thus risk the loss of amplifiable RNA (4, 5). If RNA-based assays for the detection of minimal residual disease in blood or bone marrow are to be introduced into routine laboratory procedures with the potential to complement and/or substitute for immunocytochemical assays, the time between sample collection and processing in the laboratory must be taken into account. Techniques requiring less time, such as direct mRNA isolation and subsequent RT-PCR, might offer advantages to time-consuming enrichment strategies.

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Time for Troponin T? Implications from Newly Elucidated Structure

To the Editor:
The recent publication of the structure of the core domain of the troponin complex in Nature (1) has prompted us to ask: “What is the cardiac troponin T (cTnT) assay actually measuring?”

Roche Diagnostics Corporation has developed an electrochemiluminescence assay for cTnT (Roche E170), which detects free cTnT and binary/ternary troponin complexes released into the serum after a myocardial infarction (2). This assay uses two antibodies (M7 and M11.7) that recognize two specific epitopes (residues 125–131 and 136–147, respectively). The cTnT protein itself consists of 288 amino acids. From the study by Takeda et al. (1), we have been able to ascertain that the amino acid sequences of these epitopes are DRIERR and EQQRINEREKE, respectively, and found that these epitopes are located on the N-terminal “tail” region (TnT1) of cTnT. Unfortunately, the exact conformations of these epitopes remain to be established.

Recently, it has been observed that hemolysis causes interference in the cTnT assay (3). We note that it is possible that when a sample is hemolyzed, intracellular proteases such as cathepsins may be released into the plasma from erythrocytes (4). Furthermore, Roche has recently issued a product alert (March 2002) informing users about the potential interference of hemolysis in the cTnT assay. Whether this interference in the assay is attributable to hemolysis per se or to the released proteases degrading the troponin is not yet known.

The TnT1 region has a high content of α-helices, rendering it resistant to proteolysis (5). However, residues 183–200, which form a “flexible linker” between TnT1 and the “T arm” in the core domain of the troponin complex, are less conserved and highly susceptible to proteolysis (6). The study by Takeda et al. (1) indicates that the epitopes are located in the TnT1 region of the protein. We suggest that if the flexible linkers are proteolytically degraded (during hemolysis), it is possible that the fragments formed may not be recognized and detected by the Roche cTnT assay. This could theoretically produce a false-negative result for cTnT in the presence of a myocardial infarction and might explain the decrease in cTnT concentrations in hemolyzed samples.

There are 10 known isoforms of troponin T that are generated by alternative splicing of the TTN2 gene. Isoform 6 (cTnT6) is expressed in the healthy adult heart, whereas isoform 7 (cTnT7) is expressed in the failing adult heart (7). The only difference in amino acid sequence between these two forms is the inclusion of a five-residue peptide (amino acids 15–19) in isoform 6, which is also located on TnT1 (8). This five-amino acid peptide is highly acidic, and its inclusion in the TnT1 structure could add an overall negative charge to the cTnT complex (7). This could in turn increase the likelihood of false-negative results caused by structural changes in the antibody binding sites and proteolytic degradation such that the TnT1 portion is not detected by the assay.

In the light of the work by Takeda et al. (1), we believe it is important to establish exactly what the assay is detecting and how it is being affected. The effect of in vitro proteolysis on cTnT should also be examined. We stress that it is not acceptable to have false-negative results in an assay that is used for critical decision-making. This should reinvigorate research in this area.

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Rapid Detection of the Factor V Leiden Mutation by Real-Time PCR with TaqMan Minor Groove Binder Probes

To the Editor:

Individuals heterozygous for the factor V Leiden mutation have a sevenfold higher risk to develop venous thrombosis than wild-type (WT) individuals (1). The allele frequency of factor V Leiden in Europeans is ~4.4%, and the mutation is currently considered as the most important genetic risk factor for venous thrombosis (2). Various methods have been published to detect the factor V Leiden mutation by real-time PCR using hybridization probes (3–5) and TaqMan probes (6, 7). Here we describe a robust and convenient procedure for the detection of the factor V Leiden mutation that uses TaqMan probes conjugated to a minor groove binder (MGB) group.

TaqMan probes are degraded during amplification by the 5′ → 3′ exonuclease activity of the polymerase. During degradation of the probe, the fluorescent group is separated from the quencher, leading to increased fluorescence. For allelic discrimination, probes matching the WT and the mutant allele are conjugated to a different fluorescent group. Discrimination between alleles is based on the difference in melting temperature (ΔTm) between the differently labeled matched and mismatch probes. For TaqMan assays, relatively long probes are required that remain annealed during the extension phase of the PCR. A 1-bp mismatch in such a probe may lead to a small ΔTm and subsequent difficult allelic discrimination. DNA probes conjugated to a MGB group form hyperstabilized duplexes with complementary DNA by folding of the MGB group into the duplex, giving a higher Tm (8, 9). The conjugation of a MGB group therefore allows the design of shorter probes with an increased specificity attributable to increased mismatch discrimination (9).

For detection of the factor V Leiden mutation, we designed MGB probes with a length of 17 bases, which is >25% shorter than the length (23–24 bases) of previously published TaqMan probes for factor V Leiden detection (6, 7) without a MGB group. In addition, the probes were designed with the mutation site located in the MGB region to achieve the maximum ability to discriminate alleles (9). The following primer and probe sequences were designed with Primer Express software (Applied Biosystems) and synthesized by Applied Biosystems: forward primer, 5′-GCC TCT GGG CTA ATA GGA CTA CTT C-3′; reverse primer, 5′-TTT CTG AAA GGT TAC TTC AAG GAC AA-3′; WT factor V probe, FAM-ACC TGT ATT CCT CGC CT-nfq-MGB, and factor V Leiden probe, VIC®-ACC TGT ATT CCT TGC CT-nfq-MGB. The primers generate a 97-bp PCR product. The 5′-fluorescently labeled probes [6-carboxylfluorescein (FAM) or VIC] were conjugated to a nonfluorescent quencher (nfq) and an MGB group at the 3′ end.

DNA was isolated from 200 μL of EDTA-treated blood with the QIAamp DNA Mini Kit (Qiagen) according to the instructions of the manufacturer. The DNA was eluted in 200 μL of elution buffer and stored at −20 °C. PCR was performed in a final reaction volume of 25 μL, which contained 12.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 450 nM each primer, 200 nM each probe, and 5 μL of DNA solution. PCR was performed in MicroAmp optical 96-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were carried out with an ABI prism 7000 sequence detection system (Applied Biosystems). The amplification conditions were 2 min at 50 °C for AmpEraser uracil-N-glycosylase activity and 10 min at 95 °C for AmpliTaq Gold activation, followed by 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. After amplification, endpoint detection of fluorescence was performed at 60 °C.

The fluorescence data were analyzed with the allelic discrimination software of the ABI prism 7000 instrument.

The genotypes of 60 clinical samples were analyzed retrospectively. These samples were submitted to the laboratory for factor V Leiden mutation detection, and the genotype was initially established by PCR followed by restriction fragment analysis based on the method described by Koeleman et al. (10). Real-time factor V Leiden mutation detection was performed in duplicate. After amplification, four fluorescence patterns are expected. In samples from homozygous WT factor V patients, FAM fluorescence attributable to degradation of the WT probe will be present. In samples from patients homozygous for factor V Leiden, VIC fluorescence attributable to degradation of the factor V Leiden probe will be present. In samples from heterozygous patients, both FAM and VIC fluorescence will be detected. In PCRs to which no DNA was added, no fluorescence is expected except for the background signal. Fig. 1 shows that these four groups are...