

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 October 2007 (18.10.2007)

PCT

(10) International Publication Number
WO 2007/117155 A1

(51) International Patent Classification:
G01N 33/53 (2006.01)

(21) International Application Number:
PCT/NZ2007/000044

(22) International Filing Date: 7 March 2007 (07.03.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
546485 7 April 2006 (07.04.2006) NZ

(71) Applicant (*for all designated States except US*): **AGRE-SEARCH LIMITED** [NZ/NZ]; East Street, Ruakura Campus, Hamilton (NZ).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **HENDERSON, Keith** [NZ/NZ]; 21 Victoria Street, Upper Hutt (NZ).

(74) Agents: **WILSON, Kathryn, S.** et al.; PriceWaterhouse-Coopers Centre, Level 11, 119 Armagh Street, P O Box 2201, Christchurch (NZ).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SOLID PHASE IMMUNO-ASSAY

(57) **Abstract:** A solid phase immuno-assay in the form of: a test strip for determining the pregnancy status of equines, via an analyte present in urine, which includes: a solid support comprising: a control zone; a test zone; and a sample zone; all of said zones being in fluid communication with each other; characterised in that: the control zone includes a ligand specific for a labelled-complex; the test zone includes the analyte or analogue thereof; the sample zone includes the labelled-complex specific for the analyte; and wherein the sample zone is located upstream of the test zone, which is upstream of the control zone.



WO 2007/117155 A1

SOLID PHASE IMMUNO-ASSAY

STATEMENT OF CORRESPONDING APPLICATIONS

- 5 This application is based on the Provisional specification filed in relation to New Zealand Patent Application Number 546584, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

- 10 The present invention relates to a solid-phase immuno-assay. In particular, the present invention relates to a solid-phase immuno-assay and related methodology suitable for determining pregnancy in *equines*.

BACKGROUND ART

For ease of reference the present invention will now be described with specific reference to equines. However, it should be appreciated that this should not be seen as necessarily restricting the scope of the present invention.

- 15 The determination of pregnancy in equines is presently problematic. For example, determining pregnancy by internal methods such as palpation or ultrasound can often be a traumatic experience for a horse, and in some cases can cause physical injury, either of which may endanger the health of mare and foetus.

- 20 It has recently been separately reported that the pregnancy status of bovines and equines biochemically indicated by increases in the levels of oestrone sulphate, a pregnancy-related steroid found in the blood, by Hirako *et. al* (2003)¹; and Henderson *et al.*(1999).² respectively.

¹ Hirako *et. al* (2003) - Changes in plasma estrogen concentrations during the first trimester of gestation in dairy cows: in comparison with the origin of embryos and foetal number *JARQ* 37 (3), 195-200

² Henderson, K.M., Perkins, N.R., Wards, R.L, Stewart, J.I. (1999). Enzymeimmunoassay of oestrone sulphate concentrations in faeces for non-invasive pregnancy determination in mares. *New Zealand Veterinary Journal*, vol 47, pages 61 to 66.

The use of oestrone sulphate provides an alternative method for determination of whether a mare is pregnant, which usually requires obtaining a blood sample for testing. However, the use of blood samples to determine pregnancy is problematic for a number of reasons including:

- 5 • the use of needles is required, which can be traumatic and painful for mares, as the blood sample is usually taken from the nostril of the mare; and
- the blood needs to be further processed in order to obtain plasma or serum before testing to determine pregnancy status.

10 The inventor has now discovered that oestrone sulphate is also present in the urine of equines.

It would therefore be an advantage if there could be provided a less invasive and traumatic methodology for determining the pregnancy status of equines which did not require the use of a blood sample or an internal physical examination.

15 All references, including any patents or patent applications cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although
20 a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this
25 specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

5 **DISCLOSURE OF INVENTION**

According to one aspect of the present invention there is provided a solid phase immuno-assay in the form of: a test strip for determining the pregnancy status of equines, via an analyte present in urine, which includes:

a solid support comprising:

- 10 - a control zone;
- a test zone; and
- a sample zone;

all of said zones being in fluid communication with each other;

characterised in that:

- 15 the control zone includes a ligand specific for a labelled-complex;
- the test zone includes the analyte or analogue thereof;
- the sample zone includes the labelled-complex specific for the analyte;

and wherein the sample zone is located upstream of the test zone, which is upstream of the control zone.

- 20 For ease of reference, the present invention will now be described in relation to equines. However this should not be seen as limiting the scope of the invention.

As used herein, the term 'analyte' refers to oestrone sulphate (OS).

- Within the present specification, the term 'analogue' refers to a compound having a structure which is similar to oestrone sulphate. In preferred embodiments the
- 25 test-zone may use Oestrogen Bovine Serum Albumin conjugate as the analogue.

The term 'ligand', as used herein, refers to a molecule which can bind specifically to another molecule.

In general, the ligand may be an immuno-molecule.

Most preferably, the ligand is an antibody.

In the present specification, the term 'immuno-molecule' refers to a molecule derived from an immune system which is capable of specifically binding to another molecule. It may include an antibody, antibody binding fragment, or a phage display molecule.

As used herein, the term 'labelled-complex' refers to a complex of compounds which includes a tracer compound bound, or otherwise associated, with an immuno-molecule specific for oestrone sulphate, and may also include oestrone sulphate once bound to the immuno-molecule portion of the complex.

10 In preferred embodiments the labelled-complex may be a conjugated gold-oestrone sulphate monoclonal antibody complex (OS detection antibody complex).

In the present specification, the term 'antibody' refers to an immunoglobulin molecule having an amino acid sequence which specifically binds to an antigen, such as oestrone sulphate. The term 'antibody' includes antibody binding fragments. In general an antibody binding fragment may include, but not be limited to, an Fr, F(ab)₂ fragment, or ScFr molecule or the like.

In preferred embodiments the antibody may be a monoclonal antibody or a binding fragment thereof.

20 As used herein, the term 'tracer compound' encompasses any compound which is capable of being visibly determined by the eye without the need for instrumentation.

It is envisaged that the solid support may come in a variety of different forms without departing from the scope of the present invention.

In general, the solid support may be in the form of a cellulose ester.

25 In preferred embodiments, the solid support may be a nitro-cellulose paper.

In preferred embodiments, the solid support may have a surface area capable of supporting the ligand in a concentration of at least 0.5mg/ml and the analyte or an analogue thereof in a concentration of 0.5 to 10 mg/ml. Most preferably the support may also have a surface area capable of supporting 0.02ml of the labelled-complex.

30 In a preferred embodiment where the solid support is nitro-cellulose paper, the surface area of the paper may be at least 25 mm x 5mm.

In preferred embodiments, to facilitate handling and ease of use, the test strip may be substantially housed within a body configured so that the control zone, test zone and sample zone are visible and/or accessible.

- 5 In use, a sample of urine may be collected from a mare 100 days post-mating, and this is added to the sample-zone where it soaks into the solid-support and releases the conjugated gold-OS monoclonal antibody complex ("OS detection antibody complex"), which starts to migrate up the solid support.

- 10 When OS is present in the urine, this can become bound to the OS detection antibody. If OS is not present, or the OS concentration is low, the antigen-binding sites in the OS detection antibody do not become filled, and as a result when the OS detection antibody passes over the test-zone, some of it binds to the analyte analogue in the solid-support and a visible dot forms. This indicates the mare is not pregnant.

- 15 Conversely, if the OS concentration is high in the urine, then all of the antigen binding sites in the OS detection antibody are filled and consequently no test-dot can form when the OS detection antibody passes over the analyte analogue in the test-zone. This indicates the mare is pregnant.

It should be appreciated that further embodiments of the present invention have a number of advantages over the prior art, which can include:

- 20 - a simple-to-use and non-invasive test for pregnancy in equines; and
- a less traumatic and/or painful method by which pregnancy can be determined in equines.

BEST MODES FOR CARRYING OUT THE INVENTION

25 **Preparation of solid phase immunoassay test.**

In accordance with one preferred embodiment and by way of example only, a solid phase immunoassay test may be prepared as follows:

Preparation of the Sample Zone

- 30 Affinity purified IgG fraction of monoclonal antibody to oestrone sulphate is dissolved in 2mM sodium tetraborate (0.01 to 1 mg antibody/ml), and 0.5 ml added

rapidly to 10 ml colloidal gold particles (40 nm) undergoing fast stirring. The solution is left to stand for 1 hour at 26° C, then 1 ml of 1% BSA in 2mM sodium tetraborate added and the incubation continued for a further 15 min. The solution is centrifuged at 4500_g (av) for 10 min at 4° C to pellet the conjugated gold-IgG particles. The supernatant is discarded, the pellet resuspended in 10 ml of 0.1% BSA in 2mM sodium tetraborate and centrifuged again, before being finally resuspended in 20 ml of 100 mM borate buffer (pH 8) containing 0.1% BSA, 0.1% Triton X-100 and 5% sucrose. Individual pieces (7x5 mm) of the sample zone which is in the form of a conjugate release pad (rapid 27Q paper, obtained from Whatman International Ltd.) each receive 0.02 ml of the conjugated gold-IgG solution. The prepared conjugate release pads are dried for 1 h at 37° C and 1 h at 26° C before being stored dry with silica gel desiccant at 4° C.

Preparation of the Test Zone

PuraBind A-FP nitrocellulose membrane test platforms (75X300mm) are purchased from Whatman International Ltd. Solutions of oestrogen-bovine serum albumin conjugate (O-BSA; 1 to 10 mg/ml) and anti-mouse IgG (1 mg/ml) are prepared in coating buffer (50 mmol/l carbonate-bicarbonate buffer, pH 9.6). A 'test' zone is generated by applying a line (1mm thick) of the O-BSA solution, 9mm from the bottom edge of the PuraBind A-FP membrane.

Preparation of a Control Zone

A 'control' zone is generated by applying a line (1mm thick) of the anti-mouse IgG solution, 5mm above the test zone 'line'. After drying for 30 minutes at 37° C, the platform is immersed in blocking solution (aqueous 1% polyvinylpyrrolidone, average molecular weight 10 000, and 0.5% Triton X-100) for 30 minutes at room temperature. The platform is then washed with 5 changes of distilled water, dried at 37° C for 30 minutes and stored dry with silica gel desiccant at room temperature.

Assembly of the Pregnancy Test

The prepared PuraBind A-FP membrane platforms are cut into 75 X 5 mm strips, and the adhesive areas above and below the nitrocellulose membrane exposed by removing the silicone release tapes. An absorbent sink (Whatman 17 ChR cellulose paper, 13x5 mm) is attached to the top of the membrane (2 mm overlap) and a prepared conjugate release pad to the bottom of the membrane (2 mm overlap). A sample application pad (Whatman GF/D glass fibre paper, 17x5 mm) is

attached fully overlapping the conjugate release pad. Each assembled strip is placed inside a plastic housing cassette (5mm) and sealed inside an aluminium pouch together with a disposable exact volume plastic pipette (100 µl) and a silica gel pack (1g). The packaged devices are stored at 4° C.

5 Use of the Pregnancy Test

To run the solid phase immunoassay test, the packaged device is allowed to warm to room temperature before being opened. A 100 µl aliquot of mare test urine is added drop by drop into the sample well of the plastic cassette using the exact volume pipette. As the urine migrates along the nitrocellulose membrane, a line in the test zone and a line in the control zone appear in the cassette window between 5 and 10 minutes, with maximum colour intensity occurring within 20 minutes. The colour intensity of the line in the test zone is inversely proportional to the concentration of oestrone sulphate in the urine sample. For pregnancy status diagnosis, the appearance of 2 visible lines is interpreted as indicative of non-pregnancy. The appearance of a control line only is interpreted as indicative of pregnancy.

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof as defined in the appended claims.

THE CLAIMS DEFINING THE INVENTION ARE:

1. A solid phase immuno-assay in the form of: a test strip for determining the pregnancy status of equines, via an analyte present in urine, which includes:
- 5 a solid support comprising:
- a control zone;
 - a test zone; and
 - a sample zone;
- 10 all of said zones being in fluid communication with each other;
- characterised in that:
- the control zone includes a ligand specific for a labelled-complex;
 - the test zone includes the analyte or analogue thereof;
 - 15 the sample zone includes the labelled-complex specific for the analyte;
- and wherein the sample zone is located upstream of the test zone, which is upstream of the control zone.
2. A solid phase immuno-assay as claimed in claim 1 wherein the analyte is oestrone sulphate.
3. A solid phase immuno-assay as claimed in claim 1 or claim 2 wherein the ligand is an antibody.

4. A solid phase immuno-assay as claimed in any one of the preceding claims wherein the labelled-complex includes a tracer compound and an immuno-molecule specific for oestrone sulphate.
5. A solid phase immuno-assay as claimed in any one of the preceding claims wherein the labelled-complex is a conjugated gold-oestrone sulphate monoclonal antibody complex.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ2007/000044

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/53 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS and Medline. Keywords: (o)estrone; pregnant, urine and related terms; equine; mare; horse; pregnancy test kit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 6187598 B1 (MAY et al) 13 Feb 2001 Col. 1, line 39 and lines 46 – 58; Col. 5, lines 8 – 26; and Figs 8 and 9 and col. 12, lines 48-61	1,3 2 – 5
Y	Monfort, S. L. et al Monitoring ovarian function and pregnancy by evaluating excretion of urinary oestrogen conjugates in semi-free-ranging Przewalski's horses (Equus przewalskii) Journal of Reproduction and Fertility, (1991 Jan) Vol. 91, No. 1, pp 155-164 See abstract	2 – 5
Y	Henderson, K. and Stewart, J. A dipstick immunoassay to rapidly measure serum oestrone sulfate concentrations in horses. Reprod Fertil Dev., (2000) Vol. 12, No. 3-4, pp 183-189 See abstract	2 – 5



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:			
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 June 2007

Date of mailing of the international search report

28 JUN 2007

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

Steven Chew
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. (02) 6283 2248

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/NZ2007/000044

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US	6187598	AU	16228/88	EP	0291194	GB	2204398
			17049/92		0560410		
			17050/92		1248112		
			44386/97		1555529		
			80489/94				
			80490/94			HK	140995
		US	5602040	JP	6160388	WO	8808534
			5622871		6180320		
			5656503		6180320		
			2001008774				
			2001041368				
			2005244986				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX