INTEGRATED REAL-TIME PCR FORMATS: METHODOLOGICAL ANALYSIS AND COMPARISON OF TWO AVAILABLE INDUSTRY OPTIONS

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Abstract

The real-time PCR methodology improved profoundly the basic amplification technology; not only by permitting the digital tracking of the reaction from the very first minutes, but also by significantly compressing the time for an amplification program, through technical improvements in expendables and hardware. Two of the integrated, end-to-end solutions available in the market, the Roche and the Applied Biosystems products, also capitalize on the advances at the multi-color dyes and the melting-curve analysis to perform products discrimination and dispose of post-amplification steps, such as electrophoresis, thus further compressing time and logistics footprint of the integrated assay. They also focus on the well-proven ability of the methodology to provide accurately quantitative results, which was always a priority throughout the field of biosciences, and one rarely tackled efficiently. Electrophoresis, though, remains popular with low-end users, who find it robust, reliable, flexible and relatively cheap since expendables are low-cost and know-how is well-established. Given that high-end users prefer true high-throughput methods (such as arrays) for simultaneous assaying, which vastly outperform real-time PCR, the ease and rapidity of the latter's results, which both are of capital diagnostic concern, remain the strongest points of the method, coupled with its quantification potential.

Key words: *Real-time PCR, FRET pairs, SYBR-Green, post-amplification procedure, DNA quantification*

Introduction

The real-time PCR is a technological advance somewhat underadvertised by its name. In fact it brings a number of advantages over previous, conventional formats. The first one is the ability to monitor, during the cycling, the production- or lack of it- of amplicons, a feature that christened the whole concept. A second, most important advantage is the compression of the time required for a typical cycler protocol to 20-30 min instead of 2-4 h, a 76-82% drop [3, 4]. This is made possible by advanced reaction chemistry, optimised volume- to- surface ratio in the capillaries used by some concepts and excellent heat exchange properties and mechanics of the hardware and the capillary, tube or plate of the reaction [15]. The third, intensely advertised feature is the prospect of eliminating post-amplification sample processing by usual means such as electrophoresis and subsequent blots. This is made possible by the inherent capabilities offered by the photometric module of the instrument and its accessorising expendables, coupled with a most robust software application.

The real-time monitoring of amplicon production is made possible by the scanning sessions after each PCR cycle. As amplicons elongate and accumulate, the DNA-binder dye (usually SYBR-Green [15] but not always- [9]) gets densely and securely fixed between the chains of each DNA molecule and thus produces visual signal after suitable excitation, which is measured by a sensor devise and plotted. This kind of "sequence-independent analysis" informs on the production or not of an amplicon as such. With proper negative and positive controls, this is just enough for some time-critical diagnostic issues [3, 4].
Moreover, melting-curve analysis (MCA) in sequence-independent formats can produce a rough discrimination between primer-dimers and specific product in the same tube/well and even relative differential specific product analysis, based on Tm differences due to length and/or sequence variation. Sequence variations may reflect to different melting curves, but, again, they may not. Thus, the fact remains that there is no firm data about absolute length and sequence of the produced amplicon, nor for its identity [2, 15, 16].

Comparative Methodology

This kind of data has been traditionally obtained by post-amplification analysis, usually electrophoresis combined with restriction digestion [23, 24], probe hybridization (the latter usually after a blotting step) [14] or other methods such as SSCP (Single Strand Conformation Polymorphism)[1], sequencing etc. Such abilities are out of the league of any method restricted in liquid phase, because homogenous liquid preparations lack positional discrimination for their ingredients. With no kind of dynamic grading to reveal product size (relative or absolute- 8, 26), the integrated post-amplification analysis of the samples could be nothing but sequence-related, [2, 21] which is usually a synonym for hybridization methodology, given that Restriction Enzyme Analysis, although sequence-dependent (or sequence-sensitive), needs an electrophoresis step for data extraction and could prove cumbersome in some respects [23, 24]. On the contrary, the new breed of hybridization protocols [20] can well be integrated in a liquid-phase product with a minimum of changes, especially in accurately controlled thermal environments -as are the cyclers in general- which allows for dedicated melting point consideration. The widening use of non-radioactive and, most importantly, polychromatic dye preparations makes the hybridization a far more robust tool for sequence-related analysis than it had been in the past [25].
Even more intriguing are the possibilities opened with the binary dies, the prime example being FRET pairs [22]. Such a format allows for increased specificity. The particulars of the Roche system allow for only two-colour separation, which is enough to resolve, under optimal design, two binary queries. This is just enough to simultaneously study two biallelic SNPs (or mutations) but obviously fails to provide usable tools for higher order discrimination problems, as are multiallelic SNPs and mutations or multi-locus genotyping [25]. The Roche system, with two probes for one-signal production offers extreme specificity (the need for it is an altogether different matter) whereas the Applied Biosystems proposal is somewhat different. It uses a single probe as carrier of both dyes, of which one is a quencher. The use of a single probe relaxes a bit the specificity obtained, since the coverage is usually less in length and more concentrated in location than in a two-probe system, but the matter of specificity is always subject to the enzyme (polymerase) tolerance in handling mismatches in the regions of the PCR primers. On the other side, the single-probe

system diminishes the cost and relaxes probe design constraints, as a two-probe system is more expensive than a single one, even if the sum of bases is the same. Moreover, a denser probe environment needs more stringent laws governing its design to avoid unwanted dimer formation and random hybridizations in a given set of conditions. The fact remains that the AB system is also able to discriminate only two colours, allowing as poor a multiplex performance as the one of Roche's.

In simplex diagnostic PCRs, as performed in Reference labs, the RealTime format is more than adequate [3, 4]. Its sequence-independent analysis provides a very timely answer, whereas the use of one probe with exitable dye (with or without quencher) offers the specificity needed in such contexts. It is true that this format cannot discriminate between full-length and some truncated sequences (i.e. pseudogenes), but one must keep in mind that simple electrophoresis, which is the current diagnostic standard, cannot discriminate in its turn between similarly sized but different amplicons, in cases of related sequences (homologues). Electrophoresis can always be performed with the Real Time PCR amplicon, [3, 4] although this negates one of the main advantages claimed by the companies, the exclusion of post-amplification procedures-especially electrophoresis [2, 15, 16]. However, most labs with Molecular Biology infrastructure are capable of electrophoresis and not very eager to phase it out, as it is a proven, well-established and extremely adaptive technique [27, 28], although this may be not the case with new high-tec labs that will be established in the near future.

More specificity- and discriminatory power- can be obtained if use of the FRET principle is made. In such cases, given that there are two FRET dyes, it is possible to resolve two diagnostic questions in one reaction (duplex PCR format) which may refer to closely related or to vastly remote pathogens. In simplex formats, the FRET procedure adds specificity and diminishes the possibility of pseudo-positives due to truncated target sequences or allows better analysis of a single variable taking multiple (more than two and, to the present, up to four) values.

The main disadvantage lies in the infrastructure for sequence-specific analyses. The design of probes, especially when opted for FRET (either simplex and the more so in duplex) is a laborious, time –consuming, high-risk, high-restriction and expensive procedure [1, 11, 12, 15, 17, 25]. Genomic data is required for truly trustworthy results in assay development, but other genomic-based procedures offer far greater robustness for similar specific cost and infrastructure investment [25]. It is rather ill-advised to invest in a technique were duplex is the definite limit of its current parallel throughput, suggesting that a triplex or quadrplex diagnostic format will not be needed in the near future of a lab's routine.

A more problematic issue is the degree of specificity. The ramping nature of the cycling and melting curve analysis permits hybridization of both the primers and the probes despite mismatches, which extends the scope of the target but reduces the specificity [25]. In the case of the probe-based melting curve analysis, the exact and precise thermal performance of the hardware and the robustness of the software allow for sharply defined profiles in melting curve analyses, which indicate a single base difference. The problem is that such data cannot indicate which is the polymorphic base/residue (not to mention the identity of the polymorph) [1]. Presumptions have to be made as to the expected sequence homology and the anticipated polymorphic position, but no hard evidence can be derived, which drastically limits the scope of applicability of such experiments since they usually have to both locate and identify the detected variability.

Roche promotes the Real-Time PCR hardware and assorted software integrated system for applications such as mutation analysis that are a hot issue in contemporary research. In such cases though, the system is plagued by inherent drawbacks, making it anything but the preferred choice. First, mutation analysis must make certain -to a degree- that the observed difference between two sequence samples is due to a difference in a specific residue presenting polymorphism. Else, the experiment shifts by default to the mutation detection application, a very important function but a very different one, which must be followed by mutation location, identification and tracking, which all form part of a mutation analysis procedure. Mismatch-based melting curve analysis (MCA) [6] is far less suitable in such cases than even simple REA (Restriction Enzyme Analysis), due to the far smaller detection footprint of the latter on the target molecule (less than a score of residues, with normal value of less than ten) [10, 19].
A restriction endonuclease "reads" a limited number of nucleotide residues ("n"), thus making the possibilities of erroneous result Po1=(n-1)/n. The melting curve dislocation may be caused by a single mismatch of any of a number of the probe's residues ("x"). x is a function of probe's design and nucleotide context, but normally x>>n. As the possibility for erroneous result is Po2=(x-1)/x, it follows that Po2>Po1.

The other drawback of Real-Time PCR-MCA in mutation analysis is the extremely limited scope of analysis even when location considerations are dealt with. Single-locus analysis may be performed, under ideal conditions, for triallelic loci and in the case of biallelic loci a maximum of two loci can be resolved in a single reaction. Such throughput potential is, at the very least, insufficient for contemporary similar experiments. Nowadays, the use of high-throughput methodologies, based on similar infrastructure (probe design and multi-dye excitation) allows easily for hundred-entity (*entity* equals to *sum of loci* X *allelic context of each locus*) analyses and sometimes for thousand-, without too much effort, due to the advantage of positional discrimination offered by solid-state nucleic acid chips [21, 25, 26]. Even if many more dyes were available, the practicability of multiplex Real-Time PCR would be limited to less than a score of loci due to colour cross-talk in a liquid phase. It is a very unfortunate coincidence that the real-time PCR, which could have made an enormous impact in research and diagnostics if invented 5 years earlier, was made available at the time of high-throughput methodologies, which revolutionized research and for which the Real-time PCR methodology is patently unable to adapt [18].

Conclusion

The undoubted strong point of Real-time PCR, except for assay speed, is the possibility for rapid and accurate quantification [16]. Roche, but especially AB, emphasize this fact. Truly, quantification actions based on photometry of dyes' emissions (probe-attached or DNA-bound) produce a linear function between signal and product quantity. Fairly accurate calculations can be made if the measurements are performed during the exponential phase of the amplification, rather than during the plateau (where dynamics are complicated due to restrictive phenomena and unknown succession of reaction endpoints). The basis is that a certain amount of signal will be produced earlier or later during the cycling by all positive samples. The timing is a function of the starting quantity, and the signal a direct relation of actual quantity. Standard curves of *signal increase* Vs *cycle number*, produced by standards,

are straight regressing lines. Appropriate standards, computation and considerations allow for relative or absolute quantification, both of which have particular uses (the former in gene switch-on, the latter in infection dynamics). Although standard-curve plotting, especially in formats with internal (endogenous or not) controls [5], is a laborious and sensitive procedure, it assists prompt results and even high throughputs, especially where such considerations have been an integral part of the system's design (automation-friendly PCR formats [7, 13]) and the lab's infrastructure (compatible lab equipment and instrumentation). The problem is that amplification efficiencies may not be uniform in the samples as DNA (and, even worse, RNA) extraction are notoriously un-reproducible procedures, especially in diagnostic applications where there might be different biological (and even environmental) samples tested for the same pathogen, not to mention slight but dynamically critical variations of the target sequence [2, 10, 23].

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Τίτλος

ΟΛΟΚΛΗΡΩΜΕΝΕΣ ΠΡΟΤΑΣΕΙΣ PCR ΠΡΑΓΜΑΤΙΚΟΥ ΧΡΟΝΟΥ ΑΝΑΓΝΩΣΗΣ: ΜΕΘΟΔΟΛΟΓΙΚΗ ΑΝΑΛΥΣΗ ΚΑΙ ΣΥΓΚΡΙΣΗ ΔΥΟ ΕΜΠΟΡΙΚΑ ΔΙΑΘΕΣΙΜΩΝ ΕΠΙΛΟΓΩΝ

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Περίληψη

Η PCR Πραγματικού Χρόνου Ανάγνωσης επέτρεψε την πολυδιάστατη βελτίωση της βασικής τεχνικής ενίσχυσης πυρηνικών οξέων. Αν και μεγάλη σημασία δίνεται την παρακολούθηση της αντίδρασης εν τω γενάσθαι, που επιτρέπει εντός ελαχίστων λεπτών την δυαδική αξιολόγηση της αντίδρασης ως θετικής ή αρνητικής, τα πλεονεκτήματα εκτείνονται πέραν και ανεξαρτήτως αυτού του βασικού γνωρίσματος. Η βασική αρετή της είναι η συμπίεση του χρόνου εκτέλεσης πρωτοκόλλων ενίσχυσης στο 20% των συμβατικών μηχανημάτων, χάρη στην βελτιωμένη σύνθεση αντιδραστηρίων, την προηγμένη κατασκευή θερμικών κυκλοποιητών και την βελτιστοποίηση περιεκτών και σωληναρίων αντίδρασης, που επιτρέπουν ταχύτατες μεταβολές της θερμοκρασίας με ομοιογενή τρόπο. Δύο από τις ολοκληρωμένες λύσεις που εμφανίστηκαν στην αγορά ήταν τα συστήματα της Roche και της Applied Biosystems. Και τα δύο βασίζουν την προώθησή τους περισσότερο στην δυνατότητα εξάλειψης μεταενισχυτικού σταδίου ανάγνωσης, στις δυνατότητες ποσοτικοποίησης των προϊόντων και στην ικανότητα διάκρισης μεταξύ διαφορετικών προϊόντων για πολυπλεκτικές ή διαζευκτικές εφαρμογές σε ένα στάδιο. Οι δυνατότητες αυτές παρέχονται από την τεχνολογία ανάλυσης καμπύλης τήξεως και τις νέες, προηγμένες και αυξημένης ευκαμψίας τεχνολογίες χρώσης. Καθώς η διακριτική ικανότητα παραμένει ιδιαίτερα χαμηλή στην εποχή των συστοιχιών και των μεθόδων υψηλού ρυθμού συνεπεξεργασίας και η κατάργηση της ηλεκτροφόρησης δεν ελκύει πολλούς από τους λιγότερο εύρωστους χρήστες που έχουν αποκτήσει υποδομή και ικανοποιητική τεχνογνωσία σε αυτή τη μέθοδο, η εμπορική προοπτική των εν λόγω προσεγγίσεων προφανώς θα στηριχθεί στην εξαιρετική ταχύτητά τους, πολύτιμη για διαγνωστική χρήση, και στην δυνατότητα ακριβούς ποσοτικοποίησης που έχει εφαρμογές σε όλο το φάσμα των βιοεπιστημών.

Λέξεις-κλειδιά: *PCR πραγματικού χρόνου ανάγνωσης, SYBR-green, διώνυμες χρωστικές FRET, ποσοτικοποίηση προϊόντος PCR*