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Review Article

Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available

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ABSTRACT

We review different methodologies to estimate the expression levels of microRNAs (miRNAs) using real-time quantitative PCR (qPCR). As miRNA analysis is a fast changing research field, we have introduced novel technological approaches and compared them to existing qPCR profiling methodologies. qPCR also remains the method of choice for validating results obtained from whole-genome screening (e.g. with microarray). In contrast to presenting a stepwise description of different platforms, we discuss expression profiling of mature miRNAs by qPCR in four sequential sections: (1) cDNA synthesis; (2) primer design; (3) detection of amplified products; and (4) data normalization. We address technical challenges associated with each of these and outline possible solutions.

1. Introduction

MiRNAs are small, non-coding RNAs that post-transcriptionally regulate gene expression [1]. They are derived from genome encoded stem-loop precursors and function through RNA induced silencing complex (RISC) mediated binding to their target mRNAs by base pairing, mostly to the 3′ untranslated region (3′-UTR). Recently, miRNAs have emerged as important regulators of metabolism, immunity and cancer [2,3]. Functionally, miRNAs regulate gene expression by mainly repressing mRNA translation and thus reducing target protein levels [4,5]. However, this activity may produce different results when applied in different contexts. For example, in early stages of development, few miRNAs are expressed. As the organism develops and becomes enriched in differentiated cell types, then the number of detectable miRNAs increases [6]. It is hypothesized that miRNAs act to induce cell type specific gene expression, which however does not address the effect of miRNA regulation in the adult organism. Through reviewing the literature, the known function of expressed miRNAs in the fully developed organism can be divided into three groups:

a) the dysfunctional expression of specific miRNAs, known as oncomiRs [7], is linked to the biogenesis of human malignancies [8,9];
b) the expression of particular miRNAs has no functional consequence unless some specific type of stress occurs [10,11];
c) the expressions of certain miRNAs are required to preserve metabolic pathways, such as cholesterol and insulin biosynthesis [12,13].

Therefore, through the determination of cell specific miRNA profiles, it may be possible to identify genes whose expression must be restricted when cellular states change, such as during differentiation, response to stress or in oncogenesis. Changes in miRNA expression in disease, and/or correlation of miRNA expression profiles with clinical parameters (such as disease progression or therapy response) may serve as clinically relevant biomarkers [14,15]. In consequence, accurate determination of the level of expression of miRNAs in a specific cell type or tissue is an essential parameter to describing the biological, pathological and clinical roles of miRNAs in health and disease.

2. Challenges of miRNA expression profiling

Determining miRNA expression profiles with high sensitivity and specificity is technically demanding as:

i) mature miRNA are short (~22 nucleotides; nts);
ii) miRNAs are heterogeneous in their GC content, which results in a relatively large interval of melting temperatures (*Tm*) of nucleic acid duplexes for the population of miRNAs;
iii) mature miRNAs lack a common sequence feature that would facilitate their selective purification [e.g., poly(A)];
iv) the target sequence is present in the primary transcript (pri-miRNA) and the precursor (pre-miRNA), in addition to the mature miRNA;

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v) miRNAs within the same family may differ by a single nucleotide (e.g., Let-7 family).

Several methodological approaches to enrich, label, amplify and profile mature miRNAs are available at present, including northern blotting with radiolabelled probes [16], oligonucleotide macroarrays [17], qPCR-based detection of mature miRNAs [18–20], single molecule detection in liquid phase [21], oligonucleotide microarrays [22–26], *in situ* hybridization [27,28] and by using massively parallel sequencing [29].

To identify global differences in miRNAs expression across comparative samples, we first perform whole-genome screening using techniques such as microarrays (i.e. Exiqon, Agilent, Illumina or miCHIP), bead based assays (Luminex) or qPCR-based methodologies (TaqMan Low density microRNA Array card, TLDA). In principle, massively parallel sequencing could also be used, as the relative abundance of sequences representing miRNAs will recapitulate miRNA expression levels in the source tissue [30].

Whole-genome screening generates a qualitative and quantitative evaluation of how experimental conditions affect miRNA profiles. Next, qPCR is used to validate observations determined by genome wide profiling of miRNA expression. The successful outcome of qPCR analysis depends upon a number of interconnected steps that require individual optimization. To perform qPCR that provides meaningful and reproducible results, several parameters such as RNA extraction, RNA integrity control, cDNA synthesis, primer design, amplicon detection, and data normalization must be taken into account.

The reliability of miRNA expression profiling depends also to the quality of the total RNA used as input material, which should have an RNA integrity number (RIN) that exceeds seven [31]. RNA isolation and assessment of its quality is beyond the scope of this article, however, robust, reproducible methods for RNA isolation and estimation of RNA quality should be employed prior to initiating the characterization of miRNA expression levels.

3. Step 1; cDNA synthesis

The first step in qPCR of miRNAs is the accurate and complete conversion of RNA into complementary DNA (cDNA) by reverse transcription. However, this step is challenging as:

i) the template has a limited length (~22 nts);
ii) there is no common sequence feature to use for the enrichment and amplification of miRNAs;
iii) the mature miRNA sequence is present in pre- and the pri-miRNAs.

To date, two different approaches to reverse transcribe miRNAs have been utilized. In the first approach, miRNAs are reverse transcribed individually by using miRNAs-specific reverse transcription primers. In the second approach, miRNAs are first tailed with a common sequence and then reverse transcribed by using a universal primer. As general consideration, the use of miRNA-specific primers (MSPs) decreases background, whereas universal reverse transcription is useful if several different amplicons (i.e. miRNAs) need to be analyzed from a small amount of starting material. As alternative it is possible to multiplex miRNAs reverse transcription by pooling stem-loop primers. This specific approach will be discussed in a separate section (see Special applications; Multiplexing stem-loop primers).

3.1. cDNA synthesis by using stem-loop and linear miRNA-specific primers

Despite the short length of mature miRNAs, specific complementary primers can be annealed to them to prime reverse transcription. The resulting cDNA is then used as a template for qPCR with one MSP and a second universal primer. While the 3′-end of the MSP has to be complementary to the miRNA, there are two different approaches to design the 5′-end of a MSP: with either a stem-loop [32] (Fig. 1A) or a linear structure [19,20] (Fig. 1B). Stem-loop primers are designed to have a short single-stranded part that is complementary to the 3′-end of miRNA, a double-stranded part (the stem) and the loop that contains the universal primer-binding sequence. Stem-loop primers are more difficult to design but their structure reduces annealing of the primer to pre- and pri-miRNAs, therefore by increasing the specificity of the assay. A particular disadvantage of a stem-loop primer is reduced ability to achieve reverse transcription of isomiR sequences [33].

The 3′-end of linear primers is designed to complement the target miRNA, to enable reverse transcription, while the 5′-end of the primer encodes a universal sequence that is used to achieve qPCR amplification. Although linear primers are simpler to design, compared to stem-loop primers, linear primers may not discriminate between mature miRNA and their precursors. Consequently, the annealing and reverse transcription steps must be optimized to prevent any interaction between the primer and the pre- and pri-miRNAs.

3.2. cDNA synthesis by tailing RNAs

In an alternative approach, the 3-ends of miRNAs are elongated to provide them with a common tail. *E. coli* Poly(A) Polymerase (PAP) [20] is used to add, in a template independent fashion, adenosine nucleotides to the 3′-end of RNA. A primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end is then used to prime reverse transcription and to amplify the target sequences in the qPCR reaction. The stretch of "dTs" between the miRNA and the universal sequence of the oligo(dT) primer is defined by using a degenerate sequences at the 5′end of the primer, that anchors the primer to the 3′-end of the miRNA (Fig. 1C).

Recently, our group has developed a novel approach to synthesize cDNA from miRNA, that we have named miQPCR (Patent application EP 09 002 587.5, manuscript in preparation). miQPCR exploits the activity of T4 RNA Ligase 1 (single-stranded RNA ligase) to covalently attach the 3′-hydroxyl group of mature miRNAs to the 5′-phosphate group of a RNA/DNA linker adaptor, which in turn contains a universal primer-binding sequence. The extended miRNAs are then reverse transcribed by using a universal primer complementary to the 3′-end of the linker (Fig. 1D). In our experience, using T4 RNA ligase1 increases the specificity, sensitivity and the efficiency of reverse transcription and of qPCR. Advantages of using primer ligation, as compared to polyadenylation are:

i) the T4 RNA ligase preferentially uses short single-stranded RNAs as substrates;
ii) the linker, containing a universal primer-binding sequence, is directly attached to the miRNA. This allows more flexibility in the design of the MSPs (see below).

4. Step 2; miRNA-specific primer design

The specificity and sensitivity of qPCR assays are dependent upon primer design. In particular, individual miRNAs have highly heterogeneous GC content, which results in a relatively large interval of predicted T_m against complementary sequences, that is, the miRNA-specific primer. The design of MSPs is linked to both the type of cDNA synthesized (see Step 1) and to the method used to detect the amplicon (see Step 3). The miRBase database [34] can be used to analyze the sequence of the miRNA under evaluation, with important factor to consider being the GC content and the existence of closely related family members. If the predicted T_m

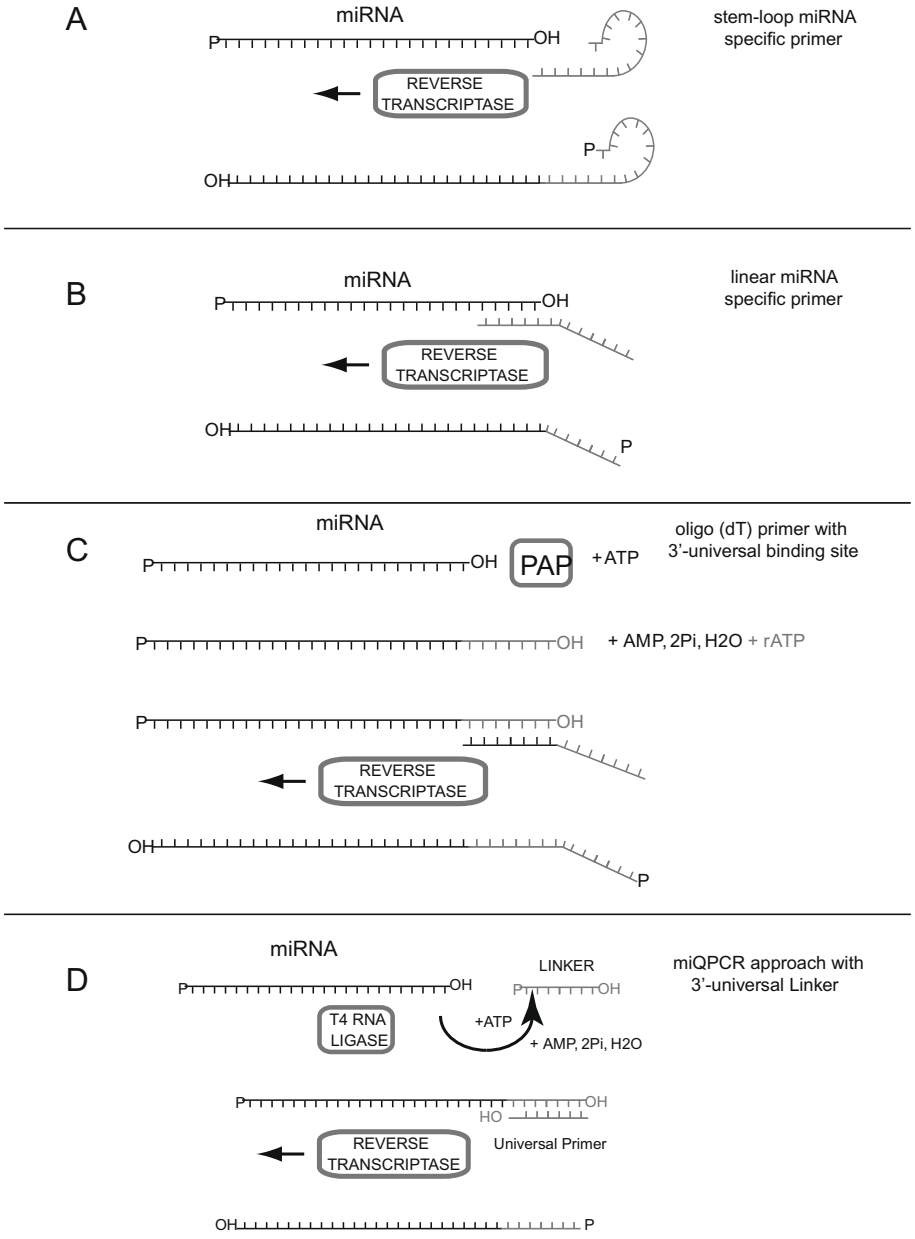

Fig. 1. Schematic representation of alternative reverse transcription methodologies to generate cDNA. Reverse transcription of individual mature miRNAs using stem-loop (A) or linear (B) MSPs. The MSPs are used to prime reverse transcription. Universal reverse transcription of all the mature miRNAs contained in the RNA sample by enzymatic tailing of the miRNAs by using Poly(A) Polymerase (C) or T4 RNA Ligase (D). miRNAs are first tailed tailing and then reverse transcribed by using oligo(dT) (C) or universal primers (D). (For detailed explanation see the text).

between the MSP and the target sequence is high (e.g., >65 °C) then shortening the length of the primer can be used to increase specificity. However, if the predicted T_m between the MSP and the target sequence is low (e.g., <55 °C) then sensitivity becomes an issue. One way to increase the T_m, which is independent of sequence, is to use Locked Nucleic Acids (LNAs). LNA is a synthetic RNA/DNA analog characterized by increased thermostability of nucleic acid duplexes [35]. Each incorporated LNA monomer increases the T_m of a DNA/DNA hybrid up to approximately 5 °C, depending upon the position of the LNA moiety in the oligonucleotide primer. Consequently, LNA-modified primers can be designed to provide primers whose T_m satisfies a required value. However, LNA nucleotides are more expensive than DNA nucleotides; furthermore, incorporation of an LNA moiety may not result in the desired result with trial-and-error often required to identify an optimal primer. A different methodology to adjust the T_m of the primer is to synthesize cDNA by using the miQPCR approach. This is achieved by extending the 3′-end of the MSP, however, it is important to prevent unspecific amplification products by ensuring that the primer will not anneal to linker sequence.

5. Step 3; detection of qPCR products

The principle of qPCR is based on the detection, in real-time, of a fluorescent reporter molecule whose signal intensity correlates with amount of DNA present in each cycle of amplification [36]. A number of fluorescent technologies exist for performing qPCR [SYBR Green I, TaqMan probes, Molecular Beacons, Light Upon eXtension (LUX) and HybProbes (LightCycler)], however, to date only two of these technologies have found application to miRNA detection; SYBR Green I [20,37]; Fig. 2A) and TaqMan probes

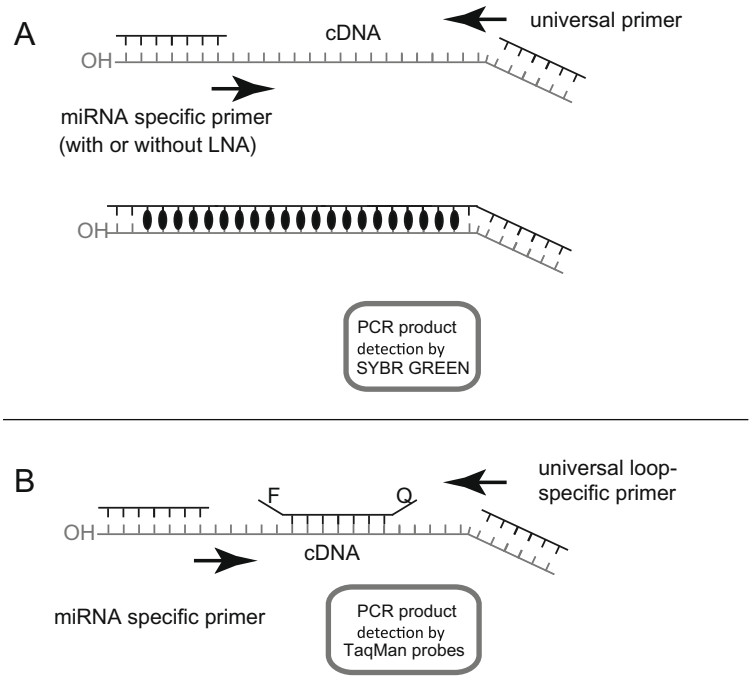

Fig. 2. Amplicon detection by using fluorescent molecules. Detection of the amplicon by using SYBR Green (A) or TaqMan probes (B). (For detailed explanation see the text).

[38,39]; Fig. 2B). Two different types of SYBR Green are available commercially, SYBR Green I and II. While SYBR Green I binds preferentially to double-stranded DNA (dsDNA), SYBR Green II binds preferentially to RNA. In this review, SYBR Green I will be referred to as SYBR Green.

5.1. Detection of qPCR products by using SYBR Green

SYBR Green is an intercalating dye whose fluorescescence increases approximately 100-times only upon association with dsDNA and this property is used to detect amplification products as they accumulate during PCR cycles. Importantly, SYBR Green cannot discriminate between different PCR products and binds to all dsDNA, including non-specific products such as primer-dimers [40]. This limits the accurate detection of the target sequence and necessitates methodologies that assess the specificity of the amplification products. To this end the use of SYBR Green enable us to perform a Melting point analysis, also referred to as dissociation curve analysis, is frequently used to monitor the homogeneity of the qPCR products. During this procedure, the fluorescence intensity emitted by SYBR Green intercalated into PCR products is recorded at temperatures rising in small increments from 65 °C to 95 °C. This increasing temperature gradually denatures dsDNA, which induces a consequent reduction of the fluorescent signal, which appears as a sharp drop in signal intensity when both strands separate completely. As the T_m of a DNA duplex is dependent upon length and base composition, the number of points of inflection in the melting curve indicates the number of PCR products (including the primer-dimers) generated. An acceptable dissociation curve has a single peak (i.e. one PCR product), while the occurrence of multiple peaks indicates the presence of non-specific amplification products.

5.2. Detection of qPCR products by using TaqMan probes

Another possibility to detect amplification products is use of dual-labeled hydrolysis probes, known as TaqMan probes. TaqMan probes, which are designed to hybridize to an internal stretch of the amplicon, contain a fluorescent reporter and quencher upon adjacent nucleotides. The close proximity of the fluorescent reporter to its quencher molecule prevents the emission of fluorescence. Taq polymerase extends the outside primer and then reaches the 5′ end of the TaqMan probe, where upon the 5′ → 3′ exonuclease activity of *Taq* polymerase hydrolyses base by base the TaqMan probe, and in consequence the fluorescent probe is no longer in close proximity to the quenching group. This results in an increase of fluorescence that is proportional to the amount of PCR products generated, which allows accurate quantification of the amplified target. Importantly, even if within the reaction. Primer-dimers or other non-specific amplification products form, they will not generate any fluorescent signal (please note that primer-dimers and non-specific amplification products will have a negative impact on both the efficiency and the sensitivity of the qPCR assay).

6. Step 4; selection of reference genes and data normalization

Comparison of miRNA expression between different samples requires standardization and normalization of whole genome approaches and of qPCR analyses. As with mRNA analysis, the reliability, reproducibility and interpretation of miRNA experiments can be improved by including reference genes (RGs) in the assay. The estimation of the amount of RGs across samples is useful to correct for sample-to-sample variation. Ideally, the identification of RG-miRNAs, analogous to Glyceraldehyde-3-phosphate-dehydrogenase dehydrogenase (GAPDH), β-Actin (ACTB) or α-Tubulin (TUBA1) that are used in expression analysis, would be useful to normalize qPCR data for miRNA expression. Unfortunately, to date, no RG-miRNAs have been identified, and in consequence, the selection of RGs to normalize miRNA levels is still rather empirical. For example, in our experimental qPCR set up, we include three randomly selected small nucleolar RNAs (snoRNAs) as RGs. Where possible, we also include one or two invariant miRNAs (i.e. miRNAs with constant expression across the samples) as endogenous controls following RGs normalization.

Table 1A
Summary of the miRNA specific reverse transcription-based methods described in this review.

	Method	Substrate	Primer type	*Tm* adjustment	Detection	References
1	TaqMan	Total RNA	Stem-loop	No	Dual-labeled hydrolysis probes	[32]
2	Exiqon GSP	Total RNA	Linear (LNA)	Yes	SYBR Green I	[19]
3	Ambion	Total RNA	Linear (DNA)	No	SYBR Green I	(This assay has been discontinued by Ambion)

Table 1B
Summary of the universal reverse transcription-based methods described in this review

	Method	Substrate	Elongating enzyme	Primer type	*Tm* adjustment	Detection	References
1	Exiqon universal	Total RNA	PAP	Linear (LNA)	Yes	SYBR Green I	(http://www.exiqon.com)
2	Ambion	Total RNA	PAP	Linear (DNA)	No	SYBR Green I	[20]
3	miQPCR	Total RNA	T4 RNA ligase	Linear (DNA)	Yes	SYBR Green I	(Manuscript in preparation)
4	TLDA	Total RNA	NA	Stem-loop	No	Dual-labeled hydrolysis probes	(http://www.appliedbiosystems.com
5	TaqMan pools	Total RNA	NA	Stem-loop	No	Dual-labeled hydrolysis probes	[45,46]

Once the qPCR run is completed, the relative quantity of each miRNA is estimated by the ΔΔCq method [41]. Dedicated qPCR software, such as Relative Expression Software Tool (REST; [42]) or qBASE [43] is used to achieve these measurements.

Recently, Vandesompele and coworkers have described an alternative approach to normalize qPCR experiments. They circumvent the difficulty in identifying reliable RGs by using the mean miRNA expression value as normalization method [44]. Although this method generates stable results, which performs better than using snoRNAs for normalization, this approach is applicable only when a large enough number miRNAs is analyzed.

7. Special applications; multiplexing stem-loop primers

The major advantages of qPCR over microarrays are (1) the speed and the sensitivity of the qPCR assays, (2) considerably larger dynamic range compared to microarray analysis and (3) a convenient requirement for low amounts of starting material (in the range of nanograms of total RNA). Currently, over 900 mature miRNAs have been identified in the human genome (miRBase v14, September 2009 [34]) and consequently the analysis of known miRNAs expression using qPCR is both time and reagent consuming. However, two different approaches to perform parallel reverse transcription for a large number of miRNAs contained in a single sample have been reported recently. In the first approach, described by Thang and collaborators [45,46] a mixture of 220 individual stem-loop primers is used to multiplex the reverse transcription step. The resulting cDNA is first pre-amplified using low amounts of qPCR primers (pre-PCR; this step is also carried out in a single tube). Subsequently, the pre-PCR amplification product is diluted and distributed into 96 or 384 well plates for qPCR using individual MSPs, universal primers and TaqMan probes.

TaqMan Low density microRNA Array card (TLDA, see Link) is another approach to increase number of miRNAs analyzed on one plate. TLDAs are customized 384-well micro fluidic cards that used to determine the expression of multiple miRNAs by using qPCR. Each individual well of a TLDA contains dried primers and TaqMan probes to amplify a single miRNA. Predefined pools of reverse transcription primers are used to synthesize cDNAs (i.e. Megaplex RT Primers, AB). Following this, the cDNAs are spun into the micro fluidic card where amplification of individual miRNA will take place. This method achieves an easy and convenient way to screen in parallel a large number of miRNAs by using qPCR.

8. Conclusions

What can be learnt from the analysis of miRNA expression profiles? The alteration of miRNA expression profiles in disease or correlation of miRNA expression profiles with clinical parameters like disease progression or therapy response can serve as clinically relevant biomarkers [14,15]. In addition, differential miRNA expression in cellular processes such as differentiation, proliferation or apoptosis is an important parameter that may allow the determination of disease associated genes that are specifically regulated by miRNAs. However, in most cases miRNA target genes (i.e. protein coding genes) remain unknown, hence determination of changes in miRNA expression remains a starting point in the characterization of physiological and pathological processes. These results require to be complemented with prediction of miRNA target genes followed by the functional validation of putative targets. In this way, meaningful diagnostic and prognostic conclusions, in addition to the provision of new clinically relevant targets will derive from the accurate quantification and normalization of miRNA expression levels.

The methods presented in this review are summarized in Table 1A (miRNA-specific primers) and Table 1B (universal reverse transcription).

Conflict of interest

VB and MC are co-applicants of the miQPCR patent applications EP 09 002 587.5 "miQPCR, A novel approach for expression profiling of mature microRNAs" submitted to the European Patent Office on February 2009.

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