



Quantitative Realtime Polymerase Chain Reaction in Cancer Drug Target Identification and Validation

a report by
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The availability of large-scale, functional genomics tools has enabled direct genome-wide RNA interference (RNAi) phenotype analysis for cancer drug target identification. Once these important targets are identified, they may be validated utilising widely applicable and readily upgradeable technologies.^{1,2} Quantitative (q) realtime polymerase chain reaction (PCR) (qPCR) is the 'gold standard' and the method of choice for validation of novel drug targets, provided proper measures are taken for generating reliable and reproducible data.

Cancer Drug Target Identification

Gene regulation networks that orchestrate the growth and survival of cancer cells are influenced by many intrinsic and extrinsic phenomena. The genetic and molecular context of a cell delineates its particular state and determines its relative dependency on certain genes, which is critical for growth and survival. Application of chemogenomics can complement drug target identification and validation overall. Chemogenomics is simply defined as a genomic response to chemical compounds. The overarching goal of chemogenomics is the rapid identification of novel drugs and drug targets, including multiple early-phase drug discovery technologies ranging from target identification and validation, prioritised over compound design and chemical synthesis to biological testing.²

A chemogenomics approach towards novel target discovery combined with RNAi-based screening are facilitating the robust, improved discovery of new targeted therapies.³ Combination strategies using compound sensitisation screens are powerful tools for cancer drug target identification and drug development. These approaches have strong potential to provide better cancer drug targets using a combination of short interfering RNA (siRNA) libraries and pre-existing chemotherapeutics, as well as a combination of siRNAs and novel compound libraries.^{4,5}

RNA Interference

A classic technique for determining the function of a gene is to experimentally inhibit the expression of endogenous genes in order to examine the resulting phenotype or effect on molecular end-points and signalling pathways. To better comprehend tumour development that occurs during the neoplastic process, RNAi profiling can be utilised to discover context-dependent vulnerabilities in cancer cells. RNAi is one of the most recent discoveries of a naturally occurring mechanism of gene regulation that is triggered by the introduction of double-stranded RNA into a cell.^{6,7} This phenomenon can be mimicked to downregulate gene expression by transfecting mammalian cells with synthetic siRNAs.⁸ To date, efforts have been put together to create large siRNA libraries targeting human genes on a genomic scale using RNAi technology.⁹ Hence, siRNAs can be designed to specifically knock down the expression

of genes harbouring a particular target sequence, and they represent an exciting therapeutical potential for inhibiting gene expression.^{10,11}

To complement the identification of candidate druggable siRNA target 'hits', a validation process utilising qPCR technology is needed to confirm the validity of the hits. Ultimately, RNAi phenotype profiling for the identification of candidate druggable gene targets that functionally regulate the response to the knock-downs would be validated. Consequently, RNAi has become the ideal research tool for studying gene function and is one of the more frequently used tools in drug discovery and development. Eventually, siRNA agents will be put to the test in clinical trials of RNAi-based therapeutics. This will most likely be in combination with chemotherapeutic drugs in the cancer field and other areas of genetic disorders, and qPCR will play a major role in defining the ultimate candidates of the siRNA players.

Quantitative Realtime Polymerase Chain Reaction

Fluorescent reporter molecules are utilised during qPCR to monitor the production of amplicons during each cycle of the PCR. Thus, nucleic acid amplification and detection steps are combined into a single assay, rendering unnecessary the need for conventional gel electrophoresis to detect amplification products.¹² Additionally, the application of suitable software from individual qPCR instruments for data analysis and the availability of appropriate chemistries rules out the need to use conventional methodologies, such as Northern blotting, *in situ* hybridisation, semi-quantitative realtime PCR, ribonuclease protection assay (RPA), competitive quantitative realtime PCR and Southern blotting.^{12,13}

There are significant advantages of qPCR compared with other nucleic acid quantification techniques. These advantages include enhanced sensitivity and accuracy, a large dynamic range, the ability to perform multiplex reactions, high-throughput capability, automation compatibility, online monitoring capacity, elimination of post-reaction analyses and faster and more reliable amplification.¹³ Among some of the major applications for qPCR in the life sciences are as follows: quantification of bacterial pathogens such as *Helicobacter pylori*, measurement of viral load in clinical samples, detection of genetic



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alterations, solid tumour diagnosis, sex determination in human blastomeres, toxicology, single nucleotide polymorphism (SNP) analysis, RNA copy number quantification, verification of microarray data and validation of siRNA knock-downs. Additional interesting applications include the diagnosis of ploidy based on the measurement of gene copy number, quantification of cytomegalovirus DNA in amniotic fluid samples from mothers with primary infection and quantification of genetic modification in food for biotechnology applications.¹⁴ This powerful technology has become the method of choice for rapid and quantitative monitoring of specific gene expression levels. Recently, a wide range of gene expression assays and qPCR instrumentation for high-throughput application of these assays in 96- and 384-well formats have become commercially available. Hence, this technology is appealing for end-point analysis and has become the method of choice for validation of siRNA targets.¹³

Quantitative realtime polymerase chain reaction assays will continue to be the gold standard and the method of choice for validation of drug target identification.

Validation by Quantitative Realtime Polymerase Chain Reaction

The delineation of the roles of the genes studied at a transcriptional level is aided by qPCR. Using qPCR, gene expression knock-downs of new chemotherapy sensitiser genes can be validated and confirmed, illustrating levels of gene silencing associated with a drug-response phenotype. The two most routinely used methods of data analysis for qPCR are absolute quantification and relative quantification.^{13–17} Absolute quantification assesses the exact copy number of the target gene using a standard curve quantification,^{18–21} whereas relative quantification presents the relative change in gene expression compared with an untreated control and/or a scrambled siRNA control sample.^{13,16,21} Due to the large sample size and cost concerns, the relative quantification method is more feasible and favourable for drug-target identification and validation when determining

chemosensitiser and/or high-throughput RNAi screening hits. Taking extreme care is crucial when preparing and setting up qPCR protocols. This set-up process involves steps including RNA extraction, RNA quality and concentration determination, complementary DNA (cDNA) synthesis, utilisation of appropriate endogenous reference genes for normalisation purposes, qPCR reaction set-up and qPCR data analysis. Any imprecision would influence the biology, and one of the most critical parts worth mentioning is the normalisation step when performing relative quantification of qPCR.^{13,22} Variability of qPCR experimental results can be the consequence of technical as well as biological alterations. It is crucial that technical variability be kept at a minimum. Preferably, one may avoid errors by using robotic handling. This makes it more feasible to identify and optimise biologically relevant variations at a transcriptional level.^{13,22} Standardisation of data normalisation for biologically relevant qPCR assays is unavoidable. There is no universally recognised method for data normalisation in the course of qPCR experiments. Hence, it is of utmost importance that data normalisation is considered a requirement for qPCR, especially the input of RNA and endogenous reference gene utilisation for normalisation purposes, which should be kept as a guideline for minimising variability in the qPCR assays.²³

Conclusions

qPCR assays will continue to be the gold standard and the method of choice for validation of drug target identification, provided that proper steps are taken for generating reliable and reproducible data measuring the extent of target gene silencing, as well as effects on various gene expression end-points. The advent of state-of-the-art technologies is helping expedite the qPCR process from small sample sizes to as many as 2,304 individual qPCR reactions per run. Such technologies facilitate the use of smaller amounts of reagents and sample materials. Equally as important, they utilise reagent mixes that can achieve faster rates of reaction in combination with appropriate instrumentation, thereby enabling ultra-high-throughput qPCR analysis. ■

Acknowledgments

The author thanks Drs Nathalie Meurice and Candice Nulsen for their perspective and critical proofreading of the manuscript. The author also would like to acknowledge the Pharmaceutical Genomics Division of The Translational Genomics Research Institute for its continued support.

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