found were codon 55 (CCC→CTC) (n=1) and codon 53 mutation (ACC→GTC) (n=3). The latter mutation type is not represented in the MFP-LRC reference panel (**Table 3**).

The real time PCR-HRM results were highly concordant with the sequencing results. All four strains with mutations *in folP1*, including those with the 53 mutation (ACC→GTC) were re-identified (**Table 4**). Furthermore, a *folP1* mutant strain [55 (CCC→CTC)] that was overlooked previously was identified. Thus the sensitivity for mutation detection was 100% for *folP1*. Samples that produced HRM variants (i.e., non WT) clusters were found at 3.3%, 4.9% and 4.9 % for the *folP1*, *rpoB* and *gyrA* DRDRs. Of these, three appeared as HRM variants in more than one DRDR locus; the amplification curves showed high C(t) values/and or early fluorescence plateau indicative of low DNA concentrations and/or PCR inhibition which can limit the reliability of HRM cluster assignments. When tested in PCR inhibition assays, the addition of these DNA samples to NHDP63 controls increased the C(t) by 2-3 cycles (data not shown). Overall, these results showing a high sensitivity rate for clinical samples and when combined with a low false mutant rate validate that HRM as established for the mutations tested is suitable for drug resistance surveillance in the clinic.

**Development of SNP typing assays based on real-time PCR-HRM analysis and assay validation with reference specimens**

Four lineages of *M. leprae* (SNP types 1-4), distinguishable on the basis of three bi-allelic SNP loci have been described (28) (**Table 6A**). The PCR conditions were standardized using NHDP63 (**Table 5**). ADML that represent each of the SNP types and all the 18 MFP reference strains were quantitated using these assays (**Table 5**). For HRM cluster analysis, by using the

NHDP63 DNA, which is of SNP type 3, for the reference cluster assignment it was easy to identify the strains which had alleles that matched or differed from it at each of the three SNP loci **(Figure 4 and Tables 6A and 6B)**. For example, for SNP locus 1, the 'C' allele amplicons cluster together with NHDP63 (reference cluster 1), while those with the alternative allele 'T' separate (cluster 2) (**Figure 4**). With this HRM cluster approach, the actual SNP haplotype of the *M. leprae* DNA could then be readily determined.

While SNP type 1 and 4 can be ascertained by genotyping at just one locus, other types require mapping of at least two loci as can be seen by the haplotypes shown in **Table 6 A**. The real-time PCR HRM cluster assignment scheme was validated for the MFP-LRC reference samples. The SNP types in these strains had been determined previously by PCR amplicon sequencing method. In this process, HRM found that the strains Airaku-3, Indonesia-1 and Thai-311 are all actually SNP type 1 and not as previously published (Airaku-3 as SNP type 3 and the other two as type 2) (22).

**Performance of the real-time PCR-HRM SNP typing assays on clinical samples:**

To verify if real-time PCR-HRM was suitable for SNP typing of clinical isolates, a set of DNA samples prepared from biopsies from Nepali patients of unknown SNP types were selected. Correct SNP types were readily obtained, as demonstrated by the concordance of the HRM clustering derived results with those from a previously described PCR-RFLP assay (**Table 7**). Only SNP types 1 and 2 were identified within this clinical sample set, consistent with the genotypes that are prevalent in this country (28).

289

290 **DISCUSSION**

291 The success of antimicrobial therapies for leprosy is central and critical to prevent morbidities
292 and disabilities, and to decrease the incidence of new cases. The low levels of relapse reported
293 after MDT indicate that this treatment is effective (41). However, the nearly stable incidence rate
294 attests to continuing transmission. Multiple factors can contribute to the emergence and spread of
295 *M. leprae* drug resistance, particularly in highly endemic countries. These include unsupervised
296 components in the multidrug therapy and possible lack of compliance or irregular use; absence
297 of standardized tests for cure at release from treatment (RFT) and long term follow up. On the
298 other hand, drug resistance may also emerge in countries that have apparently achieved
299 'elimination status', because of scant resources and attention to leprosy.

300

301 Drug resistance surveillance and strain typing of *M. leprae* are useful molecular tools for leprosy
302 control. Traditional PCR and sequencing techniques for these applications are laborious and
303 expensive. In order to improve throughput, reduce costs of molecular tests and to support
304 inclusion of all patients, new and relapse cases, we explored emerging high resolution melt
305 technologies in real-time PCR. It is now possible to discriminate genetic variants in target loci by
306 post PCR analysis of the shapes and melting temperatures of amplicon melting curves. Such
307 methods have been utilized for scanning mutations in the tumor suppressor gene TP53 for cancer
308 detection (6), LDL receptor gene for hypercholesterolemia (16), *rpoB* and *inhA* genes for
309 rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* strains (2, 36), and the 3'
310 untranslated region for typing of bronchitis viruses (9). The requirements for real-time PCR-
311 HRM are a compatible thermocycler, PCR mix containing appropriate enzymes, buffer, and

312 DNA saturating dyes and high resolution melt software. For genotyping by HRM analyses, there
313 are no operator dependent sample manipulations after the real-time PCR is assembled, or the
314 need for additional reagents.

315

316 In the context of leprosy molecular epidemiology, we developed and demonstrated real-time
317 PCR-HRM assays for two situations: to detect mutations within drug targets *gyrA*, *rpoB*, *folP1*
318 (DRDR assays) and another for strain typing based on SNPs (SNP typing assays). Real-time
319 PCR assays have the added advantage of being able to estimate template amount in the clinical
320 DNA samples.

321

322 The capability of HRM in separating DNA variants is related to mutation types. As *M. leprae* is
323 not cultivable *in vitro* and few laboratories possess facilities and expertise for the propagation of
324 clinical strains in mouse foot pad or armadillo animal models, the availability of characterized
325 strains, particularly for testing drug resistance is limited. Clinical (biopsy or slit skin smear)
326 derived DNA containing *M. leprae* with DRDR mutations is a scant resource, precluding
327 availability, sharing and testing in different laboratories. Furthermore, some DRDR mutations
328 have been reported only once or infrequently. In this context, the feasibility of HRM for DRDR
329 mutation screening in *M. leprae*, the first study of its kind in leprosy, has been explored by
330 accessing the largest publically shared mouse derived DRDR mutant *M. leprae* library,
331 maintained at LRC, Japan. This library, although not comprehensive contained several
332 mutations in each of three drug targets (*rpoB*, *folP1* and *gyrA*), allowing standardization and
333 demonstration of proof of concept. Furthermore, by adding a mutation detected when screening
334 clinical strains available in-house, all mutant strains within these combined collections could be

identified by the HRM assays developed. These include *folP1* 53 (ACC→ATC, ACC→GTC) and 55 (CCC→CTC). Together, these three mutation types cover 50% of the mutants described world-wide. The DRDR mutation types not tested by HRM are *folP1* 53 (ACC→GCC) and 55 (CCC→CGC); the former has A→G substitution which is within HRM resolution capability. For rifampicin resistance, mutations in *rpoB* codons 441, 451, 456 and 458 have been reported. We tested HRM for 441 (GAT→TAT), 451 (CAC→TAC) and 456 (TCG →TTG). More than 80% of reported mutants are covered by these three genotypes. Four mutations not included in the current studies are 441(CAC→GAC), 456 (TCG→ATG), 456 (TCG to TTC) and 458 (GTA to GTG). Of these, substitutions in the latter three should be easily detected by HRM due to the level of change expected in Tms. For *gyrA*, we tested mutation in codon 91 (GCA-GTA); this covers more than 90% of the reported mutations. The other mutation is codon 89 (GGC to TGC), the G→T transition is detectable by HRM. It is of note, that for the double mutation (AC→GT) in the *folP1* codon 53 ACC to GTC detected in the clinical strains, the Tm change is negligible, and differentiation by HRM was possible only when a small amplicon (52 bp) was generated. There is only one report for this mutation type (24), which incidentally was detected in the same studied population, i.e., leprosy patients in Cebu, Philippines. The reason for identification of multiple cases with this mutation in our study sample is due to transmission within a closely linked community, as captured and described in greater detail by Li et *al* by prospective molecular epidemiological approaches (18, 30, 31).

Singh *et al* recently reported a method based on Taqman® probe assays for *rpoB* and *folP1* (35). The allele specific assays are not suitable for unknown or new mutations and require reciprocal testing with both wild type and mutation specific primers/probes (35). For these reasons, we find

358 that HRM is convenient for the preliminary screening of DNAs and rapid classification of
359 clinical strains into wild type or variant clusters. For *folP1* and *rpoB*, three different proven drug
360 resistance mutations were tested, which could be separated from the wild type strain. Further, for
361 the three variants of each target gene, two different HRM clusters could be detected.

362

363 With regard to SNP typing, HRM was robust and straightforward due to the bi-allelic nature of
364 each of the SNPs. SNP types 1, 2, 3 and 4 are based on the finding that only four out of 64
365 possible haplotypes have been detected by mapping SNPs at three loci (28). Thus SNP types can
366 be assigned by mapping only one or two of the three loci. These four SNP types can be further
367 divided into 16 SNP subtypes; however these are also restricted within a given endemic region.
368 For e.g., Philippines carries SNP types 1A and 3K, while in Brazil, 3I and 4 P strains are found
369 (29). Thus SNP 1-4 typing by real time-HRM provides a simple, rapid and robust classification
370 and is suitable for comparison of *M. leprae* strains on a global and national level. HRM assays
371 for the discrimination of the 16 subtypes which can be designed based on principles as described
372 herein were beyond the scope of the present study goals and also restricted by the availability of
373 a representative collection of all strains subtypes. Furthermore, to date, the highest resolution of
374 strains within SNP subtypes is achieved by VNTR strain typing (7, 15, 30, 31).

375

376 Real time PCR-HRM analysis of various DRDR targets aided in the detection of discrepancies
377 between expected and/or reported DRDR genotypes for strain Airaku-2. Secondary genotyping
378 of VNTR loci clarified that the strain received for HRM testing was indeed not Airaku-2 as
379 reported, but could be Gushiken (44). The HRM analysis was also sensitive in detecting the
380 presence of both wild type and mutant alleles at the *gyrA* locus in strain Zensho-4 due to a melt

curve that differed from the wild type or the expected mutant. VNTR strain typing of Zensho-4 did not show that the sample was not contaminated with another wild type strain. Thus real-time HRM analysis may enable detection of minor populations of mutant alleles in a wild type background and emergence of drug resistance. The melt curve of Zensho-5 for *gyrA* locus, expected to be of wild type showed possibility of a variant. Careful re-examination of the sequence chromatogram showed a minor contaminant peak (Figure 3). Similarly, melt curves of the *folP1* amplicon, although very close to that of the wild type are slightly different. When VNTR profiles were reviewed, duplicate alleles in several loci were detected. Overall, these data indicate that Zensho-5 DNA was contaminated with another DNA. Zensho-9 is interesting, previously shown to be *folP1* wild type sequence and susceptible in MFP assays (21,22). Current data shows *folP1* mutation also, which may indicate emergence of this mutation. The strain type did not show discrepancies by VNTR typing, so sample contamination is not an issue (44). These examples, illustrate the potential of real time PCR-HRM analysis as a sensitive mutation screening tool and for quality control, such as when sharing reagents between researchers and for detection of sample contamination.

For each target amplicon real-time PCR-HRM assays can be accomplished for 26 different samples each performed in triplicate, in a 96 well plate system, resulting in costs of less than $3 per sample and not requiring any other post-PCR reagents or procedures. In contrast, despite multiplexing of the target *rpoB, folP and gyrA* DRDRs, which reduced time, template, PCR reagent and plastic supply costs, the cost of a single sequencing reaction per target for just one direction was $7-10 at a subsidized rate. Sequencing is often performed off-site, which separates the PCR from subsequent steps, and adding replicate tests increases costs and labor. Another

404 issue is that in sequence chromatograms, mixtures of wild type and mutant are difficult to
405 delineate above background peak heights and can be missed (as seen in **Figure 3**). As a proof of
406 principle, DNAs with two known *rpoB* and *folP1* variants (0-100%, in 10% increments) were
407 combined. Even without a heteroduplex formation step in the PCR program or COLD-PCR
408 procedure (17) mixtures with as little as 10% of one type of DNA could be separated (data not
409 shown).

410

411 Our studies also showed that leprosy clinical DNA samples are amenable for real-time PCR-
412 HRM. The majority of clinical DNA samples that showed real-time PCR C(t) values less than 35
413 were suitable for HRM. This translates to a sensitivity of ~ 30 bacilli per PCR. It is highly likely
414 that next generation parallel sequencing technologies will advance sample throughput, quantities
415 and qualities of data including numbers of gene targets, and depth of coverage. In the interim, the
416 real-time PCR-HRM assays described here are viable, simple options, and can be easily
417 integrated into practice, by centralization of tests in a reference laboratory.

418

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428 **REFERENCE:**


429 1. **Balagon, M. F., R. V. Cellona, E. D. Cruz, J. A. Burgos, R. M. Abalos, and G.**
430 **P.Walsh.** 2009. Long-term relapse risk of multibacillary leprosy after completion of 2 years
431 of multiple drug therapy (WHO-MDT) in Cebu, Philippines. Am. J. Trop. Med. Hyg.
432 **81**:895-899.

433 2. **Choi G. E., S. M. Lee, J. Yi, S. H. Hwang, H. H. Kim, E. Y. Lee, E. H. Cho, J. H. Kim,**
434 **H. J. Kim, and C. L. Chang**. 2010. High-resolution melting curve analysis for rapid
435 detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical
436 isolates. J. Clin. Microbiol. **48**:3893-3898.

437 3. **Colston, M. J., G. R. F. Hilson, and D. K. Banerjee.** 1978. The 'proportional bactericidal
438 test': a method for assessing bactericidal activity of drugs against *Mycobacterium leprae* in
439 mice. Lepr. Rev. **49**:7-15.

440 4. **dela Cruz, E., R. V. Cellona, M. V. Balagon, L. G. Villahermosa, T. T. Fajardo, Jr., R.**
441 **M. Abalos, E. V. Tan, and G. P. Walsh. 1996. Primary dapsone resistance in** Cebu, The
442 Philippines; cause for concern. Int. J. Lepr. Other Mycobact Dis. **64**:253-256.

443 5. **Fukuda, H., and K. Hiramatsu.** 1999. Primary targets of fluoroquinolones in
444 *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **43**:410-412.

445 6. **Garritano S., F. Gemignani, C. Voegele, T. Nguyen-Dumont, F. Le Calvez-Kelm, D. De**
446 **Silva, F. Lesueur, S. Landi, and S. V. Tavtigian**. 2009. Determining the effectiveness of
447 High Resolution Melting analysis for SNP genotyping and mutation scanning at the TP53
448 locus. BMC Genet. **10**:5.

449 7. **Groathouse N. A., B. Rivoire, H. Kim, H. Lee, S.-N. Cho, P. J. Brennan, and V. D.**
450 **Vissa**. 2004. Multiple polymorphic loci for molecular typing of strains of *Mycobacterium*
451 *leprae*. J. Clin. Microbiol. **42:**1666-1672.

452 8. **Gupta U. D., K. Katoch, and V. M. Katoch**. Study of rifampicin resistance and
453 comparison of dapsone resistance of *M. leprae* in pre- and post-MDT era. Indian J. Lepr.
454 **81:**131-134.

455 9. **Hewson K. a, G. F. Browning, J. M. Devlin, J. Ignjatovic, and a H. Noormohammadi**.
456 2010. Application of high-resolution melt curve analysis for classification of infectious
457 bronchitis viruses in field specimens. Aust.Vet. J. **88:**408-413.

458 10. **Honore, N., and S. T. Cole.** 1993. Molecular basis of rifampin resistance in *Mycobacterium*
459 *leprae*. Antimicrob. Agents Chemother. **37:**414-418.

460 11. **Honore, N., E. Perrani, A. Telenti, J. Grosset, and S. T. Cole.** 1993. A simple and rapid
461 technique for the detection of rifampin resistance in *Mycobacterium leprae*. Int. J. Lepr.
462 Other Mycobact. Dis. **61:**600-604.

463 12. **Honore, N., P. W. Roche, J. H. Grosset, and S. T. Cole.** 2001. A method for rapid
464 detection of rifampicin-resistant isolates of *Mycobacterium leprae*. Lepr Rev. **72:**441-448.

465 13. **Kai, M., N. H. Nguyen Phuc, H. A. Nguyen, T. H. B. D. Pham, K. H. Nguyen, Y.**
466 **Miyamoto, Y. Maeda, Y. Fukutomi, N. Nakata, M. Matsuoka, M. Makino, and T. T.**
467 **Nguyen**. 2011. Analysis of drug-resistant strains of *Mycobacterium leprae* in an endemic
468 area of Vietnam. Clin. Infect.Dis. **52:**e127-132.

469 14. **Kapur, V., L. L.Li, S. Iordanescu, M. R. Hamrick, B. N. Kreiswirth, and J. M. Musser.**
470 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*)

471 encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium*
472 *tuberculosis* strains from New York City and Texas. J. Clin. Microbiol. **32:**1095–1098.

473 15. **Kimura, M., R. M. Sakamuri, N. A. Groathouse, B. L. Rivoire, D. Gingrich, S.**
474 **Krueger-Koplin, S.-N. Cho, P. J. Brennan, and V. Vissa**. 2009. Rapid variable-number
475 tandem-repeat genotyping for *Mycobacterium leprae* clinical specimens. J. Clin. Microbiol.
476 **47:**1757-1766.

477 16. **Laurie, A. D., and P. M. George**. 2009. Evaluation of high-resolution melting analysis for
478 screening the LDL receptor gene. Clin. Biochem. **42:**528-535.

479 17. **Li, J., and G.M. Makrigiorgos**. 2009 "COLD-PCR: a new platform for highly improved
480 mutation detection in cancer and genetic testing". Biochem. Soc. Trans. **37:** 427–432.

481 18. **Li, W., R. M. Sakamuri, D. E. Lyons, F. M. Orcullo, V. Shinde, E. L. Dela Pena, A. A.**
482 **Maghanoy, I. B. Mallari, E. V. Tan, I. Nath, P. J. Brennan, M. Balagon, V.Vissa.** 2011
483 Transmission of dapsone-resistant leprosy detected by molecular epidemiological
484 approaches Antimicrob, Agents Chemother. **55:**5384-5387.

485 19. **Maeda, S., M. Matsuoka, N. Nakata, M. Kai, Y. Maeda, K. Hashimoto, H. Kimura, K.**
486 **Kobayashi, and Y. Kashiwabara.** 2001. Multidrug resistant *Mycobacterium leprae* from
487 patients with leprosy. Antimicrob. Agents Chemother. **45:**3635-3639.

488 20. **Markham N. R., and M. Zuker.** 2005 DINA Melt web server for nucleic acid melting
489 prediction. Nucleic. Acids Res. **33:**W577-581

490 21. **Matsuoka, M.** 2010. Drug resistance in leprosy. Jpn. J. Infect. Dis. **63:**1-7.

491 22. **Matsuoka M.** 2010. History and characteristics of isolates maintained at the leprosy
492 research center. Nihon Hansenbyo Gakkai Zasshi. **79:**247-256.

493 23. **Matsuoka, M., K. S. Aye, K. Kyaw, E. V. Tan, M. V. Balagon, P. Saunderson, R.**
494 **Gelber, M. Makino, C. Nakajima, and Y. Suzuki.** 2008. A novel method for simple
495 detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA
496 microarray, and its applicability in developing countries. J. Med. Microbiol. **57**:1213-1219.

497 24. **Matsuoka, M., T. Budiawan, K. S. Aye, K. Kyaw, E. V. Tan, E. D. Cruz, R. Gelber, P.**
498 **Saunderson, V. Balagon, and V. Pannikar.** 2007. The frequency of drug resistance
499 mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from
500 Myanmar, Indonesia and the Philippines. Lepr. Rev. **78**:343-352.

501 25. **Matsuoka, M., Y. Kashiwabara, and M. Namisato.** 2000. A *Mycobacterium leprae*
502 isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int. J. Lepr. Other
503 Mycobact Dis. **68**:452-435.

504 26. **Matsuoka M., Y. Suzuki, I. E. Garcia, M. Fafutis-Morris, A. Vargas-González, C.**
505 **Carreño-Martinez, Y. Fukushima, and C. Nakajima.** 2010. Possible mode of emergence
506 for drug-resistant leprosy is revealed by an analysis of samples from Mexico. Jpn. J.
507 Infect.Dis. **63**:412-416.

508 27. **Molyneux, D. H.** 2004. "Neglected" diseases but unrecognised successes--challenges and
509 opportunities for infectious disease control. Lancet. **364**:380-383.

510 28. **Monot, M., N. Honoré, T. Garnier, R. Araoz, J.Y. Coppée, C. Lacroix, S. Sow, J.S.**
511 **Spencer, R.W. Truman, D.L. Williams, R. Gelber, M. Virmond, B. Flageul, S.N. Cho,**
512 **B. Ji, A. Paniz-Mondolfi, J. Convit, S. Young, P.E. Fine, V. Rasolofo, P.J. Brennan,**
513 **S.T. Cole.** 2005. On the origin of leprosy. Science. **308**:1040-1042.

514 29. **Monot M., N. Honoré, T. Garnier, N. Zidane, D. Sherafi, A. Paniz-Mondolfi, M.**
515 **Matsuoka, G. M. Taylor, H. D. Donoghue, A. Bouwman, S. Mays, C. Watson, D.**

516 **Lockwood, A. Khamesipour, A. Khamispour, Y. Dowlati, S. Jianping, T. H. Rea, L.**
517 **Vera-Cabrera, M. M. Stefani, S. Banu, M. Macdonald, B. R. Sapkota, J. S. Spencer, J.**
518 **Thomas, K. Harshman, P. Singh, P. Busso, A. Gattiker, J. Rougemont, P. J. Brennan,**
519 **and S. T. Cole.** 2009. Comparative genomic and phylogeographic analysis of
520 *Mycobacterium leprae*. Nat. Genet. **41:**1282-1289.

521 30. **Sakamuri R.M., J. Harrison, R. Gelber, P. Saunderson, P.J. Brennan, M. Balagon, V.**
522 **Vissa.** 2009. Continuation: study and characterization of *Mycobacterium leprae* short
523 tandem repeat genotypes and transmission of leprosy in Cebu, Philippines. Lepr. Rev.
524 **80:**272-279.

525 31. **Sakamuri R. M., M. Kimura, W. Li, H.-C. Kim, H. Lee, M. D. Kiran, W. C. Black, M.**
526 **Balagon, R. Gelber, S.-N. Cho, P. J. Brennan, and V. Vissa**. 2009. Population-based
527 molecular epidemiology of leprosy in Cebu, Philippines. J. Clin. Microbiol. **47:**2844-2854.

528 32. **Sapkota B. R., C. Ranjit, K. D. Neupane, and M. Macdonald**. 2008. Development and
529 evaluation of a novel multiple-primer PCR amplification refractory mutation system for the
530 rapid detection of mutations conferring rifampicin resistance in codon 425 of the *rpoB* gene
531 of *Mycobacterium leprae*. J. Med. Microbiol. **57:**179-184.

532 33. **Sapkota, B. R., C. Ranjit, and M. Macdonald**. 2006. Reverse line probe assay for the
533 rapid detection of rifampicin resistance in *Mycobacterium leprae*. Nepal Med. Coll. J.
534 **8:**122-127.

535 34. **Shepard, C. C.** 1967. A kinetic method for the study of the activity of drugs against
536 *Mycobacterium leprae* in mice. Int. J. Lepr. **35:**429-435.

537 35. **Singh, P., P. Busso, A. Paniz-Mondolfi, N. Aranzazu, M. Monot, N. Honore, A. D. F. F.**
538 **Belone, M. Virmond, M. E. Villarreal Olaya, C. Rivas, and S. T. Cole**. 2011. Molecular

539 drug susceptibility testing and genotyping of *Mycobacterium leprae* from South America.
540 Antimicrob. Agents Chemother. **55:**2971-2973.

541 36. **Ramirez, M. V., K. C. Cowart, P. J. Campbell, G. P. Morlock, D. Sikes, J. M.**
542 **Winchell, and J. E. Posey.** 2010. Rapid detection of multidrug-resistant *Mycobacterium*
543 *tuberculosis* by use of real-time PCR and high-resolution melt analysis. J Clin Microbiol.
544 **48:**4003-4009.

545 37. **Rozen, S and J. Helen**. Skaletsky. 2000, Primer3 on the WWW for general users and for
546 biologist programmers, pp 365-386. *In* S. Krawetz, and S.A. Misener (ed.), Bioinformatics
547 Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

548 38. **Williams, D. L., and T. P. Gillis.** 2004. Molecular detection of drug resistance in
549 *Mycobacterium leprae*. Lepr. Rev. **75:**118-130.

550 39. **Williams, D. L., L. Spring, E. Harris, P. Roche, and T. P. Gillis. 2000**. Dihydropteroate
551 synthase of Mycobacterium leprae and dapsone resistance. Antimicrob. Agents Chemother.
552 **44:**1530-1537.

553 40. **Williams, D.L., and T. P. Gillis.** 1999. Detection of drug-resistant *Mycobacterium leprae*
554 using molecular methods. Indian J. Lepr. **71:**137-153.

555 41. **World Health Organization**. 2010. Global leprosy situation, 2010. Wkly. Epidemiol. Rec.
556 **85:**337–348.

557 42. **World Health Organization.** 2009. Drug resistance in leprosy: reports from selected
558 endemic countries.Wkly. Epidemiol. Rec. **84:**264-267.

559 43. **World Health Organization.** 1982. Chemotherapy of leprosy for control programmes.
560 Geneva: WHO. Technical Report Series. 768.

561 44. **Zhang, L., T. Budiawan, and M. Matsuoka.** 2005. Diversity of potential short tandem
562 repeats in *Mycobacterium leprae* and application for molecular typing. J. Clin. Microbiol.
563 **43**:5221-5229.
564 45. **Zuker M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction.
565 Nucleic Acids Res. **31**:3406-3415.

566

567 **FIGURE LEGENDS**

568 **Figure 1: The *M. leprae* targets of dapsone (ML0224, *folP1*), rifampicin (ML1891c, *rpoB*),**
569 **and fluoroquinolones (ML0006, *gyrA*).** The partial nucleotide (upper) and corresponding amino
570 acid (lower) sequences containing the drug resistance determining regions (DRDR) of the target
571 genes are presented. The nucleotides and the amino acid numbers are with reference to the open
572 reading frames of the genes for the *M. leprae* TN strain as found in the Leproma website
573 (http://genolist.pasteur.fr/Leproma/). The codons/amino acids implicated in drug resistance are
574 shown within boxes. The primer sequences selected for real-time PCR- HRM are underlined.

575 **Figure 2: Real-time PCR-HRM analysis for detection of mutations in *M. leprae* drug**
576 **resistance determining regions (DRDR assays).**

577 Representative real-time-PCR normalized melt curves (A) and the differential curves (B) for *M.*
578 *leprae* strains analyzed at the DRDRs of *folP1*, *rpoB* and *gyrA*. The green color was assigned to
579 NHDP63 strain serving as the wild type for each DRDR. The mutants or mixed genotype strains
580 are shown in red, orange and blue. The genotypes of the mutants are indicated within parentheses
581 next to the sample name.

582 **Figure 3: Sequence chromatograms of samples depicting multiple alleles in *gyrA* and *folP1***
583 **DRDR**. Arrows indicate nucleotide positions where mixed alleles were detected for samples
584 named in the chromatograms.

585 **Figure 4: Real-time PCR-HRM analysis for SNP detection for *M. leprae* typing (SNP typing**
586 **assays).** Representative real-time-PCR normalized melt curves (A) and the differential curves
587 (B) for *M. leprae* strains analyzed at three SNP loci as indicated beside the panels. The green
588 color was assigned to NHDP63, and the corresponding alleles for each locus are indicated (this is
589 referred to as Cluster 1 in Tables 6 and 7). The red curves indicate strains with the alternative
590 allele at each locus (this is referred to as Cluster 2 in Tables 6 and 7).

Partial sequence of *M. leprae* |ML0224|*folP1*

```
 91 - gct gtc cag cac ggc ctg gca atg gtc gcg gaa ggc gcg gcg att gtc gac gtc ggt ggc
 31 -  A   V   Q   H   G   L   A   M   V   A   E   G   A   A   I   V   D   V   G   G
151 - gaa tcg acc cgg ccc ggt gcc att agg acc gat cct cga gtt gaa ctc tct cgt atc gtt
 51 -  E   S   T   R   P   G   A   I   R   T   D   P   R   V   E   L   S   R   I   V
```

Partial sequence of *M. leprae* |ML1891c|*rpoB*

```
1261 - cgt ccg gtg gtc gcc gct atc aag gaa ttc ttc ggc acc agc cag ctg tcg cag ttc atg
 421 -  R   P   V   V   A   A   I   K   E   F   F   G   T   S   Q   L   S   Q   F   M
1321 - gat cag aac aac cct ctg tcg ggc ctg acc cac aag cgc cgg ctg tcg gcg ctg ggc ccg
 441 -  D   Q   N   N   P   L   S   G   L   T   H   K   R   R   L   S   A   L   G   P
1381 - ggt ggt ttg tcg cgt gag cgt gcc ggg cta gag gtc cgt gac gtg cac cct tcg cac tac
 461 -  G   G   L   S   R   E   R   A   G   L   E   V   R   D   V   H   P   S   H   Y
```

Partial sequence of *M. leprae* |ML0006|*gyrA*

```
181 - tta gac tcc ggt ttc cgc ccg gac cgt agc cac gct aag tca gca cgg tca gtc gct gag
 61 -  L   D   S   G   F   R   P   D   R   S   H   A   K   S   A   R   S   V   A   E
241 - acg atg ggc aat tac cat ccg cac ggc gac gca tcg att tat gac acg tta gtg cgc atg
 81 -  T   M   G   N   Y   H   P   H   G   D   A   S   I   Y   D   T   L   V   R   M
```