Leprosy is an infectious disease of skin and nerves caused by *Mycobacterium leprae*. The disease remains endemic in many parts of the world, and is now listed as a neglected tropical disease (27) by the World Health Organization. The drug dapsone was introduced in 1950, and administered in the form of long term monotherapy for treatment of leprosy; unfortunately, drug resistance emerged during the 1960s and 1970s (4). For this reason, in 1982, the World Health Organization (WHO) formally recommended multidrug therapy (MDT) which includes dapsone, rifampicin and clofazimine for the treatment and control of multibacillary (MB) leprosy (43). Sporadic reports of clinical resistance to dapsone and rifampicin started appearing in several countries such as Vietnam, Mexico, India and Philippines (1, 8, 13, 19, 24, 25, 26). Noncompliance and inadequate therapy may be the causes, particularly for MB leprosy. The drug targets and the mutations in the coding genes *folP1*, *rpoB* and *gyrA* that lead to clinical resistance to dapsone, rifampicin and the fluoroquinolones (used in an alternative leprosy drug regimen) respectively have been identified and characterized (5, 10, 14, 39). The *in vivo* drug susceptible or resistance phenotypes of various mutations seen in clinical strains in patient skin biopsies have been determined empirically by the traditional mouse foot pad inoculation assays (3, 20, 34). Mice are given drugs (in diet or by gavage) at different concentrations, and bacterial growth in the foot pads is measured at different time points. These assays have corroborated that clinical resistance to rifampicin, dapsone and oxfloxacin highly correlates (almost always) with detection of specific mutations within *M. leprae rpoB*, *folP1* and *gyrA* genes (21). For *M. leprae*, mutations have been reported in one or several codons located within short DNA regions in each of the target genes, thus defining the drug resistance determining regions (DRDRs). Although, it is possible that there are mutations outside the DRDRs of *rpoB*, *folP1* and *gyrA* or in other genes; or alternative mechanisms of resistance exist, these have yet to be identified. The MFP assays are labor, time and cost intensive; moreover the results are not available in time to

66 influence treatment options. Therefore, the MFP methods have given way to molecular methods
67 for screening proven resistance related mutations (42).

68 Furthermore, despite global MDT programs, the new case detection rates have not declined as
69 expected in many of the highly endemic countries (41), which indicates continued transmission
70 of the pathogen. In 2001, the first reference *M. leprae* genome of the 'TN' strain from a Tamil
71 Nadu, India leprosy patient was sequenced, offering new insight and opportunities for
72 development of tools in investigating bacteriology, pathogenesis and epidemiology. Mapping
73 polymorphic loci, such as 'variable number tandem repeats' (VNTRs) (7, 15) and single
74 nucleotide polymorphisms (SNPs) have applications in strain typing for tracing transmission of
75 leprosy. Four *M. leprae* lineages (SNP type 1, 2, 3 and 4) have been described, on the basis of
76 unique haplotypes derived from three SNPs which were identified by comparative genome
77 sequencing following the availability of TN strain genome sequence (28, 29).

78

79 Although it has become possible to perform amplification of the target loci by PCR followed by
80 DNA sequencing of the amplicons for detecting genetic variants (10, 11, 12, 32, 38, 40), the
81 labor and costs involved in PCR-DNA sequencing are still limiting factors for routine drug
82 resistance surveillance and SNP strain typing. Several surrogate methods such as single strand
83 conformation polymorphism (SSCP), reverse hybridization on membranes or microarrays have
84 been developed for allele specific detection of DRDR mutations from clinical specimens (23,
85 33), which require dedicated reagents or kits. PCR-Restriction fragment length polymorphism
86 assays developed by us expedited SNP typing by eliminating the sequencing steps, and replacing
87 them with conventional DNA electrophoresis for distinguishing the variants (31). DRDRs are not
88 suitable for PCR-RFLP, as there are different mutations at one or more positions. Numerous

89 PCR assays exist for allele discrimination, but often require multiple PCRs, allele target specific
90 primers or additional expensive probes (35).

91

92 In this context, we exploited the emerging real-time PCR technologies which can eliminate post
93 PCR procedures for genotyping any *M. leprae* genomic target of interest, particularly those
94 suitable for leprosy epidemiology applications. Real-time PCR-high resolution melt (HRM)
95 analysis is a novel simple post-PCR step that exploits thermal characteristics of the amplicons for
96 detection of sequence variants. This report describes the method development and validation of
97 real-time PCR-HRM assays for two applications: global drug resistance surveillance and SNP
98 based strain typing of *M. leprae*. These will be referred to as DRDR and SNP typing assays in
99 this study. The technical and practical considerations of the methods, and the advantages and
100 current limitations are discussed in the context of leprosy.

101

102 **MATERIALS AND METHODS**:

103 ***M. leprae* Reference and Clinical Specimens:**

104 *M. leprae* clinical strains maintained in mouse foot pad (MFP) system and in armadillo animal
105 systems were utilized as reference strains. Eighteen MFP strains were obtained from the Leprosy
106 Research Centre (LRC), National Institute of Infection Diseases, Tokyo, Japan (22); these strains
107 are therefore designated as MFP-LRC in this study. Suspensions of bacilli (10^8) were preserved
108 in 1ml of 70% ethanol. The armadillo derived *M. leprae* (ADML) clinical strains NHDP63,
109 Br4923, and 3039/210 obtained from infected tissues have been described previously (15).

110

The clinical skin biopsy samples used in this study were obtained from patients consulting at the Cebu Skin Clinic, Leonard Wood Memorial Leprosy Research Centre, Philippines as previously described (n=121) (18, 31), and at the Anandaban Hospital, Kathmandu, Nepal (n=25, during 2000-2010). The Philippine samples were stored in 70% ethanol at the time of collection. The Nepal samples were obtained from a repository of homogenized skin biopsies stored frozen in phosphate saline buffer containing 0.1% BSA. An aliquot of the homogenate was transferred to a fresh vial and suspended in 70% ethanol. All of the procedures involving biological sample collections and testing were performed following approval from the governing human research ethical committees and informed consent procedures as necessary.

DNA extraction from reference *M. leprae* cells and clinical tissue specimens:

As template DNA quality affects the amplification and the analysis by HRM, all the DNAs were prepared under uniform conditions using the DNeasy® tissue kit (QIAGEN, Valencia, CA) as described previously (15). The method involves proteinase K digestion and spin column chromatography. DNA was eluted in 100-200 µl AE buffer provided in the kit. For use as DNA concentration standards and as reference genotypes for the DRDR and SNP typing assays, approximately 1 mg of purified cells NHDP63 *M. leprae* purified from infected armadillo tissues as described previously were processed by DNeasy® tissue kit. These cells were prepared and obtained *via* the Leprosy research support-NIH-NIAID Contract at Colorado State University. The DNA was eluted in AE elution buffer. The DNA concentration was estimated by measuring UV light absorbance at wavelength of 260 nm. An aliquot of the stock DNA was adjusted with AE buffer to a final working stock of 10µg/ml, from which the DNA standards (1ng/ µl, 100pg/µl, 10pg/ µl, 1 pg/ µl and 0.1pg/µl) were prepared by 10 fold serial dilutions in

AE buffer. The DNA derived from MFP-LRC *M. leprae* (~10^8 cells) was diluted 1:100 in AE buffer; typically 1 µl of DNA was sufficient for one PCR.

Primers for real-time PCR-HRM DRDR and SNP typing assays:

Primer sets used for amplification of the DRDRs in *rpoB*, *folP1* and *gyrA* genes for real-time PCR-HRM are shown in **Figure 1**. The primers for the SNP typing assays are shown in **Table 1**. The primer sequences and nucleotide numbering system refer to those of the genome sequence of the TN strain (http://genolist.pasteur.fr/Leproma/). Primers for target loci were designed per the recommended amplicon size (less than 200-bp) for optimal real-time PCR-HRM genotyping using the freely available software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (20, 37). The target sequences were analyzed by MFOLD software to select the products with least secondary structure (http://mfold.rna.albany.edu/?q=mfold) (45).

Real-time PCR conditions and DNA template quantitation:

The real-time PCR (20 µl) was composed of 10 µl 2X Precision Melt Supermix (Bio-Rad Laboratories, Hercules, CA), forward and reverse primers (except for *rpoB* DRDR, 0.5 µl each, from 10 µM working stocks was used for the other five PCR amplicons; for *rpoB* 0.4 µl of each primer was used), nuclease-free water (8 or 8.2 µl) and DNA extracted from clinical and reference materials (1 µl). The reactions were set up in triplicate, in 96 well PCR plate and run on a CFX96® Real-Time PCR system (Bio-Rad). The CFX Manager™ software (Bio-Rad) was utilized to set up the sample arrangement on the PCR plate, to define PCR conditions, to monitor

the amplification in real time, to view melting curves and to calculate DNA concentrations and other PCR parameters.

The cycling parameters of PCR were as follows: 95 °C for 2 min followed by 45 cycles of 95 °C for 10 sec, 60 °C for 30 sec, 72 °C for 30 sec, and then a hetero-duplex formation step including 95 °C for 10 sec and 60 °C for 1 min. After the PCR amplification steps, melt curves for the products were generated by heating in 0.2 °C increments at a rate of 10 sec/step for the temperature range 65-95 °C.

DNA quantitation of templates was based on a standard curve developed with the five NHDP63 standards, each tested in triplicate for each target. The results were presented as C(t) values and starting quantity (SQ).

Real-time PCR-HRM analysis for cluster detection:

Post-PCR HRM analyses of the melt curves were performed using Precision Melt Analysis ™ software (Bio-Rad) which analyzes the temperature and shapes of the melting curves. The software classifies the data into different clusters. Data that are similar to each other are 'clustered' by the software and assigned a cluster number. The melt curves corresponding to each cluster are color coded for easy visualization. For each of the three targets, in DRDR (*folp1*, *rpoB* and *gyrA*) and SNP typing (locus 1, 2 and 3) assays, NHDP63 DNA was used to select the reference cluster. Clustering results are influenced by the melt region selected and cluster detection settings. The melt region can be auto-detected or manually defined by selecting the pre- and post melt temperature ranges. The cluster detection settings include melt curve shape sensitivity (default value of 50% clustering) and Tm difference threshold (default of 0.15

177 degrees). These settings can be adjusted to determine the stringency of the clusters. The
178 instrument and software manual indicates that for most applications, default settings produce
179 acceptable results; these were used as the starting points for analyzing melt curves obtained for
180 each of the six amplicons and mutation types within the reference and clinical samples.

181

182 **RESULTS**

183 **Development of DRDR assays based on real-time PCR-HRM analysis and assay validation**
184 **with reference specimens**

185 First, the performance characteristics of the real-time PCR assays using the primers, reagents and
186 reaction conditions were investigated using NHDP63 DNA. As shown in **Table 2**, it can be seen
187 that all three DRDRs were reliably amplified using the primer sequences shown in **Figure 1,**
188 with high quality performance values for PCR efficiency and correlation of determination (see
189 foot note, **Table 2**). A standard DNA curve (0.1 to1000 pg) using NHDP63 DNA was
190 established to enable the estimation of the *M. leprae* DNA in test samples and also used for assay
191 evaluation. The corresponding C(t) values ranged from 39 to 22. The cycle quantification C(t)
192 values obtained for the three DRDRs are comparable.

193

194 Next, the melt curves were analyzed by HRM. For the five concentrations of NHDP63 tested in
195 triplicate, the melt curves clustered together as a tight group; occasionally the standard with the
196 least input DNA (0.1 pg) separated into a different cluster. The normalized relative fluorescence
197 units (RFU) and differential RFU curves produced by the HRM Precision Melt Analysis software
198 in the melt region are shown (**Figure 2**). These results indicated that the PCR assay conditions

199 are suitable for testing other DNA samples, and that cluster classification for amplicons produced
200 at C(t) values of >35 may not be reliable and should be individually analyzed, repeated and
201 assessed by other tests.

202

203 The objective of the real-time PCR HRM assays was to determine if the samples that have
204 DRDR mutations of interest could be identified. By using NHDP63 DNA, which is of 'wild
205 type' sequence for the DRDRs in *folP1*, *rpoB and gyrA*, melt curves from the test samples that
206 do not 'cluster' with NHDP63 have putative sequence variations occurring anywhere in the
207 amplicon (hence designated as Cluster V), while those that cluster are of wild type sequence
208 (hence designated as Cluster WT). Prior to testing HRM assays on samples derived from leprosy
209 patients, a panel of 18 mouse foot pad (MFP) and one or more armadillo derived *M. leprae*
210 (ADML) of known DRDR mutations were utilized for validating the assays (**Table 3).**

211

212 The reference MFP-LRC strains that were available for this study represented several wild type
213 and two, three and one unique sequence variant(s) for the *folP1*, *rpoB and gyrA* amplicons
214 respectively (**Table 3)**. Furthermore, in this panel, one or multiple isolates represented each of
215 these genotypes. The DNA quantitation and HRM cluster classification (wild type, WT or
216 variant, V) are shown in **Tables 2 and 3**. The HRM cluster classifications were concordant in 8
217 WT and 10 mutant *folp1*; 12 WT and 6 mutant *rpoB;* and 14WT and 4 mutant *gyrA* DRDRs
218 (**Table 3**). Four situations are of note: 1) the strain Airaku-2 was expected to carry mutations in
219 both *rpoB* and *folP1*. Repeated HRM assays did not indicate these mutations, as was confirmed
220 from the amplicon DNA sequences. Therefore, VNTR strain typing was performed for all MFP-
221 LRC strains to verify strain identity (14, 44). The VNTR strain type of the sample received as

Airaku-2 was indeed not consistent with the expected VNTR (44) (data not shown). 2) For strain Zensho-4, the *gyrA* amplicon was separated into a different cluster (blue curve in **Figure 2**, *gyrA* panel) when compared to other strains that carry the expected mutation type. To verify if this was due to a sample quality or quantity artifact, the PCR products was sequenced, which confirmed presence of the mixed alleles at codon 91 (see **Figure 3)**. 3) For strain Zensho-5, the HRM clustered the *folP1* and *gyrA* amplicons , as being different from NHDP63 and another strain (Kusatsu-6) with the expected mutation type (blue and orange curves in **Figure 2,** *folP1* and *gyr*A panels). Closer inspection of the sequence detected mixed alleles (in 53 and 55 codons in *folP1* and codon 91 in *gyrA*) as shown in **Figure 3.** 4) For Zensho-9, HRM detected a variant for *folP1*, which when sequenced verified the presence of a *folP1* DRDR mutation.

Altogether, these results validate that real-time PCR-HRM is suitable for distinguishing wild type strains from those that carry some of the known mutations in the DRDRs of *folP1*, *rpoB* and *gyrA*. Furthermore, HRM clustering can be sensitive to the presence of multiple alleles.

Performance of the real-time PCR-HRM DRDR assays on clinical samples:

Having validated that wild type and several mutation types in the DRDRs can be identified in reference strains, the real-time HRM-PCR assays were tested on clinical samples. For this, 121 DNA samples extracted from clinical biopsies from Philippine patients that were analyzed previously by conventional PCR-DNA sequence based mutation surveillance of *folP1* and *rpoB* DRDRs were selected (18). The *gyrA* DRDR had not been sequenced. This sequence based survey detected mutations in *folP1,* but not in *rpoB* DRDRs (18). The *folP1* mutation types

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

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

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

Real time PCR-HRM analysis of various DRDR targets aided in the detection of discrepancies between expected and/or reported DRDR genotypes for strain Airaku-2. Secondary genotyping of VNTR loci clarified that the strain received for HRM testing was indeed not Airaku-2 as reported, but could be Gushiken (44). The HRM analysis was also sensitive in detecting the 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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