Serology: Interpretation of test results

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INTRODUCTION

Serological tests are important test methods used to assist in diagnosing infectious diseases. They are usually done to determine the level of antibody that is present in a serum sample, but in some tests an antibody preparation is used to demonstrate the presence of antigen in a serum or tissue sample. In antibody detection tests, serum (or an antibody preparation) is mixed with antigen, and there is an indicator system to demonstrate whether an interaction between antibody and antigen has occurred. Although some serological tests are qualitative tests, most provide a quantitative measure of the antibody present in the serum sample. The level of antibody present is usually critical to the interpretation of the test because in most tests, the level of antibody must exceed a critical level before the test is regarded as a positive test.

When a new test is developed an essential part of the process is the establishment of standards for interpretation. It is important to determine the cut-off (threshold) levels for classifying test results as negative, suspicious and positive. However, it is also important that test results are not just mechanically classified as positive or negative, according to test titres. Careful consideration should be given to the epidemiological principles that are important for interpreting the results of a particular case and the reason for which the test is being done.

DETERMINATION OF POSITIVE/NEGATIVE CUT-OFF POINTS

Two important characteristics of diagnostic tests for infectious diseases are their sensitivity (ability to detect infected animals) and specificity (ability to not give false positive results). Sensitivity and specificity are discussed in more detail in Chapter 3. The level at which a test result is classified as positive or negative (test cut-off point) is the most important single factor in determining the sensitivity and specificity of the test. The level of antibody found in a serum is often expressed as the highest dilution of serum (titre) giving a positive reaction in the test. For most tests the distribution of the level of antibody found in sera from infected and non-infected populations overlap. Figure 1 gives an example of a plot of the frequency distribution against the log10 antibody content of sera from a group of animals infected with Brucella ovis and a group of non-infected animals (taken from reference 5 of the recommended reading list). It should be noted that the frequency distribution is usually skewed and that expressing the antibody content as a log function makes the distribution closer to a normal distribution. Since serological tests are often done by testing doubling dilutions of serum, it is a common practice to use log2, but log10 or any other log function can be used. Because of the overlap of titres in the positive and negative populations there is usually a trade-off between test sensitivity and specificity. If the cut-off point is set at a low level there is a tendency to increase the sensitivity by classifying the maximum number of infected animals as positive. However, this will decrease the specificity since several non-infected animals will be classified as positive (false positive reactions).
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Figure 1. Frequency distributions for *B. ovis* ELISA values from negative (n = 2,535) and positive (n = 589) sera with fitted distribution curves. Reproduced with the permission of the authors and publisher from Reichel MP., Ross G., Drake J., Jowett JH. 1999. Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. *New Zealand Veterinary Journal*, 47, 71-76.

The decision about where to set the cut-off point can be made in several ways:

- Ideally a large number of sera, from proven infected and non-infected animals, from the population of animals under test should be tested and the frequency distribution of the reactions, plotted as shown in Figure 1. More than or less than cumulative frequency curves can also be plotted from the data for positive and negative populations, so that the percentage of reactors above or below a particular titre and hence specificity and sensitivity, can be easily determined for any chosen cut-off point. The numbers of reactions in the region of overlap must be small; otherwise the test may be unsuitable for use. In the overlap region all reactions are low titre reactions and if tests are repeated small test result variations that would ordinarily be regarded as insignificant, may because of their proximity to the cut-off point, move sera from one category to another. Therefore, in deciding where the cut-off should be the best-fit distribution curve should be considered rather than the careful tallying of the few false positive or negative test results in the critical region. Taking into account the requirements for sensitivity and specificity a number of possible cut-off points can be considered and by trial and error the most suitable one can be found. Because of the overlap of the positive and negative distributions limits may be set for a suspicious category, if this is required. Decisions made by this simple approach will give similar results to those calculated by more sophisticated mathematical approaches.

- A more sophisticated approach is to use a receiver-operating characteristic (ROC) plot of the data. In this approach sensitivity and specificity are calculated for a range of cut-off values. These estimates can be made from the actual data, but should preferably be made from the fitted
distribution curves for the positive and negative populations. Plots are then made of the sensitivity and specificity against a range of cut-off values as shown in Figure 2.

![Image](image_url)

**Figure 2.** Receiver-operating characteristic (ROC) plot for an actual set of data for an ELISA test for *Brucella ovis*, from the set of data given in Figure 1. The red line is the sensitivity and the black line represents the specificity. Reproduced with the permission of the authors and publisher from Reichel MP., Ross G., Drake J., Jowett JH. 1999. Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. *New Zealand Veterinary Journal*, 47, 71-76.

The intercept of the two lines in a ROC plot is the point, at which sensitivity and specificity are equal. At this cut-off point the error rate (false positives + false negatives) will remain constant at all prevalence rates. However, for some prevalences lower error rates can be achieved at different cut-off points. If a population of infected animals has a low prevalence of infected animals, say 5%, then increasing the cut-off point would reduce the number of false positive reactions in the negative animals which make up 95% of the population. It will also increase the number of false negative reactions in the infected animals that constitute only 5% of the population. The overall effect will be a reduction in the error rate. It is possible to plot the cut-off point that gives the minimal error against the prevalence (Figure 3). This gives rise to the possibility of using different cut-off points to suit different circumstances as discussed below.
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Figure 3. Optimising cut-off values (minimising error rates) in the *B. ovis* ELISA depending on the prevalence of *B. ovis* infection. Reproduced with the permission of the authors and publisher from Reichel MP., Ross G., Drake J., Jowett JH. 1999. Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. *New Zealand Veterinary Journal, 47*, 71-76.

- Where it is not possible to assemble a suitable bank of sera from known infected animals, a cut-off point can be determined by testing a suitable number of non-infected animals from known disease free herds or flocks. The decision on where the cut-off point will be in this case is based only on the required specificity of the test and does not take sensitivity into account. In this case the data is analysed and the cut-off point is generally set at 2 to 3 standard deviations above the mean for the log transformed data. If the distribution of reactions amongst the negative animals is normally distributed, the percentage of uninfected animals included in the negative category will be 97.5% or 99.9% depending on whether 2 or 3 standard deviations are used. This by definition implies a specificity of 97.5% or 99.9. It is a simple matter to choose a cut-off point that will give any required specificity by using Tables for the area under the normal probability curve. However, these estimates of specificity are only correct if the distribution of log transformed data for the negative specimens are normally distributed, and although the distribution may be close to the normal distribution, in practice it may not fit a normal distribution. In this case the type of distribution curve that most closely fits the experimental data can be found and used to make the estimates.

- Mathematical methods are also available to determine the cut-off point from data gathered by testing a mixed population of infected and non-infected animals.
In deciding where the cut-off point should be a conscious decision may be made about whether to err on the side of sensitivity or specificity. Therefore, the purpose of the test and the requirements for sensitivity and specificity are important and should be considered. Some of the factors that may be considered are:

- In the case of eradication programmes, especially large national programmes it is more important to try to identify all infected animals, than to be concerned about the slaughter of a few non-specific reactors. The cost of leaving infected animals in a herd and risking re-infection and consequentially several more re-tests and the culling of additional newly infected animals, are high compared to the costs of sacrificing a few non-specific reactors. The same considerations also apply to eradication programmes in individual herds and flocks. Therefore, in these cases the requirement is for high sensitivity and the threshold may be set slightly lower than would be the case in other circumstances.

- Where testing is being done for export/import certification the importing country’s requirements are generally for high sensitivity, while the exporters demands are for maximum specificity. Testing laboratories should remember that their primary clients and the party to whom they are ethically responsible, is the importing country even if the exporter is paying for the tests. Test interpretations specified by the importing country that may be biased toward high sensitivity and low specificity, should be strictly adhered to.

- In the case of accredited herds or herds with a long history of freedom from a disease and no indication that the disease could have been recently introduced, the requirements are for high specificity. The unnecessary revocation of accredited status when all indications are that the herd is not infected, should be avoided unless the reactions provide clear evidence of infection.

- Serological testing of a statistically significant numbers of animals is often used in Surveillance programmes designed to provide evidence of a country’s freedom from specific diseases. False positive diagnoses may have significant national economic impacts as other countries may impose trade restrictions due to the perceived presence of infection. In this case the requirements should be for maximum specificity to avoid the making of false positive diagnoses in doubtful cases. This is an acceptable policy decision to make because if infection is really present this will quickly become obvious as the disease spreads and further high titre reactions are found in future testing. For Surveillance programmes serological tests are often used as screening tests, with all positive or suspicious cases being followed up by more intensive investigations of individual reactor animals and herds or flocks. Proof of a correct diagnosis would usually be required by the use of a highly specific test method such as the isolation of the infectious agent.

- Testing of individual animals with no herd history may be easy in clear-cut cases but may equally be difficult for cases with low titres around the cut–off point. The cut-off point should be set to give the best balance between sensitivity and specificity and the use of a suspicious category is warranted.

In the discussion above it was shown that the cut-off rate that gives the minimal overall error rate can be estimated (Figure 3). Therefore, it would be possible to use a floating cut-off point to suit the particular case. In each case it would be necessary to estimate the prevalence of disease before deciding the cut-off point. For example the estimated prevalence in herds or flocks that have for some time been accredited free from a disease would be close to zero and a cut-off point in the vicinity of
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500 ELISA units, might be selected (Figure 3). At a cut-off point of 500 ELISA units the sensitivity is 50% (Figure 1). A judgement must then be made about what sensitivity and specificity are acceptable for the particular case. In the case of a disease eradication campaign the cut-off point could be low, until accreditation is achieved, and then increased as deemed appropriate. There is certainly a lot of merit in moving in this direction but there are also disadvantages that must be considered. The situations that arise in practice are not possible to incorporate into mathematical analyses. The reason periodic (often annual) accreditation tests are done, is to guard against events such as unauthorised introduction of animals into a flock, broken fences that allow animals onto a neighbour’s infected property, taking animals to shows or sales where they may come into contact with infected animals etc. There are clearly advantages to using different cut-off points under different circumstances, but it should be done with discretion and the clinician’s knowledge of the clinical status of the animals and the management practices used on the farm should also be considered. On farms where untested animals have been introduced, fences are down, and hygiene and management practices are not suitable, the tendency should be to use a strict interpretation. A reasonable compromise is probably to have two interpretation levels: one for situations requiring high specificity (high cut-off), and another where high sensitivity is needed (low cut-off).

Another difficulty in setting interpretation standards is whether to have a suspicious category included in the interpretation standards or whether only a positive/negative diagnosis is allowed. Regulatory authorities often seek to have a cut-off point that allows for a simple yes/no decision to be made and find that a “may be” category is an inconvenient complication to the practical application of control programmes, export certification etc. They may word regulations to say that only negative animals can be exported or animals that are not negative must be slaughtered etc. Some practitioners may also want to have a clear-cut answer from the laboratory that relieves them of the responsibility of difficult decision-making. However, titres falling within the suspicious category are unavoidable and other ways of resolving the problem should be sought.

Fortunately the numbers of cases falling in the suspicious category are generally low and therefore only rarely cause serious interpretation dilemmas. Consideration of epidemiological evidence in interpretation of results should be used to increase the accuracy of diagnosis and the ability to recognise false positive reactions.
PATHOGENESIS OF DISEASES AND TEST INTERPRETATION

Optimal interpretation of test results should involve more than mechanical sorting of titres above or below a particular threshold point. Veterinarians are trained professionals and should be able to add their knowledge and skills to the making of a diagnosis. Some field veterinarians adhere to the principle that a laboratory result is a test result and only an aid to diagnosis and that they should interpret the test result and make the diagnosis. Others believe that laboratory staff are the experts in testing and make minimal additional input into the final diagnosis. This latter view denigrates the veterinarians role to that of a blood collector and dispatcher, while the former ignores the professional input and knowledge of highly skilled laboratory staff. Clearly a partnership involving the sharing of skills is preferable.

Laboratory veterinarians should have extensive knowledge of the diseases for which they are testing and the test methods they are using. They should also keep records of previous testing and occurrence and geographical distribution of diseases and of non-specific reactor problems, both nationally and internationally. They should draw on their experience and knowledge to comment usefully on test results and interpretation, and not just copy titres and positive and negative results onto a test result form. Additionally laboratory staff should be constantly vigilant for technical, clerical and sample identification errors made by the laboratory or by the submitter. Excellent Quality Control and Quality Assurance procedures should operate in the laboratory.

Field veterinarians should have detailed knowledge of the clinical characteristics of the cases they are investigating as well as a knowledge of the disease history, stock movement and management methods used on the farm. Ideally they should also have knowledge of disease problems and non-specific reactor problems that have occurred on individual farms and in their areas of operation.

All parties involved in using serological test results should have extensive knowledge of the pathogenesis and manifestations of the diseases concerned and extrapolating from this the meaning of serological titres. For example in chronic diseases such as brucellosis, Aujeszky’s disease, leptospirosis, maedi visna or bovine pleuropneumonia a positive serological titre usually indicates infection and serological tests are ideal for identifying infected animals. Animals with positive titres may be clinically infected (maedi visna) or may be symptomless carriers of infection (cattle following an abortion caused by *Brucella abortus*). High rising titres may indicate recent infection (leptospirosis) which may be followed by a carrier state in which titres persist at a lower level (leptospirosis) or by recovery with the animals no longer carrying the infectious agent (bovine viral diarrhoea -BVD). Some carrier animals may excrete the organism continuously or intermittently (bovine brucellosis) or only during periods when the infection is reactivated (herpes virus infections such as Aujesky’s disease and infectious bovine rhinotracheitis - IBR). In some diseases intrauterine infection of the foetus leads to the development of immune tolerant carriers that do not develop antibody but carry the virus for the rest of their lives (BVD and border disease) or for protracted periods before the infection is reactivated and antibodies develop (bovine brucellosis). In some infections the host may overcome the infection in a percentage of cases and become transiently serologically positive before eliminating the infection (*Brucella ovis* infection). In yet other infections animals may only develop the disease in a small percentage of cases while the majority become
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carriers of infection (enzootic bovine leukosis - EBL). With some agents infected animals invariably develop into symptomless carriers (bovine aids or BIV). In some infections the incubation period may be short and the course of the disease short and acute so that antibodies are generally only detected in the convalescent phase or in sub-acute cases (foot and mouth disease). In yet other cases the incubation period may extend for years and antibody may be hard to detect until the animal has reached an advanced stage of the disease (Johne’s disease). In some diseases the development of antibodies coincides with the elimination of the infectious organism and serologically positive animals are immune and not infected (BVD).

In each of the above cases the meaning of a serological test result is different. A positive test for bovine brucellosis indicates probable infection. It also means that if the animal is a milking cow she may be excreting the organism intermittently or continuously in her milk, or if dry will probably again shed the organism in her uterine discharges next time she calves. A positive test for BVD means a recovered case that is no longer infectious, and a negative means either an animal that has not been exposed to infection or an immunotolerant carrier. A positive test for Aujeszky’s disease generally suggests an animal that is carrying but not excreting the organism, but that it may become an active excretor if subjected to stress. A clear cut positive for Johne’s disease often means that the animal is in the clinical phase of the disease, and a negative may indicate no previous contact with the disease or an animal in the incubation phase.

Unfortunately there is no logical method of predicting how diseases develop and a sound knowledge of the pathogenesis of individual diseases is necessary if serological results are to be meaningfully interpreted. Other permutations of possibilities may be found and a detailed knowledge of animal diseases and access to literature is needed for good interpretation of serological results.

FALSE POSITIVE, SPECIFIC, NON-SPECIFIC AND CROSS REACTIONS

Three terms that are often used as synonyms are false positive, non-specific and cross-reactions.

From a diagnosticians point of view any result that suggests a wrong diagnosis is a false positive reaction. However, even this concept is not clear-cut. As discussed above positive reactions may indicate past or present contact with the infectious agent. Therefore a positive reaction may indicate active infection, a carrier state, or previous contact with a disease agent. A reaction in an animal that has been exposed to an infection is therefore a specific reaction although it does not necessarily indicate infection. A typical example would be a ram that has been transiently infected with *Brucella ovis*. Antibodies developed in response to exposure to *Brucella ovis* cause positive reactions that are therefore specific reactions. However, to someone involved in eradicating the disease it may be regarded as a false positive reaction because it does not indicate an infected animal that must be culled. Similarly in bovine brucellosis, if the disease has been effectively eradicated from an infected herd by test and slaughter, there may still be a residue of very low reactions in the herd. This can be demonstrated by doing agglutination tests on the animals and it may be found that a greater than
usual number of animals have detectable reactions at a level below those normally regarded as positive. These background reactions disappear with time and are presumably the result of exposure to sub-infectious doses of organisms or dead organisms or animals with increased ability to resist the infection that have overcome an episode of infection. Again these reactions could be considered as false positive reactions by someone using the test for the diagnosis of cases of brucellosis.

Reactions caused by antibody developed in response to a vaccine that has identical antigens and epitopes to the disease reagent, are specific reactions and should be referred to as vaccine reactions rather than non-specific reactions.

True non-specific reactions are reactions that occur between a molecule that has an affinity for and interacts with part of the antibody at a site other than its antigen-binding site. Such reactions are rare but some proteins such as Protein A, produced by specific strains of *Staphylococcus aureus*, react with the Fc portion of the antibody molecule and cause agglutination. Other similar proteins are known and there are probably many unidentified substances capable of such interactions. In the agglutination test and to a lesser extent the ELISA test for *Brucella abortus* low level reactions are known to occur and are thought to be due to the interaction of the FC part of some agglutinins with *Brucella abortus* cells. In the case of brucellosis these reactions are significantly reduced in the presence of EDTA.

The term non-specific is also often applied to reactions that should more correctly be termed cross-reactions. These are reactions caused by antibodies that have been produced in response to exposure to epitopes that are identical or similar to epitopes on the antigen being used for the particular test. In this case the interaction is between the epitope in question and the antigen-binding site (paratope) of the antibody. In the case of *Brucella abortus* bacteria other than *Brucella* that have epitopes containing perosamine may stimulate the production of antibodies that cross react with *Brucella abortus* epitopes.

The diagnostician needs to develop methods of distinguishing false positive reactions from specific ones. Sometimes a laboratory can solve the problem by the use of highly specific tests, such as tests based on a monoclonal antibody or immunoblot tests with specificity for an epitope that is unique to the antigen in question. However, the molecules and epitopes involved in causing the cross-reactions may be very similar or even identical to those involved in the “specific” reaction. It is therefore unreasonable to expect that in the short term, laboratory tests can be developed that will solve all problems. For this reason it is necessary to use other aids. Knowledge of the pathogenesis and epidemiology of diseases is a powerful tool that should be utilised to assist with interpretation of results.

In practice there is often no way to determine the exact cause of a false positive reaction or even to determine with certainty whether they are non-specific or cross-reactions. The terms are therefore often used in an interchangeable manner.

**Recognition of false positive reactions**

When testing sera from single animals it may be difficult or impossible to know whether a suspicious reaction in a single test is a specific reaction. Generally the reaction will be classified as suspicious
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until repeat tests on the animal are able to resolve the issue. If a reaction is due to a recent infection with the animal in the first stages of an immune response the antibody titre will rise rapidly and re-testing the animal after a suitable interval (2-4 weeks) will resolve the issue. On the other hand if the reaction is a non-specific or cross reaction it is likely that the titre will fall or disappear after a time interval varying from 2 weeks to several months. From a practical point of view specific reactions caused by transient sub-clinical infections will often also decline to negativity over a period of several months. Re-testing of animals after an appropriate time interval is therefore an important tool for distinguishing specific and non-specific reactions.

Fortunately in veterinary medicine many of the most important applications of serological testing involve the testing of herds or flocks of animals rather than single animals. This often simplifies the interpretation of tests. The first decision that should be made when analysing the data from a herd/flock test should be whether the herd/ flock is infected or not. Only when this decision has been made should consideration be given to the interpretation of individual test results.

Once it has been established that a herd/flock is infected with a disease interpretation of test results should be very strict. Consideration that reactions may be non-specific should be put aside and suspicious reactions are generally classed as positive. In disease eradication programmes two different sets of cut-off points for interpretation may be used. For accredited herds/flocks or those with a long history indicating freedom from disease a higher cut-off point may be used and for herds that are classed as infected a lower threshold may be chosen.

Studying the distribution of reactions may give useful clues as to the cause of the reactions. When a large infected population of animals in which non-specific reactions also occur is tested, the frequency distribution of reactions will be trimodal. One peak of distribution will include the low titre results representing the uninfected part of the population, the other main peak will represent the infected population and they will be distributed around a clearly higher mean. Intermediate between these two peaks there will be a peak of low titre non-specific reactions. The three peaks may overlap with each other and if the numbers of non-specific reactions is low they may be swamped by the other reactions and not clearly distinguishable. Data from a single herd that includes both infected and non-infected reactors, may not show clear distribution peaks of specific and non-specific reactors, especially because the prevalence of non-specific reactions is often low. It may appear to be just a skewed distribution of reactors. In a herd in which there is non-specific sensitisation of animals and no infected animals, the distribution of reactors will be located at a low level and probably have a mean in the low positive or suspicious or even high negative range. However, the number of reactors is often so low that a distinct peak of distribution is not obvious. In practice it is not necessary or usual to plot a frequency distribution curve of the data for individual herds, but the knowledge of these distributions should be used to assist interpretation. If a herd/flock is infected the majority of reactors will have clearly positive titres and there will be at least some with strong positive titres. If the titres that occur are few in number and nearly all are in the suspicious or low positive range the case probably involves non-specific or cross-reactions.

Infectious diseases will spread to other animals in the herd and the finding of a single reactor animal in a herd is a rare event. Singleton reactor animals in a herd should always be viewed with some suspicion. They are more likely to be true reactors if they occur in a previously infected herd,
especially for diseases that have a long incubation period and for diseases where latently infected animals occur, or when there have been recent introductions of new animals.

In cases of doubt thorough herd and laboratory examinations should be done. The type of investigation will depend on the particular case but would typically include at least some of the following lines of investigation:

- Tests should be repeated on the same serum sample to make sure the result was not a laboratory error and sample identification should be carefully checked. This check should be done before the results are issued.
- Alternative test methods may be used to confirm the test result. Where possible more specific confirmatory tests should be used.
- Herd history, history of stock movements and animal introductions should be carefully investigated to establish any basis for the introduction of the particular disease.
- Where samples from single animals have been found to be suspicious the test should be repeated after a suitable time interval (2-4 weeks). Depending on the nature of the disease the animal may be kept isolated in the intervening period. The investigation should be extended to include a significant sample of other animals in the herd or the whole herd.
- Where possible repeat tests should be done on all animals in the herd or at least on all the reacting animals and a sample of non-reacting or previously untested animals. Any sample of sera should include sera from animals in the various groupings of animals on the farm e.g. calves, heifers, milking herd, dry cow herd, bulls etc. Repeat tests are best done at least one month after the initial test but where there is urgency, may be done sooner. In the case of infection the virtually all reactors are likely to remain reactors and titres will not vary a great deal although some recently infected animals may have rising titres and some new reactors can be expected. In the case of non-specific reactors there are likely to be several animals showing decreasing titres or that have become negative. There may also be some new reactors. Variable low titre reactions of short duration are typical for many cross-reaction and non-specific reaction problems. Where possible repeat test should be done using more than one serological test.
- Reactors should be examined clinically for signs of infection and specimens taken for demonstration of infectious agents by agent isolation and identification, antigen capture ELISA, PCR tests or electron microscopy.
- In the case of herd investigations particularly for economically important diseases, it may be possible to sacrifice one or more animals for detailed postmortem examination and collection of tissue samples for histological examination and demonstration of infectious agents.

The problem of non-specific reactions has been discussed in some detail because problems of poor specificity of tests can have far reaching effects for individual herd/flock owners, sales of stud animals, national accreditation schemes and international trade. However, it needs to be emphasised that these problems are the exception not the rule and that serological testing is generally a very reliable method of testing. Because of their simplicity, comparatively low cost, adaptability for mass testing, the ability to easily collect and transport samples, the fast turnaround time for testing and the high reliability of the test results, serological tests are extremely important for diagnostic and certification testing.