The development and evaluation of a colloidal gold immunosorbent assay for the detection of Human Chorionic Gonadotrophin

A dissertation submitted for the degree of Master of Science

by

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Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Master of Science, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Aileen Conway ID No.: 98971107

Aileen Conway

Date: 12th Sept 2000

Statement of Confidentiality

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Finally, this thesis is dedicated to my mother, Leshia, as poor repayment for the love, encouragement and support I have always received from her, in particular over the last few years. I would also like to dedicate this thesis to the memory of my father, Tom, it's times like this I miss you most.

Abbreviations

A_{280nm}	Absorbance at 280nm
A_{520nm}	Absorbance at 520nm
Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
Au	Gold
BCIP	5-Bromo-4-chloro-3-indoxyl phosphate
Bd.	Boulevard
Cat. No	Catalogue number
C.I.	Colour Intensity
CL	Control Line
Conc.	Concentration
Corpn.	Corporation
Da	Dalton
DAB	3,3'- Diaminobenzidine
DH_2O	Distilled water
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunoassay
FBS	Foetal Bovine Serum
FSH	Follicle stimulating hormone
g	gram
GCDB	Gold Conjugate Dilution Buffer
gp	Glycoprotein
HAMA	Human anti-mouse antibody
HAs	Heterophilic antibodies
hCG	Human Chorionic gonadotropin
HepB	Hepatitis B
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
Inc.	Incorporated
IVD	*In vitro* diagnostics

L	Litre
LH	Luteinising Hormone
Ltd.	Limited
M	Molar
mg	Milligram
min.	Minutes
mL	Millilitre
m.w.	Molecular weight
µL	Microlitre
n/a	Not applicable
Neg.	Negative
nm	Nanometre
No.	Number
OTC	Over-the-counter
p.a.	Per annum
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
POC	Point-of-care
Pos.	Positive
Prod. No.	Product Number
Rd.	Road
RIA	Radioimmunoassay
RNase	Ribonuclease
RO water	Reverse osmosis water
Soln.	Solution
St.	Street
TL	Test line
TMB	3,3',5,5'-Tetra methyl benzene
TSH	Thyroid stimulating hormone
UV	Ultra violet
Vol.	Volume
vs.	Versus
v/v	volume per volume
WHO	World Health Organisation
w/v	weight per volume

Table of Contents

Abstract

The aim of the work described in this thesis was to develop and evaluate two rapid, colloidal gold-based, immunochromatographic assays, a 'cassette' and 'wick' type, both for the qualitative detection of the glycoprotein hormone, human chorionic gonadotrophin (hCG) in human urine and serum. hCG is present in large quantities in the urine and serum of pregnant women and thus, provides a means for diagnosing pregnancy. A key element of this work was to develop assays, which would be capable of competing strongly in this market.

The outcome of this project was a highly sensitive and specific cassette and wick hCG assay, having the capability to detect as low as 25 IU/L hCG on the first day of a missed period. These assays also had the capacity to detect levels up to 250,000 IU/L hCG before a 'hook' effect was observed. This analytical range was equal to the majority of the tests already on the market. Evaluation of the two assays yielded 100% sensitivity and specificity for the cassette assay with urine samples and 98.5% sensitivity and 100% specificity for serum samples. A sensitivity of 99.4% (urine) and 100% (serum) and a specificity of 100% (urine) and 98% (serum) were obtained for the wick assay. It should be emphasised that these assays are limited to the qualitative detection of hCG and subsequently, all positive results should be confirmed by a doctor. It is a measure of the success of the assays that predicted sales for the first six months of 2000 are estimated at ~$60,000 for the cassette assay and ~$100,000 for the wick assay.

Section 1:

Introduction

1.1 History of hCG detection methods

In 1905 Halban suggested that the placenta was an endocrine organ. The investigations, which followed, led to the discovery, in 1920, of the placental formation of steroid, protein and polypeptide hormones, of which one was human chorionic gonadotropin (hCG). This was deduced by a Japanese scientist, Hirose, who transplanted placental tissue under the skin of female rabbits, which resulted in the stimulation of the ovaries and other reproductive organs (Saxena, 1983). In 1927 Aschheim and Zondek confirmed these findings. They also provided a detailed characterisation of hCG and its presence in the urine of pregnant women. This work by Aschheim and Zondek, as reported by Saxena (1983), established the measurement of hCG as the basis of pregnancy tests. It has since been recognised that hCG is secreted by the syncytiotrophoblast cells of the placenta and is present in the serum and urine during pregnancy (Gabbe *et al.*, 1996).

During the ensuing seventy years a number of biological, immunological and radioreceptor assays have been developed for measuring hCG in the urine and blood of pregnant women. The first reliable bioassay for hCG (the AZ test) was introduced with the publication of Aschheim and Zondek's work in 1928. This bioassay involved injecting mice with urine over a two-day period and four days later examining their ovaries for corpus luteum formation. According to Beers (1981), this method served as a source of qualitative as well as quantitative information for at least 20 years. In 1931 the Friedman and Haplan test reduced the time-span involved in the AZ test by using mature female rabbits. A number of variations of these time-constricting bioassays were developed over the next 20 years.

In 1944 Salk published his work on haemagglutination and in 1951 Boyden applied a method of protein attachment following acid treatment of sheep red blood cells, thus paving the way for the first immunological hCG assay introduced in 1960 by Wide and Gemzell. This assay was based on haemaglutination inhibition (see Figure 1.1.1, page 3). Various latex and radioisotope versions of this test were introduced in the 1960's. For the determination of hCG in urine, the tube and slide method was used with a sensitivity of ~0.200-1.250 IU/mL. Quantitation of hCG was carried out using radio-immunoassays (RIA's), enzyme-linked immunosorbent assays (ELISA's) and

by immunoassay particle counting (IMPACT) as well as by fully automated turbidometric methodology. Latex-based tests have also been popular, one method being the latex inhibition procedure. In the early 1980s dyed latex particles were utilised for rapid 'one-step' pregnancy tests (e.g.: Clearblue™ One Step by Unipath). Colloidal gold and carbon labels have also been used for similar purposes (Price *et al.*, 1997).

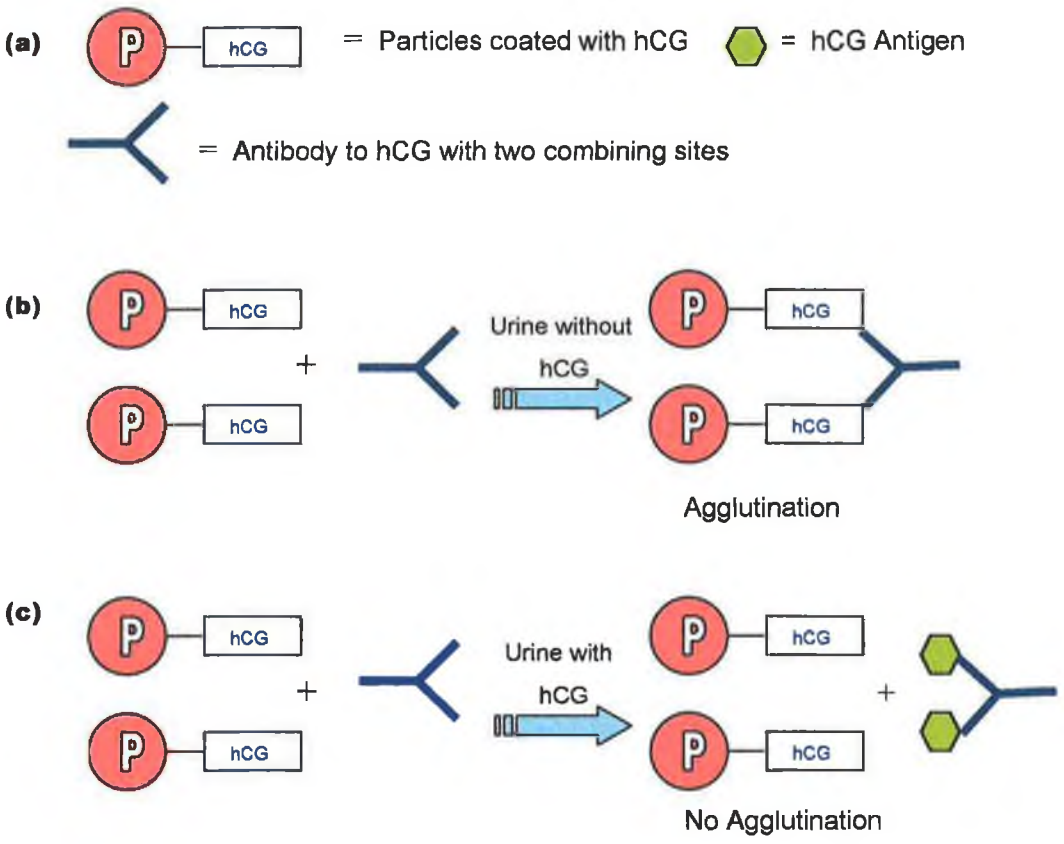


Figure 1.1.1: *The Agglutination Inhibition Assay. (a) Shows diagram key. (b) The agglutination of particle labelled anti-hCG antibodies when no hCG is present in the sample. (c) The presence of hCG in the sample prevents agglutination of these labelled anti-hCG antibodies (Adapted from Chamberlain, 1995).*

Within 7 to 10 days after conception hCG is detectable in the urine of pregnant women using current methods, thus, providing the ideal tool for pregnancy diagnosis. The older assays typically yielded inaccurate results due to the reliance on older and relatively impure hCG standards. These tests were unable to differentiate hCG from the pituitary hormones. Thus, they would only indicate a positive result when the concentrations of hCG were much higher than normal physiological levels of either luteinising hormone (LH), follicle stimulating hormone (FSH) and thyroid

stimulating hormone (TSH). This meant that many of the tests would not give a positive pregnancy test result until about the third month of the pregnancy (Gard, 1998). Since then, the reliability and ease of use of pregnancy tests has greatly improved due to technical advances. The refinement of enzyme immunoassays and other non-radiometric methods coupled with automated instrumentation has greatly improved the accuracy of test results. A major landmark in the optimisation of hCG assays was the development of rapid one-step assays, which can be used at home as they do not require specialised equipment or training. While an ELISA may prove to be more sensitive than these one-step assays, the time involved in obtaining a result from an ELISA was found to be a disadvantage, particularly in emergency situations where potentially embryotoxic procedures, such as x-ray studies, might be carried out. Generally, the sensitivity and specificity of the rapid assays are good, as the average level of hCG at time of presentation for pregnancy testing (invariably around the time of the first missed period) is approximately 100-200mIU/mL. This level is well within the detectable range for the majority of the rapid assay tests. This thesis describes the development of two such assays for use with either urine or serum samples.

1.2 Properties and Chemical Characteristics of hCG

1.2.1 The Glycoprotein Hormones

The purpose of any pregnancy test is to detect human chorionic gonadotropin in human urine and/or serum. The glycoprotein hormones comprise a group of pituitary hormones bearing carbohydrate moieties; luteinising hormone (LH), thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH), and the placental hormone human chorionic gonadotropin (hCG). The average molecular weight, carbohydrate content and pI of these hormones are shown in Table 1.2.1 below.

Hormone	Molecular Weight	Carbohydrate Content (g/100g)				
		Neutral Sugar	Acetyl Hexosamine	Sialic Acid	Fucose	pH
hCG	*36700*	*11.0-11.2*	*10.8-11.1*	*8.5-9.0*	*1.2*	*4.9*
FSH	*35500*	*3.9-12.2*	*2.9-9.0*	*1.4-5.1*	*-*	*5.5*
LH	*28500*	*11.3*	*4.9*	*2*	*-*	*7.4*
TSH	*25000*	*5.9*	*4.1*	*-*	*0.5*	*-*

Table 1.2.1: *Approximate molecular weight, carbohydrate content and pI of the glycoprotein hormones. (From George, 1996)*

These glycoprotein hormones are composed of two non-covalently linked, non-identical peptide chain subunits, designated α and β (Cole, 1999b; Bischof *et al.*, 1997; Morgan and Canfield, 1971 and Ross, 1977). The amino acid sequence of the α-subunit is identical among all four hormones and is encoded for by the same gene. The β-subunit genes are distinct for each hormone but they share many conserved sequences. Their gene products are 40-45% homologous in their primary structure. However, it is the differences in the β-subunit sequences which confers the unique biological activity and immunologic specificity of each hormone (Cole *et al.*, 1991 and Morgan *et al.*, 1974). It has also been suggested that the biological activity is dependent on reversible tertiary or quaternary structural configuration of subunits in the intact molecule (Ross, 1977). Recombination of an α and β-subunit of the four

glycoprotein hormones gives rise to a molecule with biological activity characteristic of the hormone from which the β-subunit was derived (Cunningham *et al.*, 1989).

1.2.2 The Structure of hCG

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone which is secreted by trophoblastic tissue of the placenta and by certain tumours. In comparison with the other glycoprotein hormones, hCG has a unique amino acid extension at the carboxy terminal end (-COOH-terminal peptide, 109-145), which is less important for its intrinsic biological activity, but has a strong bearing on its metabolic clearance rate (Hoermann, 1996).

hCG is composed of two dissimilar subunits, α and β, associated non-covalently (see Figure 1.2.1, page 7). They are held together by electrostatic and hydrophobic forces and can be separated by treatment with acidified urea (Cunningham *et al.*, 1989). The molecular weight of the dimer is approximately 36.7kDa, the α-subunit contributing 14.5kDa and the β-subunit 22.2kDa. The α-subunit of hCG consists of a 92 amino acid sequence essentially identical and shared in common with the pituitary glycoprotein hormones (see Figure 1.2.2, page 8) (Cole et *al.*, 1991 and Gabbe *et al.*, 1996). Within this α-chain 5 intrachain disulfide bonds and 2 N-linked glycosylation sites (asparagines 52 and 78) are found (Bischof *et al.*, 1997). The α-subunit has been shown to have a biological activity of less than 1% of the native hormone (Morgan and Canfield, 1971).

The β-subunit, although structurally similar to the α-subunit, differs enough to confer specific biological activity on the intact (dimer) hormone (Gabbe *et al.*, 1996). The β-subunit itself has no intrinsic biological activity, appearing to be unable to bind to the receptor (Cunningham *et al.*, 1989). The β-subunit of hCG is composed of 145 amino acids (see Fig. 1.2.2, page 8), 6 intrachain disulfide bonds, 2 N-linked oligosaccharides (asparagines 13 and 30) and 4 O-linked oligosaccharides (serines 121,127,132 and 145) (Cole *et al.*, 1991 and Bischof *et al.*, 1997). The β-subunits of hCG, LH, TSH and FSH exhibit considerable homology. This is particularly true of the β-subunits of hCG and LH (Bischof *et al.*, 1997).

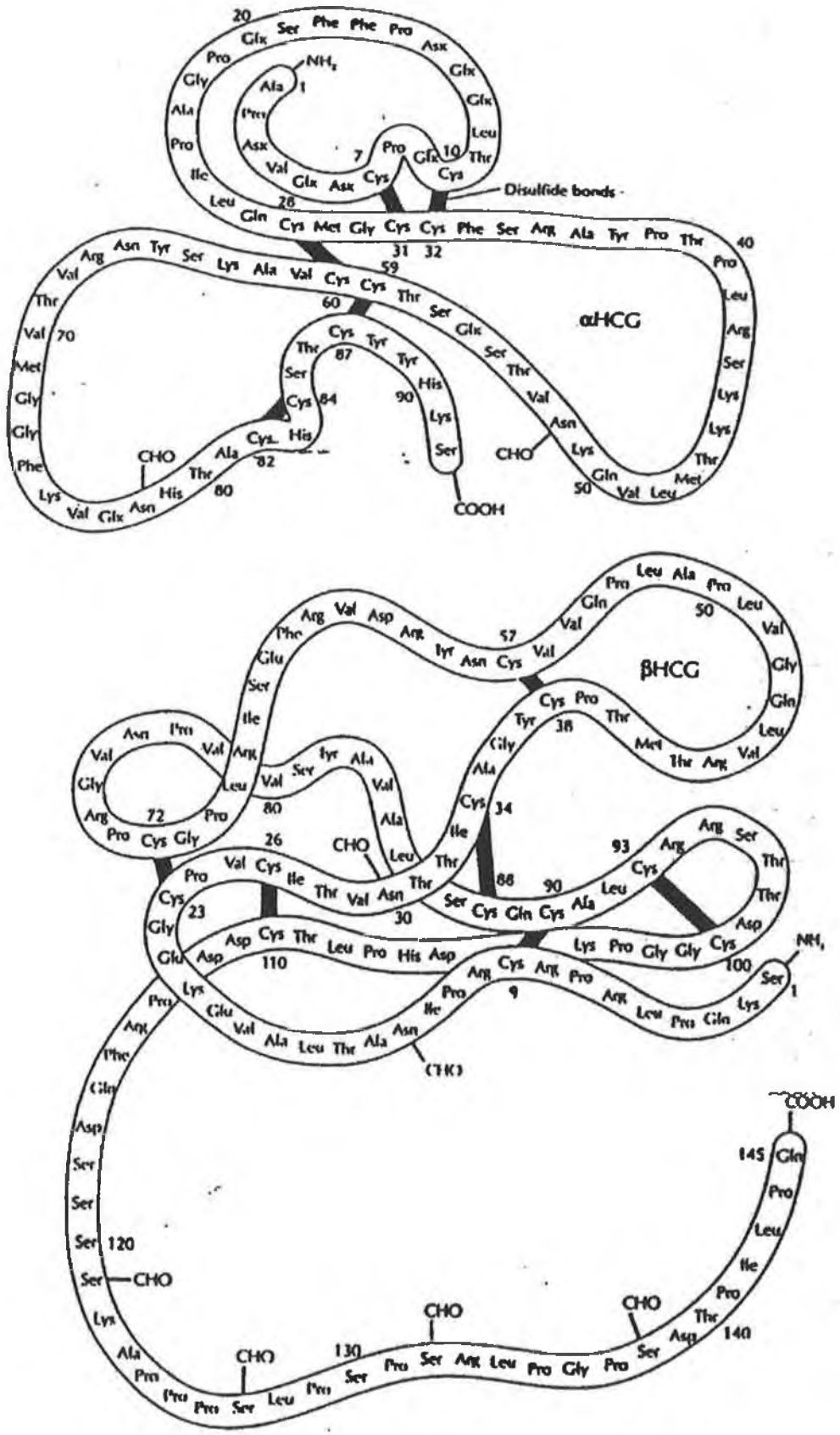


Figure 1.2.1: *The structure of hCG. Disulfide bonds are marked with dark lines. (Taken from Gabbe et al., 1996, with permission).*

A review of the amino acid and carbohydrate compositions of the β-subunits of hCG and LH reveals similarities. hCG, however, possesses more total carbohydrates and specifically much more sialic acid. At the C terminus of the peptide β-hCG is 23 amino acids longer than β-LH, the remainder of the β-chain differs only by 24 residues out of 121 (80% homology) and none of these residues is involved in disulfide bonds. It is thus apparent why many hCG assays have fallen victim to the high immunologic cross-reactivity with LH (Bischof *et al.*, 1997).

(a)

Ala ^{1} *– Pro – Asp – Val – Gln – Asp – Cys – Pro – Glu – Cys* ^{10} *– Thr – Leu – Gln – Glu – Asp – Pro – Phe – Phe – Ser – Gln* ^{20} *– Pro – Gly – Ala – Pro – Ile – Leu – Gly – Cys – Met – Gly* ^{30} *– Cys – Cys – Phe – Ser – Arg – Ala – Tyr – Pro – Thr – Pro* ^{40} *– Leu – Arg – Ser – Lys – Lys – Thr – Met – Leu – Val – Gln* ^{50} *– Lys – Asn* ^{CHO} *– Val – Thr – Ser – Glu – Ser – Thr – Cys – Cys* ^{60} *– Val – Ala – Lys – Ser – Tyr – Asn – Arg – Val – Thr – Val* ^{70} *– Met – Gly – Gly – Phe – Lys – Val – Glu – Asn* ^{CHO} *– His – Thr* ^{80} *– Ala – Cys – His – Cys – Ser – Thr – Cys – Tyr – Tyr – His* ^{90} *– Lys – Ser*

(b)

Ser ^{1} *– Lys – Glu – Pro – Leu – Arg – Pro – Arg – Cys – Arg* ^{10} *– Pro – Ile – Asn* ^{CHO} *– Ala – Thr – Leu – Ala – Val – Glu – Lys* ^{20} *– Glu – Gly – Cys – Pro – Val – Cys – Ile – Thr – Val – Asn* $^{CHO\ 30}$ *– Thr – Thr – Ile – Cys – Ala – Gly – Tyr – Cys – Pro – Thr* ^{40} *– Met – Thr – Arg – Val – Leu – Gln – Gly – Val – Leu – Pro* ^{50} *– Ala – Leu – Pro – Gln – Val – Val – Cys – Asn – Tyr – Arg* ^{60} *– Asp – Val – Arg – Phe – Glu – Ser – Ile – Arg – Leu – Pro* ^{70} *– Gly – Cys – Pro Arg – Gly – Val – Asn – Pro – Val – Val* ^{80} *– Ser – Tyr – Ala – Val – Ala – Leu –Ser – Cys – Gln – Cys* ^{90} *– Ala – Leu – Cys –Arg – Arg – Ser – Thr – Thr – Asp – Cys* ^{100} *– Gly – Gly – Pro – Lys – Asp – His – Pro – Leu – Thr – Cys* ^{110} *– Asp – Asp – Pro – Arg – Phe – Gln – Asp – Ser – Ser – Ser* ^{120} *– Ser* ^{CHO} *– Lys – Ala – Pro – Pro – Pro – Ser* ^{CHO} *– Leu – Pro – Ser* ^{130} *– Pro – Ser* ^{CHO} *– Arg – Leu – Pro – Gly – Pro – Ser – Asp – Thr* ^{140} *– Pro – Ile – Leu – Pro – Ser* $^{CHO\ 145}$

Figure 1.2.2: *Amino acid sequences of (a) the α-subunit of hCG and (b) the β-subunit of hCG. Residue numbers and glycosylation sites are superscripted in red (Saxena, 1983).*

The folding of the peptide and carbohydrate extensions that are attached to certain amino acids dictates the three-dimensional structure of the glycoprotein hormones (O'Connor *et al.*, 1994). The polypeptide portion accounts for 66% of the molecular weight of hCG and amino acid analysis reveals remarkably high values for proline, cysteine and serine, with no free SH groups (Reid *et al.*, 1972). The carbohydrate moiety is abundant in hCG in comparison to the other glycoprotein hormones, (refer

to Table 1.2.1, page 5), making up 30% of the weight of the hCG molecule. This is the largest carbohydrate content of any human hormone, with 11 residues of N-acetyl glucosamine, 9 of mannose and galactose, 8 of sialic acid, 3 of N-acetylgalactosamine and 1 residue of fucose per glycopeptide chain (Reid *et al.*, 1972). The hCG molecule contains four N-linked carbohydrate antennae bound to two asparagine residues of the α-subunit and the β-subunit and four O-linked sugar chains bound to serine residues at the carboxyterminus of the β-subunit, all of which terminate with sialic acid. The carbohydrate moiety, especially the terminal sialic acid, plays an important role in prolonging the half-life of hCG in plasma and preserving the biological activity of the molecule by protecting it from catabolism. It is essential for biological activity since enzymatic removal of sialic acid abolishes the hormones biologic properties (Reid *et al.*, 1972). Furthermore, it forms the basis for glycosylation variants of the hormone (Hoermann, 1996).

1.3 Cellular Origin of hCG

hCG mRNA is detectable in blastomeres of 6-8 cell embryos. It is widely believed that the complete hCG molecule is produced by the syncytial trophoblastic cells of the placenta and is secreted into the intervillous space (see Fig. 1.3.1 below) (Gabbe *et al.*, 1996; Cunningham *et al.*, 1989 and Braunstein et al., 1976).

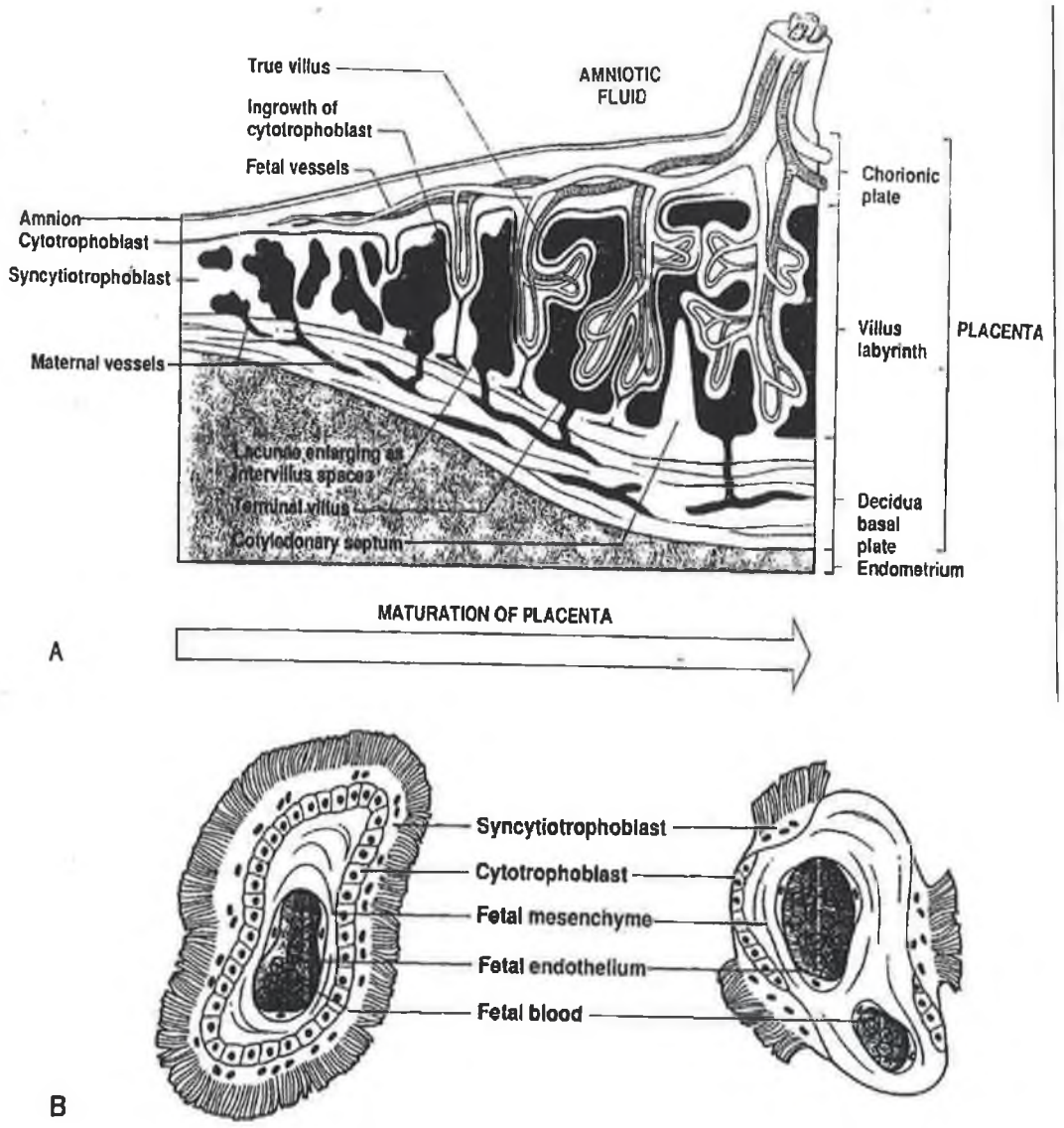


Figure 1.3.1: *(A) Gross morphology of the human placenta. The changes, which occur in the placental morphology as gestation advances, evolve from left to right. (B) Anatomic structure of early (left) and late (right) terminal villi. (Taken from Gabbe et al., 1996, with permission).*

It was demonstrated after implantation that mRNA for hormonal hCG is present in cytotrophoblasts and syncytiotrophoblasts in the outer syncytial layer, whereas mRNA for α-hCG is present primarily in cytotrophoblasts with none in the syncytial layer. hCG secretion is related to the mass of hCG-secreting trophoblastic tissues. Therefore, the release of hCG *in vivo* can be correlated with trophoblast layer widths from weeks 4 to 20 and with placental weights from 20 to 38 weeks. Proliferation of immature trophoblastic villi and an extensive syncytial layer occurs between weeks 3 and 9. This coincides with rapidly rising hCG levels. Between 10 and 18 weeks gestation there is a decline in hCG which has been associated with a relative reduction in syncytiotrophoblasts and cytotrophoblasts at this time. From 20 weeks gestation to term there is a gradual increase in dimer hCG which corresponds with a gradual increase in placental weight and villus volume. In short, it is apparent that as the placenta begins to proliferate, the levels of hCG begin to rise and hCG levels decline as the placenta undergoes morphological changes which results in a reduction in trophoblasts, the source of hCG.

1.4 Biosynthesis of hCG

The biosynthesis of hCG is a quite a complex process. The synthesis of the α and β chains of hCG are regulated independently. A single gene, located on chromosome 19, codes for the α-subunit of all of the glycoprotein hormones (Bischof *et al.*, 1997; Cunningham *et al.*, 1989). There are eight separate genes that code for the β-subunit of hCG and LH. Seven genes code for β-hCG and one codes for β-LH. These genes are located on chromosome 7. It has been suggested that the seven β-hCG genes evolved from an ancestral gene for β-LH. Only two, possibly three of the genes for the β-subunit are expressed (Cunningham *et al.*, 1989). The rate at which the β-subunit of hCG is synthesised limits the rate of formation of the complete hCG molecule (Cunningham *et al.*, 1989). Intact hCG and α and β-subunits of hCG are secreted by the trophoblasts of normal placenta, hydatidiform moles and choriocarcinoma tissues (Cunningham *et al.*, 1989). Generally, there is an excess of α-subunits detectable in the placenta and plasma of normal pregnant women, while the hCG β-subunit is usually present in small or even undetectable amounts. The signal sequences for both the α and β-subunits are cleaved by microsomal endopeptidases since they are synthesised as larger molecular weight precursors.

It should be noted that eight different molecular forms of hCG have been identified: holo hCG, nicked hCG, holo hCG missing the C terminus, free β-hCG, free β-hCG missing the C terminus, nicked free β-hCG, free α-hCG and different glycosylation forms (Gabbe *et al.*, 1996) (see Figure 1.4.1). Following translation of the hCG mature RNA (i.e.: any non-coding regions have been removed) into polypeptides, the peptides are further processed by glycosylation of the subunits. This is followed by cleavage of the signal peptide, folding of the subunits into their three-dimensional shape, formation of the αβ complex, attachment of the O-linked oligosaccharides and 'trimming' of the N-linked oligosaccharides (Cole and Kardana, 1992). This is a complex biosynthetic pathway which results in the secretion of biologically active hCG (holo hCG). This pathway is not 'fixed' so that besides holo hCG and free α and β-subunits, aberrant glycosylation products can appear in the circulation, some of which have clinical significance. In recent years, hCG or free β-subunits missing

the peptide bonds 44-45, or 47-48 in β-hCG, called nicked hCG (see Fig. 1.4.1) have been detected in serum and urine (Bischof *et al.*, 1997; Kardana and Cole, 1994; Cole *et al.*, 1991 and Nishimura *et al.*, 1988).

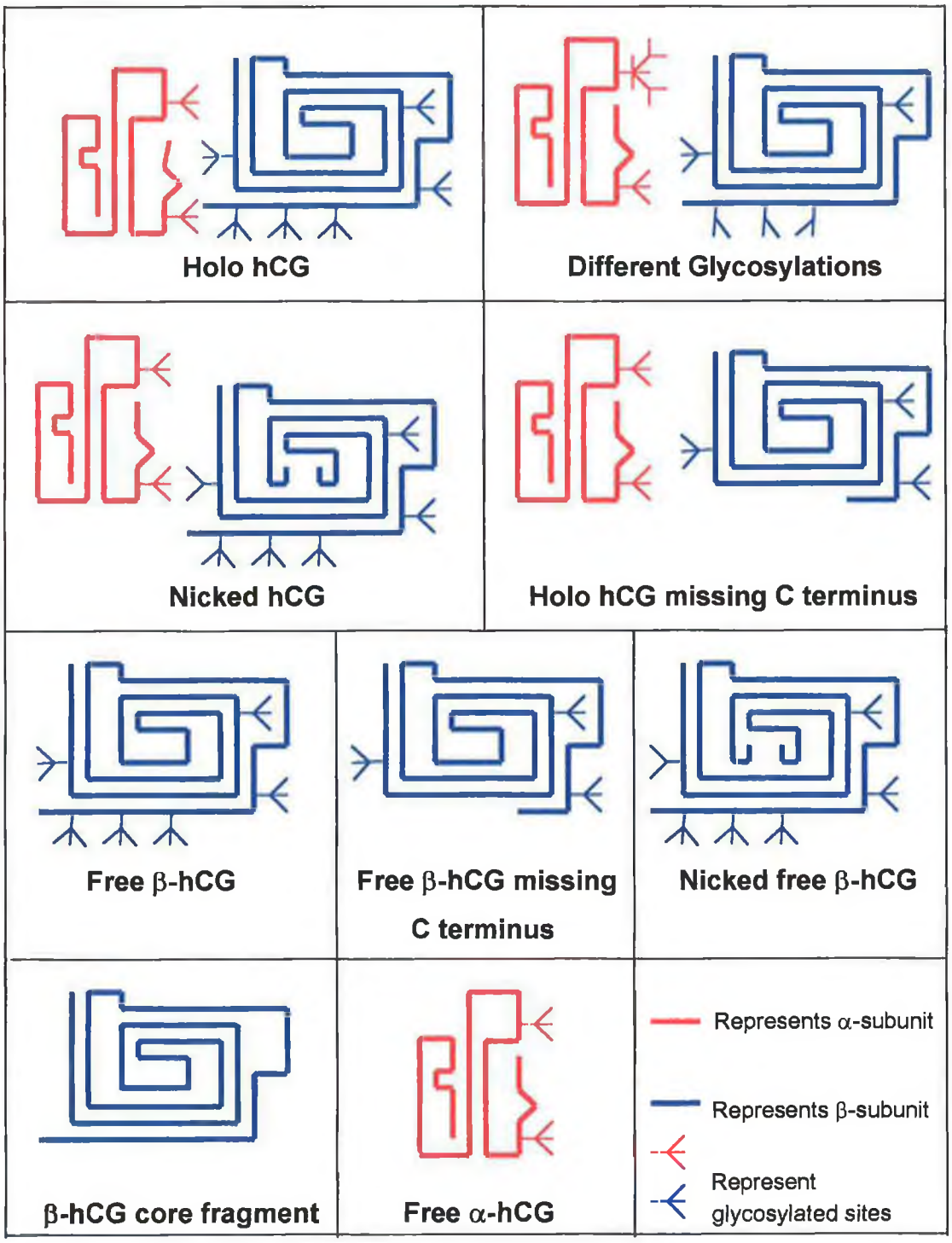


Figure 1.4.1: *Schematic representation of the known isoforms of the hCG molecule. Underneath each diagram is the relevant isoform name. A diagram key is included (see bottom right-hand corner). (Adapted from Cole et al., 1993b).*

1.5 Regulation of hCG Biosynthesis

hCG secretion exhibits a 24 hour rhythm in the first trimester of pregnancy during which the hCG levels are secreted in a pulsative manner . A nadir is observed at 1400 hours (12% below the 24 hour mean), followed by a progressive rise in the evening, reaching a peak at 0700 hours. hCG secretion is related to placental gonadotropin releasing hormone (GnRH). When an *in vitro* trophoblast perfusion system is used, hCG is released in 11 to 22 minute pulses where the pulse frequency and amplitude are linked to the release of placental GnRH (Gabbe *et al.*, 1996).

1.5.1 cAMP

It has been demonstrated that hCG secretion can be stimulated in purified cytotrophoblasts in culture using 8-bromo-cAMP. This is followed by an increase in mRNA levels encoding for the α and β-subunits of the hormone. The demonstration that cytotrophoblasts possess adenylate cyclase activity identifies the adenylate cyclase-cAMP pathway in the activation of cellular responses to stimulatory agents *in vitro*. Stimulatory agents *in vivo* which may trigger cytotrophoblasts' adenylate cyclase are not known.

1.5.2 Gonadotropin Releasing Hormone

The cytotrophoblasts of the placenta produce and secrete gonadotropin-releasing hormone (GnRH). Similar mechanisms of regulation act on placental and hypothalmic GnRH. It has been observed that circulating GnRH levels are higher in pregnant women than in non-pregnant women. Placental GnRH stimulates hCG release through a dose-dependent paracrine mechanism (see Figure 1.5.1, page 15). The time course and pattern of serum GnRH levels are parallel to those of hCG and are correlated with placental GnRH content, suggesting a placental origin for circulating GnRH and a regulatory role for GnRH in the secretion of hCG from the placenta. The addition of a GnRH antagonist reduces hCG secretion in placental cells in culture and reverses the stimulatory effect of GnRH. First trimester placental trophoblasts in culture appear to be more sensitive to GnRH stimulation of hCG secretion than those of late pregnancy; this is possibly due to the relative reduction of

trophoblasts as the placenta transforms into an organ of transfer during gestation (Gabbe *et al.*, 1996). There also appears to be a local regulatory loop between the secretion of GnRH, hCG and placental steroids (see Figure 1.5.1). Evidence for this theory is seen in the stimulation of progesterone production by hCG, while GnRH inhibits the formation of progesterones and oestrogens.

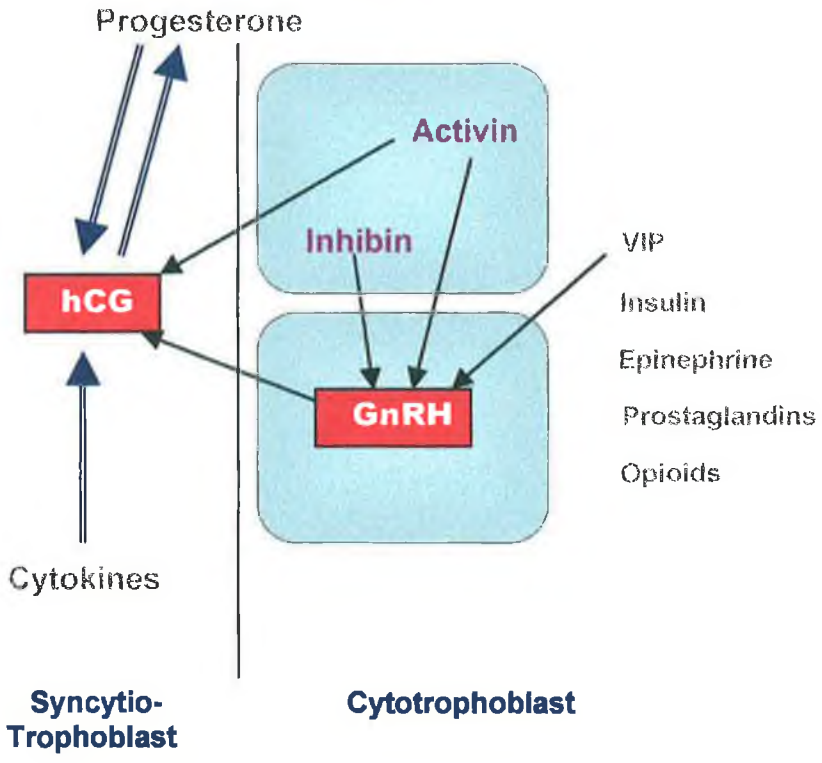


Figure 1.5.1: *The regulatory system of GnRH and hCG between cytotrophoblasts and syncytiotrophoblasts. The autocrine and paracrine modes of regulation of GnRH by inhibin (inhibitory) and activin (stimulatory) are depicted (Adapted from Chamberlain, 1995).*

1.5.3 Inhibin and Activin

Inhibin and activin, which are synthesised in the cytotrophoblast, may also partake in the regulation of hCG secretion by modulating GnRH activity. Activin is a nonsteroidal regulator, which stimulates the secretion of FSH. The actions of activins are the opposite of those of inhibin. Inhibin contributes to the control of gametogenesis, embryonic and foetal development and haematopoiesis. The actions of inhibins are opposed by activins (Dorland, 1994). Adding inhibin antiserum to placental cell culture causes a significant increase in hCG secretion with a

simultaneous rise in GnRH release. This suggests an inhibitory action by inhibin on hCG secretion. The suppressive effect of inhibin on hCG secretion only acts in the placenta in the later part of pregnancy and not in the first trimester. An important point to note is that addition of purified inhibin to cultured placental cells has no effect on either hCG or GnRH release. Thus, placental inhibin may function as both an autocrine and paracrine factor in regulating hCG via GnRH.

Activin has the reverse effect to inhibin (see Fig. 1.5.1). By adding purified activin to placental culture the GnRH-induced release of hCG is augmented. It has also been observed that this effect can be significantly reduced by the addition of inhibin. It thus appears that placental GnRH plays a fundamental role in the regulation of hCG secretion, but the functional activity of GnRH seems to be regulated by inhibin (inhibitory) and activin (stimulatory).

1.6 Function of hCG

Biologically inactive, isolated hCG is present in normal and pathological states. Biologically active hCG is present normally only during pregnancy, secreted by the syncytial trophoblastic cells of the placenta (Braunstein *et al.*, 1976). The preimplantation embryo produces hCG and represents a signal of maternal-embryo dialogue at the time of implantation. The role of hCG is of importance in the endocrine control of early pregnancy in maintaining an ovarian source of steroid hormones until the placental endocrine activity is capable of replacing it (Reid *et al.*, 1972).

One of the functions of hCG is to stimulate the growth and action of the corpus luteum, thereby stimulating enhanced production of progesterone and relaxin by the ovaries. This luteotropic activity of hCG rescues corpus luteal function that otherwise would regress. Concomitantly, there appears to be a heightened production of inhibin by the corpus luteum of early pregnancy, thereby supressing pituitary FSH secretion. It has also been proposed that hCG may suppress LH secretion by the maternal hypothalamic-pituitary unit (Cunningham *et al.*, 1989). It is known that hCG possesses intrinsic thyrotropic activity, binding to the TSH receptor and stimulating iodide uptake and DNA synthesis in the thyroid gland (Gabbe *et al.*, 1996).

hCG enhances placental progesterone biosynthesis and provides gonadotropic and adrenotropic input to the foetus during the first trimester of pregnancy (Reid *et al.*, 1972). Of special interest is the stimulation of Leydig cells in the male foetus during organogenesis to produce testosterone, which, in turn, is instrumental in the development of the external genitalia. Foetal testicular testosterone secretion is maximum at the same time in gestation as the maximum levels of hCG secretion. Thus, at a critical time in male foetal development, hCG serves as an LH surrogate on foetal testes to promote testosterone synthesis and secretion and, thereby, male sexual differentiation. When testosterone is converted to dihydrotestosterone by 5-α-reductase in the external genital tissue the development of male genitalia is determined (Gabbe *et al.*, 1996) (see Figure 1.6.1).

Adult human ovaries primed with FSH can be induced to ovulate by hCG. hCG is, therefore, often used therapeutically, in combination with FSH, as an LH surrogate in the treatment of infertility caused by anovulation due to hypogonadotropic hypogonadism (Cunningham *et al.*, 1989).

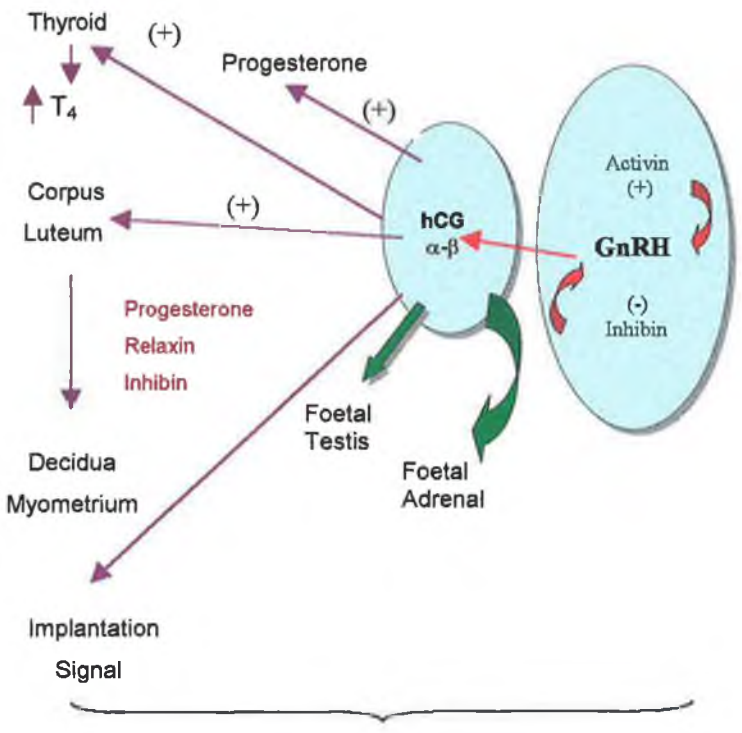


Figure 1.6.1: *Diagrammatic representation of the functional role of hCG in the maternal and foetal compartments during the first 10 weeks of gestation. The GnRH/inhibin/activin system of the cytotrophoblast and the biosynthesis of hCG in the syncytiotrophoblast are depicted by ovals (Gabbe et al., 1996).*

1.7 Levels of hCG

1.7.1 Normal Pregnancy

hCG appears in the maternal circulation soon after implantation, as early as 6-10 days after conception (Cole, 1999b and Braunstein *et al.*, 1978) and its levels rise rapidly with a doubling time of 30.9 hours. This exponential rise reaches a peak of 5,000-150,000 IU/L at the end of the first trimester, making hCG an ideal indicator for early confirmation of pregnancy (Cole, 1999b). This is followed by a fall to between 2,000-50,000 IU/L where it remains until the end of pregnancy (Cole, 1999b). The levels of hCG in the second and third trimesters are approximately one third to one sixth of peak hCG levels (see Figure 1.7.1). There is a parallelism between values in serum and urine, there being an equivalent unitage per litre of serum and urine per 24 hours (Loraine and Bell, 1966). The levels of the α and β-subunits of hCG are substantially different from those of the intact molecule (see 1.7.4.2). The levels of the β-subunit are low or undetectable, while the α-subunit increases gradually until about 36 weeks gestation when a plateau is reached (Cunningham *et al.*, 1989). In twin pregnancies hCG levels 4-5 weeks after the last menstrual period (LMP) are more than two-fold higher than in singleton (one child) pregnancies. There are also significantly higher levels of hCG detectable in women with hydatidiform moles and choriocarcinoma (Reid *et al.*, 1972).

1.7.2 Ectopic pregnancies

Abnormal pregnancies can be predicted by examination of hCG levels. In abnormal (ie: ectopic) pregnancies hCG may fall below 200 IU/L. A very high concentration of hCG is seen for molar pregnancies following surgery. Clinical observations have shown that tubal pregnancies develop individually different levels of β-hCG (Klein *et al.*, 1995). A pregnancy can be considered abnormal if two or more serum samples collected at two-week intervals show hCG levels above or below normal expected levels. Those with ectopic or those whose pregnancies are destined to terminate in a first or second trimester spontaneous abortion will have abnormally low serum hCG levels (Braunstein *et al.*, 1978).

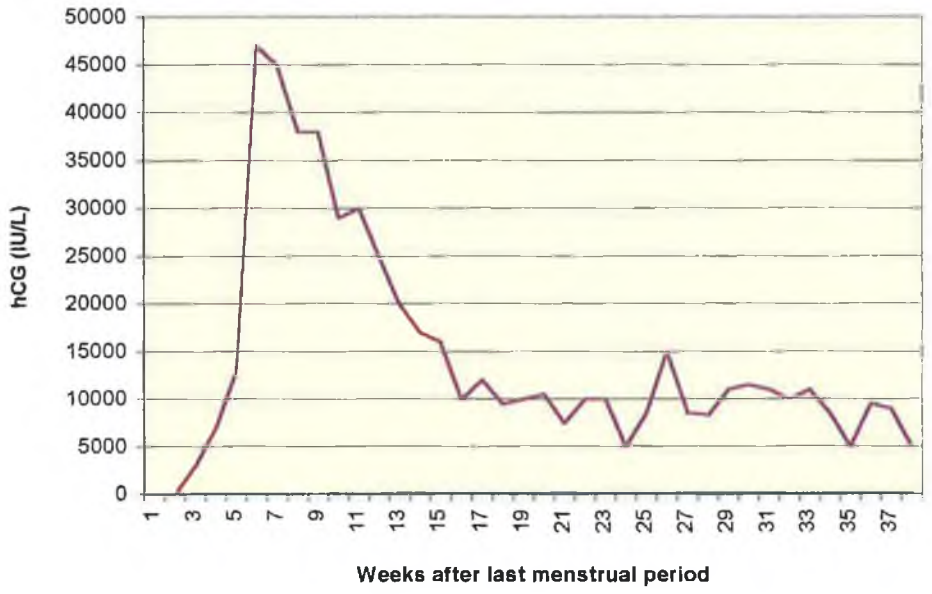


Figure 1.7.1: *Graph of mean serum hCG levels (IU/L) throughout normal pregnancy (i.e.: 39 weeks) (Braunstein et al., 1976).*

1.7.3 Non-pregnant Levels

hCG is not unique during pregnancy. A remarkable characteristic of hCG is its broad distribution. It has been found in most, if not all, human tissues (Braunstein *et al.*, 1978 and Cunningham *et al.*, 1989) and in the urine and serum of normal men and women and in many types of cancer cells. Free α and β-subunits have been detected in the human pituitary gland. In normal nonpregnant women the median level of hCG increases more than tenfold from reproductive age (median 0.05 IU/l) to post-menopausal age (median 1.1 IU/l).

1.7.4 Levels of various isoforms in normal pregnancy and disease

There are three groups of hCG configuration (refer to Figure 1.4.1, page 13) that effect the performance and interpretation of hCG assays.

1.7.4.1 Dimeric hCG

In the first trimester of pregnancy there is a sharp rise in the levels of dimeric hCG and both subunits, α-hCG and β-hCG. The dimeric hCG is usually measured for the diagnosis and monitoring of early pregnancy, either directly by sandwich-type assays

or indirectly by the detection of β-hCG (Gabbe *et al.*, 1996). hCG is detectable in serum in approximately 5% of patients by 8 days after conception and in virtually all patients at day 11 (Cunningham *et al.*, 1989). Ectopic pregnancies or spontaneous abortions are suspected if there is any deviation from the normal doubling pattern. Therefore, if the hCG levels fail to rise 66% in two days further investigation is required to determine if the patient is presenting with one of the above conditions.

1.7.4.2 Free α-hCG and β-hCG Subunits

Free α-hCG subunits are detectable at gestational week 6 and rise in sigmoid fashion to peak at gestational week 36 (Gabbe *et al.*, 1996). The levels of free α-hCG subunits are rarely used for clinical interpretation; however, it should be noted that the levels of this subunit are elevated in persistent gestational trophoblastic neoplasia and appear to be significantly lower in women with insulin-dependent diabetes. Free β-hCG can be detected as early as 7 days after implantation. Persistently elevated levels of free β-hCG in patients with trophoblastic disease may be an indication of a poor prognosis.

1.7.4.3 Nicked hCG Subunits

Nicked hCG is a hCG subunit with deleted peptide linkages as outlined in section 1.4. In the β-subunit these deleted linkages are between residues 47-48 and between 44-45 or 46-47 (Hoermann, 1996). In the α-subunits they are found between 70-71. It has been observed that as nicking increases receptor binding decreases (Cunningham *et al.*, 1989), making nicked hCG less biologically active. Much dissimilarity has been observed between the levels of hCG detected for the same samples on various assays, this is due to assays detecting subunits such as the free β-subunit, nicked hCG or the β core fragment. Many of these assays will give higher than expected readings by picking up these isoforms of hCG.

1.7.5 hCG International Standards

The concentration of hCG may be measured in IU/L or ng/mL in urine or serum. The relationship between IU/L and ng/mL is expressed as:

$$1 \text{ ng/mL} = 9 \text{ to } 11 \text{ IU/L}$$

The International Unit (IU) is based on hCG bioassays, with the standard being the gonadotropin maintained at the WHO in London. (Flynn and Seifer, 1996). Two World Health Organisation (WHO) standards for hCG are commonly encountered while running hCG assays. These standards are utilised for reference, in order to ensure reproducibility and correlation between all of the various hCG assays on the market. The first of these standards is the Third International Standard (3rd IS) which is the same as the First International Standard (1st IS). Most assays are now calibrated against one of these two standards. Clinical specimens tested using the older, relatively impure Second International Standard (2nd IS) typically yield results that are about 50% lower. This is particularly important for assays which recognise the β-subunit part of the molecule as the 2nd IS was quite highly contaminated with free α and β-subunits.

1.8 Clinical Applications of hCG Measurement

1.8.1 Pregnancy Diagnosis

Using very sensitive hCG assays, hCG is first detectable in the maternal blood and urine at approximately 8 to 11 days after conception. A sensitivity of approximately 0.1 to 0.3 IU/L is required due to the low levels of hCG. The concentration of hCG increases exponentially approximately one week after conception. hCG levels rise rapidly with a doubling time of 1.5 to 2.5 days for the first 6 weeks of gestation (Klee, 1994 and Flynn and Seifer, 1996). Most of the current home use tests have a sensitivity of 25 IU/L and, therefore, would not show a positive result until 12-13 days for most subjects (Gabbe *et al.*, 1996). False positive results are common in the range 5-25 IU/L. Such false positive results can occur in perimenopausal and postmenopausal women because of endogenous pituitary hCG secretions. This phenomenon should, therefore, serve to discourage the current trend in rapid home tests to detect 10 IU/L. In practice, a level less than 5 IU/L can be confidently stated as negative and a level exceeding 25 IU/L can be confidently stated as positive. If any uncertainty arises, repeating the test 2 days later normally shows an increase in hCG levels, confirming a pregnancy.

1.8.2 Ectopic Pregnancy

Since 1970 the incidence of ectopic pregnancies has increased six fold in the United States (Flynn and Seifer, 1996). Approximately 10% of all maternal mortality can be attributed to ectopic pregnancies and only one third of women who have sustained an ectopic pregnancy will go on to deliver a live-born infant. The reported risk of sustaining a subsequent ectopic pregnancy ranges from 4% to 25% (Grimes, 1994). Up to day 10 after ovulation, the hCG levels in ectopic pregnancy are similar to those seen for a normal pregnancy. However, these levels then begin to decline to one fifth to one fiftieth of the median levels of hCG in a normal intrauterine pregnancy of comparable duration (Cole, 1999b). Direct information to diagnose ectopic pregnancy is available by comparing hCG concentrations with the results of transvaginal ultrasonography. Klee (1994) recommended that if an embryo is large

enough to produce more than 1600 IU/L hCG, the gestational sac should be visible on transvaginal sonography. Therefore, if the levels of hCG are >1600 IU/L and there is no evidence of a gestational sac in the uterus, the presence of an ectopic pregnancy should be suspected. This evidence combined with symptoms such as missed period, lower abdominal tenderness, or an adenexal mass and vaginal staining are good indicators of ectopic pregnancy.

1.8.3 Spontaneous Abortion

In patients with spontaneous abortion the serum hCG levels fall or fail to double. Serial quantitative titres of β-hCG are indicative of an abnormal pregnancy if a plateauing or a decrease from the expected doubling time is displayed by a normal intrauterine pregnancy. The incidence of early pregnancy loss has been cited as high as 31% (Wilcox *et al.*, 1988). HCG concentration reaches a peak two weeks after conception at 10-100 IU/L, then rapidly declines (Cole, 1999b). After spontaneous abortion the serum β-hCG levels fall to near zero within 9 to 35 days, with a median of 19 days. This is much less than for induced abortion in which titres become non-detectable within 16 to 60 days, with a median of 30 days. This is attributed to the amount of residual vital trophoblastic tissue remaining after evacuation of the uterus (Steier, 1984).

1.8.4 Gestational Trophoblastic Disease

Gestational trophoblastic disease (GTD) (choriocarcinoma, hydatidiform mole) occurs in approximately 1 in 1000 pregnancies. Clinical presentation may include vaginal bleeding after a missed period, discrepancy between uterine size and gestational age, nausea, vomiting, pre-eclampsia, thyrotoxicosis and neurologic symptoms. A classic case of GTD would be a woman with an enlarged uterus and positive β-hCG, without foetal heartbeat (Flynn and Seifer, 1996). Generally, in GTD abnormally high levels of hCG are encountered, mostly intact hCG; however, one third of patients with GTD may have hCG titres that are below the sensitvity of the conventional pregnancy tests (Saxena, 1983). The concentration of hCG corresponds to the mass of the viable tumour which is useful to monitor the effect of surgery or therapy (Klee, 1994). Patients with GTD also exhibit higher than normal levels of free β-hCG (2-10% of intact hCG level); nicked hCG after therapy or

surgery (up to 100% of hCG molecules) and possibly higher proportions of hyperglycosylated hCG (up to ~40% of hCG molecules) (Cole, 1999b).

1.8.5 Testicular Tumours

hCG levels in serum are useful in monitoring the efficacy of treatment for nonseminomatous germ cell testicular tumours. Serial measurement of both hCG and α-fetoprotein (AFP) is the most sensitive gauge for monitoring treatments. Both hCG and AFP should be measured as some tumours only produce one of these markers. It is also important to detect both intact hCG and β-hCG as some tumours may only produce select forms (Klee, 1994).

1.8.6 Screening for Foetal Down's Syndrome

hCG levels are elevated in women carrying a foetus affected by Down Syndrome. The median serum hCG and free β-hCG level in a Down Syndrome pregnancy is twice the level in a normal pregnancy (Cole, 1999b). Generally these measurements are used in combination with maternal age, serum AFP, unconjugated estriol, pregnancy associated protein A and inhibin to provide a more accurate diagnosis (Cole, 1999b and Haddow *et al.*, 1998). Using such serum analytes, two thirds of affected foetuses can be identified with a 6% false positive rate. It has been suggested that β-hCG is a superior marker for Down's Syndrome (Gabbe *et al.*, 1996). Recently, a potentially more accurate indicator of Down's Syndrome pregnancies in the second trimester has been reported. This marker is hyperglycosylated hCG, the levels of which are nine fold higher than seen for normal pregnancies (Cole, 1999b).

1.9 Pregnancy Diagnosis and Monitoring

The term 'pregnancy test' is actually a misnomer, since most of the assays described in this thesis measure the hormone hCG and not the presence of a foetus. There are a variety of techniques available for the both the qualitative and quantitative detection of hCG. Section 1.9.1-1.9.5 describes some of the assay methods, which have been used for the detection of hCG. One step rapid hCG assays are described in greater detail in section 1.10.

1.9.1 Precipitation Assays

Precipitation assays were the first immunoassays to be developed. Agglutination, nephelometric and turbidimetric immunoassays are examples of precipitation assays, which are still in use today, albeit to a lesser extent than in the first half of the 20th century. These assays all share a common endpoint, the direct detection of an insoluble immune complex. When a soluble antigen and its antibody are in contact in solution, the resultant antigen-antibody complexes may become insoluble and precipitate. Nephelometry, the measurement of scattered light, provides a means of quantifying immunoprecipitation reactions. A variation of this method, called nephelometric inhibition, is used for the measurement of haptens. Investigators are pursuing the use of this technique for the measurement of hormones (Albertson and Swanson, 1996).

Particle-enhanced immunoassays, employing inert solid phases such as covalently linked latex beads or erythrocytes are used to increase the sensitivity of these assays. The basis of these assays is the measurement of the agglutination of the antigen and its corresponding antibody as a function of the concentration of one of these components. Currently, there are commercially available kits and automated instrumentation systems for either the qualitative or semi-quantitative β-hCG assay (Albertson and Swanson, 1996). The haemagglutination inhibition (HAI) and latex particle agglutination inhibition assays belong to this group and are discussed since they represent commonly used pregnancy tests.

In the HAI assay, erythrocytes coated with hCG are incubated with anti-hCG serum and the patient's urine. In pregnancy, the hCG in the urine neutralises the hCG antibody and thus no agglutination occurs. Agglutination will occur in the absence of hCG in the urine which results in a uniform film at the bottom of the test tube. The assay time for HAI is approximately 2 hours. LAI is similar in principle to HAI, using hCG-coated latex particles instead of hCG-coated erythrocytes. Most of these assays are carried out on glass slides and take only a few minutes to perform (e.g.: pregnancy test from Cortéz Diagnostics, refer to Table 1.10.1, page 37) (Flynn and Seifer, 1996).

1.9.2 Radioimmunoassay (RIA)

Radioimmunoassays (RIA) were first developed for the detection of hormones and are known to be sensitive, specific and straightforward to perform. The basic principle of RIAs is the competitive inhibition of the binding of a labelled antigen to a 'specific' antibody by the same unlabelled antigen, which is contained within samples. A variation on this technique is the immunoradiometric assay (IRMA). These assays differ from the classic RIA in that the antibody is labelled instead of the antigen. The IRMA is generally of the 'sandwich' format. Despite the advantages of these assays there are concomitantly several drawbacks with these assays. These include the short shelf-life of the radioactive tracer component, long assay times (generally in the region of several hours), the cost of equipment for measuring the gamma or beta particles emitted, the exposure of users to radiation and the environmental problem of isotope disposal (Albertson and Swanson, 1996).

Traditionally this clinical laboratory technique has measured hCG through its β-subunit. RIA's measure the combined concentrations of intact hCG and free β-subunits. hCG is labelled with a radioactive isotope and is displaced from binding sites on an anti-hCG antibody by unlabelled hCG in the patient's serum. If all the labelled hCG is displaced, the patient's serum contains a high concentration of hCG. These assays are quantitative and are useful in the diagnosis of ectopic pregnancy, spontaneous abortion or gestational trophoblastic neoplasia, as they are suitable for monitoring doubling times (Gabbe et al., 1996). Despite the disadvantages associated with these assays they continue to be relatively popular, particularly the IRMA tests, for the quantitative detection of hCG.

1.9.3 Immunoradiometric Assay (IRMA)

In IRMAs the sandwich principle is used to detect dimeric hCG. These assays are quick to run, usually ~30 minutes and are very sensitive. IRMAs utilise a radioactive antibody to detect hCG in the serum. Anti-hCG antibodies are bound to a test tube, the patient's sample is added and a second labelled antibody binds to the hCG-antibody complex already bound to the test tube. The amount of hCG in the patient's sample is therefore proportional to the amount of labelled antibody bound to the test tube. The hCG molecule is thus, 'sandwiched' between two antibodies. Some IRMAs are based on the use of antibodies against the α and β-subunits of hCG. These assays have been known to exhibit significant cross-reaction with both LH and FSH. Antisera against the β-subunit result in an IRMA that is much more specific for hCG (Flynn and Seifer, 1996).

1.9.4 Enzyme-Linked Immunosorbent Assay (ELISA)

The evolution from radiometric to nonradiometric assays is best typified by the current widespread use of various enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA). This move from isotopic to nonisotopic methodologies is as a result of the factors outlined in section 1.9.2 with respect to the disadvantages involved in the use of radiometric assays.

The principle used in these assays is the same as for IRMAs. These use an enzyme-labelled second antibody instead of a radioisotope-labelled second antibody. This enzyme-labelled antibody can be detected by a colour change once binding has occurred and a substrate is added. The basic ELISA procedure first described by Engvall and Perlman (1971) has been subject to many modifications and refinements. An example of a heterogenous ELISA procedure is the 'sandwich antigen format', which entails 'sandwiching' the antigen between two specific and different antibodies (Albertson and Swanson, 1996). The primary or capture antibody is usually linked to a solid phase matrix, for example, a microtitre plate (96 wells) or a polystyrene tube. A separate secondary antibody is conjugated to the enzyme tracer. With respect to hCG EIAs or ELISAs, it is usual that the antibody attached to the solid phase (the microtitre plate) would be directed toward an epitope of the α-

subunit of hCG and the secondary (labelled) antibody would be directed at an epitope of the β-subunit (Flynn and Seifer, 1996).

Although not as precise, these assays are sensitive and quick and are ideal for early diagnosis of pregnancy as they can detect concentrations as low as 10 IU/L. More rapid versions of such 'sandwich' assays are available as home pregnancy kits for the qualitative or semi-quantitative detection of hCG. The principle of these assays, colloidal gold-based in particular, will be described in more detail in section 1.10 and 1.11.

1.9.5 Fluoroimmunoassay (FIA)

Fluoroimmunoassays (FIA) and immunofluorometric assays (IFMA) are other examples of nonradiometric assays and were developed concurrently with EIAs and ELISAs. These particular assays are also known for possessing good sensitivity, being inexpensive, stable and safe for the end user. FIAs and IFMAs are based on the labelling of the immunoreactants with fluorescent probes. This is by no means a new technique, as early as 1941 Coons *et al.* described the immunological properties of antibodies containing fluorescent groups (Albertson and Swanson, 1996). This technique is also a 'sandwich' assay, using a fluorescent label on the secondary antibody. The amount of fluorescence emitted is proportional to the concentration of hCG in the sample. Levels as low as 1 IU/L can be detected using this method and it takes approximately 2 to 3 hours to complete.

1.9.6 Chemiluminescence assays

The chemiluminescent assay also consists of a similar format to that of the RIAs, EIAs and FIAs, utilising a chemiluminescence end point. Chemiluminescence differs from fluorescence in that it involves excited state formation as the product of an exoergic chemical reaction. The excited state product molecule relaxes to a ground state and in so doing emits photons. The photoefficiency of this reaction or the number of photons emitted per molecule of reactant is called the chemiluminescence quantum yield. Chemiluminescent molecules (e.g.: luminol) are covalently linked to either the assay antigen or antibody. The acridinium derivative labels are favoured for gonadotropin assays (Albertson and Swanson, 1996).

1.10 Disposable immunoassay devices

The last decade has seen an enormous increase in the availability and range of point of care (POC) and over the counter (OTC) products. One of the biggest success stories of this branch of immunodiagnostics is the pregnancy test. The current trend in immunodiagnostics is to develop less time consuming, cheaper and more user-friendly tests, which would be particularly suited for use in the home, doctor's office or health clinic. Such assays also lend themselves to emergency situations, in ambulances and casualty departments where a quick diagnosis is often required. Similarly, these tests have application in third world countries where, ordinarily, neither the necessary finance nor equipment is available to carry out elaborate clinical assays. Additionally, many of these assays use unprocessed samples, which are then absorbed within the test device during testing. This requires less manipulation of potentially infectious samples and ensures a reduced risk of exposure to infectious diseases during disposal. This is of particular advantage to areas where the rate of infectious disease is high and/or the presence of proper disposal facilities for hazardous materials is negligible.

1.10.1 Classification of devices

Any immunoassay which is to be sold for the POC or OTC sector must incorporate the following design features: reproducibility, low cost, short assay time, an internal assay control, the use of unprocessed sample, no requirement for either specialised equipment or skilled operators, be user-friendly, no requirement for refrigeration and have as few steps as possible within the assay.

According to Price *et al.* (1997) there are four types of disposable devices:

① Encapsulated wet chemistry.

For this device all of the components of the test, including any diluents, are encapsulated in a complex disposable plastic unit, which only requires the addition of sample to run the assay. Basically, this assay format directly mimics the fluidics of a wet chemistry immunoassay. In general, these devices require an instrument for reading a photometric end point of the test reaction.

② Simple immobilised analyte capture.

Typically, this format involves the immobilisation of a capture antibody to a solid porous matrix. The sample is added to this and additional reagents are added to detect captured antigen analyte. The liquid phase is trapped within the porous matrix. These assays are sometimes termed 'flow-through' or 'cross-flow' devices.

③ Chromatographic and liquidic circuits.

With this assay format all reaction components are impregnated or immobilised on a porous solid phase and are brought into contact with the samples in sequence, sometimes after addition of a diluent. The impregnation/immobilisation of the test components obviates the need for the addition of any critical reagents. These assays may be termed 'lateral-flow' devices.

④ Immunosensors

Antigen is captured in these assays by addition of the sample to the surface of the biosensor. Upon binding of the antigen to the sensor, the antigen is directly detected by the change in the immediate environment of the surface of the sensor.

Devices of either class ② or ③ are of most interest for the purposes of this thesis, since the majority of POC and OTC pregnancy tests fall within these categories. Immunosensor assays are still somewhat complex in comparison to the relatively straightforward 'flow-through' or 'lateral-flow' devices and do not lend themselves to the target sector of the market relevant to this thesis, due to their requirement for equipment. The wet chemistry devices are similarly unsuitable for this market due to the general prerequisite for a photometric reader for the assay end point.

Early disposable immunoassay devices for pregnancy testing were typically based on latex agglutination or colorimetrically monitored enzyme-labelled conjugates and occurred either on a slide or within a test tube (Flynn and Seifer, 1996). These assays were fraught with problems in that the interpretation of results was very 'operator-dependent'. This problem is now eliminated with the new one-step devices, which are easy to interpret and contain their own in-built procedural control.

1.10.2 'Lateral-flow' and 'Flow-through' devices

The majority of current disposable devices rely on the binding of at least one reagent to a solid support. In general, a membrane (e.g.: nitrocellulose) is used. This is true for both the 'lateral-flow' and 'flow-through' devices.

'Lateral-flow' devices

Immunochromatographic or 'lateral-flow' devices involve the unidirectional flow of a pre-impregnated antibody-coated particle (e.g.: latex or colloidal gold particles) along a membrane. These coated particles capture the antigen of interest, if present, and subsequently are captured by an antibody, which is immobilised in certain defined areas of the membrane. Since the antigen/antibody-coated particle complex is concentrated in only defined areas of the membrane a signal is generated, which can be seen by the naked eye (see Figure 1.10.1). The construction of the 'lateral-flow' device is dependent on the end use of the test. These devices may be held directly in the sample without any plastic housing. They can also be constructed within plastic 'cassette' housing to which the sample is directly added. The 'wick' device is a combination of both of these formats in that the wick of the test is immersed directly in the sample but an outer plastic housing, which lends robustness to the assay, protects the device. The construction of a 'lateral-flow' test is described and illustrated in section 1.11.

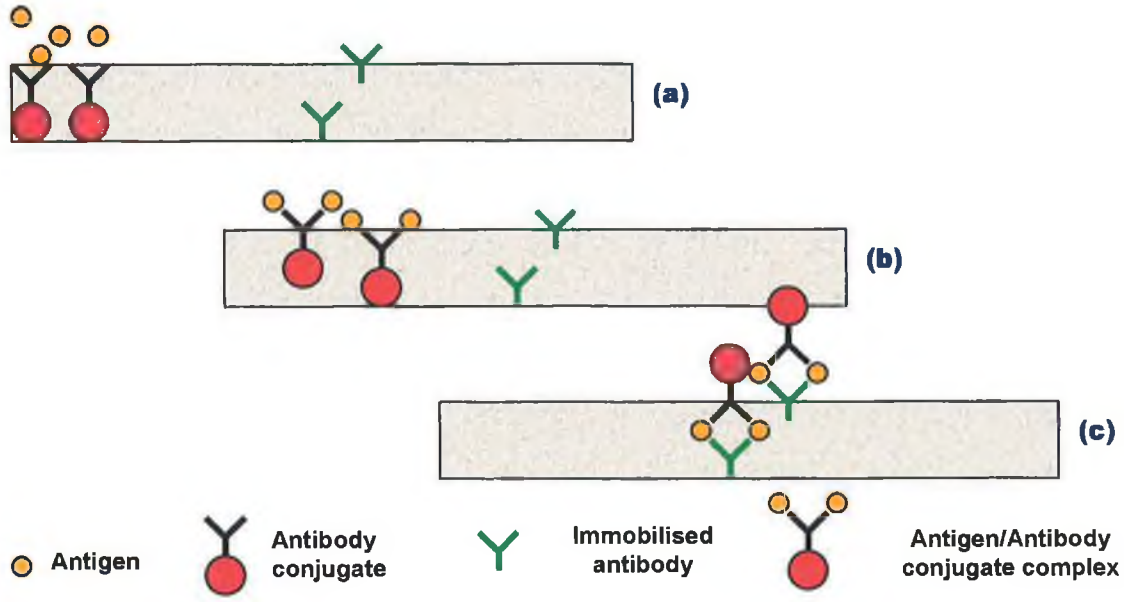


Figure 1.10.1: *(a) Sample addition to the test strip. (b) Antibody conjugate binds the antigen whilst progressing up the strip. (c) A 'sandwich' is formed with the antigen/conjugate complex and the antibody immobilised in the membrane.*

'Flow-through' devices

'Flow-through' devices are actually multi-step procedures, which have proven to be very successful in the laboratory and outpatient clinic. With these assays the capture antibody is immobilised onto a membrane, which is attached to an absorbent reservoir (see Figure 1.10.2 below). The sample is allowed to flow through the membrane and is followed at defined time intervals by the addition of set volumes of the test reagents. One of these reagents is an enzyme-labelled antibody conjugate. If the antigen is captured, which yields a colour change, the intensity of the colour is proportional to the concentration of the antigen being detected (Price *et al.*, 1997). These devices supply a qualitative result when visually interpreted. A semi-quantitative result can also be achieved by using a calibrated colour comparison chart. 'Flow-through' devices have also been used to provide quantitative results by the use of a reflectance meter (Price *et al.*, 1997).

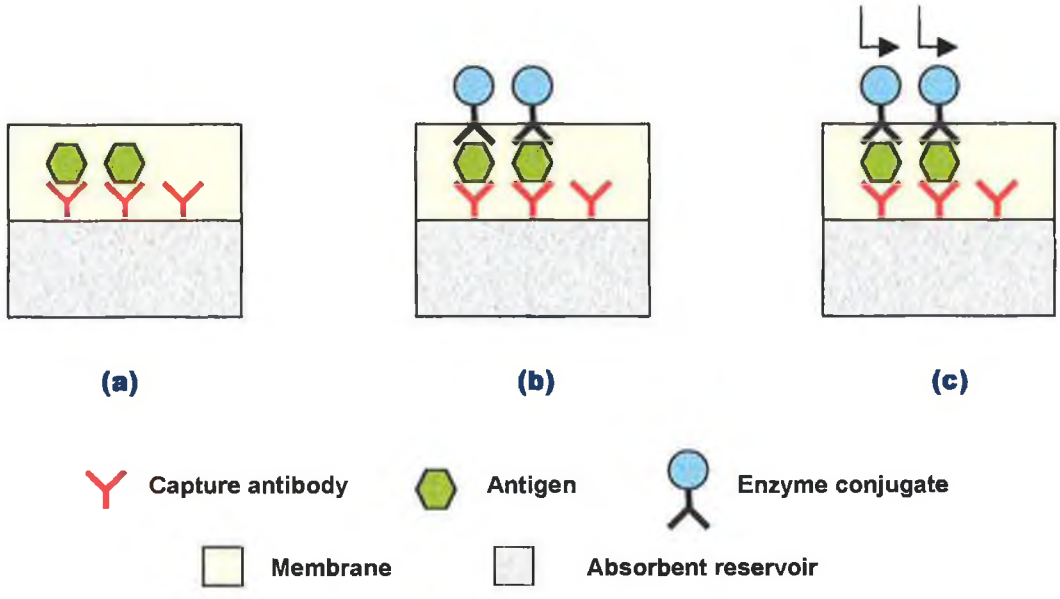


Figure 1.10.2: *Schematic representation of a 'flow-through' device. (a) Addition of sample to the device. (b) An enzyme conjugate solution is added to the test and incubated for a defined period of time. (c) Visualisation of the enzyme label is by addition of a substrate, which produces a coloured product (denoted by the arrow).*

An example of a 'flow-through' device is the Cambridge Biotech recombigen HIV-1/HIV-2 test. This test can be used to simultaneously detect antibodies to both the HIV-1 and HIV-2 viruses. The test is made up of a small asymmetrical plastic chamber, which contains an absorbent reservoir to which glass fibre membrane is fused at the top. Coated on the surface of this membrane, in three discrete circular

spots, are antigen or human IgG-coated latex beads. These spots are not visible to the user before addition of reagents. 7 drops of human serum are added to the centre of the device, followed by 4 drops of conjugate solution. 800µl of wash buffer and 4 drops of substrate solution are then added. This is incubated for 3 minutes and 400µl of stop solution is then added. The results are interpreted qualitatively, a negative result is indicated by the presence of the control spot only, which was coated with human IgG latex. A sample positive for HIV-1 will show 2 spots, one control and one which reacted with HIV-1 antigen-specific-coated latex beads. A similar result is obtained for HIV-2 positive samples, only with a different test spot, which had reacted with HIV-2 antigen-specific-coated latex beads, giving visible colour. Co-infected or cross-reactive samples display 3 spots (see Figure 1.10.3) (Gosling, 1996).

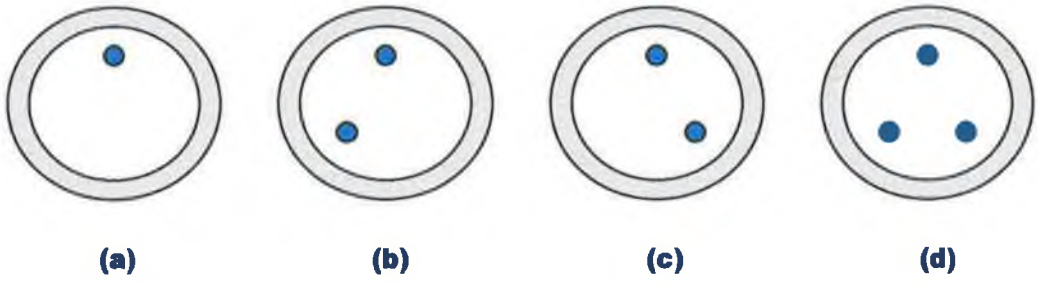


Figure 1.10.3: *Top view of Cambridge Biotech HIV-1/HIV-2 'flow-through' device. Test and control spots are denoted by a blue spot, the shaded ring denotes the plastic housing of the device. (a) Negative result, showing the control spot. (b) HIV-1 positive result, showing the control and HIV-1 spots. (c) HIV-2 positive result, showing the control and HIV-2 spots. (d) Co-infected or cross-reactive sample result, showing all 3 spots.*

The Trinity Biotech HIV-1/HIV-2 Serocard, while not a 'flow-through' device, does fall under the same category as 'flow-through' devices, i.e.: group ②, simple immobilised analyte capture. This test takes 7 minutes to run and serum, plasma or whole blood samples can all be used with this device. No sample pretreatment is necessary. Any anti-HIV-1 or anti-HIV-2 antibodies present in the sample are captured within 1 minute by the HIV-1 and HIV-2-specific peptides, which are immobilised on a discrete portion of the membrane. A goat anti-human IgG alkaline phosphatase conjugate solution is then incubated in the test and control ports of the

device for 1 minute. The alkaline phosphatase then converts its substrate, BCIP, from a clear to a blue colour within 5 minutes. Results are interpreted semi-quantitatively.

1.10.3 Advantages and disadvantages of 'lateral-flow' and 'flow-through' devices

When choosing a format suitable for the development of a rapid pregnancy test, the advantages and disadvantages of both the 'lateral-flow' and 'flow-through' formats were examined.

1.10.3.1 'Flow-through'

One of the most important advantages of the 'flow-through' device is obviously, the ability to adapt these devices for qualitative, semi-quantitative or quantitative measurement of the analyte, while still maintaining a relatively 'rapid' assay time. However, this positive aspect of such assays is not amenable for use by the lay person at home in that the number of steps involved in running these assays are too great and would almost certainly lead to inaccurate results due to incorrect use of the device. It is no surprise to see from the list of pregnancy tests, currently on the market (see Table 1.10.1, page 37) that only three of the tests listed required four or more steps. Only one assay was described as a 'flow-through' device and this test is limited to use within the doctor's office or in a laboratory situation.

1.10.3.2 'Lateral-flow'

From Table 1.10.1, page 37 it is clear that the majority of pregnancy tests on the market at the moment are 'lateral-flow' devices of either the cassette or wick formats. The popularity of these devices is due to the number of advantages this format possesses over the 'flow-through' device. With the 'lateral-flow' device the sample addition site is separate from the reactant surface. This allows for good separation of the sample, particularly for viscous samples such as serum, and also the antigen/antibody interaction is maximised as the entire sample volume comes in contact with the immobilised reactants. This is not so for vertical flow ('flow-through') devices where only some of the sample comes in contact with the immobilised reactants (see Figure 1.10.4). Typically, with 'lateral-flow' any contaminants within the sample are retained at the sample addition site, whereas for

'flow-through' devices any contaminants remain on the membrane surface, this could potentially lead to non-specific reactivity with the reactants of the test.

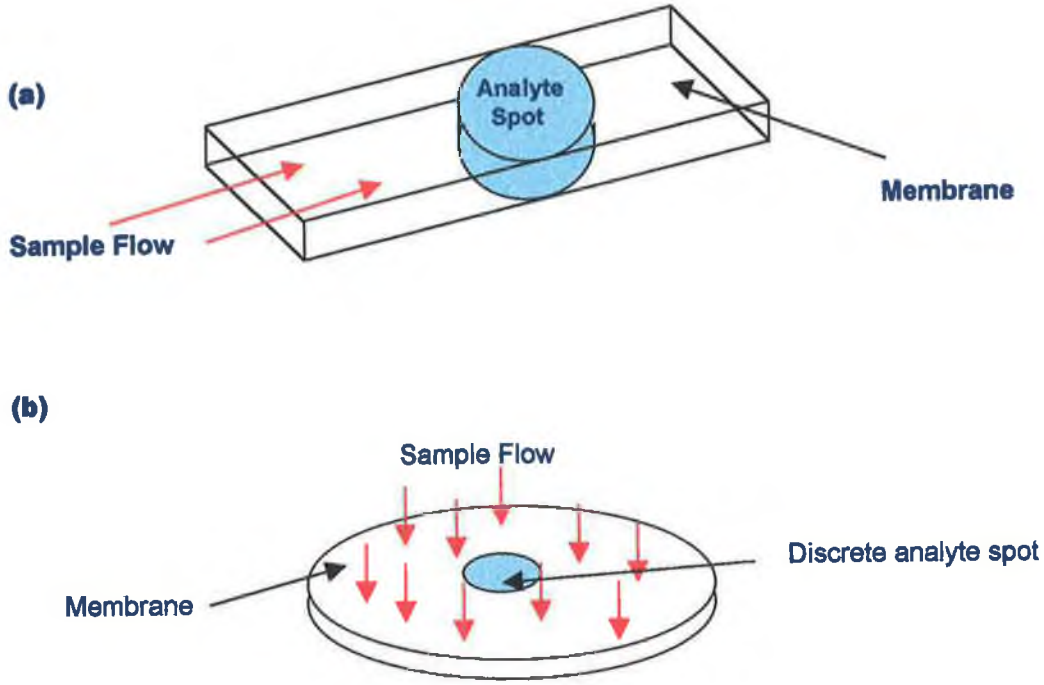


Figure 1.10.4: *(a) Sample flow through a 'lateral-flow' device. (b) Sample flow through a 'flow-through' device*

Company	Test Name	Sample	Format	Assay Time	Detection Limit	Comments
Abbott Laboratories	TestPack® +Plus™	Urine	Cassette	5 min.	≥25 IU/L	
ABI	Surestep	Urine/ serum	Cassette	5 min.	≥20 IU/L	
Acon Biotech	Pregnancy test	Urine	Strip	3-5 min.	≥ 25 IU/L	
ANI Biotech	Biocard hCG	Urine	Cassette & Wick	5 min.	≥ 25 IU/L	
Chembio Diagnostic Systems	HCG STAT-PAK	Urine/ serum	Cassette	5 min.	≥ 25 IU/L	
Cortez Diagnostics Inc	Pregnancy Test	Urine	Indirect Latex	3 min.	30-50 IU/L	2-8°C storage
	Direct Pregnancy Test	Urine	Direct Latex	3 min.	≥ 30 IU/L	2-8°C storage
	Creschek	Urine	Cassette	5 min.	≥ 25 IU/L	
	Serachek	Serum	Cassette	10 min.	≥ 25 IU/L	
	Combichek	Urine/ Serum	Cassette	3 min.	≥ 25 IU/L	4step test
Hybritech	ICON®II hCG	Urine	Flow Through Device	~5 min.	≥ 20 IU/L	5 step test
Millenium Health Resources Inc	i-hCG Test 2000	Urine	Cassette	3-5 mins	≥ 30 IU/L	
Medix Biotech Inc.	Contrast hCG	Urine/ Serum	Cassette	4 min.	≥ 20 IU/L	
Mizuho USA Inc.	Quick Checker	Urine	Wick	5 min.	≥ 25 IU/L	
Operon	Stick hCG	Urine	Wick	5-10mins	≥ 25 IU/L	
Quidel	Quickvue hCG	Urine/ serum	Cassette	3 min.	≥ 25 IU/L	TL pink, CL blue
Randox	Pregnancy Strip Test	Urine	Cassette	5 min.	≥ 25 IU/L	
Rapid Diagnostic Technologies	Concise PLUS	Urine	Cassette	3-5 min.	≥30	
Savyon Diagnostics	Quckstripe hCG	Urine/ serum	Cassette	3-5 min.	≥ 25 IU/L	
Syntron Bioresearch	Quikpac II Onestep	Urine/ serum	Cassette	5 min.	≥ 25 IU/L	Latex
TECO Diagnostics	One step	Urine/ serum	Cassette & Wick	2 min.	≥ 25 IU/L	
Unipath	Clear Blue	Urine	Wick	1 min.	Not given	
	Clearview hCG II	Urine	Cassette	5 min.	≥ 25 IU/L	
	Clearview hCG Duo	Urine/ serum	Cassette	5 min.	≥25 IU/L urine ≥50 IU/L serum	
Vedalab	Babycheck -1	Urine/ serum	Strip	5-10mins	≥ 10 IU/L	
V-TECH Inc	Target hCG	Urine/ serum/ plasma	Cassette	3 min.	≥ 50 IU/L urine.≥ 25 IU/L serum/ plasma	6 step test
Warner Lambert	1 step e.p.t	Urine	Wick	3 min.	Ist day missed period	

Table 1.10.1: *List of OTC/POC pregnancy tests currently available. (TL = test line, CL = control line).*

1.11 Characteristics and manufacture of 'lateral-flow' immunochromatographic assays

1.11.1 Feasibility of the development of a 'lateral-flow' hCG test

Section 1.10 illustrated the four different types of disposable immunoassays and provided reasons as to why a 'lateral-flow' assay was the most appropriate format for the development of a pregnancy test suitable for the POC/OTC sector of the immunodiagnostics market (see section 1.10.3). This sector is expected to be worth in excess of $6 billion by the year 2000, with 30% growth in POC diagnostics and 25% growth for OTC products (Shine, 1998). Within Europe, there is a potential market value of £149 million available by 1999 if a test was to meet the criteria listed below (Kingshott, 1995).

1.11.2 Principles of the procedure

The test is based on the colloidal gold-linked immunosorbent assay technique and employs an anti-α-hCG antibody solution sprayed on the lower half of a nitrocellulose strip (test line) and a goat anti-mouse antibody solution sprayed on the upper half of the strip (control line) (See Figure 1.11.1, below).

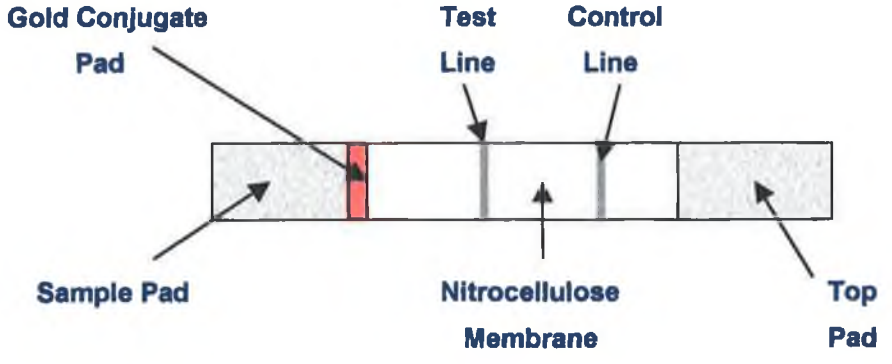


Figure 1.11.1: *Schematic representation of a 'lateral-flow' test strip.*

The antibody lines are sprayed onto the nitrocellulose membrane during manufacture using an Acculine spraying machine, which is described in section 1.11.3.4. Nitrocellulose is a porous material, the properties of which are described in section 1.11.3. The gold conjugate, which is dried onto a glass fibre pad (see Figure 1.11.1 and 1.11.2), is pink/red in colour and each gold particle is coated with anti-β-hCG antibodies. Once sample is added to the test strip it passes through the sample pad, some of the larger molecules present which might otherwise interfere with the assay remain on this pad, the resultant filtrate passes into the conjugate pad and rehydrates the gold conjugate. hCG present in the sample will bind to the gold anti-β-hCG conjugate forming a complex which laterally migrates up the test strip by capillary action (see Figure 1.11.2). These gold conjugate/hCG complexes will bind to the anti-hCG test line forming a sandwich (i.e.: a pink/red line is formed). Excess gold conjugate bound to hCG and any unbound conjugate continue to migrate up the test strip and deposition of these complexes appears in the form of a pink/red line at the control line site as they are bound by the anti-mouse IgG antibodies of the control line (see Figure 1.11.2). The visual formation of one or two pink/red lines allows for easy interpretation of results for negative (one line) or positive (two lines) specimens (see Figure 1.11.3). The control line should always appear and any test which does not possess a control line, should be considered invalid and should be repeated.

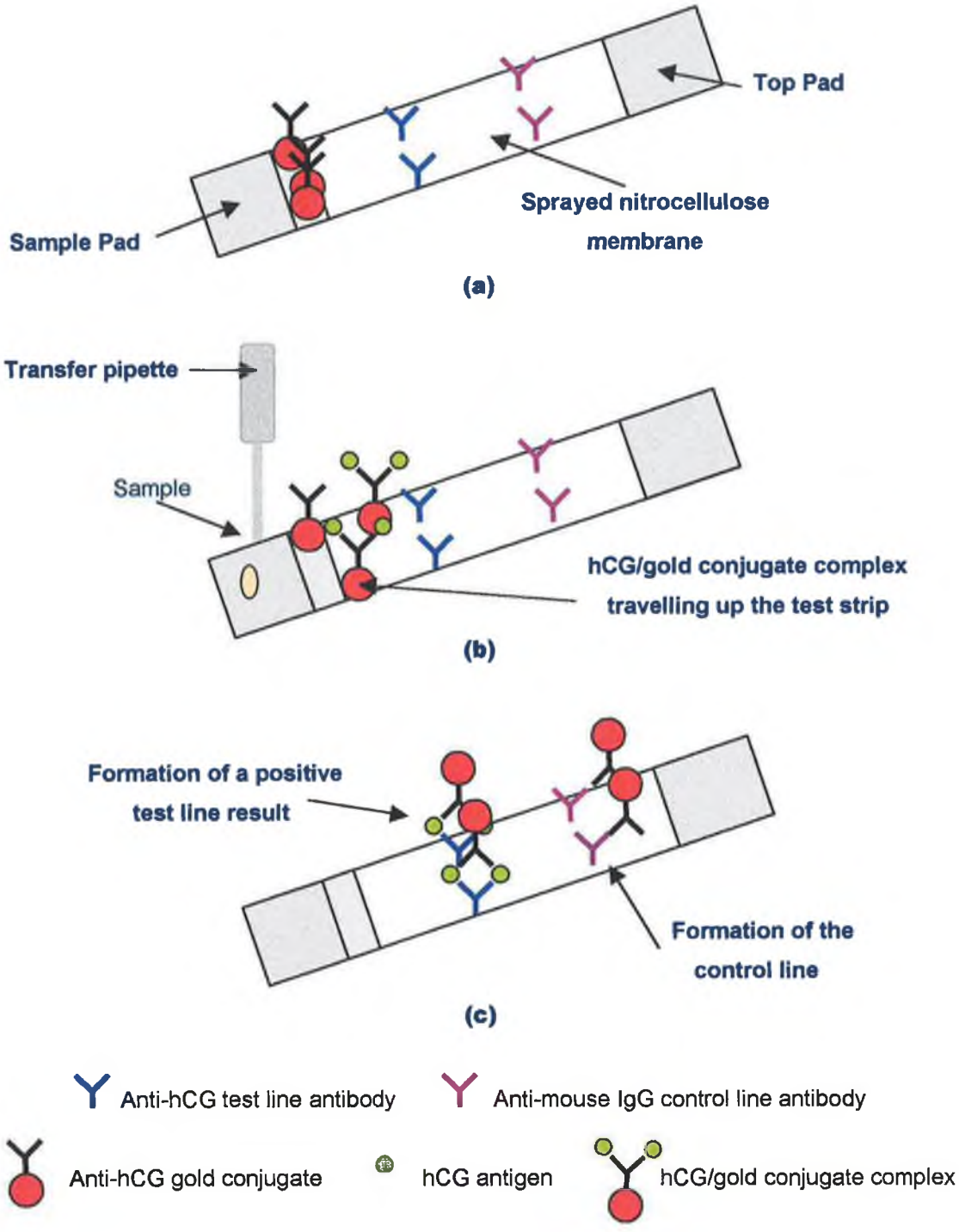


Figure 1.11.2: *(a) hCG test strip showing the gold conjugate impregnated pad and the nitrocellulose sprayed with anti-α-hCG antibody (test line) and anti-mouse IgG (control line). (b) Sample is added and the hCG antigen binds to the anti-β-hCG gold conjugate and flows up the test strip. (c) The hCG antigen/gold conjugate complex binds to the test line antibody and any gold conjugate not bound to the hCG antigen binds to the anti-mouse IgG control line.*

Figure 1.11.3: *Photograph showing the test device, which was developed for this thesis. The device on the left shows a negative result (one line) and the device on the right displays a low positive result (two lines).*

1.11.3 Properties of the test components

1.11.3.1 Nitrocellulose Membrane

The type of solid phase chosen for use in a rapid immunoassay is critical. The solid phase should not be considered an inert part of the test as the surface and structure play an important part in the performance of the assay. The choice of the most suitable solid phase will ensure the desired flow of reagents (and, therefore, reaction time between the sample and test components) the mixing of constituents and the minimisation of non-specific binding. Additionally, the chemistry of the solid phase will determine the effectiveness of the immobilisation of capture reagents while also affecting the release rate of the impregnated (temporarily immobilised) mobile reagents (Price *et al.*, 1997).

Typically, for rapid immunoassays the solid phase used is one of several different membranes. The binding of protein to the membrane is very much dependent on the polymer from which the membrane is made. Membranes can also be treated during manufacture sometimes with secondary polymers. These post-treatments can dramatically lower the ability of the membrane to bind protein. Table 1.11.1 shows the binding properties of three different types of membrane.

Membrane	Mechanism of binding
Nitrocellulose	*Electrostatic*
Polyvinylidene Fluoride (PVDF)	*Hydrophobic*
(Charge modified) Nylon	*(Ionic) Electrostatic*

Table 1.11.1: *Binding properties of three different membrane polymers (Millipore, 1996).*

Nitrocellulose is the most suitable membrane for 'lateral-flow' immunoassays and is suitable for use with both colloidal gold and latex conjugates. It is used as the solid phase due to the following favourable characteristics which it possesses: ① high protein binding, ② consistent 'lateral-flow' characteristics, ③ in general, low non-specific binding, ④ a smooth white surface for better visibility of results and ⑤ typically, uniform thickness.

Protein binding levels can be influenced by membrane formulation, the lateral flow rate of liquid up the membrane and the membrane pore structure. Performance varies with different proteins. As mentioned earlier, the polymer of a membrane affects its protein binding capacity, but this characteristic is also influenced by the porosity of the membrane (porosity is a measure of the amount of pores in a membrane). As the pore size of the membrane increases, the available polymer surface area declines and subsequently, the membrane binds less protein. Surface area decreases with increasing pore size (non-linearly), increases with increasing thickness (linearly) and increases with increasing porosity (non-linearly) (Millipore, 1996). Increasing pore size also has other effects, such as increased flow rates (Harvey, 1991). Slower flow rates in general increase the sensitivity of an assay since the test reagents are in contact with one another for longer periods of time. Faster flow rates allow a shorter assay time but typically at the expense of sensitivity. It is these pores which determine the flow rate of the assay.

Nitrocellulose membranes bind proteins by an electrostatic mechanism. The extemely strong dipole of the nitrocellulose ester interacts with the strong dipole of the peptide bonds of the protein (see Figure 1.11.4). Nitrocellulose membranes contain no acidic protons and are completely neutral. The binding of protein to the surface of the nitrocellulose is independent of the pH of the immobilisation solution for the capture antibody. However, it should be noted that the pH of this solution can

have an effect on both the solubility and immobilisation efficiency of a particular protein (Millipore, 1996).

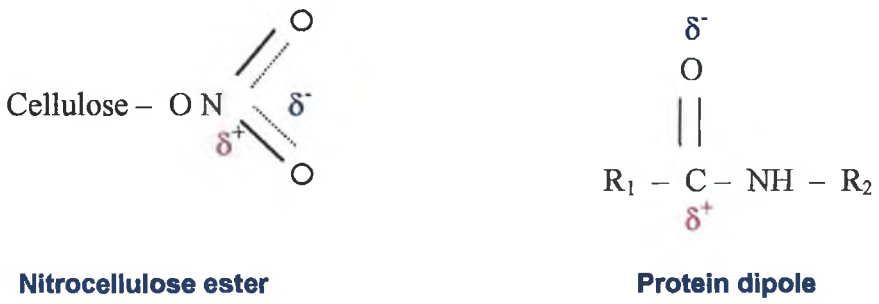


Figure 1.11.4: *The structure of nitrocellulose ester and protein dipoles (Millipore, 1996).*

Another key area in protein binding to nitrocellulose is the actual application of the antibody solution to the membrane. If an efficient and optimised application process is not utilised, the intensity of the signal obtained will be diminished and hence the functionality of the test produced. There are four factors which influence the application of the antibody solution to the membrane: ① humidity effects, ② wicking effects, ③ the properties of the protein being applied and ④ the drying conditions. For maximum reproducibility the humidity under which the membrane is being handled should be kept constant. The ideal humidity conditions for nitrocellulose membrane are between 45 and 55% RH. Wicking affects the application of protein in that depending on the membrane used, the width of the protein capture line is not necessarily as wide as the liquid line applied. The intensity of the result obtained for a given antibody concentration is inversely proportional to the line width (Jones, 1999a). With regard to the protein being applied, the conditions that maximise the electrostatic interactions necessary for binding also lower the stability in solution of the protein. Generally, it is advised to avoid high salt content (although a certain amount is required) and the use of chaotropic detergents (e.g.: Tween® or Triton®) (Jones, 1999b). The ideal conditions to work at are around the isoelectric point of the protein. Finally, the drying of the sprayed membrane is critical. Failure to dry sufficiently could result in the capture antibody being removed from the membrane.

1.11.3.2 Gold Conjugate

The 25th anniversary of the introduction of colloidal gold as an immuno-specific probe in electron microscopy was celebrated in 1996. Due to its excellent detection qualities, colloidal gold has, since 1971, been used in immunoblotting, flow cytometry or to detect nitrocellulose-immobilised proteins. The use of colloidal gold in pregnancy tests and other rapid one-step assays is a relatively recent development. Colloidal gold conjugates are suitable for this type of test device as they are sensitive and specific, give good colour definition, are relatively inexpensive to use, easy to manufacture and have good stability on storage. The use of gold conjugates avoids the disadvantages involved in the use of radioactive labels, as gold probes are essentially non-toxic. Gold-based assays, as for all rapid one-step tests lend themselves to 'on-site' and 'in-home' use by virtue of their simplicity, speed and ease of use and interpretation. The properties and theory of colloidal gold use are discussed overleaf.

When viewed under a light or electron microscope colloidal gold sols are found to be made up of uniformly dispersed gold particles, which appear as solid spheres. It is possible to prepare suspensions of small particles of gold of known diameter (measured in nanometres). In general, gold particles of diameters of 15nm-40nm are used for nitrocellulose-based rapid assays. The processes involved in the interactions of gold particles with various macromolecules, which are exploited during gold conjugation procedures, are poorly understood. The surface of the gold particles in a stable sol is negatively charged due to overlap of the electrical double layer of each particle. This leads to gold-gold repulsion, thereby preventing aggregation of the particles. The preparation of a stable protein-gold complex is dependent on three interactions; ① the attraction which exists between the negatively charged gold particles and the protein molecule which in general possesses many positively charged sites, ② the adsorption of hydrophobic pockets on the protein to the metal surface and ③ dative binding (i.e.: covalent binding) of the gold occurs if sulphydryl groups are present on the protein surface, (see Figure 1.11.5) (Hermanson, 1996). Another phenomenon, which comes into play during the labelling of macromolecules with gold, is the potential which exists for Van der Waals forces between the metal surface and other molecules.

All gold conjugates are created by means of electrolyte-mediated coagulation. In a gold sol, coagulation of the gold particles is prevented as a result of the balance which exists between the attractive Van der Waals forces between gold particles and the gold-gold repulsive forces, which is caused by the negative charge which exists on each gold particle of a stable sol. For aggregation to occur this energy barrier, which prevents coagulation, must first be breached. This is possible by the addition of electrolytes to the sol which mask the negative charge on the surface of each particle. The colloid will collapse with the aggregation of the gold particles in the absence of macromolecules, at a certain concentration of these electrolytes. However, if macromolecules are present when the negative repulsive forces are overcome the gold particles will adsorb to the protein molecules instead of one another, ie: the protein becomes labelled with gold (Hermanson, 1996). NaCl or other buffer salts are the most common electrolytes used. Aggregation within a sol minus macromolecules is accompanied by a colour change from orange/red or red/violet to blue (Roth and Binder, 1978).

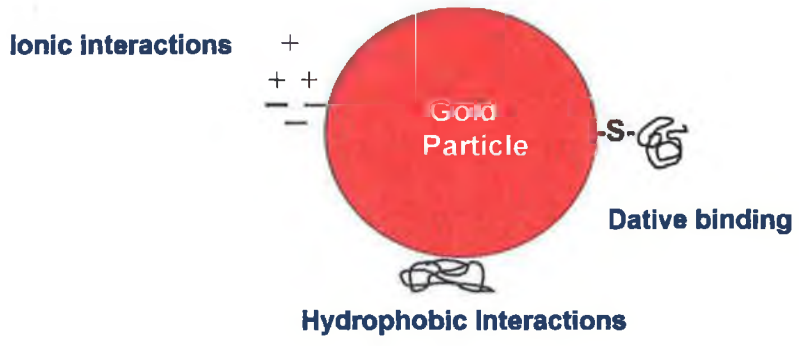


Figure 1.11.5: *The various interactions involved in protein-gold binding.*

1.11.3.3 Other important test components

There are several other components of the test, all of which play a very important role in the dynamics of the assay. These are ① the sample pad, ② the conjugate pad and ③ the top pad.

The sample pad, as the first test component to come into contact with the sample, is critical for the progression of the assay. The function of the sample pad within the test is to remove any particulate matter that may be present in the sample and in some assays it may also be used to adjust either the pH or the viscosity of the sample.

The pad, depending on its structural makeup can also be used if necessary to separate plasma or serum from whole blood samples. Since the sample pad is responsible for the even distribution of the sample to the rest of the test it is important to choose the most suitable material for this purpose. The sample being used for the test will determine which material is the most appropriate. The materials available are glass fibre, cellulosics (paper), woven fibres (meshes) and non-woven filters. In general, the cellulosics are selected for urine and serum assays (Weiss, 1997b). The glass fibre pads have a very large void volume and thus would release the sample far too quickly (Marsden, 1999). There is only a limited product range for the woven fibres and the non-woven fibres are not intrinsically wettable (Weiss, 1997b).

The conjugate release pad requires similar properties to that of the sample pad. It must be capable of transferring the sample volume efficiently and consistently to the membrane. In addition, it is required to take up and hold a consistent amount of the conjugate solution to be impregnated and to also maintain the stability of this conjugate. Most importantly, it must allow for good rehydration of the conjugate and deliver this conjugate consistently and quantitatively to the test strip (Weiss, 1997a). Similar materials to those listed above for the sample pad are also used for the conjugate release pad. Glass fibre is the chosen material for the test developed within this thesis as it releases a large volume of reagent quickly and evenly.

The absorbent top pad, despite having no direct involvement in the assay result, actually could significantly affect the result of the test were it absent. This pad ensures the attraction of liquid towards the top of the test strip due to its high fluid capacity. Its high absorbence rate can influence the speed of migration of the sample/reagents through the preceding substrates. The fact that no liquid waste is generated during the running of these assays highlights the advantage of the presence of a good quality absorbent top pad within the device, as it acts to draw the sample and reagents across the membrane and traps any waste liquid (Wells, 1997).

1.11.3.4 Equipment required for test manufacture

There are three main techniques involved in the manufacture of both test components and the test itself. These are ① spraying nitrocellulose, ② assembling the test strips and ③ cutting the test strips to size.

The Acculine sprayer is used for immobilising antibodies on nitrocellulose membrane. This machine dispenses the antibody solutions at relatively high speed by the use of air pressure, thus creating an aerosol of the test and control line, which can be controlled by changing the air jet opening width, by the use of a moveable needle (see Figure 1.11.6). The test and control line antibody solutions bind by electrostatic adsorption to the nitrocellulose membrane, which is passing under the air jets via rollers at a fixed speed (see Figure 1.11.7).

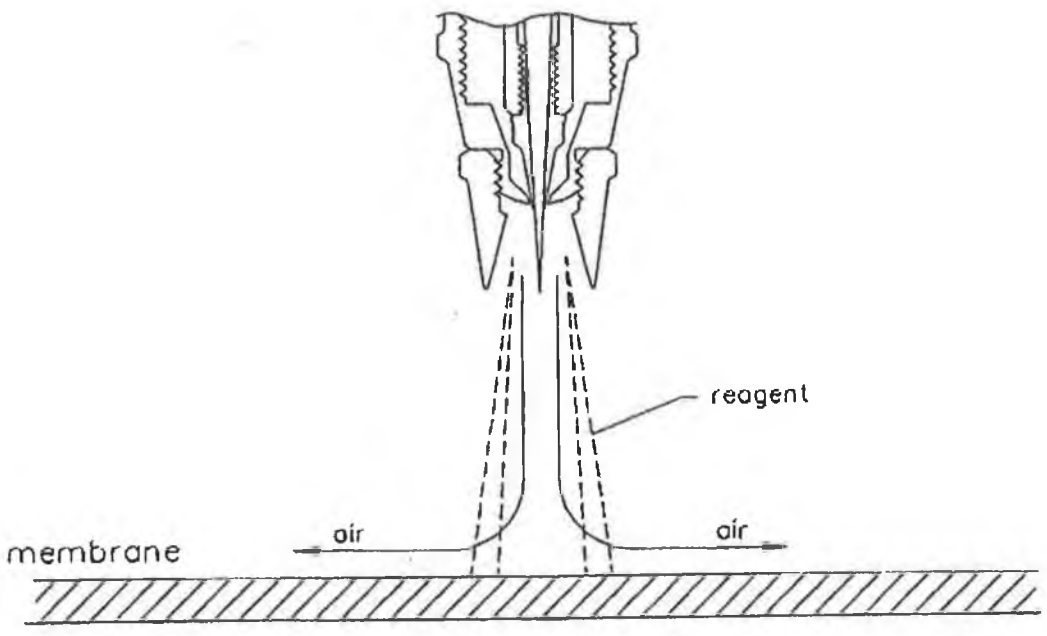


Figure 1.11.6: *Diagram of air jet needle similar to that used with the Acculine sprayer. Diagram shows test line reagent being sprayed through the needle opening onto the nitrocellulose membrane (Tisone, 1997).*

For the assembly of a test laminate (i.e.: approximately 30 tests) an assembly device is required (see Figure 1.11.8). This device ensures that each of the test components is overlapping to the degree required to allow the test to proceed. It is vital that all of the porous media (membrane, sample pad, conjugate pad and top pad) used within the test are in intimate contact with one another in order to achieve an even transfer of the sample throughout the test. The BioDot cutter is used once the test laminates are manufactured to cut each laminate into approximately 30 x 8mm test strips. A photograph of this machine is shown in Figure 1.11.9.

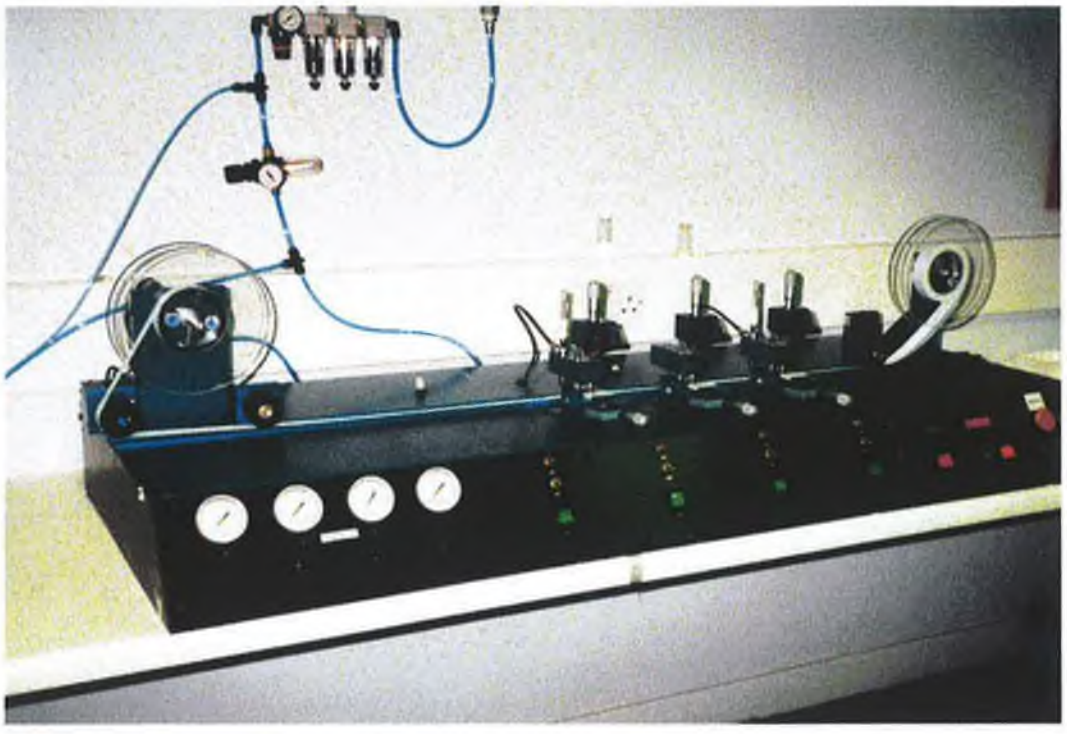


Figure 1.11.7: *Photograph of the Acculine sprayer, showing a roll of nitrocellulose membrane in position for spraying.*

Figure 1.11.8: *Photograph displaying the assembly device, which shows a test laminate with the nitrocellulose membrane, gold conjugate and top and bottom pads in position. A section of the bottom pad has been cut away to reveal the pink gold conjugate impregnated strip.*

Figure 1.11.9: *Photograph of the BioDot cutter, which is used to cut test laminates into test strips (8mm wide).*

1.11.4 Desired parameters of the test

There are several obvious advantages to developing a rapid POC/OTC hCG assay as opposed to an ELISA or IRMA for hCG, designed for use within the clinical laboratory. POC/OTC products give almost immediate results and are easy to use and interpret. They also provide confidentiality, in that customers can run the test in the privacy of their own home. The risk of the spread of disease is reduced, as there is little or no sample preparation required for these tests. They are relatively inexpensive to manufacture. Finally, and perhaps most importantly for the lay person, the cost of such tests is low.

Before the development process had begun the following test parameters were laid out based on studies, which examined competitor tests that were available for use in either the home or by the healthcare professional (refer to Table 1.10.1, page 37). The test parameters selected were ① 3-5 minute assay time, ② single step only (i.e.: sample addition), ③ no prerequisite for extra reagents, ④ no necessity for equipment, ⑤ no technical skills required to run assay, ⑥ room temperature storage ⑦ low cost and ⑧ user-friendly format. It was the aim of the work described within this thesis to meet all of these requirements.

Section 2:

Materials and Methods

2.1 Materials

2.1.1 List of Suppliers

Abbott Laboratories	c/o Langanbach, Unit 4, Cedar Industrial Estate, Killarney Road, Bray, Co. Wicklow.
ABI Products	10237 Flanders St., San Diego, CA 92121, USA.
Advanced Microdevices	21 Industrial Area, Ambala, Cantt 133001, India.
AFC American Filtrona	8401 Jefferson Davis Highway, Richmond, USA.
Ahlstrom Filtration Inc.	122W Butler St., P.O. Box A, Mount Holly Springs, PA 17065-0238, USA.
Alex Mitchel Ltd.	Unit 5, IDA East Wall Road, Dublin 3.
Bio Sciences Ltd.	3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin.
British Biocell	Gold Gate, Tyglas Ave., Cardiff, CF4 5DX, UK.
Calbiochem	Boulevard Ind. Est., Padge Road, Beeston Nott., NE9 2JR, UK.
Coombe Women's Hospital	The Coombe, Dublin 8.
Cork Blood Bank	St. Finbar's Hospital, Douglas Road, Cork.
Dako Ltd.	16 Manor Court Yard, Hughendon Ave., High Wycombe, Bucks, HRP13 5RE, UK.
Dublin City University	Collins Ave., Glasnevin, Dublin 9.
Gelman Science	24 Kill Ave., Dun Laoghaire, Co. Dublin.
Genzyme/ Medix Biochemica	50 Gibson Drive, Kings Hill, W. Malling, Kent, ME19 6HG, UK.
Gibco BRL.	c/o Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, Scotland, UK.
G & L Precision Die Cutting	1766 Junction Ave., San Jose, CA 95112, USA.
International Enzymes	c/o Aalto BioReagents Ltd., 14 Main St., Rathfarnham, Co. Dublin.
JBC Inc.	147 Early Court, Elyria, Ohio, 44035, USA.
Maine Biotechnology	1037R Forest Ave., Portland, Maine 04103, USA.

Medix Biochemica	See Genzyme/Medix Biochemica, page 50.
Millipore (UK) Ltd.	The Boulevard, Blackmoor Lane, Watford, Herts, WD1 8YN, UK.
Nalge Europe	Foxwood Court, Rotherwas, Hereford, HR2 6JQ, UK.
NIBSC	Blanche Lane, South Mimms, Potter's Bar, Herfordshire, EN6 3QG, UK.
Pelican House	40 Mespil Road, Dublin 4.
Propak	Unit 4, Turnpike Ind. Est., Ballymount, Dublin 22.
Riedel de Haen	c/o Sigma (Ireland), Airton Road, Tallaght, Dublin 24.
Rotunda Hospital	Parnell Square, Dublin 1.
Sarsteadt Ltd.,	Sinnotstown Lane, Drinagh, Co. Wexford.
Scantibodies	9336 Abraham Way, Santee, CA 92091, USA.
Schleicher & Schuell	c/o Carl Stuart Ltd., Tallaght Business Park, Whitestown, Dublin 24.
Sigma (Ireland)	Airton Road, Tallaght, Dublin 24.
St. James Hospital	No. 1 St. James Street, Dublin 8.
Trinity Biotech Plc.	IDA Business Park, Southern Cross Route, Bray, Co. Wicklow.
Wyntek Inc.	6146 Nancy Ridge Drive, Suite 101, San Diego, CA 92121, USA.

2.1.2 Chemicals

Boric Acid, ACS Reagent	Sigma (Cat. No. B0394)
Bovine Serum Albumin (BSA), minimum 98% purity	Sigma (Cat. No. A7030)
EDTA, free acid, ACS Reagent	Sigma (Cat. No. E9884)
DAB tablet set (Sigma Fast™)	Sigma (Cat. No. D4418)
Gold Chloride (Trihydrate), ACS Reagent	Sigma (Cat. No. G4022)
Goldline	British Biocell (Cat. No. not available)

Hydrochloric Acid, ACS Reagent	Sigma (Cat. No. H7020)
Polyethylene Glycol Compound 200K	Sigma (Cat. No. P3015)
Polyethylene Glycol Compound 1000K	Sigma (Cat. No. P3515)
Polyethylene Glycol Compound 3350K	Sigma (Cat. No. P4338)
Polyethylene Glycol Compound 8000K	Sigma (Cat. No. P4463)
Polyethylene Glycol Compound 10000K	Sigma (Cat. No. P6667)
Polyethylene Glycol Compound 15-20K	Sigma (Cat. No. P2263)
Polyvinylpyrrolidone (PVP K-30), ACS Reagent	Calbiochem (Cat. No. 9003-39-8)
Potassium Phosphate, monobasic, ACS Reagent	Sigma (Cat. No. P0662)
Potassium Phosphate, dibasic, ACS Reagent	Sigma (Cat. No. P3786)
Sodium Azide, minimum 99.5% purity	Sigma (Cat. No. S2002)
Sodium Borate, ACS Reagent	Sigma (Cat. No. S9640)
Sodium Chloride, ACS Reagent	Sigma (Cat. No. S9888)
Sodium citrate, Trisodium Salt Dihydrate, ACS Reagent	Sigma (Cat. No. S4641)
Sodium Hydroxide, ACS Reagent	Sigma (Cat. No. S0899)
Sucrose, D(+) – saccharose, extra pure	Riedel-de-Haen (Cat. No. 16104)
Trizma Base (Tris Base), Primary Standard & Buffer Reagent Grade	Sigma (Cat. No. T1503)
Triton X-100®, Laboratory Grade.	Sigma (Cat. No. X-100)
Tween 20® (Polyoxyethylenesorbitan, Monolaurate), Protein Grade.	Calbiochem (Cat. No. 655206)

2.1.3 Interference Study Reagents

Acetylsalicylic acid, minimum 99.5% purity	Sigma (Cat. No. A5376)
Albumin, Human, Fraction V Powder	Sigma (Cat. No. A1653)
Ascorbic acid, ACS Reagent	Sigma (Cat. No. A1417)
Caffeine, SigmaUltra Grade	Sigma (Cat. No. C8960)
Estriol, minimum 99% purity	Sigma (Cat. No. E1253)

Gentisic acid, Free Acid	Sigma (Cat. No. G5254)
Glucose, Anhydrous, ACS Reagent	Sigma (Cat. No. G5767)
Haemoglobin, Human, Lyophilised Powder	Sigma (Cat. No. H7379)
Phenothiazine, SigmaUltra grade	Sigma (Cat. No. P4889)

2.1.4 Sources of Serum

Goat Serum	Bio Sciences (Cat. No. 16210)
Rabbit Serum	Bio Sciences (Cat. No. 16210/040)
Foetal Bovine Serum	Gibco BRL (Cat. No. 10108)

2.1.5 Anti-hCG Antibodies

Anti-hCG (Intact) Monoclonal Antibody	Maine Biotechnology, (No.105)
Anti-hCG (Beta) Monoclonal Antibody	Maine Biotechnology, (No.106)
Anti-hCG (Beta) Monoclonal Antibody	Maine Biotechnology, (No.107)
Anti-hCG (Beta) Monoclonal Antibody	Maine Biotechnology, (No.108)
Anti-hCG (Beta) Monoclonal Antibody	Medix Biochemica, (No.5006)
Anti-hCG (Intact) Monoclonal Antibody	Medix Biochemica, (No.5008)
Anti-hCG (Beta) Monoclonal Antibody	Medix Biochemica, (No.5009)
Anti-hCG (Intact) Monoclonal Antibody	Medix Biochemica, (No.6601)
Purified Rabbit Anti-α–hCG	Wyntek Inc., (PN 210)
Purified Anti-β–hCG	British Biocell (No.1817)
Anti-hCG Monoclonal Antibody	Genzyme (No.9812)

2.1.6 Miscellaneous Antibodies

Purified Goat Anti-Mouse, IgG fraction	International Enzymes, (Cat. No. 6405)
Rabbit Anti-Mouse antibody	Dako Ltd., (Prod. No. Z0109)
Anti-Luteinising Hormone antibody	Genzyme (D11675)

2.1.7 Hormone Standards

Human Chorionic Gonadotropin, from human pregnancy urine	Sigma (Cat. No. CG-10)
Human Chorionic Gonadotropin, WHO 3rd International Standard.	NIBSC (Cat. No. 75/537),
Luteinizing Hormone, Antigen grade	International Enzymes, (Cat. No. 4207)
Follicle Stimulating Hormone, Antigen grade	International Enzymes, (Cat. No. 4202)
Thyroid Stimulating Hormone, Antigen grade	International Enzymes (Cat. No. 4221)

2.1.8 Gold Conjugates

Monoclonal Anti-hCG, 40nm gold conjugate	British Biocell (No Cat. No. available)
Mouse Anti-HBsAg, 40nm gold conjugate	British Biocell (No Cat. No. available)

2.1.9 HRP Conjugates

Rabbit anti-mouse IgG-HRP conjugate	Sigma (Cat. No. A9044)
Goat anti-rabbit IgG-HRP conjugate	Sigma (Cat. No. A6154)

2.1.10 Miscellaneous

Intact hCG ELISA Test Kit	Genzyme (Cat. No. KIF4013)
Unigold PT-hCG Test	ABI (Cat. No. 3260)
TestPack® +Plus™ hCG Test	Abbott (Cat. No. not available)
Gold Conjugate Dilution Buffer	Trinity Biotech, (Part No. 2979)

2.1.11 Plastics/Filters

Pre cut filter wicks	AFC American Filtrona
One step bottom pad	Gelman (No. 51334)
One step top pad	Ahlstrom (Grade 222)
Laminated backing material	G & L Precision Die Cutting Inc., (No.187)
Glass Fibre Membrane	JBC Inc.

Membranes

AE 105 8μm nitrocellulose membrane (Unbacked)	Schleicher & Schuell (Ref. No.549909)
AE 98 5μm nitrocellulose membrane (Unbacked)	Schleicher & Schuell (Ref. No.549916)
8μm nitrocellulose membrane (Backed)	Advanced Microdevices Inc. (Cat. No. CNPF-S_1)
10μm nitrocellulose membrane (Backed)	Millipore (Cat. No. SRHF 04)

General Plastics

Foil pouches	Propak (Cat. No. VF58)
Dessicants 1.0g	Propak (Cat. No. 40AG37)
C8 Maxi Breakapart Microtitre plate	Nalge Europe (Cat. No. 473768)
One Step cassette housing device (Top)	Alex Mitchel (Cat. No. 1295)
One Step cassette housing device (Bottom)	Alex Mitchel (Cat. No. 1296)
One Step wick housing device (Top)	Alex Mitchel (Cat. No. 1295w)
One Step wick housing device (Bottom)	Alex Mitchel (Cat. No. 1296w)
One Step wick splash guard	Alex Mitchel (Cat. No. 1297)
3.5ml graduated plastic transfer pipettes	Sarsteadt (Cat. No. 86.1172)

2.1.12 Samples

hCG-free serum	Scantibodies (Cat. No. T1503)
hCG-Negative Serum	Cork Blood Bank
hCG-Negative Serum	Rotunda Hospital

hCG-Negative Serum	Pelican House
hCG-Positive Serum	Rotunda Hospital
hCG-Positive Serum	In house samples
hCG-Positive Serum	The Coombe Hospital
hCG-Negative Urines	Collected in house
hCG-Negative Urines	St. James Hospital
hCG-Negative Urines	Dublin City University
hCG-Positive Urines	Rotunda Hospital
hCG-Positive Urines	The Coombe Hospital
HAMA-Positive samples	Scantibodies, Part No. 3PH498
Heterophilic Antibody positive samples	Scantibodies, Part No. 3PH498

2.1.13 Equipment

General Equipment

Mettler Toledo Analytical Balance.

Corning pH Meter 245 with calibration buffers at pH 4.0, pH 7.0 and pH 10.0.

Mettler Toledo Magnetic Stir Plate & bars.

Stuart Scientific Vortex.

Jenway 6105 Spectrophotometer (UV Monitor).

Eppendorf single channel pipettes: 2-20µL, 10-100µL, 50-250µL and 200-1000µL.

Disposable pipettes, 10mL and 25 mL.

Pi-pump.

Dialysis Tubing.

Sterile plastic syringes, 5mL, 10mL, 20mL.

Syringe filters 0.2µm, 0.45µm, 1.2µm.

Duran glass flasks, 100mL, 500mL, 1L.

Glass universals.

Glass test tubes.

Sterile urine containers, 30mL and 60mL.

Latex gloves.

Face masks.

Safety glasses.

Spraying/Conjugating Equipment

Acculine Sprayer.

Vacuum dryer.

Erlenmyer conical flasks, 500mL and 1L.

Sorvall Centrifuge.

Centrifuge tubes, 50mL and 200mL.

Disposable glass transfer pipettes.

Gauze Tray.

Stainless Steel Tray.

Flat tipped Tweezers.

Guillotine.

Calipers.

UniGold™ Test Assembly Device.

Biodot Test Strip Cutter.

2.2 Buffers and Solutions

2.2.1 Spraying Solutions

2.2.1.1 10mM EDTA Solution

EDTA	3.722g

Made up to 1L with deionised water.

2.2.1.2 1X Phosphate Buffered Saline (PBS), pH 7.2

Sodium chloride	8.7g
25x Phosphate Buffer	40mL
1N NaOH	as required
1N HCl	as required

The pH was adjusted, if necessary, to 7.2± 0.1 using either the 1N HCl or the 1N NaOH, and made up to 1L with deionised water.

Note: This 1X phosphate buffer was 0.0008M with respect to the potassium phosphate, monobasic and 0.0064M with respect to the potassium phosphate, dibasic.

2.1.1.3 25X Phosphate Buffer

Potassium phosphate, monobasic	2.72g
Potassium phosphate, dibasic	27.9g
1N NaOH	as required
1N HCl	as required

The pH was adjusted, if necessary, to 7.8± 0.2 using either the 1N HCl or the 1N NaOH, and made up to 1L with deionised water.

Note: This 25X phosphate buffer was 0.02M with respect to the potassium phosphate, monobasic and 0.16M with respect to the potassium phosphate, dibasic.

2.2.1.4 20% (w/v) Sucrose/0.05% (w/v) Sodium Azide

Sucrose	20g
Sodium azide	0.05g

Made up to 100mL with deionised water.

2.2.1.5 Spraying solutions

Control Line spraying solution as per Method 2.3.2.1.

Test Line spraying solution as per Method 2.3.2.1.

2.2.2 Gold Conjugate solutions

All the solutions for the gold conjugate preparation were made up in cleaned boiled glassware using 0.2µm filtered RO water (For cleaning method, see Method 2.3.1.1).

2.2.2.1 10% (w/v) Gold Chloride (Trihydrate)

See Method 2.3.1.2.

2.2.2.2 1N Sodium Hydroxide (NaOH)

Sodium Hydroxide	40g

Made up to 1L with deionised water.

2.2.2.3 1N (M) Hydrochloric Acid (HCl)

Deionised water	917mL
Hydrochloric Acid	83mL

Note: Acid should always be added to water and never in the reverse order.

2.2.2.4 5% (w/v) BSA Solution

Dialysed BSA (65mg/mL)	38.5mL
Reverse Osmosis water (0.2µm filtered)	11.5mL

Mix gently.

2.2.2.5 10% (w/v) Sodium Chloride solution

Sodium chloride	2g

Made up to 20mL with deionised water.

Final solution was 0.2µm filtered.

2.2.2.6 0.25M Boric Acid

Boric acid	7.73g

Made up to 500mL with deionised water.

Final solution was 0.2µm filtered.

2.2.2.7 0.1M Sodium Borate

Sodium borate	19.07g

Made up to 500mL with deionised water.

Final solution was 0.2µm filtered.

2.2.2.8 0.5M Tris Buffer, pH 7.5

Trizma Base	60.55g
1N HCl	as required
1N NaOH	as required

The pH was adjusted, if necessary, to 7.5± 0.1 using either the 1N HCl or the 1N NaOH, and made up to 1L with deionised water.

2.2.2.9 Dialysed BSA (65mg/mL)

See Method 2.3.1.5.

2.2.2.10 Gold Conjugate Wash Buffer

0.25M Boric Acid, 0.2µm filtered	50mL
0.1M Sodium Borate, 0.2µm filtered	4mL
Polyethylene Glycol (PEG)	1g
Sodium Azide	0.5g

The pH was adjusted to pH 7.75 ± 0.1 if necessary using 0.25M Boric Acid and 0.1M Sodium Borate and made to 1L with deionised water.

The GCWB was 0.2µm filtered and stored at 2-8°C for up to three months.

2.2.2.11 12% (w/v) Sodium citrate

This solution was made up fresh for each manufacture of gold sol.

Sodium citrate	1.2g

Made up to 10mL with 0.2µm filtered deionised water. The solution was then 0.2µm filtered into a cleaned glass test tube and used on the same day.

2.2.2.12 0.25M Borate Buffer, pH 7.5

0.25M Boric Acid	10 parts
0.1M Sodium Borate	1 part

Note: a sterile 10mL pipette was used to make this solution.

The pH was adjusted, if necessary to 7.5 $\pm$ 0.1 using 0.25M Boric acid or 0.1M Sodium Borate. The buffer was 0.2µm filtered and stored at 2-27^{o}C for up to one year.

2.2.2.13 1% (w/v) Polyethylene Glycol Solution

1.0g of PEG 15-20K was dissolved in 100mL of distilled water.

2.2.2.14 Gold Conjugate Impregnation Solution

See Method 2.3.1.6.

2.2.3 Miscellaneous Buffers

2.2.3.1 2% (w/v) Triton® X-100 Solution

Triton® X-100	20g

Made up to 1L with deionised water.

2.2.3.2 hCG Control Solutions

See Method 2.3.5.3.

2.3 Methods

2.3.1 Gold Conjugation Methods

2.3.1.1 Preparation of Glassware for Gold Work

1. The presence of any particulate matter on equipment or in solutions used in the manufacture of gold sols and gold conjugates could potentially destabilise the gold binding. Therefore, glassware was kept especially for gold-labelling-related work. Half of the glassware was dedicated solely for the preparation of colloidal gold and the remainder for protein-gold conjugations.
2. The glassware was first cleaned with hot water and anti-microbial hand soap, then rinsed with distilled water and finally boiled for 10-15 minutes with 0.2µm filtered RO water.
3. Immediately before use the glassware was rinsed again with 0.2µm filtered RO water.

2.3.1.2 Formulation of 10% (w/v) Gold Chloride (Trihydrate)

10mL of 0.2µm filtered distilled water was added to an amber glass container of 1.0g of Gold Chloride. The bottle was swirled gently and the gold chloride was allowed to dissolve at room temperature. This was timed for 1 hour $\pm$ 5 minutes. The final homogenous solution was 0.2µm filtered into a glass universal tube, which was covered in tin foil to exclude light.

2.3.1.3 Preparation of 0.01% (v/v) Colloidal Gold Sol, 40nm

1. 1L of 0.2µm filtered RO water was added to an Erlenmyer glass flask, which had been cleaned according to method 2.3.1.1.
2. A stirring bar (used only for gold sol preparation) was added and the flask was placed on a heating stirring plate.
3. The water was heated while stirring to 85-90°C.
4. 1.0mL of 10% (w/v) Gold Chloride solution (see 2.3.1.2) was added while stirring.

5. The solution was heated to boiling temperature (until multiple bubbles were observed). The solution was boiled for a further 2 minutes.
6. 1.0mL of 12% (w/v) Sodium Citrate was added and the solution was allowed to boil for 6 minutes.
7. The flask was then removed from the stirring plate and placed in a dark place at room temperature for 12-18 hours.
8. The absorbance was read and recorded at 520nm using the spectrophotometer.
9. The gold sol was stored at 2-8°C in a dark place and given an expiry of one month.

2.3.1.4 Manufacture of Anti-hCG Gold conjugate

1. 20mL of 0.01% (w/v) Colloidal gold was added to 0.8mL of 0.25M Borate buffer and mixed by manual horizontal rotation.
2. 1mL of the gold-borate buffer and 50mL of 1% (w/v) PEG solution was added to a glass test tube and mixed well. The pH was determined and should be within the range 7.3-7.6.
3. The absorbance of the Anti-hCG antibody solution was read at 280nm to determine the concentration of antibody (Ig) in the solution by using the following equation:

$$\frac{\text{mg of Ig}}{\text{ml}} = \frac{\text{Absorbance 280nm of solution}}{\text{1.35 x dilution factor}}$$

Note: 1.35 = the absorption coefficient for Ig molecules at 280nm (Aslan and Dent, 1998). The dilution factor was only taken into account if it was necessary to dilute the stock antibody solution before reading at 280nm.

4. The solution was diluted to 1mg/mL using distilled water and any unused solution was stored at 2-8°C for up to 1 week.

Adsorption Isotherm

5. The anti-hCG antibody was diluted to 100mg/mL using distilled water to give a volume of ~ 400mL.
6. 1.0mL of colloidal gold-borate buffer was added to each of six cleaned 10mL glass test tubes.

7. The specified volume of antibody solution was added to each tube as outlined in the example in Table 2.3.1.4 and each tube was mixed briefly and incubated for 5 minutes.
8. The colour change was observed and recorded in a table like that shown in 2.3.1.4. 100mL of 10% (w/v) NaCl was then added to each tube and mixed briefly.
9. The solutions were incubated for 5 minutes and the colour change, if any was visually interpreted and then recorded in the same table as used for step 8.
10. The tube containing the minimum amount of anti-hCG antibody solution without a colour change being observed was selected as the amount of antibody required to stabilise 1mL of gold sol.
11. If none of the tubes were seen to be stable or all tubes remained the same colour the titration was repeated with higher/lower concentrations of the antibody.
12. 300mL of 0.01% (v/v) gold sol was weighed into an Erlenmyer flask (it was assumed that 1mL of gold sol weighed approximately 1g).
13. 12mL of 0.25M Borate buffer was added using a sterile 10mL pipette and mixed manually as the buffer was being added.

Tube No.	Gold Sol-Borate Buffer (mL)	Anti-hCG Soln (µL)	Colour before NaCl at 5 mins	Colour after NaCl at 5 mins
1	1	5		
2	1	10		
3	1	20		
4	1	30		
5	1	40		

Table 2.3.1.4: *Example of a plan for an adsorption isotherm showing examples of the amount of gold sol-borate buffer and antibody which could be added to each tube is displayed. The volume of antibody required to stabilise the gold sol-borate buffer solution may vary depending on the antibody being conjugated. The respective colour for each tube before and after the addition of NaCl would be recorded in the two right hand columns.*

14. From the adsorption isotherm, the volume of anti-hCG antibody (mL) required to stabilise 300mL of gold sol was calculated as follows:

{Vol. of antibody* (μL)(step 10)}x{300mL (i.e.: vol. of gold sol)}

*volume added to the selected tube from step 10.

15. The volume calculated in step 14 was multiplied by 1.1 to allow for a 10% excess of antibody solution to be added to the gold sol. This volume was added to the flask as one aliquot while manually mixing horizontally.
16. The solution was incubated for 10 minutes at room temperature and the colour was recorded.
17. 1mL of the solution was removed and 100mL of 10% (w/v) NaCl was added to the aliquot and was allowed to incubate for 5 minutes and the colour was recorded.
18. The above electrolyte test was repeated and if the aliquot retained its pink/purple colour, the conjugation was allowed to proceed.
19. 12mL of 5% (w/v) BSA was added to the flask and mixed by manual horizontal rotation and incubated for 60-90 minutes in a dark place.
20. 100mL of this solution was transferred to a 250mL centrifuge tube and centrifuged at 10,000 rpm for 60 minutes at 2-8°C.
21. The supernatants were aspirated and discarded and the pellets resuspended in ~100mL of GCWB and centrifuged as above.
22. Steps 20-21 were repeated once more and the supernatants aspirated the pellets were combined and reconstituted with GCDB to a final volume of 30mL.
23. The absorbance at 520nm was recorded and the conjugate solution was further diluted if necessary, with GCDB until an absorbance at 520nm of 3.0 was acheived.
24. The conjugate was stored at 2-8°C in a dark place.

2.3.1.5 Manufacture of a 65mg/mL dialysed BSA solution

1. A 65mg/mL solution of BSA was made up in distilled water as follows:
   Note: The total amount of BSA required was initially added to 60% of the total amount of distilled water required to make up the 65mg/mL solution. The BSA was added slowly and allowed to dissolve without stirring, as this would cause frothing. Once the BSA had dissolved, the solution was made up to its required volume using distilled water.
2. Some dialysis tubing was pre-wet in distilled water for a minimum of 10 minutes. One end of the tubing was knotted tightly several times as close to the end as possible and the BSA solution was transferred to the tubing using a pasteur pipette. The other end of the tubing was then knotted several times to form a tight seal, keeping the air space above the liquid to a minimum. Note: gloves were worn at all times while handling the dialysis tubing.
3. The dialysis tubing was placed in 2.0L of deionised water. This deionised water was replaced three times a day for two days, keeping the dialysis tubing in the replacement buffer for a minimum of two hours.
4. The dialysed solution was removed from the tubing and first 1.2µm filtered into a sterile container. The resulting solution was then 0.2µm filtered in the laminar hood into a sterile container. The volume was noted.
5. A 1 in 20 dilution of the dialysed BSA was carried out using 1X PBS, pH 7.2.
6. A dilution series was prepared using a BSA protein standard and 1X PBS, pH 7.2 as shown in Table 2.3.1.5.
   The absorbance at 280nm was recorded for each BSA solution.

Tube No.	BSA Conc. (mg/mL) per tube	Volume of BSA added to tube	Volume of PBS, pH 7.2, added to tube
1	*0.700*	*1.0mL of 1.400 mg/mL standard*	*1.0 mL*
2	*0.350*	*1.0mL of 0.700 mg/mL standard*	*1.0 mL*
3	*0.175*	*1.0mL of 0.350 mg/mL standard*	*1.0 mL*
4	*0.087*	*1.0mL of 0.175 mg/mL standard*	*1.0 mL*

Table 2.3.1.5: *BSA Protein Standard Dilutions*

7. The absorbance at 280nm for each BSA solution was plotted against the BSA concentration (mg/mL) to create a standard curve.

8. The 1 in 20 dilution of the dialysed BSA was serially diluted to give the following dilutions: 1 in 40,
1 in 80,
1 in 160,
1 in 320,
1 in 640,
1 in 1280.

9. The absorbance at 280nm was read and the concentration of BSA was calculated for the 1 in 320-1 in 1280 dilutions. The mean of these concentrations was used to dilute the original undiluted dialysed BSA solution to obtain a concentration of 65mg/mL, using 0.2μm filtered water in the laminar flow hood.

10. This readjusted solution was serially diluted as previously outlined in step 9 and the absorbance values used to determine if the solution was within the range 65 ± 1mg/mL. If not, the dilution step was repeated.

11. The 65mg/mL dialysed BSA was aliquotted into 1.1mL lots into 2mL polypropylene vials and stored at -20°C for up to 1 year.

2.3.1.6 Impregnation of Gold Conjugate Strips

1. In order to manufacture gold conjugate strips, it was necessary to impregnate glass fibre strips with a gold conjugate solution of a specified A_{520nm} with respect to the gold conjugate component. The absorbance of the stock Anti-hCG gold conjugate was read at 520nm. Using the desired A_{520nm} of the gold conjugate the volume of this stock conjugate required was calculated as follows:

$$\frac{\text{Required } A_{520nm} \text{ of gold conjugate} \times \text{Vol. of Gold Conjugate Solution required*}}{A_{520nm} \text{ of stock gold conjugate}}$$

* 1.5mL of gold conjugate solution required per gold conjugate strip.

2. The volume of stock conjugate required was added to a clean test tube and the solution was made up to volume using gold conjugate dilution buffer (GCDB).

3. Strips of glass fibre membrane were cut to measure 8mm x 28mm using a paper guillotine. Their widths were checked using a measuring calipers.
4. The glass fibre strips were placed on a cleaned stainless steel tray and numbered with a ball point pen.
5. 750µL of the diluted gold conjugate solution was added in drops along the length of each strip and allowed to absorb. A further 750µL was added per strip and the solution was spread evenly along the strip using the tip of the Eppendorf.
6. The impregnated strips were placed on a cleaned gauze tray using a flat tipped tweezers and placed in the vacuum dryer overnight (see 2.3.5.1).
7. Once dried, all strips were stored with indicating desiccants at 20–25°C in an airtight container until required for test assembly.

2.3.2 Spraying of Nitrocellulose Membrane

2.3.2.1 Formulation of Spraying Solutions

Test Line

1. The volume of the stock anti-hCG solution required for the test line was calculated as follows:

$$\frac{\text{Concentration required}}{\text{Concentration of stock solution}} \times \text{Volume of spraying solution required}$$

Note: The volume of spraying solution required is calculated by assuming that 1ml of solution is required per test.

2. The amount of 10mM EDTA required was calculated as follows:
   Spraying volume required x 0.03 = Vol. of 10mM EDTA required.
3. The volume of 20% (w/v) sucrose required was calculated by using the following formula:
   Spraying volume required x 0.1 = Vol. of 20% (w/v) Sucrose required.
4. These three reagents were added to a clean test tube and the solution was made up to the required volume using 1X PBS, pH 7.2.

Control Line

5. The formulation of the control line solution was carried out exactly as for the test line solution apart from the use of different antibody solutions.

2.3.2.2 Spraying of Nitrocellulose

1. The starting temperature and humidity were recorded.
2. The nitrocellulose membrane and airbrushes were mounted on the Acculine Sprayer according to the instructions outlined in the Acculine Manual, Section 5, pages 12 and 13.
3. The parameters of the sprayer were set as follows:

Membrane Speed: 4 cm/sec

Airbrush Pressure: 24 psi

Airbrush Position:

Test Line:	12mm (Cassette)	9.75mm (Wick)
Control Line:	19mm (Cassette)	20mm (Wick)

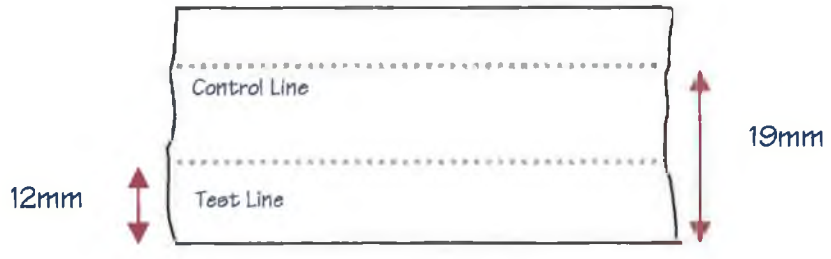


Figure 2.3.2.2: *Diagram showing the distance (mm) of the sprayed test & control lines from the bottom of the nitrocellulose membrane. Spraying positions shown are for the cassette device. The relevant distances for the wick device are shown above.*

Airbrush Opening for spraying needle:

Test Line:	Began with 6mm
Control Line:	Began with 6mm

4. The relevant reagents were connected to their airbrushes and the brushes were primed as outlined in the Acculine Manual.
5. The test and control line solutions were sprayed until the solutions ran out, maintaining a mean line thickness of 1-1.2mm.

6. The membrane was vacuum dried for 2-16 hours as per method 2.3.5.1 depending on the length of membrane sprayed.

2.3.3 Assembly of Test Strips and Devices (Cassette & Wick)

1. The following were required to assemble test strips:
   - Laminated Backing Material
   - Top and Bottom pads
   - Sprayed Nitrocellulose
   - Impregnated Conjugate Pad
   - Assembly Device
2. The laminated backing material was placed between the guide rails of the assembly device and the larger top cover strip removed to reveal the adhesive surface.
3. A section of sprayed membrane was measured and cut to fit the length of the laminated strip and was applied as evenly as possible to the adhesive surface, ensuring that the sprayed side was orientated correctly (i.e.: with the sprayed control line to the top side).
4. The larger cover strip (just removed) was used to press down the membrane thus avoiding direct contact with the sprayed surface.
5. The centre guide rail was placed in the device and the top pad was placed on top of the membrane meeting the top edge of the centre guide rail and overlapping the membrane.
6. The lower cover strip was removed from the laminated backing and the conjugate strip (pink) was placed overlapping the membrane (by at least 2mm) against the bottom edge of the centre guide rail.
7. The bottom pad was placed over the conjugate strip keeping its bottom edge against the top edge of the bottom guide rail of the assembly device.
8. The resulting ~30cm strip was cut into 8mm test strips using a Biodot Cutter (see section 1.0, Introduction, Figure 1.11.8.)

2.3.3.1 Device Assembly (Cassette)

9. An 8mm test strip was placed in a bottom housing device, ensuring that the conjugate (pink) strip was at the bottom.

10. The top housing device was placed carefully over the bottom piece with the sample port at the bottom.
11. The pieces were snapped closed, ensuring a tight fit.

2.3.3.2 Device Assembly (Wick)

12. The wick test was assembled as above in step 9 and an impregnated wick was placed within the wick guide walls of the wick housing device leaving ~20mm of the wick outside the housing device.
13. The top housing piece was placed over this and snapped shut, ensuring all pins are closed down firmly.

2.3.4 Assay Formats

2.3.4.1 Test format for the 'in-house' assay

Cassette Assay

1. The test was removed from its foil pouch and was allowed to come to room temperature along with the sample being run.
2. Four drops of urine/serum were added to the sample port and the timer was started.
3. The test was read at 3 minutes for urine and 5 minutes for serum and results interpreted as per 2.3.4.2.

Wick Assay

1. The test was removed from its foil pouch and was allowed to come to room temperature along with the sample being run.
2. The splash guard was pushed back and the 2/3 of the wick was immersed in the urine/serum until the sample flow was seen at the bottom of the test window.
3. The test was read at 3 minutes for urine and 5 minutes for serum and results interpreted as per 2.3.4.2.

2.3.4.2 Results Interpretation

Negative

A line is visible only at the Control Line

Positive

A line is visible at both the Test & Control Line

Inconclusive

No line is visible at either the Test or Control Line

Figure 2.3.4.1: *Interpretation of Assay results*

2.3.4.3 ABI Assay Format

Cassette Device

1. Four drops of urine/serum were added to the sample port of the device.
2. Once the required assay time had elapsed (3 minutes for urine, 5 minutes for serum), the device was interpreted in a manner identical to that described for the 'in-house' assay in Figure 2.3.4.1, shown above.

2.3.4.4 Assay Format for the Genzyme hCG ELISA

1. 50μL of each serum sample to be run and each of the following kit standards were dispensed in duplicate 0, 5, 25, 50, 100 and 200 IU/L to the required number of coated wells. The plate was gently shaken for 30 seconds.
2. 100μL of the supplied sample/standard dilution buffer was added to each well and the plate was shaken thoroughly for 10 seconds and incubated for 30 minutes at room temperature.

3. The plate was hand washed x 5 with running tap water and tapped dry.
4. 150µL of the enzyme conjugate solution was then added to each well of the plate and the plate was shaken gently for 5 seconds and incubated at 15 minutes at room temperature.
5. The washing step was repeated as in step 3.
6. 200µL of TMB substrate was dispensed into each well and gently shaken for 5 seconds. The plate was incubated for 15 minutes at room temperature.
7. The reaction was stopped with 50µL of 4N H_2SO_4 solution per well and the absorbance was read at 450nm.
8. A standard curve was plotted using the absorbance reading at 450nm for the kit standards and a value was calculated for each sample using this curve.

2.3.5 Miscellaneous Methods

2.3.5.1 Vacuum Drying Procedure

1. The vacuum dryer refrigeration unit was turned on approximately 15 minutes before use to ensure that the temperature had reached –56°C.
2. Once the temperature had reached –56°C the gold conjugate strips/sprayed membrane were placed on cleaned gauze trays in the vacuum dryer.
3. The door of the vacuum dryer was sealed and the vacuum pump was switched on. The opening of the outlet tube was covered until a sufficient vacuum was pulled.
4. The strips/membrane were left vacuum drying overnight under these conditions.

2.3.5.2 Impregnation Prodedure for Wicks

1. The wicks were placed in a large container and covered completely with a 2% (w/v) Triton X-100® solution and allowed to soak for 1hour ± 10 minutes at room temperature or overnight at 2-8°C.
2. Once the required time had elapsed the solution was drained off and the wicks were placed in a single layer on a gauze tray specifically set aside for this purpose and placed in the vacuum dryer overnight.

2.3.5.3 Manufacture of hCG controls

The hCG used was a lyophilised sample of the WHO 3rd International Standard. This standard contained 650 IU of hCG/ampoule. The contents of each ampoule were diluted in 6.5mL of deionised water to give a concentration of $1x\ 10^5$ IU/L. A 1 in 100 dilution was made of this stock to give a solution of 1000 IU/L. This was then diluted as follows in either serum or urine to give the required concentration of hCG.

Volume of 1000 IU/L required =

$$\frac{\text{Required hCG Concn. (IU/L)}}{\text{1000 IU/L (i.e.: Stock Concn.)}} \times \text{Vol. required}$$

The volume of 1000 IU/L stock calculated above was added to a sterile container and the solution was made up to the desired volume with either hCG negative urine or serum and mixed gently.

Prozone Solutions

These solutions were known as 'Prozone Solutions' as a result of their high hCG concentrations and the subsequent potential prozone effect (false negative result, see Discussion, section 4.9.4) that would most likely be observed for the test due to these high concentrations of hCG.

The solutions were manufactured using the 1×10^5 IU/L hCG stock as described above. This stock was diluted as shown overleaf to give the required concentration:

Volume of 1×10^5 IU/L required =

$$\frac{\text{Required hCG Concn. (IU/L)}}{1 \times 10^5\ \text{(IU/L)(i.e.: Stock Concn.)}} \times \text{Vol. required}$$

The volume of 1×10^5 IU/L stock calculated above was added to a sterile container and the solution was made up to the desired volume, with either hCG-negative urine or serum, and mixed gently.

2.3.5.4 Testing of Sample Pads

Absorbance Test for sample pads

Distilled water was added in 20μL increments to each square until the maximum amount that the pad could absorb was reached. This was observed as being the point where the water was seen to leach from the edges of the pad and to form a film on the surface of the pad.

Flow Test for sample pads

Distilled water was placed in a weigh boat and each pad was held vertically and the edge of its short side dipped into the liquid. The time taken for the water to travel to the top of the strip of absorbent pad was recorded and used as a crude method for determining the flowrate of the pad.

2.3.5.5 Analysis of Clinical Trial Results (Hart, 1980)

For all calculations using the sample results from clinical trials the following apply:

$$\% \text{ Sensitivity} = \frac{\text{Number of True positives}}{\text{Number of True positives} + \text{Number of false negatives}} \times 100$$

$$\% \text{ Specificity} = \frac{\text{Number of True negatives}}{\text{Number of True negatives} + \text{Number of false positives}} \times 100$$

2.3.5.6 Dot Blot for comparison of various antibody affinities for the hCG antigen

1. hCG solutions were manufactured as per Method 2.3.5.3 to ensure that hCG was titered out from 100 IU/dot to 5 IU/dot (Each dot consisted of 10μL of solution).
2. Each dot was added carefully to membrane no. 1 (see Materials, section 2.1) to ensure that each dot was of the same size and the membrane was dried at 37°C for 10 minutes.
3. Each membrane strip was blocked for 30 minutes at room temperature on a shaker in a solution of 5% (w/v) BSA in 1X PBS, pH 7.2.
4. The strips were repeatedly washed in 1X PBS, pH 7.2.

5. Each of the antibodies being examined for their affinity to hCG were diluted to 5μg/mL in 1% (w/v) BSA in 1X PBS, pH 7.2 and incubated with the relevant membrane strip for 30 minutes on a shaker at room temperature. This was to allow an accurate comparison to be made between results.
6. The strips were repeatedly washed in 1X PBS, pH 7.2.
7. The rabbit anti-mouse-HRP conjugate at 1μg/mL in 1% (w/v) BSA in 1X PBS was added to all the strips which had been incubated with mouse monoclonal antibodies. Only one rabbit polyclonal antibody was examined and therefore this was incubated with goat anti-rabbit-HRP also at 1μg/mL in 1% (w/v) BSA in 1X PBS, pH 7.2. All strips were incubated for 30 minutes at room temperature on a shaker.
8. The strips were repeatedly washed in 1X PBS, pH 7.2.
9. All strips were incubated with a DAB solution for 10 minutes to provide colour. The DAB solutions were disposed of safely and the strips were washed in deionised water and examined for colour intensity.

2.3.5.7 Formulation of Variations of the Gold Conjugate Dilution Buffer

The following applied for Section 3.8.5 of the Results section, entitled 'Buffer evaluations':

1. 10% (w/v) Tween 20® and Triton X-100® were added into the GCDB to a final concentration of 0.001% (v/v).
2. Each of the different sized Polyethylene glycols (PEGs) to a final concentration of 5mg/mL in the GCDB.

The following applied for Section 3.8.5i of the results section, entitled 'Further buffer evaluation':

1. GCDB was supplemented individually with each of Triton X-100® and Tween 20® to give a final concentration of 50g/L. Each of these solutions was serially diluted 1 in 2 to give 6 solutions, with the lowest concentration being 1.25g/L.
2. Similarly, a 60g/L stock of PVP K-30, a 50g/L stock of PEG 15-20K, a 250g/L stock of sucrose and a 40g/L stock of BSA were made up in the GCDB. Each of these stock solutions were also serially diluted 1 in 2 as

described in step 1, to give 6 solutions, with the lowest concentrations of each stock solution as follows: 1.5g/L PVP K-30, 1.25g/L PEG 15-20K, 12.5g/L sucrose and 1g/L BSA.

2.3.5.8 'Comb' Method

1. The nitrocellulose membrane was sprayed in the usual manner with test line and control line antibodies (see method 2.3.2).
2. This membrane was placed on the laminate plastic backing but no gold conjugate strip, top or sample pads were stuck down with this membrane. This laminated membrane was then cut into 4mm strips.
3. Any excess laminate was trimmed from these strips at the test line bottom edge so that the membrane exactly reached this edge.
4. The laminate cover was removed from the top edge and each 4mm strip was arranged on a piece of card using the now exposed adhesive. This formed a 'comb' of strips with enough space between each strip so that the 'comb' could fit into the wells of a blank microtitre plate (See Figure 2.3.5.8).
5. The following was placed in each well:

   40µL of Gold conjugate

   80µL of the relevant GCDB (1-16)

   40µL of sample*

   * Both negative and spiked positive samples were used.
6. The plate was shaken for 1 minute to allow mixing of the solutions.
7. The 'comb' was dipped into the wells and the flow characteristics were observed over time.

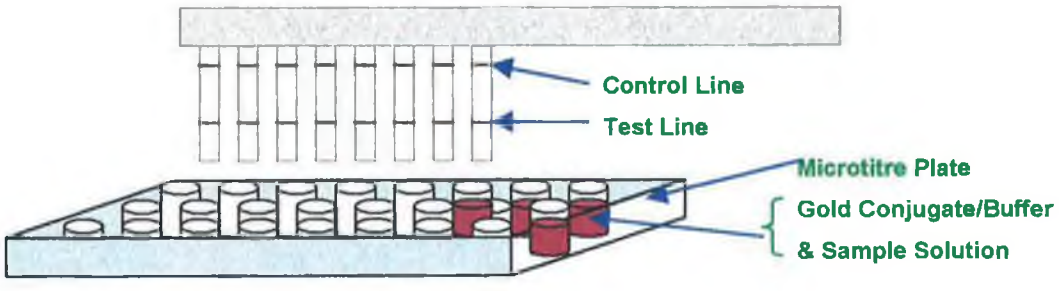


Figure 2.3.5.8: *The Microtitre 'Comb' Method. Schematic representation of the 'comb' made up of several test strips being inserted into the microtitre plate wells, which contain liquid components to run a test.*

2.3.5.9 Stability Study Method

Accelerated stability studies were carried out to determine an expected shelf-life of both the cassette and wick 'in-house' tests. This was carried out by placing tests at 37°C for the periods of time specified in Table 2.3.5.9 and testing these against devices, which were stored at 20-25°C (control devices) to decipher how stable the tests were (Anderson and Scott, 1991). A shelf-life equivalency table is shown in Appendix II. Devices were tested on day 0, 7, 14, 28 and month 2, 3, 4, 5, 6 and 7 where applicable.

Weeks at 37°C	Equivalent time at 20-25°C
14	*12 months*
21	*18 months*
28	*24 months*

Table 2.3.5.9: *Accelerated time stability study: reference table used for calculating approximate shelf life of the 'in-house' tests.*

1. The number of devices to be placed at 37°C was calculated for each individual study. These devices were then manufactured in one lot using identical components for both the control devices and the devices to be placed on stability study (test devices). All devices were pouched to ensure they remained dry throughout the study.
2. The required number of test devices was placed at 37°C for the required length of time. The required number of control devices was placed at 20-25°C.
3. The test and control devices were tested in duplicate on day 0 with a negative urine and/or serum control and low and high hCG-positive urine and/or serum controls.
4. The same lot of hCG controls were run on the test and control devices on the subsequent testing days to ensure minimum variability. The results for the test devices were compared to those for the control devices. The shelf-life was calculated for the test based on the shelf-life equivalency table (see Table 2.3.5.9 above and Appendix II) and on the length of time taken for the test devices to deteriorate in comparison to the control devices.
5. The shelf-life calculated by this accelerated time stability method was confirmed by 'real-time' studies in a similar manner.

Section 3:

Results

3.1 Summary of common parameters throughout the development and evaluation of the 'in-house' hCG assay

This section outlines methods, which were used throughout the development of the assay. These methods did not change and were common for all experiments during the course of this project unless otherwise specified in the relevant sections. Various parameters of the assay, such as sample volume, assay time, etc., evolved with the development of the test. These changes are recorded in the relevant sections of the results and also in the summary tables (see 3.6, 3.10 and 3.13).

(A) The Manufacture of Assay Components

❶	Nitrocellulose membrane spraying:	Methods 2.3.2.1 – 2.3.2.2.
❷	Gold conjugate manufacture:	Methods 2.3.1.1 – 2.3.1.5.
❸	Gold Conjugate impregnation:	Method 2.3.1.6.
❶	Membrane and conjugate strip drying:	Method 2.3.5.1.
❺	Assembly of test devices:	Methods 2.3.3.1 – 2.3.3.2.
❻	Manufacture of hCG controls:	Method 2.3.5.3.

(B) Assay Format

The assay format for the initial developmental process is laid out in Table 3.1.1.

Sample Volume	*200µl (4 drops, using transfer pipette)*
Assay Time	*5 minutes*
Assay Temperature	*Room Temperature*

Table 3.1.1: *'In-house' assay parameters during early development.*

Note: In some cases the assay time was varied from 3 to 15 minutes for observation purposes. Any deviation from the assay time stated in Table 3.1.1 is noted in the relevant results section. The final assay formats are shown in method 2.3.4.1.

(C) Interpretation of Results

All results for the duration of the development and evaluation of the assay were interpreted as follows:

❶ The assay result was deemed negative, positive and/or inconclusive as per Method 2.3.4.2.

❷ The intensity of the test and/or control line (where relevant) was then read against a chart similar to that shown below (see Figure 3.1.1).

Line Intensity	Result	Score
	Negative	*0*
	Positive	*0.5*
	Positive	*1*
	Positive	*2*
	Positive	*3*
	Positive	*4*
	Positive	*5*
	Positive	*6*
	Positive	*7*
	Positive	*8*
	Positive	*9*
	Positive	*10*

Figure 3.1.1: *Example of a Colour Interpretation Chart*

❸ Use of P-N Value

In an attempt to facilitate more straightforward comparisons it was decided to employ a *'P-N value'*, which was essentially the positive result minus the negative result obtained for a particular test combination.

(D) Miscellaneous Common Components

❶ Reference hCG Assay: ABI Unigold PT-hCG assay (see Materials, section 2.1.10)

❷ Sample Pad B (see Appendix I): used throughout the initial development to Section 3.11.2.

3.2 Initial Selection of Suitable Test Line Antibodies

3.2.1 Assessment of the performance of nine anti-hCG antibodies when sprayed as the test line antibody.

Nine anti-hCG antibodies (one polyclonal and eight monoclonals) were selected initially for examination as suitable capture antibodies for the test line. Each antibody solution was sprayed on membrane no.2 (see Appendix I) at the concentrations outlined in Table 3.2.1.

A monoclonal anti-βhCG antibody (antibody no.5, Appendix I) was conjugated to 40nm colloidal gold (conjugate C, see Appendix I) and conjugate strips were made up at an A_{520nm} of 0.6. Test strips were then manufactured using each of the sprayed nitrocellulose strips with the 40nm gold conjugate strip. Each test combination was assayed, as described in section 3.1, and the results interpreted accordingly. The results are shown below in Table 3.2.1.

Test Line Antibody No.	Test Line Conc. (mg/mL)	0 IU/L hCG Urine (C.I.)		50 IU/L hCG Urine (C.I.)	
		3 min	5 min	3 min	5 min
1	4mg/mL (undiluted)	0	0	1	2
	1mg/mL	0	0	1	1
2	4mg/mL (undiluted)	0	0	0	1
	1mg/mL	0	0	0	1
3	4mg/mL (undiluted)	1	1	1	2
	1mg/mL	1	1	1	2
4	4mg/mL (undiluted)	0	0	0	1
	1mg/mL	0	0	0	1
5	1mg/mL (undiluted)	1	1	1	2
6	1mg/mL (undiluted)	2	2	1	2
7	1mg/mL (undiluted)	2	2	0	1
8	1mg/mL (undiluted)	0	1	1	2
9	6mg/mL (undiluted)	0	1	1	2
	1mg/mL	0	0	1	2

Table 3.2.1: *Results for Test Line Antibody Comparison Study ~ Evaluation of nine test line antibodies with 40nm gold conjugate (C). Colour intensity results at three and five minutes for positive and negative samples.*

In examining the results shown in Table 3.2.1, it is apparent that three of the sprayed antibodies, no.1, no.8 and no.9 exhibited satisfactory colour intensity (C.I.) results for both the positive and negative samples. No colour was observed for the negative sample at 3 minutes for each of the devices assembled using nitrocellulose sprayed with these three antibodies. At five minutes antibody no.1 was still clear of colour for both the 4mg/mL and 1mg/mL concentrations with this negative sample. Antibody no.8 demonstrated a C.I of 1 at five minutes for a sprayed concentration of 1mg/mL, as did antibody no.9 at the 6mg/mL concentration. This positive C.I. result was deemed acceptable, despite its presence for a negative sample since the C.I of 1 was very faint and was only apparent outside the proposed assay time of 3 minutes for urine samples. It was also noted that for antibody no.9 this positive result was only observed for the 6mg/mL spraying concentration and not for the 1mg/mL concentration, i.e.: it was proposed that further titering of the test line concentrations would eliminate the false positive C.I. result.

Antibodies no.1, no.8 and no.9 all provided a C.I. of 1 at 3 minutes and a C.I. of 2 at five minutes for the 50 IU/L hCG urine sample. These results, although faint, gave satisfactory positive results, with a distinct difference observed in the C.I's obtained for the positive and negative samples using these three antibodies as the test line.

No positive/negative distinction was apparent for the remaining test line antibodies. Antibody no.2 and no.4 demonstrated poor sensitivity while antibodies no.3, no.5, no.6 and no.7 exhibited poor specificity.

- **Conclusion**

As a result of these observations antibodies no.1, no.8 and no.9 were selected for further examination as the test line antibody.

3.2.2 Further Evaluation of Three Test Line Antibodies.

Antibodies no.1, no.8 and no.9 were sprayed as described in Table 3.2.2 on nitrocellulose membrane no. 2 with gold conjugate C (see Appendix I).

Male urine was collected in house, pooled into different lots (A, B and C) and used as hCG negative controls. 50 IU/L hCG positive controls were manufactured using each of these three hCG negative controls. Each lot of controls was run on each device. The results are shown below in Table 3.2.2.

Test Line ID No.	Test Line Concentration (mg/mL)	Pooled Sample ID No.	Colour Intensity (C.I.) Results			
			Negative Pool		Positive Pool	
			5 min	10 min	5 min	10 min
1	4mg/mL (Undiluted)	A	0	0	0	1
	1mg/mL		0	0	0	1
8	1mg/mL (Undiluted)		0	0	1	1
9	6.2mg/mL (Undiluted)		0	0	1	2
	1mg/mL		0	0	0	1
1	4mg/mL (Undiluted)	B	0	0	0	0
	1mg/mL		0	0	0	0
8	1mg/mL (Undiluted)		0	0	0	1
9	6.2mg/mL (Undiluted)		0	1	1	2
	1mg/mL		0	0	1	2
1	4mg/mL (Undiluted)	C	0	0	1	2
	1mg/mL		0	0	0	1
8	1mg/mL (Undiluted)		0	0	2	2
9	6.2mg/mL (Undiluted)		0	0	3	3
	1mg/mL		0	0	2	2

Table 3.2.2: *Comparison of three Test Line Antibodies ~ Colour intensity results for three 0 IU/L & 50 IU/L hCG sample pools at five and ten minutes.*

Negative Sample Results:

Each of the three test line antibodies showed no colour (C.I. of 0) for all three negative sample pools, apart from a C.I. of 1 which was noted at ten minutes for antibody no.9 sprayed at 6.2 mg/mL, with pool B. This false positive result was therefore considered non-applicable since no colour was observed with the same antibody no.9 at the lower concentration of 1mg/mL. The positive sample for pool B gave a C.I. result of 2 with the test line antibody no.9 at 1 mg/mL.

Positive Sample Results:

Antibody no.8 and no.9 demonstrated good C.I results for each of the positive sample pools with antibody no.9 exhibiting the higher C.I. results. Antibody no.1 showed only faint C.I. results at 10 minutes for two out of three of the sample pools. For the positive sample pool A the highest C.I. (C.I. of 2) was observed for antibody no.9 at a concentration of 6.2 mg/mL at 10 minutes, a C.I. of 1 was seen for antibody no.8 at 5 and 10 minutes. Similarly, C.I. results of 2 were seen for antibody no.9 at 10 minutes with positive sample pool B, a C.I. of 1 was only detected at 10 minutes for antibody no.8, while no colour was noted for antibody no.1. Positive sample pool C provided C.I.'s of 2 at 5 and 10 minutes for both antibody no.8 and the 1mg/mL concentration of antibody no.9. The devices manufactured with nitrocellulose sprayed with undiluted antibody no.9 gave C.I.'s of 3 at both 5 and 10 minutes. Once again the lowest results were obtained with antibody no.1 with a C.I. of only 1 noted at 5 minutes for the undiluted test line. It should be noted that antibody no.1 did not perform as well as observed in Table 3.2.1. Therefore, due to inconsistent performance with different samples this antibody was eliminated from inclusion into further test line antibody studies.

- **Conclusion**

Antibodies no.8 and no.9, at 1 mg/mL, were selected for further examination with six different gold conjugates to determine the optimum test line/gold conjugate antibody pair (see section 3.3).

3.3 Combined Evaluation of Various Gold Conjugate and Test Line Combinations

3.3.1 Assessment of six gold conjugates with two test line antibodies

Selection of an antibody for conjugation to a 40nm gold colloid was initially based on six monoclonal antibodies A, B, C, D, E and F (see Appendix I). Each gold conjugate was impregnated at the A_{520nm} readings as outlined in Table 3.3.1 below, which were formerly shown to be optimal from previous studies (results not shown). Test devices were manufactured and evaluated using these gold conjugate strips and membrane no.2 was sprayed with antibodies no.8 and no.9 in an attempt to choose the most suitable test line/gold conjugate antibody pair.

Gold Conjugate Antibodies		Test Line Antibodies	
Gold Conjugate I.D.	**A_{520nm}**	**Test Line I.D.**	**Concentration**
A	*0.3*	*no. 8*	*1mg/mL*
B	*0.3*	*no. 9*	*1mg/mL*
C	*1.0*	--	--
D	*0.43*	--	--
E	*0.3*	--	--
F	*1.0*	--	--

Table 3.3.1: *List of six 40nm gold conjugate and two test line antibodies evaluated in combination. The A_{520nm} at which the gold conjugates were impregnated and the test line spraying concentrations are included.*

The results are shown in Table 3.3.2, 3.3.3 and 3.3.4 and graphed on pages 87-88 (Figure 3.3.1 and 3.3.2).

The results from these tables reveal that the gold conjugates C and D performed best with both of the test lines no. 8 and no. 9 and exhibited comparable results to those observed for the ABI reference test. Gold conjugates A and B in combination with test line no. 8 manifested little differentiation between positive and negative samples. The same conjugates with test line no. 9 provided acceptable results (P-N value of 0.5 for both conjugates, see Table 3.3.4, page 87) but exhibited slight false positives for the negative sample (C.I. of 0.5 for both conjugates at 5 minutes). The C.I. results

for the positive samples were also slightly lower than those noted for conjugate C and D (C.I. of 1 for both, see Figure 3.3.2).

Test Line I.D. No.	Test Line Conc. (mg/ml)	Conjugate & A_{520nm}	Result (C.I.)	
			at 5 min	at 10 min
8	1 mg/ml	A at 0.3	1	1
		B at 0.3	1	1
		C at 1.0	0	0.5
		D at 0.43	0	0
		E at 0.6	2	2
		F at 1.0	2	2
9	1 mg/ml	A at 0.3	0.5	1
		B at 0.3	0.5	1
		C at 1.0	0	0.5
		D at 0.43	0	0
		E at 0.6	1	2
		F at 1.0	2	2
ABI			0	0.5

Table 3.3.2: *C.I. results for the gold conjugate/test line combinations outlined in Table 3.2.1, using a negative (0 IU/L hCG) urine sample.*

Test Line I.D. No.	Test Line Conc. (mg/ml)	Conjugate & A_{520nm}	Result (C.I.)	
			at 5 min	at 10 min
8	1 mg/ml	A at 0.3	1	1
		B at 0.3	1	2
		C at 1.0	2	2
		D at 0.43	2	2
		E at 0.6	2	2
		F at 1.0	2	2
9	1 mg/ml	A at 0.3	1	2
		B at 0.3	1	1
		C at 1.0	3	3
		D at 0.43	4	4
		E at 0.6	2	2
		F at 1.0	2	2
ABI			2	2

Table 3.3.3: *C.I. results for the gold conjugate/test line combinations outlined in Table 3.2.1, using a positive (50 IU/L hCG) urine sample.*

Conjugate E and F were eliminated from further study since strong false positives were a factor for these two conjugates with both test lines. No differentiation was

apparent between positive and negative samples for these combinations (all samples provided a C.I. result of 2).

Test Line I.D. No.	Test Line Conc. (mg/ml)	Conjugate & A_{520nm}	P-N Value at 5 min
8	1 mg/ml	A at 0.3	0
		B at 0.3	0
		C at 1.0	2
		D at 0.43	2
		E at 0.6	0
		F at 1.0	0
9	1 mg/ml	A at 0.3	0.5
		B at 0.3	0.5
		C at 1.0	3
		D at 0.43	4
		E at 0.6	1
		F at 1.0	0
ABI			2

Table 3.3.4: *Comparison of P-N values for the gold conjugate/test line combinations outlined in Table 3.3.1, using the negative and positive C.I. results from Tables 3.3.2 and 3.3.3*

Figure 3.3.1: *Comparison of C.I. values for six gold conjugates & two test lines using a hCG negative urine at 5 minutes*

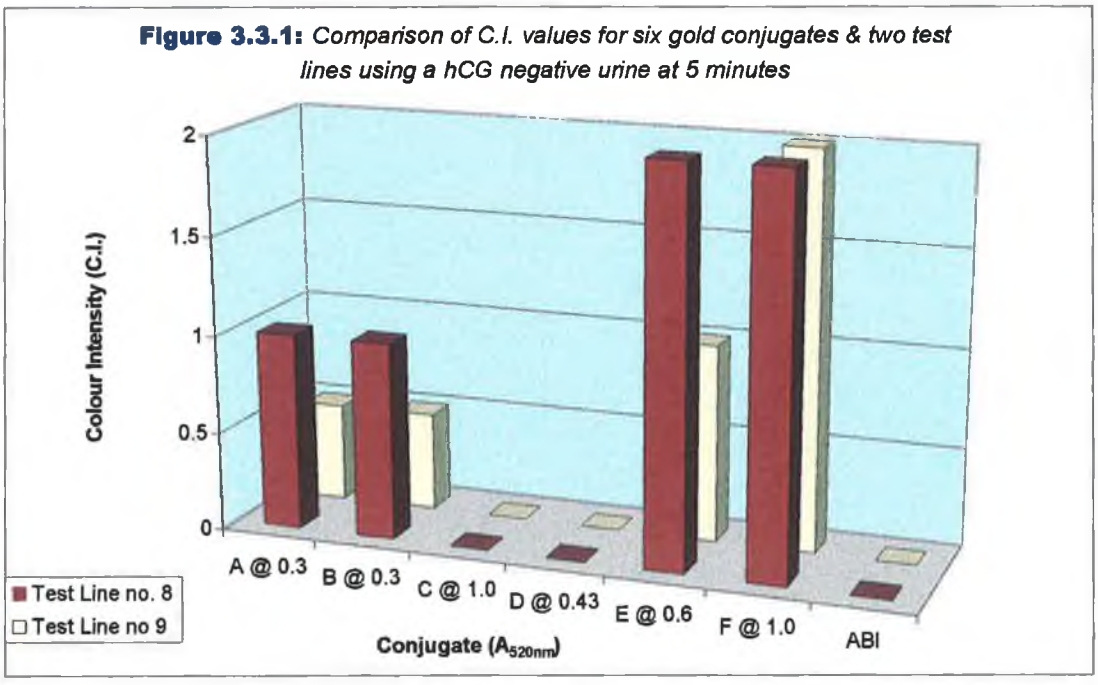
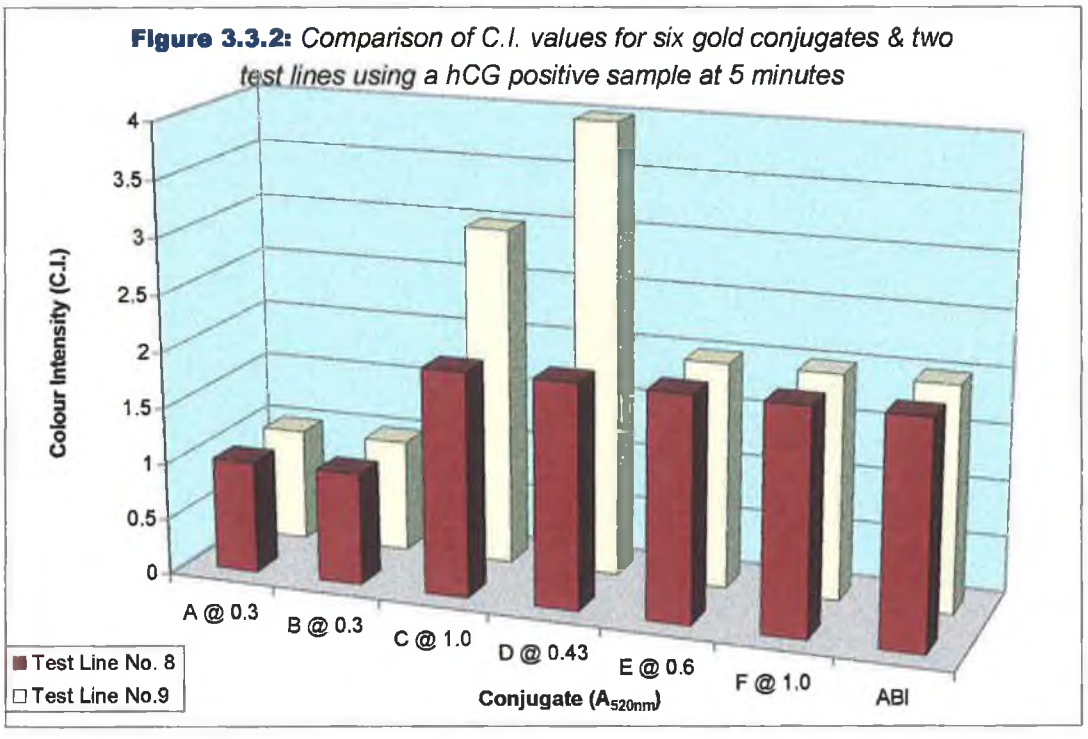


Figure 3.3.2: *Comparison of C.I. values for six gold conjugates & two test lines using a hCG positive sample at 5 minutes*



Based on these results the test line chosen for further evaluation was no. 9 as this test line gave stronger positive C.I. results for conjugate C and D compared to the ABI test. No. 9 also demonstrated cleaner negative results than no. 8, with those for conjugate C and D matching those for the ABI reference test.

The gold conjugates selected as the most applicable for further use within this assay were C and D, based upon the P-N values which pertained to their positive and negative C.I. results. Peaks for P-N values were seen with both conjugates C and D from Table 3.3.4. This result confirms their suitability as conjugates.

- **Conclusion**

Test line no.9 was chosen as the test line antibody at this point. Two monoclonal anti-β-hCG antibody-gold conjugates (C and D) were selected for further testing with the new test line based on the above results.

3.3.2 Direct Comparison of Conjugate C and Conjugate D

The performance of the two selected gold conjugates, C and D, were assessed with varying concentrations of the test line antibody (results not shown). Consequently, two test combinations were chosen:

1. Conjugate C at an A_{520nm} of 1.0 with the test line antibody at 1.0mg/mL.
2. Conjugate D at an A_{520nm} of 0.6 with the test line antibody at 2.0mg/mL.

Both of these combinations were compared using the same urine samples to determine the most suitable final format for the test. The results are presented in Figure 3.3.3 overleaf.

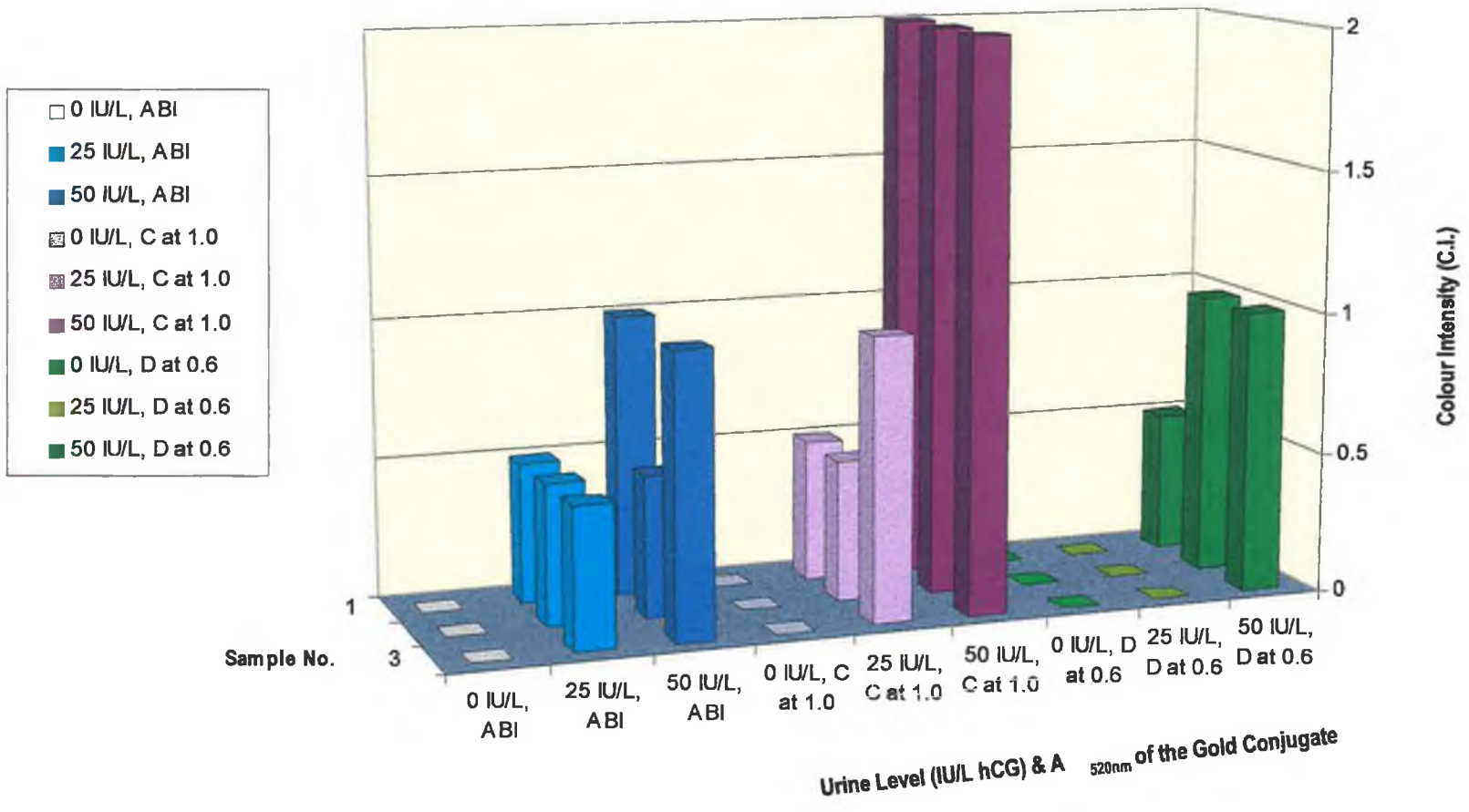
As observed from Figure 3.3.3 conjugate D did not perform as expected. None of the 25 IU/L samples were detected and the 50 IU/L samples resulted in quite low colour intensity. The C.I. results for the 50 IU/L samples were, however, of similar intensity as the ABI reference test. The conjugate C combination gave the most satisfactory results, giving better results than the ABI test, particularly for the 50 IU/L samples.

Conjugate D was re-evaluated at an A_{520nm} of 0.75, 1.0 and 1.5 with a test line antibody concentration of 2.0 mg/mL (results not shown). Even at the A_{520nm} of 1.5 the colour intensity of these positive samples was very faint. It appeared that the conjugate became unstable over time.

- **Conclusion**

Conjugate C at an A_{520nm} of 1.0, with the test line antibody at 1.0 mg/mL, was selected for use as the format for the in house assay at this stage in the development of the test.

Figure 3.3.3: *Comparison of C.I. values for Conjugate C & D vs the ABI test using 3 urines , each tested at 0IU/L hCG and spiked with 25 IU/L and 50 IU/L hCG.*



3.4 The discovery of specificity problems with female urines & methods to resolve this issue

3.4.1 Initial evaluation of in house test combination using male and female urine samples

The previous experimentation with gold conjugates had been carried out using male urine samples for evaluation. It was, however, noted at a later date that negative female urine samples gave a higher proportion of false positives than that observed for male urines. A summary of these results is shown below in Table 3.4.1. Since the assay was being developed for use as a rapid pregnancy test (i.e.: the projected market was 100% female) it was vital to re-examine the gold conjugate/test line antibody combination again using female urine samples.

	Samples Assayed	
	Male Urine	Female Urine
Total number of Samples	25	25
Number of negative results	25	12
Number of false positive results	0	13
% Specificity	100%	48%

Table 3.4.1: *Summary of evaluation of the in house test combination with male & female urine samples.*

Conjugate C was impregnated at the A_{520nm} readings outlined below and tested with membrane no. 2 sprayed at 1mg/mL with test line antibody no. 9. Each conjugate/test line antibody combination was re-evaluated with positive and negative male and female urines (obtained in-house), some of the results of which are shown overleaf in Table 3.4.2 and illustrated in Figure 3.4.1 - 3.4.3.

The problem of false positives with female samples was apparent from the results shown in Table 3.4.2 and Figures 3.4.1, 3.4.2 and 3.4.3. The results for the 'in-house'

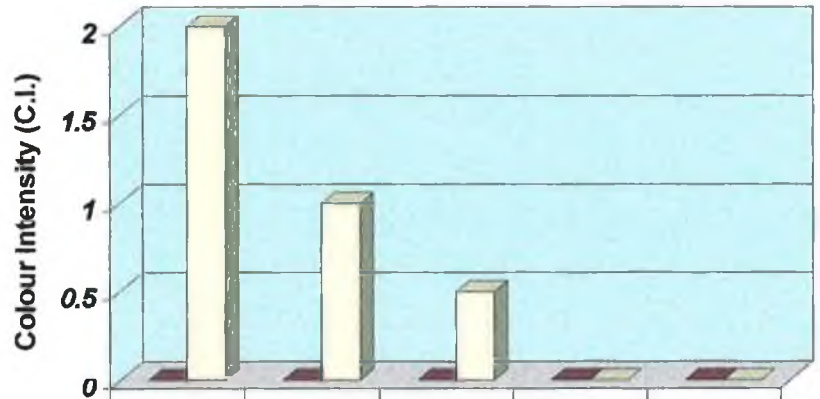
Conjugate A_{520nm}	C.I. Results at 5 minutes					
	Male Samples			Female Samples		
	Negative	Positive	P-N Value	Negative	Positive	P-N Value
C at 1.0	0	2	2	2	2	0
C at 0.6	0	2	2	1	1	0
C at 0.3	0	1	1	0.5	1	0.5
C at 0.15	0	1	1	0	0.5	0.5
ABI (Reference)	0	2	2	0	2	2

Table 3.4.2: *Comparison of C.I. results and P-N values with both male and female negative (0 IU/L) and positive (50 IU/L) using four dilutions of the same gold conjugate (c). Results read at 5 minutes.*

assay with female samples vary substantially from those detected when a male urine pool was used (see Table 3.4.2 and Figure 3.4.1 and 3.4.2). Figure 3.4.3 clearly demonstrates these different reactivities of conjugate C with male and female urines. The P-N value dropped considerably from 2 (with male samples) to 0 (with female samples) with an A_{520nm} of 1.0 for this conjugate. At an A_{520nm} of 1.0 conjugate C gave equal results for both the positive and negative female samples (C.I. of 2, see Table 3.4.2) and consequently a poor P-N value of 0. It was necessary to dilute out conjugate C to an A_{520nm} of 0.15 before the false positive was eliminated (C.I. of 0), however, the colour intensity of the positive result was also reduced to a C.I. of 0.5. This elimination of the false positive result provided an improved P-N value of 0.5 (see Figure 3.4.3).

While conjugate C at an A_{520nm} of 0.15 was the only combination to supply a negative C.I. result for the female negative sample, the C.I. results with positive female samples were too low to be deemed useful. The positive C.I. of 0.5 for the female positive sample compared poorly with that recorded for the ABI assay. The ABI test provided a C.I. of 2 for the same sample while maintaining a C.I. of 0 for the hCG-negative sample. It was noted from Figure 3.4.3, that the *best* P-N value obtained for the *female* samples was only half of the *worst* P-N value calculated for the *male* samples. It was, as a result, necessary to determine a method of eliminating the false positive result with negative female samples without jeopardising the positive C.I. result.

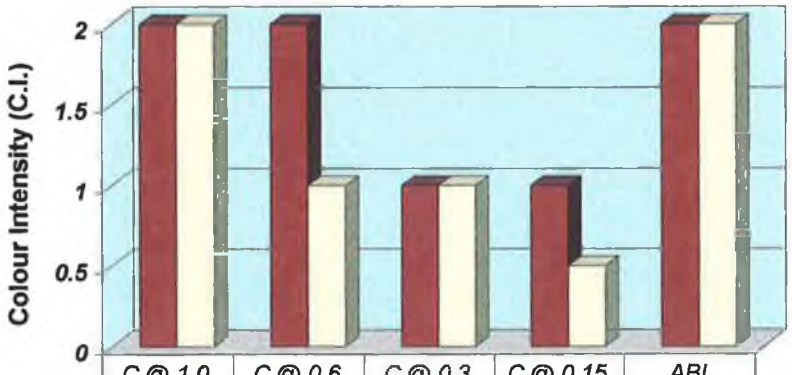
Figure 3.4.1: *Comparison of C.I. results at 5 minutes using male and female hCG-negative (0 IU/L) urine samples on tests with four dilutions of the gold conjugate C*



	C @ 1.0	C @ 0.6	C @ 0.3	C @ 0.15	ABI
Male Samples	0	0	0	0	0
Female Samples	2	1	0.5	0	0

Conjugate (A_{520nm})

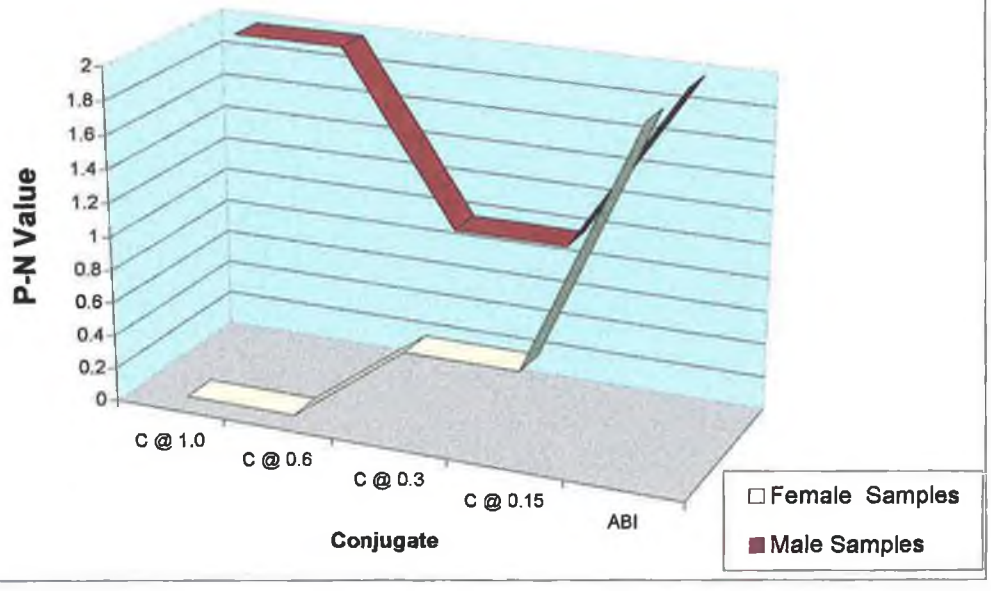
Figure 3.4.2.: *Comparison of C.I.results at 5 mins using male and female hCG-positive (50 IU/L) urines with tests manufactured with four dilutions of Conjugate C*



	C @ 1.0	C @ 0.6	C @ 0.3	C @ 0.15	ABI
Male Samples	2	2	1	1	2
Female Samples	2	1	1	0.5	2

Conjugate (A_{520nm})

Figure 3.4.3 : *Comparison of P-N Values for four gold conjugates using male and female test samples*



- **Conclusion**

It was concluded that, in an attempt to eliminate false positives with conjugate C, it was necessary to examine the use of various non-specific serums in the conjugate. It was hoped that the inclusion of such serums would absorb the non-specific component in the female urine, which was binding to the test line antibody (see Discussion, section 4.5.2).

3.4.2 Assessment of the inclusion of various serums in the gold conjugate strip to reduce non-specific results.

Outlined overleaf in Table 3.4.3 are five different serums and the relevant percentages to which they were supplemented into the gold conjugate solution. Each gold conjugate solution was manufactured using conjugate C at an A_{520nm} of 1.0 in GCDB (see Materials, section 2.1). A control conjugate strip was manufactured by impregnating conjugate C at the same A_{520nm} but without the inclusion of any non-specific serums. The test line antibody no. 9 was sprayed at 1mg/ml on membrane no. 2.

The test combinations were tested with both a negative and positive female urine sample. The results are outlined in Table 3.4.3 below and compared in Figure 3.4.4.

Conjugate/Serum	C.I. Results		P-N Value
	Negative (0 IU/L)	Positive (50 IU/L)	
C at 1.0 / No added serum	2	2	0
C at 1.0 / 15% (v/v) Goat Serum	1	2	1
C at 1.0 / 20% (v/v) Goat Serum	0	2	2
C at 1.0 / 5% (v/v) Human Serum	1	2	1
C at 1.0 / 10% (v/v) Human Serum	1	2	1
C at 1.0 / 15% (v/v) Rabbit Serum	1	2	1
C at 1.0 / 20% (v/v) Rabbit Serum	0.5	2	1.5
C at 1.0 / 5% (v/v) Mouse IgG	0.5	2	1.5
C at 1.0 / 10% (v/v) Mouse IgG	0	1	1
C at 1.0 / 15% (v/v) FBS	1	1	0
C at 1.0 / 20% (v/v) FBS	2	2	0

Table 3.4.3: *Evaluation of the addition of non-specific serums to gold conjugate C ~ Colour intensity results for a 0 IU/L and a 50 IU/L hCG female urine sample at an assay time of 5 minutes.*

The inclusion of all of the non-specific serums, with the exception of FBS, to the impregnation solution for Conjugate C (A_{520nm} 1.0) reduced non-specific binding when compared to the control strip. This is clearly demonstrated in Figure 3.4.4 and by the P-N values illustrated in Table 3.4.3.

The greatest improvement in the P-N value and in the reduction of the C.I. for the negative sample was noted for the strip supplemented with 20% (v/v) goat serum. This combination appeared to be the most effective in reducing the false positive result (from a C.I. of 2 to a C.I. of 0). This lowering of the negative C.I. was achieved without diminishing the positive C.I. result of 2 (see Figure 3.4.4). The P-N value for this combination was therefore seen to increase from 0 to 2 (see Table 3.4.3).

The performance of the 20% (v/v) goat serum combination was followed closely by that of the 20% (v/v) rabbit serum combination and the 5% (v/v) mouse IgG. Both of these combinations displayed only a very faint positive line (C.I. of 0.5) for the negative urine while maintaining the positive sample C.I. at 2 (see Figure 3.4.4). This allowed for a satisfactory P-N value of 1.5 (see Table 3.4.2). 10% (v/v) mouse IgG eliminated the false positive result, however, the C.I. for the 50 IU/L positive sample was reduced by 50% (v/v) from 2 down to 1. This decrease, thereby, negated any expected positive effect that the reduction in the negative sample C.I. may have had on the P-N value (see Table 3.4.2).

The supplementation of the gold conjugate strip with human serum reduced the false positive result seen for the negative sample by 50% (v/v) (i.e.: the C.I. dropped from 2 to 1). As for the other two serums the positive result remained the same, at a C.I. of 2. It is important to note, however, that the inclusion of 10% (v/v) human serum did not reduce the false positive result any further than that seen for the addition of 5% (v/v) human serum (i.e.: both displayed a negative sample C.I. of 1). Therefore, the incorporation of twice as much human serum in the 10% (v/v) human serum strip as was added to the 5% (v/v) strip, did not have any effect on the negative sample result. Since the addition of greater amounts of human serum did not produce the desired negative result (i.e.: a C.I. of 0) it was decided to reject the use of human serum as a 'blocker' in the gold conjugate strip for this assay.

Both percentages of FBS produced poor results, showing no differentiation between positive and negative samples. This is clearly illustrated in Table 3.4.2 since both percentage solutions yielded a P-N value of 0. For this reason FBS was also rejected for inclusion in the gold conjugate impregnation solution.

Despite their satisfactory results, rabbit serum and mouse IgG were excluded for use as blockers. Rabbit serum was removed from further use since the 20% (v/v) solution did not completely eliminate the specificity problem and it was not feasible to add a higher percentage of serum to the conjugate strip. Mouse IgG was eliminated due to the effect observed on the C.I of the positive sample. These results combined with the results

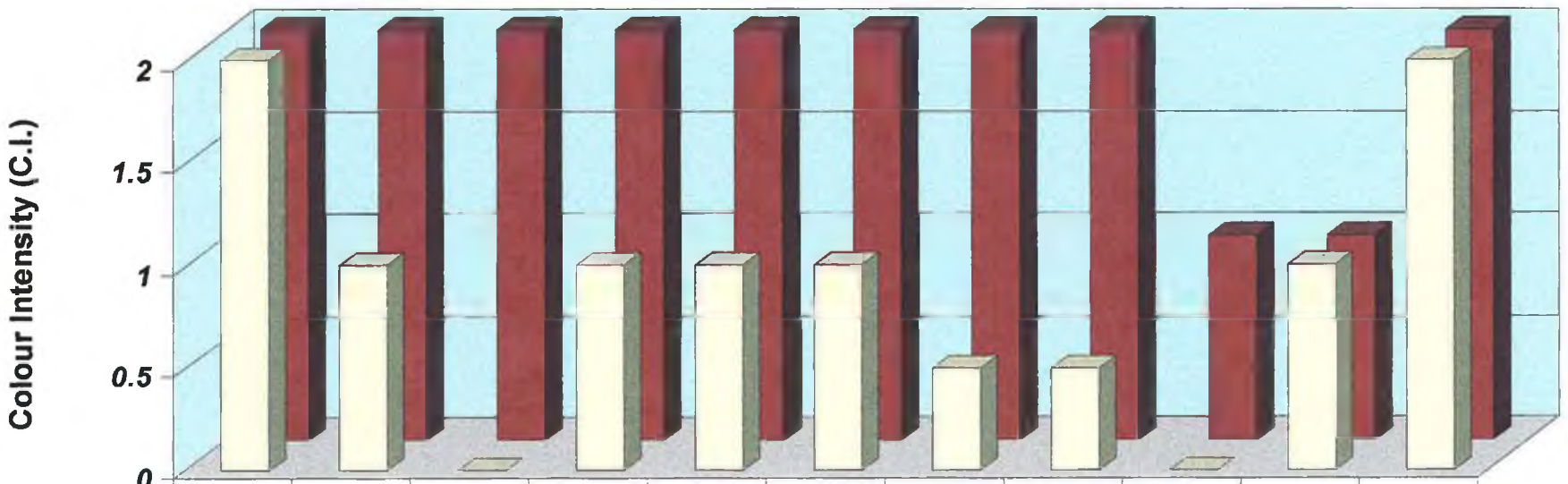
outlined on the previous page and the knowledge that purified mouse IgG would prove to be an expensive blocker at high percentages, ensured the selection of goat serum for addition to the gold conjugate solution to eliminate non-specific binding.

- **Conclusion**

A substantial difference was noted in the results achieved with male and female urine samples using the chosen format of the in house assay.

The inclusion of 20% (v/v) goat serum to the gold conjugate C, impregnation solution was found to reduce the non-specific binding seen with the use of female samples.

Figure 3.4.4 : Comparison of C.I. results for hCG-negative and hCG-positive urines using tests with five different sera included in the conjugate strip at concentrations ranging from 0-20% (v/v)



	No added serum	15% (v/v) Goat	20% (v/v) Goat	5% (v/v) Human	10% (v/v) Human	15% (v/v) Rabbit	20% (v/v) Rabbit	5% (v/v) Mouse IgG	10% (v/v) Mouse IgG	15% (v/v) FBS	20% (v/v) FBS
□ Negative (0 IU/L)	2	1	0	1	1	1	0.5	0.5	0	1	2
■ Positive (50 IU/L)	2	2	2	2	2	2	2	2	1	1	2

% (v/v) Serum

3.5 Selection of a suitable Control Line

As outlined in Section 1.11.3, Introduction, the control line is an intrinsic part of the rapid assay. The necessary properties for a suitable control line are described in section 4.5.1, Discussion. Three potential control lines were selected for comparison with the in house assay:

1. Rabbit anti-mouse IgG (RAM)
2. Goat anti-mouse IgG (GAM)
3. Goldline (GL)

Membrane no. 2 was sprayed with each of the above antibodies at the required control line position (see Method 2.2.2.2). The test line antibody (no. 9) was sprayed at the test line position (see Method 2.2.2.2) and gold conjugate C was used at an A_{520nm} of 1.0. Spraying concentrations for the test and control lines are outlined in Table 3.5.1 along with the corresponding results.

From the results shown in Table 3.5.1 it is apparent that the colour intensity for each of the control line combinations were affected by the strongly positive hCG sample. The intensity of the GL control line was almost diminished completely by this sample, giving a reading of only 1 at five minutes and a C.I. of 2 at the same assay time for the negative sample. The C.I. results for control line with the negative and low positive (50 IU/L) samples were far too low for this control line to be considered acceptable. The GL solution had been applied to the membrane undiluted, i.e.: the *maximum* amount of GL possible was available to bind any unbound gold conjugate in each test. Therefore, it would have been expected that this GL control line would have given stronger colour intensities than those observed. GL was thus eliminated from any further evaluation for use as a control line.

Both the RAM and GAM gave excellent control lines for the negative and 50 IU/L hCG positive samples, giving colour intensities of 10 with each sample. There was, however, a marked decrease in intensity when the strongly positive (100,000 IU/L) sample was run. For the RAM at 0.5 mg/mL an intensity of 4 was observed at 3 minutes, less than half the intensity observed for the negative and 50 IU/L hCG samples at the same assay time. The colour intensity increased to 6 at 3 minutes with

Control Line	Conc. of Control Line (mg/ml)	Sample (IU/L)	C.I. Result for Control Line	
			3 mins	5 mins
RAM	0.5	0	10	10
		50	10	10
		100,000	4	6
	1.0	0	10	10
		50	10	10
		100,000	6	6
	1.5	0	10	10
		50	10	10
		100,000	6	6
GAM	0.5	0	10	10
		50	10	10
		100,000	6	6
	1.0	0	10	10
		50	10	10
		100,000	8	8
	1.5	0	10	10
		50	10	10
		100,000	8	8
Goldline*	Undiluted	0	2	2
		50	2	2
		100,000	0	1

Table 3.5.1: *Comparison of three control lines, rabbit anti-mouse (RAM), goat anti-mouse (GAM) and Goldline*, at various concentrations using negative (0 IU/L hCG), positive (50 IU/L hCG) and strongly positive (100,000 IU/L hCG) urine samples. C.I. results at 3 and 5 minutes. *Reacts with the gold particle on the gold conjugate*

the 1.0 and 1.5mg/mL concentrations of RAM. These lines were quite distinct but were not as intense in colour as those observed with the GAM at concentrations of 1.0 and 1.5 mg/mL. These control lines both gave colour intensities of 8 when the strongly positive hCG sample was used, which was only slightly less intense than that noted for the same GAM concentrations using the negative and 50 IU/L hCG samples.

- **Conclusion**

Goat anti-mouse antibody at 1 mg/mL was selected for use as the control line for the in house assay format.

3.6 Format of the Prototype 'In-House' Assay following Initial Development Work

Outlined below in Table 3.6.1 is a summary of all the test components and the amounts of each of these that are utilised for the manufacture of the 'in-house' assay at this point in the development of the test. The aim of this section is to clarify, in one table, the format of the assay which was used for the first clinical trial (see following section, 3.7). Further on in this results section similar tables will be presented to exhibit the changes that occur in the test format as the development progresses (see sections 3.10 and 3.13).

Component	Component Details	Used at:
Test Line Antibody	*Antibody no. 9*	*1 mg/mL*
Control Line Antibody	*Goat Anti-Mouse Antibody*	*1 mg/mL*
Nitrocellulose Membrane	*Membrane no. 2*	*n/a*
Gold Conjugate Antibody	*Anti β-hCG Monoclonal (Conjugate C)*	*A_{520nm} 1.0*
Gold Conjugate Impregnation Buffer	*GCDB With Goat Serum*	*Goat Serum at 20% (v/v)*
Sample Pad	*Sample Pad B*	*n/a*
Sample Volume	*n/a*	*200μl (4 drops)*
Assay Time	*n/a*	*5 minutes*

Table 3.6.1: *Format of Prototype Assay: List of test components, relevant quantities and test parameters. Full details of components are found in Appendix I and Materials, section 2.1.*

3.7 First Internal Clinical Trial

The first clinical trial was carried out internally using 116 female urine samples from the Rotunda Hospital, Dublin. All samples were assayed using the ABI assay (reference test) and the 'in-house' assay. The 'in-house' hCG test format utilised during this trial is outlined in Section 3.6.

An analysis (see Method 2.2.5.5) of the results obtained is shown below in Table 3.7.1 and 3.7.2. The frequency of various C.I. results obtained with each test is plotted overleaf in Figure 3.7.1.

Test	'In-house' hCG assay			Total
		Positive	Negative	
Rotunda Hospital	Positive	95	1	96
	Negative	6	14	20
Total		101	15	116

Table 3.7.1: *Comparison of the 'in-house' assay versus the Rotunda Hospital results*

Test	ABI Assay			Total
		Positive	Negative	
Rotunda Hospital	Positive	95	1	96
	Negative	1	19	20
Total		96	20	116

Table 3.7.2: *Comparison of the ABI assay versus the Rotunda Hospital results*

The results obtained for the 'in-house' hCG assay were satisfactory in comparison to those observed for the ABI assay. As demonstrated in Table 3.7.1 the 'in-house' assay detected all but one of the positive samples which matched the results obtained with the ABI assay for the same samples (see Table 3.7.2) i.e.: both displayed a sensitivity of 98.9% (see Method 2.2.5.5). However, six of the twenty negative samples read as positive on the 'in-house' assay giving a specificity of only 70% (see Figure 3.7.1). This was in contrast to a specificity of 95% for the ABI test since only

one of these samples resulted in a false positive C.I. result. For some of the samples run, the 'in-house' assay demonstrated quite an uneven release of gold conjugate from the glass fibre pad. It was, therefore, deemed necessary to examine different methods of improving the release of the gold conjugate from the pad. It was also decided to examine other methods of optimising the assay parameters in an attempt to reduce the number of false positive results thereby improving the specificity of the assay.

Another issue, which was noted for the in-house assay was that 24 of the 116 tests run, gave faint control lines. It was concluded that further work was necessary to obtain a control line giving good colour intensities for strong positive samples.

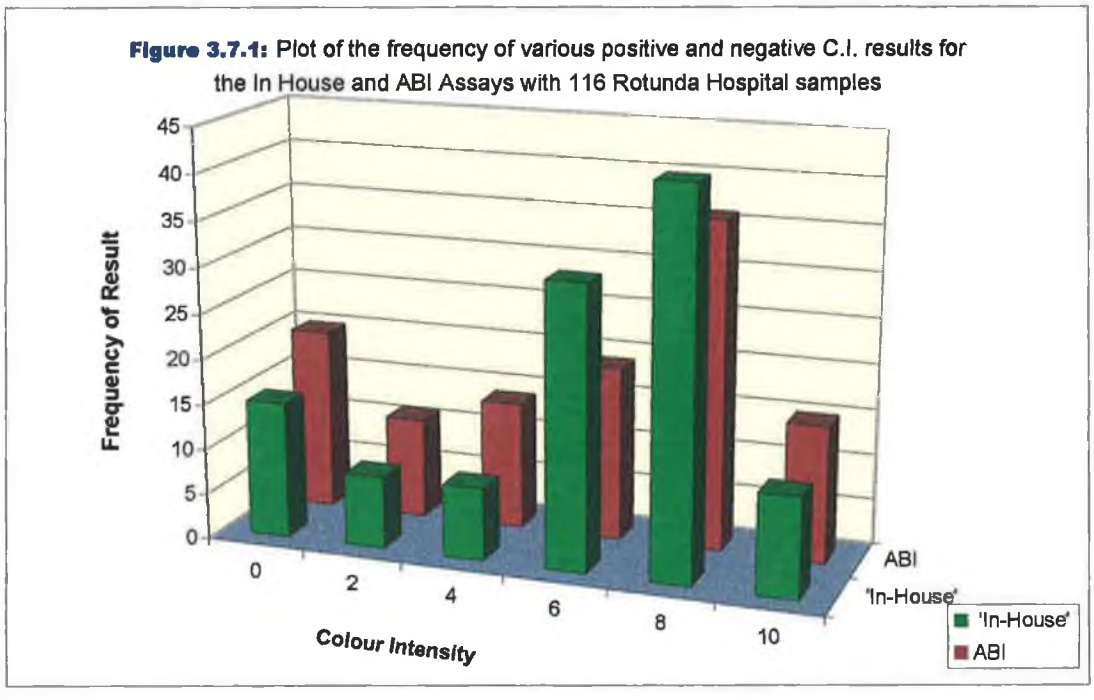


Figure 3.7.1: Plot of the frequency of various positive and negative C.I. results for the In House and ABI Assays with 116 Rotunda Hospital samples

- **Conclusion**

It was decided to evaluate the components of the Gold Conjugate Dilution Buffer (GCDB), which was used for the impregnation of the gold conjugate, in an attempt to improve the flow of gold conjugate from the glass fibre pad.

Based on the poor specificity of the 'in-house' assay it was also concluded that further optimisation was necessary to alleviate the problem of false positives which was observed for this trial and that the control line required further optimisation.

3.8 Optimisation of Prototype test based on first clinical trial results

3.8.1 Improvement of the Control Line

Following the observation of faint control lines for the in house assay during the clinical trial (Section 3.7) it was decided to supply excess gold conjugate within the assay to improve the intensity of the control line. This was carried out by adding a non-specific gold conjugate to the conjugate pad. The ideal non-specific gold conjugate was one which would bind only to the control line and would have little or no affinity for the test line antibody.

A commercially available anti-hepatitis B antibody (see Materials, section 2.0) was conjugated to 40nm gold. This gold conjugate was impregnated to give a solution of A_{520nm} 1.0 (i.e.: the same A_{520nm} as used for the anti-β-hCG gold conjugate in the 'in-house' assay). This conjugate was also impregnated at the A_{520nm} readings as outlined in Table 3.8.1 with conjugate C at an A_{520nm} of 1.0. An anti-βhCG gold conjugate strip was manufactured without any anti-hepatitis B gold conjugate for use as the negative control strip. Each of these gold conjugate strips were assembled using membrane sprayed with GAM IgG control line and the test line antibody no. 9 at 1 mg/mL. The results are shown in Table 3.8.1.

It is clear from Table 3.8.1 that the addition of a non-specific gold conjugate to the conjugate strip improved the intensity of the control line, especially when a strongly positive sample was used. The test strip devoid of the non-specific gold conjugate gave a colour intensity of only 6 at three minutes assay time for the 100,000 IU/L sample.

As can be seen from the results this colour intensity was improved upon for all of the conjugate strips containing the anti-Hepatitis B gold conjugate up to an A_{520nm} of 0.075. The conjugate strips that possessed the anti-Hepatitis B gold conjugate at an A_{520nm} of 0.15 and 0.1 each gave a colour intensity of 10 for each of the samples run. The strips manufactured at an A_{520nm} of 0.08 and 0.06 gave lower C.I. results for the

100,000 IU/L sample. Since a C.I. result of 10 was the maximum colour intensity attainable, according to the colour interpretation chart (see section 3.1), the anti-Hepatitis B gold conjugate at A_{520nm} 0.1 was selected for inclusion into the gold conjugate impregnation solution. This A_{520nm} was also chosen for cost reasons, as less anti-Hepatitis B gold conjugate would be required at this dilution.

Conjugate Strip (A_{520nm})	Sample (IU/L)	C.I. Result for Control Line	
		3 mins	5 mins
No anti-HepB Au & anti-hCG Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*6*	*8*
No anti-hCG Au & anti-HepB Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*10*	*10*
Anti-HepB Au at 0.15 & anti-hCG Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*10*	*10*
Anti-HepB Au at 0.1 & anti-hCG Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*10*	*10*
Anti-HepB Au at 0.08 & anti-hCG Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*8*	*10*
Anti-HepB Au at 0.06 & anti-hCG Au at 1.0	*0*	*10*	*10*
	50	*10*	*10*
	100,000	*6*	*8*

Table 3.8.1: *Comparison of the control line (CL) colour intensity for gold conjugate strips with varying levels of a non-specific gold conjugate. Colour intensity results for the CL with 0, 50 & 100,000 IU/L hCG urines at 3 and 5 mins.*

It should be noted that the conjugate strip which only contained anti-Hepatitis B gold conjugate did not interfere with the test line for any of the samples, i.e.: a colour intensity of 0 was obtained for each sample (results not shown). This indicated that the anti-Hepatitis B gold conjugate did not have an affinity for hCG and would, therefore, not interfere in the assay.

- **Conclusion**

Anti-hepatitis B gold conjugate was selected for inclusion in the conjugate strip at an A_{520nm} of 0.1.

3.8.2 Determination of the affinity of various Anti-hCG antibodies for the hCG antigen and confirmation that an Anti-LH antibody has no affinity for hCG, by the employment of a Dot Blot Method.

It was decided to examine the affinity of the antibodies used in the current format of the 'in-house' assay for the hCG antigen as a consequence of the poor specificity of the assay as observed during the first clinical trial. The following antibodies were evaluated using the dot blot method (see method 2.3.5.6) to determine their affinity, if any, for the hCG antigen: test line antibodies nos. 1, 8 and 9 and gold conjugate antibody C (see Appendix I for further details). An anti-LH antibody was also examined for its affinity for the hCG antigen as it was planned (see section 3.8.3) to determine whether its inclusion in the assay would have an effect on the false positive results seen during the first clinical trial (see section 3.7). Each antibody was incubated with membrane, which had been 'dotted' with hCG concentrations ranging from 5-100 IU/dot (see Method 2.3.5.6). Two negative control blots were performed in combination with the antibodies being examined for affinity. These negative control blots used the goat anti-rabbit –HRP and rabbit anti-mouse-HRP conjugates, which were used to detect the other antibodies. The results are shown overleaf in Figure 3.8.1.

From the results shown in Figure 3.8.1 it is evident that the antibody no. 9 possessed the highest affinity for the hCG antigen at all levels of hCG. The dots for this antibody were several times more intense in colour than those observed for any of the other antibodies tested. From their dot blots it could be seen that antibodies no. 8, no. 1 and C all have similar affinities for the hCG antigen but none as strong as that observed for antibody no. 9. The colour intensities for the blots of these antibodies were much fainter than those achieved with antibody no. 9, but were similar in intensity to one another. From this it can be concluded that the test line antibody used for the clinical trial was indeed the most suitable, as it possesses the highest affinity for the antigen being detected.

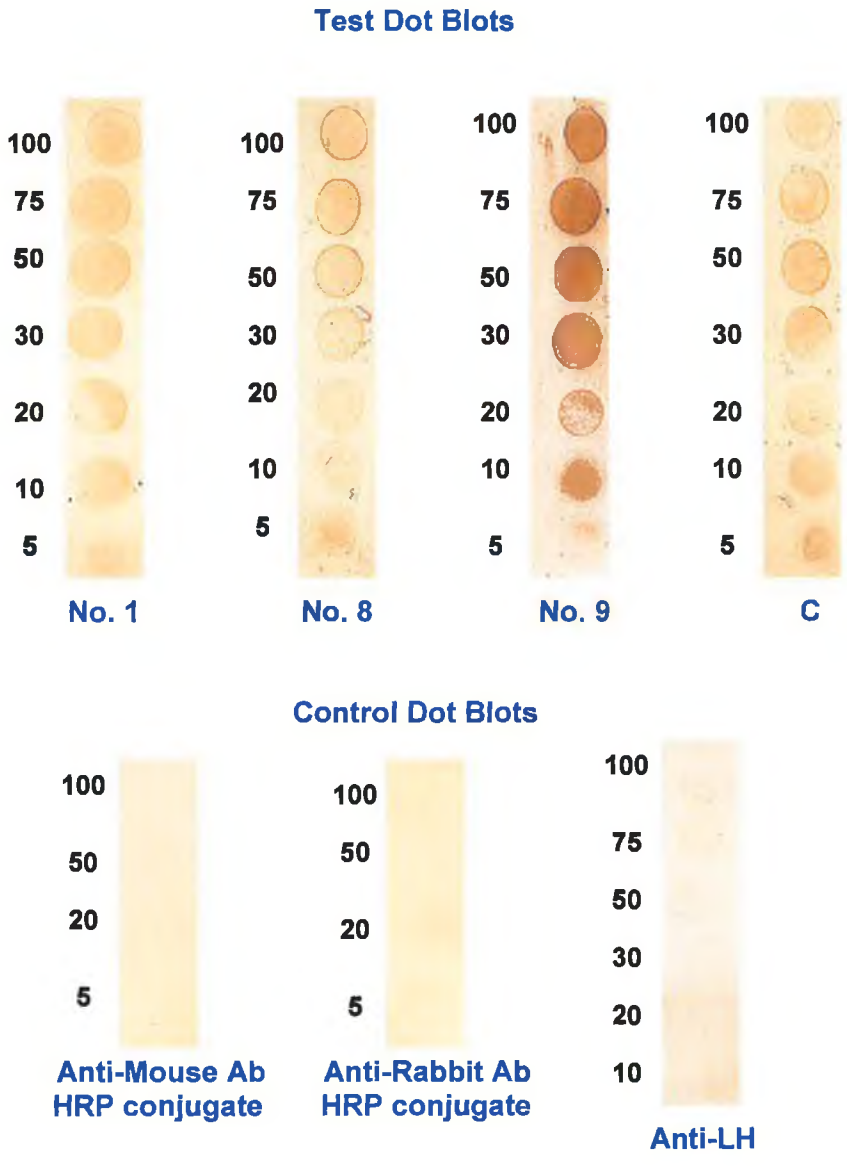


Figure 3.8.1: *Dot blot results demonstrating the affinities of the antibodies listed on page 106, for the hCG antigen at levels varying from 100 IU hCG per dot to 5 IU hCG per dot (shown by the numbers to the left of the blots). The blue writing beneath each blot indicates the antibody, which was incubated with that particular blot. All antibodies were examined at a concentration of 5μg/mL using HRP conjugates and probed with a DAB substrate.*

Based on these results the spraying concentration of the test line antibody (no. 9) was re-evaluated, as was the A_{520nm} of the gold conjugate C, both of which had been selected for use in the 'in-house' assay format during previous studies. The re-evaluations demonstrated that the chosen test line concentration of 1 mg/mL and the A_{520nm} of 1.0 for the gold conjugate provided the most acceptable, sensitive and specific results (results not shown).

Minimal cross-reactivity was detected for the anti-LH, anti-mouse-HRP and anti-rabbit-HRP antibodies. It can be concluded, therefore, that the colour intensity seen for antibodies no. 9, no. 8, no. 1 and C was due to these antibodies only binding to the hCG on the membrane and not through non-specific binding of the conjugates. It was also decided that the anti-LH could be used in the assay without interfering in the binding of hCG to the test line (see section 3.8.3 and Discussion, section 4.5.2).

- **Conclusion**

Antibody no. 9 was proven to be the most suitable test line antibody and was thus retained for use as the test line. The anti-LH antibody was shown to have minimal interaction with the hCG antigen and was deemed suitable for inclusion in the assay.

3.8.3 Examination of the inclusion of an Anti-Luteinising Hormone (LH) line below the test line

Also as a result of the disappointing % specificity noted during the first clinical trial it was postulated that LH may have been binding to the test line (see Discussion, section 4.5.2). An anti-LH antibody was received from Genzyme and was sprayed at a concentration of 1mg/mL 3mm beneath the test line with the intention that this antibody could bind any LH in the urine, thereby reducing the specificity problem. Negative urine, which resulted in a false positive C.I. in the clinical trial, was assayed, along with a 50 IU/L and 100,000 IU/L-positive urine sample on strips assembled using the membrane sprayed with and without the anti-LH line. The results were read at 3, 5, 10 and 15 minutes and are shown in Table 3.8.2.

The results obtained from this study were not as expected, as both the specificity and sensitivity worsened with the inclusion of an anti-LH line below the test line (see Discussion, section 4.5.2). Without the anti-LH line, only faint colour was beginning to appear at 10 minutes for the negative urine. On the other hand, with the anti-LH line colour appeared by 3 minutes for the same sample, which increased to a C.I. of 2 by 15 minutes. It is interesting to note that for the tests sprayed with the anti-LH line the 50 IU/L and 100,000 IU/L samples displayed lower colour intensities than that of the tests devoid of an anti-LH line.

Test	Sample (IU/L)	Anti-hCG Line				Anti-LH Line			
		3 min	5 min	10 min	15 min	3 min	5 min	10 min	15 min
Without LH Line	*0*	*0*	*0*	*0.5*	*0.5*	*n/a*	*n/a*	*n/a*	*n/a*
	50	*0*	*2*	*4*	*4*	*n/a*	*n/a*	*n/a*	*n/a*
	100,000	*2*	*2*	*4*	*4*	*n/a*	*n/a*	*n/a*	*n/a*
With LH Line	*0*	*0.5*	*0.5*	*1*	*2*	*0*	*4*	*6*	*6*
	50	*0*	*1*	*2*	*2*	*4*	*6*	*6*	*6*
	100,000	*1*	*1*	*2*	*2*	*6*	*6*	*6*	*8*

Table 3.8.2: *Colour intensity results for the test line (anti-hCG) and where applicable, the anti-LH line for tests manufactured with and without an Anti-LH line sprayed below the test line. Colour intensity results are shown for a negative (0 IU/L), a positive (50 IU/L) and a strongly positive (100,000 IU/L) hCG urine.*

Quite intense colour was seen for the anti-LH line on tests which possessed such a line (see Table 3.8.2 above, C.I. results ranging from 4 – 8). Reasons as to why this line was visible and as to why the colour intensity increased for the negative sample and decreased for the positive samples are discussed section 4.5.2, Discussion.

- **Conclusion**

It was decided not to carry out further studies using the anti-LH line due to the worsening of sensitivity and specificity levels as a result of the presence of this line below the anti-hCG test line. It was concluded that further work would be necessary examining various agents capable of blocking non-specific binding (i.e.: 'blockers') in an attempt to alleviate the false positive problem.

3.8.4 Evaluation of Gold Conjugate Release Pads

The importance of the characteristics of the gold conjugate release material are outlined on section 4.5.3, Discussion. In this study it was decided to examine six different release pads for their release qualities and their volume capacity. These were the release pad, which had been in use up until this point (no. 1) and five

release pads of various grades supplied by Millipore (pads 2-6) (see Materials, section 2.1 and Appendix I).

An 8mm x 30cm strip was cut from each of the release materials. Each strip was impregnated with conjugate C at an A_{520nm} of 1.0 and the volume of gold absorbed was recorded in Table 3.8.3 below for each pad. The strips were dried and assembled into test devices, keeping all other components common. The results are outlined in Table 3.8.4.

Pad ID no.	Volume Absorbed (mL)	Ease of Absorption
1	1.35	Quick Absorption
2	0.35	Uneven, patchy
3	0.40	Very slow
4	0.50	Poor, hydrophobic
5	0.40	Good
6	0.45	Poor

Table 3.8.3: *Comparison of various release pads nos. 1-6 (see Appendix I and Materials, section 2.1) for the gold conjugate strip: volume of gold conjugate absorbed and absorption characteristics for each release pad are recorded.*

Pad I.D no.	Negative Sample		Positive Sample	
	Test Line	Control Line	Test Line	Control Line
1	0	8	4	8
2	2	4	4	4
3	2	4	4	4
4	2	4	4	4
5	2	4	4	4
6	4	4	4	4

Table 3.8.4: *Comparison of release pads nos. 1-6 (see Appendix I and Materials, section 2.1) for the gold conjugate strip: Colour intensity results for the test and control lines using a positive (50 IU/L hCG) and negative (0 IU/L hCG) urine sample.*

The results shown in Table 3.8.3 and Table 3.8.4 illustrated that pad no. 1 possessed the most satisfactory characteristics required for a gold release pad. Good absorption, release and good positive/negative discrimination were properties of this pad. The remaining pads demonstrated inconsistent absorption (see Table 3.8.3) and poor

release of the gold conjugate, which resulted in poor specificity for negative samples (see Table 3.8.4).

- **Conclusion**

Pad no. 1 was confirmed as the most suitable gold conjugate release material due to its satisfactory absorption traits, sensitivity and specificity. Pads no. 2-6 were disregarded based on the results outlined above.

3.8.5 Buffer Evaluations

Polyethylene glycol (PEG) (size range 0.2-20.0K), Triton® X-100 and Tween® 20 were added to the Gold Conjugate Dilution Buffer (GCDB) (see Materials, section 2.1) in the combinations listed overleaf in Table 3.8.5. The purpose of the addition of these reagents was to examine their effect on the reactivity, release and flow of the anti-hCG gold conjugate from the vacuum-dried glass fibre strip. The reasons why these particular components were selected for inclusion in the GCDB are outlined in the Discussion, section 4.5.3. Initially, due to the large number of buffers (i.e.: 16, see Table 3.8.5) these 16 buffers were examined in liquid form, i.e. the gold conjugate and sample were added to the buffer and the test strip was 'dipped' into the solution. The effect if any, of the reagent on the test result was thereby monitored and the selected buffers were examined in greater detail (see 3.8.5i and 3.8.5ii).

The Triton® X-100, Tween® 20 and PEG's were added to the relevant buffers (See Table 3.8.5) at the concentrations outlined in Method 2.2.5.7 (i.e.: 0.001% (w/v) for Triton® X-100 and Tween® 20 and 5mg/mL for each of the PEG's). All of the above buffers were evaluated by employing the ELISA plate 'Comb' Method (see Method 2.3.5.8). Each buffer was assessed using both a negative and positive (25 IU/L) urine sample. The intensity of both the control and test lines were recorded along with the flow rates for the samples. The results are recorded in Table 3.8.6.

It was observed from this study that the addition of PEG (15-20K) in combination with either of the detergents (Tween® 20 or Triton® X-100) exhibited the best test and control lines. Both of these buffers (no. 15 and no. 16 overleaf) gave control

lines of a colour intensity of 10 and test lines of colour intensity 0.5 for the positive urine sample. Buffers no. 1-no. 4, which did not contain any detergent but contained the smaller sized PEG's (0.2 – 8K) demonstrated faint control lines of colour intensity ranging from 2 to 4. This was due to their faster flow rates, the average flow rate being 1 minute 7 seconds. An uneven release of gold was also observed for these four buffers and significant amounts of gold conjugate remained at the bottom of these strips. The amount of residual gold, which remained at the bottom of the strip decreased with the addition of Tween® 20 (buffers no. 10-no. 13). It was, therefore, concluded that the addition of detergent assisted the even flow and release of the gold conjugate up the strip.

Identification no.	Buffer
1	GCDB with PEG 0.20 K
2	GCDB with PEG 1.0 K
3	GCDB with PEG 3.35 K
4	GCDB with PEG 8.0 K
5	GCDB with PEG 10.0 K
6	GCDB with PEG 15.0 – 20.0 K
7	GCDB with Triton® X-100
8	GCDB with Tween® 20
9	GCDB no additives
10	GCDB with PEG 0.2 K and Tween® 20
11	GCDB with PEG 1.0 K and Tween® 20
12	GCDB with PEG 3.35 K and Tween® 20
13	GCDB with PEG 8.0 K and Tween® 20
14	GCDB with PEG 10.0 K and Tween® 20
15	GCDB with PEG 15.0 – 20.0 K and Tween® 20
16	GCDB with PEG 15.0 – 20.0 K and Triton® X-100

Table 3.8.5: *Description of sixteen gold conjugate buffers examined for their effect on the gold conjugate. The amount of each component added to the GCDB is given in Method 2.3.5.7, section 2.2.*

No false positive result was observed for any of the 16 buffers. It was also noted that the GCDB without additives (buffer no. 9) had high background colour and did not detect the 25 IU/L hCG-containing urine sample.

Fast flow rates were also seen for buffers no. 9-no. 14. It was, therefore, deduced that the best test and control lines were only observed with the buffers, which were

supplemented with PEG of sizes greater than 10K and with either Tween® 20 and Triton® X-100.

- **Conclusion**

It was decided to further examine the effects on the assay of the use of the gold conjugate dilution buffer (GCDB) supplemented with PEG (15–20K), Tween® 20 and Triton® X-100.

Buffer	Negative Urine				Positive Urine			
	C.I. Result		Flow Time	Comments	C.I. Result		Flow Time	Comments
	TL	CL	(min,sec)		TL	CL	(min,sec)	
1	0	2	1,02	Flow too fast &	0	2	1,05	Uneven flow of
2	0	2	1,07	poor flow of Au.	0	2	1,01	gold. Residual
3	0	4	1,09	Residual Au at bottom	0	2	1,10	Au at bottom of strip.
4	0	4	1,12	"	0	4	1,06	"
5	0	8	1,30	Good flow	0.5	8	1,27	Good flow
6	0	8	1,36	Good flow	0.5	8	1,32	Good flow
7	0	8	1,06	Flow too fast	0	8	1,09	Flow too fast
8	0	8	1,39	Good flow	0.5	8	1,26	Good flow
9	0	6	1,10	High Background	0	4	1,13	Flow too fast
10	0	6	1,20	Uniform Flow, some	0	4	1,23	Even flow,some
11	0	4	1,23	residual Au left.	0.5	6	1,21	residual Au observed.
12	0	6	1,25	"	0	6	1,19	"
13	0	8	1,26	"	0	8	1,27	"
14	0	8	1,29	"	0	8	1,26	"
15	0	10	1,41	Cleanest	0	10	1,40	Best test & control
16	0	10	1,38	backgrounds.	0	10	1,39	lines. Good flow

Table 3.8.6: *The comparison of sixteen different gold conjugate buffers. Colour intensity results for the test line (TL) and control line (CL) and flow rates for each buffer using a negative (0 IU/L hCG) and positive (50 IU/L) urine sample.*

3.8.5 i Further Buffer Evaluation

From the results of the previous study carried out in relation to buffers (see section 3.8.5) it was decided to further examine the effect of Triton® X-100 and Tween® 20 on the assay. It was also postulated that the addition of BSA and sucrose should confer stability on the gold conjugate in its dried state and thus might possibly benefit the GCDB. This topic is discussed further in section 4.5.3 of the Discussion.

Thus, BSA and sucrose were also examined along with PEG 15–20K and PVP K-30 to determine their effect on the test and control lines.

Each of the components listed below was added to a Tris-based buffer (see Method 2.3.5.7) at a range of concentrations, with the higher concentrations providing an excess of the component, thereby forcing an effect on the assay. The GCDB was used as the reference diluent. Each buffer, including the GCDB was spiked to a level of 50 IU/L hCG to give a hCG positive solution. Each unspiked buffer was used as a negative sample. 200μL (4 drops) of each buffer were added per test unless stated otherwise. All buffers were run on the same test combination. Each test was examined for C.I. result, flow rate, overall visual impression as to how the test ran (e.g.: release of gold conjugate, intensity of control lines) and buffer front. The buffer front is defined as the distance observed between the 'top' of the liquid sample flow and the 'top' of the gold conjugate flow as both components travel up the sprayed membrane (see section 4.5.3, Discussion). The ideal buffer front is one where both the sample and gold conjugate flow together thereby allowing an optimum of conjugate binding to the antigen. It is important to note that the chemicals and biologicals contained within the GCDB play a significant role in determining the size of the fronts for this type of assay (see Discussion, section 4.5.3).

The results are shown in Table 3.8.7 (pg 118) and Table 3.8.8 (pg 119). These results are further examined in section 4.5.3, Discussion, the conclusions drawn from the results shown in tables 3.8.7 and 3.8.8 are presented below:

The inclusion of Triton® X-100 in the Tris-based buffer improved the release of gold conjugate from the pad. The buffer front improved with increasing concentrations of Triton® X-100. However, the flow was too slow at the higher concentrations of Triton® X-100, which resulted in weak control lines. Positive results were observed (C.I. of 0.5) for the hCG-positive solution on those strips, which were run with Triton® X-100 at concentrations higher than 2.4 g/L. It was decided to include Triton® X-100 at a concentration of 2.4 g/L in the current GCDB since this concentration gave a positive result for the positive sample, provided good buffer fronts and the flow was not as slow as that seen for the higher concentrations.

Tween® 20 produced similar results to Triton® X-100. The release of the gold conjugate from the glass fibre pad improved with the addition of increasing amounts of Tween® 20 to the Tris-based buffer. Even flow was noted for the strips run with Tween® 20-containing buffers, which further improved with the higher concentrations of Tween® 20. These higher concentrations in turn slowed the flow from the glass fibre pad. The intensity of the control line was proportional to the concentration of Tween® 20 in the Tris-based buffer run on the strip. It was, therefore, concluded that the inclusion of Tween® 20 at 5 g/l would provide the best release of gold conjugate from the pad with good fronts and acceptable flow rates (slower flow rates were observed for 10 g/L and higher concentrations). Since no positive result was noted with the Tween® 20 buffers it was anticipated that the addition of other components from this study would allow the detection of the hCG. This assumption was based on the previous buffer study (see section 3.8.5) in which C.I.'s of 0.5 were observed for the positive solution of buffers containing Tween® 20.

With regard to PVP K-30, false positive results were noted for all of the negative solutions. However, it was hoped that, in its dried state in the impregnated gold conjugate strip, the PVP K-30 would not react with the test line to such an extent. This was assumed since the function of the PVP K-30 is to form a protective coating around the gold conjugate as the liquid is evaporated off the glass fibre strip during the drying procedure. In liquid form, as in this case, the PVP K-30 is not forced to form such a coating due to the abundance of water molecules surrounding the gold conjugate. Thus, the PVP K-30 is free in solution to bind non-specifically to the test line of the membrane strip.

The gold conjugate front was very good for all concentrations of PVP K-30 (i.e.: there was minimal distance between the top of the liquid sample flow and the gold conjugate flow. Flow rates were also good and similar for each buffer, i.e.: no effect was observed on flow rate with increasing levels of PVP K-30. PVP K-30 appeared to play a more significant role in the release of gold conjugate from the glass fibre strip since it was apparent that the addition of greater amounts of PVP K-30 allowed for cleaner background with more distinct test lines. However, this phenomenon may have partly been as a result of the interaction of the PVP K-30 with the test line and

gold conjugate as viewed for the negative solution which demonstrated false positive results at all concentrations of PVP K-30. As a consequence of the occurrence of this non-specific interaction with the test antibodies it was decided that the PVP K-30 should be added to the GCDB at the lowest concentration examined in this study, i.e.: 1.5 g/L. It was unnecessary to include the PVP K-30 at a higher concentration because, as noted earlier from the results, the most important effect of the addition of this component was in providing a good release of the conjugate. As all of the concentrations of PVP K-30 demonstrated a similar gold conjugate release, there was no apparent benefit in adding more than 1.5g/L PVP K-30 to the GCDB.

In examining the results for the Tris-based buffers supplemented with PEG 15-20K it was clear that the concentration of PEG 15-20K was inversely proportional to the width of the gold conjugate front observed, i.e.: the gold front was seen to improve as higher concentrations of PEG 15-20k were added. False positive results were also observed for the negative solutions containing PEG 15-20K. The flow rate decreased with higher concentrations of PEG 15-20K and no effect was apparent on the intensity of the control line. Therefore, it was concluded that the PEG 15-20K served only to decrease the gold conjugate front and had no bearing on the release of gold. Due to the false positive results noted for concentrations higher than 2.5g/L and because the flow rate of the test was too slow at the same concentrations of PEG 15-20K, 1.25 g/L of PEG 15-20K was chosen as the most suitable concentration for addition to the GCDB. The fronts observed at this concentration were as good as those seen for higher concentrations but without the slower flow rates and higher false positive C.I. results.

Poor fronts and fast flow featured for the strips run with buffers containing various levels of sucrose. Since the sucrose was included in the buffer as a stabilising agent in the dried gold conjugate strip and not to improve flow or release of gold conjugate only the positive /negative result was important in this case. No false positives were apparent and a concentration of 12.5 g/L of sucrose was selected because high concentrations would be required to confer stability in the dried state. A higher concentration was not selected because the flow slowed considerably as the amount of sucrose increased. More significantly, as a gold conjugate strip was dried the

removal of liquid from the strip would, as a result, cause an increase in the concentration of the chemicals present in the strip.

No false positives were detected for the BSA containing buffers and the gold conjugate front was not affected by increasing amounts of BSA. The release of gold conjugate from the pad was, nevertheless reduced with the addition of high concentrations of BSA. This poor release could be assumed to be a consequence of the very slow flow rate noted with these buffers. Due to the diminished release of gold conjugate and slow flow rates, the addition of 2 g/L of BSA to the GCDB was deemed the most appropriate concentration for this constituent.

- **Conclusion**

The following components were selected for inclusion in the GCDB formulation at the concentrations specified below in Table 3.8.9:

Component	Concentration
Triton® X-100	*2.4 g/L*
Tween® 20	*5.0 g/L*
PVP K-30	*1.5 g/L*
PEG 15-20K	*1.25 g/L*
Sucrose	*12.5 g/L*
BSA	*2.0 g/L*

Table 3.8.9: *Concentrations of 6 components for addition to the gold conjugate dilution buffer (GCDB)*

Component	Conc. (g/L)	hCG Status	C.I. Result		Buffer front	Flow	Visual
			3 min	5 min			
Triton® X-100	0.3	Negative	0	0	Fronts quite	Had to add 2	Good release of
	0.6		0	0	pronounced	drops extra to	gold, improves
	1.2		0	0		release gold	with increasing
	2.4		0	0	Good fronts	from pad for	Triton X-100®
	6		0	0	"	concns of	
	12		0	0	"	2.4 g/L and	
GCDB			0	0	"	upwards	
Triton® X-100	0.3	Positive	0	0	Good control	Very slow	
	0.6		0	0	lines.	flow. Extra	Release of gold
	1.2		0	0	"	drops of sample	increases with
	2.4		0	0.5	Faint control	required above	increasing
	6		0	0.5	lines due to	2.4 g/L.	Triton X-100®
	12		0	0.5	slower flow		
GCDB			0	0.5			
Tween® 20	1.25	Negative	0	0	Good fronts	Good flow but	Good release of
	2.5		0	0	observed	slightly slower	gold. C.I of
	5		0	0	for all	than GCDB.	control line higher
	10		0	0	combinations.	The flow for	with more T-20®.
	25		0	0		50g/L was too	25 & 50g/L had
	50		0	0		slow.	background
GCDB			0	0			problems.
Tween® 20	1.25	Positive	0	0	Good fronts.	Good Flow	
	2.5		0	0	Fronts	"	Increasing
	5		0	0	decreased	"	Tween®
	10		0	0	with higher	"	increases release
	25		0	0	levels of	Slow Flow	of gold from pad.
	50		0	0.5	Tween 20®	Slow Flow	
GCDB			0	0			
PVP K-30	**1.5**	Negative	0.5	0.5	good front	Similar to	False
	3		0.5	0.5	"	GCDB	positives
	6		2	2	"	apart from	appeared
	12		2	2	"	60g/L	on high conc.
	30		2	4	"		buffers
	60		2	4	"		immediately
GCDB			0	0			
PVP K-30	**1.5**	Positive	0.5	1	v.good front	Similar to	Background
	3		0.5	1	"	GCDB	clears up as
	6		1	1	"	apart from	more PVP is
	12		2	2	"	60g/L.	added. Test lines
	30		2	2	"		more distinct as
	60		2	2	"		PVP increases.
GCDB			0.5	0.5			

Table 3.8.7: *Examination of the inclusion of two different detergents (Triton® X-100 and Tween® 20) and PVP K-30 in the GCDB. Best results are highlighted in red.*

Component	Conc. (g/L)	hCG Status	C.I. Result		Buffer front	Flow	Visual
			3 min	5 min			
PEG 15-20K	1.25	Negative	0	0.5	Front		Strong false
	2.5		0.5	0.5	decreases	Flow slows	positives
	5		1	2	as conc of	as PEG is	on all except
	10		2	2	PEG is	increased.	1.25 & 2.5g/L
	25		4	4	increased.		No control line
	50		4	4			seen for 50g/L.
GCDB			0	0			
PEG 15-20K	1.25	Positive	0.5	0.5	As above	As above	No control line
	2.5		0.5	0.5	for PEG	for PEG	seen for 50g/L.
	5		2	2	negative	negative	
	10		2	2	solution	solution	
	25		4	4			
	50		4	6			
GCDB			4	4			
Sucrose	12.5	Negative	0	0	Front	Fast flow	Control line
	25		0	0	decreases as	Flow slows	faint for all.
	50		0	0	sucrose conc	as sucrose is	Needs to be used
	100		0	0	is increased.	increased	in combination
	250		0	0	Best front with	Good release	with other
GCDB			0	0	GCDB.		components
Sucrose	12.5	Positive	0	0	As above	As above	As above
	25		0	0	for sucrose	for sucrose	for sucrose
	50		0	0	negative	negative	negative
	100		0	0	solution	solution	solution
	250		0	0.5			
GCDB			2	2			
BSA	1	Negative	0	0	V. good front	Extremely	
	2		0	0	"	slow flow	
	4		0	0	"		
	8		0	0	"		
	20		0	0	"		
	40		0	0.5	"		
GCDB			0	0.5	Worst front		
BSA	1	Positive	0	0	Appears to	Very slow	
	2		0	0	be very	flow	No control lines
	4		0	0	poor release	Very slow to	visilble except
	8		0	0	of gold	adsorb off pad	for GCDB
	20		0	0		2 drops extra	
	40		0	0		required	
GCDB	40		0..5	0.5			

Table 3.8.8: *Examination of the inclusion of PEG 15-20K, Sucrose & BSA in the GCDB. Best results are highlighted in red.*

3.8.5ii Evaluation of the performance of gold conjugate strips manufactured using the GCDB substituted with various components

Gold conjugate strips were manufactured using the GCDB with and without the substitution of the components selected in section 3.8.5 and 3.8.5i (see Table 3.8.9). These components were supplemented, where relevant, into the GCDB at their optimum concentrations as determined in section 3.8.5i. All devices were tested with samples from the Rotunda Hospital to determine if any differences were apparent in the C.I. result for both the test and control line. The results are shown in Table 3.8.10 and overleaf in Figure 3.8.2.

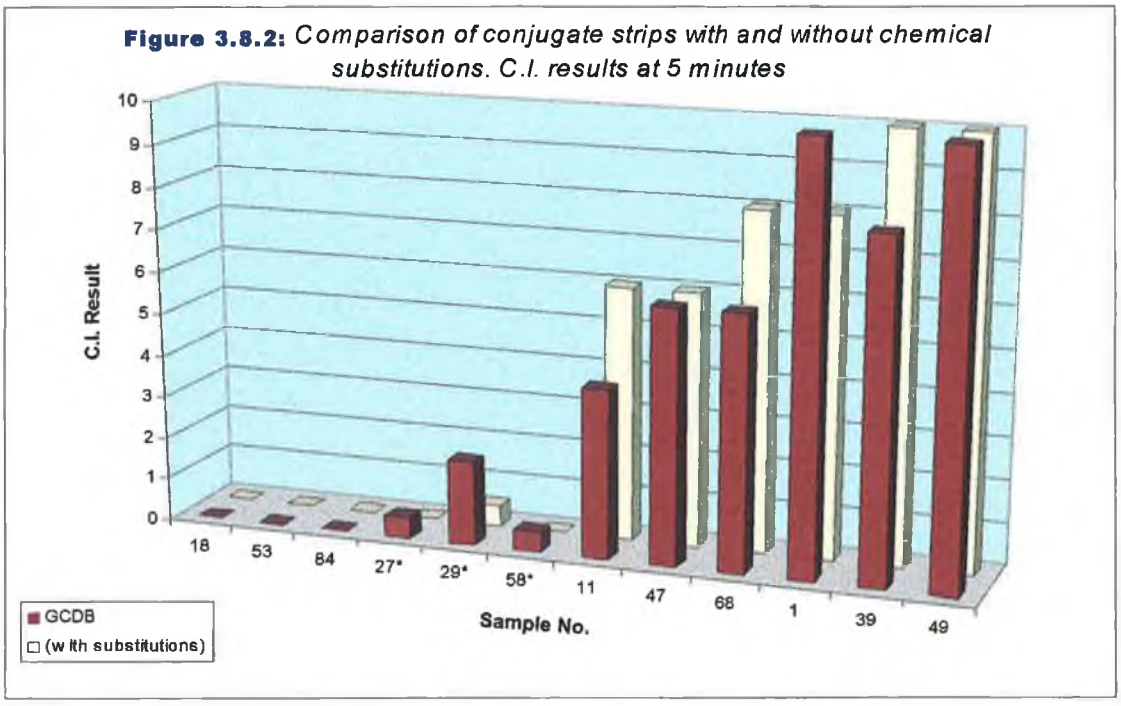
Sample No.	Sample Status	C.I. Result @ 5 min		Comments
		GCDB (no substitutions)	GCDB (with substitutions)	
18	negative	0	0	
53	negative	0	0	
84	negative	0	0	
27*	negative	0.5	0	
29*	negative	2	0.5	
58*	negative	0.5	0	
11	positive	4	6	
47	positive	6	6	
68	positive	6	8	
1	strong positive	10	8	Stronger C.lines on GCDB with substitutions
39	strong positive	8	10	
49	strong positive	10	10	

Table 3.8.10: *Comparison of the performance of conjugates with & without chemical substitutions as decided in section 3.8.5 & 3.8.5 i. Evaluation of 6 negative & 6 positive samples from the Rotunda Hospital. Colour intensity results at 5 minutes. * = Previously gave false positive results with the 'in-house' assay.*

As observed from Figure 3.8.2, the gold conjugate strip made up with GCDB including substitutions gave superior results to those obtained for the strip using GCDB without any chemical additions. The GCDB with substitutions demonstrated only one false positive for sample no. 29, which was of a low C.I. of 0.5. Conversely, the GCDB without substitutions exhibited three false positives, for samples no. 27, no. 29 and no. 58. These three samples were selected for testing as they had demonstrated false positive C.I. results with the prototype 'in-house' assay during the clinical trial described in section 3.7. The results obtained for the positive samples were also better for the strips using the GCDB with substitutions. Higher C.I. results

were noted for three of the positive samples (no. 11, no. 58 and no. 39) with this strip in comparison to those obtained with strips manufactured with unsupplemented GCDB. Only one positive sample gave lower results with the supplemented GCDB strip (no. 1).

Figure 3.8.2: *Comparison of conjugate strips with and without chemical substitutions. C.I. results at 5 minutes*



The aim of the inclusion of additional components to the GCDB was to reduce the false positive problem seen during the first clinical trial. Samples no. 27, 29 and 58 had demonstrated the worst false positive C.I. results from this trial. Strips containing the GCDB with substitutions were tested with 30 negative urines and 30 negative serums from the Cork Blood Bank. 100% specificity was observed with these strips (results not shown). Therefore, it was decided that the GCDB could be supplemented with the chosen chemicals since the worst false positive results were eliminated in two cases and much reduced in the third. Also, no detrimental effect was observed on the other negative samples tested and an improvement in C.I. results was observed for three of the positive samples. It should be noted that all of the samples examined were run in triplicate, each sample giving the same C.I. result on each occasion and thereby strengthening the decision to favour the 'new' GCDB.

- **Conclusion**

The addition of the chemicals outlined in section 3.8.5i was approved based on the above results.

3.9 Assessment of the Effect on Specificity of the Position and Thickness of the Test Line

Considerable success was achieved in improving the specificity of the assay by the addition of various components to the GCDB (see sections 3.8.5-3.8.5ii). However, from section 3.8.5ii it was observed that the specificity could be improved further as one of the negative urines still exhibited a positive C.I. result, albeit a low one. It was decided to examine the effect of varying the thickness and position of the sprayed test line on the sensitivity and specificity of the assay in preparation for further clinical trials.

Test lines were sprayed at 8, 9, 10, 11 and 12 mm on membrane no. 2. The C.I. results for three samples with each of these sprayed membranes are recorded in Table 3.9.1 below.

Test Line Position (mm)	Sample (IU/L)	C.I. Result @ 5 min
8	0	1
	25	2
	50	4
9	0	0.5
	25	2
	50	4
10	0	0.5
	25	1
	50	3
11	0	0
	25	1
	50	2
12	0	0
	25	1
	50	2

Table 3.9.1: *Determination of the effect of varying the position of the test line. Colour intensity results for a 0, 25 and 50 IU/L hCG urine sample at 5 minutes, using conjugate C at A_{520nm} of 1.0 and test line antibody no. 9 at 1.0 mg/ml.*

From Table 3.9.1 it can be seen that the closer the test line was to the bottom edge of the nitrocellulose membrane the higher the C.I. result for each of the three samples

due to the extension of the reaction time (see Discussion, section 4.6). Consequently, positive C.I. results were noted for the negative sample on membrane sprayed at 8, 9 and 10 mm. The C.I. results for the positive samples were approximately two-fold greater with test lines sprayed at 8, 9 and 10mm, than the C.I. results for the same samples with membrane sprayed at 11 and 12 mm. Membrane sprayed at both 11 and 12 mm gave good positive-negative differentiation with both membranes exhibiting the same C.I. results for all three samples assayed. Since, from this experiment, the closer the test line was sprayed to the edge of the membrane the more likely the occurrence of false positive results, it was decided to select a spraying position of 12 mm for use in the 'in-house' assay.

Membrane no. 2 was then sprayed at 12 mm with a 1mg/mL solution of antibody no. 9 at the thicknesses described in Table 3.9.2. The results are presented in Table 3.9.2.

Test Line Thickness (mm)	Sample (IU/L)	C.I. Result @ 5 min
0.5	0	0
	25	0
	50	1
1.0	0	0
	25	1
	50	2
1.5	0	0.5
	25	2
	50	3
2.0	0	2
	25	2
	50	4

Table 3.9.2: *The effect of test line thickness on the sensitivity & specificity of the 'in-house' assay. Colour intensity results at 5 minutes for 0, 25 and 50 IU/L hCG urine samples, with conjugate C at an A_{520nm} of 1.0 & test line no. 9 at 1 mg/ml at a position of 11 mm.*

From Table 3.9.2 it is evident that the only line thickness which supplied the required sensitivity and specificity was that at 1 mm. The 0.5 mm thick test line was not sensitive enough as exhibited by the poor C.I. result obtained for the 25 and 50 IU/L samples (C.I. result of 0 for 25 IU/L and 1 for the 50 IU/L). The 1.5 and 2 mm thick test lines presented specificity problems with the appearance of a C.I. of 0.5 for the 0

IU/L sample with the 1.5 mm test line and a C.I. of 2 for the same sample with the line of 2 mm thickness. Conversely, the test line sprayed at a thickness of 1 mm gave a C.I. of 0 for the negative sample and a C.I. of 1 for the 25 IU/L sample and 2 for the 50 IU/L sample.

- **Conclusion**

A line position of 12 mm from the bottom edge of the membrane and a line thickness of 1 mm was selected for use in the in-house assay.

3.10 Format of the 'In-House' Assay following adjustments to solve specificity problems

Table 3.10.1 and 3.10.2 are presented in this section in order to summarise the changes made to the 'in-house' assay format during the optimisation of the original assay format (see 3.6) to improve the test's specificity. As for section 3.6 the tables outline the test components and the quantities of each component used for the manufacture of the 'in-house' assay at this stage in the test development.

Component	Component Details	Used at
Test Line Antibody	*Antibody no. 9*	*1 mg/mL*
Test Line Position	*n/a*	*12 mm*
Test Line Thickness	*n/a*	*1 mm*
Control Line Antibody	*Goat Anti-Mouse Antibody*	*1 mg/mL*
Control Line Position	*n/a*	*19 mm*
Nitrocellulose Membrane	*Membrane no. 2*	*n/a*
Gold Conjugate Antibody	*Anti β-hCG Monoclonal ~ Conjugate C*	*A_{520nm} 1.0*
Gold Conjugate Impregnation Buffer	*Trinity Biotech ~ GCDB With Goat Serum, Anti-Hep B gold conjugate, Triton X-100, Tween 20, PVP K-30, PEG 15-20K, Sucrose & BSA*	*# see overleaf for amounts added per constituent*
Sample Pad	*Sample Pad B*	*n/a*
Sample Volume	*n/a*	*200µl (4 drops)*
Assay Time	*n/a*	*5 minutes*

Table 3.10.1: *Format of Assay following improvements on specificity. List of test components, relevant quantities and test parameters. For further component details see Appendix I and Materials, section 2.1.*

 # Added to the GCDB as follows:

Component	Concentration
Triton x-100	*2.4 g/L*
Tween 20	*5.0 g/L*
PVP K-30	*1.5 g/L*
PEG 15-20K	*1.25 g/L*
Sucrose	*12.5 g/L*
BSA	*2.0 g/L*
Goat Serum	*20%*
Anti Hep B gold conjugate	*A_{520nm} @ 0.1*

3.11 Methods to increase the flow rate of the assay with both urine and serum samples

This area in the development of the 'in-house' assay was concerned with the introduction of the use of serum samples and the improvement of the flow rate for both urine and serum samples. Therefore the aim of this section was to increase the sample flow rate while maintaining the sensitivity and specificity inherent to the in house assay.

This was carried out by examining the flow rate of the sample with the following:

① Three nitrocellulose membranes of various pore sizes.

② Different sample pads.

③ Two different sample volumes

These studies are described in this section.

It should be clarified that throughout the assay development that follows, the flow rate was defined as the distance travelled (cm) per second by the urine/serum sample (unit: cm/sec). The flow time was defined as the length of time taken (unit: min,sec) for the sample to travel from the bottom edge of the test window to the top edge of the test window (see Figure 3.11.1).

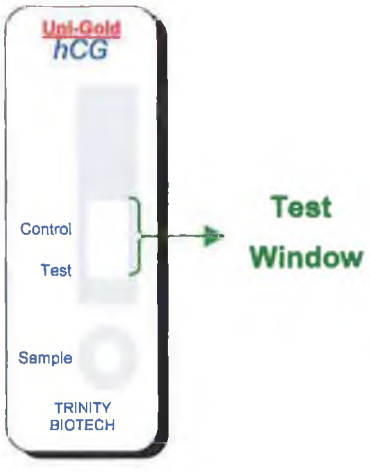


Figure 3.11.1: *Device showing test window, used for the calculation of flow times.*

3.11.1 Examination of the flow rates of urine and serum sample with various membranes.

The flow rates of several membranes were examined initially with deionised water (to mimic urine samples) and a 5% (w/v) BSA solution (to mimic serum samples) to aid in the selection of an appropriate nitrocellulose membrane for further flow rate studies (results not shown). As the assay time was required to be within 3 minutes for urine samples and 5 minutes for serum samples, large pore size membranes were examined. These membranes were expected to have intrinsically faster flow rates.

From this study the nitrocellulose membranes, as described for the combinations in Table 3.11.1, were selected for evaluation with urine and serum samples. Because membranes no. 1 and no. 3 possessed different pore sizes and protein binding capacities (see Discussion, section 4.7) to membrane no. 2 it was necessary to optimise the test line concentration and gold conjugate A_{520nm} for each of these membranes (results not shown). Membrane no. 2 had been used throughout the development of the test to this point. These new test line and gold conjugate combinations for the two membranes to be tested are outlined in Table 3.11.1 below along with the combination used to date.

Test Combination Identification no.	Membrane No.	Optimised Test Line Conc.	Optimised Gold Conjugate
1	No. 2	1mg/mL	A_{520nm} 1.0
2	No. 1	1mg/mL	A_{520nm} 1.0
3	No. 3	1.5mg/mL	A_{520nm} 0.9

Table 3.11.1: *List of test combinations assayed using 31 urine samples & 30 serum samples from the Rotunda Hospital.*

Each of the above test combinations were assayed with 31 urine and 30 serum samples from the Rotunda Hospital, Dublin. The sample flow time was recorded for each of the urine and serum samples with each of the three test combinations. The C.I. result was read for each combination at 3 minutes for urine samples and at 5 minutes for serum samples as these were the assay times for the competitor kits being run in parallel with the in-house assay. The C.I. result was also recorded for

the competitor kit (ABI) to ensure that the sensitivity and specificity characteristics of the in-house assay did not decline with the faster flow rates.

The results are illustrated overleaf in Tables 3.11.3 and 3.11.4. A summary of the average flow time results is outlined in Table 3.11.2 below.

Sample	Average Flow Time (min,sec)		
	Combination no. 1	Combination no. 2	Combination no. 3
Urine	*2,12*	***1,52***	*1,57*
Serum	*3,22*	***2,55***	*3,03*

Table 3.11.2: *Summary of Average Flow Time results for the three test combinations assayed to evaluate three different nitrocellulose membranes.*

From the summary table above it is clear that Combination no. 2 possesses the fastest average flow times for both urine and serum samples. Each of the three 'in-house' test combinations performed well in comparison to the ABI assay with regard to C.I. results. Despite the faster flow times for test combination no. 2 it is important to note that 9 out of 31 urine samples evaluated had a flow time of 2 minutes or greater while 13 out of the 30 serum samples demonstrated a flow time of 3 minutes or more. It was the aim of this section to reduce the sample flow time for urines to a maximum of 2 minutes since the assay was to be read at 3 minutes. Likewise, it was the intention to establish a maximum flow time of 3 minutes for serum samples since these samples were read at 5 minutes. These flow times would ensure that all samples could run to completion within the specified assay time and allow good sample clearance from the membrane.

As quite a large percentage of both the urine and serum samples fell outside these maximum flow times it was concluded that it was necessary to examine the effect of changing the sample pad and sample volume to bring the majority of samples within these assay time zones.

- **Conclusion**

Membrane no. 1 was selected for use in the assay due to its more favourable flow characteristics with both urine and serum samples.

In house no.	C.I. Result at 3 mins & Flow time for urine samples on 3 combinations						ABI Test
	Combination no. 1		Combination no. 2		Combination no. 3		
	C.I. Result	Flowrate (min,sec)	C.I. Result	Flowrate (min,sec)	C.I. Result	Flowrate (min,sec)	C.I. Result
1	4	2,14	7	1,59	6	2,01	8
3	8	1,58	8	1,48	6	1,45	6
4	7	1,42	8	1,35	6	1,42	6
5	8	2,31	8	2,12	7	2,14	8
6	8	2,10	5	1,52	6	2,03	4
7	6	2,06	8	1,47	6	1,45	8
8	8	2,19	6	2,01	7	1,58	6
9	5	1,43	6	1,27	6	1,36	5
10a	8	2,37	7	2,08	6	2,10	6
11	8	1,54	6	1,39	5	1,42	7
12	7	1,39	8	1,24	6	1,33	6
13	8	2,22	6	2,03	8	2,13	5
14	8	1,48	4	1,32	7	1,27	4
15	8	2,16	7	1,54	6	2,05	6
16	8	2,27	6	1,59	7	2,14	4
17	8	2,05	8	1,42	8	1,35	8
18	8	2,17	6	2,00	5	2,06	6
19	9	1,52	6	1,26	6	1,37	3
20	8	1,59	6	1,41	8	1,49	5
21	8	1,45	6	1,22	6	1,20	6
22	7	2,03	4	1,50	8	1,56	4
23	8	1,58	8	1,33	8	1,42	2
24	8	2,34	8	2,07	6	2,11	8
25	5	2,13	4	1,59	4	1,54	2
26	8	2,24	8	2,04	8	2,15	6
27	8	2,11	8	1,57	6	2,01	8
28	8	2,36	9	2,09	6	2,03	8
29	7	1,41	8	1,21	6	1,38	6
30	8	1,50	8	1,35	6	1,44	6
31	6	2,27	6	2,02	6	2,10	6
Average Flowrate		2,12		1,52		1,57	

Table 3.11.3: *Comparison of colour intensity results at 3 minutes and flow rates (min,sec) for 31 urine samples from the Rotunda Hospital using three 'in-house' assay formats (Combination no. 1, no. 2 and no. 3). The average flow rate (min,sec) for the 31 samples is shown for each test combination.*

In house no.	C.I. Result at 5 mins & Flow time for urine samples on 3 combinations						ABI Test
	Combination no. 1		Combination no. 2		Combination no. 3		
	C.I. Result	Flowrate (min,sec)	C.I. Result	Flowrate (min,sec)	C.I. Result	Flowrate (min,sec)	C.I. Result
1	8	2,57	8	2,20	6	2,15	8
2	6	3,56	8	2,59	7	3,06	8
3	8	4,24	7	3,44	8	3,57	8
4	8	3,22	9	2,54	9	2,44	10
5	6	3,09	9	2,36	6	2,45	10
6	6	2,59	6	2,22	8	2,51	8
7	6	3,35	7	3,01	6	3,15	8
8	7	3,29	8	3,06	8	3,22	8
9	8	3,47	8	3,12	8	3,32	8
10	6	4,18	6	3,56	6	4,01	8
11	8	2,54	8	2,33	8	2,47	8
12	8	3,36	6	3,05	6	2,59	7
13	8	3,12	6	2,52	6	3,11	8
14	8	2,45	8	2,27	8	2,34	8
15	8	2,59	6	2,31	8	2,28	8
16	8	2,19	8	2,06	8	2,14	8
17	8	3,42	6	3,15	8	3,26	8
18	8	3,18	7	3,04	6	3,07	8
19	6	3,06	6	2,54	6	3,01	8
20	6	2,47	6	2,23	6	2,33	8
21	8	3,44	6	3,21	6	3,24	8
22	6	3,16	6	2,45	6	3,02	8
23	6	2,34	6	2,12	6	2,25	8
24	8	2,56	6	2,55	8	2,48	8
25	8	3,54	6	3,34	6	3,42	8
26	6	4,01	6	3,57	6	4,00	8
27	8	3,22	9	2,41	9	2,52	10
28	8	3,52	8	3,18	8	3,20	10
29	6	3,41	7	3,02	6	3,16	8
30	8	2,58	6	2,38	8	2,41	8
Average Flowrate		3,22		2,55		3,03	

Table 3.11.4: *Comparison of colour intensity results at 5 minutes and flow rates (min,sec) for 30 serum samples from the Rotunda Hospital using three 'in-house' assay formats (Combination no. 1, no. 2 and no. 3). The average flow rate (min,sec) for the 30 samples is shown for each test combination.*

3.11.2 Evaluation of the effect of the sample volume and Sample Pad on the assay flow time.

From 3.11.1 it was noted that an increase in the sample flow rates was achieved by changing the nitrocellulose membrane to one of a larger pore size, i.e.: membrane no. 1 replaced membrane no. 2. However, this new membrane did not sufficiently reduce the sample flow time for all samples and accordingly it was decided to attempt to increase the flow time by the addition of more sample per test. The ABI assay employed a sample volume of 250µl whereas up until this point a sample volume of 200µl was added to each in house test. A comparison of these two sample volumes using urine and serum samples with the 'in-house' assay was carried out and the results are recorded below in Table 3.11.4. The 'in-house' assay flow times were compared to those obtained with the ABI test.

Sample		Flow Time (mins, sec)		
		ABI	Sample Volume 200µl	Sample Volume 250µl
Urine:	no. 1	1,50	2,20	1,58
	no. 2	1,38	2,30	1,50
	no. 3	1,44	2,10	1,33
	no. 4	1,29	2,00	1,47
	no. 5	1,32	1,50	1,38
Serum:	no. 1	1,40	3,10	2,20
	no. 2	1,42	2,30	2,10
	no. 3	1,53	2,50	1,56
	no. 4	1,50	2,23	1,41
	no. 5	1,52	2,14	1,52

Table 3.11.4: *Evaluation comparing sample volumes of 200µl & 250µl against the ABI reference test (sample volume ~ 250µl)*

From the above results there is a significant difference noted for the urine and serum samples run with a sample volume of 200µl as opposed to 250µl. Excellent flow rates were achieved with a sample volume of 250µl. The flow times noted for the 250µl volume on the in-house assay were very similar to those seen for the ABI assay for all of the urine samples and for 3 out of the 5 serum samples. Much slower flow times were recorded for both urine and serum samples with the sample volume of 200µl.

The selected sample volume (i.e.: 250µl) was used below in combination with various sample pads to further improve the flow time for all the serum samples.

Sample pads B, D, E, F and G (see Appendix I) were examined for their effect on assay flow with the sample volume of 250µl. The function of the sample pad is to absorb and uniformly deliver the sample to the test membrane at a flow rate that is consistent with the flow time required for the assay being developed. The sample pads examined here possessed varying absorbancies and sample release rates. This study aimed to choose the pad most suited to the required assay times of 3 minutes for urine and 5 minutes for serum samples. Note: sample pad B had been used in the 'in-house' assay format up until this point. Devices were assembled using the same sprayed membrane (membrane no. 1) and gold conjugate strips. Only the sample pad varied in each case. The same five urine and serum samples as used to evaluate sample volume were used in this instance also. The flow times for each pad are laid out in Table 3.11.5 below.

Sample		Flow Time (mins, sec)				
		Pad D	Pad E	Pad F	Pad G	Pad B
Urine:	no. 1	1,34	1,40	1,40	2,23	1,40
	no. 2	1,30	1,26	1,29	1,57	2,14
	no. 3	1,27	1,32	1,10	1,30	2,21
	no. 4	1,30	1,33	1,27	2,07	2,20
	no. 5	1,44	1,53	1,24	2,26	1,55
Avg Flow Time		1,33	1,37	1,26	2,05	2,06
Serum:	no. 1	4,00	4,24	1,56	3,29	3,03
	no. 2	4,11	3,22	2,05	3,43	3,14
	no. 3	5,00	3,07	1,52	4,20	1,58
	no. 4	3,23	2,42	1,59	2,42	2,01
	no. 5	4,36	3,58	2,02	3,24	1,54
Avg Flow Time		4,14	3,31	1,59	3,32	2,26

Table 3.11.5: *Flow times of 5 urine and serum samples with 5 different sample pads. Average flow times are shown for each pad with both urine and serum.*

The results illustrated in Table 3.11.5 show that the fastest flow times for the 'in-house' assay were seen when devices were assembled using sample pad F. The flow times recorded with this sample pad were in line with those seen in Table 3.11.4 for the ABI assay. Pad B, which was the sample pad utilised with the 'in-house' assay throughout the development of the test until this point, gave results similar to those seen in Table 3.11.4. Sample pad F and B were directly compared as outlined in Table 3.11.6 overleaf.

It was necessary to evaluate the effect, if any that the increased flow rate may have had on the sensitivity and specificity of the 'in-house' assay. As demonstrated in Table 3.11.6, no adverse effect was apparent on the specificity or sensitivity of the

assay by the reduced flow times observed when sample pad F was used. While the flow times for the urine and serum samples were acceptable with sample pad B also, it was necessary to select sample pad F with faster flow times. This was in order to meet the projected assay times for both urine and serum samples.

Device Description	Sample	Flow Time (min,sec)	C.I. Result (TL,CL)*
with sample pad B	*0 IU/L urine*	*2,25*	*0,6*
with sample pad F	*0 IU/L urine*	*1,42*	*0,6*
with sample pad B	*25 IU/L urine*	*2,16*	*0.5,5*
with sample pad F	*25 IU/L urine*	*1,37*	*0.5,6*
with sample pad B	*50 IU/L urine*	*2,19*	*2,6*
with sample pad F	*50 IU/L urine*	*1,39*	*3,8*
with sample pad B	*Serum no. 4*	*4,19*	*0,6*
with sample pad F	*Serum no. 4*	*3,25*	*0,8*
with sample pad B	*Serum no. 6*	*4,44*	*0,6*
with sample pad F	*Serum no. 6*	*3,13*	*0,8*
with sample pad B	*Serum no. 8*	*4,59*	*0,6*
with sample pad F	*Serum no. 8*	*2,45*	*0,8*
with sample pad B	*Serum no. 11*	*4,33*	*0,5*
with sample pad F	*Serum no. 11*	*3,10*	*0,7*
with sample pad B	*Serum no. 12*	*3,58*	*0,5*
with sample pad F	*Serum no. 12*	*2,37*	*0,8*

Table 3.11.6: *Evaluation of sample pad F in the 'in-house' assay in comparison with sample pad B which had been previously used. Colour intensity results for the test line (*TL) and control line (*CL) at 3 minutes for urine and 5 minutes for serum.*

- **Conclusion**

Sample pad F and a sample volume of 250µl were selected to increase the flow rate for both urine and serum samples.

3.12 Evaluation of the In House Assay Wick Format

The home-use test was to consist of a test format as pictured below in Figure 3.12.1. The test strip employed for the wick assay was the same as used for the cassette device apart from a test line position of 9.75mm and a control line position of 20mm to facilitate the structure of the plastic housing devices. The only significant difference between the two assays was, therefore, the wick itself, which served to deliver the sample to the test strip.

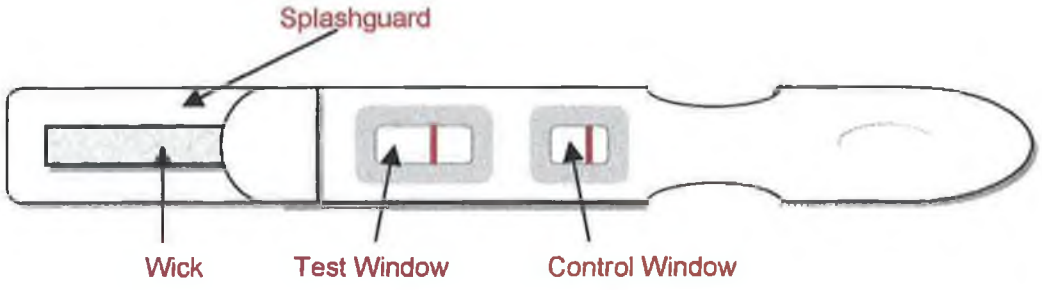


Figure 3.12.1: *Wick Format of In House Assay*

3.12.1 Selection of a suitable detergent for wick impregnation

It was deemed necessary to examine the performance of the wick assay using several detergents impregnated on the wick (see Method 2.2.5.2) to allow for an even and fast delivery of the sample to the test strip within the device housing.

Wicks were impregnated with varying levels of Triton® X-405, Triton® X-100 and Tween® 20 and compared with the unimpregnated wick and wicks taken from the ABI wick assay and assembled into the in house assay. The test strip for each of the wick devices tested was identical. Both positive and negative urine samples were assayed on each wick combination. The results are described overleaf in Table 3.12.1 and Table 3.12.2.

As illustrated in Table 3.12.1, the 'in-house' wicks which provided the most uniform flow, with clean negative results and control lines of strong intensity, were those impregnated with Triton® X-100 and Triton® X-405 at concentrations of 2% (w/v).

Wick	C.I. Result (*TL, *CL)		Flow	Comments
	At 3 min	At 5 min		
ABI wick	0,6	0,8	Fastest Flow	Flow observed for 2% (w/v) Triton® X-100 equal to that seen with ABI wick Cleanest test & control line seen for 2% (w/v) Triton® X-100 & 2% (w/v) Triton® X-405 2% (w/v) Triton® X-100 Still negative at 40 minutes
Unimpregnated	0,5	0,5	Fastest Flow	
2%(w/v) Triton® X-100	0,6	0.5,8	Fastest Flow	
5%(w/v) Triton® X-100	1,6	1,6		
10%(w/v) Triton® X-100	1,5	2,6		
2%(w/v) Triton® X-405	0,6	0,8	Fast Flow	
5%(w/v) Triton® X-405	1,4	2,6		
10%(w/v) Triton® X-405	2,3	2,4		
2%(w/v) Tween® 20	0,5	0,6	Slow Flow	
5%(w/v) Tween® 20	0,4	0,5		
10%(w/v) Tween® 20	0,4	0,5	Faster than 2% (w/v) Tween® 20	

Table 3.12.1: *Comparison of wicks impregnated with various detergents. Colour intensity results for the test line (*TL) and control line (*CL) at 3 and 5 minutes and flow observations with negative urine no. 27 (Rotunda Hospital).*

A slight false positive result (C.I. of 0.5) was noted for the negative urine sample using the wick impregnated with 2% (w/v) Triton® X-405. However, this false positive result was not present at three minutes and was of a much less colour intensity than those observed for the higher concentrations of both Triton® X-100 and Triton® X-405. The unimpregnated wick developed control lines of low colour intensity (C.I. of 5) due to slow transfer of the sample from the wick onto the sample pad of the test strip. All of the wicks impregnated with Tween® 20 yielded similarly low intensity control lines also as a result of slow sample flow. The flow was much slower for the wicks impregnated with Tween® 20 than observed for the wicks impregnated with either Triton® X-100 and Triton® X-405.

Likewise, it can be seen from Table 3.12.2 that similar results were obtained for the positive sample with the unimpregnated wick. In contrast, 2% (w/v) Triton® X-100 and 2% (w/v) Triton® X-405 demonstrated uniform flow arising in C.I. results similar to those recorded with the ABI wick. The flow of the gold conjugate for all concentrations of Tween® 20 and for the higher concentrations of both of the

Tritons® was too fast and subsequently the test line gave low C.I. readings. It was most likely that some of the sprayed test line was being washed away by this rapid flow, resulting in diminished binding and, therefore, lower C.I. results.

Wick	C.I. Result *TL, *CL)		Flow	Comments
	At 3 min	At 5 min		
ABI wick	8,8	8,8	Fastest Flow	Best test line seen for 2% (w/v) Triton® X-100 & 2% (w/v) Triton® X-405 Flow decreases with higher % (w/v) of Triton® X-100 & 2% (w/v) Triton® X-405 Increases with higher % (w/v) of Tween® 20
Unimpregnated	2,5	2,5	Fastest Flow	
2%(w/v) Triton® X-100	8,8	8,8	Fastest Flow	
5%(w/v) Triton® X-100	6,7	6,8		
10%(w/v) Triton® X-100	6,7	6,8		
2%(w/v) Triton® X-405	8,8	8,10	Fast Flow	
5%(w/v) Triton® X-405	6,5	6,8		
10%(w/v) Triton® X-405	4,6	6,6		
2%(w/v) Tween® 20	6,8	6,9	Slow Flow	
5%(w/v) Tween® 20	4,6	4,6		
10%(w/v) Tween® 20	4,5	4,5	Faster than 2% (w/v) Tween® 20	

Table 3.12.2: *Comparison of wicks impregnated with various detergents. Colour intensity results at 3 and 5 minutes for the test line (*TL) and control line (*CL) and flow observations with positive urine no.37 (Rotunda Hospital)*

Based upon the results described above it was decided to evaluate wicks impregnated with Triton® X-100 and Triton® X-405 at concentrations ranging between 1–3% (w/v) with a low positive sample to determine the optimum conditions for maximum sensitivity. The results are outlined in Table 3.12.3 overleaf.

As can be seen from Table 3.12.3 overleaf, wicks impregnated with 2% (w/v) Triton® X-100 gave C.I results with the most acceptable differentiation between the positive and negative samples and 2% (w/v) Triton® X-100 was thus selected for the impregnation of wicks for use in the wick assay. Tests possessing wicks with lower concentrations of Triton® X-100 were not sensitive enough and those with higher concentrations (i.e.: 2.5 and 3% (w/v) exhibited false positive results of varying intensities. False positive C.I. results were also a factor with Triton® X-405

Wick Description	C.I. Result for 0 IU/L (*TL, *CL)		C.I. Result for 25 IU/L (*TL, *CL)	
	At 3 min	At 5 min	At 3 min	At 5 min
ABI wick	0	0	1	2
Unimpregnated	0	0	0	0
1%(w/v) Triton® X-405	0	0	0	0
1.5%(w/v) Triton® X-405	0	0	0	0
2%(w/v) Triton® X-405	0	0.5	0	1
2.5%(w/v) Triton® X-405	0.5	1	1	1
3%(w/v) Triton® X-405	1	1	1	2
1%(w/v) Triton® X-100	0	0	0	0.5
1.5%(w/v) Triton® X-100	0	0	0	0.5
2%(w/v) Triton® X-100	0	0	0.5	1
2.5%(w/v) Triton® X-100	0	0.5	0.5	1
3%(w/v) Triton® X-100	0.5	0.5	1	1

Table 3.12.3: *Comparison of wicks impregnated with 1–3% (w/v) Triton® X-405 & Triton® X-100. Colour intensity results at 3 and 5 minutes for the test line (*TL) and control line (*CL) using a 0 IU/L and 25 IU/L hCG urine sample.*

concentrations of 2% (w/v) and higher (3% (w/v)). Concentrations lower than this did not possess the required sensitivity. It was necessary to evaluate the chosen wick with a large number of urine and serum samples to monitor performance of the 'in-house' wick assay.

29 hCG negative and 37 hCG positive urine samples and 50 hCG positive sera were run on the 'in-house' wick assay and the ABI wick assay. The results are listed in Tables 3.12.4 and 3.12.5. All devices flowed within the desired time specifications (i.e.: 3 minutes for urine samples and 5 minutes for serum samples). 100% specificity was observed for both the ABI and the 'in-house' assays. Similar colour intensity results were achieved for both assays using the 37 positive urines.

- **Conclusion**

The in-house wick device utilising 2% (w/v) Triton® X-100 matched the ABI device with regard to performance and flow times.

In House No.	'In-House' Assay at 3 min				ABI Assay at 3 min			
	C.I. Result (TL,CL)		Flow Time (min, sec)		C.I. Result (TL,CL)		Flow Time (min, sec)	
	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
1	0,7	6,7	1,20	1,11	0,7	6,7	1,30	1,25
2	0,7	6,8	1,42	1,16	0,7	6,7	1,26	1,26
3	0,7	5,8	1,09	1,22	0,7	6,8	1,03	1,35
4	0,7	6,7	1,29	1,41	0,7	6,7	1,19	1,43
5	0,7	6,8	1,16	1,36	0,7	6,7	1,10	1,30
6	0,7	6,7	1,10	1,33	0,7	6,9	1,07	1,54
7	0,7	6,10	1,15	1,21	0,7	6,8	1,06	1,24
8	0,7	6,7	1,08	1,17	0,7	6,7	1,07	1,22
9	0,7	8,6	1,14	1,06	0,7	8,7	1,14	1,12
10	0,7	6,6	1,14	1,14	0,7	6,7	1,22	1,22
11	0,7	6,7	1,14	1,46	0,7	6,8	1,34	1,34
12	0,7	6,9	1,24	1,35	0,7	6,9	1,35	1,26
13	0,7	7,10	1,33	1,28	0,7	8,8	1,15	1,10
14	0,7	6,8	1,12	1,44	0,7	6,7	1,07	1,39
15	0,7	6,9	1,05	1,39	0,7	6,8	1,12	1,33
16	0,7	9,10	1,24	1,23	0,7	8,9	1,46	1,32
17	0,7	5,8	1,22	1,20	0,7	5,6	1,07	1,34
18	0,7	6,8	1,10	1,47	0,7	6,7	1,10	1,39
19	0,7	6,7	1,40	1,50	0,7	6,8	1,20	1,41
20	0,7	6,10	1,21	1,08	0,7	6,7	1,08	1,02
21	0,7	4,8	1,13	1,13	0,7	5,7	1,14	1,20
22	0,7	6,6	1,20	1,24	0,7	6,7	1,13	1,33
23	0,7	5,5	113	1,22	0,7	6,7	1,13	1,19
24	0,7	6,9	1,18	1,34	0,7	7,8	1,13	1,42
25	0,7	7,10	1,24	1,18	0,7	7,8	1,17	1,26
26	0,7	6,10	1,13	1,03	0,7	6,7	1,19	1,10
27	0,7	6,7	1,30	1,33	0,7	6,8	1,20	1,45
28	0,7	8,8	1,18	1,45	0,7	7,8	1,14	1,51
29	0,7	8,9	1,20	1,27	0,7	8,8	1,15	1,33
30		6,7		1,14		6,7		1,17
31		6,7		1,07		6,7		1,06
32		7,6		1,15		6,8		1,21
33		7,10		1,20		7,8		1,15
34		6,9		1,55		6,7		1,47
35		6,7		1,23		6,6		1,30
36		6,8		1,27		6,7		1,24
37		8,9		1,19		8,8		1,14

Table 3.12.4: *Assessment of the Performance of the 'In-House' Wick assay in comparison with the ABI wick assay. Colour intensity results for the test line (TL) and control line (CL) at 3 minutes and flow times (min,sec) for 29 hCG-negative urine samples from St. James Hospital, Dublin & 37 hCG-positive urine samples from the Coombe Hospital, Dublin.*

In House No.	'In-House' Assay at 5 minutes		ABI Assay at 5 minutes	
	C.I. Result (TL,CL)	Flow Time (min, sec)	C.I. Result (TL,CL)	Flow Time (min, sec)
1	6, 8	2,20	7, 8	2,14
2	6, 8	2,12	5, 8	3,00
3	6, 8	2,15	8, 8	2,10
4	4, 6	1,58	8, 8	2,12
5	7, 8	2,04	6, 8	2,16
6	6, 8	2,01	6, 8	2,18
7	6, 8	2,13	6, 8	2,30
8	6, 8	2,21	6, 8	2,40
9	6, 8	2,05	6, 8	2,34
10	6, 8	1,56	6, 8	2,50
11	6, 8	2,07	6, 8	2,14
12	6, 8	1,58	6, 8	2,36
13	6, 8	2,02	6, 8	2,26
14	6, 8	2,06	6, 8	2,34
15	8, 8	2,02	6, 8	2,20
16	6, 8	2,16	6, 8	2,22
17	6, 8	2,27	6, 8	2,07
18	6, 8	2,04	7, 8	2,09
19	6, 8	2,12	6, 8	2,14
20	6, 8	1,59	7, 8	2,30
21	6, 8	2,00	6, 8	3,00
22	6, 8	2,40	6, 8	3,30
23	6, 8	2,21	6, 8	2,30
24	6, 8	2,07	6, 8	2,30
25	7, 8	2,11	6, 8	3,15
26	6, 8	2,17	6, 8	2,42
27	8, 8	2,04	8, 8	2,06
28	8, 8	2,08	8, 8	2,35
29	6, 8	2,10	8, 8	2,33
30	8, 8	2,14	8, 8	2,29
31	8, 8	2,20	8, 8	2,25
32	6, 8	2,06	6, 8	3,00
33	0, 8	1,57	0, 8	2,56
34	6, 8	2,03	8, 8	2,30
35	6, 8	2,14	6, 8	2,40
36	6, 8	2,17	6, 8	2,30
37	6, 8	2,22	6, 8	2,30
38	6, 8	2,05	8, 8	2,24
39	6, 8	2,07	6, 8	2,28
40	6, 8	2,13	8, 8	2,34
41	8, 8	2,29	8, 8	2,38
42	6, 8	2,14	8, 8	2,24
43	6, 8	1,56	6, 8	2,17
44	6, 8	2,01	8, 8	2,43
45	6, 8	2,08	8, 8	2,28
46	8, 8	2,11	6, 8	3,22
47	8, 8	2,15	6, 8	2,40
48	6, 8	2,06	6, 8	2,20
49	8, 8	2,06	8, 8	2,46
50	7, 8	2,03	7, 8	2,39

Table 3.12.5: *Assessment of the Performance of the 'In-House' Wick assay in comparison with the ABI wick assay. Colour intensity results for the test line (TL) and the control line (CL) at 5 minutes and flow times (min,sec) for 50 hCG positive serum samples from the Coombe Hospital, Dublin.*

3.13 Format of the Cassette and Wick 'In-House' Assay following the introduction of serum samples

The function of this section is to highlight the 'new' format of the 'in-house' assay following the changes made to the test as a result of the evaluation of serum samples and the assessment of the 'wick' assay during the development which is described in sections 3.11-3.12. The format is summarised below in Table 3.13.1 and is an update on the format shown in Table 3.10.1.

Component	Component Details	Used at:
Test Line Antibody	*Antibody no. 9*	*1.0 mg/mL*
Test Line Position	*n/a*	*12 mm (Cassette)* *9.75mm (Wick)*
Test Line Thickness	*n/a*	*1 mm*
Control Line Antibody	*Goat Anti-Mouse Antibody*	*1 mg/mL*
Control Line Position	*n/a*	*19 mm (Cassette)* *20mm (Wick)*
Nitrocellulose Membrane	*Membrane no. 1*	*n/a*
Gold Conjugate Antibody	*Anti β-hCG Monoclonal (Conjugate C)*	*A_{520nm} 1.0*
Gold Conjugate Impregnation Buffer	*As per formulation outlined in Section 3.10.*	*See Table 3.10.2, Section 3.10.*
Sample Pad	*Sample Pad F*	*n/a*
Sample Volume	*n/a*	*250µl (5 drops)*
Assay Time	*n/a*	*3 minutes (Urine)* *5 minutes (Serum)*
Wick Impregnation Solution	*Triton® X-100 in deionised water*	*2% (w/v)*

Table 3.13.1: *Format of assay following the introduction of serum samples and the improvement of sample flow times. See Appendix I and Materials, section 2.1 for further details on components.*

3.14 Validation Trials on the Final 'In-House' Assay Format

3.14.1 Testing samples positive for heterophilic antibodies and Human anti-mouse antibodies (HAMA) on the 'In-House' Assay.

Heterophilic and human anti-mouse antibody (HAMA) positive samples are a range of samples, which bind non-specifically in many immunoassay formats. hCG assays are particularly prone to non-specific interference by such samples (see Discussion, section 4.9.1). 25 known heterophilic positive samples and 24 HAMA positive samples (see Materials, section 2.1) were run on the 'in-house' assay and on the ABI assay. Each test was examined for colour intensity, if any, at the time intervals shown below in Table 3.14.1 and 3.14.2 where the relevant C.I. results are described. At each time interval, the number of tests, if any, which developed a false positive result within this time frame was recorded in Table 3.14.1 and 3.14.2.

Heterophilic Positive Samples

Time elapsed (min)	Number of False Positive Results	
	'In-House'	ABI
5	0	0
10	0	0
15	0	0
20	0	0
30	0	1
40	2	2
60	3	4
Total	**5**	**7**

Table 3.14.1: *Examination of the appearance of false positives with known heterophilic samples on the 'In-House' assay and the ABI reference assay over 60 minutes.*

HAMA Positive Samples

Time elapsed (min)	Number of False Positive Results	
	'In-House'	ABI
5	0	0
10	0	1
15	0	0
20	0	1
30	0	2
40	1	0
60	0	0
Total	**1**	**4**

Table 3.14.2: *Examination of the appearance of false positives with known HAMA positive samples on the 'In-House' assay and the ABI reference assay over 60 minutes.*

Five false positive results were noted for the 'in-house' assay when heterophilic positive samples were run and the first of which was not visible until 40 minutes after the sample had been added. 7 false positives were present with the same samples on the ABI assay, the first of which appeared at 30 minutes.

Only one false positive result was recorded for the 'in-house' assay at 40 minutes with the addition of HAMA positive sample no. 30. Conversely, the ABI assay resulted in a total of 4 false positives, which were first noted at 10 minutes (sample no. 30).

- **Conclusion**

The 'in-house' assay demonstrated superior specificity to that observed for the ABI device upon evaluation with heterophilic and HAMA positive samples.

3.14.2 Interference Study for the 'In-House' Cassette and Wick Assays

The objective of this study was to ensure that no interference occurred with the 'in-house' assay when everyday occurring substances were present in either the urine or serum sample being assayed.

Potentially interfering drugs and physiological substances common to urine/serum (see Discussion, section 4.9.2) were spiked into female negative urine and serum and into both 25 IU/L and 50 IU/L hCG positive urine and serum standards at the levels outlined in Table 3.14.4 – 3.14.9. The same negative, 25 IU/L and 50 IU/L hCG positive urine and serum samples which had not been supplemented with any of the interfering substances were run in parallel with the samples described above. The results were determined by comparison to the non-supplemented negative, 25 IU/L and 50 IU/L hCG positive urine and serum standards. The study was carried out for both the cassette and wick formats of the assay.

The tests were scored visually by comparison to the negative control and a 25 IU/L samples. All samples whose test line was fainter than that of the 25 IU/L sample were determined as negative.

A summary of the results obtained with the various interfering substances with their corresponding supplementation level are outlined for urine in Table 3.14.4 and in Table 3.14.6 and for serum in Table 3.14.5 and 3.14.7. The flow times for the urine and serum samples with the average C.I. results at 3 and 60 minutes for urine and 5 and 60 minutes for serum are summarised with the ABI assay in these tables. Tables 3.14.10-3.14.20 show the actual C.I. results and flow times for both tests with both sample types.

No interference was observed for any of the urine or serum negative or positive samples with either the cassette or wick assay. All of the negative urine/serum samples, both supplemented and unsupplemented, with both 'in-house' assay formats yielded their expected negative results. Similarly, all positive samples supplemented and unsupplemented gave positive results on both formats. Each test was re-examined at 60 minutes for colour intensity. All tests from both formats still remained without interference at this point.

Substance Added	Conc. (mg/dl)	'In-House' Assay Visual Result		
		Urine 0 IU/L	Urine 25 IU/L	Urine 50 IU/L
Acetaminophen	20	*Negative*	*Positive*	*Positive*
Ascorbic Acid	20	*Negative*	*Positive*	*Positive*
Acetylsalicylic Acid	20	*Negative*	*Positive*	*Positive*
Albumin	2000	*Negative*	*Positive*	*Positive*
Caffeine	20	*Negative*	*Positive*	*Positive*
Estriol	2	*Negative*	*Positive*	*Positive*
Gentisic Acid	20	*Negative*	*Positive*	*Positive*
Glucose	2000	*Negative*	*Positive*	*Positive*
Haemoglobin	50	*Negative*	*Positive*	*Positive*
Phenothiazine	20	*Negative*	*Positive*	*Positive*

Table 3.14.4: *Summary table of Interference study on the **'In-House' hCG Cassette assay** with **urine** samples spiked with various substances.*

Substance Added	Conc. (mg/dl)	'In-House' Assay Visual Result		
		Serum 0 IU/L	Serum 25 IU/L	Serum 50 IU/L
Acetylsalicylic Acid	20	*Negative*	*Positive*	*Positive*
Albumin	2000	*Negative*	*Positive*	*Positive*
Estriol	2	*Negative*	*Positive*	*Positive*
Glucose	2000	*Negative*	*Positive*	*Positive*
Haemoglobin	50	*Negative*	*Positive*	*Positive*

Table 3.14.5: *Summary table of Interference study on the **'In-House' hCG Cassette assay** with **serum** samples spiked with various substances.*

Substance Added	Conc. (mg/dl)	'In-House' Assay Visual Result		
		Urine 0 IU/L	Urine 25 IU/L	Urine 50 IU/L
Acetaminophen	*20*	*Negative*	*Positive*	*Positive*
Ascorbic Acid	*20*	*Negative*	*Positive*	*Positive*
Acetylsalicylic Acid	*20*	*Negative*	*Positive*	*Positive*
Albumin	*2000*	*Negative*	*Positive*	*Positive*
Caffeine	*20*	*Negative*	*Positive*	*Positive*
Estriol	*2*	*Negative*	*Positive*	*Positive*
Gentisic Acid	*20*	*Negative*	*Positive*	*Positive*
Glucose	*2000*	*Negative*	*Positive*	*Positive*
Haemoglobin	*50*	*Negative*	*Positive*	*Positive*
Phenothiazine	*20*	*Negative*	*Positive*	*Positive*

Table 3.14.6: *Summary table of Interference study on the 'In-House' hCG Wick assay with urine samples spiked with various substances.*

Substance Added	Conc. (mg/dl)	'In-House' Assay Visual Result		
		Serum 0 IU/L	Serum 25 IU/L	Serum 50 IU/L
Acetylsalicylic Acid	*20*	*Negative*	*Positive*	*Positive*
Albumin	*2000*	*Negative*	*Positive*	*Positive*
Estriol	*2*	*Negative*	*Positive*	*Positive*
Glucose	*2000*	*Negative*	*Positive*	*Positive*
Haemoglobin	*50*	*Negative*	*Positive*	*Positive*

Table 3.14.7: *Summary table of Interference study on the 'In-House' hCG Wick assay with serum samples spiked with various substances.*

Sample Added	'In-House' Assay			ABI Assay		
	C.I. Result at 3 min	C.I. Result at 60 min	Average Flow time (min,sec)	C.I. Result at 3 min	C.I. Result at 60 min	Average Flow time (min,sec)
0 IU/L Urine	*0*	*0*	*1,18*	*0*	*0*	*1,17*
25 IU/L Urine	*1*	*3*	*1,15*	*1*	*4*	*1,18*
50 IU/L Urine	*2*	*6*	*1,13*	*2*	*5*	*1,16*
0 IU/L Serum	*0*	*0*	*2,10*	*0*	*0*	*2,15*
25 IU/L Serum	*1*	*4*	*2,15*	*2*	*4*	*2,15*
50 IU/L Serum	*3*	*5*	*2,18*	*3*	*5*	*2,18*

Table 3.14.8: *Summary table of Interference study data for the 'In-House' and ABI hCG Cassette assays.*

Sample Added	'In-House' Assay			ABI Assay		
	C.I. Result at 3 min	C.I. Result at 60 min	Average Flow time (min,sec)	C.I. Result at 3 min	C.I. Result at 60 min	Average Flow time (min,sec)
0 IU/L Urine	*0*	*0*	*1,30*	*0*	*0*	*1,18*
25 IU/L Urine	*1*	*4*	*1,16*	*1*	*4*	*1,15*
50 IU/L Urine	*2*	*5*	*1,15*	*2*	*5*	*1,13*
0 IU/L Serum	*0*	*0*	*2,25*	*0*	*0*	*2,14*
25 IU/L Serum	*2*	*3*	*2,21*	*2*	*4*	*2,19*
50 IU/L Serum	*4*	*5*	*2,15*	*3*	*5*	*2,14*

Table 3.14.9: *Summary table of Interference study data for the 'In-House' and ABI hCG Wick assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	0, 8	0, 6	0, 9	0, 8	1,16	1,19
Acetaminophen	0, 8	0, 6	0,9	0, 8	1,18	1,16
Ascorbic Acid	0, 8	0, 6	0, 9	0, 8	1,17	1,20
Acetylsalicylic acid	0, 8	0, 6	0, 9	0, 8	1,17	1,14
Albumin	0, 8	0, 6	0, 9	0, 7	1,15	1,20
Caffeine	0, 8	0, 6	0, 9	0, 8	1,17	1,17
Estriol	0, 8	0, 6	0, 9	0, 8	1,20	1,19
Gentisic acid	0, 8	0, 6	0, 9	0, 8	1,22	1,15
Glucose	0, 8	0, 6	0, 9	0, 8	1,18	1,23
Haemoglobin	0, 7	0, 8	0, 8	0, 8	1,15	1,17
Phenothiazine	0, 8	0, 6	0, 9	0, 8	1,19	1,11

Table 3.14.10: *C.I. results and flow times (min,sec) for 0 IU/L hCG urine samples (supplemented & unsupplemented) with the 'In-House' and ABI cassette assays.*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	0, 8	0, 6	0, 9	0, 8	2,24	2,06
Acetylsalicylic acid	0, 8	0, 6	0, 9	0, 8	2,07	2,14
Albumin	0, 8	0, 8	0, 9	0, 9	2,13	2,45
Estriol	0, 8	0, 6	0, 9	0, 8	2,05	2,02
Glucose	0, 8	0, 7	0, 9	0, 8	2,06	2,18
Haemoglobin	0, 8	0, 8	0, 9	0, 9	2,08	2,03

Table 3.14.11: *C.I. results and flow times (min,sec) for 0 IU/L hCG serum samples (supplemented & unsupplemented) with the 'In-House' and ABI cassette assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	0, 8	0, 6	0, 9	0, 8	1,42	1,30
Acetaminophen	0, 8	0, 6	0, 8	0, 8	1,50	1,16
Ascorbic Acid	0, 8	0, 6	0, 8	0, 8	1,32	1,22
Acetylsalicylic acid	0, 7	0, 6	0, 8	0, 8	1,33	1,14
Albumin	0, 8	0, 6	0, 9	0, 7	1,15	1,20
Caffeine	0, 8	0, 6	0, 8	0, 8	1,26	1,17
Estriol	0, 8	0, 6	0, 8	0, 8	1,26	1,16
Gentisic acid	0, 8	0, 6	0, 8	0, 8	1,30	1,15
Glucose	0, 8	0, 6	0, 8	0, 8	1,33	1,23
Haemoglobin	0, 7	0, 8	0, 8	0, 8	1,15	1,17
Phenothiazine	0, 8	0, 6	0, 8	0, 8	1,28	1,08

Table 3.14.12: *C.I. results and flow times (min,sec) for 0 IU/L hCG urine samples (supplemented & unsupplemented) with the 'In-House' and ABI wick assays.*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	0, 8	0, 6	0,8	0, 8	2,16	2,06
Acetylsalicylic acid	0, 8	0, 6	0, 8	0, 8	2,41	2,14
Albumin	0, 8	0, 7	0, 9	0, 8	2,11	2,18
Estriol	0, 8	0, 6	0, 8	0, 8	2,21	2,00
Glucose	0, 8	0, 5	0, 8	0, 8	2,45	2,31
Haemoglobin	0, 8	0, 7	0, 9	0, 8	2,11	2,16

Table 3.14.13: *C.I. results and flow times (min,sec) for **0 IU/L hCG serum** samples (supplemented & unsupplemented) with the 'In-House' and ABI **wick** assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	1, 8	1, 7	3, 9	4, 8	1.18	1,21
Acetaminophen	1, 8	1, 7	3, 9	4, 8	1,12	1,16
Ascorbic Acid	1, 8	1, 6	4, 9	4, 8	1,11	1,18
Acetylsalicylic acid	1, 8	1, 8	4, 9	3, 8	1,15	1,20
Albumin	1, 8	1, 8	4, 9	4, 8	1,18	1,19
Caffeine	1, 8	1, 7	4, 9	4, 8	1,16	1,17
Estriol	1, 8	1, 8	3, 9	4, 8	1,14	1,20
Gentisic acid	1, 8	1, 6	3, 9	3, 8	1,13	1,15
Glucose	1, 8	1, 7	4, 9	4, 8	1,19	1,20
Haemoglobin	1, 8	1, 8	4, 9	4, 9	1,19	1,21
Phenothiazine	1, 8	1, 7	3, 9	3, 8	1,14	1,16

Table 3.14.14: *C.I. results and flow times (min,sec) for **25 IU/L hCG urine** samples (supplemented & unsupplemented) with the 'In-House' and ABI **cassette** assays.*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	2, 8	2, 6	4, 8	4, 8	2,21	2,11
Acetylsalicylic acid	2, 8	1, 6	4, 8	3, 8	2,41	2,19
Albumin	1, 8	2, 7	3, 9	4, 8	2,11	2,15
Estriol	1, 8	1, 8	4, 8	3, 8	2,04	2,03
Glucose	1, 8	1, 5	4, 9	3, 8	2,01	2,20
Haemoglobin	1, 9	2, 8	3, 9	4, 8	2,11	2,24

Table 3.14.15: *C.I. results and flow time (min,sec) for **25 IU/L hCG serum** samples (supplemented & unsupplemented) with the 'In-House' and ABI **cassette** assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	1, 9	1, 7	3, 9	3, 8	1,20	1,19
Acetaminophen	1, 8	1, 7	4, 9	4, 8	1,16	1,13
Ascorbic Acid	1, 8	1, 6	4, 8	3, 8	1,15	1,12
Acetylsalicylic acid	1, 8	1, 8	3, 8	3, 9	1,18	1,14
Albumin	1, 8	1, 8	3, 9	4, 8	1,25	1,27
Caffeine	1, 8	1, 7	4, 9	4, 8	1,10	1,17
Estriol	1, 8	1, 8	3, 9	4, 8	1,06	1,16
Gentisic acid	1, 8	1, 6	4, 9	3, 8	1,08	1,15
Glucose	1, 9	1, 7	4, 9	4, 8	1,20	1,11
Haemoglobin	1, 8	1, 8	3, 9	4, 9	1,23	1,11
Phenothiazine	1, 8	1, 7	3, 8	3, 8	1,12	1,14

Table 3.14.15: *C.I. results and flow times (min,sec) for **25 IU/L hCG urine** samples (supplemented & unsupplemented) with the 'In-House' and ABI **wick** assays.*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	2, 8	2, 7	4, 9	4, 8	2,13	2,15
Acetylsalicylic acid	2, 8	2, 6	4, 8	3, 8	2,41	2,19
Albumin	1, 8	2, 7	3, 9	3, 8	2,42	2,39
Estriol	1, 8	1, 8	4, 8	3, 8	2,04	2,09
Glucose	2, 8	2, 6	4, 9	4, 8	2,01	2,18
Haemoglobin	2, 9	2, 8	4, 9	4, 8	2,11	2,13

Table 3.14.16: *C.I. results and flow time (min,sec) for **25 IU/L hCG serum** samples (supplemented & unsupplemented) with the 'In-House' and ABI **wick** assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	2, 7	2, 7	5, 9	5, 8	1,18	1,15
Acetaminophen	2, 8	2, 7	6, 9	5, 8	1,11	1,11
Ascorbic Acid	2, 8	2, 7	5, 7	5, 8	1,19	1,20
Acetylsalicylic acid	2, 8	2, 8	5, 9	5, 9	1,04	1,17
Albumin	2, 7	2, 8	6, 8	4, 8	1,18	1,20
Caffeine	2, 7	2, 8	6, 9	5, 8	1,10	1,13
Estriol	2, 8	2, 8	6, 9	4, 9	1,10	1,14
Gentisic acid	2, 7	2, 7	5, 8	5, 8	1,09	1,19
Glucose	2, 8	2, 8	5, 9	5, 8	1,14	1,16
Haemoglobin	2, 8	2, 8	5, 9	5, 9	1,14	1,18
Phenothiazine	2, 8	2, 7	6, 9	5, 9	1,20	1,14

Table 3.14.17: *C.I. results and flow times (min,sec) for **50 IU/L hCG urine** samples (supplemented & unsupplemented) with the 'In-House' and ABI **cassette** assays*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	3, 8	3, 6	5, 8	5, 8	2,22	2,10
Acetylsalicylic acid	4, 8	3, 6	5, 9	5, 8	2,16	2,19
Albumin	3, 8	3, 7	4, 8	4, 7	2,41	2,37
Estriol	3, 8	2, 6	5, 8	5, 8	2,11	2,03
Glucose	4, 8	3, 7	6, 9	5, 8	2,15	2,22
Haemoglobin	3, 8	3, 8	5, 8	5, 8	2,01	2,16

Table 3.14.18: *C.I. results and flow time (min,sec) for **50 IU/L hCG serum** samples (supplemented & unsupplemented) with the 'In-House' and ABI **cassette** assays.*

Substance Added	C.I. Result at 3 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	2, 8	2, 8	5, 9	5, 8	1,13	1,12
Acetaminophen	2, 8	2, 7	5, 9	5, 8	1,21	1,13
Ascorbic Acid	2, 8	2, 7	5, 7	5, 8	1,17	1,11
Acetylsalicylic acid	2, 8	2, 8	5, 9	5, 9	1,12	1,17
Albumin	2, 7	2, 8	6, 8	5, 8	1,22	1,25
Caffeine	2, 7	2, 8	5, 9	5, 8	1,08	1,05
Estriol	2, 8	2, 7	6, 8	4, 7	1,15	1,04
Gentisic acid	2, 7	2, 7	5, 8	5, 8	1,13	1,10
Glucose	2, 8	2, 8	5, 9	5, 8	1,19	1,17
Haemoglobin	2, 8	2, 8	5, 9	5, 9	1,05	1,18
Phenothiazine	2, 8	2, 7	6, 9	5, 9	1,16	1,12

Table 3.14.19: *C.I. results and flow times (min,sec) for **50 IU/L hCG urine** samples (supplemented & unsupplemented) with the 'In-House' and ABI **wick**assays.*

Substance Added	C.I. Result at 5 min (TL,CL)		C.I. Result at 60 min (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
None	3, 7	3, 7	5, 8	5, 8	2,11	2,09
Acetylsalicylic acid	3, 8	3, 7	5, 9	5, 8	2,04	2,14
Albumin	3, 8	3, 6	5, 8	5, 7	2,33	2,26
Estriol	3, 8	3, 7	5, 8	5, 8	2,15	2,08
Glucose	3, 8	3, 7	5, 9	5, 8	2,16	2,14
Haemoglobin	3, 8	3, 8	5, 8	5, 8	2,09	2,10

Table 3.14.20: *C.I. results and flow time (min,sec) for **50 IU/L hCG serum** samples (supplemented & unsupplemented) with the 'In-House' and ABI **wick** assays.*

3.14.3 Specificity study of the 'In-House' Assay

The hormones described below were examined with the 'in-house' cassette and wick assay to determine if any of these physiological hormones would cross react with the test line and/or gold conjugate used in these assay formats.

The following hormones were added, at the levels quoted, to both negative and positive (25 IU/L and 50 IU/L) urine and serum samples:

1. Follicle Stimulating Hormone (FSH) – 1000 mIU/mL
2. Thyroid Stimulating Hormone (TSH) – 1000 μIU/mL
3. Lutenising Hormone (LH) – 500 mIU/mL

The urine and serum samples (spiked with hCG to the following levels: 0, 25 and 50 IU/L) to which the above hormones (1–3) were added, were directly compared with samples (spiked to the same levels with hCG) without any addition of the above hormones (1–3). The tests were scored visually by comparison to the negative control and a 25 IU/L samples. All samples showing C.I. results lower than that observed for the 25 IU/L sample were recorded as negative.

The results are illustrated in Table 3.14.21–3.14.24.

None of the three hormones supplemented into either urine or serum samples affected the specificity of the 'in-house' cassette or wick assay. All samples devoid of hCG, whether supplemented or not with the three hormones, provided negative C.I. results as expected. All positive samples spiked with the three hormones displayed C.I. results I line with those observed for the control positive samples (i.e.: those without FSH, TSH and LH).

- **Conclusion for Section 3.14.1 – 3.14.3**

From the results outlined in this section, none of the interfering substances examined affected the performance of either the cassette or wick 'in-house' assay with positive and negative urine or serum samples.

Sample	C.I. Result at 3 mins. (TL,CL)		C.I. Result at 30 mins. (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
0 IU/L (hCG) only	0, 8	0, 8	0, 9	0, 9	1,24	1,12
25 IU/L (hCG) only	1, 8	1, 8	2, 9	2, 8	1,30	1, 14
50 IU/L (hCG) only	2, 8	2, 8	4, 9	3, 9	1,26	1, 17
0 IU/L (hCG), 1000 mIU/mL FSH	0, 8	0, 8	0, 9	0, 9	1,20	1, 19
25 IU/L (hCG), 1000 mIU/mL FSH	1, 8	1, 8	2, 9	3, 8	1,37	1, 14
50 IU/L (hCG), 1000 mIU/mL FSH	2, 8	2, 9	4, 9	2, 9	1,20	1, 15
0 IU/L (hCG), 1000 µIU/mL TSH	0, 8	0, 9	0, 9	0, 9	1,28	1, 19
25 IU/L (hCG), 1000 µIU/mL TSH	1, 8	1, 9	2, 9	2, 9	1,27	1, 18
50 IU/L (hCG), 1000 µIU/mL TSH	2, 8	2, 8	4, 9	3, 9	1,21	1, 21
0 IU/L (hCG), 500 mIU/mL LH	0, 8	0, 8	0, 8	0, 9	1,27	1, 14
25 IU/L (hCG), 500 mIU/mL LH	1, 8	1, 8	2, 9	2, 9	1,28	1, 16
50 IU/L (hCG), 500 mIU/mL LH	2, 8	2, 8	4, 9	3, 9	1,23	1 18

Table 3.14.21: *C.I. results and flow times (min,sec) for **urine** samples (with hormones and without) for the **'In-House' and ABI wick assays**.*

Sample	C.I. Result at 5 mins. (TL,CL)		C.I. Result at 30 mins. (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
0 IU/L (hCG) only	0, 8	0, 8	0, 9	0, 9	2,10	2, 16
25 IU/L (hCG) only	1, 8	1, 8	2, 8	2, 8	2,11	2, 18
50 IU/L (hCG) only	2, 8	2, 8	3, 9	3, 9	2,14	2, 21
0 IU/L (hCG), 1000 mIU/mL FSH	0, 8	0, 8	0, 9	0, 9	2,12	2, 14
25 IU/L (hCG), 1000 mIU/mL FSH	1, 8	1, 8	2, 8	2, 8	2,16	2, 17
50 IU/L (hCG), 1000 mIU/mL FSH	2, 8	2, 9	3, 9	2, 9	2,25	2, 20
0 IU/L (hCG), 1000 µIU/mL TSH	0, 8	0, 8	0, 9	0, 8	2,22	1, 19
25 IU/L (hCG), 1000 µIU/mL TSH	1, 8	1, 8	2, 8	2, 8	2,02	1, 18
50 IU/L (hCG), 1000 µIU/mL TSH	2, 8	2, 8	3, 9	3, 9	2,00	2, 01
0 IU/L (hCG), 500 mIU/mL LH	0, 8	0, 8	0, 8	0, 9	2,08	1, 14
25 IU/L (hCG), 500 mIU/mL LH	1, 8	1, 8	2, 8	2, 9	2,04	1, 16
50 IU/L (hCG), 500 mIU/mL LH	2, 8	2, 8	3, 9	3, 9	2,08	2, 10

Table 3.14.22: *C.I. results and flow times (min,sec) for **serum** samples (with hormones and without) for the **'In-House' and ABI wick assays.***

Sample	C.I. Result at 3 mins.(TL,CL)		C.I. Result at 30 mins. (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
0 IU/L (hCG) only	0, 8	0, 8	0, 9	0, 9	1,20	1,18
25 IU/L (hCG) only	1, 8	1, 8	2, 8	2, 8	1,20	1,16
50 IU/L (hCG) only	2, 8	2, 8	3, 9	3, 9	1,16	1,20
0 IU/L (hCG), 1000 mIU/mL FSH	0, 8	0, 8	0, 9	0, 9	1,18	1,13
25 IU/L (hCG), 1000 mIU/mL FSH	1, 8	1, 8	2, 9	3, 8	1,19	1,17
50 IU/L (hCG), 1000 mIU/mL FSH	2, 8	2, 8	3, 9	2, 9	1,21	1,10
0 IU/L (hCG), 1000 µIU/mL TSH	0, 8	0, 9	0, 9	0, 9	1,16	1,21
25 IU/L (hCG), 1000 µIU/mL TSH	1, 8	1, 8	2, 9	2, 9	1,20	1,19
50 IU/L (hCG), 1000 µIU/mL TSH	2, 8	2, 8	3, 9	3, 9	1,12	1,14
0 IU/L (hCG), 500 mIU/mL LH	0, 8	0, 8	0, 8	0, 9	1,14	1,16
25 IU/L (hCG), 500 mIU/mL LH	1, 8	1, 8	2, 9	2, 9	1,16	1,14
50 IU/L (hCG), 500 mIU/mL LH	2, 8	2, 9	3, 9	3, 9	1,27	1,20

Table 3.14.23: *C.I. results and flow times (min,sec) for **urine** samples (with hormones and without) for the **'In-House' and ABI cassette assays.***

Sample	C.I. Result at 5 mins. (TL,CL)		C.I. Result at 30 mins. (TL,CL)		Flow Time (min,sec)	
	'In-House'	ABI	'In-House'	ABI	'In-House'	ABI
0 IU/L (hCG) only	0, 8	0, 8	0, 9	0, 9	2,20	2, 02
25 IU/L (hCG) only	1, 9	1, 8	3, 8	2, 8	2, 20	2, 16
50 IU/L (hCG) only	2, 8	2, 8	4, 9	3, 9	2, 23	2, 14
0 IU/L (hCG), 1000 mIU/mL FSH	0, 8	0, 8	0, 9	0, 9	2, 17	2, 06
25 IU/L (hCG), 1000 mIU/mL FSH	1, 8	1, 8	2, 8	2, 8	2, 15	2, 12
50 IU/L (hCG), 1000 mIU/mL FSH	2, 9	2, 9	3, 9	2, 9	2, 22	2, 15
0 IU/L (hCG), 1000 µIU/mL TSH	0, 8	0, 8	0, 8	0, 8	2,18	2, 04
25 IU/L (hCG), 1000 µIU/mL TSH	1, 9	1, 8	2, 8	2, 8	2, 24	2, 11
50 IU/L (hCG), 1000 µIU/mL TSH	2, 8	2, 9	3, 9	3, 9	2, 01	2, 01
0 IU/L (hCG), 500 mIU/mL LH	0, 8	0, 8	0, 9	0, 9	2, 48	2, 14
25 IU/L (hCG), 500 mIU/mL LH	1, 8	1, 9	2, 9	2, 9	2, 54	2, 04
50 IU/L (hCG), 500 mIU/mL LH	2, 8	2, 8	3, 8	3, 9	2, 08	2, 08

Table 3.14.24: *C.I. results and flow times (min,sec) for **serum** samples (with hormones and without) for the **'In-House' and ABI cassette assays.***

3.14.4 Prozone Study

hCG assays are prone to prozone results caused by the very high levels of hCG present in a substantial number of samples during the first trimester of pregnancy (see Discussion, section 4.9.4). Due to this phenomenon it was necessary to determine at what level of hCG did this prozone effect occur.

Negative urine was spiked with hCG according to Method 2.2.5.2 to the levels outlined in Table 3.14.25 and the effect on C.I. results was recorded in the same table.

hCG Concentration (IU/L)	C.I. Result at 3 minutes
1,000,000	0
750,000	0
500,000	0
250,000	0.5
200,000	1
125,000	2
100,000	4
10,000	6
1,000	8
100	4
0	0

Table 3.14.25: *Examination of the effect of prozone samples on the 'In-House' Assay.*

- **Conclusion**

From the C.I. results illustrated in Table 3.14.25 it is clear that negative C.I. results are obtained when samples contain over 250,000 IU/L of hCG, i.e.: the prozone effect is apparent with this level of analyte.

3.14.5 Clinical Trials for the Final Format of the 'In-House' Assay

The cassette and wick formats of the 'in-house' assays were evaluated separately for performance in both external and internal clinical trials. This section details the results achieved with both of these assay formats for several clinical trials using both urine and serum samples. The ABI test (see section 2.1, Materials) was used throughout the clinical trials as the predicate device.

Internal and External Clinical Trials for the Cassette Device

302 hCG-negative urines were examined on the cassette device. 135 of these samples were sourced internally at Trinity Biotech Plc., 117 samples were received from St. James Hospital, Dublin and a further 50 hCG-negative samples were obtained during a customer perception study at Dublin City University (D.C.U.) (see page 157-158). All of the urines tested yielded negative results on both the 'in-house' cassette assay and on the ABI test.

275 hCG-positive urine samples were evaluated on the cassette device. Of these, volunteers assayed 100 samples as part of the customer perception study carried out in D.C.U. Since the results were interpreted as either positive or negative, no C.I. result grading was determined for these 100 samples. Subsequently, they are not included in the histogram shown overleaf in Figure 3.14.1. All of the hCG-positive urines tested gave positive results on the cassette device.

313 hCG-negative serum samples were assayed on the cassette device. All of these samples were received from Pelican House, Dublin and all displayed negative C.I. results.

260 hCG-positive serum samples were tested on cassette format of the 'in-house' assay. Four of these samples were interpreted as negative for hCG. However, all four of these samples were heavily blood-stained and should have been centrifuged before testing since the blood made it difficult to read the test result. The remaining 256 positive C.I. results are summarised in the histogram in Figure 3.14.2.

Overall, for the sample populations examined on the cassette device demonstrated 100% sensitivity and specificity with urine and 100% specificity and 98.5% sensitivity with serum samples. This concurred with the results obtained for the ABI device as can be clearly seen from the similar sample distributions in both of the histograms shown in Figures 3.14.1 and 3.14.2.

Figure 3.14.1: *Histogram results displaying the frequency at which particular C.I. results occurred for both the 'in-house' cassette assay and the ABI test for hCG-positive urines from the same sample population.*

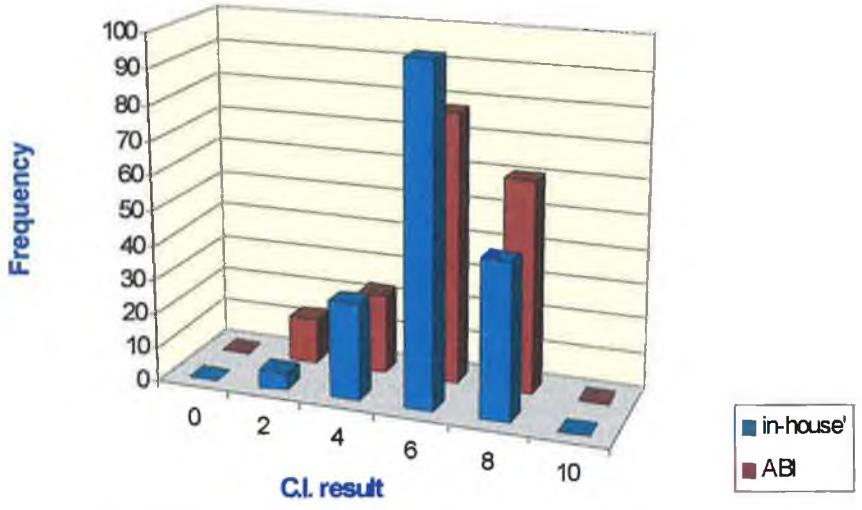
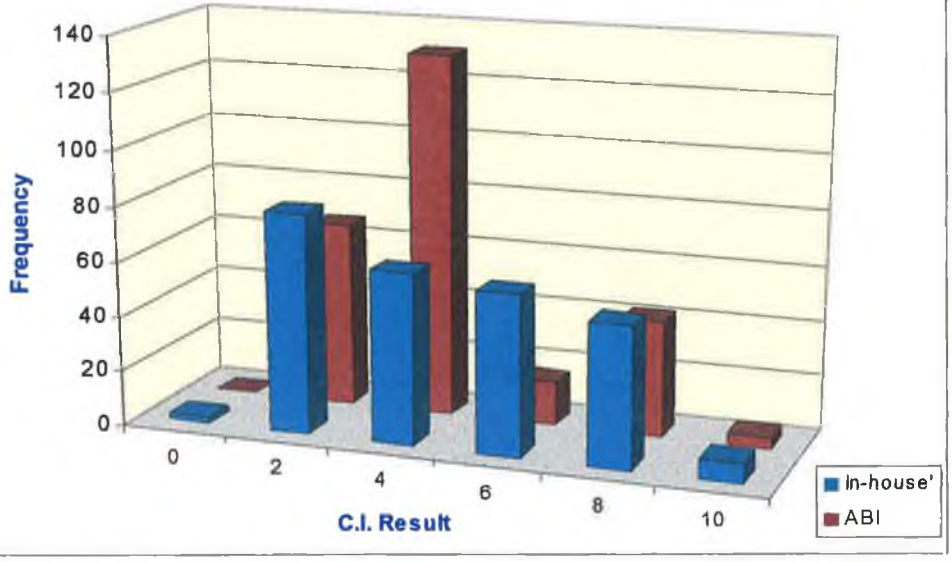


Figure 3.14.2: *Histogram results displaying the frequency at which particular C.I. results occurred for both the 'in-house' cassette assay and the ABI test for the same sample population of hCG-positive serum samples.*

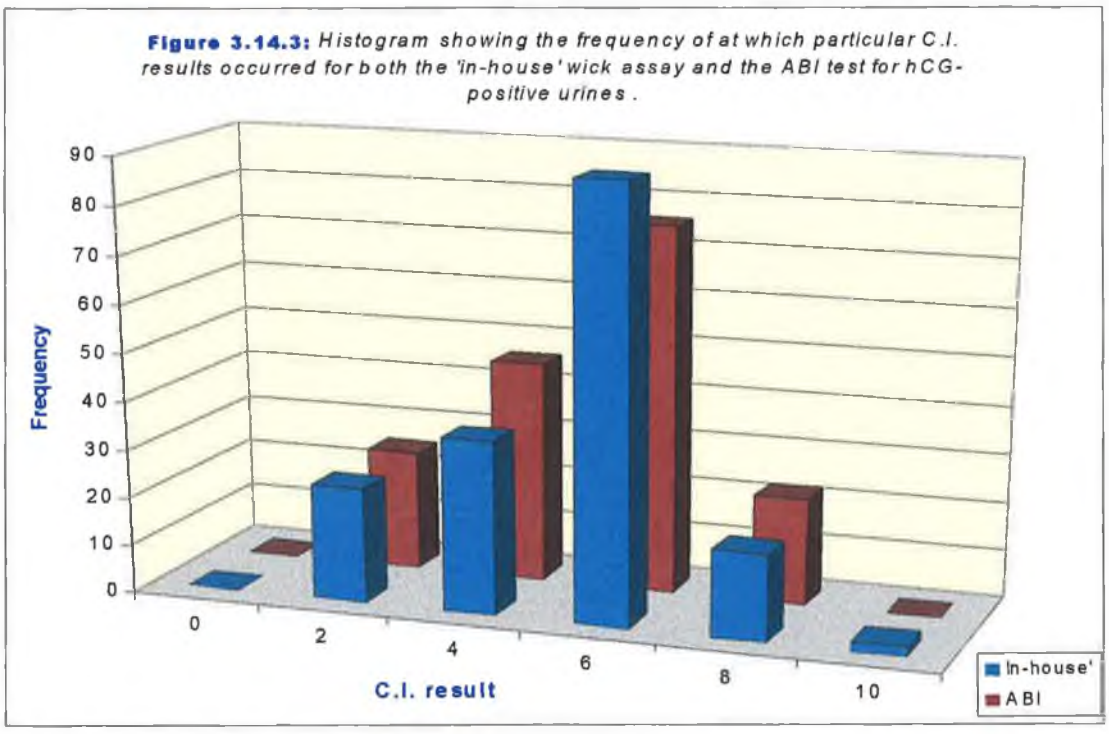


Internal and External Clinical Trials for the Wick Device

As carried out for the cassette device, the wick device was tested alongside the ABI wick device. 129 hCG-negative urine samples were evaluated on this format. Volunteers from the customer perception study at D.C.U. ran 100 of these hCG-negative urines; these are described overleaf. A further 29 samples were obtained from St. James' Hospital, Dublin. All samples tested negative, as expected.

With regard to hCG-positive urine, 169 samples were run. 100 of these samples were tested as part of the customer perception study in D.C.U., another 32 of the samples were supplied by the Rotunda Hospital, Dublin and the remaining 37 samples were obtained from The Coombe Hospital, Dublin. The results are summarised in Figure 3.14.3. It should be pointed out that one of the samples tested at D.C.U. gave a negative result when first tested. A positive result was achieved upon repeat testing and this result was included in the histogram below.

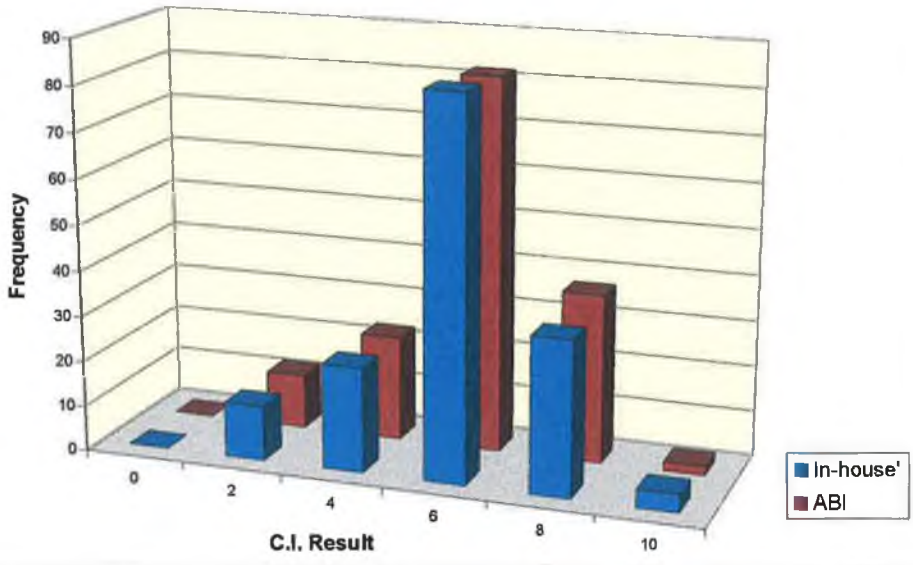
Figure 3.14.3: *Histogram showing the frequency of at which particular C.I. results occurred for both the 'in-house' wick assay and the ABI test for hCG-positive urines.*



100 hCG-negative serums were tested on the wick device. These samples were received from the Cork Blood Bank. Two of these samples yielded low positive C.I. results. It should, be pointed out, however, that these samples were among a group of samples, which had been thawed from –20°C one week previous to testing and displayed some signs of deterioration.

156 hCG-positive sera were assayed on the 'in-house' wick device. 100 of these had been received from the Rotunda Hospital, Dublin and 56 were obtained from the Coombe Hospital, Dublin. All 156 samples gave positive C.I. results and as can be seen from Figure 3.14.4 below, quite similar sample distributions were observed for the 'in-house' and ABI tests.

Figure 3.14.4: *Histogram showing the frequency at which particular C.I. results occurred for both the 'in-house' wick assay and the ABI test for hCG-positive serums .*



In summary, the wick device demonstrated 100% specificity and 99.4% sensitivity for urine samples. 98% specificity and 100% sensitivity were achieved with serum samples on the wick device. It is probable that by using fresh samples and by centrifuging any bloodstained samples that 100% specificity and sensitivity could have been achieved.

Clinical Trial and Customer Perception Study

The 'in-house' wick assay was evaluated against the ABI wick assay by fifty volunteers at Dublin City University, Glasnevin. These volunteers provided a urine sample each (all of which were negative for hCG) and ran their own sample on both the 'in-house' assay and the ABI test.

Each volunteer was then supplied with a set of three coded hCG controls, one negative (0 IU/L), one low positive (50 IU/L) and one high positive control (200 IU/L) to run on the 'in-house' assay. The control bottles were labelled with code numbers in such a way as to allow for a completely blind study regardless of how many volunteers were running the assay at the one time. Each volunteer was asked to interpret the results obtained as positive or negative based on a diagram similar to that shown in the provided instruction sheet (see Appendix II). Comments were also invited from volunteers on their perceptions of the assay, a summary of which is illustrated in Figure 3.14.5.

Therefore in total, 100 negative samples (50 from the volunteers and 50 from the coded controls), 50 low positive samples and 50 high positive samples were assayed on the 'in-house' assay.

A summary of the results obtained is shown below:

	Performance of Assay	
	'In-House'	ABI
% Sensitivity	*100 (100/100)*	*100 (50/50)*
% Specificity	*99 (99/100)*	*n/a**

Table 3.14.26: *Summary of the 'In-House' Assay Performance*

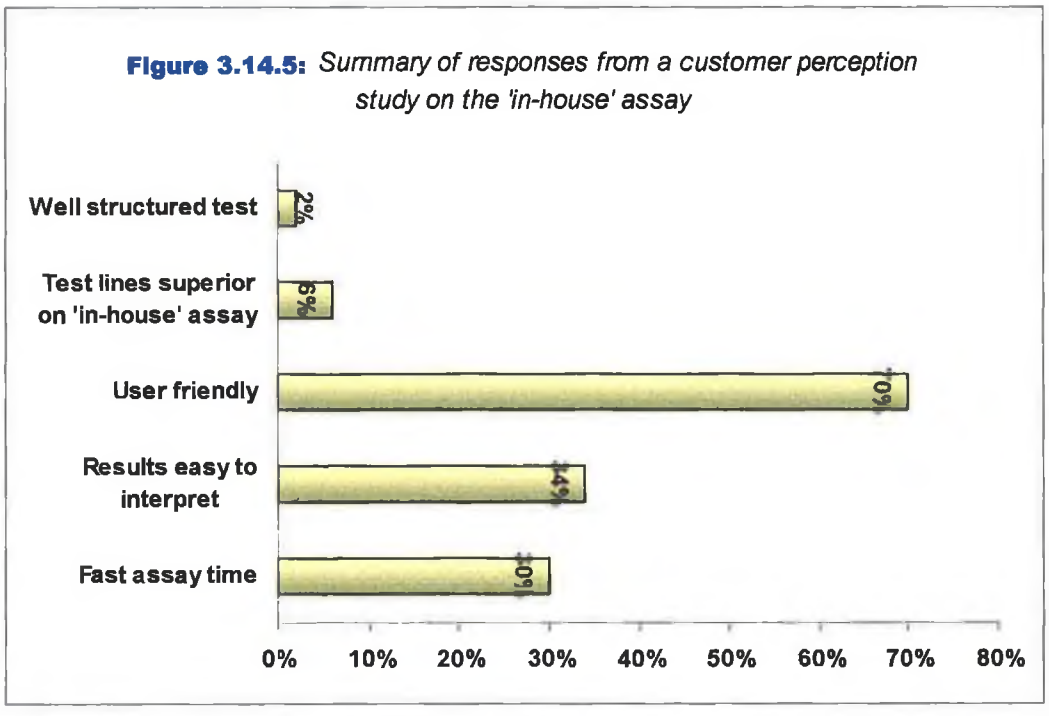
**Volunteers ran positive controls on the 'in-house' assay only.*

No false positive results were obtained on either the 'in-house' assay or the ABI hCG wick tests. All the 100 negative samples were interpreted as negative by the 50 volunteers. All 50 of the high positive controls (200 IU/L hCG) were interpreted as positive by the volunteers, while 49 of the 50 low positive samples resulted in

positive interpretations by the volunteers (i.e.: only one false positive result was observed with the 'in-house' assay.

It is important to note that many volunteers commented on the ease of use and rapidity of the test and some expressed a preference for the 'in-house' assay over the competitor device (See Figure 3.14.5 below).

Figure 3.14.5: *Summary of responses from a customer perception study on the 'in-house' assay*



- **Conclusion**

Excellent specificity and sensitivity were achieved for the sample populations tested with the 'in-house' assay. The cassette and wick formats of the 'in-house' assay compared favourably to the predicate device, i.e.: the ABI test. Positive feedback was received when potential customers were asked to give their views on the test, with most participants (70%) declaring that the test was 'user-friendly'.

3.15 Stability of the 'In-House' Assay

The stability of both the cassette and wick formats of the 'in-house' hCG assay was evaluated in this study in accordance with method 2.3.5.9, which was adapted from that described by Anderson and Scott (1991). The stability studies carried out employed the accelerated time method as summarised in Table 3.15.1 below.

Weeks at 37°C	Equivalent Time at 20 – 25°C
14	12 months
21	18 months
28	24 months

Table 3.15.1: *Accelerated Time Stability Study*

'In-House' Cassette Assay Stability Study

The cassette devices were placed in a 37°C incubator and tested at the time intervals outlined in Table 3.15.1 above. Therefore the devices were tested at an accelerated time of 12 months, 18 months and 24 months. The C.I. results and flow times for the devices are recorded in the tables, which follow, for urine and serum samples with unstressed (Control) devices and at accelerated times of 12, 18 and 24 months (see Table 3.15.2 and 3.15.3). The results for ABI devices incubated under the same conditions are also shown. These results are summarised in Figures 3.15.1 and 3.15.2.

Sample	C.I. Result at 3 mins				Flow Time			
	Control*	12 mths	18 mths	24 mths	Control*	12 mths	18 mths	24 mths
Urine								
0 IU/L	0, 8	0, 8	0, 8	0, 6	1,11	1,35	2,04	2,57
0 IU/L	0, 8	0, 8	0, 8	0, 6	1,24	1,37	1,59	2,52
25 IU/L	0.5, 8	0.5, 8	0.5, 8	0.5, 6	1,19	1,29	2,11	2,41
200 IU/L	3, 8	3, 8	3, 8	1, 6	1,22	1,40	2,13	2,54
2000 IU/L	6, 7	7, 7	8, 7	9, 7	1,31	1,39	2,21	2,58
Serum								
0 IU/L	0, 7	0, 8	0, 7	0.5, 6	2,35	2,47	3,59	4,59
0 IU/L	0, 7	0, 7	0, 7	0.5, 6	2,40	2,54	3,45	4,35
25 IU/L	1, 7	1, 7	1, 6	1, 6	2,33	2,57	3,52	4,24
200 IU/L	6, 7	6, 7	7, 6	6, 6	2,42	2,55	3,24	4,37
2000 IU/L	8, 6	8, 8	9, 6	9, 6	2,37	3,05	3,46	4,40

Table 3.15.2: *C.I. results & flow times for test and control 'in-house' devices, which were stressed at 37°C for time periods equivalent to 12, 18 & 24 months at 20-25°C. (* = unstressed device results at Day 0)*

Sample	C.I. Result for the 'in-house'control device at 3 mins				C.I. Result for the ABI test at 3 mins			
	Day 0	12 mths	18 mths	24 mths	Day 0	12 mths	18 mths	24 mths
Urine								
0 IU/L	*0, 8*	*0, 8*	*0, 7*	*0, 7*	*0, 8*	*0, 8*	*0, 6*	*0, 6*
0 IU/L	*0, 8*	*0, 8*	*0, 7*	*0, 7*	*0, 8*	*0, 8*	*0, 6*	*0, 6*
25 IU/L	*0.5, 8*	*0.5, 8*	*1, 7*	*0.5, 7*	*0.5, 8*	*0.5, 8*	*0.5, 8*	*0.5, 6*
200 IU/L	*3, 8*	*4, 8*	*6, 8*	*6, 8*	*3, 6*	*3, 7*	*3, 6*	*3, 6*
2000 IU/L	*6, 7*	*8, 6*	*8, 6*	*8, 6*	*7, 6*	*7, 6*	*8, 6*	*8, 7*
Serum								
0 IU/L	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0.5, 8*
0 IU/L	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 7*	*0, 8*
25 IU/L	*1, 7*	*1, 7*	*1, 7*	*1, 7*	*1, 7*	*1, 7*	*1, 6*	*1, 6*
200 IU/L	*6, 7*	*6, 8*	*6, 8*	*6, 8*	*6, 7*	*6, 7*	*8, 6*	*6, 6*
2000 IU/L	*8, 6*	*8, 6*	*8, 6*	*8, 6*	*8, 6*	*8, 7*	*10, 6*	*10, 3*

Table 3.15.3: *C.I. results for the 'in-house' control devices (i.e.: unstressed) and ABI devices, used for comparison with the test devices which were being examined at accelerated time periods at 37°C, which were equivalent to 12, 18 & 24 months at 20-25°C.*

Figures 3.15.1 and 3.15.2 (see page 161, 162) demonstrate a predicted stability of the 'in-house' assay for up to 18 months at room temperature. The 'in-house' device presented satisfactory C.I. results at 12 and 18 months in comparison to those recorded for the control devices. It is important to note that positive C.I. results were noted for the negative urine samples at 24 months (accelerated). For the same time interval the 200 IU/L serum sample resulted in much lower colour intensity than seen for the same sample at 18 months (accelerated). The C.I. result for the 2000 IU/L serum sample was seen to increase significantly with each time interval.

From Figure 3.15.2, it should be noted that the flow time for the assay increased gradually with increasing storage periods at 37°C. Most importantly, the flow time required to see the 'in-house' assay to completion with both the urine and serum samples increased dramatically at 24 months (accelerated).

• Conclusion

These results show the cassette to be stable for 18 months according to the shelf life equivalency table, however the specificity declines at 24 months and the time taken for the assay to complete at this time interval was unsatisfactory.

Figure 3.15.1: Plot of C.I. results for urine and serum samples for the 'in-house' control and test (stressed) devices, following accelerated stability studies at 37°C, equivalent to 12, 18 and 24 months at 20-25°C.

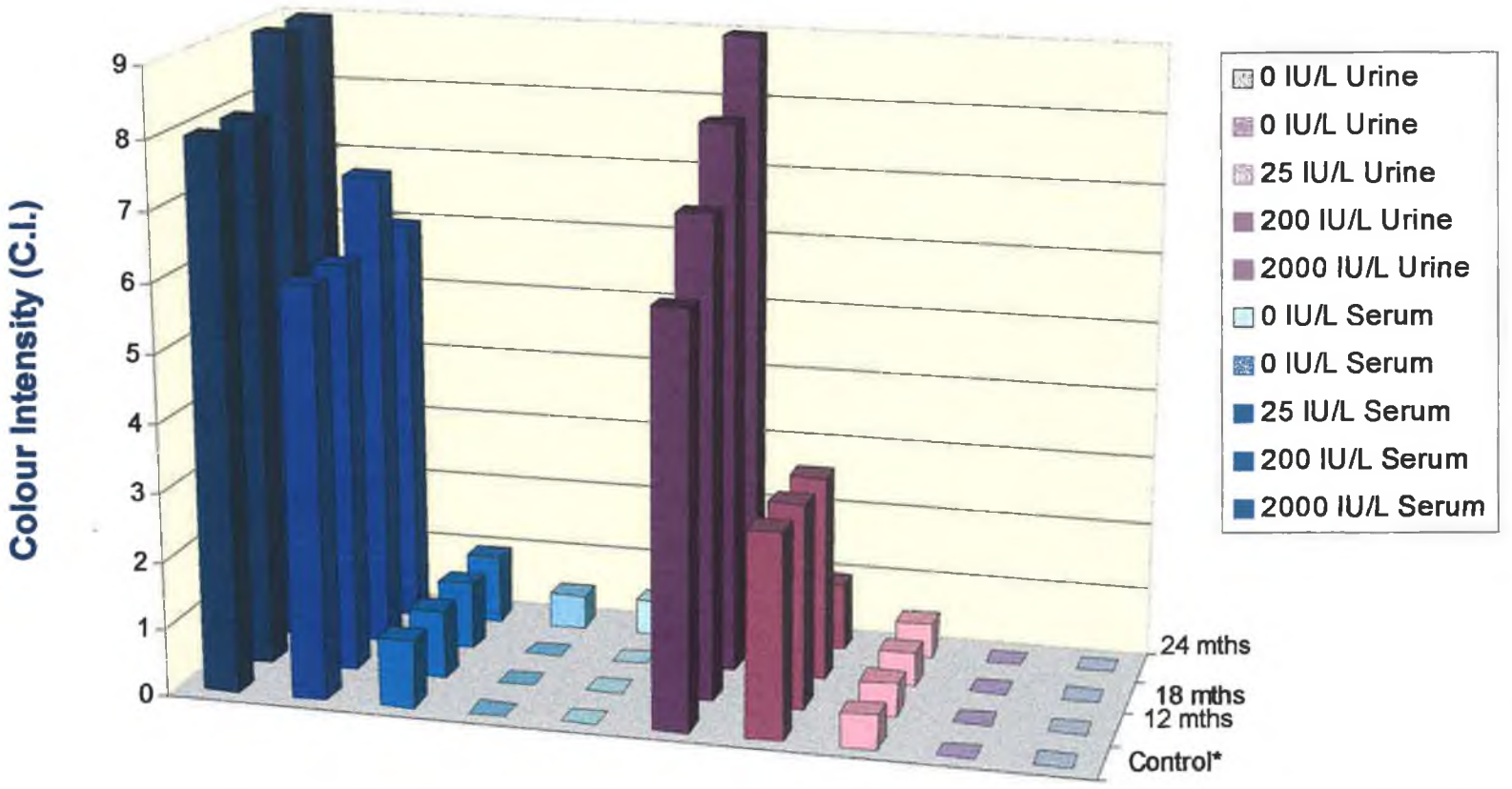
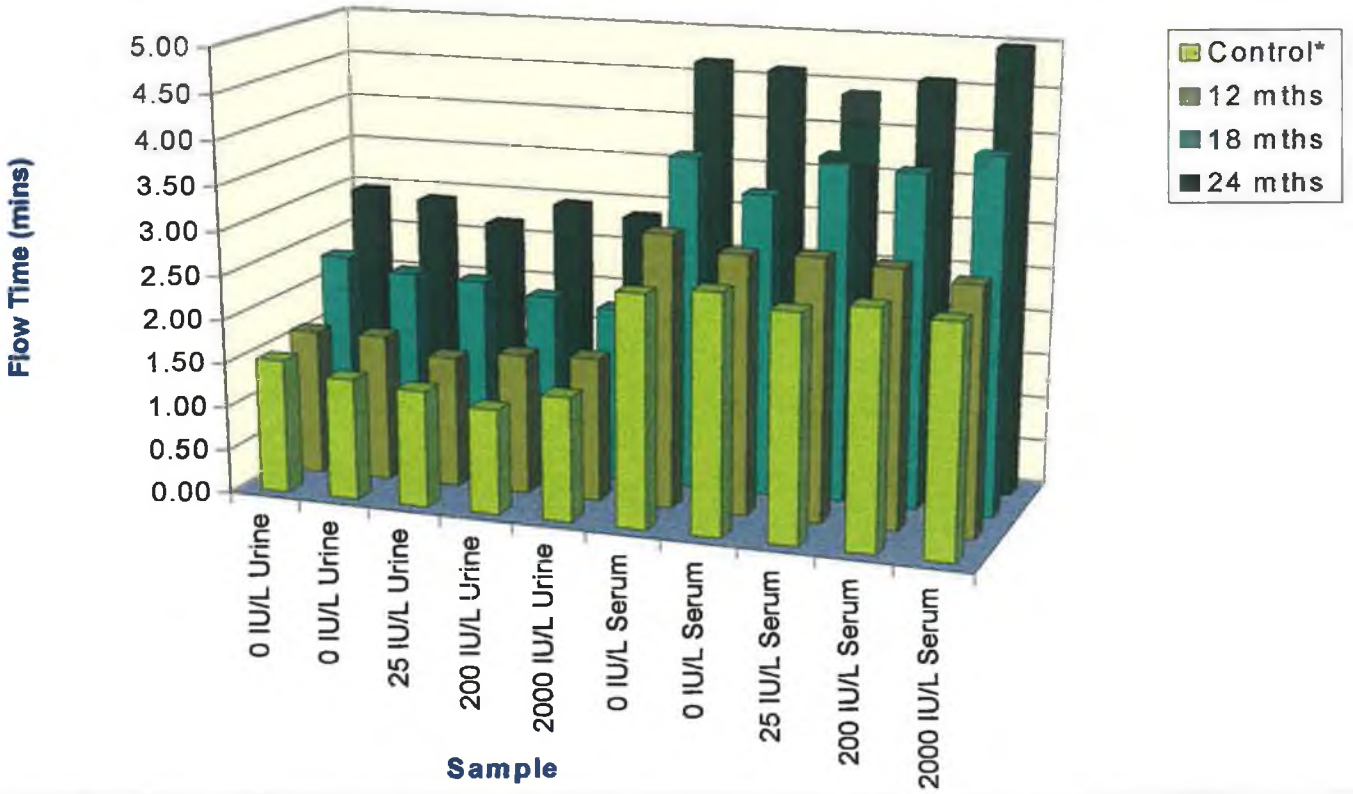


Figure 3.15.2: *Plot of flow times for urine and serum samples with the 'in-house' control and test (i.e.: stressed) devices following accelerated stability studies at 37 ° C, equivalent to 12, 18 and 24 months at 20-25 ° C.*



Section 4:

Discussion

4.0 Discussion

The presence of the hormone, human chorionic gonadotropin (hCG), in urine and serum is normally indicative of pregnancy. As detailed in section 1.2 (Introduction), hCG is a dimeric protein, consisting of an α and β subunit. As reported by Gosling (1990) the greatest variety and sophistication in the formulation of immunoassays are seen for over the counter (OTC) kits such as pregnancy tests. A wide range of pregnancy tests, which qualitatively detect this hormone, are currently available on the market. These vary from the over the counter 'stick' or 'pen' devices available from chemist shops for home use to the point of care (POC) cassette devices commonly used in the GP's office. Various EIA hCG assays are also commercially available for use in the clinical laboratory situation where the quantitation of exact hCG concentrations is important. Inferences can be made about the origin of the hCG and its related molecules in a given sample (e.g.: pregnancy, hydatidiform mole, persistent trophoblast disease, choriocarcinoma and 'phantom' or false positive hCG) (Cole, 1999b).

The OTC pregnancy tests were some of the first immunoassays to find application in the home with an ease of use suitable to the untrained or lay person. Due to their simplicity of operation, these tests enable a woman to determine for herself in the privacy of her own home whether or not she is pregnant without the expense of a blood test and a doctor's visit (Price *et al.*, 1997). New technology has ensured that these tests are now highly reliable and easy to use with results taking as little as 3 minutes to read and capable of detecting hCG as early as the first day of a missed period.

The reasons for developing a new rapid pregnancy immunoassay were several fold. It would be prudent to question the wisdom of placing another OTC/POC pregnancy test on the market when so many already exist (see Table 1.10.1, section 1.10, Introduction). The decision to develop a new gold-based immunochromatographic assay was mainly based on predicted figures researched by the marketing department at Trinity Biotech, where all of the research and development of the test took place. It was forecast that by the year 2000 the worldwide market for POC/OTC *in vitro* diagnostics (IVD) would be worth $6 billion from a figure of $2 billion in 1995 (30%

growth p.a.) (Shine, 1998). The scale of the growth in the POC/OTC market deemed the development of such a test feasible. It was also the aim of Trinity Biotech to develop a test that was faster for urine testing than competitors on the market (3 minutes for the 'in-house' urine test vs. 5-10 minutes for other assays already on the market, see Table 1.10, section 1.10, Introduction). This knowledge from the pre-development survey justified the proposed inclusion of the rapid hCG test on the market.

As discussed in section 1.11.4 of the Introduction the aim was to develop a test, which could match or exceed those already on the market (see Table 1.10.1, Introduction) with regard to several parameters: ① assay time, ② ease of use, ③ no requirement for equipment/reagents ④ low skill factor, ⑤ no refrigeration required, ⑥ low cost and ⑦ safety.

This discussion will examine the results achieved during the development and evaluation of a cassette and wick hCG test, both of which fulfil the above criteria.

4.1 The selection of the test line antibody

This section of the discussion details the initial developmental work on the selection of a suitable test line antibody, which was described in sections 3.2–3.3.

Newman and Price, 1997a, state that in order to select a suitable antibody for immunoassays there are four main characteristics to consider in connection with final assay performance: specificity, sensitivity, reaction time and stability. It is known that the desired specificity, sensitivity and reaction time are dependent on the affinity constant (K_a) and paratopic conformation of the antibody. Although thermal stability can vary between different antibodies, thereby conferring stability at higher temperatures for some antibodies, the overall stability of the antibody will probably be largely dependent on the solid phase to which it is bound (Newman and Price, 1997a).

For the initial test line antibody selection studies (see section 3.2, Results), it was necessary to assume that all of the potential test line antibodies being examined would be stable on the nitrocellulose membrane. This was a logical assumption since

many papers have reported on the robustness of immunoglobulin molecules and on their retention of immunological activity when conjugated to solid phases (Butler, 1991; Thakkar *et al.*, 1991). Once a test line antibody had been chosen, interim stability studies were performed throughout the development of the test to verify this assumed stability (results not shown). The binding of antibody to nitrocellulose membrane occurs non-covalently (Harvey, 1991), therefore, no harsh coating and/or washing procedures are required to bind the antibody to the membrane. It should be pointed out that while it was known that the manufacture of the test would involve vacuum drying procedures, the assumption was made that once the antibody was dried in a suitable stabilising buffer (Jones, 1999(a), Millipore, 1996) no adverse effects would be apparent on the chosen antibody. This assumption can be substantiated by reports that the coupling of antibodies to solid phases which are subsequently dried and stored desiccated ensures that the antibody is stable almost indefinitely (Newman and Price, 1997b).

As mentioned on the previous page, the specificity, sensitivity and reaction time of the antibody can be assessed by determining the affinity constant and paratopic conformation for the antibody in question. For the purposes of this test, however, such investigations were neither required nor practicable due to time limitations and insufficient instrument resources. The affinity was indirectly evaluated by spraying each of nine antibodies, sourced as test line antibodies (one polyclonal and eight monoclonal IgG antibodies) at similar concentrations on the same nitrocellulose membrane, using the same gold conjugate. This also provided sufficient indication with respect to the specificity and sensitivity of the antibodies. The C.I. results at 5 and 10 minutes were examined (see section 3.2, results). It should be emphasised that the results were read at 5 and 10 minutes as the other components were not optimised at this point and it was more likely that significant assay observations would be obtained with a slightly extended assay time.

The various antibodies evaluated were raised against different isoforms of the hCG antigen (see Table 4.1, overleaf). The results from the evaluation showed that antibody nos. 1, 8 and 9 gave the most satisfactory positive/negative discrimination for the samples evaluated (see section 3.2, Results). These three antibodies consisted of two monoclonal antibodies raised against intact hCG (nos. 1 and 8) and a

Antibody No.	Antigen raised against
1	*Intact hCG molecule*
2	*β-hCG*
3	*Intact hCG molecule*
4	*β-hCG*
5	*β-hCG*
6	*β-hCG*
7	*β-hCG*
8	*Intact hCG molecule*
9	*α-hCG*

Table 4.1: *List of antibodies evaluated as the test line antibody (see section 3.2) and the antigen to which each of these antibodies was raised.*

polyclonal antibody (no. 9) raised against α-hCG. The benefit of a test line antibody raised against intact hCG was apparent since it appeared that no potential steric hindrance problems arose between the binding of the antigen to both the test line antibody and the gold-conjugated antibody simultaneously. This was because the gold-conjugated antibody bound only to the β-hCG subunit of the molecule and the test line bound to the intact hCG molecule (i.e.: α and β subunits).

With regard to the polyclonal antibody, it was initially thought that an antibody raised against the α-subunit of hCG might cause crossreactivity problems with the homologous α-subunits of the other peptide hormones (see section 1.2, Introduction). It should be noted, however, that it is generally easier to produce high-affinity polyclonal antibodies (Newman and Price, 1997a). Also if a high affinity anti-β-hCG monoclonal antibody was used for the gold conjugate this would ensure that only molecules of intact hCG would bind to the test line. This is explained by the fact that free β-hCG isoforms and other hCG metabolites present in some samples would not be capable of binding to the anti-α-hCG test line antibody. It is preferable to have a pregnancy test for the of the detection of intact hCG only. This is due to the significant variation and discordant results reported for tests which rely on anti-β-hCG antibodies for the detection of hCG (Cole *et al.*, 1993a). It should be noted that free β-hCG is an extremely minor component of normal pregnancy serum, making up <1% of the hCG concentration (Cole, 1997). Many commercial pregnancy tests rely on the use of anti-β-hCG detector reagents because of the homology that exists between the α-subunits of the glycoprotein hormones. Specificity for a particular

glycoprotein hormone is governed by its β-subunit (see section 1.2, Introduction). However, it is known that many isoforms of the hCG molecule can exist (see section 1.4, Introduction). It is also known that some of these isoforms, particularly β-hCG, can be present at different levels in the urine and blood of the individual at the same time (Norman *et al.*, 1987). Obviously, for the purposes of the assay being developed within this thesis (i.e.: one, which detects hCG in both urine *and* serum) such a situation must be avoided. This, then, can only be possible by the use of an antibody pair which detects intact hCG only.

Quite variable results were achieved with antibodies raised against the same isoform of the hCG molecule. This was noted, in particular, for the antibodies raised against β-hCG (antibodies no. 2 and nos. 4-7). However, this variation in affinity for the hCG antigen corresponds with current theory. It is possible to have more than one non-identical antibody reactive to an antigenic site, each with a different affinity depending on the composition of the idiotype of the antibodies in question (Steward and Steensgaard, 1983). Alternatively, it is also possible that the antibody molecules were reacting with different antigenic determinants on the β-hCG molecule (Bassion, 1989). Also, since the gold-conjugated antibody was raised against β-hCG it is probable that, due to the positioning of some of the antigenic determinants, steric hindrance played a role in the poor results obtained for antibodies nos. 2 and 4 (Newman and Price, 1997a). Antibody nos. 3, 5, 6 and 7 all displayed poor positive/negative discrimination, therefore, the low affinity of these antibodies for the antigen (i.e: the hCG molecule) was not sufficient for this assay.

Following further evaluation (section 3.2.2 and 3.3) the polyclonal anti-α-hCG antibody (no. 9) was chosen as the test line antibody. Consequently, since a polyclonal antibody was selected for use as the test line antibody, it was possible to avoid the licensing restrictions and legal ramifications on the use of paired monoclonal antibodies in assays which use the 'sandwich' format (i.e.: the assay format being utilised for this test). This would have increased the cost per test due to royalties incurred by licensing U.S. patent no. 4376110 assigned to Hybritech, in respect of the detection of antigens by the use of two monoclonal antibodies in a 'sandwich' format.

4.2 The selection of a suitable gold conjugate

The question arises at this point in the discussion as to why a gold-labelled conjugate was used for this rapid pregnancy test since so many other market leaders have found success using latex conjugates (e.g.: Clearview by Unipath; OSOM hCG Combo by Wyntek Diagnostics) (see Table 1.10.1, section 1.10, Introduction).

There are several important reasons as to why gold labelling was chosen over latex beads. Due to the significantly larger size of latex beads, generally 0.1-0.4μm for diagnostic strip tests (Singer, 1992), it is possible to achieve very clear and dense lines on a lateral flow assay. However, because of the size of the particles the sensitivity of the assay can be compromised due to significant steric hindrance of the large antibody-coated latex beads. The use of antibodies conjugated to such large labels can also be limited by the high non-specific binding of the label (Gosling, 1997). This effect is much reduced for gold-conjugated antibodies. Throughout the development of this test 40nm gold particles were used for the conjugation based on recommendations from gold conjugate manufacturers (Chandler, 1997). Typically, an antibody of approximately 150,000 Da molecular weight will have a linear dimension of 8nm (Chandler, 1999). Thus a 1nm gold particle, attached to the Fc portion of the antibody will hardly impede the antibody's activity. A 40nm gold particle, albeit more visible, will produce increased steric hindrance by its proximity to the antigen binding region of the antibody (Chandler, 1999). It is clear then, that the attachment of a latex particle of size 0.1-0.4μm would have considerably greater steric hindrance effects on the binding of the antibody to the antigen (in this case, the hCG molecule) than that seen for a 40nm gold label. Despite that, theoretically, there are less antibodies bound to the smaller gold label than to the larger latex bead due to the smaller available surface area, the sensitivity is better with the smaller gold-labelled conjugates. The increased sensitivity with the smaller particles is directly as a result of more of the antibodies on the gold label being free to bind to the hCG antigen due to the smaller nature of the label. Obviously, the steric hindrance effect could be reduced when using latex conjugates if suitable spacer arms were utilised (Griffin *et al.*, 1994). However, the cost and time factors involved in producing such complex labels were not acceptable. The aim was to provide the customer with a low cost assay and to develop a test which was relatively straightforward to manufacture

Criteria for the selection of a conjugate
① *Sensitivity*
② *Colour desired*
③ *Amount of antibody required for conjugation*
④ *Membrane type used in device*
⑤ *Ease of preparation and reproducibility*
⑥*Stability*
⑦*Scale-up issues*

Table 4.3: *The criteria for selecting a conjugate for a rapid membrane-based immunoassay. (Recommendations from Colanduoni, 1997 and Gosling, 1990).*

as opposed to the highly labour intensive procedure, which would be required for the manufacture of such latex conjugates.

The criteria used for the selection of a suitable conjugate are detailed in Table 4.3 above. These criteria can be applied to the selection process, which was conducted during the development of the 'in-house' assay.

① Section 3.3 of the results section dealt with the evaluation of six gold conjugates with two different test lines and with the subsequent selection of the most sensitive and specific gold-labelled antibody. As described on the previous page, the sensitivity of a labelled antibody can be dependent on the size of the attached label. It should be noted with regard to conjugate sensitivity that the size of the gold label used can easily be controlled by various reduction methods (DiScipio, 1996; Hermanson, 1996 and Handley, 1989).

② Since only one analyte was to be detected in this assay only one colour was required for the detector label therefore, a 40nm gold label, which provided strong red colour due to its high label content (Colanduoni, 1997) was ideal for use in this assay with regard to label colour.

③ During the conjugation procedure antibody is added to gold sols in order to stabilise the sol, thus resulting in binding of the antibody to the gold beads (i.e.: the formation of gold conjugates) (see section 1.11.4.2, Introduction). The amount of antibody required to stabilise each mL of gold sol for this test was similar for all of the gold-labelled antibodies examined in section 3.3 (results not shown). Typically, the average amount of antibody required per litre of gold sol was 6-10mg, which would provide enough gold conjugate for approximately 17,000 tests.

④ The membrane type (no. 2, see Appendix I), which was kept constant for these initial studies, provided a fast flow of sample/reagents (refer to section 1.11.4.1, Introduction). It has been suggested that the pore size of the membrane used should be $1/10^{th}$ of the size of the detector label to allow free movement of the labelled antibody on the membrane (Bangs and Meza, 1995). As the gold particle was only 40nm in diameter it was not anticipated that the gold conjugate would be hindered in its progression up the test strip as a result of the pore size of the membrane.

⑤ No reproducibility problems had been observed for gold conjugates that had previously been manufactured in Trinity Biotech. These gold conjugates were constantly under validation by Trinity Biotech staff, who regularly monitored the sensitivity and specificity of completed tests using these reagents. It was, therefore, decided to continue with the use of a similar conjugate for this assay.

⑥ The stability of the selected gold-labelled antibody was monitored as the development of the assay progressed and was seen to be satisfactory (see section 3.15, Results and section 4.9.6, Discussion).

⑦ Scale-up issues did occur in the manufacture of the gold conjugate, leading to sensitivity and specificity problems for the final product (results not shown). These problems pertained to the difficulty in manufacturing batches of more than one litre of gold conjugate at a time. This was due to difficulties in determining a method suitable for such conjugates, which ensured homogeneity of the gold conjugate solution. However, as 1 litre of gold provided approximately 17,000 tests this did not impede the production of this gold conjugate once its manufacture was kept to batch sizes of one litre and below.

Section 3.3 of the results section describes the comparison of six monoclonal anti-hCG gold conjugates with two test line antibodies. It also details the further assessment of various test line and gold conjugate antibody pairs until conjugate C (see Appendix I for details) was selected for use with test line antibody no. 9.

In order to carry out the determination of usable conjugates a positive minus negative value (P-N value) was employed. In the early stages of the development of the 'in-house' assay it was necessary to compare the positive and negative results obtained for different combinations of sprayed membrane and gold conjugate. In an attempt to facilitate more straightforward comparisons it was decided to employ a 'P-N value',

which was essentially the positive result minus the negative result obtained for a particular test combination. This P-N value was used as opposed to a P/N ratio as, for this type of assay, a negative result of 0 is not uncommon and therefore could not be used to determine a value based on ratios. This P-N value served as an indicator of the strength of the colour intensity of the positive result, in comparison with that of the negative. Therefore, it is obvious that greater P-N values were more advantageous for this assay and consequently, the aim of the testing carried out in section 3.3 was to choose the antibody which provided the highest P-N value.

Since the assay parameters were not at an optimum, all of the results for this section were recorded at 5 and 10 minutes, instead of 3-5 minutes, as is ordinarily required for rapid chromatographic hCG assays. This longer assay time allowed for a longer reaction time, thereby increasing the probability of sample being bound to the membrane and detected by the various gold conjugates under examination.

Four of the monoclonal antibodies used for conjugation were raised against β-hCG (A, C, E and F) while two were raised against the intact hCG molecule (B and D). Each of the gold conjugates had been previously evaluated with hCG positive and negative solutions to determine a working A_{520nm} at which they should be impregnated. Poor P-N values were obtained for conjugates A, B, E and F. No positive/negative discrimination was apparent for these conjugates using test line no. 8 with only marginally better P-N values seen with test line no.9. It was also observed that conjugates C and D presented much higher P-N values than the other conjugates with test line no. 8. However, these values were almost half of those seen with test line no.9. It should be emphasised at this point that test line antibody no. 8 was directed against the intact hCG molecule while test line no. 9 was directed against the α-hCG subunit. For reasons similar to those described in section 4.1 for the selection of the test line antibody it is possible that poor affinity for the analyte was responsible for the high false positive results seen for conjugates A, B, E and F with both test line antibodies. Why one antibody might have stronger affinity for the antigen than another has already been discussed in section 4.1 in relation to the test line antibodies. The same principles apply here with the possibility of greater steric hindrance problems due to the attached 40nm gold label. This is most likely what is

being demonstrated with the lower P-N value, which was seen for conjugates C and D with test line antibody no. 8, an anti-intact hCG antibody.

Gold conjugates C and D were chosen for further evaluation with test line antibody no. 9. It was found that gold conjugate D did not possess the required sensitivity (see Figure 3.3.3, Section 3.3, Results). This was most likely due to the steric hindrance between the anti-α-hCG test line and the anti-intact hCG gold conjugated antibody, which may have been binding to an antigenic determinant on the hCG molecule in close proximity to that also used by the test line antibody. From the studies carried out in section 3.2 and 3.3, gold conjugate C was optimised with the chosen test line antibody no. 9 to give the desired signal response.

4.4 Resolving specificity problems with female hCG-negative urines

Section 3.4.1 of the results section demonstrated 100% specificity when hCG negative *male* urine samples were used. However, only 48% specificity was observed with hCG-negative *female* urine samples. Up to this point male urines had been used to manufacture controls since it was difficult to attract female urine donors due to emotional fears associated with donating samples for a pregnancy test. Since the intended sample for use with this assay was either female urine or serum, this specificity problem was of great concern. The gold conjugate/test line antibody combinations were re-evaluated using only female urine samples.

What is termed as 'non-specific' binding in immunoassays, is in some cases, in actual fact, unintended *specific* reactions. In general, three types of binding reactions can be witnessed in immunoassays. These are (a) the *intended specific* reactions, (b) the *unintended specific* reactions and (c) the *unintended non-specific* reactions (Leunissen, 1999). Reaction (a) consists of the desired reaction, i.e.: the binding of the antigen, if present, to the detector antibodies (in this case the test line antibody and the gold conjugate). There may be several possible reasons for the manifestation of reaction (b) in immunoassays: the test antibodies may recognise epitopes in molecules other than the antigen the test was intended to detect (i.e.: crossreactivity). The relationship between an antibody and its antigen is never of an exclusive nature. In addition to recognising the antigen against which it was raised, an antibody will

always bind to a variety of related antigens that share some structural features with the antigen used for immunisation (Regenmortel, 1997). Immunological non-specificity is due to the presence of the same (shared reactivity) or similar (cross-reactivity) epitopes on different antigen molecules (Tijssen, 1985). This was a logical explanation for what was occurring with the 'in-house' assay at this point since the reaction could have been due to the presence of hormones such as luteinising hormone (LH) in significant quantities in female urine and which has a structure very similar to that of hCG (see section 1.2, introduction). Consequently, this molecule would be likely to cross-react with the assay test line and/or gold conjugate. Reaction (b) can also be caused by the presence in the sample material of circulating IgG to various antigens to which the subject has been exposed. Normal adult serum IgG levels are in the range 8-16mg/mL (Jackson, 1989). Some of this circulating IgG may have been capable of recognising an antigenic determinant on the antigen being detected. This was unlikely to be the reason in this instance as urine samples were used, which contain little or no IgG in solution (Boehringer Mannheim, 1999). Reaction (c) is governed by general physico-chemical properties such as hydrophobicity and charge determined forces. The characteristics of the sample, solid phase (for this test, nitrocellulose) and the antibodies utilised in the assay have considerable influence, causing the labelled antibody to bind *non-specifically* to the solid phase or test line (Leunissen, 1999). Porous materials such as nitrocellulose should not be considered inert as the pretreatment of the surface of this membrane can largely affect whether or not non-specific binding will be a problem for a particular assay (Price *et al.*, 1997). This phenomenon is generally removed by 'blocking' or coating the unreacted sites on the nitrocellulose membrane with one or more of: proteins, surfactants and man-made polymers (Jones, 1999(c); Esser, 1997; Liddell and Cryer, 1991; Jitsukawa, 1989 and Harlow and Lane, 1988). Some have reported that this 'blocking' is unnecessary and sometimes harmful (Gosling, 1990; Mohammed and Esen, 1989). The nitrocellulose membrane was not 'blocked' for this assay as blocking generally reduces the flow rate of the sample and reagents on the test surface. This would not be satisfactory for a test to be interpreted at 3 minutes for urine and 5 minutes for serum. It was unlikely that reaction (c) had caused the disappointing results seen with female urines because it would be expected that a general 'background' problem would exist for the assay if this was

so. Since 'background' problems were not an issue with this particular gold conjugate it was concluded that the false positives were due to reaction (b).
The problem of reaction (b) can be avoided by the use of antibodies with higher affinity for the antigen being detected. This can be achieved by the examination of other monoclonal antibodies or by the preparation of antisera with the purest available form of the antigen (Leunissen, 1999). Nevertheless, it was known that the antibodies selected for the test line and conjugate had demonstrated the highest affinity for the hCG antigen in male urine. Additionally, gold conjugates A, B and D were re-examined with female urines and as seen for male urines, displayed notably lower affinity for hCG than the chosen antibodies. These conjugates also demonstrated strong background binding to the nitrocellulose membrane. This phenomenon would have undoubtedly created difficulties in the interpretation of low positive and negative results. Consequently, it was decided to maintain the selected antibodies within the assay format but to attempt to 'block' the occurrence of reaction (b) as described earlier with the use of various non-specific serums in the conjugate in an attempt to eliminate false positives (Harlow and Lane, 1988).

Various serums, goat, human and rabbit serum and FBS and mouse IgG were included in the gold conjugate solution (refer to section 3.4.2, results). It was hoped that the inclusion of such serums, which ordinarily contain substantial levels of IgG molecules, generally about 10mg/mL of total IgG (Harlow and Lane, 1988), would ensure that the unknown component contained in female urine which was responsible for the non-specific binding would be bound by the free IgG in these serums. This unknown component would thus be unable to bind to the test line antibody.

It should be noted that the human serum used was from a non-pregnant patient from the Rotunda Hospital, Dublin. This serum sample was included at lower concentrations than the goat and rabbit serum due to the possibility of the presence of potentially interfering human serum proteins or substances (e.g.: luteinising hormone (LH) or follicle stimulating hormone (FSH)). It was also possible that safety issues may have arisen with the use of large volumes of human serum in the manufacture of the test. Male serum was not used because of the conflicting results observed with male and female urines in the assay. The mouse IgG was added at similarly lower percentages since the active component being sought (i.e.: IgG) was in its purified state and therefore, lower concentrations of this solution were required in comparison

with that used for the various serums. This is because the reactive molecule (i.e.: IgG) in this solution is not masked by other serum constituents, as would possibly occur with the serum solutions.

The results for each sample were recorded at 3, 5 and 10 minutes. Since the test was at a more advanced stage it was feasible to test at 3 minutes, i.e. the proposed reading time for the test with urine samples. It was necessary to read the tests at 5 and 10 minutes to monitor the results for the negative samples to observe whether or not there were false positive problems with the combinations being tested.

20% (v/v) goat and rabbit serum gave good P-N values for female urines as did 5% (v/v) mouse IgG. It is possible that the result seen for the 20% (v/v) rabbit serum could have been improved even further by the addition of a higher percentage of serum. However, since excess serum in the conjugate solution could contribute to the destabilisation of the gold conjugate (Leunissen, 1999; Hermanson, 1996) it was not feasible to examine such higher percentages of this serum. Due to its reducing effect on the positive C.I result and equally due to its cost element (i.e.: purified IgG as opposed to untreated serums) the mouse IgG was not selected as the 'blocker'.

4.5 The first clinical trial and methods to resolve issues arising from this trial

Section 3.7 of the results section describes the outcome of the first internal clinical trial. In summary, while the 'in-house' assay displayed identical sensitivity (98.9%) to that observed for the ABI reference assay, three problems were identified with the assay format at this point in the development. These were ① weak control lines (observed for 24/116 samples), ② false positive results ('in-house' specificity was only 70% (ABI-95%)) and ③ uneven release of the gold conjugate. This section of the discussion deals with attempts to overcome these problems and the reasons why such approaches were taken.

4.5.1 Optimisation of the control line

A good control line is a vital part of any rapid assay, since it protects the consumer from relying on invalid test results. The ideal control line for a rapid assay is one which reacts with equal colour intensity for both negative and strongly positive

samples. The principle by which the control line works is described in section 1.11.3 of the Introduction.

There were two possible reasons why the control line yielded low colour intensities. Firstly, the release of the rehydrated gold conjugate from the gold conjugate pad, upon the addition of the liquid sample for the 'in-house' assay was unsatisfactory, thereby resulting in control lines and test lines which were *less* intense than those observed for the ABI reference test. This problem is addressed in section 4.5.3. Several test results demonstrated faint control lines, but unlike the tests described above, these were accompanied by test lines which were *more* intense in colour than those noted for the ABI test with the same samples. In other words, not enough excess gold conjugate was available to bind to the goat anti-mouse (GAM) antibody on the control line. The high concentration of the antigen was bridging most of the available conjugate with the test line antibody, leaving little gold conjugate free to bind to the GAM antibody. These particular 24 samples, which displayed faint control lines, were most likely strong positives. This would leave smaller amounts of unbound gold conjugate available to bind with the control line, which concurs with the theory outlined above. It appeared that the test line antibody used in the 'in-house' assay had a higher affinity for the hCG antigen than that used in the ABI assay. Since it was not desirable to employ a test line antibody of lesser affinity, due to the effect this might have had on the detection of lower levels of hCG, it was decided to supply an excess of gold conjugate by adding a non-specific gold conjugate to the gold conjugate solution impregnated on the gold conjugate pad. The ideal non-specific gold conjugate was one which would bind only to the control line and would have little or no affinity for either the antigen or test line antibody. This was successfully achieved by the addition of anti-HepB gold conjugate in the gold strip, which was shown not to interfere with the performance of the assay (see section 3.8.1).

4.5.2 Examination of problems associated with false positives

With regard to the false positive issue, several approaches were taken. These are described in the following pages.

① The affinity of the 'in-house' assay antibodies and antibodies previously evaluated were examined by dot blotting to determine if the correct test antibodies had been selected (section 3.8.2). This study confirmed that test line antibody no. 9 had high affinity for the hCG antigen. The antibody used for gold conjugate C had satisfactory, albeit somewhat lower, affinity than that observed for antibody no.9. Ideally, these antibodies should also have been evaluated against the LH antigen to ensure that no cross-reactivity existed; however, the reagents for such an experiment were not available at the time. Both the rabbit anti-mouse and goat anti-rabbit-HRP conjugates were used at identical concentrations to ensure that there was no variation in the method. However, it should be pointed out that it was stated in the relevant product catalogue that the rabbit anti-mouse-HRP conjugate required a minimum titre of only 1 in 40,000 (ELISA) whereas the goat anti-rabbit-HRP conjugate required a minimum titre of 1 in 12,000 (ELISA). Although these figures are with reference to ELISA tests, they indicate that the rabbit anti-mouse-HRP conjugate has a higher affinity for its antigen than the goat anti-rabbit-HRP conjugate. Therefore, the true affinity of the rabbit anti-α-hCG is not fully reflected in the blot seen in section 3.8.2, Results. As the test line antibody displayed such high affinity for the hCG antigen and was known to be a highly purified polyclonal, it was decided to retain these previously chosen antibodies as the test line and gold-conjugated antibodies.

② The inclusion of an anti-luteinising hormone (anti-LH) line sprayed 2mm below the test line was also examined. LH is a glycoprotein hormone possessing considerable structural similarities to hCG. The α-subunits of the glycoprotein hormones are essentially identical (Gabbe *et al.*, 1996). The glycoprotein hormones also exhibit significant similarity for their β-subunits. Most importantly, the β-subunits of hCG and LH display 80% homology in their amino acid sequences (Bischof *et al.*, 1997) (see section 1.2.2, Introduction). The potential for LH interference in hCG assays has been a subject of much scientific debate. In some cases it is reported that many hCG assays have fallen victim to the high immunologic cross-reactivity with LH (Bischof *et al.*, 1997). Others suggest that while this may have been the case for earlier generations of hCG assays, today's immunoassays have little or no cross reactivity with LH (Cole and Kardana, 1992). However, since no evidence was available to confirm or reject either hypothesis, it was decided to

evaluate the benefits (if any) of spraying an anti-LH line beneath the sprayed test line antibody.

By placing an anti-LH line beneath the test line it was intended that this line would bind out any LH, which may have been present in considerable quantities in some samples and consequently, may have been cross-reacting with the test line antibody due to the structural similarities between LH and hCG. Some of the negative samples which gave positive readings were from gynaecology patients who may have been undergoing fertility treatment with hormones or who may have donated their samples at the peak of their LH cycle. It was hoped that the presence of this anti-LH line would reduce the positive C.I. seen for the negative samples, which were evaluated in the first clinical trial, without affecting the true positive results. Indeed, from section 3.8.3 it is apparent that the opposite was reported. In attempting to explain these results it is presumed that since the positive results were reduced on the anti-hCG line, and quite high on the anti-LH line, that the anti-LH antibody has a certain affinity for the hCG antigen, thus removing some of the available hCG antigen for binding to the hCG test line. This is quite possible if the antibody was raised against a region of the LH molecule, which may be homologous with certain areas on the hCG antigen (see section 1.2.2, introduction). The high C.I. results seen for the anti-LH line with the negative sample would indicate that this sample did, as suspected, contain a significant amount of LH. The increased C.I. results for the anti-hCG line with the same sample is most likely an exaggeration of the false positive seen without the anti-LH line due to the proximity of high concentrations of bound LH on the anti-LH line, which was positioned just below the anti-hCG line.

4.5.3 Improvement of the release of the gold conjugate from the gold conjugate pad

Of fundamental importance to any immunoassay is the availability of the detector reagents employed to capture the antigen of choice. In immunochromatographic assays, such as the one developed within this thesis, the solid phase detector reagent, i.e.: the test line antibody is readily available for binding of the antigen, in this case, hCG. The availability of the second detector reagent, i.e.: the gold-conjugated antibody is dependent upon the extent to which it is rehydrated within the sample pad and subsequently released from the gold conjugate pad and further still on its

progression, if any, up the nitrocellulose test strip to reach the test line antibody. Sufficient sample volume (250μL) was added to each test to ensure that all of the impregnated strip (gold conjugate pad) beneath the sample pad (see Figure 1.11.2, Introduction) was completely rehydrated and available for release from the gold conjugate pad. The release from this pad is affected by several parameters. The mesh structure and composition of the material used as the gold conjugate pad play a significant role in the release of the conjugate, since they can physically hinder the progress of the gold conjugate through the pad. Another critical choice concerned with the release from the pad is the selection of the buffer in which the gold conjugate is dried and impregnated, as this can alter the flow characteristics and stability of the resuspended gold conjugate solution (Weiss, 1997a; Wells, 1997).

The most suitable material with regard to release of the gold conjugate and end test result (Jones, 1999d; Weiss, 1997a; Wells, 1997) was selected for use as the gold conjugate pad in section 3.8.4, Results. This study verified that optimum release was obtained with a glass fibre pad. The importance of a good conjugate release material cannot be stressed enough. According to Weiss (1997a), the role of the conjugate pad is to ① hold a consistent volume of the detector reagent, ② transfer the sample volume consistently onto the membrane, ③ maintain the stability of the detector reagent, ④ to release the detector reagent consistently and quantitatively, ⑤ to control the volume of sample to be analysed (i.e.: the volume which migrates *before* or *with* the detector reagent) and ⑥ if blocked, to prevent non-specific binding. For this assay a glass fibre material was employed as the gold conjugate release pad. Glass fibre is known to be very wettable, moderately low protein binding with a moderate to high bed volume (Jones, 1999d). From section 3.8.4 it was confirmed that glass fibre was the most suitable release pad, as it absorbed the greatest volume with ease and delivered good positive/negative discrimination when used in an assay.

Following this, studies were carried out determining the effect on the release of the gold conjugate by various gold conjugate dilution buffers (GCDB), in which the gold conjugate was suspended before impregnation. It is obvious that the components contained within this buffer could play a significant role in determining the stability, release upon hydration and performance of the gold conjugate from the dried glass fibre pad (Wells, 1997). Several components were added to this buffer in order to

rectify the poor gold conjugate release and to decrease the frequency of false positive results, as observed in the first clinical trial.

In section 3.8.5 it is described how Triton® X-100, Tween® 20 and PEG ranging from 0.2 up to 20K in size were added in various combinations to the GCDB. These buffers were examined for flow rates on the strip and end test result, in liquid format as opposed to within the dried conjugate strip. The buffers were examined in liquid format as the examination of such an array of buffers (16 in total) with various samples would have proven far too time consuming and would have significantly depleted large quantities of other test components. The reasons why these reagents were selected for inclusion in the GCDB are many.

Triton® X-100 and Tween® 20 are both non-ionic surfactants of low molecular weight. The term surfactant is generally used when referring to wetters, solubilisers and emulsifiers. Surfactants have a bipartite structure consisting of a distinct hydrophobic and hydrophilic part (Neugebauer, 1992; Harlow and Lane, 1988). At this point it should be noted that gold conjugates have a net negative charge and are hydrophobic (Leunissen, 1999) and similarly the nitrocellulose membrane is also hydrophobic. In most cases, the membranes are pre-treated with surfactants during or post manufacture to make them more wettable (Harvey, 1991), but it is not uncommon for these manufacturing treatments to evaporate from the membrane over time, thereby exposing the hydrophobic membrane surface once again (Millipore, 1996). Because of their bipartite structure, as mentioned above, surfactants have the ability to disperse hydrophobic molecules in aqueous medium. It was hoped that the ability of Triton® X-100 and Tween® 20 to disperse such molecules would aid in the even distribution of the gold conjugate upon addition of the sample to the test, thereby allowing for a consistent flow of the detector reagent up the membrane strip. Additionally, surfactants are almost always employed to reduce attractive interactions between like particles and bring them to unlike surfaces (Neugebauer, 1992; Esser, 1997). Clearly, this effect would aid in the distribution of the gold conjugate (hydrophobic) to the nitrocellulose membrane (hydrophilic, when post-manufacture treated). Since most surfactants possess wetting properties (i.e.: they reduce the surface tension of a liquid), the presence of surfactants in the gold conjugate strip would, therefore, improve the re-wetting of the membrane upon

sample addition. Surfactants can also suppress many hydrophobic interactions, which could cause non-specific binding and are capable of breaking up existing hydrophobic complexes due to surface forces (Leunissen, 1999; Liddell and Cryer, 1991). All of these above properties have, therefore, significant application in the immunochromatographic test system and would assist in the aim of these experiments i.e.: to improve the release and flow of the gold conjugate. Triton® X-100 was selected not only for its properties (described above) but because it is reported as being very compatible for applications using enzymes (Neugebauer, 1992), which was perceived as being beneficial to the application for this assay. Tween® 20 was also chosen for the reasons previously mentioned and also because it is a non-haemolytic surfactant. This latter property was thought to be an advantage should the test ever be required to run using whole blood samples, a requirement which is becoming ever more popular (Newman and Price, 1997c). Additionally, it was known that some surfactants (e.g: Tween® 20) have a significant impact on the membrane's ability to bind proteins (Millipore, 1996).

The PEG molecule consists of repeating units of ethylene oxide, which terminate in hydroxl groups on either end of a linear chain. Most of the forms of PEG useful in bioconjugate applications have molecular weights less than 20000 (Hermanson, 1996). PEG coupled to other molecules has been used to increase the stability of proteins in solution and to prevent protein adsorption to surfaces. The properties of PEG in solution are especially unusual, frequently displaying amphiphilic tendencies and having the ability to solubilise on hydrophobic membranes. Whether in solution or attached to other insoluble surfaces, PEG has a tendency to exclude other polymers and forms a protein-rejecting region that is effective in preventing non-specific protein binding (Hermanson, 1996). It is for these solubilising properties and its capabilities to reduce non-specific binding that PEG was chosen for addition in the GCDB.

The study carried out (section 3.8.5) confirmed the hypothesis that the addition of Tween® 20 and Triton® X-100 would be beneficial for the assay. The test results with the lowest non-specific binding of the gold conjugate to the membrane were buffers nos. 15 and 16, which contained Tween® 20 (no.15) and Triton® X-100 (no. 16). Both of these buffers also contained PEG 15-20K. These buffers provided an

even flow and distribution of the gold conjugate on the strip and the highest intensity control lines. It was also observed from the results of this study that the larger PEG polymer (i.e.: 15-20K) was required to add viscosity to the sample/gold solution and to provide an even and consistent flow of gold conjugate (Bangs and Meza, 1995). The addition of PEG (15-20K) in combination with either of the detergents (Tween® 20 or Triton® X-100) gave rise to the most satisfactory test and control line C.I. results. Those tests run using buffers with smaller sized PEG molecules displayed faster flow and an uneven release of the gold conjugate from the gold conjugate pad.

Due to the success of this study it was decided to examine these and other reagents at various concentrations (see section 3.8.5i). The reagents examined were Triton® X-100, Tween® 20, PEG (15-20K), PVP K-30, BSA and sucrose. The concentrations selected for evaluation were based on experimental results obtained when using these components in other Trinity Biotech rapid assays.

PVP K-30 was selected due to its known functions as a film former, a protective colloid and suspending agent and binder and stabiliser (ISP, 1994). It was anticipated that these properties would have the benefit of keeping the gold conjugate stable under the harsh conditions of the drying procedure carried out after impregnation. It has already been reported that attachment of such polymers to labelled proteins can provide cryoprotection for proteins, which are sensitive to freezing (Hermanson, 1996). The viscosity that this polymer would lend to the reconstituted dried gold conjugate would ensure that the solution would travel up the membrane in an even manner (Williams, 1997).

BSA was included in the study as it was already used in the gold conjugation method as a blocking agent. Therefore, its addition to the GCDB was justified in that it would be expected to mask any remaining sites of gold-gold or protein-gold interactions, thus preventing aggregation of the gold conjugate or non-specific binding during the assay (Hermanson, 1996).

Sucrose was added to the GCDB since it has been suggested that protecting the antibody-coated gold from aggregation by 'treating' the gold conjugate and/or the membrane where they would be dried with a hydrophilic substance, such as sucrose,

would allow the dried conjugate to readily rehydrate upon addition of the sample (Bangs and Meza, 1995). Sucrose also confers some viscosity on the rehydrating solution enabling the solution to travel evenly towards the top of the test strip.

Each buffer at each concentration was examined for sensitivity and specificity, the flow speed and appearance and the buffer front. The buffer front is the distance between the flow 'front' of the sample and the gold conjugate solution as they progress up the test strip. Large sample/gold conjugate fronts are to be avoided. This is where the sample washes ahead of the gold conjugate while flowing up the membrane and can result in the test line antibody being washed off the membrane. The concentrations of Triton® X-100 and Tween® 20 chosen for the final buffer were intermediate levels (see Table 3.8.8). These were selected as they balanced the positive effects (such as keeping the gold conjugate monodispersed) resulting from their addition with their possible negative effects, such as the removal of the membrane bound antibody (Harvey, 1991; Millipore, 1996; Wells, 1997). The sucrose and BSA levels chosen corresponded to concentrations which were already being used in Trinity Biotech for other similar assays. The selected concentration for sucrose was also within the range, 5-15% (w/v) as recommended by Wells (1997). The concentrations of PEG (15-20K) and PVP K-30 chosen were the lowest concentrations examined. This was based on advice from Millipore, a nitrocellulose manufacturer and ISP, a PVP manufacturer, that as little of these polymers should be used as possible with an absolute maximum of 0.5% (w/v) recommended (Millipore, 1996; ISP, 1994). This is because both PVP K-30 and PEG interfere with the electrostatic binding of proteins to the nitrocellulose membrane. While this is an advantage at lower concentrations for removing non-specifically bound proteins, it can be detrimental to the sprayed test line antibody at higher concentrations.

This new GCDB was validated in section 3.8.5ii by directly comparing gold conjugate strips dried in the GCDB substituted with these chemicals with those in the original GCDB, keeping all other test components constant. Higher C.I. results were observed with the new GCDB for both the positive test results and the control line. This confirmed that better gold conjugate release was obtained. Significantly, improved specificity was observed with negative urines from the first clinical trial, which had previously caused false positive results with the original GCDB. Also,

100% specificity was noted when 30 negative urines and 30 negative serums were evaluated. This confirmed that some of the added chemicals functioned as blocking agents.

4.6 Evaluation of test line thickness and position

Despite the improved specificity achieved by the optimisation processes described in section 4.5, one out of the three negative urines from the Rotunda Hospital gave a low positive result. This indicated that the issue of specificity was not fully resolved. Another method for improving the specificity of the assay was to determine the effect of moving the spraying position of the test line antibody and the thickness of the line sprayed. The principle behind this idea was that the closer the test line is to the gold conjugate pad the longer the period of exposure to the gold conjugate/sample solution. For example, a test line at the top of the membrane would be in contact with the gold conjugate/sample solution, which is running up the test strip, for a much smaller percentage of the assay time than a line, which is sprayed at the bottom of the membrane. In other words the reaction time for the antigen/gold conjugate to bind to the test line is longer for strips closer to the bottom of the membrane. A test line position of 12mm from the bottom edge of the membrane was shown to be the most suitable, which verifies this theory. Less non-specific binding was also observed at this position due to the shorter reaction time. The test line thickness was set at 1mm as this provided the most acceptable sensitivity and specificity. Specificity problems were noted for thicker test lines as a result of the higher amounts of antibody present, which increases the chances of non-specific binding occurring.

4.7 Methods to decrease the assay time for serum samples

An aim of this project was to develop a test suitable for use with either urine or serum samples. It was necessary to reduce the flow time of the in-house assay so that it would conform to assay times used by competitor kits on the market (see Table 1.10.1, Introduction). For example, the ABI reference kit allowed urine samples to be read at 3 minutes and serum samples to be read at five minutes. Due to the different nature of the samples themselves (i.e.: serum being significantly more viscous than

urine) a compromise with regard to assay flow had to be achieved in order that the above assay times be met for both samples on the one test.

This was achieved on three fronts: ① by changing the nitrocellulose to a faster flowing membrane, ② by selecting a sample pad which allowed a faster throughput of sample and ③ by increasing the sample volume.

As can be seen in section 3.11.1, three membranes were examined. Each had to be individually optimised because as the pore size of the membrane changes, so too does its protein binding capacity. Increasing the porosity in a membrane decreases the available surface area for protein binding (Harvey, 1991); therefore, this must be compensated by adding more test line antibody to achieve similar results to the smaller pore size membrane. In addition, a faster flowing membrane would require more antibody to achieve the same result in its shorter assay time than a more slow flowing membrane with a longer reaction time. These principles are laid out in more detail in section 1.11.4.1, Introduction. From the testing carried out it was decided to change from a membrane with 5μm pore size to one with an 8μm pore size.
The replacement of the original membrane with a faster flowing membrane reduced the assay time. However, the new assay time was still not fast enough. Five sample pads were evaluated together with two sample volumes, initially on the basis of flow time. The sample pad plays an important role in the flow dynamics of the rapid chromatographic test. Its function is to control the flow of the gold conjugate and sample onto the strip, to remove particulate matter from the sample and in some cases it can be used to adjust the pH or viscosity of the sample before reaching the membrane (Weiss, 1997b). A sample pad which allowed for faster release of the sample and test reagents onto the nitrocellulose, accompanied by the use of a larger sample volume, was shown to provide the desired assay times for both urine and serum without affecting the end test result. It was important that the sample volume was not increased beyond the absorption capacity of the sample pad material, as this would have resulted in leaching of the sample off the sample pad and directly onto the membrane without having passed through the gold conjugate pad. Additionally, a very short flow time which would have reduced the signal for the positive results and control lines, would have resulted.

4.8 The selection of a suitable detergent for the wick

The wick or 'stick' assay was essentially identical to the cassette assay with the exception of the wick and the plastic housing. The same test strip was contained in both versions of the test. Since the housing was inert and played no significant role in the use of the assay, the only important element of the wick test, which required examination was the wick itself. Its function was purely to deliver the sample as quickly as possible to the test strip so that the assay time could be identical for both test formats. The benefits of surfactants and their wetting and solubilising properties are described in detail in section 4.5.3. Due to these beneficial properties it was decided to impregnate the wick (see section 3.12) with various detergents to determine which would deliver the sample quickly and evenly, without affecting the test results. From the studies carried out in section 3.12, 2% (w/v) of Triton® X-100 fulfilled the above criteria and this concentration was incorporated into the wick before assembly with the test strip.

4.9 Validation trials on the final 'in-house' assay format

4.9.1 Evaluation of HAMA and HA positive samples with the 'in-house' cassette and wick assay formats

The importance of assessing a hCG 'sandwich' assay for interference by human anti-mouse antibodies (HAMA) or heterophilic antibodies (HAs) was clearly demonstrated recently (Cole *et al.*, 1999a). This case, involving HAMA and/or HA interference in a hCG assay is currently the subject of a lawsuit in the USA involving several women who were allegedly misdiagnosed based on their hCG test results. Based on persistent elevated hCG results for several months using the Abbott AxSym and Abbot IMX hCG assays, four patients received multiple courses of chemotherapy, two patients underwent hysterectomies and one other underwent an oophorectomy. It was discovered at a later date that all six cases contained no intact hCG, subunits or metabolites. Such persistently positive results on hCG assays, which are later shown to be incorrect are termed 'phantom' hCG results. The 'phantom' hCG results were most likely due to either HAMA or HAs as previously demonstrated by Cole (1998).

The effects of HAMA and HAs in patient plasma and serum is well documented (Cole *et al.*, 1999a; Fitzmaurice *et al.*, 1998; Boerman *et al.*, 1990; Nahm and Hoffman, 1990; Boscato and Stuart, 1988). These antibodies generally arise from treatment given to patients involving mouse monoclonal antibodies (e.g.: radioimaging of tumours). Significantly, it should be pointed out that such antibodies can also arise as a result of incidental or occupational exposure to foreign proteins, as in the case of veterinarians, farm workers and food preparers or by the presence of domestic animals in the home environment (Fitzmaurice *et al.*, 1998). It has been estimated that the prevalence of HAMA and HAs in the general population is as high as 40% (Boscato and Stuart, 1988 and Nahm and Hoffman, 1990). These HAMA and HAs are typically IgG antibodies which recognise epitopes on the Fc portion of the foreign immunoglobulin; some, however, have also been known to bind to the Fab portion (Fitzmaurice *et al.*, 1998). The presence of high titers of such antibodies in some samples leads to false positive results in 'sandwich' assays by cross-linking the capture and label antibodies in the absence of the specific antigen (see Figure 4.1 (a)). These antibodies can also falsely decrease assay results by affecting analyte recognition by the capture and label antibodies. This is possibly due to steric hindrance as a result of the HAMA and HAs binding to the capture and/or label antibodies which may form complexes of various sizes (Boscato and Stuart, 1988) (see Figure 4.1 (b)).

No interference was observed for the 'in-house' assay with any of the HAMA or HA-positive samples run on the assay (see section 3.14). Despite these promising results there should be no complacency with regard to HAMA and HA interference. The majority of commercially available kits, including those described as being susceptible to HAMA and HA interference, already contain some type of non-specific 'blocker' immunoglobulins, such as mouse IgG, MAK-33 (polymerised murine IgG_1, Boehringer Mannheim), HBR (monoclonal anti-human HA, Scantibodies) and IIR (formulation of anti-HAMA Ig's, Bioreclamations). It should be emphasised that failure to observe interference for these HAMA and HA samples does not guarantee against such interference occurring when the test is on the market. Many different forms of such antibodies exist and some have also been known not to be species-specific; therefore, cross-linking between antibodies of different species

can occur (Fitzmaurice *et al.*, 1998). It is probably to the advantage of the 'in-house' assay that

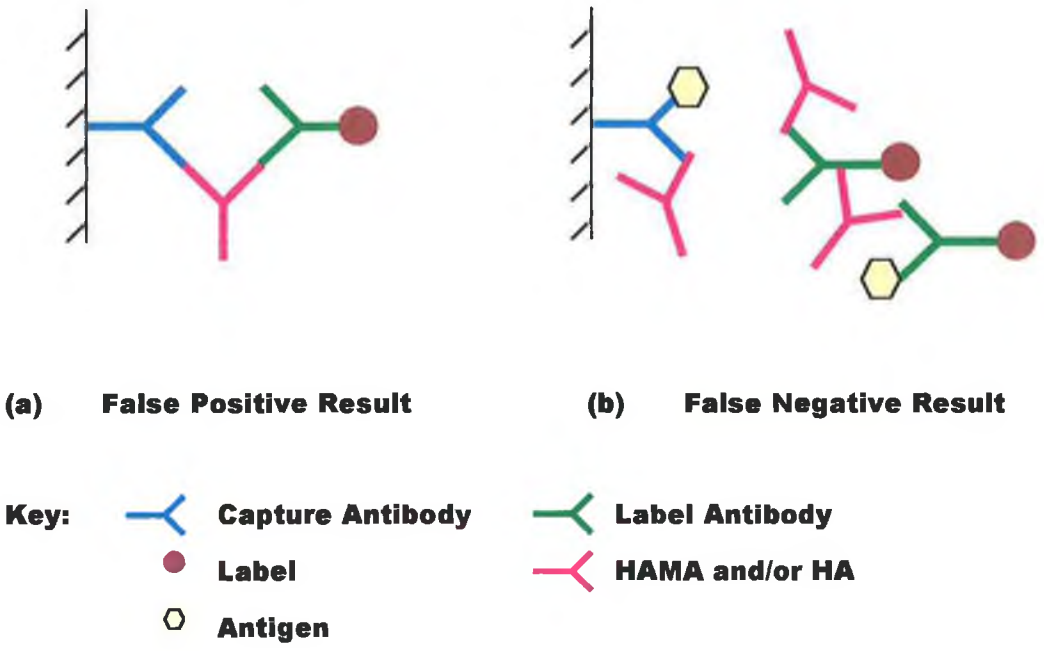


Figure 4.1: *The possible effects of human anti-mouse antibodies (HAMA) and heterophilic antibodies (HAs) on 'sandwich' assays. (a) False positive result due to the cross-linking of the capture and label antibodies. (b) False negative result due to the complexing of the labelled antibody and/or steric hindrance effects.*

such a high percentage of goat serum, 20% (v/v), is contained in the GCDB. Such a solution would subsequently consist of substantial quantities of Ig, which may non-specifically absorb HAMA and HA which may be present in the test samples.

4.9.2 Examination of potentially interfering substances on the cassette and wick 'in-house' assays

Female hCG-negative (0 IU/L) and hCG-positive (25 IU/L and 50 IU/L) urine and serum samples were supplemented with each of the substances outlined in Table 4.4. All test samples were then examined on both the 'in-house' and ABI cassette and wick assays. The substances added were either physiological components of urine and/or serum or potentially interfering drugs, which could possibly be present in significant quantities in the urine and/or serum of women who might use this test. It was, therefore, necessary to demonstrate that these potentially interfering substances did not adversely affect the sensitivity and specificity of either the cassette or wick assays being developed in this thesis. These substances may have interfered in the

assay results through unintended non-specific reactions, which are described in section 4.4, Discussion. Table 4.4 lists the substances added with a brief description in order to clarify why each particular substance was added. No interference was observed for any of the substances added on either the cassette or wick assay.

Substance	Description*
Haemoglobin	*Oxygen bearing protein found in red blood cells.*
Albumin	*Any of several water soluble proteins that are found in blood serum and various animal tissues.*
Bilirubin	*A compound derived from haemoglobin during normal and pathological destruction of red blood cells.*
Estriol	*Found in the ovaries and in the urine of pregnant mammals.*
Glucose	*A mixture of dextrose, maltose and dextrins. Fasting plasma glucose levels are between 80-100mg/dL of serum.*
Acetylsalicylic Acid	*Also known as Aspirin, used in tablet form as an antipyretic and analgesic.*
Caffeine	*Found in tea, coffee and kola nuts and used as a stimulant and diuretic.*
Ascorbic Acid	*A vitamin found in citrus fruits, tomatoes, potatoes and leafy green vegetables.*
Phenothiazine	*Used in insecticides, livestock anthelminitics (a compound to destroy intestinal worms), and dyes.*
Acetoaminophen	*Used in medicine to reduce pain and fever.*
Gentisic Acid	*Used to season tobacco and in perfumes, dentrifices, and germicides.*

Table 4.4: *List of potentially interfering substances and their description.*

(Descriptions obtained from Sigma-Aldrich Co., 1999).*

It should be noted that other possible interferants exist which are sometimes implicated as interfering agents in urinary assays (e.g.: creatinine, urate, urea). These potential interferants were not evaluated on the in-house assay within the scope of this thesis as these interferants were not known to interfere in hCG assays. Only those interferants which were known from literature and other competitor hCG test kit inserts to interfere in hCG assays, were investigated here.

4.9.3 Cross reactivity study examining the effect of adding the glycoprotein hormones LH, FSH and TSH to the 'in-house' cassette and wick assays.

As for the interference study, the substances being examined were added to 0 IU/L, 25 IU/L and 50 IU/L hCG female urine and serum samples and run on both the cassette and wick assays to determine if they would cross-react with the test line antibody. Such a reaction would be classed as an unintended specific reaction, which is outlined in section 4.4, Discussion. The substances tested were three hormones, which possess considerable homology with the hCG antigen (see section 1.2.2, Introduction), which could potentially bind non-specifically to the test line. These

hormones were: ① Follicle Stimulating Hormone (FSH) at 1000 mIU/mL, ② Thyroid Stimulating Hormone (TSH) at 1000 μIU/mL and ③ Lutenising Hormone (LH) at 500 mIU/mL. The hormones were added to the samples at these concentrations since these were their normal physiological levels. No cross reactivity was observed for any of the urine or serum samples spiked with these hormones on either the cassette or wick assay.

4.9.4 Study to determine when the prozone effect occurred in the assay

The prozone or hook effect can best be described as a false negative result occurring due to an excess amount of antigen (in this case, hCG) binding to all of the available detector antibodies (for this assay, the anti-hCG gold conjugate), thereby leaving no available antigen to bind to the solid phase antibody (here, the test line antibody) (Shine, 1998). Since the hCG levels in the urine of pregnant women in their first trimester can peak to 100,000 IU/L (see section 1.7, Introduction) it is apparent why the determination at what hCG level this effect may be seen is so important for a pregnancy test.

An experiment is described in section 3.14.4 to determine this point. A female hCG-negative urine was spiked with a hCG solution to give samples ranging from 100-1,000,000 IU/L hCG. It was observed that levels greater than 250,000 IU/L caused a false negative result. This was a satisfactory result and was on a par with competitor devices. One of the market leaders, the Clearview test by Unipath was reported as possessing 'an impressive detection limit and analytical range' (Price *et al.*, 1997) when it successfully detected hCG levels ranging from 19-47,800 IU/L in 130 samples (Price *et al.*, 1997). From this study, it is apparent that an analytical range which exceeded that reported for the Clearview test was well within the capabilities of the assay developed in this thesis.

4.9.5 Results from clinical trials on the final assay formats

Section 3.14.5 of the Results section describes both internal and external clinical trials, which were undertaken to determine the performance characteristics of both the 'in-house' cassette and wick assays using both urine and serum samples. Table 4.5 overleaf lists the % sensitivity and specificity obtained for both the 'in-house'

cassette and wick assays in comparison with the ABI cassette and wick devices, which were used as the reference tests.

Excellent sensitivity and specificity were obtained with both formats of the 'in-house' assay when compared with results from the ABI reference tests. These % sensitivity and specificity values were comparable with most of the rapid pregnancy tests on the market (e.g.: from ABI cassette pack insert: 100% sensitivity and specificity with urine and serum; from Wyntech Diagnostics OSOM test pack insert: > 99% concordance with another commercial assay and from BioTipp pregnancy test pack insert: 99% accurate results). Therefore, these clinical trial data in combination with the results obtained from the cross reactivity study inferred that excellent sensitivity and specificity was a feature of both of these 'in-house' assays. It would be prudent to bear in mind that these % sensitivity and specificity values are calculated for the sample population tested only.

Test	Sample	Sensitivity	Specificity
'In-house' Cassette	*Urine*	*100%*	*100%*
'In-house' Cassette	*Serum*	*98.5%*	*100%*
'In-house' Wick	*Urine*	*99.4%*	*100%*
'In-house' Wick	*Serum*	*100%*	*98%*

Table 4.5: *% sensitivity and specificity for the 'in-house' assay following internal & external clinical trials using hCG-positive & negative urine & serum samples.*

Impressive feedback was also obtained with regard to the overall appearance, operation and presentation of the assays (see Figure 3.14.5, Results). 50 volunteers from Dublin City University (DCU) were asked to assay negative and positive urine samples and controls on the 'in-house' wick test. This study was carried out with the intention of establishing a customer perception of the product and to anticipate any potential difficulties that the customer may encounter in performing and interpreting the assay results. It was preferable to discover such problems at this stage in the test development than during the transfer of the test to full production, where it would prove more difficult and costly to rectify. No problems were noted by the volunteers with regard to the test methodology and the interpretation of test results. 34% of volunteers commented that the test was easy to interpret and 30% praised the 'fast assay time'. Encouragingly, 70% of test volunteers described the test as 'user-

assay time'. Encouragingly, 70% of test volunteers described the test as 'user-friendly'. Based on the results of the clinical trials, the interference and cross reactivity studies and the customer perception study it was concluded that the development of the cassette and wick 'in-house' assays was a success.

4.9.6 Stability of the final test format

Section 3.15 concerns itself with the determination of the stability of the final format of the 'in-house' assay by accelerated means. The importance of generating stability data on a completed assay cannot be stressed enough. Such data is required by the developers of an immunoassay in order to assign a realistic shelf-life to the product and also to identify any limitations that the assay might possess.

While 'real-time' stability studies are obviously the 'gold standard' in determining the shelf life of a product, this is often not feasible, particularly if the product is new and no idea exists of how the test might vary over long periods of time. Stability is the inverse of degradation and the dominant variable affecting the degradation rate is temperature. Anderson and Scott, 1991 and Zakowski, 1991 state that the shelf-life of a product can be defined as the time that essential performance characteristics are maintained under specific handling conditions. Accelerated stability testing (stressing) is often carried out during the development of clinical reagents to provide an indication of product shelf life and, thereby shorten the development schedule. For this purpose the product is placed at high (warmer than ambient) temperatures and the amount of heat input required to cause product failure is determined (Anderson and Scott, 1991). This information can then be applied to predict product shelf life. Generally the stressed material is compared to unstressed material from the same batch. The sample recovery for the stressed product is then expressed as a percentage of the unstressed material. This utilisation of the product itself as a control is extremely valuable when no suitable reference material is available.

From the results shown in section 3.15 it can be seen that the final format of the 'in-house' assay is stable with regard to sensitivity and specificity for an accelerated time of 18 months. Specificity problems were encountered at 24 months accelerated time. Based on these results the assays could be sold with a shelf life of up to 18 months. According to Porterfield and Capone (1984), the release of a product based

on accelerated stability studies can be reasonable but the importance of regular monitoring of multiple lots at real time/temperature conditions should be acknowledged.

4.10 Future Developments

4.10.1 The continuing importance of hCG and the standardisation of hCG assays

Traditionally, hCG has been qualitatively detected for the diagnosis of pregnancy by the use of various forms of rapid immunoassays. Quantitative hCG assays have long been used to monitor the progress of pregnancy; to screen for ectopic pregnancy, hydatidiform mole and Down's Syndrome and to follow the course of trophphoblast disease, testicular cancer and certain nontrophoblastic maligancies (Cole and Kardana, 1992). The capability now exists to adapt the rapid hCG immunoassay for the detection of those conditions previously only detected by the quantitative assays. This could be achieved by the use of more sensitive rapid assays using the relevant antibody combinations for the detection of the isoform of hCG common for the condition in question (see below). The use of semi-quantitative rapid assays and what are termed as multi-analyte tests (Price *et al.*, 1997) may also prove useful.

The relationship between hCG and trisomy 21 (Down Syndrome) has been well documented (Cole, 1999b; Cole *et al.*, 1999c). The median serum hCG and free β-subunit level in a Down Syndrome pregnancy is twice the level in a normal pregnancy. This, however, would not enable a test to distinguish between normal and Down Syndrome pregnancies due to the wide variation in hCG levels in normal pregnancy (see Figure 1.7.1, Introduction). In general, what is employed to determine such genetically abnormal pregnancies is a combination of elevated hCG and free β-hCG levels with other biochemical agents such as alpha-fetoprotein, unconjugated estriol and inhibin (Haddow *et al.*, 1998). Theoretically, it would be possible to manufacture a test which detected all of these markers. As the levels of these markers in the patient's serum are important, the test capture and tracer reagents could be formulated in such a way that they would only detect levels equal to or above a pre-determined cut-off value. A new marker has also been determined for Down syndrome. This is an isoform of hCG called hyperglycosylated hCG, the

levels of which are elevated 9-fold in Down Syndrome pregnancies when compared to normal pregnancies (Cole, 1999b; Cole *et al.*, 1999c). This may prove to be a more accurate indicator of genetic abnormality in the second trimester of pregnancy (Cole *et al.*, 1999c).

The detection of ectopic pregnancies is quite difficult and can be of extreme importance in the hospital emergency room situation. The levels of hCG in such cases are generally one fifth to one fiftieth of normal pregnancy hCG levels (Cole, 1999b). As described above, it would be difficult to diagnose ectopic pregnancies based on lower than average hCG levels due to the range of levels that exists in normal pregnancy. Progesterone is another biochemical marker for ectopic pregnancy. Progesterone has been shown to have a cut off value of 5ng/mL at 4 weeks gestation, at 5 weeks 10ng/mL and at 6 weeks 20ng/mL (Stern *et al.*, 1993) while most viable pregnancies have a serum concentration of >25ng/mL (Stovall *et al.*, 1992). Clearly a test which semi-quantitatively detected a combination of these two markers, could give an indication of ectopic pregnancy in emergency situations. Such a test has already been welcomed once its result is followed up with further clinical examination (Grudzinskas and Stabile, 1993). A rapid test has been evaluated with complicated pregnancies with some success (Ikomi *et al.*, 1998).

Persistent trophoblast disease and choriocarcinoma both display higher than average levels of free β-hCG and nicked hCG (2-10% of intact hCG level in trophoblast disease and 5-40% of intact hCG level in choricarcinoma) (Cole, 1999b). By changing the antibody pair combination used for the detection of hCG one could develop a test specific for the nicked hCG and free β-hCG found for these conditions.

It should noted, however, that considerable variation exists between commercial assays due to poor selection of the antibody pair. Over eight different antibody binding sites have been identified on different parts of the hCG molecule. Each commercial test uses a different combination of capture and tracer antibodies, which recognise any two of the eight different binding sites (Cole, 1999b). Each assay measures intact hCG and one of seven other hCG-related molecules (Cole, 1997). This is a source of much interassay variation. While more than two-fold variation can be noted for pregnancy samples between different manufacturers' assays, even

greater discordance is observed for results from samples from irregular gestations, cancers and trophoblast disease (Cole, 1997). One problem with hCG assays is the heterogeneity of hCG, its different isoforms and degradation products. This problem is also compounded by the wide variation in hCG concentrations observed in serum and urine samples throughout pregnancy.

The variation seen in rapid pregnancy tests, similar to the tests developed within this thesis, is not significant enough to affect the qualitative detection of normal pregnancy urine or serum levels of hCG. However, considerable variation might be observed should such tests be adapted for the diagnosis of cancers or irregular gestations.

4.10.2 New applications for hCG

Evidence has been produced that hCG may protect the developing foetus from HIV (Notkins, 1999). This phenomenon may be explained by the fact that researchers have isolated three proteins in the urine of pregnant women. The proteins were distinct from hCG but tended to adhere to the pregnancy hormone (Bayer, 1999). Could this be the future for hCG, as a new method of AIDS treatment with few side effects due to the abundance of hCG, naturally occurring in the urine of pregnant women. (Bayer, 1999).

Also in relation to HIV, a pregnancy test which combines the detection of the HIV virus may be of use in third world countries. Rapid tests are currently available for the detection of the HIV virus. With increasing numbers of the population in African countries presenting with HIV infection, it would be useful to screen women who present with positive pregnancy test results for HIV so that treatment can begin immediately.

Another use for rapid hCG assays is in the area of drugs testing in athletes. A ban has been imposed on hCG administration by the International Olympic Committee. Athletes use hCG because it stimulates testicular steroidogenesis, especially testosterone. It is also abused by body builders in order to counteract the feedback effects related to the abuse of anabolic steroids (Delbeke *et al.*, 1998). The concentration at which a sportsperson might be considered positive has been set at 5 IU/L (Delbeke, 1998). The test developed within this thesis could be redeveloped to

allow for more sensitive detection of the hCG molecule (i.e.: a minimum of 5 IU/L) for the purposes of a test which could be used for hCG misuse in sport.

4.10.3 The introduction of automated hCG test devices and readers

The use of tests such as the cassette and wick tests developed in this thesis, while popular in the home and at various non-laboratory healthcare situations (e.g.: GP's office, health clinic, emergency departments etc.), do not lend themselves to the large scale screening required in the clinical laboratory setting. In order to make these tests more 'user-friendly' for the clinical laboratory technician an automated system for the reading and interpretation of such devices is necessary. Test strips and automated readers already exist for the rapid urine testing market, for example the Boehringer Mannheim Supertron® Fully Automated Urine Analyser, which can throughput a maximum of 300 samples per hour (Boehringer Mannheim, 1999).

Such a system exists, albeit in its infancy stage. This system, manufactured by a company called Cozart, is currently used for a drugs of abuse test only and is known as the COZART Rapiscan Saliva DoA (Cozart, 1999). The system consists of a disposable test cartridge which is inserted into the Rapiscan instrument for reading once the test is completed. The Rapiscan instrument is similar in size and weight to a mobile phone and is capable of being linked to a computer for data analysis. The possibility exists of adapting such a reader to read several devices at once and to store and analyse this information for hospital records, thereby allowing an avenue for these POC tests to be introduced into the clinical laboratory. The screening of large amounts of samples quantitatively for hCG may become a reality in the future as a marker for conditions such as Down's Syndrome, ectopic pregnancies and choriocarcinoma.

In conclusion, what was initially offered as a rapid pregnancy test could be evolved into a sophisticated diagnostic tool aiding clinicians in the differential diagnosis of a broad spectrum of diseases. The rapid hCG test need not be limited to pregnancy testing. As described earlier, with improvements in sensitivity or changes in the choice of antibody pairs used to detect hCG isoforms relevant to particular conditions or diseases, the format used for hCG detection by the assays developed here can be manipulated to provide a wide range of clinically useful immunoassays.

Section 5:

Conclusion

5.0 Conclusion

The detection of the dimeric glycoprotein hormone, hCG, has long been used for the diagnosis of pregnancy in female urine and/or serum (Gabbe *et al,* 1996). Qualitative measurements of urine/serum hCG are used to diagnose pregnancy, while quantitative measurements are used for monitoring various conditions and malignancies, all of which express either hCG or one of its metabolites (Cole and Kardana, 1992). The aim of the work described in this thesis was to develop a qualitative cassette and wick test for the detection of hCG in urine and serum. Both tests developed in this study detect either nicked or non-nicked intact hCG. This detection method is preferable for a pregnancy test, as these forms of hCG are present in varying quantities (see section 1.7, Introduction) during the course of pregnancy, peaking in the first trimester. In general, the nicked hCG component makes up one quarter of the total hCG content in any given sample from a patient presenting with a normal pregnancy. The intact hCG constitutes the remaining three quarters of the total hCG content (Cole *et al.*, 1991).

Both assays developed were based on lateral flow immunochromatography, using the 'sandwich' format, which was made up of a polyclonal anti-α-hCG antibody and a gold conjugated monoclonal anti-β-hCG antibody. This assay technology lends itself to the requirements of the test being designed in this thesis. Specified parameters for such a test are laid out in section 1.11, Introduction and section 4.1, Discussion. Success was achieved in the development of the rapid cassette and wick assays as all of these parameters were fulfilled. Conveniently, both assays utilise either urine or serum as the sample medium. The test possesses a sensitivity of 25 IU/L with results available in 3 minutes for urine and 5 minutes for serum. This compares favourably with other commercially available tests for hCG detection (see Table 1.10.1, Introduction). Untrained individuals can easily perform the assay and no refrigeration of the product is required for storage. The cost of the test has been maintained at a minimum (approximately $1.50 for either a cassette or wick device) and there are no safety risks involved in running the assay, as the sample is absorbed within the sample pad and thus, contained within the test device.

Following optimisation of both assays, external and internal clinical trials were performed. 100% sensitivity and specificity were achieved for the cassette assay with urine samples and 98.5% sensitivity and 100% specificity were noted for serum samples on the same test format. The wick assay generated a sensitivity of 99.4% (urine) and 100% (serum) and a specificity of 100% (urine) and 98% (serum). None of the potentially interfering or cross reacting substances tested on both formats showed a negative effect on either assay using either urine or serum. The tests developed are equal to those on the market in relation to sensitivity and specificity.

These excellent performance characteristics and the knowledge that the test is estimated, from accelerated stability studies, to remain stable for approximately 18 months will ensure that these assays will compete well with the other tests on the market. It should be emphasised that a positive result on these assays indicates the presence of hCG in the urine/serum rather than the impending arrival of a baby. It would be prudent to follow all positive results with further clinical examination as there are other physiological conditions, some serious, which can show elevated levels of hCG (e.g: choriocarcinoma) (Cole, 1999b). It is a measure of the success of the assays that predicted sales for the first six months of the year 2000 are ~$60,000 for the cassette assay while ~$100,000 worth of sales are forecast for the wick assay (Shine, 1999).

Section 6:

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Section 7:

Appendices

Appendix 1

ID No.	Membrane Cat No.	Supplier
1	AE105	Schleicher and Schuell
2	AE98	Schleicher and Schuell
3	n/a	Advanced Microdevices
4	n/a	Millipore

Table 1: *Codes for Nitrocellulose Membranes*

ID No.	Antibody Cat No.	Supplier
1	5008	Medix Biochemica
2	5006	Medix Biochemica
3	6601	Medix Biochemica
4	5009	Medix Biochemica
5	1817	British Biocell
6	108	Maine Biotechnology
7	106	Maine Biotechnology
8	105	Maine Biotechnology
9	Polyclonal	Wyntech Diagnostics

Table 2: *Codes for Antibodies*

ID No.	Antibody used for conjugation	Supplier of antibody
A	107	Maine Biotechnology
B	5008	Medix Biochemica
C	1817	British Biocell
D	9812	Genzyme
E	106	Maine Biotechnology
F	108	Maine Biotechnology

Table 3: *Codes for Gold Conjugates*

Appendix 2

The following pages display a copy of the test information and procedure handed out to volunteers of the external trial for the wick assay, which took place in Dublin City University (see Results, Section 3.14.5).

PREGNANCY TEST CONSUMER STUDY

You will receive one test device (labelled ABI) and four test devices (labelled TB) , a urine sample container and a copy of the product insert. Please read the instructions carefully. You will then provide a urine sample which will be brought back to the reception area , where you will run the sample on the ABI test and a TB test. After performing the test , you will be asked to interpret the results. Three possible results, A, B and C are shown on page 3 . You will record which one of these results you obtained in table 1 on page 4 in the relevant columns.

You will then be given three further samples to run on the remaining TB tests. You will interpret these results as before and record the results in table 2 on page 4. There is a simple questionnaire to fill out at the end of the study.

Trinity biotech

PREGNANCY TEST CONSUMER STUDY

I.D Number : _____

Date of Birth : _____

Informed Consent :

I agree to voluntarily participate in the Trinity Biotech Pregnancy Test consumer study. I fully understand that all results and communication relating to my participation in this study will be kept confidential.

_____ _____

Signature of Participant Date

PREGNANCY TEST CONSUMER STUDY

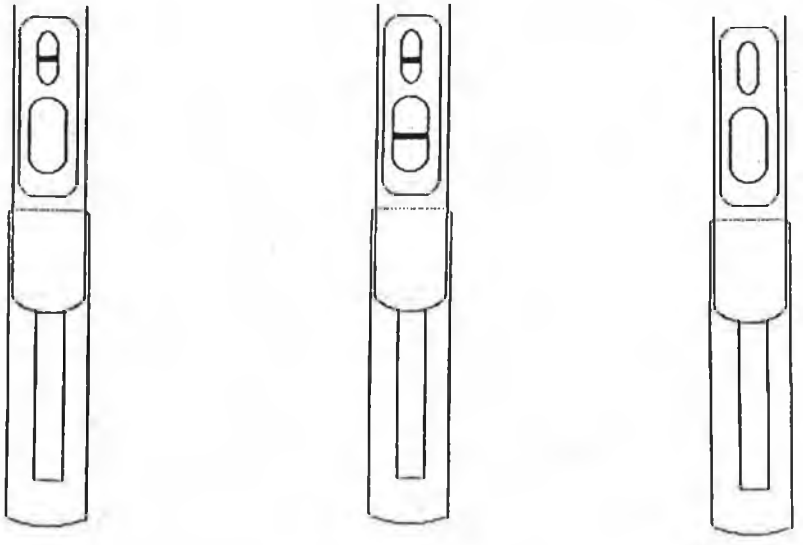
One Step hCG Pregnancy Test

1. Objectives:

Trinity Biotech PLC. are conducting a consumer clinical trial of the Unigold hCG pregnancy test. Your results and comments will be used to evaluate the performance of the test and to determine how well consumers can perform this new home pregnancy test. You will be provided with a number of devices , asked to read the instructions and to perform the test using the given procedure , just as would happen in the home. Once you have carried out the test , you are asked to record the test results on this form in the tables overleaf.

2. Interpretation of Results :

The following are three possible test results which you may see. Please record the relevant result code (i.e : either A, B or C) in the results tables.



A B C

PREGNANCY TEST CONSUMER STUDY

3. Test Results :

Your sample :

Table 1

Sample ID	Result – ABI test	Result – TB test

Samples Provided :

Table 2

Sample Code	Result – TB test

Test performed and result recorded by : _____
Signature of Participant

Comments : _____
_____
_____
_____

Checked by: _____