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Rapid sequencing of the *p53* gene with a new automated DNA sequencer

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***p53* is the most commonly mutated gene in human cancers. Approximately 90% of the *p53* gene mutations are localized between domains encoding exons 5 to 8. Sequencing methods currently available are tedious and time-consuming and are not suitable for routine laboratory testing. In an effort to identify a simple and rapid sequencing method, we analyzed 16 preselected breast tumors and 18 preselected ovarian tumors, using a newly developed automated DNA sequencer. *p53* gene mutations had been previously identified in these tumors, using a conventional automated sequencing procedure. Exons 5 to 8 were amplified by PCR, and the PCR products were subsequently subjected to cycle sequencing with the Sanger chain termination method, using Cy5.5-labeled primers. The sequencing mixture was then resolved on a newly developed automated DNA sequencer that can sequence ~300 bases of DNA in 30 min. Of these 16 breast tumors, two had mutations in exon 5, four in exon 6, three in exon 7, and three in exon 8. Of the 18 ovarian tumors, two had mutations in exon 5, five in exon 6, two in exon 7, and three in exon 8. In all cases, we identified the same mutations by both the new and the conventional sequencing procedures. Most mutations affected an arginine codon. These data demonstrate that the new method has the capability to provide accurate sequencing information in a fraction of the time and labor in comparison with current automated sequencing techniques. When such procedures are used, DNA sequencing may become a routine tool for identifying clinically important mutations for diagnosis and prognosis of patients with genetic, malignant, infectious, and other diseases.**

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p53 is a 53-kDa protein that is frequently mutated in human cancer *(1)*. Many studies have confirmed that the mutations at the *p53* locus constitute the single most common genetic alteration observed in human cancer *(2)*. This tumor suppressor gene is known to control cellular growth after DNA damage through mechanisms involving cell cycle arrest and programmed cell death *(3)*. The wild-type p53 functions as a cell cycle check point, activated by insults against the integrity of the genome. Cellular stresses such as DNA damage induce p53 protein accumulation, which transcriptionally modulates the expression of genes, triggering either growth arrest or apoptosis. p53 inactivated by mutations is generally nonfunctional, leading to a failure to arrest or delete cells harboring potentially tumorogenic mutations. Some mutated forms of *p53* gene may even gain the ability to stimulate cell growth *(4)* and promote tumor induction and progression *(5)*.

The human *p53* gene is located on chromosome 17p13 and is composed of 11 exons and 10 intervening introns. The protein consists of a highly charged acidic amino-terminal domain, which can interact with components of the transcriptional machinery, and a highly charged basic carboxy-terminal region, which can mediate oligomerization, nuclear localization, and possibly the recognition of damaged DNA. The central portion contains the DNA-binding core, within which the vast majority of single amino acid substitutions occur when the *p53* gene is mutated. A database assembled from nearly 1500 p53 mutations from cancers of different cell or tissue types indicated that almost 85% are point missense mutations leading to a functionally defective protein *(6)*. Approximately 90% of these missense mutations have been shown to be localized in the DNA-binding domain of p53, which is encoded by exons 5 to 8 *(7)*. Another consequence of missense mutation in this domain is the increased stability of the encoded mutant p53 protein, which accumulates in tumor cell nuclei. Amino acids R248 and R273, the most frequently mutated residues in the *p53* gene, exhibit direct contact with DNA in the consensus p53 binding sequence. In total, ~40% of the missense mutations are localized at the "hotspot" residues R175, G245, R248, R249, R273, and

R282 *(8)*, which play a vital role in the structural integrity of the DNA-binding domain. Sequencing of the core region alone allows the detection of the majority of the mutations *(9)*.

Many techniques have been used to identify p53 abnormalities at either genetic or protein levels. These procedures, including immunohistochemistry and single-strand conformation polymorphism, are plagued with inconsistencies and often are difficult to interpret *(6, 10)*. Therefore, the use of sensitive and reproducible methods is imperative if the objective is to gain definitive and reliable assessment of the mutational status of the *p53* gene. Such information is very useful and has established prognostic implications for the cancer patient *(11–14)*. Direct sequencing is the most accurate technique to assess the mutational status of the *p53* gene, but many clinical laboratories do not use it because of the complexity, cost, and labor-intensive nature of the currently available techniques. This study evaluates a new DNA-sequencing method for assessing the *p53* gene status. The method is based on new instrumentation that simplifies some steps of the procedure and appears to be suitable for routine DNA diagnostic applications.

Materials and Methods

TUMOR EXTRACTION

Breast and ovarian tumor tissues that had been previously sequenced using the ALFexpress automated DNA sequencer (Pharmacia Biotech) were used in this study *(15)*. Approximately 200 mg of the tumor tissue, which contained >70% tumor cells as determined by histologic examination, was pulverized to a fine powder at −70 °C. DNA was extracted and purified using a conventional phenol-chloroform-based procedure *(16)*, quantified by measurements at 260 nm, and stored at 4 °C until analysis.

PCR AMPLIFICATION

The paired primer sequences flanking each of the exons 5 to 8 of the *p53* gene are shown in Table 1. All oligonucleotide primers were designed using computer software Oligo 5.0 (National Biosciences), according to the *p53* sequence deposited in GenBank by Chumakov et al. (accession no. 54156). PCR amplification of each exon was performed in a final volume of 25 μL, containing ~100 ng of template DNA, 10 mmol/L tromethamine (Tris) buffer, pH 8.3, 50 mmol/L KCl, 1 unit of AmpliTaq Polymerase (Hoffmann-La Roche), 250 μmol/L deoxynucleoside triphosphates, 1.5 mmol/L $MgCl_2$, and 1 μmol/L of each primer. PCR primers and other PCR reagents were part of the *p53* gene sequencing kit that was used in this study and provided by Visible Genetics Inc., Toronto, Ontario, Canada (www.visgen.com or info@visgen.com). The thermal cycling profile consisted of a 30-s denaturation step at 94 °C, a 30-s annealing step at 60 °C, and a 30-s extension step at 70 °C, for a total of 35 cycles. Each PCR was initiated with a 5-min denaturation at 94 °C and terminated with a 5-min extension at 70 °C. The success of the PCR was verified by running a 5-μL aliquot of the PCR product on a 0.8% agarose gel containing ethidium bromide.

DNA SEQUENCING

The primers used for sequencing the PCR-amplified *p53* exons 5 to 8 with the Visible Genetics system (see below) were labeled at the 5′ end with the fluorescent dye Cy5.5 (Amersham International). Their sequences are shown in Table 1. The same primers, labeled with Cy5 fluorescent dye, were used to sequence the PCR products on the Pharmacia ALFexpress system. The detailed procedure for sequencing these samples with the ALFexpress system has been described elsewhere *(15)*.

The sequencing primers, labeled with Cy5.5, are also part of the Visible Genetics *p53* gene sequencing kit. The primers were prepared as follows. During standard phosphoramidite synthesis of the primers, the 6-(trifluoroacetylamino)propyl-(2-cyanoethyl)-(*N*, *N*-diisopropyl)-phosphoramidite (5′-amino modifier, C3-TFA) was introduced at the 5′ end of each sequencing primer. The base-labile TFA protecting group of the 5′-amino modifier TFA was removed by ammonium hydroxide treatment. The free amino group of the primer was then reacted with an *N*-hydroxysuccinimide ester of the fluorescent dye Cy5.5 (Amersham) to obtain the labeled primer, which was purified by reversed-phase high performance liquid chromatography.

In the sequencing procedure, we used reagents from Visible Genetics. Briefly, an aliquot of the PCR product (1–8 μL depending on the yield, no purification necessary) was mixed with Sequenase™ buffer, dimethyl sulfoxide, sequencing primer, water, and Thermoseque-

Table 1. Primers for PCR amplification and DNA sequencing of *p53* exons 5 to 8.

Exon	Strand	PCR primers	Sequencing primers
5	Sense	5′-CACTTGTGCCCTGACTTT-3′	5′-TCTTTGCTGCCGTGGTTCC-3′
	Antisense	5′-CCTGGGACCCTGGGCAA-3′	5′-CCTGGGACCCGTTGGTCG-3′
6	Sense	5′-TGTTCACTTGTGCCCTGACT-3′	5′-TGGTTGCCCAGGGTCCCC-3′
	Antisense	5′-GGAGGGCCACTGACAACCA-3′	5′-CCACCCTTACCCCTCC-3′
7	Sense	5′-GGCGACAGAGCGAGATTCCA-3′	5′-CTCCCCTGCTTGCCACA-3′
	Antisense	5′-GGGTCAGCGGCAAGCAGAGG-3′	5′-TCAGCGGCAAGCAGAGG-3′
8	Sense	5′-GACAAGGGTGGTTGGGAGTAGATG-3′	5′-ATGGGACAGGTAGGACC-3′
	Antisense	5′-GCAAGGAAAGGTGATAAAAGTGGAA-3′	5′-CATAACTGCACCCTTGG-3′

nase™ enzyme (Amersham). This mixture was then distributed into four tubes (5 μL/tube, labeled A, C, G, or T), and the nucleotide termination mix was added. The mixture was then cycled for 35 cycles on a PCR machine as follows: 94 °C for 30 s, 55 °C (exon 5) or 60° C (exons 6 and 8) or 68° C (exon 7) for 30 s, and 70 °C for 60 s. The thermal cycling was initiated with a 5-min denaturation step at 94 °C and terminated with a 5-min extension step at 70 °C. At the end, 6.0 μL of the loading dye was added to each of the four tubes to stop the sequencing reaction. The sequencing samples were heated at 95 °C for 5 min and then placed on ice before 2.0 μL was loaded on the sequencing gel. Details of the sequencing mixes, including concentrations of dNTPs and ddNTPs, are described in the insert of the *p53* gene sequencing kit.

The Microgene Blaster™ automated DNA sequencer is a compact sequencing device containing a visible light laser diode (675 nmol/L) as the excitation source. The laser power is 0.5 mW/lane. The machine applies 1500V across the sequencing gel and can complete a run in ~30 min, resolving ~300 bases of sequence. The instrument accepts a 14 × 14 cm sequencing gel, 50 μm thick. The gel is cast between two disposable glass plates (MicroCel™ cassette). In our study, each gel was capable of analyzing four patient samples (16 lanes; 4 lanes per sample for A, C, G, and T sequencing reaction tubes). The cassette was filled with 6% acrylamide solution containing a photoinitiator that was activated by UV light on a special unit (Gel Toaster™ polymerizing unit). The process of gel filling and casting took ~5 min.

Once the gel was ready, it was positioned into the sequencer, and the buffer chambers were filled with 150 mL of a 1× Tris-borate-EDTA buffer, pH 8.3. The gel temperature and voltage were set at 50 °C and 1500 V, respectively. A 5-min prerun was initiated to bring the gel and the temperature to the set values. Each of the 16 lanes was loaded with 2 μL of the cycle sequencing reactions. The electrophoresis time was 30 min. Data acquisition and interpretation was achieved with Gene Objects™ software (Visible Genetics).

The Gene Objects software includes modules for both base calling and fragment analysis. Part of the base calling algorithm is the selection of thresholds for heterozygote detection, i.e., no threshold, 50%, 20%, and 15% thresholds. When the instrument detects two peaks of different intensities on the same position, it will call it an ambiguity (possible heterozygosity) if the intensity of the second peak, in comparison with the main peak, is more than the specified threshold. In addition, the software includes a comparator tool that compares two sequences and automatically lists all the differences including insertions, deletions, and base changes. Once a difference is identified, the comparator can realign downstream bases of the differing allele to produce a clear comparison. This option allows the operator to compare a generated sequence with a reference sequence without the need for manual com-

Table 2. *p53* mutations in breast and ovarian tumors detected by automated sequencing.

Sample number	Code/tumor type	Exon	Codon	Nucleotide change	Predicted amino acid change
1	Breast	5	132	A—G	Lys to Glu
2	Breast	5	163	A—G	Trp to Cys
3	Breast	6	213	A—G	Arg to Arg
4	Breast	6	213	A—G	Arg to Arg
5	Breast	6	220	A—G	Trp to Cys
6	Breast	6	220	A—G	Trp to Cys
7	Breast	7	245	G—A	Gly to Ser
8	Breast	7	248	C—T	Arg to Trp
9	Breast	7	249	G—C	Arg to Thr
10	Breast	8	278	C—T	Pro to Ser
11	Breast	8	280	G—C	Arg to Thr
12	Breast	8	283	C—T	Arg to Cys
13	Ovarian	5	175	G—A	Arg to His
14	Ovarian	5	175	G—A	Arg to His
15	Ovarian	6	193	C—G	His to Asp
16	Ovarian	6	194	T—G	Leu to Arg
17	Ovarian	6	196	C—T	Arg to stop
18	Ovarian	6	213	A—G	Arg to Arg
19	Ovarian	6	213	A—G	Arg to Arg
20	Ovarian	7	248	C—G	Arg to Gly
21	Ovarian	7	248	G—T	Arg to Leu
22	Ovarian	8	272	G—A	Val to Met
23	Ovarian	8	273	G—A	Arg to His
24	Ovarian	8	280	A—G	Arg to Gly

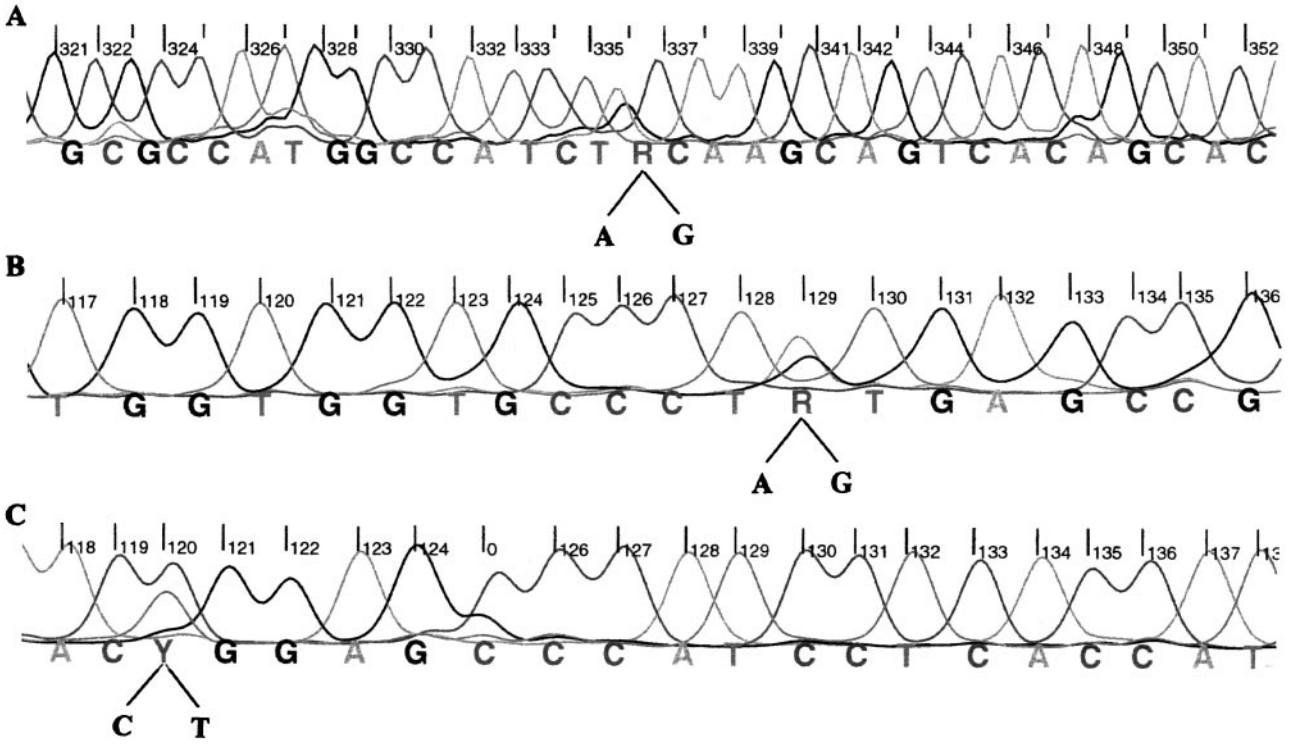

Fig. 1. DNA sequencing of the *p53* gene with the Microgene Blaster automated DNA sequencer: examples of point mutations in breast tumors. (*A*) A → G point missense (Tyr → Cys) mutation in exon 5, sample 2; (*B*) A → G point missense (Tyr → Cys) mutation in exon 6, sample 5; (*C*) C → T point missense (Arg → Trp) mutation in exon 7, sample 8. R: A or G; Y: C or T.

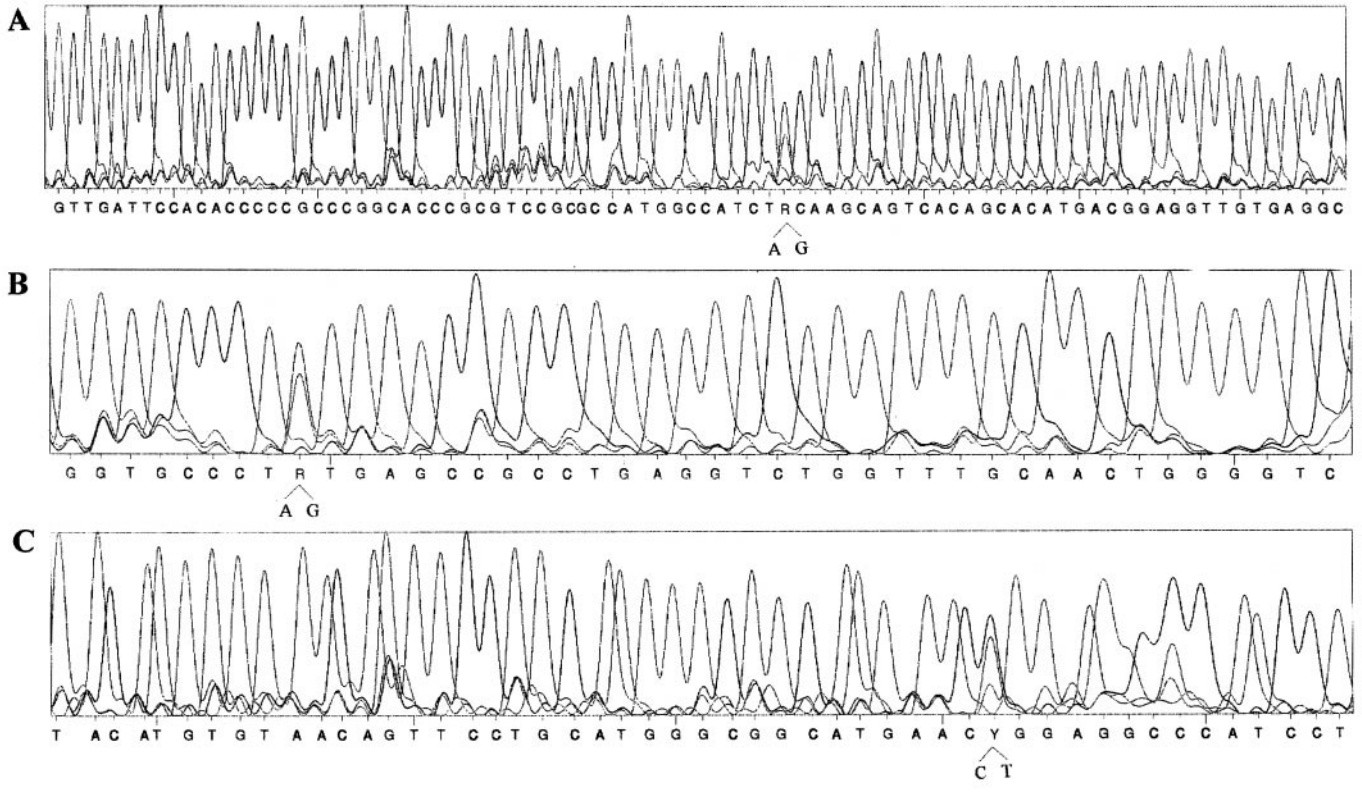

Fig. 2. DNA sequencing of the *p53* gene with the ALFexpress automated DNA sequencer.
Mutation detection of the same patients described in Fig. 1 with this comparative method.

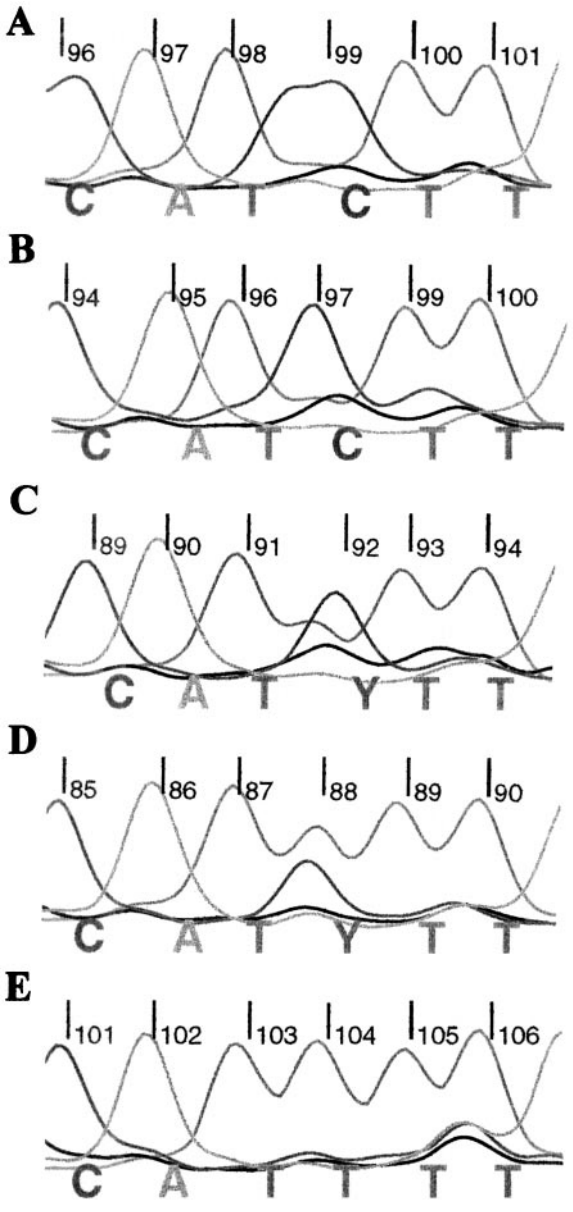

Fig. 3. Ability of the new sequencing method to detect heterozygosity.

(*A*) Wild-type *p53* gene sequence of part of exon 6, using DNA from the breast carcinoma cell line MCF-7. (*E*) Homozygous mutation in exon 6 of the *p53* gene (C to T), using DNA from the breast carcinoma cell line T-47D. (*B–D*) Sequencing of a mixture of MCF-7/T-47D DNA at ratios of 75%, 50%, and 25%, respectively. The software efficiently detects heterozygosity in (*C*) and (*D*) but not (*B*). Y: C or T.

parison. The Gene Librarian tool is also part of the software and allows comparison of a newly generated sequence with a whole database of known genes stored in the library.

Results

We present in detail all the mutations identified by the new sequencing methodology in Table 2 . Among the total of 34 specimens tested (16 breast and 18 ovarian tumors), 24 (71%) were found to harbor a mutation (12 breast and 12 ovarian tumors). The rest of the specimens had the wild-type *p53* gene sequence. These data do not reflect mutation rates in serial samples from breast and ovarian carcinoma, because the specimens were preselected from a larger series to contain ~60–70% mutant and 30–40% wild-type *p53* gene sequences. This optimized the comparison between the new method and the conventional sequencing technique in terms of ability to detect mutations. Among all tumors, the new sequencing method agreed in terms of *p53* gene status in all cases. Representative examples of sequencing tracings obtained with the new technique as well as with the ALFexpress comparative method are shown in Figs. 1 and 2.

To check the ability of the new sequencing method to detect heterozygotes, we sequenced the exon 6 of the *p53* gene in the breast carcinoma cell lines MCF-7 (wild-type sequence) and T-47D (homozygous mutation within exon 6). We then mixed DNA from these cell lines at various proportions and repeated the sequencing. The data (Fig. 3) confirm the ability of the new technique to detect the heterozygous state. However, in Fig. 3B, the presence of ~25% of mutant DNA was not detected, presumably because the intensity of the mutant DNA signal in the final mixture was below the threshold (15%) of the base-calling algorithm.

Discussion

DNA sequencing is one of a few fundamental techniques that have revolutionized the generation of new genetic knowledge. This method has been used in research settings for many years. More recently, the needs of the Human Genome Project necessitated the introduction of instrumentation that can generate large amounts of sequencing information in an automated fashion, without using radioactivity. Such instruments, which are mainly based on gel electrophoresis and laser-induced fluorescence detection, now have been introduced in clinical laboratories for studying genetic, infectious, and malignant diseases. The number of genes that are cloned every year is expanding exponentially. Many of these genes are altered by mutations, causing various human diseases. Examples of genes that are implicated in carcinogenesis and harbor mutations include *p53 (17)*, *ras (18)*, *BRCA1* and *BRCA2 (19–21)*, and *Rb (22)*. To study such mutations, clinical laboratories may apply an array of genome-screening techniques, recently reviewed in this journal *(10)*. None of these methods is as reliable as DNA sequencing. Thus, current and future clinical applications will require simple, reliable, and cost-effective methods for DNA sequencing.

The sequencing protocol starts from DNA preparation, which is generally a manual procedure. The same applies to the PCR amplification step and the performance of the sequencing reactions. The new protocol described here does not need a PCR product purification step because the PCR reactions have been carefully optimized to produce a single PCR band, and only a fraction of PCR product is

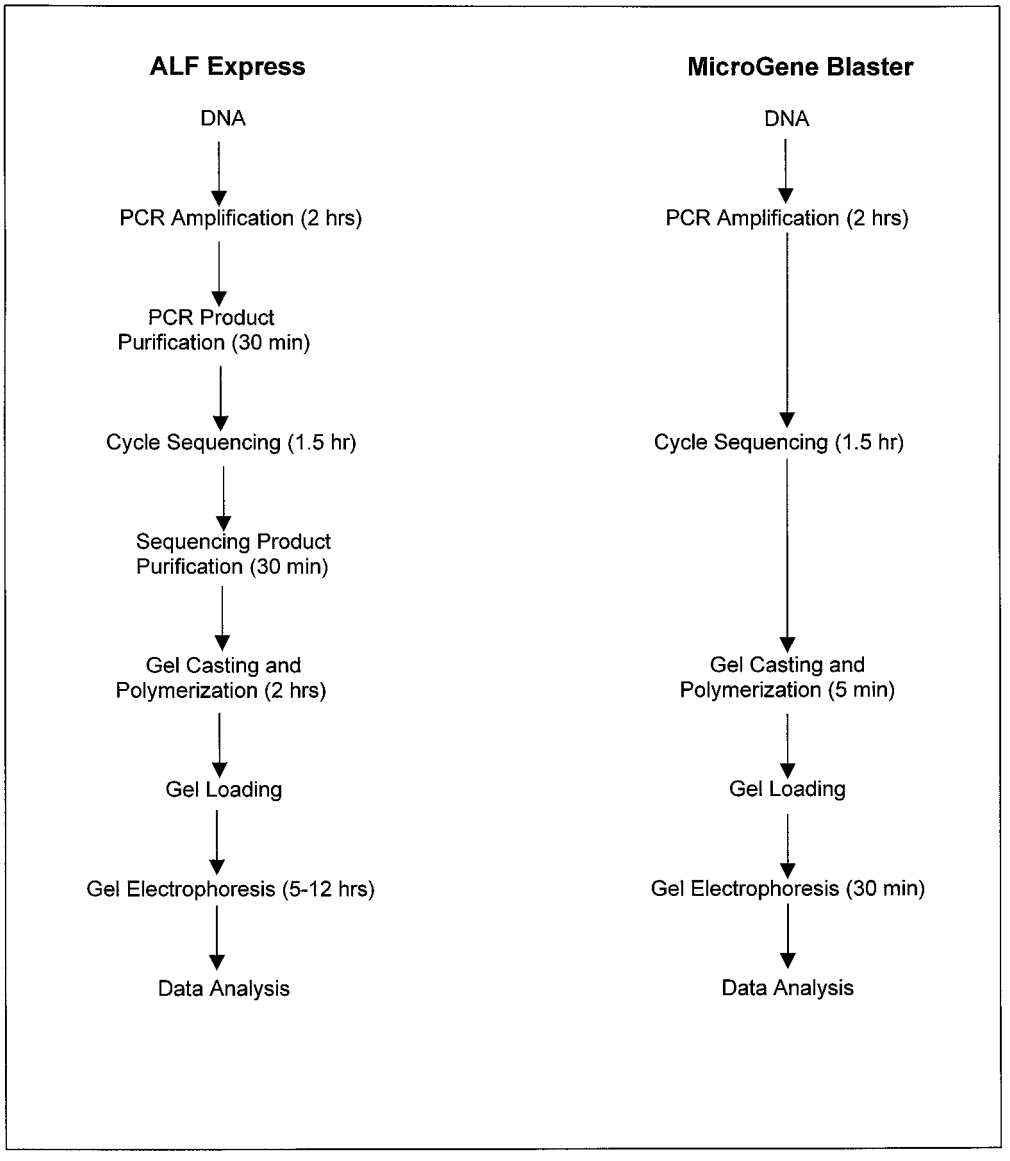

Fig. 4. Flowchart describing approximate times and steps necessary to complete a sequencing experiment with the new method and the ALFexpress procedure.

The new method provides sequencing data within 1 working day; the comparison method within 2 days.

used for sequencing. In some sequencing protocols, PCR product purification is recommended (Fig. 3). In addition, the sequencing reaction products do not need precipitation for the purpose of preconcentration and cleaning from unincorporated labeled primer or nucleotides. The use of the more sensitive label, Cy5.5, in comparison with Cy5, allows for loading of only a fraction of the sequencing reaction product.

The major contributions of this new technology involve the gel casting procedure and the electrophoresis time. Gel casting using low cost disposable cassettes can be completed within 5 min and is a major advantage over conventional reusable plates, which need more time to prepare and clean. Running time of the gel can be completed within 35 min, an order of magnitude faster than conventional DNA sequencers like the ALFexpress. This is mainly due to the use of thinner gels. In general, sequencing data with the new system may be obtained within 1 working day, whereas with a conventional system, data are usually generated in 2 working days. Hands-on time with the new procedure is relatively limited. Importantly, the new system provides faster data output while maintaining the accuracy of the provided information, as outlined in Table 2 and Fig. 1. An outline of the workflow comparing the new system with the ALFexpress is shown in Fig. 4.

The sequencing data of Table 2 were generated by selecting specimens from a series of ~80 tumors (50 ovarian and 30 breast). Because the type of the mutation was not preselected in this study, it is remarkable that in 15 of 24 cases with missense mutations, the affected codon encodes for arginine [CG (ACGT)]. These mutations will

probably affect the biological activity of p53, because many arginine molecules are necessary for DNA binding of p53 *(23)*. These observations were also made by others *(8)*.

In conclusion, we here provide evidence that the new instrumentation efficiently detects mutations in the *p53* gene while reducing the complexity of the technique and substantially decreasing the electrophoresis time. This system has the capability to be used for detecting mutations in routine diagnostic laboratories.

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