recommended CV at this concentration. We believe that this change will significantly increase the sensitivity for detecting myocardial necrosis (1, 3).

In summary, we have shown that the upper reference limit for a healthy population is <0.16 μg/L for TnI concentrations measured using the Bayer ACS:Centaur method. The difference between TnI values obtained for serum or heparin-plasma specimens is only just significant at the low concentrations found in a healthy reference population. This difference is not likely to alter the clinical response and so does not justifying having a different reference interval related to each specimen type.

References
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Unexplained DNA Melting Behavior in a Genotyping Assay

To the Editor:

A common methionine/valine polymorphism at codon 129 of the prion protein gene modulates disease susceptibility and phenotypic variability of human prion diseases (1, 2). All patients with the new variant of Creutzfeldt-Jakob disease (vCJD) were homozygous for methionine at codon 129 (3). We developed a genotyping assay that uses rapid-cycle PCR and melting point analysis of fluorogenic hybridization probes, but we encountered an unexplained artifact of melting behavior that may lead to misinterpretation when using this method. Asymmetric PCR allowed reliable genotyping results.

Primers (5'-CACAGTCAGG-AACAAG-3' and 5'-GTACATGTTTG-GGT-3'); GenBank accession no. HSU29185, positions 25735–25752 and 25935–25919, respectively) and probes (anchor probe, 5'-CCGAAATGTAT-GTGCCGTCTCAT-fluorescein-3'; detection probe, 5'-LC-Red640-CAC-TCCAGCAGTAGCC-phosphate-3'; GenBank accession no. HSU29185, positions 25874–25849 and 25846–25828, respectively) were designed using a beta version of the LightCycler probe design software (Ver. 0.99.11; Roche Molecular Biochemicals). This software uses recommended methods for probe design (4) and calculates melting temperatures (Tm) by a nearest-neighbor method. PCR reactions were carried out in a final volume of 10 μL in glass capillaries (Roche Molecular Biochemicals). Each reaction mixture contained 5 pmol of forward primer, different amounts of reverse primer (0.5–5 pmol), 2 pmol each of anchor and detection probe, 3 mmol/L MgCl2, 1 μL of LightCycler-FastStart DNA Master Hybridization Probes (which includes reaction buffer, nucleotides, and Taq polymerase), and 2.5 μL of genomic DNA. The thermocycling conditions were as follows: 95°C for 10 min for initial denaturation and activation of Taq polymerase and 45 cycles of 95°C for 0 s, 55°C for 5 s, and 72°C for 10 s, with a ramping rate of 20°C/s. Melting analysis was performed by heating the capillary at 95°C for 10 s, followed by incubation at 45°C for 1 min and then slow (0.1°C/s) heating to 80°C.

The results of the melting point analysis were unexpected. Both a methionine/valine heterozygous sample and a valine homozygous sample lacked a valine peak, which was expected to occur at ~65°C (i.e., the calculated Tm for the detection probe without mismatch).

To evaluate the quality of the hybridization probes, we used single-stranded oligonucleotides complementary to the probe set in a melting point experiment without prior PCR amplification. Both so-called complements, one corresponding to the valine genotype (no mismatch) and the other corresponding to the valine genotype (one mismatch), showed the calculated melting peaks of ~65 and 58°C, respectively (Fig. 1A).

Because the probes had now been shown to work well on a single-stranded template, an asymmetric PCR system was devised to increase the amount of single-stranded PCR product available as target for the probes. In this system, the target DNA was amplified in the presence of a fixed amount of forward primer and various dilutions of reverse primer. The heterozygous (methionine/valine) sample could be successfully amplified with all concentrations of the reverse primer, from 5 pmol (equal to forward primer) to only 0.5 pmol (Fig. 1B). However, the reaction containing equal amounts of primers (Fig. 1C, curve 1), revealed only one peak at ~58°C in the melting point analysis. The correct result for the heterozygous sample, with the two expected peaks (at ~58 and
68 °C), was obtained only when the concentration of the reverse primer was 1/5 to 1/10 that of the forward primer (Fig. 1C, curves 3 and 4). In the presence of 2.5 pmol of reverse primer, the valine peak at 68 °C was present but was substantially smaller than the methionine peak at 58 °C (Fig. 1C, curve 2).

To check the influence of the probe composition, we designed a second set of hybridization probes (anchor probe, 5'-LC-Red640-GCCGGCAGCCTG-3'; detection probe, 5'-AGACGTAGCCGCC-f640-3'; GenBank accession no. HSU29185; positions 25821–25805 and 25838–25824, respectively). This alternative probe system produced identical results in the melting point analysis of the heterozygous sample, i.e., only asymmetric PCR revealed the correct genotype. The results were also reproduced when a different reverse primer (5'-CGTGCA-CAAATGTCG-3'; GenBank accession no. HSU29185; position 25981–25962) was combined with the first hybridization probe system.

Our results suggest that a reannealing of equimolar amounts of the PCR product strands inhibited the binding of the detection probe to the valine genotype. However, the replacement of G with A in the methionine genotype may have allowed probe hybridization. The net result was a single melting peak for a heterozygous sample (Fig. 1C, curve 1).

The asymmetric PCR protocol with more forward than reverse primer allowed fast and specific genotyping of the human prion protein polymorphism at codon 129 with the hybridization probes we designed and obviated the need for redesign and expensive ordering of new probes. An assay for prion protein genotyping that apparently works under symmetric PCR conditions was published recently (5). Since its introduction, many publications have shown the superior performance of the LightCycler™ in rapid genotyping of single-nucleotide polymorphisms [e.g., see Refs. (6–8)]. Several reports have documented the robustness of this type of assay. However, these have not mentioned the optimization procedure for assay development and that changes in reaction conditions might cause misleading genotyping results.

Fig. 1. PCR and genotyping by melting point analysis of the methionine/valine polymorphism at codon 129 of the prion protein gene. (A), melting point analysis of artificial complements [5'-CCTTGCGGTACG/AATGCTGGAGTCCATGAGGAGG-GCCCATCATATTTCGGCAGTGA-3'; GenBank accession no. HSU29185; position 25821–25880]. The numbered curves show results obtained with a complement with one mismatch, corresponding to the methionine genotype (A at position 25834; curve 1), or a complement without a mismatch, corresponding to the valine genotype (G at position 25834; curve 2). Melting peaks are at ~58 and 65 °C. (B and C), rapid-cycle PCR and genotyping by melting point analysis of a heterozygous sample. In each amplification, 5 pmol of forward primer was used. The numbered curves show results obtained in the presence of 5 pmol (curve 1), 2.5 pmol (curve 2), 1 pmol (curve 3), and 0.5 pmol (curve 4) of reverse primer. (B), fluorescence signal detected at the annealing step of each cycle during the amplification. (C), melting point analysis of the PCR products. Melting peaks are between 57 and 59 °C and ~67/68 °C. --d(F2)/dT, negative derivative of fluorescence with respect to temperature.
Our findings suggest the value of careful evaluation of new LightCycler genotyping assays and of controls for each genotype.

References


Heterophilic Antibody Interference with CARDIAC T Quantitative Rapid Assay

To the Editor:
Point-of-care troponin assays are promoted as providing rapid, reliable results to support best practice management of acute chest pain (1). We report a case of false-positive cardiac troponin T (cTnT) results that caused inappropriate clinical management.

A 46-year-old man consulted his general practitioner after 10 h of central chest pain and tingling in the left arm after a heavy fall. An electrocardiogram showed possible ST elevation. A repeat electrocardiogram in the local hospital showed sinus rhythm and an early repolarization pattern. Whole-blood cTnT (lithium heparinate) was increased both on admission and the next day in a CARDIAC T Quantitative Rapid Assay (third generation; Roche Diagnostics). The patient was a moderately heavy smoker, was mildly hypercholesterolemic, and had a family history of ischemic heart disease. The patient was transferred to the tertiary hospital where a coronary angiogram and plasma (lithium heparinate) was increased both on DIAC T and T STAT results, we retrieved the first admission plasma specimens (4°C storage) and analyzed them by both methods. The whole blood and serum specimens were collected from the patient 4 weeks after discharge and similarly analyzed (see Table 1). Serum rheumatoid factor was <20 kIU/L.

All specimens showed increased serum cTnT by CARDIAC T and undetectable concentrations by T STAT. The measured cTnT value for the later whole-blood specimen was 0.52 μg/L by CARDIAC T, whereas the plasma value was 0.39 μg/L (Table 1), which is within the within-run imprecision (CV, 10–15%) reported for the method (1).

The T STAT reagents sheet stated that they contain additives to minimize erroneous findings in samples from patients treated with monoclonal mouse antibodies. The CARDIAC T assay strips contain a mouse antibody as a human anti-murine antibody (HAMA) blocking agent (Rainer Zerback, Near Patient Testing Group, Roche Diagnostics, GmbH, personal communication). We found no record of our patient receiving mouse monoclonal antibodies.

We incubated (1 h at room temperature) the patient serum with increments of normal mouse serum (cat. no. M-5905; Sigma Chemicals) and then measured cTnT by the CARDIAC T method. Mouse serum abolished the apparent detection of cTnT well beyond a dilution effect, suggesting the presence of heterophilic murine antibodies (Table 1). Two precipitin bands were observed against mouse serum, and a single band was observed against mouse IgG (cat. no. I-5381; Sigma Chemicals) after counter immunoelectrophoresis of the patient’s serum on agarose gel (data not shown). These bands were not observed with the normal human serum control.

We conclude that the third-generation CARDIAC T assay is susceptible to positive heterophilic mouse

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