



(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/EP2010/057871

(22) International Filing Date:
7 June 2010 (07.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09162425.4 10 June 2009 (10.06.2009) EP

(71) Applicant (for all designated States except US): **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ALBERS, Andreas** [NL/DE]; Oberanger 3a, 82377 Penzberg (DE). **BAHLE, Beatrix** [DE/DE]; Waldstrasse 53, 82386 Oberhausen (DE). **BOBADILLA, Maria** [FR/FR]; 50b rue de Village-Neuf, F-68128 Rosenau (FR). **ECABERT, Barbara** [CH/CH]; Im Hofacker 17, CH-4106 Therwil (CH). **LOHMANN, Sabine** [DE/DE]; Primelstrasse 1, 82377 Penzberg (DE). **PALLAUD, Céline** [FR/FR]; 25, rue de Delle, F-68220 Follgensbourg (FR). **QUIRK, Jeremy** [GB/CH]; Gundeldingerrain 110, CH-4059 Basel (CH). **SCHINDLER, Thomas** [DE/DE]; Lisa-Rees-Strasse 10, 79540 Loerrach (DE). **SUCHANKOVA, Gabriela** [CZ/CH]; Bachlettenstrasse 29, CH-4054 Basel (CH).

(74) Agent: **KUENG, Peter**; Grenzacherstrasse 124, CH-4070 Basel (CH).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, 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, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD FOR DETECTING GLUCOSE TRANSPORTER GENE EXPRESSION

(57) Abstract: The present invention provides an in vitro for determining the expression level of a glucose transporter protein in renal cells comprising: a) isolation of renal cells from urine by filtration and b) determining the expression level of the glucose transporter, wherein the isolated cells are not cultivated *in vitro* prior to step b).



METHOD FOR DETECTING GLUCOSE TRANSPORTER GENE EXPRESSION

The present invention provides a method for the measurement of glucose transporter gene expression in renal cells.

Glucose is the main source of energy for the mammalian cells and its entry is mediated via various transporters. About 7 facilitative (GLUT-1 to -7) and 2 concentrative glucose transporters (SGLT-1 and -2) have been identified. The facilitative glucose transporters allow the glucose entry into the cell interior due to the concentration gradient and the latter via the Na⁺-dependent electrochemical gradient. They have similar structural motifs with 12-14 putative transmembrane domains with a predicted protein size varying from 50 to 76kDa. Some of the facilitative glucose transporters (GLUT-1, -2, -4 and -5) and both the sodium glucose co-transporters (SGLT-1 and -2) are expressed in the kidney. The transporters that are involved in the major transport of glucose in the kidney include GLUT-2 and SGLT-2.

The bulk of glucose that is filtered by the renal glomerulus is reabsorbed by the glucose transporters of the proximal convoluted tubular epithelium. 90% of the glucose is reabsorbed by the low-affinity/high capacity sodium glucose co transporter (SGLT) 2 located in the S1 segments of proximal tubule. Available methods for the detection of glucose transporter expression in renal cells either involve a renal biopsy or the *in vitro* cultivation of exfoliated renal cells isolated from urine prior to the determination of the SGLT expression level.

It is an object of the present invention to provide an *in vitro* method for determining the expression level of a glucose transporter protein in renal cells comprising:

- a) isolation of renal cells from urine by filtration and
- b) determining the expression level of the glucose transporter, wherein the isolated cells are not cultivated *in vitro* prior to step b).

In a preferred embodiment, the expression level of the glucose transporter protein is determined by RT-PCR.

In another preferred embodiment, the expression level of the glucose transporter protein is determined by RT-PCR using housekeeping genes selected from the group consisting of CD13, MRPL19 (mitochondrial ribosomal protein L19) and ALAS1 (aminolevulinate, delta-, synthase 1).

In a further preferred embodiment, the glucose transporter protein is selected from the group consisting of GLUT-1, GLUT-2, SGLT-1 and SGLT-2, preferably SGLT-2.

In another preferred embodiment, the renal cells are proximal tubular epithelial cells.

5 In yet another preferred embodiment, the cells are isolated from urine using a polypropylene membrane filter having a pore size of approximately 1.6 μm . A suitable filter for the isolation of renal cells from urine is the ZRC GFTM filter which is commercially available from Zymo Research Corporation.

10 Techniques for the detection and quantification of gene expression of a glucose transporter gene include, but are not limited to northern blots, RT-PCR, real time quantitative PCR, primer extension, RNase protection, RNA expression profiling and related techniques. These techniques are well known to those of skill in the art see e.g. Sambrook J et al., Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor Press, Cold Spring Harbor, 2000).

Techniques for the detection of protein expression of a glucose transporter include, but are not limited to immunohistochemistry (IHC).

15 **Short description of the figures:**

Fig. 1 shows SGLT-2 and CD13 expression in T2D (Type 2 Diabetes) urine samples. Urine from 10 (T2D) volunteers was collected. 30 ml urine was processed with the Zymo Research kit to extract the RNA according the instructions of the manufacturer. RNA Expression of CD13 and SGLT-2 was analysed with Real-time PCR using the LightCycler® 480 Instrument
20 II and

Fig. 2 shows the linearity of SGLT-2 quantification in urine samples spiked with SGLT-2 transfected Chinese hamster ovary (CHO) cells. RNA isolation was performed with the Zymo Research Kit. The linear measuring range for 0 to 50.000 CHO/SGLT-2 cells spiked in urine sediment are shown and corresponds to a cp-range from 31,19 (only urine) to 17,13.

25 **Example:**

Isolation of Cells from Urine and subsequent isolation of RNA from cells:

RNA isolation from fresh clean-catch urine using a Urine RNA Isolation Kit (Zymo research Inc, Orange CA)

30 Clean-catch urine was freshly collected into a 200 ml beaker. Cells were resuspended by carefully swirling the beaker and filtered through the ZRC GF filter according to the manufacturer's instructions. A total of 30 ml urine was filtered. Cells retained by the filter were

extracted using the Zymo Research extraction buffer. The cell extract was stored at -80°C before further processing.

RNA was isolated from the thawed cell extract. After adding one volume 95% ethanol to the cell extract the solution was mixed and applied to a Zymo-spin IC column and processed according to the manufacturers instructions. In brief, the column was washed with RNA wash buffer and RNA was eluted using 20 mL RNA Elution Buffer. The RNA was stored at -80°C.

Amplification and detection of SGLT-2 RNA from total RNA isolated from urinary cells

RNA of SGLT-2 and 3 potential housekeeping genes (CD13, MRPL19, ALAS1) were amplified in real-time reverse transcription – polymerase chain Taqman reaction using the following instruments, reagents and reaction conditions:

- **Instrument:** LightCycler® 480 II: Serien 25200, Software version: LCS480 1.5.0.39
- **Primers and probes sequences:**

Primer	Primer Sequence (5'-3')	Seq. Id. No.
SGLT2 fwd 84	ATC CTG CTG ACA TCC TAG TCA	1
SGLT2 rev144	GTT GGT TCT GCA CAT GGA CC	2
SGLT2 P 106	TG CTG CAT ATT TC CTG CTG GTC ATT GGC GTT GG	3
CD13 fwd 1795	GTC CTA CCT CCA CAC CTT TGC	4
CD13 rev1855	GTT GTT CAC AGC CTC CTG C	5
CD13 P 1823	AA CAC CAT CTA CC TGA ACC TGT GGG ACC ACC	6

SGLT-2 was labeled by Dye1 and BHQ2, Housekeeping genes were labeled by Dye2 and BHQ2.

Mastermix:

Mn2+ (25mM Manganacetat), Ident. No. 05 384 567. 5x RNA Mix TaqMan RNA Amp Kit, Ident. No. 05 384 583

Preparation of the Amplification Mix:

<u>Reagents:</u>	<u>µl</u>	<u>final amount</u>
H2O	6,56	ad 15µl
Fwd Primer (10µM)	1,00	500nM
Rev Primer (10µM)	1,00	500nM
Probe (10µM)	0,20	100nM
RNA-Mix (5x)	4,00	1x
Mn2+ (25mM)	2,24	2.8mM

-4-

Manganacetat)		
total volume	15,00	
Isolated RNA	5,00	

Amplification conditions and settings on Lightcycler:

	Reaction Volume			
Dual Color Hydrolysis Probe	20µl			
Program Name	Cycles	Analysis Mode		
Reverse Transcription	1	None		
Amplification Program 1	2	Quantification		
Amplification Program 2	45	Quantification		
Cooling	1	None		

Target [°C]	Acquisition Mode	Hold	Ramp Rate [°C/s]	Acquisitions [per °C]
Reverse Transcription				
50	None	5 min	4,4	---
95	None	1 min	4,4	---
61	None	5 min	2,2	---
Amplification Program 1				
95	None	15s	4,4	---
60	Single	50s	2,2	---
Amplification Program 2				
92	None	15s	4,4	---
60	Single	50s	2,2	---
Cooling				
40	None	30	2,2	

5

Result calculation:

Results are calculated based on relative crossing point values of SGLT-2 relative to potential housekeeping gene crossing points.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

10

Claims

1. An *in vitro* method for determining the expression level of a glucose transporter protein in renal cells comprising:

- 5 a) isolation of renal cells from urine by filtration and
- b) determining the expression level of the glucose transporter, wherein the isolated cells are not cultivated *in vitro* prior to step b).

2. The method of claim 1, wherein the expression level is determined by RT-PCR.

3. The method of claim 2, wherein the expression level is determined by RT-PCR using
10 housekeeping genes selected from the group consisting of CD13, MRPL19, and ALAS1.

4. The method of claim 1 – 3, wherein the glucose transporter protein is selected from the group consisting of GLUT-1, GLUT-2, SGLT-1 and SGLT-2.

5. The method of claim 4, wherein the glucose transporter protein is SGLT-2.

6. The method of claim 1 – 5, wherein the renal cells are proximal tubular epithelial cells.

15 7. The method claim 1 – 6, wherein the cells are isolated from urine using a polypropylene membrane filter having a pore size of about 1.6 μm .

8. The methods substantially as hereinbefore described, especially with reference to the foregoing examples.

Fig. 1

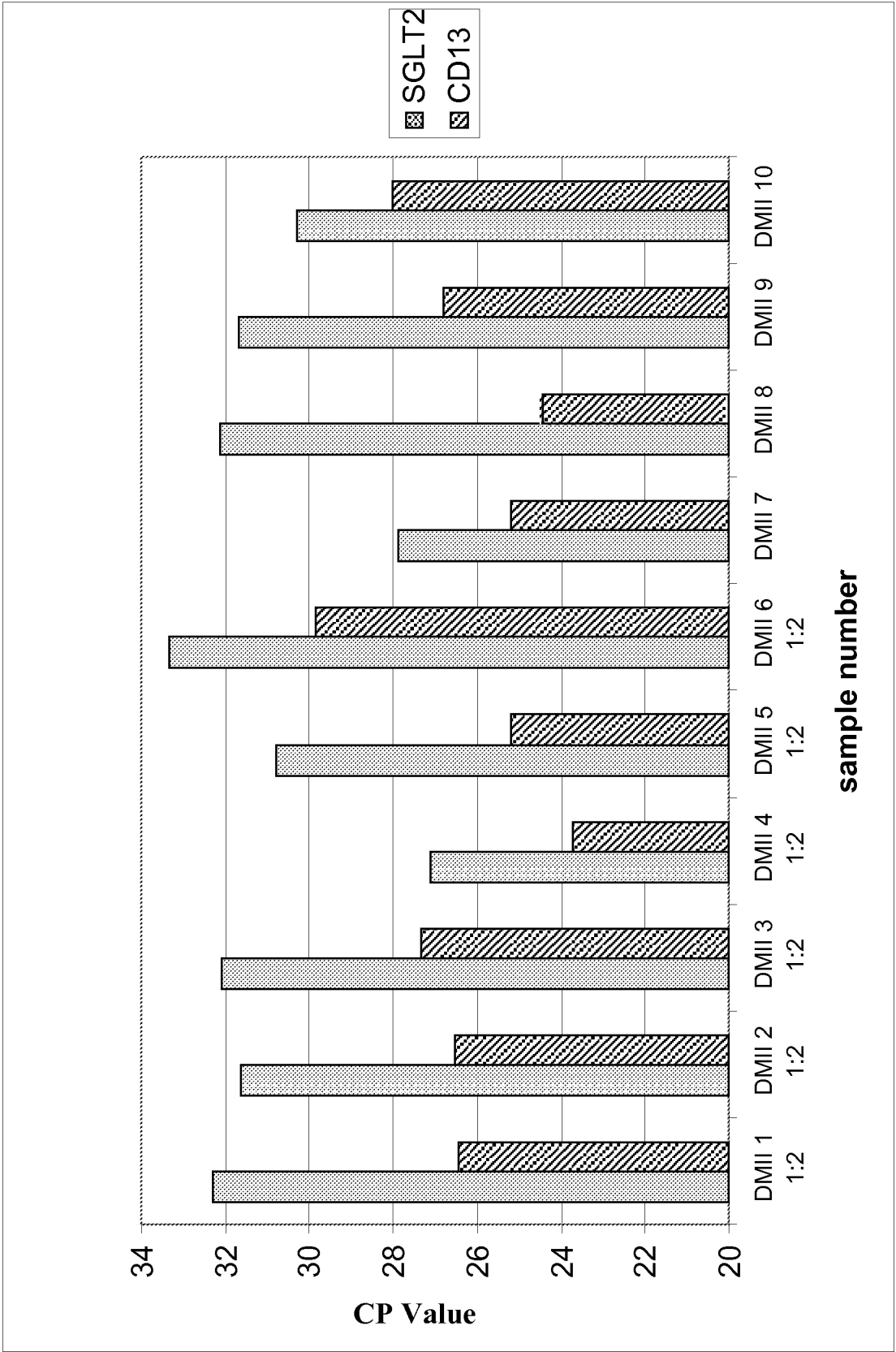
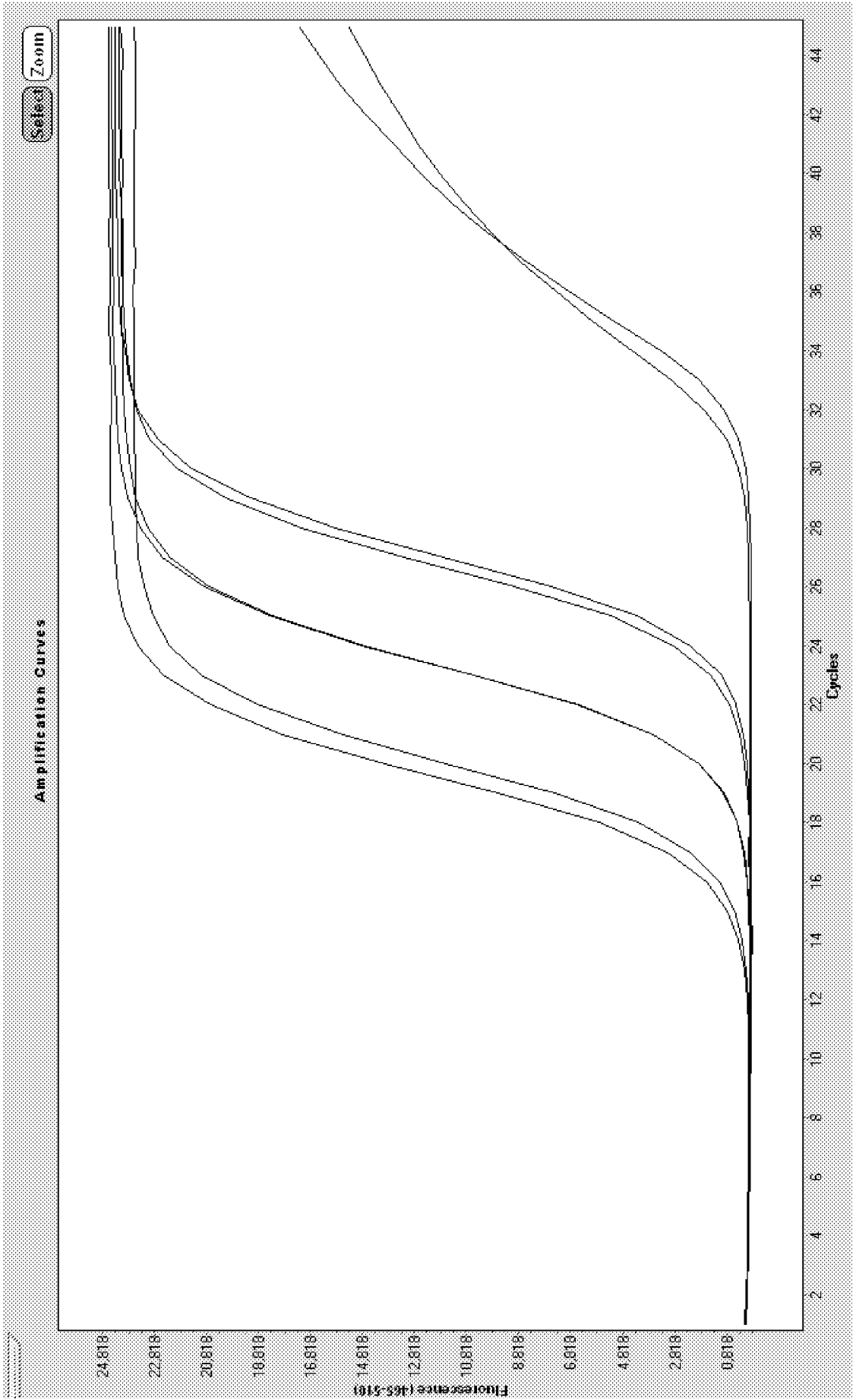


Fig. 2



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/057871

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

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)

C12Q C12N

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.
A	RAHMOUNE HASSAN ET AL: "Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes" DIABETES, vol. 54, no. 12, December 2005 (2005-12), pages 3427-3434, XP002596589 ISSN: 0012-1797 the whole document ----- -/--	1-8



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

"E" earlier document but published on or after the international filing date

"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)

"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

17 August 2010

Date of mailing of the international search report

01/09/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

NL - 2280 HV Rijswijk

Tel. (+31-70) 340-2040,

Fax: (+31-70) 340-3016

Authorized officer

Guarinos Viñals, E

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/057871

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TABATABAI N M ET AL: "Enhanced expressions of sodium-glucose cotransporters in the kidneys of diabetic Zucker rats" DIABETES RESEARCH AND CLINICAL PRACTICE 200901 IE LNKD- DOI:10.1016/J.DIABRES.2008.11.003, vol. 83, no. 1, January 2009 (2009-01), pages E27-E30, XP002596590 ISSN: 0168-8227 the whole document -----	1-8
A	VESTRI S ET AL: "Changes in sodium or glucose filtration rate modulate expression of glucose transporters in renal proximal tubular cells of rat." THE JOURNAL OF MEMBRANE BIOLOGY 15 JUL 2001 LNKD- PUBMED:11447502, vol. 182, no. 2, 15 July 2001 (2001-07-15), pages 105-112, XP002596591 ISSN: 0022-2631 the whole document -----	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2010/057871

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☒ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: