is important to risk-stratify patients (i.e., high relapse risk), so that KIT cleophosmin (NPMI), and CD117 include mutations in FMS-related tyrosine kinase 3 (FLT3), nusitive markers in these normal karyotype AML patients. These mutations have been identified as useful diagnostic and prog-
ducts detected in patients [6-9]. The insertion of the repeated sequence leads to the abnormal regulation of the kinase and its constitutive activation, which results in the abnormal expan-
sion of the mutated stem cell clone. These mutations have been

**INTRODUCTION**

Acute Myeloid Leukaemia (AML) is a clonal stem cell disor-
der, which is characterised by an expansion of myeloid cells arrested at various stages of differentiation. Although initial treat-
ment responses are fairly successful, with 80% of younger patients achieving complete remission, the 5 year survival rate is as low as 40%[6,2]. To improve the rate of long-term survival, it is important to risk-stratify patients (i.e.: high relapse risk), so that different treatment approaches can be administered[8]. Although cytogenetic abnormalities are often used to subgroup these patients, some AML subtypes demonstrate a normal karyotype, preventing further stratification[9]. In recent years, several gene mutations have been identified as useful diagnostic and prognostic markers in these normal karyotype AML patients. These include mutations in FMS-related tyrosine kinase 3 (FLT3), nucleophosmin (NPMI), and CD117 (KIT)[10].

**FLT3** is the most frequently mutated gene in AML, occurring in at least 30% of patients. It is most frequently detected in patients with normal karyotype, although it is also associated with Acute Promyelocytic Leukaemia (APL) cases[11,12]. This tyrosine kinase is a class III receptor, normally expressed on haematopoietic stem cells, which has been shown to be essential in stem cell proliferation by allowing signaling between the stroma and stem cells[13]. Two different types of FLT3 mutations have been shown to be clinically relevant in AML patients: point mutations within the activating loop (AL) and internal tandem duplications (ITD) within the juxtamembrane (JM) domain. ITD mutations, which are in-frame 3bp repeats, account for the majority of FLT3 mutations detected in patients[14-16]. The insertion of the repeated sequence leads to the abnormal regulation of the kinase and its constitutive activation, which results in the abnormal expansion of the mutated stem cell clone. These mutations have been shown to be a vital link in the disease progression of AML[17,18]. The presence of FLT3 ITD mutations in patients with normal cy-
togenetics indicates a poor prognosis, with higher relapse risk, lower event free survival and decreased overall survival[6,8-12]. FLT3 ITD mutations vary in size from 15-231bp, in a variety of different positions within the area spanning exons 14 and 15 FLT3[6,8-12], for this reason, DNA encoding part of the JM domain of FLT3 (including exons 14, 15 and the intervening intron) is normally amplified in a standard PCR reaction using well-characterised primers[12,13]. However, there are several ways the mutation can be detected in these amplification products, with the tendency these days towards using the more advanced technology approach. The premise being that although these methods are more expensive, they are at least faster and more sensitive. The cost however, makes it difficult for basic molecular laboratories to offer these important molecular tests. We compared the performance of the most commonly used capillary electrophoresis method of FLT3 ITD mutation detection[13], with high resolution melt technology (HRM) and a basic agarose gel electrophoresis assay modified for higher resolution capabilities. Parameters such as sensitivity limits, accuracy, cost and processing time were evaluated for each methodology.

MATERIAL AND METHODS

Samples:
A) FLT3 ITD sensitivity panel: homozygous FLT3 ITD mutant DNA (30bp ITD) (InVivoScribe Technologies, SanDiego, USA) diluted into WT DNA to give 50%, 20%, 10%, 5%, 2% and 1% mutant alleles;
B) AML patient panel: DNA extracted from PB-EDTA from 15 newly diagnosed AML patients. These patients were not aged biased (21-50yrs) and consisted of patients with a range of cytogenetic abnormalities, including normal karyotype. DNA was extracted and purified using the Invivoscan genomic DNA extraction KIT (Invitex, Berlin, Germany) and diluted to 25ng/µl with nuclease-free water (Promega, USA). Controls for the patient analysis included: WT, 100% FLT3 ITD, 2%, 5% or 20% FLT3 ITD sensitivity samples and a reagent blank in each experimental run. This study was approved by the University of Cape Town Research and Ethics committee.

PCR and amplicon analysis:
The same exon14-15 region of FLT3 was amplified in each assay using 11F:GCAAATTTAGGTATGAAAGCAGC and 12R:CTTTTCAGCATTTTGACGGCAA PCR primers[12,13]. Expected product sizes were 329bp for the WT allele and >331bp for the mutant ITD allele for all assays. All samples were amplified and analyzed at least in duplicate on two separate occasions. Different PCR conditions and analysis modes were used based on the specific parameters of each methodology:

A) Agarose gel electrophoresis analysis: Amplification was performed on a GeneAmp 9700 thermocycler, using 1.5U GoTaq and GoTaq green Flexibuffer (Promega, USA), 1.5mM MgCl2, 1.33mM dNTP (ABgene, Surry, UK), 0.5µM each primer and 75ng DNA. PCR conditions: 30 cycles – 94°C/40s, 56.5°C/30s, 72°C/20s, followed by a 72°C-90°C melt at 0.05°Cs. Re-melt analysis: 72°C-90°C at 0.05°C/s, after 1-4 hours at 4°C. Automated genotyping was performed using a leading edge of 75.4-75.8°C and a trailing edge of 82.2-82.6°C, with a confidence coefficient of 75%. A difference of >5 units or peak shift was used to indicate “variant” on the difference graphs.

DNA sequence analysis and melt domain predictions:
Amplication products were extracted from MS-8 agarose using the Nucleospin extract II KIT (Macherey-Nagel, Germany). Sequencing reactions were performed using the Big Dye terminator V3.1 cycle sequencing KIT (Applied Biosystems, CA, USA) with 10ng purified amplification product and 3.2pmoles 11F and 12R primers in separate reactions. Reactions were separated and analyzed on an ABI3730 DNA analyzer (Applied Biosystems, CA, USA). Predicted melting domains were generated using the POLAND program[14], with the 75mM NaCl algorithm (predicted Tm closely mimicked the observed Tm). A 75°C-82°C theoretical melt at 0.05°C intervals was analyzed to closely resemble the actual HRM analysis, with differential plots being used for the analysis. The predicted secondary structures for WT and FLT3 ITD sequences were calculated using the DINAMELT server using both the quikfold and two-state folding options[15], with a melting temperature of 60°C (same as annealing temperature for HRM PCR analysis), Na+ = 50mM and Mg2+ = 1.5mM.

RESULTS

Sensitivity comparison
To determine the reliable detection limit of FLT3 ITD mutations using each methodology, a dilution panel of a homozygote FLT3 ITD DNA sample (30bp ITD) was analysed (Figure 1).

The agarose gel electrophoresis method reproducibly gave the lowest detection limit using this panel. The 2% ITD/WT mix was easily detected in all experiments (Figure 1A), while the 1% ITD/WT mix could be seen in at least 50% of replicated experiments (results not shown). The capillary electrophoresis method was capable of reliably detecting the 5% mutant peak, with peak heights that were consistent with 5% of the total FLT3 signal (WT and mutant) (Figure 1B). Efforts to increase sensitivity by analyzing more PCR product were hampered by higher background levels and difficulty in interpreting the data due to double peaks and “channel bleeding” (results not shown). An alternative to improve sensitivity would be to use a dual-labeled PCR product (each primer labeled with either FAM or HEX)[14], which allows distinction between real amplification products
Figure 1: Comparison of methodology detection limits.

A) Agarose gel electrophoresis: Representative gel photograph of an ethidium bromide stained 3% MS-8 agarose gel showing the amplification and detection of FLT3 amplicons using DNA from WT (329bp), 100% FLT3/ITD (359bp), as well as 5% (329 and 359bp) and 2% (329 and 359bp) FLT3/ITD/WT dilutions (in triplicate).

B) Capillary electrophoresis: Representative electrophoretograms showing the successful detection of single FLT3 amplicons using WT and 100% FLT3/ITD DNA, as well as dual peaks for the 20% FLT3/ITD/WT and 5% FLT3/ITD dilutions.

C) Predicted melt profiles: Differential melting curves generated from WT and homozygous FLT3/ITD sequences using the POLAND14 analysis software. Absorption curves at both 260nm and 280nm are presented. The WT sequence shows one melting domain/peak, while two distinct domains/peaks are present in the homozygous mutant.

D) HRM: Difference and normalized graphs showing the melting profiles of various FLT3/ITD dilutions. 100%, 50% and 20% are indicated by dashed lines, while the solid lines represent WT, 2%, 5% and 10% mutant dilutions. Only those dilutions containing >20% mutant DNA produced curves that are readily discernible from the WT (>5 difference units).
Figure 2: AML patient DNA analysis: Agarose gel versus capillary electrophoresis.

A) Agarose gel electrophoresis: Representative gel photographs of ethidium bromide stained 3% MS-8 agarose gels showing the amplification and detection of FLT3 amplicons in patient samples: F7, F8, F9, F13 and F14 (in duplicate), as well as the relevant controls (WT (329bp), 100% FLT3 ITD (359bp), 5% (329 and 359bp) and 2% (329 and 359bp) FLT3/WT dilutions, reagent blank (BLK).

B) Capillary electrophoresis: Representative electrophoretograms showing the analysis of patient samples F1, F9 and F10, with the arrows indicating the relevant WT and FLT3/ITD peaks.

C) Sequence analysis of patient FLT3/ITD amplicons: The WT JM-domain sequence contained within the FLT3 amplicon is shown (NCBI accession number: NC_000013), with the positions of the switch, zipper and binding domains (including corresponding amino acid sequence numbers) indicated. The insertion sites of three patient ITD sequences (F1, F9, F13) are indicated by arrows and the inserted ITD sequence, the size and domain area duplicated is shown. Duplicated tyrosine residues Y589 and 591 are indicated in bold type (F1 and F13). F13 contains an inserted, unrelated 3bp insert prior to the FLT3 duplication, which is indicated by a * symbol.

Table 1: FLT3/ITD mutation analysis of AML patients: Methodology comparison.

<table>
<thead>
<tr>
<th>Method</th>
<th>F1</th>
<th>F2</th>
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<th>F15</th>
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<th>Time</th>
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<td>FLT3+ITD</td>
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<td>Variant</td>
<td>WT</td>
<td>R500</td>
<td>2.5hr re-melt: +2hr*</td>
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†Genotypes were only assigned when agreement was obtained between duplicate samples.

The cost for a single patient result also included the cost of the controls: WT, 100% FLT3/ITD, 2/5 or 20% FLT3/ITD and extraction blank. These values were calculated according to the costs of the reagents available in South Africa. R1 (RSA) = ± $8.

This indicates the processing time required from the PCR setup to obtaining a reportable result. For the HRM, the time indicated does not include the sequence confirmation.

When a re-melt analysis was required, a minimum time of 30 mins at 4°C was followed by an HRM program (1.5hrs).
and background noise. The advantages of both the agarose gel and capillary methodologies were the ease of data interpretation, with no complex analysis required to achieve consistent results and the robust nature of the assays, which allowed consistent replicate concordance.

Despite the use of a hot-start enzyme, the HRM protocol proved to be very sensitive to pre-amplification conditions, such as temperature and standing time. It was also imperative that both amplification and HRM data be thoroughly checked to ensure that each sample complied with strict inclusion/exclusion criteria (i.e. similar amplification curves, crossing point<30, similar HRM starting fluorescence)[17,18]. Failure to exclude non-adherent samples resulted in unreliable results. We also found that due to automated calling (based on difference graphs) relying solely on the input of one WT HRM profile, errors were often encountered. To prevent false calling, it was necessary to inspect the normalized curves, which clearly showed genotype groupings to correlate the automated results. Genotypes were only scored if there was concordance between the two graph types. As a result of this complexity and unusual sensitivity of the assay, several samples (especially duplicates) failed in each experimental run and had to be repeated.

To improve concordance between the normalized data and difference graphs, we found that a re-melt was often required to generate correctly clustered genotypes, especially with regards to the WT samples. It was postulated that this may be due to complex folding and melting dynamics that may be caused by secondary structures or multiple melt-domains in the large amplicon[17,18]. DNA sequence analysis of the WT and FLT3 ITD products, using DINAMESL[15] and POLAND[14], showed that although very little secondary structure was predicted and was identical between the amplicons (results not shown), the insertion of the 30bp ITD had created two melt-domains in the FLT3 ITD product, rather than a preferred single shifted peak (Figure 1C). Multiple melt-domains interfere with HRM efficiency[17,18], especially in a clonal environment and although the effects would be ITD-specific, we predicted that the sensitivity of the assay would be affected. This was indeed reflected in the results of this sensitivity analysis, with the lowest ITD dilution that could reproducibly indicate the presence of a mutant/variant being 20% (Figure 1D).

It is important to note, that when the amplification products generated by each PCR method were analysed using the 3% MS-8 agarose gel protocol, all showed similar sensitivities (2%), indicating that the amplicon analysis method rather than the PCR amplification was the limiting factor (results not shown).

**AML Patient analysis**

A small panel of DNA from 15 AML patients was analysed for FLT3 ITD mutations by all three methodologies, to investigate the correlation between them. We also determined the estimated cost of each method to generate a single patient result (including controls) and the processing time for complete FLT3 ITD analysis. A summary of the results is presented in Table 1. Figure 2 shows typical results from the gel and capillary analysis, while Figure 3 details the HRM analysis.

FLT3 ITD mutations were detected in DNA extracted from four AML patients (F1, F9, F13, F14), using both the MS-8 gel and capillary based analysis methods. All four patients had single ITD mutations. The advantage of the MS-8 gel method was the ability to easily isolate and purify the mutant product for sequence confirmation, which was performed on the F1, F9 and F13 ITD products (Figure 2C). They all showed a predicted clustering of the in-frame insertions within 30bp on exon14, with duplication of part of the JM zipper region (at least D593-L601), F1 and F13 also contained duplicated tyrosine residues Y589 and Y591 of the JM switch domain. The advantage of the capillary electrophoresis method was the determination of the insert size without sequence analysis, which in this instance showed ITD’s ranging from 21bp-75bp (Table 1). However, amplicon sequencing revealed the potential inaccuracy of this sizing, which has also been observed previously[13], when it consistently showed a different size for the F1 ITD (66vs63bp). Although both assays were capable of generating a result within the same time frame (± 4hrs), the agarose gel assay was half the price of the standard capillary assessment method (Table 1).

As with the previous analysis, despite protocol modifications such as holds at 50°C, 25°C or 95°C[17-19] to ensure complete renaturation prior to HRM, re-melt analysis was often required to generate consistent results, by causing tighter clustering of the WT samples (Figure 3A). For the patient analysis, this HRM protocol identified five variants in the patient cohort: F1, F9, F13, F14, as well as F6 (Figure 3B). F6, which generated a single amplicon, was sequenced and as no point mutations or homozygous ITD mutations were identified, was deemed to be a false positive.

F9 again demonstrated that the unpredictable nature of the ITDs is not well-suited to the HRM system. Although the FLT3 ITD allele in this sample represented approximately 30% of the total FLT3 alleles (calculated from the peak areas shown in Figure 2B), both the normalised and difference HRM curves for F9 showed only a subtle variation from WT (Figure 3B) and if a confidence co-efficient >75% had been used for automated genotyping, this variant would have been missed. Predicted melt-domain analysis of the FLT3 ITD sequences of F9, as well as F1 and F13, showed that all three generated different two melt-domain patterns, when compared to the WT amplicon (Figure 3C). As previously mentioned, the sequence and location of the ITD may affect the assay sensitivity unpredictably, reducing it below the 20% level seen earlier and lowering its detection reliability further. Although the HRM method was theoretically capable of producing a result within 2.5hrs, the frequent necessity to re-melt and re-analyze the data resulted in a similar analysis time to the other methods. The cost of the assay was however ± 5X higher than the gel methodology, making it the most expensive assay. Sequence analysis needed to confirm the result would also increase the cost further.

**DISCUSSION**

Most modern pathology facilities, especially within academic centers, have access to expensive molecular capillary analysers and real-time amplification instruments. These systems are deemed to be faster, more sensitive or capable of a higher sample throughput, which is particularly useful in a clinical-trial setting. These methodologies are then employed for the development of protocols that are widely published for the routine identification of mutations such as FLT3ITD’s. However, smaller more basic molecular laboratories, especially in non first-world countries have neither the advanced equipment, nor the customer base that are able to afford these tests and therefore do
Figure 3: AML patient DNA analysis: HRM analysis.

A) Normalized HRM curves showing the effects of a re-melt: The left-hand panel shows the clustering of WT and FLT3/ITD negative samples (grey lines) (retrospectively identified), as well as duplicate F14 patient samples (black) following the standard HRM analysis. The right-hand panel shows the analysis of the same sample cohort following a 2hr period at 4°C and a re-melt. This result demonstrates the tighter WT clustering that is obtained after a re-melt.

B) Normalized and difference graphs highlighting the FLT3/ITD positive AML patient samples (black), compared to the clustered FLT3/ITD negative samples (grey). This result indicates the variation in the melt curves due to the insertion of various sized ITD fragments. The insert shows the subtle curve shape difference between F9 and WT.

C) Predicted melt domains: Differential melting curves generated from the sequence data of the WT FLT3 amplicon, as well as the FLT3/ITD sequences of three AML patient samples (F1, F9 and F13). These curves represent predicted melting domains of homozygote populations only and clearly demonstrate the presence of different two-phased melting domains in these mutant amplicons.
not offer these important molecular assays. In order to make this assay more accessible, we evaluated the use of a modified gel electrophoresis assay for the detection of FLT3 ITD mutations, in comparison to the standard capillary electrophoresis, or HRM assays. This assessment included comparisons of assay sensitivity, accurate identification of mutations within a small patient cohort, as well as the processing time required to obtain a result and the cost factor.

Although agarose gel electrophoresis analysis of FLT3 ITD products has been used previously, we chose to enhance the discriminatory power of this technique with the use of MS-8 agarose, which improves the gel clarity and PCR product resolution. Using this agarose, the gel electrophoresis method gave the lowest reliable sensitivity limit of the three methods tested, being able to consistently detect 2% mutant alleles, which represent 1:50 mutant cells (homozygote). The assessment of the small AML patient cohort gave 100% concordant results with the standard capillary electrophoresis assay, with sequence analysis confirming the correct identification of mutant alleles. Not surprisingly, this method was considerably cheaper than the other technologies and required no complex molecular equipment or specially trained personnel for data interpretation. The processing time comparison also indicated that a FLT3 ITD mutation could be identified by both the capillary electrophoresis and agarose gel methods within four hours. Overall, in spite of the lure of advanced technology to provide faster and more sensitive analysis, in the case of FLT3 ITD mutation detection, basic molecular methodology proved more sensitive and cost-effective.

In several recent studies of large AML patient cohorts, FLT3 ITD mutations ranging from 15-231bp have been found, which occur in clonal populations representing 2-97% of FLT3 alleles. The agarose gel electrophoresis method presented here would be capable of detecting clones within these size and population parameters in a diagnostic environment.

What is still the subject of debate is whether additional information such as the extent (%) of the mutant clone, the accurate size of the ITD or the location of the insertion has further clinical impact. Most studies indicate that mutant populations representing >50% (which indicates homozygosity in some clones) confer a slightly worse prognosis compared to smaller ones. However, whether this further stratification would aid in patient management is not clear, with Gale et al., suggesting that irrespective of the size of the mutant population, FLT3 ITD positive patients would probably benefit from FLT3 inhibitors. As FLT3 ITD mutations seem to change in type and number unpredictably during disease progression, they cannot be used for minimal residual disease monitoring and therefore accurate quantification is unnecessary.

The size and location of the insert is more controversial, with the possibility that the involvement or duplication of more than one domain (switch, zipper or binding domains) and/or duplication of key residues such as the phosphorylation sites (YS89 and Y591), may impact more on the auto-inhibitory effects of the JM-binding domain and hence disease status than the ITD size itself. Due to the current lack of consensus, routine sequence analysis and accurate size determination has not yet been definitively proven to be clinically relevant. Thus although the agarose gel assay can easily be enhanced using basic gel-analysis software to accurately determine size and band intensities, the protocol presented here is adequate to provide data that is currently clinical relevant.

HRM technology is now being commonly used for the detection of SNPs, as well as small insertions and deletions in inherited diseases and some cancer types. However, its use in the detection of larger, unpredictable ITD’s in clonal populations has only been tested in a very limited number of studies, on a restricted HRM instrumentation platform (Roche). In this study we investigated the use of HRM using the Rotorgene and Quantace systems to detect FLT3 ITD mutations. As with previous HRM-FLT3 ITD studies, we found that this methodology was not very sensitive, only being able to detect mutations in clones ≥20%. This may well be due to the problem of ITDs causing the formation of multiple melting domains. In a homozygous population this would be an advantage, as the multiple domains would potentially generate not only a different shape to the melting curve, but also a temperature shift. The clonal environment (2%-97%) of these mutations however, complicates the analysis, as the ability to detect heterozygous populations is reduced when multiple melt-domains are present, leading to lower sensitivities for specific ITD’s. Unlike previous HRM studies, we found that the accuracy was not 100%, with a certain degree of false positivity (limited analysis). Several SNPs within exon14 of FLT3 have also recently been identified (clinical relevance not yet established) and HRM analysis would be able to detect these variants. It is therefore necessary to sequence all possible HRM “variants” to distinguish FLT3 ITD mutants from these SNP’s and false positives. Unfortunately this adds to the cost and processing time involved in using HRM for the detection of FLT3 ITD mutations. Despite the advantages of a single tube, one instrument system, the HRM assay investigated here is not well-suited for the detection of FLT3 ITD mutations in a smaller molecular laboratory.

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