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Notes & Tips

A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods

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Before generating cDNA in a molecular biology lab, the researcher is confronted with choosing the appropriate priming method for the previously isolated RNA. The most common methods utilize the poly(A) tail of the mRNA either by priming with an oligo(dT) primer or by using random hexamers that statistically bind to the mRNA pool. Another method uses gene-specific primers, but this shall not be discussed here, as the resulting cDNA is limited to one transcript. To obtain a good representation of sequences at the 3′ end of the transcripts, it is desirable for the priming to be as far toward the 3′ end as possible, for instance by using an oligo(dT) primer to the poly(A) tail of eukaryotic mRNA. On the other hand it is a known and common problem for this method to get a good representation of transcript regions that are upstream at the 5′ end of the transcripts [1]. Secondary structures of mRNA can often cause difficulties for the reverse transcriptase, causing the enzyme to stall and end its synthesis well ahead of the 5′ end [2]. The benefit of random hexamers is a cDNA synthesis occurring along the entire length of the transcripts, avoiding possible secondary structures such as loops and stems and resulting in a more even representation of the whole mRNA sequence.

Here we describe the effect of these two different cDNA priming methods on the transcript variety and length, when used individually or in combination, by evaluating the cDNA synthesis efficiency using a quantitative real-time PCR approach.

Material and methods

RNA preparation. Total RNA was prepared from 15 mg of mouse thymus tissue using the RNeasy Mini Kit (Qiagen, Germany) according to the supplier's protocol. RNA quantity was determined by UV spectrophotometry (Ultrospec 3000; APBiotech, Germany) in 10 mM phosphate buffer. RNA quality was accepted with an OD 260/280 ratio of 2.2. RNA integrity was determined by denaturing agarose gel electrophoresis according to [3]. Visual assessment of 28S and 18S rRNA comparative brightness (2:1) was performed after gel imaging (Imago, B&L Systems, Netherlands).

cDNA synthesis. For cDNA synthesis, 5 μg of total RNA and either 1 μl of oligo(dT) primer (500 ng/μl, Invitrogen), 1 μl of random hexamers (500 ng/μl, Promega), or a combination of both (500 ng/μl each) were brought to a final volume of 11 μl with RNase-free water. The RNA was denatured at 70 °C for 10 min and cooled to room temperature for 5 min. Then 9 μl of a cDNA synthesis mastermix (2 μl dithiothritol (0.1 M; Invitrogen), 1 μl dNTPs (10 mM; Invitrogen), 4 μl 5× first-strand buffer (Invitrogen), 1 μl RNasin (40 U/μl; Promega), and 1 μl SuperScript II reverse transcriptase (200 U/μl; Invitrogen) were added. The reactions were incubated at 42 °C for 1 h and heated to 70 °C for 15 min to inactivate the enzyme. The cDNAs were diluted with 80 μl of RNAse-free water prior to real-time PCR.

Standard control PCR. A control PCR to check for the presence of genomic DNA contamination was conducted using a Biometra T-personal Cycler (Biometra, Göttingen). Primers (mHPRT-sense, cctgctggattacattaaagc; mHPRT-antisense, gtcaagggcatatccaacaac) were designed to span across two introns of the mouse hypoxanthine guanine phosphoribosyl transferase (HPRT)[1] gene, giving PCR products that are 180 bp for

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[1] *Abbreviations used:* HPRT, hypoxanthine guanine phosphoribosyl transferase; RT, reverse transcription

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the cDNA product and 1.1 kb for the genomic DNA product.

PCRs contained 2 μl of diluted cDNA, 5 μl of 10× PCR Buffer (Takara, Japan), 1 μl 10 mM dNTPs (Takara), 1 U ExTaq DNA polymerase (Takara), and 1 μl of each gene-specific primer (10 pmol/μl, MWG Biotech, Germany) and were brought to a final reaction volume of 50 μl with PCR-grade water. Reactions were incubated at 95 °C for 5 min initial denaturation and then cycled at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min for a total of 35 cycles followed by a final extension of 5 min at 72 °C.

Real-time PCR. Real-time PCR was conducted using a LightCycler instrument (Roche, Switzerland) and quantification was accomplished with the accompanying software package.

Real-time PCRs were composed of the following reagents: 10 μl QuantiTect Mastermix (Qiagen), 1 μl (10 pM/μl) gene-specific primers, 6 μl of RNase free-water (Qiagen), and 2 μl of template cDNA. Gene-specific primers used were as follows: primer combination A, mouse ribosomal protein S27, 415 nt from poly(A), PCR product size 296 bp, T_m 62 °C, sense, ccaggataaggaaggaattcctcc, antisense, ccagcaccacattcatcagaagg; primer combination B, mouse dystrophin, 4006 nt from poly(A), PCR product size 401 bp, T_m 55 °C, sense, gccctactatatcaaccacgagac, antisense, tgatgccagttttaaaagacagga; and primer combination C, mouse dystrophin, 12053 nt from poly(A), PCR product size 475 bp, T_m 60 °C, sense, gcttctttctgccgaggatacatt, antisense, gtgagtg agcgagttgaccctgac.

An initial denaturation was conducted for 20 min at 95 °C to activate the enzyme. Forty cycles of amplification were performed with a denaturation at 95 °C for 30 s, annealing at primer-specific temperatures for 20 s, and elongation at 72 °C for 25 s, followed by a fluorescent data acquisition for 5 s at 75°C for combination C, 80 °C for combination B, and 77 °C for combination A. Following the cycling program, a melting curve was performed by cooling to 40 °C for 2 min and then increasing the temperature to 95 °C with a slope of 0.1 °C/s while measuring the fluorescence continuously. The melting peak was obtained by plotting the negative first derivate of fluorescence against temperature. The threshold cycle in which the fluorescence rises significantly above background level was determined by a second derivate maximum method with the use of the LightCycler quantification software. Fold differences were calculated by a mathematical model described by Pfaffl [4].

In addition to the verification of a single PCR product by the presence of only one melting peak, the PCR cocktail was spun out of the glass capillaries (2000*g*, 1 min) and resolved by electrophoresis on a 1.3% agarose/TAE gel. Gels were imaged with the Imago System (B&L Systems).

Results and discussion

In this study we tested the differences in cDNA synthesis efficiency dependant on the priming method using a quantitative real-time PCR approach.

The PCR with mHPRT control primers and subsequent agarose gel electrophoresis revealed a single band of 180 bp, indicating good quality and absence of genomic contamination in the template cDNA (data not shown). A positive control with genomic DNA and deliberately contaminated cDNA was included. Whereas the genomic DNA revealed a single band of 1.1 kb size, the contaminated cDNA revealed two bands of 180 bp and 1.1 kb (data not shown).

All quantification experiments were conducted with four independently synthesized cDNAs per group, measured in duplicates for statistical precision. Samples from the three priming groups were subjected to real-time PCR simultaneously (with the same PCR primer combination) to reduce interassay variation. Fig. 1 illustrates the course of the real-time PCR for the specified primer combinations and cDNA used for calculating the cDNA synthesis efficiencies. The slope of the amplification curve represents the efficiency of the reaction. Amplification of oligo(dT)/hex-mixed cDNA with primer combination A resulted in more end product (approximately 0.6-fold) than amplification of the other cDNAs with the same primer combination. In the other amplifications, the total amount of end product did not differ so strongly, as can be seen by the amount of total fluorescence when reaching saturation. Nevertheless, the increased end product may be explained by a generally higher PCR efficiency for this cDNA and primer combination. In addition to the melting curves (data not shown) to demonstrate the resulting fluorescence of the real-time PCR due to the presence of a single PCR product, the PCRs were resolved by agarose gel electrophoresis (Fig. 1, inset).

Fig. 2 shows the average cycle number at which the cDNA level is determined (C_T value), depending on priming type and PCR primer location. For PCR primers located close (415 bp) to the poly(A) tail, the most efficient methods were oligo(dT) (16.72 ± 0.1)- and oligo(dT)/hex (17.14 ± 0.28)-primed cDNA versus hexamer-primed cDNA (21.61 ± 0.06). Taking the PCR efficiency into account (as calculated from the slope of the amplification curve), this is a 20.7-fold increase in detection sensitivity for the oligo(dT)-primed over that of the hexamer-primed cDNA and 22-fold for the mixed oligo(dT)/hex-primed over the hexamer-primed cDNA. The discrepancy of the oligo(dT)/hex mix-primed cDNA having a higher C_T value but also a higher x-fold sensitivity over the hexamer-primed cDNA is explainable by the efficiency of the PCR.

For primers situated further upstream from the poly(A) tail (4006 bp), the superiority of the oligo(dT)

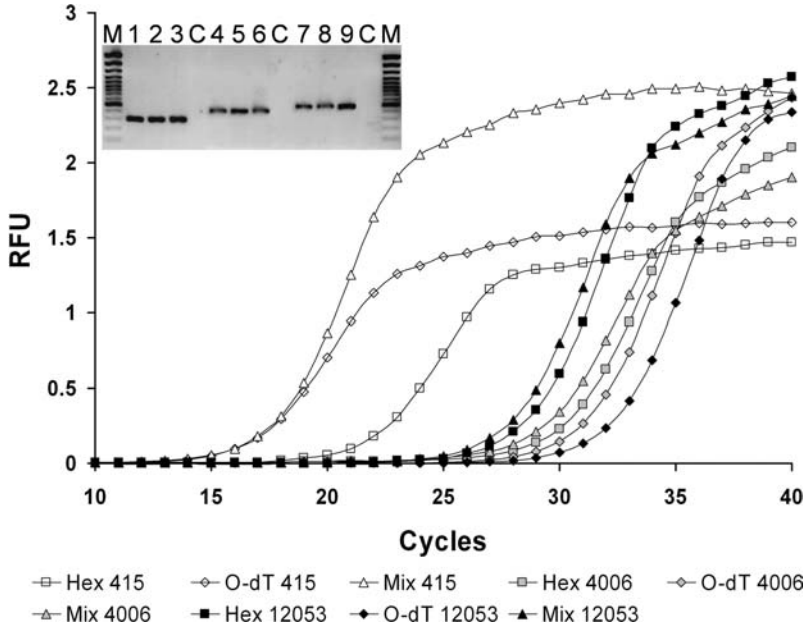

Fig. 1. Real-time PCR amplification curves of cDNAs reverse-transcribed by different methods. The three different groups of cDNA were subjected to 40 cycles of amplification with the respective primer combinations in a LightCycler. The primer locations from the poly(A) tail are 415 bp (transparent icons), 4006 bp (gray icons) and 12053 bp (black icons). Oligo(dT)-primed cDNA abbreviated as O-dT, hexamer primed as Hex, oligo(dT)/hexamer mix primed as Mix. The depicted amplification curves were the basis for data evaluation seen in Fig. 2. (Inset) Evaluation of single PCR products by agarose gel electrophoresis. The letter C indicates a control for each primer pair, without cDNA. Lanes 1–3, primer combination A, 293-bp PCR product (oligo(dT); oligo(dT)/hex mixed; hexamer); lanes 4–6, primer combination B, 401-bp PCR product (oligo(dT); oligo(dT)/hex mixed; hexamer); lanes 7–9, primer combination C, 475-bp PCR product (oligo(dT); oligo(dT)/hex mixed; hexamer). A 100-bp DNA size marker is loaded on the far left and far right sides of the gel.

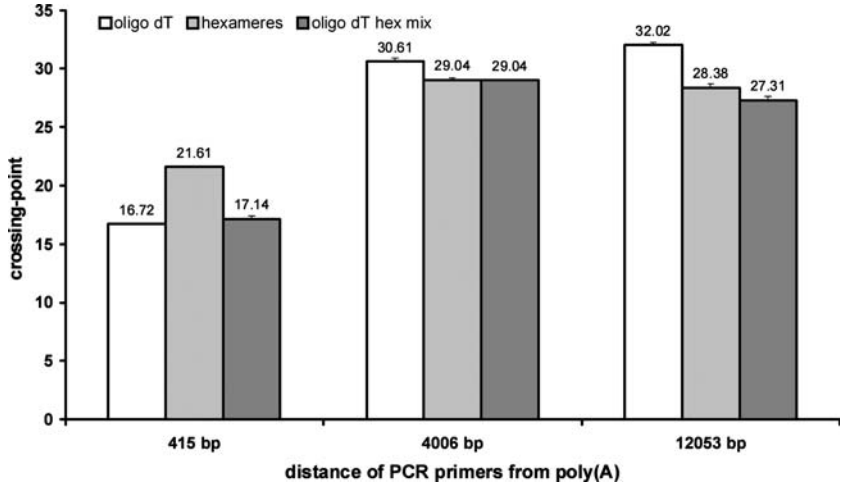

Fig. 2. Average cycle number at which the cDNA level is determined (C_T value), depending on priming type and PCR primer location. C_T values are shown for the three different priming methods in combination with the three different locations of the primers from the poly(A) priming site. A smaller C_T value means more PCR product, i.e., more efficiently synthesized cDNA.

priming method over the other methods decreases. Both the hexamer- and the mixed oligo(dT)/hex-primed cDNA yielded 1.57 ± 0.02 (hexamer) and 1.57 ± 0.03 (oligo(dT)/hex mix) cycles lower C_T values. Interestingly this does not agree with an increased sensitivity if we take the PCR efficiency of the reactions into account. As the efficiency of the hexamer-primed cDNA is significantly lower than that in the other methods (2.38-fold increased PCR sensitivity compared to that of the hexamer-primed cDNA), there is no positive effect.

PCRs conducted with primers located 12 kb away from the poly(A) tail were most efficient for the cDNA with combined oligo(dT)/hex priming (27.31 ± 0.33 cycles) compared to the relatively similar hexamer-primed

cDNA (28.38 ± 0.34 cycles) and the far less efficient oligo(dT)-primed cDNA (32.02 ± 0.21 cycles). Here the C_T values and the calculated x-fold increase in PCR sensitivity are in good accordance, leading to the largest increase in sensitivity for the mixed oligo(dT)/hex-primed cDNA with 12.5-fold, followed by hexamer-primed cDNA with 10.9-fold, compared to oligo(dT)-primed cDNA. The advantage of oligo(dT)/hex and hexamer over oligo(dT)-primed cDNA synthesis is obvious for transcripts of this length.

By assessing the average C_T value for the oligo(dT)-primed group separately it is evident that this value increases with the distance of PCR primer pairs from the poly(A) tail. It would be expected that the amount of amplifiable PCR target would decrease with increasing RT/PCR primer spacing toward the 5′ end of the cDNA, as the amount of secondary structure leading to a stalled cDNA synthesis increases with synthesis length.

It must be mentioned at this point though that oligo(dT) priming can also lead to truncated cDNAs through internal priming in adenine-rich regions, which seems to occur relative frequently, as a large amount of data in the EST databases seems to originate from internally primed cDNA sequences [5]. This effect can be reduced by using anchored oligo(dT) primers [6], ensuring that the 5′ end of the primer anneals to bases other than arginines. It is not sensible to scale up the amount of RT primers, as increasing the concentration of oligo(dT) primers results in an increase of internally primed cDNAs [5]. In addition, increasing the amount of hexamer primers shifts the equilibrium of the first-strand reaction to products of <500 bp length (Fermentas product guide, Kit K1611).

Using hexamers in combination with oligo(dT) primers in RT has already been demonstrated to be suitable for the detection of rare transcripts by PCR compared to specific priming [7]. However, the target mRNA copy number transcripts may be overestimated by using random hexamers compared to gene-specific RT priming [8], although the severeness of this effect in oligo(dT)/hexamer mix-primed cDNA was not assessed in that study. By normalizing samples to an internal standard, this overestimation should not be of great concern, as the unspecific priming and the resulting alteration of the real expression occurs in all transcripts and is counterbalanced after normalization.

We observed a strong bias of reverse transcription efficiency that depends on the priming method. Generally it is not possible to suggest the one over the other method, as this must be considered for each experiment separately. The type of priming should be determined by the distance of the PCR primers used in the experiment from the poly(A) tail of the mRNA (i.e., the spacing between RT and PCR primers). There are a variety of parameters that can be changed in cDNA synthesis for optimization. To obtain longer cDNA transcripts, the inclusion of betaine alone and in combination with trehalose has been shown to result in longer cDNA synthesis products [9]. It has also been demonstrated that omitting dithiothritol from the cDNA synthesis reaction results in a lower C_T value for the resulting cDNAs, i.e., increasing the cDNA quality and subsequent PCR efficiency [6]. There may also be an up to 100-fold increase in sensitivity depending on the kind of enzyme used [10].

The choice of priming seems to be crucial for best PCR results. In real-time PCR, transcripts of low expression may be difficult to quantify, so it is necessary to generate the best possible cDNA to start with, as the entire subsequent experiment depends on its quality. If only one transcript shall be quantified, a gene-specific primer may be used for cDNA synthesis to obtain higher target amounts for amplification [6,8]. To generate a cDNA suitable for most versatile real-time PCR applications, we have demonstrated that the combination of oligo(dT) primers and random hexamers is an ideal approach. The usage of gene-specific primers is not suitable for all experimental procedures, for instance DNA-array post validation, as routinely performed in our group. After electronically evaluating up to 10,000 clone signals, it is much more effective (and less costly) to prepare a cDNA from the corresponding samples that is suitable for all following real-time PCRs. A further advantage of the "one cDNA for all" method is the need to normalize it only once. After determining the quantification values for the household gene of interest for each sample, normalization can be applied to any other transcript of interest.

In conclusion, we recommend the oligo(dT)/mix-primed cDNA approach to obtain the most versatile cDNA with regard to the analysis of transcript representation. If the designed PCR primers are close enough to the 3′ end of the transcript, oligo(dT)-primed cDNA will also give sensitive results.

Acknowledgments

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