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LESIONS OF LEPROSY PATIENT USING REAL TIME QPCR**



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## DETECTION AND QUANTITATION OF MYCOBACTERIUM LEPRAE DNA IN BIOPSY LESIONS OF LEPROSY PATIENT USING REAL TIME QPCR

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### Objectives:

- 1 To Quantitate *Mycobacterium leprae* bacilli in Biopsy samples using Real-Time QPCR
- 2 To assess the efficacy of QPCR results as compared to those obtained using regular PCR and Microscopy
- 3 To assess the optimal activity of QPCR Kits under diagnostic conditions
- 4 To assess the medical status of follow-up patients suffering from *Mycobacterium leprae* infection
- 5 To differentiate between actual positives and suspected positives

### Abstract:

Quantitative Real time PCR of the *Mycobacterium leprae* in biopsy sample was carried out in order to evaluate its use in the detection and quantitation of *Mycobacterium leprae* in patients suffering from Leprosy at Anandaban Hospital. In experiment carried out in this laboratory, PCR results from biopsy samples were compared with a set of known standards. Actual number of gene copies was obtained by plotting samples of unknown concentration to a standard curve and was calculated to be 7.0640E+4copies/ml. The result of this study appears to demonstrate higher specificity and sensitivity of the QPCR results over both microscopic examination and in vivo detection (foot pad inoculation) of the organisms.

## Introduction:

Leprosy, caused by *Mycobacterium leprae*, is still considered a major health problem in many developing countries, including Nepal. It is a chronic infectious disease of skin, nasal cavity, and peripheral nerves which eventually leads to disability, disfiguration, and socioeconomic problems. There is no efficient serologic test for the diagnosis of leprosy. Early detection of the causative microorganisms is, therefore, the key element to early identification and treatment of patients and subclinical cases before the disease progresses and neural damage occurs. These organisms can not be cultivated on artificial media, and attempts to identify them by inoculating a susceptible animal such as the armadillo and mouse footpads have proved cumbersome and time-consuming. The routine bacteriological diagnostic test as well as the demonstration of acid-fast bacilli in skin smears, is not sufficiently sensitive or specific.

Molecular techniques for viability estimation of *M. leprae* have been recently developed that are based on a quantitative estimation of target gene levels by direct hybridization or hydrolysis of specific probes, or by amplification of the target gene by PCR. Nucleic acid sequence-based amplification (NASBA) targeting genes of particular interests have been reported to be useful for the determination of viability of *M. leprae*. *M. leprae* genes (Templates) used in the development of PCR assays have been identified as

- Gene: *hsp18* , *ag36*, *groEL1* , 16S rRNA
- Noncoding sequences: RLEP
- rRNA -16S rRNA

## Principle of Real-Time PCR:

The robust assay exploits the so-called Taqman principle. During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A Taq polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

Absolute quantification is performed by plotting samples of unknown concentration to a standard curve generated from a dilution series of template DNA of known concentration. Typically the standard curve is a plot of the threshold cycle, C(t) against the logarithm of the amount of DNA. A linear regression analysis of the standard plot is used to calculate the amount of DNA in unknown samples. The following formula is applied to convert the values determined using the standard curve to the copies/ml of the sample material or else direct value can be generated from the operating software which is again based on the formula below.

$$\text{Result (Copies/ml)} = \frac{\text{Result (Copies/}\mu\text{l)} \times \text{Elution Volume (}\mu\text{l)}}{\text{Sample Volume (ml)}}$$

In this study the direct detection of PCR product during the exponential phase of the reaction was carried out. This technique, due to its specificity and sensitivity, is ideal for detection of *Mycobacteria* spp., and in the differentiation between different *Mycobacterial* spp or drug resistance in *Mycobacterial* spp. infection as well as in the quantification of *Mycobacterial* spp. This study was mainly undertaken to evaluate the use of QPCR as a means of diagnosis of leprosy and its potential to help assess the bacterial load in patients during the course of treatment.

## Materials and methods

QPCR assay involved the following steps as per protocol outlined by Manufacturer Kits;

- 1 DNA extraction (always use an extraction method with a higher DNA yield)
- 2 Quantitation by Spectrophotometry
- 3 Preparation of PCR Mastermix
- 4 Preparation of PCR amplification
- 5 Programming of the software
- 6 Running QPCR Programme
- 7 Data Interpretation and Analysis

### Specimen collection:

The Biopsy specimen was collected from the leprosy patient attending Anandaban Hospital. The sample was ground and dissolved in 0.1% BSA and then immediately stored at -20°C. The bacterial index (BI) of this patient was recorded at the time of the sample collection by AFB Staining.

### History and clinical background of the patient (is this the only patient ? need to be clear here)

Gender	Male	First Diagnosis - 03 July 2002  Clinical Classification(( Redly and Jopling classification) -Lepromatous Leprosy (LL)  WHO classification - Multibacillary(MB)
Age	29	
Initial mean BI value in Slit Skin Smear (MB)	3.75+	
Follow ups	2.75+	
25-12-03:	2.75+	
26-11-03:	2.75+	
2-06-04:	1.25+	
9-05-05:	neg	
22-02-06:		

### Template preparation and quality;

The *M. leprae* DNA was isolated and extracted from the specimens using Freeze and Boil method. 100µl of specimen was first aliquoted in a sterile Eppendorf tube. It was placed in a heat block at 100°C for 1 min. and transferred to the liquid Nitrogen (maintained at -196°C) for 1 min. The above process was repeated 4 times i.e. the total 5 cycles of repetitive heat and cold process was applied. The DNA was quantified and suspended in Tris EDTA buffer (10Mm tris; 1Mm EDTA P<sup>H</sup> 8.0)

The sample was transported from Hospital in ICE PACKED container. Upon arrival of the sample it was immediately frozen at -20°C. The sample was stored until it was further processed.

### Analysis of total DNA

The total DNA and its purity were quantified by Nano-drop Spectrophotometry. Concentration of the DNA was found to be 27ng/µl. The 260/280 ration was 1.66 and the total volume of the sample at the time of receipt was around 100 µl.

## Preparation of PCR Mastermix: and Sample loading

The working Mastermix was prepared by mixing Leprosy super mix 24 µl, Mg. Sol Leprosy 5µl and IC 1µl. Primers and probe(s) for qPCR step and Reference genes were already incorporated in the mix. The sample DNA was then added to the mix at a volume of 20µl, positive controls (LEPROSY S1-5), or negative controls (Water, PCR grade) in separate 200µl PCR tubes. The total of 5 reactions were carried out, calculation for whole sets of reaction is listed below. Negative control was included in all experimental protocols. GLP was strictly maintained throughout this and other procedures.

## Reaction set up for PCR amplification/Fluorescence detection

Leprosy Master mix	1 rxn	6 rxn	Comments
LEPROSY Super Mix (R1)	24 µL	144 µL	Mixed thoroughly and dispensed appropriate volume (30µL) of the master mix in each test reaction followed by the addition of the negative, samples and standards
LEPROSY Mg Sol. (R2)	5 µL	30 µL	
IC-1 (R3) RG	1 µL	6 µL	
Total	30µL	180µL	

## PCR amplification:

The amplifications were carried out in a programmable temperature control system FTC-2000 (Funglyn Biotech) as follows: an initial activation of the Hot start Taq polymerases Enzyme step at 95°C for 10 min, followed by Setting up cycling Profile of 45 cycles of 15 secs denaturation set at 95°C, 20secs primer annealing at 60C, and 15 secs extension at 72°C. The gain value for appropriate background for the fluorescence was adjusted before the running the thermal cycle. The Plate set up was also set up and wells were defined for the given set of the reactions. The Quality control of the whole process including PCR mix and the performance of the amplification was maintained excellent.

## Cycling Protocol for Hydrolysis Probes (TaqMan)

Step	Purpose	Temp (°C)	Time (secs)	Number of Cycles
1	Hot Start	95	600	1
2	Denaturation	95	15	45
3	Annealing (Data acquiring Step for FAM and JOE)	60	20	45
4	Extension	72	15	45

## Results

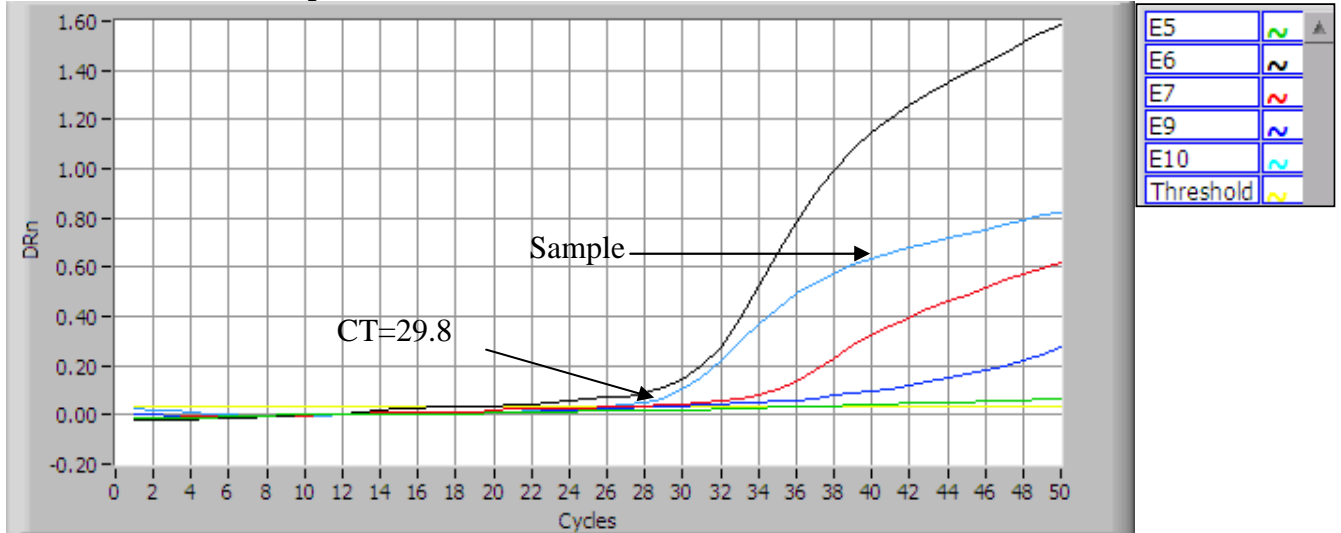
We analysed only one sample from Anandaban leprosy patient for QPCR diagnosis and quantitation. The analysis was partly Qualitative using FAM as a reporter dye which signifies that the particular pathogen is present. The absolute quantitation for the exact copy number of leprae bacilli was done with reference to the standard Curve (shown below). The standard curve was generated based on the Copy number of each standard plotted against the Threshold Cycle. The first significant increase in the amount of PCR product (CT - threshold cycle) correlates to the initial amount of target template. The software examines and then calculates an unknown sample, the quantities of which are determined automatically and displayed in the experimental report. The optimal threshold was selected such that the noise was filtered from the raw fluorescence readings by smoothing, baseline

subtraction and amplitude normalization. The unknown sample after normalization process was calculated and thus found  $7.0640E+4$  copies/ml. PCR inhibition was not observed as was evident by the fluorescence detection in the second channel. The reporter dye for the PCR inhibition was used as JOE.

**Table for the QPCR assay**

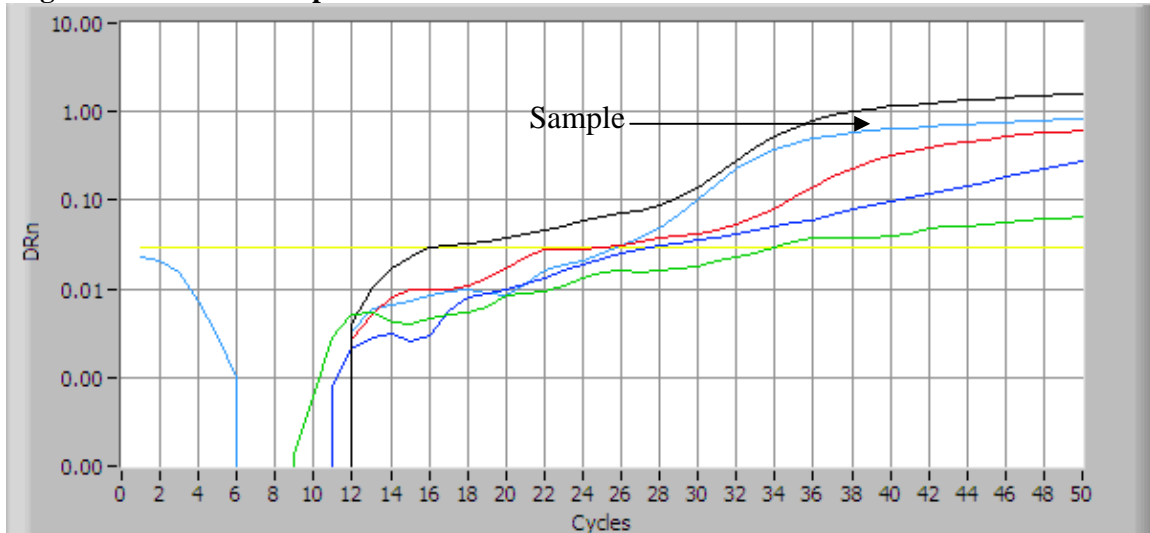
<b>Sample ID</b>	<b>Sample Type</b>	<b>Primer</b>	<b>Report</b>	<b>Ct</b>	<b>Copies</b>	<b>PCR Efficiency</b>
neg	S I /Negative Control		FAM	NTC	-	0.00
s1	S I x10 <sup>5</sup>		FAM	28.9058	1.0000E+5	1.4820
s2	S I x10 <sup>4</sup>		FAM	34.8277	1.0000E+4	1.3576
s3	S I x10 <sup>3</sup>		FAM	40.7385	1.0000E+3	1.0895
unk	S I /Unknown Sample		FAM	29.8007	7.0640E+4	1.4670

### Linear View of PCR amplification

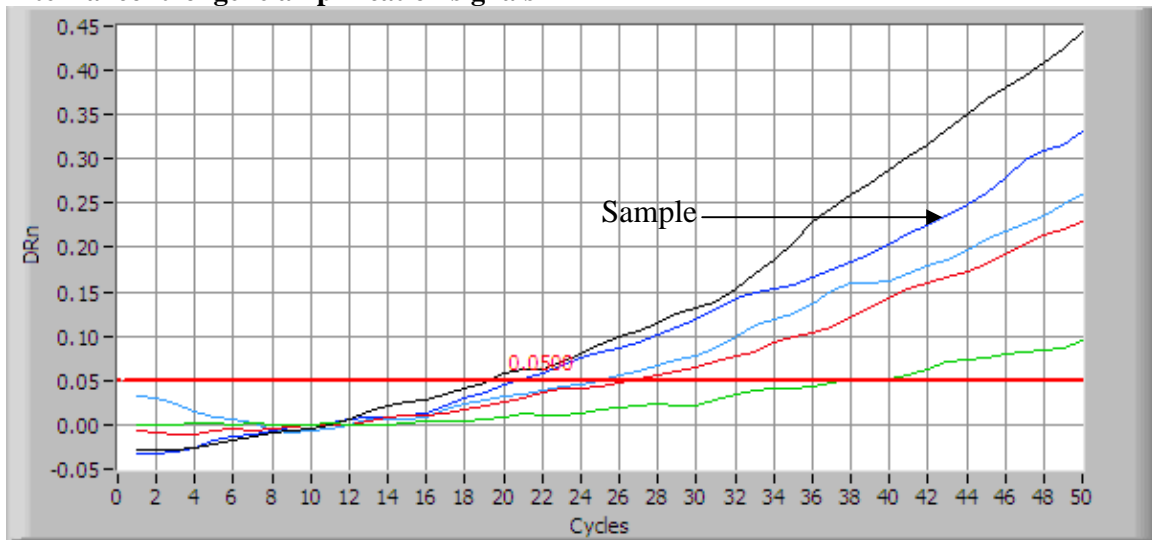


This is linear view of PCR amplification. The exponential amplification of three standards have been shown in the graph, whereas an unknown has its fluorescence curve detected in between the standards, indicative of its quantity in between the range of the standards. The  $C_t$  value 29.8 assigned to a particular well for the sample reflects the point during the reaction at which a sufficient number of amplicons have accumulated.

### Log view of the PCR amplification

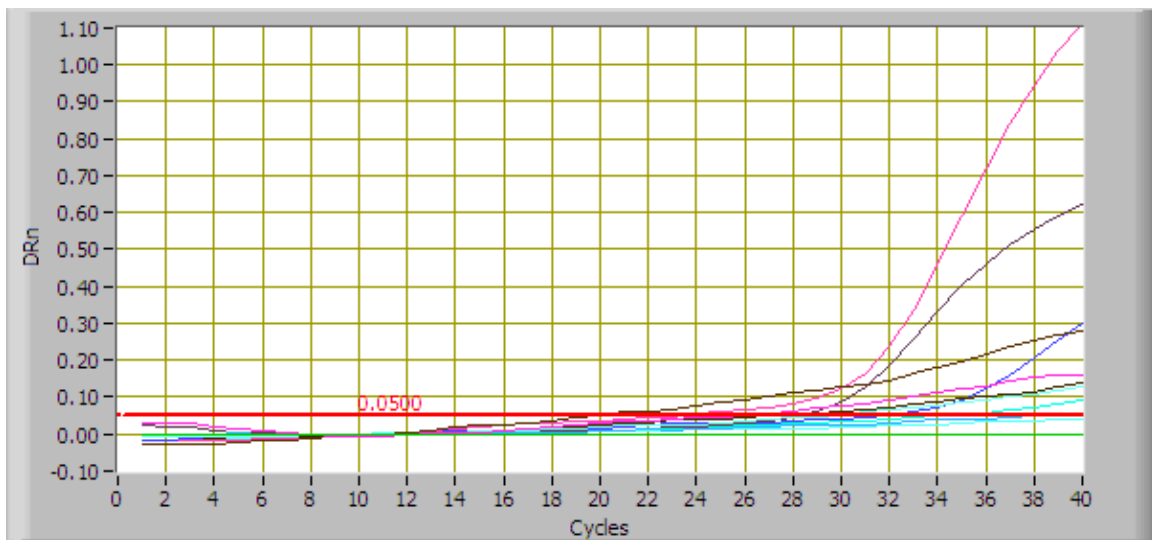


### Internal control gene amplification signals

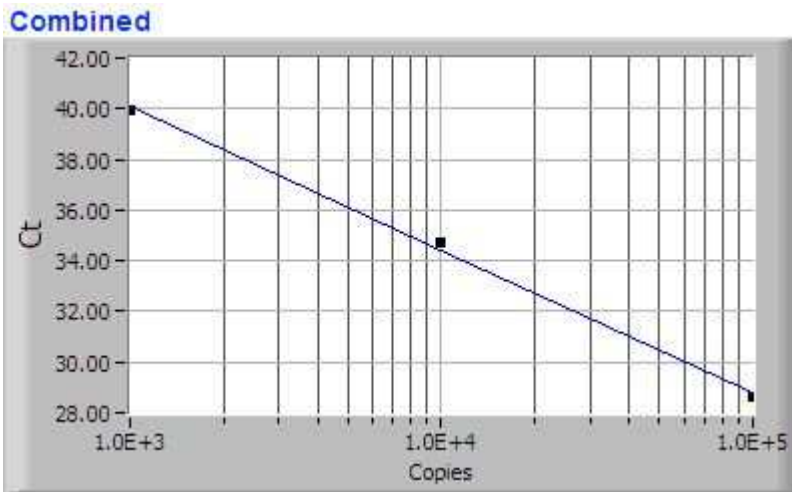


All Standards, negatives and samples showing the fluorescence detection curves in channel 2; indication for no PCR inhibition or no false results

### Fluorescence detection DRn in multichannel (using both channels)



## Standard Curve for Absolute Quantitation



This is the standard curve generated based on the QPCR data and experimental design. The standard curve is used in a reaction to determine the unknown quantities. The software displays the standard curves as well as the unknowns in the standard curve view (unknown has not been shown in the curve above). The slope and mse meet standard values; therefore the copy number found for the given sample has provided concurrent and valid results

## Discussion

The definitive and rapid diagnosis of leprosy in routine clinical work is difficult since culturing is not available and lack of standard histological and immunological testing to provide reliable and reproducible results. Immunological techniques for eliciting Delayed Type Hypersensitivity reactions, as well as serological responses in case of leprosy, although present, only suggest exposure to the infecting organism, as the antigens persist for a long time after subsidence of clinical or subclinical disease. Demonstration of acid-fast bacteria in skin smears is also often not sufficiently sensitive, and in histology assessments, some granuloma characteristics can suggest nonspecific dermatitis. Extensive information about the molecular structure and function of *M. leprae* is now available, and this has helped in developing molecular techniques for early diagnosis, monitoring of treatment and detection of drug resistance. Rapid and confirmatory results are mandatory for early detection, and prompt therapeutic prescription of medication against infecting pathogens. This holds very true in the case of *M. leprae*.

In the present study, a patient who was diagnosed and classified as Multibacillary Leprosy long time back in 2003, was used as a subject in this experiment for QPCR assessment. The results demonstrated that the patient was still harboring huge number of the bacilli and was estimated to be  $7.0640E+4$  copies/ml. He was although confirmed microscopy negative in 2006, his biopsy sample was found to be highly positive for leprae bacilli by Real time PCR. Positive results in such case indicate the presence of viable organisms, and therefore, may be considered for further anti-leprosy treatment. This type of diagnosis may be helpful in cases of late reactions and relapses. In his previous tests, his sample also showed a strong band in a typing for 12 loci tested for strain identification.

This experiment successfully determined the number of bacilli in the patient. The kits that were utilized in this experiment appear to have worked well under laboratory conditions; there was no PCR inhibition as an exogenous IC was added to the PCR master mix to determine whether inhibitory substances are present in the mix. The reactions showed no PCR inhibition. However, Owing to the relatively low efficiency of the PCR reactions, mainly because of improper transportation and storage conditions, all five standards could not be included in this study as lower standards did not show appreciable amplification curves. The efficiency of the reaction can be calculated by the following equation:  $E = 10^{(-1/\text{slope})} - 1$ . The efficiency of the PCR should be 90-100% meaning doubling of the amplicon at each cycle. This corresponds to a slope of  $-3.1$  to  $-3.6$  in the  $C_t$  vs log-template amount standard curve. In order to obtain accurate and reproducible results, reactions should

have efficiency as close to 100% as possible. Our PCR efficiency was quite low below 1.5. A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, presence of inhibitors, secondary structure and primer design.

Although valid data can be obtained from our experiment, the quantitative real-time PCR should be further optimized using new sets of kits as well as a large pool of samples are required to confirm its sensitivity and specificity. For diagnostic purposes, at least replicates of the same samples have to be analysed for more accurate and consistent results.

Real time QPCR has greater advantages over other techniques in various ways. It has enhanced sensitivity of a large dynamic range and accuracy with elimination of post reaction analyses and faster and more reliable amplification. With the use of new techniques for the detection of mutations directly from clinical specimens, surveillance programmes to determine the exact magnitude of drug-resistant mutants to rifampicin, and possibly other drugs, can be undertaken from the biopsies. Target genes for *M. leprae* drug resistance PCR-based assays include *gyrA folP rpoB gyrA rpoB*

## **Conclusion**

Standard immunological and histological approaches for assessing leprosy in suspect cases have limited value for diagnosing new cases at the 'suspicious' and early I stages and for monitoring treatment. Real-time PCR has demonstrated the advantage of accurate measurement of mycobacterial load measurement by template quantification in the clinical sample leading to precise diagnosis and also estimation of load of infection. It is a fully controllable, fast, high throughput diagnostic tool for the rapid identification of mycobacterial infection.

The sample tested yielded significant number of leprosy bacilli (7.0640E+4 copies/ml), which provides a clear picture on the status of the disease and continuation of the antimicrobial therapy. This can be valuable to physicians since QPCR is known to detect potentially viable microorganisms. It can also give an indication of patient compliance, of the presence of drug-resistant organisms or persists, or of relapsed cases. Based on its accuracy, this technique can be established as a diagnostic procedure for leprosy patients and sub clinical cases or as a tool for drug assessment. However, investigation of a larger number of clinical specimens needs to be carried out for comparative evaluation with other diagnostic techniques.

## **Acknowledgement:**

SPECIAL THANKS TO Anandban Leprosy Mission Hospital for their active participation in our study and a part of their help in sample processing and providing patient information.

# References

## Description of the Product

- PCR reagents
  - Leprosy Super mix
  - Mg Sol
  - IC-1
  
- Leprosy Standards
  - S1 (1\* 10<sup>5</sup>Copies/μl)
  - S2 (1\* 10<sup>4</sup>Copies/μl)
  - S3 (1\* 10<sup>3</sup>Copies/μl)
  - S4 (1\* 10<sup>2</sup>Copies/μl)
  - S5 (1\* 10<sup>1</sup>Copies/μl)
  
- Molecular Grade H<sub>2</sub>O

## Importance of controls

- 1 negative control (no DNA)
  - checks reagents for contamination
  
- 2 Internal Control
  - detects if there is any PCR inhibition
  - If inhibitors are present – no amplification occurs or efficiency is usually altered
  - Thus low level ambient DNA serves as an internal control for inhibitors
  
- 3 positive control
  - Generate Standard Curve
  - Absolute Quantification
  - PCR efficiency

## Data Interpretation and Analysis

- 1 Qualitative analysis: The detection of the pathogen is recorded based on the fluorescence detection. Following conditions were taken into consideration for qualitative analysis
  - Signal is detected in fluorescence Cycling FAM- Positive
  - Signal is not detected in fluorescence Cycling FAM- Negative i.e No Leprosy DNA detectable.
  
- 2 Quantitative analysis
  - Quantitation using Ct Value
  - The log of DNA template concentration vs Ct Plotted using a series of stds yielding a calibration
  - The unknown is then run and the number of cycles required to reach threshold, Ct is compared to the calibration curve.
  
3. Inhibition Control Gene amplification
  - In fluorescence channel Cycling A. joe no signal is detected
  - But signal detected in FAM: The sample is positive for Leprosy DNA
  
  - In fluorescence channel Cycling A. Joe no signal is detected
  - No signal detected in FAM as well: A possible PCR inhibition has occurred.

### Indications and suitable specimens for *M. leprae* PCR

QPCR has applications for the detection of *M. leprae* in following human specimens

- 1 Tissue Sample
- 2 Ear Dropping
- 3 Lesion Curatings
- 4 Biopsy of Patched, Scarpe from Nasal Mucosa

Category	Description
PCR indicated	Identification of acid-fast organisms when bacilli are numerous but tissue site, clinical history, or other circumstances are questionable; bacilli are sparse and tissue site, clinical history, or other circumstances are questionable
QPCR indicated	For diagnosis and for assessment of viable load of <i>M. leprae</i> , for monitoring treatment, drug resistance, in early and suspected cases, and when the diagnosis may be doubtful by using traditional methods