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(54) Title: USE OF REAL TIME PCR FOR DETECTION OF ALLELIC EXPRESSION

(57) Abstract: The present invention relates, e.g., to a method for quantitating the relative amounts of a plurality of different RNA transcripts expressed from a gene of interest (such as expression products of two alleles of a gene of interest), e.g. in a subject or cell, comprising performing real time quantitative PCR, in a single container, of cDNAs of the transcripts, in the presence of probes specific for each of the different transcripts, wherein the probes for each transcript are labeled with distinguishable fluorophores. The amount of expression of each of the plurality of transcripts may be quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the plurality of transcripts. Methods for validating the effectiveness and specificity of allele-specific siRNAs, and methods of diagnosis, are also described, as are kits for performing methods of the invention.

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USE OF REAL TIME PCR FOR DETECTION OF ALLELIC EXPRESSION

FIELD OF THE INVENTION

This invention relates, *e.g.*, to a method for simultaneously detecting the levels of expression of a plurality of alleles of a gene of interest. The method comprises the use of a plurality of distinguishable allele-specific reporter probes and real time PCR amplification kinetics. Kits for performing such assays and diagnostic methods are also disclosed.

BACKGROUND INFORMATION

Allelic variation in gene expression is common in eukaryotic, including human, genomes. See, *e.g.*, Lo *et al.* (2003) *Genome Research* 13, 1855-1862. The phenomenon of imprinting, in which one allele is suppressed early during development while the other is expressed, is widespread. It is of interest to study experimentally the occurrence and the mechanisms of this and other types of allelic variation in non-pathological tissues. Furthermore, a variety of disease conditions appear to be mediated by, or accompanied by, aberrant expression, such as over-expression, of one allele (often a mutant) of a gene. Such differences in allelic expression can serve as the basis for diagnostic tests for those conditions. In addition, the ability to specifically silence the expression of detrimental alleles with agents such as siRNAs provides both a method to study the role of the allelic expression in the disease phenotype and, in some cases, a therapeutic method to treat the disease. It would be desirable to have a method to measure such differences in allelic expression, as well as to validate an agent for its ability to specifically and efficiently silence the expression of an allele of interest.

Among the available methods to quantify differences in allelic expression are restriction fragment length polymorphism analysis (RFLP) (Botstein *et al.* (1980) *Am. J. Hum. Genet.* 32, 314-331); pyrosequencing (Wasson *et al.* (2002) *Biotechniques* 32, 1144-1152); Taq MAMA (Glaab *et al.* (1999) *Mutat. Res.* 430, 1-12); and fluorescence-based automated DNA sequencing (Qui *et al.* (2003) *Biochem. Biophys. Res. Commun.* 309, 331-338). These methods have drawbacks, including limited accuracy (*e.g.*, manual analysis of chromatogram peaks), limited applicability (*e.g.*, enzyme recognition sites not always available), labor intensiveness (*e.g.*, enzyme digestions followed by analysis on ethidium bromide stained gels followed by densitometry), or the requirement for relatively large amounts of RNA.

There is a need for an improved method for quantifying differences in allelic expression, as

well as for quantitating other instances in which a plurality of RNA transcripts are expressed from a gene of interest, such as alternate splicing.

DESCRIPTION OF THE DRAWINGS

Figures 1A-1E show a validation of the allelic discrimination of dual-probe *GNE* Taqman assays.

5 For each assay, endpoint fluorescent readings (R_n , normalized reporter signal) for allele-specific fluorescent probes specific for 5 different *GNE* target mutations were determined and visualized in a scatter plot. In each plot, the DNA samples cluster in three groups: homozygous wild type, heterozygous patient DNA, and no template control (ntc). Primer/probe sets for five different *GNE* target mutations were validated (see also Table 1). **Fig. 1A:** c.735C>T heterozygous in HIBM patient H1; **Fig. 1B:** c.403G>T heterozygous in HIBM patient H1; **Fig. 1C:** c.646T>C heterozygous in HIBM patient H2; **Fig. 1D:** c.2018C>T heterozygous in HIBM patient H2; **Fig. 1E:** c.787G>T heterozygous in sialuria patient S1.

15 **Figures 2A-2C** show standard regression curves for quantifying mutation-dependent allelic expression of *GNE*.

For each *GNE* mutation-specific assay, a standard regression curve was constructed (left panels) by mixing two allelic cDNA samples and determining the ΔC_t for the each allelic ratio ($\Delta C_t = C_t \text{ VIC normal allele} - C_t \text{ FAM mutated allele}$). The measured $2^{\Delta C_t}$ was plotted against mixed allelic ratios of the specific *GNE* target mutation. Solid lines: experimental curves; dotted lines: expected curves. Right panels show amplification plots of each assay (changes of reporter fluorescence ΔR_n against PCR cycle number) for allelic ratios 0.5 and 1.0.

Fig. 2A: Regression curve for c.735C>T assay ($r^2 = 0.99$). Measured $2^{\Delta C_t}$ for patient H1 is 1.1, extrapolating to 0.78 T-allele/C-allele ratio.

25 **Fig. 2B:** Regression curve for c.646T>C assay ($r^2 = 0.99$). Measured $2^{\Delta C_t}$ for patient H2 is 1.2, extrapolating to 0.83 C-allele/T-allele ratio.

Fig. 2C: Regression curve for c.787G>T assay ($r^2 = 0.98$). Measured $2^{\Delta C_t}$ for patient S1 is 0.54, extrapolating to 1.1 T-allele/G-allele ratio.

30 **Figures 3A-3E** illustrate the Sialic acid pathway, *GNE* defect and siRNA design

Fig. 3A: Intracellular sialic acid metabolism. The synthesis of sialic acid (Neu5Ac) is initiated in the cytosol, where glucose undergoes several modifications to eventually become sialic acid. The UDP-GlcNAc 2-epimerase (GNE)/ ManNAc kinase (MNK) enzyme is the central and rate-limiting enzyme in this cytosolic process. GNE activity is feedback inhibited by the downstream product
 5 CMP-sialic acid. Sialic acid is converted in the nucleus to CMP-sialic acid, which is utilized by the Golgi complex to sialylate oligosaccharides (OGS). Sialylated OGS are degraded in lysosomes.

Fig. 3B: Schematic of the *GNE* gene structure (not to scale) and the allosteric site residing in exon 5, which codes for the epimerase enzymatic domain of GNE. Sialuria patient S2 (Ferreira *et al.* (1999) *Mol Genet Metab* 67, 131-7) presented with mildly coarse facies, slight motor delay, and urinary
 10 excretion of large quantities (>1 gram/day) of free sialic acid. Mutation analysis revealed a c.797G>A nucleotide mutation, resulting in amino acid change R266Q (CGG>CAG).

Fig. 3C: Two siRNAs were designed, one targeting the normal allele (siRNA normal) and one specifically targeting the mutant allele (siRNA mutant). The upper sequence is SEQ ID NO:23. The lower sequence is SEQ ID NO:24.

Fig. 3D: Regression curve for c.797G>A assay, constructed by mixing two allelic cDNA samples (see Figure 2 for details) and determining the ΔC_t for the each allelic ratio ($\Delta C_t = C_t \text{ VIC normal allele} - C_t \text{ FAM mutated allele}$). The measured $2^{\Delta C_t}$ was plotted against mixed allelic ratios of the
 15 *GNE* target mutation.

Fig. 3E: Patients fibroblasts were transfected with siRNA (mock, normal and mutant) and cultured
 20 for 48 hours, after which total RNA was isolated and subjected to the quantitative real-time PCR assay. $2^{\Delta C_t}$ values were determined and extrapolated on the regression curve to A-allele/G-allele ratios for both siRNA constructs. Transfection with a mock siRNA did not result in a significant difference in allelic expression. However, transfection with siRNA_{normal} resulted in an *increase* of the allele ratio mutant allele:wildtype allele from 1.1 to 1.7 ± 0.2 (n=4), and transfection with
 25 siRNA_{mut} resulted in a significant *decrease* of the allele ratio mutant allele:wildtype allele from 1.1 to 0.4 ± 0.2 (n=4). These PCR analyses demonstrating allele-specific functionality of the target-sequence specific siRNAs. Therefore, this assay can be used for validation of allele-specific knock-down of the dominant allele RNA.

DESCRIPTION OF THE INVENTION

This invention relates, *e.g.*, to a method for simultaneously determining, in a single container

(such as a tube), the relative amounts of a plurality of different RNA transcripts expressed from a gene of interest. The different RNA transcripts may be, *e.g.*, RNAs expressed from different alleles of a gene of interest (*e.g.* from two alleles, wherein one copy is wild type and the other has a mutation or other variant sequence); splice variants; RNAs expressed from variant virus species; etc.

5 One of the transcripts may be the wild type counterpart of a variant of interest (*e.g.*, a wild type splice form, or a wild type counterpart of an allelic mutation). The different RNA transcripts contain "defining features," which are nucleic acid sequences that distinguish a variant form of an RNA transcript from its wild type counterpart, or from another RNA variant. The defining feature can be, *e.g.*, the site of an allelic variation, such as a mutation; a variant splice site; or the like. The term
10 "gene," as used herein, refers to all forms of a gene, including allelic variants.

The method employs real-time PCR amplification in the presence of reporter probes which are specific for each of the RNA transcripts and which are distinguishable from one another (*e.g.* comprise different fluorophores). A probe that is "specific for" an RNA transcript binds preferentially to that RNA transcript, in comparison to another RNA transcript, *e.g.* an RNA
15 transcript expressed from the same gene (*e.g.*, a wild type sequence, another allelic variant or another splice variant). Generally, one makes a cDNA copy of the RNA transcript, using reverse transcriptase, then amplifies the cDNA by real time PCR in the presence of special, variant-specific, single stranded probes, such as "TaqManTM probes." Each such probe has a fluorophore (fluorescent reporter probe) on one end and a quencher on the other end. For example, the fluorophore may be on
20 the 5' end and the quencher on the 3' end, or vice-versa. Much of the discussion herein is directed to probes with the fluorophore on the 5' end and the quencher on the 3' end; but it is to be understood that probes with the fluorophore on the 3' end and the quencher on the 5' end are also included. Each sequence-specific (*e.g.* allele-specific) probe contains a different fluorophore, so the expression of the plurality of nucleic acid transcripts (*e.g.* allelic variants) can be detected independently. As used
25 herein, the expression "comprising performing real time PCR" generally includes a step of converting the RNA to DNA before PCR amplification of the DNA (*e.g.*, a method of reverse-transcriptase (RT)-PCR).

The assay is designed such that the labeling moieties on the 5' and/or 3' ends of each probe do not fluoresce unless PCR amplification of the sequence to which the probe binds has occurred,
30 followed by hybridization of the probe to the amplified sequences, in which case fluorescence of the probe can be seen. The labeling moieties on both ends of a probe are fluorescent molecules, which

quench one another. For simplicity, the labeling moiety on one end (*e.g.*, the 5' end) is sometimes referred to herein as a "fluorophore," and the labeling moiety on the other end (*e.g.*, the 3' end) as a "quencher." When a single stranded probe is not hybridized to a target and is free in solution, the probe molecule is flexible and folds back partially on itself, so that the quencher and the fluorophore are close together; the quencher thus prevents the probe from fluorescing. Furthermore, when a probe of the invention is hybridized to a single stranded target, the two labeling moieties are close enough to one another to quench each other. However, without wishing to be bound by any particular mechanism, it is suggested that when the probe is hybridized to its target to form a perfect double stranded DNA molecule, a 5'->3' exonuclease which recognizes perfect hybrids, and which is an activity of the enzyme used for PCR, cleaves the duplex, releasing the fluorophore. The fluorophore is thus separated from the quencher, and will fluoresce. The amount of detected fluorescence is proportional to the amount of amplified DNA.

In a method of the invention, the released fluorescent emission is measured continuously during the exponential phase of the PCR amplification reaction. Since the exponential accumulation of the fluorescent signal directly reflects the exponential accumulation of the PCR amplification product, this reaction is monitored in real time ("real time PCR").

In methods of the invention, the relative amounts of the plurality of RNA transcripts are measured by comparison to a standard curve. Several suitable methods for generating standard curves are described herein. In general, the standard curve is generated by mixing isolated DNAs representing each of the plurality of RNA transcripts (rather than using genomic DNA) in a series of ratios, then amplifying those mixtures by real time PCR in the presence of variant-specific probes.

Advantages of the method of the invention include that it is rapid, sensitive and accurate, can be performed simply and conveniently in one vessel (*e.g.*, tube), and requires only small quantities of RNA (*e.g.*, less than about 25 ng). The method can be applied to small tissue samples (*e.g.* biopsies and tumor samples), and can be readily miniaturized and/or adapted for use in a high throughput format. The method can be used for a wide variety of applications, including research purposes (*e.g.* to study X-chromosomal inactivation, genetic imprinting, epigenetics, dominant disorders, and cancer gene expression); to validate allele-specific inhibition (*e.g.* allelic silencing by RNAi techniques); and in diagnostic methods (*e.g.* to detect differential expression of alleles which is correlated with a pathological condition).

One aspect of the invention is a method for quantitating (quantifying) the relative amounts of a plurality of different RNA transcripts expressed from a gene of interest in a subject or cell, comprising performing real time quantitative PCR, in a single container, of cDNAs of the transcripts, in the presence of probes specific for each of the different transcripts (*i.e.*, specific for a defining feature of each transcript), wherein the probes specific for each of the different transcripts are labeled with distinguishable fluorophores. The RNA transcripts can be, *e.g.*, RNAs expressed from two or more alleles of a gene of interest (*e.g.* RNAs expressed from two alleles, wherein one copy is wild type and the other has a mutation or other variant sequence); splice variants transcribed from a gene of interest; RNAs expressed from a plurality of variant microorganism (*e.g.* virus) species; etc. RNA viral genomes from related species can also be detected by methods of the invention. In methods of the invention, the relative amounts of the different transcripts is quantitated by comparison to a standard curve generated from isolated DNAs representing each of the plurality of transcripts.

The PCR is carried out by selecting PCR primers which flank a defining feature of the RNA transcript (or a cDNA thereof) (*i.e.*, a feature which distinguishes a variant RNA from its wild type counterpart, or from other RNA variants). For example, the PCR primers can flank the site of a mutation which distinguishes an allelic variant from its wild type counterpart. Or the PCR primers can flank the splice site of an alternatively spliced variant. The flanking PCR primers are selected such that they amplify a suitable length of the cDNA template. Factors to be considered for determining a suitable amplicon length are discussed elsewhere herein.

A preferred embodiment of the invention is a method for quantitating the relative amounts of the two transcripts expressed from two alleles of a gene of interest (allelic RNAs) in a subject or cell, comprising performing real time quantitative PCR, in a single container, of cDNAs of each of the transcripts, in the presence of probes which are specific for each of the two allelic transcripts, wherein the probes for each transcript are labeled with distinguishable fluorophores, and wherein the relative amounts of expression of the two transcripts is determined by comparison to a standard curve generated from isolated DNAs representing each of the two alleles. In one embodiment of the invention, a cDNA copy is generated from RNA expressed from each of the alleles, and the cDNA is amplified by real time PCR. In one embodiment of the invention, the two alleles differ by a single nucleotide.

In embodiments of the invention, a standard curve is used to quantitate the relative amounts

of the plurality of RNA transcripts. The curve may be generated by various methods, each of which employs isolated DNAs representing the RNA transcripts (one of which may be a wild type counterpart of a particular variant of interest). An isolated DNA which "represents" an RNA transcript is a DNA which comprises a defining feature of the transcript, or a complete complement thereof. For example, the DNA may be a full-length clone of a gene, or it may be a fragment of the gene, provided that the fragment comprises the defining feature of the transcript, such as a mutation or other allelic variation. In one embodiment, the standard curve is generated by expressing RNA from cloned DNA encoding each of the RNA transcripts; generating cDNA from each of the RNAs; and amplifying mixtures comprising different ratios of the cDNAs by real time quantitative PCR in the presence of distinguishable, variant-specific probes. Generally, the PCR primers and probes for amplifying and detecting a DNA representing an RNA transcript of interest are the same as those used to amplify and detect the variant transcript which is being quantitated. If desired, when preparing a standard curve to quantitate expression of mammalian sequences, non-mammalian cDNA, such as insect or *E. coli* cDNA, may be included during the PCR reactions to generate the standard curve; this procedure simulates the conditions found in mammalian (*e.g.* patient) samples and in some circumstances can enhance the accuracy of a standard curve. In another embodiment, the standard curve is generated by providing mixtures comprising different ratios of isolated DNAs representing each of the RNA transcripts. For example, amplicons as described above which comprise defining features of the RNA transcripts can be used. For example, each amplicon can be cloned and then excised for use in preparing a standard curve. The mixtures of DNA are then amplified by real time PCR in the presence of distinguishable, variant-specific probes. By comparing the amounts of the plurality of RNA variants in a test sample to points on the standard curve, one can readily determine the relative amounts of the transcripts in the sample. When allelic expression is being measured, one can determine the relative expression of the alleles.

Another aspect of the invention is a kit for quantitating the relative amounts of RNA expression from a plurality of alleles of a gene of interest. Of course, one of the "alleles" may be a wild type counterpart of a variant(s) (*e.g.*, a wild type splice form, or a wild type counterpart of an allelic mutation). The kit comprises one or more of the following elements:

- (a) reagents for reverse transcribing the RNAs expressed from the alleles, to produce cDNAs (*e.g.*, reverse transcriptase and, optionally, suitable buffers, dNTPs, or the like);
- (b) suitable primers and fluorophore-containing probes for amplifying and detecting the

cDNAs, wherein the primers and probes are preferably in a single container; and

(c) the components for generating a standard curve of the invention, comprising varying ratios of isolated DNAs representing each of the plurality of alleles. Optionally, when the gene of interest is mammalian, and DNAs used to generate the standard curve are generated by reverse transcribing RNAs expressed from the alleles, non-mammalian cDNA may be included in the kit to be included in this reverse transcription reaction.

A method of the invention may be used for a variety of applications.

For example, a method of the invention can be applied to validating a method for specifically inhibiting expression of an allele of interest, *e.g.* using an siRNA. One aspect of the invention is a method for detecting the inhibition of RNA expression in a cell or subject from a first allele of a gene by a putative inhibitor of gene expression which is specific for that first allele (an "allele-specific" inhibitor or silencer), *e.g.* an siRNA, compared to the expression from a second allele of the gene, using a method of the invention to quantitate the relative expression of the two alleles in the presence of the putative inhibitor. In an embodiment of this method, the amount of expression of each allele is determined by comparison to a standard curve generated from isolated DNAs representing each of the two alleles. An illustration of such a method is shown in Example IV and Figure 3.

Kits for validating that an siRNA is specific for an allelic variant of interest and that it is able to inhibit (*e.g.*, silence) expression of that allele are also included. In such a kit, the primers and fluorophore-containing probes are selected so as to amplify and detect cDNA copies of RNAs expressed from the allele of interest, *e.g.* in the presence or absence of the siRNA. Optionally, reagents for reverse transcribing the RNA are present in the kit. The kit can be sold, for example, in conjunction with the siRNA which is to be validated. Alternatively, the RNAi to be validated can be included as part of the kit.

In another example, a method of the invention can be used as a diagnostic assay.

One aspect of the invention is a method for detecting an autosomal-dominant disorder (a disorder which results from a mutation in a first allele, which causes reduced expression from that allele compared to the expression of a wild type allele) in a subject, comprising performing real time quantitative PCR, in a single container, of cDNA copies of RNA expressed from each of the mutant and the wild type alleles, in the presence of a distinguishable allele-specific probe for each of the two alleles, by a method of the invention. An example of such a method is illustrated in Example III

and Figure 2C.

Another aspect of the invention is a method for detecting a recessive disorder (*e.g.*, a compound heterozygous autosomal recessive disorder) which is characterized by differential expression of more than one RNA from a gene of interest, in a subject, comprising performing real time quantitative PCR, in a single container, of a cDNA of each of the RNAs, in the presence of distinguishable probes specific for each RNA, by a method of the invention. An example of such a method is illustrated in Example III and Figures 2A and 2B. An X-linked disorder, either dominant or recessive, can also be detected by such a method.

Another aspect of the invention is a method for detecting a disorder mediated by an alternately spliced RNA from a gene of interest, in a subject, comprising performing real time quantitative PCR, in a single container, of cDNA copies of the alternately spliced RNA and the wild type RNA, in the presence of a probe specific for each of the RNAs, by a method of the invention.

This invention relates, *e.g.*, to a method for quantitating the relative amounts of a plurality of different RNA transcripts from a gene of interest in a subject or cell. As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, "a" gene of interest, as used above, includes two or more genes. The RNA transcripts can take any of a variety of forms.

For example, a method of the invention can be used to distinguish among two or more known splice variants (including alternative splice variants) transcribed from a gene of interest. In this embodiment of the invention, PCR primers are designed which extend across unique exons or fused exon borders of the splice variants. The probes used to detect the amplified DNA contain, at their 5' and 3' ends, a fluorophore and a compatible quencher, such that unique, distinguishable fluorophores are present on the probes for each of the splice variants to be quantitated. In some forms of cancer, for example, aberrant isoforms are translated from aberrant splice variants. A method of the invention can be used to study the alternative splicing experimentally, and/or to diagnose the presence of a disease condition characterized by alternative splicing, such as a cancer.

Another embodiment of the invention is a method to distinguish among transcripts of two or more closely related, but distinguishable, infective organisms, such as microorganisms. For example, a method of the invention can be used as a diagnostic assay to detect expression of multiple variants of HIV or other viruses.

Another embodiment of the invention is a method to distinguish among the RNA genomes of closely related, but distinguishable, infective organisms, such as mutant viruses or virus species.

A preferred embodiment of the invention is a method to quantitate the relative expression from a plurality of (*e.g.* two) alleles of a gene of interest in a subject or cell. The method may be used to compare the levels of expression of two alleles (*e.g.* a wild type and a mutant allele), or it may be used to compare the levels of expression of three or more alleles. The latter may occur, for example, if RNA is expressed from a pseudogene as well as from the full-length allele. Typical (non-limiting) disease conditions characterized by differential allelic expression from pseudogenes and/or full length genes include Gaucher disease and congenital adrenal hyperplasia. Expression from three or more alleles can also occur, for example, in a sample from a patient, such as a blood sample, which contains different cells, which, taken together, contain three or more expression products of allelic forms of a gene of interest.

Much of the discussion herein is directed to comparing (quantitating) the relative expression from a plurality of alleles. It is to be understood that this discussion also relates to comparing (quantitating) the relative amounts of other types of RNA transcripts expressed from a gene of interest, such as splice variants.

The RNA transcripts whose expression is quantitated comprise nucleic acid sequence variants which differ in at least one base within a binding site for a specific probe. For example, consider the case of RNAs expressed from two alleles of a gene of interest. The RNAs transcribed from each allele (RNA allelic variants) comprise the same nucleic acid sequence variation as the DNAs from which they are transcribed (alleles), wherein T residues in the DNA are comparable to U residues in the RNA. Therefore, a probe that is specific for an allelic RNA of interest is also specific for the DNA from which the allelic RNA is expressed, or for a cDNA copy of the RNA. A probe that is specific for an RNA expressed from a gene of interest (*e.g.*, an allelic expression product) will form a match with one sequence variation but a mismatch with the other(s). When expression from multiple alleles is to be distinguished, a different allele-specific probe is used for each allele whose expression is to be measured. These probes form a match only with the allele they are specific for, but form mismatches with all the other alleles.

The RNA transcripts may differ from one another by a single base, two or more non-contiguous bases, a deletion, an insertion, an inversion, combinations thereof, etc. Such differences comprise the defining features of the alleles. A method of the invention can readily distinguish

between expression from alleles which differ from one another by only a single base mismatch.

The term "allele" as used herein, is not limited to variants of a gene in a diploid DNA genome, but is used in a broader manner. The term allele additionally comprises a variety of other nucleic acid sequences with point mutations, deletions, insertions, etc. of one or more base pairs, such as mitochondrial DNA (mtDNA), messenger RNA (mRNA), viral DNA or RNA genomes, or DNA of microorganisms, including viruses. Consequently, the method of the present invention is applicable for the quantification of nucleic acid species in a variety of systems. A method of the invention can be used to detect a variety of RNAs other than mRNA, including, *e.g.*, tRNA, rRNA, microRNAs, etc. As used herein, any of those types of RNAs, or others, or cDNA copies thereof, can be "gene products" of a gene of interest. RNA transcripts which are quantitated by a method of the invention can be expressed from a gene of interest in a subject or cell, or they can be expressed in a cell-free system.

In embodiments of the invention, expression of allelic variants or splice variants from two or more genes is assayed simultaneously. For example, if a disease condition, such as a cancer, is characterized by differential expression of alleles from two (or more) genes, expression from the alleles of both of the genes can be analyzed simultaneously in a sample taken from a subject (*e.g.* a patient). Disease conditions where such an assay can be employed include multigenic cancers (see, *e.g.*, Tayebi *et al.* (2003) *Am. J. Hum. Genet.* 72, 519-534; Felix-Lopez *et al.* (2003) *J. Pediatr. Endocrinol. Metab.* 16, 1017-1024; Guo *et al.* (2005) *Am. J. Pathol.* 166, 877-890; Kibel *et al.* (2003) *Cancer Res.* 63, 2033-2036; Modugno *et al.* (2001) *Clin. Cancer Res.* 7, 3092-3096). Examples of allelic expression of tumor genes or oncogenes that contain heterozygous polymorphisms or mutations have been described, *e.g.*, by Venables (2004) *Cancer Res.* 64, 7647-7654; Kalnina *et al.* (2005) *Genes Chromosomes Cancer* 42, 342-457; Prochownik (2005) *Cell Mol Life Sci.* Sep 7 Epub ahead of print; Steele *et al.* (2005) *Surgeon* 3, 197-205.

In methods of the invention, real time quantitative PCR is carried out. PCR primers are selected which will amplify a segment of a nucleic acid containing the "defining feature" of an RNA transcript (*e.g.*, the site of an allelic variation, a variant splice site, or the like). Typical amplicons range in size from about 50 base pairs (bp) to about 1200 bp, preferably from about 50 to about 150 bp. (All ranges used herein include the end points of the range.) A skilled worker will recognize how to select an amplicon of an appropriate size. Factors to take into account include the possibility that too long a sequence may include polymorphisms other than particular sequence difference of

interest, giving rise to reduced specificity compared to a shorter sequence; and that relatively short sequences can be amplified and analyzed more rapidly than relatively long sequences.

Methods for designing PCR primers and for carrying out PCR reactions (*e.g.* real time PCR), including reaction conditions, such as the presence of salts, buffers, ATP, dNTPs, etc. and the times and temperature of incubation, are conventional and can be optimized readily by one of skill in the art. See, *e.g.*, Innis *et al.*, editors, PCR Protocols (Academic Press, New York, 1990); McPherson *et al.*, editors, PCR: A Practical Approach, Volumes 1 and 2 (IRL Press, Oxford, 1991, 1995); Barany (1991) *PCR Methods and Applications* 1, 5-16; Diffenbach *et al.*, editors, PCR Primers, A Laboratory Manual (Cold Spring Harbor Press); etc.

The "TaqManTM PCR" assay (PE Applied Biosystems) was suggested for detecting polymorphic forms of DNA by Livak *et al.* (1995) *PCR Methods and Applications* 4, 357-362. See also Livak *et al.* (1999), *Genetic Analysis: Biomedical Engineering* 14, 143-149; Lie *et al.* (1998) *Curr. Opin. Biotechnol.* 9, 43-48; Suda *et al.* (2002) *International Journal of Molecular Medicine* 12, 243-246; and Lo *et al.* (2003) *supra*.

Any strand-displacing thermostable DNA polymerase which exhibits a 5'→3' exonuclease activity can be used in a method of the invention. A preferred DNA polymerase is the Taq polymerase isolated from *Thermus aquaticus*. In addition to the wild type enzyme, many variants of Taq are available, including Hot-start Taq, Hi fidelity Taq, Platinum Taq, and many others. Furthermore, many Taq-related enzymes which exhibit the desired properties can be used, including *Pfu* (*e.g.*, Pfu Ultra, Pfu Turbo, Hot start Pfu) and *Herculase*. Suitable polymerases are available from commercial sources, such as Stratagene (La Jolla, CA), Sigma-Aldrich (St. Louis, MO), Roche Diagnostics (Indianapolis, IN), Promega (Madison, WI), Invitrogen (Carlsbad, CA), and Applied Biosystems (Foster City, CA).

In variations of the methods of the invention, the amplification need not be carried out by a thermostable DNA polymerase (PCR). Other DNA polymerases may be used, which operate at lower temperatures, provided that the polymerase is strand-displacing and is associated with a 5'→3' exonuclease activity, and that the amplification can be measured in real time. Typical amplification procedures that can be adapted include ligase based amplification schemes, such as ligase chain reaction (LCR); Q-beta replicase-based amplification schemes; and strand displacement amplification (SDA) schemes. Among the types of strand displacing DNA polymerases which can be used in methods of the invention are: M2 DNA polymerase; VENTTM DNA polymerase; Klenow

fragment of DNA polymerase I; T5 DNA polymerase; modified T7 DNA polymerase; SequenaseTM; and T4 DNA polymerase holoenzyme. Much of the discussion herein is directed to PCR amplification; it is to be understood that amplification by other methods is included.

PCR reactions of the invention are carried out in a single reaction mixture (reaction buffer), in a single reaction chamber (*e.g.*, container, well of a plate, etc.), and/or in a single thermocycling protocol. Any of a variety of reaction chambers can be used, in any of a variety of formats, provided that the material is compatible with the detection of fluorescence. Suitable "optical" plasticware is available from several distributors, including Applied Biosystems (Foster City, CA), Axygen, and Bio Rad. Preferable containers can be closed to form a leak-proof seal, in order to reduce or prevent cross-contamination of samples. Suitable formats for performing the PCR reactions include computer-controlled thermal cyclers with a fluorescence detectors, *e.g.*, ABI7300, ABI7500, ABI7900 (Applied Biosystems, Foster City, CA), Biorad iCycler (Biorad, Hercules, CA), Mx3000P (Stratagene, La Jolla, CA), Lightcycler (Roche, Indianapolis, IN), and Smart Cycler (Cepheid, Sunnyvale, CA).

The sequences of interest are amplified in the presence of distinguishable probes specific for each of the RNA transcripts whose expression is to be measured. The probes have a fluorophore, sometimes referred to herein as a "reporter," at one end but do not fluoresce when free in solution because they have a "quencher" at the other end that absorbs fluorescence from the reporter. Without wishing to be bound by any particular mechanism, it is suggested that during PCR, the polymerase (*e.g.* Taq) encounters a probe specifically base-paired with its target and unwinds it. The polymerase cleaves the partially unwound probe and liberates the reporter fluorophore from the quencher, thereby increasing net fluorescence. The presence of two probes, each labeled with a different fluor, allows one to detect both variants (*e.g.* alleles) in a single tube. Because only perfect double stranded nucleic acids are cleaved by the exonuclease, the method can readily distinguish between RNA transcripts that do or do not bind to a given probe.

Conventional procedures can be used to design an oligonucleotide probe that is specific for an RNA transcript of interest. The terms "variant-specific" probe or "allele-specific" probe, as used herein, refer to a probe which binds specifically to a predetermined sequence of a variant or allele. A probe which binds "specifically" to a sequence binds selectively to that species (*e.g.*, hybridizes to it, or duplexes with it) in comparison to its binding to other sequences, *e.g.* when the target sequence is present in a preparation comprising other alleles expressed from the gene of interest, splice variants

expressed from the gene of interest, or other, unrelated sequences. The phrases "specific for" or "specific to" a polynucleotide have a functional meaning that the polynucleotide can be used to identify the presence of one or more target genes in a sample and distinguish them from non-target genes. It is specific in the sense that it can be used to detect polynucleotides above background noise ("non-specific binding"). A specific sequence is a defined order of nucleotides which occurs in the polynucleotide, *e.g.*, in the nucleotide sequence of an allelic variant or splice variant of interest, and which is characteristic of that sequence, and substantially no non-target sequences. The probe can be of any size which is necessary to confer specificity, *e.g.* about 10, 15, 20, 25 or more nucleotides. In the present case, the probe binds specifically to the variant for which it is designed under the conditions of PCR amplification used in a method of the invention.

The probes used in methods of the invention are designed so that they cannot be extended in the 3' direction by the polymerase in the reaction. For example, the quenchers bound to the 3' end of a probe may prevent it from being extended. The probe and primers generally bind at different temperatures. In general, the probe binds in the range of about 65-94°C, while the primers bind from about 55-65°C, depending on their sequence. See, *e.g.*, the discussion in the Applied Biosystems manual (K.J. Livak, ABI Prism 7700 Sequence Detection System, User Bulletin no. 2, PE Applied Biosystems, 1977). If desired, a minor groove binding protein may be attached to a probe; this assists in more efficient binding of the probe and will help to bring the annealing temperature of the probe within PCR conditions. Furthermore, by using a minor groove binder, probe sequences can be shortened.

With regard to the labeling moieties at the 5' and 3' termini of the specific (*e.g.*, allele-specific) fluorophore-quencher reporter oligonucleotide probes, a wide variety of conventional fluorophores can be used. A skilled worker will recognize sets of spectrally resolvable fluorophores which emit at different frequencies and thus can be readily distinguished. To distinguish among a plurality of alleles or variant sequences, a plurality of fluorescent probes can be used. For example, three or more nucleic variants can be quantitated simultaneously. When quantitating three or more variants, the conditions can be optimized to reduce potential problems with specificity and cross hybridization, using conventional procedures.

Suitable fluorescer-quencher dye sets will be evident to the skilled worker. Some examples are described, *e.g.*, in Holland *et al.* (1991) *Proc. Natl. Acad. Sci.* 88, 7276-7280; WO 95/21266; Lee *et al.* (1993) *Nucleic Acids Research* 21, 3761-3766; Livak *et al.* (1995), *supra*; U.S. Pat. No.

4,855,225 (Fung *et al.*); U.S. Pat. No. 5,188,934 (Menchen *et al.*); PCT/US90/05565 (Bergot *et al.*), and others. Suitable fluorophores include rhodamine dyes and fluorescein dyes, including, *e.g.*, fluorescein; 6-carboxyfluorescein (FAMTM), 2',4',5',7',-tetrachloro-4,7-dichlorofluorescein (HEXTM), 2',7'-dimethoxy-4',5'-6-carboxyrhodamine (JOETM), N',N',N',N'-tetramethyl-6-carboxyrhodamine (TAMRATM) and 6-carboxy-X-rhodamine (ROXTM). Other dyes which can be used include TETTM; VICTM; Texas Red®, Cy3TM, Cy5TM, SYBR® Green I, NEDTM, CAL Fluor Orange 560, BHQ-1, and others. Various combinations of fluorophores can be used. Suitable pairings include, *e.g.*, FAMTM/ROXTM; FAMTM/SYBR® Green I; VIC®/JOETM; NEDTM/TAMRATM/ROX HEXTM FAMTM/SYBR® Green I; VIC®/JOETM; NEDTM/ TAMRATM/ Cy3TM; ROXTM/Texas Red®; Cy5TM dyes; and CAL Fluor Orange 560/ BHQ-1. These and other suitable dyes are available commercially, *e.g.* from Invitrogen (Carlsbad, CA), Applied Biosystems (Foster City, CA), Biosearch Technologies (Novato, CA), and others.

Methods for detecting and quantitating the fluorescence signals are conventional. For example, an ABI PRISMTM Sequence Detection System (PE Applied Biosystems, Germany) can be used. See the ABI user manual (*supra*) for further guidance concerning methods of quantitative real time PCR.

To quantitate the relative levels of expression of a plurality different RNA transcripts, the amount of expression of the transcripts is compared to a standard curve, which is prepared by mixing "isolated" DNAs representing each of the plurality of RNA transcripts in a series of ratios, then amplifying the DNA in those mixtures by real time quantitative PCR. Procedures for preparing such isolated DNAs will be evident to the skilled worker. In one embodiment of the invention, RNA is generated from a DNA clone of each RNA transcript to be quantitated (*e.g.* allelic or splice variants), preferably *in vitro*; the RNA is converted to cDNA; and the cDNAs are mixed in a series of ratios and amplified by real time PCR in the presence of probes that are specific for the RNA transcripts, as described above. In another embodiment, cloned DNAs comprising the defining (distinguishing) features of each allele are optionally amplified (*e.g.* by PCR) and are mixed in a series of ratios and amplified by real time PCR in the presence of specific probes as described above. By comparing the amounts of the plurality of RNA transcripts in a test sample to points on the standard curve, one can readily determine the relative expression of the plurality of RNA transcripts in the sample.

An "isolated" DNA, as used herein, refers to a DNA molecule (*e.g.* a polynucleotide or

oligonucleotide) which is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring), and is isolated or separated from at least one other component with which it is naturally associated. For example, a naturally-occurring polynucleotide present in its natural living host is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a composition, and still be isolated in that such composition is not part of its natural environment. An isolated DNA representing an RNA transcript of interest (*e.g.*, a full-length gene or cDNA, or a fragment thereof which comprises a defining feature of the RNA transcript) can be prepared by PCR-amplifying and/or cloning the sequence, synthesizing it chemically, or other methods which will be evident to a skilled worker. An "isolated" sequence, as used herein, is to be distinguished from that sequence when it is present in genomic DNA.

The present inventors have found that using such isolated DNAs to generate a standard curve, rather than using genomic sequences, has many advantages. For example, standard curves generated with isolated sequences are generally more accurate and consistent. The reasons for this improvement are not well-understood. Without wishing to be bound by any particular mechanism, it is suggested that in genomic DNA, a sequence of interest may be obscured or masked by other sequences in the genomic DNA, or that other homologous sequences present in the genomic DNA may be amplified by the PCR primers, skewing the amplification.

A standardization curve prepared as above may not be reliable. To address this problem, particularly when the DNA to be measured is from a mammalian source, one can optionally include non-mammalian DNA (*e.g.* from an insect or *E. coli*) in the real time PCR reactions of the various ratios, in order to simulate the conditions found in mammalian tissue (*e.g.* in patient samples).

Samples for analysis can be obtained from any suitable source. For example, cells or tissues from eukaryotes (*e.g.*, plants or animals, such as mammals, including humans), including tumor samples, biopsy samples or other tissues, or bodily fluids, such as blood or blood fractions, urine, seminal fluid, etc., can be isolated from a subject (*e.g.* a patient); and RNA (*e.g.* expressed RNA) can be isolated by conventional procedures and subjected to a method of the invention. Methods of isolating RNA and other molecular biology methods used in the invention can be carried out using conventional procedures. See, *e.g.*, discussions in Sambrook, *et al.* (1989), *Molecular Cloning, a Laboratory Manual*, Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel *et al.* (1995). *Current Protocols in Molecular Biology*, N.Y., John Wiley & Sons; Davis *et al.* (1986),

Basic Methods in Molecular Biology, Elsevier Sciences Publishing, Inc., New York; Hames *et al.* (1985), *Nucleic Acid Hybridization*, IL Press; Dracopoli *et al.* (current edition) *Current Protocols in Human Genetics*, John Wiley & Sons, Inc.; and Coligan *et al.* (current edition) *Current Protocols in Protein Science*, John Wiley & Sons, Inc.

5 The disclosed methods can be used to quantitate the relative amounts of RNA expressed from a variety of templates. For example, in addition to measuring the expression of RNA transcripts from cellular or tissue nucleic acid samples, a method of the invention can be used to measure nucleic acids, or expression from nucleic acids, which include cloned fragments or subclones thereof, chemically synthesized nucleic acids, genomic nucleic acid samples, cDNAs,
10 nucleic acid molecules obtained from nucleic acid libraries, etc.

 Methods of the invention can be readily adapted to a high throughput format, using automated (*e.g.* robotic) systems, which allow many measurements to be carried out simultaneously. Furthermore, the methods can be miniaturized (*e.g.*, carried out in reaction buffers of about 25 μ l, 1 μ l, 0.1 μ l, or less).

15 The order and numbering of the steps in the methods described herein are not meant to imply that the steps of any method herein must be performed in the order in which the steps are listed or in the order in which the steps are numbered. The steps of any method disclosed herein can be performed in any order which results in a functional method. Furthermore, the method may be performed with fewer than all of the steps, *e.g.*, with just one step.

20 A variety of applications for methods of the invention will be evident to the skilled worker. For example, a method of the invention can be substituted for any method in which the relative amounts of a plurality of RNA transcripts expressed from a gene of interest is quantitatively measured. In one embodiment, the relative expression of an imprinted gene and a non-imprinted correlate can be monitored. See, *e.g.*, the imprinted genes discussed in Lo *et al.* (2003) *Genome*
25 *Research* 13, 1855-1862.

 Methods of the invention can be used to monitor the relative amounts of a plurality of RNA transcripts from a gene of interest (*e.g.* the relative expression of variant alleles) which are associated with a disease condition (*e.g.* a pathological disorder). Such measurements can be used in experimental studies of the disease condition, in methods of prognosis, or in diagnostic methods,
30 such as methods to detect the presence of the condition, to follow the progression of the condition or its response to a therapeutic treatment; etc.

Among the conditions which can be detected by a method of the invention are autosomal-dominant disorders (disorders which result from a mutation in one allele, which causes reduced expression from that allele compared to the expression of a wild type allele). Among the many types of mutations that can be involved in such autosomal-dominant disorders are nonsense, missense and frame-shift mutations which result in RNA decay. Typical (non-limiting) examples of human autosomal-dominant disorders include incontinentia pigmenti (Berlin *et al.* (2002) *J. Am. Acad. Dermatol.* 47, 169-187), Hutchinson-Gilford progeria syndrome (Scaffidi *et al.* (2005) *Nat. Med.* 11, 440-445), neurofibromatosis (Eisenbarth *et al.* (2000) *Am. J. Hum. Genet.* 66, 393-401), myotonic dystrophy (Korade-Mirnic *et al.* (1999) *Hum. Mol. Genet.* 8, 1017-1023), sialuria, Machado-Joseph disease/spinocerebellar ataxia, frontotemporal dementia (Miller *et al.* (2003) *Proc. Natl. Acad. Sci. U.S.A.* 100, 7195-7200), amyotrophic lateral sclerosis (ALS) (Maxwell *et al.* (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 3178-3183), slow channel congenital myasthenic syndrome (Abdelgany *et al.* (2003) *Hum. Mol. Genet.* 12, 2637-2644), and spinobulbar muscular atrophy (Caplen *et al.* (2002) *Hum. Mol. Genet.* 11, 175-184). Other disease conditions characterized by differential allelic expression include various types of cancers. For example, a cancer suppressor gene, such as p53, may be down-regulated, or an oncogene may be upregulated (Venables (2004) *Cancer Res.* 64, 7647-7654; Kalnina *et al.* (2005) *Genes Chromosomes Cancer* 42, 342-457; Prochownik (2005) *Cell Mol Life Sci.* Sep 7 Epub ahead of print; Steele *et al.* (2005) *Surgeon* 3, 197-205).

Other conditions that can be detected by methods of the invention are autosomal recessive disorders (*e.g.*, compound heterozygous autosomal recessive disorders), which are characterized by differential expression of more than one RNA transcript from a gene of interest, in a subject. Such differential expression can result from a variety of mechanisms, including *e.g.* a nonsense, missense or splice site mutation in a first allele which results in RNA decay or alternate splicing and thus altered expression compared to expression of the comparable wild type allele. Other mechanisms that can be responsible for up or down regulation of RNA expression include insertions, deletions, frame shift mutations, etc, within coding or non-coding sequences of a gene, or mechanisms such as genetic imprinting, X-inactivation, etc. Typical (non-limiting) examples of such autosomal recessive disorders include diabetes (Pugliese *et al.* (2002) *Diabetes Metab. Res. Rev.* 18, 13-25), cystic fibrosis, homocystenuria, hereditary inclusion body myopathy, Hermansky-Pudlak syndrome, cystinosis, Zellweger syndrome, beta-thalassemia, alkaptonuria, individual genetic variations on

drug disposition, efficacy and safety (Lamba *et al.* (2002) *Adv. Drug Deliv. Rev.* 54, 1271-1294), certain cancers (Chi *et al.* (1999) *Cancer Res.* 59, 2791-2793), and a variety of other human genetic diseases (Holbrook *et al.* (2004) *Nat. Genet.* 36, 801-808; Culbertson (1999) *Trends Genet.* 15, 74-80). A number of cancers, for example, have been reported to be associated with specific mutations and/or alternative splice variants.

A method of the invention can be used to characterize the progression of, or the staging of, a disease condition of interest. It has been suggested that differences in allelic expression can modify the phenotype of a number of diseases. For example, expression of the Decorin gene 179 allelic variant is associated with a slower progression of renal disease in patients with type I diabetes (DeCosmo *et al.* (2002), *Nephron* 92, 72-76); differences in allelic expression of the CLCN1 gene influence the myotonia congenital phenotype (Duna *et al.* (2004) *Eur. J. Hum. Genet.* 12, 738-743); and allelic variation in normal FBN1 expression has been suggested to modify phenotype in family members with Marfan syndrome (Hutchinson *et al.* (2003) *Hum. Mol. Genet.* 12, 2269-2276). Other examples will be evident to the skilled worker.

Examples II-III herein show the results of a study to determine whether mutation-dependent variations in allelic expression of the *GNE* gene can account for the variable disease phenotypes of sialuria and Hereditary Inclusion Body Myopathy (HIBM).

Hereditary Inclusion Body Myopathy (HIBM; MIM 600737) and sialuria (MIM 600737) are two distinct disorders resulting from mutations in the same gene, *GNE*, coding for the bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase (GNE)/ *N*-acetylmannosamine kinase (MNK). GNE/MNK catalyzes the first two committed, rate-limiting steps in the biosynthesis of sialic acid, and is feedback-inhibited at its allosteric site by the end product, CMP-sialic acid.

Sialuria exhibits autosomal dominant inheritance; patients are heterozygous for a missense mutation in the allosteric site of *GNE* (codons 263-266), leading to a loss of feedback-inhibition. Consequently, cytoplasmic accumulation and urinary excretion of large quantities of free sialic acid occur. In contrast, HIBM is inherited in an autosomal recessive fashion; patients harbor two recessive *GNE* mutations (mostly missense) outside of the allosteric site. HIBM mutations lead to decreased GNE/MNK enzyme activity and, in some patients, decreased sialylation of muscle glycoproteins. Both sialuria and HIBM have a variable phenotypic expression and disease progression, not only among patients with the same *GNE* mutations but also among affected patients within the same family.

Although the mutations tested in the Examples herein did not reveal changes in allelic expression, these experiments provide proof of principle for the use of the rapid, convenient methods of the invention.

Because a variety of disease conditions appear to be mediated by expression of an undesirable (detrimental) allele, *e.g.* aberrant expression, such as over-expression, of one allele (often a mutant) of a gene, it is often desirable, both for experimental purposes and in clinical applications, to specifically inhibit expression of the undesirable allele. The gene may be inhibited partially, or it may be substantially inhibited, *e.g.* silenced or knocked out. Methods which can be used for such specific inhibition of expression include, *e.g.*, the use of antisense molecules, ribozymes or the like. A method of great current interest is the inhibition of the expression of an undesirable (detrimental) allele by RNA silencing, using an allele-specific siRNA. For example, Machado-Joseph disease/spinocerebellar ataxia, frontotemporal dementia, ALS, slow channel congenital myasthenic syndrome, and spinobulbar muscular atrophy have been reported to be responsive to RNAi silencing.

Presently, methods for confirming *in vitro* that such inhibition has occurred rely on crude, cumbersome techniques such as visual inspection of the treated cells under a microscope to observe whether morphological changes have taken place. The inventive methods can be used, as an alternative, improved, procedure to determine the level of expression from the allele before and after specific inhibition of expression (*e.g.* siRNA treatment), thereby validating the efficacy of the inhibitory agent (*e.g.* an siRNA). Furthermore, the ability of an siRNA to inhibit the expression of the targeted gene compared to a control can be tested, to validate the specificity of the inhibitory agent (*e.g.*, siRNA).

Example IV illustrates allele-specific silencing of an allele (the dominant disease allele in siluria) with an allele-specific siRNA; and shows that, using a method of the invention, one can validate that an allele-specific siRNA of interest is specific and effective.

Any combination of the materials useful in the disclosed methods can be packaged together as a kit for performing any of the disclosed methods. For example, PCR primers and/or fluor-containing probes for one or more RNA transcripts of interest can be packaged individually or in various combinations. In one embodiment, PCR primers and probes suitable for the detection of two or more RNA transcripts expressed from a gene of interest are packaged together in a single container (such as an Eppendorf tube). Components for reverse transcribing an RNA to be measured

(e.g. a reverse transcriptase and, optionally, suitable reaction components) may also be included. If desired, components for generating a standard curve may be included (e.g., a set of isolated DNA molecules, each of which represents one of the RNA transcripts of interest). If desired, the reagents are packaged in single use form, suitable for carrying one set of quantitation comparisons.

5 Kits may supply reagents in pre-measured amounts so as to simplify the performance of the subject methods. Optionally, kits of the invention comprise instructions for performing the method. Other optional elements of a kit of the invention include suitable buffers, packaging materials, a thermostable strand-displacing DNA polymerase suitable for use in fluorescer-quencher probe assays, *i.e.*, having 5'-3' exonuclease activity, such as Taq DNA polymerase, etc. The kits of the
10 invention may further comprise additional reagents that are necessary for performing the subject methods. Such reagents include, but are not limited to dNTP mixtures, buffers, etc. The reagents of the kit may be in containers in which they are stable, *e.g.*, in lyophilized form or as stabilized liquids.

15 DNAs used in methods of the invention (*e.g.*, for generating standard curves, or as probes or primers) can have one or more modified nucleotides, provided that the functionality of the DNA is not destroyed.. For example, they may contain one or more modifications to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T as well as different purine or pyrimidine bases, such as uracil-5-yl,
20 hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-
25 thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch *et al.* (1991) *Angewandte Chemie*,
30 *International Edition* 30, 613, and Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such

as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex formation. Base modifications often can be combined with for example a sugar modification, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability. There are numerous United States patents such as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, which detail and describe a range of base modifications.

Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety would include natural modifications of the ribose and deoxyribose as well as synthetic modifications. Sugar modifications include but are not limited to the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10, alkyl or C2 to C10 alkenyl and alkynyl. 2' sugar modifications also include but are not limited to --O[(CH₂)_nO]_mCH₃, --O(CH₂)_nOCH₃, --O(CH₂)_nNH₂, --O(CH₂)_nCH₃, --O(CH₂)_n--ONH₂, and --O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10.

Other modifications at the 2' position include but are not limited to: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂, CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars would also include those that contain modifications at the bridging ring oxygen, such as CH₂ and S. Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. There are numerous United States patents that teach the preparation of such modified sugar structures such as U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein

incorporated by reference in its entirety.

Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate moieties include but are not limited to those that can be modified so that the linkage between two nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkyl-phosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkages between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Numerous United States patents teach how to make and use nucleotides containing modified phosphates and include but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

It is understood that nucleotide analogs need only contain a single modification, but may also contain multiple modifications within one of the moieties or between different moieties.

Nucleotide substitutes are nucleotides or nucleotide analogs that have had the phosphate moiety and/or sugar moieties replaced. Nucleotide substitutes include molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes include molecules that will recognize and hybridize to complementary nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

Substitutes for the phosphate can be for example, short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate back-

bones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts. Numerous United States patents disclose how to make and use these types of phosphate replacements and include but are not limited to U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

It is also understood in a nucleotide substitute that both the sugar and the phosphate moieties of the nucleotide can be replaced, by for example an amide type linkage (aminoethylglycine) (PNA). U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262 teach how to make and use PNA molecules. See also Nielsen *et al.* (1991) *Science* 254, 1497-1500.

DNA molecules of the invention can be made up of different types of nucleotides or the same type of nucleotides. For example, one or more of the nucleotides in a primer can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 10% to about 50% of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 50% or more of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; or all of the nucleotides are ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides. The nucleotides can be comprised of bases (that is, the base portion of the nucleotide) and can comprise different types of bases. For example, one or more of the bases can be universal bases, such as 3-nitropyrrole or 5-nitroindole; about 10% to about 50% of the bases can be universal bases; about 50% or more of the bases can be universal bases; or all of the bases can be universal bases.

In the foregoing and in the following example, all temperatures are set forth in uncorrected degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

EXAMPLES

Example I - Materials and Methods

A. Patients and Cells

HIBM patient H1 was a 27-year-old male of English/Irish/Scottish descent who had

progressive weakness beginning at 20 years of age and recently required crutches to ambulate. He was compound heterozygous for two missense *GNE* mutations; c.735C>T (Arg246Trp) and c.403G>T (Gly135Val). HIBM patient H2 was a 37-year old female of non-Jewish descent. She had onset of leg weakness in her early twenties and became wheelchair-bound in her mid thirties. She was compound heterozygous for *GNE* mutations c.646T>C (Val216Ala) and c.2018C>T (Ala631Val) (Vasconcelos *et al.* (2002) *Neurology* 59, 1776-1779). Sialuria patient S1 (Krasnewich *et al.* (1993) *Biochem. Med. Metab. Biol.* 49, 90-96) presented with mildly coarse facies, slight motor delay, and urinary excretion of large quantities (>1 g/d) of free sialic acid. Patient S1 was heterozygous for the missense mutation c.787G>T (Arg263Leu), located within the allosteric site of *GNE*.

Primary cultures of skin fibroblasts of all patients were obtained from a skin biopsy and grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum containing 100 U/ml penicillin and 0.1 mg/ml streptomycin.

B. In Vitro Transcription

The human *GNE* coding sequence (GenBank accession number NM_005476) was amplified from cDNA of normal human fibroblasts and cloned, using *EcoRI* and *XhoI* restriction sites, into the *pET17b* vector (EMD Biosciences, San Diego, CA), which contained an N-terminal T7-tag and a T7 promoter sequence. This *GNE-pET17b* plasmid was used to create each of the patient-specific *GNE* target mutations by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were verified by sequencing before experimental use. PCR amplifications of the *GNE* coding sequence, including the T7-promotor sequence of *pET17b*, were performed using pure Taq Ready-To-Go PCR beads (Amersham, Piscataway, NJ). Amplifications were performed in a 25 µl reaction volume containing 25 ng *GNE-pET17b* plasmid DNA (normal or mutated) and 0.4 µM of each primer flanking the T7-promotor (forward, 5'-GACTCACTATAGGG-3') (SEQ ID NO:21) and the *GNE* termination codon (reverse, 5'-GCTAGTTATTGCTCAGCGG-3') (SEQ ID NO:22).

After amplification, the T7-promotor sequence on each PCR fragment was employed for RNA transcription of normal and mutated *GNE* using the MAXIscript *In Vitro* Transcription Kit (Ambion, Austin, TX).

C. DNA, cDNA, and RNA Preparation

Skin fibroblasts of patients and normal controls were plated and grown to 80-90% confluency after which genomic DNA or total RNA was isolated. Genomic DNA was obtained using the Wizard genomic DNA purification kit (Promega, Madison, WI). Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA). Bacterial total RNA was isolated from *TOP10* chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA) using the Trizol Max Bacterial RNA isolation kit (Invitrogen). All RNA samples were treated with RNase-free DNase (rDNase; Ambion, Austin, TX), and purified using RNeasy mini columns (Qiagen, Valencia, CA). First strand cDNA synthesis was performed using the High Capacity cDNA Archive kit employing oligo-dT primers (Applied Biosystems, Foster City, CA), after which the cDNA was purified using RNeasy mini columns (Qiagen Valencia, CA). Concentrations of RNA, genomic DNA and cDNA were measured with a GeneQuant Pro spectrophotometer (Amersham, Piscataway, NJ).

D. Allelic Discrimination by Real-Time RT-PCR

TaqManTM primers and probes (Table 1) were designed for each specific target mutation in the *GNE* gene (Applied Biosystems, Foster City, CA). For each assay, the region flanking the *GNE* target mutation was PCR amplified by a specific primer set in the presence of two probes, each selectively hybridizing to the normal (5' VIC-labeled) or the mutated (5' FAM-labeled) allele (Table 1). A non-fluorescent quencher and a minor groove binder (MGB) were connected to the 3' ends of each probe. During the PCR amplification, the 5'-exonuclease activity of *Taq* DNA polymerase cleaves fully hybridized probes, separating the reporter dye (VIC or FAM) from the quencher, resulting in fluorescence. The internal reference dye (ROX) is used to correct for non-PCR related fluorescence fluctuations. PCR-generated fluorescent VIC and FAM signals can be measured either at their endpoint (after the last PCR cycle, performed for Figure 1) or in 'real time', where the increase in fluorescence is followed on a per-cycle basis (performed for Figure 2).

For each allele-specific real-time PCR reaction a total reaction mixture of 25 µl contained 50 nM primers and 50 nM allele-specific VIC/FAM probes, 25 ng of genomic DNA or 50 ng of cDNA, and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). All real-time PCR reactions and subsequent analyses were performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The pre-run thermal cycling conditions were 10 min at 95°C to activate the *Taq* DNA-polymerase, followed by 40 cycles of 95°C for 15 s and 60°C

annealing/extension for 1 min. Each experiment was performed in duplicate. Within each experiment, reactions were run in duplicate.

For each target *GNE* mutation, a standard curve was constructed by mixing two allelic cDNA samples to allelic ratios of 8, 4, 2, 1, 0.5, 0.25 and 0.125 in a total concentration of 50 ng/μl. In addition, 10 ng/μl bacterial cDNA was added to each standard curve reaction mixture.

Example II - Validation of the Taqman assays

The Taqman real-time assays for each of the five *GNE* target mutations (Table 1) were validated using genomic DNA from normal controls and from HIBM (H1, Figures 1A and 1B; H2, Figures 1C and 1D) and sialuria (S1, Figure 1E) patients. The endpoint readings of each fluorescent probe (Rn, normalized reporter signal) after the final PCR cycle were determined and visualized in a scatter plot. Figure 1 shows that the patients' samples cluster in the heterozygous area of each plot (3 independent experiments), while the normal control samples cluster in the homozygous area for the normal allele, demonstrating accurate allele-specificity of each assay. Non-template control assays (ntc) show very minimal fluorescence. Since none of the patients was homozygous for the tested *GNE* mutations, no results are shown in the homozygous mutated allele area on each plot (indicated by dotted circles).

Example III - Allele-specific RNA expression

To determine whether any of the patients' *GNE* mutations resulted in different allelic mRNA expression levels, standard curves were prepared for each assay. Allelic cDNAs were prepared from cell-free transcribed RNA for each target *GNE* mutation and mixed in various ratios (normal allele:mutated allele ranging from 8 - 0.125) to a total cDNA concentration of 50 ng/μl. Since each standard curve mixture consisted of 'pure' cDNA produced from *in vitro* transcribed RNA, we added bacterial cDNA (non-specific cDNA) to reflect the situation in the patient's samples as closely as possible. BLAST analysis (see the web site at ncbi.nih.gov/BLAST/) showed that our primer-probe assays were not located in an area homologous to an expressed bacterial gene.

For each standard-curve dilution the real-time PCR generated fluorescent VIC and FAM signals that were followed on a per-cycle basis. The comparative Ct method was used for analysis of the PCR results [K.J. Livak (1997) Comparative Ct method. ABI Prism 7700 Sequence Detection System. User Bulletin no. 2. PE Applied Biosystems] Ct is the PCR cycle number at which the

fluorescence generated by cleavage of a probe reaches a fixed threshold above baseline. At a given threshold, a higher Ct value indicates a lower starting copy number. The Ct was manually assigned or determined by the auto-Ct option using the SDS 2.1 software of Applied Biosystems. For all cDNA samples the Ct's fell within the range of the standard curves. The difference in Ct's between the different probes is ΔCt ($\Delta Ct = Ct_{VIC} - Ct_{FAM}$ for our assays) and is a measure of the initial concentration ratio of each allele. A one cycle difference ($\Delta Ct = 1$) relates to a ratio of one allele to the other of 1:2, a two cycle difference ($\Delta Ct = 2$) to 1:4, a three cycle difference ($\Delta Ct = 3$) to 1:8; or in general, the ratio of the expressed amount of RNA of one allele to the other is $1:2^{\Delta Ct}$ (Livak, 1997, *supra*)

Figure 2 (left panels) shows the regression curves of observed $2^{\Delta Ct}$ plotted against mixed allelic ratios for each *GNE* target mutation. For each of the assays, the experimental curves (solid lines) closely resembled the expected curves (dotted lines) ($r^2 > 0.98$), indicating that the probes showed minimal cross-reactivity.

To further validate this quantitative assay, for each of the allelic ratios (8, 4, 2, 1, 0.5, 0.25) that comprised the standard curve, a 1x, 2x, 4x, and 8x dilution series was made. The Ct values for both probes in this dilution series were 0, 1, 2, and 3 cycles different respectively. This indicates that 1 cycle difference relates to approximately a two-fold concentration difference, thereby justifying the use of the $1:2^{\Delta Ct}$ formula for allelic expression.

Next, the standard regression curves were used to determine allele-specific RNA expression in fibroblasts of patients with HIBM and sialuria (Fig. 2). cDNA was prepared from DNA-free RNA of each patient and control and subjected to the *GNE* target mutation-specific Taqman assays. The measured $2^{\Delta Ct}$ values were then used to determine the allele ratios from the regression curve (dotted lines in Figure 2). For example, the allele-specific assay for the c.735C>T mutation in HIBM patient H1 (Fig. 2A), yielded a $2^{\Delta Ct}$ reading of 1.1 (average of 2 independent experiments); this extrapolated in the regression curve in Fig. 2A (dotted line) to an allele ratio of 0.78.

This ratio means that the C allele and the T-allele at position 735 of *GNE* mRNA in this patient are expressed in similar amounts. Allelic ratios of approximately 1 were also obtained for the other tested *GNE* mutations, *i.e.*, 0.8 for the H1 c.403G>T allele, 0.83 for H2 c.646T>C (Fig. 2B), and 1.1 for the S1 c.787G>T allele (Fig 2C), indicating that the *GNE* gene is not susceptible to allelic mutation-specific alterations in expression, at least for the tested mutations. The cDNA obtained from wild type controls yielded $2^{\Delta Ct}$ readings outside the standard curve readings for

each assay. For some assays, the regression curve diverged slightly from the expected curve (as in Fig. 2C), which can be due to reduced quality of the control cDNA and/or to cross reactivity of the allele-specific probes. In fact, for the c.2018C>T allelic assay of patient H2 the observed and expected standard curves deviated too much from each other, probably due to extensive cross-hybridization of the probes, and accurate readings could not be made. In this case, further optimization of the primer/probe set would be needed for reliable measurements of allelic expression.

Although the tested mutations did not reveal changes in allelic expression, these experiments precisely measured allelic expression and provide a proof of principle for the use of this rapid, convenient method.

Example IV - Allele-specific silencing of the dominant disease allele in sialuria

Sialuria ('French type' sialuria; MIM 269921) is an autosomal dominant disorder characterized by mild coarse facies and slight motor delay, and profound cytoplasmic accumulation and urinary excretion of large quantities (>1 gram/day) of free sialic acid. Additional sporadic clinical features are hepatosplenomegaly, delayed skeletal development, microcytic anemia, and mild intellectual impairment.

The molecular defect of sialuria is a failure to regulate sialic acid synthesis, due to impaired allosteric feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-sialic acid (Figure 3A). The loss of feedback inhibition due to a mutation in one allele is enough to constitutively produce cytoplasmic free sialic acid. All described sialuria patients are heterozygous for a missense mutation in one of two amino acids, arginine at position 263 (R263L) or arginine at position 266 (R266Q; R266W). The clustering of these mutations in the region of codons 263–266 supports the hypothesis that this region is part of the allosteric site for CMP-sialic acid binding.

Currently, only symptomatic therapy is available for sialuria patients. Very few other human disorders with dominant inheritance due to failed allosteric inhibition are reported, *e.g.*, hyperinsulinism and hyperammonia in infants with regulatory mutations of the glutamate dehydrogenase gene (see, *e.g.*, Stanley *et al.* (1998) *N. Engl. J. Med.* 338, 1352-1357; Fang *et al.* (2002) *Biochem. J.* 363, 81-87). Therapies for these disorders are also lacking. However, recent *in vitro* studies have suggested that allele-specific silencing of dominant disease genes might be future (gene-) therapies for treatment of dominant disorders (Miller *et al.* (2003) *Proc. Natl. Acad. Sci.*

U.S.A. 100: 7195-7200; Abdelgany et al. (2003) *Hum. Mol. Genet.* 12, 2637-2644; Saito et al. (2005) *J. Biol. Chem.* 280, 42826-42830; Rodriguez-Lebron E et al. (2006) *Gene Ther.* 13, 576-581). Sialuria would be a good candidate for such a therapy.

In this Example, we employed siRNA specifically targeting the dominant mutation c.797G>A (R266Q) in fibroblasts from a sialuria patient (Figures 3B and 3C). We then confirmed allele specific silencing at the RNA level by allele-specific real time PCR (Figure 3D,E), using a method of the invention. Furthermore, this allele-specific silencing in sialuria fibroblasts resulted in decreased total sialic acid levels and recovery of feed-back inhibition of the epimerase enzyme by CMP-sialic acid.

Our findings indicate that allele-specific silencing of the mutated *GNE* allele is a viable therapeutic strategy for autosomal dominant diseases, including sialuria. The present Example further supports the therapeutic potential of RNAi-based methods for the treatment of inherited human diseases, including sialuria.

Table 1. Primer and probe sequences for each *GNE* target mutation

<u><i>GNE</i> target mutation^a</u>	<u>Patients</u>	<u>Forward primer</u>	<u>Reverse primer</u>	<u>Tagman probes^b</u>
c.735C>T	H1	5'-GTTTGAATTAAACATTGGATGGCACTTATCTCA-3' (SEQ ID NO 1)	5'-TCCCTGCGTCAATATTTGGAAACA-3' (SEQ ID NO 2)	5'-NFQ-CTAGGGTCCGCTTGT-T-VIC-3' (SEQ ID NO 3) 5'-NFQ-CTAGGGTCCACTTGT-T-FAM-3' (SEQ ID NO 4)
c.403G>T	III	5'-GCCTTGATGAACATCCGAATCCTT-3' (SEQ ID NO 5)	5'-TTTTTGTATGGCATGTCTGATAGATCA-3' (SEQ ID NO 6)	5'-NFQ-ACCTCCCACTTCAA-VIC-3' (SEQ ID NO 7) 5'-NFQ-ACCTCCCACTTCAA-FAM-3' (SEQ ID NO 8)
c.646T>C	H2	5'-GCAATGTGGCTAGGTGATGATGATAA-3' (SEQ ID NO 9)	5'-GGAATGCTTAATGTCAGTGGTCACA-3' (SEQ ID NO 10)	5'-NFQ-TGTAGTGCAACAATGTA-VIC-3' (SEQ ID NO 11) 5'-NFQ-TAGTGCAGCAATGTA-FAM-3' (SEQ ID NO 12)
c.2018C>T	H2	5'-GGTGGCTCCATCTCATC-3' (SEQ ID NO 13)	5'-CTGGGCGCTTGGCATTTGC-3' (SEQ ID NO 14)	5'-NFQ-CAAGTTTTCGACGCTTG-VIC-3' (SEQ ID NO 15) 5'-NFQ-CAAGTTTTCGACGCTTG-FAM-3' (SEQ ID NO 16)
c.787G>T	S1	5'-ATTGACCGGAGGACCAAGA-3' (SEQ ID NO 17)	5'-GGATGATGCTCAATGCCCTTCTT-3' (SEQ ID NO 18)	5'-NFQ-CATCATCTGGAACCATC-VIC-3' (SEQ ID NO 19) 5'-NFQ-CATCATCTGGAACCATC-FAM-3' (SEQ ID NO 20)

NFQ = non-fluorescent quencher.

^aThe A nucleotide of the *GNE* initiation codon ATG is assigned number 1.

^bThe base substitution at the target mutation sites are underlined.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding
5 description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications cited above and in the figures are hereby incorporated by reference.

We claim:

1. A method for quantitating the relative amounts of a plurality of different RNA transcripts expressed from a gene of interest in a subject or cell, comprising performing real time quantitative PCR, in a single container, of cDNAs of the transcripts, in the presence of probes specific for each of the different transcripts, wherein the probes for each of the different transcripts are labeled with distinguishable fluorophores,

wherein the relative amounts of the plurality of transcripts is quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the plurality of transcripts.

2. The method of claim 1, wherein the PCR is performed on cDNAs that are less than full-length copies of the transcripts, but that comprise a defining feature of the transcripts; and wherein the specific probes are specific for the defining features.

3. The method of claim 1, wherein the different RNA transcripts are expressed from allelic variants of the gene of interest, are splice variants of the gene of interest, or are expressed from the genomes of variant species of an organism of interest.

4. The method of claim 3, wherein the organisms of interest are different variants of a virus.

5. The method of claim 1, wherein the different RNA transcripts are expressed from two different alleles of a gene of interest.

6. The method of claim 5, further wherein the relative RNA expression from different alleles of one or more additional genes of interest is also quantitated, in the same container.

7. The method of claim 5, wherein the amount of expression from each allele is determined by generating a cDNA copy of RNA expressed from the allele and amplifying the cDNA by real time PCR.

8. The method of claim 5, wherein the standard curve is generated by expressing RNA from a cloned

DNA of each of the two alleles; generating cDNA from each of RNAs; and amplifying mixtures comprising different ratios of the two cDNAs by real time PCR in the presence of variant-specific probes.

9. The method of claim 5, wherein the standard curve is generated by providing mixtures comprising different ratios of DNA representing each of the two alleles, and amplifying those mixtures by real time PCR in the presence of variant-specific probes.

10. The method of claim 8, wherein the gene is mammalian, and the method for generating the standard curve further comprises including non-mammalian cDNA in the mammalian cDNA mixtures which are amplified.

11. The method of claim 1, wherein two different RNA transcripts, which differ by a single nucleotide, are quantitated.

12. The method of claim 5, wherein the two different RNA transcripts differ by a single nucleotide.

13. A kit for quantitating the relative RNA expression from a plurality of alleles of a gene of interest, comprising,

(a) a single container comprising suitable primers and fluorophore-containing probes for amplifying and detecting cDNAs that are reverse transcribed from the RNA expressed from the alleles.

14. The kit of claim 13, further comprising

(b) the components for generating a standard curve, comprising varying ratios of isolated DNAs representing each of the plurality of alleles.

15. The kit of claim 14, further comprising reagents for reverse transcribing the RNA expressed from the alleles, to produce cDNAs.

16. A method for detecting the inhibition of RNA expression in a cell or subject from a first allele of a

gene by a putative inhibitor of gene expression which is specific for said first allele, compared to the expression from a second allele of the gene, comprising quantitating the relative expression of the first allele and the second allele, in the presence of the putative inhibitor, by the method of any of claims 1-12.

17. The method of claim 16, wherein the inhibitor is an siRNA specific for the first allele.

18. The method claim 16 or 17, further wherein the relative expression of the first allele compared to the second allele is determined in the absence of the inhibitor, and the ratio of the expression of the first allele in the absence of, and in the presence of, the inhibitor is determined.

19. A method for detecting the inhibition of RNA expression in a cell or subject from a first allele of a gene by a putative inhibitor of gene expression which is specific for said first allele, compared to the expression from a second allele of the gene, comprising performing real time quantitative PCR, in a single container, of cDNAs of the RNA transcripts, in the presence of the putative inhibitor and probes specific for each of the two transcripts, wherein the probes for the two different transcripts are labeled with distinguishable fluorophores.

20. The method of claim 19, wherein the inhibitor is an siRNA specific for the first allele.

21. The method of claim 19 or 20, further wherein the relative RNA expression from the first allele compared to the second allele is determined in the absence of the inhibitor, and the ratio of the expression of the first allele in the absence of, and in the presence of, the inhibitor is determined.

22. The method of claim 19 or 20, further wherein the gene is mammalian, and the standard curve comprises non-mammalian cDNA.

23. The method of claim 21, further wherein the gene is mammalian, and the standard curve comprises non-mammalian cDNA.

24. A kit for validating that an siRNA of interest is specific for an allele of a gene of interest and is able to substantially inhibit expression from the allele, compared to the expression from a second allele of the gene, comprising

(a) the siRNA; and

(b) in a single container, suitable primers and fluorophore-containing probes for amplifying and detecting cDNAs that are produced by reverse transcription of RNA expressed from each of the alleles in the presence of the siRNA.

25. The kit of claim 24, further comprising

(c) the components for generating a standard curve of the invention, comprising varying ratios of DNAs generated from isolated DNAs representing each of the two alleles.

26. The kit of claim 25, further comprising

(d) reagents for reverse transcribing RNA expressed from each of the alleles in the presence of the siRNA.

27. The kit of claim 24, wherein the gene is mammalian, and wherein the components for generating the standard curve further comprise non-mammalian cDNA.

28. A method for detecting an autosomal-dominant disorder, which results from a mutation in a first allele that causes reduced RNA expression from that allele compared to the expression of a wild type allele, in a subject, comprising

performing real time quantitative PCR, in a single container, of cDNA copies of RNA expressed from each of the mutant and the wild type alleles, in the presence of an allele-specific probe for each of the two alleles, wherein the two allele-specific probes are labeled with different fluorophores.

29. The method of claim 28, further

wherein the amount of expression from each of the two alleles is quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the two alleles.

30. The method of claim 28 or 29, wherein the autosomal-dominant disorder is Hutchinson-Gilford progeria, incontinentia pigmenti, neurofibromatosis, myotonic dystrophy, sialuria, Machado-Joseph disease, spinocerebellar ataxia, frontotemporal dementia, amyotrophic lateral sclerosis (ALS), slow channel congenital myasthenic syndrome, or spinobulbar muscular atrophy.

31. A method for detecting a recessive disorder, which is mediated by differential expression of one or more RNA transcripts from a gene of interest, in a subject, comprising

performing real time quantitative PCR, in a single container, of cDNA copies of each of the RNA transcripts, in the presence of probes specific for each of the transcripts, wherein the probes for each of the transcripts are labeled with distinguishable fluorophores.

32. The method of claim 31, wherein a mutation in a first allele results in RNA decay or alternate splicing, and thus altered expression, compared to expression of the comparable wild type allele, or wherein one allele is genetically imprinted or subject to X inactivation.

33. The method of claim 31, wherein

the amount of expression from each of the two alleles is quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the two alleles.

34. The method of any of claims 31-33, wherein the disorder is diabetes, cystic fibrosis, homocystinuria, hereditary inclusion body myopathy, Hermansky-Pudlak syndrome, cystinosis, Zellweger syndrome, beta-thalassemia, alkaptonuria, or a cancer

35. A method for detecting a disorder mediated by an alternately spliced RNA from a gene of interest, in a subject, comprising

performing real time quantitative PCR, in a single container, of cDNA copies of the alternately spliced RNA and a wild type RNA expressed from the gene of interest, in the presence of a probe specific for each of the RNAs, wherein the two probes are labeled with different fluorophores.

36. The method of claim 35, further

wherein the relative amount of the alternatively spliced and the wild type RNAs is quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the two RNAs.

37. The method of claim 35 or 36, wherein the disorder mediated by an alternately spliced RNA is a cancer.

38. A method for quantitating the relative amounts of a plurality of RNA transcripts expressed from a gene of interest in a subject or cell, comprising amplifying cDNAs generated from the plurality of RNA transcripts with a strand-displacing DNA polymerase which is associated with a 5' → 3' exonuclease activity, in a single container, in the presence of probes specific for each of the RNA transcripts, and quantitating the amount of each of the PCR products in real time, using probes for each RNA transcript which are labeled with distinguishable fluorophores,

wherein the relative amounts of expression of the plurality of RNA transcripts is quantitated by comparison to a standard curve obtained by amplifying mixtures of different ratios of isolated DNAs representing each of the plurality of transcripts.

39. A method for quantitating the relative amounts of a plurality of different RNA transcripts expressed from a gene of interest, comprising performing real time quantitative PCR, in a single container, of cDNAs of the transcripts, in the presence of probes specific for each of the different transcripts, wherein the probes for each of the different transcripts are labeled with distinguishable fluorophores,

wherein the relative amounts of the plurality of transcripts is quantitated by comparison to a standard curve obtained by real time quantitative PCR of mixtures of different ratios of isolated DNAs representing each of the plurality of transcripts.

Figure 1

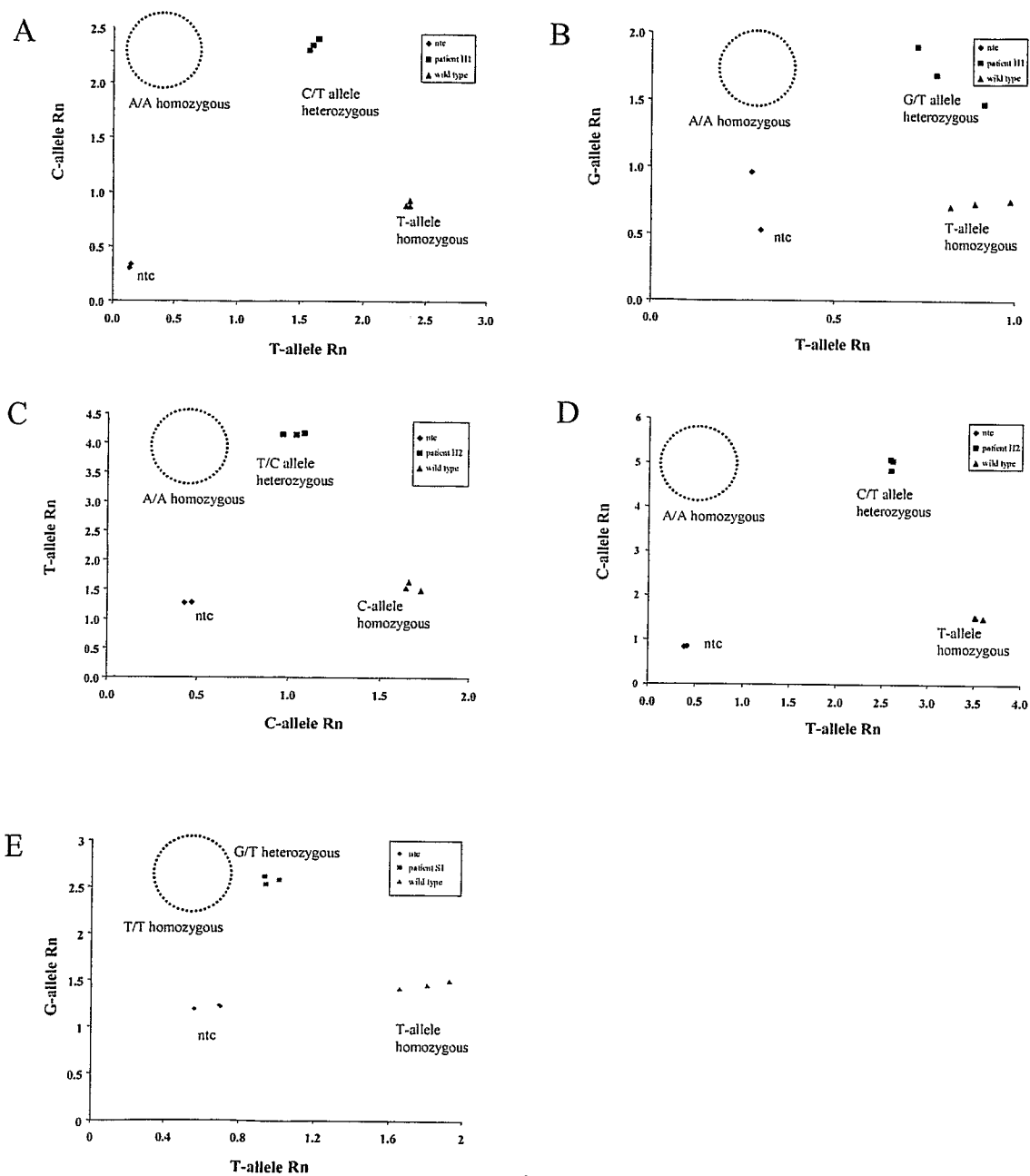
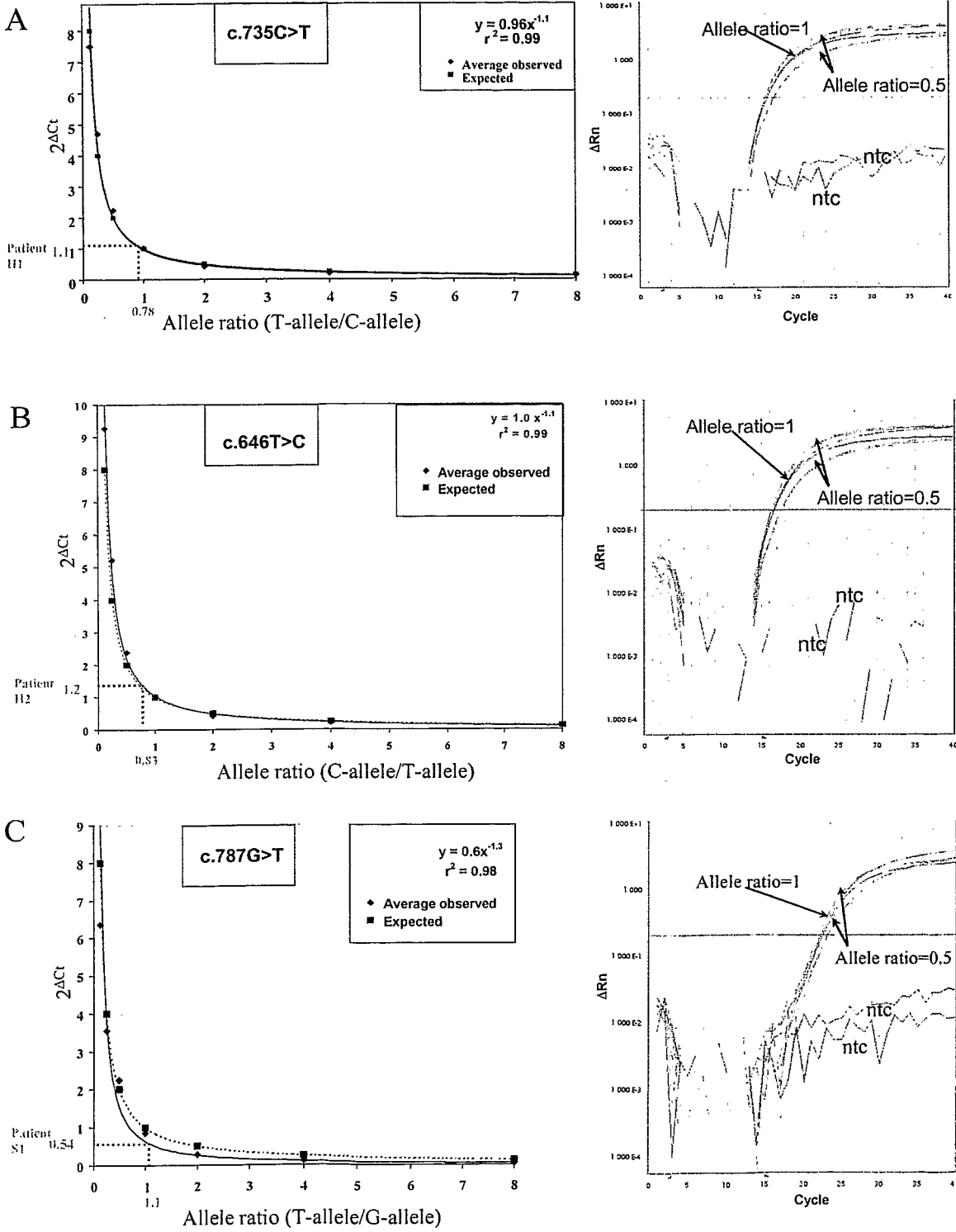


Figure 2



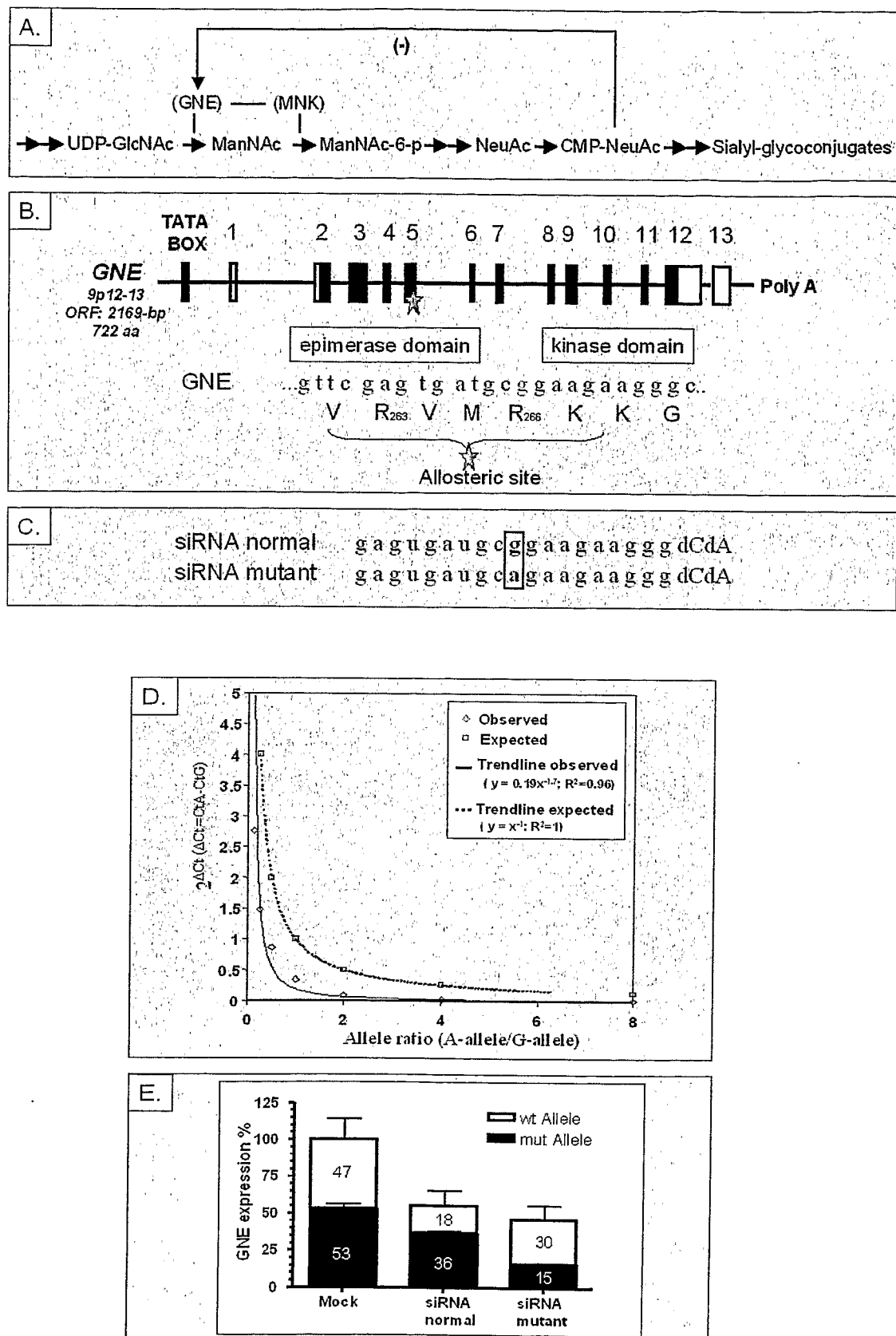


FIG. 3

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SEQUENCE LISTING

<110> GOVERNMENT OF THE UNITED STATES OF AMERICA, AS
REPRESENTED BY THE SECRETARY, HEALTH AND HUMAN
SERVICES

<120> USE OF REAL TIME PCR FOR DETECTION OF ALLELIC
EXPRESSION

<130> 31978-236195

<140>

<141>

<150> 60/718,321

<151> 2005-09-20

<160> 26

<170> PatentIn Ver. 3.3

<210> 1

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
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31

<210> 2

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
primer

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24

<210> 3

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
primer

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16

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<210> 4
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<212> DNA
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<220>
<223> Description of Artificial Sequence: Synthetic
primer

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<210> 5
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
primer

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<210> 6
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
primer

<400> 6
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<210> 7
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
primer

<400> 7
acttccccac cttcaa 16

<210> 8
<211> 16
<212> DNA
<213> Artificial Sequence

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<220>

<223> Description of Artificial Sequence: Synthetic primer

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16

<210> 9

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

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<212> DNA

<213> Artificial Sequence

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<211> 17

<212> DNA

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<210> 12

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<212> DNA

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<223> Description of Artificial Sequence: Synthetic primer

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<210> 13
<211> 19
<212> DNA
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<220>
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primer

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<210> 14
<211> 17
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<220>
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<210> 15
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<220>
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<223> Description of Artificial Sequence: Synthetic
primer

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<210> 17
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<220>
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20

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23

<210> 19
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<220>
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<400> 19
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16

<210> 20
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
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<400> 20
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16

<210> 21
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<210> 22

<211> 19

<212> DNA

<213> Artificial Sequence

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<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(24)

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Val Arg Val Met Arg Lys Lys Gly
1 5

24

<210> 24

<211> 8

<212> PRT

<213> Homo sapiens

<400> 24

Val Arg Val Met Arg Lys Lys Gly
1 5

<210> 25

<211> 21

<212> RNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic oligonucleotide

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21

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<210> 26

<211> 21

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
oligonucleotide

<400> 26

gagugaugca gaagaagggc a

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/036411

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

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

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

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUDA TETSUJI ET AL: "Use of real-time RT-PCR for the detection of allelic expression of an imprinted gene." INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE, vol. 12, no. 2, August 2003 (2003-08), pages 243-246, XP002412537 ISSN: 1107-3756	1-15, 38, 39
Y	page 244, column 1; figure 2	16-37
X	LO H S ET AL: "Allelic variation in gene expression is common in the human genome" GENOME RESEARCH, COLD SPRING HARBOR LABORATORY PRESS, WOODBURY, NY, US, vol. 13, no. 8, 2003, pages 1855-1862, XP002996587 ISSN: 1088-9051	1-15, 38, 39
Y	figure 2	16-37

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

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- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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G document member of the same patent family

Date of the actual completion of the international search

16 January 2007

Date of mailing of the international search report

22/01/2007

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/036411

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	MILLER V M ET AL: "Targeting Alzheimer's disease genes with RNA interference: An efficient strategy for silencing mutant alleles" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 32, no. 2, 2004, pages 661-668, XP002315762 ISSN: 0305-1048 the whole document	16-37
Y	DING H ET AL: "Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis" AGING CELL, BLACKWELL PUBLISHING,, GB, vol. 2, 2003, pages 209-217, XP002389534 ISSN: 1474-9718 the whole document	16-37
A	XIA H ET AL: "RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia" NATURE MEDICINE, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 10, no. 8, August 2004 (2004-08), pages 816-820, XP002311180 ISSN: 1078-8956 page 820, column 1, paragraph 2	16-27
A	CHEN C ET AL: "REAL-TIME PCR: ADVANCING RNA INTERFERENCE AND MICRORNAs STUDIES" INTERNET CITATION, [Online] 1 May 2005 (2005-05-01), XP002365817 Retrieved from the Internet: URL: http://www.appliedbiosystems.com/about/presskit/pdfs/pharma_discovery.pdf [retrieved on 2006-02-02] page 4, last paragraph - page 5	16-27
A	WILHELM JOCHEN ET AL: "Real-time polymerase chain reaction." CHEMBIOCHEM : A EUROPEAN JOURNAL OF . CHEMICAL BIOLOGY. 7 NOV 2003, vol. 4, no. 11, 7 November 2003 (2003-11-07), pages 1120-1128, XP002412539 ISSN: 1439-4227	

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International application No

PCT/US2006/036411

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