

TUMOR DETECTION REAGENT AND KIT

TECHINICAL FIELD

The present disclosure belongs to the field of gene detection, and more specifically, the present disclosure relates to a tumor detection reagent and a kit.

BACKGROUND ART

With the development of social economy, the number of patients suffering from tumors is increasing, and the incidence is becoming younger. Early, painless, rapid and accurate detection of tumors has become an increasingly urgent need.

The existing conventional tumor diagnosis and follow-up methods mainly include *in vitro* imaging, *in vivo* microscopy, tissue biopsy, and exfoliative cytology or excremental cytology. *In vitro* imaging techniques mainly include computed tomography, nuclear magnetic resonance imaging, transabdominal ultrasound, *etc.*, and these techniques often have a high false positive rate in the early diagnosis of cancer. *In vivo* microscopy, as the golden standard for the diagnosis of some tumors, has been improved and uses soft materials in recent years, but it is still highly invasive and brings great pain to patients, who will suffer from pain, bleeding, and other problems within few days after detection. Furthermore, *in vivo* microscopy has low specificity and sensitivity for the early diagnosis of some tumors. Tissue biopsy is used to detect suspected diseased tissues and mainly used to detect the morphology of tumor cells and biomarkers, with high specificity and sensitivity. However, tissue biopsy is somewhat invasive to patients during sampling, and has defects such as long sample pre-treatment time and complicated steps. Exfoliative cytology or excremental cytology is a non-invasive test, and therefore is widely applied to the diagnosis of several tumors. However, some exfoliative cytology or excremental cytology tests cannot exclude the presence of low-grade tumors, and result in low sensitivity. Detection methods, such as nuclear matrix protein-22, tumor-associated antigens, Immuno Cyt assay, and Uro Vysion assay, are not applied to routine clinical testing due to their low sensitivity and/or specificity.

Epigenetics is a rapidly growing field in cancer biology and holds great potential for clinical and translational medicine research. Studies have found that biochemical pathways critical for tumorigenesis are partially regulated by epigenetic phenomena, such as changes in DNA methylation in tumor cells, abnormal histone modifications, miRNA-mediated silencing of various target genes, and reconstruction of hamartomatous nucleosomes. Aberrant DNA methylation is the most extensively studied epigenetic change associated with all types of human cancer. Hypermethylation silencing transcription factors, such as RUNX3, GATA-4, and GATA-5, cause the inactivation of their downstream targets involved in various cellular processes. RUNX3 is an important member of a family of transcription factors, and studies have revealed that in lung cancer cell lines and primary lung cancer specimens, RUNX3 is inactivated by aberrant DNA hypermethylation. Similarly, the GATA family of transcription factors is associated with the pathogenesis of gastrointestinal diseases, and it is observed that among promoters of colorectal cancer, promoter regions of genes GATA-4 and GATA-5 are frequently methylated. Promoters of some genes in bladder cancer are also highly methylated with the frequency of DNA methylation of 48%-96%, which include genes such as A2BP1, NPTX2, POU4F2, HOXA9, MEIS1, GDF15, TMEFF2, VIM, STK11, MSH6, BRCA1, TBX2, TBX3, GATA2, ZIC4, PAX5A, MGMT, and IGSF4^[1].

In recent years, detection of the methylation of a specific region of a tumor-associated gene in a blood, sputum, saliva, faeces or urine sample has been widely applied to aspects such as the diagnosis and early diagnosis of cancer, the prediction of the progress of cancer, the prediction of the prognosis of cancer, post-treatment monitoring, and the prediction of response to anticancer therapy.

SUMMARY

In some embodiments, the present disclosure provides use of a nucleotide sequence (hereinafter “*nucleotide sequence*” or “*nucleic acid fragment*”) that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96%

or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in preparation of a tumor detection reagent or a kit.

In some embodiments, the present disclosure provides use of a reagent for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in preparation of a tumor detection reagent or a kit.

In some embodiments, the present disclosure also provides a primer, which includes a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 46, or SEQ ID NO: 47, or complementary sequences thereof.

In some embodiments, the primer includes multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 37 and SEQ ID NO: 38, SEQ ID NO: 40 and SEQ ID NO: 41, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47.

In some embodiments, the primer includes multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47.

In some embodiments, the primer includes multiple nucleotide sequences that respectively

have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of a primer pair shown as SEQ ID NO: 43 and SEQ ID NO: 44.

In some embodiments, the present disclosure also provides a probe, which includes a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 39, SEQ ID NO: 42, SEQ ID NO: 45, or SEQ ID NO: 48, or complementary sequences thereof.

In some embodiments, the probe includes a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 36, SEQ ID NO: 45, or SEQ ID NO: 48, or complementary sequences thereof.

In some embodiments, the probe includes a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a sequence shown as SEQ ID NO: 45.

In some embodiments, the primer or probe is an isolated primer or probe.

In some embodiments, the present disclosure also provides use of the abovementioned primer and/or probe in preparation of a tumor detection reagent or a kit.

In the present disclosure, the term “*detection*”, is synonymous with *diagnosis*, includes not only early, but also mid and late diagnosis of tumors, and also includes tumor screening, risk assessment, prognosis, disease identification, diagnosis of disease stages, and selection of therapeutic targets.

In an optional embodiment in a disease stage, diagnosis is available by detecting the degree

of methylation of the nucleotide sequence in a sample according to the progression of a tumor in different stages or periods. A specific tumor stage of a sample can be detected by comparing the degree of methylation of the nucleotide sequence isolated from tumor samples in different stages to the degree of methylation of the nucleotide sequence of one or more nucleic acids isolated from a sample without abnormal cell proliferation.

In some embodiments, the present disclosure provides a tumor detection reagent, which includes a reagent for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

Methylation refers to addition of a methyl group to cytosine. After being treated with a hydrosulfite or a bisulfite or a hydrazine salt, cytosine is transformed into uracil. Because uracil is similar to thymine, it is recognized as thymine during PCR amplification. Thus, in a PCR-amplified sequence, unmethylated cytosine is transformed into thymine (C is transformed into T), and methylated cytosine (C) is not transformed. MSP is the commonly used PCR technique for detecting gene methylation, in which primers are designed for a treated methylated fragment (i.e., untransformed C in the fragment), and then PCR amplification is performed. If the fragment is amplified, it is indicated that the fragment is methylated, and if the fragment is unamplified, it is indicated that the fragment is unmethylated.

In some embodiments, the methylation level detection reagent is used to detect a sequence modified with a hydrosulfite or a bisulfite or a hydrazine salt of the nucleotide sequence.

In some embodiments, a sequence modified with a bisulfite of the nucleotide sequence is detected.

In some embodiments, the methylation level detection reagent includes primers and a probe for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at

least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

In some embodiments, a forward primer of the primers has any one of the following nucleotide sequences:

I. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with nucleotide sequences shown as SEQ ID NO: 31, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO: 43, or SEQ ID NO: 46; or

II. complementary sequences of the sequences shown in I.

In some embodiments, a reverse primer of the primers has any one of the following nucleotide sequences:

III. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with nucleotide sequences shown as SEQ ID NO: 32, SEQ ID NO: 35, SEQ ID NO: 38, SEQ ID NO: 41, SEQ ID NO: 44, or SEQ ID NO: 47; or

IV. complementary sequences of the sequences shown in III.

The primers are used to amplify the nucleotide sequence. It is well known in the art that the successful design of primers is critical for PCR. Compared with common PCR, the design of primers is more critical in methylation level detection. The reason is that methyl sulfurization induces the transformation of “C” in a DNA strand to “U”, resulting in reduction of the GC content and the presence of long contiguous “T” in a sequence after PCR. It is easy to cause DNA strand breakage, and thus it is difficult to select stable primers with appropriate T_m values. On the other hand, in order to distinguish sulfurized DNA from unsulfurized and incompletely treated DNA, primers need to have a sufficient number of “C”, which increases the difficulty of selecting stable primers. Therefore, in DNA methylation level detection, selection of a fragment to be amplified with primers, such as the length and position of the fragment to be amplified,

selection of primers, *etc.* have an influence on the detection sensitivity and specificity. By experiments, the inventors find that different target fragments to be amplified and primers make a difference in detection results. Many times, some genes or nucleotide sequences have been found to be differentially expressed in tumors and non-tumors, but there is a long way to transform these genes into tumor markers and apply the tumor markers clinically. The main reason is the limitation of detection reagents, which makes it difficult for the detection sensitivity and specificity of these potential tumor markers to meet the requirements of detection, or a detection method is complicated in operation and high in cost, and is difficult to be applied clinically on a large scale.

In some embodiments, the probe has any one of the following nucleotide sequences:

V. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with nucleotide sequences shown as SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 39, SEQ ID NO: 42, SEQ ID NO: 45, or SEQ ID NO: 48; or

VI. complementary sequences of the sequences show in V.

In some embodiments, the reagent includes a reagent for detecting a reference gene.

In some embodiments, the reference gene is β -actin (ACTB).

In some embodiments, the reagent for detecting the reference gene is primers and a probe for the reference gene.

In some embodiments, the reagent also includes at least one of a hydrosulfite, a bisulfite, and a hydrazine salt for modifying the nucleotide sequence, and of course, the reagent may not include the hydrosulfite, the bisulfite or the hydrazine salt.

In some embodiments, the reagent includes one or more of a DNA polymerase, dNTPs, Mg^{2+} ions, and a buffer; and optionally, the reagent includes a PCR reaction system that includes a DNA polymerase, dNTPs, Mg^{2+} ions, and a buffer and is used for amplifying a modified nucleotide sequence.

A sample to be detected with the detection/diagnosis reagent of the present disclosure may be selected from at least one of tissue, body fluid, and excrement.

Optionally, the tissue is bladder tissue.

Optionally, the body fluid is at least one of blood, serum, plasma, extracellular fluid, tissue fluid, lymph fluid, a cerebrospinal fluid, and an aqueous humour.

Optionally, the excrement is selected from at least one of sputum, urine, saliva, and faeces.

Optionally, the excrement is selected from urine.

In some embodiments, the present disclosure also provides a kit, which includes the above tumor detection reagent. In some embodiments, the kit also includes instructions. In some embodiments, the kit also includes a nucleic acid extraction reagent. In some embodiments, the kit also includes a sampling apparatus.

In some embodiments, tissue to be detected with the detection reagent of the present disclosure is selected from tumor tissue and para-carcinoma normal tissue (or benign tumor tissue).

In some embodiments, the present disclosure also provides a method for detecting a methylation level of the nucleotide sequence in a sample, characterized by including the following steps: (1) treating a sample to be detected with a hydrosulfite, a bisulfite, and a hydrazine salt to obtain a modified sample to be detected; (2) detecting a methylation level of the nucleotide sequence, for example, using the above reagent or kit to detect the methylation of the nucleotide sequence in the sample to be detected that is modified at step (1). In an optional embodiment, at step (2), real-time fluorescence quantitative methylation-specific polymerase chain reaction is used for detection.

In some embodiment, the present disclosure also provides a tumor detection method, which includes: detecting a methylation level of a nucleotide sequence in a sample to be detected, optionally, by the above method for detecting a methylation level of a nucleotide sequence; and indicating whether a subject has or is at risk of having a tumor according to the deviation,

optionally, a methylation level difference, of the methylation level detected, optionally, by the above method for detecting a methylation level of a nucleotide sequence, from a corresponding methylation level in a normal control sample. The term “*deviation*” in the above steps refers to the deviation of a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

In some embodiments, the present disclosure also provides a method for treating a tumor in a subject, which includes: detecting a tumor in a subject, including detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in a sample to be detected from the subject, and treating the tumor in a case that the detection of the tumor in the subject indicates that the subject has or is at risk of having the tumor. Optionally, the methylation level is detected by the above method for detecting a methylation level of a nucleotide sequence. Optionally, the tumor in the subject is detected by the above tumor detection method. Optionally, the treatment is the administration of surgery, chemotherapy, radiotherapy, chemoradiotherapy, immunotherapy, oncolytic virus therapy, any other kind of tumor treatment method used in the art, or a combination of these treatment methods.

In some embodiments, the present disclosure also provides a method for designing primers, which includes steps of designing primers for a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

In some embodiments, the present disclosure also provides a system for designing primers, which includes:

1) an input component; 2) a processing component; and 3) an output component. Optionally, the input component is configured to read a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4. Optionally, the processing component is loaded with a program for designing primers based on information read by the input component. Optionally, the output component is configured to output primer sequences designed by the processing component.

In some embodiments, the present disclosure also provides a tumor detection system. The system includes: (1) a component for detecting a methylation level of a nucleotide sequence that at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4, and (2) a result determination system.

In some embodiments, the methylation level detection component includes the above detection reagent or kit.

In some embodiments, the result determination component is configured to output the risk of having a tumor and/or a tumor type according to a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 that is detected by the detection system.

In some embodiments, the risk of having a tumor is determined by comparing a methylation level of a sample to be detected to a methylation level of a normal sample, and it is determined that the sample to be detected has a high risk of having a tumor if there is a significant difference or an extremely significant difference between the methylation level of the sample to be detected and the methylation level of the normal sample.

In some embodiments, if the methylation of the nucleotide sequence is positive, it is indicated that a donor of the sample to be detected is a patient with a tumor or at high risk of

having a tumor. In an optional embodiment, the positive refers to that when a detection result of a sample to be detected is compared to a detection result of a normal sample, if there is a significant difference or an extremely significant difference between an amplification result of the sample to be detected and an amplification result of the normal sample, a donor of the sample to be detected is positive.

In some embodiments, determination criteria of the determination system include: whether a specimen is a tumor specimen or a normal specimen is determined according to a cut-off value.

In some embodiments, the tumor is selected from urothelial tumors. Optionally, the tumor is selected from bladder cancer, ureteral cancer, renal pelvis cancer, and urethral cancer. Optionally, the tumor is selected from bladder cancer.

The detection method of this application can be used before and after tumor treatment or used in combination with tumor treatment. After treatment, the detection method is used to, for example, evaluate whether the treatment is successful, monitor the relief, relapse and/or progress (including metastasis) of tumors after treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A to 1F respectively show ROC curves of detection of nucleic acid fragments 1 to 6 in 108 clinic tissue specimens (including 66 bladder cancer tissue specimens and 42 bladder cancer para-carcinoma tissue specimen);

Fig. 2 shows ROC curves of detection of the nucleic acid fragment 4 and MEIS1 in 97 urine samples (including 45 bladder cancer samples and 52 control samples);

Fig. 3 shows statistical results of detection of the nucleic acid fragment 4 in different types of tumor samples including 299 urine samples (including 1 low-malignant-potential inverted urothelial tumor sample, 16 low-malignant-potential papillary urothelial tumor samples, 105 bladder cancer samples, 31 prostate cancer samples, 17 renal pelvic cancer samples, 10 ureteral cancer samples, and 119 control samples) of different types of tumors with the nucleic acid fragment 4, wherein Fig. 3A shows comparison of ROC curves of a control group and a “bladder

cancer & ureteral cancer & renal pelvic cancer” group; Fig. 3B shows comparison of ROC curves of a control group and a ureteral cancer group; Fig. 3C shows comparison of ROC curves of a control group and a renal pelvic cancer group; Fig. 3D shows comparison of ROC curves of a control group and a bladder cancer group; Fig. 3E shows comparison of ROC curves of a control group and a “low-malignant-potential inverted urothelial tumor & low-malignant-potential papillary urothelial tumor” group; and Fig. 3F shows comparison of ROC curves of a control group and a prostate cancer group;

Fig. 4 shows amplification curves and melting curves of different primer and probe sets; and

Fig. 5 shows ROC curves of detection of CG441 in 193 urine samples.

DETAILED DESCRIPTION

The technical solutions of the present disclosure will be further described below with reference to specific examples, and the specific examples are not intended to limit the scope of protection of the present disclosure. Some nonessential modifications and adjustments made by others on the basis of the idea of the present disclosure shall still fall within the scope of protection of the present disclosure.

In this application, a “*primer*” or a “*probe*” refers to an oligonucleotide, which includes a region complementary to a sequence of at least 6 contiguous nucleotides in a target molecule (e.g., a target nucleic acid fragment). In some embodiments, at least a portion of the primer or probe sequence is not complementary to an amplified sequence. In some embodiments, the primer or probe includes a region complementary to a sequence of at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19 or at least 20 contiguous nucleotides in the target molecule. In a case that the primer or probe includes a region “complementary to at least x contiguous nucleotides in the target molecule”, the primer or probe is at least 95% complementary to at least x contiguous or discontinuous block nucleotides in the target molecule. In some embodiments, the primer or probe is at least

80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% complementary to the target molecule.

In this application, a “*normal*” sample refers to the same type of sample isolated from an individual known to be free of the cancer or tumor.

In this application, samples for methylation level detection include, but are not limited to, DNA, RNA, mRNA-containing DNA and RNA samples, and DNA-RNA hybrids. DNA or RNA may be single-stranded or double-stranded.

In this application, a “*subject*” is a mammal, such as a human.

In this application, the term “*methylation level*” is synonymous with “the degree of methylation”, and is usually expressed as the percentage of methylated cytosine, which is calculated by the number of methylated cytosine divided by the sum of the number of methylated cytosine and the number of unmethylated cytosine. At present, a methylation level is generally calculated by the number of methylated target genes divided by the number of reference genes, or calculated by other formulas in other prior arts.

In this application, the term “*sample*” is synonymous with “*specimen*”.

As used in this application, the term “*and/or*” refers to and covers any and all possible combinations of one or more of the associated listed items. When used in a list of two or more items, the term “*and/or*” means that any one of the listed items can be used alone or any combination of two or more of the listed items can be used. For example, if a composition, combination, construction, *etc.* is described as including (or containing) components A, B, C and/or D, the composition may include A alone; include B alone; include C alone; include D alone ; include a combination of A and B; include a combination of A and C; include a combination of A and D; include a combination of B and C; include a combination of B and D; include a combination of C and D; include a combination of A, B, and C; include a combination

of A, B, and D; include a combination of A, C, and D; include a combination of B, C, and D; or include a combination of A, B, C, and D.

Example 1

The inventors screened hundreds of gene markers and nucleic acid fragments to study the distribution of methylated sites of genes.

Six nucleic acid fragments screened out by the inventors and screening results are listed herein.

Sequences of nucleic acid fragments 1 to 6 are respectively shown as SEQ ID NO: 1 to SEQ ID NO: 6.

Experimental process:

UM-UC-3, J82, SW780, T24, RT4, 5637, SCaBER, UM-UC-3, and J82 cell lines were obtained from the National Infrastructure of Cell Line Resource, and other cell lines were purchased from ATCC. All the cell lines were resuscitated in recommended media. All the cell lines were negative in mycoplasma detection, and had normal cell morphology. After being expanded, cells were collected, subpackaged at a density of 5×10^6 cells/tube, and stored in a cryogenic refrigerator at -80°C for DNA extraction.

1) DNA extraction

DNA was extracted from 17 bladder cancer cell lines by using DNA extraction kits (QIAGEN DNA Mini Kit, #51306) purchased from QIAGEN.

2) DNA modification

The DNA was modified with a sulfite by using a DNA transformation kit (EZ DNA Methylation Kit, D5002) purchased from ZYMO RESEARCH.

The modified sequences are shown in Table 1.

Table 1

Original fragment	After modification
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Nucleic acid fragment 1 (SEQ ID NO: 1)	SEQ ID NO: 25
Nucleic acid fragment 2 (SEQ ID NO: 2)	SEQ ID NO: 26
Nucleic acid fragment 3 (SEQ ID NO: 3)	SEQ ID NO: 27
Nucleic acid fragment 4 (SEQ ID NO: 4)	SEQ ID NO: 28
Nucleic acid fragment 5 (SEQ ID NO: 5)	SEQ ID NO: 29
Nucleic acid fragment 6 (SEQ ID NO: 6)	SEQ ID NO: 30

3) Amplification and detection

Primers and probes for determining whether the nucleic acid fragments are methylated are shown in Table 2.

Table 2 Primer and probe sequences for nucleic acid fragments

Nucleic acid fragment	Primer and probe sequences
ACTB (reference gene)	F: TTTTGGATTGTGAATTTGTG (SEQ ID NO: 49) R: AAACCTACTCCTCCCTTAAA (SEQ ID NO: 50) P: TTGTGTGTTGGGTGGTGGTT (SEQ ID NO: 51)
Nucleic acid fragment 1	F: CGAGAGGTTATATAAGTTTACG (SEQ ID NO: 7) R: AATTTCTTAACAAATAATTTCCG (SEQ ID NO: 8) P: CGCCCGAAAACGTATAAATTACTCC (SEQ ID NO: 9)
Nucleic acid fragment 2	F: TTTAAGGATTAATAATAGAGCG (SEQ ID NO: 10) R: GAACCGCGTAATTAAAAC (SEQ ID NO: 11) P: CTAACCTCTCGCCGTACCGAATC (SEQ ID NO: 12)
Nucleic acid fragment 3	F: GGACGGAGATATAAGGAT (SEQ ID NO: 13) R: ATAAACGAACACCAAATC (SEQ ID NO: 14) P: CGCAACCCCATAAAACCCAA (SEQ ID NO: 15)
Nucleic acid fragment 4	F: GTAGTCGAGAAGTATTCG (SEQ ID NO: 16) R: GATAAACGTACACTTAACG (SEQ ID NO: 17)

	P: ATCCTTCCAACGACAATAACG (SEQ ID NO: 18)
Nucleic acid fragment 5	F: TTCGTTTATTTATGGATTACG (SEQ ID NO: 19) R: CGTAAATCGACTCCTTATAC (SEQ ID NO: 20) P: AACAAACGACGACCGAACGAT (SEQ ID NO: 21)
Nucleic acid fragment 6	F: GAAAATAATGTCGAATTTCGT (SEQ ID NO: 22) R: AAAATCGAAAATCCCACG (SEQ ID NO: 23) P: CTCGAACAAACGTCTCCTTAAC (SEQ ID NO: 24)

A reaction system is shown in Table 3.

Table 3

Reaction component	Addition amount (μ L)
iTaq Universal SYBR Green supermix (2 $\times$)	10
Primers (F+R, 12.5 μ M)	0.2
Nuclease-free Water	8.8
Template DNA	1
Total volume	20

An amplification protocol is shown in Table 4.

Table 4

Step	Temperature and time	Number of cycles
Pre-denaturation	95°C for 10 min	1
Amplification	95°C for 30 s	45
	55°C for 30 s	
	72°C for 30 s, collect fluorescence	
Melting curve	95°C for 5 s	1

	65°C for 60 s, continuously collect fluorescence until 97°C	
Cooling	40°C for 3 min	1

After detection was completed, the number of copies of each nucleic acid fragment quantitative system in each cell line was quantitatively calculated by using the standard curve, and the degree of methylation of each nucleic acid fragment in 17 bladder cancer cell lines was calculated by a formula of “the number of copies of a nucleic acid fragment ÷ the number of copies of reference gene ACTB × 100”.

Methylation levels of the 6 nucleic acid fragments in the 17 bladder cancer cell lines were detected. Detection results are shown in Table 5.

Table 5 Detection results of methylation levels of nucleic acid fragments in 17 bladder cancer cell lines

Cell line	Nucleic acid fragment 1	Nucleic acid fragment 2	Nucleic acid fragment 3	Nucleic acid fragment 4	Nucleic acid fragment 5	Nucleic acid fragment 6
BFTC-905	14%	34%	56%	58%	73%	55%
CAL-29	57%	59%	81%	51%	39%	50%
SCABER	10%	8%	3%	90%	83%	95%
VM-CUB-1	88%	87%	87%	87%	56%	97%
RT-112	78%	71%	83%	76%	66%	84%
SW780	44%	51%	62%	72%	75%	80%
DSH1	89%	85%	78%	92%	54%	95%
RT4	90%	83%	86%	74%	80%	92%
SW1710	86%	67%	81%	70%	58%	77%
639-V	35%	62%	91%	85%	76%	95%
UM-UC-3	92%	83%	85%	89%	88%	84%
LB831-BLC	78%	81%	89%	82%	71%	72%
KU-19-19	56%	68%	84%	60%	63%	34%

647-V	13%	50%	83%	83%	60%	85%
5637	1%	5%	36%	84%	86%	98%
J82	79%	80%	76%	74%	35%	80%
T-24	62%	83%	87%	76%	55%	83%

It can be seen from Table 5 that in the 17 bladder cancer cell lines, the 6 nucleic acid fragments are methylated to different extents, among which, the nucleic acid fragments 3, 4, and 6 are methylated in more cell lines compared with other fragments.

Example 2

The sensitivity and the specificity of each nucleic acid fragment of Example 1, to 108 clinic tissue specimens (including 66 bladder cancer tissue specimens and 42 bladder cancer para-carcinoma tissue specimens), were detected.

Experimental process:

1) DNA extraction

DNA was extracted from each tissue slice specimen by using a DNA extraction kit (HiPure FFPE DNA Kit, D3126-03) purchased from Magen.

2) DNA modification, amplification, and detection, and primers and probes for the fragments were the same as those in Example 1.

3) Calculation method

After detection was completed, the number of copies of each nucleic acid fragment quantitative system in each cell line was calculated by using the standard curve, and the degree of methylation of each nucleic acid fragment in each tissue specimen was calculated by a formula of “*the number of copies of a nucleic acid fragment* ÷ *the number of copies of reference gene ACTB* × 100”. Finally, a threshold was selected as a criterion for distinguishing a cancer group from a control group. If a converted ratio is greater than the set threshold, the methylation is determined as positive, and if the converted ratio is equal to or less than the set threshold, the

methylation is determined as negative. According to the criterion, statistical results of detection of the 6 nucleic acid fragments in the 108 clinic tissue samples are shown in Table 6, and ROC curves are shown in Fig. 1A to Fig. 1F.

Table 6 Detection results of 6 nucleic acid fragments in clinic tissue samples

Fragment	Sensitivity	Specificity	AUC (P<0.001)
Nucleic acid fragment 1	65.2	80.5	0.783
Nucleic acid fragment 2	77.3	83.3	0.854
Nucleic acid fragment 3	81.8	90.5	0.915
Nucleic acid fragment 4	84.8	92.2	0.925
Nucleic acid fragment 5	63.6	91.1	0.773
Nucleic acid fragment 6	78.8	92.9	0.896

The results show that different fragments have differences in the sensitivity and the specificity to the tissue samples. Among them, the sensitivity and the specificity of the nucleic acid fragments 3 and 4 are higher than those of other fragments.

Example 3

The nucleic acid fragment 4 of the present disclosure was compared to bladder cancer gene methylation markers MEIS1, NKX6-2, OTX1, SIM2, SOX1, BARHL2, ZNF154, and RUNX3 that were often reported in documents.

Twenty paraffin-embedded bladder tissue samples including 10 bladder cancer samples and 10 cystitis glandularis control samples were taken.

Experimental process:

1) DNA extraction

DNA was extracted from each tissue slice by using a DNA extraction kit (HiPure FFPE DNA Kit, D3126-03) purchased from Magen.

2) DNA modification

The DNA was modified with a sulfite by using a DNA transformation kit (EZ DNA Methylation Kit, D5002) purchased from ZYMO RESEARCH.

3) Amplification and detection

A reaction system and an amplification protocol were the same as those in Example 1.

Primer sequences are shown in Table 7.

Table 7

Name	Sequence
ACTB (reference gene)	Forward primer: TGGTGATGGAGGAGGTTTAGTAAGT (SEQ ID NO: 52) Reverse primer: AACCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO: 53)
Nucleic acid fragment 4	Forward primer: GCGTTCGGAGTTGTTTAGC (SEQ ID NO: 54) Reverse primer: CACCGACGCCACAAACG (SEQ ID NO: 55)
MEIS1	Forward primer: GTTCGGGATAAGATTTTCGGGG (SEQ ID NO: 56) Reverse primer: TAATTAAAACTACGCAACCCGACT (SEQ ID NO: 57)
NKX6-2	Forward primer: AGAAGAAGTATTCGCGTTCGAT (SEQ ID NO: 58) Reverse primer: GATCATACCCAACGAATAAACG (SEQ ID NO: 59)
OTX1	Forward primer: GTTAGTAGTAGTAGAGCGGGAGC (SEQ ID NO: 60) Reverse primer: GACGTAAATTAACCACTACTTTTCG (SEQ ID NO: 61)
SIM2	Forward primer: GTAGGCGTAGAGGGGATAATTCG (SEQ ID NO: 62) Reverse primer: ACCCCGCGCTAAATCTACAAC (SEQ ID NO: 63)
SOX1	Forward primer: TAATTAGGATCGGGTTAAACGGT (SEQ ID NO: 64) Reverse primer: AAACGCTTACTAATCTCCGAAT (SEQ ID NO: 65)
BARHL2	Forward primer: CGTTAGTAGTCGGATTATAAGCGAAC (SEQ ID NO: 66) Reverse primer: AAAAATTACGAAACAAACACGACCG (SEQ ID NO: 67)
ZNF154	Forward primer: GTTAGGTTTGGGATAGGGATCG (SEQ ID NO: 68) Reverse primer: CGCTACCATCAAACCTCTACG (SEQ ID NO: 69)

RUNX3	Forward primer: GAGGTTTAGTACGCGTTCG (SEQ ID NO: 70)
	Reverse primer: CCCGCCTCCTAAATCTATCG (SEQ ID NO: 71)

4) A calculation method was the same as that in Example 2.

Detection results of the nucleic acid fragment 4 and the markers in the 20 tissue samples are shown in Table 8, and statistical analysis results are shown in Table 9.

Table 8 Detection results of methylation levels of 9 genes in bladder tissue specimens

Sample No.	Sample type	MEIS1	NKX6-2	OTX1	SIM2	SOX1	BARHL2	Nucleic acid fragment 4	ZNF154	RUNX3
BA01	bladder cancer	54.1%	0.0%	3.7%	39.8%	0.0%	0.0%	21.6%	0.0%	0.0%
BA02	bladder cancer	133.0%	0.0%	0.9%	68.5%	9.7%	1.3%	1.7%	0.0%	11.0%
BA03	bladder cancer	87.5%	0.0%	50.6%	2.6%	19.4%	34.8%	46.8%	75.8%	23.0%
BA04	bladder cancer	8.6%	36.4%	46.5%	48.6%	66.7%	36.7%	46.1%	79.9%	37.8%
BA05	bladder cancer	98.6%	0.0%	0.6%	28.8%	6.0%	0.0%	0.0%	7.7%	8.1%
BA06	bladder cancer	52.5%	21.5%	53.2%	60.4%	8.1%	20.3%	45.7%	87.9%	3.2%
BA07	bladder cancer	0.0%	17.7%	119.5%	57.5%	25.8%	70.8%	12.7%	161.5%	23.4%
BA08	bladder cancer	53.8%	57.7%	48.5%	64.7%	14.1%	54.8%	29.0%	94.0%	43.4%
BA09	bladder cancer	54.1%	21.1%	47.5%	28.4%	8.9%	23.8%	43.6%	55.9%	55.9%
BA10	bladder cancer	74.1%	2.3%	63.9%	59.0%	52.8%	67.1%	42.8%	76.8%	85.8%
BB01	control	3.4%	0.0%	1.2%	26.4%	11.4%	0.0%	1.4%	11.5%	42.3%
BB02	control	5.3%	2.4%	1.1%	11.1%	6.7%	8.9%	0.7%	7.0%	54.3%
BB03	control	0.4%	0.0%	2.2%	7.3%	3.7%	0.6%	0.0%	3.9%	37.7%
BB04	control	0.9%	0.0%	7.1%	2.1%	12.8%	0.0%	0.0%	23.3%	19.6%
BB05	control	0.7%	0.0%	0.6%	4.7%	10.0%	0.9%	1.2%	18.0%	14.2%
BB06	control	3.2%	1.4%	2.0%	11.3%	5.1%	0.9%	1.0%	9.8%	43.2%
BB07	control	5.3%	0.0%	4.4%	1.8%	9.9%	0.0%	1.0%	6.8%	17.2%

BB08	control	2.1%	0.0%	33.7%	18.7%	3.9%	0.0%	0.0%	8.2%	15.9%
BB09	control	0.9%	0.0%	1.4%	3.1%	0.0%	0.0%	0.0%	15.4%	1.2%
BB10	control	3.5%	0.0%	0.9%	25.9%	40.3%	24.0%	7.8%	3.8%	124.0%

In the table, methylation percentage = the quantitative concentration of a target gene / the quantitative concentration of a reference gene $\times 100\%$.

Table 9 Statistical results

	MEIS1	NKX6-2	OTX1	SIM2	SOX1	BARHL2	Nucleic acid fragment 4	ZNF154	RUNX3
Sensitivity	90.0%	60.0%	70.0%	90.0%	50.0%	70.0%	90.0%	70.0%	20.0%
Specificity	90.0%	90.0%	90.0%	90.0%	90.0%	90.0%	90.0%	90.0%	90.0%

The results show that MEIS1, SIM2, and the nucleic acid fragment 4 of the present disclosure can well distinguish the cancer tissue samples from patients with bladder cancer and the bladder tissue samples from people without bladder cancer, and their sensitivity and specificity to the bladder cancer tissue specimens are both 90%.

Example 4

Sample information: 97 urine samples, including 45 bladder cancer samples and 52 control samples.

In the present example, DNA extraction and transformation were the same as those in Example 3.

Primer and probe sequences are shown in Table 10.

Table 10

Name	Sequence
ACTB (reference gene)	Forward primer: TTTTGGATTGTGAATTTGTG (SEQ ID NO: 49) Reverse primer: AAACCTACTCCTCCCTTAAA (SEQ ID NO: 50) Probe: TTGTGTGTTGGGTGGTGGTT (SEQ ID NO: 51)

Nucleic acid fragment 4	Forward primer: GCGTTCGGAGTTGTTTAGCG (SEQ ID NO: 72) Reverse primer: CACCGACGCCACAAACGA (SEQ ID NO: 73) Probe: CTATTACCGCCGCCGCGTCG (SEQ ID NO: 74)
MEIS1	Forward primer: GTTCGGGATAAGATTTCGGGG (SEQ ID NO: 75) Reverse primer: TAATTAAACTACGCAACCCGACT (SEQ ID NO: 76) Probe: CGAGAGGGGTCGGGCGAGTTAG (SEQ ID NO: 77)

An amplification system of the present example is shown in Table 11.

Table 11

Reaction component	Addition amount (μL)
5× buffer	4
Magnesium ions (25 mM)	4
dNTPs (10 mM)	0.8
Forward primer F (25 μM)	0.4
Reverse primer R (25 μM)	0.4
Probe P (10 μM)	0.4
Taq polymerase (5 units/μL)	0.4
H ₂ O	7.6
Template DNA	2
Total volume	20

An amplification protocol of the present example is shown in Table 12.

Table 12

step	temperature and time	number of cycles
pre-denaturation	95°C for 5 min	1
amplification	95°C for 20 s	45
	65 °C for 20 s	

	62 °C for 40 s	
cooling	40°C for 3 min	1

In the 97 urine samples, if a Ct value of methylation level detection is greater than a cut-off value, the methylation is determined as negative, and otherwise, the methylation is determined as positive. A cut-off value for detection of the nucleic acid fragment 4 is 34.65, and a cut-off value for detection of MEIS1 is 33.82. Detection results are shown in Table 13.

Table 13 Detection results of the nucleic acid fragment 4 and MEIS1 in 97 urine samples

Sample no.	Sample type	Nucleic acid fragment 4		MEIS1	
		Ct value	Detection result	Ct value	Detection result
UF001	control	35.21	negative	34.82	negative
UF002	control	35.59	negative	35.67	negative
UF003	control	45	negative	36.27	negative
UF004	control	45	negative	38.84	negative
UF005	control	37.64	negative	36.16	negative
UF006	control	35.74	negative	33.77	<i>positive</i>
UF007	control	35.29	negative	36.04	negative
UF008	control	35.78	negative	35.24	negative
UF009	control	38.69	negative	33.45	<i>positive</i>
UF010	control	36.29	negative	35.19	negative
UF011	control	45	negative	37.9	negative
UF012	control	45	negative	38.24	negative
UF013	control	35.66	negative	34.53	negative
UF014	control	36.01	negative	33.99	negative
UF015	control	45	negative	37.7	negative
UF016	control	35.51	negative	43	negative

UF017	control	38.13	negative	37.21	negative
UF018	control	36.81	negative	36.47	negative
UF019	control	35.47	negative	38.45	negative
UF020	control	38.94	negative	36.76	negative
UA009	control	37.26	negative	34.56	negative
UA011	control	35.81	negative	35.53	negative
UA013	control	39.42	negative	38.74	negative
UA018	control	37.11	negative	35.1	negative
UA022	control	37	negative	35.63	negative
UA023	control	34.74	negative	36.69	negative
UA027	control	45	negative	35.29	negative
UA028	control	36.54	negative	33.88	negative
UA029	control	38.01	negative	34.08	negative
UA030	control	37.43	negative	35.35	negative
UA032	control	37.35	negative	35.88	negative
UA037	control	45	negative	36.94	negative
UA040	control	39.99	negative	35.97	negative
UA042	control	36.98	negative	35.32	negative
UA046	control	45	negative	34.91	negative
UA049	control	36.78	negative	34.18	negative
UE001	control	36.62	negative	34.69	negative
UE002	control	40.11	negative	33.89	negative
UE003	control	38.04	negative	32.22	<i>positive</i>
UE004	control	35.21	negative	32.48	<i>positive</i>
UE005	control	35.85	negative	33.81	<i>positive</i>

UE006	control	34.83	negative	35.28	negative
UE008	control	34.7	negative	33.87	negative
UE009	control	45	negative	35.98	negative
UE010	control	36.34	negative	35	negative
UE013	control	45	negative	35.5	negative
UE014	control	36.1	negative	32.03	<i>positive</i>
UE016	control	37.54	negative	34.6	negative
UE017	control	37.84	negative	31.55	<i>positive</i>
UE018	control	38.09	negative	33.71	<i>positive</i>
UE019	control	39.16	negative	35.08	negative
UE020	control	36.56	negative	36.16	negative
UC107	bladder cancer	26.19	positive	30.05	positive
UC108	bladder cancer	27.43	positive	31.65	positive
UD043	bladder cancer	34.26	positive	38.77	<i>negative</i>
UD044	bladder cancer	28.51	positive	31.87	positive
UD046	bladder cancer	31.21	positive	33.72	positive
UD047	bladder cancer	28.08	positive	32	positive
UD049	bladder cancer	34.4	positive	35.46	<i>negative</i>
UD051	bladder cancer	27.47	positive	31.34	positive
UD054	bladder cancer	28.2	positive	31.28	positive
UD056	bladder cancer	27.87	positive	31.01	positive
UD057	bladder cancer	26.69	positive	30.22	positive
UD059	bladder cancer	31.27	positive	33.76	positive
UD060	bladder cancer	28.24	positive	37.69	<i>negative</i>
UD063	bladder cancer	28.89	positive	33.08	positive

UD065	bladder cancer	30.45	positive	33.72	positive
UD068	bladder cancer	31.03	positive	33.82	<i>negative</i>
UD070	bladder cancer	30.46	positive	33.93	<i>negative</i>
UD072	bladder cancer	34.65	<i>negative</i>	34.76	<i>negative</i>
UD076	bladder cancer	27.43	positive	31.16	positive
UD084	bladder cancer	31.18	positive	38.69	<i>negative</i>
UD089	bladder cancer	35.94	<i>negative</i>	31.28	positive
UA001	bladder cancer	39.38	<i>negative</i>	37.19	<i>negative</i>
UA002	bladder cancer	34.58	positive	33.91	<i>negative</i>
UA003	bladder cancer	30.83	positive	29.99	positive
UA004	bladder cancer	27.54	positive	30.54	positive
UA063	bladder cancer	27.48	positive	29.67	positive
UA066	bladder cancer	27.38	positive	30.61	positive
UA067	bladder cancer	33.04	positive	35.16	<i>negative</i>
UA068	bladder cancer	31.82	positive	33.7	positive
UA069	bladder cancer	34.33	positive	38.53	<i>negative</i>
UA070	bladder cancer	29.89	positive	32.33	positive
UA071	bladder cancer	25.65	positive	28.54	positive
UA072	bladder cancer	36.09	<i>negative</i>	27.93	positive
UC018	bladder cancer	30.49	positive	34.31	<i>negative</i>
UC035	bladder cancer	25.15	positive	27.93	positive
UC045	bladder cancer	34.14	positive	38.61	<i>negative</i>
UC062	bladder cancer	34.52	positive	31.73	positive
UC068	bladder cancer	33.77	positive	33.31	positive
UC070	bladder cancer	26.54	positive	29.28	positive

UC080	bladder cancer	26.07	positive	30.74	positive
UC082	bladder cancer	38.23	<i>negative</i>	37.59	<i>negative</i>
UC083	bladder cancer	32.52	positive	33.67	positive
UC087	bladder cancer	29.51	positive	32.2	positive
UC092	bladder cancer	29.64	positive	31.9	positive
UC095	bladder cancer	26.63	positive	31.88	positive

Statistical analysis results of the above detection results are shown in Table 14, and ROC curves are shown in Fig. 2.

Table 14

	Nucleic acid fragment 4	MEIS1
Cut-Off	34.65	33.82
Sensitivity	91.1	71.1
Specificity	100.0	84.6
AUC	0.956	0.790

The results show that in detection of methylation levels of the nucleic acid fragment 4 in the urine samples to detect bladder cancer, the detection specificity is as high as 100%, and the sensitivity is 91.1%. In the detection of methylation levels of gene MEIS1 in the urine samples to detect bladder cancer, the detection specificity is 84.6% only, and the sensitivity is 71.1% only.

Example 5

Sample information: 299 urine samples, including 1 low-malignant-potential inverted urothelial tumor sample, 16 low-malignant-potential papillary urothelial tumor samples, 105 bladder cancer samples, 31 prostate cancer samples, 17 renal pelvis cancer samples, 10 ureteral cancer samples, and 119 control samples.

In the present disclosure, DNA extraction and transformation, primer and probe sequences for the nucleic acid fragment 4, an amplification system, and an amplification protocol were the

same as those in Example 4.

Detection results of the nucleic acid fragment 4 in different types of tumor samples are shown in Table 15.

Table 15 Detection results of the nucleic acid fragment 4 in different types of tumors in urine samples

	Sample type	Ct value of the nucleic acid fragment 4	Detection result of the nucleic acid fragment 4
UA126	low-malignant-potential inverted urothelial tumor	37.89	negative
UD007	low-malignant-potential papillary urothelial tumor	45	negative
UD035	low-malignant-potential papillary urothelial tumor	39.18	negative
UD055	low-malignant-potential papillary urothelial tumor	36.92	negative
UD058	low-malignant-potential papillary urothelial tumor	36.77	negative
UD061	low-malignant-potential papillary urothelial tumor	31.52	positive
UD091	low-malignant-potential papillary urothelial tumor	40.48	negative
UD106	low-malignant-potential papillary urothelial tumor	33.11	positive
UD123	low-malignant-potential papillary urothelial tumor	37.45	negative
UD159	low-malignant-potential papillary urothelial tumor	34.93	positive
UD160	low-malignant-potential papillary urothelial tumor	35.19	positive
UD181	low-malignant-potential papillary urothelial tumor	38.92	negative
UD230	low-malignant-potential papillary urothelial tumor	37.64	negative
UD232	low-malignant-potential papillary urothelial tumor	45	negative
UD319	low-malignant-potential papillary urothelial tumor	38.82	negative
UG043	low-malignant-potential papillary urothelial tumor	36.51	negative
UC071	low-malignant-potential papillary urothelial tumor	28.56	<i>positive</i>
UA010	control	37.29	negative
UA012	control	45	negative

UA014	control	45	negative
UA015	control	45	negative
UA016	control	36.49	negative
UA017	control	37.42	negative
UA020	control	36.91	negative
UA021	control	45	negative
UA024	control	45	negative
UA025	control	36.85	negative
UA026	control	45	negative
UA031	control	45	negative
UA033	control	45	negative
UA034	control	35.75	positive
UA035	control	38.62	negative
UA036	control	45	negative
UA039	control	45	negative
UA041	control	34.56	<i>positive</i>
UA043	control	45	negative
UA044	control	37.17	negative
UA045	control	37.63	negative
UA047	control	33.25	<i>positive</i>
UA048	control	37.92	negative
UA050	control	45	negative
UA051	control	37.24	negative
UA052	control	38.46	negative
UA108	control	36.84	negative

UA109	control	40.73	negative
UA114	control	37.47	negative
UA117	control	38.63	negative
UA118	control	39.81	negative
UA128	control	42.86	negative
UC001	control	34.94	<i>positive</i>
UC002	control	36.77	negative
UC005	control	37.97	negative
UC006	control	45	negative
UC007	control	37.7	negative
UC009	control	38.15	negative
UC011	control	38.43	negative
UC012	control	38.92	negative
UC013	control	37.17	negative
UC017	control	37.49	negative
UC019	control	37.67	negative
UC020	control	37.79	negative
UC022	control	37.67	negative
UC023	control	38.4	negative
UC024	control	39.64	negative
UC026	control	37.81	negative
UC027	control	45	negative
UC030	control	37.23	negative
UC031	control	45	negative
UC032	control	36.74	negative

UC033	control	38.47	negative
UC039	control	37.73	negative
UC040	control	38.62	negative
UC041	control	37.8	negative
UC044	control	38.03	negative
UC046	control	34.15	positive
UC047	control	37.57	negative
UC048	control	36.51	negative
UC051	control	45	negative
UC052	control	37.56	negative
UC055	control	45	negative
UC056	control	45	negative
UC059	control	45	negative
UC060	control	39.64	negative
UC061	control	45	negative
UC063	control	37.51	negative
UC064	control	38.07	negative
UC081	control	36.53	negative
UC097	control	37.37	negative
UC098	control	37.81	negative
UD008	control	45	negative
UD040	control	39.98	negative
UD041	control	35.93	positive
UD045	control	37.53	negative
UD050	control	38.19	negative

UD071	control	38.29	negative
UD073	control	37.69	negative
UD108	control	37.99	negative
UD124	control	39.18	negative
UD126	control	37.49	negative
UD200	control	42.77	negative
UD236	control	45	negative
UD238	control	37.68	negative
UD242	control	37.06	negative
UD259	control	34.49	<i>positive</i>
UD263	control	45	negative
UD265	control	45	negative
UD266	control	38.52	negative
UD269	control	37.42	negative
UD270	control	36.66	negative
UD274	control	38.54	negative
UD276	control	45	negative
UD277	control	38.73	negative
UD281	control	39.23	negative
UD301	control	38.06	negative
UD304	control	45	negative
UD306	control	36.66	negative
UD316	control	38.57	negative
UD318	control	36.85	negative
UD323	control	39.11	negative

UD325	control	38.66	negative
UD326	control	38.6	negative
UD328	control	40.71	negative
UD331	control	37.89	negative
UD336	control	32.95	<i>positive</i>
UG004	control	37.95	negative
UG009	control	45	negative
UG021	control	45	negative
UG036	control	38.4	negative
UG039	control	45	negative
UG066	control	38.08	negative
UG093	control	45	negative
UD164	control	36.5	negative
UG035	control	38.61	negative
UD346	control	37.55	negative
UD357	control	37.33	negative
UD364	control	45	negative
UA002-2	bladder cancer	35.86	positive
UA006	bladder cancer	33.2	positive
UA008	bladder cancer	29.04	positive
UA053	bladder cancer	30.97	positive
UA055	bladder cancer	29.19	positive
UA056	bladder cancer	28.83	positive
UA057	bladder cancer	37.26	<i>negative</i>
UA060	bladder cancer	32.61	positive

UA061	bladder cancer	37.8	<i>negative</i>
UA062	bladder cancer	29.53	positive
UA073	bladder cancer	29.92	positive
UA074	bladder cancer	34.94	positive
UA077	bladder cancer	32.69	positive
UA078	bladder cancer	29.86	positive
UA079	bladder cancer	35.04	positive
UA090	bladder cancer	36.6	<i>negative</i>
UA091	bladder cancer	29.57	positive
UA092	bladder cancer	30.71	positive
UA094	bladder cancer	31.85	positive
UA097	bladder cancer	29.41	positive
UA100	bladder cancer	36.99	<i>negative</i>
UA104	bladder cancer	37.5	<i>negative</i>
UA111	bladder cancer	31.03	positive
UA115	bladder cancer	34.47	positive
UA116	bladder cancer	31.71	positive
UA119	bladder cancer	33.26	positive
UA121	bladder cancer	31.05	positive
UA122	bladder cancer	30.28	positive
UA124	bladder cancer	29.37	positive
UA134	bladder cancer	29.32	positive
UA135	bladder cancer	36.85	<i>negative</i>
UA137	bladder cancer	31.16	positive
UA138	bladder cancer	30.48	positive

UA139	bladder cancer	28.75	positive
UA141	bladder cancer	28.14	positive
UA147	bladder cancer	45	<i>negative</i>
UA149	bladder cancer	29.24	positive
UA154	bladder cancer	31.17	positive
UB001	bladder cancer	31.57	positive
UB003	bladder cancer	29.23	positive
UB005	bladder cancer	28.74	positive
UB006	bladder cancer	31.12	positive
UB008	bladder cancer	32.25	positive
UC096	bladder cancer	36.11	positive
UC101	bladder cancer	30.2	positive
UD003	bladder cancer	35.16	positive
UD004	bladder cancer	29.16	positive
UD009	bladder cancer	29.79	positive
UD011	bladder cancer	28.87	positive
UD014	bladder cancer	32.92	positive
UD015	bladder cancer	34.54	positive
UD016	bladder cancer	30.08	positive
UD018	bladder cancer	32.11	positive
UD028	bladder cancer	34.16	positive
UD030	bladder cancer	28.73	positive
UD038	bladder cancer	29.54	positive
UD039	bladder cancer	36.31	negative
UD078	bladder cancer	33.22	positive

UD080	bladder cancer	28.36	positive
UD095	bladder cancer	31.15	positive
UD099	bladder cancer	35.08	positive
UD109	bladder cancer	30.96	positive
UD110	bladder cancer	36.1	positive
UD111	bladder cancer	32.91	positive
UD134	bladder cancer	33.59	positive
UD135	bladder cancer	33.21	positive
UD136	bladder cancer	28.59	positive
UD137	bladder cancer	35.82	positive
UD141	bladder cancer	34.57	positive
UD142	bladder cancer	34.63	positive
UD145	bladder cancer	29.86	positive
UD150	bladder cancer	34.84	positive
UD161	bladder cancer	30.01	positive
UD162	bladder cancer	35.01	positive
UD174	bladder cancer	34.83	positive
UD175	bladder cancer	32.68	positive
UD205	bladder cancer	30.64	positive
UD210	bladder cancer	34.01	positive
UD220	bladder cancer	29.11	positive
UD221	bladder cancer	29.06	positive
UD225	bladder cancer	37.17	<i>negative</i>
UD237	bladder cancer	33.03	positive
UD243	bladder cancer	36.29	positive

UD244	bladder cancer	30.27	positive
UD257	bladder cancer	29.87	positive
UD268	bladder cancer	29.51	positive
UD293	bladder cancer	32.53	positive
UD298	bladder cancer	30.48	positive
UD303	bladder cancer	32.98	positive
UD308	bladder cancer	34.96	positive
UD312	bladder cancer	35.02	positive
UD314	bladder cancer	31.95	positive
UD330	bladder cancer	30.06	positive
UD349	bladder cancer	32.5	positive
UD355	bladder cancer	30.59	positive
UD361	bladder cancer	33.99	positive
UD366	bladder cancer	31.61	positive
UD367	bladder cancer	31.71	positive
UG003	bladder cancer	32.28	positive
UG025	bladder cancer	35.58	positive
UG046	bladder cancer	34.94	positive
UG053	bladder cancer	33.5	positive
UG059	bladder cancer	30.67	positive
UG064	bladder cancer	32.83	positive
UG080	bladder cancer	32	positive
UA088	prostate cancer	35.19	positive
UA089	prostate cancer	34.53	positive
UA093	prostate cancer	36.14	positive

UA105	prostate cancer	36.46	<i>negative</i>
UD064	prostate cancer	34.91	positive
UD102	prostate cancer	45	<i>negative</i>
UD127	prostate cancer	36.5	<i>negative</i>
UD138	prostate cancer	37.08	<i>negative</i>
UD151	prostate cancer	36.07	positive
UD154	prostate cancer	36.95	<i>negative</i>
UD165	prostate cancer	37.88	<i>negative</i>
UD179	prostate cancer	35.7	positive
UD206	prostate cancer	39.03	<i>negative</i>
UD233	prostate cancer	35.56	positive
UD240	prostate cancer	34.29	positive
UD254	prostate cancer	37.11	<i>negative</i>
UD255	prostate cancer	45	<i>negative</i>
UD294	prostate cancer	45	<i>negative</i>
UD310	prostate cancer	37.97	<i>negative</i>
UD338	prostate cancer	37.18	<i>negative</i>
UD341	prostate cancer	38.13	<i>negative</i>
UD351	prostate cancer	38.71	<i>negative</i>
UG007	prostate cancer	34.69	positive
UG011	prostate cancer	37.87	<i>negative</i>
UG037	prostate cancer	38.11	<i>negative</i>
UG038	prostate cancer	45	<i>negative</i>
UG044	prostate cancer	33.04	positive
UG054	prostate cancer	36.13	positive

UG061	prostate cancer	36.66	<i>negative</i>
UA110	prostate cancer	45	<i>negative</i>
UD292	prostate cancer	45	<i>negative</i>
UA080	renal pelvis cancer	30.98	positive
UA085	renal pelvis cancer	34.92	positive
UA086	renal pelvis cancer	35.11	positive
UA106	renal pelvis cancer	33.1	positive
UA142	renal pelvis cancer	37.85	<i>negative</i>
UD013	renal pelvis cancer	35.76	positive
UD023	renal pelvis cancer	30.23	positive
UD042	renal pelvis cancer	33.77	positive
UD062	renal pelvis cancer	35.71	positive
UD067	renal pelvis cancer	38.29	<i>negative</i>
UD079	renal pelvis cancer	31.58	positive
UD113	renal pelvis cancer	28.25	positive
UD125	renal pelvis cancer	30.63	positive
UD251	renal pelvis cancer	31.07	positive
UD290	renal pelvis cancer	36.56	<i>negative</i>
UD291	renal pelvis cancer	31.54	positive
UD053	renal pelvis cancer	31	positive
UD167	ureteral cancer	29.78	positive
UD094	ureteral cancer	33.57	positive
UD156	ureteral cancer	36.74	<i>negative</i>
UD176	ureteral cancer	29.94	positive
UD177	ureteral cancer	31.76	positive

UD246	ureteral cancer	31.02	positive
UD253	ureteral cancer	34.98	positive
UD350	ureteral cancer	31.66	positive
UG078	ureteral cancer	34.14	positive
UG085	ureteral cancer	30.01	positive

In the detection, if a Ct value of detection of ACTB is less than 32, it is indicated that the sample is qualified or the operation is correct. If a Ct value of detection of a methylation level of the nucleic acid fragment 4 is less than 36.3, it is indicated that a detection result is positive, and otherwise, the detection result is negative.

Statistical analysis results of the above detection results are shown in Table 16, and ROC curves are shown in Fig. 3.

Table 16

Analysis group	Indicator	Nucleic acid fragment 4
Comparison of a control group and a bladder cancer & ureteral cancer & renal pelvis cancer group	Specificity	93.3%
	Sensitivity	90.9%
	AUC	0.962 (P<0.001)
Comparison of a control group and a ureteral cancer group	Specificity	92.4%
	Sensitivity	90.0%
	AUC	0.979 (P<0.001)
Comparison of a control group and a renal pelvis cancer group	Specificity	92.4%
	Sensitivity	82.4%
	AUC	0.927 (P<0.001)
Comparison of a control group and a bladder cancer group	Specificity	93.3%
	Sensitivity	90.4%
	AUC	0.966 (P<0.001)
Comparison of a control group and	Specificity	92.4

a low-malignant-potential inverted urothelial tumor & low-malignant-potential papillary urothelial tumor group	Sensitivity	83.6
	AUC	0.923 (P<0.001)
Comparison of a control group and a prostate cancer group	Specificity	80.7
	Sensitivity	58.1
	AUC	0.671 (P=0.006)

It can be seen from the results in the above tables, the nucleic acid fragment 4 has high sensitivity and specificity for detection of various types of tumors in the urine samples.

Example 6

Primers and probes also have a great influence on detection effects of tumor markers. In the course of the research, the inventors designed multiple pairs of primers and their corresponding probes to screen out probes and primers that can improve the detection sensitivity and specificity as much as possible in order to enable the detection reagent of the present disclosure to be practically applied to clinical detection. Some primers and probes (6 sets) are shown in Table 17, and detection results are shown in Table 18. All the primers and probes were designed by the inventors using a methylated sequence of the nucleic acid fragment 4 that was obtained by transformation with a sulfite as a template. They were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Table 17 Primers and probes

Name	Sequence No.	Sequence
CG443-F	SEQ ID NO.: 31	AGGTTTCGTTTACGAGGTTTTTC
CG443-R	SEQ ID NO.: 32	CCTACGCCAACTACTCCG
CG443-P	SEQ ID NO.: 33	CGAACGCTCCCGCTCCAAA
CG447-F	SEQ ID NO.: 34	TGCGTTAAGTGTACGTTTATC
CG447-R	SEQ ID NO.: 35	CGTAAAACAACACTACAACCTCGCG
CG447-P	SEQ ID NO.: 36	TTCTCCTCCTACGCCTACTACCTA

CG190-F	SEQ ID NO.: 37	GCGTTGCGTAGGTAGTAGGC
CG190-R	SEQ ID NO.: 38	GAACCTCGAAAAATAATACCGTT
CG190-P	SEQ ID NO.: 39	AGGAGAACGAGGCGCGCGA
CG437-F	SEQ ID NO.: 40	GCGTTCGGAGTTGTTTAGCG
CG437-R	SEQ ID NO.: 41	CACCGACGCCACAAACGA
CG437-P	SEQ ID NO.: 42	CTATTACCGCCGCCGCGTCG
CG441-F	SEQ ID NO.: 43	GCGGTCGTTGTATCGTTATC
CG441-R	SEQ ID NO.: 44	GAATACTTCTCGACTACCCG
CG441-P	SEQ ID NO.: 45	TAACGACCCCCGCAACAAACCG
CG446-F	SEQ ID NO.: 46	CGTTGTATCGTTATCGGTGAGC
CG446-R	SEQ ID NO.: 47	GCGCACTTAAAAATCCGCG
CG446-P	SEQ ID NO.: 48	CTTCTCGACTACCCGCAACAACAATAACG
A1-F	SEQ ID NO.: 78	TTGGATTGTGAATTTGTGTTTGT
A1-R	SEQ ID NO.: 79	CAATAAAACCTACTCCTCCCTTA
A1-P	SEQ ID NO.: 51	TTGTGTGTTGGGTGGTGGTT
A2-F	SEQ ID NO.: 80	GATGGAGGAGGTTTAGTAAGTT
A2-R	SEQ ID NO.: 81	CAATAAAACCTACTCCTCCCTTA
A2-P	SEQ ID NO.: 51	TTGTGTGTTGGGTGGTGGTT
A3-F	SEQ ID NO.: 82	GGAGGTTTAGTAAGTTTTTTGGATT
A3-R	SEQ ID NO.: 83	CAATAAAACCTACTCCTCCCTTA
A3-P	SEQ ID NO.: 51	TTGTGTGTTGGGTGGTGGTT

Screening of the primer and probe sets was performed using a 5637 bladder cancer cell line (positive) and an SK-N-BE cell line (negative). By mycoplasma detection and cell morphology analysis, it was determined that the cells were normal. The cells were expanded and collected, and DNA was extracted by using a DNA extraction kit (QIAGEN DNA Mini Kit, #51306) purchased from QIAGEN. The extracted DNA was quantified by using a UV spectrophotometer

and then modified with a sulfite by using a DNA transformation kit (EZ DNA Methylation Kit, D5002) purchased from ZYMO RESEARCH. The DNA was diluted with a 1× TE buffer to 6,000 copies/μL. A positive reference P0 (100% positive DNA, that is, the degree of methylation of the nucleic acid fragment 4 was 100%) and a negative reference N0 (100% negative DNA, that is, the degree of methylation of the nucleic acid fragment 4 was 0) were respectively obtained. P1 was obtained by mixing positive DNA with negative DNA in a ratio of 1:9, P2 was obtained by mixing positive DNA with negative DNA in a ratio of 1:99, and P3 was obtained by mixing positive DNA with negative DNA in a ratio of 1:999.

Primer melting curves were obtained according to the system and the amplification protocol of Example 1, and further, amplification results of the primer and probe sets were obtained according to the detection system and the amplification protocol of Example 4. Amplification curves and melting curves of the primer and probe sets are shown in Fig. 4.

Detection result data of the primer and probe sets in different references is shown in Table 18.

Table 18 Ct values of detection of primer and probe sets in different references

Detection of DNA	CG190	CG437	CG441	CG443	CG446	CG447	A1 (internal reference)	A2 (internal reference)	A3 (internal reference)
P0 (LOD 100%)	24.973	25.809	24.934	25.090	25.238	25.355	24.582	24.363	24.371
P1 (LOD 10%)	28.340	29.020	28.035	28.285	28.637	28.949	24.691	24.270	24.715
P2 (LOD 1%)	31.520	32.309	31.090	31.145	32.637	31.902	24.504	24.285	24.559
P3 (LOD 0.1%)	35.293	33.738	35.012	34.949	34.738	34.824	24.449	24.230	24.566
N0 (negative)	37.176	34.520	No amplification	35.754	40.895	No amplification	24.793	24.449	24.840

A cut-off value for result determination is 38, if a Ct value is less than or equal to 38, it is indicated that a detection result is positive, and if the Ct value is greater than 38, it is indicated

that the detection result is negative.

As shown in Table 18, all the 6 primer and probe combinations can detect the 5637 positive bladder cancer cell line at the limit of detection (LOD) of 0.1%, and only the primer and probe sets CG441, CG446, and CG447 do not cause nonspecific amplification in the SK-N-BE negative cell line or misjudge the cell line as positive. For practical application in clinical, the inventors designed and selected three sets of primer and probe sequences for detecting the reference gene ACTB, the detection and assessment results of the references show that detection results of the primer and probe detection systems A1, A2, and A3 are consistent.

Fig. 4 shows that melting curves of the detection systems CG190, CG437, and CG443 have two peaks, positions of melting peaks in the detection results of the references P0, P1, and P2 are consistent, positions of melting peaks of some detection systems in the detection results of the references P3 and N0 greatly differ from that in the detection result of P0, Ct values of the quantified amplification curves indicate that the detection systems cannot well distinguish the negative reference from the reference P3, and the limit of detection cannot reach one thousandth. A melting curve of the detection system CG446 also has two peaks, a position of a melting peak of CG446 in the detection result of N0 greatly differs from that in the detection result of P0, a Ct value of the quantified amplification curve of CG446 indicates that the detection system can well distinguish the negative reference from the reference P3, and the limit of detection can reach one thousandth. Melting curves of the detection systems CG441 and CG447 have a single peak, there is no amplification curve and no product melting peak in the detection result of N0, Ct values of the quantified amplification curves of CG441 and CG447 indicate that the detection systems can well distinguish the negative reference from the reference P3, and the limit of detection reaches one thousandth. The fluorescence signal intensity of CG447 in the platform stage is significantly lower than those of CG441 and CG446.

Example 7

The primer and probe set CG441 was detected in 193 urine specimens (including 89 bladder cancer specimens and 104 contrast specimens), and an amplification system and an amplification protocol were the same as those in Example 4. Detection results are shown in Table 19.

Table 19 Detection results of the primer and probe set CG441 in 193 urine samples

Sample No.	Sample type	ACTB (reference gene)	CG441	Detection result
UA141	bladder cancer	25.77	28.79	positive
UD080	bladder cancer	27.98	28.55	positive
UD136	bladder cancer	28.61	28.55	positive
UD030	bladder cancer	28.62	28.97	positive
UB005	bladder cancer	28.09	29.01	positive
UA139	bladder cancer	27.84	29.53	positive
UA056	bladder cancer	27.94	30.66	positive
UD011	bladder cancer	27.88	28.89	positive
UA008	bladder cancer	28.02	31.41	positive
UD221	bladder cancer	27.97	29.18	positive
UD220	bladder cancer	28.07	29.25	positive
UD004	bladder cancer	28.18	29.54	positive
UA055	bladder cancer	28.58	31.55	positive
UB003	bladder cancer	28.53	29.88	positive
UA149	bladder cancer	27.98	29.49	positive
UA134	bladder cancer	28.73	32.23	positive
UA124	bladder cancer	27.25	32.72	positive
UA097	bladder cancer	28.43	30.24	positive
UD268	bladder cancer	28.16	29.72	positive
UA062	bladder cancer	27.8	30.46	positive

UD038	bladder cancer	28.16	30.23	positive
UA091	bladder cancer	29.07	33.89	positive
UD009	bladder cancer	28.09	30.74	positive
UD145	bladder cancer	28.26	29.84	positive
UA078	bladder cancer	27.87	31.69	positive
UD257	bladder cancer	28.27	30.65	positive
UA073	bladder cancer	28.04	31.23	positive
UD161	bladder cancer	28.58	30.16	positive
UD016	bladder cancer	29.27	30.28	positive
UC101	bladder cancer	28.5	30.95	positive
UD244	bladder cancer	27.84	30.99	positive
UA122	bladder cancer	28.31	31.12	positive
UD298	bladder cancer	28.52	30.43	positive
UA138	bladder cancer	28.26	31.49	positive
UD205	bladder cancer	28.63	31.62	positive
UA092	bladder cancer	28.84	31.46	positive
UD109	bladder cancer	28.69	31.26	positive
UA053	bladder cancer	27.98	32.22	positive
UA111	bladder cancer	28.61	31.82	positive
UA121	bladder cancer	28.76	31.62	positive
UB006	bladder cancer	28.5	31.92	positive
UD095	bladder cancer	28.02	31.17	positive
UA137	bladder cancer	31.55	31.86	positive
UA154	bladder cancer	28.33	33.49	positive
UB001	bladder cancer	28.79	31.74	positive

UA116	bladder cancer	28.36	32.16	positive
UA094	bladder cancer	30.78	33.13	positive
UD018	bladder cancer	30.32	33.01	positive
UB008	bladder cancer	28.58	32.81	positive
UD293	bladder cancer	28.53	32.7	positive
UA060	bladder cancer	28.7	36.1	positive
UD175	bladder cancer	28.82	33.06	positive
UA077	bladder cancer	28.14	33.82	positive
UD111	bladder cancer	32.89	33.47	positive
UD014	bladder cancer	28.35	32.94	positive
UD336	control	29.06	39.21	negative
UD303	bladder cancer	28.76	36.95	positive
UD237	bladder cancer	30.92	34.63	positive
UA006	bladder cancer	28.13	40.73	<i>negative</i>
UD135	bladder cancer	28.59	33.82	positive
UD078	bladder cancer	28.63	33.74	positive
UA047	control	28.44	33.44	<i>positive</i>
UA119	bladder cancer	28.85	34.58	positive
UD134	bladder cancer	28.85	34.18	positive
UD210	bladder cancer		34.47	positive
UC046	control	28.98	35.98	<i>positive</i>
UD028	bladder cancer	28.32	34.14	positive
UA115	bladder cancer	30.82	35.14	positive
UD259	control	28.64	37.1	<i>positive</i>
UD015	bladder cancer	28.21	36.08	positive

UA041	control	28.9	38.6	negative
UD141	bladder cancer	29.8	34.97	positive
UD142	bladder cancer	29.92	35.47	positive
UD174	bladder cancer	29.03	36.32	positive
UD150	bladder cancer	29.44	36.45	positive
UA074	bladder cancer	32.62	36.47	positive
UC001	control	28.89	36.78	<i>positive</i>
UD308	bladder cancer	28.52	35.41	positive
UA079	bladder cancer	29.33	40.29	<i>negative</i>
UD099	bladder cancer	28.14	36.62	positive
UD003	bladder cancer	28.46	40.21	<i>negative</i>
UA034	control	28.95	39.11	negative
UD137	bladder cancer	30.71	39.03	<i>negative</i>
UA002-2	bladder cancer	29.72	37.66	positive
UD041	control	28.7	38.65	negative
UD110	bladder cancer	28.6	37.43	positive
UC096	bladder cancer	30.93	44	<i>negative</i>
UD243	bladder cancer	31.46	36.57	positive
UD039	bladder cancer	28.43	39.76	<i>negative</i>
UA016	control	28.94	43	negative
UD164	control	28.73	40.47	negative
UC048	control	28.21	44	negative
UC081	control	27.9	40.39	negative
UA090	bladder cancer	28.71	40.83	<i>negative</i>
UD270	control	29.85	37.27	<i>positive</i>

UD306	control	28.91	44	negative
UC032	control	28.93	38.9	negative
UC002	control	28.58	37.82	<i>positive</i>
UA108	control	28.66	38.81	negative
UD318	control	29.83	39.14	negative
UA025	control	28.91	43	negative
UA135	bladder cancer	28.79	39.61	<i>negative</i>
UA020	control	28.82	43	negative
UA100	bladder cancer	28.14	44	<i>negative</i>
UD242	control	29.58	44	negative
UA044	control	29.17	44	negative
UC013	control	28.33	44	negative
UD225	bladder cancer	30.63	39.58	<i>negative</i>
UC030	control	29.2	44	negative
UA051	control	28.72	39.49	negative
UA057	bladder cancer	30.11	44	<i>negative</i>
UA010	control	28.93	39.99	negative
UD357	control	27.73	44	negative
UC097	control	28.88	44	negative
UD269	control	30.34	40.72	negative
UA017	control	29.56	44	negative
UA114	control	28.04	43	negative
UD126	control	28.81	38.62	negative
UC017	control	28.92	44	negative
UA104	bladder cancer	28.79	44	<i>negative</i>

UC063	control	28.27	44	negative
UD045	control	28.29	44	negative
UD346	control	29.17	44	negative
UC052	control	28.07	44	negative
UC047	control	29.03	44	negative
UA045	control	28.45	44	negative
UC022	control	28.93	38.73	negative
UC019	control	28.94	39.08	negative
UD238	control	28.2	39.73	negative
UD073	control	28.31	40.61	negative
UC007	control	28.61	44	negative
UC039	control	28.04	41.53	negative
UC020	control	28.66	44	negative
UC041	control	28.16	44	negative
UA061	bladder cancer	28.57	39.92	<i>negative</i>
UC026	control	29.23	39.74	negative
UC098	control	28.68	43	negative
UA048	control	29.52	44	negative
UC005	control	29.43	40.93	negative
UD108	control	28.36	44	negative
UC044	control	28.83	39.09	negative
UC064	control	28.87	39.75	negative
UC009	control	28	44	negative
UD050	control	28.79	44	negative
UD071	control	29.67	44	negative

UC023	control	29.51	44	negative
UC011	control	28.65	44	negative
UA052	control	29.08	39.13	negative
UC033	control	28.84	44	negative
UD266	control	29.92	39.91	negative
UD274	control	28.81	44	negative
UC040	control	29.21	40.05	negative
UA035	control	31.25	44	negative
UA117	control	29.24	44	negative
UD277	control	28.64	44	negative
UC012	control	28.89	44	negative
UD124	control	30.65	39.12	negative
UD281	control	28.62	44	negative
UC024	control	29.61	44	negative
UC060	control	31.53	44	negative
UA118	control	33.15	44	negative
UD040	control	28.75	44	negative
UA109	control	31.61	44	negative
UD200	control	32.69	44	negative
UA128	control	31.09	44	negative
UA147	bladder cancer	30.53	44	<i>negative</i>
UA021	control	28.93	39.63	negative
UD364	control	30.84	39.72	negative
UC061	control	31.94	40.68	negative
UA050	control	28.88	43	negative

UC059	control	30.63	43	negative
UD263	control	30.59	43	negative
UD008	control	30.29	44	negative
UC056	control	32.65	44	negative
UC055	control	28.32	44	negative
UC027	control	32.18	44	negative
UD265	control	30.04	44	negative
UD236	control	28.35	44	negative
UA015	control	29.47	44	negative
UG093	control	29.74	44	negative
UA014	control	31.76	44	negative
UA012	control	31.47	44	negative
UA024	control	32.56	44	negative
UA036	control	30.78	44	negative
UA043	control	30.74	44	negative
UA033	control	29.55	44	negative
UA031	control	29.48	44	negative
UA026	control	28.84	44	negative
UA039	control	33	44	negative
UC006	control	31.89	44	negative
UC031	control	30.49	44	negative
UC051	control	31.51	44	negative
UD276	control	32.49	44	negative

A cut-off value for result determination is set as 38.6, if a Ct value is less than or equal to 38.6, a detection is determined as positive, and if the Ct value is greater than 38.5, the detection result is determined as negative. The obtained detection specificity of the nucleic acid fragment 4

to the bladder cancer samples in the 193 urine samples is 93.3%, the sensitivity is 84.3%, and the area under an ROC curve is 0.931 ($P < 0.001$). The ROC curve is shown in Fig. 5.

References:

1. Mbeutcha, A., Lucca, I., Mathieu, R., Lotan, Y. & Shariat, S. F. Current Status of Urinary Biomarkers for Detection and Surveillance of Bladder Cancer. *Urologic Clinics of North America* 43, 47–62 (2016).

CLAIMS

What is claimed is:

1. A primer, comprising:

a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 46, or SEQ ID NO: 47, or complementary sequences thereof;

optionally, the primer comprising multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 37 and SEQ ID NO: 38, SEQ ID NO: 40 and SEQ ID NO: 41, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47;

optionally, the primer comprising multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47;

optionally, the primer comprising multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of a primer pair shown as SEQ ID NO: 43 and SEQ ID NO: 44.

2. A probe, comprising:

a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 39, SEQ ID NO: 42, SEQ ID NO: 45, or SEQ ID NO: 48, or complementary sequences thereof;

optionally, the probe comprising a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown as SEQ ID NO: 36, SEQ ID NO: 45, or SEQ ID NO: 48, or complementary sequences thereof;

optionally, the probe comprising a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a sequence shown as SEQ ID NO: 45.

3. The primer according to claim 1 and/or the probe according to claim 2, for use to detect tumors.

4. Use of the primer according to claim 1 and/or the probe according to claim 2 in preparation of a tumor detection reagent or a kit.

5. Use of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in preparation of a tumor detection reagent or a kit.

6. Use of a reagent for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in preparation of a tumor detection reagent or a kit.

7. A method for detecting the methylation of a nucleotide sequence in a sample, comprising:
treating a sample to be detected with a hydrosulfite or a bisulfite or a hydrazine salt to obtain a modified sample to be detected; and

using a reagent for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 to detect a methylation level of the nucleotide sequence in the modified sample to be detected; optionally, using real-time fluorescence quantitative methylation-specific polymerase chain reaction for detection.

8. A method for detecting a tumor in a subject, comprising:
detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in a sample to be detected from a subject; optionally, a methylation level of the nucleotide sequence in the sample being detected by the detection method according to claim 7; and

indicating whether the subject has or is at risk of having a tumor according to the deviation, optionally, a relative difference, of the methylation level of the nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a

nucleotide sequence shown as SEQ ID NO: 4 in the sample from the subject that is obtained by the methylation level detection method, optionally, the methylation level of the nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in the sample from the subject that is detected by the detection method according to claim 7, from a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in a normal control sample.

9. A method for treating a tumor in a subject, comprising:

detecting a tumor in a subject, comprising: detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 in a sample to be detected from the subject; and

treating the tumor in case that the detection of the tumor in the subject indicates that the subject has or is at risk of having the tumor;

optionally, the methylation level of the nucleotide sequence in the sample is detected by the detection method according to claim 7;

optionally, the tumor in the subject is detected by the detection method according to claim 8;

optionally, the treatment is selected from one or more of surgery, chemotherapy, radiotherapy, chemoradiotherapy, immunotherapy, oncolytic virus therapy, and a combination thereof.

10. A tumor detection reagent, comprising a reagent for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

11. A kit, comprising the tumor detection reagent according to claim 9.

12. The method, the tumor detection reagent, the kit or the use according to any one of claims 6 to 11, wherein the methylation detection reagent comprises primers and/or a probe for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4;

optionally, a forward primer of the primers has any one of the following nucleotide sequences:

I. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with nucleotide sequences shown as SEQ ID NO: 31, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO: 43, or SEQ ID NO: 46, or

II. complementary sequences of the sequences shown in I;

optionally, a reverse primer of the primers has any one of the following nucleotide sequences:

III. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with nucleotide sequences shown as SEQ ID NO: 32,

SEQ ID NO: 35, SEQ ID NO: 38, SEQ ID NO: 41, SEQ ID NO: 44, or SEQ ID NO: 47, or

IV. complementary sequences of the sequences shown in III;

optionally, the primers comprise multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 37 and SEQ ID NO: 38, SEQ ID NO: 40 and SEQ ID NO: 41, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47;

optionally, the primers comprise multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of any primer pair shown as SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 43 and SEQ ID NO: 44, or SEQ ID NO: 46 and SEQ ID NO: 47;

optionally, the primers comprise multiple nucleotide sequences that respectively have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with each primer of a primer pair shown as SEQ ID NO: 43 and SEQ ID NO: 44;

optionally, the probe has any one of the following nucleotide sequences:

V. nucleotide sequences that have at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with sequences shown as SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 39, SEQ ID NO: 42, SEQ ID NO: 45, or SEQ ID NO: 48; or

VI. complementary sequences of the sequences shown in V;

optionally, the probe comprises a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with any one of sequences shown

as SEQ ID NO: 36, SEQ ID NO: 45, or SEQ ID NO: 48, or complementary sequences thereof;

optionally, the probe comprises a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a sequence shown as SEQ ID NO: 45.

13. The use, the method, the tumor detection reagent or the kit according to any one of claims 3 to 12, wherein the sample to be detected is selected from tissue, body fluid, and excrement;

optionally, the tissue is selected from bladder tissue;

optionally, the body fluid is selected from at least one of blood, serum, plasma, extracellular fluid, tissue fluid, lymph fluid, cerebrospinal fluid, and aqueous humor;

optionally, the excrement is selected from at least one of sputum, urine, saliva, and faeces;

optionally, the excrement is selected from urine.

14. A method for designing primers, comprising steps of designing primers for a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4.

15. A system for designing primers, comprising:

1) an input component;

2) a processing component; and

3) an output component;

optionally, the input component being configured to read a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at

least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4;

optionally, the processing component being loaded with a program for designing primers based on information read by the input component;

optionally, the output component be configured to output primer sequences designed by the processing component.

16. A tumor detection system, comprising:

a. a component for detecting a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4; and

b. a result determination system;

optionally, the methylation level detection component comprising the detection reagent or the kit according to any one of claims 10 to 12;

optionally, the result determination component being configured to output the risk of having a tumor and/or a tumor type according to a methylation level of a nucleotide sequence that has at least 85% or at least 90% or at least 91% or at least 92% or at least 93% or at least 94% or at least 95% or at least 96% or at least 97% or at least 98% or at least 99% or 100% identity with a nucleotide sequence shown as SEQ ID NO: 4 that is detected by the detection system;

optionally, the risk of having a tumor being determined by comparing a methylation level of a sample to be detected to a methylation level of a normal sample, and determining that the sample to be detected has a high risk of having a tumor if there is a significant difference or an extremely significant difference between the methylation level of the sample to be detected and the methylation level of the normal sample.

17. The primer, the probe, the use, the nucleotide sequence, the tumor detection reagent, the kit, the method or the system according to any one of claims 3 to 13, and 16, wherein the tumor is selected from urothelial tumors;

optionally, the tumor is selected from bladder cancer, ureteral cancer, renal pelvis cancer, and urethral cancer;

optionally, the tumor is selected from bladder cancer.