Detection of Mycobacterium tuberculosis by Real-Time PCR Using Pan-Mycobacterial Primers and a Pair of Fluorescence Resonance Energy Transfer Probes Specific for the M. tuberculosis Complex, Christian Drosten,* Marcus Panning, and Stefanie Kramme (Bernhard-Nocht Institute of Tropical Medicine, National Reference Centre for Tropical Infections, 20359 Hamburg, Germany; * address correspondence to this author at: Bernhard Nocht Institute of Tropical Medicine, National Reference Centre for Tropical Infections, 20359 Hamburg, Germany; fax 49-40-42818378, e-mail drosten@bni-hamburg.de)

PCR is widely used in clinical laboratories to diagnose pulmonary, extrapulmonary, and disseminated tuberculosis. A multitude of primer pairs have been successfully applied, one of which has been studied most extensively because it is included in the Roche Amplicor MTB assay, whereas the adjacent downstream probe hybridized to a region conserved within the rDNA genes of all mycobacteria. After optimization of PCR, the probes gave specific fluorescence for MTC when combined with the Amplicor primers in the LightCycler. However, the sensitivity of the LightCycler protocol was inferior to that of a conventional thermal cycler protocol using the same reagent formulation (detectability of DNA in a limiting-dilution series reduced by a factor of 100).

In earlier experiments we had observed insufficient amplification of DNA fragments longer than 500 bp in LightCycler glass reaction capillaries (our unpublished data). We therefore adjusted the test to an Applied Biosystems 7700 SDS instrument that uses polypropylene reaction tubes similar to a conventional cycler. Because the long (640 nm) wavelength of FRET probes usually cannot be processed in this instrument, a spectral calibration run was performed according to the instrument manual with four replicate reaction vials containing 1 μM probe MTBP3 (carrying LCre640, a dye emitting fluorescence at 640 nm) in 50 μL of 1× PCR buffer.

After reoptimization of the amplification protocol, a 50-μL reaction volume contained 20 μL of nucleic acids extract, 5 μL of 10× PCR buffer II, 200 μM each of the deoxy nucleotide triphosphates, 4 mΜ MgCl₂, 200 μM each of primers KY18 [5'-catctgatgagccaggagaagg-3' (1)] and KY75 [5'-gccctctacgccagcctca-3' (1)], 100 μM probe MTBP3 (5'-acccgatgacccagggatcgttc-3', 5'-labeled with 6-carboxyfluorescein), 80 nM probe MTBP3 (5'-ggttgaagccgctttccg-3', 5'-labeled with LCre640 and 3'-phosphorylated), and 1.25 U of AmpliTaQ Gold DNA polymerase (all reagents from Applied Biosystems). Thermal cycling was as follows: 15 min at 95 °C, 50 cycles of 15 s at 95 °C, 20 s at 60 °C, and 40 s at 72 °C. The signal of LCre640 was read at 60 °C and divided by the signal for 6-carboxyfluorescein (495 nm) for normalization.

The efficiency of this protocol in detecting M. tuberculosis DNA was assessed by amplifying various defined amounts of a photometrically quantified plasmid containing the cloned target region of the assay (for details, refer to the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue10/). The results were subjected to probit regression analysis using the Statgraphics 5.0 software package (Statistical Graphics) with default set-
tings. A mean of 2.32 plasmids per PCR were calculated to yield a positive result with 95% probability. According to the Poisson distribution formula \( P(a) = e^{-m}(m^a/a!) \), with \( P \) being the probability of \( a \) occurrences per test at a mean of \( m \) objects per volume unit; set \( P = 0.05, a = 0 \), \( \sim 2.99 \) plasmids would theoretically be required to achieve 95% positivity in a 100% efficient test. We therefore assumed a 100% efficiency of DNA amplification in our optimized PCR protocol for further calculations.

Mycobacterial DNA was extracted from sputum samples or tissue biopsies by proteinase K digestion and lysozyme treatment in combination with the Puregene Blood Kit (Gentra Systems). In brief, 2-mm\(^3\) pieces of skin biopsies or, alternatively, bacteria pelleted from 1 mL of sputum samples treated with of 25 mL/L NaOH–N-acetylcysteine (17), were incubated overnight at 55 °C in 300 μL of buffer CLS (included in the reagent set) containing a final proteinase K concentration of 300 mg/L (Sigma). After the samples were heated to 95 °C for 15 min and cooled to room temperature, egg-white lysozyme (Sigma) was added to a final concentration of 250 mg/L and incubated for 1 h at 37 °C. The further procedure was according to the manufacturer's instructions. Pelleted DNA was resuspended in 200 μL of Tris-EDTA buffer.

Compared with nearly cell-free specimens such as cerebrospinal fluid, urine, or decontaminated sputum samples, MTC DNA extracted from biopsies would contain an interfering background of tissue DNA. The preparation procedure was therefore tested with human skin biopsies (3 mm\(^3\) in size) to which we added during the lysis step photometrically counted stock suspensions of cultured \( M. \) tuberculosis. Samples containing different amounts of bacteria were each extracted three times in parallel; the obtained DNA solutions were then analyzed in five parallel PCR reactions each (15 reactions per concentration). Probit analysis of these data (see the online Data Supplement) showed that a positive result could be expected in 95% of tests at a mean copy number of 7.36 copies/assay. Knowing that, theoretically, 2.99 bacteria per test would have achieved the same positivity rate if their DNA had been liberated with 100% efficiency in the purification procedure and that the recovery of liberated DNA is 100% efficient in our procedure (as determined above using pure plasmids), the liberation of DNA from mycobacteria would be 41% efficient by relating these numbers (2.99/7.36).

The analytical detection limit of \( \approx 7.36 \) mycobacteria/test was in the same range as that of the commercial Roche Amplicor assay [\( \approx 20 \) mycobacteria/test (3)]. The sensitivity was further assessed by use of a small collective of samples from 20 inpatients of the Bernhard-Nocht Institute treated for pulmonary or extrapulmonary tuberculosis. Twelve, 13, and 10 of 20 samples were positive by PCR, culture, and Ziel-Neelsen-stained smear, respectively. The resulting sensitivity of real-time PCR in culture positive samples was 92% (95% confidence interval, 66–99%; Table 1). Similar values have been found in large studies comparing the Amplicor PCR with culture and microscopy (3). To directly compare the clinical sensitiv-

### Table 1. Clinical sensitivity.

<table>
<thead>
<tr>
<th>PCR method</th>
<th>Samples tested</th>
<th>No. of positive results</th>
</tr>
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<tbody>
<tr>
<td>Amplicor</td>
<td>Stored samples(^a)</td>
<td>29</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Stored samples(^a)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Routine samples(^a)</td>
<td>13</td>
</tr>
</tbody>
</table>

\(^a\) Composition of panel: 22 sputum samples, 2 bronchial secretion specimens, 2 lung tissue biopsy specimens, 2 gastric aspirate samples, 1 urine sample. Four of 29 samples were positive in one method only (2 in the Amplicor only and 2 in the real-time PCR only).

\(^a\) Sputum specimens tested on a routine basis in culture and real-time PCR.

We are grateful to Gisela Bretzel and Elvira Richter for providing cultured and clinical mycobacteria samples. This work was funded by the German Ministry of Health.

### References

1. Tevere VJ, Hewitt PL, Dare A, Hocknell P, Keen A, Spadaro JP, et al. Detection of Mycobacterium tuberculosis by PCR amplification with pan-
16. Landt O. Selection of hybridization probes for real-time quantification and genetic analysis. In: Meuer S, Wittwer C, Nakagawara K, eds. Rapid polymerase chain reaction and rate-limiting enzyme in the three-step degradation of thymine-uraciluria (9). This is characterized by mental retardation and is sometimes accompanied by other neu- rologic disorders, such as microcephaly, motor retardation, and autism (9). In addition, increased DPD activity leads to toxicity from 5-FU even when typical doses are administered. This is often accompanied by severe diarrhea, neutropenia, and sometimes, neurotoxicity (9).

The DPD gene located on chromosome 1p20 encodes DPD (14). At least 13 variant alleles of the DPD gene have been described (13). In particular, three genetic variants have been associated with severe toxicity after 5-FU therapy: DPD*2A, DPD*5, and DPD*6 (9, 12, 13). DPD*2A is a result of a G-to-A transition in a splice donor site that causes skipping of exon 14 (9). DPD*2B includes the exon 14 splice SNP and A1627G in exon 13 (I543V), but it is extremely rare in the general population. DPD*5 and DPD*6 are point mutations (A-to-G and G-to-A polymorphisms, respectively) at codons 543 and 732, respectively (9). DPD*9A is a point mutation in exon 2 (T to C) with variable effect on DPD activity, but it has a high frequency in the general population (12). This makes it a useful marker for haplotype construction.

To better predict toxicity in patients receiving 5-FU, the first step is to find a reliable detection method to identify SNPs in the DPD gene. Pyrosequencing, a relatively recent method used to analyze SNPs based on the utilization of ATP to produce light (15), is an efficient new technology. The pyrosequencing reaction is based on the release of a pyrophosphate molecule with the sequential incorporation of bases to the DNA template.

This study describes the use of pyrosequencing to assay four previously described SNPs in the DPD gene:

Common human diseases, such as cancer, have been associated with multiple types of variation in the genome, including sequence repeats and deletions and single-nucleotide polymorphisms (SNPs) (1). Of these, SNPs are the most abundant in the human genome (2, 3). As a result of the efforts of many groups (4), an estimated 5 million SNPs are now deposited in public databases (5), providing a resource for determining how genomic variation affects human biology. Building on this work, many groups have shown that drug response is also influenced by genomic variation (6). Multiple SNPs have been identified that have a major impact on response to chemotherapy (7–11); it is therefore necessary to have rapid and efficient SNP evaluation techniques to analyze genes that influence chemotherapy response.

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the three-step degradation of uracil to β-alanine (12). This is the only endogenous pathway for production of the neurotransmitter β-alanine (13). DPD also degrades >80% of the anticancer agent 5-fluorouracil (5-FU), a pyrimidine analog used to treat colorectal cancer, and limits the oral absorption of the drug (13). DPD activity is found to be highest in liver and mononuclear cells, but it is also present in most other human tissues (14).

Variation in DPD activity can lead to nonbeneficial physiologic conditions. A complete absence of DPD has been associated with the hereditary metabolic disorder thymine-uraciluria (9). This is characterized by mental retardation and is sometimes accompanied by other neurologic disorders, such as microcephaly, motor retardation, and autism (9). In addition, decreased DPD activity leads to toxicity from 5-FU even when typical doses are administered. This is often accompanied by severe diarrhea, neutropenia, and sometimes, neurotoxicity (9).