# From saliva to faeces and everything in between- a guide to biochemical analysis using animal samples for biomarker detection.

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Abstract

Over the last decade, interest in the emotional states and stress levels of animals has grown. These emotional states can have secondary effects for owners, for example if an animal becomes aggressive this can lead to relinquishment or even euthanasia. In addition, long term stressful situations can have serious health impacts upon animals and can affect meat quality in livestock. A variety of methods can be used to investigate biomarkers in animals, and many different sample types can be taken to facilitate this. The choice of assay will often depend on the animal under investigation and practicalities of obtaining the sample. The assay choice can also be dependent on testing conditions such as the field vs laboratory, samples taken, costs, and the desired results. There is also the question of the timescales of the investigated response- do you want to test what happened over the last month? Last week? Yesterday? Or within the last hour?

This review highlights some of the pros and cons of the different samples, and the different methods for biomarker analysis in animals. Studies can be made or broken based on the type of samples taken, and what aspects are to be investigated, and this simple decision can make a world of difference to the results of an investigation. Careful planning and thought before starting a study, can make the difference

between a scientific breakthrough with animal welfare and husbandry implications, or poor results which are of little use to man nor beast.

Keywords: Biochemical analysis, ELISA, saliva, blood, hair, faecal

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Careful selection and analysis of biological samples is vital for accurate quantification of animal health, condition, and welfare. This review will cover some of the key considerations for selecting the biological sample for analysis and the type of biochemical test(s) required to analyse the biological markers (biomarkers) present. As an extra complexity, multiple biomarkers can be used for similar types of analysis; for example to evaluate stress; cortisol, amylase, or even IgA are potential biomarkers (Mack & Fokidis 2017; Hong, Oh, Kim & Seo, 2019; Muneta, et al., 2010). As you would imagine, these different biomarkers are all present in the host animal, but they will be at different levels or even totally absent depending on the choice of biological samples (blood plasma, saliva, urine, faeces, etc). The type of biological assay, or the number of steps needed to process/extract the biomarker varies both with the type of sample and the selected biomarker. These biomarkers are often hormones, but can also be enzymes, other proteins or metabolites (including hormone metabolites). These factors might even require compromises between selecting accuracy over easy, rapid, or in-field testing, when selecting the sample and/or biomarker to be investigated.

This review is divided to two major sections;

The first section covers the aspects of selecting samples, different sample types and examples of biomarkers that can be measured from these samples.

The second section will mostly focus on the different types of biochemical assays that can be used to measure biomarkers and simple descriptions of how these assay work or maybe be used. Given the enormous breadth of the review, we have provided general examples and citations for more specific examples covering the major sample types, commonly used biomarkers and biochemical assays. The goal

of the review is to provide a first resource for researchers interested in studying biomarkers to complement behavioural analysis, and should not be seen as a complete guide. We urge readers planning to use any of the techniques described here to read more details in the many papers we have cited because many of our general rules of thumb may not apply to specific implementations.

## Choosing the ideal sample

Selecting the ideal physical sample is incredibly important. While most assays will be available either to run on site or in a laboratory, this may not be the case for all samples. The selection of sample type is dependent on the biomarker(s) to be measured, the types of assays available to measure these biomarkers (see types of biochemical test section for further information) and the ability/availability to collect the sample type. Availability will depend on many factors, not least the ability to collect invasive samples, such as blood, and the research project questions/aims (Mormede et al., 2007). It is important to mention that the correct ethical and legal licences must be in place before samples are obtained from animals. In addition, licences may also be needed for working with certain species, such as Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) species, or for transportation of samples across borders or into countries due to biosecurity concerns (Animal Science Procedure Act (1986)). As an example, a schematic diagram for selecting the ideal sample for studying stress responses is shown in Figure 1.

### *Blood*

Blood is generally regarded as the gold standard sample, but sampling is highly invasive, and as such may require licenses, such as a Home Office Licence in the UK (Animal Science Procedure Act (1986)). It is also important to remember that the process of taking blood can alter the level of stress hormones in the animal, particularly where an animal is not keen on being handled, such as in sheep (Romero and Read, 2005). In other cases, where animals require capture in the wild, the stress of the process may induce glucocorticoid hormone release, so, samples should ideally be taken within a few minutes of capture, whilst ensuring sample collector safety (Romero & Read, 2005).

Blood samples have to be processed correctly, commonly by low-speed centrifugation or clotting into sedimentation layers such as the cells, buffy coat, or plasma/serum, to ensure that you retain the layer with the biomarker (Bielohuby et al., 2012). It is also worth noting that there is a substantial difference, especially for protein concentrations like fibrinogen, between serum and plasma, and so care must be taken to ensure that the correct one of these two are chosen (plasma has fibrinogen and clotting factors, but are absent or greatly reduced in serum samples). Blood samples can be stored with relative ease at room temperature or chilled while sampling, before ideal storage at -80C for sera and plasma (Bielohuby et al., 2012; Lombardi et al., 2012). However, biomarker stability can vary with temperature, allowing conformational changes or proteolytic cleavage (Reimers et al., 1983). Freeze thaw cycles can also cause degradation of some hormones, so ideally, samples which are to be used to detect multiple hormones should be aliquoted out into several smaller volumes for single use (Bielohuby et al., 2012).

It is also important that dilution of blood or serum sample, even by introduction of an anti-coagulant as commonly occurs in a vacutainer, is taken into account with

final calculations as it may affect the results (Bowen et al., 2010; Lippi et al., 2006; Kontny et al., 2011). Haemolysis, which can be caused by inappropriate sample handling and storage, can also lead to dilution and potential contamination of the sample and should be prevented where possible (Lippi et al., 2006b; Koseoglu et al., 2011; Bellomo et al., 2012).

Additionally, and importantly, there is a difference in measurable blood analytes, especially with rodents where there is a large difference in the site where the blood sample is obtained from (e.g. tail vain, tail tip sampling, jugular vein, cardiac puncture etc; Arola et al., 1980; Fitzner et al., 2006; Aasland et al., 2010; Christensen et al., 2009; Vahl et al., 2005). It is, therefore, worth considering where you would take the terminal blood sample from if an animal is to be euthanised. There are also biomarker variations on a daily, monthly, post-feeding, and even sometimes a yearly circadian rhythm with hormones such as testosterone and progesterone, as has been shown in maned wolves, and in seals (Greig et al., 2007) which show increased peaks in certain seasons (Maia et al., 2008).

Although blood is a complicated mixture of a large number of proteins, it offers a rapid or even instantaneous insight into stress hormones. Indeed, it may be so fast that unless samples are obtained very rapidly, the stress of being handled may create issues and inflate stress hormone levels.

*Saliva*

Saliva is a relatively easy to obtain sample, which can contain a variety of different hormones, including cortisol (Wenger-Riggenbach et al., 2010; Cook et al., 2013; Cobb et al., 2016). As cortisol is a lipid soluble hormone, it crosses cellular membranes, allowing for its detection in saliva within 15 minutes of a stressor being

applied, making it an ideal sample for rapid stress hormone detection (Kirschbaum et al., 1993; Dickerson & Kemeny, 2004; Gunnar & Vazquez, 2006; Shirtcliff et al., 2015). This rapid release and ease of testing has led to development of point of care devices to assess stress in both humans and animals (Nara et al., 2010; Choi et al*.,* 2014; Kaushik et al., 2014; Zangheri et al., 2015).

It is also worth noting that, depending on how sampling is done, it may also require a licence. For example, within the UK, the Animal Science Procedure Act (1986) (ASPA) means that you can swab around the outside of the teeth but a licence is required to enter the buccal cavity as this is considered an invasive sample (Animal Science Procedure Act (1986)). In addition, the volume of saliva can sometimes only be small, from around 100µl from dogs (personal experience), to around 500-750 µl from equids using the Equisal ®Saliva collection swab (Austin Davis Biologics Ltd).

A wide variety of studies have used saliva from a variety of different animals to test for different hormones associated with behaviour and stress, including cortisol (Wenger-Riggenbach et al., 2010; Cook et al., 2013; Cobb et al*.,* 2016), luteinising hormone (Srinivasan et al., 2020), oxytocin (MacLean et al*.,* 2018), vasopressin (MacLean et al*.,* 2018), prolactin (Gutiérrez et al., 2019), and testosterone (Kutsukake et al., 2009).

Studies have also shown a strong correlation between cortisol in blood and in saliva, suggesting that saliva may be a useful proxy for blood sampling, avoiding invasive sampling (Fell et al.,1985; Greenwood & Shutt, 1992; Negrão et al*.,* 2004), although other studies disagree (Dzviti et al*.,* 2019). It does however have the advantage of being repeatable multiple times in a relatively short period (Koyama et al., 2003). However, this can be difficult in field situations for wild animals, but novel

methods of saliva collection have been devised and used successfully (Higham et al., 2010; Smiley et al., 2010).

Care also needs to be taken when choosing the method for collection of saliva. There is potential for contamination of swabs with food debris and even plant hormones can interact with immunoassay antibodies (Dabbs, 1991; Granger et al., 1999), and some biomarkers may adhere to the cotton swab and thus escape detection (Shirtcliff et al., 2001). Processing of saliva can be difficult if the sample is highly viscous, and samples can precipitate after a freeze thaw cycle (Riad-Fahmy et al., 1982; Read et al., 1990). Once again, several freeze thaw cycles can alter the concentration of hormones (Gröschl, 2001).

Additionally, salivary flow rate can influence results by diluting potential hormones of interest, and this can be exacerbated by many factors, including increased temperature, food and liquid intake (Jacques et al., 1989; Ito et al., 2001; Elmer & Ohlin, 1971) as well as exercise (Colussi et al., 2018). Food intake can also cause errors in hormone measurement (Magnano et al., 1989; Laudenslager et al., 2006), and care also needs to be taken to avoid causing bleeding or getting blood contamination of the swab as this can alter the concentrations of hormones (Dzviti et al., 2019).

So even an easy to obtain sample, may require considerably more planning than initially thought (Kalliokoski et al., 2019; Koren et al., 2019).

*Hair or other tissue*

Hair and tissue are rapidly developing areas of interest for studies associated with hormones and behaviour/cognition. Hair presents a simple, relatively easy to

obtain sample, but can prove difficult if working with some animals, such as wild cats because of issues in safely collecting samples.

Although mainly used so far for human medicine, hair can act as a nice proxy for long term analysis of hormones which are encompassed into the hair during its growth (Schweikert and Wilson, 1974). Studies often focus on a variety of different hormones, including testosterone, oestradiol, cortisone, progesterone, cortisol, and androstenedione (Chen et al., 2013; Gao et al., 2013; 2015; Grass et al., *2016*; Kapoor et al., 2014; Yang et al., 1998).

Complications exist with using hair for analysis of some hormones, for example cortisol levels can vary with location of the hair on the body, so standardisation of sampling area is important to allow for comparisons (Carlitz et al., 2015; Terwissen et al., 2013; Yamanashi et al., 2013; Mesarcova et al., 2017; Heimbürge et al*.,* 2019). This means that collection of random hair from nests, traps, or on fences such as from rodents or sheep, can prove problematic (Heimbürge et al*.,* 2019). The reasons for these variations remain uncertain, but may be associated with factors such as the level of sunlight a part of the body is exposed to (Grass et al., 2016).

Hormone degradation does not appear to be a big issue with hair, meaning that the sample can easily be stored at room temperature without the need for desiccation or freezing (Accorsi et al., 2008), but washing hair samples is important to limit contamination with sweat, urine, or faecal matter (Chen et al., 2013; Gao et al., 2015; Musshoff & Madea, 2007; Ferrero & Liberles, 2010; Sheriff et al., 2011).

The hormone concentration can vary with factors which influence the growth rate of the hair, and these can include age, part of the body, sex, breed, and species (Heimbürge et al*.,* 2019). In addition, concentrations can vary with hair colour, as

seen in dogs and chimpanzees (Bennett & Hayssen, 2010; Taylor et al., 2015; Wennig, 2000; Yamanashi et al., 2013). There are also questions about the effectiveness of measuring hormones in hair strands. Most hormones are concentrated in the bulb of the hair, and not completely incorporated into the hair strand as it grows, so hormone concentration is highly dependent on the part of the hair analysed (Keckeis et al., 2012; Stubsjøen et al., 2015).

As an alternative to hair, using claw tissue, such as that obtained during routine trimming of nails in animals, may offer another sample to work with. This tissue provides assessment of stress hormones in the animal over a long time period (Matas et al., 2016). There has also been a correlation shown between hair and claw cortisol in new-born dogs (Veronesi et al., 2015). While, for avian species, feathers can be used rather than hair (Matas et al., 2016).

So, hair, feathers, and claws can offer a simple, in some cases easy to collect sample, but there is some level of planning required to ensure that results obtained from the samples taken are comparable, and that differences are not due to different types of samples.

*Faeces*

This is possibly the easiest sample to obtain, as it is left naturally by all animals. It may be obvious, but this is a dirty sample, filled with a variety of hormones and enzymes at different stages of metabolism, animal gut microbiota, food remains and other bits which may interfere or inhibit certain reactions like polymerase chain reaction (PCR; El-Bahr et al., 2005; Lepschy et al., 2007; Palme, 2005). Indeed, differences in the gut microbiota can lead to differences in hormone

metabolism and breakdown, so this may affect results of faecal hormone analysis (Antwis et al., 2019; Hooda et al., 2013).

Faecal sampling has proven particularly useful for a variety of different research focuses, including long-term stress, seasonal hormone patterns or pregnancy status (Nemeth et al., 2016, Hadinger et al., 2015; Hernandez et al., 2018; Cizauskas et al., 2015; Wheeler et al., 2013; Isobe et al., 2005; Garnier et al,. 1998; Wasser et al., 1991; Schwartzenberger et al., 1996). It is commonly used to investigate the presence of steroidal hormones, such as oestrogens, androgens and progestins for reproductive status determination and glucocorticoids for stress analysis (Amaral et al., 2010; Kotrschal et al., 1998, Harper & Austad, 2000, Goymann et al., 2002).

However, the use of faeces for hormone detection is somewhat limited by heavily degraded samples due to the harsh conditions of the gut as well as the actions of the bacteria in the microbiome (Antwis et al., 2019; Hooda et al., 2013). As such, it is important that faecal samples are rapidly collected post defecation and frozen to prevent further bacterial degradation of hormones (Hodges & Heistermann, 2011; Millspaugh & Washburn, 2004), as this will affect the results (Palme, 2005; Palme, 2019). Alternatively, where freezing is not possible, such as in field situations, rapid drying of the sample may prevent significant degradation, such as can be achieved by using alcohol to remove water (Palme, 2005), but this is not always ideal. Addition of sodium azide or other acids can aid preservation of hormones in alcohol at room temperature, although, these are harmful chemicals which are dangerous to the environment, so may not be suitable in the field (Whitten *et al*., 1998). More recently, rapid field extraction has given a more accurate picture of the hormones which are present in a faecal sample (Beehner & Whitten, 2004; Kalbitzer & Heistermann, 2013; Whitten et al., 1998, Ziegler et al., 2005).

Biomarkers are not always evenly distributed throughout a faecal sample, and so the part of the sample taken needs to be carefully planned. This is particularly true for large animals which produce a large faecal sample such as cattle or elephants. The same part of the sample should be taken to allow for appropriate comparison between samples (Peter et al., 2018, Hadinger et al., 2015). Then homogenisation is required prior to analysis, removal of large pieces of non-digested foods such as seeds and insects will aid in this (Millspaugh & Washburn, 2004; Palme, 2005).

Difficulties can also arise in collection and potential contamination, especially urine contamination, which can increase concentrations of some hormones, skewing results (Hay et al., 2016; Schonning et al., 2002). Despite difficulties, studies have been carried out on aquatic animals (Amaral et al., 2010). A major positive to this sample is that there are very limited diurnal variations of hormones within faeces, unless there is a very rapid gastrointestinal tract transit time (Goymann, 2005, 2012; Millspaugh & Washburn, 2004).

Although a simple sample to collect in many cases, faeces needs some thought, and some standardising to ensure that the results can be appropriately analysed and compared.

*Urine*

Urine is a mixture of waste products and hormones which are commonly excreted as conjugated water-soluble forms, they are often an indication of the presence of hormones over a few hours, rather than an instantaneous indication as seen with saliva (Bouatra et al., 2013).

Urine is already widely used for detection of pregnancy hormones in humans and detecting human chorionic growth hormone (Chard, 1992) is used in home pregnancy test kits. Urine is also useful in disease diagnostics, such as monitoring diabetes in humans and in dogs (Hess et al., 2000).

Urine can be relatively easy to obtain from some animals, such as through a swab of the bottom of the cage, but relatively difficult from others and may require some training of the animals (Laule et al., 1996; Kurien et al*.,* 2004). When working with wild animals, collection of this sample can be even more difficult, but methods have been developed (Danish et al., 2015; Knott, 1997). Urine has been used to detect a variety of different peptide hormones, such as cauxin (McLean et al*.,* 2007), trypsinogen activation peptide (Allen et al., 2006), c-peptide (Polonsky et al., 1984), oxytocin (Nagasawa et al*.,* 2009; Mitsui et al., 2011), vasopressin (Dolph et al., 1962), thyroid hormones (Goff et al., 1986), as well as certain immune parameters, particularly linked with infection with a variety of different pathogens (Doward et al., 1991; Ravnik et al*.,* 2014).

Although some biomarkers are considered to be stable in urine at room temperature for up to 24 hours, some degradation may occur due to the bacteria within the sample (Grant & Beastall, 1983). Equally, urine samples should be centrifuged to remove particulate matter, and care should be taken when freezing urine samples, as this may lead to further sample degradation (Heistermann, 2010).

It is also worth bearing in mind that the volume of urine, which is linked to the hydration of the animal, will have an impact on the concentration of hormones. For example, a well hydrated animal will produce higher volumes of urine than a dehydrated animal and more dilute urinary hormones (Miller et al*.,* 2004). To counter this issue, creatinine is sometimes used as a counterbalance, but this is also subject

to many fluctuations based on diet, age, sex, breed, kidney function, etc (Carrieri et al., 2000; Thompson et al., 2012;  Miller et al*.,* 2004).

Urine also allows for an investigation into varying concentrations of hormones over a day, with a sample which is easier to collect than saliva in some cases (Heistermann, 2010). This, however, can cause issues as certain hormones can be naturally increased at certain times of the day and this may not be in response to a stressor, for example, cortisol levels can be higher in the morning in some species of monkey, than they are at other times of the day (Davis et al., 2005; Mueller and Lipson, 2003; Smith and French, 1997). This is also true for dogs, which show variation during the day and night (Gordon et al., 1985; Kolevská et al., 2003). Therefore, timing of sampling should be kept as similar as possible throughout the study, and if this is not possible, urinary hormones should be analysed with caution. So, although urine is an easy to obtain sample for some animals, there are several other factors which need to be factored into studies to make sure that the results are reliable and easy to interpret.

Whatever sample is chosen, the extraction method is important, and this is discussed later within this document.

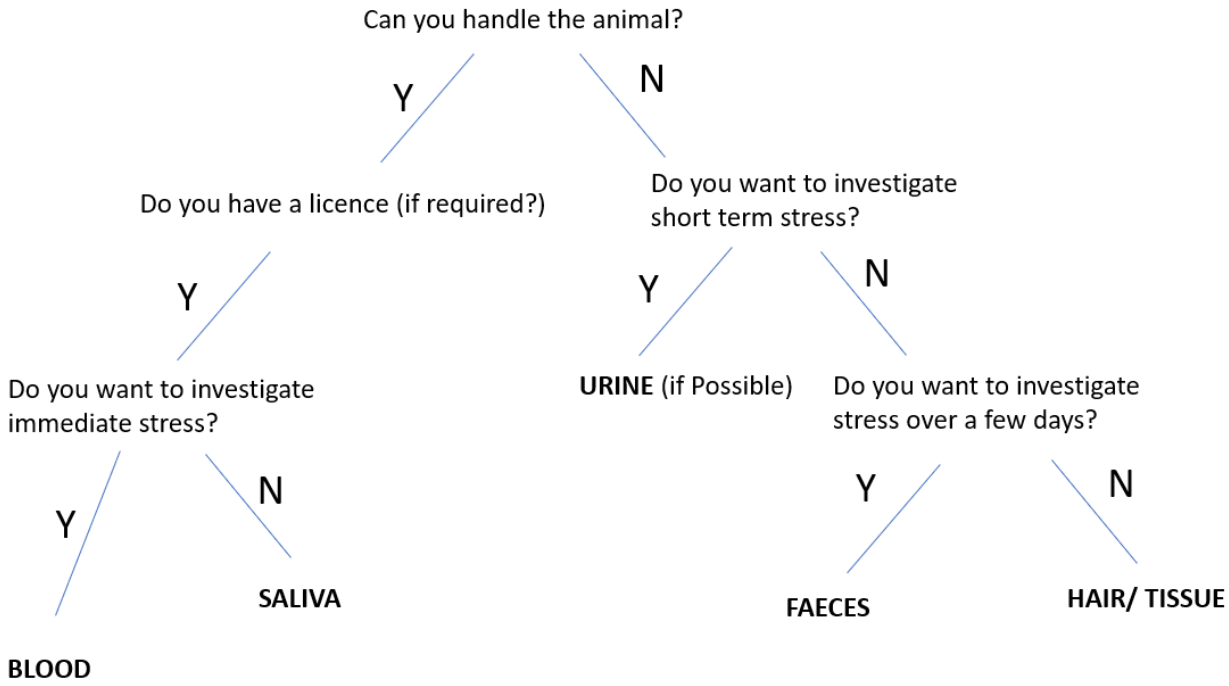


Figure 1. A flow chart diagram to aid with the selection of the ideal sample for the study which you wish to undertake. Y is yes and N is no. Note that this may differ from country to country as licencing requirements vary.

## Types of biochemical tests and their applications

Commonly, the selection of a biochemical test is determined by the choice of biomarker and the type of sample available for its isolation. Some of the key considerations in selecting the most appropriate test(s) are briefly discussed below and summarised in Table 1. These considerations include the type of biomarker (chemical, protein, DNA/RNA), how much sample can be collected (as some

methods require as little as 1-10µl of sample, but other tests require several mL's of sample), budget, and whether the test needs to be performed *in situ.*

Table 1. A table of considerations for selecting biochemical test types or biomarkers.

| **Test type** | **Biomarker** | **Method output** | **Limitations** |
|---|---|---|---|
| Chemical | Chemicals and proteins | Mostly colorimetric reporter systems or colour change | Sensitivity of the test. Amount of sample required. |
| Enzyme linked assay | Chemicals, proteins | Mostly colorimetric reporter systems | Sensitivity of the test. Amount of sample required. |
| Antibody tests | Biomolecules and chemicals | Lateral flow positive/negative readout or colorimetric reporter systems | Availability of antibody test being commercially available. |
| Enzyme activity | Enzymes | Mostly colorimetric reporter systems | Sensitivity of the test. Amount of sample required. |
| PCR Test | RNA or DNA | Graphic or gel band. Indicator of presence or copy number. | Availability of RT-PCR facilities/ equipment. Ability to extract RNA/DNA from the samples. |
| Proteomics | Proteins | Mostly colorimetric reporter systems or identification of modifications by affinity isolation then either mass spectrometry or western analysis using PTM specific antibodies | Cost. Analysis of PTMs/services. |
| Mass spectrometry | Chemicals and proteins | Graphic/tabulated data. Indicator of presence or amount present. | Potentially required as an external service due to expertise and equipment cost. |

## *Chemical assays*

Chemical detection of biomarkers directly measures a chemical in a biological sample by causing a reaction with the chemical to report the presence or amount of

the chemical. Chemical assays are routinely used to detect and measure the levels of sugars or proteins in urine for example (Boag et al., 2019; Shropshire et al., 2018). They are comparatively inexpensive compared to antibody test assays, quick to perform but can lack sensitivity. An example of rapid and easy to use chemical assays are “dipstick” type assays, where the “dipstick” is dipped briefly (usually for around 1-15 seconds) into the biological liquid being assayed, then left for around 15 seconds for the reaction to occur. The read-out is colorimetric and the amount of colour produced is proportional to the amount of chemical present, a common example are the systems used for diabetes testing in animals. The dipstick colour can be compared to a colour range chart supplied with the assay kit to provide a semi-quantitative data, which allows for rapid in-field measurements of proteins, chemicals and pH by comparatively untrained researchers (Boag et al., 2019; Anthanasious et al., 2018).

*Enzyme linked assays*

Enzyme linked assays are commonly used as either a reporter system “linked” to another chemical reaction, so that you get a quantitative read-out, or to amplify the read-out of another reaction to increase assay sensitivity. Enzyme linked assays are, in general, more complex than standard chemical or enzymatic assays. They are commonly used to measure enzymatic activity and have the ability to amplify the reporter system so that low levels of activity can be more easily measured. These assays have multiple stages and require dedicated laboratory systems/equipment. Not all enzyme-linked assays require the use of antibodies but simply use linked enzyme systems as a method of reporter amplification for

detecting the activity of enzymes at very low concentrations. Commonly, antibody detection systems such as Enzyme Linked Immunosorbent Assays (ELISAs) rely on enzyme linked reporter systems to amplify the colorimetric quantitative read-out systems. These ELISA systems are now becoming veterinary-specific, instead of re-protocoling human ELISA kits and systems (Lane et al., 2018).

*Antibody test assays*

Antibody test systems are normally divided into two categories, those that test for the presence of an antibody in a biological sample or those that use antibodies to test for chemicals in biological samples. One of the simplest tests that uses antibodies to detect chemicals present in biological samples are the lateral flow test kits, which are commonly used as pregnancy test devices, examples of which can be used for early detection of conception on-site or in the field (Ambrose et al., 2007). However, with the massive increase in the range of ELISAs available in recent years for detecting and the quantification of molecules, most key biomolecules can be quantified using ELISAs. These systems vary enormously in sensitivity and the types of samples they are suitable for, while costs range from around £200 for the commonly used/available kits all the way up to £1000 or more for specialist kits.

ELISA assays are most commonly used in 96 well microplate format and normally allow around 80 samples (~40 samples if performed in duplicate or ~24 samples in triplicate; figure 2) to be analysed. This is because up to 16 wells are required for calibration standards and controls (these are important to confirm that the assay works correctly, and should not be skipped or reduced, however tempting it may seem). As well as standard protocols, ELISAs can be modified for use in novel

techniques, for example, in assessing chronic stress in dogs using cortisol as a biomarker extracted from nails as the biological source (Mack & Fokidis, 2017).

Sadly, the choice of ELISA is not always simple, as there are many different types to choose from. Generally, they can be classified into four main groups:

- Direct ELISA- Antigen (target) is bound directly to the well of the plate and directly assayed by a conjugated reporter antibody.
- Indirect ELISA- Similar to the direct ELISA, but a second, conjugated antibody is used to detect the first unlabelled-antibody that bound the antigen.
- Sandwich ELISA- Antigen (target) is captured between two antibodies, one is bound to the plate and captures the antigen and the other is added later for detection, thus forming a complex like a “sandwich”.
- Competitive ELISA- Measures the amount of sample by quantification of its interference/competition with an expected signal.

Potentially, the competitive ELISA is most useful for quantification of biomarkers because it allows accurate quantification of the levels present in biological samples (Cell Signaling Technology Ltd, Types of ELISA Tests) though it depends on the biomarker. Plotting calibration curves and calculating values for unknown samples can be time consuming and potentially challenging. However, there are many free to use ELISA analysis websites that will plot the data and perform the calculations directly from the raw plate reader data (elisaanalysis.com).

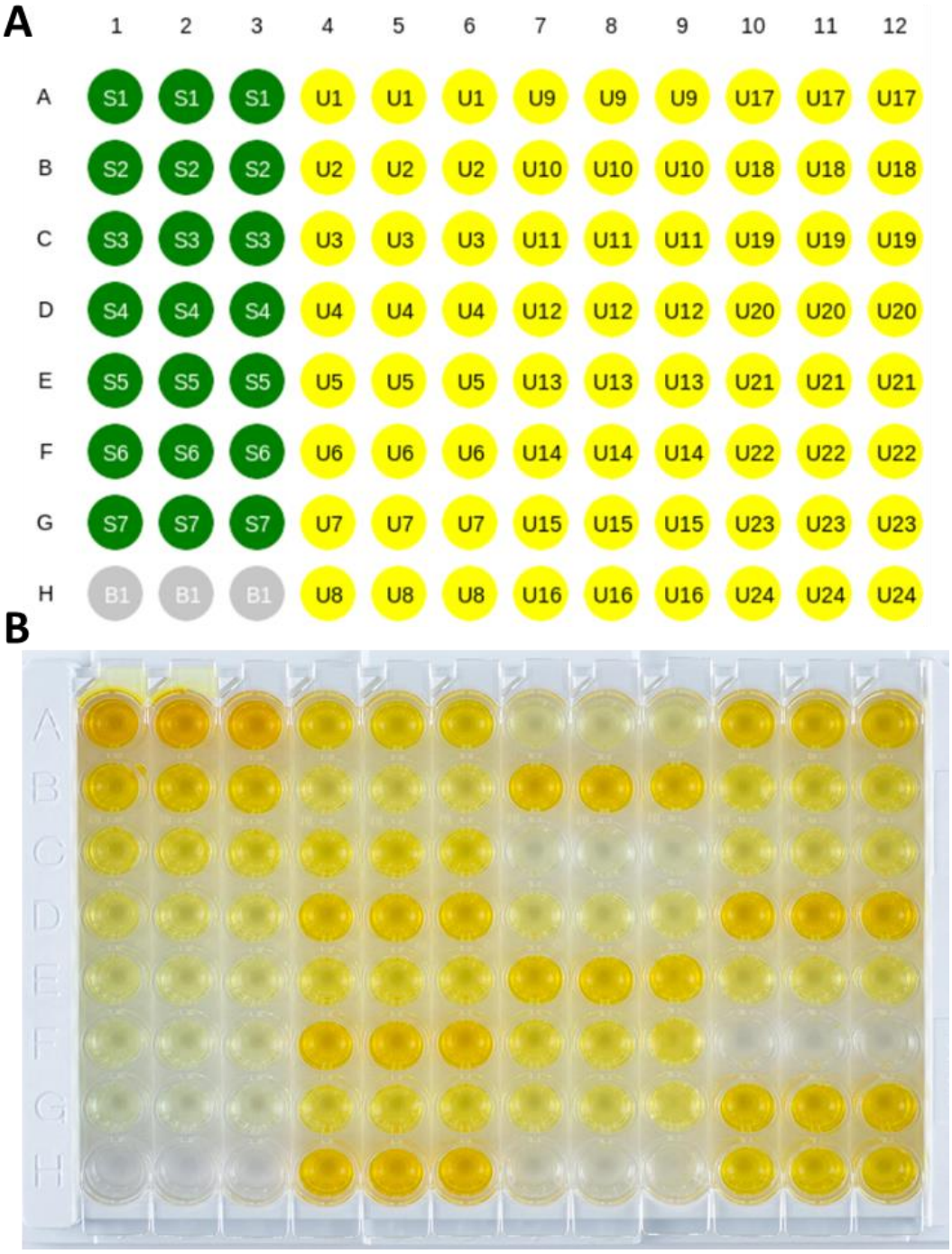


Figure 2. An example ELISA 96 well plate layout. A). Triplicates for known standards S1-7 (green), Blank (B1, grey) and unknown samples (U1-40, yellow). B). ELISA plate with TMB substrate producing a yellow colour measurable at 450nm on a plate reader.

## *Enzyme activity*

Unlike chemical and antibody assays, enzyme activity assays measure the activity of an enzyme, not just its concentration in a biological sample. Different isoforms of enzymes, may vary in their rate of activity (Sulakhe & Lautt, 1987), so it can be important to measure how much usefully active enzyme is present. Enzyme activity is the key biological process causing modifications to other proteins, such as cleaving or changing sugars; in essence controlling and regulating all key responses within an organism. The activity levels of certain enzymes are altered with changes in an animal's environment; for example, quality of care or stress. In its simplest form, enzyme assays measure substrate cleavage, for example, amylase activity can be measured using the substrate blue starch. Amylase cleaves the sugar starch into smaller sugar molecules releasing a blue dye, that can then easily be measured. The amount of blue dye released is proportional to the amount of enzyme activity present within the sample (Abe et al., 1996).

However, with enzymes being an active biological molecule, their activity will change with temperature and the presence of certain chemicals. EDTA (ethylenediaminetetraacetic acid), a powerful chelating agent sequesters ions such as calcium, iron and zinc that are required by some enzymes for activity (Sabeur, Vo & Ball, 2001). So, it is extremely important when designing enzyme assays, to check that any buffer being used for dilution is compatible with that enzyme assay.

## *Polymerase Chain Reaction (PCR) Tests*

Polymerase chain reaction (PCR) tests amplify DNA from either a DNA or RNA template to aid in the identification of genes, mutations, or viral infection. These

tests have a wide variety of uses from identifying dogs that have mutations in the oxytocin receptor (OXTR) that can influence animal behaviour (Bence et al., 2017), to identifying species or breed types (Wu et al., 2018) and can identify animals infected with a virus such as coronavirus (Chan et al., 2020). These PCR reactions take around 30 minutes to 3 hours to run, the reagent cost per reaction is comparatively inexpensive (from ~£0.30) but running the test requires a thermal cycler, which is a comparatively expensive piece of equipment.

RNA samples must be converted to DNA prior to PCR amplification, so an additional reverse transcriptase (RT) step is needed. This is important for identifying RNA based viruses, such as those currently being used to detect COVID-19 infections in both humans and animals (Chan et al., 2020). The good news is that all-in-one RT-PCR kits are commercially available, that can work from RNA in a single combined reaction step, but at a higher cost per sample.

### *Proteomics*

Proteomics studies the variation in protein levels within a biological sample or even changes in the post-translational modification (PTM) status of proteins. Many factors increase/decrease protein levels to control processes in an organism, but at a cellular level, proteins can also be regulated once synthesised by PTM. For example, amino acid residues can undergo phosphorylation or acetylation within protein molecules. These PTMs can then alter the protein's behaviour, for example, changing its affinity for a target receptor, changing the proteins it binds, and even changing its half-life (speed of degradation). Most proteomic analysis is through western blotting analysis using specialised antibodies that are specific to certain

proteins, isoform specific antibodies, and even antibodies that only bind and recognise proteins with certain PTMs on specific amino acid residues/sites. Proteomics has even been used to identify changes in PTM/phosphorylation at a global protein level in saliva samples of dogs infected from suspected tick borne babesiosis (Galan et al., 2018).

### *Mass spectrometry*

Mass spectrometry identifies the presence and relative amounts of chemicals and proteins in a biological sample. Mass spectrometry accurately determines the exact molecular mass of the molecules present in biological samples and these are then compared to known standards. An example of its use is an investigative study on dogs treated with cannabidiol for canine epilepsy that used a simple and fast gas-chromatography mass spectrometry assay (GC/MS) to accurately quantify cannabidiol metabolite levels in serum samples (Rotolo et al., 2019). Mass spectrometry is usually provided as a service, either academically or commercially, due to both the high level of expertise required to undertake the analysis and the cost of the equipment. Mass spectrometry (LC-MS/MS) can be extremely useful in identifying PTMs on proteins and small molecules in biological samples that cannot easily be determined by antibody methodologies (Koivunen et al., 2006).

## **Factors that might influence selection of biomarkers**

Biological samples have a wide variety of influencing factors that are outside the researcher's control. Therefore, these factors might influence biomarker or sample selection (Figure 1) and can be summarised as follows:

- Length of time the marker might be present
    - Half-life, excretion time, potential collection times (Wolff et al., 1999)

- Compounds that might affect readings
    - Medications or contamination, for example lysed blood samples will have falsely high levels of lactate dehydrogenase (Solberg, Holme & Little 1986)

- Medical conditions
    - For example, pancreatic disease can raise blood amylase levels, giving falsely high reading if using amylase is used as a marker of stress (Murtaugh & Jacobs, 1985)

The use of biomarkers to evidence animal cognitive, welfare, and health issues has vastly influenced the ability of a researcher to accurately and easily quantify biomarkers and directly correlate these to observed behaviours. In conclusion, no one size fits all solution exists for the analysis of biomarkers, rather the selection of sample type and biomarker is dependent on the biological samples available and the ability to analyse the biomarkers. Care should be taken when deciding on the sample to ensure that the results will answer the research question. The correct assay needs to be chosen to give the precise, required information. Making the wrong choices here can lead to confusing results, and incorrect conclusions, and we all know how bad science can be propagated.

After all, the old saying rings true- garbage in equals garbage out.

**Biochemical Tips:**

- Extraction of saliva from swaps - Saliva can be extracted and collected in Eppendorf microcentrifuge tubes by cutting the swab ~5mm from the end of the swab and inverting it, so the stick is at the bottom of the tube. The tube can then be centrifuged briefly (~4-6000xg for 10-30 seconds) to pellet the saliva at the bottom of the tube for easy pipetting.
- Dilution of samples – Some samples, for example saliva samples, might have to be diluted (in our experience, this is very common for the larger breeds of dogs), as the viscosity of the saliva can be too high to allow for pipetting and analysis. Commonly these dilutions are around 1:4 with the assay buffer.
- More is better- Although it adds to the costs, it is always better to carry out the analysis more than once, so run a single sample on an ELISA plate in duplicate or triplicate. That way, any single anomaly is removed, and the results are more reliable. No assay is perfect, so there will always be some in built errors, and this helps to reduce them (but never removes them).
- Keep your samples appropriately- if they need to be at 4$^{o}$C, -20$^{o}$C or -80$^{o}$C, keep them there, and avoid freeze thawing on multiple occasions where possible as this can affect the results. Although this takes up space, the samples are there to go back to if needed, and can even be used for other studies, such as testing for antibodies in a pandemic. But make sure that your ethical approval states this.
- Remember safety comes first- e.g., collection of saliva swabs from rabid dogs is never a good idea, but from ordinary dogs is fine. Faecal samples often hide hidden pathogens and dangers such as *Salmonella*, which is zoonotic and can make people very ill.

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