DRUG CLASS	CAT NO.	DRUG RESIDUE	APPLICATION
Anabolic steroids	TB2106	Trenbolone	• To increase the quality and efficiency of meat production • Performance enhancement in horse racing / dog racing
	NT2105	19 Nortestosterone/trenbolone	
	BD2382	Boldenone	
	SW2418	Stanozolol	
Oestrogenic hormones	SJ2152	Stilbenes (diethylstilbestrol, hexestrol, dienestrol)	• Growth promotion in cattle
	ZR2421	Zeranol	
Corticosteroids	DM2156	Corticosteroids (dexamethasone, flumethasone, betamethasone)	• Cattle fattening • Performance enhancement in horse racing / dog racing
β-agonists	CB1418	Clenbuterol/brombuterol	• To increase the quality and efficiency of meat production • Performance enhancement in horse racing / dog racing
	SU2148	β-agonists (clenbuterol, salbutamol, mabuterol, brombuterol, mapenterol, terbutaline, pirbuterol, methylclenbuterol, carbuterol)	
	RT3451	Ractopamine	
Anti-microbial	SQ2145	Sulphaquinoxaline	Treatment of: • Scours • Pneumonia • Atrophic rhinitis • Mastitis • Fungal, bacterial and parasitic infections
	SZ2147	Sulphadiazine	
	SM2146	Sulphamethazine	
	CN1469	Chloramphenicol	
	BL3448	β-lactams	
	BL1371	β-lactams standard accessory kit	
	QL3454	Quinolones	
Nonsteroidal Anti-inflammatory Drugs	PB3454	Phenylbutazone	Used to treat chronic pain or lameness in Horses
Urine control	CB3449	Clenbuterol	Size: 5 low: 5 elevated

Enzyme Linked Immunosorbent Assay or ELISA is one of the most common tests conducted in immunobiochemistry. The principle of the assay involves immobilising antibodies onto a microtitre plate. These antibodies show specificity for an analyte and therefore allow qualification and quantification of the analyte in question.

There are 2 main types of ELISA commonly available:

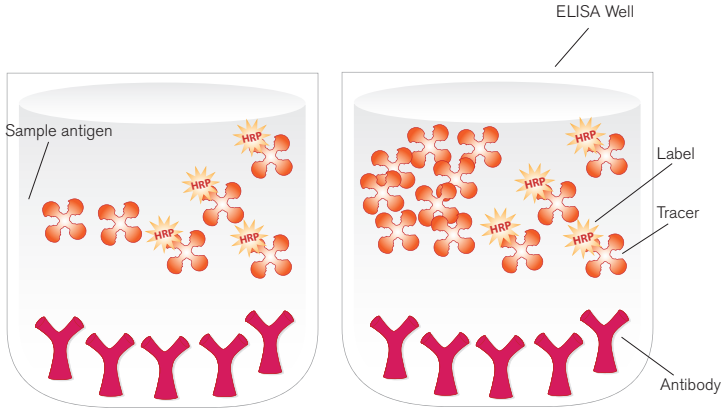
Competitive
Competitive ELISA is principally used for the detection of small molecules, which do not have multiple epitopes available for the simultaneous binding of two antibodies.

Sandwich
Sandwich ELISA is principally used for larger protein analytes, which have multiple epitopes that can be accessed by two antibodies simultaneously.

COMPETITIVE

In a competitive ELISA, antibody that is specific for the analyte is immobilised on the micro-titre plate. Then enzyme-conjugated antigen is incubated with capture antibody in the presence of a competing, unconjugated form of the same antigen (which can be sample or calibrator). A solution containing chromophore/substrate specific to the enzyme is added. The colour intensity produced is directly proportional to the amount of conjugate enzyme bound, which in turn is inversely proportional to the amount of unconjugated antigen present. Absorbance values are determined for a range of antigen concentrations (standard range) and the data generated used to construct a standard curve (calibration). The actual standard range chosen will depend upon the required measuring range for that particular assay/analyte.

The principal determinant for this assay is the sensitivity, below which the concentration of antigen cannot be accurately measured. Thus, initial studies are performed to compare differences in absorbance readings when low antigen concentrations are present across a range of conjugate dilutions. A target optical density range of 1.8-2.2 is optimal for the zero standard i.e. in the absence of any competing antigen.



Low concentration of sample antigen present
Antibodies bind majority of enzyme linked antigen
Signal output / OD = High

High concentration of sample antigen present
Antibodies bind majority of unlabelled antigen
Signal output / OD = Low

CASE STUDY 1:

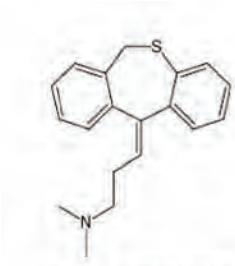
Tri-cyclic anti-depressants (TCA's) detection on ELISA using Randox TCA capture antibody (MAB9960) in combination with in-house conjugate ESC2275 labelled with horseradish peroxidase.

Randox manufacture a monoclonal antibody NT1.3C11.B11.D4.B8 (MAB9960) that can quantify a number of TCAs. TCAs are indicated for the treatment of clinical depression, neuropathic pain, nocturnal enuresis and ADHD. Whilst therapeutic drug monitoring (TDM) of TCAs is well established in the treatment of depression to optimise outcome and safety. TCA overdose remains the most common cause of death from prescription drugs.

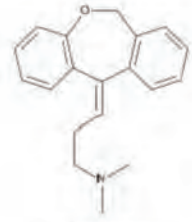
In this case study the monoclonal antibody binds a specific epitope that is common to a number of the TCAs. This allows the antibody to cross react with various molecules with a similar overall structure, examples of five such TCA drugs are shown below.

TRI-CYCLIC ANTIDEPRESSANTS (TCAs)

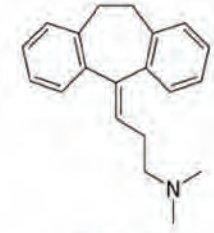
The 2D drawings below are an example of 5 of the drugs from the family.



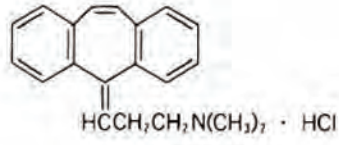
Dosulepin hydrochloride



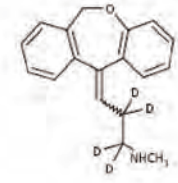
Doxepin



Amitriptyline



Cyclobenzapine

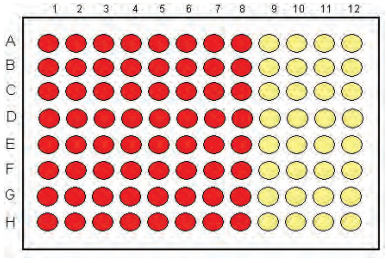


Nordoxepin

TCA competitive ELISA Development

MICROTITRE PLATE PREPARATION:

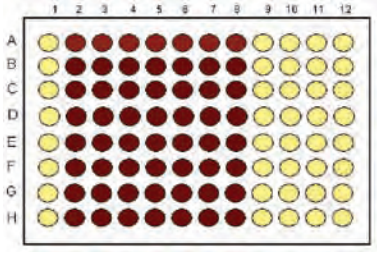
1. Add coating antibody (MAB9960) at 0.9µg/ml in phosphate buffered saline (PBS) to A1-A8 through to H1-H8 wells inclusive on 2 plates (100µl/well and incubate for 1 hour at 37°C).
2. Wash in phosphate buffered saline + 0.1% tween (PBST) (x3)
3. Block with 0.2% Block/PBS (200µl/well).
4. Wash in PBST (x2)



ONE SHOT ASSAY PROTOCOL:

Diluent addition

1. Add 50µl of 0.2% Block/PBS to A2-H2 through to A8-H8 inclusive.



Calibrator addition

1. Add 73µl/well of Impramine at 1000ng/ml to H1 material

Cross-reactant addition

2. Add 73µl/well of cross reactant as shown in results chart on page 178 to each of the corresponding wells A1-H1 on both plates

Conjugate addition

3. Add 75µl of the enzyme labelled detector tracer (ESC2275) at 1:1000 to wells A1-H1-A8-H8 inclusive.
4. Incubate for 1 hour at 37°C.

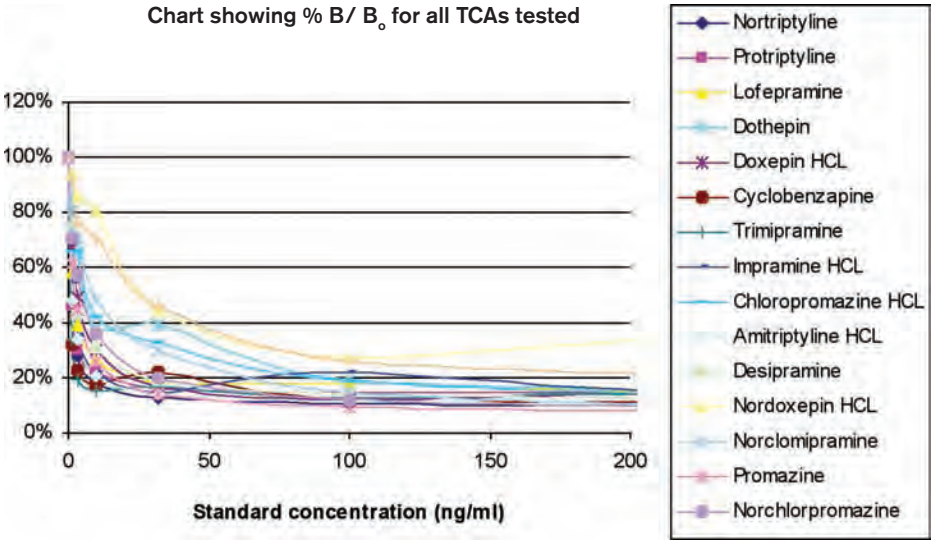
Substrate/Read

5. Wash in PBST (x5)
6. Add 100µl/well TMB substrate at room temperature in the dark for 20 minutes
7. Add 100µl/well 0.2M sulphuric acid and read plate immediately at 450nm on a photo-spectrometer.

RESULTS INTERPRETATION:

The table below shows the percentage cross reactivity profile for other members of the TCA family when the monoclonal antibody NT1.3C11.B11.D4.B8 and the tracer ESC2275 where used.

All data based on monoclonal NT1.3C11.B11.D4.B8										Randox
	1000	320	100	32	10	3.2	1	0	IC50 NG/ML	IC50 ng/ml % CR to Imipramine
Nortriptyline	9.33%	9.50%	10.42%	12.44%	17.90%	28.32%	46.30%	100.00%	0.95	129.47
Protriptyline	12.45%	13.84%	14.48%	16.20%	22.32%	30.26%	45.82%	100.00%	0.68	180.88
Lofepramine	16.45%	14.25%	18.08%	18.08%	26.88%	39.51%	57.82%	100.00%	1.57	78.34
Dothepin	15.33%	14.69%	19.44%	39.20%	37.80%	65.12%	83.15%	100.00%	2.69	45.72
Doxepin HCL	9.07%	15.66%	11.83%	16.55%	29.72%	49.29%	67.26%	100.00%	2.77	44.40
Cyclobenzapine	14.51%	11.85%	12.29%	21.82%	17.17%	22.81%	31.67%	100.00%	0.41	300.00
Trimipramine	13.50%	15.02%	12.92%	16.41%	15.13%	19.21%	33.88%	100.00%	0.38	323.68
Impramine HCL	19.44%	11.36%	22.04%	14.63%	22.33%	35.51%	54.48%	100.00%	1.23	100.00
Chloropromazine HCL	9.46%	12.78%	18.84%	32.68%	42.06%	65.36%	65.20%	100.00%	3.22	38.20
Amitriptyline HCL	10.81%	10.62%	11.56%	14.31%	20.76%	33.74%	48.15%	100.00%	1.41	87.23
Desipramine	11.52%	11.71%	13.92%	18.62%	30.41%	42.58%	74.75%	100.00%	3.03	40.59
Nordoxepin HCL	14.76%	36.45%	27.26%	44.84%	80.48%	85.08%	93.71%	100.00%	14.13	8.70
Norclomipramine	6.42%	8.81%	14.41%	29.68%	48.93%	70.98%	73.27%	100.00%	1.51	81.46
Promazine	6.47%	7.25%	9.19%	14.17%	26.21%	44.60%	61.55%	100.00%	2.76	44.57
Norchlorpromazine	7.29%	8.38%	12.00%	19.77%	35.99%	57.46%	70.69%	100.00%	2.86	43.01
2-hydroxyimipramine	9.37%	19.43%	25.82%	46.27%	70.57%	75.70%	92.28%	100.00%	9.94	12.37



In this case the binding affinity has a proportionally inverse relationship with the measure of colour intensity following a colorimetric reaction carried out on ELISA. A photo-spectrometer is then used to measure the absorbance at 450nm (A450nm). These results are expressed as % B/B_0 i.e. $(B/B_0)^*$ 'x'100 where B equals A450nm at 'x' ng/ml standard concentration and B_0 equals A450nm at 0ng/ml standard concentration. The term IC_{50} is a measure of the concentration of a specific drug, which produces a 50% drop in A450nm relative to B_0. This allows the specificity of the antibody to a series of similar drugs to be measured by calculating percentage cross reactivity (%CR) i.e. $(IC_{50}^{[Drug\ X]}/IC_{50}^{[Drug\ Y]})^*100$.

DISCUSSION

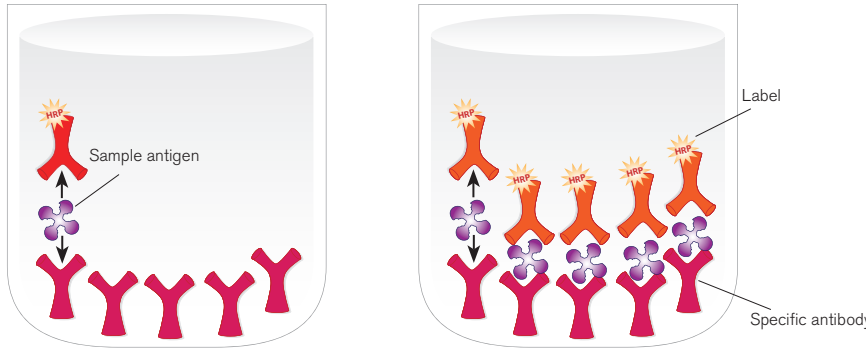
IC_{50} values were <1ng/ml for Nortriptyline, Protriptyline, Cyclobenzapine and Trimipramine; <3.5ng/ml for Lofepramine, Dothepin, Doxepin, Imipramine, Chlorpromazine, Amitriptyline, Desipramine, Norclomipramine, Promazine and Norchlorpromazine; 9.94ng/ml for 2-Hydroxyimipramine; and 14.13ng/ml for Nordoxepin.

Therefore this generic monoclonal antibody exhibits broad specificity for a wide range of TCAs and their metabolites, which could be of value in developing more effective immunoassays for both TDM and toxicological applications.

SANDWICH

In a sandwich ELISA, antibody that is specific for the analyte is immobilised on the microtitreplate. Then antigen (which can be sample or calibrator) is bound to the immobilized capture antibody. A second, enzyme-conjugated, antibody is added, followed by a solution containing chromophore/substrate specific to the enzyme. The colour intensity produced is directly proportional to the amount of antigen present. Absorbance readings are determined over a range of antigen concentrations (standard range), and used to construct a standard curve (calibration). The actual standard range chosen will depend upon the required measuring range for that particular assay/analyte.

The principal determinant in this type of ELISA is the full dynamic range of antigen over which the conjugate effectively binds. Initial studies are performed to compare differences in absorbance readings between low (or zero) and high concentrations. Generally, an OD peak around 1.8-2.2 is optimal for the linearity of the assay.



Low concentration of sample antigen present
Limited Secondary Antibody binding available
Signal output / OD = Low

High concentration of sample antigen present
High Secondary Antibody binding to antigen
Signal output / OD = High

CASE STUDY 2:

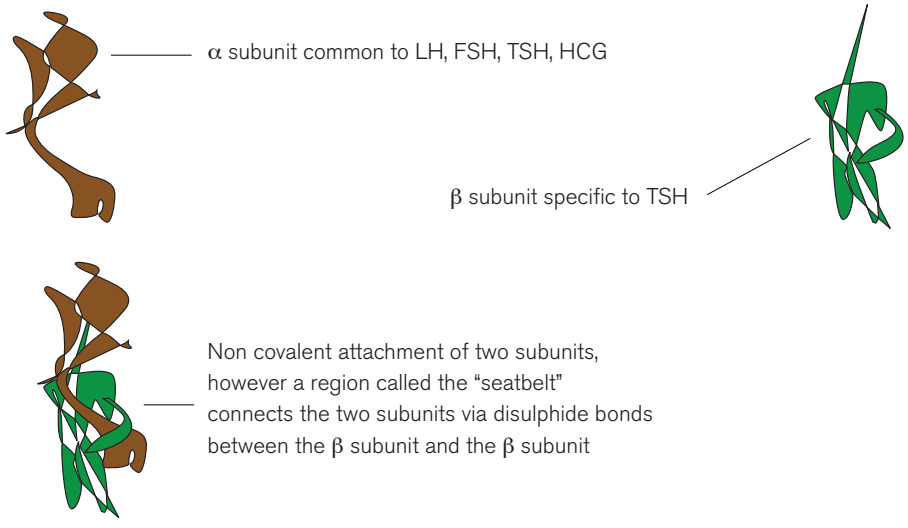
Thyroid Stimulating Hormone (TSH) detection on ELISA using Randox TSH β Specific capture antibody (MAB9932) in combination with Randox TSH β specific detector antibody (MAB9930) labelled with horseradish peroxidase.

Randox manufacture a range of β specific TSH monoclonal antibodies. TSH is quantified to determine an individual's thyroid status. It is a heterodimeric glycoprotein belonging to a hormone family known as the cysteine-knot growth factor super family. This family includes the hormones:

1. Thyroid stimulating hormone (TSH)
2. Follicle stimulating hormone (FSH)
3. Human Chrorionic gonadotropin (HCG)
4. Lutienizing hormone (LH)

Evolution tends to re-use protein scaffolds and modify existing genes to perform different tasks. Here, for example, the α subunit of the protein is homologous throughout four different proteins, whilst the β subunit is distinct to TSH. Therefore if an antibody were raised to the α subunit it would bind to not only the hormone in question but would also bind to the other proteins. This could not be used when trying to detect the presence of a particular protein, as small amounts of the other three proteins, if present in the sample, could skew the results.

Diagrammatic representation of TSH protein structure



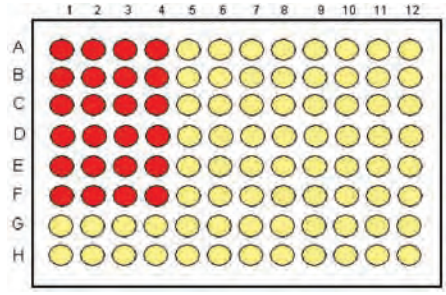
Therefore in order for an antibody to be useful it must recognise the β subunit of the heterodimeric molecule and not cross-react with other members of the super family.

Case Study

TSH SANDWICH ELISA DEVELOPMENT

Microtitre Plate Preparation:

1. Add coating antibody (MAB9933) at 1µg/ml in phosphate buffered saline (PBS) to wells A1-A4 through to F1-F4 inclusive (100µl/well for 2 hours at 37°C).
2. Wash in Phosphate buffered saline + 0.1% tween (PBST) (x3)
3. Block with 0.2% Block/PBS (200µl/well).
4. Wash in PBST (x2)



ONE SHOT ASSAY PROTOCOL:

Calibrator addition

1. Add 50µl/well of calibrator material, set up four analogous six-point calibration curves at 0, 8, 26, 83, 264 and 834µIU/ml in wells A1-F1, A2-F2, A3-F3 and A4-F4.

Cross-reactant addition

2. Add 50µl/well of PBS to each of the points on the calibration curve A1-F1
3. Add 50µl/well of LH at 1000 mIU/ml LH to each of the points on the calibration curve A2-F2
4. Add 50µl/well of HCG at 200 IU/ml to each of the points on the calibration curve A3-F3,
5. Add 50µl/well of FSH at 1000 mIU/ml to each of the points on the calibration curve A4-F4

Conjugate addition

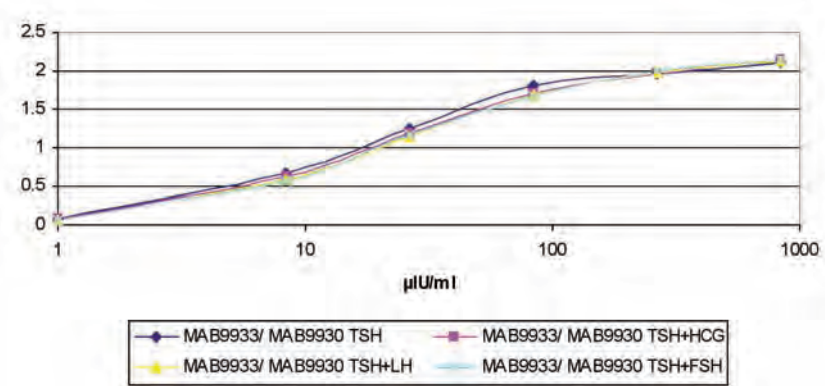
6. Add 50µl of the enzyme labelled detector antibody (MAB9930) at 1:1000 to wells A1-A4 through to F1-F4 inclusive.
7. Incubate for 1 hour at 37°C.

Substrate/Read

8. Wash in PBST (x5)
9. Add 100µl/well TMB substrate at room temperature in dark for 5 minutes
10. Add 100µl/well 0.2M sulphuric acid and read plate immediately at 450nm a photo-spectrameter.

RESULTS INTERPRETATION:

Chart showing Randox in house monoclonal capture and detector set detecting TSH specifically when cross reactants are present



- Chart showing a calibration curve for a monoclonal sandwich pair showing β specificity for thyroid stimulating hormone.
- The chart also includes the calibration curve response when HCG, LH and FSH are added as cross reactants.

DISCUSSION

For this test to be of use, it must offer a high degree of specificity and allow the user to perform this test in the shortest time possible. Hence this test was developed using a One Shot method. The term "one shot" means that the sample and the detecting conjugate are added at the same time, as both capture and detecting antibody are β specific, only TSH is pulled from the sample and quantified.

Consequently note that even when FSH, LH and HCG are present in the sample at maximal *in vivo* levels, the calibration curve is not affected.

ELISA TROUBLESHOOTING GUIDE

POOR PRECISION	
POSSIBLE REASON	RECOMMENDED ACTION
Pipetting errors.	Ensure pipettes are calibrated on a regular basis and that operators have been adequately trained in pipetting techniques.
Incomplete washing of plate.	Ensure that plates are washed according to the IFU and that wash buffer has been correctly prepared.
Interruption of assay set-up.	Assay set-up should be continuous. Ensure required reagents are prepared before starting set-up of the ELISA.

NO COLOUR DEVELOPMENT OR LOW ABSORBANCE VALUES	
POSSIBLE REASON	RECOMMENDED ACTION
Omission of one or more reagents.	Check all reagents have been added.
Kit reagents have expired.	Check expiry dates of all reagents. No individual reagent expiry should be less than the overall expiry of the kit.
Reagents added in the wrong order.	Check reagents have been added in the correct order.
Reagents incorrectly prepared or contaminated.	Follow reagent preparation as indicated in the IFU. Ensure that the reagents are not contaminated.
The wrong volumes of reagents were used.	Re-run the assay, taking care to use correct volumes.
The conjugate was not prepared correctly.	Follow instructions on the conjugate dilution sheet. Ensure conjugate is pipette diluted with the conjugate diluent and not sonicated.
The reagents were not brought to the correct temperature before using.	Ensure reagents are brought up to the appropriate temperature before use.
Laboratory temperature was too low.	Maintain the laboratory temperature between 20 - 25°C. Do not run assays under air conditioning vents or near cold windows.
Inadequate incubation times and temperatures.	Refer to IFU for correct incubation times and temperatures for each step of the ELISA.
The correct wash protocol was not followed.	Refer to IFU for wash protocol.
Incorrect wavelength used to read the plate.	Ensure the wavelength specified in the IFU is used to read the plate (e.g. 450nm).

HIGH ABSORBANCE VALUES	
POSSIBLE REASON	RECOMMENDED ACTION
The wrong volumes of reagents were used.	Re-run the assay, taking care to use correct volumes of reagents.
The reagents were prepared incorrectly or contaminated.	Ensure that reagents are prepared according to instructions and that they are not contaminated.
The conjugate was not prepared correctly.	Check instructions on the conjugate dilution sheet. Ensure conjugate is pipette dilutes with the conjugate diluent and not sonicated.
Substrate solution has deteriorated or become contaminated.	Make sure the substrate is colourless before adding to the plate.
Sonication of buffers.	It is not necessary to sonicate any of the buffers used, follow exact procedure documented in the IFU.
Laboratory temperature was too high.	Maintain the laboratory temperature between 20 - 25°C. Do not run assays in direct sunlight or near sources of heat.
Incubation temperatures too high or incubation times too long.	Refer to IFU for correct incubation temperatures and times.
Plate inadequately washed.	Refer to IFU for detailed plate washing procedure.
Use of a plate shaker.	A gentle tap on the side of the microtitre plate is adequate. Use of a plate shaker is not necessary.
Plate reader was not functioning properly.	Ensure the plate reader is calibrated regularly.

POOR STANDARD CURVE	
POSSIBLE REASON	RECOMMENDED ACTION
Standards were added in the wrong order.	Re-run the assay ensuring the standards are applied to the microtitre plate in the correct order.
If appropriate, standards may not have been correctly prepared.	Prepare standards as instructed in the IFU.
Standards and other reagents were not added to the microtitre plate simultaneously.	Ensure standards and other reagents are ready to use before starting the assay to avoid disruption in assay set-up.
Inadequate incubation times.	Refer to the IFU for the correct incubation times for each step of the ELISA and ensure it is incubated in a dark environment

LOW SAMPLE ABSORBANCE	
POSSIBLE REASON	RECOMMENDED ACTION
Concentration of analyte in the sample is too high to be detected.	Further dilute the sample so that it can be read from the existing standard curve.
The sample may not have been adequately diluted.	Ensure samples are diluted according to instructions.