

Comparison of two commercially available single radial immunodiffusion kits for quantitation of llama immunoglobulin G

Jennifer M. Hutchison, Mowafak D. Salman, Franklyn B. Garry, LaRue W. Johnson, James K. Collins, Thomas J. Keefe

Abstract. Immunoglobulin G (IgG) quantitation was performed by a commercially available single radial immunodiffusion kit on 528 plasma samples obtained from llamas. Fifty samples encompassing the range of values obtained were selected for further evaluation. The IgG concentration of these samples was measured by a second commercially available single radial immunodiffusion kit and a sodium sulfite precipitation kit. γ -Globulins also were measured. IgG values predicted by the single radial immunodiffusion kits were not in agreement. Failure to appreciate this lack of agreement could result in misinterpretation of a llama's IgG level derived from one kit when clinical guidelines established using the other kit are followed.

Circulating immunoglobulin G (IgG) concentration is commonly measured to help evaluate the immune system. In species for which passive transfer of Igs is important, serum or plasma IgG concentration assesses passive Ig transfer during the early neonatal period. In animals sufficiently mature for endogenous IgG production, serum or plasma IgG concentration provides information about humoral immune function. Low serum IgG concentration is one of several characteristics of an immunodeficiency syndrome described in juvenile llamas (juvenile llama immunodeficiency syndrome, JLIDS).¹⁰ Single radial immunodiffusion measurement of IgG using a commercially available kit⁴ in conjunction with other testing currently is used as a screening test for JLIDS.

IgG concentration can be estimated by techniques^{1,4,12,15,17} including total protein and γ -globulin measurement, zinc sulfate turbidity, sodium sulfite precipitation, latex agglutination, glutaraldehyde coagulation, enzyme-linked immunosorbent assay (membrane filter or dipstick), immunoturbidimetry, and single radial immunodiffusion. Common to most of these methods is comparison of measured values with a reference standard. Sources of inaccuracy or incongruity between methods include laboratory error, differences in calibration of standards, or true method-related differences. Lack of agreement between methods complicates interpretation of a patient's status, particularly if standards or cut-off points for interpre-

tation have been established using an alternative test method.

Previous studies have evaluated the performance of tests to quantitate equine^{1,4,12,17} or bovine¹⁵ IgG. Single radial immunodiffusion measurement has frequently been chosen as the basis for comparison. For some studies^{15,17} SRID kits were developed in the laboratory and standards prepared and calibrated against commercially available, purified bovine or equine IgG. Later workers^{1,4,12} used commercially available SRID kits as the standard for comparison. Between-technique differences are difficult to interpret when reference standards are not equivalent in each technique. In such cases,⁴ different calibration standards can account for some reported differences between techniques. Reagents used in several commercial kits for equine IgG measurement may not be in agreement.³ These kits are labeled for experimental, not diagnostic use, so they are not subject to USDA regulation.

Measurement of human serum proteins, including IgG, was addressed by the Standards Committee of the College of American Pathologists, Ad Hoc Committee on Reference Preparations for Serum Proteins, and the US Centers for Disease Control.¹⁶ Briefly, 2 reference preparations were submitted to 24 collaborators for determination of 12 analytes. The results obtained were grouped and the mean estimates for mass and international units were assigned to the analytes in each of the preparations. These reference preparations were made widely available to laboratories to serve as "gold standards" with the goal of unifying interlaboratory results.

A similar collaborative project could resolve the problems of dissimilar standards used as references for analytes in other species. No "gold standard" exists as

From the Department of Clinical Sciences (Hutchison, Salman, Garry, Johnson), the Diagnostic Laboratory (Collins), and the Department of Environmental Health (Keefe), Colorado State University, Fort Collins, CO 80523.

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Table 1. Prediction of IgG concentrations using SRID kits.

Plate	Reference solutions	Equation*	R ²	Equation number
SRID-A	SRID-A	$c = 46.55 \cdot d^2 - 410.94$	99.9	1
SRID-B	SRID-B	$c = \text{antilog}(0.21 \cdot d + 1.03)$	99.7	2
SRID-A	SRID-B	$c = 23.53 \cdot d^2 - 204.68$	99.2	3
SRID-B	SRID-A	$c = \text{antilog}(0.23 \cdot d + 1.25)$	99.8	4

* Where c = predicted concentration and d = zone diameter.

a reference for the measurement of IgG in llamas. As in the equine, the available llama SRID kits are labeled for research use only and are not subject to federal regulation. The goal of this study was to compare the 2 commercially available llama SRID kits with each other and with other techniques for estimation of llama IgG.

Materials and Methods

Blood from each of 528 llamas was collected in tubes containing lithium heparin. Plasma was obtained by centrifugation and frozen at 0 C until analysis. Each sample was thawed and applied to 1 well of an SRID plate (SRID-A), according to manufacturer's instructions. Zone diameters were measured at 24 hr. Three reference solutions were supplied with this kit (label concentrations 172, 878, and 2,500 mg/dl). Samples were divided into 50 strata on the basis of the numerical result from SRID-A. One sample was randomly selected from each stratum.

For each of these 50 samples, plasma IgG concentration was measured both by single radial immunodiffusion using the other SRID kit (SRID-B^b) and by sodium sulfite precipitation (SSP). The reference proteins for SRID-B were labeled 125,500, and 1,000 mg/dl. Zone diameters were measured at 18 hr according to manufacturer's recommendations. No reference standards were provided with SSP; samples processed as directed by the manufacturers were visually compared with pictures of solutions containing 0, 300, 600, and 1,200 mg/dl of IgG. Also, for each of these 50 samples, γ -globulin concentration was determined by protein electrophoresis (PE) on cellulose acetate. The biuret^d technique⁷ was used to obtain the values for total protein used in the calculation of γ -globulin concentration. Additionally, reference solutions provided with SRID-A and SRID-B were assessed by these techniques.

Statistical analysis. For determination of IgG concentration from SRID kits, regression analyses¹³ were performed for each kit to determine the model best describing the relationship between label concentration and zone diameter. A univariate ANOVA for repeated measures⁵ having 2 within factors (plates, reference solutions) was performed on the ranks of the data to assess differences between IgG concentrations predicted by the 4 combinations of reference solutions and SRID plates. The coincidence of regression models was assessed by a single model approach¹³ to evaluate the differences between IgG values predicted when the 2 sets of reference solutions were applied to 1 SRID plate. The Wilcoxon signed rank test⁶ was used to evaluate differences be-

Table 2. Analysis of standard proteins supplied with SRID-A and SRID-B kits.

Standard	SRID-A kit (mg/dl)	SRID-B kit (mg/dl)	SSP kit (mg/dl)	γ -Globulins (PE) (mg/dl)	Total proteins (biuret) (mg/dl)
SRID-A1 (172 mg/dl)	172	86	0	—*	200
SRID-A2 (878 mg/dl)	878	423	600	408	2,400
SRID-A3 (2,500 mg/dl)	2,500	1,007	600–1,200	918	5,100
SRID-B1 (125 mg/dl)	298	125	0	322	1,400
SRID-B2 (500 mg/dl)	898	500	300	560	3,500
SRID-B3 (1,000 mg/dl)	2,004	1,000	600–1,200	1,224	7,200

* Unmeasurable.

tween predicted IgG concentrations when 1 set of reference solutions was used on both SRID plates. The ability of 1 SRID kit to predict the test result of the other was assessed by regression analyses, using IgG concentrations predicted by each kit for the 50 test samples. Friedman's 2-way ANOVA with multiple comparison testing was used for comparison of SRID-A and SRID-B results with γ -globulin measurements. The criterion for statistical significance was $P < 0.05$.

Results

Prediction of IgG concentrations using SRID kits. Models were developed to describe the relationships between reference solution label IgG concentration and measured zone diameter for the 4 combinations of SRID-A plates, SRID-B plates, SRID-A reference solutions, and SRID-B reference solutions (Eqs. 1-4, Table 1).

Analysis of reference solutions supplied with SRID kits. Reference solutions supplied with SRID-A and SRID-B kits were analyzed using SRID-A or SRID-B kits and SSP. Total protein and γ -globulin concentrations were measured (Table 2). The label IgG concentration of reference solutions supplied by 1 kit did not match the IgG concentration predicted when the other SRID kit was used for measurement of the solution's IgG content. In all cases, the total protein concentration of the reference solutions exceeded that of the IgG concentration. The label IgG concentrations of the SRID-A solutions were higher than the γ -globulin concentrations of the 2 solutions for which γ -globulins could be measured. The label IgG concentrations of the SRID-B solutions were lower than the γ -globulin concentrations in all 3 cases.

Agreement between SRID-A and SRID-B. Equations 14 were used to derive predicted IgG concentrations for each of 50 samples. Plate effects were not significant ($P = 0.252$); effect of reference solutions

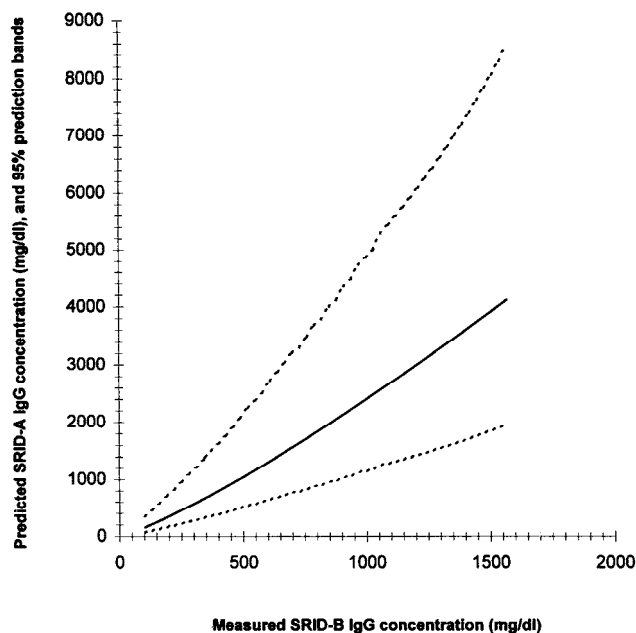


Figure 1. Prediction of SRID-A IgG concentration from measured SRID-B IgG concentration ($n = 50$).

used was highly significant ($P < 0.0001$), and a significant interaction between plates and reference solutions was detected ($P < 0.0001$).

The difference in predicted IgG concentration resulting from use of the 2 different sets of reference solutions was further evaluated by comparison of regression Eqs. 1 and 3 (IgG concentrations derived from the SRID-A plate), and regression Eqs. 2 and 4 (IgG concentrations derived from the SRID-B plate). In both cases, the equations were determined to be noncoincident ($P < 0.001$), due to significant differences in both the slopes and the intercepts.

The overall effect of using different plates was not statistically significant; however, the significant interaction term warranted further investigation. The effect of using different plates was evaluated separately for each set of reference solutions. Predicted IgG concentrations obtained using SRID-A reference solutions on SRID-A and SRID-B plates were not different ($P = 0.858$); however, when SRID-B reference solutions were used, predicted IgG concentrations differed significantly between SRID-A and SRID-B plates ($P = 0.038$).

Prediction of SRID results. A model for prediction of SRID-A results (when SRID-B results are known) was developed using IgG concentrations predicted by Eqs. 1 and 2 for the 50 test samples.

$$\text{SRID-A} = \text{antilog}[1.2 \log(\text{SRID-B}) - 0.221] \quad (R^2 = 78.3\%). \quad (5)$$

The corresponding model for prediction of SRID-B results (when SRID-A results are known) is as follows:

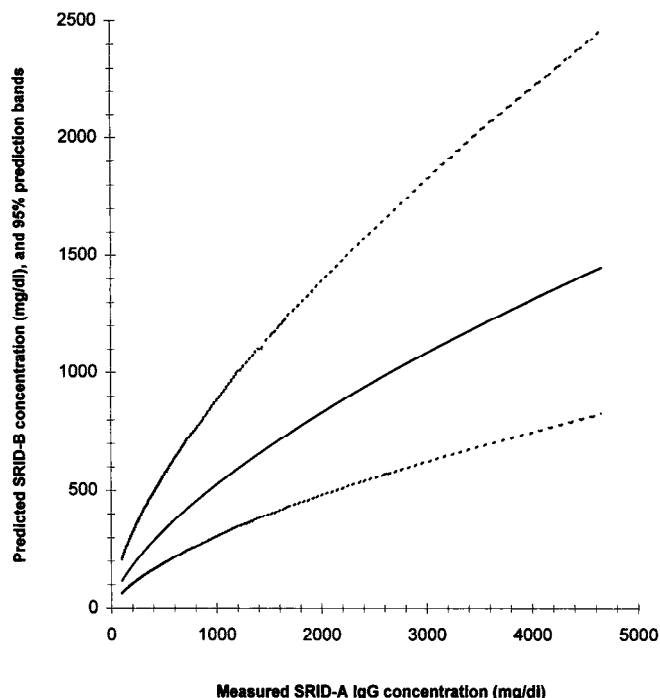


Figure 2. Prediction of SRID-B IgG concentration from measured SRID-A IgG concentration ($n = 50$).

$$\text{SRID-B} = \text{antilog}[0.66 \cdot \log(\text{SRID-A}) + 0.741] \quad (R^2 = 78.3\%). \quad (6)$$

The relationships described by Eqs. 5 and 6 are depicted in Figs. 1 and 2.

Comparison of IgG concentrations (predicted by SRID-A, SRID-B, and SSP) with sample γ -globulin concentration. SRID-A, SRID-B, and γ -globulin results differed significantly from each other, with SRID-A > γ -globulin > SRID-B. A summary of IgG values predicted by SRID-A, SRID-B, and SSP, and γ -globulin concentration for the 50 samples is shown in Fig. 3. IgG concentration predicted by SSP was always less than the γ -globulin concentration and IgG concentration predicted by SRID-A but appeared more consistent with IgG results predicted by SRID-B.

Discussion

Using single radial immunodiffusion, wells containing small amounts of antigen will reach endpoint or antigen-antibody equivalence earlier than those containing large amounts of antigen. The antigen concentration at point of equivalence is proportional to the square of zone diameter.^{8,14} However, prior to equivalence, a linear relationship exists between the log of the antigen concentration and the zone diameter. It has been suggested that SRID results will be more accurate if the reaction is read at the point of equivalence²; however, the demand for early results often necessitates the reading of plates prior to this time. It is im-

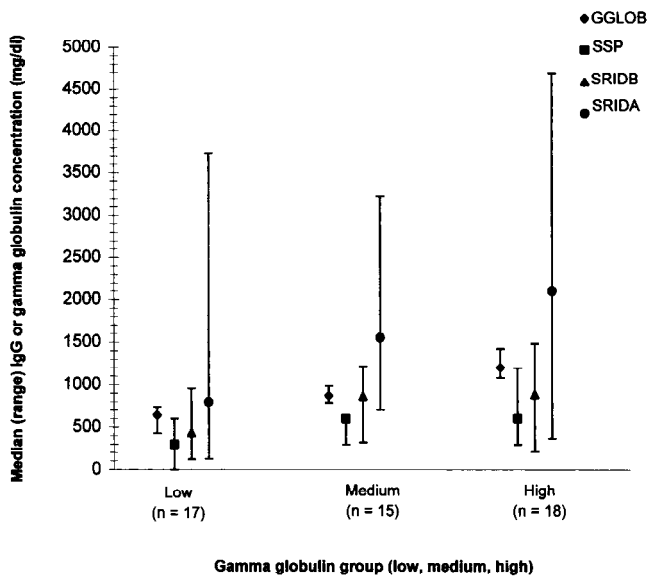


Figure 3. Comparison of γ -globulin concentrations and IgG concentrations measured by SSP, SRID-B, and SRID-A ($n = 50$).

portant, therefore, that the manufacturer's instructions be carefully followed and that the form of equation used is appropriate for the time of measurement. Reference standards should be run every time a sample or batch of samples is processed with the appropriate equation developed on each occasion.

Zone diameters from samples run on SRID-A kits were read at 24 hours, when samples had reached equivalence. This was demonstrated by lack of further change in zone diameter 12 hours later (data not shown) and the excellent fit of the quadratic curve of the form $c = \beta_1 * d^2 + \beta_0$ ($\beta_1 =$ slope, $\beta_0 =$ intercept, $c =$ IgG concentration; $d =$ zone diameter). The excellent fit of this curve (model 1, $R^2 = 99.9\%$) at equivalence demonstrates that the label concentrations of the standard proteins supplied were consistent with each other. Manufacturer's instructions provided with SRID-A indicated that zone diameters could be read prior to equivalence. In this case, IgG concentration would be predicted using an equation of the form $c = \text{anti-log}(\beta_1 * d + \beta_0)$. The manufacturer, however, suggested that endpoint readings were preferred (D. Jorgensen, personal communication).

Zone diameters from samples run on SRID-B were recorded at 18 hours in accordance with the manufacturers' suggestion. These samples had not reached equivalence, as demonstrated by the excellent fit of the exponential curve of the form $c = \text{anti-log}(\beta_1 * d + \beta_0)$ (model 2, $R^2 = 99.7\%$). Again, the label concentrations of the standard proteins supplied with SRID-B were consistent with each other.

Examination of the 6 reference solutions (3 from each SRID kit) and the 50 test samples suggested that differences would exist between results reported for a sample analyzed by both SRID-A and SRID-B. The

reference standards do not consist of purified IgG; it is apparent that proteins in addition to IgG were present in the reference solutions.

IgG concentrations were predicted for each sample using each of the 4 combinations of SRID-A plates, SRID-B plates, SRID-A reference solutions, and SRID-B reference solutions. If the reference solutions are calibrated to the same standard and there is no difference between plates, then all 4 results should be in agreement. The ANOVA did not support this hypothesis. Overall, the effects of reference solutions, but not plates, were found to be significant. The highly significant interaction term between plates and reference solutions indicates that the reference solutions (or plates) behave differently, depending on which plate (or set of reference solutions) is used. The test of coincidence of regressions confirmed that the 2 sets of reference solutions are calibrated to different standards but provided no information concerning the plate-reference solution interaction. Although the overall plate effect was determined to be not significant, further analysis revealed that SRID-A and SRID-B plates return equivalent IgG values when SRID-A, but not SRID-B, reference solutions are used. This explains the interaction term detected in the ANOVA. These results may indicate that the SRID-A reference solutions are seen in the same manner by both SRID-A and SRID-B plates, but that there is some component in the SRID-B reference solutions that is perceived differently by the 2 plates. Each manufacturer isolated and purified IgG individually. Anti-IgG antibodies were then developed for incorporation into the agar of the SRID plate. The significant effect of the reference solution as well as the plate-reference solution interaction must indicate that not only are the 2 sets of reference solutions calibrated differently, but that the "purified" IgG is not identical between manufacturers.

It is apparent that the 2 SRID plates will provide different IgG values for the same sample. Models 5 and 6 were developed to allow estimation of the value for SRID-A when that for SRID-B is known and vice versa. Failure to recognize and account for the differences between kits could have clinical consequences. For instance, the manufacturer of SRID-A suggests that achieving a minimum serum concentration of 800 mg/dl IgG will avoid the consequences of FPT of immunoglobulins in most crias (baby llamas), based on observations of his own crias and those from neighboring areas.¹¹ A sample assessed at 800 mg/dl by SRID-A would measure approximately 440 mg/dl by SRID-B. Use of guidelines established by one kit to assess IgG values derived from another would result in inappropriate interpretation. The relationships between the SRID kits described in Figs. 1 and 2 should have general application unless the characteristics of the kits are changed. The 95% prediction bands are

presented to indicate the uncertainty involved in making predictions. The width of these bands depends on the number of samples involved in the calculation (here, $n = 50$) and the variability of the data; therefore because their application is not general, their derivation is not presented here.

Most Ig subclasses are expected to migrate in the γ region of the electrophoretogram.¹⁸ The migration pattern for llama Igs has not yet been defined; however, **alpaca IgG has been reported to migrate in the γ region.**⁸ IgG is the predominant Ig subclass in blood; therefore, **γ -globulin concentration can be used to estimate IgG status roughly.** The calculation of r-globulin concentration is independent of any kit or standard. However, error involved in calculating the total protein concentration of the sample, as well as error in defining the γ fraction of the electrophoretogram, will affect the accuracy of the measurement. Unfortunately, the distinction between γ and β fractions is not always clear in the llama, particularly when the γ fraction is small. Analysis of the 50 samples (and the 6 reference solutions) showed that IgG concentrations predicted by SRID-A were consistently very much greater than the r-globulin fraction (Fig. 3). This may imply either that some llama IgG migrates outside of the γ fraction, or, more likely, that SRID-A overestimates the IgG content of the sample.

The SSP kit provides results that are categorical, making problematic the comparison with results from SRID-A, SRID-B, and γ -globulin tests, which are continuous in outcome. Figure 3 shows that SRID-B and SSP results are more similar to each other than to SRID-A.

In summary, we have shown that 2 commercially available llama SRID kits are not in agreement. The reference solutions provided with the kits appear to have been calibrated to different standards. It is not possible to determine which, if any of these kits, most accurately predicts true IgG concentrations, as no "gold standard" for llama IgG is currently available. Publication of the techniques used for the isolation and purification of llama IgG, and widespread dissemination of representative samples of such a preparation to allow for "standardization" of reference proteins, would do much to encourage uniformity of results between techniques.

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Sources and manufacturers

- a. Llama IgG Test Kit, Triple J Farms, Redmond, WA.
- b. Llama VET-RID Kit, Bethyl Laboratories, Montgomery, TX.
- c. Llama-STM, VMRD Inc, Pullman, WA.

- d. Hitachi 704 Automated Serum Chemistry Analyzer, Boehringer Mannheim Diagnostics, Indianapolis, IN.

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