

Taqman PCR In The Detection And Quantification Of Chromosomal Translocations In Follicular Lymphoma, Mantle Cell Lymphoma And Chronic Myeloid Leukemia: Taqman Real-time PCR Assay

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Citation

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Abstract

Real-time polymerase chain reaction (PCR) methods are relatively recent technological advances that allow routine quantitative analysis of nucleic acid sequences (DNA and RNA). In this review, we provide a brief overview of 5'-3' exonuclease-based real-time PCR assays (also known as TaqMan real-time PCR) used in own and in many other laboratories. We discuss the theory, protocols, advantages, drawbacks and utility of these methods in the assessment and quantification of some of the common chromosomal translocations associated with hematopoietic tumors.

I. TAQMAN REAL-TIME PCR ASSAY

The real-time polymerase chain reaction (PCR) assay is a relatively recent technological advance that is quickly becoming widely accepted for routine quantitative analysis of nucleic acid sequences (DNA and RNA). TaqMan Real-time PCR, sometimes referred to as kinetic PCR, or 5' exonuclease-based PCR assay, exploits the 5'-3' exonuclease activity of Taq polymerase first described by Holland and collaborators (1) in 1991. This assay integrates fluorogenic PCR with a laser-based instrumentation system, the PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA), to detect and quantitate specific PCR amplicons as the reactions proceed.

The real-time PCR reaction includes all the components of a conventional PCR reaction (the four nucleotides in an appropriate buffer containing an optimum concentration of magnesium, primers and DNA polymerase), however, a TaqMan probe is also required. The TaqMan probe is the key feature of the real-time PCR assay and consists of a non-extendable oligonucleotide complementary to the target sequence that is labeled with a 5'-reporter dye and a 3' quencher dye. The fluorescent dye 6-carboxy-fluorescein (FAM), generally used as reporter dye is covalently linked to the 5' end of the oligonucleotide. Other dyes such as JOE (2,7 dimethoxy-4,5-dichloro-6-carboxyfluorescein) and VIC

may also be used as reporter dyes. The reporter dye is quenched by TAMRA (6-carboxy-tetramethylrodamine), located at the 3' end. When the probe is intact, the fluorescence emission of the reporter dye is quenched owing to the physical proximity of the reporter and quencher dyes (Foster-type energy transfer) (2,3). During PCR, forward and reverse primers hybridize to a specific sequence of the target of DNA and the TaqMan probe hybridizes to the target sequence internal to the primer sequences. During the extension phase of PCR, the 5' exonuclease activity of Taq polymerase hydrolyzes the TaqMan probe. The reporter dye and quencher dyes are separated upon cleavage resulting in increased fluorescence of the reporter (Figure 1).

Figure 1

Figure 1a: Denaturing

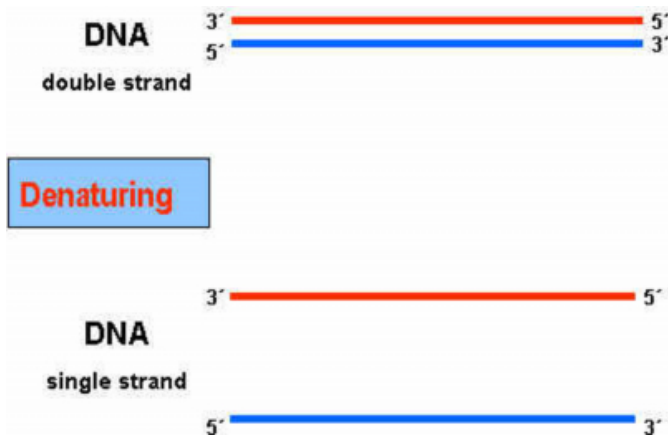


Figure 2

Figure 1b: Annealing Primers and Probe

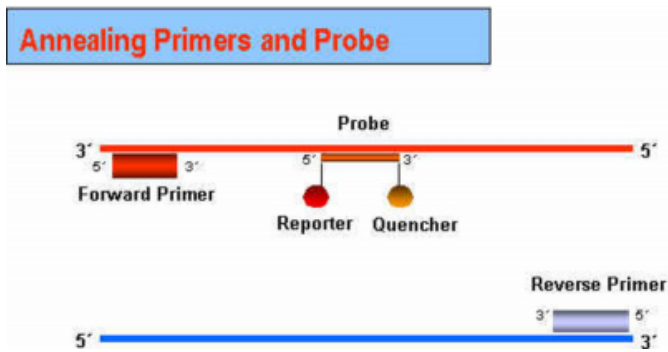


Figure 3

Figure 1c: Taq Polymerase Extends Primers

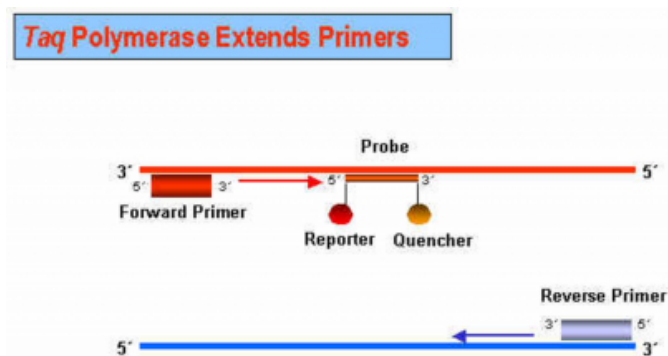


Figure 4

Figure 1d: Initiation of Cleavage of the Probe

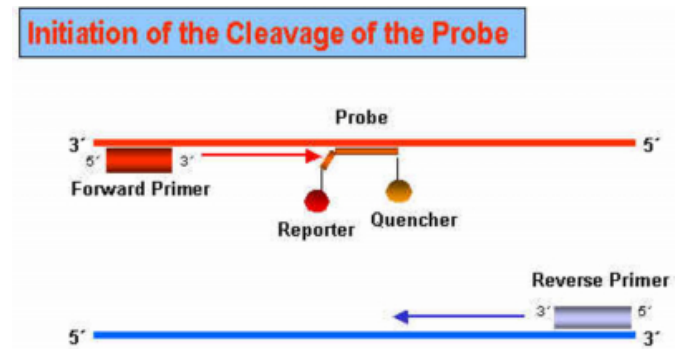


Figure 5

Figure 1e: Cleavage of the Probe

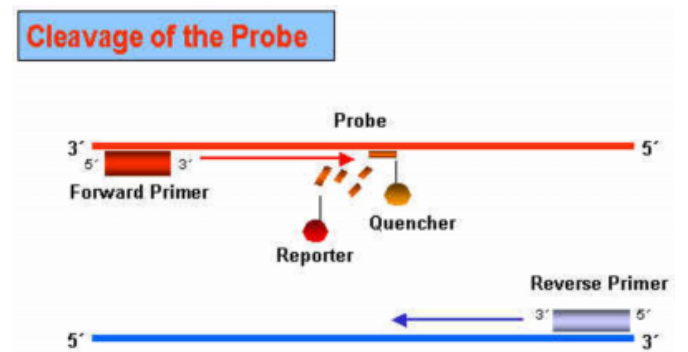
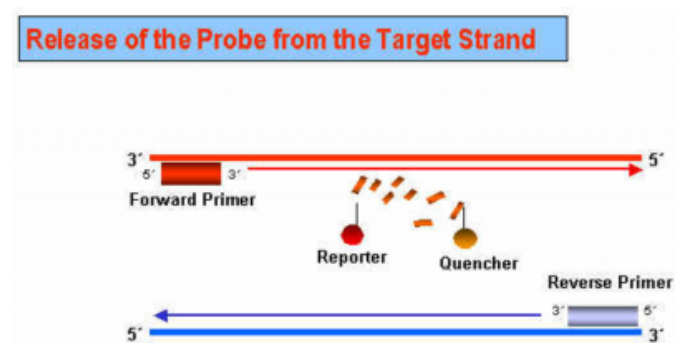


Figure 6

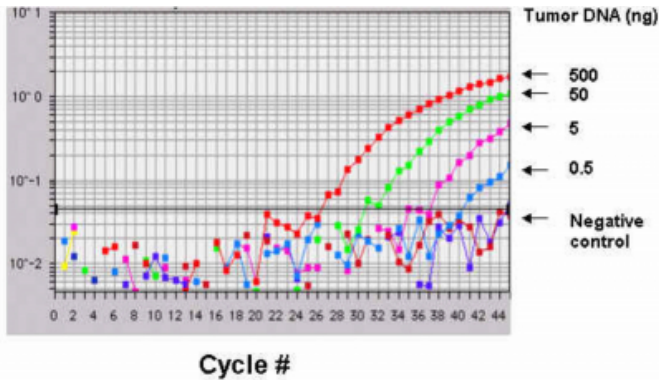
Figure 1f



This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence is measured, and is proportional to the amount of target amplification during PCR. Both primer and probe must hybridize to the target for amplification and cleavage to occur. Because of this requirement, non-specific amplification is not detected. Fluorescence increases in proportion to the concentration of the DNA templates, which rises geometrically in the presence of DNA amplification, but only linearly in its absence (Figure 2).

Figure 7

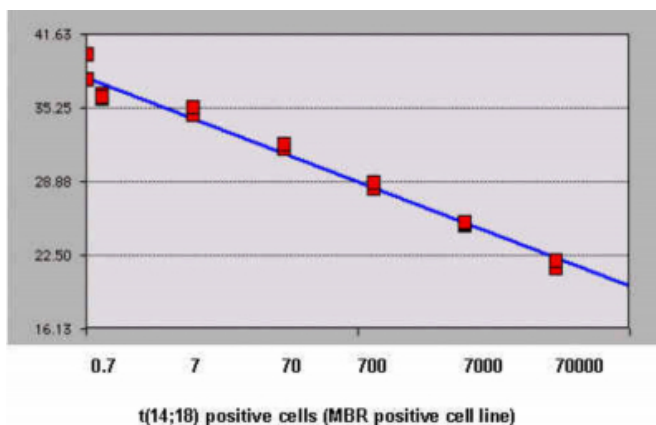
Figure 2: Detection sensitivity of real-time PCR assay for t(11;14). Genomic DNA derived from a mantle cell lymphoma sample with t(11;14) was diluted with DNA from nonneoplastic tissue to achieve the final quantity indicated and then subjected to real time PCR. DNA obtained from nonneoplastic tissue served as negative control. Only one plot for reaction is shown.



As the amount of amplicon produced in any given cycle within the exponential phase of PCR is proportional to the initial number of template copies, a standard curve can be generated using the fluorescence data from the serial dilution study (Figure 3). The threshold cycle (y-axis) is the cycle at which the fluorescence signal of the reporter dye rises above the baseline signal of the dye. Such standard curves can be utilized to determine the relative number of cells in a test sample carrying tumor associated fusion sequences, such as t(14;18), t(11;14) or t(9;22) chromosomal translocations.

Figure 8

Figure 3: Standard curve showing the initial quantity vs threshold cycle. Each point represents the mean of triplicate PCR amplifications. Threshold Cycle (CT) is defined as 10 S.D. above baseline. CT is proportional to log of input copy number. Slope is efficiency of PCR reaction (-3.3 indicates doubling each cycle). y axis intercept indicates the theoretical CT values at 1 copy of target.



II. ADVANTAGES AND DRAWBACKS OF THE REAL-TIME PCR ASSAY

Real-time PCR methods have several advantages over conventional PCR techniques that require gel electrophoresis-based amplicon detection and quantitation techniques. The major advantages are:

- No post-PCR manipulation is required. Thus, the results are available as soon as PCR is completed (i.e., within two hours) decreasing the turnaround time and additionally reducing the risk of PCR contamination.
- This methodology allows co-amplification and detection of a normalizer gene (housekeeping gene and/or positive internal control) in the same tube to obtain accurate quantitative measurements and to confirm the presence of amplifiable DNA in a test sample.
- Decreased variability, because the collection of the data is performed during the exponential phase of the PCR, thus the results are not influenced by limited reagents.
- As the TaqMan probe is complementary to the target, the assay is target specific.
- High sensitivity, 1 tumor cell in 100,000 normal cells can be detected.

The major disadvantages are:

- Real-time PCR methods do not provide size determination of the PCR products. Due to this drawback, one cannot easily exclude contamination between samples and compare amplicon sizes when multiple samples from the same patient are analyzed.
- The assay requires that the size of the target to be amplified to be limited to a less than 150 base pairs to obtain a maximum efficiency.

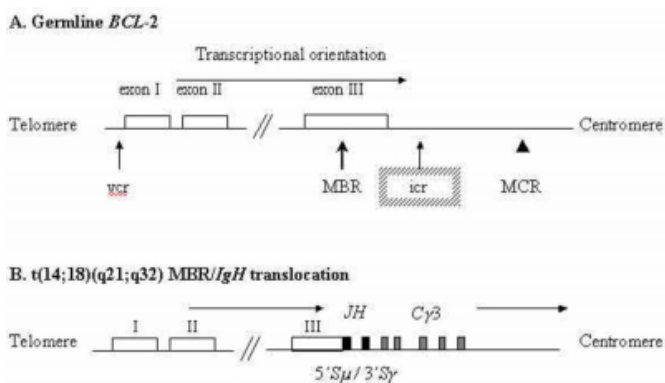
III. TAQMAN PCR IN THE DETECTION AND QUANTIFICATION OF CHROMOSOMAL TRANSLOCATIONS

DETECTION OF T(14;18)(Q32;Q21) IN FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is a neoplasm of follicle center B cells and represents 22% of non-Hodgkin's lymphomas (NHL) (4). Most cases of FL are cytogenetically characterized by the t(14;18)(q32;q21) translocation detected by conventional cytogenetics in approximately 90% of FL, as well as 20-30% of diffuse large B-cell lymphomas (DLBCL), the latter presumably of follicular center origin (5,6). In the t(14;18), the bcl-2 gene on 18q21 is juxtaposed with the IgH gene on 14q32. The breakpoints on chromosome 14 are tightly clustered, occurring immediately 5' to the IgH joining regions, usually JH5 or JH6. The breakpoints on chromosome 18 occur predominantly in two tight clusters, the major (MBR) (Figure 4) and minor (MCR) breakpoint regions (7). The MBR is involved in 50-70% and the MCR is involved in 10 to 15% of the cases of FL (8). Other small breakpoint cluster regions have been reported and are widely scattered in FL. The tight clustering of the MBR and MCR breakpoints is amenable to standard PCR assays, which are commonly used as tests of choice.

Figure 9

Figure 4: A) Germline configuration of chromosome 18q21 and bcl-2 gene involved in t(14;18)(q32;q21). The exons of bcl-2 are indicated as open boxes. Most of the breakpoints occur in the major (MBR) and minor (MCR) breakpoint cluster regions. The variant translocation cluster region (vcr) is involved in t(2;18) and t(18;22) reported in ~5% of cases of B-cell chronic lymphocytic leukemia. Intermediate cluster region (icr).



(B) IgH-bcl2 translocation involving the bcl2 MBR within the 3' UTR of the gene and the JH segments. In the case illustrated IgH class-switching has also occurred.

REAL-TIME PCR AND FOLLICULAR LYMPHOMA

Although the majority of patients with indolent FL achieve clinical remission after induction therapy, they continue to have low-level disease that eventually leads to clinical

relapse. Several studies have recently demonstrated that achievement of molecular complete remission is a desirable goal of new therapies because patients with molecular complete remission have longer disease free status (9,10,11). The clinical importance of achieving molecular response has been demonstrated in FL patients treated with several regimens, including rituximab (11,12) and stem cell therapy (13).

Since our original report on the application of real-time PCR for t(14;18), several investigators have demonstrated the utility and reliability of this technique in quantifying tumor burden in FL patient samples (8,13,14,15,16,17). For example, Hirt and Dolken (13) have demonstrated that quantitative detection of circulating t(14;18) by real-time PCR in follow up samples of FL patients after autologous bone marrow transplantation predicts clinical course of the disease. Similarly, Ladetto and collaborators (17) have shown that tumor burden in stem cell harvests detected by real-time PCR for t(14;18) can predict the effectiveness of therapeutic intervention in FL patients. These studies demonstrate that real-time PCR is a reliable tool that can be used for monitoring minimal residual disease and in the evaluation of treatment effectiveness in FL patients.

DETECTION OF T(11;14)(Q13;Q32) IN MANTLE CELL LYMPHOMA

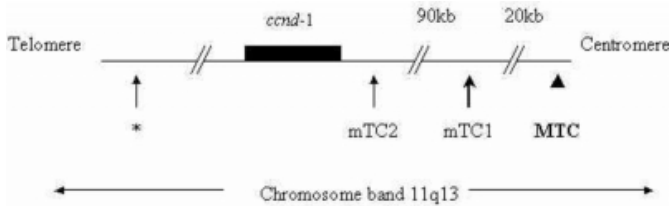
Mantle cell lymphomas (MCL) represent approximately 6% of NHLs (4). The t(11;14)(q13;q32) is present in virtually all cases of MCL and is also found in a small subset of cases of plasma cell myeloma (18A). The t(11;14) also has been rarely reported in other types of NHL and lymphoid leukemias. However, most of these cases, in retrospect, are examples of MCL that were misclassified because they were either high-grade or had marked leukemic involvement (18B).

As a result of the t(11;14), ccnd-1 protooncogene (also known as bcl-1, prd1, cyclin-D1) is juxtaposed with an enhancer sequence of the immunoglobulin heavy chain gene at 14q32 (IgH) (18C). Nearly half of these translocations cluster within an 80-100 base pair region of the major translocation cluster (MTC) region on chromosome 11q13 (Figure 5). However, the remainder of chromosome 11 breakpoints are widely scattered over approximately 120 kb. The breakpoints on chromosome 14 occur within the 5' region of one of six joining regions of the IgH gene (19). The ccnd-1 gene is not disrupted by the translocation and thus the t(11;14) results in a quantitative increase of normal

cyclin D1 protein (20).

Figure 10

Figure 5: Germline configuration of chromosome 11q13 region involved in (11;14)(q13;q32). The *ccnd1* gene is indicated as a closed box. The major translocation cluster (MTC) region accounts for 30-40% of all translocations. Minor breakpoint clusters are indicated as mTC1 and mTC2. The asterisk indicates breakpoints that occur far 5', which have been reported in cases of plasma cell myeloma



Translocations involving the MTC are amenable to routine PCR analysis, and thus 30% to 40% of cases of MCL can be shown to carry the t(11;14) translocation by PCR methods.

REAL-TIME PCR AND MANTLE CELL LYMPHOMA

Our study using real-time PCR for the detection of the t(11;14) offered promising results. This assay effectively identified *bcl-1* MTC/JH DNA fusion sequences in 24 of 25 MCLs shown to be positive by conventional PCR but not in other types of NHL or reactive lymphoid proliferations shown to be negative by conventional PCR (21).

Concordance between real-time and conventional PCR methods for MCL was 96% and for all the samples was 98% (21). Although the real-time PCR for genomic t(11;14) is sensitive enough for the detection of minimal residual disease, the application is restricted to the patients with a detectable breakpoint.

An alternative approach to obtain support for the diagnosis of MCL is to determine expression levels of the cyclin-D1 gene, as all t(11;14) translocations result in overexpression of cyclin-D1 gene. As lymphoid cells express very low levels of cyclin-D1, not detectable by immunohistochemical methods, these methods are convenient and specific for distinguishing MCL from other NHL. However, a negative immunoreaction does not exclude the diagnosis of MCL, as some cases have relatively low levels of cyclin D1 (22). Detection of cyclin-D1 mRNA by reverse transcriptase-mediated polymerase chain reaction (RT-PCR), in contrast, is very sensitive. Unfortunately, the RT-PCR assay is likely to amplify faint physiological cyclin-D1 derived from nonoverexpressing lymphomas or normal cells.

Quantification of cyclin-D1 mRNA from microdissected tissue sections would be an ideal solution coupling the requirements of high sensitivity and specificity. However, this approach is time consuming and is not considered convenient for routine diagnostic use.

Several investigators have demonstrated the utility and reliability of the real-time PCR assay detection of cyclin-D1 is particularly useful for the diagnosis of MCL when the cyclin-D1 signal is normalized to a housekeeping gene (23,24,24B). These works demonstrate that this assay is rapid, technically less demanding than previously available methods, and with a high specificity.

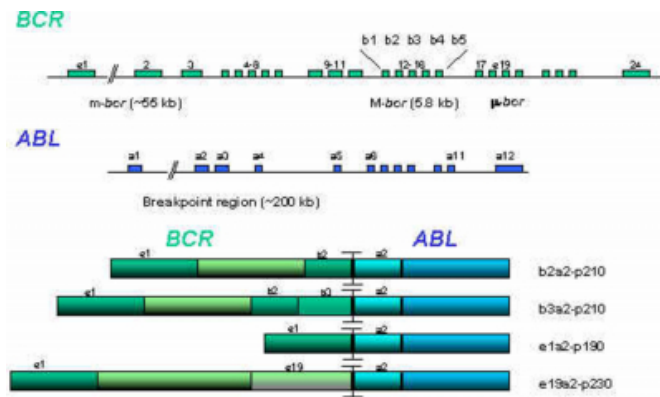
DETECTION OF T(9;22)(Q34;Q11) IN CHRONIC MYELOID LEUKEMIA

The Philadelphia (Ph) chromosome, which can be found in 90-95% of cases of chronic myeloid leukemia (CML), is the cytogenetic hallmark of this disease. The t(9;22)(q34;q11) in CML involves juxtaposition of sequences from *c-abl* located at 9q34, the cellular homologue of the transforming sequence of Abelson murine leukemia virus (*v-abl*), with the *bcr* locus on 22q11 (25,25B) creating a *bcr-abl* fusion gene and protein.

Depending on the breakpoint in *bcr*, the fusion gene produces proteins of either 210 kD or 190 kD. In 95% of patients with CML, p210 *bcr-abl* is seen. Additionally, a rare larger fusion protein that results from splicing at a downstream *bcr* locus, p230 *bcr-abl*, is associated clinically with the chronic neutrophilic leukemia variant and thrombocytosis (26,27). The three principal isoforms of the resulting hybrid *bcr-abl* oncogene differ in the portions of *bcr* juxtaposed to the nearly invariable *abl* fragment (Figure 6) and contribute to the development of distinct Ph-positive leukemias.

Figure 11

Figure 6



The major forms of bcr-abl fusion gene. Translocations involving the M-bcr region of the bcr gene lead to joining of the second or third exons of this region (b2,b3) with the second exon of the abl gene (a2) to form the b2a2 or b3a2 transcripts and the p210 product. Breakpoints in the m-bcr region of the bcr gene result in fusion of the first exon of this gene (e1) with the second exon of the abl gene (a2) to form e1a2 bcr-abl transcript which encodes the p190 hybrid protein. Breakpoints in the m-bcr region of the bcr gene juxtapose the exon 19 (e19) of this gene to the second exon of the abl gene (a2) to produce the e19a2 transcript which encodes the p230 protein

The (9;22) translocation is detected by conventional cytogenetics in approximately 90% of patients with a clinical presentation consistent with CML. Three to five percent of patients show a normal chromosome 22 but molecular evidence of the bcr-abl translocation. Conventional cytogenetics has been considered to be the “gold standard” for evaluating patient response to treatment but this technique is laborious. The use of cytogenetic analysis may be considerably reduced by using molecular methods such as fluorescence in situ hybridization (FISH) (28), Southern blot analysis (29), Western blot analysis (30), RT-PCR (31), and real-time PCR (32). These methodologies also can be performed on peripheral blood specimens, which may result in less use of invasive bone marrow aspiration, and also they are applicable to Philadelphia negative, bcr-abl positive cases.

REAL-TIME RT PCR AND CHRONIC MYELOID LEUKEMIA

Real-time RT PCR technique is a reliable method for monitoring CML patients before and after therapy (33-37). As new therapies improve the rate of complete cytogenetic

response, molecular monitoring is likely to become increasingly important in the management of patients with CML.

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