REAL TIME PCR;
APPLICATIONS IN DIAGNOSTICS AND RESEARCH

REVIEW
PROF-1947

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ABSTRACT… In recent years, real-time PCR has come forward as a robust and widely used molecular technique in clinical and biological settings. Although it can detect very minute quantities of target nucleic acid, but quantification of specific nucleic acids is not an easy task. Accurate and precise quantification is hampered by a number of factors that may include assay development and validation, fluorophores selection, handling during sample preparation, storage, reaction procedures, and batch analysis conditions. Even minor variations are significantly magnified by the exponential nature of this technique. Current review gives an insight of the advantages, limitations, assay chemistries, quantitation parameters, and quality control issues related to this technology. Moreover it will also highlight the utilization of Real time PCR in clinical oncology, virology, microbiology, and gene expression studies.

Key words: Real time PCR, molecular diagnostics, quality control issues, Real time chemistries

INTRODUCTION

In 1983, the most significant molecular biology technique, polymerase chain reaction (PCR) was first conceptualized by Dr. Kary Banks Mullis at Cetus[1], who was awarded Nobel Prize in chemistry in 1993 for his invention. Later on, the technique became the most popular and extensively used techniques in biological research. Now it plays a vital role in the identification of inherited disorders, molecular diagnostics of cancer and infectious diseases, forensic laboratory, poultry for investigation purpose.

After the historical development of polymerase chain reaction technique, a breakthrough came when Bob Griffith and Russell Higuchi gave the concept of amplification in real time through fluorescence dye. This was an accidental discovery as PCR ran with intercalating dye ethidium bromide and post-PCR processing was done[2]. PCR turned into quantitative category, when fluorescence signal cross threshold value by observing amplification cycles and amount of end-product generated during a reaction. Practically, the name real time PCR is based on the fact of measuring the fluorescence of dye during a reaction as observed in real time environment. This new emerging technique, Real-Time PCR, is an advanced form, modification to conventional or traditional PCR technique which has enormous applications in biomedical, agriculture and veterinary. In this technique amplification and quantification of targeted DNA molecule is done simultaneously as reaction progress in Real time.

Both specific and non specific dyes can be used as a detection fluorophore. Non-specific fluorescent dyes based detection follow the phenomenon of intercalation where any double-stranded DNA is available (whether it is amplified gene of interest product or any primer dimer), whereas sequence based DNA probes allows the detection of only complementary DNA target. Real-Time PCR application includes quantification of specific sequences in complex mixtures, for example viral or bacterial genotyping[3-5], quantifying viral nucleic acid and screening of infectious diseases in patients[4,6] pathogen detection, gene expression profiling by coupling it with reverse transcription, DNA damage measurement, drug therapy rationale and for RNA interference measurement[7-8] technique. In short real-time PCR is an advance, sophisticated, accurate, precise, rapid, sensitive and less time consuming technique.

Conventional versus Real-Time PCR

Real-Time methodology detects amplification of PCR products as they accumulate in early phase and statistically significant fluorescence is captured by the instrument. Threshold cycles are calculated on the basis of exponential phase whereas in traditional PCR

amplification is measured at the completion of the reaction (also called end point phase) followed by agarose gel electrophoresis. The principal disadvantages of end point technique, which make conventional PCR less popular are, low accuracy and sensitivity, poor resolution, short or limited dynamic range, and requirement of post PCR processing. Conventional PCR offers size based discrimination, not very quantitative and very time consuming. Agarose has poor resolution while Real time PCR has high detection capability even two-fold change.

The end point approach vary for each sample and amplification results on gels are unable to determine these variabilities, but real-time PCR is giving a powerful insight to detect these sensitive changes[9-10]. Real time PCR deals in the template with which you have started. Major difference between the two technologies is that endpoint assays bisects the assay perpendicular to x-axis (no. of cycles), while real time assays bisects assay horizontal to x-axis. For qualitative results classic PCR is quite good for diagnostics and different gene assays for medical, poultry, plant assays[11-16] while real time PCR can give quantitative results that can be used in checking treatment response and quantification of different genes[17-19].

ADVANTAGES OF REAL-TIME PCR

A PCR amplification reaction usually has three phase, exponential, linear, and plateau.

In **exponential phase** amplification of PCR product is analyzed in terms of doubling of the product which is accumulating in each cycle. It is possible to assume 100% reaction efficiency during this phase if factors affecting the reactions are fully optimized. This includes run profile, nucleic acid purity, length of the target region, and primer characteristics. **Linear phase** is highly variable in which most of the reaction reagents are consumed, and the products starts to degrade, so the reaction is becomes slow. **Plateau** is the final phase of the end-point detection methods for ethidium bromide gel based detection assays.

Now there is no more reaction process, so no further products would be made and if the cycling profile is quite long, the amplified products may start to degrade. Area of detection for Real time PCR is exponential phase that is why it is more accurate and precise[8,10]. It has nothing to do with plateau or end of the reaction in contrast to conventional techniques. Many nucleic acid quantification techniques that require post PCR processing are now replaced by real time PCR e.g. different hybridization assays like Northern, Southern and in situ hybridizations are thought as obsolete techniques because of very laborious, time consuming and complicated ones. Real time PCR has an edge of quantification of nucleic acids over extensive range, high detection sensitivity of a target sequence, quick with high-throughput automation, less chance of carry over and cross contamination as reaction is performed in a close vial[20].

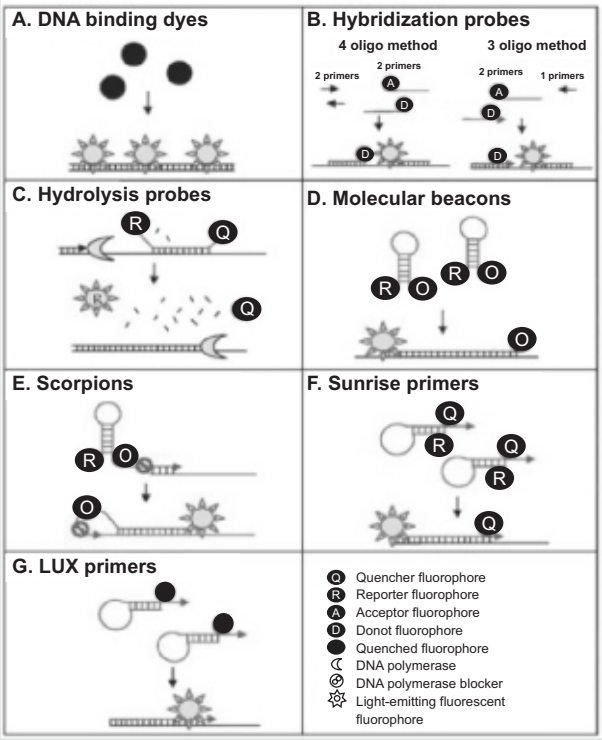
LIMITATION OF REAL TIME PCR

The major disadvantage of real time PCR is its failure to examine amplicon size. Most of the time real time PCR systems are not compatible with each fluorogenic chemistry. Multiplex capabilities are also less than conventional thermal cyclers. The cost for the establishment of real time PCR setup also offers hurdles. Limitations in system hardware and selection of fluorophores are probably most promising problems to overcome. Figure V gives details of the instruments being in use all over the world. Moreover sample preparation is also very tricky. Amplification can be inhibited if nucleic acid is not extracted properly. Biological samples like body fluids may contain inhibitors, food samples for microbe detection are not so easy because of the presence of so many things in the sample like phenolic and organic inhibitors, forensic samples are also very difficult to perform[9]. Gene expression assays are many times halted because of the wrong selection of specimen, selection of inappropriate housekeeping genes, reaction normalization processes and many others[21-22].

REAL TIME PCR CHEMISTRIES

Real time PCR chemistries generally divided into three main categories: a. Hydrolysis probes; b. Hybridization probes and c. DNA-binding agents although it also

Fig-I. Real time PCR chemistries



includes use of molecular beacons, scorpions, Sun rise and Lux primers (Figure. I).

Hydrolysis probes

The chemistry behind hydrolysis probe is based on quencher and reporter fluorochrome conjugated with probe. As long as quencher dye is intact with probe, it absorbs the fluorescence of reporter fluorochrome. When target sequences are amplified, Taq polymerase uses its 5' to 3' exonuclease activity and reporter dye is detached from quencher fluorochrome making the fluorescence of the reporter fluorochrome detectable. During exponential phase of successive PCR, progressive accumulation of free reporter fluorochrome will increase.

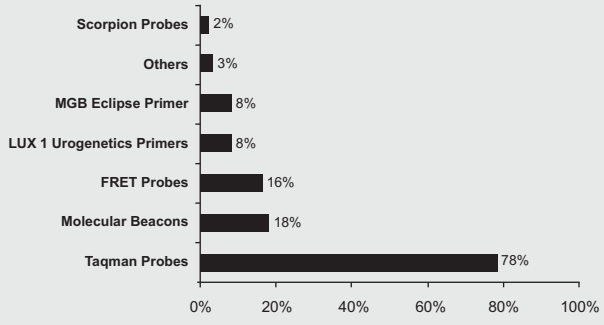
Hybridisation probes

This method relies on labelling of donor fluorochrome at the 3' end of one probe and acceptor fluorochrome at second adjacent probe. Donor fluorochrome emitted light when acceptor fluorochrome comes in close vicinity (1 to 5 nucleotides apart), which excite the acceptor fluorochrome resulting in the emission of fluorescence. This is called fluorescent resonance energy transfer or FRET analysis to differentiate among various products. That can be observed during annealing and first part of the extension phase. During each consecutive cycle annealing of hybridization probes will increase, resulting in higher fluorescence signals.

DNA binding dyes

SYBR Green dye is the first choice when someone want to develop an assay because of its low cost. This dye is mostly used in melt curve analysis assays to detect mutations or for typing. This dye gives fluorescence when it get bound with double strand DNA. As the reaction progresses, more dye will bind, resulting in increased fluorescence. More fluorescence signal will detect when more SYBER Green I will bind to PCR product at extension phase[8]. But this dye is rather nonspecific. In every cycle; syber green frequently used DNA binding dye and is the first choice when you are going to develop the assay. SYBR Green I gives when bind to double-stranded DNA. A detailed use of probes all over the world is stated in Figure II.

Fig-II. Real time PCR probes used all over the world



Assay Development

This includes; a. Sequence selection: For disease of interest, first you have to select sequences that are specific in nature to disease phenotype;

b. Primer & probe selection: For gene expression quant use intron-spanning primers to avoid genomic contamination or use DNase treatment after RNA purification. All primers must be optimized under required

temperature. Efficient primers can be designed by using following instruction as mentioned in table and avoiding the following flaws: primer-dimer formation, self-complementarity, too low Tm of the primers, and/or their incorrect internal stability profile (Table I);

c. Reporter and Quencher fluorophores and use of internal reference: Fluorescein (6-FAM) is a classical reporter dye, however other reporters like Joe and Vic are also used for multiplexing. Stratagene Mx4000 Real time PCR system can use red dyes as reporters. TAMRA (Rhodamine) has been a classic quencher dye many years but some newer quenchers DABYCL and the black hole quenchers are also available now but these are the dark dyes. There is added benefit of TAMRA-quenched probes that they do not require a reference dye because TAMRA can itself be used. But if you are using dark dyes, an additional dye as an internal reference dye is also needed, most likely ROX (dark red). In multiplex reactions mostly this combination is used. An internal reference choice is usually based on the selection of those genes that are most abundant and are constantly expressed. Moreover these should be reliable and offer a valid test for the selected endogenous control. Most importantly probes are selected depending upon their absorption and emission spectrum (Figure III); d. Assay validation: It is very much important to check the primers in different combination of probes for amplification of known templates. Standard assay conditions can be used initially for optimization. This includes 300-400 nM primer concentration, 100nM probe and 3 mM MgCl2.

Fig-III. Selection of quencher and reporter dyes for a real time PCR
(IDT integrated DNA technologies, www.idtdna.com)

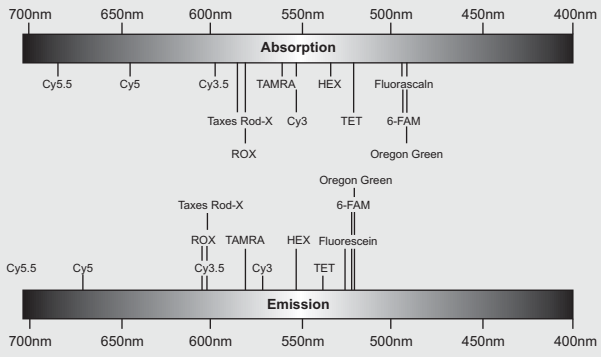
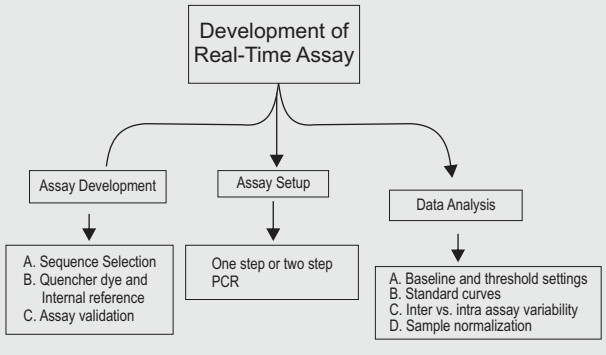


Table-I. Guidelines for designing primers and probes for real time PCR assay

Primer	Probe	Amplicons
Tm 58-60˚C	Tm 10˚C higher than Primer Tm (7˚C for Allelic Discrimination)	50-150 bp in length
20-80% GC	20-80% GC	As close to the proobe as possible without overlapping
Length 9-40	Length 9-40	----------
<2˚C difference in Tm between the two primers	No G on the 5' end	----------
Maximum of 2G or C at 3' end	<4 contiguous G's, must not have more G's than C's	----------

After running comparative reactions the primer pair giving highest Delta Rn with the lowest Ct is selected. Now a standard curve can be prepared by making dilutions of a template. Template can be a single strand RNA, single strand DNA or total RNA. Analysis parameters include the values of R, slope, and intercept. Slope of the standard curve can guide towards selection of $MgCl_2$ concentration. Ideal assay should have a slope value of -3.3, if it is around -3.5 then an increase in $MgCl_2$ to 5 mM can solve the issue while a value higher than -3.6 shows that primers are not specific.

Fig-IV. Flowchart guidance for the developing a real time PCR Assay



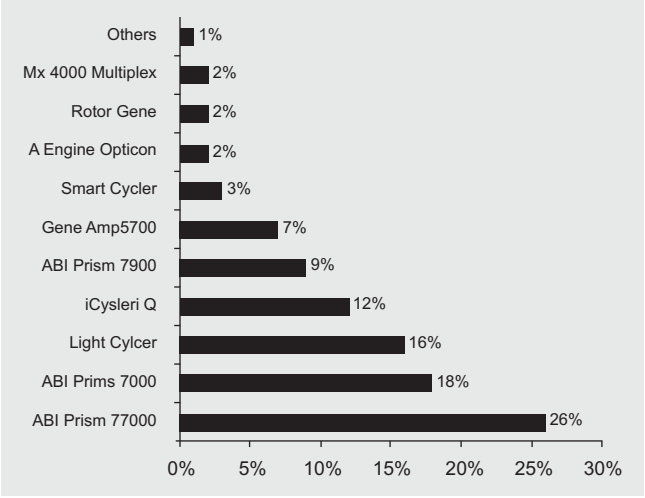
Assay Setup

Real time PCR assay can be one step or two step (Figure IV). In one step assay, reverse transcription is followed by amplification and detection step in a single buffer system in one tube while in two-step assay both steps are performed separately in two different tubes, performing reverse transcription product as a template for amplification and detection. Thermo cycler settings includes usual denaturing, annealing, and amplification steps but an extra step is the acquiring of the fluorescence of dyes at specific amplification steps depending upon the use of selected dye.

DATA ANALYSIS

Data analysis settings vary instrument to instrument. Most commonly used instruments detail is given in Figure V.

Fig-V. Commonly used real time PCR instruments all over the world



Parameters to be kept in mind during analysis are slope correction, dynamic tube settings, intra standard %age variance calculated automatically within run (accuracy), intra run %age variance (precision) calculated manually, threshold settings (calculated manually keeping in view the actual well fitted data), threshold cycles (statistically significant fluorescence as compared to back ground), elimination of early cycles, difference of handling, R^2 value of standard curve (must be between 1 to 0.98), r value of the run (a measure of how well the actual data fit to the standard curve) that can be obtained by dividing the "explained variation" by "total variation", slope value of the run must be within a certain range/value (-3.76 to -3.04), efficiency of reaction can be calculated from slope value using the formulae [10(-1/slope)]–1. For an efficient and reliable measurement system, it must based on accuracy and precision to give authentic results, if any of these parameter is missing the results are not valid for further considerations

QUANTITATION OF RESULTS

Real time PCR results are obtained mainly by using two board strategies; the Absolute quantification and the Relative quantification which are mentioned below.

ABSOLUTE QUANTIFICATION

This method follows a reference standard (standard curve) which is used to quantify unknown concentration of target mRNA. As the name indicates the reference standard are build on known RNA concentrations and the comparison is done based on known RNA concentration with unknown target RNA concentration. However, the stability of RNA standards can cause variation in the final analysis. In contrast to RNA, other nucleic acid samples i.e. purified plasmid dsDNA, in vitro generated ssDNA or any cDNA sample may use as standard curve because they expressed target gene. Further evaluation of these DNAs, spectrophotometric measurements at 260nm can be used, which convert a copy number value based on molecular weight of sample used. Preferred standards are based on cDNA plasmids however, cDNA plasmids will not capable to manage variants in reverse transcription, so it only gives relative change in mRNA expression. To overcome these variations, house keeping genes can be use[9,21].

Relative quantification

Another mathematical derived quantification named as comparative Ct method used a control or calibrator (RNA sample form normal tissue, or non-treated sample) with Ct values of target sample. Endogenous housekeeping gene is used to standardize the Ct values of both calibrator and target sample. The comparative Ct method is also known as the $2^{-[\text{delta}]\,[\text{delta}]}$ Ct = method, where [delta] [delta] Ct = [delta] $C_{t,\text{sample}}$ - [delta] $C_{t,\text{reference}}$ Here, [delta] $C_{t,\text{sample}}$ is the Ct value for any sample normalized to the endogenous housekeeping gene and [delta]Ct,

reference is the Ct value for the calibrator also normalized to the endogenous housekeeping gene. For valid calculation of [delta][delta]C_t, target and endogenous reference amplification, must be equal such that [delta]C_t varies with template dilution. Target and the housekeeping genes show close similarity if the plot of cDNA dilution versus delta Ct is close to zero and if both show dissimilarity than standard curve method is preferred[24].

QUALITY CONTROL ISSUES

For diagnostics selection of instrument, methodology and kits is very much important. Four controls are used in each batch including known value standards, no template control, negative control, and positive controls that can tell you how precise and accurate you are in your handling, contamination free area, accuracy of your results, and validity of your results respectively. It includes following parameters

The optimization of new batches of primers

Different variation in primers and probes will affect assay sensitivity. For a new batch of reagents, a PCR run must be conducted based on new and old primers with similar positive quality control that runs parallel. Results of Ct and Rn values are examine if the results are comparable then the new reagents are used for routine test, but if the assay reduces its sensitivity then optimization of probes and primers should take palace.

Routine validation of each real time PCR runs

For validation of each real time PCR run Ct of the positive control must be documented with each run to recognize the user error or primers degradation. Moreover overall fluctuation in fluorescence must be observed because decrease fluorescence signal may be the indication of some problem which occurs during PRC reaction. Internal control should include for ideal results to make sure confidence in negative controls.

Quality control of quantitative assays

There must be standard quantitative controls to the assay by using uniform sample type to achieve significant results for research purpose but for molecular diagnostics specified positive and negative controls along with known standards are used.

Continual assessment of the sensitivity of real time PCR primer and probe

There must be a need to re-access the sensitivity of assays by using positive samples detected by an alternative test or by comparing primers and probe to new sequences stored in surveillance databases. Serial dilutions of reference are usually used to check the sensitivity limit of the assay.

Interpretation of results

Numbers of factors are responsible for signal drift. In case of true positive samples, signal drift can be generated due to sub optimal PCR conditions, inhibition and primer mismatches. Signal drift for negative sample is base on the fact that probe breakdown resulting in fluorescence increase 23. Interpretation of the captured fluorescence can be in international units (IU), or in copies per ml. For some kits there is no specific coefficient value to calculate the IU and copies inter conversion. Summary of quality control issues is stated in table II.

There are other parameters that can affect the real time PCR results. This includes the handling errors, sample processing techniques, time duration, storage conditions etc.[24]

Clinical Application of real time PCR technology

PCR are widely used technique in molecular diagnostics and essential tool in research laboratories to identify nucleic acids form biological samples. Real time PCR is beneficial in order to differentiate specific sequence form DNA mixture. Quantitative analysis of target genome or other signature sequences within a sample can be carried out.

At NORI we are offering services for the viral load quantification of HCV and HBV for antiviral therapy monitoring by detecting changes in viral load[17,25].

Antiviral therapies effectiveness and disease progress can also be calculated by real time PCR which depend on viral load and disease severity relation. Individual and epidemiological studies of viral co infections or quasi

Table II. Summary of quality control measures

Protocol	Measures
RT-PCR quality control	The experiment should include no template and no reverse transcriptase. Use RNAse-free tube that is appropriate for the thermal cycler. Barrier tips and gloves to avoid RNA contamination
Real time PCR quality control	Set the threshold to detect the PCR product manually according to standard set by the laboratory. If setting the threshold manually, set it as low as possible to ensure that one is detecting the product during exponential phase of amplification. The melting curve of all samples should be checked to ensure that they are producing one peak. The slope of sample when plotted in log view should be parallel. Samples that can be detected at 10 cycle or earlier should be diluted.
Standard curve quality control	The nucleic acid being used for standard curve should be carefully quantified and serially diluted by using calibrated micropipettes. Remove those dilutions whose fluorescence measurement cross the threshold line together the correlation coefficient of how well a series of diluted samples should fit a straight line. The log plot of the template should be equal to or higher than 0.99.
Reproducibility/Repetition/precision	Random use of negative and positive samples in random runs, intra run STD validation
Accuracy	No. of true positives + No. of true negatives/No. of true positive + false positive + true Negative Must be 100% for dual labeled probe
Sensitivity & specificity	Calculation is based on binary classification test
Sensitivity	No. of true positive / no. of true Positive + false Negative
Specificity	No. of true negative / no. of true negative + No. of False Positive

species can be carried out by mutation analyses using melt curve analysis[26]. The technology is in routine use for identification of bacteria[27], or to solve problematic bacterial diseases[11]. Fast and early diagnosis make possible for clinicians to recommend better antibiotic therapies and minimize the extensive use of antibiotic, which may cause to develop antibiotic resistance strains[28-29]. Mutational analysis is used to monitor antibiotic resistance among Staphylococcus and Enterococcus species[30] or detection of spores of Bacillus anthracis, the well-known causative agent of anthrax and a potential weapon of biological warfare[31]. In microbiological diagnostic, real time PCR has made revolutionary changs for variant determination based on sensitivity and specificity for target samples. It is quite efficient strategy to overcome traditional cultural method and reducing the cost and time in clinical investigations. lymph node micro metastases detection in prostate cancer and bone metastases in breast cancer is recently been exploited by RT-PCR technology[32].

In addition, real time PCR make possible to identify minimum residual disease (MRD) , or disease progression by using quantification approach for chromosomal translocation in patient sample[33], find out copy number variation that are responsible for malignancy [36], or useful for gene expression analysis in solid tumors with fine-needle aspirates[34]. To determine the pathogenesis of cancer and tumor biology, real time PCR analysis gives accurate and responsive prediction which is quite helpful to understand the disease mechanism, recurrence risk and development of cancer therapies. Drug discovery can be facilitated through this technology by establishing response between target gene expression to drugs and transporters[35-37].

In conclusions recent developments in fluorogenic dyes and labeling chemistries has made real time PCR technology most widely accepted. Advanced technology instrumentation has made incredibly short cycling times possible with an enhanced ability of detecting minute quantities and differentiates among multiple amplicons. Technology is quite flexible to allow the use of any of the chemistries described in this article making nucleic acid amplification an increasingly attractive and workable

proposition for routine diagnostics and research.

REFERENCES

1. Mullis K, Faloona F, Scharf S, R. S, Hom G, Erlich H. **Specific enzymatic amplification of DNA in vitro: The Polymerase Chain Reaction.** Cold Spring Harb Symp Quant Biol, 1986.
2. Higuchi R, Dollinger G, Walsh PS, Griffith R. **Simultaneous amplification and detection of specific DNA sequences.** Biotechnol. 1992;10(4):413-17.
3. Alker AP, Mwapasa V, Meshnick SR. **Rapid real-time PCR genotyping of mutations associated with sulfadoxine-pyrimethamine resistance in Plasmodium falciparum.** Antimicrobial Agents Chemo 2004;48(8):2924.
4. Ward CL, Dempsey MH, Ring CJA, Kempson RE, Zhang L, Gor D, et al. **Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement.** J Clini Virol 2004;29(3):179-88.
5. Gibson NJ. **The use of real-time PCR methods in DNA sequence variation analysis.** Clinica Chimica Acta 2006;363(1-2):32-47.
6. Sails AD. **Real-Time PCR: An Essential Guide.** Horizon Bioscience, Norfolk, 2004.
7. Gibson UE, Heid CA, Williams PM. **A novel method for real time quantitative RT-PCR.** Genome Research 1996;6(10):995.
8. Livak KJ, Schmittgen TD. **Analysis of relative gene expression data using Real-Time quantitative PCR and the 2-[Delta][Delta] CT method.** Methods 2001;25(4):402-08.
9. Wilson IG. **Inhibition and facilitation of nucleic acid amplification.** Appli Environ Microbiol 1997;63(10):3741.
10. Fraga D, Meulia T, Fenster S. **Real-Time PCR.** John Wiley & Sons, Inc., 2008.
11. Haque A, Naeem A, Anwar P, Abida R, Samina B, Abbas. G. **Utility of PCR in diagnosis of problematic cases of typhoid.** Jpn J Infect Dis 2001;54:237-39.
12. Raza A, Abdul H, Saadia K, Javed AQ, Amer J, Sheikh MA. **In vitro relationship between differing levels of virulence and antibiotic resistance in Salmonella typhi.** Int. J. Agri. Biol 2003;5(4):566-70.
13. Bashir S, Abdul H, Nasir A, Abida R, Asma H, Yasra S, et al. **Significant incidence of typhoid in hepatitis C patients.** Professional Med J 2005;12:287-94.
14. Islam A, A. Aslam, Z.I. Chaudhry, M.U.D. Ahmed, H.U. Rehman, K. Saeed, et al. **Pathology of Mycoplasma gallisepticum in naturally infected broilers and its diagnosis through PCR.** Int. J. Agric. Biol. 2011;13(4):835-37.
15. Al-Rawashdeh IM, Al-Rawashdeh NQ. **Exploring genetic diversity in Jordanian wheat landraces collected from different agro-ecological regions using random amplified polymorphic DNA analysis.** Int. J. Agric. Biol., 2011;13(3):325-31.
16. Ramessur AD, Ranghoo-Sanmukhiya VM. **RAPD Marker-assisted identification of genetic diversity among mango (Mangifera indica) varieties in Mauritius.** Int. J. Agric. Biol. 2011;13(2):167-73.
17. Aziz H, Gil ML, Waheed Y, Adeeb U, Raza A, Bilal I, et al. **Evaluation of prognostic factors for Peg Interferon alfa-2b plus ribavirin treatment on HCV infected patients in Pakistan.** Infec Genetics Evol 2011;11(3):640-45.
18. Raza A, Aziz H, Irfan J. **Predictive Factors to Response to Interferon Therapies for Treatment-naive Patients Infected with Hepatitis C Virus in Islamabad and Vicinity Areas: A Study from Pakistan.** Anti Res 2009;82(2):A31-A31.
19. Raza A, Aziz H, Zaman N, Yousuf I, Din F, Murtaza S, et al. **Therapeutic response guided interferon therapy among patients chronically infected with hepatitis c virus.** Anti Res 2010;86(1):A43-A43.
20. Valasek MA, Repa JJ. **The power of Real-Time PCR.** Advan Physiol Edu 2005;29(3):151-59.
21. Bustin SA. **Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.** J Mol Endocrinol 2000;25(2):169.
22. Bustin SA, Nolan T. **Pitfalls of quantitative RealTtime reverse-transcription polymerase chain reaction.** JBT 2004;15(3):155.
23. Wong ML, Medrano JF. **Real-time PCR for mRNA quantitation.** Biotech 2005;39(1):75.
24. Raza A, Zamir A, Javaid I, Shahnaz M, Samina Q. **Analytical variables influencing the HCV RNA determination by Taq Man Real-Time PCR in routine**

clinical laboratory practice. Mol Biol Rep 2012;DOI: 10.1007/s11033-012-1574-3.

25. Arshad BG, Raza A, Shakeel S, Anwar MA. **Pegasys as a second line of effective treatment plan for g3 non responders of conventional therapy.** Anti Res 2011;90(2):A53.

26. Gunson RN, Collins TC, Carman WF. **Practical experience of high throughput real time PCR in the routine diagnostic virology setting.** J Clin Virol 2006;35(4):355-67.

27. Mackay IM, Arden KE, Nitsche A. **Real-time PCR in virology.** Nucleic Acids Res 2002;30(6):1292.

28. Mackay IM. **Real-time PCR in the microbiology laboratory.** Clini Microbiol Infec 2004;10(3):190-212.

29. Cleary TJ, Roudel G, Casillas O, Miller N. **Rapid and specific detection of Mycobacterium tuberculosis by using the smart cycler instrument and a specific fluorogenic probe.** J Clin Microbiol 2003;41(10):4783.

30. 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(3):361-68.

31. Whiley DM, Buda PP, Freeman K, Pattle NI, Bates J, Sloots TP. **A real-time PCR assay for the detection of Neisseria gonorrhoeae in genital and extragenital specimens.** Diag Microbiol Infect Dis 2005;52(1):1-5.

32. Gibson JR, Saunders NA, Burke B, Owen RJ. **Novel method for rapid determination of clarithromycin sensitivity in Helicobacter pylori.** J Clin Microbiol 1999;37(11):3746.

33. Martineau F, Picard FJ, Lansac N, Menard C, Roy PH, Ouellette M, et al. **Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of Staphylococcus aureus and Staphylococcus epidermidis.** Antimicrobial Agents Chemo 2000;44(2):231.

34. Tefferi A, Wieben ED, Dewald GW, Whiteman DAH, Bernard ME, Spelsberg TC. **Primer on Medical Genomics Part II: Background Principles and Methods in Molecular Genetics.** Mayo Clinic Proceedings 2002;77(8):785-808.

35. Krauter J, Heil G, Ganser A. **The AML1/MTG8 fusion transcript in t (8; 21) positive AML and its implication for the detection of minimal residual disease.** Hematol 2001;5(5):369-81.

36. Flohr T, Schrauder A, Cazzaniga G, Panzer-Grümayer R, Van der Velden V, Fischer S, et al. **Minimal residual disease-directed risk stratification using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the international multicenter trial AIEOP-BFM ALL 2000 for childhood acute lymphoblastic leukemia.** Leukemia 2008;22(4):771-82.

37. Barthe C, Mahon F-X, Gharbi M-J, Faberes C, Bilhou-Nabera C, Hochhaus A, et al. **Expression of interferon-á receptor 2c at diagnosis is associated with cytogenetic response in IFN-á-treated chronic myeloid leukemia.** Blood 2001;97(11):3568-73.

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"Anyone can deal with victory.
Only the mighty can bear defeat."

Adolf Hitler