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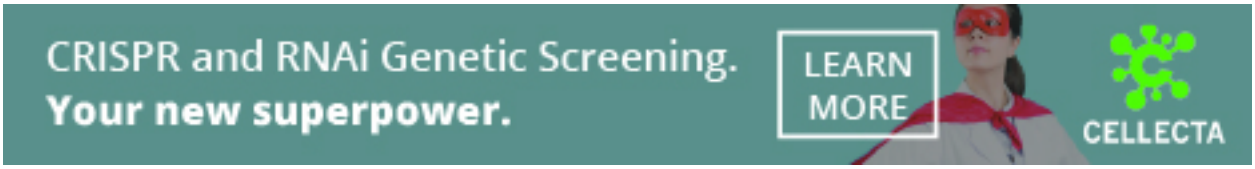

Quantitative Determination of Rare mRNA Species by PCR and Solid-phase Minisequencing

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We present a new method for quantification of mRNA, in which the limitations of the current quantitative PCR methods can be overcome. A known amount of a synthetic RNA standard differing from the mRNA to be quantified by a single nucleotide is reverse-transcribed and amplified together with the mRNA template using a biotinylated primer. The biotinylated PCR product is immobilized on a streptavidin-coated solid support and denatured. The ratio between the two amplified sequences is determined by separate "minisequencing" reactions, in which a detection step primer annealing immediately adjacent to the site of the variable nucleotide is elongated by a single labeled dNTP complementary to the nucleotide at the variable site. The ratio between the incorporated labels accurately determines the ratio between the two sequences in the original RNA sample. We applied this method to quantify the mRNA of human aspartylglucosaminidase (AGA) in tissues and cultured cells. AGA is a lysosomal enzyme participating in the degradation of glycoproteins. A mutation in the AGA gene abolishes the enzyme activity and leads to aspartylglucosaminuria (AGU), a recessively inherited metabolic disorder. The mRNA quantification revealed that the normal and mutant genes are expressed at similar levels in kidney, liver, and cultured fibroblast, whereas the amount of AGA mRNA in normal placenta and brain is significantly higher than that found in the corresponding samples from AGU patients. The method presented here is generally applicable for PCR-based quantification of rare mRNAs and DNA as well.

Quantitative analysis of a specific mRNA species is of major importance when analyzing the expression of genes in different biological systems and for studying the consequences of mutations resulting in human genetic diseases. When the mRNA population under study is expressed at a low level or in a small proportion of cells, conventional Northern blot hybridization is often not sensitive enough. Furthermore, Northern blot hybridization allows only semiquantitative comparison of mRNA levels that differ from each other significantly. Consequently, the transcription levels of many genes have not been determined accurately.

Aspartylglucosaminidase (AGA, E.C. 3.5.1.26) is a lysosomal acid hydrolase essential in the degradation of glycoproteins in all cells.[1] Analyses of the expression level of AGA in different tissues and cell cultures have so far been based on determination of the enzymatic activity. As expected for a housekeeping gene, only slight variations between tissues have been found.[2,3] Deficient AGA activity in humans leads to aspartylglucosaminuria (AGU; McKusick 208400), a recessively inherited lysosomal storage disease.[4] The AGU disease is enriched in the genetically isolated population of Finland.[5] The majority of these AGU cases are caused by a single point mutation in the AGA gene[6,7] which leads to substitution of Cys-163 for Ser in the AGA polypeptide, practically abolishing its enzymatic activity.[8] Consequently, enzyme assays cannot be applied to study the expression of the mutated AGA gene in tissues of AGU patients. Analysis of AGA mRNA in tissue samples by Northern blot hybridization requires the isolation of poly(A)$^+$ RNA. Even then, it is difficult to detect quantitative differences in AGA mRNA levels between different tissues from either AGU patients or control individuals (Ikonen et al., unpubl.).

The PCR technique[9] combined with a first-strand cDNA synthesis step[10,11] significantly improves the sensitivity of analyzing rare mRNA populations. However, this approach does not allow direct quantification due to several factors. First, the PCR amplification is exponential only at low concentrations of template.[12,13] Second, differences in the reaction conditions may cause significant sample-to-sample variation. Therefore, an internal standard should be coamplified with the sample in the same reaction.[14] The internal standard should preferably be RNA that is included before the reverse transcription step to control also the efficiency of the cDNA synthesis. Third, the efficiency of the PCR depends on the se-

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quence of the PCR primers and on the sequence and size of the template. Therefore, the internal standard should be as similar as possible to the template to ensure that the ratio between the two sequences remains constant during the PCR.[15,17] On the other hand, the two PCR products must differ from each other by some property so that their relative amounts can be determined after the amplification. In most of the quantitative PCR methods developed so far, this is accomplished by size separation in gel electrophoresis, although other approaches have been suggested.[18–19] Usually a label is incorporated during PCR followed by electrophoretic separation, either directly if the products differ by size[14–16] or after digestion with a restriction enzyme if the products differ by a restriction enzyme cleavage site.[15,17] Correct determination of the ratio between two amplified sequences of different size requires multiple sampling to confirm that the amplification of both sequences is in the exponential phase of PCR.[16,20] In the separation of the PCR products based on restriction site variation, errors are caused by incomplete restriction enzyme digestion due to heteroduplexes between the products, which are formed during the PCR.[17,21] The relative amount of the separated products is determined by densitometric scanning of autoradiographs of the gels, or by liquid scintillation counting of bands excised from the gel. These detection procedures involve several steps resulting in further inaccuracy of the quantification.

We introduce a new method, in which the problems associated with quantitative PCR analysis listed above are avoided. First, a known amount of standard RNA differing in sequence from the mRNA to be quantified only by a single nucleotide is reverse-transcribed and coamplified with the RNA. Then the ratio between the two coamplified sequences is determined by the solid-phase minisequencing technique.[7,22] The results of this method are obtained as numeric values reflecting the ratio of the PCR products without the need for a gel electrophoretic separation step. We applied this method to determine the amount of AGA mRNA in tissues and

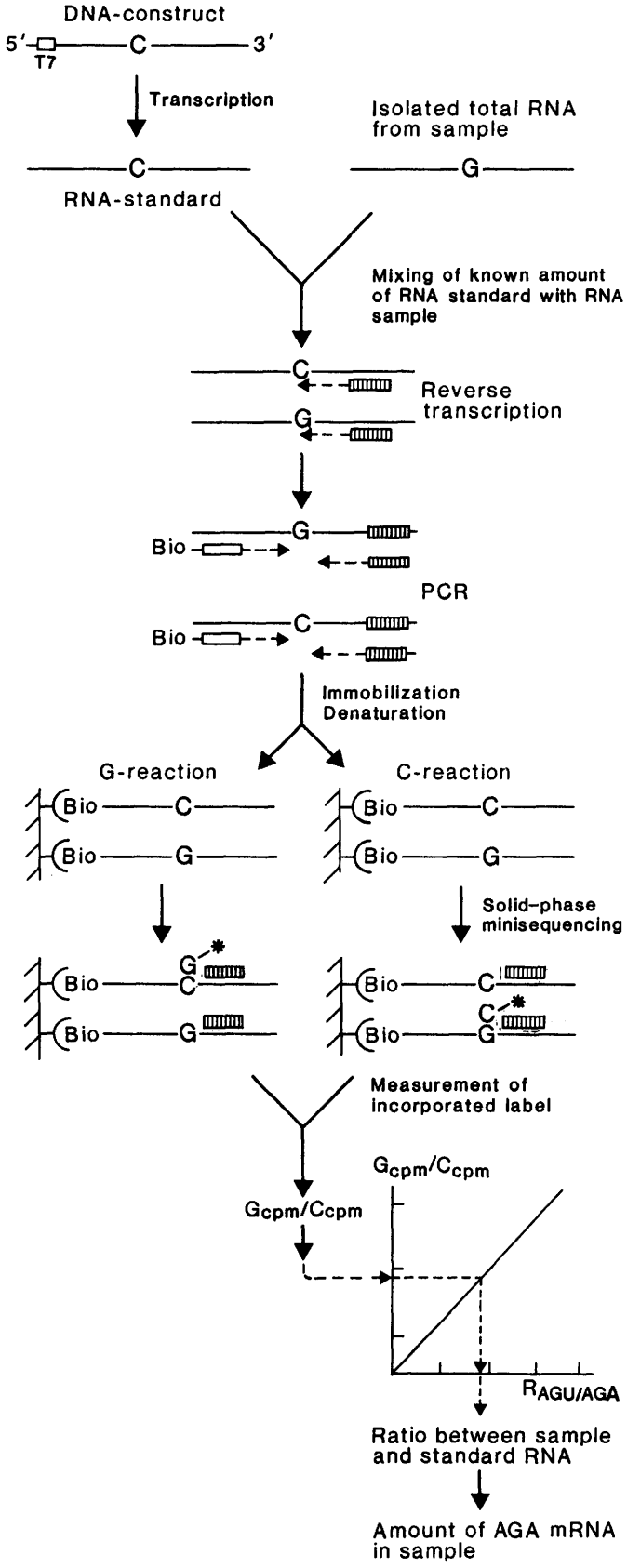

FIGURE 1 Steps of the procedure for quantitative analysis of AGA mRNA.

cultured cells from normal individuals and AGU patients.

MATERIALS AND METHODS

RNA Samples

Control tissue samples were obtained in the autopsy of a 1-year-old child who died from congenital heart disease and from a fetus aborted because of chromosome 21 trisomy. The AGU tissue samples were from the autopsy of a 32-year-old AGU patient and from an aborted AGU fetus of a carrier female. The placental tissue from this pregnancy is here referred to as AGU placenta. The samples were frozen at -80°C or in liquid nitrogen within 24 hr after death. Fibroblast cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Total RNA was extracted from tissue samples and cultured cells by the guanidinium isothiocyanate/cesium chloride gradient procedure.[23] The amount of extracted RNA was determined spectrophotometrically at 260 nm, and the RNA preparations were stored at -80°C in 70% ethanol until use.

Primers

Three oligonucleotides, one to serve as primer for reverse transcription of the AGA mRNA and as downstream PCR primer (543: 5′-AGCAATATGTCCTGTCTTATGG, nucleotides 657–636 of the cDNA sequence), an upstream PCR primer (695: 5′-AGTGCTTCTCAAGCTCTTCA, nucleotides 445–464), and a detection step primer for the minisequencing reaction (D163: 5′-CCTCCAATAATTTGGCTGG, nucleotides 507–489) were designed according to the published AGA cDNA sequence.[6] The primers were synthesized on an Applied Biosystems 381A DNA synthesizer.[24] The 5′ end of the primer 695 was biotinylated as previously described.[25]

Preparation of RNA Standards

Normal AGA cDNA extending from nucleotide 13 to nucleotide 1108 of the full-length cDNA and the corresponding cDNA containing a C_{488} to G transition as inserts in the plasmid pGEM 7Z were constructed as previously described.[8] Two micrograms of the plasmids linearized with the restriction enzyme *Mlu*I were used as template for transcription with T7 RNA polymerase. The reactions, in which 1.2-kb transcripts are produced, were carried out according to Melton et al.[26] using reagents from Promega Biotech. The excess of NTPs was removed by gel filtration on a Bio-Gel P30 spin column. After extraction with phenol-chloroform and precipitation by ethanol, the amount of synthesized RNA was determined spectrophotometrically at 260 mn. Typically, 20–40 μg of RNA was produced per reaction. The RNA was diluted to 10^{11} molecules/μl in water containing 1 unit/ml of RNasin (Promega Biotech) and was stored at 4°C until use. The RNA preparations were stable at these conditions for about 4 weeks.

Reverse Transcription and Polymerase Chain Reaction

Immediately prior to the reverse transcription reaction, the RNA standards were diluted to 10^8–10^2 molecules/μl in water containing 20 μg/ml of yeast transfer RNA and 1 unit/ml of RNasin. One microgram of total cellular RNA together with a known amount of RNA standard, or mixtures of known amounts of both RNA standards, were reverse-transcribed in 50-μl reactions with the primer 543 at 1 μM and the dNTPs at 0.4 μM concentrations, 1 unit/μl of RNasin and 1 unit/μl of AMV reverse transcriptase (New England Biolabs) in 50 mM Tris-HCl (pH 8.1), 2 mM dithiotreitol, 5 mM $MgCl_2$, 40 mM KCl. The reaction mixtures were incubated at 22°C for 10 min followed by 60 min at 42°C.

A 212-bp fragment of the AGA cDNA was amplified by the PCR. Each reaction contained 15–25 μl of the reverse transcription mixture, 20 pmoles of the biotinylated primer 695 and an additional 100 pmoles of the primer 543, the four dNTPs at 0.2 μM concentrations, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega Biotech) in 100 μl of 50 mM Tris-HCl (pH 8.8), 15 mM $(NH)_4SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.01% gelatin (*Taq* DNA polymerase buffer). The PCR was initiated with a "hot start," by first heating the samples for 5 min at 95°C, followed by addition of the *Taq* DNA polymerase at 80°C. Thirty PCR cycles of 1 min at 95°C, 1 min at 55°C, and 1 min for 72°C were carried out.

Quantification by Solid-phase Minisequencing

The solid-phase minisequencing procedure was carried out essentially as described for detection of the AGU muta-

TABLE 1 Approximation of the Amount of AGA mRNA in 1 μg of Total Cellular RNA from Normal and AGU Fibroblasts

Number of molecules added		Radioactivity incorporated (cpm)		
AGU RNA[a]	AGA RNA[b]	^{3}H[dGTP]	^{3}H[dCTP]	G_{cpm}/C_{cpm}
10^8		17,700	42	>200
10^7		20,100	76	>200
10^6		10,100	200	51
10^5		7,550	1,200	6.2
10^4		3,120	3,930	0.79
10^3		601	5,710	0.10
10^2		197	5,550	0.036
-		246	8,043	0.033
	10^8	317	31,500	0.011
	10^7	337	32,300	0.010
	10^6	790	28,500	0.036
	10^5	3,530	10,300	0.34
	10^4	7,500	3,360	2.2
	10^3	10,700	8,990	12
	10^2	12,900	380	34
	-	11,100	363	30

[a]Added to 1 μg of RNA isolated from normal fibroblasts.
[b]Added to 1 μg of RNA isolated from fibroblast of an AGU patient.

tion in genomic DNA.[7] One-tenth of the 5′-biotinylated PCR products were captured in microtitration wells that had been coated with streptavidin, and the bound DNA fragment was denatured by treatment with 50 mM NaOH. The primer annealing and extension reactions were carried out simultaneously in a minisequencing reaction mixture containing the detection step primer D163 at 0.4 μM and either ^{3}H[dGTP] (TRK 627, 26–35 Ci/mmole, Amersham) or ^{3}H[dCTP] (TRK 625 52–64 Ci/mmole) at 0.2 μM concentration and 0.2 unit of *Taq* DNA polymerase in 50 μl of *Taq* DNA polymerase buffer for 10 min at 50°C. The primer was released by treatment with 50 mM NaOH, and the eluted radioactivity was measured in a liquid scintillation counter. For each sample, the ratio between the incorporated ^{3}H[dGTP] and ^{3}H[dCTP] was calculated.

Enzyme Assay

The AGA activity in normal brain, kidney, liver, and placental tissues was determined using an assay based on colorimetric measurement of liberated *N*-acetylglucosamine.[1] The tissue samples were those used for the isolation of RNA. The amount of total protein in the samples was determined using the microassay version of the Bio-Rad protein assay.

RESULTS AND DISCUSSION

Principle of the Quantitative Analysis

The solid-phase minisequencing technique allows determination of the ratio between two sequences, which differ from each other by a single nucleotide and are present as a mixture in a sample.[7] A DNA fragment spanning the variable site is amplified with one biotinylated and one unbiotinylated PCR primer. The synthesized biotinylated PCR product is immobilized on a streptavidin-coated solid support and denatured. Each of the nucleotides at the variable site in the immobilized DNA strands is detected separately by a primer extension reaction, in which a single labeled nucleoside triphosphate is incorporated. The ratio between the incorporated labels is a measure of the ratio between the two amplified sequences.

We tested the applicability of solid-phase minisequencing to quantify the mRNA of a housekeeping enzyme (AGA) in tissues and cultured cells of normal individuals and AGU patients. For this analysis we prepared two synthetic RNA standards by transcription with T7 RNA polymerase from two cloned DNA constructs, which differ from each other by one nucleotide corresponding to the $C_{488}\rightarrow G$ transversion in the AGA cDNA. This is the most common mutation causing the AGU disease.[6,7]

To determine the amount of normal AGA mRNA, a known amount of synthetic RNA with the AGU RNA sequence is added to the samples. Analogously, mRNA in tissues of AGU patients is quantified using an RNA standard corresponding to the normal sequence of the AGA gene. The RNA mixtures are subjected to reverse transcription and PCR amplification, and the ratio between the two sequences in the PCR product is determined by the ratio between [^{3}H]dGTP and [^{3}H]dCTP incorporated in the solid-phase minisequencing reaction. Comparison of the obtained G_{cpm}/C_{cpm} ratio with a standard curve constructed with mixtures of known amounts of the RNA standards gives the initial ratio between the sample and standard RNA. Figure 1 outlines the steps of the procedure. Because the variable nucleotide of the two RNA standards is the same as the mutation in the AGA gene causing the AGU disease, one standard curve can be used for analyzing both types of samples.

Determination of the Range of Detection

First, an experiment to obtain a preliminary estimate of the amount of AGA transcript in RNA isolated from cultured fibroblasts was carried out. Tenfold dilutions ranging from 10^8 to 10^2 of the AGU and AGA RNA standard were added to 1 μg of total cellular RNA isolated from fibroblasts of a normal individual and an AGU patient, respectively. The G_{cpm}/C_{cpm} ratios obtained by the solid-phase minisequencing assay (Table 1) indicate that in both fibroblast cell lines the amount of AGA mRNA is in the range of 10^4–10^5 molecules/μg RNA.

In principle, the absolute amount of RNA template present in the sample could be calculated directly from the G_{cpm}/C_{cpm} ratio obtained in the solid-phase minisequencing test. This ratio is affected by the specific activities of the ^{3}H[dNTP]s used, which is a factor easily accounted for. In addition, a small

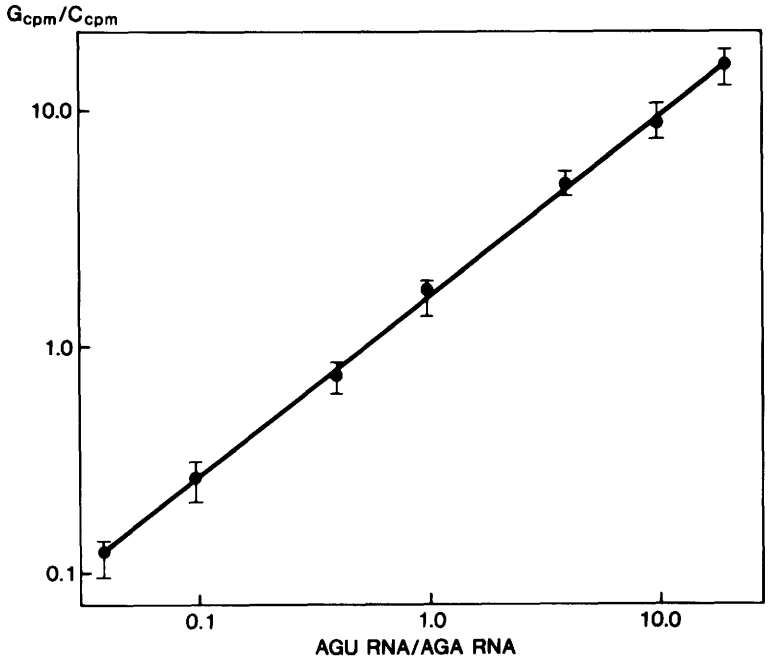

FIGURE 2 Solid-phase minisequencing standard curve. Mean G_{cpm}/C_{cpm} values and variation range of three separate assays, in which labels of the same specific activity were used, are given.

amount (<3%) of background caused by misincorporation of ^{3}H[dNTP] by the DNA polymerase affects the ratio. This background is partially due to other dNTPs present as impurities in the labeled dNTPs and is thus sequence dependent. The specificity of the DNA polymerase may also be reduced by the chemical moiety by which the label is introduced into the dNTP. Therefore, we prefer to use a standard curve constructed with mixtures of known amounts of the RNA standards analyzed in parallel with each series of samples.

For preparation of a standard curve, 10^5 molecules of the AGA RNA standard were mixed with AGU RNA standard in ratios ranging from 0.04 to 20. Plotting of the G_{cpm}/C_{cpm} ratio obtained in the minisequencing test as a function of the initial ratio between the standards yielded a linear standard curve over the whole range (Fig. 2). Identical G_{cpm}/C_{cpm} ratios were obtained when the two standards were mixed in the same ratios, but using 10^3-fold larger or 10^3-fold smaller absolute amounts of them (data not shown). According to the standard curve in Figure 2, the smallest detectable proportion of one of the sequences is 4–5%.

Determination of AGA mRNA Levels

Based on the preliminary estimate of the amount of mRNA in the cultured fibroblasts, we analyzed 1 μg of total cellular RNA extracted from tissues of normal individuals by adding 10^5 molecules of the AGU RNA standard. Tissues from AGU patients were analyzed using 10^4 molecules of the AGA RNA standard. The number of molecules (N) of AGA mRNA per 1 μg of total RNA was calculated with the aid of the ratio ($R_{AGU/AGA}$) obtained from the standard curve as follows:

N_{AGA} = 10^5 molecules AGU RNA / ($R_{AGU/AGA}$)

N_{AGU} = ($R_{AGU/AGA}$) × 10^4 molecules AGA RNA

So far, the only available information on the expression of the AGA gene is based on the measurement of the enzymatic activity of AGA in human autopsy tissues or biopsy samples.[2,3] Only slight differences in the AGA activities between different tissues were reported in these studies; e.g., in kidney tissue the AGA activity was twofold compared to that of liver and three- to fourfold that of brain.[2,3] When we determined the AGA activity from the tissues used here for quantification of the AGA mRNA, the activities were comparable to the previous reports. The lowest AGA activity was measured in brain tissue, where the activity was only one-seventh of that in liver and kidney (data not shown). Contrary to these data, we found in our quantitative mRNA analysis that the AGA mRNA level was highest in brain tissue, being two to three times that of liver and 10 times that of kidney (Fig. 3). Evidently, the transcription level is not directly reflected in the amount of enzyme activity. As could be expected for the metabolically highly active placental tissue, the AGA mRNA levels exceeded those of the other tissue samples 10- to 100-fold.

When comparing the expression of the AGA gene between AGU patient and control samples, the amount of transcript in AGU brain and placental tissue of an AGU fetus was only about one-hundredth of that in the corresponding normal tissues. Opposite to this, the amounts of AGA mRNA in kidney and liver were at comparable levels in AGU and control samples. Additionally equal amounts of AGA mRNA were detected in kidney tissue from an AGU fetus and an adult AGU patient, favoring the idea of age-independent expression typical of a housekeeping gene. Equal transcriptional efficiency of the normal and AGU allele was also found in cultured AGU and control fibroblasts (Table 2). The variation in mRNA levels between the fibroblasts from individuals of different AGU genotype was not greater than between the normal fibroblasts obtained from four control individuals.

In this study, we used the solid-phase minisequencing technique for determining the transcription levels of a housekeeping-type gene in different human tissues and cultured cells. The method is generally applicable for quantification of any RNA species, provided that sequence information for preparing the RNA standard and design of the PCR and detection step primers is available. We used a cloned template prepared by site-directed mutagenesis for preparation of internal RNA standards for the analysis. This procedure could be simplified using PCR to prepare the mutagenized template and for introduction of an RNA polymerase promoter sequence.[27–29]

Our method has several advantages compared to other detection methods used in quantitative PCR analysis. The minisequencing reaction is carried out on a solid support, which allows determination of the relative amounts of PCR product from the RNA template and an internal RNA standard without a gel electrophoretic separation step. Because the detection is not based on difference in size between the two amplified sequences, an internal stan-

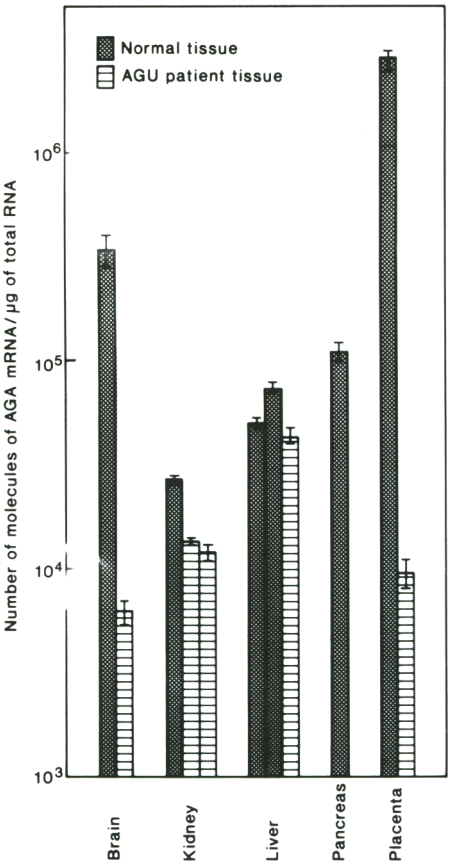

FIGURE 3 Amount of AGA mRNA in tissue samples from normal individuals (shaded columns) and AGU patients (striped columns). The middle column for kidney and the left-hand column for liver represent fetal samples. The sample from normal placenta was analyzed using 10^6 molecules of the AGU RNA standard. The mean value of two assays and the variation range is given.

TABLE 2. Amount of AGA mRNA in Cultured Cells from Individuals of Different Genotype

Genotype of patient	Number of molecules ($\times 10^4$) of mRNA in 1 μg of total cellular RNA[a]	
1. AGU homozygote	3.4	4.5
2. AGU homozygote	5.0	5.3
3. AGU homozygote	1.2	1.0
4. Compound heterozygote[b]	1.7	1.3
5. Compound heterozygote[b]	2.4	1.9
6. Compound heterozygote[b]	1.9	1.3
7. Normal homozygote	3.1	2.2
8. Normal homozygote	1.9	1.3
9. Normal homozygote	6.1	9.0
10. Normal homozygote (fetal)	1.4	1.3

[a]The result of two separate determinations are given.
[b]Compound heterozygotes with the major AGU mutation in one allele, in which only this allele is expressed (Ikonen et al., unpubl.).

dard differing from the template only at a single nucleotide can be used to ensure that both sequences are amplified with equal efficiency. Identification of the variable nucleotide by the minisequencing primer extension reaction is independent of restriction sites in the sequence to be quantified, and consequently size separation of restriction fragments is avoided. The result of the analysis is directly obtained as an accurate numeric value reflecting the initial ratio between the two RNA sequences. Thus, errors introduced into the analysis by scanning methods or excision of separated fragments from gels are avoided.

In conclusion, the solid-phase mini-sequencing technique is a relevant alternative for quantitative analysis of small amounts of RNA. Obviously, the method is also applicable for quantification of DNA by the PCR.

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REFERENCES

1. Makino, M., T. Kojima, T. Ogushi, and I. Yamashina. 1966. Enzymatic cleavage of glycoproteins. *Biochem. Biophys. Res. Commun.* **24:** 961–966.
2. Palo, J., P. Riekkinen, A.U. Arstila, S. Autio, and T. Kivimäki. 1972. Aspartylglucosaminuria II: Biochemi-cal studies on brain, liver, kidney and spleen. *Acta Neuropath.* **20:** 217–224.
3. Palo, J., P. Riekkinen, A. Arstila, and S. Autio. 1971. Biochemical and fine structural studies on brain and liver biopsies in aspartylglucosaminuria. *Neurology* **21:** 1198–1204.
4. Pollitt, R.J., E.A. Jenner, and H. Merskey. 1968. Aspartylglucosaminuria. An inborn error of metabolism associated with mental defect. *Lancet* **ii:** 253–262.
5. Aula, P., S. Autio, K.O. Raivio, and J. Rapola. 1982. *Aspartylglucosaminuria. Genetic errors of glycoprotein metabolism* (ed. P. Durand and J.S. O'Brien), pp. 122–152. Springer Verlag, Berlin.
6. Ikonen, E., M. Baumann, K. Grön, A.-C. Syvänen, N. Enomaa, R. Halila, P. Aula, and L. Peltonen. 1991. Aspartylglucosaminuria: cDNA encoding human aspartylglucosaminidase and the missense mutation causing the disease. *EMBO J.* **10:** 51–58.
7. Syvänen, A.-C., E. Ikonen, T. Manninen, M. Bengtström, H. Söderlund, P. Aula, and L. Peltonen. 1992. Convenient and quantitative determination of the frequency of a mutant allele using solid-phase minisequencing: Application to aspartylglucosaminuria in Finland. *Genomics* (in press).
8. Ikonen, E., N. Enomaa, I. Ulmanen, and L. Peltonen. 1991. In vitro mutagenesis helps to unravel the biological consequences of aspartylglucosaminuria mutation. *Genomics* **11:** 206–211.
9. Mullis, K.B. and F.A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.
10. Kawasaki, E.S., S.S. Clark, M.Y. Coyne, S.D. Smith, R. Champlin, O.N. White, and F.P. McCormick. 1988. Diagnosis of chromic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified *in vitro*. *Proc. Natl. Acad. Sci.* **85:** 5698–5702.
11. Chelly, J., J.-C. Kaplan, P. Maire, S. Gautron, and A. Kahn. 1988. Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature* **333:** 858–860.
12. Bloch, W. 1991. A biochemical perspective of the polymerase chain reaction. *Biochemistry* **30:** 2735–2747.
13. Syvänen, A.-C., M. Bengtström, J. Tenhunen, and H. Söderlund. 1988. Quantification of polymerase chain reaction products by affinity-based hybrid collection. *Nucleic Acids Res.* **16:** 11327–11338.
14. Chelly, J., D. Montarras, C. Pinset, Y. Berwald-Netter, and J.-C. Kaplan. 1990. Quantitative estimation of minor mRNAs by cDNA-polymerase chain reaction. Application to dystrophin mRNA in cultured myogenic and brain cells. *Eur. J. Biochem.* **187:** 691–698.
15. Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci.* **87:** 2725–2729.
16. Wang, A.M., M.V. Doyle, and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* **86:** 9717–9721.
17. Becker-André, M. and K. Hahlbrock. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res.* **17:** 9437–9446.

18. Lundeberg, J., J. Wahlberg, and M. Uhlén. 1991. Rapid colorimetric quantification of PCR-amplified DNA. *BioTechniques* **10:** 68–75.
19. Syvänen, A.-C. and H. Söderlund. 1992. Quantification of polymerase chain reaction products by affinity-based collection. *Methods Enzymol.* (in press).
20. Lubin, M.B., J.D. Elashoff, S.-J. Wang, J.I. Rotter, and H. Toyoda. 1991. Precise gene dosage determination by polymerase chain reaction: Theory, methodology, and statistical approach. *Mol. Cell. Probes* **5:** 307–317.
21. Shoffner, J.M., M.T. Lott, A.M.S. Lezza, P. Seibel, S.W. Ballinger, and D.C. Wallace. 1990. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA $tRNA^{Lys}$ mutation. *Cell* **63:** 931–937.
22. Syvänen, A.-C., K. Aalto-Setälä, L. Harju, K. Kontula, and H. Söderlund. 1990. A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. *Genomics* **8:** 684–692.
23. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299.
24. Beaucage, S.L. and M.H. Caruthers. 1982. Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* **22:** 1859–1862.
25. Bengtström, M., A. Jungell-Nortamo, and A.-C. Syvänen. 1990. Biotinylation of oligonucleotides using a water soluble biotin ester. *Nucleosides & Nucleotides* **9:** 123–127.
26. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12:** 7035–7056.
27. Higuchi, R., B. Krummel, and R.K. Saiki. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res.* **16:** 7351–7367.
28. Sarkar, G. and S.S. Sommer. 1990. The "megaprimer" method of site-directed mutagenesis. *BioTechniques* **8:** 404–407.
29. Perrin, S. and Gilliland, G. 1990. Site-specific mutagenesis using assymetric polymerase chain reaction and a single mutant primer. *Nucleic Acids Res.* **18:** 7433–7438.

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