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Time Is an Important Factor when Processing Samples for the Detection of Disseminated Tumor Cells in Blood/Bone Marrow by Reverse Transcription-PCR

To the Editor:

Despite histopathologic evidence of tumor-free margins, many breast cancer patients suffer from recurrence of their disease. This may reflect the presence of disseminated, premetastatic tumor cells at the time of initial diagnosis and therapy. The detection of disseminated tumor cells in bone marrow/blood has been associated with increased mortality in breast cancer patients (1). Reverse transcription-PCR (RT-PCR) for cytokeratin 19 (CK19) may be a useful alternative or complement to immunocytochemical detection (2). Unfortunately, results of RT-PCR studies vary widely. The reasons for this are conceptual as well as technical.

Because RNAses in samples can degrade RNA before analysis, we measured mRNA by RT-PCR in 15 sets of four samples each that were processed at different time points after collection and addition of a known amount of MCF-7 cells. One sample was processed immediately (≤5 min after blood drawing); the other three blood samples were processed after being left in their original tubes at room temperature (10 sets of samples) and on ice (5 sets of samples) for 1, 2, and 8 h, respectively, to simulate routine handling.

After mRNA extraction, RT-PCR for CK19 was performed on the LightCycler® System. Primers and probes were specific for the CK19 gene (Tib Molbiol) and avoided amplification of the CK19 pseudogene (3). We used a calibration curve and the Fit Points Method of the LightCycler quantification software. Experimental details are available in the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vo150/issue4/. We repeated this set-up 10 times for experiments with samples kept at room temperature and 5 times for experiments with samples kept on ice.

The apparent concentration of tumor cells decreased with increasing time of storage (Fig. 1). In 10 of 15 cases, the “1 h” samples yielded results that showed fewer or an equivalent amount of cells compared with the “0 h” results. In 12 of 15 cases, fewer cells were amplified in the “2 h” samples than in the “1 h” samples. Recovery of any cells was possible in only 4 of 15 “8 h” samples. We observed no difference in terms of recovery for samples that were kept at room temperature and those that were kept on ice at each time point.

Our results demonstrate that the time between sample collection and RNA stabilization is an important factor when the amounts of cellular RNA to be detected are small, as in the case of disseminated tumor cells. Despite our expectations, results from experiments on samples stored on ice did not differ from results for those kept at room temperature. Detection of very small amounts of specific mRNA species, i.e., at the limits of detectability, might therefore be highly sensitive to even small amounts of RNase activity.

Most research groups use enrichment methods such as density gradient centrifugation over Ficoll or epithelial antigen magnetic bead enrichment before RNA isolation and/or freezing. These are lengthy procedures, which take at least 60–90 min before cells can be added to a RNA-stabilizing environment, and

![Fig. 1. Degradation over time of mRNA from MCF-7 cells isolated from 5 mL of blood. The y axis indicates the mean number of cells detected in 15 experiments (whiskers, 2SD); the x axis represents the four time points (0, 1, 2, and 8 h) at which samples were processed.](http://www.clinchem.org/content/vo150/issue4/)
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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References

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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 DRIERRR and EQQRIRNEREKE, 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 troponins 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 TnT2 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.

References
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