Design of Clone-Specific Quantitation Standards for Competitive PCR


The polymerase chain reaction (PCR) is an exquisitely sensitive technique, which can be used in semiquantitative assays to determine the relative concentrations of target and reference sequences (9). Quantitation in acute lymphoblastic leukemia (ALL) is of the utmost importance for the evaluation of minimal residual disease and prognosis (2,10). To develop quantitative PCR assays, a number of approaches have been applied, most of which use chromosomal translocations or the somatic gene rearrangements occurring at the antigen receptor gene loci. Some systems rely on amplification of a range of dilutions of diagnostic material to produce a calibration curve (1,3,8,10). This method is reliable only if all amplifications proceed with the same efficiency. Furthermore, all data must be obtained before the reactions reach the plateau phase of product formation (6).

The most reliable quantitation is achieved by competitive PCR (cPCR), which uses internal standards with the same primer recognition sites as the target and similar internal DNA sequence (6,14). This type of reference sequence can be produced by modifying a cloned PCR target by site-directed mutagenesis so that it contains a restriction site (13). Known amounts of a modified target are included in a cPCR, and the products are digested to yield fragments of distinct sizes, the intensities of which can be compared following gel electrophoresis. By utilizing cell line T-cell receptor gene rearrangements, a range of competitor molecules sharing the same limited number of proximal and distal (V and J) segments, but with variable intermediate DNA sequences, have been prepared. These competitor molecules have then been used in cPCR to quantify residual disease (5). Another approach uses T4 DNA ligase to bind a PCR primer, which has been made double stranded by hybridization to its complementary oligonucleotide sequence, to a double-stranded DNA fragment (12). It may be possible to use these approaches for immunoglobulin heavy chain (IgH) gene consensus primer PCR, but most IgH assays now rely on clone-specific PCR. By their nature, clone-specific primers present a problem for the design of cPCR quantification standards since there are too many possible alternative sequences.

We have developed a simple yet effective strategy for the production of double-stranded quantitation standards for clone-specific PCR analysis. This methodology is especially useful for precursor-B ALL evaluation using

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Table 1. Generation of Clone-Specific Standards for Competitive PCR

(A) Steps Involved in Generating of Clone-Specific Standards for Competitive PCR

1. Digest V_H3-J_H amplified *Daudi* IgH rearrangement with *Hae*III enzyme.

2. Save J_H end for ligation to clone-specific primers for all subsequent ALL patients.

3. Amplify and sequence patient IgH rearrangement. Design clone-specific primer.

4. Make clone-specific primer double stranded by DNA polymerase.

5. Ligate double-stranded clone-specific primer to saved *Daudi* fragment.

6. Amplify above with clone-specific and JH primers, and isolate and quantify product.

For nested cPCR:

7. Make clone-specific primer double stranded by DNA polymerase.

8. Ligate double-stranded clone-specific primer to saved *Daudi* fragment.

9. Amplify above with clone-specific and JH primers, isolate and quantify product.

(B) Primer Primer Annealing Name: Sequence: Temperature:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>J_H</td>
<td>5′ ACCTGAGGAGACGGTGACCAGGGT 3’</td>
<td></td>
</tr>
<tr>
<td>V_H1</td>
<td>5′ CCTCAGTGAAAGTCTCCTGCAAGG 3’</td>
<td>60°C</td>
</tr>
<tr>
<td>V_H3</td>
<td>5′ GTGCCCTGAGACTCTCTGTGCA 3’</td>
<td>60°C</td>
</tr>
<tr>
<td>FR3</td>
<td>5′ ACACGCGC(T/G)(G/C)TGATTACTGT 3’</td>
<td>58°C</td>
</tr>
<tr>
<td>J_H2</td>
<td>5′ GACCGAGTTCCCTTGGCC 3’</td>
<td></td>
</tr>
<tr>
<td>Raji-specific (NR5')</td>
<td>5′ CTATTACTTGCGAGACAGA 3’</td>
<td>60°C</td>
</tr>
<tr>
<td>Patient 41-specific (41:5')</td>
<td>5′ TGCAAGCCGAGAAATAGCAGCT 3’</td>
<td>60°C</td>
</tr>
</tbody>
</table>

(C) Map of the PCR-amplified *Daudi* cell line IgH gene rearrangement showing the single *Hae*III restriction enzyme recognition site, the location and direction of amplification of the consensus PCR primers and the V3NR5’D3HL construct showing the orientation of a clone-specific PCR primers.

(A) Flowchart for generation of clone-specific standards for competitive PCR. (B) Primers used for PCR of Ig gene rearrangements and their annealing temperatures. Primers J_H, V_H1 and V_H3 were previously described by Deane and Norton (7), and primer FR3 was previously described by Brisco et al. (4). (C) Map of the PCR-amplified *Daudi* cell line IgH gene rearrangement showing the single *Hae*III restriction enzyme recognition site, the location and direction of amplification of the consensus PCR primers and the V3NR5’D3HL construct showing the orientation of a clone-specific PCR primers.
rearranged IgH genes, where the variability of primer target sequences would make it costly or impractical to generate competitor molecules using the methods described above. The rearranged IgH gene of the B-lineage cell line Daudi was amplified using consensus VH3 and JH primers. The restriction enzyme HaeIII was used to digest the V region of this fragment, to yield a product similar in structure and length to the target sequence, but without an internal VH primer recognition site. This DNA fragment was stored and used as a template for generating PCR-competitors for all subsequent B-lineage ALL patients. Single-stranded clone-specific PCR primers were made double stranded by DNA polymerase and were then ligated to the V end of the Daudi fragment containing the consensus JH region at its distal end. This construct therefore contains the necessary primer recognition sites for clone-specific PCR and is of similar length and structure to the clonal target sequence. This can then be PCR-amplified, isolated and stored for use in a quantitative co-amplification PCR assay, which employs only one set of amplimers.

The B-lineage lymphoblastoid cell lines Raji and Daudi were cultured at 37°C in CO2-independent medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 0.5 µg/mL fungizone, 20 IU/mL penicillin/streptomycin and 0.4 mM L-glutamine (all from Life Technologies, Paisley, Scotland, UK). DNA was isolated from 3–5 million Raji, Daudi, mononuclear cells from the bone marrow of ALL patients or blood donor peripheral blood mononuclear cells using the PuraGene™ DNA isolation kit (Gentra, Minneapolis, MN, USA), with a minor alteration to the manufacturer’s instructions. All restriction and modifying enzymes, nucleotides and buffers were from Promega (Southampton, England, UK). PCR primers (shown in Table 1) were constructed by OsweI DNA (Southampton, England, UK). The PCR was performed, in a programmable thermal cycler (MJ Research, Watertown, MA, USA), with a 30-µL reaction volume consisting of 1× reaction buffer, 1.5 mM MgCl₂, 100 µM each dNTP, 10% (vol/vol) dimethyl sulfoxide and 100 nM of the 3′ (JH) and 5′ primers. The reaction mixture was overlaid with two drops (ca. 40 µL) of liquid paraffin. Initial denaturation was at 92°C for 10 min during which 1 U of Taq DNA polymerase was added to each reaction. This was followed by 35 cycles of 1-min denaturation at 90°C and 1-min annealing at 60°C. No extension step was required for amplification of the IgH PCR fragments of length ≤350 bp. Amplification was confirmed, using 8–10 µL of the PCR volume, by electrophoresis in 5% (wt/vol) agarose gel (NuSieve®; FMC BioProducts, Risingevej, Denmark). The amplification products were excised, isolated by Wizard® PCR Prep kit and sequenced directly, using the above PCR primers and a Silver Sequence™ kit both essentially according to manufacturer’s instructions (Promega). The sequence information was used to design clone-specific primers corresponding to the highly variable CDRIII region (Table 1). These were synthesized by OsweI DNA. The VH3-JH PCR product from the Daudi cell line was approximately 300 bp in length. Two pooled PCR products were digested overnight at 37°C by 90 U of HaeIII restriction enzyme according to standard protocols. This DNA was electrophoresed, and the restriction fragments were isolated as above. PCR with the internal IgH consensus FR3 primer and the JH primer was used to confirm that the smaller of the two fragments produced by HaeIII digestion contained the JH end (see Table 1C). This fragment was approximately 140 bp in length. It is hereafter referred to as D3HL. Two-and-one-half microliters of 20 µM single-stranded Raji-specific (NR5′) or patient 41-specific (41:5′) primer were made double stranded by the addition of 0.5 µL DNA polymerase Klenow enzyme, 4 µL 5× buffer, 1 µL bovine serum albumin, 1 µL each dNTP (Prime-a-gene®; Promega) and 11 µL ddH₂O. The reactants were incubated at room temperature for 1 h. Six microliters of the mixture were then added to 1 µL 10× ligase buffer, 1 µL D3HL DNA fragment and 2 µL T4 DNA ligase enzyme (6 U) or 2 µL ddH₂O as a negative control and incubated at 15°C for 2 h, followed by 10 min at 70°C. PCR using JH and either NR5′ or 41:5′ primers was then performed as above to yield either NR5′D3HL or 41:5′D30HL constructs.

Electrophoresis of the products from two sets of PCRs: one with a Raji-specific primer (NR5′) and one with a patient 41-specific primer (41:5′) showed that amplification with Raji- and patient 41-specific primers was successful in those reactions where T4 DNA ligase and ligase buffer had been added. Four identical PCRs were pooled and electrophoresed, and the products were isolated from the agarose as before. They were then quantified by optical density (11) and stored for use as standards of known concentration in cPCR assays.

We processed further one of these standards for use in a nested PCR system. Briefly, we ligated the double-
stranded consensus V_{H}3 primer to the 
NR5’ end of the NR5’D3H3 construct 
to produce a new construct designated 
V3NR5’D3H3 (see Table 1C). This 
construct was amplified using V_{H}3 and 
J_{H} primers, and the products were iso-
lated from agarose gels and quantified as 
before. Known amounts of this standard 
were introduced into a cPCR with 
Raji genomic DNA diluted with DNA 
from normal peripheral blood mononu-
clear cells. One set of 35 cycles of PCR 
was performed with V_{H}3 and J_{H} 
primers, followed by a second set of 35 
cycles with the Raji-specific primer 
(NR5’) and an internal J_{H} region primer 
designated J_{H}β2 (see Table 1 for se-
quence and location of this primer). Gel 
electrophoresis of the final PCR prod-
ucts indicated that competitive amplifi-
cation had taken place (Figure 1). The 
sensitivity and specificity of this meth-
ology is currently being evaluated.

Although the addition of T4 DNA 
ligase and ligase buffer alone is suffi-
cient to yield amplifiable target and re-
moves the requirement for a 1-h DNA 
polymerase incubation (data not shown), we recommend the use of the 
full labeling procedure for optimal re-
Sults. As alkaline phosphatase was not 
used to restrict the possible binding 
partners of the ligation step, a variety of 
ligation products were presumed to be 
present. However, only constructs, 
which join the 3’ end of the primer se-
quence to the V end of the DNA frag-
ment will contain the PCR primer 
recognition sites in the correct orienta-
tion to permit amplification (see Table 
1C). PCR products isolated from 
agarose gels will therefore consist of 
purified clone-specific quantitation 
standard. In summary, the simple stra-
gy described allows the production of 
clone-specific quantitation standards 
for use in cPCR assays.

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Benchmarks