# Chapter 2 Materials and Methods

Methodology common to more than one experimental chapter has been included below. Experimental protocols specific to a chapter have been included in that relevant chapter.

## 2.1 Blood collection

Blood was collected from either the coccygeal or the jugular vein into evacuated plain silicone spray coated or lithium heparin vacutainers respectively (Becton, Dickson Ltd., Oxford, UK). Serum and plasma samples were analysed immediately or following a single freeze thaw.

## 2.2 Plasma progesterone enzyme-linked immune-sorbent assay (ELISA)

Plasma progesterone concentrations were determined using pre-coated ELISA plates specific for bovine plasma (Ridgeway Science, Gloucestershire, UK). Manufacturer's protocol was followed and no alterations were made to reagents or plates (as this assay was used in two of the following chapters inter- and intra- assay % coefficient of variation (CV) values are quoted there).

***Progesterone ELISA procedure***

Plates and reagents were removed from 4˚C storage and allowed to come to room temperature before commencing the assay. All wells were emptied and the plate was inverted and tapped on towel until dry. Unknown samples (10µl), standards (10µl) and quality controls (QCs) (10µl) were added to the plate in duplicate. The progesterone-enzyme label (200µl) was added to all wells and left to incubate for 2-2.5 hours at room temperature (18-20˚C). The

photosensitive alkaline phosphatase substrate was reconstituted at this stage and left in a dark cupboard until required. Following incubation, wells were washed three times using the wash buffer provided. The previously reconstituted substrate was now added to each well (200µl) and allowed to incubate for 20-30 minutes. Plates were then read at 570nm on a Multiskan Ascent 96/384 plate reader (MTX Lab Systems, Inc., Vienna, VA, USA).

2.3 Oestradiol radioimmunoassay (RIA)

Plasma oestradiol concentrations were determined using a solvent extracted radioimmunoassay based on the method outlined in Mann *et al.*, (1995) adapted for use in bovine samples using Radim MAIA Estradiol RIA kits 370001 and Radim MAIA Estradiol Tracer 370037 (Alere Ltd., Cheshire, UK).

***Extraction of $^{125}$I-labelled oestradiol***

As a result of interference caused by the buffer solution in which the [$^{125}$I]-oestradiol was diluted, the tracer needed to be extracted. A silica based column (Sep-Pak® Vac C18 20cc/5g 55-105µg, Water Ltd., Elstree, Hertfordshire, UK) was attached to a glass jar, connected to a vacuum pump within a fume hood. Initially, to activate the column, 20ml of methanol (analytical grade, Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) was added and eluted into the glass jar, followed by 40mls of distilled water, added and eluted into the jar. The column was now ready for the tracer, so the [$^{125}$I]-oestradiol was added to the column and eluted into a borosilicate tube. The [$^{125}$I]-oestradiol had been trapped into the column and the buffer causing the interference in the assay had been eluted into the jar. Next, 2mls of

acetone (analytical grade, Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) was added to the column and eluted into pre-labelled borosilicate tubes (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK), this was repeated until six or seven 2ml fractions had been collected. Using an automatic gamma counter (1277 Gammamaster, Wallac OY, Turku, Finland) 50µl of each fraction was used to determine radioactivity. The fractions containing the highest radioactivity were dried by evaporation under a stream of nitrogen at room temperature (24-25˚C). Each fraction was then reconstituted in 250µl of assay buffer (0.01M phosphate buffered saline (PBS) with 0.1% gelatin, 0.2% sodium azide and 0.3% EDTA, pH to 7.6). The resulting solution was pooled and used as tracer for the subsequent RIAs.

***Oestradiol radioimmunoassay procedure***

Plasma samples and QCs needed to be extracted prior to the radioimmunoassay. Samples and controls (0, 2 and 5pg/ml) were pipetted out in duplicate (2 x 500µl) into ether washed tubes (analytical grade di-ethyl ether stabilised with ethanol, Romil Ltd, Cambridge, UK), to which 3ml of diethyl ether (Romil Ltd., Cambridge, UK) was added, including two ether blanks to monitor potential contamination of reagents. All tubes were mixed using a vortex mixer for 60 seconds before being snap frozen using a dry ice and diethyl ether bath in a fume hood. The supernatant was poured off into pre-labelled glass borosilicate tubes, evaporated at room temperature under a stream of nitrogen, and later reconstituted with assay buffer (Table 5.2, page 136).

A set of standards were set up in triplicate, using a 16ng/ml top standard and double diluting to 1.25pg/ml, as well as non-specific binding (NSB), total count (TC) and total bound (TB), all also in triplicate. An appropriate amount of assay buffer was added to each tube (see Table 2.1) and all samples were mixed using a vortex mixer to recover the extracted oestradiol. To all tubes except NSB and TC, 50µl of the primary antibody was added. Tracer was used at 10-12,000 counts per minute (cpm), obtained by adding small amounts of tracer to assay buffer and counting 50µl until 10-12,000cpm was achieved. To all tubes, 50µl of tracer was added and all tubes were mixed using a vortex mixer and incubated overnight at 4˚C. After 24 hours, 100µl of the magnetic secondary antibody was added to all tubes except TC, and all tubes were mixed using a vortex mixer and incubated at room temperature for 1 hour. This assay was designed to be used with a magnetic test tube rack, which following centrifugation would hold the pellet in place to facilitate the decanting of all tubes simultaneously. As a magnetic test tube rack was not available, 5% (w/v) PEG 1000 (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) was added to the wash buffer (0.01M PBS with 0.2% sodium azide and 0.3% EDTA, pH to 7.6) in order to achieve a more compact pellet. After incubation 1ml of wash buffer + PEG 1000 (5%) was added to all tubes except TC, and all tubes except TC were centrifuged for 30 mins at 3000g at 4˚C. Tubes were aspirated and counted for 60 seconds (1277 Gammamaster, Wallac OY, Turku, Finland).

Preliminary oestradiol concentrations were obtained however; the data demonstrated inadequate and unreliable binding of the tracer to the known and unknown samples, QCs and standards, leading to inconsistent results. After

extended efforts trying but failing to achieve good quality binding from the multiple lots of tracer supplied, and considering the time restrictions of this project, it was decided that any results obtained in this manner would be unreliable, and have therefore regrettably been omitted from this thesis.

Table 2.1: Quantity of reagents, radioactive tracer and antibodies in non-specific binding (NSB), total count (TC), total bound (TB), standard curve (STD), quality controls (QCs) and samples for the oestradiol MAIA RIA. *NB: Samples (500µl) and QCs (500µl) were previously extracted and dried prior to reconstitution with assay buffer as stated in the table.

| | No. of Tubes | Assay Buffer (µl) | STD (µl) | Tracer (µl) | 1˚Ab (µl) | 2˚Ab (µl) | Wash Buffer (ml) |
|---|---|---|---|---|---|---|---|
| NSB | 3 | 300 | - | 50 | - | 100 | 1 |
| TC | 3 | - | - | 50 | - | - | - |
| TB | 3 | 250 | - | 50 | 50 | 100 | 1 |
| STD | 3 | 200 | 50 | 50 | 50 | 100 | 1 |
| QC/Sample* | 2 | 250 | - | 50 | 50 | 100 | 1 |