CHAPTER 2

Assays for *Methylenetetrahydrofolate Reductase* Polymorphisms

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Polymorphisms, Allelic Frequencies and Linkage of Alleles

To date, two functional polymorphisms, 677C→T in exon 4[1] and 1298A→C in exon 7,[2] in the gene encoding the enzyme methylenetetrahydrofolate reductase (MTHFR) have been found and characterized. Both lead to an amino acid change, and different biochemical properties between the normal and variant enzyme have been demonstrated.[3] In addition, a missense mutation (1793G→A in exon 11[4]), three silent polymorphisms (129C→T in exon 1,[5] 1068T→C in exon 6,[6] and 1317T→C in exon 7[7]), and three intronic polymorphisms (IVS2+533G→A,[8] IVS6+31T→C[9] and IVS10+262C→G[8]) have been reported, but their functional implications are unknown.

Allele frequencies of the polymorphisms 677C→T and 1298A→C vary considerably according to ethnicity (4-58%, and 9-37%, respectively,[10-12] a more detailed description of the frequency of the 677 variant is provided in chapter 2). The 1793G→A allele frequency varied from 15.5 to 32.2% in four distinct ethnic populations,[4] and the 1317T→C change was common (39 %) in an African American cohort,[7] but essentially absent in German Caucasians.[13] Data on frequencies of the remaining polymorphisms are limited.

The1298C-allele is in linkage disequilibrium with the 677C allele. Among the 9 possible genotype combinations, the 677TT-1298AC, 677CT-1298CC, and 677TT-1298CC are rarely observed, although a few cases have been reported.[7,14]

Figure 1 shows a graphical overview of the SNPs and their relative positions along the gene.

Methods for Genotyping *MTHFR* SNPs

Currently, there are some 30 different reported methods for the detection of polymorphisms of the *MTHFR* gene, but methods for the detection of 677C→T are by far the most frequently described. These methods can, broadly, be divided into four categories: PCR/restriction fragment length polymorphism based assays (PCR/RFLP), allele-specific PCR assays, heteroduplex assays, and real-time PCR with fluorogenic probes. In addition, the technique of minisequencing,[15] and the use of mass spectrometry[16] has been reported.

Restriction Enzyme Based Assays, the 677C→T Variant

Figure 2 depicts some features of the assay reported by Frosst et al in the paper that first described the 677C→T polymorphism.[1] The enzyme *Hinf*I recognizes the sequence GANTC (N being any base). This implies that the variant sequence (GAGTC) is cleaved, whereas the wild-type sequence GAGCC remains undigested. The forward primer was placed close to the cleavage site and the reverse primer at some distance in the 3′ direction, producing a fragment length of 198bp, and 175 and 23bp after cleavage. Figure 3, panel A, shows a schematic representation of the fragments in a sieving gel. Alongside the three possible genotyping outcomes

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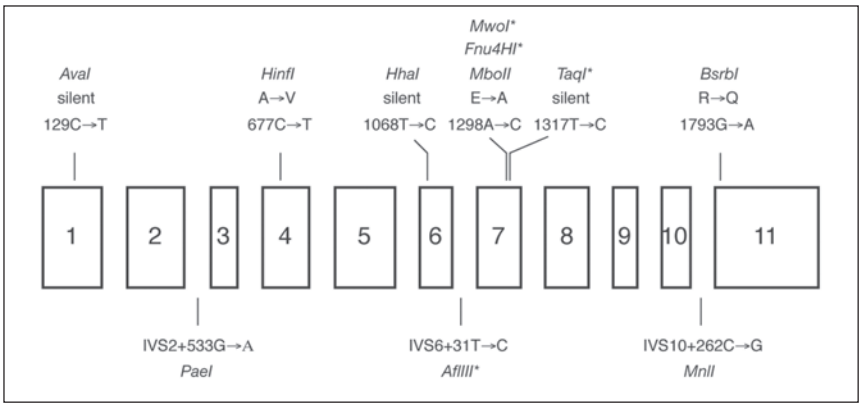

Figure 1. Polymorphisms of the *MTHFR* gene. Exons are numbered, and the positions of SNPs are indicated along with the associated amino acid change (if any) and restriction enzyme(s) used for detection. *Artificially created restriction site.

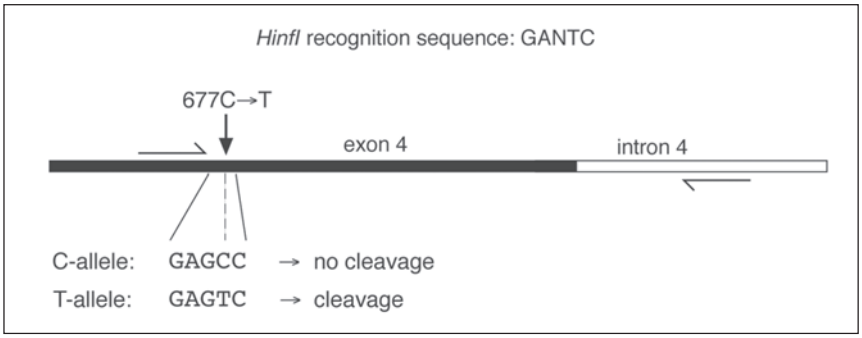

Figure 2. Some features of the method by Frosst et al.[1] Distances along the horizontal axis are drawn to scale, and the PCR-primers are drawn as half-arrows.

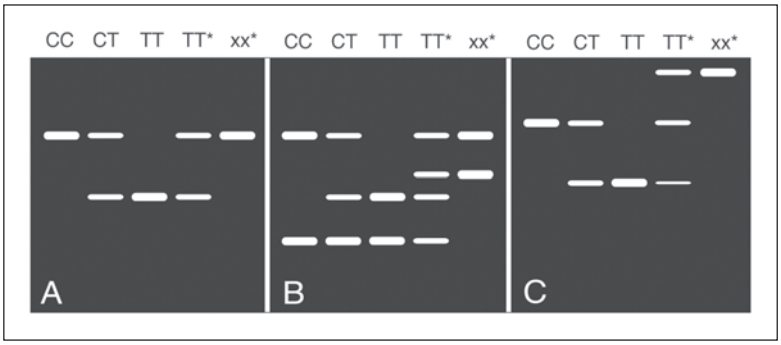

Figure 3. Schematic drawing of gel electrophoresis, showing characteristics of different RE-based assays. Panel A) Assay by Frosst et al.[1] Panel B) Assay by Ulvik et al.[18] Panel C) Assay by Bravo-Osorio et al.[19] The lanes labeled TT* and xx* denote a TT genotype that is partially digested, and any genotype with no digestion, respectively. The distances between fragments are proportional to the differences in the logarithm of the fragment sizes.

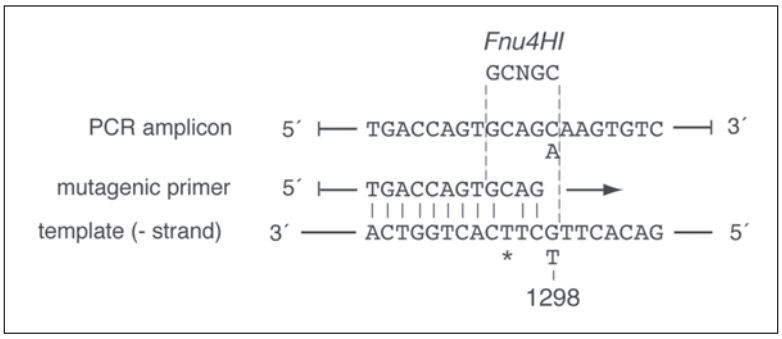

Figure 4. Artificially-created restriction sites. The strategy used by Weisberg et al[7] for detecting the 1298A→C variant is shown. The mutagenic primer changes one base in the template (marked by an asterisk), and the resulting PCR amplicon contains the *Fnu*4HI recognition sequence when the 1298C-allele is present.

(CC, CT and TT), profiles obtained after partial digestion of a homozygous TT genotype or after no digestion (any genotype) are shown. The figure demonstrates that if *Hinf*I digestion fails, it is possible to make an erroneous genotype assignment. This problem, which is an inherent feature of this assay design, was addressed in several subsequent publications. Van Amerongen et al suggested coamplification with another fragment containing a *Hinf*I recognition sequence,[17] whereas Ulvik et al included a preamplified standard amplicon containing the recognition sequence before addition of the restriction enzyme.[18] The latter assay was optimized for capillary electrophoresis with multiple injections, but the strategy is applicable to traditional gel formats as well (Fig. 3, panel B). Yet another design was presented by Bravo-Osorio et al They engineered an additional restriction site into the amplified fragment by using a reverse primer that included an extra 25 bases containing the *Hinf*I recognition sequence (Fig. 3, panel C).[19] Benson et al demonstrated a multiplexing strategy where fluorescent primers of different colors were incorporated into amplicons of the 677C→T and other polymorphisms in separate PCR reactions.[18] Restriction enzyme digestion was performed as appropriate, and products were then pooled and separated by color as well as by size on an automated capillary electrophoresis system. A digestion control for each SNP was added, similar to Ulvik et al.[20]

Restriction Enzyme Based Assays, the 1298A→C Variant and Other SNPs

An assay for the 1298A→C variant was first described by van der Put et al using the enzyme *Mbo*II that cuts the 1298A allele.[2] Weisberg et al, as they discovered the 1317T→C polymorphism, noted that the presence of 1317C generates a *Mbo*II recognition sequence that (using the assay by van der Put et al) produces a restriction pattern almost identical to 1298A. To overcome this problem, they used artificially- generated restriction sites (explained in Fig. 4) to produce an alternative assay for 1298A→C as well as an assay for 1317T→C using the enzymes *Fnu*4HI and *Taq*I, respectively.[7] Yi et al, modified the assay for 1298A→C by van der Put et al so that fewer fragments were generated after cleavage. Moreover, they coamplified fragments for the 677 and 1298 polymorphisms, but performed separate cleavages using the *Hinf*I and *Mbo*II enzymes, respectively, followed by pooled gel electrophoresis.[21] However, they failed to take into account the interference by 1317T→C. Meisel et al used two allele-specific long range PCRs targeting the 677C and 677T alleles separately, followed by the analysis of 1298A→C devised by Weisberg et al to establish allelic association between the two polymorphic sites.[13] A new assay for 1298A→C has recently been developed by Leclerc et al using the enzyme *MwoI* (described in Chapter 1). An assay has been reported for 1793G→A using the enzyme *Bsrb*I which cuts the G variant.[4] The 129C→T variant creates a *Ava*I site,[5] and the 1068T→C variant creates a *Hha*I recognition site,[6] but has also been analysed using the enzyme *Cfo*I.[5] Figure 1 lists the reported restriction enzymes used for the analysis of each polymorphism.

Allele-Specific PCR Assays, General Description

This type of assay, as well as the PCR/RFLP assay, was first described in the 1980s. Different variations are known under such acronyms as ARMS (amplification refractory mutation system), ASA (allele-specific amplification), and MS-PCR (mutagenically- separated PCR). The idea behind these assays is that a PCR primer that ends at the polymorphic position will only bind completely to one of the variants, which will then be amplified at normal efficiency, whereas the other variant will not be amplified, due to the mismatch generated at the last base. In order to design effective assays, some knowledge of the refractoriness of different mismatches to amplification is advisable. A few studies have been reported (summarized in ref. 22). General findings are that G : T mismatches confer low specificity, and the sequence-specific primer should not end with an A. In some cases (sequence contexts), substantial amounts of PCR product from the wrong allele may be generated if reaction conditions (annealing temperature and number of PCR cycles) are not carefully optimized. Strategies such as shortening the primer, adding a competitive primer,[23] and, most often, adding additional mismatches close to the 3′end[24] have been used to enhance selectivity.

Assays have been designed where the wild-type and mutant alleles are detected in separate reactions, or the two alleles are detected in the same tube (MS-PCR). Both strategies can be multiplexed to include several SNPs.

Allele-Specific PCR Assays for MTHFR *Genotyping*

Hill et al described a MS-PCR assay detecting both 677 alleles in the same tube.[25] The same strategy was used by Ulvik et al who designed a multiplex assay for the simultaneous detection of the 677C→T and factor V 1691G→A variants.[26] This was later extended to include 1298A→C (unpublished). Endler et al extended the assay to also encompass the prothrombin 20210G→A polymorphism.[27] Two other studies report multiplexing of three SNPs (including 677C→T), carried out in two reaction tubes.[28,29] The advantage of allele-specific methods over the restriction enzyme assays is that allele-specific reaction products, directly detectable by electrophoresis, are generated during PCR. Furthermore, these methods are readily amenable for multiplexing. Multiplex MS-PCR, however, rapidly reaches a limit of complexity. A three-way multiplex MS-PCR assay involves the simultaneous amplification, in an allele-specific and balanced manner, of up to 6 products, requiring careful control of the relative amount of each primer. The two-tube strategy is more straightforward, but does not involve the competitive priming of allele-specific primers as in MS-PCR. In some cases, this could increase the risk of spurious priming and false results. In addition, depending on the assay design, there may be a need for an extra control amplicon for confirmation of adequate reaction conditions.[29] As long as the reaction conditions/primers are properly optimized, allele-specific PCR is a rapid and reliable assay.

Heteroduplex Assays

These assays are robust and well suited for multiplexing. However, they usually require the formation of so-called heteroduplex generators (HDGs). This is a fairly complex process that involves the generation of an artificial DNA construct, using site-directed mutagenesis of the sequence encompassing the polymorphism, followed by confirmation and testing of the construct. Figure 5 illustrates the effect of a heteroduplex generator. This construct is usually coamplified with the DNA of interest and the assay is somewhat sensitive to the ratio of the amount of HDG-construct to DNA. Enhanced resolution by separation on polyacrylamide gels combined with long electrophoresis times is usually necessary. At least three reports using this method are found in the literature: Clark et al[30] for the 677C→T mutation, Bowen et al[31] for the 677C→T polymorphism multiplexed with two other SNPs, and Barbaux et al[32] for 677C→T with 1298A→C as well as two other polymorphisms. Once the heteroduplex generators are prepared, and their effects verified, these assays, similarly to the allele-specific PCR assays, require no post-PCR processing (other than electrophoresis).

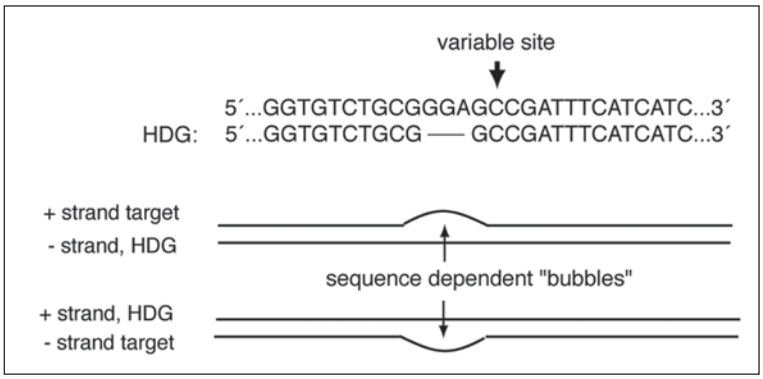

Figure 5. Principle of a heteroduplex assay. A heteroduplex generator (HDG) is constructed by site-directed mutagenesis to contain a short deletion 1-3 bases from the variable site. This construct is coamplified with target DNA, and after the last cycle of PCR the amplicons are melted and allowed to reanneal. Apart from correctly annealed homoduplexes (target amplicons and HDG amplicons), heteroduplexes are formed, with a short bubble due to the deletion in the HDG. The size of the bubble depends on the surrounding sequences including the variant base. Thus, the heteroduplexes containing the variation are seen by their differential electrophoretic migration.

Real-Time PCR with Fluorogenic Probes—Homogenous Assays

The development of fluorescent labeling technology, and systems for detection of fluorescence during amplification, have facilitated the development of these techniques, which are fairly recent additions to the methodological repertoire (see refs. 33,34 for reviews). Some of the reported methods require sophisticated, yet increasingly available, equipment for real-time PCR detection, but for some methods, a post-PCR reading in an ordinary fluorimeter is adequate. An important reason for the rapid gain in popularity of these methods is that they are homogenous, meaning that all ingredients for genotyping are added to one tube, and results are obtained without further manipulations. This also removes the most important source of contamination in PCR: the reintroduction of PCR products to the PCR setup. At least seven reports, using these techniques to detect *MTHFR* polymorphisms, have been published, divisible into two main categories as described below.

5´Exonuclease and Molecular Beacon Assays

The molecular beacon and 5´-exonuclease (also known as TaqMan) assays have a number of similarities. Both use probes that are doubly labeled oligonucleotides with a reporter fluorophore at the 5´end and a quencher at the 3´end. The beacon probes contain a short additional sequence at both ends, with internal homology, so that a hairpin loop is created. The probe will then be in an equilibrium between intramolecular hybridization, and hybridization to a target. In the former state, fluorescence is quenched whereas opening the hairpin structure allows the reporter to fluoresce.[35] Signal generation from the 5´exonuclease probe stems from cleavage of the probe by the 5´exonuclease activity of the DNA polymerase during PCR, thereby releasing the fluorophore from the quencher.[36]

Both types of assays depend on the different binding strengths of the probes to the normal and variant sequences on target DNA. Giesendorf et al used molecular beacons in separate reactions for the determination of the 677C→T alleles,[37] whereas Happich et al used the TaqMan format and two differently labeled probes in the same tube for the simultaneous determination of both 677C→T alleles.[38] Ulvik et al demonstrated that the homogenous format (TaqMan) is compatible with direct analysis on blood without DNA purification. Included were the 677C→T and 1298A→C polymorphisms.[39]

The competition between target and internal hybridization of the molecular beacons is said to enhance the selectivity of this format. A study comparing the 5′exonuclease probes and molecular beacons, however, found only a marginal difference in the ability to discriminate between variants[40] A recent development of the TaqMan probes is the addition of a minor groove binder which allows shorter and thereby more selective probes.[41] Ulvik et al however, showed that short (16-20bp) probes can function without the aid of minor groove binders.[39]

Hybridization Probe Assays

In hybridization probe assays, singly labeled hybridization probes are used. The labeled probe hybridizes in close proximity to a second fluorophore, either attached to one of the PCR primers, or an additional hybridized oligonucleotide. Signal is detected as fluorescence resonance energy transfer (FRET) from the probe fluorophore to the second (acceptor) fluorophore. Genotyping is done by performing a melting curve analysis after PCR. A completely matched probe melts at a higher temperature than one with a mismatch against the target. The data are analyzed and plotted as the derivative of signal with respect to temperature (-dF/dT) against temperature. The probe may be homologous to the normal, or variant allele, and one probe is sufficient for genotyping.[42]

Two variants of the assay format outlined above for the analysis of 677C→T have been published.[42,43] Von Ahsen et al demonstrated a hybridization probe assay multiplexed by using different fluorophores for the two SNPs 677C→T and factor V 1691G→A.[44] Crockett et al, on the other hand, showed that quenching of the probe fluorophore mediated by proximal guanosines in the target sequence was sufficient as a hybridization-dependent signal for the generation of melting temperature curves. In their report, the 677C→T was among the included SNPs.[45]

Comparison of the Different Homogenous Formats

The singly labeled probes used in the hybridization probe assays are easier and less costly to produce than the doubly labeled probes associated with the TaqMan and molecular beacon (and some other) formats. On the other hand, the melting temperature analysis carried out with hybridization probes requires specialized equipment and software, whereas the assays using doubly labeled probes are all compatible with a one-time reading of fluorescence after PCR (although all three quoted reports make use of real-time PCR equipment). Using the principles outlined in the paper by Crockett et al,[45] hybridization probe assays may be designed, using one singly labeled probe per SNP, as opposed to two doubly labeled probes per SNP for the TaqMan/molecular beacon format. Also, the potential for multiplexing several SNPs in the same tube are greater when melting curves and differently labeled fluorophores are combined,[44,46] (reviewed in ref. 33). Finally, with the hybridization probe assay, additional base substitutions within the boundary of the probe may be more easily discovered, and not compromise the interpretation of results.[33] A minor drawback with the hybridization probe assay has been that the equipment involves capillary tubes, which do not conform to the industry standard 96-well format. Recently, other melting curve-based assays have been published,[47,48] and a wider variety of equipment has been introduced.

Assessment of Methods

Frequently, when a new polymorphism is detected, a PCR/RFLP assay is designed. These assays are easy to perform and do not require expensive or specialized equipment. If a suitable restriction enzyme is not available, the format is still applicable by the generation of artificial restriction sites. A digestion control should be included, and, among the different variants described above, perhaps the most simple and elegant solution was presented by Bravo-Osorio et al.[19]

Allele-specific PCR is an alternative characterized by less handling time, as the step involving the restriction enzyme digestion is obviated.

Generally, some of the reported PCR/RFLP and allele-specific PCR assays may benefit from moving one of the primers closer to the variant position, thereby making the relative difference of fragment sizes larger. This would enhance resolution and shorten migration time in electrophoresis.

A minor but finite problem associated with many of the described assays is additional substitutions near the position of the SNP of interest. This may affect the recognition sequence of the restriction enzyme, the binding of allele-specific primers, and the binding of fluorogenic probes.[49] The heteroduplex method and hybridization probe assay are probably the most resilient to this influence. If anomalies in the migration pattern of heteroduplexes, or melting peaks of hybridisation probes are detected, reanalysis by sequencing should be performed. An additional strength of the heteroduplex assay is its multiplexing capability, although multiplexing can also be obtained with allele-specific PCR and with the real-time PCR strategy described by von Ahsen et al and others.[33,44] The homogenous assay formats are characterized by speed, ease of operation, and contamination control.

Preparation of DNA Material

An important part of genotyping is the preparation of DNA for subsequent processing (usually involving PCR). If traditional purification methods, such as phenol-chloroform extraction, are used, this part of the overall workload may require more time and effort than the actual genotyping. Recently, a number of DNA purification techniques have been developed, which involve no hazardous chemicals and with the potential for automation. It is often overlooked that PCR-based genotyping may not require highly purified DNA. Also, the amount of DNA needed for successful genotyping is small. A few nanograms are sufficient in most cases. This is less than the amount contained in 1 μl of whole blood. There have been a number of reports where blood and other biological fluids have been used directly for PCR, either without, or with minimal treatment.[50,51] This includes complicated multiplexed assays,[26,29] as well as one example referred to above using a homogenous assay format.[39] Also, in studies using archival material such as paraffin-embedded tissue slices, simple boiling protocols have been described.[52,53] When using blood as template, it is necessary to use a DNA polymerase that is tolerant to inhibiting substances. If such information for a particular enzyme is lacking, it can easily be obtained by appropriate tests.

In laboratories where automated DNA purification has been established, there may be logistic reasons for sample purification. A convenient storage format for future analyses, e.g., microtiter plates with dissolved DNA and identifier tags, may then be established. In our laboratory, we have had good experience with aliquoting purified DNA into PCR tube strips or PCR plates and letting it air-dry. Plates can then be stored at ambient temperature or 4°C for months, shipped to another laboratory, or processed immediately by adding a PCR master mix. This works with purified DNA (2-20 ng) as well as with unpurified blood (≤ 1μl).

Throughput Considerations

When more than a few hundred SNPs per week need to be analyzed, throughput, or time spent per sample, becomes important. The way this is addressed partly depends on whether 600 SNPs refers to one SNP in 600 samples, 3 SNPs in 200 samples, or 20 SNPs in 30 samples. In the first case, the workload falls heavily on the DNA preparation step, and genotyping without template purification could be considered as a means of increasing throughput and decreasing cost. The second case seems to be ideal for some of the multiplexed methods described above. Multiplexing also ensures that the correct ensemble of SNPs is assigned for any given sample.

None of the assays described thus far seems ideal for the last case. Most of the PCR/RFLP, allele-specific PCR, and heteroduplex methods quoted above involve a fair amount of manual handling (e.g., preparation and loading of gels, photographing etc.) All these assays, however, are compatible with automated capillary electrophoresis, or equivalent, for fragment analysis.

The homogenous assays require minimal sample handling, mainly the setup of reagents, which can be carried out by a robotic workstation, and genotype annotation is usually automated.

New Technologies and Future Developments

Thus far, fairly established methods of genotyping have been described. The great interest in determination of single nucleotide polymorphisms has motivated the development of new assay formats offering unprecedented levels of automation and throughput. Among recently developed technologies are the Invader assay,[54] fluorescence polarization detection,[55] Pyrosequencing, which is a form of chemical sequencing without subsequent gel-separation,[56] DNA microarrays, PNA based probing, and mass spectrometry detection.[16] Many of the new methodologies are aimed at large-scale, genome-wide mapping of SNPs, which are beyond the scope of this chapter. However, the reader should be aware of the rapidly expanding possibilities, including assays on-demand that are currently offered by several companies.

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