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(54) Title: NOVEL LINKER FOR NUCLEOTIDES

(57) Abstract: The present invention relates to a linker for oligonucleotide. The linker of this invention is useful for tagging a fluorescent molecule as a reporter or quencher or linking a solid support to oligonucleotide.



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NOVEL LINKER FOR NUCLEOTIDES

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

5 The present invention relates to novel linkers for nucleotides. More particularly, the present invention relates to novel linkers for tagging a fluorescent molecule as a reporter or quencher or linking a solid support to oligonucleotide and methods for preparing the
10 same.

DESCRIPTION OF THE RELATED ART

For recent years, automated DNA synthesizers have made breakthrough in molecular biology and biochemistry
15 providing commercial linear DNA nucleotides with aimed sequences. Currently, the most general method for oligonucleotide synthesis is the phosphite-triester method synthesizing oligonucleotides on a solid support using nucleoside phosphoamidites. Generally, the process
20 contains the steps of deblocking, coupling, oxidation and capping.

Applicability of oligonucleotides is limitless, e.g. DNA oligonucleotides provide primers in cDNA synthesis and real-time PCR, templates for RNA transcription, linkers
25 for plasmid manipulation and hybridization probes for diagnosis.

Further modification of oligonucleotide enhances its applicability in various fields. Among several

modifications of the oligonucleotide, the most feasible method in current is attaching a label on the oligonucleotide using the phosphoramidite method.

In general, the oligonucleotide synthesized is then
5 labeled for chasing of oligonucleotide-target complex and quantitative analysis. Such a specific labeling on oligonucleotide is pivotal process for DNA-probe synthesis (Goodchild, *J. Bioconjugate Chem.*, 1:165 (1990)).

Isotopes for labeling of nucleotides have been rapidly
10 substituted by fluorescent materials owing to their hazardous and tricky property for treatment (Smith, L. M. et al., *Nature*, 321: 674-679 (1986); and Douglas, M. E. et al., *BioMed. Chem. Lett.* 4(8):995-1000 (1994)). For labeling oligonucleotide with fluorescent materials, a
15 variety of methods for oligonucleotide derivatives with amino or thiol group has been developed (Ono, K. et al., *Bioconjugate Chem.* 4:499-508(1993); and Douglas, M. E. et al., *BioMed. Chem. Lett.* 4(8):995-1000(1994)).

The synthesized oligonucleotides can be tagged with
20 reporter etc., either by chemical and enzymatic methods, and a reporter or quencher can also be incorporated using usable material in the course of phosphoramidite method (Kempe, T. et al., *Nucleic Acid Res.* 13:45-57 (1985); WO 91/17169; and Gibson, K. J. et al., *Nucleic Acid Res.*
25 15:6455-6467 (1987)).

There are currently several types of linkers for tagging the reporter etc. on oligonucleotides. Nelson et al. have stepped their pioneer feet by developing a linker

based on 2-(4-aminobutyl)-1,3-propanoldiol capable to be covalently linked with 3 functional groups (*Nucleic Acid Res.* 20: 6253-6259 (1992)), and Mullah B. et al. have followed them with a improved linker introducing amide
5 groups on their predecessors' creation (*Nucleic Acid Res.* 26: 1026-1031 (1998)).

The linkers aforementioned, however, exist in racemic mixture and then are very likely to generate 2 diastereomers upon linking to oligonucleotide, thereby
10 leading to difficulty in purification of the synthetic oligonucleotides and complexity in analysis using the synthetic oligonucleotides.

Throughout this application, various patents and
15 publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entitles are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which
20 this invention pertains.

SUMMARY OF THE INVENTION

Under such situation, the present inventors have made intensive studies to be from the shortcomings of the
25 conventional linkers, and as a result, we have developed novel linkers to enable more convenient purification of and reliable analysis with oligonucleotide linked to the

present linkers.

Accordingly, it is an object of this invention to provide a novel linker useful in modification such as labeling or synthesis of oligonucleotide.

5

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjunction with the appended claims.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a represents 4 sorts of 35S promoter sequences identified in cauliflower mosaic viruses;

Fig. 1b represents primers and probe designed on the basis of the 4 sorts of 35S promoters.

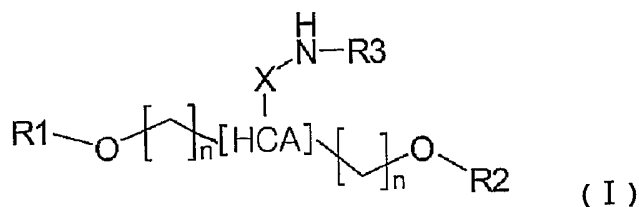
15 Fig. 2 represents intensity of fluorescence depending on the number of PCR cycles.

Fig. 3 represents quantification of transformed plants using standard curve obtained according to the present invention.

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DETAILED DESCRIPTION OF THE INVENTION

In one aspect of this invention, there is provided a novel linker for oligonucleotide represented by the following formula (I):

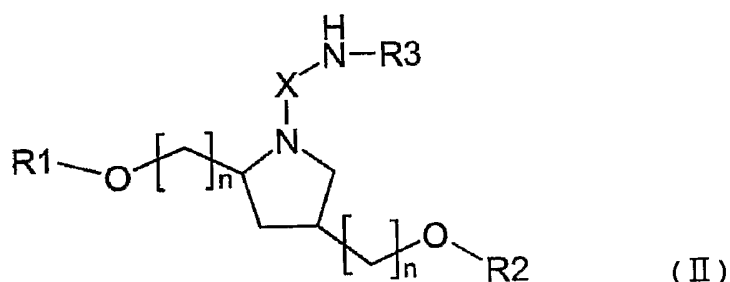


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wherein HCA represents a heterocyclic amine containing 5 or 6 atoms; n is an integer of 0-10; X is linked to nitrogen atom of the heterocyclic amine and represents one selected from the group consisting of saturated C₁-C₁₀ hydrocarbon chain, saturated C₁-C₁₀ hydrocarbon chain in which carbon atoms are substituted with 1-5 heteroatoms, and saturated C₁-C₁₀ hydrocarbon chain containing amide, ester, ether, amine, sulfonyl or combination thereof; R₁ is a hydrogen or hydroxyl-protecting group; R₂ is one selected from a group consisting of hydrogen, phosphoramidite group, H-phosphate and solid support; and R₃ is one selected from a group consisting of reporter, quencher, amino-protecting group, amino acid and peptide.

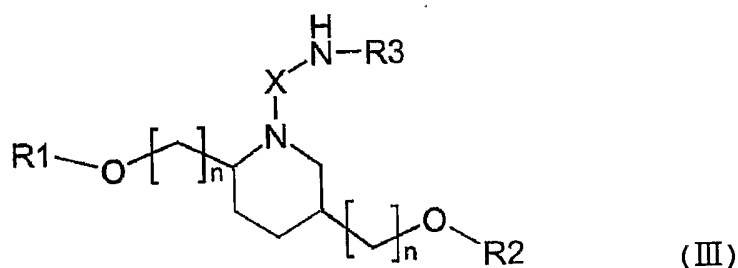
The heterocyclic amine centered in the linker according to the present invention is non-aromatic one comprising heterocyclic amine containing 5 atoms e.g. pyrrolidine or 6 atoms e.g. piperidine. Furthermore, the heterocyclic amine can contain either one or more nitrogen atoms (e.g. piperazine) or other additional heterogeneous atom as well as one or more nitrogen atoms (e.g. morpholine).

According to a preferred embodiment of the present invention (where the central heterocyclic amine is pyrrolidine), the linker is represented by formula (II)



wherein n, X, R1, R2 and R3 are the same as described in the formula I.

5 According to another preferred embodiment of the present invention (where the central heterocyclic amine is piperidine), the linker is represented by the following formula (III).



10

wherein n, X, R1, R2 and R3 are the same as described in the formula I.

The linker of the present invention can be linked to
 15 any nucleotide in oligonucleotide with or without reporter molecule. The term "oligonucleotide" used herein means substance comprising several nucleotides and may contain one or more derivatives of nucleotide and/or linker allowing for adequate gap. The "nucleotide", as used
 20 herein, refers to ribonucleotide or deoxyribonucleotide

containing adenine, cytosine, thymine, guanine, uracil and their derivatives.

The molecules incorporated into oligonucleotide by the linker of the present invention includes reporter, quencher, amino-protecting group, amino acid and peptide, but not limited to.

A reporter may comprise reporter molecule with oligonucleotide, permitting photometrical detection, linkage and quantification of oligonucleotide. Preferably, the reporter is a fluorescent reporter molecule. Where amine group is linked to the reporter, the reporter can contain spacer not to hinder the function of the amine group.

The preferred fluorescent molecules includes 5-carboxyfluorescein (5-FAM), 6-carboxyfluorescein (6-FAM), 2',4',1,4-tetrachlorofluorescein (TET), 2',4',5',7',1,4-hexachlorofluorescein (HEX) and 2',7'-dimethoxy-4',5'-dichloro-6-carboxyrhodamine (JOE).

Furthermore, the quencher may comprise quencher molecule with oligonucleotide, and can quench the function of reporter in a proximal position and restore the function of reporter in a distal position e.g. by separation from reporter.

Preferably, the quencher is a fluorescent quencher molecule. Where amine group is linked to the reporter, the reporter can contain spacer not to hinder the function of the amine group.

The preferred fluorescent quencher includes

tetramethyl-6-carboxyrhodamine (TAMRA), tetrapropano-6-carboxyrhodamine (ROX), cyanine, androquinone, nitrothiazole and nitroamidazole.

It is prerequisite to protect functional groups (e.g. functional groups of 4 bases (e.g. amino group), phosphate group or 5-hydroxyl group) for permitting the aimed chemical reaction in the course of nucleotide synthesis.

To begin with, the amino group in bases should be protected for prevention of acetylation or phosphorylation during oligonucleotide synthesis. Generally, a protecting group stable in acid and removable conveniently in alkaline condition may be used.

For amino-protecting group in the linker of the present invention, benzyloxycarbonyl group is preferred.

5'-hydroxyl group should be protected during condensation, oxidation and capping steps and the protecting group is required to be removed by weak acid (e.g. trichloroacetic acid: TCA) before the following nucleotide is added.

As the hydroxyl-protecting group, 4,4-dimethoxytrityl (DMT), 9-phenylxanthen-9-yl (pixyl) or 9-fluorenylmethoxycarbonyl (Fmoc) is recommendable and DMT works better than others.

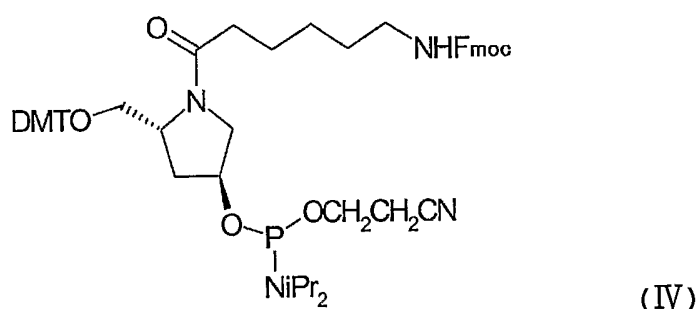
According to another preferred embodiment of the present invention, n is an integer of 0-10. More preferably, n is an integer of 0-1, and the smaller n results in shorter-lengthen saturated hydrocarbon chain, which is linked to central heterocyclic amine,

contributing to less steric hindrance and simpler linker structure.

According to another preferred embodiment of the present invention, X is C₄-C₈ hydrocarbon chain containing amide group and more preferably, X is C₆ hydrocarbon chain containing 1 amide group. The amide group is likely to be prepared in easier manner, stable in condition of oligonucleotide synthesis and enable analysis to be easier. In the case that X contains a heterogeneous atom, preferred is either O or N.

The applicability of the linker of the present invention is limitless; however, the representatives are incorporation of fluorescent molecule into oligonucleotide and application in oligonucleotide synthesis.

In the case for the linker of the present invention being used for the purpose of incorporation of fluorescent molecules into oligonucleotide, one example of the present invention is represented by the following formula (IV).



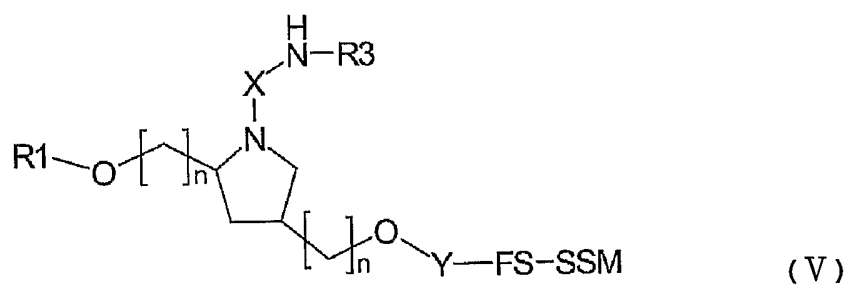
wherein iPr represents isopropyl group.

In the linker represented by formula (IV), a reporter molecule or a quencher molecule is linked to in place of Fmoc, an amino-protecting group.

In the case that the linker according to present invention is employed in the process of oligonucleotide synthesis, for typical example, phosphoramidite DNA synthesis, the linker can be attached to 3'-end of nucleotide or oligonucleotide and employed instead of the protected deoxynucleoside phosphoramidite added into DNA synthesizer.

Moreover, protected deoxynucleoside derivatized lcaa-CPG (long chain alkylamine-controlled pore glass) column, which is generally employed in phosphoramidite DNA synthesis, can be successfully replaced with a column packed with resin having the linker of the present invention.

Another example of linker according to the present invention is an immobilized linker represented by the following formula (V).



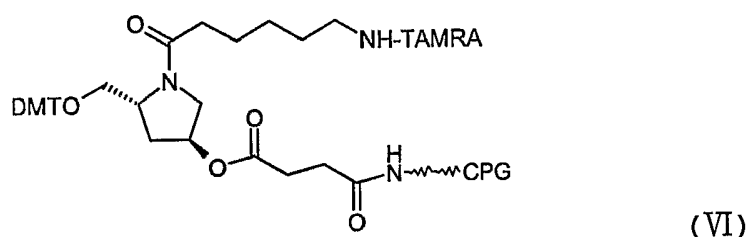
wherein n, X and R1 are the same as described in the formula (I), Y is single bond, nucleotide or oligonucleotide; FS is functional spacer; SSM is solid support; and R3 is amino-protecting group, reporter or quencher.

In the formula (V), non-limiting example includes long chain alkylamine CPG (1cca-CPG), 3-amino-propyl silica gel, aminomethyl polystyrene resin and TengaGel™ (polyethylene glycol-TENTAcles grafted on low cross-linked
5, gelatinous polystyrene matrix).

The SSM is separated from final oligonucleotide and a functional spacer is located between the SSM and the central part of the linker. The functional spacer includes any spacer useful for chemical synthesis of
10 oligonucleotide, e.g., succinyl group and glutaryl group.

According to the most preferable embodiment of the present invention, n is 0 or 1, X is C₆ hydrocarbon chain containing 1 amide group, Y is single bond, FS is succinyl spacer, SSM is CPG, R3 is reporter or quencher and R1 is
15 DMT in the linker of formula (V).

One example of the linker of the formula (V) is represented by the following formula (VI):

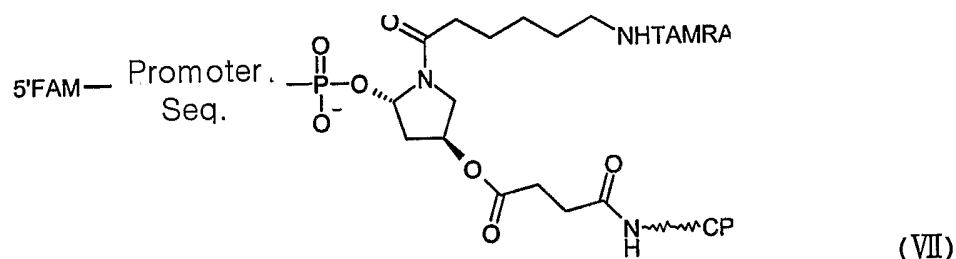


20 The nucleotide synthesis employing the linker of the present invention can be performed by conventional methods described by Caruthers, M.H. *Science*, 230: 281-285 (1985), Itakura, K. et al. *Ann. Rev. Biochem.* 53; 323-356 (1984) and Hunkapiller, M. et al. *Nature*, 310: 105-111 (1984),
25 and these publications are incorporated herein by

reference.

The oligonucleotide with the linker of the present invention can be employed for real-time monitoring of DNA amplification, typically for real-time PCR. The method for the real-time PCR is disclosed in U.S. Pat. 5,538,848, incorporated herein by reference.

Where the oligonucleotide with the linker of the present invention is employed as probe in real-time PCR, the probe is represented by the following formula (VII).



10

As described above, the linker according to the present invention is useful for tagging oligonucleotide with fluorescent molecule or linking solid resin to oligonucleotide.

Moreover, the present linker can be prepared in pure enantiomer, removing the difficulty in purification and analysis caused by conventional linkers that are generally in racemic mixture. Such pure enantiomer is prepared using natural-occurring chiral compound (e.g. proline with certain stereoisomeric property). The process for preparing the enantiomeric linker according to the present invention is exemplified in the following example.

It is obvious for ones skilled in the art that the following specific examples are intended to be

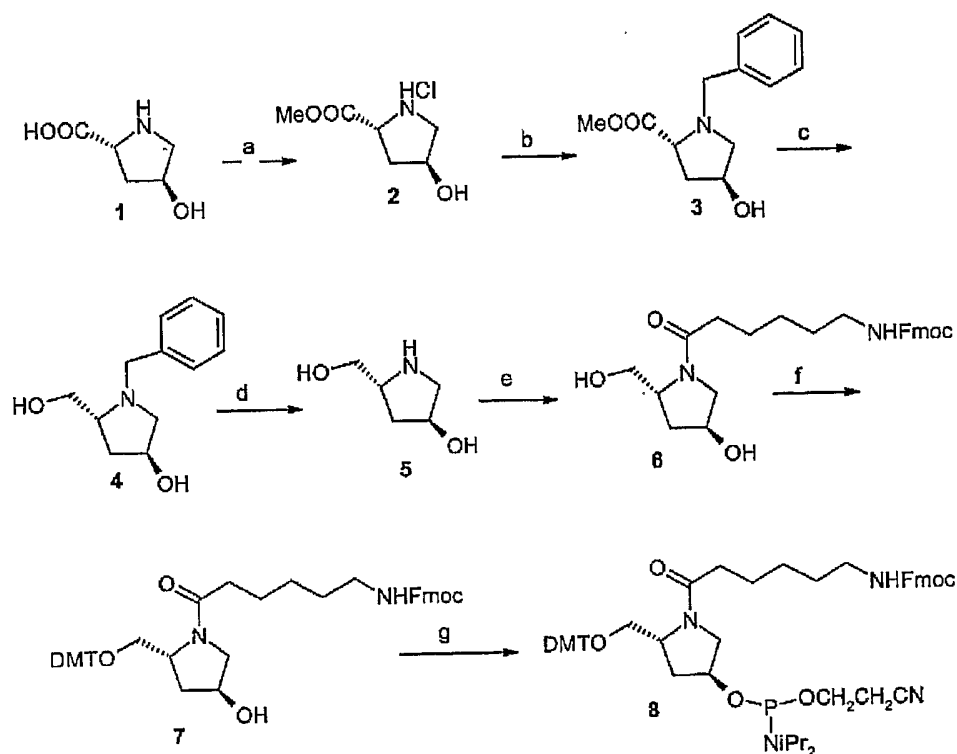
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illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

5 **EXAMPLE I: Synthesis of Linker Tagged with Phosphoramidite**

The following scheme 1 shows a process for preparing the linker tagged with phosphoramidite according to the present invention. In scheme 1, the linker capable of attaching fluorescent material on oligonucleotide can be synthesized and linked either to 3'-end or intra-position of oligonucleotide. Referring to scheme 1, the elaborate process for preparing the linker tagged with phosphoramidite according to the present invention is described as follows:

15 **Scheme 1**



a: MeOH, acetylchloride; b: BnCl, $t\text{Bu}_4\text{NI}$, CH_3CN ; c: LAH,

THF, N₂; d: Pd-C, EtOH, H₂; e: 6-N-Fmoc-ε-aminocaproic acid, HOAT, HBTU, DIPEA; f: DMTCI, DMAP, CH₂Cl₂, TEA; and g: DIPEA, CH₂Cl₂, 2-cyanoethyl-N,N-diisopropylamino chlorophosphoramidite

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Step 1: Synthesis of Trans-4-hydroxy-L-proline methyl ester hydrochloride (2)

Trans-4-hydroxy-L-proline (1; 10 g, 76.3 mmol) was dissolved in 200 ml of methanol, acetyl chloride (7.6 ml, 106.8 mmol) was added therein and the mixture was refluxed with stirring up for overnight. The mixture was cooled to RT (room temperature) and 500 ml of ether was added to form white crystal followed by filtration and drying under vacuum to yield the compound 2 (12.7 g, 87.6 %): ¹H NMR (CDCl₃) δ2.07-2.30 (m, 1H, C3-H), 2.33-2.50 (m, 1H, C3-H), 3.29-3.34 (m, 2H, C2,C5-H), 3.45-3.50 (m, 1H, C5-H), 3.84 (s, 3H, CO₂CH₃), 4.43-4.60 (br, 1H, NH), 4.62-4.72 (m, 1H, C4-H); Mass EI m/z 146.80, 87.00, 68.75.

15

20 Step 2: Synthesis of 1-Benzyl-4-hydroxy-pyrrolidine-2-carboxylic acid methyl ester (3)

The above-prepared compound 2 (12.7 g, 66.6 mmol), K₂CO₃ (26.4 g, 199.8 mmol), BnCl (6.5 ml, 56.6 mmol) and tBu₄NI (cat.) were dissolved with dried CH₂CN (100 ml) and refluxed with stirring up for overnight. The mixture was cooled to RT, diluted with 200 ml of DW, extracted three times with 50 ml of AcOEt and washed with DW. The final concentrate in oil phase was obtained by drying organic

25

phase with MgSO_4 . The concentrate was separated through a column chromatography (MC:MeOH = 20:1/15:1) and colorless oily compound 3 (10.57 g, 67.5%) was yielded: ^1H NMR (CDCl_3) δ 2.03-2.11 (m, 1H, C3-H), 2.20-2.29 (m, 1H, C3-H),
5 2.46 (dd, 1H, $J=3.82, 10.14$ Hz, C5-H), 3.32 (dd, 1H, $J=5.62, 10.14$ Hz, C5-H), 3.60 (m, 1H, C2-H), 3.65 (s, 3H, CO_2CH_3), 3.66 (d, 1H, $J=12.87$ Hz, $\underline{\text{CH}_2}\text{Ph}$), 3.89 (d, 1H, $J=12.87$ Hz, $\underline{\text{CH}_2}\text{Ph}$), 4.44 (m, 1H, C4-H), 7.24-7.32 (m, 5H, Ph); Mass EI m/z 235.20, 176.10, 91.15.

10

Step 3: Synthesis of 1-Benzyl-5-hydroxymethyl-pyrrolidin-3-ol (4)

The compound 3 (11.2 g, 47.6 mmol) was added dropwise into 150 ml of THF containing LAH (2.16 g, 57.12 mmol) for
15 20 min at 0°C under nitrogen atmosphere and the mixture was stirred for 3 hrs at RT. The excess LAH was treated with acetone and DW. The gray precipitate yielded was removed with cellite and the remaining solution was concentrated. The residual water was removed by use of constant boiling
20 point with toluene, the remnant was vacuum-dried to obtain compound 4 (9.54 g, 97.0 %). The compound 4 was employed without further purification in the subsequent reaction:
 ^1H NMR (CDCl_3) δ 1.77-1.90 (m, 1H, C3-H), 2.07-2.22 (m, 1H, C3-H), 2.38 (dd, 1H, $J=4.88, 10.17$ Hz, C5-H), 3.04-3.13 (m, 1H, CH_2OH), 3.24 (dd, 1H, $J=5.70, 10.17$ Hz, C5-H), 3.41 (dd, 1H, $J=2.03, 11.39$ Hz, C2-H), 3.48 (d, $J=13.02$ Hz, $\underline{\text{CH}_2}\text{Ph}$), 3.67 (dd, 1H, $J=3.26, 10.99$ Hz, $\underline{\text{CH}_2}\text{OH}$), 3.99 (d, 1H, $J=13.02$ Hz, $\underline{\text{CH}_2}\text{Ph}$), 4.28-4.80 (m, 1H, C4-H), 7.25-7.33 (m,

25

5H, Ph); Mass EI m/z 207.35, 176.15, 90.85.

Step 4: Synthesis of 5-hydroxymethyl-pyrrolidin-3-ol (5)

The compound 4 (9.54 g, 46.0 mmol) was dissolved in
5 50 ml of absolute ethanol and 10% Pd-C (1.0 g) was added
to, followed by stirring for 5 hrs at 25°C under 40 psi of
H₂ gas. The residual Pd-C was discarded by filtration and
raw compound 5 (4.8 g, 88.9%) was prepared by
concentration under vacuum: ¹H NMR (D₂O) δ1.93-2.14 (m, 1H,
10 C3-H), 2.15-2.25 (m, 1H, C3-H), 3.34-3.40 (m, 1H, C5-H),
3.46-3.54 (m, 1H, C5-H), 3.71-3.81 (m, 1H, CH₂OH), 3.94-
4.02 (m, 1H, CH₂OH), 4.05-4.10 (m, 1H, C2-H), 4.69-4.78 (m,
1H, C4-H).

15 Step 5: Synthesis of 1-N-(N-Fmoc-6-aminohexanoyl)-5-
hydroxy methyl pyrrolidin-3-ol (6)

The compound 5 (0.3 g, 2.5 mmol), 6-N-Fmoc-ε-
aminocaproic acid (0.88 g, 2.5 mmol), 1-hydroxy-7-
azabenzotriazole (HOAT) (0.34 g, 2.5 mmol) and 2-(1H-
20 benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium
hexafluorophosphate (HBTU) (0.95 g, 2.5 mmol) were
dissolved in 1 ml of DMF, diisopropylethyl amine (0.44 ml,
2.5 mmol) was added dropwise therein at 25°C under nitrogen
atmosphere, followed by stirring for 2.5 hrs. The reaction
25 mixture was concentrated under vacuum, dissolved in 100 ml
of chloroform and washed with 5% HCl (50 ml), DW (50 ml)
and NaCl solution (50 ml) consecutively. The organic phase
was dried with MgSO₄, vacuum-dried to prepare oily

residual and 2 ml of ethanol was then added to, followed by standing the resultant in a freezer. The precipitate thus obtained was dried to yield compound 6 (0.98 g, 86.7%): ^1H NMR (CDCl_3) δ 1.27-1.35 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.41-1.50 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.55-1.64 (m, 4H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 2.10 (dd, 1H, $J=7.50$, 13.59 Hz, C3-H), 2.13-2.32 (m, 2H, C3,C5-H), 3.08-3.16 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 3.40-3.49 (m, 3H, C2-H, CH_2ODMT), 3.59-3.65 (m, 1H, C5-H), 4.14 (dd, 1H, $J=6.77$, 13.53 Hz, C4-H), 4.25-4.33 (m, 2H, NHCO_2CH_2), 4.93 (s, 1H, NH), 5.34 (d, 1H, $J=8.21$ Hz, Fmoc), 7.19-7.26 (m, 2H, Fmoc), 7.30-7.35 (m, 2H, Fmoc), 7.50-7.53 (m, 2H, Fmoc), 7.67-7.70 (m, 2H, Fmoc); Mass FAB+ cal. 452.2311 found 453.2390 (+H).

15 Step 6: Synthesis of 1-N-(N-Fmoc-6-aminohexanoyl)-5-O-DMT methyl-pyrrolidin-3-ol (7)

The compound 6 (0.3 g, 0.66 mmol), DMTCl (0.22 g, 0.66 mmol) and DMAP (cat.) were dissolved in 2 ml of absolute CH_2Cl_2 , TEA (0.18 ml, 1.32 mmol) was added thereto at -20°C under nitrogen atmosphere and the mixture was stirred for 4 hrs. The mixture was then treated with methanol, diluted with CH_2Cl_2 (10 ml), rinsed with NaCl solution (10 ml), dried with Na_2SO_4 and concentrated under vacuum. The remnant was separated by column chromatography (50:1 = MC:MeOH, containing TEA 5%) to obtain compound 7 (0.32 g, 64.2%): ^1H NMR (CDCl_3) δ 1.12-1.17 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.20-1.33 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.34-1.48 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.49-1.60 (m, 2H, $\text{CH}_2-\text{CH}_2\text{NHFMoc}$), 1.83-

1.91 (m, 1H, C3-H), 2.09-2.33 (m, 2H, C3,C5-H), 2.99-3.08 (m, 2H, CH₂-CH₂NHFmoc), 3.32-3.36 (m, 1H, C5-H), 3.55-3.60 (m, 1H, C2-H), 3.65-3.66 (brs, 8H, CH₂ODMT, PhOCH₃), 4.01-4.12 (m, 1H, C4-H), 4.26-4.31 (m, 2H, NHCO₂CH₂), 5.16 (s, 1H, Fmoc), 5.20-5.22 (br, 1H, NH), 6.67-6.73 (m, 4H, DMT), 7.05-7.17 (m, 9H, DMT), 7.22-7.29 (m, 4H, Fmoc), 7.48-7.51 (m, 2H, Fmoc), 7.62-7.66 (m, 2H, Fmoc); Mass FAB⁺ cal. 754.3618 found 777.3514 (+Na).

10 Step 7: Synthesis of 1-N-Fmoc-5-O-DMT methyl pyrrolidine-3-O-2-cyanoethyl-N,N-diisopropyl phosphoramidite (8)

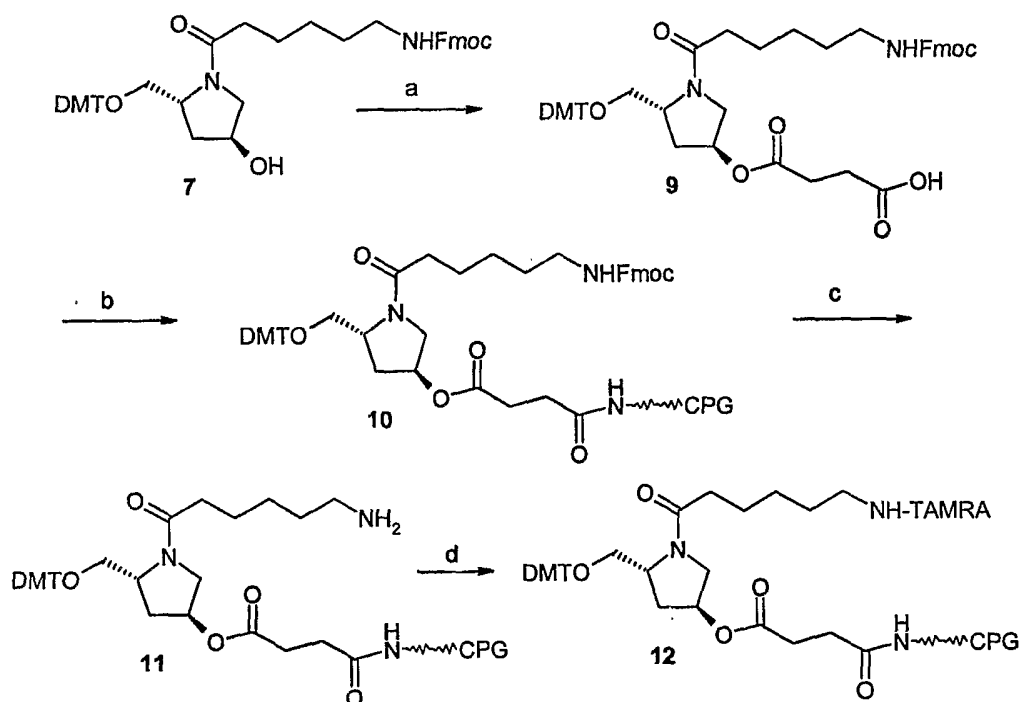
The compound 7 (0.10 g, 0.13 mmol) was dissolved in 1 ml of CH₂Cl₂, diisopropylethylamine (DIPEA; 67 μ l, 0.39 mmol) and 2-cyanoethyl-N,N-diisopropylchloro
15 phosphoramidite (43 μ l, 0.20 mmol) were added and then the mixture was stirred for 1 hr at 0°C and for 2 hrs at RT subsequently. The reactant mixture was treated with methanol and concentrated under vacuum. The remnant was resuspended in AcOEt, rinsed with NaHCO₃ and NaCl solution
20 consecutively, and concentrated under vacuum after desiccation with NaHSO₄. The remnant was separated to obtain compound 8 (0.076 g, 58.8%) through column chromatography (Hex:AcOEt = 3:2, containing TEA 2%): ¹H NMR (CDCl₃) δ 1.10-1.15 (m, 2H, CH₂-CH₂NHFmoc), 1.16 (d, 6H, J=0.9Hz, iPr), 1.18 (d, 6H, J=1.0Hz, iPr), 1.21-1.31 (m, 2H, CH₂-CH₂NHFmoc), 1.30-1.46 (m, 2H, CH₂-CH₂NHFmoc), 1.46-1.57 (m, 2H, CH₂-CH₂NHFmoc), 1.80-1.90 (m, 1H, C3-H), 2.07-2.32 (m, 2H, C3,C5-H), 2.64 (t, 2H, J=5.9Hz, OCH₂CH₂CN),

2.95 (m, 1H, iPr), 2.96-3.07 (m, 2H, CH₂-CH₂NHFmoc), 3.12 (m, 1H, iPr), 3.30-3.34 (m, 1H, C5-H), 3.54-3.59 (m, 1H, C2-H), 3.65-3.66 (brs, 8H, CH₂ODMT, PhOCH₃), 3.75 (m, 2H, OCH₂CH₂CN), 4.00-4.11 (m, 1H, C4-H), 4.24-4.30 (m, 2H, NHCO₂CH₂), 5.15 (s, 1H, Fmoc), 5.20-5.22 (br, 1H, NH), 6.66-6.73 (m, 4H, DMT), 7.05-7.17 (m, 9H, DMT), 7.22-7.29 (m, 4H, Fmoc), 7.48-7.51 (m, 2H, Fmoc), 7.62-7.66 (m, 2H, Fmoc); Mass FAB⁺ cal. 968.4853 found 991.4754 (+Na).

Example II: Synthesis of Linker Attached to CPG

The process for preparing the linker attached to CPG (controlled pore glass) according to the present invention is schematically described in the following scheme 2. According to scheme 2, a linker attached to solid support can be obtained.

Scheme 2



a: succinic anhydride, DMAP, TEA, CH₂Cl₂; b: aminopropyl CPG, HOBT, HBTU, DIPEA; c: acetic anhydride/lutidine-THF, piperidine/DMF; and d: TAMRA NHS ester, acetic anhydride/lutidine-THF

5

Step 1: Synthesis of 1-N-(N-Fmoc-6-aminohexanoyl)-5-O-DMT-pyrrolidine-3-succinate (9)

The compound 7 (0.10 g, 0.13 mmol), succinic anhydride (17 mg, 0.17 mmol) and DMAP (cat.) were dissolved in absolute CH₂Cl₂ (0.5 ml), TEA (18 μ l, 0.13 mmol) was added thereto and the mixture was stirred overnight. The reacted mixture was diluted with 5 ml of CH₂Cl₂ and rinsed with 5% citric acid solution (5 ml) and NaCl solution (5 ml) sequentially. The organic phase was dried with MgSO₄, the residual after concentration under vacuum was separated to yield compound 9 (0.079 g, 71.8 %) through column chromatography (CH₂Cl₂:MeOH = 50:1/20:1, 5% TEA contained): ¹H NMR (CDCl₃) δ 1.38-1.48 (m, 4H, CH₂-CH₂NHFmoc), 1.55-1.66 (m, 4H, CH₂-CH₂NHFmoc), 1.97-2.04 (m, 1H, C3-H), 2.09-2.24 (m, 2H, C3,C5-H), 2.46-2.48 (m, 4H, CH₂CH₂CO₂H), 3.04-3.11 (m, 2H, CH₂-CH₂NHFmoc), 3.38-3.48 (m, 1H, C5-H), 3.54-3.60 (m, 1H, C2-H), 3.69 (brs, 8H, CH₂ODMT, PhOCH₃), 4.13-4.15 (m, 1H, C4-H), 4.28-4.33 (m, 2H, NHCO₂CH₂), 5.18-5.23 (m, 1H, Fmoc), 5.32 (br, 1H, NH), 6.70-6.75 (m, 4H, DMT), 7.10-7.23 (m, 9H, DMT), 7.26-7.30 (m, 4H, Fmoc), 7.50-7.53 (m, 2H, Fmoc), 7.66-7.69 (m, 2H, Fmoc); Mass FAB⁺ cal. 854.3778 found 877.3679 (+Na).

Step 2: CPG Attachment (10)

The compound 9 (0.025 g, 0.029 mmol), aminopropyl CPG (164 mg, 89.2 μ mol/g amine loading, 14.6 μ mol), 1-hydroxybenzotriazole (HOBt) (3.9 mg, 29 μ mol) and 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tertramethyluronium hexafluorophosphate (HBTU) (11 mg, 29 μ mol) were dissolved in absolute DMF (4 ml), diisopropylethylamine (DIPEA) (8.4 μ l, 49.3 μ mol) was added under nitrogen atmosphere, and the mixture was stirred with vibrational stirrer for 3 days. Solid support was rinsed with DMF (5 ml x 3) and CH₃CN (2 ml x 2) sequentially, and dried overnight under vacuum to prepare compound 10 (158 mg, 71.6 μ mol/g).

Step 3: Removal of Fmoc group (11)

With vibrational stirring, the compound 10 (158 mg, 71.6 μ mol/g) was capped in acetic anhydride/lutidine-THF (10 % solution, 3 ml) for 1 hr and washed with CH₃CN (3 ml x 3). Fmoc group was detached by treating residual 4 times with 20% piperidine/DMF (4 ml) for 10 min. The detachment of Fmoc was continuously checked by UV (302 nm) spectrophotometer. The residual was washed with DMF (4 ml x 3) and dried under vacuum, thereby obtaining compound 11 (150 mg, 71.6 μ mol/g, 10.7 μ mol).

Step 4: Synthesis of Compound 12

The compound 11 (150 mg, 71.6 μ mol/g, 10.7 μ mol), TAMRA N-hydroxysuccinimide (NHS) ester (30 μ l, 57 μ mol)

and TEA (15 μ l, 171 μ mol) were stirred for 3 days in absolute DMF (4 ml). The reacted mixture was rinsed with DMF (1 ml x 3) and then CH₃CN (1 ml x 2). The residual was capped in acetic anhydride/lutidine-THF (10%, 2 ml) for 1
5 hr with vibrational stirring, washed with CH₃CN (5 ml x 3) and vacuum-dried for 3 days to obtain the final compound 12 (145 mg, 71.6 μ mol/g, 10.4 μ mol).

In an effort to evaluate the applicability of the
10 oligonucleotide tagged with the linker of the present invention as probe, the probe was applied to assay for quantitative analysis of transformed plants as follows: The plants were transformed with 35S promoter isolated from cauliflower mosaic virus.

15

Experimental Example 1: Isolation of Plant DNA

DNA from several plants including non-transformed Korean native maize, transformed maize (BT-11 maize, Fluka), non-transformed Korean native soybean, transformed
20 soybean (Roundup Ready Soybean, Fluka) and unknown maize or soybean, was isolated respectively using the method described by Edwards K., et al. (*Nucleic Acids Research*, 19: 1349(1991)). Firstly, 200 μ l of extraction buffer (200 mM Tris-Cl, pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS)
25 was added to plant tissue and the tissue was crushed with a pestle. And then, additional 200 μ l of extraction buffer was added and was centrifuged for 10 min. 300 μ l of the

extract were transferred into a tube, 300 μl of chloroform:isoamylalcohol (24:1) were added thereto, then inverted and centrifuged for 5 min. at 13,000 rpm. The supernatant was transferred into a new microtube and mixed
 5 with 300 μl of isopropanol. After centrifugation, the precipitated DNA was rinsed twice with 70% ethanol and resuspended with 50 μl of ddH₂O. Each isolated DNA was diluted to 50 ng/ μl , and serial dilution was performed to indicated in Table 1. The diluents in Table 1 are prepared
 10 as a positive control for real-time RCR in Experimental Example 4.

Since plant DNA sample with known concentration must be highly purified for fidelity of standard curve, DNA samples with 99.99% purity were employed as positive
 15 control in the instant Examples.

Table 1

Content of transformed plant (%)	DNA of transformed plant(50 ng/ μl)	DNA of non-transformed plant(50 ng/ μl)	Final volume
1	10 μl	990 μl	1000 μl
3	30 μl	970 μl	1000 μl
5	50 μl	950 μl	1000 μl
10	100 μl	900 μl	1000 μl
50	500 μl	500 μl	1000 μl

Experimental Example 2: Construction of Primers

For preparing primers for PCR to detect transformed
 20 plant containing the 35S promoter from of cauliflower

mosaic virus, the adequate sequences for primers were selected referring to known sequences (GenBank accession No. AJ251014, X84105, AF044029 and X04879). The known sequences were aligned and the conserved sequences were identified (Fig. 1a and 1b). In Fig. 1a and 1b, TBI251014, Astdnabv, Af044029 and Arcamvpr indicate sequence of GenBank accession No. AJ251014, X84105, AF044029 and X04879, respectively and the shaded sequences adjacent to 3'-end indicate the conserved sequence. The sequence comprising the conserved sequence is shown in SEQ ID NO:1. Considering application to DNAs from various plants, the sequence comprising the conserved sequence was selected as amplified region in PCR. And then, the forward (SEQ ID NO:2) and the reverse primers (SEQ ID NO:3) were synthesized for amplification of the selected region. Other primers hybridizable to the conserved sequence or its proximal region (e.g., SEQ ID Nos:4, 5, 6 and 7) were synthesized and shown to be effective as a primer in Experimental Example 4.

20

Experimental Example 3: Preparation of Probe for Real-Time PCR Using Linker Attached to CPG

To prepare the probe to be employed in PCR of the present invention, the adequate region of template DNA which is hybridized to the probes was selected. The probe was designed in such a manner that it may be located between the forward and reverse primers and more adjacent to the forward primer to evaluate amplified DNA more

rapidly.

All probes employed in Example were synthesized at 1 μ mol scale in trityl-on using Expedite 8909 DNA/PNA Synthesizer (PerSeptive Biosystems) according to instructions of manufacturer. The compound 12 was employed for the incorporation of TAMRA quencher at 3'-end. And for the incorporation of FAM at 5'-end, 5-fluorescein phosphoramidite ([(3',6'-dipivaloylfluoresceinyl)]-6-carboxyamidoethyl)-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite, Glen Research) was used. As nucleotides added, dA^{bz}, dC^{bz}, dG^{dmf} and T phosphoramidite (ABI) were used. The condensation of 5'-end FAM was done for 3 min. whereas the general duration of condensation was 1 min. Solid support and protecting groups were heated in t-butylamine:methanol:DW (1:1:2) at 65°C for 3 hrs, isolated and lyophilized. The residual was dissolved in 1 ml of triethylammonium acetate (TEAA, pH 7, 100 mM) and purified through reverse phase HPLC (Hamilton PRP-1, 300 mm x 7 mm, 18-38% acetonitrile/100 mM TEAA, pH 7, UV monitor at 260 nm and 560 nm). The fraction of interest was lyophilized, dissolved in 1 ml of DW twice, lyophilized again and the residual TEAA salt was removed thoroughly. The remnant was dissolved in 1 ml of DW and the absorbance of UV was measured at 70°C. For calculation of concentration from absorbance, the absorbance of natural nucleotide is as follows: dAMP, 15200: dCMP, 7700: TMP, 8830: dGMP, 11500: FAM, 20958: TAMRA, 31980.

The composition of oligomers was confirmed by HPLC

(Hewlett Packard, ODS hypersil, C-18; 20 mM K₂HPO₄, pH 5.6(A), MeOH(B), 100% A:40% B, 20 min.) and Laser Desorption molecular weight analysis after enzymatic treatment of alkaline phosphatase and snake venom phosphodiesterase. The final nucleotide is 5'FAM-aag gaa
5 agg cca tcg ttg aag atg c-TAMRA-3' (SEQ ID NO:8).

Experimental Example 4: Real-Time PCR Using Probes

Prepared in Experimental Example 3

10 3 PCR reactions per each unknown and standard sample were prepared. The total volume of each PCR reaction was 50 μ l composed of 39.25 μ l cocktail, 0.25 μ l AmpliTaq GoldTM DNA polymerase (5 U/ μ l, Perkin-Elmer), 0.5 μ l AmpErase Uracil N-glycosylase (UNG, 1 U/ μ l, Perkin-Elmer), 10 μ l DNA
15 templates (unknown DNA or DNA for positive control). The composition of the reaction cocktail is: PCR reaction buffer (10x, Roche), MgCl₂ (3.5 mM), dATP (200 μ M), dCTP (200 μ M), dGTP (200 μ M), dUTP (400 μ M), forward primer (300 nM), reverse primer (300 nM) and probe (200 nM). The PCR
20 reaction buffer contains 100 mM Tris/HCl, 400 mM KCl, 15 mM MgCl₂, 10 mM DTT and 5 μ l/ml BSA.

0.25 μ l AmpliTaq GoldTM DNA polymerase and 0.5 μ l AmpErase uracil N-glycosylase (UNG) were freshly mixed in 39.25 μ l PCR cocktail at the brink before PCR. And then,
25 the mixture of PCR cocktail and enzymes was aliquot in each tube with each template DNA. The template DNAs added were positive controls (i.e. 10 μ l of 1, 3, 5, 10 or 50% transformed maize or soybean DNA with concentration of 50

ng/ μ l), negative control (10 μ l of DW, non-transformed maize or soybean DNA with concentration of 50 ng/ μ l) or 10 μ l of unknown DNA A or B with concentration of 50 ng/ μ l, which were obtained in Experimental Example 1.

5 Thereafter, pre-denaturation at 50°C for 2 min. and denaturation at 95°C for 10 min. were done consecutively, and total 40 cycles of PCR was done in which each cycle is composed of the denaturation step at 95°C for 15 sec., annealing and extension step at 64°C for 1 min. Real-time
10 PCR was performed in Rotor Gene 2000 (Corbett) under checking of copy number of amplified template DNA using florescence measurement and the PCR result was also analyzed using the program equipped in the PCR device.

 The real-time PCR results are shown in Figs. 2 and 3.
15 As shown in Fig. 2, the intense of fluorescence is increased in a sigmoidal pattern depending on the PCR cycles, which indicates the primers and probes of the present invention work their functions.

 Fig. 3 shows the standard curve drawn based on the
20 results of the positive and negative controls. As shown in Fig. 3, correlation coefficient of the standard curve is next to 1 (over 0.999) enhancing fidelity of the results on unknown samples. As indicated in Fig. 3, samples A and B are proved to have 0.35% and 0% of transformed plants.
25 The above results are summarized in the following Table 2.

Table 2

No	Sample	Class of sample	Known conc. of transformant (%)	Measured conc. of transformant(%)	C _v	C _t	C _t st. deviation
1	H ₂ O	- control	0	0			
2	1% soy	+ control	1.00	1.00	0.06%	31.45	0.01
3	1% soy	+ control	1.00	1.05	5.49%	31.38	0.01
4	3% soy	+ control	3.00	2.79	6.88%	30.09	0.11
5	3% soy	+ control	3.00	2.86	4.74%	30.06	0.11
6	5% soy	+ control	5.00	5.23	4.56%	29.26	0.03
7	5% soy	+ control	5.00	5.19	3.77%	29.27	0.03
8	10% soy	+ control	10.00	9.71	2.91%	28.44	0.12
9	50% soy	+ control	50.00	50.34	0.69%	26.26	0.09
10	50% soy	+ control	50.00	5.034	0.69%	26.26	0.09
11	Sample A	Unknown	-	0.37		32.78	-
12	Sample A	Unknown	-	0.33		32.92	-
13	Sample B	Unknown	-	0		38.95	-
14	Sample B	Unknown	-	0.01		37.94	-

In Table 1, C_t is threshold cycle number that indicates the number of PCR cycle showing overtly distinguished fluorescence from background.

5

Experimental Example 5: Comparison of Taqman Probe and Probe of the Present Invention in their Efficacy

Each real-time PCR was performed using the probes in Experimental Example 3 and Taqman probe (ABI) in the same manner as Experiment 4 to compare their efficacy.

10

The results of comparison are summarized in Table 3.

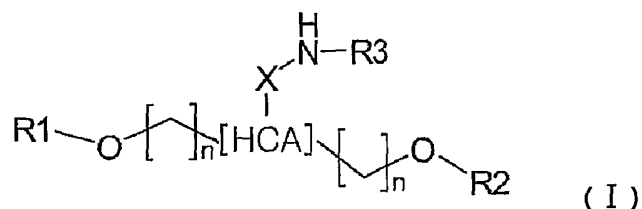
Table 3

Sample	C _t	
	Taqman probe	Present Probe
5% soybean	31.5	31.33
10% soybean	30.67	30.41
50% soybean	28.14	27.87

As shown in Table 3, the probe of the present invention is proven to get better sensitivity compared to Tagman probe. In a result, the probe according to the present invention can be applied to real-time PCR with improved efficacy.

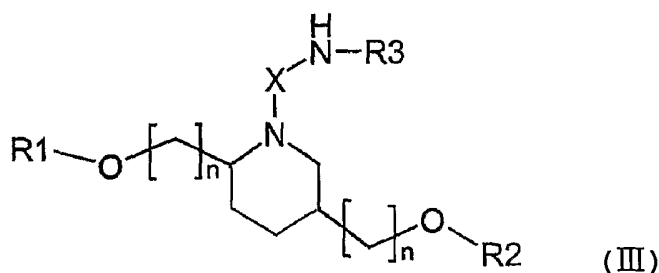
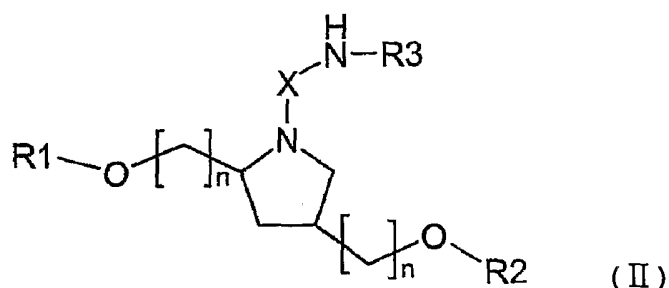
What is claimed is:

1. A linker for oligonucleotide represented by the following formula (I):



5 wherein HCA represents a heterocyclic amine containing 5 or 6 atoms; n is an integer of 0-10; X is linked to nitrogen atom of the heterocyclic amine and represents one selected from the group consisting of saturated C₁-C₁₀ hydrocarbon chain, saturated C₁-C₁₀ hydrocarbon chain in
 10 which carbon atoms are substituted with 1-5 heteroatoms, and saturated C₁-C₁₀ hydrocarbon chain containing amide, ester, ether, amine, sulfonyl or combination thereof; R1 is a hydrogen or hydroxyl-protecting group; R2 is one selected from a group consisting of hydrogen,
 15 phosphoramidite group, H-phosphate and solid support; and R3 is one selected from a group consisting of reporter, quencher, amino-protecting group, amino acid and peptide.

2. The linker for oligonucleotide according to claim 1,
 20 wherein the linker is represented by the following formula (II) or (III):



wherein n is an integer of 0-10; X is linked to
 5 nitrogen atom of the heterocyclic amine and represents one
 selected from the group consisting of saturated C_1 - C_{10}
 hydrocarbon chain, saturated C_1 - C_{10} hydrocarbon chain in
 which carbon atoms are substituted with 1-5 heteroatoms,
 and saturated C_1 - C_{10} hydrocarbon chain containing amide,
 10 ester, ether, amine, sulfonyl or combinations thereof; R_1
 is a hydrogen or hydroxyl-protecting group; R_2 is one
 selected from a group consisting of hydrogen,
 phosphoramidite group, H-phosphate and solid support; and
 R_3 is one selected from a group consisting of reporter,
 15 quencher, amino-protecting group, amino acid and peptide.

3. The linker for oligonucleotide according to claim 1,
 wherein the n is 0-1.

20 4. The linker for oligonucleotide according to claim 1,

wherein the X is C₄-C₈ hydrocarbon chain containing amide group.

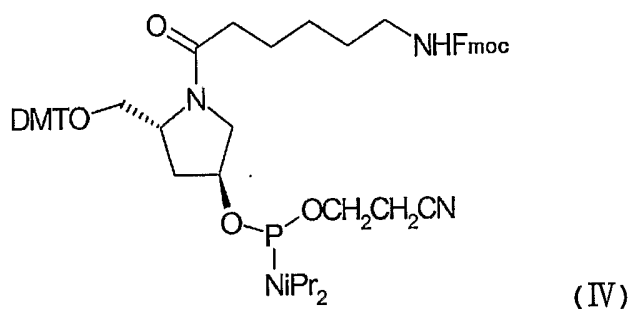
5. The linker for oligonucleotide according to claim 4,
 5 wherein the X is C₆ hydrocarbon chain containing 1 amide group.

6. The linker for oligonucleotide according to claim 1,
 wherein the R₁ is hydroxyl-protecting group.

10

7. The linker for oligonucleotide according to claim 6,
 wherein the R₁ is dimethoxytrityl group.

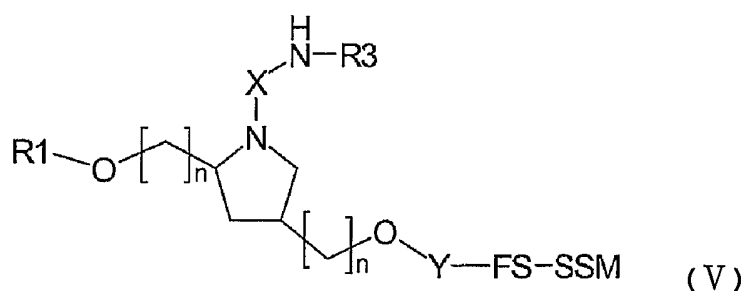
8. The linker for oligonucleotide according to claim 2,
 15 wherein the linker is represented by the following formula
 (IV):



wherein iPr represents isopropyl group.

20

9. The linker for oligonucleotide according to claim 2,
 wherein the linker is represented by the following formula
 (V):



wherein n is an integer of 0-10; X is linked to nitrogen atom of the heterocyclic amine and represents one selected from the group consisting of saturated C₁-C₁₀ hydrocarbon chain, saturated C₁-C₁₀ hydrocarbon chain in which carbon atoms are substituted with 1-5 heteroatoms, and saturated C₁-C₁₀ hydrocarbon chain containing amide, ester, ether, amine, sulfonyl or combinations thereof; R₁ is a hydrogen or hydroxyl-protecting group; Y represents single bond, nucleotide or oligonucleotide; FS represents functional spacer; SSM represents solid support; and R₃ is one selected from a group consisting of amino-protecting group, reporter and quencher.

1/4

Fig. 1a

TBI251014		0
Astlnabv	TTTCAGAAAGAATGCTAACCACAGATGGTTAGAGAGGCTTACGCAGCAGGTCATCA	60
Af044029	TTTCAGAAAGAATGCTAACCACAGATGGTTAGAGAGGCTTACGCAGCAGGTCATCA	60
Arcamvpr		0
Consensus		
TBI251014		0
Astlnabv	GACGATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTCCCAAGAAGGTTAAAGA	120
Af044029	GACGATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTCCCAAGAAGGTTAAAGA	120
Arcamvpr		0
Consensus		
TBI251014		0
Astlnabv	TGCAGTCAAAAGATTTCAGSACTAACTGCATCAAGAACACAGAGAAAGATATATTTCTCA	180
Af044029	TGCAGTCAAAAGATTTCAGSACTAACTGCATCAAGAACACAGAGAAAGATATATTTCTCA	180
Arcamvpr		0
Consensus		
TBI251014		0
Astlnabv	GATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTCACAAACCAAGGCAAGT	240
Af044029	GATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTCACAAACCAAGGCAAGT	240
Arcamvpr		0
Consensus		
TBI251014	GATTCCCATGGAGTCAAA	19
Astlnabv	AATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCCACTGAATCAAAGGCCATGGAGTCAAA	300
Af044029	AATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCCACTGAATCAAAGGCCATGGAGTCAAA	300
Arcamvpr		0
Consensus		
TBI251014	GATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACAGTTCATACAGAG	79
Astlnabv	GATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACAGTTCATACAGAG	360
Af044029	GATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACAGTTCATACAGAG	360
Arcamvpr		0
Consensus		
TBI251014	TCTCTTACGACTCAATGACAAGAGAAAATCTTCGTCAACATGGTGGAGCACCACACGCT	139
Astlnabv	TCTCTTACGACTCAATGACAAGAGAAAATCTTCGTCAACATGGTGGAGCACCACACGCT	420
Af044029	TCTCTTACGACTCAATGACAAGAGAAAATCTTCGTCAACATGGTGGAGCACCACACACT	420
Arcamvpr		0
Consensus		
TBI251014	TGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATIGAGACTTT	199
Astlnabv	TGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATIGAGACTTT	480
Af044029	TGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATIGAGACTTT	480
Arcamvpr		0
Consensus		

2/4

Fig. 1b

TBI251014	TCACAAAGGGTAATATCCGGAAACCTCCTCGGATCCATTGCCAGCTATCTGTCACTT	259
Astdnabv	TCACAAAGGGTAATATCCGGAAACCTCCTCGGATCCATTGCCAGCTATCTGTCACTT	540
Af044029	TCACAAAGGGTAATATCCGGAAACCTCCTCGGATCCATTGCCAGCTATCTGTCACTT	540
Arcamvpr		0
Consensus		
TBI251014	TATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCTACAAATGCCATCATTGCCATAAAGG	319
Astdnabv	TATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCTACAAATGCCATCATTGCCATAAAGG	600
Af044029	TATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCTACAAATGCCATCATTGCCATAAAGG	600
Arcamvpr	GAAAAGGAAGGTGGCACTACAAATGCCATCATTGCCATAAAGG	43
Consensus	Primer-1 tagggaaaaggaaggtggc ctacaaatgccatcattgccataaagg probe	
TBI251014	AAAGGCCATCGTTGAAGATGCCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCAC	...
Astdnabv	AAAGGCCATCGTTGAAGATGCCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCAC	660
Af044029	AAAGGCCATCGTTGAAGATGCCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCAC	660
Arcamvpr	AAAGGCTATCGTTCAAGATGCCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCAC	103
Consensus	aaaggc atcgtt aagatgcctctgccgacagtgggtcccaaagatggacccccacccac	
TBI251014	GAGGAGCATCGTGGAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATG	439
Astdnabv	GAGGAGCATCGTGGAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATG	720
Af044029	GAGGAGCATCGTGGAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATG	720
Arcamvpr	GAGGAGCATCGTGGAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATG	163
Consensus	gaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatg	
TBI251014	TGATA..... Primer-2 ←	444
Astdnabv	TGATACT.....	728
Af044029	TGATACT.....	728
Arcamvpr	TGATACTCCACTGACGTAAGGGATGACGCACAATCCACTATCCTTCGCAAGACCCCTC	223
Consensus	tgatact	
TBI251014	...	444
Astdnabv	...	728
Af044029	...	728
Arcamvpr	CTC	226
Consensus		

Fig. 2

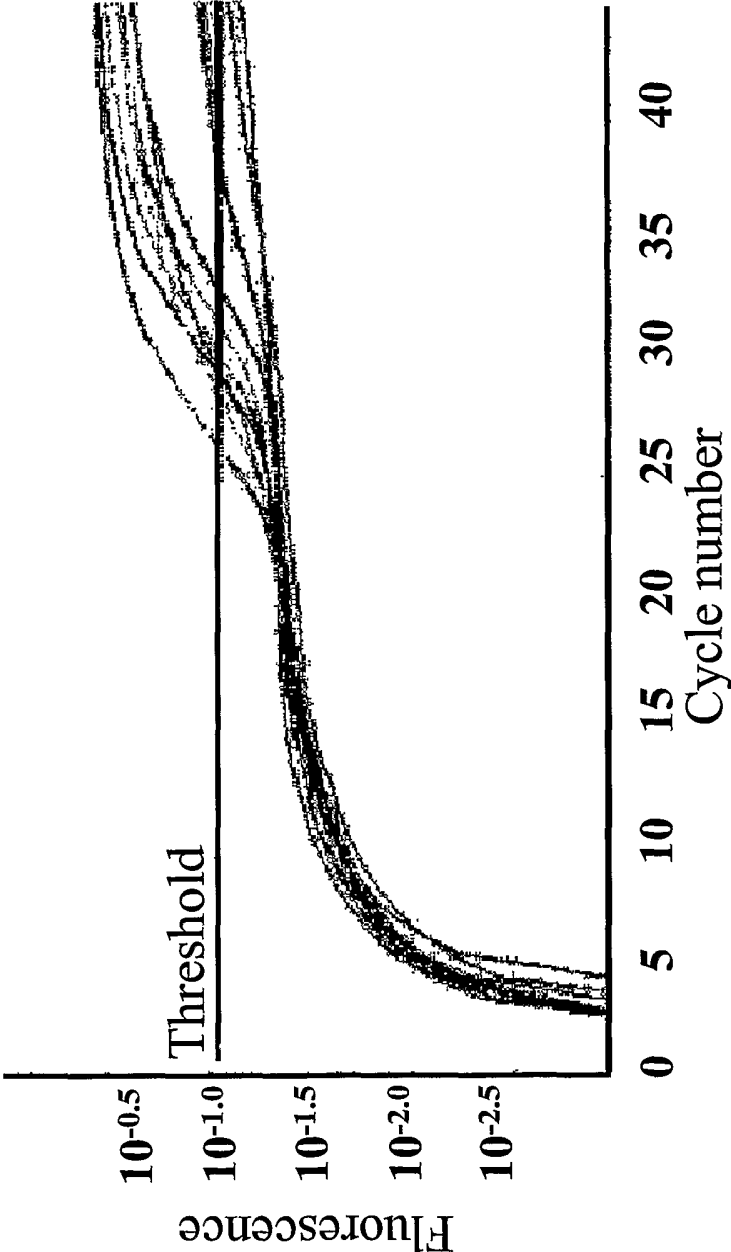
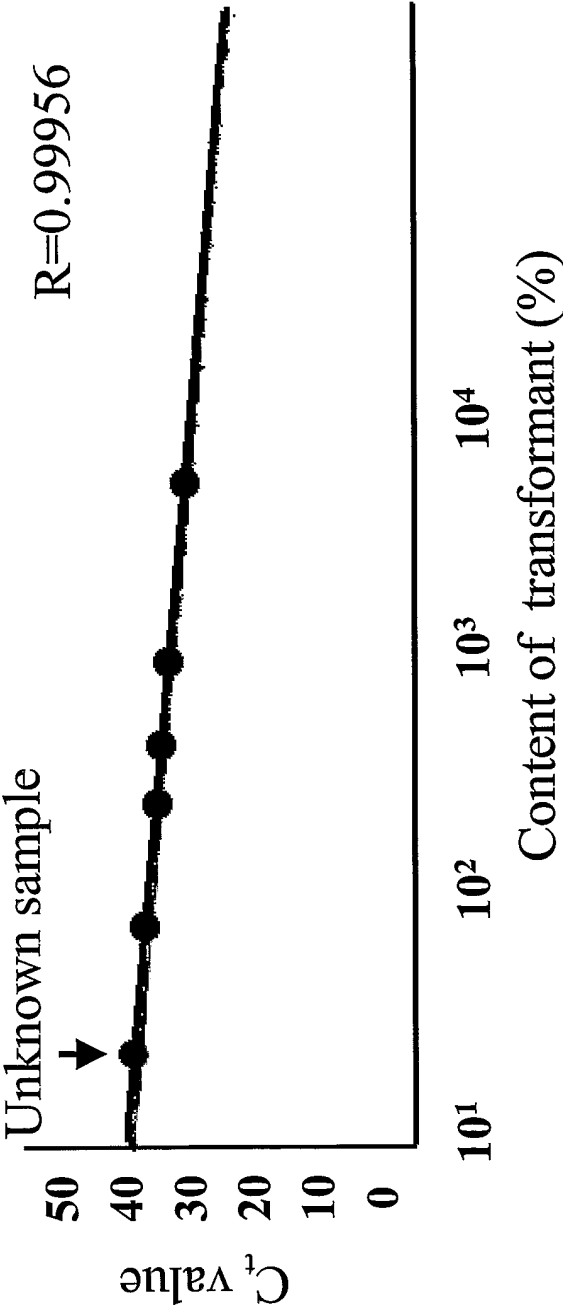


Fig. 3



Sequence Listing

<110> Nexgen Biotechnologies, Inc.

<120> Novel Linker for Oligonucleotides

<130> PCT0125

<150> KR2002-31978

<151> 2002-06-07

<160> 8

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<210> 1

<211> 169

<212> DNA

<213> Cauliflower mosaic virus

<220>

<221> promoter

<222> (1) .. (169)

<223> 3'-terminal sequence of 35S promoter

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gatgcctctg ccgacagtgg tcccaaagat ggacccccac ccacgaggag catcggtgaa 120

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<213> Artificial Sequence

<220>

<223> primer

Sequence Listing

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<210> 3
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<400> 3
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<210> 4
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<212> DNA
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<223> primer

<400> 4
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Sequence Listing

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<220>
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<400> 7
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<210> 8
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<220>
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<400> 8

Sequence Listing

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25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 02/01456

CLASSIFICATION OF SUBJECT MATTER

IPC⁷: C07H 19/10, 19/16, C07D 207/12, 207/08, 211/22, 211/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁷: C07H, C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY, CAPLUS, WPI, EPODOC, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5714606 A (ISIS Pharmaceuticals, Inc., USA) 3 February 1998 (03.02.98) <i>compounds formula I, col. 4, lines 8-50.</i>	1-9
X	<i>examples 13, 48, 64, 72, 91.</i>	1-3
X	US 6316626 B1 (Isis Pharmaceuticals, Inc., USA) 13 November 2001 (13.11.01) <i>compound RN 221138-32-5.</i>	1
X	Swayze, Eric E. "The solid phase synthesis of trisubstituted 1,4-diazabicyclo[4.3.0]nonan-2-one scaffolds: on bead monitoring of heterocycle forming reactions using 15N NMR" Tetrahedron Letters (1997), 38(50), 8643-8646 (abstract). Chemical Abstracts [online] Copyright 2003 American Chemical Society [retrieved on 3 March 2003 (03.03.03)]. Retrieved from STN International, Karlsruhe. Chem. Abstr. No. 1997:790867. <i>compound RN 200623-15-0.</i>	1

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&“ document member of the same patent family

Date of the actual completion of the international search

4 March 2003 (04.03.2003)

Date of mailing of the international search report

26 March 2003 (26.03.2003)

Name and mailing address of the ISA/AT

Austrian Patent Office
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 02/01456

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
US	A	5714606	03-02-1998	CA	AA	2180978	13-07-1995
				EP	A1	739335	30-10-1996
				EP	A4	739335	02-04-1997
				JP	T2	9509408	22-09-1997
				US	A	5519134	21-05-1996
				WO	A1	9518792	13-07-1995
US	BA	6316626	13-11-2001	AU	A1	93831/98	22-03-1999
				WO	A1	9912034	11-03-1999