−80 °C. For a storage period of up to 30 days, we believe that the best procedure of obtaining stable AA concentrations is to deproteinize samples with TCA immediately and neutralize the supernatant of the acidified CSF after centrifugation. After this treatment the sample can be stored at −20 °C. Thus, this method of processing and storing CSF is proposed to avoid artifactual changes of AA concentrations caused by in vitro modifications of the AAs.

## Table 1. Effects of temperature and duration of sample storage on the measurement of neuroactive amino acids in CSF treated in different ways.

<table>
<thead>
<tr>
<th>CSF processing</th>
<th>Amino acid</th>
<th>°C</th>
<th>Fresh</th>
<th>2</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-treated CSF</td>
<td>ASP</td>
<td>−20</td>
<td>0.13 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>0.16 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GLU</td>
<td>−20</td>
<td>0.60 ± 0.21</td>
<td>0.68 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.52 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GLY</td>
<td>−20</td>
<td>5.60 ± 0.75</td>
<td>5.60 ± 0.67</td>
<td>5.55 ± 0.68</td>
<td>5.46 ± 0.68</td>
<td>5.44 ± 0.68</td>
<td>4.86 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>TAU</td>
<td>−20</td>
<td>6.54 ± 1.32</td>
<td>6.56 ± 1.30</td>
<td>6.56 ± 1.34</td>
<td>6.60 ± 1.28</td>
<td>6.40 ± 1.34</td>
<td>6.08 ± 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acid-treated CSF</td>
<td>ASP</td>
<td>−20</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.19 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GLU</td>
<td>−20</td>
<td>0.60 ± 0.21</td>
<td>0.83 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.49 ± 0.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.78 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.48 ± 1.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GLY</td>
<td>−20</td>
<td>5.60 ± 0.75</td>
<td>5.58 ± 0.74</td>
<td>5.62 ± 0.79</td>
<td>5.60 ± 0.76</td>
<td>5.56 ± 0.78</td>
<td>4.96 ± 0.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>TAU</td>
<td>−20</td>
<td>6.54 ± 1.32</td>
<td>6.56 ± 1.34</td>
<td>6.56 ± 1.28</td>
<td>6.56 ± 1.30</td>
<td>6.38 ± 1.31</td>
<td>6.02 ± 1.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutral CSF</td>
<td>ASP</td>
<td>−20</td>
<td>0.13 ± 0.03</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>GLU</td>
<td>−20</td>
<td>0.60 ± 0.21</td>
<td>0.60 ± 0.21</td>
<td>0.61 ± 0.20</td>
<td>0.62 ± 0.21</td>
<td>0.63 ± 0.19</td>
<td>0.64 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>GLY</td>
<td>−20</td>
<td>5.60 ± 0.75</td>
<td>5.60 ± 0.79</td>
<td>5.60 ± 0.75</td>
<td>5.60 ± 0.76</td>
<td>5.62 ± 0.71</td>
<td>5.42 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>TAU</td>
<td>−20</td>
<td>6.54 ± 1.32</td>
<td>6.54 ± 1.27</td>
<td>6.56 ± 1.28</td>
<td>6.52 ± 1.29</td>
<td>6.68 ± 1.52</td>
<td>6.66 ± 1.28</td>
</tr>
</tbody>
</table>

* Results are expressed in μmol/L, mean ± SD.

<sup>b</sup> Significantly different (P < 0.001) vs freshly analyzed samples (ANOVA).

References


Genotyping Method for Methyleneetetrahydrofolate Reductase (C677T Thermolabile Variant) Using Heteroduplex Technology, Zoe E. Clark,1 Derrick J. Bowen,2 Sharon D. Whalley,3 Michael F. Bellamy,3 Peter W. Collins,2 and Ian F. W. McDowell4* (Departments of 1 Medical Biochemistry, 2 Haematology, and 3 Cardiology, University of Wales College of Medicine, Cardiff CF4 4XN, United Kingdom; 4 author for correspondence: fax 44-0-1222-766276, e-mail Mcdowell@cf.ac.uk)

Low flow status, with associated increases of plasma homocysteine concentrations, has been implicated as a cause of neural tube defects and premature cardiovascu-
lar disease (1, 2). The production of 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR) enables remethylation of homocysteine to methionine, which thereby reduces homocysteine concentrations. A common mutation in the MTHFR gene (C677T) (3) produces an enzyme with partially reduced activity, which may be characterized by thermolability in vitro (4). Homozygotes (TT) are prone to developing mild to moderate hyperhomocysteinemia if dietary folate intake is below average (5). This may constitute an interaction between inheritance and diet for neural tube defects and cardiovascular disease.

The original technique described for C677T genotyping was restriction digestion, which exploits the creation of a restriction site for Hinfl (6). We have developed an alternative method based on heteroduplex technology and applied this to genotyping studies in a healthy population.

The principle of heteroduplex analysis is production of double-stranded DNA containing a short mismatched sequence, which will differ in conformation depending on the specific base sequence (7). A 147-bp region containing the mutation site (position 677) is amplified from genomic DNA in PCR, which includes an additional fragment of DNA known as a heteroduplex generator (HG). The HG is identical to the genomic PCR product except for a 3-bp deletion one nucleotide upstream from the mutation site. During PCR, genomic DNA and HG are both amplified. The genotyping PCR contains 0.4 mmol/L upstream primer MTHFR1 (5′AGGGAGCTTTGAGGCTGACCT-GAA3′), 0.4 mmol/L downstream primer MTHFR2 (5′AC-GATGGGGCAAGTGATGCCCATG3′), 0.2 mmol/L each dNTP, 2.5 mmol/L reaction buffer, 1.25 units of Taq polymerase, genomic DNA (0.5 mL for blood DNA and 5 mL for buccal cell DNA), and 1 μL of an appropriate dilution of HG in a total volume of 25 mL. The PCR program comprises 95 °C for 5 min for one cycle, followed by 95 °C for 30 s, 71 °C for 30 s, and 72 °C for 1 min for 35 cycles. The dilution of HG used in the PCR was established by titration. Serial dilutions of the HG were amplified in a PCR also containing 0.5 μL of genomic DNA. A 1:55 000 dilution produced similar amplification of both genomic DNA and HG target sequences and has been used for all genotyping with this HG preparation.

The heteroduplexes formed by annealing between amplified HG and genomic PCR product give profiles of DNA bands characteristic of each genotype, which can be visualized on a 10% polyacrylamide gel. The PCR appears to be tolerant of a wide range of concentrations of patient DNA such that it is not necessary to estimate the DNA concentration before setting up the reaction. The first-time success rate is ~96%.

The HG was constructed using site-directed mutagenesis by PCR using a modification of a method described previously (8). Briefly, primers MTHFR1 and MTHFR2 were used in an initial PCR (PCR I) to amplify a 147-bp fragment from genomic DNA. In a second reaction (PCR II), a mutagenic upstream primer, MTHFR3 (5′GAAGGAGAAGGTTGTCGTGGCGCCATTTCCATCATCA3′), specifying a 3-bp deletion in the HG was used in combination with MTHFR2 to amplify a 114-bp fragment spanning the mutation site from genomic DNA. Both fragments were electrophoresed and isolated from a 2% agarose gel. The 114- and 147-bp fragments were mixed and amplified in PCR III using all three primers MTHFR1, MTHFR2, and MTHFR3. This reaction yielded a number of products, one of which corresponded to the HG of size 144 bp containing the specified 3-bp deletion. The HG was extracted after polyacrylamide gel electrophoresis, and a uniform stock of HG was produced by cloning into Escherichia coli. The sequence was confirmed in both directions, and aliquots were stored frozen (available from I.F.W.M.).

Genotyping by restriction enzyme digestion was carried out using different primers (MTHFR1 and MTHFR2) as described by Frosst et al (6). Restriction fragments (96 bp and 51 bp) and/or undigested product were separated by electrophoresis on a 1% SeaKem–3% Nusieve agarose gel and visualized using ethidium bromide (Fig. 1).

In the comparison of the restriction digestion and heteroduplex techniques on 97 consecutive DNA samples, there was complete agreement (TT, 6; CT, 42; and CC, 49). Both methods can give reliable results; however, the restriction enzyme gels are more difficult to interpret and
do carry a risk of error should the restriction enzyme digestion not be complete. The heteroduplex genotyping technique is straightforward to perform and produces gel patterns that are easily interpreted without ambiguity. In heterozygotes both alleles are visualized to the same extent. Heteroduplex analysis requires only three steps to obtain a result (DNA extraction, PCR, and electrophoresis). The speed, economy, and robustness of the method make it particularly suitable for screening studies requiring large sample throughput. Although this method requires the initial synthesis of the HG, once it is synthesized and cloned theoretically unlimited supplies are available. Whole EDTA blood or buccal cells are both suitable sources of DNA for genotyping. The recovery of cells from the cheek lining using a “cytobrush” is a convenient method for both patients and laboratory.

In a study of 605 healthy subjects from South Wales, the genotype frequency was TT, 73 (12.1%); CT, 242 (40.0%); and CC, 290 (47.9%). The gene frequency for the T allele (0.32 ± 0.03, 95% confidence interval) is similar to that described in other Northern European studies (9–11) and confirms the high prevalence of this genetic variant in the general population. This economical and rapid method for MTHFR genotyping will facilitate further research on the role of folate and homocysteine in human disease.

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References


Is Prostate-specific Antigen a Marker for Pregnancies Affected by Down Syndrome? Kevin Spencer and Paul Carpenter (Endocrine Unit, Clinical Biochemistry Department, Harold Wood Hospital, Gubbins Lane, Romford, Essex, RM3 0BE United Kingdom; * author for correspondence: fax 44 (0) 1708381486, e-mail Kevin_Spencer@Compuserve.com)

Prenatal screening for trisomy 21 (Down syndrome) based on the analysis of biochemical markers in maternal serum during the second trimester of pregnancy is becoming an established part of obstetric practice (1, 2). Several markers have been investigated in the second trimester, and of these, a combination of α-fetoprotein and free β-human chorionic gonadotropin (hCG) has been shown in retrospective (3) and prospective studies (4, 5) to achieve detection rates of 65–75% (with a 5% false-positive rate) in routine practice. Similarly, combinations including α-fetoprotein, total hCG, and unconjugated estriol have been shown in prospective studies to perform almost as well, with detection rates on average of 65% (6).

Recent developments in this area of prenatal screening have focused on three specific areas. The first area is that of refinements to the risk algorithm, leading to improved detection efficiency by taking into account a variety of factors that influence the analyte concentrations, for example, multiple pregnancies (7), maternal weight (8–10), insulin-dependent diabetes mellitus (11, 12), gravidity (13), ethnicity (9), previous pregnancy results (14, 15), and smoking (16). Correction for these factors seeks to reduce the between-patient variance, leading to reductions in the false-positive rate. The second area involves the investigation of possible new markers of trisomy 21, such as dimeric inhibin A, which has been reported to add anything from 3% (17) to 22% (18) to the detection rate using existing double or triple marker approaches. In addition, beta core in urine (19) and free β-hCG in urine (20) have both been proposed as possible markers of trisomy 21. Neither the urine approach nor the addition of dimeric inhibin A have yet been shown conclusively to be better than existing procedures, and indeed their use is still somewhat controversial. The third area of investigation is the development of screening programs in the first trimester, in which a combination of ultrasound (nuchal translucency) and the biochemical markers pregnancy-associated plasma protein A and free β-hCG enabled detection rates of ~90% (21).

Recently, Lambert-Messerlian et al. (22) in this Journal reported increased concentrations of prostate-specific antigen (PSA) in the maternal serum of second trimester pregnancies affected by trisomy 21. Although PSA was originally thought to be a marker specific to the prostate, a variety of biological fluids and extracts of female tissues has now been shown to contain and produce a material that reacts positively in sensitive PSA immunoassays. The original observation leading to the study of PSA in cases of trisomy 21 was a study in amniotic fluid (AF) that observed low values of PSA in four of six cases (23).

In this study, we endeavored to reproduce these initial