A Comparison of Two Methods for Screening *CEBPA* Mutations in Patients with Acute Myeloid Leukemia

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The goal of the study was to compare the performance of a fluorescence-based multiplex PCR fragment analysis to a direct sequencing method for detecting *CEBPA* mutations in patients with acute myeloid leukemia. Thirty-three samples were selected from a larger study of 107 cases of acute myeloid leukemia by screening for *CEBPA* mutations by sequence analysis. Of ten identified mutations, six (insertions and deletions) were detected by both sequencing and fragment methods. The fragment analysis method did not detect the remaining four base substitutions because the method cannot detect changes that result in identically sized products. The multiplex PCR fragment length analysis method therefore failed to detect substitution mutations accounting for 40% of total *CEBPA* mutations in our patient set. Our results indicate that fragment length analysis should not be used in isolation, and that direct sequencing is required to evaluate *CEBPA* gene mutational status in acute myeloid leukemia. A combination of the two assays may offer some advantages, chiefly in permitting more sensitive detection by fragment length analysis of insertions and deletions. (J Mol Diagn 2009, 11:319–323; DOI: 10.2353/jmoldx.2009.080121)

CEBPA encodes a protein exclusively expressed in the myelomonocytic lineage. It is important for commitment to the granulocytic lineage and for differentiation of mature neutrophils.¹ The ability of *CEBPA* to induce granulocytic differentiation is enhanced by RAS-dependent phosphorylation of serine 248 of the *CEBPA* transactivation domain.² Leukemia-associated *CEBPA* gene mutations can be divided into two groups. One group of mutations consists of in-frame insertions in the basic/leucine zipper (bZIP) region that contains DNA-binding as well as dimerization domains. The other group of mutations consists of truncating out-of-frame insertions or deletions in the N-terminus, resulting in premature termination of the normal 42-kDa protein.³ Most of the frame-

shifting N-terminal mutations cause enhanced translation of a dominant-negative 30-kDa protein, which may be responsible for the differentiation block observed in acute myeloid leukemias expressing this protein.³

Mutations in the *CEBPA* gene are among the most common mutations in AML, particularly in patients with French-American-British (FAB) subtypes M1 and M2.^{4–6} The majority of leukemias with *CEBPA* mutations have a normal karyotype.^{7,8} Several studies of the prognostic significance of *CEBPA* mutations in patients with acute myeloid leukemia (AML) have shown that the presence of a *CEBPA* mutation is associated with significantly increased event-free survival, overall survival, and remission duration.^{2,6,7} In addition, sequential analysis of *CEBPA* mutations revealed that the same mutation was present at diagnosis and relapse, but not in complete remission.^{4,9}

Accurate testing for *CEBPA* mutations is important for identification of this prognostically significant AML subtype. AML with mutated *CEBPA* is a provisional disease entity in the 2008 World Health Organization classification of leukemias.¹⁰ Many prior studies have used direct sequencing of PCR products for mutation screening. Although it is a reliable gold standard for detection of mutations, direct sequencing of PCR product is a relatively labor-extensive, time-consuming, and expensive procedure. To overcome these limitations, a novel multiplex fluorescence-based capillary electrophoresis analysis method for screening of *CEBPA* mutations has been recently described. This multiplex PCR with fluorescence fragment analysis assay has been described as a simple, highly sensitive, accurate, and reproducible method for screening for *CEBPA* mutations. This method reportedly detects mutant DNA percentages as low as 5%.¹¹

In the present study, we tested the validity of the fluorescence-based multiplex PCR fragment analysis method for the detection of *CEBPA* mutations in AML patient samples when compared with direct sequencing results, and found that it failed to detect a significant proportion of patient mutations.

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Table 1. Primers Used for Sequencing Method

Primer	Sequence
Set 1 FWD	5'-TCGCCATGCCGGGAGAACTCTAAC-3'
Set 1 REV	5'-CCTGCTGCCGGCTGTGCTGGAAC-3'
Set 2 FWD	5'-CTTCAACGACGAGTTCCTGGCCGA-3'
Set 2 REV	5'-AGCTGCTTGGCTTCATCCTCCT-3'
Set 3 FWD	5'-CCGCTGGTGATCAAGCAGGA-3'
Set 3 REV	5'-CCGTACTCGTTGCTGTTCT-3'
Set 4 FWD	5'-CAAGGCCAAGAAGTCGGTGGACA-3'
Set 4 REV	5'-CACGGTCTGGGCAAGCCTCGAGAT-3'

Materials and Methods

Subjects

Patient samples of bone marrow or peripheral blood were collected at the time of diagnosis before initiation of treatment, in the Department of Pathology, Stanford University Medical Center from 2003 to 2007. Thirty-three samples were selected from a larger study of AML in which 107 cases were originally studied by screening for *CEBPA* mutations by sequence analysis alone. To compare the performance of fragment analysis and the sequencing method, samples were chosen from the sequence positive and negative cases solely on the basis of DNA availability. Among 33 AML patients, according to the 2001 World Health Organization classification, 21 were AML not otherwise categorized (including 1 M0, 3 M1, 5 M2, 1 M4, 8 M5, 1 M6, and 2 not further classified in the FAB classification), 8 were AML with multilineage dysplasia, 2 were acute promyelocytic leukemia, 1 was therapy-related AML, and 1 was biphenotypic leukemia (myeloid/T-cell). Twenty-nine of the 33 cases had cytogenetic data available, and 16 of those showed a normal karyotype. Archived genomic DNA was retrieved for the mutation analysis methods used in this study. The study was approved by the Stanford University Institutional Review Board.

CEBPA Mutation Analysis

Direct Sequencing of PCR Products

For *CEBPA* mutational analysis, the entire coding region of human *CEBPA* was PCR amplified using four pairs of primers as described previously (Table 1 and Figure 1).⁵ The total reaction volume of 50 μ l contained 100 ng of

Table 2. Primers Used for Multiplex-PCR Fragment Analysis

Primer	Sequence
TAD1 FWD	HEX-5'-TCGGCCGACTTCTACGAGGC-3'
TAD1 REV	5'-GCGCCCGGGTAGTCAAAGT-3'
TAD2 FWD	NED-5'-TACCTGGACGGCAGGCTGGA-3'
TAD2 REV	5'-TGCAGGTGCATGGTGGTCT-3'
bZIP FWD	FAM-5'-AGAAGTCGGTGGACAAGAACA GCAA-3'
bZIP REV	5'-AGTTGCCCATGGCCTTGAC-3'

DNA, 200 μ mol/L of each dNTP, 0.2 μ mol/L of primers, 1.5 mmol/L (for primer sets 1 and 4) or 2.75 mmol/L (for primer sets 2 and 3) $MgCl_2$, 1 $\times$ buffer, 2.5 U of HotStarTaq DNA Polymerase, 1 $\times$ Q solution especially for GC-rich template (Qiagen, Valencia, CA). PCR conditions were as follows: 95°C for 15 minutes; 35 cycles of 94°C for 60 second, 59°C (primer sets 1 and 4) or 55°C (primer sets 2 and 3) for 40 seconds, 72°C for 90 seconds or 60 seconds, respectively; 72°C for 10 minutes. PCR products were electrophoresed in 2% Tris-Acetate-EDTA agarose gels and then purified with a QIAquick PCR purification kit (Qiagen). The purified products were sequenced using BigDye Terminators with ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). After initial sequencing analysis, ambiguous or abnormal sequences were sequenced again or cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Ten colonies, randomly picked, were cultured in 2 ml Lennox L broth Base medium overnight and plasmid DNA was isolated using pureLink quick plasmid miniprep kit (Invitrogen) and then sequenced. The criterion for a clonal mutation was that a case should have two or more mutated colonies. The sample sequences were compared with *CEBPA* genomic sequences (U34070) using SeqScape Software Version 2.1 (ABI).

Multiplex-PCR Fragment Analysis

For fragment analysis, the previously published primers with slight modification (Dye change from VIC to HEX for TAD1- FWD primer) were used (Table 2 and Figure 1).¹¹ Three labeled PCR fragments were generated using 100 ng of genomic DNA, 1 $\times$ PCR buffer containing 1.5 mmol/L $MgCl_2$, 1 $\times$ Q solution, 200 μ mol/L of each dNTP, 2.5 U of HotStartTaq DNA polymerase, 0.2 μ mol/L of primers (TAD1 and bZIP) and 0.25 μ mol/L of primer (TAD2). The PCR program conditions were an initial incubation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 90 seconds, and elongation at 72°C for 60 seconds. The last cycle was followed by a 10-minute elongation step at 72°C. After amplification, 1 μ l of PCR product was added to 9 μ l of highly deionized formamide and 0.5 μ l of GENE scan 400HD internal size standard (Applied Biosystem, Foster City, CA). The mixture was denatured at 95°C for 2 minutes, immediately chilled on ice and analyzed by capillary electrophoresis on an ABI3100 Genetic Analyzer using 36-cm capillaries and POP-4 polymer. Data were analyzed with GeneMapper v3.0 software (Applied BioSystem). To establish cutoff values, +1 or -1 bp of average of 10 sequence-confirmed, wild-type fragments

Figure 1. Schematic representation of the location of multiplex-PCR fragment and direct sequencing primers on *CEBPA*.

Table 3. Comparison between Direct Sequencing and Multiplex-PCR Fragment Analysis in Patients with *CEBPA* Mutations and Polymorphisms

Sample ID	Age	Sex	WHO (FAB) classification	Cytogenetic results	Direct sequencing of PCR product*	Multiplex PCR Fragment analysis
D25	24	M	AML, NOC (M5)	46,XY [20]	nt 733-734 ins GGGG, nt 1516-1517 ins CCAAGCAGCGCAA CGTGG	TAD1: +4 bp, bZIP: +18 bp
D31	50	F	AML, NOC (M1)	46,XX [20]	nt 1159 ins T	TAD2: +1 bp
E5	67	M	AML, NOC (M2)	46,XY [20]	nt1440 sub CG>GT	Wild type
G6	69	F	AML, NOC (M2)	46,XX [20]	nt 1490 sub G<T	
G9	58	F	AML, NOC (M2)	46,XX [20]	nt 708 del C and nt 1528-1529 ins AGA	bZIP: +3 bp
A026	47	F	AML, NOC (M5)	46,XX,t (4;13;15) (q21;q14;q26) [cp17] /46,XX [3]	nt 939 sub C>T	TAD1: -1 bp, Wild type
A027	43	M	AML, NOC (M1)	46,XY,del(920) 9 9q11.20 [2] /46,XY [18]	nt 787 sub GCCT>CTAC	Wild type
C005	46	M	AML, NOC (M5)	46,XY [20]	nt 1505 ins AGG	bZIP: +3 bp
A010	49	F	APL ⁺ (M3)	46,XX,t (15;17) (q22;q210) [20]	nt 1175-1180 dup [‡]	TAD2: +6 bp
G2	70	M	AML with multilineage dysplasia	Complex including abnormalities chromosome 5 and 7	nt 1175-1180 dup [‡]	TAD2: +6 bp

*Sequencing numbering according to GenBank accession U34070.

[†]APL, acute promyelocytic leukemia.

[‡]Polymorphism.

were calculated. If the fragment sizes fell within the wild-type range (bZIP: 238.5-240.5 bp, TAD1: 313.7-312.7 bp, TAD2: 243.6,-245.6 bp), we considered the case to be negative.

Results

Characterization of Patients with *CEBPA* Mutations

CEBPA mutations were identified in the AML specimens from eight patients, all of which were considered AML not otherwise categorized by the 2001 World Health Organization classification and would be considered M1 (n = 2), M2 (n = 3), or M5 (n = 3) in the FAB classification. Cytogenetic data were available in all *CEBPA*-mutated patients: two patients (M1 and M5 in the FAB classification) had an abnormal karyotype. The other six patients had a normal karyotype. All karyotypes were in the intermediate Medical Research Council cytogenetic risk subgroup (Table 3).

Figure 2. Schematic representation of the ten *CEBPA* mutations detected in the current study.

Characterization of *CEBPA* Mutations

We identified 10 sequence changes compared with the reference sequence for *CEBPA* in eight patient samples by direct sequencing (Figure 2 and 3, Table 3). An additional two patient samples demonstrated known insertional polymorphism sequence variants. Of the 10 non-polymorphism sequence variants, only six were detected by multiplex-PCR fragment analysis. Fragment length analysis detected two mutations in the TAD1 region, three in the bZIP domain, and one in the TAD2 region. Among

Figure 3. Gene scan electropherogram from multiplex PCR method and partial sequence of *CEBPA* from sequencing method (numbering according to GenBank access number U34070). **A:** Detected mutation (G9: nt 1528 to 1529 ins AGA) by sequencing method and fragment analysis methods. **B:** Detected mutation (A027: nt 787 sub GCCT>CTAC) by sequencing method alone. * W: wild (normal) peak, M: mutation peak.

the 10 mutations detected by sequencing, six were the insertions or deletions that were detected by the multiplex PCR fragment analysis, while the remaining four mutations were substitutions. The four substitution mutations were: nt 1440 (CG→GT), nt 1490 (G→T), nt 939 (C→T), and nt 787 (GCCT→CTAC) with numbering according to GenBank accession number U34070. One case with a single base substitution mutation in the bZIP region (patient G6) initially appeared to demonstrate a single base insertion in the fragment analysis assay, but on repeat testing showed a wild-type length fragment, indicating that the fragment length assay can have variable reproducibility. Samples from two patients had a TAD2 (1175 to 1180dup: +6 bp) insertion previously reported as a polymorphism.^{9,12,13} One polymorphism (patient G2) was initially detected only by fragment analysis. That case was classified as wild-type without a polymorphism by initial sequencing analysis, but the polymorphism was confirmed after sequencing more colonies.

Discussion

Within the hematopoietic system, *CEBPA* encodes a leucine zipper transcription factor that is important for normal myeloid cell differentiation. Expression of *CEBPA* is detectable in early myeloid precursors and is up-regulated as cells commit to granulocytic differentiation and maturation.¹⁴ Mature granulocytes are not observed in *CEBPA*-deficient mice, whereas other cell types are present in normal proportions. These studies indicate a crucial role for *CEBPA* in granulocytic lineage development, and make it unsurprising that *CEBPA* gene mutations can be involved in the pathogenesis of myeloid leukemias.¹⁵ Mutations of *CEBPA* are found in 7% to 15% of AML cases (most frequently categorized in FAB-M1 and M2 and 2001 World Health Organization AML not otherwise categorized), and are present in as many as 15% to 18% of AML cases with normal karyotype.⁶⁻⁹ In this study, all *CEBPA*-mutated patients were in the intermediate MRC cytogenetic risk subgroup. Among those *CEBPA*-mutated patients, there were six with a normal karyotype and two with abnormal karyotypes. Our data confirm the prevalence of *CEBPA* mutations in AML patients with intermediate MRC cytogenetic risk subgroup.

Ideally, screening tests for genetic mutations in patients with AML should be accurate, rapid, inexpensive, and automated, since this testing is used both for initial diagnosis and in the evaluation of possible relapse. Multiplex PCR fragment analysis might be considered an appealing method for screening of *CEBPA* mutations in patients with AML given its relative ease, rapidity, and sensitivity for detecting insertions and deletions. This method can detect mutant DNA representing only 5% of total DNA,¹¹ and it could potentially be used to detect early relapse in AML patients after complete remission. However, there are several important limitations of multiplex PCR fragment analysis in detecting *CEBPA* mutations. Most critically, size-based fragment analysis cannot detect base substitutions. It is reported that 18% to 44% of *CEBPA* mutations are base substitutions.^{4,8} Our

results showed that of ten detected mutations from eight samples, four (40% of mutations and half of patients) were substitutions that could be identified only by direct sequencing. Therefore, fragment analysis alone is not a thorough way to screen for *CEBPA* mutation in AML patients. Additional problems with the published fragment length assay are the lack of full coverage of the coding region of *CEBPA* (a GC-rich region of the gene is omitted), and the possibility of missing insertion or deletion mutations if they lie at the sites of PCR primer binding.¹⁶ Given that mutations are spread through all areas of the *CEBPA* gene, some clinically relevant mutations may be present in regions of the gene that are not analyzed using this method. Fragment analysis is also unable to distinguish between mutations and polymorphisms and a positive result would require sequencing to determine whether a clinically relevant mutation is present. Finally, the fragment analysis method may have a higher rate of false positive results when compared with sequencing (see below).

One case (patient G6) in our study showed a discrepancy between the fragment analysis and sequencing methods, which proved to be due to a non-reproducible fragment length assay result. According to Lin's study, the between-run reproducibility for fragment length analysis may show a coefficient of variation of 1.5% to 3.7%.¹¹ It is important to note that there are several factors affecting the accuracy and reproducibility of this method such as instrumentation, gel formulation, size standards, and gel running conditions for automated fluorescent fragment analysis.

Typically, direct sequencing is a useful and reliable gold standard for the detection of mutations. Although it is not always the most sensitive or successful method,¹⁷⁻¹⁹ it allows immediate and detailed analysis of PCR amplified DNA fragments. Direct sequencing is a more time-consuming and laborious method than fragment analysis, particularly if an ambiguous or abnormal result necessitates cloning of the PCR amplicon and sequencing of multiple subclones for result confirmation. The increased time and labor required for the sequencing method makes it suboptimal for screening and follow-up for early detection of relapse in patients with AML. Additionally, the results of direct sequencing are detected by direct visualization of sequence information, which can have limited sensitivity because of background noise in the generated chromatogram. For instance, DNA mixing experiments have demonstrated that for most point mutations, automated sequencing is only sensitive down to about 20% of mutant DNA in a wild-type background.²⁰ In our study, the polymorphic six bp insertion in the sample from patient G2 was detected in only 1 of 10 colonies, but was evident in the fragment length analysis. Although this particular insertion was a polymorphism, it is possible that a true mutation could be missed by a sequencing method alone due to low detection limit.^{9,11,12}

Taken together, our results suggest that the optimal approach for detecting *CEBPA* mutations in AML may be a combination of direct sequencing and a modified version of the fragment length analysis assay. Direct se-

quencing is absolutely necessary to detect substitution mutations, but concurrent fragment length analysis offers greater sensitivity for detecting insertion or deletion mutations. Fluorescently tagged primers with the same sequences as those used to generate amplicons for sequencing could generate fragments for length analysis, allowing direct correlation between the two methods, and combining the sensitivity of fragment length analysis with the thoroughness of direct sequencing. Finally, improved high-throughput DNA sequencing methods may soon offer an even better solution by permitting deep sequencing of amplicons and detection of low levels of prognostically significant mutations.

References

1. Tenen DG, Hromas R, Licht JD, Zhang DE: Transcription factors, normal myeloid development and leukemia. *Blood* 1997, 90:489–519
2. van Waalwijk van Doorn-Khosravani SB, Erpelinck C, Meijer J, van Oosterhout S, van Putten WLJ, Valk PJM, Beverloo HB, Tenen DG, Löwenberg B, Delwel R: Biallelic mutations in the *CEBPA* gene and low *CEBPA* expression levels as prognostic markers in intermediate-risk AML. *Hematol J* 2003, 4:31–40
3. Schwieger M, Löhler J, Fischer M, Herwig U, Tenen DG, Stocking C: A dominant-negative mutant of C/EBP α , associated with acute myeloid leukemias, inhibits differentiation of myeloid and erythroid progenitors of man but not mouse. *Blood* 2004, 103:2744–2752
4. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG: Dominant-negative mutations of *CEBPA*, encoding CCAAT/enhancer binding protein- α (C/EBP α), in acute myeloid leukemia. *Nat Genet* 2001, 27:263–270
5. Snaddon AJ, Smith ML, Neat M, Cambal-Parrales M, Dixon-McIver A, Arch R, Amess JA, Rohatiner AZ, Lister TA, Fitzgibbon J: Mutations of *CEBPA* in acute myeloid leukemia FAB types M1 and M2. *Genes Chromosomes Cancer* 2003, 37:72–78
6. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, Thomas X, Raffoux E, Lemandin C, Castaigne S, Fenaux P, Dombret H: Favorable prognostic significance of *CEBPA* mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood* 2003, 100:2717–2723
7. Fröhling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Döhner H, Döhner K: *CEBPA* mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. *J Clin Oncol* 2004, 15:1–10
8. Bienz M, Ludwig M, Mueller BU, Leibundgut EO, Ratschiller D, Solenthaler M, Fey MF, Pabst T: Risk assessment in patients with acute myeloid leukemia and a normal karyotype. *Clin Cancer Res* 2005, 15:1416–1424
9. Lin LI, Chen CY, Lin DT, Tsay W, Tang JL, Yeh YC, Shen HL, Su FH, Yao M, Huang SY, Tien HF: Characterization of *CEBPA* mutations in acute myeloid leukemia: most patients with *CEBPA* mutations have biallelic mutations and show a distinct immunophenotype of the leukemic cells. *Clin Cancer Res* 2005, 11:1372–1379
10. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press, Lyon, 2008
11. Lin LI, Lin TC, Chou WC, Tang JL, Lin DT, Tien HF: A novel fluorescence-based multiplex PCR assay for rapid simultaneous detection of *CEBPA* mutations and NPM mutations in patients with acute myeloid leukemias. *Leukemia* 2006, 20:1899–1903
12. Wouters BJ, Louwers I, Valk PJM, Löwenberg B, Delwel R: A recurrent in-frame insertion in a *CEBPA* transactivation domain is a polymorphism rather than a mutation that does not affect gene expression profiling-based clustering in AML. *Blood* 2007, 109:389–390
13. Fuchs O, Provaznikova D, Kocova M, Kostecka A, Cvekova P, Neuwirtova R, Kobylka P, Cermak J, Brezinova J, Schwarz J, Markova J, Salaj P, Klamova H, Maaloufova J, Lemez P, Novakova L, Benesova K: *CEBPA* polymorphisms and mutations in patients with acute myeloid leukemia, myelodysplastic syndrome, multiple myeloma and non-Hodgkin's lymphoma. *Blood Cells Mol Dis* 2008, 40:401–405
14. Radomska HS, Huettnner CS, Zhang P, Cheng T, Scadden DT, Tenen DG: CCAAT/enhancer binding protein α is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998, 18:4301–4314
15. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG: Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α deficient mice. *Proc Natl Acad Sci* 1997, 94:569–574
16. Antonson P, Xanthopoulos KG: Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). *Biochem Biophys Res Commun* 1995, 215:106–113
17. Ley TJ, Minx PJ, Walter MJ, Ries RE, Sun H, McLellan M, DiPersio JF, Link DC, Tomasson MH, Graubert TA, McLeod H, Khoury H, Watson M, Shannon W, Trinkaus K, Heath S, Vardiman JW, Caligiuri MA, Bloomfield CD, Milbrandt JD, Mardis ER, Wilson RK: A pilot study of high-throughput, sequence-based mutational profiling of primary human acute myeloid leukemia cell genomes. *Proc Natl Acad Sci* 2003, 100:14275–14280
18. Strausberg RL, Simpson AJG, Wooster R: Sequence-based cancer genomics: progress, lessons and opportunities. *Nat Rev Genet* 2003, 4:409–418
19. Weber BL: Cancer genomics. *Cancer Cell* 2002, 1:37–47
20. Smith TA, Whelan J, Parry PJ: Detection of single-base mutations in a mixed population of cells. *Genet Anal Tech Appl* 1992, 9:143–145