Novel multiplex RT-PCR assay to detect BCR/ABL mRNA variants

K Shires, A Rust

Division of Haematology, Department of Pathology, University of Cape Town and NHLS, Groote Schuur Hospital, South Africa

Corresponding author, email: karen.shires@uct.ac.za

Introduction

BCR/ABL is a novel oncogene that is formed by a reciprocal translocation involving the long arms of chromosomes 9 and 22 (t(9;22)(q34;q11)), which results in the fusion of the break-point cluster region (BCR) gene and the Abelson murine leukaemia viral oncogene homologue 1 (ABL) gene, forming the characteristic Philadelphia chromosome (Ph+) (elongated chromosome 22). The BCR/ABL oncogene on the Ph+ chromosome gives rise to the oncoprotein BCR/ABL, a constitutively active tyrosine kinase involved in many signalling pathways, while the reciprocal ABL/BCR on chromosome 9 does not yield a protein product. The translocation is most commonly associated with the development of Chronic Myeloid Leukaemia (CML), a clonal stem cell disorder that is characterised by abnormal myeloid proliferation proceeding through three distinct phases: chronic, accelerated and blastic crisis. The t(9;22) event is usually reported to occur in > 95% of CML cases, however as the primary oncogenic event in CML is now known to be the synthesis and action of BCR/ABL, the lack of detection in some historical cases of CML is probably a detection methodology issue rather than representing a true alternate disease mechanism in these cases. This oncoprotein also plays a role in the pathogenesis of subtypes of two other leukaemias, namely: Ph+ Acute Myeloid Leukaemia (AML) and Ph+ Acute Lymphoid Leukaemia (ALL), however its role in these cancers is likely to be as a secondary event rather than the initiating event and is part of a much more complex pathogenesis picture. It has been reported that as many as 30% of adult ALL cases are Ph+, while < 5% of AML cases express the BCR/ABL oncoprotein.

Due to the unique nature of the BCR/ABL oncoprotein in these diseases, targeted drugs belonging to the class of tyrosine kinase inhibitors (TKI) are now used to effectively treat CML and aid in the induction responses of both Ph+ AML and ALL. Stable expression of BCR/ABL mRNA transcripts in each diseased cell has also led to novel monitoring methods, allowing detection of minimal residual disease (MRD) at levels as low as 0.001% disease. However, detection and monitoring of mRNA transcripts is complicated by the fact that the BCR/ABL translocation can occur at several places within the BCR gene (all fused to exon 2 of ABL), resulting in the formation of different transcript variants, and it is essential that the variant/isofrom type is known for the effective monitoring of treatment responses, due to the different methodologies required for each transcript type. The three major variants are M-bcr variants (major), which involve the fusion of the first 13 or 14 exons of BCR to exon 2 of ABL and are known as e13a2 (b3a2) and e14a2 (b2a2) transcripts, or p210, referring to the size of the protein produced, 210kDa; m-bcr variant (minor), which is the e1a2 translocation, often referred to as p190, as a smaller 190kDa is translated; and µ-bcr variant (micro), which contains 19 exons of BCR fused to exon 2 of ABL (e19a2), and produces the largest BCR/ABL protein of 230kDa, known simply as p230. Little is known about the clinical consequences of the different proteins, as, if there are differences, they are subtle, as they appear to have identical functions within the cell, fundamentally those of a constitutively activated and cytoplasmic-restricted ABL protein. CML is most usually associated with the p210 translocation types (M-bcr), 2008, but p190 and p230 variants have also been identified, with the p230 variant usually being associated with a high neutrophil count and a few limited studies suggesting that the p190 CML variant (associated with increased monocytes) may have a more aggressive disease course. Ph+ ALL is more commonly associated with the p190 protein isoform, with only 20% of BCR/ABL positive cases being p210 positive. Ph+ AML cases, while rare, are predominately associated with the p210 transcript type, although p190 cases have been reported.

Diagnosis and treatment of these BCR/ABL-positive diseases does not require the identification of the specific mRNA variant, as the simple presence of the t(9;22) translocation (detected...
via karyotyping or BCR/ABL FISH) is sufficient for diagnosis and initiation of TKI therapy. However, the identification of the specific mRNA variant is essential for the monitoring of the patients during therapy, as the quantitative reverse transcription PCR (QRT-PCR) assays are highly specific to each variant, and false negative results will be obtained if the wrong test is used. Unfortunately, as a diagnostic laboratory we have observed the limited requests for testing for the alternate transcripts, which in turn has resulted in several cases of incorrect diagnoses (when cytogenetics is not performed in addition to QRT-PCR) or poorly controlled disease due to the apparent lack of transcript (incorrect test requested). This is primarily due to confusion regarding the prevalence and thus relevance of testing for the alternate variants and the cost that is associated with testing for each variant, which currently involves single variant RT-PCR assays.

We aimed to simplify the important step of BCR/ABL transcript identification, by designing a multiplex RT-PCR assay that is capable of detecting all mRNA BCR/ABL variants (involving exon 2 of ABL), in a more cost-effective, single test format. Standardised guidelines have been recommended by the Europe Against Cancer (EAC) programme and others for the detection and quantification of the various BCR/ABL translocations, for use in MRD monitoring. We combined the recommended primer sets for p190 (primers ENF402/ENR561) and p210 (primers ENF501/ENR561) transcript amplification to develop a single multiplex RT-PCR assay that is capable of simultaneously detecting all of the common BCR/ABL transcripts, including p210, p190 and p230. We assessed the assay for specificity, accuracy and sensitivity to establish its applicability in the diagnostic environment.

**Materials and methods**

**Multiplex BCR/ABL RT-PCR assay**

Total RNA was extracted from the white cell component of 5–10 ml EDTA PB or 5 ml BM (received on ice, within 36 hours) using TRIzol (Invitrogen) or equivalent methodology. The following specific reaction conditions and reagents were carefully designed to generate optimal, robust amplification in the multiplex setting specifically. RT was performed as a two-step reaction, with step 1 being the RNA denaturation and annealing of the cDNA primer: 2 ug RNA (5 μl maximum volume), 50 pmol Oligo dt (1 μl), 1 μl 10 mM dNTP (aids in primer-binding) and nuclease-free water to 13 μl final reaction volume, incubation at 65 °C for 10 mins, followed by immediate incubation on ice (not cooling block) for 5 mins. Step 2 represents the cDNA synthesis: 1 μl 0.1 mM DTT, 0.5 μl RNAse Out (40 U/μl, Invitrogen), 1 μl SuperScript™ III (200 U/μl Invitrogen), 4 μl 5X SuperScript™ RT buffer, 0.5 μl nuclease-free water and 13 μl from step 1. This provides an RT reaction (20 μl) containing reagents at the final concentrations of 2.5 pmol/μl Oligo DT, 0.5 mM dNTP, 5 mM DTT, 20 U RNAse inhibitor, 200 U SuperScript™ III and 10% CDNA. The reaction was incubated as follows: 50 °C for 60 mins (cDNA synthesis), 70 °C for 15 mins (denatures SuperScript™II), 4 °C for 5 mins, then immediately on ice (important step to ensure secondary/tertiary structures are prevented).

The final multiplex BCR/ABL PCR reaction contained the following (25 μl final volume): 1.5 mM MgCl₂, 0.8 mM dNTP, 1X AmpliTaq Gold buffer (Applied Biosystems), 2.5 U AmpliTaq Gold (Applied Biosystems), 0.4 pmol/μl ENF402, 0.4 pmol/μl ENF501, 0.4 pmol/μl ENR561 and 2 μl cDNA. The final AB1 PCR reaction contained the following: 1.5 mM MgCl₂, 0.8 mM dNTP, 1X AmpliTaqGold buffer (Applied Biosystems), 2.5 U AmpliTaq Gold (Applied Biosystems), 0.4 pmol/μl ENF1003 and 0.4 pmol/μl ENR1063 and 2 μl cDNA. Primer sequences are shown in Table I. The multiplex PCR reactions are easily setup using pre-prepared mastermixes (MM) that can be stored at -20 °C in aliquots until required. Details of reagents that can be incorporated into a MM are shown in the supplementary data, as well as the simplified setup using these MM. The following PCR reaction conditions were applied: 95 °C for 10 mins, 40 cycles of 94 °C for 30 sec/65 °C for 60 sec/68 °C for 150 sec, 68 °C for 10 mins (using a ProFlex PCR machine, with a ramp time of 0.5 °C/secs). Amplicons were electrophoresed on a 3% TAE/agarose in 1X TAE for approx. 90 minutes at ~80–100 v, stained with ethidium bromide and photographed under UV. DNA molecular ladders should be placed on either side of the samples to enable easier product sizing and a mixture of RNA containing p210 (e13a2) and p190 (e1a2) should be used as a positive control for the assay to ensure adequate separation between the 72 bp and 90 bp products.

**Table I: Primer sequences used in the amplification of BCR/ABL variants and control ABL cDNA**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENF402²</td>
<td>CCGCCCAACGATGGGCA</td>
</tr>
<tr>
<td>ENF501²</td>
<td>TCCGCTGACCATCAAYAGGA</td>
</tr>
<tr>
<td>ENR561²</td>
<td>CACCTGAGCCCTGAGGCTCA</td>
</tr>
<tr>
<td>ENF1003¹³</td>
<td>TGGAGATAACACTCCTAAGCTAAGGT</td>
</tr>
<tr>
<td>ENR1063¹³</td>
<td>GATGTGTGCTTGGGACCCA</td>
</tr>
</tbody>
</table>

**Validation assessment**

A) Assay specificity was assessed by sequence analysis of the amplicons produced by the multiplex assay using commercial RNA samples (Invivoscribe) containing BCR/ABL transcript types: e13a2 (IVS0003), e14a2 (IVS0011), e1a2 (IVS0032) and e19a2 (sequenced patient sample), as well as analysis of BCR/ABL negative RNA controls: IVS0035 (Invivoscribe), human testis RNA (Clontech), Jurkat/HL60/RPMI8226 RNA (cultured cell lines). Amplicons were excised from the multiplex assay agarose gel, purified using a standard PCR clean-up kit (Nucleobond) and the fragments sequenced using the ENR561 primer and the Bigdyte Terminator cycle sequencing kit (Applied Biosystems), followed by separation on an AB13500XL capillary analyser. Generated sequences were compared to the reference BCR transcript sequence (NM_004327.5) and ABL transcript sequence (NM_005157.5) to determine the break point in BCR and thus the transcript variant type.

![Image](image-url)
B) Accuracy of transcript variant calling was determined by analysis of 10 external quality control samples (NEQAS), in duplicate, in a technologist-blinded study using the multiplex RT-PCR assay and comparing the results to the official EQA reports.

C) The limit of detection for the assay was determined by analysing stored RNA that had previously been quantified using our SANAS accredited BCR/ABL p210 quantification assay, covering disease levels over 4 logs: 43.233%, 3.977%, 0.505%, 0.050%, 0.005%. These samples were reanalysed in duplicate on two separate occasions using the multiplex RT-PCR to determine the lowest level that could be readily detected.

**Results**

**Assay design**

This novel multiplex RT-PCR BCR/ABL assay was designed to be able to identify BCR/ABL transcript variants at diagnosis in a single simple assay, in patients suspected of suffering from CML, Ph+ AML or Ph+ ALL. Briefly the multiplex assay should be performed as follows: RNA is extracted from either bone marrow (BM) or peripheral blood (PB) at diagnosis and a specialised RT is performed in duplicate. BCR/ABL (primers ENF402/ENF501/ENR561) and ABL (primers: ENF1003/ENR1063) reactions are then performed separately using a high fidelity Taq polymerase,
A: Specific BCR/ABL variant amplification products

B) Sequences generated from the multiplex RT-PCR BCR/ABL amplicons were sequenced using primer ENR561 and resulting sequences aligned to the BCR and ABL reference sequences (NM_004327.5 and NM_005157.5 respectively), with the raw data shown here annotated to indicate the specific BCR exon fused to exon 2 of ABL.
with the cDNA as the input sample, and ABL amplification acting as an RNA integrity control. Following the PCR reaction, the amplicons are separated on an agarose gel to identify the transcript variant based on the resulting amplicon size. Figure 1 shows the binding positions of the three primers in the BCR/ABL multiplex reaction and the expected amplicon sizes of the various transcript variants.

Assay specificity

To confirm that the assay only produces amplicons from RNA samples containing BCR/ABL transcripts and that specific amplicon sizes correlate to the correct BCR/ABL transcript variants, commercial RNA samples and RNA extracted from BCR/ABL-negative cell lines were analysed using the multiplex assay, with any resulting amplicons being sequenced to identify the corresponding translocation points. Figure 2A shows the different amplicons produced from two known BCR/ABL-positive transcript variants, as well as the lack of amplification with a known BCR/ABL-negative RNA. Figure 2B shows the sequence analysis of several of the amplicons produced from this analysis, with break-points identified by comparison to the NCBI reference sequences for BCR and ABL. In total, nine different RNA types were analysed, with no amplicons being produced for IVS0035, human testis, Jurkat, HL60 and RPMI8226 as expected and amplicons sized as e1a2 (90 bp), e13a2 (72 bp), e14a2 (147 bp) and e19a2 (650 bp) variants producing sequences that corresponded with the expected translocation points (i.e: BCR exon 1 fused to ABL exon 2 for e1a2). In combination with the analysis of EQA samples described below, the results of this exercise confirmed the specificity of the assay.

Assay accuracy

NEQAS EQA samples from five separate “AML translocation identification trials” were analysed in a blinded study using the multiplex RT-PCR BCR/ABL assay (10 separate samples in total) to determine the accuracy of variant calling. Figure 3 shows the BCR/ABL-specific amplicons produced from the analysis of five of these samples and the corresponding variant call based on the amplicon sizing, while Table II shows the comparison between results obtained (in duplicate) and those reported by NEQAS for all 10 samples. Full correlation was observed between the multiplex results and those officially reported by NEQAS.

Table II: Comparison of BCR/ABL variant calling using multiplex assay to reported NEQAS EQA report

<table>
<thead>
<tr>
<th>EQA sample name</th>
<th>Multiplex amplicon size (bp)</th>
<th>Multiplex BCR/ABL variant call</th>
<th>NEQAS EQA trial number</th>
<th>Consensus achieved</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR136</td>
<td>A and B: 147</td>
<td>p210 – e14a2</td>
<td>BCR/ABL pos- e14a2/p210</td>
<td>151602</td>
</tr>
<tr>
<td>BCR138</td>
<td>A and B: 90</td>
<td>p190 – e1a2</td>
<td>BCR/ABL pos- e1a2/p190</td>
<td>151603</td>
</tr>
<tr>
<td>BCR142</td>
<td>A and B: none</td>
<td>BCR/ABL negative</td>
<td>No BCR/ABL</td>
<td>161702</td>
</tr>
<tr>
<td>BCR144</td>
<td>A and B: 90</td>
<td>p190 – e1a2</td>
<td>BCR/ABL pos- e1a2/p190</td>
<td>161703</td>
</tr>
<tr>
<td>BCR146</td>
<td>A and B: 90</td>
<td>p190 – e1a2</td>
<td>BCR/ABL pos- e1a2/p190</td>
<td>171801</td>
</tr>
<tr>
<td>BCR150</td>
<td>A and B: none</td>
<td>BCR/ABL negative</td>
<td>No BCR/ABL</td>
<td>171803</td>
</tr>
<tr>
<td>BCR152</td>
<td>A and B: 147</td>
<td>p210 – e14a2</td>
<td>BCR/ABL pos- e14a2/p210</td>
<td>181901</td>
</tr>
<tr>
<td>BCR154</td>
<td>A and B: 90</td>
<td>p190 – e1a2</td>
<td>BCR/ABL pos- e1a2/p190</td>
<td>181902</td>
</tr>
<tr>
<td>BCR156</td>
<td>A and B: none</td>
<td>BCR/ABL negative</td>
<td>No BCR/ABL</td>
<td>181903</td>
</tr>
<tr>
<td>BCR158</td>
<td>A and B: 90</td>
<td>p190 – e1a2</td>
<td>BCR/ABL pos- e1a2/p190</td>
<td>192001</td>
</tr>
</tbody>
</table>

* – A and B represent duplicate analysis
Assay sensitivity

While this assay was designed to be used primarily at diagnosis to determine the BCR/ABL transcript variant, it is possible that queries regarding the isoform type may be made several weeks or months into TKI therapy, during which time the levels of transcript may have been reduced by several logs. It may also be requested when cytogenetic results are not available, thus aiding in the initial diagnosis of CML or Ph+ ALL and AML. For these reasons it is important to know the limit of detection of the assay to be able to report relevant qualitative pos/neg results. For this assessment BCR/ABL p210-positive samples (e14a2 and e13a2) that have been previously quantified using our accredited BCR/ABL quantitative QRTPCR assay (NHLS) and covered a 4 log range of disease levels (43 – 0.005%) were analysed by the multiplex RT-PCR BCR/ABL assay. Figure 4 shows the amplicons produced from one of the repeat assessments of the sample set.

The results of this analysis showed that p210 transcripts could be reproducibly and unequivocally detected at levels as low as 0.05%, which allows for detection of transcripts over at least a 3 log range, therefore including patients who have achieved a complete cytogenetic response whilst on TKI therapy. Detection of levels below this gave inconsistent results between replicates (see Figure 4). It is important to note that as this is an endpoint PCR analysis, the BCR/ABL amplicon band intensities are not necessarily related to the amount of starting material and thus cannot be used for quantification in any way (i.e: 0.050% vs 3.977% in Figure 4).

Discussion

BCR/ABL is an oncoprotein that not only causes disease, but also fortuitously provides a target for a specific group of inhibitors known as TKIs. While there are still off-target effects, these are limited as the drugs can be designed as closely as possible to the unique nature of this specific cancer-protein, resulting in a very effective treatment for CML and Ph+ acute leukaemias. Unfortunately, the t(9;22) translocation, which forms BCR/ABL, can occur at different breakpoints, resulting in several mRNA variants. While TKI therapy is not dependent upon the transcript type, highly sensitive MRD monitoring is transcript specific and false negative results will be obtained if the incorrect method is used. While the majority of CML patients express the common p210 BCR/ABL transcript variant and Ph+ ALL is associated primarily with the p190 mRNA variant, there are exceptions and these are unfortunately often missed. This can lead to a delay in diagnosis and inaccurate disease monitoring, all of which can affect patient outcome. This is fundamentally due to the lack of education of clinicians and pathologists regarding the limitations of the newer molecular techniques and the additional testing that is required.

Currently, using either commercial kits or in-house methods, it is possible to test for the different variants using separate qualitative RT-PCR methods, which can be performed in a sequential manner. However, this can be both costly and time consuming and clinicians are not always aware that alternate transcripts can be expressed. We thus aimed to develop a single assay that can simultaneously detect the vast majority of the reported BCR/ABL transcript variants, ensuring that the correct monitoring method can be used at the start of therapy and that a diagnosis of variant CML or BCR/ABL positive ALL and AML is not missed. Our assay is capable of detecting all of the known BCR/ABL variants that have breakpoints involving exon 2 of ABL and any of the BCR exons. This includes variants p210 (e13a2 and e14a2), p190 (e1a2) and p230 (e19a2). We validated the assay by testing it with a series of known BCR/ABL positive and negative EQA samples, cell lines and commercial RNA and found the assay to be highly specific, accurate and reproducible. While the assay should preferably be used at diagnosis, the detection sensitivity limit showed that it is still capable of calling the correct variant when disease levels were as low as 0.05%, when a patient has effectively just achieved a major molecular response to TKI therapy.

The testing algorithm for CML, AML and ALL cases should include karyotyping, so that the existence of the Ph chromosome can be established, as well as additional large DNA mutations. However this often fails due to technical issues and t(9;22) FISH
is sometimes requested instead. If the BCR/ABL translocation has been confirmed using either method, then it can be assumed that patients diagnosed with CML will be expressing the p210 transcript and monitoring via p210 QRT-PCR can be initiated. However, if the p210 transcript is NOT detected at the first time point (usually within three months), it is imperative that the patient be tested for the expression of the alternate transcript. TKI responses in CML are relatively gradual and it is virtually impossible to have a > 4 log response to TKI therapy within a three-month time frame, resulting in undetectable transcript. Continued testing using the p210 QRT-PCR assay will result in the ineffective treatment monitoring of the patient, leading to possible haematological relapse without any warning, which may have dire consequences for the patient. We have, in fact, seen this exact scenario in two separate CML patients, one who was expressing the p230 variant and the other a p190 variant. In the cases where CML is suspected but cytogenetics is not available, then it is highly advised that the multiplex assay be used, instead of the p210 QRT-PCR as a diagnostic test, to ensure that all possible BCR/ABL variants are tested simultaneously for a definitive diagnosis. Thus far, using our multiplex BCR/ABL variant assay we have detected two CML patients expressing the p230 variant, four with p190 and a novel p210/p190 dual expression. Whilst this represents < 1% of the CML cases that we are monitoring through the p210 QRTPCR assay, which is in agreement with other studies,8,9 another 6% (24/400) of cases continue to be regularly monitored by clinicians using this particular MRD assay despite never having the p210 variant confirmed, leaving these patients potentially at risk and possibly indicating a higher level of CML variants than usually reported. BCR/ABL positive (Ph+) AML and ALL cases are considerably less common than CML, however they can be treated with TKIs and monitored using QRT-PCR assays, thus transcript identification is required. Similar to other international reports, the few Ph+ AML patients that we have tested have all been expressing p210 mRNA transcripts, and 20% of the Ph+ ALL cases have been p210 positive, with the remainder expressing p190 transcripts. Thus in the case of ALL cases, it is very important that p190 expression is not automatically assumed.

On a final note, while the optimal TKI response of CML patients expressing p210 transcripts has been very well described, with results being expressed on the International Scale (European Leukemia Network guidelines),10,11 the optimal response patterns of those patients expressing the alternate transcripts is poorly described. Although similar log reductions over time would obviously be preferable, exact optimal time frames have not been standardised. In addition, both CML and Ph+ acute leukaemia patients may not respond to TKI therapy appropriately due to the presence of ABL kinase mutations, mutations that block the binding of the TKI. The ABL kinase domain from all of the BCR/ABL transcript variants can however be sequenced and alternate TKI therapy administered if mutations are detected.

References