(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 January 2016 (28.01.2016)

(10) International Publication Number
WO 2016/014307 A1

(51) International Patent Classification:
C12N 1/04 (2006.01) *C12N 1/20* (2006.01)

(21) International Application Number:
PCT/US2015/040508

(22) International Filing Date:
15 July 2015 (15.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/026,903 21 July 2014 (21.07.2014) US
62/041,367 25 August 2014 (25.08.2014) US

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(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, BN, 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, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, 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, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title**: COMPOSITIONS AND METHODS FOR EXTENDING STORAGE TIME OF COMPETENT CELLS AT -20 C

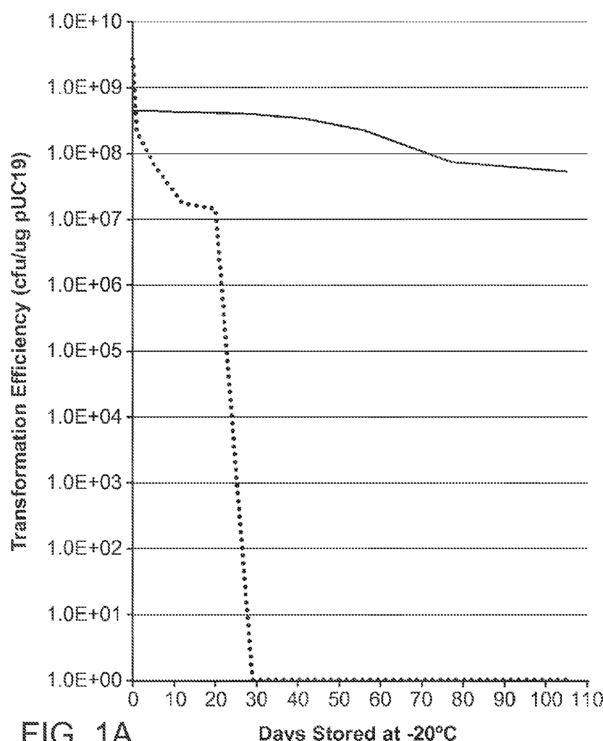

FIG. 1A

(57) **Abstract**: Compositions and methods are provided for storing prokaryotic cells including competent prokaryotic cells at -20°C in a buffer so that the cells are suitable for transformation at 0°C with a foreign molecule.

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COMPOSITIONS AND METHODS FOR EXTENDING STORAGE TIME OF COMPETENT CELLS AT -20°C

BACKGROUND

Competent cells are available for cloning vectors of interest for molecular biology research. A variety of competent cells are provided commercially for various types of laboratory procedures such as routine cloning, protein expression, and library production. Bacterial cells are rendered competent in the presence of high salt and are then washed and resuspended in a high salt solution for storage at -80°C. Storage at -80°C is at best a costly inconvenience.

SUMMARY

In general, a method is provided that includes removing salts from prokaryotic cells optionally induced to competency by a first salt containing buffer; adding a storage buffer comprising glucose or an oligosaccharide containing glucose; storing cells at -20°C for a period of time; and adding a salt containing buffer to the cells at a time proximate to transformation of the cells with a foreign molecule.

In one aspect, the foreign molecule is a DNA, an RNA or a protein. In another aspect, the prokaryotic cells are *E.coli.* In another aspect, the storage buffer further comprises glycerol and may further include DMSO. In one aspect, where glucose or an oligosaccharide is added to the storage buffer, its concentration is within a range of 10 mM-500 mM. In one aspect the period of time may be greater than 5 days.

In general, a composition is provided having stabilized competent bacterial cells in a buffer including a monosaccharide and/or an oligosaccharide such as for example trehalose or sucrose or glucose.

In various aspects, the concentration of the one or more monosaccharides or oligosaccharides may be the range of 50 mM-500 mM. The competent bacterial cells may have a concentration of OD600=2-500.

In general, a method is provided that includes storing a composition described above at about -20°C for at least 5 days; adding a salt containing buffer to the cells in the composition at a time proximate to transformation of the cells with a foreign molecule; and transforming the cells.

DESCRIPTION OF THE FIGURES

FIG. 1A-1B shows *E.coli* cells which were stored at 0, 28, 42, 56, 77 and 105 days at -20°C in a storage buffer containing oligosaccharides and no salt without any significant loss of transformation efficiency compared with a control sample stored at -20°C in the standard competent cell storage buffer containing salt used for routine storage at -80°C.

FIG. 1A shows the effect of storage at -20°C of competent cells

(●●●) Competent *E.coli* cells which were previously stored at -80°C with substantially diminished activity after being stored at -20°C for 29 days. At the end of the selected time period, the sample was moved to 0°C and transformed with pUC19.

(—) Competent *E.coli* which was made by removing the buffer used to prepare cells at time zero followed by storage of the cells in a novel buffer containing 200 mM trehalose, as well as standard DMSO and glycerol and lacking salt did not lose significant activity after being stored up to 105 days at –20°C.

FIG. 1B shows a comparison of competency of *E.coli* cells which were stored at -20°C in the novel storage buffer formulation that lacked salt and contained various concentrations of different oligosaccharides in addition to glycerol and DMSO in a standard buffer over various time periods- 1, 28, 42, 56, 77 and 105 days showing remarkable stability.

At the end of the selected time period (0, 28, 42, 56, 77 and 105 days), the sample was moved to 0°C and transformed with pUC19. The data shows that cells could be stored at -20°C for as long as 105 days with little or no change in transformation efficiency. Several examples of oligosaccharides were shown to have similar preservative activities. A=trehalose at 200 mM, B=sucrose at 250 mM, C=sucrose at 200 mM and D=sucrose at 150 mM. Data is provided for transformation efficiency after storage.

DESCRIPTION OF EMBODIMENTS

The term “competent” bacteria refers to bacteria having altered cell walls so that DNA can pass through more easily. Generally competent cells are fragile and may lose viability rapidly after thawing from -80°C so that they are no longer available to take up DNA.

The term "stable" or "stabilized" refers to a preparation of bacterial cells that are capable of retaining a transformation efficiency of at least 1.00E+08 cfu/ug pUC19 when stored for at least 5 days at -20°C.

The term "-20°C" is intended to refer to a temperature suitable for storage that is preferable at -20°C but may vary according to various factors so that the temperature may represent a range of -15°C to -30°C.

In embodiments of the invention, bacterial cells can optionally be rendered competent when prepared under standard conditions in a salt containing buffer using standard protocols for preparing competent cells after which the salt is preferably removed (See for example, Hanahan, et al., *Methods in Enzymology*, 204:63 (1991)). The competent cells can then be stored in the novel storage buffer described herein at -20°C and after thawing can remain competent for an extended period of time when a salt containing buffer is added to the thawed cells. Such competent bacterial cells are suitable for transformation by biological macromolecules such as DNA, RNA and protein. The examples provided herein measure transformation efficiency with pUC19 DNA.

Competent bacterial cells (for example those tested in FIG. 1B), can be washed (for example, with water) to remove salt which may optionally be utilized prior to placing cells at -20°C in a storage buffer containing glycerol, DMSO and sugars. Glycerol may be used at a concentration of 1%-50%, and DMSO may be used at a concentration of 1%- 25%. By way of an example, FIG. 1A shows loss of competency of cells stored in a conventional storage buffer at -20°C where the conventional storage buffer includes 10% glycerol and 7% DMSO suitable for standard conditions of storage at -80°C. In contrast, FIG. 1B shows sustained levels of competency at -20°C in the novel storage buffer which contains 20% glycerol, 14% DMSO, one or more sugars and optionally ethylene glycol and/or propylene glycol. In FIG. 1B, the assay for competency included moving the cells to 0°C and adding an equal volume of a salt buffer to the storage buffer. The cells were then transformed with biological macromolecules.

The sugars in the storage buffer comprise one or more glucose monosaccharides or oligosaccharides comprising glucose and having a size of 2-10 monosaccharides. In one embodiment , the monosaccharide or oligosaccharide in an amount of 10 mM-500 mM, for example, 50 mM-500 mM for example 50 mM-200 mM of the monosaccharide or oligosaccharide was added to cells having

a concentration in the range of OD_{600}=1-500, for example, OD_{600}=2-250, OD_{600}=3-100, OD_{600}=4-75, or OD_{600}=5-50. It should be understood by a person of ordinary skill in the art that a monosaccharide or oligosaccharide for use herein might include something other than glucose such as xylose or ribose. While not intended to be limiting, FIG. 1B shows the advantageous effect of a single concentration of a disaccharide, trehelose that is composed of two alpha glucose units, and sucrose at 3 different concentrations. In addition to the above, ethylene glycol and/or propylene glycol to the storage buffer may be added at concentrations in the range of 0.1 mM-1000 mM.

Storage preferably occurs in the absence of salts of the amount and type used to generate the competent cells.

The storage conditions used herein are suitable for any competent *E.coli* strain including *E.coli* K-12, *E.coli* B, *E.coli* W and *E.coli* C.

Cells stored in the manner described herein were found to be capable of retaining competency for at least 5, 10, 15, 20, 25, 30, 35, 40, 42 days.

What is claimed is:

1. A method; comprising:

   optionally inducing prokaryotic cells to competency in a buffer containing salt;

   adding a storage buffer comprising glucose or an oligosaccharide containing glucose;

   storing cells at -20°C for a period of time; and

   adding a salt containing buffer to the cells at a time proximate and prior to transformation of the cells with a foreign molecule.

2. A method according to claim 1, wherein the foreign molecule is a DNA, an RNA or a protein.

3. A method according to claim 1 or 2, wherein the prokaryotic cells are *E.coli.*

4. A method according to any of claims 1 through 3, wherein the storage buffer further comprises glycerol.

5. A method according to any of claims 1 through 4, wherein the storage buffer further comprises DMSO.

6. A method according to any of claims 1 through 5, wherein the glucose or the oligosaccharide comprising glucose is present within a range of 10 mM-500 mM.

7. A method according to any of claims 1 through 6, wherein the period of time is greater than 5 days.

8. A composition, comprising: stabilized competent bacterial cells in a buffer comprising a monosaccharide or an oligosaccharide.

9. A composition, according to claim 8, wherein the oligosaccharide is trehalose or sucrose.

10. A composition according to claim 8, wherein the monosaccharide is glucose.

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11. A composition according to claims 9 or 10, wherein the concentration of monosaccharide or oligosaccharide is in the range of 50 mM-500 mM.

12. A composition according to any of claims 9 through 11 wherein the competent
10 bacterial cells have a concentration of OD600=2-500.

13. A method comprising:

storing a composition according to any of claims 9 through 12 at -20°C for at least 5 days; and

15 adding the salt containing buffer to the cells in the composition at a time proximate to transformation of the cells with a foreign molecule; and

transforming the cells.

14. A kit comprising a storage buffer in a first reaction tube wherein the storage
20 buffer comprises glucose or an oligosaccharide containing glucose and optionally glycerol and/or DMSO; and a salt solution in a second reaction tube capable of inducing competence in thawed cells.

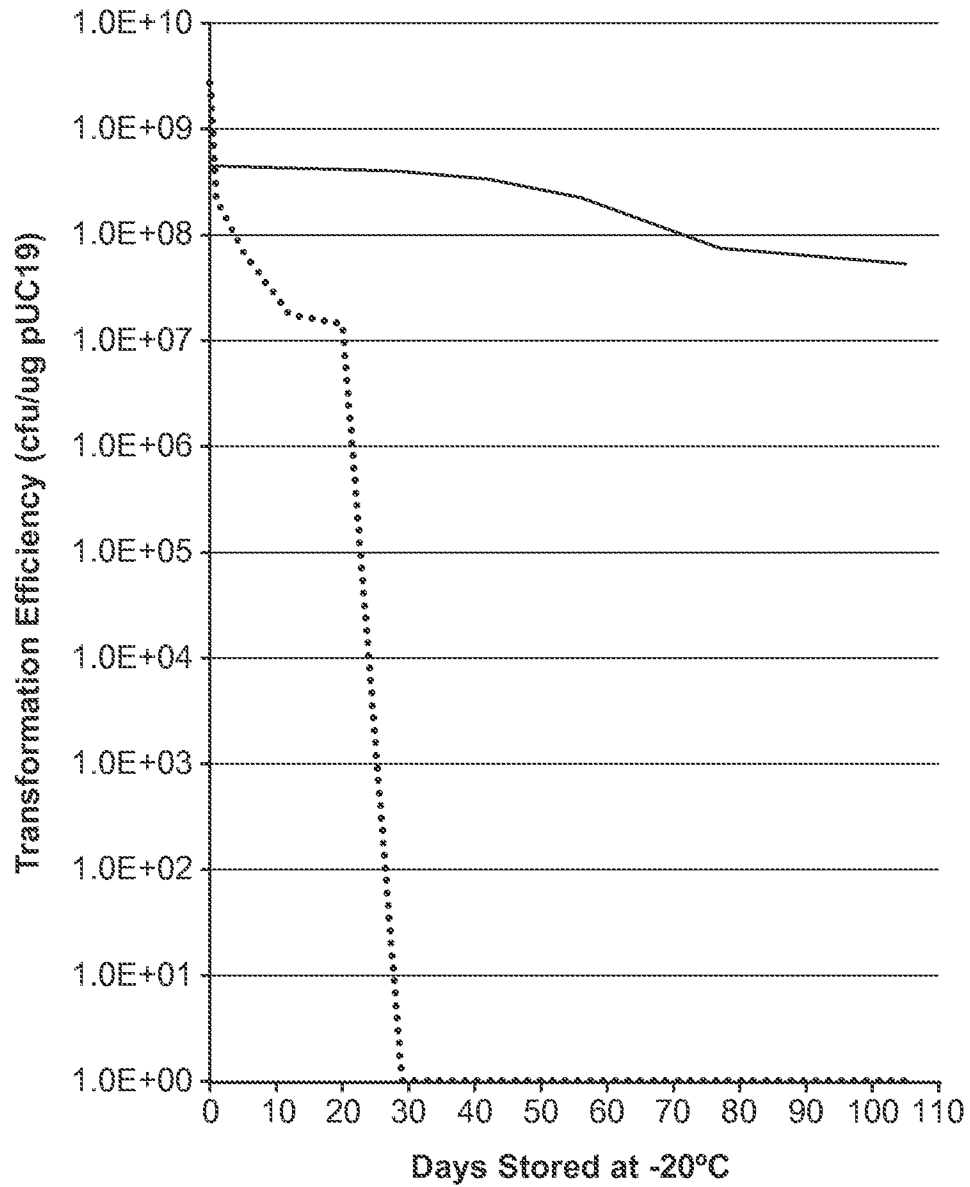

FIG. 1A

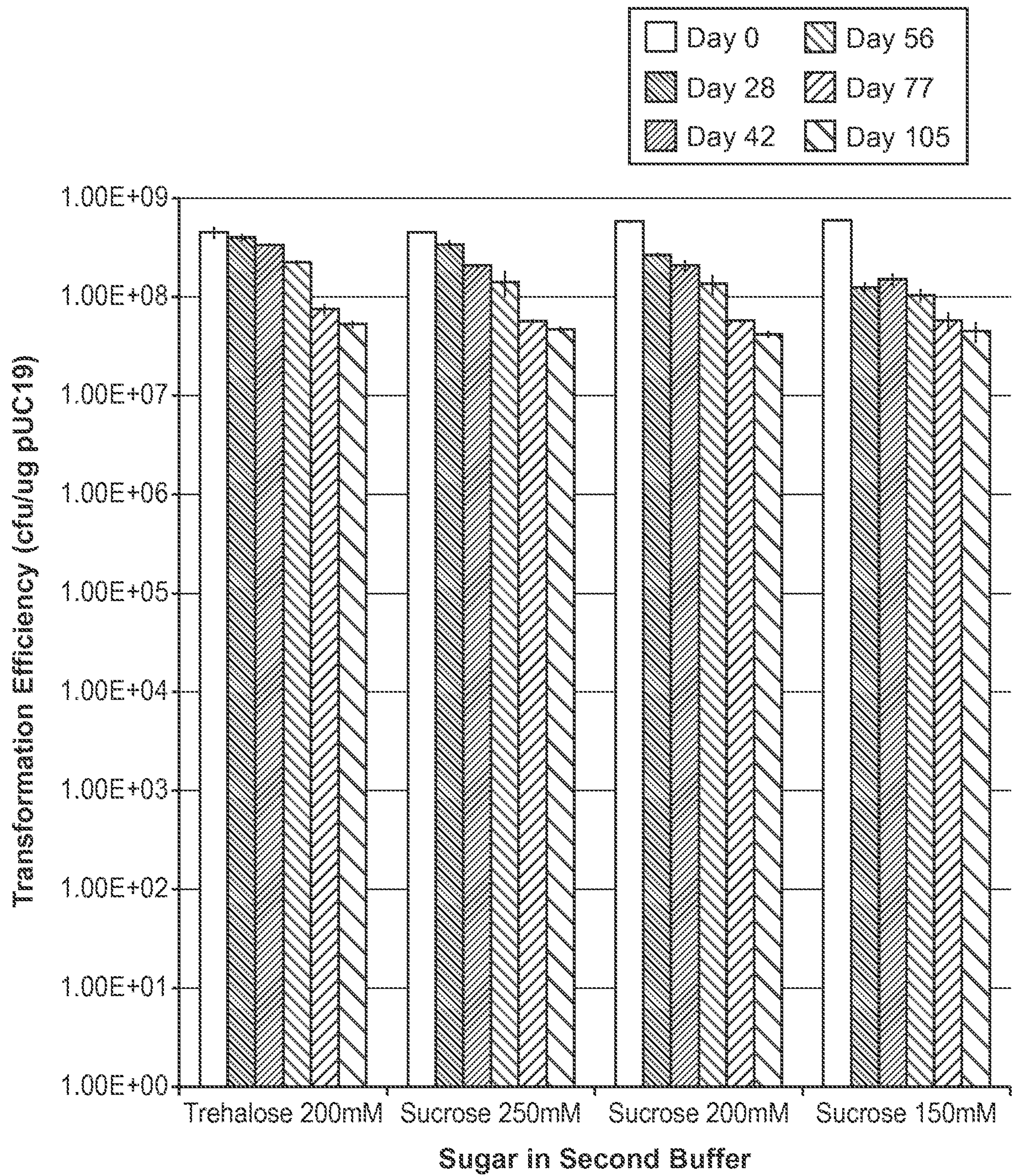

FIG. 1B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/040508

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/04 C12N1/20
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)
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 practicable, search terms used)

EPO-Internal, BIOSIS, 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	WO 98/35018 A1 (LIFE TECHNOLOGIES INC [US]) 13 August 1998 (1998-08-13) the whole document page 9, line 14 - page 11, line 30 examples 1-4 claims 1,16-18,22,31,35 -----	1-14
X	WO 02/36745 A2 (SIGMA ALDRICH CO [US]; BARNEA EFRAT [IL]; ASSCHER YAEL [IL]; WATTAD CA) 10 May 2002 (2002-05-10) the whole document page 9, line 30 - page 12, line 5 examples 1-5 claims 1,15,16,23-25 -----	8-12

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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

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

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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
16 September 2015	28/09/2015

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/040508

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/031363 A2 (STRATAGENE INC [US]; SUNDAR LATHA [US]) 15 April 2004 (2004-04-15) the whole document page 20, line 8 - page 21, line 24 examples 1-3,7 claims 1,8-10,30,48	1-14
A	TAEHO AHN ET AL: "Improved long-term cryostorage of Escherichia coli competent cells using trehalose", BIOTECHNOLOGY LETTERS, vol. 26, no. 20, 1 October 2004 (2004-10-01), pages 1593-1594, XP055213717, ISSN: 0141-5492, DOI: 10.1023/B:BILE.0000045659.41621.de the whole document	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/040508

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9835018	A1	13-08-1998	AT	294229	T	15-05-2005
			DE	69829976	D1	02-06-2005
			DE	69829976	T2	27-04-2006
			DK	1005529	T3	13-06-2005
			EP	1005529	A1	07-06-2000
			ES	2241120	T3	16-10-2005
			JP	2001512310	A	21-08-2001
			US	2002164771	A1	07-11-2002
			US	2006014255	A1	19-01-2006
			US	2010081171	A1	01-04-2010
			WO	9835018	A1	13-08-1998
WO 0236745	A2	10-05-2002	AU	1333702	A	15-05-2002
			US	2002081565	A1	27-06-2002
			WO	0236745	A2	10-05-2002
WO 2004031363	A2	15-04-2004	AU	2003277273	A1	23-04-2004
			US	2004110267	A1	10-06-2004
			WO	2004031363	A2	15-04-2004