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(Also referred to as FORM PTO-1465)

REQUEST FOR EX PARTE REEXAMINATION TRANSMITTAL FORM

U.S. PTO

Address to:
**Mail Stop Ex Parte Reexam
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450**Attorney Docket No.: **BLG-RE-00002**Date: **6/25/10**

06/25/10

1. ☒ This is a request for *ex parte* reexamination pursuant to 37 CFR 1.510 of patent number 5,808,045 issued 8/15/1998. The request is made by:
☐ patent owner. ☒ third party requester.
2. ☐ The name and address of the person requesting reexamination is:

3. ☒ a. A check in the amount of \$ 2520.00 is enclosed to cover the reexamination fee, 37 CFR 1.20(c)(1);
☐ b. The Director is hereby authorized to charge the fee as set forth in 37 CFR 1.20(c)(1) to Deposit Account No. _____; or
☐ c. Payment by credit card. Form PTO-2038 is attached.
4. ☐ Any refund should be made by ☐ check or ☐ credit to Deposit Account No. _____ 37 CFR 1.26(c). If payment is made by credit card, refund must be to credit card account.
5. ☒ A copy of the patent to be reexamined having a double column format on one side of a separate paper is enclosed. 37 CFR 1.510(b)(4)
6. ☐ CD-ROM or CD-R in duplicate, Computer Program (Appendix) or large table
☐ Landscape Table on CD
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
If applicable, items a. - c. are required.
 - a. ☐ Computer Readable Form (CRF)
 - b. Specification Sequence Listing on:
 - i. ☐ CD-ROM (2 copies) or CD-R (2 copies); or
 - ii. ☐ paper
 - c. ☐ Statements verifying identity of above copies
8. ☐ A copy of any disclaimer, certificate of correction or reexamination certificate issued in the patent is included.
9. ☒ Reexamination of claim(s) 1-7 is requested.
10. ☒ A copy of every patent or printed publication relied upon is submitted herewith including a listing thereof on Form PTO/SB/08, PTO-1449, or equivalent.
11. ☐ An English language translation of all necessary and pertinent non-English language patents and/or printed publications is included.

[Page 1 of 2]

This collection of information is required by 37 CFR 1.510. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Ex Parte Reexam, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Columbia Exhibit 2009
 Illumina, Inc. v. The Trustees of Columbia
 University in the City of New York
 IPR2018-00322

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12. ☒ The attached detailed request includes at least the following items:
- a. A statement identifying each substantial new question of patentability based on prior patents and printed publications. 37 CFR 1.510(b)(1)
 - b. An identification of every claim for which reexamination is requested, and a detailed explanation of the pertinency and manner of applying the cited art to every claim for which reexamination is requested. 37 CFR 1.510(b)(2).

13. ☐ A proposed amendment is included (only where the patent owner is the requester). 37 CFR 1.510(e)

14. ☒ a. It is certified that a copy of this request (if filed by other than the patent owner) has been served in its entirety on the patent owner as provided in 37 CFR 1.33(c).

The name and address of the party served and the date of service are:

LERNER DAVID LITTENBERG KRUMHOLZ & MENTLIK600 SOUTH AVENUE WESTWESTFIELD NJ 07090-1497Date of Service: 6/25/10; or

- ☐ b. A duplicate copy is enclosed because service on patent owner was not possible. An explanation of the efforts made to serve patent owner is **attached**. See MPEP 2220.

15. Correspondence Address: Direct all communications about the reexamination to:

- ☒ The address associated with Customer Number:

35938**OR**

- ☐ Firm or
Individual Name _____

Address _____

City _____

State _____

Zip _____

Country _____

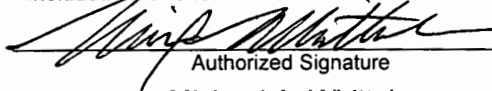
Telephone _____

Email _____

16. ☐ The patent is currently the subject of the following concurrent proceeding(s):

- ☐ a. Copending reissue Application No. _____
- ☐ b. Copending reexamination Control No. _____
- ☐ c. Copending Interference No. _____
- ☐ d. Copending litigation styled: _____

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Authorized Signature

Michael A. Whittaker

Typed/Printed Name

6/25/10

Date

46,230Registration No. ☐ For Patent Owner Requester☒ For Third Party Requester

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number : 08/486,897 Confirmation No.: 7142
Filed : June 7, 1995
Applicant : Andrew C. HIATT
Title : COMPOSITIONS FOR ENZYME CATALYZED TEMPLATE-INDEPENDENT
CREATION OF PHOSPHODIESTER BONDS USING PROTECTED
NUCLEOTIDES
TC/Art Unit : 1623
Examiner: : James O. WILSON

Docket No. : 68205.000103
Customer No. : **21967**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**REVOCATION OF POWER OF ATTORNEY WITH NEW POWER
OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS**

Sir:

Illumina, Inc., a corporation, having a place of business at Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA 92121 is the assignee of total interest in the above-captioned patent application pursuant to an Assignment recorded August 24, 2010 at Reel 024864, Frame 0900.

Pursuant to 37 C.F.R. § 3.73(b), the undersigned, who is empowered to act on behalf of the Assignee, hereby declares that he has reviewed the evidentiary documents of the chain of title and certifies that, to the best of Assignee's knowledge and belief, title to the above-captioned patent application is with the Assignee.

Assignee hereby revokes any and all previous Powers of Attorney in the above-identified application and hereby appoints, both jointly and severally, the practitioners associated with

CUSTOMER NUMBER 21967

as its attorneys and agents with full power of substitution and revocation, to prosecute said patent, including any and all continuation, divisional, renewal, substitute, reexamination and reissue applications based in whole or in part on said patent application, before the U.S. Patent and Trademark Office, to transact all business in the U.S. Patent and Trademark Office connected therewith, including receiving any Letters Patent issuing thereon, and to take any and all other legal action with regard to this patent application.

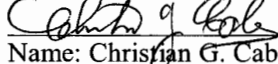
All correspondence and telephone communications should be addressed to:

CUSTOMER NUMBER 21967

which corresponds to the following:

Intellectual Property Department
Hunton & Williams LLP
1900 K Street, N.W.
Suite 1200
Washington, DC 20006-1109
(202) 955-1500 (telephone)
(202) 778-2201 (facsimile)

Dated: August 31, 2010

By: 
Name: Christian G. Cabou
Title: Senior Vice President
and General Counsel
Illumina, Inc.
9885 Towne Centre Drive
San Diego, CA 92121

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Control Number : 90/009,769 Confirmation No.: 5114
Inventors : Andrew C. HIATT et al.
Patent Number : 5,808,045
Issue Date : September 15, 1998
Title : COMPOSITIONS FOR ENZYME CATALYZED TEMPLATE-
INDEPENDENT CREATION OF PHOSPHODIESTER BONDS
USING PROTECTED NUCLEOTIDES
TC/Art Unit : 3991
Examiner: : Gary L. KUNZ
Docket No. : 68205.000103
Customer No. : **21967**

MAIL STOP *EX PARTE* REEXAM

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**AMENDMENT AND RESPONSE TO NON-FINAL OFFICE
ACTION IN *EX PARTE* REEXAMINATION**

Sir:

This amendment and response is responsive to the non-final Office Action issued December 21, 2010 in the above-captioned *ex parte* reexamination. A one-month extension of time was granted on February 7, 2011, thereby extending the due date for responding up to and including March 21, 2011.

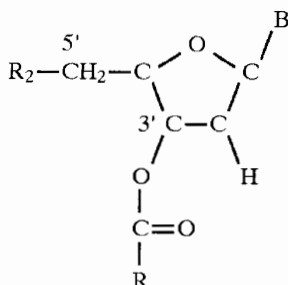
Amendments to the Claims begin on page 2 of this Amendment.

Remarks begin on page 9 of this Amendment.

AMENDMENTS

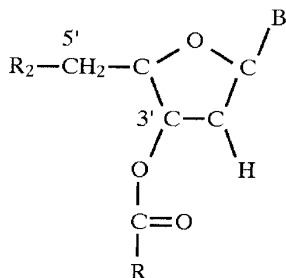
In the Claims

1. (original) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:

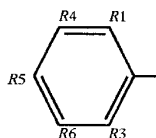


wherein R₂ is a triphosphate and R is selected from the group consisting of: formate, benzoylformate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamanioate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α-naphthanoate.

2. (original) A mononucleoside 5' triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



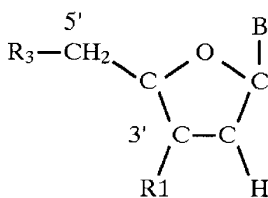
wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: H , CH_3 , $CH_3(CH_2)_N$ where N is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x$ where x is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x and n are independent integers from 1 to 12, $C_x(CH_3)_{3x-(x)1}(CH_2)_n$ where x and n are independent integers from 1 to 12, and



where R_1 , R_3 , R_4 , R_5 and R_6 are selected from the group consisting of CH_3 , H or NO_2 .

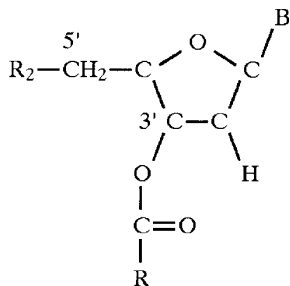
3. (original) The mononucleoside 5'-triphosphate of claim 1 or 2 wherein R_2 is a triphosphate and said mononucleoside 5'-triphosphate is a deoxynucleoside.

4. (original) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:



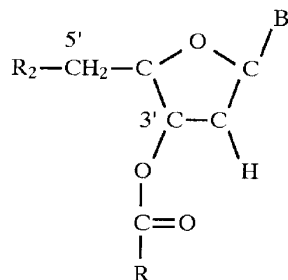
wherein R_2 is triphosphate; and wherein R_1 is toluic, benzoic or acetic acid ester.

5. (original) The mononucleoside 5'-triphosphate of claim 1, 2 or 4 wherein said removable blocking moiety is removable by an enzyme.
6. (original) The mononucleoside 5'-triphosphate of claim 1, 2 or 4 wherein said removable blocking moiety is removable by a reaction which occurs within 2 to 10 minutes.
7. (not reexamined) The mononucleoside 5'-triphosphate of claim 1, 2 or 4 wherein said removable blocking moiety is linked to a solid support.
8. (new) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:

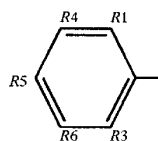


wherein R₂ is a triphosphate and R is selected from the group consisting of: benzoylformate, methoxyacetate, triphenylmethoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamanioate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenolate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α-naphthanoate.

9. (new) A mononucleoside 5' triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



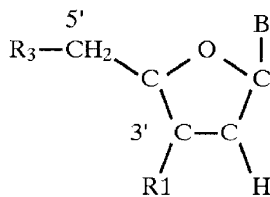
wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: H, CH_3 , $(CH_2)_N$ where N is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x$ where x is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x and n are independent integers from 1 to 12, $C_x(CH_3)_{3x-(x)1}(CH_2)_n$ where x and n are independent integers from 1 to 12, and



where R1, R3, R4, R5 and R6 are selected from the group consisting of CH_3 or NO_2 .

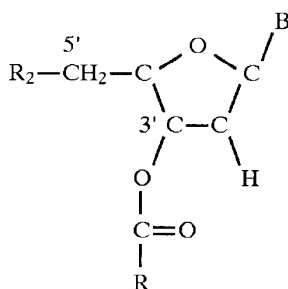
10. (new) The mononucleoside 5'-triphosphate of claims 8 or 9 wherein R_2 is a triphosphate and said mononucleoside 5'-triphosphate is a deoxynucleoside.

11. (new) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:



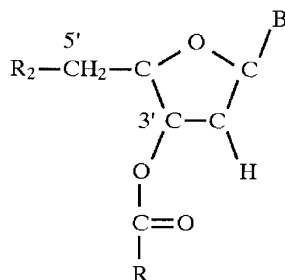
wherein R_2 is triphosphate; and wherein R_1 is toluic or benzoic acid ester.

14. (new) A composition comprising a mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:

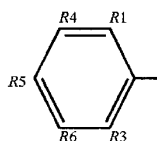


wherein R₂ is a triphosphate and R is selected from the group consisting of: formate, benzoylformate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamanioate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenolate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α-naphthanoate, wherein said composition further comprises a template independent polynucleotide polymerase.

15. (new) A composition comprising a mononucleoside 5' triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



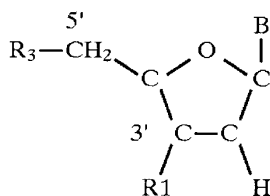
wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: H, CH_3 , $CH_3(CH_2)_N$ where N is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x$ where x is an integer from 1 to 12, $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x and n are independent integers from 1 to 12, $C_x(CH_3)_{3x-(x)1}(CH_2)_n$ where x and n are independent integers from 1 to 12, and



where R1, R3, R4, R5 and R6 are selected from the group consisting of CH_3 , H or NO_2 , wherein said composition further comprises a template independent polynucleotide polymerase.

16. (new) The composition of claim 14 or 15 wherein R_2 is a triphosphate and said mononucleoside 5'-triphosphate is a deoxynucleoside.

17. (new) A composition comprising a mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:



wherein R_2 is triphosphate; and wherein R_1 is toluic, benzoic or acetic acid ester, and wherein said composition further comprises a template independent polynucleotide polymerase.

18. (new) The composition of claim 14, 15 or 17 wherein said removable blocking moiety is removable by an enzyme.
19. (new) The composition of claim 14, 15 or 17 wherein said removable blocking moiety is removable by a reaction which occurs within 2 to 10 minutes.
20. (new) The mononucleoside 5'-triphosphate of claim 1, wherein R is 3-benzoylpropionate.

REMARKS

I. Status of Claims and Support for Claim Changes

In the Claims

Reconsideration of the rejections in the reexamination proceeding is respectfully requested. Claims 1-6 are subject to reexamination and stand rejected. Claim 7 is granted and not subject to reexamination. New claims 8-20 are presented for entry and consideration. Upon entry of the foregoing amendments and new claims 8-20, claims 1-6 and 8-20 will be pending and subject to reexamination.

Patent Owner respectfully requests entry of the claim amendments and new claims, and submits that these do not introduce new matter. Support for the amendments to the claims and for the new claims can be found throughout the specification (considered as a whole) and in the claims as originally granted. In particular, support for new claims 8-20 can be found, *inter alia*, in the granted claims and in the specification as originally filed on page 17, line 25 extending to page 18, line 25.

New claims 14-19 are directed to compositions comprising the claimed mononucleoside 5'-triphosphates having a removable blocking moiety, and further comprising a template independent polynucleotide polymerase. Patent Owner submits that new claims 8-20 do not enlarge the scope of the claimed subject matter. For example, claims 14-19 are based on claims 1-6, respectively, and at least recite all of the elements of claims 1-6, respectively.

Based on the above amendments and the following remarks, Patent Owner respectfully requests that the Examiner reconsider all outstanding rejections and that the rejections be withdrawn.

II. Claim Rejections Under 35 U.S.C. 102(b)

A. Claims 1-4 and 6 were rejected under 35 U.S.C. § 102(b) as allegedly anticipated by the disclosure of Tsien.

Patent Owner respectfully disagrees and traverses.

Claims 1, 2, and 4 are drawn to mononucleoside 5'-triphosphates having a removable blocking moiety protecting the 3' position which is an ester, having the formula set forth in claim 1 and a recitation of blocking groups at the 3' position as set forth in those claims. Claim 3 depends from claims 1 and 2 and further specifies that the mononucleoside 5'-triphosphate is a deoxynucleoside triphosphate. Claim 6 depends from claims 1, 2, or 4, and sets forth attributes of the removable blocking moiety. Claims 1-4 and 6 were rejected as allegedly anticipated by the disclosure of Tsien. According to the office action, Tsien describes ester blocking groups such as lower alkyl (1-4 carbon), alkanolic acid and substituted low alkanolic acid esters, for example formyl, acetyl, isopropanoyl, alpha-chloroacetyl esters and the like.

It is acknowledged by Patent Owner that certain of the claimed blocking groups are recited by name in Tsien. For example, Tsien does mention formyl, isopropanoyl, alpha fluoro- and alpha chloroacetyl ester blocking groups by name. Nevertheless, Tsien does not disclose methods for the preparation of formyl, isopropanoyl, alpha fluoro- and alpha chloroacetyl ester 3' blocked mononucleoside 5'-triphosphates. Moreover, Tsien does not disclose working examples demonstrating the preparation of any of the claimed 3' ester blocking groups, as Tsien is a prophetic disclosure.

It is well established that a mere naming of a genus of compounds in a paper disclosure is not sufficient to stand as an anticipatory reference under 35 U.S.C. § 102(b) where an applicant can show that members of that genus are not enabled, *even where that reference specifically names some of the compounds claimed by an Applicant*. In order to anticipate a claimed invention, a prior art reference must enable one of ordinary skill in the art to make the invention without undue experimentation.¹ The Federal Circuit's predecessor upheld claims as patentable in light of an allegedly anticipating reference where the reference merely mentioned compounds by name.² In Wiggins, claims directed to compounds having anti-Parkinsonism activity selected

¹ Impax Labs. v. Aventis Pharma., Inc., 88 USPQ2d 1381, 1383 (Fed. Cir. 2008).

² In re Wiggins, James, and Gittos, 179 USPQ 421 (C.C.P.A. 1973).

from a broad class of compounds were rejected under 35 U.S.C. § 102(b) over a prior art reference by Giudicelli. Giudicelli mentioned by name two compounds falling within the rejected claims, but whose syntheses were unsuccessfully attempted.³

The CCPA disagreed that the claims were anticipated, stating that

The defect in the issue ... is that it presumes that the naming of the compounds by Giudicelli constitutes a description of the invention within the meaning of § 102(b). We do not accept this presumption. In our view Giudicelli's listing of the compounds by name constituted nothing more than speculation about their potential or theoretical existence. The mere naming of a compound in a reference, without more, cannot constitute a description of the compound, ...⁴

As with the prior art in Wiggins, the disclosure of Tsien encompasses broad classes of compounds, perhaps hundreds or more potential blocking groups. Tsien states that “[t]he most common 3'-hydroxyl blocking groups are ... esters ... preferred embodiments focus on ... ester blocking groups such as lower (1-4 carbon) alkanolic acid and substituted lower alkanolic acid esters, for example ...”⁵ This disclosure constitutes speculation about the existence of mononucleoside 5'-triphosphates having removable blocking moieties protecting the 3' position of said mononucleoside 5'-triphosphate.

As stated previously, Tsien is a purely prophetic disclosure, including only prophetic examples. Examples 1 and 2 of Tsien are prophetic in nature, as is the prophetic discussion of the preparation of “representative blockers” on pages 21-23. Not surprisingly, Tsien's PCT publication never ultimately issued into a patent in apparent recognition of the general inoperability of the disclosure at that time. Undue experimentation would be required to determine which of the multitude of potential blocking groups would be expected to be removable blocking moieties that also protect the 3' position of said mononucleoside 5'-triphosphates, as required by the instant claims. Thus, Tsien is a paper disclosure merely mentioning certain claimed blocking groups by name, and fails to anticipate claims 1-4 and 6 at least for the reason that Tsien fails to provide an enabling disclosure of the rejected subject

³ The Examiner cited a second reference as evidence that one skilled in the art could make the named compounds, thereby making them available to the public.

⁴ *Id.*, at pp. 424-425 (emphasis added).

⁵ Tsien, p. 21, ll. 12-26.

matter of claims 1-4 and 6. Patent Owner respectfully requests reconsideration and withdrawal of this rejection of claims 1-4 and 6 under 35 U.S.C. § 102(b).

Patent Owner submits that new claims 8-20 are not anticipated by Tsien for at least the same reason. New claims 14-19 are based on the granted claims and further recite the presence of a template independent polynucleotide polymerase. Patent Owner submits that Tsien does not teach this claim element. Accordingly, Patent Owner respectfully requests reconsideration and withdrawal of this rejection.

B. Claims 2-5 were rejected under 35 U.S.C. § 102(b) as being anticipated by the disclosure of Dower *et al* (“Dower”).

Patent Owner respectfully disagrees and traverses.

Claims 2-5 are drawn to mononucleoside 5'-triphosphates having a removable blocking moiety protecting the 3' position which is an ester, having the formula set forth in claims 2 and 4 and a recitation of blocking groups at the 3' position as set forth in claims 2 and 4, respectively. Claim 3 depends from claim 2 and further specifies that the mononucleoside 5'-triphosphate is a deoxynucleoside triphosphate. Claim 5 depends from claims 2 or 4 and sets forth attributes of the removable blocking moiety. Claims 2-4 and 5 were rejected as allegedly anticipated by the disclosure of Dower. According to the office action, Dower describes ester blocking groups possessing a removable protecting group on the 3'-hydroxyl moiety that includes acetyl, t-butanoyl (tBOC) and nitrobenzyl acetic acid.⁶ The office action asserts that only the acetyl protecting group reads on rejected claims 2 and 4.

It is acknowledged by Patent Owner that the acetyl blocking group is recited by name in Dower. Nevertheless, Dower does not disclose methods for the preparation of acetyl ester 3' blocked mononucleoside 5'-triphosphates. Moreover, Dower does not disclose methods for the general preparation of any of the claimed 3' ester blocking groups. Dower is a prophetic disclosure, lacking even working examples.

It is well established that a mere naming of a genus of compounds in a paper disclosure is not sufficient to stand as an anticipatory reference under 35 U.S.C. § 102(b) where an applicant can show that members of that genus are not enabled, *even where that reference specifically names some of the compounds claimed by an Applicant*. In order to anticipate a claimed

⁶ Dower, p. 44, ll. 20-22.

invention, a prior art reference must enable one of ordinary skill in the art to make the invention without undue experimentation.⁷ The Federal Circuit's predecessor upheld claims as patentable in light of an allegedly anticipating reference where the reference merely mentioned compounds by name.⁸ In Wiggins, claims directed to compounds having anti-Parkinsonism activity selected from a broad class of compounds were rejected under 35 U.S.C. § 102(b) over a prior art reference by Giudicelli. Giudicelli mentioned by name two compounds falling within the rejected claims, but whose syntheses were unsuccessfully attempted.⁹

The CCPA disagreed that the claims were anticipated, stating that

The defect in the issue ... is that it presumes that the naming of the compounds by Giudicelli constitutes a description of the invention within the meaning of § 102(b). We do not accept this presumption. In our view Giudicelli's listing of the compounds by name constituted nothing more than speculation about their potential or theoretical existence. The mere naming of a compound in a reference, without more, cannot constitute a description of the compound, ...¹⁰

As with the prior art in Wiggins, the disclosure of Dower encompasses broad classes of compounds, perhaps hundreds or more potential blocking groups. Dower states that "[e]xamples of these compounds are deoxynucleotide triphosphates with small blocking groups such as ..." ¹¹ This disclosure constitutes speculation about the existence of mononucleoside 5'-triphosphates having removable blocking moieties protecting the 3' position of said mononucleoside 5'-triphosphate.

As stated previously, Dower is a purely prophetic disclosure, including only prophetic examples. Undue experimentation would be required to determine which of the multitude of potential blocking groups would be expected to be removable blocking moieties that also protect the 3' position of said mononucleoside 5'-triphosphates, as required by the instant claims. Thus, Dower is a paper disclosure merely mentioning certain claimed blocking groups by name, and fails to anticipate claims 2-5 at least for the reason that Dower fails to provide an enabling

⁷ Impax Labs. v. Aventis Pharma., Inc., 88 USPQ2d 1381, 1383 (Fed. Cir. 2008).

⁸ In re Wiggins, James, and Gittos, 179 USPQ 421 (C.C.P.A. 1973).

⁹ The Examiner cited a second reference as evidence that one skilled in the art could make the named compounds, thereby making them available to the public.

¹⁰ *Id.*, at pp. 424-425 (emphasis added).

¹¹ Tsien, p. 21, ll. 12-26.

disclosure of the rejected subject matter of claims 2-5. Patent Owner respectfully requests reconsideration and withdrawal of this rejection of claims 2-5 under 35 U.S.C. § 102(b).

Patent Owner submits that new claims 8-20 are not anticipated by Dower for at least the same reason. New claims 14-19 are based on the granted claims and further recite the presence of a template independent polynucleotide polymerase. Patent Owner submits that Dower does not teach or suggest this claim element. Accordingly, Patent Owner respectfully requests reconsideration and withdrawal of this rejection.

III. Claim Rejections Under 35 U.S.C. 103(a)

Claims 1-6 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Tsien in view of Greene *et al.* (“Greene”).

Patent Owner respectfully traverses these rejections.

The office action asserts that Tsien discloses certain 3'-blocking groups, and that the majority of the claimed blocking groups not mentioned in Tsien are listed in Greene. The office action states that it would have been obvious for one of ordinary skill in the art to modify the 3'-blocked deoxynucleotides of Tsien by using a different blocking group as taught by Greene. Citing o-(dibromomethyl-(benzoate ester)) as an example, the office action states that the skilled artisan would have used the information provided by Greene for each functional group to select those most likely to satisfy the criteria set forth by Tsien.

The office action further asserts that one of ordinary skill in the art could have readily determined whether any modified nucleotide was a suitable substrate for polymerase enzymes, and that there would have been a reasonable expectation of success because Tsien teaches that blocking groups may be modified to incorporate bulky substituent groups.¹²

Patent Owner submits that there is ample evidence to rebut these assertions.

A. Tsien and Greene Teach Away from Using Certain 3' Blocking Groups

According to Tsien, “... the highly electronegative group trifluoroacetate is cleaved rapidly from 3' hydroxyls in methanol at pH 7 (Cramer et al., 1963) and thus would **not be**

¹² Office action, pp. 9-10.

stable during coupling at that pH.”¹³ On pages 19-20, Tsien teaches preferred exemplary reaction conditions having a pH of 7.3-7.7 and a pH of 7.0-7.4 for the polymerase enzymes Sequenase™ and Klenow, respectively. Tsien’s earlier statement suggests that 3’-blocked deoxynucleotides of Tsien using the trifluoroacetate blocking group of Greene would not work for Tsien’s intended purpose, as Tsien states that this blocking group would not be stable during the coupling process at pH 7, the very pH exemplified by Tsien as preferred. The asserted instability of trifluoroacetate at pH 7 is inconsistent with the use of a 3’ trifluoroacetate blocking group for Tsien’s intended purpose, and teaches away from its use thereof. This supports the non-obviousness of trifluoroacetate as a 3’-blocking group.

Similarly, Tsien states that:

Phenoxyacetate groups are cleaved in less than one minute but require substantially higher pH such as is achieved with NH₃/methanol ... To prevent significant premature deblocking and DNA degradation, the ester deblocking rate is advantageously selected so as to exhibit a deblocking rate of less than 10⁻³s⁻¹ during the incorporation, and at least 10⁻¹s during the deblocking stage. Ideally, this rate change is achieved by changing the buffer pH from 7 to about 10, but care must be taken not to denature the DNA.¹⁴

These statements of Tsien express concern with the use of phenoxyacetate groups as blocking groups, and teach deblocking conditions that appear to deviate from the “mild conditions” set forth as one criteria for the successful use of 3’-blocking groups. These deblocking conditions “require substantially higher pH”, and a buffer of pH 10 is purportedly ideal for controlling the deblocking rate so long as care is taken “... not to denature the DNA.” While Tsien does not define what is intended by “mild conditions”, Patent Owner submits that this teaching of Tsien discourages the use of phenoxyacetate at these conditions (i.e., a pH of 10). Accordingly, this teaches away from the use of phenoxyacetate as a 3’ blocking group and supports the non-obviousness thereof.

The office action also states that “Greene also provides the reaction conditions for addition and removal of each of the protecting groups disclosed ... the skilled artisan would have used the information provided by Greene for each functional group to select those most likely to

¹³ Tsien, p. 24, ll. 10-13 (emphasis added).

¹⁴ *Id* at ll. 13-23.

satisfy the criteria set forth by Tsien.”¹⁵ A review of Tsien reveals that Tsien states that his deblocking method should proceed rapidly and also yield a viable 3’-OH function in high yield.¹⁶ While Tsien does not specify what is intended by rapid deblocking and high yield, Patent Owner submits that it is reasonable to interpret this assertion as minutes of time for deblocking and greater than 95% deblocking yield. Such is likely the case, for example, in an automated process. Tsien’s own prophetic disclosure does suggest minutes for deblocking time: p. 24, ll. 13-14; and p. 38, ll. 34-36.

Based at least on these deblocking criteria, Tsien teaches away from the use of certain blocking groups set forth in Greene. For the Examiner’s convenience, Patent Owner provides the following table in Appendix A, listing certain claimed blocking groups and Greene’s deblocking reaction condition(s). For certain (but not every) deblocking reactions, Greene provides the time for deblocking and/or the percent yield. Patent Owner asserts that Tsien teaches away from the use of most, if not all, of Greene’s blocking groups set forth in Appendix A. For most if not all of these deblocking reactions set forth in Appendix A, the percent yield and/or time to deblock data provided is inadequate to meet Tsien’s deblocking criteria, namely rapid deblocking and high yield.

To exemplify, Greene references two deblocking (or cleavage) reactions for the formate ester blocking group on page 87. In the first reaction, three days are indicated as necessary for the cleavage reaction. In the second reaction, the stated reaction yield is 62%. Patent Owner submits that both the deblocking reaction time and the yield provided in Greene for the formate ester blocking group are insufficient to meet Tsien’s criteria, particularly in an automated system. The other blocking groups set forth in Appendix A have similar deficiencies in most or all of Greene’s referenced deblocking reactions. Thus, Tsien teaches away from using these blocking groups due to the inadequate deblocking reactions set forth in Greene.

On page 114, Greene provides a mere listing of miscellaneous esters, including the claimed 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, (E)-2-methyl-2-butenate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, and α -naphthoate esters. According to Greene, these miscellaneous esters “... have seen little use since publication

¹⁵ Office action, page 8.

¹⁶ Tsien, p. 23.

of the first edition,” and are listed in Greene simply for completeness.¹⁷ The first edition of Greene published in 1981. Hence, Greene asserts that these miscellaneous esters saw little use for approximately 13 years before Patent Owner’s priority date.

Greene teaches away from the use of these esters as 3’ blocking groups, as is apparent from Greene’s disclosure, or more specifically lack thereof. As stated by Greene, these miscellaneous esters are not described in detail, and there is no express teaching of conditions for forming and cleaving these blocking groups. Moreover, certain references cited in Greene for purposes of reaction conditions are not even directed to the field of nucleotides. Had Greene intended one of ordinary skill in the art to consider the use of these “miscellaneous esters” as 3’ blocking groups, rather than reciting them by name for the sake of completeness, Greene would have provided reaction conditions associated with these miscellaneous esters.

B. The Cited Prior Art Fails to Appreciate an Unexpected Discovery

The claims encompass a substantial number of aromatic 3’-O esters of deoxynucleotide triphosphates.¹⁸ Patent Owner directs the Examiner’s attention to the disclosure located on page 32, lines 13-27 of the specification as originally filed. This disclosure summarizes the unexpected discovery that certain removable blocking moieties, namely the aromatic 3’-O esters of deoxynucleotide triphosphates, are unstable in commonly used buffers containing divalent cations. The specification attributes this instability problem to the presence in commonly-used buffers of cacodylic acid, tris(hydroxymethyl) aminomethane, sodium acetate or sodium phosphate and the presence of a divalent cation, such as cobalt, manganese or magnesium. Examples of such buffers used in the field of molecular biology is available in the literature. For example, Appendix B provides copies of reference materials discussing the use of buffers such as Cacodylate-HCL, Tris(hydroxymethyl)aminomethane (Tris), and sodium phosphate buffers.

¹⁷ Greene, p. 114.

¹⁸ For example, claim 1 includes the following aromatic 3’-O esters: benzoylformate, triphenylmethoxyacetate, phenoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α -naphthanoate, while claim 4 includes toluic and benzoic acid esters.

Appendix B also demonstrates that the current Taq polymerase buffer from New England Biolabs Inc. (NEB) contains magnesium, a divalent cation.

It is asserted that the inventors are the first individuals to appreciate this unexpected discovery. The cited prior art references certainly fail to appreciate the existence of this problem, when said aromatic 3'-O ester-linked dNTPs are used in the presence of common buffers discussed above. In the absence of this unexpected discovery, one of ordinary skill in the art attempting to use these blocked mononucleoside 5'-triphosphates in the presence of these buffers under appropriate conditions likely would have failed. For example, utilization of a buffer of this nature under appropriate conditions in a sequential coupling and cleavage reaction likely would have lead to failure by preventing formation of a stable removable aromatic blocking group, which would otherwise allow for multiple phosphodiester bond formations and hence repeated mononucleotide additions. The unappreciated nature of this discovery in the art speaks to the non-obviousness of using aromatic 3'-O esters of deoxynucleotide triphosphates.

It is noted that Example 2 of the specification as originally filed identifies conditions associated with instability, and provides direction for addressing these concerns.¹⁹ For example, the specification teaches that "[d]egradation of the esters was first observable after about three minutes of incubation."²⁰ Tsien, on the other hand, teaches exemplary coupling reaction times of from about 1 to about 20 minutes and preferably 1 to 5 minutes for Sequenase™, and from about 1 to about 40 minutes for Klenow.²¹ Were one of ordinary skill in the art to use Tsien's reaction times in the presence of one of these buffers, it is reasonably likely that one of ordinary skill in the art would exceed the three minute mark at which time degradation of aromatic 3'-O esters was first observable.

C. No Expectation of Success Using Greene's Deblocking Conditions

KSR assumes a starting reference point or points in the art, prior to the time of the invention, from which a skilled artisan might identify a problem and pursue potential solutions, KSR presupposes that the record would give some reasons to make particular modifications to achieve the claimed compound, and KSR presumes that the record would supply some reasons

¹⁹ Specification as originally filed, pp. 60-61.

²⁰ Id. at p. 61, ll. 7-8.

²¹ Tsien, pp. 19-20.

for narrowing the prior art universe to a finite number of identified, predictable solutions.²² According to the USPTO, where there was reason to select and modify the lead compound(s) to obtain the claimed compound, but no reasonable expectation of success, the claimed compound would not have been obvious.²³

The office action states that Greene provides the reaction conditions for addition and removal of each of the protecting groups disclosed, and that the skilled artisan would have used the information provided by Greene for each functional group to select those most likely to satisfy the criteria set forth by Tsien.²⁴ Tsien identifies “the availability of mild conditions for rapid and quantitative deblocking” as one of the criteria for the successful use of 3’-blocking groups.²⁵

A review of Greene demonstrates that a multitude of blocking groups set forth in Greene are presented with deblocking conditions not considered mild. For example, Greene provides deblocking conditions for several blocking groups that are not considered by one of ordinary skill in the art to be mild, such as the use of elevated temperature, high pH and/or organic solvents.

One of ordinary skill in the art, relying on the criteria set forth in Tsien, would not have had a reasonable expectation of success in using 3’-blocked mononucleoside 5’-triphosphates, harboring a large number of the blocking groups of Greene, in the method of Tsien. This is because Tsien states that one of the criteria for the successful use of 3’-blocking groups is the availability of mild conditions for rapid and quantitative deblocking. Since Greene does not teach mild deblocking conditions for many of the blocking groups, it logically flows that one of ordinary skill would not have a reasonable expectation of success in using these blocking groups. Greene does not teach the conditions necessary for the successful use of these blocking groups.

In contrast, the specification as originally filed sets forth “... a convenient and gentle method for the rapid removal of these esters from the growing polynucleotide chain after a coupling reaction. These deprotection conditions are sufficiently gentle to enable the synthesis

²² *Eisai Co. Ltd. v. Dr. Reddy’s Labs, Ltd.*, 87 U.S.P.Q.2d 1452 (Fed. Cir. 2008).

²³ Examination Guidelines Update: Developments in the Obviousness Inquiry After *KSR v. Teleflex*, 75 Fed. Reg. 53643, 53659 (Sept. 1, 2010).

²⁴ Office action, p. 8.

²⁵ Tsien, p. 20, ll. 33-34.

of an object polynucleotide onto a pre-existing double stranded DNA without denaturation of the DNA at every cycle.”²⁶

Furthermore, Patent Owner submits that certain blocking groups encompassed by the claims are not taught or suggested in the cited references, and are accordingly non-obvious.

Thus, Patent Owner submits that the claims are not rendered obvious over the disclosure of the cited documents for all of the reasons set forth above. Accordingly, Patent Owner respectfully requests reconsideration and withdrawal of this rejection under 35 U.S.C. § 103(a).

Patent Owner submits that new claims 8-20 are not rendered obvious over the cited references for at least the reasons set forth above. Patent Owner submits that new claims 14-19 are not rendered obvious over the cited references, as these references fail to teach or suggest for example compositions comprising a template independent polynucleotide polymerase with one or more claimed mononucleoside 5'-triphosphates having a removable blocking moiety protecting the 3' position.

²⁶ Specification as originally filed, pp. 61, ll. 17-23.

CONCLUSION

For at least the reasons stated above, claims 1-6 and 8-20 are in condition for confirmation and allowance. Accordingly, Patent Owner respectfully requests that these claims be confirmed and allowed, and passed to issue. Should the Examiner believe anything further is desirable in order to place the claims in even better condition for allowance, the Examiner is invited to contact Patent Owner's undersigned representative.

While Patent Owner is hopeful that the instant response will result in an *ex parte* Reexamination Certificate, Patent Owner wishes to express its availability to interview with the Examiner(s) should the Examiner(s) consider an interview a useful means to resolve any rejections prior to issuance of a further Office Action. Patent Owner respectfully requests a personal interview in the event of a further Office Action. It is believed that no fees are necessary in connection with this Amendment and Response. However, in the event that the U.S. Patent and Trademark Office determines that fees are due with this response, the Commissioner is hereby authorized to charge such fees to the undersigned's Deposit Account No. **50-0206**.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Date: March 21, 2011

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Appendix A

Greene's Deblocking Conditions Incompatible
with Tsien's Asserted Deblocking Criteria

| Greene's Ester Blocking Group (Number of Asserted Cleavage Processes)²⁷ | Percent Yield (Cleavage Process No.) | Time to Deblock (Cleavage Process No.) | Greene Reference Page Number |
|---|---|---|---|
| 1.) Formate (2 cleavage processes) | (2) 62% | (1) 3 days | 87 |
| 2.) Benzoylformate (1 cleavage processes) | 31% | 12 hrs | 88 |
| 3.) Chloroacetate (5 cleavage processes) | (2) 70% (4) 88-99 % | (1) 1 hr (2) 5 hrs (3) 20 hrs (4) 2-20 min | 92 |
| 4.) Dichloroacetate (3 cleavage processes) ²⁸ | | (1) 30 min | 93-94 |
| 5.) Trichloroacetate (2 cleavage processes) | (1) 81% (2) 72% | (1) 6 hrs | 94 |
| 6.) Methoxyacetate (1 cleavage process) | 78% | | 95 |
| 7.) p-P-phenylacetate (1 cleavage process) | | 48 hrs | 96 |
| 8.) 3-phenylpropionate (1 cleavage process) | 70-90% | 8-16 hrs | 97 |

²⁷ For certain blocking groups and the respective deblocking reactions, insufficient information is provided in the Greene reference to ascertain the percent yield and/or time to deblock.

²⁸ Insufficient information is provided for reactions 2 and 3.

| | | | |
|--|---|---|-------|
| 9.) pivaloate (9 cleavage processes) ²⁹ | (3) 58% (4) 70-85% (6) 94% (7) 95% (8) 64% (9) 78% | (1) 4 hrs (2) 3 hrs (t_{1/2}) (3) 12 hrs (6) 3 hrs (8) 6 hrs | 98-99 |
| 10.) adamantate (2 cleavage processes) ³⁰ | | (1) 20 min | 100 |
| 11.) crotonate (1 cleavage process) | | 2 hrs | 100 |
| 12.) 4-methoxycrotonate (1 cleavage process) | | 2 hrs | 100 |
| 13.) 2,4,6-trimethylbenzoate (4 cleavage processes) | (2) 50-72% | (1) 2 hrs (2) 24-72 hrs (3) 20 hrs (4) 15 min | 104 |

²⁹ Insufficient information is provided for reaction 5.

³⁰ Insufficient information is provided for reaction 2.

Appendix B

Copies of Buffer Reference Materials

BIOCHEMISTRY

LAB FAX

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CHAPTER 1

BUFFERS, CHELATING AGENTS AND DENATURANTS

J. A. A. Chambers

1. BUFFERS

1.1. Introduction

Buffers maintain pH within a limited range (*Figures 1 and 2*) despite the generation or loss of hydrogen ions by the reactions going on within the system. Buffers are weak acids or bases that are not fully dissociated in solution. Within a limited pH range the equilibrium between the dissociated and undissociated forms of the buffer remains constant. Many such substances have been used but comparatively few remain widely used. Some unusual buffers remain in use for specific systems. *Table 1* summarizes the properties of non-zwitterionic buffers.

1.2. Goods' buffers

Goods' buffers (1) are a group of zwitterionic organic compounds, primarily sulfonates and tertiary amines, that show excellent buffering properties, solubility and biological and chemical inertness. As a group these buffers show excellent compatibility with biological systems. The properties of these are summarized in *Table 2*.

1.3. Preparation of buffers

Buffers are prepared by two methods:

- (i) bringing a dissolved solution of the buffer to the correct pH by titrating with a strong acid or base; or
- (ii) mixing of stock solutions; this is used frequently for phosphate and acetate buffers.

When the mixing of stock solutions is used confusion can sometimes arise over what the molarity refers to. In the case of phosphate and acetate buffers the molarity refers to all phosphate or acetate ions in the medium (see Sections 1.8 and 1.9 for the preparation of these buffers). On the other hand, for most buffers the concentration refers to the buffer substance alone and the concentration of the titrating acid or base is ignored. *Tables 3 and 4* give the composition of phosphate and acetate buffers, respectively.

1.4. Counter ions

Buffers with an acid pK_a are brought to their working pH with a strong base (e.g. NaOH, KOH), and those with a basic pK_a use a strong acid (e.g. HCl). The counter ion used is not always important and may be chosen by criteria such as convenience and compatibility with the system. Sometimes compound buffers such as Tris-acetate or Tris-phosphate may be used.

1.5. Effects of temperature and concentration

All buffers are sensitive to temperature to some extent. This is indicated in tables by the value $\Delta t^\circ\text{C}$. Some buffers, such as Tris are comparatively temperature sensitive and should be brought to the required pH at the temperature to be used for the experimental work.

BUFFERS

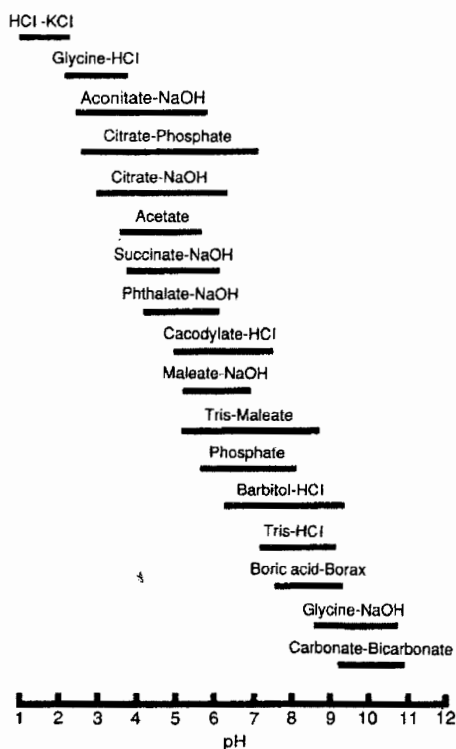
1

Similarly, some buffers are very sensitive to dilution of the stock solution (e.g. phosphate). After a concentrated stock has been prepared an aliquot should be diluted to the working concentration to check the final pH.

1.6. Biological compatibility and chemical reactivity

Most buffers, in their optimum pH ranges, are compatible with most biological systems; however, there can be exceptions. When used outside their optimum ranges some buffers can become reactive, insoluble, or may complex metal ions in addition to acting as a poor buffer. Some of the classical buffers have deficiencies that led to the development of zwitterionic buffers. These include reactivity, metabolism in some systems (glycine, citrate and aconitate) and limited buffer capacity. Tris, for example, inhibits phospholipase C, the Hill reaction of chloroplasts, complexes metal ions and is toxic to cells. The amino group of Tris or glycine reacts with aldehydes (e.g. formaldehyde) and even enzyme-bound aldehyde intermediates (2-6). Carboxyl and hydroxyl groups of aconitate, citrate, glycine and maleate are also reactive and these compounds have extensive chemistries, as does imidazole. Cacodylate reacts with thiol groups and is moderately toxic (7). Phosphate and borate buffers at high ionic strengths can chelate inorganic cations, and form insoluble salts (especially phosphate). In addition, borate complexes 1,2-*vic*-diols including nucleosides (8).

Figure 1. pH ranges of non-zwitterionic buffers.



Despite their otherwise excellent behavior, the following reactivity and incompatibility problems have been found for zwitterionic buffers:

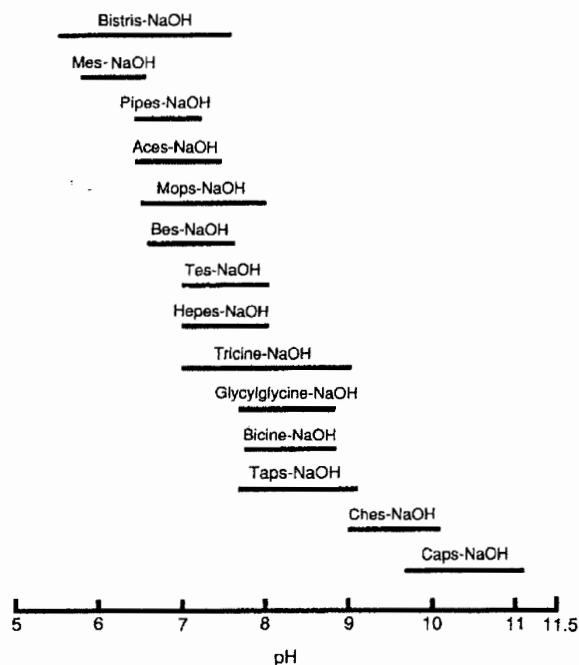
Compatibility

Zwitterionic buffers, including Hepes, are not compatible with all tissue cultures (e.g. chondrocytes (9)). Hepes also acts as an antagonist of γ -aminobutyric acid (inhibits action of the receptor (10)). Pipes interferes with tubulin polymerization into microtubules but Mes does not (11).

Reactivity

Zwitterionic buffers with ethanolamine groups interfere with the Lowry protein assay (12). Bicine is reactive in the acylation of tertiary amino alcohols with active esters (e.g. *N*-hydroxysuccinimide (13)); similarly, Tes reacts with succinic anhydride (14). Bistris interferes with the diaminobenzoic acid (DABA) fluorimetric assay for DNA and is reactive with the superoxide anion in heme-containing systems (15, 16). Caps, Ches, Taps and Mes are reactive, or catalytically active in some proton transfer reactions (17, 18). If zwitterionic buffers have a weakness, it is transition metals. Hepes is auto-oxidized in the presence of some aromatic compounds (19) and by copper (II) (20). Cyclohexyl groups of some of these buffers are prone to chemiluminescent oxidation in the presence of certain metal ions, for example Ce(IV) (21). Those with piperazine rings can form long-lived radicals in the presence of Fe(III) (22).

Figure 2. pH ranges of zwitterionic buffers.



It must be pointed out that these problems are minimal in comparison to the advantages they offer over non-zwitterionic buffers.

1.7. Terms used in the tables

pK_a

\log_{10} apparent dissociation constant for the acid component of the buffer substance. A buffer is usually most effective at about 1 pH unit above or below the value of pK_a . pH is related to pK_a and the concentration of the buffer by the well-known Henderson-Hasselbach equation.

$\Delta t^\circ C$

Temperature coefficient of change of pK_a . A large value, e.g. for Tris, implies that the buffer is temperature sensitive and should be brought to the correct pH at the working temperature.

pH range

The pH values over which the buffer is effective. Outside this range it will not only be ineffective but may also become highly reactive.

Counter ion

The preferred acid or base for bringing the buffer to the correct pH.

Risk and Safety

The numbers given in these columns refer to the European Commission Risk and Safety Phrases (see Chapter 14, Table 3).

Table 1. Components of non-zwitterionic buffers

| Name | Formula | Mol. wt | Solubility (20°C) (g/100 ml) | pK _a (20°C) | | | Titrate with | Risk | Safety |
|--|--|---------------|------------------------------|------------------------|-------|--------------|---------------------------------|---------|--------|
| | | | | 1 | 2 | 3 | | | |
| Boric acid [10043-35-3] | H ₃ BO ₃ | 61.8 | 6.4 | 9.14 | 12.74 | 13.8 | Tris, NaOH | — | — |
| Citric acid [77-92-9] | C ₆ H ₈ O ₇ | 192.4 | 146.0 | 3.14 | 4.77 | 6.39 (18°C) | Sodium phosphate, NaOH, KOH | — | — |
| Imidazole [288-32-4] | C ₃ H ₄ N ₂ | 68.1 | v.sol. | 6.95 (25°C) | — | — | NaOH, KOH | R21/R22 | S25 |
| Potassium dihydrogen phosphate [7778-77-0] | KH ₂ PO ₄ | 136.1 | 33 | 2.12 | 7.21 | 12.61 (25°C) | K ₂ HPO ₄ | — | — |
| di-Potassium hydrogen phosphate [7758-11-4] | K ₂ HPO ₄ | 174.2 | 167 | 2.12 | 7.21 | 12.61 (25°C) | KH ₂ PO ₄ | — | — |
| Potassium dihydrogen phthalate [877-24-7] | C ₈ H ₆ KO ₄ | 204.2 | 10 | 2.89 | 5.51 | — | KOH | — | — |
| Sodium acetate anhydrous trihydrate [1227-09-3] | CH ₃ COONa CH ₃ COONa·3H ₂ O | 82.0 136.1 | 119.0 (0°C) 76.2 (0°C) | 4.75 | — | — | Acetic acid | — | — |

Buffers

6

Table 1. Continued

| Name | Formula | Mol. wt | Solubility (20°C) (g/100 ml) | pK _a (20°C) | | | Titrate with | Risk | Safety |
|---|---|------------|------------------------------------|------------------------|-------|----------------|----------------------------------|-------------|--------------------------------|
| | | | | 1 | 2 | 3 | | | |
| Sodium cacodylate [124-65-2] | (CH ₃) ₂ AsO ₂ Na·3H ₂ O | 214.0 | 200 | 6.21 | — | — | NaOH | R23/R25-R33 | S1/S2-S7 S20-S21 S28-S44 |
| Sodium carbonate anhydrous | Na ₂ CO ₃ | 106.0 | 7.1 (0°C) | 6.37 | 10.25 | — | CO ₂ , carbonates | — | — |
| decahydrate [497-19-8] | Na ₂ CO ₃ ·10H ₂ O | 286.1 | 21.5 (0°C) | | | | | | |
| tri-Sodium citrate [68-04-2] | Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O | 294.1 | 72.0 | 3.09 | 4.75 | 5.41 (18°C) | Phosphates, hydroxides | — | — |
| Sodium dihydrogen phosphate [7558-80-7] | NaH ₂ PO ₄ ·2H ₂ O | 156.0 | v.sol. | 2.12 | 7.21 | 12.67 | Na ₂ HPO ₄ | — | — |
| Sodium hydrogen carbonate [144-55-8] | NaHCO ₃ | 84.0 | 9.6 | 6.37 | 10.25 | — | CO ₂ , carbonates | — | — |
| di-Sodium hydrogen phosphate anhydrous | Na ₂ HPO ₄ | 142.0 | | 2.12 | 7.21 | 12.67 | NaH ₂ PO ₄ | — | — |
| dihydrate | Na ₂ HPO ₄ ·2H ₂ O | 178.0 | 60.0 | | | | | | |
| decahydrate [7558-79-4] | Na ₂ HPO ₄ ·10H ₂ O | 358.1 | 4.2 | | | | | | |

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Table 1. Continued

| Name | Formula | Mol. wt | Solubility (20°C) (g/100 ml) | pK _a (20°C) | | | Titrate with | Risk | Safety |
|---------------------------------|---|------------|------------------------------------|------------------------|-------|-------|---------------------------------|------|--------|
| | | | | 1 | 2 | 3 | | | |
| Sodium tetraborate anhydrous | Na ₂ B ₄ O ₇ | 201.2 | 1.1 (0°C) | 9.14 | 12.74 | 13.80 | Boric acid | — | — |
| decahydrate | Na ₂ B ₄ O ₇ ·10H ₂ O | 381.4 | 2.0 (0°C) | | | | | | |
| [1330-43-4] | | | | | | | | | |
| Triethanolamine | C ₆ H ₁₅ NO ₃ | 149.2 | Liq. freely misc. with water | | | | | | |
| [102-71-6] | | | | | | | | | |
| Tris ^a | C ₄ H ₁₁ NO ₃ | 121.1 | 39.6 | 8.3 | — | — | HCl, acetic, phosphoric acid | — | — |
| [77-86-1] | | | | | | | | | |

^a 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Abbreviations: v.sol., very soluble; liq., liquid; misc., miscible.

Buffers

Table 2. Zwitterionic (Good's) buffers

| Name | Mol. wt | Solubility (0°C) (mol l ⁻¹) | pK _a | | Useful pH range |
|-----------------------------|---------|---|-----------------|--------|-----------------|
| | | | at 20°C | Δt°C | |
| Aces [7365-82-4] | 182.2 | 0.22 | 6.88 | -0.02 | 6.4-7.4 |
| Bes [10191-18-1] | 213.3 | 3.2 | 7.17 | -0.016 | 6.6-7.6 |
| Bicine [150-25-4] | 163.2 | 1.1 | 8.35 | -0.018 | 7.8-8.8 |
| Bis-Tris [6976-37-0] | 209.2 | — | 6.5 | — | 5.5-7.5 |
| Caps [1135-40-6] | 221.3 | 0.47 | 10.4 | 0.032 | 9.7-11.1 |
| Ches [103-47-9] | 207.1 | 1.15 | 9.55 | -0.011 | 9.0-10.1 |
| Glycylglycine [556-50-3] | 132.1 | 1.01 (25°C) | 8.25 | -0.026 | 7.7-8.8 |
| Hepes [7365-45-9] | 238.3 | 2.25 | 7.55 | -0.014 | 7.0-8.0 |
| Hepps [16052-06-5] | 252.3 | 1.6 | 8.0 | -0.011 | 7.6-8.6 |
| Mes [4432-31-9] | 213.3 | 0.65 | 6.15 | -0.011 | 5.8-6.5 |
| Mops [1132-61-2] | 209.3 | 3.1 | 7.2 | -0.011 | 6.5-7.9 |
| Pipes [5625-37-6] | 302.4 | 1.4 | 6.82 | 0.009 | 6.4-7.2 |
| Taps [29915-38-6] | 243.3 | v.sol. | 8.4 (25°C) | 0.018 | 7.7-9.1 |
| Tes [7365-44-8] | 229.3 | 2.6 | 7.5 | -0.02 | 7.0-8.0 |
| Tricine [5704-04-1] | 179.2 | 0.8 | 8.15 | -0.028 | 7.0-9.0 |

Abbreviations: Aces, *N*-(2-acetamido)-2-aminoethanesulfonic acid; Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Ches, 2-(cyclohexylamino)-ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid; Mes, 2-morpholinoethanesulfonic acid monohydrate; Mops, 3-morpholinopropanesulfonic acid; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid); Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminomethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; v. sol., very soluble.

1.8. Preparation of phosphate buffers

The following table is used for the preparation of 200 ml of 0.1 M sodium phosphate buffer. The buffer may be made as a five- or tenfold concentrate simply by dissolving the appropriate masses of the phosphates in a final volume of 200 ml. Phosphate buffers show concentration-dependent changes in pH, so the pH of the concentrate should be checked by diluting an aliquot to the final concentration.

Table 3. Preparation of 200 ml of 0.1 M sodium phosphate buffer (adapted from ref. 23, with permission from CRC Press, Inc.)

Solution A: 0.2 M NaH_2PO_4 ^a (27.8 g in 1000 ml water). †

Solution B: 0.2 M Na_2HPO_4 ^a (53.65 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71.7 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml water).

The appropriate volumes of solutions A and B are mixed, and water is added to a final volume of 200 ml:

| Vol. A (ml) | Vol. B (ml) | pH |
|----------------|----------------|-----|
| 93.5 | 6.5 | 5.7 |
| 92.0 | 8.0 | 5.8 |
| 90.0 | 10.0 | 5.9 |
| 87.7 | 12.3 | 6.0 |
| 85.0 | 15.0 | 6.1 |
| 81.5 | 18.5 | 6.2 |
| 77.5 | 22.5 | 6.3 |
| 73.5 | 26.5 | 6.4 |
| 68.5 | 31.5 | 6.5 |
| 62.5 | 37.5 | 6.6 |
| 56.5 | 43.5 | 6.7 |
| 51.0 | 49.0 | 6.8 |
| 45.0 | 55.0 | 6.9 |
| 39.0 | 61.0 | 7.0 |
| 33.0 | 67.0 | 7.1 |
| 28.0 | 72.0 | 7.2 |
| 23.0 | 77.0 | 7.3 |
| 19.0 | 81.0 | 7.4 |
| 16.0 | 84.0 | 7.5 |
| 13.0 | 87.0 | 7.6 |
| 10.5 | 90.5 | 7.7 |
| 8.5 | 91.5 | 7.8 |
| 7.0 | 93.0 | 7.9 |
| 5.3 | 94.7 | 8.0 |

^aThe corresponding potassium salts may also be used.

1.9. Preparation of acetate buffers

Acetate buffers are prepared in the same way as phosphate buffers. The following recipe is for 100 ml of a 0.1 M acetate buffer.

Table 4. Preparation of 100 ml of 0.1 M acetate buffer (adapted from ref. 23, with permission from CRC Press, Inc.)

Solution A: 0.2 M acetic acid (11.55 ml glacial acetic acid in 1000 ml water).

Solution B: 0.2 M sodium acetate (16.4 g $C_2H_3O_2Na$ or 27.2 g $C_2H_3O_2Na \cdot 3H_2O$ in 1000 ml water).

The appropriate volumes of solutions A and B are mixed, and water is added to a final volume of 100 ml:

| Vol. A (ml) | Vol. B (ml) | pH |
|----------------|----------------|-----|
| 46.3 | 3.7 | 3.6 |
| 44.0 | 6.0 | 3.8 |
| 41.0 | 9.0 | 4.0 |
| 36.8 | 13.2 | 4.2 |
| 30.5 | 19.5 | 4.4 |
| 25.5 | 24.5 | 4.6 |
| 20.0 | 30.0 | 4.8 |
| 14.8 | 35.2 | 5.0 |
| 10.5 | 39.5 | 5.2 |
| 8.8 | 41.2 | 5.4 |
| 4.8 | 45.2 | 5.6 |

1.10. Buffered salines, balanced salts and osmotic supports

Salines and buffered salines

Used to keep animal tissue in good condition before analysis, also in immunochemistry, as a perfusant, as an osmotic support, and as a diluent.

Physiological saline. 0.9% w/v NaCl.

Phosphate-buffered saline.

| | |
|---|------------------------|
| NaCl | 7.2 g l ⁻¹ |
| Na ₂ HPO ₄ (anhydrous) | 1.48 g l ⁻¹ |
| KH ₂ PO ₄ (anhydrous) | 0.43 g l ⁻¹ |

Tris-buffered saline. Physiological saline containing 10–50 mM Tris-HCl (pH 7.2). However, Tris is toxic to some cells, so with cells it is better to use Tricine as a buffer. Table 5 lists the composition of selected balanced salt solutions.

Sugars and sugar alcohols as osmotic supports

Non-reactive sugars and sugar alcohols are used as osmotic supports during the formation of spheroplasts of plant, fungal or microbial cells, because of the very high internal osmotic pressures in these systems. Usually they are used at a hypertonic concentration in the range 0.3–0.8 M. Those used most frequently are glucose, mannitol and sorbitol (mol. wt 182.18) and sucrose. All are readily soluble in water and solutions can be sterilized by autoclaving but sucrose is toxic to some cells.

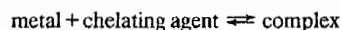
Table 5. Balanced salt solutions

| Compound (mg l ⁻¹) | Dulbecco's | Earle's | Hank's I | Hank's II | Ringer's mammalian | Ringer's amphibian | Tyrodé's |
|---|------------|---------|-------------|--------------|-----------------------|-----------------------|----------|
| CaCl ₂ | 100 | 200 | 200 | 140 | 250 | 120 | 200 |
| MgCl ₂ ·6H ₂ O | 100 | — | — | — | — | — | 100 |
| MgSO ₄ | — | 100 | — | — | — | — | — |
| MgSO ₄ ·7H ₂ O | — | — | 200 | 200 | — | — | — |
| KCl | 200 | 400 | 400 | 400 | 420 | 140 | 200 |
| KH ₂ PO ₄ | 200 | — | 100 | 60 | — | — | — |
| NaHCO ₃ | — | 2200 | 1273 | 350 | — | 200 | 1000 |
| NaCl | 8000 | 6800 | 8000 | 8000 | 9000 | 6500 | 8000 |
| Na ₂ HPO ₄ | 1150 | — | — | — | — | — | — |
| Na ₂ HPO ₄ ·2H ₂ O | — | — | 100 | 60 | — | — | — |
| NaH ₂ PO ₄ ·H ₂ O | — | 140 | — | — | — | — | 50 |
| Glucose | — | 1000 | 2000 | 1000 | — | 1000 | — |

Adapted from the *Biology Data Book, Vol. 1 (2nd edn)*, with permission from the Federation of American Societies for Experimental Biology.

2. CHELATING AGENTS

Chelating agents are used to control metal ion concentrations in reactions or to remove metal ions essential for structural or functional integrity. The affinity of a chelating agent for a metal is indicated by the stability constant. This is the log₁₀ of the dissociation constant for the complex. Although it is generally thought that there is a simple binary reaction:



involved in the formation of a complex, in practice a series of minor complexes may also be formed. In such cases it is the stability constant for the most stable complex that is reported. For some ligands (e.g. EDTA) the complex contains one chelating agent molecule and one metal ion, but for others there may be more than one chelating agent molecule with a series of intermediates; in these cases the stability constant for the most stable form is indicated in *Table 7* and the presence of intermediates is marked.

The ratio of stability constants for a chelating agent with a pair of metal ions shows how effectively the two would be bound in a mixture. For EDTA, the stability constants for Ca²⁺ and Mg²⁺ are 10.61 and 8.83. The difference between these two is ~1.8, which is log₁₀ of ~60. Accordingly, the ratio of complexed Ca²⁺ to Mg²⁺ in an equimolar mixture is about 60:1. For EGTA, with a lower stability constant for Mg²⁺, this ratio is about 4 × 10⁵.

Tables 6 and *7* include the two most widely used chelating agents, EDTA and EGTA, and two others that have some specialized uses. Citrate is used as a weak chelating agent in some systems and phosphate is included for comparison.

BUFFERS

Robert K. Scopes

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Appendix C

Buffers for Use in Protein Chemistry

Buffers with pK_a values between 4 and 10 have been presented. For pHs significantly below 4, a range of carboxylic acids (e.g., formic acid) is available for $n = -1$ buffers, and glycine and other simple amino acids for $n = +1$ buffers. For pHs significantly above 10, volatile amines such as triethylamine can be used ($n = +1$).

A Selection of Useful Buffers

| Name | n | pK_a^0 (25°C) | Comments |
|--|-----------------|--------------------|--------------------------------------|
| Lactic acid | -1 | 3.86 | |
| Acetic acid | -1 | 4.76 | |
| Pivalic acid (trimethylacetic acid) | -1 | 5.03 | Not very soluble; unpleasant odor |
| Pyridine | +1 | 5.23 | Volatile, poisonous |
| Picolinic acid | -1 ^a | 5.4 | Strong UV absorption |
| Succinic acid | -3 | 5.64 | |
| Histidine | +1 ^a | 6.0 | Complexes Me^{2+} strongly |
| <i>N</i> -Morpholinoethane sulfonic acid (Mes) | -1 ^a | 6.15 | |
| Bis-(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane (Bis-Tris) | +1 | 6.5 | |
| <i>N</i> -(2-acetamido)-2-aminoethane sulfonic acid (Aces) | -1 ^a | 6.9 | |
| Imidazole | +1 | 6.95 | Complexes Me^{2+} |
| Phosphate | -3 | 7.20 | Stabilizes many enzymes |
| <i>N</i> -Morpholinopropane sulfonic acid (Mops) | -1 ^a | 7.2 | |

(continued)

| | | | |
|---|-----------------|------|-----------------------------------|
| <i>N</i> -Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Tes) | -1 ^a | 7.5 | |
| Carbonic acid | -1 | 6.3 | |
| Triethanolamine | +1 | 7.75 | |
| Tris(hydroxymethyl)aminomethane (Tris) | +1 | 8.06 | |
| <i>N</i> -Tris(hydroxymethyl)methyl-glycine (Tricine) | -1 ^a | 8.15 | |
| Tris(hydroxymethyl)aminopropane sulfonic acid (Taps) | -1 ^a | 8.4 | |
| 2-Amino-2-methyl-1,3-propanediol | +1 | 8.8 | |
| Diethanolamine | +1 | 8.9 | |
| Taurine | -1 ^a | 9.1 | |
| Ammonia | +1 | 9.25 | Volatile |
| Boric acid | -1 | 9.23 | Complexes with many carbohydrates |
| Ethanolamine | +1 | 9.5 | |
| Glycine | -1 ^a | 9.8 | |
| 1-Aminopropan-3-ol | +1 | 9.95 | |
| Carbonate | -3 | 10.3 | |

^aIndicates zwitterionic buffer in either acidic or basic form.

The following buffers, which used to be quite widely used, are now usually replaced with more suitable buffers, for reasons of cost, toxicity, solubility, or *n* value.

Old buffer

Veronal (barbitone, 5:5'-diethyl barbituric acid) pK_a 8.0, $n = -1$

Cacodylic acid (dimethyl arsonic acid)

pK_a 6.2, $n = -1$

Citric acid (third pK_a , 6.4, $n = -5$)

Maleic acid (second pK_a , 6.3, $n = -3$)

Has UV absorption

Suggested replacement

Tricine, pK_a 8.1, $n = -1$

Tris, pK_a 8.1, $n = +1$

Mes, pK_a 6.2, $n = -1$

Histidine, pK_a 6.0, $n = +1$


Bis-Tris, pK_a 6.5, $n = +1$

Mes, pK_a 6.2, $n = -1$

Histidine, pK_a 6.0, $n = +1$

Bis-Tris, pK_a 6.5, $n = +1$

as above



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- Isolated from a recombinant source
- Supplied with 10X Reaction Buffer
- Robust and reliable reactions
- Tolerates a wide range of templates
- Incorporates dUTP, dITP and fluorescently-labeled nucleotides
- Exceptional value in terms of cost per unit

Description:
Taq DNA Polymerase is a thermostable DNA polymerase that possesses a 5' → 3' polymerase activity (1,2,3) and a 5' flap endonuclease activity (4,5).

It is supplied with 10X Standard Taq Reaction Buffer, which is detergent-free and designed to be compatible with existing assay systems.

Source:
An *E. coli* strain that carries the Taq DNA Polymerase gene from *Thermus aquaticus* YT-1

Applications:

- PCR
- Primer Extension
- DHPLC
- Microarray Analysis
- High-Throughput PCR

Reagents Supplied:
Standard Taq Reaction Buffer Pack (10X)

Enzyme Properties

Heat Inactivation:
No

Reaction & Storage Conditions

Unit Definition:
One unit is defined as the amount of enzyme that will incorporate 15 nmol of dNTP into acid-insoluble material in 30 minutes at 75°C.

Unit Assay Conditions: 1X ThermoPol Reaction Buffer, 200 µM dNTPs including [³H]-dTTP and 200 µg/ml activated Calf Thymus DNA.

Reaction Conditions:

1X Standard *Taq* Reaction Buffer, DNA template, primers, 200 μ M dNTPs (not included) and 1.25 units of *Taq* DNA Polymerase in a total reaction volume of 50 μ L.

1X Standard Taq Reaction Buffer:

10 mM Tris-HCl
50 mM KCl
1.5 mM $MgCl_2$
pH 8.3 @ 25°C

Concentration:

5,000 units/ml

Storage Conditions:

10 mM Tris-HCl
100 mM KCl
1 mM Dithiothreitol
0.1 mM EDTA
50% Glycerol
0.5% Tween-20
0.5% NP-40
pH 7.4 @ 25°C

Storage Temperature:

-20°C

FAQs

1. Can *Taq* DNA Polymerase be used in other buffers?
2. How should I set up a PCR reaction using *Taq* DNA Polymerase?
3. Why is the product a smear when visualized on an agarose gel?
4. Why is there no product when visualized on an agarose gel?
5. What type of DNA ends result from a primer extension reaction or a PCR reaction using LongAmp *Taq* DNA Polymerase?
6. The product sequence doesn't completely match the expected sequence. How can this result be improved?
7. When should *Taq* DNA Polymerase be used in a primer extension reaction or for PCR?
8. Does the presence of Ca^{2+} inhibit PCR reactions?
9. Will the 5' \rightarrow 3' exonuclease activity of *Taq* DNA Polymerase degrade primers?
10. Can *Taq* DNA Polymerase be used for nick translation?

Protocols

Protocols for *Taq* DNA Polymerase with Standard *Taq* Buffer

Quality Control for Current Lot

Quality control values for a specific lot can be found on the datacard which accompanies each product.

5 kb Lambda PCR:

25 cycles of PCR amplification of 5 ng Lambda DNA with 1.25 units of *Taq* DNA Polymerase in the presence of 200 μ M dNTPs and 0.2 μ M primers in Standard *Taq* Reaction Buffer results in the expected 5 kb product.

3' \rightarrow 5' Exonuclease Activity:

Incubation of a 20 μ L reaction in ThermoPol Reaction Buffer containing a minimum of 20 units of *Taq* DNA Polymerase with 10 nM fluorescent internally labeled oligonucleotide for 30 minutes at either 37°C or 75°C yields no detectable 3' \rightarrow 5' degradation as determined by capillary electrophoresis.

Endonuclease Assay:

Incubation of a 50 μ L reaction containing 20 units of *Taq* DNA Polymerase with 1 μ g of supercoiled ϕ X174 DNA for 4 hours at 75°C resulted in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

References

1. Chien, A., Edgar, D.B. and Trela, J.M. (1976) *J. Bact.*, 127, 1550-1557.
2. Kaledin, A.S., Sliusarenko, A.G. and Gorodetskii, S.I. (1980) *Biokhimiya*, 45, 644-651.
3. Lawyer, F.C. et al. (1993) *PCR Methods and Appl.*, 2, 275-287.
4. Longley, M.J., Bennett, S.E. and Mosbaugh D.W. (1990) *Nucleic Acids Res.*, 18, 7317-7322.
5. Lyamichev, V., Brow, M.A. and Dahlberg, J.E. (1993) *Science*, 260, 778-783.
6. Saiki R.K. et al. (1985) *Science*, 230, 1350-1354.

7. Powell, L.M. et al. (1987) *Cell*, 50, 831-840.
8. Sun, Y., Hegamyer, G. and Colburn, N. (1993) *Biotechniques*, 15, 372-374.
9. Sarkar, G., Kapelner, S. and Sommer, S.S. (1990) *Nucleic Acids Res.*, 18, 7465.

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Deoxynucleotide Solution Mix
Deoxynucleotide Solution Set
Diluent F
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Quick-Load® *Taq* 2X Master Mix
Standard *Taq* (Mg-free) Reaction Buffer Pack

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Appendix C

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| | | | |
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| Control Number | : 90/009,767 | Confirmation No.: | 1812 |
| Patent Number | : 5,872,244 | | |
| Filed | : September 13, 1999 | | |
| Title | : 3' PROTECTED NUCLEOTIDES FOR ENZYME CATALYZED TEMPLATE-INDEPENDENT CREATION OF PHOSPHODIESTER BONDS | | |
| TC/Art Unit | : 3991 | | |
| Examiner: | : Bruce R. CAMPELL | | |
| Docket No. | : 68205.000104 | | |
| Customer No. | : 21967 | | |

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Molly He, Ph.D.

I, Molly He, Ph.D., declare that:

1.) I am the Director of Protein Engineering at Illumina, Inc. It is my understanding that Illumina, Inc., is the current owner of all right, title and interest in U.S. Patent No. 5,872,244, the patent involved in the reexamination control no. 90/009,767 proceeding ("the '767 reexamination"). I have received a Bachelor of Sciences degree in Biochemistry from Nankai University, China in 1992 and a Doctor of Philosophy degree in Biochemistry and Molecular Genetics from the University of California, Los Angeles in 1997.

2.) I have been associated with teaching and research in the field of Protein Engineering for approximately fifteen years, and am an author of approximately twenty five publications including peer-reviewed articles, conference proceedings, and published patent applications. A recitation of some of these publications, together with details of my education, are given in my *curriculum vitae* which is attached as the Appendix.

3.) I am not a named inventor of U.S. Patent No. 5,872,244.

4.) I **do** currently have a financial interest (i.e., stock) in my employer Illumina, Inc. I **am not** being directly compensated for my efforts regarding this declaration, and I **do not** have a financial interest *immediately* associated with the outcome of this reexamination proceeding.

5.) I have read, and am familiar with, the following documents from the '767 reexamination: U.S. Patent No. 5,872,244; the non-final office action dated December 16, 2010; International patent application publication No. WO 91/06678 to Tsien ("Tsien"); Greene's protective Groups in Organic Synthesis, P.G.M. Wuts and T.W. Greene, John Wiley and Sons, New York, 2nd Ed., Chap. 2, pp. 10-117 (1991) ("Greene"); Hayakawa et al., J. Org. Chem., 58:5551-5555 (1993) ("Hayakawa").

6.) I have also read, and am familiar with, the following documents from the prior reexamination control no. 90/008,149 proceeding ("the '149 reexamination"): the non-final office action dated February 28, 2007; Patent Owner's response dated April 30, 2007; EPO office action dated March 30, 2005 and Applicant's response dated October 10, 2005 in EP 1,337,541 B1 ("EP '541"); the final office action dated January 7, 2008; Patent Owner's response dated March 11, 2008; and the April 11, 2008 Examiner interview summary record.

7.) I understand that certain claims in the '767 reexamination have been rejected as obvious over Tsien in view of Hayakawa, and also rejected as obvious over Tsien in view of Greene. I have been asked to provide my opinion whether one of ordinary skill in the art at the time of the invention in 1994 would have had a reasonable expectation of success in using 3' blocked mononucleoside 5'-triphosphates having a blocking group larger than an allyl group in Tsien's method requiring polymerase incorporation of the 3' blocked mononucleoside 5'-triphosphates into DNA.

8.) It is my understanding that agreement was reached with the USPTO in the '149 reexamination proceeding that the person of ordinary skill in the art would not have expected that a nucleoside 5'-triphosphate with a 3'-OH protected by an allyl group

would have been an acceptable substrate for a DNA polymerase because of steric considerations.

9.) Initially, I note that the crystal structure of a polymerase enzyme may be useful to one of ordinary skill in the art in determining suitable substrates therefor. In 1994, the crystal structures of template-independent polymerase enzymes were generally unknown.

10.) Based on my experience with polymerase enzymes, and with the understanding that one of ordinary skill in the art in 1994 would not have expected that a nucleoside 5'-triphosphate with a 3'-OH protected by an allyl group would have been an acceptable substrate for a DNA polymerase because of steric considerations, in my opinion one of ordinary skill in the art in 1994 would also not have had a reasonable expectation of success in using the following claimed 3' blocking groups in Tsien's method requiring polymerase incorporation of the 3' blocked mononucleoside 5'-triphosphates into DNA:


CH₃ (CH₂)_N, wherein N is an integer from 1-10, methoxymethyl, methylthiomethyl, benzyloxymethyl, t-butyloxymethyl, 2-methoxyethoxymethyl, 2,2,2-trichloroethoxymethyl, bis(2-chloroethoxy)methyl, 2-(trimethylsilyl)ethoxymethyl, tetrahydropyranyl, 3'-bromotetrahydropyranyl, tetrahydrothiopyranyl, 4'-methoxytetrahydropyranyl, 4'-methoxytetrahydrothiopyranyl, 4'-methoxytetrahydrothiopyranyl S,S-dioxido, tetrahydrofuranlyl, tetrahydrothiofuranlyl, 1-ethoxyethyl, 1-methyl-1-methoxyethyl, 1-(isopropoxy)ethyl, tri(p-methoxyphenyl)methyl, di(p-methoxy)phenylmethyl, 2,2,2-trichloroethyl, 2-(phenylselenyl)ethyl, butyl, allyl, cinnamyl, p-chlorophenyl, benzyl, p-methoxybenzyl, o-nitrobenzyl, p-nitrobenzyl, p-halobenzyl, p-cyanobenzyl, 3-methyl-2-picolyl N-oxido, diphenylmethyl, 5-dibenzosuberyl, triphenylmethyl, α-naphthyl diphenylmethyl, p-methoxyphenyl diphenylmethyl, p-(p'-bromophenacyloxy)phenyl diphenylmethyl, 9-anthryl, 9-(9-phenyl-10-oxo)anthryl, benzisothiazolyl S,S-dioxido, trimethylsilyl, triethylsilyl,

isopropyldimethylsilyl, t-butyl dimethylsilyl,
(triphenylmethyl)dimethylsilyl, methyldiisopropylsilyl, methyl di-t-
butylsilyl, tribenzylsilyl, tri-p-xylylsilyl, triisopropylsilyl and
triphenylsilyl.

11.) This is because the afore-mentioned 3' blocking groups are similar to or larger than an allyl group, and would have been expected to at least present the same steric hindrance considerations to one of ordinary skill in the art as an allyl group. For example, many of the afore-mentioned 3' blocking groups contain one or more ring structures that would have been expected to produce the same steric hindrance as an allyl group.

12.) I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the patent.

Signed:


Molly He, Ph.D.

Place:

Illumina San Diego

Executed on:

3/16/2011

Appendix

Curriculum vitae of Molly He, Ph.D.

CURRICULUM VITAE

Molly Min He

QUALIFICATIONS

- Seasoned protein biochemist with 10+ years of experiences in protein engineering using rational design approaches
- Proven track record of success in structure-based design of enzymes and substrates/inhibitors
- Extensive hands-on experiences in structural biology especially x-ray crystallography and structure-function relationship studies of various enzymes
- Proficient in molecular biology, enzyme expression, purification, and characterization
- Working knowledge of enzyme kinetics and assay development
- Familiar with various cutting-edge next generation sequencing platforms
- Familiar with molecular modeling and visualization software
- Good understanding of directed evolution and infrastructure setup for high throughput screening
- 7+ years of industrial experiences in group and project management
- Ph.D. in Biochemistry and Molecular Genetics

EXPERIENCES

1. 08/2009 - Present, Director

Protein Engineering, Illumina

Lead a large team of molecular biologists, biochemists and biophysicists to develop new and improved polymerases for various Illumina platforms and assays.

2. 05/2008 – 08/2009, Manager

Protein Sciences, Pacific Biosciences

Oversee a dynamic team comprised of structural biologists, molecular modeler, and protein biochemists to develop new and improved polymerases for single molecule real time (SMRT) DNA sequencing platform.

- Successfully used structural-based approach in the design of mutations to improve DNA polymerase properties for SMRT sequencing. These properties included

enzyme substrate binding kinetics, stability, and solubility.

- Lead the team to express and produce DNA polymerases in large quantity to support in-house and future customer use.
- Lead the team to characterize the properties of polymerase mutants by biochemical and spectroscopic methods. Quantify how these properties influence and interact with other subsystems of the DNA sequencing platform

3. *09/2007 – 05/2008, Consultant*

Protein Engineering, Trinity Biosystems

- Improve stability and solubility of protein therapeutics by protein engineering approaches including site-directed mutagenesis, chemical modification and solution chemistry
- Protein characterization by HPLC, peptide-mapping, and SPR using ForteBio System

4. *04/2002 – 09/2007, Senior Scientist and Project Leader*

Protein Sciences, Sunesis Pharmaceuticals, Inc.

- Lead a group of scientists in molecular cloning, protein production, high-throughput assay development, structural biology and molecular modeling to study structure-activity relationships of various proteins including kinases, phosphatase, and cytokines
- Used structure-based design approaches to improve properties of enzyme inhibitors
- Identified a novel mechanism of inhibition of TNF α by a small molecule by crystallography and biochemical approaches (published on Science).
- Used limited proteolysis and mass spectrometry to engineer protein targets with desired features for inhibitor discovery

5. *07/2000 – 04/2002, Scientist*

Structural Biology and Computational Chemistry, Chiron Corporation

- Used x-ray crystallography and molecular modeling to support structure-based drug design
- Had extensive experiences with various polymerases.

6. *08/1997 – 07/2000, Postdoctoral Research Associate*

*Howard Hughes Medical Institute
University of Oregon
Dr. Brian Matthews, P.I.*

- Studied the structure-function relationship of glyoxalase I of *Escherichia coli* by structural biology, biochemical assays and enzyme kinetics studies.
- Investigated the structure basis of protein stability and folding by utilizing a combination of structural biology, thermodynamic and mutational analysis of phage T4 lysozyme.

7. *09/1993 – 06/1997, Graduate Research Assistant*

*Howard Hughes Medical Institute
University of California, Los Angeles
Dr. Ronald Kaback, P.I.*

- Investigated the structure, molecular dynamics, and refolding of a membrane protein, the Lactose Permease of *Escherichia coli*, by using various biochemical and biophysical methods including engineered metal-binding sites, site-directed spin labeling, fluorescence spectroscopy, and site-detected mutagenesis.

EDUCATION

Ph.D.

9/93 - 6/97

Biochemistry

Department of Microbiology and Molecular Genetics,
University of California, Los Angeles.
Howard Hughes Medical Institute
Dr. H. Ronald Kaback, Advisor

B.S.

9/88 - 7/92

Biochemistry

Department of Biochemistry

PUBLISHED PATENTS AND PATENT APPLICATIONS

1. GENERATION OF MODIFIED POLYMERASES FOR IMPROVED ACCURACY IN SINGLE MOLECULE SEQUENCING

US patent 12/384,112

2. TWO SLOW-STEP POLYMERASE ENZYME SYSTEMS AND METHODS

US patent 12/414,191

3. Characterization of the GSK-3 β protein and methods of use thereof

US patent 60/233,538

PUBLICATIONS

1. Cancilla MT, **He MM**, Viswanathan N, Simmons RL, Taylor M, Fung A., and Erlanson DA. (2008) Discovery of an Aurora Kinase Inhibitor through Site-Specific Dynamic Combinatorial Chemistry. *Bioorg Med Chem Lett.* 18(14):3978-81
2. Frazier K, Jazan E, McBride CM, Pecchi S, Renhowe PA, Shafer CM, Taylor C, Bussiere D, **He, M.M.**, Jansen JM, Lapointe G, Ma S, Vora J, Wiesmann M. (2006) Design and structure-activity relationship of heterocyclic analogs of 4-amino-3-benzimidazol-2-ylhydroquinolin-2-ones as inhibitors of receptor tyrosine kinases. *Bioorg Med Chem Lett.* 16(8):2247-51.
3. **He, M. M.**, Smith A., Oslob J.D., Flanagan W.M., Braised, A. C., Whitty, A., Cancilla M. T., Wang, J., Lugovskoy, A.A., Yoburn, J.C., Fung, A. D., Farrington G., Eldredge, J.K., Day, E.S., Cruz, L.A., Cachero, T. G., Miller, S.K., Friedman, J.E., Choong I. C, and Cunningham, B. C. (2005) Small Molecule Inhibition of TNF- α . *Science* 310: 1022-1025.
4. **He, M. M.**, Wood Z.A., Basse, W. Xiao, H. & Matthews B. W. (2004) Alanine-scanning mutagenesis of the beta-sheet region of phage T4 lysozyme suggests that tertiary context has a dominant effect on beta-sheet formation. *Protein Sci.* 13(10):2716-24.
5. Erlanson, D.A., McDowell, R.S., **He, M.M.**, Randal, M., Simmons, R.L., Kung, J., Waight, A., & Hansen, S. K. (2003) Discovery of a new phosphotyrosine mimetic for PTP1B using breakaway tethering. *J Am Chem Soc* 125 (19):5602-3

6. **He, M. M.**, Clugston, S., Honek, J., & Matthews B. W. (2000) Determination of the structure of *Escherichia coli* glyoxalase I suggests a structural basis for differential metal activation. *Biochemistry* 39, 8719-8727
7. **He, M. M.**, & Kaback, H.R. (1998) *In vitro* folding of a membrane protein: effect of denaturation and renaturation on substrate binding by the lactose permease of *Escherichia coli*. *Molecular Membrane Biology* 15, 15-20
8. **He, M. M.**, & Kaback, H.R. (1997) Interaction between residues Glu269 (Helix VIII) and His322 (Helix X) of the lactose permease of *Escherichia coli* is essential for substrate-binding. *Biochemistry* 36, 13688-13692.
9. **He, M. M.**, Voss, J., Hubbell, W. L., & Kaback, H. R. (1997) Arginine 302 (Helix IX) in the lactose permease of *Escherichia coli* is in close proximity to Glutamate 269 (Helix VIII) as well as Glutamate 325 (Helix X). *Biochemistry* 36, 13682-13687.
10. Voss, J., **He, M. M.**, Hubbell, W. L., & Kaback, H. R. (1996) Site-directed spin-labeling demonstrates that transmembrane domain XII in the lactose permease of *Escherichia coli* is an α -helix. *Biochemistry* 35, 12915-12918.
11. **He, M. M.**, Sun, J., & Kaback, H. R. (1996) Cysteine-scanning mutagenesis of transmembrane domain XII and the flanking periplasmic loop in the lactose permease of *Escherichia coli*. *Biochemistry* 35, 12909-12914.
12. **He, M. M.**, Voss, J., Hubbell, W. L., & Kaback, H. R. (1995) Use of designed metal-binding sites to study helix proximity in the lactose permease of *Escherichia coli*. 2. Proximity of helix IX (Arg302) with helix X (His322 and Glu325). *Biochemistry* 34, 15661-15666.
13. **He, M. M.**, Voss, J., Hubbell, W. L., & Kaback, H. R. (1995) Use of designed metal-binding sites to study helix proximity in the lactose permease of *Escherichia coli*. 1. Proximity of helix VII (Asp237 and Asp240) with helices X (Lys319) and XI (Lys358). *Biochemistry* 34, 15667-15670.
14. Jung, K., Voss, J., **He, M.**, Hubbell, W. L., & Kaback, H. R. (1995) Engineering a metal binding site within a polytopic membrane protein, the lactose permease of *Escherichia coli*. *Biochemistry* 34, 6272-77.

INVITED SPEAKER AND CONFERENCE PROCEEDINGS

1. **Invited speaker:** Department of biochemistry, **University of Texas Austin**. May 8, 2009
2. **Invited speaker:** Protein-Protein Interactions as Drug Targets. Drug Discovery Chemistry, **Cambridge Healthtech Institute Conference**, La Jolla CA. May 13 – 17, 2007
3. **Invited speaker.** 17th **World Molecular Engineering Network Conference**, Cabo San Lucas. April 30th-May 4th, 2006
4. **He, M. M.**, Basse, W. & Matthews B. W. Polyalanine substitution of the β -sheet region of phage T4 lysozyme: effects on stability and structure. **The Twelfth Protein Society Meeting**. San Diego, CA, July 27-30th, 1998.
5. **He, M. M.**, Clugston, S., Honek, J., & Matthews B. W. Structural evidence for the differential metal activation observed in the

- detoxification isomerase *Escherichia coli* Glyoxalase I. **The Thirteenth Protein Society Meeting.** Boston, MA, July 26-29th, 1999.
6. **He, M. M., & Kaback, H.R.** Interaction between residues Glu269 (Helix V) and His322 (Helix X) of the lactose permease of *Escherichia coli* is essential for substrate-binding. **Gordon Conference 1997: Protons and Membrane Proteins.** Ventura, CA, Feb. 20-24th, 1997.

AWARDS AND HONORS

Patently Offensive Award Pacific Biosciences
2009

Spot Award Sunesis Pharmaceuticals
2005

Graduate Woman of the Year Department of Microbiology and Molecular Genetics
University of California, Los Angeles
6/97

Warsaw Fellowship Department of Microbiology and Molecular Genetics
9/96 - 6/97 University of California, Los Angeles

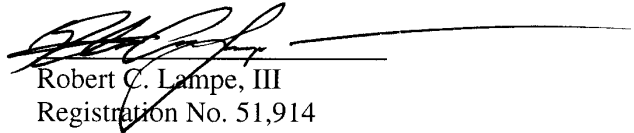
Non-resident Tuition Fellowship Department of Microbiology and Molecular Genetics
9/93 - 8/96 University of California, Los Angeles

Wang Kechang Scholarship Department of Biochemistry
8/1988 – 6/1992 NanKai University, TianJin, China

CERTIFICATE OF SERVICE

The undersigned representative for the patent owner hereby certifies that a true and correct copy of this **Amendment and Response to Non-Final Office Action in Ex Parte Reexamination** for *Ex Parte* Reexamination 90/009,769, as filed in the United States Patent and Trademark Office on March 21, 2011 was served by first class mail on March 21, 2011, on counsel for the third party requester at the following address:

Mr. Michael A. Whittaker
BIOTECHNOLOGY LAW GROUP
12707 High Bluff Drive
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San Diego, CA 92103


Robert C. Lampe, III
Registration No. 51,914
HUNTON & WILLIAMS LLP
1900 K Street, N.W.
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Telephone: (202) 419-2046
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E-mail: rlampe@hunton.com

67274 U.S. PTO



REEXAMINATION CONTROL NO.: 90/009,769
ATTORNEY DOCKET NO: 68205.000103

Re-Exam

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Control Number : 90/009,769 Confirmation No.: 5114
Inventors : Andrew C. HIATT et al.
Patent Number : 5,808,045
Issue Date : September 15, 1998
Title : COMPOSITIONS FOR ENZYME CATALYZED TEMPLATE-
INDEPENDENT CREATION OF PHOSPHODIESTER BONDS
USING PROTECTED NUCLEOTIDES
TC/Art Unit : 3991
Examiner: : Gary L. KUNZ
Docket No. : 68205.000103
Customer No. : **21967**

MAIL STOP *EX PARTE* REEXAM

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**SUPPLEMENTAL AMENDMENT AND RESPONSE TO
NON-FINAL OFFICE ACTION IN *EX PARTE* REEXAMINATION**

Sir:

This Amendment supplements Patent Owner's prior submission of March 21, 2011, and is responsive to the non-final Office Action issued December 21, 2010 in the above-captioned *ex parte* reexamination.

Amendments to the Claims begin on page 2 of this Amendment.

Remarks begin on page 9 of this Amendment.

AMENDMENTS

In the Claims

Please cancel claim 1:

1. (cancelled)

Please cancel claim 2:

2. (cancelled)

Please cancel claim 3:

3. (cancelled)

Please cancel claim 4:

4. (cancelled)

Please cancel claim 5:

5. (cancelled)

Please cancel claim 6:

6. (cancelled)

7. (not reexamined) The mononucleoside 5'-triphosphate of claim 1, 2 or 4 wherein said removable blocking moiety is linked to a solid support.

Please cancel claim 8:

8. (cancelled)

Please cancel claim 9:

9. (cancelled)

Please cancel claim 10:

10. (cancelled)

Please cancel claim 11:

11. (cancelled)

Please cancel claim 12:

12. (cancelled)

Please cancel claim 13:

13. (cancelled)

Please cancel claim 14:

14. (cancelled)

Please cancel claim 15:

15. (cancelled)

Please cancel claim 16:

16. (cancelled)

Please cancel claim 17:

17. (cancelled)

Please cancel claim 18:

18. (cancelled)

Please cancel claim 19:

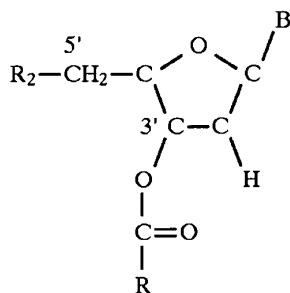
19. (cancelled)

Please cancel claim 20:

20. (cancelled)

Please add new claim 21 as follows:

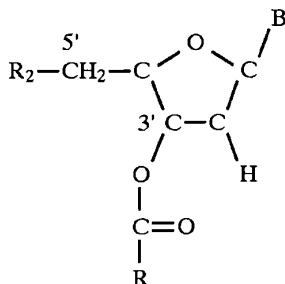
21. (new) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



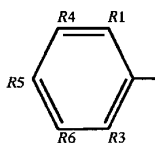
wherein R₂ is a triphosphate and R is selected from the group consisting of: benzoylformate, methoxyacetate, triphenylmethoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamantoate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α-naphthanoate.

Please add new claim 22 as follows:

22 (new) A mononucleoside 5' triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



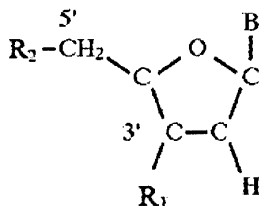
wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: H ; $CH_3(CH_2)_n$ where n is an integer from 1 to 12; $(CH_3)_{x+1}(CH)_x$ where x is an integer from 1 to 12; $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x and n are independent integers from 1 to 12; $C_x(CH_3)_{3x-(x-1)}(CH_2)_n$ where x and n are independent integers from 1 to 12; and



where R_1 , R_3 , R_4 , R_5 and R_6 are selected from the group consisting of CH_3 or NO_2 .

Please add new claim 23 as follows:

23. (new) A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:

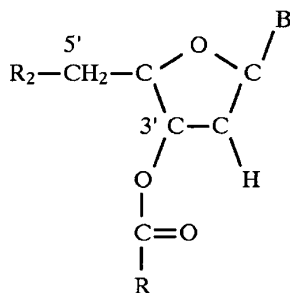


wherein R_2 is triphosphate; and wherein R_1 is toluic or benzoic acid ester.

Please add new claim 24 as follows:

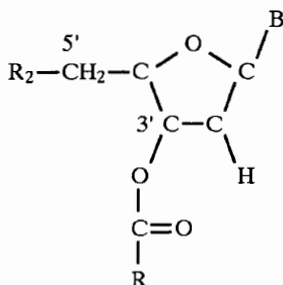
25. (new) The mononucleoside 5'-triphosphate of claims 21, 22, or 23 wherein said removable blocking moiety is removable by a reaction which occurs within 2 to 10 minutes.

26. (new) A composition comprising a mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:

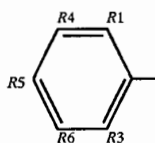


Please add new claim 27 as follows:

27. (new) A composition comprising a mononucleoside 5' triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



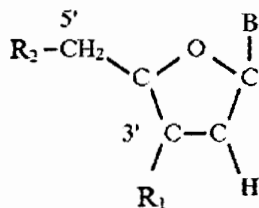
wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: H; CH_3 ; $CH_3(CH_2)_n$ where N is an integer from 1 to 12; $(CH_3)_{x+1}(CH)_x$ where x is an integer from 1 to 12; $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x and n are independent integers from 1 to 12; $C_x(CH_3)_{3x-(x-1)}(CH_2)_n$ where x and n are independent integers from 1 to 12; and



where R1, R3, R4, R5 and R6 are selected from the group consisting of CH_3 , H or NO_2 ; wherein said composition further comprises a template independent polynucleotide polymerase.

Please add new claim 28 as follows:

28. (new) A composition comprising a mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:



wherein R_2 is triphosphate; and wherein R_1 is toluic, benzoic or acetic acid ester, and wherein said composition further comprises a template independent polynucleotide polymerase.

Please add new claim 29 as follows:

29. (new) The composition of claim 26, 27 or 28 wherein said removable blocking moiety is removable by an enzyme.

Please add new claim 30 as follows:

30. (new) The composition of claim 26, 27 or 28 wherein said removable blocking moiety is removable by a reaction which occurs within 2 to 10 minutes.

Please add new claim 31 as follows:

31. (new) The mononucleoside 5'-triphosphate of claim 21, wherein R is 3-benzoylpropionate.

REMARKS

This amendment and response supplements Patent Owner's prior submission of March 21, 2011, and is further responsive to the non-final Office Action issued December 21, 2010 and the Examiner interview held on April 6, 2011 in the above-captioned *ex parte* reexamination. To the extent necessary, the arguments and evidence set forth in Patent Owner's March 21, 2011 response are herein incorporated by reference in their entireties. Patent Owner submits that the instant claims comply with 37 C.F.R. § 1.530(f).

I. Status of Claims and Support for Claim Changes

In the Claims

Reconsideration of the rejections in the reexamination proceeding is respectfully requested. Granted claims 1-6 are subject to reexamination and stand rejected. Claim 7 is granted and not subject to reexamination.

Claims 8-20 were previously presented as new claims for entry and consideration in Patent Owner's March 21, 2011 response and amendment.

In this supplemental amendment and response, claims 1-6 and 8-20 are cancelled without prejudice or disclaimer, and new claims 21-31 are presented for entry and consideration. Following entry of this amendment and response, claims 21-31 will be pending and subject to reexamination.

Patent Owner respectfully requests entry of new claims 21-31, and submits that these new claims do not introduce new matter. Patent Owner presents new claims 21-31 to correct obvious errors contained in the claims presented in Patent Owner's March 21, 2011 response and amendment, as reflected in the interview summary. Support for the new claims can be found throughout the specification as originally filed (considered as a whole) and in the claims as originally granted. For example, support for new claim 21 can be found in granted claim 1 and in the specification as originally filed on page 21, lines 3-19. Support for new claim 22 can be found in granted claim 2 and in the specification as originally filed on page 21, line 20 extending to page 22, line 7. Support for new claim 23 can be found in granted claim 4. Support for new claim 24 can be found in granted claim 5. Support for new claim 25 can be found in granted claim 6. Support for new claim 26 can be found in granted claim 1 and in the specification as

originally filed on page 21, lines 3-19, and on page 40, lines 2-8. Support for new claim 27 can be found in granted claim 2 and in the specification as originally filed on page 21, line 20 extending to page 22, line 7, and on page 40, lines 2-8. Support for new claim 28 can be found in granted claim 4, and in the specification on page 40, lines 2-8. Support for new claim 29 can be found in granted claim 5. Support for new claim 30 can be found in granted claim 6. Support for new claim 31 can be found in granted claim 1. Accordingly, Patent Owner submits that no new matter is introduced into the specification by way of the new claims, and that the new claims do not enlarge the scope of the claimed subject matter.

Based on the new claims and the following remarks, Patent Owner respectfully requests that the Examiner reconsider all outstanding rejections and that the rejections be withdrawn.

II. Interview Summary

An Examiner interview was held at the USPTO on April 6, 2011. The interview was attended by Examiners Gary Kunz and Dwayne Jones, Patent Owner's in-house representative John Murphy, Ph.D. (telephonically), and Patent Owner's representatives Robert M. Schulman and Robert C. Lampe III. Patent Owner and its representatives thank Examiners Kunz and Jones for the courtesies extended during the interview and for their time and attention.

The interview was directed to claims 1-6 and 8-20, and the rejections and art raised in the office action issued December 21, 2010. As an initial matter, Patent Owner's representatives appreciate the Examiners' suggestions regarding the following proposed claim amendments set forth in the interview summary:

- 1.) the recitation of "p-P-phenylacetate" in claims 1, 8, and 14 should be rewritten to recite "phenylacetate";
- 2.) the variable "n" should be added to the formula $\text{CH}_3(\text{CH}_2)_n$ as a subscript in claims 2, 9, and 15;
- 3.) the word "adamanioate" should be rewritten to recite "adamantoate" in claims 1, 8, and 14;
- 4.) the formula on line 2 of claims 2, 9, and 15 should recite $\text{C}_x(\text{CH}_3)_{3x-(x-1)}(\text{CH}_2)_n$; and
- 5.) claims 3, 10, and 16 appear not to further limit the claims from which they depend.

During the interview, Patent Owner's representatives stated that new claims 8-20 do not improperly broaden original claims 1-6 because any infringement of the new claims 8-20 would also require infringement of original claims 1-6. Patent Owner's representatives referred the Examiners to MPEP § 1412.03(I) (*via* MPEP § 2258(III)(A)).

In this regard, "[a] new claim enlarges if it includes within its scope any subject matter that would not have infringed the original patent."¹ Patent Owner notes the existence of the two Board of Patent Appeals and Interferences (BPAI) cases of Ex parte Wikdahl and Ex parte Logan, both of which are distinguishable from the facts of the instant reexamination.² In Wikdahl and Logan, the Patent Owners were deemed by the BPAI to have improperly broadened

¹ Thermalloy Inc. v. Aavid Engineering Inc., 43 USPQ2d 1846, 1847 (Fed. Cir. 1997) (*citing* Quantum Corp. v. Rodime, PLC, 36 USPQ2d 1162, 1165 (Fed. Cir. 1995))

² Ex parte Wikdahl, 10 USPQ2d 1546 (BPAI 1989); Ex parte Logan, 38 USPQ2d 1852 (BPAI 1994)

the scope of the claims through presentation of claims to different inventions (e.g., apparatus and method claims). In contrast, the claims presented herein are all directed to subject matter falling within the same statutory class of invention, namely compositions of matter.³ Wikdahl and Logan are non-precedential decisions of the BPAI and are therefore restricted to their facts, which are not on-point with the instant reexamination. Furthermore, the claims presented in this response do not include within their scope any subject matter that would not have infringed the original patent. Therefore, Patent Owner submits that Wikdahl and Logan should not drive a conclusion that the instant claims have an improperly broadened scope in violation of 35 U.S.C. § 305.

Patent Owner's representatives discussed the anticipation rejections over Tsien and Dower, reiterating from Patent Owner's response that the Wiggins case supports Patent Owner's position that the paper disclosures of broad classes of compounds in Tsien and Dower constitute speculation about the existence of mononucleoside 5'-triphosphates having removable blocking moieties protecting the 3' position of said mononucleoside 5'-triphosphates. In other words, undue experimentation would be required to determine which of the multitude of potential blocking groups would be expected to be removable blocking moieties that also protect the 3' position of said mononucleoside 5'-triphosphates.

In this respect, Patent Owner's representatives noted that Tsien teaches away from making and using trifluoroacetate because, according to Tsien, "... the highly electronegative group trifluoroacetate is cleaved rapidly from 3' hydroxyls in methanol at pH 7 ... and thus would not be stable during coupling at that pH."⁴ Tsien's exemplified trifluoroacetate is purportedly unstable during coupling at pH 7, and therefore likely would not protect the 3' position of a mononucleoside 5'-triphosphate as required by the claims.

Patent Owner's representatives further stated that it is reasonable to extend this to other electronegative groups encompassed by the claims, such as for example dichloroacetate and trichloroacetate. Patent Owner's representatives also noted that Tsien discouraged the use of phenoxyacetate due to the recommended high pH. Patent Owner's representatives discussed how the rejected claims are distinguished from the facts of the In re Gleave case, in which

³ Under 35 U.S.C. § 101, the statutory classes of patentable subject matter are process, machine, manufacture, or composition of matter, or any new and useful improvement thereof.

⁴ Tsien, p. 24, ll. 10-13.

counsel for Gleave admitted that Gleave's claims did not recite functional language.⁵ In contrast, the instant claims require removable blocking moieties that also protect the 3' position of said mononucleoside 5'-triphosphate.⁶

Patent Owner's representatives also discussed that Tsien teaches away from using certain blocking groups of Greene because those blocking groups do not satisfy Tsien's criteria that blocking groups should rapidly deblock and yield a viable 3'-OH function in high yield. A non-limiting listing of these groups was provided in Appendix A of Patent Owner's March 21, 2011 response.

It was agreed that Patent Owner may submit a supplemental response that may argue the non-obviousness of the rejected claims on the basis of: 1.) the failure of certain 3'-OH blocking groups to rapidly deblock and in high yield under the criteria of Tsien, and/or 2.) the expectation of one of ordinary skill in the art in 1994 that a nucleoside 5'-triphosphate with a 3'-OH protected by a large blocking group would not have been an acceptable substrate for a DNA polymerase due to steric hindrance considerations. It is noted that the former was sufficiently addressed in Patent Owner's response of March 21, 2011. The latter point with supporting evidence is addressed below.

III. Claim Rejections Under 35 U.S.C. 102(b)

Claims 1-4 and 6 were rejected under 35 U.S.C. § 102(b) as allegedly anticipated by the disclosure of Tsien. Claims 2-5 were also rejected under 35 U.S.C. § 102(b) as allegedly anticipated by the disclosure of Dower *et al* ("Dower").

Claims 1-6 are cancelled herein, thereby rendering these rejections moot. Nevertheless, Patent Owner submits that new claims 21-31 are not anticipated by the disclosure of Tsien or Greene, as neither of these references teaches all of the elements of new claims 21-31.

⁵ In re Gleave, 90 USPQ2d 1235, 1239 (Fed. Cir. 2009).

⁶ Patent Owner notes that in Gleave, the anticipatory polynucleotides were actually made. In contrast, Tsien does not prepare any actual molecules.

IV. Claim Rejections Under 35 U.S.C. 103(a)

Claims 1-6 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Tsien in view of Greene *et al.* ("Greene").

Patent Owner respectfully traverses this rejection.

The office action asserts that Tsien discloses certain 3'-blocking groups, and that the majority of the claimed blocking groups not mentioned in Tsien are listed in Greene. The office action states that it would have been obvious for one of ordinary skill in the art to modify the 3'-blocked deoxynucleotides of Tsien by using a different blocking group as taught by Greene. The office action further asserts that one of ordinary skill in the art could have readily determined whether any modified nucleotide was a suitable substrate for polymerase enzymes, and that there would have been a reasonable expectation of success because Tsien teaches that blocking groups may be modified to incorporate bulky substituent groups.⁷

As noted previously, Patent Owner provided responsive arguments and evidence in Patent Owner's March 21, 2011 response, which are herein incorporated by reference in their entireties to the extent necessary. Briefly, Patent Owner previously argued in its March 21, 2011 response that: 1.) Tsien and Greene teach away from using certain 3' blocking groups; 2.) the cited prior art fails to appreciate an unexpected discovery; 3.) one of ordinary skill in the art would not have an expectation of success in using Greene's deblocking conditions in Tsien's methods; and 4.) certain blocking groups encompassed by the claims are not taught or suggested in the cited references, and are accordingly non-obvious. Patent Owner submits that these arguments are equally applicable to new claims 21-31, which are distinguished over the cited prior art. For the Examiner's convenience, these arguments are reproduced below as sections A, B and C. Supplemental responsive remarks and evidence regarding the absence of a reasonable expectation of success in combining Tsien and Greene to arrive at the claimed invention are provided in section D.

A. Tsien and Greene Teach Away from Using Certain 3' Blocking Groups

According to Tsien, "... the highly electronegative group trifluoroacetate is cleaved rapidly from 3' hydroxyls in methanol at pH 7 (Cramer et al., 1963) and thus would **not be**

⁷ Office action, pp. 9-10.

stable during coupling at that pH.”⁸ On pages 19-20, Tsien teaches preferred exemplary reaction conditions having a pH of 7.3-7.7 and a pH of 7.0-7.4 for the polymerase enzymes Sequenase™ and Klenow, respectively. Tsien’s earlier statement suggests that 3’-blocked deoxynucleotides of Tsien using the trifluoroacetate blocking group of Greene would not work for Tsien’s intended purpose, as Tsien states that this blocking group would not be stable during the coupling process at pH 7, the very pH exemplified by Tsien as preferred. The asserted instability of trifluoroacetate at pH 7 is inconsistent with the use of a 3’ trifluoroacetate blocking group for Tsien’s intended purpose, and teaches away from its use thereof. This supports the non-obviousness of trifluoroacetate as a 3’-blocking group.

Similarly, Tsien states that:

Phenoxyacetate groups are cleaved in less than one minute but require substantially higher pH such as is achieved with NH₃/methanol ... To prevent significant premature deblocking and DNA degradation, the ester deblocking rate is advantageously selected so as to exhibit a deblocking rate of less than 10⁻³s⁻¹ during the incorporation, and at least 10⁻¹s during the deblocking stage. Ideally, this rate change is achieved by changing the buffer pH from 7 to about 10, but care must be taken not to denature the DNA.⁹

These statements of Tsien express concern with the use of phenoxyacetate groups as blocking groups, and teach deblocking conditions that appear to deviate from the “mild conditions” set forth as one criteria for the successful use of 3’-blocking groups. These deblocking conditions “require substantially higher pH”, and a buffer of pH 10 is purportedly ideal for controlling the deblocking rate so long as care is taken “... not to denature the DNA.” While Tsien does not define what is intended by “mild conditions”, Patent Owner submits that this teaching of Tsien discourages the use of phenoxyacetate at these conditions (i.e., a pH of 10). Accordingly, this teaches away from the use of phenoxyacetate as a 3’ blocking group and supports the non-obviousness thereof.

The office action also states that “Greene also provides the reaction conditions for addition and removal of each of the protecting groups disclosed ... the skilled artisan would have used the information provided by Greene for each functional group to select those most likely to

⁸ Tsien, p. 24, ll. 10-13 (emphasis added).

⁹ *Id* at ll. 13-23.

satisfy the criteria set forth by Tsien.”¹⁰ A review of Tsien reveals that Tsien states that his deblocking method should proceed rapidly and also yield a viable 3’-OH function in high yield.¹¹ While Tsien does not specify what is intended by rapid deblocking and high yield, Patent Owner submits that it is reasonable to interpret this assertion as minutes of time for deblocking and greater than 95% deblocking yield. Such is likely the case, for example, in an automated process. Tsien’s own prophetic disclosure does suggest minutes for deblocking time: p. 24, ll. 13-14; and p. 38, ll. 34-36.

Based at least on these deblocking criteria, Tsien teaches away from the use of certain blocking groups set forth in Greene. For the Examiner’s convenience, Patent Owner provides the following table in Appendix A, listing certain claimed blocking groups and Greene’s deblocking reaction condition(s). For certain (but not every) deblocking reactions, Greene provides the time for deblocking and/or the percent yield. Patent Owner asserts that Tsien teaches away from the use of most, if not all, of Greene’s blocking groups set forth in Appendix A. For most if not all of these deblocking reactions set forth in Appendix A, the percent yield and/or time to deblock data provided is inadequate to meet Tsien’s deblocking criteria, namely rapid deblocking and high yield.

To exemplify, Greene references two deblocking (or cleavage) reactions for the formate ester blocking group on page 87. In the first reaction, three days are indicated as necessary for the cleavage reaction. In the second reaction, the stated reaction yield is 62%. Patent Owner submits that both the deblocking reaction time and the yield provided in Greene for the formate ester blocking group are insufficient to meet Tsien’s criteria, particularly in an automated system. The other blocking groups set forth in Appendix A have similar deficiencies in most or all of Greene’s referenced deblocking reactions. Thus, Tsien teaches away from using these blocking groups due to the inadequate deblocking reactions set forth in Greene.

On page 114, Greene provides a mere listing of miscellaneous esters, including the claimed 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, (E)-2-methyl-2-butenolate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, and α -naphthoate esters. According to Greene, these miscellaneous esters “... have seen little use since publication

¹⁰ Office action, page 8.

¹¹ Tsien, p. 23.

of the first edition,” and are listed in Greene simply for completeness.¹² The first edition of Greene published in 1981. Hence, Greene asserts that these miscellaneous esters saw little use for approximately 13 years before Patent Owner’s priority date.

Greene teaches away from the use of these esters as 3’ blocking groups, as is apparent from Greene’s disclosure, or more specifically lack thereof. As stated by Greene, these miscellaneous esters are not described in detail, and there is no express teaching of conditions for forming and cleaving these blocking groups. Moreover, certain references cited in Greene for purposes of reaction conditions are not even directed to the field of nucleotides. Had Greene intended one of ordinary skill in the art to consider the use of these “miscellaneous esters” as 3’ blocking groups, rather than reciting them by name for the sake of completeness, Greene would have provided reaction conditions associated with these miscellaneous esters.

B. The Cited Prior Art Fails to Appreciate an Unexpected Discovery

The claims encompass a substantial number of aromatic 3’-O esters of deoxynucleotide triphosphates.¹³ Patent Owner directs the Examiner’s attention to the disclosure located on page 32, lines 13-27 of the specification as originally filed. This disclosure summarizes the unexpected discovery that certain removable blocking moieties, namely the aromatic 3’-O esters of deoxynucleotide triphosphates, are unstable in commonly used buffers containing divalent cations. The specification attributes this instability problem to the presence in commonly-used buffers of cacodylic acid, tris(hydroxymethyl) aminomethane, sodium acetate or sodium phosphate and the presence of a divalent cation, such as cobalt, manganese or magnesium. Examples of such buffers used in the field of molecular biology is available in the literature. For example, Appendix B provides copies of reference materials discussing the use of buffers such as Cacodylate-HCL, Tris(hydroxymethyl)aminomethane (Tris), and sodium phosphate buffers.

¹² Greene, p. 114.

¹³ For example, claim 1 includes the following aromatic 3’-O esters: benzoylformate, triphenylmethoxyacetate, phenoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α -naphthanoate, while claim 4 includes toluic and benzoic acid esters.

Appendix B also demonstrates that the current Taq polymerase buffer from New England Biolabs Inc. (NEB) contains magnesium, a divalent cation.

It is asserted that the inventors are the first individuals to appreciate this unexpected discovery. The cited prior art references certainly fail to appreciate the existence of this problem, when said aromatic 3'-O ester-linked dNTPs are used in the presence of common buffers discussed above. In the absence of this unexpected discovery, one of ordinary skill in the art attempting to use these blocked mononucleoside 5'-triphosphates in the presence of these buffers under appropriate conditions likely would have failed. For example, utilization of a buffer of this nature under appropriate conditions in a sequential coupling and cleavage reaction likely would have lead to failure by preventing formation of a stable removable aromatic blocking group, which would otherwise allow for multiple phosphodiester bond formations and hence repeated mononucleotide additions. The unappreciated nature of this discovery in the art speaks to the non-obviousness of using aromatic 3'-O esters of deoxynucleotide triphosphates.

It is noted that Example 2 of the specification as originally filed identifies conditions associated with instability, and provides direction for addressing these concerns.¹⁴ For example, the specification teaches that "[d]egradation of the esters was first observable after about three minutes of incubation."¹⁵ Tsien, on the other hand, teaches exemplary coupling reaction times of from about 1 to about 20 minutes and preferably 1 to 5 minutes for Sequenase™, and from about 1 to about 40 minutes for Klenow.¹⁶ Were one of ordinary skill in the art to use Tsien's reaction times in the presence of one of these buffers, it is reasonably likely that one of ordinary skill in the art would exceed the three minute mark at which time degradation of aromatic 3'-O esters was first observable.

C. *No Expectation of Success Using Greene's Deblocking Conditions*

KSR assumes a starting reference point or points in the art, prior to the time of the invention, from which a skilled artisan might identify a problem and pursue potential solutions, KSR presupposes that the record would give some reasons to make particular modifications to achieve the claimed compound, and KSR presumes that the record would supply some reasons

¹⁴ Specification as originally filed, pp. 60-61.

¹⁵ Id. at p. 61, ll. 7-8.

¹⁶ Tsien, pp. 19-20.

for narrowing the prior art universe to a finite number of identified, predictable solutions.¹⁷ According to the USPTO, where there was reason to select and modify the lead compound(s) to obtain the claimed compound, but no reasonable expectation of success, the claimed compound would not have been obvious.¹⁸

The office action states that Greene provides the reaction conditions for addition and removal of each of the protecting groups disclosed, and that the skilled artisan would have used the information provided by Greene for each functional group to select those most likely to satisfy the criteria set forth by Tsien.¹⁹ Tsien identifies “the availability of mild conditions for rapid and quantitative deblocking” as one of the criteria for the successful use of 3'-blocking groups.²⁰

A review of Greene demonstrates that a multitude of blocking groups set forth in Greene are presented with deblocking conditions not considered mild. For example, Greene provides deblocking conditions for several blocking groups that are not considered by one of ordinary skill in the art to be mild, such as the use of elevated temperature, high pH and/or organic solvents.

One of ordinary skill in the art, relying on the criteria set forth in Tsien, would not have had a reasonable expectation of success in using 3'-blocked mononucleoside 5'-triphosphates, harboring a large number of the blocking groups of Greene, in the method of Tsien. This is because Tsien states that one of the criteria for the successful use of 3'-blocking groups is the availability of mild conditions for rapid and quantitative deblocking. Since Greene does not teach mild deblocking conditions for many of the blocking groups, it logically flows that one of ordinary skill would not have a reasonable expectation of success in using these blocking groups. Greene does not teach the conditions necessary for the successful use of these blocking groups.

In contrast, the specification as originally filed sets forth “... a convenient and gentle method for the rapid removal of these esters from the growing polynucleotide chain after a coupling reaction. These deprotection conditions are sufficiently gentle to enable the synthesis

¹⁷ Eisai Co. Ltd. v. Dr. Reddy's Labs, Ltd., 87 U.S.P.Q.2d 1452 (Fed. Cir. 2008).

¹⁸ Examination Guidelines Update: Developments in the Obviousness Inquiry After KSR v. Teleflex, 75 Fed. Reg. 53643, 53659 (Sept. 1, 2010).

¹⁹ Office action, p. 8.

²⁰ Tsien, p. 20, ll. 33-34.

of an object polynucleotide onto a pre-existing double stranded DNA without denaturation of the DNA at every cycle.”²¹

Furthermore, Patent Owner submits that certain blocking groups encompassed by the claims are not taught or suggested in the cited references, and are accordingly non-obvious.

Patent Owner further provides the following supplemental responsive remarks and evidence.

D. One of Ordinary Skill in the Art did not have a Reasonable Expectation of Success

KSR assumes a starting reference point or points in the art, prior to the time of the invention, from which a skilled artisan might identify a problem and pursue potential solutions, KSR presupposes that the record would give some reasons to make particular modifications to achieve the claimed compound, and KSR presumes that the record would supply some reasons for narrowing the prior art universe to a finite number of identified, predictable solutions.²² According to the USPTO, where there was reason to select and modify the lead compound(s) to obtain the claimed compound, but no reasonable expectation of success, the claimed compound would not have been obvious.²³

Patent Owner directs the Examiner’s attention to Appendix C containing copies of certain patent prosecution documents from prior Reexamination Control Number 90/008,149 (“the ‘149 reexamination”). During prosecution in the ‘149 reexamination, Patent Owner discussed the cited Tsien reference in light of the prosecution of EP 1,337,541 B1 (“EP ‘541”).²⁴ This evidence is probative of a determination that Tsien does not suggest that certain removable blocking moieties would have been effective from the standpoints of hydrolyzability and steric hindrance on a nucleic acid polymerase during nucleic acid synthesis.²⁵

As presented in the ‘149 reexamination, during prosecution of EP ‘541 the European Patent Office (“EPO”) determined that the prior art (including the closest prior art, Tsien) did not

²¹ Specification as originally filed, pp. 61, ll. 17-23.

²² Eisai Co. Ltd. v. Dr. Reddy’s Labs, Ltd., 87 U.S.P.Q.2d 1452 (Fed. Cir. 2008).

²³ Examination Guidelines Update: Developments in the Obviousness Inquiry After KSR v. Teleflex, 75 Fed. Reg. 53643, 53659 (Sept. 1, 2010).

²⁴ EP 1,337,541 is in the name of the Trustees of Columbia University in the City of New York.

²⁵ See Appendix C, April 30, 2007 response, p. 15, last full paragraph.

suggest that the methoxymethyl or the allyl ether groups possessed the advantages of being hydrolysable and having little steric effect on the polymerase during an elongation step. In light of this evidence, Patent Owner successfully argued that the inventors in the '149 reexamination proceeded against the accepted wisdom in the art at the time of the invention. The accepted wisdom was that a person of ordinary skill in the art, at the time the claimed invention was made, would have expected that blocking moieties such as allyl and methoxymethyl ether-linked groups used at the 3'-position on a nucleoside 5'-triphosphate would not have been effective in nucleic acid synthesis from the standpoints of hydrolysability or steric hindrance, and thus would not have used them in the synthesis of nucleic acids.²⁶

In a subsequent interview the USPTO Examiner stated:

The examiner acknowledges that at the time of the invention, the person of ordinary skill in the art would not have expected that a nucleoside 5'-triphosphate with a 3'-OH protected by an allyl group would have been an acceptable substrate for a DNA polymerase because of steric considerations. Consequently, claims limited to nucleoside 5'-triphosphates with a 3'-OH protecting group of comparable size to allyl are deemed allowable.²⁷

The Examiner agreed to allow claims reciting protecting groups of comparable size to allyl, such as methoxymethyl, ethoxymethyl, methylmethoxyethyl, methylthiomethyl, carbonitrile, substituted ethyl, and thio-substituted methyl groups as removable blocking moieties linked to the 3' carbon of nucleoside triphosphates *via* an ether linkage.

Patent Owner submits that one of ordinary skill in the art at the time of filing also would not have had a reasonable expectation of success in using most if not all of the claimed removable blocking groups for the purpose asserted in the office action due to anticipated steric hindrance. This is supported by the Declaration of Dr. Molly He submitted herewith as Appendix D.

In her Declaration, Dr. He states that in her opinion one of ordinary skill in the art in 1994 would not have had a reasonable expectation of success in using the following claimed ester-linked 3' blocking groups in Tsien's method requiring polymerase incorporation of the 3' blocked mononucleoside 5'-triphosphates into DNA: benzoylformate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate,

²⁶ See Appendix C, March 7, 2008 response, p. 14, first full paragraph.

²⁷ See Appendix C, April 11, 2008 Interview Summary.

phenoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, p-P-phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamanioate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenolate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate, α -naphthanoate, toluic acid, benzoic acid, and acetic acid. Furthermore, Dr. He states that she would reasonably expect other ester-linked 3' blocking groups not set forth above, but having a similar or larger size than an allyl group, to raise steric hindrance considerations.

In Dr. He's opinion, this is because the afore-mentioned 3' blocking groups are similar to or larger than an allyl group, and would have been expected to at least present the same steric hindrance considerations to one of ordinary skill in the art as an allyl group. For example, many of the afore-mentioned 3' blocking groups contain one or more ring structures that would have been expected to produce the same steric hindrance as an allyl group. Moreover, according to Dr. He, the use of an ester linkage further contributes to the steric concerns due to the increased size of the linkage group, relative to an ether linkage.

In the instant reexamination, because one of ordinary skill in the art at the time of the invention did not have a reasonable expectation of success in using certain removable 3' blocked, ester-linked dNTPs as a polymerase enzyme substrate for incorporation into DNA, one of ordinary skill in the art would not have sought to modify the teachings of Tsien to incorporate certain blocking groups of Greene in an attempt to reach the claimed invention.

It is further noted that new claims 26-31 are based on the granted claims and further require the presence of a template independent polynucleotide polymerase. Patent Owner submits that neither Tsien nor Greene, alone or in combination, teach or suggest this claim element, and therefore these claims are nonobvious.

Patent Owner submits that the claims are not rendered obvious over the disclosure of Tsien in view of Greene for the reasons set forth above. Accordingly, Patent Owner respectfully requests reconsideration and withdrawal of this rejection under 35 U.S.C. § 103(a).

CONCLUSION

For at least the reasons stated above, claims 21-31 are in condition for confirmation and allowance. Accordingly, Patent Owner respectfully requests that these claims be confirmed and allowed, and passed to issue. Should the Examiner believe anything further is desirable in order to place the claims in even better condition for allowance, the Examiner is invited to contact Patent Owner's undersigned representative.

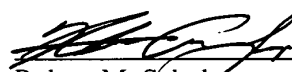
While Patent Owner is hopeful that the instant response will result in an *ex parte* Reexamination Certificate, Patent Owner wishes to express its availability to interview with the Examiner(s) should the Examiner(s) consider an interview a useful means to resolve any rejections prior to issuance of a further Office Action. Patent Owner respectfully requests a personal interview in the event of a further Office Action. It is believed that no fees are necessary in connection with this Amendment and Response. However, in the event that the U.S. Patent and Trademark Office determines that fees are due with this response, the Commissioner is hereby authorized to charge such fees to the undersigned's Deposit Account No. **50-0206**.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Date: May 3, 2011

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67274 U.S. PTO



05/03/11

REEXAMINATION CONTROL NO.: 90/009,769

ATTORNEY DOCKET NO: 68205.000103

CERTIFICATE OF SERVICE

The undersigned representative for the patent owner hereby certifies that a true and correct copy of this **Supplemental Amendment and Response to Non-Final Office Action in Ex Parte Reexamination** for *Ex Parte* Reexamination 90/009,769, as filed in the United States Patent and Trademark Office on May 3, 2011 was served by first class mail on May 3, 2011, on counsel for the third party requester at the following address:

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EXAMINER

ART UNIT PAPER NUMBER

DATE MAILED: 06/24/2011

Please find below and/or attached an Office communication concerning this application or proceeding.



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EX PARTE REEXAMINATION COMMUNICATION TRANSMITTAL FORM

REEXAMINATION CONTROL NO. 90/009,769.

PATENT NO. 5808045.

ART UNIT 3991.

Enclosed is a copy of the latest communication from the United States Patent and Trademark Office in the above identified *ex parte* reexamination proceeding (37 CFR 1.550(f)).

Where this copy is supplied after the reply by requester, 37 CFR 1.535, or the time for filing a reply has passed, no submission on behalf of the *ex parte* reexamination requester will be acknowledged or considered (37 CFR 1.550(g)).

**Notice of Intent to Issue
Ex Parte Reexamination Certificate**

Control No.

90/009,769

Patent Under Reexamination

5808045

Examiner

GARY L. KUNZ

Art Unit

3991

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☒ Prosecution on the merits is (or remains) closed in this *ex parte* reexamination proceeding. This proceeding is subject to reopening at the initiative of the Office or upon petition. Cf. 37 CFR 1.313(a). A Certificate will be issued in view of

- (a) ☒ Patent owner's communication(s) filed: 5/31/11.
(b) ☐ Patent owner's late response filed: _____.
(c) ☐ Patent owner's failure to file an appropriate response to the Office action mailed: _____.
(d) ☐ Patent owner's failure to timely file an Appeal Brief (37 CFR 41.31).
(e) ☐ Other: _____.

Status of *Ex Parte* Reexamination:

- (f) Change in the Specification: ☐ Yes ☒ No
(g) Change in the Drawing(s): ☐ Yes ☒ No
(h) Status of the Claim(s):

- (1) Patent claim(s) confirmed: _____.
(2) Patent claim(s) amended (including dependent on amended claim(s)): _____.
(3) Patent claim(s) canceled: 1-6.
(4) Newly presented claim(s) patentable: 21, 23, 31 and 35 - 37.
(5) Newly presented canceled claims: 8-20, 22, 24-30 and 32-34.
(6) Patent claim(s) ☐ previously ☐ currently disclaimed: _____.
(7) Patent claim(s) not subject to reexamination: 7.

2. ☒ Note the attached statement of reasons for patentability and/or confirmation. Any comments considered necessary by patent owner regarding reasons for patentability and/or confirmation must be submitted promptly to avoid processing delays. Such submission(s) should be labeled: "Comments On Statement of Reasons for Patentability and/or Confirmation."

3. ☐ Note attached NOTICE OF REFERENCES CITED (PTO-892).

4. ☒ Note attached LIST OF REFERENCES CITED (PTO/SB/08 or PTO/SB/08 substitute).

5. ☐ The drawing correction request filed on _____ is: ☐ approved ☐ disapproved.

6. ☐ Acknowledgment is made of the priority claim under 35 U.S.C. § 119(a)-(d) or (f).

- a) ☐ All b) ☐ Some* c) ☐ None of the certified copies have
☐ been received.
☐ not been received.
☐ been filed in Application No. _____.
☐ been filed in reexamination Control No. _____.
☐ been received by the International Bureau in PCT Application No. _____.

* Certified copies not received: _____.

7. ☐ Note attached Examiner's Amendment.

8. ☐ Note attached Interview Summary (PTO-474).

9. ☐ Other: _____.

GARY L. KUNZ

PRIMARY EXAMINER

DWAYNE C. JONES

PRIMARY EXAMINER

Deborah D. Jones

Supervisory Patent Examiner
CRU - Art Unit 3991

cc: Requester (if filed and requested)
U.S. Patent and Trademark Office
PTOL-469 (Rev. 05-10)

Notice of Intent to Issue Ex Parte Reexamination Certificate

Part of Paper No 20110504

REEXAMINATION

REASONS FOR PATENTABILITY / CONFIRMATION

Reexamination Control No. 90/009,769

Attachment to Paper No. 20110504.

Art Unit 3991.

In the compliant Fourth Supplemental Amendment filed May 31, 2011, the Patent Owner cancelled claims 1 - 6, 8 - 20, 24-30 and 32-34. New claims 21, 23, 31 and 35-37 are allowable for the following reasons.

Tsien et al. and Dower are no longer applicable to the new 3'-O-protected nucleotide claims because the new 3'-O-protected nucleotides are narrower than the original claims 1 - 6.

Tsien et al. in view of Greene is not applicable to new claims 35 - 40 for the reasons presented in the Patent Owner's response of March 21, 2011. Two arguments are especially persuasive.

a) Tsien and Greene teach away from using the 3'-O-protecting groups which cannot be removed under mild conditions. See Appendix A in Response of March 21, 2011, for 13 different protecting groups which cannot be removed rapidly and quantitatively under the required mild conditions of Tsien et al.

b) There is no expectation of success in using large 3'-OH protecting groups in nucleotides being used as an enzyme substrate. This is due to the increased steric hindrance effects that would be predicted from large protecting groups.

The Patent Owner filed an IDS with the Fourth Supplemental Amendment on May 31, 2011, which cited US Patent No. 6,214,987. This newly cited patent is part of a family of patents including the Hiatt '045 patent. The 6,214,987 patent is directed to compositions comprising 3'-O-protected nucleotides and a template independent enzyme. Therefore, there exists an Obviousness-Type Double Patenting relationship between any composition in Hiatt '045 and 6,214,987. The Patent Owner has obviated this problem by cancelling all claims directed to compositions.

PTOL-476 (Rev. 03-98)

Continuation of Description of the general nature of what was agreed to if an agreement was reached, or any other comments: The Examiner pointed out that new claim 22 still encompasses the 3'-O-protecting groups disclosed in Tsien at page 21, lines 20 - 24. Claim 22 must be amended to overcome this anticipatory reference. At the interview on April 6, 2011, the Examiner had emphasized that Tsien is an anticipatory reference for those claims which encompass the 3'-O-protecting groups in Tsien at page 21, lines 20 - 24. Specifically, the Examiner indicated that all references to the 3'-O-ester nucleotides taught in Tsien et al. at page 21, lines 20 - 31 must be deleted from claim 22, i.e., formyl, acetyl, propyl, isopropyl, butyl (lower alkyl).



US005808045C1

(12) **EX PARTE REEXAMINATION CERTIFICATE** (8569th)
United States Patent
Hiatt et al. (10) **Number:** **US 5,808,045 C1**
(45) **Certificate Issued:** **Sep. 27, 2011**

(54) **COMPOSITIONS FOR ENZYME CATALYZED
 TEMPLATE-INDEPENDENT CREATION OF
 PHOSPHODIESTER BONDS USING
 PROTECTED NUCLEOTIDES**

(75) Inventors: **Andrew C. Hiatt**, San Diego, CA (US);
Floyd Rose, Del Mar, CA (US)

(73) Assignee: **Solexa, Inc.**, Hayward, CA (US)

Reexamination Request:
 No. 90/009,769, Jul. 23, 2010

Reexamination Certificate for:
 Patent No.: **5,808,045**
 Issued: **Sep. 15, 1998**
 Appl. No.: **08/486,987**
 Filed: **Jun. 7, 1995**

Certificate of Correction issued Mar. 16, 1999.

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/300,484, filed on
 Sep. 2, 1994, now Pat. No. 5,990,300.

(51) **Int. Cl.**
C07H 19/00 (2006.01)
C07H 19/10 (2006.01)
C07H 19/20 (2006.01)
C07H 21/00 (2006.01)
C12P 19/00 (2006.01)
C12P 19/30 (2006.01)

(52) **U.S. Cl.** **536/26.26; 536/26.7; 536/26.71;
 536/26.72; 536/26.74; 536/26.8**

(58) **Field of Classification Search** **536/26**
 See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | |
|-----------|----|---------|--------------|
| 4,711,955 | A | 12/1987 | Ward et al. |
| 5,118,605 | A | 6/1992 | Urdea |
| 5,302,509 | A | 4/1994 | Cheeseman |
| 5,872,244 | A | 2/1999 | Hiatt et al. |
| 6,214,987 | B1 | 4/2001 | Hiatt et al. |
| 7,459,275 | B2 | 12/2008 | Dower et al. |

FOREIGN PATENT DOCUMENTS

| | | |
|----|----------------|---------|
| WO | WO 90/15070 | 12/1990 |
| WO | WO 91/06678 | 5/1991 |
| WO | WO 92/10587 | 6/1992 |
| WO | WO 2004/018497 | 3/2004 |

OTHER PUBLICATIONS

Amit et al., "Photosensitive Protecting Groups of Amino Sugars and Their Use in Glycoside Synthesis, 2-Nitrobenzyloxycarbonylamino and Nitroveratryloxycarbonylamino Derivatives," *Journal of Organic Chemistry*, vol. 39, No. 2, pp. 192-196 (1974).

Cantoni et al., "Characterization of DNA Lesions Produced by HgCl₂ in Cell Culture Systems," *Chemico-Biological Interactions*, vol. 49, pp. 209-224 (1984).

Cantoni et al., "Mechanism of HgCl₂ Cytotoxicity in Cultured Mammalian Cells," *Molecular Pharmacology*, vol. 26, pp. 360-368 (1984).

Chinault et al., "Preparation of *Escherichia coli* tRNAs Terminating in Modified Nucleosides by the Use of CTP(ATP): tRNA Nucleotidyltransferase and Polynucleotide Phosphorylase," *Biochemistry*, vol. 16, No. 4, pp. 756-765 (1977).

Costa et al., "DNA Damage by Mercury Compounds: An Overview," In *Advances in Mercury Toxicology*, pp. 255-273 Plenum Press New York (1991).

Dale et al., "Conversion of Covalently Mercurated Nucleic Acids to Tritiated and Halogenated Derivatives," *Nucleic Acids Research*, vol. 2, No. 6, pp. 915-930 (1975).

Dale, et al., "The Synthesis and Enzymatic Polymerization of Nucleotides Containing Mercury: Potential Tools for Nucleic Acid Sequencing and Structural Analysis," *Proceedings of the National Academy of Science USA* vol. 70, No. 8, pp. 2238-2242 (1973).

Eichhorn et al., "The Reaction of Mercury (II) with Nucleosides," *Journal of the American Chemical Society*, vol. 85, No. 24, pp. 4020-4024 (1963).

Gait, "Oligonucleotide Synthesis: A Practical Approach," IRL Press, Oxford p. 5 (1984).

Gigg et al., "The Allyl Ether as a Protecting Group in Carbohydrate Chemistry," Part II, *Journal Chemical Society*, pp. 1903-1911 (1968).

Gruenwedel et al., "Mercury-Induced DNA Polymorphism: Probing the Conformation of Hg(II)-DNA via Staphylococcal Nuclease Digestion and Circular Dichroism Measurements," *Biochemistry*, vol. 29, No. 8, pp. 2111-2116 (1989).

Hayakawa et al., "O-Allyl Protection of Guanine and Thymine Residues in Oligodeoxyribonucleotides," *Journal of Organic Chemistry*, vol. 58, pp. 5551-5555 (1993).

Henner et al., "Enzyme Action at 3' Terminal of Ionizing Radiation-induced DNA Strand Breaks," *The Journal of Biological Chemistry*, vol. 258, No. 24, p. 15198-15205 (1983).

(Continued)

Primary Examiner—Gary L. Kunz

(57) **ABSTRACT**

A method for the stepwise creation of phosphodiester bonds between desired nucleosides resulting in the synthesis of polynucleotides having a predetermined nucleotide sequence by preparing an initiation substrate containing a free and unmodified 3'-hydroxyl group; attaching a mononucleotide selected according to the order of the predetermined nucleotide sequence to the 3'-hydroxyl of the initiating substrate in a solution containing a catalytic amount of an enzyme capable of catalyzing the 5' to 3' phosphodiester linkage of the 5'-phosphate of the mononucleotide to the 3'-hydroxyl of the initiating substrate, wherein the mononucleotide contains a protected 3'-hydroxyl group, whereby the protected mononucleotide is covalently linked to the initiating substrate and further additions are hindered by the 3'-hydroxyl protecting group. Methods in which a mononucleotide immobilized on a solid support is added to a free polynucleotide chain are also disclosed.

OTHER PUBLICATIONS

- Honda et al., "Deprotection of Allyl Groups with Sulfonic Acids and Palladium Catalyst," *Journal of Organic Chemistry*, vol. 62, pp. 8932-8936 (1997).
- Hovinen, et al., "Synthesis of 3'-O-(ω -Aminoalkoxymethyl)thymidine 5'-Triphosphates, Terminators of DNA Synthesis that Enable 3'-Labeling," *Journal Chemical Society Perkins Trans.*, Issue 2, pp. 211-217 (1994).
- Ju et al., "Four-Color DNA Sequencing by Synthesis Using Cleavable Fluorescent Nucleotide Reversible Terminators," *PNAS*, vol. 103, No. 52, pp. 19635-19640 (2006).
- Karakawa et al., "The Utility of a 3'-O-Allyl Group as a Protective Group for Ring-Opening Polymerization of a α -D-Glucopyranose 1,2,4-Orthopivalate Derivatives," *Biomacromolecules*, vol. 3, pp. 538-546 (2002).
- Katz, "The Reversible Reaction of Sodium Thymonucleate and Mercuric Chloride," *Journal of the American Chemical Society*, vol. 74, No. 9, pp. 2238-2245 (1952).
- Kraevskii et al., "Substrate Inhibitors of DNA Biosynthesis," *Molecular Biology*, vol. 21, pp. 25-29, (1987).
- Kruse et al., "Use of (Thio)Acetal Esters as Reagents for the Protection of Alcohols Synthesis of 2-Tetrahydrothienyl Ethers," *Journal of Organic Chemistry*, pp. 43, No. 18, pp. 3548-3553 (1978).
- Letsinger et al., "2,4-Dinitrobenzenesulfonyl as a Blocking Group for Hydroxyl Functions in Nucleosides," *Journal of American Chemistry*, vol. 29, pp. 2615-2618 (1964).
- Matsuoka et al., "Mercury Chloride Activate c-Jun N-Terminal Kinase and Induces c-jun Expression in LLC-PK1 Cells," *Toxicological Sciences*, vol. 53, pp. 361-368 (2000).
- Metzker, "Emerging Technologies in DNA Sequencing," *Genome Research*, vol. 15, pp. 1767-1776 (2005).
- Patchornik, et al., "Photosensitive Protecting Groups," *Journal of American Chemical Society*, vol. 92, No. 21, pp. 6333-6335 (1970).
- Prober et al., "A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides," *Science*, vol. 238, pp. 336-341 (1987).
- Ruparel et al., "Design and Synthesis of a 3'-O-allyl Photocleavable Fluorescent Nucleotide as a Reversible Terminator for DNA Sequencing by Synthesis," *PNAS*, vol. 102, No. 17, pp. 5932-5937 (2005).
- Welch et al., "Syntheses of Nucleosides Designed for Combinatorial DNA Sequencing," *European Journal of Organic Chemistry*, vol. 5, No. 3, pp. 951-960 (1999).
- Yamane, "On the Complexing of Desoxyribonucleic Acid (DNA) by Mercuric Ion," *Journal of the American Chemical Society*, vol. 83, No. 12, pp. 2599-2607 (1961).
- Young et al., "Binding of Mercury(II) to Poly(dA-dT) Studied by Proton Nuclear Magnetic Resonance," *Biochemistry*, vol. 21, pp. 62-66 (1982).
- Corrected Third Party Request for Ex Parte Reexamination filed Jul. 23, 2010, in Reexamination Control No. 90/009, 767.
- Greene and Wutz, *Protective Groups in Organic Synthesis*, 2nd Ed. 1991, pp. 10-119, John Wiley & Sons, New York, USA.
- Gait, "Oligonucleotide Synthesis: A Practical Approach," IRL Press, Oxford, 225 pages (1984).
- Chambers, et al., "Buffers, Chelating Agents And Denaturants," *Biochemistry Labfax*, Bios Scientific Publishers, pp. 1-11 (1993).
- Scopes, Robert K., "Buffers For Use In Protein Chemistry," *Protein Purification Principles and Practice*, Third Edition, Springer-Verlag, pp. 351-352 (1994).
- New England Biolabs On-Line Catalog, "Taq DNA Polymerase with Standard Buffer(M0273), Routine PCR, NEB," New England Bio Labs Inc., <http://www.neb.com/nebecomm/products/productm0273.asp>, pp. 1-3 (2011 (printed Mar. 20, 2011)).
- Losse, et al., "Der 2-Benzoyloxycarbonyl-benzoyl-Rest als OH-Schutz für Dipeptid-Synthesen," *Chem. Ber.*, vol. 98, pp. 1522-1530 (1965), and English language Abstract thereof.
- Reese, "Tetrahedron Report No. 56: The Chemical Synthesis Of Oligo- and Poly-Nucleotides By The Phosphotriester Approach," *Tetrahedron*, vol. 34, pp. 3144-3179 (1978).
- Büchi et al., "CV. Total Synthesis of The Structural Gene For An Alanine Transfer Ribonucleic Acid From Yeast. Chemical Synthesis Of An Icosadeoxyribonucleotide Corresponding To The Nucleotide Sequence 31 to 50," *J. Mol Biol.*, vol. 72, pp. 251-288 (1972).
- Watanabe, et al., "Synthesis of a Lividomycin B Analogue, 5-O-[3-O-(2-Amino-2-deoxy- α -D-glucopyranosyl)- β -D-ribofuranosyl]-3'-deoxyparomamine," *Bulletin of the Chemical Society of Japan*, vol. 50 (9), pp. 2369-2374 (1977).
- Cook et al., "Use of Chloroacetic Anhydride for the Protection of Nucleoside Hydroxyl Groups," *J. Org. Chem.*, vol. 35, No. 6, pp. 1940-1943 (1970).
- Kupchan et al., "The Tiglate Ester as an Alcohol Blocking Group in Organic Synthesis," *J. Org. Chem.*, vol. 27, pp. 3103-3106 (1962).
- Axelrod, et al., "Specific Termination of RNA Polymerase Synthesis As A Method of RNA and DNA Sequencing," *Nucleic Acids Research*, vol. 5, No. 10, pp. 3549-3564 (1978).
- Kutateladze, et al., "3'-Hydroxymethyl 2'-deoxynucleoside 5'-triphosphates Are Inhibitors Highly Specific For Reverse Transcriptase," *FEBS 4130*, Elsevier Science Publishers B.V. (Biomedical Division), vol. 207, No. 2, pp. 205-212 (1986).

1
EX PARTE
REEXAMINATION CERTIFICATE
ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS
INDICATED BELOW.

Matter enclosed in heavy brackets [] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in *italics* indicates additions made to the patent.

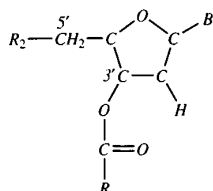
AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

Claims 1-6 are cancelled.

New claims 8-13 are added and determined to be patentable.

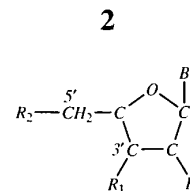
Claim 7 was not reexamined.

8. A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



wherein R_2 is a triphosphate and R is selected from the group consisting of: benzoylformate, methoxyacetate, triphenylmethoxyacetate, p-chlorophenoxyacetate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, phenylacetate, 3-phenylpropionate, 3-benzoylpropionate, isobutyrate, 4-oxopentanoate, pivaloate, adamantate, crotonate, 4-methoxycrotonate, (E)-2-methyl-2-butenate, o-(dibromomethyl)benzoate, o-(methoxycarbonyl)benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate and α -naphthanoate.

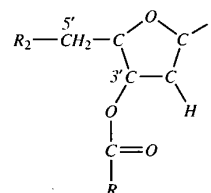
9. A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which has the following formula:



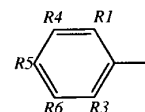
wherein R_2 is triphosphate; and wherein R_1 is toluic or benzoic acid ester.

10. The mononucleoside 5'-triphosphate of claim 21, wherein R is 3-benzoylpropionate.

11. A mononucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position which is an ester and which has the following formula:



wherein R_2 is triphosphate; and wherein R is selected from the group consisting of: $CH_3(CH_2)_n$ where n is an integer from 4 to 12; $(CH_3)_{x+1}(CH)_x$ where x is an integer from 2 to 12; $(CH_3)_{x+1}(CH)_x(CH_2)_n$ where x is an independent integer from 1 to 12 and n is an independent integer from 2 to 12; $C_x(CH_3)_{3x-(x-1)}(CH_2)_n$ where x and n are independent integers from 1 to 12; and



where R1, R3, R4, R5, and R6 are selected from the group consisting of CH_3 or NO_2 .

12. The mononucleoside 5'-triphosphate of claim 21, 23, or 11 wherein said removable blocking moiety is removable by an enzyme.

13. The mononucleoside 5'-triphosphate of claim 21, 23, or 11 wherein said removable blocking moiety is removable by a reaction which occurs within 2 to 10 minutes.

* * * * *