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(54) **Title**: RELEASE REAGENT FOR VITAMIN D

(57) **Abstract**: Disclosed is an invention in the field of conducting an immunoassay of 25(OH) vitamin D in blood or blood components, notably serum or plasma. The invention employs a perfluoro alkyl acid, or a salt thereof,to release 25(OH) vitamin D from vitamin D binding protein. Thereafter the binding protein comprising the 25-OH vitamin D is subjected to a competitive binding assay with a labeled vitamin D compound.

Title: RELEASE REAGENT FOR VITAMIN D

Field of the invention

The present invention pertains to an immunoassay method, including point-of-care tests, for assaying a sample of blood or blood components for total vitamin D or Vitamin D metabolites, in particular 25-hydroxy vitamin D using an agent to release Vitamin D from endogenous binding proteins.

Background of the invention

The substances referred to as "vitamin D" encompass a group of fat-soluble prohormones, as well as metabolites and analogues thereof. The main forms in which vitamin D occurs in the body are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The latter is the endogenous form of vitamin D, which humans can form in the skin under the influence of sunlight. The former is an exogenous form of vitamin D, taken up with food. In the US, Vitamin D2 is used as the pharmaceutical vitamin D supplement.

Whilst vitamin D_2 and D_3 differ in the molecular structure of their side-chains, they share the same biological activity in being prohormones, metabolized in two steps to, ultimately, 1,25 dihydroxy vitamin D (calcitriol, or 1,25 dihydroxy cholecalciferol). The preceding metabolite, 25-hydroxy vitamin D or calcidiol, results from conversion in the liver, and is considered the storage form of vitamin D in the body.

Circulating vitamin D consists mainly of 25(OH)vitamin D3 and 25(OH)vitamin D2. Biologically, 25(OH)vitamin D2 is as effective as 25(OH)vitamin D3. The half-life of 25(OH)vitamin D2 in the circulation is shorter. For clinical practice the use of an 25(OH)vitamin D assay that

measures both 25(OH)vitamin D3 as well as 25(OH)vitamin D2 is recommended (1).

Vitamin D has long been recognized as an important substance, the active form of which plays a role in the formation and maintenance of bone, as well as in other processes in the human or animal body. Thus, it serves to increase the concentration of calcium in the bloodstream, by promoting absorption of calcium and phosphorus from food in the intestines, and re-absorption of calcium in the kidneys; enabling normal mineralization of bone and preventing hypocalcemic tetany. It is also necessary for bone growth and bone remodeling by osteoblasts and osteoclasts.

Vitamin D deficiency results in impaired bone mineralization and leads to bone softening diseases, rickets in children and osteomalacia in adults, and possibly contributes to osteoporosis.

In recent years it has been recognized that Vitamin D plays a number of other roles in human health. It can modulate the immune function and reduce inflammation. It has also been suggested that Vitamin D may prevent colon, breast and ovarian cancer.

Thus, it is of the essence for a person's or animal's health to have an adequate level of vitamin D.

Yet, excess of vitamin D (which may occur as a result of overdosing) is toxic. Some symptoms of vitamin D toxicity are hypercalcaemia (an elevated level of calcium in the blood) caused by increased intestinal calcium absorption. Vitamin D toxicity is known to be a cause of high blood pressure. Gastrointestinal symptoms of vitamin D toxicity can include anorexia, nausea, and vomiting. These symptoms are often followed by polyuria (excessive production of urine), polydipsia (increased thirst), weakness, nervousness, pruritus (itch), and eventually renal failure.

Clearly, it is important to be able to diagnose subjects for a possible vitamin D deficiency. It is also important, particularly for subjects that are on vitamin D supplementation, to be able to test subjects for a potential excess of

vitamin D. In clinical practice, the serum level of 25-hydroxy-vitamin D is considered to be the primary indicator of the vitamin D status. (2).

Almost all circulating 25(OH)-vitamin D in serum is bound by vitamin D binding protein (88%) and Albumin (12%). Vitamin D binding protein (DBP) is an abundant protein, with a concentration of 250-400 mg/L of serum. Vitamin D is bound to DBP with a relatively high affinity, close to that of antibodies ($5*10^8 M^{-1}$).

An accurate measurement of the concentration of Vitamin D in serum requires the release of bound vitamin D from the DBP.

Early methods for the determination of Vitamin D included an extraction step using organic solvents such as acetonitrile. Other approaches have relied on dissociation of Vitamin D-DBP complex using a high or low pH (WO2004/063704). Other methods rely on the competitive displacement of Vitamin D from endogenous binding proteins using ANS (US 7,482,162). Recently methods including proteolytic digestion of DBP have been published (WO 2008/092917 A1). Armbruster has published a method for direct measurement of Vitamin D using displacement by hydroxylated aromatic carboxylic acid (WO 2003/023391). The method described by Kyriatsoulis relies on the release of Vitamin D from Vitamin-D binding protein by using a reagent with a pH from 3.8 to 4.8 and 5-30% DMSO, a liquid organic amide and optionally 0.5-5% of a short chain alcohol. Kobold presented a method for the release based on a salt with a cation having a quaternary nitrogen based ion.EP2007/140962. US 2008/0182341 mentions stabilizing agents and capture ligands for use in assays measuring analyte concentrations. These stabilizing agents are disclosed against the background of certain alkyl amino fluoro surfactants. The inventors suggest that this surfactant facilitates the measurement of free unbound analyte versus bound analyte by stabilizing the equilibrium. Fluorocarbon octanoic acid is mentioned as a potential hazardous substance.

Background references on assaying Vitamin D include Hollis BW. Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs. Am J Clin Nutr. 2008 Aug;88(2):507S-510S; Holick MF. Vitamin D: extraskeletal health. Endocrinol Metab Clin North Am. 2010 Jun;39(2):381-400.

Summary of the invention

This invention, in one aspect, presents an *in vitro* method for the qualitative assaying of blood or blood components for the presence of 25-hydroxy vitamin D, comprising:

(a) adding to the sample a perfluoro alkyl acid with a carbon chain length of from 4 to 12 carbon atoms, or a salt thereof, in order to enable the release of Vitamin D from Vitamin D binding protein;

(b) optionally diluting the sample with a diluent;

(c) subjecting the mixture to incubation with an immobilized binding protein, notably an anti-Vitamin D antibody;

(d) contacting the sample with a conjugate of Vitamin D and a functional label that binds to the anti-Vitamin D antibody in a competitive way

(e) determining the concentration of labeled vitamin D compound bound to the binding protein

In another aspect, the invention resides in a kit for conducting the foregoing method.

In yet another aspect the method can be used for "point-of-care" testing. The latter refers to testing at or near the site of patient care, i.e. rather than drawing blood samples and sending these to a diagnostic laboratory, a sample can be immediately introduced into a portable, preferably handheld device which is able to perform the assay in as limited a number of

steps as possible, and with as limited a number of manual operations as possible.

Brief Description of the Drawings

Fig. 1 depicts a 25(OH)Vitamin D calibration curve.

Fig. 2 presents a graph comparing results obtained with perfluorhexanoic (PFHxA) acid and with Perfluoroctanoic acid (PFOA) release.

Detailed Description of the Invention

In a broad sense, the invention concerns the determination of Vitamin D in blood or blood components, notably serum or plasma, by an immunoassay using a perfluoroalkyl acid or salt thereof with a carbon chain length of 4 to 12 to release Vitamin D from endogenous binding proteins.

Conceivably, in the invention use can be made of perfluoroalkyl carboxylic acid or perfluoro sulfonic acid or salts thereof. In particular Perfluorhexanoic acid (PFHxA) or perfluoroctanoic acid (PFOA) can be used.

The assay generally involves

(a) adding a diluent/assay buffer to sample

(b) adding magnetic particles coated with anti-vitamin D antibody

(c) incubating the sample for an amount of time

(d) adding a conjugate of vitamin D and a functional label;

(e) determining the amount of the conjugate of vitamin D and the functional label bound to the antibody.

Such samples can be drawn, in any manner known in the art, from a subject, particularly a human, in whose blood it is desired to assay the presence of 25-OH vitamin D.

The sample preferably is diluted with an aqueous diluent. Preferably the diluent is an assay buffer. The dilution can take place before, during, or after the addition of the antibody. The sample diluent or assay buffer can be aqueous-based, and preferably will be a buffer solution. Preferably, the buffered pH is in the range of from 6.0 to 8.0. Suitable diluents include, e.g. phosphate citrate buffer. The concentration of the perfluoro alkyl acid in the buffer should be 0.1%-3%, preferably 0.5%. Suitable buffer solutions are customary in the art and do not require elucidation here.

The perfluoro alkyl acid can be added in a separate manner, but preferably is comprised in the sample diluent, preferably in the assay buffer.

The sample is contacted with an anti- 25(OH)Vitamin D antibody The latter can be added to the sample, or the sample can be transferred to a reaction vial containing the binding protein.

Antibodies for vitamin D are known in the art, and are widely used in the existing immunoassays for vitamin D. These same antibodies, as well as other binding proteins, can be used in the present invention as well. E.g., in the place of an antibody for Vitamin D an antibody fragment can be used such as produced with phage display technology. Suitable antibodies can be monoclonal or polyclonal antibodies. They can be obtained in known manner, e.g. polyclonal goat anti-vitamin D, polyclonal rabbit anti-vitamin D, or any other suitable antibody for vitamin D as known in the art from application in immunoassays for vitamin D. Suitable antibodies are known, e.g. from the following references: Hollis, Clin.Chem 31/11, 1815-1819 (1985); Hollis, Clin.Chem 39/3, 529-533 (1993).

The antibodies as used are preferably immobilized. They are preferably used in a particulate form comprising solid carriers. Typically, the antibody is coated on a solid phase, e.g. on a microtiter plate. In a preferred embodiment, the antibodies are coated onto magnetic particles, which facilitates their separation in a magnetic field.

After addition of the antibody, the sample is allowed to incubate. The required time will depend on circumstances such as the concentration of the reagents, the type of binding protein, and conditions during incubation, e.g. shaking and temperature. Generally, the incubation time will be in a range of from 10 seconds to several hours, preferably 1 minute to 1 hour. For automated platforms, short incubation times (10 seconds to 10 minutes, preferably 30 seconds to 30 minutes) are preferred.

After the first incubation period, a conjugate of vitamin D with a functional label is added. Numerous labeled compounds are known that are capable of serving as competitive binding antigens in immunoassays for the determination of vitamin D. Typical labels are radiolabels, fluorescent labels, luminescent labels, biotin labels, gold labels, enzyme labels. Competitive binding assays are known to the skilled person, and do not require elucidation, notably since this part of the method of the invention can be carried out using any label known to be suitable for the determination of vitamin D. Labels that can be used are, inter alia, those disclosed in the foregoing references on existing vitamin D immunoassays.

With the label allowing measuring a concentration, as a result, the concentration of vitamin D in the sample is determined. It will be understood that the interpretation of the values measured, is determined by a calibration measurement, i.e. by the response – in the same assay – of calibrators.

The calibration for the assay of the invention can be done by providing calibrators comprising a predetermined concentration of 25-OH vitamin D. The concentration of Vitamin D in the calibrators is preferably determined using an LC-MS-MS method.

The invention, in another aspect, presents a product in the form of an immunoassay for the determination of 25-OH vitamin D in blood or blood components, wherein the assay makes use of a method according to any one of preceding embodiments. More particularly, such a product will be provided in the form of a kit for conducting the immunoassay. Such a kit may comprise the

loose reagents involved, i.e. the antibody, the labeled vitamin D compound and the diluents/assay buffer. These reagents can be provided separately, and thus form a kit only upon their use in the assay of the invention. Preferably, the reagents are provided together, preferably packaged together, as one kit of parts. The kit optionally comprises a container for a sample of blood or blood components, but as is customary this may also be provided separately. Typically a kit comprises a binder immobilized on a solid phase and a separate conjugated vitamin D. Other kit components will depend, as is customary in the art, on the label chosen, as different labels may require different reagents.

It is to be understood that the invention is not limited to the embodiments and formulae as described hereinbefore. It is also to be understood that in the claims the word "comprising" does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

The invention will be illustrated with reference to the following, non-limiting Example and the accompanying non-limiting Figures.

Example

Measurement of Vitamin D

The assay was performed using an automated platform. To 15 µl of sample 255 µl of sample diluent/assay buffer was added. An aliquot of 100 µl of the diluted sample was transferred to a second incubation well. A volume of 50 µl of magnetic particles coated with monoclonal antibody was added to the diluted sample and incubated for 45 minutes at 37°C. Subsequently 50 µl of a solution biotinylated Vitamin D was added and incubated for 7 minutes at 37 °C. Then 50 µl of a solution streptavidin-Acridinium ester was added and again incubated for 7 minutes at 37 °C. After magnetic separation of bound and free biotinylated Vitamin D, the bound acridinium ester was quantified.

Materials

Paramagnetic particles Magnetic particles (Invitrogen, M280 tosyl-activated, 2.8 µm) were coated with a polyclonal antibody against Mouse IgG (5 µg/mg of magnetic particles). The particles were coated on a roller at a concentration of 0.5 mg/ml in 0.01M PBS, 0.138M NaCl of pH 7.4 during 16 hours. Finally particles were blocked with 0.05M Tris / 0.05% BSA containing 0.1% Proclin-950. The particles were coated during 16 hrs at 37 °C with a second layer of anti-Vitamin-D monoclonal antibody at a concentration of 0.4 µg/mg particles.

The sample diluent consisted of 0.1M TRIS buffer of pH 8.0 containing 0.05% BSA and 0.5% PFOA.

The conjugate (i.e. the labeled vitamin D compound) is a biotinylated Vitamin-D. The conjugate was presented at a concentration of 0.5 ng/ml in a 0.1M Tris buffer of pH 8.0 containing 0.05% Bovine Serum Albumin.

Protocol

To 15 µl of sample 255 µl of sample diluent/assay buffer was added. An aliquot of 100 µl of the diluted sample was transferred to a second incubation well. A volume of 50 µl of magnetic particles coated with monoclonal antibody was added to the diluted sample and incubated for 45 minutes at 37°C. Subsequently 50 µl of a solution biotinylated Vitamin D was added and incubated for 7 minutes at 37 °C. Then 50 µl of a solution streptavidin-Acridinium ester was added and again incubated for 7 minutes at 37 °C. After magnetic separation of bound and free biotinylated Vitamin D, the bound acridinium ester was quantified. A chemiluminescent signal was generated by addition of trigger reagent. The signal generated in the cuvette is

inversely proportional to the concentration of 25 (OH)Vitamin D in the sample or calibrator. The concentration of 25(OH) vitamin D in the original sample can be calculated by comparing the signal of unknowns with the response of calibrators.

Results

In the table below, and in Fig. 1, a calibration curve is shown. 25(OH)Vitamin D3 calibrators were prepared in Vitamin D-free serum and ranged from 0-136 ng/ml.

Biotinylated 25(OH)Vitamin D is displaced to a level of 7% at 136 ng/ml.

A set of samples was measured using perfluoroctanoic acid release and with perfluorhexanoic acid release. Results correlate very well (r = 0.990) indicating that both compounds can be used (Fig 2).

Table

Table 1. 25(OH)Vitamin D calibration curve.

Standard curve	St 0	St A	St B	St C	St D	St E
dose (ng/mL)	0	4.1	13.5	24.9	52.1	136
RLU (1)	733227	618731	432378	254588	105251	41737
RLU (2)	754974	637612	422703	228484	106879	41870
RLU mean	744101	628172	427541	241536	106065	41804
RLU SD	15377	13351	6841	18458	1151	94
RLU %CV	2.1%	2.1%	1.6%	7.6%	1.1%	0.2%
Binding %	**100.0%**	**84.4%**	**57.5%**	**32.5%**	**14.3%**	**5.6%**

Claims

1. A method for the qualitative *in vitro* assaying of blood or blood components for the presence of 25-hydroxy vitamin D, comprising:

(a) adding to the sample a perfluoro alkyl acid with a carbon chain length of from 4 to 12 carbon atoms, or a salt thereof;

(b) optionally diluting the sample with a diluent;

(c) subjecting the mixture to incubation with an anti-Vitamin D antibody;

(d) contacting the sample with a conjugate of Vitamin D and a functional label that binds to the anti-Vitamin D antibody in a competitive way;

(e) determining the concentration of labeled vitamin D compound bound to the binding protein

2. A method according to claim 1, wherein the perfluoro alkyl acid is selected from the group consisting of perfluoro hexanoic acid, perfluoro octanoic acid, and mixtures thereof.

3. A method according to claim 1 or 2, wherein the perfluoro alkyl acid is comprised in the diluent.

4. A method according to any one of the preceding claims, wherein the sample is diluted before incubation.

5. A method according to any one of the preceding claims, wherein the sample is human serum or plasma.

6. A method according to any one of the preceding claims, wherein the binding protein is provided in a form coated on magnetic particles.

7. A method according to any one of the preceding claims, wherein the functional label is selected from the group consisting of radiolabels, fluorescent labels, luminescent labels, biotin labels, gold labels, enzyme labels.

8. A method according to any one of the preceding claims, wherein the concentration is determined with reference to a calibrator concentration for total 25-OH vitamin D.

9. A method according to any one of the preceding claims, wherein the the perfluoro octanoic acid, or the derivative thereof, is selected from the group consisting of perfluoro octanoic acid, perfluoro octanoic acid ammonium salt, and perfluoro octane sulfonate.

10. An immunoassay for the determination of 25-OH vitamin D in blood or blood components, wherein the assay makes use of a method according to any one of the preceding claims.

11. A kit for conducting an immunoassay using the method of any one of the claims 1-10, the kit comprising an antibody specific for 25-OH vitamin D immobilized on a solid phase, and a conjugate of Vitamin D and a functional label.

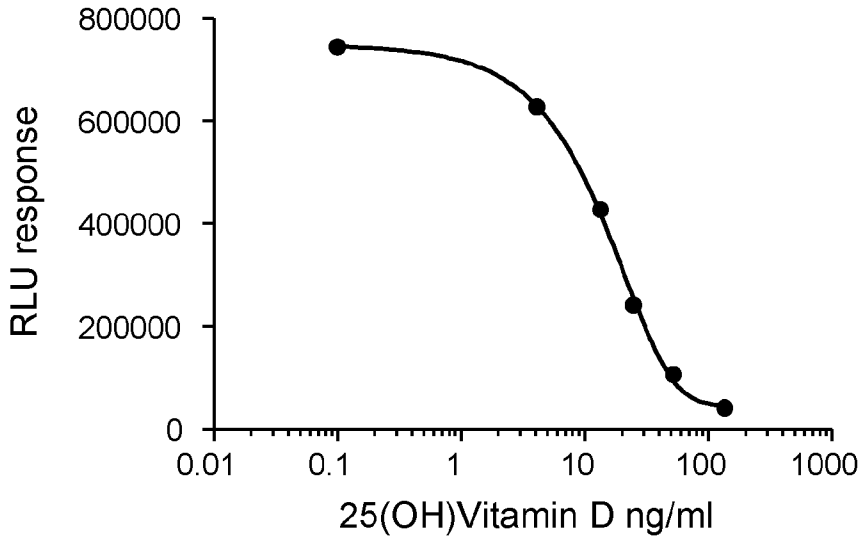
800000
600000
400000
200000
0
RLU response
0.01
0.1
1
10
100
1000
25(OH)Vitamin D ng/ml

Fig. 1

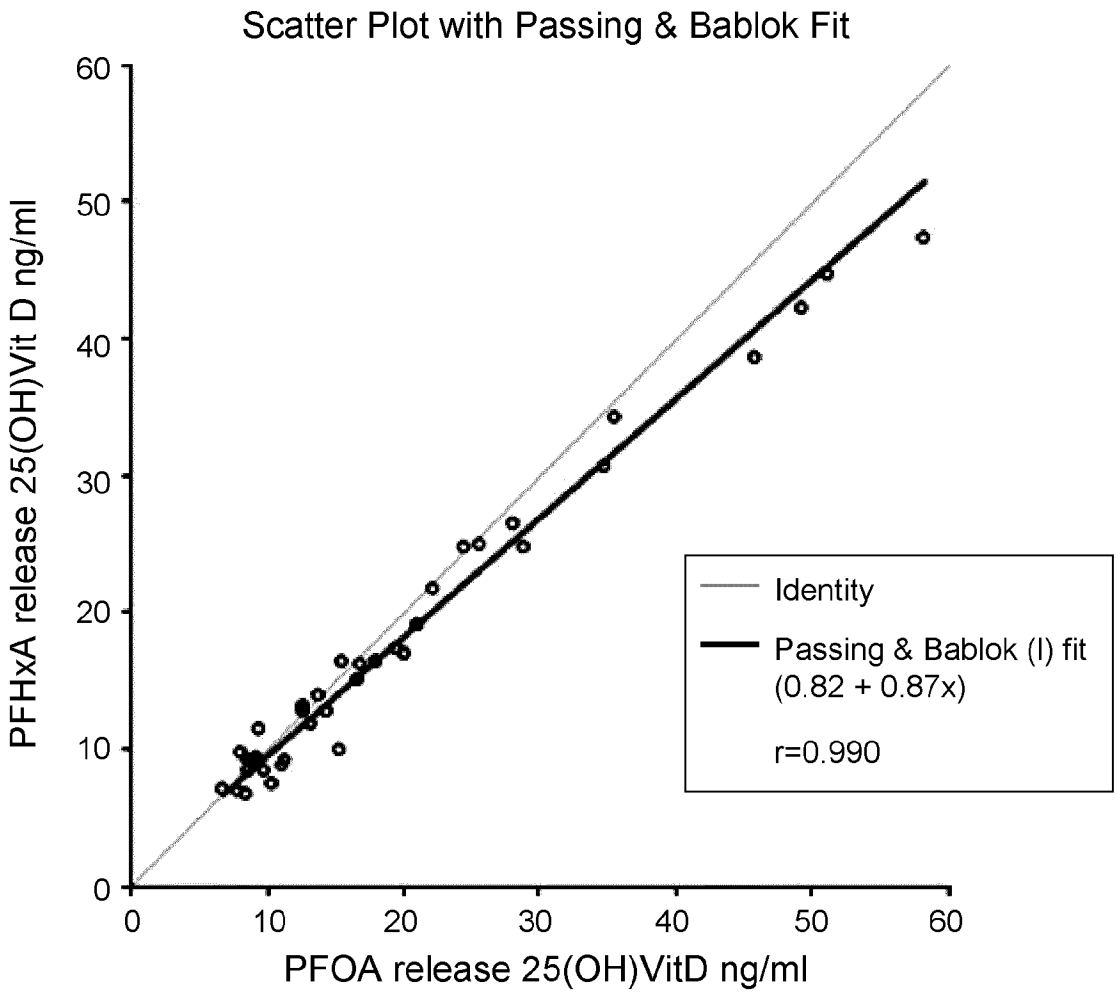
Scatter Plot with Passing & Bablok Fit
PFHxA release 25(OH)Vit D ng/ml
PFOA release 25(OH)VitD ng/ml
0
10
20
30
40
50
60
Identity
Passing & Bablok (I) fit
(0.82 + 0.87x)
r=0.990

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050905

A. CLASSIFICATION OF SUBJECT MATTER
INV. C08F14/26 G01N33/82 G01N33/53
ADD.

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)
C08F G01N

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2011/122948 A1 (FUTURE DIAGNOSTICS B V [NL]; MARTENS MICHAEL FRANCISCUS WILHELMUS CORN) 6 October 2011 (2011-10-06) claims 1, 2, 5-12, 15, 16 abstract the whole document	1-11

X	WO 03/023391 A2 (IMMUNDIAGNOSTIK AG [DE]; ARMBRUSTER FRANZ PAUL [DE]; FRIEDL SABINE [DE]) 20 March 2003 (2003-03-20)	10,11
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Y	WO 02/057797 A2 (QUEST DIAGNOSTICS INC [US]) 25 July 2002 (2002-07-25) abstract; examples 3-8; p. 24, l. 8-10; claims 1-84 the whole document	1-11

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[X] Further documents are listed in the continuation of Box C.

[X] 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

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2012	12/04/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schindler-Bauer, P

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050905

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	US 5 981 779 A (HOLICK MICHAEL F [US] ET AL) 9 November 1999 (1999-11-09) the whole document line 24 - column 6, line 37	1-11
Y	ZHANG XIAN ET AL: "Binding of PFOS to serum albumin and DNA: insight into the molecular toxicity of perfluorochemicals", BMC MOLECULAR BIOLOGY, vol. 10, no. 16, 2009, XP002620518, ISSN: 1471-2199 the whole document	1-11
Y	WU LING-LING ET AL: "Interaction of perfluorooctanoic acid with human serum albumin", BMC STRUCTURAL BIOLOGY, vol. 9, no. 31, 2009, XP002620519, ISSN: 1472-6807 the whole document	1-11
Y	WO 2007/140962 A2 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]; KOBOLD UWE [DE];) 13 December 2007 (2007-12-13) the whole document	1-11
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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