METHODS FOR RAPID AND SENSITIVE DETECTION OF HOTSPOT MUTATIONS

TECHNICAL FIELD OF THE INVENTION

[01] This invention is related to the area of genetic and biochemical assays. In particular, it relates to assays of neoplastic samples and their components.

BACKGROUND OF THE INVENTION

[02] Malignant gliomas are the most common primary central nervous system (CNS) malignancy in adults, responsible for >14,000 deaths in the U.S. in 2012 [2]. The World Health Organization (WHO) has established a number of histologic and clinical criteria used for classifying gliomas into various subtypes and grading them I to IV, indicating their degree of malignancy. Diffuse gliomas (WHO grade II-IV), which include astrocytomas, oligodendrogliomas, oligoastrocytomas, and glioblastomas (GBM)[3] are of particular clinical importance as they account for 80% of all primary malignant brain tumors. These tumors are diffusely infiltrative, which makes curative surgical resection impossible. Additionally, grade II-III diffuse gliomas also have the ability to progress to higher WHO grade IV GBM. GBM is the most common malignant brain tumor in adults and has the worst survival (median overall survival 12-15 months)[4]. Additionally, even among entities with identical histology, patient outcome can vary substantially. This is best exhibited by primary GBM, which occurs *de novo* as compared to secondary GBM, which progresses from lower grades. Both tumors histologically are indistinguishable, but genetically and clinically these diseases are distinct, as the survival of patients with secondary GBM is almost double that of primary GBM[5].

[03] Accurate diagnosis of diffuse glioma is particularly challenging due to heterogeneity, invasiveness, reactive parenchyma, and ambiguity among morphologic features. These diagnostic challenges are reflected by the high degree of inter-observer variability seen in

clinical use of these criteria. In a study of 244 gliomas reviewed independently by four neuropathologists, concordance rates were as low as 52%[6]. Accurate diagnosis of diffuse glioma is critically important for clinical decision-making for patients. This diagnosis determines the treatment regimen, and particular subtypes are known to show increased treatment response to particular chemotherapies (*e.g.,* procarbazine, CCNU, and vincristine for oligodendroglioma treatment). Additionally, histologic subtype dictates patient prognosis. Objective, tumor specific markers are clearly needed for more accurate diagnosis, prognosis and delivery of personalized care to glioma patients.

[04] To address these needs, large-scale sequencing studies have profiled the genetic alterations found in diffuse glioma. Many alterations were noted, such as frequent mutations in *isocitrate dehydrogenase 1 and 2 (IDH1/2)*[1], the promoter of *telomerase reverse transcriptase (TERT)*[7]*, alpha thalassemia mental retardation syndrome X-linked* (*ATRX*)[8,9], *homolog of Drosophila capicua* (*CIC), far upstream element binding protein 1 (FUBP1)*[10]*,* among others. These findings have helped to establish clear objective molecular subtypes of glioma. In terms of relevance to diagnosis, alterations in the *TERT* promoter and *IDH1/2* are the most promising due in large part to their frequency and their occurrence as single nucleotide substitutions at specific genomic loci ("hotspots"). In diffuse glioma, we found that the degree to which these mutations co-occur or occur exclusively defines glioma subtypes: *e.g., IDH1/2* mutations occur in >50% of secondary GBMs but are infrequent in primary GBMs (<5%) (Fig. 1), while *TERT* promoter mutations are found in >80% of primary GBMs as well as in >70% of oligodendrogliomas (Fig. 2). Furthermore, we found that the genetic subtypes of glioma established by *TERT/IDH* status effectively stratifies glioma patients into subtypes with distinct prognoses, more effectively than by histology alone, providing physicians with an objective test to guide more appropriate treatment (Fig. 3). For example, patients with gliomas harboring *TERT* promoter mutations have median overall survival (OS) of 11.5 months, while those with both *TERT* promoter and *IDH1/2* mutations exhibit a median OS of 125 months. Additionally, our studies have revealed that the "mixed histology" oligoastrocytomas, which likely reflect the ambiguity of histologic classification most, genetically stratify into either a astrocytic ($TERT^{WT}IDH^{MUT}$) or oligodendroglial ($TERT^{MUT}IDH^{MUT}$) signature (Fig. 3). We have expanded our study of these mutations to a

number of other tumor types and found that *TERT* promoter mutations are also frequent in other cancers, most notably liver cancer (44.2%), bladder cancer (66%), myxoid liposarcoma (79.1%), and medulloblastoma (21%), in which early diagnosis, recurrence and therapeutic response monitoring is critical[7,11]. (Fig. 2) Similarly, *IDH1* and *IDH2* mutations have been found in high frequencies in other types of cancers as well, including chondrosarcoma (56%)[12], enchondroma (87%), spindle cell hemangioma (70%)[13,14], acute myeloid leukemia (15%)[15], and cholangiocarcinoma of intrahepatic origin (22-28%)[16].

[05] These glioma subtype-specific and highly recurrent mutations call for diagnostic assays that are able to rapidly, sensitively, and specifically detect these mutations in *IDH1/2* and the *TERT* promoter. Such a tool would aid neuropathologists in these challenging diagnoses, provide patients with more precise prognostic information, and allow physicians to tailor therapy to a patient tumor's unique molecular signature. Additionally, for the many other aforementioned cancer types with frequent mutations in *IDH1/2* and the *TERT* promoter at these loci, such a diagnostic tool would assist in rapid and sensitive detection of these mutations also.

[06] Current diagnostic efforts for mutation detection are based on Sanger sequencing, which is time-consuming, costly, and most importantly is limited by poor sensitivity (limit of detection ~20% mutant alleles)[17]. Samples of low tumor percentage (<40% tumor, for heterozygous mutations implies <20% mutant alleles) can be misdiagnosed as lacking mutations due to limited sensitivity (Figure 4, 5). Such scenarios of low tumor percentage are very realistic in the context of diffuse glioma and other malignancies. In addition to the inherent heterogeneity and invasive nature of diffuse glioma, tumor biopsies can contain minimal tumor tissue, leading to poor sampling. Necrosis is a feature common to many cancers that can also limit usable tissue. These limitations have significant diagnostic and prognostic implications and can result in misguided therapy, making them inadequate for clinical use.

[07] There is a continuing need in the field to make clinical analyses faster, more sensitive, and more specific.

SUMMARY OF THE INVENTION

[08] According to one embodiment of the invention a method is provided for testing a body sample of a human with a tumor. Tumor DNA of a body sample of the human is amplified with a set of amplification primers. Each amplification primer comprises a sequence selected from SEQ ID NO: 1-14. Amplification products comprising TERT promoter and IDH1 and IDH2 sequences are thereby generated. Thereafter, the amplification products are detected.

[08a] Disclosed herein is a method of testing a body sample of a human with a tumor, comprising: amplifying tumor DNA of a body sample of the human with a set of amplification primers, wherein each primer consists of a sequence selected from:
sequences of SEQ ID NO: 3, 6, 9, 10, 13, and 14 that optionally further contain a tag sequence selected from SEQ ID NO: 28 and 29; and
sequences of SEQ ID NO: 1, 2, 4, 5, 7, 8, 11, and 12;
to generate amplification products comprising at least one TERT promoter sequence and amplification products comprising at least one IDH1 sequence and amplification products comprising at least one IDH2 sequence, wherein when said primers are used in amplification reactions, they are used in pairs that consist of:
a sequence of SEQ ID NO: 1 and a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 2 and a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 4 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 5 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 7 and a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 8 and a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 11 and a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 12 and a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 10 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29; and
a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 14 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;
wherein primers consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 11, and 12 contain a modified LNA nucleotide at their 3' ends;
wherein each of SEQ ID NO: 1-14 is represented in a primer in the set of amplification primers; and
detecting the amplification products.

[09] According to another embodiment of the invention a heterozygous calibrator plasmid for TERT/IDH1 is provided. The plasmid comprises (a) a *TERT* C228T segment and a *TERT* C250T segment, and (b) an *IDH1* R132H segment and an *IDH1* wild type segment, wherein each of the TERT segments is present in equal amounts and sizes, and wherein each of the IDH1 segments is present in equal amounts and sizes. The plasmid has restriction sites between each segment that enables selective linearization and addition of other loci of interest, such as, but no limited to, other *IDH1* and *IDH2* mutations, and other genetic loci of interest.

[10] One embodiment of the invention comprises a method for detecting mutations in *IDH1/2* and/or *TERT* promoter in a subject comprising, consisting of, or consisting essentially of (a) obtaining a body sample from the subject, the sample comprising at least one tumor DNA; (b) providing at least one nucleic acid primer to the sample; (c) providing enzymes and reagents for amplification of the at least one DNA template using the at least one primer; (d) incubating the sample, enzymes, reagents, at least one primer under conditions suited for amplification of the at least one DNA template; (e) detecting the amplified nucleotides; and (f) identifying mutations in *IDH1/2* and/or *TERT* promoter.

[11] In some embodiments, the at least one primer is selected from the group consisting of those primers found in Table 1A, Table 1B and Table 2, and combinations thereof.

[12] In other embodiments, the enzymes and reagents comprise those found in Table 4.

[13] In yet another embodiment, the sample is incubated under conditions according to Table 3.

[14] In some embodiments, the subject is a mammal. In other embodiments, the subject is a human.

[15] In other embodiments, the biological sample is selected from the group consisting of cerebral spinal fluid (CSF), tissues, cells, biopsies, blood, lymph, serum, plasma, urine, saliva, mucus, and tears. In some embodiments, the sample comprises a tissue biopsy. In some embodiments, the biological sample comprises CSF. In some embodiments, the biological sample comprises urine. In some embodiments the biological sample comprises plasma.

[16] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with assays which are robust, rapid, and reproducible.

BRIEF DESCRIPTION OF THE DRAWINGS

[17] FIG. 1. Mutations in IDH1 and IDH2 across different tumor types. IDH1 and IDH2 mutations occur frequently in diffuse glioma (WHO grade II-III) and secondary GBM (WHO grade IV).[1]

[18] FIG. 2. Frequency of TERT promoter mutations across a large panel of tumor subtypes.[7]

[19] FIG. 3. TERT/IDH based genetic classification for diffuse glioma. The predominant genetic signatures include: $TERT^{MUT}IDH^{MUT}$, made up primarily of histologic oligodendrogliomas; $TERT^{WT}IDH^{MUT}$, made up of progressive astrocytomas; $TERT^{MUT}IDH^{WT}$, made up of primary GBMs; and $TERT^{WT}IDH^{WT}$, made up of GBMs.

Red indicates TERT promoter mutation at either C228T or C250T; Green indicates mutation at either R132 of IDH1 or R172 of IDH2.

[20] FIG. 4. Potential issues with sampling tissue from cancer patients. False negatives can result from low percentages of tumor cells in a background of normal tissue or from a subclonal tumor cell population in a background of other cancer cells.

[21] FIG. 5. Sanger sequencing chromatograms on dilutions of genomic DNA with specified mutation. At <20% mutant allele fraction, the peak for the mutant allele becomes indistinguishable from background, limiting use of this approach for clinical samples, in particular for diffuse glioma which is heterogeneous and invasive.

[22] FIG. 6 is a schematic showing the overview of allele-specific qPCR using LNA primers.

[23] FIG. 7 is a schematic drawing showing TERT promoter C228/C250T and IDH1 R132H heterozygous calibrator plasmid based on PGL3-enh vector. TERT C228T, C250T and IDH1 R132H are present in ratios of 50% in each plasmid, with 300 bp of each target cloned into pGL3-enh plasmid. Loci are distanced 3kb from each other to limit cross reactivity of primers. Additional genetic loci of interest can be added alongside these segments to generate reproducible mutant allele fractions of mutations.

[24] FIG. 8. Detailed schematic of TERT promoter C228T/C250T and IDH1 R132H heterozygous calibrator plasmid, cloned into sites ~3kb away from each other in pgl3-enh vector

[25] FIG. 9. Dilution of target mutation to varying mutant allele fractions (10%, 1%, 0.1%). Across these ranges, the allele-specific assays show excellent linearity (R^2>0.994).

[26] FIG. 10. TERT/IDH Allele-specific LNA qPCR test detects 0.1% mutant allele fraction (~15 mutant copies).

[27] FIG. 11. TERT/IDH Allele-specific LNA qPCR in nested format retains high discrimination, while using only 50 ng of gDNA pre-amplified as a template.

DETAILED DESCRIPTION OF THE INVENTION

[28] The inventors have developed a highly sensitive quantitative PCR (qPCR)-based assay that can detect the *TERT* promoter, *IDH1/2* hotspot mutations swiftly, specifically, and sensitively. This qPCR-based diagnostic assay can detect mutant DNA in high backgrounds of normal DNA, such as when the mutant DNA is as low as 0.1% mutant alleles. This is a 200-fold higher sensitivity than traditional methods based on Sanger sequencing. Such detection limits are similar to other expensive, time consuming and complex techniques such as BEAMing, however the qPCR assay can be done in a matter of a few hours, requiring a single PCR step. Due to this assay's sensitivity, it permits detecting mutations in circulating tumor DNA (ctDNA). ctDNAs are often found in very limited amounts in the blood, urine, and CSF of cancer patients, but hold the promise of being used as a "liquid biopsy," through which patients can be diagnosed without surgical intervention. Tumor recurrence or drug resistance development can be monitored by examining these body fluids. The assay is not limited to using body fluids, however, and can similarly be used on more traditional tissue and biopsy samples.

[29] This technology can be applied to detect hotspot mutations in *IDH1, IDH2, TERT* promoter mutations in DNA extracted from tumor tissues as well as from "liquid biopsy" samples, including DNA extracted from CSF, plasma, serum, urine, and other body fluids. Further, the identification of these mutations is relevant not only to brain tumors, but also to many other tumor types in which these mutations are frequent, including liver and bladder cancer, skin cancers (melanoma, squamous and basal cell carcinoma), acute myeloid leukemia, cholangiocarcinoma, soft tissue tumors (echondroma, chondrosarcoma, spindle cell hemangioma, myxoid liposarcoma, atypical fibroxanthoma, myxoid liposarcoma) and thyroid cancer.

[30] Body samples can be any convenient and expendable part of the body which contains tumor DNA. This may be a tumor tissue, margin tissue, biopsy sample, metastatic sample, lymph, cerebra spinal fluid, blood, including serum or plasma, urine saliva, mucus, and tears. Other fluids which drain a particular organ containing a tumor may also be sampled

and tested. The DNA may be tested in the body sample, or it can be extracted using techniques known in the art. The DNA may be pre-amplified prior to the amplification to test for a mutation in TERT promoter, IDH1, and IDH2. The DNA may be depleted of extraneous sequences to render the target sequences a larger proportion of the analyte. The DNA that is tested may be genomic DNA, including without limitation mitochondrial DNA, amplified DNA, and cDNA.

[31] As part of the assay, amplification cycles may be conducted at a higher temperature that is customary for PCR, to introduce a higher level of stringency and specificity. Higher temperatures may be at least 66°C, at least 67°C, at least 68 °C, at least 69°C, at least 70°C, at least 71°C, at least 72°C, at least 73°C, at least 74°C, or at least 75°C, for example. Similarly, it may be desirable to conduct the pre-amplification step, if used, at such an elevated temperature. Low temperature amplification cycles can be used after initial higher temperature cycles. Low temperature cycles may be conducted at 60 degrees, at less than 60°C, at less than 59°C, at less than 58 °C, at less than 57°C, at less than 56°C, at less than 55°C, at less than 54°C, at less than 53°C, at less than 52°C, or at less than 51°C, for example.

[32] Multiplex reactions can be used in the assays if convenient in the particular setting. In a multiplex setting more than one set of primer pairs is used simultaneously in the same reaction mixture. In some cases it may be preferable to separate amplifications so that the reactions have less complexity, using fewer primer pairs, or even using a single primer pair. As described below, single reactions of the assay may use primer trios, *e.g.,* having wild-type and mutant specific primers and a common primer. This can be considered a single reaction, rather than a multiplex for multiple different genomic segments.

[33] LNA-modified nucleotides in the assays are typically used at the 3' end of primers to enhance specificity. The primers can also have additional sequences that are not complementary to the target genomic segments. The additional sequences or tags, can be used for convenience of assaying. As an example, and as discussed below, M13 sequences are added to the 5' end of the primers to facilitate sequencing of the amplicons formed using the primers. Tag sequences can be used as hybridization tags for identifying and/or

quantifying. Tag sequences can be used for any additional functionality or for no functionality. Additional nucleotides complementary to the target can also be added to the 5' end of the primers with minimal effect on sensitivity.

[34] High fidelity polymerases may be used to contribute to the accuracy of the amplification reactions. Many such polymerases are known in the art and can be selected by the skilled artisan. Exemplary DNA polymerases include FideliTaq™ DNA polymerase, Easy-A™ High-Fidelity PCR Cloning Enzyme, Herculase® II Fusion DNA Polymerase, Herculase® Enhanced DNA Polymerase, PfuUltra™ High-fidelity DNA Polymerase, ACCUZYME™ DNA Polymerase, VELOCITY™ DNA Polymerase, Vent™ (exo-) DNA Polymerase, KAPA HiFi HotStart™ DNA polymerase, and *Pfx50*™ DNA Polymerase.

[35] While the mutations that are the targets of the assays are referred to as *TERT* C228T and C250T, the complement of these designations can also be assayed at the same position to assess the complementary nucleotides. Similarly for IDH1/IDH2 although R132 and R172 are mentioned throughout, the assay can be done on the complementary strand to detect the complementary nucleotides of the named mutations on the sense strand.

[36] The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLES

TERT Promoter and IDH1/2 hotspot mutations AS LNA q-PCR assay:

A. Background:

[37] Allele-specific PCR is a form of DNA template amplification used for selective amplification of template containing a variation of interest and thus a method for SNP genotyping[18]. Most methods rely on discriminating primers that have higher complementarity to a target sequence with the genotype of interest. This is most often done by forcing the primer's 3' to be at the location of the mutation and complementary only to the target variant or wild type nucleotide. In this way, PCR efficiency is reduced when the primer binds to non-target alleles, delivering selective amplification (Figure 6).

[38] A major advance in this area has been the use of an alternate nucleic acid at the 3' end of these allele-specific primers known as locked nucleic acids (LNA). LNA is a nucleic acid analog with a methylene bridge between the 2'-O and 4'-C of a nucleic acid, which generates a bicyclic structure that locks the ribose moiety into a C3'-end conformation. This confirmation increases the T_m when the LNA base hybridizes with its complement, greatly increasing the specificity of the primer for its target mutation or single nucleotide polymorphism (most sources measure at least 9 cycle greater difference LNA vs. DNA)[19]. This platform has been employed for allele discrimination in the context of identification of bacterial species[20], cystic fibrosis genetic alterations[19], *BRAF* mutations[21], HBV drug resistance mutations[22], mitochondrial mutations (in MELAS and NARP) [23], among other applications.

Although other approaches have been used for detecting mutations in the *TERT* promoter and *IDH1/2* as alternatives to Sanger sequencing, including TaqMan LNA probes[24], High Resolution Melting Curve Analysis (traditional[25] and FRET-based[26]), SNaPshot[27,28], pyrosequencing[29], COLD PCR HRM[30], and SafeSeq[31], these techniques are either resource intensive or lack sensitivity and therefore are not practical as true clinical diagnostics.

B. TERT promoter and IDH1/2 hotspot mutation AS LNA q-PCR assay:

Primer Design

[39] We have designed and tested a number of allele-specific primers for use as diagnostics for detection of the most frequent mutations in both the *TERT* promoter and *IDH1/2* as well as their non-allele specific opposing primers needed for amplification (Table 1A). Of note, to establish these high performance, allele-specific (AS) primers we have designed over 10 different candidate AS primers which have varying length, forced mismatches introduced (3'-1 and -2 positions), and varied position of the LNA in an attempt to improve the discrimination of these primers. To test this discrimination ability, we used standard dilutions of tumor DNA in a background of normal DNA down to 15 copies or 0.1% and assessed the primers with the greatest ΔCt on qPCR while still producing a specific product. In addition, we have tested over 20 different candidate opposing primers again to produce a PCR product without background that specifically amplifies this region of the TERT promoter and that generates a small enough amplicon (<160 bp) capable of amplifying fragmented DNA sources (*i.e.,* formaldehyde fixed paraffin embedded (FFPE), ctDNA). Other opposing primers also work with the candidate AS primers, however we have selected the smallest amplicons to facilitate qPCR (Table 1,2). Finally we have also developed similar allele-specific primers for the extremely rare IDH1 R132 mutations R132C,G,S,L) and IDH2 R172 mutations (R172M,W,G) however although these can be used, for application in glioma, the IDH1 R132H primer set is of most use.

Table 1A: High performance allele-specific primers for detection and quantification of most common TERT promoter (C228T, C250T), IDH1 (R132H), and IDH2 (R172K) hotspot mutations (*Note: + indicates). M13 tags (TGTAAAACGACGGCCAGT; SEQ ID NO: 28) and CAG GAA ACA GCT ATG ACC; SEQ ID NO: 29) were added to the 5' end of the common primers to facilitate sequencing.

SEQ ID NO:	Primer Name	Primer Sequence (5'-> 3')	Length
1	TERT C228 AS LNA WT	CTGGGAGGGCCCGGA+G	16
2	TERT C228T AS LNA MUT	CTGGGAGGGCCCGGA+A	16
3	TERT C228 Common	GTCCTGCCCCTTCACCTTC	19
4	TERT C250 AS LNA WT	CCCGTCCCGACCCCT+C	16
5	TERT C250T AS LNA MUT	CCCGTCCCGACCCCT+T	16
6	TERT C250 Common 2	CAGCGCTGCCTGAAACTC	18
7	IDH1 R132 AS LNA WT	GGGTAAAACCTATCATCATAGGTC+G	25
8	IDH1 R132H AS LNA MUT	GGGTAAAACCTATCATCATAGGTC+A	25
9	IDH1 R132 Common	AACATGCAAAATCACATTATTGCC	24
10	IDH1 R132 Common 2	ATCCCCCGGCTTGTGAGT	18
11	IDH2 R172 AS LNA WT	AAGCCCATCACCATTGGCA+G	20
12	IDH2 R172K AS LNA MUT	AAGCCCATCACCATTGGCA+A	20
13	IDH2 R172 Common	AGGTCAGTGGATCCCCTCTC	20
14	IDH2 R172 Common 2	GGACCAAGCCCATCACCATT	20

Table 1B: High performance allele-specific primers for detection of rare IDH1 and IDH2 mutations (note these primer sets use the same opposing Common primers as in 1A).

SEQ ID NO:	Primer Name	Primer Sequence (5'-> 3')	Length
15	IDH1 R132C AS LNA MUT	GGGTAAAACCTATCATCATAGGT+T	24
16	IDH1 R132G AS LNA MUT	GGGTAAAACCTATCATCATAGGT+G	24
17	IDH1 R132S AS LNA MUT	GGGTAAAACCTATCATCATAGGT+A	24
18	IDH1 R132L AS LNA MUT	GGGTAAAACCTATCATCATAGGTC+T	25
19	IDH2 R172M AS LNA MUT	AAGCCCATCACCATTGGCA+T	20
20	IDH2 R172W AS LNA MUT	AAGCCCATCACCATTGGC+G	19
21	IDH2 R172G AS LNA MUT	AAGCCCATCACCATTGGC+T	19

Table 2: Primer sets for allele-specific qPCR assay for genotyping the TERT promoter and IDH1/2

TERT: C228 and C250

Primer 1	Primer 2	Target size	Amplicon size*	Purpose
TERT C228 AS LNA WT or C228T AS LNA MUT	TERT C228 Common	101 bp	119 bp	**WT/MUT TERT C228 Detection**
TERT C250 AS LNA WT or C250T AS LNA MUT	TERT C250 Common 2	115 bp	133 bp	**WT/MUT TERT C250 Detection**
TERT C228 Common	TERT C250 Common 2	163 bp	199 bp	***TERT* promoter copy number/normalization. Pre-amplification of *TERT* promoter**

IDH1: R132

Primer 1	Primer 2	Target size	Amplicon size*	Purpose
IDH1 R132 AS LNA WT or R132H AS LNA MUT	IDH1 R132 Common	80 bp	98 bp	**WT/MUT R132 Detection**
IDH1 R132 Common 2	IDH1 R132 Common	102 bp	138 bp	**IDH1 exon 4 copy number/normalization. Pre-amplification of *IDH1*.**

IDH2: R172

Primer 1	Primer 2	Target size	Amplicon size*	Purpose
IDH2 R172 AS LNA WT or R172K AS LNA MUT	IDH2 R172 Common	72 bp	90 bp	**WT/MUT R172 Detection**
IDH2 R172 Common 2	IDH2 R172 Common	77 bp	113 bp	**IDH2 exon 4 copy number/normalization. Pre-amplification of *IDH2*.**

*Note target regions amplified are 18 bp shorter for AS amplicons and 36 bp shorter for non-AS amplicons due to M13 sequence tags added

C. High performance PCR program and reagents

[40] The PCR program and reagents efficiently amplify the *TERT* promoter and *IDH1/2* exon 4 both in an allele-specific and non-AS fashion and in two different contexts: (1) PCR of genomic DNA and (2) nested PCR of pre-amplified genomic DNA. For applications in which mutation status is needed rapidly and there is sufficient sample to perform PCR with

replicates, the first program is recommended. For applications in which there is limited DNA or the DNA is of poor quality, the second program is recommended.

a. 1-step AS-LNA qPCR of genomic DNA for *TERT/IDH status*

[41] It is challenging to create a PCR program to allow for efficient allele-specific PCR in particular because of the limitation in location of the primers. The *TERT* promoter is notoriously challenging to amplify due to its high GC content (> 80% through the region of interest and 88% in the stretch from C228 to C250) and repetitive sequence (10 runs of 4G's in the region of interest). For maximum discrimination between wildtype and mutant alleles, the amplification program we employ uses an initial phase of amplification at a high annealing temperature ($\geq 66^0$C) followed by a second phase of amplification at a lower annealing temperature ($\leq 60^0$C). This program on our thermocycler with ramp rate 5°C/sec, takes < 1 hour excluding the melt curve (Table 3). This can be adjusted with shorter denaturation with higher temperature (*e.g.,* 1 min at 98°C) and shorter annealing times to facilitate even more rapid detection, which may be applicable in scenarios of intraoperative diagnosis. We have also used traditional single annealing temperature programs, which work best at higher annealing tepmeratures ($\geq 66^0$C), but they are less sensitive. Below we have listed the program for maximum discrimination that works best for all primer sets. Allele-specific (AS) primers were purchased from Exiqon (Woburn, MA, USA) as custom oligonucleotides that had been subjected to dual HPLC purification. Non-AS primers were purchased from IDT (Coralville, Iowa, USA) as custom oligonucleotides with standard purification

Table 3. PCR program 1 for AS-qPCR based genotyping

	Stage	**Temperature**	**Duration**	**cycles**
Step 1	Initial Denaturation	95 °C	180 s	1x
Step 2	Denaturation	95 °C	15 s	19x
Step 3	Annealing #1	68 °C	20 s	
Step	Denaturation	95 °C	15 s	20-30x

	Stage	Temperature	Duration	cycles
4				
Step 5	Annealing #2	57.6 °C	20 s	
Step 6	Melt Curve	65 °C - 95 °C	increase 0.5°C/ 5 s	
Note: Plate reads occur at Step 5 and Step 6 (FAM)				

[42] The reaction conditions for this PCR are shown in Table 4. Many GC-rich kits and other polymerases were used and had difficulties in particular with the TERT promoter. The read out for our assay is a SYBR green signal, using the KAPA SYBR Fast 2X MasterMix (KK4600, Boston, MA, USA). The primer concentration has been varied and works across a large range of concentrations. Additionally, input >50 ng can be used, but begins to inhibit the reaction.

Table 4. PCR reagents for 1-step qPCR using SYBR for TERT/IDH mutation detection

	Final concentration
KAPA SYBR FAST 2X MasterMix	1X
dH2O	Up to 15 µL
Primer 1 (10 µM)	400 nM
Primer 2 (10 µM)	400 nM
Template	1 - 50 ng
Total	15 µL

b. Nested AS LNA qPCR of pre-amplified DNA for *TERT/IDH* status

[43] A second approach for mutation detection is a nested qPCR approach that works very well for: low quality samples (*i.e.,* ctDNA, FFPE gDNA), and samples of low analyte quantity

that are insufficient for necessary replicates (*i.e.,* fine needle biopsies). This nested qPCR assay also has highly reliable quantification as PCR inhibition and differences in primer efficiencies are much less significant in a nested PCR context. To use this approach, the locus of interest is amplified with a non-biased (NB) primer set in *TERT* promoter, *IDH1*, or *IDH2*, or all three in a multiplex fashion, using a high fidelity enzyme for limited cycle number (<20 cycles) at a high annealing temperature (≥ 66^0C) (Table 5,6). Then, the resulting PCR product is purified (either using column-based or bead-based approach) and diluted (usually 1:1000) and serves as the template for the next round of amplification, which is using the allele-specific LNA-modified primers described above. The nested PCR is performed at an annealing temperature ≥ 66ºC as this offers the highest discrimination (Table 7, 8). The benefit of this nested approach is that there is a much greater supply of template, all generated from 1-50 ng of gDNA, which can be screensed for many more mutations than would otherwise be possible. Below we describe the high performance PCR programs for nested qPCR. It is beneficial to split pre-amplification reactions into multiple reactions (*e.g.,* 50 µl split into 5x10 µl), which can then be pooled. Following this step, one can purify PCR products using a column or bead-based approach.

Table 5. PCR program for high-fidelity pre-amplification

	Stage	**Temperature**	**Duration**	**cycles**
Step 1	Initial Denaturation	95 °C	180 s	1x
Step 2	Denaturation	98 °C	15 s	20x
Step 3	Annealing #1	68 °C	20 s	
Step 4	Extension	72 °C	2 s	
Step 5	Storage	4 °C	20 s	

Table 6. PCR reagents for high fidelity pre-amplification of TERT/IDH loci

	Final concentration
KAPA SYBR FAST	1X
KAPA HiFi HotStart DNA polymerase	1 unit

10 mM dNTP mix	0.3 mM each
dH2O	Up to 15 µL
Primer 1 (10 µM)	200-500 nM
Primer 2 (10 µM)	200-500 nM
Template	1 - 50 ng
Total	50 µL

Table 7. PCR program for nested Allele-Specific qPCR of Non Bias PCR product

	Stage	**Temperature**	**Duration**	**cycles**
Step 1	Initial Denaturation	95 °C	3 – 5 min	1x
Step 2	Denaturation	95 °C	15 s	40-50x
Step 3	Annealing #1	69 °C	20 s	
Step 4	Extension	72 °C	1 s	
Step 5	Melt Curve	65 °C - 95 °C	increase 0.5°C/ 5 s	Only if using SYBR
Plate read occurs at step 3, and if using SYBR and a melt curve, step 5 for FAM/SYBR				

Table 8. PCR reagents for allele-specific qPCR using LNA modified primers

	Final concentration
KAPA SYBR Fast/KAPA PROBE Fast/KAPA PROBE FORCE, all 2X	1X
Probe (10 µM) (if probe used instead of SYBR)	250 nM
Primer 1 (10 µM)	500 nM
Primer 2 (10 µM)	500 nM
dH2O	Up to 25 µl
Template	1 - 50 ng
Total	25 µL

[44] If using a probe for detection in nested qPCR, the protocol should use either of the two probe mixtures mentioned in Table 8 and either of these probes depending on TERT or IDH detection: TERT probe: /56FAM/CGGGTCCCC/ZEN/GGCCCAGC/3IaBkFQ/ (SEQ ID NO: 22-23); IDH1 probe: /56-FAM/ATGACTTAC/ZEN/TTGATCCCCATAAGCATGA/3IABkFQ/ (SEQ ID NO: 24-25). The TERT probe is designed in the common region between C228T and C250T, whereas the IDH1 probe is designed in the region between the allele-specific primer and the common primer, and as such can be used for all allele-specific IDH reactions (R132S, C, L, G and H).

D. Standards, copy number and mutation % quantification

[45] Alongside the AS *TERT* and *IDH1/2* primer sets, we use primers to amplify in a non-allele-specific fashion the TERT promoter region surrounding the two mutations (C228T and C250T), IDH1 exon 4 surrounding the R132H mutation, and also primers designed to amplify the highly repetitive human line-1 elements (hline1Fwd: 5' TCACTCAAAGCCGCTCAACTAC-3' (SEQ ID NO: 26), hline1Rev: 5'-TCTGCCTTCATTTCGTTATGTACC-3') (SEQ ID NO: 27). This information can be used to normalize the Ct value from allele-specific PCR amplifying the WT or MUT allele and provide a quantitative readout of the mutant allele fraction, enabling cross comparison of samples. As is mentioned in section E, this is not necessary if samples are all at identical concentrations. In addition to this, a number of standardized samples are run alongside tested samples for the purpose of validation, including samples such as: 50% mutant sample (positive control), 1% mutant sample (low mutation positive control), 0% wildtype sample (negative control), and a no template control. These standards were generated by isolation of genomic DNA (gDNA) from cell lines with known *TERT/IDH* status. As an example, we use gDNA from the following cell lines: DAOY (C228T), A375 (C250T), HCT116 (wildtype) and HCT116 IDH1 R132H knockin #2 (IDH1 R132H). Dilution of these samples with gDNA wildtype at the target locus yields the 1% and lower standards needed. Another source of standard is plasmid DNA with the target mutations, which we have generated as "100% standards" using TOPO cloning (K4810-01, Life technologies).

We used inserts generated by PCR amplification of a portion of the TERT promoter/IDH1/2 from tumor samples with the mutations of interest. Additionally, to serve as a more realistic quantitative control, we have generated a perfect heterozygote plasmid with both the TERT and IDH1 mutations of interest (see details below, Figure 7), which can be expanded to other target mutations by cloning these loci into the same plasmid or in a similar fashion into independent plasmids.

[46] Each primer set is run in triplicate on the samples. An example run for detecting the mutant alleles for TERT and IDH1 (*i.e.,* the most common glioma-related mutations) is using the following primer sets in Table 9. The wildtype AS primer sets can also be used as an alternative to the non-biased (NB) amplicons for more accurate quantification. Primers for non-biased amplicons show no specificity for a particular allele (mutant or wild type) and should amplify both types of alleles equally.

Table 9. Primer combinations for testing for *TERT* promoter C228T, C250T and *IDH1* R132H

Primer combination	Forward Primer Name	Reverse Primer Name	Purpose
TERT AS 228 MUT	TERT C228T AS LNA MUT	TERT C228 Common	TERT promoter C228T detection and quantification
TERT AS 250 MUT	TERT C250T AS LNA MUT	TERT C250 Common 2	TERT promoter C250T detection and quantification
TERT NB	TERT C228 Common	TERT C250 Common 2	Reference for copy number at TERT promoter locus
IDH1 R132H AS MUT	IDH1 R132H AS LNA MUT	IDH1 R132 Common	IDH1 R132H detection and quantification
IDH1 NB	IDH1 R132 Common	IDH1 R132 Common 2	Reference for copy number at IDH1
hline1	hline1 forward	hline1 reverse	Reference for copy number using line1 elements, do not use with nesting

[47] We designed the heterozygote calibrator to serve as a 50% mutation/50% WT sample, as dilutions can only approximate relative ratios of mutant and wildtype allele fractions. This

standard works best as a standard for the nested PCR approach mentioned previously. A heterozygous calibrator plasmid has been previously generated for single mutations, such as BRAF V600E mutation[32], however here we made a single calibrator for multiple mutations: IDH1 R132H and TERT C228T/C250T, greatly facilitating quantification and workflow (Figure 7). The two target regions are separated by > 3100 bp and the respective mutations are roughly equidistant from each other to ensure limited primer interference. These standards were generated by first designing genes for synthesis by GENEWIZ where the portion of the TERT promoter (the 228 and 250 loci +/- ~150 bp) and the portion of the IDH1 exon 4 (R132 or c.395G locus +/- ~ 150 bp) in pUC-57-ampR plasmids, along with synthetic restriction enzyme sites separating each gene fragment in case of desire to separate the loci from each other (some authors cite this as improving its performance as a standard). These were cloned into the vector pGL3-enh (Promega) which is only used as it has cloning sites that are approximally equidistant from each other, ensuring that the two TERT-IDH blocks making up the "heterozygote" are maximally separated. Additionally we generated an equivalent plasmid where all loci were wildtype as a negative control, with two copies of each gene locus. This system can be expanded to other mutations of interest, for example rarer *IDH1* mutations and *IDH2* mutations. We have introduced restriction enzyme sites surrounding each locus to facilitate expansion with other loci and to enable selective linearization.

E. Data analysis

[48] There are several options for data analysis. One approach that is most straightforward is to ensure all samples have equal concentrations and use a trend-line generated from the dilution of standard cell line DNA to various mutation percentages to back-calculate the mutant allele fraction (Figure 9). As shown below, the trend-lines have excellent linearity (R^2>0.994).

[49] For use of samples with varying DNA input amounts, we have employed approaches that normalize the Ct value to a measurement of the sample's copy number (using Ct value of the non-bias/common amplicon or of hline1), and use the positive control sample as a

mutant allele percentage standard. (The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level). This type of normalized calculation additionally requires an estimation of the primer efficiency. Based on dilutions of genomic DNA, we have estimated the primer efficiencies for each primer combination (Table 10).

Table 10. Primer combinations and their efficiencies

Primer Combination Name	Efficiency
TERT C228T AS LNA MUT+ TERT C228 Common	106.5%
TERT C250T AS LNA MUT + TERT C250 Common 2	91.80%
TERT C228 Common + TERT C250 Common 2	82.33%
IDH1 R132H AS LNA MUT + IDH1 R132 Common	105.8%
IDH1 R132 Common + IDH1 R132 Common 2	91.05%

[50] The triplicate Ct values are averaged for each primer set and normalized by primer efficiency values. For each sample, the average Ct value for the AS mutant primers are then normalized by the average Ct value for the respective common (NB) primer set. Using the equation below (by Pfaffl et al.), where E_{AS} refers to the efficiency of the AS mutant primer combination, control refers to the mean Ct value for the positive control of known mutation percentage (in this case 50%), and sample refers to the mean Ct value for the sample being assessed. The same information is in the denominator, but all with reference to the apporopriate NB primer set being used for normalization (*e.g.,* TERT common, IDH1 common, line1). For more accurate values, we recommend adjusting the equation using the AS mutant vs. AS wildtype for comparson, which yields a ratio of mutant:wildtype allele fraction which can be adjusted to yield the mutant allele fraction alone.

$$\text{Mutant allelle\%} = \frac{(E_{AS})^{\Delta Ct(\text{50\% control-sample})}}{(E_{NB})^{\Delta Ct(\text{50\% control}-\text{sample})}}$$

[51] These approaches work for both allele-specific qPCR of gDNA and nested qPCR of pre-amplified, purified PCR product. In the context of nesting qPCR, we recommend using the pre-amplified heterozygous calibrator plasmid as a highly accurate standard with 50% of each target mutation, and normalizing to this as the reference, although pre-amplified genomic DNA of known mutation percentage can also be used.

F. Performance

[52] Using the above primer sets and conditions, we have tested the limit of detection of these primers in high levels of background and found that it can reliably detect down to 15 mutant genomic DNA copies in a background of 15,000 wildtype copies (0.1%), and distinguishes this from 0% (wildtype) samples (Figure 10). Using nested qPCR, high discrimination can also be achieved with allele-specific PCR (Figure 11).

G. Application of the qPCR diagnostic

[53] To test our assay in a clinically relevant context, we have taken DNA from 43 glioma samples that have been previously genotyped using Sanger sequencing and identified using this method as TERT promoter WT and IDH1 R132 WT. We used our qPCR assay and identified 9 samples with TERT promoter C228T mutations and 3 samples with IDH1 R132H mutations, totaling 12 samples or 28% of the total, all with percentages <10% that had been previously undetectable by traditional methods (Table 11). In addition, this assay was completed within ~1 hr with quantitative results, whereas Sanger sequencing involves an additional sequencing step following PCR.

Table 11. Cases with low level mutations detected by qPCR but not by Sanger sequencing (false negatives).

Case ID	***TERT* Sanger**	***TERT* qPCR**	**Histology**
A	WT	**C228T**	GBM
B	WT	**C228T**	GBM

C	WT	**C228T**	GBM
D	WT	**C228T**	Anaplastic Astrocytoma
E	WT	**C228T**	GBM
F	WT	**C228T**	GBM
G	WT	**C228T**	GBM
Case ID	***IDH1* Sanger**	***IDH1* qPCR**	**Histology**
H	WT	**R132H**	Astrocytoma
K	WT	**R132H**	Astrocytoma

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The disclosure of each reference cited is expressly incorporated herein.

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We claim:

1. A method of testing a body sample of a human with a tumor, comprising:

amplifying tumor DNA of a body sample of the human with a set of amplification primers, wherein each primer consists of a sequence selected from:

sequences of SEQ ID NO: 3, 6, 9, 10, 13, and 14 that optionally further contain a tag sequence selected from SEQ ID NO: 28 and 29; and

sequences of SEQ ID NO: 1, 2, 4, 5, 7, 8, 11, and 12;

to generate amplification products comprising at least one TERT promoter sequence and amplification products comprising at least one IDH1 sequence and amplification products comprising at least one IDH2 sequence, wherein when said primers are used in amplification reactions, they are used in pairs that consist of:

a sequence of SEQ ID NO: 1 and a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 2 and a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 4 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 5 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 7 and a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 8 and a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 11 and a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 12 and a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 3 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 6 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29;

a sequence of SEQ ID NO: 9 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 10 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29; and

a sequence of SEQ ID NO: 13 that optionally further contains a tag sequence selected from SEQ ID NO: 28 and 29 and a sequence of SEQ ID NO: 14 that optionally further

contains a tag sequence selected from SEQ ID NO: 28 and 29;
wherein primers consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 11, and 12 contain a modified LNA nucleotide at their 3' ends;
wherein each of SEQ ID NO: 1-14 is represented in a primer in the set of amplification primers; and
detecting the amplification products.

2. The method of claim 1 further comprising amplifying tumor DNA in the body sample with a set of amplification primers comprising sequences of SEQ ID NO: 15-21.

3. The method of claim 1 wherein the tumor DNA is genomic DNA.

4. The method of claim 1 wherein the tumor DNA is genomic DNA that has been subjected to pre-amplification.

5. The method of claim 1 wherein at least a portion of cycles of the amplifying are conducted at ≥ 66˚ C.

6. The method of claim 5 wherein a portion of the cycles of the amplifying are conducted at ≤ 60˚C.

7. The method of claim 1 wherein separate aliquots of the tumor DNA of the body sample are amplified with sets of primers comprising sequences of: (a) SEQ ID NO: 1-3; (b) SEQ ID NO: 4-6; (c) SEQ ID NO: 3 and 6; (d) SEQ ID NO: 7-9; (e) SEQ ID NO: 9 and 10; (f) SEQ ID NO: 11-13; and (g) SEQ ID NO: 13-14.

8. The method of claim 1 wherein the amplification is performed as a quantitative PCR.

9. The method of claim 4 wherein the pre-amplification employs a high fidelity DNA polymerase.

10. The method of claim 4 wherein the pre-amplification employs an annealing temperature of ≥ 66˚ C.

11. The method of claim 4 wherein the pre-amplification employs an annealing temperature of ≥ 68˚ C.

12. The method of claim 4 wherein the pre-amplification is conducted as a multiplex reaction.

13. The method of claim 1 wherein the body sample is selected from the group consisting of cerebral spinal fluid (CSF), blood, lymph, serum, plasma, urine, saliva, mucus, and tears.

14. The method of claim 1 wherein the body sample is a biopsy sample.

15. The method of claim 1 wherein the body sample is a needle aspirate.

16. The method of claim 2 wherein at least one of said primers comprising sequences of SEQ ID NOs: 15-21 comprise an LNA-modified nucleotide at its 3' end.

17. The method of claim 2 wherein each of said primers comprising sequences of SEQ ID NOs: 15-21 comprise an LNA-modified nucleotide at its 3' end.

18. The method of claim 1 wherein the tag sequence is located at the 5' end of at least one of the primers.

19. The method of claim 2 wherein the tag sequence is located at the 5' end of at least one of the primers.