PML-RARα gene detection method optimization for quantitative PCR

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Otimização do método de detecção do gene PML-RARα para PCR quantitativo

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key words

APL

PML-RARα

Q-PCR

SYBR® Green

abstract

Hybrid gene PML-RARα is the molecular target found in most cases of acute promyelocytic leukemia (APL) and has been used for diagnosis and minimal residual disease studies. The standard molecular technique employed is qualitative reverse transcriptase-polymerase chain reaction (RT-PCR), but with the emergence of real time PCR (Q-PCR), PML-RARα gene detection approaches have been described allowing transcript detection, with the methodological advantage of eliminating post-PCR processing. However, current protocols report the use of expensive fluorescent labeled probes, limiting its routine application in the laboratory. The objective of this study was to optimize PML-RARα gene detection method for Q-PCR, using SYBR® Green fluorescent dye. The analysis was performed with NB4 cellular lineage cDNA. Thermal cycling protocols, cDNA synthesis with random or specific *primer* and different $MgCl_2$ and amplification *primers* concentrations were tested. Results show that amplification improved in the following conditions: 2 mM $MgCl_2$, 10 pmol *primers* and cDNA synthesized with specific primer. There were no significant differences using annealing temperature (58°C/30 s) followed by extension (72°C/30 s) or annealing associated with extension as a single step (60°C/45 s). This paper demonstrates the optimization of PML-RARα gene detection for Q-PCR studies using a technique considered sensitive and less expensive for routine use in the laboratory.

resumo

O gene híbrido PML-RARα é o marcador molecular presente na maioria dos casos de leucemia aguda promielocítica (LAP), sendo útil ao diagnóstico e ao estudo da doença residual mínima. A técnica molecular empregada como rotina laboratorial é a reação em cadeia da polimerase com transcrição reversa (RT-PCR) qualitativa, porém com o surgimento da PCR em tempo real (Q-PCR), foram descritas abordagens de detecção do gene PML-RARα possibilitando a quantificação de transcritos, com a vantagem metodológica da eliminação do processamento pós-PCR. No entanto, os protocolos relatam o uso de sondas fluorescentes de custo elevado para a rotina clínica, limitando sua aplicação. Este estudo teve como objetivo otimizar o método de detecção do gene PML-RARα para Q-PCR, utilizando como sistema de marcação fluorescente o intercalante SYBR® Green. A análise foi realizada com cDNA da linhagem celular NB4, tendo sido testados protocolos de termociclagem, síntese de cDNA com primer *randômico ou específico e diferentes concentrações de* $MgCl_2$ *e* primers *para amplificação. Os resultados mostraram amplificação mais eficiente nas seguintes condições: 2 mM* $MgCl_2$*, 10 pmol de* primers *e cDNA sintetizado com primer específico. Não houve diferença na utilização de etapas para anelamento (58°C/30 s) seguido de extensão (72°C/30 s) ou etapa única de anelamento associado à extensão (60°C/45 s). Esses resultados demonstram a otimização da detecção do gene PML-RARα para Q-PCR através de um método considerado sensível e de baixo custo para a rotina laboratorial.*

unitermos

LAP

PML-RARα

Q-PCR

SYBR® Green

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Introduction

Acute promyelocytic leukemia (APL) is characterized by rearrangements that include retinoic acid receptor alpha (RARα) in chromosome 17[9]. In almost 98% of APL cases the t(15;17)(q22;q12) is detected, involving the promyelocytic leukemia (PML) gene in chromosome 15, and generates the hybrid gene PML-RARα, the molecular target of APL[16, 12, 14].

PML-RARα fusion protein has a negative dominant function in the retinoic pathway; it is important in myeloid differentiation arrest and apoptosis inhibition, and is also involved in APL pathogenesis[8].

APL cellular lineage used as a model for *in vitro* studies is called NB4. It was isolated from long-term leukemic blast cells culture from a patient in disease relapse[11]. NB4 validation as APL cellular lineage was established by the presence of t(15;17), treatment response to all-trans retinoic acid and PML-RARα gene detection[11].

Qualitative PCR has been used for the investigation of gene rearrangements at diagnosis and for minimal residual disease studies[2, 19], but the authors have published different protocols for the quantification of PML-RARα transcripts[21, 4, 20, 6, 7, 18], employing quantitative PCR (Q-PCR) technique. Taken together, these papers have demonstrated that transcription levels can be evaluated by Q-PCR and can differentiate patients with good or poor prognosis. Nevertheless, up to now quantitative detection methods for APL have been based on expensive fluorescent labeled probes. The purpose of this study was to optimize PML-RARα gene detection method for Q-PCR, using the SYBR® Green fluorescent dye.

Materials and methods

RNA from NB4 cell lineage was isolated by the phenol-guanidine technique[5] – Trizol® (Invitrogen, Carlsbad, CA, USA). RNA analysis was done by spectrophotometry and electrophoresis in 1.5% ethidium bromide-stained agarose gels. cDNA synthesis was performed with 1μg of RNA through reverse transcription (RT) reaction using reverse transcriptase enzyme (Gibco-BRL, Grand Island, NY, USA/Promega, Madison, WI, USA), random (New England BioLabs, Beverly, MA, USA) and specific primers, dNTP's (Pharmacia, Piscataway, NJ, USA), water and Tris-HCl/KCl buffer. The specific primer used for RT reaction was described by van Dongen *et al.*[19]. After a five-minute incubation at room temperature RT conditions were: 42°C/60 min. and 70°C/15 min.

Amplification was done by the Q-PCR method[10] using the Smart Cycler® thermocycler (Cepheid, Sunnyvale, CA, USA). Reagents used were Taq DNA Polymerase diluted in Tris-HCl/KCl buffer with $MgCl_2$ (VJR, São Paulo, SP, Brazil); 10 mM dNTP's (Pharmacia, Piscataway, NJ, USA); SYBR® green (Molecular Probes, Eugene, OR, USA); cDNA and Milli-Q water treated with DEPC (diethylpyrocarbonate) added to a 25 μL final volume. This Milli-Q water was also used as contamination control. PML-RARα primers used were also described by van Dongen *et al.*[19]. The products were detected through amplification curves constructed during the reaction with the threshold set to 30. The specificity of amplified products was verified through melting curve analysis, constructed between 60°C and 95°C and with a rampage of 0.2°C/s.

Results and discussion

Two amplification protocols were tested (**Table**) with different $MgCl_2$ concentrations (2 and 3 mM) and specific primers (5, 10, 15 and 20 pmols). The first assay was tested in duplicate with different $MgCl_2$ concentrations (2 and 3 mM) and primers (5 and 10 pmols). The reactions with $MgCl_2$ 3mM had a zero cycle threshold (Ct), which means that the fluorescent signal was not sufficient to reach the threshold. With the $MgCl_2$ 2 mM concentration both thermocycling protocols were efficient (Cts of 29.46 and 32.2), but smaller Ct values were detected with 10 pmol primers.

Based on these results, a second assay was performed with $MgCl_2$ 2 mM concentration and increased primer concentrations of 15 and 20 pmols with both protocols of amplification. In this assay, Ct values had no significant difference from the latter assay. Primers and $MgCl_2$ were the reagents in which the concentration was analyzed. $MgCl_2$ concentration affects many aspects of PCR, including Taq DNA polymerase activity and primer annealing. The PCR

Table Steps and amplification protocols used in optimization assays

Steps	Protocol I	Protocol II
Initial denaturation	95°C/120 s	95°C/120 s
Denaturation*	94°C/15 s	94°C/15 s
Annealing*	58°C/30 s	-
Extension*	72°C/30 s	-
Annealing + extension*	-	60°C/45 s

**Steps were repeated 40 times in both protocols.*

enzyme used here (VJR, São Paulo, SP, Brazil) demonstrated high specificity with $MgCl_2$ 2 mM final concentration. Ideal primer concentration varies according to its sequence and amplification target. Results demonstrated no significant differences between 10, 15 and 20 pmols. Therefore, 10 pmol was selected because high primer concentrations have a limited effect on PCR and can affect the specificity of the reaction.

In the third assay, from the parameters established, the amplification efficiency from cDNA synthesized with random (0.2 μg/μL) and specific primers (5 pmols) was compared. Results demonstrated more efficient amplification from cDNA synthesized with specific primer (Ct of 24.9) than with random primers (Ct of 28.07). However, considering the relative efficiency of random primers, their use has the advantage of amplifying many genes with a single transcription reaction.

The variables selected in this study are the most important ones for an efficient Q-PCR reaction[13, 17, 15]. Reverse transcription is an important step in Q-PCR. The cDNA synthesized must be representative of the sample transcripts pool[1]. Amplicons were 381 base pair length, and annealing temperatures, 59°C and 57°C for forward and reverse primers, respectively. At first, the two amplification protocols showed similar efficiency, analyzed from Ct values, with Cts of 33.74 and 33.87.

SYBR® Green is a sensitive but non-specific dye. It intervenes in double strand molecules and because of this property its specificity can be compromised by the formation of primer dimers or secondary structures in PCR products[3]. Thus, the specificity of the amplification reaction should be analyzed through melting curves. In this study, NB4 cellular lineage had 92°C as standard melting temperature, ensuring the specificity of amplification with SYBR® Green.

In conclusion, PML-RARα gene detection method was optimized in the following conditions: 2mM $MgCl_2$, 10 pmol primers and cDNA synthesized with specific primer. The use of SYBR® Green as fluorescent dye allowed a sensitive and less expensive laboratory analysis for diagnosis and minimal residual disease studies in APL patients.

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