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Essential Practice Lab Tips, Tricks and Techniques Mini Series

Session One: Blood Sampling and Analysis: How to Get the Best from a Drop of Blood

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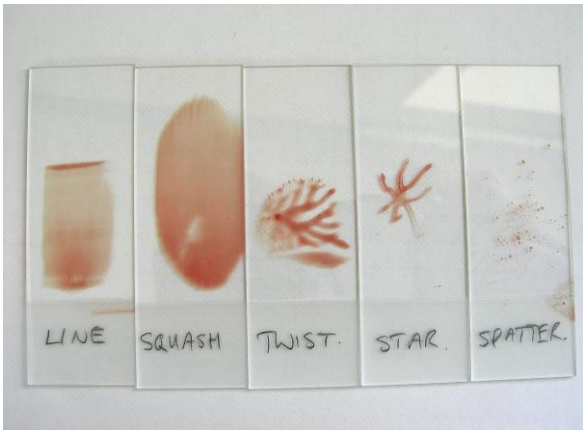


BLOOD SAMPLING

Biochemistry

In many ways, because many biochemical analysers are a 'black box' when it comes to understanding how a result, such as ALP level, is produced they are more open to abuse and misuse. This is compounded by the lack of visual checking that can be performed, as opposed to the blood film that provides a degree of 'quality control' and checking for the haematology analyser.

Many of the principles and shortcomings of biochemical analysis have already been outlined in the laboratory concepts section earlier. There are too many analysers and too many ways to measure each analyte, from electrolytes to enzymes and metabolites, for a comprehensive and specific discussion here. Certain broad ideas and points though will be addressed.

Sampling:

Most in house analysers use lithium heparinised blood that is spun to produce plasma, which is then analysed for electrolyte levels and various biochemical parameters, usually within a set or panel of tests. The following are some guidelines when taking blood for biochemical analysis.

- venupuncture technique and tube filling is similar to that described for haematology earlier. Haemolysis can be even more significant and limiting for these tests.
- it is advisable to **correctly fill the heparin (or serum) tube first** before filling the EDTA tube. If the syringe tip touches the side of the EDTA tube, EDTA can contaminate its tip. EDTA works by binding/chelating divalent ions (such as calcium and magnesium) and these are essential for some test enzyme reactions. If unavailable due to the EDTA, the enzyme reaction may be slowed or unable to proceed, artificially lowering the level measured (*e.g.* ALP). The calcium levels will naturally be very low due to the chelation. The potassium levels will be artificially high, as EDTA is most commonly present as a potassium salt. **The combination of a low total calcium and high potassium should ring alarm bells for EDTA contamination.** However, some diseases such as antifreeze poisoning can produce a similar, but usually less dramatic pattern.
- reference values are primarily based on animals that have been fasted for at least 8 - 12 hours. If the animal is not fasted, certain parameters, such as glucose, will naturally/physiologically be high. Also, dietary fats can produce a lipaemic sample that will interfere with analysis. Unless a result is needed immediately (unlikely if the animal is eating), then it is best to wait and analyse a fasted sample.
- If measuring electrolytes, especially if whole blood 'I-STAT' type analysers, ensure that any anticoagulant used is not sodium or potassium based. This is especially true if using a catheter that has been 'flushed' or is administering intravenous fluids. In this instance, ensure that enough blood is withdrawn and discarded to clear the catheter before sampling. Alternatively and ideally, use a different vein (or artery).

- If the animal is known/suspected to be hyperlipidaemic, either due to endocrine disease or drug effects, prolonged fasting is recommended to minimise potential lipaemic interference. This can be between 24-48 hrs without any detriment to the animal generally. If lipaemia persists, other techniques, such as ultracentrifugation and refridgeration or chemical clearing may be required.
- Serum samples can be required for certain tests and can usually be analysed in place of heparinised blood on most analysers. However, reference values can be very different as the clotting process alters serum composition relative to plasma. Changes include increased potassium that is released from platelets, and reduction in plasma proteins as fibrinogen is used to form the clot. Glucose levels are also often higher in serum relative to plasma (see PBGM discussion later for why).
- **When submitting to external referral labs, in house centrifugation and separation is recommended**, the separated plasma being placed in a plain tube and refridgerated until posted. Alternatively serum gel tubes can be used for most analytes (except trace elements and drugs commonly). Once the blood has clotted (15-20 minutes at room temperature) the sample is then spun, the gel preventing any cellular in vitro changes. Again refrigeration before posting is recommended. If the sample is cooled too early or not allowed to clot properly, a jelly-like fluid forms which greatly reduces the amount of serum obtained.

Presuming the analyser is working correctly, it will produce a series of results to which one of the first questions asked, especially when evaluating any abnormality (high or low result), is whether this value is artefactual? Again, some potential artefacts and sources have already been discussed, but some markers than can be useful are listed later.

1. Does the value fit the animal clinically? For example, a high potassium result should produce supportive clinical signs. Values >10 mmol/l would result in the animal's death due to cardiac arrest. This level is therefore likely an artefact.
2. Are there any other obvious artefacts, as problems and interferents often affect more than one test. For example a very low glucose with significant white cell degeneration supports prolonged storage with in vitro consumption and degradation.
3. Significant variation in a test result that is repeated supports an artefactual change, unless the animal has changed clinically or been treated.
4. Haemolysis, lipaemia and/or icterus may be present, with their associated significant but variable affects on many tests.

Certain tests require more specific understanding of sample requirements and processing and common examples will be discussed briefly later.

Glucose:

This is commonly measured in house by one of two methods – portable blood glucose meter (PBGM) on whole blood or by the practice biochemistry analyser on heparin plasma/serum. These two methods are measuring the glucose in different liquids – whole blood versus plasma – and thus each method requires its own reference values. This is especially true at high glucose levels as many PBGM used are designed for human blood measurements that very rarely get as high as certain animals like cats. **Typically the level of glucose measured by a PBGM is lower than that measured in plasma**. This is because the PBGM measures the glucose in both cells and plasma, so there is relatively less water compared to plasma. This also means that if the sample's cellularity is altered (*e.g.* in anaemia) the glucose level measured will be affected – overestimated in this example. Therefore when doing a blood glucose curve, only one analyser should be used for the serial monitoring. In many instances, it is more likely the biochemistry analyser is better maintained and less prone to variation and may therefore be recommended.

If delayed analysis or submission to an external lab is expected, the sample must either be spun and separated immediately, or placed into specific tubes that contain a fluoride anti-coagulant. This substance inhibits enzymes such as enolase and their in vitro consumption of glucose and are also an anti-coagulant. If not used, or left unseparated, glucose levels will artificially fall 3-10 % per hour, in part depending on the sample's cellularity – increased numbers of red or white cells will speed up this *in vitro* consumption. However, incorrectly filling the tube allows the cells to carry on consuming glucose (overfilled) or the excess fluoride can inhibit the analyser method (also often an enzyme) to suppress the glucose (underfilled).

Bile acids:

These are analysed on serum samples, and are variably affected by haemolysis and lipaemia. Depending on the animal and disease in question, it is often inappropriate to measure bile acid levels in icteric animals as it provides no further useful information. Fasted and post prandial levels are measured, the latter being done 2 hours after feeding. The principle of feeding is to stimulate gall bladder contraction, increasing the bile acid levels in the blood to 'test' the liver's ability to remove them from the blood back into the gall bladder. People often give a meal that is rich in fats, as the dietary fat content is a potent stimulus for gall bladder contraction. However, unfortunately this often produces a sample that is too lipaemic for analysis or interferes with the level of bile acids measured. **A (small) meal of canned food with a moderate fat content is fine. An animal can have significant/primary hepatic disease and not have elevated bile acids as this is afunctional marker, not a 'damage enzymatic' one.**

It is always worth noting whether the animal was easy to sample or not, especially if significant manual restraint or chemical sedation was required. Manual restraint of any kind will always release some creatine kinase (CK) from muscles.

Increased adrenaline during sampling has several natural/physiological effects, one of the most prominent being an increased glucose level, especially in cats.

Chemical sedation in fractious patients can have profound effects. Intramuscular injection will release muscle enzymes (CK and aspartate transferase - AST) and if using an alpha 2 agonist such as medetomidine ('Domitor'), can stimulate the pancreas and so elevate glucose levels.

Concurrent or recent drug therapy, and even supplements such as essential fatty acids, should also be noted as these can have dramatic effects. Taking essential fatty acids as an example, if these are supplemented for skin diseases, they can cause altered protein binding and artificially elevate levels of various hormones like free thyroxine (T4) levels. Diuretic therapy such as frusemide can affect electrolytes (such as low potassium and calcium), whereas steroid therapy will especially affect the liver and ALP levels.

General Concepts for Laboratory Testing

When looking at a result/number produced by an analyser, reaction or method (such as a PCV, ALP level or dipstick test), several questions should be asked before any interpretation/diagnosis can be made. These can include:

- Was the analyser functioning properly? *See Quality management*
- Was the test performed correctly? *See Standard Operating Procedure (SOP)*
- Are there any sources of artefact/error? *See Laboratory error*.
- Is this result abnormal? *See Reference values*
- What does this result mean? *See Sensitivity and Specificity*.

Unfortunately, too often these questions are ignored and the 'black and white' result simply used and interpreted, potentially leading to the wrong clinical diagnosis or wasted time and money. The assumed answer to the first two is often 'yes' whilst the third is 'no'. These assumptions can be wrong or at least only partly true. The first three will be discussed in more detail as nurses are typically integral to, and responsible for, the answer. The final two questions are much more clinically driven/orientated, and thus beyond the scope of this day course. They will be discussed briefly though, as an understanding or appreciation is very useful.

Quality Management

It is inevitable that procedural mistakes will be made or analysers will malfunction. It is therefore essential that these errors are identified correctly and as early as possible to ensure they are not interpreted as genuinely abnormal results that indicate a diseased animal. Quality management ensures this, incorporating SOPs (see later) as well as continual monitoring of analyser function and performance. A simple first point is to always follow the manufacturer's instructions, storage conditions and expiry dates.

Unless the user has very detailed knowledge of the analyser, reagent, reaction *etc.* deviations from these guidelines are dangerous and can often produce incorrect results. That is not to say deviation is not possible, but as a general rule, would be considered inadvisable.

Quality management also involves correct analyser maintenance, on a day to day basis within the practice, but also less frequent but more rigorous cleaning *etc*. by trained engineers. As long as the analyser is cleaned and maintained following the manufacturer's instructions, it should function properly.

Alongside these somewhat obvious points, is so called **quality control and assessment**, the former using known reagent concentrations/solutions (calibrators and control reagents) and the latter monitoring this process both in the short and longer term.

Quality control in practice is typically "internal quality control", using day to day control reagents to check the analyser, along with less frequent calibration checks using separate calibration reagents. If an analyser is used infrequently, these reagents should always be run before analysing any patient sample. If this is not done, the result produced from the patient sample may not be 'true', with factors such as temperature change and reagent expiration/exhaustion, altering the analysis and affecting the result produced. (Please see ***analytical accuracy*** and ***precision*** later).

Ideally practice lab equipment would also use "external quality control" whereby their analysers analyse known solutions sent by an external assessment centre which then compiles the results from many practice labs, comparing methods and results. In this fashion, your lab methodology and analyser is compared against a peer 'average' and 'reference' method. If your analyser is producing erroneous results occasionally or consistently, these are identified and can then be rectified. However, this requires a critical and large number of practices to subscribe and do this, which is unlikely at the moment in the veterinary field. It does though make "internal/in house" quality control that much more vital. Without this/these control(s), errors may not be noted and can therefore wrongly be attributed to disease situations rather than the reality of analyser or human error.

Quality assessment involves the monitoring of the results produced by running quality control solutions/systems. Within an "external" system, this is inherently part of the service and benefit. The vast majority of practices do not take part in an external quality scheme, although one such limited scheme is offered by a commercial lab to those practices using its own equipment. This quality assessment is to a degree laborious and expensive, when using external assessment schemes. However, it is useful to ensure confidence in your performance and the results your analysers produce. As mentioned, using an external scheme in reality can be impractical or too expensive for veterinary practices.

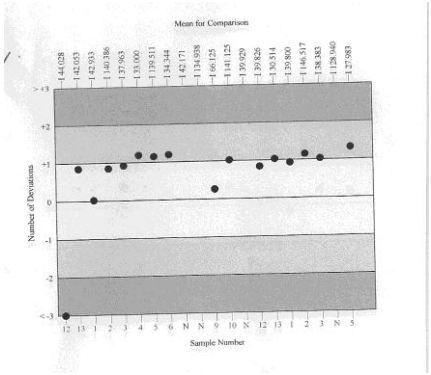
However assessing and monitoring your internal quality control results should be achievable and is recommended. A full discussion of monitoring and assessment is beyond the scope of this introductory day, but simply put, involves plotting/analysing the results of daily control runs over time, such that trends and random human/analyser errors can be identified easily. Depending on the error seen, sources for the error can be evident and therefore corrected before they adversely affect any of your patients or tests.

A crude way of identifying certain (primarily random) errors can be to run a patient sample in house and compare this to the results produced by a commercial lab on the same sample. This method though does have certain pitfalls:

- the in house analysis must be done after the same storage time and conditions (often 24 hrs for postal delay) as some parameters (especially haematological) will change with time. This timing can be (very) difficult to achieve.
- ideally the commercial lab uses the same methodology *i.e.* same reaction to measure ALP levels. This is rarely the case and so direct comparison is very limited. The changes in profile pattern though (such as liver enzyme elevations) or individual unexpected results may be assessed/compared.

This method is generally only advisable for checking individual patient profiles/samples rather than routine quality management of a practice lab.

It is the inherent difficulty of establishing and running such external schemes for the multitude of veterinary practice labs that has in part prevented widespread participation and use. 'Internal' assessment is arguably therefore more crucial, but is also rarely performed. This in part reflects lack of appreciation of sources of analytical error, but also the perceived time and effort required.



Many modern analysers store all the control data they produce which can then easily be printed out and assessed. Initiating the analytical process is intensive and time consuming, but when established, continued monitoring is relatively simple and definitely provides very valuable information and confidence in the results your analysers produce.

Again, detail of what this 'internal' assessment/analysis involves is too much for this introduction, but a noteworthy point is the use of Levey-Jennings plots (figure above), where the control results are plotted over time against a background scale showing the mean and standard deviations. In this way, error points are obvious and evident, and trends can be seen that can allow pre-emptive intervention to correct a problem. This allows many random and systematic errors (bias), both human and analyser, to be detected and addressed.

A final noteworthy point is that a few, somewhat unique, analysers are not subject to this quality control. An example is the I-STAT. Every time this is used, it is essentially a 'new' machine, and therefore cannot be monitored in the same fashion. Batches of test material can be checked against each other, but otherwise, presuming the machine is used and maintained properly, it should perform as expected and less commonly produce errors.

Standard Operational Procedures

Whenever a test is run, it involves humans and analysers/equipment interacting. As people and machines vary from one to the next, variables are introduced that can affect the final result produced. The more variables that are/can be controlled, the more likely the only variable that is changing in the test is the one that is being tested for (*e.g.* ALP). It also concerns the production of reliable results that are reproducible and can then be trusted, allowing confident interpretation by the clinician. No test or procedure is foolproof and mistakes will be made. To minimise this personnel must be trained and familiar with the analysers and methods they are using, understanding in some fashion what they are doing so that any problems can be identified as early as possible and rectified.

This whole concept often takes the form of standard operating procedures (SOPs), which strive to minimise or eliminate the variation that can be introduced by various/different people performing a test. SOPs are instructions written down such that 'anyone' can walk in, locate the necessary equipment, reagents etc, perform the test and produce a reliable result that can be used/trusted. SOPs may incorporate or simply be the analyser's instruction/operating manual. Certain tests, such as blood film examination require a degree of training and specialisation, but many tests, such as a manual PCV, should be able to be performed by any person in the practice when following the SOP for that test, obviously after initial supervision and familiarisation.

Whilst it is certainly laborious and painstaking producing SOPs for (all) the tests run in a lab, writing them down ensures that the process is fully understood (by everyone) so all necessary steps are done and staff confident they are performing the test correctly. This standardised approach produces a result that everyone knows they can trust. Too many times people assume a test is done in a certain fashion by everyone in the practice when it isn't. This can and does:

- create discontent in staff;
- make serial monitoring difficult if different people perform the test;
- falsely create a medical problem (*e.g.* anaemia) that doesn't exist;
- waste valuable time and money.

Once a SOP is written, each person performs the test, and if correctly and confidently performed, is 'signed off'. Everyone follows the SOP when performing the test, and if new information, reagents etc are obtained, it can be modified as necessary. Having a SOP also ensures continuity and cover if one person who is often responsible for the lab work is away or unavailable.

Laboratory Error

This is commonly broken down into three components: pre-analytical, analytical and post analytical. This separation is useful to identify potential sources of error that may otherwise be ignored or missed.

Pre-analytical

This is primarily to do with sample collection and then handling in house or submission of the sample to an external/reference lab. Much of this will be discussed specifically within areas covered later, but emphasis is placed on good communication between the clinician/practice and the lab regarding sample requirements, timing etc, especially if unfamiliar with the desired test. For example, submitting a sample for clotting times in a sodium citrate anticoagulant tube requires the tube to be filled to the correct level - too much may artificially prolong clotting times due to incomplete reversal when tested.

This stage incorporates physiological variation, such as the age of the animal, recent feeding or drug therapy. For example drug monitoring is typically performed at peak levels (commonly 4-6 hours after dosing). However, if this timing is not adhered to or noted, the level measured may be 'wrong' simply due to incorrect sample timing rather than poor therapeutic dosing. In some ways, sample timing depends on the question being asked. For example, if the clinician is worried about toxicity (*e.g.* digoxin), assessing the peak level is best. However, if inadequate dosing is of concern (*e.g.* epileptic fits in the face of therapy) assessing the lowest drug level (just before the next tablet is due) may be more appropriate. Occasionally multiple sample times may be required for accurate interpretation. Once the sample has been taken, certain substances can require immediate processing or analysis as delayed analysis alters their level in the sample. For example glucose levels can decrease at about 3-10% per hour if the serum/plasma is not separated from the cells (see earlier). This rate of reduction is increased if the cell counts are higher (*e.g.* increased red or white cell numbers) or the ambient temperature is raised (such as in the summer).

Legible and complete sample labelling is vital and unfortunately can often be missed or incorrect. Whilst this error can often be overcome, it creates uncertainty in the result produced or prevents analysis altogether. Ideally, labelling is best done before the sample is taken, especially with multiple samples or patients

For haematology, one of the commonest errors is sample ageing, especially if there is no fresh blood film made. In biochemistry, haemolysis, lipaemia and icterus are the major problems but are often avoidable or can be minimised by appropriate sample handling.

Analytical

This may be introduced by pre-analytical factors already mentioned, such as haemolysis, but also encompasses the actual method used to measure the required parameter. Intrinsically analytical error is minimised by quality management as already discussed. For example, a new reagent or batch of test strips should be tested on a sample alongside those already in use to ensure the new material performs in a similar fashion. This is often assumed as they are provided by the manufacturer, but variation can be seen even with these. The calibrator and control material for this has already been mentioned, and these are used to ensure the *accuracy* and *precision* of each test. Ideally a test is both accurate and precise. Even if it is, every time it is used there will be some (minimal) variation even when measuring exactly the same thing each time.

Accuracy – is my value the correct value?

Accuracy is a measure of the systematic error or bias. If a sample has a known level of something, such as ALP, and your analyser measures the ALP in this sample, it will produce a slightly different level/value. The closer this value is to the actual value/level, the more accurate your analyser is for that test. Another way of looking at this is how close your method (of measurement) can get to the 'true' value. If your method is inaccurate, you will find it will consistently give too high or too low a value, potentially producing or missing an abnormal value (or animal).

Precision – can I always get the correct value?

Precision is a measure of the random error. Following on from above, if ALP is measured a number of times, the closer each of the values is to each other, the more precise the method is. Another way of looking at this is the reproducibility of the method. If your method is imprecise, the more variation there is every time the test is performed, and the more chance there is that it is the wrong value.

If your analyser is both accurate and precise for a test, it should produce the 'true' value every time it is run. In reality no test method is 100% accurate and 100% precise. Thus you will find that if you run a sample several times through an analyser (immediately after each other and also on sequential days), the numbers/levels/values produced will vary slightly every time it is run. This concept is very important when serially monitoring a patient. If you find that initially you had an ALT (alanine aminotransferase) level of 145, and then you check the level again in a week's time and find it is now 200 there are two interpretations.

- If your analyser is precise and accurate, you can be confident that this change indicates a genuine increase and possibly worsening liver disease.
- However, if your analyser is not accurate or precise you do not know whether this change is just your analyser or a genuine change in the patient.

Ideally each practice should assess its analysers and every test in this fashion, from measuring a PCV through to a complete biochemical profile. Whilst this is obviously time consuming, and to a degree expensive in terms of reagent use, it gives you maximum confidence in what your lab is actually capable of detecting. Manufacturers of the analyser should provide much of this information if requested. A detailed discussion of what is involved to generate all this is not possible here, but simplistically it involves taking samples and for each test (enzymes, electrolytes, PCV *etc.*) making repeated measurements(10-20) immediately after each other, then ideally with different operators and finally over a couple of days; all on the same sample(s). This need only be done once, presuming conditions do not change. It will give you a minimum and maximum value for each test and allow you to assess what your analyser/test can do. For example, is my ALP value of 145 actually anywhere between 125 and 155?

Very few, if any, practices have done this with their lab, not because it doesn't provide very useful information, but because of time constraints, finances potentially and also lack of appreciation how much this can influence the final clinical interpretation. However, you will find many practices and their staff, vets and nurses, know that some of their tests are 'good' and others are 'bad' (or not trustworthy). This 'experience led' assessment demonstrates the principle, but a more detailed and systematic approach would provide much more confidence and certainty.

Interference:

This primarily covers a sample that is haemolysed (red), lipaemic (white) or icteric (yellow), but drugs *etc.* can also be a factor. Again, specific examples will be covered later, but this can be an overlooked when interpreting laboratory results. Many practice lab analysers provide a result printout that includes a scoring system for haemolysis, lipaemia and icterus. **You cannot interpret a result without taking these into account**. Every analyser will be affected to varying degrees by the interference these produce. Most in house analysers are 'dry chemistry' methods, and so less affected by these interferences, but they can still be a factor. The interference can either be indirect due to simple physical light scattering or direct due to substances, such as bilirubin, which participate in the reaction being measured. For example, all three will typically falsely elevate the total protein level measured via refractometry, whilst bilirubin can inhibit the measurement of creatinine directly, creating a falsely low creatinine value. A lipaemic sample is often due to a non fasted sample. As most reference values/intervals are based on fasted samples, levels of substances such as glucose and cholesterol will naturally be elevated if the animal has just eaten but do not mean the animal has diabetes mellitus.

Whilst this interference can be similar from analyser to analyser, and reaction to reaction, it is often very specific, artificially elevating an enzyme level such as ALP in one analyser/reaction whilst on another it falsely decreases the ALP level. Therefore each lab and analyser should have these effects evaluated. Analysers that produce a scoring system can provide an 'interferogram' – a list that grades the interfering factor, such as haemolysis, and identifies which substances/tests are affected at which level. Analyser manufacturers should again have or publish this information. The interference can be great enough to prevent the sample/test being measured. If an interferogram is not available, a practice can and should create one itself unless all such samples are to be rejected. This is relatively straight forward, involving:

- a subjective visual scoring system (grading from (+) to +++ commonly) based on reading standard newsprint through the plasma/serum.
- adding varying amounts of the interferent to the serum/plasma and then running a profile or tests
- lipaemia and icterus can be created by purchasing standard stock solutions of lipids and bilirubin respectively. Haemolysis can be created by physically lysing a blood clot. A more variable way of creating lipaemia, but arguably better as it represents a more genuine model, is to health screen 'consenting' animals that have recently been fed a fatty meal.
- Products such as oxyglobin and colloids should also be assessed if used in the practice, as these can also affect many test measurements.
- Lipaemia and haemolysis can usually be avoided or minimised. Icterus is part of a disease state, so unavoidable but still of note due to its potential interference.

Post analytical

This commonly involves transcriptional errors, with the wrong results going to the wrong patient, incorrect typing or incorrect reference values. Often all the results are attached to the patients file electronically which is generally safest. They may be manually typed into the file, which is laborious, or only the abnormal results summarised. The last style requires both consistency within the practice about what is transferred as well as what is deemed abnormal – anything outside a reference interval or only those thought to be significant? Post analytical error can therefore incorporate the actual interpretation of the values/results, which requires detailed knowledge of the clinical context, signs, history, drugs *etc.*

Reference Intervals / 'Ranges'

These are required to judge whether a result is 'normal' or 'abnormal' and thus allow interpretation as to whether disease is present or not. Typically most in practice analysers are provided with appropriate and presumably accurate reference intervals/ranges. It can be worth enquiring how these were actually produced, especially when first purchasing an analyser, as it is the author's experience that some analysers have very unusual reference intervals compared to the 'average' values seen with most analysers.

Haematological and electrolyte values are often very similar from analyser to analyser, as the technology is fundamentally the same. However there are a huge number of enzyme reactions that can be used to measure biochemical parameters, and thus you should generally only use the reference intervals/values provided for your analyser. Direct comparison from analyser to analyser may not be possible.

There are various features when creating and using a reference interval that will not be discussed. The vast majority of intervals/ranges though describe 95% of the population/animals in question. This means that 5% of healthy animals will fall outside the reference interval/range without having clinical disease – 2.5 % above and 2.5% below the interval/range. One in twenty healthy animals will have an abnormal result and if a biochemical profile/panel has twenty tests within it, there is a 64% chance that one result will be 'abnormal' for every animal. This raises the question of when a result is genuinely 'abnormal' or not. This is especially true for screening healthy individuals, such as occurs with pre-anaesthetic profiles. They can identify serious medical problems, but can also be grossly misused if the true significance of any abnormal result is not appreciated. Assessing this significance relies upon and introduces the concept of sensitivity and specificity.

Sensitivity and specificity

These two words in part describe the clinical usefulness to which all lab tests are subject. Typically they are expressed as a percentage whereby one test is compared and assessed, relative to a 'gold standard' test, in identifying a certain disease. Sensitivity describes the ability of the test to identify animals with the disease versus healthy animals (*i.e.* a positive result with the test means the animal **has** the disease). In other words, this measures the true positives. Specificity is related to this, but describes the ability of the test to identify healthy animals from those that are diseased. In other words it measures the true negatives – those that do **not** have the disease.

Ideally any test we perform would be both very (100%) sensitive and specific, but this is rarely, if ever, achieved. Typically as a test becomes more sensitive, it also becomes less specific *i.e.* the more likely we are to identify a diseased animal, the more likely we are also to falsely identify a healthy animal as diseased (false positives). Conversely if a test is not very sensitive, many diseased animals are missed (false negatives). **If a test is only 50 % specific or sensitive, it is just as useful to toss a coin as perform the test**.

This idea of specificity and sensitivity is vital when considering screening tests. Ideally a screening test is highly sensitive so that diseased animals are not missed. However, as using this test will probably falsely identify healthy animals as diseased, it must be followed by further confirmatory tests that are ideally more specific, especially if the consequences of the test are serious (*e.g.* euthanasia).

Both these factors (sensitivity and specificity) are inherent to every test, but their use and application vary depending on the population tested, creating what is called the positive and negative predictive values for a test. Take FIV screening as an example.

If we assume that FIV has a prevalence of 10%, then if 1000 animals are tested, 100 will have FIV. If an FIV screening test has a sensitivity of 90% and a specificity of 95%, it will identify 90 animals as positive for FIV, with 10 being false negatives. Of the 900 animals that are negative for the virus, this test will correctly identify 855 whilst 45 will be falsely positive
These values create a negative predictive value of 99% and a positive predictive value of 67%. **This test therefore provides a very confident negative result but a positive result is going to be wrong a third of the time**. This predictive value is dramatically altered if we test a different population where FIV is only seen in 1% of animals, such as may occur when screening healthy cats. In this situation, the negative predictive value increases to 99.9% whereas the positive predictive value falls to only 16%. **We can be even more confident that with a negative result the cat does not have the disease, but with a positive result, most of the time this will be wrong (a false positive). Just because a result is positive or negative, does not necessarily diagnose or exclude a disease**.

Getting to grips with and understanding these concepts is difficult but is crucial and vital to understanding what a test tells you and also whether it is even appropriate to perform the test. Examples could include pre-anaesthetic screening and viral testing all stray cats that come into the practice. These concepts are not directly related to actually performing the tests, more their interpretation. They can though be compounded by poor understanding of the actual test being performed. For example, continuing the FIV scenario, as in house 'snap' FIV tests are serological (they detect the cat's anti-FIV antibodies), a period of time is required from virus exposure to the cat mounting a response and seroconverting (*i.e.* for us to be able to detect the cats antibodies). This can take weeks to months and so testing for FIV in an acutely ill cat can be inappropriate, or at the least, require retesting in the near future to confirm a negative result is 'genuine'.

HAEMATOLOGY

This course will focus on the sampling, processing and examination of samples for haematology, but will only briefly address the clinical interpretation or significance of any findings or tests.

Sampling

As a general rule, venupuncture should be **clean and quick**, using as **large** a needle as possible with **minimal** or gentle suction only. **Typically this involves using the jugular vein with a 21-23 gauge needle and 2.5-5 ml syringe**. A peripheral vein, especially the cephalic, can be used in medium to large breed dogs as it can be easier to locate and sample (in overweight animals), with some animals also being easier to restrain for this sampling site. It is better to get a clean and quick sample than make repeated attempts at any single vein. If the first attempt fails, it is advisable to use a new needle (and possibly syringe), changing the venupuncture site slightly or using a different vein. Unless the animal is very small (*e.g.* less than 2.5 kg) a 21 gauge needle is recommended.

All these guidelines aim to minimise trauma to the cells and therefore the risk of artefact and cell lysis/haemolysis. Haemolysis will reduce red cell numbers, PCV and artificially elevate the cell haemoglobin concentrations. A clean technique also minimises tissue damage and activation of platelets during sampling. Therefore platelet clumping and clot formation is prevented. If either is present, platelet numbers will be artificially low and potentially white cells too, especially neutrophils.

The blood is typically placed into EDTA (ethylenediamine tetra-acetic acid) anticoagulant for many veterinary species. **The tube should be filled, without the needle attached, and to the correct level** for several reasons:

- underfilling results in a relative anticoagulant excess, especially if it is in liquid form. This excess shrinks the cells and alters their morphology. It can thus also artificially reduce the MCV and PCV. **If the tube is less than half full, the PCV can be reduced by 5%.** These effects are compounded if the animal is actually anaemic or cytopoenic (reduced cell numbers).
- overfilling will allow clotting to occur, which can prevent automated analyser analysis, and alter cell numbers as outlined above.
- if the needle is still in place, the cells are traumatised and more likely to lyse when in the tube.

The blood should be gently mixed with the anticoagulant by inversion and rolling in your hand, **not** shaking**. At least one fresh blood smear/film should now be made**. This is especially true if the sample is going via the post to an external lab. Cells begin to degenerate as soon as they leave the body, with significant changes being seen after 12 hours.

Whilst one can nearly always interpret morphology in aged samples, subtle features are often lost, artificial features are created or clinical interpretation is hampered. Once the smears are made, the sample can be processed or stored in the fridge if being sent to an external referral lab. The slides should **not** be stored in the fridge though, as condensation following removal can damage the cells or wash the sample off.

If chemical restraint or sampling occurs under general anaesthesia, this must be noted. Changes in blood pressure can have very significant effects on circulating cell numbers. This is seen naturally/physiologically in animals that are agitated during sampling. Increased blood pressure (and splenic contraction) results in increased numbers of circulating white cells (especially neutrophils) and red cells. Conversely, sedation or anaesthesia lowers systemic blood pressure and causes splenic sequestration that can reduce the number of circulating cells, both red cells (falsely low PCV) and white cells (false leukopaenia).

In summary:

Haemolysis is caused by:

- excessive suction
- narrow gauge needle (especially if left on when filling the tube)
- shaking the tube
- prolonged storage, especially at high temperature or with large temperature fluctuations
- lipaemic samples, especially over time / with postage

Sampling technique:

1. Label EDTA tube.
2. Use 2.5-5 ml syringe and 21 gauge needle in the jugular vein.
3. Ensure the venupuncture is clean and quick.
4. Remove the needle and gently expel the blood into the EDTA tube.
5. Fill to the correct level (indicated by a small black line commonly). Filling >50% at least is recommended.
6. Gently mix by rolling and inverting the tube.
7. Make a fresh blood film from the EDTA tube.
8. Store the tube in the fridge if not analysing immediately

N.B. Ideally, leave the blood in the EDTA at least 10 mins before analysing. This prevents unknown artifacts being introduced as the blood and EDTA have time to equilibrate.

COMPLETE/ FULL BLOOD COUNT (CBC / FBC)

In house analysis varies from a simple microhaematocrit and plasma protein measurement to a full analysis - five part white cell differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils), red cell parameters (RBC, Hb, HCT/PCV, MCV, MCH and MCHC) including reticulocytes, along with platelets - all depending on the equipment available. **Both scenarios though must involve assessment of a fresh blood film.** A huge amount of information can be obtained from only a microhaematocrit tube and blood film examination.

The numbers produced are only a summary of the analysed information. Looking at the graphs produced is also very important as it can show many significant changes that would otherwise be missed or highlight possible pitfalls that require additional checks and blood smear examination to confirm or change the numbers.

Packed Cell Volume (PCV)

This refers to the volume of red cells that is produced after high speed (11-15000 rpm) centrifugation of whole blood in a microhaematocrit tube for 5 minutes. Whilst not identical, it is equivalent to the calculated Haematocrit (HCT) value produced by an automated haematology analyser. Unlike the PCV that can be affected by excess EDTA significantly (see earlier), HCT from an analyser can be less affected as the cells are diluted and 're-expand' when being analysed. However, HCT can be affected artificially by factors such as agglutination and prolonged EDTA exposure, as these affect the measured MCV.

Centrifugation produces a variable column of cells in the form of bands, as shown later. At the bottom (A) is the clay plug.; band B is the red cells; band C is the buffy coat - white cells and platelets – and band D is the plasma. The width of band B is expressed as a percentage of the total length (B + C +D) to give the PCV (*i.e.* B / (B+C+D) x 100). A crude estimate of the white cell count can similarly be obtained from expressing band C as a percentage of the total length. The first 1 % equates to 10 x 10^9/l, with every additional 2 % being an extra 20 x 10^9/l.

A B C D

When performing a manual PCV in this fashion, the microhaematocrit tube **must be filled 2/3 to 3/4** as the centrifugal force produced and required is based on this volume of fluid. If there is too little, the relative force is increased, whereas if there is too much, the relative force is reduced. This is often not clinically significant, but can be, artificially altering the PCV value when compounded by changes in cell numbers of the animal itself. For example, an anaemic animal (< 25%) that is also under filled can result in an artificially lower PCV. Similarly, if the red cell numbers are increased (PCV >50-55%), the PCV can be artificially high as the cells are not packed very well by standard centrifugation.

Two other important pieces of information are also readily available once the tube has been spun. These are the plasma protein level and plasma colour. The plasma protein is measured after the tube is gently broken (with a diamond cutter commonly) at the mid-level of the plasma column. A drop of plasma is then placed onto a refractometer. As with urinary specific gravity, light is bent to varying degrees depending upon the amount of substances dissolved in the liquid or plasma. A different scale is used from urine, with the value/level being multiplied by 10 to give the plasma protein in g/l. As with urinary SG, if the liquid is not 'clear', the level/value will be artificially raised. Thus, haemolysis, lipaemia and to a lesser degree, icterus, can all falsely elevate the protein level measured via refractometry. Measuring the biochemical total protein level is often more accurate, but can be similarly affected, although to a lesser degree.

Assessing the plasma colour is therefore useful to assess the accuracy of your plasma protein level. If the plasma is grossly haemolysed or icteric and the animal is anaemic, haemolytic diseases are immediately suggested. Assessing the blood film can then provide further evidence. Similarly, if the plasma is lipaemic, and the animal has been fasted appropriately, underlying hormonal diseases may be suggested. It must be emphasised that even if the plasma looks clear, free haemoglobin (haemolysis), increased lipids (lipaemia) or bilirubin (icterus) can still be present. For example it requires about 20 µmol/l of bilirubin (healthy levels typically being <7 µmol/l) to be visible to the naked eye. Therefore biochemical analysis will detect increased bilirubin before we can see it.

Whilst these may not seem useful, or better evaluated via an analyser, if the plasma is discoloured or the protein levels elevated, they can provide a cheaper (and quicker) way of monitoring disease states (such as icterus in liver disease or hyperglobulinaemia in multiple myeloma).

Finally, the colour of the plasma also allows you to assess whether there is any potential interference to automated analyser analysis.

- **Haemolysis** – this falsely reduces the red cell (RBC) numbers (and potentially PCV), whilst falsely elevating the Hb, MCH and MCHC.
- **Lipaemia** – can induce haemolysis. In itself, it can falsely elevate the haemoglobin parameters and artificially elevate platelet numbers due to lipid droplets or cell fragments being miscounted as platelets
- **Icterus** – this does not usually have any significant effect.

Automated analyser cell counting

Before any blood is run through an analyser, it should be:

- checked for clots using either a microhaematocrit tube or cocktail stick run around the tube to locate any clotted material. If not done, cell counts can be artificially affected or the analyser blocked/damaged.
- gently and evenly resuspended, especially if the tube has been left standing for a period of time. If this is not done, cell counts can be artificially affected.

There are a large number of haematology analysers available, **none of which can provide results that can be interpreted without also looking at a fresh blood film**. Most analysers provide 6 red cell parameters, a white cell count and platelet count. Most also provide a variable white cell differential that can be very inaccurate or misleading, although newer laser cytometer analysers giving a 5 cell differentiation (neutrophils, lymphocytes, monocytes, eosinophils and basophils) can be good in many healthy and some diseased situations. Even haematology analysers in commercial referral labs, whilst costing many thousands of pounds more, can and do make differential errors. For this reason, **confirming white cell differentials and assessing cell morphology in a blood film is a vital part of the complete blood count**.

However, if you have a good analyser, especially the newer laser cytometers, as the analyser counts thousands of cells, the numerical accuracy of the analyser is superior to any manual count that typically uses only 100-500 cells. An automated differential from such analysers is therefore only altered if your manual film differential is significantly different. Other analysers that cannot reliably differentiate cells should only be used to provide total cell counts and then the differential has to be provided by your film examination.

Impedance Counters

These analysers count cells using impedance, whereby the cells are diluted in an electrolyte solution and then drawn through an aperture within an electrode. As each cell passes through, it creates a pulse of resistance – the more pulses, the more cells there are (Coulter principle). The size of each resistance (impedance) pulse equates to the size of cell. Impedance counters must therefore be adjusted for each species so that the threshold size for each cell type is known. If there is overlap between two cells types, impedance counters can fail to distinguish the cells and ascribe them to the wrong type. In this fashion, the red cells are counted and only distinguished from platelets by platelets being smaller than red cells. This is true for many veterinary species, especially dogs. However, cats have smaller red cells and larger platelets, and thus the analyser can confuse platelets for red cells, or vice versa. Similarly, if a disease creates small red cells or very large platelets (such as iron deficiency or inflammation respectively) this error can be exaggerated.

The white cells are also counted in this fashion, but only after the red cells have been lysed in another solution. This lysing solution can be used to produce variable effects on white cells allowing white cell differentials to be automatically produced. However, this lysis works primarily on anucleate cells (such as red cells), but some are resistant, especially immature nucleated red cells. The analyser therefore 'assumes' that only white cells are present which may not be true, especially in diseased states. In this example, a nucleated red cell appears 'identical' to a mature lymphocyte. Therefore most impedance 'white cell counts' are in fact a nucleated cell count, and so if nucleated red cells are present in significant numbers, the white cell count is artificially elevated and needs correcting. Newer, very expensive commercial analysers can inherently do this, but otherwise it relies on blood film examination. The calculation is as follows:

corrected (true) white cell count = 100 / (nRBC + 100) x nucleated cell count.

Once cells are counted and the sizes and haemoglobin (Hb) levels measured, the analyser calculates several red cell parameters. The HCT, Mean Cell Haemoglobin Concentration (MCHC) and Mean Cell Haemoglobin (MCH) are calculated as follows:

HCT (l/l) = MCV (fl) x RBC (x 10^{12} /l) / 1000

MCHC (g/dl) = Hb (g/dl) / HCT (l/l)

MCH (pg) = Hb (g/dl) x 10 / RBC (x 10^{12} /l)

Laser flow cytometers

These are the basis for most commercial referral lab analysers, but are only recently available as affordable bench top in house analysers. A stream of single file cells is passed through a laser beam, resulting in scattering of the light. This scatter is both forward (related to the size of the cell) and also to the side (related to the complexity/granularity of the cell). This scattering effect can be enhanced by use of certain lysing solutions to leave only a few cell types for analysis, and also the use of dyes for granules that fluoresce under laser light. A number of graphical plots are produced to aid analysis and interpretation. These produce reliable red and white cell analysis in some situations, but are still subject to artefacts or problems during many diseases.

All the analyser problems outlined above are more significant and dramatic the more diseased an animal is, and especially if the disease is haematological (*e.g.* leukaemia). A blood film must therefore **always** be examined to check that what the analyser is telling you is correct. **Blood film examination also allows you to assess cell morphology that can provide additional valuable information about disease processes.**

MCHC is a useful parameter for quality control and monitoring the analyser's performance. This parameter is calculated from the Haemoglobin (Hb) and haematocrit (HCT) in many machines (excluding the QBC) by using two independent techniques/systems. As the concentration is very similar from species to species, it therefore provides a good marker. Values less than 28 are likely artifactual, as even diseases such as iron deficiency rarely produce values this low. Similarly if the value is elevated, artefact is again supported, such as agglutination, haemolysis or lipaemia. If this value falls outside the reference values, assessing for potential artefact or interference would be recommended initially, before ascribing the value to a disease.

A good general rule related to this is that the Hb value should be one third the HCT/PCV +/- 3.

BLOOD FILM EXAMINATION

Preparation:

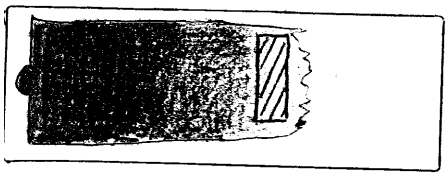
This is made from a small drop of anti-coagulated blood placed at one end of a clean glass slide and then dragged with another slide to create a thin film of blood with an even feathered edge and cell monolayer. This is the 'wedge' or 'pull & drag' technique. The slide should be labelled with the patient name or number *etc.* at one end.

1. Slides: Label one slide with the identification and date. A 'manufactured' spreader slide is ideal to create the film, although a normal slide can be used at an angle to create the same effect. It ensures the blood is not spread over the edge of the labelled slide. It can be created by breaking the corner off a normal slide.

N.B. The spreader slide should be cleaned after each use and intermittently with water, being replaced on a regular basis. Otherwise dried deposits and gradual roughening of the spreading edge occurs, creating an uneven and poor blood film.

2. The EDTA sample is gently resuspended and mixed and then a small drop of blood is placed at one end of the labelled slide using a fine pipette or capillary tube.
3. The spreader slide is held between your thumb and second finger, your index finger being placed gently on top to ensure even pressure is maintained. It is then held at **about 30°** and slid back to meet the drop of blood.
4. The blood is allowed to spread along this edge. When fully along the edge, the spreader slide is advanced **smoothly and briskly**. An even 'square' feathered edge should naturally be produced about 2/3 to 3/4 of the way along the labelled slide. Do not lift the spreader slide off until the feathering is produced. If this does not occur, please see troubleshooting later.

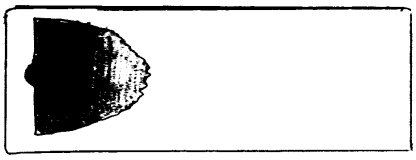
5. Allow the smear to air-dry fully before staining. This process can be quickened if necessary by gently heating the reverse side of the slide with warm air from a hairdryer. Ensure the air is not hot or directly onto the smear itself. Generally though this quickening is not required.



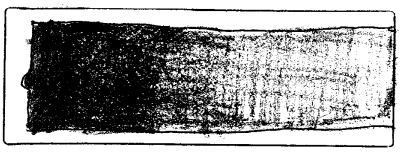
Slides with a frosted end are ideal as they allow easier labelling. The slide should be clean and handled only at the edges. Grease from fingerprints will impair even blood dispersal and contaminants (such as bacteria, fungi or skin cells) from the slide box or environment can complicate interpretation. **Producing a good quality film requires practice and consistency, but is essential for reliable examination and subsequent interpretation**. Artefacts can be introduced both in numbers, due to uneven dispersal, and cell morphology, such as slow drying due to an excessively thick film. Examples of poor films are shown below, alongside possible reasons and solutions.

Troubleshooting:

Film too short: too little blood – use a slightly larger drop
spreader slide angle too steep – check angle is near 30°
advancement is too fast – slow speed down.



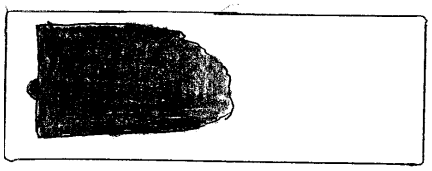
Film too long: too much blood – use a slightly smaller drop.
spreader slide too shallow – check angle is near 30°
advancement is too slow – speed up slightly



Film too thick: too much blood – use a slightly smaller drop.

spreader slide angle too steep – check angle is near 30°

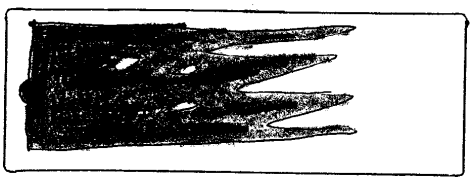
advancement is too fast – slow speed down.



Film has holes or streaks:

greasy slide – clean slides (with acetone/alcohol), ensuring no fingerprints and only sides are handled.

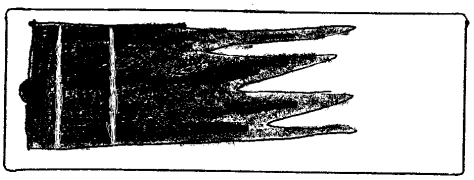
spreader slide is dirty or damaged. Clean or replace it.



Thick line(s) seen:

Uneven pressure, allows blood to escape in front of the spreader slide. Ensure index finger is placed on top of the spreader slide.

Advancement not smooth – ensure constant speed is maintained.



If the animal is anaemic or has increased numbers of red cells then the above technique, especially the angle, may need modifying to produce a good quality film. Anaemic blood has a tendency to spread too fast and far, whereas increased numbers of red cells make the film excessively thick or short. If the problem is severe enough, it may not be possible to produce a standard good quality film.

Staining

In house staining invariably involves three separate liquids into which the slide is 'dunked' a variable number of times ('Diff Quik'). There are a large number of these stains available. They should be replaced (not just topped up) and the containers (*e.g.* Coplin jars) cleaned on a regular basis, as the staining material becomes depleted with use and stain precipitates form with time. Both these factors will prevent or hamper examination of the film and its cells. The first clear liquid is a fixative, the second is an orange dye and the third a blue dye.

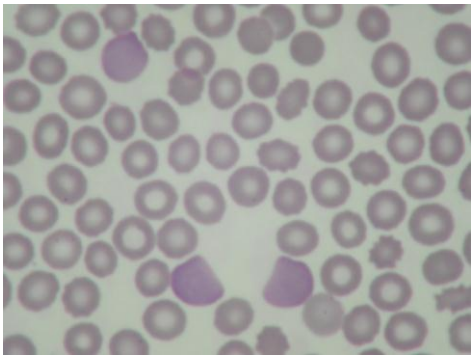
Typically the manufacturer's guidelines should be followed for the timing and number of 'dunks' required. However, it is worth checking that the colour scheme produced is acceptable for you. For example, eosinophil granules should be orange to pink, neutrophil cytoplasm clear and nuclei dark purple. Often people use too little blue dye or too much orange dye. As a rough rule, after fixing in the first liquid, dunk in the orange dye until an even orange covering is produced on the slide, and then similarly with the blue dye.

Many people rinse any remaining dye off with tap water. This can be fine, especially if gently applied to the back of the slide and not directly to the cells. However, it is better to use specific buffered or distilled water as this is gentler to the cells, is consistent and creates little artefactual change. Tap water can wash out the dyes and alter the cell shape artificially. Buffered water is easily made by adding buffer tablets to distilled water. Once rinsed, the slide is allowed to air-dry. As with initial smearing, this can be quickened by judicial use of a hairdryer.

Slide examination

The slide is now ready for examination at low power (x4, x10 or x20). Many practice microscopes have a x40 objective lens that is designed to be used with mounted slides. Without this covering, cells viewed at this magnification will appear blurred and you will be unable to see fine detail within the cells. Whilst mounting slides with glued coverslips is an approach, few, if any, practices will have the time or inclination to do this. A slightly messier alternative, but as good, is to place a drop of immersion oil on the slide and gently lower a coverslip onto this, ensuring no air bubbles are trapped in between. As long as the immersion oil is good quality, you will still be able to place oil on the coverslip itself when using the oil immersion lens (x100). Unfortunately unless carefully used, this often leads to oil contaminating the x40 lens itself, which can eventually damage it and hampers its use elsewhere. Also, if the oil is too thick and poor quality, you will find it difficult to use the x100 lens on an oil mounted coverslip, as moving the slide, moves the coverslip too, blurring the picture viewed. It will not affect use of the low power objectives (x4, 10 or 20).

Any slide examination starts with a low power (x4) scan, followed by sequentially higher powered examination. Much of the blood film examination can be performed at x40, or even x10 or 20 with experience, with x100 only needed for specific detailed examination. Find a way that suits you which is consistent and logical, but covers examination of cell numbers and morphology, from red cells to white cells and platelets. **Examination of cell morphology (size and shape *etc.)* must take place in the monolayer –** the area just behind the feathered edge where the red cells are just touching each other (see hatched area on model blood film earlier and picture below.



If examined in the feathered edge, cells are often distorted, whereas in the thicker areas the cells are poorly spread out and contracted. The following is one way of examining a blood film, with specific cell lines and factors being discussed later.

1. At low power, check the feathered edge and sides for platelet clumps primarily and rarely (larger) abnormal cells and parasites such as Dirofilaria larvae. Check white cells are evenly distributed.
2. Roughly estimate white cell numbers and perform a white cell differential count (see later). Then look at each white cell line specifically for any abnormality or changes. Check for inclusions and cell clumping.
3. At higher power (x40 or x100), first look at the red cells, checking cell shape and colour. Look for any inclusions and check for clumping.
4. At high power (x100) estimate platelet numbers (see next section), checking their size and shape. Check for any inclusions.
5. Check the smear for any atypical or abnormal cells. These are often larger and therefore more commonly found towards the edge of the film, with smaller cells remaining nearer the centre.
6. Check the background colour and appearance. Lipaemic samples often appear slightly blue with fine vacuoles, the cells appearing slightly blurred or out of focus. Elevated protein levels, especially if marked, will give a smooth light blue background, often with increased rouleaux.

Any changes or features noted, such as variation in size, should be subjectively quantified. This allows accurate serial monitoring and detailed clinical interpretation. A standardised practice scheme should be adopted that everyone follows. This may simply be from “occasional, mild, moderate to marked”, or take the form of a grading system like + to ++++. As long as everyone follows and understands it, the exact nature doesn’t matter. Often red cell features are done at x100 with the affected numbers seen in an average field equalling the grade; whereas white cell features are based more specifically on the numbers or percentages affected, say per 100 cells examined.

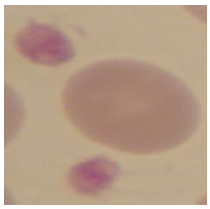
Platelets

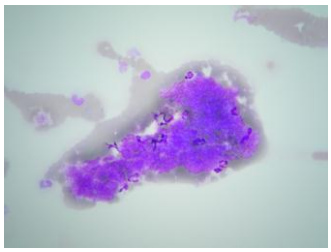
These do not have a nucleus. They are smaller than erythrocytes, often less than a quarter the diameter. They are cytoplasmic fragments, which have an irregular circular outline with a pale purple-pink, granular appearance. In healthy dogs and cats, numbers can be estimated from the blood film. At x100, every platelet seen roughly equates to a platelet count of 15 x 10^9/l. Numbers are counted per five x100 fields and then averaged. Healthy animals typically have between 10-30 platelets per x100 field.

As already stated, platelet clumping must be excluded initially – large clumps at the feathered edge and small (micro-) clumps within the monolayer. The size of the platelets should be assessed. In inflammation or infection, or following increased production of platelets, larger forms can be seen. These so called shift or macro- platelets can be as large, or larger, than an erythrocyte, especially in cats.

Cavalier King Charles dogs are somewhat unique because many have relatively low numbers of very large platelet naturally in health. Automated analysis may give a falsely low count as the platelets are miscounted as red cells. However, they often have low platelet numbers compared to other breeds and usual reference values. This though is not associated with any bleeding tendency.

Certain breeds naturally have lower platelet counts, although the size and shape are similar to other breeds. Greyhounds are an example.





Red cells (erythroid)

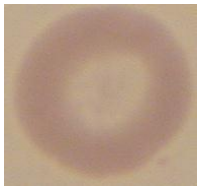
The first recognisable red cell is in the bone marrow and is an **erythroblast.** This matures into a **prorubricyte**, to a **rubricyte**, then **metarubricyte**. At this stage, further cell division cannot occur, the nucleus is lost, and visible haemoglobinisation occurs. This creates a polychromatic **reticulocyte** (see later) that in 24-48 hrs matures into an **erythrocyte**.

Typically the maturation and cell division up to the reticulocyte stage occurs in the bone marrow. In health, small numbers of reticulocytes are released into the circulation, often sequestering in the spleen whilst they mature. During periods of increased demand, when anaemic following haemolysis or haemorrhage for example, the bone marrow releases earlier precursors into the circulation to compensate for the loss of erythrocytes. This maturation process can therefore be seen occurring in the circulation and its features are identified when evaluating anaemic patients for expected and appropriate regeneration.

If the erythrocyte loss is minimal, no early release is seen, but as the severity of loss worsens, the bone marrow will start to release more and earlier immature precursors. This maturation process though should still occur in an 'orderly and regular' fashion, so reticulocytes should dominate, with reducing numbers of earlier immature stages. If this is not seen, the anaemia is inappropriately regenerative and often means bone marrow disease. **Thus the presence of nucleated immature cells alone, without appropriate and associated polychromatic reticulocytes, is not a regenerative process.**

Erythrocytes

In dogs and cats, mature red cells (erythrocytes) do not have a nucleus. They appear as a smooth circle and are slightly orangey pink to brown. In dogs they are slightly larger and have a central pale area (central pallor) that is usually not seen in cats. In dogs red cells are normally roughly equal in size, whereas in cats there is always mild variation in their relative sizes. The central pallor in dogs is usually less than 50% of the cell volume. Descriptively they are called **normocytic** (normal size) and **normochromic** (normal colour). **Hypochromic** erythrocytes have a rim of haemoglobin only, the central pallor filling more than 75% of the cell volume.



Dog

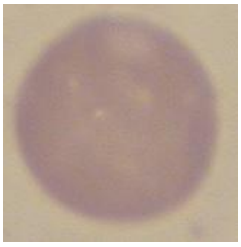
Cat

Anisocytosis –this is variation in cell size. The more relative variation there is in red cell sizes, the greater the anisocytosis. Immature red cells are typically larger than mature ones, and thus increased anisocytosis is often seen during regeneration.

Polychromasia

This means 'many coloured', and causes the cell to appear blue-purple. It is a feature of immature red cells and specifically reticulocytes in the circulation. Reticulocytes are slightly larger than erythrocytes, have this blue-purple colour, often without any central pallor. Only occasional reticulocytes are seen in healthy dogs (1%), with even fewer to none in cats (<0.5%). Increased numbers are seen during regenerative anaemia. Polychromasia grading is essential when assessing whether the anaemia is regenerative or not, presuming 2-3 days has elapsed for the bone marrow to respond.

Normal



Polychromasia

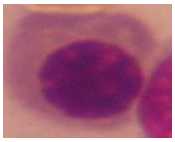
If the anaemia does not appear regenerative because no polychromasia is seen, and presuming the clinical history is more than 2-3 days, the next step may be to perform a reticulocyte count. This is a more sensitive and quantified way of monitoring and evaluating regeneration, but if polychromasia is present, there is little point in performing a reticulocyte count, especially as it is time consuming even though more objective. **Bear in mind if poorly stained, such as too little blue dye, you will not be able to see polychromatic reticulocytes and so misdiagnose an anaemia as non regenerative and potentially then perform different and misleading investigations.**

When required, it can be done in house using a separate supravital stain, such as new methylene blue, but can be best sent to your reference laboratory for accurate counting and more detailed smear evaluation.

Some laser based fluorescent analysers offer a reticulocyte count which if increased supports regeneration (given no software/analytical 'flags') but studies have shown these have a variable or porportional bias so may mis-diagnose mildly regenerative anaemias as non regenerative (ie the analyser has a negative bias).

Nucleated Red Cells

There are four types of nucleated red cell precursor (outlined earlier), the commonest seen in the blood being the metarubricyte. (shown below). The nucleus is typically more central and a neat circle, being a denser more black-purple colour that has fine white fracture lines, occupying about 50% of the cell's volume. Their cytoplasm varies from red cell colour and browny-red to more blue-purple and polychromatic. They are occasionally in health and also in low numbers alongside polychromasia during regenerative anaemias.

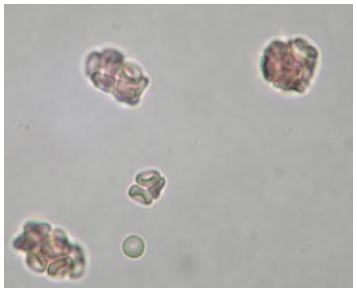


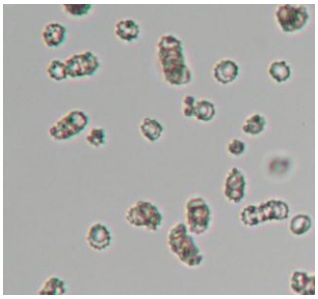
Rouleaux & Agglutination

Rouleaux describe a physiological phenomenon when erythrocytes are clumped together in a chain, similar to a stack of coins. It is commonly seen incidentally in cats but only rarely in dogs. In both, increased and marked rouleaux formation can be seen when the plasma protein levels increase due to globulin production in inflammation, infection or gammopathies associated with diseases like myeloma. **Rouleaux must be distinguished from agglutination.**

Agglutination describes when erythrocytes are seen in tight groups, similar to a bunch of grapes and is associated with immune mediated haemolytic disease (IMHA). It can be visible grossly as flecks or clumps of red cells within the tube. If present, there is no need for further diagnostic testing for IMHA. However, grossly in the tube rouleaux formation can mimic this (to the naked eye). To confirm and distinguish, the sample is examined microscopically after the addition of saline. Rouleaux formations disperse, but agglutination persists. Initially a drop of saline is mixed gently with a drop of EDTA blood on a slide.

Coverslipping aids dispersal and examination microscopically to confirm agglutination.
This process should be repeated with greater saline dilution (up to 1:10, so 1 drop of blood to 9 drops of saline) to confirm genuine agglutination and therefore IMHA. As a general rule, 1:5 for dogs and 1:10 for cats.





There are many breed specific red cell idiosyncrasies that should be excluded before diagnosing disease. For example: Poodles typically have macrocytic erythrocytes, whereas Akitas have microcytic erythrocytes; several breeds, such as Greyhounds, have naturally 'high' PCVs (55-65%).

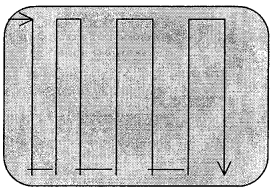
White cells/Leukocytes

White cells derive from two cells of origin – lymphoid and myeloid, myeloid stem cells also generating erythroid/red cells as described earlier. Lymphoid maturation is very different and will not be discussed. The other cell lines eventually differentiate into specific and identifiable precursors in a variable fashion. The granulocytes mature similarly, the earliest recognisable cell being a **myeloblast**, which divides and matures into a granular **promyelocyte**. From here, the three lines split, with distinctive secondary granules forming at the next **myelocyte** stage (so neutrophils become clear (neutral), eosinophils red-orange and basophils blue-purple). **Metamyelocytes** are seen next, which then mature further into **band** forms and finally the mature leukocyte (*i.e*. eosinophilic myelocyte, eosinophilic metamyelocyte, band eosinophil and eosinophil). Cell division continues until the myelocyte stage, so with maturation and storage, there are many more mature cells than immature cells.

Neutrophils are different in having a significant storage pool/buffer within the bone marrow whereas other leukocytes are produced on demand. Excluding lymphocytes, which are essentially recycled/recirculating, leukocytes exist in healthy blood for only a few hours, either destined for tissue pools (such as eosinophils and monocytes) or mucosal loss in the case of neutrophils. Neutrophils also differ in having a significant marginating pool (especially in cats) whereby they transiently adhere and roll along the blood vessel walls. It is this marginating pool that accounts for the sometimes marked physiological increase and decrease that is seen with blood pressure changes as mentioned earlier. It must be remembered that blood sampling typically only involves assessment of the circulating pool at that time, which will be affected or altered by a number of mechanisms.

It is useful initially when evaluating leukocytes in the blood film to estimate at low power (x10) by counting the number of white cells seen per field. An average from at least five such fields should be taken. If the count falls between 18-50 WBCs/x10 field, the total WBC count is likely normal, and correlates well with the automated count. However, at lower and higher numbers, this estimation becomes inaccurate and unreliable.

A differential count is performed from the edge of the monolayer towards the centre, moving in a progressive and systematic fashion, ensuring the same areas is not revisited. This can take the form of a so called 'battlement meander' method (shown below) within the examination area/monolayer (hatched area shown earlier)



A minimum of 100, or ideally 200 or more, cells are counted. Nucleated red cells are included in this differential but then numbers are adjusted as outlined earlier. The percentage of white cells for each type is then multiplied by the total white cell number to given the absolute number of that type.

It is this absolute number that is crucial and should be compared to healthy reference values. If you compare and interpret the percentages, these are inherently relative and therefore reduction in one line must 'increase' another cell line, even if that cell line hasn't altered at all.

In healthy dogs and cats, mature neutrophils are the dominant white cell, followed by lymphocytes, with low numbers of monocytes and eosinophils, with rare to no basophils seen in most differential counts.

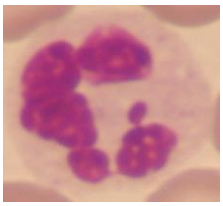
When assessing cell morphology, identifying cell types and any potential abnormality, the following three features must be assessed:

- cell size – relative to the mature erythrocyte
- nuclear shape, texture and colour
- cytoplasmic shape, colour and features

Neutrophil:

These are granulocytes and are also known as polymorphonuclear leukocytes. They are 1.5-2 times the size of an erythrocyte. They have:

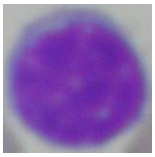
- nucleus – dark purple with indistinct white lines. It is elongated and forms thick segments joined by thinner sections. Typically there are 3-5 segments in cats and dogs.
- cytoplasm – this is smooth and light grey to clear. With some stains it can appear pale pink (due to the granules), but typically the granules do not stain. Inflammation or infection can lead to 'toxic' changes – see p.



Lymphocyte:

These are the smallest white cell, being about 1-1.5 times the size of an erythrocyte. They have:

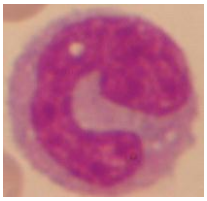
- nucleus – circular to cleaved or indented with smudged to smooth dark purple colour. It is eccentric (off centre).
- cytoplasm – minimal and on one side of the nucleus. Typically light blue and smooth.

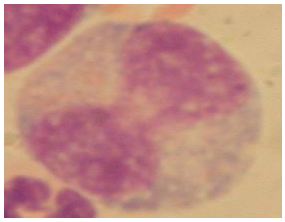


Monocyte:

These are the largest leukocyte and the most variable in appearance. They are at least twice the size of an erythrocyte. They have:

- nucleus – paler purple with more open white lines. Its shape is very variable, from U shaped to dumbbell, convoluted or bean shaped.
- cytoplasm – this is irregular. It is often a variable blue-grey with a pale pink hue and often contains small white vacuoles (holes).

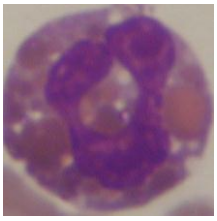




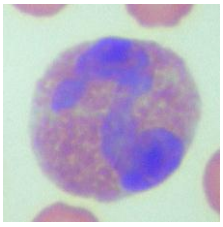
Eosinophil:

These are also granulocytes, and their granules are visible. They are 1.5-2 times the size of an erythrocyte. They have

- nucleus – moderately purple with indistinct white lines (lighter than a neutrophil). It appears segmented but much less so, with thicker segments. Usually only 1-3 lobes are seen.
- cytoplasm – this is variably filled with distinct orange-pink granules, the background being pale grey-blue. Dog granules are usually circular but very variable in size. Cat granules are very uniform, finer and rod shaped, filling the cytoplasm.
- certain dogs, especially Greyhounds, have so called 'grey' eosinophils where the typical orange-pink granules are seen instead as clear white vacuoles. This is incidental as long as the vacuoles are not seen in other cell lines to suggest storage diseases.



Dog

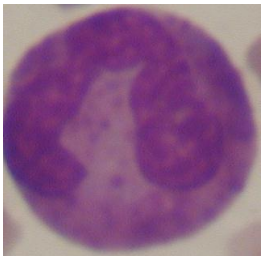


Cat

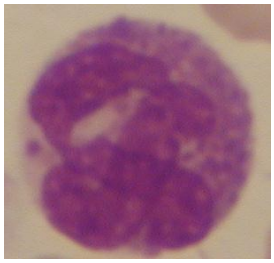
Basophil:

These are also granulocytes and their granules are also visible. They are 1.5-2 times the size of an erythrocyte. They have:

- nucleus – moderately purple with indistinct white lines. It is variably lobed or convoluted, with thicker segments.
- cytoplasm – this is variably filled with distinct purple granules, and the background is pale blue-grey. In the dog they are dark purple granules, but finer lilac/lavender in cats.



Dog



Cat

INFLAMMATION OR INFECTION

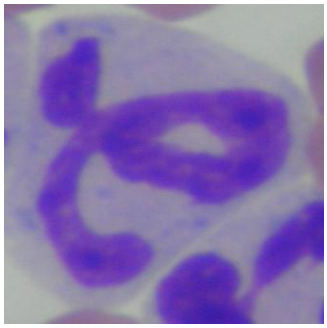
When inflammation or infection is present, one of the primary features can be an increase (or decrease) in the number of certain white cells seen (*e.g.* neutrophilia with increased numbers of mature neutrophils). This change can give an indication as to the disease process (*e.g.* increased eosinophils - eosinophilia – suggests parasitic or allergic disease most commonly). However, changes in the cell morphology can also be useful. This primarily focuses on neutrophils and lymphocytes, but monocytes can show changes to a lesser degree. Increases in certain cell lines (such as neutrophils and lymphocytes) do not necessarily indicate disease.

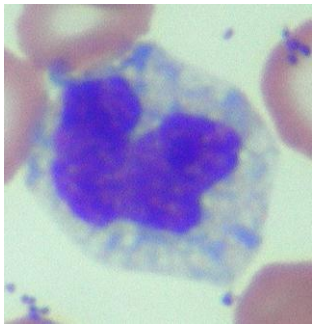
Neutrophils:

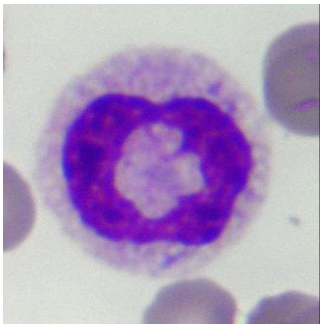
These can show changes that are termed 'toxic' changes when more neutrophils are needed during inflammation or infection. These changes are mainly cytoplasmic, but rarely and if severe enough, can affect the nucleus. They are:

- Dohle bodies can be seen. These are irregular, indiscrete darker blue deposits. In cats, they are often seen in low numbers in healthy animals so must be prominent and seen in many or all neutrophils to indicate toxicity.
- cytoplasm becomes more blue (basophilic)
- cytoplasmic vacuolation, with a foamy appearance.
- toxic granulation with dark pink indistinct granules.
- nucleus can become paler purple or form ring/doughnut shapes.

These changes actually reflect maturation defects that are seen as the bone marrow produces neutrophils so fast that maturation does not occur normally. They are not a specific product of inflammatory or infectious toxins, and are rarely seen in inflammatory/infectious tissue aspirates such as an abscess.







Band neutrophils:

These are immature neutrophils that are only rarely seen in healthy animals. They can be seen during inflammation or infection as demand is so great that the bone marrow has to release immature cells earlier than normal – a so called left shift. This principle is similar to reticulocytes (polychromasia) seen in regenerative anaemia. In these cells and situations, toxic changes can also be seen. The principal defining feature of a band neutrophil is its nucleus.

- nucleus – dark purple with indistinct white lines. It is classically U or C shaped with parallel sides. Uneven indentations can be seen, but these should not form distinct segments or be greater than 50% of the width of the nucleus. If any segments or prominent/full indentations are seen, it is classified as a mature neutrophil.

The number of band neutrophils is usually appropriate and less than the number of mature neutrophils, indicating that whilst increased, the body is able to meet the demand. Neutropoenia or dominant band neutrophilia is significant, especially if persistent, as it indicates that the demand is too great for marrow production and the disease is more severe.