Radial Immunodiffusion Kits Protocol - *For Research Use Only*

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Contents

1. Intended Use

This product is intended for measuring the specific protein in biological fluids, as described in the Specific Instruction Sheets.

2. Summary and Explanation

Radial immunodiffusion is a technique that is routinely used for measuring the concentrations of various soluble antigens (usually proteins) in biological fluids. It is principally derived from the work of Mancini *et al.* (refs. 1, 2) and Fahey and McKelvey (ref. 3).

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 conditions, 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 Binding Site RID products (see Section 8.4). Procedures ONE and TWO require that the 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 These contain monospecific antibody in an agarose gel. Certain plates contain more than one specific antibody - see Specific Instruction Sheets. Up to fourteen samples can be run per plate (including calibrator(s)). Preservatives: 0.099% sodium azide, 0.1% E-amino-n-caproic acid (EACA), 0.01% thiomersal (sodium ethylmercurithiosalicylate) (Nanorid™ plates only), 0.01% benzamidine.

4.2 Calibrator(s) Supplied either as a single calibrator or as a set of three, containing comparatively high, medium and low concentrations of the specific protein. These are provided either lyophilised or in liquid form. The specific protein concentration given on the vial label has been obtained by comparison with national and international standards, where available. Calibrators are manufactured to be physically similar to the intended sample fluid, e.g. serum, urine etc. Preservatives: 0.099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.3 Control (not included in all kits). Supplied either lyophilised or in liquid form. Preservatives: 0099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.4 Diluent (e.g. 7% bovine serum albumin, 1% sheep albumin etc.) Supplied in liquid form for diluting test samples and for preparing calibrator dilutions. Preservatives: 0.099% sodium azide, 0.1% EACA, 0.01% benzamidine.

4.5 Distilled water For reconstitution of lyophilised calibrators and/or controls. Preservatives: 0.099% sodium azide.

5. Caution

All donors of human serum or plasma supplied in this kit have been 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. NANORID plates also contain 0.01% thiomersal as a preservative (see Section 4). Appropriate precautions should therefore be observed.

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.

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 opened or 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). All other reagents should be stored at 2-8°C.

7. Specimen Collection and Preparation

Use appropriate fresh or deep frozen serum samples - see Specific Instruction Sheets. If sample dilution is recommended or required the diluent included in the kit should be used to maintain sample viscosity. A slight loss of accuracy may be experienced if samples of high or low viscosity (compared with the calibrators) are used on RID plates (ref. 4).

8. Methodology

A summary is provided at the end of the insert.

8.1 Materials provided - see Specific Instruction Sheets

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 lyophilised calibrators and dilutions of samples etc.

8.2.3 Micropipettes for calibrator and sample application. These should be capable of accurately delivering volumes in the range 5-20µL. Binding Site Micropipettes (code AD041) or 'Hamilton' syringes are recommended.

8.2.4 Jeweller's Eyepiece (Code AD040) or Digital RID Reader (code 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. Note: 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(s)

Lyophilised calibrators should be reconstituted with the volume of distilled water indicated on the vial label (use the distilled water provided in the kit). Before use, all material in the vial, including any adhering to the bung, must be completely dissolved (by inversion) over a minimum period of thirty minutes. All calibrators are prediluted and should be applied neat. If only a single calibrator is provided, dilutions of this must be made using the diluent provided if a calibration curve is required (as for Procedures TWO and THREE). These dilutions should normally be a medium dilution (60%) and a low dilution (10%). It is recommended that 120µL of calibrator is mixed with 80µL of the diluent for a 60% dilution, and 25µL of calibrator is mixed with 225µL of the diluent for a 10% dilution.

8.3.3 Control/test sample(s)

Control samples, where provided, should be treated like test samples. (Lyophilised controls should initially be reconstituted with the volume of distilled water indicated on the vial label). Dilutions (using the diluent provided) should normally only be made if recommended in the Specific Instructions Sheets. However, test samples known or thought to contain abnormally high concentrations of the specific protein should be diluted appropriately. To obtain adequate accuracy, the minimum recommended sample volume for dilution is 20µL. For test samples having a known or suspected low concentration of the specific protein one of the following is recommended:

i) Apply sample in a more concentrated form (e.g. for a specificity with a recommended sample dilution of 1/10, a low concentration sample can be applied undiluted, effectively increasing the concentration of the specific protein 10-fold).

ii) Concentrate the sample.

iii) Make a double fill of the well (see section 8.5).

iv) Use a kit with a lower measuring range if available (see Catalogue).

8.4 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. Minimum recommended diffusion times for completion are given in the Specific Instruction Sheets. The neat/high 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 three calibrators (or the neat calibrator plus two dilutions, see Section 8.3.2) are used to produce a linear calibration curve. Rings must be allowed to develop to completion. Minimum recommended diffusion times for completion are given in the Specific Instruction Sheets. To conserve wells, one calibration curve can be used for several plates of the same batch used concurrently. In such cases, the neat/high 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 three calibrators (or the neat calibrator plus two dilutions, see Section 8.3.2) are used to produce a calibration curve which is non-linear, as the rings are measured before completion. Minimum recommended diffusion times are given in the Specific Instruction Sheets. It is advisable that a separate calibration curve be constructed for each plate used.

8.5 Application of calibrators and samples

All calibrators, controls and test samples should be gently mixed immediately before use. Fill the required number of wells with the recommended volume of the neat/high calibrator using a micropipette. If Procedure TWO or THREE is being followed, also fill the required number of wells with the medium and low calibrators (or the medium and low dilutions of the neat calibrator). The remaining wells should then be filled with the recommended volume of appropriately diluted 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 the specific protein, a 'double fill' of the well may be made. The well is initially filled with the recommended volume 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 the recommended volume) 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 with the lid uppermost, at room temperature (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 and for complete diffusion (Procedures ONE and TWO) are given in the Specific Instruction Sheets. Final ring diameters may be affected by temperature; the expected ring size given for neat/high calibrators in the Specific Instruction Sheets refer to incubations carried out at 20-24°C. Extremes of temperature should be avoided.

8.7 Quality control

A control (if included) should be treated exactly like a test sample. Values obtained for the control should be within 10% of the concentration stated on the vial.

9. Ring Measurement and Result Processing

After the required diffusion time, ring diameters should be measured to the nearest 0.1mm, using a jeweller's eyepiece or a RID reader. When reading with an eyepiece, use bright side lighting and a dark background. The rings of some specificities are faint. If difficulties are experienced in measuring these, 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 measured more easily.

Note: For Procedures ONE and TWO ring diameters must have developed to completion. If there is any doubt, rings should be re-measured after a further 24 hours to ensure there has been no increase in their diameters.

Procedure ONE

Read the sample concentrations directly from the RID Reference Table. The neat/high calibrator should give the expected ring diameter given in the Specific Instruction Sheet (±0.3mm). If the ring diameter is outside this range, see Trouble Shooting (Section 10.3). Concentrations obtained for samples giving ring diameters greater than the neat/high 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 8.3.3). **Use of the RID Reference Table assumes the test samples have been diluted as recommended in the Specific Instruction Sheet. Any changes from this must be taken into account and results adjusted accordingly.**

Note: The table value corresponding to the expected neat/high calibrator ring diameter takes into account any dilution factor recommended for the test samples.

Procedure TWO

Plot the square of the diameters of the precipitin rings formed by the three calibrators (or the neat calibrator plus two dilutions) versus their specific protein concentration (given on the calibrator vial label). Antigen concentrations should be along the horizontal (x) axis, ring diameters squared along the (y) axis. A line of best fit is drawn through the three points; the y-intercept should be as indicated below:

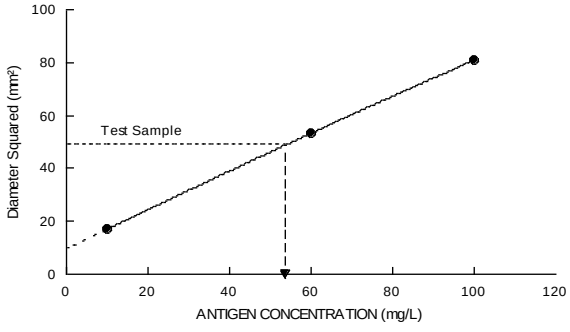
Recommended sample volume (µL)	Well diameter (mm)	y-Intercept (mm^2)
5	2.5	10-12
10	3.5	17-23
20	4.0	25-30

If the y-intercept is outside the given range, refer to Trouble Shooting (Section 10.3).

The neat/high calibrator should give the expected ring diameter quoted in the Specific Instruction Sheets (±0.3mm). If the ring diameter is outside this range see Trouble Shooting (Section 10.3).

Test sample protein concentration is read off the calibration curve; remember to adjust the sample concentration obtained by any dilution factor used.

Calibration curve example: 5µL calibrator volume, 2.5mm well diameter, high calibrator specific protein concentration = 100mg/L, giving a 9mm ring diameter at completion.

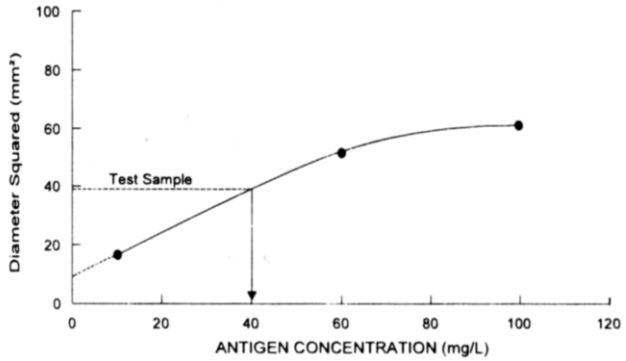


A test sample applied at the recommended sample dilution of 1/10 gave a ring diameter of 7.0mm. From the above graph this corresponds to a specific protein concentration of 53mg/L. Therefore the specific protein concentration in the original sample = 53 x 10 = 530mg/L.

Procedure THREE

Plot the calibration curve as for Procedure TWO. This will be non-linear, the gradient of the line decreasing with increasing protein concentration. The y-intercept should be as indicated for Procedure TWO. Test sample protein concentrations are read off the calibration curve; remember to adjust the sample the sample concentration obtained by any dilution factor used.

Calibration curve example: 5µL calibrator volume, 2.5mm well diameter, high calibrator specific protein concentration = 100mg/L, giving a 9mm ring diameter at completion. Recommended minimum diffusion time = 18h.



A test sample applied at the recommended dilution of 1/10 gave a ring diameter of 6.1mm after 18 hours. From the above graph this corresponds to a specific protein concentration of 40mg/L. Therefore the specific concentration in the original sample = 40 x 10 = 400mg/L.

10. Limitations of Procedure

10.1 For Procedure ONE, For Procedure ONE, results obtained from ring diameters greater than the neat/high calibrator should be regarded as approximate (See Section 9). For Procedures TWO and THREE, accurate results are limited to the calibration curve between the neat/high calibrator and the low calibrator/low dilution 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 8.3.3).

10.2 Every RID plate specificity has been shown to give positive results with a range of appropriate samples. However, it is not possible to exclude the occasional phenotype or allotype with unusual determinants (particularly monoclonal proteins) that may be undetected by this technique.

10.3 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	i) Plate deterioration.	a. Storage damage. Repeat assay using new plate.
		b. Product expired.
	ii) Poor lighting when reading.	Ensure bright side lighting and dark background when using eyepiece.
B. Oversize rings for		
1. Neat/high calibrator (more than 0.3mm above quoted size at completion)	i) Inaccurate ring measurement.	Remeasure using eyepiece or RID Plate Reader.
	ii) Incorrect volume applied.	Check recommended volume applied (see Specific Instruction Sheet).
	iii) Inaccurate volume applied.	a. Micropipette malfunction – check operation and repeat assay.
		b. Poor technique – repeat assay.
	iv) Inaccurate calibrator reconstitution (if lyophilised).	a. Pipette malfunction - check operation and calibration then repeat assay using new calibrator.
		b. Poor technique - repeat 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 plate/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 recommended volume applied (see Specific Instruction Sheets.

Problem	Possible Causes(s)	Suggested Action(s)
C. Undersized rings for		
1. Neat/high calibrator (more than 0.3mm below quoted size at completion).	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 calibrator/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 8.3.3) and repeat assay.
	ii) Incorrect volume applied.	Check recommended volume applied (see Specific Instruction Sheet).
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) Allotypes in sample.	Read obvious ring.
	iv) Calibrator deterioration.	a. Storage damage. Repeat assay using new calibrator.
		b. Product expired. Repeat assay using new calibrator/kit.
	v) Sample deterioration.	Reassay using fresh sample.

Problem	Possible Causes(s)	Suggested Action(s)
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 adjacent high concentration test samples).	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 re-measure the rings.
	ii) Calibrator rings under/oversize.	As for B1 or C1 above. (Similar explanations apply to the medium and low calibrator dilutions).
	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 calibrator dilutions).
	ii) Calibration curve constructed incorrectly.	Check calibration curve construction.

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

11. Bibliography

1. Mancini, G, Vaerman, JP *et al.* (1964). Protides of the biological fluids (XI Colloquium). Peters H. (ed), Amsterdam, Elsevier Publishing Co., p370-373.
2. Mancini, G, Carbonara, AO *et al.* (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem. **2**, 235-254.
3. Fahey, J L & McKelvey, E M (1965). Quantitative determination of serum immunoglobulins in antibody-agar plates. J. Immunol., **94**, 84-90.
4. Scott BJ & Burnett D (1978). The effect on the protein content of diluents on peak height in rocket electrophoresis. Clin. Chim. Acta **89**, 475-478.

12. Summary of Procedure

12.1 Select Procedure ONE, TWO or THREE.

12.2 Reconstitute calibrator(s) (if lyophilised) and prepare dilutions (if required).

12.3 Dilute samples and controls (if recommended or required).

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

12.5 Apply calibrator(s), controls and samples to RID plate(s).

12.6 Replace lid(s) and incubate at room temperature (20-24°C) for the minimum recommended diffusion time (Procedure THREE) or until rings are complete (Procedures ONE and TWO).

12.7 Measure the ring diameters.

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