HUMAN COMPLEMENT C2 NL BINDARID™ RADIAL IMMUNODIFFUSION KIT

For *in vitro* diagnostic use only

Product Code: RN022.3

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1 INTENDED USE

This kit is intended for quantifying human C2 in serum as an aid in the diagnosis of complement deficiencies.

2 SUMMARY AND EXPLANATION

C2 is a β1-glycoprotein which forms part of the classical complement pathway. It is cleaved by activated C1s into two fragments, C2a and C2b. C2a, a serine protease, then combines with C4b to produce C3 or C5 convertase. Reduced C2 serum concentrations result from classical complement pathway activation, i.e. from immune complex mediated activation. C2 deficiency is the most common inherited complement component deficiency, and is associated with systemic lupus erythematosis, glomerulornephritis and vasculitis (ref. 1).

Radial immunodiffusion (RID) is a technique that is routinely used for measuring the concentration of various soluble antigens in biological fluids. It is principally derived from the work of Fahey & McKelvey (ref. 2) and Mancini *et al*. (refs. 3 & 4).

3 PRINCIPLE OF THE ASSAY

The method involves antigen diffusing radially from a cylindrical well through an agarose gel containing an appropriate mono-specific antibody. Antigen-antibody complexes are formed which, under the right condition, will form a precipitin ring. The ring size will increase until equilibrium is reached between the formation and breakdown of these complexes, this point being termed 'completion'. At this stage, a linear relationship exists between the square of the ring diameter and the antigen concentration. By measuring the ring diameters produced by a number of samples of known concentration, a calibration curve may be constructed. The concentration of the antigen in an unknown sample may then be determined by measuring the ring diameter produced by that sample and reading off the calibration curve.

There are three different procedures that may be used with this kit (see Section 8.4). Procedures ONE and TWO require that rings are measured at completion. A linear calibration curve is constructed for Procedure TWO, whereas for Procedure ONE a reference table (based upon the ideal linear calibration curve) is provided, which converts ring diameters directly to protein concentrations. Using Procedure THREE, ring diameters are measured before completion; the calibration curve produced will be non-linear.

4 REAGENTS

4.1 RID plates (supplied in foil pouches). These contain monospecific antibody to C2 in agarose gel. Up to fourteen samples can be run per plate (including calibrators). Preservatives: 0.099% sodium azide, 0.1% E-amino-n-caproic acid (EACA), 0.01% benzamidine.

4.2 Calibrator. This is supplied lyophilised. The concentration of C2 is given on the vial label. Preservatives: 0.099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.3 7% Bovine Serum Albumin (BSA) solution. This is supplied in stabilised liquid form and is included for use as a diluent. Preservatives: 0.099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.4 Control. This is supplied lyophilised. The expected C2 concentration is marked on the vial label. Preservatives: 0.099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.5 Distilled water. For reconstituting the lyophilised calibrator and control. Preservative: 0.099% sodium azide.

5 CAUTION

All donors of human serum supplied in this kit have been serum tested and found negative for hepatitis B surface antigen (HBsAg) and antibodies to human immunodeficiency virus (HIV1 and HIV2) and hepatitis C virus. The assays used were either approved by the FDA (USA) or cleared for *in vitro* diagnostic use in the EU (Directive 98/79/EC, Annex II); however, these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material including (but not limited to) users wearing suitable protective equipment and clothing at all times. Only personnel fully trained in such methods should be permitted to perform these procedures.

WARNING: This product contains sodium azide and must be handled with caution; suitable gloves and other protective clothing should be worn at all times when handling this product. Do not ingest or allow contact with the skin (particularly broken skin or open wounds) or mucous membranes. If contact does occur wash with a large volume of water and seek urgent medical advice. Explosive metal azides may be formed on prolonged contact of sodium azide with lead and copper plumbing; on disposal of reagent, flush with a large volume of water to prevent azide build up.

This product should only be used by suitably trained personnel for the purposes stated in the Intended Use. Strict adherence to these instructions is essential at all times.

Reagents from different batch numbers of kits are **NOT** interchangeable. If large numbers of tests are performed care should be taken to ensure that all the reagents are from the same batch.

6 STORAGE AND STABILITY

The unopened kits should be stored at 2-8°C and can be used until the expiry date given on the kit box label. DO NOT FREEZE. The expiry dates of individual components are given on the component labels. RID plates should be stored at 2-8°C and are damaged by temperature extremes. Freezing will destroy the gel; therefore RID plates should be kept away from cooling elements in refrigerators. High temperatures should also be avoided as this will result in moisture loss from the gel, affecting performance. Unopened plates should be stored flat and upside down (pouch label uppermost) to prevent condensation accumulating in the wells. Handle plates with care to prevent gel damage.

Unopened calibrators and controls should be stored at 2-8°C. Once reconstituted they are stable for at least one week at 2-8°C, but for longer storage they should be aliquoted and frozen (-20°C or below) (do not store in self-defrosting freezer). All other reagents should be stored at 2-8°C.

7 SPECIMEN COLLECTION AND PREPARATION

Use fresh or deep frozen (-20°C or below) serum samples. Microbially contaminated, haemolysed and very lipaemic serum and samples containing particulate matter should not be used. Blood samples should be collected by venepuncture, allowed to clot naturally and the serum separated as soon as possible to prevent haemolysis. The serum may be stored at 2-8°C for up to 48 hours prior to assay, or for prolonged storage, aliquoted and kept at -20°C or below. Repeated freezing and thawing should be avoided.

The BSA included in the kit should be used as diluent when required, as this will maintain the viscosity of the material. Results can therefore be accurately compared with the calibrator which has a similar viscosity to normal serum.

8 METHODOLOGY

A summary of the entire procedure is given at the end of this instruction leaflet.

8.1 Contents

8.1.1 3 x *Human Complement C2 NL Bindarid* (radial immunodiffusion plates in foil pouches)
8.1.2 8 x *Gel Dividers*
8.1.3 1 x *Human C2 NL Calibrator (*lyophilised)
8.1.4 1 x 5mL *7% BSA Solution*
8.1.5 1 x *Human C2 Control Serum* (lyophilised)
8.1.6 1 x 5mL *Distilled water*
8.1.7 1 x instruction leaflet, including RID reference table

8.2 Materials required but not provided

8.2.1 Equipment for collection and preparation of test samples, e.g. sample tubes, centrifuge etc.
8.2.2 Pipettes for accurate reconstitution of calibrators and control and dilution of samples.
8.2.3 Micropipettes for sample application. These should be capable of accurately delivering 10µL volumes. Binding Site Micropipettes (code AD041) or 'Hamilton' syringes are recommended.
8.2.4 Jeweller's Eyepiece (code AD040) or Digital RID Plate Reader (AD400) for magnifying and accurately measuring the precipitin ring diameters to 0.1mm.
8.2.5 Graph paper.

8.3 Reagent preparation

8.3.1 RID Plate(s)

To avoid contamination of the gel, plates should be used in a dust-free environment. Take the plate from the foil pouch and remove the lid. If condensation is visible the plate should be kept upside down until the lid has been removed to prevent droplets falling onto the gel. Check the plate to ensure that no damage has occurred in storage or transit, e.g. splits in the gel. Leave the plate open for 10-15 minutes (or longer if necessary) at room temperature to allow any condensation in the wells or on the gel surface to evaporate. Samples should never be applied to wells in which moisture is still visible.

Plate partitioning: The plates may be partitioned into up to four sections using the gel dividers provided prior to use. Each divider should be positioned carefully on the gel, cutting edge downward, with the stabilising arm resting on the central plate label. Press firmly on the arm to cut the gel and leave in position.

Plate partitioning is recommended if only part of the plate is to be used initially or when measuring suspected high concentration samples which could (by diffusing over a wide area) result in antibody depletion occurring elsewhere on the plate. After initial use, partitioned plates should be resealed in their foil pouches and stored at 2-8°C with the gel divider(s) in place. Store partitioned plates right side up and use within four weeks.

8.3.2 Calibrator

The lyophilised calibrator should be reconstituted with the volume of the distilled water indicated on the vial labels – use the distilled water provided in the kit. Before use, all material in the bottle, including any adhering to the bung must be completely dissolved (by inversion) over a minimum period of thirty minutes. The calibrator is pre-diluted and should be applied to the plates neat, mixing gently immediately before use. Dilutions of the calibrator must be made if a calibration curve is required (as for Procedures TWO and THREE). These dilutions should normally be a medium dilution (60%, i.e. 6 parts in 10) and a low dilution (20%, i.e. 2 parts in 10). It is recommended that 120µL of calibrator is mixed with 80µL of the diluent provided (7%BSA) for a 60% dilution and 40µL calibrator is mixed with 160µL of the diluent for a 20% dilution.

8.3.3 Control

The C2 lyophilised control serum should be reconstituted with the volume of distilled water indicated on the vial label. It should be mixed gently by inversion until the contents are completely dissolved. It should then be applied to the plate(s) neat.

8.3.4 Sample

Samples should be applied neat to the plates. Mix gently before use. If samples containing high levels of C2 concentrations are to be measured, a dilution will be necessary. In such cases it is suggested that to obtain adequate accuracy a minimum volume of 10µL of test

sample is mixed with the appropriate volume of BSA. For samples having C2 concentrations below the detection limit of the plates one of the following is recommended:

i) Concentrate the sample
ii) Make a double fill of the well (see Section 8.5)
iii) Procedures

8.4.1 Procedure ONE: RID reference table

This method does **not** require the construction of a calibration curve – sample concentrations corresponding to each ring diameter are read directly off the RID reference table. Rings must be allowed to develop to completion which will require a minimum diffusion time of 120 hours. The neat calibrator should be run on each plate used to ensure all are performing correctly.

8.4.2 Procedure TWO: Calibration curve at completion

In this method, the neat calibrator plus the two dilutions are used to produce a linear calibration curve. Rings must be allowed to develop to completion which will require a minimum diffusion time of 120 hours. To conserve wells, one calibration curve can be used concurrently for several plates of the same batch. In such cases, the neat calibrator should be run on each plate used to ensure all are performing correctly.

8.4.3 Procedure THREE: Calibration curve prior to completion

In this method, the neat calibrator plus the two dilutions are used to produce a calibration curve which is non-linear, as the rings are measured before completion. The minimum recommended diffusion time is 18 hours. It is advisable that a separate calibration curve is constructed for each plate used.

8.5 Application of calibrators and samples

The calibrator (including the two dilutions if required), control and test samples should be mixed gently immediately before use. Fill the required number of wells with 10µL of the high calibrator using a micropipette. If Procedure TWO or THREE is being followed fill the required number of wells with the medium and low calibrators as well. The remaining wells should then be filled with 10µL test samples and controls. Plates should not be left open for long periods during calibrator / test sample application, as this will cause excessive drying of the gel.

Note: For those samples suspected of containing low concentrations of C2, a 'double fill' of the well may be made. The well is initially filled with 10µL of the sample and this is allowed to completely diffuse into the gel, which can take up to 30 minutes. The lid should be kept in place during this period. The second fill (again using 10µL) may then be made, and the plate incubated as normal. Results obtained must be corrected for the double sample volume and will be less accurate than those obtained by the normal 'single fill' procedure.

8.6 Incubation

After sample application, the lid is tightly closed and the plate stored flat at room temperature (approximately 20-24°C). It is essential that the gel is not allowed to dry out during incubation. To minimise evaporation, it is suggested that plates should either be resealed in their foil pouches or stored in a moist box (a sealed plastic box containing damp tissue paper) during incubation. The minimum incubation time for Procedure THREE is 18 hours and for complete diffusion (Procedures ONE and TWO) is 120 hours. Final ring diameters may be affected by temperature; the expected ring size for the high calibrator is 8mm (±0.3mm) when incubated at 20-24°C. Extremes of temperature should be avoided.

8.7 Quality control

The control serum should be treated exactly like a test sample. Values obtained for the control should be within ±10% of the concentration stated on the vial label.

9 RING MEASUREMENT AND RESULT PROCESSING

After the required diffusion time, ring diameters should be measured to the nearest 0.1mm, using a jewellers' eyepiece or digital RID plate reader. When reading with an eyepiece, use bright side lighting and a dark background. If difficulties are experienced, view the plate macroscopically and mark the edges of the rings on the back of the plate using a needle. The distance between these marks may then be more easily measured.

Note: For Procedures ONE and TWO ring diameters must have developed to completion. If there is any doubt, rings should be remeasured after a further 24 hours to ensure there has been no increase in their diameters. The neat calibrator should give a ring diameter of 8.0mm ± 0.3mm at completion. If the ring diameter is outside this range, see TROUBLE SHOOTING (Section 10.2).
A faint outer precipitin ring may be visible with some samples; this does not adversely affect the quantification of C2 which should be determined by measurement of the inner, stronger ring.

Procedure ONE

The concentration of the C2 in each test sample can be read directly from the RID reference table.

Concentrations obtained for samples giving ring diameters greater than the neat calibrator should be regarded as approximate, due to the possibility of incomplete diffusion; they may also cause local antibody depletion thereby affecting adjacent ring sizes. Such samples should preferably be diluted appropriately and retested. Samples giving ring diameters below the lower limit on the RID reference table should be retested in a more concentrated form (see Section 8.3.4).

Example:

Test sample	Dilution	Ring diameter (mm)	Table value (mg/L)	Original sample conc. (mg/L)
C2 Serum A	Neat	6.4	17.9	17.9
C2 Serum B	Neat	>10	>64.2	>64.2
C2 Serum B (Repeat)	1/2	7.7	32.3	64.6*

*Calculated as follows: Table value x Recommended diln/Actual diln, i.e. 32.3mg/L x (1)/(1/2).

Procedure TWO

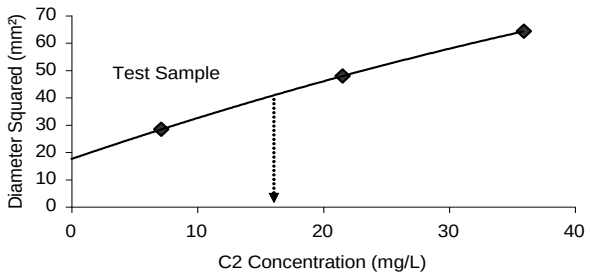
Plot the square of the diameters of the precipitin rings formed by the neat calibrator plus the two dilutions, versus their C2 concentrations (given on the calibrator vial label). C2 concentrations should be along the horizontal (x) axis, ring diameters squared along the vertical (y) axis. A line of best fit is drawn through the three points; the y-intercept should be in range 17-22mm^2. The C2 concentration is determined from the calibration curve; remember to adjust the sample concentration obtained by any dilution factor used.

Sample calculation:

C2 calibrators (i.e. the neat calibrator plus the two dilutions) gave the following ring diameters on a C2 test plate at completion:

Calibrator	Conc. (mg/L)	Diameter (D) of ring (mm)	D squared (mm^2)
Neat	36	8.0	64.00
60%	21.6	6.9	47.61
20%	7.2	5.3	28.09

A calibration curve was plotted using these results:



An unknown sample, applied neat as recommended, gave a 6.3mm diameter ring on this plate. From the above curve, this corresponds to a C2 concentration of 16.1mg/L.

Procedure THREE

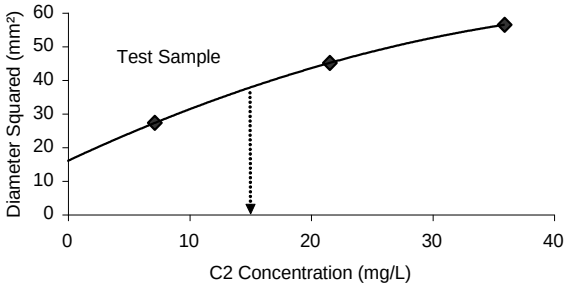
Plot the calibration curve as for procedure TWO. The graph will not be a straight line but a curve, the gradient of which decreases with increasing protein concentration. The y-intercept should be as indicated for Procedure TWO. Test sample protein concentrations are read off the calibration curve.

Sample calculation:

C2 calibrators F (i.e. neat calibrator plus the two dilutions) gave the following ring diameters on a C2 plate after 18 hours:

Calibrator	Conc. (mg/L)	Diameter (D) of ring (mm)	D squared (mm^2)
Neat	36	7.5	56.3
60%	21.6	6.7	44.9
20%	7.2	5.2	27.0

A calibration curve was plotted using these results:



An unknown sample, applied neat as recommended, gave a 6.1mm ring on this plate. From the above curve, this corresponds to a C2 concentration of 14.9mg/L.

10 LIMITATIONS OF PROCEDURE

10.1 For Procedure ONE, results generated from ring diameters greater than the neat calibrator ring diameter (i.e. 8mm) should be regarded as approximate (see Section 9). For Procedure TWO and THREE, accurate results are limited to the calibration curve between the high and low calibrator values – extrapolation beyond these points is not valid. Samples giving results outside these ranges must be diluted or concentrated as appropriate and retested (see Section 8.3.4).

10.2 TROUBLE SHOOTING

Problem	Possible Causes(s)	Suggested Action(s)
A. No ring for:		
1. Calibrator(s)	Calibrator omitted.	Repeat assay.
2. Test sample	i) Sample omitted.	Repeat assay.
	ii) Concentration too high/low	Dilute/concentrate and reassay.
3. Calibrator(s) and test samples	Plate deterioration.	a) Storage damage. Repeat assay using new plate.
		b) Product expired. Repeat assay using new plate/kit.
B. Oversize rings for:		
1. Neat calibrator (more than 8.3mm)	i) Inaccurate ring measurement.	Remeasure using eyepiece or digital RID plate reader.
	ii) Incorrect volume applied.	Check 10µL volume applied.
	iii) Inaccurate volume applied.	a) Micropipette malfunction – check operation and repeat assay.

Problem	Possible Causes(s)	Suggested Action(s)
		b) Poor technique – repeat assay.
	iv) Inaccurate calibrator reconstitution.	a) Pipette malfunction – check operation and calibration, then repeat assay using new calibrator.
		b) Poor technique – repeat assay using new calibrator.
	v) Partial evaporation of reconstituted calibrator on storage.	Repeat assay using new calibrator/kit.
	vi) Plate deterioration.	a) Storage damage. Repeat assay using new plate.
		b) Product expired. Repeat assay using new kit.
	vii) Local antibody depletion due to adjacent high concentration test samples.	Dilute the sample(s) responsible and repeat assay using new plate.
	viii) Incubation temperature too high (see Section 8.6).	Repeat assay, incubating at 20-24°C.
2. Test samples (above acceptable range - see section 10.1)	i) Concentration too high.	Dilute and reassay.
	ii) Incorrect volumes applied.	Check 10µL volume applied.
C. Undersized rings for:-		
1. Neat calibrator (less than 7.7mm)	i) Inaccurate ring measurement.	As for B1 above
	ii) Incorrect volume applied.	
	iii) Inaccurate volume applied.	
	iv) Inaccurate calibrator reconstitution.	
	v) Calibrator deterioration.	a) Storage damage. Repeat assay using new calibrator.
		b) Product expired. Repeat assay using new kit.
	vi) Incubation temperature too low (see Section 8.6).	Repeat assay, incubating at 20-24°C.
2. Test samples (below acceptable range – see Section 10.1)	i) Concentration too low.	See section 8.3.4 and repeat assay.
	ii) Incorrect volume applied.	Check 10µL volume applied.
D. Double/Multiple rings	i) Non-specific precipitation close to well (due to PEG in gel).	Read outer ring.
	ii) Poor sample application.	Repeat assay.
	iii) Calibrator deterioration.	a) Storage damage. Repeat assay using new calibrator.
		b) Product expired. Repeat assay using new kit.
	iv) Sample deterioration.	Reassay using fresh sample.
	v) Faint outer ring.	Read inner stronger ring. See Section 9.
E. Non-circular rings	i) Poor sample application.	Repeat assay.
	ii) Gel dried out before use.	a) Storage damage. Repeat assay using new plate.
		b) Product expired. Repeat assay using new plate/kit.
	iii) Gel dried out during sample application or incubation.	Repeat assay minimising the time the plate is left open. Incubate with lid on tight in a moist box or sealed foil pouch.
	iv) Local antibody depletion (due to high concentration samples on the plate).	Dilute samples and repeat assay.
F. Cloudy gel	i) Plate has been frozen.	Repeat assay using new plates. Review storage.
	ii) Gel dried out before use.	As for E(ii) above.
	iii) Gel dried out during sample application or incubation.	As for E(iii) above.
G. Weak, pitted gel	Plate has been frozen.	Repeat using new plate. Review storage.
H. Poor calibration curve:		
1. Curve non-linear (Procedure TWO)	i) Incomplete diffusion.	Incubate for further 24 hours and remeasure the rings.
	ii) Calibrator rings under/oversize.	As for B1 or C1 above. (Similar explanations apply to the medium and low calibrators.)
	iii) Calibration curve constructed incorrectly.	Check calibration curve construction.
2. y-intercept out of range (Section 9)	i) Calibrator rings under/oversize.	As for B1 or C1 above. (Similar explanations apply to the medium and low calibrators.)
	ii) Calibration curve constructed incorrectly.	Check calibration curve construction.

10.3 Diagnosis cannot be made and treatment must not be initiated on the basis of C2 measurements alone. Clinical history and other laboratory findings must be taken into account.

10.4 If an unexpected result is obtained, the assay should be repeated, preferably with a fresh sample.

If a problem cannot be resolved, please refer to supplier.

11 EXPECTED VALUES

The following C2 serum concentrations results were obtained using this kit using individual blood donors.

	Mean (mg/L)	SD (n-1)	Median (m)	95 Percentile range	No of samples
C2	19.61	3.33	19.2	14 - 25	80

The data provided above has been obtained from limited numbers of British blood donors and is intended for guidance purposes only. It is strongly recommended that each user should generate his/her own C2 concentration ranges for appropriate clinical conditions.

12 PERFORMANCE CHARACTERISTICS

12.1 Precision

The precision (repeatability) of this kit is expressed as the mean and the percentage coefficient of variation (CV) which had been determined using human serum preparation containing high, medium and low concentrations of C2. All analyses were performed in our laboratory. Each value was calculated from 10 measurements (duplicate determinations on five separate plates from a typical batch) unless otherwise stated. For Procedures ONE and TWO, rings were measured after 120 hours. For Procedure THREE, rings were read after 18 hours.

SAMPLE POOL C2	Procedure 1		Procedure 2		Procedure 3	
	Mean conc. mg/L	CV	Mean conc. mg/L	CV	Mean conc. mg/L	CV
High	34.47	2.14	35.06	2.72	30.98	5.43
Medium	24.3	3.71	23.48	4.42	21.1	4.69
Low	14.6	4.57	12.48	6.68	13.16	5.59

12.2 Within-plate and inter-batch variation

The within-plate variation is expressed as the mean ± standard deviation of determinations of CV made using 5 plates from separate batches. Six measurements were made per plate, using a human serum pool as the sample.

The inter-batch variation is expressed as the CV of mean diameter values obtained for a human serum pool sample using 5 recent batches of plates. The mean diameter for each batch was determined from six ring measurements per plate, one plate per batch.

	Within-plate variation	Inter-batch variation
C2	Mean CV% ± SD	CV (%)
	0.86 ± 0.41 (N=5)	1.37 (N=5)

13 BIBLIOGRAPHY

13.1 PRU Handbook (1999), 6th Ed, Milford Ward, A *et al* (ed), PRU Publications, Sheffield, UK.

13.2 Fahey, JL & McKelvey, EM (1965). Quantitative determination of serum immunoglobulins in antibody-agar plates. J. Immunol,. **94**, 84-90.

13.3 Mancini, G, Vaerman, J P *et al.* (1964). Protides of the biological fluids (XI Colloquiumi). Peters H. (ed), Amsterdam, Elsevier Publishing Co., p 370.

13.4 Mancini, G, Carbonara, A O *et al* (1965). Immunochemical quantification of antigens by single radial immunodiffusion. Immunochem. **2**, 235-254.

14 SUMMARY OF PROCEDURE

14.1 Select Procedure ONE, TWO or THREE. Procedure THREE must be used if results are required quickly.

14.2 Reconstitute calibrator and control with the distilled water provided.

14.3 Prepare calibrator dilutions (required for Procedures TWO and THREE).

14.4 Allow condensation to evaporate from RID plate(s).

14.5 Apply calibrator(s), control and samples to RID plate(s) in 10µL volumes.

14.6 Replace lid and incubate at room temperature (approximately 20-24°C) for fixed time period (minimum 18 hours) (Procedure THREE) or until rings are complete (minimum 120 hours) (Procedure ONE and TWO).

14.7 Measure the ring diameters.

14.8 Read results off RID Reference Table (Procedure ONE) or plot calibration curve and read off results (Procedures TWO and THREE).

15 RID REFERENCE TABLE

RID reference table for Human C2
Concentrations in mg/L

Diameter of ring (mm)	Conc.
4.5	1.72
4.6	2.43
4.7	3.16
4.8	3.91
4.9	4.67
5.0	5.44
5.1	6.23
5.2	7.04
5.3	7.86
5.4	8.70
5.5	9.55
5.6	10.4
5.7	11.3
5.8	12.2
5.9	13.1
6.0	14.1
6.1	15.0
6.2	16.0
6.3	17.0
6.4	17.9
6.5	19.0
6.6	20.0
6.7	21.0
6.8	22.1
6.9	23.2
7.0	24.2
7.1	25.4
7.2	26.5
7.3	27.6
7.4	28.8
7.5	29.9
7.6	31.1
7.7	32.3
7.8	33.5
7.9	34.7
8.0	36.0
8.1	37.3
8.2	38.5
8.3	39.8
8.4	41.1
8.5	42.5
8.6	43.8
8.7	45.2
8.8	46.5
8.9	47.9
9.0	49.3
9.1	50.7
9.2	52.2
9.3	53.6
9.4	55.1
9.5	56.6
9.6	58.1
9.7	59.6
9.8	61.1
9.9	62.6
10.0	64.2

Note: The above values assume that test samples are applied undiluted in 10µL volumes. The neat calibrator should give a ring diameter of 8.0 ± 0.3mm at completion when incubated at 20-24°C.