Simultaneous Detection of Multiple Transcription Factors in Hematopoietic Progenitors Using iCycler iQ™ Multiplex Real-Time PCR

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Introduction

The development of committed B cells from multipotent progenitors requires the coordinated activity of several transcription factors (TF). Not only is the temporal pattern of expression of TFs important, but also a number of studies have confirmed that the level of expression, as well, is important in the cell fate decisions of the developing progenitors (Kee and Murre 2001). Semi-quantitative analysis of TF gene expression by traditional reverse transcription polymerase chain reaction (RT-PCR) methods, while widely used, often lacks sufficient sensitivity to discern small differences in mRNA levels. The introduction of real-time RT-PCR provided a method for quantitatively measuring small differences in mRNA expression (Boeckman et al. 2001). However, particularly with small samples, the ability to assess the relative levels of multiple TFs is limited.

Multiplex real-time PCR, which can measure up to four target cDNAs simultaneously in a single tube, provides a method for comparing relative TF gene expression levels based on one sample of mRNA. Currently, a number of different fluorophores are available for real-time PCR, and color multiplexing is possible with oligonucleotide probes labeled with different fluorophores. Here we describe the development of a multiplex four-color real-time PCR method to detect the TFs EBF, E2A, and PU.1 using the iCycler iQ system.

Methods

cDNA Preparation

IgM$^-$B220$^+$ cells and Mac-1$^+$ cells were harvested from bone marrow and spleens of 8-week-old male C57Bl/6J mice by magnetic cell sorting with indirect MicroBeads (Miltenyi Biotec, Auburn, CA). RNA was prepared using an RNA extraction kit (QIAGEN, Valencia, CA), and cDNA was then generated using the ThermoScript RT-PCR kit (Invitrogen Life Technologies, Rockville, Maryland), both according to the manufacturers' instructions.

Multiplex Real-Time PCR

External well factors were collected before each run using iCycler iQ external well factor solution (Bio-Rad). Collection of well factor data optimizes fluorescent data quality and analysis in multiplex PCR.

Reactions for multiplex real-time RT-PCR contained primers and probes for the three TF cDNAs, as well as for GAPDH cDNA, which served as an internal standard for calculation of relative expression levels of the TFs. Reactions were prepared for each cDNA sample as follows:

12.5 µl iQ™ supermix (Bio-Rad)

1.0 µl 50 mM $MgCl_2$

0.5 µl 5 U/µl iTaq™ DNA polymerase (Bio-Rad)

2.5 µl 10 mM dNTP mix

2 µl cDNA template

1 µl PCR-grade ddH_2O

0.5 µl 10 µM GAPDH forward primer (5'-CCCCAATGTGTCCGTCGTG-3')

0.5 µl 10 µM GAPDH reverse primer (5'-GCCTGCTTCACCACCTTCT-3')

0.25 µl 10 µM GAPDH probe (5'-HEX-CGTGCCGCCTGGAGAAACCTGC-BHQ-1-3')

0.5 µl 10 µM EBF forward primer (5'-CTTGCTAACACTTCGGTCCAT-3')

0.5 µl 10 µM EBF reverse primer (5'-ACCTTGATTGGTGGCTTGTG-3')

0.25 µl 10 µM EBF probe (5'-6-FAM-TGCCTCCGAGACATTCACAGCCAG-BHQ-1-3')

0.5 µl 10 µM E2A forward primer (5'-CCCGGATCACTCCAGCAATAA-3')

0.5 µl 10 µM E2A reverse primer (5'-TGGAGACCTGCATCGTAGTTG-3')

0.5 µl 10 µM E2A probe (5'-BHQ-3-TCTCACCTAGCCCCTCAACGCCT-Cy5-3')

0.5 µl 10 µM PU.1 forward primer (5'-GGGCATCCAGAAGGGCAA-3')

0.5 µl 10 µM PU.1 reverse primer (5'-GGTAGGTGAGCTTCTTCTTGAC-3')

0.5 µl 10 µM PU.1 probe (5'-Texas Red-TCTTCACCTCGCCTGTCTTGCCGT-BHQ-2-3')

Duplicate 25 µl samples of each reaction were added to individual wells of a 96-well thin-wall PCR plate. PCR conditions were 3 min at 95°C followed by 60 cycles of 10 sec at 95°C and 60 sec at 55°C.

Results

Expression of three TFs (EBF, E2A, PU.1) in IgM$^-$B220$^+$ and Mac-1$^+$ cells from both mouse bone marrow and spleen (see figure) was detected using multiplex real-time PCR. The

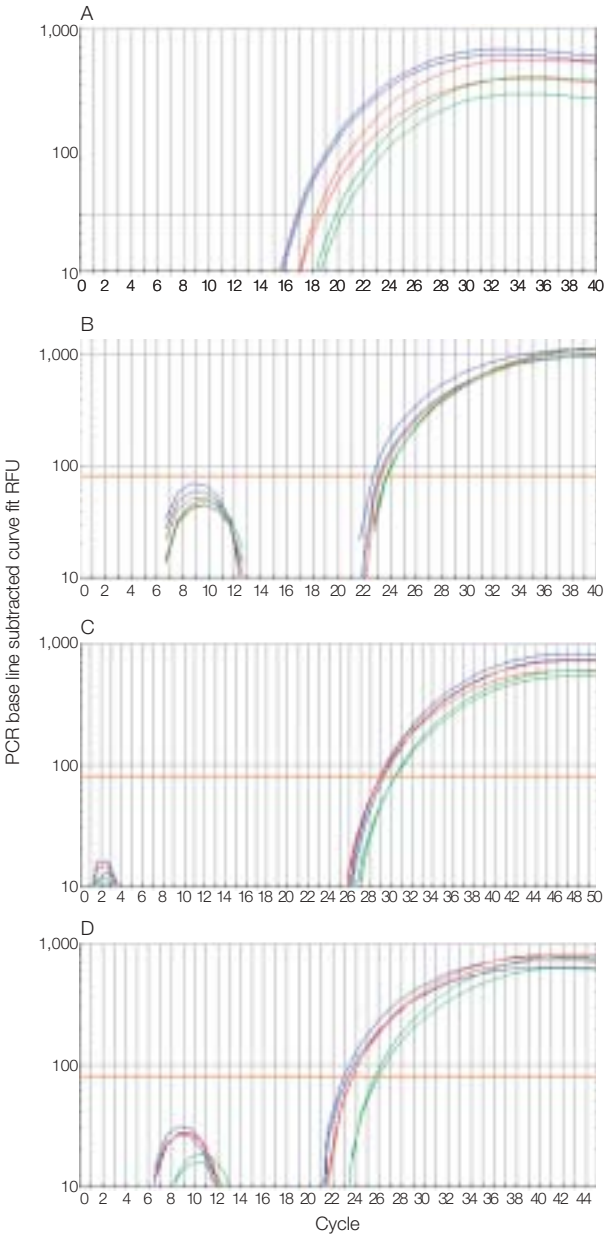

Figure. Multiplex real-time PCR of TF cDNAs. A, GAPDH; B, EBF; C, E2A; D, PU.1. Blue, red, and green traces represent cDNA derived from bone marrow IgM$^-$B220$^+$ cells, bone marrow Mac-1$^+$ cells, and spleen IgM$^-$B220$^+$ cells, respectively. Threshold and C_T values were automatically calculated for each sample. TF probes were titrated and standard curves were generated. The slopes and corresponding efficiency for these reactions were –3.51 (93%), –3.35 (98%), –3.22 (~100%), and –3.50 (93%) for GAPDH, EBF, E2A, and PU.1 cDNA, respectively.

relative amount of each TF cDNA was calculated by determining its ΔC_T value: ΔC_T = (threshold cycle for the TF) – (threshold cycle for GAPDH); i.e., the number of additional cycles required for a cDNA to reach the threshold value after the GAPDH threshold cycle. A ΔC_T value of 1.0 represents a 2-fold lower expression relative to GAPDH. The table shows the ΔC_T value of TFs in each group of cells. The results of these analyses show that multiplex PCR can be used to determine the relative expression of multiple genes in a single sample.

Table. ΔC_T value of TFs in different groups of cells.

Sample	EBF	E2A	PU.1
Bone marrow IgM$^-$B220$^+$ cells	7.1	13.4	7.4
Bone marrow Mac-1$^+$ cells	6.4	11.9	6.6
Spleen IgM$^-$B220$^+$ cells	5.2	11.8	7.3

Discussion

The data in the figure show that the iCycler iQ system can be used to quantitate cDNAs simultaneously even though their relative expression levels differ by as much as 2^8, as indicated by the difference between the highest and lowest ΔC_T values shown in the table. Points that are critical for successful analysis of multiple cDNAs include: 1) increasing the concentration of DNA polymerase, dNTPs, and $MgCl_2$; 2) adjusting the primer/probe concentrations in order to yield C_T values of the target genes that are not significantly different from the value calculated in a single-gene real-time PCR reaction (data not shown); 3) testing primer sets with SYBR Green I to generate a melting curve to detect primer-dimers before use in multiplex real-time PCR; 4) titrating the probe and generating a standard curve to ensure that the efficiency of the reaction with the probe is >90%.

References

Boeckman F et al., Real-time multiplex PCR from genomic DNA using the iCycler iQ detection system, Bio-Rad bulletin 2679 (2001)

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Supported in part by NIAA grant AA09876.

Information in this tech note was current as of the date of writing (2002) and not necessarily the date this version (Rev A, 2003) was printed.

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