be sure washing is thorough and that wash volumes exceed 200 µL.
6) Standard curve is erratic. Practice serially diluting standards. Additional standard is supplied. Avoid foaming and do not scrape the bottom of the wells during the mixing and transfer steps. (see 4 and 5).

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ALBUWELL PERFORMANCE CHARACTERISTICS

Comparison to radioimmunoassay: A regression analysis was performed to compare the urinary albumin values obtained from ALBUWELL and Diagnostic Product Corporations Double Antibody Albumin radioimmunoassay. Albumin values were obtained using the procedures described in the kit inserts, and were compared as micrograms/milliliter (µg/mL). Urine samples from 29 diabetic and 15 normal subjects, (ranging from 0.75-1,800 µg/mL) gave a correlation coefficient of 0.9995.

Intraassay variability between duplicate wells: The inherent percent coefficient of variation (%CV) within duplicate wells that was attributable to equipment and instrumentation alone (ELISA plate reader, pipettors and assay plates) was determined by reading plates in which all of the wells contained a uniform volume of developed chromogenic substrate. The average %CV was found to be 0.7% and 2.7% at absorbances of 1.37 and 0.20, respectively. The average %CV for duplicates in an actual assay, where standards and urine samples gave absorbances between 0.05 and 1.30 was 3.26%.

Intraassay variability within a dilution series: the percent coefficient of variation (%CV) between dilutions (1:4 and 1:8) of 12 different samples of normal urine run in a single assay plate is shown below. Values shown are in µg/mL.

	DILUTION				
SAMPLE	1:4	1:8	MEAN	S.D.	%CV
1)	5.06	5.43	5.25	0.18	3.53
2)	7.42	8.68	8.05	0.63	7.83
3)	7.97	7.40	7.69	0.28	3.71
4)	6.68	7.58	7.13	0.45	6.31
5)	6.01	6.18	6.10	0.09	1.39
6)	8.51	8.84	8.68	0.16	1.90

7)	5.88	6.09	5.99	0.10	1.75
8)	9.03	10.77	9.90	0.87	8.79
9)	12.73	13.40	13.07	0.33	2.56
10)	4.21	5.67	4.94	0.73	14.78
11)	7.21	7.28	7.25	0.03	0.48
12)	6.90	6.42	6.66	0.24	3.60
MEAN COEFFICIENT OF VARIATION, N=12					4.72%

Consistency throughout a greater dilution series was determined for a urine sample that contained added albumin in:

Dilution	Raw µg/mL	CORRECTED µg/mL
1:2	>10	out of range
1:4	>10	out of range
1:8	6.29	50.36
1:16	3.26	52.12
1:32	1.61	51.38
1:64	0.76	48.79
1:128	0.40	50.95
1:256	0.21	54.48
		51.34 MEAN
		1.74 S.D.
		3.38 % CV

Intraplate Reproducibility: A single sample of normal urine was assayed in ALBUWELL at various locations on the same plate. Close agreement of the values indicated that the assay reproducibility quantitates urinary albumin, and that sample location does not influence the results.

LOCATION*	µg/mL*
$A_{3,4}$ - $D_{3,4}$	.28
$E_{3,4}$ - $H_{3,4}$	5.48
$E_{5,6}$ - $H_{5,6}$	5.55
$A_{7,8}$ - $D_{7,8}$	6.19
$A_{9,10}$ - $D_{9,10}$	5.53
$E_{9,10}$ - $G_{9,10}$	5.26
$E_{11,12}$ - $G_{11,12}$	5.35
	5.52 MEAN
	0.29 S.D
	5.31 % CV

* Values were derived from dilutions of 1:2, 1:4, 1:8, and 1:16 as described in the ALTERNATE PROCEDURE.

Interassay Variability: The degree of variability to be expected between assay plates was determined by comparing binding inhibition curves generated by standards that were run on ten different assay plates on ten different days over a period of three months.

STANDARDS µg/mL	MEAN % INHIBITION	S.D.	% CV
10.00	94.57	3.47	3.67
5.000	88.42	2.47	2.79
2.500	80.92	3.02	3.73
1.250	70.12	3.45	4.91
0.625	54.70	4.28	7.83
0.313	36.59	4.70	12.86

Spiking Recovery: Three urine samples containing 0, 10 and 50 µg/mL of added albumin were assayed by the ALBUWELL ALTERNATE PROCEDURE.

ALBUMIN ADDED	OBSERVED	EXPECTED	% RECOVERY
0 µg/mL	12.26	-	-
10 µg/mL	22.37	22.26	100.49
50 µg/mL	67.76	62.26	108.83

Specificity: The antibody used in Albuwell is highly specific for human albumin. Cross-reactivity with albumin from other species and human proteins other than albumin was not apparent.

PROTEIN	CROSS-REACTIVITY
RAT ALBUMIN	<0.01%
BOVINE ALBUMIN	<0.01%
OVALBUMIN (CHICKEN)	NOT DETECTABLE
RABBIT ALBUMIN	<0.05%
HUMAN IgG	<0.01%

Interference: Several substances that may be found in human urine were tested in ALBUWELL. None of these interfered with binding in the assay, even at concentrations of 10 mg/mL, which are considerably higher than those possible in human samples.

SUBSTANCE	INTERFERENCE
UREA	NOT DETECTABLE
CREATININE	NOT DETECTABLE
GENTAMYCIN	NOT DETECTABLE

Kinetics: Incubation times can be varied, if necessary, in order to accommodate laboratory schedules. Standards were run in ALBUWELL using 15, 30 and 60 minute incubation times, giving superimposable results.

A total of 42 timed urine samples representing collection intervals of 2 - 8 hours were collected from normal volunteers at geographically separate sites and assayed with the ALBUWELL kit. The results, expressed as micrograms of albumin excreted per minute (µg/min), ranged from 1.68 to 16.14, with a mean of 4.28 µg/min. 95% had excretion rates less than 9.5 µg/min. When 24 hour urine specimens collected from the same individuals were assayed, all of the results were below 9µg/min. A total of 18 timed urine samples were collected from athletically active individuals for collection intervals of three to six hours immediately following periods of rigorous exercise. The results ranged from 2.6µg/min to 25.4 µg/min with a mean approximately 7.1 µg/min. The subjects of this study were between 22 and 42 years of age and were ambulatory, rather than at rest, both before and during the time of collection, and no attempt was made to regulate their water intake. Moreover, although all subjects were in apparent good health, they were not screened for proteinuria, or for a history of diabetes, hypertension or other conditions sometimes associated with increased microalbuminuria. Even so, the results of this preliminary study are in good agreement with published findings. The normal range limits suggested by this study should be regarded as guidelines only. Because of differences that may exist between laboratories and locales with respect to population, diet, laboratory technique and selection of reference groups, it is important for each laboratory to establish by similar means the appropriateness of adopting the normal ranges suggested by this study.

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