methods for elucidating β-thalassaemia mutations. Blood Rev 2001;15:19–29.
3. Syvanen AC. From gels to chips: "minisequencing" primer extension for analysis of point mutations and single nucleotide polymorphisms. Hum Mutat 1999;13:1–10.
4. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: a review. Hum Mutat 2001;17:439–74.
5. Oefner PJ. Allelic discrimination by denaturing high-performance liquid chromatography. J Chromatogr B 2000;739:345–55.
6. Devaney JM, Pettit EL, Kaler SG, Vallone PM, Butler JM, Marino MA. Genotyping of two mutations in the HFE gene using single-base extension and high-performance liquid chromatography. Anal Chem 2001;73:620–4.
7. Wang W, Kham SKY, Yeo GH, Quah TC, Chong SC. Mulitplex minisequencing screen for common Southeast Asian and Indian β-thalassemia mutations. Clin Chem 2003;49:209–18.
8. Premstaller A, Xiao W, Oberacher H, O'Keefe M, Stern D, Willis T, et al. Temperature-modulated array high-performance liquid chromatography. Genome Res 2001;11:1944–51.
9. Bobadilla JL, Macek M Jr, Fine JP, Farrell PM. Cystic fibrosis: a worldwide analysis of CFTR mutations—correlation with incidence data and application to screening. Hum Mutat 2003;19:575–606.

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, Bernhard-Nocht-Strasse 74, 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, the only Food and Drug Administration-cleared PCR-based test for clinical detection of *Mycobacterium tuberculosis (1–3)*. The test amplifies a 584-bp fragment of the 16s rDNA of all mycobacteria and identifies members of the *M. tuberculosis* complex (MTC) by hybridization of a specific DNA probe. However, the hybridization step extends the turnaround time of this test, and obligatory license fees render it unaffordable for application in experimental studies or resource-limited settings.

Real-time detection technology has made it possible to establish noncommercial, probe-based PCR systems that provide stable operation, low contamination risk, and semiautomated interpretation of results *(4–11)*. We therefore aimed at adapting the Roche Amplicor assay to a real-time PCR protocol.

We did not modify the primers of the Amplicor test because of their demonstrated performance; thus, only the detection probe was adapted to the requirements of real-time PCR. Classic real-time probes [5′-nuclease, "TaqMan" *(12)*] require cleavage by *Taq* polymerase *(13)*, which could not be accomplished efficiently in our assay because the amplicon was too long and the only probe binding site specific for MTC was too distant from any of the primers *(9, 12, 14)*. As an alternative approach, we chose a pair of fluorescence resonance energy transfer (FRET) probes that do not have to be cleaved, making them less dependent on the above-mentioned factors *(15)*. On neighboring hybridization to the target DNA, an excited FRET probe system generates long-wavelength fluorescent emission that can be read by a Roche LightCycler instrument.

A suitable pair of probes was identified based on empirical design guidelines *(16)* and use of Primer Express software (Applied Biosystems) for calculation of melting points. The binding site for the upstream probe was the same as for the probe used in the Roche Amplicor 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 inefficient 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 LCRed640, 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 deoxynucleotide triphosphates, 4 mM $MgCl_2$, 200 nM each of primers KY18 [5′-cacatgcaagtcgaacggaaagg-3′ *(1)*] and KY75 [5′-gcccgtatcgcccgcacgctcaca-3′ *(1)*], 100 nM probe MTBP5 (5′-accggataggaccacgggatgcatgtctt-3′; 3′-labeled with 6-carboxyfluorescein), 80 nM probe MTBP3 (5′-ggtggaaagcgctttagcggtgt-3′, 5′-labeled with LCred640 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 LCRed640 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-

Downloaded from https://academic.oup.com/clinchem/article/49/10/1659/5642101 by guest on 23 April 2024

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 $P = 0.05$, $a = 0$], ~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 ≥7.36 mycobacteria/test was in the same range as that of the commercial Roche Amplicor assay [≥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 sensitivity with that of the Roche Amplicor assay, we tested 29 stored clinical samples in both assays. Instructions for the Amplicor assay were strictly followed. The results were equivalent, as shown in Table 1. To confirm the specificity of our test, we tested strains of *M. africanum*, *M. bovis*, *M. leprae*, *M. ulcerans*, *M. chelonae*, *M. smegmatis*, *M. gordonae*, and *M. avium* (one suspension of cultured bacteria each, pretested with a pan-*Mycobacterium* PCR). Fluorescent signals were obtained with *M. africanum* and *M. bovis*, but not with any other strain.

Table 1. Clinical sensitivity.

PCR method	PCR results in culture-positive samples: Samples tested	No. of positive results
Amplicor		
Stored samples[a]	29	22
Real-time PCR		
Stored samples[a]	29	22
Routine samples[b]	13	12

[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).
[b] Sputum specimens tested on a routine basis in culture and real-time PCR.

Overall expenses for reagents and consumables are US $5–6 per sample, as opposed to approximately US $30 in the Roche Amplicor assay. Hands-on time in our assay is the same as with the Roche test, but the time required for thermal cycling and product analysis is considerably reduced (2.5 h in our assay compared with ~5 h in the Roche assay).

Real-time PCR has already been shown to be appropriate for testing for MTC *(8–11)*, but all published assays use novel primers that have not been studied as extensively as the primers used here. Because the analytical sensitivity of our test was in the same range as that of the Roche Amplicor assay and the clinical sensitivity was equivalent, we believe that it will also yield a comparable clinical sensitivity when applied to larger patient collectives. Reconfiguration for reading out FRET probes extends the capabilities of the ABI 7700, an instrument that is in operation in many laboratories. The novel approach of using these probes on such an instrument may also be useful in similar settings in which a compatible 5′-nuclease probe cannot be combined with established primers. We believe that this low-cost approach could enable experimental studies and diagnostics in resource-limited settings.

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, Spadoro JP, et al. Detection of *Mycobacterium tuberculosis* by PCR amplification with pan-

Downloaded from https://academic.oup.com/clinchem/article/49/10/1659/5642101 by guest on 23 April 2024

Mycobacterium primers and hybridization to an *M. tuberculosis*-specific probe. J Clin Microbiol 1996;34:918–23.
2. D'Amato RF, Wallman AA, Hochstein LH, Colaninno PM, Scardamaglia M, Ardila E, et al. Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR *Mycobacterium tuberculosis* PCR test. J Clin Microbiol 1995;33: 1832–4.
3. Soini H, Musser JM. Molecular diagnosis of mycobacteria. Clin Chem 2001;47:809–14.
4. Guiver M, Borrow R, Marsh J, Gray SJ, Kaczmarski EB, Howells D, et al. Evaluation of the Applied Biosystems automated TaqMan polymerase chain reaction system for the detection of meningococcal DNA. FEMS Immunol Med Microbiol 2000;28:173–9.
5. Drosten C, Weber M, Seifried E, Roth WK. Evaluation of a new PCR assay with competitive internal control sequence for blood donor screening. Transfusion 2000;40:718–24.
6. Drosten C, Seifried E, Roth WK. TaqMan 5′-nuclease human immunodeficiency virus type 1 PCR assay with phage-packaged competitive internal control for high-throughput blood donor screening. J Clin Microbiol 2001;39: 4302–8.
7. Drosten C, Göttig S, Schilling S, Asper M, Panning M, Schmitz H, et al. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, Dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol 2002;40:2323–30.
8. Desjardin LE, Chen Y, Perkins MD, Teixeira L, Cave MD, Eisenach KD. Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis. J Clin Microbiol 1998;36:1964–8.
9. Garcia-Quintanilla A, Gonzalez-Martin J, Tudo G, Espasa M, Jimenez de Anta MT. Simultaneous identification of *Mycobacterium* genus and *Mycobacterium tuberculosis* complex in clinical samples by 5′-exonuclease fluorogenic PCR. J Clin Microbiol 2002;40:4646–51.
10. Kraus G, Cleary T, Miller N, Seivright R, Young AK, Spruill G, et al. Rapid and specific detection of the *Mycobacterium tuberculosis* complex using fluorogenic probes and real-time PCR. Mol Cell Probes 2001;15:375–83.
11. Lachnik J, Ackermann B, Bohrssen A, Maass S, Diephaus C, Puncken A, et al. Rapid-cycle PCR and fluorimetry for detection of mycobacteria. J Clin Microbiol 2002;40:3364–73.
12. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995;4:357–62.
13. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc Natl Acad Sci U S A 1991;88: 7276–80.
14. Lunge VR, Miller BJ, Livak KJ, Batt CA. Factors affecting the performance of 5′ nuclease PCR assays for *Listeria monocytogenes* detection. J Microbiol Methods 2002;51:361–8.
15. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997;22: 130–1,134–8.
16. Landt O. Selection of hybridization probes for real-time quantification and genetic analysis. In: Meuer S, Wittwer C, Nakagawara KI, eds. Rapid real-time PCR. Heidelberg: Springer, 2001:35–41.
17. Ratnam S, Stead FA, Howes M. Simplified acetylcysteine-alkali digestion-decontamination procedure for isolation of mycobacteria from clinical specimens. J Clin Microbiol 1987;25:1428–32.

Use of Pyrosequencing to Detect Clinically Relevant Polymorphisms in Dihydropyrimidine Dehydrogenase, *Ranjeet Ahluwalia, Robert Freimuth, Howard L. McLeod, and Sharon Marsh*[*] (Washington University School of Medicine, Division of Oncology, St. Louis, MO 63110; * address correspondence to this author at: Washington University School of Medicine, 660 South Euclid Ave., Campus Box 8069, St. Louis, MO 63110; fax 314-362-3764, e-mail smarsh@im.wustl.edu)

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 severe toxicity from 5-FU even when typical doses are administered. This is often accompanied by severe diarrhea, neutropenia, and sometimes, neurotoxicity *(9)*.

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

Downloaded from https://academic.oup.com/clinchem/article/49/10/1659/5642101 by guest on 23 April 2024