



(86) Date de dépôt PCT/PCT Filing Date: 2008/06/16
(87) Date publication PCT/PCT Publication Date: 2008/12/24
(85) Entrée phase nationale/National Entry: 2009/12/08
(86) N° demande PCT/PCT Application No.: GB 2008/002055
(87) N° publication PCT/PCT Publication No.: 2008/155529
(30) Priorités/Priorities: 2007/06/16 (GB0711685.8);
2007/06/16 (GB0711689.0)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2006.01),
C12N 9/96 (2006.01), *G01N 33/58* (2006.01)

(71) Demandeur/Applicant:
ENIGMA DIAGNOSTICS LIMITED, GB

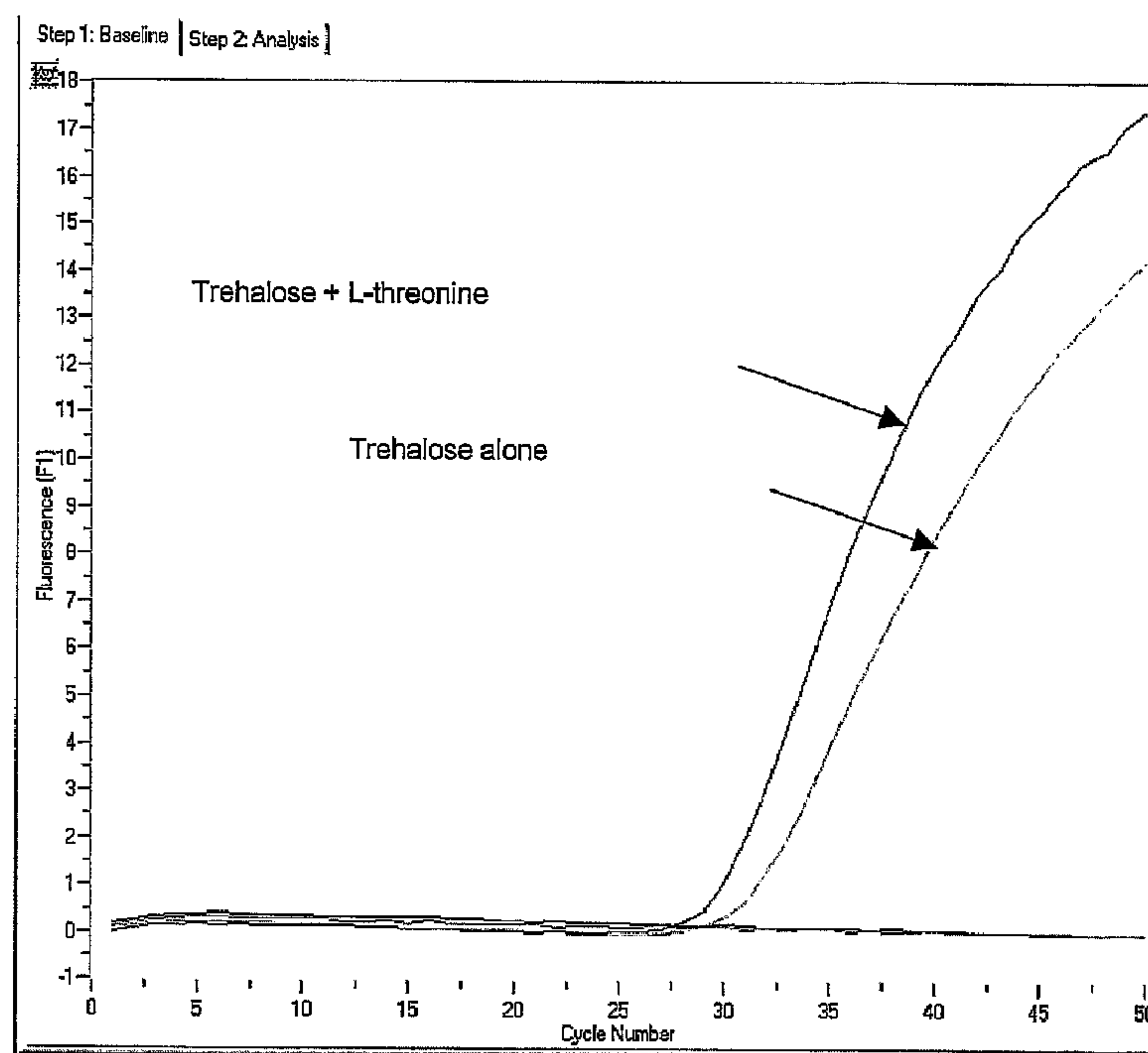
(72) Inventeurs/Inventors:
LEE, MARTIN ALAN, GB;
MITCHELL, JENNIFER, GB;
ADAMS, GERALD (DECEASED), GB

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : COMPOSITIONS

(54) Title: COMPOSITIONS

Figure



(57) Abrégé/Abstract:

A composition for carrying out a chemical or biochemical reaction, said composition being in a freeze-dried form and comprising (i) a set of reagents comprising at least some of the chemical or biochemical reagents necessary for conducting said chemical or

(57) **Abrégé(suite)/Abstract(continued):**

biochemical reaction, including at least one reagent which is fluorescent (ii) a glass forming agent, and (iii) threonine. Kits comprising these compositions and methods of using them form a further aspect of the invention.

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

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
24 December 2008 (24.12.2008)

PCT

(10) International Publication Number
WO 2008/155529 A1

(51) International Patent Classification:

C12Q 1/68 (2006.01) *G01N 33/58* (2006.01)
C12N 9/96 (2006.01)

(21) International Application Number:

PCT/GB2008/002055

(22) International Filing Date: 16 June 2008 (16.06.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0711689.0 16 June 2007 (16.06.2007) GB
0711685.8 16 June 2007 (16.06.2007) GB

(71) Applicant (for all designated States except US): **ENIGMA DIAGNOSTICS LIMITED** [GB/GB]; Building 224, Tet-ricus Science Park, DSTL, Porton Down, Salisbury, Wilt-shire SP4 0JQ (GB).(71) Applicant (for US only): **ADAMS, Valerie** (legal rep-resentative of the deceased inventor) [GB/GB]; 17 Pauls Dean Crescent, Salisbury, Wiltshire SP1 3QU (GB).(72) Inventor: **ADAMS, Gerald** (deceased).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LEE, Martin, Alan**

[GB/GB]; Enigma Diagnostics Limited, Building 224, Tet-ricus Science Park, DSTL, Porton Down, Salisbury, Wilt-shire SP4 0JQ (GB). **MITCHELL, Jennifer** [GB/GB]; 4 Atlas Way, Rivacre Village, Ellesmere Port CH66 1TL (GB).

(74) Agent: **GREAVES, Carol, Pauline**; Greaves Brewster LLP, Indigo House, Cheddar Business Park, Wedmore Road, Cheddar, Somerset BS27 3EB (GB).

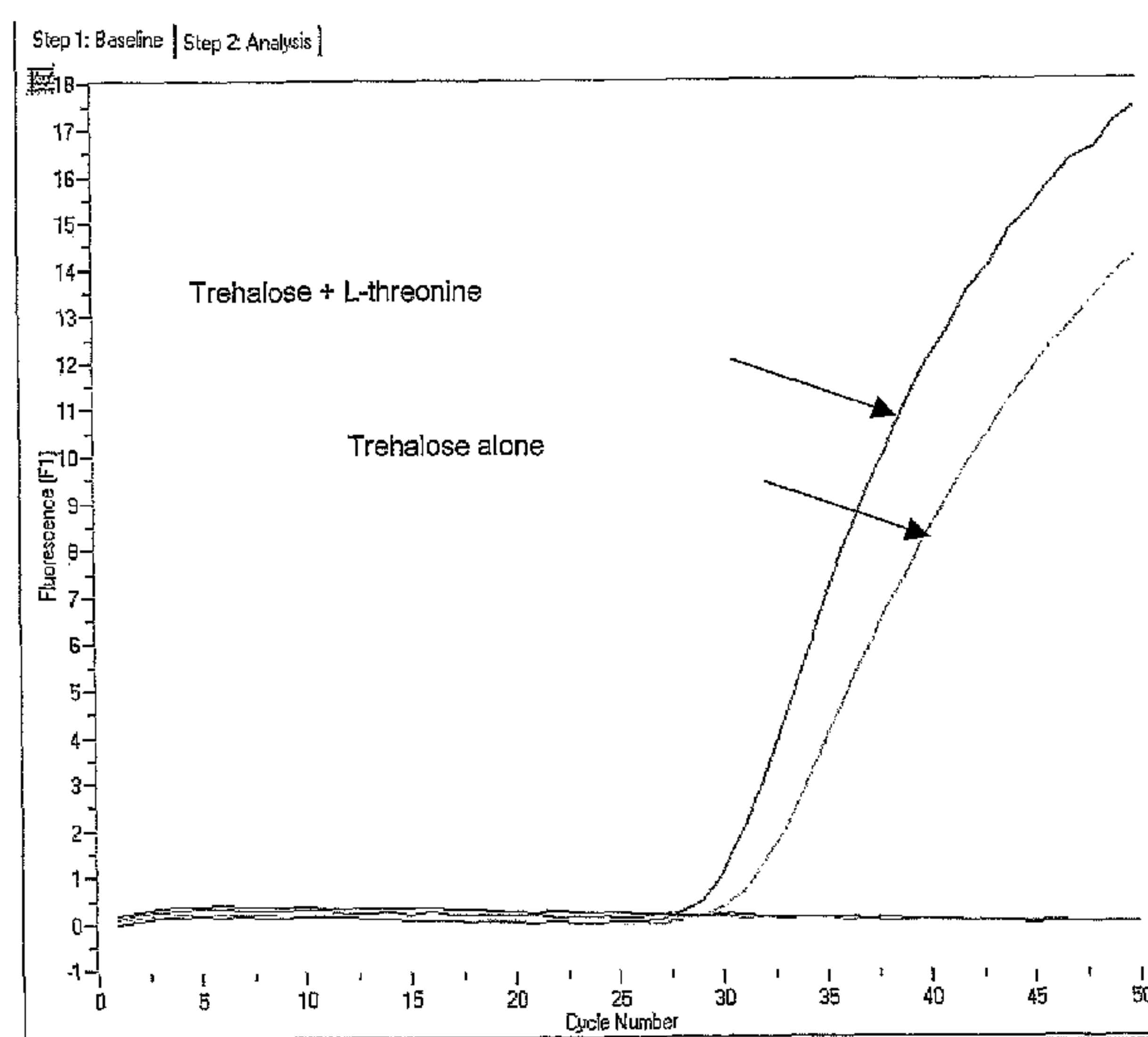
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, 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, 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,

[Continued on next page]

(54) Title: COMPOSITIONS

Figure 1



(57) Abstract: A composition for carrying out a chemical or biochemical reaction, said composition being in a freeze-dried form and comprising (i) a set of reagents comprising at least some of the chemical or biochemical reagents necessary for conducting said chemical or biochemical reaction, including at least one reagent which is fluorescent (ii) a glass forming agent, and (iii) threonine. Kits comprising these compositions and methods of using them form a further aspect of the invention.

WO 2008/155529 A1

WO 2008/155529 A1



FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,
NO, 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*

Compositions

The present invention relates to compositions comprising test reagents for use in chemical or biochemical reactions such as the polymerase chain reaction and to methods for preparing these.

Various preparations are available which provide for pre-determined mixtures of reagents that are routinely used together. For instance, the widely used polymerase chain reaction (PCR) (including reverse transcriptase (RT)-PCR) utilises a range of standard reagents including salts such as magnesium chloride (MgCl_2) and potassium chloride, a polymerase enzyme such as Taq polymerase, buffers such as Tris-HCl, and nucleotides required for an amplification of a nucleic acid. Such preparations are available for example as "ready-to-go PCR beads" from Amersham BioSciences (UK) or Pharmacia.

Generally these are prepared by freeze drying methods, which are conducted in the presence of glass-forming agents and stabilisers for the structures formed as well as optionally fillers (see for example US Patent No. 5,250,429, US Patent No. 5,763,157 and EP-0726310) although other preparation types such as those which use wax carriers are also known (see for example US Patent No. 5,599,660).

These provide a convenient and readily available means for laboratories to conduct PCR reactions of their choice, when required. Generally, the specific reagents which tailor a PCR to the particular target, such as the primers and any probes required for example for use in connection with a Real-time PCR, are added on site.

However, in many cases, in particular in the diagnostics field, the targets are the same in many cases, and therefore the

inclusion of probes and primers into the bead, so that the bead becomes assay specific is desirable for ease of use.

5 A problem with all such beads and preparations is that the components do not always remain stable over long periods of time. As the nature of assays becomes more complex, further reagents including reagents which may include relatively sensitive chemical moieties such as labels in an in particular optical labels such as fluorescent labels or dyes may be
10 required to be added. These in particular are used for conducting assays in "real-time". The sensitive moieties are frequently attached to oligonucleotides which are designed to act as probes or labelled primers. These will hybridise to amplified nucleic acids during the course of the PCR. The fate
15 of the probes during the course of the PCR and changes in the associated signal from the label is used in various ways to monitor the progress of the PCR.

However, the presence of such moieties can exacerbate the
20 problems associated with the stability of the compositions. Furthermore, the presence of additives in compositions may result in a reduction of signalling function in particular from fluorescent labels.

25 L-threonine has been proposed for use in freeze-dried enzyme compositions as a stabiliser for enzymes such as alcohol oxidase and catalase (see JP 61015685). It has further been proposed as a possible co-reactant for chemiluminescent labels such as transition metals (WO2007/005626).

30 The applicants have found discovered a means of providing improved freeze-dried compositions, in particular those which include fluorescent labels.

According to the present invention there is provided the use of L-threonine as a stabiliser for freeze-dried compositions comprising fluorescent reagents. The applicants have found that L-threonine may act as a stabiliser for these
5 compositions, but also, that fluorescent signalling function may be better retained if this is included in the compositions, even when these are stored at unrefridgerated temperatures.

Suitable freeze-dried compositions will include those used for
10 chemical or biochemical reactions which utilise fluorescent labels or moieties as signalling or indicator means.

The present invention provides a composition for carrying out a chemical or biochemical reaction, said composition being in a
15 freeze-dried form and comprising (i) a set of reagents comprising at least some of the chemical or biochemical reagents necessary for conducting said chemical or biochemical reaction including at least one reagent which is fluorescent, (ii) a glass forming agent, and (iii) threonine.

20 The applicants have found that threonine works as an effective anti-oxidant and/or anti-maillard agent, which appears to enhance the stability of the freeze-dried composition. In particular, L-threonine is used. Without being bound by
25 theory, the threonine appears to react with any oxygen produced and therefore assist in the stabilisation of the reaction mixture.

Furthermore, it has been found that the presence of threonine
30 may enhance the signalling achievable from fluorescent labels included in the composition, in particular when stored at elevated temperatures.

The amount of threonine in the composition will vary depending
35 upon the precise nature of the composition. It is suitably selected so that it does not affect the pH of the composition,

which may be important in some chemical or biochemical reactions. Typically however, it may be present in the composition in an amount of from 2-10mM, for example at about 2.5mM.

5

When a composition is freeze dried in the presence of a glass-forming reagent (ii), it generally forms a "cake" type 3-dimensional structure. This structure is optionally supported by the inclusion of a suitable stabiliser (iv) for the cake structure, and so this is a further component of the mixture.

10

Suitable glass-forming reagents include sugars, in particular a non-reducing sugar, for example, trehalose, sucrose or mannose. This is suitably present in the composition in an amount such that it represents from about 1-10%w/w and suitably about 5%w/w in the final composition.

15

Examples of suitable stabilisers that may be included in the composition include polymeric compounds such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and or polysaccharides such as Ficoll or Dextran. In a particular embodiment, however, the stabiliser is omitted from the composition as it has been found that compounds such as PEG may contribute to the inhibition of fluorescent signals.

25

In some cases also, gelatine may be used to add stability to the cake. Gelatine may be obtained from a variety of sources including bovine, pig, seaweed (carrageenan) or fish gelatine. (Any bovine material used is suitably from a certified BSE free source.)

30

The set of reagents (i) above will be selected depending upon the particular nature of the chemical or biochemical reaction being effected. They may include reactions carried out on multiple or repeated occasions such as diagnostic tests, screening tests, nucleic acid amplification reactions,

35

sequencing reactions etc. The compositions may be suitable for use in any assays or reactions in which fluorophores or fluorescent moieties are employed, in particular those which rely upon the use of enzymes to effect the procedures. A
5 specific group of such assays are nucleic acid sequencing reactions and nucleic acid amplification reactions (including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-mediated amplification (TMA), loop-mediated isothermal
10 amplification (LAMP), rolling circle DNA amplification, multiplex ligation-dependent probe amplification (MLPA) and multiple displacement amplification.)

Suitable fluorescent reagents include fluorescent dyes or
15 intercalators such as SYBR Green such as SYBR Green I, SYBR Gold, ethidium bromide, YOPRO-1, and the SYTO dyes including green dyes such as SYTO 9 and red SYTO dyes such as SYTO® 17, SYTO® 59, SYTO® 60, SYTO® 61, SYTO® 62, SYTO® 63 and SYTO® 64.

20 They may also include probes and primers labelled with fluorescent labels. Suitable labels include fluorescein or fluorescein derivatives such as carboxyfluorescein compounds, such as 5-carboxyfluorescein, 6-carboxyfluorescein, or their succinimidyl esters, cyanine dyes or rhodamine dyes.
25 Particular examples of such dyes include fluorescein, JOE, FAM, HEX, TET, TAMRA, ROX Cy5, Cy3, Cy5.5, BoDIPY FL, rhodamine, rhodamine green, rhodamine red, Oregon Green 488, 500 or 514, Texas red, LightCycler Red 610, 640, 670 or 705.

30 Dark quenchers may also be present. These are generally used in assay systems to modify fluorescent signals but without emitting detectable signals themselves. These are essentially nonfluorescent dyes include in particular azo dyes (such as
35 DABCYL or DABSYL dyes and their structural analogs), triarylmethane dyes such as malachite green or phenol red,

6

4',5-diether substituted fluoresceins (as described for example in U.S. Patent No. 4,318,846, or asymmetric cyanine dye quenchers (as described for example in WO 99/37717, the content of each of which is incorporated herein by reference).

5

In particular, quenching moiety is DABCYL (4-(dimethylaminoazo) benzene-4-carboxylic acid) or a derivative thereof, such as the halide or amide derivative, which facilitates attachment of the moiety to an amino acid of an oligonucleotide.

10

In another embodiment, the quenching moiety is an essentially nonfluorescent derivative of 3- and/or 6-amino xanthene that is substituted at one or more amino nitrogen atoms by an aromatic or heteroaromatic ring system (for example as described in US Patent No. 6,399,392, the content of which is incorporated herein by reference). These quenching dyes typically have absorption a maximum above 530 nm, have little or no observable fluorescence and efficiently quench a broad spectrum of luminescent emission, such as is emitted by chemilumiphores, phosphors, or fluorophores. In one embodiment, the quenching dye is a substituted rhodamine. In another embodiment, the quenching compound is a substituted rhodol.

15

20

25

30

35

40

45

50

55

60

65

70

75

80

85

90

95

100

105

110

115

120

125

130

135

140

145

150

155

160

165

170

175

180

185

190

195

200

205

210

215

220

225

230

235

240

245

250

255

260

265

270

275

280

285

290

295

300

305

310

315

320

325

330

335

340

345

350

355

360

365

370

375

380

385

390

395

400

405

410

415

420

425

430

435

440

445

450

455

460

465

470

475

480

485

490

495

500

505

510

515

520

525

530

535

540

545

550

555

560

565

570

575

580

585

590

595

600

605

610

615

620

625

630

635

640

645

650

655

660

665

670

675

680

685

690

695

700

705

710

715

720

725

730

735

740

745

750

755

760

765

770

775

780

785

790

795

800

805

810

815

820

825

830

835

840

845

850

855

860

865

870

875

880

885

890

895

900

905

910

915

920

925

930

935

940

945

950

955

960

965

970

975

980

985

990

995

1000

1005

1010

1015

1020

1025

1030

1035

1040

1045

1050

1055

1060

1065

1070

1075

1080

1085

1090

1095

1100

1105

1110

1115

1120

1125

1130

1135

1140

1145

1150

1155

1160

1165

1170

1175

1180

1185

1190

1195

1200

1205

1210

1215

1220

1225

1230

1235

1240

1245

1250

1255

1260

1265

1270

1275

1280

1285

1290

1295

1300

1305

1310

1315

1320

1325

1330

1335

1340

1345

1350

1355

1360

1365

1370

1375

1380

1385

1390

1395

1400

1405

1410

1415

1420

1425

1430

1435

1440

1445

1450

1455

1460

1465

1470

1475

1480

1485

1490

1495

1500

1505

1510

1515

1520

1525

1530

1535

1540

1545

1550

1555

1560

1565

1570

1575

1580

1585

1590

1595

1600

1605

1610

1615

1620

1625

1630

1635

1640

1645

1650

1655

1660

1665

1670

1675

1680

1685

1690

1695

1700

1705

1710

1715

1720

1725

1730

1735

1740

1745

1750

1755

1760

1765

1770

1775

1780

1785

1790

1795

1800

1805

1810

1815

1820

18

generally be such that the pH is between 8.3 and 9, for instance between 8.5 and 8.8. However, it is possible that one or more of these elements may be missing in particular where these elements can be readily added later, for example in a rehydration buffer used to reconstitute the dried composition ready for use. In particular, the necessary salts may be added in this way and so the set of reagents of (i) may omit the salts. Where this is done, the composition may be supplied in the form of a kit with rehydration buffer, containing the necessary salt supplements.

The composition may further comprise a fluorescently labelled oligonucleotide useful in monitoring the progress of a polymerase chain reaction in real time. Also as used herein, the expression "real-time" means that the polymerase chain reaction can be monitored as it progresses and without halting or opening the reaction vessel. By monitoring how the amplification occurs and in particular at which cycles exponential increase in amplicon becomes significant allows the amount of target nucleic acid present in the sample being subject to the PCR to be quantitated as is well known and understood in the art.

The amounts of the various components included in the composition will vary depending upon factors such as the precise nature of the particular component, the nature of the PCR which it is intended should be conducted etc. However, this will be determinable in each case using established protocols and procedures as would be understood in the art.

Suitable labelled oligonucleotides are any of the labelled probes or labelled primers which may be used in the monitoring of polymerase chain reactions in real time. Thus in a particular embodiment they will comprise probes which are capable of hybridising to the amplified nucleic acid sequence and which carry fluorescent labels in particular, which provide

a signal which varies in accordance with the progress of the PCR.

Thus for probes intended to be utilised in a TAQMAN™ assay, for example, they will generally comprise a probe which carries two labels, one of which is able to act as a donor of energy and particularly fluorescent energy, and one of which is able to act as an acceptor of that energy or "quencher". Whilst the probe is intact, these labels are held in close proximity to each other so that interaction of energy occurs. In the case of fluorescent labels, this is known as fluorescent energy transfer (FET) or fluorescent resonant energy transfer (FRET).

The probes are designed to bind to a specific region on one strand of a template nucleic acid. Following annealing of the PCR primer to this strand, *Taq* enzyme extends the DNA with 5' to 3' polymerase activity. *Taq* enzyme also exhibits 5' to 3' exonuclease activity. TaqMan™ probes are protected at the 3' end by phosphorylation to prevent them from priming *Taq* extension. If the TaqMan™ probe is hybridised to the product strand, an extending *Taq* molecule will hydrolyse the probe, liberating the donor from acceptor. This means that the interaction between the donor and the acceptor is broken, so the signal from each, changes, and this change can be used as the basis of detection. The signal in this instance is cumulative, the concentration of free donor and acceptor molecules increasing with each cycle of the amplification reaction.

Hybridisation probes are available in a number of forms and these may also be included in the compositions. Molecular beacons are oligonucleotides that have complementary 5' and 3' sequences such that they form hairpin loops. Terminal fluorescent labels are in close proximity for FRET to occur when the hairpin structure is formed. Following hybridisation of molecular beacons to a complementary sequence the

fluorescent labels are separated, so FRET does not occur, and this forms the basis of detection during a polymerase chain reaction.

- 5 Pairs of labelled oligonucleotides may also be used as probes in the detection of a polymerase chain reaction. These hybridise in close proximity on a PCR product strand-bringing donor and acceptor molecules together so that FRET can occur. Enhanced FRET is the basis of detection. Methods of this type
- 10 are described for example in European Patent Application No.0912760 the entire content of which is incorporated herein by reference. Variants of this type include using a labelled amplification primer with a single adjacent probe.
- 15 WO 99/28500 (the entire content of which is incorporated herein by reference) describes a very successful assay for detecting the presence of a target nucleic acid sequence in a sample. In this method, a DNA duplex binding agent and a probe specific for said target sequence, is added to the sample. The probe
- 20 comprises a reactive molecule able to absorb fluorescence from or donate fluorescent energy to said DNA duplex binding agent. This mixture is then subjected to an amplification reaction in which target nucleic acid is amplified, and conditions are induced either during or after the amplification process in
- 25 which the probe hybridises to the target sequence. Fluorescence from said sample is monitored.

Thus, compositions adapted for use in this assay, known as "Resonsence"TM may also be prepared. In this instance, the

30 composition will suitably further comprise a DNA duplex binding agent such as an intercalating dye.

An alternative form of this assay, which utilises a DNA duplex binding agent which can absorb fluorescent energy from the

35 fluorescent label on the probe but which does not emit visible

light, is described in WO2004/033726, the entire content of which is incorporated herein by reference.

In general, all probes used in these types of assays are
5 blocked to extension at the 3' end for example by phosphorylation, or by having a label directly attached at the 3' hydroxyl group. This prevents the probe from acting as a secondary primer, and being extended during the PCR, and so eliminates interfering products.

10

The amounts of probe utilized in any particular composition will vary depending upon factors such as whether it is used up or hydrolysed during the PCR, as well as the nature of the signaling system. These would be understood by the skilled
15 person. Generally however, the amount of the or each probe added to a composition will be sufficient to ensure that the concentration of probe in the final composition is between 0.05 μ M to 1 μ M, for example at about 0.2 μ M.

20 Other real-time assays utilize labeled primers in order to provide a monitoring system. Some of these primers may include a self-probing "tail" and are known as "Scorpion" primers. A labelled probe is linked to a DNA sequence which acts as a primer to the reaction by way of a "blocking group" which is
25 suitably a chemical linker or non-amplifiable monomer such as hexethylene glycol and which prevents an extension reaction amplifying the probe region of the oligonucleotide. Probe/primer combinations of this general type are well known as "Scorpions" and these are described for instance in WO
30 99/66071. The Scorpion may along its length comprise a donor/quencher pair so that FRET signalling is possible as described above.

A further class of real-time probes called LUXTM (light upon
35 extension) fluorogenic primers are also available. These are "hairpin" like probes, similar to the molecular beacons as

described above. However, LUX primers adopt a stem-loop structure in solution, and like Scorpion probes, LUX primers are intended for use as PCR primers. They do not contain a quencher moiety as they are fluorescent oligonucleotides which are designed to self-quench based on sequence context. LUX primers quench when free in solution, fluoresce weakly when denatured, and emit light strongly when incorporated into DNA. These also may be included in the compositions of the invention.

10 The polymerase included in the set of reagents (i) is selected so that it is useful in conducting the desired "real-time" assay. Thus for assays such as TAQMAN™, where hydrolysis of the probe is essential in order to initiate a detectable signal, a polymerase having a high level of 5'-3' exonuclease activity is suitably employed, whereas for assays such as Resonance™ assays, where probe hybridization is employed, such activity may be low or absent. The polymerase is suitably a thermostable polymerase which will operate and withstand the elevated temperatures needed for conducting a polymerase chain reaction. The amount of polymerase added should be sufficient to effect a PCR reaction, as is understood in the art. Typically, the amount of polymerase added will be sufficient to provide a concentration of from 0.02 to 1.0U/μl composition and typically about 0.025U/μl.

25 Suitably, the composition may further comprise reagents which are used in ensuring that the polymerase chain reaction does not start prematurely. So called "Hot-Start" PCR may be effected by various methods.

30 The problem addressed by a "Hot-Start" PCR arises because a successful PCR relies on the sequence of steps, denaturation, annealing and extension, occurring in a very precise order and at the precise temperature required for the operation of that step. A problem arises when reagents are mixed together, even

35

for short periods of time, at different temperatures, for example prior to the start of the reaction. Primers may interact with nucleic acid template, resulting in primer extension of the template. This can lead to a reduction in the overall yield of the desired product as well as the production of non-specific products.

Initial attempts to overcome the problem used a wax barrier to separate the various PCR reagents from each other in a test tube (see for example USP 5,565,339). The wax melted as the reaction mixture was heated to the initial denaturation temperature, allowing the reagents to mix together at the last possible moment, so that the possibility of side-reactions was minimized, and this gave rise to the expression "Hot Start".

Other chemical methods for achieving the suppression of side-reactions have been attempted. For example, US Patent No. 5,677,152 describes a method in which the DNA polymerase is chemically modified to ensure that it only becomes active at elevated temperatures. In order to effect this method, it is necessary only to include an appropriately modified DNA polymerase in item (i) above.

In another embodiment, a monoclonal antibody to *Thermus aquaticus* (Taq) DNA polymerase such as the anti-Taq DNA polymerase antibody available from Sigma, is including into the composition. The antibody binds to the enzyme, so as to inactivate it, at ambient temperature. However, the antibody denatures and dissociates from the enzyme at elevated temperatures used during the amplification cycles and so the enzyme becomes active.

The relative amount of any anti-Taq antibody included in the composition is suitably sufficient to ensure that it is able to fulfill the function of inhibiting the Taq enzyme until it is required. Generally therefore an excess of anti-Taq antibody

as compared to Taq enzyme will be used. Thus for example for every unit of Taq enzyme in the composition, at least 1.5 and preferably at least 2 units of anti-Taq antibody will be included. Taq anti body is usually sold by the μg and the concentration is very dependant upon the source and quality of the antibody as well as the nature of the assay. Too much antibody may be detrimental and can actually cause more primer dimmer in some assays. However, the precise amount of Taq antibody will be determined in accordance with usual practice and will typically be in the range of 0.001 to 0.004 $\mu\text{g}/\mu\text{l}$ final reaction mixture.

Yet another Hot-Start methodology involving the use of a combination of an inhibitory amount of a pyrophosphate salt to prevent primer extension taking place, and a pyrophosphatase enzyme which digests this pyrophosphate at elevated temperatures, to allow the PCR to progress is described in WO 02/088387, the entire content of which is incorporated herein by reference.

20

In this case, the pyrophosphate salt and the pyrophosphatase enzyme may be included as further components of the composition of the invention.

25 The use and precise selection of optional stabiliser (iv) will depend to some extent on the particular assay intended to be carried out using the final composition and this can be tested using routine methods. For example, it has been found that dextran is less preferred when the composition includes DNA duplex binding agents and labelled probes intended and is intended to be used to conduct a ResonSense™ assay as described above. However, PEG is a particularly suitable stabiliser for most of these compositions. Stabiliser is suitably added in an amount such that it represents from about 1-3%w/w in the final composition.

35

As discussed above, the set of reagents of item (i) may comprise components such as buffers, primers, nucleotides and optionally also salts, in the amounts which are generally understood for the preparation of PCR reaction mixtures.

5 Primers are suitably present in excess and this is typically achieved by including sufficient primers to ensure that the concentration of each primer in the final composition is of the order of 0.1 μ M to 1 μ M.

10 Compositions of the invention may further comprise an RNase inhibitor. The applicants have surprisingly found that addition of RNase inhibitors has a stabilising effect on the composition, even where the composition contains no RNA elements or is intended for use in amplification reactions in
15 which RNA is involved, such as RT-PCR. Its addition improves the stability of the composition, even over prolonged time periods, at the end of which, the composition is still able to operate in an effective manner when used in real-time PCR methods.

20

Without being constrained by theory, it is possible that they are assisting in preventing attack of the probe by the polymerase or otherwise controlling the activity of the polymerase, for reasons that are not understood. The number of
25 units of RNase inhibitor (for example the RNase inhibitor available commercially as RI Out available from Invitrogen), is suitably should be sufficient to control the activity of the polymerase in the composition. Thus generally the number of units of RNase inhibitor will be of the same general order or
30 preferably be higher than the amount of polymerase present in the composition to ensure effective inhibition. For example, where 0.05U/ μ l polymerase is included in a composition, this will contain from 0.04 to 0.1U/ μ l RNase inhibitor.

15

In a particular embodiment, a blocking compound, as is conventional in PCR reaction mixtures, may be included in the composition. The blocking compound is believed to function by preventing inhibition of the PCR by interaction with the vessel
5 walls, for example by preventing leaching of metals or sequestering any metals which may leach from the walls in the course of the reaction. The nature of the blocking compound will depend upon the nature of the vessel into which it is intended that the reaction should be conducted.

10

Particular examples of blocking compounds are glass coating or glass blocking compounds such as bovine serum albumin (BSA) either alone or in combination with other blocking materials such as gelatine. As described above, gelatine may be obtained
15 from a variety of sources including bovine, pig, seaweed (carrageenan) or fish gelatine.

Blocking agents are suitably included in effective amounts which will depending upon the particular compound selected.
20 However, for BSA for instance, the amount is suitably sufficient to provide from 0.1 to 1mg/ml and preferably about 0.25mg/ml in the final composition. Gelatines will suitably be present in an amount in the range of from about 0.0025%-
0.01%w/w. Care should be taken that the amount of blocking
25 agent is not sufficiently high so as to significantly inhibit the final reaction.

Further components may be included in the composition as would be understood in the PCR art. These might include sequences
30 used as internal controls as well as primers for amplifying these sequences and signalling systems such as those outlined above for detecting amplification of the internal control sequences.

35 Compositions of the invention are suitably prepared by mixing together the required components as described above to form a

composition, and adding water, preferably sterile water which
been treated with diethyl pyrocarbonate (DEPC) to the
composition to allow for mixing, for example by adding at least
equivalent volume and preferably from 1-1.5 times the volume of
5 the composition. The thus formed mixture is, if necessary
dispensed into suitable aliquots each of which contains
sufficient material for a PCR in an individual reaction pot,
and then subjected to a freeze drying process. If freeze
drying does not take place immediately, the final mixture is
10 suitably stored at low temperatures, for example on ice, or in
a freezer if the delay is prolonged beyond about 0.5 hours,
until freeze drying takes place.

The freeze-drying protocol used will depend to some extent upon
15 the particular composition being dried and will be determined
in each case using routine procedures. Typically, the
composition will be subject to a freezing step in which it is
cooled to a low temperature for example from about -20°C to -
60°C and generally at about -40°C at a pressure of from 300-
20 400torr, and held at this temperature for a sufficient period
of time to ensure that complete freezing occurs.

The pressure is then reduced to an appropriate level depending
upon the particular freeze-dryer used. Some may operate a
25 pressures as low as 6Mtorr but for current purposes, pressures
of from 10 to 100mTorr may be suitable to allow the water to
sublimate. Suitably then the composition is brought gradually
back up to room temperature under reduced pressure, before the
vacuum is released to minimise condensation effects.

30 Optionally, the vacuum is released in the presence of an inert
atmosphere such as nitrogen, so that the product is maintained
in an inert environment. This also prevents moisture ingress.

Freeze-dried product obtained in this way, it is suitably
35 packaged immediately for example in foil wrappers, to minimise
the contamination risk. If the composition is contained within

containers such as reagent pots, these are suitably sealed before the vacuum is released.

Care needs to be taken to ensure that all reagents utilised in the composition do not contain materials or contaminants which could inhibit or prevent freeze drying in the levels in which they are found. Thus for example, it may be necessary to remove substances such as glycerol which are sometimes included in commercially available enzymes such as polymerases, reverse transcriptase polymerases and RNase inhibitors, and or to reduce the levels of substances such as dimethyl sulphoxide (DMSO) which may be found in intercalating dyes which may be used as DNA duplex binding agents.

Compositions as described above have been found to be stable for extended periods of time, including up to 3 months, at the end of which, no activity loss at all was seen.

Methods for forming compositions described above form a further aspect of the invention. In a particular embodiment, the invention provides a method for preparing a freeze dried composition, which comprises mixing together at least items (i) to (iv) above and freeze drying the resultant mixture.

In use the compositions of the invention are hydrated using conventional methods, for example using a rehydration buffer and then subject to the appropriate chemical or biochemical reaction. Generally, the composition will be mixed with a chemical or biochemical sample before the reaction is conducted. For example, in the case of a polymerase chain reaction, the reaction mixture is combined with a sample which contains or is suspected of containing a target nucleic acid, and the final mixture subjected to PCR conditions. Fluorescence from the fluorescent reagent is monitored either before, during or after the process as required. In particular, the signal is monitored in real-time as required,

so as to allow the progress of the reaction to be monitored and the amount of target in the sample quantified, as is understood in the art. Such methods form a further aspect of the invention.

5

Thus in a further aspect, the invention provides the use of an RNase inhibitor as a stabiliser in reaction compositions.

10

Thus, for example, a composition for carrying out a polymerase chain reaction to amplify a target DNA sequence in a sample in such a way that the reaction may be monitored in real time, is prepared, said composition comprising (a) a set of reagents suitable for conducting a polymerase chain reaction comprising a polymerase capable of extending a primer when adhered to a template DNA sequence during a polymerase chain reaction, (b) a labelled oligonucleotide useful in monitoring the progress of a polymerase chain reaction in real time; and (c) an RNase inhibitor. The RNase inhibitor may be advantageous, even where the polymerase chain reaction is not a reverse transcriptase polymerase chain reaction, and so there are no RNA elements present or taking part in the reaction, and no reverse transcriptase used in the reaction.

20

Suitably the number of units of RNase inhibitor will be at least the same or higher than the amount of polymerase present in the composition. These compositions may further comprise (d) a glass forming agent and optionally (e) a stabilising agent therefore, and be freeze-dried as described above. Furthermore the reagents (a) may be PCR reagents as described above in relation to (i).

30

The invention will now be particular described by way of example with reference to the accompanying Figure which is a graph showing a fluorescence vs cycle number of obtained using a composition of the invention, adapted to carry out a

35

conventional TaqMan assay, as compared to a similar composition lacking the threonine component.

Example 1

- 5 Preparation of Composition for conducting Resonsense™ assay for detecting *Bacillus subtilis* var. *globigii* (BG) DNA and spores
The following reagents were mixed together. Primers were designed using conventional primer design software to amplify a BG specific DNA sequence. The probe was designed to hybridise
10 to the amplified BG sequence intermediate the primers, and fluorescently labelled with a conventional FAM label.

Table 2

Reagent	Conc.	Vol. (μ l per 50 μ l reaction volume)	Final concentration in reaction
Tris pH 8.8	500mM	2.5	50mM
BSA	20mg/ml	0.3	0.25mg/ml
MgSO ₄	100mM	0.75	3mM
KCl	500nM	1.25	25mM
dUTP mix	2mM	2.5	0.2mM
Forward primer	10 μ M	2.5	1 μ M
Reverse primer	10 μ M	2.5	1 μ M
FAM labelled probe	2 μ M	2.5	0.2 μ M
Syto 63 dye*	50 μ M	2.5	5 μ M
Trehalose	50%w/v	2.5	5%
PEG 20,000	10%w/v	7.5	3%
RI Out ribonuclease inhibitor	5U/ μ l filtered	0.5	0.1U/ μ l
L-threonine	400mM	0.62	10mM
Taq antibody	5U/ μ l filtered	0.5	0.1U/ μ l
Taq polymerase	5U/ μ l filtered	0.5	0.05U/ μ l
Water	DEPC treated	21.03	

*Available from Invitrogen

Once these reagents had been combined in a reaction tube, it was stored on ice and dispensed in 50µl aliquots into reagent pots which had been pre-chilled in a fridge within half an hour. These were then placed inside a freeze dryer (Virtis Advantage), which was set to carry out the program summarised in Table 3.

Table 3

Step	Temp°C	Time (min)	Pressure (Torr)	Ramp/Hold
Thermal Treatment				
1	+10	15	3-400	H
2	-40	55	3-400	R
3	-40	120	3-400	H
Freeze, condenser, vac	040	0	100mTorr	
Primary Drying				
1	-40	45	100mTorr	H
2	+5	55	100mTorr	R
3	+5	30	100mTorr	H
4	+20	25	100mTorr	R
5	+20	300	100mTorr	H
6	+5	25	100mTorr	R
7	+5	300	100mTorr	H
8	+20	25	100mTorr	R
9	+20	15	100mTorr	H
10	+10	20	100mTorr	R
11	+10	1000	100mTorr	H
Secondary Drying				
	+27 set point			
Post Heat Settings				
	+10	1000	100mTorr	

10

The pots were then removed from the freeze dryer and foil sealed immediately. They were stored at room temperature, and retained full activity when tested after 6 weeks.

Example 2Preparation of Composition for conducting Dual Hybridisation assay for detecting ATX DNA

- 5 The procedure of Example 1 was broadly followed, except that a dual hybridisation probe pair were used instead of the single FAM labelled probe, and using the reagents listed in Table 2. In this case, the probes were designed to hybridise to the amplified ATX DNA so that the FAM and Cy5 labels were brought
- 10 into close proximity to each other.

Table 4

Reagent	Conc.	Vol. (μ l per 50 μ l reaction volume)	Final concentration in reaction
Tris pH 8.8	500mM	2.5	50mM
BSA	20mg/ml	0.31	0.25mg/ml
MgCl ₂	100mM	0.75	3mM
dUTP mix	2mM	2.5	0.2mM
Forward primer	10 μ M	2.5	1 μ M
Reverse primer	10 μ M	2.5	1 μ M
FAM labelled Donor probe	2 μ M	2.5	0.2 μ M
Cy5 labelled acceptor probe	2 μ M	2.5	0.2 μ M
L-Threonine	400mM	0.31	5mM
Trehalose	50%w/v		10%
PEG 20,000	10%w/v	7.5	1%
RI Out ribonuclease inhibitor	5U/ μ l filtered	0.3	0.06U/ μ l
Taq antibody	5U/ μ l filtered	0.5	0.1U/ μ l
Taq polymerase	5U/ μ l filtered	0.5	0.05U/ μ l
Water	DEPC treated	27.9	

As before, once these reagents had been combined in a reaction tube, it was stored on ice and dispensed in 50 μ l aliquots into reagent pots which had been pre-chilled in a fridge within half an hour. These were then placed inside a freeze dryer (Virtis Advantage), which was previously chilled to +5°C and then set to carry out the program summarised in Table 5.

Table 5

Step	Temp°C	Time (min)	Pressure (Torr)	Ramp/Hold
Thermal Treatment				
1	+10	15	3-400	H
2	-40	65	3-400	R
3	-40	120	3-400	H
Freeze, condenser, vac	040	0	100mTorr	
Primary Drying				
1	-40	45	100mTorr	H
2	+5	55	100mTorr	R
3	+5	600	100mTorr	H
4	+20	25	100mTorr	R
5	+20	180	100mTorr	H
6	+5	25	100mTorr	R
7	+5	180	100mTorr	H
8	+20	25	100mTorr	R
9	+20	15	100mTorr	H
10	+10	20	100mTorr	R
11	+10	1000	100mTorr	H
Secondary Drying	+27 set point			
Post Heat Settings	+10	1000	100mTorr	

The pots were then removed from the freeze dryer and foil sealed immediately. They were stored at room temperature, and retained full activity when tested after 6 weeks.

Example 3Preparation of Composition for conducting Taqman™ assay for detecting BG DNA and spores

The procedure of Example 1 and Example 2 was generally followed, but in this case, a Taqman™ assay for the BG DNA was prepared. A mixture of the reagents listed in Table 6 was prepared.

Table 6

Reagent	Conc.	Vol. (μ l per 50 μ l reaction volume)	Final concentration in reaction
RI Out ribonuclease inhibitor	5U/ μ l filtered	0.3	0.06U/ μ l
Tris pH 8.8	500mM	2.5	50mM
BSA	20mg/ml	0.31	0.25mg/ml
MgCl ₂	100mM	0.75	3mM
dUTP mix	2mM	2.5	0.2mM
Forward primer	10 μ M	2.5	1 μ M
Reverse primer	10 μ M	2.5	1 μ M
Dual labelled Taqman probe	2 μ M	2.5	0.2 μ M
Trehalose	50%w/v	2.5	5%
PEG 20,000	10%w/v	2.5	1%
L-threonine	400mM	0.16	2.5mM
Taq antibody	5U/ μ l filtered	0.5	0.1U/ μ l
Taq polymerase	5U/ μ l filtered	0.5	0.05U/ μ l
Water	DEPC treated	35.24	

Once these reagents had been combined in a reaction tube, it was stored on ice and dispensed in 50 μ l aliquots into reagent pots which had been pre-chilled in a fridge within half an hour. These were then placed inside a freeze dryer, which had

previously been chilled to +5°C and was set to carry out the program summarised in Table 5 above.

The pots were then removed from the freeze dryer and foil sealed immediately. They were stored at room temperature, and retained full activity when tested after 6 weeks.

Example 4

Preparation of Composition for conducting Lux™ assay for detecting BG DNA and spores

The procedure of Examples 1-3 was generally followed, but in this case, a Lux™ assay for the BG DNA was prepared. A composition comprising the reagents listed in Table 7 was prepared

Table 7

Reagent	Conc.	Final concentration in reaction
RI Out ribonuclease inhibitor	5U/μl filtered	0.1U/μl
Tris pH 8.8	500mM	50mM
BSA	20mg/ml	0.25mg/ml
MgCl ₂	100mM	3mM
dUTP mix	2mM	0.2mM
Fish gelatine		0.006%
LUX labelled primer	10μM	0.5μM
Reverse primer	10μM	0.5μM
Trehalose	50%w/v	10%
PEG 20,000	10%w/v	1%
L-threonine	400mM	10mM
Taq antibody	5U/μl filtered	0.1U/μl
Taq polymerase	5U/μl filtered	0.05U/μl
Water	DEPC treated	

Once these reagents had been combined in a reaction tube, they were freeze dried as described above.

Example 5

5 Preparation of Composition for conducting Scorpion™ assay for detecting CHL DNA

The procedure of Examples 1-4 was generally followed, but in this case, a Scorpion™ assay for the CHL DNA was prepared. A composition comprising the reagents listed in Table 8 was
10 prepared

Table 8

Reagent	Conc.	Final concentration in reaction
RI Out ribonuclease inhibitor	5U/ μ l filtered	0.1U/ μ l
Tris pH 8.8	500mM	50mM
BSA	20mg/ml	0.25mg/ml
MgCl ₂	100mM	3mM
dUTP mix	2mM	0.2mM
Fish gelatine		0.006%
Scorpion labelled primer	10 μ M	1 μ M
Reverse primer	10 μ M	1 μ M
Trehalose	50%w/v	10%
PEG 20,000	10%w/v	2%
L-threonine	400mM	10mM
Taq antibody	5U/ μ l filtered	0.1U/ μ l
Taq polymerase	5U/ μ l filtered	0.05U/ μ l
Water	DEPC treated	

Once these reagents had been combined in a reaction tube, they were freeze dried as described above.

Example 6Comparative study using Taqman™ assay for detecting BG DNA and spores

A TaqMan™ mixture was formulated with the components listed in
 5 Table 9:

Table 9

Reagent	Conc.	Vol per reaction	Final concentration in reaction
Tris pH 8.8	500mM	2.5	50mM
BSA	20mg/ml	0.31	0.25mg/ml
MgCl ₂	100mM	0.75	3mM
dUTP mix	2mM	2.5	0.2mM
Forward primer	10μM	2.5	1μM
Reverse primer	10μM	2.5	1μM
Trehalose	50%w/v	2.5	5%
L-threonine	400mM	0.16	2.5mM
Taq antibody	5U/μl filtered	0.4	0.08U/μl
Taq polymerase	5U/μl filtered	0.2	0.04U/μl
TaqMan probe	2μM	2.5	0.2uM
Internal control probe	2μM	2.5	0.2uM
Water	DEPC treated	to 50μl	

10 A similar mixture was created without the L-threonine. Both mixtures were then freeze-dried as described in Example 1.

Once dried they were then resuspended in a sample containing template DNA (or water in the control) and a conventional
 15 TaqMan™ assay on BD positive samples, carried out on a LightCycler™, set to cycle in the following cycling regime:

Initial Denaturation	- 95°C for 2 minutes
Cycling	-95°C for 15 seconds
	-55°C for 20 seconds

5

Fluorescent data was aquired once in each cycle. The results are shown graphically in the attached Figure. The signal from the L-threonine containing composition was clearly less inhibited that that from the similar composition from which L-

10 threonine was omitted. Hence, it appears that the use of L-threonine will not only assist the stability as a result of the anti-oxidant/anti maillard properties, but also that it will reduce the inhibition of fluorescent signals.

Claims

1. A composition for carrying out a chemical or biochemical reaction, said composition being in a freeze-dried form and
5 comprising (i) a set of reagents comprising at least some of the chemical or biochemical reagents necessary for conducting said chemical or biochemical reaction, including at least one reagent which is fluorescent (ii) a glass forming agent, and (iii) threonine.
- 10 2. A composition according to claim 1 wherein the threonine is present in the composition in an amount of from 2-10mM.
3. A composition according to claim 1 or claim 2 wherein the
15 glass-forming reagent (ii) is a non-reducing sugar.
4. A composition according to any one of the preceding claims which further comprises a stabiliser (iv) for the glass forming agent.
- 20 5. A composition according to claim 5 wherein the stabiliser (iv) is selected from polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) or polysaccharide.
- 25 6. A composition according to any one of the preceding claims wherein the set of reagents (i) is a set of reagents which is specifically adapted to carry out a polymerase chain reaction (PCR) that includes a fluorescent reagent.
- 30 7. A composition according to claim 6 wherein the set of reagents comprises a polymerase capable of extending a primer when adhered to a template nucleic acid sequence during a polymerase chain reaction.
- 35 8. A composition according to claim 6 or claim 7 wherein the set of reagents includes all of buffer, salts, primers and

nucleotides suitable for conducting a polymerase chain reaction to amplify a DNA sequence.

9. A composition according to claim 6 or claim 7 wherein
5 wherein the mixture of (i) includes buffer, primers and nucleotides suitable for conducting a polymerase chain reaction to amplify a DNA sequence.

10. A composition according to any one of the preceding
10 claims wherein the fluorescently labelled reagent comprises a labelled oligonucleotide useful in monitoring the progress of a polymerase chain reaction in real time.

11. A composition according to claim 10 wherein the labelled
15 oligonucleotide is a probe which carries two labels, one of which is able to act as a donor of energy and one of which is able to act as an acceptor of that energy.

12. A composition according to any one of claims 10 or claim
20 11 wherein the labelled oligonucleotide is in the form of a molecular beacon.

13. A composition according to claim 10 which comprises a
pair of labelled probes one of which carries a label with is a
25 fluorescent energy donor and one of which carries a label which is able to accept fluorescent energy from said energy donor, and wherein the probes hybridise in close proximity on a PCR product strand.

30 14. A composition according to any one of claims 10 to 13 wherein the composition further comprises a DNA duplex binding agent which is able to exchange energy with said fluorescently labeled oligonucleotide.

15. A composition according to any one of claims 5 to 14 which further comprises one or more reagents able to control the initiation of the PCR reaction.

5

16. A composition according to claim 15 wherein the reagent is an anti-Taq antibody.

17. A composition according to any one of claims 5 to 16
10 which further comprises an RNase inhibitor.

18. A composition according to claim 17 wherein the number of units of RNase inhibitor will be at least the same or higher than the amount of polymerase present in the composition.

15

19. A composition according to any one of the preceding claims which further comprises an anti-oxidant and/or anti-maillard agent.

20. A composition according to any one of the preceding claims which further comprises a blocking compound.

21. A method for preparing a composition according to any one of the preceding claims, said method comprising mixing together
25 components of the composition and freeze drying the resultant mixture.

22. A kit comprising a composition according to claim 5 and a rehydration buffer.

30

23. A kit according to claim 22 wherein the composition does not contain salts necessary for carrying out a PCR, and the rehydration buffer includes said salts.

35 24. A method for conducting a chemical or biochemical reaction which comprises hydrating a composition according to

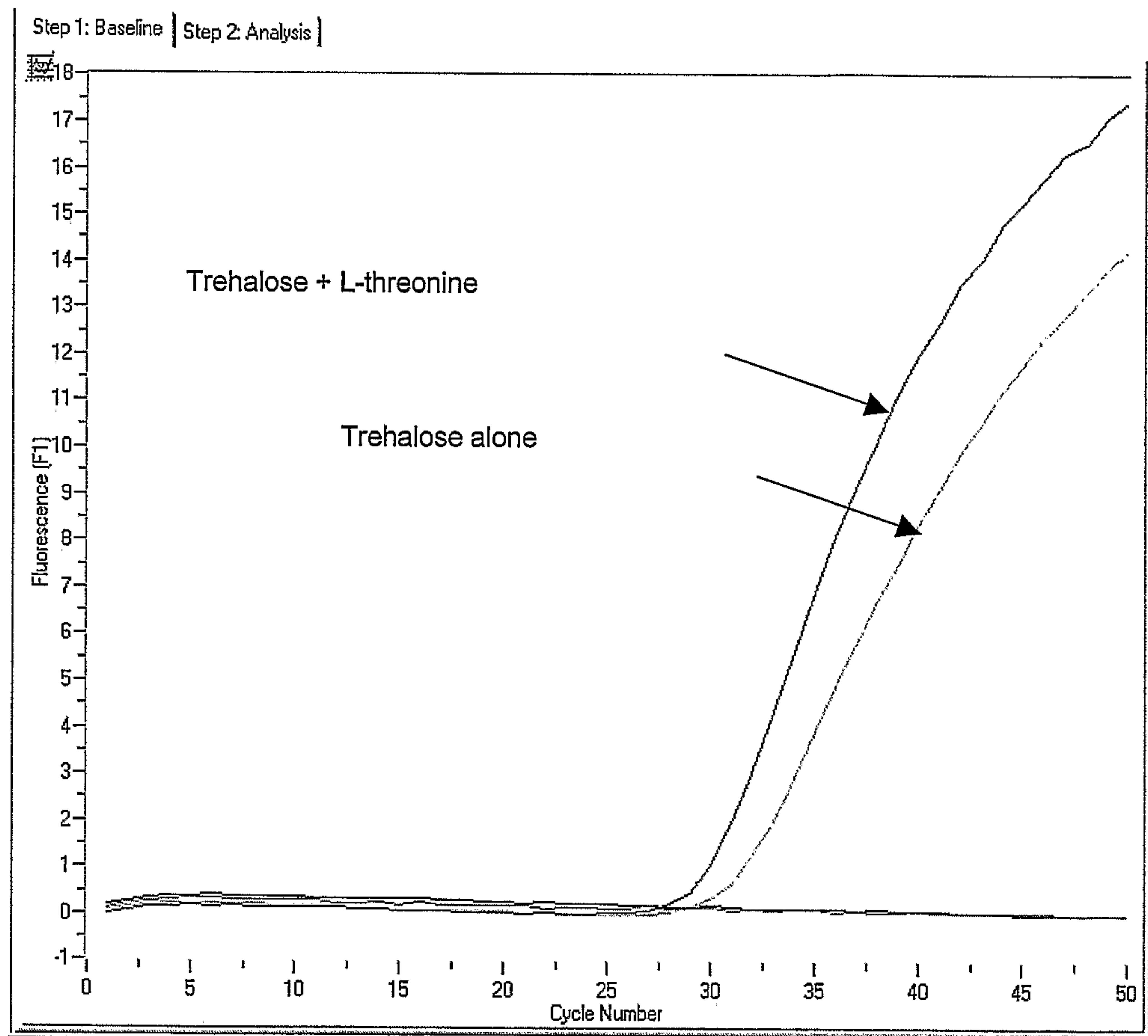
any one of claims 1 to 20 and subjecting this to conditions under which a chemical or biochemical reaction will occur, and monitoring a fluorescent signal therefrom.

5 25. The use of L-threonine as a stabiliser for freeze-dried compositions comprising fluorescent reagents.

26. The use of an RNase inhibitor as a stabiliser in a freeze-dried composition.

1/1

Figure



Figure

